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DIFFERENTIAL RESPONSE OF PCNA-ASSOCIATED CDK ACTIVITY TO PHYTOHORMONES DURING MAIZE GERMINATION

María de la Paz Sánchez[1], Sunitha H. Gurusinghe[2], Kent J. Bradford[2] and Jorge M. Vázquez-Ramos[1]*.

[1]Departamento de Bioquímica, Facultad de Química, UNAM, México D.F. 04510, México and [2]Department of Vegetable Crops, One Shields Ave., University of California, Davis, CA 95616-8631, USA.

*Corresponding author: jorman@servidor.unam.mx

Tel and fax: (52) 56225284

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ABSTRACT

The proliferating cell nuclear antigen (PCNA) is a protein factor required for processive DNA synthesis that has been found to be associated with G1 cell cycle proteins. We have demonstrated previously that in germinating maize embryonic axes, PCNA forms protein complexes with two types of Cdk-A proteins (32 and 36 kDa) and with a putative D-type cyclin, and that these complexes exhibit protein kinase activity on histone H1 and on the maize homologue of the pRB (retinoblastoma) protein. We have used flow cytometry to study the influence of the phytohormones benzyladenine (BA) and abscisic acid (ABA) on cell cycle advancement during maize germination and found that while BA accelerates the passage of cells from G1 to G2, ABA delays cell cycle events so that most cells seem to remain in G1. The amount of PCNA and Cdk-A proteins also varies according with the treatment, as PCNA amount reaches a peak by 6 h of imbibition in BA-treated axes, compared to 20 h for control embryonic axes and no increase occurs in ABA-treated axes. On the other hand, of the two Cdk-A proteins, the 32 kDa protein disappears after 15 h of imbibition in control axes while in BA- and ABA-treated axes this protein seems to be stabilized. The PCNA-associated protein kinase activity in BA-treated axes falls after 6 h of imbibition, while in ABA-treated axes, PCNA-associated kinase activity remains constant during a 24 h hydration period. Conversely, a $p13^{Suc1}$-associated Cdk-A kinase is activated after 6 h of imbibition under all treatments and in control axes. These results suggest that in maize, ABA delays the germination process by affecting cell cycle advancement, stopping cells mostly in a G1 state.

INTRODUCTION

Seed germination is a developmental process of reactivation of seed metabolism to originate a new plant. This process starts with seed imbibition and ends with the protrusion of the embryonic axis (normally the root) through the seed coats (Bewley and Black, 1994). During this time cells in a seed repair their DNA and meristematic cells are committed to start the cell cycle.

In mammals, progress through the cell cycle is regulated by distinct families of cyclin-dependent kinases (Cdks) whose activities are coordinated by different types of cyclins. These basic mechanisms of cell cycle control are conserved in plant cells (Mironov et al., 1999). Several genes coding for Cdk proteins from maize, rice, alfalfa, soybean, pea, tobacco and Arabidopsis have been reported (Colasanti et al., 1991; Ferreira et al., 1991; Hashimoto et al., 1992; Hirt et al., 1993; Miao *et al*., 1993; Fober et al., 1994; Setiady et al., 1996) and these have been divided in two main families, Cdk-A and Cdk-B. Cdk-B function is important in G2/M phase, whereas Cdk-A participates both in the G1/S and in the G2/M transitions. Cdk-A protein is the homologue of mammalian Cdk1 and contains the characteristic PSTAIRE amino acid motif in the cyclin-binding domain that defines Cdc2 type kinases (Mironov et al., 1999; Joubés et al., 2000). In tobacco and Arabidopsis cells, Cdk-A is activated by cyclin D-type proteins and this protein complex is able to phosphorylate the plant homologue of the retinoblastoma protein (pRB) (Nakagami et al., 1999) and also histone H1 (Healy et al., 2001). These results and the isolation of homologues of the E2F transcription factors from wheat, tobacco, carrot and Arabidopsis (Ramírez-Parra et al., 1999; Sekine et al., 1999; Albani et al., 2000; Magyar et al., 2000) indicate that the promotion of G1 phase in plants is controlled by cyclin D-Cdk-A type complexes and that these are able to inactivate the growth-suppressive activity of pRb.

In maize two different types of Cdk-A of 32 and 36 kDa were found and both proteins form complexes with the proliferating cell nuclear antigen (PCNA) protein (Sánchez et al., 2002). PCNA protein is the sliding clamp of the replicative DNA polymerase δ (Tan et al., 1986; Prelich et al., 1987; Shivji et al., 1992); however, during the G1 phase in higher eukaryotes, PCNA has been found associated with other cell cycle proteins like Cdk4, cyclin D and p21 (Xiong et al., 1992). During maize germination, PCNA not only associates with Cdk-A proteins, but it was also found associated with a putative cyclin D (Herrera et al., 2000). This PCNA-associated protein complex exhibits kinase activity that is able to phosphorylate both histone H1 and the maize pRB-related protein (ZmRBR) (Sánchez et al., 2002). Moreover, the PCNA-associated kinase activity is higher during the early hours of germination (0-6 h) than at later times (15-24 h) when the S phase begins, suggesting that it is a G1 phase kinase. Interestingly, in *in vitro* assays, the fission yeast Cdk-binding protein $p13^{Suc1}$ that is active during the G2 phase in yeast cells (Hayles et al., 1986) binds with the maize Cdk-A protein from maize protein extracts, and the protein complex formed shows kinase activity preferentially with Cdk-A obtained from germinating seeds (by 15 h and onward), in contrast with the PCNA-associated kinase (Sánchez et al., 2002).

