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Suppression of UDP-glycosyltransferase-coding *Arabidopsis thaliana* UGT74E2 Gene Expression Leads to Increased Resistance to *Pseudomonas syringae* pv. *tomato* DC3000 Infection

Hyo Jun Park¹, Chang Seob Kwon², Joo-Yong Woo¹, Gil-Je Lee¹, Young Jin Kim¹ and Kyung-Hee Paek^{1*}

¹Plant Signaling Network Research Center, School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, Korea

²Department of Chemistry and Biology, Korea Science Academy of KAIST, Busan 614-822, Korea

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Plants possess multiple resistance mechanisms that protect themselves against pathogen attack. To identify unknown components of the defense machinery in *Arabidopsis*, gene-expression changes were monitored in *Arabidopsis thaliana* under 18 different biotic or abiotic conditions using a DNA microarray representing approximately 25% of all *Arabidopsis thaliana* genes (www.genevestigator.com). Seventeen genes which are early responsive to salicylic acid (SA) treatment as well as pathogen infection were selected and their T-DNA insertion mutants were obtained from SALK institute. To elucidate the role of each gene in defense response, bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 was inoculated onto individual T-DNA insertion mutants. Four mutants exhibited decreased resistance and five mutants displayed significantly enhanced resistance against *Pst* DC3000-infection as measured by change in symptom development as compared to wild-type plants. Among them, member of uridine diphosphate (UDP)-glycosyltransferase (UGT) was of particular interest, since a *UGT* mutant (*At1g05680*) showed enhanced resistance to *Pst*-infection in *Arabidopsis*. In systemic acquired resistance (SAR) assay, this mutant showed enhanced activation of SAR. Also, the enhanced SAR correlated with increased expression of defense-related gene, *AtPRI*. These results emphasize that the glycosylation of UGT74E2 is a part of the SA-mediated disease-resistance mechanism.

Keywords : *Arabidopsis*, *Pseudomonas syringae*, salicylic acid, systemic acquired resistance, UDP-glycosyltransferase, UGT74E2

In nature, plants and pathogens keep up with developing a variety of weapons to attack each other for their successful survival during life time. Plants, as a defender, are under continuous biotic stresses derived from diverse invaders

including bacteria, fungi, viruses, and insect herbivores. Plant pathogens can generally be divided into three classes: biotrophs, necrotrophs, and hemibiotrophs according to their lifestyles. Biotrophic pathogens cause minimal damage to the host cell, although disease symptoms usually occur as a result of nutrient exhaustion. Necrotrophs, in contrast, rely upon dead host cell for reproduction and nutrient. However, many pathogens behave as both biotrophs and necrotrophs. Hemibiotrophs are biotrophic in one stage of the infection cycle and necrotrophic in another stage of invasion cycle (Glazebrook, 2005; Hancock and Husiman, 1981; Spoel et al., 2007). In response to those invaders, plants operate complex defense mechanisms at the level of molecular, cellular, and whole-plant (Beckers and Spoel, 2006; Jones and Dangl, 2006). These sophisticated defense responses commonly involve the harmonious transcriptional activation of multiple genes and the phytohormones including salicylic acid, jasmonic acid, and ethylene are involved. The specific disease resistance response often includes the activation of the hypersensitive response (HR) and development of systemic acquired resistance (SAR) (Dangl and Jones, 2001).

Salicylic acid (SA) is a well known crucial component that is associated with plant disease resistance. SA level increases in plant tissue following pathogen infection, and exogenous treatment of SA triggers both induced resistance to broad range of pathogens and the accumulation of pathogenesis-related proteins (Kunkel and Brooks, 2002). Outbreak of attack by certain pathogens generates activation of SA-dependent signaling. Plant resistance to biotrophic pathogens is normally thought to be mediated through SA signaling (Loake and Grant, 2007). In *Arabidopsis thaliana*, two genes, *PAD4* and *EDS1*, are needed for activating SA accumulation in response to SA-inducing stresses (Falk et al., 1999). Previous reports indicate that the majority of SA is produced by way of *SID2* which encodes isochlorismate synthase (Wildermuth et al., 2001). *EDS5* is also essential for production of SA against pathogen attack (Ferrari et al., 2003; Rossi et al., 1998). The experiment of transgenic

*Corresponding author.

Phone) +82-2-3290-3440, FAX) +82-2-928-1274

E-mail) khpaek95@korea.ac.kr

NahG, which encodes a bacterial (*Pseudomonas putida*) salicylate hydroxylase, reveals that SA is required for activation of defense-related genes such as *pathogenesis-related gene1 (PR1)* and for induction of SAR (Delaney et al., 1994; Gaffney et al., 1993). When SA levels are increased, *NPR1*, which acts downstream from SA, is converted to active monomer and this signaling regulated the conformation of *NPR1* by S-nitrosylation (Mou et al., 2003; Tada et al., 2008). Activated *NPR1* then is localized to the nucleus, where it interacts with TGA transcription factor, ultimately leading to the activation of *PR1* (Spoel et al., 2003). Even though there are many efforts to dissect SA mechanism in plants, the complete mechanism is not fully defined yet.

Recognition of pathogen often causes a localized resistance reaction, including HR, a programmed cell death (PCD), at site of invasion. Previous report showed that this local resistance spreads to the non-infected site (Ross, 1961). SAR requires transmittance of a signal from infected tissue to the systemic leaves. At first, SA was thought to be a SAR-inducing signal because application of exogenous SA triggers defense response including expression of *pathogenesis-related (PR)* genes, HR, and restriction of pathogen movement (Dempsey et al., 1999). However, the grafting experiment in tobacco showed that transgenic root stock, although unable to accumulate SA, was completely capable of delivering a signal that rendered non-transgenic scions resistant to secondary pathogen infection (Vernooij et al., 1994). Moreover, previous studies suggested that signaling might occur through the conversion of SA to the volatile compound methyl salicylate in tobacco (Park et al., 2007; Shulaev et al., 1997). These results indicate that SA itself is not a mobile signal for SAR.

