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RESEARCH PAPER

S phase of the cell cycle: a key phase for the regulation of thermodormancy in barley grain

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Abstract

The aim of the present work was to investigate the occurrence of the cell cycle during germination as related to thermodormancy in barley (*Hordeum vulgare* L., cv. Pewter) grains in relation with abscisic acid (ABA) by: (i) flow cytometry to determine the progression of the cell cycle; and (ii) reverse transcription-PCR to characterize the expression of some important genes involved in cell-cycle regulation. In dry embryos, cells are mostly (82%) arrested in G1 phase of the cell cycle, the remaining cells being in the G2 (17%) or S phase (0.9%). Germination at 20 °C was associated with an increase in the nuclei population in G2 and S (up to 32.5–44.5 and 9.2–11.3%, respectively, after 18–24 h). At 30 °C, partial reactivation of the cell cycle occurred in embryos of dormant grains that did not germinate. Incubation with 50 mM hydroxyurea suggests that thermodormancy resulted in a blocking of the nuclei in the S phase. In dry dormant grains, transcripts of *CDKA1*, *CYCA3*, *KRP4*, and *WEE1* were present, while those of *CDKB1*, *CDKD1*, *CYCB1*, and *CYCD4* were not detected. Incubation at 30 °C resulted in a strong reduction of *CDKB1*, *CYCB1*, and *CYCD4* expression and overexpression of *CDK1* and *KRP4*. ABA had a similar effect as incubation at 30 °C on the expression of *CDKB1*, *CYCB1*, and *CYCD4*, but did not increase that of *CDK1* and *KRP4*. Patterns of gene expression are discussed with regard to thermodormancy expression and ABA.

Key words: Abscisic acid, barley, cell cycle, cyclin, cyclin-dependent kinase, dormancy, germination, *Hordeum vulgare* L., KIP related protein.

Introduction

The germination of seeds is essential to the life cycle of plants. It is initiated upon uptake of water by the dry seeds and is considered complete upon initiation of elongation of the embryonic root (Bewley and Black, 1994), this process not requiring mitotic activity (Gornik *et al.*, 1997; Gendreau *et al.*, 2008). The involvement of cell-cycle activation in germination is debatable. A current hypothesis is that cell-cycle activation, especially progression into the G1 phase, is required for germination (Vázquez-Ramos and Sanchez, 2003). Seed dormancy is a trait that is imposed during the latter stages of seed development and prevents germination in an apparently suitable environment (Bewley, 1997). In cereals originating from temperate climates, such as barley, oat, and wheat, primary dormancy of mature grains is temperature dependent and is generally expressed above 15–20 °C (Lenoir *et al.*, 1983; Simpson, 1990; Corbineau and Côme, 1996; Benech-Arnold, 2004). There is little evidence for a role on the cell cycle in maintenance of the dormant state. However, nuclei of imbibed dormant seeds of tomato have been reported to remain in a G1-like state until dormancy is released (De Castro *et al.*, 2001).

Cell-cycle progression is controlled by different protein complexes associating cyclin-dependent kinase (CDK) and a catalytic subunit, cyclin (Inzé and De Veylder, 2006; Berckmans and De Veylder, 2009). The association of a CDK

with a specific cyclin partner determines the activity of the cyclin–CDK complex (Pines, 1995; Dewitte and Murray, 2003). Five types of CDK have been identified in plants, from CDKA to CDKE (Joubès *et al.*, 2000). The CDKA group, characterized by a PSTAIRE motif, plays a role at both the G1-to-S and G2-to-M transition points. The CDKB class, characterized by a PPTALRE motif, is required to progress through mitosis (Inzé and De Veylder, 2006). In plants, cyclins have been classified into five major groups: A, B, C, D, and H (Vandepoele *et al.*, 2002). The A-type cyclins accumulate during S–M phases and the B-type cyclins accumulate during G2–M phases and regulate entry into the M phase. The D-type cyclins are expressed earlier in the cell cycle and control the progression from the early G1 phase to S phase in response to external growth signals, some D-type cyclins may also act in G2/M (Meijer and Murray, 2000; Koroleva *et al.*, 2004; Planchais *et al.*, 2004). Active cyclin–CDK complexes can be inhibited by another regulating class of protein: cyclin-dependent kinase inhibitors, also known as Kip-related protein (KRP) (Oakenfull *et al.*, 2002). In *Arabidopsis*, seven genes have been identified as KRPs (De Veylder *et al.*, 2001). All KRPs, except KRP5, interact with CDKA1 and none interact with CDKB1, these interactions inducing a possible inhibition of cyclin–CDK complex activity. Futhermore, KRPs regulate both DNA synthesis and mitosis by binding and inhibiting A-type CDKs through their conserved CDK-binding domain (Oakenfull *et al.*, 2002). To complete the regulation of cell-cycle protein, it is important to notice that proteolysis of cell-cycle proteins, especially cyclins, is a common mechanism leading to an irreversible moving forward of the cycle (Genschik *et al.*, 1998; De Veylder *et al.*, 2007).

