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Temperature-dependent Apoptosis Detected in Hybrids between *Nicotiana debneyi* and *N. tabacum* Expressing Lethality

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Abstract

Interspecific hybrids of *Nicotiana debneyi* × *N. tabacum* L. show hybrid lethality, which is one of the mechanisms for reproductive isolation. Apoptotic changes were detected in the cells of hybrid seedlings expressing lethality at 28 ℃, but not at high temperature (34 ℃) indicating lethality was suppressed. When hybrid seedlings cultured at 34 ℃ were transferred to 28 ℃, lethal symptoms appeared. Chromatin condensation and nuclear fragmentation were observed in leaf protoplasts isolated from hybrid seedlings expressing lethality. Agarose gel analysis of DNA extracted from leaves of hybrid seedlings revealed a specific ladder pattern, suggesting nucleosomal fragmentation. Nuclear fragmentation was correlated with lethal symptoms in hybrid seedlings, as confirmed by flow cytometry. These results were observed in hybrid seedlings transferred to 28 ℃ from 34 ℃ but not in hybrid seedlings maintained at 34 ℃. This phenomenon suggests that apoptosis in hybrid seedlings from the cross *N. debneyi* × *N. tabacum* is temperature dependent.

Key words: apoptosis, chromatin condensation, DNA fragmentation, hybrid lethality, *Nicotiana*, nuclear fragmentation, temperature

Hybrid lethality is a postzygotic mechanism for the reproductive isolation of species (Stebbins 1966). This phenomenon has been observed in interspecific hybrids of several genera, including crop plants (Oka 1962; Phillips and Reid 1975; Zeven 1981). Hybrid lethality is also a significant obstacle preventing wide hybridization in plant breeding programs.

In the genus *Nicotiana*, Yamada *et al.* (1999) performed hybridization of 186 cross combinations among 17 species and observed that the hybrids of 14 cross combinations died at the seedling stage. *N. glutinosa* × *N. repanda* is one of those cross combinations and the seedlings of the cross were analyzed with respect to cell death. Marubashi *et al.* (1999) revealed apoptotic features in the cells of hybrid seedlings of *N. glutinosa* × *N. repanda* and concluded that apoptotic cell death induced hybrid lethality. Yamada *et al.* (2000) reported that the cell death inducing hybrid lethality of *N. suaveolens* × *N. tabacum* was apoptosis and apoptotic changes were detected in the cells of hybrid seedlings at 28 ℃ but not under high temperature (36 ℃), when the lethality was suppressed.

Hybrid seedlings of *N. debneyi* × *N. tabacum* showed hybrid lethality at the cotyledonary stage (Yamada *et al.* 1999). We detected apoptotic changes of nucleus, cytoplasm and DNA in hybrid seedlings of *N. debneyi* × *N. tabacum* at 28 ℃ but not at high temperature (34 ℃), when the lethality was suppressed (Marubashi and Kobayashi, 2002). In this study, we analyzed apoptotic changes of hybrid seedlings of *N. debneyi* × *N. tabacum* that were transferred to 28 ℃ from 34 ℃, when the lethality was suppressed.

Flowers of *Nicotiana debneyi* Domin., emasculated before anthesis, were pollinated with fresh pollen of *N. tabacum* L. cv. Hicks-2 (seeds from Japan Tobacco Inc., Iwata, Japan). F_1 seeds were sown on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.2% gelrite, pH 5.8, and were cultured at 28 ℃ under continuous illumination (ca. 3000 lx) for germination. Under these conditions, hybrid seedlings expressed lethality. To suppress lethality, the seedlings were transferred to 34 ℃ immediately after germination. The surviving seedlings (45 day-old hybrid seedlings) at 34 ℃ were exposed to 28 ℃ to induce lethality.

For detection of apoptotic changes of nuclei, protoplasts were isolated from the leaves of hybrid seedlings. The leaves were sectioned and treated

with enzyme solution containing 2% Onozuka R-10 cellulase (Yakult Co., Japan), 0.2% Macerozyme R-10 (Yakult Co., Japan), 0.7 M mannitol, and 10 mM $CaCl_2$, pH 5.6, at 60 rpm for 3-4 h at 30 ℃. The protoplasts were separated from cellular debris by using a 42-mm nylon sieve, and after centrifugation for 5 min at 100 g (room temperature), the supernatant was discarded and the protoplast pellet was resuspended in 0.7 M mannitol. The protoplasts, stained with 0.5% 4'-6-diamino-2-phenylindole dihydrochloride (DAPI), were observed under a fluorescence microscope (AX70; Olympus, Japan) using U excitation (330-385 nm) and photographed by a cooled CCD camera (Quantix; Photometrics).

