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Functional analysis of *Mob-like* genes in *Arabidopsis thaliana*

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Giada Marino

Supervisor
Dr. Sandra Citterio

CONTENTS

1. Introduction

1.1 Plant cell cycle

The cell cycle is a fundamental biological process whereby one cell grows and divides in two daughter cells. It is a highly ordered process that comprises four main phases: G1-phase (first gap); S-phase (DNA synthesis phase); G2-phase (second gap); and M-phase (karyo- and cytokinesis) (Figure 1.1).

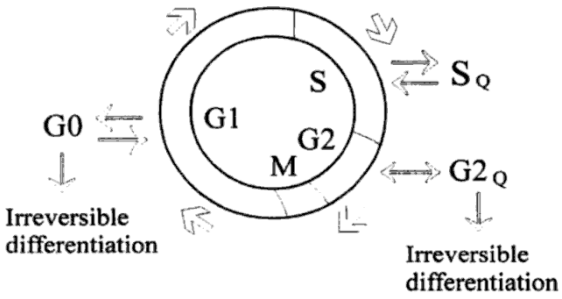

Figure 1.1: Schematic representation of cell cycle phases

During G1 phase, the cell has to grow and reach a minimum size and then replicate its genome during S-phase. When replication is complete, the genome can be divided into two complete and equal copies (M-phase). After the daughter nuclei are formed the cell can then divide into two by a process called cytokinesis. Daughter cells can enter directly in a new G1 phase or enter in a quiescent phase (G0) for a time. Cells in G0 phase are viable and metabolically active and can be stimulated to re-enter into G1 phase at any time and start the cell cycle again. Plant cells can also arrest in G2 phase and enter in the quiescent phase G2Q. G0 and G2Q can be

followed by irreversible differentiation of tissue cells, that do not divide anymore, although regression to unindifferentiated and newly proliferating cells has sometimes been observed in mesophyll cells (Marie D and Brown SCA, 1993).

All the cell cycle processes must executed in a spatially and temporally controlled manner in order to obtain two viable daughter cells with complete genomes. The gap phases allow the operation of controls that ensure that the previous phase has been accurately and fully completed, and so the major regulatory points in the cell cycle operate at the G1/S and G2/M boundaries.

The basic control mechanisms that regulate the cell cycle progression are conserved through evolution. As in all eukaryotes, the main protein family involved in plant cell cycle is that of cyclin-dependent kinases (CDKs) which associate to noncatalytic partners, the cyclins. These complexes are further controlled by a number of mechanisms including phosphorylation/dephosphorylation, interaction with inhibitory proteins, proteolysis, and intracellular trafficking (Morgan DO, 1997) (Figure 1.2).

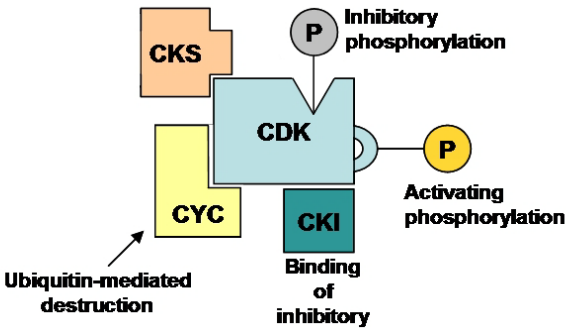

Figure 1.2: CDK complex and its regulatory mechanisms. Cyclin-dependent kinase (CDK) activity is regulated at multiple levels. Monomeric CDK lacks activity until it is complexed with cyclins (CYC) and activated by phosphorylation by CDK-activating kinase (CAK). In addition, activity can be inhibited by phosphorylation by WEE1 kinases or the binding of inhibitor proteins (CKI). Inhibitors may block the assembly of CDK/cyclin complexes or inhibit the kinase activity of assembled dimers. CDK subunit (CKS) proteins scaffold interactions with target substrates.

So the progression through the cell cycle involves the successive formation, activation and subsequent inactivation of CDKs. The kinases bind sequentially to a series of cyclins, which are responsible for differential activation of the kinase during cell cycle progression. As shown in the Figure 1.3, the G1 to S transition is thought to be controlled by CDKs containing D-type cyclin that phosporylate the retinoblastoma protein, releasing the E2F transcription factors. E2F are involved in the transcription of the genes needed for the G1 to S transition. The G2 to M transition is carried by CDK complexes containing cyclins A and cyclins B. This CDK complexes are kept in inactive state by phosphorylation by the Wee kinase, and by interaction with inhibiting proteins (CKIs) and at the G2 to M boundary the activation of the CDK complex is trigged by release of CKI protein, by positive phosphorylation (by CAK kinase) and by an unknown protein phosphatase.

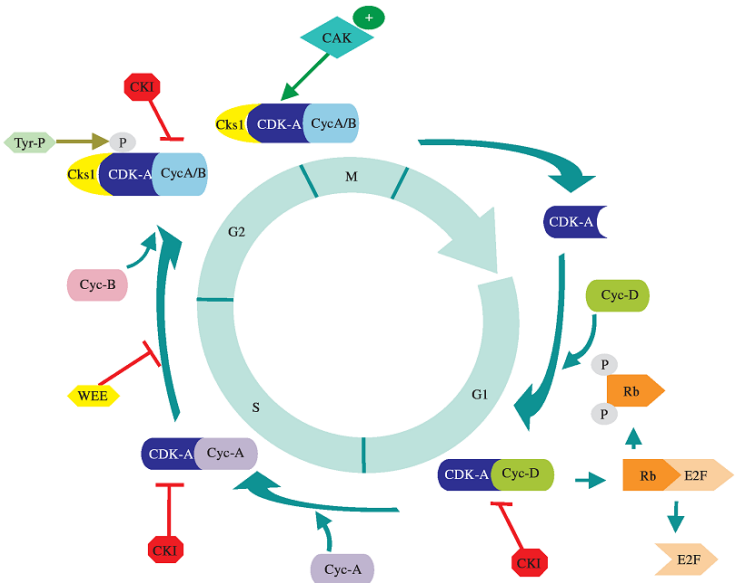

Figure 1.3: Plant cell cycle overview (Andrietta *et al,* 2001).

In the next sections, the components of the basic cell cycle machinery and the principal regulatory points will be discussed in details.

1.1.1 The basic cell cycle machinery

Cyclin-Dependent Protein Kinases

Cyclin-dependent kinases (CDKs) are a class/family of highly conserved serine/threonine kinases. In yeast, a single CDK (cdc2 in the budding yeast *Schizosaccharomyces pombe* or CDC28 in the fission yeast *Saccharomyces cerevisiae*) is responsible for cell-cycle control (Hartwell LH *et al,* 1974; Stern B and Nurse P, 1996; Nasmyth K, 1996). As shown in Table 1.1, in higher eukaryotes there are multiple cdc2/CDC28-related genes that have roles at different points in the cell cycle. The CDKs are not conserved between animals and plants and have variant sequences in their cyclin-binding domain. So in plants the nomenclature is based on a combination of letters and numbers (Joubès J *et al*, 2000, Vandepoele K *et al*, 2002), whereas in animals numbers have been used to distinguish different classes (Pines J, 1995).

	S.cerevisiae **(Budding yeast)**	***S. pombe*** **(Fission Yeast)**	***H. sapiens*** **(human)**	***A. thaliana*** **(plant)**
Cyclin-dependent kinases (CDKs)	**Cdk1** (Cdc28): all stage	**Cdk1** (Cdc2): all stage	**Cdk1**: M phase	**CdkA**: G1/S, G2/M
			Cdk2: G1/S, S	**CdkB1:** S and M phases **CdkB2**: G2/M
			Cdk4, Cdk6: G1	

Table 1.1: principal CDK involved in the cell cycle.

Plant CDK-related genes have been classified into seven classes, A–G. Of these, As, Bs, Ds and Fs have been shown to be involved in the regulatory mechanisms of the plant cell cycle (Dewitte W and Murray JAH, 2003; Inzé D and De Veylder L, 2006; Doonan JH and Kitsios G, 2009). The major drivers of the plant cell cycle are the A- and B-type CDKs.

A-type CDKs (CDKA), containing the cyclin-binding motifs PSTAIRE, are most closely related to mammalian CDK1 and CDK2 (Joubès J *et al*,

2000). At the transcript and proteins levels, plant CDKA is present throughout the cell cycle and regulates both G1/S and G2/M transitions (Menges M *et al*, 2005; Vandepoele K *et al,* 2002). Previous studies with dominant negative variants showed CDKA activity is required for entering both mitosis and DNA synthesis phase in *Arabidopsis*, maize and tobacco (Hemerly AS *et al,* 2000; Leiva-Neto JT *et al*, 2004; Hemerly A *et al*, 1995). In particular, in *Arabidopsis* only one CDKA (CDKA;1) has been idenfied and can rescue the fission yeast cdc2 mutants (Ferreira *et al*, 1991, Hirayama T *et al*, 1991). Specifically in *Arabidopsis* CDKA;1 has an essential function in early developmental stages such as gametogenesis and embryogenesis. Knockout mutants of CDKA;1 expression result in embryo lethality, whereas in the heterozygous CDKA;1 mutant, only male gametogenesis is affected (Nowack MK *et al*, 2006; Iwakawa H *et al*, 2006). On the other side, plants overexpressing a dominant negative form of CDKA;1 have severe defects during embryogenesis (Hemerly AS *et al*, 2000). It has also been reported that CDKA;1 plays an important role in post-embryonic development. Tobacco seedlings overexpressing a dominant negative form of Arabidopsis CDKA;1 consist of larger and fewer cells (Hemerly A *et al*, 1995), and *Arabidopsis* mutants with the weak cdka;1 allele show a dwarf phenotype with fewer and larger leaf cells (Dissmeyer N *et al*, 2007). However, in both cases, plants undergo normal morphogenesis with normal developmental timing.

The B-type CDK (CDKB) is a plant-specific group and is divided into subgroups CDKB1 containing PPTALRE motif or CDKB2 containing PPTTLRE motif (Joubes J *et al*, 2000; Vandepoele K *et al*, 2002). In contrast with A-type CDKs, B-type CDK transcription is cell cycle regulated. Studies conducted in *Antirrhinum* (cdc2c) (Fobert PR *et al*, 1996), alfalfa (cdc2MsD) (Magyar Z *et al*, 1997), tobacco (Sorrell DA *et al*, 2001), and *Arabidopsis* (Menges M and Murray JAH, 2002; Segers G *et al,* 1996) showed that the CDKB1 subgroup is expressed from the onset of S phase until mitosis; whereas the CDKB2 type is expressed only in G_2-M cells.
Arabidopsis harbors two CDKB1 (CDKB1;1 and CDKB1;2) and two CDKB2 (CDKB2;1 and CDKB2;2) family members (Vandepoele K *et al*, 2002). CDKB1;1 has been shown to be essential for the correct stomatal development in *Arabidopsis,* in particular down-regulation of its expression results in aberrant stomatal complexes with cells arrested in G2 phase

(Boudolf V *et al*, 2004a). In tobacco the overexpression of a dominant-negative CDKB1 interferes with cell cycle progression and causes a G2 arrest (Porceddu A *et al*, 2001). CDKBs may also function outside the cell cycle, as downregulation of *Arabidopsis* CDKB1;1 reduces the growth of etiolated seedlings primarily by inhibiting the elongation rates, independently of cell division or endoreduplication (Yoshizumi *et al*,. 1999). In CDKB2 overexpressing or knock-down lines, the shoot apical meristem (SAM) contains considerably fewer and larger cells, and the strict organisation into three distinct cell layers was disrupted caused severe defects in meristem functions (Andersen SU *et al*, 2008). Lastly, it is worth to note that CDKB, like CDKA, colocalises to microtubular structures and consequently these kinases may regulate cytoskeleton reorganisation by phosphorylation of microtubule associated proteins (MAPs) and kinesin-related proteins (Criqui MC and Genschik P, 2002).

In addition to A and B-type CDKs, there are other two CDKs involved in cell cycle, CDKD and CDKF. They are involved in the regulation of CDK activation,. In yeast and animals proteins known as CDKs-activating kinases (CAKs) are able to phosphorylate CDKs at Thr60 (or the functionally equivalent residue in the T-loop region) inducing a conformational change that allows proper recognition and binding of substrates (Kaldis P 1999; Saiza JE and Fisher RP, 2002; Fisher RP, 2005). In plants there are two types of CAK: CDKD, that is functionally related to CAKs in animals, and CDKF, that is a plant specific CAK displaying unique enzyme characteristics (Umeda M *et al*, 2005). The first plant CAK, R2, was identified in rice plants (Hata S, 1991). R2, structurally related to CDK7 (human CAK kinase), can interact with CycH-like proteins and this complex can phosphorylate the human CDK2, the rice CDKA1 and the carboxy-terminal domain (CTD) of *Arabidopsis* RNA polymerase II (Yamaguchi M *et al*, 1998, 2000). In *Arabidopsis* three CDKD genes, namely CDKD;1–CDKD;3 were found (Vandepoele K *et al*, 2002; Shimotohno A *et al*, 2003). Both CDKD;2 and CDKD;3 exhibit CDK and CTD kinase activities, whereas CDKD;1 shows neither CDK nor CTD kinase activity in vitro (Shimotohno A *et al*, 2004). CDKD;2 also forms a stable complex with the H-type cyclin CYCH;1 (Shimotohno A *et al*, 2004, 2006). *Arabidopsis* has a single gene for CDKF, named CDKF;1, which has been identified as a suppressor of the cak1 mutation in budding yeast. It has CDK-activating kinase activity but not CTD

kinase activity in vitro and the activation of CDKF requires no association with H-type cyclin (Umeda M *et al*, 1998; Shimotohno A *et al*, 2004). CDKF;1 can phosphorylate CDKD;2 and CDKD;3 and function as a CAK-activating kinase (CAKAK), thereby regulating basal transcription and CDK activation (Shimotohno A *et al*, 2004; Umeda M *et al*, 2005). Recently it was shown that CDKF;1 plays an important role in post-embryonic development by regulating the protein stability of CDKD;2 (Takatsuka H *et al*, 2009).

CDKs are also implicated in regulation of transcription during cell cycle progression and during gene expression, mainly through the phosphorylation of CTD of RNAPII (Doonan JH and Kitsios G, 2009). Plants contain C-type and E-type *CDK*-related genes, designated *CDKC* (PITAIRE or SPTAIRE motif) and *CDKE* (SPTAIRE motif), with no clear role in cell cycle control (Joubès J *et al*, 2000; Inzé D and De Veylder L, 2006). CDKCs interact with CYCT and play a presumed role in transcription elongation by phosphorylating the CTD of RNA polymerase II (Barroco RM *et al,*, 2003, Fulop K *et al*, 2005a). In *Medicago truncatula* the CDKC/CYCT complex phosphorylates the retinoblastoma-related (RBR) protein and consequently might control cellular differentiation through RBR inactivation (Fulop K *et al*, 2005a). In agreement with this postmitotic role for CDKC, in *Arabidopsis* transcripts were found mainly in differentiated tissues (Barroco RM *et al*, 2003). Moreover loss of function mutants in CDKC suggesting that in *Arabidopsis* this protein kinase is required for plant growth and development (Cui X *et al*, 2007). CDKC and CycT mutants are highly resistant to infection by the *Cauliflower mosaic virus* (CaMV) indicating that the CDKC–CycT complexes are required for transcriptional activation of CaMV viral genes (Cui X *et al*, 2007). In plants, CDKE was identified in alfalfa and *Arabidopsis (*Magyar Z *et al*, 1997*,* Vandepoele K *et al,*,2002*).* The *Arabidopsis* CDKE is encoded by HUA ENHANCER 3 (HEN3), a gene required for regulation of developmental events like leaf cell expansion and cell-fate specification in floral meristems (Wang W *et al*, 2004). Interestingly, the cyclin partner of CDKE seems to be D-type cyclins (Wang W *et al*, 2004). Considering that the cyclin-binding motif in CDKE is very similar to CDKA, it was speculated that CDKE might link cell division with cell fate specification in the floral meristems. Like CDKCs, CDKE phosphorylates C-terminal domain of RNA polymerase II (Wang W *et al*, 2004).

Cyclins

CDKs require the binding of cyclins for activation and the cell-cycle progression requires the sequential association of the CDKs with different types of cyclin. Previous studies indicated that cyclins contain a conserved region of 250-amino acid called cyclin core, organized in two domains: cyclinN and cyclinC. The cyclinN domain, also called cyclin box. is about 100-amino acid long and contains the CDK-binding site and is found in all known cyclins. The cyclinC domain is less conserved and is present in most but not all, cyclins, suggesting a specific but perhaps not a critical functions of this domain. (Wang G *et al*, 2004).

Plants contain more cyclins than other organisms (Vandepoele K *et al*, 2002; Wang G *et al*, 2004). The large number of plant cyclins may reflect the high developmental plasticity of sessile plants essential to respond to both intrinsic developmental signals and environmental cues. The different cyclins have a wide range of expression patterns and might confer different substrate specificities. As many as ten classes of cyclins have been distinguished in plants (Wang G *et al*, 2004) but just the A-, B-, D- and H-type cyclins have been shown to have a primary role in plant cell cycle regulation.

CYCA and CYCB are similar to each other and to the animal mitotic cyclins (Renaudin JP *et al*, 1996). A-type cyclins (CYCA) are mainly present from S phase to M phase, whereas B-type cyclin (CYCB) levels peak at the G2–M transition and during M phase .

A-type cyclins are subdivided into three different subclasses: CYCA1, CYCA2, and CYCA3. In *Arabidopsis* 10 sequences encoding CYCA have been identified (Vandepoele K *et al*, 2002). A-type cyclin expression is induced sequentially from G1/S through S-phase (Dewitte W *et al*, 2003). The members of those groups are sequentially expressed at different time points of the cell cycle and they show distinct localization profiles, suggesting compartment-specific functions (Roudier F *et al*, 2000; Wang G *et al*, 2004, Chaubet-Gigot N, 2000; Criqui MC *et al*, 2001). In *Arabidopsis* CYCA3 expression begins at the G1/S boundary and precedes that of members of the CYCA1 and CYCA2 subgroups (Menges M *et al*, 2005). CYCA1 and CYCA2 play an analogous role to vertebrate cyclin A and are expressed from mid-S-phase regulating the cell cycle progression (Menges

M *et al*, 2005). *Arabidopsis* plants overproducing the tobacco CYCA3;2 show ectopic cell division and delayed differentiation, correlated with an increase in expression of S phase–specific genes and CYCA3;2-associated CDK activity. In addition, overproduction of CYCA3;2 impairs shoot and root regeneration in tissue culture (Yu Y *et al*, 2003). In *Arabidopsis,* the substitution of a conserved amino acid residue probably results in an incorrect folding of CYCA1;2, causing a slower cell cycle progression during male meiosis (Magnard JL *et al*, 2001, Wang Y *et al*, 2004). Whereas *Arabidopsis* knockouts mutants for *CYCA2;3* display a slight increase in their DNA ploidy level (Imai KK *et al*, 2006). Recently, it has been demonstrated that antisense expression of *CYCA2;2* in alfalfa inhibits shoot and root development in tissue culture (Roudier F *et al*, 2003).

B-type cyclins control primarily the G2/M transition (Dewitte W and Murray JAH, 2003). Also B-type cyclins are subdivided into three subclasses, CYCB1 CYCB2 and CYCB3 (Vandepoele K *et al*, 2002). In *Arabidopsis* nine genes were identified and CYCB3s lacks a destruction box (Vandepoele K *et al*, 2002) In *Arabidopsis* CYCB1;1 interacts with both CDKA and CDKB in vitro and the expression of a nondegradable cyclin B1 affects plant development by inhibiting the formation of phragmoplast (Weingartner M *et al*, 2004). Lastly, the CYCB expression is found only in proliferating tissues, with transcription increasing during G2, and peaking in early mitosis (Dewitte W and Murray JAH, 2003).

The plant D-type cyclins have low homology to vertebrate D-type cyclins, but most plant CYCD have, like the animal cyclin D, the amino acid motif LxCxE, responsible for the interaction with retinoblastoma-related (Rb) proteins (Meijer M and Murray JAH, 2000). In Arabidopsis, ten CYCD sequences are grouped into seven sub-classes (Vandepoele K *et al*, 2002). Over-expression of *CYCD3;1* drives cell cycle progression in cell culture (Menges M *et al, 2006*) and ectopic cell division in plants (Dewitte W *et al*, *2003*). Furthermore, CYCD3;1 is a highly unstable protein that is degraded via the ubiquitin-proteasome pathway (Planchais S *et al*, 2004). Arabidopsis CYCD2;1, CYCD3;1 and CYD4;2 have been shown to interact with CDKA but not with CDKB in vivo (Healy JM *et al*, 2001; Kono *et al*, 2006). CYCD4;2 lacks the Rb binding motif and the PEST sequence, but it is able to rescue G1 cyclin-deficient yeast (Kono A *et al*, 2006). Lastly, the expression of D-type Cyclin genes is modulated by plant growth factors, such as cytokinins,

auxins, brassinosteroids, sucrose, and gibberellins (Inzé D and Veylder L, 2006; Stals H and Inzé D, 2001). The overexpression of *CYCD3;1* is sufficient to compensate for the lack of cytokinins in the culture medium (Riou-Khamlichi C *et al*, 1999).

The levels of cyclins are generally determined by highly regulated transcription as well as by specific protein-turnover mechanisms. A- and B-type cyclins possess a “destruction box” (D-box) sequences which is susceptible to ubiquitination that leads to rapid proteolytic destruction. Most of the D-cyclins contain a PEST motif (region rich in proline, glutamate/aspartate, serine/threonine) in the C-terminal, which are markers of short-lived proteins (Wang G *et al,* 2004; Rechsteiner M and Rogers SW, 1996).

In all cases, the route to destruction runs via the ubiquitin-proteasome system, which uses the highly conserved polypeptide ubiquitin as a tag to mark target proteins for degradation by the 26S proteasome. Ubiquitination requires the generation of polyubiquitin chains on target proteins through the combined action of ubiquitin-carrying enzymes (or E2s) and ubiquitin-protein ligases (or E3s) that bring targets and E2s together (Pickart CM, 2001). Two related E3 complexes are most intimately dedicated to basic cell cycle control, namely the anaphase-promoting complex (APC) and the Skp1/Cullin/F-box (SCF)-related complex (Vodermaier HC, 2004).
APC controls most cell cycle events and the activation and substrate specificity of the APC complex is in part determined by two adaptor proteins, CDC20 and CDH1/fizzy-related (FZR).
Cdc20 directs the ubiquitination of Securin, mitotic cyclins, and other substrates for anaphase onset. Cdc20 is responsible for the separation of sister chromatids because the degradation of Securin leads to the activation of the Separase protease, which cleaves the cohesin complex ring responsible for physically attaching the sister chromatids. Cdh1 targets mitotic cyclins and additional substrates for degradation in mitotic exit and G1 (Pesin JA and Orr-Weaver TL, 2008). The *Arabidopsis* genome encodes five *CDC20* genes, as well as three CDH1-related proteins, designated CCS52A1, CCS52A2, and CCS52B, that are plant-specific (Tarayre S *et al*, 2004). In *Schizosaccharomyces pombe*, expression of the three *Arabidopsis CCS52* genes elicits distinct phenotypes, supporting a nonredundant function of the CCS52 proteins. Consistent with these different functions,

CCS52B is expressed from G2/M to M, whereas *CCS52A1* and *CCS52A2* are from late M until early G1, suggesting consecutive actions of these APC activators in the plant cell cycle. In addition, the CCS52 proteins interact with different subsets of mitotic cyclins, either in free or CDK-bound forms (Fulop K *et al*, 2005b).

Arabidopsis contains at least 694 different F-box proteins **(**Vierstra RD, 2003) and just very few substrates have been identified (Lechner E *et al*, 2006).

CDK phosphorylation

Similarly to that in yeast and animals, the activity of plant CDK/cyclin complexes is regulated by phosphorylation/dephosphorylation and the interaction with regulatory proteins.

Yeast CDK/cyclin complexes are subject to an inhibitory phosphorylation of an N-terminal Tyr residue in the CDK partner, whereas in vertebrates CDKs are phosphorylated on both an N-terminal Tyr or Thr residue (Stark GR and Taylor WR, 2006). This phosphorylation is catalyzed by the WEE1 kinase. In plants, a WEE1-related kinase has been described for maize (*Zea mais*), tomato (*Solanum lycopersicum*), and *Arabidopsis* (Sun Y *et al,*, 1999; Sorrell DA *et al*, 2002; Gonzalez N *et al,*, 2007). Although the plant WEE1 gene is unable to complement mutations in its yeast homolog, its overexpression inhibits cell division in fission yeast. Additionally, recombinant purified WEE1 protein from maize is capable of inhibiting the kinase activity of biochemically purified CDKs (Sun Y *et al,*, 1999). In *Arabidopsis*, WEE1 kinase can phosphorylate CDKA;1 at tyr15, and CDKD;1, CDKD;2 and CDKD;3 at tyrosine 23/24 in vitro (Shimotohno A *et al,*, 2006). The in vivo role of WEE1 in plant cell cycle progression and growth is not well defined. However, in *Arabidopsis* the knockout mutants of WEE1 showed no obvious phenotype when grown under normal growth conditions (De Schutter K *et al*, 2007). This suggests that unlike in yeast and animals, WEE1 is not a critical CDK modifier in plants.

In yeast and mammals a specific phosphatase, CDC25, dephosphorylates the CDK at the G2/M checkpoint, resulting in the fully activation of kinase and the onset of mitosis (Stark GR and Taylor WR, 2006). In plants, the first CDC25 was identified in the primitive unicellular algae, *Ostreococcus tauri*

(Khadaroo B *et al*, 2004). However, in both *Arabidopsis* and rice, no genes with high homology to yeast or animal *CDC25* genes have been identified (Vandepoele K *et al*, 2002). A small protein CDC25-like phosphatase has been identified in *Arabidopsis*(Landrieu I *et al*, 2004). However, although the plant CDC25-like phosphatase displays structural homology with the mammalian CDC25 proteins within its catalytic domain and can activate CDKs in vitro, it lacks the complete N-terminal regulatory domain (Sorrell DA *et al*, 2005). Moreover Arath;CDC25 is homologous to *Arabidopsis* arsenate reductase (ACR2) (Bleeker PM *et al*, 2006) and ACR2 knockdowns make *Arabidopsis* hypersensitive to arsenate (Dhankher OP *et al*, 2006). A role for the *Arabidopsis* CDC25-like protein is unclear, because no effects on cell cycle progression can be seen in overexpressing or knockout mutants under normal growth or stress conditions (Bleeker PM *et al*, 2006; Dhankher OP *et al*, 2006).

CDK Inhibitor

Cyclin-dependent kinase inhibitors (CKIs) regulate cell cycle progression by binding and inhibiting CDKs (Besson A *et al*, 2008; De Clercq A and Inze D, 2006).

Budding yeast has three CKIs: Far1p inhibits G1 CDK activity; Sic1p controls S-phase entry by regulating G1/S CDK complexes; and Pho81p inactivates a CDK/cyclin complex that plays a role in regulating gene expression under low-phosphate conditions. The situation in fission yeast (*Schizosaccharomyces pombe*) is considerably simpler because only one CKI, designated Rum1, is known to control mitotic CDK complexes. Mammals have seven CKIs, which are subdivided into two very different classes, the INK4 and the Kip/Cip families, each with its own CDK binding specificity and protein structure (Besson A *et al*, 2008; De Clercq A and Inzé D, 2006).

In plants, the first CKIs have been characterized in a yeast two-hybrid screen for CDKA-interacting proteins (Inzé D and De Veylder L, 2006). Additional plant CKIs have been identified in silico through genome data mining. All known plant CDK inhibitors share a 31-amino-acid domain in the C-terminus, wich is also similar to the CDK inhibitory region of the mammalian CKI $p27^{Kip1}$ (Wang H *et al*, 1998; Lui H *et al*, 2000; De Veylder L

et al, 2001a; Zhou Y *et al*, 2002). This conserved domain is essential for the interaction between CDKs and cyclins and is essential for the inhibitory activity of the proteins (Wang H *et al*, 1997; De Veylder L *et al*, 2001a). Based on this similarity, plant CDK inhibitors were designated Kip-Related Proteins (KRPs) (De Veylder L *et al*, 2001a) although the two founding members are also known as inhibitor/interactor of CDK (ICK). This family is now referred to as the ICK/KRP family (Wang H *et al*, 2008).

ICK/KRP-related genes have been identified from *Chenopodium rubrum* (Fountain MD *et al*, 1999), tobacco (Jasinski S *et al*, 2002a), maize (Coelho CM *et al*, 2005), alfalfa (Pettko-Szandtner *et al*, 2006), tomato (Bisbis B *et al*, 2006) and rice (Barroco RM *et al*, 2006; Guo J *et al*, 2007). In *Arabidopsis*, the ICK/KRP family has seven members (De Veylder L *et al*, 2001a) and in addition to the conserved C-terminal domain, this proteins family show a second and shorter conserved motif, which is adjacent to the conserved C-terminal domain, that is important for the interaction with D-type cyclin (Wang H *et al*, 1998). Moreover, it has been reported that the N-terminal region of ICK1 can interact with CYCD3;1 (Jakoby MJ *et al*, 2006). Excluding the two conserved motifs in the C-terminal region, plant CDK inhibitors diverge greatly among themselves and from the mammalian and yeast CDK inhibitors. In terms of sequence similarity, the plant CDK inhibitors are likely the most different, among the core cell cycle regulators, from their animal counterparts (Wang H *et al*, 2006). Arabidopsis ICK/KRPs can be grouped into three more closely related sub-sets (ICK1 and ICK2), (KRP3, KRP4 and KRP5) and (KRP6 and KRP7) (Wang H *et al*, 2008). In rice, a molecular model species for monocots, seven KRP (Oryza;KRP1 to Oryza;KRP7) genes have been reported (Barroco RM *et al*, 2006; Guo J *et al*, 2007). In *Arabidopsis* results show that ICK1 is able to interact with CDKA;1 and CYCD3;1 directly (Wang H *et al*, 1998). Studies of *Arabidopsis* ICK/KRPs in the yeast two-hybrid system show that all of them can interact with D-type cyclins and most of them can also interact with CDKA (but not CDKBs) (De Veylder L *et al*, 2001a; Zhou Y *et al*, 2002a). The specific interaction of ICK2/KRP2 with CDKA has also been confirmed in vivo (Verkest A *et al*, 2005). Over-expression of ICK1 and ICK2 also inhibits the CDK activity in plants (Wang H *et al*, 2000; De Veylder L *et al*, 2001a). The removal of the C-terminal conserved domain from ICK1 abolishes the interaction with the CDK complex and its ability to inhibit CDK activity (Zhou Y *et al*, 2003a). Based on the interactions with CDKA and D-type cyclins, the

CDKA-CYCD complexes should be the major target of ICK/KRP proteins. Results from independent studies also show that they may inhibit the activities of other types of CDK complexes (Coelho CM *et al*, 2005; Nakai T *et al*, 2006; Pettko-Szandtner *et al*, 2006). Results from several studies show that transgenic over-expression of an ICK/KRP CDK gene inhibits cell divisions (Wang H *et al*, 2000; De Veylder L *et al*, 2001a; Jasinski *et al*, 2002b, 2003; Zhou Y *et al*, 2002a; Barroco RM *et al*, 2006). Furthermore, it has been shown that abscisic acid (ABA) and stress conditions, like low temperature and salt, induce the expression of ICK1 (Wang H *et al*, 1998; Ruggiero B *et al*, 2004) and the alfalfa CDK inhibitor KRPMt (Pettko-Szandtner A *et al*, 2006). These results suggest that ABA and stress conditions may induce the expression of a CDK inhibitor such as ICK1, causing arrest of the cell cycle (Wang H *et al*, 1998). No plant homologs to the INK4 family of inhibitors have been found yet (Vandepoele K *et al*, 2002).

Recently, it was shown that plants may have another small family of CDK inhibitors, SIAMESE (SIM) family (Churchman *et al*, 2006; Peres A *et al*, 2007). Although SIM-related proteins share the cyclin-binding motif with ICK/KRP CDK inhibitors, they do not have the CDK-binding motif present in ICK/KRPs. SIM protein, like ICK1, interacts with both CDKA and D-type cyclins, but not with CDKB, CYCA and CYCB (Churchman ML *et al*, 2006). The ability to inhibit CDK activity has been demonstrated for a rice SIM-related protein, Oryza;EL2 (Peres A *et al*, 2007). Analysis of a development mutant showed that mutations in the SIM gene led to multi-cellular trichomes instead of the wild-type unicellular trichomes and also to reduced levels of ploidy (Walker JD *et al*, 2000).

CDK Subunit

Genetic studies in yeast have reveald the existence of a third protein component of the CDK complexes called SUC1 in fission yeast, CKS1 in budding yeast, human and plant. Binding of SUC1/CKS1 to CDK/cyclin complexes stimulates the ability of the CDK complex to be phosphorylated by its key upstream activators, such as CDC25 and Wee1 (Morgan DO, 1997). Therefore the SUC1/CKS1 proteins may serve as docking factors on CDKs for both positive and negative regulators of kinase activity. This model is supported by the crystal structure of a human SUC1/CKS1 homologue,

CKSHs1, in complex with CDK2 (Bourne Y *et al*, 1996). CDK subunit (CKS) proteins are proposed to act as docking factors also in plants, the interaction of *Arabidopsis* CKS1 with CDKA and CDKB has been demonstrated (De Veylder L *et al*, 1997). The expression of CKS1 is associated with dividing cells as well as with endoreduplicating cells (Jacqmard A *et al*, 1999) Overproduction of CKS1 in *Arabidopsis* causes an increase in cell-cycle duration, which is in agreement with earlier results in both animal and yeast systems (De Veylder L *et al*, 2001b). Recently, a second homologue in *Arabidopsis* has been identified by genomic analysis (Vandepoele K *et al*, 2002). The two CKS gene products do not contain both the N- and C-terminal extensions compared with the yeast Suc1p/Cks1p homologs (Vandepoele K *et al*, 2002).

The RB/E2F/DP Pathway

Rb/E2F/DP pathway is involved in the regulation of many cellular processes such as cell proliferation and differentiation and was found in both animals and plants. Most E2F (adenovirus E2-promoter-binding factor) proteins associate with a DP (dimerization partner) protein and form heterodimeric complexes that bind to DNA in a sequence-specific manner. Different types of E2F complexes either activate or repress transcription. RB (retinoblastoma) protein inhibits E2F-mediated activation and increases E2F-mediated repression. The ability of RB proteins to repress E2F-dependent transcription is controlled by cyclin-dependent kinases (CDKs) through phosphorylation (van den Heuvel S and Dyson NJ, 2008;Dewitte W and Murray JAH, 2003; Inzé D and Veylder L, 2006).

In mammals, seven E2F genes (E2F1–7), two DP (DP1 and DP2) genes and three genes encoding RB-related proteins (RB, p107 and p130) have been identified to date. E2F1–E2F6 contain conserved dimerization domains and form DNA-binding heterodimers with DP proteins. E2F1–E2F5 can activate or repress transcription. E2F6 seems to function exclusively as a repressor. E2F7 and E2F8 lack a DP-binding domain but contain the tandem repeats of an E2F DNA-binding domain. Homodimers and heterodimers of E2F7 and E2F8 suppress the transcription of a subset of E2F-regulated targets (van den Heuvel S and Dyson NJ, 2008).

Arabidopsis has three E2Fs (E2Fa, E2Fb, E2Fc), two dimerization partners (DPa and DPb) and three DP-E2F-like proteins, known as DEL/ELP (E2Fd/DEL2, E2Fe/DEL1, and E2Ff/DEL3) and only one Rb-related gene (*RBR*) (Inzé D and De Veylder L,2006). E2Fs and DPs contain only one DNA-binding domain and, therefore, require dimerization to interact with the canonical E2F motif. *E2Fa* and *E2Fb*, are transcriptional activators and homologous to human E2F1-E2F5. E2Fc is a proposed transcriptional repressor homologous to human E2F6 (de Jager SM *et al*, 2009). In addition, the three atypical E2Fs, E2Fd/DEL2, E2Fe/DEL1 and E2Ff/DEL3 proteins contain two DNA-binding domains, that allow them to bind DNA as a monomer in a DP-independent manner and are homologues of human E2F7 (de Jager SM *et al*, 2009).

Expression of E2Fa–c and DP is constant during the cell cycle, although highest at S-phase, and is associated with cell proliferation and endoreduplication (Shen WH, 2002). Like the mammalian transcriptional activators, E2Fa/DPa has been shown to drive S-phase entry in differentiated, non-dividing leaf cells (Rossignol P *et al,, 2002*) and both E2Fa and E2Fb drive cell division in cultured cells, regulating both the G1-to-S and G2-to-M transitions (Magyar Z *et al,, 2005*). Constitutive overexpression of *E2Fa/DPa* or *E2Fb* in plants led to ectopic cell proliferation, and *E2Fa/DPa* also to enhanced endoreduplication (De Veylder L *et al, 2002*; Kosugi S and Ohashi Y *2003*; Sozzani R *et al, 2006*). In contrast, overexpression of a stabilized form of E2Fc reduced cell division and increased endoreduplication (del Pozo JC *et al, 2006*). Recently, it was reported that E2Fa can regulate gene expression both positively and negatively and E2Fc/DPa is a transcriptional repressor but not an antagonist of E2Fa, and does not involve consensus E2F-sites (de Jager SM *et al*, 2009). Roles for plant E2F family members in developmental processes have also been suggested. E2Fc is regulated at the level of protein degradation in a light-dependent manner, and postulated to function in the transition from skotomorphogenesis to photomorphogenesis, with a stable form of E2Fc repressing cell division in the dark (del Pozo JC *et al, 2002*). E2Fa and E2Fb have also been implicated in the regulation of cell size, although through different mechanisms (Sozzani R *et al, 2006*; He Y *et al,* 2004). In *Arabidopsis* knock-out mutants of RBR expression result in female gametophyte lethality (Ebel C *et al*, 2004). RBR gene regulates stem cell maintenance in the roots (Wildwater M *et al*, 2005).

Regarding atypical E2Fs, they do not interact with the DP proteins and do not transactivate gene expression, but are able to antagonize E2F/DP function and repress gene expression (Kosugi S and Ohashi Y, *2002a*). E2Fd/DEL2 expression is not strongly regulated during the cell cycle, while E2Fe/DEL1 and E2Ff/DEL3 show expression in S- and G2-phase (Mariconti L *et al*, 2002; Menges M *et al*, 2005). In addition, E2Ff/DEL3 has been shown to play a possible role in repressing cell wall biosynthesis during cell elongation in differentiated cells (Ramirez-Parra E *et al*, 2004) and E2Fe/DEL1 plays an important role in the control of endoreduplication (Vlieghe K *et al*, 2005).

The consensus E2F-binding site for mammalian E2F/DP heterodimers has been identified as TTT(C/G)(C/G)CG(C/G) and a slightly longer sequence has been reported for plant (Ramirez-Parra E *et al, 2003*). 5765 *Arabidopsis* genes -approximately 23% of genome- contain this sequence in their promoters(Ramirez-Parra E *et al, 2003*), but, only a few plant E2F targets have been validated experimentally, and these genes encode proteins involved in cell cycle regulation, DNA replication, and chromatin dynamics (Inzé D and De Veylder L, 2006; de Jaeger SM *et al*, 2005).

In animals, a number of experimentally established E2F target genes do not have E2F-binding sites matching the above consensus (Wells J *et al, 2000*; Weinmann AS *et al, 2002*), and indeed E2Fs can be recruited to promoters through interaction with other transcription factors (Schlisio S *et al, 2002*; Giangrande PH *et al, 2003*) Recently it was postulated that it also occurs in plants (de Jager SM *et al*, 2009). In plants, overexpression of *DEL1* and of *E2Fb*, as well as *del1* mutants, confirm that not all DNA replication genes containing E2F-sites in their promoters respond to modulation of E2F activity (Vlieghe K *et al,* 2005*;* Sozzani R *et al,* 2006).

1.1.2 Checkpoints

All the cell cycle processes must executed in a spatially and temporally controlled manner in order to obtain two viable daughter cells with complete genomes. The cell cycle checkpoints are a network of mechanisms that control that the previous phase has been accurately and fully completed, and the main checkpoints in the cell cycle operate at the operate

at the G1/S and G2/M boundaries.

G1 Entry and G1-S Transition

As in animals, the plant cell cycle is stimulated by extracellular signals (such as sucrose or hormones) that result in the synthesis of CycDs that bind to CDKA1 and form active complexes (Riou-Khamlichi C *et al*, 1999;2000; Healy JM *et al,* 2001). These complexes facilitate two major functions: (i) they disable the inhibitors of CDKs (ICKs) by phosphorylating them and (ii) activate a battery of genes whose functions are required for the G1/S transition and/or S-phase progression (Sherr CJ *et al*, 2004). Specifically, plant CDKA–CycD complexes modulate an E2F-dependent transcriptional program (Huntley R *et al*, 1998; Meijer M and Murray JAH, 2000): phosphorylation of Rb by CDKA–CycD activates the transcription of E2F-regulated genes and S-phase progression (De Veylder L *et al,* 2003). The sequential expression of the CycD-family members, from early G1 to the onset of S-phase (Menges M *et al*, 2005), is thought to drive the cell through G1 and the G1/S boundary (figure 1.4).

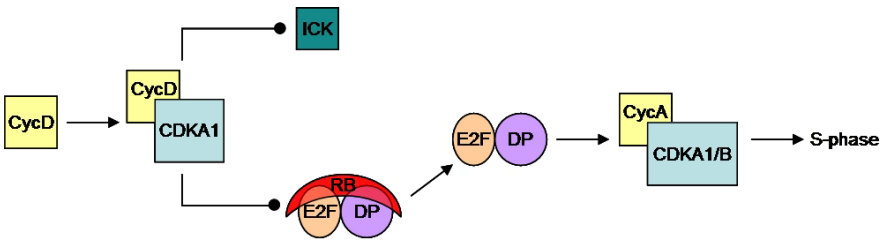

Figure 1.4: The G1/S transition in plants. Arrows indicate activation and dot-ended lines indicate inhibition.

G2-M Transition

Two classes of plant CDKs participate in the G2/M transition: CDKA1 and the plant-specific CDKBs. Mitotic cyclins, such as CycAs and CycBs, can form complexes with both CDKA and CDKB to facilitate mitotic events (Menges M *et a*, 2005, Schnittger A *et al*, 2002a, Weingartner M *et al*, 2003), but the precise role of each complex remains to be determined. As shown in

figure 1.5, at the onset of mitosis CDKs are phosphorylated (CDK-p) and kept at an inactive state. Progression from G2 to mitosis in plants requires CDK activation by the CAK complex CDKD-CycH, as well as dephosphorylation on residues T14/Y15 by a phosphatase (De Veylder L *et al*, 2003), but the identity of this phosphatase (cdc25-like) is unclear and the role of WEE1 kinase in the normal cell cycle remains to be clarified .

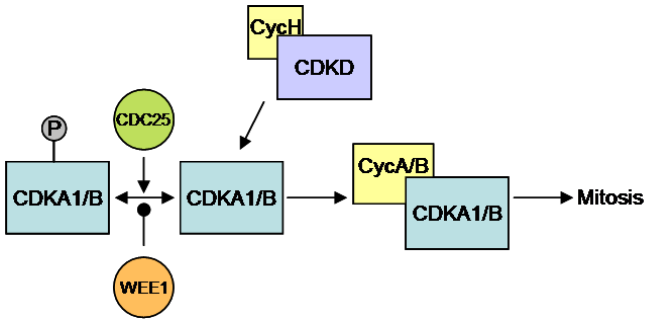

Figure 1.5: The G2/M transition in plants. Arrows indicate activation and dot-ended line indicates inhibition.

Spindle Assembly Checkpoint (SAC)

Another important regulatory checkpoint operates at the metaphase-anaphase transition, the spindle assembly checkpoint (SAC). This is a sophisticated surveillance mechanism that ensures the fidelity of chromosome segregation during mitosis (Musacchio A and Salmon ED, 2007). The SAC monitors the interaction between chromosomes and microtubules (MTs) at specialised chromosomal regions, the kinetochores. In response to unattached kinetochores and to kinetochores lacking tension, the SAC is activated and localised to unattached kinetochores. The SAC transmits a “wait anaphase” signal until all chromosomes achieve bipolar attachment. Specifically, the SAC negatively regulates the ability of CDC20 to activate the APC/C-mediated polyubiquitylation of two key substrates, cyclin B and Securin, thereby preventing their destruction by the 26S proteasome. Securin is a stoichiometric inhibitor of a protease known as separase and the degradation of Securin leads to the activation of the

Separase which cleaves the cohesin complex ring responsible for physically attaching the sister chromatids. The cohesin cleavage is required to execute anaphase. On the other hand, the proteolysis of cyclin B inactivates the mitotic CDK which promotes exit from mitosis (Musacchio A and Salmon ED, 2007).

SAC components were first identified through genetic screens in budding yeast and include the MAD and BUB proteins. In metazoans and yeast, the mitotic checkpoint complex (MCC), which contains the three SAC proteins MAD2, MAD3 (equivalent of BUBR1, for BUB1-related, in higher eukaryotes) and BUB3 together with CDC20, is regarded as the SAC effector (Musacchio A and Salmon ED, 2007). In metazoans, the SAC is an essential pathway, the integrity of which is required to prevent chromosome mis-segregation and cell death (Musacchio A and Salmon ED, 2007). In plants, SAC protein homologs have been identified *in silico* (Menges M *et al*, 2005), but function has been investigated only for MAD2, which localisation to unattached kinetochores has been demonstrated by immunolocalisation (Yu HG *et al*, 1999; Kimbara J *et al*, 2004). Recently Caillaud and collaborators have demonstrated physical interactions between *A. thaliana* BUBR1, BUB3.1 and MAD2 and their dynamics at unattached kinetochores, suggesting that plant BUBR1, BUB3.1 and MAD2 have both the SAC protein functions conserved from yeast to humans. BUB3.1 was also found in the phragmoplast midline during the final step of cell division in plants (Caillaud MC *et al*, 2009).

1.1.3 Coordination between mitosis exit and cytokinesis

The final stages of mitosis are governed by two main regulatory mechanisms: ubiquitination of APC/C substrates and dephosphorylation of CDK substrates (Sullivan M and Morgan DO, 2008).

The APC/C ubiquitinates the mitotic cyclins, the destruction of which inactivates CDKs and allows phosphatases to dephosphorylate the many CDK substrates in the cell. Dephosphorylation of CDK substrates is required for normal chromosome and spindle movements in anaphase, as well as for the subsequent events of telophase: spindle disassembly, reformation of nuclei and decondensation of chromatin. (Sullivan M and Morgan DO, 2008).

In *S. cerevisiae*, exit from mitosis is coupled to cytokinesis by the mitotic exit network (MEN). MEN is a GTPase-activated kinase cascade that promotes the full release from the nucleolus and subsequent activation of the Cdc14 phosphatase during anaphase (Luca FC *et al,* 2001; Stegmeier F and Amon A, 2004). Cdc14 dephosphorylates numerous CDK substrates, including Cdh1, which promotes association with the APC/C, thereby activating it. Although inactivation of the mitotic CDK-cyclin complex is required for mitotic exit, the MEN was shown to be essential for cytokinesis, and in particular for actomyosin ring contraction and septum deposition, also independently of its role in mitotic exit. In fact, when MEN function is abrogated in conditions where mitotic exit is allowed by artificial suppression of mitotic CDK activity cytokinesis does not take place (Shou WY *et al,* 1999; Lippincott J *et al,* 2001; Park CJ *et al,* 2003). The MEN senses the position of the spindle pole bodies (SPBs) and delays CDK inactivation until the SPB enters the bud (Bardin AJ *et al*, 2000) (figure 1.6).

