Zeatin is indispensable for the G_2-M transition in tobacco BY-2 cells

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Abstract The importance of N_6-isoprenoid cytokinins in the G_2-M transition of *Nicotiana tabacum* BY-2 cells was investigated. Both cytokinin biosynthesis and entry in mitosis were partially blocked by application at early or late G_2 of lovastatin (10 μM), an inhibitor of mevalonic acid synthesis. LC-MS/MS quantification of endogenous cytokinins proved that lovastatin affects cytokinin biosynthesis by inhibiting HMG-CoA reductase. Out of eight different aminopurines and a synthetic auxin tested for their ability to override lovastatin inhibition of mitosis, only zeatin was active. Our data point to a key role for a well-defined cytokinin (here, zeatin) in the G_2-M transition of tobacco BY-2 cells.

Key words: G_2-M transition; Cytokinin; Synchronisation; Lovastatin; Tobacco BY-2 cell

1. Introduction

Comparison of the plant cell cycle regulation with animal and yeast models has resulted in the appreciation of large homologies, however with some plant-specific cell cycle control mechanisms [1–6]. Families of cyclin dependent kinases (CDKs) and their regulatory subunits (cyclins) [5–7] control transitions through and between stages of the cell cycle, in the animal as well as in the plant kingdom. According to Zhang et al., it is in the direct coupling of mitotic control to hormonal signals, that plant cells differ from somatic animal cells [8]. To enable plant cells to proceed through different stages of the cell cycle, the continuous presence of both cytokinins and auxins is essential [9]. The stimulating effect of cytokinins on cell division has been observed in numerous cases ranging from the initiation of root primordia in planta to induction of cell division in protoplast suspension cultures [9–14]. It has been shown that cytokinins can control the cell cycle by stimulating the tyrosine dephosphorylation of a tobacco $p34^{cdc2}$-like kinase [8,15] during mitosis as well as by regulating the expression of the cdc2 gene [16]. Recent data point to a possibility for physical interaction between cytokinins and kinases [17,18]. Structural cytokinin analogues such as olomoucine [19,20] and roscovitine [21] were shown to act as competitive inhibitors of specific plant CDKs. A general conclusion emerging from all these studies is that cytokinins are among the major controlling factors of the cell cycle.

The rapid and cytokinin-autonomous cell cycle of synchronised tobacco BY-2 cell suspension cultures [22] makes them ideal tools to study cytokinin control mechanisms on cell cycle progression. The cytokinin-autonomous character of the BY-2 cells is linked to the capacity of these cells to synthesise cytokinins [23,24]. Using LC-MS/MS techniques, Redig et al. [25] were able to show a distinct transient accumulation of Z-type cytokinins at G_2-M transition.

It was the main aim of this study to determine the causal relationship between the transient accumulation of Z-type cytokinins and the entry in mitosis of BY-2 cells.

In order to investigate this relationship, lovastatin was chosen as a putative inhibitor of cytokinin biosynthesis. Lovastatin is a highly potent competitive inhibitor of HMG-CoA reductase [26] that mimics the mevaldyl-CoA thiohemiacetal in the two-step reduction of HMG-CoA to mevalonic acid (MA) [27]. Previous research by Crowell and Salaz [26] suggested the capacity of low lovastatin concentrations (up to 10 μM) to specifically inhibit the cytokinin isoprenoid side-chain synthesis. At higher concentrations (>20 μM) lovastatin also inhibits the less sensitive MA-derived pathways for the production of abscisic acid, gibberellins, ubiquinone, sterols, carotenoids and many other isoprenoid compounds [26–28], causing an irreversible arrest of cellular development [26].

In this study, we show that the addition of low lovastatin concentrations during G_2 of an aphidicolin-synchronised tobacco BY-2 suspension culture, drastically reduces both the accumulation of Z and the G_2-M transition. Out of various cytokinins as well as cyclic AMP and a synthetic auxin, only zeatin was proven to effectively reverse the inhibiting effect of lovastatin on mitosis. Our findings point to Z as a highly specific triggering factor for G_2-M transition in tobacco BY-2 cells.

