A low content in zeatin type cytokinins is not restrictive for the occurrence of G_1/S transition in tobacco BY-2 cells

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Abstract Theories on the importance of cytokinins in G_1/S transition control are manifold and contradictory. By establishing a double Aφ-PZ block, maximal synchronization of a BY-2 suspension culture was obtained to investigate the effect of cytokinin depletion on G_1/S transition. Lovastatin was used as a specific inhibitor of cytokinin biosynthesis. Flow cytometry showed that the G_1/S transition occurred regardless of the cytokinin drop. This observation indicates an extremely low dose requiry for that stage of the cell cycle. It is very likely that precisely the downregulation of zeatin type cytokinins matters for the G_1/S transition to occur, since cytokinin addition at early G_1 blocked the cycle at G_1/S.

Key words: Cell cycle; G_1/S transition; Lovastatin; Endogenous cytokinin

1. Introduction

Discovering the role of cytokinins in cell division control is of vital importance for both fundamental and applied biology. Skoog et al. [1] concluded that cytokinins are required at three stages of the cycle: G_1/S, G_2/M and Cy (cytoplasmic division). More recent work also indicates that S, G_2 and M are possible points of control [2–4].

Because of their pleiotropic effects, it is likely that if cytokinins are indeed indispensable at these different stages, their mode of action might be totally different for each situation. Several studies demonstrated that withdrawal of cytokinins in cell suspension cultures results in the arrest of cell cycle and the partially synchronous recovery upon exogenous addition of cytokinins [5–8]. On the other hand, application of cytokinins to whole plants often does not promote but rather inhibits its growth [9]. This might be due to the fact that plants normally contain optimally balanced cytokinin levels and extra supply is unnecessary or even detrimental.

Although older results seem to indicate that cytokinin addition does not influence total protein synthesis [10–12], experiments on *Sinapis* showed that addition of cytokinins at the start of the S-phase activated all replication origins synchronously [13,14]. Moreover, BA succeeded in shortening S-phase markedly. Cytokinins are thus supposed to increase the availability of one key protein component common to all replication complexes. In this perspective, the induction of CycD3 by cytokinins just before S-phase entry has been illustrated in *Arabidopsis* [15]. This induction, which probably involves regulatory phosphorylations according to Riou-Khamlichi et al., is independent of progression through G_1 and involves signal transduction by proteins already present in stationary phase cells.

Since both G_1/S and G_2/M transitions are likely controlled by a series of coupled protein phosphorylations initiated by cyclin dependent kinases (CDKs), one may speculate that cytokinins exert their control via these proteins in some way. The fact that addition of BA increased RNA levels of G_1, S and G_2 nuclei and particularly influenced mRNA for CDKs [16,17] supports that assumption. According to Zhang et al. [18], it is during late G_2 exclusively that cytokinins act as activators of the $p34^{cdc2}$-like histone kinase through the dephosphorylation of the enzyme.

The rapid and cytokinin-autonomous cell cycle of synchronized tobacco BY-2 cell suspension cultures [19] makes them ideal tools to study cytokinin control mechanisms on cell cycle progression. This cytokinin-autonomous character of BY-2 cells is linked to their capacity to synthesize the necessary cytokinin amounts at the precise moment cell cycle events require them [20,21].

Our previous work showed that it is at G_2/M in particular that zeatin is indispensable for the normal course of cell division [22]. This conclusion was linked to the observation that in suspension cultures of BY-2 cells, two transient high peaks in endogenous zeatin type cytokinins occur: around G_1/S and at G_2/M [23,24].

The control of G_1/S by cytokinins is undoubtedly an important issue, since related theories are manifold. First, there is the observation that soybean cells in suspension deprived of cytokinins were apparently able to undergo several rounds of DNA replication without cell division [3]. Endomitosis is a clear indication that one single step of the cycle requires cy-

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Abbreviations: Aφ, aphidicolin; BA, benzyl adenine; DAPI, 4-6′-diamidino-2-phenylindole; 2,4-D, 2,4-dichlorophenoxyacetic acid; DZ, dihydrozeatin; DZR, dihydrozeatin riboside; DZRP, dihydrozeatin riboside phosphate; ES, electrospray; gfw, grams freshweight; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; iP, isopentenyladenine; iPA, isopentenyladenosine; iP9G, isopentenyladenine-9-glucoside; L, lovastatin; LC, liquid chromatography; MA, mevalonic acid; MI, mitotic index; MS, mass spectrometry; PI, propidium iodide; PZ, propyzamide; Z, zeatin; Z9G, zeatin-9-glucoside; ZRP, zeatin riboside-5′-monophosphate; ZNG, zeatin-*N*-glucoside; ZOG, zeatin-*O*-glucoside; ZR, zeatin riboside; ZROG, zeatin riboside-*O*-glucoside

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tokinins stringently and that this step occurs after S-phase, possibly at G_2/M. This interpretation has been challenged by more recent findings showing that several stages of the cell cycle require cytokinins [6,10]. Until now, such discrepancies remain unsolved, but it might be a matter of differences in origin, culture conditions or synchronization methods of the cells.

