Maize Cyclin D2 Expression, Associated Kinase Activity and Effect of Phytohormones During Germination

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A cDNA corresponding to 16 kDa of the maize cyclin D2 N-terminus was cloned and this polypeptide was overexpressed to produce homologous antibodies. This antibody recognized a 38 kDa protein in extracts from maize embryonic axes which corresponds to the predicted size for cyclin D2 protein. Expression of cyclin D2 was followed at the transcriptional and protein levels, and the effect of cytokinins and abscisic acid (ABA) was followed during maize germination. Cyclins importantly stimulated cyclin D2 gene expression at late germination times and sucrose was necessary for stimulation, whereas the effect of ABA was not different from that in controls. However, cyclin D2 protein levels in control axes reached a peak at 6 h germination, declining thereafter, and neither cytokinins nor ABA modified this behavior. Two cyclic-dependent kinase A (Cdk-A)-type proteins and proliferating cell nuclear antigen (PCNA) were found co-immunoprecipitating with cyclin D2, and these immunoprecipitates were able to phosphorylate both histone H1 and the maize retinoblastoma-related protein (RBR). This protein kinase activity differed from the pattern of protein accumulation during germination, and the activity was not modified by either cytokinins or ABA. We discuss these findings in terms of the importance of the cell cycle for the germination process.

Keywords: Cdk — Cyclin D2 — Germination — Kinase activity — Phytohormones — Zea mays.

Abbreviations: BA, benzyladenine; Cdk, cyclin-dependent kinase; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; pRB, retinoblastoma protein; RBR, retinoblastoma-related protein.

Introduction

The cell cycle is an important event where cells multiply and thus play a role in the growth and development of an organism, and its regulation strongly depends on phosphorylation and dephosphorylation events. Protein complexes formed by cyclin proteins and cyclin-dependent protein kinases (Cdks) are responsible for phosphorylating specific substrates, allowing progress through the cell cycle. The main control points during the cell cycle are in the G1/S and in the G2/M boundaries (Pines 1995).

In mammals, the kinase complex present during G1 is formed by cyclin D and Cdk4 or Cdk6. This complex phosphorylates the retinoblastoma protein (pRb) that is bound to the transcriptional factor E2F. The Rb–E2F complex blocks the transcription of E2F-regulated genes (Kaelin 1999). Cyclin–Cdk-hyperphosphorylated pRb releases E2F and this can now promote gene transcription, allowing the cell cycle to continue to S phase. In plants, the essential proteins and mechanisms needed for the G1/S transition have been conserved (Gutierrez 1998).

Cyclin D has been proposed to be one of the first proteins to perceive the environment and can act as a growth factor sensor to integrate the external signals with the cell cycle machinery (Sherr and Roberts 1999). It is therefore natural that the expression of cyclin D is activated by growth factors (Prober and Edgar 2001, Dewitte and Murray 2003).

Similarly to mammals, cyclin D in plants responds to signals such as hormones and carbohydrate levels. In Arabidopsis cell suspension cultures, addition of cytokinins to the sucrose-containing medium induced transcription of the cyclin D3 gene, but expression of cyclin D2 and cyclin D4 was induced with only sucrose (Soni et al. 1995, De Veylder et al. 1999, Riou-Khamlichi et al. 1999, Riou-Khamlichi et al. 2000).

D cyclins in metazoans are only present during G1 phase, forming an active kinase complex with Cdk4 or Cdk6, and are the target for proteolysis thereafter (Diehl et al. 1997). In plants, on the other hand, cyclins D are apparently present throughout the plant cycle (Sorrell et al. 1999), although recent reports indicate that cyclin D3;1 is unstable and the target of the proteasome (Plan chais et al. 2004). The cyclin D-associated protein kinase in plants is Cdk-A. This protein contains the PSTAIRE motif which is common to Cdc2-related protein kinases (Mironov et al. 1999, Meijer and Murray 2000); it is a stable protein during the cell cycle, but its kinase activity peaks at the G1/S and G2/M boundaries (Mironov et al. 1999), therefore suggesting that cyclin D is responsible for regulating the kinase activity, giving this complex stability, cellular localization and specificity to phosphorylate different substrates (Pines 1995).

