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Ultrastructural Changes During Programmed Cell Death of Tobacco Leaf Tissues Infected with *Tobacco mosaic virus*

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Tobacco (*Nicotiana tabacum* cvs. Xanthi-nc and NC 82) plants infected with *Tobacco mosaic virus* (TMV) were examined ultrastructurally. Local lesions produced by TMV were sunken and withered. The plants were subjected to temperature shift (TS), a method to produce programmed cell death (PCD), by placing the infected plants initially at high temperature (35°C) for 2 days and then shifting them to greenhouse temperature (22-27°C). As a result, expanded lesions around the original necrotic lesions were produced. The expanded area initially had no symptoms, but it withered and became necrotic 15 h after TS. No ultrastructural changes related to PCD were noted at 0 h after TS in Xanthi-nc tobacco tissues as well as in healthy and susceptible tobacco tissues infected with TMV. At 6 h after TS, chloroplasts were convoluted and cytoplasm began to be depleted; however, no necrotic cells were found. At 17 h after TS, ground cytoplasm of affected cells was completely depleted and chloroplasts were stacked together with bent cell wall or dispersed in the intracellular space. Necrotic cells were also observed, containing virus particles in the necrotic cytoplasm. There were initially two types of symptoms in the expanded lesions: chlorosis and non-chlorosis (green). Abundant TMV particles and X-bodies were only found in the chlorotic tissue areas. These results suggest that PCD by TMV infection may start with the wilting of cells and tissues before necrotic lesion formation

Keywords **:** cell death, chlorosis, necrosis, *Tobacco mosaic virus*, ultrastructure.

Tobacco mosaic virus (TMV) is a well-known virus belonging to the genus *Tobamovirus*, found all over the world where tobacco plants are grown. The most characteristic symptoms in tobacco are: mosaic, which consists of various degrees of mottling; chlorosis; curling; distortion; and dwarfing of leaves, flowers and the entire plant (Agrios, 1997; Gooding, 1986). In some tobacco cultivars resistant to TMV, necrotic lesions develop on the inoculated leaves. In plant cells with mosaic symptoms, layers of TMV particles are accumulated in paracrystaline arrays, and aggregated cell constituents with or without virus particles form X-bodies (Edwardson and Christie, 1986; Lucas, 1975).

TMV accumulation occurs not only in susceptible tobacco plants but also in resistant plants with the N gene when the plants are placed in high temperatures (above 28°C) (Kim et al., 1996; Lucas, 1975). However, at lower temperature, viral multiplication is restricted to a few cells around the initial infection site probably not by the inhibition of multiplication but by the inhibition of viral spread (Kim et al., 1996). Even in the necrotic cells, TMV continue to reproduce for a short time.

The formation of necrotic lesion on tobacco leaves in resistant plants is a controlled cellular suicide that is an important process occurring during incompatible pathogen interactions. Programmed cell death (PCD) can be readily observed in the necrotic cells (Pennell and Lamb, 1997). Although several biochemical and morphological parameters have been described for various types of cell death in plants, the relationships existing between those different types of PCD events remain unclear. This phenomenon could correspond to the establishment of a second barrier against pathogens.

The hypersensitive response (HR) is induced by certain plant pathogens and involves PCD to restrict the spread of pathogens from the infection site. Concurrent with the induction of cell death, the host activates a defense response. The cell death associated with the HR in several plant-pathogen systems has morphological similarities with animal apoptosis, which indicates that cell death mechanisms in plants and animals may share common components that lead to similar cellular events.

Some of the morphological and biochemical events that accompany PCD during the HR of tobacco plants infected with TMV have been studied. In the study of Mittler et al. (1997), condensation and vacuolization of the cytoplasm

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and cleavage of nuclear DNA to 50 kb fragments were observed. A unique aspect of PCD during the hypersensitive response of tobacco to TMV involved an increase in the amount of monomeric chloroplast DNA. Gross changes in nuclear morphology and significant chromatin cleavage followed. However, Mittler et al. (1997) only studied the ultrastructures of PCD at 1 and 2 days after PCD induction by temperature shift of TMV-infected tobacco plants. Initial structural phenomena before these periods have not been studied. Therefore, in this study, some morphological changes related to earlier phenomena of cell death were examined by electron microscopy of tobacco cells. Also, some cytopathic phenomena implemented in chlorosis and necrosis were investigated to understand the viral symptomatology.

Materials and Methods

Plants and TMV inoculation. Tobacco plants (*Nicotiana tabacum* cvs. Xanthi-nc and NC 82) were planted in plastic pots (8 cm in diameter) and grown until 8-10 leaf stages. Xanthi-nc is a resistant cultivar, while NC 82 is a susceptible cultivar to TMV. Tobacco plants were mechanically inoculated with 1000 x dilution of TMV-diseased NC 82 tobacco leaf sap in 0.01 M phosphate buffer, pH 7.0, using cotton swab after dusting 600-mesh carborundum. The inoculated plants were placed at about 27°C at daytime and at 22°C at nighttime in a greenhouse.

Scanning electron microscopy (SEM) of local lesion. Four days after inoculation in the greenhouse, the resistant plants showing necrotic local lesions on the inoculated leaves were sampled and fixed with Karnovsky's fixative in 0.01 M cacodylate buffer (pH 7.2) for 2 h, and post-fixed with 1% osmium tetroxide in the same buffer for another 2 h. The fixed specimens were dehydrated in an ethanol series, dried in a critical point dryer, and sputter-coated with gold before examining under a scanning electron microscope (DSM 960A, Zeiss, Germany).

