Instituto Juan March de Estudios e Investigaciones

93 CENTRO DE REUNIONES INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Cell Cycle Regulation and Cytoskeleton in Plants

Organized by

N.-H. Chua and C. Gutiérrez

N. Chaubet-Gigot
N.-H. Chua
P. Doerner
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N. V. Raikhel
A. Staehelin
C. J. Staiger
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The lectures summarized in this publication were presented by their authors at a workshop held on the 22nd through the 24th of March, 1999, at the Instituto Juan March.

Depósito legal: M-17.856/1999
Impresión: Ediciones Peninsular. Tomelloso, 27. 28026 Madrid.

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Introduction

N-H. Chua and C. Gutiérrez

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Progression through the cell cycle stages occurs as a temporally coordinated series of events that involve the concerted action of both positive and negative acting effectors. In addition, the cellular decision to enter and/or exit the cell cycle is the physiological response to a series of external and internal stimuli.

The basic eukaryotic cell cycle is well exemplified by the situation found in yeasts, unicellular eukaryotic organisms with a powerful genetics, where many details of the molecular framework regulating the transits through the G1/S and the G2/M boundaries are known.

Multicellularity requires the superimposition of a number of extra control pathways derived from the need to integrate signals as well as from the specific developmental patterns. In animals, cell cycle regulators also participate actively in the maintenance of certain differentiated status. Initial studies revealed that basic cell cycle regulators, such as some cyclins or cyclin-dependent kinases (CDKs), are strikingly conserved in animal and plant cells. However, plants have unique growth and developmental properties and display a specific response to environmental signals and plant hormones. Therefore, it is likely that the regulatory pathways controlling cell cycle progression and the exit from active proliferation to differentiation in response to growth signals are different from those operating in animal cells.

In the last 3-4 years, an enormous advance in this field has occurred regarding several aspects of the plant cell cycle in terms of the identification of new genes encoding cell cycle regulators, such as homologs of the human retinoblastoma tumor suppressor protein, novel CDKs, or specific CDK inhibitors. Now, we are facing an extremely exciting future since very recent results are providing us with hints which clearly point to novel roles of cell cycle regulators in plant development and body architecture. Which is the molecular basis for the significant plasticity of plant cells? Why plant cells are extremely refractary to neoplastic transformation? How cell cycle regulators contribute to the maintenance of a particular differentiated state and how they contribute to shape the plant body? These are just a few examples.

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Another level of complexity, unique to plants, comes from the immobility of cells within the plant body. The importance of the plane of division and the significant role of cell expansion in the generation of plant organs make necessary a strict coordination between cell cycle control and the cellular components which provide the framework for cell division, i. e., the cytoskeleton. Cytoskeletons are likely involved in the establishment and maintenance of patterns during plant cell morphogenesis. Cytokinesis in plant cells is distinct from that in animal cells in several important aspects. The plane of cell division is presaged by a preprophase band which is comprised of microtubules. At the zone marked by the preprophase band, the phragmoplast, a complex array of microtubules, microfilaments and secretory vesicles, grows through a centrifugal process giving rise finally to the cell plate that divides the two daughter cells. Because cell differentation in plants is often presaged by asymmetric cell divisions, the placement of the preprophase band must be regulated by signalling events. Actin, tubulins and dynamin-like proteins contribute to develop a functional cell plate which is the basis for plant cytokinesis.

In summary, several interesting aspects related to the topics outlined above have been the subject of this workshop. A significant amount of novel results and unpublished data were presented. As expected from their potential impact in the field, they triggered active discussions throughout the meeting. Based on the directions where the plant cell cycle field is moving, we look forward to very exciting developments in the near future.

Crisanto Gutierrez and Nam-Hai Chua

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Session 1: Cell cycle regulatory proteins - 1

Chair: Dirk Inzé

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Role of D-type cyclins in cell cycle, development and the control of plant growth

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D-type cyclins have been identified in a number of plant species by their ability to complement yeast mutants defective in G1 cyclins, or by homology-based criteria. They have greatest similarity to D-type cyclins of mammals, although the level of sequence homology is low. However they maintain the functional motifs of the cyclin box (involved in cyclin-CDK interaction), the retinoblastoma protein interaction motif (LxCxE in single amino acid code) and generally PEST sequences believed to cause protein instability.

D-type cyclins are a major interface between extra-cellular signals and the process of commitment to the cell cycle. They are involved in the decision to re-enter the cell cycle, and also in the ongoing cycling of cells participating in plant growth.

Three groups of D-type cyclins have been identified, termed CycD1, CycD2 and CycD3. In many species, more than one member is present, particularly in the CycD3 subgroup. We have investigated the roles of CycD genes by their expression regulation in response to external stimuli, and by modulating their expression in transgenic plants.

CycD3 is strongly regulated by the growth regulator cytokinin, and overexpression of CycD3 in transgenic Arabidopsis makes callus outgrowth from leaf explants independent of cytokinin. In addition, widespread developmental abnormalities are observed. We conclude that CycD3 is involved in the cell division response to cytokinin, and its perturbation can affect the pattern of plant growth.

In contrast the overexpression of CycD2 in transgenic tobacco has been found to increase growth rate without altering the structure, dimensions, cell size or morphology of the plants. This developmental acceleration results from an increased rate of leaf initiation from the earliest stages of growth.

These results will be discussed in the context of the regulation of the pattern and rate of plant growth.

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Components of the retinoblastoma pathway and their role in cell cycle regulation and growth

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A variety of internal and external effectors controls plant cell proliferation, growth and differentiation. We are interested in understanding the molecular mechanisms operating during cell proliferation and the exit/entry from quiescence, during the G1/S transition, and, in general, the pathways conferring plasticity to the plant cells. The identification of regulators which control cell cycle transitions has been difficult and elusive. We have taken a novel approach by studying geminiviruses, small plant DNA viruses, and the coordination of their replicative cycle to cell cycle regulation. We identified in the wheat dwarf geminivirus (WDV, Mastrevirus genus) RepA protein an amino acid motif (LXCXE) which mediates its binding to the human retinoblastoma (Rb) tumor suppressor protein (1). This, together with the isolation of plant D-type cyclins (2), also containing the LXCXE motif, provided a strong evidence suggesting that a Rb-like protein(s) could act as a crucial regulator of plant cell cycle. This prediction was confirmed with the isolation of a maize cDNA clone encoding ZmRb1, a plant Rb protein, as well as with the observation that overexpression of ZmRb1 strongly inhibits WDV DNA replication in cultured wheat cells (3). Other Rb-related proteins have been cloned (4). The availability of plant Rb is currently facilitating the study of different aspects of the Rb pathway in cell cycle and differentiation (5).

Although Mastrevirus RepA protein interacts with plant Rb (ZmRb1), Rep protein, which also contains the LXCXE motif in its primary sequence, does not. Rep protein from other geminivirus genera do not contain a LXCXE Rb-binding motif in their primary sequence but, still they interact with Rb (4). Our results support the idea that different geminivirus members have evolved two strategies to impinge on the Rb pathway: some of them use a LXCXE motif while others rely on a different motif, which we speculate that may be related to that involved in the interaction between Rb and E2F.

To identify Rb-interacting proteins, we have used ZmRb1 as a bait in a yeast two-hybrid screening. This has allowed us to isolate a plant E2F transcription factor (6). The amino acid sequence of plant E2F reveals unique features, in particular the Rb-binding domain. E2F mRNA is abundant in proliferating cells but can also be detected in differentiated cells, perhaps suggesting a role for the Rb/E2F pathway in the maintenance of the differentiated stage. Small differences in the levels of Rb and E2F proteins can be detected throughout the cell cycle. We are currently determining whether cell cycle progression might be associated with changes in the Rb phosphorylation level and the dependence of Rb and E2F expression and accumulation on the nutrient and hormone balance in suspension cultured cells. Progress towards identifying the CDK(s) responsible for Rb phosphorylation will be reported. Altogether, our results indicate that plants contain G1/S regulators strikingly related to those of animal cells, and unrelated to those of yeast cells, and set the ground to define their function as well as to define the consequences that disruption of this pathway might have on cell cycle, differentiation and plant architecture.

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THE FUNCTION OF PLANT RRB AND RbAp48/MSI PROTEINS IN CELL CYCLE REGULATION, CHROMATIN REMODELING, AND DEVELOPMENT

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The product of the retinoblastoma gene, pRB, functions at the transition of G1 to S phase in the cell cycle (1). Its primary role is thought to be in the regulation of the E2F family of transcription factors which activate genes that are central to the onset of DNA synthesis and S phase progression (2). Recent discoveries of genes for pRB-related proteins in *Drosophila* (3), *C. elegans* (4), and plants (5, 6, 7) indicate that pRB is a central part of an evolutionary conserved regulatory system. We showed that the maize RRB1 protein shares significant biochemical similarity with the human pRB, including binding of D-cyclins, T-antigen and E1A. Mutations in the pocket domains and a conserved cysteine in RRB1 have similar effects on binding (5).

A maize line containing a Mutator (*Mu*) transposable element in the *RRB1* locus was identified in collaboration with PIONEER HI-BRED, Int. using the Trait Utility System for Corn (TUSC). Genomic blot analysis of DNA from RA6 and control seedlings suggested that an *rrb1* allele segregating in the F2 population of maize line RA6 contained a *Mu* transposable element. The *Mu9* element was inserted in an exon just proximal to the B domain, creating the *rrb1::Mu9 allele.* Preliminary analysis of *rrb1::Mu9* homozygous ($rrb1::Mu9^{+/+}$) and heterozygous ($rrb1::Mu9^{+/-}$) F2 plants shows that the development of vegetative and reproductive structures appears to be normal, and both male and female flowers are partially fertile. In view of the pseudotetraploid origin of maize that resulted in the redundancy of *RRB* genes in maize, the lack of a phenotype was not unexpected. In contrast, genetic manipulation of the single *RRB* gene in Arabidopsis results in developmental phenotypes.

Recently, the interaction between chromatin and cell cycle regulation was renewed with the discovery of histone acetyltransferases (HAT) and deacetylases (HDA) that can modulate chromatin function (8) and interact with Rb (9). HAT and HDA enzymes have now been shown to assemble into complexes with RbAp48 and RbAp46, a new subfamily of WD-40 proteins that has also been found in association with the Retinoblastoma tumor suppressor protein (pRb), specific transcription factors, and chromatin assembly factor (CAF). This subfamily of WD-40 protein is highly conserved in plants, and interaction with pRRB1, a Rb-related protein recently discovered in plants, has been confirmed (10). A related WD-40 protein in *S. cerevisiae*, Msi1p, associates with CAF and is required for gene silencing in telomeric regions. Genetic disruption of *AtMSI1* expression, the first plant RbAp48 homologue analyzed in a multicellular context, reveals that the protein is required for normal development in Arabidopsis. Similarly, *lin-53*, a gene in C. *elegans* that antagonize the Ras-signaling pathway during vulval induction, encodes a protein similar to pRbAp48. Together, it emerges that the RbAp48/MSI subfamily of WD-40 proteins has an important role in linking together key regulatory proteins that are involved in cell cycle control, gene expression, and development. Many important questions remain, however, especially

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how the observed interactions target specific genes to control their transcription during cell differentiation.

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GEMINIVIRUSES AND THE PLANT CELL CYCLE

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Geminiviruses are single-stranded DNA viruses that replicate their genomes through double-stranded DNA intermediates in nuclei of differentiated plant cells. Geminiviruses specify only a few proteins for their replication and rely on host DNA polymerases to amplify their genomes. Mature plant cells, which have exited the cell cycle, do not contain detectable levels of DNA polymerases or associated factors. Hence, geminiviruses must induce the requisite host proteins prior to their replication. We showed that the geminivirus, tomato golden mosaic virus (TGMV), causes the accumulation of the DNA polymerase processivity factor, proliferating cell nuclear antigen (PCNA), and its mRNA in differentiated cells of *Nicotiana benthamiana*. We also demonstrated that TGMV infection activates the homologous *N. benthamiana* PCNA promoter and the corresponding heterologous rice promoter. Together, these results established that geminiviruses can induce transcription of genes encoding components of the plant DNA replication apparatus and that the induction mechanism is conserved for dicot and monocot genes.

Although the mechanism of host induction is not known, ultrastructural and *in planta* labeling studies suggested that TGMV alters cell cycle controls in mature plant cells. Geminivirus infection alters nuclear morphology of mature cells to resemble meristematic cells. Chromatin condensation typical of cells in prophase is also observed in a significant proportion of infected cells. The nucleotide analog, bromodeoxyuridine (BrdU) is efficiently incorporated into both viral and chromosomal DNA of infected cells. Altered nuclear morphology, chromatin condensation and high levels of BrdU incorporation cannot be detected in uninfected cell populations of the same plants.

Like all geminiviruses, TGMV encodes one essential replication protein. TGMV "Rep" or AL1 is a multifunctional protein that mediates initiation of rolling circle replication, regulates viral gene expression, and acts as a plant cell regulator to create a replication-competent environment. Analysis of transgenic plants that express AL1 demonstrated that it is sufficient to induce PCNA expression in mature cells in the absence of other geminivirus proteins. This observation suggested that AL1 is analogous to the DNA tumor antigen proteins of mammalian viruses, which induce replication machinery in their hosts by altering cell cycle and transcriptional controls. The ability of TGMV AL1 to bind plant homologues of the animal tumor suppressor protein, retinoblastoma (Rb), indicated that it may use similar strategies to modify plant cells.