For detection of DNA fragmentation, genomic DNA was extracted from the leaves of hybrid seedlings using the modified CTAB method (LoSchavw *et al.*, 2000). To detect DNA fragmentation, extracted DNA was separated on a 2% agarose gel and was visualized using SYBR Gold (Wako Chemical Co., Japan) and UV light.

For cytometric analysis, nuclei were isolated from leaves, stems and roots of hybrid seedlings by chopping them in ice-cold buffer (Michaelson *et al.*, 1991) and filtering the macerated tissue through 70- and 20-mm nylon sieves. The nuclei were collected from the filtrate by centrifugation for 5 min at 700 g and suspended in sheath fluid (FACSFlow; Becton Dickinson) supplemented with 5 g ml^{-1} propidium iodide (PI) and 10 mg ml^{-1} RNase and incubated for 15 min at 37 ℃. The DNA contents of the isolated nuclei were analyzed by a flow cytometer (FACSCalibur; Becton Dickinson).

The hybrid seedlings from the cross *N. debneyi* × *N. tabacum* exhibited hybrid lethality at 28 ℃ (Yamada *et al.*, 1999; Kobayashi and Marubashi, 2001). When F_1 seeds were sown on MS medium and cultured at 28 ℃, the seeds germinated normally but all the seedlings expressed lethality at the cotyledonary stage. The lethal symptoms shown by the seedlings at 28 ℃ were browning of hypocotyls, roots and cotyledons. The hybrid seedlings transferred to 34 ℃ immediately after germination at 28 ℃ did not express any lethal symptoms and grew normally. **Fig. 1A** shows a hybrid seedling cultured at 34 ℃ for 45 days after germination. When 45-day-old hybrid seedlings cultured at 34 ℃ were transferred to 28 ℃, their growth stopped, their stems turned brown within a few days and their leaves turned yellow within a week (**Fig. 1B, C**). All of the leaves of hybrid seedlings cultured at 28 ℃ for 21 days turned yellow (**Fig. 1D**). All of the hybrid seedlings died by 40 days after exposure to the lower temperature (**Fig. 1E**).

Apoptotic changes of nuclei were detected in leaves of hybrid seedlings transferred to 28 ℃ from 34 ℃. **Fig. 2** shows progressive changes of nuclear structures. Normal chromatin structure was observed in the protoplasts isolated from hybrid seedlings cultured at 34 ℃, and not exposed to 28 ℃ (**Fig. 2A**). Chromatin condensation was observed in the protoplasts isolated from hybrid seedlings cultured at 28 for 14 days (**Fig. 2B**). Nuclear fragmentation was observed in the protoplasts isolated from hybrid seedlings cultured at 28 ℃ for 30 days (**Fig. 2C**). Electrophoresis of total DNA isolated from the leaves of hybrid seedlings cultured for 14 days at 28 ℃ expressing lethality showed a distinctive ladder pattern on the agarose gel after staining with SYBR Gold, suggesting nucleosomal fragmentation of DNA (**Fig. 3** lane 2). Chromatin condensation,

