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Olomoucine, an inhibitor of the cdc2/cdk2 kinases activity, blocks plant cells at the G1 to S and G2 to M cell cycle transitions

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Abstract The cdc2/cdk2 protein kinases play key roles in the cell cycle at two control points: the G1/S transition and the entry into mitosis. Olomoucine, a specific inhibitor of these kinases, was tested in two plant cell systems: *Petunia* mesophyll protoplasts induced to divide and *Arabidopsis thaliana* cell suspension cultures. The cell cycle status was analysed from DNA histograms or through continuous labelling of cells with 5-bromodeoxyuridine (BrdUrd) followed by double staining with bis-benzimide (Hoechst 33258) and propidium iodide (PI). Such analyses resolve cells from several generations according to the extent of their DNA replication. Olomoucine was shown to reversibly arrest differentiated *Petunia* cells induced to divide at G1 phase and cycling *Arabidopsis* cells in late G1 and G2. A comparison of the effects of aphidicolin, oryzalin and olomoucine suggests that in the *Arabidopsis* cell suspension culture, a cdc2/cdk2-like kinase is activated at a restriction point in late G1.

Key words: Olomoucine; Aphidicolin; Oryzalin; Cell cycle; cdc2; cdk2; *Petunia*; *Arabidopsis*

1. Introduction

The $p34^{cdc2}$ protein in yeast *Schizosaccharomyces pombe* and the homologous $p34^{CDC28}$ protein in *Saccharomyces cerevisiae* are necessary for entry into S phase and mitosis [1,2]. cdc2-homologous genes have been found in all plant species analysed [3–9]. In *Arabidopsis* [10], alfalfa [11], *Anthirrinum majus* [12] and soybean [9], distinct cdc2-homologues have been found, but their respective functions remain to be determined. Activation of the CDC2 protein kinase requires its association with a member of the cyclin protein family at the two cell cycle transition points START and G2/M [13]. In plants, $p34^{cdc2}$ kinase activity has been detected at G2/M in *Petunia* [14] and during S and G2 in alfalfa [15].

In this work we have used olomoucine, recently shown in vivo and in vitro to be a specific inhibitor of cell cycle regulating cdc2/cdk2 kinases, although it did not inhibit the cdc2-activating phosphatase cdc25. Olomoucine acts as a competitive inhibitor for ATP binding [16] and preliminary analysis of cdk2/olomoucine crystal shows that the purine group of olomoucine is located in the pocket where ATP binds to cdk2 (Schulze-Gahmen, U., Jones, H.D., Meijer, L., Vesely, J., Morgan, D.O. and Kim, S.H., unpublished results). The requirement of cdc2-related kinases during the cell cycle was studied in two different systems: differentiated mesophyll *Petunia* cells induced to divide and an *Arabidopsis* cell suspension culture. Exposure of relatively uniform populations of cells to a drug simplifies the interpretation of cell cycle distributions. Mesophyll *Petunia* protoplasts are arrested (up to 90%) at a 2C DNA state; when growth factors are added to the medium they progress to G2 through S [14,17]. This natural synchrony was exploited in this study to investigate cell cycle progression following exposure to olomoucine in order to determine whether a cdc2-like kinase is involved in the G1/S transition.

The same kinase plays another key role for entry into mitosis [18]. Asynchronously growing *Arabidopsis* cultures provide a multiplicity of targets for drug action. The distribution of an asynchronous population following drug treatment is likely to depend on the fraction of cells existing in each cell cycle phase upon drug addition, the duration of each phase relative to the duration of drug exposure and the target of the drug. Incorporation of the thymidine analog 5-bromodeoxyuridine (BrdUrd) in cells quenches the fluorescence of Hoechst 33258: a DNA stain with A–T base specificity. DNA synthesizing cells will therefore not show the expected increase in fluorescence. Upon division, daughter cells will display a reduced fluorescence intensity, enabling one to follow drug effects from one cycle to the next [19]. This powerful multiparametric approach was applied to an asynchronous *Arabidopsis thaliana* cell suspension culture. Our results show that *Petunia* and *Arabidopsis* cells are blocked, in a reversible manner, both in G1 and G2 by olomoucine. This suggests that in plants, as in yeast, cdc2 and cdk2 related kinases are involved in both G1/S and G2/M transitions.

