Planta (2003) 217: 690–698
DOI 10.1007/s00425-003-1035-2

ORIGINAL ARTICLE

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Overproduced ethylene causes programmed cell death leading to temperature-sensitive lethality in hybrid seedlings from the cross *Nicotiana suaveolens* × *N. tabacum*

Received: 9 December 2002 / Accepted: 15 March 2003 / Published online: 1 May 2003

Abstract Reproductive isolation mechanisms (RIMs) often become obstacles in crossbreeding. Hybrid lethality is a subtype of RIM but its physiological mechanism remains poorly elucidated. Interspecific hybrids of *Nicotiana suaveolens* Lehm. × *N. tabacum* L. cv. Hicks-2 expressed temperature-sensitive lethality. This lethality was induced by programmed cell death (PCD) that was accompanied by the characteristic changes of animal apoptosis in hybrid seedlings at 28 °C but not at 36 °C. When hybrid seedlings were cultured at 28 °C, DNA fragmentation started in the cotyledon, and nuclear fragmentation subsequently progressed with lethal symptoms spreading throughout the seedlings. At 28 °C, ethylene production in hybrid seedlings was detectable at a high level compared with the level in parental seedlings. In contrast, the ethylene production rate in hybrid seedlings cultured at 36 °C was equal to that in parental seedlings. Treatment with ethylene biosynthetic inhibitors, amino-oxyacetic acid and amino-ethoxyvinyl glycine, suppressed lethal symptoms and apoptotic changes, and also prolonged survival of hybrid seedlings. Thus, the increase in the ethylene production rate correlated closely with expression of lethal symptoms and apoptotic changes in hybrid seedlings. From these observations, we conclude that overproduced ethylene acts as an essential factor mediating PCD and subsequent lethality in hybrid seedlings. Furthermore, the present study has provided the first evidence that ethylene is involved in the phenomenon of hybrid lethality.

Keywords Apoptosis · Ethylene · Hybrid lethality · *Nicotiana* (programmed cell death) · Programmed cell death · Temperature sensitivity

Abbreviations AOA: amino-oxyacetic acid · AVG: amino-ethoxyvinyl glycine · CTAB: cetyltrimethylammonium bromide · DAG: days after germination · PCD: programmed cell death · RIMs: reproductive isolation mechanisms

Introduction

According to the biological species concept (Mayr 1963), identity of the species in sexual organisms is based on an isolation mechanism, which is a reproductive characteristic inducing a genetic barrier and preventing hybridization between species. Reproductive isolation mechanisms (RIMs) have been classified into two broad kinds, prezygotic and postzygotic, together with a number of subtypes (Stebbins 1966). Hybrid lethality belongs to a subtype of postzygotic RIMs that induce inviability of hybrids after fertilization. This type of RIM has been detected in certain cross-combinations of species within several genera, including *Oryza* (Oka and Doida 1962), *Triticum* (Zeven 1981), *Gossypium* (Samora et al. 1994), *Nicotiana* (Yamada et al. 1999) and *Drosophila* (Provine 1991).

RIMs often become obstacles in agricultural improvement through plant breeding. In particular, postzygotic RIMs preclude the possibility of hybridization between cultivars and wild species. Some useful rescue methods, i.e., embryo culture (Sharma and Ohm 1990) and ovule culture (Iwai et al. 1986), have been developed and have overcome the immaturity of hybrid embryos in many cross-combinations. However, there

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are few cases of overcoming lethality of hybrid seedlings by use of tissue culture methods (Lloyd 1975). These methods therefore have not been in common practice and other useful methods for overcoming hybrid lethality have not been developed. Thus, hybrid lethality remains a serious adverse event in agricultural exploitation of wild species as a genetic resource by breeding.

The physiological genetics and cell biology of hybrid lethality have been studied in some interspecific hybrids of plants. Temperature sensitivity of hybrid lethality, i.e., lethality expressed by culturing the hybrid seedlings at 28 °C but suppressed under high-temperature conditions (32–36 °C), was observed in crosses of *Nicotiana* (Yamada et al. 1999). Such a temperature-sensitive lethality has also been reported in other genera (Phillips 1977), but the mechanism of temperature sensitivity has not been explained. However, changes characteristic of apoptosis (Afford and Randhawa 2000), including DNA fragmentation, chromatin condensation, and nuclear fragmentation, have been detected in hybrid seedlings from interspecific crosses of *Nicotiana* expressing lethality but not in the parental seedlings (Marubashi et al. 1999; Yamada et al. 2000; Marubashi and Kobayashi 2002). These observations strongly suggest that programmed cell death (PCD) with similarities to animal apoptosis leads to lethality in interspecific hybrids of the genus *Nicotiana*. However, the mechanisms of PCD-induced lethality in hybrid seedlings of *Nicotiana* have remained poorly identified.

In a previous study, we confirmed a high level of endogenous auxin in hybrid seedlings from the cross *N. glutinosa* × *N. repanda* expressing lethality, and a suppressive effect of auxin and ethylene inhibitors on the expression of lethality (Yamada et al. 2001a). From these results, we proposed that auxin-induced ethylene is involved in the lethality expressed by hybrid seedlings from the cross *N. glutinosa* × *N. repanda*. However, such a suggestion has not been generalized to all types of lethality (Yamada et al. 1999) in interspecific hybrids of *Nicotiana* because results supporting this idea had not been obtained from other cross-combinations expressing lethality. Furthermore, changes in ethylene production by hybrid seedlings during expression of lethality were not examined in the previous study. Thus, we intended to obtain reliable evidence of the involvement of ethylene in hybrid lethality.

