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Temperature-Dependent Programmed Cell Death Detected in Hybrids between *Nicotiana langsdorffii* and *N. tabacum* Expressing Lethality

Hitoshi WATANABE and Wataru MARUBASHI*

School of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393, Japan
**Corresponding author* E-mail address: marubasi@mx.ibaraki.ac.jp

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Abstract

Interspecific hybrids of *Nicotiana langsdorffii* × *N. tabacum* showed hybrid lethality when they were transferred to 24℃ after cultured at 34 ℃ for 50 days. Characteristic features of programmed cell death (PCD) were detected in these plants, but no such features were observed when seedlings were transferred to higher than 26℃. Chromatin condensation and nuclear fragmentation were observed in protoplasts isolated from yellow and brown leaves of hybrid seedlings expressing lethality. Electrophoresis of total DNA isolated from the leaves of hybrid seedlings showed a DNA ladder patterns, suggesting nucleosomal fragmentation of DNA. These results suggest that PCD is accompanied by temperature-dependent lethality of hybrids between *N. langsdorffii* and *N. tabacum*.

Key words: chromatin condensation, DNA fragmentation, hybrid lethality, *Nicotiana*, nuclear fragmentation, programmed cell death, temperature.

In the genus *Nicotiana*, interspecific hybrids die at the seedling stage. The hybrid lethality can be suppressed by culturing the seedlings at high temperature. The high temperature that suppresses lethality varies by the cross combination; it is 36℃, 34℃ and 32℃ for *N. suaveolens* × *N. tabacum* (Manabe *et al.*, 1989), *N. debney* × *N. tabacum*, (Marubashi and Kobayashi, 2002a) and *N. glutinosa* × *N. repanda* (Yamada *et al.*, 1999), respectively. In these reports, the expedient temperature to induce lethality was 28℃. The characteristic features of programmed cell death (PCD) in plants undergoing hypersensitive response, such as chromatin condensation, nuclear fragmentation and DNA fragmentation, were detected in cells of these hybrid seedlings expressing lethality (Ryerson and Heath, 1996; Wang *et al.*, 1996). Agarose gel electrophoresis, fluorescent microscopy and terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay are widely used as diagnostic tools for PCD (or apoptotic cell death) in plant cells undergoing hybrid lethality (Marubashi *et al.*, 1999; Yamada *et al.*, 2000; Marubashi and Kobayashi, 2002b).

Using thin layer cell culture system for *N. suaveolens* × *N. tabacum*, Masuda *et al.* (2003) concluded that expression of hybrid lethality is divided into two processes: the former process requires expression of hybrid lethality-related gene(s), and it is reversible and temperature-sensitive, and the latter process uses the existing mechanism of the apoptotic cell death execution phase.

In the present study, we investigated the temperatures inducing and suppressing lethality of hybrid seedlings from a new cross, *N. langsdorffii* × *N. tabacum*, we then determined whether the characteristic features of PCD can also be detected in these hybrid seedlings expressing lethality.

Flowers of *N. langsdorffii* that had been emasculated before anthesis were pollinated with fresh pollen of *N. tabacum* L. cv. Hicks-2 (seeds were provided from Japan Tobacco Inc., Iwata, Japan). The hybrid seeds were treated with a 0.5% gibberellin solution (GA_3; Wako Chemical Co., Japan), then sterilized with 5% sodium hypochlorite, sown on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.2% Gelrite, pH 5.8, and then cultured at 28℃ under continuous illumination (2000 lux). Immediately after germination, they were transferred to 22℃, 24℃, 26℃, 28℃, 30℃, 32℃, 34℃ or 36℃ to investigate the temperatures inducing and suppressing lethality. The seedlings were cultured at temperatures suppressing lethality for 50 days after germination, then were transferred to temperatures inducing lethality.

To detect morphological changes in cells of hybrid seedlings expressing lethality, protoplasts

were isolated from the leaves of hybrid seedlings and observed under a fluorescence microscope. The leaves were sectioned and treated with an enzyme solution containing 2% (w/v) Onozuka R-10 cellulase (Yakult Co., Japan), 0.2%(w/v) Macerozyme R-10 (Yakult Co., Japan), 0.7 M mannitol, and 10 mM $CaCl_2$, pH 5.6, for 3-4 h at 30℃. The protoplasts were separated from cellular debris by filtering through a 53 μm nylon sieve. After centrifugation at 1300 rpm for 5 min at room temperature, the supernatant was removed, and then the pellet was resuspended in 0.7 M mannitol. Following the method of Shivanna and Rangaswamy (1992), 3 μl of 4′-6-diamino-2-phenylindole dihydrochloride (DAPI) solution was added to a drop of the cell suspension. Then the suspension was observed under a fluorescence microscope (BX50; Olympus, Japan) using U excitation (330-385 nm) and photographed by a digital camera (FinePix S1 Pro, Fujifilm, Japan).