We are characterizing AL1/Rb interaction and determining its role in the geminivirus infection process. TGMV AL1 and the Rep proteins of other dicot-infecting geminiviruses do not contain the canonical LXCXE motif associated with Rb binding by mammalian DNA tumor antigen proteins and monocot-infecting geminiviruses. Thus, TGMV AL1 must bind Rb through a novel motif. This hypothesis is supported by several lines evidence. First, unlike LXCXE-containing proteins, mutation of a conserved cysteine residue in the B domain of Rb does not impair AL1 interaction. Second, AL1/Rb interaction is dependent on the ability of AL1 to form oligomers. Third, the Rb interaction domain, which has been mapped to an 80 amino acid region of TGMV AL1, contains a 16 amino acid sequence of near identity among the Rep proteins of dicot-infecting geminiviruses. This motif is not found in the LXCXE-containing Rep proteins of monocot-infecting viruses. Mutations in the conserved sequence that impair AL1/Rb binding have been identified and are currently being tested in viral replication, infectivity and PCNA promoter activation experiments. Together, these studies will provide valuable insight into the significance of AL1/Rb interactions and may identify a novel Rb regulatory mechanism.

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GENE INDUCTION AT THE G1/S TRANSITION OF THE PLANT CELL CYCLE

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The major interest of our group is to elucidate the molecular mechanisms involved in the induction of gene expression at the G1/S transition of the plant cell cycle. We are working on two alternative model-genes, the genes encoding the histones necessary for packaging the S phase-replicated DNA into chromatin, and those encoding the two subunits of ribonucleotide reductase (RNR), the enzyme involved in the synthesis of the dNTPs necessary for DNA replication.

Analyses of gene expression in synchronised tobacco cell suspension have shown that RNR genes are induced slightly before histone genes during the G1/S transition and exhibit a different response towards inhibitors of DNA replication. These results suggested that the two model-genes are induced by two different regulatory mechanisms.

Cis-elements have been identified in histone and RNR promoters by structural and functional approaches. Relevant with our hypothesis about different regulation of the two model-genes, the cis-elements found in the histone and RNR promoters were not the same. Repeated motifs which are known to bind the cell cycle-specific transcription factor E2F in animal genes were found in the RNR promoters. Specific octameric and nonameric cis-elements were found in the histone promoters from various plant species. The persistence of protein footprints on these cis-elements throughout the cell cycle indicated that transcriptional activation of the histone genes at the G1/S transition is mediated by modification of prebound proteins rather than by the direct binding of G1/S transition-specific proteins. Band-shift experiments suggested that this modification may result from a phosphorylation event.

This hypothesis led us to investigate the cyclins capable of activating cyclin-dependent kinases (cdk) at the G1/S transition. Three distinct subtypes of cyclins A were identified whose genes are induced sequentially from late G1 to mid S phase. Two cyclin genes belonging to the "early-expressed" A3-group showed slightly different expression patterns relative to their induction times and their responses towards inhibitors of DNA replication. One of them appeared coregulated with RNR and the other one was coregulated with histone genes. Based on these results, a model is proposed for the sequential induction of genes during the G1/S transition : whereas the earliest set of genes might be induced by a mechanism involving an E2F-like factor, the second set might be activated by phosphorylation of transcription factors by a kinase complex involving the earliest-expressed cyclin.

Antibodies were raised against the three cyclins A and used to analyse cyclin expression at the protein level and to check for the presence of cyclins in the transcriptional complex of histone genes. Surprisingly, the amount of the three cyclins remained at a high level throughout the cell cycle. The massive degradation of cyclin A which occurs in animal cells at early mitosis due to the ubiquitin/proteasome destruction pathway was not observed. Immunofluorescence-localisation experiments confirmed that the tobacco cyclins A are stable during mitosis. The three cyclins were found in the mitotic spindle zone but not associated to the chromosomes. They were also detected in vesicular structures which were further defined as amyloplasts by immunogold microscopy. In addition, the "earliest-transcribed" cyclin was predominant in the nucleus of S and G2 phase cells, in keeping with our hypothesis that it might participate to transcriptional complexes.

In order to investigate the potential participation of this cyclin to transcriptional complexes, a method was developped to characterize large nuclear protein complexes on native gels. The native complexes were incubated with antibodies against the "earliest-transcribed" cyclin, or with a histone promoter fragment. Complexes of similar molecular weights were revealed by the two approaches, thus reinforcing our hypothesis that this cyclin might be part of the transcription complex of histone genes. To further characterize the cyclin-containing complex and the histone transcriptional complex, a method is being developped to isolate these complexes on immuno- or DNA-affinity columns. The eluted subunits will be identified by immunoblot analysis and microsequencing.

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Session 2: Cell cycle regulatory proteins - 2

Chair: Gerd Jürgens

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CDK/cyclins complexes of *Arabidopsis thaliana*

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Cell division is central to growth and development of higher plants. The major, long-term goal of my laboratory is to understand the molecular regulation of cell division and the mechanisms by which both intrinsic developmental signals and environmental cues impinge on it. Considerable progress was made in detailing the regulation of the cell cycle in the modelplant Arabidopsis. For the first time, it became possible to assign specific roles for Cyclin Dependent Kinases (CDKs) , Cyclins, CKS and Cyclin dependent Kinase Inhibitors (CKIs) during the various phases of the cell cycle (Burssens et al, 1998 and Mironov et al, 1999).

A highlight was the demonstration that the Arabidopsis CKS has not only a role in the mitotic cycle, but also appears to be of importance in endoreduplication, a process often observed in plants in which cells repeatedly replicate their DNA without mitosis. Furthermore, a detailed analysis revealed that endoreduplication is very early established during leaf development (Jacqmard et al, 1999). Research on CKS is currently focussing on the analysis of transgenic plants with altered CKS expression levels and on the determination of its 3-D structure by NMR (Landrieu et al, 1999).

Intensive two-hybrid screens allowed the isolation of several novel cell cycle regulators, amongst which CKIs and a novel D-type cyclins are the most important. Detailed in situ hybridisations showed that the novel D-type cyclin has a central role in allowing cells to re-enter the cell cycle. A model was proposed describing the sequence of events by which quiescent cells re-enter the G1 phase of the cell cycle (De Veylder et al, 1999).

Considerable progress was also made in unravelling the function of the two classes of plant CDKs in cell cycle progression. A first class comprises CDKs able to complement yeast cdc2/CDC28 ts nmutants. All contain the PSTAIRE amino-acid motif and are constitutively expressed during the cell cycle. The second class of CDKs contains the PPTALRE or PPTTLRE motif, not found as such in any other known CDK. Their transcription, protein levels and associated kinase activity show an oscillating pattern, being most abundant during G2 and M phases. By regenerating transgenic tobacco plants overproducing dominant negative CDKs we could unequivocally demonstrate that the PSTAIRE containing CDKs control both the G1/S and G2/M transitions, while the other class of CDKs solely regulate the G2/M transition. In order to further define the various functional domains of both classes of CDKs, an extensive mutation analysis has been undertaken (Porceddu et al, 1999).

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MULTIPLE CYCLIN-DEPENDENT KINASE COMPLEXES AND PHOSPHATASES CONTROL G_2/M PROGRESSION IN ALFALFA CELLS

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Parallel with cloning of large number of plant cell cycle genes (CDK, cyclin, CKS, CKI, Rb) limited progress has been made so far in biochemical and functional characterization of these key regulatory elements. Only few experimental data support the complex formation between the kinase and cyclin partners in plants (Magyar Z. *et al.* 1997). Due to the complexity of CDK and cyclin gene families in a single species such as alfalfa, we have to predict multi-component regulatory pathways functioning even during G_2/M transition. Indeed, the Cdc2MsA, Cdc2MsB, Cdc2MsD and Cdc2MsF genes are expressed in G_2 and M cells. The kinase assays also show that at least three Cdc2-related kinase complexes (Cdc2MsA/B, D and F) are active in G_2/M cells after synchronization. These kinase complexes differ in their substrate preferences, thus Cdc2MsD preferentially phosphorylates the topoisomerase II, while the Cdc2MsA/B and F kinases are highly active in histone H1 phosphorylation. The activity of Cdc2MsF kinase exhibiting the PPTTLRE motif is restricted to the mitotic cells, while the Cdc2MsD kinase with PPTALRE motif shows elevated activity in G_2-M cells. Roscovitine can differentially inhibit the various complexes. The Cdc2MsA/B kinase is very sensitive against the inhibition (1μM) while the Cdc2MsD and F kinases are inhibited only by higher concentration (100 μM) of roscovitine. Especially, the Cdc2MsD kinase activity is insensitive to this inhibitor.

Since complex formation between Cdc2-related kinase and cyclin partners is a limiting regulatory step in eukaryotic cell division, it is a basic requirement to prove that plant CDKs can bind cyclins and the specific interaction between the partners ensures differential role for various complexes. In these attempts, we have tested the potential interaction between the various alfalfa Cdc2 kinases and cyclins in yeast two-hybrid system, GST pull-down assay after *in vitro* transcription/translation or by immunodetection of kinase partners in the *in vitro* or *in vivo* complexes. Up to now, with the different methods we were able conclusively demonstrate the complex formation between the mitotic Cdc2-F kinase and cyclin Ms4. More

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importantly, the GST-Cdc2MsF kinase showed *in vitro* histone H1 phosphorylation activity only in the presence of the cyclin Ms4 partner. These data suggest that defined Cdc2-related kinases can form complex with cyclins and their kinase activity may depend on cyclin binding. From these studies it seems likely that a D-type cyclin is involved in G_2/M events.
The pivotal role of Cdc2MsF kinase in mitotic cells was further confirmed by immunolocalization studies. Using anti-C-terminal peptide antibodies, the Cdc2MsF kinase can be detected in preprophase bands, as nuclear ring in early prophase, in mitotic spindles and phragmoplasts.
Regulation of eukaryotic mitotic cycle includes reversible protein phosphorylation, so different phosphatases may be considered as important components in the control of cell cycle progression. In tobacco pith parenchyma tissues the cytokinin stimulated tyrosine dephosphorylation induced rapid, synchronous entry into mitosis (Zhang *et al.* 1996). In synchronized alfalfa cells the tyrosine phosphorylation was detectable only in the case of the Cdc2A kinase and the $p13^{suc1}$ bound kinase activity was increased by *Drosophila* Cdc25 treatment. Involvement of other phosphatases than Cdc25 was indicated by inhibitor experiments. Endothall as a serine/threonine phosphatase blocker reduced both the PP1 and PP2A activities in alfalfa cells that cannot proceed the prophase-metaphase transition point. Exposing synchronized alfalfa cells to high (10 μM) concentration of endothall resulted in early chromosome condensation and abnormal nuclear membrane formation as well as micro nuclear formation. In these cells, we found increased H1 histone phosphorylation in the $p13^{suc1}$-bound fraction. Therefore, we propose that serine/threonine phosphatases may play a role in G_2/M transition. Experiments are in progress to analyse the components of this phosphorylation pathway.

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CDK-activating kinases of *Arabidopsis* and rice

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Cyclin-dependent protein kinases (CDKs) play key roles in the progression of the cell cycle in eukaryotes. Activation of CDKs is controlled by phosphorylation, as well as by binding to cyclins. Most CDKs have a conserved phosphorylation site within the T-loop, and phosphorylation of this residue, which is catalyzed by a CDK-activating kinase (CAK), is required to allow substrates to gain access to the entrance of the active-site cleft. In animals, CAK consists of the catalytic subunit Cdk7/p40^{MO15}, the regulatory subunit cyclin H, and the assembly factor MAT1. The Cdk7-cyclin H complex phosphorylates not only CDKs but also the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. Phosphorylation of the CTD is required for the release of RNA polymerase II from the preinitiation complex and the initiation of transcription.

It is likely that plant CAKs also play an essential role in the regulation of cell division, but thus far no CAK activity had been reported in higher plants. To reveal the function of CAK in cell cycle activation, we started to search for CAK activity in *Arabidopsis* and rice plants. The *Arabidopsis* CAK, which we call Cak1At, was related to animal CAKs, but similarities were restricted to the conserved kinase domains[1]. Cak1At phosphorylated human CDK2 at the threonine residue within the T-loop but did not phosphorylate the CTD of *Arabidopsis in vitro*, an indication that CDK and CTD kinase activities are separately controlled in *Arabidopsis*. In contrast, rice CAK, named R2, is closely related to Cdk7/p40^{MO15} of animals. R2 phosphorylated human CDK2, rice Cdc2Os1, and also the CTD of *Arabidopsis in vitro*, suggesting that R2 is functionally analogous to CAKs of metazoans but is distinct from Cak1At of *Arabidopsis*[2]. We revealed that R2 formed at least two protein complexes of 190 kDa and 70 kDa, respectively, whereas the CDK- and CTD-kinase activities associated with R2 were found in a complex of 105 kDa. This is a unique feature of R2 when compared with animal CAKs.