The fission yeast *S. pombe* cells divide by medial fission using an actomyosin based contractile ring. The septum forms in late mitosis at the position of the ring as it closes. In *S. pombe*, orthologues to the MEN components were identified as regulators of septation, a signaling network called SIN for septation initiation network. (Krapp A and Simanis V, 2008) (figure 1.6). Mutants in the SIN failed to form a division septum and rounds of nuclear division occur in the absence of cytoplasmic division, yielding multiple nuclei inside a cell. Similarly to the MEN, the SIN consists in a GTPase-activated kinase cascade. The SIN is also required to keep CDK activity low in order to prevent entry in a next cell cycle. This function is mediated by the phosphatase CLP1p/FLP1p that, similarly to the *S. cerevisiae* CDC14, is regulated by sequestration inside the nucleolus during interphase (Chen CT *et al*, 2008). Most of the SIN/MEN components are bound together in a large protein complex that localizes to the SPB.

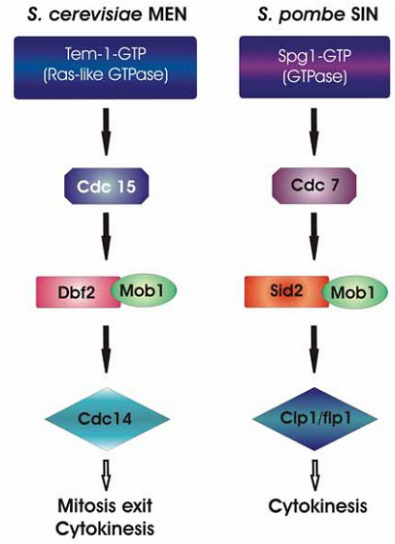

Figure 1.6: Components of the mitotic exit network (MEN) and septation initiation network (SIN) in yeasts. Exit from mitosis and co-ordination with cytokinesis is driven through a GTPase signaling network, where Mob1p is an essential regulator of the localization and activity of Dbf2 and Sid2 protein kinase. The network promotes the inactivation of the mitotic Cdk1-cyclin B complex and drives mitotic exit by leading to the release of the Cdc14p phosphatase from the nucleolus and its subsequent activation during anaphase.

Additional proteins nud1p in *S. cerevisiae* and sid4p/cdc11p in *S. pombe* function as docking proteins for dynamic localization to the SPB (Bardin AJ and Amon A, 2001). In dividing cells, the two spindle poles are not equivalent, with an old and a new SPB. This ensures that the cell machinery is correctly oriented (Simanis V, 2003). So the SIN/MEN cascade appears to function by transducing the septum promoting signal from the spindle pole body to the division site.

In conclusion, the main role of SIN is to control cytokinesis by initiating contraction of the actin ring and synthesis of the septum. Nevertheless experimental results also reveal a link between SIN and mitotic control (Krapp A and Simanis V, 2008). Whereas the primary concern of the MEN is to coordinate mitotic exit with cytokinesis by controlling inactivation of CDK (Bardin AJ and Amon A, 2001).

In *Arabidospsis*, signaling elements (GTPase, kinases), closely related to the core elements of the SIN and MEN pathways were identified, suggesting that a signaling pathway must be used in plants to coordinate

mitotic exit with cytokinesis (Jouannic S *et al*, 2001;Champion A *et al*, 2004a) (Figure 1.7).

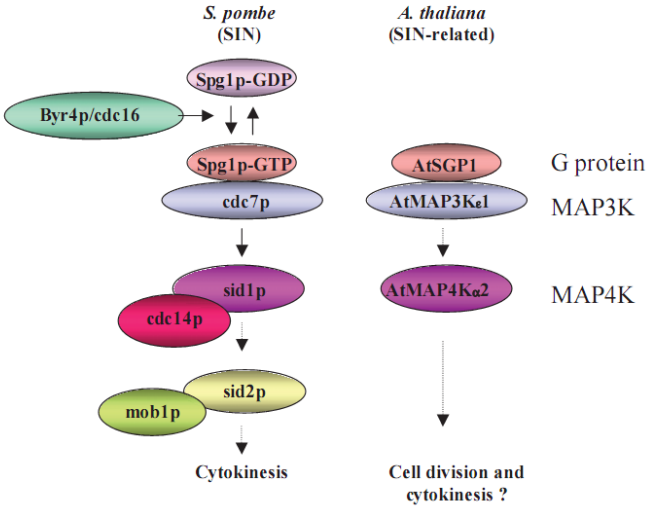

Figure 1.7: Model of the SIN pathway in *S. pombe* and homologues from *A. thaliana*. A parallel is rawn between the fission yeast and plants (Bedhomme *et al,*, 2008).

A. thaliana possess two paralogues for each of the yeast orthologue gene spg1p, cdc7p, sid1p and mob1p. Additionally, about the DBF2p/sid2p for which both budding and fission yeast possess two redundant genes, *A. thaliana* possess two gene clusters.

The plant signaling elements AtSGP, AtMAP3Kepsilon and AtMAP4Kalpha exhibit primary structure different from their *S. pombe* orthologues. For example AtSGP1/2 possess an amino-terminal extension of about 90 amino acid residues, AtMAP4Kalpha1/2 a carboxy-terminal extension of about 180 amino acid residues (Champion A *et al*, 2004a), and AtMAP3Kepsilon1/2 an interstitial extension of about 270 amino acid residues (Jouannic S *et al,* 2001). Through these additional domains, the plant proteins possess new motives, such as perfect consensus sites for phosphorylation by CDK (AtSGP2, AtMAP3Kepsilon1) or putative nuclear localization signal (AtMAP4Kalpha1). These features suggest that the plant SIN-related proteins might perform additional functions compared to their yeast counterparts (Bedhomme M *et al,* 2008).

Overexpression of the plant genes *AtSGP1*, *AtSGP2*, *MAP3Kepsilon1* and *MAP4Kalpha2* in yeast cells has septation effects identical to that of the putative orthologue SIN elements *spg1*, *cdc7* and *sid1* (Champion A et al, 2004a). Complementation tests conducted, in *spg1* mutant yeast cells expressing *AtSGP1* or *AtSGP2*, shows that septation occurs, indicating that the plants sequences are orthologues of the *S. pombe* gene(Champion A *et al*, 2004a). The phenotype of *sid1* mutant yeast cells is fully reverted by a functional complementation with *MAP4Kalpha2*: the septation of the cells is repaired (Champion A *et al*, 2004a). In contrast, in the *S. pombe cdc7* mutant cells, complementation with the plant protein MAP3Kepsilon1 produces a large amount of septum material, which is abnormally localized, demonstrating that MAP3Kepsilon1 interacts only partially with elements of the SIN pathway (Jouannic S *et al*, 2001).

Thus, it was tempting to speculate that the plant SIN-related elements AtSGP1, AtMAP3Kepsilon1 and AtMAP4Kalpha2 constitute the central core of a SIN-related signaling pathway. This pathway could control directly cell division in plant or act as a coordinator of the functions of the others MAP kinase controlling cell plate formation and cytoskeletal components in order to ensure a correct cell plate expansion and cell division (Bedhomme M *et al*, 2008).

1.1.4 Endoreduplication

Endoreduplication is a modified cell cycle missing the G2 and M phases which results in an increase in the ploidy level.
Endoreduplication is a common process in eukaryotes and it is particularly prominent in dicotyledonous plants (Edgar BA and Orr-Weaver TL, 2001). The level of ploidy varies between plant species and tissues (Sugimoto-Shirasu and Roberts, 2003).The endoreduplication process has been postulated to control various biological processes, including cell differentiation, cell expansion, metabolic activity and fitness for survival (Inzé D and De Veylder L, 2006; John PC and Qi R, 2008).

At the molecular level, the onset of endoreduplication coincides with the loss of mitotic regulators and endoreduplicated cells do not re-enter the mitotic cell cycle (Inzé D and De Veylder L, 2006; John PC and Qi R, 2008). Therefore the major component of the switch to endoreduplication is the

prevention of mitosis by the reduction of mitotic CDK/CYCLIN complex activity to a level that fails to initiate mitosis but is able to drive replication of DNA (Boudolf V *et al,* 2004b; Grafi G and Larkins BA, 1995). The decrease in mitotic kinase activity can be trigged by an increased expression of the cyclin-dependent kinase inhibitory proteins *KRP2* (De Veylder L *et al*, 2001a, Jasinski S *et al*, 2002b) and *SIM* (Churchman ML *et al*, 2006). In particular it was shown that the ICK/KRP protein has a dual effect on endoreduplication: in transgenic *Arabidopsis* plants the strong expression of CDK inhibitor blocks endoreduplication in both tissues and trichomes by the inhibition of both mitotic and S-phase CDKs (Zhou Y *et al*, 2002; Schnittger A *et al*, 2003) whereas the weak ICK/KRP-expression results in a ploidy level increase by the inhibition of mitotic CDKs (Verkest A *et al*, 2005; Weinl C *et al*, 2005).

The inhibition of M-phase CDK complexes activity can originate also via dominant negative forms of the mitotic kinase *CDKB1;1* (Boudolf V *et al*, 2004b) or via T-DNA mutants of the A-type cyclin *CYCA2;1* or *CYCA2;3* without apparent effects on the population of proliferating cells (Yoshizumi T *et al,* 2006, Imai KK *et al,* 2006). Moreover, it has been shown that the over-expression of the cyclin *CYCD3;1* blocks endoreduplication (Schnittger A *et al, 2002b*), whereas the loss of its activity enhances endoreduplication (Dewitte W *et al, 2007*).

The inactivation of the CDK activity by phosphorylation mediated by the WEE1 inhibitory kinase has been also postulated to contribute to the endoreduplication process in maize endosperm (Sun Y *et al,* 1999) as well as in the endoreduplicating tissues of developing tomato fruit (Gonzalez N *et al,* 2007).

Finally, also the RB–E2F pathway has a crucial role in the endocycle. Transgenic plants that are deficient in retinoblastoma-related protein (RBR) show an enhanced endoreduplication in the leaves (Wildwater M *et al,* 2005; Park JA *et al*, 2005). Constitutive overexpression of *E2Fa/DPa* or *E2Fb* in plants led to ectopic cell proliferation and *E2Fa/DPa* also enhances/promotes endoreduplication. (De Veylder L *et al*, 2002; Kosugi S and Ohashi Y, 2003; Sozzani R *et al,* 2006). In contrast, overexpression of a stabilized form of E2Fc reduced cell division and increased endoreduplication (del Pozo JC *et al*, 2006). Moreover the overexpression of the E2F target genes (such as CDC6 and CDT1) increased ploidy levels and suggests that endocycle onset through RB–E2F occurs in part through the transcriptional activation of replication proteins (Castellano MM *et al*, 2001,

2004). E2Fe/DEL1 has been implicated in the suppression of endoreduplication in *Arabidopsis* (Vlieghe K *et al, 2005*) through regulation of the anaphase-promoting factor (APC/C) component *CCS52A2* (Lammens T *et al,* 2008). In *Medicago* endoreduplicating cells show an increases of proteolysis of mitotic cyclin through high expression level of CCS52A, whereas the downregulation of CCS52A results in reduced endoreduplication (Cebolla A *et al,*, 1999; Kondorosi E and Kondorosi A, 2004). Analogously in *Arabidopsis* the controlled inactivation of CDKB1;1-CYCA2;3 by APC/$C^{CCS52A1}$ directs the endoreduplication process (Boudolf V *et al*, 2009). Nevertheless, FZR2/CCS52A1 expression is involved not only in endoreduplication but it is also need for a correct cell expansion (Larson-Rabin Z *et al,* 2009).

1.2 Plant Cytokinesis

In plant cells, division planes are determined before mitosis. As shown in Figure 8, two plant-specific-cytoskeletal arrays, the preprophase band (PPB) and the phragmoplast, play central roles in this process. During G2, the PPB, a band of cortical microtubules and actin filaments, marks the future cortical-division site (CDS) as the nucleus migrates into this plane, and it is disassembled at the transition from prophase to prometaphase. After chromosome segregation, cytokinesis is accomplished through the action of the phragmoplast, a microtubule and actin-based structure that play an essential role in the targeted delivery of membrane vesicles to the plane of division (Raven PH, 2002).

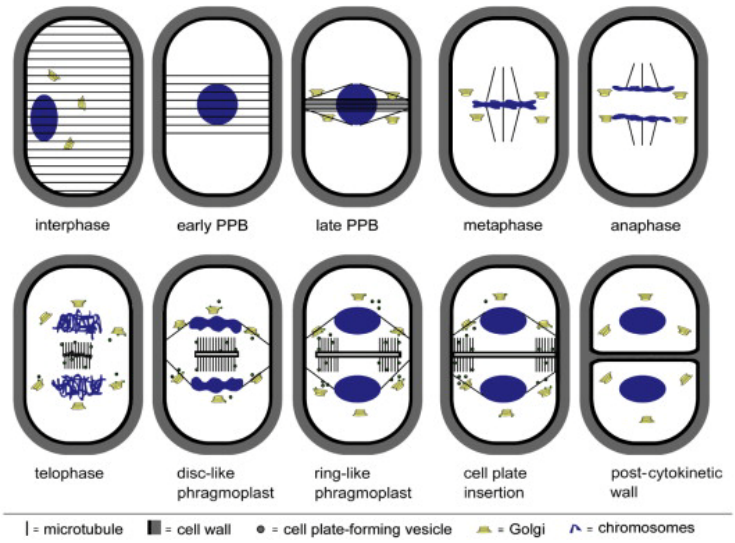

Figure 1.8: Plant somatic cytokinesis model (Van Damme and Geelen, 2008).

Little is known about mechanisms governing the establishment of the division plane or the guidance of expanding phragmoplasts to the CDS during cytokinesis. However, a variety of proteins have recently been

implicated in these processes by their localization at or exclusion from the division plane and/or by functional studies.

Plant cells lack central microtubule nucleators, such as the centrosomes found in animal cells, and their microtubules are nucleated at a variety of surfaces including the nuclear envelope and the cell cortex. During interphase, microtubules are distributed throughout the cell cortex but at preprophase, they become restricted to the future plane of division via selective depolymerization of non-PPB microtubules (Dhonukshe P and Gadella TWJ 2003) and/or selective stabilization of microtubules in the PPB zone (Vos JW *et al*, 2004). So the position at which the new cell plate will become attached to the parental wall is predicted by a cortical PPB encircling the nucleus (Mineyuki Y, 1999). One of the important issues unsolved is what mechanism controls the positioning of the PPB. Nuclear position has been shown in some but not all cases to influence the placement of the PPB (Van Damme D *et al*, 2007; Müller S et al, 2009). Other factors including cell polarity, cell geometry and extracellular cues can also have a role, but the mechanisms by which nuclear position and other factors impact division plane selection are completely unknown (Müller S *et al*, 2009). It is not entirely clear how nuclear migration occurs and it can be an actin- or a microtubule-dependent process (Chytilova E *et al*, 2000, Ketelaar T *et al*, 2002 and Van Bruaene N *et al*, 2003). Once the nucleus is centered in the middle of the PPB, its position is stabilized. A strong connection between the nucleus and the PPB must exist because the nucleus and the PPB remain associated when cells are broken (Tiwari SC *et al*, 1984; Wick SM and Duniec J, 1984).
The PPB has an essential role not only in division plane establishment (Mineyuki Y, 1999, Smith LG 2001, Wright AJ *et al*, 2009) but also in spindle assembly and orientation (Chan J *et al*, 2005; Yoneda A *et al*, 2005). Yoneda and co-workers have observed that most cells with double preprophase bands form multipolar spindles at prophase. However, by metaphase only bipolar spindles were found in these cells, indicating that there are two stages in bipolar spindle formation, one that is controlled by the PPB and a second, a correctional mechanism, at prometaphase (Yoneda A *et al*, 2005).

PPB promotes spindle pole morphogenesis and its positional stability during cell division and consequently a proper spindle orientation

facilitates subsequent orientation of the phragmoplast (Marcus AI *et al*, 2005). The *Arabidopsis* Aurora kinases are possible candidates to translate the positional information from the PPB to the formation of the prophase spindle microtubules. In mammalian cells, AURORA B and C are part of the chromosomal passenger complex and their functions overlap during cytokinesis (Ruchaud S *et al*, 2007). Chromosomal passenger proteins act in a complex that is involved in coordinating the chromosomal and cytoskeletal events of mitosis, including bipolar spindle formation and cytokinesis (Ruchaud S *et al*, 2007). In plants, three Aurora kinase genes (AtAURORA1, AtAURORA2 and AtAURORA3) have been identified in *Arabidopsis*. AtAURORA1 and 2 display characteristics of the mammalian chromosomal passenger proteins (Demidov D *et al*, 2005). In BY-2 cells the AtAURORA1 protein localizes to the PPB, to the prophase spindle microtubules during the bipolar cap formation and eventually, it ends up in the forming cell plate. Moreover, the AtAURORA3 protein localizes to the metaphase plate (Demidov D *et al*, 2005; Kawabe A *et al*, 2005) and over-expression of AtAURORA3 in BY-2 cells caused an alteration of the orientation of cell division, together with a disorganization of the spindle microtubules (Kawabe A *et al*, 2005). Recently it has been shown that in tobacco BY-2 cells plant Aurora kinases have a dual role: correction of aberrant kinetochore-microtubule attachment and dissociation of cohesion during chromosome alignment and segregation (Kurihara D *et al*, 2008).

Although plant cells lack centrosomes, plant proteins related to two animal centrosome proteins are essential for PPB formation. Plant cells lacking *Arabidopsis* FASS (also known as TON2) (Camilleri C *et al*, 2002), or its maize homologues DCD1 and ADD1 (Wright AJ *et al*, 2009), do not make PPBs and divide in abnormal orientations because fail to organize and orient the cortical microtubular array. These proteins are putative regulatory B" subunits of the PP2A phosphatase complex, which are thought to target the complex to particular sub-cellular locations. The *C. elegans* homolog of FASS–DCD1–ADD1, RSA-1, localizes to the centrosome where it interacts with proteins that mediate microtubule outgrowth and stability (Schlaitz AL *et al*, 2007). DCD1 and ADD1 localize to the PPB, suggesting that PP2A-mediated protein dephosphorylation promotes the local assembly and/or stabilization of microtubules in the PPB zone of the cortex (Wright AJ *et al*, 2009). DCD1 and ADD1 persist at the CDS after PPB disassembly, at least

through metaphase, suggesting that these proteins might have other functions in addition to promoting PPB assembly (Wright AJ *et al*, 2009). TON1a and TON1b are candidates to act in the same pathway because they are also required for PPB formation and co-localize with PPBs (Azimzadeh J *et al*, 2008). TON1a and TON1b contain domains related to the human centrosomal proteins FOP and OFD1 and interact with the *Arabidopsis* homologues of another animal centrosomal protein, centrin (Azimzadeh J *et al*, 2008).

Several highly conserved microtubule-binding proteins have also been implicated in stabilizing the PPB microtubules. MOR1 is the plant homologue of animal XMAP215 (Whittington AT *et al*, 2001). *mor1*-1 mutants show defects in PPB formation in *Arabidopsis* roots (Kawamura E *et al*,2006). MOR1 localizes to PPBs and other mitotic microtubule arrays in both *Arabidopsis* and tobacco cells (Kawamura E *et al*, 2006*;* Yasuhara H *et al*, 2002; and Hamada T *et al*, 2004*)*. XMAP215 accelerates both microtubule elongation and shortening *in vitro* (Brouhard GJ *et al*, 2008). Also in *Arabidopsis mor1* mutants, it was showed that MOR1 accelerates microtubule growth and shortening rates (Kawamura E and Wasteney GO,2008).

Another microtubule-binding protein implicated in PPB formation is the *Arabidopsis* homolog of animal CLASP, a regulator of microtubule dynamics (Mimori-Kiyosue Y *et al*, 2005). *Arabidopsis* CLASP binds to microtubule plus ends and to discrete spots along microtubule walls, localizing to PPBs along with other mitotic microtubule arrays (Ambrose JC *et al, 2007* and Kirik V *et al, 2007)*. Demonstrating a role for CLASP in PPB formation and maturation, PPBs in *clasp* mutants tend to be disorganized and fail to narrow as wild-type PPBs do during prophase (Ambrose JC *et al, 2007)*. CLASP also localizes to the sidewalls of microtubules and seems to mediate the MTs interactions with the cell cortex (Ambrose JC and Wasteneys GO, 2008).

MAP65 is a third microtubule-binding protein implicated in PPB formation. Nine different MAP65 proteins have been identified in *Arabidopsis* and some have been shown to localize to the PPB and other mitotic microtubule arrays (Smertenko AP *et al*, 2004*;* Chang HY *et al*, 2005, Gaillard J *et al,* 2008, Smertenko AP *et al,* 2008). MAP65s bundle microtubules by forming cross bridges between overlapping microtubules and thus could potentially

stabilize PPB microtubules via bundling (Smertenko AP *et al*, 2004, Sasabe M and Machida Y, 2006; Gaillard J *et al,* 2008).

The PPB is dismantled at the beginning of prophase. The use of general kinase inhibitors like staurosporine and K252a have led to the conclusion that a kinase is directly involved in PPB degradation (Katsuta J and Shibaoka H, 1992; Nogami A and Mineyuki Y, 1999) and this kinase could be CDKA (Hush J *et al*, 1996 Weingartner M *et al*, 2001).

A variety of observations have indicated that after the PPB is disassembled, some type of 'memory' of its location remains throughout mitosis and cytokinesis, i.e. the PPB leaves behind a landmark that will guide the expanding phragmoplast to the site where the PPB was positioned.

For many years, the only known marker of the CDS during mitosis and cytokinesis was the actin-depleted zone (ADZ) of the cell cortex. When PPB is disassembled, actin filaments are depleted just/only from PPB zone to create an ADZ, which persist until the conclusion of cytokinesis and is flanked by actin enriched regions called "actin twin peaks" (Hoshino H *et al*, *2003*, Sano T *et al*, 2005). The presence of an ADZ during cytokinesis is not crucial for phragmoplast guidance, but the ADZ and/or PPB F-actin has an important role in the establishment of the CDS (Hoshino H *et al*, 2003; Sano T *et al*, 2005). A recent review stresses the point that the ADZ should be viewed as a zone of low actin abundance rather than one with complete loss of the filaments (Panteris E, 2008).

The *Arabidopsis* kinesin KCA1 (PM-associated kinesin) is a second negative marker of the CDS. In tobacco BY-2 cells, GFP–KCA1 localizes to the plasma membrane and cell plate and it is locally depleted at the CDS during mitosis and cytokinesis, creating a KCA1-depleted zone (KDZ) (Vanstraelen M *et al,* 2006). So KDZs and ADZs coincide, but the KDZ seems to form earlier because it is already present in cells with PPBs~~,~~ suggesting that KDZ formation depends on the microtubule PPB (Vanstraelen M *et al,* 2006).

More recently, two proteins, TAN and RanGAP1, have been identified as positive markers of the division plane, continuously localizing there from preprophase through the completion of cytokinesis. An analysis of *tan* mutants of maize demonstrated an important role for TAN in guidance of

expanding phragmoplasts to former PPB sites (Cleary AL and Smith LG, 1998). *Arabidopsis tan* mutants form normal and correctly oriented PPBs, but some phragmoplasts are not guided back to former PPB sites, resulting in misoriented cell divisions (Walker JD *et al*, 2007). *Arabidopsis* TAN–yellow fluorescent protein (YFP) co-localizes with PPBs in preprophase and prophase cells. Initial recruitment of TAN–YFP requires PPB microtubules, but maintenance through prophase of already formed rings does not. TAN–YFP fails to form cortical rings in *fass* mutants, which do not form PPBs. (Walker JD *et al*, 2007).

Arabidopsis RanGAP1 also positively marks the CDS (Xu XM *et al*, 2008). RanGAP1 is a negative regulator of the small GTPase Ran, which functions in nucleocytoplasmic transport during interphase and in several aspects of mitosis in animal cells (Ciciarello M *et al*, 2007). Like TAN, RanGAP1 is recruited to the division plane in a FASS-dependent manner, co-localizing with the PPB and remaining at the CDS throughout mitosis and cytokinesis (Xu XM *et al*, 2008). Unlike TAN, RanGAP1 is also localized elsewhere in dividing cells including the cell plate. Inducible disruption of RanGAP1 and its close relative RanGAP2 results in occasional misoriented and incomplete divisions (Xu XM *et al*, 2008). But it is not yet clear whether RanGAPs are required for PPB assembly or disassembly (a role that would be consistent with known functions for RanGTPases in microtubule nucleation during mitosis in animal cells), or for phragmoplast guidance during cytokinesis (a role that would fit with RanGAP1 localization at the CDS throughout mitosis and cytokinesis).

In *Arabidopsis* the correct localization of TAN and RanGAP1A requires two closely related kinesins, POK1 and POK2 (one of which, POK1, interacts with TAN and RanGAP1 in yeast). Although neither *pok1* nor *pok2* single mutants have obvious defects, in *pok1;pok2* double mutants PPBs are formed but phragmoplasts are not consistently guided back to former PPB sites, so these mutants exhibit a high frequency of misoriented (but complete) cell divisions (Muller S *et al, 2006)*.

POK1 and POK2 in combination are needed for localization of TAN to the PPB and CDS, suggesting that TAN becomes associated with the division plane as cargo of POK1 and POK2 (Walker JD *et al*, 2007). By contrast, RanGAP1 does not require POK1 and/or POK2 for co-localization with PPBs, but does require these kinesins for its maintenance at the CDS after PPB disassembly (Xu XM *et al*, 2008).

At the end of prophase, the PPB is disassembled as the mitotic spindle forms. The spindle forms so that its axis is perpendicular to that of the former PPB and the eventual division plane of the cell. After separation of the chromosomes, the phragmoplast arises between the daughter nuclei and expands centrifugally toward the mother cell cortex directing the deposition of the new cell plate (Jürgens G, 2005). Disassembly of the anaphase spindle and formation of the phragmoplast occur only if mitotic cyclin B1 has been degraded (Weingartner M *et al*, 2004).

The phragmoplast contains two antiparallel arrays of co-aligned microtubules and actin filaments, without overlap of their plus ends in the plane of division (Seguí-Simarro JM *et al*, 2004). The dynamic stability of the microtubular phragmoplast is maintained perpendicular to the plane of division by kinesin-related motor proteins (KRPs) in addition to the microtubule-associated proteins (MAPs).

The *Arabidopsis* genome encodes ~60 (KRPs) (Reddy AS and Day IS, 2001), several of which localize to the phragmoplast and are implicated in its organization (Lloyd C and Hussey P, 2001; Liu B and Lee YRJ, 2001). Bipolar KRPs such as tobacco TKRP125 and carrot DcKRP120–2 are plus-end-directed motors that can slide antiparallel microtubules against each other and might, thus, compensate for microtubule growth at their plus ends (Lloyd C and Hussey P, 2001). However, their precise role in phragmoplast organization needs to be clarified if the plus ends of antiparallel microtubules do not overlap (Seguí-Simarro JM *et al*, 2004). Other *Arabidopsis* KRPs such as PAKRP1 (Lee YR and Liu B, 2000) and its homolog PAKRP1L (Pan R *et al*, 2004) form dimers and might maintain the organization of the phragmoplast. These KRPs localize specifically to the interzonal microtubules at anaphase and to the plus ends of phragmoplast microtubules (Lee YR and Liu B, 2000; Pan R *et al*,2004). The action of bipolar KRPs might be counteracted by minus-end-directed KRPs such as ATK1 (Chen C *et al*, 2002) and the kinesin calmodulin-binding protein (KCBP) (Vos JW *et al*, 2000, Abdel-Ghany SE *et al*, 2005). KCBP appears to be regulated negatively by calcium, the concentration of which rises transiently during cytokinesis (Hepler PK *et al*, 2002; Vos JV *et al*, 2000).

Two orthologous plant-specific, kinesin-related proteins, HINKEL (HIK) in *Arabidopsis* and NPK1-activating kinesin-like protein 1 (NACK1) in tobacco BY-2 cells, have comparable roles in phragmoplast dynamics during

cell-plate expansion (Strompen G *et al*, 2002; Nishihama R *et al*, 2002). NACK1 or NACK2 binds the mitogen-activated protein kinase kinase kinase (MAP3K) NPK1, activating it and the subsequent MAPK cascade. This pathway consists of NACK1 and NACK2, NPK1, the mitogen-activated kinase kinase (MAP2K) NQK1 and the mitogen-activated kinase (MAPK) NRK1, and is designated the NACK–PQR pathway (Nishihama R *et al*, 2001, Nishihama R *et al*, 2002; Soyano T *et al*, 2003). The HINKEL, ANP1 (ANP2 and ANP3) and ANQ1 genes of *Arabidopsis,* which are orthologs of the tobacco NACK1, NPK1, and NQK1 genes, respectively, are also required for cytokinesis, indicating that the NACK–PQR pathway is conserved in *Arabidopsis* (Nishihama R *et al*,2002, Soyano T *et al*, 2003, Krysan PJ et al, 2002, Strompen G *et al*, 2002 and Tanaka H *et al*, 2004). The components of the NACK-PQR pathway localize to the phragmoplast midzone, in which the dynamic processes of MTs proceed. The overexpression of dominant-negative NACK1, NPK1 and NQK1 in BY-2 cells causes defects in cytokinesis that manifest as inhibition of phragmoplast expansion (Nishihama R *et al*, 2001, Nishihama R *et al*,2002, Soyano T *et al*, 2003). In addition, this MAPK cascade is rapidly inactivated by the depolymerization of phragmoplast MTs (Soyano T *et al*, 2003), which indicates that MAPK signaling is regulated by a negative-feedback loop. MAPK signaling is a key regulator of the dynamics of phragmoplast MTs. Recently, it has been revealed that several MAPs, one of which has been identified as NtMAP65-1a, are phosphorylated by NRK1 (MAPK) in tobacco (Sasabe M and Machida Y, 2006). Then, the NACK–PQR pathway appears to play a role in phragmoplast expansion through the functions of MAPs including MAP65.

The phosphorylation of plant MAP65–1 downregulates its microtubule-bundling activity and is required for timely progression through mitosis and cytokinesis (Mao G *et al*, 2005, Sasabe M *et al*, 2006 and Smertenko AP *et al*, 2006). Moreover it is probable that a phosphatase of type 1 or 2A dephosphorylates AtMAP65-1 because okadaic acid, a phosphatase type 1 and 2A inhibitor, reduced the binding of AtMAP65-1 to phragmoplast microtubules (Smertenko AP *et al*, 2006). These results suggest that the phosphorylation/dephosphorylation of MAP65s by MAPK cascade in coordination with a phosphatase regulates the expansion of phragmoplasts by controlling MT turnover. Lastly, plant MAP65s also appear to be phosphorylated by CDKs (Mao G *et al*, 2005, Sasabe L *et al*, 2006).

Phosphorylation of MAP65s by CDKs might regulate the timing of MAP65 localization to phragmoplast MTs (Mao G *et al*, 2005). However, if cyclin B1 is not degraded, MAP65-1 does not accumulate at the midline, which may account for the failure to form a stable phragmoplast (Weingartner M *et al*, 2004). If phosphorylation of AtMAP65-1 abolishes its binding to microtubules during metaphase, then anaphase onset must be accompanied by a phosphatase activity that will dephosphorylate MAP65s, restoring its ability to bind microtubules (Smertenko AP *et al*, 2006).

Membrane fusion is mediated by SNARE proteins that associate into a complex that forms a four-helical bundle between opposing membranes (Jahn R *et al*, 2003). A major component of homotypic fusion during cytokinesis is a plant-specific syntaxin (Qa-SNARE) named KNOLLE (KN), which was identified originally in mutants that accumulate unfused vesicles in the plane of division (Lauber MH *et al*, 1997). KN is expressed during M phase only and localizes to the cell plate (Lauber MH *et al*, 1997). A KN-interacting SNAP25 homolog (Qb,c-SNARE) called SNAP33 also localizes to the cell plate as well as to the plasma membrane (Heese M *et al*, 2001). Inactivating SNAP33 causes only a minor defect in cytokinesis, possibly because of functional redundancy with two other homologs of SNAP25 (Heese M *et al*, 2001). Although a VAMP (R-SNARE) that interacts with KN and SNAP33 to complete the SNARE complex has not been identified, several VAMP7-related proteins might be candidates (Uemura T *et al*, 2004). Alternatively, the cytokinetic SNARE complex might be unusual and consist only of Q-SNAREs. In this case, it might contain the plant-specific Qb-SNARE NPSN11, which also localizes to the cell plate and interacts with KN (Zheng H *et al*, 2002). Inactivation of NPSN11 causes no obvious defects, possibly because of functional redundancy with two closely related proteins(Zheng H *et al*, 2002).
Thus, the syntaxin KN is the only specific component of the cytokinetic SNARE complex that has been identified. Finally, KN interacts with the SM-family protein KEULE (KEU) (Assaad FF *et al*, 2001), which was identified originally in mutants that accumulate unfused vesicles in the plane of division (Waizenegger I *et al*, 2000). Although KEU seems to have an additional role in nonproliferating tissues (Asaad FF *et al*, 2001), the interaction between KEU and KN, and the similarity in mutant phenotypes

indicate that activation of KN by KEU might be necessary for vesicle fusion in cytokinesis.

Proteins connecting vesicle**s** to phragmoplast microtubules have been visualized during endosperm cellularization and pollen cytokinesis (Otegui MS *et al*, 2001; Otegui MS and Staehelin LA, 2004). The kinesin AtPAKRP2 is so far the best candidate motor protein to transport the cell plate-building vesicle**s** to the division plane (Lee SE *et al*, 2001), although genetic data for this are lacking.

Recently, a lot of attention has been given to the origin of the cell plate-building vesicles. Evidence has been presented that both secretory and endocytosed vesicles contribute to cell plate formation (Baluška F *et al*, 2006). Morphological analysis over the past decade has suggested that the primary trafficking of vesicles to the cell plate is via the Golgi biosynthetic pathway: Golgi-derived vesicles are delivered toward the site of secretion via the cytoskeleton and fuse with other vesicles or membrane tubules with the aid of the exocyst complex and other fusion factors (Segui-Simarro JM *et al*, 2004, Reichardt I *et al*, 2007). However, several different groups have assembled evidence suggesting that endocytic trafficking is directed to the cell plate in dividing cells (Baluška F *et al*, 2005; Dhonukshe P *et al*, 2006). Dhonukshe *et al.* (Dhonukshe P *et al*, 2006) estimated that endocytic traffic doubles in dividing cells and found that the same plasma membrane proteins that were shown to be endocytosed by clathrin-dependent endocytosis (Dhonukshe P *et al*, 2007) are also present at very young, developing cell plates (Dhonukshe P *et al*, 2006). Moreover cross-linked cell wall pectins and xyloglucans are found both within endosomes and within the early cell plate (Baluška F *et al*, 2005; Dhonukshe P *et al*, 2006) but not within the Golgi apparatus-derived vesicles (Baluška F *et al*, 2005). An endosomal origin of the new cell wall matrix components explains how it is possible that cytokinetic plant cells manage to generate 1/3 of their surface within a few minutes (Mayer U and Jürgens G, 2004). The view of an inherently endocytic nature of the cell plate (Dhonukshe P *et al*, 2006) is strengthened also by the fact that *bona fide* dynamin, as well as several dynamin-like proteins, plays essential roles in cell plate formation (Verma DPS and Hong Z, 2005; Konopka CA *et al*, 2006). As a consequence, cell plate vesicles fuse together using finger-like protrusions (Seguí-Simarro JM *et al*, 2004; Verma DPS and Hong Z, 2005) which are characteristic features of endosomes but not of Golgi-based secretory vesicles. The endocytic

connection to cell plate was confirmed also in studies revealing the early endosomal nature of the *trans*-Golgi network (TGN) in *Arabidopsis* (Robinson DG *et al*, 2008). The TGN as grand central at the intersection of the secretory and endocytic pathways and the polarization of vesicle transport during cytokinesis could easily explain the presence of both secretory and endocytosed PM proteins at the forming cell plate (Van Damme D *et al*, 2008).
However, endosomes have not been detected in electron tomographs of the early stages of cell plate formation in somatic, endosperm, or pollen cytokinesis (Seguí-Simarro JM *et al*, 2004, Otegui MS *et al*, 2001, Otegui MS and Staehelin LA, 2004; Seguí-Simarro JM and Staehelin LA, 2006). Instead, a cloud of vesicles of the typical diameter of post-Golgi anterograde traffic is seen (Seguí-Simarro JM *et al*, 2004, Otegui MS *et al*, 2001, Otegui and Staehelin, 2004). Prevacuolar compartment/multivesicular bodies (MVBs), a late compartment in the endocytic pathway, were detected around the cell plate by electron microscopy in later stages of its development, but not until the maturation phase when there is substantial membrane recycling, at which point the total number of MVBs in the cell quadruples (Seguí-Simarro JM and Staehelin LA, 2006). By contrast, the number of Golgi stacks doubles before G2 phase and are concentrated around the cell plate in cytokinesis (Seguí-Simarro JM and Staehelin LA, 2006). In conclusion, evidence has been presented that both secretory and endocytosed vesicles contribute to cell plate formation, but it remains a challenge to distinguish between the contributions of secretory and retrograde transport pathways in the process of cell plate formation

The cell plate is guided toward the CDS by unknown mechanism that likely involves actin filaments and microtubule cytoskeleton that connect the phragmoplast and/or the daughter nuclei to the pre-defined-division zone (Van Damme D and Geelen D, 2008; Muller S *et al*, 2009). Evidence for a role of the actin cytoskeleton in guidance of the phragmoplast comes from the observation that in some cells, actin-containing cytoplasmic strands have been seen to link the edges of the phragmoplast to the cortical division zone (Lloyd CV and Traas JA, 1988; Valster AH and Hepler PK, 1997). A role for actin in guidance is also suggested from drug studies: actin drugs treatments do not interfere with cell plate formation, but cause misorientation of cell plates suggesting a role for actin: in guidance of the phragmoplast (Van

Damme D and Geelen D, 2008). However selective application of actin depolymerizing drugs at different cell cycle stages has suggested that the most important contribution of F-actin to the spatial regulation of cytokinesis occurs before cytokinesis (Hoshino H *et al*, 2003; Sano T *et al*, 2005). Other studies report that the actin cytoskeleton plays a role in consolidation of the cell plate rather than guidance (Gunning BE and Wick SM, 1985) and no major role for actin in guidance was found in meristematic cells (Cleary AL, 1995). So far, there is no genetic evidence that supports a role for actin filaments in somatic cytokinesis. *Arabidopsis* encodes eight functional actin genes and highly conserved variants are expressed in the same tissue, hampering the analysis of loss-of-function mutations. Analysis of a dominant negative form of actin (ACT2-2D) that disturbs actin polymerization revealed no effect on cell division patterns in the root meristem (Nishimura T *et al*, 2003). Discordia mutant *dcd1* is disrupted in an actin-dependent process required for the guidance of the phragmoplast in the formation of guard cells, where a strong displacement of the spindle is required (Wright AJ *et al*, 2009).

The role of microtubules in guidance of the phragmoplast was also widely investigated. In living preprophase and prophase cells, microtubules labeled at their plus ends with EB1::GFP grow out from the nuclear surface in all directions, contacting the PPB and other areas of the cortex (Chan EH *et al*, 2005). Pharmacological studies support the view that these microtubules position the nucleus in the plane of the PPB (Muller S *et al*, 2009). Spindle-radiating microtubules are short and few in number during metaphase, but become longer and increasingly abundant as cells progress through anaphase. During telophase, microtubules were observed to connect daughter nuclei to the cortex mainly at the cell poles in *Arabidopsis* tissue culture cells (Chan EH *et al*, 2005), but made frequent contacts at the CDS as well in tobacco BY-2 cells (Dhonukshe P *et al*, 2005).

As discussed earlier, the *Arabidopsis* kinesins POK1 and POK2 in combination are required for the proper localization of two CDS components, but POK1 and/or POK2 could also mediate microtubule-dependent interactions between the dividing nucleus and cell cortex during cytokinesis, perhaps via direct interaction with TAN and/or RanGAP1 (Muller S *et al*, 2006).

So, the cell plate grows outward to the cortex of the cell by peripheral vesicle fusion directed by the depolymerization of the central microtubules and polymerization of microtubules at the periphery (Jurgens G 2005; Van Damme D *et al*, 2008). Classic experiments demonstrated that when the expanding cell plate was forced experimentally to attach to the mother cell surface somewhere other than the CDS, the new cell wall failed to mature normally, suggesting that cell plate interaction with the CDS or adjacent cell wall promotes proper wall maturation (Gunning BE and Wick SM, 1985; Mineyuki Y and Gunning BES, 1990). A recently identified microtubule-associated *Arabidopsis* protein, AIR9, has been implicated in this interaction. In tobacco BY-2 cells, AIR9 is recruited specifically to the CDS when the cell plate comes into contact with it and its subsequent dispersal across the cell plate might promote wall maturation, perhaps via a microtubule-dependent mechanism. (Buschmann H *et al*, 2006).

Recent work has also implicated the *Arabidopsis* protein TPLATE in cell plate attachment (Van Damme D *et al*, 2006). TPLATE contains domains with similarity with adaptins and β-COP coat proteins, which are involved in vesicle formation, and is localized to both the cell plate and the site of fusion. Downregulation of TPLATE in *Arabidopsis* plants and BY2 cells cause the formation of ectopic and incomplete cell walls because of a failure to anchor the expanding cell plate at the correct PM-docking site (Van Damme D *et al*, 2006). The localization pattern and loss-of-function phenotype observed for TPLATE are similar to those reported earlier for RSH, a structural hydroxyproline-rich glycoprotein of *Arabidopsis* (Hall DB and Cannon K, 2002; Cannon MC *et al*, 2008). Thus, TPLATE might facilitate cell plate attachment by promoting the localized deposition of RSH into the cell wall.

When the cell plate reaches the cortex, it fuses with the parental PM and the fusion activates the maturation of the plate to a rigid cell wall, a process that includes closure of the plate fenestrae, removal of excess membrane, and replacement of callose by cellulose (Samuels AL *et al*, 1995; Segui-Simarro JM *et al*, 2004). Immature cell plate are also rich in xyloglucans and devoid of pectins, whereas mature cross walls are rich in pectins and have low xyloglucan content (His I *et al*, 2001). Pectins and xyloglucans are targeted to the cell plate, in contrast callose is synthesized within the cell plate and cellulose microfribils are synthesized by cellulose

synthase embedded in the plasma membrane, which explain why cell plate is flattening and maturation only occur after fusion with the parental membrane and wall (Samuels AL *et al*, 1995, Saxena IM and Brown RM, 2005).
Callose is essential for the completion of plant cytokinesis. Arabidopsis mutants for a putative callose synthase, MASSUE, show a delay in callose deposition at the cell plate and a incomplete cytokinesis (Thiele K *et al*, 2009). The nascent cell plate must not only expand rapidly, but must also be sufficiently fluid to respond to the guidance mechanisms that insert it at specific sites on the parental wall. The issues of speed and fluidity may in part be resolved by the transient presence of callose at the cell plate (Thiele K *et al*, 2009).
Four genes have found to be directly or indirectly implicated in cellulose biosynthesis: KORRIGAN encoding a membrane-bound endo-1,4-β-glucanase, PROCUSTE and RSW1 that encode cellulose synthases, and CYT1 that encodes a mannose-1-phosphate guanylyltransferase (Nickle TC and Meinke DW, 1998; Fagard M *et al*, 2000; Zuo J *et al*, 2000; Lukowitz W *et al*, 2001; Sato S *et al*, 2001; Williamson RE *et al*, 2001). The mutants are characterized by cell-wall stubs and radial swellings, which may also be due to a disruption of weakened walls during cell expansion (Fagard M *et al*, 2000; Söllner R *et al*, 2002).

It is estimated that about 70% of cell plate membrane is removed during the maturation process of the cell plate to a cell wall (Otegui MS *et al*, 2001). The dynamic translocation of the same molecules from one distinct plasma membrane domain to another via recycling endosomes is called transocytosis and in animal epithelial cells is important for the establishment and maintenance of cell polarity (Rodriguez-Boulan E *et al*, 2005, Leibfried A and Bellaïche Y, 2007). Also in plants, membrane removal by endocytosis is necessary to establish the identity of the cell plate membrane as PM and to re-establish polarity within the cell **(**Kleine-Vehn J and Friml J, 2008**)**.
In plants, polarities of tissue and of individual cells are closely connected by the flow of auxin and the cell biological processes depending on vesicle trafficking and polar targeting have an immediate developmental output related to auxin-mediated signalling. Auxin is distributed through tissues by a directional cell-to-cell transport system, termed polar auxin transport, that depends on specific auxin carrier proteins family. Auxin efflux carriers of the

PIN-FORMED (PIN) family show a polar subcellular localization that correlates with and determines the direction of auxin flow through tissues (Kleine-Vehn J and Friml J, 2008).
Sterols seem to play a crucial role in the reestablishment of apical PIN2 polarity (Men S *et al,* 2008).
The PIN2 proteins are initially accumulated at either side of the cell plate during the early phases of cell plate formation. Once the cell plate is constructed, side-specific removal of PIN2 from the basal PM is controlled by the sterol composition of the membrane, as the sterol biosynthesis mutant *cyclopropylsterol isomerase1-1* (*cpi1-1*) fails to remove PIN2 from the basal side of the fused cell plate following cytokinesis (Boutte Y *et al*, 2006; Men S *et al*, 2008). Next to failure in establishing PIN2 polarity, *cpi1-1* shows cytokinesis defects, which are also reported for several other sterol biosynthesis mutants like *fackel* (*fk*), *hydra1* (*hyd1*), and *sterol methyltransferase1/cephalopod* (*smt1/cph*; Schrick K *et al*, 2004; Men S *et al*, 2008). For *fk*, *hyd1*, and *smt1/cph*, these defects are likely caused by defective cell plate maturation, as these mutants contain reduced levels of cellulose, together with ectopic callose and lignin deposits (Schrick K *et al*, 2004).

1.3 Gamete formation and plant reproduction

Higher plants have a complex life cycle that alternate the growth of a diploid sporophytic organism and a highly reduced haploid gametophytic form. Gametophytes and sporophytes differ morphologically and functionally. The major function of diploid sporophyte generation is to produce haploid spores, which are the product of meiosis. Spores undergo cell proliferation and differentiation to develop into gametophytes. The major function of gametophyte generation is to produce haploid gametes. The fusion of egg and sperm gives rise to zygote, which is the beginning of diploid sporophyte generation, thereby completing the life cycle (Raven PH, 2002).