Plant hormones play essential roles in plant metabolism and have been shown to influence cell cycle proteins. Cytokinins stimulate Cdk-A activity in the G2/M phases (Zhang et al., 1996) and, at the G1/S transition, regulate cell cycle progression partly by inducing CycD3,1 transcription (Meijer and Murray, 2001). During maize germination, benzyladenine (BA) a synthetic cytokinin, accelerates the germination process, affecting the amounts of G1/S cell cycle proteins like PCNA and a putative cyclin D (Cruz-García et

al., 1998) and stimulating the activity of replicative DNA polymerases during germination (Vázquez-Ramos and Reyes, 1990; Gómez-Roig and Vázquez-Ramos, 2003). On the other hand, abscisic acid (ABA) inhibits germination, perhaps through inhibition of cell cycle processes, since this phytohormone can induce the expression of cyclin-dependent kinase inhibitors (ICKs, Wang et al., 1998), which bind and inhibit Cdk-A activity, very likely during the G1 phase.

If we consider establishment of the G1 phase as an important physiological process during germination, and the PCNA-associated kinase as being involved in the G1 phase, then it becomes fundamental to know if this kinase complex participates in the regulation of the germination process by phytohormones. We show here that there is a differential cell cycle and kinase response when axes are imbibed in the presence of BA or ABA, which may indicate that these phytohormones influence the germination status of seeds at least in part by effects on the cell cycle.

MATERIAL AND METHODS

Materials

Protein A-agarose and protease inhibitor cocktail tablets (Complete^{TM}) were from Roche; hyperfilm ECL, ECL western blotting kit, glutathione-Sepharose 4B, anti-rabbit IgG peroxidase conjugate and [γ-^{32}P] ATP (3000 Ci mmol^{-1}) were from Amersham Pharmacia Biotech; histone H1 and glutathione were from Gibco BRL; diamine-2-phenylindol (DAPI), benzyladenine (BA) and abscisic acid (ABA) were from Sigma-Aldrich Chemical; Immobilon PVDF membranes were from Millipore; anti-PSTAIRE rabbit polyclonal IgG

cat No. sc53 was from Santa Cruz Biotech; Lambda protein phosphatase and $p13^{Suc1}$ sepharose were from Upstate Biotechnology.

Flow cytometry

DNA content in maize root tips was measured by flow cytometry following imbibition in water, 1 μM BA or 20 μM ABA. To obtain nuclei, maize root tips were chopped with a razor blade in the presence of 1.5 ml of a buffer containing 50 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 0.1% Triton X-100 and 2 mg DAPI, and samples were filtered through a 30 μm nylon membrane. Nuclei were analyzed by flow cytometry in a Partec CA II instrument (Partec GMBH, Munster, Germany); 1500 nuclei were counted for each sample at a speed of 10 nuclei sec^{-1}. The resulting data were processed using the Multicycle program, version 2.53 (Phoenix Flow Systems, San Diego, CA).

Protein extraction

Proteins were obtained from maize embryonic axes from dry seeds or after imbibition in buffer for 3, 6, 15, and 24 h with or without 1 μM BA or 20 μM ABA. Protein extraction buffer contained 25 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 75 mM NaCl, 25 mM KCl, 5 mM EDTA pH 8.0, 1 mM DTT, 0.2 % Triton X-100, 0.25 M sucrose, 60 mM β-glycerolphosphate, 50 mM NaF, 200 μM Na_3VO_4, 1 mM EGTA and a tablet of protease inhibitor cocktail/50 ml buffer. Protein extracts were centrifuged at 150,000g for 30 min at 4^{o}C and protein concentration was determined by the method of Bradford (1976).

Western blot

Proteins (25 μg) were separated by SDS-PAGE, gels were blotted onto PVDF membranes and these were incubated either with anti-maize PCNA polyclonal antibody (1:1500 dilution) or with anti-PSTAIRE polyclonal antibody (1:1500 dilution) for 12 h at 4^{o}C, washed twice in PBS buffer and once in PBS buffer containing 0.5 M NaCl, 15 min each at room temperature, and membranes were incubated for 2 h with peroxidase-conjugated anti-rabbit antibody in a 1:10,000 dilution. Membranes were washed again with PBS buffer and the peroxidase reaction was detected by the Enhanced Chemiluminescence method (ECL). All western blots were repeated a minimum of three times using independent protein extracts.

Immunoprecipitation and pull down assays

Proteins (100 μg) were incubated with anti-maize PCNA antibody conjugated with Protein A-agarose or with p13^{Suc1}-Sepharose beads overnight at 4ºC; after incubation, beads were washed five times with 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) and once with kinase assay buffer (see below). Resulting protein precipitates or pulled down proteins were used as the source of kinase activity.

Phosphatase assay

Proteins (25 μg) were incubated with phosphatase buffer that contained 50 mM HEPES pH 7.5, 100 μM EDTA, 2 mM $MnCl_2$, 5 mM DTT, 100 μg ml^{-1} BSA and 4U of lambda protein

phosphatase for 30 min at 37ºC. Histone H1 phosphorylated by the PCNA-associated kinase activity was used as a control for lambda protein phosphatase activity.

Protein kinase activity

PCNA immunocomplexes or $p13^{Suc1}$-associated proteins were resuspended in 15 µl of kinase buffer (70 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT, 5 mM EGTA, 20 µM ATP and 5 µCi [γ-^{32}P] ATP). As substrate, 4 µg GST-ZmRBR-C fusion peptide (the C-terminal domain of maize RBR protein) was added per sample. Purification of GST-ZmRBR-C fusion peptide was performed according to Ramirez-Parra et al. (1999). Reactions were performed for 30 min at 30^{o}C and were stopped by adding SDS loading buffer. After boiling for 5 min, the reaction products were separated by SDS-PAGE.

