Cell Death Processes during Expression of Hybrid Lethality in Interspecific F_1 Hybrid between *Nicotiana gossei* Domin and *Nicotiana tabacum*

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Hybrid lethality, a type of reproductive isolation, is a genetically controlled event appearing at the seedling stage in interspecific hybrids. We characterized the lethality of F_1 hybrid seedlings from *Nicotiana gossei* Domin and *Nicotiana tabacum* cv Bright-Yellow 4 using a number of traits including growth rate, microscopic features of tissues and cells, ion leakage, DNA degradation, reactive oxygen intermediates including superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), and expression of stress response marker genes. Lethal symptoms appeared at 4 d after germination in the basal hypocotyl and extended toward both the hypocotyl and root of the plants grown at 26°C. Microscopic analysis revealed a prompt lysis of cell components during cell death. Membrane disruption and DNA degradation were found in the advanced stage of the lethality. The death of mesophyll cells in the cotyledon was initiated by the vascular bundle, suggesting that a putative factor inducing cell death diffused into surrounding cells from the vascular tissue. In contrast, these symptoms were not observed in the plants grown at 37°C. Seedlings grown at 26°C generated larger amounts of reactive oxygen intermediate in the hypocotyl than those grown at 37°C. A number of stress response marker genes were expressed at 26°C but not at 37°C. We proposed that a putative death factor moving systemically through the vascular system induced a prompt and successive lysis of the cytoplasm of cells and that massive cell death eventually led to the loss of the hybrid plant.

Hybrid lethality is a mechanism of reproductive isolation among the distantly related species (Stebbins, 1958; Coyne, 1992). A defect in mitotic cell division in relation to a profound failure of mitotic chromosome condensation was reported to be major cause of hybrid lethality in *Drosophila* spp. (Orr et al., 1997). In plants, the process results in the abortion of a zygote after fertilization in the embryo sac and/or cell death in the tissue of hybrid seedlings after germination (Adachi, 2001). In interspecific F_1 hybrids of the genus *Nicotiana*, four distinct types of hybrid lethality were reported including necrotic browning of the shoot apex and hypocotyl tissue in the seedlings, the type of lethality depending on the combination of parental species (Yamada et al., 1999). The lethality was suppressed at higher temperatures (32°C–36°C) but proceeded at 28°C (Manabe et al., 1989). These findings suggest that cell death in hybrid lethality is controlled genetically and that certain factors in the hybrid cells whose functions are influenced by temperature induce a prompt and broad-ranging cell death reaction in the tissue. Thus, the processes of cell death would be important when monitoring the expression of lethality in hybrid plants.

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Recent progress in the study of programmed cell death (PCD) in plants has revealed a role in defense against pathogens and normal development (Jones, 2001). The cell death that is associated with the active defense of plants against pathogens including virus, bacteria, fungi, and nematodes is known as the hypersensitive response (HR; Keen, 1990; Dangl et al., 1996). In terms of developmental features, cell death causes the deletion of suspensor and aleurone cells, the formation of leaf lobes and the xylem tracheary element (TE), and the senescence of leaf and petal (Pennell and Lamb, 1997). During development of the TE in a zinnia (*Zinnia elegans*)-cultured cell system, vacuole collapse was central to cell death, releasing the insulated hydrolytic enzymes to attack organelles and leading to the degradation of cell components within a very short time (Fukuda, 2000). Thus, none of the features of apoptosis-like cell death are observed in the TE of zinnia, although it is a developmentally regulated form of PCD (Obara et al., 2001). On the other hand, typical apoptotic features, including chromatin condensation and DNA fragmentation into nucleosomes, were found in pollination-induced petal senescence of petunia (*Petunia hybrida*; Xu and Hanson, 2000), old cultured cells of Arabidopsis (Callard et al., 1996), aleurone layers of germinating barley (*Hordeum vulgare*) seed (Wang et al., 1998) and carpel senescence in pea (*Pisum sativum*; Orzaez and Granell, 1997). These findings indicate that PCD in plants has diverse features of expression.

Although it was reported that the temperature-sensitive lethality of seedlings and cultured cells from F_1 hybrids of *Nicotiana* spp. is apoptosis (Marubashi et al., 1999; Yamada et al., 2000, 2001b), little information was obtained on the temporal and spatial features of cell death in the hybrid plants. Here, we employ the F_1 hybrid of *Nicotiana gossei* × *Nicotiana tabacum*, which also showed temperature-sensitive lethality, to characterize the cell death reaction in the seedlings. The results of the present investigation indicate that cell death induces a rapid degradation of cell complements, accumulation of brown pigments, and termination of shoot growth. It is also suggested that a putative death factor originating from a defined part of the tissue is systemically transported to distant tissues through the vascular system, successively inducing cell death and eventually leading to the death of the plant. The expression patterns of stress-responsive genes suggested that the cell death of the hybrid plants induced a response similar to systemic acquired resistance (SAR).

RESULTS

Growth and Cell Death Symptom of the Hybrid

To exclude the possibility that a qualitative difference in the seeds affects germination and subsequent growth, we used seeds collected from plants grown under the same conditions in the same year.

At 3 weeks after germination, the hybrid plants grown at 26°C showed signs of necrotic death, whereas the parents grew normally. However, at 37°C, the hybrids showed enhanced growth compared with the parents (Fig. 1). Growth analysis indicated that the shoot growth of hybrids at 26°C was arrested at between 4 and 6 d after germination (DAG; Fig. 2). On the other hand, retardation or inhibition of growth was observed in parental species but not in the hybrid. The growth of *N. gossei* was severely inhibited at 37°C but returned to normal after transferring to 26°C, indicating that the plant was not dead at the elevated temperature condition (data not shown). The results support previous data showing that hybrid lethality was suppressed by

