Chemistry

Biochemistry fields

Okayama University *Year* 2008

Modulation of defense signal transduction by flagellin-induced WRKY41 transcription factor in Arabidopsis thaliana

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Mol. Genet Genomics

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ii) Running title: *Arabidopsis* WRKY41 in defense response

iii) Key words: flagellin, flg22, FLS2, MAMP signaling pathway, WRKY41

Abstract

Flagellin, a component of the flagellar filament of *Pseudomonas syringae* pv. *tabaci* 6605 (*Pta*), induces hypersensitive reaction in its non-host *Arabidopsis thaliana*. We identified the *WRKY41* gene, which belongs to a multigene family encoding WRKY plant-specific transcription factors, as one of the flagellin-inducible genes in *A. thaliana*. Expression of *WRKY41* is induced by inoculation with the incompatible pathogen *P. syringae* pv. *tomato* DC3000 (*Pto*) possessing *AvrRpt2* and the non-host pathogens *Pta* and *P. syringae* pv. *glycinea* race 4 within 6 hr after inoculation, but not by inoculation with the compatible *Pto*. Expression of *WRKY41* was also induced by inoculation of *A. thaliana* with an *hrp*-type three secretion system (T3SS)-defective mutant of *Pto*, indicating that effectors produced by T3SS in the *Pto* wild-type suppress the activation of *WRKY41*. *Arabidopsis* overexpressing *WRKY41* showed enhanced resistance to the *Pto* wild-type but increased susceptibility to *Erwinia carotovora* EC1. *WRKY41*-overexpressing *Arabidopsis* constitutively expresses the *PR5* gene, but suppresses the methyl jasmonate-induced *PDF1.2* gene expression. These results demonstrate that WRKY41 may be a key regulator of defense signals, and may contribute to optimization of defense-signal transduction in response to different pathogens that possess different infection strategies.

Introduction

It is well known that plants have the ability to perceive environmental microorganisms and to mount defense responses (Chisholm et al. 2006; Jones and Dangl 2006). Plant defense responses include the hypersensitive reaction (HR) involving rapid and localized plant cell death, oxidative burst, and defense gene expression, which are triggered by specific avirulence (*Avr*) gene-mediated elicitors and also by non-specific elicitors. Studies of resistance (*R*) gene-mediated defense responses based on a gene-for-gene theory have focused on the specific interactions between a particular pathogen carrying a specific *Avr* and a plant cultivar that has a corresponding *R* gene. This highly specific recognition system, which includes an Avr protein and an R protein, is genetically determined without flexibility (Jones and Dangl 2006).

Recently, we found that flagellin from *Pta* acts as a major inducer of HR in non-host plants. Namely, 1) the purified flagellin of *Pta* induced HR cell death in its non-host tomato plant but not in the host tobacco plant (Taguchi et al. 2003a, 2003b), 2) Δ*fliC*, a flagellin-defective mutant of *Pta*, lost its motility and the ability to induce HR cell death in the tomato plant (Shimizu et al. 2003), 3) a recombinant flagellin polypeptide including the N-terminal domain showed elicitor activity (Naito et al. 2007), and 4) the HR-inducing ability of flagellin of *Pta* was independent of the type three secretion system (T3SS) (Marutani et al. 2005). On the other hand, flagellins of *P. syringae* pv. *glycinea* race 4 (*Pgl*) and *P. syringae* pv. *tomato* DC3000 (*Pto*) induced HR cell death, oxidative

burst, defense gene expression, and DNA fragmentation in the non-host tobacco plant (Taguchi et al. 2003b). Thus, the effects of flagellin from *Pta* and *Pgl* on tobacco plant are distinct, although the amino acid sequences of flagellins from *Pta* and *Pgl* are identical (Taguchi et al. 2003a). We found that flagellins from *P. syringae* are glycoproteins and that the flagellin glycosylation affects the virulence and ability to induce HR (Ishiga et al. 2005; Taguchi et al. 2006; Takeuchi et al. 2003).

The flagellin from *Pta* has been reported to induce one of the plant defense responses, alkalinization of the culture medium, in a variety of dicotyledonous plants (Felix et al. 1999). Felix et al. further found that a synthetic 22 amino acid oligopeptide, flg22, the sequence of which is conserved near the N-terminus in the flagellin of *P. aeruginosa*, induced alkalinization more strongly than the flagellin protein did (Felix et al. 1999). Flg22 induced not only alkalinization but also oxidative burst, callose deposition, ethylene production, expression of defense-related genes, and resistance against *Pto* in *A. thaliana* (Felix et al. 1999; Zipfel et al. 2004); however, it did not induce HR cell death (Gomez-Gomez and Boller 2002). The recognition of flg22 was mediated by a leucine-rich-repeat (LRR) receptor-like kinase (RLK), FLS2, in *A. thaliana* ecotype Col-0 but not in Ws-0 due to the lack of a functional FLS2 (Zipfel et al. 2004). The Flg22 signal is transmitted thorough the MAPK cascade, and induces gene expression encoding downstream transcription factors, WRKY22 and WRKY29 (Asai et al. 2002). Transient overexpression of *WRKY29* leads to enhanced resistance to *P. syringae* pv. *maculicola* and *Botrytis cinerea* (Asai et al. 2002), indicating

that flg22-induced transcription of WRKY29 effectively provides resistance to both fungal and bacterial pathogens.

