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Petunia p34^{cdc2} protein kinase activity in G_2/M cells obtained with a reversible cell cycle inhibitor, mimosine

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Protoplasts isolated from petunia leaf mesophyll are non-cycling cells mostly with 2C content. Cells regenerating from protoplast culture enter mitosis after 48 h. This experimental model is used to relate p34^{cdc2} kinase activity to cell cycle phase. Our results show that the histone H1 phosphorylation, and hence p34^{cdc2} kinase activity, peaks with G_2+early M cell cycle phase. However, a trace kinase activity was already present when most cells were entering S phase. To obtain a maximum of cells in G_1+S phases, the protoplast culture was treated with the rare amino acid, mimosine. Mimosine blocked plant cells derived from protoplast culture both at G_1 and in early and mid S phase. Despite the increased G_1+S level, p34^{cdc2} kinase activity did not increase. This suggests that the trace activity appearing when the majority of cells are entering S does not correspond to any putative p34^{cdc2} activation at G_1/S transition but to the activation of the minor 4C population initially present in the leaf: the hypothesis remains that p34^{cdc2} kinase activity is solely related to G_2+M phase in petunia.

Cell cycle; p34^{cdc2}; Protoplast; Mimosine; Petunia

1. INTRODUCTION

A universal intracellular factor, the 'M phase-promoting factor' (MPF), triggers the G_2/M transition of the cell cycle in all eukaryotic organisms. MPF displays a kinase activity which is calcium-, diacylglycerol- and cyclic nucleotide independent. It is a complex of at least two subunits, p34^{cdc2} and cyclin B (see [1,2] for review). p34^{cdc2} is a serine-threonine protein kinase, the activity of which increases dramatically at onset of mitosis and indeed determines the timing of entry into mitosis [3,4]. Plant cell homologues of both cdc2 genes [5–10] and cyclin B [11–13] have been identified. The p34 kinase activity is modulated in several ways, one being by phosphorylation. The protein is partially dephosphorylated on threonine and tyrosine upon entry into and progression through mitosis; the G_2/M transition has been shown to be dependent upon tyrosine dephosphorylation [14].

Petunia mesophyll protoplast preparations comprise predominantly cells with 2C DNA content: 4C cells constitute about 5–20%, while S phase cells are virtually absent [10,15,16]. Simultaneous staining of DNA and RNA with Acridine orange has shown that mesophyll protoplasts in both 2C and 4C states require an increase in RNA content before DNA synthesis can occur and before they enter into mitosis [17]; it was concluded that these cells are not cycling cells at the time of their isolation. Thus differentiation in petunia occurs at both 2C and 4C DNA levels, as in many, if not most, plant species. These two kinds of cells are fully transcriptionally activated after 18 h in culture medium [17]; the frequency of cell division observed in such cultures is positively correlated with the frequency of 4C cells in the initial leaf tissue [15]. Only 4C cycling cells can be considered in G_2 phase; they are in a differentiated 4C stage. It has been shown with protoplast-derived cells that the level of expression of the petunia cdc2 homologue, cdc2Pet (as cdc2Pet mRNA or p34^{cdc2}), was similar in 2C cycling nuclei and 2C (differentiated) leaf nuclei [10]: this indicates that 2C petunia leaf cells, although not proliferating, are metabolically in G_1 and not in the fully quiescent G_0 cell-cycle phase.

To gain a better understanding of division induction from such differentiated cells, it would be advantageous to determine the state of regulatory proteins, such as p34^{cdc2} kinase, its phosphorylation and its associated activity. Compounds that perturb the cell cycle at or near the G_1/S transition will be essential in determining the role of the subsequent events between G_1 and G_2 cell cycle phases and in the induction of DNA synthesis. Towards this end, mimosine has been reported to be more effective than aphidicolin at preventing entry into the S period in mammalian cells [18,19]. Mimosine is one compound of a class of inhibitors that apparently blocks cell cycle traverse by suppressing the formation of the rare amino acid, hypusine, in the eukaryotic translation factor 4D [18], while aphidicolin is an inhibitor of DNA α-polymerase. Mimosine prevented entry into the S period when delivered to cells after release

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from a G_0+G_1 block with drugs by preventing initiation at individual origins of replication. However, mimosine added to non-synchronized cell cultures did not allow reproducible arrest of Chinese hamster cells in late G_1 [21].

