Flagellin from an Incompatible Strain of *Pseudomonas avenae* Induces a Resistance Response in Cultured Rice Cells*

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The host range of *Pseudomonas avenae* is wide among monocotyledonous plants, but individual strains can infect only one or a few host species. The resistance response of rice cells to pathogens has been previously shown to be induced by a rice-incompatible strain, N1141, but not by a rice-compatible strain, H8301. To clarify the molecular mechanism of the host specificity in *P. avenae*, a strain-specific antibody that was raised against N1141 cells and then absorbed with H8301 cells was prepared. When a cell extract of strain N1141 was separated by SDS-polyacrylamide gel electrophoresis and immunostained with the N1141 strain-specific antibody, only a flagellin protein was detected. Purified N1141 flagellin induced the hypersensitive cell death in cultured rice cells within 6 h of treatment, whereas the H8301 flagellin did not. The hypersensitive cell death could be blocked by pretreatment with anti-N1141 flagellin antibody. Furthermore, a flagellin-deficient N1141 strain lost not only the induction ability of hypersensitive cell death but also the expression ability of the *EL2* gene, which is thought to be one of the defense-related genes. These results demonstrated that the resistance response in cultured rice cells is induced by the flagellin existing in the incompatible strain of *P. avenae* but not in the flagellin of the compatible strain.

During their lifetime, plants are subjected to thousands of microbial attacks, but actual infection occurs only in certain limited cases. Besides preformed physical and chemical barriers that prevent infection, a wide variety of defense responses is induced only after pathogen attack (1, 2). When these defense responses are triggered rapidly and coordinately, the plant becomes resistant to pathogen invasion. Susceptible plants respond more slowly with their defense mechanisms after infection. Thus, the timely recognition of an invading microorganism, as well as the rapid and effective induction of the defense responses, appears to make a key difference between resistant and susceptible plants (3, 4). Among the many resistance responses induced by microbial pathogen attack, hypersensitive response (HR)\(^1\) is one of the most dramatic events in plant-microbe interactions. HR is characterized by rapid and localized death of tissues at the site of microbial attack and is associated with the defense of plants against invading microorganisms (5–10). Since elicitation of the HR in a nonhost plant and virulence in a host appear to be linked, hypersensitive cell death is considered to be a hallmark of the resistance response.

The initial requirement of any defense response is the perception of the pathogen by the plant (11). Specific recognition molecules called elicitors often play an important role in this recognition by the plant. Elicitors involve substances of diverse chemical structure such as polygalacturonides, β-glucans, chitosan, lipids, and proteins. Elicitors have been demonstrated to correlate the interactions between plants and viruses (12), bacteria (4, 13), and fungi (14–16). Elicitors have also been categorized as general elicitors, which do not exhibit differences in cultivar sensitivity within a plant species, and specific elicitors, which function only in cultivars carrying matching disease resistance genes (17). These two types of elicitors appear to trigger a common network of signaling pathways that coordinate the overall defense response. The molecular mechanisms of elicitor perception and signal transduction have been studied extensively, and some specific elicitor-binding components have been characterized (18, 19). Nevertheless, the detailed molecular mechanisms of elicitor perception and transduction of the perception signal are not fully understood.

*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 (20). The host range of *P. avenae* is wide among monocotyledonous plants including rice, oats, Italian millet, and maize; however, individual strains of the pathogen infect only one or a few host species (20–22). For example, strains isolated from rice such as H8301 (MAFF 301505) or K1 can infect only rice plant, while the N1141 (MAFF 301141) strain isolated from finger millet cannot infect rice even after being inoculated into rice tissues (20, 21, 23). We recently reported that the rice-incompatible strain, N1141, caused rapid cell death, while the rice-compatible strain, H8301, did not induce it (23). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) showed that DNA cleavage occurred during rapid cell death induced by N1141 strain. Furthermore, the N1141 strain caused cytoplasmic condensation and shrinkage, all of which

\(^1\) The abbreviations used are: HR, hypersensitive response; CE, cell extract(s); DW, distilled water; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; PCD, programmed cell death; PCR, polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; MOPS, 4-morpholinepropanesulfonic acid; bp, base pair(s); cfu, colony-forming units.
Bacterial Flagellin as Specific Elicitor

are important morphological characteristics of programmed cell death (PCD). In contrast, rice cells inoculated by the H8301 strain appeared to cause disruption of the cell wall instead of the above morphological changes associated with PCD. These results indicated that hypersensitive cell death, which is one form of PCD, was induced only in N1141-inoculated rice cells. When cultured rice cells were inoculated with the incompatible N1141 and compatible H8301 strains, several different phenomena other than induction of hypersensitive cell death were observed. The EL2 gene is known to be expressed with N-acetylchitoheptaosamine, which induces a set of defense reactions in cultured rice cells within 15 min (24, 25). The accumulation of EL2 mRNA is detectable 3 h after inoculation with the incompatible strain, N1141, whereas in H8301-inoculated cultured cells it is not detected. From these results, we concluded that the N1141 strain causes a defense response in cultured rice cells but that the compatible strain H8301 does not (23). However, the perception mechanism of the compatible or the incompatible strain by rice and the induction mechanism of the defense response caused by the incompatible strain of \( P. \text{avenae} \) remain unsolved.