The family of WRKY transcription factors is plant-specific, and all known WRKY proteins contain either one or two WRKY domains (Eulgem and Somssich 2007). They can be classified on the basis of both the number of WRKY domains and features of the Zinc-finger-like motif. WRKY proteins with two WRKY domains belong to group I, whereas most WRKY proteins with one WRKY domain and a C2H2 zinc finger motif belong to group II, and WRKY proteins with one WRKY domain and a C2HC zinc finger motif belong to group III. Previously, cyclopedic expression profiles of the *Arabidopsis WRKY* gene superfamily were investigated during defense responses (Dong et al. 2003), and thus it was found that 49 of the 72 *WRKY* genes were regulated by pathogens and/or salicylic acid (SA). Furthermore, *WRKY group I* genes such as *WRKY25* and *33* (Andreasson et al. 2005; Zheng et al. 2006; Zheng et al. 2007; Lippok et al. 2007) and many *WRKY group II* genes, such as *WRKY7, 11, 17, 18, 22, 29, 40* and *60* (Chen and Chen 2002; Asai et al. 2002, Journot-Catalino et al. 2006; Kim et al. 2006; Xu et al. 2006; Shen et al. 2007) were shown to be involved in the regulation of plant disease resistance. As mentioned above, genes encoding the WRKY group II transcription factors WRKY22 and 29 were also reported to be involved in the flg22-mediated signaling pathway leading to resistance to *P. syringae* and *B. cinerea* (Asai et al. 2002). Furthermore, *WRKY18, 40* and *60* have been implicated in repressing basal

defense to virulent *P. syringae* (Xu et al. 2006; Shen et al. 2007).

The expression profiles of all 13 *WRKY group III transcription factor* genes have been investigated (Kalde et al. 2003). The results indicated that the expression of almost all *WRKY group III* genes was attributable to pathogen infection and SA production. Recently, energetic analyses of the WRKY group III subfamily have been performed. For example, expression of *WRKY70* was activated by SA and repressed by jasmonic acid (JA) and was reported to function as a node of the convergence for JA- and SA-mediated signal transductions in plant defense (Li et al. 2004). It is well known that the SA-mediated signaling pathway activates resistance to biotrophic pathogens, whereas the JA-mediated pathway activates resistance to necrotrophic pathogens (Kunkel and Brooks 2002). WRKY70 acts as an activator of SA-induced genes and also as a repressor of JA-responsive genes, and it enhanced resistance to the fungal biotroph *Erysiphe cichoracerum*, but reduced resistance to fungal necrotroph *Alternaria brassicicola* (Li et al. 2004; Li et al. 2006). Very recently, the functions of two members of the WRKY group III transcription factors were reported: WRKY53 is involved in leaf senescence (Miao and Zentgraf 2007), and WRKY62 negatively regulates JA-responsive gene expression (Mao et al. 2007). However, very little is known about signal transduction pathways leading to the expression of *WRKY group III* genes and the target genes of each WRKY group III factor.

In this study, we investigated expression of one *WRKY group III* gene, *WRKY41*, to reveal a

flagellin-mediated signaling pathway and the effect of overexpression of *WRKY41* to clarify the downstream signaling pathway.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. *P. syringae* strains were grown and maintained on King's B (KB) agar medium at 27ºC. For the inoculation experiments, two overnight cultures of *P. syringae* strains on MG agar medium (agar 15 g/l, mannitol 10 g/l, L-glutamic acid 2 g/l, KH_2PO_4 0.5 g/l, NaCl 0.2 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, pH 7.0) were harvested using a cell scraper (Iwaki, Tokyo, Japan); then, the cell density was appropriately adjusted with 10 mM $MgSO_4$ for use as inocula. *E. carotovora* strains were grown and maintained on YP agar medium (agar 15 g/l, yeast extract 5 g/l, peptone 10 g/l, pH 6.8) at 27ºC. For the inoculation experiments, overnight cultures of *E. carotovora* strains on YP medium were harvested using a cell scraper; then the cell density was appropriately adjusted with 0.9% NaCl for use as inocula.

Preparation of flagellin protein and flg22 oligopeptides

Flagellin protein was prepared from *Pta* as described by Taguchi et al. (2003b) with minor modifications. Briefly, *Pta* was incubated in LB containing 10 mM $MgCl_2$ for 48 hr at 25ºC.

Harvested bacteria cells were suspended and further incubated for 24 hr in minimal medium (50 mM potassium phosphate buffer, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl [pH 5.7]) supplemented with 10 mM (each) mannitol and fructose. Then, flagella were sheared off by vortexing and separated from the bacteria by centrifugation at 10,000 x g for 30 min, and the supernatant was filtered through a 0.45 μm pore filter for sterilization. The filtrate was further centrifuged at 100,000 x g for 30 min, and the resultant pellet was suspended in water as a crude flagella fraction. To obtain the further-purified flagellin, the crude flagella fraction was treated with 100 mM glycine-HCl buffer (pH 2.0) for 30 min on ice, then centrifuged at 100,000 x g for 30 min. The supernatant was collected, and the buffer was exchanged to 50 mM potassium phosphate buffer (pH 7.0) with Vivaspin6 (Vivascience, Hannover, Germany). An oligopeptide, flg22 (QRLSTGSRINSAKDDAAGLQIA), used by Felix et al. (1999) was chemically synthesized by Funakoshi (Tokyo, Japan) and Hokkaido System Science (Sapporo, Japan).