In higher plants, the phytohormone abscisic acid (ABA) regulates many processes of plant development, including synthesis of seed storage proteins, promotion of seed desiccation tolerance, and dormancy (Bewley, 1997; Feurtado and Kermode, 2007). ABA is also known to inhibit cell-cycle processes in G1 (Liu *et al.*, 1994; Swiatek *et al.*, 2002; Sánchez *et al.*, 2005), since it can induce genes such as *KRP* (Wang *et al.*, 1998). Thus, *KRP* genes may be good candidates to block cell-cycle progression via ABA in dormant seeds.

The objectives of the present study were: (i) to investigate whether cell-cycle progression in dormant barley grains is related to temperature; (ii) to determine the effects of high temperature, which inhibits the germination, on the expression of the main genes involved in cell-cycle activity; and (iii) to study whether ABA interferes with cell-cycle progression as related to dormancy expression, and if so at which cell-cycle step. Experiments were performed with mature dormant grains at 20 °C, a temperature at which germination can occur, and at 30 °C, a temperature at which germination is impossible (Corbineau and Côme, 1996; Benech-Arnold *et al.*, 2006). It is shown here that expression of dormancy at 30 °C is associated with a blocking of the nuclei in the S phase that is related to a strong reduction in *CDKB1*, *CYCB1*, and *CYCD4* transcript accumulation, and an overexpression of *CDKD1* and *KRP4*. Application of exogenous ABA at 20 °C induced a similar effect to incubation at 30 °C but did not induce expression of *CDKD1* and *KRP4*.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L., cv. Pewter) seeds were harvested in July 2004 at the end of the maturation drying phase, i.e. when their water content reached 0.10–0.12 g H_2O (g dry weight)$^{-1}$ (DW), and were provided by the 'Coopérative de Toury' (Eure et Loir, France). Seeds were stored at –18 °C in order to maintain their dormancy (Corbineau and Côme, 1996) until experiments started.

Germination assays

Germination assays were performed at 20 or 30 °C in darkness, in three replicates of 50 grains placed in 9-cm diameter Petri dishes on a layer of cotton wool imbibed with deionized water. A grain was regarded as germinated when the radicle had protruded through the seed-covering structures. Germination counts were conducted regularly for 7 d. The results presented correspond to the mean ± SD of the germination percentages obtained in three replicates.

Experiments with 1 mM ABA or 50 mM hydroxyurea, an inhibitor of the S phase (Planchais *et al.*, 2000) were done by incubating seeds with these compounds from the beginning of imbibition.

Radicle elongation measurement

Radicle lengths of seedlings were measured for seeds incubated at 20 °C with water, 1 mM ABA or 50 mM hydroxyurea after 24, 48, and 72 h. The results correspond to the mean ± SD of 30 measurements.

Flow cytometry

Amounts of nuclear DNA were quantified using radicles of embryos isolated from grains incubated for various times at 20 °C and 30 °C with water or at 20 °C with 1 mM ABA. Samples of 15 radicles including coleorhiza were chopped on ice with a razor blade in 400 µl nuclear isolation buffer (20 mM MOPS, 30 mM Na citrate, 45 mM $MgCl_2$, 1% Triton X-100, w/v; adapted from Galbraith *et al.*, 1983). The suspension was sieved through a 48 µm nylon mesh. After 15 min digestion with RNase (1 µg in 10 µl) at room temperature, the samples were stained with propidium iodide (1 mg ml^{-1}) to allow measurement of the amount of nuclear DNA by fluorescence. DNA analyses were performed using an EPICS XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA) equipped with an argon ion laser at 488 nm and fluorescence was detected over a range 605–635 nm. The amount of DNA is proportional to the fluorescent signal and is expressed as an arbitrary C value, in which the 1C value represents the amount of DNA of the unreplicated haploid chromosome complement. Three experiments were performed independently and the results presented correspond to a representative single experiment. The frequency of 2C, 4C, and S-phase nuclei was calculated as [2C nuclei/(2C nuclei + 4C nuclei + S-phase nuclei)] × 100, [4C nuclei/(2C nuclei + 4C nuclei + S-phase nuclei)] × 100, and [S-phase nuclei/(2C nuclei + 4C nuclei + S-phase nuclei)] × 100, respectively. The average variation between two measurements of a population of nuclei was ~3%. The different nuclei populations were measured for 10,000 nuclei.

RNA extraction

Embryos were isolated from the endosperm using a sharp scalpel blade, immediately frozen in liquid nitrogen, and then stored at –80 °C. For each extract, 30 embryos were ground in liquid N_2, and total RNA was extracted by a hot phenol procedure according to Verwoerd *et al.* (1989).

EST database search

In order to analyse cell-cycle transcription activity, the expression of related genes as markers of cell-cycle phase was studied. The TIGR barley expressed sequence tag (EST) databank (http://www.ncbi.

nlm.nih.gov/blast/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn&BLAST_PROG_DEF=megaBlast&BLAST_SPEC=Plants_EST) was screened using specific protein motifs for each class to identify barley genes. To complete and confirm the databank annotation, specific protein motifs were identified using the translation software from infobiogene (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). A class could be attributed for each gene with specific motifs, i.e. for *CDKDA* (*CDKA1*), *CDKB* (*CDKB1*), *CDKD* (*CDKD1*), *CYCA* (*CYCA3*), *CYCB* (*CYCB1*), and *CYCD* (*CYCD4*) (Vandepoele *et al.*, 2002), and *KRP* (*KRP4*) and *WEE* (*WEE1*) (Sorrell *et al.*, 2002). Specific primers were then designed using the software Primer3 for each EST (Supplementary Table S1) to perform reverse-transcription (RT) PCR. As an internal standard, a fragment of the barley actin gene (GI24496451) was used. Each expression profile presented was repeated at least three times in three independent experiments.

