Faculteit Wetenschappen
Vakgroep Moleculaire Genetica
Laboratorium voor Genetica
VIB – Plant Systems Biology

Involvement of Cell Cycle Regulators in Seed Germination and Cell-type Differentiation: an Expression Analysis

Rosa Maria de Pinho Barrôco

Promoter Prof. Dr. Dirk Inzé

Co-promoter Dr. Gilbert Engler

Proefschrift voorgelegd tot het bekomen van de graad van
Doctor in the Wetenschappen, richting Biotechnologie
Academiejaar 2002-2003

Promoter

Prof. Dr. Dirk Inzé

Department of Plant Systems Biology
Interuniversity Institute for Biotechnology (VIB)
Ghent University

Co-promoter

Dr. Gilbert Engler

Laboratoire Associé de l'Institut National de la Recherche Agronomique (France)
Ghent University

Members of the Examination Committee

Chairman Prof. Dr. Erik Remaut

Department for Molecular Biomedical Research
Interuniversity Institute for Biotechnology (VIB)
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Wageningen University
The Netherlands

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Department of Plant Systems Biology
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Ghent University

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Department of Molecular Biotechnology
Ghent University

Prof. Dr. Koen Goethals

Department of Plant Systems Biology
Interuniversity Institute for Biotechnology (VIB)
Ghent University

Dr. Janice de Almeida Engler

Department of Plant Systems Biology
Interuniversity Institute for Biotechnology (VIB)
Ghent University

Dr. Sigrid Cornelis

Department for Molecular Biomedical Research
Interuniversity Institute for Biotechnology (VIB)
Ghent University

Aos meus pais e meus irmãos, pelo amor e infinita generosidade.

Aos meus amigos, companheiros de sonhos e de luta.

Á Elisa, fonte de todas as alegrias.

Ao Wim, meu porto de abrigo.

"I was like a boy playing on the sea-shore, and diverting myself now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me."

Isaac Newton (1642-1727)

"The great tragedy of science – the slaying of a beautiful hypothesis by a ugly fact."

Thomas H. Huxley (1825-1895)

"Aprender é descobrir o que já sabes,
fazer é demonstrar que sabes,
ensianar é lembrar aos outros que sabem tão bem como tu.
Somos todos aprendizes, fazedores, professores."

Autor desconhecido

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Frequently used ABBREVIATIONS

ABA	Abscisic acid
ABI	ABA-insensitive
BR	Brassinosteroids
BY-2	Bright-yellow 2 tobacco cell cultures
CDK	Cyclin-dependent kinase
CKS	Cyclin-dependent kinase subunit
CMTs	Cortical microtubules
CTD	Carboxyl-terminal domain
Cyc	Cyclin
DP	Dimerization partner
E2F	Adenovirus E2 promoter binding factor
EST	Expressed sequence tag
GA	Gibberellic acid
GCs	Guard cells
GFP	Green-fluorescent protein
GMC	Guard mother cells
KRP	KIP-related protein
Lec	Leafy cotyledon
M	Meristemoid
MAPK	Mitogen-activated protein kinase
MCM	Minichromosome maintenance
MMC	Meristemoid mother cell
ORC	Origin recognition complex
PCR	Polymerase chain reaction
P-TEFb	Positive transcription elongation factor b
RBR	Retinoblastoma-related
RGA	Repressor of GA1-3
RNAP II	RNA polymerase II
RNP	Ribonucleoprotein
SAM	Shoot apical meristem
SCs	Subsidiary cells

Preface

This PhD thesis was initiated when this laboratory joined an EC research project untitled "Genetic and Molecular Markers for Seed Quality" in January of 1998. The main objective of the project was to discover molecular identifiers or markers for different seed germination processes and to study their relation with seed quality. Many different molecular genetic approaches were undertaken in order to access several of the physiological processes implicated in seed germination. Our task in that project was to obtain knowledge on the regulation of cell cycle activities through germination, and for that it was expect that we perform an extensive expression analysis of cell cycle control genes. It was also our task in this project to study the expression patterns of the marker genes isolated by other partners during the course of the project. Unfortunately, the project could not be initiated at the expected date. And so, I got (partially) involved in some ongoing cell cycle projects, like the isolation and characterization of the *Arabidopsis CDKC* genes or the analysis of transgenic tobacco plants overexpressing a *CDKB1* gene. For me, it seems obvious to me that this thesis can contribute to the further understanding of the cell cycle machinery functions during specific developmental processes. On the other hand, the isolation of the *CDKC*;1 and *CDKC*;2 has lead to the characterization of two potentially interesting genes from *Arabidopsis* encoding CDK9-like homologues. In analogy with the animal CDK9 proteins, CDKC might be implicated in the control of trancription elongation, an important regulatory step in the control of gene expression.

This thesis is essentially a survey of many expression studies, in different tissues and developmental contexts, but simultaneously it covers a set of rather important topics such as transcription control, stomata formation, embryo formation and seed germination.

Chapter 1

General Introduction

The recent model elucidating cell cycle involves CDK/cyclin complexes that, by phosphorylating different substrates, govern not only cell division, but also several cellular pathways such as signal transduction, differentiation and apoptosis. CDKs, cyclin-dependent kinases, are the catalytic subunits of complexes whose regulatory subunits are the cyclins. During the cell cycle, CDKs are sequentially activated through binding to their corresponding cyclin regulatory subunits. Cyclin expression is strictly confined to particular cell cycle stages, and is, therefore, the crucial factor in determining the activity of individual CDKs during cell cycle progression. Cell cycle progression seems to influence transcription by RNA polymerase II (RNAP II). RNAP II transcriptional activity is modulated during the cell cycle and depends upon the phosphorylation status of the carboxyl-terminal domain (CTD) of the largest subunit of RNAP. Moreover, several CTD kinases are members of the cyclin-dependent kinase (CDK) superfamily, including CDC2, CDK7, CDK8, and CDK9. Each of these CDKs, with their respective cyclin partners, might be also linked to cell cycle regulatory events.

CELL DIVISION CONTROL

The Basic Cell Cycle

The cell cycle in eukaryotes consists of four phases, G1, S, G2 and M, allowing the formation of two fully functional daughter cells. DNA-replication (S-phase) and the segregation of the chromosomes (M-phase) are separated by two gaps. The G1-phase (first gap) separates mitosis from the next S-phase, and the G2-phase (second gap) is interposed between S- and M-phases. Consequently, G2 is discriminated from G1 by the doubled DNA content of the cells. Plant cells seem to be able to arrest and/or re-enter cell cycle at the G1/S or the G2/M boundaries according to the plant species or tissue, indicating the importance of these transitions in the control of the cell cycle and cell growth (reviewed by den Boer and Murray, 2000). Additionally, these transitions have been shown to act in plant cells as primary checkpoints for some kinds of stress (Reichheld et al., 1999). However, G1 represents generally the major control point in the commitment for cell cycle progression or arrest. Before the commitment to divide, integration of intracellular and extracellular signals must occur in order to determine whether or not conditions are favourable for division. The integrity of DNA is monitored, and when necessary repair must occur prior to the onset of DNA synthesis (reviewed by den Boer and Murray, 2000; Rossi and Varotto, 2002).

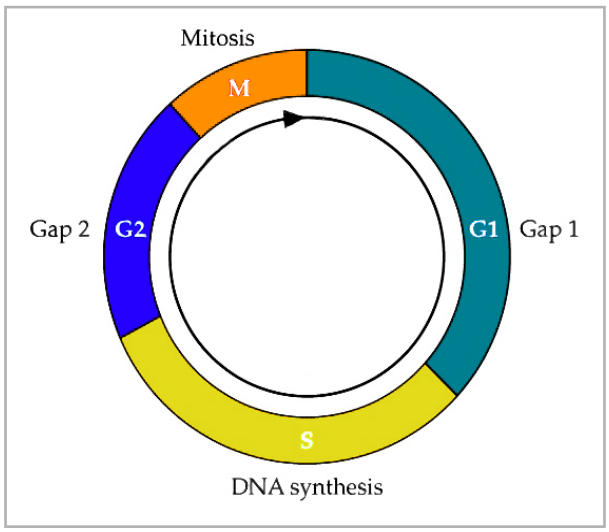

Figure 1. *The four successive phases of a standard eucaryotic cell cycle. During M-phase the cell divides and during S-phase DNA replication takes place. G1-phase is the gap between M-phase and S-phase. G2 is the gap between S-phase and M-phase. (Adapted from Doerner, 2000).*

Cyclin-Dependent Kinases as Major Players

Cyclin-dependent kinases (CDKs) are serine/threonine kinases playing an essential role in the control of cell cycle progression (reviewed by Morgan, 1997). Additionally, CDKs were found to be involved in several cellular processes including differentiation and apoptosis (reviewed by Kasten and Giordano, 1998).

The first evidence of the molecular basis underlying cell division arose more than 20 years ago, from studies involving both budding and fission yeast (reviewed by Morgan, 1997). After discovering CDC2 in *Schizosaccaromyces pombe* and CDC28 in *Saccharomyces cerevisiae*, two different research groups characterised the human homologue of these yeast regulatory proteins, which was collectively referred as to CDC2 (Draetta et al., 1987; Lee and Nurse, 1987). Since that

time, a multitude of CDC2-related kinase genes have been cloned in all eukaryotes based on sequence similarity. Comparison of the proteins encoded by their cDNAs has permitted the classification of plant CDKs into six classes (Joubès et al., 2000; Vandepoele et al., 2002). Members of the CDK-A class share the PSTAIRE motif found in the cyclin-binding domain of yeast CDC2, and are able to rescue the fission yeast temperature-sensitive *cdc2* mutant. CDK-B is a plant specific group distinguished by the PPTALRE or PPTTLRE amino acid motif in their cyclin-binding domain, and is divided into the subgroups CDK-B1 and CDK-B2, according to which of these two possible amino acids signature they possess. These CDKs are unable of complementing the yeast *cdc2* mutation and display cell cycle phase specific expression, which is not observed for any other CDKs in other organisms. Very few members have been identified from CDK-D, CDK-E and CDK-F groups. Members of the CDK-C group are marked by the PITAIRE motif but their function is, until this moment, poorly documented (reviewed by Joubès et al., 2000). The characterisation of this group of CDKs was one of the goals of our work.

Many works have demonstrated the role of specific CDKs in cell cycle progression in plants, a role common to other organisms. Constitutive expression of a dominant negative mutation of CDKA;1 resulted to be presumably lethal in *Arabidopsis*, as no transgenics could be obtained, while tobacco plants overexpressing the mutant CDKA protein presented a decreased number of larger cells, resulting in just slightly smaller plants when compared to wild-type (Hemerly et al., 1995). These results have demonstrated the capacity of cells to uncouple growth and division, allowing cell growth to continue in order to compensate for a reduced cell division rate. Also, it was shown that the plant anatomical structure was almost unaffected in these mutants. This illustrates the flexibility of plant development in relation to cell number, indicating that positional information and differentiation are not necessarily associated to the number of cells.

Mironov et al. (1999) describe that CDKA protein levels do not oscillate throughout the cell cycle, although kinase activity resulting from this protein is lowest in G1-phase. Still, chemical inhibition of CDKA;1 in cell suspension cultures resulted in the cell cycle arrest both at the G2/M and G1/S transitions (Planchais et al., 1997). In *Arabidopsis*, CDKA;1 expression was detected not only in dividing cells but also in cells that have a ability to resume division when an appropriate signal is received, resulting in to a rather widespread expression. In contrast, *Arabidopsis CDKB1;1* transcripts are distributed in a cell cycle dependent manner, which is reflected by a patchy-pattern distribution when detected by *in situ* hybridisation (Segers et al., 1996). The activity of CDKB complexes was shown to peak during M-phase. Additionally, constitutive expression of a dominant negative mutation of the tobacco CDKB1;1 resulted in delayed G2/M transition. These data showed that CDKB1;1 activity is limiting the duration of G2 or entry into M-phase (Porceddu et al, 2001).

The control of CDK activity during cell cycle

During the cell cycle, CDKs are sequentially activated through binding to their corresponding cyclin regulatory subunits. Cyclins are named for their cyclic expression and degradation and they play an

important role in regulating cell division. Cyclins are synthesised immediately before they are active and their levels fall abruptly after their action because of degradation via the ubiquitin pathway (Genschik et al., 1998). In plants, a diverse range of cyclins has been cloned, and classified into A-type, B-type or D-type according to the cell cycle phase they were assigned (reviewed by Renaudin et al., 1998). A- and B-type cyclins are mitotic whereas D-type comprises G1-specific cyclins. Using sequence information from known cyclins, it was possible to identify a total of 30 cyclins in the *Arabidopsis* genome (Vandepoele et al., 2002). Among those, one *Arabidopsis* gene was found with high sequence similarity to the cyclin H of poplar and rice. H-type cyclins are the regulatory subunits of CDK-activating kinase (CAK), which positively controls the kinase activity of CDKs and the carboxy-terminal domain (CTD) of RNA polymerase II (Yamaguchi et al., 2000). In *Arabidopsis*, experimental evidence is still required for the characterization of this recently identified cyclin subunit.

In addition to the association with their corresponding cyclin partners, full activation of CDK-cyclin complexes generally requires phosphorylation of a conserved threonine residue (corresponding to T161 in human CDC2) within their so-called T-loop region by a CDK-activating kinase (CAK) (Morgan, 1997; Kaldis, 1999). In plants, genes encoding CAKs have been recently cloned in *Arabidopsis* and rice (Umeda et al., 1998; Yamaguchi et al., 2000).

Association with another class of factors, CKS proteins, offers an additional control of CDK activity. In *Arabidopsis*, two CKS proteins have been isolated that have high affinity towards cell cycle-associated CDKs. However their function is still not completely clear, it has been suggested that they can act as assembly or docking factors, or alternatively mediate binding to (positive and negative) regulators, or perhaps be involved in protein degradation (reviewed by Stals and Inzé, 2001).

The activity of cyclin/CDK complexes is also mediated by a family of CDK protein inhibitors, or CKIs (Sherr and Roberts, 1999). In *Arabidopsis* genome were identified seven genes encoding for CKIs (or KRPs for Kip-related proteins) (De Veylder et al., 2001), and the inhibitory activity of the proteins they encode on some cyclin/CDK complexes has been demonstrated (Lui et al., 2000; De Veylder et al., 2001).

Cell Division vs Cell Differentiation

The commitment of plant cells to division may be initiated when expression of D-types cyclins is increased by extracellular signals, such as hormones or sugar. In animal cells is now fully demonstrated that (re)activation of cell cycle progression is initiated when D-type cyclins bind to CDKAs. When activated, this complex phosphorylates the Rb (retinoblastoma) proteins resulting in the liberation of E2F transcription factors. E2F binds to DP (dimerization partner) protein, thereby triggering entry into S-phase and promoting the expression of genes involved in DNA replication (reviewed by Rossi and Varotto et al., 2002). With the isolation of Rb homologues in different plant species, it became possible to define for plants a similar mechanism for control of the G1/S

transition similar to that described for animals (reviewed by Gutierrez et al., 2002). Genes encoding plant homologues of E2Fs and DP have been also recently cloned in several species (Ramírez-Parra et al., 1999; Sekine et al., 1999; Albani et al., 2000; Magyar et al., 2000; Ramírez-Parra and Gutierrez, 2000; Mariconti et al., 2002). Moreover, the presence of the putative E2F-binding motifs in D-type cyclins suggests the involvement of retinoblastoma homologues in plant cell cycle activation (de Jager et al., 2001). The binding of Rb to E2F results in the recruitment of the Rb protein to promoters containing E2F-binding sites. This binding results in chromatin reorganisation, through histone deacetylase activity in such promoters, and so making the DNA less accessible to transcription (Williams and Grafi, 2000). Upon the interaction of the CDK/CycD complexes with the Rb protein and subsequent phosphorylation of Rb by these active kinases, the protein loses its association with E2F, leading to activation of E2F-regulated genes and entry into S-phase (Gutierrez et al., 2002; Rossi and Varotto, 2002). Indeed, it has been recently shown that the decision of plant cells to proliferate is largely dependent on the heterodimeric E2F/DP transcription factor. Increased E2F/DP protein levels up-regulate the expression of S-phase-specific genes, resulting in ectopic cell divisions correlated with delayed cell differentiation (De Veylder et al., 2002).

Recent studies suggest that, as in animal cells, plant D-type cyclins integrate signals, such as nutrient status and hormones, during the G1-phase and transduce these signals via the retinoblastoma pathway. Cyclin-dependent phosphorylation of Rb proteins results in the release of transcription factors, such as the E2F/DP complex, which promote entry into S-phase (Gutierrez et al., 2002; Rossi and Varotto, 2002) (Fig. 2). Therefore, Rb proteins are considered as molecular switches between cell proliferation and differentiation.

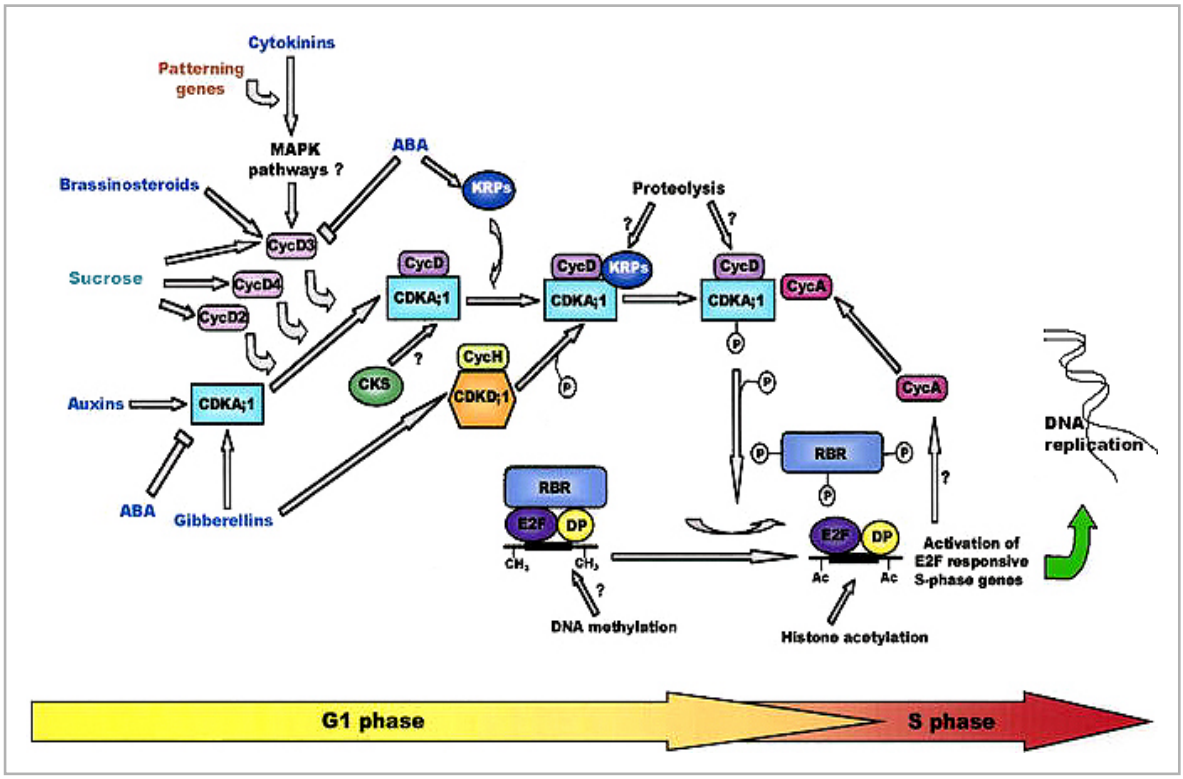

***Figure 2**. G1/S transition control of the plant cell cycle. In this schematic pathway are represented the components and mechanisms of the cell cycle core machinery as well as factors triggering cell cycle entry and progression throughout the G1-phase. The question marks indicate elements and processes that have not yet been fully demonstrated experimentally. ABA, abscisic acid; CDK, cylin-dependent kinase; CKS, CDK subunit; Cyc, cyclin; DP, dimerization partner; E2F, adenovirus E2 promoter binding factor; KRP, KIP-related protein; MAPK, mitogen-activated protein kinase; RBR, retinoblastoma-related. (Adapted from Rossi and Varotto, 2002).*

TRANSCRIPTION REGULATION

CDKs and the Control of RNA polymerase II Activity

CDK/cyclin complexes are able, through phosphorylation of different substrates, to govern not only cell division but also several cellular pathways such as signal transduction, differentiation and apoptosis (reviewed by Gao and Zelenka, 1997). Indeed, recent studies have introduced many new characteristics of CDK/cyclin function confirming the concept of a multifunctional kinase group. CDK9/cyclin T (complexed as P-TEFb), CDK7/cyclin H (forming TFIIH) CDK8/cyclin C, all are involved in transcription, and CDK5/p35, is active during neural differentiation, confirming that CDK/Cyc complexes are multifunctional kinases and not only cell cycle regulators.

Occasionally, this multifunctionality of CDKs might be a tool to attach apparently unrelated functional mechanisms. As a clear example, a relationship between cell cycle and transcription by RNA polymerase II (RNAP II) becomes slowly perceptible, not only because they use shared components but additionally because RNAP II transcriptional activity is modulated during the cell cycle. Cell cycle dependent changes in the phophorylation status of the carboxy-terminal domain (CTD) of the largest subunit of RNAP II alter transcription (Fig. 3).

CTD phosphorylation has been observed during the course of the cell cycle, for example in response to growth factors or to DNA damage (review by Oelgeschlager, 2002). The shutting down of RNAP II complexes in response to lesions helps to stimulate p53 accumulation which largely determines the cells to respond to DNA damage, including cell cycle arrest (Bregman et al., 2000). Consistent with those observations, the major cellular kinase complexes involved in CTD phosphorylation have been directly or indirectly implicated in cell cycle regulatory events (reviewed by Napolitano et al., 2002). These include CDC2, CDK7, CDK8, and CDK9, members of the cyclin-dependent kinase (CDK) superfamily, which associate with transcription initiation complexes. Mainly, the discovery of the multifunctional nature of CDK7 has prompted numerous studies to address the question whether this CDK provides a functional link between cell cycle regulation and the general transcription machinery (review by Oelgeschlager, 2002). As it has been largely described, CDK7 is part of a CDK-activating kinase (CAK complex) responsible for the activation of CDKs through threonine phosphorylation of their T-loop regions. Additionally this kinase is also implicated in transcription regulation both as a free CAK complex and as a component of the general transcription

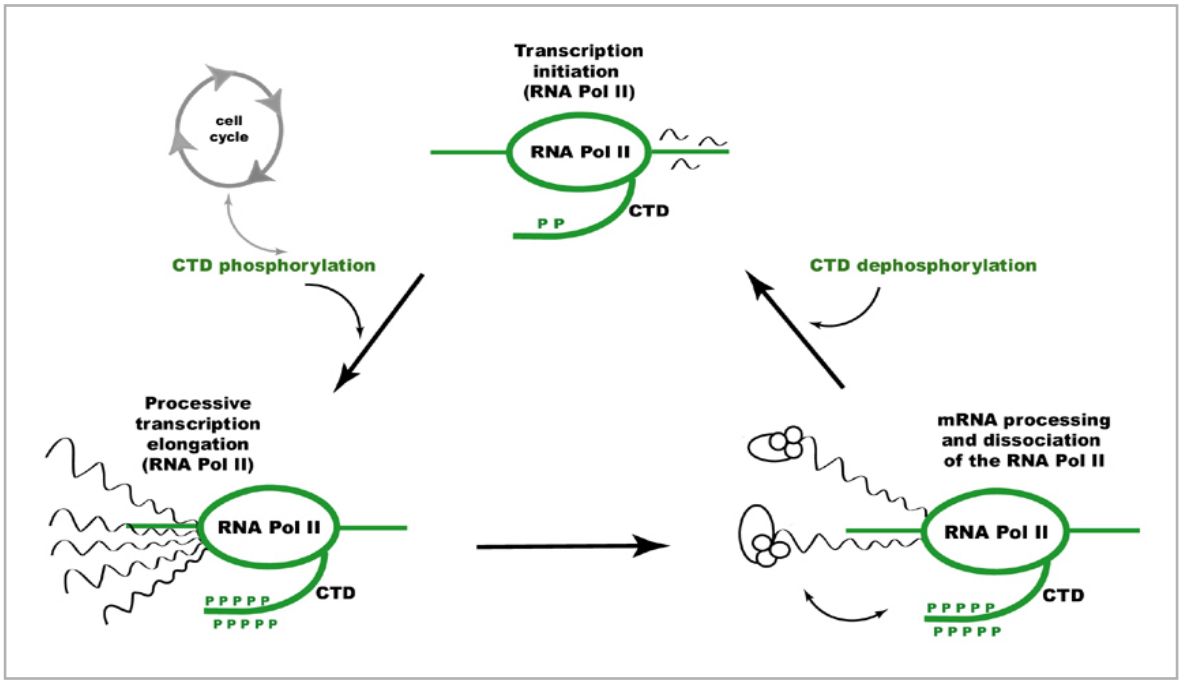

Figure 3. *Model on the transcription cycle by RNA polymerase II (RNA Pol II). The regulation of CTD (carboxyl-terminal domain) phosphorylation/dephosphorylation is responsible for the recruitment and assembly of active transcription/RNA processing complexes, but probably also for the temporal control of those processes during cell cycle. (Adapted from Napolitano et al., 2002).*

factor TFIIF through phosphorylation of the RNAP II CTD and a number of general and regulatory transcription factors. This duality of functions could serve an indication for a linkage between both cellular processes. Contrary to this idea, initial studies revealed that CDK7 activity and protein levels of both CDK7 and cyclin H do not significantly fluctuate during cell cycle. Thus, CDK7 kinase appeared not to be limiting for cell cycle progression (Nigg, 1996). However, more recent studies have presented evidences for a positive feedback loop by reciprocal T-loop phosphorylation of CDC2 and CDK7 (Garrett et al., 2001). CDK7 phosphorylation by CDC2 might differentially affect free CAK complexes and CAK associated with protein complexes such as TFIIF. Therefore, it is suggested that cell cycle-dependent regulation of CDK7 kinase activity towards specific substrates such as the RNAP II CTD occurs in the context of distinct CAK-containing protein complexes while the activity of free CAK remains constant.

At least another two CDKs, CDK8 and CDK9, have been identified which are associated with the control of transcription by RNAP II. However, their implication in cell cycle control appears to be only indirect. CDK8 associates with cyclin C and this complex is responsible for inhibition of transcription through phosphorylation of the RNAP II CTD prior to transcription initiation. CDK8/cyclin C complex can also repress transcription by phosphorylating cyclin H and in this way abolishing CDK7/cyclin H kinase activity and their ability to support transcription (Akoulitchev et al.,

2000). Therefore, CDK8 might be implicated in the control of cell proliferation through negative regulation of CDK7 kinase activity.

The other CTD kinase of the CDK family found to be associated with transcription control, CDK9, might be only very indirectly associated to cell cycle.

Elongation Control during Transcription

Cyclin-dependent kinase 9 (CDK9) is a CDC2-related serine/threonine kinase previously named PITALRE, which was isolated based on its sequence similarity to CDC2 (Grana et al., 1994). The CDK9 gene is widely expressed in human and murine tissues with higher levels found in terminally differentiated cells (Bagella et al., 1998; Bagella et al., 2000). The regulatory units of CDK9 are the T-family cyclins (T1, T2a and T2b) and cyclin K (Peng et al., 1998; Lin et al., 2002). Interestingly, cyclin K was originally identified in a yeast screen based on its ability to restore cell cycle progression and to rescue the lethality of G1 cyclin gene deletions (Fu et al., 1999). However, the elevated levels of CDK9 and its regulatory subunits (cyclins T1 and T2) in terminally differentiated cells, together with the fact that CDK9/cyclinT complexes are not cell cycle-regulated, distinguish CDK9 from the majority of the other CDKs (reviewed by Price, 2000). Also, as cyclin T levels are not cell cycle-regulated, CDK9 kinase activity does not change during the different phases of the cell cycle. Moreover, unlike the other CDKs, which regulate cell cycle progression and phosphorylate histone H1, CDK9 fails to phosphorylate H1. Notably, CDK9 phosphorylates *in vitro* the C-terminus of pRb, the protein product of the retinoblastoma gene, showing a phosphorylation pattern involving only Ser residues (Grana et al., 1994; Simone et al., 2002). This possible association of CDK9 with the retinoblastoma pathway has served as an evidence for the involvement of CDK9/cylin T in determining cell differentiation rather than its involvement in cell proliferation.

The elongation phase of transcription by RNA polymerase II is one of the many steps during the generation of mature mRNAs that is subject to regulation. Studies carried out by Dahmus *et al.* (1996) revealed that the transition from initiation to elongation during RNA synthesis is accompanied by hyperphosphorylation of repeats in the C-terminal domain (CTD) of the largest RNA polymerase subunit, which is carried out by positive transcription elongation factor (P-TEF) complex. P-TEFb is a cyclin-dependent kinase complex composed of CDK9 and one of several cyclins including T1, T2 and K (Marshall *et al.*, 1996; Peng *et al.,* 1998; Fu *et al.*, 1999).

The changes in CTD phosphorylation observed in response to cell cycle regulation events and to extracellular stimuli raise the intriguing possibility that the RNAP II CTD serves to connect multiple signalling pathways that affect transcription either globally or in a promoter-specific fashion. Ongoing investigations seek to dissect these different signalling pathways, to identify possible relevant CTD kinases involved, and to define their mode of action.

RESEARCH OBJECTIVES

Cell division and proliferation of eukaryotic cells follows a genetic program, designated cell cycle. To ensure cell cycle progression, proteins that are involved in its regulation must be periodically synthesized and activated within an appropriate period of that cycle. Cyclin and cyclin-dependent kinases are the basic component units of the core cell cycle machinery. Activation of CDKs is essential for the progression through the cell cycle. Besides a key role in cell cycle control, CDK/Cyc kinase complexes are now considered as important regulators of the transcription mechanisms.

One of the aims of the present work was to further contribute to the functional understanding of CDK proteins. New aspects of CDK function were addressed by the characterization of the *Arabidopsis* CDKC;1 and CDKC;2 proteins. For the CDKC subclass of plant CDKs no precise function has been found previously. Gene expression analysis and the searching of the interacting partners of these proteins were undertaken to provide a possible function of this CDK class.

The other aim of this study was to further contribute to the understanding of the cellular and developmental roles of CDKB, a plant-specific CDK. To achieve that goal, we have evaluated the plant cell responses to the down-regulation of CDKB1;1 activity by constitutive overexpression of a dominant negative CDKB1;1 in tobacco plants. Additionally, CDK-related kinase activity was monitored in synchronized tobacco BY-2 cells in order to understand how CDKB1;1 protein accumulates during the cell cycle and better access the role of B-type kinases on cell cycle progression. The expression pattern of CDKB1;1 in *Arabidopsis* seedlings points towards the implication of this kinase in stomatal development. The possible involvement of CDKB1;1 in this particular developmental process was further investigated by evaluating the effects of the ectopic production of CDKB;1 and its mutant inactive form on *Arabidopsis* plants.

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Chapter 2

A novel complex of cyclin-dependent kinase- and cyclin-like proteins from *Arabidopsis thaliana* with a function unrelated to cell division

Adapted from "A novel complex of cyclin-dependent kinase- and cyclin-like proteins from *Arabidopsis thaliana* with a function unrelated to cell division" by Rosa Maria Barrôco, Lieven De Veylder, Zoltan Magyar, Gilbert Engler, Dirk Inzé and Vladmir Mironov. Accepted in Cellular and Molecular Life Sciences.

Abstract

Although the majority of the cyclin-dependent kinases (CDKs) play a key role in cell cycle progression, recent evidence has shown that CDKs are also implicated in transcription regulation. Here, we describe two *Arabidopsis* CDKs designated Arath;*CDKC*;1 and Arath;*CDKC*;2. These CDKs share a PITAIRE signature in the cyclin-binding domain and the structural characteristics of the mammalian CDK9. Yeast two-hybrid screens and immunoprecipitation assays identified CDKC-interacting proteins with homology to the animal cyclin T/cyclin K group. We suggest that these *Arabidopsis* CDKCs may be part of a kinase complex similar to the animal positive transcription elongation factor b, whose activity is essential for transcription control. Expression studies showed that Arath;*CDKC* transcripts are mainly confined to epidermal tissues and most abundant in flower tissues. No expression was detected in actively dividing *Arabidopsis* tissues, suggesting a role for the CDKC proteins in differentiated cells.

Key words: *Arabidopsis thaliana*, cell cycle, cyclin-dependent kinases (CDKs), cyclin T, transcription control, two-hybrid interaction.

Abbreviation*s*: CDK, cyclin-dependent protein kinase; CTD, carboxyl-terminal domain; CYC, cyclin protein; EST, expressed sequence tag; P-TEFb, positive transcription elongation factor b; PCR, polymerase chain reaction; RNAP II, RNA polymerase II; RNP, ribonucleoprotein.

Introduction

Cyclin-dependent kinases (CDKs) have first been identified as major cell cycle regulators and their characterization has progressed considerably. CDK activity has been shown to depend on the interaction with regulatory proteins that are known as cyclins.

In *Arabidopsis thaliana*, two CDKs have been identified that clearly play a role in cell cycle regulation (Mironov et al., 1999). These CDKs have recently been designated Arath;CDKA;1 and Arath;CDKB1;1 and are representatives of two major plant CDK families, the CDKA and CDKB groups (Joubès et al., 2000). The CDKA group has a characteristic PSTAIRE motif and is involved in cell proliferation, as well as in maintenance of cell division competence in non-proliferating tissues (Hemerly et al., 1993). The CDKB group contains a PPT(A/T)LRE motif that is found only in plant CDKs. The CDKB1;1 protein has been shown to play a specific role in G2/M transition (Porceddu et al., 2001).

Although most CDKs in association with their cyclin partners are involved in cell cycle regulation, other CDK/cyclin complexes have been found to govern multiple cellular pathways, including signal transduction and differentiation. Some complexes, such as CDK7/cyclin H, CDK8/cyclin C, and CDK9/cyclin T, are responsible for the control of the transcription machinery in animals (Morgan, 1997).

The elongation control of nascent transcripts plays a major role in the expression regulation of most genes. The conserved carboxyl-terminal domain (CTD) of the RNA polymerase II (RNAP II) large subunit has been found to be a docking place for positive and negative elongation factors. A determining role in processive elongation has been attributed to the positive transcription elongation factor b (P-TEFb) complex. P-TEFb has been proposed to determine the fate of RNAP II at the early phase of elongation, causing RNAP II to become competent to produce full-length transcripts. Phosphorylation and dephosphorylation of the conserved CTD domain of RNAP II seem to be the key regulatory steps that control the entry of this RNA polymerase into the elongation process (Dahmus, 1996; Majello and Napolitano, 2001). P-TEFb is a CDK complex that consists of CDK9 (also referred to as PITALRE kinase) and one of several cyclins, including T1, T2, and K (Marshall et al., 1996; Peng et al., 1998; Lin et al., 2002).

The elevated levels of CDK9 and its regulatory subunits (cyclins T1 and T2) in terminally differentiated cells, together with the fact that CDK9/cyclin T complexes are not cell cycle regulated, distinguish CDK9 from the majority of the other CDKs (reviewed by Price, 2000). Also, because cyclin T levels are not cell cycle regulated, CDK9 kinase activity does not change during the different phases of the cell cycle. Moreover, unlike the other CDKs, which regulate cell cycle progression, CDK9 fails to phosphorylate H1, but it phosphorylates the C-terminus of the protein product of the retinoblastoma gene (pRb) (Simone et al., 2002).

We characterized a new *Arabidopsis thaliana* CDK group, designated Arath;CDKC. Two-hybrid screening revealed that Arath;CDKC;2 interacts with a protein homologous to the animal cyclin T. The formation of CDKC/cyclin T complexes in *Arabidopsis* was confirmed by the binding *in vitro* of both

CDKC;1 and CDKC;2 to the cyclin T protein during immunoprecipitation**.** The association of the Arath;CDKC proteins with a cyclin T-like protein indicates that plant CDKCs might form a kinase complex with functional characteristics of the animal P-TEFb. Based on their sequence similarities to the animal CDK9 and binding to a cyclin T-like protein, we propose that the *Arabidopsis* CDKC proteins are functional homologues of the animal CDK9.

Experimental Procedures

cDNA cloning

Arath;*CDKC;2* cDNA was isolated from cell suspension cultures of *Arabidopsis thaliana* (L.) Heynh., using sequence information of expressed sequence tag (EST) for primer design. Full-length cDNA was cloned by 5'end amplification, with the 5'end CapFinder Kit according to the manufacturer's protocol (Clontech). A second *Arabidopsis* gene coding for a putative CDKC protein (Arath;*CDKC*;1) was cloned based on its sequence homology to the identified *CDKC*;2.

Sequence analysis and phylogeny

Alignment was edited and reformated with BioEdit (Hall, 1999). Similarity between proteins was based on a BLOSUM62 matrix (Henikoff and Henikoff, 1993). Phylogenetic analysis was performed on the more conserved positions of the alignment. A phylogenetic tree was constructed with the neighbor-joining algorithm using the software package TREECON (Van de Peer and De Wachter, 1994).

Yeast two-hybrid assays

Library screening was performed as described previously (De Veylder et al., 1997). Vectors and strains (HF7c) were provided by the Matchmaker two-hybrid system (Clontech). The Arath;CDKC;2 bait was constructed by cloning a polymerase chain reaction (PCR)-amplified *CDKC;*2 fragment (740 bp) cut with *Eco*RI/*Bam*HI, in-frame with the GAL4 DNA-binding domain of the pGBT9 vector (Clontech), which was digested with the same enzymes.

For additional pair-wise two-hybrid assays using the cyclin T, the corresponding full length-coding region was inserted into a GATEWAY vector (Invitrogen, Carlsbad, CA) containing the GAL4 activation domain. The cyclin gene was inserted by homologous recombination between the *attB* sequence in the GATEWAY vector and at both ends of the cyclin T fragment, which was amplified by PCR with primers containing the terminal *attB* sites (according to the manufacturer's instructions).

Plasmids encoding baits and preys were co-transformed into a yeast reporter strain and their association was determined by the ability of the co-transformed strains to grow on histidine-deficient medium.

In vitro *transcription/translation and immunoprecipitation*

Influenza hemagglutinin (HA)-tagged CDKC;1 and CDKC;2 were constructed by cloning into the pSK plasmid (Stratagene) containing the HA-tag (Magyar et al, 2000). The coding sequences of both genes were amplified by PCR and cloned into the *Eco*RI and *Bam*HI sites of the pBluescript plasmid (Stratagene) containing a HA-tag (HA-pSK). The *c-myc*-tagged cyclin T and DAG-like (DAG; differentiation and greening) genes were generated by PCR amplification of the respective coding sequences, followed by cloning into the *Eco*RI and *Spe*I sites of pSK containing a doubled *c-myc* tag (*c-myc*-pSK). All cloning steps were carried out according to standard procedures. The reading frames were verified by direct sequencing.

The mutant forms of HA-tagged CDKC;1 and CDKC;2 ($CDKC^{1-217}$) as well as the deleted versions of *c-myc*-tagged cyclin T ($cycT^{1-153}$ and $cycT^{1-213}$) were obtained by PCR amplification of the respective full-length versions cloned in pSK (see above), using T7 (forward) primer and specific end (reverse) primers.

In vitro transcription/translation was performed using the TNT T7-coupled wheat germ extract kit (Promega, Madison, WI) primed with appropriate plasmids for 2 h at 30°C. For immunoprecipitation, 10 μl of the total *in vitro* translated extract (50 μl) was diluted at 1:5 in Nonidet P40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin/aprotinin/pepstatin) and incubated for 2 h at 4°C with anti-c-myc (9E10; BabCo) or anti-HA (HA.11; Covance) antibodies. Protein-A-Sepharose beads (40 μl 25% [v/v]) were added and incubated for 1 h at 4°C. After, the beads were washed four times with Nonidet P40 buffer. Immune complexes were eluted with 10 μl 2x sodium dodecyl sulfate (SDS) sample buffer and analyzed by 12% or 15% SDS-polyacrylamide gel eletrophoresis (PAGE) and by autoradiography. These experiments did not attempt to precisely map the region of interaction between the CDK and cyclin partners under study, but to better characterize and consolidate the existence of a truly new CDK/cyclin complex.

Reverse transcriptase-PCR analysis

Total RNA was extracted from young seedlings, roots, rosettes, stems, and flowers of *Arabidopsis thaliana* ecotype Columbia according to standard protocols. Two micrograms of each sample were reverse-transcribed (RT) into cDNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Semi-quantitative RT-PCR amplification of the cDNA was carried out in a LightCycler real-time PCR (Roche Diagnostics, Brussels, Belgium) with the gene-specific primers 5'-ACATTCTCGTTTACCTCCACAG-3' (forward) and 5'-AAAATCACAACTGCCTTAAAGAC-3' (reverse) for *CDKC;1*; 5'-ACCCAGCCACAACTTCTATG-3' (forward) and 5'-CTAGTATCACATTAAATGTAAGAGTAAG-3' (reverse) for *CDKC;2*; and 5'-TGTCGTTGTAGCGTCTTATG-3' (forward) and 5'-TCCTTCTGTCCACTTCTATC-3' (reverse) for cyclin T. The amount of target cDNA used for PCR was standardized by quantification of actin 2 transcripts present in all the samples.

In situ hybridization

Plant material was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (12 h at 4°C). Fixed tissues were dehydrated through standard ethanol series and embedded in paraffin. Tissue serial sections of 10 μm were attached to coated microscope slides. ^{35}S-UTP-labeled sense (control) and antisense RNA probes were generated by *in vitro* transcription with T7 and Sp6 RNA polymerases, according to the manufacturer's protocol (Roche Diagnostics). *CDKC*;2 DNA-coding sequence was used as template for CDKC riboprobe synthesis. Because the sequence dissimilarity between *CDKC;1* and *CDKC;2* is extremely subtle and overall spread, it is probably impossible that this riboprobe can specifically bind to *CDKC*;2 RNA in the tissue. On the contrary, this probe must be able to recognize both the *CDKC*;1 and *CDKC*;2 RNA targets. Full-length transcripts were reduced to 300 bp fragments through alkaline hydrolysis. Plant material was hybridized with the appropriated antisense and control probes as previously described (Magioli et al., 2001). Autoradiographs were taken with a Diaplan microscope (Leitz, Heerbrugg, Switzerland) using dark-field illumination.

Results

Isolation of CDKC;1 and CDKC;2 genes and sequence analysis

Arath*;CDKC;*2 kinase was first identified as an EST based on its sequence similarity to other known CDKs. Using the EST sequence information, a full-length cDNA was cloned. The cDNA (1738 bp) encodes a relatively long CDK protein of 505 amino acids (56.7 kDa predicted molecular mass). Based on sequence homology it was possible to identify a second *Arabidopsis* gene coding for a putative CDKC protein (CDKC;1). Both genes are located on chromosome V and are predicted to be the result of gene duplication (Vandepoele et al., 2002). *CDKC;1* and *CDKC;2* gene sequences are 86% identical, whereas the encoded amino acid sequences share 92% similarity. These CDKC proteins are homologous to CDKs identified in alfalfa, tomato and pea (approximately 80% identity) and all of them bear a PITAIRE signature motif in the cyclin-binding domain (Feiler and Jacobs, 1990; Magyar et al., 1997; Joubès et al., 2001). In addition to their large protein kinase domain, CDKC;1 and CDKC;2 present a nuclear localization signal at the C-terminal end (position 350 to 367) (Fig. 1). Comparison of the *Arabidopsis* CDKC sequences with all known non-plant CDK proteins showed that these proteins are closely related to CDK9 (50% identity) (Fig. 1).