RESULTS

Effect of phytohormones on cell cycle progression during maize germination

Root tips of embryonic axes were used to follow the cell cycle status during maize imbibition and germination, since cells in these tissues are the first to enter to the cycle (Baiza et al., 1989). Among nuclei from cells of dry seeds, 82% had a G1 (2C) DNA content and 17% had a G2 (4C) DNA content (Table 1). The cell cycle is activated during the first hours after imbibition and by 15 h of imbibition, the percentage of G1 nuclei dropped significantly to 63% whereas the number of G2 nuclei increased to 35% (Fig. 1A; Table 1). Growth of water-imbibed axes is first evident at 15-20 h of imbibition. Incubation of maize embryonic axes in the presence of the synthetic mitogenic hormone benzyladenine (BA) advances the initiation of growth by 6-9 h (Vázquez-Ramos, unpublished

observations) and caused an earlier fall in the percentage of nuclei in G1 and increase in the percentage in G2 already by 6 h; by 15 h the G1 proportion was 58%, while the proportion of nuclei in G2 increased to 40% (Fig. 1; Table 1). Abscisic acid (ABA) is known as an inhibitor of germination (Bewley and Black, 1994), and maize germination is highly inhibited (more than 75%) by ABA during the first 48 h of imbibition (our unpublished results). Incubation of maize embryonic axes in the presence of ABA notably reduced the number of cells that move from G1 to G2 so that by 15 h of germination only 26% of nuclei were in G2 while 68% were in G1 (Fig. 1; Table 1). Nuclei with an apparent S-phase (intermediate) DNA content had increased after 24 h of imbibition and the effect of ABA was evident with only 5% of nuclei with S-phase DNA content, compared to 12% of nuclei from water- or BA-imbibed axes (Table 1).

PCNA and Cdk-A content in BA- and ABA-treated maize embryonic axes

The mechanism by which phytohormones influence seed germination is not well known, although it may involve modulating the activity of cell cycle proteins (Liu et al., 1994; Cruz-García et al., 1998; Riou-Khamlichi et al., 1999; de Castro et al., 2001). To relate the effect of phytohormones on cell cycle advancement during germination with specific G1/S phase proteins, we followed PCNA and Cdk-A proteins by western blotting (Fig. 2). During imbibition of embryos in water, PCNA showed the previously reported gradual increase to reach a peak by 20-24 h of imbibition (Fig. 2A; Herrera et al., 2000). A faster increase in PCNA amounts was observed in BA-treated embryonic axes so that by 6 h of imbibition, the amount of PCNA was similar to that of control axes at 15 h. On the contrary, only a marginal increase was noticeable by 15 or 24 h of imbibition when

embryonic axes were imbibed in the presence of ABA (Fig. 2A), consistent with the reduced or delayed cell cycle progression (Table 1).

An antibody against the conserved PSTAIRE sequence, characteristic of Cdc2-related proteins, was used to follow maize Cdk-A proteins. As reported previously (Sánchez et al., 2002), two proteins of 32 and 36 kDa are detected with this antibody in protein extracts from maize embryonic axes, and, whereas the amount of the 36 kDa protein increased as germination advances, the amount of the 32 kDa protein was reduced at 15 and 24 h of imbibition (Fig. 2B). When imbibed in BA, the abundance of the 36 kDa protein was similar to that in control axes; however, the 32 kDa protein appeared to be stabilized at least until 15 h of imbibition and then decreased (Fig. 2B). A third protein is clearly visible at 15 and 24 h of imbibition, running between the 36 and 32 kDa bands. This 34 kDa protein could be a modified form, perhaps by phosphorylation or dephosphorylation, of either the 36 or the 32 kDa proteins.

The behavior of the Cdk-A proteins when axes are incubated in the presence of ABA shows some differences from the controls, as the amount of the 36 kDa protein increased slightly at 6 and 15 h, then decreased at 24 h, while the 32 kDa protein decreased earlier than in control axes but remained present at low levels through 24 h (Fig. 2B). The 34 kDa band, present in BA-treated axes, is also visible in proteins from ABA-treated axes at 15 and 24 h of imbibition.

To determine whether this 34 kDa band appearing at 15 h of germination in BA- and ABA-treated axes is the product of a phosphorylated 36 or 32 kDa protein, protein extracts were treated with lambda protein phosphatase. This protein has been reported to dephosphorylate human Cdk-2 (Golan et al., 2002). First, the ability of this phosphatase to dephosphorylate a previously phosphorylated substrate, histone H1, was tested. Histone H1

was incubated in the presence of the PCNA-associated Cdk-A (Sánchez et al., 2002) and [γ-^{32}P]-ATP and the labeled histone H1 served as substrate for the lambda phosphatase. An aliquot of radioactively labeled histone H1 (Fig. 3A, lane 1) was divided in half, one half receiving the lambda phosphatase (Fig. 3A, lane 2) and the other only receiving the corresponding buffer (Fig. 3A, lane 3). Lambda phosphatase almost completely removed the labeled P from histone H1. Next, lambda phosphatase was mixed with protein extracts from 6 h and 24 h BA-treated embryonic axes. However, no variation in band intensity, or in the electrophoretic mobility, of the 32, 34 or 36 kDa proteins was observed (Fig. 3B), suggesting that the different mobilities of the three Cdk-A proteins are not due to differential phosphorylation. Similar results had been obtained previously using alkaline phosphatase (Sánchez et al., 2002).

