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Benzyladenine arrests cell cycle progression in G1 phase in tobacco BY-2 cells preceding induction of cell death

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Abstract Micromolar concentrations of exogenously applied cytokinins can inhibit the growth of tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cells and induce cell death. To determine whether the antiproliferative and cell death-inducing mechanisms of cytokinins are linked, we investigated the inhibition of cell cycle transition by the cytokinin benzyladenine using BY-2 cells. Mitotic index and flow cytometric analyses revealed that benzyladenine decreased the rate of cells entering the G2 and M phases a few hours after treatment of unsynchronized cells. Additionally, for cells synchronized in M phase, benzyladenine delayed (at 10 μM) or arrested (at 50 μM) cell cycle progression at G1. Expression patterns of cell cycle-related genes (*PCNA* and *A3*-, *A1*- and *B1-type cyclins*) also indicated G1 arrest by a 50 μM benzyladenine treatment at the M or G1 phase. Cell cycle arrest was detected prior to the induction of cell death by the treatment. Increase in number of dead cells was observed 16 h after each treatment at M or G1, suggesting that cell death may not be induced when cells reach a specific time point in G1 phase but, rather, in a time-dependent manner following benzyladenine treatment.

Key words: Cell cycle, cytokinin, *Nicotiana tabacum* BY-2 cells, programmed cell death, synchronous culture.

Cytokinins are plant hormones that regulate various physiological events during plant growth and development. They were discovered based on their ability to promote cell division in cultured tobacco cells (Miller et al. 1955); however, their regulatory effects on the cell cycle have since been reported. In *Arabidopsis*, cytokinins promote the expression of CYCD3, a D-type cyclin that regulates the G1 to S phase transition (Soni et al. 1995; Riou-Khamlichi et al. 1999). In tobacco (*Nicotiana tabacum* L. cv. Bright Yellow 2 [BY-2]) cells, the endogenous zeatin-type cytokinin level increases during S phase and M phase (Redig et al. 1996). Lovastatin, an inhibitor of cytokinin biosynthesis, blocks the entry of cells into mitosis, whereas exogenous application of *trans*-zeatin restores the rate of cellular division (Laureys et al. 1998). These data indicate that cytokinins also regulate the G2 to M phase transition. The transition from G2 to M phase is regulated by the activation of cyclin-dependent kinases (CDKs). In tobacco BY-2 cells, activation of $p34^{cdc2}$-like H1 histone kinase (a CDK) by dephosphorylation is needed for G2-M transition. Cytokinins are thought to activate a Cdc25-like phosphatase, an activator of CDK, which dephosphorylates $p34^{cdc2}$-like H1 histone kinase (Zhang et al. 1996).

Suppressive effects on cellular proliferation have also been demonstrated for cytokinins. Micromolar concentrations of exogenously applied cytokinins inhibit the growth of tobacco callus (Skoog et al. 1967) and cultured sycamore cells (MacKenzie et al. 1972). Additionally, cytokinins interrupt the progression of the cell cycle at specific phases. Van't Hof (1968) reported that exogenous kinetin impairs the transition through G1 and G2 in pea root meristem cells, and Li et al. (2006) showed that cytokinins block the transition from G2 to M phase, but not from G1 to S phase, in *Arabidopsis* root pericycle founder cells, inhibiting lateral root formation. These data indicate that the growth-inhibitory effects of cytokinins are caused by a delay (or arrest) at specific phases of the cell cycle; however, which phase is impaired may vary by tissue or growth conditions.

Several types of cytokinins and cytokinin ribosides have been shown to induce cell death and inhibit cellular proliferation at micromolar concentrations in tobacco, carrot, and *Arabidopsis* (Mlejnek and Prochazka 2002; Mlejnek et al. 2003, 2005; Carimi et al. 2003, 2004, 2005; Bolduc et al. 2007). The cell death induced by cytokinins is similar to apoptosis in animals; cytoplasmic shrinkage, chromatin condensation, and genomic DNA fragmentation into nucleosomes have all been observed.

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; FAM-Asp-Glu-Val-Asp-FMK, fluoromethyl ketone peptide inhibitor of caspase-3/7; FLICA, fluorochrome inhibitor of caspase; G1, gap 1; G2, gap 2; M, mitosis; NO, nitric oxide; PCD, programmed cell death; ROS, reactive oxygen species; S, synthesis.

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Evidence also suggests that cytokinins induce cell death in plants via biochemical pathways involving the activation of caspase-like proteases, production of reactive oxygen species (ROS) or nitric oxide (NO), release of cytochrome *c* from mitochondria, and up-regulation of Bax inhibitor-1 expression and cytosolic Ca^{2+} fluxes, which are similar to those of animal apoptosis. Therefore, cytokinin-mediated cell death is considered to be a programmed cell death (PCD) with conserved pathways in plants and animals, which is also observed after stress treatments such as heat shock (Vacca et al. 2004, 2006) or hydrogen peroxide (Houot et al. 2001). However, the pathways through which cytokinins induce cell death are mostly unknown. Moreover, there remains one problem that whether the antiproliferative mechanism, which seems to occur simultaneously with the cell death, is correlated to the induction of cell death.

Recent studies have suggested that the cell cycle regulates the induction of cell death. Oxidative stress mediated by menadione slows DNA replication and delays cellular entry into mitosis by inhibiting the G1 to S phase transition, and a high concentration of menadione induces cell death in tobacco BY-2 cells (Reichheld et al. 1999). Ethylene treatment increases BY-2 cell mortality at the G2/M phase (Herbert et al. 2001). The mechanism of elicitor-induced cell death has been described as cell cycle-regulated cell death. Cryptogein, a proteinaceous elicitor derived from *Phytophthora cryptogea*, induces cell cycle arrest at G1 and G2 prior to the induction of cell death in BY-2 cells (Kadota et al. 2004). Cryptogein-induced cell death occurred only in those cells that recognized the elicitor during G1 or S phase, whereas elicitor recognition during G2 or M phase did not induce cell death. This difference in cell death-inducing activity is caused by different patterns of MAP kinase activation and ROS production (Kadota et al. 2005). Therefore, cryptogein-induced cell death may be the first model of cell cycle-dependent cell death. However, in the case of cytokinin-induced cell death, it is unknown whether the mechanism of induction is related to the cell cycle.

We investigated the inhibitory effect of the cytokinin benzyladenine on tobacco BY-2 cell growth by determining its effect on cell cycle progression. We also examined whether the growth-inhibiting effect of benzyladenine was correlated with the induction of cell death to clarify the cell death regulatory function of cytokinins.