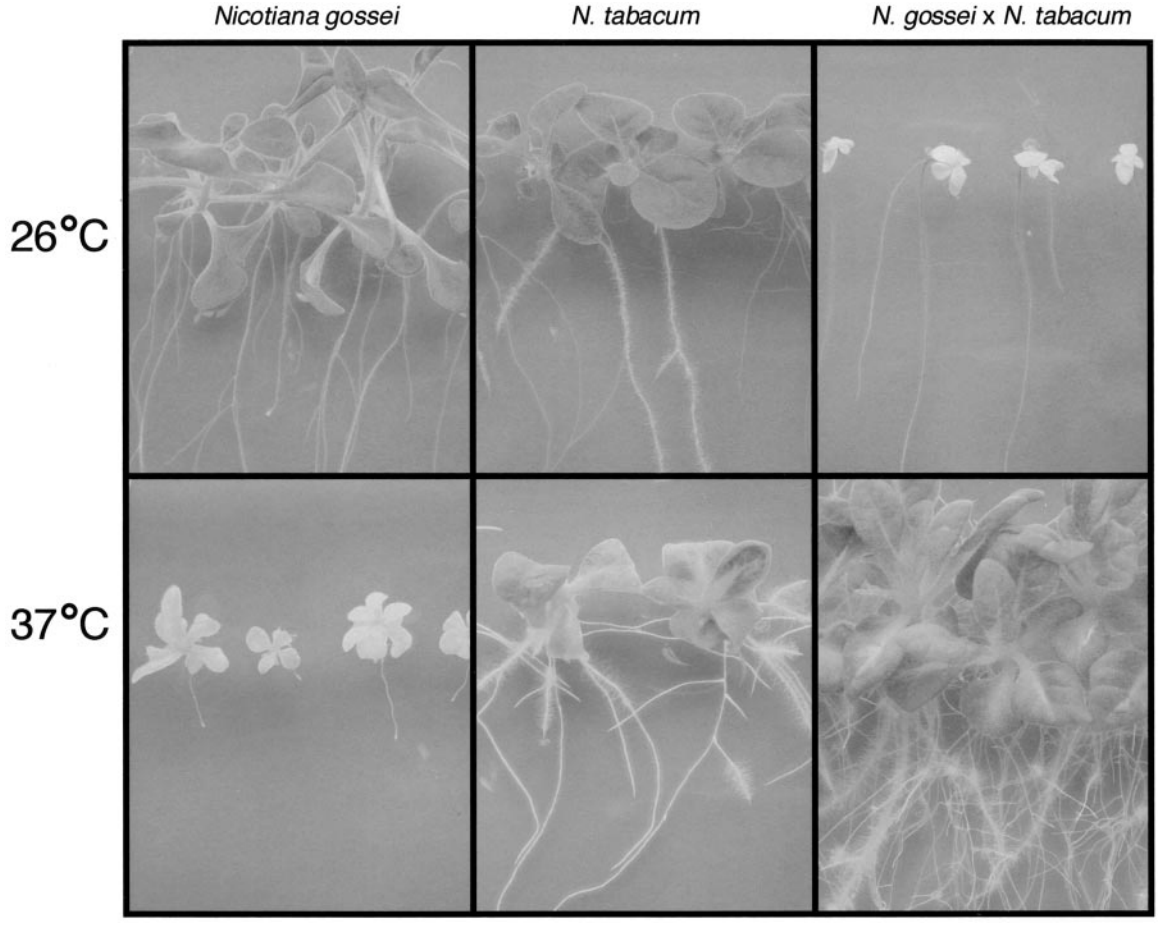

Figure 1. Seedling growth of the F_1 hybrid (*N. gossei* × *N. tabacum*) and the parental species at 26°C or 37°C. The features of 3-week-old seedlings are shown.

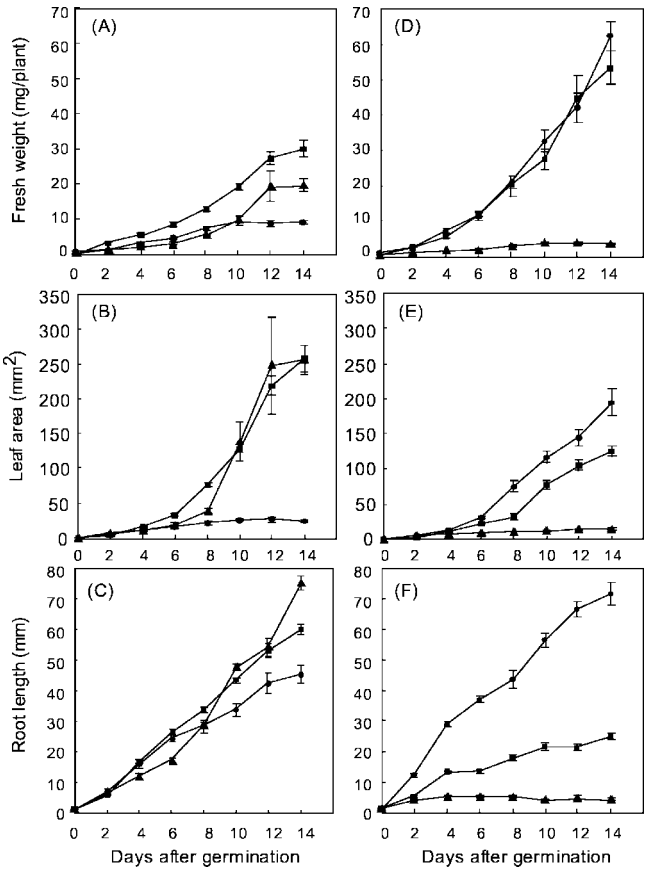

Figure 2. Seedling growth of the F_1 hybrid (*N. gossei* × *N. tabacum*) and the parental species grown at 26°C (A–C) and 37°C (D–F). ▲, *N. gossei*; ■, *N. tabacum*; ●, F_1 hybrid. Vertical bars represent SE.

high temperature in hybrids of other crosses in the genus *Nicotiana* (Yamada et al., 1999). However, in the current study, the roots of the hybrid continued to elongate (Fig. 2C).

The first evidence of lethality in the hybrid plants was tissue browning at the basal hypocotyl at 4 DAG (Fig. 3A). The browning extended toward both the cotyledon and the taproot, increasing in intensity (Fig. 3, A–D and I–L). To test whether the browned tissue was dead, plants were stained with trypan blue. This experiment showed that the brownish areas coincide with the sites deeply stained with trypan blue (Fig. 3, E–H). Thus, the spatial distribution of brown pigment in the tissue showed the sites of cell death. We have observed that browning starting at base of the hypocotyl reached the top of the hypocotyl within 5 h. This indicated that the cell death reaction extends quickly in the tissue of hybrid plants. Closer inspection showed that the brown pigment accumulated inside the tissue and in the apoplastic region of epidermal cells of the basal hypocotyl (Fig. 3I). The increase in ion leakage from the hybrid seedlings at 26°C supported that cell death proceeded with membrane disruption of the cells (Fig. 4A). On the other hand, no such increase in ion leakage was observed in normally growing hybrids at 37°C (Fig. 4B).

At an advanced stage, browning extended to the cotyledon along the vascular bundle, and mesophyll cells of the cotyledon started to die by the vascular tissue, as witnessed by trypan blue staining (Fig. 5). Browning also affected taproot tissue, although it did not reach the root tip (data not shown). To examine whether root growth of the hybrid at 26°C was sustained by active cell division, the mitotic index, and the pattern of staining with Evans blue in root tip cells were analyzed. The results indicated that the mitotic indices of F_1 hybrid (approximately 2%) were almost the same as those of *N. tabacum* and that the tissue of root tip was not stained by Evans blue (Fig. 6, A and B). These findings clearly indicated that the root tip cells were not dead. Two possibilities would explain the insensitivity of root tip tissue to cell death: The cells of meristematic tissue are insensitive to cell death, or a putative death factor translocating through the vascular bundle does not reach the root tip tissue because of the absence of a developed vascular system. The first possibility relates to a difference of cell type, differentiated or undifferentiated. In the cultured cells from hypocotyl tissue of *N. gossei* × *N. tabacum*, we also observed a temperature-sensitive cell death reaction (M. Mino, Y. Misaka, and M. Inoue, unpublished data). Because dedifferentiated cells in vitro are comparable with meristematic cells in situ (Fukuda, 2000), the first possibility can be eliminated. To test whether a putative death factor does not reach meristematic tissue, cell death in the shoot meristem was analyzed by FDA staining. The analysis indicated that the shoot meristem was still alive at an advanced stage (Fig. 6, C and D). Taken together with the fact that the death of mesophyll cells was initiated by the vascular bundle, these results suggest that a putative death factor in cells of the basal hypocotyl moves systemically through the vascular system, diffuses to surrounding cells, and induces cell death.

