Induction of Apoptotic Cell Death Leads to the Development of Bacterial Rot Caused by *Pseudomonas cichorii*

Akinori Kiba,¹ Yasutaka Sangawa,¹ Kouhei Ohnishi,² Nan Yao,³ Pyoyun Park,³ Hitoshi Nakayashiki,³ Yukio Tosa,³ Shigeyuki Mayama,³ and Yasufumi Hikichi¹

¹Laboratory of Plant Pathology and Biotechnology, Faculty of Agriculture, Kochi University, Nankoku 783-8502, Japan;

²Research Institute of Molecular Genetic, Kochi University, Nankoku 783-8502, Japan; ³The Graduate School of Science and Technology, Kobe University, Rokkodai, Nada, Kobe 657-8501, Japan

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Pseudomonas cichorii is the major causal agent of bacterial rot of lettuce. Collapse and browning symptoms were observed in lettuce leaf tissue from 15 to 24 h after inoculation (HAI) with *P. cichorii*; superoxide anion generation was detected at 1 to 6 HAI; and cell death was induced at 6 HAI, reaching a maximum at approximately 9 and 12 HAI. Heterochromatin condensation and DNA laddering also were observed within 3 HAI. Pharmacological studies showed that induction of cell death and DNA laddering was closely associated with de novo protein synthesis, protein kinase, intracellular reactive oxygen species, DNase, serine protease, and caspase III-like protease. Moreover, chemicals, which inhibited the induction of cell death and DNA laddering, also suppressed the development of disease symptoms. These results suggest that apoptotic cell death might be closely associated with the development of bacterial rot caused by *P. cichorii*.

Additional keywords: programmed cell death.

Bacterial rot and soft rot diseases are economically important worldwide. Soft rot bacteria constitute one of the most important groups of bacterial phytopathogens that are destructive to a wide variety of plants in both culture and storage (Perombelon and Kelman 1980). One of the major pathogens is *Pectobacterium carotovora* subsp. *carotovora*, which can infect a number of plant species. *Pectobacterium carotovora* subsp. *carotovora* produces pectate lyase, polygalacturonase, pectin lyase, and cellulase, which are thought to have an important role in its pathogenicity (Pirhonen et al. 1991). Thus, bacterial soft rot diseases are characterized by extensive maceration of the infected plant tissues with a variety of plant cell wall-degrading enzymes produced by *Pectobacterium carotovora* subsp. *carotovora*.

Pseudomonas cichorii is the major causal agent of bacterial rot disease of lettuce. Lettuce tissues infected with *P. cichorii* first show browning symptoms followed by the development of bacterial rot. The disease symptoms are similar to those of soft rot caused by *Pectobacterium carotovora* subsp. *carotovora*; however, in culture medium and on lettuce leaves, *P. cichorii* does not produce pectate lyase, the most important

extracellular plant cell wall-degrading enzyme in the development of soft rot (*unpublished data*). Therefore, development of bacterial rot disease caused by *P. cichorii* is thought to be mediated by unknown mechanisms.

The mechanisms of plant defense have been analyzed extensively in various plant–pathogen interactions. A large variety of microorganism-associated products, called general elicitors molecules, trigger defense responses (Nurnberger and Brunner 2002). In phytopathogenic bacteria, lipid and lipopolysaccharide fractions from gram-negative bacteria stimulate or modulate a subset of defense-associated plant responses such as accumulation of pathogenicity response proteins, phytoalexin, and hydroxycinnamoyl-tyramine conjugate (Dow et al. 2000). Effector proteins secreted through the type III secretion system and flagellins are thought to be effective elicitor molecules that trigger nonhost resistance (Felix et al. 1999; Gomez-Gomez and Boller 2002; Kjemtrup et al. 2000). The cell death-inducing protein elicitor harpin, secreted via the type III secretion system, has been isolated from many gram-negative bacteria (Nurnberger 1999). Substantial evidence also indicates that avirulence proteins translocated through the type III secretion system have a crucial role in eliciting race- and cultivar-specific resistance accompanied by a hypersensitive reaction (HR) (Nimchuk et al. 2001).

In contrast to our knowledge about the mechanisms of plant resistance, little has been known about the mechanisms related to disease susceptibility and development. HR-like cell death is reported to facilitate infection by the necrotrophic pathogen *Botrytis cinerea* (Govrin and Levine 2000). It has been reported that protein synthesis of plant cells is thought to be involved in the toxic effect of certain host-specific toxins produced by phytopathogenic fungi. The toxic effect of PC-toxin, produced by *Periconia circinata*, is inhibited by cycloheximide (CHX) and cordycepin, inhibitors of protein and RNA synthesis, respectively (Wolpert and Dunkle 1983). CHX also has been demonstrated to inhibit the toxic effects of AK-toxin and PtrA ToxA protein, produced by *Alternaria alternata* Japanese pear pathotype and *Pyrenophora tritici-repentis*, respectively (Ciuffetti and Touri 1999; Kohmoto et al. 1989). In addition, it recently was reported that the development of the disease symptoms caused by *Pseudomonas cichorii* depends on protein synthesis of lettuce cells, because tissue collapse is suppressed by the concomitant presence of CHX (Hikichi et al. 1998). However, the detailed role of plant metabolism in induction of disease susceptibility and development remains unknown.