In plant biological processes, modification of phytohormones, including salicylic acid, jasmonic acid, and ethylene, by glycosylation, methylation, and amino acid conjugation, is regarded as an integral control process.

It was known that most pathogen-induced SA is glycosylated by uridine diphosphate (UDP)-glycosyltransferase (UGT) to form non-toxic SA 2-O- β -D-glucoside (SAG). Recent studies suggest that methylation and amino acid conjugation as well as glycosylation of SA play a specific role in plant defense (Dean et al., 2005; Loake and Grant, 2007). UGTs convey the transfer of glycosyl residues from activated nucleotide sugars to a wide range of acceptor molecules such as secondary metabolites including SA and phytoalexins. Secondary metabolites accomplish multiple functions in plants, including lignifications, UV protection, herbivore protection, and disease resistance. It is supposed that UGTs are involved in bioactivity, solubility and transport of such secondary metabolites within the cell and throughout the plant. This protein constitutes a large gene

family in higher plants. It was reported that there are over 120 and 165 UGT genes in *Arabidopsis thaliana* and *Medicago truncatula*, respectively. The UGTs are classified by the presence of a carboxy-terminal 42 amino acid consensus sequence that is thought to be involved in binding of the protein to the UDP part of the sugar nucleotide. Recently, the PSPG-box, that is a signature sequence of glycosyltransferase, was found in *Dorotheanthus bellidiformis*. This consensus sequence also can be identified in ORFs from animal, plant, yeast, and bacterial genomes. A phylogenetic analysis reveals the presence of 14 distinct groups (A to N) of UGTs in *Arabidopsis*. Among these family members, at first only group D was reported to be associated with disease resistance against some pathogens in *Arabidopsis*. A UGT group D encompasses 13 members in *Arabidopsis*. And it was reported that expression of *UGT73B3* and *UGT73B5* genes is necessary for resistance to *Pseudomonas syringae* pv. *tomato* in *Arabidopsis*. In recent years, a number of UGTs were characterized in *Arabidopsis* including *UGT73C5*, *UGT73C6*, *UGT73B2*, and so forth. And also, down-regulation of tobacco glycosyltransferase gene (*TOGT1*) showed decreased resistance to *Tobacco mosaic virus* (TMV). (Chong et al., 2002; Gachon et al., 2005; Langlois-Meurinne et al., 2005; Ma et al., 2007; Ross et al., 2001). Despite of these efforts for connecting UGTs in plant defense mechanism, the exact role of UGTs remains unclear.

In this study, we performed a reverse genetic screening for finding SA- and *Pst*- inducible genes, and functional analysis of one of *Arabidopsis* UGT gene family members, *UGT74E2*. *UGT74E2* was obtained throughout T-DNA insertion mutant screening of bacterial growth assay by using *Arabidopsis-Pseudomonas* interaction as a model system during the process of local- and systemic-resistance. Our finding suggested that *UGT74E2* plays an important role in local and systemic resistance in *Arabidopsis*.

Materials and Methods

Plant materials and treatments. *Arabidopsis thaliana* ecotype Columbia-0, 21 different kinds of T-DNA insertion mutants, *npr1*-mutants, and *NahG* transgenic plants were grown in pot containing soil. The plants were grown in growth room with 16 h light and 8 h dark (long-day condition) at 23-25°C for 4-5 weeks before carrying out experiments. For sterile culture, seeds were surface-sterilized in 70% ethanol for 3 min, treated with 25% commercial clorax containing 0.05% Tween 20 (ICI Americas Inc., USA) for 5 min, and rinsed five times with sterile water. Then, the seeds were planted onto MS medium and grown in growth chamber for 3-4 weeks before bacterial inoculation. The pots and the plates were stored at 4°C for 72 h to ensure uniform germination.

Table 1. Arabidopsis gene-specific primer pairs for mutant genotyping

AGI	Polymorphism	Sequence
<i>At5g11920</i>	SALK_152299	Forward: AAGTCACAGTTGTTGCCGAAC Reverse: TGGTTTACTGCATTACCTCAAG
<i>At1g29020</i>	SALK_062045	Forward: GGTTTCTTTGGCGGAATTATC Reverse: CAGAATCAAATAAAAAGAGCGTTTC
<i>At5g45070</i>	SALK_143717	Forward: TATGGTTCGCGTTTGCAG Reverse: TTCATGAACTCTCTTTCGTTCC
<i>At1g50090</i>	SALK_070362	Forward: CAACTTTGAGGTTCAAGCCTG Reverse: ATGCGAATGTAAAATGGGATG
<i>At4g35180</i>	SALK_027033	Forward: TTGACCAAAACATCCGAAGAG Reverse: AAACCAAGAAGCATTGCACTG
<i>At4g10500</i>	SALK_059907	Forward: ATTCCATCCCTCTGATCGATC Reverse: ATCACTGCATCTGTTGAAGGG
<i>At3g23190</i>	SALK_123883	Forward: CTACAAAACGTGAGAATCCCG Reverse: CAACACAAATGATCGGTTGTG
<i>At3g56400</i>	SALK_025198	Forward: GGTGTTACACGTGTGGTTTCC Reverse: CAAACCACACCAAGAGGAAAG
<i>At1g17170</i>	SALK_034472	Forward: CAGTGGCCTAAGCAACTAACG Reverse: TAACTCCTTGGCTGCTTCTTG
<i>At1g44050</i>	SALK_011264	Forward: GAACATTTTATCTTCGCCGTG Reverse: CAGACGTTTGTAAGCTCCCTG
<i>At1g15640</i>	SALK_001121	Forward: AAAATTATCCAAAATAACCTAGATATCC Reverse: CAGCCACCGGTATAACAGATG
<i>At5g59490</i>	SALK_080547	Forward: GAGGGACTTTTAGGAATCTTCTTG Reverse: GCTCTCACAAGACTTGGCATC
<i>At2g40750</i>	SALK_095417	Forward: ACTTTTCCGGCGTTTTTAATG Reverse: TGGATTTCTGTGTTTACTATTTCG
<i>At2g04050</i>	SALK_142350	Forward: GAGAGAAAGGAGTCCACGACC Reverse: AAAATACTGAACCCGAAACG
<i>At1g05680</i>	SALK_016116	Forward: TCTTGGAACCATTTGGAGATG Reverse: TAAGCTGGAGTCTCAGCTTG
<i>At3g53480</i>	SALK_035704	Forward: TTTGTTAAAGGCCTTGTCTGG Reverse: GCGAAACAAGTACAGTAGCGC
<i>At2g14560</i>	SALK_039201	Forward: GCACAACAATAACGAGCATACTC Reverse: TTTGATACATCTGGAATAAACATGTG