Transmission electron microscopy (TEM) of healthy tobacco (Xanthi-nc) and TMV-infected susceptible tobacco plants (NC 82). The leaves of the susceptible plant (*N. nicotiana* cv. NC 82) with mosaic symptoms induced by TMV (7 days after inoculation) were cut in pieces and used as specimen for the TEM study. Also, healthy Xanthi-nc tobacco leaves were prepared for TEM. The leaf tissues excised were fixed with Karnovsky's fixative in 2% paraformaldehyde and 2% gulutaraldehyde in 0.05 M cacodylate buffers at pH 7.2, washed in the same buffer, and post-fixed with 1% osmium tetroxide for 2 h. The fixed samples were washed briefly in distilled water and stained *en bloc* with 0.5% uranyl acetate overnight. These were then dehydrated in an ethanol series (30%, 50%, 70%, 80%, 95%, and 100%), and embedded in Spurr's epoxy resin (Spurr, 1969). Ultrathin sections of 80-90 in thickness were made with a diamond knife. The sections were stained with uranyl acetate and lead citrate, and observed under a JEM-1010 electron microscope (JEOL, Japan) at 80 kV.

Temperature shift (TS) for induction of resistance in newly expanded region at high temperature (35°C). Three days after inoculation, the resistant Xanthi-nc tobacco plants showing necrotic local lesions on the inoculated leaves were transferred into a growth chamber with fluorescent light, illuminated 12 h a day at 35°C, and kept for 48 h. The heat-treated plants were again moved to the greenhouse, known as temperature shift (TS), for the induction of resistant response (or necrosis formation) of the expanded region at high temperature. At first, there were no visible symptoms in the expanded region around the local lesion previously formed by the viral infection. However, about 15 h after inoculation the surrounding expanded area became withered and necrotic.

Light and electron microscopy of tobacco cells after TS. In the resistant tobacco plants treated as above, symptom-expanded leaf tissues surrounding the local lesion were excised 0, 6, and 17 h after TS (ATS), which corresponded to: 0 h ATS = no pathogenesis-related (PR) gene was induced and no necrotic response occurred; 6 h ATS = PR gene induced, but no necrosis formed; and 17 h ATS = PR gene induced and necrosis formed (Kim et al., 1996). At 0 ATS, there were two types of symptoms around the local lesions: light green or yellow (chlorotic) area and normal green area. These two types of tissues were sampled separately.

Sample preparation was the same as described above. Embedded materials were semithin-sectioned 1-2 μm in thickness, and stained with toluidine blue O (Harris, 1978) before observation under the light microscope (Axiophot, Zeiss, Germany). Also, ultrathin sections 80-90 nm in thickness were made with a diamond knife. The sections were stained with uranyl acetate and lead citrate, and observed under the JEM-1010 electron microscope.

Results

Scanning electron microscopy of local lesions. Local lesions induced by TMV had sunken regions in the center (Fig. 1A), which were necrotic. Also, the surrounding area around the necrotic center was sunken. Between the center and periphery sunken areas, there was a boundary made up of relatively raised tissue. Healthy adaxial tobacco leaf surface consisted of epidermal cells and guard cells without subsidiary cells (anomocytic configuration of stomatal complex) (Fig. 1B). The surfaces of lesion areas were shrunken, having distorted epidermal cells (Fig. 1C, D). The surface shrinkage was more pronounced in the center than on the periphery of the lesion. In the center of the lesion, guard cells were also severely distorted (Fig. 1D).

Light microscopy. Tobacco plants (cv. Xanthi-nc) infected with TMV were placed at 35°C in an incubator for 2 days, and shifted to lower normal temperature (22-27°C). Initially, the expanded area surrounding the local lesion had no visible necrotic symptoms but showed necrotic and wilting symptoms 15 h after TC, indicating that the dis-

