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Changes in Cellular Polyamine Contents and Activities of Their Biosynthetic Enzymes at Each Phase of the Cell Cycle in BY-2 Cells

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We analyzed changes in polyamine contents and the activities of biosynthetic enzymes during each phase of the cell cycle for a synchronized population of BY-2 cells. Based on our analysis of H^3-thymidine incorporation flow cytometry, and the mitotic index, the M and G_2 phases seemed to occur at 8 h and from 2.5 to 8 h, respectively, after the release of aphidicolin. The respective activities of arginine decarboxylase (ADC), ornithine decarboxylase (ODC), and *S*-adenosyl methionine decarboxylase (SAMDC) at the beginning (7.4, 11.2, and 5.5 nmol mg^{-1} protein h^{-1}) were increased to 22.6, 22.1, and 15.1 nmol mg^{-1} protein h^{-1}. However, those increases do not coincide with the general change in polyamines reported from animal cells. In addition, the bi-phasic activation of polyamine biosynthetic enzymes, such as those found in the general animal model, was observed with ADC and ODC but not with SAMDC. These results suggest that the general animal model for explaining polyamine changes and SAMDC activation in the cell cycle cannot be applied to BY-2 cells. Further, our flow-cytometric analysis of cell populations may be a useful tool for evaluating the effects of polyamines on cell cycle progression in BY-2 cells.

Keywords: BY-2 cells, cell cycle, flow-cytometric analysis, *Nicotiana tabacum*, polyamines, *S*-adenosyl methionine decarboxylase

Cellular polycationic polyamines (spermidine, spermine, and their precursor, putrescine), which are ubiquitous in nature and absolutely required for eukaryotic cell growth, are synthesized in well-regulated biochemical pathways (Walden et al., 1997; Ackermann et al., 2003; Oredsson, 2003). The amino acids arginine and ornithine are converted to putrescine in a decarboxylation reaction catalyzed by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC), respectively (Martin-Tanguy, 2001). Spermidine is synthesized in a reaction in which an aminopropyl group derived from decarboxylated *S*-adenosyl methionine (DcSAM) is coupled to putrescine by spermidine. A similar reaction is catalyzed by spermine synthase, converting from spermidine to spermine. DcSAM is generated by the action of *S*-adenosyl methionine decarboxylase (SAMDC), which is the rate-limiting step in the polyamine biosynthetic pathway (Anderson et al., 1998; Martin-Tanguy, 2001; Ackermann et al., 2003).

Polyamines promote plant growth and development by activating the synthesis of nucleic acids and proteins (Amarantos et al., 2002; van Dam et al., 2002). They have been implicated in such processes as cell proliferation, differentiation, flowering, and senescence (Walden et al., 1997; Martin-Tanguy, 2001; Jang et al., 2002; Ahn and Jin, 2004). Cell proliferation ensures the production of two new healthy daughter cells at the end of a cell cycle (Oredsson, 2003). To understand the roles of polyamines in that cycle, researchers use either the inhibitors of various polyamine biosynthetic enzymes, e.g., difluoromethyl arginine (DFMA), difluoromethyl ornithine (DFMO), and methyl-glyoxal-*bis*-guanylhydrazone (MGBG), or mutants and transgenic cells that lack polyamine biosynthetic genes, such as ADC, ODC, or SAMDC. For example, cells of *Schizosaccharomyces pombe*, which cannot synthesize spermidine or spermine because of a deletion-insertion in the gene coding for SAMDC, show an overall delay in cell cycle progression and an accumulation of cells in the G_1 phase (Chattopadhyay et al., 2002). In addition, polyamine starvation via inhibition of biosynthetic enzymes prolongs the S and G_2 phases in polyamine-dependent Chinese hamster ovary cells (Anehus et al., 1984). ODC and SAMDC activities, as well as polyamine contents, have been investigated during the cell cycle in populations synchronized by various methods. For animal systems, the

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Abbreviations: ADC, arginine decarboxylase; DcSAM, decarboxylated S-adenosyl methionine; ODC, ornithine decarboxylase; SAM, S-adenosyl methionine; SAMDC, S-adenosyl methionine decarboxylase.

general conclusions are that ODC and SAMDC are activated in a bi-phasic manner, with a first activation phase in late G_1 close to the G_1/S transition, and then a second activation phase in conjunction with S/G_2 transition. Moreover, the doubling of putrescine, spermidine and spermine contents mainly occurs during the late S phase, S/G2 transition, and the G1 and S phases (Oredsson, 2003).