The natural partial synchronization of leaf mesophyll cell preparations, enhanced by mimosine delivery, was used here to examine the cell cycle phase-dependent activation of $p34^{cdc2}$. Our results show that $p34^{cdc2}$ activation occurs at G_2+M in petunia. A slight activation of $p34^{cdc2}$ after 24 h culture, when many protoplasts are entering S, should in fact be attributed to reactivation of the subpopulation of 4C-arrested mesophyll cells always present in freshly isolated protoplasts.

2. MATERIALS AND METHODS

2.1. *Protoplast culture and cell synchronization*

Mesophyll protoplasts were isolated from the 4th (numbered from the cotyledons to the apex) leaf of petunia hybrida (hybrid F1 P × PC6; Dr. Cornu, INRA Dijon). 10^5 protoplasts/ml were cultured according to previous conditions [22]. Mimosine (Aldrich Chemical Co.) was dissolved to 10 mM aqueous stock by adding 1 drop of 0.2 N HCl. 18 h after protoplast isolation, mimosine, 200 μM was added for 24 h to the protoplast culture. Mimosine-treated cells, washed by centrifugation, were either centrifuged for protein extraction or resuspended in conditioned medium obtained by protoplast culture for the same period and returned to culture. One control consisted of 42 h uninterrupted culture; another control was washed, untreated protoplasts resuspended in the conditioned medium.

2.2. *Preparation and use of $p9^{CKShs1}$–Sepharose beads*

By virtue of their specific and strong interaction with $p34^{cdc2}$, $p13^{suc1}$ or $p9^{CKShs1}$ (its human homologue) can be used, after immobilization on Sepharose beads, to isolate and purify $p34^{cdc2}$ from cellular extracts [23,24]. Both the protein recognized by the anti-PSTAIR antibody and the H1 histone kinase activity were retained from different plant species on beads conjugated with $p13^{suc1}$ [7,25,26]. $p9^{CKShs1}$ proteins were purified from an over-producing strain of *E. coli* then conjugated to CNBr–Sepharose beads according to [27]. A pellet from a nitrogen freeze of 10^7 cells in Eppendorf tubes was sonicated in 500 μl ice-cold extraction buffer consisting of 20 mM HEPES (pH 7.3), 100 mM β-glycerophosphate, 20 mM $MgCl_2$, 10 mM EGTA, 5 mM NaF, 1 mM DTT and 1 mM PMSF. The cell debris was discarded after 15 min centrifugation at 15,000 rpm at 4°C. A 20 μl aliquot of packed-p^{9CKsh1}protein–Sepharose beads was washed with 1 ml bead buffer and added to the protein extract. The tubes were kept under constant rotation at 4°C for 1 h. After a brief centrifugation at 10,000 × *g* and removal of the supernatant, the beads were carefully washed three times with bead buffer and used for histone H1 kinase.

2.3. *Histone H1 kinase assay*

Samples (30 μl) containing the initial 20 μl of packed beads were incubated 30 min at 30°C with 1 μCi [γ-^{32}P]ATP (1–3 Ci/mmol, 1–30 μM) (Amersham) in the presence of 1 mg of histone H1/ml in a final volume of 35 μl of buffer C according to [27]. Assays were terminated by transferring the tube onto ice. After brief centrifugation at 10,000 × *g*, 8 μl of Laemmli sample buffer were added to 30 μl of supernatant. Samples were electrophoresed on a 12.5% polyacrylamide gel followed by autoradiography.

2.4. *Flow cytometry*

From the cell pellet, nuclei were released in Galbraith buffer [28] 45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morpholine- propane sulfonate, 0.1% Triton X-100 [29]. After 5 min, the remaining membranes were mechanically disrupted by repeated passages of the suspension through a Pasteur pipette. If nuclei were not immediately analysed, 1% formaldehyde was added for 20 min, then nuclei were centrifuged and stored at 4°C in 500 μl of protoplast culture medium; before analysis 500 μl of Galbraith's buffer was added. The sample was filtered through nylon (pore size, 30 μm). The nuclei in the filtrate were stained with 2 μg/ml final concentration of bisbenzimide Hoechst 33342. Cytometric analysis was made of 20,000 nuclei on an EPICS V flow cytometer (Coulter) according to previous conditions [29], and histograms processed with Multicycle (Phoenix Flow Systems, San Diego).