During the angiosperm life cycle, the sporophyte produces two types of spores, microspores and megaspores, that give rise to male gametophytes and female gametophytes, respectively. The angiosperm gametophytes develop within sporophytic tissues that constitute the sexual organs of the flower (Figure 1.9). The male gametophyte, also referred to as the pollen grain or microgametophyte, develops within the stamen's anther and is composed of two sperm cells encased within a vegetative cell (McCormick, 1993, 2004; Borg M *et al*, 2009). The female gametophyte, also referred to as the embryo sac or megagametophyte, develops within the ovule, which is found within the carpel's ovary. The most common female gametophyte form consists of seven cells and four different cell types: three antipodal cells, two synergid cells, one egg cell, and one central cell , as shown in Figure 1.9 (Raven PH, 2002; Drews GN and Yadegari R, 2002; Yadegari R and Drews GN 2004).

The gametophytes play a central role in angiosperm reproductive process. Sexual reproduction is initiated when the male gametophyte is transferred from the anther to the carpel’s stigma. Shortly thereafter, the male gametophyte forms a pollen tube that grows great distances through the carpel’s internal tissues to deliver its two sperm cells to the female gametophyte. One sperm cell fertilizes the egg cell, and the second sperm cell fuses with the central cell. Following fertilization, the egg cell gives rise to the seed’s embryo, which is the beginning of the sporophyte generation, and the central cell’s polar nuclei give rise to the seed’s endosperm, which

surrounds and provides nutrients to the developing embryo. Embryo and endosperm comprise the major portion of the seed (Raven PH, 2002).

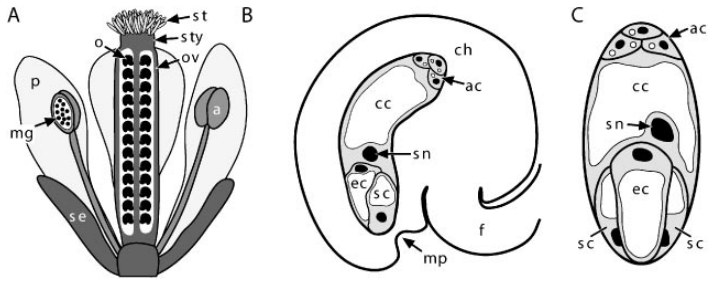

Figure 1.9: Representation of the Arabidopsis reproductive structures. (*A*) Flower. (*B*) Ovule. (*C*) Female gametophyte. The view in (*C*) is perpendicular to that in (*B*). The mature female gametophyte in Arabidopsis is approximately 105 µm in length and approximately 25 µm in width. In (*B*) and (*C*), the gray areas represent cytoplasm, the white areas represent vacuoles, and the black areas represent nuclei. Abbreviations: a, anther; ac, antipodal cells; cc, central cell; ch, chalazal region of the ovule; ec, egg cell; f, funiculus; mg, male gametophyte; mp, micropyle; o, ovule; ov, ovary; p, petal; sc, synergid cell; se, sepal; sn, secondary nucleus; st, stigma; sty, style. (Drews GN and Yadegari R, 2002)

In higher plants, the formation of the gametophyte from the sporophyte is the result of two sequential processes, sporogenesis and gametogenesis. In particular the female gametophyte development occurs over two phases referred to as megasporogenesis and megagametogenesis, whereas the male gametophyte development occurs over two phases referred to as microsporogenesis and microgametogenesis. In the next sessions are described in details the female and male gametophytes, the fertilization process and the mutations that affect this processes.

1.3.1 Development of the female gametophyte

Female gametophyte development occurs over two phases referred to as megasporogenesis and megagametogenesis. More than 15 different patterns of female gametophyte development have been described. The developmental pattern exhibited by *Arabidopsis* is referred to as the Polygonum type because it was first described in *Polygonum divaricatum*.

The Polygonum-type female gametophyte is exhibited by >70% of flowering plants (Haig, 1990; Huang BQ and Russell SD, 1992) and and is thought to be the ancestral type (Huang BQ and Russell SD, 1992).

Megasporogenesis

In *Arabidopsis thaliana*, the megasporogenesis starts with the differentiation of only one hypodermal cell to form archesporial cell in each ovule. This archesporial cell differentiates directly into the megasporocyte or megaspore mother cell (MMC) without undergoing any mitotic division.
The diploid MMC undergoes meiosis and gives rise to four haploid nuclei. Both meiotic divisions are accompanied by cell plate formation, resulting in four one-nucleate megaspores. Subsequently, three megaspores, generally the micropylar-most megaspores, undergo cell death. Thus, this pattern gives rise to a single functional megaspore that contains one meiotic nuclei.

Megagametogenesis

During megagametogenesis, the functional megaspore undergoes three rounds of mitosis without cytokinesis to produce a coenocytic megaspore with eight nuclei. After the third mitosis, simultaneous cytokinesis (cellularization) takes place and yields the mature megagametophyte.

In particular, as shown in Figure 1.10, megaspore undergoes two rounds of mitosis, producing a four-nucleate cell with two nuclei at each pole. During a third mitosis, phragmoplasts and cell plates form between sister and nonsister nuclei, and soon thereafter, the female gametophyte cells become completely surrounded by cell walls. During cellularization, two nuclei, one from each pole (the polar nuclei), migrate toward the center of the developing female gametophyte and fuse together after cellularization is completed. These events result in a seven-celled structure consisting of three antipodal cells, one central cell, two synergid cells, and one egg cell. At maturity, the antipodal cells undergo cell death immediately before fertilization (Figure 1.10) (Drews GN and Yadegari R, 2002).

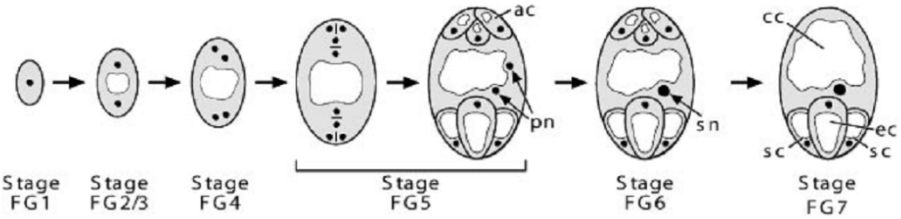

Figure 1.10: Megagametogenesis (female gametophyte, FG, stages) in Arabidopsis. The gray areas represent cytoplasm, the white areas represent vacuoles, and the black areas represent nuclei. In the depiction of early stage FG5, the black lines between the nuclei represent partial cell walls. Abbreviations: ac, antipodal cells; cc, central cell; ec, egg cell; pn, polar nuclei; sc, synergid cell; sn, secondary nucleus (fused polar nuclei) (Drews GN and Yadegari R, 2002).

Throughout development, the female gametophyte exhibits a polarity along its chalazal-micropylar axis. During Polygonum-type megasporogenesis, the chalazal-most megaspore survives and the other three megaspores undergo cell death (Figure 1.11).

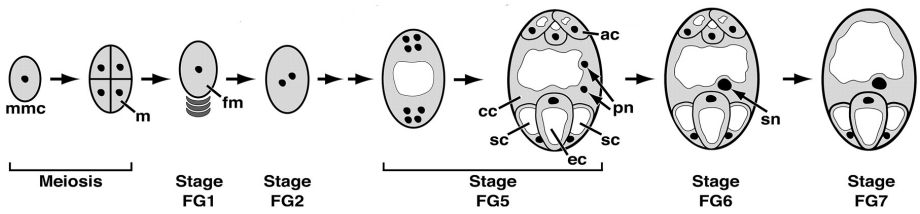

Figure 1.11 Female gametophytic development in Arabidopsis. The gray areas represent cytoplasm, the white areas represent vacuoles, and the black areas represent nuclei. In the depiction of early stage FG5, the black lines between the nuclei represent partial cell walls. Abbreviations: ac, antipodal cells; cc, central cell; ec, egg cell; pn, polar nuclei; sc, synergid cell; sn, secondary nucleus (fused polar nuclei) (Drews GN and Yadegari R, 2002).

During cell differentiation, the nuclei at the micropylar end become specified to develop into the egg cell, the micropylar polar nucleus, and the synergid cells; the chalazal nuclei develop into the three antipodal cells and the chalazal polar nucleus (Figure 1.11). Furthermore, all of the cells within the female gametophyte differentiate into polar structures. In particular, the egg cell's nucleus is located toward the chalazal end and its vacuole occupies the micropylar end; by contrast, the synergid and central cells have the opposite

polarity (Figure 1.11) (Willemse MTM and van Went JL, 1984; Huang BQ and Russell SD, 1992; Christensen CA *et al*, 1997). Thus, the establishment of polarity within the female gametophyte corresponds to the asymmetric development of the surrounding ovule layers, suggesting that female gametophyte polarity is regulated, at least in part, by the surrounding sporophytic tissues. Sporophytic factors that influence female gametophyte development have yet to be identified.

The female gametophyte plays a critical role in every step of the reproductive process. During pollen tube growth, the female gametophyte participates in directing the pollen tube to the ovule (Higashiyama T, 2002; Johnson MA and Preuss D, 2002; Higashiyama T *et al*, 2003). During fertilization, cytoskeletal components within the female gametophyte direct the sperm cells to the egg cell and the central cell (Russell SD, 1992, 1993; Lord EM and Russell SD, 2002). Upon fertilization, female gametophyte–expressed genes control the initiation of seed development (Chaudhury AM *et al*, 2001). During seed development, female gametophyte–expressed gene products play a role in controlling embryo and endosperm development (Ray A 1997; Chaudhury AM and Berger F, 2001).

1.3.2 Development of the male gametophyte

Formation of the male gametophyte in flowering plants takes place within specialized male reproductive organs called stamens and consists of two distinct sequential phases, microsporogenesis and microgametogenesis (Figure 1.12).

Microsporogenesis

In *Arabidopsis thaliana*, the microsporogenesis starts with the differentiation of hypodermal cells to form archesporial cells. The archesporial cells undergo several periclinal divisions to form endothecium, middle layer, and tapetum at the periphery and the pollen mother cells (PMCs) at the center. During microsporogenesis, diploid PMCs undergo meiotic division to produce tetrads of haploid microspores. This stage is completed when distinct unicellular microspores are released from the tetrad

by the activity of a mixture of enzymes secreted by the tapetum, the inner nutritive layer of the stamen (Scott RJ *et al.*, 2004).

Microgametogenesis

During microgametogenesis, the released microspores enlarge and a single large vacuole is produced (Owen HA and Makaroff CA, 1995; Yamamoto Y *et al.*, 2003). This is accompanied by migration of the microspore nucleus to a peripheral position against the cell wall. The microspore then undergoes an asymmetric cell division known as Pollen Mitosis I (PMI). The small germ cell, representing the male germline, is subsequently engulfed within the cytoplasm of the larger vegetative cell to create a novel cell-within-a-cell structure. This engulfing process involves degradation of the hemispherical callose wall that separates the newly formed vegetative and germ cells. The fully engulfed germ cell forms a spindle-like shape that is maintained by a cortical cage of bundled microtubules (Palevitz BA and Cresti M, 1989; Cai G and Cresti M, 2006). The asymmetric division at PMI is essential for the correct cellular patterning of the male gametophyte, since the resulting two daughter cells each harbour a distinct cytoplasm and possess unique gene expression profiles that confer their distinct structures and cell fates (Twell D *et al*, 1998). Induction of symmetrical division at PMI has demonstrated that vegetative cell gene expression is the default developmental pathway and that division asymmetry is critical for correct germ cell differentiation (Eady C *et al*, 1995).

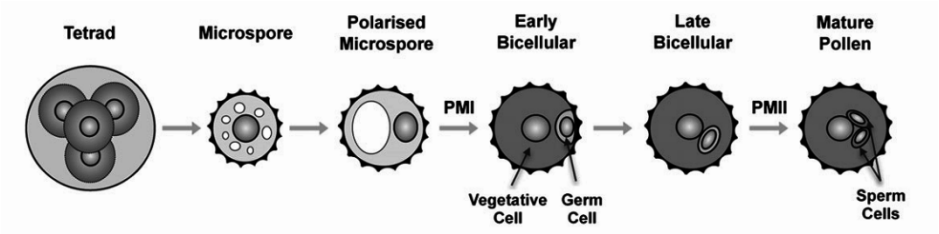

Figure 1.12: Schematic diagram representing the distinct morphological stages of male gametophyte development in Arabidopsis. VC, vegetative cell; GC, germcell; SC, spermcell.

After PMI, the large vegetative cell has dispersed nuclear chromatin and exits the cell cycle in G_1. The vegetative cell nurtures the developing

germ cell and gives rise to the pollen tube following successful pollination. This pollen tube grows through the stylar tissues of the gynoecium to deliver twin sperm cells to the embryo sac. During pollen maturation, the vegetative cell accumulates carbohydrate and/or lipid reserves along with transcripts and proteins that are required for rapid pollen tube growth (Pacini E, 1996). Osmoprotectants, including disaccharides, proline and glycine-betaine, which are thought to protect vital membranes and proteins from damage during dehydration, are also accumulated (Schwacke R *et al*, 1999).

The smaller germ cell has condensed nuclear chromatin and continues through a further round of mitosis, called Pollen Mitosis II (PMII), to produce twin sperm cells. The timing of PMII varies in different plant species. In the case of Arabidopsis, PMII occurs within the anther prior to anthesis and the mature pollen grains are tricellular (Owen HA and Makaroff CA, 1995). This is in contrast to the majority of species that shed their pollen in a bicellular state, such as *Lilium longiflorum*, with PMII taking place in the growing pollen tube. At the end of this division (PMII) the vegetative nucleus and the two sperm cells will assume a specific structural arrangement, the male germ unit (MGU). The MGU is common to both bicellular and tricellular pollen systems and is thought to be important for the co-ordinated delivery of the gametes and sperm cell fusion events (Dumas *et al*, 1998).

1.3.3 Fertilization and induction of seed development

Soon after pollen is transferred from anther to stigma, the male gametophyte forms a pollen tube that grows via a tip-growth process through the carpel's sporophytic tissue to reach the female gametophyte. The pollen tube enters the female gametophyte by growing into one of the two synergid cells through a structurally elaborated portion of the micropylar cell wall known as the filiform apparatus. The penetrated synergid cell undergoes cell death soon before or upon pollen tube arrival. Immediately after arrival, pollen tube growth ceases, an aperture forms at or near the pollen tube tip, and the contents of the pollen tube, including the two sperm cells, are released rapidly into the degenerating synergid cytoplasm.

Synergid cell death may be a prerequisite for normal fertilization in angiosperms. For example, the process of degeneration itself could decrease resistance to both pollen tube penetration and sperm cell migration

during fertilization (Huang BQ and Russell SD, 1992). In addition, synergid degeneration generally is accompanied by cytoskeletal reorganizations that may facilitate male gamete transfer from the pollen tube to the egg and the central cell (Russell SD, 1993; Fu Y *et al*, 2000). In some species, synergid cell death appears to be a final step of the megagametogenesis developmental program. By contrast, in other species, including *Arabidopsis*, synergid degeneration is not an inherent feature of the megagametogenesis process per se, because synergid cell death does not occur if pollination is prevented (Willemse MTM and van Went JL, 1984; Russell SD, 1992; Christensen CA *et al*, 1997; Faure JE, 2001). Two general mechanisms might be responsible for synergid cell death in species such as *Arabidopsis*. First, synergid degeneration may be a purely physical process (e.g., the release of pollen tube contents into the synergid cell may physically rupture the synergid membrane). Second, synergid cell death may be a physiological process induced by pollen. Pollen could induce synergid cell death via a diffusible signal (from the pollen tubes or from female tissue) or through direct contact with the synergid cell (Russell SD, 1992; Higashiyama T, 2002). All of these mechanisms may act in Angiosperms. For example, in some species, the synergid cell appears to be completely intact at the moment of pollen tube discharge, suggesting degeneration via a physical process. By contrast, in other species, synergid cell death appears to be initiated before pollen tube arrival at the female gametophyte, suggesting that a long-range, diffusible signal induces synergid cell death (Russell SD, 1992; Higashiyama T, 2002). In *Arabidopsis*, the status of synergid cell death at the moment of pollen tube arrival at the female gametophyte has not been determined. However, as discussed below, analysis of several female gametophyte mutants suggests that synergid cell death in *Arabidopsis* is an induced, physiological process.

The two sperm cells, released into the degenerating synergid cytoplasm, migrate to the egg and central cells and their plasma membranes fuse with the respective target cell to transport the sperm nuclei for karyogamy (Russell SD, 1992, 1996). Once double fertilization is complete, the embryo and endosperm act in concert to produce the mature seed.

During seed development, the fertilized egg cell develops into the embryo, the fertilized polar nuclei give rise to the endosperm, the sporophytic ovule integuments expand to form the seed coat, and the sporophytic ovary develops into the fruit (Raven PH, 2002). In sexually

reproducing plants, development of seed and fruit occurs only following fertilization, i.e., under normal circumstances, fertilization induces development of embryo, endosperm, seed coat, and fruit in angiosperms. The mechanism by which fertilization induces seed and fruit development remains to be determined (Drews GN and Yadegari R, 2002).

1.3.4 Apomixis

An exception to this mode of reproduction is apomixis, in which the embryo develops without a paternal genome, thought in most apomictic species the endosperm still requires fertilization (Richards AJ, 2003; Spielman M *et al*, 2003; Kultonow AM and Grossnuklaus U, 2003).

Apomixis has been reported in more than 300 taxa distributed over 35 families, and is most common in the Poaceae, Asteraceae and Rosaceae (Richards AJ, 2003; Spielman M *et al*, 2003; Kultonow AM and Grossnuklaus U, 2003). As shown in Figure 1.13, there are three main pathways for apomixis:

- adventitious embriony, the embryos develops directly from the sporophytic cells outside the embryo sac;
- diplospory, the MMC either bypass or fails to complete meiosis, and divides to form an unreduced embryo sac,
- apospory, the unreduced embryo sac develops from sporophytic cell that differentiates from nucellus later than the MMC.

Both diposplory and aposplory involve modifications to female gametophytic development, and are therefore sometimes described as gametophytic apomixis. In both types of gametophitic apomixis, the circumvention of chromosome reduction before embryo sac formation is known as apomeiosis (Richards AJ, 2003; Spielman M *et al*, 2003; Kultonow AM and Grossnuklaus U, 2003).

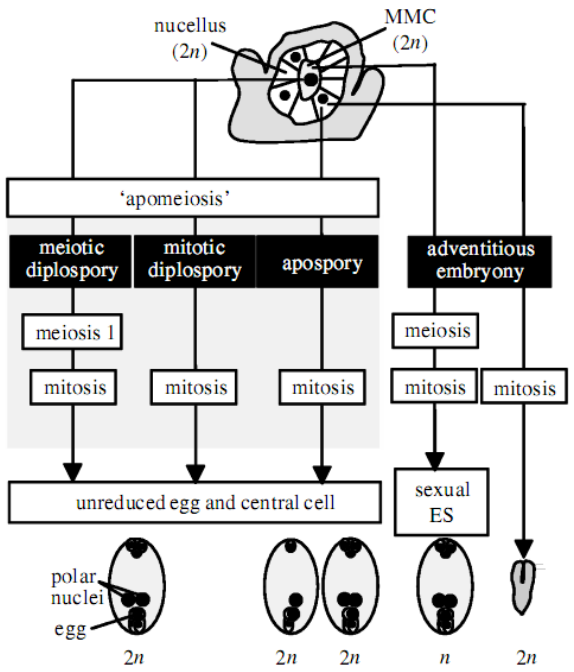

Figure 1.13: The figure show the major apomictic pathways: the adventitious embriony, the diplospory and the apospory (Spielman M *et* al,2003).

It is worth to note that in adventitious embriony a normal sexual embryo sac (n) is also produced, but this sexual embryo may abort because the sexual endosperm is required to nourish the asexual embryo (Kultonow A, 1993). Autonomous endosperm formation is rare in apomicts (autonomous apomicts). Whereas most apomicts require fertilization of the central cell by a male sperm to form functional endosperm (pseudogamous apomicts). Both these types of apomict differ from their sexual counterparts in the relative contribution to the endosperm of the maternal and paternal genomes. In diploid plants, the embryo is typically diploid while the endosperm is triploid, receiving two maternal genomes and one paternal genome (2m:1p). In apomicts, the relative genomic contribution of the maternal and paternal genomes are altered, because the central cell contains unreduced nuclei, whereas the male gametophytes are normal, resulting in a endosperm with 4m:1p ratio. In many plants, genome dosage is critical to seed development. In maize and probably the most other

cereals, normal development of the endosperm requires a maternal to paternal genome ratio of 2m:1p, deviations leads to seed abortion (Bircher JA, 1993). In *Arabidopsis* the situation is less rigid with ratios other 2m:1p affecting seed size and only extreme deviations leading to abortion (Scott RJ *et al*, 1998).

The switch from normal sexual pathway to an apomictic pathway entails at least three major steps:

- circumvention of meiosis
- development of the embryo independently of fertilization
- formation of a functional endosperm.

These requirements are met by a variety of means in apomictic plants, and as a result the mechanisms of apomixis are numerous, although they share common characteristics. First, most if not all apomicts are polyploid. Second, apomixis affects only the female reproductive pathway and male gametes are still produced though meiosis. Third, most apomicts are facultative, in the sense that a proportion of the progeny still results from sexual reproduction. Hence, apomixis does not replace sexuality; rather it coexists with sexual development within the same plant. Finally the apomixis correspond to a short-circuiting of sexual pathways, where gametes formation occurs without meiosis and embryogenesis without fertilization (Richards AJ, 2003; Spielman M *et al*, 2003; Kultonow AM and Grossnuklaus U, 2003). It has been also suggested that apomixis results from deregulation of the sexual programme, however the molecular mechanism controlling apomixis remain undeciphered (Kultonow AM and Grossniklaus U 2003). One hypothesis is that genes controlling apomixis may variant alleles of genes that act during normal sexual development (Mogie M, 1988). Such genes may be revealed by an analysis of model sexual plants. In *Arabidopsis*, the female gametophyte containing the embryo and the formation of functional endosperm egg and associated cells develops from one off our haploid megaspores formed by meiotic divisions of a single cell within the ovule, the megasporocyte. SWI1 is required for sister chromatid cohesion and centromere organization during meiosis. Mutation sin swi1 cause a single equational division in place of normal female meiosis, followed by arrest in further progression (Mercier R et al, 2001; Agashe B et al, 2002). These defects lead to the production of two diploid cells in place off our haploid megaspores, and failure to form a female gametophyte. The dyad allele of

SWI1 causes female specific sterility without affecting pollen development (Siddiqi I et al, 2000).

1.3.5 Mutations that affect female gametophyte development and function

Because of their two-staged life cycle, plants possess two broad classes of mutations: sporophytic mutations and gametophytic mutations. Sporophytic and gametophytic mutations affect different aspects of female gametophyte development.

Sporophytic mutations affect those aspects that occur during the diploid phase, including megaspore mother cell development, meiosis, and control of female gametophyte development by the surrounding sporophytic tissue (e.g., female gametophyte polarity; discussed above). Sporophytic mutations that affect these processes are identified in screens for female-sterile mutants (Chaudhury AM *et al*, 1998; Gasser CS *et al*, 1998; Grossniklaus U and Schneitz K, 1998; Schneitz K *et al*, 1998; Schneitz K, 1999). The sporophytic tissue surrounding the female gametophyte plays a role in controlling megagametogenesis. In facts megagametogenesis is affected in most sporophytic ovule-development mutants (Chaudhury *et al*, 1998; Gasser *et al*, 1998; Grossniklaus and Schneitz, 1998; Schneitz *et al*, 1998; Schneitz, 1999). Mutants defective specifically in the sporophytic control of megagametogenesis exhibiting defects in megagametogenesis but not in the sporophytic parts of the ovule have been reported (Schneitz *et al*, 1997). However, the genes affected in these mutants have not been identified; thus, the molecular basis of these sporophytic effects remains to be determined.

Gametophytic mutations affect those aspects of female gametophyte development that occur after meiosis, including megagametogenesis and functioning of the mature female gametophyte (pollen tube guidance, fertilization, induction of seed development, or maternal control of seed development). Gametophytic mutants typically are identified using two criteria: reduced seed set and segregation distortion. Reduced seed set results because on a plant heterozygous for a female gametophyte mutation,

approximately half of the female gametophytes are mutant and nonfunctional; thus, they fail to undergo normal seed development. Segregation distortion results because gametophytic mutations are transmitted to subsequent generations at reduced frequency (Moore JM *et al*, 1997; Drews GN *et al*, 1998; Drews GN and Yadegari R, 2002; Page DR and Grossniklaus U, 2002). During the last few years, many gametophytic mutants affected in female gametophyte development have been identified and analyzed (Christensen CA *et al*, 2002; Drews GN and Yadegari R, 2002; Grini PE *et al*, 2002; Huck N *et al*, 2003; Rotman N *et al*, 2003). The identification and the analysis of these mutants has allowed a genetic dissection of the female gametophyte developmental pathway (Christensen *et al*, 2002).

It has been suggested that the ovule and female gametophyte play a role in guiding pollen tube growth. During the final stages of pollen tube growth, the pollen tube grows toward an ovule and then up the surface of the funiculus until it enters the micropyle to penetrate the female gametophyte (Higashiyama T, 2002; Johnson MA and Preuss D, 2002; Higashiyama T *et al*, 2003). Many studies suggested that pollen tube guidance is controlled by both sporophytically expressed and gametophytically expressed factors (Palanivelu R and Preuss D, 2000; Franklin-Tong VE, 2002; Higashiyama T, 2002; Johnson MA and Preuss D, 2002; Higashiyama T *et al*, 2003). Signaling molecules involved in the sporophytic phase of pollen tube guidance have been identified. Long-chain lipids from the pollen coat (Fiebig A *et al*, 2000) and hydrophobic stigma (Wolters-Arts M *et al*, 1998) have been implicated in pollen hydration and germination, together with the abundant pollen coat protein GRP17 (Mayfield JA and Preuss D, 2000) and the water channel protein aquaporin (Ikeda S et al., 1997). Polar pollen tube growth is supported by arabinogalactans (Wang H *et al*, 1993; Cheung AY *et al*, 1995, 2000; Wu HM *et al*, 1995, 2000; Sommer-Knudsen J *et al*, 1996, 1998), pectin (Mollet JC *et al*, 2000), the lipid transfer protein SCA (Jauh G *et al*, 1997; Park SY *et al*, 2000), chemocyanin (Kim S *et al*, 2003; Dong J *et al*, 2005), and γ-aminobutyric acid (Palanivelu R *et al*, 2003), providing nutrition and guidance within the transmitting tract of the gynoecium. These sporophytic cues are not sufficient, however, to ensure successful guidance and fertilization. The female gametophyte plays a major role in guiding the pollen tube to the mycropylar opening of the ovule and the subsequent

double fertilization event. Although the female gametophyte (embryo sac) is the source of these final pollen tube guidance cues (Hulskamp K *et al*, 1995; Ray A *et al*, 1997; Couteau F *et al*, 1999; Shimizu KK and Okada K, 2000), little is known about the signal molecules or how these molecules are perceived. The precise origin of some signals was shown by cell ablation experiments in *Torenia fournieri* to be associated with the synergid cells of the embryo sac (Higashiyama T *et al*, 1998, 2003; Higashiyama T, 2002). Further supporting evidence showed that loss of a synergid-expressed *MYB98* gene abolished the ovule's ability to attract pollen tubes (Kasahara RD *et al*, 2005). These data suggested that the synergid cells are essential for pollen tube guidance, although it is not clear the chemical nature of the guidance signal. Calcium has been proposed to be a guidance signal because it can attract pollen tubes in vitro in some species (Mascarenhas JP and Machlis L, 1962, 1964; Reger BJ *et al*, 1992), is present in high concentrations in synergid cells (Chaubal R and Reger BJ, 1990, 1992a, 1992b, 1993; Tian HQ and Russell SD, 1997), and is necessary for pollen tube growth (Pierson ES *et al*, 1994, 1996; Holdaway-Clarke TL *et al*, 1997; Li H *et al*, 1999). Recently it has been reported that LUREs, secreted cysteine-rich polypeptides (CRPs) and subgroup of defensin-like proteins, are attractants derived from the synergid cells in *Torenia fournieri* (Okuda S *et al*, 2009).

Another important aspects affected by female gametophyte–expressed genes is the fertilization. However, because the targets of double fertilization are physically inaccessible, a molecular understanding of the angiosperm fertilization process has been lagging. In Arabidopsis several female gametophyte mutants affected in the fertilization process have been reported, including *gametophytic factor2* (*gfa2*) (Christensen CA *et al*, 2002), *feronia* (*fer*) (Huck N *et al*, 2003), and *sirene* (*srn*) (Rotman N *et al*, 2003). In all three mutants, embryo sac development is normal (*fer* and *srn*) or essentially normal (*gfa2* female gametophytes also have defects in fusion of the polar nuclei) and mutant female gametophytes attract pollen tubes but fail to become fertilized. In response to pollination, *fer* embryo sacs undergo synergid cell death (Huck N *et al*, 2003); by contrast, *gfa2* and *srn* embryo sacs fail to undergo synergid cell death after pollination (Christensen CA *et al*, 2002; Rotman N *et al*, 2003). The *gfa2* and *srn* mutations do not affect megaspore or antipodal cell death, suggesting that synergid cell death has

unique features. The *srn* and *fer* mutants have an additional defect: wild-type pollen tubes enter mutant female gametophytes but fail to cease growth, rupture, and release their contents (Huck N *et al*, 2003; Rotman N *et al*, 2003). The phenotypes of these two mutants clearly indicate that they are defective in some aspect of pollen–embryo sac interaction. In particular, *GFA2* encodes a chaperone that functions in the mitochondrial matrix, and the yeast ortholog is required for mitochondrial function, suggesting that synergid cell death requires functional mitochondria, which also are required for cell death in animals (Christensen CA *et al*, 2002). However molecular aspects of the synergid cell death process remain to be determined. The proteins encoded by *FER* and *SRN* are unknown. Analysis of the *gfa2*, *fer*, and *srn* mutants reveals several aspects of the fertilization process. First, *gfa2* and *srn* do not affect pollen tube attraction and should not prevent the physical rupture of the synergid cell, suggesting that synergid cell death in Arabidopsis is not a purely physical process. Second, *gfa2* and *srn* embryo sacs fail to undergo synergid cell death and yet attract pollen tubes, suggesting that synergid cell death is not required for pollen tube attraction. Third, the abnormal pollen tube behavior within *fer* and *srn* female gametophytes suggests that the presence of synergid cells per se does not ensure normal pollen tube termination and discharge within the female gametophyte (Christensen CA *et al*, 2002; Huck N *et al*, 2003; Rotman N *et al*, 2003).

Lastly, genetic data indicate that the female gametophyte controls also seed development at several levels. First, the female gametophyte controls the initiation of seed development by expressing a set of proteins that repress this process in the absence of fertilization. Second, the female gametophyte contains factors before fertilization that are required for embryo and endosperm development after fertilization. Third, the female gametophyte plays a role in controlling the imprinting of genes required for seed development (Drews GN and Yadegari R, 2002; Yadegari R and Drews GN 2004, Huh JH *et al*, 2008).

Female gametophyte mutations that affect the initiation of seed development include *fertilization-independent endosperm* (*fie*) (Ohad N *et al*, 1996; Chaudhury AM *et al*, 1997), *medea* (*mea*) (Chaudhury AM *et al*, 1997; Grossniklaus et al., 1998), and *fertilization-independent seed2* (*fis2*) (Chaudhury AM *et al*, 1997). In all three mutants, endosperm development

occurs in the absence of fertilization. The FIE, MEA, and FIS2 proteins are related to Polycomb group proteins involved in the heritable silencing of homeotic gene expression in *Drosophila* and mammals (Grossniklaus U *et al*, 1998; Kiyosue T *et al*, 1999; Luo M *et al*, 1999; Ohad N *et al*, 1999). The *FIE*, *MEA*, and *FIS2* genes are expressed in the female gametophyte, primarily in the central cell, before fertilization (Vielle-Calzada JP et al., 1999; Luo M *et al*, 2000; Spillane C *et al*, 2000; Yadegari R *et al*, 2000). Together, these data suggest that the female gametophyte expresses a set of proteins that repress endosperm development before fertilization (Gehring M *et al*, 2004). By this scenario, fertilization could lead to the initiation of endosperm development by inactivating the FIE/FIS2/MEA repressive complex (Ohad N *et al*, 1999). The FIE/FIS2/MEA complex most likely represses endosperm development by preventing the transcription of target genes involved directly in this process. One such target gene, *PHERES1* (*PHE1*), was identified (Kohler C *et al*, 2003). *PHE1* encodes a MADS domain–containing protein. In the wild type, *PHE1* expression occurs in the early stages of endosperm development and is not detected in the female gametophyte before fertilization. *PHE1* also is expressed in preglobular-stage embryos, suggesting a function in both embryo and endosperm. However, *PHE1* expression is upregulated strongly in *mea* and *fie* seeds and is activated inappropriately in *fie* female gametophytes. After fertilization, *mea* seeds exhibit endosperm overproliferation and embryo abortion (Grossniklaus U *et al*, 1998; Kiyosue T *et al*, 1999). These postfertilization defects are attributable, in part, to the activity of *PHE1*, because reduced levels of *PHE1* expression partially rescue the *mea* seed phenotype (Kohler C *et al*, 2003).

After the initiation step, the female gametophyte controls seed development by providing maternal cues required for this process; specifically, the female gametophyte contains maternal factors before fertilization that are required for embryo and endosperm development after fertilization. Female gametophyte–expressed genes required for embryo and endosperm development are referred to as gametophytic maternal-effect genes (Ray A 1997; Drews GN *et al*, 1998; Drews GN and Yadegari R, 2002). The clearest examples of gametophytic maternal-effect mutants described to date include the Arabidopsis *capulet1* (*cap1*) and *cap2* mutants (Grini PE *et al*, 2002), the Arabidopsis *prolifera* (*prl*) mutant (Springer PS *et al*, 1995, 2000), and the maize *maternal effect lethal1* (*mel1*) mutant (Evans

and Kermicle, 2001). With the exception of *prl*, which shows arrest predominantly at the four-nucleate stage of development (Springer PS *et al*, 1995), female gametophytes in these mutants appear normal, whereas the development of the embryo or endosperm or both is affected severely very early during seed development. For example, embryos arising from *cap1* female gametophytes exhibit defects as early as the zygote stage and fail to progress beyond the one-cell proembryo stage (Grini PE *et al*, 2002). The *CAP1*, *CAP2*, and *MEL1* genes have not been isolated; thus, the molecular basis for the gametophytic maternal effects observed in these mutants remains to be determined. However, *PRL* encodes a highly conserved homolog of the DNA replication licensing factor Mcm7, whose accumulation in the female gametophyte appears to be required for normal embryo development (Springer PS *et al*, 1995, 2000).

A third level at which the female gametophyte influences seed development is through the control of genomic imprinting. An important aspect of seed development is the parent-specific expression of genes required during this process. Parent-specific expression is achieved by imprinting the alleles inherited from the male or female gametophyte. For example, with *FIS2* and *MEA*, the maternal alleles are active but the paternal alleles are inactive during endosperm development (Kinoshita T *et al*, 1999; Luo M *et al*, 2000). The imprinting is achieved via epigenetic modification of the maternal or paternal alleles, a process generally associated with the methylation of cytosine residues within and flanking the coding region of the target gene (Jaenisch R and Bird A, 2003). An important question is when the inhibitory modifications are established during development. With paternally imprinted genes, the simplest model is that the inhibitory modifications are established during the male gametophyte lineage. An alternative possibility is that both alleles become modified at some point during the life cycle and the inhibitory modifications are removed during the female gametophyte lineage. The latter possibility appears to be the case with the *MEA* gene. It has been suggested that inhibitory modifications of the maternal allele of *MEA* are removed in the female gametophyte's central cell by a protein called DEMETER (DME). DME is a DNA glycosylase/lyase related to the superfamily of base excision DNA repair proteins (Choi Y *et al*, 2002). *DME* is expressed in the female gametophyte's central cell before fertilization. *MEA* expression is reduced in *dme* female gametophytes and the endosperm of seeds derived from *dme* embryo sacs. DME likely

activates the *MEA* gene by modifying chromatin structure through the removal of inhibitory methylated cytosine residues from its gene-regulatory sequences and counteracting *MEA*'s imprinted/silenced state in the female gametophyte. In effect, the female gametophyte marks the maternal allele of *MEA* and presumably other regulatory genes for continued activity after fertilization. The function of *DME* in derepressing gene expression may not be unique, because mutations in a related gene, termed *REPRESSOR OF SILENCING1*, have been shown to cause transcriptional silencing of a transgene and a homologous endogenous gene (Gong Z *et al*, 2002).

1.3.6 Genes regulating asymmetric division and male germline formation

The asymmetry of division at PMI is critical for the formation of the male germline as induced symmetric division results in two daughter cells that both exhibit vegetative cell fate (Eady C *et al*, 1995). Several mutants have been isolated that demonstrate the importance of genes and cellular processes in patterning male gametophyte development. *sidecar pollen (scp)* is a male-specific mutant affecting microspore division and cellular pattern (Chen Y and McCormick S, 1996). *scp* microspores undergo a symmetrical division, followed by asymmetric division of only one of the daughter cells to produce mature pollen with an additional vegetative cell. *gemini pollen1 (gem1)* affects both male and female transmission and displays a range of microspore division phenotypes including equal, unequal, and partial divisions (Park SK *et al*, 1998). GEM1 is identical to MOR1 (Whittington AT *et al*, 2001). MOR1/GEM1 belongs to the MAP215 family of microtubule-associated proteins and plays a vital role in microspore polarity and cytokinesis by stimulating growth of the interphase spindle and phragmoplast microtubule arrays (Twell D *et al*, 2002). In the *two-in-one* (*tio*) mutant, microspores complete nuclear division but fail to complete cytokinesis resulting in binucleate pollen grains. TIO is the plant homologue of the Ser/Thr protein kinase FUSED (Oh SA *et al*, 2005), which is a key component of the hedgehog-signalling pathway in fruitflies and humans (Lum L and Beachy PA, 2004). TIO localizes to the phragmoplast mid-line where it plays an essential role in centrifugal cell plate expansion. PAKRP1/Kinesin-12A and PAKRP1L/Kinesin-12B are two functionally redundant microtubule

motor kinesins that also localize to the middle region of the phragmoplast (Lee YRJ *et al*, 2007). Dividing microspores of double *kinesin-12A/kinesin-12B* mutants show disrupted microtubule organization and fail to form an antiparallel microtubule array between reforming nuclei. Although nuclear division is not affected in *gem1, tio*, and *kinesin-12A/kinesin-12B* microspores, symmetrical cell divisions and cytokinetic defects disrupt patterning of the male gametophyte and lead to failure of germline formation. These observations strengthen the hypothesis that correct differentiation of the germ cell lineage depends on persistent cell fate determinants being correctly segregated between the unique daughter cells at PMI (Borg M *et al*, 2009).

1.3.7 Genes required for germ cell division

Following asymmetric division at PMI, the vegetative cell exits the cell cycle in G_1 while the germ cell continues through a further round of mitosis at PMII. This differential control of cell cycle progression is important in order to ensure that the germ cell produces the twin sperm cells required for double fertilization. A number of mutants have been described in *Arabidopsis* in which bicellular pollen (a single germ cell within the vegetative cell) is produced due to failure of germ cell division. Analysis of T-DNA insertion mutants in the single A-type cyclin-dependent kinase (*CDKA;1*) in *Arabidopsis* revealed an essential role in germ cell division (Iwakawa H *et al*, 2006; Nowack MK *et al*, 2006). In *cdka;1* mutants, germ cell division fails and DNA synthesis (S) phase of the cell cycle is delayed. This single germ cell, however, is able to fertilize exclusively the egg cell. This preferential fertilization may arise from positional constraints, signalling within the embryo sac, or involve incomplete gamete differentiation (Nowack MK *et al*, 2006). Moreover, the fact that the single germ cell is capable of fertilization demonstrates that key features of germ cell differentiation can be uncoupled from cell division. A very similar phenotype to the *cdka;1* mutant is observed when the *F-box-Like 17* (*FBL17*) gene is disrupted (Kim HJ *et al*, 2008). F-box proteins associate with Skp1 and CUL1 to form SKP1-CUL1-F-box protein (SCF) E3 ubiquitin protein ligase complexes. These SCF complexes are involved in the ubiquitination of proteins targeted for proteasome-dependent degradation (Petroski MD and Deshaies RJ, 2005; Smalle J and

Vierstra RD, 2004). FBL17 is transiently expressed in the male germline after PMI and targets the CDK inhibitors KRP6 and KRP7 for proteasome-dependent degradation, enabling the germ cell to progress through S-phase. Conversely, vegetative cell cycle progression is inhibited since FBL17 is not expressed in the vegetative cell and persistent levels of KRP6/7 continue to inhibit CDKA;1 (Kim HJ *et al*, 2008).

Recent analysis of Chromatin Assembly Factor-1 (CAF-1) pathway mutants (*fas1, fas2, msi1*), indicates that chromatin integrity is also important for germ cell division (Chen Z *et al*, 2008). CAF-1 pathway mutants display a range of phenotypes with some failing to divide at PMI, some failing to divide at PMII, and some successfully dividing to produce tricellular pollen. This indicates that the CAF-1 pathway has a wide role in male gametophyte cell division that could involve direct or epigenetic deregulation involving nucleosome and chromatin reassembly following replication (Chen Z *et al*, 2008). CAF-1 deficient pollen are able to fertilize and the bicellular pollen correctly expresses germ cell-fate markers (Chen Z *et al*, 2008). Interestingly, while *cdka;1* and *fbl17* mutants preferentially fertilize the egg cell, CAF-1 deficient pollen can fertilize either the egg or central cell. The reason for this difference is unclear, but it could relate to incomplete specification of the germ cell in *cdka;1* and *fbl17* mutants, or to some tricellular CAF-1 deficient pollen containing only one functional sperm cell that is able to fertilize either the egg or central cell. A single germ cell phenotype is also present in *duo pollen* (*duo*) mutants. In these mutants, asymmetric microspore division at PMI is completed, however, the resulting germ cell fails to undergo cell division at PMII (Durbarry A *et al*, 2005). Heterozygous *duo1* and *duo2* mutants produce approximately 50% bicellular pollen containing a single germ cell showing complete penetrance of the mutations. *duo2* mutant germ cells enter mitosis but arrest at prometaphase suggesting a specific role for DUO2 in mitotic progression (Durbarry A *et al*, 2005). By contrast, mutant germ cells in *duo1* complete S-phase but fail to enter mitosis (Durbarry A *et al*, 2005). DUO1 encodes a novel R2R3 MYB protein specifically expressed in germline cells (Rotman N *et al*, 2005). Unlike *fbl17*, *cdka;1* and CAF-1 pathway-deficient mutant pollen, *duo1* pollen cannot fertilize. This suggests that, in addition to cell cycle defects, key features of gamete differentiation and function are incomplete in *duo1*. DUO1 may therefore act as a germ cell fate determinant linking cell division and gamete specification. *DUO1* orthologues are present throughout the

angiosperms (Rotman N *et al*, 2005) and recent identification of *DUO1* orthologues in basal angiosperms indicates the evolutionary conservation of this critical male germline specific regulator (Borg M *et al*, 2009).

1.4 Mps One Binder (MOB) family

Normal development of multicellular organisms requires appropriate cell numbers and organ sizes, and it is determined by coordinated cell proliferation, cell growth and programmed cell death (Danial NN and Korsmeyer SJ, 2004; Murray AW, 2004; Sherr CJ, 2004). Disruption or malfunction of these processes can cause diseases, such as cancer. Recent studies in yeasts and higher eukaryotes have led to the identification of a number of proteins and their interactors as key components of specific metabolic pathways that control the coordination between cell proliferation, morphogenesis and programmed cell death (Lai ZC *et al*, 2005).

Members of the NDR (nuclear Dbf2-related) family, a subclass of AGC-type serine/threonine protein kinases, are essential components of pathways that control important cellular processes, such as mitotic exit, cytokinesis, cell proliferation and morphogenesis, and apoptosis (reviewed by Hergovich A *et al*, 2006a; 2008). Human cells express four related NDR kinases, NDR1 (also known as serine/threonine kinase 38 or STK38), NDR2 (or STK38L), LATS1 (large tumour suppressor-1) and LATS2. Members of the NDR family can also be found in *Drosophila melanogaster* (Trc, tricornered, and Warts/Lats], *Caenorhabditis elegans* (SAX-1, sensory axon guidance-1, and LATS), *Saccharomyces cerevisiae* (Dbf2p, Dbf20p and Cbk1p), *Schizosaccharomyces pombe* (Sid2p and Orb6p), and some other fungi, protozoan and plants (Hergovich A *et al*, 2006a).

It has been shown that the function of several NDR kinases is dependent on association with proteins of the MOB (MPS one binder) family. Indeed, genetic, structural, and biochemical studies in yeast, worms, flies, mice and human cells strongly indicate that this interaction is essential for the activation of NDR kinases. In facts, all NDR kinases have an N-terminal regulatory (NTR) domain that contains a number of conserved basic hydrophobic residues and this positively charged cluster mediates the interaction between NDR kinases and a negatively charged area on the surface of the co-activator MOB (Hergovich A *et al*, 2006a; 2008). Interestingly, MOB proteins do not function solely as co-activators, but are

also required for the localization of NDR kinases, thereby bringing this protein into close proximity with its upstream activating kinase. However, the mechanisms that regulate selective translocation of MOBs are unknown (Hergovich A *et al*, 2006a; 2008.

So the MOB family includes a group of cell cycle-associated, non-catalytic proteins highly conserved in eukaryotes. Two distinct MOB proteins, MOB1 and MOB2, are known in yeasts, while an expansion in metazoans gives rise to six in human, four in *D. melanogaster*, and four in *C. elegans* (Mrkobrada M *et al*, 2006). Members of the MOB family are implicated in several important processes that will be described in the next sections.

1.4.1 Cell cycle progression and cytokinesis

The involvement of MOB proteins in cell proliferation was first suggested by Luca FC and Winey M in 1998. They demonstrated that *Mob1* is an essential yeast gene required for the completion of mitosis and maintenance of ploidy, as yeast *Mob1* mutations resulted in a late nuclear division arrest at restrictive temperature. Following studies better elucidated the biological role of this protein in budding and fission yeasts. In *Saccharomyces cerevisiae* MOB1 is an essential regulator of the localization and activity of DBF2 protein kinase, a component of MEN. In *S. pombe* MOB1 is part of the SIN and interacts with SID2, the ortholog of *S. cereviasae* DBF2, regulating its localization and kinase activity. However, yeast MOB1 proteins do not function solely as activators of DBF2/SID2, but are also required for DBF2/SID2 localization to activation sites (Frenz LM *et al*, 2000; Lee SE *et al*, 2001). It has been extensively reported that, in agreement with their functions in mitosis exit and cytokinesis, DBF2/SID2-Mob1 complexes localize to the spindle pole body (SPB) in anaphase and move to the division site in late mitosis (Stegmeier F and Amon A, 2004).

Recent findings suggest also an involvement of MEN-MOB in coordinating chromosome segregation and/or spindle integrity with mitotic exit and cytokinesis via regulation of chromosome passenger proteins. MOB1 has been demonstrated to be essential for maintaining the localization of Aurora, INCENP, and Survivin chromosomal passenger proteins on anaphase spindles and for dissociating Aurora from the kinetochore region (Stoepel J *et al*, 2005). Consistent with these functions,

the MEN protein kinase complex MOB1-DBF2 localizes to mitotic nuclei and partially co-localizes with CDC14 and kinetochore proteins.

