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INVITED REVIEW

The cell cycle and seed germination

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Abstract

The cell cycle is the series of molecular events that allows cells to duplicate and segregate their chromosomes to form new cells. The finding that a protein kinase, the product of the yeast *cdc2* gene, was fundamental in the regulation of the G_2/M and G_1/S transitions, associated with unstable proteins named cyclins, opened a very exciting and dynamic research area. The number of gene products that participate in the development and regulation of the cell cycle may be in the hundreds, and there is a high degree of conservation in protein sequences and regulatory pathways among eukaryotes. Although there are clear differences between plants and animals in cell structure, organization, growth, development and differentiation, the same types of proteins and very similar regulatory pathways seem to exist. Seed germination appears to be an excellent model system for studying the cell cycle in plants. Imbibition will reactivate meristematic cells – most initially with a G_1 DNA content – into the cell cycle in preparation for seedling establishment. Early events include a thorough survey of DNA status, since the drying process and seed storage conditions reduce chromosomal integrity. The initiation of cell cycle events leading to G_1 and S phases, and of the germination process itself, may depend on a G_1 checkpoint control. Most, if not all, cell cycle proteins appear to be already present in unimbibed embryos, although there is evidence of protein turnover in the early hours, suggesting the need for *de novo* protein synthesis. Regulation also may occur at the level of protein modification, because existing G_1, S and G_2 cell cycle proteins appear to be activated at precise times during germination. Thus, cell cycle control during seed germination may be exerted at multiple levels; however, knowledge of cell cycle events and their importance for germination is still scarce and fragmentary, and different species may have developed unique control mechanisms, more suited to specific germination characteristics and habitat.

Keywords: cell cycle, DNA metabolism, germination, seeds

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Introduction

Seed germination has the ultimate purpose of reproducing the plant that originated from the seed. The germination process involves the metabolic awakening of the latent seed and resumption of developmental processes. This implies that seeds already contain, or must soon acquire, the biochemical memory for the morphogenetic programme that recreates a plant. As a consequence of water entry, cells in seed tissues elongate and finally divide, a process that is concomitant with, or follows, radicle protrusion, the event that defines the end of the germination process. In general, cells in the root tip are the first to be activated to enter the cell cycle and proliferate. Different experimental approaches have indicated that, whereas cell division is not necessary for visible germination to occur, the establishment of the cell cycle is an essential event for further growth and, thus, its study during germination is fundamental.

Our purpose in this review is to give a general description of the cell cycle in eukaryotes, including relevant aspects of the cell cycle in plants, and then to discuss the importance of cell cycle events during seed germination, with particular emphasis on DNA metabolism. Excellent reviews on specific aspects of the cell cycle in plants have been published previously (Renaudin *et al*., 1996; Huntley and Murray, 1999; Mironov *et al*., 1999; Joubés *et al*., 2000; Meijer and Murray, 2000, 2001).

The cell cycle

Cell proliferation requires cell division, and to achieve this cells must first grow in size, then duplicate their chromosomes and, finally, separate the chromosomes for exact distribution between the two daughter cells. These processes are coordinated in the cell cycle, which consists of several phases (Fig. 1). In the first phase (G_1) the cell grows and becomes larger. After reaching a certain size, cells enter the next phase (S), in which DNA synthesis takes place (DNA replication) to duplicate the hereditary material. During the next phase (G_2) the cell ensures that DNA replication is completed, and only then are cells prepared for cell division. The chromosomes are separated during the M phase (mitosis), and the cell divides into two daughter cells, each receiving identical chromosome sets. After division, the cells are back in G_1 and the cell cycle is completed. The phases must proceed in the correct order, and one phase must be completed before the next phase can begin. For this, it is essential that the different phases of the cell cycle are precisely coordinated.

The cell cycle is regulated by the activity of kinase complexes formed by a cyclin protein (named after its cyclical appearance) and a cyclin-dependent kinase protein (Cdk). Research using several animal models has shown that stimulation of cell proliferation by different means leads to the activation of a cyclin–Cdk complex that is responsible for the initiation of the cell cycle (Nurse, 1994). This complex, formed by a D-type cyclin and Cdk4 (or Cdk6), appears to be the molecular gate to the cycle (Xiong *et al*., 1992). The presence and kinase activity of cyclin D/Cdk4(6) are tightly regulated and respond to both external and internal stimuli. It is at the cyclin D level that signal transduction, elicited by external stimuli, such as growth factors, hormones, nutrients and other external cues, and cell cycle metabolism converge (Matsushime *et al*., 1991). Cyclin D gene expression itself is dependent on addition of serum or growth factors to cell cultures. Cyclin D is either absent in non-cycling cells, or it is inhibited, complexed with Cdk4(6), during early G_1. The most important and best-documented task performed by this cyclin/Cdk complex is to phosphorylate and thereby inhibit a cell cycle inhibitor, the pRB protein (Brehm and Kouzarides, 1999). During early G_1, before cells are committed to enter into the cell cycle, the pRB protein (originally found in *R*etino*B*lastoma tumours) sequesters a very important S-phase transcription factor, composed of the proteins E2F and DP1 (Sherr, 1994). Therefore, cells are restricted from entering the S phase ahead of time. This constitutes a very important control mechanism to prevent cells from passing into a cell cycle phase before having finished the previous phase. This is called a 'checkpoint control'.

The cyclin D/Cdk4(6) kinase complex is the target of at least two types of kinase inhibitors grouped into two families: the Cip/Kip family of inhibitors represented by $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$; the other is the Ink4 (*In*hibitor of Cd*k4*) family of inhibitors that have as members $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$ (Sherr and Roberts, 1999). The expression of these inhibitors depends on the metabolic or developmental states of the cells, or even the integrity of the genome, implying that different effectors control their presence and accumulation. The wide variety of protein inhibitors affecting this G_1 cyclin/Cdk complex clearly indicates the stringent control modulating commitment to completion of the cell cycle.

During G_1, the cyclin D/Cdk4(6) complex is often found associated with one of the kinase inhibitors, $p21^{Cip1}$ and also to PCNA (*P*roliferating *C*ell *N*uclear *A*ntigen) (Xiong *et al*., 1992). The latter, an auxiliary factor to a replicative DNA polymerase (see below), may also serve as a meeting point for multiple DNA metabolism enzymes (replication, repair, recombination) and regulatory factors such as cyclins/Cdks (Kelman, 1997).

The release from pRB and activation of the E2F/DP1 transcription factor promotes the activation of genes whose protein products participate in the late G_1–S phase. One of these gene products is cyclin E, the cyclin partner of another kinase, Cdk2 (Black and Azizkhan-Clifford, 1999). Cyclin E/Cdk2 helps cyclin D/Cdk4(6) phosphorylate the pRB protein, establishing a positive loop that enhances the release of the E2F/DP1 factor, and thus initiates the S phase. Other targets for cyclinE/Cdk2 might be proteins that regulate the initiation of chromosome replication, but these have not been identified unambiguously.

Genes whose products participate in recognition and activation of chromosomal origins and DNA replication are also regulated by E2F/DP1. Examples are the genes encoding for Orc proteins, MCM proteins, PCNA, DNA polymerase α and DNA ligase. Other cell cycle-related gene products are cyclin A, pRB, Cdc25, Cdk2 and p34Cdc2 (Cdk1). E2F/DP1 complexes are considered part of the mechanism by which cells will either proliferate or differentiate and specialize (Black and Azizkhan-Clifford, 1999).

