

Pathogen-Responsive Expression of Glycosyltransferase Genes *UGT73B3* and *UGT73B5* Is Necessary for Resistance to *Pseudomonas syringae* pv *tomato* in *Arabidopsis*^[W]

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The genome sequencing of *Arabidopsis* (*Arabidopsis thaliana*) has revealed that secondary metabolism plant glycosyltransferases (UGTs) are encoded by an unexpectedly large multigenic family of 120 members. Very little is known about their actual function in planta, in particular during plant pathogen interactions. Among them, members of the group D are of particular interest since they are related to UGTs involved in stress-inducible responses in other plant species. We provide here a detailed analysis of the expression profiles of this group of *Arabidopsis* UGTs following infection with *Pseudomonas syringae* pv *tomato* or after treatment with salicylic acid, methyljasmonate, and hydrogen peroxide. Members of the group D displayed distinct induction profiles, indicating potential roles in stress or defense responses notably for *UGT73B3* and *UGT73B5*. Analysis of UGT expression in *Arabidopsis* defense-signaling mutants further revealed that their induction is methyljasmonate independent, but partially salicylic acid dependent. T-DNA tagged mutants (*ugt73b3* and *ugt73b5*) exhibited decreased resistance to *P. syringae* pv *tomato-AvrRpm1*, indicating that expression of the corresponding UGT genes is necessary during the hypersensitive response. These results emphasize the importance of plant secondary metabolite UGTs in plant-pathogen interactions and provide foundation for future understanding of the exact role of UGTs during the hypersensitive response.

Plant resistance to pathogen infection is often associated with the hypersensitive response (HR) characterized by localized cell death at the site of infection allowing the restriction of pathogen spread. Main events occurring during the HR are the rapid production of reactive oxygen species (ROS), ion fluxes across the plasma membrane, and transcriptional activation of defense genes as those involved in production of secondary metabolites like salicylic acid (SA) and phytoalexins (Hammond-Kosack and Jones, 1996). Secondary metabolites perform multiple functions in plants, including roles in UV protection, lignification, herbivore protection, and disease resistance (Vet and Dicke, 1992; Li et al., 1993; Dixon, 2001; Anterola and Lewis, 2002). These compounds rarely accumulate in their free form but are often conjugated to sugars, particularly Glc, through the action of glycosyltransferases (UGTs; EC 2.4.1.128; Vogt and Jones, 2000). Glc conjugation is thought to regulate bioactivity of aglycones, to enhance their solubility, to protect their reactivity toward cellular oxidases, and to alter

their transport properties throughout the whole plant (Jones and Vogt, 2001).

UGTs are encoded by a ubiquitous gene family (family 1 of UGTs; Lim and Bowles, 2004) and are as plentiful as they are diverse. The *Arabidopsis* (*Arabidopsis thaliana*) genome contains 120 UGT genes classified into 14 groups (A–N) according to their sequence similarity (Ross et al., 2001). Some of them contain enzymes sharing similar catalytic properties. For instance, enzymes forming SA Glc ester and indole-3-acetic acid Glc ester in tobacco (*Nicotiana tabacum*) and maize (*Zea mays*) exhibit a high sequence homology to group L from *Arabidopsis*, which comprises enzymes producing hydroxycinnamoyl Glc esters (Jackson et al., 2001; Lim et al., 2001). However, group D encompasses enzymes glucosylating substrates as diverse as the steroid solanidin (Moehs et al., 1997), the betalain betanidin (Vogt et al., 1999), the hydroxycoumarin scopoletin (Fraissinet-Tachet et al., 1998), the flavonoid baicalein (Hirotsu et al., 2000), and the sesquiterpenoid abscisic acid (Xu et al., 2002; Fig. 1), and no definite conclusion can be drawn on the function of a UGT from its primary sequence. Determination of UGT substrate specificities in vitro has been intensively studied in many plants for decades, but the characterization of their target in planta is still in its infancy. Therefore, their physiological roles in plants have been inferred mainly from their substrate specificity in vitro and partially from their activity in planta.

