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***Original Paper***

# High levels of the cytokinin BAP suppress programmed cell death in hybrid tobacco cells (*Nicotiana suaveolens* × *N. tabacum*) expressing hybrid lethality

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**Abstract** In *Nicotiana suaveolens* × *N. tabacum* hybrid cells incubated with high levels (0.8, 4.0 or 20 $\mu$M) of BAP at 28°C, the percentage of dead cells and extent of nuclear fragmentation were suppressed and some cultured cells remained green compared with those incubated with a standard level (0.04 $\mu$M) of BAP. Though DNA ladders were detected in all hybrid cells incubated at 28°C, positive signals for TUNEL assays were detected less frequently in hybrid cells incubated with high levels of BAP than in those incubated with a standard level of BAP. The localized accumulation of formazan, indicating production of reactive oxygen species, in hybrid cells with high levels of BAP was less than that in hybrid cells with a standard level of BAP. These results suggest that high levels of BAP suppress programmed cell death in hybrid lethality.

**Key words:** Cytokinin, hybrid lethality, *Nicotiana*, nuclear fragmentation, programmed cell death.

Hybrid lethality is a means of reproductive isolation of species that prevents wide crosses in higher plants. In the genus *Nicotiana*, hybrid lethality has been reported in several interspecific crosses, such as *N. glutinosa* × *N. repanda* (Marubashi et al. 1999), *N. suaveolens* × *N. tabacum* (Yamada et al. 2000) and *N. gossei* × *N. tabacum* (Mino et al. 2002). Additionally, features of programmed cell death (PCD), characterized by nuclear fragmentation, chromatin condensation and internucleosomal fragmentation of DNA, have been detected in hybrid tobacco seedlings (Marubashi et al. 1999; Yamada et al. 2000) during expression of hybrid lethality.

Masuda et al. (2003) reported that hybrid cells cultured from *N. suaveolens* × *N. tabacum* using a thin layer cell culture (TLCC) system express hybrid lethality at 28°C but not at high temperature (36°C). Moreover, they detected features of PCD, including nuclear fragmentation, chromatin condensation and internucleosomal fragmentation of DNA, in hybrid cells during expression of hybrid lethality. Additionally, the production of reactive oxygen species (ROS) was observed in hybrid lethality of *N. gossei* × *N. tabacum* (Mino et al. 2005). Production of ROS is a major signal in the process of PCD (Lam 2004).

In plants, cytokinins play important roles in many developmental processes, including cell division, cell differentiation, apical dominance, flower and fruit development (Haberer and Kieber 2002) and delay of leaf senescence (Orzaez and Granell 2004). Tobacco plants transformed with the *Agrobacterium tumefaciens tmr* gene (encoding isopentenyl transferase, which catalyzes the initial step in cytokinin biosynthesis) under the control of the soybean heat shock promoter HS6871, show inhibition of senescence (Smart et al. 1991). Gan and Amasino (1995) examined tobacco plants transformed with the *ipt* gene, which codes for isopentenyl transferase, under the control of a senescence-specific promoter ($P_{SAG12}$-IPT), and reported that the endogenous increase in cytokinin delays senescence. The process of senescence reportedly accompanies features of PCD (Yen and Yang 1998).

In this study, we investigated whether high levels of the cytokinin 6-benzylaminopurine (BAP) suppress PCD in hybrid cells from *N. suaveolens* × *N. tabacum* expressing hybrid lethality. Moreover, we determined whether these hybrid cells produce ROS.