Because the resistance response in cultured rice cells, such as hypersensitive cell death, was only induced by the incompatible N1141 strain, we postulated that such induced resistance in rice cells is mediated by the recognition of a specific molecule produced by the incompatible strain of \( P. \text{avenae} \). Based on this hypothesis, we undertook studies to identify such a specific elicitor molecule of \( P. \text{avenae} \). We report here that flagellin, which possesses the flagellum filament of bacteria, is the specific elicitor molecule and that the resistance response in cultured rice cells is induced by flagellin in incompatible strain N1141 of \( P. \text{avenae} \).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Cell Extracts—**\( P. \text{avenae} \) strains H8301 (MAFF 301505) and K1 isolated from rice and strain N1141 (MAFF 301141) isolated from finger millet were maintained on Pseudomonas F at 30 °C on a rotary shaker. Bacterial cells were collected by centrifugation at 6,000 \( \times g \) for 25 min at 4 °C and then resuspended in 30 ml of 20 mM Tris- HCl (pH 7.5) containing 150 mM NaCl. The flagellin were removed from the cells by shearing twice for 1 min in a homogenizer (Ultra F Homogenizer HF-93F, TAITEC, Saitama, Japan). Cells and cell debris were removed by two-step centrifugation at 6,000 \( \times g \) for 25 min and 16,000 \( \times g \) for 60 min at 4 °C. The supernatants were amended with kanamycin (100 \( \mu \text{g/ml} \)) and placed at 25 °C overnight to kill contaminating bacteria. Flagella were pelleted by ultracentrifugation at 20,000 \( \times g \) for 30 min at 4 °C, washed three times with DW to remove kanamycin.

**Cell Death Detection in Cultured Rice Cells—**Suspension cultures of rice cells, line Oc (28), were grown at 30 °C under light irradiation (28). The assay for cell death was performed as described previously (23, 29). N1141 and H8301 strains were cultured in liquid Pseudomonas F (Difco) on a rotary shaker for 3 days at 30 °C. Bacterial cells were collected by centrifugation at 5000 \( \times g \) for 10 min at 4 °C, and the pellet was resuspended in 10 ml of 0.2 M LiCl, agitated with 710-1, 180- \( \mu \text{m} \) glass beads (Sigma) in flasks on a rotary shaker for 2.5 h at 45 °C, and centrifuged at 5000 \( \times g \) for 20 min. The supernatant was ultracentrifuged at 40,000 \( \times g \) for 40 min at 4 °C to remove intact bacterial cells and other insoluble debris and then centrifuged at 100,000 \( \times g \) for 2 h at 4 °C. The resulting pellet was washed with distilled water (DW) and suspended in a small amount of DW. This sample was named as cell extract (CE).

**Immunoblot Analysis—**To obtain strain-specific antibody, rabbit antisera (anti-N1141 and -H8301) were cross-absorbed with whole cells of the other strain according the previously published method (22). IgG from cross-absorbed antisera was purified with HiTrap Protein A (Amersham Pharmacia Biotech) (22). These antibodies were named as N1141 strain-specific antibodies. Several different antibodies were detected using a 12.5% (w/v) SDS-polyacrylamide gel (26) and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore). After staining with 0.1% (w/v) Coomasie Brilliant Blue R-250 (Bio-Rad) in 50% methanol, the 50-kDa bands indicated by N1141 or H8301 strain-specific antibodies were excised. For determination of internal sequence, 50-kDa bands were eluted from SDS-polyacrylamide gel with a microelectroluter (Cenitrolator; Amicon, Beverly, MA) at 100 V for 4 h. Eluted protein was ultrafiltrated by Centricon-10 (Amicon) at 3000 \( \times g \) for 1 h at 4 °C. DW was added to the concentrated protein, and the resuspended sample was centrifuged to remove SDS and other impurities. This sample was digested with bacteriophage T7 lysozyme and SDS-PAGE. Two peptide fragments were collected. The N-terminal sequences were determined with a pulse sequencer with the protein sequencer model 492A (PerkinElmer Life Sciencees). Protein concentrations were determined using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

**Determination of N-terminal and Internal Peptide Sequences—**CEs isolated from strains N1141 and H8301 were separated by SDS-PAGE as above, and electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore). After staining with 0.1% (w/v) Coomasie Brilliant Blue R-250 (Bio-Rad) in 50% methanol, the 50-kDa bands indicated by N1141 or H8301 strain-specific antibodies were excised. For determination of internal sequence, 50-kDa bands were eluted from SDS-polyacrylamide gel with a microelectroluter (Cenitrolator; Amicon, Beverly, MA) at 100 V for 4 h. Eluted protein was ultrafiltrated by Centricon-10 (Amicon) at 3000 \( \times g \) for 1 h at 4 °C. DW was added to the concentrated protein, and the resuspended sample was centrifuged to remove SDS and other impurities. This sample was digested with bacteriophage T7 lysozyme and SDS-PAGE. Two peptide fragments were collected. The N-terminal sequences were determined with a pulse sequencer with the protein sequencer model 492A (PerkinElmer Life Sciencees). Protein concentrations were determined using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard.
molecule in N1141 CE that elicits hypersensitive cell death is a protein. For 3'-end sequence analysis, primary PCR was performed using cassette primer C1 and specific primer II (5'-CTGTTAGCCCGTTACGGTTTGGAGGAAGT-3') and specific primer III (5'-CTGTTAGCCCGTTACGGTTTGGAGGAAGT-3') and specific primer IV (5'-AAACGCAACCTCCGCTGACGGGCGAAGGCAAGC-3'). The amplified fragments for 3'-end PCR (0.5 kb) and for 5'-end PCR (1.2 kb) were cloned with a TA cloning kit (Invitrogen), and 10 independent clones from each PCR were sequenced as described above. These PCR and cloning procedures were independently repeated twice, and DNA inserts were sequenced on both strands to ensure that no mutation had been introduced during amplification.