In our present study, to establish that ethylene plays an important role in regulation of the PCD-induced lethality expressed by hybrid seedlings from the cross *Nicotiana suaveolens* Lehm. × *Nicotiana tabacum* L. cv. Hicks-2, we determined changes in ethylene production by hybrid seedlings expressing lethality and detected the effect of ethylene biosynthetic inhibitors, amino-oxyacetic acid (AOA) and amino-ethoxyvinyl glycine (AVG), on the expression of lethality. Furthermore, we discuss how changes in ethylene production are related to the temperature sensitivity of lethality and to the general regulation of PCD in plants.

Materials and methods

Plant materials

Interspecific hybrid seedlings were obtained from the cross between *Nicotiana suaveolens* Lehm. and *N. tabacum* L. cv. Hicks-2 (seeds from Japan Tobacco, Iwata, Japan). Plants were grown in a greenhouse of the School of Agriculture, Ibaraki University (Japan). Flowers of *N. suaveolens*, emasculated before anthesis, were pollinated with fresh *N. tabacum* pollen. Seedpods were harvested about a month after pollination and preserved in a desiccator under low-temperature conditions (4 °C). F1 seeds were soaked in a 0.5% (w/v) gibberellic acid (GA_3) solution for 30 min, sterilized with 5% (v/v) sodium hypochlorite solution (0.25% liberated chloride) for 15 min, and then rinsed three times with sterilized water. Sterilized F1 seeds were sown in Petri dishes (90 mm diameter, 20 mm deep) that contained 20 ml of a medium consisting of half-strength Murashige and Skoog salts, Murashige and Skoog vitamins (Murashige and Skoog 1962), 1% (w/v) sucrose and 0.2% (w/v) Gelrite (pH 5.8), and were cultured at 28 °C with a 16-h-light, 8-h-dark regime with approximately 150 μmol photons m^{-2} s^{-1} for germination. Under these conditions, hybrid seedlings of this cross spontaneously expressed temperature-sensitive lethality, as occurred under other conditions in our previous study (Yamada et al. 2000). To suppress expression of lethality, some of the cultures were transferred to 36 °C under the same light regime immediately after seed germination. Furthermore, to induce expression of lethality artificially, some of the surviving seedlings were transferred from 36 °C to 28 °C under the same light regime 6 days after germination (DAG). As a control, seedlings of the parental species, *N. suaveolens* and *N. tabacum*, were also cultured under the same temperature conditions and light regime.

Treatment with ethylene biosynthesis inhibitors

Before the expression of lethality, in order to suppress ethylene production, hybrid seedlings from the cross *N. suaveolens* × *N. tabacum* were treated with AOA and AVG at a range of concentrations (0.5, 1, 5, 10, and 25 μM). One-millimolar AOA and AVG stock solutions were prepared in ultrapure water and sterilized through a 0.22-μm filter unit (Millipore, Bedford, MA, USA). The germinating hybrid seeds were transferred to Petri dishes (90 mm diameter, 20 mm deep) that contained 20 ml of a control medium consisting of half-strength Murashige and Skoog salts, Murashige and Skoog vitamins, 1% (w/v) sucrose and 0.2% (w/v) Gelrite (pH 5.8), or a medium also including AOA or AVG. Seedlings were cultured at 28 °C with a 16-h-light, 8-h-dark regime with approximately 150 μmol photons m^{-2} s^{-1}. In order to evaluate the effect of AOA and AVG on lethality, hybrid seedlings were sampled from each culture and used in experiments to measure ethylene production, nuclear fragmentation and DNA fragmentation as described below. Furthermore, to confirm a life-extending effect of ethylene biosynthetic inhibitors on hybrid seedlings, survival time was judged on the basis of days from germination until all leaves had turned brown in the seedlings.

Measurement of ethylene production

Ethylene production of hybrid seedlings was determined during various stages of development of lethal symptoms (3, 6, 9, 12, and 15 DAG) under different temperature conditions (28, 36, or 36/28 °C), with or without treatment with ethylene biosynthetic inhibitors (15 DAG at 28 °C). We carefully collected 10 uninjured seedlings from each culture and enclosed them in 3.5-ml glass vials flushed with ethylene-free air. After 4 h of incubation at the same temperature as the cultures, a 2-ml sample of

headspace gases was removed from the glass vials using a Luer-loc tip syringe (GL Science, Tokyo, Japan) for gas chromatograph injection. Ethylene was detected using a gas chromatograph (model GC-14B; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (model C-R6A; Shimadzu) and a 100-cm stainless-steel column packed with activated alumina. The column temperature was 50 °C and the detector was 100 °C, with gas flow rates of 50, 65, and 40 kPa, for air, hydrogen, and nitrogen (carrier), respectively. The ethylene concentration was computed from standard curves prepared with ethylene gas standards (GL Science).

Quantitative analysis of fragmented nuclei

According to a previous study (Yamada et al. 2001b), nuclear fragmentation, a specific change observable in apoptotic cells, was quantitatively estimated by flow cytometry and correlated with ethylene production of hybrid seedlings. We prepared 10 seedlings from each culture and used them immediately for analysis. Seedlings were chopped in 500 µl of ice-cold nuclear extraction buffer, which was included in the High Resolution Kit for Plant DNA (Partec, Münster, Germany), a reagent set suggested by the manufacturer of the flow cytometer. The extract was filtered through a 50-µm nylon mesh. The flow-through, including isolated nuclei, was collected and then 2.5 vol. of ice-cold nuclear staining buffer of the reagent set (Partec) was added and mixed well. The DNA content of the isolated nuclei was analyzed using a flow cytometer (model Ploidy Analyzer; Partec). On the basis of histograms obtained from flow cytometry of a total of 10,000 nuclei, peak areas indicating fragmented nuclei (M1) and normal nuclei (M2) were determined, and then nuclear fragmentation rates (%) were calculated by the formula {(M1/M1 + M2)×100} provided by Win MDI Version 2.8 software for flow cytometry analysis.