In Arabidopsis, cyclins D2 and D3 interact with Cdk-A and phosphorylate histone H1 (Healy et al. 2001). The cyclin D3–Cdk-A complex also phosphorylates tobacco pRb-related protein in vitro (Nakagami et al. 1999). Arabidopsis cyclin D2–
Cdk-A can phosphorylate *Zea mays* RB-related protein (RBR), with the highest kinase activity at the G1/S boundary, and decaying thereafter (Boniotti and Gutiérrez 2001). Overexpression of Arabidopsis cyclin D2 in tobacco reduces the duration of the G1 phase and increases the plant growth rate (Cockcroft et al. 2000).

Cytokinins are phytohormones that promote germination and plant growth, and stimulate cell division (Binns 1994). During germination, cytokinins induce the activation of proteins that participate in cell cycle regulation (Cruz-García et al. 1998) and DNA replication (Vázquez-Ramos and Reyes 1990). It is not well understood how cytokinins activate the cell cycle, although it is clear that they can induce D-type cyclins (Riou-Khamlichi et al. 2000). On the other hand, abscisic acid (ABA) inhibits both seed germination and DNA synthesis (Roberston et al. 1990). In proliferating tobacco BY-2 cell cultures, ABA blocks cells in G1 phase (Swiatek et al. 2002). It has been reported that ABA can induce expression of ICKs (inhibitors of Cdk kinases) and thus inhibits the cell cycle (Wang et al. 1998).

Cyclin D–Cdk complexes can also associate with the DNA replication processivity factor proliferating cell nuclear antigen (PCNA). In protein extracts from maize seed axes, immunoprecipitates using an antibody against maize PCNA indicated that a similar protein complex exists in plants (Sánchez et al. 2002). This protein complex shows kinase activity over histone H1 and, most importantly, over Zm RBR, and its activity is highly inhibited by specific Cdc2 and Cdk-A kinase inhibitors. However, the type of cyclin D present in this complex has not been demonstrated.

Following the reported sequences of three cDNAs for maize D-type cyclins in gene banks (cyclins D1, AF351190; D2, AF351189; D4, AF351191, NCBI), we cloned a partial cDNA corresponding to the N-terminal fragment of a D2-type cyclin that differs in sequence from maize D1 and D4 D-type cyclins, and produced antibodies against the coded peptide. Here we present evidence of its association with Cdk-A and PCNA, its ontogeny, the pattern of the associated kinase activity and its regulation during maize germination.
Results

The cDNA sequences of three different D-type cyclins in *Z. mays* have been reported recently: cyclin D1 (AF351190), cyclin D2 (AF351189) and cyclin D4 (AF351191, NCBI). In *Arabidopsis thaliana*, it has been shown that cyclin D2 associates with Cdk-A during G\(_1\) and this complex phosphorylates the maize RBR (Boniotti and Gutierrez 2001). Since our goal is to study cyclin–Cdk complexes during the early stages of germination, in which cells are in G\(_1\) phase, we decided to clone the cDNA coding for a 16 kDa peptide of the cyclin D2 N-terminus, a sequence that is different among the three maize D-type cyclins, except for the presence of the LXCXE sequence, the pRB-binding motif that is characteristic of D cyclins (Gutierrez 1998, Huntley et al. 1998, Kaelin 1999). Fig. 1 shows the comparison of the maize cyclin D2 16 kDa protein sequence with the corresponding sequence in different plant species, indicating an average homology >50% (*Triticum*, 64%; *Oryza*, 51%; *Nicotiana*, 50%; *Arabidopsis*, 71%; *Chenopodium*, 40%; and *Daucus*, 42%). The cyclin D2 peptide was fused to GST and the resulting recombinant protein was overproduced and purified. Thrombin protease was used to cleave the fused recombinant protein and the purified cyclin D2 peptide was used to raise polyclonal antibodies. This antibody (1:3,000 dilution) recognized the GST–cyclin D2 fused protein and the 16 kDa cyclin D2 peptide (Fig. 2B) and in maize protein extracts it recognized a 38 kDa protein, which corresponds to the expected molecular mass for cyclin D2 according to the reported cDNA sequence (Fig. 2B). A competition assay, adding the cyclin D2 peptide to anti-cyclin D2 antibodies before performing the immunorecognition assay, prevented identification of cyclin D2 in maize protein extracts and, importantly, reduced the intensity of the recombinant cyclin D2 signal (Fig. 2C). No signal was detected when pre-immune serum was used (Fig. 2A).