For detection of a DNA ladder pattern, suggestive of nucleosomal fragmentation of DNA, genomic DNA was extracted from the leaves of hybrid seedlings using a CTAB method with some modifications (LoSchiavo *et al.*, 2000). DNA extracts were separated on a 2% agarose gel, and then were stained with SYBR Gold according to the manufacturer's instruction (Wako Chemical Co., Japan) and photographed under UV exposure.

Chromosome observation was performed by the Feulgen method (Gahan, 1984) using hybrid seedlings that had been acclimatized. Collected root tips were fixed in 45% acetic acid for 20 s, and hydrolysed in 1 N HCl for 8 min at 60℃. Chromosomes were stained with Schiff's reagent (Sigma) for 12 min and counted in squashed preparations.

Fig. 1 shows hybrid seedlings cultured at 22-36℃ for 40 days immediately after germination at 28℃. All of hybrid seedlings transferred to 30-36℃ after germination did not express lethality and grew normally. The seedlings developed faster at 34℃ than at the other temperatures. All hybrid seedlings at 26℃ and 28℃ grew slowly but did not die. All seedlings at 22℃ and 24℃ expanded first and second true leaves by 30 days after germination. However, they exhibited lethal symptoms, specifically, browning of hypocotyls by 70 days after germination, and browning of roots and leaves by 150 days after germination. According to this result, we considered 22℃ and 24℃ as temperatures inducing lethality, and 34℃ as the temperature suppressing lethality.

Seedlings surviving at 34℃ (50 days-old after germination) were divided into 4 groups (25 or 26 seedlings). Each group was transferred to 22℃ and 24℃, and to 26℃ and 28℃ for comparison with previous reports (Marubashi *et al.*, 1999; Yamada *et al.*, 2000; Marubashi and Kobayashi, 2002b). Hybrid seedlings cultured at 22℃ and 24℃ died at 40 days and 50 days after transfer from 34℃, respectively (**Fig. 2A, B**). In all of the hybrid seedlings at 22℃, green leaves began to pale the day after transfer, and all of the leaves turned yellow at 20 days and brown at 40 days after transfer from 34℃. Browning of roots and stems accompanied yellowing of the leaves (**Fig. 2A**). The leaves of all hybrid seedlings except one turned yellow and brown by 30 and 50 days, respectively, after transfer from 34℃ to 24℃ (**Fig. 2B**). The exceptional seedling, that its leaves color did not change, stopped growing and expressed browning of roots, stem and the bottom leaves, although it did not show progression of lethality during the subsequent culture. All of the hybrid seedlings at 26℃ and 28℃ grew slowly without lethal symptoms, and the hybrid seedlings at 26℃ were acclimatized (**Fig. 2C**). The acclimatized seedlings had a predicted 33 chromosomes (*N. langsdorffii*, 2n = 18; *N. tabacum*, 2n = 48) (**Fig. 2D**).

Fluorescence microscopy of DAPI-stained protoplasts showed morphological changes of nuclei from the leaves of hybrid seedlings transferred to 22℃ and 24℃ from 34℃ (**Fig. 3**). Normal nuclei with a nucleolus and chloroplasts with bright autofluorescence were observed in protoplasts isolated from green leaves of hybrid seedlings cultured at 34℃ (**Fig. 3A**). From yellow leaves of hybrid seedlings cultured at 22℃ for 20 days and at 24℃ for 30 days after transfer from 34℃, protoplasts were isolated with the same efficiency as that from green leaves. More than half of the protoplasts showed chromatin condensation, and autofluorescence of chloroplasts was weak (**Fig. 3B, D**). Brown leaves of hybrid seedlings cultured at 22℃ for 40 days and at 24℃ for 50 days after transfer from 34℃ had a very low yield of protoplasts, most of which showed nuclear fragmentation (**Fig. 3C, E**). Such changes were not observed in protoplasts isolated from hybrid seedlings cultured at 26℃ and 28℃ after transfer from 34℃ (data not shown).

Electrophoresis of total DNA isolated from the leaves of hybrid seedlings expressing lethal symptoms at 22℃ and 24℃ showed DNA ladder patterns, suggesting nucleosomal fragmentation of DNA (**Fig. 4A, B**). No such DNA ladder patterns were detectable in hybrid seedlings transferred to 26℃ and 28℃ (**Fig. 4C, D**) or those cultured at 34℃ (data not shown).