Corresponding author: Akinori Kiba; Telephone: +81 88 864 5196; Fax: +81- 88 864 5219; E-mail: akiba@cc.kochi-u.ac.jp

In this article, we analyze the mechanism of the development of bacterial rot caused by *P. cichorii*. In particular, we analyze the involvement of plant intracellular signal transduction cascades and de novo protein synthesis in disease development by biochemical and cytochemical observations. As far as we know, this is the first report to present the involvement of plant metabolism and apoptotic cell death in the development of bacterial rot disease.

RESULTS

Phenotypic observation of bacterial rot caused by *P. cichorii*.

Water-soaking lesions were observed in lettuce leaves at 9 h after inoculation (HAI) with *P. cichorii*. Collapse and browning symptoms were observed in lettuce leaves at 12 to 24 HAI

with *P. cichorii*, and then became characteristic symptoms of bacterial rot at 48 HAI (Fig. 1).

Pectate-lyase activity in culture medium and lettuce tissues.

Plant cell wall-degrading enzymes are thought of as virulence factors for bacterial rot disease (Liao et al. 1992, 1997; Pirhonen et al. 1991). To clarify the relationship between production of plant cell wall-degrading enzymes and bacterial virulence, pectate-lyase activity was estimated in vitro and in planta. *Erwinia carotovora* subsp. *carotovora*, the bacterial pathogen for soft rot disease, secreted strong pectate-lyase activity in culture medium. In contrast, no detectable pectate-lyase activity was observed in extracellular protein fractions of *P. cichorii* throughout the experiments (Fig. 2A).

Fig. 1. Disease development of bacterial rot caused by *Pseudomonas cichorii*. Lettuce leaves were infiltrated with water (W) or *P. cichorii* (Pc), then incubated at 25°C for 0, 6, 9, 12, 24, 36, and 48 h.

Fig. 2. Pectate-lyase activity secreted into culture medium and lettuce tissues. **A**, *Pseudomonas cichorii* or *Erwinia carotovora* subsp. *carotovora* was incubated in PY medium for 3, 6, 9, and 24 h. Extracellular protein fraction was subjected to pectate-lyase activity. **B**, Leaf disks were infiltrated with water (open circles), *P. cichorii* (closed circles), or *E. carotovora* subsp. *carotovora* (closed boxes), then incubated at 25°C for 0, 3, 6, 9, and 24 h. Total protein fraction was used for pectate-lyase assay. Activity of pectate-lyase was estimated by spectrophotometric measuring the absorbance optical density at 232 nm. Each value represents the mean with standard deviation of results from triplicate experiments.

Pectate-lyase activity was first detected at 3 HAI and the activity increased continuously until 24 HAI in total protein fractions from lettuce leaves inoculated with *E. carotovora* subsp. *carotovora*. In total protein fractions from *P. cichorii*-inoculated lettuce leaves, pectate-lyase activity also was detected, but the activity was under the level of water control (Fig. 2B).

Induction of programmed cell death in lettuce leaves.

Cell death was induced in lettuce leaf disks infiltrated with *P. cichorii* at 6 HAI, and reached a maximum at approximately 9 to 12 HAI (Fig. 3A). On the other hand, there were no significant changes in cell viability in water-infiltrated leaf disks. These results indicate that cell death is induced rapidly (6 h), but the lesions are observed 12 HAI with *P. cichorii*.

It has been reported that CHX completely suppresses the tissue collapse in lettuce leaf disks induced by *P. cichorii* infiltration (Hikichi et al. 1998). We examined the role of protein synthesis on *P. cichorii*-induced cell death. The cell death was markedly inhibited by the concomitant presence of CHX to the level of water-infiltrated leaf disks (Fig. 3B). These results indicate that *P. cichorii*-induced cell death is dependent on de novo protein synthesis in lettuce cells in a manner similar to tissue collapse. This is thought to be programmed cell death (PCD) in lettuce, and to be closely associated with disease development.

Induction of superoxide anion-generation in lettuce leaf disks.

Reactive oxygen species (ROS) is considered to be an important signal molecule triggering PCD. To determine ROS

production in lettuce leaf disks infiltrated with *P. cichorii*, reduction of 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-3H-tetrazolium-5-carboxanilide (XTT) was determined. Reduction of XTT (XTT-formazan formation) was first detected in lettuce leaf disks within 1 HAI and increased until 6 HAI (Fig. 4A). Scavengers of superoxide anion, superoxide dismutase (SOD), and 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron) inhibited XTT-formazan formation to the level of leaf disks infiltrated with water at 1 to 3 HAI. Approximately 50% of XTT-formazan also was reduced by concomitant presence of SOD and Tiron with *P. cichorii*, even at 4 to 6 HAI. In contrast, there were no significant changes in XTT-formazan formation in leaf disks infiltrated with water. These results indicate that generation of superoxide anion is induced in lettuce leaves infiltrated with *P. cichorii*.

The *P. cichorii*-induced superoxide-generation (XTT-formazan formation) was markedly inhibited by the concomitant presence of an NADPH oxidase inhibitor, diphenylene iodonium (DPI), to the level of leaf disks infiltrated with water. On the other hand, *P. cichorii*-induced superoxide-generation was scarcely affected in the concomitant presence of CHX, suggesting that de novo protein synthesis is not crucial for superoxide generation. (Fig. 4B). These results suggest that *P. cichorii*-induced superoxide generation is mediated by an NADPH oxidase-like enzyme.