Two-hybrid protein interaction assays

To understand the possible function of the Arath;CDKC;2 protein, a yeast two-hybrid screen was undertaken to search for its protein partners. One of the interacting clones identified encodes a protein (designated *Arabidopsis* cyclin T, or AtCycT) with sequence similarity to animal cyclins from the T and K group. The evolutionary relationship between animal cyclins from the T and K group and their potential plant counterparts is represented by a phylogenetic tree in Figure 2. Sequence

comparison of the *Arabidopsis* cyclin T-like proteins with related proteins from different kingdoms illustrates that these proteins are structurally not directly linked to the cyclin T or cyclin K groups.

Figure 1. Amino acid sequence alignment of CDK-like proteins related to the animal CDK9. Arath;CDKC;1, CDKC;1 protein from Arabidopsis thaliana; Arath;CDKC;2, CDKC;2 protein from Arabidopsis thaliana; Medsa;CDKC;1, CDK protein from Alfalfa (Medicago sativa); Lyces;CDKC;1, CDK protein from tomato (Lycopersicon esculentum); CDK9_Human, CDK9 protein from Homo sapiens; and CDK9_Mus, CDK9 protein from mouse (Mus musculus). The amino acid residues common to the six aligned proteins are indicated with asterisks, and the characteristic PITA(L/I)RE motif is boxed. The shadowed regions in the CDKC;1 and CDKC;2 proteins correspond to the amino acid residues not shared by both sequences.*

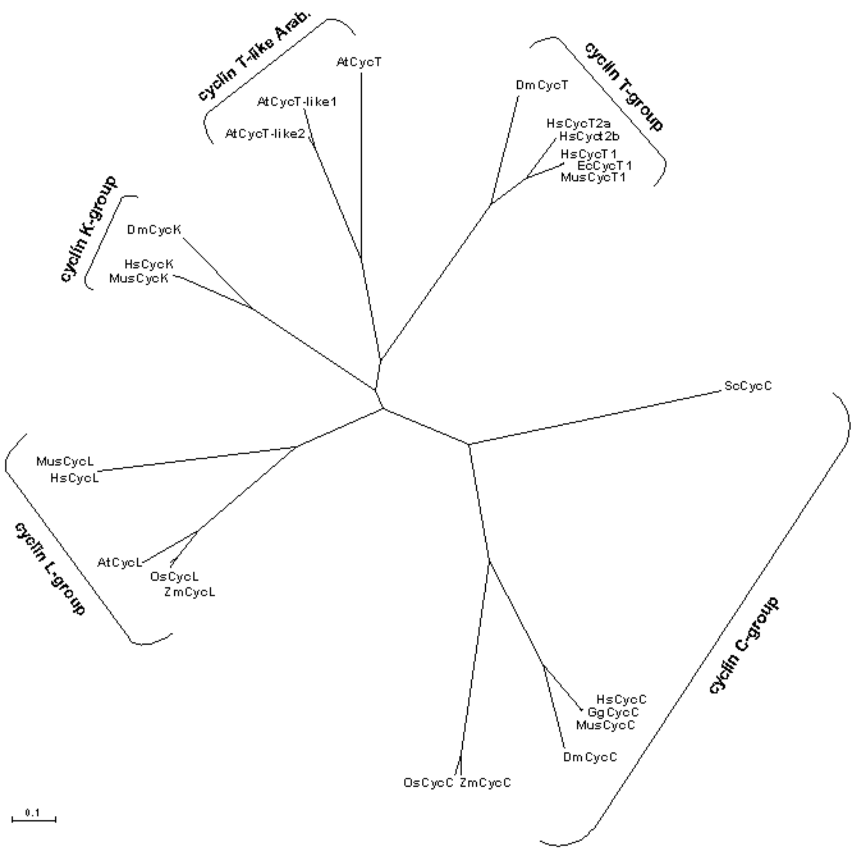

***Figure 2**. Sequence comparison of the Arabidopsis cyclin T protein with related proteins from different kingdoms. Neighbor-Joining tree of the T, K and their structurally related C and L groups of cyclin proteins with Poisson correction for evolutionary distance calculation. The scale indicates evolutionary distance. CycT, cyclin T (or T-like) proteins; CycK, cyclin K proteins; CycC, cyclin C (or C-like) proteins; CycL, cyclin L (or L-like) proteins. At, Arabidopsis; Ds, Drosophila melanogaster; Ec, Equus caballus; Gg, Gallus gallus; Hs, Homo sapiens; Mus, Mus musculus ; Os, Oryza sativum; Sc, Schizosaccharomyces pombe; and Zm, Zea mays.*

The phylogenetic analysis shows that these two cyclin groups do not include plant members. On the contrary, *Arabidopsis* cyclins form a separate group sharing common structural characteristics

with both the cyclins T and K. Presumably, the *Arabidopsis* T-like proteins take up the role of both the T and K animal cyclins. Alternatively, the T-like cyclin genes could have undergone extensive changes during evolution, so that they no longer can be recognized as functional homologues of the animal counterparts. However, the two-hybrid interaction of the *Arabidopsis* cyclin T with a protein related to CDK9, tends to exclude this hypothesis. Therefore, T-like proteins from *Arabidopsis* might have gone through particular modifications during evolution, which allow one single protein to assume the role of both the animal cyclin T and K groups.

The two-hybrid analysis showed that Arath;CDKC;2 interacts with other proteins, such as a ribonucleoprotein (RNP), a DNA-binding protein (GT-1), and a DAG-homologous protein.

To confirm the results of the screen and to assess the specificity of the interactions, we performed additional pair-wise two-hybrid assays. Vectors containing Arath;CDKC;2, Arath;CDKA;1, or Arath;CDKB1;1 fused to the GAL4-binding domain were co-introduced into a yeast reporter strain together with plasmids encoding the *Arabidopsis* cyclin T protein fused to the GAL activation domain. Figure 3 illustrates that the cyclin T protein can interact with Arath;CDKC;2, but not with Arath;CDKA;1 or Arath;CDKB1;1.

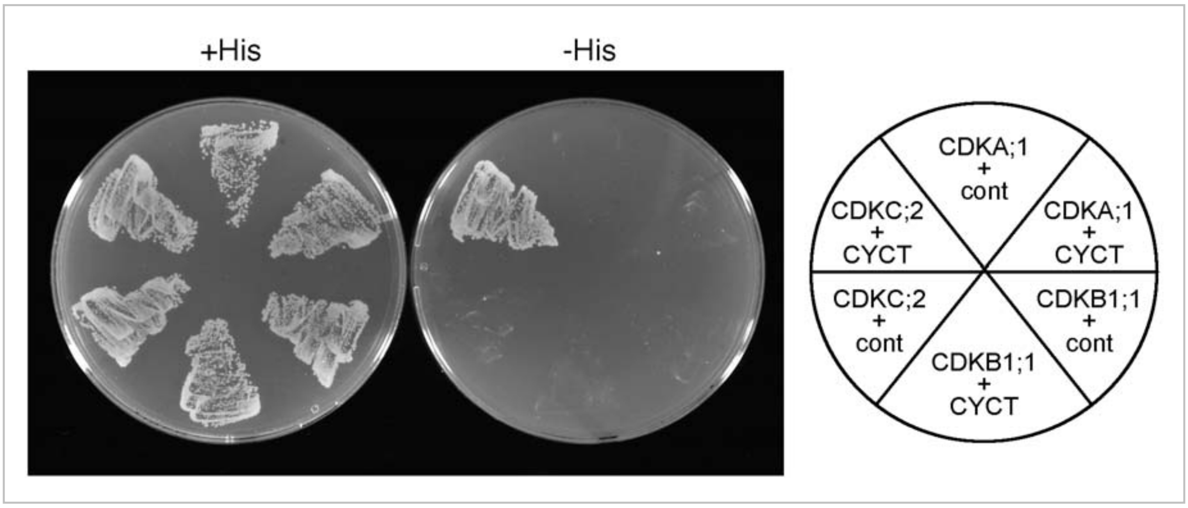

***Figure 3**. Yeast two-hybrid interaction of CDK proteins (CDKA;1, CDKB1;1, and CDKC;2) with the cyclin T (CYCT) from Arabidopsis. HF7c transformant cells of yeast were streaked on plates with (His^+) and without (His^-) histidine. Reconstitution of the GAL4 activity in the positive transformants restored the ability of yeast to grow in histidine-deficient medium. This shows that the plant cyclin T homologous protein can interact with Arath;CDKC;2 but not with Arath;CDKA;1 or Arath;CDKB1;1. cont, control two-hybrid assay performed with an empty pGBT9 vector.*

In vitro *transcription/translation and immunoprecipitation*

In order to validate the two-hybrid protein interaction results, we tested the ability of CDKC;1 and CDKC;2 to heterodimerize *in vitro* with cyclin T and DAG-like proteins. For this purpose, HA-tagged CDKC;1 and CDKC;2, and *c-myc*-tagged cyclin T and DAG-like proteins were produced using the coupled *in vitro* transcription/translation system. One part of each sample was resolved by SDS-PAGE (Fig. 4, top), while the other part was subjected to immunoprecipitation with monoclonal anti-HA

antibodies (Fig. 4, bottom). In the absence of CDKC;1 and CDKC;2 proteins, no cyclin T or DAG-like protein was precipitated by the anti-HA antibodies (data not shown). However, both cyclin T and DAG-like proteins co-precipitated reproducibly with HA-tagged CDKC;1 and CDKC;2 (Fig. 4, bottom left). Identical results were obtained in a reciprocal experiment with anti-*c-myc* monoclonal antibodies (data not shown). These data revealed that both *Arabidopsis* CDKC proteins interacted *in vitro* with the cyclin T and DAG-like proteins, as revealed by two-hybrid interaction analysis.

To further investigate the structural requirements for heterodimerization of the CDKCs and cyclin T proteins, we have tested for the *in vitro* interaction of deleted forms of both proteins, tagged with the HA or *c-myc* epitope, respectively (Fig. 4, bottom right). The interactions between the deleted forms of CDKC and cyclin T were analyzed in immunoprecipitation experiments with the specific anti-HA antibodies (Fig. 4, bottom right). These experiments showed that the CDKC N-terminal part (amino acids 1-217, $CDKC^{1-217}$) comprising the PITAIRE-binding domain and the T-loop region is sufficient to bind the full-length cyclin T protein (Fig. 4, bottom right, lane 1).

In analogy with its mammalian counterparts, the *Arabidopsis* cyclin T sequence comprises two characteristic α-helical domains, each containing five helices. The deletion of 104 amino acids at the carboxyl-terminal end (cyclin T amino acids 1-213, $cycT^{1-213}$) does not seem to significantly influence the binding of the cyclin to CDKC (Fig. 4, bottom right, lane 2). However, the further truncation to amino acid 153 ($cycT^{1-153}$) completely abolishes the binding to CDKC (Fig. 4, bottom right, lane 3). The first deletion removes most of the second α-helical domain, while the second deletion destroys also the first repeat. These results indicate that the protein region between amino acids 153 and 213 is required for the interaction with the CDKC partner. This assumption seems valid for both CDKC proteins because no differences were observed in their individual abilities to bind the tested cyclin T protein forms (data not shown). Thus, the first of the two helical domains of cyclin T must contain the crucial sites for dimerization with the CDKC proteins.

Gene expression analysis

The expression of *CDKC;1*, *CDKC;2*, and the *cyclin T* was quantified by real-time PCR. Total RNA from seedlings, roots, rosettes, stems, and inflorescences of *A. thaliana* was reverse transcribed and cDNA samples were amplified by a LightCycler PCR using gene-specific primers. The results show that *CDKC;1*, *CDKC;2*, and *cyclin T* transcripts, although present in all tested organs, were most abundant in flower tissues. Transcript levels detected in flowers for the three genes were approximately two-fold higher than in all other organs tested (Table 1).

CDKC expression was confirmed by Northern analysis, using an antisense riboprobe cross-hybridizing with both *CDKC* genes. Hybridization to total RNA from various tissues revealed the existence of two similarly sized bands of approximately 1.8 kb, corresponding to the *CDKC;1* and *CDKC;2* transcripts (data not shown).

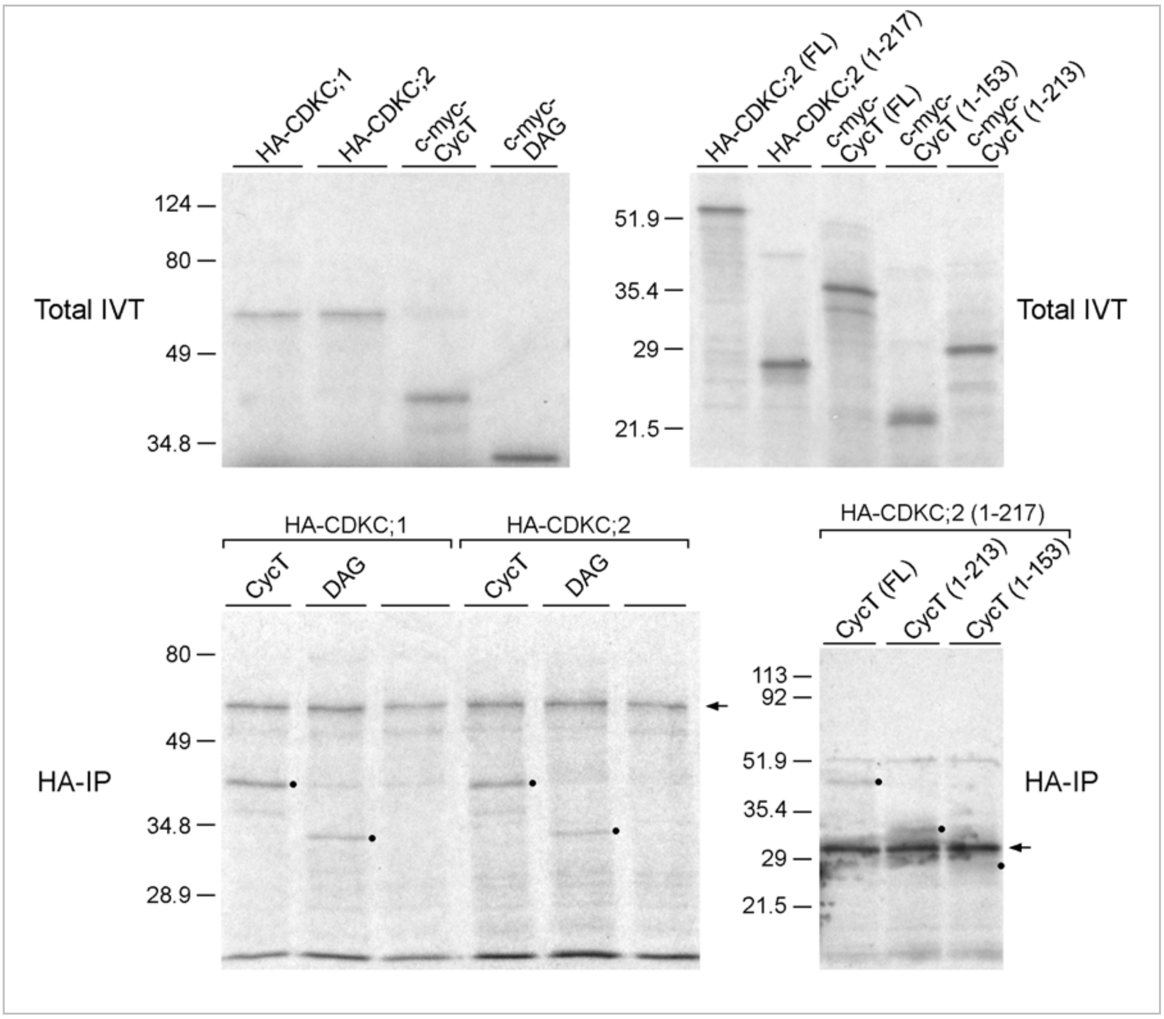

Figure 4. *Total in vitro translation (total IVT) extracts of the HA-tagged CDKC;1 (HA-CDKC;1) and CDKC;2 (HA-CDKC;2), as well as the c-myc-tagged cyclin T (c-myc-cycT) and DAG-like protein (c-myc-DAG) (top left gel). In the top right gel are shown, together with the full-length versions of both CDKC;2 and cyclin T (lane 1 and 3, respectively), the in vitro translated deleted forms of HA-CDKC;2$^{1\text{-}217}$ (lane 2), c-myc-cycT$^{1\text{-}153}$ and c-myc-cycT$^{1\text{-}213}$ (lanes 4 and 5, respectively). Aliquots (2.0 and 2.5 μl, left gel and right gel, respectively) of each sample were directly analyzed by SDS-PAGE and autoradiographed. Another aliquot of the same samples was subjected to immunoprecipitation with anti-HA monoclonal antibodies (bottom). Immunoprecipitation of cyclin T and DAG proteins by HA-CDKC;1 (lane 1 and 2) and HA-CDKC;2 (lane 4 and 5) are shown in the bottom left gel. An arrow marks the position of HA-CDKC;1 (lane 1-3) and HA-CDKC;2 (lane 4-6); dots mark the position of cyclin T (lanes 1 and 4) and DAG (lanes 2 and 5) proteins. Immunoprecipitation of cyclin T full-length and mutant forms by HA-CDKC;2$^{1\text{-}217}$ are shown in the bottom right gel. The positions of cyclin T full-length (lane 1) and mutant forms (lane 2 and 3) precipitated by HA-CDKC;2$^{1\text{-}217}$ are marked by dots. The position of HA-CDKC;2$^{1\text{-}217}$ is indicated by an arrow. In the case of mutants, the numbers refer to the amino acid sequence contained in these constructs. Molecular mass markers are indicated on the left side of all gels.*

Tissues	Relative amount of amplified DNA[1]		
	CDKC;1	CDKC;2	cyclin T
Seedlings	141,300	12,160	17,430
Root	97,340	13,330	16,290
Rosettes	103,700	11,860	25,090
Stems	130,500	9,130	30,620
Flowers	240,600	23,480	50,140

Table 1. *Semi-quantitative transcript analysis by real-time RT-PCR Total RNA from seedlings, roots, rosettes, stems, and flowers of* Arabidopsis thaliana *was reverse transcribed and cDNA samples were amplified in a LightCycler PCR with gene-specific primers. [1]Amounts after amplification of equal amount of target cDNA from all tested organs. Independent experiments have shown that the values are subject to maximum 20% error.*

A more detailed expression profile of the *CDKC* genes was obtained by *in situ* hybridization on sections of *Arabidopsis* tissues and radish roots. The transcript turned out to be mainly confined to the endodermis in roots and to the epidermis in petals (both inner and outer cell layers) and sepals (only outer epidermis) (Fig. 5). *CDKC* gene expression was clearly developmentally regulated in flowers. At young stages transcripts were only visible in sepals (mainly the distal part) (Fig. 5A and 5B). In fully mature flowers, transcripts accumulated preferentially in petals, while in sepals the expression slowly disappeared (Fig. 5C). *CDKC* transcripts were detectable in the epidermis of the anthers and the anther filaments in fully mature flowers (Fig. 5C and 5D). *CDKC* mRNA was never detected in carpels (Fig. 5C and 5D). However *CDKC* transcripts were observed in the outer epidermis of siliques (data not shown). In roots, *CDKC* mRNA was confined to the endodermis. Expression was never detected in developing and mature leaves, neither in the shoot apical meristem. These tissue expression results indicate that CDKC proteins might have a function in fully differentiated cells, rather than being involved in cell division control.

In order to confirm a functional relation between CDKC and cyclin T, we attempted to localize cyclin T transcripts in *Arabidopsis* tissues using *in situ* hybridization. Unfortunately, this study was fruitless, because no *in situ* signals had been observed in any of the studied tissues (flowers and

young seedlings). The failure to localize cyclin T mRNAs *in situ* might be the result of very low expression levels. Alternatively, this cyclin gene may be expressed in tissues other than those analyzed.

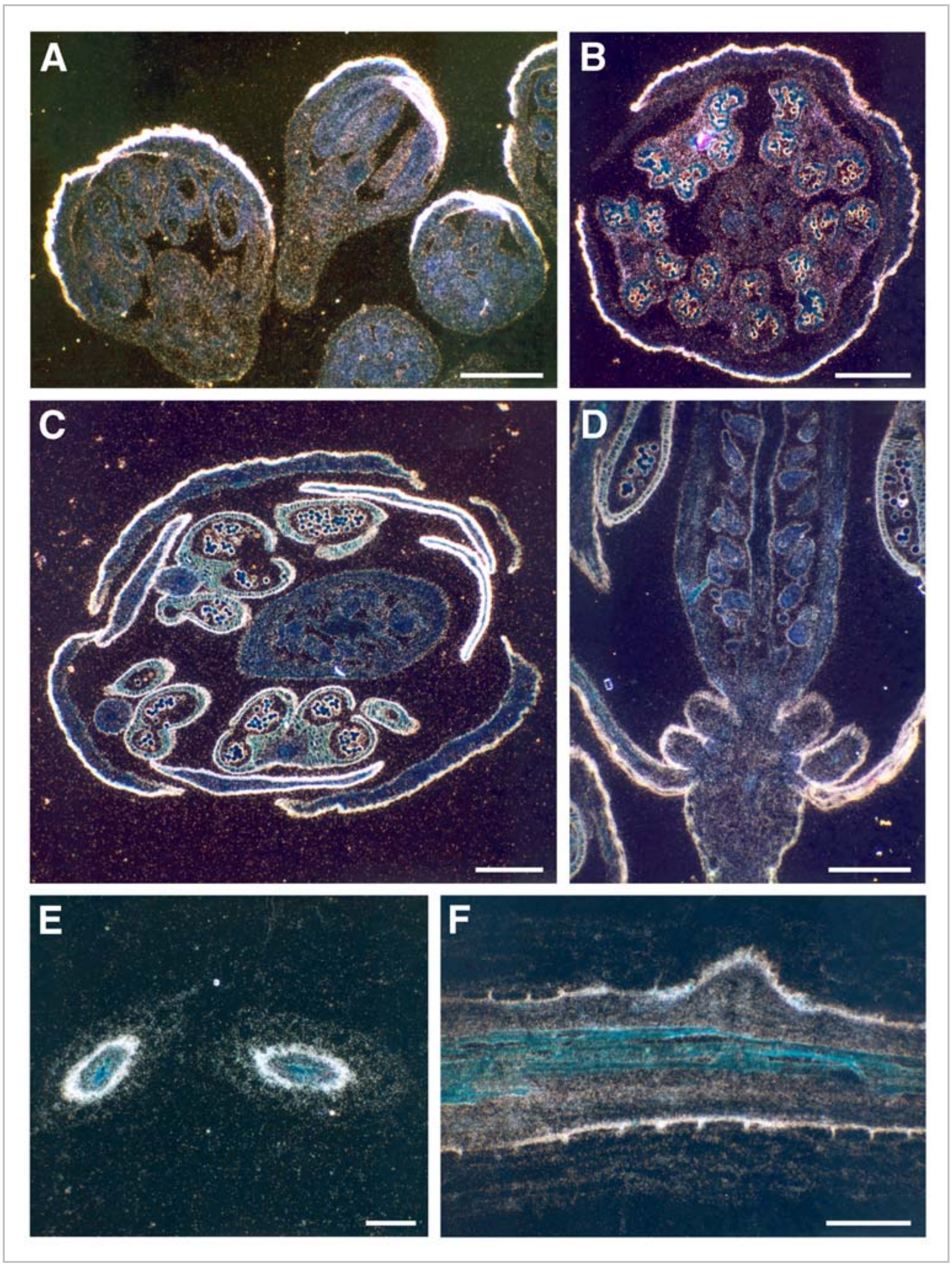

Figure 5. *Arath;CDKC;2 mRNA accumulation pattern in Arabidopsis flowers and radish roots, as shown by in situ hybridization. In flowers, CDKC;2 is confined to the epidermal cells. These floral expression studies also indicate that CDKC;2 is developmentally regulated. In young flowers, transcripts are visible only in sepals (mainly the distal part)* ***(A, B)****, whereas in fully mature flowers the transcripts accumulate preferentially in petals and the expression in sepals slowly disappears* ***(C)****. In fully mature flowers, CDKC;2 transcripts are also visible in the epidermis of the anthers and the anther filament, but never in the carpels* ***(C, D)****. Arath;CDKC;2 transcripts were also observed in the*

endodermis of radish roots ***(E, F)****.Scale bar, 200 µm.*

Discussion

The activity of CDKs has been widely studied in mammals and yeast. In these organisms, CDKs play a well-established role in the regulation of the cell cycle but are involved in the control of gene transcription and other regulatory mechanisms as well (Morgan, 1997). Intensive efforts over the past years identified many CDK homologues from plant species. However, some of these CDKs show only a limited sequence similarity to kinases from the well-characterized A- and B-types. Their relevance for the cell cycle control is still unknown (Joubès et al., 2000). Plant kinases containing a PITAIRE motif have been identified previously, but their function has not been assigned (Feiler and Jacobs, 1990; Magyar et al., 1997). The PITAIRE motif present in Medsa;CDKC;1 of alfalfa, as well as its constitutive gene expression pattern (Magyar et al., 1997) point toward Medsa;CDKC;1 as being an homologue of the *Arabidopsis* CDKC protein. In tomato (*Lycopersicon esculentum*), a CDK protein (Lyces;CDKC;1) has also been identified that comprises a PITAIRE motif in its signature region. Expression studies have shown that its RNA transcripts accumulate in actively dividing tissues as well as in non-dividing cells (Joubès et al., 2001).

Our results led us to hypothesize that the *A. thaliana* CDKC;2 may represent a functional homologue of the animal CDK9. CDK9 is implicated in transcription regulation as a core component of the P-TEFb complex. The P-TEFb complex is composed of a catalytic kinase subunit (CDK9) and a cyclin subunit (cyclin T1, cyclin T2, or cyclin K), as well as other yet unidentified proteins (reviewed by Simone and Giordano, 2001). P-TEFb has been shown to stimulate transcription elongation, through phosphorylation of the CTD domain of the largest RNAP II subunit (Marshall et al., 1996; Napolitano et al., 2000; Price, 2000).

In contrast to the CDKs implicated in cell cycle, CDK9 does not phosphorylate H1. But notably, this CDK phosphorylates the C-terminus of the retinoblastoma gene product (Graña et al., 1994; De Luca et al., 1997; Simone et al, 2002). This association of CDK9 to the retinoblastoma pathway together with its expression in terminally differentiated tissues, point toward the involvement of the CDK9/cyclin T complex in determining cell differentiation rather than taking part in cell proliferation (Simone et al., 2002). As such, the identification and characterization of CDK9 has revealed CDKs as a multifunctional group that not only governs cell division but is also implicated in pathways that promote differentiation and inhibit cell cycle progression.

When the CDK9 amino acid sequence is compared with the complete genome of *Arabidopsis*, the highest score is observed with CDKC;1 and CDKC;2. In agreement with a possible role in transcription control, CDKC proteins harbour a nuclear localization signal. Furthermore, CDKC;2 interacts with the *Arabidopsis* cyclin T that, in turn, bears the strongest similarity to the cyclin partners

of CDK9. Immunoprecipitation assays confirmed the ability of the two CDKC proteins to heterodimerize with the cyclin T. Moreover, it was shown that the carboxyl-terminal region of the CDKC proteins might not be required for the interaction with the cyclin T partner. On the other hand, the stable interaction of cylin T to CDKC seems to depend on the integrity of its N-terminal end, comprising the first α-helical domain. Analogous results have been reported after analyzing the binding ability of cyclin T1 mutants to CDK9, using yeast two-hybrid protein interaction assays (Fraldi et al. 2001). Although kinase activity of the CDKC;2/cyclin T complex remains to be demonstrated, the specificity of interaction suggests that the two proteins are functional partners.

CDK9 has been suggested to take part in many different protein complexes, each with different functional properties (De Falco and Giordano, 1998; Simone and Giordano, 2001). Has Arath;CDKC;2 similar functional properties? As a first approach to address this question, we performed a two-hybrid screen to discover potential partners of CDKC proteins. Typically, most proteins identified in the screen are implicated in transcription.

Our two-hybrid results showed that Arath;CDKC;2 binds to RNPs, a DAG-like protein, a GT-1 protein, and a novel (unknown) protein with structural features shared with transcription factors. These findings strengthen the hypothesis that Arath;CDKC;2 may be a plant CDK9 homologue, belonging to a complex implicated in transcription regulation. Indeed, RNP proteins were shown to provide a functional coupling between transcription control and RNA processing (reviewed by Bentley, 1999). RNPs are very abundant RNA-binding proteins that play an important role in the metabolism of pre-RNAs (Dreyfuss et al., 1993). They bind to pre-mRNAs attached to RNAP II elongation complexes and influence pre-mRNA maturation, such as alternative splicing and mRNA export (Krecic and Swanson, 1999; Lorkovic et al., 2000). The RNAP II CTD plays a central role in the coupling mechanism that links transcription to processing by making protein-protein contacts with both the transcription control elements and with the splicing machinery (Proudfoot et al., 2002). This connection is established when the CTD of the RNAP II is phosphorylated, shifting the enzyme from initiation to elongation mode. The phosphorylated RNAP II domain interacts directly with factors that promote mRNA processing, recruiting them to the transcription apparatus (Bentley, 1999). The interaction of Arath;CDKC;2 with RNP might be essential for the regulation of diverse processing events, including mRNA splicing and transport.

The evidence that a DAG-like protein binds to Arath;CDKC;2 suggests that this kinase might regulate proteins involved in plastid development. DAG protein has been shown to be targeted to the plastids (Chatterjee et al., 1996). However, expression of DAG is required for the expression of nuclear genes, such as *CAB* (chlorophyll a/b-binding protein) and *RBCS* (ribulose biphosphate carboxylase), which encode proteins implicated in light-regulated gene expression (Chatterjee et al., 1996). Our data indicates that DAG proteins may directly interact with nuclear proteins, such as CDKC;2. We propose that a subpopulation of DAG proteins escapes chloroplast localization being targeted to the nucleus (for instance as a complex with CDKC;2) where it probably interacts with the transcription machinery. The feasibility of such behaviour has been demonstrated recently by H. Hirt

(personal communication), who cloned a protein kinase that localizes in both the nucleus and the chloroplast. Another mechanism for the dual localization could be the nuclear translation of the DAG-like protein. Recent evidence indicates that up to 10-15% of the total protein in mammalian cells is synthesized in the nucleus (Stern et al., 1997; Iborra, et al., 2001). Whether DAG proteins might display this type of behaviour, as well as whether the interaction with DAG proteins implicates CDKC in the cross-talk between plastid and nuclear gene expression, remains unclear.

The interaction with GT-1 supports the involvement of Arath;CDKC;2 in transcriptional control. GT-1 belongs to the class of trihelix DNA-binding proteins that attach to promoter sequences of many different plant genes. *Arabidopsis* GT-1 was found to bind, among others, to the pea RBCS and CAB2 light-responsive elements (reviewed in Zhou, 1999). Lagrange et al. (1997) identified the S2 element, a binding site related to GT-1, present in the promoter region of many nuclear genes that encode plastid proteins. Le Gourrierec et al. (1999) suggested that GT-1 activates transcription by direct interaction with transcriptional complexes. We speculate that GT-1 might interact with the transcription machinery, involving RNPs, DAG and CDKC;2, whose activity results in the control of chloroplast protein synthesis.

An additional interacting (yet unknown) protein comprises a number of structural features, characteristic for nuclear proteins involved in (chromatin-mediated) transcription regulation, including a bipartite nuclear localization signal, PEST determinants of protein instability, and plant homeodomain (PHD) motifs (data not shown).

Expression analysis showed that Arath;*CDKC* transcripts are most abundant in flower tissues. mRNA *in situ* localization revealed that *CDKC* genes are confined to epidermal tissues. These results argue against the direct involvement of CDKCs in cell division control. On the contrary, it indicates that this protein may be involved in some specialized functions that take part in differentiated tissues, as previously proposed for the animal CDK9.

All together, our data led us to consider that the plant CDKC/cyclin T complex is not involved in cell cycle control but rather interacts with specific components of the transcription machinery. According to our expression studies and in conformity with what has been shown for CDK9, we propose that CDKC is implicated in molecular mechanisms implicated in cell differentiation.

Accession Numbers for the proteins *(or putative proteins) referred are*: BAA97308 (Arath;CDKC;1), T50815 (Arath;CDKC;2), T09572 (Medsa;CDKC;1), CAC51391 (Lyces;CDKC;1), NP_570930 (CDK9_Human), P50750 (CDK9_Mus) for CDKs in Fig. 1; AAD46000 (AtCycT), BAB11392 (AtCycT-like1), CAB40377 (AtCycT-like2), O60563 (HsCycT1), AAC39665 (HsCycT2a), AAC39666 (HsCycT2b), AAD13656 (MusCycT1), AAF49325 (DmCycT), Q9XT26 (EcCycT1), AAH15935 (HsCycK), AAD09979 (MusCycK), XP_079707 (DmCycK) for cyclins in Fig. 2; AF249734 (AtCycL), CAC17050 (ZmCycL), BAB39257 (OsCycL), AAD53184 (HsCycL), AAD43568 (MusCycL), NP_565622 (AtCycC), BAA13181 (OsCycC), CAC17049 (ZmCycC), CAA44720 (DmCycC), A40268 (HsCycC), AAB18947 (GgCycC), CAA19367 (SpCycC). G71404 (ribonucleoprotein (RNP)); AAG09542 (DNA-binding protein

(GT-1)), BAA97063 (DAG homologue protein), BAB08556 (unknown protein) for the two-hybrid CDKC;2 interacting proteins.

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Chapter 3

A plant-specific cyclin-dependent kinase is involved in the control of G2/M progression in plants

Adapted from "A plant-specific cyclin-dependent kinase is involved in the control of G2/M progression in plants" by Andrea Porceddu, Hilde Stals, Jean-Philippe Reichheld, Gerda Segers, Lieven De Veylder, Rosa de Maria Barrôco*, Peter Casteels, Marc Van Montagu, Dirk Inzé, and Vladimir Mironov. J Biol Chem 276: 36354-36360 SEP 28 2001.

(*)contributed to the present work with the phenotypical analysis of the tobacco transgenic plants overexpressing dominant-negative CDKB1;1 protein.

Abstract

Cyclin-dependent kinases (CDKs) control the key transitions in the eukaryotic cell cycle. All the CDKs known to control G2-to-M progression in yeast and animals are distinguished by the characteristic PSTAIRE motif in their cyclin-binding domain and are closely related. Higher plants contain in addition a number of more divergent non-PSTAIRE CDKs with still obscure functions. Here we show that a plant-specific type of non-PSTAIRE CDKs is involved in the control of G2/M progression. In synchronized tobacco BY-2 cells, the corresponding protein accumulated in a cell cycle-regulated fashion, peaking at G2-to-M transition. The associated histone H1 kinase activity reached a maximum in mitosis and required a yet unidentified subunit to be fully active. Down-regulation of the associated kinase activity in transgenic tobacco plants using a dominant-negative mutation delayed G2-to-M transition. These results provide the first evidence that non-PSTAIRE CDKs are involved in the control of G2/M progression in plants.

Abbreviations: BY-2 cells, bright yellow-2 tobacco cell cultures; CDK, cyclin-dependent kinase.

Introduction

Progression through the major transitions of the eukaryotic cell cycle is driven by a family of serine/threonine kinases known as cyclin-dependent kinases (CDKs). The catalytic activity of these protein kinases is regulated by the association with their regulatory subunits, cyclins. The activity of the complexes is further controlled by a number of mechanisms including phosphorylation/dephosphorylation, interaction with inhibitory proteins, proteolysis, and intracellular trafficking (Morgan, 1997). In yeast, a single CDK (cdc2 in *Schizosaccharomyces pombe* or CDC28 in *Saccharomyces cerevisiae*) governs both the G1/S and G2/M transitions (Nasmyth, 1996; Stern and Nurse, 1996). In animal cells, distinct CDKs that associate sequentially with different cyclins monitor the cell cycle progression (Pines, 1996). Of the five mammalian CDKs strongly implicated in cell cycle control, three (CDC2/CDK1, CDK2, and CDK3) are closely related to the prototypical yeast cdc2 and have the same characteristic motif PSTAIRE in the cyclin-binding domain (De Bondt et al., 1993). The other two CDKs, CDK4 and CDK6, form a distinct subfamily of CDKs in which PSTAIRE is substituted with either PISTVRE or PLSTIRE, respectively. Both CDK4 and CDK6 are known to function exclusively in G1 phase (Morgan, 1997).

Plants, like animals, possess also an array of CDK-like kinases (referred to as CDKs hereafter), but their functions are mostly poorly defined (Mironov et al., 1999). Based on sequence similarity, plant CDKs can be subdivided into a few distinct groups (Segers et al., 1997). The best characterized group (A-type) comprises plant CDKs that are most closely related to the mammalian CDC2 and CDK2 and that contain the same PSTAIRE motif. A-type CDKs can typically complement partially yeast cdc2/CDC28 mutations and are therefore supposed to be functional homologues of the yeast CDKs (Colasanti et al., 1991; Ferreira et al., 1991; Hata, 1991; Hashimoto et al., 1992; Imajuku et al., 1992; Setiady et al., 1996). Supporting this notion, a dominant-negative mutant of an A-type CDK from *Arabidopsis thaliana* was found to negatively affect cell cycle progression, most probably both at G1/S and G2/M transitions (Hemerly et al., 1995).

Currently, functions of plant CDKs other than A-type remain essentially not understood. Here, we studied the function of a plant-specific subfamily of CDKs (B-type; Segers et al., 1997). B-type CDKs form a group of closely related kinases from diverse plant species, sharing approximately 70-80% identity and comprising a distinct cluster of CDKs. Instead of PSTAIRE, all of them bear the unique PPT(A/T)LRE motif (Fobert et al., 1996; Hirayama et al., 1991; Kidou et al., 1994; Magyar et al., 1997). Unlike typical CDKs, the expression of B-type CDKs is under strict cell cycle control and attempts to complement yeast CDK-deficient mutants have been unsuccessful.

We have chosen tobacco as the experimental system because of the high degree of synchronization attainable in the tobacco Bright Yellow-2 (BY-2) cell line (Nagata et al., 1992). The close similarity among B-type CDKs across species has allowed us to use polyclonal antibodies against CDKB1;1 from *Arabidopsis* to identify and characterize a B-type CDK in tobacco. In synchronized BY-2 cells, the accumulation of the protein and the related kinase activity is cell cycle regulated and is linked to mitosis. We further demonstrate that the specific activity is present in the form of high-molecular

weight complexes, whereas the fraction corresponding to the monomeric protein is inactive, what implies the presence of a yet unidentified activating subunit. To assess the function of B-type CDKs *in vivo*, we have expressed a dominant-negative mutant of the CDKB1;1 in transgenic tobacco. These plants were found to have an increased fraction of 4C cells. Accordingly, we propose that B-type plant CDKs play a unique role in the G2/M progression.

Experimental Procedures

Plasmid Construction and Plant Transformation

The cDNA for *CDKB1*;1-*D161N* was obtained by mutating the codon GAT (D161) for AAT (N161) by *in vitro* mutagenesis (Porceddu et al., 1999). Both *CDKB1;1* and *CDKB1;1-D161N* cDNAs were fused to the Triple-Op promoter, which is a derivative of the cauliflower mosaic virus (CaMV) 35S promoter and has an activity comparable to that of the CaMV 35S promoter (Gatz et al., 1992). The expression cassettes Triple-Op-*CDKB1;1-D161N*-3'Nos and Triple-Op-*CDKB1;1WT*-3'Nos were ligated into pGSC1704 binary vector (Hérouart et al., 1994). The resulting plasmids were transferred into *Agrobacterium tumefaciens* C58C1Rif^R by conjugation. Tobacco plants were transformed by leaf disc transformation (Horsch et al., 1985) and 15 independent T0 primary transformants were analyzed by protein gel blotting. The T0 plants were selfed and the segregating T1 first generation was subjected to further analysis.

Maintenance of the Cell Suspension and Synchronization

The tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow-2) suspension was maintained at weekly dilution (1.8/100) of cells in fresh Murashige and Skoog (MS) medium modified according to Nagata *et al.* (Nagata et al., 1992) and cultured at 28°C and 130 rpm in the dark. The cells were synchronized as described (Reichheld et al., 1995). A stationary culture was diluted 1/5 in fresh medium supplemented with 4 μg/ml aphidicoline (Sigma, St. Louis, MO). After 24 h of culture, the drug was removed by extensive washes and the cells were resuspended into fresh medium. DNA synthesis was determined by pulse-labeling with ^{3}H-thymidine as described (Reichheld et al., 1995). Mitotic index was determined by ultraviolet light microscopic analysis of 500 cells stained with 0.1 μg/ml 4',6-diamino-2-phenylindole (Sigma) in the presence of 0.2% Triton X-100.

Purification and Flow Cytometrical Analysis of Nuclei

Tobacco BY-2 protoplasts were obtained from 5x10^4 cells by digestion with 1 ml of enzyme solution (2% cellulase Onozuka R10, 0.1% pectolyase Kikkoman Co, 0.66 M sorbitol) for 1 h at 37°C. After treatment, the protoplasts were pelleted by centrifugation (5 min, 1000 *g*), washed once with MS medium supplemented with 4.5% mannitol. Nuclei were released from the protoplast pellet in Galbraith's extraction buffer (Galbraith et al., 1983). After addition of 1% formaldehyde, nuclei were

stored at 4°C until further analysis by flow cytometry. Before analysis, nuclei were filtered through a 10-μm nylon filter, treated with RNase A, and stained with propidium iodide (50 μg/ml). Cytometrical analyses were performed on 10^4 nuclei with a FACS scan flow cytometer (Becton Dickinson, Bedford, MA). For flow cytometrical analysis of nuclear DNA content in plant tissues, cotyledons or individual calli were chopped with a razor blade in Galbraith's buffer and analyzed as described above.

Antibody Preparation, Immunoblotting, and Histone H1 Kinase assays

Polyclonal antibodies against Nicta;CDKA;1 (courtesy of P. John, Australian National University, Canberra) and CDKB1;1 were raised using the peptides CRNALEHEYFKDIGYVP and SAKTALDHPYFDSLDKSQF derived from the C-termini of the respective proteins. The sera were purified with protein A-Sepharose (Amersham Pharmacia Biotech, Little Chalfont, UK).

Protein extracts from BY-2 cells were prepared by grinding cells with sea sand in homogenization buffer as described (27). Tobacco plants were ground in liquid nitrogen. Protein concentrations were determined using the Protein Assay kit (Bio-Rad, Hercules, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein gel blots were performed according to standard procedures with primary anti-Nicta;CDKA;1 and anti-CDKB1;1 antibodies diluted 1/500 and 1/2500, respectively, and a secondary peroxidase-conjugated antibody (Amersham Pharmacia Biotech) diluted 1/5000.