Detection of fragmented DNA

DNA fragmentation, a distinct process of apoptosis, was detected by agarose gel analysis. Total DNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method. Samples were collected from each organ, i.e., root, hypocotyl and cotyledon, or from the whole hybrid seedling plant. Starting materials were adjusted to 100 mg fresh weight, frozen in liquid nitrogen, and ground into a fine powder with a mortar and pestle. The powder was suspended in 1 ml of chilled buffer containing 1% (w/v) polyethylene glycol 6000, 350 mM sorbitol, 100 mM Tris–HCl (pH 8.0), and centrifuged for 10 min at 20,000 *g*. The precipitate was resuspended in 400 µl of lysis buffer containing 2% (w/v) CTAB, 100 mM Tris–HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 5 µg ml^{-1} RNase, and incubated at 55 °C for 60 min. The suspension was shaken for 5 min after the addition of an equal volume of chloroform:isoamyl alcohol (24:1, v/v) and centrifuged for 15 min at 13,200 *g*. The supernatant was mixed with an equal volume of 2-propanol and centrifuged for 10 min at 6,000 *g*. Precipitated DNA was washed with 1 ml of 70% ethanol, drained well, and dissolved in 30 µl of TE buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA. After the addition of 1/10 vol. of loading buffer containing 50% (v/v) glycerol, 0.05% (w/v) Bromophenol Blue, 10 µl of the DNA solution was immediately loaded onto a gel of 2 or 3% (w/v in 1× TAE buffer) NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) prepared according to the manufacturer's instructions, and electrophoresed using Mupid, a mini electrophoresis unit (Cosmo Bio, Tokyo, Japan), at 100 V for 50 min. The gel was stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA), and then the DNA banding pattern was visualized and photographed using an electronic UV transilluminator system (model FAS-III mini + DS-30; Toyobo, Tokyo, Japan).

Results

Symptoms of lethality expressed by hybrid seedlings

Hybrid seedlings from the cross *N. suaveolens* × *N. tabacum* expressed lethality at 28 °C (Fig. 1a). We succeeded in interspecific hybridization between *N. suaveolens* and *N. tabacum* by artificial pollination and obtained F1 seeds. When F1 seeds were sown on MS medium and cultured at 28 °C, over 90% of them germinated. However, all of the seedlings stopped growing at the cotyledonary stage and expressed symptoms leading to death at 12 DAG. The process of hybrid seedling death was distinguishable into three stages by direct observation of the lethal symptoms. During the first stage (0–3 DAG), the cotyledons of hybrid seedlings showed a slight epinasty but no other differences from the seedlings of parental species. During the second stage (4–6 DAG), the cotyledons of the hybrid seedlings showed a slight chlorosis, and the hypocotyls turned brown. In this stage, the primary root of the hybrid seedlings was elongating, but true leaves did not expand. During the last stage (7–12 DAG), the cotyledons and the primary root turned brown. In this stage, browning of the cotyledons started from the midrib and extended to the whole blade. Meanwhile, primary roots did not grow lateral roots, and browning started from the proximal region and extended to the tip.

Fragmentation of nuclei in hybrid seedlings as determined by flow cytometry

Nuclear fragmentation, a change characteristic of apoptotic cells, was detected in hybrid seedlings expressing lethality (Fig. 1b). Histograms of 4′,6-diamidino-2-phenylindole (DAPI) fluorescence values, indicating relative size of nuclear DNA masses, were obtained from flow cytometry of isolated nuclei of hybrid seedlings. In hybrid seedlings at 3 DAG, the histogram showed two peaks that differed 2-fold in fluorescent intensity. These two peaks likely correspond to nuclei at the G_1 and G_2 phases of the cell cycle; the combined peak area is marked in the histograms as M2. At 6 DAG, the G_1 and G_2 peaks of the hybrid seedlings decreased slightly but other changes in the histogram were not detected in this stage. At 12 DAG, the G_1 and G_2 peaks decreased substantially, and additional peaks with lower fluorescence intensity values appeared. The lower intensity of these latter peaks suggests that they correspond to fragmented nuclei; the combined peak area in this region is marked in the histograms as M1.

Fragmentation of DNA in hybrid seedlings as detected by agarose gel analysis

A ladder-like DNA banding pattern, indicating fragmentation of DNA into multimers of nucleosomal base