Production and Purification of Anti-N1141 Flagellin Antibody—The flagellin (5 mg) fraction from N1141 cells was used to immunize rabbits. Full-length N1141 flagellin DNA was cloned in-frame in the pGEX-3X vector (Amersham Pharmacia Biotech). The resulting GST fusion protein was overproduced in the BL21 (DE3) strain of Escherichia coli (Invitrogen) and purified on glutathione-Sepharose 4B according to the manufacturer's protocol (Amersham Pharmacia Biotech). The anti-N1141 flagellin antibody was purified by affinity chromatography using a HiTrap N-hydroxy sulfide-activated Sepharose column (Amer- sham Pharmacia Biotech) immobilizing the recombinant GST-fused N1141 flagellin.

Construction of Flagellin-deficient Mutants—To make a flagellin-deficient mutant of P. avenae strains N1141 and K1, insertion mutants were constructed. pbLuescript SK(–) (Strategene, Victoria, Canada) was cleaved with DraI to remove the ampicillin resistance gene. A 1.4-kbp EcoRI–AvfI fragment containing the tetracycline resistance gene from pBR322 (32) was ligated into the cleaved pbLuescript SK(–), generating the plasmid pYN501. Mismatched cohesive termini were blunted before ligation. An internal region of the flagellin genes from N1141 and K1 (nucleotides 62–1097) was amplified by PCR with oligonucleotide primers 5'-AGTCGTCGCTCAACACCTCCAT-3' and 5'-TCGGAGCTTGTACCTGCACCTCCAT-3'. Each amplified fragment was cloned into pGEM-TEasy (Promega) and then excised by restriction with AvaI and SpeI and ligated to the AvaI–SpeI site of pYN501. The resulting plasmids pYN107 containing internal flagellin fragment of N1141 and pYN108 containing internal flagellin fragment of K1 (electro)-transformed into N1141 and K1 cells, and transformants were selected on LB plates supplemented with 20 μg/ml tetracycline and 28 μg/ml ampicillin at 30 °C for 2 days. Integration of the construct into the chromosomes of strains N1141 and K1 were confirmed by PCR analysis followed by sequencing of the PCR product to verify the correct junctions between the interrupted gene and vector.

RESULTS

Identification of Perception Molecules—Viable cells can exclude Evans blue dye, but dead cells cannot because of a loss of function of the plasma membrane. Therefore, it can be used to monitor cell death. We first examined whether cell death was induced by CE from P. avenae. After an 8-h co-incubation of exponentially growing cultured rice cells with both N1141 and H8301 CE, cell death in cultured rice cells as detected by Evans blue staining was found to be induced by N1141 CE at the lowest tested dose (10 μM/ml) (Fig. 1). In contrast, 10 μM/ml CE isolated from compatible H8301 cells caused no host cell death and only minimal cell death in comparison with N1141 CE at higher doses (100 and 1000 μM/ml) (Fig. 1). Such specificity of cell death induction in cultured rice cells corresponded to the cell death pattern induced by living bacteria of the N1141 strain (25), suggesting that cell death induced by N1141 CE was not an error in cell death and that elicitor or elicitor-like substance(s) were present in the N1141 CE but absent from H8301 CE. The cell death induction activity of N1141 CE was inactivated by treatment with trypsin and Proteinase K (data not shown), indicating that the predicted elicitor-like substance(s) in N1141 CE is a protein.

The above experiments suggested that the proteinaceous molecule in N1141 CE that elicits hypersensitive cell death is absent from H8301 CE or that there are significant structural differences between some components of N1141 and H8301 CE. To identify which protein(s) in CE are involved, we performed Western blot analysis using two strain-specific antibodies (N1141 strain-specific antibody and H8301 strain-specific antibody; see “Experimental Procedures”). The protein band pattern in CEs visualized with silver staining showed close correlation between strains H8301 and N1141 (Fig. 2A). However, when the blotted membrane was stained with the N1141 strain-specific antibody, a single band of the 50-kDa protein was detected in N1141 CE, and no significant bands were observed in H8301 CE (Fig. 2A). In contrast, H8301 strain-specific antibody stained a 50-kDa band only in H8301 CE (Fig. 2A).