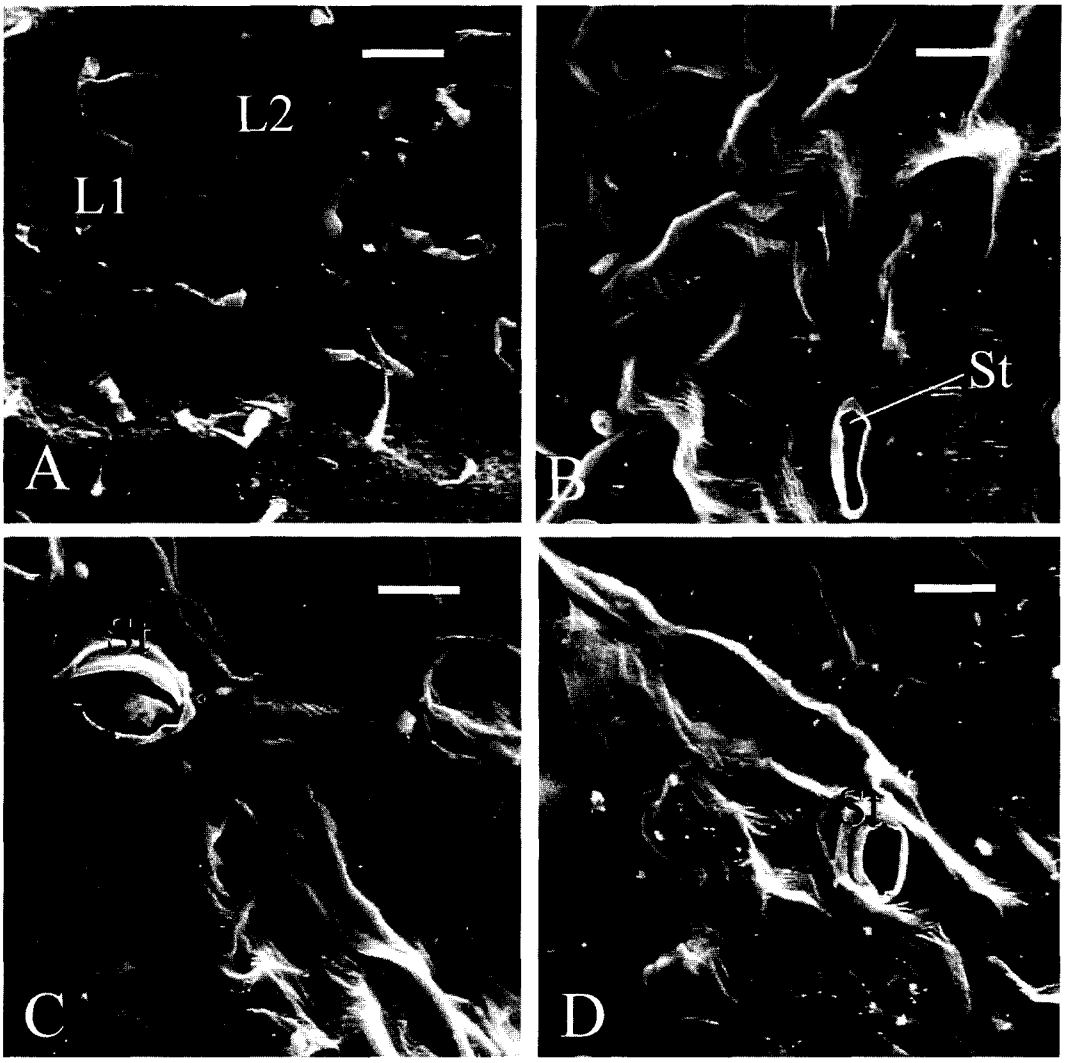

Fig. 1. Scanning electron microscopy of tobacco (cv. Xanthi-nc) leaf surface. (**A**) Local lesion formed by Tobacco mosaic virus (TMV) infection 4 days after inoculation. L1: necrotic lesion initially formed by TMV infection (located at the center of the lesion), L2: necrotic lesion formed later at the periphery. The lesions are sunken relative to the outer healthy area. (**B**) Healthy tobacco leaf surface showing epidermal cells and stoma (St). (**C**) Higher magnification of L2 area of (**A**), showing distorted epidermal cells. The guard cell around the stoma (St) looks intact. (**D**) Higher magnification of L1 area of (**A**), showing that both epidermal cells and stomata are severely distorted. Bars = 200 μm (**A**), 10 μm (**B**, **C**, **D**).

eased area was expanded outward from the original local lesion (Fig. 2A). Some of the expanded areas showed yellowish (chlorotic) symptoms, but most had normal green color, both of which were wilted and became necrotic later.

In LM, the expanded area formed at high temperature had no noticeable alteration of the leaf tissue 0 h after TC, and had normal-looking chloroplasts and nuclei (Fig. 2B). However, 6 h after TC, most chloroplasts were distorted and convoluted, sometimes becoming spherical, but nuclei were still intact (Fig. 2C). At 17 h after TC, necrosis was formed, nuclei and chloroplasts became dense, and chloroplasts sometimes were stacked together (Fig. 2D, E).

Transmission electron microscopy. In healthy plant tissues of Xanthi-nc NN treated with high temperature, cellular features appeared to be normal, having intact cytoplasm with healthy-looking chloroplasts and mitochondria (Fig. 3A, B). Chloroplasts and mitochondria contained well-developed membrane structures, grana and cristae. Other cellular organelles such as microbodies and endoplasmic reticulum (ER) also appeared intact (Fig. 4B). Lipid globules were often found in the cytoplasm.

TEM of susceptible tobacco plants (NC 82) infected with TMV showing mosaic symptoms revealed the accumulation of TMV particles in mesophyll cells (Fig. 3C, D).