Collapse of Cells, Tissues, and DNA

To better understand the structural changes in hypocotyl tissue, hybrids grown at 26°C and 37°C were compared in terms of structure of the basal hypocotyl. Analysis of surface structure using scanning electron microscopy (SEM) shows that epidermal cells started to disappear at 4 DAG in the plants grown at 26°C and that this destruction had extended over the entire surface of the hypocotyl tissue at 7 DAG (Fig. 7, A and B). Microscopic analysis of transverse sections of hypocotyl at an advanced stage showed that cortical layer cells of the plants grown at 26°C were larger than those of the plants grown at 37°C, were skewed in shape, and had lost most of their protoplasts (Fig. 7, C and D). Fluorescence microscopy clearly revealed the disappearance of cell

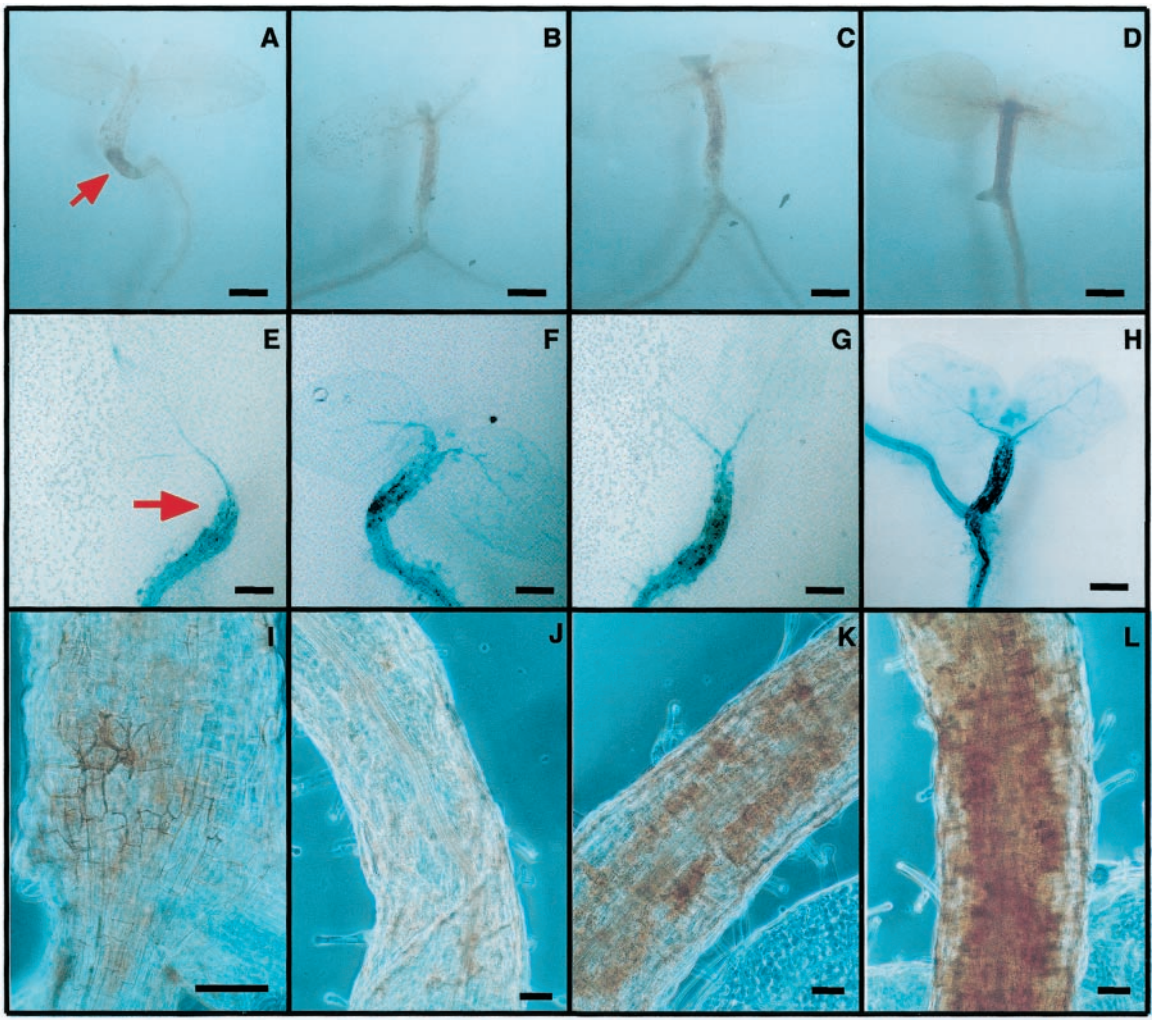

Figure 3. Process of browning and cell death in the tissue of F_1 seedlings at 4 (A, E, and I), 5 (B, F, and J), 6 (C, G, and K), and 7 (D, H, and L) DAG. A to D, The sequential process of browning initiating at the basal hypocotyl (an arrow in A) and expanding to whole tissue. I to L, A closer view of the browning (I, basal hypocotyl; J–L, middle of the hypocotyl). E to H, Dead tissue stained with trypan blue. Note that cell death initiated at the basal hypocotyl (an arrow in E) and expanded concomitantly with tissue browning. Bars in A through H = 1,000 μm and in I through L = 100 μm.

components by loss of autofluorescence of chloroplasts, which were normally found in cells of the plants grown at 37°C (Fig. 7, E and F). These results indicated that a loss of turgor pressure in the cells induced broad subsidence of epidermal cells of hypocotyl tissue.

In the central cylinder and intercellular space of cortical layer cells, brown pigments were deposited (Fig. 7D). In necrotic lesions as a result of HR, the deposition of lignin plays an important role in increasing the mechanical strength of cell walls and inhibiting pathogen invasion (Hammerschmidt and Kuc, 1982; Hammerschmidt et al., 1984). On the basis of evidence of a rapid increase in enzymatic activity for the biosynthesis of lignin, it was speculated that lignin formed in cells that were injured by ozone treatment (Galliano et al., 1993). These studies suggest that lignin is deposited at sites where cell death is under way. To analyze whether the lignin was localized to areas with browning, the hypocotyl was stained with phloroglucinol-HCl. However, this treatment did not stain browned tissue, only the xylem (data not shown).

At an advanced stage of hybrid lethality, the DNA extracted from whole seedlings was completely degraded, suggesting that a nuclease(s) with DNase activity was expressed during the cell death process (data not shown). On the other hand, no such degradation of DNA was observed in the hybrid plants grown at 37°C. We failed to detect a specific ladder pattern of DNA degradation during several trials, although it was reported that fragmented DNA was an apoptotic feature in leaf tissue of *Nicotiana glutinosa* × *Nicotiana repanda*, the hybrid showing lethality (Marubashi et al., 1999).

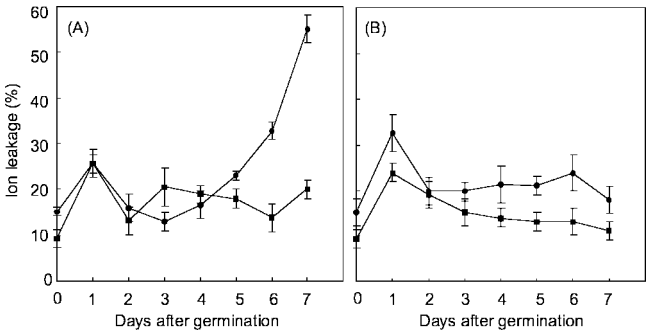

Figure 4. Ion leakage from seedlings of the F_1 hybrid and *N. tabacum* grown at 26°C (A) and 37°C (B). ■, *N. tabacum*; ●, F_1 hybrid. Vertical bars represent SE.