Changes of peroxidase activity in lettuce leaf disk.

Peroxidase activity was increased in lettuce leaf disks 1 HAI with *P. cichorii* (Fig. 5). Maximum activity of peroxidase was observed 3 HAI and then decreased to the level of water-infiltrated leaf disks 9 HAI with *P. cichorii*. No drastic activation

Fig. 3. Induction of cell death in lettuce leaf disks infiltrated with *Pseudomonas cichorii*. **A**, Leaf disks were infiltrated with water (open circles) or *P. cichorii* (closed circles), then incubated at 25°C for 0, 3, 6, 9, and 12 h. **B**, Effect of an inhibitor of eukaryotic protein synthesis on induction of cell death induced by *P. cichorii*. Leaf disks were infiltrated with water (W) or *P. cichorii* (Pc) in the absence or presence of cycloheximide (+CHX) at 10 µg/ml and incubated at 25°C for 6 (gray bar) and 9 (black bar) h. Cell death was monitored by spectrophotometric estimations of Evans blue uptake into dead cells by measuring the absorbance optical density (OD) at 600 nm. Each value represents the mean with standard deviation (SD) of results from triplicate experiments.

of peroxidase was observed in water-infiltrated leaf disks. These results suggested that drastic changes of cellular metabolisms occurred in lettuce leaf disks inoculated with *P. cichorii*.

Role of ROS in induction of cell death.

The production of superoxide anion was rapidly induced in lettuce leaf disks infiltrated with *P. cichorii* (Fig. 4A and B). To examine the relationship between ROS and cell death, the effects of several scavengers and an inhibitor on *P. cichorii*-induced cell death were analyzed. *P. cichorii*-induced cell death was slightly inhibited by SOD and Tiron, scavengers of superoxide anion; catalase, a scavenger of hydrogen peroxide; and dimethyl sulfoxide, a scavenger of hydroxyl radical. On the other hand, DPI, an inhibitor of NADPH oxidase, and *N*-methyl-*L*-arginine monoacetate (NMMA), an inhibitor of nitric oxide synthesis, did not show any inhibitory effect on *P. cichorii*-induced cell death. Interestingly, *N*-acetylcysteine (NAC), a membrane-permeable radical scavenger, markedly inhibited *P. cichorii*-induced cell death in lettuce leaf disks (Fig. 6). These results suggest that intracellular ROS might have a crucial role in induction of cell death induced by infiltration with *P. cichorii*.

Role of protein kinase in induction of cell death.

To elucidate the signal transduction cascade leading to cell death, the involvement of protein phosphorylation was tested.

Treatment of *P. cichorii* with K-252a, a Ser/Thr protein kinase inhibitor, significantly suppressed *P. cichorii*-induced cell death (Fig. 7). Therefore, protein phosphorylation is thought to be involved in induction of the cell death induced by *P. cichorii* infiltration.

Effects of protease and DNase inhibitors on induction of cell death.

It has been reported that, in plant cells, some types of DNase and protease are involved in the signaling pathways leading to PCD. We examined involvement of DNase and protease in the cascade of *P. cichorii*-induced cell death. Inhibitors of *r*-DNase, such as aurintricarboxylic acid (ATA), significantly inhibited *P. cichorii*-induced cell death. Moreover, di-sodium hydrogen citrate (diSHC), inhibitor for DNase, completely suppressed *P. cichorii*-induced cell death to the level of water control (Fig. 8A). Aprotinin, a serine protease inhibitor, also suppressed *P. cichorii*-induced cell death. A specific peptide inhibitor of caspase III in mammalian cells, acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD), also markedly suppressed *P. cichorii*-induced cell death, and L-3-trans-carboxyxiran-2-carboxyl-L-leucyl-agmatin (E-64), a cysteine protease inhibitor, slightly inhibited cell death (Fig. 8B). These results suggest that a certain type of DNase, serine protease and caspase III-like protease, might have a cru-

Fig. 4. Generation of superoxide anion on lettuce leaf disks infiltrated with *Pseudomonas cichorii*. **A**, Lettuce leaf disks were infiltrated with water (open circles) or *P. cichorii* in the absence (close circles) or presence of 100 units of superoxide dismutase (SOD, open squares) or 1 mM 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron, closed squares), and incubated in 0.5 mM 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-3H-tetrazolium-5-carboxanilide (XTT) at 25°C for 1, 2, 3, 4, 5, and 6 h. **B**, Lettuce leaf disks were infiltrated with water (open circles) or *P. cichorii* in the absence (closed circles) or presence of 100 µM diphenylene iodonium (DPI, closed triangles) or cycloheximide (CHX, open triangles) at 10 µg/ml and incubated in 0.5 mM XTT at 25°C for 1, 2, 3, 4, 5, and 6 h. Formation of XTT-formazan was estimated spectrophotometrically by measuring the absorbance optical density (OD) at 470 nm. Each value represents the mean with standard deviation of results from triplicate experiments.

cial role in induction of the cell death induced by *P. cichorii* infiltration.

Ultrastructural features of lettuce cells.

Heterochromatin started to aggregate in the lettuce cells at 3 HAI and drastically aggregate into a few large masses in the lettuce cells 6 HAI with *P. cichorii* (Fig. 9C and D). The percentage of chromatin-condensed cells significantly increased during the experiments, and chromatin condensation was observed in 80% of cells at 9 HAI (Fig. 9E). On the other hand, heterochromatin was scattered evenly in the cells of lettuce tissues infiltrated with water throughout the experiment (Fig. 9A and B).