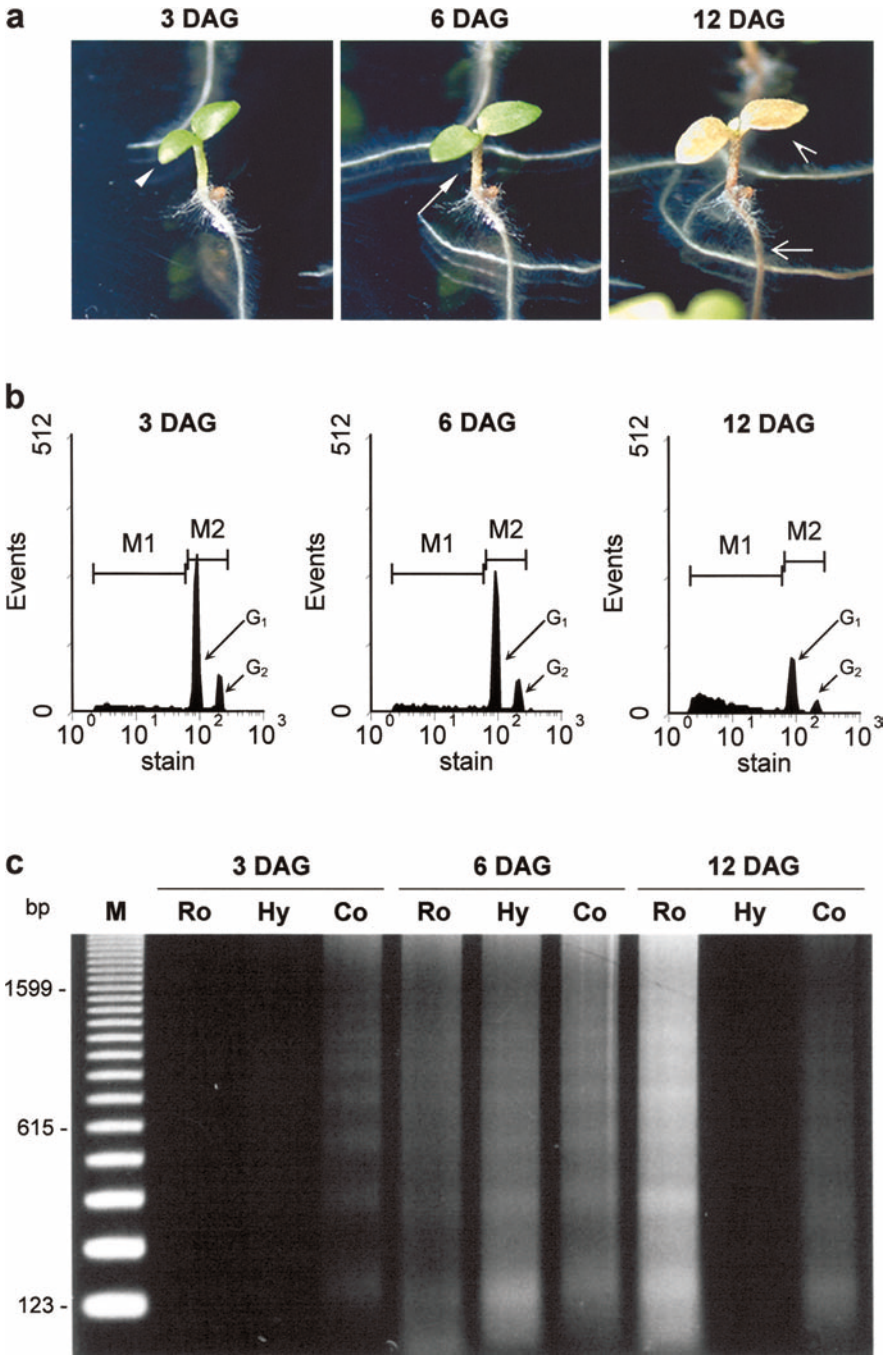

Fig. 1a–c Lethal symptoms and apoptotic changes shown by hybrid seedlings from the cross *Nicotiana suaveolens* × *N. tabacum* cultured at 28 °C. **a** Progressive changes in appearance of the seedlings. The same seedling was photographed 3, 6, and 12 DAG. In the seedlings, epinasty of the cotyledon (*closed arrowhead*), and browning of the hypocotyl (*closed arrow*), cotyledon (*open arrowhead*), and primary root (*open arrow*) were observed. **b** Nuclear fragmentation detected in the seedlings. Histograms indicate an increase in fragmented nuclei (*M1*) and a decrease in normal nuclei (*M2*), as measured by flow cytometry of a total of 10,000 nuclei. The *arrows* indicate the peaks of nuclei presumably corresponding to the G_1 and G_2 phases of the cell cycle. **c** DNA fragmentation detected in the seedlings. Total DNA was isolated from primary roots (*Ro*), hypocotyls (*Hy*), and cotyledons (*Co*) using the modified CTAB method, then electrophoresed in a 2% agarose gel and stained with SYBR Gold nucleic acid gel stain. *Lane M* 123-bp DNA ladder marker

size, was visible following agarose gel electrophoresis of total DNA extracted from each organ (i.e., roots, hypocotyls and cotyledons) of the hybrid seedlings (Fig. 1c). After staining the agarose gel with SYBR Gold, a distinctive ladder pattern was shown by the DNA extracted from the cotyledons of hybrid seedlings at 3 DAG, which ranged from approximately 125 to 1,600 bp as indicated by a DNA size marker. At this stage, roots and hypocotyls did not show the ladder pattern. At 6 DAG, all organs of the hybrid seedlings showed the ladder pattern shown by cotyledons at the first stage. At 12 DAG, hybrid seedlings showed the ladder pattern in DNA from roots and cotyledons. At this stage, the intensity of the ladder-like DNA bands increased for roots but decreased for cotyledons compared to the band intensity at the second stage. Furthermore, the DNA bands disappeared from hypocotyl samples.

Temperature-sensitive lethality expressed by hybrid seedlings

The lethality expressed by hybrid seedlings was temperature sensitive (Fig. 2a). When F1 seeds were sown on MS medium and cultured at 28 °C, all of the