For immunoprecipitation experiments, 100-200 μg protein extracts were preincubated for 1 h at 4°C on a rotating platform with 25% (v/v) protein A-Sepharose (Amersham Pharmacia Biotech). After centrifugation, equal amounts of the supernatant were incubated for 4 h with purified antibodies (1/50 diluted anti-Nicta;CDKA;1 or 1/50 diluted anti-CDKB1;1) and subsequently for 1 h with 25% (v/v) protein A-Sepharose beads. Beads were washed 3 times with homogenization buffer and a fourth time with kinase buffer (Magyar et al., 1993). The histone H1 kinase assay was carried out by incubating 25 μl of the packed beads with 5 μCi [γ-^{32}P]ATP (3000 Ci/mmol) in the presence of 1 mg/ml histone H1 (Sigma), 50 mM Tris.HCl (pH 7.8), 15 mM $MgCl_2$, 5 mM EGTA, 10 μM ATP, and 1 mM dithiothreitol, for 20 min at 30°C in a final volume of 35 μl. Samples were analyzed on 12% SDS-PAGE gel, stained with Coomassie Brilliant Blue, and autoradiographed.

For competition experiments the C-terminal CDKB1;1 peptide was purified to homogeneneity by reverse-phase chromatography on a PRLP-S column (8 μm, 300 Å) (Polymer Laboratories) equilibrated in 0.1% trifluoroacetic acid, and eluted with a 0-7-% acetonitrile gradient in 0.1% trifluoroacetic acid. The competion was achieved by preincubation of the antiserum with the purified CDKB1;1 peptide at 4°C overnight before the antiserum was used for immunoblotting and immunoprecipitation.

Biochemical Separation and Analysis of Active Kinase Complexes

Proliferating tobacco BY-2 cell suspension culture cells were collected 3 days after subculturing. Proteins were extracted in homogenization buffer (HB) as described previously (Stals et al., 1997) and

bound to a Q-ceramic HyperD column (10 x 25cm; Biosepra, Marlborough, MA), equilibrated with HB. Proteins were eluted by a one-step elution using 1 M NaCl in 0.5xHB and further fractionated by size on a gel filtration column (Superdex S200, Amersham Pharmacia Biotech, 1.7x100 cm Omnifit column). The column was equilibrated with Tris buffer (50 mM, pH 7.8) containing 15 mM $MgCl_2$, 5 mM EGTA, 5 mM β-glycerophosphate, 1 mM NaF, 1 mM dithiothreitol, 0.1 mM $NaVO_4$, 100 mM NaCl, and protease inhibitors (Roche Diagnostics, Brussels, Belgium). Associated kinase activity of Nicta;CDKA;1 and Nicta;CDKB1;1 was isolated by imununoprecipitation with anti-Nicta;CDKA;1 and anti-CDKB1;1 antisera, respectively. The activity of 100-μl aliquots of 5-ml fractions was assayed *in vitro* in the presence of histone H1 (1 mg/ml), cAMP-dependent kinase inhibitor, 15 μM ATP, and 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol) in a final volume of 35 μl at 30°C. The reactions were terminated after 20 min by heating the samples in sample buffer at 95°C for 10 min. The proteins were separated on a 15% SDS-PAGE gel, stained with Coomassie Brilliant Blue, and the incorporated radioactivity was measured using a PhosphoImager (Amersham Pharmacia Biotech).

Results

Identification of B-type CDK Activity in Tobacco

To study the function of B-type CDKs, we first analyzed the associated kinase activity in the course of the cell cycle in the tobacco BY-2 cell suspension (Nagata et al., 1992). Because B-type CDKs had not been described in tobacco, we decided to use an antibody raised against the C-terminal SAKTALDHPYFDSLDKSQF peptide of CDKB1;1 of *Arabidopsis* (Arath;CDKB1;1). This region is well conserved among the known PPTALRE kinases with 17 out of 19 amino acids being identical. Thus, we expected the anti-CDKB1;1 antibody to recognize tobacco orthologues. Indeed, the antibody recognized specifically a single protein in a crude protein extract of BY-2 cells fractionated by SDS-PAGE (Fig. 1a). The apparent molecular mass of the protein, 34.5 kDa, is close to that of the *Arabidopsis* CDKB1;1. Furthermore, the interaction was specific, as the addition of the peptide used to raise the antibody completely obliterated the signals corresponding to the protein (Fig. 1b). We also analyzed by protein gel blotting, with anti-CDKB1;1 antibody, immunoprecipitates obtained with the use of either anti-CDKB1;1 or anti-PSTAIRE antibody and found that the 34.5-kDa protein was easily detectable in the former but not the latter precipitate (data not shown). These results suggested that tobacco possessed a CDKB1;1 homologue, referred to as Nicta;CDKB1;1 hereafter.

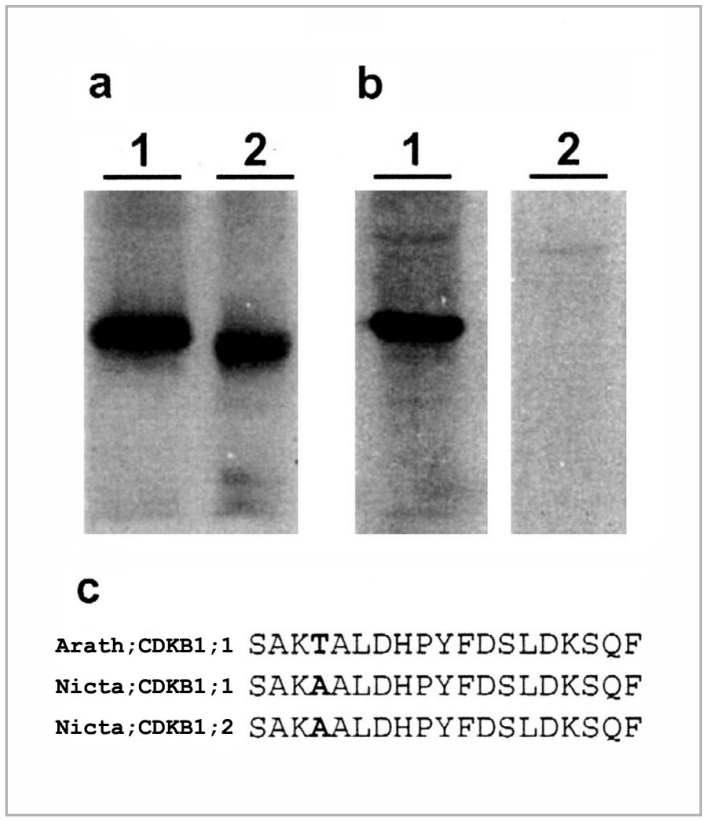

Figure 1. *The interaction of antibodies against the C-terminal peptide of CDKB1;1 with a 34.5-kDa protein in tobacco cells.* ***A****, The total protein extracts from Arabidopsis suspension and tobacco BY-2 cells (lanes 1 and 2, respectively) were used for protein gel blot with anti-CDKB1;1 antibodies.* ***B****, The total protein extract from BY-2 cell was probed either with anti-CDKB1;1 antibodies or with the same antibodies preincubated with the C-terminal peptide (lanes 1 and 2, respectively).***C***, Comparison of the C-terminal peptides of the Arabidopsis CDKB1;1 (accession numbers D10851) and two B-type CDKs from tobacco (accession numbers AF289465 and AF289466).*

Characteristically, the protein level of Nicta;CDKB1;1 fluctuated through the cell cycle (CDC2b; Fig. 2b), thus displaying the most conspicuous feature of B-type CDKs. The protein was hardly detectable in early S phase, started to accumulate in late S phase, reached the maximal level at the G2-to-M transition, and declined afterwards. After immunoprecipitation with anti-CDKB1;1 serum, the kinase activity associated with Nicta;CDKB1;1 was measured by *in vitro* phosphorylation of histone H1. In the course of the cell cycle, Nicta;CDKB1;1 kinase activity peaked later than the corresponding protein level with a maximum in the middle of mitosis (Fig. 2b). For comparison, we analyzed also the Nicta;CDKA;1 protein and associated kinase activity using a specific antibody. The level of Nicta;CDKA;1 protein was constant during the whole cell cycle (Fig. 2b). Its activity rose in early S phase and declined during mitosis, slightly earlier than that of Nicta;CDKB1;1.

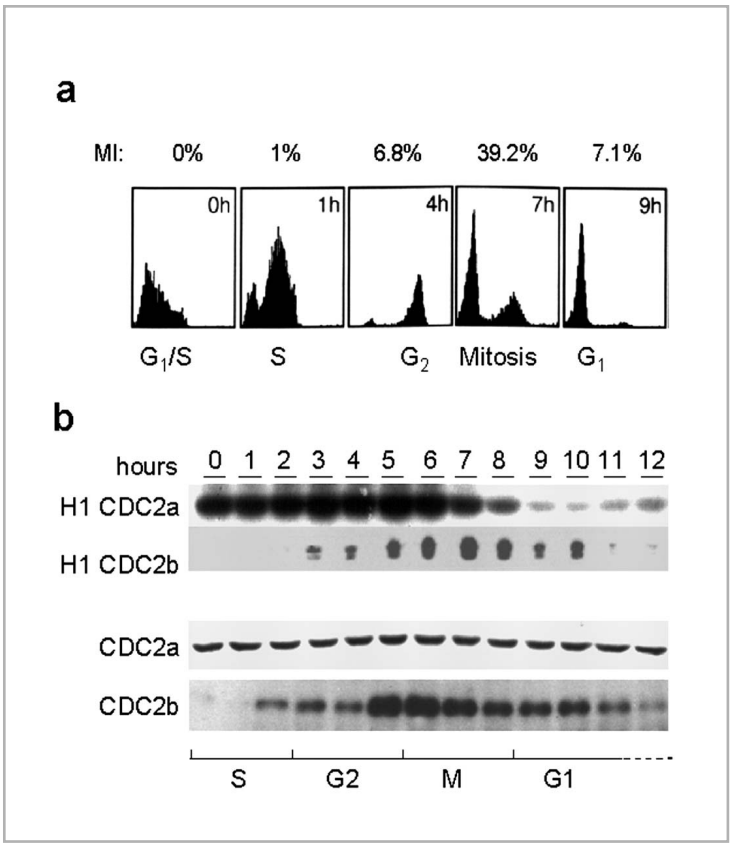

Figure 2. *Nicta;CDKA;1 and Nicta;CDKB1;1 protein levels and histone H1 kinase activity in synchronized BY-2 cell suspensions. BY-2 cells were synchronized with aphidicolin (see Experimental procedures) and samples were taken every hour.* ***A****, Cell cycle progression was monitored by flow cytometry and by measurement of the mitotic index (MI).* ***B****, Crude protein extracts were used for protein gel blot with anti-Nicta;CDKA;1 (CDC2a) and anti-CDKB1;1 (CDC2b) antibodies or to prepare immunoprecipitates for histone H1 kinase measurements (H1 CDC2a and H1 CDC2b). The duration of the different cell cycle phases, as estimated on the basis of the flow cytometrical analysis and mitotic index, is depicted at the bottom.*

Active Nicta;CDKB1;1 is Present in High-Molecular Mass Complexes

To biochemically characterize Nicta;CDKB1;1, the proteins present in a total extract of actively proliferating BY-2 suspension cells were separated in two fractions using ion exchange chromatography. The bound fraction contained most of the kinase activity (approximately 95% and 75% for Nicta;CDKA;1 and Nicta;CDKB1;1, respectively) and was further fractionated by size exclusion chromatograhpy. The CDK-associated complexes were immunoprecipitated with either anti-Nicta;CDKA;1 or anti-CDKB1;1 polyclonal antibodies and assayed in an *in vitro* kinase assay with histone H1 as a substrate. The Nicta;CDKB1;1-associated kinase activity eluted as two minor peaks of approximately 250 kDa and more than 700 kDa and one major broad peak in the range of 65-100 kDa, referred to as the 80-kDa fraction hereafter (Fig. 3a). In contrast, Nicta;CDKA;1 activity eluted essentially as a single peak of approximately 200 kDa (Fig. 3a). Immunoblotting showed that most of the Nicta;CDKB1;1 protein was present in high-molecular mass complexes with a relatively low kinase activity compared with that of the major Nicta;CDKB1;1 activity peak, whereas the majority of the Nicta;CDKA;1 protein comigrated with the peak of activity (Fig. 3b). The fractions corresponding

to monomeric Nicta;CDKA;1 and Nicta;CDKB1;1 (20-21; Fig. 3) contained detectable, albeit low, amounts of the corresponding proteins and were essentially inactive.

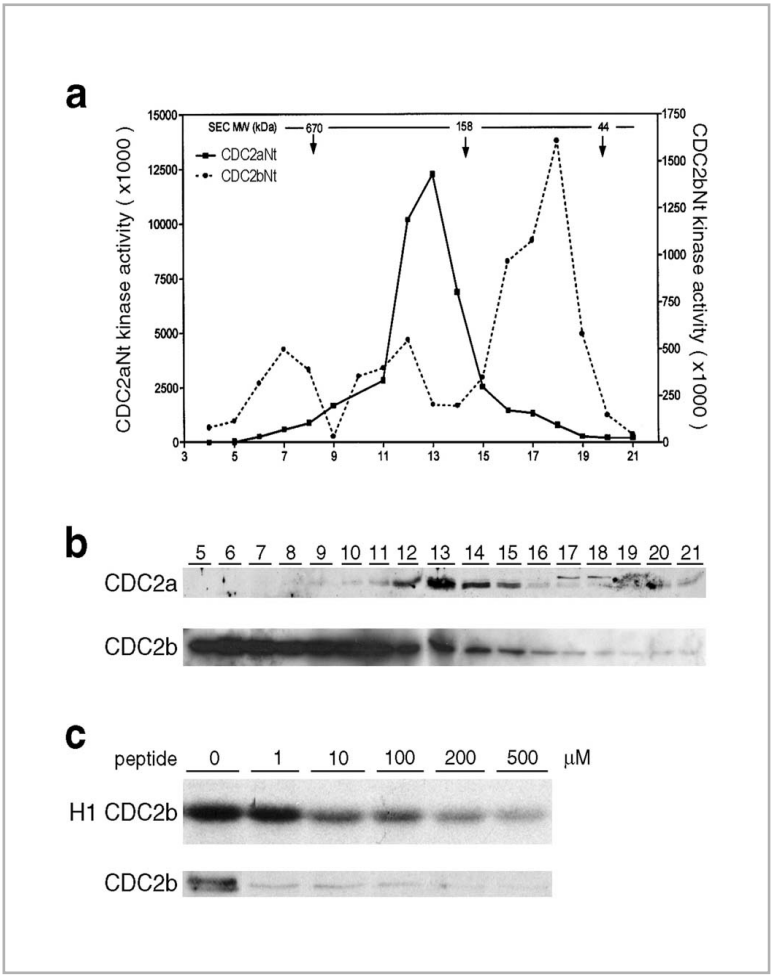

Figure 3. *Characterization of protein complexes with Nicta;CDKA;1 and Nicta;CDKB1;1-associated kinase activity in BY-2 cell suspension. The total protein extracts were purified and fractionated by size exclusion chromatography (SEC) as described in the experimental procedures.* ***A****, Nicta;CDKB1;1 (CDC2bNt) and Nicta;CDKA;1(CDC2aNt)-associated kinase activity (in relative units) as determined by histone H1 phosphorylation in immunoprecipitated complexes. Position of the size markers is indicated at the top of the panel.* ***B****, The level of Nicta;CDKA;1 (CDC2a) and Nicta;CDKB1;1 proteins (CDC2b) in individual fractions as assessed by immunoblotting with Nicta;CDKA;1- and CDKB1;1-specific antibodies.* ***C****, Specificity of the immunoprecipitated complexes as assayed by peptide competition. The bound fraction prior to size exclusion chromatography was subjected to immunoprecipitation using the anti-CDKB1;1 antiserum pre-incubated with increasing concentrations of the competing peptide followed by histone H1 kinase activity assay (H1 CDC2b) and immunodetection (CDC2b) of the B-type CDKs.*

Gel filtration separation of the flow-through fraction showed that the majority of Nicta;CDKA;1 protein was present in the inactive monomeric fractions (corresponding to fractions 20-21 in Fig. 3) and accounted for approximately half of the total Nicta;CDKA;1 protein. In contrast, only a very limited amount of the total Nicta;CDKB1;1 protein was found in the flow-through fraction and it eluted after gel filtration as a single peak of approximately 200 kDa, co-migrating with the peak of kinase

activity (data not shown). Thus, the pool of monomeric Nicta;CDKB1;1 is very small compared with that of Nicta;CDKA;1. Our data demonstrate that large amounts of both Nicta;CDKA;1 and Nicta;CDKB1;1 are kept in inactive forms, albeit by different means.

Expression of the Mutant CDKB1;1-D161N *in Tobacco Down-Regulates Endogenous Nicta;CDKB1;1 Activity*

The residue D161 of *Arabidopsis* CDKB1;1 belongs to the triad of catalytic residues that are conserved in all eukaryotic protein kinases (R-33, E-51, and D-145 in the human CDK2) and are strictly required for the kinase activity by providing vital contacts for correct ATP orientation and Mg^{2+} coordination (Jeffrey et al., 1995). The mutation of the D residue to N results in loss of activity and, in the case of CDKs has, upon overproduction, a dominant negative effect (Labib et al., 1993; van den Heuvel and Harlow, 1993), presumably because of the competition of the mutant proteins for the association with the rate-limiting interacting proteins, such as cyclins. As it is well known, orthologous CDKs, even from very divergent species, are functionally interchangeable; hence, dominant-negative mutants are efficacious in heterologous systems (Hemerly et al., 1995). To see whether kinase-negative mutants of B-type CDKs are dominant negative as well, we generated transgenic tobacco plants that express the *D161N* mutant of *CDKB1;1* under control of a strong constitutive promoter (Fig. 4).

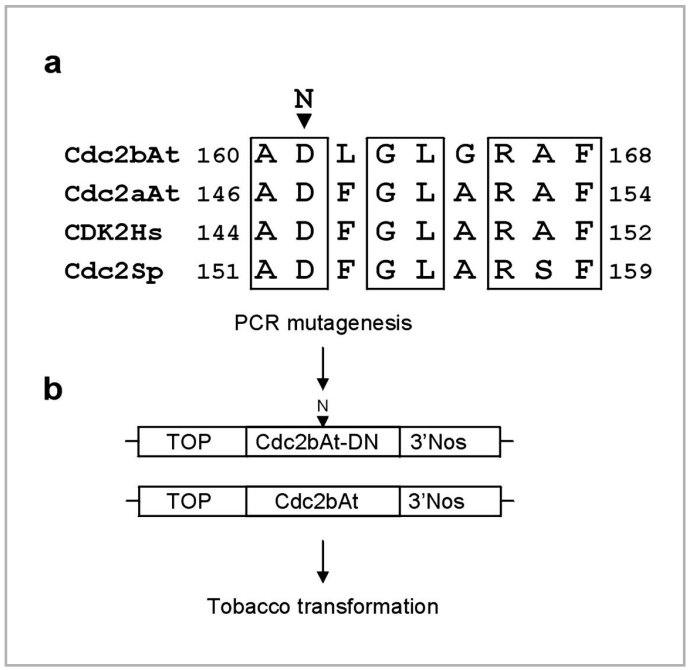

Figure 4. *Construction of transgenic tobacco expressing either CDKB1;1 or the CDKB1;1-D161N mutant.* ***A****, Aligned amino acid sequence of regions of the Arabidopsis (At) CDKB1;1 and CDKA;1, human (Hs) CDK2, and S. pombe (Sp) cdc2. The mutated residue in CDKB1;1 is indicated. Boxes are drawn around residues conserved in the four kinases.* ***B****, Schematic diagram of the expression cassettes used for transformation of tobacco SR-1 (see Experimental procedures). CDKB1;1-D161N mutant cDNA was created by PCR mutagenesis. Both the mutant and wild-type cDNAs were cloned under the control of the Triple-Op (TOP) promoter and introduced into tobacco plants (see Experimental procedures).*

We also produced tobacco plants expressing wild-type *CDKB1;1* under control of the same promoter. For 15 independent lines from each transformation, the level of the protein was analyzed in 2-week-old plantlets in the primary T0 transformants and in the self-fertilized T1 population. The level of CDKB1;1-D161N was consistently lower than that of CDKB1;1 (data not shown), suggesting that a higher level of CDKB1;1-D161N is incompatible with plant regeneration and/or development.

Two lines from both transformations that segregated the T-DNA as a single locus were selected for further analysis. We compared histone H1 kinase activity of protein complexes immunoprecipitated with CDKB1;1 antiserum in the transgenic and parental lines (Fig. 5). Production of the CDKB1;1-D161N protein correlated with an approximately 5-fold inhibition of the B-type CDK activity in both DN-1 and DN-27 lines (Fig. 5). In contrast, ectopic production of the wild-type CDKB1;1 (WT-14 and WT-23; Fig. 5) did not affect the corresponding kinase activity (in this case cumulative for Nicta;CDKB1;1 and CDKB1;1), even when produced to a much higher level than the mutant forms (WT-23). Effectively, these results also mean that at least 80% of the kinase activity in the immunoprecipitates is asociated with Nicta;CDKB1;1 and thus that cross-reacting contaminants (if any) contribute to less than 20% of the total activity.

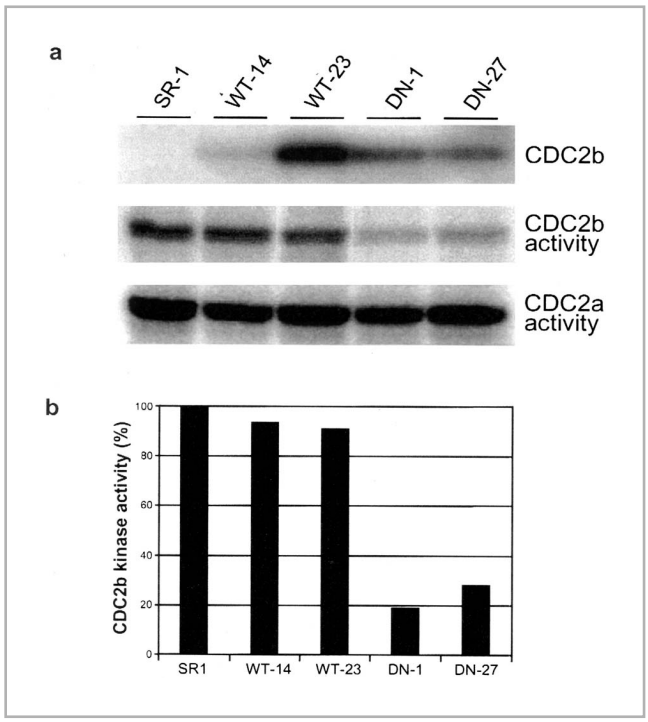

Figure 5. *Transgene expression and histone H1 activity in transgenic tobacco. Seedlings of transgenic plants expressing CDKB1;1 (lines WT14 and WT23) or CDKB1;1-D161N (lines DN-1 and DN-27) grown for 2 weeks on selective medium as compared with the SR-1 control of the same age.* ***A****, Protein gel blot analysis of total proteins with anti-CDKB1;1 antibodies (CDC2b) and the kinase activity in protein complexes immunoprecipitated either with anti-CDKB1;1 antibodies (CDC2b activity) or with anti-Nicta;CDKA;1 antibodies (CDC2a activity) (see Experimental procedures).* ***B****, Relative CDKB1;1 kinase activity estimated by quantification of the radioactively phosphorylated H1 protein.*

To see whether CDK activities other than B-type are affected in the transgenics, we analyzed histone H1 kinase activity in protein complexes either immunoprecipitated with Nicta;CDKA;1-specific antibodies or purified with $p13^{SUC1}$ affinity matrix ($p13^{SUC1}$ binds CDKA;1 but not CDKB1;1 in tobacco). In neither case, any reliable difference among the lines could be detected (Fig. 5a; data not shown).

Production of CDKB1;1-D161N Increases the Number of Cells in G2 Phase

To analyze whether the down-regulation of B-type CDK activity affects cell cycle progression, we compared the nuclear DNA content in segregating T1 plants grown on nonselective medium for 2 weeks. For each of the lines, 20 randomly chosen plants were analyzed. For each plant, half of one cotyledon was transferred to selective medium to identify the siblings that lost the transgene, while the other half was used to regenerate calli on nonselective medium. The regenerated calli and the other cotyledon were subjected to the flow cytometric analysis of nuclear DNA content. The cotyledons and calli were chosen as representatives of terminally differentiated and undifferentiated tissues, respectively. The fractions of 2C (G1 phase) and 4C (G2 phase) cells were determined for individual plants and the siblings with and without the T-DNA were compared. As shown in Figure 5b, the expression of *Nicta;CDKA;1-D161N* correlated with an approximately 2- and 1.5-fold increase in the 4C/2C ratio in cotyledons and calli, respectively. The ectopic expression of *CDKB1;1* did not modify the G2/G1 ratio. Thus, these data showed that ectopic expression of *CDKB1;1-D161N*, but not *CDKB1;1*, affects cell cycle in transgenic tobacco.

Ectopic Production of Either CDKB1;1 or CDKB1;1-D161N Does not Affect Plant Morphology

We further studied whether ectopic production of the CDKB1;1 protein and its D161N mutant affects the morphology of plants. T0 seeds were germinated on nonselective medium for 2 weeks and the phenotypes of the segregating populations of plants were compared. The plants ectopically expressing *CDKB1;1* showed no phenotypic alterations (WT-23; Fig. 6a). Neither were the plants expressing *CDKB1;1-D161N* affected, despite a 5-fold reduction of the B-type CDK activity (Fig. 6a, DN-1).

Microscopic analyses revealed no cytological modifications in any of the tissues analyzed, including epidermal cells in leaves and cotyledons and all the root cell files, nor particular modifications in the organization of the shoot and root meristems (the primary sources of all plant cells). Because the ploidy level and morphogenesis of seedlings is known to be under light control (Gendreau et al., 1997), we compared also seedlings grown in the dark and could not find any changes either. Furthermore, we did not observe any significant differences in the rate of *callus* regeneration from the cotyledons (data not shown).

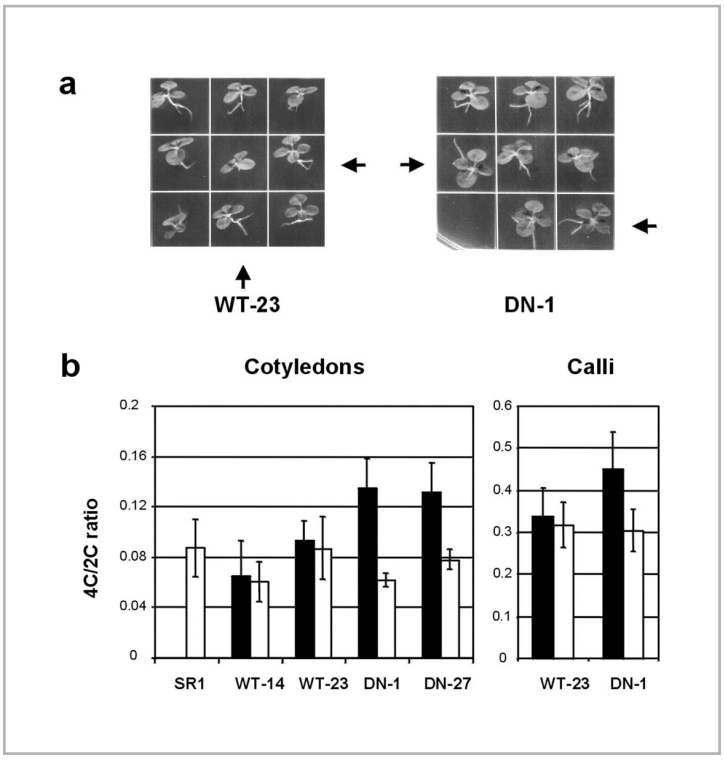

Figure 6. *Segregation analyses of transgenic plants expressing CDKB1;1 or CDKB1;1-D161N. Segregating populations of T1 plants grown on medium without selection were used for the analysis.* ***A****, The phenotypes of seedlings (representative lines WT-23 and DN-1) 2 weeks after in vitro germination. Arrows point to the siblings with no T-DNA. No morphological differences can be observed.* ***B****, Flow cytometric analysis of transgenic plants. Cotyledons of 2-week-old seedlings were subjected to flow cytometrical analysis (see Experimental procedures). Calli were regenerated from 2-week-old cotyledons and flow cytometrical experiments were performed after 2 weeks. For each line, 20 plants were analyzed individually and the results are the mean value for the particular lines, calculated separately for the siblings bearing T-DNA, both homo- and hemizygous (filled bars, transgenic lines only) and without T-DNA (open bars, SR-1 included).*

Discussion

In the present study, we have attempted to elucidate the function of the group of CDKs distinguished by a PPT(A/T)LRE motif, which are specific for plants (referred to as B-type; Segers et al., 1997). We identified a B-type CDK in tobacco on the basis of its similarity to the *Arabidopsis* CDKB1;1 protein, the founding member of the B-type CDKs (Hirayama et al., 1991). The single tobacco CDK characterized to date (Hashimoto et al., 1992) belongs to the A-group of plant CDKs (PSTAIRE-type). Here, we show that tobacco possesses a putative homologue of the CDKB1;1 kinase, recognized by a CDKB1;1-specific antibody and displaying the expression pattern characteristic of this type of CDKs. As shown before for the alfalfa PPTALRE kinase Medsa;CDKD;1 (CDC2MsD) (Magyar et al., 1997), the CDKB1;1Nt protein accumulates at the G2/M transition. Our unpublished results with partially synchronized *Arabidopsis* suspension cells, albeit not with the same degree of resolution, confirm that

the expression pattern of the tobacco CDK described here is also shared by the *Arabidopsis* homologue.

Along with PPTALRE kinases, plant B-type CDKs include PPTTLRE kinases, of which only alfalfa Medsa;CDKF;1 (CDC2MsF) has been analyzed so far in synchronized cells through the complete cell cycle. The protein level of this kinase reaches a maximum in mitosis distinctively later than PPTALRE CDKs (Magyar et al, 1997). This difference between PPTALRE and PPTTLRE kinases may reflect a general dichotomy of B-type CDKs.

The oscillation of the CDKB1;1 protein level during cell cycle suggests that it may be a rate-limiting factor for the activity, what is not typical for CDKs. To address this question, we have pursued three different approaches. First, we compared the temporal profiles of the CDKB1;1 protein and the associated CDK activity. The regulation of PPTALRE CDK-associated activity in the course of cell cycle had not been analyzed before, although it was shown that the protein complexes containing a PPTALRE CDK from rice possess histone H1 kinase activity *in vitro* (Umeda et al., 1999). Here, we demonstrate that the histone H1 kinase activity associated with Nicta;CDKB1;1 peaks in mitosis considerably later than the corresponding protein. This result indicates that post-translational mechanisms may control the B-type CDK activity. The timing of mitosis is almost universally controlled by the phosphorylation of Y15 in the PSTAIRE CDKs (Lew and Kornbluth, 1996). Taking into account that Nicta;CDKB1;1 activation occurs at the end of G2, it would be interesting to see whether this regulation is relevant for B-type CDKs. The conservation of Y15 in these CDKs supports this suggestion.

To see whether interaction with other proteins may regulate CDKB1;1 kinase activity, we have partially purified protein complexes that contain B-type CDKs from tobacco BY-2 suspension cells. Our results demonstrate that monomeric B-type CDKs (fractions 20-21; Fig. 3) have negligible kinase activity, whereas the highest activity was found in the 70-80 kDa fractions (fractions 17-18; Fig. 3) containing a comparable amount of the Nicta;CDKB1;1 protein. This implies that B-type CDKs require an activating subunit with a molecular mass of approximately 40-50 kDa. Even though the composition of the protein complexes is unknown, our results indicate that plant B-type CDKs may depend on additional components (most probably cyclins) for their full kinase activity.

It is worth noting that a high amount of the CDKB1;1 protein in tobacco was sequestered in high-molecular mass complexes (particularly 160-200 kDa and approximately 530 kDa) almost devoid of histone H1 kinase activity (Fig. 3). Recently, Yoshizumi *et al.* (Yoshizumi et al., 1999) presented evidence that *Arabidopsis* CDKB1;1 plays a role in regulating seedling growth in the dark. It is tempting to speculate that the sequestering of Nicta;CDKB1;1 in the inactive high-molecular mass complexes is due to complete inhibition of the corresponding developmental pathways in the dedifferentiated BY-2 cell. At the same time it is important to stress that histone H1 may not be a physiological substrate of Nicta;CDKB1;1. The high-molecular mass complexes we have detected, while being inactive towards histone H1, may nevertheless possess high activity towards the natural substrates, still to be identified.

Finally, we overexpressed *CDKB1;1* in transgenic tobacco under control of a strong constitutive promoter to see whether the cell cycle modulated production of B-type CDKs is fundamental for the regulation of the associated activity. In none of the transgenic lines could a significant increase in B-type CDK activity be found in spite of the high protein level. This result clearly demonstrates that, like for most other CDKs, the protein level of B-type CDKs is not a limiting factor for their activity. Correspondingly, plants overproducing CDKB1;1 show no discernible phenotype. Although the results of CDKB1;1 overproduction might be simply accounted for by assuming that CDKB1;1 does not interact with tobacco cyclins, the dominant-negative effect of the *CDKB1;1-D161N* mutant argues strongly against this interpretation (see below).

The above results negate the major argument that has been recurrently invoked to substantiate a cell cycle function of plant B-type CDKs (i.e. the cycling expression) and leaves us with the question whether they are involved in cell cycle control at all. To approach it, we resorted to kinase null mutants that had proved to be a useful tool to dissect the function of CDKs (Labib et al., 1995; van de Heuvel and Harlow, 1993; Meyer et al., 2000). Kinase-inactive mutants of CDKs have a dominant-negative effect, presumably because of the competition of the mutant proteins for the association with the rate-limiting interacting proteins, such as cyclins. We have generated a number of transgenic lines expressing the kinase null mutant *CDKB1;1-D161N* to analyze its impact on the phenotypes and cell cycle progression. We have found that in the lines with highest expression levels, the Nicta;CDKB1;1-associated kinase activity is reduced approximately by a factor of 5. Importantly, the effect was specific for Nicta;CDKB1;1-associated activity and exerted only by the mutant, but not the wild-type, protein. This result indicates that the mutant protein can titrate out a rate-limiting protein required for Nicta;CDKB1;1 activity, whereas the complexes formed by wild-type CDKB1;1 are fully active in tobacco cells. These results strongly augment the above biochemical evidence for an activating subunit of B-type CDKs.

The relatively high residual B-type CDK activity in the *CDKB1;1-D161N* transgenic plants suggests that lines with higher expression levels have been counterselected in the process of transformation, indicating that B-type CDKs may have an essential function. To get insight into that function, we analyzed these transgenic plants at the level of morphology, cytology, and cell cycle progression. While morphologically and cytologically no difference could be observed, the transgenic lines with a decreased level of CDKB1;1 activity showed a considerable increase in the size of G2 cell population.

Depending on the type of cells analyzed, our results have different implications. In the rapidly dividing *callus* cells, the increase in the G2 cell population means that the progression through and/or the exit from the G2 was compromised. In case of cotyledons, composed mainly of nondividing cells, the higher proportion of G2 cells indicates that more cells exited cell cycle in the G2 phase in the course of embryogenesis. The apparently weaker impact of the mutation in *callus* than in cotyledons (approximately 50% vs 100% G2 increase) is probably a consequence of the much higher representation of G2 cells in the *callus* than in cotyledons (approximately 4-5 fold). Given that a similar

effect has been observed in tissues as different as cotyledons and *callus*, we expect this observation to be rather general for any tissue.

We believe that the observed changes in the cell cycle progression are attributable to the down-regulation of Nicta;CDKB1;1 activity and that this activity is involved in G2/M progression. The molecular mechanism of CDKB1;1 action in the cell cycle is still to be elucidated. It is difficult to completely exclude at present the possibility that the mutant protein perturbs cell cycle indirectly for example interfering with the function of an important regulator of G2/M progression unrelated to CDKB1;1. However, this is a very improbable scenario, because no changes were observed in the case of overproduction of the wild-type protein; this means that even if the *Arabidopsis* protein (no matter wild type or mutant) is able to titrate out an essential regulatory molecule in tobacco cells, the complexes formed by CDKB1;1 with that molecule fully substitute for the endogenous counterparts and thus fulfill the same function.

While this work was in progress, Yoshizumi *et al*. (Yoshizumi *et al*., 1999) described transgenic *Arabidopsis* plants with the expression of *CDKB1;1* gene inhibited by means of antisense technology. The expression of the antisense construct correlated with short hypocotyl and open cotyledon phenotypes when transgenic seedlings were grown in the dark. We did not observe this type of changes in our transgenic tobacco lines. On the other hand, no changes in the DNA content were detected in the CDKB1;1 antisense *Arabidopsis* plants. One of the probable explanations for the discrepancy is the presence of residual B-type kinase activity in our transgenic lines, which is presumably sufficient to enable normal developmental programs in the dark. However, the comparison between the results of Yoshizumi *et al*. (Yoshizumi *et al*., 1999) and ours is compounded by the major differences in experimental set-up. First of all, different technologies were used in the two studies and it is unknown to what extent CDKB1;1 activity was influenced by the production of antisense RNA. Plants are notorious for having multiple paralogous genes and this seems to be the case for CDKB1;1 both in *Arabidopsis* and tobacco. The implication is that the antisense technology, being rather DNA sequence specific, most probably inactivated only one of the paralogues, without altering the activity of the others. In contrast, the use of dominant-negative mutants is expected to affect the activity of all the paralogous proteins. Second, different organs were used to analyze the DNA contents. Finally, the specifics of the plant species used may have also affected the outcome. The results of the two works may be rather complementary than contradictory. Obviously, many more experiments are required to define more precisely the functions of this plant-specific type of kinases.

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Chapter 4

A plant-specific CDK is involved in stomatal development in *Arabidopsis*

Adapted from: "A plant specific CDK is involved in stomatal development in *Arabidopsis*" by Véronique Boudolf, Rosa Maria Barrôco*, Dirk Inzé and Lieven de Veylder. In preparation.

(*)contributed to the present work with the expression analysis data and the preparation of the manuscript as it stands.

Abstract

Cyclin-dependent kinases (CDKs) are keys players in the coordination of cell cycle. Although, CDKs are highly conserved among eukaryotes, plants have developed a unique class of CDKs, the B-type CDKs. These kinases are believed to regulate plant specific cell division events, but till now, the specificity of their function is not understood. A dominant negative form of a B-type CDK was constitutively overexpressed, in order to elucidate the role of these plant specific kinases in plant development. The transgenic plants present an overall growth reduction. The leaves and cotyledons were smaller, and they contain fewer but significantly larger cells. Also ploidy levels were increased. Surprisingly the stomatal index was strongly reduced. Furthermore, the cotyledons showed aberrant stomatal morphology, displaying a moon-shape phenotype, as revealed by electron scanning microscopy. A role for B-type CDKs in stomatal development is corroborated by expression studies, that showed a highly specific *CDKB1*;1 transcript localization in stomata and stomatal precursors. More detailed analysis of the CDKB1;1 dominant negative transgenic plants revealed also reduced lateral root formation, suggesting a possible role for CDKB1;1 in asymmetric cell division, necessary for the formation of stomata and lateral root primordia.

Key words: *Arabidopsis thaliana*, B-type cyclin-dependent kinases (B-type CDKs), cell cycle, stomata development.

Abbreviations: CDK, cyclin-dependent protein kinase; GCs, guard cells; MMC, meristemoid mother cells; M, meristemoids; GMC, guard mother cells; PCR, polymerase chain reaction; SCs, subsidiary cells.

Introduction

Stomata are essential for plant survival, and the evolution of the genes necessary for the stomatal development was a key event in the emergence of land plants. The stoma is an epidermal structure responsible for the regulation of gas and water vapor exchange between the plant and the atmosphere. A pair of stomatal guard cells responds to short-term atmospheric changes by controlling the size of the stomatal pores. Gas exchange can also be influenced by the adjustment of the stomatal density during the leaf development depending on the environmental conditions.

Studying the development and differentiation of stomatal complexes proved invaluable for the understanding of cellular processes in plants. In *Arabidopsis*, stomata formation has been used as a model system to study the genetic and molecular control of cell fate specification and pattern formation. In this context, key elements associated with cell differentiation, such as nuclear migration, cytoskeleton rearrangements and asymmetric cell division have been investigated (von Groll and Altmann, 2001; Zhao and Sack, 1999).

The stoma consists of a pair of guard cells (GCs) that delimit the stomatal pore, and is surrounded by a number of subsidiary cells (SCs). In *Arabidopsis*, the stomata are derived from a sequence of unequal divisions and the guard cells are surrounded by three unequally-sized SCs, which defines anisocytic stomatal complexes (Berger and Altmann, 2000; Serna and Fenoll, 2000). The formation of stomata results from a series of predictable divisions with a well-defined geometry (reviewed by Serna and Fenoll, 2000). Three successive types of stomatal precursor cells are involved: meristemoid mother cells (MMC), meristemoids (M) and guard mother cells (GMC). The first two divide asymmetrically, whereas GMC divide symmetrically. In *Arabidopsis*, stomatal formation begins with an unequal cell division of a protodermal cell (MMC), resulting in the formation of one (first) subsidiary cell and the meristemoid. The meristemoid undergoes up to two further asymmetric divisions, resulting in the formation of a centrally located cell, which acquires a new shape and assumes guard mother cell identity (Zhao and Sack, 1999). Morphological and biochemical changes are observed during conversion of a meristemoid into a GMC. Meristemoids are more triangular and have evenly thickened walls, whereas GMCs have a more spherical or oval shape, increased starch accumulation, and thickened cell walls on the opposite ends of the cells (Zhao and Sack, 1999).

The GMC divides symmetrically giving rise to two equal-sized guard cells, producing two GCs. The neighbouring SCs, predominantly the most recently formed, have the potential to divide asymmetrically to form SMs (subsidiary or satellite meristemoids). SMs may undergo up to two further asymmetric divisions, thereby producing secondary anisocytic stomatal complexes (Serna et al., 2002). The newly formed meristemoids are usually positioned away from the pre-existing stoma, preventing the formation of stomatal clusters (stomata placed in direct contact). Therefore, proper stomatal complex formation implies mechanisms regulating the correct orientation of the subsidiary cell plane of division, to avoid intersection of the new cell plate with the existing stoma. A mechanism is necessary to determine the cell fate of the two daughter cells, so that the cell placed

away from the stoma acquires meristemoid identity (Yang and Sack, 1995; Serna and Fenoll, 2002; von Groll and Altmann, 2001). Three *Arabidopsis* mutants, *too many mouths* (*tmm*), *stomatal density and distribution1-1* (*sdd1-1*) and *four lips (flp),* have largely contributed for the understanding of the cellular events associated to the formation of stomatal complexes and the establishment of the stomatal pattern (Yang and Sack, 1995; Berger and Altmann, 2000; Geisler et al., 2000). Stomatal clusters occur in all three mutants, but the characteristics of the clusters formed in these mutants differ. TMM was found to encode a leucine-rich repeat containing receptor like protein expressed in stomatal precursors, both meristemoids and GMC (Nadeau and Sack, 2002). TMM controls the orientation of asymmetric divisions necessary for correct stomatal patterning and may also participate in regulating cell proliferation and differentiation based on positional context. SDD encodes a subtilisin-like serine protease generating an extracellular signal by meristemoids and GMCs and its function is dependent on TMM activity (von Groll et al., 2002). The two genes probably belong to the same signaling pathway. In contrast FLP appear to determine guard mother cell fate, and acts independently of the SDD1 pathway (Yang and Sack, 1995; von Groll et all, 2002).