Keeping this in mind, we chose to perform a double block with Aφ and PZ [25,26] to obtain maximum synchronization rates in BY-2 suspension cultures. Because lovastatin specifically inhibits MA biosynthesis, we used it to affect formation of endogenous cytokinins downstream in the pathway [28–31]. In this fashion, the influence of zeatin type cytokinin content on G_1/S transition could be investigated. Flow cytometry allowed us to monitor the occurrence of G_1/S in the cells with or without inhibition of cytokinin biosynthesis.

2. Materials and methods

2.1. Chemicals

Aφ batches from ICN (Asse, Belgium) were used for synchronization purposes. All cytokinin-deuterated tracers ([$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]iP, [$^2H_6$]iPA, [$^2H_6$]iP9G, [$^2H_3$]DZ, [$^2H_3$]DZR) were purchased at Apex (Honiton, UK). Lovastatin lactone was kindly provided by Merck (Rahway, NJ, USA). The lactone rings of lovastatin were hydrolyzed before application as described by Crowell and Salaz [28]. PI, like all other chemicals used for nuclei isolation, was bought at Sigma (Bornem, Belgium). PZ was provided by Chemservice (West Chester, PA, USA).

2.2. Culture maintenance and synchronization

BY-2 cultures were maintained in a Murashige and Skoog-based medium (Duchefa, Haarlem, The Netherlands), according to the method of Nagata et al. [19]. To obtain a maximum synchronization rate, a sequential Aφ-PZ block was performed.

In a first step, Aφ was added to an end concentration of 5 μg/ml in fresh medium, to which 1:10 (v/v) of stationary culture was added. After 24 h, the drug was removed by extensive washing and the shifted cells were released in fresh medium containing 1.54 μg/ml of PZ for the second step of synchronization [25,26]. Once an MI of minimum 85% was reached (6 to 7 h after release from Aφ), the cells were immediately washed and released in fresh medium supplemented with vitamins and 2,4-D [19].

2.3. Determination of the MI

Four to 8 h after release from Aφ, aliquots of cells were fixed with ethanol/acetic acid (3:1, v/v) and stained with 1 μg/ml DAPI for immediate evaluation of the MI. A fluorescence microscope (Leitz, Germany) was used to count the number of nuclei arrested in prometaphase due to the presence of PZ. An average MI value of 92% (±4.5%) was reached during the six independent double synchronizations carried out in this paper. Synchronization rates within the 90% range (obtained after a sequential Aφ-PZ block) have already been experienced in several other cases [25,26]. The addition time of the second blocking agent (PZ) is crucial for the attainment of very high synchronization rates. The method proposed by Samuels et al. [27] proposes PZ addition 3 h after release from Aφ. This 3 h lag-phase might account for a loss in sychronization percentage already established with Aφ, since MI under these conditions did never exceed 81% [27].

The MI was counted a second time between 17 and 20 h after release from Aφ.

2.4. Sampling of the cells

After completion of the M-phase (8 h after release from Aφ), the culture was split up in a control batch and a batch treated with 10 μM lovastatin. From that moment onward, samples were taken every hour either for nuclei isolation or for LC-MS/MS analysis. While the latter were frozen in liquid nitrogen and stored at −70°C until extraction, the first samples were immediately processed for nuclei purification.