2. Materials and methods

2.1. Protoplast and cell culture

Mesophyll protoplasts were isolated from 4th to 6th leaves (numbered from the cotyledons to the apex) of *Petunia hybrida* (hybrid F1 PxPC6, Dr. Cornu, INRA, Dijon). 10^5 protoplasts/ml were cultured in the light at 26°C as previously described [20].

Olomoucine was dissolved to 10 mM in dry DMSO, stored at −20°C. It was administered for 24 h to *Petunia* protoplast cultures 14 h after protoplast isolation. Olomoucine-treated cells, washed once by centrifugation, were either used for cell cycle analysis or resuspended in ½ volume of conditioned medium obtained from protoplast cultures of the same age and returned to culture. Controls consisted of freshly isolated protoplasts (0 h) and 36 h or 48 h uninterrupted cultures.

An *Arabidopsis thaliana* cell suspension culture (supplied by M. Axelos, Toulouse) was subcultured every 7 days in Gamborg B5 media (Sigma) supplemented with 0.2 mg/l α-naphthaleneacetic acid. The pellet from 10 ml of a 2-day-old refreshed cell suspension was digested in 5 ml of 2% cellulase RS (Onozuka), 0.1% pectolyase (Sigma) and 0.66 M sorbitol during 1 h at 35°C. The fresh *Arabidopsis thaliana* proto-

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plasts were washed twice under the same conditions as *Petunia* protoplasts.

A 30 mM aqueous bromodeoxyuridine (BrdUrd, Sigma) stock was added to 30 μM final concentration, to a refreshed two-day-old *Arabidopsis* cell suspension culture. Aphidicolin (Sigma) dissolved in dry DMSO was added to a final concentration of 30 μM. The spindle toxin [21] oryzalin (3,5-dinitro-*N*,*N*-dipropylsulfanilamide, a gift from Dow Elanco, Belgium) was dissolved in dry DMSO and added to a final concentration of 3 μM.

2.2. Flow cytometry

Nuclei were released from the protoplast pellet in Galbraith buffer [22] (45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate supplemented with 1% (w/v) Triton X-100, pH 7). After 1 min, the remaining membranes were mechanically disrupted by repeated passage of the suspension through a Pasteur pipette. Finally, 1% formaldehyde (37%) was added and nuclei were stored at 4°C. The samples were filtered through nylon (pore size, 30 μm). In a simple analysis without BrdUrd treatment, the *Petunia* nuclei in the filtrate were stained directly with 2 μg/ml (final concentration) of bisbenzimide Hoechst 33342. Cytometric analysis was performed on 2×10^4 nuclei with an EPICS V flow cytometer (Coulter) according to conditions described in [14]. Histograms were processed with Multicycle (Phoenix Flow Systems, San Diego). In the case of BrdUrd experiments, nuclei were treated for 10 min with 15 μg RNase A/ml. Then these nuclei were stained for 15 min with 1 μg Hoechst (HO) 33258/ml. Finally, 3 μg propidium iodide (PI)/ml was added for a further 15 min. In the biparametric analysis according to Kubbies et al. [23], the nuclei were excited with ultra-violet (351–364 nm) light and a bivariate cytogram of red (PI > 610 nm) vs. blue (408 nm < HO < 500 nm) fluorescence was recorded. Care was taken to eliminate both debris and doublets though light scatter and pulse shape analysis.

2.3. Cytology

Cells were fixed in methanol at −20°C for 24 h before they were stained with 1 μg Hoechst 33342/ml in Galbraith's buffer supplemented with 1% Triton X-100.