Induction of DNA fragmentation in lettuce cells.

Ultrastructural observations indicated that cells of *P. cichorii*-infiltrated lettuce tissues showed morphological characteristics for apoptotic cell death (Fig. 9). DNA cleavage of nucleosomal fragments, which are multiple sizes of 180 bp, usually is observed as DNA ladders during apoptotic cell death. To examine whether a similar phenomenon occurs with *P. cichorii*-induced cell death in lettuce, DNA was extracted from lettuce leaves 0 to 6 HAI with *P. cichorii*. DNA laddering first was observed at 1 HAI with *P. cichorii* and became clear at 3 HAI (Fig. 10A). Remarkable DNA degradation was observed 6 HAI with *P. cichorii*; in contrast, DNA degradation was not detected in cells of water-infiltrated lettuce tissues throughout the experiments (data not shown).

Fig. 5. Changes of peroxidase activity of lettuce leaf disks infiltrated with *Pseudomonas cichorii*. Lettuce leaf disks were infiltrated with water (open circles) or *P. cichorii* (closed circles), then incubated at 25°C for 0, 1, 3, 6, 9, and 12 h. Total protein fraction was used for peroxidase assay. Activity of peroxidase was estimated by spectrophotometric measuring the absorbance optical density at 470 nm. Each value represents the mean with standard deviation of results from triplicate experiments.

To clarify the relationship between cell death and DNA fragmentation, the effects of inhibitors and scavengers on DNA fragmentation were examined 3 HAI with *P. cichorii* at which DNA fragmentation was most clearly observed. DNA fragmentation was prevented completely in the concomitant presence of inhibitors and scavenger, CHX, NAC, K252a, ATA, diSHC, aprotinin, and DEVD, which inhibited *P. cichorii*-induced cell death (Fig. 10B). These results suggest that DNA fragmentation is mediated by de novo protein synthesis, protein kinase, intracellular ROS, serine protease, and caspase III-like protease in a manner similar to the induction of the cell death induced by infiltration with *P. cichorii*. Therefore, cell death induced by *P. cichorii* is thought to be apoptotic cell death.

Effects of inhibitors and scavenger on disease development.

To analyze the relationship between apoptotic cell death and the development of bacterial rot disease, the effects of inhibitors and scavengers on disease development were examined. Browning symptoms were observed in lettuce leaves infiltrated with *P. cichorii* only (Fig. 11). Inhibitors that did not inhibit *P. cichorii*-induced cell death showed no inhibitory effects on disease development (data not shown). On the other hand, CHX, NAC, K252a, ATA, diSHC, aprotinin, and DEVD inhibited not only *P. cichorii*-induced cell death and DNA fragmentation, but also disease development caused by *P. cichorii*. These results suggest the close relationship between apoptotic cell death and disease development.

DISCUSSION

PCD is the physiological process of cell destruction involved in the development of multicellular organisms, the immune system, maintenance of tissue homeostasis, and responses to biotic or abiotic stresses. In animal cells, one of the most commonly characterized forms of PCD is known as apoptosis. Apoptotic events include condensation of chromatin, fragmentation of nuclear DNA at internucleosomal regions, and fragmentation of cells into apoptotic bodies (Saraste and Pulkki 2000). Chromatin condensation and DNA fragmentation are hallmarks of apoptosis (Nagata 2005). In plant cells, one of the most characteristic forms of PCD is caused during HRs, which are induced during the defense response of plants. During the development of the HR, the hallmarks of apoptosis have been observed in plants such as BY-2 tobacco cells, cowpea leaves, tobacco leaves, and oat leaves (Ryerson and Heath 1996; Sasabe et al. 2000; Yao et al. 2002). Therefore, apoptotic cell death is thought to be a common response during hypersensitive cell death in plants.

Recently, it has been reported that apoptotic cell death is induced during susceptible interactions. A compatible race of *Puccinia coronata* f. sp. *avenae* and *Magnaporthe grisea* induced cell death which showed characteristic features of apoptotic cell death, such as chromatin condensation and DNA cleaving in oat cells (Yao et al. 2002). The host-specific AAL toxins secreted by *A. alternata* f. sp. *lycopersici* are reported to induce hallmarks of apoptosis during cell death in tomato plants (Wang et al. 1996). Victorin, a host-specific toxin produced by *Cochliobolus victoriae*, also induces apoptotic responses in susceptible oat plants (Navarre and Wolpert 1999; Tada et al. 2001; Yao et al. 2001). In bacteria and plant interactions, apoptotic cell death is induced by inoculation with a toxin-produced strain of *Pseudomonas syringae* pv. *atropurpurea* and *P. syringae* pv. *coromafaciens* (Yao et al. 2002). Thus, most necrotic lesions are thought to be the result of apoptotic cell death and occur by a mechanism similar to development of an HR. Moreover, our present study showed that chromatin condensation and DNA laddering in lettuce cells

also were induced by infiltration with *P. cichorii* (Figs. 9 and 10). These results indicate that apoptotic cell death is induced in lettuce cells during the development of bacterial rot disease caused by *P. cichorii*. Thus, induction of apoptotic cell death is observed not only with the development of necrotic disease symptoms but also with rot disease symptoms.