Reverse-transcription PCR

Total RNA (4 µg) was treated with DNase I (Sigma, St Louis, MO, USA) and then was reverse transcribed with Revertaid H minus M-MuLVRT (Fermentas, Vilnius, Lithuania). All oligonucleotides were obtained from Eurogentec (Seraing, Belgium). For semiquantitative PCR, amplifications were performed using a Mastercycler (Eppendorf, Hamburg, Germany) with 1 U Taq polymerase (New England Biolabs, Beverly, MA, USA), 0.4 mM dNTP, and 0.8 µM each primer in a 25 µl reaction volume. The thermal cycling program was initiation at 94 °C for 2 min, 25 cycles for CDK and actin genes or 30 cycles for cyclin genes at 94 °C for 30 s, 55 °C for 30 s, and termination at 72 °C for 5 min. Amplification products were detected on an agarose gel with ethidium bromide and quantified with Quantity One software (Bio-Rad, Hercules, CA, USA). The actin level in each lane was used as an internal standard. the results presented correspond to representative profiles of gene expression obtained in three independent experiments.

Results

Germination of dormant grains at 20 and 30 °C

At harvest, seeds were considered dormant since only 10% of the seed population germinated at 30 °C although almost all (90%) were able to germinate within 48 h at 20 °C (Fig. 1). Incubation of the grains with 1 mM ABA resulted in an inhibition of the germination rate: 58% of grains germinated within 72 h against 90% with water. The time to obtain 50% germination (T_{50}) was about 20 and 48 h with water and ABA, respectively (Fig. 1). Hydroxyurea (50 mM) only slightly inhibited seed germination at 20 °C; T_{50} was 34 h and more than 80% of the grains germinated within 48 h.

The mean length of radicles of seeds incubated with water was 30.5 mm after 48 h at 20 °C, and radicle length did not exceed 0.9 and 1.4 mm with hydroxyurea and ABA, respectively (Fig. 1).

Cell-cycle activity during germination

In dry grains, approximately 82 and 17% of the nuclei of the embryonic axes gave 2C and 4C signals, respectively, indicating that the majority of cells were arrested at the G1 phase of the cell cycle (Table 1). Only 0.9% of the nuclei gave an intermediate signal and were considered as being in the S phase (Table 1).

At 20 °C, germination was associated with an increase in the 4C nuclei population up to 32.5% after 18 h, i.e. when 30% of the grains germinated, and 56.8% after 48 h, i.e. when germination rate was maximal (90%) (Table 1). The 4C/2C ratio increased

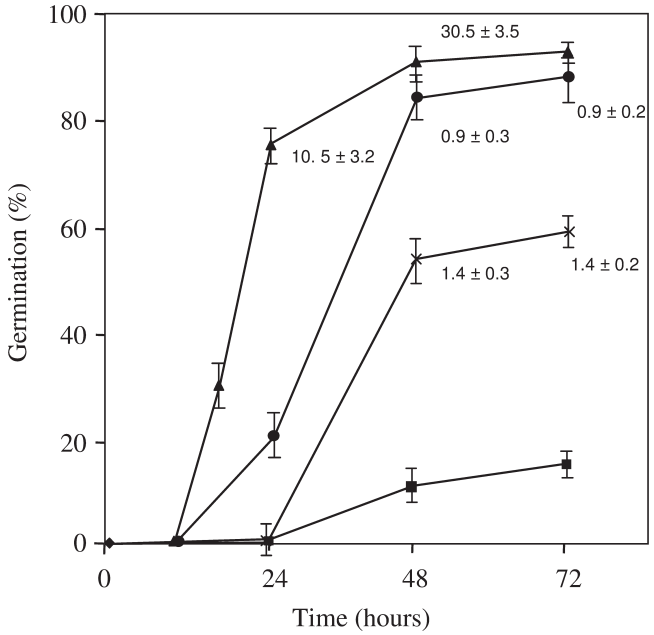

Fig. 1. Time course of germination of dormant grains at 20 °C with water (▲), with 1 mM ABA (×) or 50 mM hydroxyurea (●) and at 30 °C with water (■). Values are mean ± SD of three replicates. Numbers on the curves correspond to radicle length in mm (mean ± SD of 30 measurements).

from 0.20 in dry seeds up to 0.56 after 18 h of imbibition at 20 °C and reached 1.01 and 1.66 after 24 and 48 h, respectively. The percentage of nuclei in S phase remained between 9.2 and 11.3 (Table 1). After 24 h, 44.1, 44.5, and 11.3% of of radicle-tip nuclei were in the G1, G2, or S phase of the cell cycle (Table 1, Fig. 2A). Incubation of grains with 50 mM hydroxyurea, an inhibitor of the S phase, did not suppress radicle protrusion (cf. Fig. 1), but resulted in a blocking of the cell cycle (Fig. 2D); a second shoulder in the 2C peak of nuclei was then observed, representing cells re-entering the cell cycle but blocked in the S phase (Fig. 2D).