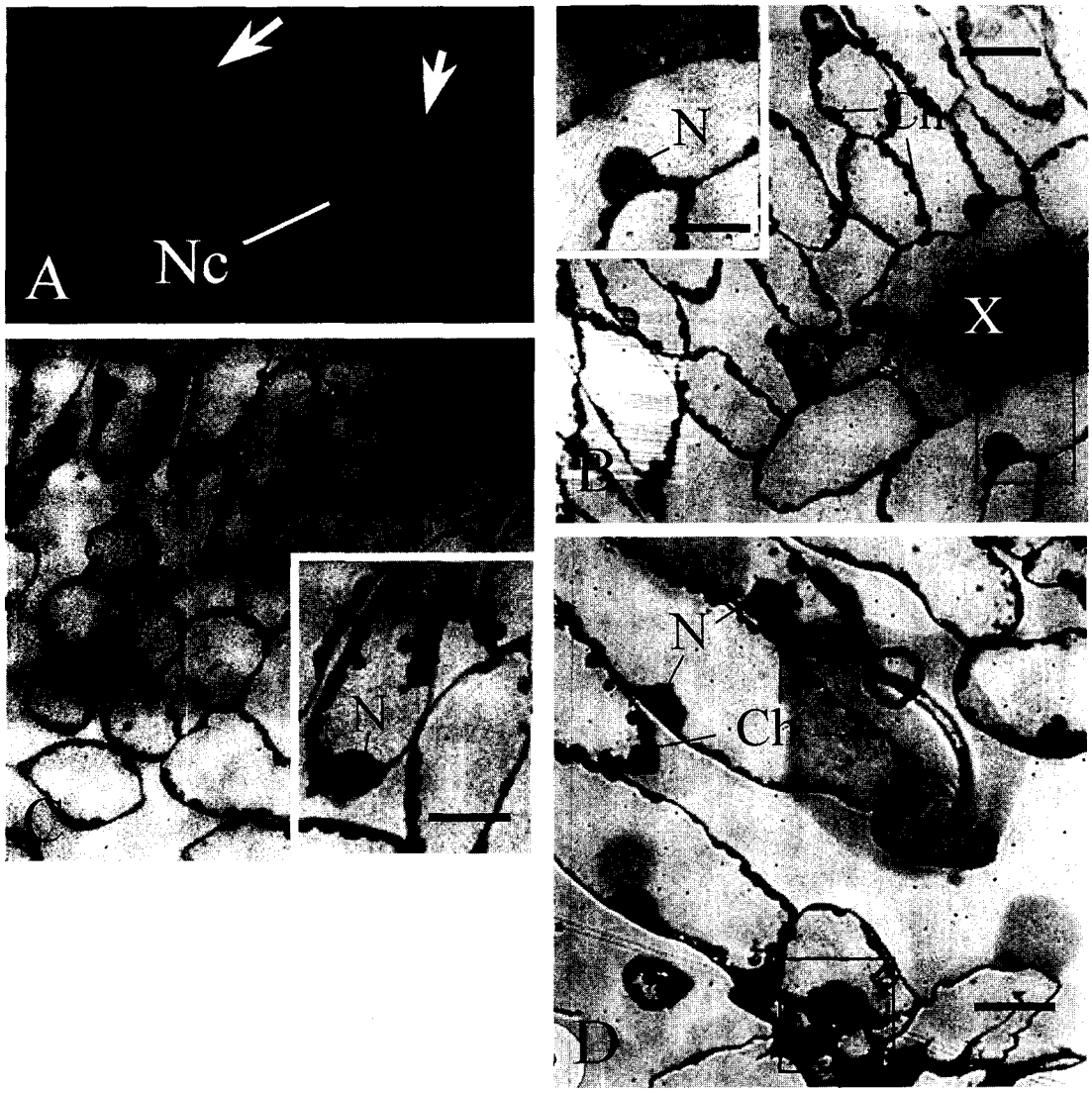

Fig. 2. Infection area expansion (outer lesion, arrow) around the original local lesion (Nc, necrosis) on the Xanthi-nc tobacco leaf at high temperature for 2 days (**A**) and light microscopy of the expanded area at 0 (**B**), 6 (**C**), and 17 h (**D**) after temperature shift (TS) from high to normal temperature following the high temperature treatment for 2 days. Note spherical chloroplasts 6 h (inset of **B**) and necrosis (Nc) 17 h after TS. Insets are higher magnifications of rectangular areas. Rectangle in **D** shows necrosis, nucleus, and chloroplasts associated with the nucleus. N: nucleus, Ch: chloroplast, X: Xylem vessel. Bars = 20 μm (**B**, **C**), and 10 μm (**D** and insets of **B** and **C**).

Oftentimes X-bodies were also accumulated adjacent to virus aggregates (Fig. 3D). The cytoplasmic features were intact, having normal-looking chloroplasts and mitochondria as in healthy cells. Central vacuole was intact, sometimes containing virus particles. Nucleus was also intact.

Tobacco leaf tissues (Xanthi-nc) around the necrotic lesions, in which TMV infection occurred at high temperature, were sampled at 0, 6, and 17 h after TS. At 0 h after TS, ultrastructures of tobacco cells of the expanded regions showed no signs of cell death, regardless of chlorosis and non-chlorosis (Fig. 4). The organelles of nucleus, chloroplasts, and mitochondria, and cytoplasm were intact. In the non-chlorotic region, no virus particles were observed (Fig. 5A), while in the region showing yellowish (chlorotic) symptoms, virus particles were abundantly accumulated in the cytoplasm and/or in the vacuole. Also, X bodies were readily observed in the infected cells (Fig. 4B, C, D, E). The inclusion bodies were composed of double membrane structures (X tubules), and sometimes semi electron-dense materials were accumulated (Fig. 4D).

At 6 h after TS, no visible symptoms were developed on the expanded region. However, two remarkable structural changes different from those of 0 h after TS were observed:

