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Apoptotic Cell Death Observed during the Expression of Hybrid Lethality in Interspecific Hybrids between *Nicotiana tabacum* and *N. suaveolens*

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Hybrid seedlings from the cross *Nicotiana tabacum* × *N. suaveolens*, obtained by test-tube pollination and ovule culture, expressed lethality at 28℃. Characteristic lethal symptoms in these hybrid seedlings consisted of browning of hypocotyls and roots. One hundred and seventeen hybrid seedlings were eventually obtained by the use of test-tube pollination and ovule culture. Hybrid seedlings maintained at 36℃ did not express any lethal symptoms. Hybrid seedlings used for further experiments were transferred to 36℃ immediately after germination at 28℃. When hybrid seedlings cultured at 36℃ were transferred to 28℃, their growth stopped and lethal symptoms were expressed. During the progressive expression of lethality, apoptotic features such as chromatin condensation, nuclear fragmentation and DNA fragmentation were detected. On the other hand, there was no sign of apoptotic cell death in the hybrid seedlings at 36℃. Based on the observation that the same lethal symptoms and the same apoptotic features were observed in the reciprocal cross, *N. suaveolens* × *N. tabacum*, we suggest that not only the underlying causes of hybrid lethality but also the underlying causes of apoptotic cell death are due to the interaction of coexisting heterogeneous genomes, rather than to the effect of cytoplasmic genes. Furthermore, the progression of apoptotic cell death in the cross *N. tabacum* × *N. suaveolens* began in stems and roots, followed by leaves.

Key Words: hybrid lethality, interspecific hybrid, reciprocal cross, test-tube pollination, ovule culture, apoptotic cell death, temperature sensitivity.

Introduction

Wide hybridization is an effective method for introducing certain desirable genes into cultivated species in plant breeding. Such attempts, however, are often thwarted by reproductive isolation. Hybrid lethality is a postzygotic mechanism for reproductive isolation that has been observed in several interspecific hybrids (Oka and Doida 1962, Phillips and Reid 1975, Zeven 1981). In the genus *Nicotiana*, hybridization has been performed between *N. tabacum* and wild species, and hybrid seedlings showing lethality were reported in several cross combinations (Reed and Collins 1978, Gerstel *et al.* 1979, DeVerna *et al.* 1987). The genetics of hybrid lethality has been studied in two interspecific crosses of the genus *Nicotiana*. Using monosomic analysis, Gerstel *et al.* (1979) suggested that in the cross between *N. tabacum* and *N. africana*, a gene or genes on the H chromosome of *N. tabacum* was related to the expression of lethality. In the cross between *N. tabacum* and *N. suaveolens*, Inoue *et al.* (1996) used two progenitors of *N. tabacum* for the crossing with *N. suaveolens* and reported that the S subgenome in *N. tabacum* was responsible for the lethality. Furthermore, Marubashi and Onosato (2002) carried out crossing between *N. tabacum* monosomic lines of the S subgenome and *N. suaveolens* by test-tube pollination and ovule culture, and reported that the Q chromosome of *N. tabacum* was responsible for the lethality observed in the cross between *N. tabacum* and *N. suaveolens*.

The expression of hybrid lethality is influenced by the temperature. Manabe *et al.* (1989) reported that hybrid lethality in the cross *N. suaveolens* × *N. tabacum* was expressed at 28°C and was suppressed at high temperatures (32–36°C). In other crosses in the genus *Nicotiana*, lethality was similarly suppressed at high temperatures (Yamada *et al.* 1999, Marubashi and Kobayashi 2002a, Mino *et al.* 2002). Temperature sensitivity of hybrid lethality was also confirmed in interspecific crosses of *Gossypium* (Phillips 1977).

Apoptosis is a distinct type of cell death characterized in animals by the condensation of chromatin, fragmentation of nuclei, cytoplasmic reduction and fragmentation of DNA (Kerr *et al.* 1972, Wyllie *et al.* 1980, Cohen 1993). In plants, apoptotic cell death has been observed during cell death associated with development (Wang *et al.* 1996a, Wang *et al.* 1996b) and senescence (Orzáez and Granell 1997, Yen and Yang 1998, LoSchiavo *et al.* 2000, Yamada *et al.* 2001c). It has also been reported during cell death induced by pathogens (Ryerson and Heath 1996) or a pathogenic toxin (Wang *et al.* 1996a) and several stresses (Koukalová *et al.* 1997, McCabe *et al.* 1997, Katsuhara 1997).