Materials and methods

Plant material

Tobacco BY-2 (*N. tabacum* L. cv. BY-2) cells were cultured in modified Linsmaier and Skoog (LS) medium (Nagata et al. 1992) on a rotary shaker at 120 rpm in the dark at 25°C. The cultures were maintained by subculturing 1 ml of stationary-phase cells in 100 ml of fresh medium every seven days.

Cytokinin treatment

The cytokinins benzyladenine, isopentenyladenine, kinetin, and *trans*-zeatin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Each cytokinin was dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted in distilled water to obtain 1 mM stock solution. The solution was sterilized by passage through a 0.22-μm filter unit and then added to the cell suspension. For the treatment of unsynchronized cells, 2-day-old cultures were used after subculturing. The final concentration of DMSO in the culture medium was approximately 0.1% (v/v). Exposure was carried out for a given period of time under the same conditions. Cell density was calculated by counting the number of cells in 20 μl of the suspension, which was diluted with LS medium as needed, under a microscope.

Cell viability assay

Selective staining with Evans blue (Wako Chemicals, Osaka, Japan) was used to discriminate between dead and living cells (Turner and Novacky 1974). Evans blue was added to each cell suspension at a final concentration of 0.02 μg ml^{-1}. After 15 min, the percentage of blue cells, indicating a loss of integrity at the plasma membrane, was determined under a microscope.

Assay of genomic DNA fragmentation

Cells were harvested, frozen in liquid N_2, and stored at −80°C. Genomic DNA was extracted from the cells as described by Liu et al. (1995). For detection of DNA fragmentation, 10 μg of extracted DNA was electrophoresed on a 2% (w/v) agarose gel, then stained with ethidium bromide, and the DNA fragments were visualized under UV light.

Detection of active caspase-like protease

Active caspase-like protease was detected using a CaspaTag caspase-3/7 *in situ* assay kit (Chemicon International Inc., Temecula, CA, USA). A fluorochrome inhibitor of caspase (FLICA) reagent, carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-3/7 (FAM-Asp-Glu-Val-Asp-FMK), contained in the kit, selectively binds to active caspase-3-like protease, which recognizes the four amino acids Asp-Glu-Val-Asp. FLICA reagent was added to cell suspensions and incubated for 1 h. The percentage of cells containing the active caspase-like protease, which produced green fluorescence in their nuclei, was evaluated under a fluorescence microscope.

Cell cycle synchronization

Stationary-phase tobacco BY-2 cells were diluted at a ratio of 1 : 10 in fresh LS medium containing 5 μg ml^{-1} aphidicolin (Wako Chemicals). After 24 h of culture, the aphidicolin was removed by extensive washing with LS medium, and the cells were resuspended in fresh medium. To analyze the post-mitotic phases, 0.8 μg ml^{-1} propyzamide (Wako Chemicals) was added prior to pre-prophase, which was approximately 6 h after the removal of aphidicolin. The cultures were maintained for 4 h before the removal of propyzamide through extensive washing.

Determination of the mitotic index

Cells were fixed in 2% (v/v) glutaraldehyde and their nuclei were stained with 1 μg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, Wako Chemicals). The mitotic index was determined by measuring the percentage of cells undergoing mitosis under a fluorescence microscope.

Flow cytometric analysis

Cells were harvested, fixed in 70% ethanol, and stored at 4°C. Each cell pellet was rehydrated with Galbraith's buffer (45 mM $MgCl_2$, 30 mM Na-citrate, 20 mM MOPS, and 1 g l^{-1} Triton X-100, pH 7.0; Galbraith et al. 1983) and chopped with a razor blade. The nuclei were separated from the cells by filtration through a 100-μm nylon filter. For nuclear staining, 2 μg ml^{-1} DAPI was added to the nuclear suspension. Flow cytometry was performed using a Ploidy Analyzer PA (Partec GmbH, Münster, Germany). Counts with weak fluorescence intensity contained fluorescence from DNA fragmented during the extraction of the nuclei; thus, all counts below a cut-off value (much weaker fluorescence intensity than that of the G1 nuclei) were disregarded.

RNA extraction and RT-PCR

Cells were harvested, frozen in liquid N_2, and stored at −80°C. Total RNA was extracted from the cells using an RNAqueous Kit (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). PCR was carried out using a 1-μl aliquot of the cDNA as a template in a 20-μl reaction volume with a GeneAmp PCR Kit (Applied Biosystems, Foster City, CA, USA) and gene-specific primers. The primer sequences and predicted product sizes are as: *PCNA* (GenBank accession number AB025029, 5′-ATGTTGGA-GTTGCGGCTTGTTCAGG-3′ and 5′-CATCTTGCTTGCTC-TCTTGCTTGCC-3′, 896 bp), *Nicta;CycA3;3* (X93467, 5′-GGAATTTGTCCAATCAGATTCAG-3′ and 5′-TTTGTTCG-AGGCCCAC-3′, 798 bp), *Nicta;CycA1;1* (D50735, 5′-CTCGGACGGATCATTGGTCTC-3′ and 5′-TTCAAGTAA-TTTAAAACGGTAGAC-3′, 639 bp), *Nicta;CycB1;3* (D89635, 5′-GGAGTTCTACCGGTTAAAGG-3′ and 5′-CCATCTGT-GCATTGGAAAC-3′, 733 bp) and *Actin* (AB158612, 5′-TCTGGCATCATACCTTTTACAATGAG-3′ and 5′-AAACA-TTGTTGTTCCACCACTAAGG-3′, 665 bp). *Actin* was used as an internal standard. The PCR conditions were as follows: 94°C for 30 s, followed by 23 (*PCNA*, *Nicta;CycA1;1* and *Actin*) or 25 cycles (*Nicta;CycA3;3* and *Nicta;CycB1;3*) of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The products were separated on 1% (w/v) agarose gels, stained with ethidium bromide, and visualized under UV light.

Results

We determined the effects of four cytokinins, benzyladenine, isopentenyladenine, kinetin and *trans*-zeatin on cell growth and viability in tobacco BY-2 cells. All of the cytokinins prevented an increase in cell number in a concentration-dependent manner at concentrations exceeding 5 μM and they completely blocked cellular proliferation at 50 μM (Figure 1A). Evans blue staining revealed that the percentage of dead cells was dramatically increased 2 days after treatment with each cytokinin at 30–50 μM (Figure 1B). Additionally, several indicators of apoptosis-like cell death, including genomic DNA fragmentation (Figure 2), caspase-like protease activity (Figure 3A, B), cytoplasmic shrinkage (Figure 3A) or nuclear degradation (data not shown) were observed during cell death. Benzyladenine had the strongest cell death-inducing effect of the four cytokinins; therefore, we used benzyladenine in our subsequent experiments. Bolduc et al. (2007) reported similar effects on cell growth and viability for these four cytokinins on cultured tobacco (*N. tabacum* cv. Xanthi) cells.