PCNA immunoprecipitation from phytohormone-treated axes

To test the effect of phytohormones on the PCNA-associated kinase activity, PCNA was first immunoprecipitated from protein extracts obtained from maize embryonic axes imbibed in water, 1 μM BA or 20μ ABA. Immunoprecipitated PCNA protein from protein extracts from axes imbibed for different times in water increased gradually (Fig. 4), similar to the pattern found without immunoprecipitation (Fig. 2A). On the other hand, extracts from BA-treated axes showed a peak of immunoprecipitated protein at 3 h of germination, followed by a gradual decrease until 24 h (Fig. 4), a behavior that differs from the pattern of continuous protein accumulation observed by western blotting during imbibition in the presence of BA (Fig. 2A). It is possible that under mitogenic conditions, after 3-6 hours of imbibition association of PCNA with the DNA replication apparatus could hide the protein

from antibody binding. Notably, a constant amount of PCNA was immunoprecipitated from protein extracts obtained from ABA-treated axes (Fig. 4), correlating with the virtually invariable amount of PCNA observed during imbibition in the presence of ABA (Fig. 2A).

Protein kinase activity in PCNA-immunoprecipitates and the influence of phytohormones

PCNA-associated Cdk-A kinase activity was measured in PCNA immunoprecipitates from control or phytohormone-treated embryonic axes. The carboxy-terminal 120 amino acids of the maize pRB homologue, GST-ZmRBR-C, was used as substrate. As reported previously (Sánchez et al., 2002), kinase activity in control PCNA immunoprecipitates was similar between 0 and 6 h of imbibition and activity subsequently declined (Fig. 5A). It is of interest to note that whereas the immunoprecipitated PCNA protein (Fig. 4) and the 36 kDa Cdk-A protein (Fig. 2B) increased and then remained constant with imbibition time, both the 32 kDa protein (Fig. 2B) and kinase activity declined over time..

Protein kinase activity in PCNA immunoprecipitates from BA-treated axes also showed some differences with respect to the amount of immunoprecipitated PCNA. At 0 h of imbibition, when the amount of PCNA was low, kinase activity was higher than at 3 h of imbibition when there was a peak of immunoprecipitated PCNA (Figs. 4, 5A). Thereafter, the decreasing amount of PCNA correlated with decreasing kinase activity. The pattern of immunoprecipitated kinase activity also contrasted with the increasing abundance of the 36 kDa Cdk-A protein on western blots during imbibition; however, there was a positive correlation with the amount of the 32 kDa protein (Fig. 2B).

In ABA-treated embryonic axes, on the contrary, the amount of immunoprecipitated PCNA roughly correlated with kinase activity and there was little variation in kinase

activity during the imbibition period (Figs. 4, 5A), consistent with the relatively constant amounts of the Cdk-A proteins detected by western blotting (Fig. 2B).

For comparison purposes, we also measured the activity of the Cdk-A protein associated to $p13^{Suc1}$. In previous work, we found that an agarose resin containing the $p13^{Suc1}$ peptide pulled down only the 36 kDa Cdk-A protein and the associated kinase activity had a contrasting behavior to the PCNA-associated Cdk (Sánchez et al., 2002). Here, the $p13^{Suc1}$- associated kinase activity from control extracts was very low at early imbibition times and increased several-fold as germination advanced (Fig. 5B). BA induced a higher $p13^{Suc1}$- associated kinase activity at earlier times, while ABA had relatively little effect on this activity.

DISCUSSION

For some time, a working hypothesis in our lab and in others has been that cell cycle initiation (particularly the G1 phase) is required for germination to advance and allow seedling growth (Cruz-García et al., 1998; de Castro et al., 2000, Sánchez et al., 2002; Vázquez-Ramos and Sánchez, 2003). Although seed metabolism during germination may vary enormously, depending on species, some results would substantiate this hypothesis: 1) cells in imbibed dormant seeds, although metabolically active, remain in a G1-like state for the imbibition period until dormancy is broken (de Castro et al., 2001); 2) meristematic cells in osmoprimed seeds tend to remain in a G1-like state for days or even weeks, although in some seed species, a number of cells move into G2, but no further (Lanteri et al., 1994; Cruz-García et al., 1995; Liu et al., 1996; Sánchez-Jiménez et al., 1997; Gurusinghe et al., 1999; de Castro et al., 2000); after removal of the osmotic agent and imbibition in water, a burst of DNA synthesis is rapidly triggered (Cruz-García et al., 1995;

de Castro et al., 2000); 3) in deteriorated or in low viability seeds, initiation of the S phase in meristematic cells can be extremely delayed compared to the time it takes in cells from high viability seeds, and radicle emergence is similarly delayed (Sen and Osborne, 1974; Elder et al., 1987; Gutiérrez et al., 1993). Thus, reactivation of at least the early stages of the cell cycle in meristematic cells is generally associated with advancement toward germination. On the other hand, radicle protrusion in the absence of cell cycle progression has been reported for cabbage seeds imbibed in the presence of hydroxyurea (an inhibitor of the S phase; Górnik et al., 1997) and in maize embryonic axes in the presence of colchicine (an inhibitor of the M phase; Baiza et al., 1989). Some varieties of tomato seeds also exhibited germination rate advancement due to priming without increases in G2 nuclei (Gurusinghe et al., 1999). While initiation of cell cycle activity normally accompanies germination, entry into the cell cycle may not be absolutely required for the early embryo expansion associated with germination, but cell proliferation is an absolute requirement for continued growth and seedling establishment.