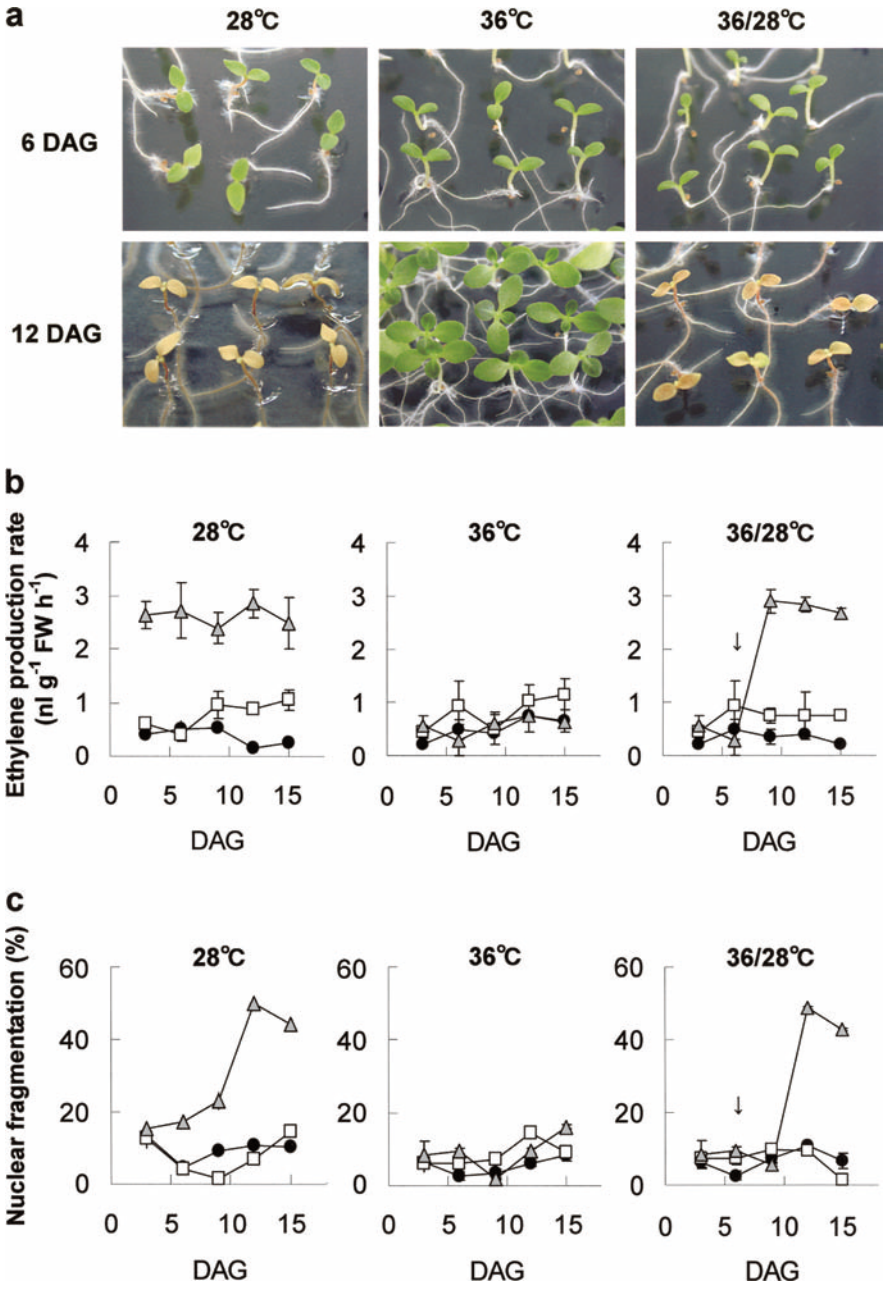

Fig. 2a–c Effect of temperature on ethylene production and nuclear fragmentation detected in hybrid seedlings from the cross *N. suaveolens* × *N. tabacum*, which express temperature-sensitive lethality. F1 seeds germinated at 28 °C were transferred to continuous temperature conditions (28 or 36 °C). Some of the seedlings cultured at 36 °C were exposed to 28 °C beginning 6 DAG (36/28 °C). **a** Growth of hybrid seedlings cultured at different temperatures. **b** Changes in ethylene production in hybrid and parental seedlings cultured at different temperatures. Ethylene production rate (nl g^{-1} FW h^{-1}) was measured by gas chromatography. *FW* Fresh weight. **c** Changes in nuclear fragmentation in hybrid and parental seedlings cultured at different temperatures. The nuclear fragmentation rate was calculated based on histograms obtained by flow cytometry of nuclei isolated from the seedlings. In **b** and **c**, the *arrows* indicate when the seedlings were exposed to 28 °C. Values are means ± SD of five separate experiments. ▲, hybrid seedlings (*N. suaveolens* × *N. tabacum*); □, maternal seedlings (*N. suaveolens*); ●, paternal seedlings (*N. tabacum*)

seedlings exhibited browning of the hypocotyl until 6 DAG and most died by 12 DAG. Meanwhile, when hybrid seeds germinating at 28 °C were immediately transferred to continuous high-temperature conditions (36 °C), none of them showed any lethal symptoms and they had developed true leaves by 12 DAG. At 36 °C, the hybrid seedlings developed vigorously and grew to maturity (data not shown). When the hybrid seedlings cultured at 36 °C were exposed to 28 °C at 6 DAG, they did not develop true leaves and exhibited lethal symptoms, such as leaf epinasty, chlorosis in the cotyledon, and browning of the hypocotyl. Finally, the hybrid seedlings exposed to 28 °C completely browned and did not grow further (data not shown).

Ethylene production in hybrid seedlings cultured at different temperatures

The levels of ethylene production were determined in hybrid and parental seedlings cultured at different temperatures: 28, 36, or 36/28 °C (Fig. 2b). Measurement of ethylene by gas chromatography (GC) revealed that ethylene production by the hybrid seedlings was more than that of parental seedlings through each growth stage at 28 °C. In hybrid seedlings 3–15 DAG, the ethylene production rate was relatively high, ranging between 2.39 and 2.85 nl g^{-1} FW h^{-1}. At the same stages, the ethylene production of parental seedlings was relatively low, ranging between 0.15 and 0.54

(*N. suaveolens*), or 0.40 and 1.68 nl g^{-1} FW h^{-1} (*N. tabacum*). In contrast, at 36 °C, ethylene production of hybrid seedlings was similar to that of parental seedlings. The levels of ethylene production in hybrid and parental seedlings at 36 °C were also close to the levels of parental seedlings at 28 °C. When hybrid seedlings cultured at 36 °C were exposed to 28 °C within 6 DAG, their ethylene production increased and was more than that of parental seedlings between 9 and 15 DAG. During the period of exposure to 28 °C, the levels of ethylene production in the hybrid seedlings were also close to those of hybrid seedlings continuously cultured at 28 °C.