Abbreviations: CTAB, cetyltrimethylammonium bromide; BAP, 6-benzylaminopurine; PCD, programmed cell death; ROS, reactive oxygen species; TLCC, thin layer cell culture; TUNEL, TdT-mediated dUTP nick-end labeling
This article can be found at http://www.jspcmb.jp/

## Materials and Methods

### Plant materials and culture conditions

Hybrid cells were cultured in liquid MS medium (pH 5.8) supplemented with 50 $\mu$M $\alpha$-naphthaleneacetic acid, 0.04 $\mu$M 6-benzylaminopurine (BAP) and 3% sucrose, and grown in conical flasks with constant shaking (130 rpm) at high temperature (36°C), which suppresses lethality in hybrid seedlings from the cross *N. suaveolens* × *N. tabacum* (Manabe et al. 1989), under continuous illumination (ca. 3,000 lux). Cell suspensions were maintained under the same conditions on a 7 day subculture cycle and used for experiments 3 days after subculturing. In this study, hybrid cells were used within 6 months after induction from seedlings.

### Treatment with cytokinin BAP

To remove old medium from cells maintained in suspension culture, hybrid cells were sieved through a 200 $\mu$m nylon mesh. For a TLCC system (Masuda et al. 2003), about 0.5 g (FW) of cells was transferred to culture dishes ($\phi$60 mm) at high temperature (36°C). Two milliliters of fresh liquid medium with standard level (0.04 $\mu$M) or high levels (0.8, 4.0 or 20 $\mu$M) of BAP was added and cells were added to form a single layer of cells. Then about 1 ml of the excess culture medium was removed to expose the cells to air in order to keep the dish under observation. For long-term culture, about 0.5 g (FW) of cells was transferred to solid MS medium supplemented with 0.2% Gelrite and standard or high levels of BAP at high temperature (36°C). Hybrid cells cultured at 36°C were transferred to 28°C, which is a lethal temperature for hybrid seedlings (Manabe et al. 1989), and maintained at 28°C on the TLCC system for 24 h or on solid medium for 3 days at various concentrations of BAP.

### Measurement of cell death

For measurement of cell death, hybrid cells exposed to 28°C were sieved through a 200 $\mu$m nylon mesh to remove the clustered cells and were resuspended in 50 $\mu$l fresh medium after centrifugation (2,000 rpm, 10 min). Ten microliters of this cell suspension was dropped onto a glass slide and observed by light microscopy. The progression of lethality in hybrid cells was estimated from the percentage of dead cells after 28°C treatment. Dead cells were scored under a light microscope after staining with 2.5% (w/v) Evans Blue. Each experiment was repeated three times, and differences among means were tested by Tukey's test ($P$=0.05).

### Detection of nuclear fragmentation

For cytometric analysis, nuclei were isolated from hybrid cells cultured with a standard level or high levels of BAP for 3 days at 28°C by chopping in ice-cold buffer (Michaelson et al. 1991) and filtering through a 70 and a 20 $\mu$m nylon mesh. The nuclei were collected from the filtrate by centrifugation for 5 min at 2500 rpm and suspended in FACSFlow sheath fluid (Becton Dickinson) supplemented with 250 $\mu$M SYTOX Green. The DNA contents of the isolated nuclei were analyzed by a FACSCalibur flow cytometer (Becton Dickinson). Based on the histograms obtained from flow cytometry, the nuclear fragmentation percentage was calculated by the formula [(Area of typical peak/Area of total count) × 100] provided by WIN MDI version 2.8 software for flow cytometric analysis. Each experiment was repeated three times, and differences among means were tested by Tukey's test ($P$=0.05).

### Detection of internucleosomal fragmentation of DNA

For detection of a DNA ladder pattern, following LoSchiavo et al. (2000), genomic DNA was extracted from hybrid cells cultured with a standard level (0.04 $\mu$M) or high levels (0.8, 4.0 or 20 $\mu$M) of BAP for 3 days using a CTAB (hexadecyltrimethyl ammonium bromide) method with some modifications (the pellet was washed with 70% v/v ethanol and resuspended in TE). The DNA solution was electrophoresed in a 2% agarose gel and was visualized using SYBR Gold (Wako Chemical Co., Japan) and UV light.