The initiation of the S phase is the result of many independent molecular processes that prepare origins of replication to be recognized and activated. At least 14 different proteins participate directly in origin recognition: six Orc proteins (Orc 1–6, *O*rigin *r*ecognition *c*omplex), six MCM proteins (MCM 2–7, *M*ini*C*hromosome *M*aintenance), and the products of the genes *cdc6* and *cdt1* (Fujita, 1999). Recognition of the origin is necessary but not sufficient for origin firing. Almost all of these proteins bind to the origin DNA in telophase or in early G_1, as if waiting for a

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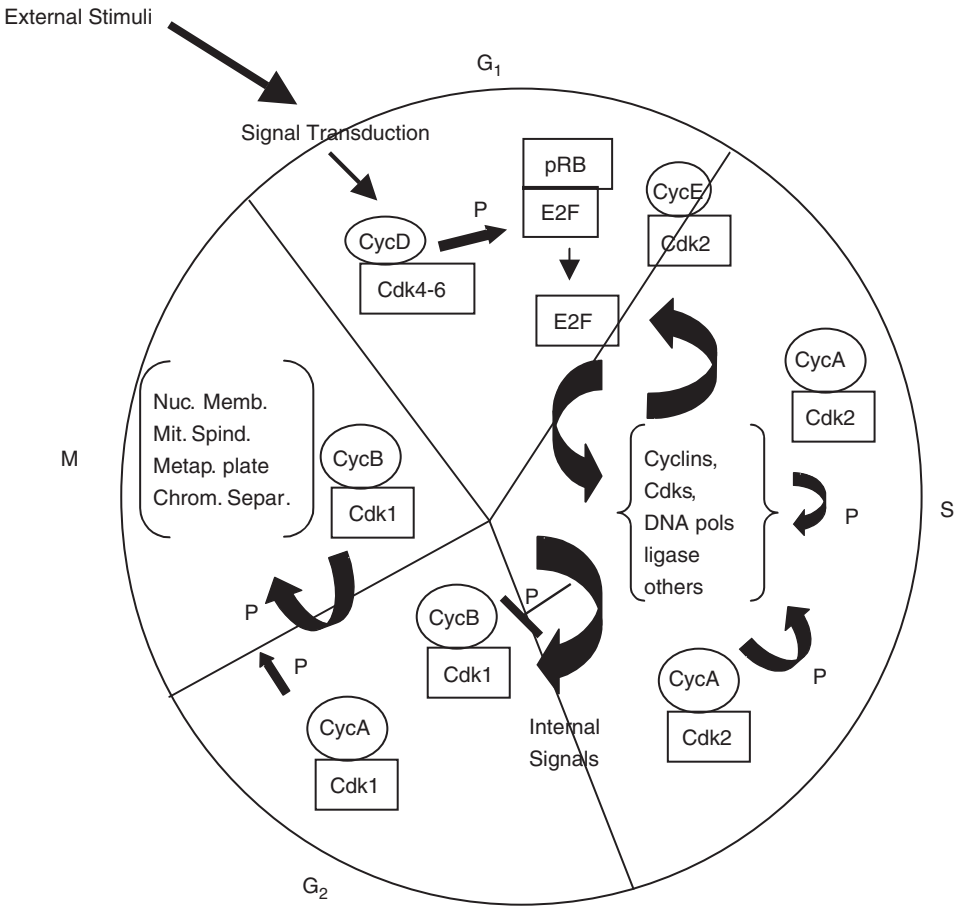

Figure 1. Model of the eukaryotic cell cycle. Only the critical action of the different cyclin/Cdk complexes is illustrated here, and, in the case of S and M complexes, the real or suggested targets of phosphorylation. DNA pols, DNA polymerases; Nuc. Memb., nuclear membrane; Mit. Spind., mitotic spindle; Metap. plate; metaphase plate; Chrom. Separ., chromosome separation; Cyc, cyclin; Cdk, cyclin-dependent kinase.

signal to proceed. This signal is, apparently, the activation of two kinase activities in late G_1 that, in a way not yet fully understood, phosphorylate target proteins and promote the initiation of DNA replication. One of these kinases is cyclin E/Cdk2 and the other kinase is the complex formed by Cdc7/Dbf4 (or DDK, *D*bf4 *D*ependent *K*inase), a non-cyclin-containing kinase. Target proteins are one or more of the Orc proteins, most of the MCM proteins and the Cdc6 protein (Takisawa *et al*., 2000). DDK kinase itself appears to be activated after phosphorylation by a cyclin/Cdk. As a result, the MCM 4, 6 and 7 proteins display helicase activity that separates the DNA double strands in a bidirectional fashion (Fujita, 1999). The resultant disassembly of the origin-bound proteins promotes DNA synthesis but, most importantly, precludes re-initiation of chromosome replication, i.e. allows DNA replication to occur once, and only once, per cell cycle (Fujita, 1999; Takisawa *et al*., 2000).

The unwinding of the origin region allows replication proteins to interact with single-strand DNA. Perhaps, the loading of the heterotrimeric RPA protein (*R*eplication *P*rotein *A*) is the first event. The presence of RPA ensures the entrance of the eukaryotic DNA primase, the enzyme responsible for synthesizing the oligoribonucleotide (primer) that will function as the anchor (the 3′OH end) for the DNA polymerizing reaction catalysed by DNA polymerases (Kornberg and Baker, 1992). DNA primase is a heterodimeric protein that is part of the heterotetrameric replicative DNA polymerase α. This enzyme, in turn, is loaded to the DNA by the product of the *cdc45* gene (Mimura and Takisawa, 1998). The DNA primase activity in DNA polymerase α synthesizes an RNA primer of 15–30 nucleotides that

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is extended with 20–30 deoxynucleotides by the DNA polymerase activity of DNA polymerase α (Waga and Stillman, 1998).

An unavoidable characteristic of truly replicative DNA polymerases should be the capacity to add at least hundreds of deoxynucleotides without disengaging from the DNA template. DNA polymerases δ and ε are the replicative enzymes in eukaryotes that possess this characteristic. DNA polymerase δ is a heterotrimeric, high-fidelity enzyme that owes its continuous engagement to the DNA template by association with a sliding clamp, the PCNA protein. Apparently, DNA polymerase ε, a heteropentameric protein, can also make use of PCNA (Kelman, 1997; Waga and Stillman, 1998). The role played by PCNA in these complexes is that of forming a homotrimer that encircles the DNA in the structure formed by a primed single-stranded DNA, leaving a protruding 3′OH end (Kelman, 1997). DNA polymerase δ (or ε) recognizes this DNA–PCNA complex and, using PCNA as the sliding clamp, slides along the DNA adding new deoxynucleotides. Mounting PCNA on to DNA requires the action of a heteropentameric protein complex, RF-C (*R*eplication *F*actor *C*) that can also dismount PCNA from terminated or stalled replication forks (Kornberg and Baker, 1992). This mechanism applies equally well in both the leading or the lagging replicating DNA chains. In the lagging chain, replication advances in the form of Okazaki fragments that are joined together by the action of DNA ligase 1, the RNA primers being removed previously by RNase H-type enzymes (Waga and Stillman, 1998).

Cyclin/Cdks also seem to participate in regulating events during the DNA replication phase. The complex formed by cyclin A/Cdk2 has been implicated in the phosphorylation of proteins such as RF-C, and probably also the MCM proteins and DNA polymerases (Schub *et al*., 2001). The role of this kinase complex is still obscure, but evidence suggests that the activity of at least some of the phosphorylated proteins is reduced or inhibited. Perhaps the role of cyclin A/Cdk2 during S phase is to allow enzymatic turnover in maturing Okazaki fragments or at the end of replicons.