To assess whether cytokinins interrupt the cell cycle at a specific point, BY-2 cells were treated with 50 μM benzyladenine, and the effect on cell cycle progression was determined. Fluorescence microscopy of DAPI-stained cells revealed that benzyladenine decreased the number of mitotic cells (Figure 4A). We also used flow cytometry to examine the effect of benzyladenine on other phases of the cell cycle. Benzyladenine decreased the number of cells in the G2 and M phases and slightly increased those in the G1 and S phases (Figure 4B, C), suggesting that high concentrations of benzyladenine mainly inhibit cell cycle progression prior to entering G2. Therefore, in this study, we focused on the inhibitory effect on G1-S phase. A slight increase in the number of dead cells was detected 24 h after benzyladenine treatment (Figure 1B), but not at 12 h after treatment (data not shown), indicating that inhibition of the cell cycle occurred prior to the induction of cell death.

To determine the effect of benzyladenine on the progression of the cell cycle in greater detail, BY-2 cells were synchronized at prometaphase with aphidicolin and propyzamide (Nagata et al. 1992) and then treated with benzyladenine. First, 10 or 50 μM benzyladenine was added at 0 h (M phase) after propyzamide release. Cell cycle progression was then monitored based on the mitotic index (Figure 5A) and flow cytometry (Figure 5B). The control cells progressed from M phase to G1 phase in approximately 4–8 h; they then continued from S phase to G2, reaching their second mitotic peak within 18 h after treatment. In contrast, cells treated with 10 μM benzyladenine exhibited an apparent delay of the second mitotic peak (22 h after treatment) and a reduction in the maximum mitotic index (Figure 5A). They also exhibited a prolonged G1 phase (at least 2 h) compared to the controls (Figure 5B). Once the cells entered S phase (12 h), they progressed to G2 phase without delay (14–16 h), suggesting that the transition from S phase to G2 may not be inhibited by benzyladenine. When treated with 50 μM benzyladenine, the cells were completely arrested at G1 (Figure 5B). No effect was observed on

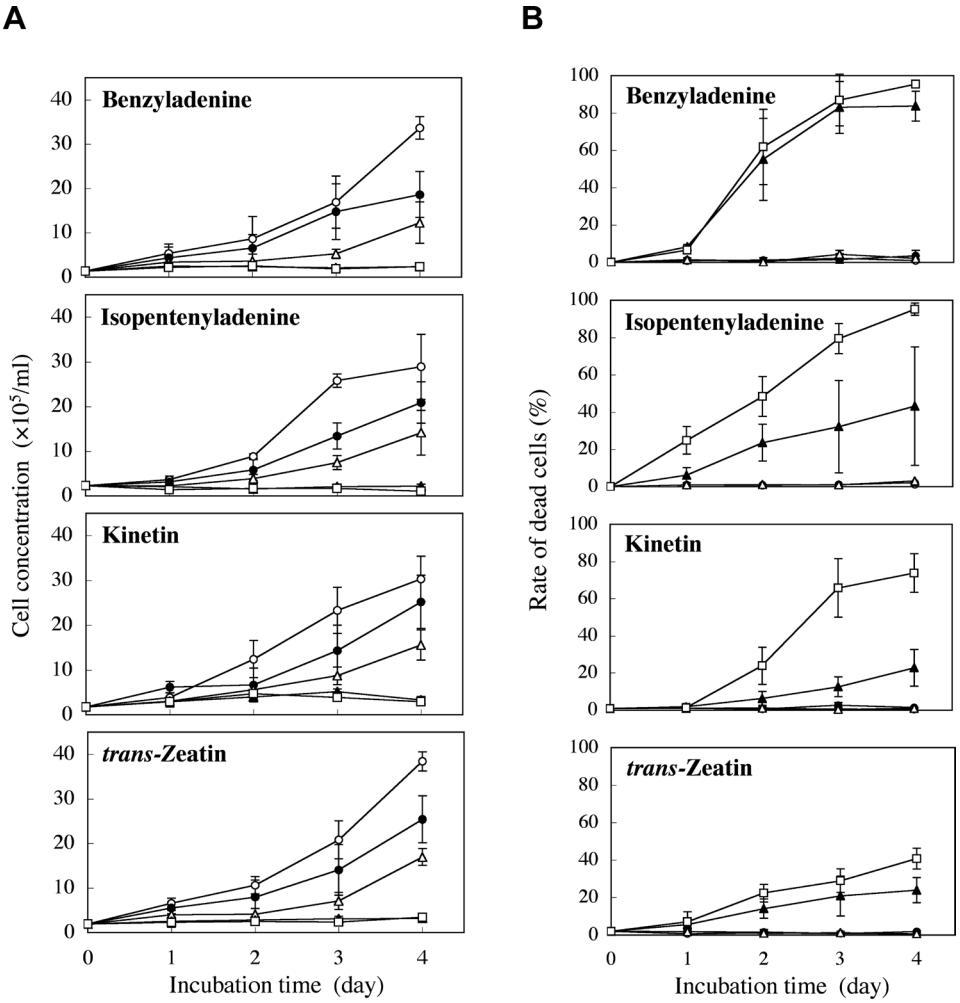

Figure 1. The effects of four cytokinins: benzyladenine, isopentenyladenine, kinetin and *trans*-zeatin on tobacco BY-2 cell growth and viability. Cells were treated with 5 μM (closed circles), 10 μM (open triangles), 30 μM (closed triangles), or 50 μM (open squares) cytokinin. Cells treated with distilled water containing 0.1% (v/v) DMSO were used as controls (open circles). The data represent the mean±SD of three replicate experiments. (A) Cytokinin-induced growth inhibition. At the indicated time intervals, cell concentration was evaluated under a microscope. (B) Cytokinin-induced cell death. At the indicated time intervals, the cells were stained with Evans blue and the number of dead (blue-stained) cells was evaluated under a microscope. At least 300 cells were observed.

the transition in mitotic index from 0 to 8 h (the M phase to G1 transition) by 10 or 50 μM benzyladenine (Figure 5A).

