Programmed Cell Death Detected in Interspecific Hybrids of *Nicotiana repanda* × *N. tomentosiformis* Expressing Hybrid Lethality

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Interspecific hybrids of *Nicotiana repanda* × *N. tabacum* die shortly after germination. Seedlings that are hybrids between *N. repanda* and *N. tomentosiformis*, which is a progenitor of *N. tabacum*, express hybrid lethality when they are cultured at 28°C for 90 days after germination, but seedlings that are hybrids between *N. repanda* and *N. sylvestris*, another progenitor of *N. tabacum*, grow normally. We detected several features of programmed cell death (PCD) in hybrid seedlings of *N. repanda* × *N. tomentosiformis* expressing hybrid lethality. Nuclear fragmentation was detected in nuclei isolated from dying shoots and roots of hybrid seedlings expressing lethality. In addition, electrophoresis of total DNA extracted from the shoots and roots of hybrid seedlings showed a distinctive DNA ladder pattern, suggesting cleavage of nuclear DNA into oligonucleosomal fragments. These results indicate that hybrid seedlings of *N. repanda* × *N. tomentosiformis* undergo PCD in the process of hybrid lethality. However, in hybrid seedlings of *N. repanda* × *N. sylvestris*, no features of PCD were detected. These results suggest that PCD might also be expressed in hybrid seedlings of *N. repanda* × *N. tabacum* because *N. tomentosiformis* is a progenitor of *N. tabacum*.

Key Words: programmed cell death, hybrid lethality, nuclear fragmentation, oligonucleosomal fragments, interspecific hybrid, *Nicotiana*.

Introduction

Hybrid lethality is one of the important devices whereby higher plants prevent wide crosses. In the genus *Nicotiana*, the hybrid lethality expressed in two interspecific crosses has been studied genetically. Using monosomic analysis, Gerstel *et al.* (1979) suggested that in the cross *N. africana* × *N. tabacum*, a gene(s) on the H chromosome of *N. tabacum* is involved in the expression of lethality in the hybrid seedlings. In the cross *N. suaveolens* × *N. tabacum*, the Q chromosome of *N. tabacum* is responsible for lethality (Marubashi and Onosato 2002).

Recently, hybrid lethality has been expressed in several

interspecific crosses, such as *N. glutinosa* × *N. repanda* (Marubashi *et al.* 1999), *N. suaveolens* × *N. tabacum* (Yamada *et al.* 2000), *N. debneyi* × *N. tabacum* (Marubashi and Kobayashi 2002a, 2002b), *N. tabacum* × *N. suaveolens* (Tezuka and Marubashi 2004), and *N. langsdorffii* × *N. tabacum* (Watanabe and Marubashi 2004). In addition, features of PCD (or apoptotic cell death), including nuclear fragmentation and cleavage of nuclear DNA into oligonucleosomal fragments, which are considered the key characteristics of PCD in plant cells undergoing a hypersensitive response (Ryerson and Heath 1996, Wang *et al.* 1996), were observed during hybrid lethality. Cytometric analysis and agarose gel electrophoresis are generally applied as diagnostic tools for PCD in plant cells during expression of hybrid lethality (Marubashi *et al.* 1999, Yamada *et al.* 2000, Marubashi and Kobayashi 2002a, 2002b, Tezuka and Marubashi 2004, Watanabe and Marubashi 2004).

N. repanda Willd. is considered a valuable source of resistance to several diseases of cultivated tobacco (*N. tabacum* L.). Reed and Collins (1978) reported that hybrid seedlings of *N. repanda* × *N. tabacum* obtained through in vitro culture of fertilized ovules die shortly after germination. When *N. repanda* was crossed with *N. tomentosiformis*, which is a progenitor of *N. tabacum*, hybrid seedlings expressed hybrid lethality at 28°C by 90 days after germination (Suda *et al.* 1991). However, when *N. sylvestris*, another progenitor of *N. tabacum*, was used as the male plant, hybrid seedlings grew normally. In the present study, we examined whether PCD features, including nuclear fragmentation and cleavage of nuclear DNA into oligonucleosomal fragments, are detected in hybrid seedlings of *N. repanda* × *N. tomentosiformis* expressing hybrid lethality.