In the last few years, apoptotic features such as chromatin condensation, nuclear fragmentation and DNA fragmentation have been detected during the expression of hybrid lethality in some crosses, such as *N. glutinosa* × *N. repanda* (Marubashi *et al.* 1999) and *N. debneyi* × *N. tabacum*

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(Marubashi and Kobayashi 2002a, 2002b). Additionally, Yamada *et al.* (2001b) suggested that auxin and ethylene were involved in apoptotic cell death related to hybrid lethality resulting from the cross *N. glutinosa* × *N. repanda*. In the studies mentioned above, it was not determine whether apoptotic cell death was also observed in reciprocal crosses. Hybrid seedlings and hybrid cells of the cross *N. suaveolens* × *N. tabacum* showed apoptotic cell death during the expression of lethality (Yamada *et al.* 2000, 2001a). Furthermore, Yamada and Marubashi (2003) confirmed that overproduction of ethylene acted as an essential factor mediating apoptotic cell death in hybrid seedlings from the cross *N. suaveolens* × *N. tabacum*. When *N. suaveolens* was used as a male parent, hybrid seedlings were obtained by test-tube pollination but they also expressed lethality at the cotyledonary stage (Marubashi *et al.* 1987). In this study, we investigated the lethality of hybrid seedlings from the cross *N. tabacum* × *N. suaveolens* in detail and determined whether these hybrid seedlings also showed apoptotic cell death during the expression of lethality. The underlying causes of apoptotic cell death during the expression of hybrid lethality were examined based on the results obtained.

Materials and Methods

Plant materials

Nicotiana tabacum L. cv. Red Russian (2n = 48) and *N. suaveolens* Lehm. (2n = 32) were used as parents in this experiment. Seeds of these species were supplied by Japan Tobacco, Inc. Plants were cultivated under greenhouse conditions.

Test-tube pollination and ovule culture

Test-tube pollination and ovule culture were carried out according to the method of Marubashi and Nakajima (1985). Anthers of *N. suaveolens* were aseptically excised from still-closed flowers and stimulated to dehisce in an incubator (28°C). Flowers of *N. tabacum* were emasculated one day before anthesis. On the next day, flowers of *N. tabacum* were collected and their sepals, petals and styles were removed. Their ovaries were surface-sterilized with 70% ethanol for 30 sec and a 5% sodium hypochlorite solution for 5 min. The ovary wall was peeled to expose the placenta with intact ovules and then the placenta was placed on the medium (3% sucrose and 0.8% agar, pH 5.8). Pollen of *N. suaveolens* was spread on the surface of the placenta. Pollinated placentas were maintained at 28°C under continuous illumination (ca. 3000 lux). Fertilized and enlarged ovules at 10 to 14 days after pollination were excised and cultured on 1/2 MS medium (Murashige and Skoog 1962) supplemented with 3% sucrose and 0.8% agar (pH 5.8) at 28°C under continuous illumination (ca. 3000 lux).

Culture under high temperature conditions to suppress hybrid lethality

Onosato and Marubashi (1998) reported that hybrid lethality observed in seedlings from the cross *N. tabacum* × *N. suaveolens* was suppressed at 36°C and that the seedlings continued to grow. Thus, hybrid seedlings obtained by test-tube pollination and ovule culture at 28°C were transferred to fresh 1/2 MS medium supplemented with 1% sucrose and 0.2% Gelrite (pH 5.8) immediately after germination and exposed to 36°C under continuous illumination (ca. 3000 lux). Hybrid seedlings surviving at 36°C more than 30 days after germination (DAG) were exposed to 28°C to induce lethality.

Protoplast isolation

Leaves of hybrid seedlings were sectioned and treated with an enzyme solution containing 2% cellulase Onozuka R-10 (Yakult Pharmaceutical Ind. Co.), 0.2% Macerozyme R-10 (Yakult), 0.7 M mannitol and 10 mM $CaCl_2$, pH 5.6, for 3–4 h at 30°C. Protoplasts were separated from cellular debris using a 42 μm nylon mesh. After centrifugation for 5 min at 100 g, the supernatant was discarded and the protoplast pellet was resuspended in 0.7 M mannitol. Protoplasts stained with 0.5% 4',6-diamino-2-phenylindole dihydrochloride (DAPI) were observed under a fluorescence microscope (BX50; Olympus, Japan) with U excitation (330–385 nm) and photographed using an automatic photomicrography system (PM30; Olympus, Japan).

Flow cytometry

Nuclei were isolated from the leaves, stems and roots of the hybrid seedlings. The seedlings were chopped in ice-cold buffer (Michaelson *et al.* 1991) and the extract was filtered successively through 70 and 20 μm nylon mesh. Nuclei were collected from the filtrate by centrifugation for 5 min at 700 g, suspended in sheath fluid (FACSFlow; Becton Dickinson) supplemented with 3 μl of 50 μg ml^{-1} propidium iodide [PI] and 1 μl of 100 μg ml^{-1} RNase and incubated for 15 min at 37°C. The DNA content of the isolated nuclei was analyzed using a flow cytometer [FACSCalibur; 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.