Nuclear fragmentation in hybrid seedlings cultured at different temperatures

Nuclear fragmentation was determined in hybrid and parental seedlings cultured at different temperatures: 28, 36, or 36/28 °C (Fig. 2c). Measurement of nuclear fragmentation by flow cytometry revealed that the percentage of fragmented nuclei in the hybrid seedlings increased with the growth stage at 28 °C. In hybrid seedlings 3–9 DAG, fragmented nuclei gradually changed from 15.5% to 23.0%. After 9 DAG, fragmented nuclei in the hybrid seedlings increased sharply and reached a maximum of 50.1% by 12 DAG. In the parental seedlings, fragmented nuclei were detected at low levels ranging between 4.7% and 13.3% (*N. suaveolens*), or 1.4% and 14.7% (*N. tabacum*) over the same growth stages. In contrast, at 36 °C, nuclear fragmentation of hybrid seedlings was similar to that of parental seedlings. The percentages of fragmented nuclei in hybrid and parental seedlings at 36 °C were also close to those of parental seedlings at 28 °C. When the seedlings cultured at 36 °C were exposed to 28 °C within 6 DAG, fragmented nuclei in the hybrid seedlings increased sharply from 9 to 12 DAG and reached 48.7%, the same level as that of the hybrid seedlings continuously cultured at 28 °C.

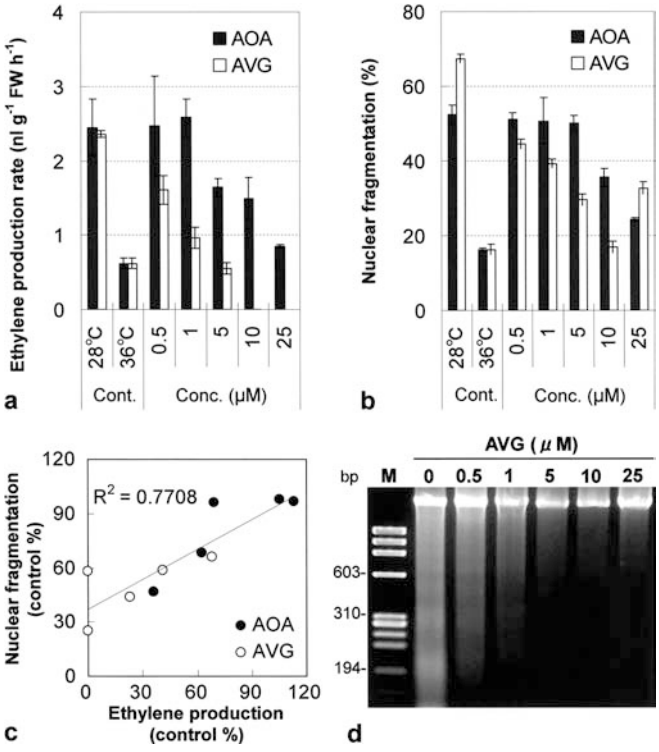

Fig. 3a–d Effects of ethylene inhibitors on apoptotic changes detected in hybrid seedlings from the cross *N. suaveolens* × *N. tabacum*. The germinating seeds were cultured on medium containing AOA or AVG at 28 °C. **a** Ethylene production in seedlings treated with ethylene inhibitors. The ethylene production rate was measured 15 DAG. **b** Nuclear fragmentation in seedlings treated with ethylene inhibitors. The nuclei were isolated from the seedlings used for measurement of ethylene production in **a**, and nuclear fragmentation rate was determined 15 DAG. In **a** and **b**, values are means ± SD of five separate experiments. **c** Correlation between ethylene production and nuclear fragmentation detected in seedlings treated with ethylene inhibitors. Data were obtained from **a** and **b** as the deviation of each treatment from controls at 28 °C (%). Statistical work was performed on the data after an arcsine transformation. **d** DNA fragmentation in seedlings treated with ethylene inhibitors. Total DNA was isolated from whole plants 15 DAG, then electrophoresed in a 3% agarose gel. *Lane M*, ΦX174/*Hae*III DNA marker

The influence of ethylene inhibitors on apoptotic changes in hybrid seedlings

We observed the effect of two inhibitors of ethylene biosynthesis (AOA and AVG) on apoptotic changes in the hybrid seedlings. Initially, their effectiveness on ethylene inhibition was confirmed by determination of ethylene production in hybrid seedlings treated with the inhibitors at different concentrations (Fig. 3a). When hybrid seedlings were cultured at 28 °C on medium containing AOA, ethylene production was distinctly reduced at concentrations of 5–25 μM compared to controls. Treatment with AVG also had a suppressive effect on ethylene production in hybrid seedlings at lower concentrations of 0.5 and 1 μM. Ethylene production in hybrid seedlings cultured on medium containing a high concentration of AVG (5, 10, and 25 μM) was further or completely suppressed, and production was lower than for controls at 36 °C.

Ethylene inhibitors had a suppressive effect on nuclear fragmentation, which is one of the characteristics of apoptosis and was detected in hybrid seedlings 12 DAG at 28 °C (Fig. 3b). In hybrid seedlings cultured at 28 °C on medium containing AOA (10 and 25 μM), the percentage of fragmented nuclei was reduced (35.6 and 24.3%, respectively) compared to controls (52.4%). In hybrid seedlings cultured at 28 °C on medium containing AVG (0.5–25 μM), nuclear fragmentation occurred at low levels ranging between 17.0% and 44.5% compared to controls (67.5%). In addition, treatment with 10 μM AVG suppressed nuclear fragmentation in hybrid seedlings at levels similar to that of controls at 36 °C. Furthermore, nuclear fragmentation correlated with ethylene production in hybrid seedlings

treated with ethylene inhibitors ($r^2 = 0.77$, obtained after arcsine transformation, $P < 0.01$) (Fig. 3c).