The most easily synchronizable plant system (Ehsan et al., 1999) is from a tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) cell line (Nagata et al., 1992; Nagata and Kumagai, 1999). Those cells have been used successfully to study the effects of stress on cell cycle progression (Reichheld et al., 1999; Świąte et al., 2002). For example, the expression of cyclin-dependent kinases (CDKs) in the plant cell cycle has been examined in synchronized BY-2 cells (Setiady et al., 1996; Reichheld et al., 1999; Sorrell et al., 2001; Świąte et al., 2002). Likewise, flow-cytometrical analysis also has established a cell cycle for BY-2 cells (Świąte et al., 2002).

Plant cells store polyamines in their vacuoles, but ADC also has a minor role in the synthesis of putrescine in animal cells (Martin-Tanguy, 2001). Antibodies against oat ADC have revealed that this enzyme is present in the thylakoid membranes of chloroplasts (Borrell et al., 1996). SAMDC and spermidine synthase are localized to the cytoplasm, whereas ODC is found in the nucleus (Martin-Tanguy, 2001). Additional functions for plant polyamines may include the maintenance of photosynthetic activity and the prevention of osmotic stress-induced senescence (Borrell et al., 1996; Martin-Tanguy, 2001), both of which not occurring in animal cells. These reports support the possibility that metabolism differs between plants and animals for polyamine biosynthesis or storage. No reports have been published about how plant polyamines and their biosynthetic enzymes can regulate each phase of the cell cycle. Therefore, the objective of our current work was to clarify the changes in polyamine contents and the activation of their biosynthetic enzymes during cell cycle progression in BY-2 cells. Here, we present data on the effect of exogenous polyamine on that progression, as well as changes in ADC, ODC, and SAMDC activities and polyamine contents at various cycle checkpoints.

MATERIALS AND METHODS

Chemical Reagents and Protein Determinations

Standard chemicals were purchased commercially. Protein contents were estimated according to the method of Bradford (1976), as described by Wi and Park (2002).

Cell Culture, Synchronization, and Flow-cytometrical Analysis

Tobacco BY-2 suspension cells were used. Cell maintenance and synchronization with aphidicolin at the G_1/S phase were achieved as described by Nagata et al. (1992). The flow-cytometric analysis was executed according to Świąte et al. (2002). Suspension cells were washed with 0.66 M sorbitol in an MS (Murashige and Skoog) medium, and then incubated with 0.1% (w/v) pectolyase and 2% (w/v) cellulase for 1 h at 37°C. After isolation via centrifugation, DNA from the released nuclei was stained with a solution containing propidium iodide, and analyzed on an FACS analytical flow cytometer (Coulter, USA). To investigate the effects of exogenous polyamines on the cell cycle of BY-2 cells, subcultures were treated with 1 mM of putrescine, spermidine, and spermine for 12 h. Fractionation of each phase followed the flow-cytometric data, according to the method of Menges and Murray (2002).

