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DNA Laddering during Hypersensitive Cell Death in Cultured Rice Cells Induced by an Incompatible Strain of *Pseudomonas avenae*

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Abstract

Clear 180-bp nucleosomal DNA laddering in cultured rice cells was induced only by an incompatible strain of *Pseudomonas avenae*. This supports the current view that the hypersensitive cell death of a plant is programmed cell death. The flagellin-deficient mutant of the incompatible strain did not induce DNA laddering, suggesting that DNA laddering was mediated by incompatible flagellin perception.

Keywords: Apoptosis, DNA Ladder, Flagellin, Hypersensitive cell death, Pathogen, *Pseudomonas avenae*, Resistance response, Rice.

Abbreviations

HR, hypersensitive response; PCD, programmed cell death; TMV, tobacco mosaic virus; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

The hypersensitive response (HR) is characterized by rapid, local death of plant cells at the sites of pathogen infection and is a common feature of incompatible plant-pathogen interactions (Dangl *et al.*, 1996; Greenberg, 1997). The HR is often associated with the activation of plant defense responses, including expression of a large number of defense-related genes, leading to the development of systemic acquired resistance (Dangle *et al.*, 1996). Hypersensitive cell death requires active plant metabolism and depends on the activity of transcriptional and translational machinery in the host plant (He *et al.*, 1994). Induction of HR is associated with certain signaling events such as protein phosphorylation and the generation of reactive oxygen species (ROS) (Jabs *et al.*, 1996; Alvarez *et al.*, 1998). Furthermore, the spontaneous activation of hypersensitive cell death in the absence of pathogens has been reported in transgenic plants that express foreign genes (Hammond-Kosack *et al.*, 1994; Mittler *et al.*, 1995). All these reports suggest that hypersensitive cell death during HR is a form of programmed cell death (PCD).

Apoptosis is one of the most widely studied forms of PCD in animal cells. One common feature of apoptosis is the degradation of high-molecular-weight genomic DNA into smaller oligonucleosomal fragments. The characteristic appearance of 180-bp oligonucleosomal ladders on agarose gels in cells undergoing apoptosis contrasts the pattern of random DNA digestion occurring at the setting of necrosis (Arends *et al.*, 1990; Mesner *et al.*, 1997). In plants, there are two inconsistent experiment results concerning the formation of approximately 180-bp DNA ladders. The cell death triggered in intact leaves by the cowpea rest fungus is accompanied by the cleavage of nuclear DNA into oligonucleosomal fragments (Ryerson and Heath, 1996), whereas DNA laddering was not found during the response of tobacco cells to pathogens (Mittler and Lam, 1995).

Pseudomonas avenae (*Acidovorax avenae*) is a Gram-negative bacterium that causes a seedling disease characterized by the formation of brown stripes on the sheaths of infected plants (Kadota *et al.*, 1991). The species *P. avenae* can infect a wide range of monocotyledonous plants including rice, oats, Italian millet, and maize. However, individual strains of the pathogen can infect only one or a few host species (Nishiyama *et al.*, 1979; Kadota *et al.*, 1991; Kadota *et al.*, 1996). We recently reported that a rice-incompatible strain such as N1141 caused hypersensitive cell death in cultured rice

cells, while rice-compatible strains such as H8301, H8201, or K1 did not (Che *et al.*, 1999; Che *et al.*, 2000). Furthermore, the purified N1141 flagellin also induced hypersensitive cell death in cultured rice cells, and a flagellin-deficient N1141 mutant, constructed by the marker exchange method, lost the ability to induce hypersensitive cell death, suggesting that the flagellin from the incompatible strain of *P. avenae* functions to elicit hypersensitive cell death specifically (Che *et al.*, 2000).

Hypersensitive cell death in cultured rice cells induced by the incompatible strain or incompatible flagellin was accompanied with apoptosis-like morphorogical changes, such as cytoplasmic condensation and plasma membrane shrinkage (Che *et al.*, 1999; Che *et al.*, 2000). However, it was still not clear whether DNA laddering is occurred in cultured rice cells undergoing the hypersensitive cell death. To elucidate the induction mechanism of hypersensitive cell death in cultured rice cells by the incompatible strain, information concerning DNA laddering and the activation of nucleases in this system is important. We therefore examined the DNA laddering during the hypersensitive cell death of rice cells.

