Instituto Juan March
de Estudios e Investigaciones

129 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Co-sponsored by

INSTITUTE OF MOLECULAR
AGROBIOLOGY (Singapore)

Workshop on

Cross Talk Between Cell Division Cycle and Development in Plants

Organized by

V. Sundaresan and C. Gutiérrez

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INDEX

Introduction
C. Gutiérrez

Cell proliferation is a highly regulated process that plays a crucial role in generating the cells that make up an organism. At the cellular level, progression through the cell cycle occurs as a temporally coordinated series of events that involve the concerted action of both positive and negative signals. At the organismal level, in turn, maintenance of cell number in the body organs also requires cell renewal and, therefore production of new cells. Terminal differentiation and initiation of a particular developmental pattern implies that cells leave the cell cycle and restrict their proliferative capacity. This is frequently accompanied by activation of tissue-specific sets of genes and by repression and/or modulation of cell cycle regulatory functions.

Plants possess unique growth characteristics, developmental patterns and body architecture. These are the consequences of a number of plant-specific features: the plasticity of plant cells, which contribute to their capacity to dedifferentiate and regenerate new organs, the continuous post-embryonic body remodelling, which needs continuous proliferative potential and the lack of cell migration, among others. The past decade, and in particular the last three or four years, has witnessed a significant progress in the identification of plant cell cycle regulators and many aspects of their interactions are now becoming to be understood at the molecular level. Furthermore, the impact of cell division control on developmental pathways has concentrated the effort of many laboratories. Therefore, it is now the time to ask what are the roles played by cell cycle regulators and by the process of cell division in plant growth, morphogenesis and development. In other words, plant development and body architecture can be manipulated through the targeted action of cell cycle regulators and, conversely, developmental trends have a direct effect on the cell division process.

The topics outlined above were the subject of excellent presentations, containing a significant amount of unpublished data, as well as of active discussions throughout this workshop. The mainstream conclusion was that the interplay between the core cell cycle machinery and the developmental regulators is complex and finely regulated. Consistent with this, the initial structure of talks covering studies of cell cycle transitions, DNA replication and endoreplication, mitosis, exit to cell differentiation, hormonal effects and organ development was clearly surpassed. It was revealed that current approaches involve studies integrating studies at the molecular, cellular, physiological, genetic and developmental levels. We are facing an extremely attractive time where exciting ideas are very likely to contribute to a deeper understanding of the interplay between plant cell proliferation and development. In addition, comparison with eukaryotes where other growth and developmental strategies have evolved should prove very enlightening for a global understanding of cell proliferation and differentiation, cellular plasticity and, perhaps, neoplastic transformation.

Crisanto Gutiérrez

Session 1: G1/S transition, DNA replication and differentiation
Chair: Nam-Hai Chua

The roles of D-type cyclins in plant cell cycle control, growth and development

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Cell division plays important roles in plant growth and development, and can affect the pattern and rate of growth. The decision of cells to enter into the division cycle is likely to be of primary importance in both the execution of developmental programmes and in the responses of plants to the environment. The D-type cyclins (CycD) play an important role in the molecular control of this process by forming the regulatory component of cyclin-dependent kinases complexes, which phosphorylate plant homologues of the retinoblastoma protein. A primary event in the stimulation of cell division is the activation of cyclin D transcription by external signals. In addition, analysis of overexpressers of CycD3 and CycD1 suggest that CycD levels may control the differentiation of certain cell types. The general roles of CycD genes in cell cycle regulation and cellular differentiation, and potential interactions between CycD and the *SHOOTMERISTEMLESS* gene will be discussed.

Myb genes and control of the plant cell cycle

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Cell growth and proliferation is spatially and temporally regulated during plant development, responding to both intrinsic and environmental cues.

Cell proliferation is regulated at a number of levels, including the production and destruction of rate limiting activators such as cyclins. We have previously shown that different cyclin genes are expressed in patterns consistent with a role in either cell cycle progression or in development of the shoot apical meristem. Thus the cyclin B1 gene is expressed in G2 and early M phase of the cell cycle. D cyclin genes are expressed throughout the cell cycle but some genes show spatially restricted expression in the meristem. Much of the information required to produce such patterns of gene expression is present in the 5' promoter regions of these genes and in some cases are regulated by meristem control genes.

Evidence that *myb*-related genes may be directly or indirectly involved in regulating cyclin gene expression will be presented.

The retinoblastoma / E2F pathway and pre-replicative complex formation in proliferating and differentiating cells

Crisanto Gutiérrez, María del Mar Castellano, Carlos del Pozo, Elena Ramírez-Parra, M. Beatrice Boniotti, Corinne Fründt and Sara Díaz

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Plant cell proliferation and differentiation are regulated by a variety of internal and external effectors. We are interested in understanding the mechanisms operating at the G1/S transition of the cell cycle, which control the expression of genes required for S-phase entry and progression, and the assembly and function of pre-replicative DNA replication complexes. The retinoblastoma-related (RBR) pathway plays crucial roles in these processes (1). RBR interacts with E2F/DP transcription factors to regulate the expression of genes required for cell cycle progression, DNA replication and, most likely, maintenance of particular differentiated states and the re-entry of differentiated cells into the cell cycle. We have studied one mechanism to supress RBR function that involves phosphorylation of RBR by CDK/cyclin complexes (2).

The *Arabidopsis* genome encodes one RBR and, at least, six E2F and two DP proteins. We have found that the availability of one of the AtE2F proteins in proliferating and differentiated cells is regulated, among other pathways, through proteosome-mediated degradation (3). Consensus sequences for E2F binding are present in the promoters of a variety of genes. One of these genes encodes for a CDC6 protein, which in yeast and animal cells is crucial for loading the MCM proteins and the activation of pre-replicative DNA replication complexes. We have studied *AtCDC6* expression in relation to cell cycle as well as the regulation of DNA replication events in connection with *Arabidopsis* growth and development. *AtCDC6* is expressed not only in cycling cells but also in cells undergoing endoreplication cycles (4). We will discuss the expression patterns of *AtCDC6* in response to dark in wild type and mutant *Arabidopsis* plants, and the possible mechanisms by which AtCDC6 triggers DNA replication.

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Involvement of CCS52 WD-repeat proteins in cell differentiation

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Plant organ development relies on a constant interplay between cell cycle and differentiation programs. Cell differentiation necessitates an irreversible cell cycle exit that is co-ordinated by the expression of unique genes to specify tissue identity. Terminal differentiation can occur by complete loss of cell cycle activity or it could remain partially active. Exit from proliferation might be achieved by different mechanisms. One of them can be the ubiquitin-mediated proteolysis that leads to rapid and irreversible removal of target proteins. The anaphase promoting complex (APC) is one of the cell cycle-regulated ubiquitin ligases that is active from M to G1 phases during the cell cycle. Recently we identified a 52 kDa WD40-repeat cell cycle switch protein, CCS52 that is involved in mitosis arrest and cell differentiation. This plant protein similarly to other WD-repeat proteins of the same family acts as an APC activator and targets D-box or KEN motif-containing proteins for degradation.

In *Medicago truncatula* and *Arabidopsis thaliana* multiple forms of CCS52 were found that were classified as A- and B-types. The expression pattern of the *ccs52A* and *ccs52B* genes was different in synchronised alfalfa cell cultures, as well as in the different organs. We identified functional domains in the MtCCS52A proteins as well as proteins interacting either with CCS52A or CCS52B in the yeast two hybrid system. The effect of *ccs52* on plant and organ development or specific cell type differentiation was studied in transgenic *Medicago truncatula* and *Arabidopsis thaliana* plants. Our data show that MtCCS52A is required for endoreduplication that is essential for symbiotic cell differentiation during nodule development and for nematode-induced giant cell formation. The distinct activities of the *ccs52A* promoter-GUS and *ccs52B* promoter-GUS fusions in transgenic plants suggest non-redundant functions of these genes in plant development.

The destruction box pathway in higher plants

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The destruction box (D box) is a nine-amino acid sequence critical for the stability of different cell cycle regulatory proteins (among them, the mitotic cyclins and the "securin" proteins). Previously, we demonstrated the existence of the Dbox pathway in plants by showing that the N-terminal domains of two tobacco mitotic cyclins (Nicta;CycA3;1 and Nicta;CycB1;1) direct the specific degradation of the chloramphenicol acetyltransferase (CAT) reporter protein at the exit of mitosis and that this degradation is proteasome-dependent (Genschik et al., 1998). Mutations within the Dbox abolished the cell cycle-specific proteolysis of the fusion proteins. More recently we showed that endogenous cyclin B1 (Nicta;CycB1;1) is a target of the Dbox pathway (Criqui et al., 2000). Here, we will present our results showing the subcellular localisation of two GFP-tagged mitotic cyclins (B1 and A3), during the time course of the cell cycle and after spindle checkpoint activation. We will also present our unpublished data showing that the Dbox pathway is maintained active in several differentiated plant tissues.