### TUNEL assays

For TdT-mediated dUTP nick-end labeling (TUNEL) assays, hybrid cells incubated with standard (0.04 $\mu$M) or a high level (20 $\mu$M) of BAP at 36°C or 28°C for 3 days were fixed with 4% paraformaldehyde in PBS at 4°C overnight. Samples were embedded in Paraplast Plus embedding medium (Oxford) and sectioned at a thickness of 10 $\mu$m. The sections were attached to silane-coated microscope slides and dewaxed. TUNEL assays were carried out with the DeadEnd fluorometric TUNEL system (Promega) according to the manufacturer's instructions.

### Detection of ROS

For the detection of ROS, the localized accumulation of superoxide radical ($O_2^-$) in the hybrid cells was detected by blue-formazan precipitation with 0.1% (w/v) Nitroblue tetrazolium (NBT). Hybrid cells incubated with standard (0.04 $\mu$M) or high levels (0.8, 4.0 or 20 $\mu$M) of BAP at 36°C or 28°C for 24 h were stained with 0.1% NBT and observed. The degree of blue-formazan precipitation was classified into three categories: +, clear accumulation of formazan; ±, little accumulation of formazan; −, no accumulation of formazan.

## Results and Discussion

When hybrid cells were incubated in the TLCC system with the standard level (0.04 $\mu$M) of BAP at 36°C for 12 or 24 h, the percentage of dead cells, based on Evans Blue staining, was less than 40%, but when they were incubated at 28°C for 12 or 24 h, the percentage of dead cells increased to about 70 and 75%, respectively (Figure 1). On the other hand, when hybrid cells were incubated with high levels (0.8, 4.0 or 20 $\mu$M) of BAP at 28°C for 12 or 24 h, the percentage of dead cells was lower than that of hybrid cells with a standard level of BAP (Figure 1). These results indicate that high levels of BAP partially suppress lethality in hybrid cells incubated in the TLCC system.

The TLCC system is not suitable for long-term culturing of hybrid cells. Therefore, hybrid cells were incubated on solid medium with a standard level or high lev-

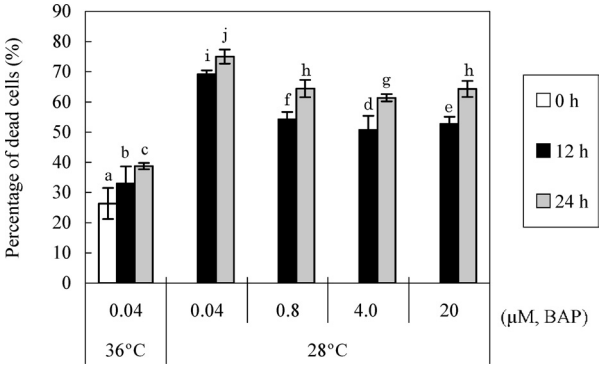


Figure 1. Changes in the percentage of dead cells in hybrid cells (*N. suaveolens* × *N. tabacum*). Hybrid cells were incubated in a TLCC system with various concentrations of BAP for 0 h (white columns), 12 h (black columns) and 24 h (gray columns). The percentage of dead cells was based on staining with Evans Blue. Values are means with SD (vertical bars) from three independent determinations. A P value less than 0.05 was considered statistically significant.