In this paper, we show that ABA, a known inhibitor of seed germination, slows down cell cycle advancement during maize germination, as compared with control or BA-treated embryonic axes, in which cell cycle activation is evident. This is consistent with reports that seeds deficient in ABA synthesis exhibit more rapid germination and advancement of the cell cycle prior to radicle emergence (Liu et al., 1997; Downie et al., 1999). Accumulation of a G1/S marker like PCNA is stimulated by BA, and this accumulation occurs earlier than in control axes, reflecting an earlier entry into the S phase, as has been reported before (Herrera et al., 2000). On the other hand, PCNA amount in ABA-treated axes remains constant, probably indicating that cells are not being stimulated to enter the S phase, corroborating the flow cytometry results. A differential effect was also observed in

the amount of another G1/S marker, the Cdk-A proteins. In both control and BA-treated axes, the amount of the 36 kDa Cdk-A increases after 15 h of germination, while in ABA-treated axes there is virtually no change during the 24 h period measured. This 36 kDa protein, and not the 32 kDa Cdk-A, is preferentially bound by the $p13^{Suc1}$ protein and in these complexes kinase activity is notably enhanced by 15 h of imbibition (Fig. 5B; Sánchez et al., 2002), suggesting that it acts during the G2 phase. This result may indicate that cells in ABA-treated axes are restricted to go beyond the G1 phase. The behavior of the 32 kDa Cdk-A is also contrasting, as the protein seems to be stabilized by the hormonal treatments, whereas it disappears during germination in water. However, a major distinction is observed when kinase activity is measured (see below). The appearance of a third protein of 34 kDa in BA- or ABA-treated axes could indicate that either the 32 or the 36 kDa protein was phosphorylated as a result of the hormonal treatment. However, this protein band did not disappear after dephosphorylation with lambda phosphatase. Whether this is a new Cdk-A kinase, or whether the 32 or 36 kDa proteins are modified in ways other than phosphorylation, is still a matter of speculation.

As is well known, the complexes formed by Cdks and D-type cyclins bound to other proteins like PCNA and $p21^{Cip1}$ are central to the activation of the cell cycle in mammals (Xiong et al., 1992). Similar complexes have been found in germinating maize axes. An antibody raised against maize PCNA co-immunoprecipitates two different Cdk-A proteins and a putative cyclin D protein, and this complex contains a protein kinase activity that is active mainly during the early hours of imbibition, a period corresponding to the G1 phase (Cruz-García et al., 1998; Sánchez et al., 2002). The PCNA-associated kinase is of the Cdk-A type, since this protein is recognized by anti-PSTAIRE antibodies and kinase activity is inhibited by specific Cdk-A inhibitors (Sánchez et al., 2002). Here we show that PCNA

immunoprecipitates from BA-treated axes also show kinase activity that follows a pattern very similar to that described in control axes, i.e., a reduction in kinase activity after 6 h of imbibition. This activity is in contrast to kinase activity from ABA-treated axes, which remains unchanged during imbibition. Thus, ABA appears to stabilize G1 protein complexes formed by PCNA that apparently should be stable and active only during early imbibition and that, on the contrary, remain stable while germination is inhibited. Therefore, ABA could be blocking cell cycle progression with most cells remaining in an extended G1 phase with an active G1 kinase that, in the absence of ABA, should be inactivated so that the next cell cycle phase is triggered. ABA does not seem to produce an "all or nothing" effect, since the $p13^{Suc1}$-associated Cdk-A, very likely a G2/M marker, is active during late germination in control, BA- or ABA-treated embryonic axes, although kinase activity is lower in ABA-treated axes. Of interest is that the $p13^{Suc1}$-associated Cdk-A from BA-treated axes is activated at earlier times than kinase activity in control axes, consistent with the cell cycle acceleration caused by this mitogenic phytohormone.

Our results suggest that establishment of the G1 phase of the cell cycle is an early component of the germination process in maize embryos. BA may accelerate germination in part by stimulating cell cycle progression, while ABA has opposite effects on both cell cycle progression and germination. Conflicting results on the role of and requirement for cell cycle activity during germination of diverse seeds may be due in part to reliance on flow cytometry, which cannot detect cell cycle activities prior to S/G2 phase. The proteins and molecular processes associated with entry into and progression through the G1 phase may constitute new and perhaps highly specific molecular markers for seed germination.

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Table 1. Cell cycle progression in maize embryonic radicle tips following imbibition in water, benzyladenine (BA) or abscisic acid (ABA).

Radicle tips were excised from dry embryos or embryos imbibed in water, in 1 μM BA or 20 μM ABA for various periods. Nuclei were isolated and DNA contents were assessed by flow cytometry. Percentages of the total nuclei counted were assigned 2N (G1), 4N (G2) or intermediate (S) DNA contents using Multicycle software. Means ± Standard deviations (n = 3) are shown; letters indicate significant differences at $p < 0.05$ among treatments within each time and cell cycle phase category.