3. Results

3.1. Increasing olomoucine concentrations block Petunia *protoplast cultures in G1*

Protoplasts isolated from *Petunia* mesophyll, differentiated mostly at 2C DNA content, were used to test for a G1 block by olomoucine (Fig. 1). In order to start with only active G1 cells with no initiation of DNA synthesis, we delivered olomoucine 14 h after protoplast isolation, as in successful studies with mimosine [14]. However during these 14 h a size increase was observed in these cells derived from protoplasts (Fig. 1). After 38 h the cell size had increased even more and 49% of the cells had reached G2 but few divisions could be observed; at 52 h almost all cells had undergone one division and some cells had divided twice. The effect of various concentrations of olomoucine upon the cell cycle status of nuclei (2C, S, 4C) from *Petunia* protoplast populations was determined by flow cytometry (Table 1). 10 μM olomoucine was sufficient to observe a decrease in S and G2; 50 μM to 100 μM olomoucine stopped cell cycle progression. The size of the G1 blocked cells (100 μM olomoucine) increased during the 24 h block compared to the 14 h state, but less so than untreated controls cultivated for the same period (38 h, 49% G2 cells). When 3 μM oryzalin was administered after 38 h during a 14 h period, the frequency of G2 cells increased to 66% as expected. However, no obvious metaphase block was observed during this period and after 52 h, 10% of the nuclei showed a DNA content greater than 4C (Fig. 1 and Table 2).

When olomoucine-treated cells (100 μM for 24 h) were washed and cultivated in olomoucine-free medium for 10 h (Table 2), 59% of the cells reached the S and G2 phases. After a 24 h wash in olomoucine-free medium, as compared to the 10 h wash, the frequency of G2 nuclei decreased in a similar way to the control protoplasts cultured for 48 h. This decrease corresponded to cells going through mitosis to G1′ (second generation). This indicates that, after olomoucine treatment, *Petunia* cells derived from protoplasts were able to resume normal cycling.