Determination of Polyamine Contents and Activities of Biosynthetic Enzymes

Polyamines were analyzed as described by Wi and Park (2002). The supernatant of leaf extracts (0.2 mL) was mixed with saturated sodium carbonate (0.2 mL) and dimethylaminonaphthalene-1-sulfonyl chloride (0.4 mL), and then incubated at 60°C for 1.5 h. Afterward, the dansylated products were extracted with benzene. Aliquots were separated by thin layer chromatography in 25:2 (v:v) chloroform:triethylamine. The separated polyamines were quantified on an RF-1501 (Shimadzu, Japan) spectrofluorophotometer (wavelengths of excitation/emission: 350/495 nm). Enzyme activities were determined according to the method of Park and Lee (1994). Briefly, frozen powder that had been ground in liquid nitrogen was re-suspended in a buffer containing 25 mM Tris-Cl (pH 7.6), 1 mM EDTA, and 15 mM β-mercaptoethanol, using a ratio of 2:3 (w/v) for tissue to buffer. The supernatant fraction served as the enzyme source for measuring the activities of SAMDC, ODC, and ADC. A 300 uL aliquot of the extract, containing 0.1 μL of [carboxyl-^{14}C] SAM, 0.05 or 0.1 μCi of L-[1-^{14}C] Orn, and 0.1 μCi of DL-[1-^{14}C] Arg, was added to a series of flasks with center wells into which were placed filter papers

with 2 N KOH. After incubation at 37°C for 60 min, the reaction was stopped with 5% TCA. The filter paper that had adsorbed the $^{14}CO_2$ was evaluated for radioactivity in a liquid-scintillation counter.

RESULTS AND DISCUSSION

Cyclin A and B are Specifically Expressed in the S and G2/M Phases, Respectively, of the BY-2 Cell Cycle

Phase progression in the cell cycle of BY-2 cells was analyzed by flow cytometry (Fig. 1A); the results produced were similar to those previously reported with *Arabidopsis* (Porceddu et al., 2001) and other BY-2 cells (Jang et al., 2005). Quantification of ratios for individual cycle phases was based on data from the flow-cytometrical analysis (Fig. 1B), and showed that 69.8% of the cells were in the G_2 phase at 3 h after the release of aphidicolin. The respective percentages for cells in the G_1 and G_2 phases versus total cells dramatically changed from 1.5% and 69.7% at hour 6 to 59.8% and 27.2% at hour 9. Our results for ^{3}H-thymidine incorporation and the change in mitotic index (Fig. 2A) subsequently confirmed those obtained from the flow cytometry (Fig. 1B). Therefore, we can

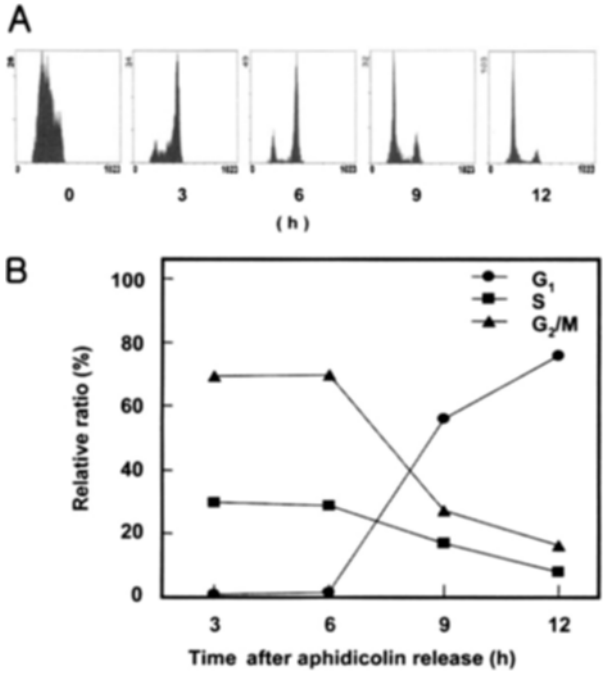

Figure 1. Changes in population of BY-2 cells after the release of aphidicolin. **A**, Flow-cytometric analysis. **B**, Relative ratio of cell population for each incubation period.