Once DNA replication has been completed, cells enter the G_2 phase. In this, cells thoroughly survey that all replication forks have gone to completion and that no DNA remains to be replicated or has accumulated structural defects of any kind (Nurse, 1994). The main control point is another cyclin/Cdk complex, formed by cyclin B and p34Cdc2. This p34Cdc2 protein is the equivalent of the original protein found in yeast cells (Simanis and Nurse, 1986). p34Cdc2 is a relatively stable protein, found at similar levels during the cell cycle. Cyclin B, however, starts accumulating in the S phase, reaching a maximum level in G_2, and binds to p34Cdc2 progressively (Morgan, 1997). Cyclin/Cdk complexes are none the less inactive until the S phase has been concluded and there is no sign of DNA defects (Smits and Medema, 2001). This is achieved by signals emanating from incomplete or faulty chromosomes, a phosphorylation cascade that ultimately phosphorylates and inhibits p34Cdc2 kinase activity. Two amino acid residues in p34Cdc2 are involved in this negative regulation: Thr 14 and, principally, Tyr 15. The products of the *wee1* and the *myt1* genes are responsible for these inhibitory phosphorylations, and it is these kinases that are the target of the phosphorylation cascade (Morgan, 1997), an event that constitutes another example of a checkpoint control. A p34Cdc2 stimulatory phosphatase, the product of the *cdc25* gene, is also the target of this checkpoint control, and will remain inhibited by phosphorylation until signals of a completed S phase or repaired DNA are released. Activated Cdc25 protein can then remove the inhibitory phosphates in p34Cdc2, together with a stimulatory phosphorylation by another cyclin/Cdk-like protein, the CAK complex (*C*dk *A*ctivating *K*inase), in residue Thr 160 of p34Cdc2. The G_2 cyclin/Cdk complex can now perform as the inducer of the G_2/M transition and M phase set up (Morgan, 1997).

Proposed targets for this kinase are the internal nuclear protein layers, formed by laminins and cytoskeletal proteins. The nuclear envelope disassembles, and the cytoskeleton is reorganized into the new structure required during the M phase, the mitotic spindle. Cyclin B and p34Cdc2 are also required to trigger the mechanism by which the sister chromatids in the metaphase plate are suddenly separated and transported to the opposite poles in the cell (Smits and Medema, 2001). This mechanism involves activation of the M-phase proteasome, the *A*naphase *P*romoting *C*omplex (APC), that ultimately will also be responsible for degradation of cyclin B itself, a necessary requisite for cells to leave the M phase and enter G_1 again (Page and Hieter, 1999). Degradation of cyclin B is also required to allow positioning of the proteins that activate the replication origins during S phase, an event that takes place in late telophase.

The cell cycle in plants

Cell cycle studies in plants have increased exponentially over the past years, and have demonstrated that the basic principles of the cell cycle have been conserved. Despite the fact that there are clear differences between plants and animals in the way cells are structured and organized, grow, develop and differentiate – aspects that could have produced

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profound differences in cell cycle regulation – similar types of proteins and very similar mechanistic processes are being discovered.

Following the discovery of the p34Cdc2 protein kinase in yeast and human cells, evidence for the existence of a similar protein in higher plants was soon produced (John *et al*., 1989) and the corresponding maize (*Zea mays*), *Arabidopsis*, alfalfa (*Medicago sativa*) and pea (*Pisum sativum*) genes were cloned by sequence homology and their expression was studied (Colasanti *et al*., 1991; Feiler and Jacobs, 1991; Ferreira *et al*., 1991; Miao *et al*., 1993). The first plant cyclin protein was also discovered at this time (Hata *et al*., 1991). Due to the protagonist role of cyclins and Cdks in cell cycle regulation, the search for the corresponding plant proteins was exhaustive. Procedures to isolate Cdk or cyclin genes consisted mainly of sequence amplification or complementation of mutant yeast cells. To date, plant cyclins have been grouped into four different families and plant Cdks into five.

Plant cyclins

The majority of plant cyclins described to date share, to some degree, homology to cyclins A and B, including the characteristic cyclin core sequence, which contains the cyclin box required for Cdk binding. According to structural characteristics, A-cyclins have been classified in three groups: CycA1, CycA2 and CycA3; and B-cyclins in two groups: CycB1 and CycB2. D-cyclins have also been found and have been classified in four groups: CycD1, CycD2, CycD3 and CycD4, although the latter may belong to the CycD2 group (Renaudin *et al*., 1996; De Veylder *et al*., 1999).

A- and B-type cyclins appear to be associated with the G_2/M phases. In cell cultures of tobacco (*Nicotiana tabacum*; Reichheld *et al*., 1996), *Arabidopsis* (Fuerst *et al*., 1996), *Catharanthus* (Ito *et al*., 1997) and rice (*Oryza sativa*; Hashimoto *et al*., 1992), B-type cyclin transcripts accumulated in late G_2 and M phases, whereas A-type cyclin transcripts started to increase a little earlier, following S phase. Destruction of A- and B-type cyclins during mitosis, as in animal cells, is essential for a successful cell cycle conclusion, and plant A- and B-type cyclins contain destruction box-like sequences similar to those found in the animal counterpart (Genschik *et al*., 1998). Proteasomal subunits in plants have been cloned recently, and studies of their activity and distribution are accumulating (Yanagawa *et al*., 2002). Regulation of mitotic cyclin gene expression by phytohormones has been demonstrated for rice CycA1, which responds to gibberellins (Fabian *et al*., 2000).

A defining characteristic of D-type cyclins is the presence of the sequence LxCxE near the N-terminus, a motif that is important for interaction with the pRB protein (Sherr, 1994). Plant D-type cyclins contain this sequence, or a much conserved one, near the N-terminus, and plant pRB-related proteins bind to cyclin D. A cyclin box is also present, although homology is rather low (Meijer and Murray, 2000). G_1 cyclins are short-half-life proteins and this depends on the presence of a sequence rich in the four amino acids, PEST. Plant D-type cyclins contain PEST sequences (Sorrel *et al*., 1999) and, therefore, these proteins should have a short half-life.

As for human D cyclins, plant D-type cyclins show a cell-cycle-dependent expression and participate in cell cycle entry in response to growth factors and nutrients. In tobacco BY-2 cell suspension cultures, CycD3 is induced in G_1 after synchronization (Sorrell *et al*., 1999). Sucrose, the most important metabolite produced during photosynthesis, induces both CycD2 and CycD4 in starved *Arabidopsis* suspension cells (Soni *et al*., 1995; De Veylder *et al*., 1999). *Arabidopsis* CycD3 is induced by sucrose and cytokinins in whole plants, and leaf explants overexpressing CycD3 will proliferate in the absence of cytokinins, implying that the cell cycle is activated through expression of CycD3; in fact, cytokinin induction of CycD3 is dependent on the presence of sucrose (Soni *et al*., 1995; Riou-Khamlichi *et al*., 1999). CycD3 transcripts are also induced by brassinosteroids in callus and suspension cultures, although the mechanism of stimulation appears to be different from that due to cytokinins (Hu *et al*., 2000). Perhaps *cycD* genes respond to different signal transduction pathways and are expressed at different levels in different plant tissues (Meijer and Murray, 2001).

Plant Cdks

Plant cyclin-dependent kinases have been classified into five groups, according to the cyclin-binding motif present in each type (Joubés *et al*., 2000). Cdk-A is structurally homologous to yeast and mammal p34Cdc2 and conserves the PSTAIRE amino acid motif. Differences between this and the mammal enzyme are that gene expression does not show variations during the cell cycle and that the protein is found at very low levels in non-proliferating tissues (Colasanti *et al*., 1991; Ferreira *et al*., 1991; Hirt *et al*., 1993). Cdk-A is active in the G_1/S and the G_2/M phases, very likely with different cyclin partners (Mironov *et al*., 1999; Joubés *et al*., 2000). *cdk-A* genes have been isolated from maize, rice, alfalfa, soybean (*Glycine max*), pea, tobacco and *Arabidopsis* (Colasanti *et al*., 1991; Ferreira *et al*., 1991; Hashimoto *et al*., 1992; Hirt *et al*., 1993; Miao *et al*., 1993; Fobert *et al*., 1996; Setiady *et al*., 1996). In maize, two virtually identical *cdk-A* genes have been cloned, differing only in the 3′ and 5′ UTRs (untranslated regions), and Southern blot

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analysis has suggested the presence of more than two genes (Colasanti *et al*., 1991). Alfalfa CycA2;1 protein binds Cdk-A and maize pRB-related protein (Roudier *et al*., 2000); tobacco CycD2;1 and CycD3;1 proteins bind Cdk-A *in vivo*, and these kinase complexes phosphorylate tobacco pRB-related protein *in vitro* (Nakagami *et al*., 1999). *Arabidopsis* CycD2 and CycD3 interact with Cdk-A, and both kinase complexes phosphorylate histone H1 protein (Healy *et al*., 2001).