Chromatin condensation, nuclear fragmentation and DNA fragmentation are considered key characteristics of PCD (Ryerson and Heath, 1996; Wang *et*

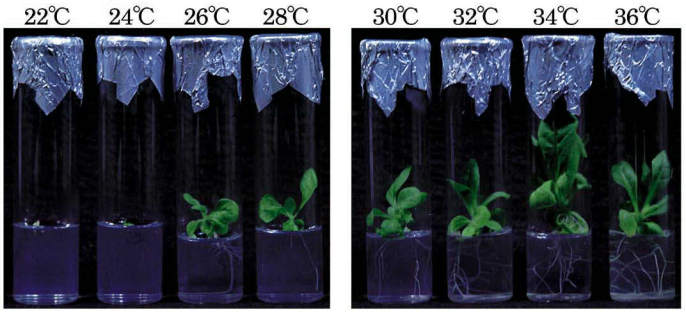

Fig. 1 *N. langsdorffii* × *N. tabacum* hybrid seedlings cultured at 22°C, 24°C, 26°C, 28°C, 30°C, 32°C, 34°C, and 36°C (left to right) for 40 days after germination at 28°C. The seedlings cultured at 22°C and 24°C died, but the seedlings cultured at other temperatures continued to grow.

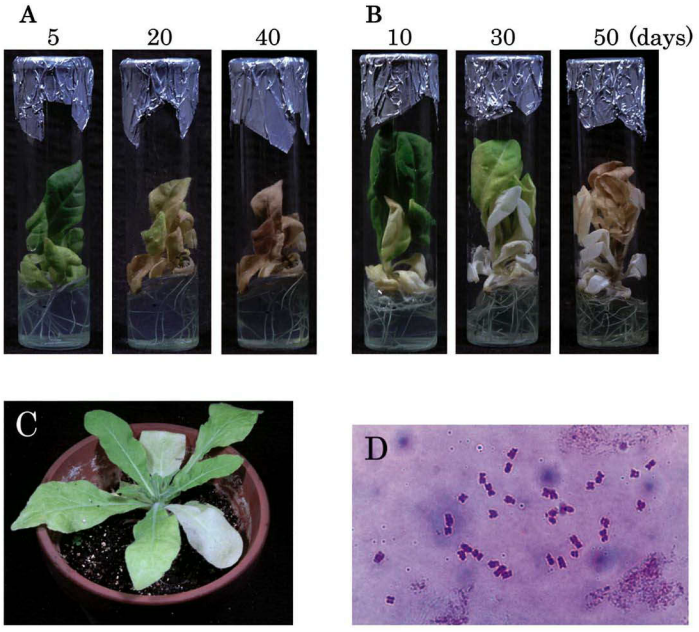

Fig. 2 *N. langsdorffii* × *N. tabacum* hybrid seedlings cultured at 22°C, 24°C and 26°C, after transfer from 34°C. (A) A seedling cultuted at 22°C for 5 (left), 20 (middle) and 40 days (right) after transfer from 34°C. All leaves of the seedlings turned yellow by 20 days and turned brown by 40 days. (B) A seedling cultured at 24°C for 10 (left), 30 (middle) and 50 days (right) after transfer from 34°C. All leaves of the seedling turned yellow by 30 days and turned brown 50 days. (C) A hybrid seedling acclimatized at 26°C. (D) Methaphase chromosomes of a root tip cell of a hybrid seedling acclimatized at 26°C (2n = 33).