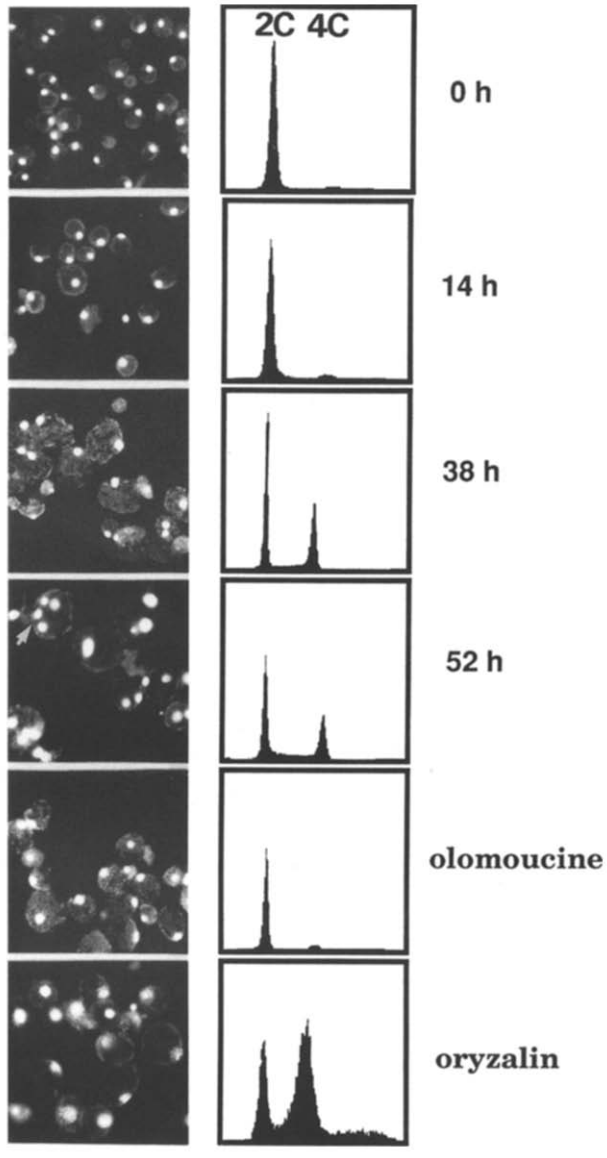

Fig. 1. Olomoucine and oryzalin cause *Petunia* mesophyll protoplast-derived cells to cease proliferation with an increased cell size and a single nucleus. Olomoucine (100 μM) was added at 14 h for 24 h, oryzalin (3 μM) was added at 38 h for 14 h. Left series: micrographs of Hoechst stained cells. Right series: histograms of nuclear DNA content, total count 20,000 nuclei.

3.2. Olomoucine block asynchronous Arabidopsis *cells in G1 and G2*

To test for a G2 block, cycling *Arabidopsis* cells were treated with olomoucine. In conventional univariate DNA-Hoechst

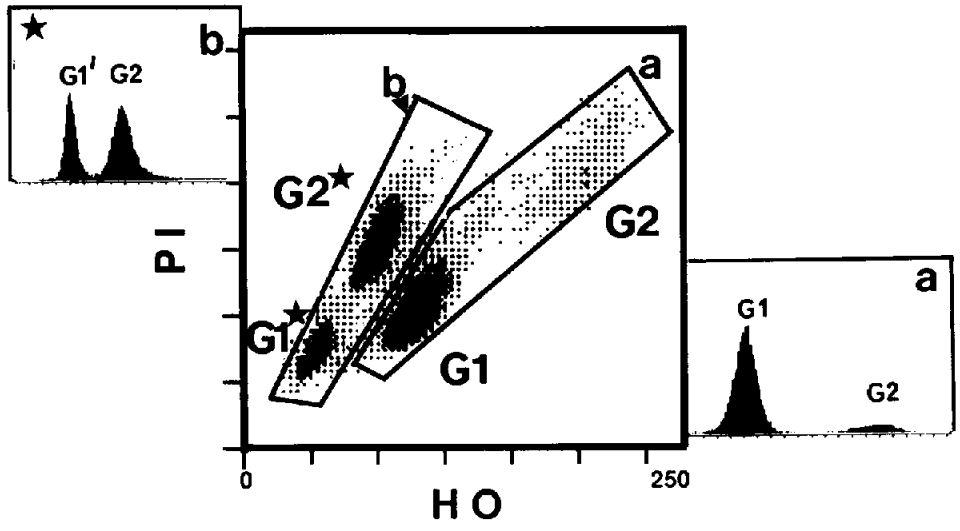

Fig. 2. BrdUrd/Hoechst-PI differential cell analysis from an asynchronous *Arabidopsis thaliana* cell suspension culture. BrdUrd was added at a concentration of 30 μM for 20 h prior to nuclear isolation and cytometric analysis. *x*-axis, HO fluorescence; *y*-axis, PI fluorescence; grey levels indicate relative frequencies of nuclei at each coordinate. BrdUrd incorporation quenches Hoechst fluorescence and allows us to distinguish the second generation of cells. Bitmap (or zone) a, G1–S–G2: initial cell cycle; Bitmap b, S*, G2*, G1*: BrdUrd labelled nuclei originating from previous G1 and S phases (S and S* comprise respectively between G1–G2 and G1*–G2*). G1′, second generation cells.