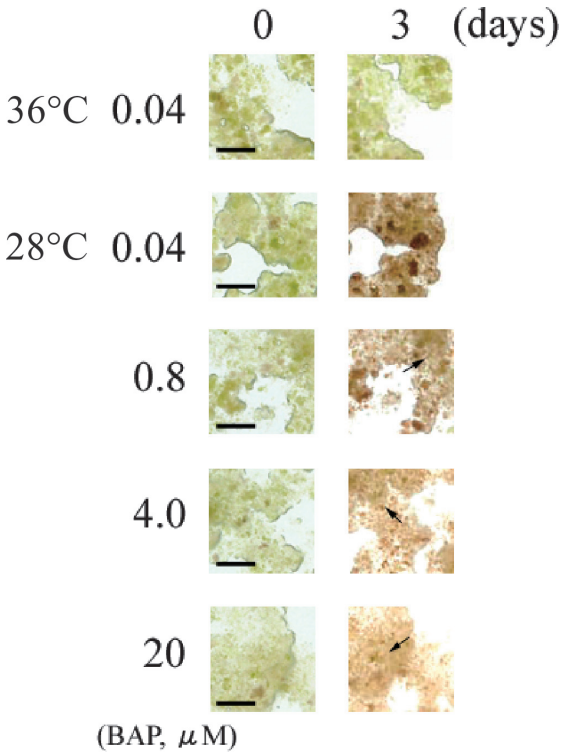


Figure 2. Morphological changes of hybrid cells (*N. suaveolens* × *N. tabacum*) incubated on solid medium with various concentrations of BAP for 3 days. Hybrid cells were incubated on solid medium with a standard level (0.04 μM) or high levels (0.8, 4.0 and 20 μM) of BAP from 0 to 3 days. Arrows indicate some hybrid cells that remained green and survived. Bars = 5 mm.

els of BAP for 3 days. Hybrid cells incubated on the solid medium with the standard level of BAP at 36°C remained green, whereas cells incubated at 28°C turned brown (Figure 2). Hybrid cells incubated on the solid medium with high levels of BAP at 28°C almost all turned brown, but some hybrid cells remained green

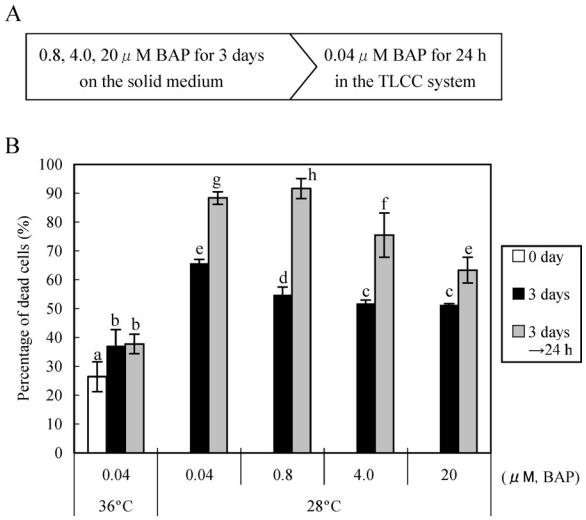


Figure 3. Changes in the percentage of dead cells in hybrid cells (*N. suaveolens* × *N. tabacum*) incubated with various concentrations of BAP. (A) Timetable of method for culture of hybrid cells to investigate suppression of lethality at high levels (0.8, 4.0 and 20 μM) of BAP. Hybrid cells were incubated in a TLCC system with a standard level (0.04 μM) of BAP for 24 h after incubation on solid medium with various concentrations of BAP for 3 days. The percentage of dead cells was measured at each time point using Evans Blue staining. (B) Changes in the percentage of dead cells in hybrid cells incubated as described in (A). Values are means with SD (vertical bars) from three independent determinations. A P value less than 0.05 was considered statistically significant.

(Figure 2). These results indicate that high levels of BAP partially suppress expression of lethality.

In hybrid cells incubated on solid medium with a standard level of BAP at 28°C for 3 days, the percentage of dead cells, determined using Evans Blue, was about 65%, while it was about 35% at 36°C (Figure 3). On the other hand, when hybrid cells were incubated with high levels of BAP at 28°C, the percentage of dead cells was about 50–55% (Figure 3). These results indicate that high levels of BAP also partially suppress lethality in hybrid cells incubated on solid medium.

