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N-terminal domain including conserved flg22 is required for flagellin-induced hypersensitive cell death in *Arabidopsis thaliana*

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Abbreviations: HR, hypersensitive reaction; PAMP, pathogen-associated molecular pattern

Abstract

Flagellin in *Pseudomonas syringae* is a potent elicitor of defense responses including hypersensitive cell death in dicot plants. Further, flg22, an oligopeptide consisting of 22 conserved amino acids near the N-terminus of flagellin is reported to induce plant defense responses. Because glycosylation of the central domain of flagellin affects its elicitor activity, we investigated whether any peptide sequence in addition to flg22 is required for flagellin-induced hypersensitive reaction. A study of recombinant flagellin polypeptides indicated that the N-terminal domain including the conserved flg22 is required for flagellin-induced hypersensitive cell death in *Arabidopsis thaliana.*

Keywords: *Arabidopsis thaliana*, flagellin, flg22, FLS2, HR cell death

Although plants have not acquired an immune system like that of animals, plants have developed a surveillance system to protect themselves against various phytopathogens. Thus, individual cells of plants possess the capacity to perceive microbial signals. The system that recognizes microbial pathogens of plants can be divided into major two phases. Cultivar-specific resistance based on a gene-for-gene theory in which resistance (R) proteins specifically detect pathogen-derived avirulence factors (Avr) is relatively well understood (Dangl and Jones, 2001). On the other hand, the common system among plant species to recognize microbial signals is perception of a pathogen-associated molecular pattern (PAMP) or "general elicitor" that induces plant defense responses to pathogens (Gómez-Gómez 2004; Zipfel and Felix 2005; Chisholm et al. 2006). The latter case is also known as nonhost resistance and the plant immune system (Jones and Takemoto 2004). Nonhost resistance has been classified into two types: type I and type II (Mysore and Ryu 2004). In type I nonhost resistance, no visible change is apparent, whereas hypersensitive reaction (HR) accompanying plant cell death in response to avirulent pathogens and/or elicitors is classified as type II nonhost resistance.

Flagellin, a major component of the flagellar filament in bacterial flagellum, is known as one of the bacterial PAMPs that induce defense responses in plants (Felix,

1999; Taguchi et al. 2003b) and animals (Ramos et al. 2004). In plants, the flg22 oligopeptide, which consists of 22 conserved amino acids near the N-terminus of flagellin, is reported to be the minimal motif required to induce defense responses in dicotyledonous plants such as tomato, *Arabidopsis thaliana*, and tobacco (Felix et al. 1999). Further, a receptor molecule for flg22, FLS2, has been identified in *A. thaliana* (Gómez-Gómez and Boller 2000).

In the classification system of nonhost resistance of Mysore and Ryu (2004), flg22 induces type I resistance, whereas flagellin induces type II resistance. Indeed, the defense responses induced by the flg22 and flagellin of *P. syringae* pv. *tabaci* on *A. thaliana* were distinct (unpublished). Flagellin rapidly induced generation of a high level of hydrogen peroxide in an Arabidopsis suspension cell culture, whereas flg22 only induced a low level of hydrogen peroxide. However, a molecular dissection of the elicitor-active domain of the flagellin protein of *P. syringae* has not been performed to date. Very recently, the amino acid sequence in the flagellin of *Xanthomonas campestris* pv. *campestris* required for elicitation in *A. thaliana* was identified in the region corresponding to flg22. Therefore, as a first step in defining the molecular motif in flagellin protein that induces plant defense, we generated recombinant flagellin polypeptides in *Escherichia coli* and investigated their elicitor activity in *A. thaliana*.