2.5. Nuclei isolation and flow cytometry

Nuclei were isolated in a stepwise protocol of enzymatic and osmotic treatments and stored at 4°C in the presence of 0.8% formaldehyde. Several currently used protocols were compared as to centrifugation speed and amount of osmotic steps [25,26,32–34]. The scope of this comparison was to obtain a method in which 'gentleness' could be combined to maximum yield, since it is known that centrifugation speed and the choice of fixative can cause clumping [34]. An independent study was carried out as to the incubation time, temperature, batch strength and concentration of the enzymatic solutions. By means of an inverted microscope (Nikon Diaphot TMD), the optimal digestion method was selected based on a judgement of when the greatest proportion of released protoplasts had assumed a spherical shape. Finally, an enzyme ratio of 0.1% pectolyase versus 2% cellulase (8.4 units/mg) was chosen for further experiments. The addition of a non-ionic detergent (1% Triton X-100) to the hypotonic buffer solution (Galbraith), allowed non-specifically stained cytoplasmic fragments to be removed [34]. The standard method developed by Bergounioux et al. [32] was thus only slightly modified, mostly in respect of centrifugation speed. The basic lysis method was followed and sorbitol was preferred to mannitol as an osmotic agent, both on account of its higher solubility and of the absence of crystals being formed which could hinder cytometry (Brown, S.C., personal communication).

Because aldehyde fixation can increase the refractive index of the cytoplasm, which could affect the precision of the fluorescence measurement [34], the nuclei were rinsed in PBS twice and centrifuged at low speed (1000 rpm) before applying the three-step Vindelov staining method. A comparison was made with a simple dye containing only PI (50 μg/ml) and RNAse (5 μg/ml), but the Vindelov method produced clearer histograms [35]. If combined with an RNAse, PI is still a very efficient DNA-specific staining agent with a broad excitation band in UV. Low speed centrifugation had the disadvantage of producing a less coherent pellet – which caused loss of nuclei during the staining procedure (treatment with trypsine and RNase A and staining with 10 μg/ml PI) – but, on the other hand, kept the nuclei intact. A FACS-scan flow cytometer was used for analysis.

2.6. Extraction, purification and quantitative electrospray LC-MS/MS analysis of cytokinins

Frozen cells were pulverized in liquid nitrogen and extracted overnight at −20°C in Bieleski solvent ($CHCl_3$, CH_2OH, H_2O, HCOOH, 5:12:2:1, v/v) [36]. Approximately 20 pmol of each deuterated cytokinin was added as an internal standard. Solid-phase extraction and immune affinity purification were conducted according to the existing protocols [37]. The samples obtained that way were stored at −70°C until analysis by (+ES)LC-MS/MS. The cytokinins could be analyzed and quantified by means of their diagnostic transitions under a multiple reaction monitoring mode [38]. The internal standard ratio method allowed the computation of endogenous cytokinin concentrations.

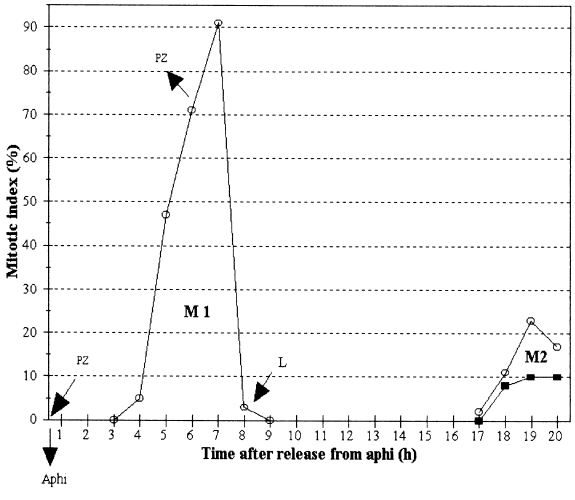

Fig. 1. Effect of lovastatin on the second round of mitosis after G_1/S transition. MI in control conditions (○) and in presence of 10 μM L (■).