The first example of the identification of a UGT function was through the isolation of the maize bronze-1

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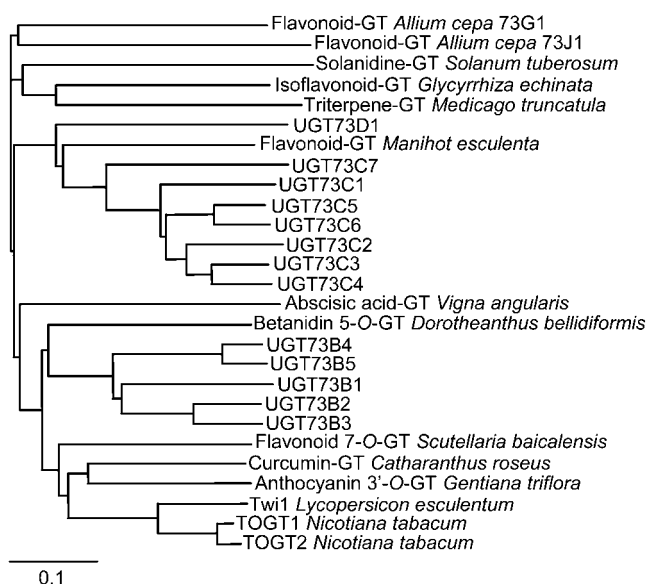


Figure 1. Neighbor-joining distance tree of Arabidopsis UGTs and other plant glucosyltransferases (GT) belonging to the group D. The amino acid sequences were aligned using ClustalX (Thompson et al., 1997). The Arabidopsis Genome Initiative codes corresponding to Arabidopsis UGTs are given in Table II. The GenBank accession numbers of other plant GTs are: *Allium cepa* 73G1 and 73J1, AAP88406 and AAP88407, respectively (Kramer et al., 2003); *Catharanthus roseus*, BAD29722 (Kaminaga et al., 2004); *Dorotheanthus bellidiformis*, CAB56231 (Vogt et al., 1999); *Gentiana triflora*, BAC54092 (Fukuchi-Mizutani et al., 2003); *Glycyrrhiza echinata*, BAC78438 (Nagashima et al., 2004); tomato, CAA59450 (O'Donnell et al., 1998); *Manihot esculenta*, CAA54610 (Hughes and Hughes, 1994); *Medicago truncatula*, AAW56091 (Achnine et al., 2005); tobacco, TOGT1 and TOGT2, AAK28303 and AAK28304, respectively (Fraissinet-Tachet et al., 1998); *Scutellaria baicalensis*, BAA83484 (Hirovani et al., 2000); *Solanum tuberosum*, AAB48444 (Moehs et al., 1997); and *Vigna angularis*, BAB83692 (Xu et al., 2002).

transposon-tagging mutant, which was altered in a flavonol-3-O-glucosyltransferase gene (Dooner and Nelson, 1977). A clear characterization for the biological role of a UGT acting on the hydroxycoumarin scopoletin was made in tobacco by using transgenic plants harboring the corresponding tobacco glucosyltransferase (*TOGT1*) gene in antisense and sense orientation. Down-regulation of *TOGT1* gene weakened resistance to *Tobacco mosaic virus* (TMV) during the HR, and the observed phenotype resulted from a decrease in the antiviral secondary metabolite scopoletin, correlated with an increase in the oxidative burst (Chong et al., 2002). Conversely, up-regulation of *TOGT1* led to the overaccumulation of scopoletin and its glucoside scopolin, to the early appearance of lesions during the HR to TMV (Gachon et al., 2004a), and to increased resistance against *Potato virus Y* (Matros and Mock, 2004). In recent years, a number of *UGTs* were characterized in Arabidopsis (Gachon et al., 2005b); metabolic profiling of T-DNA insertion lines revealed that *UGT73C6* and *UGT78D1* encode UDP-rhamnose:flavonol-3-O-rhamnosyltransferase and UDP-Glc:flavonol-3-O-glycoside-7-O-glucosyltransferase, respectively (Jones et al., 2003).