Plant materials

A. thaliana ecotype Columbia-0 (Col-0) was grown at 22ºC with an 18 hr photoperiod and used for experiments 4 weeks after cultivation. The *fls2*- and *wrky41*-null mutants were obtained by analysis of independent T-DNA insertional mutants from the Salk Institute (San Diego, CA, USA). The homozygous mutations of *FLS2* and *WRKY41* were confirmed by PCR, and the sequences of the

insertion sites were determined. T-DNA sequences were found in the first exon in two mutant lines (*fls2#4*:SALK_093905 and *fls2#9*:SALK_026801) and in the third exon in one mutant line (*wrky41*#6:SALK_068648). A suspension culture of *A. thaliana* (Col-0) cell line T87, provided by the Plant Cell Bank (RIKEN, Tokyo, Japan), was grown at 22ºC under continuous illumination and routinely inoculated into No. 5 medium every 2 weeks.

Elicitor treatment and bacterial inoculation

Flagellin protein and the flg22 oligopeptide were applied to 5-day-old suspension-cultured cells, and incubated on a rotary shaker at 22ºC or infiltrated into *Arabidopsis* leaves by a needle-less syringe infiltration method.

For preparation of the bacterial inoculum, *P. syringae* strains were suspended in 10 mM $MgSO_4$ with 0.02% Silwet L77 (OSI Specialties, Danbury, CT, USA) at a density of 2×10^8 cfu/ml. To observe visible changes of *A. thaliana* and to measure bacterial propagation, whole *Arabidopsis* plants were dipped into the bacterial suspension. After successive incubations, detached leaves were sterilized with 15% H_2O_2 to detect the bacterial population in the apoplastic spaces, and the leaves were then washed well with sterilized water and homogenized in water. Serial dilutions of the homogenates were placed on MG agar plates and the visible colonies were counted 48 hr after incubation. To analyze gene expression profiles after inoculation with *P. syringae*, bacterial strains

were suspended in 10 mM $MgSO_4$, and the bacterial suspension (2 x 10^8 cfu/ml) was infiltrated into the leaves using a needle-less syringe as described by Huang et al. (1988). *E. carotovora* strains were suspended in 0.9% NaCl, and adjusted to an OD_{600} of 0.001. To observe visible changes of the leaves in *A. thaliana*, 10 µl of bacterial suspension was dropped onto the leaves by an auto-pipette as described by Li et al. (2004). After successive incubations, the disease index was scored according to disease symptoms.

Cloning of WRKY41, vector construction and plant transformation

To clone the coding region of *WRKY41* (At4g11070), the gene was amplified by PCR with the forward primer 5'-TCTAGAATGGAAATGATGAATTGGGAGCG-3' (underlined letters indicate an artificial *Xba*I site) and the reverse primer 5'-GAGCTCTTAAATCGAATTGTGGAAAAAAG-3' (underlined letters indicate an artificial *Sac*I site). The PCR-amplified DNA fragment was cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions, and the DNA insertion was verified by restriction enzymes and sequence analyses. The recombinant pGEM-T Easy plasmid DNA was digested with *Xba*I and *Sac*I, and *WRKY41* DNA was inserted into the binary transformation plasmid pBI121 (Clontech, Palo Alto, CA, USA) by replacing the β-*glucuronidase* gene. The resultant plasmid possessing a 35S promoter governing the *WRKY41* gene was

introduced into *Agrobacterium tumefaciens* strain LBA4404.

A. thaliana wild-type (Columbia-0) was transformed with *A. tumefaciens* by the vacuum infiltration method (Clough and Bent, 1998). Seeds collected from the *A. tumefaciens*-infected plants were sterilized with 2% sodium hypochlorite and 0.01% Tween20 (Sigma, Tokyo, Japan) for 10 min and washed three times with sterilized water. The sterilized seeds were placed on Murashige and Skoog (MS) agar medium containing 50 μg/ml kanamycin (Wako, Tokyo, Japan). Transgenic T1 seedlings were selected on MS plates; after 3 weeks of growth, the existence of the transgene was confirmed by PCR analysis, and T3 homologous lines were used for further experiments.

Application of MeJA

Methyl jasmonate (50 μM in 0.25% ethanol [v/v]) was sprayed on whole plants. MeJA-treated plants were immediately placed in a tray with a transparent lid. Control plants were treated with water containing 0.25% ethanol (v/v). Leaves were collected at the time points indicated.

RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was purified from *A. thaliana* suspension-cultured cells and leaves using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

One microgram of total RNA was used to synthesize the first strand cDNAs for 2 hr at 42ºC

in a 30 µl of a reaction mixture containing 5 U of AMV RTase (Takara, Otsu, Japan), 2.5 mM each dNTPs, 1 µg oligo(dT)$_{12-18}$, 40 units of ribonuclease inhibitor (Takara), 25 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol. For semi-quantitative RT-PCR, 1 µl of first strand cDNA was diluted with four volumes of the reaction mixture, and 1 µl of the diluted cDNA was used as a template for PCR in 25 µl of 12.5 µl PCR Master Mix (Promega, Madison, USA) and 0.4 mM of each gene-specific primer. Gene-specific primers and PCR cycles are shown in Supplementary Table 1. PCR was performed with one denaturation cycle of 5 min at 95ºC and appropriate cycles of 30 sec at 95ºC, 30 sec at 60ºC, and 30 sec at 72ºC. To avoid saturation of the PCR product, the number of reaction cycles was reduced. Ten microliters of the PCR product was subjected to 1.5% agarose gel electrophoresis. To verify that equal amounts of cDNA were used in each reaction, PCR was performed with primers corresponding to the elongation factor-1α (*EF-1α*) gene (*At5g60390*), which is constitutively expressed.