The association of stomata to the cell division cycle has frequently been reported (reviewed by Croxdale, 2000; Geisler and Sack, 2002). In *Arabidopsis*, trichomes and epidermal cells can enter the endomitotic cycle, while stomata remain diploid (Melarango et al., 1993). Mature guard cells, although terminally differentiated, express genes associated with competence for cell division (Serna and Fenoll, 1997). This might explain why transgenic sugar beets were easily generated from guard cell protoplasts (Hall et al., 1996). Also isolated stomata are able to assume different fates and express different gene products when cultured (Taylor et al, 1998).

Geisler and Sack (2002) showed that in *Arabidopsis* the formation of stomatal precursor cells does not follow a steady-state kinetics. Early steps in the pathway are not necessarily synchronized, but later steps, such as the conversion of meristemoids into GMS, might be coordinated. Croxdale (2000) postulates that the targeting of cells to form stomata is related to the cell cycle. *Arabidopsis* cotyledons generate stomata in non-steady-state kinetics, meaning that the time of meristemoid formation does not correlate with its switch into a GMC (Geisler and Sack, 2002). This conversion into GMC seems to occur in partially synchronized waves. Croxdale (2000) proposes that cell cycle signals dictate whether a GMC is produced when a stomatal-inducing signal is present.

Our understanding of the cell division cycle is expanding, however little is known on the specificity of some typical plant cell cycle genes. Here we show that the plant specific B-type CDK, CDKB1;1, is specifically expressed in stomata of cotyledons and that the overexpression of a dominant negative form of CDKB1;1 has an aberrant effect on stomatal development.

Experimental Procedures

Expression analysis of pCDKB1;1::GUS transgenic plants

Cotyledons were collected everyday from seedlings that were sown at the same moment. Seeds were placed on wet germination paper or agar, transferred to darkness at 4°C overnight, and then moved to the light. Seedling age was scored starting from the moment that the seeds were placed under photoperiodic light.

The histochemical *GUS* assays were carried out according to standard protocols (Jefferson et al., 1987), with minor modifications. The young seedlings were incubated in 90% acetone for 2 h at 4°C. After washing in phosphate buffer, the material was immersed in the enzymatic reaction mixture (1 mg/mL of 5-bromo-4-chloro-3-indolyl ß-D-glucuronide, 2 mM ferricyanide, and 0.5 mM of ferrocyanide in 100 mM phosphate buffer, pH 7.4). The reaction was carried out at 37°C in the dark for a period of 4 h to overnight. Upon reaction, the material was cleared with chlorolactophenol (chloral hydrate/phenol/lactic acid 2:1:1) and observed under light microscope or stereoscope.

Regeneration and molecular analysis of CDKB1;1- and CDKB1;1.N161-overexpressing plants

The cDNA for *CDKB1;1.N161* was obtained by mutating the codon GAT (Asp-161) for AAT (Asn-161) by in vitro mutagenesis (Porceddu *et al*., 1999). The *CDKB1;1* and *CDKB1;1.N161* coding regions were amplified by PCR with the 5'-GGCCATGGAGAAGTACGAGAAGC-3' and 5'-GGGGATCCTCAGAACTGAGACTTGTCAAGG-3' primers. The obtained PCR fragments were digested with *Nco*I and *Bam*HI. Subsequently, the restriction fragments were cloned between the *CaMV35S*-promoter and the nopaline synthase (*NOS*) 3'untranslated region in the *Nco*I and *Bam*HI sites of PH35S (Hemerly *et al*., 1995), resulting in the PH35SCDKB1;1 and PH35SCDKB1;1.N161 vectors. The *CaMV35S/CDKB1;1/NOS* and *CaMV35S/CDKB1;1.N161/NOS* cassettes were released by *Eco*RI and *Sal*I and cloned into the *Eco*RI and *Sal*I sites of pBinPLUS (van Engelen *et al*., 1995), resulting in the pBINCDKB1;1 and pBINCDKB1;1.N161 vector. Both pBINCDKB1;1 and pBINCDKB1;1.N161 were mobilized by the helper plasmid pRK2013 into the *Agrobacterium tumefaciens* C58C1Rif^R harboring the plasmid pMP90 (Koncz and Schell, 1986). *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia was transformed by the floral dip method (Clough and Bent, 1998). Transgenic *CaMV35S-CDKB1;1* and *CaMV35S-CDKB1;1.N161* plants were obtained on kanamicin-containing medium. For all analyses, plants were grown under a 16h light/8h dark photoperiod at 22°C on germination medium (Valvekens *et al*., 1988). Molecular analysis of the obtained transformants was performed by protein gel blotting, and cyclin dependent kinase (CDK) activity measurements were done as described by De Veylder *et al*. (1997).

Histological analysis of leaf and cotyledon abaxial epidermis

Untransformed and transgenic plants were grown on the same petri dish to exclude different growth conditions. To exclude errors resulting from discrepancies in germination timing, seedlings that germinate at the same time were marked and harvested 3 weeks after sowing. Leaves and cotyledons were placed overnight in ethanol to remove chlorophyll, and subsequently they were cleared and stored in lactic acid for microscopy. Cell density and stomatal index were determined as described by De Veylder *et al*. (2001).

For scanning electron microscopy, the cotyledons from 3-week-old seedlings were processed and analyzed as described by De Veylder *et al*. (2002).

Fluorescent staining of nuclei was performed by fixing 3-week-old cotyledons in a mixture ethanol/acetic acid 9:1 (v/v). After the rinsing, the samples were stained for 24 h with 0.1 μg/ml DAPI and mounted in Vectashield® Mounting medium (Vector Laboratories). The samples were observed with a 63x oil immersion objective on a Zeiss Axioskop equipped with an Axiocam CCD camera (Carl Zeiss Ltd, Welwyn Garden City, UK). Images were acquired using the Axiovision software and were analyzed in grayscale using the public domain image analysis program ImageJ (version 1.28; http://rsb.info.nih.gov/ij/). Nuclei to be analyzed were individually identified as objects by thresholding manually to a range that isolate nuclei from the background image. Relative fluorescence units (RFU) were reported as integrated density, which represent the product of the area and the average fluorescence of the selected nucleus.

Results and discussion

CDKB1;1 promoter activity in cotyledons is associated with stomata formation

Previously, *in situ* hybridisation experiments have showed that Arath;*CDKB1*;1 transcripts accumulate in a patchy pattern. This patchy distribution pattern reflects the expression of *CDKB1*;1 in a cell-cycle-dependent manner (Segers et al., 1996). Also CDKB1;1 protein levels were found to oscillate during the cell cycle, and CDKB1;1 activity was suggested to be rate-limiting for G2/M transition (Porceddu et al., 2001). To further determine the role of CDKB1;1 in plant cell division, we have searched for *CDKB1*;1 promoter activity in cotyledons of transgenic plants carrying promoter-*gus* fusions.

Frindler et al., (1996) reported the absence of cell division in cotyledons till radicle has emerged. The same authors report a mitotic index of about 2% following germination, and a rapid decline in cell division about one week after sowing. These rates of division coincide with those resulting from stomata formation described by Geisler and Sack (2002). Together, these studies illustrate that post-germinative cell proliferation in *Arabidopsis* cotyledons is nearly exclusive associated with the formation of stomata, making this plant tissue particularly interesting for cell cycle associated expression studies. Obviously, stomata can also be easily identified in leaves, but young leaves present themselves large rates of cell division. The identification of cell division

associated to stomata formation is therefore more difficult in dividing leaf epidermis than in a non-dividing cotyledon. Moreover, stomatal formation in *Arabidopsis* leaves follows a tip-to-base gradient, whereas in cotyledons stomata formation is more uniformly distributed.

Histochemical analysis of p*CDKB1;1*::*gus* transgenic plants revealed that GUS activity in cotyledons of young seedlings is almost exclusively confined to stomata and stomatal precursors (meristemoids and guard mother cells). As only exception, reporter gene expression was also observed in the vascular tissue of cotyledons from young seedlings (Fig. 1A).

Meristemoids were identified based on their small size, roughly triangular shape and the pattern of surrounding cell walls, indicating an apparent asymmetric division (Fig. 1B). Mature GMCs were recognized as being larger and more rounded than meristemoids (Fig. 1C). Newly formed stomata can be distinguished from mature GMCs by the presence of a thin dividing wall.

GUS expression is observed in both the meristemoids and the guard mother cells (Fig. 1B and 1C). Highest GUS expression levels are found in GMCs. In contrast, we also have found GMCs with very low GUS activity (Fig. 1C). We postulate that after GMC formation GUS activity slowly disappears. When GMC division is about to occur, promoter activity is strongly increased resulting in the *de novo* synthesis of large transcript amount, which ends with the formation of guard cells. If this hypothesis is correct, *CDKB1;1* expression might respond to signals determining the timing of guard cell formation. Alternatively, we can postulate that *CDKB1;1* transcript level is essential to determine the timing of guard cell formation, since observed GMC divisions were always accompanied by strong GUS expression.

Strong GUS activity can be also observed in already fully formed guard cells (Fig. 1D) but stain intensity seems to slowly disappear following division. In seedlings older than 10 days GUS expression becomes restricted to a few remaining stomatal precursors. A large number of mature stomata can be observed but GUS activity is nearly absent. Although, the presence of non-stained guard cells is evident in older seedlings, they also can be observed in young seedlings (Fig. 1F), showing that stomata formation is indeed not temporally synchronized.

As described, GUS expression is present throughout the formation of the stomata. Additionally, GUS activity is also observed in subsidiary meristemoids (Fig. 1E), as well as during its development into stomatal complexes. The newly formed meristemoids originate from the asymmetrical division of subsidiary cells, resulting in the development of a new stoma. Occasionally, GUS activity can be observed in subsidiary cells (data not shown). Therefore, it can be postulated that reporter gene expression occurs already in subsidiary cells, which will give rise to secondary stomata.

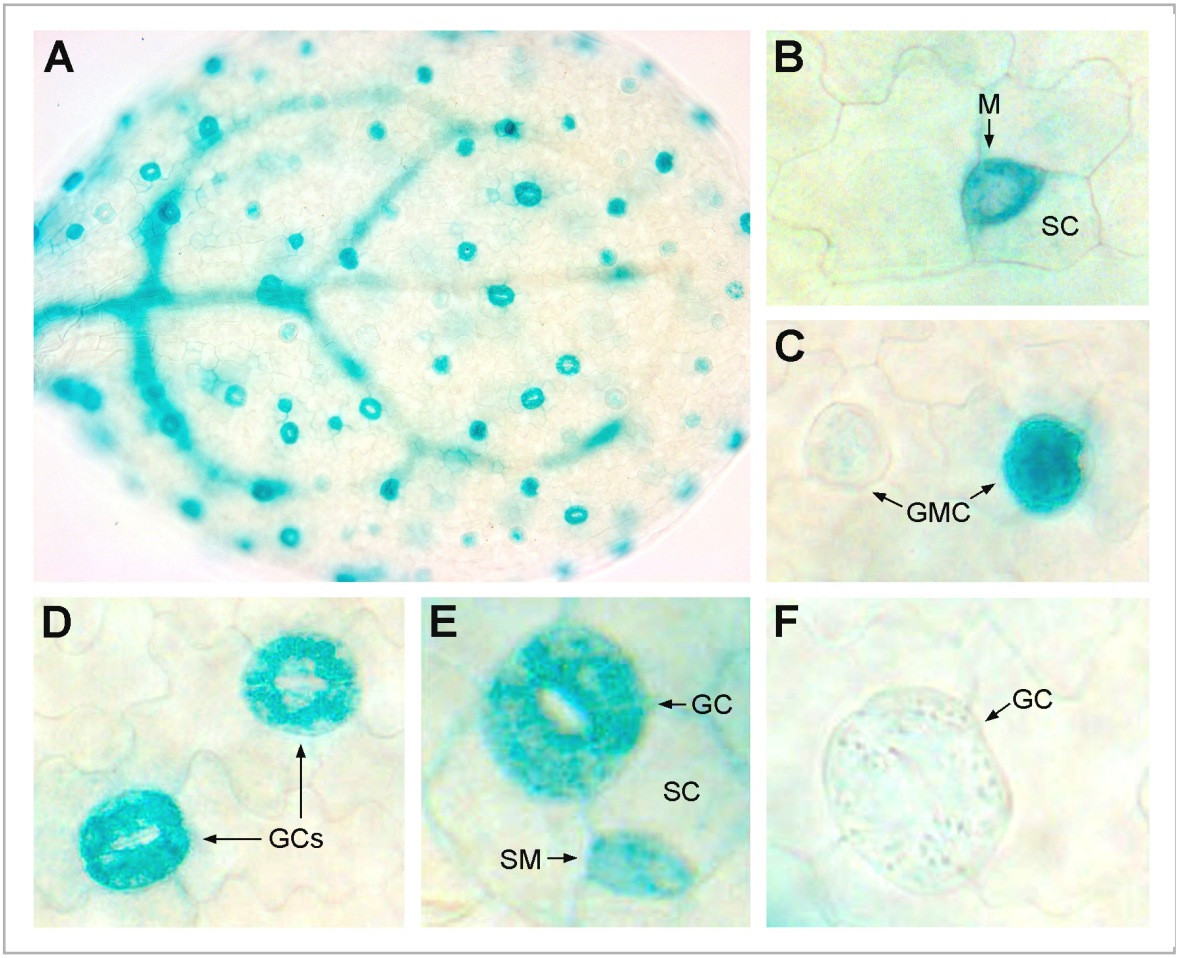

Figure 1. *Profile of CDKB1;1 promoter activity in* Arabidopsis *cotyledons, as revealed by GUS histochemical assays.* ***A****, GUS expression profile in a five day-old cotyledon. GUS activity is restricted to stomata and stomatal precursors, together with the developing vascular tissue. GUS staining is observed throughout stomatal development.* ***B****, GUS-expressing meristemoid cell (M). In neighboring subsidiary cell (SC) expression is absent.* ***C****, Guard mother cells (GMC), with and without GUS expression.* ***D****, Guard cells (GCs) after GMC division.* ***E****, Stomata and subsidiary meristemoid (SM) cell during stomatal complex formation.* ***F****, Mature stomata after GUS activity has disappeared.*

The analysis of mutants like *sdd1* and *tmm* provided evidence that stomatal spacing is under genetic control. However, the fate and activity of the subsidiary cells is probably not only controlled by the action of SDD1 and TMM proteins. Some other early factors may determine which neighboring cells undergo secondary stomatal complex formation. The existence of such factors is supported by the observation of promoter activity of genes that mark competence of cells for division (*cdc2aAt* renamed *CDKA*;1) and mitotic activity (*cyc1aAt* renamed *CYCB1*;1) (Serna and Fenoll, 1997). The promoter activity of these genes during stomatal development in leaves was assayed using GUS as reporter gene. The results showed that in leaves both promoters are not only active in meristemoids and guard mother cells, but also in small subsidiary cells, which presumably will initiate secondary stomatal complex formation (Serna and Fenoll, 1997).

The promoter activity of *CDKA*;1 and *CYCB1*;1 genes in cotyledons, and the possible role of these genes in stomata formation was also investigated (data not shown). Promoter activity of *CDKA*;1 and *CYCB1*;1 was not specific to any particular cell type in the epidermis of cotyledons. GUS expression associated to those two genes was spread overall in the epidermis, and was visibly identical in stomata and pavement cells. On the contrary and as described above, GUS expression associated with *CDKB1*;1 gene was in cotyledons restricted to stomatal precursors and stomata. The differential expression of the three genes further emphasizes the possible involvement of CDKB1;1 in stomata formation.

GUS expression in stomatal cells seems to be initiated very early during development. Immediately after root protrusion, strong GUS activity could be detected in the stomatal precursors present in the cotyledons. At this stage fully developed stomata were nearly absent but a significant number of GMCs could already be observed. Curiously, the timing of stomata formation in the abaxial and adaxial epidermis appears to be very different. In very young cotyledons, stomatal precursors (meristemoids or GMCs) are almost restricted to the adaxial side, however stomatal precursor cell formation stops much earlier in this side of the epidermis. Precursor cells are no longer present in the adaxial epidermis of one-week older cotyledons. On the contrary, reporter gene expression could still be observed in the abaxial stomatal precursor cells of cotyledons from 12 days-old seedlings. Additionally, it is clear that abaxial epidermis forms many more stomata than the adaxial side. Thus, the cotyledon abaxial and adaxial epidermis present clear differences in the rate of stomata formation, as well as discrepancies in the temporal production of precursor cells. These data highlight the non-uniformity in the number and timing of stomata formation between both sides of the cotyledon.

The stomatal number seems to dependent on the temporal regulation of precursor cell formation and its developmental progression (Geisler and Sack, 2002). Whether this temporal regulation is dependent on cell cycle activators, either directly or in response to external stimuli, still needs to be investigated.

CDKB1;1.N161 plants display an increased cell size and decreased stomatal index

In order to elucidate the role of CDKB1;1 in plant cell division, we overproduced a dominant negative form of *CDKB1;1*, *CDKB1;1.N161*, in *Arabidopsis thaliana* under control of the constitutive cauliflower mosaic virus (*CaMV*) *35S* promoter. *CDKB1;1.N161* contains a substitution of the residue D161 to N161, resulting in loss of kinase activity by misorienting the Mg^{2+}-ATP complex (Hemerly et al., 1995; Porceddu et al.,2001). We also produced *Arabidopsis* plants producing the wild type CDKB1;1 under the same promoter. Out of multiple transgenic lines, two independent lines *CaMV35S-CDKB1;1* and two *CaMV35S-CDKB1;1.N161* lines were selected, containing only one T-DNA locus and in which the protein levels exceeded those found in untransformed plants (Fig 2A). Overexpression has no effect on the level of the CDKA;1 protein. The presence of the

CDKB1;1.N161 recombinant protein correlated with a decrease in extractable CDK activity, reflecting the expected dominant negative effect (Fig. 2B).

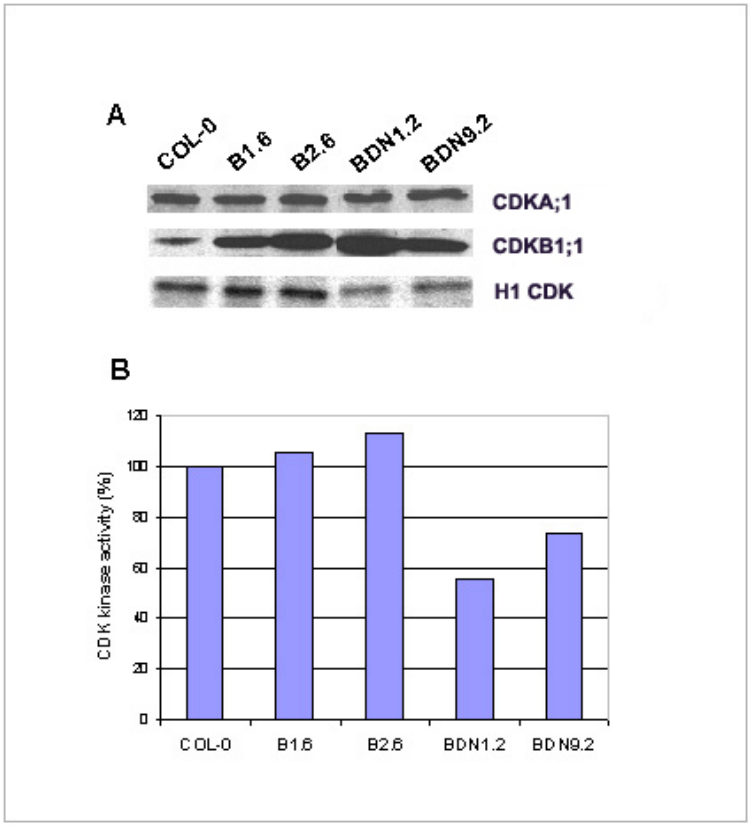

Figure 2. *Transgene expression and histone H1 activity in transgenic* Arabidopsis. *Seven-day-old seedlings of transgenic plants expressing CDKB1;1 (lines 1.6 and 2.6) or CDKB1;1.N161 (lines DN1.2 and DN9.2) are compared with the Col-0 control of the same age.* ***A****, Protein gel blot analysis of total proteins with anti-CDKB1;1 and anti-CDKA;1 antibodies and the H1 kinase activity (H1 CDK) on protein complexes purified with P10^{CKS} beads.* ***B****, Relative CDK kinase activity estimated by quantification of the radioactively phosphorylated H1 protein. For better graphic visualization the value relative to CDK kinase activity of COL-0 was arbitrarily represented as 100%.*

The transgenic *Arabidopsis* plants overexpressing the CDKB1;1 protein were morphological similar to non-transgenic controls and non-transgenic segregating siblings, however CDKB1;1.N161 transgenic plants showed an overall growth reduction (Fig. 3).

The leaves and cotyledons of CDKB1;1.N161 transgenics were smaller, containing less but larger cells (Fig. 4A and 4B). The total number of epidermal cells in the abaxial epidermis decreased up to 75% in the cotyledons (Fig. 4C) and 43% in the first leaf pair (data not shown). The increase in cell size only partially compensated for the decrease in cell number, and that justifies that the mutant plants were smaller. Furthermore CDKB1;1.N161 cotyledons exhibit up to 66% decrease in the stomatal index (Fig. 4D). This can explain the important decrease in total cell number, as stomatal lineages produce almost 70% of all pavement cells in cotyledons and 50% in leaves (Geisler et al., 2000).

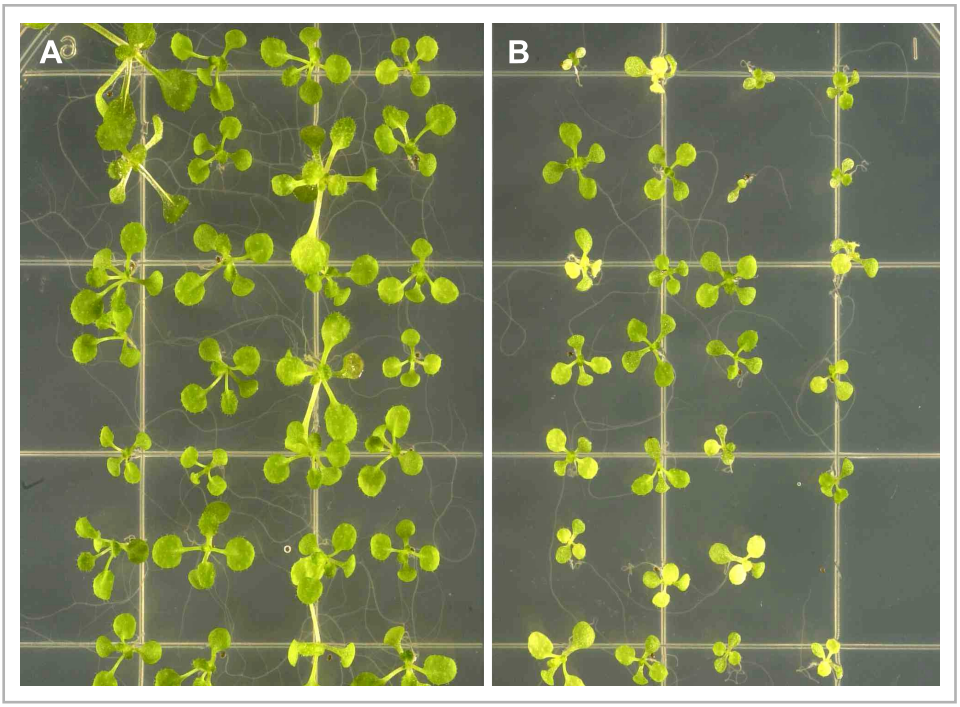

Figure 3. *Phenotype of CDKB1;1.N161 overexpressing 16-day-old seedlings.* ***A****, Untransformed control.* ***B****, CDKB1;1.N161 overexpressing plants.*

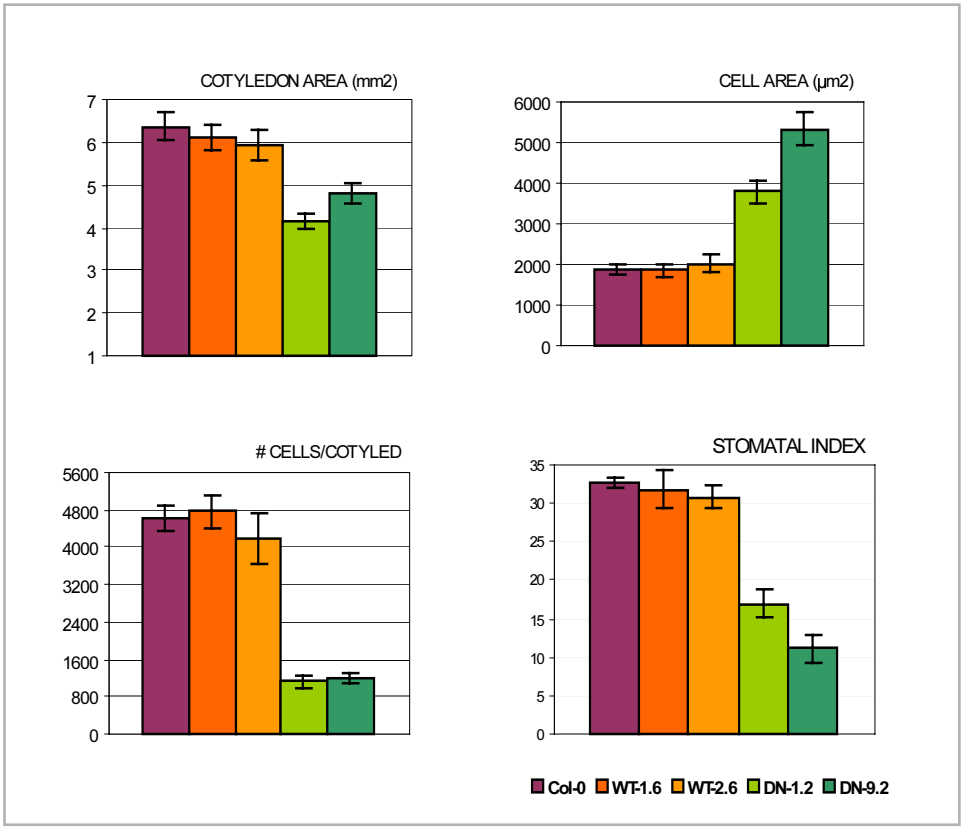

Figure 3. *Quantitative analysis of the abaxial epidermal cells of the cotyledons from CDKB1;1 transgenic plants vs. wild-type.* ***A****, Cotyledon area (mm2);* ***B****, Average cell area (um2);* ***C****, Epidermal cell number on the abaxial side of the cotyledon;* ***D****, Stomatal index. Error bars denote standard errors (n=6 to 10).*

CDKB1;1 activity is required for stomata formation but not for its differentiation

Microscopic analysis also revealed an altered stomatal development in the CDKB1;1.N161 transgenic plants, mainly in the cotyledons (Fig. 5), but also in the leaves (data not shown). In cotyledons, up to 58.5% of the stomata displayed an aberrant morphology, showing a moon-shape appearance (Fig. 5 and Fig. 6).

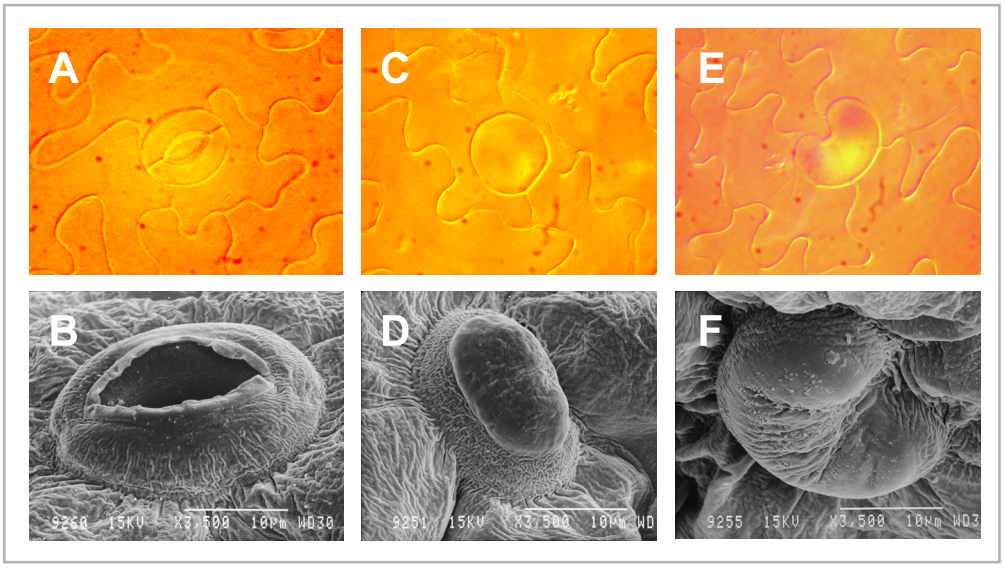

Figure 5. *Microscopic analysis of CDKB1;1.N161 overexpressing* Arabidopsis *plants.* ***A and B****, Normal stomata;* ***C to F****, aberrant stomata.* ***A,C,E****, Bright-field micrographs;* ***B,D,F****, Scanning electron microscopy micrographs.*

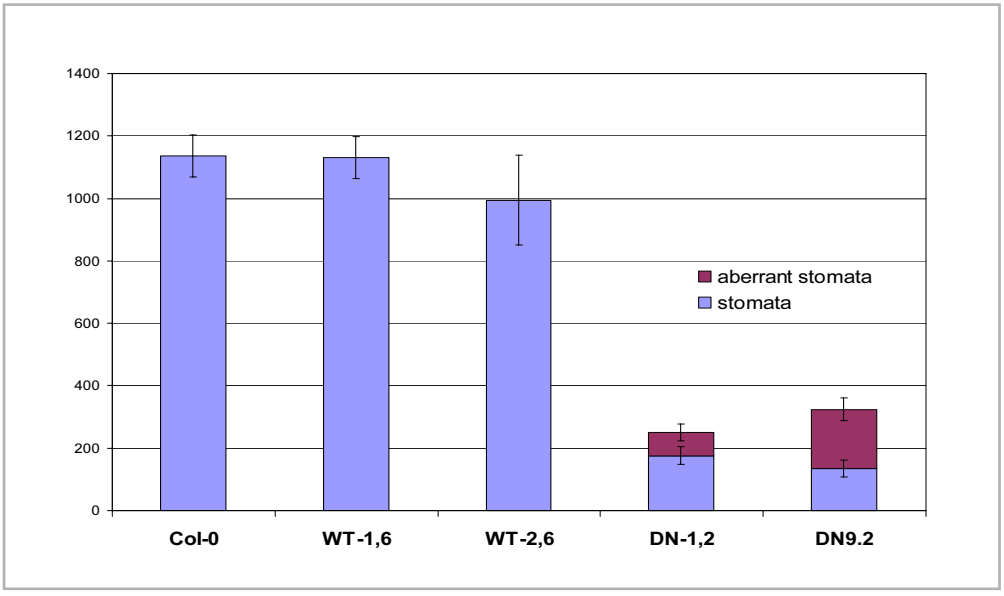

Figure 6. *Total and aberrant stomata on the abaxial epidermis of cotyledons from 3-week-old seedlings expressing CDKB1;1 (lines 1.6 and 2.6) or CDKB1;1.N161 (lines DN1.2 and DN9.2) compared to a Col-0 control of the same age.*

On the other side, electron scanning microscopy observations showed that some of the anomalous stomata present cell wall features resembling the initiation of cytokinesis (Fig. 7). These aberrant stomata possess central thickenings (Fig. 7B to 7D) similar to the ones giving rise to the stomatal pore (Fig. 7A). These observations suggest that guard cell identity does not rely on the activity of CDKB1;1 and thus, final stomatal differentiation might be independent from cell cycle activity.

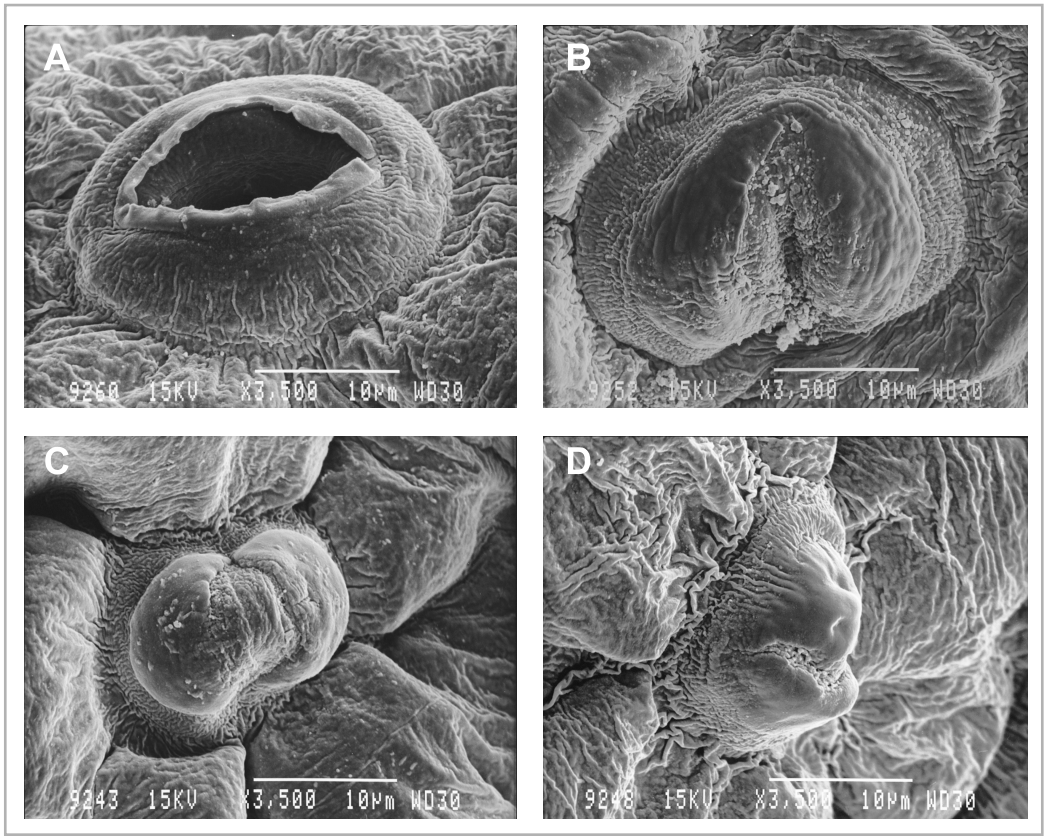

***Figure 7**. Scanning electron microscope micrographs of normal stomata (**A**) and aberrant stomata (**B-D**) on cotyledons of CDKB1;1.N161 transgenics.*

CDKB1;1.N161 aberrant stomata are blocked in G2 phase

Because CDKB1;1 is required for G2/M progression in *Arabidopsis*, the effect of CDKB1;1.N161 overexpression on cell cycle progression was analyzed. Microscopic analysis (Fig. 8A and 8B) and quantification of DAPI stained cotyledons (Fig. 8C) showed that each abnormal stomata contained one large nuclei with a 4C content. All phenotypically normal stomata, either in transgenic or in wild-type plants, contained one 2C nuclei per guard cell. The asymmetric emplacement of the 4C nuclei might derive from a blocked asymmetric cell division (Fowler and Quatrano, 1997).

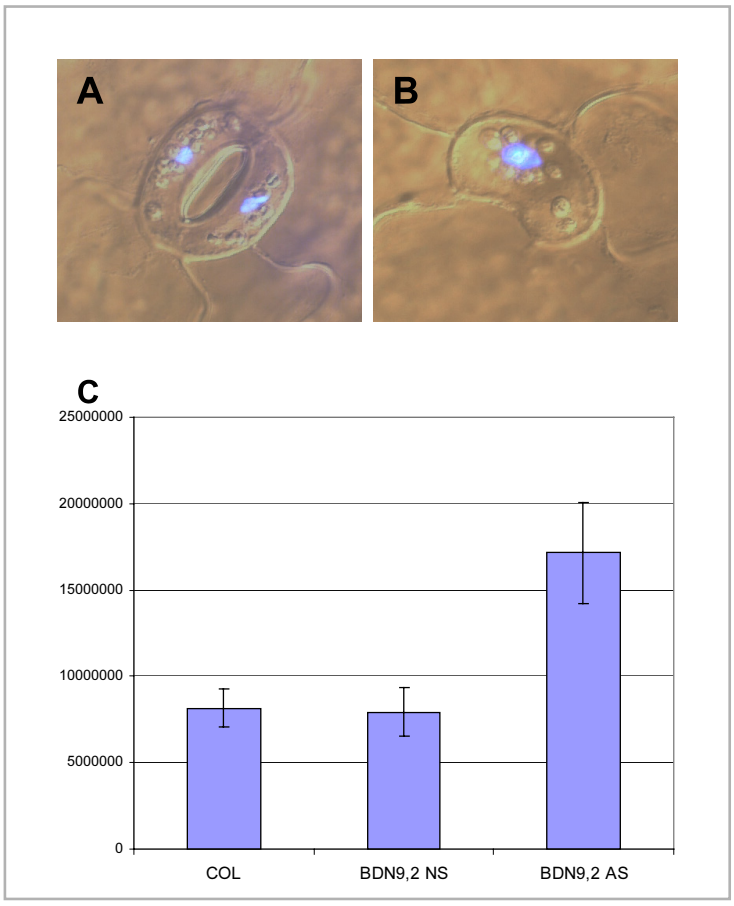

Figure 8. A *and* ***B****, Microscopic analysis of DAPI stained cotyledons of plants overexpressing CDKB1;1.N161.* ***C****, Stoma and aberrant stoma of control and CDKB1;1.N161 transgenic plants, respectively. Quantification of DAPI-intensity of nuclei in guard cells of normal stomata (NS) from control plants (COL) or from CDKB.N161 transgenic plants (BDN9.2), and from aberrant stomata (AS) of CDKB1;1.N161 transgenic plants (BDN9.2AS).*

In DAPI stained cotyledons, it was never possible to observe undivided cells with two nuclei, demonstrating that cells are blocked in the G2 phase. Moreover, using interference contrast microscopy, it was never possible to visualize in those 4C stomatal cells, morphological evidences associated with the initiation of cytokinesis, such as cell plate formation. Therefore, the guard cell shape or apparent pore formation and wall thickening of aberrant stomata could be the effect of pre-programmed differentiation information contained in each stomatal precursor. This might also justify the presence of normal substomatal cavities under aberrant stomata (data not shown). It has been shown that in cytokinesis defective mutants, like *keule* (Söllner et al., 2002) or *cyd1* (Yang et al., 1999), GMCs that are unable to form a ventral wall presents wall thickenings on the outer cell wall. Likewise, the treatment of maize seedlings with caffeine interferes with cell plate formation but does not prevent the deposition of a wall thickening on the middle of the periclinal walls (Galatis and Apostolakos, 1991). Taken together, these results indicate that the acquirement of guard cell identity occurs independently from cell division.

All together our results suggest that CDKB1;1 activity is implicated in the cell division(s) leading to stomata formation. Activity of CDKB1;1 might also be necessary to determine meristemoid specification, but does not seem to be essential for guard cell differentiation (Fig. 9).

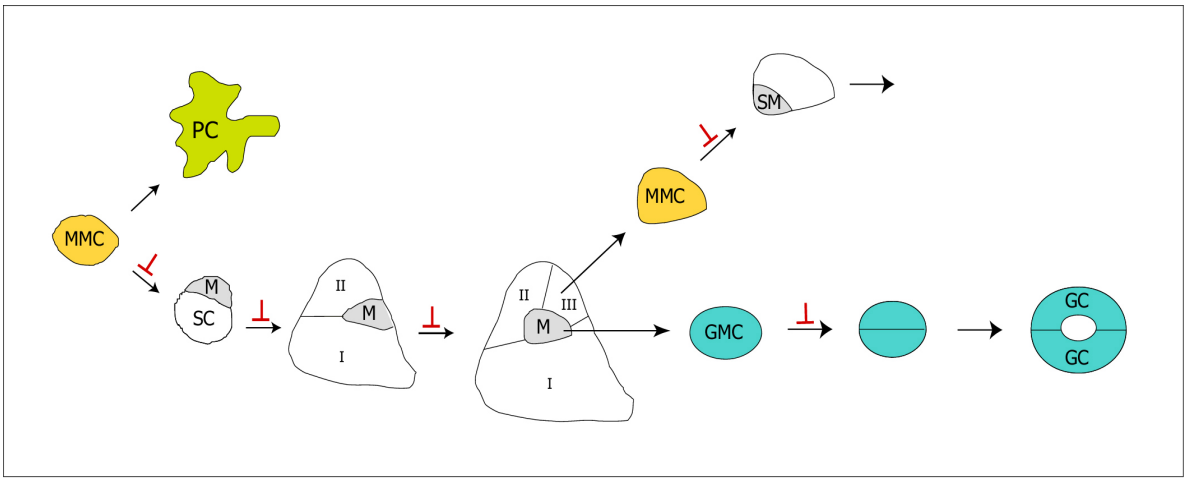

***Figure 9**. Model representing stomata development in the epidermis of* Arabidopsis *cotyledons. A meristemoid mother cell (MMC) divides asymmetrically to form a smaller meristemoid (M). The meristemoid then undergoes three rounds of asymmetric cell divisions, in which the larger daughter cell assumes the fate of a less-specialized subsidiary cell (SC). The meristemoid then becomes a guard mother cell (GMC) that divides symmetrically to form two guard cells (GC) surrounding a central pore. The most recently formed neighbouring SC, might divide asymmetrically to form SMs (subsidiary or satellite meristemoids). This pattern of division results in each stoma being surrounded by at least one pavement cell derived from the same meristemoid. Abnormal stomata development in CDKB1;1 transgenics is explained by the hold-up of the developmental transitions marked by negative arrows. PC, pavement cell.*

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Chapter 5

General Introduction

Seed germination is the transition of the quiescent embryo, which has developed from the fertilised ovule, into a new photosynthetically active plant. The visible sign that germination has been completed is the protrusion of the radicle through the seed coat. However, germination begins with water uptake by the seed (imbibition) and ends with the start of elongation of the embryonic axis inside the seed. Germination results from a combination of many cellular and metabolic events, coordinated by a complex regulatory network that triggers the breaking of seed dormancy. Dormancy is an intrinsic ability to temporarily block radicle elongation in order to optimise the timing of germination. In the field of seed biology, germination mechanisms and their control by dormancy have been investigated in a wide range of species. Nonetheless, how these processes are coordinated, how they contribute to germination, and the regulatory network leading to completion of germination remain poorly understood.

SEED DEVELOPMENT

Embryogenesis

Embryo development is a crucial part of the life cycle of plants, during which the body plan is established, storage products are accumulated in readiness for germination, and the embryo develops desiccation tolerance that enables prolonged survival in a dry state.

Recent work in the plant model *Arabidopsis* has revealed many genes participating in the early stages of body plan formation (e.g. *MONOPTEROS*, *GURKE*, *GNOM, KNOLLE* and *FACKEL*). Although the role of these genes in determining the shape of the adult plant is not yet fully understood, several different conceptual models have been proposed to account for their action, including a morphological model that emphasises developmental processes and the iterative nature of plant development (reviewed by Kaplan and Cooke, 1997). The *Arabidopsis* gene *PROLIFERA* (*PRL*) has been shown to be also essential for proper cytokinesis of embryo cells, but additionally it might contribute to the regulation of cell cycle progression. PRL is a member of the MCM (minichromosome maintenance proteins) family of genes that are required for DNA replication licensing (Holding and Springer, 2002).