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Cell division proteins in Arabidopsis

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We have used a gene trap/enhancer trap transposon mutagenesis system in *Arabidopsis* to generate a large collection of independent insertion lines (1). This collection has been examined for insertions into genes encoding cell division proteins, by screening for mutations that affect either embryo or gametophyte development. Previously, an insertion into a gene encoding the *Arabidopsis* homolog of the yeast cell cycle proteins Cdc46/Mcm1 has been described (2). This protein in yeast belongs to a class of initiation factors for DNA replication, and may act as a "licensing factor" that restricts replication to one round per cell cycle. In *Arabidopsis* the mutation results in lethality of the female gametophyte. More recently, we have isolated another mutant containing an insertion into a gene encoding the *Arabidopsis* homolog of budding yeast Cdc16 and fission yeast cut9. Both yeast proteins are required for progression through the G2/M transition and are thought to be components of the Anaphase Promoting Complex (APC). In budding yeast, Cdc16 may also be required for initiation of DNA replication, while in fission yeast, cut9 may be involved in checkpoint control since *cut9* mutants undergo cytokinesis without nuclear division. The *Arabidopsis* mutant exhibits defects in female gametophyte development. These defects are being analyzed with a view to understanding the functions of these proteins in higher plants.

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Members of the plant shaggy/GSK-3 protein kinase gene family are implicated in a signalling pathway regulating cell division and development.

The *Drosophila shaggy* (*sgg*) gene and its mammalian homologue *GSK-3* are key components of the wingless and wnt signalling pathways required for cell fate determination and the establishment of the boundaries between tissues.

The plant *shaggy/GSK-3* family of protein kinase genes is composed of at least 10 members per haploid genome. Eight genes have been isolated from *P. hybdria* and *A. thaliana*, named respectively *PSK* or *ASK*. For all the genes the cognate cDNAs have been characterized as well. Furthemore, a cDNA (*i. e. BSKθ*) was isolated from a *B. napus* microspore derived embryo library, recently the orthologue has been isolated from *A. thaliana* as well. These genes have been sequenced, characterized and phylogenetic analyses suggested that they belong to a family containing at least three ancient groups of genes that diverged early in land plant evolution (see Dornelas et al., 1998a and Tichtinsky et al., 1988). Although these genes were isolated from different species, the gene sequences of one group are closer to each other than to members of the same species of the other two groups. The members of group I (*i. e* ASKα and γ) and II (*i. e.* Askι, ASKζ and ASKη) are expressed in vegetative and reproductive organs, while genes of group III (*i. e. PSK6, PSK7, ASKθ, ASKβ*) are mainly expressed in reproductive organs. The different classes may reflect specificity of substrates and/or different biological functions. The differential expression of each member was accessed by Northern, RT-PCR and *in situ* hybridization (see Dornelas et al., 1998b and Tichtinsky et al., 1998).

Antisense gene constructs of the *PSK6* and 5 *ASK* gene sequences under the controle of the organ specific promoters *FBP7* (ovule), *LTP* (protoderm), *LAT53* (pollen specific) as well as the 35SCaMV promoter have been made and integrated into the genome of Petunia and Arabidopsis using Agrobacterium. About 20 antisense transgenic plants have been obtained for each gene construct. Severall highly interesting phenotypes have been obtained. Our results show that the above gene family has essential functions in plant development.

Analyses of the antisense phenotypes suggest that the genes play an important role in organ number determination, flower morphogenesis, ovule, embryo sac and embryo development. Based on expression patterns accessed by *in situ* hybridization and on the phenotype of Arabdopsis transgenic antisense plants, it is likely that the ASK genes are essential for defining boundaries within the meristem by controlling cell division during organ primordia growth. Hence, our results define a novel evolutionary conserved wingless signalling pathway component in plants.

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Cell cycle-dependent proteolysis in plants: identification of the destruction box pathway and metaphase arrest produced by the proteasome inhibitor MG132.

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It is widely assumed that mitotic cyclins are rapidly degraded during anaphase, leading to the inactivation of the cell cycle-dependent protein kinase Cdc2 and allowing exit from mitosis. The proteolysis of mitotic cyclins is ubiquitin/26S proteasome mediated and requires the presence of the destruction box motif at the N terminus of the proteins. As a first attempt to study cyclin proteolysis during the plant cell cycle, we have investigated the stability of fusion proteins in which the N-terminal domains of an A-type and a B-type tobacco mitotic cyclin were fused in frame with the chloramphenicol acetyltransferase (*CAT*) reporter gene and constitutively expressed in transformed tobacco BY2 cells. For both cyclin types, the N-terminal domains led the chimeric cyclin-CAT fusion proteins to oscillate in a cell cycle-specific manner. Mutations within the destruction box abolished cell cycle-specific proteolysis. Although both fusion proteins were degraded after metaphase, cyclin A-CAT proteolysis was turned off during S phase whereas that of cyclin B-CAT was turned off only during the late G_2 phase. Thus, we demonstrated that mitotic cyclins in plants are subjected to post-translational control (e.g., proteolysis). Moreover, we showed that the proteasome inhibitor MG132 blocks BY2 cells during metaphase in a reversible way. During this mitotic arrest, both cyclin-CAT fusion proteins remained stable.

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ASK **genes in** *Arabidopsis*

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Targeted protelolysis has been shown to be one of the key mechanisms regulating the cell cycle in yeast. The highly regulated proteolysis of regulatory proteins such as cyclins and Cdk inhibitors, is mediated through the ubiquitination pathway. This involves activation of ubiquitin by an E1 enzyme and transfer to an E2 enzyme, which then conjugates ubiquitin to a target protein recruited by an E3 ubiquitin-ligating enzyme. In addition to the anaphase promoting complex (APC) activated in the G2/M, another protein complex was found serving as an E3 ubiquitin ligase in the G1/M. This complex consists of Skp1, Cdc53 and an F-box protein (SCF). The F-box protein is the receptor and recruits target proteins to the E3. The F-box is important for interaction with Skp1. Homologs of the SCF proteins are found in all eukaryotes.

The *UNUSUAL FLORAL ORGANS* (*UFO*) gene from *Arabidopsis* encodes an F-box protein. Loss of function mutations in *UFO* result in the replacement of early flowers with inflorescences, as well as highly variable and homeotic transformations of floral organs in the second (petal) and third (stamen) floral whorls. In the floral meristem, *UFO* is expressed early during primordia development in a small number of non-dividing cells. It has been postulated that UFO is somehow involved in positively regulating the B-class floral organ identity genes *APETALA3* and *PISTILLATA*.

Using the yeast 2-hybrid system, UFO was found to interact with two *Arabidopsis* SKP1-like proteins ASK1 and ASK2. The interaction was verified by *in vitro* protein binding and deletion of the F-box domain abolished the interaction. The ASK genes are part of a large gene family and share homology with at least 7 more *Arabidopsis* genes. A yeast 2-hybrid screen with ASK1 and ASK2 as bait constructs resulted in the isolation of a large number of different *Arabidopsis* F-box genes. In addition to the F-box, most of these genes contained domains that are known to be involved in protein-protein interactions. Possibly ASK1 and ASK2 are components in many different protein complexes.

The genomic *ASK1* gene was isolated and the *ASK1* promoter region was cloned in front of the GUS reporter gene. *Arabidopsis* plants transformed with this gene construct revealed high ß-glucuronidase activity in lateral root meristems, stipules, mature pollen and transiently in the abscission zone of siliques after flowering. Lower activity was observed in developing pollen and in fertilised ovules. Little or no expression of the GUS gene was seen in the floral meristems.

A mutant was obtained in *ASK1* from a T-DNA tagged *Arabidopsis* population and was found to be affected in pollen maturation. The *ask1-1* allele was not transmitted through pollen, suggesting that *ask1-1* pollen are nonviable. The *ASK1/ask1-1* heterozygote exhibited a reduced percentage of viable pollen as expected from a 1:1 segregation for the *ask1-1* allele. As a result of this phenotype, we were unable to obtain an ask1-1/ask1-1 homozygote. Interestingly, the Ma laboratory (Penn State Univ) has identified a mutant *ask1* allele obtained by *Ds* element insertion, although this allele is transmitted through pollen. The homozygote is viable, but has a floral phenoptype that shares some characteristics with *ufo*.

ASK1 and ASK2 also interact with TIR1 in the yeast 2-hybrid system. TIR1 is another F-box protein involved in auxin signal transduction (Estelle laboratory, Indiana University). The phenotype and expression of *ASK1* indicate that ASK1 is indeed located in the same pathway as UFO and TIR1. Protein-interaction and phenotypic evidence supports the possibility that target ubiquitination is involved in regulating aspects of floral development in *Arabidopsis*.

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Session 3: Cell cycle and organogenesis

Chair: Denes Dudits

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Control of Cell Cycle Gene Expression during Floral Morphogenesis.

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Plant growth is the combined result of cell proliferation and cell expansion. In plants, cells tend to be produced in the (approximate) location where they are required. In the meristem, the production and elaboration of lateral organs is associated with localised proliferation and expansion. To understand the molecular basis of cell proliferation within the apical meristem we have isolated cell cycle control genes from the Snapdragon, Antirrhinum majus, with a view (a) to defining the spatial and temporal pattern of gene expression during floral morphogenesis and (b) to investigating if and how developmental regulators impinge on cell proliferation.

Key regulators of cell proliferation in eukaryotes include the cyclin dependent protein kinases (CDKs). The minimal kinase complex comprises the CDK catalytic subunit and an activating cyclin subunit. The founding member of the CDK family of protein kinases, the cdc2 gene from S.pombe, is required for cell cycle progression at both G1/S and G2/M. In higher organisms multiple CDKs can be involved at different stages in the cell cycle. We have found that Antirrhinum contains a small family of CDK genes, all of which are expressed in proliferating cells. Based on structural similarities, function in yeast and expression pattern in the plant, the CDK gene family falls into two groups. The first group is very similar to the yeast cdc2 gene, functionally complements mutations in the yeast gene and transcripts are expressed throughout the cell cycle. The second group, found only in plants, diverge in the cyclin interaction domain, do not function in yeast and are expressed in restricted phases of the cell cycle. These data indicate that CDK function in plants has diverged significantly from other groups and may undertake specialised roles in the plant cell cycle.

Cyclins also comprise a multigene family and are an essential component of the CDK kinase complex. In many cells, the activity of the CDK complex is regulated (at least in part) by cyclin abundance. Cell cycle checkpoints could act through regulation of cyclin abundance and developmental controls may be superimposed on these checkpoints. Also, a given CDK can, potentially, interact with different cyclins and this may profoundly alter function.

The different cyclin subclasses are expressed in distinct patterns within the meristem. Cyclin B1 genes are expressed in a stochastic manner, during G2 and M-phase. Cyclin A3 is expressed more generally but is elevated in isolated cells.
Three members of the D-cyclin gene family, implicated in the decision to enter or exit the cell cycle, are expressed throughout the cell cycle in meristematic cells. However, different cell populations within the shoot meristem express different combinations of D-cyclins. D-cyclin expression is also regulated by availability of a carbon source and is differently modulated by plant growth substances suggesting that these genes might be the targets of growth regulators.
We have examined how these genes are expressed in a variety of developmental mutants with either general or localised effects on cell proliferation. The cycloidea gene, which modifies development in the dorsal part of the flower, specifically represses both cell proliferation and cyclin D3b expression in the dorsal stamen.
Understanding how such genes regulate cell cycle gene expression should provide insight into the regulation of cell division in the meristem.

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Cell division in plant growth control

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Introduction

Plant growth responds flexibly to nutrient resources, particularly the availability of nitrogen, potassium and phosphate to roots. These mineral nutrients are the major factors limiting overall plant growth. When plants experience nutrient deficiencies, their responses include changes in root growth patterns and rates, for example lateral root induction or root apical growth acceleration. Our goal is to understand the mechanisms involved in root growth control, specifically how the regulation of cell proliferation affects organ growth.

Root growth involves three processes: cell growth in the apical meristem alternates with division to give rise to new cells. However, the bulk of organ growth is mediated by turgor-driven cell expansion in the adjacent post-meristematic expansion zone of the root. Plant organ growth is thus the aggregate result of cell growth, division and expansion, each of which provide independent avenues for growth regulation. We have shown that elevated expression of a mitotic cyclin in transgenic Arabidopsis plants results in enhanced root apical growth rates, likely by increased proliferation[1]. We have therefore proposed previously that changes in cell division activity can serve in graded growth control to control the number of cells produced per unit time.

Results

To determine which process – cell division or expansion – is primarily affected in plants adapting to altered nutrient availability, we examined changes in meristem organization. We used a transgenic *Arabidopsis* line in which the mitotic cyclin B1a promoter and part of the coding region was fused to the GUS reporter gene. In this line we can specifically identify cells in the G2/M phase of the cell cycle and thus delineate the extend of the proliferating zone of the apical meristem. Moreover, we can measure the volume of cells at the time of division, which reveals whether the timing of mitosis relative to the cell growth process is altered.