flow cytometry, the G1′ products of mitosis are undistinguishable from those of the original G1. The BrdUrd-HO-PI technique has however proven to be an efficient tool for studying the time course of cell division following mitogenic stimulation [19]. As previously stated, after BrdUrd incorporation, the HO fluorescence is no longer proportional to cellular DNA content. Therefore single parameter staining with HO does not yield information about the cell's location within the cell cycle. This additional information can be obtained by staining the cells simultaneously with a DNA-specific dye that is not sensitive to the BrdUrd-induced quenching, such as propidium iodide (PI). A bivariate analysis based on dual staining with HO and PI (fig. 2) can provide complete information about the cell cycle progression within the time interval between the beginning of BrdUrd incorporation and cell harvest. After 20 h of incorporation the distribution of the BrdUrd-labelled cells (those containing BrdUrd, in zone *, noted as G1*, S*, G2*) was thus easily distinguished and could be analysed separately from the first G0 generation (Fig. 2). Within BrdUrd labelled cells only G1* correspond to the second generation (G1′). If olomoucine inhibits cdc2/cdk2 kinase activity, it should block cells both in the G1 and G2 stages; only cells engaged in S should progress to G2 but without reaching G1′. *Arabidopsis* cell suspension culture was treated with either BrdUrd alone, BrdUrd plus olomoucine, BrdUrd plus aphidicolin or BrdUrd plus oryzalin. Olomoucine and aphidicolin blocked samples (without BrdUrd) were also washed in medium without inhibitors before being exposed to BrdUrd for 5 h (Table 3).

Table 1
Concentration dependence of the olomoucine induced G1 block

Olomoucine concentration (μM)	Frequency (%)		
	G0–G1	S	G2
0	54	10	36
1	49	14	37
5	56	8	36
10	63	7	30
50	91	3	6
100	92	3	5
200	97	1	2

Cell cycle distribution of nuclei from *Petunia* mesophyll protoplasts cultured for 14 h and then treated for 24 h with various concentrations of olomoucine.

3.2.1. BrdUrd alone. When cells were exposed to BrdUrd for 20 h without any blocking agent, 68% of the cells incorporated BrdUrd and 20% of the cells engaged in a new cycle, thus reaching G1′. Only a minority of the nuclei in the S and G2 positions of the first generation did not incorporate BrdUrd; after 20 h, part of the 2C nuclei (representing 28% of the total nuclei) was still unlabelled.

3.2.2. BrdUrd plus aphidicolin. When aphidicolin was added no BrdUrd incorporation was observed; however, the proportion of S and G2 cells decreased, 86% of the cells showed 2C DNA content.

3.2.3. BrdUrd puls oryzalin. From the oryzalin/BrdUrd treated cells, it is evident that when cells could not proceed through mitosis, only S or G2 cells did incorporate BrdUrd; no G1′ cells were present. Moreover, without any G1 block (as was the case with this treatment), 53% of the cells incorporated BrdUrd, indicating the percentage of G1 and S cells which were truly active during the treatment. In the control, in which 69% of the cells were in the G1+S phases, only 53% of the cells did incorporate BrdUdr in 20 h (oryzalin treated sample). Therefore about 16% of the cell suspension at the 2C position were non-active.

3.2.4. BrdUrd plus olomoucine. From the sample supplemented with olomoucine and BrdUrd, only S* and G2* cells were observed: no G1′ cells could be detected. In addition, the BrdUrd labelled fraction was smaller than in the oryzalin treated sample.

When cell suspensions were supplemented for 20 h with either aphidicolin or olomoucine, then washed and subsequently

Table 2
Reversibility of the olomoucine block

Treatment	Inhibitor (μM)	Frequency[a] (%)		
		G0–G1	S	G2
14 h + 24 h olomoucine	100	94	3	3
14 h + 24 h olomoucine + 10 h wash	100	41	19	40
14 h + 24 h olomoucine + 24 h wash	100	59	10	32
38 h + 14 h oryzalin	3	25	9	66[b]
14 + 24 = 38 h culture	0	44	7	49
14 + 34 = 48 h culture	0	49	20	31

In the time scale, 0 h corresponds to *Petunia* protoplast culture initiation. Olomoucine was added after 14 h over a period of 24 h. To release olomoucine, cells were washed in a olomoucine-free medium and then cultured during either 10 or 24 h. After each treatment, cell cycle phase distribution was determined from flow cytometric analysis performed on isolated nuclei as described in section 2.
[a] 100% = diploid cycle.
[b] 56% 4C nuclei and 10% 6C.

cultivated in medium supplemented with BrdUrd for five hours. BrdUrd labelled cells were observed in both samples, the percentage of labelled cells being higher with the aphidicolin treated cells.