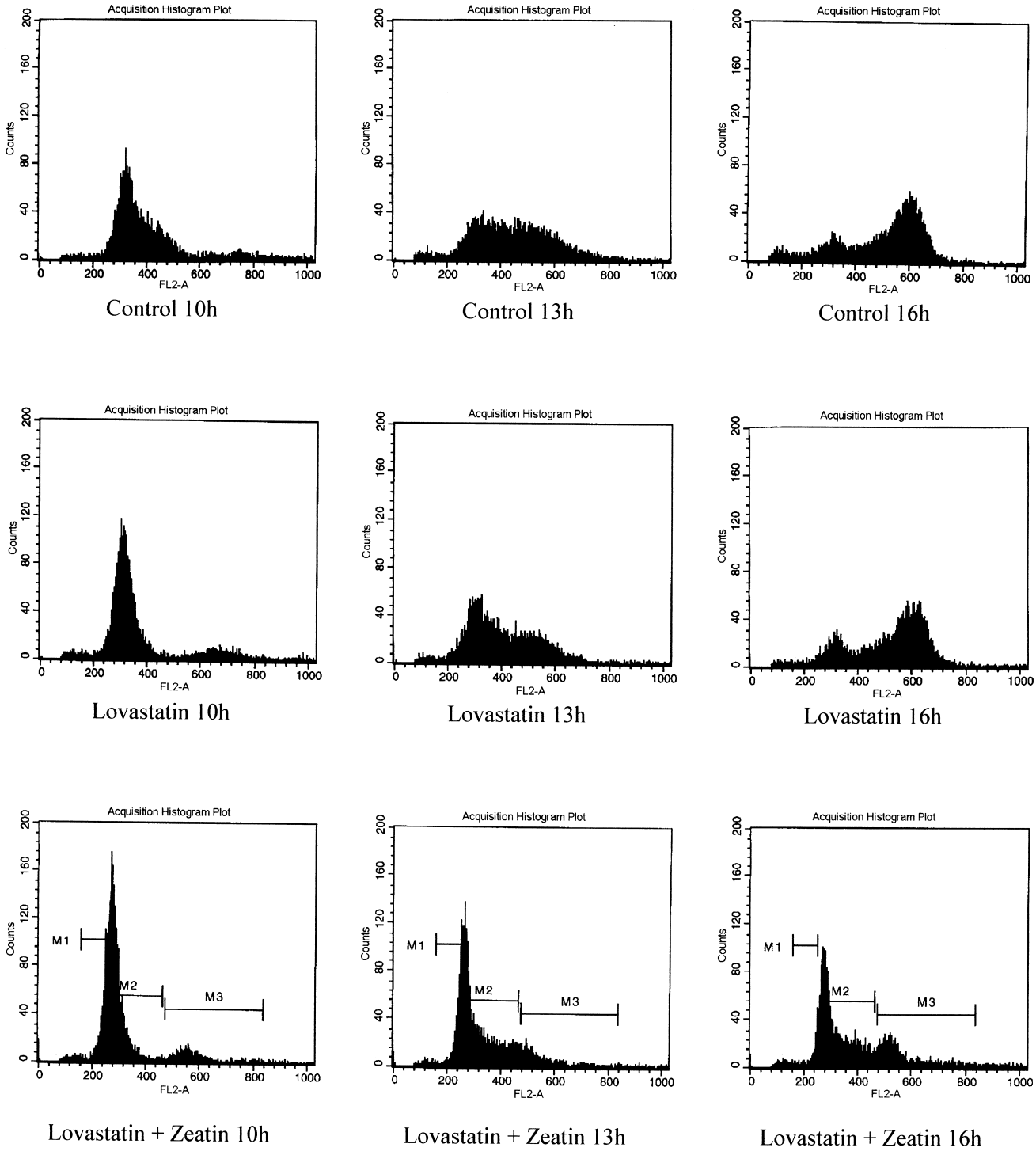

Fig. 2. Effect of the addition of L (10 μM) and L+Z (8 μM) to a cell population accumulated into the M-phase after double Aφ-PZ block. Flow cytometry analyses.

3. Results

3.1. *Effect of lovastatin (with or without cytokinins) on the G_1/S transition*

Addition of lovastatin allowed a closer look at the G_1/S transition in presence or absence of cytokinins. Sharper flow cytometry results could be obtained when maximum synchronization rates were induced by a double Aφ-PZ block. At 6 h after release from Aφ, the majority of cells was blocked in prometaphase due to the presence of PZ (Fig. 1). The second rinse allowed them to leave M-phase about 2 h later. The double blocking method easily produced synchronization rates within the 90% range [25,26] (Fig. 1). However, one must take into account that synchronization decreases rather quickly and can only be maintained for about one cycle round [19]. The synchrony at 10 h (first histogram shown) will therefore already have dropped in respect to the initial high to very high values at 7 h and this is exactly the reason why we aimed

for maximum synchronization rates by double block. After taking one sample in control conditions at 8 h, the culture was split up and lovastatin (10 μM) was added in one batch. In another batch, lovastatin (10 μM) and zeatin (8 μM) were added. The cells were in M/G_1 at the time of addition (Fig. 1). From that point, samples were taken every hour until G_2 set in. Cell cycle progression was analyzed by flow cytometry (Fig. 2). The experiment was repeated six times. The results were fully reproducible.

For the sake of uniformity, all time points are given as 'time after release from Aφ' even though in every experiment a PZ block was also performed and released 6–7 h later. Fig. 1 shows the *X*-axis as it will be considered in the entire experimental description and gives a clear view of the entire setup. The period between first and second mitosis is visualized by the histograms in Fig. 2.