3. RESULTS

3.1. *Nuclear DNA content in protoplast cultures*

To check the proliferation of protoplasts isolated from mesophyll cells, the cell cycle status of nuclei (2C, S, 4C) from protoplast populations obtained from the 4th leaf was determined during 72 h by flow cytometry (Table I). Culture for 18 h was required to observe an increase in the frequency of S phase nuclei, and for 28 h to observe an increase of the 4C nuclei. At 48 h a peak frequency of 47% 4C cells was reached; at 72 h the proportion of 4C decreased as nuclei returned through mitosis to 2C.

3.2. *$p34^{cdc2}$ histone kinase activity during induction of division*

To further understand the induction of division from isolated protoplasts, we analysed histone H1 kinase/MPF activity which triggers the entry into M phase. Cell extracts prepared from the 10^7 protoplasts immediately after their isolation from the leaf (0 h), then after 24 h and 48 h of culture, were absorbed onto p9-Sepharose beads. $p34^{cdc2}$ kinase activity was measured. Although freshly isolated protoplasts included 11% 4C nuclei, no histone H1 phosphorylation occurred. A very slight increase of $p34^{cdc2}$ activity was obvious after 24 h with cells transitting G_1-to-S but before any increase of 4C nuclei (Fig. 1b). After 48 h of culture, which corresponded to 47% 4C nuclei, there was a strong burst of histone H1 phosphorylation capacity (Fig. 1c).

3.3. *Mimosine-treated cells do not reach G_2 phase*

No increase in the 4C population was observed in

Table I

Cell cycle phase distribution of nuclei from mesophyll petunia protoplasts cultured from 0 h to 72 h

Culture	Cycle phase		
(h)	G_0–G_1	S	G_2
0	88	1	11
18	83	5	12
24	64	26	10
28	55	33	12
42	52	11	37
48	41	12	47
72	45	25	30

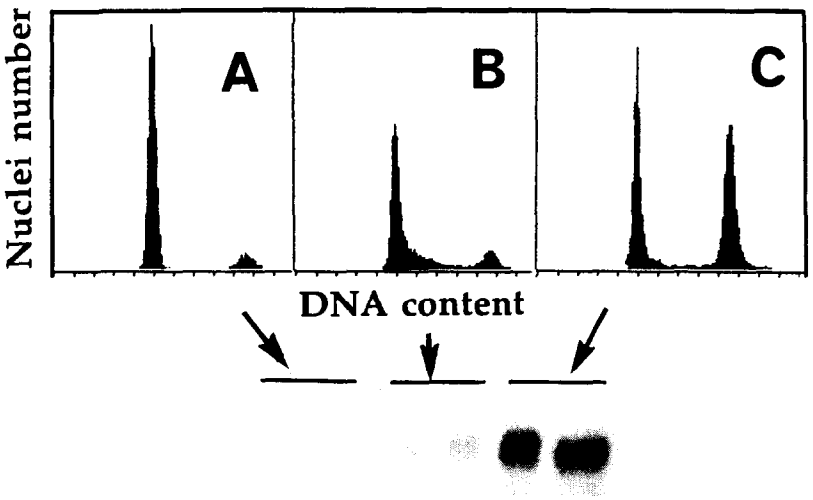

Fig. 1. Time–course of $p34^{cdc2}$ histone H1 kinase activity. Cell cycle analysis was done on 20,000 nuclei from freshly isolated petunia mesophyll protoplasts (A), cells derived from protoplasts after 24 h (B) and 48 h (C). For each sample the histone H1 phosphorytion was assessed from the protein fraction bound to $p9^{CKShs1}$–Sepharose. Two repetitions are shown.

standard culture after 24 h. However, such data could derive from a low number of cells entering G_2 phase, while a similar number exit through mitosis, but microscopy did not reveal such mitosis. The 24 h cultures are characterized by G_1-to-S transition and an S traverse, a phenomenon first observed at 18 h. Therefore, 18 h cultures were treated with mimosine for 24 h (to 42 h total) to block this progression before 4C (Table I). Compared to the control 42 h culture (Fig. 2) mimosine-treated cells did not reach G_2 (Fig. 2b, upper): the 4C frequency remained at the initial 18 h level although in S phase it increased six-fold.