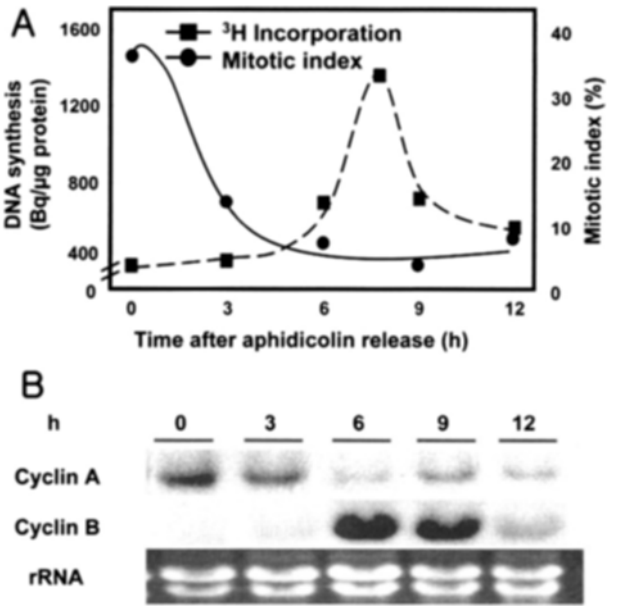

Figure 2. Changes in indicators that separate phases in cell cycle after the release of aphidicolin. **A**, H^3-thymidine incorporation and mitotic index. **B**, Expression of cyclins A and B.

conclude that the G_2 and M phases in BY-2 cells was at hour 3 and 8.

We also monitored changes in the expression of cyclin A (GenBank accession No. AF518250) and B (GenBank accession No. AF518251). Our data for the expression of cyclin A in the S phase (Fig. 2B) supports a previous result that A-type cyclin plays an essential function in DNA replication during that phase (Reichheld et al., 1999). In addition, the expression pattern observed here for cyclin B in the G_2/M phase coincides with the previous report (Sorrell et al. 2001).

Exogenous Polyamines Stimulate Accumulation of Cyclin A and B in BY-2 Cells

Based on our current data, the question arises as to whether flow cytometry could adequately analyze the changes in each cell cycle phase for BY-2 cells. Although polyamine starvation can prolong or arrest specific phases (Anehus et al., 1984; Chattopadhyay et al., 2002), treatment with polyamine can stimulate the progression of the cell cycle (Pohjanpelto et al., 1994). Therefore, it is possible that polyamine can control that progression. We conducted a series of experiments that assessed the cell population at each phase in the cycle, and compared the expression levels of cyclin A and B in BY-2 cells treated with polyamines. Exogenous applications did indeed

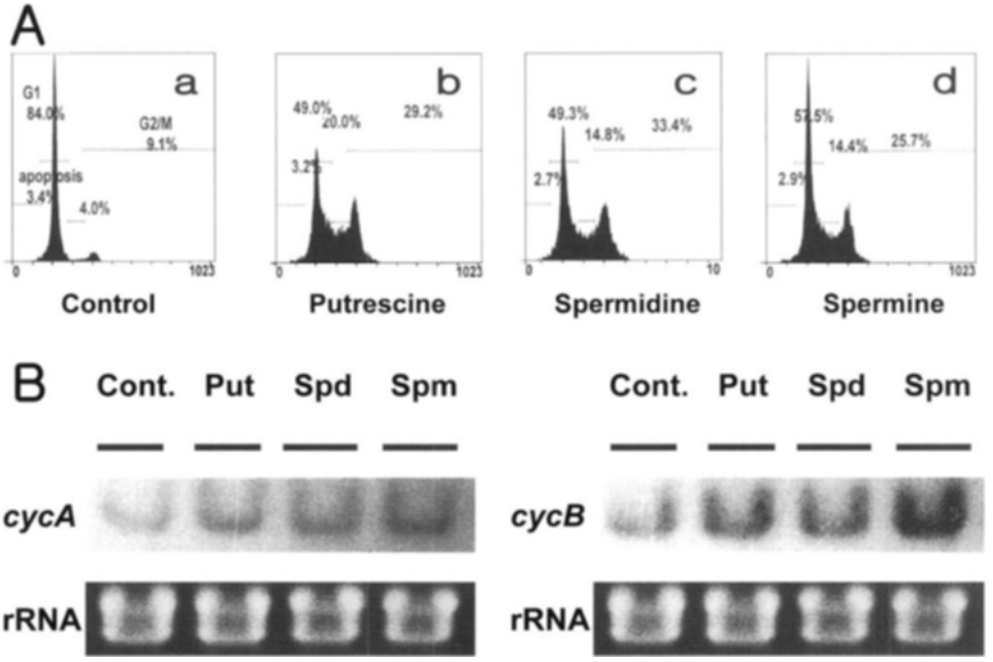

Figure 3. Effects of exogenous polyamines during phases of cell cycle. **A**,Changes in Polyamine in G_1, S, and G_2 phases from 4-day-old BY-2 cells. **B**, Effect of exogenous polyamines on expression of cyclins A and B in 4-day-old BY-2 cells.