4. Discussion

It is difficult to determine precisely the relative timing of the various events which participate in cell cycle induction in differentiated plant cells. As most molecular events cannot be studied in a single assay, this problem becomes particularly acute when attempts are made to correlate events occurring on a cellular scale (e.g. the start of S phase) with those that occur on a molecular scale (e.g. activation). However, the relationship between cellular and molecular events can be studied by using cell synchronisation to produce large numbers of cells that occupy a narrow cell cycle window.

Table 3
G1 and G2 cell cycle phase arrests of asynchronous *Arabidopsis* cell suspensions treated with olomoucine from a bivariate BrdUrd/Hoechst-PI flow cytometric analysis: comparison with aphidicolin and oryzalin blocks

Treatment		G1 G1*	S S*	G2 G2*	BrdUdr* class (%)	
		Frequencies (%)			–	+
Control (no BrdUdr)		62	7	31	100	
	*	nd	nd	nd		0
(BrdUrd) 20 h		27.6	1.5	3.2	32	
	*	20	32	14		68
(olomoucine + BrdUrd) 20 h		58	3	8	70	
	*	nd	12	18		30
(Aphidicolin + BrdUrd) 20 h		86	5	9	100	
	*	nd	nd	nd		0
(oryzalin + BrdUrd) 20 h		17.5	7.8	21.6	47	
	*	nd	29.7	23.4		53
(olomoucine 20 h then wash) + (BrdUrd 5 h)		51	4	34	89	
	*	nd	11	nd		11
(aphidicolin 20 h then wash) + (BrdUrd 5 h)		41.6	3.8	12.6	58	
	*	nd	42	nd		42

Two cases of BrdUrd incorporation were performed. In one case, BrdUrd was added at the concentration of 30 μM for 20 h at $t = 0$ h without or with either olomoucine, aphidicolin or oryzalin. In the other case, BrdUrd was added at the same concentration for 5 h after the release from olomoucine or aphidicolin 20 h treatment. Cell cycle phase distribution of BrdUrd negative (G1, S, G2) or BrdUdr positive (G1*, S*, G2*) nuclei was determined though flow cytometric analysis as described in Fig. 2. *correspond to BrdUrd labelled nuclei; nd = not detected.

Using *Xenopus* egg extracts we have shown [16] that olomoucine inhibits both M-phase Promoting Factor and DNA synthesis in vitro. In starfish oocytes, olomoucine blocks in vivo the 1-methyladenine-induced prophase to metaphase transition. In this case, olomoucine did not inhibit the activation of the kinase $p34^{cdc2}$ by the cdc2-activating phosphatase cdc25 but blocked the kinase activity of activated $p34^{cdc2}$ [16]. In collaboration with a number of colleagues we have observed the cellular effects of olomoucine on a large variety of models, ranging from Fucus embryos to mammalian cells in culture (manuscript in preparation). After combining the results, it may be shown that olomoucine arrests cells both at the G1/S and G2/M boundaries, consistent with the hypothesis that this agent acts on cdk2 and cdc2, respectively.