3.4. *p34cdc2 kinase is activated in G_2+M phase*

At 42 h $p34^{cdc2}$ activity was obviously highest in the untreated protoplast culture rich in G_2 (Fig. 2a). A weak kinase activity was still observed in blocked culture. Mimosine was then depleted from the medium and cells were cultured for 24 h more in conditioned medium (Fig. 2c). This culture then reached 61% G_2 nuclei, showing that mimosine was non-toxic for plant cells. The highest level of $p34^{cdc2}$ kinase activity was then observed in this G_2-enriched culture.

4. DISCUSSION

Petunia cells derived from protoplasts have the advantage of a partial natural synchronization, as has previously been used to obtain high mitotic indexes [30] and thence chromosome suspensions. A second advantage is that until 72 h the cell wall can easily be disrupted without enzymatic digestion to obtain isolated nuclei without any delay.

Protoplast populations from mesophyll have predominantly 2C DNA content: 5–20% of the cells have 4C DNA content, depending upon the leaf rank used [15]. This percentage also changes according to the species [28]. From the low number of S phase cells (1%) and from Acridine orange quantification of nuclear DNA and total RNA, it is evident that cells isolated from mesophyll are not cycling [17]. Cytometry with dyes specific to DNA has not clarified the distribution between quiescence and proliferation, at both 2C and 4C.

As one means to overcome this, we assessed nuclear levels of cdc2 mRNA or $p34^{cdc2}$ protein to study the passage from differentiated mesophyll cells to cycling cells [10]. Levels of cdc2 mRNA and $p34^{cdc2}$ are similar in 2C nuclei whether these come from leaf or from regenerating protoplasts. This is evidence that differentiated petunia leaf cells are essentially in a G_1 state rather than at G_0.

On the other hand, cdc2Pet mRNA increased in G_2 nuclei during culture [10], suggesting that this petunia cdc2 gene is transcriptionally regulated from G_1 to G_2. Moreover, the increase in total cdc2Pet mRNA in cycling cells derived from protoplasts may be due to G_2 cells. This indicates that the use of protoplast regeneration models to examine biochemical changes associated with cycle phase shifts must take into account the possible contribution of any initial 4C subpopulation, also capable of proliferation but out-of phase with the G_1 majority.

Most of the work on cdc2 and cdc28 regulation has focussed on post-translation modification of $p34^{cdc2}$, especially the key tyrosine dephosphorylation occurring on $p34^{cdc2}$ as cells undergo the G_2/M transition of the cell cycle [31] with DNA synthesis completed [32,33]. As $p34^{cdc2}$ becomes tyrosine dephosphorylated, the M phase-specific H1 kinase is activated, the nuclear envelope breaks down and cells enter the M phase [34]. $p34^{cdc2}$ activity thus appears as a G_2/M-specific event of active cycling cells. However, the profusion of mammalian cyclins has been accompanied by increasing evidence that cdc2 is not the sole cyclin-activated kinase