Generation of Reactive Oxygen Intermediates (ROI)

To assess whether superoxide radical (O_2^-) is required for the initiation and propagation of cell death in the hybrid, we treated the seedlings with a nitroblue tetrazolium to localize the site of O_2^- generation. This experiment showed that O_2^- was accumulated in various parts of the seedling including the basal hypocotyl where the signs of cell death symptom first appeared (Fig. 8A). To quantify the O_2^- in the basal hypocotyl, we measured the MCLA dose-dependent chemiluminescence by O_2^- using a two-dimensional photon-counting system. The evolution of O_2^- in the basal hypocotyl at 2 DAG was significantly greater in the hybrid than *N. tabacum* at 26°C, but not at 37°C (Fig. 8, B and C). The actual cell death reaction at 26°C was initiated after the rise in O_2^- (Figs. 3 and 4). To exclude the possibility that O_2^- generated from photosynthesis is involved in cell death, the plants were grown in the dark at 26°C and 37°C. Such plants showed signs of hybrid lethality at 26°C but not at 37°C (data not shown). These experiments indicated that the expression of hybrid lethality conclusively depends on temperature, not light. To analyze the role of O_2^- in the cell death processes, we treated the seedlings with a plasma membrane NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI) at various concentrations. It was shown that DPI effectively inhibited accumulation of O_2^- in the basal hypocotyl (Fig. 8D). The rate of browning in the seedlings was also reduced in a dose-dependent manner as compared with that in the untreated control (Fig. 8E). However, DPI did not completely suppress the browning.

To further dissect the role of ROI in the process of cell death, we detected endogenous hydrogen peroxide (H_2O_2) in the plants by 3,3′-diaminobenzidine oxidation (Thordal-Christensen et al., 1997; Rea et al., 2002). The accumulation of 3,3′-diaminobenzidine oxidation product in the seedlings grown at 26°C and 37°C is shown in Figure 9A. Production of H_2O_2 revealed by reddish-brown color was not detected in the basal hypocotyl at 2 DAG, but it dramatically increased in the hypocotyl at 26°C but not at 37°C. To directly test whether H_2O_2 is involved in the process of cell death, the seedlings were treated with catalase. This experiment shows that catalase reduced an amount of H_2O_2 in the hypocotyl tissue (Fig. 9A). The treatment also effectively reduced the amount of brown pigment deposited in the hypocotyl and retarded the rate of browning in the seedling (Fig. 9, B and C). However, catalase did not totally inhibit procession of browning in the tissue. Taken together, our results indicate that ROI plays some part in the cell death process of hybrid seedlings grown at 26°C.

Expression of Stress Response Genes

Quantitative reverse transcriptase (RT)-PCR showed that, compared with the plants grown at 37°C, the hybrids grown at 26°C accumulated significantly more mRNA encoding pathogenesis-related

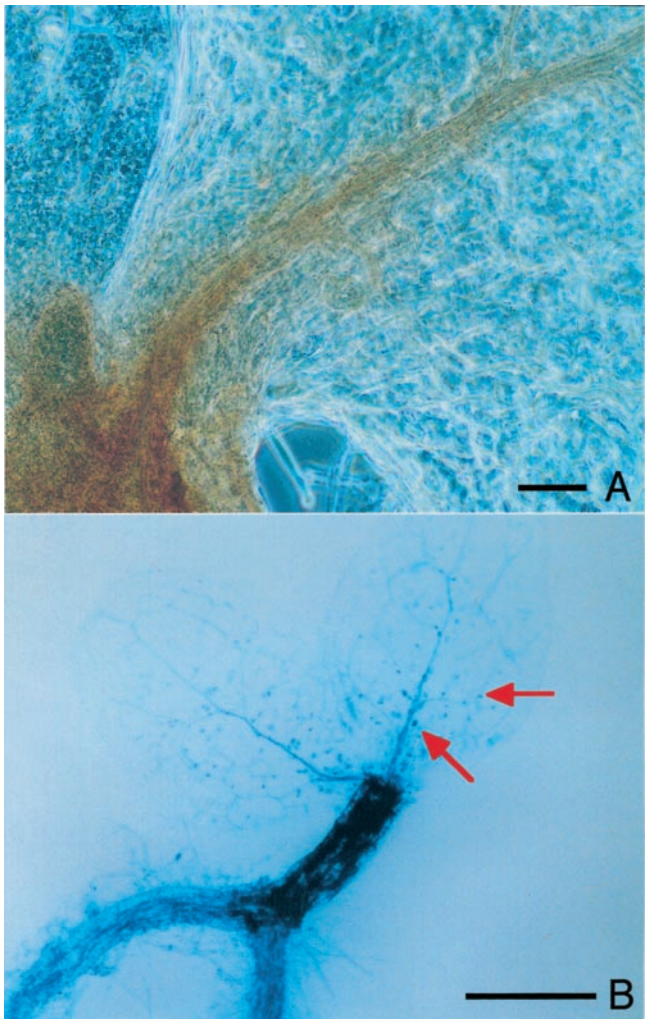

Figure 5. Browning of vascular tissue in the cotyledon (A) and staining with trypan blue (B) of F_1 seedlings at 10 DAG. Arrows in B indicate the sites of cell death (blue spots), which appeared along the vascular bundle, in mesophyll tissue of the cotyledon. Bar in A and B = 100 and 1,000 μm, respectively.

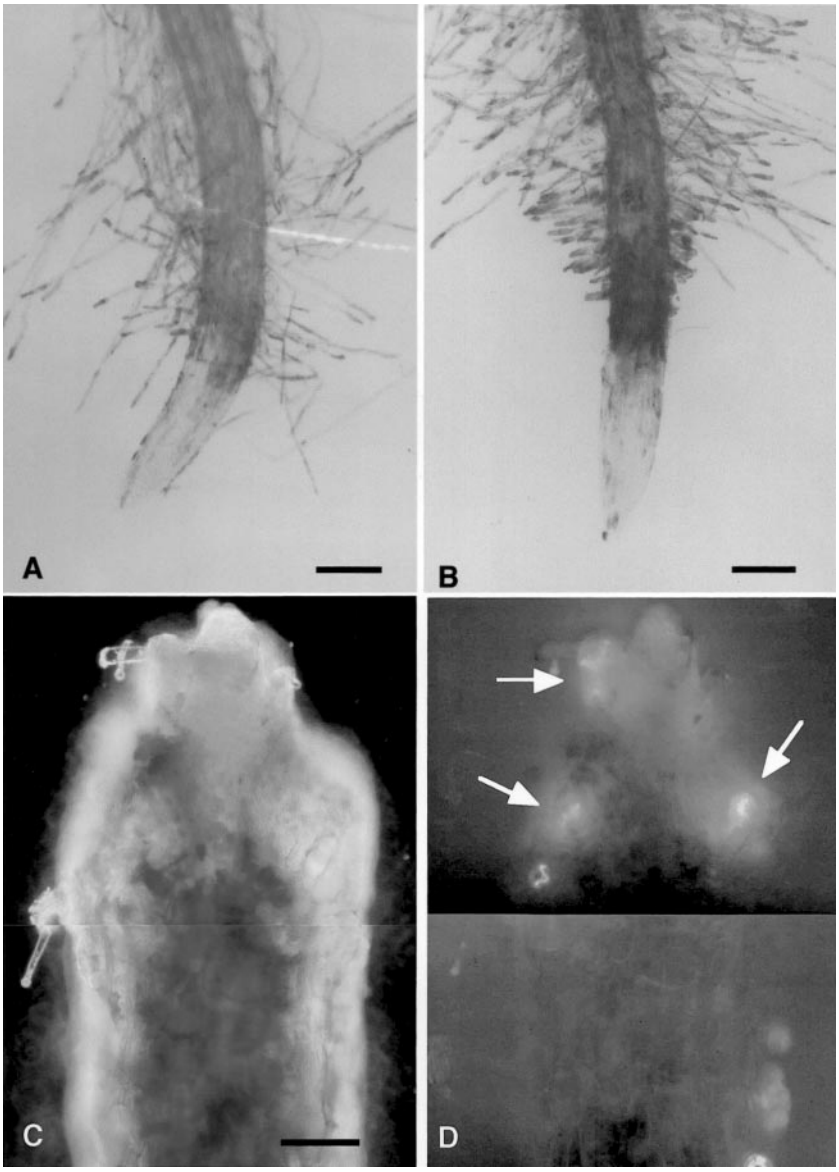

Figure 6. Patterns of Evans blue staining in the root of *N. tabacum* (A) and the F_1 hybrid (B) and images of phase contrast microscopy (C) and fluorescein diacetate (FDA) staining (D) of shoot tip tissue of the F_1 hybrid at 10 DAG. B, Note the absence of staining in tip tissue and the dark staining in the upper part of the hybrid root. C and D, Tissue beneath the shoot tip accumulated brown pigments and exhibited no fluorescence. Arrows in D indicate the sites where shoot meristem (top) and two axillary buds (bottom) show strong fluorescence. Bar in A and B = 200 μm and in C = 100 μm.