Imbibition time (h)	Treatment	G1 (%)	G2 (%)	S (%)
0	Dry axes	82.1 ± 3.03	17.6 ± 2.67	0.30 ± 0.48
6	Water	77.3 ± 1.81^{A}	19.7 ± 1.39^{B}	2.84 ± 2.03^{A}
	BA	73.9 ± 1.98^{B}	24.8 ± 1.09^{A}	1.33 ± 1.75^{A}
	ABA	77.2 ± 2.60^{A}	20.8 ± 1.68^{B}	1.98 ± 2.25^{A}
15	Water	63.4 ± 1.41^{B}	35.4 ± 3.08^{B}	1.18 ± 2.35^{A}
	BA	58.4 ± 1.39^{C}	39.7 ± 2.86^{A}	1.94 ± 3.70^{A}
	ABA	68.4 ± 3.77^{A}	25.7 ± 2.53^{C}	5.90 ± 4.90^{A}
24	Water	68.4 ± 1.50^{A}	19.9 ±1.70^{C}	11.7 ± 1.46^{A}
	BA	58.3 ± 1.88^{B}	29.7 ± 2.50^{A}	12.0 ± 1.90^{A}
	ABA	67.9 ± 1.90^{A}	26.7 ± 1.15^{B}	5.47 ± 2.90^{B}

FIGURE LEGENDS

Figure 1. Flow cytometric histograms of numbers of nuclei containing different relative DNA contents (channel numbers) from radicle tips of embryos imbibed in (A) water, (B) 1 μM BA or (C) 20 μM ABA for 15 h. The first peak in each panel represents 2C nuclei (G1 phase) while the second peak represents 4C nuclei (G2).

Figure 2. Effect of BA and ABA on the amount of PCNA and Cdk-A proteins during maize germination. Protein extracts from maize embryonic axes imbibed for 0, 3, 6, 15, and 24 hours in the presence/absence of BA or ABA (zero time sample for ABA is the same as that for BA) were prepared for western blot assays using (A) anti-maize PCNA antibodies or (B) anti-PSTAIRE antibodies. (C) Gels stained for protein to show loading of each lane.

Figure 3. Incubation of Cdk- A proteins with lambda protein phosphatase. Histone H1 protein was phosphorylated by the PCNA-associated protein kinase, using [γ-^{32}P] ATP, and then this radioactively labeled histone H1 was used as substrate for phosphatase (λ-phosphatase). Histone H1 was resolved by SDS-PAGE and (A) stained with Coomassie blue and exposed for autoradiography. (B) Extracts from maize embryonic axes imbibed 6 or 24 h in the presence of BA were treated with lambda phosphatase (λ-Pase) and then proteins were processed for western blot assay using the anti-PSTAIRE antibody.

Figure 4. Immunoprecipitation of PCNA during maize germination**.** Protein extracts from maize embryonic axes imbibed for 0, 3, 6, 15 and 24 h in the presence or absence of BA or ABA were immunoprecipitated using anti-maize PCNA antibody (anti-PCNA precipitate) or preimmune serum (PS), separated, and stained for protein. The first lane in the upper two panels contains purified PCNA protein standard (PCNA std). The PS sample was from 6 h of imbibition. IgG = immunoglobulin G.

Figure 5. Effect of phytohormones on protein kinase activity in PCNA immunoprecipitates or in $p13^{Suc1}$-associated proteins. PCNA immunoprecipitates (A) or $p13^{Suc1}$ pulled-down proteins (B) from protein extracts from maize embryonic axes imbibed for 0, 3, 6, 15 or 24 h in the presence or absence of BA or ABA were used as the kinase source for activity assays using GST-ZmRBR-C as substrate. Autoradiograms show the ^{32}P activity associated with the substrate protein after electrophoretic separation.

Figure 1

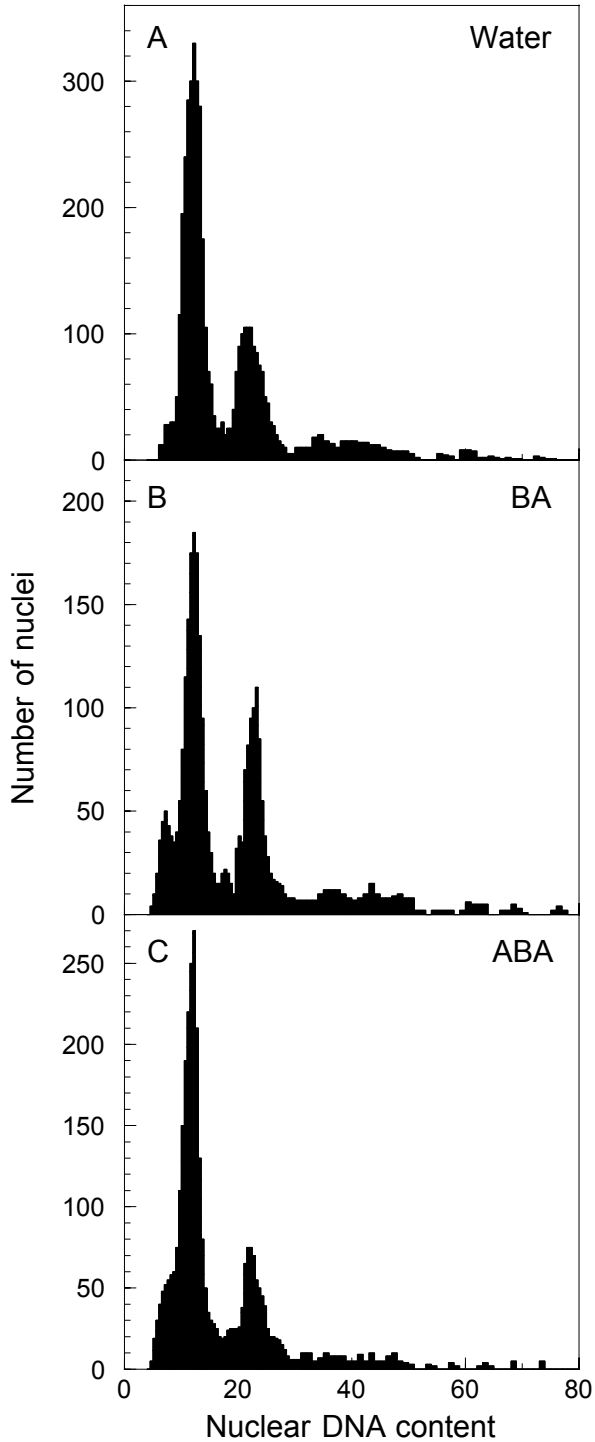

A
Water
B
BA
C
ABA
Number of nuclei
Nuclear DNA content
0
100
200
300
50
150
250
20
40
60
80