At 30 °C, there was a reactivation of the cell cycle; 30.9% of the nuclei population were in G2 phase against 17.1% for radicle tips of embryos from dry grains (Table 1). From 24 to 72 h of imbibition, nuclei in the G1, G2, or S phase represented 59.4–60.4, 29.8–30.9, and 8.8–10.6% of the population, respectively (Table 1, Fig. 2B). The 4C/2C ratio remained at 0.50. When seeds were incubated with hydroxyurea (Fig. 2E), some nuclei (12%) re-entered the cell cycle but were blocked in G1 phase, suggesting that, in the dormant state, nuclei were blocked in the S phase.

ABA (1 mM) inhibited germination (Fig. 1) and cell-cycle reactivation as compared with control grains incubated at 20 °C with water (Table 1). The percentage of nuclei with the 4C signal was between 26.2% (after 24 h) (Fig. 2C) and 29.2% (after 72 h), values that were close to those observed for grains incubated at 30 °C (Table 1), a temperature at which the seeds could not germinate (cf. Fig. 1). However, the 4C/2C ratio was lower (0.38–0.44) than that for dormant seeds incubated with water at 30 °C (0.50–0.52) (Table 1). Nuclei with an intermediate DNA signal, i.e. in an apparent S phase, corresponded to only 4–5% of the population, while they represented about 10% of the nuclei

Table 1. Percentage of nuclei giving 2C, 4C, and S-phase signal of cells of embryonic radicle tips isolated from dry seeds and seeds incubated for various durations at 20 °C with water or with 1 mM ABA, or at 30 °C with water with or without 50 mM hydroxyurea

Values are mean ± SD of three measurements carried out on 10000 nuclei. The frequency of 2C, 4C, and S-phase nuclei was calculated as [2C nuclei/(2C nuclei + 4C nuclei + S-phase nuclei)] × 100, [4C nuclei/(2C nuclei + 4C nuclei + S-phase nuclei)] × 100, and [S-phase nuclei/(2C nuclei + 4C nuclei + S-phase nuclei)] × 100, respectively. The 2C* population corresponds to nuclei of cells entering S phase but blocked from further DNA synthesis by the presence of 50 mM hydroxyurea (Hur). It was integrated to the total amount of nuclei for the hydroxyurea treatments.

Imbibition time (h)	Treatment	Nuclei (%) in:				4C/2C
		2C	2C*	4C	S-phase	
0	Dry seed	82.2 ± 3.1		17.1 ± 1.6	0.9 ± 1.3	0.20
18	Water (20 °C)	58.1 ± 2.6		32.5 ± 1.3	9.2 ± 1.4	0.56
24	Water (20 °C)	44.1 ± 2.2		44.5 ± 1.8	11.3 ± 1.6	1.01
	Water + Hur (20 °C)	46.2 ± 2.3	44.1 ± 1.2	8.5 ± 1.5	2.3 ± 1.5	0.09
	ABA (20 °C)	69.6 ± 1.5		26.2 ± 1.9	4.3 ± 1.3	0.38
	ABA + Hur (20 °C)	68.1 ± 2.2	–	27.5 ± 1.2	5 ± 1.4	0.40
	Water (30 °C)	59.9 ± 2.5		30.9 ± 1.5	8.8 ± 2.5	0.52
	Water + Hur (30 °C)	49.1 ± 1.9	12.6 ± 1.1	37.5 ± 1.8	2.3 ± 1.1	0.72
48	Water (20 °C)	34.2 ± 1.9		56.8 ± 1.5	9.5 ± 1.9	1.66
	ABA (20 °C)	66.5 ± 1.8		28.6 ± 2.5	4.8 ± 0.9	0.43
	Water (30 °C)	59.4 ± 1.9		29.8 ± 1.8	10.6 ± 2.5	0.50
72	Water (20 °C)	–		–	–	–
	ABA (20 °C)	65.3 ± 1.6		29.2 ± 1.6	5.7 ± 1.0	0.44
	Water (30 °C)	60.4 ± 2.0		30.0 ± 1.8	9.6 ± 2.2	0.50