Results

Flagellin induces expression of WRKY group III transcription factor genes in *A. thaliana*

To reveal the signal transduction pathway after perception of flagellin in *Arabidopsis*, we carried out DNA microarray analysis using *Arabidopsis* 2 (Agilent Technologies, Palo Alto, CA, USA) to screen the genes in which expression is up-regulated by flagellin treatment in T-87 *Arabidopsis*

suspension-cultured cells. DNA microarray analysis suggested that genes encoding three WRKY group III transcription factors (WRKY41, WRKY53, and WRKY55) were up-regulated after treatment of T-87 suspension-cultured cells with 2 μM flagellin for 1 hr (data not shown).

To investigate the expression profiles of these *WRKY group III* genes, we prepared total RNA from T-87 suspension-cultured cells treated with flagellin and sodium phosphate buffer (50 mM, pH 7.0) as a control for 1, 3, or 6 hr, and then carried out semi-quantitative RT-PCR. *WRKY41*, *WRKY53*, and *WRKY55* genes were immediately and strongly expressed after flagellin treatment, then diminished 3 hr after treatment. When 2 μM flg22 was applied to T-87 cells, the expression of *WRKY41* and *WRKY55* genes was not observed, and the expression of *WRKY53* was weakly induced 1 and 3 hr after treatment (Fig. 1A).

Because FLS2 was identified as a receptor kinase for flg22 in *A. thaliana* (Gomez-Gomez and Boller 2000), the expression of *WRKY41*, *WRKY53*, and *WRKY55* genes in response to the treatment with flagellin and flg22 was investigated in the leaves of *A. thaliana* Col-0 wild-type and its *fls2* mutant (Fig. 1B). Flagellin induced mRNA accumulations of *WRKY41*, *WRKY53*, and *WRKY55* at 1 and 3 hr after treatment in the Col-0 wild-type, but not in the *fls2* mutant. These results suggest that flagellin-induced expression of these *WRKY* genes depends on flg22/FLS2 interactions. The expression of these *WRKY* genes was also induced by treatment with flg22, but the induction was transient compared to the treatment with flagellin. Although the reason of differential

expression profiles of *WRKY* genes in flg22-treated cell cultures and leaves is obscure, differences in gene expression are also observed in response to treatment with flg22 between suspension cells and seedlings (Navarro et al. 2004).

Compatible strain of *P. syringae* specifically suppresses expression of *WRKY41* gene

To investigate the regulation of group III *WRKY* genes expression by *P. syringae*, we analyzed the expression of Arabidopsis group III *WRKY* genes (*WRKY41*, *WRKY53*, and *WRKY55*) in response to inoculation with different strains of *P. syringae* (Fig. 2). Inoculation of the leaves with non-host pathogens of *A. thaliana* such as *Pta* and *Pgl* induced the expression of *WRKY41* and *WRKY55* at 6 hr after inoculation, whereas inoculation with *Pto*, a compatible pathogen of *A. thaliana* Col-0, did not induce expression of these genes. To examine the involvement of T3SS effectors in the suppression of flagellin-induced expression of *WRKY41*, *A. thaliana* was inoculated with a T3SS-deficient mutant of *Pto*, Δ*hrcQ-U* (Badel et al. 2006), and investigated the expression of group III *WRKY* genes. Semi-quantitative RT-PCR analysis showed that the Δ*hrcQ-U* mutant strain induced *WRKY41* gene expression, suggesting that *Pto* suppressed the expression of *WRKY41* by the effector proteins secreted by a T3SS (Fig. 2B). When the leaves were inoculated with *Pto* possessing the *avirulence* gene *AvrRpt2,* the expression of *WRKY41* and *WRKY55* was induced, like the inoculation with *Pta* (Fig. 2A). Interestingly, expression of *WRKY53* was weakly induced by the

inoculation with any strain of *P. syringae*.

Generation of *WRKY41*-overexpressing *Arabidopsis thaliana*

To investigate function of WRKY41 during defense responses, *Arabidopsis* was transformed with CaMV 35S promoter-*WRKY41* DNA construct. Overexpression of *WRKY41* was confirmed with RT-PCR analysis in the independent lines, and no significant differences in expression and other phenotypes were observed between the lines (Fig. 3). Constitutive expression of *PR5* was also observed in *WRKY41*-overexpressing *Arabidopsis*.

Arabidopsis overexpressing *WRKY41* shows enhanced disease resistance to virulent *Pseudomonas* but decreased resistance to *Erwinia carotovora*

To determine the contribution of WRKY41 to disease resistance in *A. thaliana*, we examined the resistance of the transgenic plants that overexpress *WRKY41* in response to the virulent hemi-biotrophic bacterial pathogen *Pto* (Fig. 4) and the necrotrophic bacterial pathogen *Erwinia carotovora* subsp. *carotovora* (*Ecc*) EC1 (Fig. 5). Inoculation of wild-type *A. thaliana* with *Pto* caused obvious disease symptoms with leaf chlorosis, whereas the *WRKY41*-overexpressing *A. thaliana* was not drastically changed after inoculation with this pathogen. Furthermore, propagation of *Pto* in the *WRKY41*-overexpressing plant was significantly lower than that in the wild-type

Arabidopsis at 5 days after inoculation (Fig. 4). Enhanced disease resistance of *A. thaliana* to *P. syringae* is often accompanied by the accumulation of elevated levels of transcripts of *PR* genes associated with SA-mediated defense pathway (*PR1*, *PR2*, and *PR5*) (Uknes et al. 1992, Li et al. 2004). Elevated expression of *PR5* may contribute to enhanced disease resistance to *Pto*.