induce the relative accumulation of cells during the S and G_2/M phases (20% of S and 28% of G_2 for putrescine, 14% of S and 33.4% of G_2 for spermidine, and 14% of S and 25.7% of G_2 for spermine) compared with the corresponding control (4.2% of S and 9.1% of G_2) (Fig. 3A). We also performed northern analysis of cyclin A and cyclin B genes, which were specifically expressed in the S and G_2/M phase, respectively, to confirm this accumulation while BY-2 cells were being incubated with exogenous polyamines. The ratios for cell populations determined by flow-cytometric phase separation coincided with the accumulation of both genes (Fig. 3B). These results support that measurements of cell populations via flow-cytometric phase separation can adequately describe changes in each phase of the cell cycle for BY-2 cells. In addition, our findings may extend to the control of gene expression related with cell cycle progression by exogenous polyamines.

Polyamine Content Depends on the Cell Cycle

Using the results shown in Figure 1 and 2, we constructed a scheme to illustrate phase separation during the cell cycle (Fig. 4A). Polyamines are essential for both the synthesis of DNA in the S phase and the stabilization of the DNA structure (Fillingame et al., 1975; van Dam et al., 2002), as well as for cell

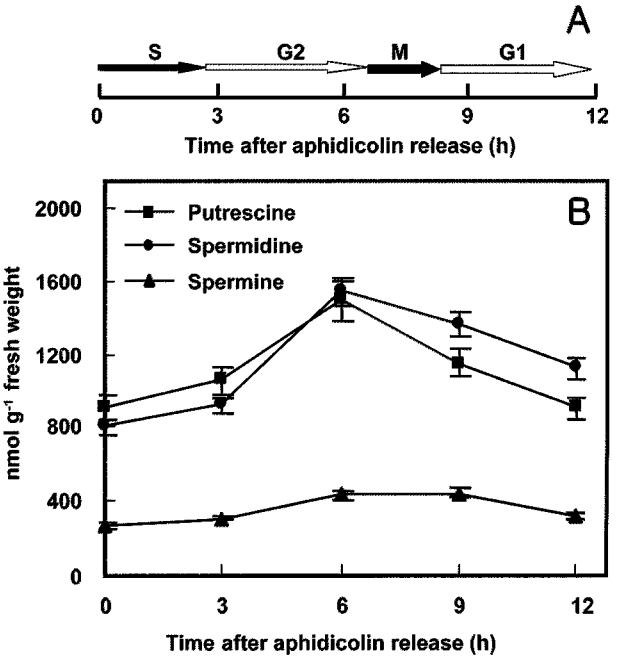

Figure 4. Comparison between phase separation scheme of aphidicolin-based synchronization **A** and changes in polyamines after releasing aphidicolin **B**.

growth and proliferation (Martin-Tanguy, 2001). These reports and our results in Figure 3 confirmed that cellular polyamines play an important role in the pro-

gression of the cell cycle. Because polyamine contents had not previously been analyzed in each phase, we did so here, and found that polyamine increased in the G_2 phase (Fig. 4B), which concurred with our schematic depiction of phase separations based on the time that elapsed after aphidicolin was released (Fig. 4A). Thus, about twice as much putrescine, spermidine and spermine were accumulated in the S (or late G_1) vs. the G_2 phases, whereas the ratio of (spermidine + spermine)/putrescine seemed to remain constant at each phase. In contrast, the doubling phase in animal systems differs, i.e., late S phase for putrescine, S/G2 transition for spermidine, and G1 and S phases for spermine (Oredsson, 2003). Therefore, our data support the conclusion that regulation of polyamine contents in BY-2 cells cannot and should not be explained by the general animal model (Oredsson, 2003).