In the *trp1-100* mutant, which is defective in Trp biosynthesis, a loss-of-function mutation in *UGT74F2* led to the disappearance of anthranilic acid-Glc, indicating that anthranilic acid is metabolized by this UGT in this particular genetic background (Quiel and Bender, 2003). Likewise, overexpression of *UGT84B1* led to the accumulation of 1-O-(indol-3-acetyl)- β -D-Glc, suggesting that indole-3-acetic acid is the main substrate in vivo (Jackson et al., 2002). Recent studies provided evidence for a biological role of *UGT74B1* in glucosinolate biosynthesis (Grubb et al., 2004) and showed that *UGT78D2* and *UGT75C1* encode flavonoid 3-O-glucosyltransferase and anthocyanin 5-O glucosyltransferase, respectively (Tohge et al., 2005).

UGT group D includes 13 members in Arabidopsis and contains a number of genes identified in other plant species as being involved in stress responses (Fig. 1). *IS5a* and *IS10a* genes from Bright-Yellow 2 tobacco cells were shown to be induced by SA and hydrogen peroxide (H_2O_2 ; Horvath and Chua, 1996), and *TOGT1* and *TOGT2* are highly expressed in tobacco reacting hypersensitively to TMV and in tobacco cell suspension cultures treated with the fungal elicitor β -megaspermin (Fraissinet-Tachet et al., 1998). *Tomato wound induced 1* is a wound- and elicitor-inducible gene in tomato (*Lycopersicon esculentum*; O'Donnell et al., 1998), whereas *UGT73B2* is responsive to the mycotoxin fumonisin B1 in Arabidopsis (Asai et al., 2000). Expression of *UGT73B5* was induced by O_2^- and during the HR of Arabidopsis to *Pseudomonas syringae* pv *tomato* (*Pst*) carrying the *AvrRpm1* gene (*Pst-AvrRpm1*; Mazel and Levine, 2002). In a powdery mildew-resistant mutant, *UGT73B2* expression is induced 2-fold higher than in wild-type plants after *Erysiphe cichoracearum* inoculation (Nishimura et al., 2003). Finally, applications of SA, methyljasmonate (MeJA), or deoxynivalenol, a mycotoxin produced by the plant pathogenic fungus *Fusarium*, induced the expression of *UGT73C5* (Poppenberger et al., 2003). Taken together, these observations point to a particular role for group D *UGTs* in stress responses. Still, except for *UGT73C6* (Jones et al., 2003), none of the endogenous substrates nor the function of these enzymes are known.

The aim of this study was to determine which *UGT* in the group D is the most relevant to plant defense, using the Arabidopsis-*Pst* interaction as a model system. We first developed an extensive gene expression analysis of 11 *UGT* genes after *Pst* infection and treatments with SA and H_2O_2 , two key modulators of HR (Delaney et al., 1994; Levine et al., 1994), or MeJA, a wound-related signal (Liechti and Farmer, 2002). To discriminate between highly similar genes, we used real-time quantitative PCR (RT-qPCR) analysis. *UGT* genes were shown to be differentially induced during the HR and after elicitation with the signaling molecules. Furthermore, we found that this induction is MeJA independent, but partially SA dependent. Members of this group are also expressed in an organ-specific manner. All these results are further

Table I. Identity/similarity matrix for group D UGTs

Identity (lower triangle) and similarity (upper triangle) percentages were determined using the needle program (<http://bioweb.pasteur.fr/seqanal/interfaces/needle.html>).