To examine whether suppression of lethality is a continuous effect, hybrid cells incubated on solid medium with a standard level (0.04 μM) or high levels (0.8, 4.0 or 20 μM) of BAP for 3 days were transferred to the TLCC system with a standard level (0.04 μM) of BAP for 24 h, and the percentage of dead cells was then determined using Evans Blue staining. When hybrid cells were incubated on solid medium with 0.8 μM BAP for 3 days before transfer to the TLCC system, the percentage of dead cells was higher than that of hybrid cells incubated with the standard level (0.04 μM) of BAP before transfer (Figure 3). On the other hand, in hybrid cells incubated on solid medium with 4.0 or 20 μM BAP before transfer, the percentage of dead cells was lower than that of hybrid cells incubated with the standard level before transfer (Figure 3). Additionally, the percentage of dead hybrid cells with 20 μM of BAP was less than those

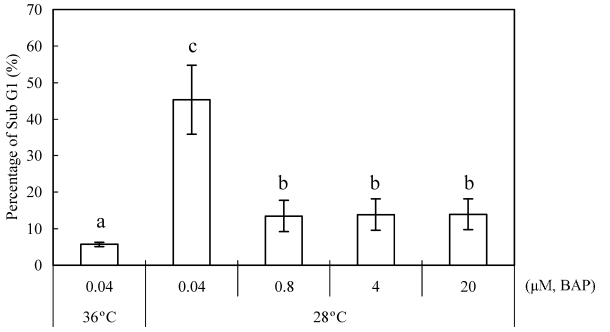


Figure 4. Changes in the percentage of nuclear fragmentation in hybrid cells (*N. suaveolens* × *N. tabacum*) incubated with various concentrations of BAP for 3 days. Percentage of fragmented nuclei was calculated based on histograms obtained by flow cytometry. Values are means with SD (vertical bars) from three independent determinations. A P value less than 0.05 was considered statistically significant.

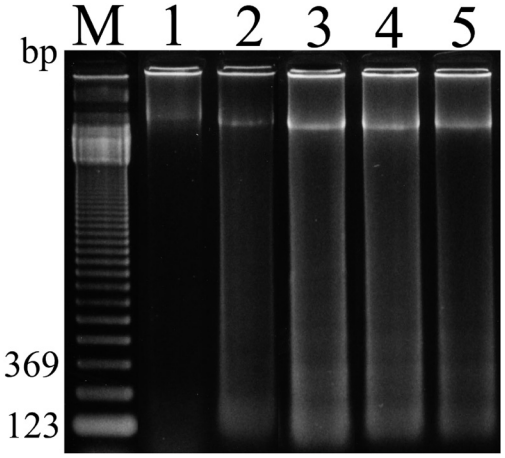


Figure 5. DNA fragmentation detected in hybrid cells (*N. suaveolens* × *N. tabacum*) incubated with various concentrations of BAP for 3 days. The DNA solution was electrophoresed in a 2% agarose gel and stained with SYBR Gold (Molecular Probes); M, 123 bp ladder marker (BRL); lane 1, hybrid cells incubated with a standard level (0.04 $\mu$M) for 3 days at 36°C; lane 2, hybrid cells incubated with a standard level for 3 days at 28°C; lanes 3–5, hybrid cells incubated with high levels (0.8, 4.0 and 20 $\mu$M, respectively) of BAP at 28°C.

with 4.0 $\mu$M of BAP. These results suggest that a higher concentration of BAP intensifies the suppression of lethality.

To examine nuclear fragmentation, nuclei isolated from hybrid cells incubated on solid medium with a standard level (0.04 $\mu$M) or high levels (0.8, 4.0 or 20 $\mu$M) of BAP were subjected to flow cytometry. The histograms of fluorescence values indicate the relative size of nuclear DNA masses. When nuclear fragmentation occurs during the process of PCD, additional peaks (Sub G1) with lower fluorescence values than that for G1 increase. In hybrid cells incubated on solid medium with the standard level of BAP at 36°C for 3 days, the percentage of Sub G1 peaks was about 5% (Figure 4). However, in hybrid cells incubated with the standard level of BAP at 28°C, the percentage of Sub G1 peaks rose to about 45% (Figure 4). On the other hand, in hybrid cells incubated on solid medium with high levels of BAP at 28°C for 3 days, the percentage of Sub G1 peaks did not exceed 15% (Figure 4). These results indicate that high levels (0.8, 4.0 and 20 $\mu$M) of BAP suppress nuclear fragmentation.