Figure 2

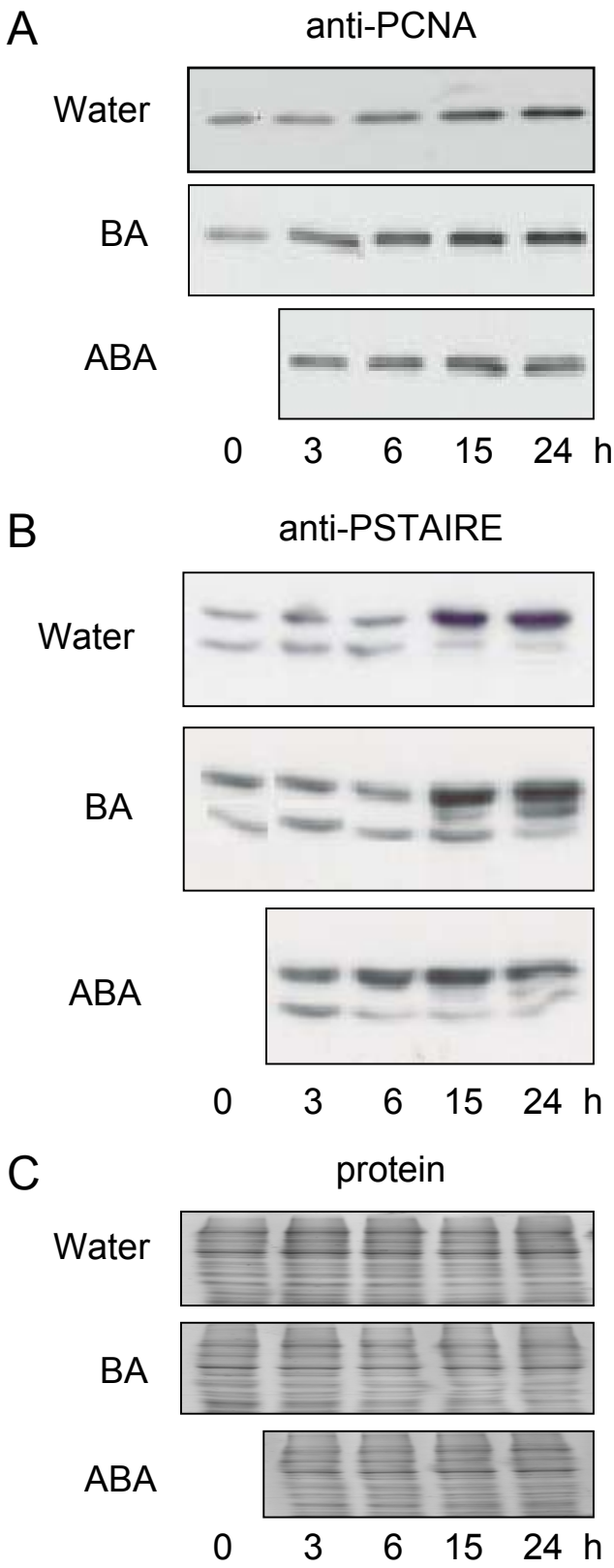

A
anti-PCNA
Water
BA
ABA
0 3 6 15 24 h
B
anti-PSTAIRE
Water
BA
ABA
0 3 6 15 24 h
C
protein
Water
BA
ABA
0 3 6 15 24 h

Figure 3

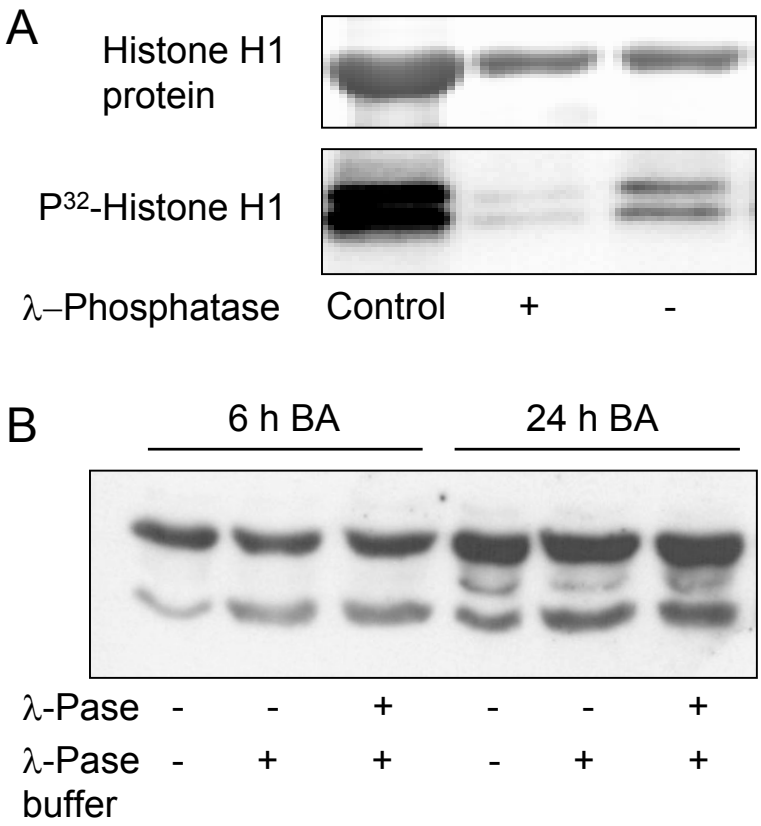

A
Histone H1 protein
P32-Histone H1
λ–Phosphatase
Control
+
-
B
6 h BA
24 h BA
λ-Pase - - + - - +
λ-Pase buffer - + + - + +