Induction of apoptotic cell death is regulated by the intracellular signaling pathway of plant cells. Induction of HR by INF1 elicitor and xylanase elicitor is reported to inhibit by the concomitant presence of serine/threonine protein kinase inhibitors in tobacco cells (Sasabe et al. 2000; Suzuki et al. 1999). In this study, pharmacological evidence indicates that protein phosphorylation/dephosphorylation cycles are crucial for the induction of apoptotic cell death induced by *P. cichorii*-lettuce interactions (Fig. 7). Victorin-induced chromatin condensation and DNA fragmentation also are prevented by inhibitors of protein kinase in susceptible oat cells (Tada et al. 2001; Yao et al. 2001). Therefore, this induction of apoptotic cell death is mediated, at least in part, by a pathway similar to those in other plant species in both disease development and induction of the HR.

P. cichorii-induced cell death was drastically inhibited by NAC, indicating the involvement of ROS in induction of PCD (Fig. 6). A recent study has demonstrated hydrogen peroxide-induced apoptotic cell death in cultured tobacco BY-2 cells (Houot et al. 2001). Induction of chromatin condensation and DNA fragmentation by victorin is reported to prevent by SOD and NAC (Tada et al. 2001), indicating the involvement of

ROS in induction of apoptotic cell death. Therefore, among intracellular signal molecules leading to apoptotic cell death, ROS is thought to be important; though it is not sufficient for apoptotic cell death induced by INF1 elicitor and harpin in tobacco cells (Ichinose et al. 2001, Sasabe et al. 2000).

Cellular sources of ROS include plasma membrane, mitochondria, and peroxisome in animal cells (Siraki et al. 2002). NADPH oxidase is localized in plasma membranes and is a major source of ROS in mammalian neutrophils (Cross and Jones 1991). Mitochondria produce ROS by respiratory chains and play a central role in controlling apoptosis via dysfunction of mitochondria (Kromer et al. 1997). Peroxisomal enzymes such as xanthin oxidase, D-amino oxidase, and uricase also are thought to be major generators of ROS (Siraki et al. 2002). In plants, an oxidative burst is catalyzed by a plasma membrane-bound NADPH oxidase-like enzyme during the defense response (Doke et al. 1996). Recent studies also have indicated that induction of apoptotic cell death is regulated by ROS produced by electron transport chains of mitochondria and chloroplasts (Curtis and Wolpert 2002; Samuilov et al. 2002). From our present results, the plasma membrane is thought to be one of the sources of ROS generated by an NADPH oxidase-like enzyme in lettuce cells challenged by *P. cichorii* (Fig. 4), whereas extracellular ROS might be related only to partial induction or acceleration of apoptotic cell death. In contrast, intracellular ROS was shown to have a crucial role in induction of apoptotic cell death (Fig. 6). The evidence suggests that intracellular organ-

Fig. 6. Effects of reactive oxygen species scavenger and inhibitor of NADPH oxidase on induction of cell death induced by *Pseudomonas cichorii*. Leaf disks were infiltrated with water (W) or *P. cichorii* (Pc) in the absence or presence of 100 units of superoxide dismutase (+SOD), 1 mM 4,5-dihydroxy-1,3-benzene-disulfonic acid (+Tiron), 100 units of catalase (+CAT), 1 mM dimethyl sulfoxide (+DMSO), 100 μ M diphenylene iodonium (+DPI), 2 mM N-acetyl cysteine (+NAC), or 5 mM N-methyl-L-arginine monoacetate (+NMMA) and incubated at 25°C for 6 (gray bar) and 9 (black bar) h. Cell death was monitored by spectrophotometric estimations of Evans blue uptake into dead cells by measuring the absorbance optical density (OD) at 600 nm. Each value represents the mean with standard deviation of results from triplicate experiments.

elles might participate in generating ROS and controlling apoptotic cell death.

During apoptosis, nucleases and proteases play a crucial role in the induction of apoptotic cell death in animal cells. The enzymes required for DNA fragmentation during apoptosis include DNA fragmentation factor and DNase. These enzymes are present in normal cells as inactive forms and are activated selectively as a result of cleavage by specific proteases. One of the major proteases responsible for DNase activation is a member of the caspase family (Saraste and Pulkki 2000). The cell death associated with HRs in several plant-pathogen systems occurs through a mechanism similar to animal apoptosis (Lam and del Pozo 2000). Induction of nuclease activity also is detected during an HR in tobacco (Mittler and Lam 1995). In tobacco cells, induction of cell death is suppressed by the presence of serine protease inhibitor (Sasabe et al. 2000). Caspase-like activity seems to be involved in HRs in tobacco and cowpea cells (del Pozo and Lam 1998; D'Silva et al. 1998). In the case of compatible interactions, victorin induces activation of nuclease and DNA laddering, and this phenomenon also is prevented by both serin and cysteine protease inhibitors (Tada et al. 2001). A tetrapeptide caspase inhibitor is shown to block disease symptoms caused by *P. syringae* pvs. *tabaci*, *angulata*, *phaseolicola*, and *pisi* and *Xanthomonas campestris* pv. *vesicatoria* (Richael et al. 2001). We also showed that inhibitors of nuclease and serine protease effectively prevented the induction of cell death and DNA fragmentation in all cells of lettuce tissue infiltrated with *P. cichorii*. Moreover, a specific peptide inhibitor for caspase III completely inhibited the induction of cell death and DNA fragmentation. Thus, certain nucleases, serine proteases, and caspase-like proteases are thought to be key components in apop-

totic nuclear disassembly and induction of cell death in plants, as observed in mammalian cells (Figs. 8 and 10).