To obtain N-terminal sequence information of these 50-kDa proteins, the Coomasie Brilliant Blue R-250-stained bands corresponding to the 50-kDa proteins were excised from polyvinylidene difluoride membranes and subjected to N-terminal sequencing. The N-terminal sequence was the same for each 50-kDa band, ASTNTNVSLSLARQLRNLSLSQSSL, and was highly homologous to the N-terminal amino acid sequences of flagellin that compose a flagellar filament from the Gram-negative bacteria P. aeruginosa, Salmonella typhimurium, and E. coli (Fig. 2B). Furthermore, the internal sequences of peptide sequences obtained by digestion of the H8301 50-kDa protein using lysylendopeptidase and V8 protease (the underlined sequence in Fig. 5A) also showed high similarity to flagellin from other bacteria (data not shown). To confirm that the 50-kDa protein is P. avenae flagellin, immunogold electron microscopic analysis was performed using N1141 strain-specific antibody. When bacterial cells of strain N1141 were incubated with N1141 strain-specific antibody and gold-conjugated anti-rabbit antiserum, many gold particles were found on the flagellum filament of N1141 strain (Fig. 2C). In contrast, no gold particles were found on the flagellum filament of strain H8301 (Fig. 2C). All other control tests, including the omission of primary antibody, yielded negative results (data not shown). These results clearly show that the N1141 strain-specific antibody can recognize N1141 flagellin but not H8301 flagellin and that structural differences exist between N1141 and H8301 flagellins.

Rice Cell Death Induced by Flagellin—The experiments using N1141 or H8301 strain-specific antibodies suggested that flagellin is a major bacterial substance with a different structure in strains H8301 and N1141. Therefore, we assumed that flagellin is a candidate for a cell death-inducing specific substance. To clarify this point, the triggering of cell death was
studied using flagellin purified from strains N1141 and H8301. The crude flagellin fractions were prepared from both strains by several centrifugation steps. SDS-PAGE analysis of the crude N1141 and H8301 flagellin fractions showed that the main component in these fractions is a 50-kDa protein, which is identified as flagellin. When the N1141 flagellin fraction was added to cultured rice cells, cell death was detected at concentrations greater than 0.1 μM (Fig. 3A), while the cell death induction activity of the H8301 flagellin fractions was much lower than that of the N1141 flagellin fraction (Fig. 3A). At a concentration of 1 μM, the N1141 flagellin fraction induced cell death within 6 h of treatment, whereas the H8301 flagellin fraction did not cause detectable cell death until 8 h after incubation (Fig. 3B).

To eliminate the possibility that a contaminating protein in the N1141 flagellin fraction could act as an elicitor, flagellin proteins were further purified with preparative electrophoresis. The purified N1141 flagellin (0.1 μM) also caused the cell death of cultured rice cells within 12 h of treatment, whereas the rate of cell death was detected at concentrations greater than 0.1 μM (Fig. 3A), while the cell death induction activity of the H8301 flagellin fractions was much lower than that of the N1141 flagellin fraction (Fig. 3A). At a concentration of 1 μM, the N1141 flagellin fraction induced cell death within 6 h of treatment, whereas the H8301 flagellin fraction did not cause detectable cell death until 8 h after incubation (Fig. 3B).

Effect of Anti-flagellin Antibody on Hypersensitive Cell Death—Several post-translational modifications of flagellin such as glycosylation (33), phosphorylation (34), methylation (35), and sulfation (36) have been reported. Among these modifications, glycosylation is the most likely because of the large mass differences (over 1,000 Da). When the flagellin fraction was separated by SDS-PAGE and stained using a glycoside detection kit (Pierce), both flagellins and horseradish peroxidase, as glycosylated control, were stained (Fig. 4B). The negative control, soybean trypsin inhibitor, could not be detected by this staining (Fig. 4B). The data demonstrated that the flagellin proteins of the N1141 and H8301 strains were modified after translation.
Death—Since ligand activity can be interrupted by binding of the ligand with specific antibody, both living bacterial strains and anti-N1141 flagellin purified by affinity column immobi-
lizing the recombinant GST-fused N1141 flagellin were mixed
and incubated with cultured rice cells. Induction of cell death
by living N1141 cells was reduced by anti-N1141 flagellin an-
tibody but did not affect the H8301 strain reaction (Fig. 5, A
and B). We previously reported that the rapid cell death caused
by the N1141 strain is defined with PCD and that the delayed
cell death caused by the H8301 strain would have catastrophic
results due to the attack of compatible strain H8301 (23). These
data indicate that the anti-N1141 flagellin antibody inhibits
the PCD-type cell death induced by inoculation of the N1141
strain but not the necrotic type-cell death caused by inoculation
of the H8301 strain.

When the N1141 flagellin fraction together with the anti-
flagellin antibody was added to cultured rice cells, the cell
death-inducing activity of N1141 flagellin was also inhibited by
the antibody in a dose-dependent manner (Fig. 5C). The pre-
immune serum did not cause any cell death, and the cell death
induced by the N1141 strain or N1141 flagellin was not affected
by the addition of preimmune serum (data not shown). These
data indicate that cell death by the N1141 flagellin fraction is
also caused by flagellin and that the cell death caused by
N1141 living cells is substantially mediated by the flagellin
molecule.