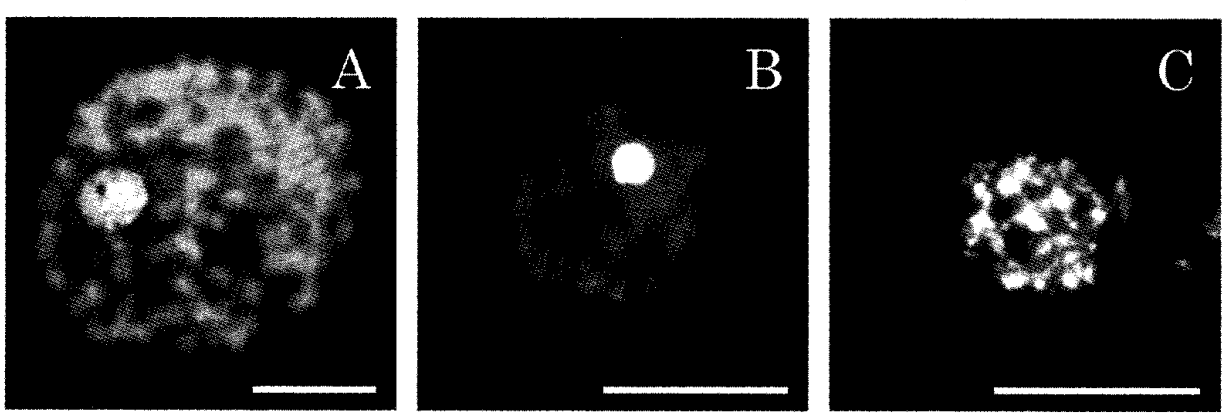

Fig. 2 Progressive changes of nuclear structure detected in protoplasts stained with DAPI. (A) Normal structure of chromatin in green leaves of a seedling cultured at 34 ℃ for 45 days after germination. (B) Chromatin condensation in yellow leaves of a seedling cultured at 28 ℃ for 14 days. (C) Nuclear fragmentation in yellow leaves of a seedling cultured at 28 ℃ for 30 days.
Bars are 5 μm.

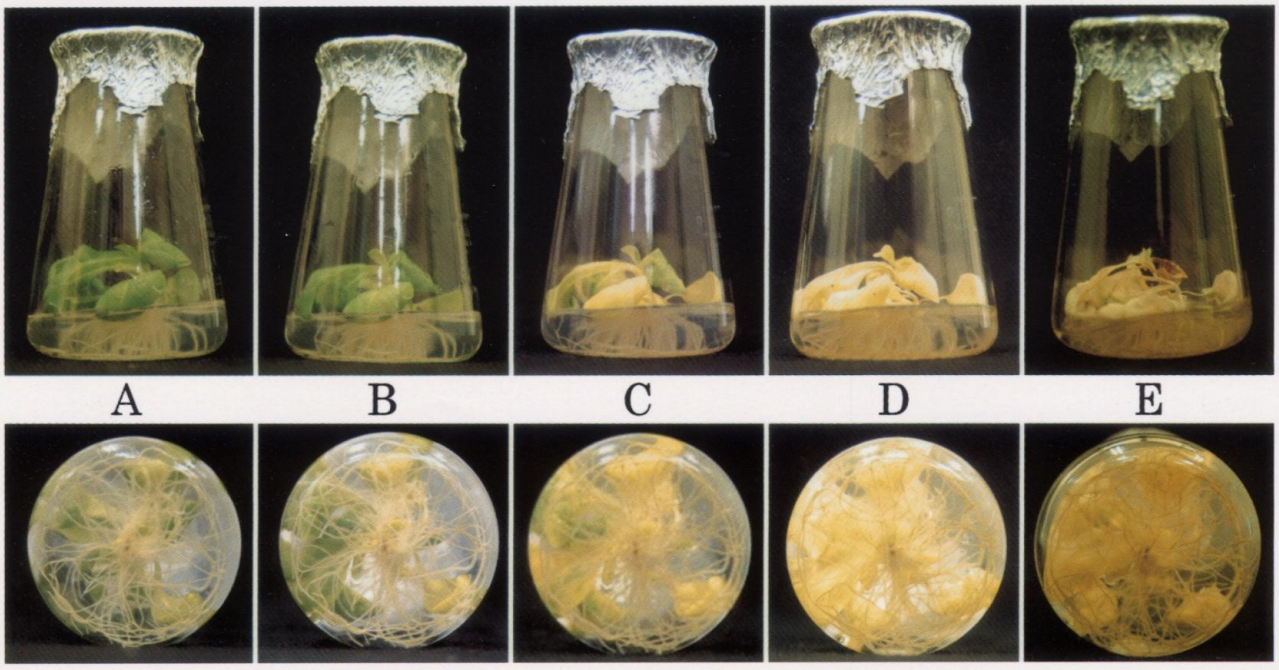

Fig. 1 Morphological changes of hybrid seedlings from the cross *N. debneyi* × *N. tabacum*. (A) A seedling cultured at 34°C for 45 days after germination grew without any lethal symptoms. (B) A seedling transferred to 28°C and cultured for 3 days stopped growth and the stem turned brown. (C) A seedling cultured at 28°C for 7 days showed chlorosis in some leaves. (D) A seedling cultured at 28°C for 21 days showed chlorosis in all the leaves. (E) A seedling cultured at 28°C for 40 days dried up and died.