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Session 2: Cell division and kinases
Chair: Mark Estelle

Cyclin dependent kinase inhibitors of the plant cell cycle

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Cyclin Dependent Kinases (CDK) plays a pivotal role in the regulation of cell cycle progression and are subject to multiple controls including transcription, proteolysis, phosphorylation/dephosphorylation and interaction with inhibitory proteins. The *Arabidopsis* genome contains seven genes encoding CDK inhibitors which were nominated, in analogy to their human counterparts, Kip-Related-Proteins or KRPs. KRP proteins specifically interact with A-type, but not with B-type CDKs. Using *S.pombe* we demonstrated that the KRP proteins are effective inhibitors of plant CDKA activity without affecting the activity of the yeast CDK. In concert, overexpression of the KRP proteins in *Arabidopsis* reduced CDK activity and resulted in a profound effect on plant growth and development. KRP overexpressing plants are reduced in size and have unexpectedly serrated leaves. We demonstrated that the overproduction of KRP proteins strongly reduces cell numbers and causes a six fold increase in average cell size. KRP overexpression also is shown to modify the degree of endoreduplication in plants. *In situ* hybridisation furthermore revealed that the different KRP proteins have unique tissue specific expression profiles consistent with a role in restricting cell division during plant development. Experiments are in progress to analyse the mechanisms by which developmental signals impinge on the levels of the KRPs.

Inhibitor studies to uncover the role of CDK complexes and serine-threonine phosphatases in the control of division cycle in cultured alfalfa cells

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In the control of the cell cycle progression, reversible phosphorylation of proteins by kinases and phosphatases belongs to the most fundamental regulatory mechanisms including also the formation of specific protein complexes and selective protein degradation. Experimental systems based on chemical or protein inhibitors of phosphorylation events can contribute to the discovery of functions of cyclin-dependent kinases (CDK) and phosphatases in regulation of plant cell division. In a yeast two hybrid screen a novel alfalfa CDK inhibitor (CKI Mt) has been recently identified that can interact with Cdc2*Ms*A kinase used as bait and D-type cyclins (*Medsa*CycD3, D4). This CKI protein shares low homology with other inhibitors of plant origin and $p27^{Kip1}$ protein. The recombinant CKI exhibited differential inhibitory effects on various CDK complexes. The Cdc2*Ms*A/B and Cdc2*Ms*F complexes were inhibited while the Cdc2*Ms*D complex was activated by this CKI. The phosphorylation of *Ms* retinoblastoma protein by Cdc2*Ms*A/B complex was more sensitive to this inhibition than histone H1 phosphorylation. The CKI protein itself is under phosphorylation control. It is especially important that phosphorylation of CKI by an alfalfa calmodulin like domain kinase (CDPK) could considerably increase the inhibitory function of the recombinant CKI. This finding can outline a possible molecular link between Ca^{2+}-centered signaling pathway and cell cycle control. Endothall, a synthetic inhibitor of various phosphatases (PP1 and PP2A) can significantly interfere with G_2/M progression in alfalfa cells. Inhibition of PP2A function caused an early activation of mitotic Cdc2*Ms*F kinase and disturbance of the co-ordination between chromosome condensation and microtubule organization.

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NPK1-mediated MAP kinase cascade: a controlling system of cytokinesis and differentiation of plant cells

Yasunori Machida*, Ryuichi Nishihama, Masaki Ishikawa, Satoshi Araki, Takashi Soyano, Tetsuhiro Asada**

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Plant cells have developed a unique system for cytokinesis, which is achieved by centrifugal formation of the cell plate, from the inside to the cortex of the cell. The formation of cell plate occurs within a phragmoplast, which is mainly composed of microtubules and microfilaments. Although candidate proteins that may control such a plant-specific inside-out mode of cytokinesis have been reported, its molecular mechanism is little understood. We report requirement of the NPK1 mitogen-activated protein kinase kinase kinase (MAPKKK) of tobacco1-3 for plant cytokinesis. Activity of NPK1 increases in late M phase of tobacco cell cycle. NPK1 is localized to an equatorial zone of the phragmoplast where a series of reactions related to formation of the cell plate occur. Expression of a kinase-negative mutant of *NPK1* (*NPK1KW*) in tobacco cells results in generation of multinucleate cells with aberrant forms of cell plates, which may be due to its dominant inhibitory effect. Kinetics of the lateral expansion of GFP::NPK1 signals localized to the phragmoplast rings in living cells shows that upon expression of *NPK1KW*, the expansion is arrested after the phragmoplast is formed4. These results indicate that NPK1 MAPKKK functions in cell plate expansion by controlling the lateral progression of the phragmoplast. In addition, our recent analysis of cell differentiation in transgenic tobacco plants showed that the system mediated by NPK1 also controls the differentiation of cell types.

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Organogenesis in plants: STRUBBELIG encodes a distinct putative leucine-rich repeat receptor kinase involved in the control of cell proliferation and cell morphogenesis in *Arabidopsis thaliana*

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A prominent biological question concerns how an organ attains its characteristic size and shape. Since there is essentially no cell movement in plants morphogenesis is largely achieved through the differential control of patterns of cell divisions and cell shape changes. Little is known about the communication between cells and groups of cells that has to occur to coordinate these processes in the shoot apical meristem and the emerging organ primordium. Here we present our analysis of the STRUBBELIG (SUB) gene. Plants defective for SUB function exhibit altered ovule development and thus female sterility. In sub mutants the outer integument undergoes extra-cell proliferation and characteristic outgrowths are formed. Genetic analysis of sub mutants indicated that SUB has a wider role than initially appreciated and is required for the control of the cell number of the apical meristem (and thus its size), floral organ initiation and outgrowth, and integument morphogenesis. We have cloned the gene by chromosomal walking. RT-PCR data showed that SUB is ubiquitously expressed in the plant. *In situ* hybridization experiments revealed that SUB mRNA accumulates in the inflorescence, floral meristems, and emerging organ primordia. Taken together, the sub mutant phenotype and the expression pattern implicate a role for SUB in cell proliferation control throughout plant development. Sequence analysis suggest that SUB encodes a member of a small but distinct family of leucine-rich repeat (LRR) transmembrane receptor-like kinases. At the N-terminal end a new protein domain can be defined which was called SUB domain. The SUB domain is followed by a small number of LRRs (5 to 7) and by a proline-rich region close to the membrane-spanning domain. The intracellular parts of the proteins carry a putative serine/threonine kinase domain. Thus, SUB defines a novel signaling pathway regulating cell proliferation and cell morphogenesis in *Arabidopsis*.

Session 3: Cell division, gametogenesis and embryogenesis
Chair: Dirk Inzé

Cell division genes in gametogenesis and embryogenesis

Venkatesan Sundaresan

We have been using large-scale gene trap transposon mutagenesis in *Arabidopsis thaliana* to identify genes controlling gametogenesis and embryogenesis. A number of the genes that we have identified appear to be genes involved in the control of cell division. It appears that gametophyte development is particularly sensitive to perturbations in cell cycle control, and mutations in these genes affect female gametophyte (embryo sac) development more severely than male gametophyte (pollen) development. For example, we find that mutation of the cdc16 gene which is required for the functioning of the Anaphase Promoting Complex (APC), causes arrest of the embryo sac at the two nuclear stage but no discernible affect on the pollen. Some mutations cause only partial gametophytic lethality, and therefore homozygous embryos can be generated, in most cases these embryos are not viable. A particularly interesting mutant called *tormoz*, appears to affect both the timing and orientation of the planes of cell division, generating arrested embryos in which longitudinal cell divisions are randomized. The gene encodes a WD40 repeat nuclear protein of unknown biochemical function, conserved in all eukaryotes, and essential for survival in yeast. These mutants highlight the importance of the regulated control of cell divisions in the early stages of plant development.