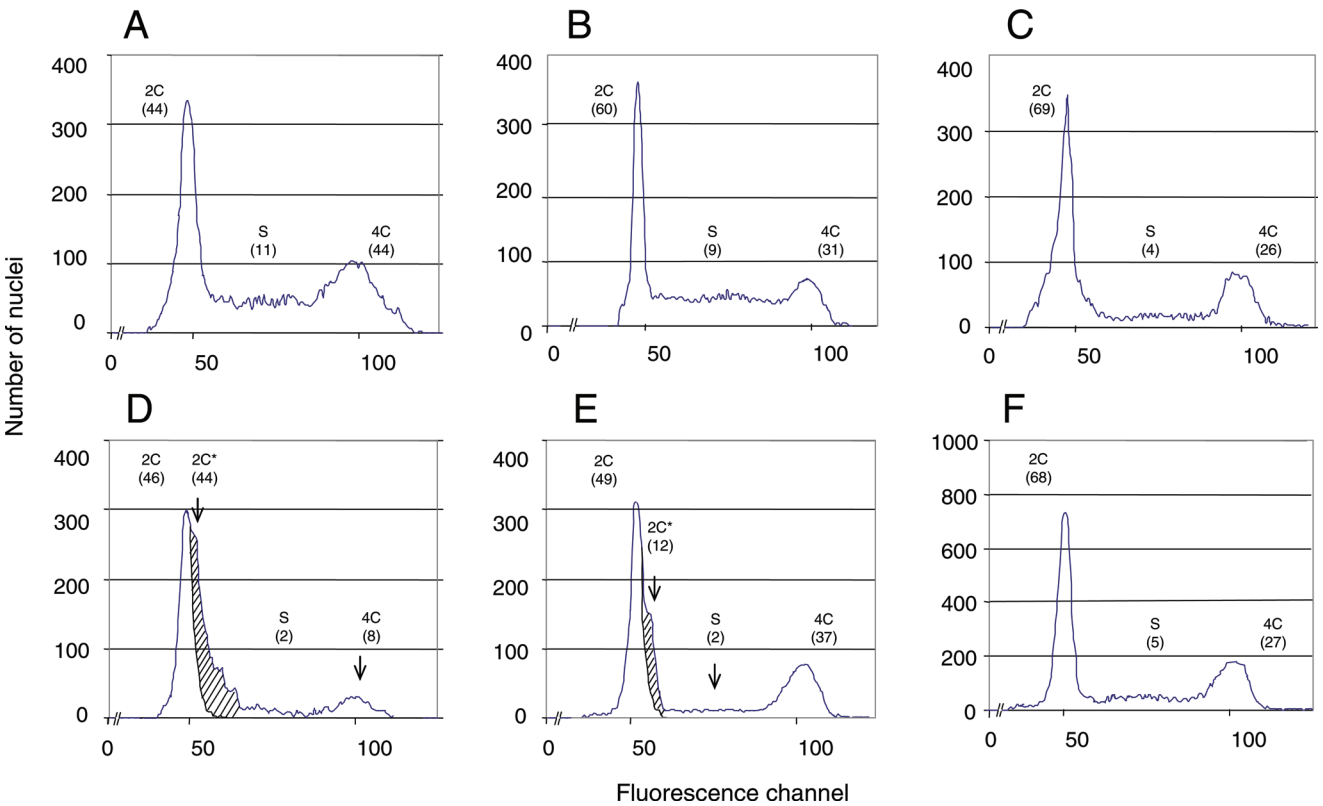

Fig. 2. Flow cytometric analysis of nuclei from embryo radicles isolated from germinating grains: (A–C) after 24 h with water at 20 °C (A) and 30 °C (B) or with 1 mM ABA at 20 °C (C); (D–F) as for A–C but also with 50 mM hydroxyurea. Numbers in parentheses indicate percentage of nuclei in 2C, 2C*, S, and 4C cell-cycle phases. Hatched area corresponds to nuclei entering S phase but blocked (2C*) from further DNA synthesis by the presence of hydroxyurea. The average variation between two independent measurements of a population of nuclei is 3% maximum. Measurements were carried out on 10,000 nuclei.

population when incubated with water either at 20 or 30 °C (Fig. 2C, Table 1). Application of hydroxyurea did not result in a shoulder in the 2C peak (Fig. 2F).

Expression of CDK, CYC, KRP, *and* WEE

Changes in transcript abundance of CDK (*CDKA1*, *CDKB1*, and *CDKD1*), cyclins (*CYCA3*, *CYCB1*, and *CYCD4*) and regulatory genes such as *KRP4* and *WEE1* were analysed by RT-PCR in embryos during incubation of seeds at 20 and 30 °C with water or at 20 °C with 1 mM ABA. The results are presented in Fig. 3, and Supplementary Fig. S1 shows the expression profiles. Transcripts of some genes (*CDKA1*, *CYCA3*, *KRP4*, and *WEE1*) were present in dry seeds, while those of *CDKB1*, *CDKD1*, *CYCB1*, and *CYCD4* were not detected. *CDKA1*, *KRP4*, and *WEE1* expression did not change significantly during seed imbibition/germination at 20 °C. In contrast, *CDKB1*, *CDKD1*, *CYCA3*, *CYCB1*, and *CYCD4* transcripts accumulated during germination, with *CYCB1* expression reaching a maximum at 18 h, while the expression of the other genes was maximal at 24 h, i.e. when about 80% of the grain population germinated (cf. Fig. 1).

Incubation of the grains at 30 °C resulted in an almost complete suppression of *CDKB1*, *CYCB1*, and *CYCD4* expression (Fig. 3) and in an overexpression of *CDKD1* and *KRP4*. Expression of *CDKA1* and *WEE1* was not significantly affected at 30 °C as compared to 20 °C, when that of *CYCA3* was higher at 18 h and then reduced from 24 h.

Application of exogenous 1 mM ABA at 20 °C, similarly to incubation at 30 °C, resulted in a suppression of the expression of *CDKB1*, *CYCB1*, and *CYCD4* (Fig. 3). However, ABA did not increase the expression of *CDKD1* and *KRP4*. With the exception of 18 h incubation, expression of *CDKA1*, *CDKD1*, *CYCA3*, *KRP4*, and *WEE1* was close to that measured in embryos of control grains incubated at 20 °C with water.