We also treated the cells with 50 μM benzyladenine at 4 h (G1 phase) after the propyzamide release. Treatment at G1 induced an immediate cell cycle arrest during G1 (Figure 5B) with the loss of the second mitotic peak (Figure 5A).

The effects of benzyladenine on the expression of four genes encoding cell cycle-related proteins (proliferating cell nuclear antigen [*PCNA*], A3-type cyclin [*Nicta;CycA3;3*], A1-type cyclin [*Nicta;CycA1;1*] and B1-type cyclin [*Nicta;CycB1;3*]), whose expression patterns are regulated by the transition of specific phases, were investigated by RT-PCR (Figure 6) using cells synchronized at M phase and treated with 50 μM benzyladenine at 0 h (M phase) or 4 h (G1 phase), whose cell cycle transition pattern is described in Figure 5. Expression of *PCNA* and A3-type cyclin genes starts at the G1 phase and their expression levels reach a maximum in S phase (Reichheld et al. 1996; Sekine et al. 1999; Jang et al. 2005); thus, we chose these two genes as markers of the G1-S transition. The accumulation of gene transcripts in control cells started at 4–6 h (*PCNA*) or at 6–8 h (*Nicta;CycA3;3*), and reached their expression peak at 10 h, when several cells entered S phase (Figure 5B). In contrast, cells treated with 50 μM benzyladenine at M phase (0 h) showed no accumulation of *PCNA* or *Nicta;CycA3;3* transcripts. In cells treated with benzyladenine during G1 (4 h), the expression level of these two genes increased up to 10 h after treatment; however, it was slightly less than that observed for the controls. After 10 h, the level of transcription was sustained in the benzyladenine-treated cells, whereas it gradually decreased in the controls. These results indicate that the expression of *PCNA* and *Nicta;CycA3;3*

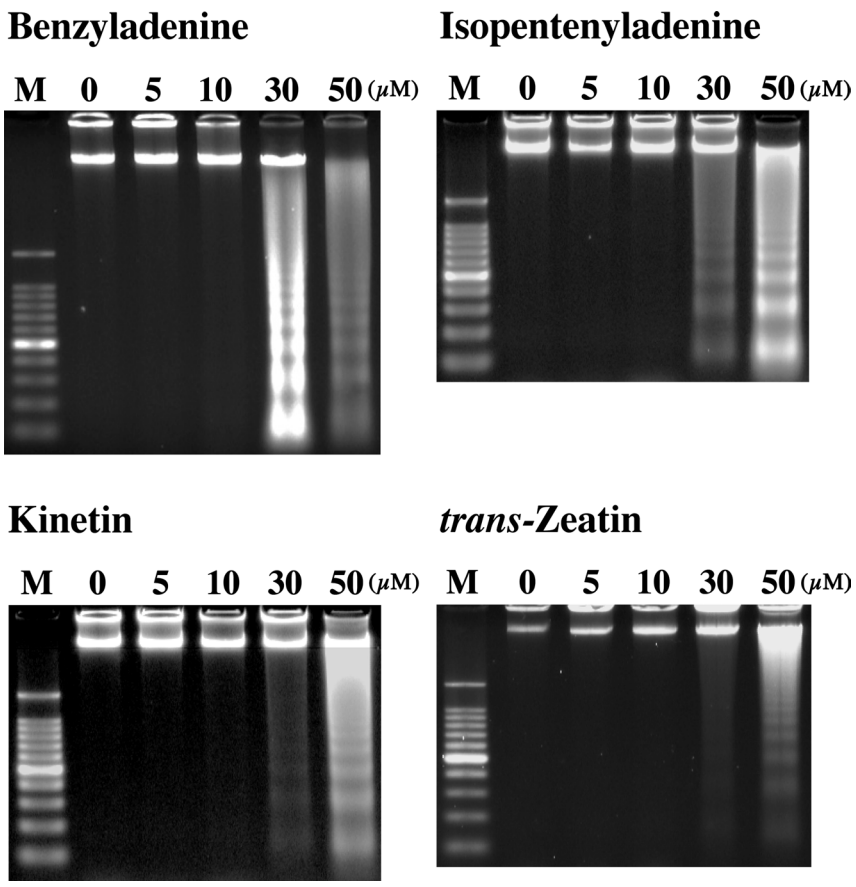

Figure 2. Genomic DNA fragmentation by cytokinins. Genomic DNA from cells incubated with 5, 10, 30 or 50 μM cytokinin (benzyladenine, isopentenyladenine, kinetin and *trans*-zeatin) for four days was electrophoresed on 2% (w/v) agarose gels. Cells treated with distilled water containing 0.1% (v/v) DMSO were used as controls (referred to as 0 μM treatment).

was not arrested by benzyladenine treatment at 4 h, although our flow cytometric results indicate cell cycle arrest at the G1 phase (Figure 5B).

Transcripts of the A1-type cyclin (*Nicta;CycA1;1*) accumulated from the S phase (Setiady et al. 1995) and those of the B1-type cyclin (*Nicta;CycB1;3*) accumulated from the G2 phase (Ito et al. 1997), and both types of transcripts were present until the end of M phase. Therefore, we used these two genes as the markers for the S-M and G2-M transitions, respectively. In the control cells, a high level of *Nicta;CycA1;1* expression was exhibited at 0–2 h (M phase) and at 12–16 h (S-M phase), and that of *Nicta;CycB1;3* at 0–2 h and at 14–16 h (G2-M phase). By contrast, in the cells treated with benzyladenine during M phase (0 h) or G1 (4 h), *Nicta;CycA1;1* and *Nicta;CycB1;3* transcription after S phase was completely suppressed. The absence of transcription may have resulted from arrest at G1 (Figure 5B). Benzyladenine treatment during M phase (0 h) did not affect the accumulation levels of *Nicta;CycA1;1* or *Nicta;CycB1;3* transcripts at 0–2 h, nor did it interrupt the decrease in transcription after 2 h.

To assess the time point where cell death occurred, we determined the proportion of dead cells by Evans blue staining following treatment with 50 μM benzyladenine at 0 h (M phase) or 4 h (G1 phase) after propyzamide release (Figure 7), the cell cycle transition patterns of which are demonstrated in Figure 5. By each treatment, an apparent increase in the number of dead cells was first detected at 16 h after each addition of benzyladenine. After the start of cell death, the percentage of dead cells increased over time following benzyladenine exposure. This result revealed that benzyladenine-induced cell death by treatment at M or G1 phase did not start at a specific point during G1, but in a time-dependent manner after addition of benzyladenine. It suggests that cell death can be induced by recognition of benzyladenine, during either M or G1 phase, and cell death-induction may not require entering a specific phase, as was observed for elicitor-induced cell death (Kadota et al. 2004).