Angiosperm embryo development can be conveniently divided into three stages (Fig. 1). During post-fertilization and the globular-heart transition stage, the single-celled zygote undergoes extensive mitotic division, and the resultant cells differentiate to form the basic body plan of the embryo composed of the axis and cotyledons. Simultaneously, the triploid endosperm is formed. After that, the embryo undergoes a process of maturation, which occurs largely in the absence of further cell divisions and is characterised by cell expansion and deposition of reserves (organ expansion and maturation). Therefore, metabolic activities are largely characterised by the massive synthesis and deposition of polymeric reserves within the storage tissues (cotyledons and endosperm).

The expression of the *Arabidopsis* dominant negative CDK;A (cyclin-dependent kinase type-A) protein under the control of a late-heart stage embryo specific promoter has underlined the importance of cell division during embryo patterning and morphogenesis. During this phase of development, cell division was shown to be necessary in order to create the embryo structure and the apical-basal pattern. However, even in severe phenotypes, radial differentiation was little affected, showing that the rate of cell division does not determine cellular differentiation of the embryo (Hemerly et al., 2000).

Seed maturation is terminated by some degree of drying, which results in a progressive reduction in metabolism as water is lost from the seed tissues and the embryo enters a metabolically inactive, or quiescent, state.

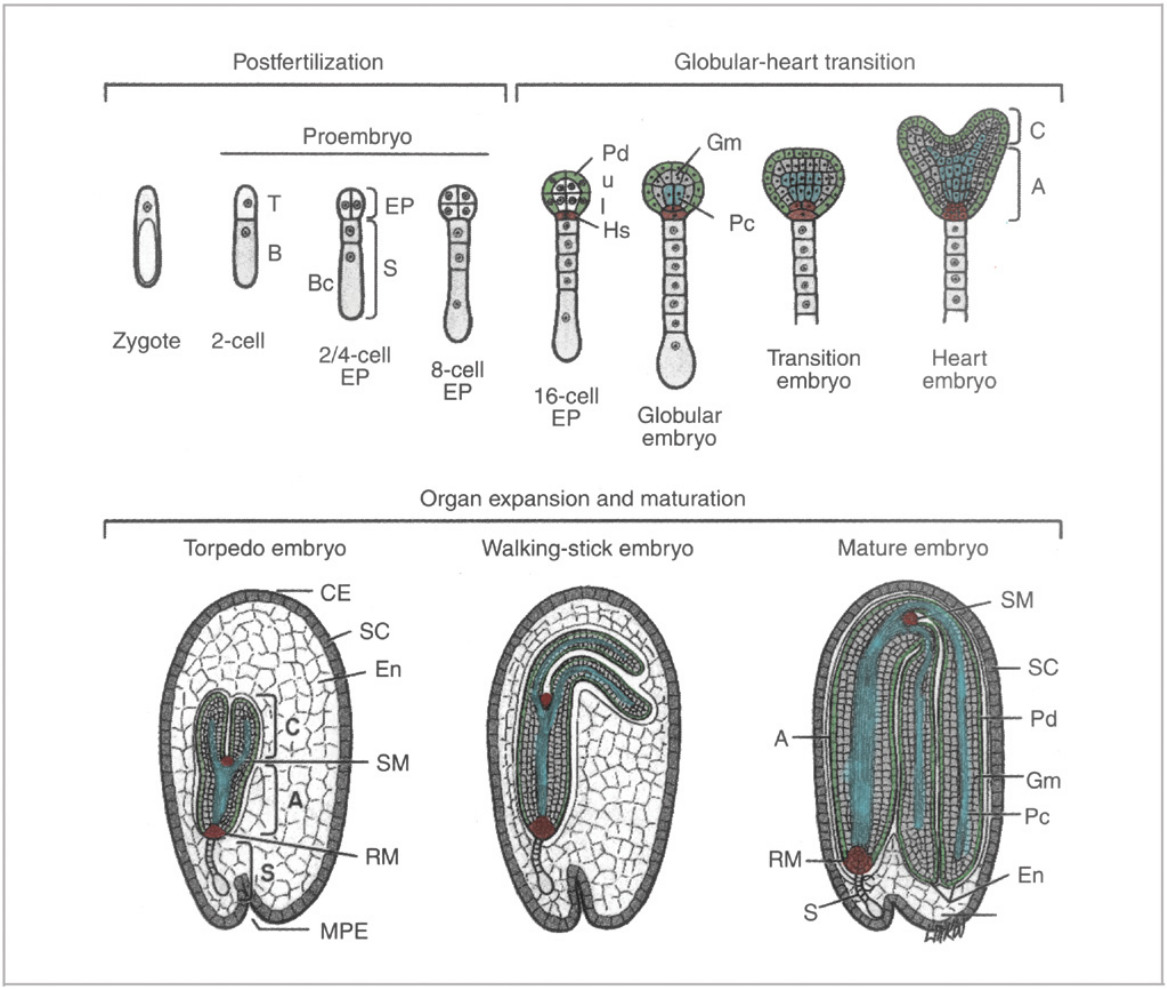

Figure 1. *Stages of development of a wild-type Arabidopsis embryo. Initially the fertilized zygote cell divides to form a terminal (apical) and a basal cell (T and B). The former divides to become the embryo proper (EP) region of the preglobular stage, and the latter becomes the suspensor (S). The protoderm (Pd) develops into the epidermal (Ed) layer of the mature embryo; the ground meristem (Gm) develops into the parenchyma; the procambium (Pc) develops into the vascular tissue; and the hypophysis (Hs) develops into root and shoot meritems (RM and SM). A, axis ; Bc ,basal cell C, cotyledons; CE,chalazal end; En, endosperm; l, lower tier; MPE, micrpylar end; SC, seed coat; u, upper tier. (Adapted from Goldberg et al., 1994).*

Hormonal regulation of late maturation events

In the vast majority of seeds, development and germination are separated by a period of dormancy that is accomplished upon maturation and drying. In this state of low metabolic activity seeds can withstand adverse conditions for extended periods. In order to serve its purpose of reproducing and propagating the plant, it is necessary that the seed presents a high degree of resistance to cold, drought, and other environmental changes. The differentiation of protective structures, such as seed coat, and the shut down of main cellular metabolic pathways constitute part of an active process that leads to dormancy.

Dormancy is an important evolutionary strategy that delays seed germination until successive seasons and, therefore, increases the potential survival of the seed progeny (Bewley and Black, 1994; Baskin and Baskin, 1998). Many species present viviparous mutants in which the seeds do not become dormant, but instead proceed directly into germination while still attached to the mother plant and continue their development to become seedlings. In many cases these mutants have been shown to fail to produce abscisic acid (ABA). It has been shown that seed dormancy is triggered by ABA, a hormone that accumulates in the seed. Therefore, disruption of ABA signal transduction clearly disturbs dormancy, affecting germination (Koornneef et al., 1998). Mutation of the *ENHANCED-RESPONSE TO ABA* (*ERA1*) locus causes an increased sensitivity to ABA, and *era1* mutant seeds have an increased level of primary dormancy (Cutler et al., 1996). The phenotypes of these mutations are not seed specific and also affect other aspects of plant growth and development.

Additionally, genetic screens for mutants presenting early germination (low dormancy) have also led to the isolation of mutants that are deficient in the synthesis of ABA or its sensitivity (Leung and Giraudat, 1998; Finkelstein et al., 2002). Analysis of those ABA mutants led to the conclusion that most of the embryonic regulators are transcription factors with complex patterns of cross-regulation. The effects of ABA and some key regulators during embryogenesis are summarized in Fig. 2. The molecular nature of those regulators, and the effects resulting from their disruption, are explained further in this chapter.

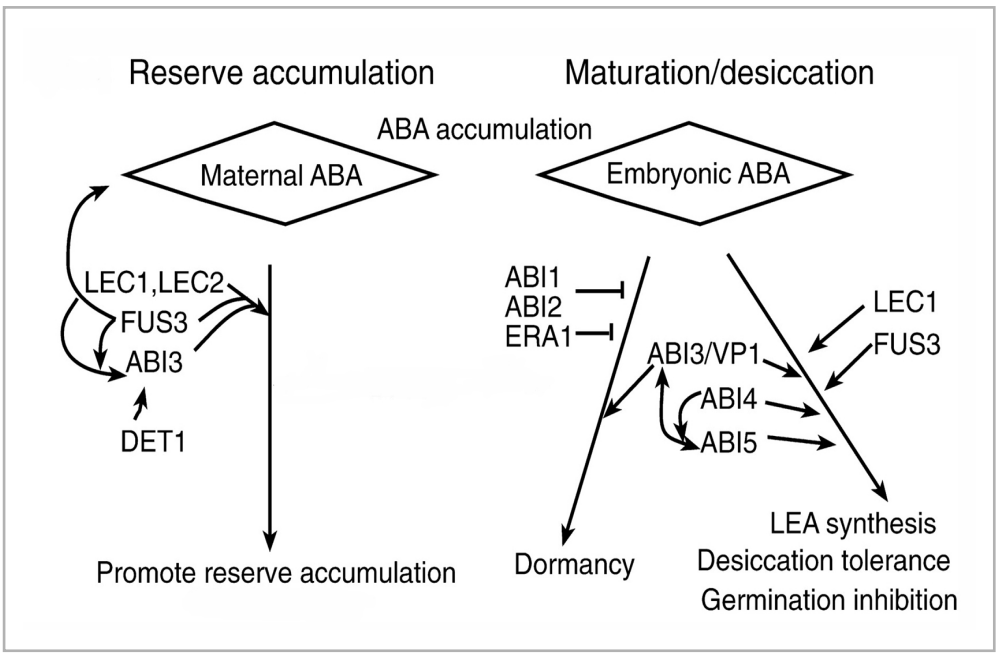

Figure 2. *Proteins identified and positioned by mutant and genetic analyses in the ABA signalling pathway, which are involved in seed development. Arrows represent a positive effect on the pathway, and bars represent negative regulatory effect. ABI, ABA-insensitive; DET, de-etiolated; ERA, enhanced-response to ABA; FUS, FUSCA; LEC, leafy cotyledon; VP, viviparous. (Adapted from Finkelstein et al., 2002).*

Decreased dormancy might also be associated to testa structure (*seed shape* mutants), testa color (*transparent testa* mutants), and in unknown downstream dormancy-inducing processes (*rdo* mutants) (Léon-Kloosterziel et al., 1996). All together, these mutants provide useful support to the results of physiological studies, and they genetically confirm the roles of hormones in the regulatory network of germination, including dormancy.

SEED GERMINATION AND DORMANCY

Genetic control mechanisms regulating the transition from embryo development to germination

The transition from embryo development to germination is a highly complex developmental process (McCarty, 1995; Bewley, 1997; Holdsworth et al., 1999). The embryo within the dry seed undergoes a process of after-ripening that is environmentally determined and results in the removal of dormancy. Therefore the environmental history of the dry seed is crucial in determining whether the embryo will remain dormant or will germinate after imbibition (water uptake by the seed). More important, the genetic background of the seed is the pre-eminent determinant of the level of embryo dormancy (Koornneef et al., 1984; Jana et al., 1988; McCarty et al., 1991; Li and Foley, 1997). Despite almost a century of investigation, the molecular and genetic mechanism(s) underlying after-ripening and the removal of embryo dormancy are poorly understood.

The genetic and molecular determinants of seed germination and dormancy have been intensively investigated in tomato, in *Nicotiana plumbaginifolia* and in *Arabidopsis* (reviewed by Koornneef et al., 2002). Several studies have led to the identification of many proteins or mRNAs, the accumulation of which is correlated with dormancy or seed germination. Many of these products have sequence homology with proteins involved in desiccation tolerance or protection against various injuries and with storage proteins (Goldmark et al., 1992; Johnson et al., 1995; Li and Foley, 1995; Stacy et al., 1996). Identifying these functions is important for understanding the differences between the dormant and non-dormant states.

In *Arabidopsis*, genetic studies have identified many loci that regulate the transition from embryo to seedling (Koornneef and Karssen, 1994; Dubreucq et al., 1996). These include a number that are related to the synthesis or perception of the hormones ABA and gibberellin (GA), and to a lesser degree ethylene (C_2H_4), and have a general effect on plant development (Giraudat et al., 1994; Koornneef and Karssen, 1994). Mutations in a second group of loci specifically affect the phenotype of the embryo. The *LEAFY COTYLEDON1 (LEC*1) (Castle et al., 1993; West et al., 1994; Lotan et al., 1998), *ABSCISIC ACID INSENSITIVE3 (ABI*3) (Koornneef et al., 1984; Giraudat et al., 1992), *ABI4* (Finkelstein et al., 1998) and *FUSCA3 (FUS*3) (Baumlein et al., 1994; Keith et al., 1994) loci all encode transcription factor or transcription factor-like proteins that control embryo development. The *LEC1* locus encodes a homologue of the yeast CAAT-box DNA binding factor

HAP3, ABI3 and FUS3 both contain a 'B3' DNA-binding domain that has so far only been identified in plants, and the *ABI4* locus encodes a protein that contains an APETALA2-like DNA binding domain. Severe alleles of *LEC*1, *ABI3* and *FUS3* cause seedling development to be advanced (Fig. 3). These genes interact in a complex manner affecting characteristics such as chlorophyll breakdown, induction of desiccation, repression of anthocyanin synthesis, accumulation of storage proteins, suppression of leafy characteristics and, more important, the repression of germination. Therefore, the positive action of these and other loci on embryo dormancy must be overcome before germination can commence (Meinke et al., 1994; Nambara et al., 1995).

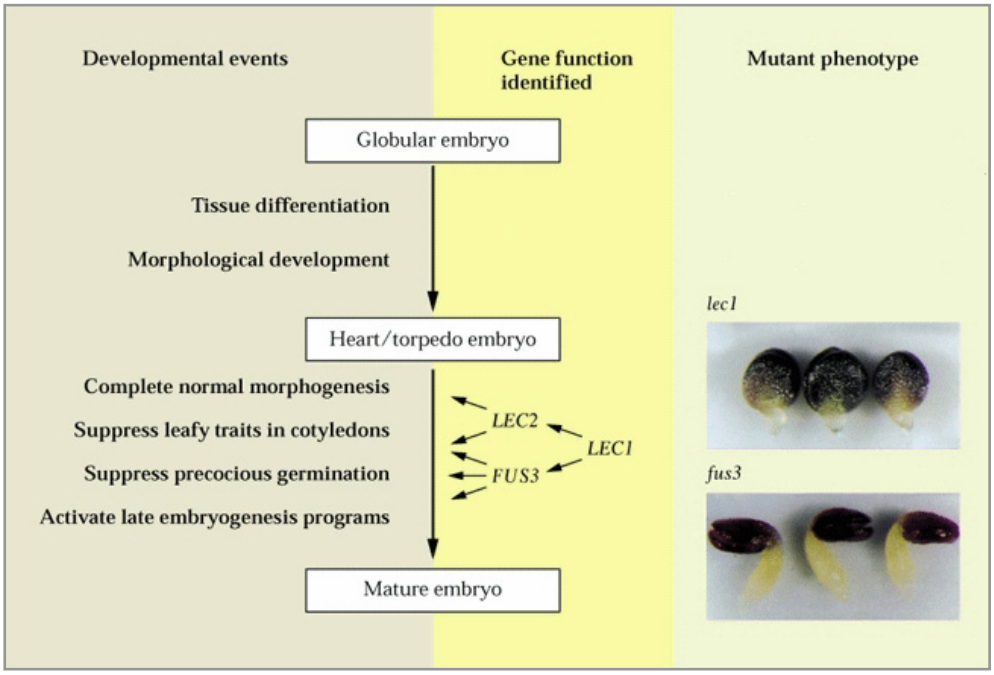

Figure 3. *Data from the analysis of lec1 and fus3 phenotypes, showed that these loci regulate the transition from seed to seedling by simultaneously activating developmental processes related to embryo maturation and repressing germination. LEC, Leafy cotyledon; FUS, FUSCA. (Adapted from Bewley et al., 2000).*

Repression of germination by *ABI3* and other loci in *Arabidopsis* indicates that these factors might interact with other loci that enhance germination potential. Genetic screens to search for regulators of germination resulted in the identification of the *COMATOSE* (*CTS*) locus. A mutation of this locus causes a disruption in the transition from embryo development to germination, but does not affect other aspects of development (Russell et al., 2000). Analysis of seed characteristics indicates that the *cts* mutation specifically blocks germination potential by enhancing after-ripening time, sensitivity to GA and pre-chilling, and by repressing the activities of loci that activate embryo maturation. The *cts* mutation requires the prior action of *ABA*1, *ABI*3, *FUS3* and *LEC1* to induce embryo dormancy, suggesting an interaction between these loci and *CT*S. Based on these observations, it has been proposed that each of these loci perform dual functions of activation of

embryo maturation culminating in embryo dormancy and simultaneously repression of germination and seedling characteristics (Russell et al., 2000; Holdsworth et al., 2001).

In addition to these mutants involving specifically the embryo, other mutants have been selected that control dormancy through the seed coat or other maternal mutants (Debeaujon and Koornneef, 2000; Debeaujon et al., 2000). Knockout mutants of the *Dof AFFECTING GERMINATION* (DAG) genes, which encode a Dof (DNA-binding with one finger) transcription factor, show contrasting effects on the regulation of dormancy versus germination (Papi et al., 2000; Gualberti et al., 2002). *DAG1* knockout results in reduced dormancy but, in contrast to other dormancy mutants, this phenotypic effect is determined by the maternal genotype. This maternal inheritance is consistent with the expression pattern of the *DAG1* gene in the vascular tissue that enters the developing seeds, which is genetically derived from the mother plant (Papi et al., 2000). The *dag2* mutant line is less sensitive to all parameters that promote germination, such as light and GAs, showing an opposite phenotype to *dag1*. However, *DAG1* and *DAG2* show an overlapping tissue-specific expression pattern, and segregation analysis showed that both *dag1* and *dag2* mutations are maternally determined. Therefore, DAG1 and DAG2 were suggested to act in the same maternal gene in order to promote or repress germination. In the model proposed, DAG1 would activate maternal genes that promote dormancy or repress germination, whereas DAG2 might either interact with DAG1 to decrease its activity or can also act in the same maternal target gene to counteract DAG1 effect. In both cases, DAG2 would counteract and in some cases neutralize the effect of DAG1 to ensure the correct levels of dormancy in seeds (Gualberti et al., 2002).

The identification and investigation of loci that clearly promote germination will allow a greater understanding of how the processes of after-ripening, the removal of dormancy and germination initiation are specifically controlled within the context of seed and seedling development.

Imbibition and early germination events

Following desiccation, the plant embryo needs an after-ripening (metabolic changes that must take place in a seed to overcome dormancy) period in order to be ready to germinate. If the embryo has not fully completed its after-ripening period (the dormancy period following seed formation, necessary for embryo changes that assure germination), it will remain in a dormant state even if subjected to water imbibition. Development is suspended during dormancy, although the seeds remain metabolically active. Finally, if after-ripening in the dry seed is completed, then imbibed embryos will germinate under suitable conditions.

Seed germination is a process by which the plant embryo within the seed resumes growth after a period of dormancy and the seedling emerges. For germination to occur, quiescent seeds need only to be hydrated under conditions that encourage metabolism, such as a suitable temperature and the presence of oxygen (Fig. 4). The uptake of water by the seed, which is considered to be a trigger for germination, and the metabolic processes that take place as a result,

are described in Figure 4. Under favourable conditions rapid growth of the embryo culminates in rupture of the covering layers and emergence of the growing radicle tip. Radicle emergence is considered as completion of germination *sensu stricto*. Uptake of water by a mature dry seed is triphasic, with a rapid initial uptake (phase I) followed by a plateau phase (phase II). Phase III is a further increase in water uptake which occurs after germination is completed, as the embryonic axes elongate. Food stored in the endosperm or in the cotyledons provides energy for these early stages, until the seedling is able to manufacture its own nutrients through photosynthesis (Mansfield and Briarty, 1996).

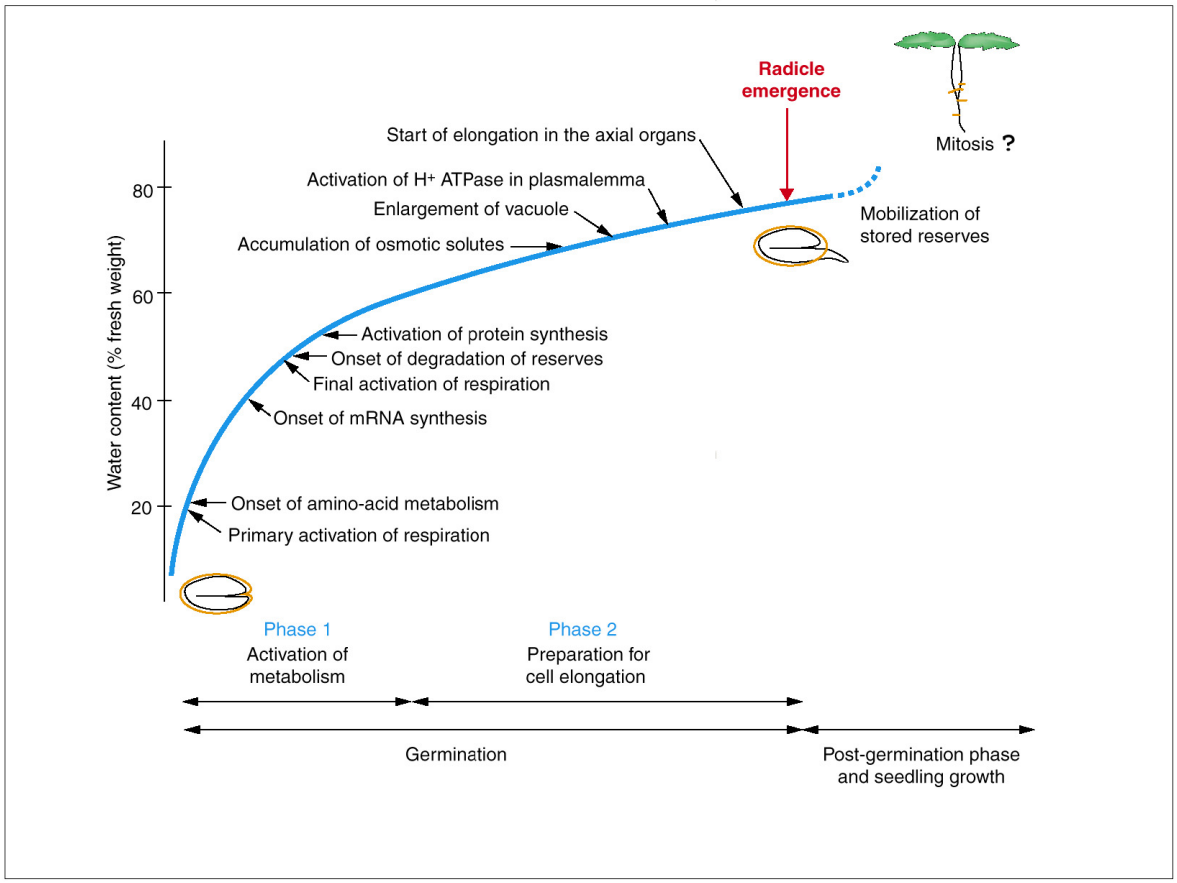

Figure 4. *The cellular and metabolic events triggered by water uptake during seed germination. A rapid imbibition phase (phase 1) initiates the resumption of basic metabolism. During this phase, known as 'physical' imbibition, the activation of metabolic pathways results from the gradual increase in hydration. Once the level of hydration exceeds 60%, the rate of hydration decelerates (phase 2) and new physiological mechanisms prepare cell expansion in the embryonic axes, culminating in the start of cell elongation. Osmotically active solutes, such as sugars and amino acids, are accumulated resulting in a further increase in water uptake that may coincide with weakening of the endosperm as the embryonic axes elongate and germination is completed. Storage nutrients (lipids, proteins or starch) accumulated in the embryo's cotyledon and endosperm start to be mobilized before completion of germination and are used in the post-germination steps to sustain the early growth stages, before the seedling becomes autotrophic. During germination, cell cycle activities are slowly resumed but the occurrence of the first cell divisions (mitosis) is still under controversy. (Adapted from Bove et al., 2001).*

Upon imbibition of the mature seed there is a reactivation of the existing metabolic systems, incremented by synthesis of new components, leading to renewed cell expansion and cell division as the seedling becomes established (Mansfield and Briarty, 1996) (Fig. 4). Seed development and germination are distinct physiological stages of the plant life cycle, in which the status of stored reserves contrast markedly. Seed germination involves the breaking of dormancy and the resumption of the embryo growth. Gene transcription is resumed, protein synthesis is reinitiated, and the rates of respiration and intermediary metabolism increase dramatically. The immediate cause of germination is often the imbibition of water by the seed, however this is certainly not the only necessary requirement. Once germination is initiated, seedling growth is fully dependent on metabolic substrates stored in the seed.

Hormonal regulation in the induction and breaking of dormancy

Studies of GA-deficient, ABA-deficient, and signalling mutants in different species have identified the crucial role of ABA in seed dormancy, as well the requirement of GA for germination.

Treating *Nicotiana* seeds with norfluorazon, an inhibitor of ABA biosynthesis, was shown to promote germination, demonstrating that the maintenance of dormancy in imbibed seeds is an active process involving *de novo* ABA synthesis (Grappin et al., 2000). This finding complements many others that emphasised the role of ABA during seed development.

Seed germination is inhibited by ABA and promoted by ethylene (reviewed by Kepczynski and Kepczynska, 1997), however studies with *Arabidopsis* mutants have shown that these two signalling pathways interact during germination (Beaudoin et al., 2000); Ghassemian et al., 2000). In addition to the well-known *abi* (ABA insensitive) and *era* (enhanced response to ABA) mutants, which present a clear seed germination phenotype, it was also found that the *ethylene insensitive2* (*ein2*) and *ethylene resistant* (*etr*) mutants of *Arabidopsis* are also hypersensitive to ABA (Beaudoin et al., 2000; Ghassemian et al., 2000). This finding coincides with the fact that *ein2* mutants were isolated as suppressors of the *abi1* mutant. The *constitutive triple response1* (*ctr1*) mutant, which is characterised by an ethylene constitutive response, was found among mutants selected as enhancers of the ABA-insensitive mutant *abi1* (Beaudoin et al., 2000). Ethylene appears to promote germination by decreasing the sensitivity to endogenous ABA and therefore seems to be a negative regulator of ABA action during seed germination.

In addition, the presence of a cross-talk between sugar signalling and ethylene signalling is suggested by the sugar-insensitive phenotype of *ctr1* and the sugar-hypersensitive phenotype of *etr* (Finkelstein and Gibson, 2002). Apparently, ABA, ethylene and sugar signalling pathways strongly interact during the regulation of germination and early seedling growth. This interaction is further supported by the observation that many sugar-signalling mutants turn out to be ABA-biosynthesis mutants or alleles of ABA-insensitive mutants (Gibson, 2000).

ABA effects on germination are also antagonized by gibberellins. GAs play a key role in grain producing species. The presence of this hormone in the aleurone tissues triggers *de novo* synthesis of a number of hydrolytic enzymes and their secretion to the endosperm (reviewed by Ritchie and Gilroy, 1998). But the importance of GAs for seed germination is not restricted to monocots. The GA-regulated enzymatic breakdown of endosperm cell walls in tomato shows interesting parallels with the GA-controlled starch hydrolysis in cereal aleurone cells (Ritchie and Gilroy, 1998; Peng and Harberd, 2002). From physiological studies on a wide variety of species, including several mutants, the importance of this hormone as a key player in germination becomes clear (reviewed by Peng and Harberd, 2002). The mutation of loci that control GA biosynthesis and signal transduction reduces germination potential of seeds showing that GA is a promoter of germination. In *Arabidopsis*, several mutations in genes acting early in GA biosynthesis (*GA1*, *GA2*, and *GA3*) display a number of GA-rescued phenotypes (Koornneef and van der Veen, 1980). These include failure to germinate, growth of plants as a green dwarf, reduced apical dominance and delayed senescence.

Over the years, a substantial collection of mutants defective in GA signalling has been isolated. Most of those mutants fall into one of the two classes: those that resemble GA-biosynthetic mutants but do not respond to exogenous application of GA; and those that resemble plants in which a GA response is constitutively activated. The first include *gai (gibberellin insensitive)*, whereas *spy (spindly)* and *rga (repressor of GA1-3)* belong to the second category. Thus, the *gai* mutation leads to GA-unresponsive dwarf plants, whereas the spy and *rga* mutations lead to plants in which a GA response appears constitutively activated (reviewed by Ogas, 1998). *spy* mutation was identified by the ability of *Arabidopsis* seeds to germinate in the presence of inhibitory concentrations of paclobutrazol, a GA biosynthetic inhibitor (Jacobsen et al., 1996). SPY is predicted to be a glycosyl transferase, and because loss of the SPY function leads to an increase in GA responsiveness, it has been proposed that protein modification of a GA signalling component by SPY acts to decrease GA signal transduction (Jacobsen et al., 1996).

The *RGA* gene was identified in a screen in which *ga1* mutants (which are defective in GA biosynthesis) were screened for second site mutations that rescued their GA-deficient phenotype. RGA and also GAI are members of a family of transcription regulators, which activity results in the downregulation of GA responses (Peng et al, 1997; Silverstone et al., 1998). Recently, the identification and characterization of RGL1 and RGL2 (RGA-like), two GAI/RGA homologous has been reported (Lee et al., 2002; Wen and Chang, 2002). GAI, RGA and RGL1 were shown to negatively affect stem elongation, whereas RGL2 and probably RGL1 were shown to be negative regulators of seed germination. So that GA must counteract RGL proteins in order seed germination to take place. Therefore, a model has been proposed to explain how GA signals control seed germination. According to that model dormancy is the default program. Biosynthesis of GA, following imbibition, down-regulates the expression of repressors (e.g. SPY and RGL2), increasing the germination potential of the embryo (reviewed by Peng and Harberd, 2002).

The identification of *Arabidopsis* GA-constitutive response mutants and GA-insensitive mutants has revealed much of the GA-response system and its importance for germination, but these studies have also contributed to uncover the close interplay between ABA and GA. *sleepy1* (*sly1*), an *Arabidopsis* mutant that has a severe germination defect, was selected in a screen for suppressors of the ABA-insensitive *abi1-1* mutant (Steber et al., 1998). Also, experiments carried out with the barley homologue of SPY1 suggest that this gene is not only involved in GA signalling but may also be implicated in enhancing ABA response in barley aleurone cells. *spy1* barley mutants are slightly insensitive to ABA and suppress *era1* (enhanced response to ABA) defective germination. These results suggest that GA and ABA interact to determine the overall dormancy of the seed in a type of push and pull fashion with GA encouraging germination while ABA encourages dormancy (Fig. 5).

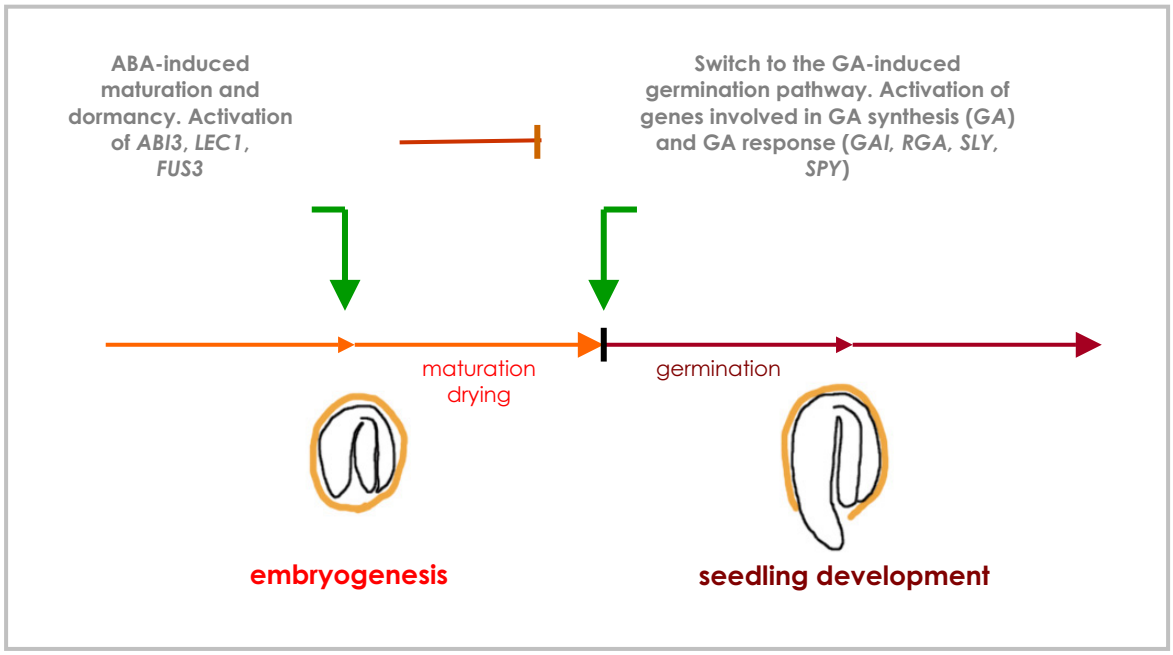

Figure 5. *An activation/repression model for the genetic control of the transitions from embryo growth to maturation, and from the quiescent dry state to germination. This schematic overview illustrates the importance of the close interplay between GA and ABA regulatory pathways, as those developmental switches are mainly regulated by loci implicated in the synthesis or sensitivity to ABA and GA plant hormones.* ABI, *ABA-insensitive;* FUS, *FUSCA;* GA, *gibberellic acid deficient;* GAI, *gibberellin insensitive;* LEC, *leafy cotyledon;* SLY, *sleepy;* SPY, *spindly.*

Seed germination is also promoted by brassinosteroids (BR). BR was shown to rescue the germination phenotype of severe GA-biosynthesis mutants and of the GA-insensitive mutant *sleepy1* (Steber and McCourt, 2001). But the strongest evidence for a role of BRs in germination is the increased ABA sensitivity of BRs mutants (*de-etiolated2* [*det2*] and *brassinosteroid insensitive1* [*bri1*]), showing that decreased BR biosynthesis or response increases sensitivity to ABA during germination. Germination of both the BR biosynthetic mutant *det2* and the BR-insensitive *bri1* is more strongely inhibited by ABA than is germination of wild-type seeds. Thus, the BRs are needed to overcome inhibition of germination by ABA. On the other side, the partial rescue of GA mutants

germination by brassinosteroids raises the possibility that BR interact also with GA. Because BR stimulates the germination of a GA-insensitive mutant, *sly1*, it was suggested that BR act by stimulating GA biosynthesis and not by increasing GA sensitivity (Steber and McCourt, 2001).

Thus, in addition to the well-characterized antagonism between ABA and GA effects on germination, recent studies demonstrated interactions between signalling by ABA and/or GA with brassinosteroids, ethylene, light or sugars (reviewed by Finkelstein et al., 2002). Therefore, transition from seed maturation to germination seems to result from a complex signalling network in which shared signalling elements interact with a variety of hormonal and environmental signals that regulate germination (Fig. 6).

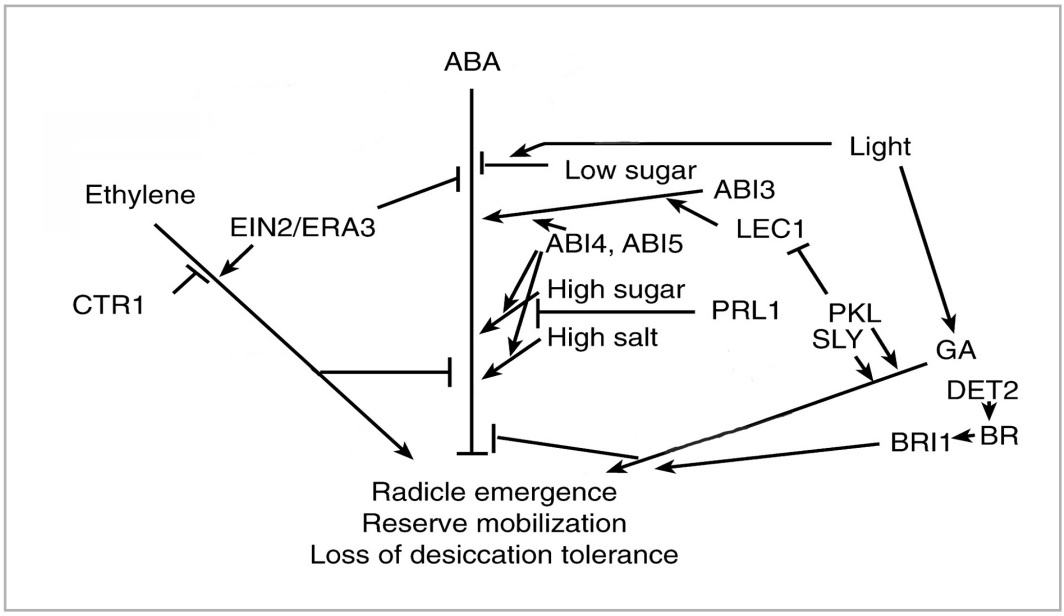

***Figure 6**. Proteins from the ABA signalling pathway which mutant and genetic analyses showed to be involved in seed germination. Arrows represent a positive effect on the pathway, and bars represent negative regulatory effect. ABI, ABA-insensitive; BRI, brassinosteroid insensitive; CTR, constitutive triple response; DET, de-etiolated; EIN, ethylene insensitive; ERA, enhanced-response to ABA; LEC, leafy cotyledon; PKL, pickle; PRL, prolifera; SLY, sleepy. (Adapted from Finkelstein et al., 2002).*

Cell cycle progression in germinating seeds

CDKs, cyclin-dependent kinases, are the catalytic subunits of complexes whose regulatory subunits are the cyclins. These are a family of proteins, named for their cyclic expression and degradation, that play an important role in regulating cell division. Cyclins (Cyc) are synthesised immediately before they are used and their levels fall abruptly after their action because of degradation via the ubiquitin pathway (Genschik et al., 1998). Interaction between CDKs and their cyclin partner occurs at specific stages of the cell cycle. The transition between cell cycle phases is controlled by one or more specific CDK/cyclin complexes, which propel the cell in the next cell cycle phase. Different complexes control successive passages through different cell cycle windows.

Activity of CDKs is positively regulated not only by binding of the cyclin partner but also by phosphorylation, and negatively regulated by proteolysis of the cyclin subunit, binding of CDK protein inhibitors (KRPs, Kip-related proteins) or also by phosphorylation (Obaya and Sedivy, 2002).

It is accepted that in plants, CDK/Cyc complexes phosphorylate the retinoblastoma-related protein (RBR), causing the activation of a set of genes that are regulated by the E2F/DP transcription factor and are necessary for S-phase entry and DNA replication. Later in G2, the activity of other CDK/Cyc complexes induce entry into mitosis. During M-phase, the degradation of the mitotic cyclins and the deactivation of the kinase complexes permit exit from mitosis. The cell cycle machinery responds to external signals such as hormones, sugars, and light, which are integrated with positional and developmental signals (reviewed by Stals and Inzé, 2001). As a result, cells can modulate their activities to maintain proliferation and differentiation, or become quiescent. (Further details on cell cycle regulation are described in the part I of the thesis).

Embryos of maturing seeds display a programmed transition from cell proliferation to quiescence. In the majority of seeds, this transition is characterised by a cell cycle arrest of most embryonic cells. Using flow cytometry, it has been shown that embryos of fully matured dry seeds contain relatively large amounts of 2C DNA, indicating that most cells are arrested in the G1-phase of the cell cycle (Bino et al., 1992; Elder and Osborne, 1993; Lanteri et al., 1993; de Castro et al., 1995; Gornik et al., 1997; Liu et al., 1997). During dormancy, cell division does not occur, although the cells of the embryo might be in a metabolically active state (Bewley and Black, 1994). Also the absence of BrdU (bromodeoxyuridine) incorporation into embryonic DNA from dry control seeds demonstrates a lack of DNA synthesis activity, whereas the almost absence of β-tubulin point towards the absence of a microtubular cytoskeleton network, probably resulting from the process of seed dehydration during maturation (de Castro et al., 2001).

The inhibitory effects of ABA on germination are correlated with the inhibition of cell division and the arrest at the G1-phase of the cell cycle (Liu et al., 1994). The effects of ABA on cell cycle progression might be explained by the discovery that ABA induces the expression of a CDK protein inhibitor that interacts with both Cdc2a (CDKA;1) and CycD3 and is correlated with decreased histone H1 kinase activity (Wang et al., 1998).

The transition from quiescence to cell proliferation during water imbibition is characterised by increasing numbers of radicle tip cells with 4C DNA, reflecting the entry in the G2-phase of the cell cycle (Bino et al., 1992; de Castro et al., 1995; de Castro et al., 2001). In tomato root tips an increase of the 4C signal after 24 hours imbibition indicates that nuclear replication occurs just prior to radicle protrusion. Moreover, it was observed that β–tubulin accumulation is reinitiated early during germination, concomitantly with the initial assembly of the cortical microtubular arrays and DNA synthesis in the radicle (de Castro et al., 2000). Prior to radicle protrusion, DNA synthesis and the appearance of microtubule arrays progresses from the embryonic radicle tip region toward the cotyledons and seems to be correlated with the commitment of cells to cell division (de Castro et al., 2000). Experiments in both tomato and Brassica germinating seeds showed that blocking of

DNA replication with hydroxyurea does not affect the accumulation of β–tubulin and does not halt radicle protrusion, even if subsequent seedling development is blocked (Gornik et al., 1997, de Castro et al., 2000). Those observations showed that in both species cell division must not be a requirement for radicle protrusion, even if essential for further seedling growth. However, the delayed completion of germination in the presence of that inhibitor suggested that seed germination in normal conditions might also involve mitotic divisions. Moreover, cell cycle activity events preceding radicle emergence might be necessary to ensure seedling growth after the completion of germination. These events are mainly associated with replication of the nuclear DNA content and the preparation of the cortical microtubular cytoskeleton. These early cell cycle events were the main target of our studies.

The behaviour of the cell cycle apparatus during seed germination remains to be revealed. Some studies have been performed on the detection of cell cycle mRNAs and proteins using heterologous probes and antibodies (Georgieva et al., 1994; Cruz-Garcia et al., 1998). Using antibodies against different mammal cell cycle proteins, Georgieva and collaborators (1994) showed the behaviour of several cell cycle markers in germinating maize. However, the specificity of those antibodies remains controversial, and the real existence of a putative plant p53 as detected in that study is questionable. In mammals, the p53 protein was shown to be crucial for inducing genes that lead to G1 arrest following DNA damage, enabling DNA repair. Although some plant cDNA sequences with partial homology to p53 were identified, the evidence that p53 mechanisms exist in plants was still not found.