On the contrary, the *WRKY41*-overexpressing plant showed decreased resistance to *Ecc* EC1 (Fig. 5). *Ecc* EC1 is a soft-rot pathogen that causes serious damage to a wide variety of crop species such as carrot, celery, chicory, and potato (Hossain and Tsuyumu 2006). *E. carotovora* also causes soft rot on *A. thaliana* (Li et al. 2004). To evaluate susceptibility of *Arabidopsis* plants to *Ecc* EC1, we categorized disease symptoms on three different levels by their severity. After inoculation with drops of 10 µl of bacterial suspension, disease symptoms were evaluated. The wild-type *Arabidopsis* developed severe diseased symptoms in only less than 10% of inoculated regions at 24 hr after inoculation with *Ecc* EC1, and in 30% at 48 hr after inoculation. Thus more than 50% of the inoculated regions in wild-type *Arabidopsis* showed no symptoms even 48 hr after inoculation. However, *WRKY41*-overexpressing *Arabidopsis* developed severe diseased symptoms in 55-70% of the inoculated regions at 24 hr after inoculation and in 70-75% at 48 hr after inoculation. These data suggest that the constitutive expression of *WRKY41* results in opposite effects on the resistance to two bacterial pathogens, *Pto* and *Ecc*.

Effect of overexpression of *WRKY41* on JA signaling pathway

Because the expression of *PDF1.2* is known to be induced by treatment with JA and methyl jasmonate (MeJA), we investigated the expression of *PDF1.2* in the leaves after treatment with MeJA. As shown in Fig. 6, the expression of *PDF1.2* was induced in wild-type *Arabidopsis* at 3 hr after treatment with MeJA, whereas *WRKY41*-overexpressing plants did not respond to MeJA at all. These results suggest that WRKY41 at least partially suppresses the JA-mediated signaling pathway.

Discussion

In this study, we found that flagellin derived from *P. syringae* rapidly and transiently induces the expression of *WRKY41, WRKY53*, and *WRKY55*, and that the flagellin-induced expression of *WRKY* genes requires *FLS2* (Fig. 1), since the expression was abolished in the *Arabidopsis fls2*-mutant.

Expression of *WRKY41* was induced by non-host pathogens such as *Pta* and *Pgl* at 6 hr after inoculation (Fig. 2). However, activation of *WRKY41* was abolished by inoculation with *Pto*, a compatible pathogen of *A. thaliana* Col-0. Recently, a number of studies revealed that bacterial effectors secreted by a T3SS suppressed host immune responses that were triggered by microbe-associated molecular patterns (MAMPs) (He et al. 2006, da Cunha et al. 2006, Nomura et al. 2006). Because the expression of *WRKY41* was observed in Δ*hrcQ-U* mutant-inoculated *A.*

thaliana, *WRKY41* also seems to be suppressed by the T3SS-derived effectors. In an incompatible interaction, expression of *WRKY41* was again induced by the inoculation with *Pto* possessing *avrRpt2,* indicating that Avr protein also induced *WRKY41* expression in a gene-for-gene theory–dependent manner. These results indicate that there are plural pathways leading to the transcriptional activation of the *WRKY41* gene.

Although the activation of *WRKY55* expression was abolished by the inoculation of *Pto*, expression of *WRKY55* was not restored by inoculation with the T3SS-defective Δ*hrcQ-U* mutant of this pathogen, indicating that there is another factor that suppresses the activation of *WRKY55* expression (Fig. 2). It is known that besides T3SS effectors, coronatine, a phytotoxin that is produced by *Pto*, also suppresses flg22-induced host immunity, including the activation of *NHO1* expression (Li et al. 2005) and stomatal closure (Melotto et al. 2006). These results indicate that coronatine is one of the plausible candidates to suppress induced plant immunity. However, AvrRpt2/RPS2-mediated resistance activated not only the expression of *WRKY41* but also that of *WRKY55*, suggesting that gene-for-gene resistance overcomes the suppression effects of effectors secreted by T3SS and other systems.

WRKY41-overexpressing plants exhibited constitutive expression of the *PR5* gene (Fig. 3). Because expression of the *PR5* gene was also up-regulated with flg22 treatment (Asai et al. 2002) and the treatment of *Arabidopsis* with flg22 resulted in enhanced disease resistance to a compatible

pathogen, *Pto* (Zipfel et al. 2004), activation of *PR5* may contribute in part to enhanced resistance in flg22-treated plants, as reported by Zipfel et al. (2004) and as we observed in *WRKY41*-overexpressing plants in this study (Fig. 4). Because overexpression of *WRKY29* enhanced resistance to *P. syringae* pv. *maculicola* and *Botrytis cinerea* (Asai et al. 2002), WRKY41 may contribute to this resistance together with other flg22-induced transcription factors such as WRKY29.