Overall the available data in yeast indicates an essential role of MOB1 in cell cycle progression, through the interaction with DBF2/SID2 protein kinases and reveals an essential temporal and spatial regulation of MOB1 activity.

MEN components are conserved through evolution and in particular MOB1 and DBF2-related proteins have been found in both animal (Stavridi ES *et al*, 2003; Ponchon L *et al,* 2004; Devroe E *et al,* 2004) and plant cells (Van Damme D *et al,* 2004; Citterio S *et al,* 2005, 2006), suggesting that their role in controlling cell cycle progression might be conserved in higher eukaryotes.

Some human proteins (for example CDC14) are functionally so conserved that they can compensate for the loss of their yeast counterpart (Vazquez-Novelle MD *et al*, 2005). Human LATS1 and LATS2 have recently been reported to play a role in mitotic exit, suggesting the presence of a MEN conserved pathway in higher eukaryotes (Bothos J *et al*, 2005; Yabuta *et al*, 2007). Nevertheless, any detailed analysis of mammalian MEN/SIN pathways is yet to be undertaken.

The presence of a MEN pathway in higher eukaryotes is also suggested by the study of MOB-like proteins in plants (Citterio S *et al,* 2006). *Medicago sativa* MOB-like proteins are mostly expressed in actively proliferating tissues and their localization pattern shares many features with that of yeast, despite the differences in mitotic entry and progression between the two organisms. The subcellular localization of MsMOB-like proteins is cell cycle-regulated. In alfalfa cells, MOB-like proteins forms grains in the cytoplasm from which fibrillar structures radiate in all directions, preferentially toward the cell mid-plane. These grains could likely correspond to sites in which microtubules are reorganized during cell cycle progression, the yeast SPBs, and barely detectable in G_1 and S cells, whereas become evident in G_2, forming clusters around the nucleus. In mitosis, they preferentially localize at the two opposite cellular poles. Differently from yeast, in alfalfa cells undefined MOB-like fibrillar structures are formed. In addition, during pre-prophase MOB-like proteins mark the inner border of the cell wall in correspondence with the outer parts of the pre-prophase band, and in cytokinesis besides the progressive labeling of the septum, forms

fibrillar structures, that partially co-localize with phragmoplast microtubules and partially form an aster, radiating from the growing septum poles.

An interesting possibility is that MOB-like proteins participate to the orientation of cell plate during cytokinesis, interacting with cytoskeletal structures and conjugating the determination of division site, marked by pre-prophase band before the onset of mitosis, with the septum formation (Citterio S *et al,* 2006). Nevertheless the expression of MsMOB-like could not rescue the lethality of the budding yeast mob1 mutant. This inability can be attributed to several reasons and does not rule out that the two genes do encode functional homologs. It is possible that MsMOB-like does not bind efficiently to budding yeast DBF2, thus explaining the lack of cross-complementation. Importantly, amino acid residues of ScMOB1, such as Thr105, Leu196 and Cys221, that are changed in mob1 mutant alleles and presumably crucial for MOB1 function (Luca FC and Winey M, 1998; Stavridi ES *et al,* 2003), are replaced in a non-conservative way in the MsMOB-like primary sequence, suggesting that in spite of their high degree of similarity the two proteins might have substantially diverged and that the interaction of Mob-like proteins with their effectors may be species-specific.

1.4.2 Coordination of cell death and proliferation

In higher eukaryotes multiple MOB members are involved in multiple pathways. An important pathway is the HIPPO (HPO) or SWH (SALVADOR/WARTS/HIPPO) signaling cascade that balances the relationship between cell size, cell proliferation and cell death in flies.

In *Drosophila*, an unknown extracellular stimulus transmits a signal through FT (Fat), MER (Merlin) and EX (Expanded) to a complex containing HPO, SAV (Salvador), dMOB1 (also termed MATS, Mob as tumour suppressor) and LATS/WARTS, which in turn phosphorylates and inactivates the transcriptional co-activator YKI (Figure 1.14).

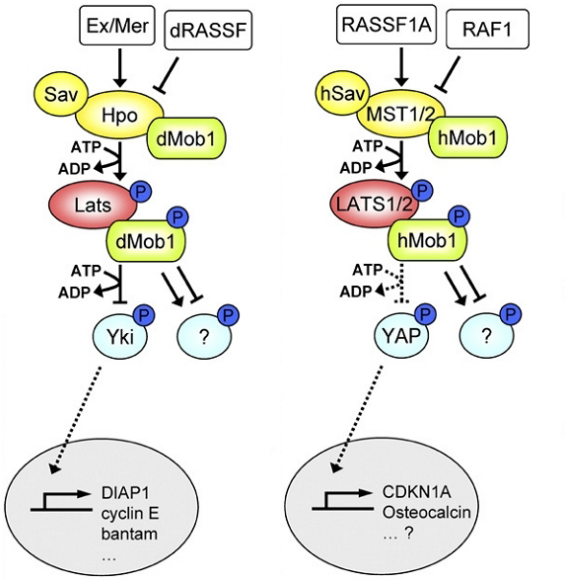

Figure 1.14: The Hippo/SWH pathway in *D. melanogaster* (left) and the putative Hpo pathway in mammals (right) (Hergovich A et al, 2008)

Normally, YKI stimulates the transcription of cyclin E (a driver of cell proliferation), DIAP1 (an inhibitor of apoptosis) and the bantam microRNA. Thus, loss of HPO signaling components results in tissue overgrowth that is associated with increased cell proliferation and decreased cell death. Current evidence strongly indicates that HPO phosphorylates and activates LATS in a SAV/HPO/LATS complex by a SAV-mediated manner (Hergovich A *et al*, 2006; Harvey K and Tapon N, 2007; Pan D, 2007). Recent data further suggest that dMOB1 associates with HPO and is phosphorylated by HPO (Wei X *et al*, 2007 Phosphorylated dMOB1 then has a higher affinity for LATS, and this activated dMOB1/LATS complex (both molecules are phosphorylated by HPO) can target the downstream factor YKI more efficiently (Wei X *et al*, 2007). Interestingly, further evidence suggests that the HPO pathway can be activated by radiation in a p53-dependent manner and that HPO is required for p53-induced cell death in flies (Colombani J *et al*, 2006). Therefore, the HPO pathway might be kept in check by p53 under conditions of aberrant activity in *Drosophila* cells.

All components of the HIPPO pathway are well conserved in mammals and researchers have hypothesized that MST/hSAV/LATS/hMOB1/YAP act as tumour suppressor signaling in

humans as shown in Figure 1.14 (Zeng Q and Hong W, 2008; Chow A *et al*, 2009). hWW45, MST1/2, and LATS1 (the human relatives of *Drosophila* Sav, Hpo, and Lats, respectively) have been reported to form a complex Chan EH *et al,* 2005; Callus BA *et al*, 2006; Guo C *et al*, 2007). Furthermore, MST1/2 phosphorylates and activates human LATS1/2 (Chan EH *et al,* 2005), and hMOB1A associates with human LATS1 (Hergovic A *et al*, 2006b; Bothos J *et al*, 2005), LATS2 (Yabuta *et al*, 2007), as well as MST1 (Wei X *et al*, 2007). However, it remains to be determined whether the proto-oncogene YAP (the human orthologue of Yki - Overholtzer M *et al*, 2006) is regulated by the HPO pathway in human cells, and whether the p53 tumour suppressor protein is required for the activation of MST1/2 by radiation. Of equal importance will be more studies that address whether the tumour suppressor RASSF1A (RAS-association domain family protein 1A) plays an activating or inhibiting role in the HPO/SAV/LATS complex. Current reports suggest that dRASSF functions as an antagonist of the HPO pathway in *Drosophila* (Polesello C *et al*, 2006), while in human cells RASSF1A activates MST2, thereby promoting the phosphorylation of LATS1 (Guo C *et al*, 2007).
The significance of functional conservation is further strengthened by the fact that human MST2, hMOB1A and LATS1 can rescue the tissue-overgrowth phenotype of HPO, dMOB1/MATS and LATS mutants in *D. melanogaster* (Wu *et al,* 2003; Lai *et al,* 2005). Moreover HIPPO components, including MOB1-like proteins are mutated in mammalian tumors (Hergovich A *et al*, 2008; Chow A *et al*, 2009). In facts, in mammalian cells LATS1/2 and NDR1/2 in association with MOB proteins appear to be tumour suppressor proteins, although some evidence indicates that mammalian NDR1/2 kinase could function as proto-oncogenes (Hergovich A *et al,* 2006a, 2008). Many more studies are needed to clarify the role(s) of the mammalian NDR/MOB complex in cancer biology, in order to fully comprehend the potential importance of these kinases in cellular transformation.

Like in animals, also in plants specific cell types undergo programmed cell death (PCD) as part of their developmental and differentiation program (Vaux DL and Korsmeyer SJ, 1999). From embryogenesis to fertilization, cell and tissue death is an integral part of plant development and morphogenesis as well as a response to the

environment (Barlow PW, 1982; Buckner B *et al,* 1988). Even though the cellular deterioration patterns described in plant tissues are in some cases similar to those observed in animal tissues, little is known of the mechanisms that control PCD in plants (Pennell RI and Lamb C, 1997; Allen RT *et al,* 1998; Vaux DL and Korsmeyer SJ, 1999). In angiosperms, PCD occurs late in the degenerative stage of the reproductive phase in both anther and pistil (Wu HM and Cheung AY, 2000). Production of functional male gametes depends largely on the deterioration and death of the anther tapetum, whose main functions appear to be the nurturing of microspores with cortical surface molecules and allowing pollen dispersion at maturity. The pathway of female gametogenesis frequently begins with the death of all but one reduced megaspores, while surrounding nucellar cells degenerate in concert with embryo sac expansion (Reiser L and Fisher RL, 1993; McCormick S, 1993; Barcaccia G *et al,* 2003).

MOB-like protein may be a component of a complex of proteins with multiple functions, not only involved in cytokinesis, cell proliferation and morphogenesis, but also operatively associated with cell death. Database searches revealed that in plants MOB domain (pfam03637) can be combined in complex proteins with elements of the NB-ARC domain (pfam00931), a signaling motif shared by animal cell death gene regulators. Proteins containing a highly conserved MOB-like domain include also receptors for ubiquitination targets (F-Box), Ser/Thr and Tyr kinases as well as CBL (Calcineurin B-Like)-interacting kinases which may be implicated in either cell proliferation or cell death. The possible involvement of MOB-like proteins in PCD is also supported by the analysis of Mob-like expression in alfalfa reproductive tissues. In the ovules during gametogenesis, both transcripts and proteins were mainly visualized in the reduced megaspores undergoing PCD or in the remnants of degenerated megaspores, whereas in the anthers, Mob-like gene products were specifically found at the end of gametogenesis in tapetum cells naturally undergoing PCD to allow pollen grain dispersal (Citterio S *et al,* 2005). Moreover, localization of MOB-domain containing proteins was also documented in alfalfa meristematic tissues of the plant roots. It is known that the root cap consists in living parenchyma cells derived continuously from the apical meristem and programmed to die: as new cells are produced in the interior, those on the root periphery are shed in an orderly manner. Hybridization signals were detected in a thin cell layer of the root apex where meristematic root tip cells

divide and differentiate in root cap. Such finding further supports the concept that MOB proteins can be related to the onset of programmed cell death in plants (Citterio S *et al,* 2006).

1.4.3 Cell polarity and morphogenesis

Co-ordinating asymmetric cell division, and establishment and maintenance of cell polarity are essential processes in growth and differentiation. Polarized morphogenesis is necessary for proper functioning of specific cell types such as neurons, epithelial cells, plant root hairs and pollen tubes and fungal hyphae and its core elements are substantially conserved across eukaryotes. Cell intrinsic polarity is established early during cell division and factors governing cell separation and cell polarity are tightly controlled and co-ordinated. Cells can differentiate by segregating molecules that direct expression of specific sets of genes to one of the two cells produced by division. This generally occurs by direct mechanical movement or asymmetric anchoring of these molecules, which act after division to influence gene expression (Horvitz HR and Herskowitz I, 1992, Roegiers F and Jan YN, 2004).

In the budding yeast transcription regulator ACE2 is asymmetrically partitioned (Nelson B *et al*, 2003). ACE2 moves from uniform distribution to strong accumulation in the daughter nucleus while mother and daughter cells are still connected, and that the enzyme CBK1 (the second NDR kinase in *S. cerevisiae*) directly controls this segregation by attaching phosphate to specific sites on ACE2. CBK1-MOB2 phosphorylation controls ACE2 in two distinct ways: by directly blocking its interaction with nuclear export machinery and by enhancing its activity as a transcription factor (Mazanka E *et al*, 2009).

The components of the budding yeast Regulation of Ace2 Activity and Morphogenesis (RAM) network are conserved in a broad range of eukaryotes and are generally involved in the control of cell architecture (Verde F *et al*, 1998; Gallegos and Bargmann, 2004; He Y *et al*, 2005; Hergovich *et al*, 2006a; Seiler *et al*, 2006). The yeast network comprises six genes: *CBK1*, *KIC1*, *HYM1*, *MOB2*, *TAO3/PAG1*, and *SOG2* (Nelson B *et al,* 2003). Cells lacking any of these proteins exhibit two phenotypes: a failure to degrade the septum between mother and daughter, resulting in large groups

of connected cells, and poor maintenance of polarized growth. The cell separation defect results from the mislocalization of ACE2 to both mother and daughter nuclei, resulting in the loss of Ace2-dependent transcription (Bidlingmaier *et al*, 2001; Colman-Lerner *et al*, 2001). However, defective polarized growth is not attributable to loss of Ace2 function: cells lacking ACE2 can maintain polarized growth (Weiss EL *et al*, 2002). Therefore, the RAM network has separate roles in regulation of ACE2 and control of polarized growth. Recently it has been proposed the CBK1 and the RAM singaling network maintain cell integrity and control polarized growth via regulating Golgi function and exocytosis (Sec2-Sec4 functions) independently of ACE2. Moreover CBK1 kinase activity is essential for bud emergence and cell growth independently of actin polarity establishment (Kurischko C *et al*, 2008).

In *S. pombe*, ORB6 is required for the coordination of morphological changes with cell cycle progression (Hergovich A *et al*, 2006). Furthemore it has been shown that ORB6 regulates cell polarity by spatially restricting the localization and activity of Cdc42 GTPase at the cell cortex (Das M *et al*, 2009). Yeast polarity establishment involves the recruitment and activation of Cdc42 GTPase to a cortical landmark. Cdc42 regulates several polarity processes, including septin assembly and actin cytoskeleton polarization (Park HO and Bi E, 2007). Polarized actin cables are necessary for delivering organelles and secretory vesicles to the cortical sites of polarized growth (for review, see Pruyne *et al*, 2004; Park HO and Bi E, 2007). Defects in yeast polarity establishment, vesicle trafficking, or exocytosis can yield a variety of phenotypes ranging from minor morphological aberrations to severe growth inhibition and cellular lysis.

Interestingly, the existence of the RAM pathway with CBK1 as centerpiece has also been described in other organisms. In the human pathogenic fungus *Cryptococcus neoformans* mutations in RAM components in this organism result in either loss of cell polarity or hyperpolarized growth (Walton FJ *et al,* 2006). Whereas in the worm and fly it has been showed that NDR kinases have important roles concerning morphological changes. In *D. melanogaster*, the NDR Kinase Trc is important for the integrity of outgrowths (such as epidermal hair) and essential for the control of non-redundant innervations (dendritic tiling) and branching of sensory neurons (summarized in more detail in Hergovich A *et al*, 2006a). The homologue of Trc in *C. elegans*, termed SAX-1, is also important for neurite growth and

neurite tiling (Gallegos ME and Bargmann CI, 2004), indicating that NDR kinases play important roles during dendritic outgrowth in invertebrates.

Lastly, the *Drosophila* Trc gene functions altering actin and microtubule organization (He Y *et al,* 2005) and has been placed on the same genetic pathway of RhoA GTPase since loss of Trc function and expression of a dominant negative form of RhoA result in similar non additive phenotypes (He Y *et al,* 2005). Rho GTPases are well known players in cell polarity establishment through the regulation of actin dynamics, however even though it has been suggested that they may be downstream components of NDR kinases in *Drosophila* (He Y *et al,* 2005) and in *C. elegans* (Zallen JA *et al,* 2000), definitive biochemical evidence is needed to fully clarify their exact hierarchical relationships. In fact, it cannot be excluded that the MOB-NDR complex may be a downstream component of Rho GTPases, also considering the similarity of NDR kinases with Rho kinases, the immediate downstream components of Rho signaling.

1.4.4 Centrosome/SPB duplication

In animal cells, the centrosome (comprised of two centrioles surrounded by pericentriolar material) functions as a primary microtubule-organising center (MTOC) and orchestrates chromosome segregation during mitosis and meiosis. The centrosome is duplicated once and only once each cell cycle to organize a bipolar spindle required for successful partitioning of the DNA. Centrosomal abnormalities occur in many cancer types and have been observed in association with genomic instability (Nigg EA, 2002; Sluder G and Nordberg JJ, 2004) . However, little is known about the co-ordination and control of centriole duplication.

Although members of the NDR family have been detected on spindle pole bodies (SPB, the equivalent of centrosomes in lower eukaryotes) and centrosomes, only human NDR1/2 kinases have been attributed a role in centrosome duplication (Hergovich *et al*, 2008). NDR family members might utilize the SPB/centrosome “only” as signaling platforms (Doxsey S *et al*, 2005; Sluder G, 2005). In fact, in yeast, Dbf2p and Sid2p reside on the SPB (summarized in Hergovich A *et al*, 2006a), whereas in mammals, LATS1 and LATS2 have been detected on interphase and mitotic centrosomes (Abe Y

et al, 2006; Hirota T *et al*, 2000; Morisaki T *et al*, 2002; Nishiyama Y *et al*, 1999; Toji S *et al*, 2004). Although LATS1/2 kinases are found on centrosomes, they are not involved directly in the regulation of centrosome duplication in human cells (Hergovich A *et al*, 2007) On the other side, mammalian NDR1/2 kinases have been found on centrosomal structures throughout the entire cell cycle (Hergovich A *et al*, 2007) and interestingly, the first known molecular function of mammalian NDR1/2 kinases has been defined as regulators of centriole duplication(Hergovich A *et al*, 2007). Overexpression of NDR1/2 resulted in centrosome/centriole overduplication, while expression of kinase-dead NDR1/2 or depletion of NDR1/2 by small interfering RNA (siRNA) negatively affected centrosome duplication Nevertheless, mammalian LATS1/2 kinases are most likely not directly involved in the control of centrosome duplication (Hergovich A *et al*, 2007).

Intriguingly, one study has already suggested that Mob1p (the yeast counterpart of human hMOB1A/B proteins) plays a role in SPB duplication (Luca FC and Winey M 1998). Recently Hergovich and co-workers analyzed all six human MOB proteins (hMOBs: hMOB1A, hMOB1B, hMOB2, hMOB3A, hMOB3B, and hMOB3C) for a potential involvement in centrosome duplication. Given that MOB proteins can function together with Ste20-like kinases in yeast, fly, and human cells (Hergovich A *et al*, 2006a), they further expanded their study by addressing all human mammalian serine/threonine Ste20-like kinases (MSTs: MST1, MST2, MST3, MST4, and SOK1) in centrosome duplication. They found that MST1/hMOB1 signaling is required for centrosome duplication and the centriole formation depends on intact MST1/hMOB1/NDR signaling (Hergovich *et al*, 2009).

Moreover MST1-hMOB1 complex plays a role in human centrosome duplication independently of its activity in apoptotic cells supporting the idea that human cells utilize similar signaling systems for the regulation of very different biological processes (e.g., programmed cell death versus centrosome duplication in the case of MST1/hMOB1/NDR signaling) (Hergovich *et al*, 2009).

In conclusion, it is not exclude the possibility that MOBs are also important in other cellular processes. Given the complexity of the interactions it is possible that different isoforms of MOB-like proteins and NDR kinases belong to specific network and/or that the activation of different pathways is organism, tissue and/or cellular context dependent. Moreover, it

is also possible that other MOBs may also interact with proteins other than NDR/LATS to trigger important cellular events. For example, a previous study has demonstrated that hMOB3 was associated with protein phosphatase 2A, which may be involved in the modulation of cytoskeleton and membrane structures (Moreno CS *et al*, 2001). Moreover, the most recent study in Drosophila has also shown that dMOB4 failed to bind to Drosophila LATS and NDR but it was able to organize spindle poles, perhaps through interacting with an unknown binding partner (Trammell MA *et al*, 2008). So, the identification of the binding partners for other MOBs may shed light in determining the functions of these proteins.

2. Aim of the work

As explained in the chapter 1.4 of the Introduction, the MOB (Mps1-One Binder) family is a group of cell cycle-associated, non-catalytic proteins highly conserved in eukaryotes. In last years it has been shown that MOB proteins are required for the activation and the localization of NDR kinases and in particular these protein complexes have been demonstrated to be essential components of some pathways controlling important cellular processes, such as mitotic exit, cytokinesis, cell proliferation and morphogenesis, and apoptosis in higher eukaryotes (reviewed by Hergovich A *et al*, 2006a; 2008).

The MOB family has been mainly studied in animals. In plants MOB-like proteins were studied only in *Medicago sativa* (Citterio *et al*, 2006).

The main aim of this PhD thesis is to gain further insight into the plant MOB protein function using *Arabidopsis thaliana* as a model organism.

In order to achieve these results, first of all a bioinformatic analysis will be performed to reconstruct the evolution of the MOB family, since the evolution of this family genes is poorly understood and a classification and nomenclature is not fully established. Then the role of MOB proteins in plants will be investigated through the development and characterization of transgenic lines with altered expression of *Mob-like* genes. Finally in order to shed light on the pathway involving MOB proteins, experiments to identify the Mob interactors in plant cells will be carried out.

3. Materials and Methods

Plant materials and growth conditions

The Columbia (Col-0) ecotype of *Arabidopsis thaliana* was used in all experiments. Surface-sterilization of wild-type (WT) and silenced lines (4G, 6M and 2F) seeds was performed by immersion in 70% ethanol for 2 min followed by 10 min in a 30% (v/v) commercial bleach solution, and finally 5 rinses with sterile water. Sterile seeds were then plated in Petri dishes (Ø 10 cm) containing 0.5 X Murashige and Skoog (1962) salts, 0.5% (w/v) sucrose (Sigma), 1% agargel (Sigma), and left for 3 days at 4°C in a dark chamber to synchronize their germination. Kanamycin (50 mg/ml) was added for selecting silenced lines and plates were incubated at 22°C in a growth chamber with a 16:8 h light:dark cycle for 10 to 12 days. Seedlings were then transplanted and grown under the same environmental conditions.

For gene expression analysis, leaves and roots were collected 30 days after germination, whereas flowers were collected at S7-8, S12 and S13 developmental stages (Smyth *et al*, 1995), corresponding to flower buds, flower with visible petals and completely opened flowers, respectively. Siliques were collected at stage 1 (half of total elongation) and stage 2 (complete elongation), corresponding to early and late S17 flower developmental stage, respectively (Smyth *et al*, 1995).

Methods for bioinformatic analyses

To perform a complete and exhaustive analysis of the Mob domain distribution and phylogenetic relationship among eukaryotes, the proteomes of 43 complete and ongoing eukaryotic genomes were downloaded from NCBI (ftp://ftp.ncbi.nih.gov/genomes/), ENSEMBL (ftp://ftp.ensembl.org/pub) and DOE Joint Genome Institute (http://genome.jgi-psf.org/euk_home.html)

sites. The hidden Markov model profile for the Mob domain (Pfam code: PF03637) was downloaded from the Pfam site (http://www.sanger.ac.uk/Software/Pfam/) (Sonnhammer *et al*, 1998) and was used to search for similarity against the proteome databases using the HMMER software (Durbin *et al*, 1998). Using a cut-off expectation value equal or 20 lower than e^{-20}, a total of 202 MOB domain containing proteins were identified. Among these, ten sequences were not considered in the subsequent analysis because of low quality problems. As many as 192 Mob domains were extracted from the original sequences and aligned using the progressive alignment algorithm implemented in CLUSTALW (Higgins *et al*, 1992), and the result was edited to remove any ambiguous region. The ProtTest software (http://darwin.uvigo.es/) (Abascal *et al*, 2005) was used to select the most appropriate amino acid substitution models for tree construction. Phylogenetic tree was generated from Mob domain amino acid sequences using the linux version of PhyML (Guindon *et al*, 2003) with JTT+I+G as protein model evolution and with a bootstrap analysis of 200 resampling runs. Proteins belonging to different branches of the phylogenetic tree were aligned using CLUSTALW software and a consensus sequence was extracted for each group. The consensus sequences reflect the most common sequences in the alignment. For a more detailed analysis and visualization of each aligned group, a web logo was created using the web version of WebLogo software (http://weblogo.berkeley.edu).

Construction of the binary vectors and plant transformation

For the production of a RNAi construct specific to the Arabidopsis *MobA* gene (*At5g45550*), a unique 158 bp cDNA fragment was amplified using specific primers designed in the 3'-UTR region: RNAMOB1FOR (5'-CACCTTGAGCAAAAGACCATTTCTG-3') RNAMOB1REV (5'-TACATAGTAAATGTTTAAATTTTACAG-3'). The forward primer contained four additional bases at the 5' end, not present in the native sequence, which were required for directional cloning. The PCR product was cloned into a pENTR™/D-TOPO_vector (Invitrogen, Carlsbad, CA), according to the recommendations of the supplier. This vector was sequenced to confirm the absence of errors and then used for LR recombination using the RNAi Gateway destination vector pK7GWIWG2(II) (Karimi *et al*, 2002) to produce the *Mob-A-RNAi* vector.

For the FLAG-MOBA construct, the *Mob-A* coding region was PCR-amplified from leaf cDNA using specific primers ESMOBAFOR (5'-CACCATGAGTCTCTTTGGGTTAGG-3') and ESMOBAREV (5'-TCAATAAGGTGAAATGATAGATT-3'). The forward primer contained four additional bases at the 5' end, not present in the native sequence, which were required for directional cloning. The PCR product was cloned into a pENTR^{TM}/D-TOPO_vector (Invitrogen, Carlsbad, CA), according to the recommendations of the supplier. This vector was transferred into the destination vector pEarleyGate 202 (Earley K *et al*, 2006) to produce the *FLAG-MOB-A* vector using the same strategy described above. Constructs were transformed into the *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Arabidopsis thaliana plants ecotype Col-0 were transformed by a modified version of the floral dip method (Clough and Bent, 1998), in which the *Agrobacterium* culture was applied directly to flower buds using a pipette.

RNA isolation and analysis

Total RNA was extracted and purified using the SIGMA-Aldrich RTN70 Mammalian RNA extraction Kit, following the manufacturer's instructions.

The samples were treated with RQ1 RNase-free DNase (Promega, Madison, WI) following the manufacturer's instructions. Reverse transcription was performed with the oligo(dT) primer using the ImProm-II Reverse Transcriptase system (Promega) according to the manufacturer's instructions.

Semi-quantitative Real-Time RT-PCR

Semi-quantitative Real-Time RT-PCR analyses were performed using an Mx3000P QPCR (Stratagene, La Jolla, CA) with the SYBR green PCR Master Mix reagent (Applera, Foster City, CA). Specific primers for *Mob-A* were designed in the 3'-UTR region: MOBAFOR (5'-CGCCTCTACAAGAGCTCATA-3') and MOBAREV (5'-ATTGGGGTTTTTAATCTGAA-3'). All Real-Time RT-PCR experiments were

performed with two independent sets of RNA samples. Each analysis was performed in a final volume of 50 µL containing 1 µL of cDNA, 0.2 µM of each primer, and 25 µL of 2XSYBR Green PCR Master Mix according to the manufacturer's instructions. The following thermal cycling profile was used for all PCR reactions: 95°C for 10 min, ~50 cycles of 95°C for 10 s, 57°C for 15 s, 72°C for 15 s. All quantifications were normalized to actin cDNA fragments amplified in the same conditions by primers ACT5RT (5'-CTCATGCCATCCTCCGTCTT-3') and ACT3RT (5'-CAATTTCCCGCTCTGCTGTT-3'). Each Real-Time assay was tested with a dissociation protocol to ensure that each amplicon was a single product. Negative template controls were run in these experiments, and no signal was observed (data not shown). The amplification efficiency was calculated from raw data using the LingRegPCR software (Ramakers *et al*, 2003). The relative expression ratio value was calculated for the first developmental stage of flowers according to the Pfaffl equation (Pfaffl, 2001).

Root and stem histological analysis

Transverse vibratome sections of 80 µm at the basal internode of stems of wild-type Col-0 and *AtMob-A*-RNAi lines were performed on plants five weeks after germination. Portions of the stem were excised and immediately embedded in 4% low-melting agarose before sectioning on a Microm HM650V vibratome. Stainings were performed for 5 min with toluidine blue 0.05%, phloroglucinol/HCl 2% and aniline blue 0.1%, in different preparations, and immediately viewed under a Zeiss Axiovert 200 microscope.

For what concerns the primary root, the effect of *Mob-A* silencing was investigated in seedlings after 48 h from imbibition. Image analysis (Image-Pro Express 6.0, Media Cybernetics) was applied to measure the length, diameter and area of the different primary root regions and the mean area of the meristematic cells.

Confocal laser scanning microscopy

Three day-old seedlings were fixed overnight at 4°C in 4% (w/v) paraformaldehyde in Tris buffer (10 mM Tris, 10 mM NaEDTA and 100 mM

NaCl, pH 7.4) and washed thoroughly in the cold buffer for 30 min. After fixation seedlings were stained with 1:100 SybrGreen (SYBR Green I nucleic acid gel stain 10,000X concentrate in DMSO, Molecular Probes). LSCFM images were obtained by using Leica microscope Mod.TCS-SP2 (Leica Microsystem). Image processing was performed with Leica Confocal Software (LCS.EXE) and Adobe Photoshop Software.

Cyto-histological observations of sporogenesis and gametogenesis by light microscopy

Ovules were dissected on a slide under a Zeiss Discovery V20 (Carl Zeiss MicroImaging, Germany) stereomicroscope and cleared with chloral hydrate prior to observation. Alternatively, whole inflorescences were stain-cleared following the protocol reported by Stelly *et al*. (1984) with some minor modifications. Briefly, the tissues were fixed in FAA (3.7% formalin, 5% acetic acid, 50% ethanol) overnight at 4°C, and then progressively rehydrated for subsequent staining steps. Samples were stained with pure Mayer's hemalum for 30 min, placed in acetic acid 2% for 30 min, then dehydrated in 25%, 50% 70%, 95% and 100% progressive ethanol solutions for 20 min every time. After dehydration, samples were cleared with mixtures of pure ethanol and methyl salicylate (2:1 and 1:2) and twice with pure methyl salicylate (10 min per step). Ovules were dissected on a slide under a Zeiss Discovery V20 (Carl Zeiss MicroImaging, Germany) stereomicroscope, and then mounted with one drop of pure methyl salicylate and coverslipped. Cytohistological observations were performed on a Zeiss Axioplan (Carl Zeiss MicroImaging, Germany) microscope under DIC optics, under 100X magnification.

Flow-cytometry

Nuclear suspensions were obtained from Arabidopsis plants at different developmental stages following the protocol of Galbraith *et al* (1983). Chicken erythrocytes were added as reference internal standard to each sample. The mixed nuclei were stained with the DNA binding fluorochrome DAPI at a final concentration of 5.5 µM. The fluorescence intensity of the nuclei was measured with an arc lamp-based flow cytometer

(Bryte-HS; Bio-Rad, Hercules, CA). At least three different independent experiments were carried out.

Statistics

Data were statistically analysed by Statgraphics plus program for Windows (Manugistic, Maryland USA): Student's t-test, for two-sample comparison, or ANOVA and Duncan test, for multiple sample comparison, were applied when normality and homogeneity of variance were satisfied. Data, which did not conform to the assumptions, were alternatively transformed into logarithms or were analysed by Mann-Withney or Kruskal-Wallis non-parametric procedures (for two or multiple sample comparison, respectively).

Protein Cross-linking

Three day-old seedlings were harvested, washed four times in cold, sterile water for 1 min, and vacuum-infiltrated in 1% (w/v) paraformaldehyde (PFA) in PBS (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7,5) for 15 min. Fixation was stopped using 125 mM glycine under vacuum for 5 min. Fixed seedlings were washed four times in cold and sterile water for 1 min, dried, ground in liquid nitrogen to a fine powder and stored at -80°C. In the absence of (PFA) fixation, seedlings were harvested and directly frozen in liquid nitrogen.

Electrophoresis and Western blot analysis

Total protein extracts were obtained from whole 3-day-old seedlings of *Arabidopsis thaliana*. 4g of plant material was frozen in liquid nitrogen, transferred to a pre-chilled mortar, ground into a fine powder and homogenized in two volumes of extraction buffer with 250 mM sucrose, 25mM BIS-TRIS/MES pH 7.8, 0.02% SDS, 0.02% casein and 1× protease inhibitor cocktail for plant (Sigma, P9599). The homogenate was centrifuged at 1,000 × g for 10 min at 4°C. The pellet was resuspended in1 volume of the same buffer and then centrifuged at 1,000 × g for 10 min at 4°C. The two

supernatants were collected and centrifuged at 12,000 × g for 10 min at 4°C. The protein concentration of the supernatant was measured with the Bradford assay with a dye reagent from Bio-Rad (Hercules). Proteins were separated electrophoretically (SDS-PAGE). Samples were mixed 1:1 (v:v) with 2× sample buffer (60 mM Tris–HCl pH 6.8, 1 mM β-mercaptoethanol, 20% glycerol, 3% SDS, 0.002% bromophenol blue) and then boiled 5 min. Paraformaldehyde cross-linked samples were heated for 10 min at 65°C in sample buffer and the reversal of formaldehyde cross-links was obtained by boiling 20 min in sample buffer. 30 µg of proteins were loaded on 12% poliacrylamide gels and either stained with colloidal Coomassie Blue or transferred to PVDF membranes (Amersham Bioscience) for subsequent Western blot analysis. For MOB-like proteins immunodetection, membranes were incubated 1 hour with 5% non-fat dry milk in TBS-T buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% Tween20) and then with 1 µg / ml anti-MOB primary antibody (Citterio *et al*, 2006) in TBS-T buffer. After 1 h, they were washed in TBS-T and probed with horseradish peroxidase conjugated secondary antibody (Amersham Bioscience). Bound antibodies were visualized using a chemiluminescent detection system ECL (Amersham Bioscience).

Immunoprecipitation

Immunoprecipitation experiments were performed with the Primary Immunoprecipitation kit (PIERCE, 45335) following the manufacturer's recommendations with minor modification in order to improve the binding of the antibody to the resin. Briefly, after the coupling of the antibody to the AminoLink plus coupling resin (PIERCE, 20501), a washing step with the IgG Eluition buffer (PIERCE, 21004) was performed in order to completely remove the uncoupled antibody. The antibody against MsMOB-like protein (Citterio *et al*, 2006) was used. Total protein extraction was performed as described above. Before adding the sample to the antibody-coupled resin, the crude lysates were spun at 20,000x*g* for 10 min at 4°C. Eluted fractions were collected and concentrated with an AmiconUltra – 10000 MWCO centrifugal filter device (Millipore, Nepean, ON, Canada). The sample was resuspended in 2× sample buffer (60 mM Tris–HCl pH 6.8, 1 mM β-mercaptoethanol, 20% glycerol, 3% SDS, 0.002% bromophenol blue) and

then loaded on 12% poliacrylamide gels. After electrophoresis proteins were stained with colloidal Coomassie Blue or transferred to PVDF membranes (Amersham Bioscience) for subsequent Western blot analysis.

Expression and purification of recombinant proteins

The pENTR^{TM}/D-TOPO_vector containing the *Mob-A* coding region was used for LR recombination using the Gateway destination vector pET160-GW/CAT (Invitrogen, Carlsbad, CA) to produce the HIS-MOB-A. Six histidine-tagged MOB-A protein (HIS-MOB-A) was expressed in the BL21 Star (DE3) One Shot Cell (Invitrogen, Carlsbad, CA) strain of *E. coli* following the manufacturer's instructions. Cells were grown to an OD600 of 0,8 and induced for 3 h at 37 °C with 1mM IPTG. Cells were harvested by centrifugation and lysed by sonication in binding buffer (50 mM NaH_2PO_4 , pH 8.0; 0.5 M NaCl) with proteases inhibitors The cell lysate was centrifuged 15 minutes at 3,000xg to rid it of cell debris and whole cells. Recombinant HIS-MOB-A protein was precipitated from the clarified cell extract by incubation with 1.5 ml of a 1:1 slurry of Ni-agarose (Invitrogen, Carlsbad, CA). The purification was carried out following the manufacturer's instructions and bound protein was eluted with a final concentration of 300mM of imidazole. Estimates of protein concentration were obtained by comparison to known amounts of bovine serum albumin on an SDS-PAGE. The purified protein was sent to PRIMM company (Milan, Italy) for the production of an antibody against MOB-A. The purified antibody was tested on total protein extracts from *Arabidopsis thaliana* leaves.

4. Results

4.1 Results – part I

Mob proteins are a small family of highly conserved proteins, found in all eukaryotes, approximately 210 to 240 amino acid residues in length. The evolution of MOB family genes is poorly understood and a classification and nomenclature of *Mob* genes is not fully established. Here we propose some insight into the evolutionary dynamics of this family and a system of classification based on a phylogenetic analysis of *Mob* genes in all complete and ongoing eukaryotic genome sequences.

4.1.1 Primary structure characteristics and classification of family members

Mrkobrada S *et al* (2006) proposed a classification based on the alignment of the core domain of MOB proteins from yeast to human, identifying three distinct groups defined by similarity between the conserved N-terminal region. On the basis of the distribution of ScMOB1 and ScMOB2 members within the clusters, they referred to the groups as MOB1-like, MOB2-like and MOB3-like. The MOB1-like group contains two subgroups (A and B): MOB1A contains the ortholog of ScMOB1 in fungal species and single proteins from *H. sapiens* and *D. melanogaster*, whereas the MOB1B group contains one or more Mob proteins from *H. sapiens*, *D. melanogaster*, *D. rerio, C. elegans* and *X. laevis*. The MOB2-like cluster contains two groups, MOB2A, consisting of the fungal ortholog ScMOB2 and a second group, MOB2B, containing metazoan genes. Finally, the MOB3-like group is the most divergent one and contains a single protein from each metazoan organism analyzed. Moreover, two mammalian homologs to yeast *Mob* genes have been described, the mammalian *Mob homolog* (MMh), that has high similarity with *S. cerevisiae Mob2* genes, and *phocein* or *mammalian Mob1* distantly related to *Mob1* and *Mob2* (Hennebold JD *et al,* 2000; Baillat

G *et al*, 2001; 2002; Moreno CS *et al*, 2001). Stavridi ES *et al* (2003) proposed that *MMh* be referred to as *Mob2* and that *phocein/mMob1* be referred to only as *phocein*.

To classify the MOB domain into related groups of sequences, a phylogenetic analysis was performed, by searching MOB domain hidden Markov model profile on all complete or ongoing available eukaryotic genomes. Figure 4.1 shows the phylogenetic tree for 192 *Mob* genes. The results highlight that MOB domain is clearly separated into five classes: MOB1, MOB2, MOB3, MOB4 and MOBp with high bootstrap support. Among the different classes, MOB3 is the most divergent clade.

The numbers of genes in class *Mob1*, *Mob2*, *Mob3*, *Mob4* and *Mobp* are 47, 28, 31, 57 and 14 respectively. Some of the *C. elegans* and *C. briggsae*, and *S. cerevisiae*, *S. pombe* and Protist MOB related proteins clustered outside these groups and they will be treated separately. *Mob4* class can be subdivided into two phylogenetic clades, corresponding to invertebrate (9 genes) and vertebrate *Mob-like* genes (48). Moreover, vertebrate *Mob-like* genes can be further subdivided into other two subgroups, *Mob4a*, containing 19 genes, and *Mob4b* with 29 MOB-like proteins.
The average amino acid identity within MOB classes is 92% (MOB1), 54% (MOB 2), 86% (MOB 3), 70% (MOB 4), 86% (MOB a), 84% (MOB 4b) and 78% (MOBp).

The results partially support the previous classification by Mrkobrada S *et al* (2006). The main differences are probably due to the higher number of genes analyzed in this study and concern the *Mob1* class which was previously subdivided into two groups, *Mob1A* and *Mob1B*. Our analysis allowed us to recognize a *Mob1* class that corresponds to *Mob1A* group and a *Mob4* class that contains the previously established *Mob1B* group (see Mrkobrada S *et al*, 2006). Moreover, both *Mob4a* and *Mob4b* groups proved to contain *Mob-like* genes previously annotated as part of the *Mob1B* group (Mrkobrada S *et al*, 2006).

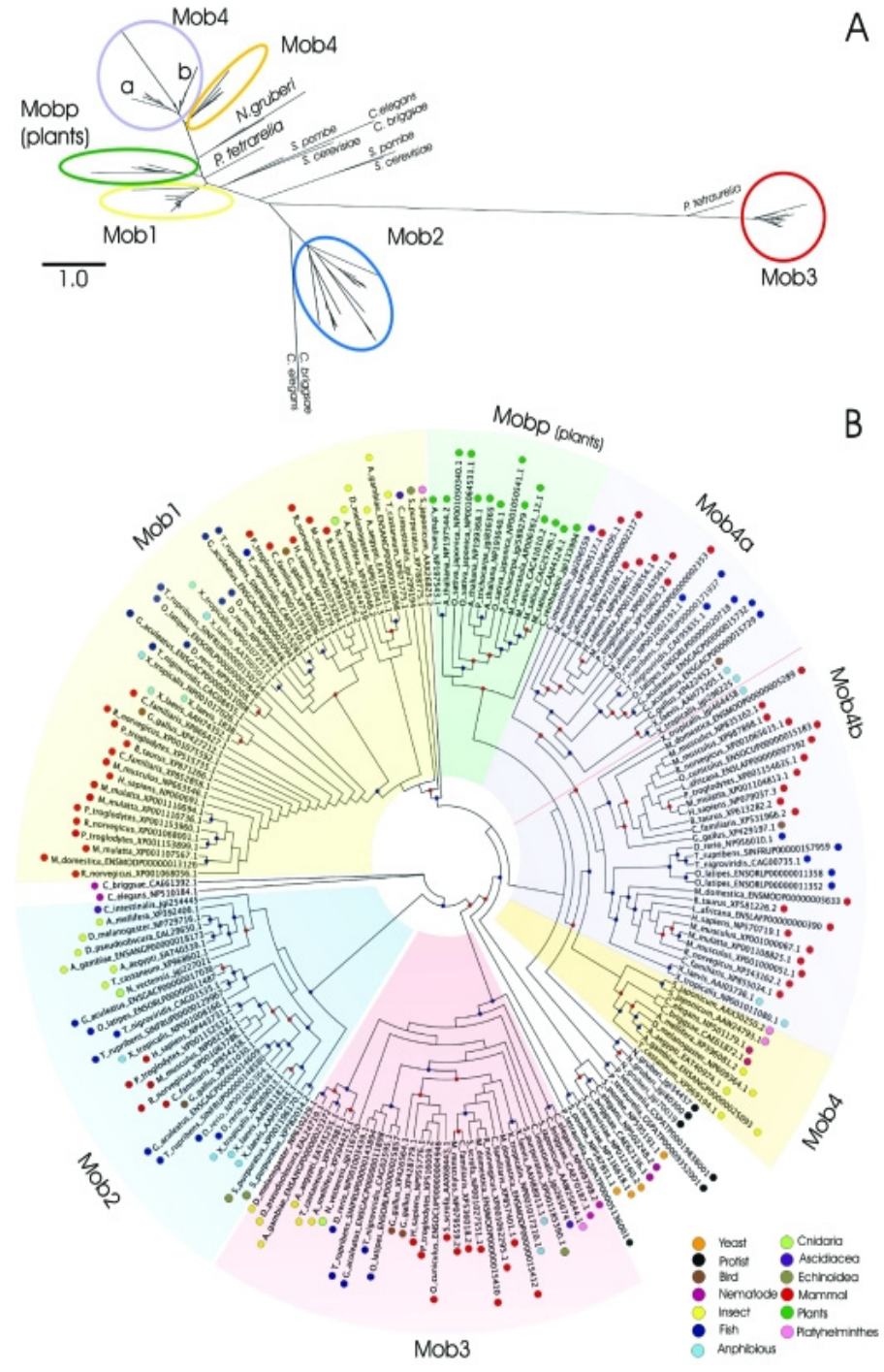
A
Mob4
Mob4
a
b
Mobp
(plants)
N.gruberi
P. tetraurelia
S. pombe
S. cerevisiae
C.elegans
C. briggsae
S. pombe
S. cerevisiae
P. tetraurelia
Mob1
Mob2
Mob3
1.0
C. elegans
C. briggsae
B
Mobp (plants)
Mob1
Mob4a
Mob4b
Mob4
Mob2
Mob3
Yeast
Protist
Bird
Nematode
Insect
Fish
Amphibious
Cnidaria
Ascidiacea
Echinoidea
Mammal
Plants
Platyhelminthes

Figure 4.1 Phylogenetic tree of the 192 MOB domain proteins. MOB groups identified with the phylogenetic analysis are shown and highlighted in different colors. The Panel A shows a maximum likelihood MOB protein phylogenetic tree (the scale represents the number of amino acid substitution per site). The Panel B shows a maximum likelihood cladogram without branch length for an easier visualization of the MOB groups (the colored dot on each organism name refers to the taxonomy classification). The red dot on each node of the tree represents a bootstrap value equal or higher than 50%, while the blue dot a bootstrap value equal or higher than 70%.

4.1.2 Phylogenesis: distribution and evolution of *Mob* genes in eukaryotic genomes

The phylogenetic tree shown in Figure 4.1 has been generated from the available proteomes of 43 complete and draft genomes. Only in two plant genomes, *Ostreococcus tauri* and *Zea mays*, it was not possible to identify MOB-like proteins. This could be due to the consensus sequence quality and to the genome assembly; both of them being quite important issues for producing a high quality alignment and a reliable counting of *Mob* genes.

Figure 4.2 shows the distribution of MOB-like proteins among the organisms used for the analysis. Vertebrates (mammals, birds, amphibian and fish) have the highest number of *Mob* genes, distributed in all the *Mob* classes. Interestingly, all the vertebrate genes of the *Mob4* class are included in a single branch that is supported by a bootstrap value of 77%. This suggests that all *Mob4-like* vertebrate genes derived from a single ancestral gene at the basis of *Mob4* chordata/hemichordata gene evolution. The two subclasses *Mob4a* and *Mob4b* found in vertebrates must have arisen from an early duplication, which further subdivided this class into two subgroups.