Cyclin D2 expression during germination and the effect of phytohormones

Expression of cyclin D2 was followed both at the transcriptional and at the protein levels. In synchronized *Arabidopsis* cell suspension cultures, cyclin D2 mRNA was expressed at very low levels and these were not affected by blocking cells at the G\(_1\)/S transition (Fuerst et al. 1996). In germinating maize, cyclin D2 mRNA was not detectable in dry seeds or at 3 h of germination; its expression notably increased by 6 h of germination and then levels fluctuated until 24 h (Fig. 3A, B). Imbition in the presence of ABA produced a similar behavior to that in control. On the other hand, cytokinins altered this pattern since the mRNA was already visible at 3 h of germination, remained at the same level at 6 h and then levels increased, reaching a peak by 24 h of germination, demonstrating cytokinin regulation of cyclin D2 mRNA expression (Fig. 3A, B), a result that differs from that observed in *Arabidopsis* cell cultures in which cyclin D2 gene expression does not seem to
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Cyclin D2 expression in the absence of sucrose was also evaluated. A reverse transcriptase-polymerase chain reaction (RT–PCR) experiment was performed to measure the level of cyclin D2 mRNA expression in the absence of sucrose, whether cytokinins were present or not, in 6 or 24 h imbibed axes (Fig. 4). In the absence of sucrose or cytokinins (or adding none), the amount of mRNA was very similar at both imbibition times (Fig. 4A, B); only when both sucrose and cytokinins were present did the levels of mRNA increase, mainly at 24 h germination, indicating the need for sucrose for cytokinin stimulation of expression. The difference in induction levels in the experiments with cytokinins and sucrose may be due to differences between maize seed lots (see Discussion).

Cyclin D2 was also followed at the protein level. It has been documented that the Arabidopsis cyclin D2 is a stable protein in cycling cells. This protein is present in dry maize seeds, and levels increase up to 6 h of germination to gradually drop until 24 h. Addition of either cytokinins or ABA did not modify this pattern importantly, indicating that regulation of cyclin D2 protein accumulation during germination is not influenced by these phytohormones (Fig. 5A, B, C).

Cyclin D2 in protein complexes

Cyclin proteins associate with Cdk kinases and thus participate in cell cycle regulation. Arabidopsis cyclins D2 and D3 associate with Cdk-A kinase but not with Cdk-B1 (Healy et al. 2001). Anti-maize cyclin D2 antibodies immunoprecipitated cyclin D2 from maize protein extracts (Fig. 6A) and, using anti-PSTAIRE antibodies, two PSTAIRE-containing Cdk-A type proteins co-immunoprecipitated, corresponding to those previously reported by our group, of 32 and 36 kDa (Fig. 6B), precipitating together with PCNA (Sánchez et al. 2002). Cyclin D2 was also retained by a resin containing p13Suc1–agarose beads, further indicating an association between cyclin D and a Cdk kinase (Fig. 6C). p13Suc1 strongly binds Cdks and was first characterized in yeast cells (Brizuela et al. 1987). Additionally, cyclin D2 and PCNA also associated (Fig. 6D). Therefore, it was of great interest to show that this cyclin–Cdk complex contains kinase activity.

