Plant Biotechnology 22, 345–348 (2005)

Short Communication

Heat treatment temporarily suppresses expression of programmed cell death in hybrid tobacco cells (*Nicotiana suaveolens*×*N. tabacum*) expressing hybrid lethality

Satoshi Kobori[1], Yu Masuda[2], Wataru Marubashi[3]*

[1] School of Agriculture, Ibaraki University, 3-21-1 Chuo, Ami, Ibaraki 300-0393, Japan; [2] Laboratory of Plant Chromosome and Gene Stock, Graduate School of Science, Hiroshima University, 1-4-3 Kagamiyama, Higashi-Hiroshima City, Hirroshima 739-8526, Japan; [3] School of Agriculture, Meiji University, 1-1-1 Higashimita, Kawasaki, Kanagawa 214-8571, Japan

*E-mail: marubasi@isc.meiji.ac.jp Tel & Fax: +81-44-934-7811

Received August 17, 2005; accepted October 14, 2005 (Edited by T. Kohchi)

Abstract Features of programmed cell death (PCD), including nuclear fragmentation and DNA ladders were detected in hybrid cells of *Nicotiana suaveolens*×*N. tabacum* expressing hybrid lethality at 28°C, but not in cells kept at 36°C. Heat treatment (HT, 50°C for 15 min) before transfer to 28°C from 36°C temporarily suppressed the increase in the percentage of dead cells. In hybrid cells without HT, the percentage of Sub G1 nuclei, corresponding to those with nuclear fragmentation increased at 6 h after transfer to 28°C and DNA ladders were detected at 9 h after transfer to 28°C. On the other hand, in hybrid cells with HT, the percentage of Sub G1 increased and DNA ladders were detected 15 h after transfer to 28°C. These results suggest that HT temporarily suppresses PCD during expression of hybrid lethality.

Key words: Heat treatment, hybrid lethality, *Nicotiana*, programmed cell death.

Hybrid lethality is one of the important devices whereby higher plants prevent wide crosses. In the genus *Nicotiana*, Marubashi et al. (1999) reported observing features of programmed cell death (PCD) such as chromatin condensation, nuclear fragmentation and nucleosomal DNA fragmentation in hybrid seedlings of *N. glutinosa*×*N. repanda* expressing hybrid lethality. Moreover, Yamada et al. (2000) reported that hybrid seedlings and calli derived from the cross *N. suaveolens*×*N. tabacum* showed lethality within 2 weeks after germination at 28°C but not under high temperature conditions (36°C). The observation that PCD during expression of hybrid lethality was detected in suspension-cultured cells derived from *N. suaveolens*×*N. tabacum* indicated that the same phenomenon is carried out among seedlings, calli and suspension-cultured cells (Yamada et al. 2001). Recently, Masuda et al. (2003) reported that hybrid cells of *N. suaveolens*×*N. tabacum* express hybrid lethality with accompanying features of PCD using a thin layer cell culture (TLCC) system at 28°C, but not at 36°C. Additionally, they revealed that PCD was suppressed by actinomycin D or cycloheximide (Masuda et al. 2003).

Chromatin condensation, nuclear fragmentation and internucleosomal fragmentation of DNA are considered the key characteristics of PCD and detection of these features is widely used as a diagnostic tool for PCD in plant cells during normal plant development (Pennell and Lamb 1997) and in response to various stresses (Swidzinski et al. 2002; Huh et al. 2002) and pathogens (Greenberg and Yao 2004). Flow cytometric analysis and agarose gel electrophoresis are used as tools for detection of PCD in plants expressing hybrid lethality (Marubashi et al. 1999; Yamada et al. 2001; Masuda et al. 2003; Yamada and Marubashi 2003).

In a human leukemic T cell line, a brief conditioning heat treatment (HT) protects the cells from PCD (Mosser and Martin 1992). In the present study, we investigated whether HT inhibits expression of PCD, including nuclear fragmentation and internucleosomal fragmentation of DNA, in hybrid cells of *N. suaveolens*×*N. tabacum*.