protein 1a (*PR1a*) and protein inhibitor II (*PI-II*) in accordance with the process of browning (Fig. 10). These two proteins are acidic (*PR1a*) and basic (*PI-II*) biochemical and molecular markers of HR and SAR. On the other hand, another basic PR protein gene, *PR5*, and *MnSOD* were not induced at significant levels in the hybrid grown at 26°C. None of these four genes was expressed in *N. tabacum* except *PI-II*, which was detected at 2 DAG in the plants grown at 37°C.

DISCUSSION

To study the cell death process in the hybrid lethality of *N. gossei* × *N. tabacum*, the growth of seedlings and the browning of the hypocotyl and root in hybrid plants grown at 26°C were analyzed. Browning has been found in other combinations of the genus *Nicotiana*, including *Nicotiana paniculata* × *N. gossei* and *N. paniculata* × *Nicotiana suaveolens*, and was classified as a feature of type II lethality (Yamada et al., 1999). A genetic study on interspecific hybridization suggested that the S genome in *N. tabacum* was responsible for hybrid lethality (Inoue et al., 1996). It was reported that irradiation of pollen of *N. tabacum* with an ion beam before crossing increased the survival rate among F_1 hybrids of *N. gossei* × *N. tabacum* (Yamashita et al., 1995). These reports indicate that the cell death associated with hybrid lethality is a genetically controlled phenomenon.

Comparing the time courses of different parameters during seedling growth provided a better understanding of the lethality. Up to 4 DAG, the growth of seedlings (Fig. 2) and the integrity of the membrane (Fig. 4) in the hybrid were comparable with the findings in the parental species. The experimental results

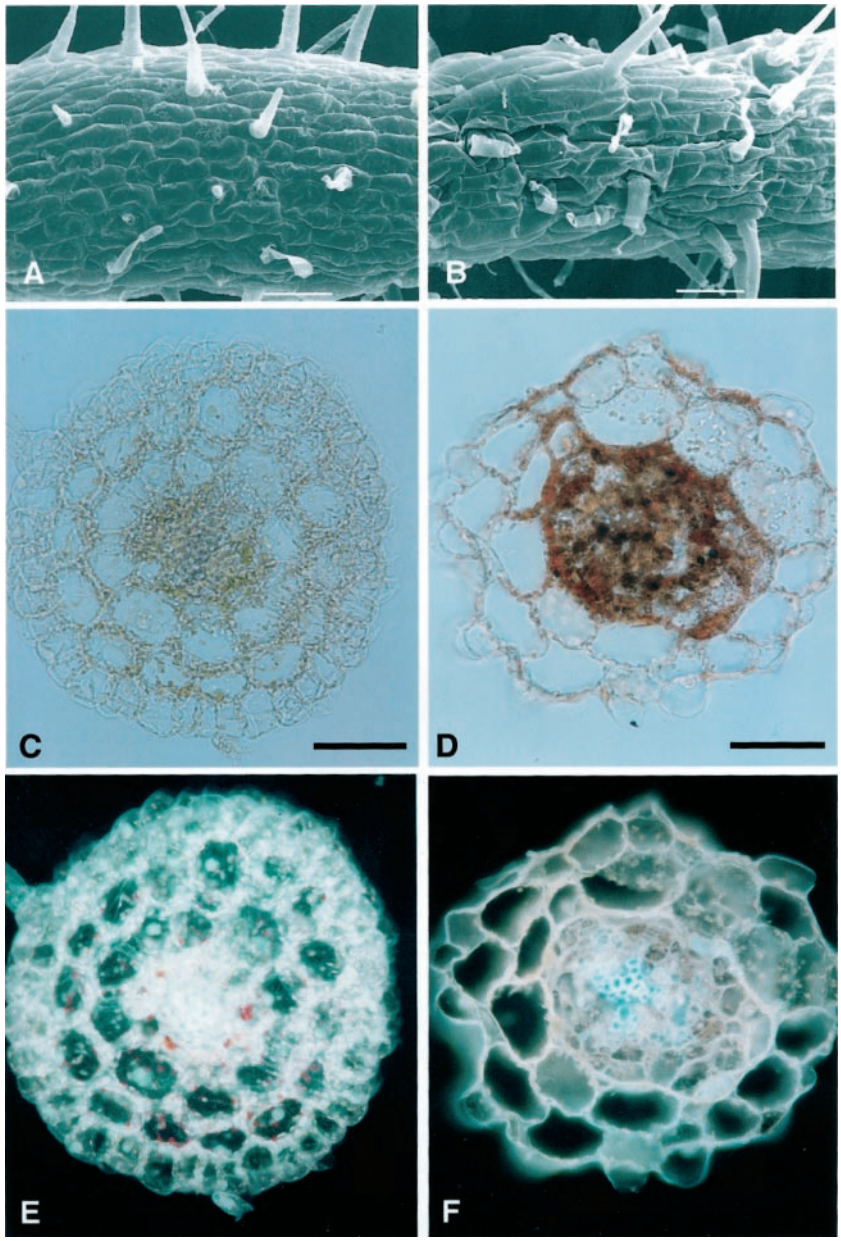

Figure 7. Histological analysis of F_1 hybrid hypocotyl. A and B, Surface structure of basal hypocotyl of F_1 seedlings grown at 37°C (A) and 26°C (B) for 7 DAG. C to F, Microscopic features of cross section of basal hypocotyl. Images of light (C and D) and autofluorescence (E and F) of seedlings grown at 37°C (C and E) and 26°C (D and F) for 8 DAG. Bars = 100 μm.

indicate that cell death accompanied by browning starts in the cells of basal hypocotyl tissue at 4 DAG (Fig. 3). At this point, leaf expansion was arrested (Fig. 2B), whereas roots continued to elongate (Fig. 2C), and browning subsequently extended very quickly along the vascular tissue with disintegration of the membrane (Figs. 3 and 4). However, cell death did not reach the meristematic tissue of roots and shoots (Fig. 6). The initiation of mesophyll cell death in the cotyledon via the vascular bundle suggests that the factor inducing the loss is highly mobile and diffusible in tissue (Fig. 5B). Plants have various signals that evoke defense responses to wounding and pathogens at sites distant from the injured cells. In tomato (*Lycopersicon esculentum*), an octadecameric peptide termed systemin derived from a 200-amino acid precursor protein prosystemin in wounded cells is rapidly translocated into intact tissue (McGurl et al., 1992). Systemic responses to pathogens and wounding are also mediated by salicylic acid, ethylene, and jasmonic acid (Niki et al., 1998). In the hybrid of *N. glutinosa* × *N. repanda*, it was suggested that auxin-induced ethylene was involved in apoptotic cell death during lethality (Yamada et al., 2001a). In the present study, a putative death factor might be present in the hybrid, which moves through the vascular bundle. Because the vascular system is not well established in meristematic tissue, the factor could not reach the shoot and root meristem. The factor is yet to be identified, but it would be temperature-sensitive given that it is not synthesized, or else it is degenerated and/or loses its function at 37°C (Figs.