Discussion

Although cytokinins promote cell division and delay leaf senescence, their growth-inhibitory and cell death-inducing effects have been reported in several plant species (Mlejnek and Prochazka 2002; Mlejnek et al.

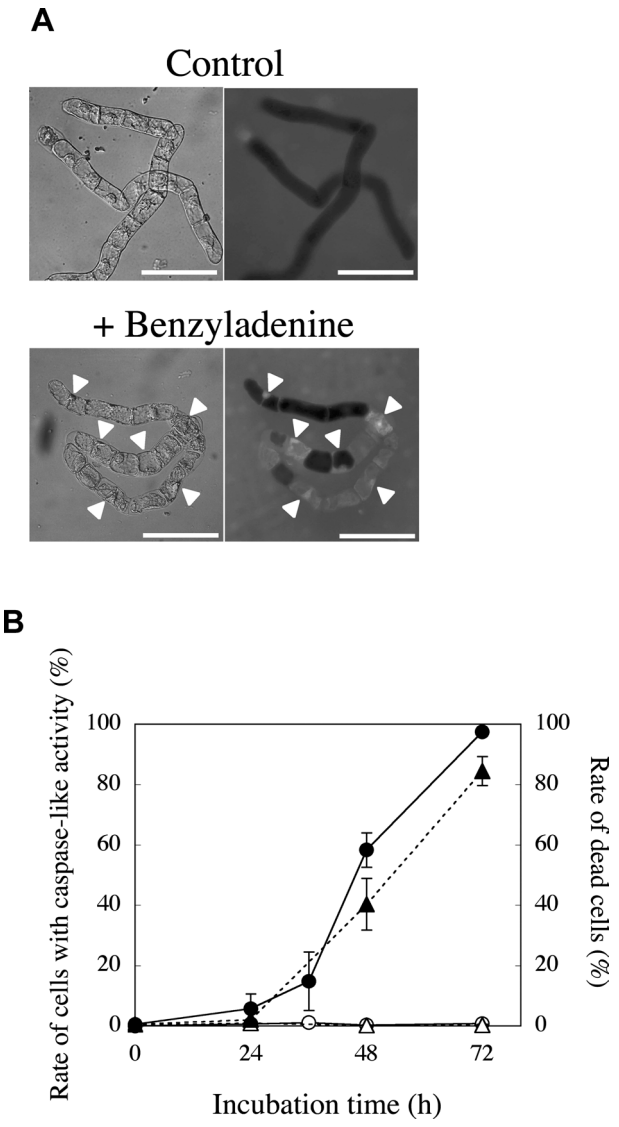

Figure 3. Activation of caspase-like protease by benzyladenine. 50 μM benzyladenine or distilled water containing 0.1% (v/v) DMSO (control) was applied to cell suspensions and incubated for 72 h. For detection of active caspase-like protease, FLICA (fluorochrome inhibitor of caspase; FAM-Asp-Glu-Val-Asp-FMK) reagent contained in the CaspaTag caspase-3/7 *in situ* assay kit, which specifically binds to the active caspase-3-like protease, was added to cell suspensions which were then processed for fluorescence microscopy. (A) Cells labeled with FLICA reagent 48 h after treatments (left, bright field; right, fluorescence). Arrowheads show the cells with nuclei containing active caspase-like protease. Figures represent typical examples. Bars represent 100 μM. (B) Percentage of cells containing active caspase-like protease in control (open circles) and benzyladenine-treated cells (closed circles), and rate of dead cells in control (open triangles) and benzyladenine-treated cells (closed triangles). The data represent the mean±SD of three replicate experiments. At the indicated time intervals, the number of cells producing green fluorescence in their nuclei (cells with active caspase-like protease) and those stained with Evans blue (dead cells) were evaluated under a fluorescence or a bright field microscope. At least 300 cells were observed for each determination.

2003, 2005; Carimi et al. 2003, 2004, 2005; Bolduc et al. 2007). Nevertheless, the mechanism underlying their negative effect on cell growth during cell death induction is unknown. Previous studies have shown that cytokinins inhibit cell cycle progression at G1 and G2 in pea root meristem cells (Van't Hof 1968) and at G2 in *Arabidopsis* root pericycle founder cells (Li et al. 2006); however, the mechanism of cell death was not addressed in these studies. Therefore, in this study, we focused on the mechanism underlying the antiproliferative effect of cytokinins during the induction of cell death in tobacco BY-2 cells.

In randomly dividing cells, inhibition of the cell cycle occurred hours after the addition of benzyladenine (Figure 4A, B), quite a bit earlier than other events that indicate cell death, such as change in the integrity of the cytoplasmic membrane (Figure 1B), genomic DNA fragmentation (Figure 2; the time-dependent data are not shown), or activation of caspase-like protease (Figure 3B). Additionally, in synchronized cells, a delay (at 10 μM) or arrest (at 50 μM) of the cell cycle at G1 was induced by benzyladenine treatment (Figure 5A, B), suggesting that benzyladenine mainly inhibits cell cycle transition at G1 phase.

We also examined the expression patterns of several cell cycle-related genes during benzyladenine treatment (Figure 6). The accumulation of *PCNA* and *Nicta;CycA3;3* mRNA was completely inhibited by benzyladenine during M phase (0 h after propyzamide release). According to our RT-PCR results, benzyladenine suppressed the expression of the *PCNA* gene for up to 4 h in cells treated at 0 h, as in the earliest case. In contrast, when benzyladenine was applied at G1 (4 h), the level of *PCNA* and *Nicta;CycA3;3* transcript accumulation increased until 10 h, and the level of expression was maintained until 16 h. Interestingly, despite the similarities (G1-phase arrest) shown by our flow cytometric results (Figure 5B), the expression patterns of *PCNA* and *Nicta;CycA3;3* were different in cells treated with benzyladenine at 0 h and 4 h. *PCNA* expression is not induced by DNA synthesis; therefore, it is possible that its expression occurs even when progression through S phase is blocked. Indeed, *PCNA* expression was not suppressed in synchronized periwinkle cells treated with aphidicolin, a DNA synthesis inhibitor, during the G1 phase (Kodama et al. 1991). Additionally, a decrease in the level of mouse *PCNA* at the end of S phase was prevented by the inhibition of DNA synthesis (Bravo 1986), suggesting that the sustained level of *PCNA* transcription between 10 and 16 h may have been caused by the absence of an S phase. Furthermore, in synchronized periwinkle cells, treatment with aphidicolin or anisomycin (a protein synthesis inhibitor) at G1 prevented DNA synthesis and altered the expression pattern of *PCNA* (Kodama et al.