To determine whether DNA laddering occurred in cultured rice cells inoculated with *P. avenae*, cultured cells (line Oc) (Baba *et al.*, 1986) were incubated with an incompatible N1141 strain (MAFF 301141) and a compatible H8201 strain (MAFF 301141) (10^8 cfu ml^{-1}) at 30℃ at different time lengths after inoculation. Cultured cells were harvested and washed with 50 mM Hepes-KOH (pH 7.2) twice, and the washed cultured rice cells were ground to a fine powder in liquid nitrogen. 0.5 ml of a DNA extraction buffer containing 2% (w/v) cetyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA, and 1% (w/v) polyvinylpyrrolidone (K-30) was added to each ground sample and then incubated for 10 min at 65℃. After adding 60 μl of 3 M sodium acetate, DNA was extracted with an equal volume of phenol/chloroform and centrifuged. The aqueous phase was transferred to a new tube, and equal volumes of isopropyl alcohol were added. DNA was washed with 70% cold ethanol, dried, resuspended in a 0.5 ml Tris-EDTA (TE) buffer supplemented with 100 μg ml^{-1} RNase A, and then incubated at 37℃ for 30 min. After adding 60 μl of 3 M sodium acetate, DNA was purified by extraction with an equal volume of phenol/chloroform, and the aqueous phase was transferred to a new tube. The aqueous phase was added with 0.6 ml isopropyl alcohol, and the DNA solution was incubated at room temperature for 5 min and then at -20℃ for 15 min. The DNA mixture was centrifuged at 4℃ for 1 s to remove the large-size DNA, and the DNA in the supernatant was then precipitated by centrifugation for 15 min at approximately 12,000*g*. After washing with cold 70% ethanol, DNA was quantitated by measuring the absorbance at 260 and 280 nm. Approximately 50 μg DNA was obtained from 0.5 g of the cultured rice cells using this preparation method. Five micrograms of the purified DNA was subjected to electrophoresis on 2% (w/v) agarose gels and stained with 0.5 μg ml^{-1} final concentration ethidium bromide. Gels were photographed using the ATTO CCD camera system (ATTO, Tokyo, Japan).

In order to examine whether the DNA laddering is occurred during the hypersensitive cell death, cultured rice cells was incubated with incompatible N1141 or compatible H8201 strains, DNA was purified from each cultured rice cells. When cultured rice cells was inoculated with incompatible N1141, clear DNA laddering was observed 10 h after inoculation (**Fig. 1A**). Using a DNA size marker, the sizes of the five smallest bands of the ladder were calculated to be around 180, 360, 540, 720 and 930-bp suggesting that they represent oligonucleosomal fragmentation. Such DNA laddering was occurred 6 h after N1141 inoculation (**Fig. 1A**). In sharp contrast, such DNA laddering did not occur in cultured rice cells inoculated with the compatible H8201 strain though a slight smearing of genomic DNA occurred 10 h after inoculation (**Fig. 1A**). Thus, only the incompatible N1141 strain causes DNA laddering in cultured rice cells.

To clarify the relationship between DNA laddering and hypersensitive cell death, hypersensitive cell death is induced in the cultured rice cells used in DNA laddering experiments. The dead cells were detected by Evans blue staining (Che *et al.*, 1999). In cultured rice cells inoculated with the incompatible N1141 strain, cell death was detected 4 h after inoculation, and the number of dead cells gradually increased (**Fig. 1B**). In contrast, no cell death was detected in cultured rice cells inoculated with the compatible strain H8201 after 8 h of incubation (**Fig. 1B**), and a comparatively small amount of cell death could be detected after 10 h of incubation. Mock-treated cultured cells showed no accumulation of Evans blue dye (**Fig. 1B**). Thus, the induction patterns of hypersensitive cell death and DNA laddering showed good agreement, and DNA laddering occurred only in the cultured rice cells undergoing hypersensitive cell death.

It has been reported that endogenous barley aleurone nucleases contaminated in purified DNA degrade aleurone DNA and that DNA degradation by