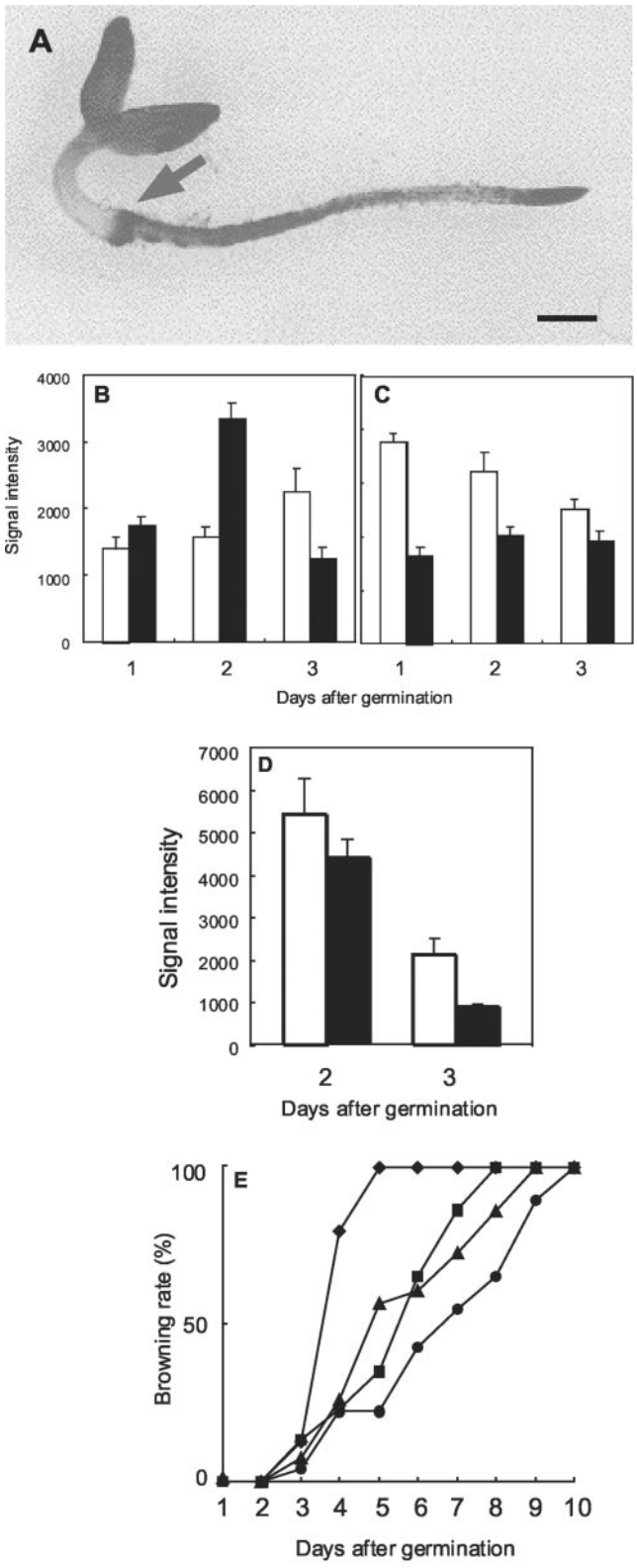

Figure 8. Detection of O_2^- in F_1 hybrid seedlings. A, Accumulation of O_2^- in the F_1 seedlings at 2 DAG. An arrow indicates purple formazan precipitation in the basal hypocotyl. Bar = 1,000 μm. B and C, Amount of O_2^- generated in the basal hypocotyl of *N. tabacum* (white columns) and the F_1 hybrid (black columns) seedlings grown at 26°C (B) and 37°C (C). Seedlings were incubated with 2-methyl-6-(*p*-metoxyphenyl)-3,7-dihydroimidazol[1,2-()]pyrazin-2-one (MCLA) at room temperature for 10 min, and MCLA-dependent chemiluminescence in the basal hypocotyl was measured by ARUGUS-50 (Hamamatsu Photonics, Hamamatsu, Japan). D, Effect of DPI (5 μM) on accumulation of O_2^- in the basal hypocotyl of F_1 hybrid seedlings. White and black columns represent control and treated group, respectively. E, Effect of DPI on the browning rate of the F_1 hybrid seedlings. ◆, 0 μM; ■, 0.5 μM; ▲, 1 μM; ●, 5 μM. Vertical bars represent SE.

1, 2, and 4). However, one cannot completely rule out the possibility that the cells of meristematic tissue are insensitive to the death reaction.

DNAaddering is widely recognized in plant PCD (Callard et al., 1996; Wang et al., 1998; Xu and Hanson, 2000). In the interspecific hybrids of *N. suaveolens* × *N. tabacum* and cultured cells of *N. glutinosa* × *N. repanda*, a DNA ladder was detected (Marubashi et al., 1999; Yamada et al., 2001b). Our experimental findings indicate that the DNA degraded without forming a ladder pattern. We did not detect nuclear fragmentation, which is another typical sign of apoptosis, in the cells of tissue showing the lethality phenomenon (data not shown). Thus, we concluded that the cell death accompanying hybrid lethality in *N. gossei* × *N. tabacum* was different from apoptosis, although it is a developmentally programmed and active process.

The disappearance of cell components of hypocotyl tissue indicates a progressive degeneration of organelles and the removal of protoplasts (Fig. 7). A similar cell death process was reported in the development of TE in zinnia culture cells (Fukuda, 2000; Obara et al., 2001). In this cell, the collapse of the large central vacuole is a key to the onset of cell death, the release of various hydrolytic enzymes into the cytoplasm induces the autolysis of cell components, and finally only a hollow TE cell is left. The collapse of the vacuole also releases insulated compounds into the cytoplasm, allowing polyphenol oxidase and probably peroxidase to catalyze the oxidation of phenolics to produce o-quinones that polymerize with amino acids and protein to yield brownish insoluble phenolic polymers (Booker and Miller, 1998). Our preliminary experiment suggests that the brown pigment was composed of proteins and phenolic compounds (M. Mino, K. Maekawa, and S. Tsujiyama, unpublished data), although it did not stain positive for lignin using phloroglucinol-HCl. These results indicate that disruption of the normal compartment of hydrolytic enzymes induces the lysis of cytoplasm to give a hollow cellular structure, and the subsequent cell death reaction leads to massive tissue damage and eventually the death of hybrid plants.

To examine whether ROI are involved in this cell death reaction, the effects of DPI and catalase, a potent inhibitor of plasma membrane NADP(H) oxidase and scavenging enzyme of H_2O_2, respectively, on the cell death process were analyzed. The gener-