DNA extraction and analysis

Genomic DNA was extracted from the leaves, stems and roots of the hybrid seedlings according to the method of LoSchiavo *et al.* (2000), with some modifications. Each organ was ground in a mortar with liquid nitrogen, then preheated (60°C) CTAB (cetyltrimethylammonium bromide) isolation buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added and the mixture incubated at 60°C for 60 min. The suspension was extracted twice with chloroform/isoamyl alcohol (24 : 1) and centrifuged for 15 min at 3000 rpm. The aqueous phase was transferred to a new tube and nucleic acids were precipitated by the addition of isopropanol (2/3 volume) and centrifuged for 20 min at 14000 rpm.

The pellet was washed with 70% ethanol and dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) supplemented with 1 µl of 100 µg ml^{-1} RNase. DNA was separated by electrophoresis on a 2.0% agarose gel in TAE buffer and stained with SYBR Gold nucleic acid gel stain (Molecular Probes).

Results

Hybridization

To obtain hybrid seedlings, test-tube pollination and ovule culture were carried out. Fertilized ovules started to develop on the placenta and became visible at about 7 days after pollination. Ninety-two placentas were pollinated and 1,343 ovules were cultured. From these ovules, 117 hybrid seedlings were obtained. There were differences among the hybrid seedlings in the days to germination; it took approximately 2 weeks to 2 months after initiation of ovule culture for germination.

Expression of temperature-sensitive lethality in hybrid seedlings

Hybrid seedlings obtained by test-tube pollination and ovule culture were transferred to fresh 1/2 MS medium immediately after germination and cultured at 28°C. Under these conditions, they expressed hybrid lethality. Lethal symptoms observed in these seedlings at an early stage consisted of browning of hypocotyls and roots. While hybrid seedlings did not exhibit any apparent lethal symptoms by about 2 DAG, the hypocotyls turned brown and the roots began to turn brown from the base to the tip at about 5 DAG (Fig. 1A and 1B). Hybrid seedlings continued to grow for about 10 DAG (Fig. 1C). At about 20 DAG, plant growth nearly stopped and the leaves began to fade and turn brown (Fig. 1D). Finally, hybrid seedlings died at 40-50 DAG (Fig. 1E).

To suppress lethality, the hybrid seedlings used for further experiments were transferred to 36°C immediately after germination at 28°C. Under these temperature conditions, the hybrid seedlings did not express any lethal symptoms and continued to grow (Fig. 2A). Hybrid seedlings were cultured at 36°C for over 30 DAG, and then they were exposed to 28°C to induce lethality. When the hybrid seedlings were exposed to 28°C, their growth stopped and lethal symptoms were expressed. At about 2 days after transfer from 36°C to 28°C, stems and roots began to turn brown from the crowns (Fig. 2B). The color of the leaves began to fade at about 5 days after transfer from 36°C to 28°C (Fig. 2C). Leaves began to turn yellow at about 10 days after transfer from 36°C to 28°C, and then gradually turned brown at about 15 days after transfer from 36°C to 28°C (Fig. 2D and 2E). Hybrid seedlings finally died at about 20 days after transfer from 36°C to 28°C (Fig. 2F).

Nuclear changes in hybrid seedlings observed by fluorescence microscopy

DAPI-stained protoplasts isolated from the leaves of the hybrid seedlings showed structural changes in the nucleus. Normal chromatin structure was observed in the protoplasts isolated from the hybrid seedlings cultured at 36°C (Fig. 3A). Chromatin condensation was observed in the protoplasts isolated from the hybrid seedlings exposed to 28°C and cultured for 10 days after development at 36°C (Fig. 3B). Nuclear fragmentation was observed in the protoplasts isolated from the hybrid seedlings exposed to 28°C and cultured for 15 days after development at 36°C (Fig. 3C).

Nuclear fragmentation evaluated by flow cytometry

Since microscopic observation was not suitable for the measurement of fragmented nuclei in protoplasts, flow cytometry was carried out after staining of DNA with PI. To investigate whether there were differences among the organs in terms of hybrid lethality, nuclei were isolated from the leaves, stems and roots of the hybrid seedlings. In the histograms of PI fluorescence values, indicating the relative mass of nuclear DNA, normal hybrid seedlings at 36°C showed two peaks that probably corresponded to nuclei at the G1 and G2/M phases of the cell cycle (Fig. 4A, 4F and 4K). In the leaves, no changes were observed in the peaks until 5 days after transfer of the seedlings from 36°C to 28°C (Fig. 4B), and in most cases no changes were detected in the peaks from the hybrid seedlings cultured at 28°C for 10 days after development at 36°C (Fig. 4C). However, the size of the G1 peaks of the leaves from the hybrid seedlings cultured at 28°C for 15 days after development at 36°C decreased slightly, and additional peaks with lower fluorescence values appeared (Fig. 4D). The size of these later-eluting peaks, corresponding to fragmented nuclei, increased further on the histograms of the leaves from the hybrid seedlings cultured at 28°C for 20 days after development at 36°C (Fig. 4E). In contrast, for the stems and roots, additional peaks with lower fluorescence values were detected from the hybrid seedlings cultured at 28°C for 5 days after development at 36°C (Fig. 4G and 4L). Subsequently, the size of these peaks increased gradually (Fig. 4H–4J and 4M–4O).