For hormone treatments, a final concentration of 1 mM was used for SA, 50 μ M for methyl jasmonate, and 20 μ M for 2,4-Dichlorophenoxyacetic acid (2,4-D). Each hormone was dissolved in water and applied to 3-week-old early seedlings grown in MS medium. The seeds of T-DNA insertion mutants were obtained from ABRC (Arabidopsis Biological Resource Center, USA). The information of SALK-line mutants and specific primer sequence for genotyping used in this study is provided in Table 1.

Mutant genotyping. Genomic DNA was isolated from 4-week-old *Arabidopsis* leaves by using CTAB method as following: Two or three *Arabidopsis* leaves were collected into 1.5 ml eppendorf tube, and the juice was released with pestle at room temperature (R/T). Then 400 μ l of CTAB buffer (3% CTAB (ICI Americas Inc., USA), 1.4 M NaCl,

20 mM EDTA, 100 mM Tris-HCl, and 200 mM β -Mercaptoethanol (Sigma-Aldrich, USA)) was added to the tube, and the tissue was completely ground with pestle. The mixture was incubated at 65°C incubator for 30 min. Four hundred microliters of chloroform was added to the mixture, and then the suspension was centrifuged at 14,000 rpm for 10 min. The supernatant was transferred into fresh tube, and 250 μ l of isopropanol was added. The mixture was incubated at R/T for 30 min to overnight. The mixture was centrifuged for 10 min at R/T. Then the pellet was ethanol precipitated. After the supernatant was discarded, the DNA was gently dissolved in TE buffer, pH 8.0.

The SALK homozygote was confirmed by PCR using a T-DNA-specific (LBa1 5'-TGGTTCACGTAGTGGGCCA-TCG-3') and an *Arabidopsis thaliana* gene-specific primer pair for the presence of the insertion site (Table 1).

Pathogens and inoculation procedures. The bacterial leaf pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000), *Pst* DC3000 containing an *AvrRpm1* (*Pst* DC3000 *avrRpm1*), and fungal pathogens (*Alternaria brassicicola* and *Botrytis cinerea*) were used in this study.

Pst DC3000 was cultured overnight in King's B medium containing 50 $\mu\text{g mL}^{-1}$ rifampicin. *Pst* DC3000 *avrRpm1* was cultured overnight in King's B medium containing 50 $\mu\text{g mL}^{-1}$ rifampicin and 50 $\mu\text{g mL}^{-1}$ kanamycin at 28°C with vigorous shaking. Prior to inoculation, bacterial cells were collected by centrifugation and were resuspended in 10 mM MgCl_2 solution. Leaves of 4-week-old *Arabidopsis* plants were inoculated by dipping, syringe infiltration, or vacuum infiltration of bacterial suspension cultures.

For the pathogenicity assay, *Arabidopsis* plants were dipped in 1×10^7 cfu of *Pst* DC3000 in 10 mM MgCl_2 . For the local resistance assay, leaves of 4-week-old *Arabidopsis* were infiltrated with 5×10^4 cfu of *Pst* DC3000 using vacuum pump. The infected plants were incubated in controlled chamber to observe the symptom development. Infected leaves were harvested at 0, 1, 3, and 5 days after inoculation. Bacterial growth experiments were repeated more than three times for each result.

A. brassicicola and *B. cinerea* were cultured on potato dextrose agar (PDA) medium at 28°C. Conidia of *A. brassicicola* and *B. cinerea* were collected from 12-day-old cultures in PDA (24 g L^{-1}), and then 10 μL suspension of 5×10^4 spores mL^{-1} in PD broth was inoculated on leaves of individual 4-week-old plants. After five days, lesion-size of *A. brassicicola* on the leaves was measured, and susceptibility to *B. cinerea* was evaluated by microscopic observation of the diameter of the symptom. All inoculated plants were covered with a transparent film to maintain humidity and then put in a growth chamber at 22°C under 16 h light.

The symptom of bacterial and fungal blight of *Arabidopsis* leaf are normally leaf wilting and discoloration, leaf drop, a little bit stunting and finally wilting and death. Disease incidence was quantified by determining the disease severity after bacterial inoculation. Disease severity ratings were expressed on the basis of symptom severity and area of chlorosis on each leaf: 1, no symptom or chlorosis on the leaf; 2, < 30% of the leaf area covered with chlorosis and no cell death observed; 3, < 60% of the leaf area covered with chlorosis; 4, > 60% of the leaf area covered with chlorosis and cell death observed; 5, leaves of heavy cell death with chlorosis and leaf collapse

Systemic acquired resistance assay. SAR was observed by comparing growth of virulent *Pst* DC3000 in upper parts of plants after SAR induction. Plants were either SAR-induced by inoculation in two or three leaves with avirulent

Pst DC3000 *avrRpm1* (10^6 cfu/ml) or mock-inoculated with 10 mM MgCl_2 using syringe infiltration method. And three days later, the plants were challenged by inoculation of upper leaves with virulent *Pst* DC3000 strain (5×10^4 cfu/ml).