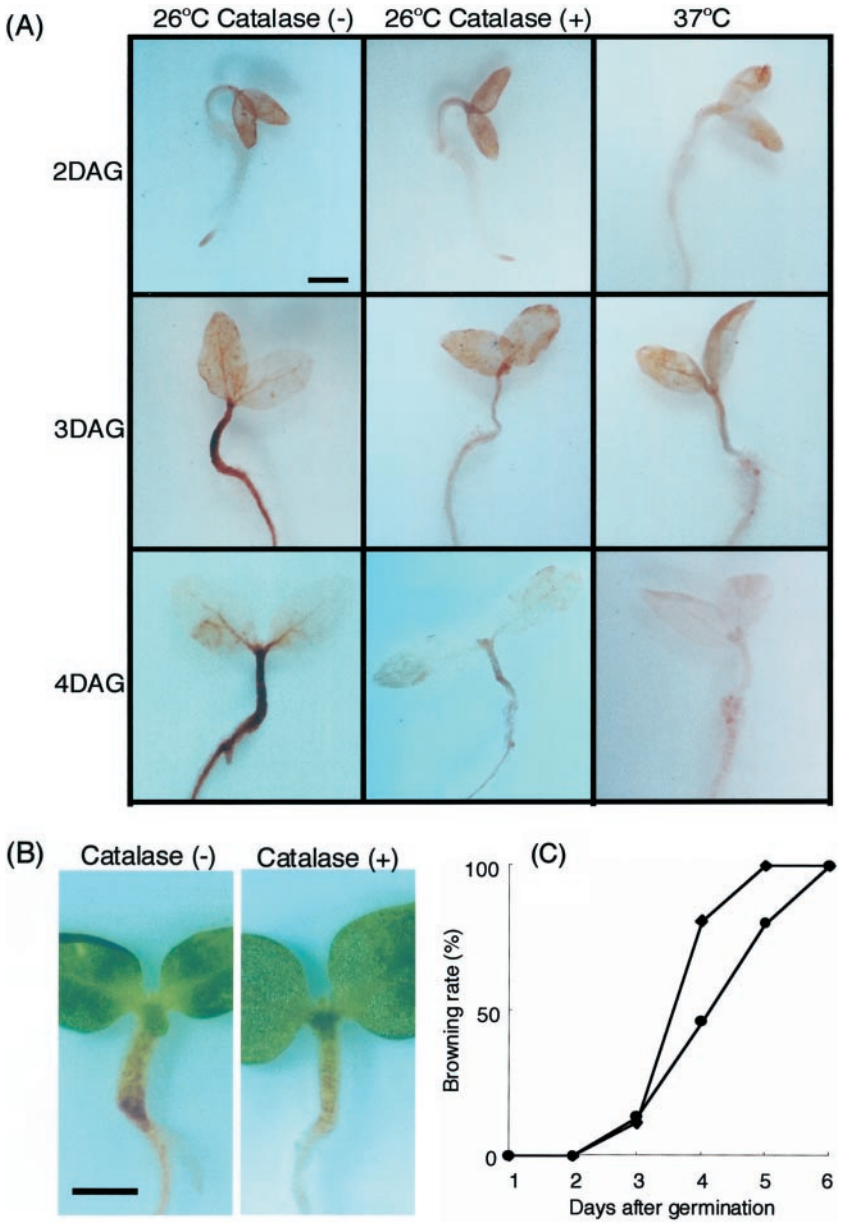

Figure 9. Detection of H_2O_2 in F_1 hybrid seedlings. A, Accumulation of H_2O_2 in seedlings grown for 2, 3, and 4 DAG at 26°C with or without catalase treatment and at 37°C. B, Browning in hypocotyl of F_1 hybrid seedlings at 4 DAG grown at 26°C with or without catalase treatment. C, Effect of catalase on the browning rate of the F_1 hybrid seedlings. ♦, Control; ●, catalase (5.2 units μL^{-1}). Bars = 1,000 (m, and all panels in A and B, respectively, are the same magnification.

ation of O_2^- by photosynthesis is not a factor in the cell death of these hybrids. DPI has been shown to inhibit effectively plant cell death reactions mediated by the generation of reactive oxygen species (Overmyer et al., 1991; Jabs et al., 1996; Orozco-Cardenas et al., 2001). The results of the present study indicated that the generation of O_2^- in the basal hypocotyl and the process of cell death as monitored by the rate of browning was suppressed by DPI treatment, suggesting the involvement of O_2^- in the reaction (Fig. 8, D and E). This is further supported by the results obtained by comparing the rate of evolution of O_2^- in the basal hypocotyl of plants grown at 26°C and 37°C (Fig. 8, B and C). The increase in O_2^- followed by a cell death reaction in the basal hypocotyl bears resemblance to oxidative bursts, which play a central role in the development of host cell death during the HR (Grant and Loake, 2000). However, DPI could not suppress the reaction completely, even though it inhibited the generation of O_2^- (Fig. 8, D and E). The establishment of defense mechanisms against a pathogen through HR require the presence or accumulation of H_2O_2 (Hammond-Kosack and Jones, 1996; Ono et al., 2001). These observations suggest that H_2O_2 plays an important role in the process of local cell death reaction. In keeping with these observations, our time-course study demonstrated that H_2O_2 accumulate in the hypocotyl of the plants grown at 26°C but not at 37°C (Fig. 9A). Although it effectively reduced the generation of H_2O_2, the treatment of catalase did not completely inhibit browning process (Fig. 9, B and C). These results indicated that

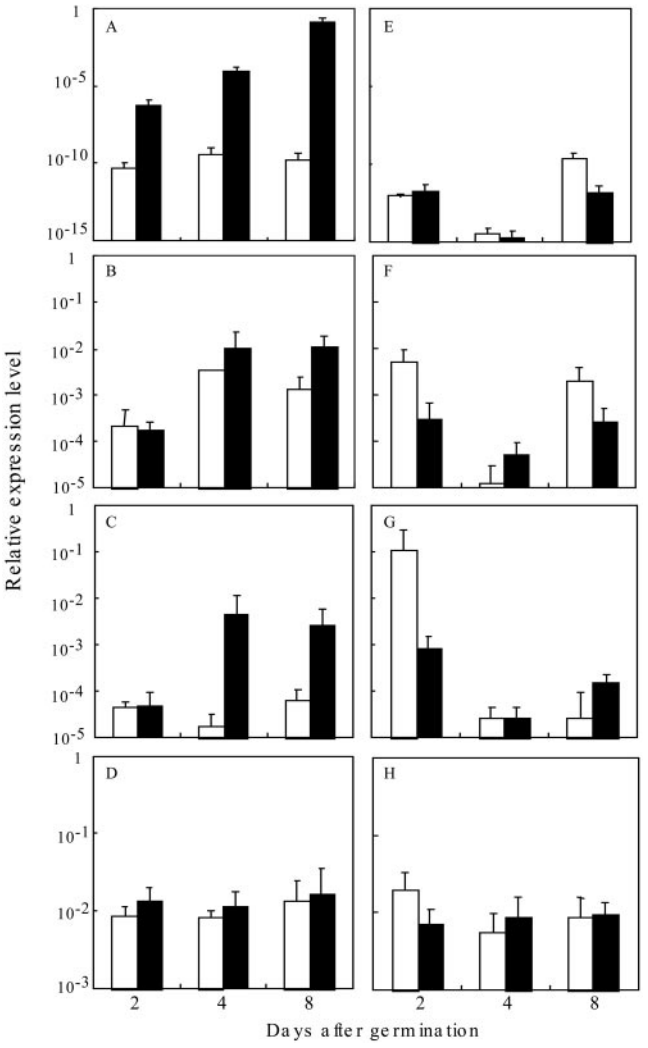

Figure 10. Induction of stress response marker gene expression. A to D, F_1 hybrid; E to H, *N. tabacum*. A and E, *PR1a*; B and F, *PR5*; C and G, *PI-II*; D and H, *MnSOD*. White and black columns represent the data at 37°C and 26°C, respectively. The level of expression for each gene is given relative to the amount of mRNA for the *Ubiquitin* gene, taken as 1. Vertical bars represent SE.

ROI might not participate throughout the sequence of cell death in the hybrid seedlings. In TE PCD of zinnia cultured cells, the oxidative burst is not involved in cell death (Fukuda, 2000).