Ethylene inhibitors also suppressed DNA fragmentation 15 DAG at 28 °C (Fig. 3d). In hybrid seedlings cultured at 28 °C on medium containing AVG (0.5–25 μM), the intensity of the ladder-like DNA bands indicating fragmented DNA was reduced compared to controls. In particular, treatment with high concentrations of AVG (5, 10, and 25 μM) completely suppressed DNA fragmentation in the hybrid seedlings. Similarly, in hybrid seedlings cultured on medium containing AOA (5–25 μM), DNA fragmentation also occurred at low levels, although AOA did not suppress fragmentation completely (data not shown).

Temperature-sensitive lethality expressed by hybrid seedlings

We observed a life-extending effect of ethylene inhibitors on hybrid seedlings at 28 °C. Expression of lethal symptoms in the hybrid seedlings was suppressed by treatment with AOA and AVG at concentrations optimal for suppressing ethylene production and apoptotic changes. When hybrid seedlings were cultured on medium containing AOA at 25 μM, browning of hypocotyls, a typical symptom of lethality, did not occur 6 DAG (Fig. 4a). In this treatment, chlorosis was visible on the cotyledons 12 DAG, but the hybrid seedlings grew for an average of 20 days without browning (Fig. 4b). Meanwhile, hybrid seedlings cultured on medium containing AVG at 10 μM did not show browning and chlorosis on their organs until 12 DAG (Fig. 4a). Hybrid seedlings treated with AVG grew for an average of 38 days without browning (Fig. 4b), although they did not reach maturity. Additionally, growth inhibition of the primary root and true leaves was commonly observed in hybrid seedlings treated with AOA and AVG.

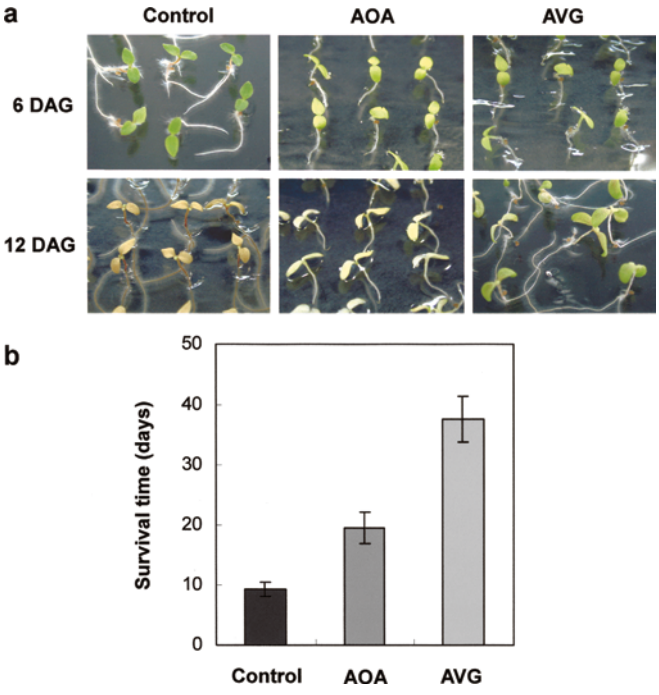

Fig. 4a, b Effect of ethylene inhibitors on the lethality detected in hybrid seedlings from the cross *N. suaveolens* × *N. tabacum*. Germinating seeds were cultured on medium containing AOA (25 μM) or AVG (10 μM) at 28 °C. **a** Growth of seedlings treated with ethylene inhibitors at optimum concentrations. **b** Life-extending effects of ethylene inhibitors on seedlings treated with ethylene inhibitors at optimum concentrations. Survival time was judged on the basis of days from germination until all leaves turned brown in 25 seedlings. Values are means ± SD of five separate experiments

Discussion

Apoptotic cell death has been identified by distinct changes in cells, such as condensation of chromatin, fragmentation of nuclei, cytoplasmic reduction, and fragmentation of DNA, and it plays major roles during animal development, homeostasis and disease (Afford and Randhawa 2000). In plants, all or some of these apoptotic changes have been detected in cases of PCD associated with senescence (Yamada et al. 2001b), development and defense (Jones 2001), and in response to chemicals (De Jong et al. 2000). In the present study, we closely examined apoptotic changes in hybrid seedlings at the organ level and made novel observations. The rate of nuclear fragmentation increased in hybrid seedlings when their cotyledons and roots were turning brown. However, a ladder-like DNA banding pattern was observed in cotyledons of hybrid seedlings that did not show any lethal symptoms, and was also detected in whole seedlings along with browning of the hypocotyl. In hybrid seedlings that turned brown, the intensity of ladder-like DNA bands decreased in the hypocotyl and cotyledon, but increased in the roots. This may be caused by movement of fragmented DNA from upper to lower parts in the hybrid seedlings. From these observations, it is clear that the progress of nuclear fragmentation paralleled that of the lethal symptoms in hybrid seedlings, but the timing of DNA fragmentation did not correlate with lethal symptoms in each organ of the hybrid seedlings. However, DNA fragmentation preceded expression of lethal symptoms in the cotyledons and roots. Thus, there is no doubt that apoptotic changes are constitutive processes of PCD leading to hybrid lethality. Although browning of organs in the hybrid seedlings has been used as a symptom for detection of lethality, there is no incontrovertible evidence of a cause-and-effect relationship between browning and apoptosis. It is possible that browning is a secondary process of hybrid lethality.