Extraction of total RNA. Approximately three to four *Arabidopsis* leaves were ground in liquid nitrogen to fine powder in pre-chilled 2 ml centrifuge tube (Sarstedt, Germany). The powder was transferred into 400 μL of RNA extraction buffer (0.2 M Tris-HCl, pH 8.0, 0.4 M LiCl_2 , 25 mM EDTA, pH 8.0, and 1% SDS), and mixed well. Four hundred microliters of water-saturated phenol were added to the tube. The tubes were centrifuged at 14,000 g for 15 min at 4°C. The supernatant was transferred into new tubes and 400 μL of chloroform were added. The tubes were centrifuged at 13,000 g for 15 min at 4°C. Then the supernatant was ethanol precipitated. The pellet was washed with DEPC-treated 70% ethanol, and dissolved in 4 M LiCl_2 . After re-precipitation, the pellet was dissolved in DEPC-treated water.

RT-PCR analysis. The cDNA was synthesized by using 3 μg of total RNA, oligo (dT) primer and superscript reverse transcriptase (Promega, USA). Semiquantitative RT-PCR was performed as described by Liu et al. (2002). The gene-specific primers for *UGT74E2*, *AtPR1*, *AtPR2*, *AtPR3*, *AtPR4*, *AtPR5*, *NPR1*, *TGA*, *IAA1*, *PDF1.2*, and *AtActin7* are listed in the Table 2. The *AtActin7* gene was used as an internal control for RNA quantity in RT-PCR analysis. The intensities of RT-PCR-amplified fragments were analyzed and quantified using Quantity One (Ver. 4.3, Bio-Rad, USA) and Multi Gauge (Ver. 3.0, Fujifilm, Japan).

Real-Time PCR analysis. Total RNA (3 μg) was converted into cDNA with the superscript reverse transcriptase (Promega, USA) using oligo (dT), yielding 20 μL of cDNA solution. Quantitative RT-PCR was performed using the Lightcycler 480 system (Roche). The amplification reaction mixture contained 2 μL of 2-fold diluted cDNA template, 2 μL of primer solution (containing 10 mM [10 pmol/mL] of each forward and reverse primer), 8 μL of SYBR Green PCR Master Mix (Takara, Japan). The primer pairs used in the real-time PCR are listed in the Supplemental Table 2. Thermal cycling conditions consisted of 10 min at 95°C, and 40 cycles of 10 sec at 95°C, 15 sec at 56°C, 15 sec at 72°C. Transcript levels were normalized to the expression of *AtActin7* measured in the same samples.

Isolation and sequence alignment of UGT74E2 cDNA fragments. To isolate *UGT74E2* cDNA fragment, RNA was extracted from *Pst* DC3000-inoculated *Arabidopsis*

Table 2. Arabidopsis gene-specific primer pairs for PCR analysis

AGI	Sequence
<i>At1g05680</i> Forward: AGAAAGAATGCTGAGAAATGGAA (UGT74E2) Reverse: TGGACTTGCAAAAGACGATG	
<i>At2g14610</i> Forward: CGGAGCTACGCAGAACAACT (<i>AtPR1</i>) Reverse: CGTTCACATAATCCCACGA	
<i>At3g57260</i> Forward: TCCTTAGAGACTTGTGGGCTTT (<i>AtPR2</i>) Reverse: CCTATAGGCCCATGCTATTTT	
<i>At3g12500</i> Forward: CTTCTTAACGGCCTCCTC (<i>AtPR3</i>) Reverse: GGGAAATAAAATCGCAACATAAA	
<i>At3g04720</i> Forward: CCATTGATCGCGTTTGATATT (<i>AtPR4</i>) Reverse: CGAAACATATATCCCTCGACAA	
<i>At1g75040</i> Forward: TCGGGTTTAATTATCTCTCACG (<i>AtPR5</i>) Reverse: TCCGTAGATCAATCATTTTGTTTT	
<i>At5g09810</i> Forward: AATGGTGAAGGCTGGTTTTG (<i>AtActin7</i>) Reverse: TGCCTCTGTGAGTAGAACTG	
<i>At4g14560</i> Forward: TGGTCGAGGCAAAAACAAAT (<i>AtIAA1</i>) Reverse: AAACCTTCGTATAATCAAACTCATCTT	
<i>At5g44420</i> Forward: TCTTTGGTGCTAAATCGTGTG (<i>AtPDF1.2</i>) Reverse: AACACAACGGGAAAATAAACA	

leaves. The cDNA of *UGT74E2* was synthesized by using 3 µg of total RNA, oligo (dT) primer and superscript reverse transcriptase (Promega, USA) according to the manufacturer's instruction. The *UGT74E2* gene-specific primer pairs were used for PCR (Supplemental Table 2). The PCR amplification was performed at 95°C for 30 S,

56°C for 30 S, and 72°C for 30 S for 33 cycles. After PCR, the amplified samples were size-fractionated on 1% agarose gel, and the resolved fragments were eluted, cloned into pGEM-T Easy vector (Promega, USA), and sequenced. DNA sequence data were assembled and analyzed with DNASTAR package (DNASTAR, USA). Database searches were performed with the NCBI BLAST program (www.ncbi.nlm.nih.gov/BLAST/).