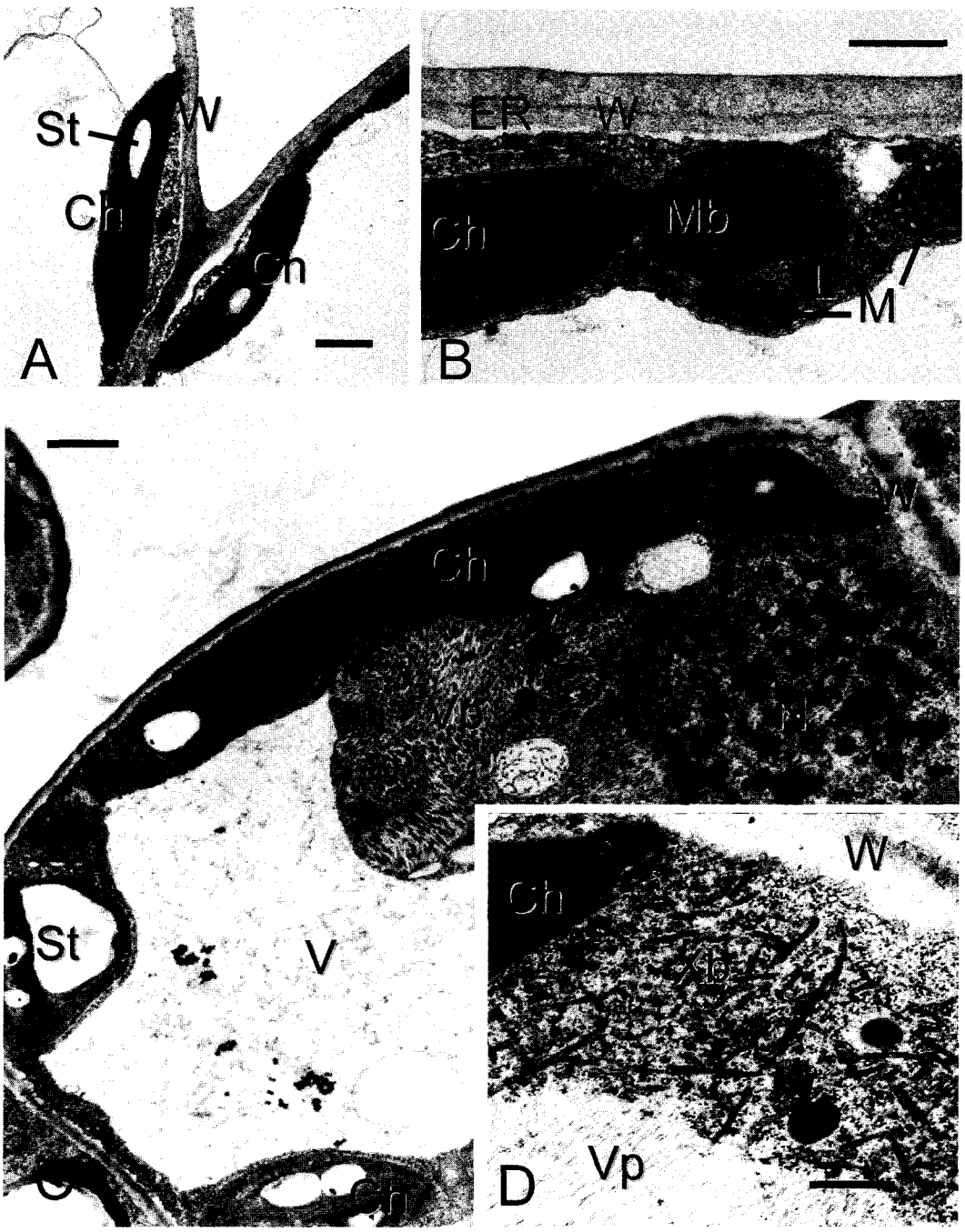

Fig. 3. Electron microscopy of healthy tobacco (Xanthi-nc) (**A**, **B**) and TMV-infected susceptible tobacco plants (NC 82) (**C**, **D**) with mosaic symptoms. Note intact cytoplasm containing chloroplasts (Ch) with well-developed grana and mitochondria (M) in **A**. Microbodies (Mb) and lipid globules (L) were often found in the cytoplasm of **B**. **C**) Infected mesophyll cells, showing proliferated virus particles (Vp) in normal-looking cytoplasm. Chloroplasts (Ch) and nucleus (N) appear to be intact. **D**) Higher magnification of another section of (**C**), showing proliferation of virus particles and X- bodies (Xb). Arrow: unknown inclusion in X-body and infected cytoplasm, V: vacuole, W: cell wall. ER: endoplasmic reticulum. Bars = 1 μm (**A**, **B**) and 0.5 μm (**C**, **D**).

first, convolution of chloroplasts; and second, depletion of cytoplasm (Fig. 5A, B, D). Chloroplasts contained developed grana and often increased lipid globules or plastoglobuli (Fig. 5A, D). In the chlorotic tissue, grana were little developed (Fig. 5D). Virus particles were observed in the yellowish region with some indication of cellular degeneration (Fig. 5C), which might lead to necrosis. Nucleus in the affected cells appeared to be intact (Fig. 5B).