To examine internucleosomal fragmentation of DNA, total DNA isolated from hybrid cells incubated on solid medium with a standard level (0.04 $\mu$M) or high levels (0.8, 4.0 or 20 $\mu$M) of BAP at 28°C for 3 days was separated on a 2% agarose gel and visualized using SYBR Gold and UV light. A distinctive DNA ladder pattern, suggesting internucleosomal fragmentation of DNA, was detected regardless of BAP concentration (Figure 5). However, electrophoresis of total DNA isolated from hybrid cells incubated on solid medium with the standard level of BAP at 36°C didn't show a distinctive ladder pattern (Figure 5).

TUNEL assays were also applied to detection of DNA fragmentation. When hybrid cells were incubated on solid medium with the standard level (0.04 $\mu$M) of BAP at 28°C for 3 days, positive signals were detected in all nuclei (Figure 6B), but when they were incubated at 36°C, few positive signals were detected (Figure 6A). On the other hand, in hybrid cells incubated with 20 $\mu$M BAP, few positive signals were detected (Figure 6C). These results suggest that high levels of BAP suppress DNA fragmentation. Based on both agarose gel electrophoresis and TUNEL assays, it is possible that DNA laddering occurred in hybrid cells incubated on solid medium regardless of BAP concentration.

For the detection of ROS, hybrid cells with a standard level (0.04 $\mu$M) or high levels (0.8, 4.0 or 20 $\mu$M) of BAP at 28°C or 36°C were incubated in the TLCC system for 24 h and then stained with 0.1% NBT. The degree of localized accumulation of formazan was classified into three categories: +, clear accumulation of formazan; ±, little accumulation of formazan; −, no accumulation of formazan. Hybrid cells producing ROS turned blue-black due to accumulation of formazan. In hybrid cells incubated in the TLCC system with a standard level of BAP, localized accumulation was detected 3 h after transfer from 36°C to 28°C (Figure 7). However, localized accumulation was not detected 6, 12, or 24 h after transfer to 28°C. In hybrid cells incubated in the TLCC system with a standard level of BAP at 36°C, localized accumulation was not detected at all during the time course. On the other hand, in hybrid cells incubated in the TLCC system with high levels of BAP, some localized accumulation was detected 3 h after transfer to 28°C. However, localized accumulation was not detected 6, 12, or 24 h after transfer to 28°C. Although in hybrid cells cultured with both a standard level and high levels of BAP the production of ROS was detected 3 h after transfer to 28°C, the degree of localized accumulation in