Ethylene production plays an important role in regulating many plant processes, ranging from germination to senescence (Yang and Hoffman 1984). In the previous study, we proposed that auxin and ethylene are involved in the lethality expressed by hybrid seedlings from the

cross *N. glutinosa* × *N. repanda*, and that the abnormal increase in endogenous auxin leads to ethylene production in hybrid seedlings during their early growth stages (Yamada et al. 2001a). However, we did not have reliable evidence that ethylene is involved in lethality because ethylene production in the hybrid seedlings was not measured quantitatively. In the present study, we confirmed that ethylene production was involved in the lethality expressed by hybrid seedlings from the cross *N. suaveolens* × *N. tabacum*. At 28 °C, ethylene production in hybrid seedlings both before and after showing of lethal symptoms indicated high levels that exceeded those in parental seedlings. When hybrid seedlings were exposed to 28 °C after culturing at 36 °C, an increase in ethylene production occurred immediately, and preceded expression of the lethality. Meanwhile, as a result of administration of AOA and AVG, ethylene production was suppressed and the lifetime of hybrid seedlings was clearly extended compared to controls without ethylene inhibitors. An increase in ethylene production thus correlates closely with expression of lethal symptoms and overproduced ethylene shortens the lifetime of the hybrid seedlings. These observations indicate that ethylene is involved in induction of lethality in this case, and also provide the first evidence that ethylene is involved in the expression of hybrid lethality.

Temperature sensitivity, which is the suppression of lethality under high-temperature conditions (32–38 °C), has been observed in several crosses of *Nicotiana* (Yamada et al. 1999) and other genera (Phillips 1977), but the physiological mechanism has not been explained. In the present study, we confirmed that ethylene production in hybrid seedlings was suppressed at 36 °C compared to 28 °C, which is the lethal temperature condition. In general, the optimum temperature for ethylene production is near 30 °C, and the rate of ethylene production declines above 30 °C until production ceases near 40 °C (Abeles et al. 2000). It is thought that such an inhibition of ethylene biosynthesis arises from inactivation and turnover of the ethylene-forming enzyme, ACC (1-aminocyclopropane-1-carboxylic acid) oxidase, under high-temperature conditions (Atta-Aly 1992; Lurie et al. 1996). From these relevant cues, we inferred that temperature sensitivity in hybrid lethality is caused by changes in ethylene biosynthesis under different temperature conditions.

In several cases of plant processes induced by PCD, ethylene is involved in the regulation of these processes, e.g., aerenchyma formation (He et al. 1996), endosperm development (Young and Gallie 2000), and petal senescence (Orzáez and Granell 1997), and responses to phytotoxins (Asai et al. 2000), elicitors (Hanania et al. 1999) and pathogens (Lund et al. 1998). It has been suggested that ethylene is a mediator of PCD in plants (Woltering et al. 1999). In the present study, we evaluated the role of ethylene in PCD-induced hybrid lethality of the cross *N. suaveolens* × *N. tabacum*. At 28 °C, DNA fragmentation and nuclear fragmentation progressed in hybrid seedlings that produced high levels of ethylene. When hybrid seedlings were exposed to 28 °C after culturing at 36 °C, nuclear fragmentation occurred in them following an increase in ethylene production and exhibition of lethal symptoms. In addition, as a result of administration of AOA and AVG, nuclear fragmentation decreased along with inhibition of ethylene biosynthesis in the hybrid seedlings. DNA fragmentation was also suppressed in hybrid seedlings treated with AOA and AVG. From these results, it is clear that an increase in ethylene production correlates closely with DNA fragmentation and nuclear fragmentation, and that overproduced ethylene causes PCD in hybrid seedlings. These observations indicate that endogenous ethylene acts as an essential factor mediating PCD and subsequent hybrid lethality.

Since ethylene is involved in PCD induction of many plant processes, including hybrid lethality, it is assumed that plants share a common mechanism by which ethylene mediates PCD. Recently, further information about the possible mechanism of ethylene-mediated PCD has been reported. When a sensitive *Arabidopsis* plant is exposed to ozone (O_3) as an abiotic elicitor, the elevated level of ethylene production results in the formation of hypersensitive response (HR)-like lesions that have the characteristics of PCD (Overmyer et al. 2000). In this case, ozone also induces production of reactive oxygen species (ROS) and results in an oxidative burst. This suggests that ROS are involved in ethylene-mediated PCD in ozone-exposed plants. In addition, camptothecin, a topoisomerase-I inhibitor, induces PCD with similarities to animal apoptosis in tomato cells (De Jong et al. 2002). The ethylene level, however, does not increase, but it is a distinct potentiator of H_2O_2 production and subsequent PCD. Calcium- and caspase-dependent stimulation NADPH oxidase activity is also involved in this cell death. In addition, although exogenous ethylene does not trigger PCD in itself, it greatly stimulates camptothecin-induced PCD independently of caspase activity. From these observations, two partly overlapping pathways have been proposed for ethylene-mediated PCD. One pathway involves caspases that require low levels of ethylene and another caspase-independent pathway is operative at high ethylene levels. In accordance with this model, it is possible that the latter pathway mainly functions in PCD-induced hybrid lethality that is mediated by overproduced ethylene.

In general, PCD or apoptosis induces elimination of dispensable or abnormal cells during growth and development of multicellular organisms, and plays a role in formation and preservation of individuals. In the present study, we showed that ethylene-mediated PCD induces destruction of whole seedlings, i.e., somatic death. Our results introduce the new idea that ethylene has an important role in conserving plant species, as well as maintaining individuals, by inducing death of abnormal individuals, e.g., interspecific hybrids that consist of cells with coexisting heterogeneous genomes. In plants, it is possible that ethylene plays a common

role in inducing PCD at each of these levels. Thus, it is important for an understanding of plant homeostasis to identify the endogenous factors controlling ethylene production in each case.

Acknowledgement This work was partly supported by Grant-in Aid for Exploratory Research No. 12876002 and Scientific Research (A) No. 13306003 from the Ministry of Education, Science, Sports and Culture, Japan.

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