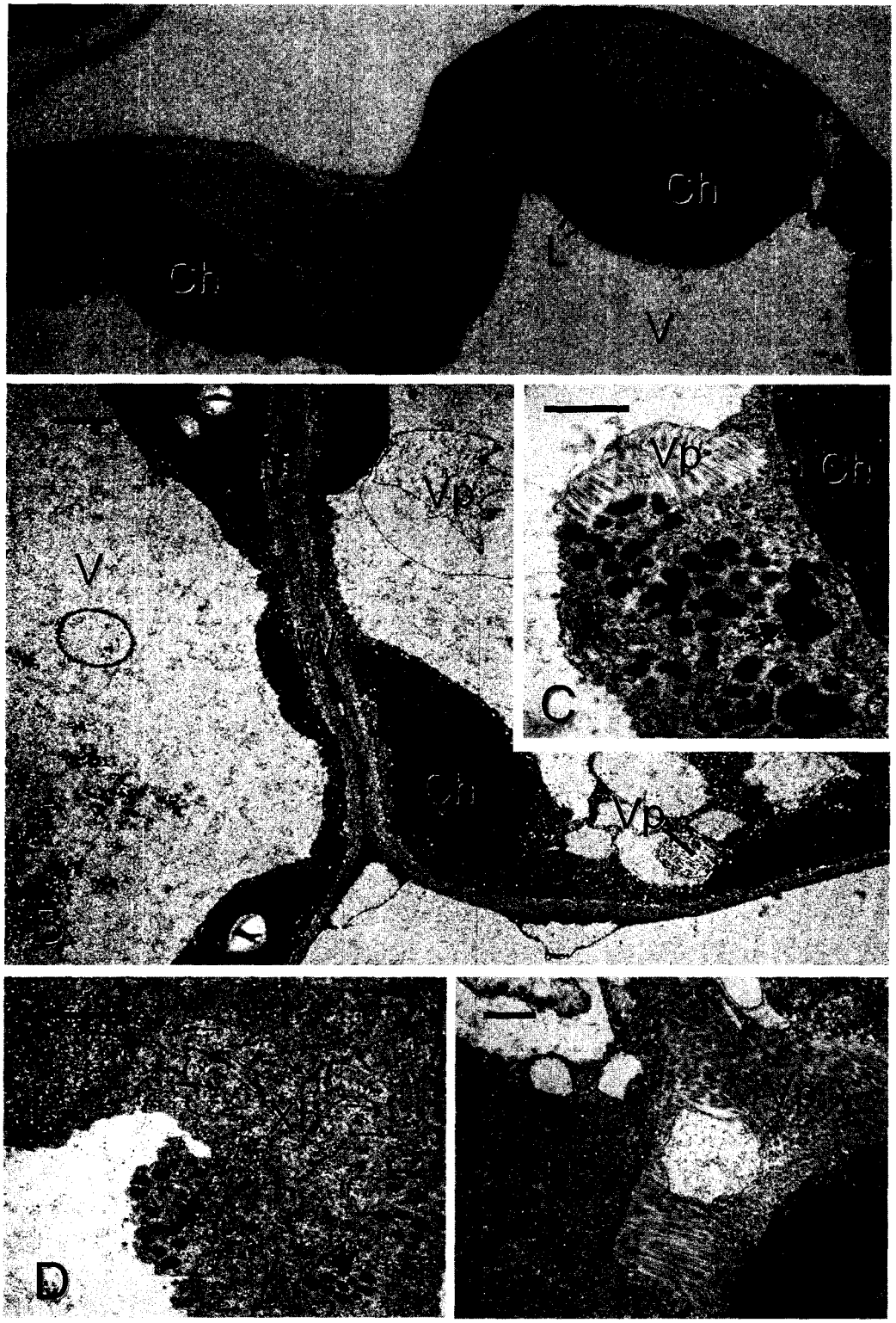

Fig. 4. Electron microscopy of expanded tissues at high temperature around the local lesions induced by TMV infection in the resistant tobacco plant (cv. Xanthi-nc) at 0 h after temperature shift (TS) for the induction of programmed cell death. **A**) Non-chlorotic expanded area with no or few virus particles found in the cell. Chloroplasts (Ch) have well-developed grana, and cytoplasmic depletion is minimal. **B-E**) Chlorotic area, showing intact cytoplasm containing aggregated virus particles (Vp) and normal-looking chloroplast (Ch) with membraneous grana. **C**) Infected cell, showing aggregated virus particles (Vp), and unknown inclusion bodies (arrow) formed in association with virus particles. X-bodies were also formed in the cells of the chlorotic area (**D**). Nucleus (N) and chloroplasts look intact. W: cell wall. Bars = 1 μm (**A**, **B**, **D**) and 0.5 μm (**C**, **E**).