Based on the histograms obtained by flow cytometry, we calculated the percentage of nuclei included in the area of the peaks with lower fluorescence values compared to the G1 peaks (Fig. 5). This percentage was correlated with the percentage of fragmented nuclei. In the leaves, the percentage of fragmented nuclei from hybrid seedlings at 36°C was less than 10%. At 5 days after transfer of the seedlings from 36°C to 28°C, the percentage of fragmented nuclei was approximately equivalent to that from the hybrid seedlings at 36°C. At 10 days after transfer from 36°C to 28°C, the percentage of fragmented nuclei increased slightly, but was still less than 10%. At 15 days after transfer from 36°C to 28°C, the percentage of fragmented nuclei exceeded previous values, showing clearly that nuclear fragmentation had already occurred. In the stems and roots, the percentage of fragment-

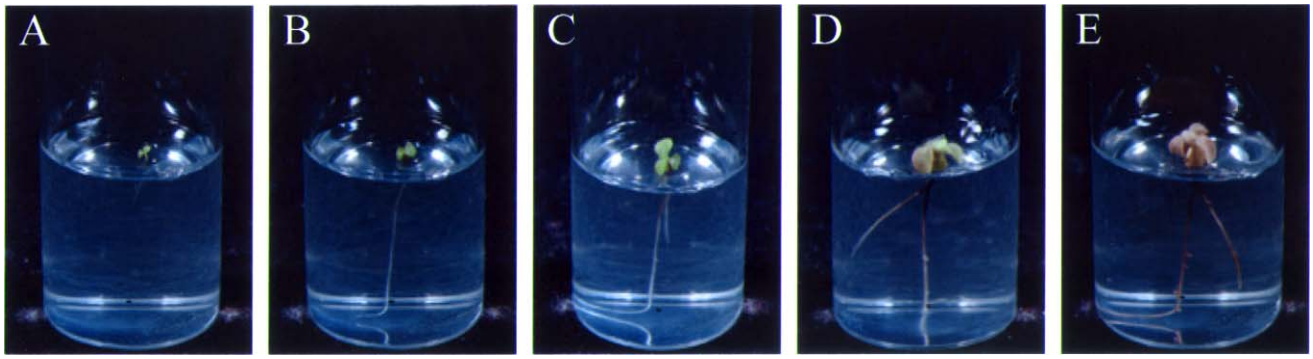

Fig. 1. Lethal symptoms observed in hybrid seedlings from the cross *N. tabacum* × *N. suaveolens* cultured at 28°C. The same seedling was photographed. (A) The seedling grew like a normal plant at 2 DAG. (B) The seedling's hypocotyl and roots began to turn brown at 5 DAG. (C) The seedling continued to develop and true leaves expanded at 10 DAG. (D) The seedling nearly stopped developing and the color of the leaves started to fade at 20 DAG. (E) The seedling turned brown all over and died at 40 DAG.