Among vertebrates, mammals reveal the highest number of Mob genes. *M. musculus* have the highest number of *Mob4b* genes (4), while *P. troglodytes* and *R. norvegicus* have the highest number of Mob1 genes (4). *L. africana*, *O. cuniculus* and *S. scrofa*, compared to the other mammals, present a smaller number of Mob genes, probably reflecting a still limited coverage of the entire gene space of these organisms. Mrkobrada S *et al* (2006) reports that the *Homo sapiens* genome contains six MOB-like proteins whereas in our analysis we found seven MOB-like proteins. Nomenclature of *Mob* genes not only is poorly established but often can be quite misleading. Proteins identified by codes NP_060691 and NP_775739 are annotated as “MOB4B” and “MOB1, Mps One Binder kinase activator-like 1A” respectively, while in

our phylogenetic tree they both fall in MOB1 group. NP_443731 is a member of the MOB2 group but it is annotated as “HCCA2 protein”. Moreover protein NP_955776 in public databases is defined as “preimplantation protein 3 isoform 2” and in our analysis belongs to the MOB3 group. Finally, NP_958805, NP_079037, NP_570719 proteins, annotated respectively as “MOB1, Mps One Binder kinase activator-like 2C isoform 2”, “MOB1, Mps One Binder kinase activator-like 2B” and “MOB-LAK”, are all members of the MOB4 group, with the first one belonging to MOB4a and the last two to MOB4b group.

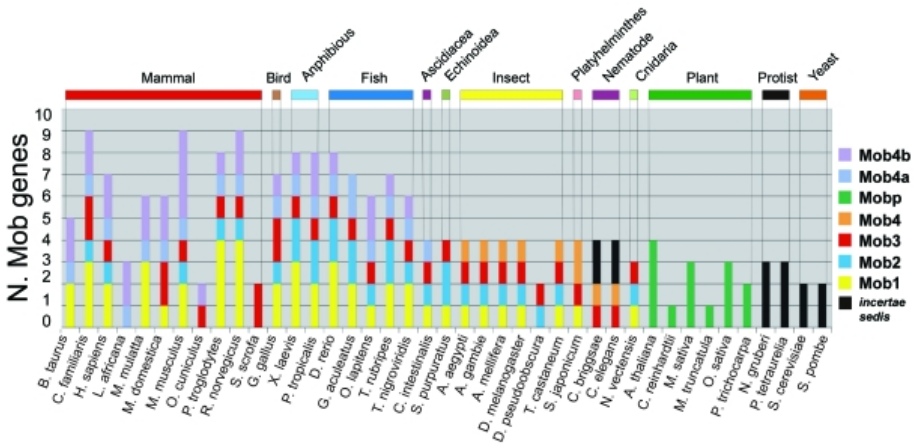

Figure 4.2 MOB protein distribution among organisms used in the analysis. Different *Mob* groups are represented with different color and the species grouped on the base of the taxonomy classification. The label “*incertae sedis*” refers to MOB proteins that have an undefined position on the phylogenetic tree

All insects show four *Mob* genes belonging respectively to *Mob1*, *Mob2*, *Mob3* and *Mob4* classes, except *D. pseudoobscura*, in which only two *Mob* genes can be found, probably due to genome assembly quality. Finally, plants represent a monophyletic group defined as MOBp class.
The phylogenetic tree shows that *S. cereviseae* (NP_012160, NP_116618), *S. pombe* (NP_595191, NP_587851), *C. elegans* (NP_502248, NP_510184), *C. briggsae* (CAE62136, CAE61392) and Protist proteins are listed as *incertae sedis*. Because of historical reasons, in the previous literature *Mob* yeast genes have been generally described as the founding members of the *Mob* family (Stavridi ES *et al,* 2003, Mrkobrada S *et al*, 2006). However, the protein sequences analyzed in this work, mostly of

multicellular organisms, do not allow a clear definition of the phylogenetic relationships existing among the yeast and the other *Mob* genes. In this regard it is interesting to point out that NP_116618 and NP_587851 yeast proteins, described as MOB2A in Mrkobrada S *et al* (2006), did not cluster with any other protein, possibly due to an early divergence of these orthologs in the lineage that generated modern Fungi.

Even if it is quite difficult to reconstruct the evolution of the *Mob* family as a whole, some possible scenarios can be drawn by looking at the distribution of genes in the so far sequenced organisms. If plants are not considered, Figure 4.2 indicates a minimum of two genes in all the eukaryotic genomes analyzed. This in turn seems to suggest a duplication of the ancestral Mob gene at an early stage of the eukaryotic evolution.

Going from unicellular to multicellular organisms there is a progressive expansion of the *Mob* family, reaching the highest number in mammals. Moreover, plant *Mob-like* genes appear to have evolved from a single ancestor, most likely due to the loss of one or more genes during the early evolution of Viridiplantae. Compared to vertebrates, plants show a significant decrease in *Mob-like* gene possibly due to the adaptation to a much more simple life style. The relationship observed among genes of the same organism and/or different organisms suggests that the *Mob* gene family evolved under a birth-and-death type of evolution. In this model new genes are created by duplication, and some duplicated genes are maintained in the genome for a long time whereas other are deleted or become non-functional through deleterious mutations (Nei M and Rooney AP, 2005).

4.1.3 MOB-like protein structure and architecture of MOB-domain

Three MOB1 protein structures have been described in literature. Human and *Xenopus laevis* structures correspond to the most conserved C-terminal core but lack the variable N-terminal region, whereas *Saccharomyces* MOB1 structure contains both the conserved C-terminal core and the variable N-terminal region (Stavridi ES *et al,* 2003; Ponchon L *et al*, 2004; Mrkobrada S *et al*, 2006).

In our phylogenetic tree, Human and *Xenopus* proteins used in structure analyses belong to the Mob1 group, while *Saccharomyces* MOB-like proteins have been assigned as *incertae sedis*.
To compare the different MOB classes, a consensus sequence for each identified group was constructed. Figura 4.3 shows the amino acid sequence conservation over all positions for each of the seven MOB groups: MOB 1, MOB2, MOB3, MOB4, MOB4a, MOB4b and MOBp. These consensus sequences were then adopted to generate a new multiple protein alignment, using three additional MOB proteins, such as the *S. cerevisiae* MOB1 and MOB2 proteins (NP_116618 and NP_012160) and one *H. sapiens* MOB1 protein (NP_775739). The latter two proteins were added in the alignment since they have been structurally characterized (Stavridi ES *et al*, 2003; Mrkobrada S *et al*, 2006).

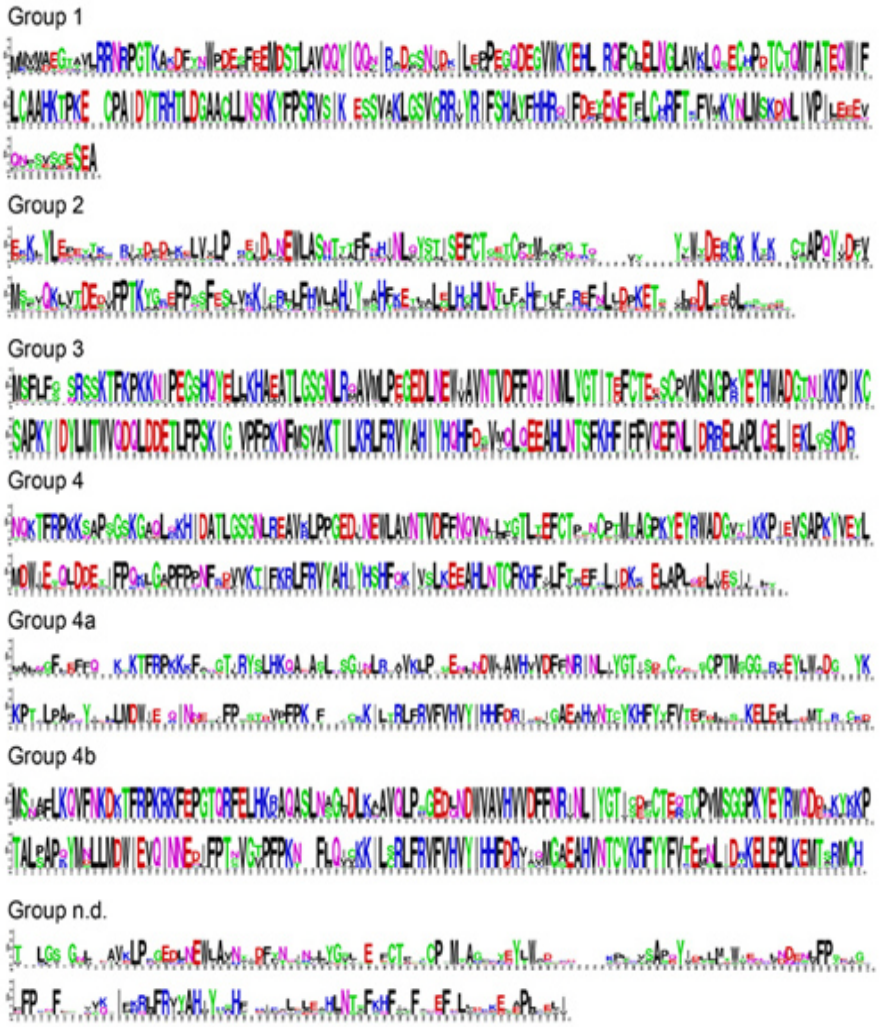

Figure 4.3. Sequence logos for each of the multiple alignment MOB groups: MOB1, MOB2, MOB3, MOB4, MOB4a, MOB4b and MOBp. Each logo consists of stacks of symbols, one stack for each position in the sequence: the overall height of the stack indicates the sequence conservation at that position, while the height of symbols within the stack indicates the relative frequency of each amino acid at that position. The yellow arrows represent the starting position adopted for the multiple alignment of MOB group consensus sequences.

The final multiple alignment of MOB group consensus sequences in shown in Figure 4.4.

MOB proteins are approximately 210 to 240 amino acid residues in length, with the exception of *S. cerevisiae* MOB1, which has a further 78 residue N-terminal extension not conserved or even present in the closely related fungal proteins.

MOB1 adopts a globular structure consisting of seven α helices, two 3_{10}-helices and a β hairpin. The core of the structure consists of a helical bundle formed by four long α helices (H2, H4, H5, and H7). This left-handed four-helix bundle, comprising the H2 and H5 helices running anti-parallel to H4 and H7 helices, is capped at one end by two short helices (H3 and H6) and the β hairpin, which are stabilized to the helical bundle via a tetrahedrally coordinated zinc (Zn) atom. The sequences N-terminal to the core contribute one α helix (H1), whereas the sequences C-terminal to the core contribute helices H8 and H9 (Stavridi ES *et al,* 2003).

On one side, the structure has a flat surface consisting of H1 and H2 and parts of H3, H4, H6, and H7. Stavridi ES *et al* (2003) reports that most of the conserved residues of MOB family members map to parts of the flat surface formed by H2 and two loops, L1 and L2, adjacent to the N-terminus of H2. Loop L1 in human MOB protein goes from residues 46 to 51 and Leu47 and Pro48 are highly conserved since are needed to stabilize the structure of the loop. These results are confirmed in our analysis, with the exception of position 47 in MOB3 consensus sequence where a Pro is present. Moreover, Stavridi ES *et al* (2003) reports that Glu51 is conserved only in MOB1 family. Figura 4.4 shows that Glu51 is conserved in MOB1 and MOB4 consensus sequences, while in MOB2 sequence is replaced by an isoleucine and in MOB3 by a glutamine. The L2 loop, consisting of residue 128–142, presents several highly conserved amino acids involved in structural interaction, such as Pro133 and Pro141 and Phe132 and Phe140 that, together with Phe144 from H5, form hydrophobic interactions with each other and with Ala58 and Ile151 from H2 and H5, respectively. Figura 4.4 shows that all these positions are conserved, except for MOB3 where various non-conservative amino acid changes can be seen in the consensus sequence (Phe140→Glu140, Phe144 →Val144, Ala58 → Tyr58). Moreover, the MOB3 consensus sequence is missing the amino acid in position 141.

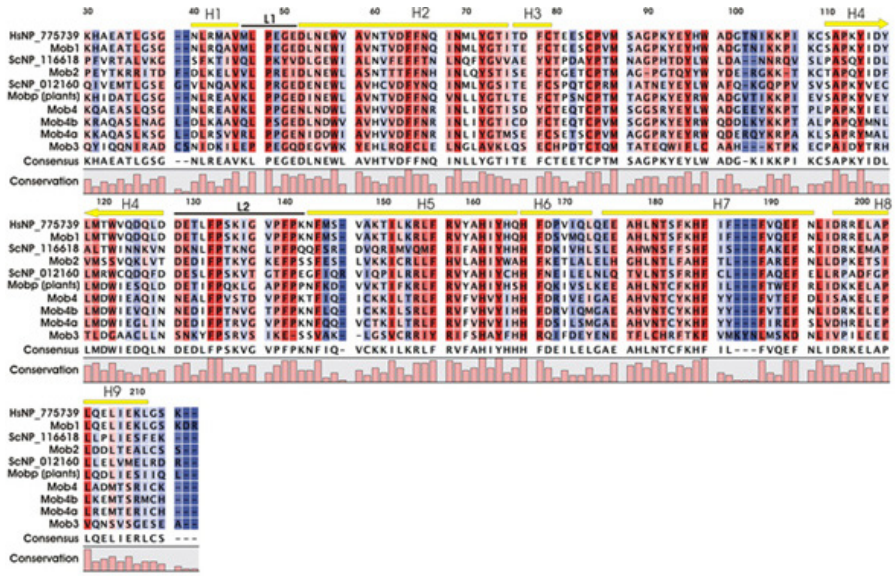

Figure 4.4. Multiple alignment of MOB group consensus sequences. The alignment was performed taking into consideration two structural defined MOB proteins, Hs NP_775739 and Sc NP_012160 plus Sc NP_116618. The helix (yellow lines) and loops (black lines) nomenclature and position on the alignment refer to Hs MOB protein as described by Stavridi et al. (2005). On each of the alignment columns, a colour scale going from red to blue represents high and low amino acid conservation, respectively.

Helix H2 has a large number of conserved residues, several of which have solvent exposed negatively charged side chains. While Stavridi ES *et al* (2003) report that Asp52 is the only charged conserved residue in all Mob families, in our analysis we found that in Mob4 and Mob4a there is an amino acid conservative substitution Asp→Asn. Moreover, we observed that Glu55, that makes a hydrogen bond with Glu51, is conserved in Mob1, Mob2 and MOBp groups while MOB4 contains aspartate and the consensus sequence of MOB3 contains a valine. Asp63 interacts with His185, that is conserved in all MOB consensus sequences except for MOB3 that contains a lysine. Interestingly, Asp63 is conserved in all MOB4, MOBp and MOB1 classes, but it is replaced by a threonine in MOB2 and by a glutamine in MOB3. Towards the C-terminal of helix H2 there is Asn69, the only polar residue other than tyrosines, that is conserved in all members of the MOB family. H2 also has several hydrophobic residues that are conserved to varying degrees in members of the MOB protein family: notably, Trp56 and Phe64,

which should have buried side chains and participate in hydrophobic interactions that stabilize the protein fold, are conserved in all MOB consensus sequences.

A Zn binding site appears to be conserved in all MOB classes, with a peculiar exception in fungi. Considering human MOB1 protein as a reference, the Zn binding site is composed by Cys79 and Cys84 from loop connecting H3 to the first strand of the β hairpin and His161 and His166 from H5 (Stavridi ES *et al,* 2003). The presence of the Zn atom contributes to the stability of the structure by anchoring H3 to the C terminus of H5. As reported in Mrkobrada S *et al* (2006) most of the yeast genes previously described as MOB2A apparently lack the Zn binding site, since the two cysteines are substituted with a valine and a tyrosine respectively, suggesting an alternative structural element for stability compensations. The consensus sequences alignment confirms these observations with the *S. cerevisiae* NP_116618 as the only MOB protein lacking the Zn binding site (Figure 4.4). To make sure that this observation was not due to a consensus artefact, we analyzed the complete 192 MOB-like protein multiple alignment and we found that essentially all the proteins analyzed contained a well conserved Zn binding site. The only exceptions, found in *M. musculus* XP_001000051, *S. purpuratus* XP_001185390 and *M. mulatta* XP_001108825, are probably due to bad quality sequences producing an unreliable alignment in the region that contains His161 and His166.

4.2 RESULTS – part II

In plants, MOB-like proteins were studied only in *Medicago sativa*, where they are expressed in a cell cycle-dependent manner and are localized in the cell division midplane during cytokinesis, as shown in the Introduction. In this plant MOB-like transcripts and proteins seem also to be associated with the onset of programmed cell death. To better understand the role of *Mob-like* genes in plant growth and development, we developed and characterized *Arabidopsis thaliana* transgenic lines with altered expression of *Mob-like* genes.

4.2.1 Arabidopsis MOB-like proteins family

Arabidopsis thaliana genome contains four MOB-like proteins as shown in Table 4.1.
In order to avoid confusion, we adopted a nomenclature based on letters for the Arabidopsis MOB-like proteins, whereas in animals a nomenclature based on a combination of numbers and letters have been used (see chapter 1.4 of the Introduction).

TAIR ID	Locus	lenght	molecular weight	name
AT5G45550	NP_199368	215 aa	24,5 kDa	MOB-A
AT4G19045	NP_001154253	215 aa	24,5 kDa	MOB-B
AT5G20440	NP_197544	216 aa	25 kDa	MOB-C
AT5G20430	NP_197543	122 aa	14.5 kDa	MOB-D

Table 4.1: MOB-related proteins in *Arabidopsis thaliana* genome.