UGT	Nucleotide Identity/Amino Acid Similarity (%)												
	73B1	73B2	73B3	73B4	73B5	73C1	73C2	73C3	73C4	73C5	73C6	73C7	73D1
73B1	–	80.4	79.6	76.5	77.5	59.6	60.6	60.7	60.7	60.4	61.2	61.3	58.2
73B2	71.5	–	90.1	78.2	78.9	57.3	58.9	59.8	60.9	58.4	59.4	60.0	57.7
73B3	71.0	88.8	–	77.5	77.9	58.5	60.2	60.2	60.3	59.3	59.3	59.6	58.8
73B4	69.1	71.2	69.0	–	94.0	59.2	60.4	59.7	61.5	60.7	61.4	60.0	58.3
73B5	70.4	71.2	70.2	90.9	–	60.0	59.8	59.8	61.5	59.8	61.2	61.7	59.7
73C1	52.9	51.4	52.1	52.1	54.4	–	85.7	87.5	87.5	88.3	86.3	79.0	66.3
73C2	53.7	52.5	53.7	55.1	53.9	79.1	–	89.5	89.5	86.1	86.3	77.6	65.0
73C3	54.7	51.7	53.8	53.8	54.3	79.5	84.8	–	93.8	88.5	88.3	77.7	68.5
73C4	56.1	51.9	53.4	54.9	54.2	79.3	83.8	91.3	–	87.7	88.3	77.9	67.1
73C5	53.9	54.7	55.7	54.9	54.5	79.3	81.1	84.0	83.8	–	93.1	77.0	68.8
73C6	54.0	53.9	54.0	52.9	53.8	77.6	80.4	82.6	82.0	91.0	–	77.0	68.4
73C7	51.9	53.9	53.2	51.5	53.0	66.9	66.4	65.7	67.1	67.2	67.0	–	64.7
73D1	50.8	51.8	53.1	52.5	55.0	53.6	55.0	54.9	54.9	57.1	55.4	54.1	–

confirmed by data-mining analyses of publicly available microarray datasets. Finally, two T-DNA insertion mutants belonging to group D (*ugt73b3* and *ugt73b5*) show a decreased resistance to *Pst-AvrRpm1*, suggesting an important role for these two UGTs during the HR.

RESULTS

Group D UGTs Are Differentially Expressed during the HR to *Pst-AvrRpm1*

To point out candidate genes involved in resistance responses, we decided to gain insight into

Table II. Primer sequences used in the RT-qPCR experiments

AGI, Arabidopsis Genome Initiative.		
Gene	AGI Code	Primer Pairs
<i>UGT73B1</i>	At4g34138	5'-TCTGAAACGAGTTCCCAAATTAGC-3' 5'-AAATGGAGCTTAGAGACTTCGACAG-3'
<i>UGT73B2</i>	At4g34135	5'-AGTTAAATTCAAATGGCAGCAACC-3' 5'-TCTTGAACCATGATTTTCTCCTAAC3'
<i>UGT73B3</i>	At4g34131	5'-ATAGCTTCATTGAAAAGACCTCAGTAAG-3' 5'-CCAAGACAAAGACTAAGCAGAATCG-3'
<i>UGT73B4</i>	At2g15490	5'-GTCCACTTTCACTATCCAACAGAGG-3' 5'-CTGTTCTGGTAAAGCCG-3'
<i>UGT73B5</i>	At2g15480	5'-GAGCTGAATGGTAGAAAAGTAGAGGAAG-3' 5'-ATGACATAAAGAACAACCTCCAAGAGG-3'
<i>UGT73C1</i>	At2g36750	5'-TGCAGATACTAAAAGCCGGTGTG-3' 5'-CTTCTTACTCCTTCTTTATCCACCAG-3'
<i>UGT73C2</i>	At2g36760	5'-CAGAACTGATCGTGCAGGTG-3' 5'-CTCTCTTTGCTTCATCACTCTCG-3'
<i>UGT73C3</i>	At2g36780	5'-TCTATATTGGAGAAATTTAAATCAGAGCC-3' 5'-ACTGATTATCTTCTGATTCAATATCC-3'
<i>UGT73C6</i>	At2g36790	5'-TTTTGTCTTCTGTGTGTTAACGTTCTG-3' 5'-TCTATCAGGAAAATAAGGAACAATGAAG-3'
<i>UGT73C7</i>	At3g53160	5'-AGGAGATAGGAGCGATGGTGAG-3' 5'-TGCTTCTCACTATACCCATTAGC-3'
<i>UGT73D1</i>	At3g53150	5'-AAGAAACCGAGTGTGTAAGC-3' 5'-TCATTATCATCATTTTCGTCTACAC-3'
<i>TUBa2</i>	At1g50010	5'-ATCTCTTGCTTGTGGCGGTAG-3' 5'-ACCCAGCTTAAATTCAGTCTTGG-3'
<i>PR1</i>	At2g14610	5'-AGGCTAACTACAACCTACGCTGCG-3' 5'-GCTTCTCGTTCACATAATCCCAC-3'
<i>PDF1.2</i>	At5g44420	5'-CTGTTACGTCCCATGTTAAATCTACC-3' 5'-CAACGGGAAAATAAACATTAAAACAG-3'
<i>lask</i>	At5g26751	5'-CTTATCGGATTTCTCTATGTTTGGC-3' 5'-GAGCTCCTGTTATTTAACTGTACATACC-3'