Results

Selection of SA-inducible genes and genetic screening of T-DNA insertion lines which show impaired response to salicylic acid treatment. To identify further components of the defense mechanism in *Arabidopsis thaliana*, we investigated expression changes in *A. thaliana* under 18 different biotic or abiotic conditions using a DNA microarray representing approximately 25% of all *A. thaliana* genes on the web site (www.genevestigator.com). Analysis using GENEVESTIGATOR software showed that expression of over 42 genes in *Arabidopsis* is affected by SA application (data not shown). Among the 42 genes, we chose genes which were early responsive to SA treatment and whose biological functions have not been characterized so far (Table 3). To investigate biological meaning, we obtained available T-DNA insertion mutants of the above 17 genes from SALK institute (<http://signal.salk.edu>). We finally obtained genuine homozygotes through the mutant geno-

Table 3. Summary of *Arabidopsis* SA-inducible gene mutants used in this study and their local resistance to *Pst* DC3000

AGI	Polymorphism ^a	Gene description	log ₂ SA ^b	Level of symptom change (%) ^c
<i>At4g35180</i>	SALK_027033	Lys/His transporter 7 (HLT7)	4.51	35.0
<i>At1g15640</i>	SALK_001121	Unknown protein	2.88	30.0
<i>At1g44050</i>	SALK_011264	DC1 domain-containing protein	2.68	23.1
<i>At5g59490</i>	SALK_080547	Haloacid dehalogenase-like hydrolase family protein	3.18	22.6
<i>At1g17170</i>	SALK_034472	Glutathione S-transferase TAU 24 (ATGSTU24)	3.68	11.8
<i>At3g23190</i>	SALK_123883	Lesion inducing protein-related	2.41	0.8
<i>At2g14560</i>	SALK_039201	Late upregulated in response to <i>Hyaloperonospora parasitica</i> (LURP1)	5.35	-1.3
<i>At5g11920</i>	SALK_152299	6- & 1-fructan exohydrolase (AtCWINV6)	3.89	-1.7
<i>At3g56400</i>	SALK_025198	WRKY DNA-binding protein 70 (ATWRKY70)	4.88	-3.8
<i>At1g29020</i>	SALK_062045	Calcium-binding EF hand protein	3.67	-4.4
<i>At4g10500</i>	SALK_059907	Subtilase family protein	4.94	-6.6
<i>At1g50090</i>	SALK_070362	Aminotransferase class IV protein	2.78	-11.8
<i>At2g40750</i>	SALK_095417	WRKY DNA-binding protein 54 (ATWRKY54)	8.54	-16.3
<i>At3g53480</i>	SALK_035704	Pleiotropic drug resistance 9 (PDR9)	2.41	-17.5
<i>At2g04050</i>	SALK_142350	MATE efflux family protein	1.93	-19.0
<i>At1g05680</i>	SALK_016116	UDP-glucuronosyl/UDP-glucosyl transferase family protein	5.1	-22.5
<i>At5g45070</i>	SALK_143717	Phloem protein 2-A8 (AtPP2-A8)	3.82	-47.2

^aAll T-DNA insertion mutants are in Columbia-0 background.

^bThis expression data reference is from GENEVESTIGATOR and designates changed expression levels of the genes treated by salicylic acid.

^cRelative disease severity on 17 T-DNA insertion mutants (SALK) at 5 dpi upon *Pst* DC3000 infection (1×10^6 cfu/ml) by dipping.

Fig. 1. Growth comparison of *P. syringae* pv. *tomato* DC3000 in *Arabidopsis* Col-0, *npr1-1*, and T-DNA insertion mutants. Leaves were vacuum infiltrated with 5×10^4 cfu/ml of bacteria and *in planta* bacterial populations were determined at 3 day post infiltration. Bacterial growth represents the bacterial titers of three pooled discs in three replicates. The experiment was repeated three times with similar results. (A) Symptoms development by *Pst* DC3000 on Col-0 and 9 homozygous T-DNA insertion lines. (B) and (C) Bacterial recovery of *Pst* DC3000 on Col-0, *npr1-1*, and mutants. According to the Duncan's multiple range test ($P = 0.05$), means with the same letter are not significantly different. Error bar indicates SD ($n = 3$).

typing.

Monitoring of disease resistance in SA-inducible gene knock-out mutants. To elucidate the biological function of each gene in defense response, virulent bacterial pathogen *Pst* DC3000 that is known to be associated in SA-dependent defense signaling pathway in *Arabidopsis* was inoculated on the leaves of knock-out plants using dipping method as described by Katagiri et al. (2002). High concentration of inoculum (1×10^7 cfu/ml) was used for observation of bacterial speck development. Then we assessed bacterial symptoms including chlorotic and necrotic lesions in rosette leaves. As shown in the Table 3, several mutants exhibited increased bacterial speck symptoms on the leaves and other mutant lines showed decreased symptoms against *Pst*-infection compared to wild type Col-0. Among these mutants, four T-DNA insertion mutants that showed more than 20% of increased-symptom and five T-DNA insertion mutants that exhibited more than 15% of decreased-symptom than those in wild type were collected for further work. To

investigate more detailed disease response change against *Pst*-infection, above nine T-DNA insertion mutants were inoculated with virulent *Pst* DC3000 using vacuum infiltration method. To recover the bacterial pathogen, a concentration of bacterial inoculum was moderate (5×10^4 cfu/ml) at this time for confirmation of the disease response change in mutant plants. When bacterial populations were recovered at 3 days post inoculation (dpi), there were actual differences between wild type and mutants. In terms of bacterial growth, there were not so much differences in symptom-increased group compared to wild-type. But the bacterial growth was lower than that of wild-type (> three-fold) in some cases of symptom-decreased group (Fig. 1). Next to check whether these genes are also associated with defense response against fungal invasion, fungal growth assay was performed *in planta*. *Alternaria brassicicola* involved in ethylene/jasmonate signaling was used in the experiments. Overall there were not so significant differences in fungal infection (Fig. 2). Similar results were obtained in the case of *Botrytis cineria* infection (data not

Fig. 2. Growth of fungal pathogens in *Arabidopsis* T-DNA insertion mutants. (A) Pathogenesis of *Alternaria brassicicola* on Col-0 and mutant plants. Picture was taken 5 days after infection. (B) Relative lesion size of *Alternaria brassicicola* growth on Col-0 and mutant plants. Leaves were inoculated with 5×10^4 spores/ml and *in planta* fungal growth was measured as a lesion diameter at 5 days post inoculation. According to the Duncan's multiple range test ($P = 0.05$), means with the same letters are not significantly different. Error bar indicates SD ($n = 3$).

shown). These results indicate that these genes may be salicylic acid dependent but jasmonate/ethylene independent, and these genes may be associated with bacterial disease resistance in *Arabidopsis*.