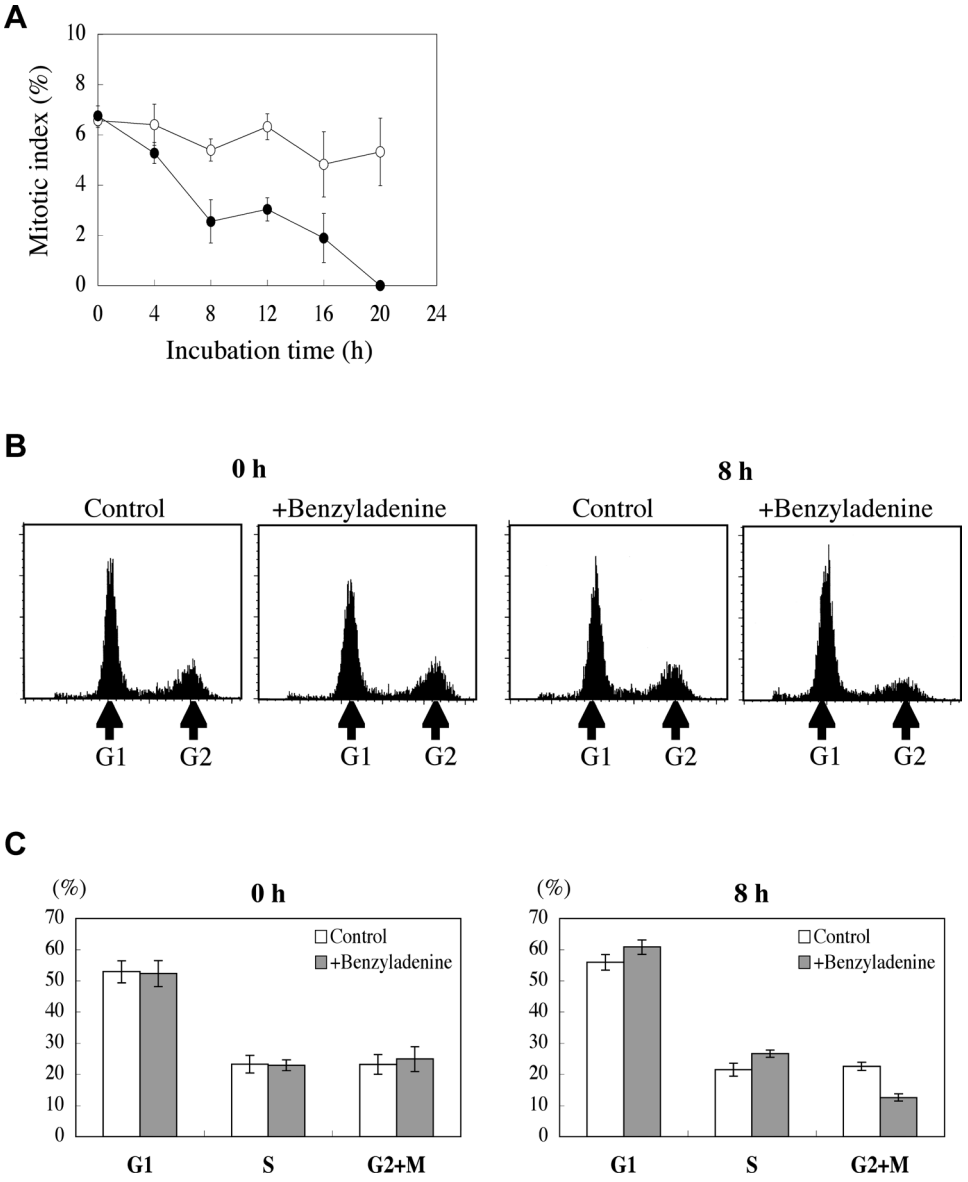

Figure 4. The effect of benzyladenine on cell cycle progression in unsynchronized tobacco BY-2 cells. Benzyladenine (50 μM) or distilled water containing 0.1% (v/v) DMSO (control) was applied to cell suspensions. (A) Changes in the mitotic index after the application of benzyladenine (closed circles) and in the controls (open circles). The cells were stained with DAPI, and the frequency of dividing cells was determined using a fluorescence microscope. At least 300 cells were observed. The data represent the mean±SD of three replicate experiments. (B) Change in the distribution of cells in the G1, S, or G2+M phase. Flow cytometry was performed using 5000 nuclei at 0 h and 8 h after the application of benzyladenine or water containing DMSO. Representative results of three replicate experiments are shown. (C) The percentage of cells in the G1, S, or G2+M phase in (B) was evaluated using the Cell Cycle Analysis mode of the flow cytometer. The data represent the mean±SD of three replicate experiments. Significant difference ($P<0.05$) between control and benzyladenine-treated cells was observed in each phase in 8 h.

1991). *PCNA* transcript accumulation was delayed and the maximum level of accumulation was reduced by the addition of aphidicolin, whereas it was completely suppressed by anisomycin, suggesting that different patterns of *PCNA* expression are the result of different pathways leading to G1 arrest. Compared with the results in periwinkle cells, the effect of benzyladenine on *PCNA* expression when applied at 0 h may be similar to that of anisomycin; however, the effect of benzyladenine when applied at 4 h was different from any of the observed patterns because transcripts were present, but the expression peak was not delayed. The pattern of *PCNA* expression following benzyladenine treatment at 4 h also differed from that after cryptogein treatment: cryptogein immediately reduced *PCNA* transcription when it was added to G1-phase BY-2 cells (Kadota et al. 2004). Similar results indicating that the levels of DNA synthesis and gene expression are uncoupled were also reported in A3-type cyclins. Reichheld et al. (1995, 1996) reported that the expression of *NtcycA59* and *NtcycA105* (renamed *Nicta;CycA3;1* and *Nicta;CycA3;2*, respectively; reviewed by Hemerly et al. 1999) was not