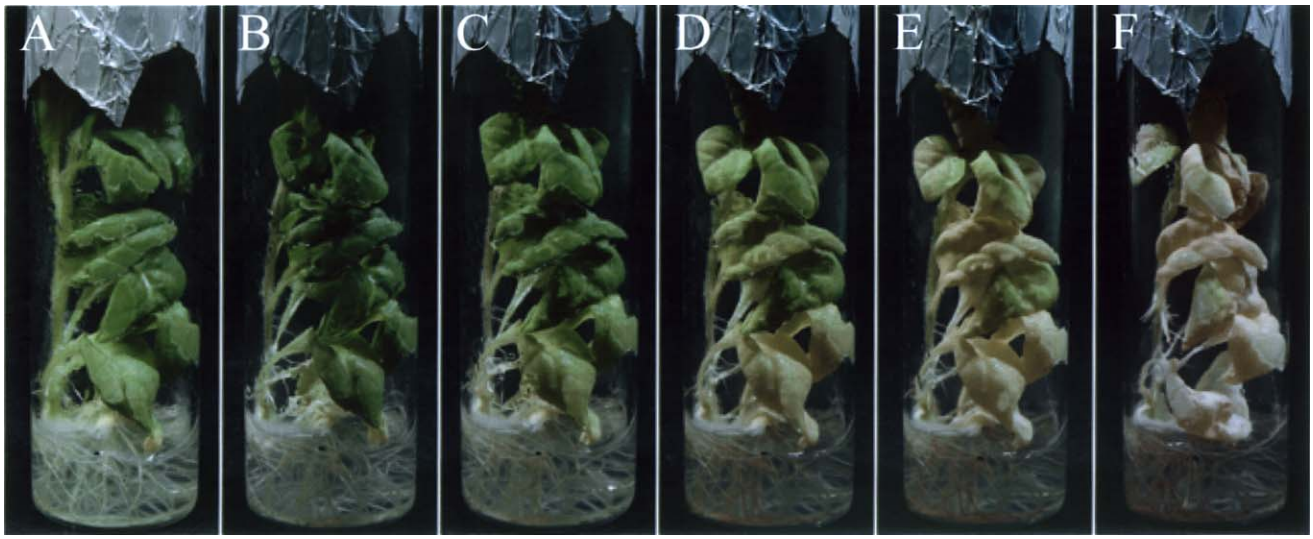

Fig. 2. Lethal symptoms observed in hybrid seedlings from the cross *N. tabacum* × *N. suaveolens* exposed to 28°C after culture at 36°C for 40 DAG. The same seedling was photographed. (A) The seedling cultured at 36°C for 40 DAG. (B) The seedling exposed to 28°C and cultured for 2 days. The stem and roots of the seedling began to turn brown. (C) The seedling exposed to 28°C and cultured for 5 days. The color of the leaves of the seedling began to fade. (D) The seedling exposed to 28°C and cultured for 10 days. Leaves of the seedling began to turn yellow. (E) The seedling exposed to 28°C and cultured for 15 days. Leaves of the seedling began to turn brown. (F) The seedling exposed to 28°C and cultured for 20 days. The entire seedling turned brown and died.

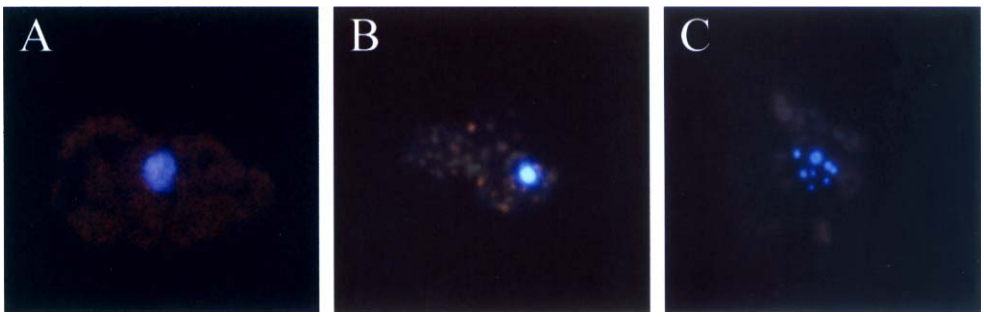

Fig. 3. Progressive changes in the nuclear structure detected in protoplasts isolated from the leaves of the hybrid seedlings from the cross *N. tabacum* × *N. suaveolens*. Protoplasts were stained with DAPI. (A) Normal structure of chromatin in the protoplasts from the seedlings cultured at 36°C for 30 DAG. (B) Chromatin condensation in the protoplasts from the seedlings exposed to 28°C and cultured for 10 days. (C) Nuclear fragmentation in the protoplasts from the seedlings exposed to 28°C and cultured for 15 days.

ed nuclei from the hybrid seedlings at 36°C was less than 10 % as in the case of leaves. At 5 days after transfer from 36°C to 28°C, however, the percentage of fragmented nuclei increased significantly.

Fragmentation of DNA detected by agarose gel analysis

Agarose gel analysis of DNA was carried out to determine whether fragmentation of DNA has occurred. Total DNA was isolated from the leaves, stems and roots of the hybrid seedlings. In the leaves, electrophoresis of DNA isolated from the hybrid seedlings that failed to express lethality at 36°C did not show any ladder pattern (Fig. 6A, lane 1); a ladder pattern would suggest the occurrence of nucleosomal fragmentation. No ladder pattern was observed in the hybrid seedlings until 10 days after transfer from 36°C to 28°C (Fig. 6A, lanes 2, 3). A distinctive ladder pattern was detected in the hybrid seedlings cultured at 28°C for 15 days after development at 36°C (Fig. 6A, lane 4), and the intensity of the ladder pattern increased in the hybrid seedlings cultured at 28°C for 20 days after development at 36°C (Fig. 6A, lane 5). On the other hand, in the stems and roots, no ladder pattern was observed in the hybrid seedlings at 36°C, as in the case of leaves (Fig. 6B, lane 1, Fig. 6C, lane1), while a ladder pattern was detected in the hybrid seedlings cultured at 28°C for 5 days after development at 36°C (Fig. 6B, lane 2, Fig. 6C, lane 2). A ladder pattern was also detected in the hybrid seedlings cultured at 28°C for 10 days after development at 36°C (Fig. 6B, lane 3, Fig. 6C, lane 3). In the stems and roots from the hybrid seedlings cultured at 28°C for 15 days after development at 36°C, the intensity of the ladder pattern decreased (Fig. 6B, lane 4, Fig. 6C, lane 4), and the ladder pattern disappeared or was hardly detectable in the hybrid seedlings cultured at 28°C for 20 days after development at 36°C (Fig. 6B, lane 5, Fig. 6C, lane 5).