Bioinformatic analysis showed a very high sequence similarity (93%) between MOB-A and MOB-B proteins (Figure 4.5), 80% similarity between MOB-C and MOB-D and less than 50% between the first group (A, B) and the second group of sequences (C, D).

```
at5g45550       MSLFGLGRNQKTFRPKKSAPSGSKGAQLRKHIDATLGSGNLREAVRLPPGEDANEWLAVN 60
at4g19045       MSLFGLGRNQKTFRPKKSAPSGTKGAELRKHIDATLGSGNLREAVKLPPGEDLNEWLAVN 60
                **********************:***:******************:****** *******

at5g45550       TVDFFNQVNLLYGTLTEFCTPDNCPTMTAGPKYEYRWADGVQIKKPIEVSAPKYVEYLMD 120
at4g19045       TVDFFNQVNLLFGTLTEFCTPENCSTMTAGPKYEYRWADGVQIKKPIEVSAPKYVEYLMD 120
                ***********:*********:**.************************************

at5g45550       WIETQLDDETLFPQRLGAPFPQNFKDVVKTIFKRLFRVYAHIYHSHFQKIVSLKEEAHLN 180
at4g19045       WIETQLDDETIFPQKLGAAFPPNFKEVVKTIFKRLFRVYAHIYHSHFQKIVSLKEEAHLN 180
                **********:***:***.** ***:************************************

at5g45550       TCFKHFILFTHEFGLIDKKELAPLQELIESIISPY 215
at4g19045       TCFKHFILFTHEFVLIDKKELAPLQELIESIIAPY 215
                ************* ******************:**
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Figure 4.5: Amino acid sequence alignment of MOB-A and MOB-B from *A. thaliana* with the assistance of the multiple alignment CLUSTALW. "*" means that the residues in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed and "." means that semi-conserved substitutions are observed.

Because of the high similarity (87%) with *Medicago sativa Mob -like* genes, we decided to characterize firstly *AtMob-A* and *AtMob-B* genes. We generated transgenic lines with altered expression of these two genes and we decided to adopt the post-transcriptional silencing approach mediated by RNA interference.

In this thesis, only the results of the characterization of the *Mob-A* mutants lines in *Arabidopsis thaliana* are described.

4.2.2 Generation of *Mob-A* RNAi Lines

A 158 bp long sequence specifically belonging to the 3'-UTR of the target *Mob-A* gene (locus At5g45550) was selected to produce the RNAi construct for gene silencing experiments. These sequences were cloned in both sense and antisense orientations in the pK7GWIWG2(II) vector, under control of the CaMV-35S promoter and terminator, and the resultant construct was used to successfully transform wild-type *Arabidopsis* (Col-0) plants. Twenty independent kanamycin-resistant transgenic lines were recovered. Several plants from all obtained transgenic lines were self-pollinated. Progeny plants (T_1) from parental lines (T_0) hemizygous for RNAi transgenes were screened for kanamycin resistance. T_2 plants derived from

individual T_1 RNAi lines were screened again by kanamycin assays. The T_3 lines whose progeny plants (over 10 plants per line) all displayed antibiotic resistance were considered to be RNAi homozygous lines and therefore were used for further molecular analysis.

Expression analysis of the *Mob-A* gene was performed by Real-Time reverse transcriptase PCR in order to measure transcript levels in distinct plant organs (*i.e.*, flowers, stems, leaves and roots) and to evaluate the effectiveness of gene silencing in all selected transgenic plants. We have also measured transcript levels of *Mob-B* gene in these selected transgenic plants, confirming that the expression of the *Mob-B* gene was not affected and that the silencing is specific only for *Mob-A* gene (data not shown).

In all selected transgenic lines, *Mob-A* transcripts were detected in analyzed tissues with different expression levels. In particular, the absolute abundance of transcripts in young flowers was significantly higher compared to that found in later stages of partially or fully open flowers. Siliques, leaves and roots were characterized by lower levels of expression with respect to the flowers (Figure 4.6).

More specifically, the expression levels measured in 2F, 4G and 6M lines decreased by as much as 50%, 50% and 70% respectively, compared to the corresponding wild-type organ (Figure 4.6).

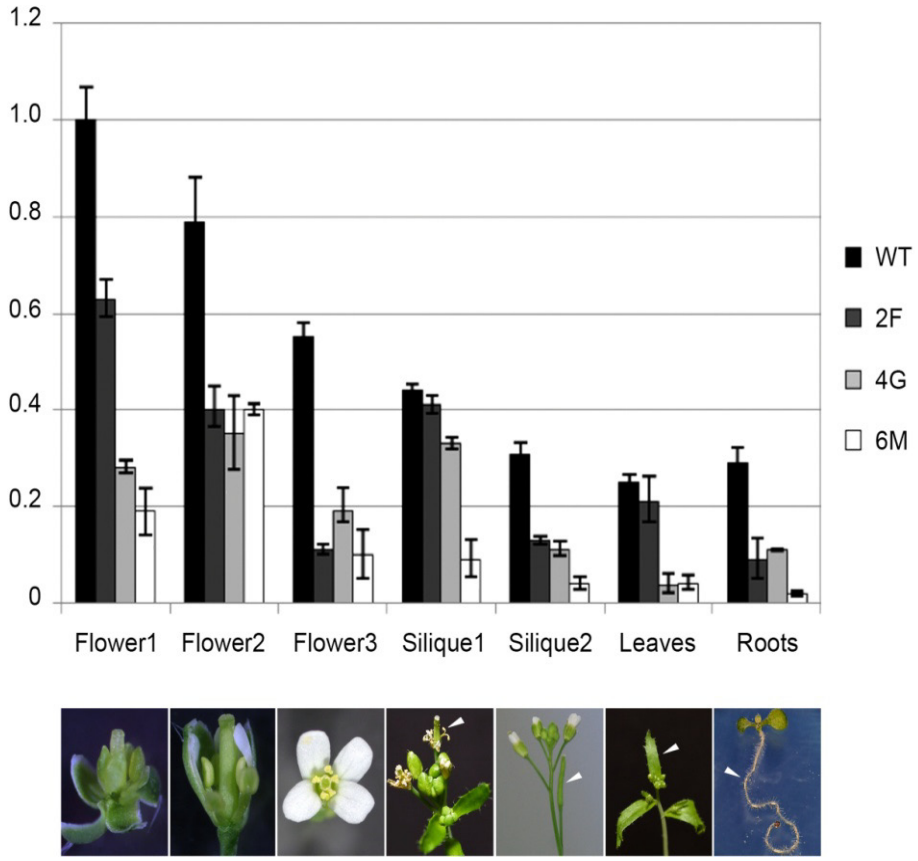

Figure 4.6: Expression analysis in *Mob-A* RNAi lines as assessed by Real-Time PCR. Three different stages of the flower development were analyzed along with young and mature siliques, leaves and roots (bottom panels). Flower and associated ovule stages are reported below the histograms. Dark grey, light grey and white histograms refer to the expression levels recorded in 2F, 4G and 6M *Mob-A* RNAi lines, respectively. The black histograms refer to the wild-type. Data are expressed in arbitrary units normalised against the level of expression detected on wild type flower stage 1.

4.2.3 Phenotype analyses

The effect of *Mob-A* gene silencing was investigated in both sporophytic and gametophytic constitutive tissues of the selected transgenic lines.

The silencing of the *Mob-A* gene resulted in a marked reduction of the seed set in all analyzed RNAi lines. Transgenic plants showed smaller siliques and much lower numbers of seeds per silique compared to wild-type plants (Table 4.2).

In particular, compared to the wild-type, line 4G showed a more deviant phenotype relative to lines 6M and 2F (Figure 4.7). As an example, wild-type plants yielded, on average, 3614±382 seeds per plant (No. of seeds/silique 54,2±4,8), whereas this number decreased to 1754±262 for line 6M (No. of seeds/silique 30,0±6,7) and to 2105±402 for line 2F (No. of seeds/silique 36,0±4,2). The number of seeds per plant was lowest in line 4G, 130±74 with a number of seeds/silique as low as 10,4±5,2.

Silique size ranged from 47,9±6,3 mm in wild-type plants, to as low as 31,0±3,5 mm and 33,0±3,0 in the lines 6M and 2F, respectively. Moreover, line 4G produced siliques approximately one third in length compared to wild-type (17,8±2,5 mm). Interestingly, for other morphological traits such as plant height, No. of stems per plant, siliques per plant, branches per stem, leaves per rosette and days to first open flower, line 4G differed significantly from wild-type plants, whereas lines 6M and 2F were comparable with the wild-types (Figure 4.7 B). Indeed, with the exception of some aberrant traits observed for line 4G, lines 2M and 6F did not significantly differ from the wild-type for most morphological traits (Table 4.2).

	Plant height (cm)	Leaves/rosette (No.)	Stems/plant (No.)	Branches/stem (No.)	Siliques/plant (No.)	Silique size (mm)	Seeds/silique (No.)	Seeds/plants (No.)	Germinability (%)	Days to flowering
WT	40.3^{a} ±2.7	13.4^{a} ±0.8	3.0^{b} ±1.1	2.9^{b} ±0.6	66.7^{a} ±4.2	47.9^{a} ±6.3	54.2^{a} ±4.8	3614^{a} ±382	98.5	$30,5^{a}$ ±1.4
2F	38.0^{a} ±4.0	13.0^{a} ±0.4	3.4^{b} ±0.8	3.4^{b} ±1.1	61.0^{b} ±6.2	33.0^{b} ±3.0	36.0^{b} ±4.2	2105^{b} ±402	96.0	$30,0^{a}$ ±1.3
4G	28.6^{b} ±2.8	9.0^{c} ±1.7	10.6^{a} ±2.5	5.4^{a} ±1.1	21.0^{c} ±7.1	17.8^{c} ±2.5	10.4^{d} ±5.2	130^{d} ±74	78.3	$24,9^{b}$ ±2.1
6M	39.0^{a} ±3.7	12.0^{b} ±0.3	3.4^{b} ±1.0	3.0^{b} ±0.6	60.0^{b} ±5.8	31.0^{b} ±3.5	30.0^{c} ±6.7	1754^{c} ±262	94.0	$29,0^{a}$ ±1.8

Table 4.2: Morphological characterization of *Mob-A* RNAi silenced lines

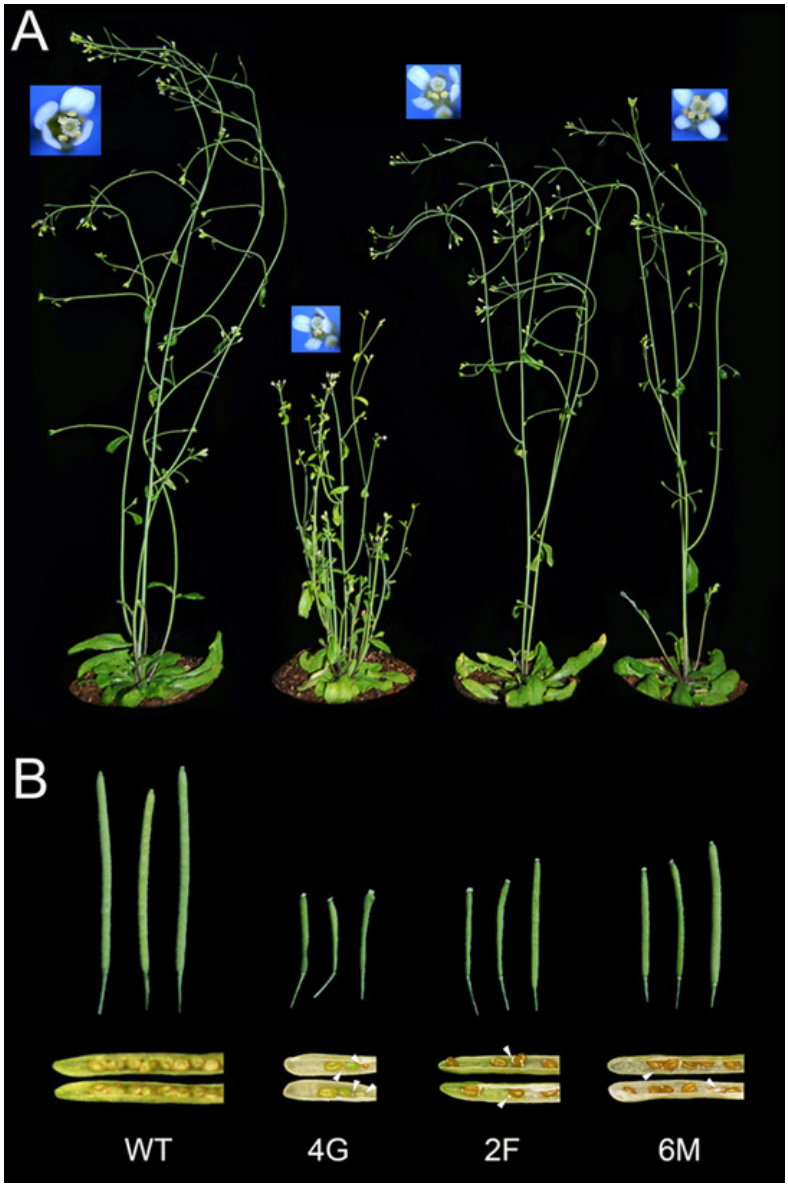

Figure 4.7:. Morphological traits of wild-type and *Mob-A* RNAi lines. Panel A shows representative pictures of five week-old flowering plants of wild-type and *Mob-A* RNAi lines 4G, 2F and 6M. Panel B shows the silique sizes of WT and RNAi lines: the presence of aborted seeds (arrow-heads) can be observed within siliques of *Mob-A* silenced lines.

However, it is worth mentioning that a thinning of the stems was observed in RNAi lines. To further investigate structure and size of stem tissues, toluidine blue, phloroglucinol and aniline blue staining were performed on transversal sections at the basal internode of stems of both wild-type and RNAi 30 days old plants.

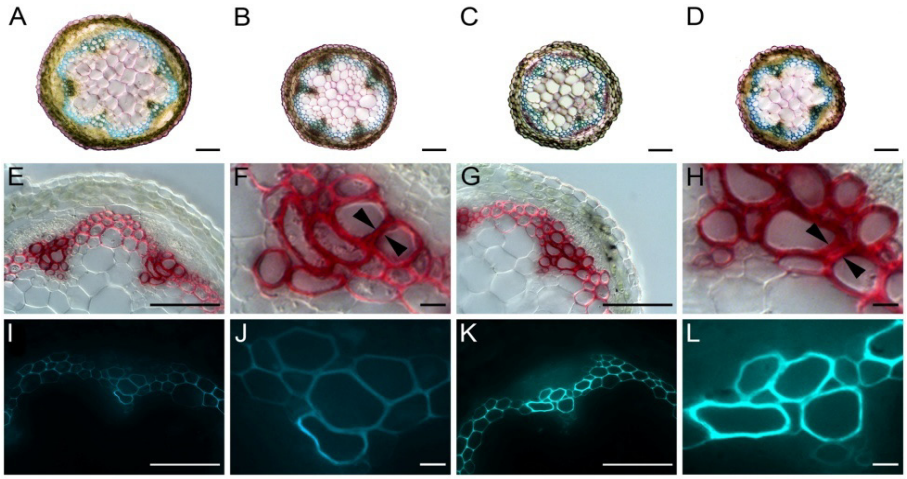

Figure 4.8. Organization and size of stems from WT (A, E, F, I, J) and *Mob-A* RNAi individuals analyzed by staining of cross sections. A, E, F, I, J: wild type line; B, G, H, K, L: line 2F; C: line 4G; D: 6M.Panels A-D: toluidine blue staining. Panels E-H: phloroglucinol staining. Panels I-L: aniline blue staining. F and H: close up images of E and G respectively. J and L: close up images of I and K respectively (Size bars: A-D, E, G, I, K = 100 µm and F, H, J, L = 10 µm).

As shown in Figure 4.8, the *Mob-A* silencing affected the dimension of the cell size, in particular we observed a decrease in the average cell size in these lines (data not shown). On the contrary, an increase in cell wall thickness was observed using either phloroglucinol (Figure 4.8 E-H) or aniline blue staining (Figure 4.8 I-L) only in a few cells of the xylem vessels of the silenced lines in comparison to the wild-type lines.

The effect of *Mob-A* gene silencing was also investigated in primary root of seedlings after 48h from imbibition. Image analysis was applied to measure the length, the diameter and the area of the different primary root regions and the mean area of the meristematic cells. Figure 4.9 shows that,

on average, only the area of the root elongation region was clearly affected by the silencing of the *Mob-A* gene. The region was significantly (P<0,05) reduced in length (Figure 4.9,C) but not in width (Figure 4.9 D).

The calculated size of the cap and the meristem was similar in wild-type and silenced seedlings whereas the hair region tended to be greater in silenced plants, although the difference was not statistically significant (Figure 4.9 B). In silenced seedlings, the greater hair region balanced the reduced elongation region and consequently for this reason the mean area of the whole root tended to be greater than in the wild-type roots (Figure 4.9 A). Most important, the measurement of the meristematic cell size was significantly reduced in silenced seedlings (Figure 4.9 E) and, as the size of the meristem was similar to that of the control, the cell number in the region was higher.

Furthermore through confocal laser scanning microscopy (CLSM) we analyzed the structure of the primary root in wild-type and RNAi mutant 3-day old plants. The structure of the root apical meristem (RAM) and the development of the *Arabidopsis* primary root are well characterized (Dolan *et al*, 1993). The *Arabidopsis* RAM contains four rarely dividing QC cells that are surrounded by actively dividing initial cells (Figure 4.10 A). Each initial cell divides in the same plane and provides new cells to each cell file. The root tips of the wild type and *Mob-A* RNAi lines were fixed and then stained with Syber-Green and observed by CLSM. The root tip of the wild-type plants was visible as organized files of cells regular in size and shape, where the QC cells were easily discernible (Figure 4.10 B). In contrast, the root tip of RNAi mutants was visible as a disturbed organization of cells irregular in size and shape, in particular in the columella region. Moreover the QC cells were not easily discernible (Figure 4.10 C, D). Irregular planes of cell division were not observed in any other cell layer.

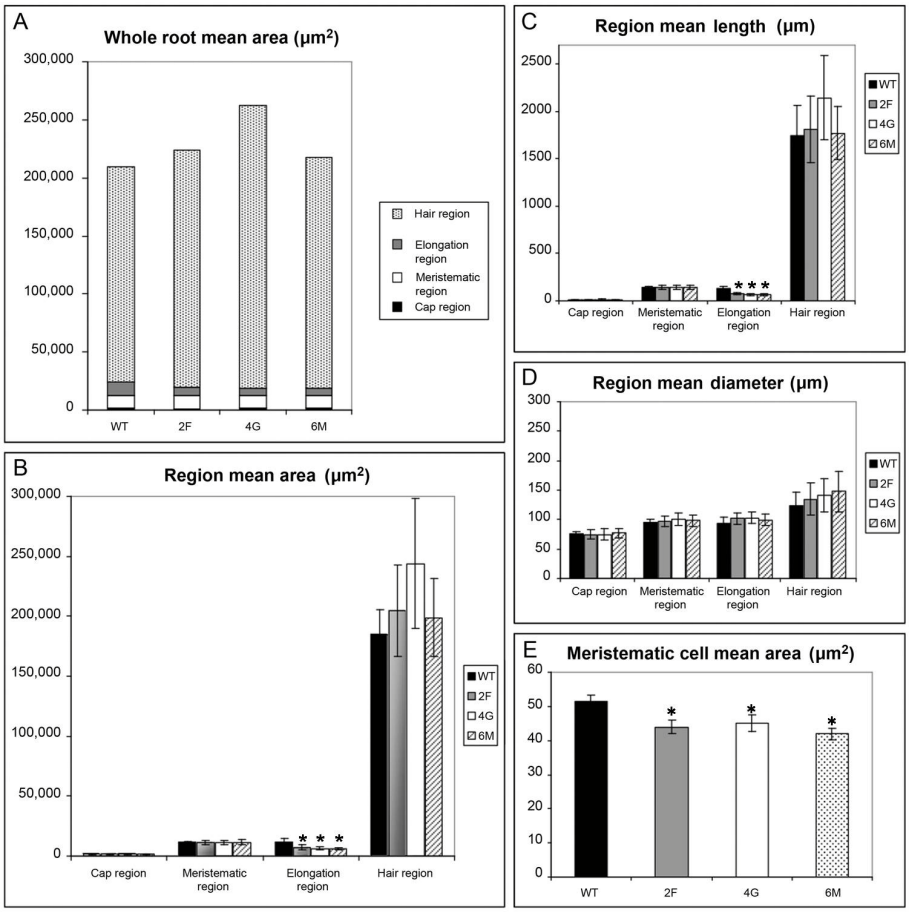

Figure 4.9. Histological analysis of the primary root in *Mob-A* silenced plants in comparison with WT after 72 h from imbibition. Histogram A shows the mean area of the whole root, measured for three independent RNAi lines (2F, 4G and 6M) and for wild-types. Histograms B, C and D show the mean area, length and diameter (±SD) of the different root regions. The size of the meristematic cells (mean cell area ± SD) is reported in histogram E.

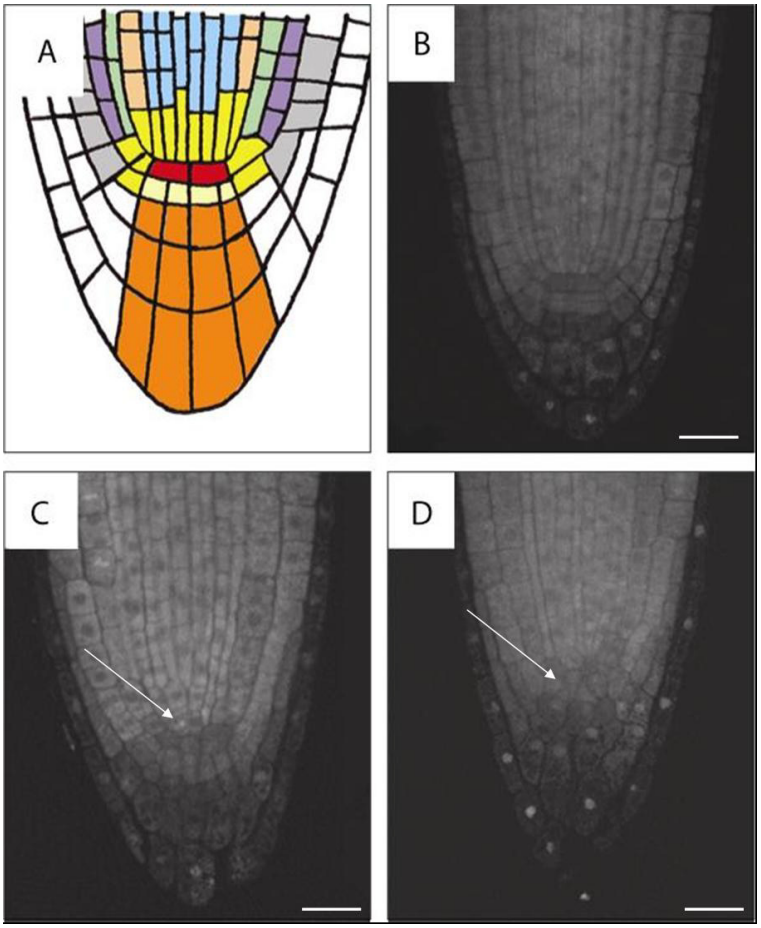

Figure 4.10*: Arabidopsis thaliana* root apical meristem organization. A. Colorized drawing of the root apical meristem region: gray: epidermis, purple: cortex, green: endodermis, peach: pericycle, blue: stele, white: lateral root cap, orange: columella, yellow: initials (stem cells), red: quiescent centre (QC). B meristem region of wild type seedling; C-D meristem region of two seedlings from two different *Mob-A* RNAi lines in which the QC region is disorganized. Bars = 25 µm (in all images).

4.2.4 Ploidy analyses

As reported in literature, *Mob1* is an essential gene required for the completion of mitosis and maintenance of ploidy in yeast (Luca FC and Winey M, 1998), so changes in ploidy level were evaluated by flow cytometry in the *Mob-A* RNAi lines. Ploidy level was measured at different developmental stages: we have isolated nuclei from whole 3 days old seedlings and from fully differentiated plant organs (e.g., leaves and differentiated portions of roots from 10-days- and 30-days-old seedlings). No significant difference was found between WT and mutants lines (Figure 4.11).

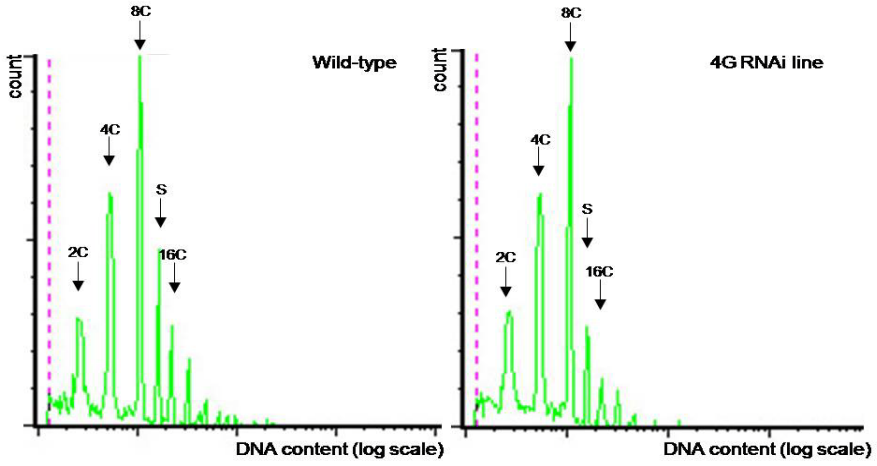

Figure 4.11: example of DNA ploidy distribution in fully differentiated leaves measured by flow cytometry. WT plants (left panels) and RNAi line (right panels). S: internal standard

The presence of multinucleated cells was also excluded by using traditional and confocal microscopy.

4.2.5 Analysis of the female sporogenesis and gametogenesis in RNAi lines

A total of 81 out of 183 ovules (44%) from the *Mob-A* RNAi lines revealed deviations from the regular female sporogenic developmental

pattern at the final stage of megasporogenesis (corresponding to stage 2-IV/V, Schneitz K *et al* Hülskamp, 1995).

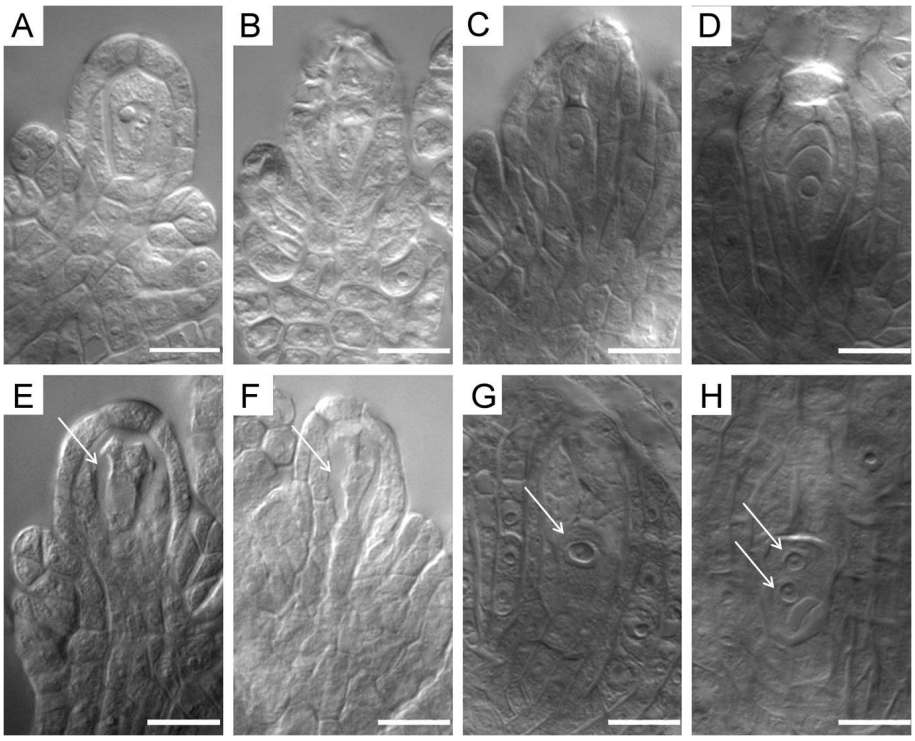

Figure 4.12. Female sporogenesis progression in *Arabidopsis* wild-type (upper panels) and *MobA* RNAi lines (bottom panels). MMC (A); tetrad (B); functional megaspore at an early stage (C) and late stage (D). Ovules containing degenerated meiotic megaspores (E, F). Ovules showing an enlarged MMC of apomeiotic origin (G) and a binucleated chalazal megaspore of meiotic origin (H) (Size bars: A-H = 10 µm).

As shown in Figure 4.12, besides the progression of normal megasporogenesis (Figure 4.12 A-D), 31 out of 110 ovules (28%) were characterized by premature degeneration of megasporocytes and megaspores at stages of the first meiotic division (Figure 4.12 E, F). We found the proportion of degenerating pre-meiotic MMCs and meiotic megaspores to be highly variable among the three independent RNAi lines, ranging from less than 10% (line 4G) to as high as 60% (line 6M). The

abortion of megaspores in post-meiotic stages was frequently associated with the presence of two clearly detectable nuclei, enclosed within a centrally contracted cytoplasm. A few ovules containing an enlarged MMC apparently not undergoing meiosis were also observed (Figure 4.12 G). Moreover, 30 out of 132 viable ovules (23%) at the final stage of megasporogenesis proved to include bi-nucleated megaspores (Figure 4.12 H).

Unlike the case in wild type where three of the four spores formed after meiosis undergo programmed cell death, both unreduced megaspores and bi-nucleated cell megaspore persist through later stages of ovule development and are present in mature ovules (data not shown).

The silencing of the *Mob-A* gene also affected later stages of embryo sac development. About 27% (50 out of 183 ovules) of ovules at the seven-celled stage (FG6, see Chapter 1.3, Figure 1.10) showed gametogenesis abnormalities eventually leading to embryo sac degeneration (16% of the total number of embryo sacs considered).

Consistent with the degeneration events observed during early developmental stages, from 3.5% to 30.2% of the ovules completely lacked an embryo sac. This phenomenon was mainly observed in the RNAi lines 2F and 6M, which were characterized by 21.0% and 30.2% of degenerated embryo sacs, respectively. The frequency of degenerated embryo sacs was much lower in line 4G (4%). Collapsed embryo sacs were observed in ovules at different developmental stages, ranging from FG2 to FG6 (see Figure 1.10, Chapter 1.3). Frequently the cavity of these aborting ovules was filled by somatic cells.

Along with fully degenerated embryo sacs, numerous ovules clearly showed non-differentiated embryo sacs (Figure 4.13). Cytological evidence suggests that these ovules were characterized by blocked embryo sacs development at early stage of female gametophyte development (Figure 4.13 B). Most of the ovules completely lacked an embryo sac, whose cavity was apparently replaced by a tissue produced from an atypical proliferation of sub-epidermally localized cells (Figure 4.13 C and D).

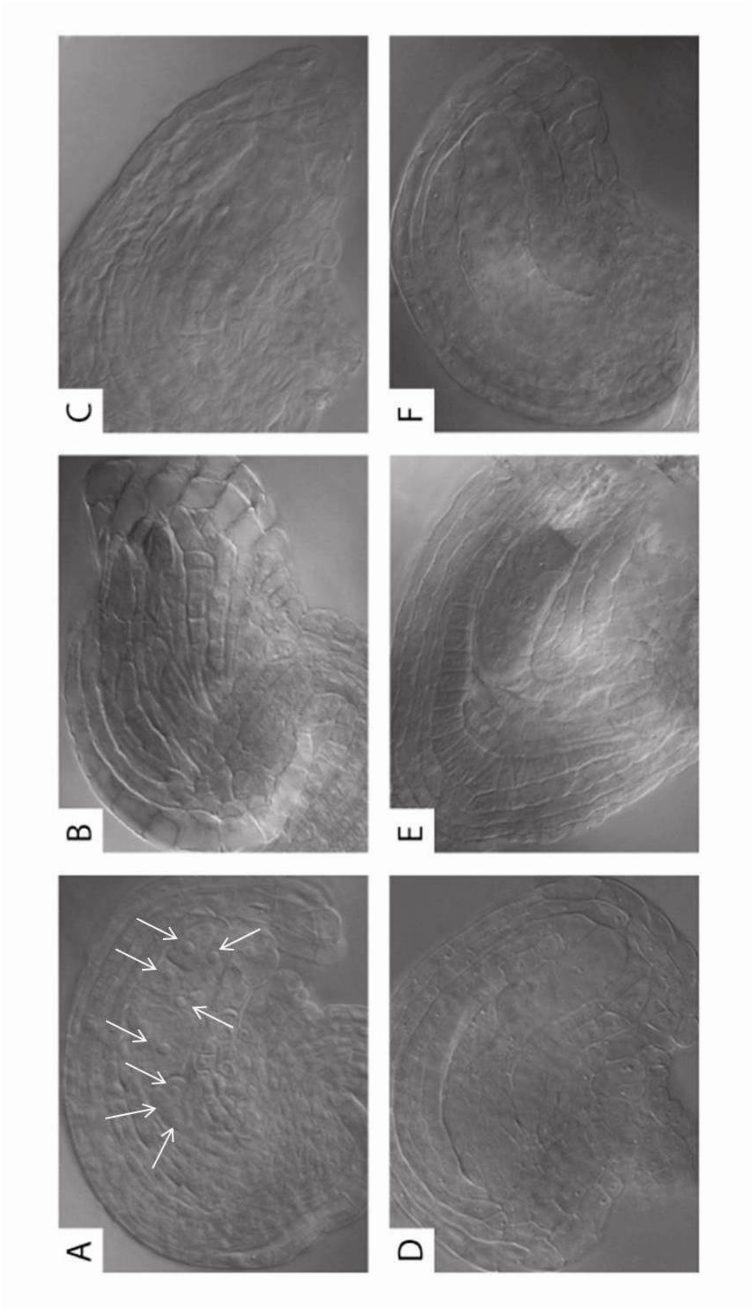

Figure 4.13. *Arabidopsis* wild-type embryo sac at the developmental stage 8N (A). B-F most frequent deviations observed within ovules of RNAi lines: embryo sac development arrest at one-nucleated stage (B); overgrowing embryo sac (C and D), embryo sac degeneration and unregulated growth of the endothelium (E); lack of cellularization of the egg cell apparatus (F) (Size bars: A-H = 10 µm).

A rare phenotype was observed in ovules lacking an embryo sac, consisting of the presence of an abnormal coenocytic structure developing from the already formed endothelium (Figure 4.13 E). This structure was observed only within ovules characterized by a complete lack of embryo sac at late developmental stages. Multinucleate cells were produced by either anticlinal or periclinal divisions of somatic cells belonging to the endothelium. Coenocytic structures included an atypical number of nuclei, up to 10, characterized by a high variation in size and distribution pattern within the cytoplasm. Moreover, about 30% of ovules bearing an FG6 embryo sac were characterized by cellularization abnormalities. In particular, from 26.4% to 28.4% (19 and 23 embryo sacs out of 72 and 81, respectively) of ovules bearing an FG6-like embryo sac showed defective cellularization due to the mis-localization, often combined with an abnormal shape, of the egg cell and synergids prior to their degeneration (Figure 4.13 F).

We have also performed experiments on pollen from RNAi lines and wild type lines. We found out that the pollen viability of *Mob-A*-RNAi plants was comparable with that of wild-type. Moreover no variations in pollen size with aceto-carmyne staining or in nucleus stainability with DAPI were detected (data not shown).

Lastly, because of the high homology between MOB-A and MOB-B, we decided to develop double mutants by crossing the *Mob-A* and *Mob-B* RNAi lines. Pollen from three *Mob-A* RNAi lines was used to fertilise three different RNAi *Mob-B* lines and *vice versa*. Only three siliques from two different cross were recovered and the progeny were analyzed through PCR, but it was not possible to find the presence of both the constructs in these plants. We supposed that a reason for the lack of double mutants could be the lack of the fecundation due to a degenerated embryosac or the sporophyte lethality due to the lack of *Mob-A/B* function.

4.3 Results – part III

As explained in the Introduction, the identification of the binding partners of MOBs may shed light in determining the functions of these proteins. So to better understand the role of MOB-like proteins in plant growth and development, we tried to identify plant DBF2-related proteins.

4.3.1 Co-immunoprecipitation experiments

As first attempt, we decided to use a commercial kit for co-immunoprecipitation experiment. In a typical immunoaffinity experiment, protein complexes are captured from a cell lysate by an immobilized antibody that recognizes an epitope on one of the known components of the complex. After extensive washing to remove unspecifically bound proteins, the complexes are eluted and analyzed by mass spectrometry (MS). Transient complexes, which are characterized by high dissociation constants, are typically lost by this approach. Loss of specific components of a protein complex may be prevented by chemically cross-linking proteins prior to purification.

Therefore firstly we tested the ability of the antibody, that we previously developed for the detection of MsMOB-like proteins, to detect *Arabidopsis* MOB-like proteins (Citterio *et al*, 2006). This polyclonal antibody recognizes the peptide LGSRNQKTFRPKKSA, located in the N terminal region of the sequence. The ability and the specificity of the antibody were tested by immunoblotting experiments with total protein extracts from 3-day-old seedlings (Figure 4.14). Monodimensional Western blots revealed that our anti MsMOB-like antibody recognized MOB-like proteins also in *Arabidopsis thaliana*. A single band at about 45 kDa and a doublet at about 25 kDa can be observed in Figure 4.14A. The pattern was identical to that characteristic of *Medicago sativa*. The 25 kDa doublet likely corresponds to different post-translational modifications of the AthMOB-A and/or AthMOB-B proteins, whereas the 45 kDa band is likely a dimer (Mrkobrada S *et al*, 2006). No signals were observed in replicated Western blots when the

affinity-purified anti-MOB antibody was saturated with the correspondent peptide (1:1) (Figure 4.14 B).

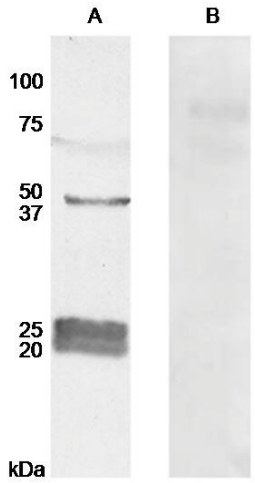

Figure 4.14: Antibody specificity assay. A, Immunoblotting using an *Arabidopsis* total protein extract from 3-day-old seedlings: a single band at about 45 kDa and a double band at about 25 kDa were recognized in the plantlet tissues. The 25 kDa doublet likely corresponds to different post-translational modifications of the AthMOB-A and/or AthMOB-B proteins, whereas the 45 kDa band is likely a dimer (Mrkobrada S *et al*, 2006). B, negative control obtained by saturating the anti-MOB antibody with the correspondent peptide (1:1). No aspecific signals were observed.

Then we tested paraformaldehyde as cross-linker. Formaldehyde and paraformaldehyde are a simple, inexpensive, and commonly used protein cross-linkers, which react primarily with Lys residues (Miernyk JA and Thelen JJ, 2008). Several properties of formaldehyde and paraformaldehyde make them useful reagents for characterizing protein–protein interactions in vivo. They are membrane permeable, characteristic that allows rapid fixation and consequently inactivation of many cellular proteases. Formaldehyde/ paraformaldehyde is also considered to be a 'zero-length' cross-linker (although the actual arm distance is 2 Å), so only close-proximity associations are characterized, minimizing non-specific protein interactions. Formaldehyde/paraformaldehyde cross-links can be reversed by boiling in SDS-PAGE sample buffer (Hall DB and Struhl K, 2002; Vasilescu J *et al*, 2004). Formaldehyde/paraformaldehyde can be

used at concentrations ranging from 0.1–1.0% depending on protein type and cell density. After quenching with 1 to 2 mM Gly, in vivo formaldehyde/paraformaldehyde cross-linked samples (PFA-treated) can be used directly with co-IP (Miernyk JA and Thelen JJ, 2008).

So, 3 day-old seedlings were cross-linked with 1% paraformaldehyde as described in Materials and Methods. We performed Western blotting on protein extracts from PFA-treated and untreated samples and after subjecting them to the reversal process. As shown in Figure 4.15, the cross-linking resulted in a 25 kDa doublet corresponding to AthMOB-like proteins and a doublet with a higher molecular weight in the 160kDa range, presumably a MOB-like protein complex. Moreover with PFA treatment, the 45 kDa band/s disappeared.

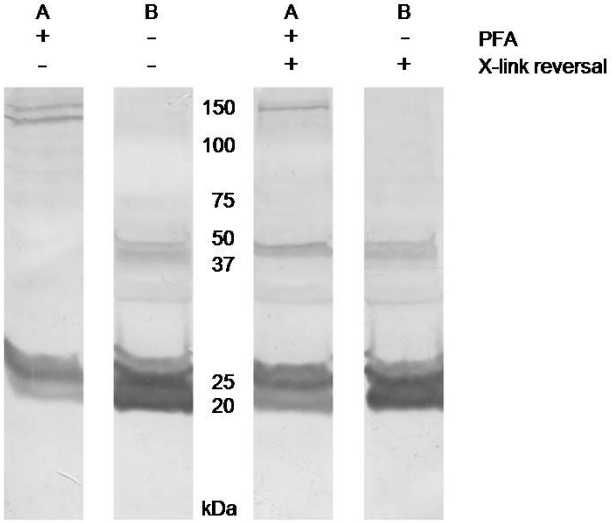

Figure 4.15: Western blot analysis of treated (A) and untreated seedlings (B) with 1% PFA before and after the reversal process (X-link reversal). PFA-treated and untreated samples were heated 10 minute at 65°C (lane 1 and 2), and for the reversal of formaldehyde cross-links were boiled for 20 min prior to SDS-PAGE (lane 3 and 4). Lane 2 and 4, corresponding to the untreated plants, showed the typical pattern of MOB-like proteins in plants. In the lane 1 corresponding to a PFA-treated plant was visible a double band with higher molecular weight at about 160kDa that partially disappeared after the boiling of the sample (lane 3). Moreover the band a 45kDa appeared just after boiling the sample.

After boiling the 45 kDa band/s re-appeared, whereas signal of one of the two higher bands produced by PFA-treatment was no longer detected, confirming successful cross-link reversal. Denaturation or modification of the MOB epitope should be excluded because the 25 kDa doublet was still clearly observed. .

As reported above, our goal was to identify protein/s interacting with MOB-like proteins in *Arabidopsis thaliana*. By treating living cells with paraformaldehyde, we wanted to purify MOB protein/s and their interactor/s by using the anti-MsMOB antibody and then identify them by LC-MS/MS.

Therefore we tested a commercial immunoprecipitation kit from PIERCE (Primary Immunoprecipitation kit, 45335) in order to evaluate the capacity of the antibody to bind MOB-like proteins under non denaturating condition.

The immunoprecipitation experiments were carried out following the manufacturer's recommendations with minor modifications. We optimized the protocol in order to avoid any contamination in the elution fractions due to uncoupled antibody chains and in order to immunoprecipitate MOB proteins.

We performed several attempts. The best result was obtained when eluates from ten successive purifications were pooled together and concentrated with an AmiconUltra – 10000 MWCO centrifugal filter device (Millipore, Nepean, ON, Canada). Proteins were then separated on a SDS-PAGE gel and visualized with a colloidal coomassie staining and blotted on PVDF membrane.

No bands were visualized on Coomassie-stained gel (data not shown). Only a very weak bands corresponding to MOB-like proteins were detected with Western blotting analysis (Figure 4.16).

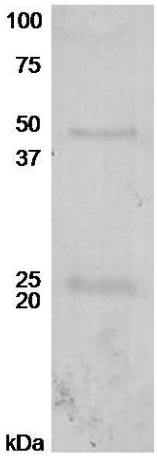

Figure 4.16: Western Blot analysis of fractions collected from the 10 subsequent purifications. The presence of the MOB-like proteins was detected as a very weak signal.

We concluded that that the antibody in our possession is not suitable for immunoprecipitation experiments in non-denaturating condition. In fact the sequence recognized is located at the N-terminal region of MOB-like proteins and this region could be important for the interaction with DBF2-related proteins, so that the antibody could not bind MOB proteins when they are interacting with their partners

Therefore we decided to produce an antibody against the whole recombinant protein His-MOB-A and performed a large-scale immunoprecipitation approach.

4.3.2 Purification of HIS-tagged-MOB for antibody production

The *Mob-A* coding region was PCR-amplified, cloned into a pENTR^{TM}/D-TOPO_vector and subsequently transferred into the destination vector pET160-GW/CAT (Invitrogen) to produce the 6xHis-MOB vector as described in Materials and Methods.

The construct was used to transform BL21 Star (DE3) *E. coli* strain, and a pilot expression was performed in order to test the protein expression. We found out that 6xHis-MOB-A was expressed as soluble protein and the largest amount was obtained after 3 hours of induction with 0,1mM of IPTG (Figure 4.17).

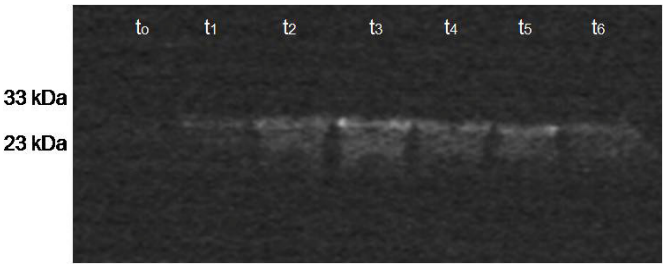

Figure 4.17: Pilot expression. The fusion proteins 6xHis-MOB-A was detected directly in SDS-PAGE gel under UV illumination. This was possible because the vector contained a Lumio-Tag. The Lumio System is based on the FlAsH (Fluorescein Arsenical Hairpin) technology which uses a biarsenical reagent to bind and detect proteins containing a tetracysteine motif (i.e. Lumio) (Griffin *et al*, 1998). The biarsenical reagent becomes strongly fluorescent only upon binding to the tetracysteine motif, allowing specific detection of fusion proteins directly in gels. T0 represents samples collected immediately before the induction of recombinant protein expression with IPTG, then cells were grown further and samples were collected after one (T1), two (T2), three (T3) four (T4), five (T5) and six (T6) hours.

It is worth to note that also 6xHis-MOB-A recombinant proteins produced in BL21 *E. coli* strain showed a Western blot pattern similar to that obtained by using the anti-MsMOB antibody (Figure 4.18).

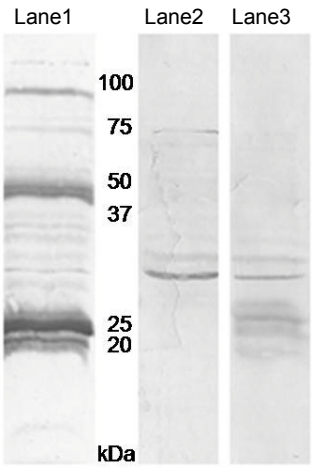

Figure 4.19: Western blot analysis of total proteins extract from BL21 Star (DE3) transformed and inducted (lane 1), untrasformed (lane 2) and transformed but not inducted (lane 3) *E. coli* cells. This resulted confirmed the signal of the alfalfa antibody against MOB is specific for the recombinant protein and that the pattern is similar to a total proteins extract from plant leaves.

As shown in Figure 4.19, the anti-MsMOB antibody recognized specifically MOB-A protein produced in *E. coli* and the presence of a 45 kDa band in *E. coli* extracts confirmed that also this band corresponds to MOB-A proteins, and is likely a dimer.

In order to purify a sufficient amount of recombinant protein (6xHis-MOB-A) we started with large colture volumes, to prepare soluble extracts and apply them on the specific Ni-NTA affinity chromatography column. After the recombinant protein purification we removed the 6xHis tag by AcTEV protease. With this cleavage we removed also the Lumio tag. As shown in Figure 4.20, after digestion with TEV protease, two different bands were visible in Coomassie stained gel: a 25 kDa band corresponding to the eluted MOB-A protein and a 4,5 kDA band corresponding to the 6xHis-Lumio tag.

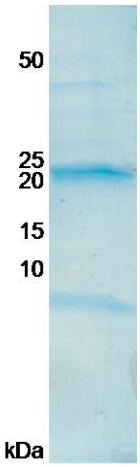

Figure 4.20: SDS-PAGE gel stained with Coomassie of column eluates treated with AcTEV protease: a 25kDa band corresponding to MOB-A and a 4,5 kDa band corresponding to the 6xHis-Lumio tag peptide can be observed.

In order to purified MOB protein from the 6xHis-Lumio tag, all the digested eluates were loaded again on the Ni-NTA column. The fractions corresponding to the MOB-A protein were collected and analyzed by SDS-PAGE (Figure 4.21).

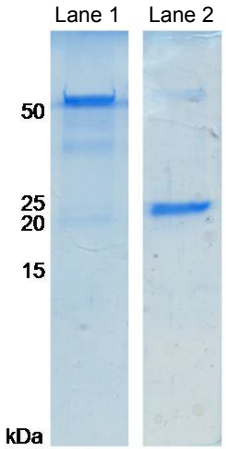

Figure 4.21: Coomassie stained gels after SDS-PAGE. In order to permit a qualitative estimation of the purified protein (cleaved MOB protein (lane 2), 1 ug of BSA was loaded on the gel (lane 1).

The purified 6xHis-MOB protein was sent to the company PRIMM S.r.l. (Milan, Italy) for antibodies production. The new produced antibody was tested on total proteins extracts from *Arabidopsis thaliana* leaves. As shown in Figure 4.22, this antibody recognized MOB-like proteins (25 kDa and 45 kDa bands), but unfortunately in the blots were presented a lot of aspecific bands. After many tests, we concluded that also this antibody was not suitable for immunoprecipitation experiments.

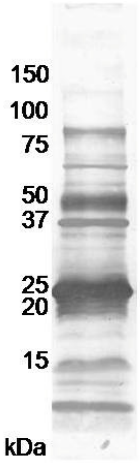

Figure 4.22: Western blot with the purified anti-ArthMOB-A antibody. This antibody recognized MOB-like proteins (25kDa and 45kDa bands) but also aspecifically many other proteins.

In the meanwhile we overexpressed *Arabidopsis* MOB-A protein tagged with Flag peptide in *Arabidopsis thaliana* plants. Our goal was to perform an immunoprecipitation with the ANTI-FLAG-M2 antibody linked to a resin, in order to purify the overexpressed ArthMobA and its interactor/s. This system has been used successfully for the identification of NDR/MOB complexes in mammalian cells.

4.3.3 Overexpression of 35S-FLAG-MOB construct in plants

The *MOB1* coding region was PCR-amplified, cloned into a pENTR^{TM}/D-TOPO_vector and subsequently transferred into the destination vector pEarleyGate 202 (Earley K *et al*, 2006) to produce the *FLAG-MOB1* vector as described in Materials and Methods. Constructs were transferred into the *Agrobacterium tumefaciens* strain EHA105 by electroporation and then *Arabidopsis* plants Col-0 ecotype were transformed. Ten independent kanamycin-resistant transgenic lines were recovered, but only 3 of them survived. The presence of the insertion in these lines was confirmed by PCR as shown in Figure 4.23 and these plants were self-pollinated.

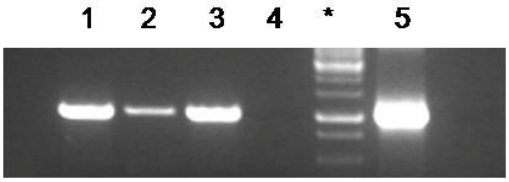

Figure 4.23: Validation of the mutant lines. PCR amplification on genomic DNA with primers specific for the insertion: lanes 1,2,3 mutants lines showing the presence of the vector, lane 4 wild-type line showing the absence of the construct; lane 5 is the positive control and lane 6 is the negative control without DNA. Lane *, 1 kb ladder.

These plants, like *Mob-A* silenced *Arabidopsis* plants, showed a reduction in seed production and only about 50 seeds were collected from the 3 survived plants. It seems that also the Arth*Mob-A* overexpression affects the plant growth and development. The progeny plants were screened for kanamycin resistance (Figure 4.24) and the selected plants are currently under investigation in order to test the presence of the protein FLAG-MOB and to confirm the phenotype.

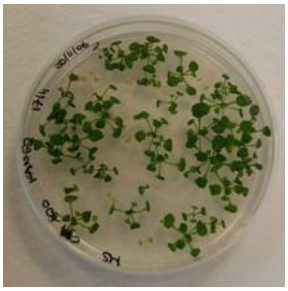

Figure 4.24: Screening of the progeny from transformed plant with FLAG-MOB-A vector. Seedlings were growing on MS medium with kanamycin; plant containing the insert were antibiotic resistant and survived whereas plants lacking the insert died.

5. Discussion and Conclusion

The aim of this thesis was to better understand the role of MOB-like proteins in plants. This protein family is a group of cell cycle-associated, non-catalytic proteins highly conserved in eukaryotes, whose founding members are implicated in several important cellular processes (see chapter 1.4 of the Introduction). This protein family has been mainly studied in animals (Luca FC and Winey M in 1998; Hergovich A *et al*, 2006; 2008; 2009); in plants MOB-like proteins were studied only in *Medicago sativa*, where they are expressed in a cell cycle-dependent manner and are localized in the cell division midplane during cytokinesis (Citterio *et al*, 2006).

Using *Arabidopsis thaliana* as a model organism, we have pursued two main aims: the development and characterization of transgenic lines with altered expression of *Mob-like* genes and the identification of plant Dbf2-related protein/s.

A post-transcriptional silencing approach mediated by RNA interference was carried out to shed light on the biological function of *Mob-like* genes in *Arabidopsis*. We have developed transgenic lines with a reduction in the *Mob-A* gene expression. This reduced expression was evident in all the mutant tissues that we have analysed in comparison to wild type. The effect of *Mob-A* gene silencing was investigated in both sporophytic and gametophytic tissues.

Concerning the vegetative part of the plant, anomalies were detected in both stem and root during development. In particular we addressed our study to the analysis of the primary root. In the RNAi mutants the root tips showed an aberrant morphology: in comparison to the wild type, the root meristem of the transgenic lines showed a disturbed organization of cells which were irregular in size and shape, particularly in the columella region. Moreover in these root tips the QC cells were not easily discernible.
In *Arabidopsis*, the root meristem can be divided into three main regions: the meristematic zone, the elongation zone, and the differentiation zone. The meristematic zone contains the stem cell niche, comprising the rarely

dividing quiescent centre (QC) cells, which control the surrounding stem cells (also called initial cells). After division of an initial cell, the daughter cell still in contact with the QC keeps its stem cell fate, whereas the other cell becomes a transit-amplifying cell, and after further divisions and expansion in the elongation zone acquires its destined cell fate in the differentiation zone (Stahl Y and Simon R, 2005). The root defects detected in our *Mob-A* RNAi mutant remember some characteristics of the SCARECROW (SCR) and SHORTROOT (SHR) mutants. In particular, like in *Mob-A* RNAi mutant, cells in the src-1 QC region are aberrant in shape and roots show defects in columella initials. However, contrary to *src* mutant roots which ultimately cease to grow, *Mob-A* mutant forms a root containing a meristem composed of a higher number of smaller cells and a very reduced elongation region.

The transcription factors SCARECROW (SCR) and SHORTROOT (SHR), have essential roles in QC establishment and stem cell maintenance (Iyer-Pascuzzi AS and Benfey PN, 2009) which are essential functions for a correct root growth (Aida K *et al*, 2004, Tucker MR and Laux T, 2007). We did not test the functionality of the QC in our *Mob-A* mutant but it is likely that at least partially QC function is maintained since the root growth is not abolished.

Another class of very important regulators of *Arabidopsis* root development are the PLETHORA (PLT) genes encoding the AP2-domain transcription factors. PLT gene family controls distinct aspects of root development in a dose-dependent manner through PLT expression gradients that culminate in the stem cell niche. For instance, the PLETHORA1 (PLT1, At3g20840) and PLT2 (At1g51190) genes have been shown to be essential for defining the root stem cell niche. plt1;plt2 mutants display stem cell loss, loss of transit-amplifying cells and reduced cell expansion. Interestingly, columella cell defects and reduced cell expansion are two evident traits of our *Mob-A* mutants. In *Arabidopsis* PLT gene functions seem to be linked to the activity of signalling elements (GTPase, kinases) closely related to the core elements of SIN/MEN pathway (Bedhomme *et al,* 2009) of which MOB is an essential component. Neverheless, we must take into account that in yeast SIN/MEN pathway link cell cycle exit to cytokinesis, whereas in plant it seems that the homolog SIN/MEN factors, although highly conserved between plants and fungi, have evolved to perform different functions. Evolution i.e. seems to have recycled ancient signalling components to derive new plant signalling pathways in Angiosperms (Bedhomme *et al,*

2007). The reason for the evolution of these new different signalling pathways is explained by the complexity of multicellular organisms. In these organisms the cell division process requires additional cellular controls associated to differentiation, organogenesis and development.
Regarding the role of *Mob-A* in *Arabidopsis* root, we can suppose that it is a component of the new emerging signalling pathway controlling cell type specification by PLT factors and plant SIN-like elements.
More specifically, Bedhomme *et al* (2009) recently showed that PLT expression is required to restrict AtSGP1 promoter activity (a putatively kinase upstream of MOB) to the QC, meaning that, genetically, PLT is upstream of AtSGP1. AtSGPs are crucial signalling components involved either in early cell fate specification, or in the final steps of cell differentiation. Bedhomme *et al* (2007, 2009) also showed that a functional QC was not necessary for AtSGP1 promoter activity and that cells expressing AtSGPs possessed limited or null mitotic activity.

On the basis of our *Mob-A* mutant root features, we can speculate that through a not yet defined pathway involving PLT and SIN-like plant factors *AtMob-A* (At5g45550) can limit mitotic activity and couple cell division with cell expansion and differentiation. It should be in agreement with the function of animal MATS (Mob as tumour suppressor protein). In these eukaryotes loss of Mats function results in increased cell proliferation, defective apoptosis, and induction of tissue overgrowth.

Concerning the analysis of reproductive organs, Real-Time PCR of wt plants showed that *Mob-A* gene expression was predominant in flowers and much higher than levels recorded in siliques, leaves and roots, thus suggesting a major activity of the *Mob-A* gene in these *Arabidopsis* reproductive organs. Analyzing both female and male organs, we found out that post-transcriptional silencing of the gene affects only the normal progression of female meiosis and megagametogenesis. In particular, our cytological results showed that the *Mob-A* gene is essential for the regular progression

of megasporogenesis and the formation of functional embryo sacs in *Arabidopsis*.

Many of the ovules analyzed displayed aberrant phenotypes emerging at multiple time points during development. Similar results have been reported in relation to meiosis and cytokinesis failure.
Indeed, similar to the phenotype of the *Mob-A* gene silencing, it was recently reported that mutation of *swi1*, a gene involved in chromatid cohesion and centromere organization, causes a single equational division in place of normal female meiosis, followed by arrest in further progression (Motamayor JC *et al*, 2000; Siddiqui I *et al*, 2000; Ravi M *et al*, 2008). Furthermore, these defects lead to the production of two diploid cells in place of four haploid megaspores, and failure to form a female gametophyte. It is particularly interesting that the dyad allele of SWI1 specifically causes female sterility, without affecting the pollen developmental pathway. Moreover recent findings suggest also an involvement of MOB proteins in coordinating chromosome segregation and/or spindle integrity with mitotic exit and cytokinesis via regulation of chromosome passenger proteins (Stoepel J *et al*, 2005). It is thus possible to suppose that also in plant cells *Mob-A* gene is involved in the correct chromosome segregation. This hypothesis could explain the resulting phenotype detected during megasporogenesis in the transgenic lines. Nevertheless we were not able to detect defects in chromosome segregation and/or cytokinesis in proliferating root meristematic cells. A possible reason could be ascribed to the gene redundance. However, a deep analysis of meiosis during female sporogenesis is needed to confirm or exclude this hypothesis.

In literature it has been reported that genes normally required for cytokinesis in sporophytic cells, are also involved in gametophytic development. In particular, it has been showed that AtNACK1/HIK and STD/TES/AtNACK2 (Tanaka H *et al*, 2004), *GEM1*, *GEM2* and *TIO* (Park SK et al, 2004) are essential for the development of the female gametophyte. These mutants showed a partial or absent cellularization in embryo sac during megagametogenesis, similar to the phenotype of *Mob-A* transgenic lines. As reported in chapter 1.4 of the Introduction, it has been showed that in *Medicago sativa* MOB-like proteins are localized in the cell division midplane during cytokinesis and partially co-localize with phragmoplast microtubules (Citterio *et al*, 2006). It is thus possible to suppose that MOB-A

proteins are likely to be involved in organisation of microtubules that are essential for the normal cellularisation of embryo sac.

Taken together, results obtained from the characterization of *Arabidopsis Mob-A* mutant, support the hypothesis that *Mob-A* gene (At5g45550) is involved in several cellular processes important for the correct growth and development of both vegetative and reproductive organs. The identification of the binding partners of MOB-A may help to identify the pathways involving this protein. As reported in chapter 1.4 of the Introduction, mammalian MOB proteins have been demonstrated to be important for the regulation, the localization and activity of Dbf2-related (NDR – nuclear Dbf2 related) protein kinase and NDR-MOB complexes are essential components of pathways that control important cellular processes, such as mitotic exit, cytokinesis, cell proliferation and morphogenesis, and apoptosis (Hergovich A *et al*, 2006; 2008; 2009).

Hence the second aim of this PhD thesis was to identify Dbf2-related proteins in plant cells through immunoprecipitation experiments. For this purpose, several immunoprecipitation experiments have been performed. However the antibody against MsMOB proteins and the antibody produced against the *Arabidopsis* recombinant protein were not suitable for immunoprecipitation experiments. Another effort to identify the interacting protein/s was to overexpress the FLAG-MOB-A in *Arabidopsis thaliana* plants. The overexpressing transgenic plants showed a phenotype with features similar to the RNAi lines. In spite of the phenotype, we hope to use these transgenic plants to set up co-precipitation experiments based on the tagged protein.

In conclusion we have demonstrated the importance of the MOB-A protein in *Arabidopsis thaliana* growth and development. Specifically we showed that the reduction of *Mob-A* level affects cell morphology, cell size and cell proliferation in root tip, the regular progression of megasporogenesis and the formation of functional embryo sacs in reproductive organs.

The whole results seem to indicate that *Mob-A* has more than one role in plant growth and development. Given the complexity of the interactions it is possible that MOB-A belongs to specific networks depending on the interactor and/or that the activation of different pathways is organism, tissue

and/or cellular context dependent. It is also likely that different isoforms of MOB-like proteins belonging to different pathways can substitute each other, making mutant analysis more complex. However the future identification of their binding partners may shed light in determining the functions of these proteins.

6. REFERENCES

Abascal F, Zardoya R and Posada D (2005) ProtTest: Selection of best-fit models of protein evolution. Bioinformatics. 21:2104–2105.

Abe Y, Ohsugi M, Haraguchi K, Fujimoto J and Yamamoto T (2006) LATS2-Ajuba complex regulates gamma-tubulin recruitment to centrosomes and spindle organization during mitosis. FEBS Lett. 580:782–788.

Abdel-Ghany SE, Day IS, Simmons MP, Kugrens P and Reddy AS (2005) Origin and Evolution of Kinesin-Like Calmodulin-Binding Protein Plant Physiol. 138(3):1711–1722.

Agashe B, Prasad CK and Siddiqi I (2002) Identification and analysis of DYAD: a gene required for meiotic chromosome organisation and female meiotic progression in Arabidopsis. Development 129:3935–3943.

Aida K, Beis D, Heidstra R, Willemsen V, Blilou I, Galinha C, Nussaume L, Noh YS, Amasino R and Scheres B (2004) The PLETHORA genes mediate patterning of the Arabidopsis root stem cell niche. Cell 119:109-120

Allen RT, Cluck MW and Agrawal DK (1998) Mechanisms controlling cellular suicide: role of Bc11 and caspases. Cell Mol. Life Sci 54:427–44.

Ambrose JC, Shoji T, Kotzer AM, Pighin JA and Wasteneys GO (2007) The *Arabidopsis* CLASP gene encodes a microtubule-associated protein involved in cell expansion and division. Plant Cell 19:2763–2775.

Ambrose JC and Wasteneys GO (2008) CLASP modulates microtubule-cortex interaction during self-organization of acentrosomal microtubules Mol. Biol. Cell 19:4730–4737.

Andrietta MH, Eloy NB, Hemerly AS and Ferreira PCG (2001) Identification of sugarcane cDNAs encoding components of the cell cycle machinery. Genetics and Molecular Biology 24:61-68.

Andersen SU, Buechel S, Zhao Z, Ljung K, Novák O, Busch W, Schuster C and Lohmann JU (2008) Requirement of B2-type cyclin-dependent kinases for meristem integrity in Arabidopsis thaliana. Plant Cell 20:88–100.

Assaad FF (2001) Plant Cytokinesis. Exploring the Links Plant Physiol 126(2): 509–516.

Assaad FF, Huet Y, Mayer U and Jürgens G (2001) The cytokinesis gene *KEULE* encodes a Sec1 protein that binds the syntaxin KNOLLE, *J. Cell Biol.* 152 531–543.