the transcriptional regulation of group D *UGTs* during the HR to *Pst-AvrRpm1*. We chose RT-qPCR to follow the transcriptional changes of *UGT* genes to ensure the highest possible specificity. The 13 *UGT* sequences exhibit between 50.8% and 91.3% identity within their coding sequences at the nucleotide level (Table I). Specific primer sets for each gene were carefully designed (Table II). *UGT73C4* and *UGT73C5* could not be unambiguously targeted with PCR primers and were not studied further. *UGT* expression levels were normalized over the constitutive tubuline

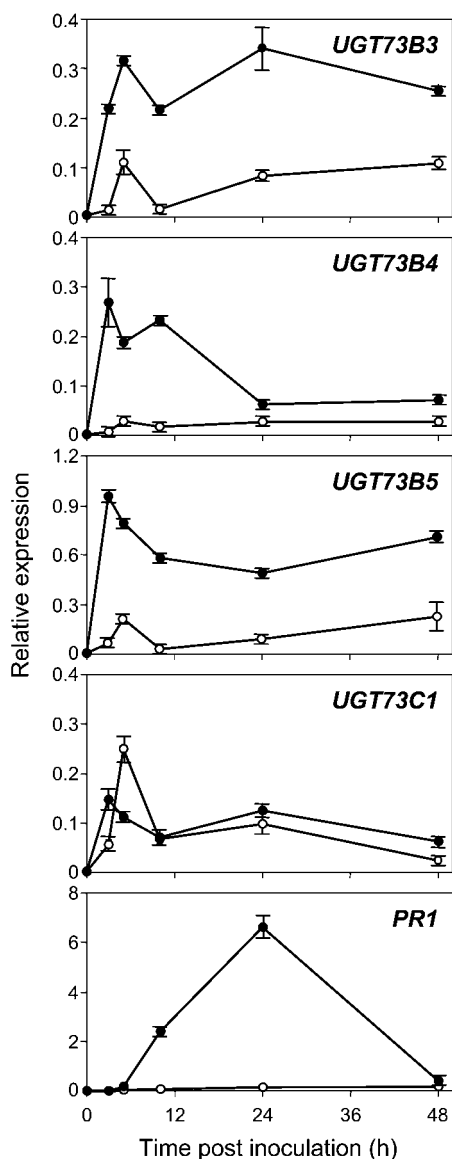


Figure 2. RT-qPCR expression profiles of *UGT73B3*, *UGT73B4*, *UGT73B5*, *UGT73C1*, and *PR1* genes after *Pst* challenge. Transcript levels were quantified after *Pst* DC3000 (white circles) or *Pst-AvrRpm1* (black circles) inoculation. The transcript level (relative expression) is represented as a ratio of the transcript abundance of the studied gene to the transcript abundance of the *TUBα2* gene. Values correspond to the mean and SD of duplicates. The experiments were repeated with similar results.