Activations of Polyamine Biosynthetic Enzymes Depend on the Cell Cycle and SAMDC

To test whether changes in cellular polyamine were a direct result of enzymatic activation, we analyzed activities of key enzymes for polyamine biosynthesis. At the starting point, ADC, ODC, and SAMDC activities were 7.4, 11.2, and 5.5 nmol mg^{-1} protein h^{-1}; at their peaks, these levels were 22.6, 22.1, and 15.1 nmol mg^{-1} protein h^{-1}, respectively (Fig. 5A). However, compared with the phase period after the release of aphidicolin, peaks in ADC and ODC activities were localized to the G_2 phase (Fig. 5A). This indicates that the observed increase in putrescine at the G_2 phase may have directly resulted from the rise in ADC and ODC activities. In contrast, the doubling in levels of spermidine and spermine was not observed at the peak of SAMDC activity, which occurred 3 h earlier than for the others (Fig. 4B, 5A). Moreover, when the changes in ADC, ODC, and SAMDC activities were re-drawn from G_1 to M, ADC and ODC were activated in a bi-phasic manner during cell cycle progression. The first and second activations of ADC and ODC were at G_1/S and G_2, respectively, which coincides with the activation of ODC reported in animals (Oredsson, 2003). However, SAMDC activity in our BY-2 cells peaked, without marked fluctuation, during the S/G_2 transition. This suggests that cell-cycle changes in SAMDC activity may differ from those reported for the general animal model (Oredsson, 2003).

As analyzed by flow cytometry, the coincidence noted between the expression of cyclin (A and B)

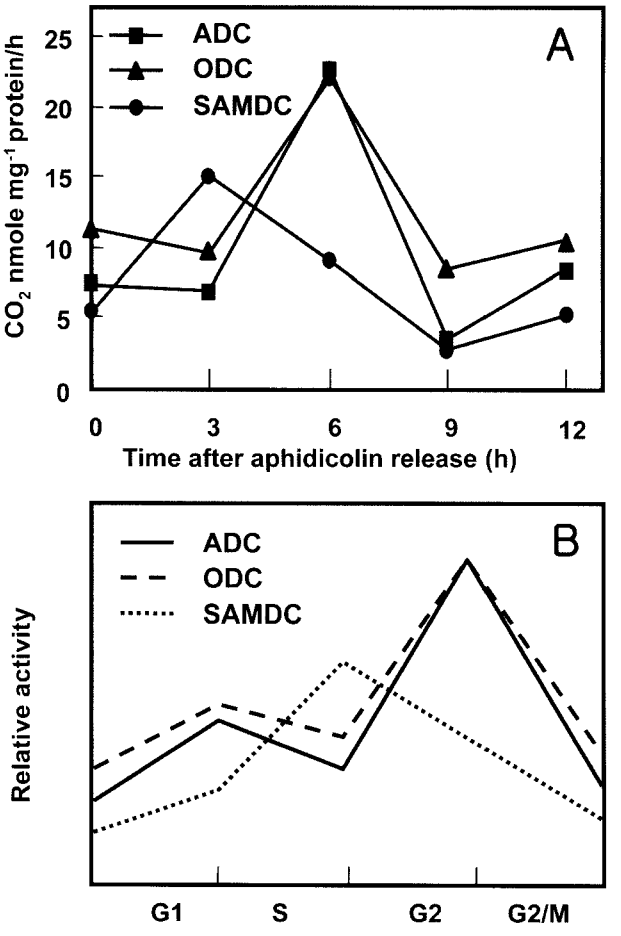

Figure 5. Fluctuations in levels of polyamine biosynthetic enzymes after the release of aphidicolin. **A**, Activities of ADC, ODC, and SAMDC. **B**, Schematic presentation of changes in ADC, ODC, and SAMDC activities with orderly progression of cell cycle. (Phase separation from G_1 to M was based on data in Figure 4A; relative activity is ratio of highest activity among ADC, ODC, and SAMDC).

with/without polyamine and the ratio of cell populations in each phase raises the possibility of using synchronized BY-2 cells to identify the role of polyamine in controlling the cell cycle in plants. In addition, our present data demonstrate that the general animal model (Oredsson, 2003) should not apply to BY-2 cells for explaining polyamine changes and SAMDC activation in the cell cycle.