***Atlg05680* encodes UGT74E2 that belongs to *Arabidopsis* UGT group L member.** Among nine mutants, one member of *AtUGT* mutant (SALK_016116; *Atlg05680*) that showed relatively strong enhanced resistance was of our particular interest (Fig. 1C). Since glycosylation is an integral step in modification of numerous secondary metabolites to stable and soluble forms by transferring sugars (Lim and Bowles, 2004) and the actual functions of *AtUGTs* in plant-pathogen interaction are still unknown, we performed further studies with this *AtUGT*. The insertion site of T-DNA in this *UGT* mutant was second exon (Fig. 3A) and the mutant

used at this study was genuine homozygous mutant, as confirmed by genotyping (Fig. 3B). In the previous reports, group D members of UGT gene family were known to be associated with defense response against pathogen *in planta* (Chong et al., 2002; Gachon et al., 2004; Langlois-Meurinne et al., 2005). Also a previous report showed the partial biological functions of *UGT73D1* that are induced by SA treatment and *Pst* DC3000-infection (Langlois-Meurinne et al., 2005). However, there was no information about exact biological functions that are involved in plant defense of other groups of UGT family. The *Atlg05680* encodes a protein of 413 amino acids. To analyze sequence homology between this *AtUGT* and other members of *Arabidopsis* UGT gene superfamily, amino acid sequence analysis was performed. In the case of *Atlg05680*, putative coding protein was completely identical with UGT74E2

that belongs to an *Arabidopsis* UGT group L member (Fig. 3D). When compared with several other *Arabidopsis* UGT group L members, this UGT74E2 protein showed strong homology with UGT74E1 throughout the entire coding

Fig. 3. Analysis of genomic organization, T-DNA insertion mutant, and phylogenetic tree of *Arabidopsis* UGT74E2. (A) Genomic structure of UGT74E2 and location of T-DNA insertion in UGT74E2 mutant. (B) Genotyping of UGT74E2 mutant by using UGT74E2 gene-specific primer pair and T-DNA border primer (LBA1). (C) Alignment analysis of UGT74E2 (At1g05680) and other AtUGTs belonging to the L group. Amino acid sequences comparison was done with the C termini of the AtUGT group L member proteins. Multiple sequence alignment was performed with the DNASTAR package. Black shading shows amino acid identities. Underlining indicates the signature sequence (PSPG box, plant secondary product glycosyltransferase) of glycosyltransferases. The conserved domain of UGT is boxed. (D) Phylogenetic tree analysis of At1g05680 and other AtUGTs belonging to the group L. DNA sequence data were assembled and analyzed with DNASTAR package (DNASTAR, USA). Database searches were performed with the NCBI BLAST program (www.ncbi.nlm.nih.gov/BLAST/).

region (about 82%), including the PSPG-box (Fig. 3C). In general, this AtUGT family member using alignment analysis showed considerable homology (about 30 to 80%) in the C-terminal region including the PSPG-box but they exhibited rather weak homology in other regions. This amino acid sequence alignment analysis indicates that UGT74E2 retained the features of the *Arabidopsis* UGT superfamily.

UGT74E2 expression is organ-specific and is induced by biotic and abiotic stress treatments. To verify whether the expression of UGT74E2 is induced in organ-specific manner, RT-PCR analysis was performed by using UGT74E2 gene-specific primer pair. RNA was extracted from typical organs of *Arabidopsis* such as root, stem, flower, and rosette leaf. In the case of UGT74E2, the gene was strongly expressed in flower compared to the other organs such as root and

Fig. 4. Analysis of UGT74E2 gene expression in Col-0. (A) Organ specific expression patterns of UGT74E2 gene in each organ of Col-0 plant. The abbreviation for *Arabidopsis* organs is following: R, Root; S, Stem; F, Flower; Ro, Rosette leaves. (B) Gene expression pattern of UGT74E2 in response to hormone treatments. Three-week-old Col-0 seedlings grown in MS media were treated with SA (1 mM), MeJA (50 μ M), and 2,4-D (20 μ M) and rosette leaves were harvested at the time points indicated. (C) Expression pattern of UGT74E2 gene upon inoculation with *Pst* DC3000.

Fig. 5. Changed disease resistance in *UGT74E2* mutant against *Pst* DC3000 infection. Multiplication of *Pst* DC3000 in Col-0 and *UGT74E2* mutant leaves. Leaves were inoculated with 5×10^4 cfu/mL of bacteria and *in planta* bacterial populations were determined at 0, 1, 2 and 3 days post infiltration. For each time frame, six leaf discs were taken from *Pst* DC3000-inoculated *Arabidopsis* leaves, ground in 10 mM MgCl₂, and plated in five 10-fold dilutions. The dilution factor of each sample is from 10^0 to 10^{-5} . The plate was incubated at 28 °C for 2 days. Multiplication of *Pst* DC3000 in each plant was plotted on a log scale (A) Determination of the bacterial population in inoculated leaf tissue at 3 dpi. (B) and (C) Quantitative real-time PCR expression profiles of *AtPRs* after *Pst* DC3000 infiltration in Col-0 and *UGT74E2* mutant. Relative transcripts level of *AtPR1* gene was determined at 0, 1, 2, and 3 dpi (B) and those of *AtPR2* and *AtPR5* genes were determined at 3 dpi (C). According to the Duncan's multiple range test ($P = 0.05$), means with the same letter are not significantly different. Error bar indicates SD ($n = 3$).