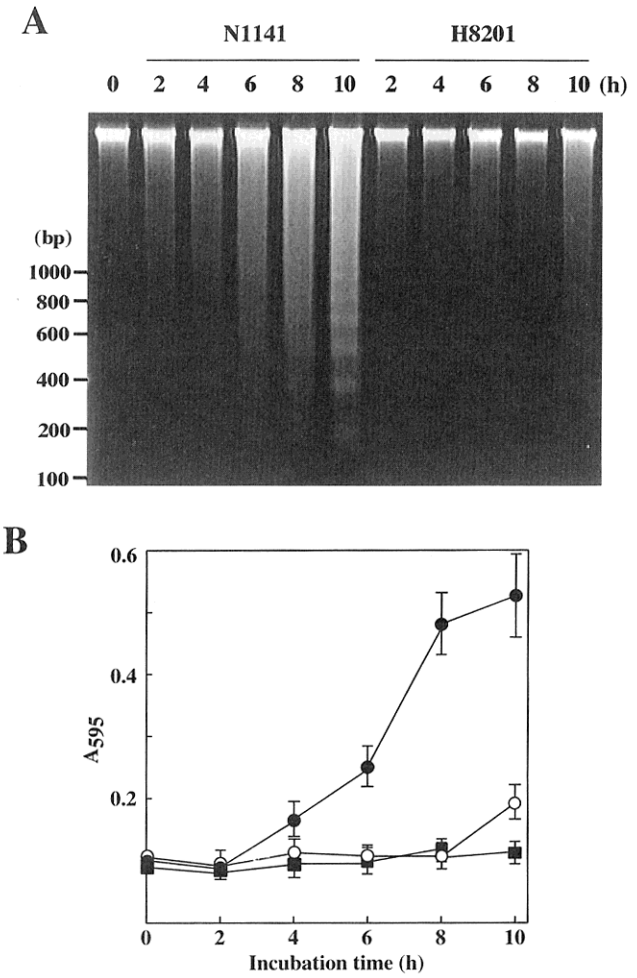

Fig. 1 DNA laddering and hypersensitive cell death in cultured rice cells inoculated with the incompatible N1141 and the compatible H8201 strains of *P. avenae*.

(A) Time-dependent induction of DNA laddering induced by N1141 and H8201 strains. Chromosomal DNA degradation assessed by agarose gels (2%) with ethidium bromide staining. DNA samples were obtained from cultured rice cells inoculated with the N1141 and H8201 strains (10^8 cfu ml^{-1}) after 0, 2, 4, 6, 8, and 10 h incubation, and 5 μg of each DNA was subjected to electrophoresis.

(B) Time-dependent induction of hypersensitive cell death induced by the N1141 and H8201 strains. Closed circles, N1141 strain, open circles, H8201 strain, closed square, control (distilled water). The values shown are averages of three independent experiments. Bars are standard deviations.

these nucleases is rapid and can result in the formation of 180-bp DNA ladders (Fath *et al.*, 1999). Moreover, this artificial DNA laddering is prevented by the use of a DNA isolation buffer containing 50 mM EDTA and 1% (w/v) SDS (Fath *et al.*, 1999). To confirm the idea that DNA laddering

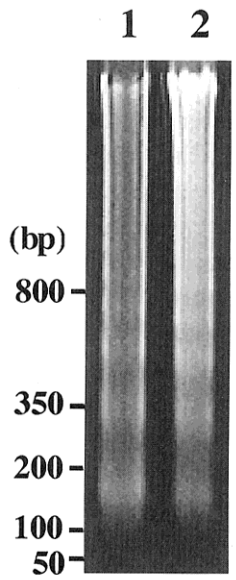

Fig. 2 Effect of lysis buffer composition on DNA laddering induced by N1141 strain.

Lane 1, DNA isolated from N1141-infected cultured rice cells 10 h after inoculation using an SDS-containing buffer to prevent endogenous nuclease activity (Fath *et al.*, 1999). N1141-inoculated cultured rice cells were frozen in liquid nitrogen, and frozen cells were thawed in a homogenizing buffer containing 0.1 M Tris-HCl (pH 7.5), 50 mM EDTA, 0.5 M NaCl, and 1% SDS. The extracts were added with an equal volume of isopropyl alcohol and then centrifuged. The pellet was resuspended in TE, and DNA was purified by the normal methods described in Methods. Lane 2, DNA isolated from N1141-infected cultured rice cells 10 h after inoculation using the normal buffer described in Methods. 5 μg of the each DNA was subjected to electrophoresis and visualized with ethidium bromide staining.

in cultured rice cells inoculated with the incompatible N1141 strain was not caused by endogenous nucleases contaminated in the DNA samples purified from cultured rice cells, genomic DNA was prepared from cultured rice cells inoculated with the N1141 strain using an isolation buffer containing 50 mM EDTA and 1% (v/w) SDS. The nucleosomal DNA fragmentation occurred in all tested samples regardless of the buffers with or without EDTA and SDS (**Fig. 2**), suggesting that DNA laddering in cultured rice cells was caused by the incompatible N1141 strain but not by contaminated endogenous nuclease during DNA preparation.

To elucidate whether DNA laddering in cultured rice cells is regulated under the signaling pathway of flagellin recognition, we performed DNA laddering detection on cultured rice cells using flagellin-deficient mutants (Che *et al.*, 2000). When the

incompatible strain N1141 of *P. avenae* (final concentration 10^8 cfu ml^{-1}) was incubated with cultured rice cells, clear DNA laddering was observed 10 h after incubation (**Fig. 3**), whereas incubation with the flagellin-deficient N1141 mutant, Δfla1141-2, did not cause DNA laddering in cultured rice cells until 10 h after inoculation (**Fig. 3**). The flagellin-deficient compatible strain, ΔflaK1-3 (Che *et al.*, 2000), induced no DNA laddering of rice cultured cells, similarly to the K1 wild type (data not shown). These results indicate that DNA laddering accompanied with hypersensitive cell death in cultured rice cells is mediated by the flagellin of the incompatible strain.