Evidence of Cdk regulation *in vivo* has been observed in tobacco cell cultures or pith parenchyma. A Cdk-A-type kinase accumulates in G_2, but it is not active in the absence of cytokinins; addition of cytokinins greatly increases kinase activity. Regulation appears to be at the level of protein phosphorylation, because the inactive enzyme is Tyr-phosphorylated in the absence of cytokinins, but can be activated *in vitro* by the yeast Cdc25 phosphatase (Zhang *et al*., 1996). This inhibitory phosphate in Tyr would be due to the enzymatic activity of the plant Wee1 kinase homologue, a protein that has been characterized in maize developing endosperm (Sun *et al*., 1999).

Cdk regulation also can be effected by inhibitory proteins, such as the recently described ICK (*I*nhibitor of *C*yclin-dependent *K*inases), the plant equivalent of animal Cip/Kip proteins. Using *Arabidopsis* Cdk-A-type kinase as bait in two-hybrid screens, two Cdk inhibitors, ICK1 and ICK2, were identified (Wang *et al*., 1997). ICK1 interacts with, and inhibits, the CycD/Cdk-A kinase complex, and it is induced by abscisic acid (Wang *et al*., 1998), suggesting a possible mechanism by which this phytohormone can inhibit cell proliferation. Overexpression of ICK1 inhibits cell number, cell growth and alters leaf morphology, indicating that ICK1 participates in the control of plant growth and development (Wang *et al*., 2000). ICK2 is also a potent inhibitor of Cdk-A. The recent finding of seven different ICKs in *Arabidopsis* has shown that they belong to two different groups: the A group contains ICK1, 2, 6 and 7, which interact with both Cdk-A and a D-type cyclin (D1, D2 or D3), while the B group consists of ICKs 4 and 5, and these interact with cyclin D but not with Cdk-A (Zhou *et al*., 2002). Overexpression of ICK members of every group strongly affected plant growth and morphogenesis, similarly to ICK1.

Cdk-B kinase possesses a divergent cyclin-binding motif, PPTALRE or PPTTLRE, and thus, has been divided in two subgroups, Cdk-B1 and Cdk-B2, respectively. These kinase proteins are exclusive for plants and have been studied in alfalfa, pea, *Arabidopsis* and rice (Feiler and Jacobs, 1991; Magyar *et al*., 1997; Fabian *et al*., 2000). In contrast to Cdk-A, the transcriptional and translational expression levels of Cdk-B are cell cycle controlled, and are present mainly in the G_2/M transition (Mironov *et al*., 1999). Cdk-B can pair and be activated by both CycA and CycB (Huntley and Murray, 1999). Expression of rice Cdk-B type kinase is regulated by gibberellins (Fabian *et al*., 2000).

Cdk-C, Cdk-D and Cdk-E belong to three different kinase families, and these proteins have not been well characterized. Cdk-C has been found in pea and alfalfa and has a PITAIRE cyclin-binding motif, also present in human Cdk-9 (Lapidot-Lifson *et al*., 1992). Alfalfa Cdk-C is expressed constitutively in synchronized cell cultures (Magyar *et al*., 1997); however, no hybridization signal has been found in dividing *Arabidopsis* cell cultures (Mironov *et al*., 1999). Cdk-D has been classified as a Cdk-activating kinase (CAK) and has a cyclin-binding motif N(I/F)TALRE; it is highly homologous to human Cdk7. Only the rice and *Arabidopsis* Cdk-D proteins have been studied, and rice Cdk-D is able to phosphorylate rice Cdk-A, human Cdk2 and the large subunit of *Arabidopsis* RNA polymerase II, a characteristic target of Cdk7 (Serizawa *et al*., 1995). A SPTAIRE-containing kinase has been found in alfalfa, sharing no features with other known kinases, and has been classified as Cdk-E. The role of this kinase is still unknown (Magyar *et al*., 1997).

The cell cycle in plants is very complex, and different species may have different members of specific cell-cycle proteins. For example, a genomic analysis in *Arabidopsis* showed the existence of several cyclin and Cdk genes, of which ten were for A-type cyclins, nine were for B-type cyclins and ten were for D-type cyclins (Vandepoele *et al*., 2002); however, only one *cdk-A* gene was found (Vandepoele *et al*., 2002), whereas in maize, two *cdk-A*-type genes have been found (Colasanti *et al*., 1991). The existence of more than two Cdk-A type kinases has also been reported in other plant species (Joubés *et al*., 2000).

Other cell cycle proteins

Plant cell cycle proteins of the group formed by the plant pRB and E2F proteins have also been studied intensely. The presence of these proteins demonstrates that the plant cell cycle is related more to that of mammals than to that of yeast. The first indication of the existence of RB-related proteins in plants was the finding of the sequence LxCxE, known to mediate RB binding in plant D-type cyclins and in geminivirus proteins (Dahl *et al*., 1995; Soni *et al*., 1995; Xie *et al*., 1995). Soon thereafter, three different groups identified RB-related proteins (RBR) in maize tissues (Grafi *et al*., 1996; Xie *et al*., 1996; Ach *et al*., 1997), and interaction between maize RBR and all types of D-like cyclins was demonstrated by yeast two-hybrid assays (Ach *et al*., 1997; Huntley *et al*., 1998). Moreover, tobacco RBR can be phosphorylated *in vitro* by tobacco Cdk-A and cyclin D3;1 (Nakagami

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et al., 1999). The finding that maize RBR is expressed differentially in plant tissues supports the notion that this protein may also be involved in cell differentiation in plants, as it is in animal cells (Huntley *et al*., 1998). This same study showed that maize RBR expressed in human cells was able to repress an E2F-responsive promoter, strongly indicating that RBR recognizes similar proteins in plants.

Demonstration of the existence of a plant E2F protein came from yeast two-hybrid assays using RBR as a probe. Wheat E2F had a sequence organization that resembled human E2F 1/2/3, although in primary sequence was more related to human E2F 4/5. Wheat (*Triticum aestivum*) E2F was expressed in proliferating cultured cells with up-regulation in early S phase (Ramírez-Parra *et al*., 1999). E2F from carrot (*Daucus carota*), *Arabidopsis* and rice have also been described (Albani *et al*., 2000, Magyar *et al*., 2000; Kosugi and Ohashi, 2002). The E2F protein partner, the DP protein, was also found in wheat and *Arabidopsis* (Ramírez-Parra *et al*., 1999; Magyar *et al*., 2000). Six different E2F and two DP proteins have recently been described in *Arabidopsis* and rice (Kosugi and Ohashi, 2002; Mariconti *et al*., 2002). Some of the E2F proteins are transcription factors that, in association with DP proteins, can transactivate genes containing E2F binding sites (Chabouté *et al*., 2000; Kosugi and Ohashi, 2002; Mariconti *et al*., 2002). The role of E2F (possibly bound to RBR proteins) as a transcriptional repressor has also been demonstrated (Egelkrout *et al*., 2001; Mariconti *et al*., 2002). Interestingly, in some of these studies, the gene promoter used has been that of the PCNA protein (an S-phase marker), with conflicting results. Perhaps differences in the action of E2Fs as promoters or repressors of transcription are due to the type of E2F isolated, as demonstrated for *Arabidopsis* E2F proteins (Mariconti *et al*., 2002).