Flagellin protein comprises 282 amino acids that can be divided into five domains, D0a, D1a, D3, D1b, and D0b. Both domains D0a and D0b are located inside the flagellar filament, and their amino acid sequences are well conserved among flagellin proteins (Ichinose et al. 2004; Taguchi et al. 2006). The flg22 oligopeptide exists in the D0a domain. Recombinant flagellin peptides were expressed in *E. coli* BL21 (DE3) cells using a pET16b system (Novagen, Madison, WI, USA). In addition to whole flagellin protein, several partial polypeptides were produced. To construct the "Full (1-282)" polypeptide, PCR was carried out using P1 (5′-ttccatatgGCTTTAACAGTAAACAC-3′) and P4 (5′-cgggatccTACTGAAGCAGTTTCAGTAC-3′) as primers with the *fliC* gene of *P. syringae* pv. *tabaci* (Taguchi et al. 2003b) as the DNA template (Fig. 1A). In the same way, to construct a "ΔN (57-282)" polypeptide, which lacks the N-terminal D0a domain, PCR was carried out using P2 (5′-ttccatatgCAGATCCGTGGTCAGACAAT-3′) and P4 as primers, and to construct a "ΔNC (57-238)" polypeptide, which lacks both N- and C-terminal D0 domains, PCR was carried out using P2 and P3 (5′-cgggatccTACAGTGCAGCACTGGCGTTTT-3′) as primers. P1 and P2 primers possessed an artificial sequence for *Nde*I digestion, whereas P3 and P4 primers possessed an artificial sequence for *Bam*HI digestion, as shown in lowercase. After

amplification, PCR products were digested with *Nde*I and *Bam*HI and inserted into the pET16b plasmid vector. To produce the "NC (1-89 + 228-282)" polypeptide, which lacks all D3 and almost all D1a and D1b domains, a pET16b::full plasmid was digested with *Pst*I and self-ligated to remove the internal fragment.

These plasmids were introduced into *E. coli* BL21 (DE3). The resultant BL21 (DE3) was cultured in LB medium at 37°C overnight and then inoculated into new LB medium at 10^{-1} volume. After 1 h of incubation, isopropyl-1-thio-β-D-galactoside was added to induce the expression of recombinant polypeptides at the final concentration of 0.4 mM. After 3 h of incubation, bacterial cells were harvested, then solubilized in B-PER™ Reagent (Pierce, Rockford, IL). After centrifugation at 14,000 × *g* for 30 min, the insoluble fraction was resuspended in B-PER™ Reagent; then inclusion bodies were solubilized by the addition of lysozyme at a final concentration of 200 μg ml^{-1}, followed by a wash with a 10^{-1} dilution of the same reagent. After centrifugation, the recombinant flagellin polypeptides with a His-tag sequence were purified with Ni-NTA Spin Columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified polypeptides were dialyzed with water, then their molecular weight, quality, and quantity were confirmed by SDS-PAGE analysis (Fig. 1B). The flg22 oligopeptide (QRLSTGSRINSAKDDAAGLQIA) used by Felix et al. (1999) was chemically

synthesized by Funakoshi (Tokyo, Japan).

Recombinant flagellin polypeptide-induced cell death was investigated in leaves of *A. thaliana* ecotype Col-0. The wild-type Col-0 possesses *FLS2*, a gene encoding a receptor molecule for flg22. The *fls2* mutant was obtained from the Salk Institute (Alonso et al. 2003). The homozygous mutation of *FLS2* and the sequence of the insertion site were confirmed by PCR and sequencing. The insertion sites of two T-DNA lines (*fls2*#4:SALK_093905 and *fls2*#9:SALK_026801) were located in the coding region in the first exon.

Full and NC recombinant flagellin polypeptides caused chlorosis 5 days after infiltration of the leaves of the wild-type Col-0 but not of the *fls2* mutant (Fig. 2A). However, all other polypeptides and flg22 caused no any visible change in leaves of both the wild-type and *fls2* mutant. The cell death-inducing ability of recombinant polypeptides was examined by trypan blue staining. The wild-type leaves treated with Full, NC, or flg22 were stained with trypan blue, while *fls2* mutant leaves were not stained after any treatment (Fig. 2B).

Callose depositions were reported to be induced by flg22 treatment of the leaves of *A. thaliana* ecotype La-er, which possesses functional FLS2 (Gómez-Gómez and Boller, 2002). The leaves of the *A. thaliana* Col-0 wild-type and *fls2* mutant were

treated with the recombinant polypeptides and flg22. Only the combinations of wild-type leaves and the Full, NC, or flg22 peptides induced remarkable callose depositions (Fig. 3).