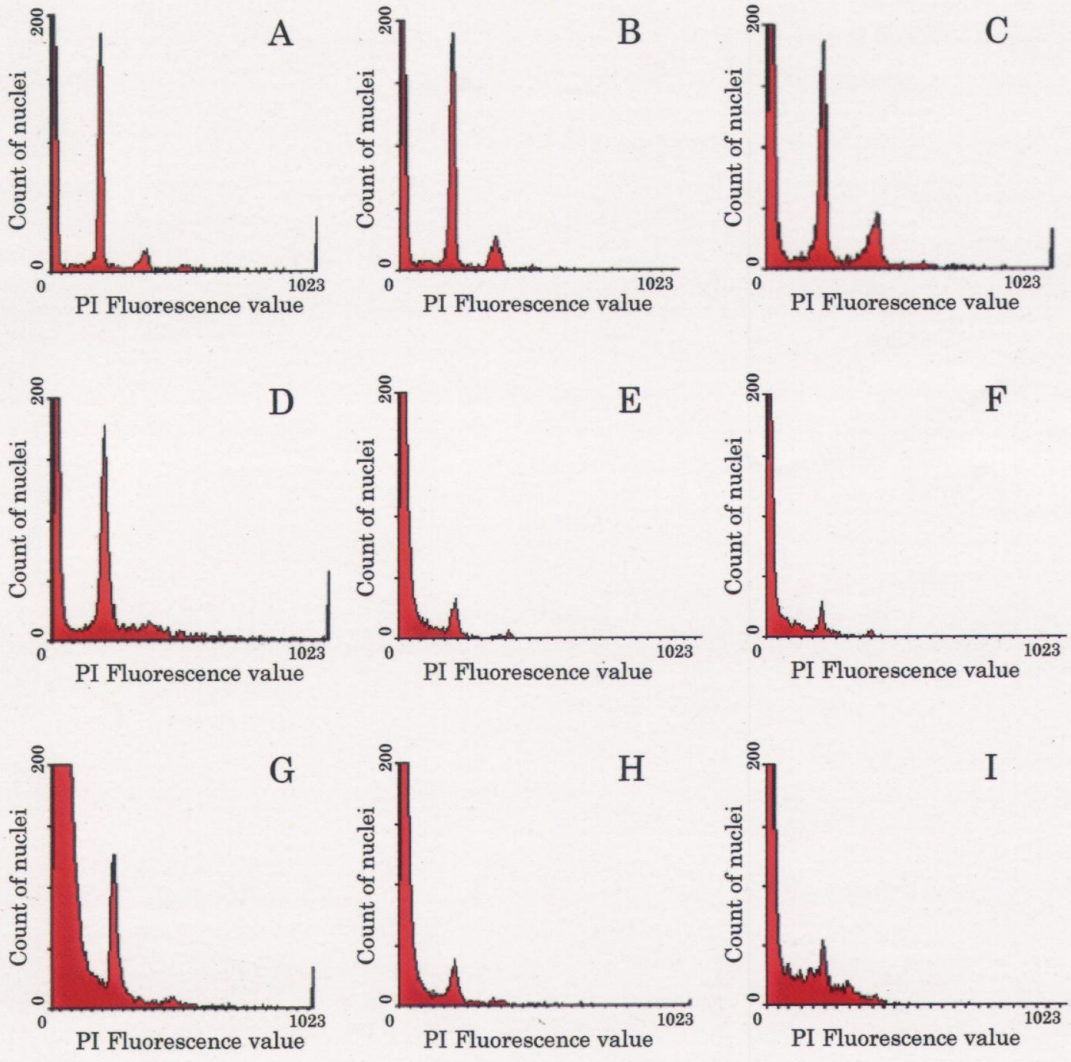

Fig. 4 Histograms indicating increased fragmentation of nuclei in leaves, stems and roots of hybrid seedlings. (A)-(C) A seedling cultured at 34°C for 45 days. (D)-(F) A seedling transferred to 28°C and cultured for 14 days. (G)-(I) A seedling transferred to 28°C and cultured for 30 days.