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Abbreviations: Aφ, aphidicolin; cAMP, 3′,5′-cyclic adenosine monophosphate; BA, 6-benzyl aminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; DAPI, 4,6′-diamidino-2-phenylindole; DZ, dihydrozeatin; DZNG, dihydrozeatin-*N*-glucoside; DZMP, dihydrozeatin riboside-5′-monophosphate; DZOG, dihydrozeatin-*O*-glucoside; DZROG, dihydrozeatin riboside-*O*-glucoside; DZR, dihydrozeatin riboside; ES, electrospray; gfw, gram fresh weight; GiP, isopentenyladenine glucoside; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HPLC, high performance liquid chromatography; 2iP, isopentenyladenine; 2iPA, isopentenyladenosine; iPAP, isopentenyladenosine-phosphate; 2iP9G, isopentenyladenine-9-glucoside; MA, mevalonic acid; MI, mitotic index; MS, mass spectrometry; NAA, α-naphtyl acetic acid; NH_4Ac, ammoniumacetate; PBS, phosphate-buffered saline; TBY-2, tobacco Bright Yellow 2; Z, zeatin; Z9G, zeatin-9-glucoside; ZMP, zeatin riboside-5′-monophosphate; ZNG, zeatin-*N*-glucoside; ZOG, zeatin-*O*-glucoside; ZR, zeatin riboside; ZROG, zeatin riboside-*O*-glucoside

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2. Materials and methods

2.1. Chemicals

Aφ-batches from Sigma (Bornem, Belgium) and ICN (Asse, Belgium) were used. Cytokinins, NAA, BA and cAMP were delivered by Sigma (Bornem, Belgium). All cytokinin-deuterated tracers ([$^2H_5$]Z, [$^2H_5$]ZR, [$^2H_5$]Z9G, [$^2H_5$]ZOG, [$^2H_5$]ZROG, [$^2H_6$]2iP, [$^2H_6$]iPA, [$^2H_6$]2iP9G, [$^2H_3$]DZ, [$^2H_3$]DZR) were purchased from Apex (Honiton, UK). Lovastatin lactone was kindly provided by Merck (Rahway, NJ, USA) and MA lactone (DL-3,5-hydroxy-3-methyl-5-valerolactone) was bought from Sigma (Bornem, Belgium). The lactone rings of lovastatin (lactone form) and MA lactone were hydrolysed before application as described by Crowell and Salaz [26].

2.2. Culture maintenance and synchronisation

Tobacco BY-2 cell suspension cultures grew in Murashige and Skoog medium (Duchefa, Haarlem, The Netherlands), which was enriched with sucrose (30 g/l), KH_2PO_4 (200 mg/l), thiamine (1 mg/l) and with 0.2 mg/l of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). Culture maintenance and synchronisation were conducted as described by Nagata et al. [22]. Briefly, an end concentration of 5 mg/ml of aphidicolin (Aφ) was added to fresh medium to which 1:10 (v/v) of stationary culture was transferred. After 24 h, the drug was removed by extensive washing and the cells were re-suspended in fresh medium. At either 3 or 4.5 h after Aφ release, aliquots of the culture were supplemented with the different substances of interest. Samples for cytokinin analysis (4 ml, corresponding to ±0.25 gfw) were taken and left to sediment on ice, the supernatant was removed and the fresh weight was recorded. Afterwards, they were immediately frozen in liquid N_2 and stored at −70°C until extraction.

2.3. Determination of the MI

Cells were fixed in PBS-buffered paraformaldehyde/glutaraldehyde (3% (w/v)/0.5% (v/v)) at 4°C and stained with DAPI after a rinse in PBS. Nuclei from late prophase to early telophase were scored in 500 cells by means of UV-fluorescence microscopy (Leitz dialux).

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

Frozen cells were ground 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) [29]. Deuterated cytokinins were added as internal standards (5 pmol each). Solid-phase extraction and immuno-affinity purification were performed as described [30]. The samples thus obtained were stored at −70°C until analysis by (+ES) LC-MS/MS.