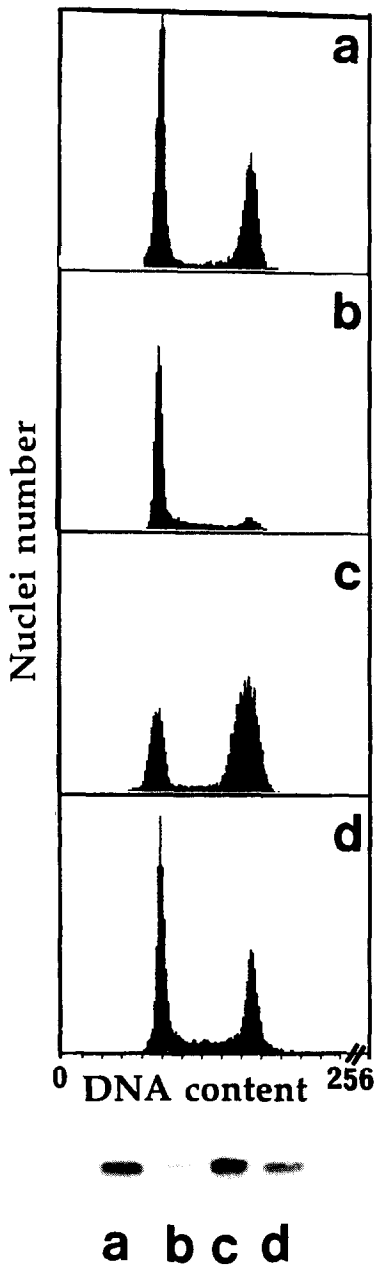

Fig. 2. Mimosine arrests G_1 and S progression and $p34^{cdc2}$ activity related to G_2+M phase. Upper panel: cell cycle analysis of untreated control cells cultivated for 42 h (a); 42 h culture, treated 24 h with 200 μM mimosine (b); 66 h culture, treated 24 h with mimosine and washed for a 24 h release (c); untreated 66 h control cells (d). Lower panel: from each sample $p34^{cdc2}$ activity was monitored by histone H1 phosphorylation from the protein fraction bound to $p9^{CKShs1}$–Sepharose beads; a–d, as above.

involved in mammalian cell cycle control. Cdk2 was found to be activated during S and G_2 phases [35]. Concerning plants, in the highly synchronized meiotic cycle of *Lilium*, the $p34^{cdc2}$ activity from bud protein extracts trapped on $p13^{suc1}$–Sepharose beads was shown to peak in the prophase 1 [26]. However, in alfalfa cell suspensions blocked by hydroxyurea, the $p34^{cdc2}$ kinase activity was shown to be highest in S rather than in G_2+M phases [36].

$p9^{CKShs1}$ was used after immobilisation on Sepharose beads to isolate and purify active $p34^{cdc2}$. This $p34^{cdc2}$ activity was compared with the different steps of induction of division: immediately after protoplasts isolation (0 h), when S phase was engaged (24 h) and when G_2 was maximum (48 h). Despite the 11% 4C nuclei present in freshly isolated protoplasts, no $p34^{cdc2}$ activity was detected immediately after isolation. It peaked after 48 h, when a majority of cells were at 4C and the first mitotic figures were observed by light microscopy. Thus, clearly, $p34^{cdc2}$ kinase is inactive in differentiated mesophyll leaf cells, and is only active in petunia cells induced to divide. After 24 h of protoplast culture, without any 4C increase being observed, kinase activity became weak but detectable in each different experiment. Either (i) there was $p34^{cdc2}$ kinase activity in G_1 or S phases, or (ii) the 11% 4C cells present in the leaf when protoplasts were isolated became 11% G_2.

Mimosine was shown to prevent S phase progression when delivered after G_0+G_1 block to Chinese hamster cells [21]. Mimosine inhibits only the new individual origins of replication. Thus, in a cell population blocked in early S, replication of DNA occurred from the engaged replication forks but new replication origins should be blocked and thus a majority of cells should be in G_1 and S. For the first time in plants, it is shown here that mimosine delivered to cells derived from protoplasts after 18 h, when early S phase cells were appearing, can block these cells in G_1 and S phases. This was not a toxic effect: 24 h after mimosine depletion 60% of cells reached G_2. On the basis of nuclear content quantification with bisbenzimide Hoechst, only 3% of cells were engaged in S phase after 18 h. With mimosine then added for 24 h to these cells; 42 h from cell culture initiation the S cell frequency reached 33%. It can be assumed that during the first 18 h, DNA replication forks were engaged in 33% of cells and consequently S phase was reached for this 33% of cells, although an increase in DNA could only be detected in 3% with fluorescent dye. This amino acid analogue may be useful for determining the rate of creation of replication origins in plant nuclei. To obtain only active G_1 cells and no initiation of DNA synthesis, mimosine would have to be delivered to protoplast cultures after 15 h (result not shown).

In mimosine-treated cells, although blocked at G_1+S with 33% S, only slight $p34^{cdc2}$ activity was observed. Thus $p34^{cdc2}$ kinase activity in petunia cells was not related to S phase. Conversely, after mimosine release, with 60% G_2+early M cells, the highest $p34^{cdc2}$ activity was observed, confirming that in petunia cells the kinase activity corresponds to G_2/early M phase. The minor kinase activity observed prior to any increase in frequency of 4C cells could be attributed to an M form of

$p34^{cdc2}$ activation in 4C cells present as a 10% subpopulation in mesophyll.

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