Based on our knowledge, SAMDC activity in carnation may be controlled by transcriptional regulation (Mad Arif et al., 1994; Lee et al., 1997; Park et al., 2001; Kim et al., 2004) or translational regulation with uORF (Raney et al., 2002). This SAMDC activity is also highly regulated by a variety of physiological, hormonal, and environmental stimuli (Lee et al., 1996; Walden et al., 1997; Martin-Tanguy, 2001;

Marco and Carrasco, 2002). Therefore, our current work suggests three questions. First, can the mechanics for cyclin-dependent kinase regulation of the cell cycle (Menges and Murray, 2002) also be used to explain the changes in SAMDC activity during the cell cycle in plants? Second, is the increase in SAMDC activity related to translational inhibition by uORF? Finally, how do changes in endogenous polyamine, due to the over-expression of SAMDC, affect cell cycle progression in BY-2 cells? Further research should involve the use of SAMDC gene-transformed BY-2 cells to determine the role of SAMDC in the cell cycle.

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LITERATURE CITED

Ackermann JM, Pegg AE, McCloskey DE (2003) Drugs affecting the cell cycle via actions on the polyamine metabolic pathway. Pro Cell Cyc Res 5: 461-468

Ahn SM, Jin CD (2004) Protective role of exogenous spermidine against paraquat toxicity in radish chloroplasts. J Plant Biol 47: 338-347

Amarantos I, Zarkadis IK, Kalpaxis DL (2002) The identification of spermine binding sites in 16S rRNA allows interpretation of the spermine effect on ribosomal 30S subunit functions. Nucl Acid Res 30: 2832-2843

Anderson SE, Bastola DR, Minocha SC (1998) Metabolism of polyamines in transgenic cells of carrot expressing a mouse ornithine decarboxylase cDNA. Plant Physiol 116: 299-307

Anehus S, Pohjanpelto P, Baldetorp B, Längström E, Heyb O (1984) Polyamine starvation prolongs the S and G2 phases of polyamine-dependent (arginase-deficient) CHO cells. Cell Biol 4: 915-922

Borrell A, Bedford RT, Altabella T, Masgrau C, Tiburicio AF (1996) Regulation of arginine decarboxylase by spermine in osmotically stressed oat leaves. Physiol Plant 98: 105-110

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dyebinding. Anal Biochem 72: 248-254

Chattopadhyay MK, Tabor CW, Tabor H (2002) Absolute requirement of spermidine for growth and cell cycle progression of fission yeast (*Schizosaccharomyces pombe*). Proc Natl Acad Sci USA 99: 10330-10334

Ehsan H, Roef L, Witters E, Reichheld JP, van Bockstaele D, Inzé D, van Onckeln H (1999) Indomethacin-induced G1/S phase arrest of the plant cell cycle. FEBS Lett 458: 349-353

Fillingame RH, Jorstad CM, Morris DR (1975) Increased cellular contents of spermidine or spermine are required for optimal DNA synthesis in lymphocytes activated by concanavalin A. Proc Natl Acad Sci USA 72: 4042-4045

Jang SJ, Choi YJ, Park KY (2002) Effects of polyamines on shoot and root development in *Arabidopsis* seedlings and carnation cultures. J Plant Biol 45: 230-236

Jang SJ, Shin SH, Yee ST, Hwang B, Im KH, Park KY (2005) Effects of biotic stresses on cell cycle progression in tobacco BY-2 cells. Mol Cells 20: 136-141

Kim YJ, Lee SH, Park KY (2004) A leader intron and 115-bp promoter region necessary for expression of the carnation *S*-adenosylmethionine decarboxylase gene in the pollen of transgenic tobacco. FEBS Lett 578: 229-235

Lee MM, Lee SH, Park KY (1997) Characterization and expression of two members of the *S*-adenosylmethionine decarboxylase gene family in carnation flower. Plant Mol Biol 34: 371-382