Discussion

In previous studies, apoptotic cell death was observed during the expression of hybrid lethality in the seedlings of *N. glutinosa* × *N. repanda* (Marubashi *et al.* 1999), *N. suaveolens* × *N. tabacum* (Yamada *et al.* 2000) and *N. debneyi* × *N. tabacum* (Marubashi and Kobayashi 2002a, 2002b). In the present study, we examined whether hybrid seedlings from the cross *N. tabacum* × *N. suaveolens*, the reciprocal cross of *N. suaveolens* × *N. tabacum*, also showed apoptotic cell death during the expression of hybrid lethality.

In the cross combination of *N. tabacum* × *N. suaveolens*, test-tube pollination was necessary to obtain hybrid seedlings because pollen tubes did not reach the base of the style and fertilization did not occur (Marubashi *et al.* 1987). After test-tube pollination and ovule culture, we obtained a total of 117 hybrid seedlings. When some of these hybrid seedlings were cultured at 28°C, they expressed lethality as reported by Marubashi *et al.* (1987). Yamada *et al.* (1999) classified hybrid lethality in the genus *Nicotiana* into four types as follows: Type I, browning of shoot apex and root tip; Type II,

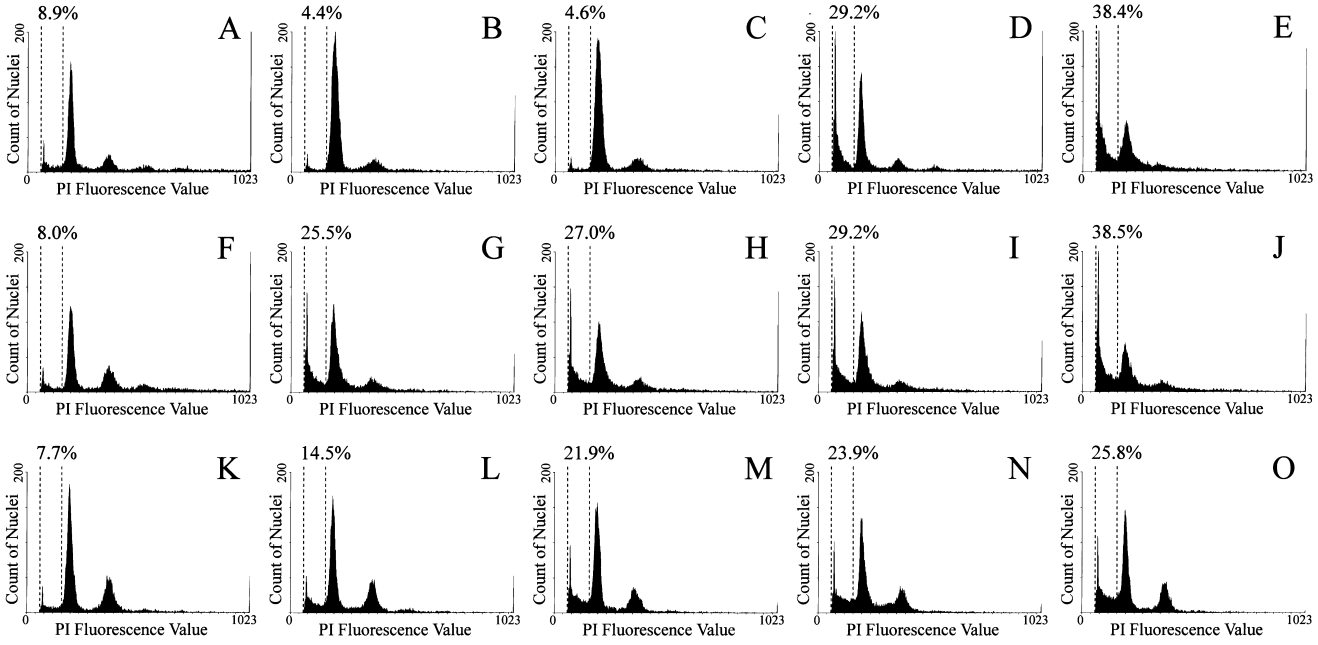

Fig. 4. Nuclear fragmentation of hybrid seedlings from the cross *N. tabacum* × *N. suaveolens*. Histograms reveal increased fragmentation of nuclei in seedlings, determined by flow cytometry in a total of 10000 nuclei. Broken vertical lines on histograms divide the area containing additional peaks with lower fluorescence values from the area depicting the G1 and G2/M phases. Nuclei were isolated from the leaves (A–E), stems (F–J) and roots (K–O) of the seedlings. (A), (F), (K), seedlings cultured at 36°C for 30 DAG. (B), (G), (L), seedlings exposed to 28°C and cultured for 5 days. (C), (H), (M), seedlings exposed to 28°C and cultured for 10 days. (D), (I), (N), seedlings exposed to 28°C and cultured for 15 days. (E), (J), (O), seedlings exposed to 28°C and cultured for 20 days.