Cell division in *Arabidopsis* embryogenesis

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Developing organisms are growing populations of cells that exchange information about their relative positions and adopt specific fates accordingly. Cell division not only increases the number of cells but also separates daughter cells from each other so that adjacent cells can acquire different fates, either by inheriting a different cytoplasm or by responding to different cues from their neighbours. We are using the *Arabidopsis* embryo as an assay system for identifying genes involved in cell division. Two classes of mutants have been obtained. Cytokinesis mutants are defective in the actual process of division whereas cell-division mutants stop dividing altogether at a very early stage of embryogenesis (Heese et al, 1998; Nacry et al., 2000). The cytokinesis mutants identify components of the machinery for generating a cell plate that physically separates the forming daughter cells. The prototype gene is *KNOLLE* which encodes a cytokinesis-specific syntaxin required for vesicle fusion at the plane of division (Lauber et al., 1997). Ectopic expression of KNOLLE in non-dividing cells is not deleterious, and KNOLLE protein is targeted to the plasma membrane, suggesting that its cell cycle-regulated expression may only serve the purpose of providing enough KNOLLE protein for efficient vesicle fusion to occur in cytokinesis (Vö lker et al., 2001). Another gene, *KEULE*, encodes a Sec1 homolog that is required for cytokinesis and interacts with the KNOLLE syntaxin (Assaad et al., 2001). Whereas ***knolle*** or ***keule*** mutant embryos can still sustain some cell divisions, resulting in lethal seedlings, cytokinesis is completely abolished in the ***knolle keule*** double-mutant embryo which consists of a single large cell with multiple synchronously cycling nuclei (Waizenegger et al., 2000). While additional cytokinesis genes identified by mutation are being cloned, the *KNOLLE* gene has been used as a bait to identify interacting components involved in vesicle fusion during cytokinesis. One of the KNOLLE interactors is a SNAP25 homolog called AtSNAP33. Analysis of an *atsnap33* knockout mutation and the subcellular localisation of a myc-tagged variant of the protein indicate that AtSNAP33 is not only involved in cytokinesis but also in vesicle fusion at the plasma membrane (Heese et al., 2001). These observations suggest that the cytokinetic SNARE complex is related to those mediating fusion at the plasma membrane, each consisting of a syntaxin, a SNAP25 homolog and an (as yet unknown) synaptobrevin. The other class of mutants with defects in cell division are arrested in embryo development although the mutant cells stay alive until late stages. The mutant embryos are mushroom-shaped (*pilz*) and consist of one or a few enlarged cells with one or more enlarged nuclei (Mayer et al., 1999). They lack microtubules altogether, both interphase cortical arrays and mitotic arrays, such as preprophase band, spindle and phragmoplast. More recent data indicate that actin microfilaments are present in *pilz* mutant embryos. Molecular analysis of the affected genes suggests a plausible interpretation of the mutant phenotypes (Steinborn et al., unpublished).

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Control of cell division during reproductive development in *Arabidopsis*

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The plant life cycle alternates between a diploid and a haploid generation, the sporophyte and the gametophyte. In a spezialized female reproductive organ, the ovule, a single cell gets committed to the reproductive pathway, undergoes meiosis, and forms a tetrad of megaspores. Only one megapore survives and forms the eight-nucleated embryo sac through a series of syncytial divisions (Grossniklaus and Schneitz, 1998; Yang and Sundaresan, 2000). The genetic control of embryo sac development and its precisely controlled cell cycle program are not well understood. In fact, as many cell division genes are expected to play an essential cellular role, it is likely that some of them affect the haploid gametophytic phase of the life cycle and cannot be recovered as homozygotes. In order to identify genes involved in embryo sac development we performed an insertional mutagenesis screen using the transposon system developed by Sundaresan et al. (1995). We performed screens for mutants affecting the development and function of the embryo sac on the basis of reduced fertility and segregation ratio distortion caused by reduced transmission of insertional mutations affecting gametophyte development (Moore et al., 1997; Howden et al., 1998). Fourteen of these mutants were characterized in detail at the genetic and morphological level and two of them were found to have a profound effect on cell division in the female gametophyte. We will present our progress on the molecular characterization of these genes and discuss their role in cell division.

A third gene with profound effects on cell division within our collection was identified as a maternal effect embryo lethal mutant, *medea* (*mea*) (Grossniklaus et al., 1998). *MEA* is expressed in the female gametophyte prior to fertilization but shows its most dramatic phenotypes during seed development. *mea* belongs to *fertilization-independent seed* (*fis*) class of mutants (Chaudhury et al., 1997; Grossniklaus and Vielle-Calzada, 1998; Kinoshita et al., 1999) which share two phenotypes whose relationship has not yet been fully elucidated. While *mea* was isolated in a sreen for gametophytic mutants displaying a maternal effect (Grossniklaus et al., 1998), *fertilization-independent endosperm* (*fie*) and *fis2* were identified based on their ability to induce endosperm formation in the absence of fertilization (Ohad et al., 1996; Chaudhury et al., 1997). Seeds derived from a female gametophyte carrying a mutant allele of any of these genes abort irrespective of the paternal contribution. Aborting seeds show abnormalities in cell proliferation of both the endosperm and embryo, which grows to a giant size (Grossniklaus et al., 1998). In addition, endosperm formation is initiated in the absence of fertilization in *mea*, *fie* or *fis2* mutants, suggesting that these genes are required to repress endosperm proliferation until fertilization triggers cell division.

MEA, *FIE* and FIS2 are similar to *Enhancer of zeste* [*E(z)*], *Extra sex combs* (*Esc*), and *suppressor of zeste12* [*su(z)12*], respectively, which belong to the *Drosophila Polycomb* group (PcG) of genes (Grossniklaus et al., 1998; Ohad et al., 1999; Luo et al., 1999; Birve et al., 2001). Members of the structurally heterogeneous PcG form mutlimeric protein complexes that are thought to regulate gene expression by modulating higher order chromatin structure (Pirotta, 1997). While PcG genes are best known for their role in the regulation of the homeotic gene complexes in animals, many PcG members also regulate growth and cell proliferation (van Lohuizen, 1999), suggesting a conservation of this function from animals to plants (Grossniklaus et al., 2001). We have further investigated the role of *MEA* in the control of cell proliferation during embryogenesis and will present some of our recent findings.

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Characterization of a novel mutation that affects megaspore cell fate in *Arabidopsis thaliana*

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Meiosis of the megasporocyte produces four megaspores, namely tetrad. In *Arabidopsis*, only the chalazal megaspore is functional and will form an embryo sac, while the remaining three non-functional megaspores undergo programmed cell death. To investigate mechanisms controlling the megaspore cell fate, a screen of our Ds(KanR) insertion lines for gematophytic mutations was performed. A line, SGT1084 was identified which showed a KanR:KanS ratio of 1:4, instead of the expected 3:1 segregation. The mutation affects both male and female gametophytes, but is more severe in the female demonstrated by reciprocal crosses with wild type. About 37% of the ovules in the heterozygous plants were aborted. The aborted ovules are arrested at different developmental stages. In some mutant embryo sacs, the polar nuclei fail to fuse, resulting in the abortion of early embryos. More intriguingly, in about 5% of the aborted ovules, instead of a single embryo sac there were four large cells varying in size. In most cases, an embryo sac at the micropylar end was observed together with additional 3 smaller cells at the chalazal end. The origin of these big cells is not clear. Preliminary data suggest that in these ovules the three micropylar megaspores most likely fail to undergo apoptosis, but survive and acquire the ability to form embryo sacs and express a synergid-specific marker gene. It is also possible that cellularization is affected in the embryo sac. The mutant line contains a single Ds element inserted between two predicted genes. Recently complementation experiments showed that the downstream gene contributes to the male sterile phenotype, whereas the upstream gene whose promoter is active in megaspores and embryo sacs contributes mainly to the ovule phenotype.

Diversity of TITAN gene functions in *Arabidopsis* seed development

David Meinke

The titan mutants of *Arabidopsis* exhibit striking defects in seed development. The defining feature is the presence of abnormal endosperm with giant polyploid nuclei. Several TTN genes encode SMC proteins (condensins and cohesins) required for chromosome function at mitosis. Another TTN gene product (TTN5) is related to the ARL2 class of GTP binding proteins. Here we identify four additional TTN genes and present a general model for the titan phenotype. TTN1 was cloned after two tagged alleles were identified through a large-scale screen of T-DNA insertion lines. The predicted gene product is related to tubulin-folding cofactor D, which interacts with ARL2 in yeast and humans to regulate tubulin dynamics. We propose that TTN5 and TTN1 function in a similar manner to regulate microtubule function in seed development. The titan phenotype can therefore result from disruption of either chromosome dynamics (ttn3, ttn7, ttn8) or microtubule function (ttn1, ttn5). Three other genes have been identified that affect endosperm nuclear morphology. TTN4 and TTN9 appear to encode plant-specific proteins of unknown function. TTN6 is related to the isopeptidase T class of deubiquitinating enzymes that recycle polyubiquitin chains following protein degradation. Disruption of this gene may reduce the stability of the SMC complex or interfere with another factor that remains to be identified. Further analysis of TITAN networks should help to elucidate the regulation of microtubule function and chromosome dynamics during seed development.