Azimzadeh J, Nacry P, Christodoulidou A, Drevensek S, Camilleri C, Amiour N, Parcy F, Pastuglia M and Bouchez D (2008) Arabidopsis TONNEAU1 proteins are essential for preprophase band formation and interact with centrin. Plant Cell 20(8):2146-59.

Baillat G, Moqrich A, Castets F, Baude A, Bailly Y, Benmerah A and Monneron A (2001) Molecular cloning and characterization of phocein, a protein found from the Golgi complex to dendritic spines. Mol. Biol. Cell, 12:663–673.

Baillat G, Gaillard S, Castets F and Monneron A (2002) Interactions of phocein with nucleoside-diphosphate kinase, Eps15, and Dynamin, I. J. Biol. Chem 277:18961–18966.

Baluška F, Liners F, Hlavaèka A, Schlicht M, Van Cutsem P, McCurdy D and Menzel D (2005) Cell wall pectins and xyloglucans are internalized into dividing root cells and accumulate within cell plates during cytokinesis. Protoplasma 225:141–155.

Baluška F, Menzela D and Barlow PW (2006) Cytokinesis in plant and animal cells: Endosomes 'shut the door'. Developmental Biology 294(1):1-10.

Barcaccia G, Tavoletti S, Mariani A and Veronesi F (2003) Occurrence, inheritance and use of reproductive mutants of alfalfa (Medicago spp). Euphytica 133:37–56.

Bardin AJ, Visintin R and Amon A (2000) A mechanism for coupling exit from mitosis to partitioning of the nucleus, Cell 102:21–31.

Bardin AJ and Amon A (2001) MEN and SIN: What's the difference? Nature review in molecular cell Biology 2: 815-826.

Barlow PW (1982) Cell death: an integral part of plant development. In: Jackson MB, Grout B, Mackenzie IA, editors. Growth regulators in plant senescence. Oxon British Plant Growth Regulator Group. Wantage 1:27–45.

Barrôco RM, De Veylder L, Magyar Z, Engler G, Inze D and Mironov V (2003) Novel complexes of cyclin-dependent kinases and a cyclin-like protein from Arabidopsis thaliana with a function unrelated to cell division. Cellular and Molecular Life Sciences 60:401–412.

Barrôco RM, Peres A, Droual AM, et al. (2006) The cyclin-dependent kinase inhibitor Orysa; KRP1 plays an important role in seed development of rice. Plant Physiol. 142:1053–1064.

Bedhomme M, Jouannic S, Champion A, Simanis V and Henry Y (2008) Plants, MEN and SIN. Plant Physiology and Biochemistry 46 1:1-10.

Bedhomme M, Mathieu C, Pulido A, Henry Y and Bergounioux C (2009) Arabidopsis monomeric G-proteins, markers of early and late events in cell differentiation. Int. J. Dev. Biol. 53:177-185

Besson A, Dowdy SF and Roberts JM (2008) CDK inhibitors: cell cycle regulators and beyond. Dev Cell. 14(2):159-69.

Bidlingmaier S, Weiss EL, Seidel C, Drubin DG and Snyder M. (2001) The Cbk1p pathway is important for polarized cell growth and cell separation in Saccharomyces cerevisiae. Mol. Cell. Biol. 21:2449–2462.

Bircher JA (1993) Dosage analysis of maize endosperm development. Annual review genet 27:181-203

Bisbis B, Delmas F, Joubès J, Sicard A, Hernould M, Inzé D, Mouras A and Chevalier C (2006) Cyclin-dependent Kinase (CDK) Inhibitors Regulate the CDK-Cyclin Complex Activities in Endoreduplicating Cells of Developing Tomato Fruit. The Journal of Biological Chemistry 281:7374-7383.

Bleeker PM, Hakvoort HW, Bliek M, Souer E and Schat H (2006) Enhanced arsenate reduction by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in arsenate-tolerant Holcus lanatus. Plant Journal 45:917–929.

Boavida LC, Becker JD and Feijó JA (2005) The making of gametes in higher plants International. Journal of Developmental Biology 49:595–614.

Borg M, Brownfield L and Twell D (2009) Male gametophyte development: a molecular perspective. Journal of Experimental Botany 60(5):1465-1478.

Bothos J, Tuttle RL, Ottey M, Luca FC and Halazonetis TD (2005) Human LATS1 is a mitotic exit network kinase. Cancer Res. 65:6568–6575.

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

Boudolf V, Vlieghe K, Beemster TSG, Magyar Z, Torres Acosta JA, Maes S, Van Der Schueren E, Inzé D and De Veylder L (2004) The Plant-Specific Cyclin-Dependent Kinase CDKB1;1 and Transcription Factor E2Fa-DPa Control the Balance of Mitotically Dividing and Endoreduplicating Cells in Arabidopsis. The Plant Cell 16:2683-2692.

Boudolf V, Lammens T, Boruc J, Van Leene J, Van Den Daele H, Maes S, Van Isterdael G, Russinova E, Kondorosi E, Witters E, De Jaeger G, Inzé D and De Veylder L (2009) CDKB1;1 Forms a Functional Complex with CYCA2;3 to Suppress Endocycle Onset. Plant Physiology 150:1482-1493.

Bourne Y, Watson MH, Hickey MJ, Holmes W, Rocque W, Reed SI and Tainer JA (1996) Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. Cell 84:863–874.

Boutte Y, Crosnier MT, Carraro N, Traas J and Satiat-Jeunemaitre B (2006) The plasma membrane recycling pathway and cell polarity in plants: studies on PIN proteins. J. Cell Sci. 119:1255–1265.

Brouhard GJ, Stear JH, Noetzel TL, Al-Bassam J, Kinoshita K, Harrison SC, Howard J, and Hyman AA (2008) XMAP215 is a processive microtubule polymerase, Cell 132:79–88.

Buckner B, Janick-Buckner D, Gray J, Johal GS. Cell death mechanisms in maize. Trends Plant Sci. 1998;3:218–223.

Burns N, Grimwade B, Ross-Macdonald PB, Choi EY, Finberg K, Roeder GS and Snyder M. (1994) Large-scale analysis of gene expression, protein localization, and gene disruption in Saccharomyces cerevisiae. Genes. Dev. 8:1087–1105.

Buschmann H, Chan J, Sanchez-Pulido L, Andrade-Navarro MA, Doonan JH and Lloyd CW (2006) Microtubule-associated AIR9 recognizes the cortical division site at preprophase and cell-plate insertion. Curr. Biol. 16:1938–1943.

Cai G and Cresti M (2006) The microtubular cytoskeleton in pollen tubes: structure and role in organelle trafficking. Plant Cell Monographs 3:157.

Caillaud MC, Paganelli L, Lecomte P, Deslandes L, Quentin M, et al. (2009) Spindle Assembly Checkpoint Protein Dynamics Reveal Conserved and Unsuspected Roles in Plant Cell Division. PLoS ONE 4(8):e6757.

Callus BA, Verhagen AM and Vaux DL (2006) Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. FEBS J. 273:4264–4276.

Camilleri C, Azimzadeh J, Pastuglia M, Bellini C, Grandjean O and Bouchez D (2002) The *Arabidopsis TONNEAU2* gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. Plant Cell 14:833–845.

Cannon MC, Terneus K, Hall Q, Tan L, Wang Y, Wegenhart BL, Chen L, Lamport DT, Chen Y and Kieliszewski MJ (2008) Self-assembly of the plant cell wall requires an extensin scaffold. Proc. Natl Acad. Sci. USA 12:2226–2231.

Castellano MM, del Pozo JC, Ramirez-Parra E, Brown S and Gutierrez C (2001) Expression and stability of *Arabidopsis* CDC6 are associated with endoreplication. Plant Cell 13:2671–2686.

Castellano MM, Boniotti MB, Caro E, Schnittger A and Gutierrez C (2004) DNA replication licensing affects cell proliferation or endoreplication in a cell type-specific manner. Plant Cell 16: 2380–2393.

Cebolla A, Vinardell JM, Kiss E, Oláh B, Roudier F, Kondorosi A and Kondorosi E (1999) The mitotic inhibitor ccs52 is required for endoreduplication and ploidy-dependent cell enlargement in plants. EMBO J. 18:4476–4484.

Chaiwongsar S, Otegui MS, Jester PJ, Monson SS and Krysan PJ (2006) The protein kinase genes MAP3K epsilon 1 and MAP3K epsilon 2 are required for pollen viability in Arabidopsis thaliana. Plant J. 48:193–205.

Chan EH, Nousiainen M, Chalamalasetty RB, Schafer A, Nigg EA and Sillje HH. (2005) The Ste20-like kinase Mst2 activates the human large tumour suppressor kinase Lats1. Oncogene 24:2076–2086.

Champion A, Jouannic S, Krapp A, Mockaitis K, Guillon S, Picaud A, Simanis V, Kreis M and Henry Y (2004) Identification of a plant G-protein, MAP3K and MAP4K involved in a putative plant septum initiation network pathway. J. Cell Sci. 117:4265–4275.

Chan J, Calder G, Fox S and Lloyd C (2005) Localization of the microtubule end binding protein EB1 reveals alternative pathways of spindle development in *Arabidopsis* suspension cells. Plant Cell 17:1737–1748.

Chang HY, Smertenko AP, Igarashi H, Dixon DP and Hussey PJ (2005) Dynamic interaction of NtMAP65-1a with microtubules *in vivo.* J. Cell Sci. 118:3195–3201.

Chaubal R and Reger BJ (1990) Relatively high calcium is localized in synergid cells of wheat ovaries. Sex Plant Reprod. 3:98–102.

Chaubal R and Reger BJ (1992a) Calcium in the synergid cells and other regions of pearl millet ovaries. Sex Plant Reprod. 5:34–46.

Chaubal R and Reger BJ (1992b) The dynamics of calcium distribution in the synergid cells of wheat after pollination. Sex Plant Reprod. 5:206–213.

Chaubal R and Reger BJ (1993) Prepollination degeneration in mature synergids of pearl millet: An examination using antimonite fixation to localize calcium. Sex Plant Reprod. 6:225–238.

Chaubet-Gigot N. (2000) Plant A-type cyclins. Plant Molecular Biology 43:659–675.

Chaudhury AM, Ming L, Miller C, Craig S, Dennis ES and Peacock WJ (1997) Fertilization-independent seed development in Arabidopsis thaliana. Proc Natl Acad Sci USA 94:4223–4228.

Chaudhury AM, Craig S, Dennis ES and Peacock WJ (1998) Ovule and embryo development, apomixis and fertilization. Curr. Opin. Plant Biol. 1:26–31.

Chaudhury AM, Koltunow A, Payne T, Luo M, Tucker MR, Dennis ES and Peacock WJ (2001) Control of early seed development. Annu. Rev. Cell Dev. Biol. 17:677–699.

Chaudhury AM and Berger F (2001) Maternal control of seed development. Semin. Cell Dev. Biol. 12:381–386 .

Chen C, Marcus A, Li W, Hu Y, Vielle Calzada JP, Grossniklaus U, Cyr RJ and Ma H (2002) The *Arabidopsis ATK1* gene is required for spindle morphogenesis in male meiosis. Development 129:2401–2409.

Chen CT, Feoktistova A, Chen JS, Shim YS, Clifford DM, Gould KL and McCollum D (2008) The SIN kinase Sid2 regulates cytoplasmic retention of the S. pombe Cdc14-like phosphatase Clp1. Current Biology 18:1594–1599.

Chen Y and McCormick S (1996) Sidecarpollen, an Arabidopsis thaliana male gametophytic mutant with aberrant cell divisions during pollen development. Development 122:3243–3253.

Chen Z, Tan JLH, Ingouff M, Sundaresan V and Berger F (2008) Chromatin assembly Factor1 regulates the cell cycle but not cell fate during male gametogenesis in Arabidopsis thaliana. Development 135:65–73.
Cheung AY, Wang H and Wu H-M (1995) A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. Cell 82:383–393.

Cheung AY and Wu H-M (2000) Pollen tube guidance – Right on target. Science 293:1441–1442.

Choi Y, Gehring M, Johnson L, Hannon M, Harada JJ, Goldberg RB, Jacobsen SE and Fischer RL (2002) DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in Arabidopsis. Cell 110:33-42.

Chow A, Hao Y and Yang X (2009) Molecular characterization of human homologs of yeast MOB1 International Journal of Cancer DOI: 10.1002/ijc.24878

Christensen CA, King EJ, Jordan JR and Drews GN (1997) Megagametogenesis in Arabidopsis wild type and the Gf mutant. Sex. Plant Reprod. 10:49–64.

Christensen CA, Gorsich SW, Brown RH, Jones LG, Brown J, Shaw JM and Drews GN (2002) Mitochondrial GFA2 is required for synergid cell death in Arabidopsis. Plant Cell 14:2215-2232.

Churchman ML, Brown ML, Kato N, et al. (2006) SIAMESE, a plant-specific cell cycle regulator, controls endoreplication onset in Arabidopsis thaliana. Plant Cell. 18:3145–3157.

Chytilova E, Macas J, Sliwinska E, Rafelski S.M, Lambert GM and Galbraith DW (2000) Nuclear dynamics in Arabidopsis thaliana *Molecular Biology of the Cell* 11(8):2733-2741.

Ciciarello M, Mangiacasale R and Lavia P (2007) Spatial control of mitosis by the GTPase Ran, *Cell. Mol. Life Sci.* 64:1891–1914.

Citterio S, Varotto S, Albertini E, Feltrin E, Soattin M, Marconi G, Sgorbati S, Lucchin M and Barcaccia G. (2005) Alfalfa Mob1-like proteins are expressed in reproductive organs during meiosis and gametogenesis. Plant Mol. Biol. 58:789–808.

Citterio S, Piatti S, Albertini E, Aina R, Varotto S and Barcaccia G (2006) Alfalfa Mob1-like proteins are involved in cell proliferation and localize in the cell division midplane during cytokinesis. Exp. Cell. Res. 312:1050–1064.

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

Cleary AL and Smith LG (1998) The *Tangled1* gene is required for spatial control of cytoskeletal arrays associated with cell division during maize leaf development. Plant Cell 10:1875–1888.

Coelho CM, Dante RA, Sabelli PA, Sun Y, Dilkes BP, Gordon-Kamm WJ and Larkins BA (2005) Cyclin-dependent kinase inhibitors in maize endosperm and their potential role in endoreduplication. Plant Physiol. 138:2323–2336.

Colman-Lerner A, Chin TE and Brent R (2001) Yeast Cbk1 and Mob2 activate daughter-specific genetic programs to induce asymmetric cell fates. Cell 107:739–750.

Colombani J, Polesello C, Josue F and Tapon N (2006) Dmp53 activates the Hippo pathway to promote cell death in response to DNA damage, Curr. Biol. 16:1453–1458.

Couteau F, Belzile F, Horlow C, Grandjean O, Vezon D and Doutriaux MP (1999) Random chromosome segregation without meiotic arrest in both male and female meiocytes of a dmc1 mutant of Arabidopsis. Plant Cell 11:1623-1634.

Criqui MC, Weingartner M, Capron A, Parmentier Y, Shen WH, Heberle-Bors E, et al. (2001) Sub-cellular localisation of GFP-tagged tobacco mitotic cyclins during the cell cycle and after spindle checkpoint activation. The Plant Journal 28:569–581.

Criqui MC and Genschik P (2002) Mitosis in plants: how far we have come at the molecular level? Current Opinion in Plant Biology 5(6)487-493.

Cui X, Fan B, Scholz J and Chen Z. (2007) Roles of Arabidopsis cyclin-dependent kinase C complexes in cauliflower mosaic virus infection, plant growth, and development. The Plant Cell 19:1388–1402.

Danial NN and Korsmeyer SJ. (2004) Cell death: critical control points. Cell 116:205–219.

Das M, Wiley DJ, Chen X, Shah K and Verde F (2009) The Conserved NDR Kinase Orb6 Controls Polarized Cell Growth by Spatial Regulation of the Small GTPase Cdc42 Current Biology 19(15):1314-1319.

De Clercq A and Inze D (2006) Cyclin-dependent kinase inhibitors in yeast, animals, and plants: A functional comparison. Critical Reviews in Biochemistry and Molecular Biology 41(5):293-313.

De Jager SM, Maughan S, DewitteW, Scofield S and Murray JAH (2005) The developmental context of cell-cycle control in plants Seminars in Cell & Developmental Biology 16(3):385-396.

De Jager SM, Scofield S, Huntley RP, Robinson AS, den Boer BG and Murray JA (2009) Dissecting regulatory pathways of G1/S control in Arabidopsis: common and distinct targets of CYCD3;1, E2Fa and E2Fc. Plant Mol Biol. 2009 Aug 7. [Epub ahead of print]

De Schutter K, Joubès J, Cools T, Verkest A, Corellou F, Babiychuk E, Van Der Schueren E, Beeckman T, Kushnir S, Inzé D and De Veylder L (2007) Arabidopsis WEE1 kinase controls cell cycle arrest in response to activation of the DNA integrity checkpoint. Plant Cell. 19(1):211–225.

De Veylder L, Segers G, Glab N, Casteels P, Van Montagu M and Inzé D. (1997) The Arabidopsis Cks1At protein binds to the cyclin-dependent kinases Cdc2aAt and Cdc2bAt. FEBS Lett. 412:446–452.

De Veylder L, Beeckman T, Beemster GTS, et al. (2001a) Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell. 13:1653–1668.

De Veylder L, Beemster GTS, Beeckman T and Inzé D (2001b) CKS1At overexpression in Arabidopsis thaliana inhibits growth by reducing meristem size and inhibiting cell-cycle progression. The plant journal 25(6):617–626.

De Veylder L, Beeckman T, Beemster GTS, de Almeida Engler J, Ormenese S, Maes S, Naudts M, Van Der Schueren E, Jacqmard A, Engler G and Inzé D (2002) Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. EMBO J 21:1360–1368.

De Veylder L, Joubes J and Inze D (2003) Plant cell cycle transitions. Current Opinion in Plant Biology *6:*536–543.

De Veylder L, Beeckman T and Inzé D (2007) The ins and outs of the plant cell cycle. Nature reviews Molecular cell biology 8(8):655-65.

Del Pozo JC, Boniotti MB and Gutierrez C (2002) Arabidopsis E2Fc functions in cell division and is degraded by the ubiquitin-SCF^{AtSKP2} pathway in response to light. Plant Cell 14:3057–3071.

Del Pozo JC, Diaz-Trivino S, Cisneros N and Gutierrez C (2006) The balance between cell division and endoreduplication depends on E2FC-DPB, transcription factors regulated by the ubiquitin-SCF^{SKP2A} pathway in Arabidopsis. Plant Cell 18:2224–2235.

Demidov D, Van Damme D, Geelen D, Blattner FR and Houben A (2005) Identification and dynamics of two classes of Aurora-like kinases in *Arabidopsis* and other plants. Plant Cell 17:836–848.

Devroe E, Erdjument-Bromage H, Tempst P and Silver PA. (2004) Human Mob proteins regulate the NDR1 and NDR2 serine-threonine kinases. J. Biol. Chem. 279:24444–24451.

Dewitte W and Murray JAH (2003) The plant cell cycle. Annual Review of Plant Biology 54:235–264.

Dewitte W, Riou-Khamlichi C, Scofield S, Healy JMS, Jacqmard A, Kilby NJ and Murray JAH (2003) Altered cell cycle distribution, hyperplasia, and inhibited differentiation in *Arabidopsis* caused by the D-type cyclin CYCD3. Plant Cell 15: 79–92.

Dewitte W, Scofield S, Alcasabas AA, Maughan SC, Menges M, Braun N, Collins C, Nieuwland J, Prinsen E, Sundaresan V and Murray JAH (2007) *Arabidopsis* CYCD3 D-type cyclins link cell proliferation and endocycles and are rate-limiting for cytokinin responses. Proc Natl Acad Sci USA 104:14537–14542.

Dhankher OP, Rosen BP, McKinney EC and Meagher RB. (2006) Hyperaccumulation of arsenic in the shoots of Arabidopsis silenced for arsenate reductase (ACR 2). Proceedings of the National Academy of Sciences, USA 103:5413–5418.

Dhonukshe P and Gadella TWJ (2003) Alteration of microtubule dynamic instability during preprophase band formation revealed by yellow fluorescent protein-CLIP170 microtubule plus-end labeling, Plant Cell 15:597–611.

Dhonukshe P, Mathur J, Hulskamp M and Gadella T (2005) Microtubule plus-ends reveal essential links between intracellular polarization and localized modulation of endocytosis during division-plane establishment in plant cells. BMC Biol 3:11.

Dhonukshe P, Baluška F, Schlicht M, Hlavacka A, Šamaj J, Friml J and Gadella Jr TW (2006) Endocytosis of cell surface material mediates cell plate formation during plant cytokinesis, Dev Cell 10 137–150.

Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, Stierhof YD and Friml J (2007) Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in Arabidopsis. Curr *Biol* 17:520–527.

Dissmeyer N, Nowack MK, Pusch S, Stals H, Inzé D, Grini PE and Schnittger A (2007) T-loop phosphorylation of Arabidopsis CDKA;1 is required for its function and can be partially substituted by an aspartate residue. Plant Cell 19:972–985.

Dong J, Kim ST and Lord EM (2005). Plantacyanin plays a role in reproduction in Arabidopsis. Plant Physiol. 138:778–789.

Doonan JH and Kitsios G (2009) Functional evolution of Cyclin-Dependent Kinases Mol Biotechnol 42:14-29.

Doxsey S, McCollum D and Theurkauf W (2005) Centrosomes in cellular regulation, Annu. Rev. Cell Dev. Biol. 21:411–434.

Drews GN, Lee D and Christensen GA (1998) Genetic analysis of female gametophyte development and function. Plant Cell 10:5-17.

Drews GN and Yadegari R (2002) Development and function of the angiosperm female gametophyte Annual Review of Genetics 36:99-124.

Du LL and Novick P (2002) Pag1p, a novel protein associated with protein kinase Cbk1p, is required for cell morphogenesis and proliferation in Saccharomyces cerevisiae. Mol. Biol. Cell. 13:503–514.

Dumas C, Berger F, Faure JE and Matthys-Rochon E (1998) Gametes, fertilization and early embryogenesis in flowering plants. Advances in Botanical Research 28:232–261.

Durbarry A, Vizir I and Twell D (2005) Male germline development in Arabidopsis: duo pollen mutants reveal gametophytic regulators of generative cell cycle progression. Plant Physiology 137, 297–307.

Durbin R, Eddy S, Krogh A and Mitchison G. Biological sequence analysis: probabilistic models of proteins and nucleic acids. Cambridge University Press; UK: 1998.

Eady C, Lindsey K and Twell D (1995) The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. The Plant Cell 7:65–74.

Earley, Keith, Jeremy R. Haag, Olga Pontes, Kristen Opper, Tom Juehne, Keming Song and Craig S. Pikaard (2006) Gateway-compatible vectors for plant functional genomics and proteomics. The Plant J. 45:616-629.

Ebel C, Mariconti L and Gruissem W (2004) Plant retinoblastoma homologues control nuclear proliferation in the female gametophyte. Nature 429:776–780.

Edgar BA and Orr-Weaver TL (2001) Endoreplication cell cycles: more for less. Cell 105:297–306.

Edgar BA (2006) From cell structure to transcription: Hippo forges a new path. Cell. 124(2):267–73.

Emoto K, He Y, Ye B, Grueber WB, Adler PN, Jan LY and Jan YN (2004) Control of dendritic branching and tiling by the Tricornered-kinase/Furry signaling pathway in Drosophila sensory neurons. Cell. 119:245–256.

Evangelista M, Pruyne D, Amberg DC, Boone C, Bretscher A. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. Nat. Cell. Biol. 2002;4:260–269.

Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, McCann M, Rayon C, Vernhettes S and Hoefte H (2000) PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of Arabidopsis. Plant Cell 12:2409–2424.

Faure JE (2001) Double fertilization in flowering plants Discovery study methods and mechanisms. C.R. Acad. Sci. Ser. III Sci.Vie. 324:551-558.

Ferreira PC, Hemerly AS, Villarroel R, Van Montagu M and Inze D (1991) The *Arabidopsis* functional homolog of the p34cdc2 protein kinase. Plant Cell 3:531–540.

Fiebig A, Mayfield JA, Miley NL, Chau S, Fischer RL and Preuss D (2000) Alterations in CER6, a gene identical to CUT1, differentially affect long-chain lipid content on the surface of pollen and stems. Plant Cell 12:2001–2008.

Frenz LM, Lee SE, Fesquet D and Johnston LH (2000) The budding yeast Dbf2 protein kinase localises to the centrosome and moves to the bud neck in late mitosis. J. Cell. Sci. 113:3399–3408.

Fisher RP (2005) Secrets of a double agent: CDK7 in cell-cycle control and transcription. J Cell Sci. 118(22):5171-80.

Fobert PR, Gaudin V, Lunness P, Coen ES and Doonan JH (1996) Distinct classes of *cdc2*-related genes are differentially expressed during the cell division cycle in plants. Plant Cell 8:1465–76.

Fountain MD, Renz A and Beck E (1999) Isolation of a cDNA encoding a G1-cyclin-dependent kinase inhibitor (ICDK) from suspension cultured photoautotrophic Chenopodium rubrum L. cells. Plant Physiol. 120:339.

Francis D (2007) The plant cell cycle – 15 years on. New phytologist. 174: 261-278.

Franklin-Tong VE (2002) The difficult question of sex: the mating game. Curr Opin Plant Biol 5:14-18.

Fu Y, Yuan M, Huang BQ, Yang HY, Zee SY and O'Brien TP (2000) Changes in actin organization in the living egg apparatus of *Torenia fournieri* during fertilization. Sexual Plant Reproduction 12:315-322.

Fulop K, Pettko-Szandtner A, Magyar Z, Miskolczi P, Kondorosi E, Dudits D, et al. (2005a). The Medicago CDKC;1-CYCLINT;1 kinase complex phosphorylates the carboxy-terminal domain of RNA polymerase II and promotes transcription. The Plant Journal 42:810–820.

Fulop K, Tarayre S, Kelemen Z, Horváth G, Kevei Z, Nikovics K, Bakó L, Brown S, Kondorosi A and Kondorosi E. (2005b) Arabidopsis anaphase-promoting complexes: multiple activators and wide range of substrates might keep APC perpetually busy. Cell Cycle. 4(8):1084–1092.

Gaillard J, Neumann E, Van Damme D, Stoppin-Mellet V, Ebel C, Barbier E, Geelen D, Vantard M.(2008) Two microtubule-associated proteins of Arabidopsis MAP65s promote antiparallel microtubule bundling. Mol Biol Cell. 19(10):4534–44.

Gallegos ME and Bargmann CI (2004) Mechanosensory neurite termination and tiling depend on SAX-2 and the SAX-1 kinase. Neuron 44:239–249.

Gasser CS, Broadhvest J and Hauser BA (1998) Genetic analysis of ovule development Annu Rev Plant Physiol Plant Mol Biol 49:1–24.

Gehring M, Choi Y and Fischer RL (2004) Imprinting and seed development. Plant Cell 16: S203–S213.

Geng W, He B, Wang M and Adler PN (2000) The tricornered gene, which is required for the integrity of epidermal cell extensions, encodes the Drosophila nuclear DBF2-related kinase. Genetics. 156:1817–1828.

Giangrande PH, Hallstrom TC, Tunyaplin C, Calame K and Nevins JR (2003) Identification of E-box factor TFE3 as a functional partner for the E2F3 transcription factor. Mol Cell Biol 23:3707–3720.

Gong Z, Morales-Ruiz T, Ariza RR, Roldan-Arjona T, David L and Zhu JK (2002) ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. Cell 111:803-814.

Gonzalez N, Gévaudant F, Hernould M, Chevalier C and Mouras A (2007) The cell cycle-associated protein kinase WEE1 regulates cell size in relation to endoreduplication in developing tomato fruit. The Plant Journal 51(4):642-655.

Grafi G and Larkins BA (1995) Endoreduplication in maize endosperm: involvement of M phase-promoting factor inhibition and induction of S phase-related kinases. Science 269:1262–1264.

Griffin BA, Adams SR and Tsien RY (1998) Specific Covalent Labeling of Recombinant Protein Molecules Inside Live Cells. Science 281:269-272.

Grini PE, Jurgens G and Hulskamp M (2002) Embryo and endosperm development is disrupted in the female gametophytic capulet mutants of Arabidopsis. Genetics 162:1911-1925.

Grossniklaus U and Schneitz K (1998) The molecular and genetic basis of ovule and megagametophyte development Semin Cell Dev Biol 9:227-238.

Grossniklaus U, Vielle-Calzada JP, Hoeppner MA and Gagliano WB (1998). Maternal control of embryo genesis by medea, a Polycombgroup gene in Arabidopsis. Science 280:446-450.

Guindon S and Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704.

Gunning BE and Wick SM (1985) Preprophase bands, phragmoplasts, and spatial control of cytokinesis, J Cell Sci Suppl 2:157–179.

Guo C, Tommasi S, Liu L, Yee JK, Dammann R and Pfeifer GP (2007) RASSF1A is part of a complex similar to the Drosophila Hippo/Salvador/Lats tumor-suppressor network. Curr. Biol. 17:700–705

Guo J, Song J, Wang F and Zhang XS (2007) Genome-wide identification and expression analysis of rice cell cycle genes. Plant Mol. Biol. 64:349–360.

Haig D (1990) New perspectives on the angiosperm female gametophyte. Bot. Rev. 56:236–274.

Hall DB and Struhl K (2002) The VP16 activation domain interacts with multiple transcriptional components as determined by protein-protein cross-linking in vivo. J. Biol. Chem. 277:46043–46050.

Hall Q and Cannon MC (2002) The cell wall hydroxyproline-rich glycoprotein RSH is essential for normal embryo development in *Arabidopsis*. Plant Cell 14:1161–1172.

Hamada T, Igarashi H, Itoh TJ, Shimmen T and Sonobe S (2004) Characterization of a 200 kDa microtubule-associated protein of tobacco BY-2 cells, a member of the XMAP215/MOR1 family, Plant Cell Physiol. 45:1233–1242.

Hammarton TC, Lillico SG, Welburn SC and Mottram JC (2005) *Trypanosoma brucei* MOB1 is required for accurate and efficient cytokinesis but not for exit from mitosis. Mol. Microbiol. 56:104–116.

Hartwell LH, Culotti J, Pringle JR and Reid BJ (1974) Genetic control of the cell division cycle in yeast. Science 1*83*:46–51.

Harvey KF, Pfleger CM and Hariharan IK (2003) The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell 114:457–467.

Harvey K and Tapon N (2007) The Salvador–Warts–Hippo pathway—an emerging tumour-suppressor network. Nat. Rev. Cancer 7 182–191.

Hata S (1991) cDNA cloning of a novel cdc2^{+}/CDC28-related protein kinase from rice. FEBS Lett. 279:149–52.

Hayles J, Fisher D, Woollard A and Nurse P (1994) Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34^{cdc2}-mitotic B cyclin complex. Cell 78:813–22.

He SS, Liu J, Xie Z, O'Neill D and Dotson S (2004) Arabidopsis E2Fa plays a bimodal role in regulating cell division and cell growth. Plant Mol Biol 56:171–184.

He Y, Emoto K, Fang X, Ren N, Tian X, Jan YN and Adler PN (2005) *Drosophila* Mob family proteins interact with the related tricornered (Trc) and warts (Wts) kinases. Mol. Biol. Cell. 16:4139–4152.

Healy JM, Menges M, Doonan JH and Murray JA (2001). The Arabidopsis D-type cyclins CycD2 and CycD3 both interact in vivo with the PSTAIRE cyclin-dependent kinase Cdc2a but are differentially controlled. The Journal of Biological Chemistry 276:7041–7047.

Heese M, Gansel X, Sticher L, Wick P, Grebe M, Granier F and Jürgens G (2001) Functional characterization of the KNOLLE-interacting t-SNARE AtSNAP33 and its role in plant cytokinesis, J. Cell Biol. 155:239–249.

Hemerly A, Engler Jde A, Bergounioux C, Van Montagu M, Engler G, Inzé D and Ferreira P (1995) Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. EMBO J. 14:3925–3936.

Hemerly AS, Ferreira PC, Van Montagu M, Engler G and Inzè, D (2000). Cell division events are essential for embryo patterning and morphogenesis: studies on dominant-negative cdc2aAt mutants of *Arabidopsis*. Plant J. 23:123–130.

Hennebold JD, Tanaka M, Saito J, Hanson BR and Adashi EY (2000) Ovary-selective genes I: the generation and characterization of an ovary-selective complementary deoxyribonucleic acid library. Endocrinology 141:2725–2734.

Hepler PK, Valster A, Molchan T and Vos JW (2002) Roles for kinesin and myosin during cytokinesis. Philos. Trans. R. Soc. Lond. B Biol. Sci. 357:761–766.

Hergovich A, Stegert MR, Schmitz D and Hemmings BA (2006a) NDR kinases regulate essential cell processes from yeast to humans. Nature Reviews. 7:253–264.

Hergovich A, Schmitz D and Hemmings BA (2006b) The human tumour suppressor LATS1 is activated by human MOB1 at the membrane. Biochem. Biophys. Res. Comm. 345:50–58.

Hergovich A, Lamla S, Nigg EA and Hemmings BA (2007) Centrosome-Associated NDR Kinase Regulates Centrosome Duplication Molecular Cell 25(4):625-634.

Hergovich A, Cornils H and Hemmings BA (2008) Mammalian NDR protein kinases: From regulation to a role in centrosome duplication Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics 1784(1):3-15.

Hergovich A, Kohler RS, Schmitz D, Vichalkovski A, Cornils H and Hemmings BA (2009) The MST1 and hMOB1 Tumor Suppressors Control Human Centrosome Duplication by Regulating NDR Kinase Phosphorylation Current Biologi doi:10.1016/j.cub.2009.09.020

Higashiyama T, Kuroiwa H, Kawano S and KuroiwaT (1998) Guidance in vitro of the pollen tube to the naked embryo sac of Torenia fournieri. Plant Cell 10:2019-2031.

Higashiyama T (2002) The synergid cell: Attractor and acceptor of the pollen tube for double fertilization. J. Plant Res. 115:149–160.

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

Higgins DG, Bleasby AJ and Fuchs R (1992) CLUSTAL: a package for performing multiple sequence alignments on a micro-computer. Comput. Appl. Biosci. 8:189–191.

Hirayama T, Imajuku Y, Anai T, Matsui M and Oka A (1991) Identification of two cell-cycle-controlling cdc2 gene homologs in *Arabidopsis thaliana*. Gene 105:159–165.

Hirota T, Morisaki T, Nishiyama Y, Marumoto T, Tada K, Hara T, Masuko N, Inagaki M, Hatakeyama K and Saya H (2000) Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumour suppressor. J. Cell Biol. 149:1073–1086.

Hisaoka M, Tanaka A and Hashimoto H (2002) Molecular alterations of h-warts/LATS1 tumor suppressor in human soft tissue sarcoma. Lab Invest. 82:1427–1435.

His I, Driouich A, Nicol F, Jauneau A and Hoefte H. (2001) Altered pectin composition in primary cell walls of *korrigan*, a dwarf mutant of *Arabidopsis* deficient in a membrane bound endo-1,4-beta-glucanase. Planta. 212:348–358.

Holdaway-Clarke TL, Feijo JA, Hackett GR, Kunkel JG and Hepler PK (1997) Pollen tube growth and the intra cellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. Plant Cell 9:1999-2010.

Horvitz HR and Herskowitz I (1992) Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. Cell. 68:237–255.

Hoshino H, Yoneda A, Kumagai F and Hasezawa S (2003) Roles of actin-depleted zone and preprophase band in determining the division site of higher-plant cells, a tobacco BY-2 cell line expressing GFP-tubulin. Protoplasma 222:157–165.

Hou MC, Wiley DJ, Verde F and McCollum D (2003) Mob2p interacts with the protein kinase Orb6p to promote coordination of cell polarity with cell cycle progression. J. Cell Sci. 116:125–135.

Huang BQ and Russell SD (1992) Female germ unit: Organization, isolation, and function. Int. Rev. Cytol. 140:233–292.

Huang J, Wu S, Barrera J, Matthews K, Pan D. (2005) The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila homolog of YAP. Cell. 122:421–434.

Huck N, Moore JM, Federer M and Grossniklaus U (2003) The Arabidopsis mutant feronia disrupts the female gametophytic control of pollen tube reception. Development 130:2149-2159.

Huh JH, Bauer MJ, Hsieh T-F and Fischer RL (2008) Cellular programming of plant gene imprinting Cell 132:735-744.

Hulskamp M, Schneitz K and Pruitt RE (1995) Genetic evidence for along-range activity that directs pollen tube guidance in Arabidopsis. Plant Cell 7:57-64.

Huntley R, Healy S, Freeman D, Lavender P, de Jager S, Greenwood J et al. (1998) The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (CycD) proteins. Plant Molecular Biology *37:*155–169.

Hush J, Wu L, John PC, Hepler LH and Hepler PK (1996) Plant mitosis promoting factor disassembles the microtubule preprophase band and accelerates prophase progression in *Tradescantia.* Cell Biol Int 20:275–287.

Ikeda S, Nasrallah JB, Dixit R, Preiss S and Nasrallah ME (1997) An aquaporin-like gene required for the Brassica self-incompatibility response. Science 276:1564–1566.

Imai KK, Ohashi Y, Tsuge T, Yoshizumi T, Matsui M, et al. (2006) The A-type cyclin CYCA2;3 is a key regulator of ploidy levels in *Arabidopsis* endoreduplication. Plant Cell 18:382–96.

Inzé D and De Veylder L (2006) Cell cycle regulation in plant development. Annual review genet 40:77–105.

Iwakawa H, Shinmyo A and Sekine M (2006) Arabidopsis CDKA;1, a cdc2 homologue, controls proliferation of generative cells in male gametogenesis. The Plant Journal 45(5):819–831.

Jacqmard A, De Veylder L, Segers G, de Almeida Engler J, Bernier G, Van Montagu M and Inzé D (1999) CKS1At expression in Arabidopsis thaliana suggests a role for the protein in both the mitotic and the endoreduplication cycle. Planta 207:496–504.

Jaenisch R and Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33:245–254.

Jahn R, Lang T and Südhof TC (2003) Membrane fusion, Cell 112:519–533.

Jakoby MJ, Weinl C, Pusch S, Kuijt SJ, Merkle T, Dissmeyer N and Schnittger A (2006) Analysis of the subcellular localization, function and proteolytic control of the Arabidopsis CDK inhibitor ICK1/KRP1. Plant Physiol. 141:1291–1305.

Jasinski S, Perennes C, Bergounioux C and Glab N. (2002a) Comparative molecular and functional analyses of the tobacco cyclin-dependent kinase inhibitor NtKIS1a and its spliced variant NtKIS1b1. Plant Physiol. 130:1871–1882.

Jasinski S, Riou-Khamlichi C, Roche O, Perennes C, Bergounioux C and Glab N (2002b) The CDK inhibitor NtKIS1a is involved in plant development, endoreduplication and restores normal development of cyclin D3;1-overexpressing plants. J. Cell Sci. 115:973–982.

Jasinski S, Leite CS, Domenichini S, et al. (2003) NtKIS2, a novel tobacco cyclin-dependent kinase inhibitor is differentially expressed during the cell cycle and plant development. Plant Physiol. Biochem. 41:667–676.

Jauh G, Eckard K, Nothnagel E and Lord E (1997) Adhesion of lily pollen tubes on an artificial matrix. Sex. Plant Reprod. 10:859–865.

Johnson MA and Preuss D (2002) Plotting a course: Multiple signals guide pollen tubes to their targets. Dev. Cell 2:273–281.

Jia J, Zhang W, Wang B, Trinko R and Jiang J (2003) The Drosophila Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. Genes. Dev. 17:2514–2519.

Jimenez-Velasco A, Roman-Gomez J, Agirre X, Barrios M, Navarro G, Vazquez I, Prosper F, Torres A and Heiniger A (2005) Down-regulation of the large tumor suppressor 2 (LATS2/KPM) gene is associated with poor prognosis in acute lymphoblastic leukemia. Leukemia. 19:2347–2350.

John PC and Qi R (2008) Cell division and endoreduplication: doubtful engines of vegetative growth. Trends Plant Sci. 13(3):121-127.

Jouannic S, Champion A, Segui-Simarro JM, Salimova E, Picaud A, Tregear J, Testillano P, Risueno MC, Simanis V, Kreis M and Henry Y (2001) The protein kinases AtMAP3Kepsilon1

and BnMAP3Kepsilon1 are functional homologues of *S. pombe* cdc7p and may be involved in cell division. Plant J. 26:637–649.

Joubes J, Chevalier C, Dudits D, Heberle-Bors E, Inze D, Umeda M and Renaudin JP (2000) CDK-related protein kinases in plants. Plant Mol. Biol. 43:607–20.

Jürgens G (2005) Plant cytokinesis: Fission by fusion. Trends Cell Biol. 15:277–283.

Kaldis P (1999) The cdk-activating kinase (CAK): from yeast to mammals Cell Mol Life Sci. 55 (2):284–96.

Karimi M, Inze D. and Depicker A. (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends in Plant Science 7(5):193–5.

Kasahara RD, Portereiko MF, Sandaklie-Nikolova L, Rabiger DS and Drews GN (2005) MYB98 is required for pollen tube guidance and synergid cell differentiation in Arabidopsis. Plant Cell 17 2981–2992.

Katsuta J and Shibaoka H (1992) Inhibition by kinase inhibitors of the development and the disappearance of the preprophase band of microtubules in tobacco BY-2 cells, J Cell Sci 103:397–405.

Kawabe A, Matsunaga S, Nakagawa K, Kurihara D, Yoneda A, Hasezawa S, Uchiyama S, and Fukui K (2005) Characterization of plant Aurora kinases during mitosis. Plant Mol Biol 58:1–13.

Kawamura E, Himmelspach R, Rashbrooke MC, Whittington AT, Gale KR, Collings DA and Wasteneys GO (2006) MICROTUBULE ORGANIZATION 1 regulates structure and function of microtubule arrays during mitosis and cytokinesis in the *Arabidopsis* root, Plant Physiol. 140: 102–114

Kawamura E and Wasteneys GO (2008) MOR1, the *Arabidopsis thaliana* homologue of *Xenopus* MAP215, promotes rapid growth and shrinkage, and suppresses the pausing of microtubules *in vivo*. J. Cell Sci. 121:4114–4123.

Kawamura K, Murray JA, Shinmyo A, and Sekine M (2006) Cell cycle regulated D3-type cyclins form active complexes with plant-specific B-type cyclin-dependent kinase in vitro. Plant Molecular Biology 61:311–327.

Ketelaar T, Faivre-Moskalenko C, Esseling JJ, de Ruijter NC, Grierson CS and Dogterom M and Emons AMC (2002) Positioning of nuclei in *Arabidopsis* root hairs: an actin-regulated process of tip growth. Plant Cell 14:2941–2955.

Khadaroo B, Robbens S, Ferraz C, Derelle E, Eychenie S, Cooke R, Peaucellier G, Delseny M, Demaille J, Van de Peer Y, Picard A and Moreau H (2004) The first green lineage cdc25 dual-specificity phosphatase. Cell Cycle 3:513–518.

Kim HJ, Oh SA, Brownfield L, Hong SH, Ryu H, Hwang I, Twell D and Nam HG (2008) Control of plant germ line proliferation by SCF^{FBL17} degradation of cell cycle inhibitors. Nature 455:1134–1137.

Kim S, Mollet J-C, Dong J, Zhang K, Park SY and Lord EM (2003) Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. Proc. Natl. Acad. Sci. USA 100: 16125–1613.

Kimbara J, Endo TR, Nasuda S (2004) Characterization of the genes encoding for MAD2 homologues in wheat. Chromosome Res 12:703–714.

Kinoshita T, Yadegari R, Harada JJ, Goldberg RB and Fischer RL (1999) Imprinting of the MEDEA Polycomb gene in the Arabidopsis endosperm. Plant Cell 11:1945–v1952.

Kirik V, Herrmann U, Parupalli C, Sedbrook JC, Ehrhardt DW and Hülskam M (2007) CLASP localizes in two discrete patterns on cortical microtubules and is required for cell morphogenesis and cell division in *Arabidopsis.* J. Cell Sci. 120:4416–4425.

Kiyosue T, Ohad N, Yadegari R, Hannon M, Dinneny J, Wells D, Katz A, Margossian L, Harada JJ, Goldberg RB and Fischer RL (1999) Control of fertilization-independent endosperm development by the MEDEA Polycomb gene in Arabidopsis. Proc Natl Acad Sci USA 96:4186-4191.

Kleine-Vehn J and Friml J (2008) Polar Targeting and Endocytic Recycling in Auxin-Dependent Plant Development Annual Review of Cell and Developmental Biology 24:447–473.

Kohler C, Hennig L, Spillane C, Pien S, Gruissem W and Grossniklaus U (2003) The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. Genes Dev 17:1540–1553.

Kondorosi E and Kondorosi A (2004) Endoreduplication and activation of the anaphase-promoting complex during symbiotic cell development. FEBS Lett. 567:152–157.

Kono A, Ohno R, Umeda-Hara C, Uchimiya H and Umeda M (2006) A distinct type of cyclin D, *CYCD4;2*, involved in the activation of cell division in *Arabidopsis.* Plant Cell Rep. 25:540–545.

Konopka CA, Schleede JB, Skop AR and Bednarek SY (2006) Dynamin and cytokinesis. Traffic 7:239–247.

Kosugi S and Ohashi Y (2002) E2Ls, E2F-like repressors of *Arabidopsis* that bind to E2F sites in a monomeric form. Journal of Biol Chem 277:16553–16558.

Kosugi S and Ohashi Y (2003) Constitutive E2F expression in tobacco plants exhibits altered cell cycle control and morphological change in a cell type-specific manner. Plant Physiology 132:2012–2022.

Krapp A and Simanis V (2008) An overview of the fission yeast septation initiation network (SIN) Biochemical Society Transactions 36:411-4115.

Krysan PJ, Jester PJ, Gottwald JR and Sussman MR (2002) An *Arabidopsis* mitogen-activated protein kinase kinase kinase gene family encodes essential positive regulators of cytokinesis, *Plant Cell* 14:1109–1120.

Kultonow A (1993) Apomixis: embryo sacs and embryos formed without meiosis or fertilization in ovules. Plant Cell 5:1425-1437.

Kultonow AM and Grossniklaus U (2003) Apomixis: a development perspective. Annual review of Plant Biology 54:547-574.

Kurihara D, Matsunaga S, Uchiyama S and Fukui K (2008) Live cell imaging plant aurora kinase has dual roles during mitosis. Plant Cell Physiology 49(8):1256-1261.

Kurischko C, Kuravi VK, Wannissorn N, Nazarov PA, Husain M, Zhang C, Shokat KM, McCaffery JM and Luca FC (2008) The Yeast LATS/Ndr Kinase Cbk1 Regulates Growth via Golgi-dependent Glycosylation and Secretion Mol Biol Cell. 19(12):5559–5578.

Iyer-Pascuzzi AS and Benfey PN (2009) Transcriptional networks in root cell fate specification, *Biochim Biophys Acta* 1789:315–325.

Lai ZC, Wei X, Shimizu T, Ramos E, Rohrbaugh M, Nikolaidis N, Ho LL and Li Y (2005) Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell. 120:675–685.

Lammens T, Boudolf V, Kheibarshekan L, Panagiotis Zalmas L, Gaamouche T, Maes S, Vanstraelen M, Kondorosi E, La Thangue NB, Govaerts W, Inzé D and de Veylder L (2008) Atypical E2F activity restrains APC/CCCS52A2 function obligatory for endocycle onset. Proc Natl Acad Sci USA 10:14721–14726.

Landrieu I, da Costa M, De Veylder L, Dewitte F, Vandepoele K, Hassan S, Wieruszeski J-M, Faure J-D, Van Montague M, Inze D and Lippens G (2004) A small CDC25 dual-specificity tyrosine-phosphatase isoform in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA 101:13380–13385.

Larson-Rabin Z, Li Z, Masson PH and Day CD (2009) FZR2/CCS52A1 Expression Is a Determinant of Endoreduplication and Cell Expansion in Arabidopsis Plant Physiology 149:874–884.

Lauber MH, Waizenegger I, Steinmann T, Schwarz H, Mayer U, Hwang I, Lukowitz W and Jürgens G (1997) The *Arabidopsis* KNOLLE protein is a cytokinesis-specific syntaxin. J. Cell Biol. 139:1485–1493.

Lechner E, Achard P, Vansiri A, Potuschak T and Genschik P (2006) F-box proteins everywhere. Current Opinion in Plant Biology 9:631–638.

Lee SE, Frenz LM, Wells NJ, Johnson AL, Johnston LH (2001) Order of function of the budding-yeast mitotic exit-network proteins Tem1, Cdc15, Mob1, Dbf2, and Cdc5. Curr. Biol. 11:784–788.

Lee YR and Liu B (2000) Identification of a phragmoplast-associated kinesin-related protein in higher plants. Curr. Biol. 10:797–800.

Lee YR, Giang HM and Liu B (2001) A novel plant kinesin-related protein specifically associates with the phragmoplast organelles. Plant Cell 13:2427–2439.

Lee YRJ, Li Y and Liu B (2007) Two Arabidopsis phragmoplast-associated kinesins play a critical role in cytokinesis during male gametogenesis. The Plant Cell 19:2595–2605.

Leibfried A and Bellaïche Y (2007) Functions of endosomal trafficking in *Drosophila* epithelial cells. Curr. Opin. Cell Biol. 19(4):446–52.

Leiva-Neto JT, Grafi G, Sabelli PA, Dante RA, Woo Y, Maddock S, Gordon-Kamm WJ and Larkins BA (2004) A dominant negative mutant of cyclin-dependent kinase A reduces endoreduplication but not cell size or gene expression in maize endosperm. Plant Cell 16, 1854–1869.

Li H, Lin Y, Heath RM, Zhu MX and Yang Z (1999) Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. Plant Cell 11:1731-1742.

Lippincott J, Shannon KB, Shou WY, Deshaies J and Li R. (2001) The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. J. Cell Sci. 114:1379–1386.

Lipschutz JH and Mostov KE (2002) Exocytosis: the many masters of the exocyst. Curr. Biol. 12:R212–R214.

Liu B and Lee YRJ (2001) Kinesin-related proteins in plant cytokinesis. J. Plant Growth Regul. 20:141–150.

Lloyd CW and Traas JA (1988) The role of F-actin in determining the division plane of carrot suspension cells – drugs studies. Development 102:211-221.

Lloyd C and Hussey P (2001) Microtubule-associated proteins in plants – why we need a map. Nat. Rev. Mol. Cell Biol. 2:40–47.

Lord EM and Russell SD (2002) The mechanisms of pollination and fertilization in plants. Annu. Rev. Cell Dev. Biol. 18:81–105.

Luca FC and Winey M (1998) MOB1, an essential yeast gene required for completion of mitosis and maintenance of ploidy. Mol. Biol. Cell. 9:29–46.

Luca FC, Mody M, Kurischko C, Roof DM, Giddings TH and Winey M (2001) *Saccharomyces cerevisiae* Mob1p is required for cytokinesis and mitotic exit. Mol. Cell Biol. 21:6972–6983.

Lui, H, Wang H, DeLong C, Fowke LC, Crosby WL and Fobert PR (2000) The Arabidopsis Cdc2a-interacting protein ICK2 is structurally related to ICK1 and is a potent inhibitor of cyclin-dependent kinase activity in vitro. Plant Journal 21:379–385.

Lukowitz W, Nickle TC, Meinke DW, Last RL, Conklin PL and Somerville CR (2001) Arabidopsis cyt1 mutants are deficient in a mannose-1–phosphate guanylyltransferase and point to a requirement of N–linked glycosylation for cellulose biosynthesis. Proc. Natl Acad. Sci. USA 98: 2262–2267.

Lum L and Beachy PA (2004) The Hedgehog response network: sensors, switches, and routers. Science 304, 1755–1759.

Luo M, Bilodeau P, Koltunow A, Dennis ES, Peacock WJ and Chaudhury AM (1999) Genes controlling fertilization-independent seed development in Arabidopsis thaliana. Proc Natl Acad Sci USA 96:296-301.

Luo M, Bilodeau P, Dennis ES, Peacock WJ and Chaudhury A (2000) Expression and parent-of-origin effects for FIS2, MEA, and FIE in the endosperm and embryo of developing Arabidopsis seeds. Proc Natl Acad Sci USA 97:10637-10642.

Magnard JL, Yang M, Chen Y-CS, Leary M and McCormick S (2001) The *Arabidopsis* gene *tardy asynchronous meiosis* is required for the normal pace and synchrony of cell division during male meiosis. Plant Physiol. 127:1157–66.

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

Magyar Z, De Veylder L, Atanassova A, Bakó L, Inzé D and Bögre L (2005) The role of the Arabidopsis E2FB transcription factor in regulating auxin-dependent cell division. Plant Cell 17:2527–2541.

Mah AS, Jang J and Deshaies RJ. (2001) Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. Proc. Natl. Acad. Sci. U.S.A. 98:7325–7330.

Mah AS, Elia A, EH, Devgan G, Ptacek J, Schutkowski M, Snyder M, Yaffe MB and Deshaies RJ (2005) Substrate specificity analysis of protein kinase complex Dbf2-Mob1 by peptide library and proteome array screening. BMC Biochem. 6:22.