The relationship between apoptotic cell death and disease development remains obscure in plants. Recently, necrotic disease symptoms were reported to be caused by results of PCD (apoptotic cell death) in plants (Govrin and Levine 2000; Richael et al. 2001). However, no report has described the role of PCD in the development of rot disease symptoms. In our present study, metabolic inhibitors and scavengers, which effectively prevented apoptotic cell death, blocked development of bacterial rot caused by *P. cichorii* (Fig. 11). Therefore, apoptotic cell death is thought to be closely associated with the development of bacterial rot disease. There are at least two possibilities with regards to the relationship between apoptotic cell death and disease development. One is that apoptotic cell death initially is induced in cells along with initial infection sites of *P. cichorii*, and has a role in controlling gene expression and protein synthesis of peripheral cells required for disease development. The other possibility is that biochemical pathways mediating both cell death and disease development could be activated in parallel by infection with *P. cichorii*.

Generally, soft rot and bacterial rot diseases are thought to be caused by the degradation of plant cell walls by extracellular enzymes produced by bacterial pathogens such as *P. carotovora* subsp. *carotovora*, *P. viridiflava*, and *P. marginalis* (Liao et al. 1992, 1997; Pirhonen et al. 1991). However, our present data suggest that active plant responses might regulate the development of bacterial rot disease (Table 1). The results described in this study are the first to indicate that plant-pathogenic bacteria induce plant cell death by induction of apoptotic cell death as a part of pathogenesis, causing symptoms of rot diseases. Further study will be required to elucidate the role of apoptotic cell death in the development of bacterial rot disease.

MATERIALS AND METHODS

Plant and bacterial materials.

P. cichorii PC9018 was cultured in potato-sucrose (PS) medium (Wakimoto et al. 1968) at 30°C for 18 h. Lettuce (*Lactuca sativa* cv. Success) plants were grown in a growth room at 25°C.

Inhibitors and scavengers.

Aprotinin, ATA, cycloheximide, diSHC, K-252a, NMMA, and NAC were obtained from Nakarai Tesque Co. Ltd., Tokyo. Catalase, DPI, E-64, DEVD, SOD, Tiron, and XTT were purchased from Sigma Co. Ltd. (St. Louis). None of the above chemicals caused reduction in the growth of *P. cichorii* in peptone-yeast extract (PY) medium and in lettuce leaves (Table 2). *P. cichorii* pretreated with these chemicals was capable of inducing cell death and disease development on lettuce leaves in a manner similar to nontreated *P. cichorii*.

Extraction of protein fractions.

A lettuce leaf disk was infiltrated with water, *P. cichorii*, or *E. carotovora* subsp. *carotovora* and incubated at 25°C for appropriate times. The leaves were homogenized with 10 mM Tris-HCl (pH 7.4) containing 3 mM MgCl₂, 2 mM β -mercaptoethanol, and 0.3 mM phenylmethylsulfoxide. The homogenates were centrifuged at 10,000 $\times$ g for 10 min and supernatants were used as total protein fractions. *P. cichorii* or *E. carotovora* subsp. *carotovora* was incubated in PY medium for 3, 6, 9, and 24 h and centrifuged at 10,000 $\times$ g for 10 min and supernatants were used as extracellular protein fractions.

Fig. 7. Effect of protein kinase inhibitor on induction of cell death after infiltration with *Pseudomonas cichorii*. Leaf disks were infiltrated with water (W) or *P. cichorii* (Pc) in the absence or presence of 5 μ M K-252a (+K252a) and incubated at 25°C for 6 (gray bar) and 9 (black bar) h. Cell death was monitored by spectrophotometric estimations of Evans blue uptake into dead cells by measuring the absorbance optical density (OD) at 600 nm. Each value represents the mean with standard deviation of results from triplicate experiments.

Assay for enzyme activity.

Assay for pectate-lyase activity was carried out according to Collmer and associates (1988) with slight modifications. The reaction mixture was composed of 60 mM Tris-HCl buffer (pH

8.5) with 0.6 mM CaCl_2 and 0.24% (wt/vol) of polygalacturonic acid (Sigma). The reactions were started by the addition of a protein fraction to the reaction mixture and measuring the absorbance optical density (OD) at 232 nm every 20 s for 5

Fig. 8. Effects of inhibitors of nuclease and protease on induction of cell death after infiltration with *Pseudomonas cichorii*. **A**, Leaf disks were infiltrated with water (W) or *P. cichorii* (Pc) in the absence or presence of 50 mM di-sodium hydrogen citrate (+diSHC) or 50 μM aurintricarboxylic acid (+ATA). **B**, Leaf disks were infiltrated with water (W) or *P. cichorii* (Pc) in the absence or presence of 500 μM Ac-DEVD-CHO (+DEVD), aprotinin (+Apro) at 150 $\mu\text{g/ml}$, or 500 μM L-3-trans-carboxyoxiran-2-carbonyl-L-leucyl-agmatin (+E64) and incubated at 25°C for 6 (gray bar) and 9 (black bar) h. Cell death was monitored by spectrophotometric estimations of Evans blue uptake into dead cells by measuring the absorbance optical density (OD) at 600 nm. Each value represents the mean with standard deviation of results from triplicate experiments.