Discussion

As previously shown in non-dormant barley grains (Gendreau *et al.*, 2008) and other species (Bino *et al.*, 1993; Lanteri *et al.*, 1994; Özbingöl *et al.*, 1999; Sánchez *et al.*, 2005), the majority of radicle-tip nuclei of dry dormant barley grains arrested in G1 phase of the cell cycle, i.e. with a 2C DNA level, the remaining cells being in G2 or S phase. In non-dormant grains, 8% of the nuclei population arrested in the cell cycle with 4C DNA levels (Gendreau *et al.*, 2008) while 4C signals were observed in 17% of the nuclei population in dormant grains at harvest (Table 1). This decrease in the G2 nuclei population during after-ripening might result from a progressive dehydration of the grain during dry storage, as observed in sugar beet seeds after drying (Sliwinska, 2003).

Seed dormancy is defined as an inability to germinate in apparently favourable conditions (Bewley and Black, 1994; Bewley, 1997). In barley, it corresponds to a difficult germination at temperatures higher than 20 °C (Corbineau and Côme, 1996; Benech-Arnold, 2004). At 20 °C, a temperature at which dormancy was not expressed in the current seed batch, the time to obtain 50% germination was about 20 h (Fig. 1), and cell-cycle activity was initiated in radicle-tip nuclei after 18 h of imbibition, i.e. before the radicle elongation of all seeds (Table 1). The induction of the cell cycle during imbibition varies among species; the G2 population reaches 56% at 14 h, when radicle protrusion occurs, in non-dormant barley grains (Gendreau *et al.*, 2008), 35% at 15 h in maize (Sánchez *et al.*, 2005), and 30–39% in sugar beet (Sliwinska, 2000). On the other hand, application of 50 mM hydroxyurea, which resulted in a blocking of the cell cycle (Fig. 2D and Table 1), did not prevent germination but inhibited radicle growth (Fig. 1), suggesting that cell-cycle initiation may not be totally required for the early phases of germination.

At 30 °C, a temperature at which dormant grains cannot germinate (Fig. 1), the dynamic of the cell cycle was different than that at 20 °C (Figs 2A and 2B and Table 1). A partial activation occurred, 30% of the nuclei moving from G1 to G2 at 24 h, but the proportion of 4C nuclei remained at this level until 72 h, versus 44.5–56.8% at 20 °C. The results obtained with hydroxyurea suggest that, at 30 °C, the cell cycle is not being completed. The G2 population remained stable (about 37.5%) even after blocking the S phase (Fig. 2E and Table 1), when it was lowered to 8.5% at 20 °C (Fig. 2D and Table 1), probably because nuclei moved to the M and G1 phases. It can be concluded that, in non-germinated dormant barley seeds at 30 °C, the nuclei of the radicle tips are blocked in the G2/M transition and in the S phase.

Similarly to numerous species (Bewley, 1997; Nambara and Marion-Poll, 2003; Nambara *et al.*, 2010), barley seed dormancy and germination are mainly regulated by ABA (Benech-Arnold *et al.*, 2006; Millar *et al.*, 2006). The inability of dormant barley grains to germinate at 30 °C is associated with a maintenance of a high concentration of ABA (Benech-Arnold *et al.*, 2006; Gubler *et al.*, 2008) and higher embryo sensitivity to ABA (Corbineau and Côme, 2000; Benech-Arnold *et al.*, 1999, 2006). In the current study, at 20 °C, exogenous ABA (1 mM) inhibited germination (Fig. 1) and resulted in an almost 2-fold reduction of the 4C nuclei population at 24 and 48 h of imbibition (Table 1). The hydroxyurea treatment revealed that ABA, similarly to incubation at 30 °C, resulted in a blocking of the G2/M and G1/S transitions.

Similarly to non-dormant seeds (Gendreau *et al.*, 2008), major changes in the transcript levels of *CDK* and *CYC* occurred in the embryo during incubation at 20 °C (Fig. 3), a temperature at which dormant seeds germinated (Fig. 1). In contrast, at 30 °C and at 20 °C with ABA, expression of *CDKA1* and *CDKD1* suggested that cell-cycle progression was possible, but the lack of expression of *CDKB1* might explain the arrest in G1/S transition observed (Menges *et al.*, 2005).