In *Arabidopsis*, root growth increases transiently up to 250% of control plants in response to reduced nitrogen availability. In N-deprived roots, the length of mature root cortical cells, taken as measure of cell expansion processes in the root, was increased by only 10% and thus is not sufficient to account for the overall increase in root growth. This suggests that changes in the rate of cell production, presumably mediated by cell division control mechanisms, are primarily responsible for the observed acceleration.

Increased proliferation in apical meristems could result from either accelerated transit through the cell cycle, or by increasing the number of dividing cells, either of which will result in a net increase of cell production per unit time. In N-limited roots, we observed an approximate 20% size increase of the region in which proliferation occurs. However, the spatial distribution of cells in G2/M, as monitored by GUS staining did not differ markedly from that observed in roots grown with adequate N. This suggested that if expansion of the proliferating zone occurred, cell division in the newly recruited population was not very frequent. If accelerated transit through the cell cycle at G2/M occurs in N-deprived roots, two predictions would have to be met: First, we expect the fraction of cells in this cell

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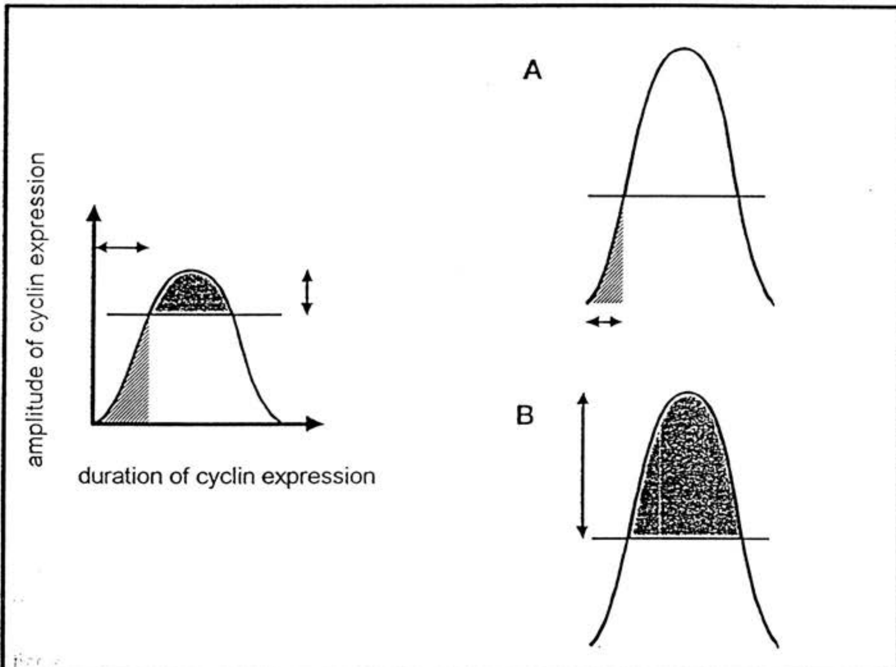
cycle phase within the whole population to decrease, and second, the cell volume at the time of mitosis should be decreased. A comparison of meristems of N-deprived roots with controls grown with adequate N, reveals that while root growth increases 2.5-fold, the number of cells in mitosis increases by only 1.5 fold. Furthermore, cell size at birth in root apices from N-deprived plants are on average 30% smaller. The simplest explanation for these observations is that cell cycle progression in N-limited roots is accelerated.

Discussion

We hypothesize that graded control of root apical meristem activity in response to N-deficiencies is mediated by quantitative changes in cyclin expression. Quantitative changes in cyclin expression can affect organ growth rate in two ways. Higher rates of transcription lead to a more rapid attainment of effective levels of cyclin (Fig. 1 A). This leads to a shortened G2 phase and concommitantly a smaller cell size at division. Increased cyclin expression also increases the absolute amount of cyclin made, perhaps allowing some cells already well on their way to quiescence to divide one further time (Fig. 1B)



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Cell cycle control during maize germination.

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Most of the cells in a dry seed embryo are in G1 phase and move into the S phase only after several hours of imbibition. In maize, cell cycle events can be accelerated if seeds are imbibed in the presence of cytokinins. We have found that under these conditions, the beginning of the S phase is shifted from 12-15h to around 3-6h of germination and replicative DNA polymerases are rapidly activated. This increase in activity seems to be due to protein modification since there is no evidence of an increase in the amount of the polymerase proteins. Mitotic figures in stimulated germinating seeds also start appearing several hours in advance with a concomitant early activation of the M-phase $p34^{cdc2}$ kinase and a possible transport into nuclei, its site of action.

We have cloned, sequenced and overexpressed PCNA, a DNA polymerase delta auxiliary protein, essential for DNA replication and also involved in cell cycle control. By using antibodies developed against this protein, we have found that expression of PCNA is enhanced by cytokinins along germination with a peak at 6h, coinciding with the replication peak. On the other hand, abscisic acid, which inhibits germination, blocks PCNA accumulation under conditions in which DNA replication is prevented.

Immunoprecipitation experiments have indicated that maize PCNA interacts with a kinase-containing putative cyclin D protein. This putative cyclin D-kinase complex, the kinase activity that controls entry into the cell cycle, is active during early germination and then the cyclin moiety is degraded, and faster if seed axes are imbibed in the presence of cytokinins. During a normal germination process, PCNA shifts between two cell cycle states: one in which it associates with the putative cyclin D-Cdk4 complex, condition in which PCNA would be unable to interact with replication proteins, and a second state in which it is released from the cyclin-kinase complex, when the cyclin is degraded. The timing of PCNA protein association to and release from the kinase complex is modified by cytokinins so that PCNA appears to be free by 6h of germination, coinciding with the time of maximal DNA replication, and suggesting that free PCNA would then interact with polymerase delta and stimulate the S phase. Maize germination is discussed in terms of control of the cell cycle.

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CELL CYCLE REGULATION DURING NODULE ORGANOGENESIS

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Symbiosis between *Rhizobium* soil bacteria and their leguminous host plants results in the formation of a novel plant organ, the root nodule, specialised for symbiotic nitrogen-fixation. Nodule development can be programmed in a well defined root zone by application of the *Rhizobium* the lipochitooligosaccharide Nod factors acting as host-specific mitogen signals.

Nodule organogenesis is based on the *de novo* formation of the nodule meristem. This requires re-entry of differentiated cortical cells in the cell cycle in the emerging root hair zone in front of the protoxylem poles. Following the initiation of the meristem, cells start to differentiate into various nodule cell types. This process is linked to cell division arrest and accompanied by gradual increase in cell size and endopolyploidy. Thus, key events of nodule development are linked to cell cycle regulation.

Studying nodule organogenesis in the *Rhizobium meliloti* - *Medicago* symbiotic model system, we aim at the identification of cell cycle components switching the cell fate either from quiescent state to proliferation or from proliferation toward differentiation. In *Medicago* roots and cell cultures Nod factors activate the cell cycle in the G0-arrested cells. To elucidate the action of Nod factors on re-entry of cell cycle and cell proliferation we have been studying different cell cycle elements, particularly cyclins regulating the transition of cells through the G0/G1-S and G2-M phases. Their involvement in nodule organogenesis will be presented.

By studying the exit from cell proliferation and differentiation of nodule cells we have identified a novel regulator of the plant cell cycle, *ccs52* that controls growth arrest, endoreduplication and cell size and is essential for differentiation of nodule cells. Our data indicate that *ccs52* is a conserved regulator of the plant cell cycle and it has a general role in plant development.

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Transport to the vacuole: receptors and *trans* elements

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The vacuolar targeting signals of several soluble plant proteins are now relatively well defined; however, few proteins involved in the targeting process have been identified. Several likely members of this trafficking machinery have been characterized from *Arabidopsis thaliana*: AtPEP12p, a t-SNARE that resides on what we now call a prevacuolar compartment (Conceicao et al., 1997; AtELP, a protein that shares many common features with mammalian and yeast transmembrane cargo receptors (Ahmed et al., 1997), AtVPS45, a peripheral membrane protein, a homologue of which in yeast is required for transport to the vacuole (Bassham and Raikhel, 1998) and two very similar proteins, AtVTI1a and AtVTI1b, v-SNAREs which maybe involved in interaction with several t-SNAREs residing on different compartments along the secretrory pathway. We have further investigated the intracellular distribution of all these proteins in comparison to one another. We have found that AtELP is located at the TGN of *Arabidopsis* root cells and can preferentially interact with the TGN-specific AP-1 clathrin adaptor complex. Further, consistent with a role in trafficking vacuolar cargo, we have found that AtELP co-localizes with AtPEP12p on a prevacuolar compartment and AtVPS45 co-fractionates with AtELP (Sanderfoot et al., 1998). The connection between these markers, as well as to other endomembrane markers which we continue to characterize, will allow us to investigate the complexity of plant secretory machinery in greater detail. This knowledge is pivotal to the effectiveness of various basic and biotechnological

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This work was supported by grants from NSF and DOE to NR.

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Session 4: Cell cycle and cytoskeleton components

Chair: Andrew Staehelin

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The functional and evolutionary significance of gene families encoding cytoskeletal proteins: diverse plant actins and profilins

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Our working hypothesis has been that the two ancient and divergent classes of plant actin genes (vegetative and reproductive) have been preserved throughout vascular plant evolution because they have unique patterns of regulation and/or encode protein isovariants with distinct functions. We made use of the compact genome, rapid life cycle, small size, ease of transformation and regeneration, and simple genetics of *Arabidopsis thaliana* to dissect different parts of this hypothesis. Based on the analysis of actin promoter/reporter fusions in hundreds of transgenic plants, RNA levels, and protein levels assayed with subclass-specific monoclonal antibodies, we determined that the two vegetative actin subclasses and three reproductive actin subclasses each have strong and distinct tissue specific and temporal expression patterns. These range from constitutive expression of *ACT2* in most vegetative tissues, to the phytohormonal induction of *ACT7* in rapidly expanding or wounded vegetative tissues, to the expression of *ACT1* in ovules and pollen, to the expression of *ACT11* in all reproductive structures and throughout fruit and seed development.

Sequences-based screening methods were used to isolate T-DNA insertion mutants in four of the eight expressed-actin genes, since no clear prediction could be made about the phenotypes of possible actin mutants. These mutants have exciting, but subtle cell and developmental phenotypes that are only revealed when plants are grown under specialized conditions. Multigenerational studies on mutants in three actin genes grown under idealized greenhouse conditions suggest that each gene is under strong selective constraint and is required for the survival of *Arabidopsis*. Thus, consistent with the high degree of sequence conservation of all eight *Arabidopsis* actins, these actin gene family members are not redundant.

But what evolutionary forces and molecular mechanisms selected out vegetative and reproductive plant actin genes in the first place? It is well established that the plant cytoskeleton is essential for physically programming the development of organs and tissues. One of the greatest puzzles in evolutionary biology is how large numbers of genes were simultaneously recruited to build new organ structures is. The vegetative and reproductive classes of actins arose concurrent with the macroevolution of leaves from duplicated and modified

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reproductive structures in the earliest land plants. The other cytoskeletal protein families that have been studied (e.g., profilins, actin depolymerizing factors, α-tubulins, β-tubulins) also have diverse members with vegetative and reproductive expression patterns. Thus, we propose that this regulatory dichotomy represents an ancient landmark event in the global regulation of hundreds of higher plant cytoskeletal genes, an event linked to the macroevolution of plant vegetative and reproductive organs. The recent availability of sequence and expression data for large numbers of plant genes should make it possible to further dissect this other important macroevolutionary events linked to novel regulation of the cytoskeleton.

There are a relatively large number of non-conservative amino acid substitutions mapping to different protein surfaces among the isovariants that may effect actin polymerization into homo- and hetero-polymers and its interactions with the numerous actin-binding proteins. It is likely that each plant actin isovariant will have its own distinct set of dissociation and rate constants for many of these interactions. Are the plant actins sequences simply under less selective constraint than animal actins or are these relatively extreme differences important? Experiments are under way to test the ectopic expression of different actins and suppression of various actin mutants with heterologous isovariants. While we expect these experiments will demonstrate the different isovariant structures are important to cellular function, it will not explain how such diversity evolved.

One explanation for the preservation of such sequence diversity is that the co-expression of diverse protein isovariants leads to more dynamic protein-protein interactions and is of selective advantage to plants. Isovariant dynamics, as defined herein, result in the temporal and biochemical expansion and buffering of responses of a biological system due to the simultaneous expression and interaction of multiple isovariants of a protein. Expression of multiple isovariants with a variety of dissociation constants would broaden the possible responses of the plant cytoskeleton to various signals and stresses. The actin gene family in *Arabidopsis thaliana* serves as a useful case study of isovariant dynamics, because the expression of all the family members has been thoroughly studied and there is detailed information about the three-dimensional structure of actin proteins. Future research will quantify these interactions and attempt to determine if the co-expressed actins are co-localized into the same filaments and bundles or separated into distinct structures in plant cells.

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Profilin and actin from maize pollen: a complex problem.