Another well-studied cell cycle protein is PCNA. Its usefulness as an S-phase marker prompted the cloning and characterization of its encoding gene in rice, carrot, *Catharanthus*, tobacco, pea and maize (Suzuka *et al*., 1989; Kodama *et al*., 1991; Hata *et al*., 1992; Matsumoto *et al*., 1994; López *et al*., 1995; Shimizu and Mori, 1998). PCNA gene and protein sequences are highly conserved in all eukaryotes so far studied, and in all cases the protein shows a cyclin D-binding domain. In animal cells, PCNA and a cyclin D/Cdk complex bind during G_1 (Xiong *et al*., 1992; Waga *et al*., 1994), and a similar complex appears to be present in plant tissues as well (Herrera *et al*., 2000; Sánchez *et al*., 2002). The nature of this association is still a puzzle, although it is speculated that PCNA, a protein required in virtually every aspect of DNA metabolism, serves as a sliding platform to mediate interaction between or among proteins that interact with the DNA: DNA polymerases, repair or recombination enzymes, and protein kinases (Kelman, 1997). Cyclins/Cdks fall within the latter category and perhaps their activity helps assembly/removal of DNA–protein complexes. Unfortunately, the role of PCNA in DNA replication in plant cells is largely unknown. This is mainly due to the scarce work and poor knowledge of plant DNA polymerases. These have only been characterized based on their biochemical properties and compared to their animal and yeast homologues, but no physiological role has yet been identified *in vivo*. Different examples of α-, δ-, ε- and β-type enzymes can be found mainly for wheat, pea, cauliflower (*Brassica oleracea*), rice and maize (Castroviejo *et al*., 1990; Richard *et al*., 1991; Bryant *et al*., 1992; Coello *et al*., 1992; Al-Rashdi and Bryant, 1994; Coello and Vázquez-Ramos, 1995a; Benedetto *et al*., 1996; Sanathkumar *et al*., 1996; García *et al*., 1997; Luque *et al*., 1998; Seto *et al*., 1998). Over the past few years, cDNA sequences for α- and δ-type DNA polymerases have been reported (Yokoi *et al*., 1997; Collins *et al*., 1998), opening the possibility for a more comprehensive study of the physiological role of these enzymes.

Maize DNA polymerase 2 is perhaps the best described example of a plant polymerase–primase complex; it is an α-like enzyme and its activity may be regulated by phosphorylation (Coello and Vázquez-Ramos, 1995b; García *et al*., 1997). Wheat and tobacco DNA primases have also been partially characterized; however, none of these enzymes has been reported unambiguously to co-purify with a DNA polymerase activity (Laquel *et al*., 1990, 1994; García-Maya and Buck, 1998). The processivity (the amount of polymerization catalyzed when the enzyme binds to a template) of a wheat δ-type enzyme is stimulated by mammalian PCNA (Laquel *et al*., 1993), and this is perhaps the only example of an association between PCNA and a plant DNA polymerase.

The complete sequencing of the *Arabidopsis* genome has uncovered the presence of multiple gene sequences with similarity to replication proteins such as Orc proteins, MCM proteins, Cdc6p, Cdt1p, Cdc45p, RP-A, RP-C, DNA repair proteins and others (Kosugi and Ohashi, 2002). Interestingly, the corresponding genes contain E2F consensus DNA sequences in their promoter regions, near the transcription start site. *Arabidopsis mcm7*- and *mcm3*-like genes have been reported and, as expected, they are expressed in proliferating cells (Springer *et al*., 1995; Holding and Springer, 2002, Stevens *et al*., 2002). Also, the *Arabidopsis cdc6* gene has been cloned and analyzed (Castellano *et al*., 2001). The gene is expressed in early S phase in proliferating cells and in endoreplicating cells. Recent studies of expression of cell cycle genes in synchronized cells from *Arabidopsis*

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and tobacco have identified the periodicity of expression of the different cyclins, Cdks and many other cell-cycle-related genes, together with genes involved in signal transduction, hormonal response, transcriptional regulation and other metabolically related pathways (Breyne *et al*., 2002; Menges *et al*., 2002). These types of studies are extremely important because they not only indicate the precise time at which cell cycle gene-products are required, but also provide clues about the genetic and developmental programmes that plant cells employ to monitor cell division, patterning and development.

The cell cycle and seed germination

Embryo development during seed formation involves high rates of cell proliferation; this process may take up to several weeks, after which seeds accumulate reserve material and mature. Cells in embryos of dry seeds of different species have mostly a G_1-phase DNA content (Deltour and Jacqmard, 1975; Conger and Carabia, 1976; Bewley and Black, 1994), implying that, during embryo formation, developmental control imposes a halt in proliferation at the end of mitosis or in early G_1. This may not be an absolute requirement, because some cells stop in G_2, or perhaps, even in S phase. The latter, however, might have accumulated chromosomal damage during maturation and drying of seeds due to the fragility of DNA in replication forks, and cells may not be able to survive.

Imbibition of seeds causes a metabolic revival. Whether cells in seeds have an imprinted biochemical memory of the processes that will follow, or they acquire this memory in the course of germination, has been the matter of intense research and debate. Inhibition of seed germination by protein or RNA synthesis inhibitors has been taken as a proof that new proteins/messengers should appear *de novo* during imbibition. However, these results could also be interpreted in terms of gene dosage, i.e. the need for greater amounts of the same products. In the early hours of seed germination, the pattern of existing proteins (determined by two-dimensional electrophoresis) does not exhibit important qualitative changes, whereas at later times, there is a changing pattern (Spiegel and Marcus, 1975; Sánchez de Jiménez *et al*., 1981; Sánchez de Jiménez and Aguilar, 1984). But again, it is very likely that regulatory proteins are produced in quantities below the electrophoretic detection levels. The molecules or molecular processes that trigger the germination process are still undefined.

Most cells in meristematic tissues of dry seed embryos, the only cell populations to proliferate, have a G_1 DNA content and are metabolically in a G_0-like state, characterized by the absence of signals that favour proliferation. However, imbibition does not cause an immediate entry into the cycle. Instead, there is a lag of up to several hours before the S phase becomes evident. The long G_0/G_1 transition must be due to the need of cells to recover from cellular damage accumulated during seed maturation/seed drying/seed storage (Osborne, 1983). Chromosomal damage must be particularly important because nuclear DNA in dry seeds loses its integrity during storage, and this damage increases as seeds age (Villiers and Edgcumbe, 1975; Cheah and Osborne, 1978, Elder *et al*., 1987; Vázquez-Ramos *et al*., 1988). Thus, DNA repair is an important and, very likely, vital process during early germination, as has been shown for seeds of lettuce (*Lactuca sativa*), barley (*Hordeum* spp.), rye (*Secale cereale*) and maize (Villiers and Edgcumbe, 1975; Osborne *et al*., 1980; Vázquez-Ramos and Osborne, 1986; Elder *et al*., 1987; Zaraín *et al*., 1987; Zlatanova *et al*., 1987).

In aged seeds the mean germination time is delayed. There is a direct correlation between seed ageing and an increasingly extended time for DNA replication to take place during germination (Sen and Osborne, 1974; Elder *et al*., 1987; Gutiérrez *et al*., 1993). Evidently, DNA repair will take longer in deteriorated seeds and this delay will affect the timing of all cell cycle events. Two conclusions derived from these results could be that: (a) DNA replication is part of the germination process; and (b) there must be some sort of checkpoint control that inhibits the setting up of the S phase if proper germination conditions have not been achieved. In yeast and mammalian cells, it has been established clearly that DNA damage blocks the establishment or progression of the S phase until damaged DNA has been repaired (Rhind and Russell, 2000; Bartek and Lukas, 2001). Is the completion of a full cell cycle a prerequisite for germination to finish? It appears to be clear that, in general, the M phase and cell division are concomitant with, or take place after, radicle protrusion (Bewley and Black, 1994). Therefore, the answer would be that only the initial phases of the cell cycle are necessary. Interestingly, cabbage seeds germinate (radicle protrusion) after 12 d of incubation in 500 mM hydroxyurea (an inhibitor of the S phase). However, flow cytometry analysis of radicle tip cells exhibits a similar pattern to that of dry seeds, indicating a total blockage of cell division activity. This result led to the conclusion that DNA replication is not a prerequisite for radicle protrusion and initial extension (Górnik *et al*., 1997). Similarly, inhibition of M phase progression with colchicine allows maize radicle protrusion, but not elongation (Baíza *et al*., 1989). Whereas these results would indicate that cell cycle events are not essential for germination to occur, they may also indicate that radicle protrusion is not, metabolically, the end of the germination process, as cell proliferation, an absolute

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requirement for seedling establishment, has not been triggered. Certainly, this is a very interesting research area to pursue, and it is possible that these may be seed-specific-controlled processes.