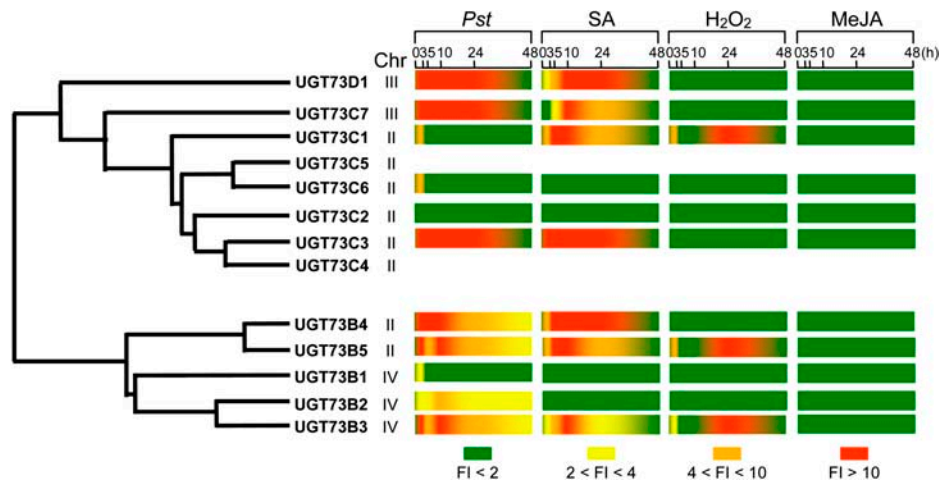
$\alpha 2$ gene (*TUBα2*; At1g50010; Carpenter et al., 1993), the expression of which was similar throughout the time-course experiments between treated and untreated conditions (Supplemental Fig. 1).

Figure 2 shows the time course of *UGT73B3*, *UGT73B4*, *UGT73B5*, *UGT73C1*, and *PR1* expression during the compatible and incompatible interactions with *Pst*. *PR1* is a well established marker gene for the defense responses of Arabidopsis against *Pst* (Uknes et al., 1992). Whereas it was barely expressed in leaves inoculated with the virulent bacteria *Pst*, it was activated to a high level 24 h after inoculation with the avirulent strain *Pst-AvrRpm1*. Among *UGT* genes, diverse patterns of expression could be observed. *UGT73B3* and *UGT73B5*, which share 77.9% similarity at the amino acid level (Table I) but do not map on the same chromosome (Fig. 3), presented highly similar expression profiles. Transcripts of *UGT73B3* accumulated early and strongly during the incompatible interaction and remained high throughout the time-course experiment. Similarly, *UGT73B5* transcripts accumulated early and to higher levels than *UGT73B3*, peaking at 5 h after *Pst-AvrRpm1* inoculation and then decreased to a steady-state level. Leaf inoculation with the isogenic virulent bacteria *Pst* resulted in a very low induction of *UGT73B3* and *UGT73B5*, indicating that the observed response is specific of HR onset. *UGT73B4* was expressed at a lower level compared to *UGT73B5* but exhibited a similar pattern of expression in both interactions until 10 h after inoculation. Thereafter, *UGT73B4* transcripts decreased to basal level whatever the interaction, whereas *UGT73B5* expression remained strongly up-regulated. These two genes share 94% similarity at the amino acid level (Table I) and are mapped in tandem on the same chromosome (Fig. 3). A different picture emerged from *UGT73C1* gene expression (Fig. 2). Here the levels of expression were low, and the patterns of induction were the same in both compatible and incompatible interactions. Altogether, these results clearly indicate that members of the group D, except *UGT73B1*, *UGT73C2*, and *UGT73C6*, are responsive to pathogen infection. They display divergent expression patterns after *Pst* challenge, which are summarized in Figure 3. Thus, genes such as *UGT73D1* exhibited a high and persistent expression level, whereas *UGT73C2* and *UGT73C6* expression was barely detectable (Fig. 3).

Group D *UGTs* Respond Differentially to SA, MeJA, and H₂O₂

A kinetic analysis of *UGT* expression was performed after treatments of Arabidopsis leaves with 1 mM SA, MeJA, or 5 mM H₂O₂. Whereas *PDF1.2*, a marker gene of the jasmonate pathway, was induced, none of the *UGT* genes under investigation responded to MeJA treatment (Figs. 3 and 4A). On the contrary, accumulation of *UGT73B3* and *UGT73B5* transcripts was apparent at 3 h after application of SA (Fig. 4B) and rose continuously until approximately 10 h. Afterward,

Figure 3. Induction of *UGT D* expression during the HR and after treatments with signaling molecules. *UGT D* relationship and position on chromosomes are presented on the left. Treatments and kinetics are indicated on the top. Induction of *UGT D* expression is represented as a ratio (fold induction) of the studied gene relative expression (*UGT* transcript abundance/*TUBα2* transcript abundance) in each inductive condition to its relative expression in the corresponding control (*Pst*: *Pst-AvrRpm1*/*Pst* DC3000; SA: SA/ K_2HPO_4 ; H_2O_2 : H_2O_2 /water; MeJA: MeJA/air). Fold induction (FI) of gene expression is represented by a color scale.