In this study, we found that FLS2 is required for the perception of not only the flg22 oligopeptide but also the flagellin polypeptide. Although flg22 in the D0a domain is expected to be localized on the inside of the flagellar tube, Arabidopsis has a surveillance system that detects microbial pathogens by recognition of a well-conserved region of the bacterial flagellum as one of the typical PAMPs. However, we also found that the flagellin of *P. syringae* is a glycoprotein (Taguchi et al. 2003a), and that the glycosylation affects the ability of flagellin protein to induce a defense response. For example, a nonglycosylated flagellin purified from the Δ*orf1* defective mutant of *P. syringae* pv. *tabaci* had reduced HR-inducing ability in its nonhost soybean plant (Taguchi et al. 2006), while a nonglycosylated recombinant flagellin of *P. syringae* pv. *tabaci* expressed in *E. coli* significantly induced HR cell death in its host tobacco (Taguchi et al. 2003b). Further, the Δ*orf1* mutant of *P. syringae* pv. *tabaci* had reduced ability to induce defense response in its nonhost *A. thaliana* (Ishiga et al. 2005). These results indicate that the efficiency of recognition of flagellin via flg22-FLS2 molecules may be affected by glycosylation of the flagellin protein. However, the recombinant

polypeptides containing potentially glycosylated residues such as ΔN and ΔNC did not induce cell death in Arabidopsis leaves (Fig. 2AB). Because the recombinant polypeptides do not possess glycans, non-glycosylated polypeptides in the central domain of flagellin do not involve in the induction of HR cell death.

The result obtained by Fig. 2AB indicates that *FLS2* is required for both induction of visible change and cell death of Arabidopsis leaves by the treatment with flagellin polypeptides, Full and NC. Thus FLS2 is indispensable for the signaling by flagellin. Although it was described that the flg22 oligopeptide did not induce hypersensitive cell death (Gómez-Gómez and Boller, 2002), it induced trypan blue staining 24 h after treatment, but not visible change 5 days after treatment. Recently it was reported that flg22 induced the endocytosis of its receptor FLS2, followed by degradation. Thus signaling triggered by flg22 is transient and attenuates after internalization of receptor (Robatzek et al. 2006). As compared with the treatment of flg22, flagellin might maintain the effect longer. The cell death detected by trypan blue staining induced by the treatment with recombinant polypeptides or flg22 was inhibited with the concomitant presence of 15 μM cycloheximide, an inhibitor of protein synthesis (Fig. 2C). This result indicates that the cell death is not passive. Further investigation is needed to elucidate the molecular mechanism of the modulation of

flagellin recognition.

Acknowledgments

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Figure legends

Fig. 1. Production of recombinant flagellin polypeptides. A, Constructs of recombinant flagellin polypeptides. Position and direction of PCR primers (P1 to P4) are shown with arrows. Numbers indicate the positions of amino acids from the N-terminus. Location of five domains and the flg22 oligopeptide are indicated. Four types of recombinant flagellin polypeptides, Full, ΔN, ΔNC, and NC, were expressed in *E. coli*. **B**, SDS-PAGE analysis of recombinant flagellin polypeptides. Recombinant polypeptides were separated on 14% SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. 1: Full, 2: ΔN, 3: ΔNC and 4: NC.

Fig. 2. Recombinant flagellin polypeptides-induced hypersensitive cell death in Arabidopsis leaves. Wild-type or *fls2* mutant of *A. thaliana* ecotype Columbia-0 (Col-0) were grown at 22°C with an 18 h photoperiod and used for experiments after 4 weeks cultivation. The left half of the leaves was infiltrated with water as a control, 2 μM recombinant polypeptides (Full, ΔN, ΔNC and NC), or flg22 peptide by syringe infiltration, then incubated for 5 days (**A**), or for 24 h, then stained with lacto-phenol trypan blue as described previously (Koch and Slusarenko 1990) (**B**). Effect of cycloheximide on flagellin- and flg22-induced cell death (**C**). The left half of the leaves

of wild-type *A. thaliana* ecotype Columbia-0 (Col-0) was infiltrated with a combination of water as a control, 2 μM recombinant polypeptides (Full) or flg22 and 15 μM cycloheximide by syringe infiltration, then incubated for 24 h, then stained with lacto-phenol trypan blue.

Fig. 3. Microscopic observation of callose deposition. Arabidopsis leaves treated with 2 μM recombinant polypeptides or flg22 peptide by syringe infiltration were incubated for 24 h, then stained with aniline blue according the method described by Adam and Somerville (1996). Scale bar is 500 μm.