Session 4: Cell division and hormones
Chair: Eva Kondorosi

The plant hormone auxin regulates SCF^{TIR1}-dependent degradation of the Aux/IAA proteins

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Some years ago we initiated a screen for *Arabidopsis* mutants with altered responses to exogenous auxin. This screen resulted in the identification of six loci called *AXR1* through *AXR6*, five of which have now been cloned. Remarkably, the genes all encode proteins that function in the same pathway. Three of the genes (*AXR2*, *AXR3*, and *AXR5*) encode members of the Aux/IAA family of proteins. Recent studies indicate that these proteins functions as repressors of auxin-regulated gene expression. The other two genes, *AXR1* and *AXR6*, function in the ubiquitin-proteasome pathway and are required for degradation of the Aux/IAA proteins. Our recent results indicate that auxin promotes an interaction between the Aux/IAA proteins and a ubiquitin protein ligase complex (E3) called SCF^{TIR1}, resulting in their degradation. Disruption of this process results in diverse, auxin-related effects on growth and development. One of the challenges of understanding auxin action is myriad of growth process that are regulated by auxin. We speculate that much of this complexity is related to the diversity in expression kinetics and patterns of the Aux/IAAs family members, as well as differences in their degradation kinetics.

Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development

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Auxin plays a key role in lateral root formation, but the signaling pathway for this process is poorly understood. We show here that *NAC1*, a new member of the *NAC* family, is induced by auxin and mediates auxin signaling to promote lateral root development. *NAC1* is a transcription activator consisting of an N-terminal conserved *NAC*-domain that binds to DNA and a C-terminal activation domain. This factor activates the expression of two downstream auxin-responsive genes, *DBP* and *AIR3*. Transgenic plants expressing sense or antisense *NAC1 cDNA* show an increase or reduction of lateral roots, respectively. Finally, overexpression can restore lateral root formation in the auxin-response mutant *tir1*, indicating that *NAC1* acts downstream of *TIR1*.

Towards dissecting the jasmonate signaling transduction pathway in *Arabidopsis*

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Jasmonates are a new class of plant hormone and regulate diverse plant developmental processes including pollen formation, fruit ripening and tuberization. Jasmonates also act as regulators to mediate plant responses to stress, wounding, insect attack and pathogen infection. Several jasmonate-insensitive mutants, including *jar1, jin1, jin4* and *coi1,* and jasmonate-supersensitive mutants, such as *cex1* and *cev1*, have been identified to study jasmonate signal pathway in *Arabidopsis*. The *COI1* gene has been isolated and provided a clue for the molecular mechanism of jasmonate action. We are currently using genetic and biochemical strategies to further dissect the jasmonate signaling transduction pathway in *Arabidopsis*. The recent results will be discussed.

Cell division and auxin signaling: AtE2F might be a connection

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Cell proliferation is controlled by a diverse group of cell cycle regulators. In animal cells, the retinoblastome protein (RB) and the E2F transcription factor play a crucial role in controlling the transition from G1 to S-phase. Recent findings have shown that HsE2F1 is regulated by specific degradation through the ubiquitin (UBQ)-SCFSKP2 pathway.

In the *Arabidopsis* genome, at least, six different E2F-related genes have been identified. One of these genes, AtE2F-I, is expressed in meristematic zones, along the young root system and during flower development. As we expected, AtE2F-I protein is able to bind, in association with DP, the consensus DNA E2F-binding site. Furthermore, we have identified an F-box protein that interacts with AtE2F-I, suggesting that the stability of AtE2F-I might be controlled through the UBQ-SCF pathway. Supporting this idea, we found that AtE2F-I accumulated when *Arabidopsis* cells were treated with MG132, a specific proteasome inhibitor. To analyze this *in vivo* and *in situ* we cloned an N-terminal region of AtE2F-I fused in frame to the GUS reporter protein. This chimeric protein is also degraded in a proteosome dependent manner. Interestingly, (N-t)AtE2F-I-GUS is more stable into axr1-12 genetic background and treatments with auxin provoke a slight reduction of the AtE2F-I protein levels. Furthermore, ectopic expression of E2F-I seems to alleviate partially the axr1 phenotype. Our results strongly suggest that AtE2F-I protein is regulated through the UBQ-SCFAUXIN pathway and might be the one of the connections between auxin and cell division.

Manipulation of leaf initiation and morphology by local modulation of cell wall extensibility and cell division

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To test the potential function of cell division and cell wall extensibility in leaf morphogenesis, we have developed a novel micro-induction technique based on a chemically-inducible promoter which allows us to transiently induce gene expression to the spatial resolution of a fraction of a meristem. We have used this system to locally modulate either cell wall extensibility (by expression of the cell wall protein expansin) or cell division patterns (by expression of both an A-type cyclin and a cdc25 protein) within the apical meristem and young leaf primordia.

The results of these manipulations indicate, firstly, that local increase in expansin gene expression leads to the initiation of phenotypically normal leaves, whereas altered cell division pattern in the meristem does not influence organogenesis. Secondly, manipulation of either expansin expression or cell division pattern on the flanks of young primordia leads to altered relative growth of the leaf lamina and, thus, altered leaf shape. However, whereas local expansin gene expression leads to local increase in tissue growth (gain of lamina), local alteration of cell division pattern leads to decreased growth (loss of lamina). These data identify cell wall extensibility as a regulator of morphogenesis and show that the influence of cell division on morphogenesis is context dependent.

Session 5: Cell division and organogenesis
Chair: Gerd Jürgens

Control of gene expression and development by MSI1

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MSI- and RbAp46/48-like proteins are components of several complexes involved in nucleosome assembly, histone acetylation or deacetylation and other chromatin modifying processes (Verreault, 2000). In addition, MSI-like proteins physically interact with the retinoblastoma cell cycle regulator protein in animals and plants (Qian et al., 1993; Ach et al., 1997). In the model plant , there are five genes encoding MSI-like proteins (Ach et al., 1997; Kenzior and Folk, 1998; own unpublished observations). Recently, one member of this family, MSI1, has been shown to form a complex with chromatin assembly complex (CAC) subunits CAC1 and CAC2 of (Kaya et al., 2001). The complex possesses nucleosome assembly activity *in vitro*. Mutants of CAC1 and CAC2 (*fas1* and *fas2*, respectively) have been isolated previously during genetic screens for meristem enlargement (Reinholz, 1966; Leyser and Furner, 1992). To elucidate the role of CAC3 *in vivo*, we constructed MSI1 overexpressor and antisense lines. While plants with strongly elevated protein levels had no obvious phenotype, plants with reduced MSI protein levels show a diverse set of developmental alterations, affecting phyllotaxy as well as leaf and flower morphology. The phenotype becomes progressively more severe through the plants life cycle, indicating a role of MSI1 in maintaining proper developmental states through successive rounds of cell divisions. Interestingly, the observed phenotypes only partially overlap with the phenotypes of the CAC1 and CAC2 mutants *fas1* and *fas2*, strongly suggesting major roles of MSI1 in addition to chromatin assembly. In order to characterize the consequences of MSI1-deficiency or overexpression on global patterns of gene expression, transcriptional profiling was performed using Affymetrix GeneChip® technology. These experiments revealed that alterations in transcript levels of specific subsets of genes, including several cell cycle regulators, form a link between gene expression and development.

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Analysis of FVE and PRE: two genes involved in the autonomous flowering promotion pathway

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The transition from the vegetative to the reproductive phase of a plant is a complex process controlled by multiple environmental and endogenous factors. The genetic and molecular dissection of this developmental switch in *Arabidopsis* has led to the involvement of at least two main flowering promotion pathways: i) the photoperiod promotion pathway, primarily involved in the photoperiodic induction of flowering and ii) the autonomous flowering promotion pathway (AFPP). Mutants in the AFPP show altered flowering phenotypes independently of the photoperiod in which plants grow, and therefore, it is speculated they might identify genes encoding central molecular elements controlling this process. In the present work we have analysed two mutants involved in the AFPP: i) the late flowering mutant fve, and ii) precocious (pre), an early flowering mutant largely suppressing the late flowering of fve. FVE has been located in the middle genomic region of chromosome 2, while PRE has been assigned to the bottom part of chromosome 1. Using map-based strategies we have isolated both genes, thus providing new molecular pieces that participate in the flowering induction process. FVE encodes AtMSI4 one protein related with the mammalian retinoblastoma associated proteins that could participate in transcriptional repression histone deacetylase complexes. PRE encodes a protein related to mammalian nucleoporin 96, a nucleoplasmic component of the nuclear pore complex. The current state of our understanding of the role of this proteins in the control of flowering time will be presented.