Decrease of Resistance Responses in Rice Cultured Cells In-
oculated with Flagellin-deficient Strains—To construct a
flagellin-deficient mutant, the efficiency of transformation in
several strains of compatible and incompatible P. avenae was
tested. The efficiency of transformation was quite low in all of
the tested strains, and the transformant of the H8301 strain
could not be obtained. Among the tested strains, the transforma-
tion was successful only in N1141 of the incompatible strain
and K1 of the compatible strain, although with very low effi-
ciency. The K1 strain caused symptoms of brown stripe in rice
and did not induce the resistant response of rice cultured cells
in the same manner as strain H8301. Furthermore, the de-
duced amino acid sequence of flagellin in the K1 strain is the
same as that of flagellin in the H8301 strain (data not shown).
Therefore, we chose strains N1141 and K1 for the construction
of the flagellin-deficient mutant.

Isogenic N1141-fla and K1-fla mutants of P. avenae were
constructed by electroporation of strains N1141 and K1 with
plasmid pYN107 and pYN108, respectively. We have isolated
two mutants of N1141 (Δfla1141–2 and -3) and three mutants of
K1 (ΔflaK1–1, -2, and -3) that are unable to move in soft agar.
The disruption of the flagellin gene and the lack of flagellin
were confirmed by PCR analysis, Western blot analysis, and
electron microscopic observation. All of the constructed defi-
cient mutants showed the same growth rate as N1141 or K1
wild-type in a liquid medium, and the phenotypes of all mutant
strains were the same.

When the incompatible strain N1141 of P. avenae (final
concentration 10^8 cfu/ml) was incubated with cultured rice
cells, cell death was detected 4 h after inoculation, and the
number of dead cells gradually increased. In contrast, incuba-
tion with the flagellin-deficient N1141 mutant, Δfla1141–2, did
not cause cell death in cultured rice cells until 6 h after inoc-
ulation, and a comparatively small amount of cell death could
be detected after 12 h of incubation (Fig. 6A). The flagellin-
deficient K1 strain, ΔflaK1–3, induced no cell death of rice
cultured cells in the same manner as the K1 wild-type (Fig.
6B).

EL2 mRNA accumulated at six times after 3-h inoculation
with the wild-type strain N1141, whereas only little accumu-
lation of EL2 mRNA was observed in cultured rice cells with
inoculation of Δfla1141–2 (Fig. 7A). In contrast, the accumula-
tion pattern of EL2 mRNA in cultured rice cells inoculated with
the K1 wild-type was quite similar in timing and intensity to
accumulation with ΔflaK1–3 (Fig. 7B).

Recently, it has been reported that a recurrent feature of HR
is the cleavage of DNA at specific chromosomal sites by DNA
endonucleases (37, 38). Terminal deoxynucleotidyl transferase-
mediated dUTP nick-end labeling (TUNEL) can be used to
quantify the accumulation of DNA 3’-OH groups caused by
DNA fragmentation and breakage (39, 40). Using TUNEL, we
have reported that DNA cleavage occurred in N1141-inoculated
cultured rice cells, whereas the compatible H8301 strain did
not cause DNA cleavage (23). We performed TUNEL on cul-
tured rice cells to determine whether the DNA is cleaved dur-
ing exposure to the flagellin-deficient mutants. Cultured rice
cells incubated with the incompatible strain N1141 had many
fluorescein-derived bright green fluorescence signals at 8 h
after inoculation, as detected by TUNEL. The number of posi-
tive nuclei in Δfla1141–2-treated rice cells was considerably
reduced. In contrast, no significant bright green signals were
observed in cultured rice cells inoculated with either K1 wild-
type or ΔflaK1–3 (all photographs not shown). The timing of
DNA cleavage after inoculation of the cultured rice cells was
examined. Fig. 8 shows the percentage of TUNEL-positive nu-
clei at several points after the addition of bacteria. In the
cultured rice cells inoculated with the incompatible strain
N1141, the number of TUNEL-positive nuclei did not increase
until 4 h after inoculation. The percentage of TUNEL-positive

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Fig. 4. Structural analysis of N1141 and H8301 flagellin of P. avenae. A, alignment of the deduced amino acid sequences of N1141 flagellin (N1141-fla1; DDBJ accession number AB040139 for the gene) and H8301 flagellin (H8301-fla1; DDBJ accession number AB040140 for the gene). The hyphens indicate amino acids identical in N1141 and H8301 flagellins. The underlined N-terminal 20-amino acid sequence was determined by sequencing the purified flagellin, and double underlined sequences were determined by internal sequencing of fragment peptides obtained by lysylendopeptidase (Ly) and V8 protease (V8). B, detection of sugar moiety in N1141 and H8301 flagellins of P. avenae. SDS-PAGE detection by Coomassie Brilliant Blue R-250 (CBB) staining (left) and glycoprotein staining (right) is shown. Lane 1, flagellin of strain N1141; lane 2, flagellin
nuclei, however, increased slightly 6 h after inoculation, and approximately 18% of the nuclei had fluorescein isothiocyanate-derived fluorescence by 10 and 12 h after inoculation (Fig. 8). In contrast, the percentage of TUNEL-positive nuclei in rice cells inoculated with the \( D \) fla1141–2, K1-wild type, and \( D \) flaK1–3 did not increase until 12 h after inoculation (Fig. 8). Approximately 1.6% of TUNEL-positive nuclei were detected in water-treated control cells by the end of the assay (data not shown).