Most cells in meristematic tissues of seed embryos seem to be arrested in G1-phase (Bino et al., 1992; de Castro et al., 1995; de Castro et al., 2001). Water imbibition, however, does not cause an immediate entry into cell cycle, instead there is a holdup before S-phase is initiated. The long G1-phase or G0/G1 transition must be due to the need of cells to recover from cellular damage accumulated during seed maturation, drying and storage (Osborne, 1983). Particularly important seems to be the chromosomal damage. Some studies showed that nuclear DNA in dry seeds has lost integrity. Therefore, DNA repair is an important process during early germination (Tuteja et al., 2001). As seeds age, germination time is delayed, and a direct correlation has been established between the level of deterioration of cabbage seeds and the time that separates start of imbibition from DNA replication (Powell et al., 2000). Thus, there must be a control point that regulates the initiation of S-phase and ensures that this metabolic point is not reached if conditions for germination are not appropriate. A parallel between the germination of aged seeds containing DNA damage and the p53-mediated G1 arrest in mammals has been proposed (Whittle et al, 2001). The extended time required for aged seeds to germinate would be an active process of prolonging G1 arrest to accomplish the repair needed in those embryos, before replication is initiated. However, a p53 homologue has not yet been identified in the entire genome of *Arabidopsis*, or in other plant species. A possible putative plant p53 might share little sequence homology with its mammalian counterparts, and therefore the immunodetection of a putative maize p53 protein using human

antibodies remains questionable. Even if, the p53 accumulation prolife found in maize germinating seeds is consistent with the pattern observed in mammalian p53 (Georgieva et al, 1994). The immunodetection results showed that following imbibition the amount of p53-like protein and also cyclin D remains constant for a few hours and then reduces abruptly to eventually disappear before root protrusion. As a consequence of this protein accumulation pattern was assumed that these proteins are essential for the establishment of G1-phase (Georgieva et al., 1994). Based on these studies was suggested that the dry embryo seeds contain at least some of the key proteins required to enter the cell cycle. After imbibition, a surveillance mechanism is induced, instigating the repair of cellular structures and DNA damage. Once this repair is completed, cell cycle proteins are activated. In case of massive damage, the repair mechanisms will persist delaying entering in the S-phase (Georgieva et al., 1994; Cruz-Garcia et al., 1998; Whittle et al., 2001).

Recently, it was found that overexpression of G protein-coupled receptor of *Arabidopsis* (GCR1) abolishes dormancy. GCR1 is modulated during the cell cycle, and was suggested to initiate signal transduction cascades leading to germination (Colucci et al., 2002). It remains a question whether GCR1 is able to respond to imbibition by triggering G1 kinase complexes, opening the gate to cell cycle activation.

The functional genomics approach

Genetic analyses identified a crucial role of ABA in seed dormancy, as well as the requirement of GAs for germination. Mutant and QTL analyses are identifying additional genes. Whether these genes with unknown functions are downstream targets of ABA and GA, or they affect seed dormancy/germination in an independent way remains mostly a question. The molecular identification of all these genes becomes important, as also the identification of more target genes. Using whole transcriptome and proteome approaches can be the most efficient way to identify target genes (Bove et al., 2002; van der Geest, 2002). Recent genomic and proteomic analysis of germinating *Arabidopsis thaliana* seeds demonstrates the effectiveness of functional genomics for investigating the complexity of developmental regulatory networks, such as the development of the embryo into a young plant (reviewed by Bove et al., 2002; van der Geest, 2002). Microarray analysis, containing genes expressed in developing *Arabidopsis* seeds, were described by Girke et al. (2000). These microarrays revealed many genes of unknown function that are highly expressed in seeds. Initial experiments showed that one quarter of the seed derived genes were preferentially expressed in seeds, as compared to roots and leaves. Gene expression in *A. thaliana* and *Brassica napus* seeds was compared by additional hybridization experiments, which showed that expression patterns correlate well between the two species, indicating that those species share high gene sequence homology (Girke et al., 2000). Also to provide a broad analysis of gene expression occurring during seed germination, a cDNA microarray was produced containing genes from immature and imbibed *Brassica napus* seeds (van der Geest, 2002). This array was used to monitor gene expression of *B. napus*, *B. oleracea* and *Arabidopsis* seeds following imbibition in water. Initial

experiments revealed that during germination, prior to radicle protrusion, the expression of about half of the genes had increased, while a decrease in mRNA levels was observed for only less than 10% of the genes (van der Geest, 2002).

On the other hand, Gallardo and coworkers (2001) have initiated a proteomic analysis of the *Arabidopsis* seed germination. Two-dimensional gel electrophoresis was used to resolve and analyze seed proteins and the changes in their abundance during germination. Several of these proteins were subsequently identified by matrix-assisted laser-desorption/ionisation time-of-flight (MALDI-TOF) mass spectroscopy. Protein extracts from mature dry seeds and from seeds imbibed for 1 or 2 days were compared in order to follow the pattern of changes in protein expression in quiescent mature seeds, in imbibed germinating seeds and after radicle protrusion, respectively. Most of the proteins found were present in dry seeds, and their abundance remained constant throughout the germination process. The germination process therefore appeared to be associated with modifications in the abundance of only a limited number of proteins, supporting the idea that dry seeds are essentially ready to germinate (Gallardo et al., 2001). Thus, those results suggested that resumption of metabolic activity during germination may rely mainly upon proteins that are stored during seed maturation. The same kind of approach was undertaken to examine the role of gibberellins in the germination of Arabidopsis seeds (Gallardo et al., 2002). A proteomic analysis of GA-deficient seeds led to the identification of proteins whose abundance depends on GA action. This study indicated that GAs do not directly participate in many of process involved in germination, such as the initial mobilization of reserves. On the contrary, GAs seems to be implicated in mechanisms that promote cell radicle protrusion. Moreover, this study has showed that GAs are not essential for the activation of cell cycle progression in the germinating embryo cells (Gallardo et al., 2002).

Comparison of proteins and mRNAs identified by proteomics and microarrays with sequence databases may give some working hypotheses about the mechanisms of seed germination, but their biological function in germination remains to be proved. Identifying knockout mutants in the gene of interest is still a promising approach for meeting this challenge. As many *Arabidopsis* knockout lines do not show altered phenotypic traits in standard growth conditions, a wide range of physiological studies and the integration of the mutation into a dormant ecotype of *Arabidopsis* are still required to find mutants with altered germination phenotypes and to obtain functional evidences.

The combination of transcriptome and proteome analysis with reverse genetics will provide the resources to characterize the regulatory genes implicated in seed germination (Bove et al., 2002; van der Geest, 2002).

RESEARCH OBJECTIVES

By definition, germination commences when the quiescent dry seed begins to take up water (imbibition) and is completed when the embryonic axes elongates. The visible sign that germination is completed is usually the penetration by the radicle of structures surrounding the embryo, which marks the start of seedling development. Apart from seed swelling, not much can be observed within a seed between the start of water uptake and protrusion of the embryonic radicle. Cell cycle activity is certainly a requirement for growth and development, but it remains to be clarified whether the initiation of cell cycle activity is one of the "hidden" events occurring prior to root protrusion. For several plant species, it has been demonstrated that particular cell cycle activities, like DNA replication and β-tubulin accumulation, precede radicle protrusion, however halting of these processes does not always prevent the completion of germination.

It was our objective to determine when the cell cycle is activated during seed germination, and what the importance is of cell cycle activity for proper seedling establishment. Three main experimental approaches were undertaken in order to achieve these goals: (1) expression analysis of cell cycle genes, (2) monitoring DNA replication using flow cytometry, and (3) study of the microtubular cytoskeleton organization following imbibition. To thoroughly assign the precise spatial and temporal expression profiles of the different cell cycle genes during seed germination, expression studies were performed during early germination in *Arabidopsis thaliana* and cabbage seeds using a large set of cell cycle control genes from *Arabidopsis*. Flow cytometry studies were performed to monitor DNA replication and identify the position of the embryo cells within the cell division cycle. Because the distribution and rearrangement of the microtubular cytoskeleton is cell cycle associated, the dynamic reorganization of microtubules during germination was also investigated. The analysis of the dynamic organization of the microtubular cytoskeleton during germination intended to determine whether cell division is implicated in the growing of the embryo leading to radicle protrusion, and additionally to clarify how the organization of microtubular cytoskeleton might be associated to seed germination.

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Chapter 6

Profiles of Cell Cycle Gene Expression in Germinating Seeds

Adapted from "Profiles of cell cycle gene expression in germinating seeds" by Rosa Maria Barrôco, Kris van Poucke, Jan HW Bergervoet, Steven PC Groot, Dirk Inzé and Gilbert Engler. In preparation.

Abstract

Seed germination is a process by which the embryo within the seed resumes growth after a period of quiescence. In an attempt to fully understand how cell cycle activities are resumed during germination, we have made use of molecular markers for studying cell cycle progression preceding and following root protrusion. We have performed an extensive expression analysis in *Arabidopsis thaliana* and *Brassica oleracea* seeds, during the process of germination by using different *Arabidopsis* cell cycle control genes. Flow cytometry studies were performed to assess the synchrony/position of the embryo cells within the cell division cycle. Since also, the distribution and rearrangement of the microtubular cytoskeleton is cell cycle associated, the dynamic reorganization of microtubules during germination was investigated using the microtubule-binding domain (MBD) of the microtubule–associated protein 4 (*MAP4*) gene fused to the green fluorescent protein (*GFP*) gene.

The cell cycle associated cellular and structural changes that accompany germination and early seedling development could be described. Before imbibition, cell division does not occur and embryonic cells remain arrested in the G1-phase of the cell cycle. Early during imbibition microtubular cytoskeleton networks are promptly assembled, and preparation for cell expansion is initiated. Cellular expansion induces the rupture of the embryo covering layers at about the same time when nuclear replication is initiated. Subsequent, growth of the radicle through the seed coat is followed by further progression in the cell cycle, and entering in mitosis.

Abbreviations: CDK, cyclin-dependent protein kinase; CKS; CDK subunit; Cyc, cyclin; E2F, adenovirus E2 promoter binding factor; GFP, green-fluorescent protein; KRP, Kip-Related Protein; ORC, origin recognition complex; RBR, retinoblastoma-related; SAM, shoot apical meristem.

Introduction

The building of plant form and function depends on the ability of the embryo cells to divide and differentiate. The decision of individual cells to re-enter the cell cycle or to remain arrested is decisive to determine the formation of a seedling. Integration of cell cycle gene networks in plant development became crucial, and understanding how the cell cycle genes work at this particular phase of plant development might mean the understanding of how a metabolic cascade brings a dried embryo to a metabolic active plant.

The control of the cell cycle in plants is the responsibility of a conserved set of protein complexes, which key components are the cyclins (Cyc) and the protein dependent kinases (CDKs) (reviewed by Mironov et al., 1999). Cyclins, named after their cyclical expression and degradation, are proteins with a supreme role in the cell division cycle. They are generally synthesized just before their activity is initiated, and after being active they are quickly degraded through ubiquitination. The cyclins play a role as catalytic subunits of many CDKs. Interaction between the cyclins and the CDKs occurs at specific stages of the cell cycle, and their activities are required for progression through the cell cycle. Additionally to cyclin binding, multiple mechanisms have evolved that specifically regulate CDK activity to maintain the correct temporal order of crucial cell cycle events such as DNA replication or nuclear division (reviewed by Stals and Inzé, 2001).

The plant cell cycle consists of alternating phases of DNA replication (S-phase) and mitosis (M-phase), and appropriate control mechanisms operate before entrance in both phases to ensure correct cell cycle progression. G1-phase defines the interval between mitosis and S-phase, and represents the major point of commitment to cell division. In this phase a cell can either continue cell cycle progression or exit from the cell cycle to become quiescent or differentiate. Cells can remain in this stage permanently or only temporarily, as it is the case of quiescent dry seeds. Therefore, it is normally assumed that G1 is the gate where cells resume cell cycle progression after a non-proliferative period. Specific CDKs and cyclins are involved in G1 entry and G1/S transition (reviewed by Rossi and Varotto, 2002). A-type CDK (CDKA;1), D-type cyclins (CycD) and some members of A-type cyclins seem to be key cell cycle drivers of G1-phase (reviewed by Stals and Inzé, 2001). CDKA;1 is the homologue of mammalian CDK1 and contains a conserved PSTAIRE amino acid motif in the cyclin-binding domain. Contrarily to B-type CDKs which specific control G2/M transition, A-type CDKs regulate both G1/S and G2/M transition (Hemerly et al., 1995; Magyar et al., 1997; Porceddu et al., 2001). CDKA;1 transcript and protein level remain constant during cell cycle progression while CDKA;1 kinase activity increases throughout S and G2 and peaks at early M-phase (Mironov et al., 1999).

The sequential action of several D-type cyclins is essential for the passage through the G1-phase. D-type cyclins act by integrating extracellular signals to mediate progression through the G1/S switch (Meijer and Murray, 2000). Plant D-type cyclins are more divergent than their animal counterparts and it has been suggested that different members of D-type cyclins alternate in

binding their CDK partner, CDKA;1, coupling nutrient availability and hormones to progression through G1 (Meijer and Murray, 2000).

CDK inhibitor proteins (CKIs) inhibit CDK activity by close association with CDK/cyclin complexes (Sherr and Roberts, 1999). Identification of *Arabidopsis* CKIs showed that all seven proteins display sequence homology to a member of the mammalian Kip/Cip family of CKIs, and therefore *Arabidopsis* CKIs have been renamed to Kip-related proteins (KRPs; De Veylder et al., 2001a). All KRP proteins bind CDKA;1, and overexpression of KRP1 and KRP2 correlates with a decrease in CDK activity (Wang et al., 2000; De Veylder et al., 2001a; De Veylder L, unpublished). The differences in domain organization and in the expression profile of the seven *Arabidopsis* KRP genes suggest that they might specifically regulate different CDK/cyclin complexes in a tissue specific manner (De Veylder et al., 2001a).

At G1 and G1/S transition one of the targets of the CDK/Cyc complexes is the pRB/E2F pathway (reviewed by Gutierrez et al., 2002; Rossi and Varotto, 2002). Further details on this interaction and the importance of pRB/E2F pathway are described in the first part of the thesis. The interaction with the pRB/E2F pathway is essential to initiate DNA replication, and therefore to enter S-phase. In S-phase, CDK/Cyc complexes seem to be crucial for the inactivation of pre-replication complexes, assuring that the DNA is faithfully duplicated only once per cycle (reviewed by Gutierrez et al., 2002). Pre-replication complexes are multiprotein structures that contain the origin recognition complex (ORC) proteins, CDC6, and the minichromosome maintenance (MCM) complex, among others. The initiation of DNA replication in strictly regulated on the pre-replication complexes, and replication licensing consists of the sequential recruitment of these proteins to the DNA replications origins (Bell and Dutta, 2002). Both, the pRB/E2F and the replication licensing pathways are connected, once E2F acts as transcription activator of the genes coding for the proteins in pre-replicative complex.

G1/S transition represents the main point of commitment to cell division. Additionally G1 seems to be the main target of the cross-talk between cell cycle activation/progression and environmental signals (Rossi and Varotto, 2002). Taking into account that seed germination implies the resumption of cell cycle progression after a period of quiescence, it seems correct to presume that entrance in G1 and activation of G1/S controls may represent an important control in early seedling development. Monitoring cell cycle gene expression, and G1- and S-phase markers in particular, will possibly help to clarify how the cell cycle is activated during seed germination, how cell cycle genes are regulated, and what their importance is for proper seedling establishment during seed germination.

The cytoskeleton plays an important role in the preparation of cell division and cell elongation (Lloyd, 1999). The microtubule monomeric subunits, tubulins, were shown to be associated with cell division and cell enlargement (Wymer and Lloyd 1996; Hasezawa and Kumagai, 2002). Microtubules (MTs) are heterodimeric polymers of α- and β-tubulin in association with other proteins know as microtubule-associated proteins (MAPs). There are many different MAPs, so their

individual nature determines which structures the microtubule may interact with and how this interaction will occur (reviewed by Lloyd and Hussey, 2001). Microtubule dynamics, their regulation during mitosis and their role in signal transduction have been largely investigated (Azimzadeh et al., 2001). Microtubules are polar polymers that continually switch between phases of elongation and shortening. This behavior has been termed dynamic instability. In higher plant cells MTs show dynamic structural changes during cell cycle progression and play significant roles in cell morphogenesis. Immunofluorescence studies using antibodies against tubulin have shown that the progression through the cell cycle is associated with changes in microtubule organization (Hussey et al., 1990; Traas et al., 1992). Even if, the direct relation between the microtubular cytoskeleton and cell cycle activity remains to be elucidated, β-tubulin accumulation has been extensively studied in relation to seed germination (de Castro et al., 1995; Górnik et al., 1997; Jing et al., 1999; de Castro et al., 2000; 2001). Indeed, a correlation between the rate of germination and the rate of β-tubulin accumulation in imbibed tomato (*Lycopersicon esculentum*) seeds has been demonstrated (de Castro et al., 1995). Here, we have tried to further understand how the microtubular cytoskeleton is associated to seed germination. Cortical microtubule array organization is distinct in elongating and dividing cells, whereas spindle, pre-prophase bands and phragmoplast mark exclusively mitotic cells. Thus, the observation of the microtubule arrays allows the identification of either elongating cells as well as dividing ones. One of the aims of this work was to determine whether cell division was implicated in the growing of the embryo that leads to radicle protrusion. Additionally, it was important to clarify which embryo cells contribute for either cell division or elongation. Therefore, the analysis of the dynamic organization of the microtubular cytoskeleton during germination became crucial for this work.

Seed germination is an essential process in the plant life cycle. However, the molecular mechanisms engaged in this important developmental process have only been poorly investigated at the molecular and plant genetic level. Our work intended to investigate how cell cycle activity is implicated in the triggering of molecular mechanisms leading to early seedling formation. In the present study *Arabidopsis thaliana* and *Brassica olearaceae* (cabbage) seeds were chosen to investigate the transition from the quiescent dry state to the first divisions. Although *Arabidopsis* has been generally accepted as the most important model organism for plant molecular biological research, the study of seed germination on this species encounters some practical disadvantages. Due to those difficulties, mainly related to the small size of *Arabidopsis* seeds, some of our studies were performed in *Brassica oleracea.* These two species are ontogenetically closely related and the morphology of the seeds is comparable. Previously, hybridization experiments in *A. thaliana* and *Brassica napus* seeds have shown that the expression patterns correlate well between the two species, indicating that *Arabidopsis* and *Brassica* genus share high gene sequence homology (Girke et al., 2000). Although the availability of many *Arabidopsis* mutants, including mutant lines expressing marker genes under the control of specific gene promoters, makes the study of

Arabidopsis very attractive, cabbage seeds are much larger making the analysis of gene expression in embryo tissues more feasible.

Experimental Procedures

Plant material

Dried *Arabidopsis thaliana* (ecotype Landsberg *erecta*) and white cabbage *(Brassica oleracea* L., cv. Bartolo) seeds were imbibed on a triple layer of filter paper saturated with distilled water. The seeds were germinated at 22-24^{o}C during the time periods specified (DAI-days after imbibition; HAI-hours after imbibition). A population of *Brassica* seeds were also germinated under the same conditions but at low temperatures (15^{o}C).

Cell cycle gene promoter-GUS analysis

GUS expression was analysed in germinating seeds of *Arabidospsis* mutant lines expressing the *GUS* gene under the control of specific cell cycle gene promoters. Seeds were imbibed in water for 0, 1, 2 or 3 DAI and subjected to *GUS* protein assays. The histochemical *GUS* detections were carried out according to standard protocols, with minor modifications. Seed embryos and young seedlings were incubated in 90% acetone for 2 h at 4^{o}C. After washing in phosphate buffer, the material was immersed in the enzymatic reaction mixture (1 mg/mL of 5-bromo-4-chloro-3-indolyl ß-D-glucuronide, 2 mM ferricyanide, and 0.5 mM of ferrocyanide in 100 mM phosphate buffer, pH 7.4). The reaction was carried out at 37^{o}C in the dark for a period of 4 h to overnight. Upon reaction, the material was cleared with chlorolactophenol (chloral hydrate/phenol/lactic acid 2:1:1) and observed using stereo- and conventional light microscopy.

Gene transcript localization by mRNA in situ *hybridization*

mRNA *in situ* localization research was performed using white cabbage seeds (*Brassica oleracea f. oleracea* L.). Seeds were imbibed in water for 0, 12, 24, 36 or 48h HAI, transferred to fixative and peeled. Dry seeds from the same lot (time-point 0 h) have been placed directly in fixative (3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2), without any pre-treatment, and subsequently peeled. After seed coat removal, all the seeds were transferred to fresh fixative and incubated during 12-16 hours (at 4^{o}C) for additional fixation. Fixed seeds were dehydrated through standard ethanol series, and embedded in paraffin. Embedded tissue was sliced into serial 10 μm sections and attached to coated microscope slides. ^{35}S-UTP-labelled sense (control) and antisense RNAs of *CycB1;*1, *CycD5*;1, *CycD6*;1, *E2Fb*, *E2Fa*, *CDC6*, *CDC7*, *CKS1*, *KRP1*, *KRP2* and *H4* genes were generated by *in vitro* transcription with SP6 or T7 RNA polymerases, according to the manufacturer's protocol (Roche Diagnostics, Brussels, Belgium). Full-length transcripts were reduced to 300 bp fragments through alkaline hydrolysis performed according to standard protocols.

Plant material was hybridized overnight at 42^{o}C with the appropriated antisense and control ^{35}S-labelled mRNA probes (5x10^{6} c.p.m. per slide). After hybridization, the slides were washed in 2x SSC at room temperature for 1 h and in 0.1x SSC/50% formamide at 42^{o}C for 1 h. All post-hybridization procedures, including RNase treatment and washes, were performed as described by de Almeida Engler *et al*. (2001). Signal detection was achieved by autoradiography, using Kodak NBT film emulsion. After developing, the slides were stained in 0.1% toluidine blue and mounted in DePex mounting media (BDH, Poole, England). Autoradiographs were taken under bright- and dark-field illumination using a Diaplan microscope (Leitz, Heerbrugg, Switzerland).

Whole-mount mRNA* in situ *analysis

Whole-mount *in situ* hybridization was performed essentially as described by de Almeida Engler et al. (1998). Arabidopis germinating seeds and young seedlings were fixed for 30 min at RT in 1x PBS solution containing 0.1% Tween 20, 0.08mM EGTA, 10% DMSO and 5% paraformaldehyde. After dehydration, samples were stored at -20°C until hybridization. Digoxigenin (DIG) labelling of *CDKA*;1, *CDKB1*;1, *E2Fa*, *CDC7*, *CDC6*, *ORC2* RNA probes was carried out using the Roche/Boehringer Mannheim nucleic acid labelling kit following the manufacturers instructions. Prehybridization and hybridization were carried out as described by Almeida Engler et al. (1998) with minor modifications. After washing, first with ethanol and then with methanol, the tissues were soaked in a 1:1 mixture of methanol/PBT (PBS containing 0.1% Tween 20) for 10 min. After three washes in PBT for 2 min each, samples were digested with 40μgml^{-1} proteinase K for 20 min. Digestion was stopped by washing twice with PBT for 5 min. Thereafter, samples were transferred stepwise into hybridization solution [HS: 50% formamide, 5x SSC (750mM NaCl, 75mM sodium citrate pH 7.0), 50μgml^{-1} heparin] by incubation for 10 min in a 1:1 PBT/HS solution, rinsing twice in HS, and prehybridization in HS for 2 h at 62°C. For hybridization samples were incubated in HS containing DIG-labelled probe (approximately 30 ngμl^{-1}kb^{-1}) and 5mgml^{-1} herring sperm DNA for 16 h at 60°C. Probe and sperm DNA were first denatured at 85°C for 5 min.

After hybridization, the material was washed twice in fresh HS for 30 min at 60°C. The tissue was then washed for 60 min in HS/NTE (1:1) at RT and repeated with NTE (0.5mM NaCl, 10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0). An RNase A digestion (40 μgml^{-1}) in NTE was carried out for 45 min at 37°C. Digestion was stopped by washing with NTE at 37°C for 15 min. Final washes were conducted in 2x SSC for 30 min at 45°C, and twice in 0.2x SSC for 45 min at 62°C. For signal detection, samples were equilibrated in PBT for 5 min and blocked for 1 h in PBT containing 2% BSA. The tissue was then incubated with gentle agitation for 16 h in PBT containing a sheep anti-DIG alkaline phosphatase-conjugated Fab fragment antibody (Roche/Boehringer Mannheim) diluted 1:2000. After four washes for 60 min with PBT, the tissue was equilibrated in detection buffer (100mM Tris-HCl pH 9.5, 100mM NaCl, 0.1% Tween 20) for 5 min. Detection solution was prepared by adding 5μl nitroblue tetrazolium chloride (100mgml^{-1} stock solution) and 3.75μl 5-bromo-4-chloro-3-indolyl-phosphate (50mgml^{-1} stock solution; both from Roche/Boehringer

Mannheim, Germany) per ml detection buffer. Formation of the stable colour precipitate took up to 16h, revealing a blue/purple colour, and the colour reaction was monitored with a stereomicroscope. The reaction was stopped by washing in PBT with 20 mM EDTA pH 8.0. When stained, the material was cleared with chlorolactophenol (chloral hydrate/phenol/acid lactic 2:1:1) and microscopically observed and imaged.

Reverse transcriptase(RT)-PCR analysis

Total RNA was extracted from dry mature seeds and seeds at different stages of germination (1, 2, 3 and 4 DAI). Seeds of *Arabidopsis thaliana* ecotype Columbia (300 mg initial weight) were imbibed during the necessary time and promptly grinded in liquid nitrogen. RNA extraction was performed as described by Chang et al. (1993), with minor modifications. After warm extraction with the appropriate buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0, 2.0 M NaCl, 0.05% spermidine) the RNA was purified with chloroform:isoamylalcohol (24:1), followed by precipitation with 10 M LiCl. The RNA pellet was then redissolved in SSTE (1M NaCl, 0.5% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and again purified using a mixture of chloroform:isoamylalchool (24:1). After ethanol precipitation for 2 h at –20^{o}C the RNA samples were resuspended in nuclease-free water. Two micrograms of each sample were reverse-transcribed (RT) into cDNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogene). Semi-quantitative RT-PCR amplification of the cDNA was carried out with the ABI Prism 7900 real-time PCR machine (Applied Biosystems; Foster City, CA, USA), using the following (forward and reverse, respectively) gene-specific primers: 5′-ATATCTTTACCAGATTCTCCGTGGAA-3′ and 5′-GAGTTTGTGCGGCGATCAAT-3′ for *CDKA*;1; 5′-TTCAGAAGTTGATGTTTCAGCTTTG-3′ and 5′-GAAGCTCTTTATCTTTCACCAGAAGAA-3′ for *CDKB1*;1; 5′-GCAAACGTACCTGAACAAGTCAGA-3′ and 5′-CTTGAACTCCGGGAACAGAAAC-3′ for *CycB1*;1; 5′-TGTAATCATCACTTGGCAAATGC-3′ and 5′-ATTGTTGACGAAACTCCGATTGA-3′ for *CycD4*;1; 5′-TCACTGGAAAGACCATTACTCTTGAA-3′ and 5′-AGCTGTTTTCCAGCGAAGATG-3′ for *UBQ14*. Direct detection of PCR products was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green I dye to double-stranded (ds) DNA (SYBR Green PCR core reagents; Applied Biosystems). Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction.

The amount of target cDNA used for PCR was standardized by quantification of ubiquitin14 transcripts present in all the samples. However, it must be emphasized that ubiquitin levels are not absolutely stable during germination. The ubiquitin14 relative expression ratio between germinating and dry *Arabidopsis* seeds is about 2.4, as detected by cDNA microarray analysis (Lonneke van der Geest, personal communication). However, this fluctuation has not been considered and therefore, the values presented must be regarded as fairly accurate values. This analysis does not intend to quantify transcript levels on seeds. On the contrary, it aims to illustrate the transcription profile of main cell cycle genes during germination.

To facilitate the comparison between the different mRNA levels of the four genes analyzed, all values have been adjusted to a relative final value of 100 (time-point 4d). This adjustment allowed the graphic representation of transcript fluctuation from the four genes in a single graph, and the easy comparison of the respective profiles.

Flow cytometry

Relative DNA content of the seed nuclei was measured in *Arabidopsis thaliana* seeds imbibed from 0 to 72 hours. Suspensions of intact nuclei were collected with an 8 hours interval. Samples of at least 20 seeds were chopped with a razor blade in ice-cold nucleus-isolation buffer (10 mM $MgSO_4.7H_2O$, 50 mM KCl, 5 mM Hepes, 1 mg/ml DTT and 2.5 mg/ml Triton X-100, 1% (w/v) PVP-40) on ice. After chopping, the suspension was sieved through an 88µm nylon mesh. After digestion with RNAse, for 30 minutes at room temperature, the samples were stained with propidium iodide (1 mg /ml) for 15 minutes. DNA analyses were performed with a Beckman-Coulter EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA) equipped with an argon ion laser at 488nm. The amount of DNA, proportional to the red fluorescent signal, is expressed as arbitrary C values in which 1C represents the amount of DNA of the unreplicated haploid chromosome complement. The number of nuclei present in each peak of the histogram, 2C, 3C, 4C, 6C and 8C, was analysed by measuring the peak area. Histograms were processed using ModFit LT (Verity, USA) for data analysis and correction of the background noise, and the volume of each histogram was calculated.

Confocal microscopy

Arabidopsis thaliana (ecotype C24) transgenic seeds carrying a chimeric gene containing the *GFP* gene fused to the microtubule-binding domain (MBD) of *MAP4,* under the control of the 35S-promoter were kindly provided by M. Karimi. Transgenic seeds expressing *35S-GFP-MDB* were imbibed in water, as described above, and carefully peeled at different time-points. For time-sequence observations, the seed embryos were sealed in an approximately 2-mm-thick slide chamber (Lab-Tek, Christchurch, New Zealand) containing 1.5% low-melting point agarose (Sigma). This environment allowed the normal development of seed embryo for at least 48 hours, as confirmed by the imaging of fresh peeled seeds after imbibition in water for the same time period. The rearrangement of CMTs was recorded at the peripheral epidermal cell layer of the embryo radicle and cotyledons. Images were obtained by confocal laser scanning microscopy (Zeiss LSM510) using an Argon laser for 488 nm excitation, a 505-530 nm emission filter and a water immersion 63x Plan-Apochromat objective, with exception of image in Fig. 13G which was obtained with a 10x Plan-Neofluar objective. Images of embryonic epidermal cells are presented as single sections or as z-stacks of consecutive sections as stated in the figure legends.

Results

Nuclear DNA content of embryo cells from **Arabidopsis** ***germinating seeds***

Flow cytometry histograms from nuclei of dry control *Arabidopsis* seeds show one large peak, corresponding to the 2C DNA content (Fig. 1). A 2C DNA content can be found in nuclei after mitotic division and during G1 arrest while cells in which DNA has replicated have two or four times the 2C DNA contents. A very small peak with slightly more fluorescence representing the nuclei with 3C DNA content can also be observed in the same histogram. This peak represents the relative DNA content of nuclei from endosperm cells. Upon 8h imbibition in water, a peak corresponding to 4C nuclei starts to become visible. However, a clear increase of the 4C percentage is only observed after 40 hours imbibition, coinciding with the onset of germination (Fig. 1; Graph 1).

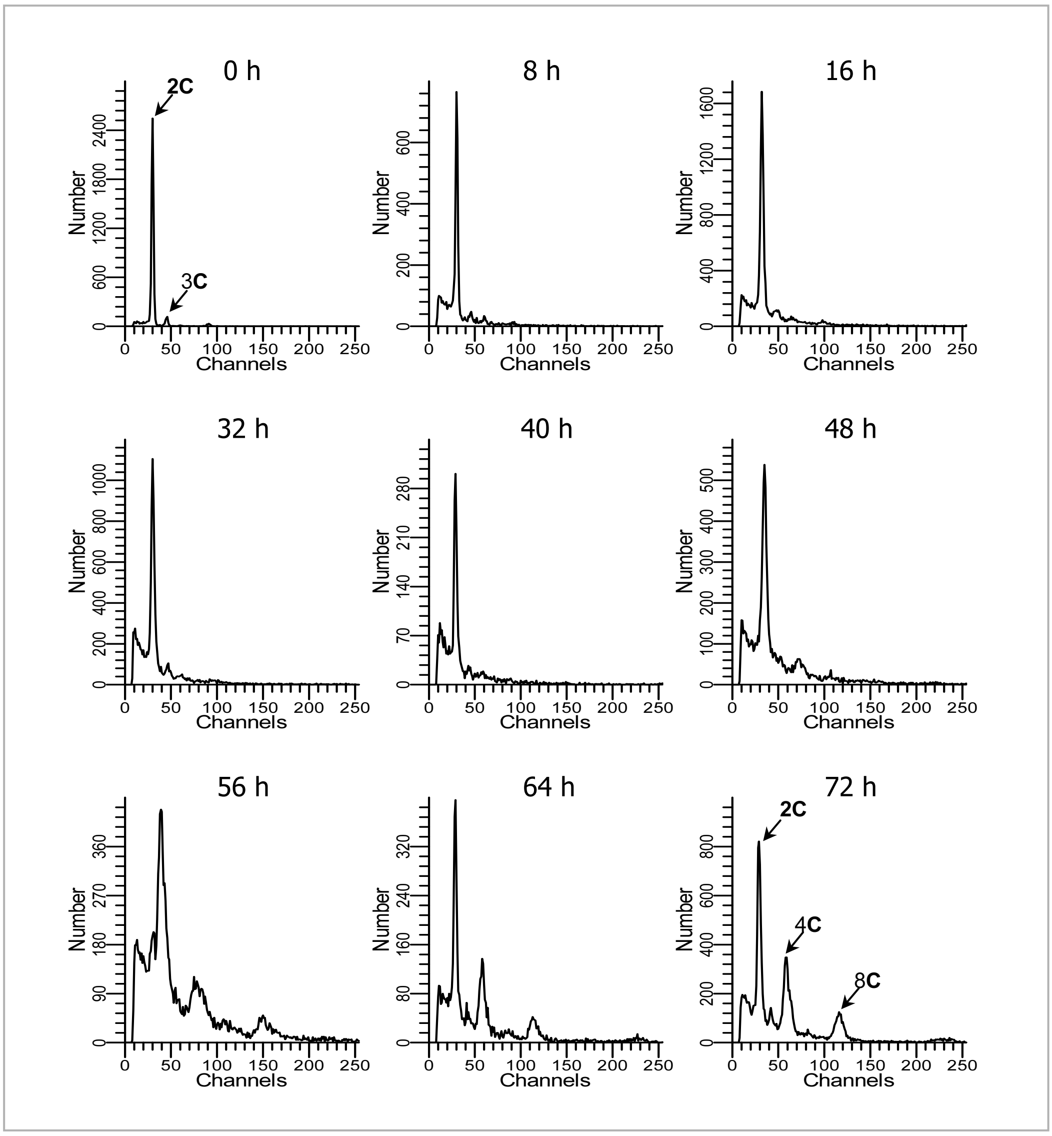
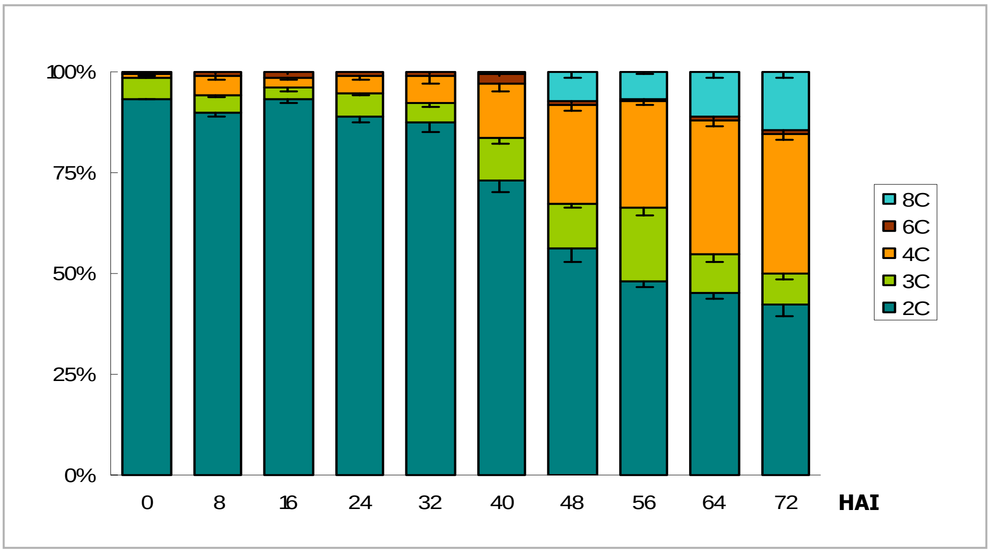
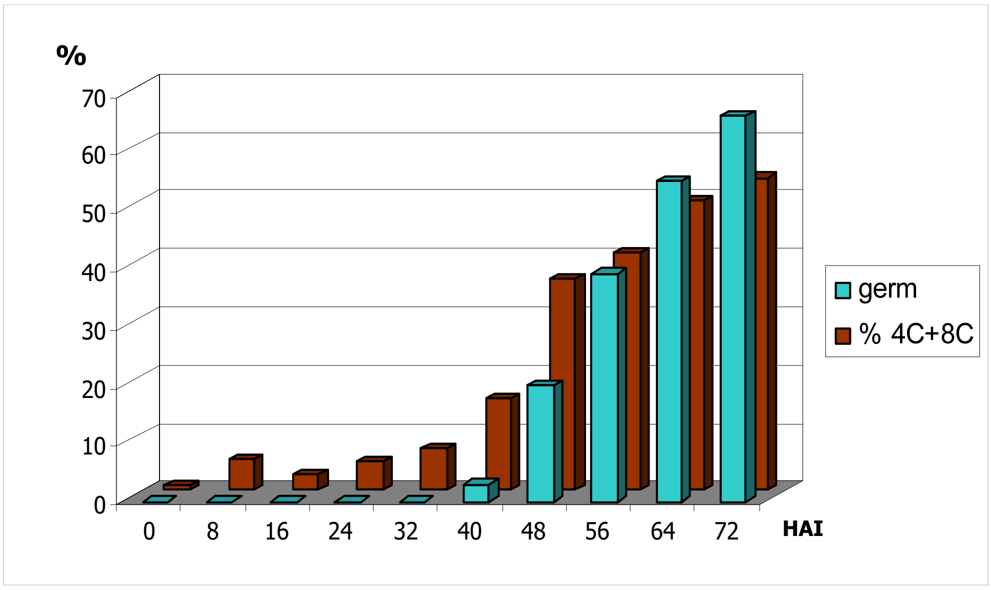


Figure 1. *Nuclear DNA content profiles of A. thaliana germinating seeds. The positions of the 2C and 3C (endosperm nuclei) are indicated in the graph from dry seeds (0 h) and the position of 2C, 4C and 8C peaks are indicated in the 72 h graph.*



Graph 1. *Percentage of cells from germinating Arabidopsis seeds displaying a 2C, 3C, 4C, 6C or 8C nuclear DNA content.*

The relative amount of nuclei from which the DNA has replicated can be expressed as the percentage of 4C+8C to the total amount of embryo nuclei [4C+8C/(2C+4C+8C)]. This coefficient can be used as a direct measure of the nuclear DNA replication activity upon seed imbibition (Graph 2). The results show that the percentage of embryo nuclei from dry seeds subject to DNA replication is negligible (0.7%). Following imbibition, DNA replication increases to up to 5-7% of the nuclei. A clear increase in the amount of nuclei going through S-phase is only visible after 40 hours of imbibition. Thus, a clear transition through S-phase towards G2 is only detected around the moment when radicle protrusion starts (Graph 2).



Graph 2. *Graphic representation of the percentage of embryonic cells from which the DNA has gone through replication [calculated as the ratio (4C+8C)/(2C+4C+8C)]. In parallel is also shown the percentage of seeds which the radicle has protruded at the defined time-points. [X-axis, time (HAI); Y-axis, percentage (%)].*

Cell cycle gene expression analysis by mRNA in situ hybridization

Extensive mRNA *in situ* localization studies of several Arabidopsis cell cycle gene transcripts (*CycB1;*1, *CycD5*;1, *CycD6*;1, *E2Fb*, *E2Fa*, *CDC7*, *CDC6*, *CKS1*, *KRP1*, *KRP2*, and the histone *H4* gene) were performed in *Brassica* seeds as target tissues (Fig. 2, Tab. 1). In *Brassica* seeds germinated at optimal temperatures radicle protrusion was initiated just after 24 hours of imbibition in water. In seeds germinated at 15^{o}C radicle protrusion was delayed for about 16 hours, occurring only at 40 hours of imbibition.

Our results illustrate that the *CycB1;1* mRNA distribution pattern corresponds to the *GUS* pattern observed in *Arabidopsis* transgenic seeds harboring *GUS* gene under the control of *CycB1;1* promoter. Expression of this mitotic cyclin is mainly confined to the meristematic regions, both in shoot and roots, but also in the pericycle cells, which will probably form lateral roots (Fig. 2A-2C). Also histone *H4* mRNA has been found in the main meristematic regions of the young seedling (Fig. 2D-2G). The induction of these genes could not be detected prior to seed coat opening and, only 12h after protrusion strong defined *in situ* signals were visible.

The *KRP* (KIP-related protein) genes encode proteins with an inhibitory role on cell cycle activity (De Veylder et al., 2001a). Our studies have shown that expression of *KRP1* gene in *Brassica* seeds is only established after radicle protrusion. This gene is expressed in epidermal tissues, predominantly in the SAM region but also in the hypocotyls (Fig. 2H-2K). No expression could be detected in the cotyledons (Fig. 2H-2J).

Like *KRP1*, also *CDC7* expression is restricted to the epidermis of the hypocotyls and basis of the cotyledons (data not shown). However, *CDC7* expression was hardly detectable. This kinase might be very weakly expressed or its expression is only significantly activated later in development.

In contrast, *KRP2* is strongly expressed in the epidermis of all seedling tissues (Fig. 2L-2P). However, *KRP2* transcripts are always not uniformly distributed throughout the epidermis but concentrated in some regions of the cotyledons or the hypocotyls (Fig. 2L, 2N and 2O). Moreover, *KRP2* expression is very early visible throughout the epidermis of leaf primordial (Fig. 2P). *KRP2* mRNAs were also observed in the hypocotyl surrounding the vascular tissue (Fig. 2N and 2O). In all tissues described *KRP2* is only observable after the radicle has emergered from the seed coat.

CKS1 mRNA transcripts were also found to be mainly confined to the embryo epidermal cell layers (Fig. 2Q-2T). Similarly to *KRP2*, also the expression of *CKS* did not uniformly distribute over all epidermal cells, and frequently a patchy expression pattern was observed (Fig. 2Q and 2R).

