Glycan moiety of flagellin in Acidovorax avenae K1 prevents the recognition by rice that causes the induction of immune responses

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Recognition of pathogen-associated molecular patterns (PAMPs) such as flagellin, a main component of the bacterial flagellum, constitutes the first layer of plant immunity and is referred to as PAMP-triggered immunity (PTI). The rice avirulent N1141 strain of gram-negative phytopathogenic bacterium, Acidovorax avenae, induces PTI including H2O2 generation, while flagellin from the rice virulent K1 strain of A. avenae does not induce these immune responses. Mass spectrometry analyses revealed that total 1,600-Da and 2,150-Da of glycan residues were present on the flagellins from N1141 and K1, respectively. A deglycosylated K1 flagellin induced immune responses in the same manner as N1141 flagellin, suggesting that the glycan in K1 flagellin prevent epitope recognition in rice. We identified three genes in K1 flagella operon, which regulate structural modification of glycan in K1 flagellin. The immature glycan-attached flagellin from three genes deletion mutant, KD3FG, induced H2O2 generation in cultured rice cells, whereas the K1 mature-type flagellin did not cause a detectable increase in H2O2. The data indicate that the immature glycan of flagellin from KD3FG cannot prevent the epitope recognition in rice.

Specific Induction of PTI by Flagellin of A. avenae

Plants are continuously confronted with diverse potential pathogens, but actual infection occurs only in certain limited cases. Plant can recognize pathogens via two perception systems. In the first layer, plant recognize pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors, leading to PAMP-triggered immunity (PTI). The second layer immune system, termed effector-triggered immunity (ETI), is caused by recognition of effector proteins, which are injected into the host cell from bacteria cell through the Type III secretion system (T3SS). By definition, PAMPs are conserved across a wide range of microbes, which may or may not be pathogenic. Because these molecules are essential for viability or lifestyle, microbes are less likely to evade host immunity through mutation or deletion of PAMPs, compared with virulence effectors. PAMPs include structures characteristic from pathogens such as β-glucan, polysaccharide chitin, ergosterol, lipopolysaccharides (LPS), flagellin and elongation factor-Tu. Among these PAMPs, has been the most extensively studied in regards to the recognition mechanism and signal transduction. We previously reported that flagellin from the rice avirulent N1141 strain of gram-negative phytopathogenic bacterium, Acidovorax avenae, induces PTI including H2O2 generation, while flagellin from the rice virulent K1 strain of A. avenae does not induce. Because recombinant flagellins of N1141 and K1 equally induced H2O2 generation in cultured rice cells, we examined posttranslational modification of flagellins from N1141 and K1. Mass spectrometry analysis revealed that N1141 flagellin and K1 flagellin had total 1,600-Da and 2,150-Da of glycan residues, respectively. Therefore, we next clarified whether the glycosylation of flagellins in A. avenae affects the induction specificity of the immune response in rice cells. Both deglycosylated flagellins derived from flagellin glycosyltransferase gene deficient mutants of N1141 and K1 induced H2O2 generation in cultured rice cells. Furthermore, we identified four glycosylated-amino acid residues (178Ser, 183Ser, 212Ser and 351Thr) in K1 flagellin. In the mutants of Ala-substituted flagellins at four glycosylated amino acid position (178Ser/Ala, 183Ser/Ala, 212Ser/Ala and 351Thr/Ala), 178Ser/Ala and 183Ser/Ala and 183Ser K1 flagellins induced the immune response in cultured rice cells, indicating that the glycans at 178Ser and 183Ser in K1 flagellins prevent epitope recognition in rice. Interestingly, mass spectrometry analysis using flagellins from the...
that causes the induction of immune responses.16

The K1 glycosyltransferase disrupts flagellin recognition by rice
lin. These data clearly indicate that the glycan moiety linked by
not induce H₂O₂ to the same degree as the K1 wild-type flagel-
genation, while the flagellin from the
gramm-of K1 flagellin, the glycosylation island, a genetic region
mated to be 550. The K-type flagellin purified from the
molecular weight of each glycan chain in N1141 flagellin is esti-
glycan chain in K1 flagellin is predicted to be 540, while the
lin deficient strain) revealed that the molecular weight of each
molecule-of K1 are involved in structural modification of glycan

Figure 1. Structures of the flagellin glycosylation island in the A. avenae K1. The predicted methyltransferase gene (Fmt) are indicated by hatched pentagon, the predicted carbamoylphosphate synthase gene (Fcs) are indicated by gray pentagon, and the predicted sugartransaminase gene (Fst) are indicated by black pentagon.

Identification of Genes that Regulate Glycan Modification of K1

To clarify the prevention mechanism of the epitope site by
glycan of K1 flagellin, the glycosylation island, a genetic region
required for flagellin glycosylation, within the flagellar gene operon of A. avenae K1 were determined with DNA sequencing. The glycosylation island of flagellin in A. avenae K1 consists of four orfs: designated Fgt, Fmt, Fcs, and Fst. Fgt encodes putative glycosyltransferase and the Fgt-deletion K1 strain produced deglycosylated flagellin.16

The proteins encoded by Fmt, Fcs, and Fst had predicted molecular masses of 27,013-, 50,796- and 41,817-Da, respectively, and showed homology to type 12
methyltransferase of Pseudomonas putida W619, carbamoylphosphate synthase of Bacillus pumilus SAFR-032, and sugartransaminase of P. fulva 12-X (Fig. 1).