Absence of germination of dormant grains at 30 °C and at 20 °C with ABA is associated with low expression of *CYCB1* and *CYCD4* (Fig. 3), which might be a cause of the arrest in the G2/M and G1/S transition of the cell cycle, respectively (Menges *et al.*, 2005). *CYCB1* was not also induced in non-dormant barley seeds incubated with low water content, which did not allow germination, while expression of *CYCD4* remained high in correlation with an increase in the 4C population (Gendreau *et al.*, 2008). Since cyclins are strongly regulated at the transcriptional level (Barrôco *et al.*, 2005), the disappearance of *CYCB1* might be a good indication of the regulation of the cell cycle at the

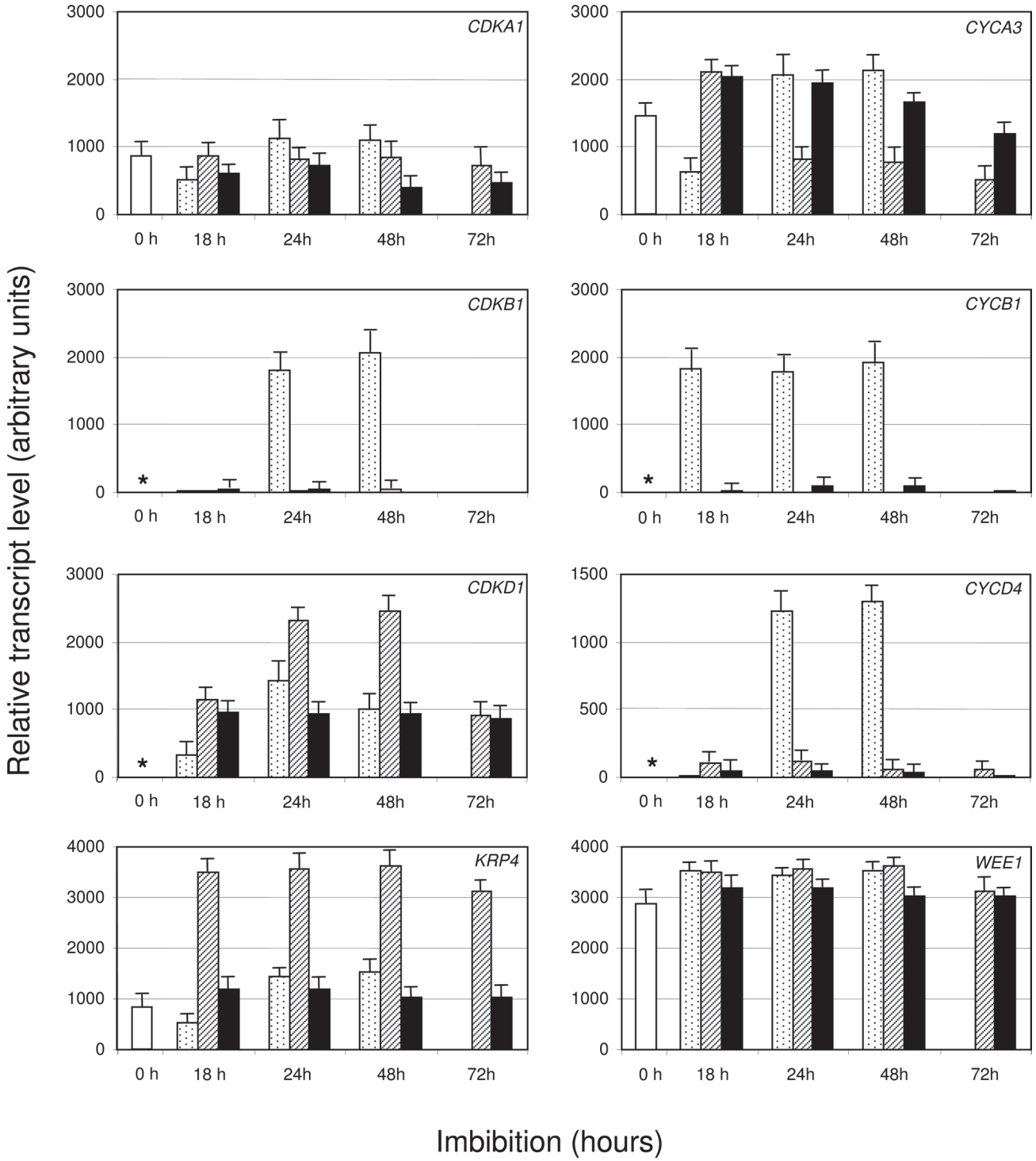

Fig. 3. Transcript abundance of the main genes involved in cell-cycle activity in embryos of grains incubated at 20 and 30 °C with water, or at 20 °C with 1 mM ABA. Data from RT-PCR (mean ± SD of three replicates) are given in arbitrary units. Dry seeds (white), seeds incubated at 20 °C with water (light grey), 30 °C with water (dark grey), or at 20 °C on 1 mM ABA (black). *, no detectable signal.

G2/M transition when no germination occurs independently of the dormancy phenomenon. In contrast, the reduced expression of *CYCD4* is related to dormancy *per se* and not to the absence of germination.

KRP negatively regulates the cyclin–CDK complex at the G1/S phase transition by interfering with formation of the complex (Oakenfull *et al.*, 2002; Cho *et al.*, 2009). In the barley databank, one KRP was found with a clear protein motif as in KRP4 and KRP6 according to the KRP classification of De Veylder *et al.* (1999). The current results indicate that *KRP4* is expressed even when the cell cycle is turning normally at 20 °C and that *KRP4* is upregulated at 30 °C. However, its expression was not increased with exogenous ABA at 20 °C, although KRPs have been shown to be induced by ABA (Wang *et al.*, 1998; Ruggiero *et al.*, 2004).