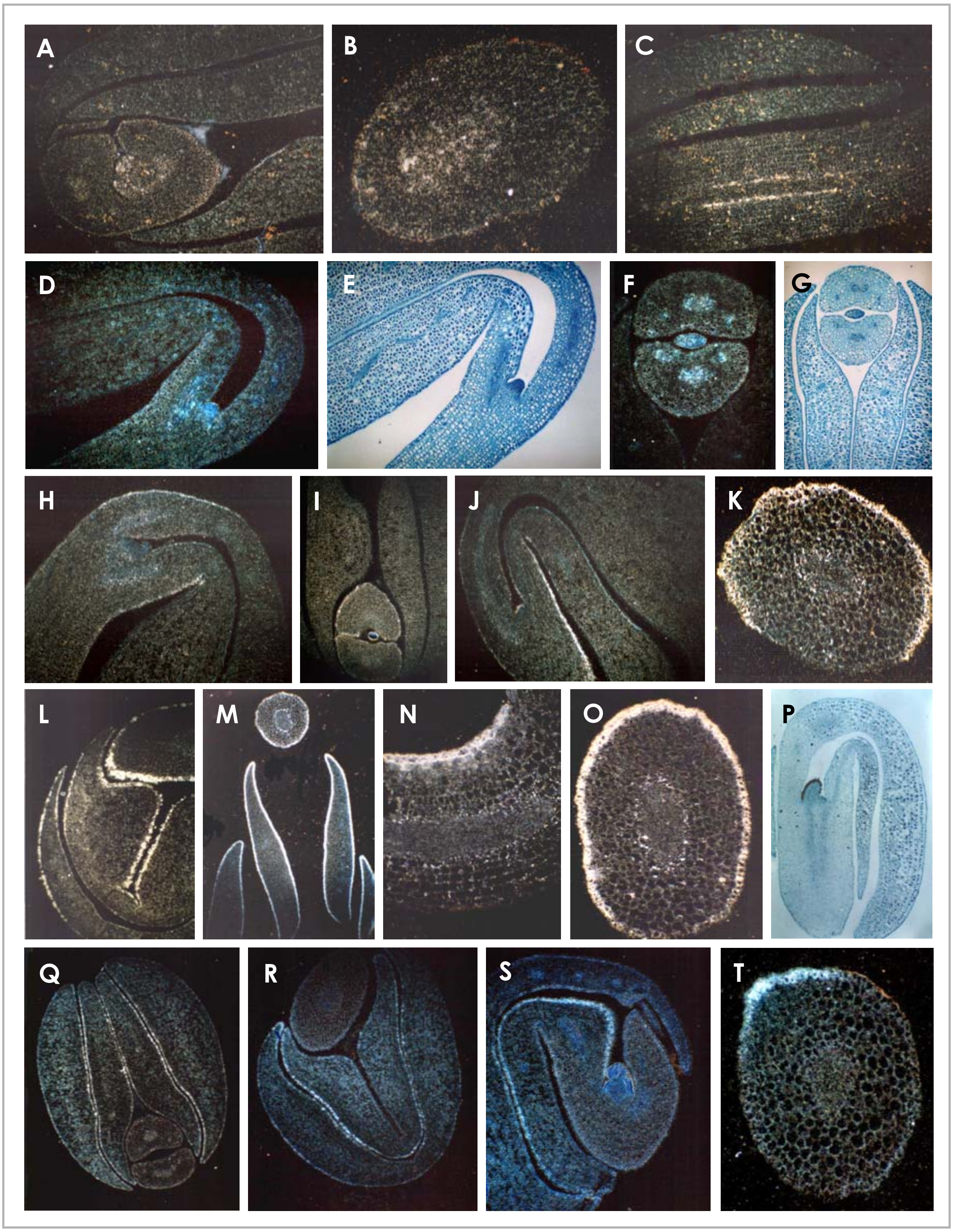
E2F genes code for transcription factors and play an important role in the regulation of proteins implicated in the G1/S transition (Gutierrez et al., 2002). In the germinating embryo, *E2Fa* gene expression seems to be excluded from the root meristem, whereas transcripts are present nearly everywhere in the root and hypocotyls (Fig. 2A′ and 2B′). In both tissues, *E2Fa* is strongly expressed in a restricted number of cells of the vascular bundle (Fig. 2C′ and D′). Expression was also observed in the peripheral endosperm both before and after germination (Fig. 2L′-2O′).

CycD5;1 gene expression could only be observed in the endosperm of both dry and imbibed seeds (Fig. 2L′-O′). Expression in embryonic tissues might only be initiated later in development. Likewise, also *CycD6*;1 and *E2Fb* mRNA transcripts accumulate in the peripheral endosperm cells (Fig. 2L′-O′). Besides, *CycD6*;1 and *E2Fb* are expressed in the embryo epidermis and in a restricted number of cells of the vascular tissues (Fig. 2E′-F′ and Fig. 2G′-H′, respectively). In these tissues expression is only established after protrusion, while in the endosperm corresponding transcripts are present at all stages. The involvement of both genes in DNA replication together with their matching spatial expression pattern might be indicative for a functional relation between both genes, during early seedling establishment.

From all genes analyzed, *CDC6* transcripts were found to be expressed most early. In the radicle meristem, this gene is expressed in a clear patchy pattern and expression could be traced back to just prior and during root protrusion (Fig. 2I′ and 2J′). In later stages, expression was also observed in the epidermis of the radicle (Fig. 2K′), the epidermis of the hypocotyl and in the vascular tissue (data not shown). The truly early expression pattern of this gene can be justified by its early involvement in DNA replication. The ability to detect this gene in early stages can also be due to its high level of expression.

Expression of the majority of the cell cycle genes mentioned above was compared in two *Brassica* lines with different germination performances. It was found that in seeds germinated at optimal temperatures (22-24^{o}C), the mRNA of those genes do not show any observable difference in distribution or signal intensities among these lines. However, *in situ* analysis has been performed using seeds of both *Brassica* lines germinated at low temperatures (15^{o}C). For both lines, distinct mRNA expression levels seem to be induced in response to germination at this temperature. Differences in terms of signal intensity can apparently be observed between the lines by *in situ* analysis. Nevertheless, we are aware that mRNA *in situ* hybridization technique is not a truly quantitative method.

Whole-mount mRNA *in situ* localization protocols were optimized to allow an extensive analysis of gene expression patterns in *Arabidopsis* seeds and young seedlings. In order to substantiate our expression studies using promoter-*GUS* fusions, we have chosen to initiate our whole-mount hybridization studies with the localization of *CDKA*;1 and *CDKB1*;1. Our results have shown that *CDKA;1* mRNA distribution pattern shown by the *in situ* experiments is very similar to the *GUS* expression pattern observed in *Arabidopsis* transgenic seeds harboring *GUS* gene under the control of *CDKA*;1 promoter (Fig. 1C-1E). Expression of this CDK is mainly confined to dividing tissues. Expression is clearly visible in the shoot apical meristem but weaker in the root tip (data not shown). *CDKB1*;1 is very weakly expressed. Expression is highly confined to the root meristem, and not observed in the shoot apical meristem (data not shown). This lack of expression in opposition to the promoter activity observed in p*CDKB1*::*GUS* transgenic embryos, could be related to the low expression of the gene and the relatively limited sensitivity of the whole-mount method.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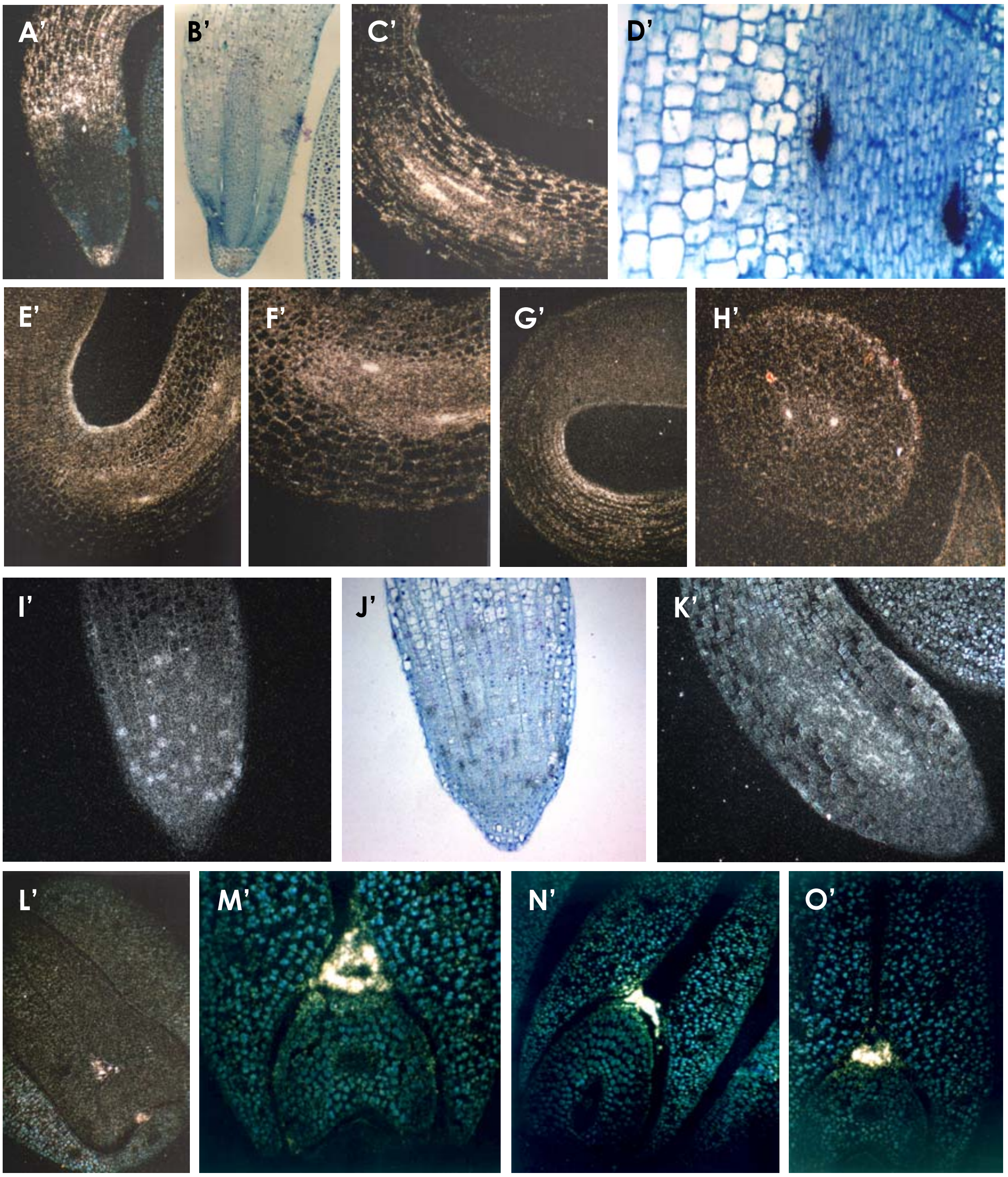


Figure 2*. Expression pattern of cell cycle genes in young cabbage seedlings as shown by mRNA* in situ *hybridization. All micrographs show sections of cabbage seedlings about 12 hours after radicle protrusion, with exception of images I' and J' which are sections of radicles just after protrusion.* ***A-C,*** *CycB1;1 transcript accumulation in the SAM region, near the radicle tip, and in the pericycle cells from which lateral roots are initiated, respectively.* ***D-G,*** *Histone H4 transcript accumulation in young cabbage seedlings. Dark- (D and F) and bright-field (E and G) micrographs of longitudinal (D and E) and transversal sections (F and G) of the SAM region, and the basis of the cotyledons.* ***H-K****, KRP1 expression pattern in cabbage seeds following root protrusion. KRP1 transcripts are localised in the epidermis of the hypocotyl (K) and basis of the cotyledons (H-J).* ***L-P,*** *KRP2 expression pattern in young cabbage seedlings. KRP2 is strongly expressed in the epidermis of all seedling tissues, including young leaf primordia.* ***Q-T****, CKS1 transcript localization in germinating cabbage seeds. Q-S, sections through the embryo cotyledons. T, transversal section of the hypocotyl.* ***A'-D'****, Expression pattern of E2Fa. Expression is spread*

throughout the root and hypocotyl cells but absent in root meristematic region (A'). E2Fa mRNAs accumulate also in some cells localised in the vascular tissue (C'and D'). ***E' and F'****. Expression of CycD6;1 in the hypocotyl epidermis (E') and in some vascular cells (F').* ***G' and H'****, Expression of E2Fb in the hypocotyls epidermis (G') and cells of the vascular tissue (H').* ***I'-K'****, Expression of CDC6 gene. Dark- (I') and bright-field micrographs (J') of the patchy accumulation pattern observed in radicle tips, soon after radicle protrusion.* ***L'-O'****, Expression in the peripheral endosperm was observed for several genes such as CycD5;1, CycD6;1, E2Fa and E2Fb.*

Genes	Expressing tissues/organs		
	Meristematic regions	Radicle/hypocotyl	Cotyledons
CycB1;1	Shoot and root (Fig 2A, 2B)	Lateral root initiation (?) (Fig 2C)	
H4	SAM and root (Fig 2D, 2E)	Vascular tissue (Fig 2D-2G)	
KRP1	Epidermis of leaf primordia (Fig 2I, 2J)	Epidermis (Fig 2H-2K)	
KRP2	Epidermis of leaf primordia (Fig 2P)	Epidermis (Fig 2L, 2M)	Epidermis (Fig 2L-2O)
CKS1		Epidermis (Fig 2T)	Epidermis (Fig 2Q-2S)
E2Fa		Radicle and hypocotyl (Fig 2A'-2D')	
CycD6;1		Epidermis and vasc. tissue (Fig 2E'-2F')	
E2Fb		Epidermis and vasc. tissue (Fig 2G'-2H')	
CDC6	Root meristem (Fig 2K')	Epidermis and cortex (Fig 2I'-2J')	

Table 1. *Schemed description of the cell cycle gene expression patterns resulting from mRNA in situ hybridisation analysis. The results in the table are followed by cross-references to images in figure 2 which illustrate the detail spatial transcript distribution of each one of the genes. The colour of the cells reflects the expression level of each gene in the different studied tissues; dark grey represents strong expression levels whereas light-grey stands for lower transcript accumulation. When no expression was observed cells were left empty.*

Expression analysis in *Arabidopsis* germinating seeds was also extended to genes implicated in DNA replication due to their early assignment to re-enter the cell cycle. We have chosen *E2Fa*, *CDC6*, *CDC7* and *ORC2*.

E2Fa expression is mainly confined to the root tip region, however, it was not possible to determine whether expression is excluded from the root meristem as observed for *Brassica*

embryos. E2Fa seems to be strongly expressed at the moment of root protrusion. When the seedling grows older, expression occurs also in other tissues and becomes gradually weaker in the radicle tip. Due to the small size and fragility of the material, it was not possible to determine if the hybridization signal is restricted to the epidermis or if the signal extends to more internal seedling tissues. Also with *Arabidopsis* seeds a strong hybridization signal was observed in the peripheral endosperm of seeds at all stages of germination.

ORC2 is very strongly expressed in the radicle tip following radicle protrusion. After germination expression is clearly visible all through the root, mainly in the root tip, but not in the hypocotyls (Fig. 3). In older seedlings strong expression can still be observed in the root tip. At this stage, *ORC2* transcripts are also observed in the hypocotyl, probably in the epidermis as suggested by the expression pattern observed in *Brassica*.

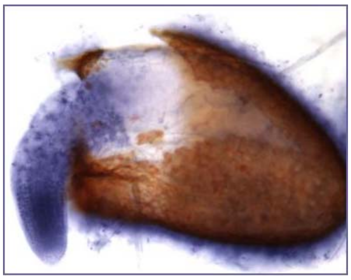


Figure 3. *Expression of ORC2 in* Arabidopsis *germinating seed as visualized by using whole-mount* in situ *hybridisation.*

The whole-mount analysis of *CDC7* expression confirms that following germination this gene is only very lowly expressed (data not shown).

CDC6 expression is apparently confined to the radicle tip at the moment of root protrusion. Shortly after germination, *CDC6* is highly expressed in the root and hypocotyl. In older seedlings expression is nearly absent. This demontrates that *CDC6* expression is early activated during protrusion, as showed by the expression studies in *Brassica*. On the other hand, this gene is also quickly down-regulated rather early during seedling development. This result might suggest that *CDC6* is subjected to strict expression control in response to germination.

Expression of cell cycle control genes by promoter-reporter gene analysis in transgenic Arabidopsis seeds

The promoter activity pattern of *CDKA*;1, *CDKB1*;1, *CycB1;1*, *CycA2;1, CycD4;1* and *CKS1* cell cycle genes was characterized in detail by performing GUS enzymatic assays. Seeds of the *Arabidopsis* mutant lines expressing the *GUS* gene under the control of the cell cycle gene promoters were imbibed in water and screened for *GUS* expression. In those seeds radicle protrusion was initiated in average at 1.75 days (42 hours) of imbibition.

For most of the genes analyzed expression is already visible in dried seeds. In p*CDKB1*::*GUS* dried seeds, expression is extremely weak and restricted to the tip of the embryo radicle (data not shown). Immediately after the root has protruded, *CDKB1;1* promoter is active in the SAM and the root meristem (Fig. 4A). Additionally, the *GUS* expression pattern reveals that *CDKB1;1* is present in the stomata cells of the cotyledons (Fig. 4A and 4B). Contrarily, *CDKA*;1 and *CycA2;*1 promoter activity is nearly overall observed in the dry seeds (data not shown). During germination their activity is slowly turned off in most of the embryonic tissues (Fig. 4C and 4F). In germinated seeds and young seedlings, *CDKA*;1 and *CycA2;1* promoter activity becomes restricted

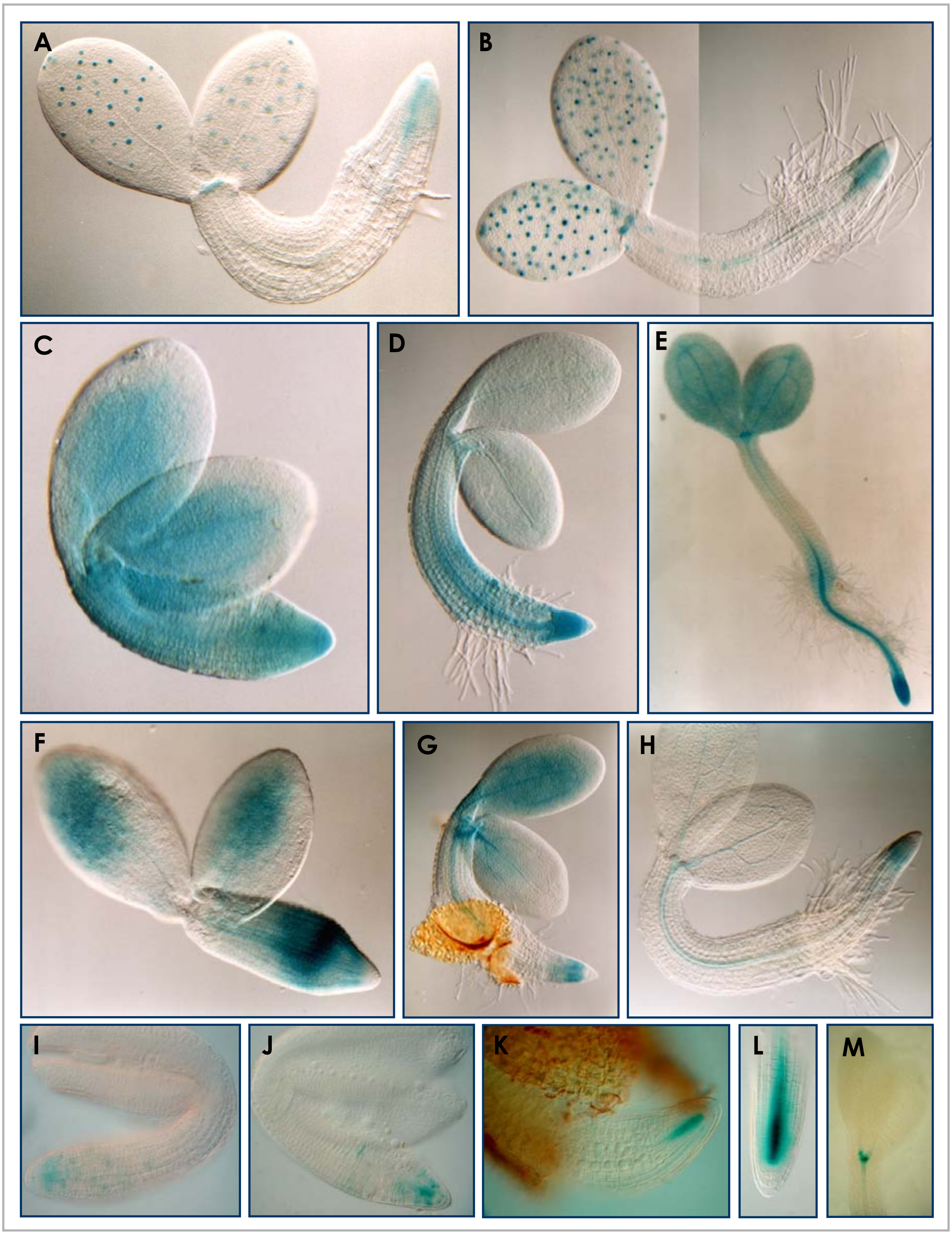
to dividing tissues (the root and shoot apical meristems and the vascular tissue) (Fig. 4D and 4E, Fig. 4G and 4H, respectively).

GUS expression driven by the *CycD4*;1 is already observed in dry seeds. Although weak, the *GUS* expression is at this stage spread through the radicle tip and a few cells of the upper part of the radicle (Fig. 4I). In seeds imbibed for one day, *CycD4*;1 promoter activity is mostly observed in the radicle meristem (Fig. 4J). Once germination is completed, *GUS* expression is exclusively confined to the radicle meristem (Fig. 4K). As the seedling starts to develop, *CycD4*;1 promoter activity remains in the radicle meristem, but is also detected in the SAM (Fig. 4L and 4M).

p*CKS1*::*GUS* activity was also present in most embryo tissues before root protrusion (data not shown). After two-days of imbibition, *CKS1* promoter activity has gradually switched off in most embryo tissues and at the moment of root protrusion activity is mainly localized in the cotyledons (Fig. 4N). After germination GUS activity becomes restricted to the cotyledons and vascular tissue of root, being fully absent in all hypocotyl tissues (Fig. 4N and 4O). About one-day later GUS activity gradually disappeared from the basis of the cotyledons (data not shown). *CKS1* promoter activity was never observed in the shoot or root meristem.

CycB1;1 is a mitotic cyclin, which expression has been shown to be confined to actively dividing meristems. However, during seed germination, this gene promoter showed to be only activated late after radicle protrusion (Fig. 4Q and 4R). Following root emergence, *GUS* expression is restricted to very few cells of the root meristem (Fig. 4S). Two to three days later *CycB1*;1 promoter activity is easily detectable in the root tip and shoot apical meristem (Fig. 4T-4W). In young seedlings, *CycB1*;1 promoter is strongly active during lateral root formation (Fig. 4Y and 4Z).

Histochemical assays in transgenic p*KRP4*::*GUS* plants showed that this cell cycle inhibitor gene is not expressed during the early steps of seedling development. This finding was confirmed by *in situ* hybridization analysis.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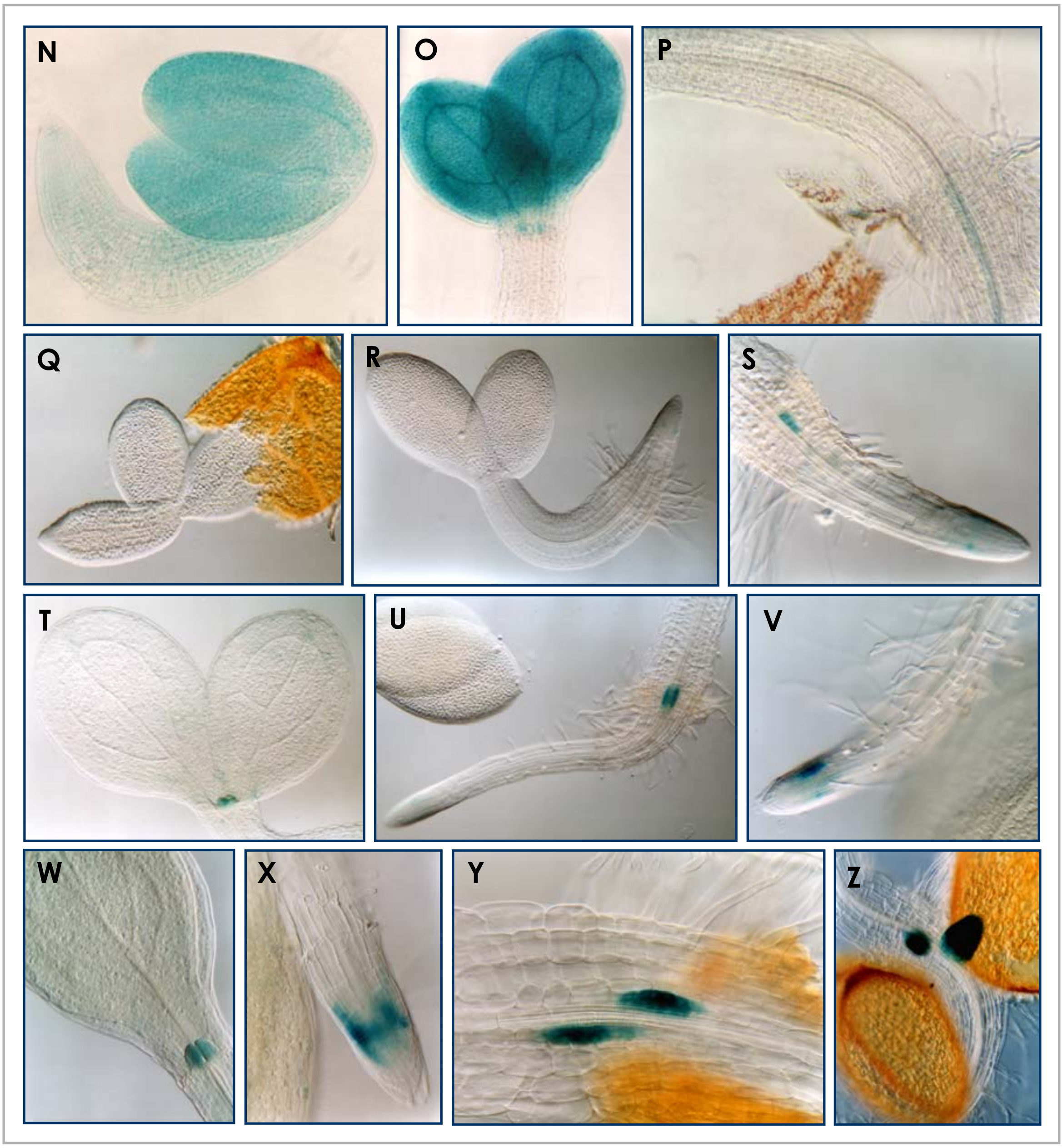
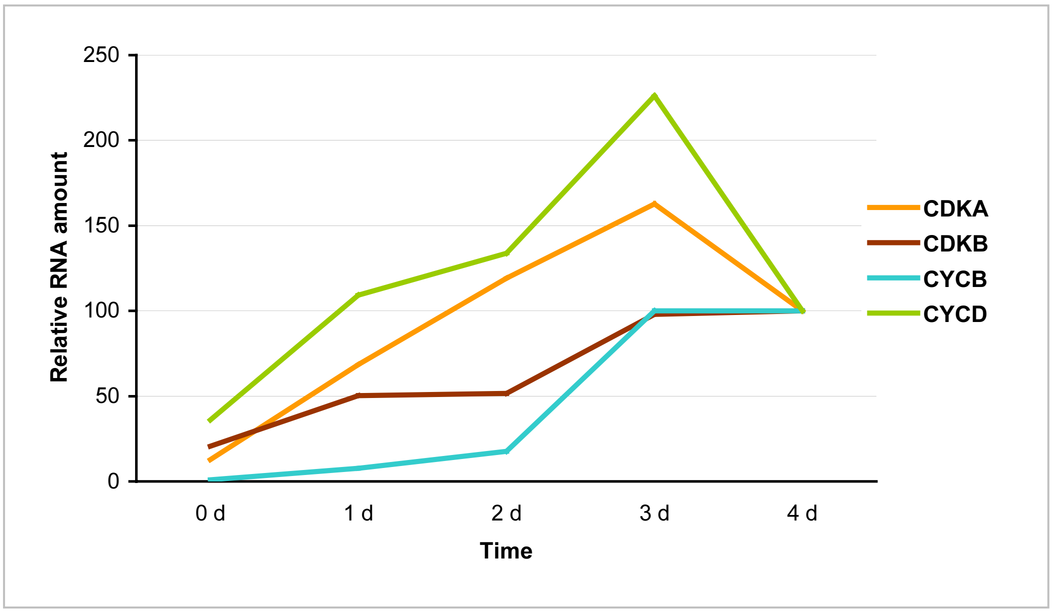


Figure 4. *Promoter activity of cell cycle genes in* Arabidopsis *seeds and young seedlings as revealed by GUS enzymatic assays.* ***A and B****, CDKB1;1 promoter activity in* A. thaliana *during early germination (2 DAI and 3 DAI, respectively).* ***C-E****, Promoter activity of CDKA;1 in germinating* A. thaliana *seeds (1 DAI, 2 DAI and 3 DAI, respectively).* ***F-H****, Histochemical GUS detection of CycA2;1 promoter activity in germinating* Arabidopsis *seeds (1 DAI, 2 DAI and 3 DAI).* ***I-M****, GUS expression driven by the CycD4;1 promoter in dry seeds (I), and seeds imbibed for 1 (J), 2 (K) and 3 days (L and M).* ***N-P****, CKS1 promoter activity in germinating* Arabidopsis *seeds, 2 DAI (N) and 3 DAI (O and P).* ***Q-Z****, CycB1;1 promoter activity in* Arabidopsis *germinating seeds and young seedlings, 1 DAI (Q), 2 DAI (R), 3 DAI (S), 4 DAI (T and U), 5 DAI (V and W), and 6 DAI (X-Z).*

Analysis of cell cycle gene transcript profiles by RT-PCR

To further validate our expression studies, the mRNA levels of key cell cycle genes were further analysed by RT-PCR. Five time-points after seed imbibition were selected, covering seed germination all through early seedling development. Specific transcript accumulation patterns have been identified for the four cell cycle regulators analysed: *CDKA*;1, *CDKB1*;1, *CycD4;1* and *CycB1*;1 (Graph 3). *CycD4;1* expression was found to abruptly rise from the instant when seed imbibition was initiated. *CycD4*;1 expression peaks at day 3 after imbibition, after which transcript levels clearly drop. Based on its expression profile, this cell cycle regulator seems to be essential for early seed germination and the resuming of embryo growth. *CDKA*;1 shows a very similar profile of gene expression. However, the increase and decrease of its expression levels is less abrupt. This gene might be also implicated in the early activation of seedling growth.

On the contrary, the rise of *CycB1;1* and *CDKB1;1* expression levels is far less abrupt. *CDKB1*;1 transcript levels steadily increase during the initial four days following seed imbibition. This increase is slightly more abrupt between day two and day three, coinciding with radicle protrusion. *CycB1*;1 expression is nearly absent during the first two days after seed imbibition. Between day three and four, the expression levels rapidly increase. Thereafter the expression of this cyclin remains unchanged. Transcription of *CycB1*;1 seems to be rigorously controlled in time. Expression suddenly peaks at the moment of radicle protrusion. This expression peak might relate to the initiation of cell division events. These expression results largely corroborate the profiles revealed by tissue expression analysis.



Graph 3*. Transcript accumulation profiles as revealed by semi-quantitative RT-PCR analysis. Total RNA isolated from dry and imbibed seeds of* Arabidopsis *thaliana was reverse transcribed and cDNA samples were amplified in a ABI-Prism 7900 real-time PCR with gene-specific primers. Transcript levels of the different samples were standardized by the quantification of ubiquitin14 gene transcripts in each sample. Independent experiments have shown that the values are subject to maximum 20% error. CDKA,* CDKA*;1; CDKB,* CDKB1*;1; CYCB,* CycB1*;1; CYCD,* CycD4*;1.*

Microtubule assembly and dynamics during seed germination

Transgenic *Arabidopsis MAP4-GFP* plants were used as a living model system in which the dynamic behaviour of microtubules was visualized. Confocal microscopy was used to simultaneously monitor growth and microtubule dynamics within epidermal embryonic cells as the seed germinates.

Based on our observations, organized cortical microtubules (CMTs) seem to be absent in dry seeds but they are rapidly assembled once imbibition is initiated. The visualization of living embryonic cells implies that the seeds are necessarily soaked, and so the visualization of embryos of dry seeds is obviously unfeasible. After 45 min imbibition in water, a reduced number of randomly aligned short CMTs could be observed (Fig. 5A). At 6-8 HAI (hours after imbibition) the number of microtubules has increased but the arrays remain short and irregularly orientated. The number and size of the microtubule arrays steadily increases as the seeds imbibed (Fig. 5B).

We have followed the *in vivo* behaviour of microtubules from living embryo cells, between 10 and 20 HAI. Time-sequence observations showed that at this stage some cortical microtubules are highly dynamic whereas others remain stable in the cell during large periods.

Until 24 HAI the cortical microtubule arrays are randomly aligned in all embryo cells (Fig. 5C). The absence of a specific array orientation is indicative of extensive radial swelling rather than cellular elongation in a particular direction.

We have followed the microtubule dynamics of several living embryos, and it was found that there is not a synchronous behaviour of the microtubule arrays between different embryos. Also, the reorientation of microtubule arrays is not synchronised among neighbouring cells, meaning that even if adjacent cells are subjected to the same external signals, the reorientation of MTs is probably dependent on each cell autonomously.

We observed that in general, at about 24 HAI cortical microtubules start to lose their seemly free organization and progressively align in transverse arrays. This orchestrated array organization seems to be preceded by a noteworthy increase in the number of microtubules.

Gradually the transverse oriented CMTs of some embryo cells start to be replaced by newly organized longitudinal arrays. After 48 HAI, when the radicle has already protruded the seed coat, the majority of the cells show a very specific orientation of the cortical microtubules (Fig. 5D). At this stage, the microtubule arrays of the majority of the cells are placed perpendicularly to the root-hypocotyl axis showing that the majority of the cells are elongating. This is particularly noticeable for the elongation zone above the root meristematic region. In the radicle, microtubules seem to be more freely organized in the meristematic region, whereas with increasing distance from the root tip transverse arrays become much more highly aligned. At this stage mitotic figures can be observed in the root cap layer (Fig. 5F). Although at this stage root protrusion has been accomplished no mitotic figures were evident in the embryo tissues, with the exception of the root cap.

In the hypocotyl the CMTs present all kind of orientations, from random to transverse and in some cases helical. Random orientation is particularly noticeable in the top of the hypocotyl close

to the shoot apex. Helical arrays are only occasional and seem to be randomly placed in the embryo epidermis. This type of arrays is in general an indication of potential twisting.

Also in the cotyledons the dispersed cortical microtubules gradually align into transverse arrays (Fig. 5E). But contrarily to what happens in the other regions of the embryo, the cortical arrays in the cotyledons once organized retain the same transversal positioning throughout further development. There are no obvious differences in the length and number of microtubule arrays between cotyledons and other embryo tissues throughout germination.

The fluorescence intensity in the cells of the root/hypocotyl transition zone is significantly lower as compared to other embryo tissues (Fig. 5G). Exhaustive observations showed that this region has fewer cortical microtubules arrays. A similar pattern of GFP accumulation has been observed in transgenic plants expressing GFP under *GLABRA2* (*GL2*) promoter control (Lin and Schiefelbein, 2001). *GL2* is a transcription factor required for proper specification of epidermal cell fate in *Arabidopsis* seedlings. Therefore, this expression pattern was suggested to be related to the unusual epidermal tissue anatomy of that region, which prevents cell-signalling events required for epidermal specification. However, this model does not seem to be able to explain the reason why the number of microtubules is highly reduced in the root/hypocotyl junction, since no obvious relation exist between epidermal specification and the microtubular cytoskeleton.

Dry MAP4 transgenic seeds were also imbibed in water containing cycloheximide (CHX). Although this drug prevents protein synthesis, the seeds presented a normal organization of cortical microtubule arrays. In these treated seeds a small number of randomly aligned short MTs was observed, both in the cotyledons and radicle (Fig. 5H). The dynamic changes of microtubule distribution after CHX treatment were followed *in vivo*. Time-sequence observations showed that CHX treatment did not cause changes in the cellular microtubule network and polymerization of microtubule arrays. An increasing number of CMTs was still observable after embryo cells were imbibed for more than 60 minutes in water containing CHX (Fig. 5I and 5J). Although, in a similar number and highly dynamic, these arrays remained much shorter than those of untreated seeds. Thus, monitoring the behavior of microtubules from seeds treated with CHX demonstrates that, although this drug disrupts protein synthesis and prevents the synthesis of tubulin monomers, it does not halt the initial assembly of CMT arrays in germinating seeds.

These studies demonstrate that, following imbibition, tubulin assembly and the microtubule rearrangement are not blocked by CHX treatment and thus are not dependent on "*de novo*" protein synthesis.

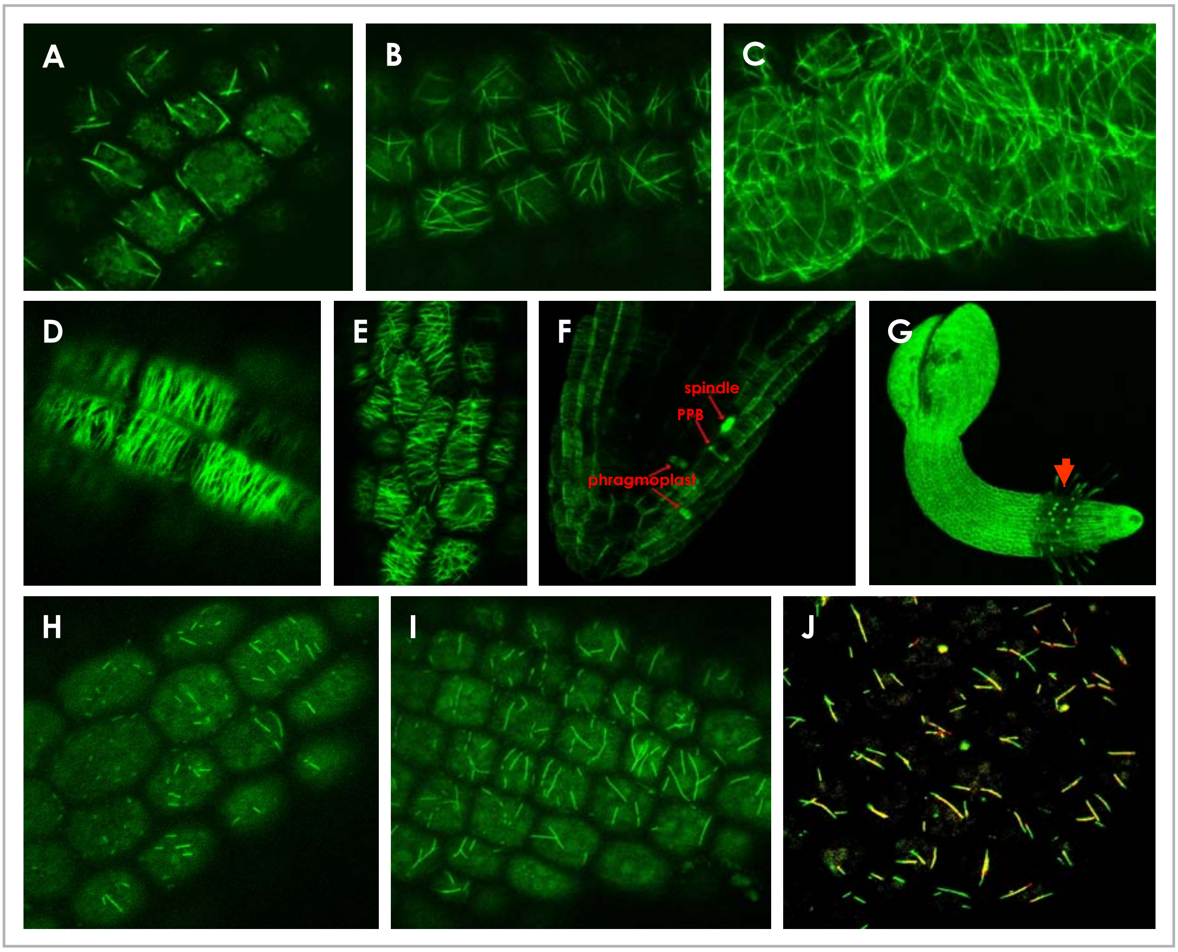


Figure 5*.* *Localization of MAP4-GFP during germination. Cortical microtubule arrays in the radicle epidermis of embryos imbibed in water for (**A**) 45 minutes, (**B**) 6 hours, (**C**) 24 hours and (**D**) 48 hours.* ***E****, Cortical microtubules in the epidermis of cotyledons from seeds imbibed for 48 hours.* ***F****, Mitotic divisions of root cap cells from embryos imbibed for 48 hours. A mitotic spindle, a pre-prophase band (PPB) and a phragmoplast are visible* ***G****, Overview of an* Arabidopsis *young seedling expressing MAP4-GFP (48 HAI). The root/hypocotyl transition zone is pointed out by an arrow.* ***H-J****, Cortical microtubule organization of embryo cells imbibed in a cycloheximide solution for (**H**) 30 minutes or (**I**) 1 hour.* ***J****, Overlay of two images from cortical microtubules of cells treated with cycloximide for 60 (red) and 70 minutes (green). The image in* ***F*** *is the result of Z-stacks from consecutive confocal sections.*

Discussion

Seed germination is a complex physiological process triggered by the imbibition of water and the release of dormancy mechanisms by appropriate signals. Under favorable conditions, rapid growth of the embryo culminates in rupture of the covering layers and emergence of the radicle. Radicle emergence is considered as the completion of germination.

Using flow cytometry, we show that embryos of fully matured dry seeds contain almost exclusively nuclei with 2C DNA content, indicating that most embryonic cells are arrested in the G1-phase of the cell cycle. Indeed, study of cell cycle events in germinating embryos of tomato, using

both quantitative and cytological analysis of DNA synthesis has shown that most embryonic nuclei of dry untreated control seeds are arrested in the G1-phase of the cell cycle (Liu et al., 1994; Groot et al., 1997; de Castro et al., 2000). Additionally, the non-existence of DNA replication during the first hours of imbibition was demonstrated by the absence of BrdU (bromodeoxyuridine) incorporation into embryonic DNA from seeds imbibed for 3 hours (de Castro et al., 2000). During imbibition in water, DNA synthesis seems to be activated first in the radicle tip and then extended towards the cotyledons, resulting in an increase in the number of nuclei in G2 (Groot et al., 1997). DNA replication occurs also in seeds imbibed in the presence of abscisic acid, even if this hormone inhibits germination. On the contrary, there is no replication of DNA upon imbibition in water of giberellin deficient mutant seeds. In these seeds cell cycle progression is only observed after the addition of exogeneous gibberellins (Liu et al., 1994; Groot et al., 1997). On the other hand, gibberellins might have only an indirect role in the activation of cell cycle activities. By performing a proteomic analysis of GA-deficient seeds, Gallardo et al. (2002) have showed that GAs are not essential for cell cycle progression.

Our flow cytometry studies showed that following imbibition in water the relative amount of nuclei with 4C DNA content slowly increases. However, a clear increase in the 4C DNA level is only observed at the moment of root protrusion. It was shown in tomato seeds that after 1 day of imbibition in water the relative amount of 4C nuclei starts to increase. In the radicle tip the 4C:2C ratio rises till 2.5 at the moment of radicle protrusion, whereas in the remaining embryo tissues the progression towards 4C DNA levels is less clear, and 4C:2C ratio does not exceed 0.2 at the moment of embryo emergence. In radicle tips of tomato, most nuclei have progressed toward late S-phase or G2, at the moment of radicle emergence (Liu et al., 1994; Groot et al., 1997). The small size of *Arabidopsis* seeds does not allow such a detailed tissue analysis, therefore we can not exclude the presence of high rates of DNA replication in particular embryo regions, even if in this plant the 4C:2C ratio never reaches values superior to 0.5. Also in *Brassica oleracea* root tips, the onset of DNA replication was shown to precede root protrusion (Górnik et al., 1997).