To examine whether the Fmt, Fcs, and Fst genes are responsi-
ble for the specific recognition by rice, we generated the three
genesis-deletion mutant using homologous recombination and
designated KΔ3FG. To determine the glycan structure in the flagellin of KΔ3FG strain, MALDI-TOF MS analysis was per-
formed. The mass spectrum of the K1 wild-type flagellin showed
that the molecular mass of the mature-type K1 flagellin is
51,225, which is greater than the calculated molecular mass by
approximately 2,150 (Fig. 2). Mass spectrometry analysis also
showed that the molecular mass of the KΔ3FG flagellin is
50,495, which are also greater than the calculated masses by
approximately 1,420 (Fig. 2). The mass spectrum data revealed
that glycan of KΔ3FG are smaller than that of glycan from K1
flagellin. Three genes, Fmt, Fcs, and Fst within flagellin glycosyla-
tion island of K1 are involved in structural modification of glycan
of K1 strain.

It previously reported that flagellin from Pseudomonas syringae
pv. tabaci 6605 (Pta 6605) was attached glycan chains composed of
disaccharide (modified viosamine (mVio)-rhamnose-rham-
nose).17 Moreover, viaA (dTDP-viosamine aminotransferase),
viOB (dTDP-viosamine acetyltransferase), viOM (methyltransfer-
ase), viOR (3-oxoacyl-(acyl-carrier protein) reductase), vioS (3-
-oxoacyl-(acyl-carrier protein) synthase III) and acp (acyl-carrier protein) genes were identified as biosynthetic genes of mVio. Flagg
ellin from ΔvioR mutant of Pta 6605 was attached with rhamnose
and glycan without modified viosamine and flagellin from
ΔvioM of Pta 6605 was attached with demethylated mVio-rham-
nose-rhamnose.18 Several common properties including pre-
dicted function and sequence homology were observed between
two genes (vioR and viaM) of Pta 6605 and three genes (Fmt,
Fcs, and Fst of A. avenae K1 strain). These suggest the possibility
that three genes (Fmt, Fcs, and Fst of A. avenae K1 strain) are
involved in modification of the glycan. In addition, molecular
weight of each glycan chain attached on flagellin from KΔ3FG
was 730 smaller than that of glycan chain attached on mature flagellin from K1 strain. These data together with the properties of
Fmt, Fcs, and Fst genes indicate that Fmt, Fcs, and Fst may be
involved in modification of the non-reducing terminal group of
glycan chain. Determination of glycan chains attached on flagellin of KΔ3FG will be helpful to confirm the possibility about
function of Fmt, Fcs, and Fst genes.
the motility of rice cells were treated with the immature glycan-attached flagellin affects the specific induction of PTI in rice. When the cultured bacteria and was similar to the parental strains, suggesting that the deletion of Fmt, Fcs, and Fit genes does not affect the swimming motility (Fig. 3A). We next examined whether structural modification of K1 flagellin glycan by deletion of Fmt, Fcs, and Fit genes affects the specific induction of PTI in rice. When the cultured rice cells were treated with the immature glycan-attached flagellin from KΔ3FG, H2O2 was rapidly generated, whereas the K1 mature-type flagellin did not cause a detectable increase in H2O2 until 3 h after treatment (Fig. 3B). These results clearly indicate that the immature glycan of flagellin from KΔ3FG cannot prevent the epitope recognition in rice. An identification of the glycan structure attached with flagellin in the A. avenae K1 will be important to further understand the specific recognition mechanism of flagellin by rice.

Prevention of Epitope Recognition by the Immature Glycan of K1 Flagellin

To clarify role of Fmt, Fcs, and Fit genes, the motility of KΔ3FG was examined based on a swimming assay on soft agar plates. KΔ3FG strain had a diffuse spreading growth pattern that is characteristic of motile bacteria and was similar to the parental strains, suggesting that the deletion of Fmt, Fcs, and Fit genes does not affect the swimming motility (Fig. 3A). We next examined whether structural modification of K1 flagellin glycan by deletion of Fmt, Fcs, and Fit genes affects the specific induction of PTI in rice. When the cultured rice cells were treated with the immature glycan-attached flagellin from KΔ3FG, H2O2 was rapidly generated, whereas the K1 mature-type flagellin did not cause a detectable increase in H2O2 until 3 h after treatment (Fig. 3B). These results clearly indicate that the immature glycan of flagellin from KΔ3FG cannot prevent the epitope recognition in rice. An identification of the glycan structure attached with flagellin in the A. avenae K1 will be important to further understand the specific recognition mechanism of flagellin by rice.

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