Cell cycle regulation during leaf development

Gerrit T.S. Beemster, Lieven de Veylder, Tom Beeckman and Dirk Inzé

We have developed a kinematic method to follow cell division, cell expansion and differentiation in the first two leaves of *A. thaliana*. We used this method to investigate the effect of overexpression of the cell cycle gene *KRP2*, which results in smaller leaves that have an altered shape, showing that cell division rate was reduced by ca 50% but that the developmental program was unaffected. We did flow cytometry on these leaves showing for the first time the developemental timing and rate of the process of endoreduplication. These data were subsequently used as a basis for molecular analysis of the cell cycle by use of cdc2a kinase activity essay, and the expression pattern of promotor activity of a number of cell cycle genes such as *Cdc2a*, *Cdc2b*, *Cks*, *Krp2*, *CycA2* and *CycB2*. These data give a comprehensive insight into the relationship between cell cycle regulation and leaf development.

Cell fate and cell division in the *Arabidopsis* root

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Saskia Folmer, Florian Frugier and Harald Wolkenfelt

The *Arabidopsis* root displays astonishing developmental flexibility despite nearly constant lineage relationships. This organ is therefore particularly well suited to study mechanisms of plant development.

Auxins, with indole-3-acetic acid as the major active form, have diverse roles in plant growth and development that have hitherto been difficult to disentangle. We have utilised an auxin-response promoter element and show that auxin is asymmetrically distributed in the root with a peak concentration in the distal tip. Mutants in auxin transport and response suggest that this distribution is required for patterning. Re-distribution of the auxin concentration peak by laser ablation and by polar auxin transport inhibitors correlates with changes in multiple cell fates and cell- and organ polarity. Thus, auxin and its transport machinery play major roles in organising pattern and polarity in the distal root tip. The role of auxin transport proteins in pattern formation in currently being investigated.

The *HOBBIT* gene is required during early embryonic development in the founder cell of the root meristem, and it is expressed throughout development in a cell-cycle dependent manner. The HOBBIT protein likely is a component of the Anaphase Promoting Complex, and the requirement of the *HOBBIT* gene for correct cell fate determination provides an interesting opportunity to investigate how cell division and cell fate may be linked in plants.

Downstream of auxin as a patterning cue, separate distal domains need to be specified. The *PLETHORA1*, *PLETHORA2*, *FEZ* and *SOMBRERO* genes are required for proper development of the columella and lateral root cap and current evidence suggests that they act downstream of auxin signaling.

Role of cell division in the shoot apical meristem

Olivier Grandjean, Teva Vernoux, Patrick Laufs and Jan Traas

The aerial parts of the plant are generated by groups of rapidly dividing cells called shoot apical meristems (SAMs). SAMs are highly stable and organised structures, which are divided into functionally distinct domains. Extensive genetic and cellular analysis has helped to identify a number of factors involved in meristem function. These include not only transcription factors defining cell identity in the different domains but also elements of signalling cascades coordinating cellular behaviour (e.g. Clark, 2001; Vernoux et. al. 2000a,b). We have been particularly interested in the spatial control of cell proliferation in the meristem. In previous studies (e.g. Laufs et al. 1998; Vernoux et al. 2000a,b) we have analysed the effects of different mutations in upstream regulators like CLAVATA or SHOOT MERISTEMLESS on cell division patterns. Here we will discuss the reverse approach, i.e. studying the effects of perturbed cell proliferation on developmental patterns. We have developed a technique to visualize living meristems in the confocal microscope. This method, which combines GFP marker lines and vital stains allows us to follow cell proliferation, cell expansion and cell differentiation in individual meristems for several days. In a first series of experiments, using primordium promoters (ANT and LFY) driving GFP expression, we followed the recruitment of meristematic cells in the incipient flower primordia. This suggested that cells preferentially activated the reporter genes just after cell division, i.e. while they are in early G1. To investigate this further, we have treated meristems with mitosis inhibiting drugs. Interestingly, when cell division was blocked using Oryzalin, (depolymerising microtubules and blocking the cells in G2/M) both cell expansion and cell differentiation proceeded in the treated meristems. In addition, cells at the periphery of the meristem and in the young primordia expanded much faster than those at the meristem centre. This showed that differential cell expansion rates and cell differentiation do not necesarily depend on the cell cycle. Implications of these and other experiments for the role of cell division in patterning at the SAM will be discussed.

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POSTERS

A cell cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in *Arabidopsis*, a CDKA/cyclin D complex

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The activity of cyclin-dependent kinases (CDK) is crucial for cell cycle transitions. We describe the identification of a CDK activity that phosphorylates the retinoblastoma-related (RBR) protein. A CDK/cyclin complex that binds to and phosphorylates RBR can be isolated from different plant sources, e.g. wheat, maize, *A. thaliana*, tobacco, and from cells growing under different conditions. The presence of a RBR-associated CDK activity correlates with the proliferative activity, suggesting that phosphorylation of RBR is one major event in actively proliferating tissues. In *A. thaliana*, this activity contains a PSTAIRE CDKA and, at least, cyclin D2. Furthermore, this CDK activity is cell cycle-regulated, as revealed by studies with highly-synchronized tobacco BY-2 cells where it is maximal in late G1 and early S-phase cells and progressively decreases until G2 phase. Aphidicolin-arrested, but not roscovitine-arrested cells, contain a PSTAIRE-type CDK that bound to and phosphorylated RBR. Thus, association with a D-type cyclin is a likely mechanism leading to CDK activation late in G1. Furthermore, we are trying to identify which of the putative phosphorylation sites present in ZmRBR are crucial for phosphorylation. We have generated point mutants of the ZmRBR phosphorylation sites and we are testing them with different kinase complexes. The results will be discussed. Our studies constitute the first report measuring the activity of CDK/cyclin complexes formed *in vivo* on RBR, an activity that fluctuates in a cell-cycle-dependent manner.

E2F-like elements mediate cell cycle regulation of tobacco RNR gene expression

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Ribonucleotide reductase (RNR), the enzyme involved in dNTPs synthesis, consists of two R1 and R2 subunits whose activities and gene expression are differentially regulated during the cell cycle in animals. We have isolated and characterized three cDNA clones from a tobacco S phase library, two encoding the large R1 subunit and one encoding the small R2 subunit (1). RNR gene expression was tightly regulated through the cell cycle progression of synchronized tobacco BY2 cells, with a maximal level in S phase. In different plant organs, RNR gene expression is highest in tissues with high mitotic activity. RNR2 promoter was cloned, and its sequence analysis revealed several potential cis-elements and more particularly E2F-like motifs, which are known to play a crucial role in cell cycle-regulated gene expression at the G1/S transition in animals. *In vivo* and *in vitro* structural analyses of this promoter have shown specific interactions between nuclear protein complexes and these E2F-like motifs. Binding activities of some of these complexes vary during cell cycle progression. A purified tobacco E2F factor could specifically interact with these E2F motifs, but with different affinities. Moreover, E2F factor was shown to be a part of protein complexes bound to E2F elements of RNR2 promoter. Functional analyses of the RNR2 promoter have highlighted the different roles played by E2F motifs in regulating RNR2 promoter activity throughout the cell cycle (2). RNR2 promoter activities driving GUS gene expression were analyzed in plantlets and various plant tissues. The results will be discussed.

Role of TCP genes in growth and development

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The TCP genes code for putative transcription factors containing the so-called "TCP domain" a predicted non-canonical bHLH region that mediates DNA binding and protein-protein interactions (1,2). The best-characterised members of this family are TEOSINTE BRANCHED 1 (TB1), responsible for the apical dominance in maize (3) and CYCLOIDEA (CYC) that controls the dorsoventral asymmetry of *Antirrhinum majus* flowers (4). Subtle changes in the expression patterns and levels of these genes have been responsible for two important evolutionary events: maize domestication (5, TB1) and the evolution of floral asymmetry in angiosperms (6, CYC). These genes affect not only the fate but also the growth patterns in the regions where they are expressed. Maize TB1 prevents the outgrowth of axillary meristems in lower nodes, causes the shortening of ear internodes and it is expressed in the stamen primordia of female florets and in upper florets of female inflorescences, structures that become arrested during development. CYC retards the growth of the young floral meristems and is responsible for the abortion of the uppermost stamen in the *Antirrhinum* flower. In the staminode its activity has been associated to a down-regulation of the genes cyclin D3b, B1, B2 cdc2c and cdc2d (7). In *Schyzanthus* (Solanaceae) the CYC ortholog is also expressed in the stamen primordia that will become aborted. On the other hand, other TCP genes are related to active cell division. PCF1 and PCF2 specifically bind to promoter elements of the PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) gene of rice that participates in DNA replication, DNA repair, and cell cycle control (8, 9) and the *Arabidopsis* TCP20 protein interacts, in two hybrid assays, with cdc2a. Moreover the expression patterns of the *Arabidopsis* genes TCP2 and TCP3 correlate with actively dividing regions in floral primordia (2).