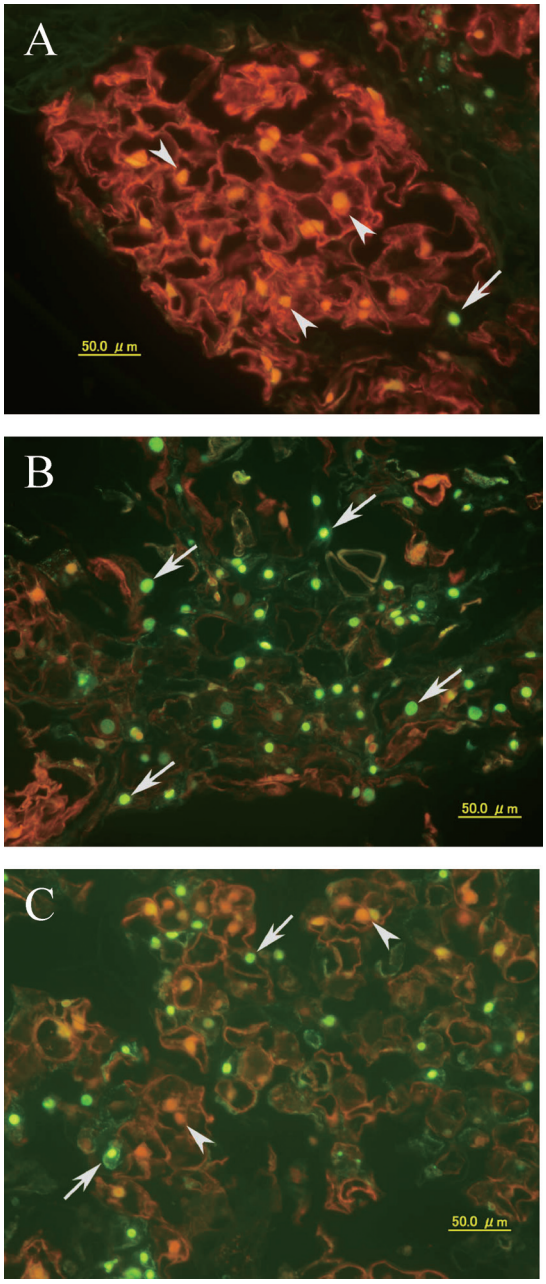


Figure 6. *In situ* detection of DNA fragmentation in hybrid cells (*N. suaveolens* × *N. tabacum*) incubated on solid medium with various concentrations of BAP for 3 days. Detection of DNA fragmentation by TUNEL assay (green fluorescence, arrows). Detection of nuclei by counterstaining the same section with PI (red fluorescence, arrowheads). (A) Hybrid cells incubated with a standard level (0.04 μM) of BAP at 36°C had few positive signals. (B) Hybrid cells incubated with a standard level of BAP at 28°C exhibited positive signals in all nuclei. (C) Hybrid cells incubated with an even higher level (20 μM) of BAP at 28°C had a few positive signals. Bars = 50 μm.

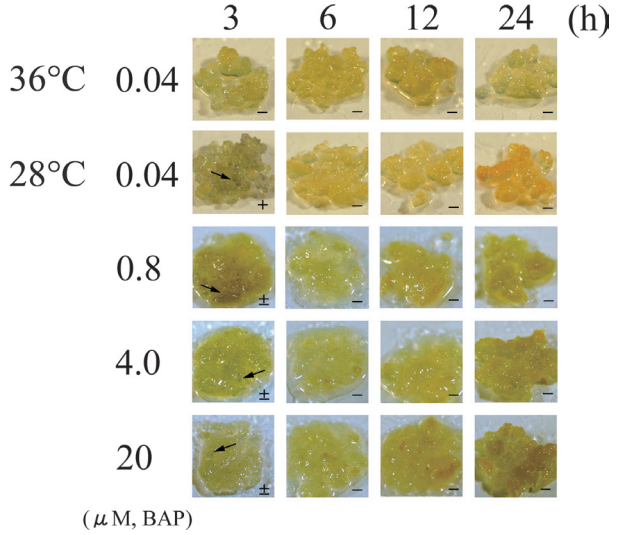


Figure 7. Localized accumulation of formazan in hybrid cells (*N. suaveolens* × *N. tabacum*) incubated in a TLCC system with various concentrations of BAP for 24 h. Hybrid cells incubated on solid medium with a standard level (0.04 μM) or high levels (0.8, 4.0 and 20 μM) of BAP at 28°C or 36°C were immersed in 0.1% NBT solution. The degree of localized accumulation was classified into three categories: +, clear accumulation of formazan; ±, little accumulation of formazan; −, no accumulation of formazan. Arrows indicate accumulation of formazan.

hybrid cells with high levels of BAP was less than in those with a standard level of BAP. Hybrid seedlings and cultured cells from *N. gossei* × *N. tabacum* produce ROS during hybrid lethality (Mino et al. 2002; Mino et al. 2005). Therefore, we considered that high levels of BAP suppress production of ROS in the hybrid cells expressing hybrid lethality.