Hybrid cells were cultured in liquid MS medium (pH 5.8) supplemented with 50 μM α-naphthaleneacetic acid, 0.04 μM 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

Abbreviations: BAP, 6-benzylaminopurine; CTAB, cetyltrimethylammonium bromide; HT, heat treatment; PCD, programmed cell death; TLCC, thin layer cell culture.

were maintained under the same conditions on a 7 day subculture cycle and used for experiments 3 days after subculturing.

To remove old medium from cells maintained in suspension culture, hybrid cells were sieved through a 200 μm nylon mesh and about 0.5 g (fresh weight) of cells was transferred to culture dishes (φ90 mm) at a high temperature (36°C). Three ml of fresh medium was added and cells were placed in order to form a single layer of cells. Then about 2 ml of the extra culture medium was removed to expose the cells to air in order to keep the dishes under observation. For HT, the dishes were placed in a water bath at 40, 50, 60 and 80°C for 15 min. Then, the dishes were placed at 28°C for 24 h.

Hybrid cells cultured at 36°C were transferred to 28°C, which is the lethal temperature for hybrid seedlings (Manabe et al. 1989), and maintained at 28°C in a thin layer cell culture (TLCC) system (Masuda et al. 2003). Hybrid cells were sieved through a 200 μm nylon mesh to remove the clustered cells and were resuspended in 50 μl fresh medium after centrifugation (2,000 rpm, 10 min). Ten microliters of this cell suspension was dropped on a glass slide and observed by light or fluorescence microscopy. The progression of lethality in hybrid cells was estimated from the percentage of dead cells after 28°C treatment for different intervals over 24 h. Dead cells were scored under a light microscope after staining with 2.5% (w/v) Evans Blue. At least three independent experiments were performed with more than 500 cells counted per condition.

For cytometric analysis, nuclei were isolated from hybrid cells cultured with or without HT by chopping them in ice-cold buffer (Michaelson et al. 1991) and filtering the macerated tissue through 70 and 20 μm nylon mesh. The nuclei were collected from the filtrate by centrifugation for 5 min at 2,500 rpm, suspended in the ice-cold buffer supplemented with 5 μg/ml propidium iodide and 10 μg/ml RNase and incubated for 15 min at 36°C. The DNA contents of the isolated nuclei were analyzed by a flow cytometer (FACSCalibur; Becton Dickinson). Based on the histograms obtained from flow cytometry of a total of 10,000 nuclei, 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.

For detection of a DNA ladder pattern, the hybrid cells frozen in liquid nitrogen were ground into a fine powder with a mortar and pestle. The powder was suspended in 2% cetyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, and incubated at 65°C for 40 min. The suspension was shaken after the addition of an equal volume of chloroform/isoamyl alcohol (24 : 1, v/v) and centrifuged for 15 min at 3,000 rpm. The supernatant was mixed with 3/4 volume of isopropyl alcohol and centrifuged for 20 min at 14,000 rpm. Precipitated DNA was washed with 70% ethanol and centrifuged for 10 min at 14,000 rpm. Precipitated DNA was dissolved in 10 mM Tris-HCl (pH 8) containing 10 mM EDTA. The DNA solution was electrophoresed in a 2% agarose gel and was visualized using SYBR Gold (Wako Chemical Co., Japan) under UV light.

Table 1. Percentage of dead cells after HT.

	Temperature of HT (°C)	Temperature of culture (°C)	Percentage of dead cells (%)
Without HT	—	36	30.4±6.2*
	—	28	44.2±2.8
With HT	40	28	57.6±3.1
	50	28	35.0±2.8
	60	28	90.1±2.7
	80	28	100±0.0

Hybrid cells were incubated at 28°C for 6 h after various HT (15 min). Percentage of dead cells was evaluated by Evans Blue staining. * Mean (%)±SD of three independent experiments.

To search for the optimum condition for suppression of lethality, hybrid cells that had been subjected to various temperatures (40, 50, 60 and 80°C) for 15 min were incubated at 28°C for 6 h and the percentage of dead cells was measured by staining with Evans Blue (Table 1). In hybrid cells without HT, the percentage of dead cells at 6 h was 30.4% at 36°C, but increased to 44.2% at 28°C. In hybrid cells with HT (40, 60 and 80°C) for 15 min before transfer to 28°C, the percentage of dead cells also increased, to 57.6, 90.1 and 100%, respectively. However, the application of HT (50°C for 15 min) before transfer to 28°C suppressed the increase in the percentage of dead cells by approximately 30% after culturing hybrid cells for 6 h at 28°C. Therefore, a HT of 50°C for 15 min was applied in all subsequent experiments.