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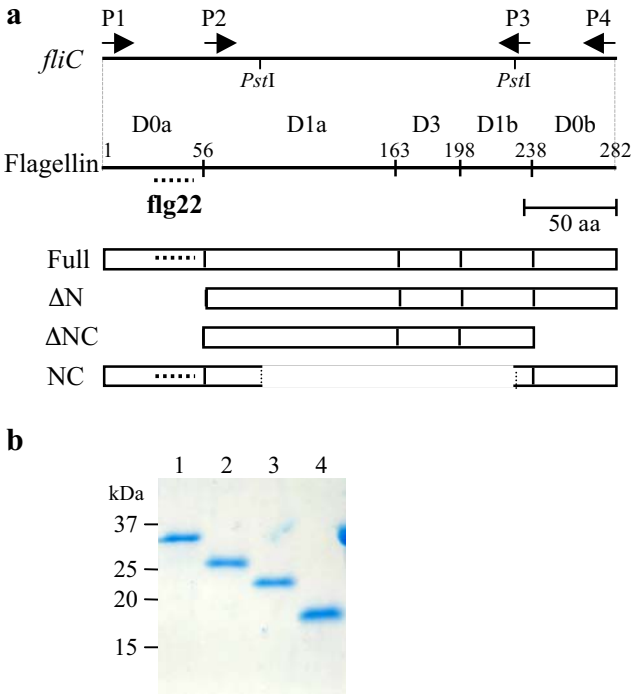
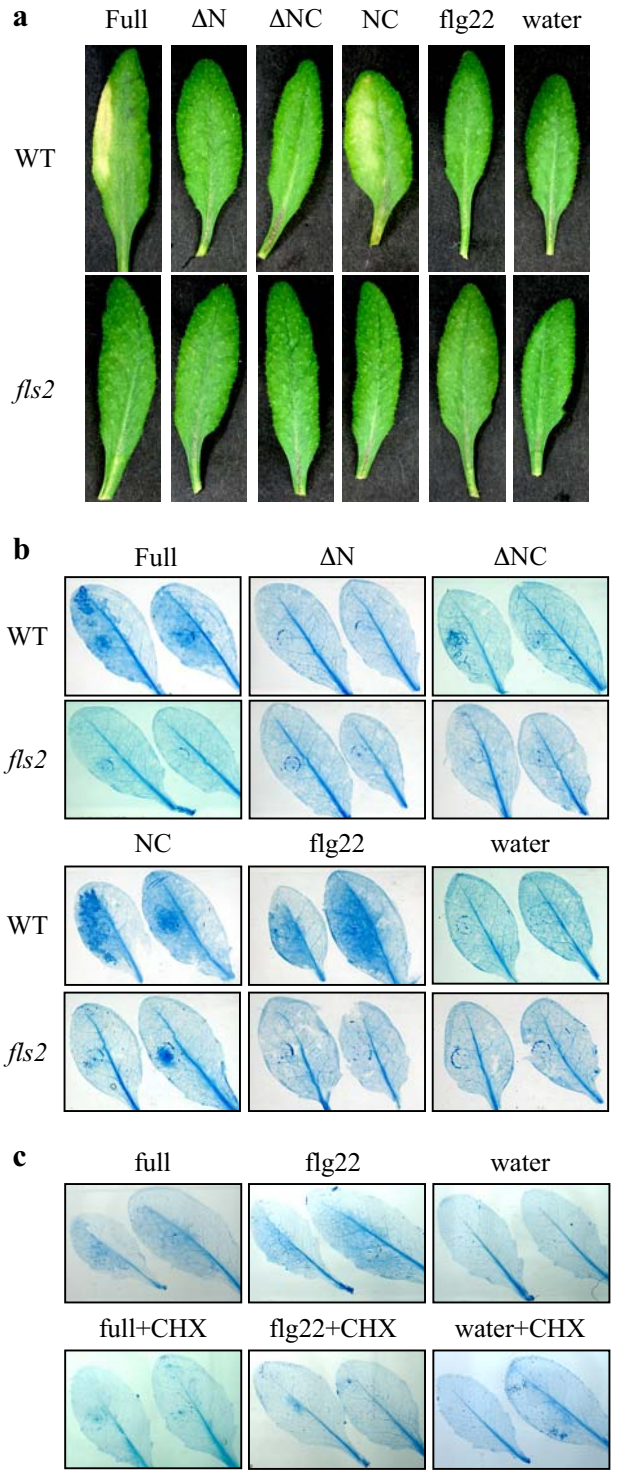
Fig. 1




a
Full
ΔN
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fls2
b
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flg22+CHX
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Fig. 3