Fig. 9. Ultrastructural changes in lettuce cells infiltrated with *Pseudomonas cichorii*. Lettuce leaves were infiltrated with **A** and **B**, water or **C** and **D**, *P. cichorii* for **A** and **C**, 3 and **B** and **D**, 6 h, after which ultrathin sections were observed by transmission electron microscopy. Arrows indicated condensed chromatin. **E**, Frequency of cells with condensed chromatin. The average percentage of nuclei with condensed chromatin was estimated at 0, 3, 6, and 9 h after infiltration.

min. Enzyme activity was estimated using molecular extinction coefficient $4,600 \text{ M}^{-1} \text{ cm}^{-1}$.

Peroxidase activity with guaiacol as the substrate was determined by the method of Shinkle and associates (1992) with slight modifications. The assay mixture contained 40 mM sodium phosphate (pH 6.0), 0.5 mM guaiacol (Tokyo Kasei Co. Ltd., Tokyo), 0.1 mM H_2O_2 (Nakarai Tesuque), and 1 μg of total protein fractions. The reaction was started by the addition of H_2O_2 . Changes of absorbance at 470 nm were recorded every

10 s for 3 min, and converted into specific enzyme activity using $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Evaluation of cell viability.

For evaluation of viability, leaf disks were stained with Evans Blue (Wako) by the method of Baker and Mock (1994). Bacterial suspensions in the absence or presence of an appropriate amount of chemicals were adjusted to give an initial OD at 600 nm of 0.1 ($1.0 \times 10^8 \text{ CFU/ml}$). Water or bacterial suspensions

Fig. 10. Induction of DNA laddering in lettuce leaves infiltrated with *Pseudomonas cichorii*. **A**, Lettuce leaves were infiltrated with *P. cichorii* and incubated for 0, 1, 3, and 6 h. **B**, Effects of inhibitors and scavenger on DNA laddering in lettuce leaves infiltrated with *P. cichorii*. Lettuce leaves were infiltrated with water (W) or *P. cichorii* (Pc) in the absence or presence of cycloheximide (+CHX) at 10 $\mu\text{g/ml}$, 2 mM *N*-acetyl cysteine (+NAC), 5 μM K-252a (+K252), 50 μM aurintricarboxylic acid (+ATA), 50 mM di-sodium hydrogen citrate (+diSHC), aprotinin (+Apro) at 150 $\mu\text{g/ml}$, or 500 μM Ac-DEVD-CHO (+DEVD) and incubated for 3 h. DNA was isolated and separated as described in the Materials and Methods. The relative migration of 100-bp DNA lengths was shown on the left as the standard.

Fig. 11. Effect of inhibitors and scavengers on disease development in lettuce leaves infiltrated with *Pseudomonas cichorii*. Lettuce leaves were infiltrated with *P. cichorii* in the absence (Pc) or presence of cycloheximide (+CHX) at 10 $\mu\text{g/ml}$, 2 mM *N*-acetyl cysteine (+NAC), 5 μM K-252a (+K252), 50 μM aurintricarboxylic acid (+ATA), 50 mM di-sodium hydrogen citrate (+diSHC), aprotinin (+Apro) at 150 $\mu\text{g/ml}$, or 500 μM Ac-DEVD-CHO (+DEVD) and incubated for 24 h.

(50 µl) with or without chemicals were infiltrated into lettuce leaf disks (0.75 cm in diameter) in vacuo with an aspirator (Iwaki, ASP-13) followed by incubation at 25°C for 6 and 9 h. The leaf disks were submerged in 1 ml of 0.25% Evans blue and incubated for 20 min. Then, they were washed several times with water to remove excess and unbound dye. The disks were homogenized and bound dye was extracted with 1% sodium dodecyl sulfate (SDS). The extracted dye was measured spectrophotometrically at 600 nm.

Detection of superoxide generation.

Determination of superoxide generation was carried out using the method described by Abel and associates (1998) with slight modifications. Water or a bacterial suspension (1.0×10^8 CFU/ml) was infiltrated into lettuce leaf disks (0.75 cm in diameter) in vacuo with an aspirator, followed by incubation in 0.5 mM XTT (Sigma) at 25°C. Formation of XTT-formazan was spectrophotometrically estimated by measuring the absorbance OD at 470 nm.

Ultrastructural analysis.

Electromicroscopic observations of lettuce cells were carried out using the method of Yao and associates (2001). Bacterial suspension (1.0×10^8 CFU/ml) or water was infiltrated into detached lettuce leaves with a needleless syringe. Leaf segments were prefixed in 2.5% glutaraldehyde with 60 mM phosphate-buffered saline and postfixed with 1% osmium tetroxide. The samples were further dehydrated in ethanol and embedded in Epon 812 resin. Then, ultrathin sections were stained with 2% uranyl acetate for 20 min followed by lead citrate for 10 min. The sections were photographed by transmission electron microscopy (Hitachi-7100; Hitachi, Tokyo) at an accelerating voltage of 75 kV. Rates of chromatin-condensed cells were estimated by counting 100 cells for each inoculation.

DNA extraction and analysis.