Materials and Methods

Plant materials

Nicotiana repanda Willd. (2n=48), *N. tomentosiformis* (2n=24) and *N. sylvestris* (2n=24) were used in this experiment. These seeds, which were supplied by Japan Tobacco Inc., were cultivated under greenhouse conditions.

Hybridization and growth conditions

Flowers of *N. repanda*, emasculated before anthesis, were pollinated with fresh pollen of *N. tomentosiformis* or *N. sylvestris*. F₁ seeds were soaked in a 0.5% (w/v) gibberellic acid solution for 40 min, sterilized with 5% (v/v) sodium hypochlorite solution for 15 min, and then rinsed three times

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with sterilized water. Sterilized 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 germinated at 28°C under continuous illumination (ca. 3,000 lx).

Hybrid seedlings of *N. repanda* × *N. tomentosiformis* cultured at 28°C were harvested at 30, 60 and 90 days after germination and applied to subsequent experiments. At 30 days after germination, hybrid seedlings of *N. repanda* × *N. tomentosiformis* maintained normal shoots and roots. However, by 60 days after germination, shoots and roots of hybrid seedlings turned yellow and brown, respectively. By 90 days after germination, both shoots and roots of hybrid seedlings had turned brown. In contrast, hybrid seedlings of *N. repanda* × *N. sylvestris* cultured for 90 days after germination were harvested and applied to subsequent experiments. Their growth was normal for more than 90 days after germination.

Flow cytometry

Nuclei were isolated from shoots 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- μ m nylon mesh. The nuclei were collected from the filtrate by centrifugation for 5 min at 2,500 rpm, suspended in ice-cold buffer supplemented with 5 μ g/ml propidium iodide and 10 μ g/ml RNase, and incubated for 15 min at 36°C. The DNA contents of the isolated nuclei were analyzed by flow cytometry (FACSCalibur; Becton Dickinson). Based on the histograms obtained by 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.

Agarose gel electrophoresis

Shoot and root materials frozen in liquid nitrogen were ground into a fine powder in a pestle with a mortar. The powder was suspended in 2% cetyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 0.2% v/v β -mercaptoethanol, 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, centrifuged for 10 min at 14,000 rpm, then dissolved in 10 mM Tris-HCl (pH 8) containing 10 mM EDTA. The DNA solution was electrophoresed in a 2% agarose gel and visualized using SYBR Gold (Wako Chemical Co., Japan) under UV light.

Results and Discussion

Hybrid seeds of *N. repanda* × *N. tomentosiformis* and *N. repanda* × *N. sylvestris* were sown on 1/2 MS medium and germinated at 28°C. Hybrid seedlings of *N. repanda* ×

N. sylvestris, which did not express lethality, grew normally until at least 90 days after germination (Fig. 1D). Hybrid seedlings of *N. repanda* × *N. tomentosiformis* also germinated normally and showed no unusual changes in appearance 30 days after germination (Fig. 1A). By 60 days after germination, however, the shoots of all the seedlings turned yellow, and the roots turned brown (Fig. 1B). By 90 days after germination, the shoots and roots had turned brown, and the hybrid seedlings died (Fig. 1C).

Nuclear fragmentation was evaluated by flow cytometric analysis of DNA contents of nuclei isolated from the hybrid shoots and roots. The histograms of PI fluorescence values indicate the relative size of nuclear DNA masses. Nuclei isolated from leaves of hybrid seedlings of *N. repanda* × *N. sylvestris*, which did not express hybrid lethality, showed two peaks, which probably correspond to the nuclei at the G1 and G2/M phases of the cell cycle (Fig. 2G). The shoots and roots of *N. repanda* × *N. tomentosiformis* seedlings at 30 days after germination also showed two peaks (Fig. 2A, D). By 60 days after germination, the hybrid shoots and roots exhibited additional peaks with lower fluorescence values than that for G1 (Fig. 2B, E). By 90 days after germination, the G1 peaks of the hybrid shoots and roots had decreased slightly, and additional peaks with lower fluorescence values appeared (Fig. 2C, F), indicating nuclear fragmentation.