At 10 h after release from Aφ (or 3 h after release from PZ), the cells were into G_1-phase (Fig. 2). A large amount of S-phase appeared consistently at about 13 h, in control conditions as well as in the presence of lovastatin. At 16 h, cells underwent G_2 regardless of the fact that lovastatin was present or not. Considering the loss of synchrony after one cell cycle [19], the apparent broader peakshape in the 16 h histogram is an expected phenomenon.

In the presence of 8 μM zeatin however, the cell cycle could only evolve very slowly towards S-phase. It was not before 14 h after release from Aφ that a minor decrease in the amount of G_1 cells was observed. Within the 16 h of observation, the S-plateau did not occur. This particular experiment was repeated two times with comparable effects.

The same experiment was repeated with 8 μM concentrations of ZR and iP in the presence of lovastatin, with similar inhibiting effects on the G_1/S transition (data not shown).

Table 1
Endogenous cytokinin levels (pmol/gfw) during the G_1/S transition of a synchronized BY-2 cell culture (C) and effect of 10 μM lovastatin (L) on endogenous cytokinin (CK) concentrations

CK	Time after release from Aφ (h)	Conditions	
		C	L
Z	9	**344**	–
	10	274	18
	11	205	9
	12	90	11
	13	**179**	**28**
DZ	9	29	–
	10	75	16
	11	0	0
	12	94	0
	13	0	0
ZR	9	193	–
	10	249	179
	11	**673**	**15**
	12	5	7
	13	54	4
DZR	9	7	–
	10	9	5
	11	20	1
	12	1	1
	13	2	1
ZNG	9	2	–
	10	9	11
	11	5	3
	12	9	10
	13	5	6
ZRP	9	93	–
	10	117	165
	11	**535**	**23**
	12	34	18
	13	45	3
DZRP	9	4	–
	10	5	5
	11	18	1
	12	1	1
	13	2	0

3.2. *Effect of lovastatin on the second round of mitosis*

In order to ensure ourselves of the stability of lovastatin in the culture medium, a double block was conducted in the way described above. MI at the first mitotic peak reached an average of 92% (Fig. 1). The culture was also split in two batches at 8 h after release from Aφ: control and lovastatin (10 μM). The cultures were allowed to grow for 8 h after treatment with lovastatin.

Then, samples were taken for the determination of the MI at the second putative mitosis. This experiment was repeated twice with comparable results. The second mitotic peak indeed occurred between 18 and 19 h after release from Aφ. An average residual MI of 25% was obtained in control conditions, whereas MI only reached an average of 12% in the presence of lovastatin.

3.3. *Effect of lovastatin on the endogenous cytokinin content*

Table 1 unequivocally shows that application of lovastatin induces a severe drop in any endogenous cytokinin level. In control conditions, the expected peaks are observed before occurrence of G_1/S [23]. More precisely, peaks in the zeatin content are observed at 9 h (cells entering G_1) and around 13 h (G_1+early S) (Fig. 2). For ZR and ZRP, one dominating peak is reached at 11 h (G_1+early S). Lovastatin almost totally succeeded in blocking the formation of all zeatin type cytokinins by the time G_1/S was expected (12 h).

4. Discussion

The requirement for high transient zeatin type cytokinin levels at the G_1/S transition of tobacco BY-2 cells was investigated by means of a sequential Aφ-PZ block followed by flow cytometry on control and lovastatin-treated samples. BY-2 cells exhibit stringently regulated kinetics of their endogenous cytokinin levels [20–22]. As stated previously, transient high peaks in zeatin type cytokinins are noticed not only at G_2/M, but also around G_1/S [23,24]. Our former research proved zeatin to be essential for G_2/M transition in the BY-2 system [22]. It was the main issue of this paper to check whether the same assumption could be made for the G_1/S transition.

Our data show that addition of lovastatin at M/G_1 did indeed block the cytokinin biosynthesis severely (Table 1), but did not affect the normal transition from G_1 to S (Fig. 2). Surprisingly, addition of 8 μM zeatin in the presence of lovastatin, prevented normal progression through S-phase (Fig. 2).