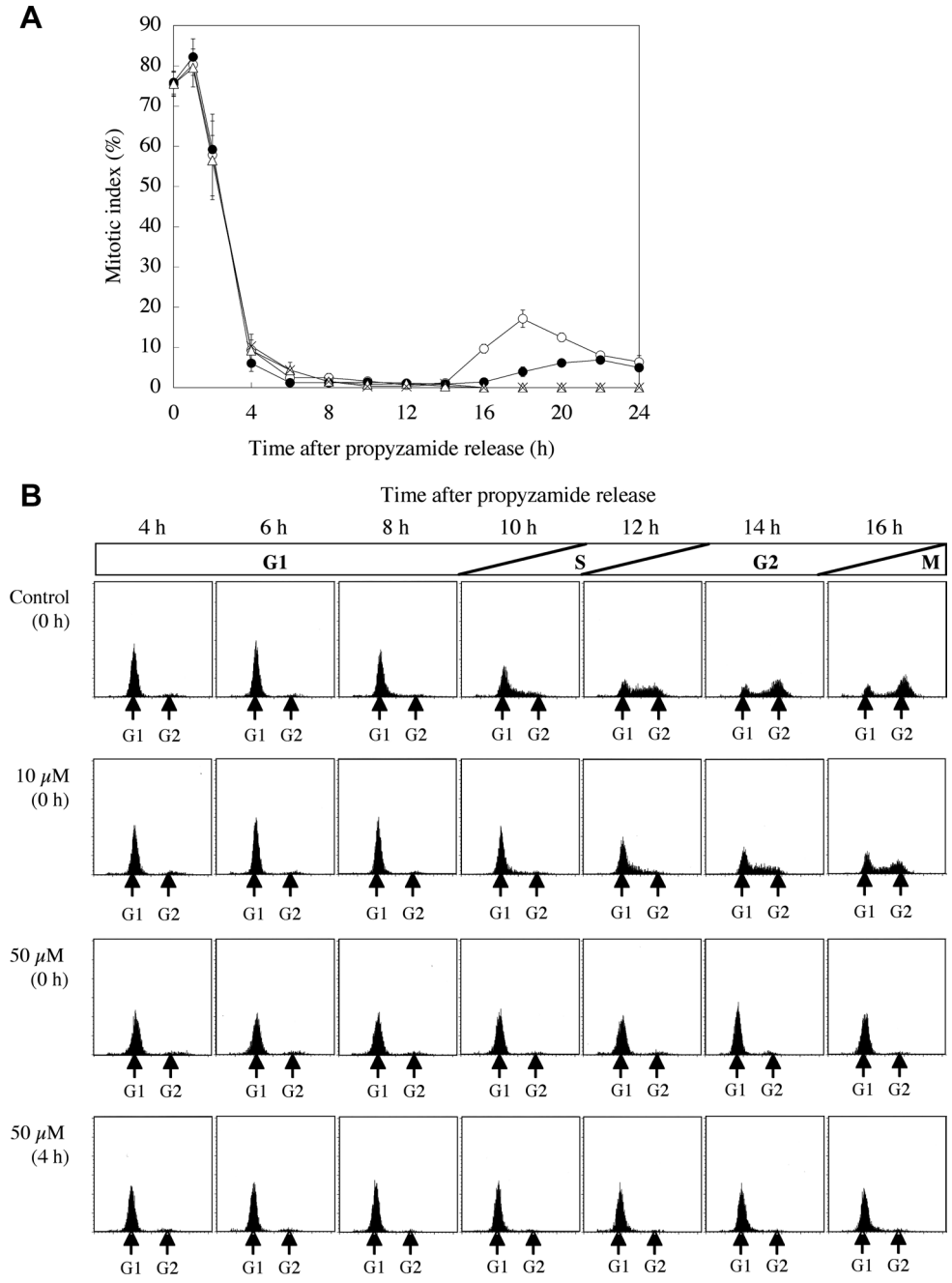

Figure 5. The effect of benzyladenine on the cell cycle progression of synchronized BY-2 cells. Cells were synchronized at M phase with aphidicolin and propyzamide and then treated with distilled water containing 0.1% (v/v) DMSO (control), 10 or 50 μM benzyladenine at 0 h (M phase), or 50 μM benzyladenine at 4 h (G1 phase) after propyzamide release. (A) Change in the mitotic index of the control cells (open circles) and cells treated with 10 μM (closed circles) or 50 μM benzyladenine (open triangles) at 0 h, or 50 μM benzyladenine at 4 h. (crosses). The data represent the mean±SD of three replicate experiments. (B) Flow cytometry was performed using 3000 nuclei from the control cells and cells treated with benzyladenine. Representative results from three independent experiments are shown.

suppressed by the addition of aphidicolin to tobacco BY-2 cells in the stationary phase, even though DNA synthesis rates decreased. This suggests that the expression level of A3-type cyclin genes and the level of DNA synthesis are not always correlated. They also demonstrated that hydroxyurea (a dNTPs synthesis inhibitor) treatment inhibited the accumulation of these gene transcripts, thus suggesting that the G1 arrest