Seed germination and cell cycle initiation

As stated above, most meristematic cells in dry embryos have a G_1 DNA content. If dormant seeds of wild oat (*Avena fatua*) are imbibed at 25°C, they resume a limited but continuous metabolic activity, synthesizing proteins and nucleic acids, without showing a net increase in DNA content (Elder and Osborne, 1993), and seeds can stay under these conditions for days or weeks without germinating. Changing the temperature to 15°C will allow germination, and root cells will duplicate their DNA within 16 h. Dormant tomato seeds (*Lycopersicon esculentum*) also contain cells in G_1, and only replicate DNA and move into G_2 if dormancy is broken (de Castro *et al*., 2001). Similar conditions to dormancy can be induced if seeds are osmoprimed. Osmopriming consists of pre-imbibing seeds in a solution containing an inert osmotic agent such as polyethylene glycol (PEG), which reduces water availability. Cells in seeds can reactivate their metabolism, but germination (i.e. radicle protrusion) will not ensue. The beneficial effect of osmopriming is manifested as a fast and uniform germination after PEG removal (Heydecker and Coolbear, 1977; Bradford, 1986). After priming, seeds can also be re-dried and stored for some time, conserving the acquired high vigour. Studies with leek, maize and bean (*Phaseolus* spp.) indicated that there was a low level of protein and DNA synthesis during osmopriming, but with no evidence of nuclear replicative DNA synthesis (Coolbear and Grierson, 1979; Bray *et al*., 1989; Ashraf and Bray, 1993; Cruz-García *et al*., 1995; Sánchez de Jiménez *et al*., 1997). In maize, [^{3}H]thymidine labelling of meristematic cells of 3 and 10 d osmoprimed seeds showed predominantly cytoplasmic localization of the label, and no evidence of mitotic figures was found. In all cases, removal of the hygroscopic agent caused a rapid increase in [^{3}H]thymidine incorporation, well before this would occur under germination conditions of the control seeds. In maize and bean, the appearance of mitotic figures was evident several hours before this was visible in germinating control seeds (Cruz-García *et al*., 1995; Sánchez de Jiménez *et al*., 1997). As under conditions maintaining dormancy, these data suggest that a control point regulates the initiation of the S phase, and this point is not reached if conditions for germination are not appropriate. The long delay of S-phase initiation in aged or deteriorated seeds could also be explained in the same terms.

However, this model may need to be modified, as not all cells in osmoprimed seeds are blocked in the G_1 phase. Flow cytometry measurements of the DNA content in root tip cells of osmoprimed tomato and pepper seeds indicated that a certain proportion of cells acquired a G_2 DNA content, i.e. 4C, without entering mitosis (Bino *et al*., 1992; Lanteri *et al*., 1993). The amount of 4C nuclei depended on both the osmotic potential of the solution used for osmopriming and the duration of the treatment. A positive correlation was established between the number of cells in G_2 and improved germination performance (Lanteri *et al*., 1994, 1996). However, it was later demonstrated that germination performance can be separated from the number of cells that can replicate their DNA during priming since, whatever the number of G_2 cells accumulating, the effect on the mean germination time following priming was the same (Gurusinghe *et al*., 1999; Lanteri *et al*., 2000). Thus, although some cells can replicate their DNA depending on the priming conditions, the conclusion seems to be that neither replication nor the 4C state is essential for advancement of germination. Flow cytometry experiments should also be performed for maize, leek and bean to obtain a more general view of the metabolic state that seeds reach during osmopriming and the importance of cell cycle advancement. Again, different types of seeds may respond in metabolically different ways to priming conditions. However, it is relevant to stress that both seeds with cells apparently blocked in G_1 and seeds with some cells passing into G_2 show a burst of [^{3}H]thymidine incorporation into DNA upon germination (Lanteri *et al*., 1993; Cruz-García *et al*., 1995; Sánchez de Jiménez *et al*., 1997). This, indeed, suggests that many cells are stopped at the G_1 phase during osmopriming, and this gives a clue about the importance of this cell cycle stage for germination control.

Additionally, the effects of phytohormones on seed germination lend support to the idea that the G_1 phase is a very important metabolic state. The G_1 phase in cytokinin-stimulated germinating maize caryopses is notably shortened when compared with the time it takes in untreated controls. The S phase starts by 4 h of germination, in comparison with around 12 h under control conditions (Baíza *et al*., 1989; Reyes *et al*., 1991). This suggests that cytokinins overrule most G_1 control points. On the other hand, abscisic acid (ABA) inhibits seed germination, possibly by blocking DNA replication. In maize kernels, ABA keeps cells in a G_1-like metabolic state (Bewley and Black, 1994; Sivritepe and Dourado, 1995; Sánchez and Vázquez Ramos, unpublished results). Since ABA induces accumulation of G_1 cyclin/Cdk inhibitors in *Arabidopsis* cells (Wang *et al*., 1998), this may be the mechanism by which S-phase establishment is blocked during seed germination.

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Cell cycle proteins during seed germination

Unfortunately, very few studies exist on cell cycle proteins and their behaviour during seed germination. Pioneering studies on detection of cell-cycle-related proteins were those of the Loidl group (Georgieva *et al*., 1994a). Using heterologous cDNA probes and antibodies against human nuclear proto-oncogenes and tumour supressors, these authors identified putative n-Myc, c-Fos, c-Myc, c-Jun and p53 proteins by Western blotting and n-Myc, c-Myc and p53 mRNAs by Northern blotting in germinating maize cayopses. Perhaps the most interesting behaviour was that of the putative p53 protein, since it was present only during the first 12 h of germination, a period corresponding to the G_1 phase and the G_1/S transition, and then disappeared. This would be the expected behaviour of a protein whose role is to inhibit cell cycle progression until chromosomal damage has been repaired (Whittle *et al*., 2001). It should be noted that Georgieva *et al*. (1994a) identified a 72 kDa protein instead of the expected 53 kDa protein. Work with G_1 cell cycle markers has established the presence of putative cyclin D, p53 and E2F proteins using heterologous antibodies, and the presence of Cdk-A and PCNA proteins using homologous antibodies, in cells from dry maize embryonic axes (Cruz-García *et al*., 1998; Herrera *et al*., 2000). With the exception of the putative E2F protein, which showed little variation during the first 24 h of germination (no radicle protrusion), all other proteins exhibited a distinctive and expected pattern. The cyclin D-like protein content remained constant for about 6 h and then declined to almost undetectable levels by 24 h of germination. The amount of the putative p53 protein, giving a 53 kDa band, also declined after 15 h of germination (Cruz-García *et al*., 1998). This behaviour resembles that reported by Georgieva *et al*. (1994a), and also reinforces the hypothesis that seed germination, especially of aged seeds, would be an ideal model system to use for the study of a p53-related pathway in plants for cell cycle arrest (Whittle *et al*., 2001).

The pattern shown by the putative cyclin D would indicate that, after establishing the G_1 phase, involvement of this protein would have ended. Further evidence that this was a cyclin-like protein came from immunoprecipitates formed with an anti-human cyclin D1 antibody, which were able to phosphorylate the typical cyclin/Cdk substrate, histone H1. In these immunoprecipitates a putative Cdk was also identified. In addition, the kinase activity in the immunoprecipitates followed the same pattern as that of the putative cyclin D, i.e. it disappeared after 15 h of germination (Cruz-García *et al*., 1998).