We observed morphological changes of hybrid cells with or without HT continuously over 24 h after transfer to 28°C (Figure 1). At 28°C, hybrid cells without HT turned brown after 3 h, while hybrid cells with HT maintained their green color for 9 h and then turned brown from 12 h to 24 h at 28°C. These results indicate that the inhibitory effect of HT is temporary.

We measured the percentage of dead cells from 0 h to 24 h after transfer to 28°C (Figure 2). In hybrid cells without HT, the percentage of dead cells increased between 3 h (32.8%) and 24 h (76.1%). In hybrid cells with HT (50°C for 15 min), the percentage of dead cells was about 35% for 12 h at 28°C, which was identical to the percentage for hybrid cells cultured at 36°C, but the percentage increased from 15 h (53.2%) to 24 h (77%) at 28°C. These results suggest that HT (50°C for 15 min) inhibits progression of lethality until 12 h after transfer to 28°C.

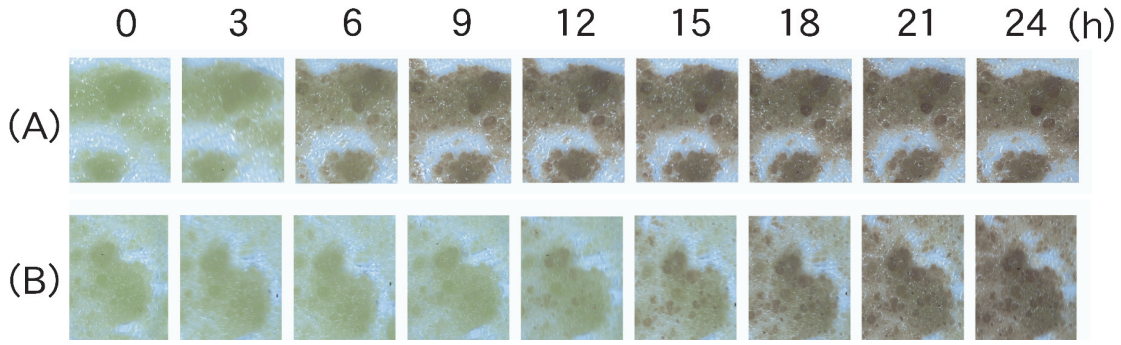

Figure 1. Morphological changes of hybrid cells from the cross *N. suaveolens*×*N. tabacum* with or without HT (50°C for 15 min). (A) Hybrid cells incubated without HT at 28°C for 24 h. (B) Hybrid cells incubated with HT at 28°C for 24 h.

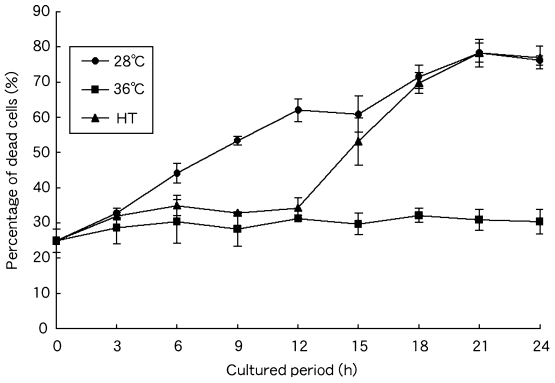

Figure 2. Change in the percentage of dead cells from the cross *N. suaveolens*×*N. tabacum* incubated with or without HT (50°C for 15 min) at 28°C or at 36°C for 24 h. Percentage of dead cells was evaluated by Evans Blue staining. Values are means with SD (vertical bars) of results from three independent determinations.