In this study, we observed that expression of lethality in hybrid cells (*N. suaveolens* × *N. tabacum*) was suppressed by high levels (0.8, 4.0 and 20 μM) of BAP. Additionally, nuclear fragmentation, which is a feature of PCD, did not increase compared to controls. This suggests that high levels of BAP suppress progression of PCD. However, internucleosomal fragmentation of DNA, which is another feature of PCD, was detected in hybrid cells incubated with standard and high levels of BAP at 28°C. TUNEL assays, which detect DNA fragmentation, showed that positive signals were detected at any concentration of BAP. Moreover, we detected production of ROS in hybrid cells during PCD in hybrid lethality, which decreased in hybrid cells incubated with high levels (0.8, 4.0 and 20 μM) of BAP. These results suggest that the cytokinin BAP suppresses PCD expressed during hybrid lethality.

In the present study, we observed that hybrid cells from *N. suaveolens* × *N. tabacum* incubated for 3 h at 28°C produced ROS during PCD in hybrid lethality. We previously reported that in hybrid cells incubated at 28°C, nuclear fragmentation and DNA fragmentation were observed 6 and 9 h after transfer from 36°C to 28°C, but not after 3 h (Kobori et al. 2005). These results indicate that production of ROS occurs earlier than nuclear fragmentation and DNA fragmentation during PCD. Mino et al. (2005) also reported that cultured cells of *N. gossei* × *N. tabacum* produced ROS

in an early phase at 26°C. In animal cells, production of ROS acts as an early signal in the process of PCD (Kazzaz et al. 1996). Moreover, in the PCD associated with the hypersensitive response (HR) in plants, ROS is produced within 1 h after infection (Draper 1997). Therefore, the production of ROS occurring prior to nuclear fragmentation and DNA fragmentation during hybrid lethality is identical with phenomena observed in animal PCD and the HR in plants.

Transgenic tobacco plants expressing a bacterial gene encoding isopentenyl transferase, a key enzyme in cytokinin biosynthesis, exhibit delayed leaf senescence (Gan and Amasino 1995). However, leaves of cytokinin-deficient engineered transgenic Arabidopsis plants show a delay of senescence, rather than an acceleration (Werner et al. 2001, Werner et al. 2003). On the other hand, cytokinins reportedly induce PCD in plants, including tobacco (Mlejnek and Prochazka 2002), carrot and Arabidopsis (Carimi et al. 2003). Additionally, Carimi et al. (2004) reported that in Arabidopsis cell suspension cultures, high levels of BAP induce features of PCD by accelerating senescence and in carrot cells, high levels of BAP and carbanilide, another cytokinin, induce some features of PCD, but kinetin and zeatin, which are also cytokinins, do not. No previously published report suggest that cytokinins suppress PCD. In the present study, we observed that high levels of the cytokinin BAP inhibited features of PCD, including nuclear fragmentation, DNA fragmentation and production of ROS. PCD in senescence may also be suppressed by cytokinins.

Masuda et al (2003) revealed that the process of hybrid lethality on the hybrid cells of *N. suaveolens* × *N. tabacum* has initiation phase (within 3 h after transfer to 28°C from 36°C), which induces hybrid lethality, and a following execution phase, which induces the process of PCD including nuclear fragmentation and DNA fragmentation. In the present study, production of ROS decreased in hybrid cells with high levels of BAP at 3 h after transfer to 28°C. Therefore, high levels of BAP act during induction of hybrid lethality within 3 h after transfer to 28°C.

The major phytohormone ethylene is also a key factor in senescence of plants (Mayak et al 1977). In petunia flowers with $P_{SAG12}$-IPT, which increases cytokinin content, sensitivity to ethylene decreases (Chang et al 2003). In addition, Yamada and Marubashi (2003) reported that hybrid seedlings (*N. suaveolens* × *N. tabacum*) expressing hybrid lethality at 28°C had increased production of ethylene compared with seedlings cultured at 36°C. Although we have not detected production of ethylene in hybrid cells (*N. suaveolens* × *N. tabacum*), high levels of BAP suppressing hybrid lethality might decrease sensitivity to ethylene.

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