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Plant reproduction depends upon the successful transmission of the male genome, contained in the sperm, to the embryo sac where double-fertilization occurs. Sperm from angiosperms are non-motile, but are deposited in the embryo sac by the unique behavior of the vegetative cell of the pollen grain. Studies using cytochalasins, compounds which bind to actin filament ends and depress actin dynamics, have been used to demonstrate that polarization of cytoplasm, selection of a germination site and pollen tube extension are all dependent upon an intact actin cytoskeleton However, relatively little is know about the factors which regulate actin polymer levels and filament dynamics in pollen. Indeed, only sparse information about the size and coordination between the monomer (G-) and polymeric (F-) pools of actin exists for plant cells generally. We have used a new class of actin cytoskeleton inhibitor to examine the fundamental requirement for a precise F-actin level during pollen development. Latrunculins are marine macrolides isolated from a red sea sponge which exhibit the potent ability to bind and sequester G-actin. Latrunculin B (LATB) binds to purified maize pollen G-actin, with a K_d value of 70 nM, and causes F-actin depolymerization *in vitro*. When applied to living pollen grains and tubes, LATB treatments revealed that there are different requirements for F-actin between key stages of pollen development and morphogenesis. Growth of the maize pollen tube was much more sensitive to LATB than was pollen germination, suggesting that tip growth was extremely sensitive to alteration of F-actin levels or to changes in the F- to G-actin ratio. To examine this further, we measured F-actin and total actin protein concentration in pollen with a sensitive fluorimetric assay for phalloidin-binding sites and with an ELISA method, respectively. The total actin protein level did not change during maize pollen germination or when pollen was treated with LATB. In contrast, treatment with LATB caused a dose-dependent reduction of F-actin in pollen. Furthermore, ungerminated grains were as sensitive to the inhibitor as growing pollen tubes. These findings suggest that pollen tip growth has a different and more stringent requirement for F-actin than does germination, and provides the first evidence that these two processes are mechanistically distinct.

Profilin, a small protein with the ability to bind to G-actin, is a complex regulator of actin organization that can cause disassembly or promote polymerization depending on cellular ionic conditions and the presence of other actin binding factors. We find that profilin is an abundant component of maize pollen, with intracellular concentrations of ~95 µM, and is present in nearly equimolar amounts with pollen actin. By measuring a dissociation constant for purified, native pollen actin binding to pollen profilin (1.4 µM), and extrapolating to the situation of pollen cytoplasm, we predict that the bulk of actin in pollen grains and tubes is present as profilin–actin complex. Additional biochemical and molecular characterization of pollen profilin reveals that this protein may be a key intermediate between components of signal transduction cascades and actin organization. We have found that at least 5 profilin transcripts are present in maize pollen. Recombinant maize pollen profilins fall into at least two classes based on their functional properties *in vitro*. Furthermore, the multiple profilin isoforms from maize pollen differ in the ability to alter cellular architecture. These differences among isoforms correlate with the ability to bind to poly-L-proline *in vitro*, which presumably reflects the ability to bind proline-rich cellular proteins. Surprisingly, the highly divergent pollen profilins also have several previously unexpected properties including the ability to modulate protein kinase activity *in*

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vitro. Our hypothesis is that profilin is a major regulator of pollen actin organization and that multiple profilins perform distinct functions during pollen development.

This work was supported by grants from the USDA-NRICGP.

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Cytoskeleton - Organizing Centers (COCs) in Higher Plant Cells.

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The striking co-distribution and co-alignment of actin filaments and microtubules in most cytoskeletal arrays of the higher plant cell, such as in the cortical bundles, the pre-prophase band, the phragmoplast, suggests cooperative activities of both polymers under cell-cycle control. This organization also suggests the presence of spatially co-distributed sites of microtubule and F-actin assemblies, that could be termed Cytoskeleton-Organizing Centers or COCs instead of restrictive MTOCs. Compelling evidence has been obtained *in vivo* for the idea that F-actin assembly in the cell cortex or in the post-mitotic interzone precedes microtubule assembly and is guiding the spatial distribution of microtubules, such as in the phragmoplast (1) or the preprophase band. The positioning of the plant mitotic spindle also involves the cooperative activity of cortical actin filaments and microtubules which interact with the cell membrane at sites where the cell-plate will later fuse.

The molecular mechanisms that control those essential events remain unknown. We address the questions of what is the nature of the higher plant COCs , where and how they are activated. The nuclear surface is so far the only site of microtubule nucleation that has been functionally identified in plants (2). Its activity for actin assembly is supported by *in vitro* experiments on isolated plant nuclei. The presence of gamma tubulin around the nucleus and surprisingly over most microtubule arrays raises the question of whether microtubule-nucleating complexes that are concentrated at the nuclear envelope could be transported along microtubules towards cortical domains where they could be co-activated with actin assembly sites. To investigate this hypothesis complementary approaches are developed. We established tobacco cell lines and plants overexpressing maize or tobacco gamma tubulin. F2 generations are now investigated. GFP-gamma tubulin constructs used for transient expression in tobacco protoplasts permitted to identify cytoskeleton reorganization consequent to transfected gamma tubulin accumulation.

In parallel, the molecular identification of cytosolic protein complexes competent for the assembly of plant microtubules and/or actin filaments is under way. We have already found that higher plant cell free extracts are capable to restore the nucleating activity of isolated mammalian centrosomes that have been inactivated by urea, indicating the presence of soluble complexes competent for microtubule nucleation when they are recruited at an organizing center (3).

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ROLE OF ACTIN CYTOSKELETON IN POLARIZED GROWTH OF PLANT CELLS

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Pollen tube cells grow rapidly and in a highly polarized manner based on actin dependent targeted secretion at the tip. Rho family small GTPases have been implicated in the regulation of related processes in animal and yeast cells. We have cloned a gene encoding an *Arabidopsis thaliana* Rac type Rho family protein that is preferentially expressed in pollen tubes. The function of this protein in the regulation of pollen tube growth was analyzed. Expression of dominant negative Rac inhibited pollen tube elongation, whereas expression of constitutive active Rac induced depolarized growth. Rac was found to accumulate at the plasma membrane specifically in the pollen tube tip. Biochemical analysis demonstrated that Rac physically associates with a phosphatidylinositol monophosphate kinase (PtdIns-P-K) activity in pollen tubes. Phosphatidylinositol 4, 5-bisphosphate (PtdIns 4, 5-P_2), the product of PtdIns-P-Ks, was observed to localize to the plasma membrane at the tip similar to Rac. Expression of the green fluorescent protein (GFP) fused to the pleckstrin homology (PH-) domain of phospholipase C (PLC)-δ_1, which binds to PtdIns 4, 5-P_2 specifically and with high affinity, strongly inhibited pollen tube elongation. Our results indicate that Rac and PtdIns 4, 5-P_2 act in a common pathway to control polar pollen tube growth and provide direct evidence for a function of PtdIns 4, 5-P_2 compartmentalization in the regulation of this process.

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Session 5: Mitosis and cytokinesis

Chair: Venkatesan Sundaresan

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Control of plant mitosis

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Plants are multicellular organisms with defined shapes and sizes. A developmentally determined body plan is elaborated by the controlled timing and orientation of cell divisions in restricted zones, the meristems. Conserved regulators of cell division in eukaryotes are the cyclin-dependent protein kinases (CDKs). The expression of cyclin genes is tightly regulated in plants, e.g. B-type cyclins are only found in mitotic cells. We used a tetracyclin-regulatable promoter construct to study if cyclin expression is limiting in the timing of cell divisions. The expression of a mitotic cyclin gene, *cycMs2* of alfalfa (Hirt et al. 1992), in suspension cultures of transgenic tobacco plants resulted in an elevated CDK activity, and cells with an increased cyclin amount entered mitosis about 2 hours earlier (M. Weingartner, I. Meskiene, M. Pfosser, K. Zwerger, P. Binarova, B. Melikan, L. Bögre, and E. Heberle-Bors, in prep.). The shortened G2 phase had no apparent effect on the phenotype of tetracyclin-treated seedlings, but interfered with root organogenesis in vitro. In auxin- and cytokinin-dependent regeneration of roots and shoots from tobacco leaf disks, the tetracyclin-induced premature passage through mitosis inhibited root formation, i. e. overexpression of the mitotic cyclin had an anti-auxin or cytokinin-like effect. We propose a model in which shoot versus root regeneration is regulated by the G1 and G2 exit points from the cell cycle.

MAP kinases have been shown to have regulatory roles in mitosis in fungi and animals. We recently isolated two MAP kinase genes, i. e. *ntf6* in tobacco and *mmk3* in alfalfa which play similar roles in mitosis (Calderini et al. 1998, Bögre et al. 1998). MMK3/NTF6 showed an activity pattern during mitosis which is very similar to the ERK1 and ERK2 MAPKs in somatic mammalian cells, and they are also found at comparable locations, i.e. at the midplane of cell division in the phragmoplast of plant cells and in the midzone between the two daughter nuclei in anaphase in animal cells (Shapiro et al. 1998). Normal microtubule dynamics were necessary for MMK3/NTF6 activation but intact microtubules were not sufficient for MAPK activity. As the phragmoplast microtubules were redistributed from the center to the periphery during telophase, MMK3 still localized to the whole plane of division, indicating that phragmoplast microtubules are not required to keep MMK3 at this location.

What might be the signal which the MMK3/NTF6 MAP kinase transmits? The tobacco MAP kinase kinase kinase NPK1 is also located at the cell plate, although unlike MMK3/NTF6, its protein level increases during mitosis (Machida et al 1998). This suggests that NPK1 may be an upstream regulator of NTF6. NPK1 is also a negative regulator of auxin signaling (Kovtun et al. 1998).

Recently, embryo-patterning genes in Arabidopsis, such as KNOLLE and KEULE have been identified as being involved in cytokinesis (Lukowitz et al. 1996, Assaad et al. 1996), and the syntaxin-related KNOLLE protein was localized at the cell plate (Lauber et al. 1997). Similar to MMK3, KNOLLE seems to be present across the entire plane of division late in mitosis when the phragmoplast reaches the lateral cortex of the cell.

The relationship of embryo patterning, organogenesis, cell divison, cytoskeleton, signal transduction and hormones constitutes a crossroads with stimulating prospects for an understanding of plant growth and development.

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ASSEMBLY OF THE CELL PLATE DURING PLANT CELL CYTOKINESIS

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We have used high pressure-freezing/freeze-substitution and immunocytochemical techniques to investigate cell plate formation in synchronized tobacco BY-2 cells as well as in Arabidopsis and tobacco root tip cells. These studies have led to the development of a model of cell plate formation (1) that distinguishes two major phases, a phragmoplast-associated phase and a post-phragmoplast phase. Each of these phases can be further subdivided into specific assembly steps which develop sequentially and can be observed adjacent to each other as the cell plate expands from the center.

The first phase commences with the delivery of Golgi-derived vesicles to the equatorial plane along phragmoplast microtubules, where they immediately begin to fuse with each other. Fusion of the vesicles is brought about by ~20 nm tubules that grow out of individual vesicles and fuse with others giving rise to a continuous, interwoven, fusion tube-generated network. Consolidation of this network into a tubulo-vesicular network is accompanied by the assembly of a dense "fuzzy" coat of unknown composition on the membranes and the appearance of numerous clathrin-coated buds. We postulate that these clathrin-coated buds remove both excess lipids and selected proteins from the tubulo-vesicular network and thereby contribute to its maturation into a smooth tubular network. The loss of the fuzzy coat from the tubulo-vesicular network during its conversion into a slimmed down tubular network coincides with the disassembly of the central phragmoplast MTs and the assembly of new ones at the leading edge of the cell plate. These new MTs bias the delivery of vesicles to the outer edge of the expanding cell plate where they fuse and initiate a new round of assembly. Meanwhile the central tubular network fills with callose produced by enzymes in the cell plate membrane, which results in its conversion into a fenestrated sheet-like structure and then into a continuous primary wall. Fusion of the cell plate with the parental plasma membrane is brought about by fusion tubes generated along the plate margins. This fusion appears to coincide with the appearance of the first cellulose fibrils and a concomitant reduction in callose deposits.

To further analyze these events we have re-examined the effects of two drugs known to inhibit cell plate formation, caffeine (2) and dichlobenil (DCB) (3, unpublished results). In the presence of 10 mM caffeine, which most likely perturbs local calcium gradients, the Golgi-derived vesicles fuse normally and give rise to a typical fusion tube generated network capable of expanding centrifugally. However, further maturation of the cell plate into a

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tubulo-vesicular network appears to be impaired as evidenced by the lack of assembly of a fuzzy coat, the lack of callose synthesis, and the lack of clathrin-coated bud formation. In the absence of these changes, the membrane network remains in a fragile state and eventually breaks up into vesicles that are reabsorbed.