Detection of DNA fragmentation was carried out using the method described by Jones and associates (2001) with slight modifications. Bacterial suspension (1.0×10^8 CFU/ml) or water was infiltrated into detached lettuce leaves with a needleless syringe. The infiltrated lettuce leaves were sliced with a laser blade and incubated in 20 mM MES-KOH (pH 5.7) buffer containing 0.3 M mannitol, 10 mM CaCl₂, 0.8% cellulase, and 0.06% macerozyme at 25°C for 3 h. Protoplasts were collected by centrifugation at $500 \times g$ for 3 min, then washed with 0.3 M mannitol. The protoplasts were lysed by suspending them with 0.1 M Tris-HCl (pH 7.5) containing 50 mM EDTA, 0.5 M

NaCl, and 0.1% SDS, and then sodium acetate was added to a final concentration of 0.3 M. Samples were mixed and incubated for 20 min on ice and centrifuged at 4°C for 1 min at $12,000 \times g$. An equal volume of ice-cold 100% isopropanol was added to the supernatant, mixed by inversion, incubated on ice for 1 min, and centrifuged at 4°C for 5 min at $12,000 \times g$. Pellets were resuspended in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and then an equal volume of cetyltrimethylammonium bromide (CTAB) buffer (0.2 M Tris-HCl, 50 mM EDTA, 2 M NaCl, and 2% CTAB) was added. After incubation at 65°C for 15 min, the samples were subjected to chloroform extraction and ethanol precipitation. Pellets were dissolved in TE buffer containing RNase at 20 µg/ml and incubated at 37°C for 30 min. Phenol extraction and ethanol precipitation were performed to recover the DNA. The DNA was separated by 2% agarose gel and visualized by ethidium bromide.

Observation of disease symptoms.

In all, 50 µl of bacterial suspension (1.0×10^8 CFU/ml) or water was infiltrated into detached lettuce leaves in the absence or presence of inhibitors and scavengers with a needleless syringe. Infiltrated lettuce leaves were incubated in a petri dish in 100% humidity at 25°C for 16 h.

Bacterial population in lettuce leaves.

Bacterial suspension (50 µl, 1.0×10^8 CFU/ml) was infiltrated into lettuce-leaf disks (0.75 cm in diameter) in vacuo with an aspirator in the absence or presence of inhibitors and scavengers followed by incubation at 25°C for 0, 6, 12, and 24 h. Each leaf disk was ground in 1 ml of distilled water with mortar and pestle. A 0.1-ml sample of original suspension or a 10-fold serial dilution was spread onto *P. cichorii* selection medium (Uematsu et al. 1982). The colonies were counted 2 days after incubation at 30°C.

Bacterial population in culture medium.

Triplicate test tubes containing PY medium in the absence or presence of the appropriate amount of chemicals were prepared and each tube was inoculated with 10 µl of bacterial suspension to give an initial OD at 600 nm of 0.2. The cultures were incubated at 30°C for 0, 3, 6, and 9 h, and the absorbance was measured at 600nm.

Table 1. Summary of inhibitory effects of inhibitors or scavengers on induction of programmed cell death, DNA fragmentation and disease development^a

Treatment	Cell death	DNA fragmentation	Disease development
CHX	–	+	+
SOD	–	–	–
Tiron	–	–	–
Catalase	–	–	–
NAC	+	+	+
DMSO	–	–	–
DPI	–	–	–
NMMA	–	–	–
K252a	+	+	+
Aprotinin	+	+	+
E64	–	–	–
DEVD	+	+	+
ATA	+	+	+
diSHC	+	+	+

^a A plus (+) indicates strong inhibition and a minus (–) indicates no inhibitory effect.

Table 2. Effect of inhibitors and scavengers (I and S) on bacterial growth^a

I and S	Bacterial population		
	Abs. OD ₆₀₀ ^b	Log CFU/disk ^c	Log CFU/disk ^d
Pc	2.86 ± 0.26	3.22 ± 0.24	2.75 ± 0.18
+CHX	2.76 ± 0.26	3.26 ± 0.24	2.70 ± 0.19
+NAC	2.78 ± 0.26	3.22 ± 0.27	2.64 ± 0.23
+K252	2.74 ± 0.22	3.26 ± 0.26	2.68 ± 0.19
+Apro	2.80 ± 0.25	3.24 ± 0.22	2.68 ± 0.18
+diSHC	2.82 ± 0.25	3.16 ± 0.22	2.70 ± 0.16
+ATA	2.74 ± 0.25	3.09 ± 0.22	2.71 ± 0.20
+DEVD	2.95 ± 0.26	3.19 ± 0.21	2.60 ± 0.20

^a Each value represents the mean with standard deviation of results from triplicate experiments.

^b Absorbance optical density at 600 nm/ml. Bacterial suspension of *Pseudomonas cichorii* was inoculated into a test tube containing PY medium in the absence (Pc) or presence of cycloheximide (+CHX) at 10 µg/ml, 2 mM *N*-acetyl cysteine (+NAC), 5 µM K-252a (+K252), 50 µM aurintricarboxylic acid (+ATA), 50 mM di-sodium hydrogen citrate (+diSHC), aprotinin (+Apro) at 150 µg/ml, or 500 µM Ac-DEVD-CHO (+DEVD) and incubated for 9 h.

^c Lettuce leaves were infiltrated with *P. cichorii* in the absence (Pc) or presence of I and S and incubated for 12 h.

^d Lettuce leaves were infiltrated with *P. cichorii* in the absence (Pc) or presence of I and S and incubated for 24 h.

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