Our working hypothesis is that some of the TCP genes, the PCF-like genes, promote cell proliferation at the transcriptional and postranscriptional level while others, such as the TB1/CYC-like genes, when accumulate at high levels in the same regions as the PCFs, could interact with them through their TCP domain and interfere or modulate their proliferation-promoting activity. The inhibition of growth observed in areas where CYC-like genes are expressed might therefore be a result from these interactions. TCP genes could couple development and cell proliferation by acting both as selector developmental genes (i.e. CYC and TB1) and as modulators of the activity and transcription of cell cycle genes.

We are trying to test this in *Arabidopsis thaliana* in which functional analysis is amenable. We have identified the complete *Arabidopsis* TCP family, formed by 24 members that map in all five chromosomes. TCP genes fall in two subclasses one related to CYC/TB1 and another one related to the PCFs We are carrying out a functional analysis of the complete *Arabidopsis* TCP family by expression pattern analysis, study of mutants and missexpression

experiments to elucidate their role in development and cell proliferation. Our results will be presented.

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Functional analysis of cyclin-dependent kinase inhibitors of *Arabidopsis*

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Cyclin-dependent kinases (CDKs) regulate the progression through the cell cycle by the phosphorylation of key substrates [1]. CDK activity is regulated upon many levels including the association of the kinases with regulatory proteins (cyclins, docking factors, ...) and posttranscriptional phosphorylation. In recent years in yeast and mammals another level of complexity in the process of CDK activation has been identified, existing under the form of families of mainly low molecular mass proteins named cyclin-dependent kinase inhibitors (CKIs). In mammals two CKI families can be recognised: the INK4 family and the Kip1/Cip1 family. Members of the latter family have been shown to inhibit CDK activity in response to anti-mitogenic stimuli. It is therefore believed that the Kip1/Cip1 inhibitors are involved in regulating the onset of cell differentiation by causing a cell cycle arrest.

Two-hybrid screens and database searches led to the identification of 7 different *Arabidopsis thaliana* gene products sharing sequence identity to the mammalian Kip1/Cip1 CKIs in a region of approximately 30 amino-acids [2]. Despite this low level of sequence conservation the plant CKIs, named Kip-related proteins (KRPs) were proven to inhibit CDK activity in planta: When overproduced, KRP2 dramatically inhibited cell cycle progression in leaf primordia cells without affecting the temporal pattern of cell division and differentiation in leaves. Mature transgenic leaves were serrated and consisted of enlarged cells. Although the ploidy levels in young leaves were unaffected, endoreduplication was suppressed in older leaves. We conclude that KRP2 exerts a plant growth inhibitory activity by reducing cell proliferation in leaves, but in contrast to its mammalian counterparts, it may not control the timing of cell cycle exit and differentiation.

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The tobacco CDK inhibitor NtKIS1a is involved in flower development

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In eukaryotes, development is partly driven by the regulation of cell cycle progression. This progression is controlled by cyclin-dependent kinases (CDKs) whose activity is regulated at several levels including inhibition by CDK inhibitors (CKIs). Here, we report the isolation and characterisation of the first tobacco CKI named NtKIS1a. Its C-terminal end shares strong sequence similarity with mammalian CIP/KIP inhibitors. The existence in planta of a variant, named NtKIS1b, suggests an alternative splicing of the NtKIS1 pre-mRNA. NtKIS1b lacks sequence similarity with mammalian CKIs. Consistent with this, we show that NtKIS1a but not NtKIS1b inhibits the kinase activity of BY-2 cell CDK/cyclin complexes. Similarly, tobacco D-type cyclins and an a-type CDK are shown to interact with NtKIS1a but not with NtKIS1b. NtKIS1a and NtKIS1b transcripts are mainly found in flowers and more precisely in stamens. Furthermore, using a recombinant potato virus X RNA, NtKIS1a and NtKIS1b proteins were overproduced in planta. Interestingly, only NtKIS1a overproducing plants show an abnormal flower development, suggesting for the first time the involvement of a CDK inhibitor in flower development.

TORMOZ is required for orientating cell division planes in early embryo development

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Precisely orientated cell division planes is a mark of early embryogenesis in *Arabidopsis*. However, relatively few plant genes have been identified to be involved with this process. We have isolated a mutant named tormoz (toz) from our Ds gene trap collection that segregates for arrested embryos which show abnormal planes of cell division in the embryo proper (EP), but not in the suspensor. The zygote of mutant embryos elongates and divides transversely as in wild type, but further cell division planes of the EP are disrupted. Unlike wild type, all cells in the mutant EP have the potential to divide longitudinally, transversely or occasionally obliquely. Thus, the plane of division that each cell adopts bears little relationship to the orientation of the previous division, or that of the neighboring cells. In addition, embryo arrest may occur at any time, with the most advanced mutant embryos consisting of less than 50 cells.

The gene disrupted in toz-1 mutants is predicted to encode a WD-40 repeat containing protein with unknown function. Through RT-PCR analysis we found that it is not a null mutation. Detailed observations of heterozygous plants using the GUS fusion from the Ds gene trap show that TOZ is expressed in tissues undergoing rapid growth and localizes to the nucleus. A uniquely conserved gene homologous with TOZ is found in many Eukaryotic species including yeast, insects and humans. Using the TOZ homologue in fission yeast we found that septum formation is not perturbed in null mutants, but disruptions to nuclear integrity and chromosome structure are observed during mitosis. It is possible that TOZ and its homologues perform an essential function during cell division in Eukaryotes.

Proteins interacting with MOM1: possible links between transcriptional gene silencing and cytokinesis

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Recently we have identified a gene of *Arabidopsis thaliana*, MOM1, which regulates transcriptional gene silencing without changes in DNA methylation (Nature 405:203-206, 2000). The MOM1 protein of 2001 amino acids shows a similarity to the ATPase region of the chromatin remodeling factors, but there is no known protein with similarity to the overall structure of MOM1. The mom1 mutant exhibits no obvious developmental defects, although it releases transcriptional gene silencing. To elucidate the function of MOM1, we have overexpressed various domains of the MOM1 protein in yeast cells. Overexpression of a C-terminal part of MOM1, which does not contain the ATPase region, in haploid yeast cells triggers accumulation of polyploid cells. Using a yeast two-hybrid system, we have detected three *Arabidopsis* proteins specifically interacting to the C-terminal part of MOM1. Their identity suggests the involvement of MOM1 in mitotic checkpoint. Possible linking of the function of MOM1 in transcriptional silencing to the mitotic checkpoint will be discussed.

NPK1 MAPKKK-related protein kinase contains a functional nuclear localization signal in the binding site of the NACK1 kinesin-like protein

Masaki Ishikawa

NPK1 gene from tobacco encodes protein kinase related to mitogen-activated protein kinase kinase kinase (MAPKKK). Recently, we showed that NPK1 is localized to the equatorial region of phragmoplast during late M phase and is necessary for the formation of the cell plate during cytokinesis of plant cells (Genes & Dev., 15, 352). Its localization during interphase is, however, yet known. In this study, we investigated the subcellular localization of NPK1 in BY-2 cells at interphase. This result showed that NPK1 localize in the nucleus of BY-2 cells. Examination of amino acid sequence of NPK1 showed that at the Carboxyl (C)-terminal terminal region of the regulatory domain, NPK1 contains a region of basic amino acids (amino acid residues 645 to 659) that resemble consensus sequences of the bipartite nuclear localization signal (NLS). Amino acid substitution mutations of the sequence caused drastic reduction in the nuclear localization of NPK1 in BY-2 cells, indicating that this sequence is functional in BY-2 cells. Furthermore, we have found that amino acid residues 627 to 690 of the C-terminal region is necessary for interaction with NACK1 kinesin-like protein which is the activator of NPK1 and transports NPK1 to the equatorial region of phragmoplast. Taken together, these results indicate that the NLS is overlapped with the NACK1-biding site at certain extent.