On the other hand, the JA-mediated signaling pathway leading to *PDF1.2* gene expression was suppressed in *WRKY41*-overexpressing plants (Fig. 6). It was reported that the activation of the JA-mediated signaling pathway contributes to resistance to the necrotrophic bacterial pathogen *E. carotovora* (Kunkel and Brooks 2002). In general, WRKY transcription factors bind to a W box existing in the promoter region of the target genes (Eulgem and Somssich 2007). However, the promoter sequences (1.1 kb ATG-upstream) of *PR5* and *PDF1.2* genes are almost completely lacking in W boxes (Supplementary Table 2), indicating that these genes are not direct targets of WRKY41.

We also observed that overexpression of *WRKY41* suppressed resistance to *Ecc* EC1, probably through repression of the JA-mediated signaling pathway, although the mechanisms of the suppression by WRKY41 are obscure. It is well known that JA and SA antagonize each other (Niki et al. 1998, Gupta et al. 2000). In this connection, WRKY70, one of group III WRKY proteins in *A.*

thaliana, is reported to repress JA-mediated defense responses (Li et al. 2004). WRKY70 functions as a node of the convergence for JA- and SA-mediated signals in plant defense. Namely, WRKY70 activates expression of SAR-related genes and resistance to the biotrophic fungal pathogen *Erysiphe cichoracearum* but suppresses expression of JA-responsive genes and resistance to the necrotrophic fungal pathogen *Alternaria brassicicola* (Li et al. 2006). Because the WRKY70-mediated suppression of JA-induced defense genes was partly mitigated in the *npr1* mutant background, NPR1 is required for the suppression of WRKY70-mediated resistance to *A. brassicicola* (Li et al. 2006). These results indicate that WRKY41-mediated JA signaling suppression may also require NPR1. Furthermore, the JA-mediated signaling pathway also contributes to the virulence of *Pto*, because coronatine and some T3SS effectors need a Coi1-dependent signaling pathway (He et al. 2004). Therefore, the reduction of the JA-signaling pathway (Fig. 6) might accompany the reduction of virulence of *Pto*, as we observed in *WRKY41*-overexpressing *Arabidopsis* (Fig. 4).

In this study, although *WRKY41*-overexpressing *Arabidopsis* had enhanced disease resistance to *Pto*, increased susceptibility to *Ecc* EC1, and altered gene expression, a *WRKY41*-knockout mutant did not show any difference in phenotype (data not shown) compared to the wild-type, indicating that other WRKY transcription factors may complement the function of WRKY41. Similar to WRKY70, WRKY41 also showed opposite effects on the hemi-biotrophic bacterial pathogen and the necrotorophic one. Recently, Wang et al. found that *WRKY70* was one of

the genes regulated by NPR1 (Wang et al. 2006). However, *WRKY41* was not identified as a target of NPR1 using *Arabidopsis* microarray analysis (Wang et al. 2006). Therefore the expression of *WRKY41* and *WRKY70* is differentially regulated, although each overexpressing *Arabidopsis* showed a similar phenotype; thus, in addition to WRKY70, WRKY41 may contribute to optimization of defense signal-transduction pathways to different pathogens that possess different infection strategies as one of the key regulators of *Arabidopsis* basal resistance.

Acknowledgments

We thank Dr. A. Collmer (Cornell University, USA), Dr. J. L. Dangl (North Carolina University, USA), and Dr. S. Tsuyumu (Shizuoka University, Japan) for providing *Pto* wild-type and its Δ*hrcQ-U* mutant, *Pto*::*avrRpt2*, and *Ecc* EC1, respectively. We are also grateful to the Leaf Tobacco Research Laboratory, Japan Tobacco Inc., the Plant Cell Bank of The Institute of Physical and Chemical Research (RIKEN), and the Salk Institute Genomic Analysis Laboratory for providing *Pta* and T-87 *A. thaliana* suspension-cultured cells and seeds for *fls2*- and *WRKY41*-mutants, respectively. This work was partly supported by Grants-in-Aid for Scientific Research (S) (No. 15108001) and (B) (No. 18380035) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) and the Okayama University COE program "Establishment of

Plant Health Science".

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Table 1 Bacterial strains used in this study

Bacterial strain	Relevant characteristics	Reference or source
Pseudomonas syringae pv. *tabaci*		
Isolate 6605	Wild type, tobacco isolate from Nagasaki Prefecture, Japan, spontaneous deletion *hrpZ*	Taguchi et al. (2001)
Pseudomonas syringae pv. *tomato*		
DC3000	Wild type, race 0, Rif^r	He et al. (1993)
DC3000::*AvrRpt2*	Wild type, race 0, containing *avrRpt2* gene, Rif^r, Km^r	Mackey et al. (2003)
CUCP5113	Δ*hrcQbRSTU*::Sp^r, Rif^r Sp^r	Badel et al. (2006)
Erwinia carotovora subsp. *carotovora*		
EC1	Wild type	Hossain and Tsuyumu (2006)

Rif^r, Km^r and Sp^r = rifampicin, kanamycin and spectinomycin resistant, respectively.

Figure legends

Fig. 1. Expression profiles of flagellin-inducible *WRKY* III genes in *A. thaliana* suspension-cultured cells (**A**) and in leaves of *A. thaliana* Col-0 wild-type and *fls2*-knockout mutant (*fls2-4*) (**B**). Suspension cultured cells and leaves were treated with 50 mM sodium-phosphate buffer (pH 7.0), as a control, 2 μM monomer flagellin, or 2 μM flg22. The *EF-1α* gene was used as an internal control for an equal volume of cDNA., Total RNA was isolated from the leaves at the times indicated and used for RT-PCR. The data presented are the representative result obtained from three replicates.