In the *N. repanda* × *N. sylvestris* seedlings, cleavage of nuclear DNA into oligonucleosomal fragments was not detected in either the leaves or the roots (Fig. 3C). In the *N. repanda* × *N. tomentosiformis* seedlings, electrophoresis of total DNA isolated from the shoots from 60 to 90 days after germination showed a distinctive DNA ladder pattern, suggesting cleavage of nuclear DNA into oligonucleosomal fragments (Fig. 3A). In the roots of *N. repanda* × *N. tomentosiformis*, this distinctive ladder pattern was observed from 45 to 90 days after germination (Fig. 3B).

Nuclear fragmentation and cleavage of nuclear DNA into oligonucleosomal fragments are considered the key characteristics of PCD (Ryerson and Heath 1996, Wang *et al.* 1996). In the present study, these features were detected in seedlings of *N. repanda* × *N. tomentosiformis* expressing hybrid lethality. However, *N. repanda* × *N. sylvestris* seedlings, which did not express lethality, grew normally without any features of PCD.

Reed and Collins (1978) reported that seedlings of *N. repanda* × *N. tabacum* obtained through in vitro culture of fertilized ovules died shortly after germination. Additionally, Suda *et al.* (1991) investigated hybrid lethality of seedlings in crosses of *N. repanda* with *N. tomentosiformis* or *N. sylvestris*, both of which are progenitors of *N. tabacum*. Hybrid seedlings showed lethality 90 days after germination only when *N. tomentosiformis* was used as the pollen parent and they were grown at 28°C. These results suggested that hybrid lethality of these crosses is caused by incompatibility of genomes between *N. repanda* and *N. tomentosiformis*. In the present study, we observed that the process of hybrid lethality in seedlings of *N. repanda* × *N. tomentosiformis*

Fig. 1. *N. repanda* × *N. tomentosiformis* and *N. repanda* × *N. sylvestris* seedlings cultured at 28°C. (A)–(C) *N. repanda* × *N. tomentosiformis* seedling cultured for 30, 60 and 90 days after germination, respectively. (D) *N. repanda* × *N. sylvestris* seedling cultured for 90 days after germination.

Fig. 2. Histograms indicating increased fragmentation of nuclei in shoots and roots of hybrid seedlings cultured at 28°C. (A)–(C) Shoots of *N. repanda* × *N. tomentosiformis* cultured for 30, 60 and 90 days after germination, respectively. (D)–(F) Roots of *N. repanda* × *N. tomentosiformis* cultured for 30, 60 and 90 days after germination, respectively. (G) Leaves of *N. repanda* × *N. sylvestris* cultured for 90 days after germination.

Fig. 3. DNA fragmentation detected in shoots and roots of *N. repanda* × *N. tomentosiformis*, and normal DNA profiles in leaves and roots of *N. repanda* × *N. sylvestris*. M, 123 bp DNA ladder marker. (A) Lanes 1–6, DNA from shoots of *N. repanda* × *N. tomentosiformis* cultured for 15, 30, 45, 60, 75 and 90 days after germination, respectively. (B) Lanes 1–6, DNA from roots of *N. repanda* × *N. tomentosiformis* cultured for 15, 30, 45, 60, 75 and 90 days after germination, respectively. (C) DNA from leaves (lane 1) and roots (lane 2) of *N. repanda* × *N. sylvestris* cultured for 90 days after germination.

seedlings is accompanied by PCD, including nuclear fragmentation and cleavage of nuclear DNA into oligonucleosomal fragments. These observations suggest that PCD observed in hybrids of *N. repanda* × *N. tomentosiformis* is induced by incompatibility of genomes between these two species and should also be detected in hybrid seedlings of *N. repanda* × *N. tabacum*.

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