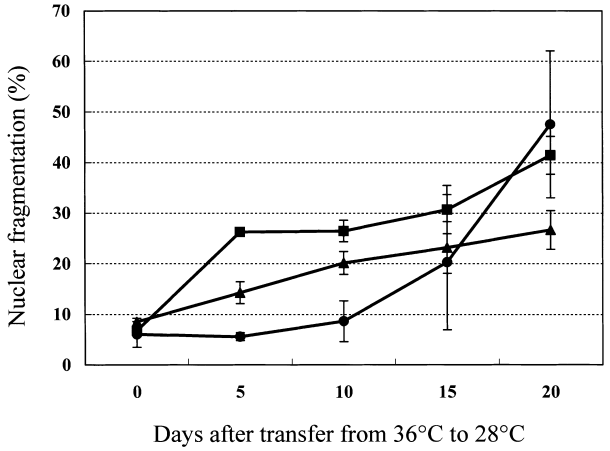

Fig. 5. Progressive changes in the nuclear fragmentation percentage detected in the hybrid seedlings from the cross *N. tabacum* × *N. suaveolens*. Percentage of fragmented nuclei was calculated based on the histograms obtained by flow cytometry in more than three independent experiments. ●, Leaves. ■, Stems. ▲, Roots.

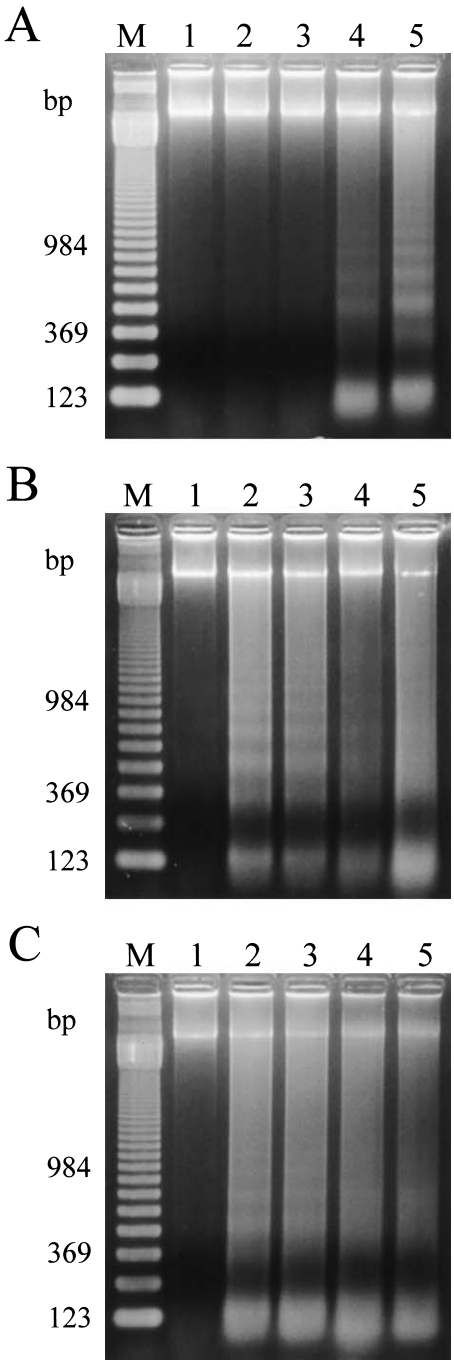

Fig. 6. DNA fragmentation in the hybrid seedlings from the cross *N. tabacum* × *N. suaveolens*. Total DNA was extracted from the leaves (A), stems (B) and roots (C) of the seedlings. M, 123 bp DNA ladder marker. Lane 1, seedlings cultured at 36°C for 30 DAG; lanes 2–5, seedlings exposed to 28°C and cultured for 5, 10, 15 and 20 days, respectively.

browning of hypocotyl and roots; Type III, yellowing of true leaves; Type IV, formation of multiple shoots. In our observation of hybrid lethality in the cross *N. tabacum* × *N. suaveolens*, since the lethal symptoms observed in these seedlings at an early stage consisted of browning of hypocotyls and roots, this lethality was identified as Type II, which is the same lethality type as that observed in the reciprocal cross, *N. suaveolens* × *N. tabacum* (Yamada *et al.* 1999).