stem (Fig. 4A). This expression pattern is identical with a dataset shown on GENEVESTIGATOR. This result indicates that *UGT74E2* gene expresses with organ-specific manner. *In silico* data suggested that *UGT74E2* is strongly expressed upon typical phytohormones (salicylic acid, methyl jasmonic acid, and ABA) and chemicals (2,4-dichlorophenoxyacetic acid, AgNO₃ and so forth) treatments. To assess whether *UGT74E2* is actually expressed upon hormone and chemical treatments, RT-PCR analysis was performed by using *UGT74E2*-specific primer after SA, MeJA, and 2,4-D treatments on early seedlings. As shown in Fig. 4b, *UGT74E2* was expressed at relatively early time points (8 h) upon hormone and chemical treatments. These results indicate that this gene is induced by wide range of stress conditions. To identify whether *UGT74E2* gene is actually induced upon *Pst*-infection, RT-PCR analysis was performed by using gene-specific primers of *UGT74E2*. For this analysis, *Pst* DC3000 inoculum (5×10^4 cfu/ml) was infiltrated into plants by using vacuum infiltration method. *Arabidopsis* leaves were collected at 0, 1, and 2 days post inoculation (dpi) and also non-treated leaves of same time points were collected as control. The *UGT74E2* expression level was increased gradually from 1 to 2 dpi. *AtPR1* as a marker gene of SA-mediated defense against biotrophs or hemibiotrophs was significantly induced by *Pst*-infection (Fig. 4C). This result suggests that *UGT74E2* is induced by *Pst* DC3000-infection and the expression patterns of this gene coincide with those of *in silico* data.

***UGT74E2* mutant shows moderately increased resistance to *Pst* DC3000-infection in local infected leaves.** To examine the function of *UGT74E2* on defense response against *Pst*-infection, defense activation was directly compared from Col-0 and *UGT74E2*-deficient mutant through the inoculation with virulent *Pst* DC3000 strain and by determination of bacterial recovery at 0, 1, 2, and 3 dpi. There was no difference in bacterial growth between Col-0 and *UGT74E2* mutant until 2 dpi. But, bacterial growth was significantly inhibited (about 7 fold) in *UGT74E2* mutant compared to those of Col-0 at 3 dpi (Fig. 5A). To explain the reason for enhanced resistance against *Pst* DC3000 in *UGT74E2* mutant, we assessed a change of typical marker gene, *AtPR1*, which is well known to be involved in SA-mediated defense signaling pathway. When the virulent strain *Pst* DC3000 was infiltrated in *UGT74E2*-deficient mutant, There was no difference in expression of *AtPR1* in mutant plants compared to those of Col-0 (Fig. 5B and 5C). Because of no difference on *AtPR1*-induction, we investigated the changes of other *pathogenesis-related genes*, *AtPR2* and *AtPR5* in Col-0 and *UGT74E2* mutant at 3 dpi. In the case of *AtPR2* and *AtPR5*, those transcripts accumulation were slightly less induced by *Pst* DC3000 in *UGT74E2* mutant plants

Fig. 6. Systemic acquired resistance assay in *UGT74E2* mutant. (A) Bacterial growth in uninduced (Mock, 10 mM MgCl₂) and induced (*Pst* DC3000 *avrRpm1* inoculation) Col-0 and *UGT74E2* mutant. SAR was monitored by comparing growth of virulent *Pst* DC3000 in Col-0 and *UGT74E2* mutant at 3 days post secondary inoculation. Quantitative real-time PCR expression profiles of *AtPRs*, (B) *AtPR2*, (C) and *AtPR5*, (D) in systemic leaves of uninduced (Mock, 10 mM MgCl₂) and induced (*Pst* DC3000 *avrRpm1*) Col-0 and *UGT74E2* mutant. SAR was monitored by comparing expression of defense-related genes at 3 days post inoculation. And at least three independent experiments were carried out. According to the Duncan's multiple range test ($P = 0.05$), means with the same letter are not significantly different. Error bar indicates SD ($n = 3$).

compared to those of Col-0 at 3 dpi (Fig. 5B and 5C). These results indicate that *UGT74E2* mutant exhibited slightly enhanced local resistance to *Pst* DC3000-infection, not through some *PR*-mediated system.

Functions of *UGT74E2* in systemic immunity. In the bacterial growth assay, *UGT74E2* mutant showed an enhanced-resistance against *Pst* DC3000 infection (Fig. 5A). Moreover, this gene was isolated from genetic screening of SA-inducible genes. So, we at first postulated that transcripts accumulation of *AtPR1* gene would be elevated in *UGT74E2* mutant compared to those of Col-0. However, the *AtPR1* expression was not changed and the expression level of *AtPR2* and *AtPR5* was even rather slightly decreased in *UGT74E2* mutant (Fig. 5B and 5C). As shown in previous report, a glycosylated form of SA plays an important role during biological processes *in planta* (Dean et al., 2005). Thus, as a next step, to see whether *UGT74E2*

is involved in SAR in *Arabidopsis*, SAR assay was performed. SAR was induced by inoculating two or three leaves with avirulent *Pst* DC3000 strain carrying *avrRpm1* (1×10^6 cfu/ml) or 10 mM MgCl₂ as mock control. And then, upper leaves were challenged with virulent *Pst* DC3000 strain (5×10^4 cfu/ml) after 3 days post primary inoculation. As shown in the Fig. 6a, bacterial growth of *Pst* DC3000 was decreased in *UGT74E2* mutant compared to Col-0 plant (> ten-fold). SAR was also monitored by comparing expression levels of *PRs* in Col-0 and *UGT74E2* mutant using RT-qPCR analysis. As shown in the Fig. 6b, the *AtPR1* as well as *AtPR2* and *AtPR5* expression levels of *UGT74E2*-deficient mutant were increased compared to Col-0 (> three-fold). (Fig. 6C and 6D).