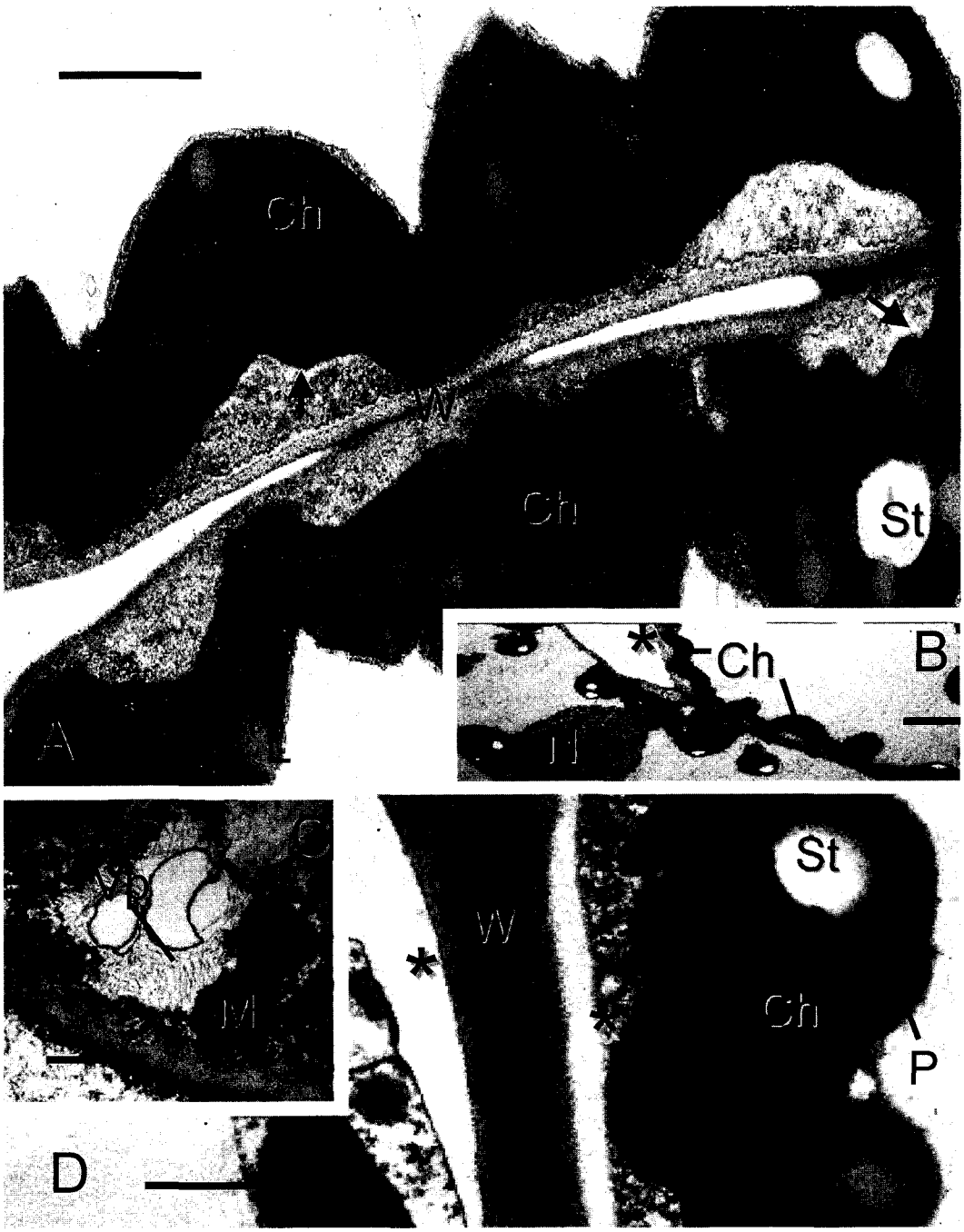

Fig. 5. Electron microscopy of chlorotic (**A**, **B**) and non-chlorotic (**C**, **D**) tissues of tobacco plants (cv. Xanthi-nc) infected with TMV at 6 h after induction of programmed cell death (PCD) by temperature shift (TS). (**A**, **B**) Infected cells showing convoluted chloroplasts (Ch) (arrow), showing no virus particles in the cells. Nucleus (N) appears to be normal. Cells are somewhat shrunken, and shizogenous intercellular space (asterisk in **B**) is noted. (**C**) The infected cell of chlorotic tissue showing virus particles (Vp) and degenerated cytoplasm (arrow). (**D**) Infected cells with PCD showing distorted chrlorplasts (Ch) and separation of plasmalemma from cell wall (W) (asterisk). M: mitochondria, St: starch granule. Bars = 0.5 μm (**A**, **C**, **D**) and 2 μm (**B**).

At 17 h after TS, when visual symptoms were observed in the expanded regions, chloroplasts were highly convoluted, often grouped together to form a complex that contained cytoplasmic remains inside or dispersed separately in degenerated intracellular space (Fig. 6A, B). However, chloroplasts had intact membrane structures, grana. Another type of structural change was cellular degeneration involving cytoplasmic degradation and necrosis (Fig. 7C). Virus