It is generally assumed that the effects of DCB on cell plate formation are related to its ability to inhibit cellulose synthesis (3). Studies of the effects of DCB on olfactory neuroepithelial cells of catfish, however, have suggested that DCB may act on regulatory G-protein systems (4). Our most recent observations are consistent with the latter theory, since the data show that DCB and/or its metabolites perturbs cell plate formation prior to the cellulose synthesis step by disrupting membrane trafficking to and from the cell plate as well as cell plate maturation in general. Micrographs of BY-2 cells exposed to 1 - 5 μM DCB demonstrate that cell plate formation is normal up to and including the assembly of the fuzzy coated tubulo-vesicular network with clathrin-coated buds. However, the images of the clathrin-coated buds become more distinct and, in contrast to the controls, high numbers of buds persist through all of the subsequent stages of cell plate formation. In addition, the tubulo-vesicular network never seems to slim down even after the disappearance of the phragmoplast MTs and the loss of the fuzzy coat. This results in highly convoluted, bulbous cell plates that expand and mature slowly and do not link up efficiently with the parental plasma membrane. All of these observations are consistent with the hypothesis that DCB inhibits the budding of clathrin-coated vesicles and thereby affects the removal of excess membrane from the cell plate, yielding cell plates with abnormally high surface to volume ratios and convoluted geometries. One possible target of DCB could be dynamin (phragmoplastin), that is known to be a G-protein and has been shown to function as a vesicle "pinchase" in mammalian cells (5, 6).

Supported by grants NIH-GM18639 and USDA96-35304-3710 to A.S.

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REGULATION OF CELL PLATE FORMATION: THE LAST STEP IN PLANT CELL CYCLE

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Formation of cell plate at cytokinesis is a unique process in plant cell division. An extracellular compartment is established at the center of phragmoplast by accumulation and fusion of Golgi-derived vesicles. These vesicles fuse by extending a tubular structure which consolidates and form a tubulo-vesicular net work at the forming cell plate. We have identified a protein (Phragmoplastin) homologous to dynamin family which is involved in fusion of vesicles at the forming cell plate. Phragmoplastin is a high molecular weight GTPase but unlike dynamin is missing the SH3 domain. The molecule self polymerizes with the help of two domains that we have identified and shown to be responsible in forming a spiral polymeric structure by interaction of domain A of one molecule with domain B of the next molecule. Such structures can wrap around the tubules at cell plate. Using GFP fused to the amino end of phragmoplastin, we have shown the dynamics of cell plate formation and have uncovered a checkpoint in the completion of cell plate. By making GTP domain mutants, we have established a possible role of GTP hydrolysis. Using two hybrid system, we have identified several proteins that interact with phragmoplastin. One of these is a glucosyl transferase which may be involved is the supply of UDP-glucose for the synthesis of beta-1-3-glucan (callose) which is initiated as soon as tubulo-vesicular structure is established. We have also shown that phragmoplastin interacts with a Rho type molecule which may provide signal for the initiation of glucan synthesis. Further characterization of proteins associated with this complex may allow us to dissect the complexity and the mechanism of cell plate polysaccharide biosynthesis. The latter leads to cellulose deposition which hardens the cell plate and permanently closes the "door" dividing the cell into two.
This work was supported by a NSF grant to DPSV.

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CYTOKINESIS IN THE *ARABIDOPSIS* EMBRYO

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Cytokinesis partitions the cytoplasm of the dividing cell. This basic biological process serves multiple purposes in plant development (Jürgens, 1996, Sem. Cell Dev. Biol. 7, 867-872). For example, cell fates can segregate during the division of a polarised cell, and cell divisions that are regulated in time or space contribute to morphogenesis since the wall-bounded cells cannot change their positions relative to their neighbors. In more general terms, the newly-formed plasma membrane and cell wall may act as a barrier to help establish separate microenvironments for the daughter nuclei. Plant cytokinesis starts in the center of the dividing cell where a specific cytoskeletal array, the phragmoplast, forms between the daughter nuclei. Golgi-derived vesicles are transported along the phragmoplast to the plane of division where they fuse with one another to form the cell plate, a membrane-bounded incipient cell wall (Staehelin and Hepler, 1996, Cell 84, 821-824). As the phragmoplast is being displaced centrifugally, the disc-shaped cell plate expands by the continuous incorporation of new vesicles and eventually fuses with the parental cell wall.

We have taken a genetic approach to analyse mechanisms that underlie the regulation and execution of cytokinesis. Two classes of mutants were isolated: cell division defective mutants collectively called *pilz* group and cytokinesis mutants. Mutations in *PILZ* group genes block cell division during embryogenesis, and mutant embryos consist of only one or a few large cells each containing one or more variably enlarged nuclei and often cell wall stubs. Cell cycle regulators, such as CDK, are expressed but microtubule assembly appears defective (Mayer et al., in press). Cytokinesis mutants which represent at least 6 genes are impaired in cell plate formation. One of these genes, *KNOLLE*, is transcribed in a cell-cycle dependent manner and encodes a syntaxin-related protein (Lukowitz et al., 1996, Cell 84, 61-71). Syntaxins are members of a family of vesicle-docking proteins, and KNOLLE protein may thus play a specific role in cytokinetic vesicle trafficking. As shown by indirect immuno-fluorescence and electron microscopy, KNOLLE protein is made during the M phase of the cell cycle and accumulates in the plane of cell division during cytokinesis, mediating the formation of the cell plate by vesicle fusion (Lauber et al., 1997, J. Cell Biol. 139, 1485-1493). Mutations in another gene, *KEULE*, also cause defects in cytokinesis, and the mutant phenotype resembles that of *knolle* embryos (Assaad et al., 1996, Molec. Gen. Genet. 253, 267-277). Whereas the single mutants are seedling-lethal, the *knolle keule* embryo develops into a huge single cell with many nuclei, suggesting that the two genes have partially overlapping functions in cytokinetic vesicle trafficking (Waizenegger et al., in prep.).

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POSTERS

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Isolation of a yeast protein that interact with the replicase of the geminivirus TYLCV.- Franco M., Castillo-Garriga A., and Bejarano E.R.; Departamento de Genética. Universidad de Málaga. 29071 Málaga (Spain)

Geminivirus are plant viruses with circular single-stranded DNA genomes, containing 6-8 genes. The gene Rep is the only virus gene essencial for replication. This gene encodes a multifunctional protein that joins specifically to the viral replication origin.

The presence of this protein in the cell induces the apearance of S phase specific cell proteins. This induction is similar to that in animal viruses with a similar genomic structure (adenovitus, polyomavirus, etc.).

The fact that pRep from geminivirus WDV and Rb from mammalians interacts suggests that geminivirus could have similar mechanism to those viruses to induce the entrance in S phase. The isolation in maize of an homologous of retinoblastoma supports this idea.

As in animal viruses, the cell cycle deregulation could involve interactions between pRep and several cell cycle regulatory proteins. We have used pRep from geminivirus TYLCV and a cDNA library from *S. pombe*. Till now, we have isolated a protein that interacts with pRep and is essencial for *S. pombe*. We have identified analogous in *S. cerevisiae*, *L. esculentum* and *A. thaliana*. We are studying the function in cell cycle regulation in *S. pombe* and its interaction with pRep.

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A *MCM3* homologue is regulated through the cell cycle and by post-transcriptional processing events during *Arabidopsis thaliana* development.

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In eukaryotes, tight regulatory mechanisms ensure the ordered progression through the cell cycle phases. The mechanisms that prevent chromosomal DNA replication from taking place more than once each cell cycle are thought to involve the function of proteins of the minichromosome maintenance (*MCM*) family. Here we characterize *AtMCM3*, a new member of the *MCM* family from*Arabidopsis. thaliana*, with regard to different regulation levels. We show that *AtMCM3* trancript is regulated during the mitotic cell cycle with a maximum level in S phase. Moreover, the AtMCM3 protein is present in highest abundance in proliferating cells. The distribution of AtMCM3 to both cytoplasm and nucleoplasm suggested that this protein is not regulated by nuclear exclusion Three different cDNA clones encoding *AtMCM3* were obtained, although they possess identical coding regions, they display heterogeneous positions of cleavage and poly(A) tail lengths. Our results indicate that the *AtMCM3* mRNA 3' untranslated regions are specific to different organs, suggesting a novel post-transcriptional regulation for MCM3 genes. During leaf growth two types of cell cycles, mitotic cell cycle then endocycle, are engaged. The*AtMCM3* transcript level was maximun for the youngest stages of leaf development, when a maximum of mitotic divisions occurs, suggesting that *AtMCM3* may not be a key factor in endoreplication. Furthermore, a new function involving *AtMCM3* in DNA repair is proposed.

Tréhin C, Glab N, Perennes C, Planchais S, Bergounioux C
M phase-specific activation of the *Nicotiana sylvestris Cyclin B1* promoter involves multiple regulatory elements.
Plant J 1999 *in Press*

Trehin C, Planchais S, Glab N, Perennes C, Tregear J, Bergounioux C
Cell cycle regulation by plant growth regulators: involvement of auxin and cytokinin in the re-entry of Petunia protoplasts into the cell cycle.

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Growth enhancement by *cyclin D2* overexpression in tobacco

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In multicellular organisms the correct timing of cell division requires the coordination of developmental signals and factors that control the cell cycle. An important control point is the G1 to S-phase transition where the decision to enter S-phase or arrest in G1 is controled by G1 cyclins (e.g. D cyclins). To study the relation between plant development and cell cycle regulators we have generated tobacco plants that express *Arabidopsis cyclin D2* under control of the cauliflower mosaic virus promoter.

In comparison with control tobacco plants *cycD2* overexpressors show an enhanced growth rate, earlier flowering and increased seed yield but a normal leaf size and stature(1). This phenotype contrasts sharply with plants that overexpress other cell cycle components like cdk's(2).

Scanning electron microscopy of shoot apical meristems shows that *cycD2* overexpressors have a reduced plastochron but similar size of the apical meristem. Growth enhancement is not only restricted to the aerial part of the plant but extends also to the root. No difference in cell size could be detected in the apical meristem or leaves. The observation that the effect of *cycD2* overexpression is restricted to dividing cells is confirmed by the fact that leaf expansion rate is similar to that seen in control plants.

When exposed to a number of stresses like cold, low light and salt transgenic plants always out-perform the control plants. To investigate the effect of *cycD2* overexpression on the cell cycle more directly we have initiated experiments to measure the cell cycle time of root meristematic cells.

(1) Cockcroft et al. in preparation.
(2) Hemerly et al. (1995). EMBO J. 14, 3925-3936.

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Involvement of MAP-kinases in the initiation of cell division and cytoskeleton reorganisation

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The microtubule-associated protein 2 kinase (MAP2-kinase), now better known as mitogen-activated protein kinase (MAPK), was initially discovered in animal cells in association with the cytoskeleton, and was later also implicated in cell division. The importance of mitogenic stimulation in plant development roused interest in finding the plant homologues of MAPKs. Here we show that the alfalfa MAPK, MMK1, is likely to be involved in cell cycle regulation. During synchronous cell division after aphidicoline block MMK1 is activated in early G2 phase as well as in G1 phase. In dividing cells we found this MAPK in association with various cytoskeleton structures. MMK1 is also activated during hormone-induced cell divisions just before G1 cyclin expression. Another alfalfa MAPK, MMK3, is induced in mitosis and localises to the cell plate during cytokinesis. Therefore, sequential activation of MAPKs might regulate multiple aspects of plant cell division such as the induction of G1-cyclin expression, reorganisation of microtubules during G2 phase, entry into mitosis and cytokinesis.

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DEPENDENCE OF RB PROTEIN LEVELS ON CELL CYCLE AND GROWTH

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In all eukaryotes, cell cycle progression is tightly controlled by a number of regulatory proteins. In human cells the retinoblastoma (Rb) gene product controls the transition through the G1 phase of the cell cycle by inhibiting transcription factors, such as those of the E2F family, which are able to activate genes required for DNA replication. Moreover, the activity of different cyclin-dependent kinases temporally regulates the Rb/E2F association. Advances in the study of the G1/S regulators in plants and the isolation of a number of homologues to human cell cycle regulators (i. e. kinases and cyclins), together with the isolation of a homologue of Rb in maize (ZmRb1) in our group, lead us to focus our efforts on the study of the importance of Rb in the G1/S transition.

First, the ZmRb1 cDNA and deletions at the N-terminal, at the C-terminal and at both domains were cloned in pGEX-KG and overexpressed as Glutathion-S-transferase fusion proteins. Purified proteins from the N and C pGSTZmRb deletion (27-557), containing the functional Rb A/B pocket, and the complementary clone, containing only the C-terminal region (558-683), were used for antibody production in rabbits. Using hyperimmune sera, a doublet of about 110 Kda is detected in total cell protein extracts of both maize and wheat cells. Interestingly, extracts also contain immunoreactive bands of about 95 Kda and about 65 Kda.

The anti-Rb antibodies were also used to determine the Rb protein levels at different stages throughout the cell cycle in synchronized cultures.

In order to determine the dependence of Rb protein levels on growth signals, we starved wheat cell culture in medium without carbon source and auxin. After the starvation period, the Rb protein level is considerably decreased. Subsequent addition of the carbon source is necessary and sufficient to restore the Rb protein to a normal level.

One direct way to study the function of plant Rb is to identify cellular factors, i.e., CDK, cyclin, E2F, that interact with the protein and that, likely are part of the Rb regulatory pathway. We have used an anti-PSTAIRE antibody to assay for the presence of a Rb specific kinase. In total wheat cell extracts, this antibody is able to detect and to immunoprecipitate a band of about 34 Kda. We are currently testing the kinase activity of the immunoprecipitation products using purified GSTRb as substrate. The results of these experiments will be discussed.