Mechanisms of G2/M-phase-specific transcription during the plant cell cycle

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Plant B-type cyclin genes are expressed specifically in late G2 and M phases during the cell cycle. Their promoters contain a common *cis*-acting element, called the MSA element, which is necessary and sufficient for periodic promoter activation. The functional MSA element is also present in the tobacco kinesin-like protein gene, *NACK1*, which is expressed with similar timing to B-type cyclin genes. Thus, in plants, a defined set of M phase-specific genes seems to be co-regulated by an MSA-mediated mechanism. We have identified three different Myb-like proteins that specifically interact with the MSA sequence. Unlike the majority of plant Myb-like proteins, these Myb proteins, NtmybA1, NtmybA2 and NtmybB, have three imperfect repeats in the DNA binding domain, as in animal c-Myb proteins. During the cell cycle, the *NtmybB* gene showed constitutive expression, while the levels of *NtmybA1* and *A2* mRNAs fluctuated and peaked at M phase, when B-type cyclin genes are maximally induced.

In situ hybridization analysis confirmed that the periodic expression of *NtmybA1* and *A2* also occurs in tobacco shoot apices. In tobacco protoplasts, NtmybA1 and A2 activated the MSA-containing promoters, while NtmybB repressed them. We propose that competitive DNA binding of Ntmyb proteins with different activities for transactivation may provide a mechanism for transcriptional regulation of plant M phase-specific genes. Indeed, a balance between activators and repressors is thought to be a common mechanism to reduce inappropriate triggering of biological responses, and such a mechanism could help to explain the unusually tight window of expression displayed by plant B-type cyclin genes.

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A recombinase-mediated transcriptional induction system for modulation of cell cycle gene expression in tobacco BY-2 cells

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Molecular mechanisms controlling cell cycle progression are conserved between the different organisms. However temporal and spatial control of cell division can diverge between organisms. Thus plants have a unique developmental program, which doesn't exist in animals or fungi. Even if several plant genes encoding cell cycle regulating proteins have been characterized, little is known about the function of the proteins and factors, which control CDK/Cyclin complex activity and regulate cell cycle progression. In order to analyze the function of these regulatory proteins and to unravel the relationship between cell division and development we have chosen to express cell cycle inhibitor/stimulatory genes using a recombinase-mediated transcriptional induction system.

The use of the site-specific DNA recombinase Cre is well established in a broad range of organisms. We have investigated the use of a Cre-lox recombination-mediated vector system to control the induction of cell cycle gene expression in BY2 cells. In this system, treatment of cells under inducing conditions mediates an excision event that removes an intervening piece of DNA between a promoter and the gene of interest. The system we developed uses a heat-shock-inducible Cre to excise gfp gene flanked by lox sites, thereby generating a constitutive expression under the control of the *Arabidopsis thaliana* CDKA promoter.

We report on the generation and characterization of a BY-2 line allowing heat shock-mediated gene transcriptional induction in synchronized BY-2 cells. Different cell cycle genes from *Arabidopsis thaliana* supposed to be involved in the transition from G2 phase to mitosis have been used. Data concerning the effects of the inducible expression of the cell cycle genes at the G2/M transition in BY2 cells will be presented.

Protein kinase ck2 from plants: structural characteristics and pattern of expression during *Arabidopsis* development

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Protein kinase CK2 is an ubiquitous Ser/Thr kinase with multiple substrates Though a lot of information is now available for the CK2 from animal and yeast cells, much less is known about the CK2 from plants. In a previous work we have used the tobacco BY2 cells to study the regulation of CK2 during the cell cycle.We have now characterized the tobacco genes families coding for the two subunits that constitute the tetrameric CK2, the a and the ß subunit. Both subunits are encoded for more than one gene in tobacco cells, confirming the existence of a heterogeneity of both the a and the ß subunits in plants.

On the other hand, we have studied the pattern of expression of CK2a and CK2ß genes by *in situ* hybridization in *Arabidopsis thaliana*. Our results reveal the presence of mRNA for both subunits mainly in meristematic cells, although they can also be detected in some differentiated tissues, such as the mesophil cells of *Arabidopsis* leaves. The functional implications of these results are discussed. The pattern of expression during *Arabidopsis* flower development suggests that both subunits are coordinately regulated at a transcriptional level, and their expression correlates well with an active proliferating state.

Regulation of floral induction and plant yield: role of the *MSI4* gene coding for a WD-repeat protein

Patrice Morel, Christophe Tréhin, Françoise Monéger and Ioan Négrutiu

Work on a dioecious plant, *Silene latifolia*, allowed the cloning and characterisation of the first gene localised on a plant sex chromosome: *SLY1*. The product of this gene is preferentially expressed in meristems and floral organ primordia and seems to be involved in the control of cellular proliferation.

Five homologues of *SLY1* - *MSI1-5,* according to yeast nomenclature - are found in *Arabidopsis* genome. All code for WD-motif proteins which are homologous to the Retinoblastoma-binding protein of mammals, involved in the cell cycle control. One mutant for the *MSI4* gene was found in the T-DNA tagged collection (INRA at Versailles France, ecotype WS). Thus, the *msi4* mutant presents a late flowering phenotype and an increase in biomass and in seed production. The phenotypic analysis shows that flowers are approx. 5-times larger in the mutant than in the WT and exibit an increased number of organs. In addition, first cytometric analyses indicate that the level of ploidy is not significantly modified in the *msi4* mutant but that S phase duration is increased. msi4 is allelic to fve, a late flowering mutant identified genetically several years ago (José Martinez-Zapater, pers. communic). Ongoing experiments suggest that MSI4 proteins are potential regulators of flowering time via mechanisms that control cell proliferation.

Further genetic, molecular and cellular work is underway to refine the functional analysis of the *MSI4* gene.

Overexpression of phosphorylated rab17 arrests germination of transgenic *Arabidopsis* seeds in the presence of salt

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Maize Rab17 is a Late Embryogenesis Abundant (Lea) protein of still unknown function. We have analyzed transgenic *Arabidopsis* plants expressing the maize Rab17 protein under a constitutive promoter. The protein is found accumulated in all tissues and shows extensive phosphorylation as occurs in maize . On the contrary other transgenic plants that express a mutated version of the protein in the consensus motif of the casein kinase 2 show minimal phosphorylation of Rab17 and lower levels of protein accumulation. Both transgenic plants show no differences in their phenotype, germination or growth rate in comparison to their untransformed counterparts. However, during germination the presence of NaCl or KCl in the medium prevents and/or arrests the germination of seeds expressing the phosphorylated version of Rab17, whereas the seeds expressing the non-phosphorylable Rab17 protein or the wild type controls are able to complete the germination process. In addition, vegetative tissues of Rab17 overexpressing plants, irrespectively of the degree of phosphorylation of the Rab17 protein, are more tolerant than untransformed controls to high salinity and drought. Thus, our results point to a distinctive role of the Rab17 protein in embryo and vegetative tissues under stress conditions; the functionality of Rab17 protein in germination arrest is dependent on its phosphorylation and on exogenous salt, whereas its protective effect on vegetative tissues under stress is phosphorylation independent.

A maize histone deacetylase and retinoblastoma-related protein physically interact and cooperate in repressing gene transcription

Vincenzo Rossi

In mammalian cells the product of the human retinoblastoma tumor suppressor gene (pRb) can recruit Rpd3-like histone deacetylases to repress transcription. In this report, we investigated whether this mechanism might also be relevant in plants and found both conserved and distinct features. The expression patterns and cellular localization of the *Zea mays* Rpd3-type histone deacetylase (ZmRpd3I) and the retinoblastoma-related (ZmRBR1) homologue were analyzed. GST pull-down and immunoprecipitation experiments showed a physical interaction between ZmRBR1 and ZmRpd3I. Because ZmRpd3I lacks a LXCXE motif, conserved in several pRb-interacting proteins, we have mapped the amino acid domains involved in the ZmRBR1/ZmRpd3I interaction. Furthermore, we observed that a maize retinoblastoma-associated protein, ZmRbAp1, facilitated this protein interaction. ZmRbAp1 is a member of the MSI/RbAp family of WD-repeat proteins that binds acetylated histones H3 and H4 and suppresses mutations negatively affecting the Ras/cAMP pathway in yeast. Co-transformations of tobacco protoplasts with plasmids expressing ZmRBR1 and ZmRpd3I showed that the two proteins cooperate in repressing gene transcription. Our findings represent the first direct indication that a regulator of important biological processes, ZmRBR1, can recruit a histone deacetylase, ZmRpd3I, to control gene transcription in plants. Finally, a model that consider the role of ZmRBR1/ZmRbAp/ZmRpd3I complex in the control of plant G1/S transition is presented.