The expression of molecular marker genes expands the observed phenotypes of cell death. An accumulation of the mRNA encoding *PR1a* and *PR5*, beginning before any sign of cell death reaction, was found in the hybrid plants grown at 27°C (Fig. 10). *PR5* was also expressed but in a developmentally regulated fashion, whereas *MnSOD* expression was not regulated developmentally or environmentally. Hybrid plants grown at 26°C still have green cotyledons at 8 DAG, and the extracted RNA was well integrated. Thus, signaling events subsequent to the cell death reaction in the hypocotyl lead to a general induction of the expression of genes associated with the establishment of SAR in living cells (Ohashi and Ohsima, 1992; Seo et al., 1997). The generation of O_2^- does not induce expression of *MnSOD* gene, one of the scavenging system of ROI. In the present experiments, we did not monitor the genes of proteins for other scavenging systems or maintenance of the cellular redox balance. Further study to analyze these genes is required.

In conclusion, we showed here that cell death during the expression of hybrid lethality in the F_1 of *N. gossei* and *N. tabacum* is a developmentally and environmentally regulated reaction. The results suggest that the breakdown of normal compartment in the cell lead to a quick and broad cell death reaction in the tissue. Initiation of the reaction induces a loss of homeostasis in the cells of the hybrid and eventually acts as a reproductive isolation mechanism among the species. What is an actual cause of this death reaction? We suggest the possibility that a putative transmissible death factor is involved in the execution of this reaction. Additional work is needed to test this hypothesis, e.g. by performing grafting experiments using lethal hybrids as stock and healthy plants as scion.

MATERIALS AND METHODS

Plant Materials and Growth Analysis

All seeds used were collected from plants grown in the same year in a greenhouse and stored at 4°C under dry conditions. Surface-sterilized seeds of *Nicotiana tabacum* L. cv Bright-Yellow 4 and F_1 hybrids (*N. gossei* Domin × *N. tabacum* L. cv Bright-Yellow 4) were sown on Murashige and Skoog medium supplemented with Suc (30 g L^{-1}) and Gelrite (2 g L^{-1}; Sigma-Aldrich, St. Louis), pH 5.8. When the radicles emerged (0 DAG), 10 germinating seeds were transferred onto the Murashige and Skoog medium in a sterile plastic container (14 × 10 × 1.5 cm). The culture was carried out at 26°C or 37°C with lighting (approximately 60 μmol m^{-2} s^{-1}). The fresh weight, height, root length, and leaf area of the 20 plants were determined. Leaf area was obtained as the product of the long and short axes of top view of the plants.

Microscopic Techniques

To observe of brown pigment in the tissue, the plants were soaked in a mixture of chloral hydrate:glycerol:distilled water (8:1:2, v/v) for 12 h to clear the tissue. Dead cells or tissues were detected by dye-staining methods. The roots were stained with a 0.25% (w/v) Evans blue solution for 30 min under reduced pressure and then washed extensively with distilled water. Leaves and hypocotyls were stained with trypan blue by the method of Bowling et al. (1997). Shoot tips were incubated in a 0.01% (w/v) FDA solution and viewed under a fluorescence microscope (BX60 Orympas, Tokyo) using a ×10 objective.

To localize lignin, samples were incubated in a phloroglucinol-HCl solution, and pressed softly between a slide and coverslip for observation. To prepare transverse sections, the plants were embedded in a 5% (w/v) melted agar solution at 40°C and cooled on ice for 1 h. The specimens were prepared as 50-μm-thick section using a microslicer (DTK-1000, Dohan-EM, Kyoto).

After the samples had been fixed in Formalin:acetic acid:50% [v/v] ethanol (5:5:90) solution, dried (critical point dryer HCP-1, Hitachi, Tokyo), and coated in gold (ion coater IB-3, Eiko, Ibaragi, Japan), SEM (JXW840, JEOL, Tokyo) observations were performed.

Ion Leakage

To prevent the medium from adhering to the plants and causing high background values, the plants were grown on a sterile nitrocellulose filter attached to the Murashige and Skoog medium. Five plants were removed from the culture, washed three times with deionized water, and incubated in 1 mL of deionized water (0.3 μs cm^{-1}) for 3 h at room temperature. The amounts of ion released into the water before and after autoclaving were measured by conductivity meter (Twin cond B-173, Horiba, Kyoto). The experiments were repeated five times.

Detection and Determination of ROI Accumulation

The accumulation of superoxide radical (O_2^-) in the plants was detected by blue-formazan precipitation with 0.1% (w/v) nitroblue tetrazolium (Wako Pure Chemical Co., Osaka), as reported by Jabs et al. (1996), with a 30-min incubation. The amount of superoxide accumulated in the basal hypocotyl was determined by MCLA (Tokyo kasei Kogyo Co., Tokyo)-dependent chemiluminescence using a two-dimensional photon-counting system (ARUGUS-50, Hamamatsu Photonics) as described by Ogawa and Iwabuchi (2001). Detection of H202 was carried out according to the methods described by Thordal-Christensen et al. (1997).

Treatment with DPI and Catalase

Various concentrations of DPI, a potent inhibitor of NAD(P) H oxidase and other flavin-containing oxidases (Cross and Jones, 1986), were added to the medium after autoclaving to inhibit the production of cellular O_2^-. The seedlings at 0 DAG were transplanted on to the medium, and the plants showing brown pigment in the hypocotyl were subsequently enumerated.

Twenty microliters of 5.2 units μL^{-1} catalase (bovine liver, Wako Pure Chemical Co.) was added to each seedling, and the materials were placed under the reduced pressure to accelerate the diffusion of the enzyme into the tissue. The treatment was carried out every 12 h during the experiments.

Gene Analysis

Ten to 20 seedlings grown at 26°C or 37°C were harvested at 2, 4, and 8 DAG in liquid nitrogen and stored at −80°C until use. The methods employed for the extraction of total RNA and the synthesis of cDNA were described elsewhere (Oka et al., 2001). Amounts of mRNA were determined by real-time quantitative RT-PCR using the Taq-Man PCR system and the computer program Sequence Detector (v1.6.3, PE Applied Biosystems, Foster City, CA). The nucleotide sequence of each tobacco gene was obtained from the DNA data bank of Japan database; *PR1a* (D90196), *PR5*; thaumatin-like protein (X15223), *PR6*; proteinase inhibitor-II (Z29537); *MnSOD* (X14482); and *Ubiquitin* (U66264). The specific sets of primers and probes used were designed using the computer program Primer Express I 1.0 (PE Applied Biosystems); set A for *PR1a*, set B for *PR5*, set C for *PR6*, set D for *MnSOD*, and set E for *Ubiquitin*. set A (primers, 5′-TGG CTG CAG ATT GTA ACC TCG T-3′ and 5′-AAA TCG CCA CTT CCC TCA GCT-3′; probe, 5′-CAT TCT CAT GGT CAA TAC GGC GAA AAC C-3′), set B (primers, 5′-CAA TTG CAA CTT CGA TGG CA-3′ and 5′-TGA CAC TCT AGC ATC CCG TTA CA-3′; probe, 5′-TGG CCG AGG TAA TTG TGA GAC TGG AGA-3′), set C (primers, 5′-TGT ACT ACG GAA TGT GAC CCT AGA GT-3′ and 5′-AAC CCT TGT CTG CGT TAC AAC A-3′; probe, 5′-CTA CCA TAA GTT GCC CGT TTT CTG GAT TGG-3′), set D (primers, 5′-CCA TTT CCA AAG GAG ATG CTC C-3′ and 5′-CCT CCG CCG TTG AAT TTG A-3′; probe, 5′-CCG TCG CCA AAT TGC ATA GCG CT-3′), set E (primers, 5′-CCA GAA AGA GTC AAC CCG TCA C-3′ and 5′-AAC GAC ATC AAC AAC AGG CAA C-3′; probe, 5′-TTG TCC TCC GTC TCC GTG GTG GTT TCT A-3′). The amounts of mRNA of each gene were normalized by the amount of *Ubiquitin* mRNA measured as an internal standard.

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