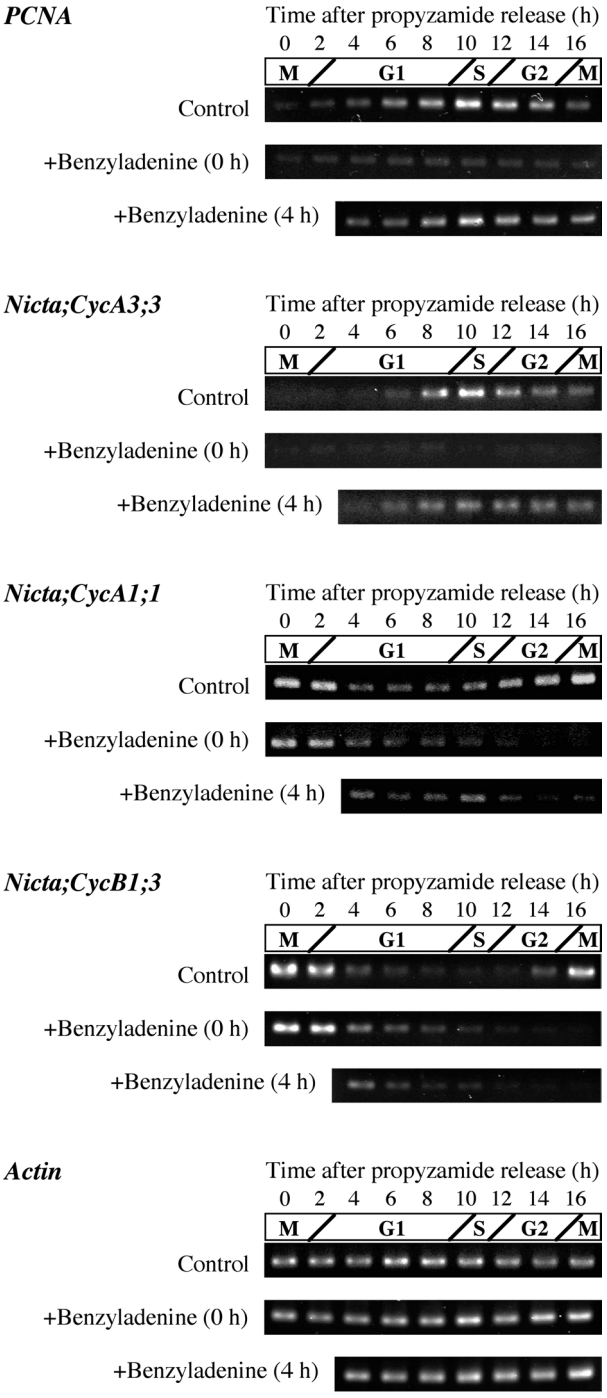

Figure 6. RT-PCR analyses of cell cycle-related gene expression (*PCNA*, *Nicta;CycA3;3*, *Nicta;CycA1;1* and *Nicta;CycB1;3*) in cells treated with distilled water containing 0.1% (v/v) DMSO (control) or 50 μM benzyladenine during M phase (0 h) or G1 (4 h). *Actin* was used as an internal standard.

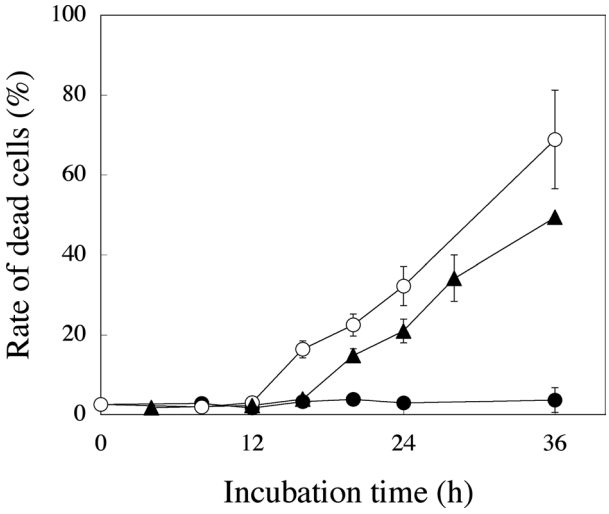

Figure 7. The induction of cell death by benzyladenine. BY-2 cells were synchronized at M phase with aphidicolin and propyzamide. Benzyladenine (50 μM) was applied at M phase (0 h, closed circles) and G1 (4 h, open triangles) after propyzamide release. Cells treated with distilled water containing 0.1% (v/v) DMSO were used as controls (open circles). Cells were stained with Evans blue, and the percentage of dead cells was evaluated under a microscope. At least 300 cells were observed. The data represent the mean±SD of three replicate experiments.

occurred at two different points. An important question for future studies is how benzyladenine inhibits G1 progression after 0 or 4 h of treatment. Dolezel et al. (2006) reported that CDK-like histone kinase activity was reduced *in vitro* by high concentrations (>50 μM) of benzyladenine and isopentenyladenine using protein kinases purified from *Arabidopsis* callus; therefore, the effects of benzyladenine on CDKs or other cell cycle-regulating factors must be considered.

Our data also indicate that cell cycle inhibition precedes cell death, but initiation of cell death may not require that cells reach a specific point in the cell cycle. Cells treated with benzyladenine at 0 or 4 h showed a G1 arrest within 10 h (Figure 5B, 6), whereas the number of dead cells increased 16 h after each treatment (Figure 7). Importantly, the increase in the number of dead cells was clearly dependent on the amount of time since benzyladenine treatment. These results are inconsistent with those that show cryptogein-induced cell cycle phase-dependent cell death (Kadota et al. 2004). In this case, cell death occurs only when cells recognize cryptogein during G1 or S phase. Therefore, cell death was rapidly induced in cells treated with cryptogein during S phase, whereas cells in G2 or M phase underwent cell death at the same time as cells treated with cryptogein in the subsequent G1 phase. Moreover, treatment with cryptogein during G1 or S phase increased the cytosolic Ca^{2+} flux more than that during G2 or M phase within minutes after addition, and this was followed by disparate patterns of MAP kinase activation and ROS production (Kadota et al. 2005). In benzyladenine-mediated cell death, a cytosolic Ca^{2+} flux has been reported (Bolduc et al. 2007). However, the addition of a cation channel blocker or Ca^{2+} chelator did not fully restore cellular viability or alter the up-regulation of Bax inhibitor-1 in tobacco Wisconsin-38

cells (Bolduc et al. 2007), suggesting that cytosolic Ca^{2+} fluxes may be only partially involved in benzyladenine-induced cell death. Studies of the relationship between Ca^{2+} signaling (or other factors involved in the early response to benzyladenine such as NO; Carimi et al. 2005) and the phase of the cell cycle during which benzyladenine was applied may explain the different mechanism of benzyladenine-induced cell death from an elicitor-mediated one.

Interestingly, the antiproliferative effect of cytokinins has also been reported in animal cells. Isopentenyladenine, a naturally occurring cytokinin, inhibits the histone kinase activity of $p34^{cdc2}$/cyclin B^{cdc13}, an M phase-specific protein kinase, in starfish oocytes *in vitro* (Rialet and Meijer 1991). Ishii et al. (2002) reported that various types of cytokinins or cytokinin ribosides inhibit the proliferation of HL-60 cells, followed by differentiation into normal granulocytes or apoptosis. Together with the above-mentioned report by Dolezel et al. (2006), that cytokinins reduce the activity of CDK-like histone kinase in *Arabidopsis*, it may be said that the antiproliferative effects of cytokinins in plant and animal cells may be mediated by similar mechanisms related to regulation of CDK activity. In future studies, the effect of cytokinins on CDK activity in plants and animals must be considered in detail.

In conclusion, growth inhibition at the G1 phase was observed prior to the induction of cell death in response to benzyladenine, and cell death was induced, dependent on time after treatment. This model is different from that proposed for elicitor-induced cell death, which is entirely dependent on the phase of the cell cycle. Additional studies of the early response to cytokinins are needed to explain the cytokinin-induced cell death.

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