Protein kinase activity in cyclin D2 immunoprecipitates

In Arabidopsis cell cultures, immunoprecipitates containing the complex formed by cyclin D2 and Cdk-A phosphorylate histone H1 (Cockcroft et al. 2000, Healy et al. 2001, Boniotti and Gutierrez 2001). Maize cyclin D2 immunoprecipitates were tested for kinase activity in the presence of histone H1 or Zm RBR as substrates. Fig. 7 shows that this kinase complex readily phosphorylated histone H1 (Fig. 7A, lanes 3 and 4) and Zm RBR (Fig. 7B, lane 3). Olomoucine, an inhibitor of Cdk-A-type kinases (Planchais et al. 2000), strongly reduced kinase activity in these immunoprecipitates, further indicating that the associated kinase is of the Cdk-A type. Residual kinase activity may be due to cyclin D2 association with olomoucine-resistant Cdks.
Cyclin D2-associated kinase activity during maize germination

Cyclin D2 immunoprecipitates from different germination times (Fig. 8A) were tested for kinase activity. Using Zm RBR as substrate, kinase activity was low in extracts from dry seeds and gradually increased to reach a peak by 15 h germination, and activity declined by 24 h germination (Fig. 8B), a behavior that differs from the protein pattern observed for cyclin D2 accumulation (Fig. 5), in which the peak is observed by 3–6 h germination, also similar to the peak observed for cyclin D2 immunoprecipitated protein (Fig. 8A). We also tested the effect of cytokinins and ABA on cyclin D2-associated kinase activity during germination, using Zm RBR as substrate; it was
found that the activity was not different from that shown by control immunoprecipitates (result not shown).

Discussion

The cloning of a partial cDNA coding for an N-terminal fragment of maize cyclin D2 whose sequence differs from cyclins D1 and D4 has allowed us to follow cyclin D2 mRNA expression and protein ontogeny during maize germination, and also to study its association with other cell cycle proteins.

At the transcriptional level, there seems to be an early mRNA accumulation by 6 h of germination and then levels tend to drop; incubation of axes in the presence of ABA does not seem to change this pattern. However, mRNA expression increases considerably if cytokinins are added, establishing that cyclin D2 gene expression is under cytokinin control. *Arabidopsis* cyclin D2 mRNA expression is stimulated by sucrose but not by cytokinins (Riou-Khamlichi et al. 1999, Oakenfull et al. 2002), whereas *Arabidopsis* cyclin D3 expression is induced with cytokinins only in the presence of sucrose (Riou-Khamlichi et al. 1999), indicating differences between maize and *Arabidopsis* in the response to biochemical factors. In fact, induction of maize cyclin D2 gene expression also requires both sucrose and cytokinins, suggesting that maize cyclin D2 may be regulated in a similar way to *Arabidopsis* cyclin D3. Thus, regulation of cyclin expression in plants may not follow unique rules. Differences observed in the increase in cyclin D2 expression between experiments may be due to variations among seed lots since the maize variety we have always used, cv. Chalqueño, is an open pollination genotype; however, the stimulatory effect of the combination cytokinin/sucrose is statistically reproducible.

Stimulation of gene expression occurs late in germination, in the 15–24 h period, when cells in maize axes meristems are engaged in the S/G2 phases or starting to move into M phase (Baíza et al. 1989). The response to phytoregulators differs notably when cyclin D2 is followed at the protein level since here there is virtually no variation whether axes are imbibed in the presence of ABA or cytokinins. In this case, there is also a peak of protein level during early germination, 3–6 h, which drops thereafter. These results would indicate that the increase in mRNA expression caused by cytokinins will have no effect on the germination process per se, but perhaps on the following seedling establishment. Thus cyclin D2 does not appear to be regulated by phytohormones at the protein level, at least during the developmental process defined by seed germination. Peaks at 6 h of cyclin D2 mRNA and protein during control germination may be indicative of an early requirement for cyclin D2 to initiate cell cycle events.