Fig. 2. Expression profiles of flagellin-inducible *WRKY* III genes in the leaves of *A. thaliana* Col-0. Wild-type Col-0 was syringe-infiltrated with 10 mM $MgSO_4$ as a mock control and with several strains at a bacterial density of 2 x 10^8 CFU/ml. The *EF-1α* gene was used as an internal control for an equal volume of cDNA. Total RNA was isolated from the leaves at the times indicated and used for RT-PCR. The data presented are the representative result obtained from three replicates.

Fig. 3. Expression profiles of *WRKY41* and *PR5* in the leaves of *A. thaliana* Col-0 wild-type and *WRKY41*-overexpressing lines. Total RNA was isolated from the leaves and used for RT-PCR. The *EF-1α* gene was used as an internal control for an equal volume of cDNA.

Fig. 4. Inoculation of *Arabidopsis* Col-0 wild-type or *WRKY41*-overexpressing line with virulent

pathogen, *Pto*. (**A**) Bacterial growth in wild-type and transgenic overexpressing plants. Plants were inoculated with a suspension of *Pto* (2 x 10^8 cfu/ml in 10 mM $MgSO_4$) by dip inoculation. The means and standard errors were calculated from four independent experiments. (**B**) Photos of representative inoculated leaves were taken 5 days post inoculation.

Fig. 5. Inoculation of *Arabidopsis* Col-0 wild-type or *WRKY41*-overexpressing lines with the necrotrophic bacterial pathogen *Ecc* EC1. (**A**) Evaluation of disease symptoms. Level 0 indicated no symptoms, level 1 indicated disease symptoms restricted to within the inoculated region, and level 2 indicated disease symptoms outside the inoculated region. (**B**) Disease symptoms were scored on at least 14 drops on each plant 24 and 48 hr post inoculation. The values represent the average of three replicate samples.

Fig. 6. Expression profiles of *PDF1.2* gene in the leaves of *A. thaliana* Col-0 wild-type and *WRKY41*-overexpressing lines. Total RNA was isolated from the leaves at the times indicated and used for RT-PCR. Leaves were treated with 50 μM methyl-jasmonate (MeJA) and 0.25% ethanol as a control. The data presented are the representative result obtained from three replicates.

Fig. 1
Higashi et al.

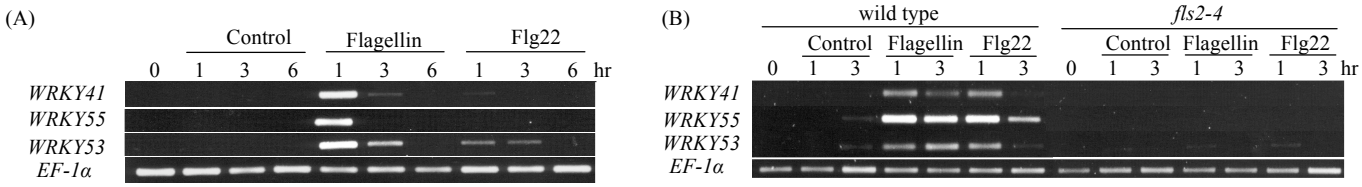
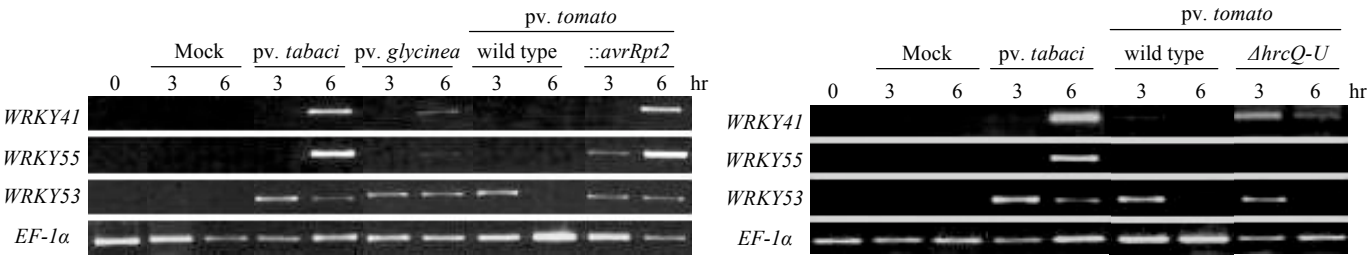


Fig. 2
Higashi et al.



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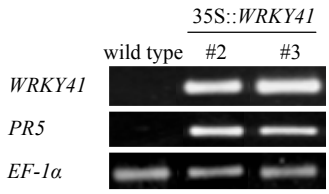
Higashi et al.