Manning G, Whyte DB, Martinez R, Hunter T and Sudarsanam S (2002) The protein kinase complement of the human genome. Science 298:1912–1934.

Mao G, Chan J, Calder G, Doonan JH and Lloyd CW (2005) Modulated targeting of GFP-AtMAP65-1 to central spindle microtubules during division. Plant J. 43:469–478.

Marcus AI, Dixit R and Cyr RJ (2005) Narrowing of the preprophase microtubule band is not required for cell division plane determination in cultured plant cells. Protoplasma 226:169–174.

Mariconti L, Pellegrini B, Cantoni R, Stevens R, Bergounioux C, Cella R and Albani D (2002) The E2F family of transcription factors from *Arabidopsis thaliana*. J Biol Chem 277:9911–9919.

Marie D and Brown SCA (1993) A cytometric exercises in plant DNA histograms, with 2C values from 70 species. Biol Cell 78:41-51.

Mascarenhas JP and Machlis L (1962) Chemotropic response of Antirrhinum majus pollen to calcium. Nature 196:292-293.

Mayer U and Jürgens G (2004) Cytokinesis: lines of division taking shape. *C*urr. Opin. Plant Biol. 7:599–60.

Mayfield JA and Preuss D (2000) Rapid initiation of Arabidopsis pollination requires the oleosin-domain protein GRP17. Nat. Cell Biol. 2:128–130.

Mazanka E, Alexander J, Yeh BJ, Charoenpong P, Lowery DM, Yaffe M and Weiss EL (2008) The NDR/LATS Family Kinase Cbk1 Directly Controls Transcriptional Asymmetry PLoS Biol. 6(8):e203.

McCormick S (1993) Male gametophyte development. Plant Cell 5:1265–1275.

McCormick S (2004) Control of male gametophyte development. Plant Cell 16:S142–S153.

Meijer M and Murray JAH (2000) The role and regulation of D-type cyclins in the plant cell cycle. Plant Mol. Biol. 43:621–33.

Men S, Boutté Y, Ikeda Y, Li X, Palme K, Stierhof YD, Hartmann MA, Moritz T and Grebe M (2008) Sterol-dependent endocytosis mediates postcytokinetic acquisition of PIN2 auxin efflux carrier polarity. Nat. Cell Biol. 10(2):237–44.

Menges M, Murray JAH (2002) Synchronous *Arabidopsis* suspension cultures for analysis of cell cycle gene activity. Plant J. 30:203–12.

Menges M, de Jager SM, Gruissem W and Murray JA (2005) Global analysis of the core cell cycle regulators of Arabidopsis identifies novel genes, reveals multiple and highly specific profiles of expression and provides a coherent model for plant cell cycle control. The Plant Journal 41:546–566.

Menges M, Samland AK, Planchais S and Murray JAH (2006) The D-type cyclin CYCD3;1 is limiting for the G1-to-S-phase transition in *Arabidopsis*. Plant Cell 18:893–906.

Mercier R, Vezon D, Bullier E, Motamayor JC, Sellier A, Lefèvre F, Pelletier G and Horlow C (2001) SWITCH1 (SWI1): a novel protein required for the establishment of sister chromatid cohesion and for bivalent formation at meiosis. Genes Dev. 15:1859 –1871.

Miernyk JA and Thelen JJ (2008) Biochemical approaches for discovering protein–protein interactions. The Plant Journal 53(4):597-609.

Mimori-Kiyosue Y, Grigoriev I, Lansbergen G, Sasaki H, Matsui C, Severin F, Galjart N, Grosveld F, Vorobjev I, Tsukita S and Akhmanova A (2005) CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. J Cell Biology 168(1):141-53.

Mineyuki Y and Gunning BES (1990) A role for preprophase bands of microtubules in maturation of new cell walls, and a general proposal on the function of preprophase band sites in cell division in higher plants. J Cell Sci 97:527–537.

Mineyuki Y (1999) The preprophase band of microtubules: Its function as a cytokinetic apparatus in higher plants. Int. Rev. Cytol. 187 1–49.

Mogie M (1988) A model for the evolution and control of generative apomixis. Biol J Linn Soc 35:127-154.

Mollet JC, Park SY, Nothnagel EA and Lord EM (2000). A lily stylar pectin is necessary for pollen tube adhesion to an in vitro stylar matrix. Plant Cell 12:1737–1750.

Moore JM, Calzada JPV, Gagliano W and Grossniklaus U (1997) Genetic characterization of hadad, a mutant disrupting female gametogenesis in Arabidopsis thaliana. Cold Spring Harbor Symp. Quant. Biol. 62, 35-47.

Moreno CS, Lane WS and Pallas DC (2001) A Mammalian Homolog of Yeast MOB1 Is Both a Member and a Putative Substrate of Striatin Family-Protein Phosphatase 2A Complexes. The Journal of Biological Chemistry 276:24253–24260.

Morgan DO (1997) CYCLIN-DEPENDENT KINASES: Engines, Clocks, and Microprocessors Annual Review of Cell and Developmental Biology 13:261–291.

Morisaki T, Hirota T, Iida S, Maremoto T, Hara T, Nishiyama Y, Kawasuzi M, Hiraoka T, Mimori T, Araki N, Izawa I, Inagaki M and Saya H (2002) WARTS tumor suppressor is phosphorylated by Cdc2/cyclin B at spindle poles during mitosis, FEBS Lett. 529:319–324.

Motamayor JC, Vezon D, Bajon C, Sauvanet A, Grandjean O, Marchand M, Bechtold N, Pelletier G and Horlow C (2000) Switch (Swi1), an Arabidopsis thaliana mutant affected in the female meiotic switch. Sex Plant Reprod 12:209–218

Mrkobrada S, Boucher L, Ceccarelli DFJ, Tyers M and Sicheri F (2006) Structural and functional analysis of *Saccharomyces cerevisiae* Mob1. J. Mol. Biol 362:430–440.

Muller S, Han S and Smith LG (2006) Two kinesins are involved in the spatial control of cytokinesis in Arabidopsis thaliana Curr Biol 16:888–894.

Müller S, Wright AJ and Smith LG (2009) Division plane control in plants: new players in the band Trends in Cell Biology 19(4):180–188.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant, 15: 473–497.

Murray AW. (2004) Recycling the cell cycle: cyclins revised. Cell. 116:221–234.

Musacchio A and Salmon ED (2007) The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol 8:379–393.

Nakai T, Kato K, Shinmyo A and Sekine M (2006) Arabidopsis KRPs have distinct inhibitory activity toward cyclin D2-associated kinases, including plant-specific B-type cyclin-dependent kinase. FEBS Lett. 580:336–340.

Nasmyth K. (1996) At the heart of the budding yeast cell cycle. Trends Genet. 12:405–12.

Nei M and Rooney AP (2005) Concerted and birth-and-death evolution of multigene families. Annu. Rev. Genet. 39:121–52.

Nelson B, Kurischko C, Horecka J, Mody M, Nair P, Pratt L, Zougman A, McBroom L, Hughes T, Boone C and Luca F. (2003) RAM: a conserved signaling network that regulates Ace2p transcriptional activity and polarized morphogenesis. Mol. Biol. Cell. 14:3782–3803.

Nickle, T.C. and Meinke, D.W. (1998) A cytokinesis-defective mutant of Arabidopsis (cyt1) characterized by embryonic lethality, incomplete cell walls, and excessive callose accumulation. Plant J. 15:321–332.

Nigg EA (2002) Centrosome aberrations: cause or consequence of cancer progression? Nat. Rev. Cancer 2:815–825.

Nishihama R, Ishikawa M, Araki S, Soyano T, Asada T and Machida Y (2001) The NPK1 mitogen-activated protein kinase kinase kinase is a regulator of cell-plate formation in plant cytokinesis. Genes Dev. 15:352–363.

Nishihama R, Soyano T, Ishikawa M, Araki S, Tanaka H, Asada T, Irie K, Ito M, Terada M, Banno H, Yamazaki Y and Machida Y (2002) Expansion of the cell plate in plant cytokinesis requires a kinesin-like protein/MAPKKK complex. Cell 109:87–99.

Nishimura T, Yokota E, Wada T, Shimmen T and Okada K (2003) An *Arabidopsis* ACT2 dominant-negative mutation, which disturbs F-actin polymerization, reveals its distinctive function in root development. Plant Cell Physiol 44:1131–1140.

Nishiyama Y, Hirota T, Morisaki T, Hara T, Maremoto T, Iida S, Makino K, Yamamoto H, Hiraoka T, Kitamura N and Saya H (1999) A human homolog of Drosophila warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis. FEBS Lett. 459:159–165.

Nogami A and Mineyuki Y (1999) Loosening of a preprophase band of microtubules in onion (*Allium cepa* L.) root tip cells by kinase inhibitors. Cell Struct Funct 24:419–424.

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

Oh SA, Johnson A, Smertenko A, Rahman D, Park SK, Hussey PJ and Twell D (2005) A divergent cellular role for the FUSED kinase family in the plant-specific cytokinetic phragmoplast. Current Biology 15:2107–2111.

Ohad N, Margossian L, Hsu YC, Williams C, Repetti P and Fischer RL (1996) A mutation that allows endosperm development without fertilization. Proc Natl Acad Sci USA 93:5319-5324.

Ohad N, Yadegari R, Margossian L, Hannon M, Michaeli D, Harada JJ, Goldberg RB and Fischer RL (1999) Mutations in FIE, a WD Polycomb group gene, allow endosperm development without fertilization. Plant Cell 11:407-415.

Okuda S, Tsutsui H, Shiina K, Sprunck S, Takeuchi H, Yui R, Kasahara RD, Hamamura Y, Mizukami A, Susaki D, Kawano N, Sakakibara T, Namiki S, Itoh K, Otsuka K, Matsuzaki M, Nozaki H, Kuroiwa T, Nakano A, Kanaoka MM, Dresselhaus T, Sasaki N and Higashiyama T (2009) Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells Nature 458:357-361.

Otegui MS, Mastronarde DN, Kang BH, Bednarek SY and Staehelin LA (2001) Three-dimensional analysis of syncytial-type cell plates during endosperm cellularization visualized by high resolution electron tomography. Plant Cell 13:2033–2051.

Otegui MS and Staehelin LA (2004) Electron tomographic analysis of post-meiotic cytokinesis during pollen development in Arabidopsis thaliana. Planta 218:501–515.

Overholtzer M, Zhang J, Smolen GA, Muir B, Li W, Sgroi DC, Deng CX, Brugge JS and Haber DA (2006) Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proc. Natl. Acad. Sci. U. S. A. 103:12405–12410.

Owen HA and Makaroff CA. (1995) Ultrastructure of microsporogenesis and microgametogenesis in *Arabidopsis thaliana* (L.) Heynh. ecotype Wassilewskija (Brassicaceae). Protoplasma 185:7–21.

Pacini E (1996) Types and meaning of pollen carbohydrate reserves. Sexual Plant Reproduction 9:362–366.

Page DR and Grossniklaus U (2002) The art and design of genetic screens: Arabidopsis thaliana. Nat. Rev Genet. 3:124-136.

Palanivelu R and Preuss D (2000) Pollen tube targeting and axon guidance: parallels in tip growth mechanisms. Trends Cell Biol 10:517-524.

Palanivelu R, Brass L, Edlund AF and Preuss D (2003) Pollen tube growth and guidance is regulated by POP2, an Arabidopsis gene that controls GABA levels. Cell 114:47-59.

Palevitz BA and Cresti M (1989) Cytoskeletal changes during generative cell division and sperm formation in *Tradescantia virginiana*. Protoplasma 150:54–71.

Pan R, Lee YR and Liu B. (2004) Localization of two homologous Arabidopsis kinesin-related proteins in the phragmoplast. Planta 220(1):156-64.

Pan D (2007) Hippo signaling in organ size control Genes Dev. 21:886–897.

Pantalacci S, Tapon N and Leopold P (2003) The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nature. Cell. Biol. 5:921–927.

Panteris E (2008) Cortical actin filaments at the division site of mitotic plant cells: a reconsideration of the ‘actin-depleted zone’. New Phytol. 179:334–341.

Park CJ, Song SG, Lee PR, Shou WY, Deshaies RJ and Lee KS (2003) Loss of CDC5 function in *Saccharomyces cerevisiae* leads to defects in Swe1p regulation and Bfa1p/Bub2p-independent cytokinesis. Genetics 163:21–33.

Park JA, Ahn JW, Kim YK, Kim SJ, Kim JK, Kim WT and Pai HS (2005) Retinoblastoma protein regulates cell proliferation, differentiation, and endoreduplication in plants. Plant J. 42:153–163.

Park HO and Bi E (2007) Central roles of small GTPases in the development of cell polarity in yeast and beyond. Microbiol. Mol. Biol. Rev. 71:48–96.

Park SK, Howden R and Twell D (1998) The Arabidopsis thaliana Gametophytic mutation gemini pollen1 disrupts microspore polarity, division asymmetry and pollen cell fate. Development 125:3789–3799.

Park SK, Rahman D, Oh SA and Twell D (2004) Gemini pollen 2, a male and female gametophytic cytokinesis defective mutation. Sexual Plant Reproduction 17 (2): 63-70

Park SY, Jauh, GY, Mollet JC, Eckard KJ, Nothnagel EA, Walling LL and Lord EM (2000) A lipid transfer-like protein is necessary for lily pollen tube adhesion to an in vitro stylar matrix. Plant Cell 12:151–164.

Pennell RI and Lamb C (1997) Programmed cell death in plants Plant Cell. 9:1157–1168.

Peres A, Churchman ML, Hariharan S, Himanen K, Verkest A, Vandepoele K, Magyar Z, Hatzfeld Y, Van Der Schueren E, Beemster GT, Frankard V, Larkin JC, Inzé D and De Veylder L (2007) Novel plant-specific cyclin-dependent kinase inhibitors induced by biotic and abiotic stresses. J. Biol. Chem. 282:25588–25596.

Pesin JA and Orr-Weaver TL (2008) Regulation of APC/C Activators in Mitosis and Meiosis Annual Review of Cell and Developmental Biology Vol. 24:475-499.

Petroski MD and Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. Nature Reviews Molecular Cell Biology 6:9–20.

Pettko-Szandtner A, Meszaros T, Horvath GV et al. (2006) Activation of an alfalfa cyclin-dependent kinase inhibitor by calmodulin-like domain protein kinase. Plant J. 46:111–123.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acid Res. 29(9): 2002–2007.

Pickart CM (2001) Mechanisms underlying ubiquitination. Annu. Rev. Biochem. 70:503–33.

Pierson ES, Miller DD, Callaham DA, Shipley AM, Rivers BA, Cresti M and Hepler PK (1994) Pollen tube growth is coupled to the extracellular calcium ion flux and the intra cellular calcium gradient: Effect of BAPTA-type buffers and hypertonic media. Plant Cell 6:1815-1828.

Pierson ES, Miller DD, Callaham DA, van Aken J, Hackett G and Hepler PK (1996) Tip-localized calcium entry fluctuates during pollen tube growth. Dev Biol 174:160-173.

Pines J (1995) Cyclins and cyclin-dependent kinases: theme and variations. Adv. Cancer Res. 66:181–212.

Planchais S, Samland AK and Murray JAH (2004) Differential stability of *Arabidopsis* D-type cyclins: CYCD3;1 is a highly unstable protein degraded by a proteasome-dependent mechanism. Plant J 38:616–625.

Polesello C, Huelsmann S, Brown NH and Tapon N (2006) The Drosophila RASSF homolog antagonizes the hippo pathway. Curr. Biol. 16:2459–2465.

Ponchon L, Dumas C, Kajava AV, Fesquet D and Padilla A (2004) NMR solution structure of Mob1, amitotic exit network protein and its interaction with an NDR kinase peptide. J. Mol. Biol. 337:167–182.

Porceddu A, Stals H, Reichheld JP, Segers G, De Veylder L, de Pinho Barrôco R, Casteels P, Van Montagu M, Inzé D and Mironov V (2001) A Plant-specific Cyclin-dependent Kinase Is Involved in the Control of G2/M Progression in Plants The Journal of Biological Chemistry 276:36354-36360.

Pruyne D, Legesse-Miller A, Gao L, Dong Y and Bretscher A (2004) Mechanisms of polarized growth and organelle segregation in yeast. Annu. Rev. Cell Dev. Biol. 20:559–591.

Racki WJ, Becam AM, Nasr F and Herbert CJ. Cbk1p, a protein similar to the human myotonic dystrophy kinase, is essential for normal morphogenesis in Saccharomyces cerevisiae. EMBO J. 19:4524–4532.

Ramakers C, Ruijter JM, Lekanne Deprez RH, Moorman AFM (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339: 62–66.

Ramirez-Parra E, Frundt C and Gutierrez C (2003) A genome-wide identification of E2F-regulated genes in *Arabidopsis*, Plant J 33:801–811.

Ramirez-Parra E, López-Matas MA, Fründt C, Gutierrez C (2004) Role of an atypical E2F transcription factor in the control of *Arabidopsis* cell growth and differentiation. Plant Cell 16:2350–2363.

Ravi M, Marimuthu MPA, Siddiqi I (2008) Gamete formation without meiosis in Arabidopsis. Nature 451: 1121–1124

Ray A (1997) Three's company: Regulatory cross-talk during seed development. Plant Cell 9: 665–667.

Rechsteiner M and Rogers SW (1996) PEST sequences and regulation by proteolysis. Trends Biochem. Sci. 21: 267–271.

Reddy AS and Day IS (2001) Kinesins in the *Arabidopsis* genome: a comparative analysis among eukaryotes. BMC Genomics 2:2.

Reger BJ, Chaubal R and Pressey R (1992) Chemotropic responses by pearl millet pollen tubes. Sex Plant Reprod. 5:47-56.

Reichardt I, Stierhof YD, Mayer U, Richter S, Schwarz H, Schumacher K and Jurgens G (2007) Plant cytokinesis requires de novo secretory trafficking but not endocytosis. Curr Biol 17:2047–2053.

Reiser L and Fisher RL (1993) The ovule and the embryo sac. The Plant Cell. 5:1291–1301.

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

Richards AJ (2003) Apomixis in flowering plants: an overview. Phil Trans R Soc Lond B 358 1085-1093.

Riou-Khamlichi C, Huntley R, Jacqmard A and Murray JAH (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. Science 283:1541–1544.

Riou-Khamlichi C, Menges M, Healy JM and Murray JA (2000) Sugar control of the plant cell cycle: differential regulation of Arabidopsis D-type cyclin gene expression. Molecular and Cellular Biology 20:4513–4521.

Robinson DG, Jiang L and Schumacher K (2008) The endosomal system of plants: charting new and familiar territories. Plant Physiol 147:1482–1492.

Rodriguez-Boulan E, Kreitzer G and Müsch A (2005) Organization of vesicular trafficking in epithelia. Nat. Rev. Mol. Cell Biol. 6(3):233–47.

Roegiers F and Jan YN (2004) Asymmetric cell division. Curr Opin Cell Biol. 16:195–205.

Rossignol P, Stevens R, Perennes C, Jasinski S, Cella R, Tremousaygue D, Bergounioux C (2002) AtE2F-a and AtDP-a, members of the E2F family of transcription factors, induce *Arabidopsis* leaf cells to re-enter S phase. Mol Genet Genomics 266:995–1003.

Rotman N, Rozier F, Boavida L, Dumas C, Berger F and Faure JE (2003) Female control of male gamete delivery during fertilization in *Arabidopsis thaliana*. Curr Biol 13:432-436.

Rotman N, Durbarry A, Wardle A, Yang WC, Chaboud A, Faure JE, Berger F and Twell D (2005) A novel class of MYB factors controls sperm-cell formation in plants. Current Biology 15:244–248.

Roudier F, Fedorova E, Gyorgyey J, Feher A, Brown S, Kondorosi A and Kondorosi E (2000) Cell cycle function of a *Medicago sativa* A2-type cyclin interacting with a PSTAIRE-type cyclin-dependent kinase and a retinoblastoma protein. Plant J. 23:73–83.

Roudier F, Fedorova E, Lebris M, Lecomte P, Gyorgyey J, Vaubert D, Horvath G, Abad P, Kondorosi A and Kondorosi E (2003) The *Medicago* species A2-type cyclin is auxin regulated and involved in meristem formation but dispensable for endoreduplication-associated developmental programs. Plant Physiol. 131:1091–1103.

Ruchaud S, Carmena M and Earnshaw WC (2007) Chromosomal passengers: conducting cell division. Nature Reviews Molecular Cell Biology 8:798–812.

Ruggiero B, Koiwa H, Manabe Y, et al. (2004) Uncoupling the effects of abscisic acid on plant growth and water relations. Analysis of sto1/nced3, an abscisic acid-deficient but salt stress-tolerant mutant in Arabidopsis. Plant Physiol. 136:3134–3147.

Russell SD (1992) Double fertilization. Int. Rev. Cytol. 140:357–388.

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

Russell SD (1996) Attraction and transport of male gametes for fertilization. Sex. Plant Reprod. 9:337–342 .

Saiza JE and Fisher RP (2002) A CDK-Activating Kinase Network Is Required in Cell Cycle Control and Transcription in Fission Yeast Current biology 12 (13):1100–1105.

Samuels AL, Giddings TH Jr and Staehelin LA (1995) Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. J Cell Biol 130:1345–1357.

Sano T, Higaki T, Oda Y, Hayashi T and Hasezawa S (2005) Appearance of actin microfilament 'twin peaks' in mitosis and their function in cell plate formation, as visualized in tobacco BY-2 cells expressing GFP-fimbrin. Plant J. 44:595–605.

Sasabe M and Machida Y (2006) MAP65: a bridge linking a MAP kinase to microtubule turnover. Curr. Opin. Plant Biol. 9:563–570.

Sato, S., Kato, T., Kakegawa, K. et al. (2001) Role of the putative membrane-bound endo-1,4–beta-glucanase KORRIGAN in cell elongation and cellulose synthesis in Arabidopsis thaliana. Plant Cell Physiol. 42, 251–263.

Saxena, I.M. and Brown, R.M. Jr (2005) Cellulose biosynthesis: current views and evolving concepts. Ann. Bot. 96:9–21.

Schlaitz AL, Srayko M, Dammermann A, Quintin S, Wielsch N, MacLeod I, de Robillard Q, Zinke, A, Yates III JR, Müller-Reichert T, Shevchenko A, Oegema K and Hyman AA (2007) The *C. elegans* RSA complex localizes protein phosphatase 2A to centrosomes and regulates mitotic spindle assembly. Cell 128:115–127.

Schlisio S, Halperin T, Vidal M and Nevins JR (2002) Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. EMBO J 21:5775–5786.

Schneitz K, Hülskamp M, Pruitt RE (1995) Wild-type ovule development in *Arabidopsis thaliana*: a light microscope study of cleared whole-mount tissue. Plant Journal 7:731–49.

Schneitz K (1999) The molecular and genetic control of ovule development. Current Opinion in Plant Biology 2:13–17.

Schneitz K, Balasubramanian S and Schiefthaler U (1998) Organogenesis in plants: the molecular and genetic control of ovule development. Trends Plant Sci 3:468–472.

Schnittger A, Schobinger U, Stierhof YD and Hulskamp M (2002a). Ectopic B-type cyclin expression induces mitotic cycles in endoreduplicating Arabidopsis trichomes. Current Biology, 12:415–420.

Schnittger A, Schöbinger U, Bouyer D, Weinl C, Stierhof Y-D and Hulskamp M (2002b) Ectopic D-type cyclin expression induces not only DNA replication but also cell division in *Arabidopsis* trichomes. Proc Natl Acad Sci USA 99:6410–6415.

Schnittger A, Weinl C, Bouyer D, Schobinger U and Hulskamp M (2003) Misexpression of the cyclin-dependent kinase inhibitor *ICK1/KRP1* in single-celled *Arabidopsis* trichomes reduces endoreduplication and cell size and induces cell death. Plant Cell 15:303–315.

Schrick K, Fujioka S, Takatsuto S, Stierhof YD, Stransky H, Yoshida S and Jurgens G (2004) A link between sterol biosynthesis, the cell wall, and cellulose in Arabidopsis. Plant J 38:227–243.

Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H, Frommer WB and Rentsch D. (1999) LeProT1, a transporter for proline, glycine betaine, and {Gamma}-amino butyric acid in tomato pollen. The Plant Cell 11:377.

Scott RJ, Spielman M, Bailey J and Dickinson HG (1998) Parent-of-origin effects in seed development in *Arabidopsis thaliana* Development 124:2049–2062.

Scott RJ, Spielman M, Dickinson HG (2004) Stamen structure and function. The Plant Cell 16:S46.

Segers G, Gadisseur I, Bergounioux C, de Almeida Engler J, Jacqmard A, Montagu M and Inzé D (1996) The *Arabidopsis* cyclin-dependent kinase gene *cdc2bAt* is preferentially expressed during S and G_2 phases of the cell cycle. Plant J. 10:601–12.

Seguí-Simarro JM, Austin II JR, White EA and Staehelin LA (2004) Electron tomographic analysis of somatic cell plate formation in meristematic cells of *Arabidopsis* preserved by high-pressure freezing. Plant Cell 16:836–856.

Seguí-Simarro JM and Staehelin LA (2006) Cell cycle-dependent changes in Golgi stacks, vacuoles, clathrin-coated vesicles and multivesicular bodies in meristematic cells of *Arabidopsis thaliana*: a quantitative and spatial analysis. Planta 223:223–236.

Seiler S, Vogt N, Ziv C, Gorovits R and Yarden O (2006) The STE20/germinal center kinase POD6 interacts with the NDR kinase COT1 and is involved in polar tip extension in Neurospora crassa. Molecular Biology of the Cell 17(9):4080–4092.

Shen WH (2002) The plant E2F-Rb pathway and epigenetic control. Trends Plant Sci 7:505–511.

Sherr CJ (2004) Principles of tumor suppression. Cell 116:235–246.

Sherr CJ and Roberts JM (2004). Living with or without cyclins and cyclin-dependent kinases. Genes & Development, 18:2699–2711.

Shimizu KK and Okada K (2000) Attractive and repulsive interactions between female and male gametophytes in Arabidopsis pollen tube guidance. Development 127:4511–4518.

Shimotohno A, Matsubayashi S, Yamaguchi M, Uchimiya H and Umeda M (2003) Differential phosphorylation activities of CDK-activating kinases in *Arabidopsis thaliana.* FEBS letters 534: 69–74.

Shimotohno A, Umeda-Hara C, Bisova K, Uchimiya H and Umeda M (2004) The Plant-Specific Kinase CDKF;1 Is Involved in Activating Phosphorylation of Cyclin-Dependent Kinase-Activating Kinases in Arabidopsis Plant. Cell 16:2954–2966.

Shimotohno A,Ohno R, Bisova K , Sakaguchi N, Huang J, Koncz C, Uchimiya H and Umeda M (2006) Diverse phosphoregulatory mechanisms controlling cyclin-dependent kinase-activating kinases in Arabidopsis. Plant Journal 47(5):701–710.

Shou WY, Seol JH, Shevchenko A, Baskerville C, Moazed D, Chen ZWS, Jang J, Shevchenko A, Charbonneau H and Deshaies RJ (1999) Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex. Cell. 97:233–244.

Siddiqi I, Ganesh G, Grossniklaus U and Subbiah V (2000) The dyad gene is required for progression through female meiosis in Arabidopsis. Development 127:197–207.

Sluder G and Nordberg JJ (2004) The good, the bad and the ugly: the practical consequences of centrosome amplification. Curr. Opin. Cell Biol. 16:49–54.

Sluder G (2005) Two-way traffic: centrosomes and the cell cycle, Nat. Rev., Mol. Cell Biol. 6:743–748.

Simanis V (2003) Events at the end of mitosis in the budding and fission yeasts, J. Cell Sci. 116:4263–4275.

Smalle J and Vierstra RD (2004) The ubiquitin 26S proteasome proteolytic pathway. Annual Review of Plant Biology 55:555–590.

Smertenko AP, Chang HY, Wagner V, Kaloriti D, Fenyk S, Sonobe S, Lloyd C, Hauser MT and Hussey PJ (2004) The *Arabidopsis* microtubule-associated protein AtMAP65-1: molecular analysis of its microtubule bundling activity. Plant Cell 16:2035–2047.

Smertenko AP, Chang, HY, Sonobe, S, Fenyk, SI, Weingartner M, Bögre L, Hussey PJ (2006) Control of the AtMAP65-1 interaction with microtubules through the cell cycle. J. Cell Sci. 119 3227–3237.

Smertenko AP, Kaloriti D, Chang HY, Fiserova J, Opatrny Z and Hussey PJ (2008) The C-terminal variable region specifies the dynamic properties of *Arabidopsis* microtubule-associated protein MAP65 isotypes. Plant Cell 20 3346–3358.

Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in Arabidopsis. Plant Cell, 2: 755–767.

Smith LG (2001) Plant cell division: building walls in the right places. Nat. Rev. Mol. Cell Biol. 2:33–39.

Sollner R, Glasser G, Wanner G, Somerville CR, Jurgens G and Assaad FF (2002) Cytokinesis-defective mutants of Arabidopsis. Plant Physiol 129:678–690.

Sommer-Knudsen J, Clarke AE and Bacic A (1996) A galactose-rich, cell-wall glycoprotein from styles of Nicotiana alata. Plant J. 9:71–83.

Sommer-Knudsen J, Lush WM, Bacic A and Clarke AE (1998) Re-evaluation of the role of a transmitting tract-specific glycoprotein on pollen tube growth. Plant J. 13:529–535.

Sonnhammer ELL, Eddy SR, Birney E, Bateman A and Durbin R (1998) Pfam: multiple sequence alignments and HMM-profiles of protein domains. Nucleic Acids Res. 26:320–322.

Sorrell DA, Menges M, Healy JM, Deveaux Y, Amano C, Su Y, Nakagami H, Shinmyo A, Doonan JH, Sekine M and Murray JA (2001) Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar bright yellow-2 cells. Plant Physiol. 126:1214–23.

Sorrell DA, Marchbank A, McMahon K, Dickinson JR, Rogers HJ, Francis D. (2002) A WEE1 homologue from Arabidopsis thaliana. Planta 215:518–522.

Sorrell DA, Chrimes D, Dickinson JR, Rogers HJ, Francis D. 2005. The Arabidopis CDC25 induces a short cell length when over expressed in fission yeast: evidence for cell cycle function. New Phytologist 165:425–428.

Soyano T, Nishihama R, Morikiyo K, Ishikawa M and Machida Y (2003) NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes Dev. 17:1055–1067.

Sozzani R, Maggio C, Varotto S, Canova S, Bergounioux C, Albani D and Cella R (2006) Interplay between Arabidopsis activating factors E2Fb and E2Fa in cell cycle progression and development. Plant Physiol 140:1355–1366.

Spielman M, Vnkenoog R and Scott RJ (2003) Genetic mechanism of apomixes Phil Trans R Soc Lond B 358:1095-1103.

Spillane C, MacDougall C, Stock C, Kohler C, Vielle-Calzada JP, Nunes SM, Grossniklaus U and Goodrich J (2000) Interaction of the Arabidopsis Polycomb group proteins FIE and MEA mediates their common phenotypes. Curr Biol 10:1535-1538.

Springer PS, Holding DR, Groover A, Yordan C and Martienssen RA (2000) The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G1 phase and is required maternally for early Arabidopsis development. Development 127:1815-1822.

Springer PS, McCombie WR, Sundaresan V and Martienssen RA (1995) Gene trap tagging of PROLIFERA, an essential MCM2-3-5-like gene in Arabidopsis. Science 268:877-880.

St John MA, Tao W, Fei X, Fukumoto R, Carcangiu ML, Brownstein DG, Parlow AF, McGrath J, Xu T (1999) Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. Nature Genet. 21:182–186.

Stahl Y and Simon R (2005) Plant stem cell niches. Int J Dev Biol 49:479–489.

Stals H and Inzé D (2001) When plant cells decide to divide. Trends Plant Sci 6:359–364.

Stark GR and Taylor WR (2006) Control of the G2/M transition. Mol Biotechnol. 32(3):227-48.

Stavridi ES, Harris KG, Huyen Y, Bothos J, Verwoerd PM, Stayrook SE, Pavletich NP, Jeffrey PD and Luca FC (2003) Crystal structure of a human Mob1 protein: toward understanding Mob-regulated cell cycle pathways. Structure 11:1163–1170.

Stegmeier F, Visintin R and Amon A (2002) Separase, polo kinase, the kinetochore protein Slk19, and Spo12 function in a network that controls Cdc14 localization during early anaphase. Cell 108:207–220.

Stegmeier F and Amon A (2004) Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. Annual Review of Genetics 38:203–232.

Stelly DM, Peloquin SJ, Palmer RG and Crane CF (1984) Mayer's hemalum-methyl salicylate: A stain clearing technique for observations within whole ovules. Stain Technol., 59: 155–161.

Stern B and Nurse P (1996) A quantitative model for the cdc2 control of S phase and mitosis in fission yeast Trends Genet. 12 (9):345–350.

Stoepel J, Ottey MA, Kurischko C, Hieter P and Luca FC. (2005) The mitotic exit network Mob1p-Dbf2p kinase complex localizes to the nucleus and regulates passenger protein localization. Mol. Biol. Cell. 16:5465–5479

Strompen G, El Kasmi F, Richter S, Lukowitz W, Assaad FF, Jürgens G and Mayer U (2002) The *Arabidopsis HINKEL* gene encodes a kinesin-related protein involved in cytokinesis and is expressed in a cell cycle-dependent manner. Curr. Biol. 12:153–158.

Sugimoto-Shirasu K and Roberts K (2003) "Big it up": endoreduplication and cell-size control in plants. Curr. Opin. Plant Biol. 6:544–553.

Sullivan M and Morgan DO (2008) Finishing mitosis, one step at a time Nature Reviews Molecular Cell Biology 8:894-903.

Sun Y, Flannigan BA and Setter TL (1999) Regulation of endoreduplication in maize (Zea mays L.) endosperm. Isolation of a novel B1-type cyclin and its quantitative analysis. Plant Molecular Biology 41:245–258.

Takahashi Y, Miyoshi Y, Takahata C, Irahara N, Taguchi T, Tamaki Y and Noguchi S (2005) Down-regulation of LATS1 and LATS2 mRNA expression by promoter hypermethylation and its association with biologically aggressive phenotype in human breast cancers. Clin. Cancer Research 11:1380–1385.

Takatsuka H, Ohno R and Umeda M (2009) The Arabidopsis cyclin-dependent kinase-activating kinase CDKF;1 is a major regulator of cell proliferation and cell expansion but is dispensable for CDKA activation. The Plant Journal 59(3):475-487.

Tanaka H, Ishikawa M, Kitamura S, Takahashi Y, Soyano T, Machida C and Machida Y (2004) The *AtNACK1*/*HINKEL* and *STUD*/*TETRASPORE*/*AtNACK2* genes, which encode functionally redundant kinesins, are essential for cytokinesis in *Arabidopsis.* Genes Cells 9:1199–1211.

Tapon N, Harvey KF, Bell DW, Wahrer DC, Schiripo TA, Haber DA and Hariharan IK (2002) Salvador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell. 110:467–478.

Tarayre S, Vinardell JM, Cebolla A, Kondorosi A, Kondorosi E (2004) Two classes of the Cdh1-type activators of the anaphase-promoting complex in plants: novel functional domains and distinct regulation. Plant Cell 16:422–34.

Terbush DR, Maurice T, Roth D, Novick P (1996) The Exocyst is a multiprotein complex required for exocytosis in Saccharomyces cerevisiae. EMBO J. 15:6483–6494.

Thiele K, Wanner G , Kindzierski V, Jürgens G, Mayer U, Pachl F and Assaad FF (2009) The timely deposition of callose is essential for cytokinesis in Arabidopsis The Plant Journal 58(1):13-26.

Tian H-Q and Russell SD (1997) Calcium distribution in fertilized and unfertilized ovules and embryo sacs of Nicotiana tabacum. L. Planta 202:93-105.

Tiwari SC, Wick SM, Williamson RE and Gunning BE (1984) Cytoskeleton and integration of cellular function in cells of higher plants, J Cell Biol 99:63s–69s.

Toji S, Yabuta N, Hosomi T, Nishihara S, Kobayashi T, Suzuki S, Tamai K and Nojima H (2004) The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. Genes Cells 9:383–397.

Traas, J., Hulskamp, M., Gendreau, E. Hofte, H. (1998) Endoreduplication and development: rule without dividing? Curr. Opin. Plant Biol. 1 , 498–503.

Trammell MA, Mahoney NM, Agard DA and Vale RD (2008) Mob4 plays a role in spindle focusing in drosophila S2 cells. J Cell Sci. 121:1284-1292.

Tucker MR and Laux T (2007) Connecting the paths in plant stem cell regulation. Trends Cell Biol. 17:403-410

Twell D, Park SK and Lalanne E (1998) Asymmetric division and cell-fate determination in developing pollen. Trends in Plant Science 3:305–310.

Twell D, Park SK, Hawkins TJ, Schubert D, Schmidt R, Smertenko A and Hussey PJ (2002) MOR1/GEM1 plays an essential role in the plant-specific cytokinetic phragmoplast. Nature Cell Biology 4:711.

Uemura T, Ueda T, Ohniwa RL, Nakano A, Takeyasu K and Sato MH (2004) Systematic analysis of SNARE molecules in *Arabidopsis*: dissection of the post-Golgi network in plant cells. Cell Struct. Funct. 29:49–65.

Umeda M, Bhalerao RP, Schell J, Uchimiya H and Koncz C (1998) A distinct cyclin-dependent kinase-activating kinase of Arabidopsis thaliana. Proc. Natl. Acad. Sci. U*SA* 95:5021–5026.

Umeda M, Shimotohno A and Yamaguchi M (2005) Control of cell division and transcritption by ciclin-dependent kinase-activating kinases. Plant Cell Physiology 46(9):1437–1442.

Valster AH and Hepler PK (1997) Caffeine inhibition of cytokinesis: effects on the phragmoplast cytoskeleton in living Tradescantia stamen hair cells. Protoplasma 196:155–166.

Van Bruaene N, Joss G, Thas O and Van Oostveldt P (2003) Four-dimensional imaging and computer-assisted track analysis of nuclear migration in root hairs of *Arabidopsis thaliana. J Microsc* 211:167–178.

van den Heuvel S and Dyson NJ (2008) Conserved functions of the pRB and E2F families Nature Reviews Molecular Cell Biology 9:713-724.

Van Damme D, Bouget FY, Van Poucke K, Inzé D and Geelen D (2004) Molecular dissection of plant cytokinesis and phragmoplast structure: a survey of GFP-tagged proteins. Plant Journal 40:386–398.

Van Damme D, Coutuer S, De Rycke R, Bouget FY, Inze D and Geelen D (2006) Somatic cytokinesis and pollen maturation in Arabidopsis depend on TPLATE, which has domains similar to coat proteins. Plant Cell 18:3502–3518.

Van Damme D, Vanstraelen M and Geelen D (2007) Cortical division zone establishment in plant cells Trends in Plant Science 12(10):458–464.

Van Damme D, Inzé D and Russinova E (2008) Vesicle Trafficking during Somatic Cytokinesis Plant Physiol. 147(4)1544–1552.

Van Damme D and Geelen D (2008) Demarcation of the cortical division zone in dividing plant cells Cell Biology International 32:178-187.

Vandepoele K, Raes J, De Veylder L, Rouze P, Rombauts S and Inzé D (2002) Genome-wide analysis of core cell cycle genes in Arabidopsis. Plant Cell 14:903-916

Vanstraelen M, Van Damme D, De Rycke R, Mylle E, Inzé D and Geelen D (2006) Cell cycle-dependent targeting of a kinesin at the plasma membrane demarcates the division site in plant cells. Curr. Biol. 16:308-314.

Vasilescu J, Guo X and Kast J (2004) Identification of protein-protein interactions using in vivo cross-linking and mass spectrometry Proteomics 4(12):3845-3854.

Vaux DL and Korsmeyer SJ (1999) Cell death in development. Cell 96:245–254.

Vazquez-Novelle MD, Esteban V, Bueno A and Sacristan MP (2005) Functional homology among human and fission yeast Cdc14 phosphatases. J. Biol. Chem. 280:29144–29150.

Verde F, Wiley DJ and Nurse P (1998) Fission yeast Orb6, a ser/thr protein kinase related to mammalian rho kinase and myotonic dystrophy kinase, is required for maintenance of cell polarity and coordinates cell morphogenesis with the cell cycle. Proc. Natl. Acad. Sci. U.S.A 95:7526–7531.

Verkest A, Manes CL, Vercruysse S et al. (2005) The cyclin-dependent kinase inhibitor KRP2 controls the onset of the endoreduplication cycle during Arabidopsis leaf development through inhibition of mitotic CDKA;1 kinase complexes. Plant Cell. 17:1723–1736.

Verma DPS and Hong Z (2005) The ins and outs in membrane dynamics: tubulation and vesiculation. Trends Plant Sci. 10:159–165.

Vielle-Calzada JP, Thomas J, Spillane C, Coluccio A, Hoeppner MA and Grossniklaus U (1999) Maintenance of genomic imprinting at the Arabidopsis medea locus requires zygotic DDM1 activity. Genes Dev 13:2971-2982.

Vierstra RD (2003) The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. 8:135–42.

Vlieghe K, Boudolf V, Beemster GTS, Maes S, Magyar Z, Atanassova A, de Almeida Engler J, De Groodt R, Inzé D and De Veylder L (2005) The DP-E2F-like gene *DEL1* controls the endocycle in *Arabidopsis thaliana*. Curr Biol 15:59–63.

Vodermaier HC (2004) APC/C and SCF: controlling each other and the cell cycle. Curr. Biol. 14:R787–96.

Vos JW, Safadi F, Reddy AS and Hepler PK (2000) The kinesin-like calmodulin binding protein is differentially involved in cell division. Plant Cell 12:979–99.

Vos JW, Dogterom M and Emons AMC (2004) Microtubules become more dynamic but not shorter during preprophase band formation: a possible “search-and-capture” mechanism for microtubule translocation. Cell Motil. Cytoskeleton 57:246–258.

Waizenegger I, Lukowitz W, Assaad F, Schwarz H, Jürgens G and Mayer U (2000) The *Arabidopsis KNOLLE* and *KEULE* genes interact to promote vesicle fusion during cytokinesis. Curr. Biol. 10:1371–1374.

Walker JD, Oppenheimer DG, Concienne J and Larkin JC (2000) SIAMESE, a gene controlling the endoreduplication cell cycle in Arabidopsis thaliana trichomes. Development 127:3931–3940.

Walker KL, Müller S, Moss D, Ehrhardt DW and Smith LG (2007) *Arabidopsis* TANGLED identifies the division plane throughout mitosis and cytokinesis, Curr. Biol. 17 1827–1836.

Walton FJ, Heitman J, Idnurm A (2006) Conserved elements of the RAM signaling pathway establish cell polarity in the Basidiomycete Cryptococcus neoformans in a divergent fashion from other fungi. Mol. Biol. Cell Vol. 17:3768–3780.

Wang G, Kong H, Sun Y, et al. (2004) Genome-wide analysis of the cyclin family in Arabidopsis and comparative phylogenetic analysis of plant cyclin-like proteins. Plant Physiol. 135:1084–1099.

Wang H, Wu H-M and Cheung AY (1993). Development and pollination regulated accumulation and glycosylation of a stylar transmitting tissue-specific proline-rich protein. Plant Cell 5 1639–1650.

Wang H, Fowke LC and Crosby WL (1997) A plant cyclin-dependent kinase inhibitor gene. Nature 386:451–452.

Wang H, Qi Q, Schorr P, Cutler AJ, Crosby WL and Fowke LC (1998) ICK1, a cyclin-dependent protein kinase inhibitor from Arabidopsis thaliana interacts with both Cdc2a and CYCD3, and its expression is induced by abscisic acid. Plant J. 15:501–510.

Wang H, Zhou Y, Gilmer S, Whitwill S and Fowke LC (2000) Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. Plant J. 24 :613–623.

Wang H, Zhou Y and Fowke LC (2006) The emerging importance of CDK inhibitors in the regulation of the plant cell cycle and related processes. Can J. Bot. 84:640–65.

Wang H, Zhou Y, Bird DA, and Fowke LC (2008) Functions, regulation and cellular localization of plant cyclin-dependent kinase inhibitors Journal of Microscopy 231(2):234-246.

Wang W and Chen X (2004) HUA ENHANCER3 reveals a role for a cyclin-dependent protein kinase in the specification of floral organ identity in Arabidopsis. Development 131:3147–3156.

Wang Y, Magnard JL, McCormick S and Yang M (2004) Progression through meiosis I and meiosis II in *Arabidopsis* anthers is regulated by an A-type cyclin predominately expressed in prophase I. Plant Physiol. 136:4127–35.

Wei X, Shimizu T and Lai ZC (2007) Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. EMBO J. 26:1772–1781.

Weingartner M, Binarova P, Drykova D, Schweighofer A, David JP, Heberle-Bors E, Doonan J and Bögre L (2001) Dynamic Recruitment of Cdc2 to Specific Microtubule Structures during Mitosis The Plant Cell 13:1929-1943.

Weingartner M, Pelayo HR, Binarova P, Zwerger K, Melikant B, de la Torre C, et al. (2003) A plant cyclin B2 is degraded early in mitosis and its ectopic expression shortens G2-phase and alleviates the DNA-damage checkpoint. Journal of Cell Science 116:487–498.

Weingartner M, Criqui MC, Mészáros T, Binarova P, Schmit AC, et al. (2004) Expression of a nondegradable cyclin B1 affects plant development and leads to endomitosis by inhibiting the formation of phragmoplast. Plant Cell 16:643–57.

Weinl C., Marquardt S, Kuijt SJ, Nowack MK, Jakoby MJ, Hulskamp M and Schnittger A (2005) Novel functions of plant cyclin-dependent kinase inhibitors, ICK1/KRP1, can act non-cell-autonomously and inhibit entry into mitosis. Plant Cell. 17:1704–1722.

Weinmann AS, Yan PS, Oberley MJ, Huang TH-M and Farnham PJ (2002) Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. Genes Dev 16:235–244.

Weiss EL, Kurischko C, Zhang C, Shokat K, Drubin DG and Luca FC (2002) The Saccharomyces cerevisiae Mob2p-Cbk1p kinase complex promotes polarized growth and acts

with the mitotic exit network to facilitate daughter cell-specific localization of Ace2p transcription factor. J. Cell Biol. 158:885–900.

Wells J, Boyd KE, Fry CJ, Bartley SM and Farnham PJ (2000) Target gene specificity of E2F and pocket protein family members in living cells. Mol Cell Biol 20:5797–5807.

Whittington AT, Vugrek O, Wei KJ, Hasenbein NG, Sugimoto K, Rashbrooke MC, Wasteneys GO (2001) MOR1 is essential for organizing cortical microtubules in plants. Nature 411:610–613.

Wick SM and Duniec J (1984) Immunofluorescence microscopy of tubulin and microtubule arrays in plant cells. II. Transition between the preprophase band and the mitotic spindle. Protoplasma 122:45–55.

Wildwater M, Campilho A, Pérez-Pérez JM, Heidstra R, Blilou I, Korthout H, Chatterjee J, Mariconti L, Gruissem W and Scheres B (2005) The RETINOBLASTOMA-RELATED gene regulates stem cell maintenance in Arabidopsis roots. Cell 123:1–12.

Willemse MTM and van Went JL (1984) The female gametophyte. In Embryology of Angiosperms, B.M. Johri, ed (Berlin: Springer-Verlag) 159–196.

Williamson RE, Burn JE, Birch R, Baskin TI, Arioli T, Betzner AS and Cork A (2001) Morphology of rsw1, a cellulose-deficient mutant of Arabidopsis thaliana. Protoplasma 215:116–127.

Wolters-Arts M, Lush WM and Mariani C (1998) Lipids are required for directional pollen tube growth. Nature 392:818–821.

Wright AJ, Gallagher K and Smith LG (2009) discordia1 and alternative discordia1 Function Redundantly at the Cortical Division Site to Promote Preprophase Band Formation and Orient Division Planes in Maize. The Plant Cell 21:234-247.

Wu H-M, Wang H and Cheung AY (1995) A floral transmitting tissue specific glycoprotein attracts pollen tubes and stimulates their growth. Cell 82:383–393.

Wu HM, Wong E, Ogdahl J and Cheung AY (2000) A pollen tube growth-promoting arabinogalactan protein from Nicotiana alata is similar to the tobacco TTS protein. Plant J. 22:167–176.

Wu HM and Cheung AY (2000) Programmed cell death in plant reproduction. Plant. Mol. Biol. 44:267–281.

Wu S, Huang J, Dong J and Pan D (2003) Hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell. 114:445–456.

Xu XM, Zhao Q, Rodrigo-Peirisa T, Brkljacic J, He CS, Müller S, and Meier I (2008) RanGAP1 is a continuous marker of the *Arabidopsis* cell division plane. Proc. Natl. Acad. Sci. U.S.A. 105:18637–18642.

Yabuta N, Okada N, Ito A, Hosomi T, Nishihara S, Sasayama Y, Fujimori A, Okuzaki D, Zhao H, Ikawa M, Okabe M and Nojima H (2007) Lats2 is an essential mitotic regulator required for the coordination of cell division, J. Biol. Chem. 282 19259–19271.

Yadegari R, Kinoshita T, Lotan O, Cohen G, Katz A, Choi Y, Nakashima K, Harada JJ, Goldberg RB, Fischer RL, and OhadN (2000) Mutations in the FIE and MEA genes that encode

interacting Polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. Plant Cell 12:2367-2381.

Yadegari R and Drews GN (2004) Female Gametophyte Development .The Plant Cell 16:S133-S141.

Yamaguchi M, Umeda M and Uchimiya H (1998) A rice homolog of Cdk7/MO15 phosphorylates both cyclin-dependent protein kinases and the carboxy-terminal domain of RNA polymerase II. Plant J. 16:613–19.

Yamaguchi M, Fabian T, Sauter M, Bhalerao RP, Schrader J, et al. (2000) Activation of CDK-activating kinase is dependent on interaction with H-type cyclins in plants. Plant 24:11–20.

Yamamoto Y, Nishimura M, Hara-Nishimura I and Noguchi T (2003) Behavior of vacuoles during microspore and pollen development in *Arabidopsis thaliana*. Plant and Cell Physiology 44:1192.

Yasuhara H, Muraoka M, Shogaki H, Mori H and Sonobe S (2002) TMBP200, a microtubule bundling polypeptide isolated from telophase tobacco BY-2 cells is a MOR1 homologue, Plant Cell Physiol. 43:595–603

Yoneda A, Akatsuka M, Hoshino H, Kumagai F and Hasezawa S (2005) Decision of spindle poles and division plane by double preprophase bands in a BY-2 cell line expressing GFP-tubulin. Plant Cell Physiol 46:531–538.

Yoshizumi T, Nagata N, Shimada H and Matsui M (1999) An *Arabidopsis* cell cycle-dependent kinase-related gene, *CDC2b*, plays a role in regulating seedling growth in darkness. Plant Cell 11:1883–96.

Yoshizumi T, Tsumoto Y, Takiguchi T, Nagata N, Yamamoto YY, Kawashima M, Ichikawa T, Nakazawa M,. Yamamoto N, Matsui M (2006) INCREASED LEVEL OF POLYPLOIDY1, a conserved repressor of CYCLINA2 transcription, controls endoreduplication in Arabidopsis plant Cell 18 2452–2468.

Yu HG, Muszynski MG, Kelly Dawe R (1999) The maize homologue of the cell cycle checkpoint protein MAD2 reveals kinetochore substructure and contrasting mitotic and meiotic localization patterns J Cell Biol 145: 425-435.

Yu Y, Steinmetz A, Meyer D, Brown S and Shen WH (2003) The Tobacco A-Type Cyclin, Nicta;CYCA3;2, at the Nexus of Cell Division and Differentiation. The Plant Cell 15:2763-2777.

Zallen JA, Peckol EL, Tobin DM and Bargmann CI (2000) Neuronal cell shape and neurite initiation are regulated by the Ndr kinase SAX-1, a member of the Orb6/COT-1/warts serine/threonine kinase family. Mol. Biol. Cell. 11:3177–3190.

Zeng Q and Hong W (2008) The emerging role of the hippo pathway in cell contact inhibition, organ size control, and cancer development in mammals. Cancer Cell 13:188-192.

Zheng H, Bednarek SY, Sanderfoot AA, Alonso J, Ecker JR and Raikhel NV (2002) NPSN11 is a cell plate-associated SNARE protein that interacts with the syntaxin KNOLLE, Plant Physiol. 129:530–539.

Zhou Y, Fowke LC and Wang H (2002) Plant CDK inhibitors: studies of interactions with cell cycle regulators in the yeast two-hybrid system and functional comparisons in transgenic Arabidopsis plants. Plant Cell Rep . 20:967–975.

Zhou Y, Li G, Brandizzi F, Fowke LC and Wang H (2003) The plant cyclin-dependent kinase inhibitor ICK1 has distinct functional domains for in vivo kinase inhibition, protein instability and nuclear localization. Plant J. 35:476–489.

Zuo J, Niu QW, Nishizawa N, Wu Y, Kost B and Chua NH (2000) KORRIGAN, an Arabidopsis endo-1,4–beta-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. Plant Cell 12:1137–115.

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