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A cell cycle regulated MAP kinase with a possible role in cytokinesis in tobacco cells

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In late anaphase/early telophase a plant-specific cytoskeletal structure, the phragmoplast, is found which is involved in the deposition of material required to form the cell plate. An intimate relationship exists between microtubules and other molecules necessary for phragmoplast and cell plate formation. Cell plate formation starts in the cell centre and then progresses towards the cell periphery. The early stages are marked by the presence of two microtubular arrays with opposite polarity. These microtubules transport Golgi-derived vesicles to the newly forming cell plate, where they fuse to form a tubulo-vesicular network that later consolidates into a smooth tubular sheet in which callose is deposited. As the forming cell plate progresses towards the periphery of the cell, microtubules are displaced from the centre and follow the leading edges of the expanding phragmoplast.

Using synchronized tobacco cell suspension cultures and a peptide antibody raised against the tobacco MAP kinase $p43^{Ntf6}$, we show that entry into mitosis appears to be necessary for the activation of the kinase, which occurs as a post-translational event. Treatment of cells with different microtubule destabilizing/stabilizing drugs and analysis of MAP kinase activities indicated that the $p43^{Ntf6}$ kinase is functionally distinct from a previously described MAP kinase whose activity is required for metaphase arrest; rather normal microtubule dynamics and/or progression into a later stage of the cell cycle is necessary for MAP kinase activation. Detailed analysis of the progression of synchronized cells through the cell cycle and the associated MAP kinase activities showed that the MAP kinase was activated later in the cell cycle than $p13^{suc1}$-associated CDK activity, corresponding to late anaphase/early telophase. Indirect immunofluorescence studies with the anti-$p43^{Ntf6}$ antibody, and double labelling with the anti-$p43^{Ntf6}$ antibody and an anti-tubulin antibody identified a transient localization of the $p43^{Ntf6}$ protein to the cell plate in anaphase cells, in between the interdigitating microtubule arrays that are characteristic of the phragmoplast. The kinetics of kinase activation and the intracellular localization of $p43^{Ntf6}$ support a role for this MAP kinase in cytokinesis in tobacco cells.

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Plasticity in nodule development on *Sesbania rostrata*.

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Leguminous plants in symbiosis with rhizobia form either indeterminate nodules with a persistent meristem or determinate nodules with a transient meristematic region. *Sesbania rostrata* is a leguminous plant that forms nodules on roots and stems upon inoculation with *Azorhizobium caulinodans*. Both stem and root nodules have been described as being of determinate type, although the development of both was found to be of a hybrid nature [1, 2, 3, 4] Developing nodules induced by *Azorhizobium caulinodans* show a transient coexistence of different developmental zones, and thus resemble indeterminate nodules. However, after a week, nodule meristem activity stops and mature nodules are determinate.

Here we show that depending on the environmental conditions, mature root nodules of *Sesbania rostrata* plants can be of the indeterminate type [5]. Molecular markers for cell division (cyclin and histone SrH4) and parenchyma formation (Srenod2) have been used to demonstrate differences between determinate *Sesbania rostrata* nodules. Those observations illustrate a case of phenotypic plasticity in plant development.

The plant hormone ethylene may be involved in determining the switch from persistent to transient meristem and the subsequent start of nodule maturation.

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Differential subcellular localizations of three A-type cyclins in tobacco BY2 cells.

The major interest of our studies is the understanding of the mechanisms involved in the G1/S transition of the cell cycle. For this purpose, the highly synchronizable tobacco cell line BY2 (Bright Yellow) has been used as a model and we have cloned three A-type cyclin's cDNAs by screening a BY2 library. Two of these cDNA belonging to group A3 of the A-type plant cyclins are G1/S induced (cyc A59 and cyc A105), whereas the third one belonging to group A1 is mid-S induced (cyc A19). Owing to oligopeptides designed specifically for each cyclin, we obtained rabbit polyclonal anti-cyclin antibodies which were used in Western blot analysis. Whereas the A-type cyclin's mRNAs are strongly cell-cycle regulated, we showed that the A-type cyclin's protein levels remain constant throughout the cell cycle. This result is surprizing because the cyclin A is degraded in the mitosis phase of the animal or yeast cell cycle. To confirm this data, we have performed immunolocalizations of the three A-type cyclins throughout the cell cycle in BY2 cells using three approaches : indirect epifluorescence, confocal microscopy and electron microscopy. Our observations showed that i. the three A-type cyclins are indeed present in the BY2 cells at every cell cycle phases including mitosis when they localize to the spindle zone, ii. all three A-type cyclins have been found to localize in the amyloplasts, precisely in the starch granules area, iii. in addition, cyc A19 is also localized in the cytoplasm and cyc A59 in the nucleus of interphase cells. These observations have been confirmed by subcellular fractionations followed by western analysis. These differential subcellular localizations in BY2 cells suggest specific roles of these three A-type cyclins in dividing plant cells.

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Modulation of cell division and primordia initiation by flower homeotic genes

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During plant development, cell division occurs mainly in the meristems. In shoot apical meristems, cell divisions are relatively unsynchronized but higher mitotic indexes are correlated with the initiation of new primordia. Within the flower meristem, a highly coordinated series of events take place that allow the formation of organ primordia in a whorled phyllotaxy. One of the genes controlling this process in Antirrhinum is Fimbriata (Fim), an F-box protein that is required to establish both proper organ identity and whorled phyllotaxy. In loss of function alleles of Fim, increased organ number and a disruption of the whorled phyllotaxy suggest that primordia initiation is not fully syncronized, allowing groups of cells to adopt a primordium fate independently of position. This might result from cells with higher mitotic index in ectopic positions. In Antirrhinum, the floral identity protein SQUAMOSA (Squa), forms ternary complexes with the organ identity proteins DEFICIENS (Def) and GLOBOSA (Glo). Flower architecture of def:squa or glo:squa double mutants is identical to fim null alleles. Double mutants of weak alleles of def and fim show an enhancement of the fim phenotype with more ectopic organs, suggesting that meristem and organ identity genes, control cell proliferation by maintaining the network that coordinates establishment of primordia. Based on the capacity of DEF-GLO and SQUA complexes to recognize the Fim promoter, and the genetic interaction observed between the genes, we hypothesize that after activation of Def and Glo by Fim, DEF and GLO form a ternary complex with SQUA at the Fim promoter maintaining Fim expression, thus allowing primordia initiation only in correct positions.

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PLANT CYCLIN D EXPRESSION IN SUSPENSION CULTURED CELLS OF *CHENOPODIUM RUBRUM* L.

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Our interest focusses on the regulation of the plant cell cycle by external and internal factors, in particular on the effects of phytohormones. However, only parts of the cell cycle control machinery of plants have been identified so far. Therefore we have examined a photoautotrophically growing cell suspension culture of *Chenopodium rubrum* L. and have identified full length cDNA clones of the following components: a D2- and D3-type cyclin, a B type mitotic cyclin, the first plant G1-type CDK-inhibitor, the cDNA of a retinoblastomaprotein and a cyclin dependent kinase (A type) aswell as its 34 kDa protein.

The sequence similarities between plant and mammalian D type cyclins suggest that plant cyclins might also have a functional role in responding to growth signals. Expression of the cyclin D2 gene correlates with the carbohydrate status of actively dividing heterotrophic *Arabidopsis thaliana* L. suspension cultured cells (1). In photoautotrophic cells of *Chenopodium rubrum* L. cyclin D2 did not respond in such a clear way even in those cells which had been stationary for more than 5 weeks. However when the cells were grown heterotrophically in the dark, addition of sucrose was immediately followed by a significant increase of the cyclin D2 transcript. The expression of cyclin D2 and in particular of cyclin D3 was also examined after cytokinin re-addition to cells deprived of exogenous cytokinin.

Furthermore the expression of these cell cycle regulating genes have been examined in young and aged suspension cultured cells. The cyclin D2 transcript decreases significantly in the progression of the cell culture whereas the concentration of the cyclin-dependent kinase and the retinoblastomaprotein did not show such significant changes. The mRNA of these genes still can be detected even 5 weeks after the last cell division. In contrast to the dynamics of the cyclin D2 transcript the CDK inhibitor in young and aged photoautotrophic cells as well as in heterotrophic cells is expressed strongly but with no significant changes. Therefore we conclude that the effects of the inhibitor is regulated on the protein level.

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CLONING AND CHARACTERIZATION OF THE 5'UTR FROM THE MA16 RNA-BINDING PROTEIN mRNA.

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The MA16 protein from maize is an abundant 16 KD RNA-binding protein and consists of two domains; the RNA-binding domain in the N-terminal half of the protein and the GAR domain in C-terminal region which is extremely rich in glycine residues . The glicine-rich region contains repeats of the RGG-box, which has been defined as an RNA-binding motif in other proteins. Homologuous proteins have been found in various other plant species and animals and also been described in cyanobacteria, indicanting that this type of RNA-binding proteins probably represents a very ancient structure, which appeared before the divergence of eukaryotes and prokaryotes.

The RNA-binding activity of these proteins has been analysed by ribohomopolymer-binding assays. Proteins from maize, tobacco, barley, cyanobacteria and animals show a high affinity for polyG and polyU, indicating that cellular RNA ligands are likely to be enriched in G and U residues. Immunoprecipitation experiments showed that MA16 binds to several RNAs, including its own mRNA. Tha transcription start site of the MA16 mRNA was determined using primer extensio analysis . It revealed that the MA16 mRNA leader sequence presents several olygopryimidine strectches (Py-stretches), the largest of which is 17 nucleotides long. Surprisingly, a pyrimidine-strech is conserved in the mRNA 5ÚTR of the MA16 group of plant protein. These made us think about of the possible function of these sequence in the control of the protein expression. For that reason in vitro translation experiments with transcripts bearing the whole MA16 mRNA leader sequence or a fragment were performed and showed that the presence of whole leader results in a decrease in the MA16 in vitro translation efficiency.

Localization studies showed that MA16 acumulate in the nucleolus. In additon, *in situ* hibridization experiments revealed a higher concentration of MA16 mRNA in different expanding tissues of maize seedlings. These results seem to indicate that glycine-rich proteins could be involved in rRNA metabolism and growth and could have a role along the cell cycle. Cell synchronization experiments are now being designed in order to study the MA16 mRNA and protein distribution along the cell-cycle.

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COORDINATION OF CELL DIVISION AND TISSUE EXPANSION IN SUNFLOWER LEAF IN VARIOUS ENVIRONMENTAL CONDITIONS.

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Patterns of cell division and tissue expansion have been followed in individual sunflower leaves from initiation to the end of expansion, in a large range of environmental conditions including temperature, radiation and soil water status. Final area of individual leaves at a given position on the stem can have 10-fold variation without changes in epidermal cell area. Spatial and temporal analyses of cell division and tissue expansion revealed that both processes followed similar patterns during leaf development and that they had common responses to temperature, radiation and water deficit. These results suggest that cell division and tissue expansion are coordinated during leaf development either because they depend one of the other or because enzymes involved in each processes have common responses to environmental changes.

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Analysis of CycD proteins in Arabidopsis

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The expression of mammalian D-type cyclins is strongly dependent on the existence of mitogenic signals, and has been implicated as the link between mitogenic stimuli and cell cycle reentry. In plants D-type cyclins have been isolated from a number of species and the expression of these genes at the mRNA level has been addressed in a number of studies. However, very little is known about the regulation of D-type cyclins at the protein level and the aim of this study is to analyse the levels and activity of D-type cyclins in plants. We have raised polyclonal antibodies against CycD2 and CycD3 from Arabidopsis, and have used these antibodies to look at the protein levels of CycD2 and CycD3 during exit from, and reentry into, the cell cycle.

The results of these experiments demonstrate that regulation of CycD2 and CycD3 protein appears to be different at a number of levels, and this suggests that the specificity of different D-type cyclins may be important in plant growth and development.

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PLANT CELL CYCLE GENES: INVOLVEMENT IN THE CONTROL OF TOMATO FRUIT ORGANOGENESIS

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Classicaly tomato fruit development can be divided in to three phases which lead to a fruit ready to ripe. The first one corresponds to the development of the ovary. It is followed by a second phase, mainly composed with active cell divisions, which begins at anthesis, probably in response to fertilization. The third phase of fruit growth is characterized by cell expansion. The phase of cell division is of prime importance as it determines the number of cells inside the fruit, and consequently at least in part the final size of the fruit. The phase of cell expansion is characterized by nuclei endoreduplication, especially inside the locular tissue (gel), where cell divisions cease rapidly and nuclei and cell enlargement occurs concomitantly.

In order to understand these phenomena at the molecular level and especially the switch between division and endoreduplication, we looked for different cDNA clones corresponding to genes involved in the plant cell cycle, namely the constituants of the mitosis promoting factor or MPF which is a key regulator of the cell cycle controlling the progression of the cell between the different phases of the cell cycle.