Figure 4

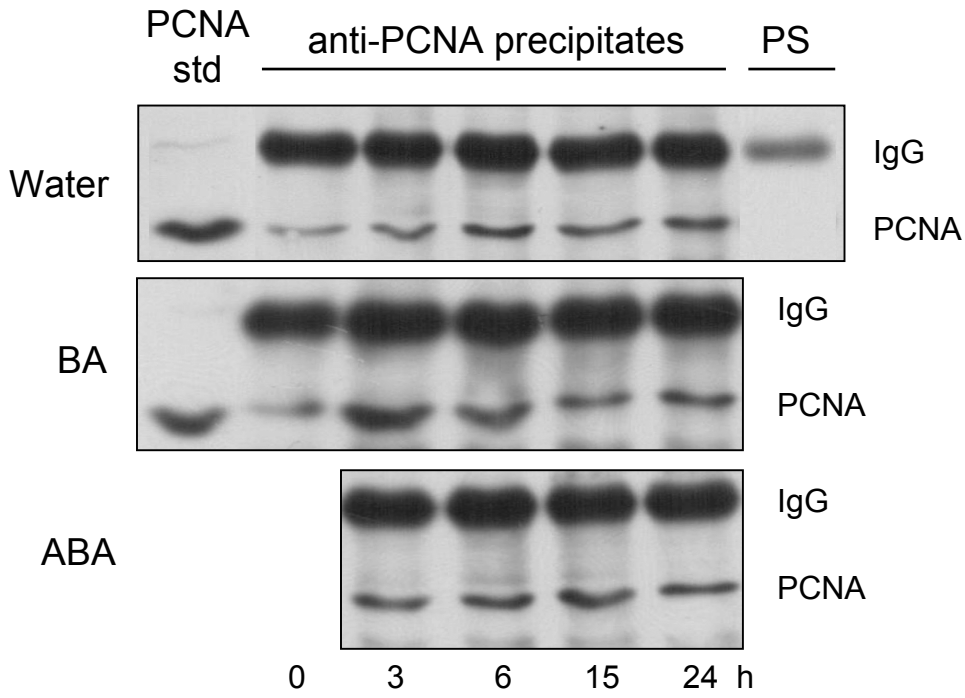
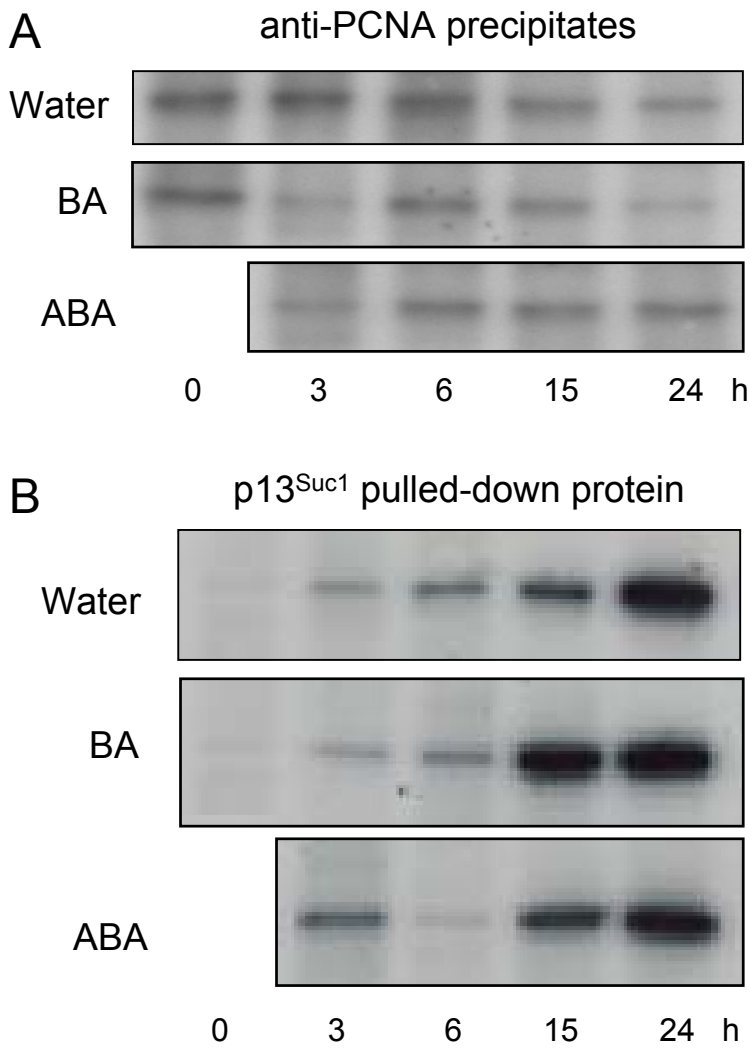

PCNA
std
anti-PCNA precipitates
PS
Water
IgG
PCNA
BA
IgG
PCNA
ABA
IgG
PCNA
0
3
6
15
24 h

Figure 5


A
anti-PCNA precipitates
Water
BA
ABA
0
3
6
15
24
h
B
p13Suc1 pulled-down protein
Water
BA
ABA
0
3
6
15
24
h