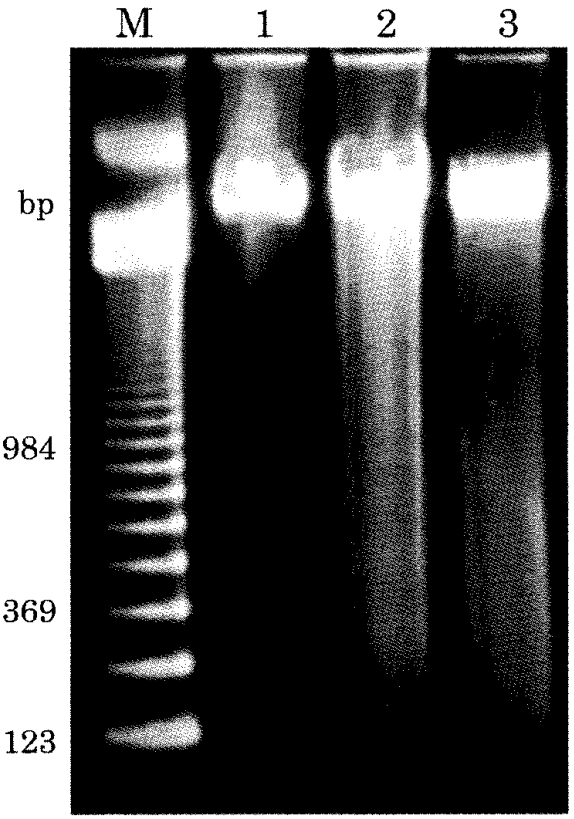

Fig. 3 DNA fragmentation detected in the leaves of hybrid seedlings. Lane 1, 123-bp DNA ladder markers; lane 2, green leaves from a seedling cultured at 34 ℃ for 45 days; lane 3, yellow leaves from a seedling transferred to 28 ℃ and cultured for 14 days; lane 4, yellow leaves from a seedling transferred to 28 ℃ and cultured for 30 days.

nuclear fragmentation and DNA fragmentation are considered typical phenomena of apoptosis in animal cells (Kerr and Harmon, 1991). These results suggest that the cell death detected in hybrid seedlings from the cross *N. debneyi* × *N. tabacum* exposed to 28 ℃ is indeed apoptosis. Apoptosis in hybrid seedlings from the cross *N. debneyi* × *N. tabacum* was temperature dependent because it was detected at 28 ℃ but not at 34 ℃ .

Manabe *et al.* (1989) reported that hybrid lethality expressed in the cross *N. suaveolens* × *N. tabacum* was suppressed at high temperature (36 ℃) and the hybrid seedlings showed browning of stems and veins within 24 h when they were transferred to 28 ℃ . In the same cross, Yamada *et al.* (2000) clarified that apoptotic cell death induced temperature-sensitive lethality in the hybrid seedlings. Marubashi *et al.* (1999) reported that apoptotic cell death induced lethality in hybrid seedlings from the cross *N. glutinosa* × *N. repanda* and Yamada *et al.* (2001) showed that high temperature (32 ℃) suppressed lethal symptoms in the same cross. In this study, we detected a new cross, *N. debneyi* × *N. tabacum*, that showed temperature-dependent apoptosis in the hybrid seedlings. Temperature-dependent hybrid lethality in the genus *Nicotina* generally might be caused by apoptosis.

The nuclear fragmentation was further evaluated by analysis of DNA contents of nuclei isolated from the hybrid leaves, stems and roots using a flow cytometer. In the histograms of PI fluorescence values, indicating the relative mass of nuclear DNA, the hybrid seedlings with green leaves, green stems and white roots maintained at 34 ℃ showed two peaks that might correspond to nuclei at the G_1 and G_2M phases of the cell cycle (**Fig. 4A-C**). The hybrid seedlings cultured at 28 ℃ for one week showed lethal symptoms. The leaves turned yellow and exhibited a smaller G_2M peak (**Fig. 4D**). The stems and roots turned brown and exhibited a smaller G_1 peak, and additional peaks with lower fluorescence values appeared (**Fig. 4E, F**). These latter peaks, corresponding to fragmented nuclei, further increased in size in histograms of seedlings cultured at 28 ℃ for 40 days (**Fig. G-I**). These quantitative results support the conclusion that distinct nuclear fragmentation progressed with lethal symptoms.

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