We showed clearly in this paper that 180-bp DNA laddering occurred during the hypersensitive cell death of cultured rice cells inoculated with the incompatible N1141 strain of *P. avenae* (**Fig. 1A**). We have previously reported that cytoplasmic condensation and plasma membrane shrinkage, which are typical morphological characteristics of animal cell apoptosis, were observed during hypersensitive cell death in cultured rice cells induced by the incompatible N1141 strain (Che *et al.*, 1999). However, nuclear condensation, which is another important morphological characteristic of apoptosis, was not observed in cultured rice cells undergoing the hypersensitive cell death (data not shown). The hypersensitive cell death of cultured rice cells would be regulated by different pathway from animal apoptosis.

DNA fragments in the range of 180-540 bp from cultured rice cells were observed 6 h after inoculation. Because DNA fragments of this size range represent the end products of a degradation process that begins with the intact genome, the initiation of internucleosomal fragmentation should have occurred at an earlier time than 6 h after inoculation. This is supported by our previous TUNEL data, in which nuclear DNA cleavage could be detected 4 h after inoculation of the incompatible N1141 strain (Che *et al.*, 1999; Che *et al.*, 2000). Because the hypersensitive cell death identified by Evans blue staining was detected 4 h after inoculation (**Fig. 2**), DNA degradation would be initiated at almost the same time as hypersensitive cell death. In the case of TMV-inoculated tobacco cells, activation of HR-related DNA degradation appeared to occur at a late stage during the cell death process, and authors have suggested that this is not the direct cause of cell death but rather a downstream event that is part of the hypersensitive cell death process (Mittler and Lam, 1995). In our case, DNA laddering occurred in cultured cells undergoing hypersensitive cell death, and cell death and DNA degradation were induced at almost the same time. Further examination is needed to understand the relationship between hypersensitive cell death and DNA degradation in cultured rice cells caused by incompatible interaction.

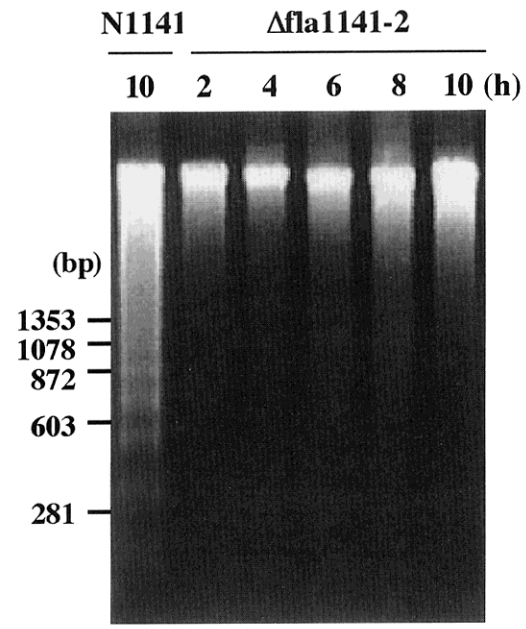

Fig. 3 DNA laddering in cultured rice cells inoculated with the incompatible N1141 strain and the flagellin-deficient N1141 mutant, Δfla1141-2, of *P. avenae*. Chromosomal DNA degradation assessed by agarose gels (2%) with ethidium bromide staining. DNA samples were obtained from cultured rice cells inoculated with the N1141 after 10 h and Δfla1141-2 strains (10^8 cfu ml^{-1}) after 0, 2, 4, 6, 8, and 10 h incubation, and 5 μg of the each DNA was subjected to electrophoresis.

The experiment using the flagellin-deficient mutant suggested that the incompatible flagellin mediated DNA laddering in cultured rice cells. This indicates the existence of a flagellin-reception system mechanism involved in the induction of hypersensitive cell death accompanied with DNA laddering. Studies of the novel perception system of the bacteria-derived elicitor flagellin have recently identified several flagellin-insensitive mutants by screening the flagellin-sensitive accession La-er of *Arabidopsis*, and the *FLS2* gene was identified by a map-based strategy. The *FLS2* gene encodes a putative receptor kinase that shares structural and functional homology with known plant resistance genes, such as the rice *Xa21* gene (Gomez-Gomez and Boller, 2000). It would be interesting to determine whether the *Xa21* or its homolog are involved in the DNA laddering mediated by the incompatible flagellin.

Nucleosomal DNA cleavage correlates with the

activation of endonucleases during apoptosis in animals (Barry and Eastman, 1992; Collins *et al.*, 1992). In plants, it has been known that two endonucleases, ZEN1 from zinnia and BEN1 from barley, may be involved in the PCD during xylem differentiation (Aoyagi *et al.*, 1998). The DNA laddering in our system should also need such endonucleases and its activations. The identification and characterization of such endonucleases remain to be addressed in the future.

Acknowledgments

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