Ectopic b-type cyclin expression is sufficient to switch from endoreduplication to mitotic cycles in *Arabidopsis* trichomes

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Cell differentiation is frequently accompanied by a switch from a mitotic division cycle to an endoreduplication cycle in which DNA replication continues but cell division does not take place. In plants more than 80 per cent of all angiosperm species undergo endoreduplication. The underlying mechanism, however, is poorly understood. One attractive scenario is that endoreduplication results from a simple short cut of the mitotic cell cycle. To test this hypothesis we misexpressed two mitotic cyclins in *Arabidopsis* trichomes, a model system for endoreduplicating cells in plants. Here we demonstrate that transgenic expression of CYCLIN B1;2 can drive the endoreduplication cycle into a mitotic cell cycle, transforming the single-celled into multicellular trichomes. This seems to be specific for CYCLIN B1;2 since the expression of CYCLIN B1;1, another member of the b-type cyclins, caused no deviation from the endoreduplication cycle. Mulitcellular trichomes also arise in the siamese mutant. Since we could not detect CYCLIN B1;2 mRNA in siamese we propose that in addition to the repression of mitotic cyclins a second factor is necessary to control the switch from a mitotic to an endoreduplication cycle.

Tobacco cyclin D3/CDKA complex phosphorylates Rb-related protein

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Progression through the mammalian cell cycle is driven by the periodic activity of cyclin-dependent kinases (CDKs). At the late G1 restriction (R) point, cells must interpret extracellular signals to decide whether to commit to a further round of division or adopt alternative differentiation pathways. The retinoblastoma (Rb) protein binds to members of the E2F transcription factor family, and the resulting Rb-E2F complex blocks the transcription of E2F-regulated genes. In response to growth-promoting signals, cyclin D is synthesized to form active complexes with CDK4/6 that phosphorylate Rb in the mid-to-late G1 phase, thereby alleviating its repressor function on E2F-controlled gene transcription, and stimulating entry into S phase. We previously demonstrated using a baculovirus expression system that tobacco cyclin D3 (Nicta;CycD3;3) forms an active complex with the PSTAIRE-containing CDKA;3, which phosphorylates both the tobacco Rb-related protein (NtRb1) and histone H1 *in vitro*.

To examine the cell cycle phase-specific activity, tobacco suspension cultured BY-2 cells were synchronized, and CycD3;3-associated kinases were immunoprecipitated with the antibody of CycD3;3. We found that NtRb1 was phosphorylated only from the cells in the G1 to S phase. Next, we generated the transgenic BY-2 cells expressing GFP fusion protein. CycD3;3-GFP also associated with the PSTAIRE CDKA in BY-2 cells and predominantly localized in the nucleus. Interestingly, expression of CycD3;3-GFP reduced the number of cells in the G1 phase. These results strongly suggest that plant cyclin D3 regulates the G1/S transition through phosphorylating the Rb-related protein in plants.

Do precise control of cell divisions function in programming stomatal fate in *Arabidopsis*?

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Pattern formation requires co-ordination between cell division and cell fate determination. We have shown that a precise control of three unequal cell divisions precedes to the stoma formation in *Arabidopsis*, and that the cell distance between neighbouring stomata is achieved through this stereotyped lineage. Does disruption of this cell division pattern affect stomatal patterning? The possibility to manipulate cell division cycle might allow us to ascertain whether precise control of cell divisions functions in programming stomatal fate.

Control of cell division and differentiation by CDK-activating kinases

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In addition to cyclins, the activation of CDKs requires phosphorylation of a threonine residue within the T-loop by CDK-activating kinases (CAKs). We have characterized three *Arabidopsis* CAKs (Cak1At, Cak2At, Cak3At) and a rice CAK (R2), and found that Cak2At, Cak3At and R2 are classified into vertebrate-type CAKs while Cak1At is a unique CAK in terms of amino acid similarity and enzyme activity. Since CAK is an upstream kinase which activates almost all CDKs, up- or down-regulation of CAK might have a crucial effect on total CDK activity in each cell of the meristems. To understand how CAK activities are controlled and how CAK regulates cell division and differentiation, we took a transgenic approach.

We investigated hormonal response of tobacco leaf sections that overexpress rice R2 in a dexamethasone (DEX)-dependent manner. They produced calli on a medium containing auxin but no cytokinin, and this phenotype was stressed by a high dose of DEX. Co-expression of cyclin H, which is a regulatory subunit of CAK, resulted in faster growth of calli. This suggests that CAK overexpression compensated for requirement of cytokinin in starting cell division. R2 is able to phosphorylate not only CDKs but also the tobacco Rb protein. Therefore, we propose that a cytokinin signal is transmitted to Rb through phosphorylation by CAK as well as by CDK/cyclin D. I will also present our recent data showing that Cak1At regulates differentiation of root initial cells in *Arabidopsis*.

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The Rb pathway in *Chlamydomonas*: A key for understanding the role of cell cycle control in the evolution of multicellularity

James G. Umen and Ursula W. Goodenough

A key pathway that controls both cell division and differentiation in animal cells is mediated by the retinoblastoma (RB) family of tumor suppressors which gate the passage of cells from G1 to S and through S phase. The role(s) of the RB pathway in plants are not yet clearly defined, nor has there been any evidence for its presence in unicellular organisms. We have identified an RB homolog encoded by the mat3 gene in *Chlamydomonas reinhardtii*, a unicellular green alga in the land plant lineage. *Chlamydomonas* cells normally grow to many times their original size during a prolonged G1, and then undergo multiple alternating rounds of S phase and mitosis (multiple fission) to produce daughter cells of uniform size. mat3 mutants produce small daughter cells and show defects in two size-dependent cell cycle controls: they initiate the cell cycle at a below-normal size, and undergo extra rounds of S phase/mitosis. However, unlike mammalian RB mutants, mat3 mutants do not have a shortened G1, do not enter S phase prematurely, and can exit the cell cycle and differentiate into gametes normally. Thus, in *Chlamydomonas*, the RB/Mat3p pathway serves specifically as a size-dependent repressor of cell cycle progression. The phenotype of mat3 mutants suggests a model in which Mat3p allows early G1 cells to initiate a new cell cycle only when they attain a specific threshold size, and blocks new rounds of S phase/mitosis when cells fall below the threshold size. This model also provides a basis for understanding how the multiple fission cell cycle could have been modified in the remarkable evolutionary process that has given rise to a diverse array of multicellular algae that are closely related to *Chlamydomonas*.

Role of the NAC368 transcription factor in *Arabidopsis* shoot meristem initiation and boundary specification

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During embryo development in *Arabidopsis*, groups of cells develop into distinct organs and precise boundaries between these organs are established. We have performed an enhancer trap screen for genes with organ- or position-specific expression patterns in developing embryos. This generated several interesting lines, including WET368, which displays GUS expression in the presumptive embryonic shoot meristem. The trapped gene encodes a putative NAC domain transcription factor, and hence was designated NAC368. NAC domain transcription factors are unique to plants. The predicted NAC368 protein shares homology with two other NAC proteins, CUC1 and CUC2, which are redundantly required for shoot meristem initiation and organ boundary specification: cuc1 cuc2 embryos do not form a shoot meristem and have fused organs. NAC368 expression starts in the 4-cell embryo, and resolves to a band between the cotyledon primordia including the shoot meristem. As soon as the shoot meristem starts to proliferate, NAC368 expression is restricted to the meristem boundaries and the boundaries of a variety of organs such as shoot and floral meristems, cotyledons, secondary inflorescences and lateral roots. This partly overlaps with expression of the CUC genes. NAC368 may act in the same pathway as the CUC genes in the restriction of cell proliferation necessary for the delineation of boundaries between organs, and possibly also for shoot meristem initiation. We are currently addressing NAC368 function by knockout and silencing approaches. This may reveal a link between restriction of cell proliferation, specification of organ boundaries and shoot meristem initiation.

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Regulation of *Arabidopsis* NAC1 in auxin signal transduction pathway

Qi Xie

Auxin plays a key role in lateral root formation but the signaling pathway for this process is poorly understood. We show here that NAC1, a new member of the NAC family (1, 2), is induced by auxin and mediates auxin signaling to promote lateral root development. NAC1 is a transcription activator consisting of an N-terminal conserved NAC-domain that binds to DNA and a C-terminal activation domain. This factor activates the expression of two downstream auxin-responsive genes, DBP and AIR3 (3). Transgenic plants expressing sense or antisense NAC1 cDNA show an increase or reduction of lateral roots, respectively. NAC1 overexpression can restore lateral root formation in the auxin response mutant tir1 (4), indicating that NAC1 acts downstream of TIR1 (5). Finally, we will demonstrate that a novel ubiquitination mechanism negatively regulates NAC1 in the auxin trasduction pathway to control the lateral root development.

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