After dissolving the samples in 40 μl of loading buffer (250 μM tetrabutyl ammonium bromide (TBAB)+1 mM NH_4Ac), cytokinins were quantified by means of a capillary column-switching chromatographic set-up combined with an on-line mass spectrometer according to Witters et al. (in press). Five microliters of cytokinin samples were injected in the LC set-up and pre-concentrated on a capillary precolumn (C18, 5×0.5 mm internal diameter) using loading buffer as a mobile phase (flow rate 20 μl/min, pH 6.6). After 5 min the precolumn was backflushed to the analytical column (C18, 150×0.3 mm internal diameter) using a mobile phase containing 0.625% (v/v) CH_3COOH and 80% MeOH (flow rate 10 μl/min, pH 3.5). Tandem mass spectrometry allowed analysis and quantification of the cytokinins by means of their diagnostic transitions under a multiple reaction monitoring mode [31]. Endogenous cytokinin concentrations were calculated following the internal standard ratio method.

3. Results

3.1. Effect of lovastatin and mevalonic acid on mitosis

Since this research focused on the importance of cytokinins for G_2-M transition, lovastatin was added at either 3 or 4.5 h after Aφ release (early or late G_2) [32]. The effect of different lovastatin concentrations (1, 5 and 10 μM), given in late G_2 (4.5 h), on the MI values of Aφ-synchronised cells is presented in Fig. 1. In absence of lovastatin, the MI reached 40% at 7 h after release from Aφ block.

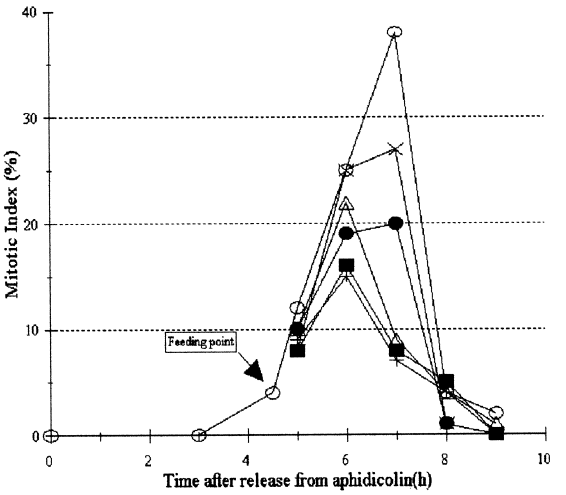

Fig. 1. Differential trial of lovastatin (L) concentrations. MI in blank conditions (○) and in presence of 1 μM L (△), 5 μM L (+), 10 μM L (■), 1 μM L+6 mM MA (×) and 10 μM L+6 mM MA (●).

Addition of 1 μM of lovastatin at 4.5 h hardly affected the MI until 6 h after Aφ release, whereas both 5 and 10 μM caused a 40% reduction in MI measured at that time. At 7 h, an abrupt drop was observed (MI < 10%) for all concentrations applied. A minor yet significant fraction of cells was not affected by lovastatin and proceeded normally through mitosis, since DAPI-staining of the DNA revealed no arrest between late prophase and telophase in presence of lovastatin.

MA (6 mM) applied together with 1 or 10 μM lovastatin was able to restore MI values at 7 h to 25% and 20% respectively.

3.2. Endogenous CK levels after lovastatin treatment

To determine the effect of lovastatin (applied in late G_2) on cytokinin biosynthesis, endogenous levels of 13 different cyto-

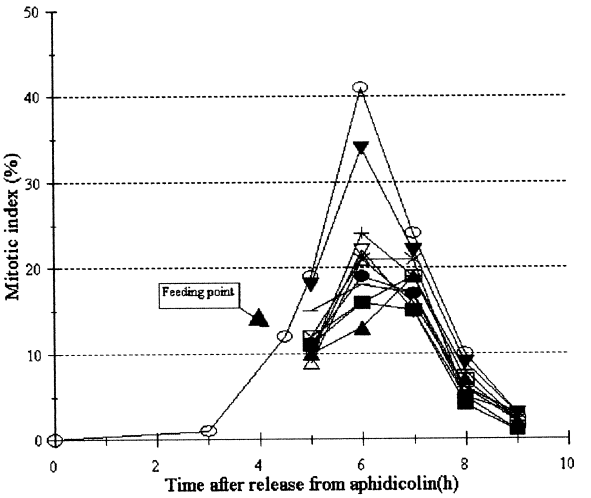

Fig. 2. Rescue of lovastatin (L)-induced inhibition of mitosis. MI in blank conditions (○) and in presence of 10 μM L (■), 10 μM L+8 μM Z (▼), 10 μM L+8 μM ZR (●), 10 μM L+8 μM iPA (▲), 10 μM L+8 μM iP (□), 10 μM L+8 μM DZ (▽), 10 μM L+8 μM kinetin (+), 10 μM L+8 μM cAMP (×), 10 μM L+8 μM BA (△), 10 μM L+8 μM NAA (−).