Lee SH, Kim YB, Lee MM, Park KY (1996) The stimulation of arginine decarboxylation by dfmo in tobacco suspension cultured cells. J Plant Biol 39: 107-112

Mad Arif SA, Taylor MA, George LA, Butler AR, Burch LR, Davis HV, Stark MJR, Kumar A (1994) Characterization of the *S*-adenosylmethionine decarboxylase (SAMDC) gene of potato. Plant Mol Biol 26: 327-338

Marco F, Carrasco P (2002) Expression of the pea *S*-adenosylmethionine decarboxylase gene is involved in developmental and environmental responses. Planta 214: 641-647

Martin-Tanguy J (2001) Metabolism and function of polyamines in plants: Recent development (new approaches). Plant Growth Reg 34: 135-148

Menges M, Murray JAH (2002) Synchronous *Arabidopsis* suspension cultures for analysis of cell-cycle gene activity. Plant J 30: 203-212

Nagata T, Kumagai F (1999) Plant cell biology through the window of the highly synchronized tobacco BY-2 cell line. Meth Cell Sci 21: 123-127

Nagata T, Nemoto Y, Hasezawa S (1992) Tobacco BY-2 cell line as the "Hela" cell in the cell biology of higher plants. Intl Rev Cytol 123: 1-30

Oredsson SM (2003) Polyamine dependence of normal cell-cycle progression. Biochem Soc Trans 31: 366-370

Park KY, Lee SH (1994) Effects of ethylene and auxin on polyamine contents in suspension-cultured cells. Physiol Plant 90: 382-390

Park WK, Lee SH, Park KY (2001) Characterization and light-induced expression of the *S*-adenosylmethionine

decarboxylase gene from *Ipomoea nil*. J Plant Biol **44**: 111-117

Pohjanpelto P, Nordling S, Knnutila S (1994) Flow cytometric analysis of the cell cycle in polyamine-depleted cells. Cytometry **16**: 331-338

Porceddu A, Stals H, Reichheld JP, Segers G, De Veylder L, Barroco RP, Casteels P, van Montagu M, Inze D, Mironov V (2001) A plant-specific cyclin-dependent kinase is involved in the control of G2/M progression in plants. J Biol Chem **276**: 36354-36360

Raney A, Lynn LG, Mize GJ, Morris DR (2002) Regulated translation termination at the upstream open reading frame in S-adenysylmethionine decarboxylase mRNA. J Biol Chem **277**: 5988-5994

Reichheld JP, Vernoux T, Lardon F, Van Montagu M, Inzé D (1999) Specific checkpoints regulate plant cell cycle progression in response to oxidative stress. Plant J **17**: 647-656

Setiady YY, Sekine M, Hariguchi N, Kouchi H, Shinmyo A (1996) Molecular cloning and characterization of a cDNA clone that encodes a cdc2 homolog from *Nicotiana tabacum*. Plant Cell Physiol **37**: 369-376

Sorrell DA, Menges M, Healy JMS, Deveaux Y, Amano C, Su Y, Nakagami H, Shinmyo A, Doonan JH, Sekine M, Murray JAH (2001) Cell cycle regulation of cyclin-dependent kinases in tobacco cultivar bright yellow-2 cells. Plant Physiol **126**: 1214-1223

Świąte A, Lenjou M, Van Bockstaele D, Inzé D, Van Onckelen H (2002) Differential effect of jasmonic acid and abscisic acid on cell cycle progression in tobacco BY-2 cells. Plant Physiol **128**: 210-211

van Dam L, Korolev N, Nordenskiöld L (2002) Polyamine: Nucleic acid interactions and the effects on structure in oriented DNA fibers. Nucl Acids Res **30**: 419-428

Walden R, Cordeiro A, Tiburicio AF (1997) Polyamines: Small molecules triggering pathways in plant growth and development. Plant Physiol **113**: 1009-1013

Wi SJ, Park KY (2002) Antisense expression of carnation cDNA encoding ACC synthase or ACC oxidase enhances polyamine content and abiotic stress tolerance in transgenic tobacco plants. Mol Cells **13**: 209-220