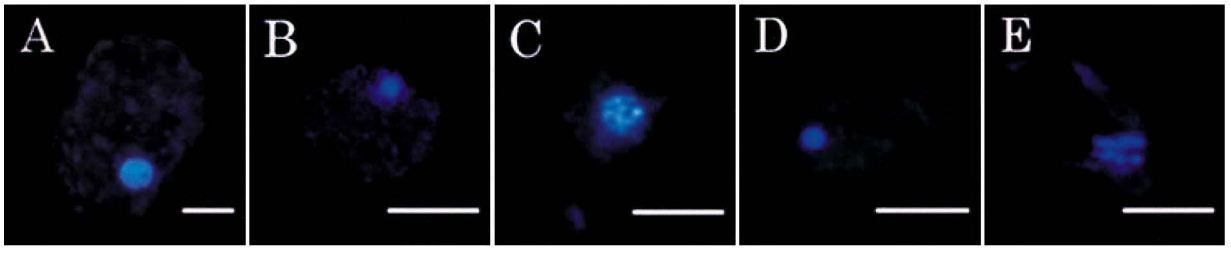

Fig. 3 Morphological changes of nuclei deteced in protoplasts isolated from leaves of *N. langsdorffii* × *N. tabacum*. (A) Normal nuclei in green leaves of a seedling cultured at 34°C for 50 days after germination. (B), (D) Chromatin condensation in yellow leaves of seedlings cultured at 22°C for 20 days and at 24°C for 30 days after transfer from 34°C, respectively. (C), (E) Nuclear fragmentation in brown leaves of seedlings cultured at 22°C for 40 days and at 24°C for 50 days after transfer from 34°C, respectively. Bar = 10μm.

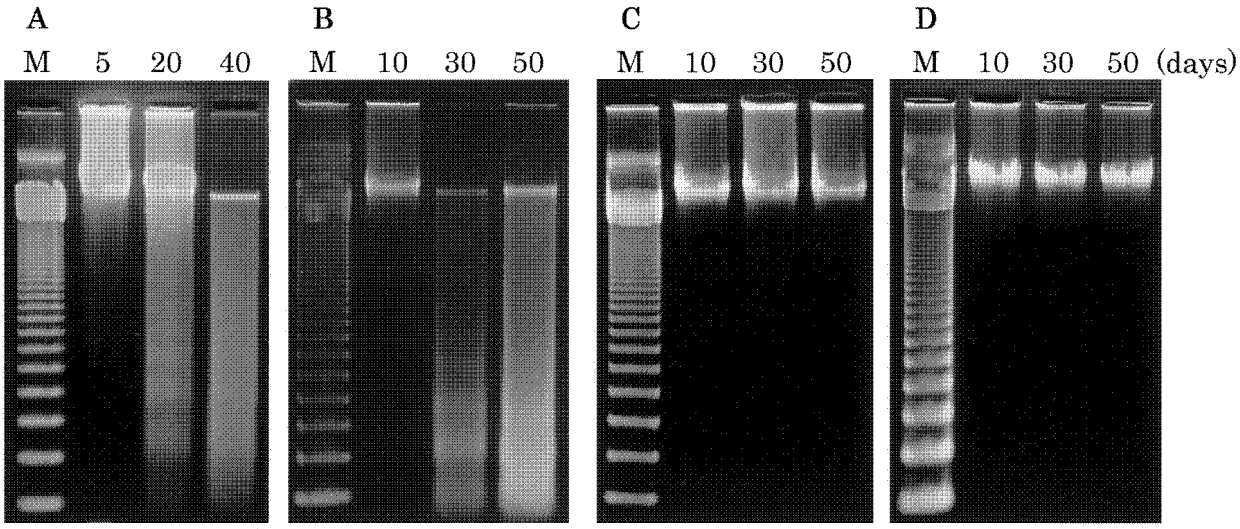

Fig. 4 DNA fragmentation detected in leaves of hybrid seedlings from the cross *N. langsdorffii* × *N. tabacum*. M, 123 bp DNA ladder marker. (A) DNA from leaves of a seedling cultured at 22℃ for 5, 20 and 40 days after transfer from 34℃. (B), (C), (D) DNA from leaves of seedlings cultured at 24℃, 26℃ and 28℃, respectively, for 10, 30 and 50 days after transfer from 34℃.

al., 1996). These features were detected in the expression of lethality of hybrid seedlings from this new cross, *N. langsdorffii* × *N. tabacum* L. cv. Hicks-2. These results suggest that PCD is accompanied by temperature-dependent lethality of the hybrid seedlings.

In the present case, hybrid lethality of hybrid seedlings from *N. langsdorffii* × *N. tabacum* was induced at 22℃ and 24℃, but not at 26℃ or 28℃. The 28℃ was the temperature inducing lethality of cross combinations reported previously (Manabe *et al.*, 1989; Yamada *et al.*, 1999; Marubashi and Kobayashi, 2002a). Yamada and Marubashi (2003) confirmed that ethylene production in hybrid seedlings from *N. suaveolens* × *N. tabacum* was increased at 28℃ and suppressed at 36℃ by inactivation of the ethylene forming enzyme, 1-aminocyclopropane-1-carboxylic acid oxidase, and concluded that overproduced ethylene acts as an essential factor causing PCD. From this consequence and the results of the present study, we propose that in the present case, ethylene biosynthesis increases below 24℃, and declines above 26℃ by inactivation of the enzyme.

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