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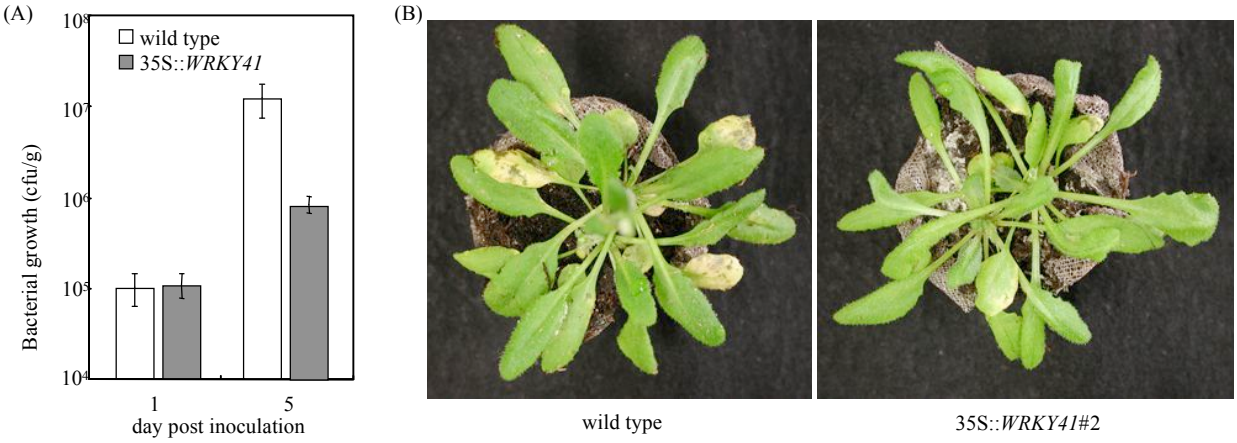

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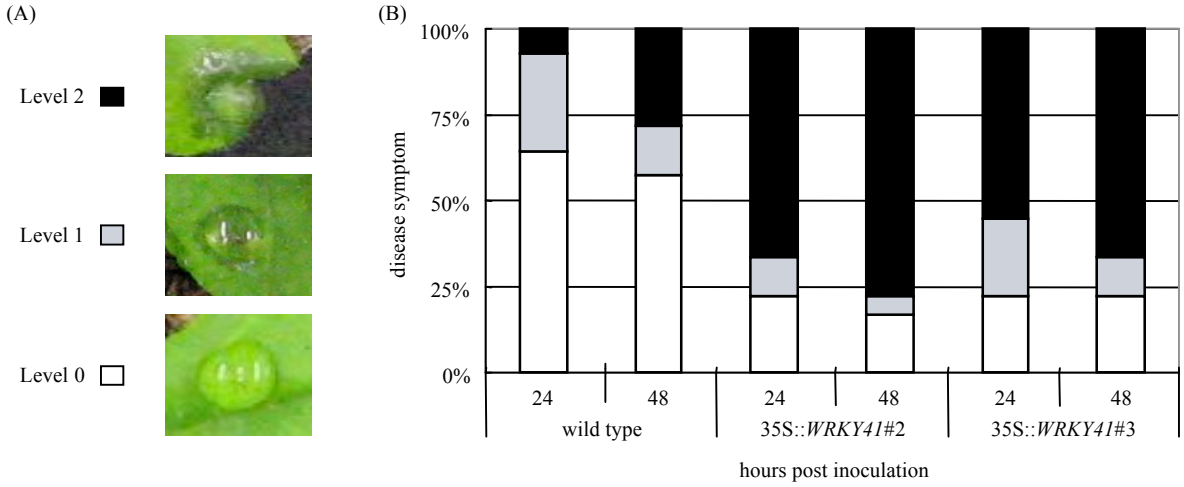

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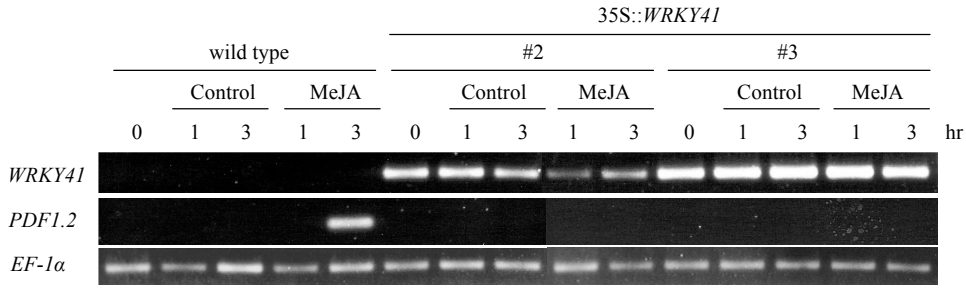

Supplementary Table 1 Primers used in this study for semi-quantitative RT-PCR

Gene name	AGI number	FW primer	RV primer	Size (bp)	PCR cycles				
					Fig. 1A	Fig. 1B	Fig. 2	Fig. 3	Fig. 6
WRKY41	At4g11070	ATTGGGAGCGGAGGAGTTTGC	CTCACTTGCTCTGTCCACTTTGG	371	26	28	32	26	26
WRKY53	At4g23810	CGGCAGTGTTCCAGAATCTC	ACCGTAGCATCCCCGTCTGA	336	24	26	26	-	-
WRKY55	At2g40740	CGCTAAGGACGGGGAACACA	TTCCGACCCCGCCGCTACCAAA	354	26	28	28	-	-
PR5	At1g75040	CTCCAGTATTCACATTCTCTTCCTCG	GCCTACTAGAGTGAATTCAGCCAG	337	-	-	-	26	-
PDF1.2	At5g44420	TAAGTTTGCTTCCATCATCACCC	GTGCTGGGAAGACATAGTTGCAT	209	-	-	-	-	26
EF-1a	At5g60390	GGTAACGGTTACGCCCCAGT	GCCTTGGTGACCTTGGCTCC	302	24	24	24	24	24