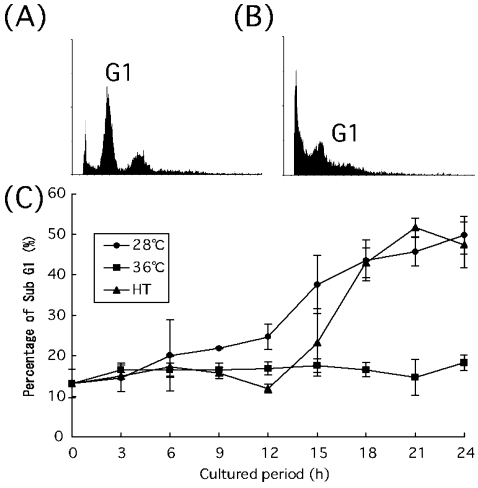

Figure 3. (A) Histogram of hybrid cells cultured at 36°C for 24 h. (B) Histogram of hybrid cells cultured at 28°C for 24 h. (C) Progressive nuclear fragmentation shown by hybrid cells from the cross *N. suaveolens*×*N. tabacum* incubated with or without HT (50°C for 15 min) at 28°C or at 36°C for 24 h. Percentage of fragmented nuclei was calculated based on histograms obtained by flow cytometry. Values are means with SD (vertical bars) of results from three independent determinations.

Features of PCD are detected in hybrid cells of *Nicotiana suaveolens*×*N. tabacum* expressing hybrid lethality (Yamada et al. 2001; Masuda et al. 2003). Consequently, we also investigated whether expression of PCD is temporarily suppressed in hybrid cells with HT (50°C for 15 min). Nuclear fragmentation was evaluated by analysis of DNA contents of nuclei isolated from hybrid cells using a flow cytometer. In hybrid cells incubated without HT at 36°C, the percentage of peaks corresponding to nuclei categorized Sub G1 was maintained at approximately 15% for 24 h (Figure 3). However, in hybrid cells incubated without HT at 28°C, the increase in the percentage of Sub G1 nuclei (the percentage of additional peaks with lower fluorescence values out of the total peaks) suggests that further nuclear fragmentation occurred from 6 h (20.1%) to 24 h (49.8%) at 28°C (Figure 3). Additional (Sub G1) peaks with lower fluorescence values than that of the G1 peak indicate the progression of PCD. On the other hand, when hybrid cells were subjected to HT, the increase in the peaks corresponding to Sub G1 was suppressed for 12 h (12%) at 28°C, and then detected from 15 h (23.3%) to 24 h (47.4%) at 28°C (Figure 3). These results indicate that HT (50°C for 15 min) inhibits nuclear fragmentation until 12 h, but not over 15 h.

The DNA ladder pattern suggested that internucleosomal fragmentation of DNA occurred from 9 h to 24 h at 28°C in hybrid cells without HT (Figure 4). In hybrid cells with HT, a DNA ladder pattern suggesting internucleosomal fragmentation of DNA, was detected from 15 h to 24 h at 28°C (Figure 4). These results indicate that HT (50°C for 15 min) inhibits internucleosomal fragmentation of DNA until 12 h, but not after 15 h.

In this study, we observed that hybrid cells with HT (50°C for 15 min) before transfer to 28°C suppressed expression of lethality for 12 h, while hybrid cells without HT expressed lethality after 3 h at 28°C. However, since hybrid cells with HT expressed lethality from 15 h, the effect of HT was temporary. Consistent with these results, expression of PCD was suppressed in