Using a RT-PCR strategy with degenerated oligonucleotides, we amplified cDNA fragments corresponding to $p34^{cdc2}$, A- and B-type cyclins. These cDNA fragments were used to screen a cDNA library prepared with fruits harvested at the « cell division stage ». Different full-length cDNAs were obtained.

The expression of these genes was studied during tomato fruit development (*Lycopersicon esculentum* cv West Virginia 106), in the different tissues of the fruit and in the different organs of the plant by RT-PCR. These different panels of expression can be compared with the variations of the $p34^{cdc2}$ histone H1 kinase activity isolated by $p9^{CKShs1}$-sepharose affinity chromatography.

These results give a new information about the temporal and spatial distribution of the mitotic activity during development of tomato fruit, cell division and cell differentiation.

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CELL-CYCLE MODULATION OF CK2 ACTIVITY IN TOBACCO BY-2 CELLS.

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Protein kinase CK2 is an ubiquitous Ser/Thr kinase essential for cell viability. It phosphorylates many substrates involved in DNA metabolism and cell division. We have used the highly synchronizable tobacco BY-2 cell line to investigate whether CK2 activity and expression is regulated in a cell-cycle specific manner in higher plants. Specific cDNA probes for tobacco CK2α and β subunits respectively, and polyclonal antibodies recognizing α and β subunits separately, were obtained to determine mRNA and protein levels of both subunits. Our data show that there is a transcriptional regulation of CK2α and CK2β genes in the transition from a resting to a proliferating state. Furthermore, β polypeptide appears to be essential for CK2 to display its full activity and it is degraded or not translated in resting cells. α and β genes' expression is constitutive in cycling cells at the transcript and protein level. However, CK2 activity oscillates throughout the cell division cycle, peaking at G1/S and M phases, in parallel with changes in the intracellular concentration of spermine, spermidine and putrescine. Our results suggest that polyamines are responsible for the post-translational activation of CK2 in dividing plant cells, acting both by stabilizing the β-subunit and by increasing the activity of the holoenzyme. Finally, the differential effects observed by depleting CK2 activity at specific cell cycle phases suggest that CK2 might play an important role at the G2/M checkpoint.

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Molecular analysis of the *tonneau* mutations in Arabidopsis

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Two classes of mutants (*ton1* and *ton2*) with a similar phenotype have been isolated from T-DNA- and EMS-mutagenized populations. Mutant plants are dwarf, and all organs show profound morphological alterations. However, the general pattern is conserved, and all organs, tissues and cell types are present at their proper location. The phenotype is correlated to defects in cell division and elongation, with a strong desorganization of cortical cytoskeleton, absence of preprophase band, and mis-orientation of cell division planes[1].

In order to understand why these mutants are still capable of partial organogenesis despite the fact that they are severely affected in basic cell processes controlling cell division and elongation, we undertook the molecular cloning of the TON genes.

Major chromosomal rearrangements occured in the *ton1* line, obtained after T-DNA insertional mutagenesis[2]. In this line, the *ton1* mutation is caused by a 1,4 kb deletion which simultaneously disrupts two genes highly similar and organised in tandem repeat (*TON1a* and *TON1b*). Complementation analysis with either *Ton1a* or *Ton1b* cDNA indicate that these two genes are functionnaly redundant. Sequence analysis of these genes does not provide a clue to the function of the TON1 proteins although significant similarities are detected with unknown proteins from several species. RNA blot and RT-PCR analysis revealed that these two genes are expressed at a low level and constituvely in Arabidopsis.

In order to get more clues about the function of these proteins at the cellular level, two approaches are now developped. Polyclonal antibodies directed against the TON1 proteins are raised to determine the subcellular localization of these proteins by immunocytolocalization. The two-hybrid system is also used to isolate the possible partner(s) of the TON1 proteins.

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CHARACTERIZATION OF PLANT E2F TRANSCRIPTION FACTOR

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Cell cycle progression is the result of a highly regulated network. Crucial for the correct passage of the cell through the different cell cycle stages is the strict regulation of the transcriptional activity of certain genes and in animals the Retinoblastoma(Rb)/E2F growth regulatory pathway is essential to cellular events related to the G1/S transition as well as to certain differentiated states.

The identification of a plant Rb member and the detection of DNA sequence elements in promoters of plant genes similar to the human E2F binding sites suggest that gene expression at the G1/S transition might be mediated by Rb-bound transcription factors.These data lead to the prediction that E2F-like proteins exist in plants which may play roles similar to those of human E2F members. However, the identification of such E2F-like proteins in plants has been elusive. Now, we describe the isolation of a plant member of the E2F family in a yeast two-hybrid screening using plant Rb as a bait.

The analysis of the amino acid sequence of plant E2F reveals a domain organization similar to other animal family members, although there are some specific characteristics of plant E2F. The homology of plant E2F to other animal family members is high in the DNA-binding domain, moderate in the homo- and heterodimerization domains and relatively low in the transactivation domain and the Rb-binding motif.

We have studied the similarities between plant and human E2F proteins and analyzed the sequence requirements of plant E2F to interact with both plant and human Rb-related proteins and with the human DP proteins.

Here, we report data of expression of plant E2F thoughout the cell cycle in synchronized cultured cells that show that the protein is up-regulated early in S-phase. We have found that plant E2F is expressed not only in cultured cells but also in different non-proliferating tissues (roots, leaves and shoots).

Other details on the characterization of plant E2F will be presented.

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Cell Cycle Regulation during Embryo Growth Arrest in *Arabidopsis*

The developmental program of the seed starts after fertilization of the egg cell and ends with a dormant seed. Seed dormancy allows the embryo to survive during restrictive conditions, and germinate when the environmental conditions permit seedling growth. During seed development embryo development and seed maturation processes are taking place. As the mature embryo does not instantly grow to a seedling until germination is induced, we suggest that a developmental pathway during embryogenesis may signal the embryo to arrest its growth. Mutants that exhibit *sle* (*sleepless embryo*) phenotype have been isolated and developmentally characterised. *sle* mutants exhibit premature germination when seeds are harvested 7-9 days after pollination. Embryo phenotype during that time is between torpedo to bent cotyledons stages, suggesting that embryo arrest is signalled before growth phase is completed.

As cell cycle might play an important role during the embryo growth phase, the expression of cell cycle regulated genes is being characterised in WT and *sle* embryos. Preliminary results shows that cell cycle activity during the growth phase is extended by 2 days in few of the *sle* mutants compared to the WT activity. Using Promoter-GUS fusion transformants, the enhanced cell cycle activity in the mutants on a cellular level is being defined.

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TRANSDUCTION OF THE MITOGENIC AUXIN SIGNAL IN TOBACCO BY-2 CELLS

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Auxin are plant growth regulators which exert a whole range of effects at the cellular level, including enlargment, mitosis and differenciation. When working with whole organs, the study of these effects is often made difficult by their cell and tissue specificity. With this respect, plant cell cultures offer the possibility to assess the effect of auxin on a whole cell population. A typical response to auxin in such a system is the control of the division rate. We have used auxin starvation to stop cell division in tobacco BY-2 cells, and we have analyzed the effects of auxin re-addition. Mitoses resume after a lag of 10 hours, and a peak of weakly synchronous cell divisions is obtained after 19 h. We have set in evidence several earlier changes in the physiology of the cells and in gene expression, which appear to be correlated with the auxin treatment and which will be reported. At the molecular level, we have addressed the possible involvement of a MAP kinase pathway in the course of the mitogenic effect of auxin. The results clearly exclude the involvement of a « classical » MAP kinase, such as it has been already reported, as a mediator of auxin effects in this system[1]. We have also studied the effects of auxin on the activity of plasma membrane H^+-ATPases, which have been shown to be one target of auxin in the context of the control of cell enlargment by this hormone. Our results indicate a dramatic effect of auxin on these enzymes in the context of the control of mitoses. Auxin treatment leads, within hours, to changes in activity and abundance of plasma membrane H^+-ATPases. The underlying mechanism appear complex as it involves both a control of mRNA abundancy, a control of protein stability and an effect on the molecular activity. The possible relationship of these effects with the control of mitotic activity will be discussed.

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REACTIVATION OF THE CELL DIVISION CYCLE DURING ROOT NODULE ORGANOGENESIS

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In plants most organs develop by post-embryonic processes from persisting or newly formed meristems. Nodule development induced in the symbiosis between *Rhizobium* soil bacteria and leguminous host plants is a well defined example of new meristem elaboration and in this respect it represents a model to understand plant developmental mechanisms. Nodule organogenesis is triggered by the external mitogenic signals of rhizobia, the Nod factors, which are capable of reactivating the cell division machinery in G_0-arrested cells, located opposite to the protoxyleme poles in the root cortex. Maintenance of cell division activity leads to the formation of the nodule meristem from which cells can develop into the different nodule cell types and form the nodule primordium.

My work aims at elucidating the re-entry of G_0-arrested cells in the cell division cycle in *Medicago*. Previously, an A2-type cyclin, CycMs3, has been shown, in phytohormones treated *Medicago* leaves, to be involved in the G_0-G_1 transition and was proposed to act upstream of G_1 cyclins.

In order to test whether Nod factors-mediated cell cycle activation implicates also A2-type cyclins, a nodule-expressed variant of CycMs3, named CycMsA2;2, was isolated in the laboratory. We have shown that the expression of this isoform was stimulated by the Nod factors, in the emerging root hair zone, within one hour after the inoculation with *Rhizobium meliloti* and preceded expression of other S and G_2 phase-specific genes. To follow the activation of CycMsA2;2 during nodule initiation and also in other plant developmental events, genomic clones were isolated both from the tetraploid *Medicago sativa* and the diploid model plant *Medicago truncatula*; the 5' regions of the different clones have been sequenced and fused to the *uidA* reporter gene. Comparison of the promoter regions and expression patterns of the corresponding reporter constructs in transgenic *M. truncatula* will be presented. Since induction of CycMsA2;2 appears to be required for the initiation of nodule organogenesis, transgenic plants overexpressing this cyclin from the 35S promoter were regenerated and are presently tested for nodulation.

To localize the CycMsA2;2 protein itself and to follow its fate in the cells, a polyclonal antibody was raised against a recombinant CycMsA2;2 expressed in *E. coli*. The results of western blots will be shown. In addition we started a two-hybrid screening to identify protein partners of CycMsA2;2 and we have been studying its direct interactions with known plant cell cycle regulators such as cdks, Rb and E2F in order to clarify its role in the G_0-to-S transition.

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Transient expression of γ-tubulin in tobacco BY-2 cells.

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γ-tubulin is known to play an essential role in microtubule nucleation. Our goal was to express it in tobacco BY-2 cells to study its function *in vivo*. Maize γ-tubulin cDNA *(Tub G2)* with the flag M2 tagg (at the N or C terminus) was expressed under the control of the CaMV 35S promotor. The tagg allowed us to distinguish between transgenic and endogenous γ-tubulin. To identify transgenic cells *in vivo*, GFP, under the control of the 35S promotor was expressed either independently or in fusion with the γ-tubulin sequence. After 3.5 days of subculture, BY-2 cells were harvested and treated for 24h with aphidicolin to increase the proportion of cells in G1/S phase. 3h after washing, the cells were plasmolysed and bombarded with DNA-coated tungsten particules using a particule gun. GFP expression was detected 4h after bombardment. The ability of transgenic cells to divide was followed over a 48h period.
After 20h, cells were fixed for immunofluorescent labelling and cell crude extracts were prepared for immunoblotting. α, β and γ–tubulin labeling was compared to Flag M2 and GFP staining. The expression of transgenic γ-tubulin was high enough to be detectable in blots at the expected MW of 59kDa for the fusion protein γ-tubulin::flag and at 85kDa for the fusion protein γ-tubulin::flag::GFP. The absence of cross-reactivity between α, β and γ–tubulin antibodies was checked. Transgenic γ-tubulin is distributed throught the cytoplasm, predominantly near the nucleus and often appeared as bright fluorescent spots. Simultaneously, microtubules progressively disassembled. The total amount of α and β tubulin increased, showing at least a ten fold higher intensity then in neighbouring non transformed cells. The distribution of actin microfilaments was also markedly disorganized indicating that microtubule and F-actin assembly sites may have cooperative activity. These results suggest that γ–tubulin plays a role in α and β tubulin regulation. The majority of cells never divided or showed an abnormal. Nucleation of new microtubules was not observed, suggesting either that overexpressed γ–tubulin was inactive or toxic.

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A cell plate-localized *Arabidopsis* cellulase is essential for cytokinesis

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The formation of the cell plate, a unique structure in dividing plant cells, is pivotal for cytokinesis. A mutation in the *Arabidopsis DEC* (*Defective Cytokinesis*) gene causes the formation of aberrant cell plates and incomplete cell walls, leading to severely abnormal seedling morphology. *DEC* encodes a novel cellulase with an N-terminal trans-membrane domain. Immunohistological studies revealed that DEC localized at growing cell plates during cytokinesis. Our results suggest that the membrane-anchored cellulase plays a critical role in cell plate maturation.

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The lectures summarized in this publication were presented by their authors at a workshop held on the 22nd through the 24th of March, 1999, at the Instituto Juan March.

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