Protein immunoprecipitates using the anti-cyclin D2 antibody show that two PSTAIRE-containing proteins (of 36 and 32 kDa) associate with cyclin D2; these proteins have also been described associated with maize PCNA, together with a putative cyclin D protein (Herrera et al. 2000, Sánchez et al. 2002). PCNA and cyclin D2 also associate, indicating the presence of ternary complexes containing cyclin D2, PCNA and either of the Cdk-A proteins in germinating maize. A resin containing p13Suc1 beads also retained cyclin D2 protein, further confirming association between cyclin D2 and PSTAIRE-containing proteins.

Protein immunoprecipitates using the anti-cyclin D2 antibody possess protein kinase activity on either histone H1 or ZmRBR, and this activity is strongly inhibited by a PSTAIRE kinase-specific inhibitor, olomoucine, corroborating that the associated kinase is of the Cdk-A type. Cyclin D2-associated protein kinase activity during germination shows a pattern that differs from the protein accumulation pattern, since cyclin D2 protein accumulates and declines earlier than the peak of kinase activity, suggesting that activity enhancement is due to post-translational modifications of cyclin D and/or the accom-
parying proteins during germination. This kinase activity may be important during G1, and the G1/S transition, as has also been reported in Arabidopsis (Boniotto and Gutierrez 2001). Additionally, this activity is not influenced by the addition of phytohormones. This result is quite unexpected since protein kinase activity associated with PCNA, and containing a Cdk-A and a putative cyclin D protein, is modified by ABA such that kinase activity over the Zm RBR remains switched on for the 24 h germination period measured, in opposition to the important reduction in activity after 6 h germination that is observed in control or cytokinin-treated maize axes (Sánchez et al. 2005). Thus, it is likely that PCNA, Cdk-As and cyclin Ds form different protein complexes that may differ in their time of appearance and/or in their localization, each having a different role in the cell cycle during maize germination. It is thus fundamental that maize cyclins D1 and D4 are cloned and antibodies against the corresponding proteins are produced to be able to establish their role, localization and associations, and their importance for the germination process.

Materials and Methods

Materials

Protein A–agarose and protease inhibitor cocktail tablest (Complete) were from Roche; Enhance chemiluminescence (ECL), ECL Western blotting kit, glutathione–Sepharose 4B, plasmid pGEX 4T2, Hybond N° membranes, anti-rabbit IgG peroxidase conjugate, [α-32P]dCTP and [γ-32P]ATP were from Amersham Pharmacia Biotech; histone H1, SuperScript (RNase H–) Reverse transcriptase, recombinant Taq DNA polymerase, Trizol, random primer kit and recombinant Taq DNA polymerase were from Life Technologies; Immunobilon PVDF membranes were from Millipore; anti-maize PCNA polyclonal antibody (1 : 1,500 dilution) or anti-maize cyclin D2 polyclonal antibody (1 : 3,000 dilution) and gels were blotted onto PVDF membranes. Membranes were incubated with anti-maize cyclin D2 polyclonal antibody (1 : 1,500 dilution) or anti-PSTAIRE polyclonal antibody (1 : 1,500 dilution) for 3 h at 25°C and washed three times with phosphate-buffered saline (PBS) for 10 min. For anti-PCNA and anti-PSTAIRE antibodies, the second wash was done with PBS containing 0.5 M NaCl. Subsequently, membranes were incubated for 1 h with peroxidase-conjugated anti-rabbit antibody at a 1 : 10,000 dilution. Membranes were washed again three times with PBS for 10 min each. Peroxidase reaction was detected by the ECL method. Densitometric analysis was performed using the Phosphoimagel software from Biorad.