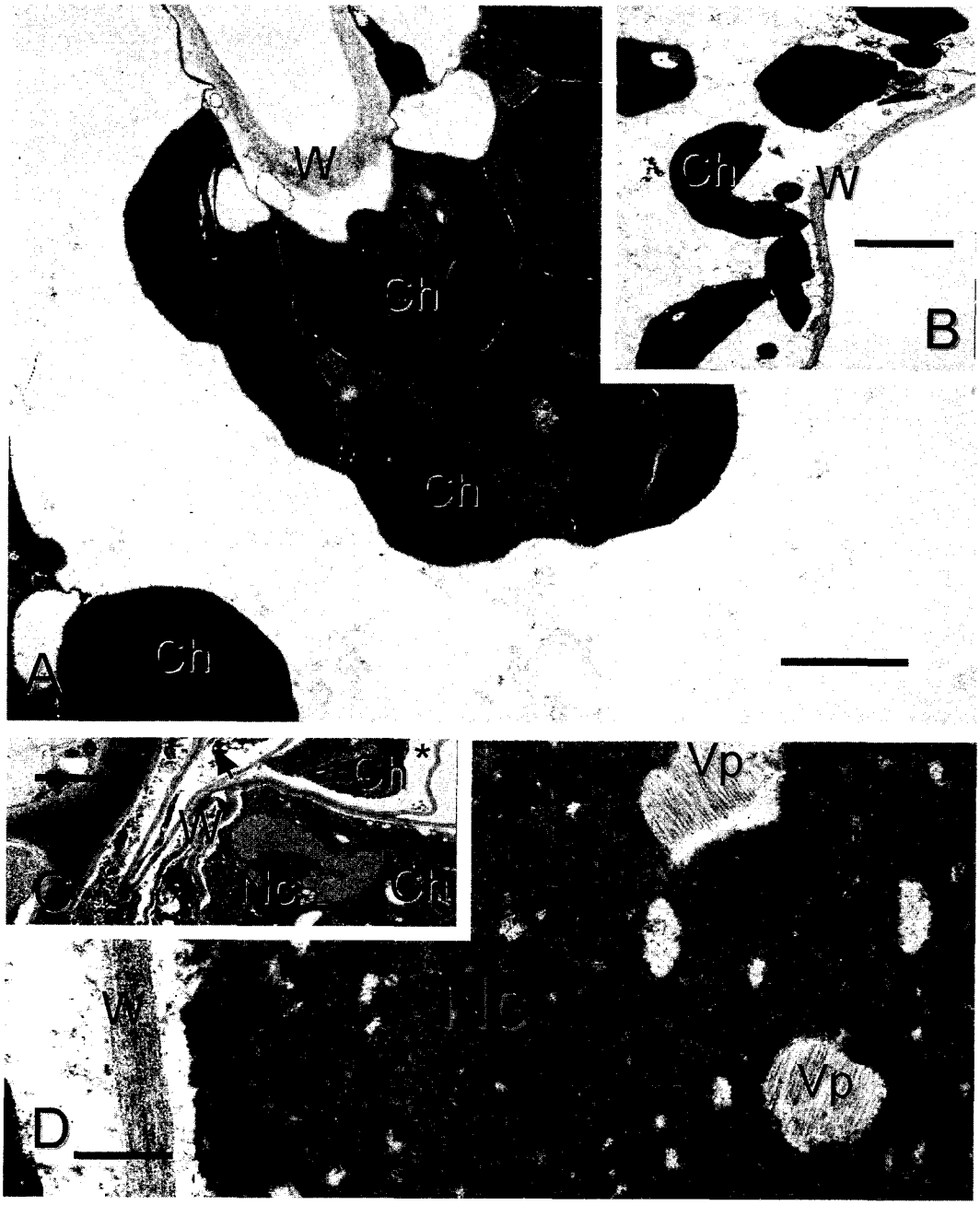

Fig. 6. Electron microscopy of withered and necrotic tissues of tobacco plants (cv. Xanthi-nc) infected with TMV at 17 h after induction of programmed cell death (PCD) by temperature shift (TS). (**A**, **B**) Cells of withered tissue showing bent cell wall (W) and stacked chloroplasts (CH) or dispersed chloroplasts. In A, cytoplasm appears to be condensed among the stacked chloroplasts. (**C**, **D**) Infected cells showing necrotic cytoplasm (Nc) containing virus particles (Vp). Bars = 0.5 μm (**A**, **D**) and 1 μm (**B**, **C**).

particles were accumulated in the necrotic cytoplasm (Fig. 7D).

Discussion

SEM, LM and TEM of the TMV-infected cells under PCD and dead cells by PCD showed that cell shrinkage occurred during cell death. SEM showed that lesions formed by TMV infection were depressed to form cave-ins on leaf surface, and their cells were shrunken and distorted. Also, these infected tissues were drier than uninfected normal tissues, and became brittle. Collapse of infected tissues is a common phenomenon during PCD and in HR, in which cells collapsed and became smaller following condensation

of cytoplasm (Mittler et al., 1997). In this study, initial symptoms after TS on the expanded area around the necrotic lesion appeared to be wilting, which indicates that the infected tissues may be deprived of water.

The loss of water was also brought about by structural changes of the expanded area after TS, showing the flinching of cytoplasm that may lead to the convolution of chloroplasts. The ultrastructural change was first observed 6 h after TS. Crooked cell wall and stacked chloroplasts inside extremely flinched cytoplasm at 17 h after TS supported the cells under PCD, and HR may have been due to deprivation of water causing the cells to wilt rapidly. Cytoplasmic mass including inconspicuous organelles was congregated, and other areas in the affected cell were almost empty with no cytoplasm at 17 h after TS, which also supports the hypothesis. This coincides with the fact that ion leakage of intercellular compartments occurs as an early step leading to PCD (Beligni and Lamattina, 1999).

In the LM of affected leaf tissues, chloroplasts, especially convoluted spherical ones at 6 h after TS, contained large starch granules. These structural features coincide with those in cells infected with TMV during PCD (Goodman and Novacky, 1994; Mittler et al., 1997). Mittler et al. (1997) suggested that the presence of large starch granules might cause breakage of chloroplast DNA during the preparation of samples for field inversion gel electrophoresis, which is an early indicative marker of TMV-induced PCD. Before necrosis formation of resistant tobacco plants infected with TMV, a decrease in the level of DS9 protein in the TMV-infected cells, resulting in a subsequent loss of function of the chloroplasts, accelerates the hypersensitive reaction (Seo et al., 2000). Caspase-like proteolytic activity was detected in tobacco tissues that developed HR following infection with TMV (del Pozzo and Lam, 1998).

Another type of structural change related to PCD was necrosis formation of infected cells. The necrosis formation was not conspicuous by TEM until 17 h after TS when visual necrosis symptoms appeared on the expanded area around the local necrotic lesion previously formed by TMV infection. Infected cells appeared to have been degenerated and to be degenerating 17 h after TS. Virus particles were found mostly in necrotic cells. Cellular degeneration also occurred in non-necrotic cells having distorted cell wall and stacked or dispersed chloroplasts as mentioned above. However, virus particles were seldom observed in these cells. In the study of Mittler et al. (1997), necrosis was not observed and virus particles were not found in tobacco cells with PCD. The fact that cell death related to HR can be activated by certain elicitors in the absence of a pathogen (Hammond-Kosack et al., 1994; He et al., 1993; Levine et al., 1994) suggests that the degenerative structural features may be derived from some factors from adjacent cells infected with TMV. Because phytoalexins including scopoletin accumulate by HR due to TMV infection (Kim et al., 2000), virus production in the cells with PCD may be inhibited. It is not clear, however, whether non-necrotic cells with PCD contain viral RNA or not. Therefore, more studies about the role of TMV infection in PCD are needed in the future.

A viral avirulence gene located in TMV coat protein (CP) was related to eliciting the N gene-mediated HR in *Nicotiana sylvestris* (Culver and Dawson, 1989, 1991, 1994). This suggests that HR symptom expression may be related to the expression of CP protein gene (CP expression). In TMV, discolored (chlorotic) areas of mosaics have much more virus particles in tobacco leaf cells than normal green areas that contain no or only minute amounts of virus particles (reviewed by Matthews, 1981). In this study, the yellowish area formed around the necrotic lesion at high temperature accumulated virus particles and X-bodies, but that of greenish area rarely contained virus particles. This indicates that coat protein gene expression is associated with symptom expression. On the other hand, there is no correlation between the intensity of symptoms and the amount of virus present in the host (Bos, 1978; Matthews, 1981), suggesting that disease symptoms may result not only from competition between the host and pathogen for metabolites, but also from interference of the host functions by virus products.

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