The cloning and overexpression of maize PCNA (López *et al*., 1995) allowed the generation of a homologous antibody against this protein. Using this antibody, it was established that PCNA is present in dry caryopses at low levels, which increase gradually to reach a peak at 24 h of germination (Herrera *et al*., 2000). Georgieva *et al*. (1994b) and Onelli *et al.* (1997), using maize and pea, respectively, produced similar results but used heterologous antibodies. More interesting was the finding that anti-human cyclin D1 antibodies co-precipitated maize PCNA, and that this association ceased when the putative cyclin D disappeared during germination (Herrera *et al*., 2000). Moreover, anti-maize PCNA antibodies co-precipitated not only the cyclin D-type protein, but also a PSTAIRE-containing Cdk-A protein with high kinase activity during the first 6 h of germination, declining to very low levels thereafter (Sánchez *et al*., 2002). This kinase efficiently used as substrate the maize pRB-related protein, ZmRBR, the *in vivo* natural template of G_1 cyclin/Cdks. These results demonstrate the existence in plants of a kinase complex equivalent to the mammalian cyclin D/Cdk4(6)/PCNA G_1 complex. They also demonstrate that germinating seeds contain, and very likely require, this complex to trigger the cell cycle during early germination. It would now be most useful to characterize the behaviour of this complex and the corresponding kinase activity in aged or deteriorated seeds, and also during osmopriming, to substantiate its relevance for the progress of germination.

Over the past years considerable progress has been made in studies of the G_1/S transition in plants, as shown by the discovery of plant orthologues to mammalian proteins that recognize and activate replication origins such as Orc, Cdc6 (Kimura *et al*., 2000; Castellano *et al*., 2001), MCM (Springer *et al*., 1995; Holding and Springer, 2002; Stevens *et al*., 2002) and other G_1/S-phase regulatory proteins. Moreover, it seems clear that two different E2F/DP-types of complexes exist, which, very likely associated with RBR, either stimulate or repress transcription of G_1/S cell cycle genes (Kosugi and Ohashi, 2002; Mariconti *et al*., 2002; Rossi and Varotto, 2002; Stevens *et al*., 2002). However, only a few G_1/S-phase markers have been followed in germinating seeds: thymidine kinase activity, histone biosynthesis, β-tubulin and PCNA accumulation, DNA polymerases, DNA primase and DNA ligase activities. H1 and core histones are absent in dry seeds and start accumulating by the time DNA replication becomes evident during maize germination, i.e. by 12 h (Georgieva *et al*., 1994b). A similar pattern is observed for thymidine kinase (Georgieva *et al*., 1994b). These authors report that, during germination, cell populations reached the S phase in a discrete, stepwise manner and not gradually, suggesting partially synchronized cell cycles. Similar

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conclusions were drawn previously, when the mitotic index was measured in meristematic cells during maize germination (Baíza *et al*., 1989).

A correlation between activation of DNA replication and β-tubulin accumulation during tomato seed germination has been reported (de Castro *et al*., 1995). No β-tubulin was detected in dry seeds or during the early stages of germination. Although a better marker for the mitotic phase, regulation of tubulin accumulation must depend on events that occur during G_1/S, since no tubulin was synthesized in low-viability seeds in which DNA replication onset was extremely delayed. Conversely, the passage from G_1 to G_2, which takes place in some root tip cells of tomato seeds during osmopriming, permits β-tubulin accumulation, despite the fact that there is no entry into mitosis, again suggesting a pre-S control (de Castro *et al*., 1995).

The S phase is characterized by DNA duplication, and DNA polymerases are the key enzymes. The knowledge of DNA polymerases in plants lags far behind that of yeast and mammal cells. However, several enzymes have been isolated and partially characterized. One of the earliest and most systematic studies on plant DNA polymerases was that of the Litvak group. Several DNA polymerases (DNA pol) from wheatgerm have been described. Among those studied, DNA pol A has some properties of an α-type enzyme (Castroviejo *et al*., 1979), whereas DNA pol B resembles DNA polymerase δ, especially since it is stimulated by mammalian PCNA (Laquel *et al*., 1993). DNA replication during wheat caryopsis germination appears to start after 7–8 h of imbibition, and reaches a peak after 12–13 h. The activity of both DNA pol A and DNA pol B increased gradually during germination, reaching a maximum around 12 h, showing a remarkable correlation with the peak of DNA synthesis (Benedetto *et al*., 1996). A DNA primase activity that co-purified with the α-type DNA pol A also increased gradually during germination, with a peak at 12 h (Benedetto *et al*., 1996). DNA polymerases are present in dry maize embryo axes (García *et al*., 1997). DNA replication during maize germination starts at 12 h after imbibition and reaches a peak at 24 h (Baíza *et al*., 1989; Georgieva *et al*., 1994b). During this time, maize DNA pol 1 activity, a δ-type enzyme, remains virtually constant, whereas activity of DNA pol 2, an α-type enzyme, gradually increases, reaching a peak by 24 h. DNA pol 2 contains a tightly associated DNA primase activity (García *et al*., 1997).

Evidence of regulation of DNA polymerase activity during germination was produced when it was found that maize DNA pol 2 was phosphorylated previous to the start of the S phase, at 9–11 h of germination (Coello and Vázquez-Ramos, 1995b), and dephosphorylated after the peak of DNA synthesis (after 24 h of germination). Maximum DNA pol 2 activity coincided with the phosphorylated state. However, *in vitro* phosphorylation of DNA pol 2 with casein kinase II or with the PCNA-associated Cdk-A type kinase (see G_1 phase during germination) did not modify polymerase activity (Coello and Vázquez-Ramos, 1995b; Gómez and Vázquez-Ramos, 2003). Phosphorylation of human DNA pol α did not modify polymerase activity either. However, initiation of replication origins required a cyclin E/Cdk2-phosphorylated DNA pol α, whereas phosphorylation by cyclin A/Cdk2 inhibited this process (Voitenleitner *et al*., 1999; Schub *et al*., 2001). The activity of both DNA pol 1 and DNA pol 2 maize enzymes was substantially enhanced after only 3 h of embryo axis imbibition in the presence of cytokinins. For DNA pol 2, this increased activity was accompanied by phosphorylation ahead of time, at 3 h of germination. These data correlate with the time of the initiation of DNA replication in cytokinin-stimulated seed axes, after 4 h of germination (Reyes *et al*., 1991; Gómez and Vázquez-Ramos, 2003). The mechanism by which cytokinins stimulate DNA polymerase activity and the S phase is largely unknown, although it probably requires RNA or protein synthesis, as stimulation was inhibited by α-amanitin and cycloheximide (Vázquez-Ramos and Reyes-Jiménez, 1990; Zúñiga-Aguilar *et al*., 1995). Stimulation of protein kinase and of poly-ADP ribosyl polymerase activities within the first 3 h of germination would suggest an early triggering of mitogenic signal transduction (Zúñiga-Aguilar *et al*., 1995). Cytokinin-induced shortening of the distance between replication origins, implying activation of silent replication origins, has been demonstrated for *Sinapis alba*, *Lolium temulentum* and tomato vegetative shoot meristems (Houssa *et al*., 1990, 1994). Controversially, these authors found no enhancement of DNA replication itself. The significance of DNA replication proteins for seed germination was confirmed when deteriorated maize caryopses, with low viability, were found to contain very low DNA pol 2 activity, due to degradation of the DNA pol 2 catalytic subunit (Coello and Vázquez-Ramos, 1996). Similarly, DNA ligase activity in deteriorated rye or maize embryos was considerably reduced (Elder *et al*., 1987; Vázquez *et al*., 1991). In all cases under these deterioration conditions, the S phase was largely delayed. Therefore, events occurring during the G_1/S transition, including DNA repair, should be considered as fundamental for germination.