Immunoprecipitation

Anti-cyclin D2 and anti-PCNA antibodies were conjugated for 2 h with protein A–agarose (1 : 10 dilution) at room temperature using buffer A (25 mM Tris–HCl pH 7.5, 125 mM NaCl, 2.5 mM EDTA pH 8.0, 2.5 mM EGTA, 2.5 mM NaF, 0.1% Triton X-100). Protein from extracts (100 µg) was added and the mixture was incubated for 3 h at room temperature with agitation; immunocomplexes were pelleted by centrifugation in a microfuge and washed three times with buffer A. The resulting protein precipitates were used as the source of kinase activity or bound proteins were eluted with SDS loading buffer and prepared for immunoblotting.

P13SU1 pull-down assays

Total protein (100 µg) was incubated with p13SU1–Sepharose beads overnight at 4°C; after incubation, beads were washed three times with buffer A.

Kinase assays

Immunocomplexes were resuspended in 15 µl of kinase buffer (70 mM Tris–HCl pH 7.5, 10 mM MgCl2, 150 mM NaCl, 1 mM DTT, 5 mM EGTA, 20 µM ATP, 5 µCi of [γ-32P]ATP). As substrate, 20 µg of histone H1 or 4 µg of GST–Zm RBR-C (the C-terminal domain of maize RBR) fusion peptide per sample was added. Purification of GST–Zm RBR fusion peptide was performed according to Ramirez-Parrá et al. (1999). Reactions were performed for 30 min at 30°C and these were stopped by adding SDS loading buffer. After boiling for 5 min, the reaction products were separated by SDS–PAGE.

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At 94°C 5 min, 94°C 1 min, 65°C 1 min, 72°C 1 min for 27 cycles, 75°C 5 min. Samples were loaded onto an agarose gel (1.5%) and transferred to Hybond N° membranes. For membrane hybridization, the Zm cyclin D2 probe was labeled with [α-32P]dCTP using the random primer reaction kit. Hybridization took place in a buffer containing 0.5 M Na2HPO4, 0.5 M NaH2PO4, 7% SDS, 10 mM EDTA, 1% bovine serum albumin (BSA) and the radioactive probe during 15 h at 60°C. The membrane was washed with 2× SSC and 0.1% SDS for 15 min, and twice with 0.1× SSC and 0.1% SDS for 15 min at 60°C.

Protein extraction

After maize embryo axes imbibition, protein extracts were produced with extraction buffer containing 25 mM Tris–HCl pH 7.5, 15 mM MgCl2, 75 mM NaCl, 25 mM KCl, 5 mM EDTA pH 8.0, 1 mM dithiothreitol (DTT), 0.2% Triton X-100, 0.25 mM sucrose, 60 mM β-glycerophosphate, 50 mM NaF, 200 µM Na2VO3, 1 mM EGTA and a tablet of protease inhibitor cocktail/50 ml buffer. Protein extracts were centrifuged at 30,000 rpm for 30 min at 4°C and protein concentration was determined by the method of Bradford.

Western blot

Protein samples (25 µg) were fractionated by SDS-PAGE (14%) and gels were blotted onto PVDF membranes. Membranes were incubated with anti-maize cyclin D2 polyclonal antibody (1 : 3,000 dilution), anti-maize PCNA polyclonal antibody (1 : 1,500 dilution) or anti-PSTAIRE polyclonal antibody (1 : 1,500 dilution) for 3 h at 25°C and washed three times with phosphate-buffered saline (PBS) for 10 min. For anti-PCNA and anti-PSTAIRE antibodies, the second wash was done with PBS containing 0.5 M NaCl. Subsequently, membranes were incubated for 1 h with peroxidase-conjugated anti-rabbit antibody at a 1 : 10,000 dilution. Membranes were washed again three times with PBS for 10 min each. Peroxidase reaction was detected by the ECL method. Densitometric analysis was performed using the Phosphoimagel software from Biorad.

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