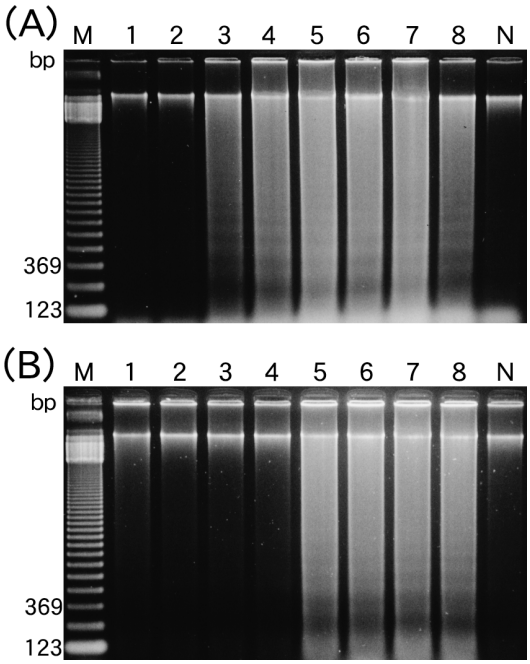

Figure 4. Nucleosomal DNA fragmentation in hybrid cells from the cross *N. suaveolens*×*N. tabacum* with or without HT (50°C for 15 min). DNA solution was electrophoresed in a 2% agarose gel and stained with SYBR Gold. (A) Lanes 1–8, hybrid cells without HT cultured at 28°C for 3, 6, 9, 12, 15, 18, 21 and 24 h, respectively. (B) Lanes 1–8, hybrid cells with HT cultured at 28°C for 3, 6, 9, 12, 15, 18, 21 and 24 h, respectively. M, 123 bp DNA ladder marker; N, hybrid cells without HT cultured at 36°C for 24 h.

hybrid cells with HT until 12 h at 28°C, but not over 15 h. These results suggest that in hybrid cells of *N. suaveolens*×*N. tabacum*, HT temporarily suppresses PCD during expression of hybrid lethality.

In a human leukemic T cell line, Mosser and Martin (1992) reported that a brief conditioning HT induced resistance to apoptosis. Moreover, they showed the synthesis of heat shock proteins after HT and suggested that heat shock proteins could protect cells from apoptotic death. Time-course analysis of synthesis of heat shock proteins might help to reveal how HT temporarily suppresses PCD in hybrid cells of *N. suaveolens*×*N. tabacum* expressing hybrid lethality.

Acknowledgments

The seeds of *Nicotiana suaveolens* and *N. tabacum* were supplied by Japan Tobacco Inc. This work was partly supported by a Grant-in-Aid for Scientific Research (C) No. 15580003 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Greenberg JT, Yao N (2004) The role and regulation of programmed cell death in plant-pathogen interactions. *Cell Microbiol* 6: 201–211

Huh GH, Damsz B, Matsumoto TK, Reddy MP, Rus AM, Narasimhan ML, Bressan RA, Hasegawa PM (2002) Salt causes ion disequilibrium-induced programmed cell death in yeast and plants. *Plant J* 29: 649–659

Manabe T, Marubashi W, Onozawa Y (1989) Temperature-dependent conditional lethality in interspecific hybrids between *Nicotiana suaveolens* Lehm.×*N. tabacum* L. *Proceedings of the 6th International Congress SABRAO*, pp 459–462

Marubashi W, Yamada T, Niwa M (1999) Apoptosis detected in hybrids between *Nicotiana* glutinosa×*N. repanda* expressing lethality. *Planta* 210: 168–171

Masuda Y, Yamada T, Marubashi W (2003) Time course analysis of apoptotic cell death during expression of hybrid lethality in hybrid tobacco cells (*Nicotiana suaveolens*×*N. tabacum*). *Plant Cell Physiol* 44: 420–427

Michaelson MJ, Pris HJ, Ellison HJ, Johnston JS (1991) Comparison of plant DNA contents determined by feulgen microspectrophtometry and laser flow cytometry. *Am J Bot* 78: 183–188

Mosser DD, Martin LH (1992) Induced thermotolerance to apoptosis in a human T lymphocyte cell line. *J Cell Physiol* 151: 561–570

Pennell RI, Lamb C (1997) Programmed cell death in plants. *Plant Cell* 9: 1157–1168

Swidzinski JT, Sweetlove LJ, Leaver CJ (2002) A custom microarray analysis of gene expression during programmed cell death in *Arabidopsis thaliana.* *Plant J* 30: 431–446

Yamada T, Marubashi W, Niwa M (2000) Apoptotic cell death induces temperature-sensitive lethality in hybrid seedlings and calli derived from the cross of *Nicotiana suaveolens*×*N. tabacum*. *Planta* 211: 614–622

Yamada T, Marubashi W, Niwa M (2001) Facile induction of apoptosis into plant cells associated with temperature-sensitive lethality shown on interspecific hybrid from the cross *Nicotiana suaveolens*×*N. tabacum*. *Plant Cell Physiol* 42: 204–213

Yamada T, Marubashi W (2003) Overproduced ethylene causes programmed cell death leading to temperature-sensitive lethality in hybrid seedlings from the cross *Nicotiana suaveolens*×*N. tabacum*. *Planta* 217: 690–698