**DISCUSSION**

*P. avenae* is a devastating plant bacterial pathogen to staple crops such as rice and corn. However, little is known about the molecular mechanisms that determine the outcome of the interactions between *P. avenae* and plants. Using strain-specific antibodies, we identified flagellin as a candidate recognition molecule. When cultured rice cells were incubated with crude flagellin fractions or purified flagellin, the incompatible N1141 flagellin induced cell death more strongly and rapidly than the compatible H8301 flagellin. The cell death induction activities of N1141 cells were inhibited by anti-flagellin antibody but did not affect the H8301 strain reaction. Moreover, induction of cell death, accumulation of \( EL2 \) mRNA, and induction of nuclear DNA cleavage were reduced by the incompatible N1141 flagellin-deficient mutant but not by the compatible K1 flagellin-deficient mutant. Because the rice chitinase mRNA (\( Cht-1 \)) was not reduced in the N1141 flagellin-deficient mutant-inoculated cultured rice cells (data not shown), the reduction of several resistant responses by the N1141 flagellin-deficient mutant are

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**FIG. 5.** Cell death blocking assay using anti-N1141 flagellin antibody. A and B, effects of anti-N1141 flagellin antibody on cell death of cultured rice cells induced by N1141 (A) and H8301 (B) living cells. Fifty \( \mu \)l of anti-N1141 flagellin antibody were added into cultured rice cells together with N1141 and H8301 cells (10\(^6\) cfu/ml). Solid circles, bacteria alone; open circles, bacteria plus anti-N1141 antibody; solid squares, anti-N1141 antibody alone. C, effect of anti-N1141 flagellin antibody on cell death induced by N1141 flagellin fraction (1 \( \mu \)M). Each amount of anti-N1141 flagellin antibody (0, 25, 50 \( \mu \)l) was added into the medium of cultured rice cells together with 0.2 \( \mu \)M N1141 flagellin fraction. After a 12-h incubation, the number of dead cells in cultured rice cells was determined by Evans blue staining. Hatched bars, N1141 flagellin fraction; open bars, DW. Each bar represents S.E. of three independent experiments.

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**FIG. 6.** Cell death induction by flagellin-deficient mutants (\( \Delta \) fla1141–2 and \( \Delta \) flaK1–3) and wild-types (N1141 and K1). A, time course of cell death in cultured rice cells inoculated with N1141 wild-type (solid circles), \( \Delta \) fla1141–2 (open circles), and DW control (solid squares). B, time course of cell death in cultured rice cells inoculated with K1 wild-type (solid triangles), \( \Delta \) flaK1–3 (open triangles), and DW control (solid triangles). Each data point is the average of three independent experiments. Bars show S.E.

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of strain H8301; lane 3, horseradish peroxidase (positive control); lane 4, soybean trypsin inhibitor (negative control). Ten \( \mu \)g of protein were loaded in each lane, and a prestained protein marker was used for identification of molecular weights.
not due to the change in the contact with cultured rice cells by the movement loss caused from flagellar absence. Based on these experiments, we concluded that the hypersensitive cell death and EL2 mRNA accumulation in cultured rice cells are induced only by flagellin in the incompatible strain of *P. avenae*. The flagellin reception system in cultured rice cells should be necessary for such specific induction of resistance response by flagellin. Our findings should provide a key to understand-

![Figure 7](image7.png)

**Fig. 7.** Accumulation of EL2 mRNA in flagellin-deficient mutants (Δfla1141-2 and ΔflaK1-3) and wild-types (N1141 and K1).

RNA gel blot analysis of EL2 mRNA after treatment with N1141 and Δfla1141-2 (A), with K1 and ΔflaK1-3 (B). Total RNA was isolated from cultured rice cells after incubation with each bacteria. Fifteen μg of RNA were analyzed in each lane with EL2 cDNA as a probe. The relative intensity of the bands is indicated in the lower bar graph. Ethidium bromide-stained gels below blots show equal RNA loading before blotting.

In the mammalian immune system, the flagellin H antigen is highly variable, with various serotypes (47). The antigenic properties of flagella have been studied by selection and genetic analysis of spontaneous serum-selected flagella antigen mutants. These studies indicate that only a small portion of the flagellin molecule carries the flagellar antigen determinants (45). Therefore, the flagellin of many mammal pathogens such as *Salmonella* is the H antigen, one of the major antigens that elicits immune responses in infected mammal hosts (45), and antibodies produced against flagellin H antigen are associated with protection against bacterial invasion. Some mammal pathogens such as *Salmonella* spp. can escape the defense response of challenged cells if they have mutations in the flagellin amino acid sequence (46). It is quite interesting that the flagellins of phytopathogenic bacteria are involved in the induction of resistance responses in the case of plants.