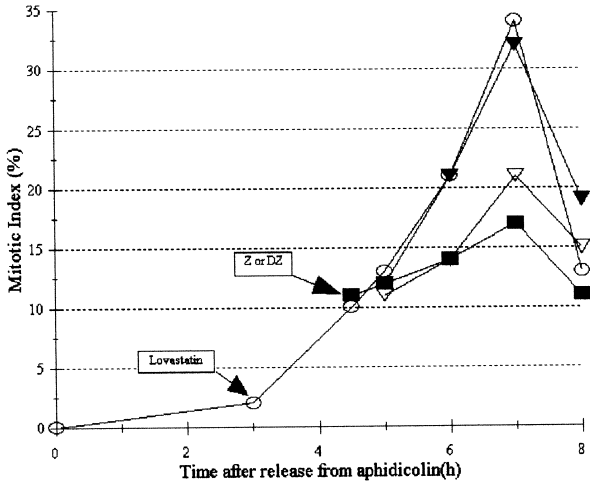

Fig. 3. Effect of addition of lovastatin (L) at 3 h instead of 4.5 h after release from Aφ. MI in blank conditions (○) and in presence of 10 μM L (■), 10 μM L+8 μM Z (▼), 10 μM L+8 μM DZ (▽).

kinins were analysed by capillary (+ES) LC-MS/MS, 6, 7 and 8 h after Aφ release (Table 1). In control conditions (no lovastatin applied), only Z (1700 pmol/gfw) and ZR (300 pmol/gfw) showed enhanced levels coinciding with the mitotic maximum at 7 h. No accumulation was recorded for any of the other cytokinins analysed. Addition of lovastatin (10 μM) at late G_2 drastically reduced the endogenous Z concentration to a maximum of 60 pmol/gfw, 7 h after release from Aφ. A minor, yet significant increase in endogenous Z (160 pmol/gfw) and ZR (30 pmol/gfw) was observed upon supplementation of lovastatin with 6 mM MA, in accordance with the observed partial rescue of the lovastatin-induced inhibition of mitosis (Fig. 1).

The rather low and steady concentrations ranging between 0 and 30 pmol/gfw that were observed under control conditions for GiP, iPA, iP, DZNG, DZR, DZ, iPAP, DZMP, OGZ, were not dramatically altered by lovastatin treatment.

3.3. Rescue of lovastatin-induced inhibition of mitosis

The ability to overcome lovastatin-induced inhibition of mitosis was tested for five isoprenoid cytokinins (Z, ZR, iPA, iP, DZ), kinetin, BA, cAMP and NAA (a synthetic auxin). Each compound was tested at a concentration of 8 μM added in combination with 10 μM of lovastatin at 4.5 h after Aφ release. For each experimental condition the MI of 500 cells was scored (Fig. 2). A mitotic peak of 41% in control conditions was reached at 6 h after release from Aφ block. Addition of 10 μM lovastatin alone reduced the MI to about 18%. Only Z could reverse the effect of lovastatin on mitosis (MI = 38%). Values measured for all the other treatments (lovastatin+ZR, iPA, iP, DZ, kinetin, cAMP, BA or NAA) remained close to those encountered when only lovastatin was added.

In order to test whether application of lovastatin in early G_2 would prevent more BY-2 cells to progress through mitosis 10 μM of lovastatin was added 3 h after Aφ release (Fig. 3). This lovastatin treatment (early G_2) resulted in a similar partial inhibition of the MI as was observed when lovastatin was added at late G_2 (Fig. 1). Again, adding Z at 4.5 h after Aφ release to lovastatin treated BY-2 cells completely restored the MI index to control values, whereas addition of DZ had no significant effect on the lovastatin-induced inhibition of mitosis.