Molecular events in the G_2/M phases have received very little attention and data are scarce. The M phase appears to take place after radicle protrusion, and, thus, it is not always considered as part of the germination process. The dominant regulator of the G_2/M transition is the cyclin

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B/p34Cdc2 complex and, as indicated before, it is regulated in plant tissues in a similar fashion as in other eukaryotes (Nurse, 1994; Zhang *et al*., 1996). In maize root tips the p34Cdc2-equivalent, one of the A-type Cdks (a PSTAIRE-containing protein), was localized and found to be bound to the pre-prophase band (Colasanti *et al*., 1993), whereas in 24 h germinated seeds, the protein was widely distributed in nuclei (Herrera-Teigeiro *et al*., 1999). Prior to this, the Cdk-A-type protein is mainly present in the cytoplasm, indicating the need for nuclear transport. Cytokinins induced an earlier entry of this protein into nuclei, by 15 h of germination, and this correlated with increasing kinase activity. Cyclin B/Cdk-A kinase activity was low during germination under normal conditions, whereas in the presence of cytokinins, there was a peak of activity by 15 h (Herrera-Teigeiro *et al*., 1999). The Cdk-A-type protein is present in dry embryo axes, and there is no variation in its amount during germination, whether axes are imbibed in the presence or absence of cytokinins. Thus, transport to nuclei and kinase activation must be part of the mechanism by which cytokinesis is promoted during germination. A summary of the cell cycle in germinating seeds is presented in Fig. 2.

Finally, the use of modern technology to analyse global gene expression, transcriptome or proteome analysis should be applied to follow the behaviour of cell cycle markers during germination. Considering the increasing availability of plant cDNA probes, this approach should yield valuable information about the cell cycle and its role during seed germination. Proteomic analysis has already been used to study gene expression during seed germination and priming in *Arabidopsis* (Gallardo *et al*., 2001), the complete genome of which has been sequenced. Although no cell cycle genes were detected among

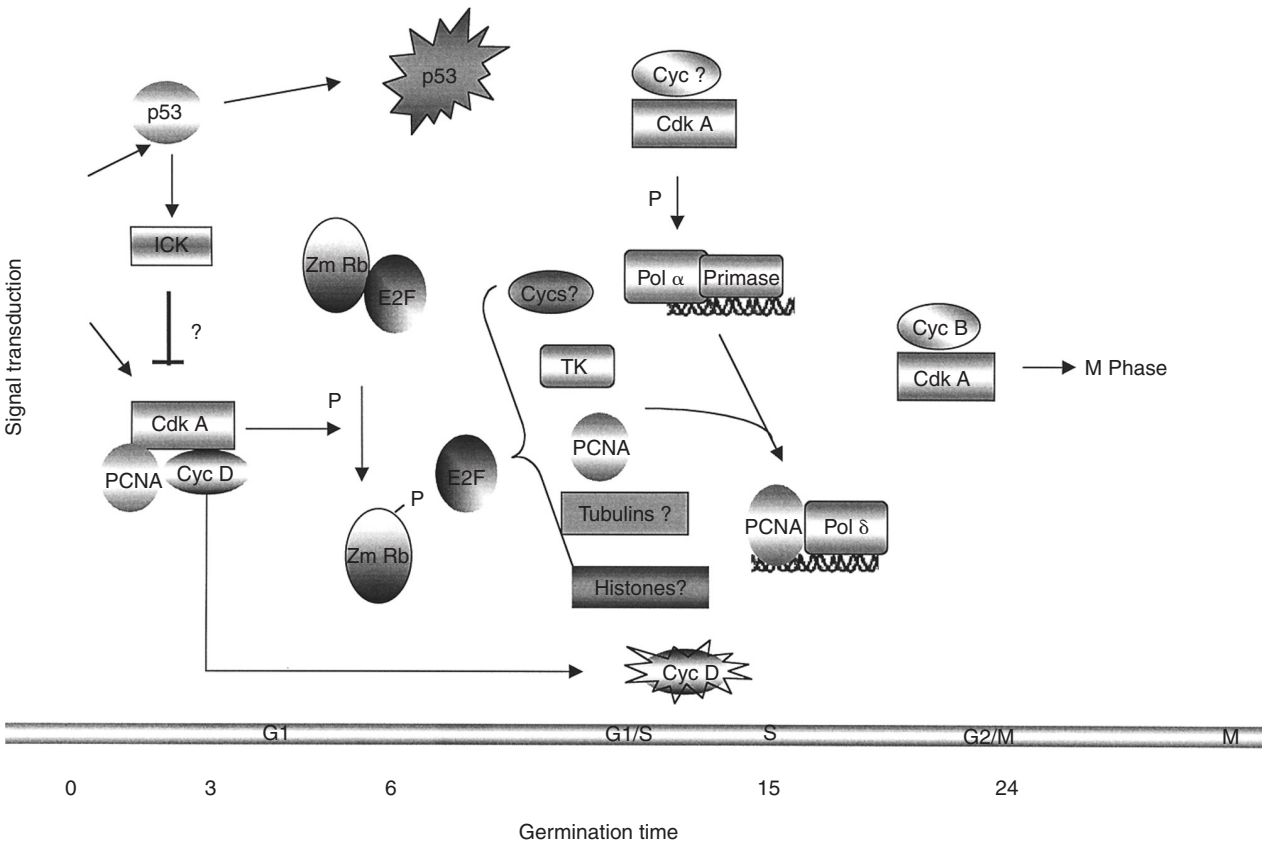

Figure 2. A model of cell cycle events during seed germination. The activity of the p53 protein would be very important in the early stages of germination, to coordinate DNA repair with the onset of the cell cycle. Then, the G_1 kinase would trigger the cell cycle, and E2F would be released from pRB to allow transcription of G_1/S genes. At this stage, it would be likely that inhibitors of transcription/translation would inhibit germination. DNA polymerases and DNA replication would be controlled by cyclin/Cdks. The timing of all these events, and thus, of germination, is modified by phytoregulators. Cyc, cyclin; Cdk, cyclin-dependent kinase; Pol, polymerase; ICK, inhibitor of cyclin-dependent kinases; PCNA, proliferating cell nuclear antigen; Zm Rb, maize pRB-related protein; TK, thymidine kinase.

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those that were up- or downregulated, the results obtained provided important information about the germination process in *Arabidopsis*.

In summary, the evidence gathered, although still scarce, suggests that dry seed embryos contain most, if not all, proteins required to enter the cell cycle. After imbibition, G_1 proteins, such as the G_1 kinase complex, are activated only when cells have finished repair of structures and macromolecules (including chromosomes), and the DNA damage-induced surveillance process has been completed (a p53-like pathway?) (Whittle *et al*., 2001). In the case of massive or unrepairable damage (deteriorated, low-viability seeds), this mechanism should go on, delaying the onset of the S phase, or even worse, eventually allowing faulty DNA replication that will result in chromosome aberrations and, very likely, organismal death. Osmopriming will allow sufficient repair time, so that seeds will recover their viability/vigour. Repair mechanisms are unscheduled processes, and thus, they will take place at any time during the cell cycle or in non-cycling cells.

Activation of already-present G_1 proteins would mean that, at some point after imbibition, a mitogenic signal transduction mechanism should be triggered that would have as a target the G_1 kinase complex, opening the gate to the cell cycle. However, activation may be more complex; addition of cycloheximide to early germinating maize axes to inhibit protein synthesis has demonstrated that both the cyclin D-like protein and PCNA virtually disappear after only 3–4 h of germination (Cruz-García *et al*., 1998; Herrera *et al*., 2000). A decrease in PCNA content during the early hours of pea germination has also been reported (Citterio *et al*., 1992). These results imply that there is turnover of cell cycle proteins accumulated in dry embryos and, at the same time, this would explain the need for transcription/translation during early germination. Newly made molecules of existing proteins, as well as *de novo* synthesis of novel proteins, may be required during germination.

Establishment of the S-phase potential appears to be vital for successful germination, as demonstrated by the direct correlation between damage to the DNA replication machinery and loss of seed viability. Knowledge of the processes that take place between G_1 set up and S-phase initiation during germination is virtually non-existing, and efforts should be focused in this direction.

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