In appropriate plants (13, 41, 42) to induce exchange of H⁺ and K⁺ across the plasma membrane (13) and to generate active oxygen species (43) when added to plant cell cultures, which are all properties of the HR elicited by live bacteria. Recently, Felix et al. (44) reported that a harpin-like protein that causes the alkalization of the tomato suspension culture medium persists in bacterial preparations of *P. syringae* pv. *tabaci* after heat denaturation by boiling. The N-terminal sequence of the harpin-like protein had no similarity to harpins but shared strong similarity with the N terminus of flagellin from other *Pseudomonas* species. The alkalization of the culture medium was also caused by a synthetic 22-amino acid peptide based on the sequence of the N-terminal conserved region of *P. aeruginosa* flagellin, suggesting that flagellin is a general elicitor produced by eubacteria because the flagellin of *P. syringae* pv. *tabaci* and the synthetic 22-amino acid peptide cause the medium alkalization of not only cultured tomato cells but also cultured cells of other species such as potato, tobacco, and *Arabidopsis*. Interestingly, neither the flagellin of *P. syringae* pv. *tabaci* nor the synthetic 22-amino acid peptide caused the alkalization of cultured rice cells (44). Our results indicate that hypersensitive cell death, EL2 mRNA accumulation, and nuclear DNA cleavage in cultured rice cells are specifically induced by flagellin molecules of the *P. avenae* incompatible strain. These two differing observations suggest that flagellin acts as both a general elicitor in some plant families and a specific elicitor in rice and perhaps other gramineae.
flagellin, indicating the possibility that the structural and functional features common to various flagella are determined by the conserved termini, whereas serological variability is determined by the middle part, especially in the hypervariable regions. On the basis of the electron density map of the flagellar filament obtained by x-ray fiber diffraction, Namba et al. (50) proposed three domains in flagellin, D1 to D3, from the center of the filament axis outwards in the radial direction. The core domain (D1) is responsible for filament assembly and polymorphism, and the middle domain (D2) may be related to the stability of the filament shape. The central domain (D3) of adjacent subunits in a filament is not connected to each other. This D3 domain corresponds with the hypervariable region located on the surface of the flagellar filament, and there are several lines of evidence showing that the exposed D3 domain contains the major epitopes of H antigen (51–54). The sequence analysis of P. avenae flagellin genes showed that all of the amino acid residues (14 amino acid residues) varying between the incompatible N1141 and the compatible H8301 strains are located in the D3 domains. In addition, the N1141 strain-specific antibody could recognize the N1141 flagellin but not H8301 flagellin (Fig. 2), indicating that the D3 domain of P. avenae flagellin contains the H antigen epitopes.

The glycosylation of flagellin protein was first confirmed in Campylobacter coli by mild periodate treatment and biotin hydrazide labeling (55, 56). It has been also reported that the glycosylation of flagellin is important in forming the specific epitope of H antigen. In variants of C. coli strain VC167, two antigenic flagellin types determined by serospecific antibodies have been described (termed T1 and T2) (56). Based on the DNA sequence of T1- and T2-encoding genes, the predicted amino acid sequences of the T1 and T2 flagellins showed that the T1 flagellin differs at two amino acid residues while T2 differs at three and that those sequence changes do not appear to be involved in the antigenic differences observed (56). Post-translational modification has been suggested to be responsible for the T1 and T2 epitopes, and flagellin from both T1 and T2 has been shown to be glycosylated. Mild periodate treatment of the two flagellins eliminated reactivity with T1- and T2-specific antibodies. However, mutation analysis demonstrated that sugar alone is not the specific epitope, suggesting that the epitope probably involves multiple glycosyl and/or amino acid residues (55). These experimental data indicate that the sugar moiety is important for flagellin recognition by antibody in the mammal defense system. The flagellins of strains N1141 and H8301 were also glycosylated (Fig. 4B), suggesting the possibility that the sugar moieties are involved in the specific recognition of flagellin by rice plants. Interestingly, induction of hypersensitive cell death in cultured rice cells was remarkably reduced in experiments with GST-fused N1141 flagellin in E. coli (data not shown). The loss of hypersensitive cell death induction of GST-fused flagellins was probably due to loss of normal conformation of the expressed flagellin by fusing the GST protein and/or absence of the sugar moiety. It seems likely that the parameters of flagellin as both general and specific elicitors lie within the N-terminal conserved region and the D3 hypervariable region of flagellin, respectively. The flagellin-switched mutants that introduced the K1 flagellin gene into ΔfliA1141 and introduced the N1141 flagellin gene into ΔfliA1K1 will provide important information to identify the sensory transduction site. To construct these switched mutants, the selection of a useful vector and promoter is necessary because such a suitable system in P. avenae has not been reported. Therefore, optimization of the transformation condition and selection of the useful vector and promoter are in progress. The sensory site of flagellin remains to be identified.

The flagellum consists of a helical filament and a hook, both of which are completely external to the cell. It also has a basal body composed of inner and outer rings that span the cytoplasmic membrane, periplasmic space, and outer membrane (57, 58). The bacterial flagellar filament is composed of a single kind of flagellin protein (59). After synthesis inside the cell, the flagellin monomer is believed to travel by a central channel through the rod, hook, and filament to be added to the filament at its tip. This fact raises the question of which type flagellin, the monomer or filament form, caused these numerous resistance responses in cultured rice cells. Usually, the reception system machinery of the elicitor is located on the cell wall or on the plasma membrane except for the receptor of a type III transfused elicitor such as the Avr protein (18, 19, 60, 61). Because the flagellin molecule is not thought to be secreted by the type III secretion system, the flagellin recognition system must be located on the cell wall or the plasma membrane. It would be difficult for the flagellin of the filament form to reach the plant cell surface without a specific transport system because the flagellum is a supramolecular structure. Since it would be easier for the flagellin of the monomer-type than for the filament-type flagellin to reach the plant cell surface, it is more likely that the resistance responses in cultured rice cells were induced by the monomer-type flagellin and that the induction of cell death by purified flagellin (Fig. 3) is due to the monomer-type flagellin contained in the flagellin fraction.