β-tubulin accumulation has been extensively studied in relation to seed germination. Indeed, a correlation has been shown between the rate of germination and the rate of β-tubulin accumulation in imbibed tomato seeds (de Castro et al., 1995). As with nuclear DNA replication, β-tubulin accumulation was strongest in the radicle tip compared to other tissues, and a possible interaction between both processes in the preparation for radicle growth and consequent protrusion has been suggested (Groot et al., 1997). Indeed, the resuming of DNA replication following imbibition of tomato seeds was proposed to correlate with an increase in β-tubulin accumulation and its assembly into microtubule cytoskeleton networks (de Castro et al., 1998). Studies with germinating tomato and cucumber (*Cucumis sativus*) seeds clearly showed that β-tubulin accumulates prior to the onset of DNA replication, and that both events precede radicle protrusion. Our studies show that in *Arabidopsis* CMT assembly might not be temporally related to DNA replication. The formation of a cortical microtubule cytoskeleton is initiated shortly after imbibition,

whereas large rates of DNA synthesis are only detected at the moment of root protrusion. Therefore, DNA replication and β-tubulin accumulation might not directly be associated. Evidently, both the rearrangement of microtubules and DNA synthesis are required for cell division (Baskin, 2000). However, there seems to be no absolute requirement of DNA synthesis for radicle growth, since it was shown that DNA replication during *Brassica oleracea* seed imbibition can be inhibited by hydroxyurea, whereas β-tubulin accumulation and radicle protrusion are unaffected (Górnik et al., 1997). Our results show that the formation of a proper CMT network might be essential for radicle protrusion, whereas DNA replication may only significantly contribute for postgerminative growth. Using flow cytometry, we show that a clear increase of the 4C percentage was measured about 40-48 h after imbibition has started, coinciding with radicle protrusion. In *Brassica* radicle tip cells an increase in the DNA content is observed already after 24 h of imbibition in water, which is before radicle protrusion (Górnik et al., 1997). In samples from entire *Arabidopsis* seeds a clear increase in 4C DNA content concomitantly with a decrease in 2C DNA was only observed at the moment of radicle emergence. In both species, breaking of the seed coat seems to correlate with the arising of an 8C peak. The discrepancy between both species in the temporal up-regulation of DNA synthesis in relation to embryo protrusion might be due to experimental variance. In *Brassica* DNA content was measured in radicle tips whereas in *Arabidopsis* the full seed was used. This might result in great discrepancies as the radicle tip can exhibit replication levels very different of those in other embryonic tissues, as it was shown for tomato seeds (Liu et al., 1994). In Arabidospsis, a substantial increase in nuclear DNA synthesis seems to be an indicative of imminent radicle protrusion. The moment of radicle protrusion is also marked by a rapid increase in cell cycle gene transcription as shown by our expression analysis results.

The promoter activity pattern of *CDKA*;1, *CDKB1*;1, *CycB1;1*, *CycA2;1, CycD6;1* and *CKS1* genes was characterised in detail by performing GUS protein assays. For some of those genes, such as *CDKA*;1, *CycA2;1, CycD6;1* and *CKS1*, *GUS* expression was already observed in the dry seed. It must be considered that GUS activity in those dry seeds might not be directly related with the expression of cell cycle genes at that developmental stage, but it might be a consequence of residual extant GUS proteins remaining in the dry seed after desiccation. This hypothesis is sustained by the presence of GUS products in most of the dry embryo tissues, instead of a more specific tissue pattern, as expected for the activity of most cell cycle gene promoters. Indeed, after imbibition in water, GUS activity in *pCDKA*;1::*GUS*, *pCycA2*;1::*GUS* or *pCycD6*;1::*GUS* seeds appears to gradually reallocate, becoming much more specific to meristematic tissues. So, the GUS detection results show a clear correlation between root protrusion and the initiation or reestablishment of cell cycle gene promoter activity. Among all genes studied, *CycB1;*1 was the least implicated in the process of germination. *CycB1*;1 is a mitotic cyclin, which expression has been shown to be confined to actively dividing meristems, suggesting that its regulation might be one of the factors for the activation of cell division in some developmental programs (Den Boer and Murray, 2000). However, during seed germination, this gene promoter showed to be only activated late after

radicle protrusion. *CKS1* promoter activity was never observed in meristematic tissues leading us to conclude that this gene is most likely not involved in cell proliferation. The preferential accumulation of GUS activity in the cotyledons indicates that *CKS1*At has a role in the endocycle. However, recent data from De Veylder *et al.* (2001b) has shown that CKS1 overproduction in *Arabidopsis* has no effect on the endoreduplication pattern. Additionally it was shown that *CKS1* overexpression equally affects the G1/S and G2/M transitions.

The tissue expression analysis observed after GUS protein assays is largely corroborated by the transcript accumulation profiles revealed by RT-PCR analysis. RT-PCR analysis was chosen in order to confirm and/or complement our tissue expression analysis, due to the high sensitivity of this technique. Moreover, expression studies in sectioned embryos revealed to be nearly unfeasible due to the small size of *Arabidopsis* seeds, therefore measuring transcript levels using RT-PCR was chosen to complement the studies on cell cycle gene expression in that species. Using RT-PCR, *CycD4* and *CDKA*;1 expression was found to abruptly rise from the instant when seed imbibition was initiated until 3 DAI. Based on these expression profiles, both *CycD4* and *CDKA*;1 seem to be necessary for early seed germination and the resuming of embryo growth, being probably implicated in the reestablishment of the G1-phase. CDKA/CycD complexes act at the G1/S checkpoint by phosphorylating the RBR (RB-related) protein, causing the release of the E2F transcription factor and entry into S-phase (reviewed by Rossi and Varotto, 2002). The plant CycD proteins seem to have a special role in the response to external signals and their integration into the cell developmental and positional contexts. Therefore, this group of plant cyclins might also play a key role in the perception of the environmental context and initiation of seed germination. The expression profile of *CycD4* suggests that this protein is indeed essential for the resuming of cell cycle progression following imbibition. In maize germinating embryo axes, it was shown that cyclin D protein levels remain constant for about 6 hours and then the protein levels decline to almost disappear after 24 hours of imbibition (still before radicle protrusion) (Cruz-Garcia et al., 1998). This accumulation pattern could be an indication that cyclin D protein is implicated in the re-establishment of cell cycle progression in the embryo cells of germinating seeds. Moreover, protein synthesis inhibition with cyclohexamide showed that although the cyclin D protein was synthesized *de novo* during germination, the balance was towards degradation (Cruz-Garcia et al., 1998). Therefore, the presence of this protein during early germination might be due to its upholding in the embryo cells following seed desiccation, remaining to be demonstrated whether these proteins are re-activated during germination. Our *CycD4*;1 transcript accumulation results show that the transcription of this gene is activated early during *Arabidopsis* seed germination.

Although the expression profile of *CDKA*;1 suggests that this protein is implicated in the early re-establishment of cell cycle progression in germinating seeds, it must be taken into account that *CDKA*;1 transcript levels do not necessarily correlate CDKA;1 protein activity. It was previously shown that *CDKA*;1 transcript and protein level remain constant during cell cycle progression while CDKA;1 kinase activity increases throughout S and G2 and peaks at early M-phase (Mironov et al.,

1999). So remains the question whether *CDKA*;1 mRNA accumulation early during germination reflects an involvement of CDKA;1 protein in the resuming of cell cycle activities in the germinating embryo. In maize a CDKA-like protein kinase was shown to be present in dry and germinating embryo axes. CDKA-like protein levels remained constant through imbibition, even when germination was accelerated by the addition of exogenous cytokinins, and as a consequence an increased kinase activity was observed. Therefore, it was proposed that CDKA activity in germinating maize is not related to protein level, but might be regulated by a post-translation mechanism (Herrera-Teigeiro et al., 1999).

Contrarily to *CDKA*;1 and *CycD4*, *CycB1;1* and *CDKB1;1* show an expression outbreak coinciding with radicle protrusion. During G2-phase, B-type cyclins and CDKs are required to trigger the entry into mitosis. As a result, the expression peak of both genes at the moment of radicle protrusion is an indication of a temporal relation between mitosis and embryo emergence.

The expression pattern of cell cycle genes was extensively analysed by mRNA *in situ* localization in *Brassica* germinating seeds. With the clear exception of *CycB1;1*, the analysed genes are implicated essentially in G1 entry and G1-S transition. Previous studies anticipated that entry in the DNA replication phase of the cell cycle might occur prior to radicle protrusion. Therefore, genes associated with the entrance of cells in S-phase are expected to become first active.

Our *in situ* expression results illustrated that the *CycB1;1* mRNA distribution pattern as shown by *in situ* hybridization is very similar to the GUS activity pattern observed in *Arabidopsis* transgenic seeds harboring the *GUS* gene under the control of the *CycB1;1* promoter. This finding not only clarifies the expression pattern of *CycB1*;1 but also validates our *GUS* expression studies.

Most of the genes studied are expressed in the epidermis. For some of these genes (*KRP1*, *ORC2* and *CDC7*) mRNA transcripts accumulate exclusively in the epidermis of the hypocotyl but not of the cotyledons, whereas others are expressed in the epidermis of both organs. In any of the cases, expression is only established after growth of the radicle through the seed coat. The large number of cell cycle related genes expressed in the epidermal cell layers of the embryos or young seedlings led us to the question whether the epidermis plays a particular role in early seedling development. Indeed, it has been shown that the epidermis is the first tissue to become metabolically active following seed imbibition (Mansfield and Briarty, 1996).

Based on the expression of the cell cycle genes it was not possible to distinguish between two *Brassica* lines with different germination performances. These two lines seem to present only a temporal disparity on cell cycle gene expression, meaning that cell cycle gene expression is indeed delayed in poor germinating seeds. However, this might be due to a delay in the activation of the general transcription machinery rather than being directly associated to the cell cycle. Based on these data, we concluded that cell division activity *per se* should not be considered as a direct measure for the ability to germinate.

As already discussed, β-tubulin accumulation has been extensively studied in relation to seed germination, using both immunofluorescence microscopy and western blot analysis (de Castro

et al., 1995; Górnik et al., 1997; Jing et al., 1999; de Castro et al., 2000; 2001). In germinating tomato and cucumber seeds was shown that cortical microtubules are formed in the radicle prior to protrusion (Jing et al., 1999; De Castro et al., 2000). In germinating embryos, the rapid organization of a microtubular cytoskeleton might be required to accomplish the large rates of cell elongation that precede embryo emergence (de Castro et al., 2000). MAP4 is a microtubule-associated protein abundantly expressed in all actively dividing cells of vertebrates. This protein promotes MT polymerisation and presents large microtubule-stabilizing activity (reviewed in Wasteneys, 2002). Seeds of transgenic *Arabidopsis* MAP4-GFP plants were used as a living model system in which the dynamic behaviour of microtubules was visualized. Confocal microscopy was used to simultaneously monitor growth and microtubule dynamics within individual cells as the seed germinates. Characteristically, microtubules in cells of division and elongation zones present different spatial patterning of cortical microtubules. Cells at the elongation zone show transverse cortical microtubule arrays characteristic of longitudinal cellular expansion, whereas dividing cells exhibit randomly aligned microtubules. However, no mitotic figures were observed before the onset of root protrusion, suggesting that radicle protrusion is probably accomplished through cell enlargement, as previously suggested for cucumber and *Brassica* seeds (Gornik et al., 1997; Jing et al., 1999). On the contrary, in tomato mitotic microtubular arrays were observed before the radicle had protruded (de Castro et al., 2000). Even though, it was suggested that in tomato, cell division is not necessary for the completion of germination, since blocking of DNA replication with hydroxyurea did not block radicle protrusion (de Castro et al., 2000).

Treating dry seeds with CHX allowed us to conclude that "*de novo*" protein synthesis is not necessary for the initial establishment of microtubule networks, given that imbibing seeds in the presence of CHX does not halt microtubule assembly and dynamics. However, the synthesis of tubulin monomers seems to be necessary for the formation of normal-sized microtubule arrays.

From our expression studies we could show that most cell cycle genes are not expressed in dry seeds and are only activated during or following radicle protrusion. These results lead us to conclude that cell division activity *per se* does not seem to be fundamental for the initiation of the seed germination process but might be essential to overpass a particular developmental stage during post-germination.

Two-dimensional gel electrophoresis was used to resolve and analyze the seed proteome and the changes in protein abundance during *Arabidopsis* seed germination (Gallardo et al., 2001). Protein extracts from mature dry seeds and from seeds imbibed for 1, 2 and 3 days were compared in order to follow the pattern of changes in protein expression in quiescent mature seeds, in imbibed germinating seeds and after radicle protrusion, respectively. Among the proteins identified whose expression levels are associated with germination, none was found to be belong to the group of cell division associated proteins, with the exception of cytoskeleton associated proteins, such as β-2 tubulin and actin-7. These results clearly indicate an absence or very low abundance of the main fundamental cell cycle proteins during the initial stages of seedling development. Furthermore,

those proteomic studies have also demonstrate that most of the proteins identified were already present in dry seeds and their abundance remained constant throughout the germination process. The germination process therefore appears to be associated with modifications in the abundance of only a limited number of proteins, supporting the idea that dry seeds are essentially ready to germinate. Thus, resumption of metabolic activity during germination may rely mainly upon proteins that are stored during seed maturation.

All together, our results indicate that the early activation of cell cycle activities in germinating seeds might result from cell cycle components that remain in the embryo after seed desiccation. This reduced machinery should be able to initiate the foremost cell cycle activity and trigger embryo specific mechanisms, resulting in the "de novo" production of the essential components of the cell cycle machinery. Our studies indicate that the "de novo" synthesis of the cell cycle apparatus mainly takes place after root protrusion. Most of the early stages of germination, prior to root protrusion, might be devoted to the reassembly and/or reorganization of pre-existing cell cycle protein complexes and cytoskeleton elements. The proposed model is summarized in Fig. 6.

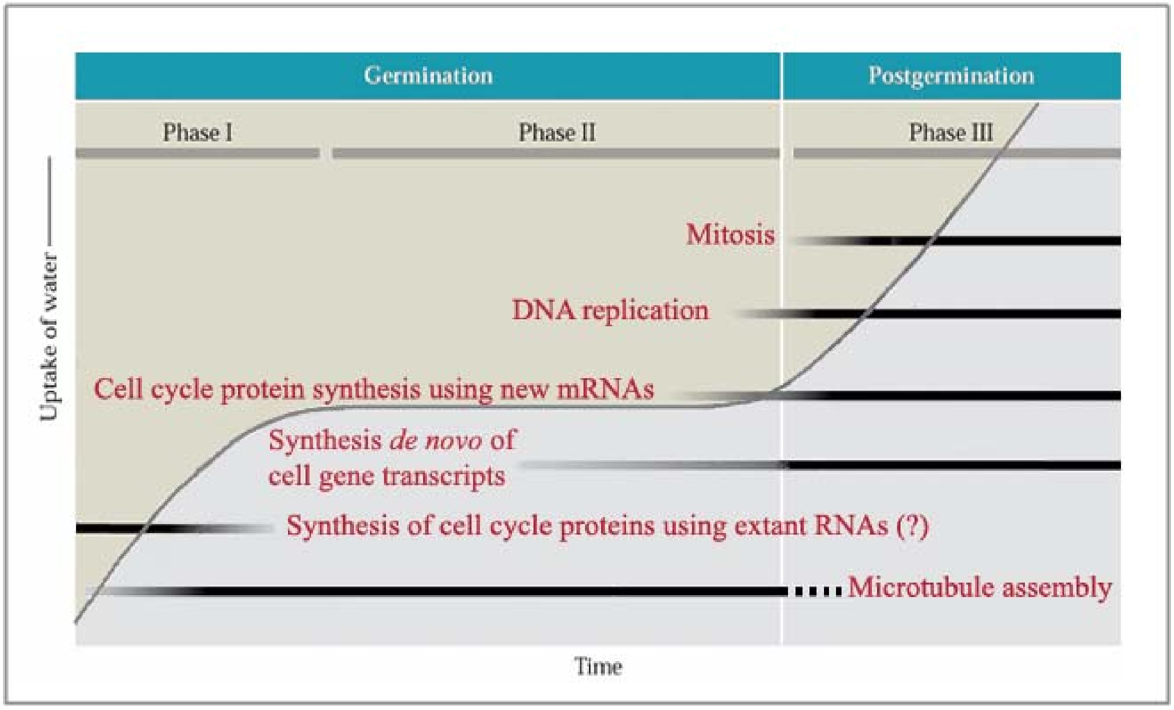


Figure 6. *Time course of some important cell cycle related events during germination and postgermination growth.*

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Summary

Cell division and proliferation of eukaryotic cells follows a genetic program, designated cell cycle. To ensure proper cell cycle progression, proteins that are involved in its regulation must be periodically activated within an appropriate window of that cycle. Cyclins (Cyc) and cyclin-dependent kinases (CDK) are the basic component units of the core cell cycle machinery. Besides a well-known role in cell cycle control, CDK/Cyc kinase complexes from animal cells are now considered as important regulators of transcription mechanisms.

The work presented in the first part I of this thesis (**Part I**) aimed to contribute to the molecular understanding of plant CDKs. This part is fully dedicated to the characterization of CDKs from the B- and C-group, with the attempt to identify their role in plant cells and/or their implication in plant development.

New aspects of CDK function were investigated by the characterization of the *Arabidopsis thaliana* CDKC;1 and CDKC;2 proteins (**Chapter 2**). For the CDKC subclass of plant CDKs no precise function has been found yet. The interaction of Arath;CDKC;1 and Arath;CDKC;2 with a cyclin T-like protein from *Arabidopsis* was demonstrated, following two hybrid interaction assays and immunoprecipitation experiments. These results suggested that *Arabidopsis* CDCKC;1 and CDCKC;2 interact with cyclin T in a manner analogous to the animal CDK9 and cyclin T. A number of other proteins with which CDKCs interact were also described. Based on our protein interaction, and tissue expression studies we suggest that the plant CDKC/CycT complex is not involved in cell cycle control. On the contrary, the CDKC/CycT complex seems to interact with specific components of the transcription machinery, regulating functions that take part in differentiated tissues, as previously proposed for the animal CDK9. Our work presents the first step towards the characterization of a plant CDKC/CycT complex analogous to the animal CDK9/CycT (P-TEFb). The P-TEFb (positive transcription elongation factor b) complex is essential in the regulation of eukaryotic mRNA transcription. This complex is responsible for the control of trancription elongation, an important regulatory step in the control of gene expression.

Several studies have now demonstrated that the role of specific CDKs in the progression through the cell cycle is generally conserved amongst many eukaryotic organisms. However, the discovery of plant B-type CDKs resulted in the identification of a CDK class which is only common to plants. The B-type CDKs is a plant-specific group distinguished by the PPTALRE or PPTTLRE amino acid signature in their cyclin-binding domain, and the inability to complement the yeast *cdc2* mutation. In order to better understand the role of CDKB kinases in plant development, a mutant inactive form of CDKB1;1 was ectopically expressed in tobacco plants (**Chapter 3**). Our studies in tobacco have shown that down-regulation of B-type CDKs by overexpressing a dominant-negative

mutant of *CDKB1*;1 has no effect on the morphology of the plant, even if the associated kinase activity was decreased. However, these transgenic tobacco plants showed a considerable increase in the size of the G2 cell population. Additionally, the detection of the histone H1 related kinase activity of the A- or B-type CDKs in synchronized tobacco BY-2 cell suspensions has shown that although A-type CDKs are constitutively expressed, their activity peaks in S, G2 and M-phase, with a pronounced decline in G1-phase. On the other hand, the activity of B-type CDKs correlate with their protein concentration and peaks during early M-phase. All together these results revealed a unique role for B-type CDKS at the G2/M transition. Since no morphological alterations could be detected in transgenic tobacco plants overexpressing a CDKB1;1 dominant negative mutation, the developmental importance of CDKB1;1 remained unclear. However, in young *Arabidopsis* seedlings, analysis of the *CDKB1*;1 promoter activity has shown that, apart from the meristematic regions, the expression of this gene is confined to stomata and stomatal precursors (**Chapter 4**). The possible involvement of CDKB1;1 on stomatal development was further addressed by evaluating the effects of the ectopic production of CDKB;1 and its mutant inactive form on *Arabidopsis* plants. In agreement with the pattern of *CDKB1*;1 promoter activity, the transgenic plants overexpressing the dominant negative CDKB1;1 protein showed a strongly reduced stomatal index and an altered morphology of a large number of stomata. DNA quantification of stomatal nuclei, using flow cytomety, showed that the aberrant stomata are blocked in the G2-phase of cell cycle. Besides, electron scanning microscopy observations showed that several anomalous stomata present cell wall features resembling the initiation of cytokinesis, suggesting that guard cell identity does not rely on the activity of CDKB1;1 and that guard cell differentiation is independent from CDKB1;1 activity. All together our results evidence that CDKB1;1 activity is required for proper cell division during stomata formation.

Cell cycle activity is certainly a requirement for plant growth and development, but its involvement in the early events that initiate seedling development remains to be clarified. The second part of this thesis (**Part II**) describes our efforts to understand when the cell cycle is activated during seed germination, and what its contribution is for proper seedling establishment. This work was part of a large European research project that aimed to understand the cellular mechanisms responsible to trigger seed germination. Seed germination is the process by which the plant embryo resumes growth after a period of dormancy. Activation of some cell cycle processes might be a requirement for germination but convincing evidence is still lacking. For different plant species, it has been demonstrated that particular cell cycle activities, like DNA replication and β-tubulin accumulation, precede radicle protrusion, even if blocking of these processes does not always prevent the completion of germination. In an attempt to understand when, during seed germination, embryo cells reinitiate cell cycle activity, we have performed flow cytometry studies and an extensive expression analysis of cell cycle genes **(Chapter 4)**.

Our flow cytometry results showed that DNA replication is initiated only a very few hours after imbibition of the seed in water. However, a large 4C cell population could only be detected at

the moment of root protrusion, when germination reaches its end. These results indicate that a number of cell cycle proteins must be activated in the seed during the first hours of imbibition in water, allowing some embryo cells to enter S-phase.

Additionally, an extensive expression analysis of cell cycle genes, using either mRNA *in situ* localization techniques, GUS detection assays or semi-quantitative RT-PCR analysis has allowed us to thoroughly describe the spatial and temporal expression profiles of a large set of cell cycle control genes in germinating seeds of *Arabidopsis thaliana* and *Brassica oleracea.* Following seed imbibition, the expression of cell cycle regulators with a clear role in G1-phase, such as *CDKA*;1 and *CycD4*;1, seems to be rapidly activated as shown by RT-PCR analysis. However, our mRNA *in situ* hybridisation analysis showed that transcription of most cell cycle genes can only be detected after completion of the germinative process. Taken together, our data suggest that the reinitiation of cell cycle activity in germinating seeds might result from the activation of some core cell cycle proteins that could have remained in the embryo after seed desiccation. This hypothesis is corroborated by our GUS histochemical assays. Indeed, the promoter activity pattern of several cell cycle genes was investigated by performing GUS protein assays in dry and germinating transgenic seeds expressing the GUS gene under the control of those cell cycle promoter genes. Our results show that GUS activity associated to some of those genes, such as *CDKA*;1, *CycA2;1, CycD6;1* and *CKS1*, can be already detected in dry seeds. However, it remains to be demonstrated whether GUS expression at early stages of germination reflects the true presence of cell cycle proteins in dry embryos.

An alternative experimental approach, based on the *in vivo* imaging of the microtubule cytoskeleton, was undertaken in order to determine whether cell division is implicated in the germinative process. Additionally, it was important to clarify which embryo cells contribute for either cell division or elongation. Indeed, the occurrence of specific arrays, such as the spindle, the pre-prophase bands and the phragmoplast, can be considered as an exclusive marker for mitosis. On the other hand, the visualization of cortical microtubule arrays allows the fast identification and observation of elongating cells. Therefore, the analysis of the dynamic organization of the microtubular cytoskeleton in living embryonic cells of germinating embryos turned out to be very relevant for our work. Studying the *in vivo* occurrence of the different types of microtubule arrays in embryo cells has shown that, although the assembly of the microtubular cytoskeleton is promptly activated once germination is initiated, mitotic specific microtubule arrays seem to occur only when the radicle has started to protrude. Therefore, we postulate that prompt assembly of a cortical microtubular network might be a preparation step towards the elongation of embryonic cells. Additionally, using a protein synthesis inhibitor drug, it was demonstrated that the dry seed contains already the required set of proteins necessary to initiate microtubule assembly. Early steps in germination therefore involve the reassembly and reorganization of cytoskeleton elements from pre-existing protein subunits.

In summary, the resuming of cell cycle progression in germinating seeds seems to be the responsibility of a reduce protein machinery, which might be promptly synthesized from the moment

imbibition in water is initiated. Alternatively, some of the proteins responsible for the embryo cells to reenter cell cycle might remain in the embryo following desiccation. From our flow cytometry analysis might be deduced that a limited set of cell cycle proteins must be activated very early in the embryo to promote cycle progression, allowing a number of embryo cells to initiate DNA replication only a few hours after germination is initiated. However, our results indicate that the "de novo" production of most cell cycle proteins mainly takes place after root protrusion, since a large production of gene transcripts can only be observed at that stage. The production of a multitude of cell cycle regulators seems to result in the activation of the cell cycle in a large number of embryonic cells, coinciding with the appearance of a large 4C population observed 40-48 hours after imbibition was initiated.

Our studies demonstrated that the early stages of seed germination, prior to root protrusion, are devoted to the synthesis/activation of only a reduced number of core cell cycle proteins. These proteins might activate cell cycle progression but not drive cells into mitosis, as no mitotic divisions are observed prior to radicle protrusion.

Samenvatting

Alle eukaryotische cellen zijn voor hun deling en proliferatie onderworpen aan een strikt genetisch programma, de celcyclus. Om een correct celcyclusverloop te verzekeren moeten een aantal sleuteleiwitten periodiek geactiveerd worden op specifieke tijdstippen van de celcyclus. Cyclines (Cyc) en cycline-afhankelijke kinasen (CDK) zijn de basiscomponenten van de celcyclusmachine. Samen vormen deze eiwitten de zogenaamde CDK/Cyc kinasecomplexen. Naast hun cruciale rol bij de controle van de celdeling wordt aan welbepaalde CDK/Cyc kinasecomplexen een belangrijke rol toegeschreven bij het reguleren van mechanismen betrokken bij de transcriptie van genen.

Het eerste deel (**Part I**) van deze thesis wil een bijdrage leveren tot het beter begrijpen van de moleculaire werking van CDKs. Dit deel is dan ook volledig gewijd aan de moleculaire karakterisering van de B- en C-type CDKs en tracht hun rol te achterhalen in de plantencel en hun eventuele betrokkenheid bij de ontwikkeling van een plant.

Een reeks nieuwe aspecten van de CDK functie werd onderzocht door het beter karakteriseren van de eiwitten CDKC;1 en CDKC;2 van *Arabidopsis* (**Chapter 2**). Tot op heden kon geen specifieke functie toegeschreven worden aan de CDKs behorende tot het CDKC-type. Door middel van het "twee-hybride systeem" en immunoprecipitatie-experimenten hebben we de interactie kunnen aantonen van het Arath;CDKC;1 en het Arath;CDKC;2 eiwit met het cycline-achtig eiwit van het T-type. Deze interactiestudies hebben aangetoond dat het *Arabidopsis* CDKC;1 en het CDKC;2 eiwit een binding aangaan met een cycline T op een wijze vergelijkbaar met het dierlijke CDK9 en cycline T. Een reeks andere eiwitten die een binding aangaan met CDKCs werden eveneens beschreven. Op basis van de resultaten bekomen met de eiwitinteractie- en expressiestudies menen we te mogen besluiten dat het CDKC/CycT complex in planten niet betrokken is bij de controle van de celcyclus. Er werd reeds aangetoond in dierlijke systemen dat het CDKC/CycT-complex een binding kan aangaan met specifieke eiwitten welke deel uitmaken van het gentranscriptiecomplex en op deze manier een regulerende functie uitoefenen in gedifferentieerde weefsels. Het P-TEFb complex (positive transcription elongation factor b) speelt een belangrijke rol bij de controle van mRNA-elongatie en is derhalve essentieel voor de regulatie van gentranscriptie bij eukaryoten. Onze resultaten bekomen in planten wijzen erop dat de CDKC/CycT eiwitcomplexen, naar analogie met het dierlijke CDK9/CycT (P-TEFb), eveneens een rol zouden kunnen spelen bij de regulatie van gentranscriptie.

Een vergelijking tussen verschillende vertegenwoordigers binnen de eukaryoten toont overtuigend aan dat de rol die welbepaalde CDKs vervullen tijdens de celdeling sterk geconserveerd zijn onder meerdere organismen. Dit geldt echter niet voor alle CDKs. Zo werden er CDKs geïdentificeerd van het B-type in planten, waarvoor er geen homologen konden worden aangetoond

in gist of dierlijke organismen. Deze B-type CDKs vormen dus een plantenspecifieke groep van CDKs die zich, onder andere, onderscheiden door de aanwezigheid van een aminozurenmotief PPTALRE of PPTTLRE in het cycline-bindend domein. Bovendien is het CDK van het B-type niet in staat een gist *cdc2* mutatie te complementeren.

Ten einde de rol van CDKB kinasen tijdens de ontwikkeling van de plant beter te begrijpen hebben we een inactieve mutantversie van *CDKB1*;1 ectopisch tot uiting gebracht in tabaksplanten (**Chapter 3**). Deze studie werd uitgevoerd in *Nicotiana tabacum* en heeft aangetoond dat de neer-regulatie van endogene B-type CDKs door middel van de overexpressie van een dominant-negatieve mutant afgeleid van *CDKB1*;1 geen invloed heeft op de zichtbaar morfologische kenmerken van de plant. Nochtans werd in deze transgene tabaksplanten een verminderde kinase activiteit waargenomen. Anderzijds hebben we wel kunnen aantonen dat het aantal cellen die zich in de G2-fase van de celcyclus bevinden, significant zijn toegenomen in deze tabaksplanten. Histon H1 kinase activiteit experimenten in de A- of B-type CDKs, uitgevoerd met gesynchroniseerde celculturen van tabak BY-2 cellen, hebben overtuigend aangetoond dat niettegenstaande de A-type CDKs constitutief tot uiting komen in BY-2 cellen, hun enzymactiviteit duidelijk piekt in de S-, de G2- en de M-fase van de celcyclus. Voor de B-type CDKs zien we een synchroon verloop van de enzymactiviteit samen met de eiwitconcentratie en dit met een uitgesproken piek in enzymactiviteit tijdens de vroege M-fase. Al deze gegevens wijzen op de unieke rol van de plantenspecifieke B-type CDKs tijdens de G2/M-transitie van de celdeling.

Wat de mogelijke rol zou kunnen zijn van de B-type CDKs tijdens de ontwikkeling van de plant is echter onduidelijk vermits we geen herkenbare morfologische wijzigingen hebben kunnen waarnemen in tabaksplanten transgeen voor de dominant-negatieve versie van *CDKB1*;1. Daarom hebben we besloten een expressie analyse van het *CDKB1*;1 gen uit te voeren in jonge *Arabidopsis* zaailingen. Deze analyse toonde aan dat naast een duidelijke promotoractiviteit in bepaalde meristematische gebieden, het *CDKB1*;1 gen zeer specifiek tot expressie komt in bladhuidmondjes en hun voorlopers. Vermits een zeer gerichte celdeling cruciaal is voor de correcte vorming van huidmondjes werd nagegaan of het *CDKB1*;1 gen daarin een essentiële rol zou kunnen spelen. Daarvoor werd het effect van de ectopische expressie van de inactieve vorm van *CDKB1*;1 bestudeerd in *Arabidopsis* planten (**Chapter 4**). Overeenkomstig met het expressiepatroon van het *CDKB1*;1 gen, zien we dat transgene planten welke het dominant negatieve CDKB1;1 eiwit tot expressie brengen, een sterk gereduceerd aantal huidmondjes vertonen, terwijl in de zaadlobben een groot aantal huidmondjes morfologisch verschillend zijn van de normale huidmondjes. Tevens werd door middel van flow-cytometrie aangetoond dat de morfologisch gewijzigde huidmondjes zich in de G2-fase bevinden van de celcyclus. Deze resultaten laten vermoeden dat het CDKB1;1 eiwit betrokken is bij de celdeling tijdens de ontwikkeling van huidmondjes.

Er bestaat geen enkele twijfel dat de regulerende celcyclusgenen noodzakelijk zijn voor de normale groei en ontwikkeling van iedere plant. Of echter al deze celcyclusgenen essentieel zijn voor de initiatie van zaailingontwikkeling is nog steeds onduidelijk. Het tweede gedeelte van deze

thesis (**Part II**) tracht dan ook een bijdrage te leveren om de link tussen de celcyclus en zaadkieming beter te begrijpen. Dit onderzoek maakt deel uit van een Europees Onderzoeksproject dat tot doel stelde de cellulaire mechanismen te besturen die verantwoordelijk zijn voor het initiëren van de zaadkieming. Zaadkieming kan men omschrijven als zijnde het proces waarbij het embryo van de plant de groei hervat na een periode van rust of "dormancy". Men gaat uit van de veronderstelling dat de zaadkieming wordt opgestart door het activeren van de celcyclus; solide argumenten zijn hiervoor echter nog niet voorhanden. Zo heeft men voor een aantal plantensoorten kunnen aantonen dat welbepaalde celcyclusactiviteiten zoals DNA-replicatie als de accumulatie van β-tubuline reeds aantoonbaar zijn tijdens de eerste stappen van de zaadkieming en dit alvorens het embryonale worteltopje de zaadhuid heeft doorboord. Om een inzicht te krijgen wanneer precies de celcyclus wordt geactiveerd tijdens de zaadkieming, werden "flow cytometrische" metingen alsook een grondige genexpressie analyse van de belangrijkste celcyclusgenen uitgevoerd (**Chapter 6**). Op basis van de "flow cytometrische" resultaten kunnen we afleiden dat reeds enkele uren na imbibitie van de zaden in water, DNA-replicatie kan aangetoond worden in de embryonale cellen. Een sterke toename van het aantal cellen in een 4C stadium werd echter enkel geobserveerd nadat de embryonale wortel de zaadhuid heeft doorboord, met andere woorden op het einde van de zaadkieming. Deze resultaten wijzen erop dat een aantal celcyclus eiwitten reeds vroeg in het zaad geactiveerd worden, meer bepaald tijdens de eerste uren na imbibitie van het zaad met water, om zodoende welbepaalde embryocellen toe te laten in de S-fase van de celcyclus.

Daarnaast werd een grondige genexpressie analyse uitgevoerd van de belangrijkste celcyclusgenen tijdens de verschillende stadia van de zaadkieming en dit zowel voor *Arabidopsis thaliana* als *Brassica oleracea*. Voor deze analyse werden drie belangrijke technieken gebruikt: de *in situ* mRNA hybridisatie methode, het β-glucuronidase (GUS) reportersysteem, en de semi-kwantitatieve RT-PCR analyse. Onze RT-PCR analyse toont overtuigend aan dat haast onmiddellijk na imbibitie van het zaad, de celcyclusregulerende genen *CDKA*;1 en *CycD4*;1 snel worden geactiveerd. Voor deze genen werd reeds aangetoond dat zij een cruciale rol spelen tijdens de G1 fase van de celcyclus. Anderzijds kunnen we afleiden uit de resultaten van de in situ mRNA hybridisatie analyse dat omzeggens alle onderzochte celcyclusgenen enkel geactiveerd worden na het beëindigen van de zaadkieming. Deze resultaten laten dan ook vermoeden dat het heropstarten van de celcyclus in kiemende zaden geïnitieerd wordt door het activeren van een beperkt aantal "kern"-celcycluseiwitten reeds aanwezig in het droge zaad. Deze hypothese wordt tot op zekere hoogte bevestigd door de resultaten bekomen met de GUS reporteranalyse. Zo konden we overtuigend β-glucuronidase activiteit aantonen in droge zaden en dit voor de constructen opgebouwd met de promotors van de volgende genen *CDKA*;1, *CycA2*;1, *CycD6*;1 en *CKS1*. Of de glucuronidase activiteit in droge zaden de aanwezigheid van de corresponderende celcycluseiwitten reflecteert is daarmee echter nog niet ondubbelzinnig aangetoond.

In een volgend luik van het onderzoek werd gebruik gemaakt van een alternatieve methode om efficiënt delende cellen aan te tonen in kiemende zaden. Hiervoor werd gebruik gemaakt van

een techniek welke berust op de "in vivo" visualisatie van het microtubulair celskelet door middel van het fluorescerende merkereiwit GFP. Bovendien leek het ons nuttig niet alleen na te gaan welke embryonale cellen tot deling overgaan maar tevens cellen te herkennen welke groeien door celstrekking. De aanwezigheid van specifieke microtubulaire structuren zoals een pre-profase band, een spoelfiguur en een fragmoplast kunnen beschouwd worden als exclusieve merkers voor de celdeling. Anderzijds laat de organisatie van het corticaal microtubulair celskelet tot op zekere hoogte toe cellen te identificeren die actief onderworpen zijn aan celstrekking.

Deze *in vivo* studie toonde overtuigend aan dat, niettegenstaande de assemblage van het microtubulair celskelet onmiddellijk plaatsgrijpt bij de start van de zaadkieming. de microtubulaire structuren kenmerkend voor delende cellen enkel werden waargenomen als het embryonale worteltopje de zaadhuid heeft doorboord. De zeer vroege assemblage van het corticaal microtubulair skelet zou derhalve een belangrijke rol kunnen spelen bij de strekking van welbepaalde embryonale cellen. Gebruikmakend van een specifieke inhibitor van de eiwitsynthese hebben we tevens kunnen aantonen dat een georganiseerd tubulair celskelet zeer waarschijnlijk opgebouwd wordt uit een pool van eiwitten welke reeds voorhanden zijn in het droge zaad. Vroege stappen in het proces van zaadkieming zijn dan ook betrokken bij het samenstellen en reorganiseren van het celskelet uit een "pool" van reeds aanwezige bouwstenen.

De bekomen resultaten suggereren dat het hervatten van de celcyclus tijdens de zaadkieming eventueel geïnitieerd wordt door de activiteit van een beperkt aantal celcycluseiwitten. Deze eiwitten worden vermoedelijk onmiddellijk aangemaakt tijdens de imbibitie van het zaad. Anderzijds is het mogelijk dat de eiwitten die verantwoordelijk zijn voor het activeren van de celcyclus in embryocellen reeds aanwezig zijn in het droge zaad. Onze "flow-cytometrische" resultaten laten vermoeden dat eerder een beperkt aantal celcycluseiwitten dienen geactiveerd te worden en dit enkele uren na de start van de zaadkieming ten einde de embryonale cellen te programmeren voor DNA-replicatie.

Dit onderzoek heeft overtuigend aangetoond dat de boodschapper mRNAs welke coderen voor het merendeel van de celcycluseiwitten enkel voorkomen in embryonale cellen nadat het embryonaal worteltopje de zaadhuid heeft doorboord. De vorming van deze celcyclusregulatoren heeft dan ook blijkbaar de activering van de celcyclus voor gevolg van een groot aantal embryonale cellen.

Samenvattend kunnen we stellen dat onze experimentele gegevens erop wijzen dat de vroege stadia in de zaadkieming, namelijk het tijdstip vóór het doorboren van de zaadhuid door de embryonale wortel, zouden betrokken zijn bij de synthese of de activering van een eerder beperkt aantal "kern" celcycluseiwitten. Deze regulatoreiwitten zouden initieel noodzakelijk zijn voor het activeren van de celcyclus waardoor DNA replicatie mogelijk wordt. De eigenlijke celdeling zou dan op gang komen nadat de embryonale wortel de zaadhuid heeft doorboord.

Thanks

Thanks

"Often we can help each other most by leaving each other alone; at other times we need the hand-grasp and the word of cheer."

Elbert Hubbard (1856-1915)

First of all I want to thank all my friends. I do believe that without them it would have been certainly much more difficult. A large part of this thesis belongs to you guys! I would prefer not to have to name you all, so for those who took care of me, brought me food, brought me cheer and ask me thousand times "how is going?", those who "seduce" me for a tea or a talk, and didn't let me feel lonely in the late working evenings, those who made me feel "I could" and, most of all, never let me go down, my big warm "thank you" (I hope I haven't forget anyone!). A huge kiss for you my friends.

Thanks also to all the others that are now somewhere around the world but have once made me enjoy many special moments in this lab and shared with us moments of great fun in Parike.

My special tanks to the ones in Portugal, that with their strength and support, made me feel that after all my heart still belongs to Portugal. You are fantastic. Sorry for all the messages without answer. Dina, Beta, Carmina, mesmo longe eu sei que voces estão sempre aqui comigo! Obrigada.

The hands and/or brain of a number of people have been essential to get this thesis to its end. Many thanks for those who help me with experiments, discussions, suggestions, reading, correcting, computer problems, etc. Even if it seems impossible not to forget any of those hands/brain's names, it is essential to thank Kris, Zoltan, Lara, Nelson, Wim, Mansour, Gil, Riet, Cristian, Janice, Lieven, Tom, Veronique, Hendrik and Martine for all the practical help, and much patience!

I wish also to express my gratitude to Dirk Inzé, whom I for the first time met in Portugal in the spring of 1995, and as a consequence so many things have happened in my life. In fact almost everything has changed from then. Thanks for the opportunity you gave me to work in this laboratory.

To Gilbert I have to specially thank for all the very good funny talks and also for the serious ones, not less funny. I think I got a bit spoiled by you!! I knew I could count on you any moment. Your contribution for these last six years is much more that I can express.

Steven (Groot) I want to thank for all the advices, all the encouragement and support, all the discussions and also for the friend that he has become during these (project) years that we have been working together. Thanks.

A nice word of appreciation also for all the colleagues that shared the same EC project. It was a real pleasure to work with such nice group of people.

My acknowledgements to all jury members for the patience, the time, and for the corrections and suggestions.

Many thanks also to Rita, Geert, Lucienne, Levi, Naomi and Remi for always taking such good care of Elisa in the busiest moments (and also the ones when some relax became essential). Thanks for all the support.

Um grande beijinho e um muito obrigada para os meus pais. A eles devo tudo. Um grande beijinho para os meus irmãos que são os melhores do mundo, e também para o(s) meu(s) sobrinho(s) (Daniel e ?) e sobrinhas (Carina e Marta) que eu sei que gostariam muito mais que eu estivesse com eles em Portugal do que ver os seus nomes neste livro. Também eu tenho muitas saudades vossas. Adoro-vos!

E finalmente ao Wim, por ser não só o meu "porto de abrigo" mas muito mais do que eu sonhei. E á Elisa, minha espécie de céu.

"Não sou nada.
Nunca serei nada.
Não posso querer ser nada.
À parte isso, tenho em mim todos os sonhos do mundo…

Que sei eu do que serei, eu que não sei o que sou?
Ser o que penso? Mas penso tanta coisa!
E há tantos que pensam ser a mesma coisa que não pode haver tantos!
Gênio? Neste momento
Cem mil cérebros se concebem em sonho gênios como eu,
E a história não marcará, quem sabe?, nem um…"

Fernando Pessoa (1888-1935)