4. Discussion

In contrast to wild-type tobacco cell cultures [33] tobacco BY-2 cells only need exogenously supplied auxin to proceed through the cell cycle [22]. This feature can be directly linked to the kinetics of endogenous cytokinins in aphidicolin-synchronised BY-2 cell suspension cultures [23,24]. LC-MS/MS analyses showed a transient accumulation of zeatin-type cytokinins at G_2-M transition [25]. Our study wishes to investigate the possible causal relationship between the accumulation of Z-type cytokinins and the progression through this particular stage of the cell cycle. Previously Crowell and Salaz [26] have shown that low concentrations of lovastatin inhibited the growth of tobacco BY-2 cells, a phenomenon that they related to a putative inhibition of the cytokinin biosyn-

Table 1
Endogenous cytokinin levels (pmol/gfw) during M phase of a synchronised TBY-2 culture (C) and effect of 10 μM lovastatin (L) and 10 μM lovastatin+6 mM mevalonic acid (MA) on endogenous cytokinin (CK) concentrations

CK	Time after release from Aφ block (h)	Conditions		
		C	L	L+MA
Z	6	**11**	**1**	**2**
	7	**1700**	**60**	**160**
	8	**14**	**31**	**14**
DZ	6	1	<1	1
	7	7	4	12
	8	<1	2	1
iP	6	<1	<1	<1
	7	15	2	3
	8	2	3	1
ZR	6	**1**	**<1**	**<1**
	7	**300**	**8**	**29**
	8	**2**	**16**	**1**
DZR	6	1	4	16
	7	9	3	3
	8	1	5	4
iPA	6	<1	1	<1
	7	4	1	1
	8	1	3	1
ZNG	6	<1	1	<1
	7	2	<1	5
	8	<1	1	<1
ZNOG	6	16	4	4
	7	3	3	3
	8	2	3	
DZG	6	<1	3	<1
	7	<1	<1	5
	8	<1	1	<1
iPNG	6	1	<1	<1
	7	1	<1	<1
	8	1	1	<1
ZRP	6	<1	<1	4
	7	10	3	3
	8	28	7	3
DZRP	6	<1	<1	<1
	7	<1	<1	<1
	8	<1	<1	<1
iPAP	6	28	9	3
	7	2	2	2
	8	2	2	1

thesis. Adding different concentrations at late G_2 (Fig. 1) confirmed the inhibitory effect of lovastatin. A large population of cells was arrested before mitosis. At the same time a clear-cut decreased endogenous concentration of zeatin-type cytokinins at the G_2-M transition was observed (Table 1). This inhibitory effect could partially be reversed by adding mevalonic acid, indicating that the observed accumulation of zeatin-type cytokinins at the G_2-M transition resulted from a sharply controlled enhanced biosynthesis. The LC-MS/MS data quantitatively prove the efficiency of lovastatin as a tool for the suppression of cytokinin biosynthesis in plant cells.

Remarkable was the occurrence of a minor yet significant population of cells, which apparently were not affected by lovastatin, even when given at early G_2. This phenomenon is possibly related to the observation that even in presence of 10 μM lovastatin, the mevalonic acid synthesis is only partially inhibited, or alternatively that a fraction of cells, due to cluster formation, was not reached by the added lovastatin. In both cases a reduced endogenous zeatin concentration in G_2-M transition, as shown in Table 1, would be the consequence. If the endogenous zeatin concentration in all cells were to be reduced, this would have as a consequence that a sub-population of cells is characterised by a higher cytokinin sensitivity. This would allow them to proceed through mitosis at lower cytokinin concentrations in comparison with the population of cells, which are arrested by lovastatin.

In order to substantiate the need of BY-2 cells for enhanced cytokinin levels to pass G_2-M transition, various cytokinins were tested for their ability to complement the lovastatin inhibition (Fig. 2). Among all tested cytokinins (Z, ZR, DZ, iP, iPA, BA, kinetin) in the presence of lovastatin, only Z effectively restored MI near control values (no lovastatin added). Neither cAMP [34] nor NAA had any effect. By means of uptake experiments with tritium labelled compounds, the conclusion could be drawn that the observed differences in behaviour of the applied cytokinins were not due to an impaired uptake of the less effective compounds (data not shown).

For the first time, all these observations point to a stringent structure-specific effect (Z only) rather than an overall function-specific effect of cytokinins on G_2-M transition in tobacco BY-2 cells. More emphasis is thereby put on the need for an accurately regulated zeatin metabolism during G_2-M transition [25] and the existence of a putative zeatin-specific receptor.

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