While hybrid seedlings were obtained by the use of test-tube pollination and ovule culture, there were differences among the hybrid seedlings in the days to germination. Hence it was difficult to collect a sufficient number of samples at the same growth stages. For this reason, we attempted to suppress hybrid lethality at high temperatures, and well-developed hybrid seedlings were transferred to 28°C to induce lethality. Onosato and Marubashi (1998) reported that hybrid lethality in the seedlings from the cross *N. tabacum* × *N. suaveolens* was suppressed at 36°C. Thus, hybrid seedlings were cultured at 36°C and allowed to develop to a certain degree. When the hybrid seedlings cultured at 36°C were exposed to 28°C, they degenerated after showing the typical symptoms observed at 28°C. We verified the occurrence of apoptosis during this expression of hybrid lethality. In the protoplasts isolated from the leaves of the hybrid seedlings expressing lethality, chromatin condensation and nuclear fragmentation were observed. We confirmed that nuclear fragmentation was correlated with lethal symptoms in the hybrid seedlings expressing lethality based on flow cytometry. Furthermore, analysis of DNA extracted from the hybrid seedlings expressing lethality revealed the presence of DNA laddering, suggesting the occurrence of nucleosomal fragmentation. These morphological and biochemical changes have been considered to be typical signs of apoptosis (Kerr *et al.* 1972, Wyllie *et al.* 1980, Cohen 1993). In previous studies, apoptotic cell death was observed during the expression of lethality in the hybrid seedlings of *N. suaveolens* × *N. tabacum* and *N. debneyi* × *N. tabacum* cultured at 28°C, and it was also observed during the expression of lethality induced by transfer from high temperatures to 28°C (Yamada *et al.* 2000, Marubashi and Kobayashi 2002a,

2002b). Therefore, we consider that the type of cell death observed in the hybrid seedlings from the cross *N. tabacum* × *N. suaveolens* must correspond to apoptotic cell death. On the other hand, the hybrid seedlings cultured at 36°C did not show any apoptotic features, such as chromatin condensation, nuclear fragmentation and DNA fragmentation, in the same way as observed with the cross *N. suaveolens* × *N. tabacum* (Yamada *et al.* 2000) and the cross *N. debneyi* × *N. tabacum* (Marubashi and Kobayashi 2002a, 2002b). These results indicate that apoptotic cell death occurs gradually at 28°C and stops under high temperature conditions.

Since the same lethal symptoms were observed in reciprocal crosses between *N. tabacum* and *N. suaveolens*, the underlying causes of hybrid lethality were attributed to the interaction of coexisting heterogeneous genomes, and not to a cytoplasmic effect. Masuda *et al.* (2003) divided the expression of lethality in hybrid cells from the cross *N. suaveolens* × *N. tabacum* into two phases; i.e., the phase before the point of no return, where the expression of hybrid lethality may or may not progress (3 h after exposure to 28°C), and the phase after the point of no return. They suggested that the former phase requires the expression of hybrid lethality-related gene(s), and the latter phase does not require gene expression and uses an existing mechanism of the apoptotic execution phase. Based on this proposal and the results of the present study, we concluded that hybrid lethality-related gene(s) were not affected by the cytoplasm. As mentioned above, hybrid seedlings from the cross *N. tabacum* × *N. suaveolens* could not be obtained by conventional crossing, and it was difficult to collect a sufficient number of samples at the same growth stages even if hybrid seedlings were obtained by the use of test-tube pollination and ovule culture. Therefore, it was considered that the cross combination of *N. tabacum* × *N. suaveolens* was not suitable for studies on cell death mechanism. The results of the present study showing that hybrid lethality-related gene(s) are not affected by the cytoplasm suggest that studies on cell death mechanism can be conducted using the reciprocal cross *N. suaveolens* × *N. tabacum*. Since hybrid seedlings from the cross *N. suaveolens* × *N. tabacum* can be easily obtained in sufficient amounts by conventional crossing, and since suspension culture cells had been derived from the hybrid seedlings previously (Yamada *et al.* 2001a), the cross combination of *N. suaveolens* × *N. tabacum* appeared to be more suitable for studies on the mechanism of cell death.

In animals, it is well known that cytochrome *c* released from mitochondria into the cytosol plays an important role in apoptosis. In plants, the release of cytochrome *c* from mitochondria into the cytosol precedes programmed cell death (PCD) accompanied by apoptotic features, suggesting that plant mitochondria also play an important role in the induction of PCD (Balk *et al.* 1999, Balk and Leaver 2001). Further studies should be conducted to determine whether organelles such as mitochondria are involved in apoptotic cell death during the expression of hybrid lethality in the cross between *N. tabacum* and *N. suaveolens*.

To determine whether there were differences among the organs of the hybrid seedlings expressing lethality, we examined the features of apoptosis using leaves, stems and roots of the hybrid seedlings. It was found that both nuclear fragmentation and DNA fragmentation could be detected in the stems and roots first, and later in leaves. These results indicate that the progression of apoptotic cell death in the cross *N. tabacum* × *N. suaveolens* was initiated in stems and roots, followed by leaves. Hence the progression of apoptotic cell death corresponded to the progression of the lethal symptoms observed. The fact that cell death in stems and roots preceded that in leaves suggests that cell death in leaves might be induced by nutrient deficiency associated with the disruption of stem and root tissues. Furthermore, these findings also suggest that the differences in the timing of apoptotic cell death among organs might be correlated with organ-specific gene expression or differential timing of gene expression. Histological and gene expression analysis may reveal why apoptotic cell death in stems and roots of hybrid seedlings occurs first.

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