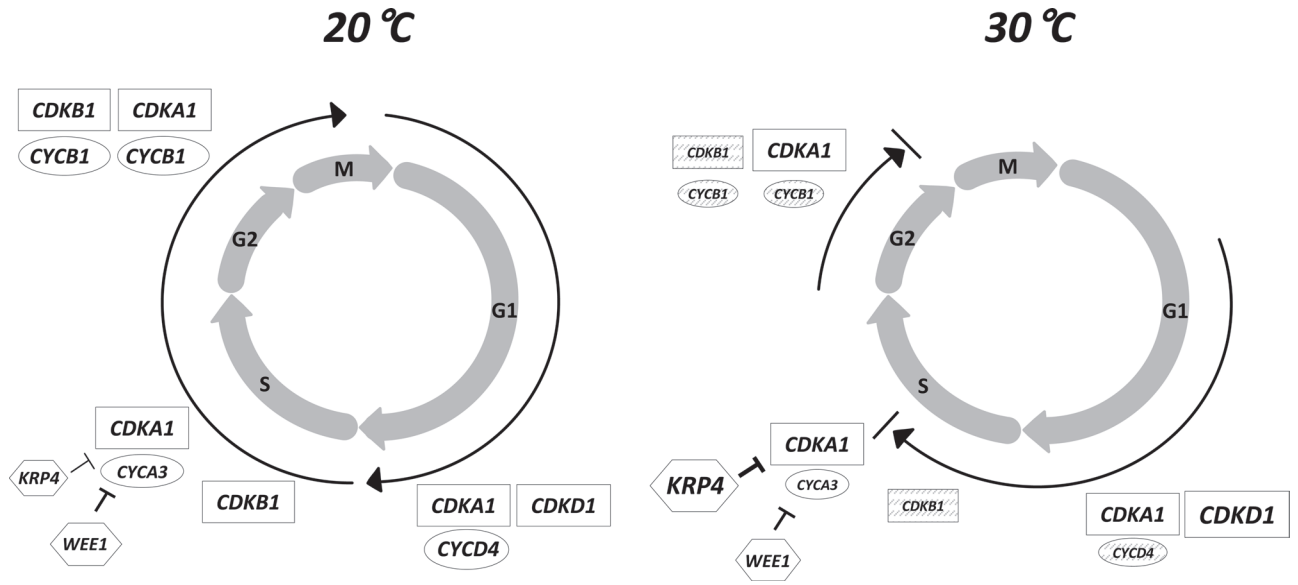

Fig. 4. The regulation of cell-cycle progression in dormant barley grains incubated at 20 and 30 °C. At 20 °C, temperature at which the grains can germinate, the cell cycle can progress through expression of *CDK* (*CDKA1*, *CDKB1*, *CDKD1*) and *CYC* (*CYCA3*, *CYCB1*, *CYCD4*); expression of *KRP4* is low. At 30 °C, the cell cycle is blocked in the S phase and at the G2/M transition which is related to a strong reduction in *CYCD4*, *CDKB1*, *CYCA3*, and *CYCB1* transcript accumulation and an overexpression of *KRP4*. Very low expression is specified in grey. ABA has the same effects as 30 °C on all genes, except on the expression of *KRP4*, which is not enhanced.

Nevertheless, *KRP4* overexpression at 30 °C could be considered a good candidate to explain cell-cycle progression in embryos of dormant seeds incubated at high temperatures. *WEE1* expression, involved in the G2/M transition, did not change as a function of incubation time either at 20 or 30 °C or even at 20 °C with ABA (Fig. 3); thus, if this gene is involved, it is probably not through its transcriptional expression.

Overall, the results reported here shed new light on the regulation of the cell cycle during incubation of dormant barley seeds as related to temperature. Fig. 4 gives a diagram depicting cell-cycle progression in dormant barley seeds incubated at 20 and 30 °C as related to the expression of *CDK*, *CYC*, and *KRP* genes. When germination can occur, for example at 20 °C, the cell cycle is initiated early, before radicle elongation, as previously shown in non-dormant barley grains (Gendreau *et al.*, 2008) and in *Arabidopsis thaliana* (Barrôco *et al.*, 2005; Inzé and De Veylder, 2006). The inability of dormant grains incubated at 30 °C to germinate is a blocking of the G2/M transition, with a downregulation of the expression of *CDKB1* and *CYC A3*, *B1*, and *D4* and an upregulation of *CDKD1* and *KRP4*. Comparison between the expression profiles of the main genes involved in cell-cycle activity in non-germinated dormant barley grains incubated at 30 °C (Fig. 3) and in non-dormant grains partially hydrated at 30 °C (Gendreau *et al.*, 2008) allows the current study to propose that the blockage in the G2/M transition and the S phase in embryos of dormant seeds incubated at 30 °C involves a specific regulation through *CYCD4* and *KRP4*. Application of 1 mM ABA inhibited germination at 20 °C (Fig. 1) but surprisingly did not simulate the effect of incubation at 30 °C at the level of gene expression (Fig. 3). Therefore, it is suggested that cell-cycle regulation during incubation of dormant seeds is only partially by the metabolism of ABA.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Oligonucleotide sequences used for reverse transcription PCR.

Supplementary Fig. S1. Expression profiles of the main genes involved in cell-cycle activity in embryos of grains incubated at 20 °C and 30 °C with water or at 20 °C with 1 mM ABA.

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