We reported earlier that $p34^{cdc2}$ kinase activity was only detected at G2/M in *Petunia* [14]. Yet the olomoucine-induced G1 block of differentiated *Petunia* cells, which had been induced to divide, suggests the involvement of a cdc2-related kinase in G1 phase. This is the first reported strong indication that a cdk2-like kinase might participate in the G1 plant cell cycle phase. In order to demonstrate the ability of olomoucine to inhibit cdk activity in plants, this hypothesis should be tested by an in vitro H1 kinase assay in the presence/absence of olomoucine. However, in our earlier work, the G1 activation of a cdc2-related kinase was too weak to be detected by histone H1 phosphorylation compared to the G2 activation [14]. Thus this in vitro assay would only be possible at the G2/M transition. Nevertheless, the structural data of cdk2/olomoucine crystal (see introduction) and the high degree of conservation of DNA sequence at the ATP binding site, among the cdc2-related kinase family in eukaryotes [5], strongly suggests that olomoucine could have a homologous behaviour toward cdc2-related kinase in plants. 10 h after eliminating olomoucine from the medium, the cells progressed through S and to G2, indicating that the olomoucine G1 induced block is easily reversed.

Growth factor stimulation of differentiated cells is a general method to obtain active G1 cells and partial synchronisation: in the case of plants, stimulation of differentiated cells such as

mesophyll protoplasts is a useful approach [14]. However, growth regulator-stimulated *Petunia* cells enter S phase over the course of 18 h to 28 h, which makes it difficult to establish relationships between particular molecular events and cell cycle progression. The pathways connecting either G1 to S phase or a differentiated stage to S phase are likely to show significant differences. After 38 h in culture (without inhibitor). *Petunia* cells derived from protoplasts show 56% (G2+S) cells, 44% in G1. In the case of the oryzalin treatment, where G2 cells cannot divide to G1′, only 25% of the cells remain in G1; while 19% of the G1 cells from the 38 h protoplast culture progress to G2 phase during the oryzalin treatment (14 h). Thus a fraction of the G1 cells are likely to be very late G1 or early S and therefore not detectable from DNA quantification analysis. Getting a maximum of cells at the G1/S transition using olomoucine appears to be an effective way to study this transition.

As shown in the BrdUrd control, the period of BrdUrd treatment is sufficiently long to allow the G2* labelled *Arabidopsis* cells to go through mitosis and become G1′ cells. However, when olomoucine was applied to cycling cells, only S* and G2* cells (BrdUrd labelled) were detected; no G1′ cells were obtained. This suggests that olomoucine blocks *Arabidopsis* cells in G2.

The percentage of G2 non-BrdUrd labelled cells was, however, lower in the olomoucine-BrdUrd sample than in oryzalin-BrdUrd, which could indicate (i) that the G2 block was delayed with olomoucine as compared to oryzalin, or (ii) that the olomoucine inducer G2 block concerns a better defined cell cycle stage than does the spindle toxin oryzalin.

Moreover, the increased proportion of G1 cells obtained with the olomoucine-BrdUrd treatment as compared to that obtained with the oryzalin-BrdUrd treatment shows that, as with differentiated *Petunia* cells, *Arabidopsis* cells cannot enter the S phase in the presence of olomoucine. Thus, if *Arabidopsis* cells are G1 blocked by olomoucine, it is likely that a cdc2-related kinase is activated before S phase initiation and therefore somewhat earlier than has been observed with alfalfa cells [15].

In contrast with the observed behaviour of aphidicolin-BrdUrd treated cells, BrdUrd uptake is evident in the olomoucine-BrdUrd sample in the form of S* and G2* cells, which indicates that olomoucine does not block cells which are already in S phase. In this sample, 30% of the cells did incorporate BrdUrd. These cells could correspond to late G1 cells which overcame a restriction-like point plus cells which were in S phase. The possibility that BrdUrd was incorporated before the olomoucine block became effective, however cannot be excluded. Nonetheless, the percentage (30%) of cells which incorporate BrdUrd is higher than the estimated percentage of cells in S phase in a cell suspension. This suggests that in a cell suspension, numerous cells are in a state between late G1 and S engagement or in a very early S stage not detectable from DNA quantification. From analysis of these two in vitro experimental plant systems, it appears that olomoucine efficiently arrests cells at a late G1 restriction point. Olomoucine provides a useful experimental tool to distinguish between the functions of the two CDC2 genes which have been obtained from several plant species [9–11] at this crucial point in the cell cycle.

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