To ensure ourselves of the stability of lovastatin after several hours in culture medium, the occurrence of a second cycle was investigated. Therefore, the second MI count started 8 h after addition of lovastatin (Fig. 1). Cells can only maintain high synchronicity for about one cycle and the MI values of this second count would never have reached a significant per-

centage without inducing a maximum synchronization rate at the start of the experiment. This is why the double Aφ-PZ blocking method was chosen. To prove that lovastatin remained effective throughout the entire experiment, a drop in MI had to be observed during the second M-phase, which was the case. This ratio of a 50% decrease in MI in the presence of lovastatin is a phenomenon also observed when lovastatin is added before the first G_2/M transition [22,31], showing that the compound is still efficient after remaining in culture medium for one complete cell cycle. Not only does this observation validate the flow cytometry data obtained; it additionally proves that lovastatin was active in the culture medium during the entire transition from G_1 to second G_2/M over S and that any stringent dose dependent effect of cytokinins during that part of the cycle can be ruled out.

Obviously, high cytokinin levels at G_1/S are not necessary for the cells to continue their cycle. This does not mean, however, that they are of no use at that time and place. A first possible explanation could be that the cells simply do not need such high endogenous concentrations to proceed with their division cycle at that point. This theory can be supported by the observation that extra addition of zeatin, which – after all – shows a prominent endogenous peak at M/G_1, prevents the occurrence of S-phase in the presence of lovastatin (Fig. 2). Moreover, the same phenomenon could be observed when ZR and iP were added. This suggests that if any cytokinin level is necessary for G_1/S transition, it is amply sufficient even after lovastatin has downregulated most of its biosynthesis. A reasonable train of thought, since literature proved over the past several years that overexposure to cytokinins can easily block the cell cycle [9]. Keeping in mind that the BY-2 cell is cytokinin autotroph and can thus perfectly regulate its own biosynthetic supplies according to the needs of the moment, these observations certainly make sense. One might even suspect that it is exactly this overexposure that is to be avoided to reach the S-phase. It could thus well be the explicit downregulation of zeatin type cytokinins at G_1 rather than the peak at M/G_1 that triggers the G_1/S transition. Additionally, the results of Hemmerlin and Bach [31] prove that G_1/S transition was only blocked by lovastatin after a 36 h treatment with the drug. After that period of time, the naturally occurring cytokinin levels were probably totally depleted, causing the cells to drop below their necessary minimal dose of cytokinins participating in the downregulation signal for G_1/S transition.

The effect of zeatin type cytokinins at G_1/S might thus be far less direct than the type of interaction observed at G_2/M [22] and even more sensitive, judging by the very low amount of cytokinins this transition requires. Whereas G_2/M transition could be attributed to a physical interaction in the periphery of specific kinases, one might think of a more basic mechanism at G_1/S, e.g. the regulation of transcription by an on-off switch mechanism. This idea can be supported by the observations of Riou-Khamlichi et al. [15] on CycD3 activation by an external cytokinin signal at that stage of the cell cycle.

The nature of different kinases and cyclins was extensively probed over the past few years and it is now accepted that very different structures act at these two distinct stages of the cell cycle ([2,16] are only two of the many references on the subject). It is therefore not surprising that processes at G_1/S or G_2/M differ in the same proportion as the active structures themselves.

Our study indeed does not prove why a peak in zeatin type cytokinins is observed at M/G_1. But it does prove the subtle and versatile nature of cytokinins in the plant cell. While we can be absolutely certain about the stringent necessity of a highly regulated zeatin peak for G_2/M, it is tempting to assume that it is the drop in zeatin type cytokinins that is essential for G_1/S to occur. The observed M/G_1 peak might therefore trigger other activities than the one just ruled out, e.g. feedback regulation of the MA pathway. A cytokinin peak in M/G_1 might also act as a signal that the indispensable hormone for the oncoming G_2/M is being properly synthesized so that it will be present at sufficient rates later on, when it is needed. With all these prerequisites fulfilled, the cell may then safely begin a new cycle round. For the G_1/S transition itself, the nature of the process will very likely be of another nature than a physical one, as opposite to the one observed at G_2/M. Then again, the peak around M/G_1 could be related to physical events on a protein basis, more similar to the events in G_2/M. Recent findings thereon showed that the effect of cytokinins might be related to the phosphorylation dephosphorylation processes activating the cyclin-kinase complexes [18]. Since also in the beginning of G_1, this type of complexes are being formed, there might be a similar function for cytokinins to be filled in yet....

So although this study does not explain or prove why the content of zeatin type cytokinins undergoes a transient high peak at M/G_1, it does prove that an extremely low dose of these compounds is required at the G_1/S transition. Additionally, it strongly emphasizes the exclusive nature of zeatin as a physical G_2/M trigger in the BY-2 system [22].

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