To confirm the role of *UGT74E2* in the SA-mediated defense signaling pathway, we investigated the expression patterns of *UGT74E2* in SA-deficient *npr1-1* mutant and *NahG* transgenic plant after *Pst* DC3000-inoculation by

Fig. 7. Expression patterns of *UGT74E2* in SA-deficient mutants, *NahG* and *npr1-1*, after *Pst*-infection. Leaves were inoculated with 1×10^7 cfu/mL of *Pst* DC3000 by spray method and transcripts level of *UGT74E2* was determined at 1, 2 and 3 days post inoculation in Col-0 and *UGT74E2* mutant. *AtPR1* was used as a positive control.

using RT-PCR analysis. We found that transcripts level of *UGT74E2* was not much different in SA-deficient mutants compared to those of Col-0 (Fig. 7). We also investigated the endogenous SA level in Col-0 and *UGT74E2* mutant during the process of SAR. However, there was no difference between them (data not shown). These results suggest that *UGT74E2* may not be directly involved in SA-dependent defense signaling pathway.

Discussion

The large-scale genomic sequencing that has been undertaken in some plants provides sequence of each gene. However, understanding of gene function remains behind the pace of genome sequencing. In *Arabidopsis*, the putative functions of more than half of its whole genes are unknown (The *Arabidopsis* Genome Initiative, 2000). For a better utilization of sequencing information, high-throughput methods for assessment of functions are required. The application of microarray technology to plant gene expression analysis has been continuously accumulated (Seki et al., 2001; Wang et al., 2000), and initial microarray experiments on plant responses to certain stimuli have already shown promise for functional characterization of important processes such as plant defense. The use of the Affymetrix ATH1 array (Redman et al., 2004) resulted in over 20,000

genes expression data. The GENEVESTIGATOR software, with the ability to query a database of 1,561 publically available *Arabidopsis* microarray datasets, has recently been available online (Zimmermann et al., 2004). Analysis using GENEVESTIGATOR software shows expression of over 42 genes in *Arabidopsis* that are specifically induced by SA application and also by *Pst*-infection (data not shown).

Salicylic acid is a crucial component in plant defense signaling network during pathogen invasion. Through the bacterial symptom counting assay and bacterial growth assay at this time with the bacterial pathogen, *Pst* DC3000, we confirmed that there was significant change in typical defense responses such as chlorosis and necrosis in some T-DNA insertional mutants of the above candidate genes compared to wild-type (Col-0) (Table 1 and Fig. 1). Through this reverse-genetic approach by using gene expression data *in silico* and T-DNA mutagenized-polymorphism, it could be identified that these SA-inducible genes are actually associated with plant defense mechanism.

As shown in Fig. 4, it is likely that *UGT74E2* is induced by many stress treatments. From these results, it is speculated that *UGT74E2*, as a member of UGT group L, is involved in stress response in *Arabidopsis*.

Even though the *UGT74E2*-deficient mutant showed enhanced resistance against *Pst* DC3000-infection (Fig. 1c; Fig. 5a), the expression patterns *PR1* genes (*AtPR1*, *AtPR2*, and *AtPR5*) in Col-0 and the mutant were not much different at the site of infection (Fig. 5B, C). Since *Pst* DC3000 is a virulent strain in this case, the expression of *PRs* would not have contributed much. We thus performed the SAR assay to determine the functional roles of *UGT74E2* gene in systemic immunity. We could confirm the increased-changes of systemic resistance of *UGT74E2* mutant during the process of SAR (Fig. 6A). Also, transcripts accumulation of *AtPR1*, *AtPR2*, and *AtPR5* was increased in *UGT74E2* mutant (> 3-fold) compared to those of mock control (Fig. 6B, 6C, and 6D). The roles of UGT in disease resistance have been reported in the past. In tobacco plants carrying the *N* gene, SA GTase (SA UDP-glycosyltransferase) mRNA was induced by bacterial or viral pathogen (Lee and Raskin, 1999). Recent report showed that *CaUGT1* is associated with disease resistance against *Tobacco mosaic virus* (TMV) in *Capsicum annuum* L. *Bugang* (Lee et al., 2009). Among the UGT super family in *Arabidopsis*, only group D members were known to be involved in disease resistance (Langlois-Meurinne et al., 2005). Moreover, it was not clearly defined what is the crucial component for SAR induction is in *Arabidopsis* to date.

UGT74E2 is a member of the group L subclass of UGTs (Ross et al., 2001), which includes *Arabidopsis* *UGT84B1*, described as auxin glycosyltransferase, and *UGT74F2*, which has been recognized previously as SA and anthranilate (a

Trp precursor) glycosyltransferase (Jackson et al., 2001; Quiel and Bender, 2003; Song, 2006).

From the results of SAR assay, it could be speculated that SAR was enhanced in *UGT74E2* mutant. These results emphasize that plant acceptor for glycosylation including hormones (ABA, auxin, cytokinin, SA, and brassinosteroids), secondary metabolites (anthranilate, caffeic acid, and flavonoids), and xenobiotics (Ross et al., 2001) plays an important role in the plant-pathogen interaction, especially in SAR system. Recently, *UGT74E2* has been reported to glycosylate auxin indole-3-butyric acid (IBA) preferentially. Also ectopic overexpression of *UGT74E2* in *Arabidopsis* altered plant architecture and improved stress tolerance (Tognetti et al., 2010).

Through the result of local and SAR assay, it could be postulated that glycosylation of auxin by *UGT74E2* contributes to disease susceptibility in local and systemic tissues. Previous studies have shown that the levels of indole-3-acetic acid (IAA), the primary plant auxin, increased in plant tissues infected by *Pst* DC3000 (O'Donnell et al., 2003). Microarray analysis has shown that infection with *Pst* DC3000 activates genes related to IAA biosynthesis and represses Aux/IAA family and auxin transporter genes (Thilmony et al., 2006). Microarray analysis in response to SA analog, benzothiadiazole S-methylester (BTH), also has shown repression of 21 genes related with auxin signal transduction, implying that BTH might affect auxin homeostasis (Wang et al., 2007). Conversely, the *Arabidopsis* miR393a is induced by the flagellin-derived peptide flg22 and represses auxin signaling through down-regulating the auxin receptor, resulting in increased resistance to *P. syringae* (Navarro et al., 2006). Together, these results suggest that auxin plays an important role in disease resistance and susceptibility.

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