The host range of P. avenae is plant-specific rather than cultivar-specific (e.g., the H8301 rice-compatible strain can infect all tested rice cultivars). Cultivar specificity is often determined by induction of a specific plant resistance response controlled by a gene-for-gene type interaction (62); i.e., a recognition event mediated by a dominant resistance plant gene and a corresponding dominant pathogen avirulence gene leads to a host-resistance response that stops the growth and spread of the pathogen. It has been demonstrated that some host-species specificity is also controlled by gene-for-gene type interactions (63, 64). The highly restricted host specificity in strains of P. avenae indicates the existence of a host determination system controlled by one or very few genes. INF1 elicitin is a 10-kDa extracellular protein produced by Phytophthora infestans and belongs to a family of host-specific elicitor proteins of Phytophthora (65, 66). INF1 elicitin induced the HR in a restricted number of plants, particularly the genus Nicotiana. Using a single-step transformation procedure with an antisense construct of the inf1 elicitin gene, Kamoun et al. (67) reported that the recognition of INF1 elicitin is a major determinant of the resistance response of N. bentamiana to P. infestans and that the recognition of the elicitor protein INF1 by plants is a major factor in the determination of host specificity. The properties of the INF-1 protein are similar to those of P. avenae flagellin in many respects. The flagellin of P. avenae may be a major avirulent factor that determines the host-species specificity of P. avenae at the species level.

The cell death-inducing activity of the N1141 flagellin was comparatively low in comparison with N1141 whole cells or CE (Figs. 1 and 3) (23). Moreover, cell death induced by the N1141 flagellin-deficient mutant was more reduced than that of the N1141-wild type but did not disappear completely. These results indicated the possibility that although flagellin is a major factor in the induction of cell death, there is another elicitor-like substance in CE or incompatible whole cells. This idea is supported by the fact that the induction of rice chitinase mRNA was the same in both cultured rice cells inoculated with the N1141 flagellin-deficient mutant or the N1141 wild-type. Identifying this other elicitor-like substance will also be essential for understanding the molecular mechanisms of resistance re-
sponse induced by the incompatible strain of \textit{P. avenae} and the determination of host-species specificity of \textit{P. avenae}.

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REFERENCES

1. Kombrink, E., and Somschke, I. E. (1995) \textit{Adv. Bot. Res.} 21, 1–34

2. Agrinos, G. N. (1997) \textit{Plant Pathology}, 4th Ed., pp. 93–114, Academic Press, Inc., San Diego, CA

3. Yang, Y., Shah, J., and Klessig, D. F. (1997) \textit{Genes Dev.} 11, 1621–1639

4. Keller, H., Pamboudjian, N., Ponchet, M., Poupet, A., Delon, R., Varrier, J. L., Roby, D., and Ricci, P. (1999) \textit{Plant Cell} 11, 223–235

5. Gabriel, D. W., and Rees, M. W. (1959) \textit{Nature} 184, 56–57

6. Wieland, F., Paul, G., and Sumper, M. (1985) \textit{J. Biol. Chem.} 260, 15180–15185

7. Ryerson, D. E., and Heath, M. C. (1996) \textit{Plant Cell} 8, 393–402

8. Wang, H., Li, J., Bostock, R. M., and Gilchrest, D. G. (1996) \textit{Plant Cell} 8, 375–391

9. Gavriel, Y., Sherman, Y., and Ben-Sasson, S. A. (1992) \textit{J. Cell Biol.} 111, 493–501

10. McCabe, P. F., Levine, A., Meijer, P. J., Tapon, N. A., and Pennell, R. I. (1997) \textit{Plant J.} 12, 267–280

11. He, S. Y., Huang, H. C., and Collmer, A. (1993) \textit{Cell} 73, 1255–1266

12. Amlt, M., von Gijsegem, F., Heut, J. C., Pernollet, J. C., and Boucher, A. (1994) \textit{EMBO J.} 13, 543–553

13. Baker, C. J., Orlandi, E. W., and Mock, N. M. (1993) \textit{Plant Physiol.} 102, 1341–1344

14. Felix, G., Duran, J. D., Volko, S., and Boller, T. (1999) \textit{Plant J.} 18, 265–276

15. Stocker, B. A. D. (1991) \textit{Bacteriol. Rev.} 55, 359–387

16. Dangl, J. L., Dietrich, R. A., and Richberg, M. H. (1996) \textit{Plant Cell Physiol.} 37, 479–501

17. Tang, X., Frederick, R. D., Zhou, J., Halterman, A. D., Jia, Y., and Martin, G. B. (1996) \textit{Science} 274, 2063–2065

18. Turner, J. G., and Novacky, A. (1974) \textit{Phytopathology} 64, 885–890

19. Gonzalez, R. G., Haxo, R. S., and Schleif, T. (1980) \textit{Biochemistry} 19, 4299–4303

20. Tsuge, R., Okimatsu, Y., Wakasaki, and J. Ishibashi for help with all experiments. We are also grateful to T. Nakanishi and H. Sato for excellent technical support.