transcript levels decreased rapidly. This pattern of expression differed from the one observed after bacterial challenge (Fig. 2) where expression was maintained to a high level throughout the time-course experiment. Levels of *PR1* transcripts were also determined during the time course. Prior to 5 h, no accumulation of *PR1* transcripts was evident, the increase occurred between 10 and 15 h after SA treatment. Surprisingly, *UGT73B2* was not induced by SA (Fig. 3), although this gene is the closest relative of the highly SA-responsive *UGT73B3* gene. Only three genes, *UGT73B3*, *UGT73B5*, and *UGT73C1*, responded to H_2O_2 application (Fig. 3). They exhibited a biphasic accumulation of transcripts with an early induction at 3 h, followed by a decrease to the background level. Then transcript levels rose dramatically, peaking at 24 h, and declined at 48 h.

Microarray Data Mining Confirms a Stress-Dependent Induction of Group D *UGTs*

Microarray data obtained under numerous stress conditions were retrieved from the publicly available datasets recently released by the AtGenExpress consortium (<http://web.uni-frankfurt.de/fb15/botanik>). Analysis of these datasets confirmed that *UGT D* members were induced differentially under stress conditions (Fig. 5). Data concerning *Pst-AvrRpm1* versus *Pst* DC3000 induction were consistent with our RT-qPCR results confirming that some *UGTs* belonging to the group D are linked with plant defense responses and particularly *UGT73B2-B3* and *UGT73B5*. Expression of these genes was also highly induced after infection with a biotrophic pathogen, *Phytophthora infestans*, and after oxidative stress caused either by paraquat application or UV treatment (Fig. 5). Interestingly, *UGT73B* genes were induced after wounding (Fig. 5), although none of these genes was expressed after MeJA treatment (Fig. 3).

The analysis of other microarray datasets available in Genevestigator (<https://www.genevestigator.ethz.ch>; Zimmermann et al., 2004) revealed that some

members of the group D, including *UGT73B3* and *UGT73B5*, were induced by the necrotrophic fungus *Botrytis cinerea* and after ozone treatment (data not shown). Furthermore, most members of group D responded to SA, whereas none of them was induced by MeJA, supporting our RT-qPCR results (data not shown).

Group D *UGTs* Exhibit a Differential SA Biosynthesis and SA-Signaling Dependence

To test whether SA is required for the induction of *UGT D* genes during the HR, we investigated *UGT* expression in plants with an altered SA-signaling pathway. *NahG* plants express constitutively a salicylate hydroxylase enzyme that degrades SA into catechol (Delaney et al., 1994) and *npr1-1* mutants are non-responsive to SA, exhibiting a loss of resistance to *Pst* and a nonexpression of *PR1* after infection (Cao et al., 1994). Figure 6 shows transcript accumulation of *UGT73B3*, *UGT73B5*, *UGT73D1*, and *PR1* in wild-type plants, *NahG* transformants, and *npr1-1* mutants. As expected, *PR1* expression was abolished in *NahG* plants and *npr1-1* mutants at 10 h after inoculation with *Pst-AvrRpm1*. *UGT73D1* expression was barely detectable after pathogen challenge in *NahG* plants, whereas it still remained detectable in *npr1-1* mutants indicating a SA-dependent and NPR1-independent induction of this gene. *UGT73B3* and *UGT73B5* were hardly expressed in *NahG* plants and *npr1-1* mutants at 3 h after pathogen challenge, suggesting an SA dependence at this time point. Strikingly, at 5 and 10 h after inoculation, *UGT73B3* and *UGT73B5* transcript levels were almost similar in wild-type plants, *NahG* plants, and *npr1-1* mutants, suggesting an induction of gene expression independent of SA at these later time points.

Organ-Specific Expression of *UGTs* Belonging to Group D

To determine the organ-specific expression pattern of each *UGT D* member, RT-qPCR was performed on

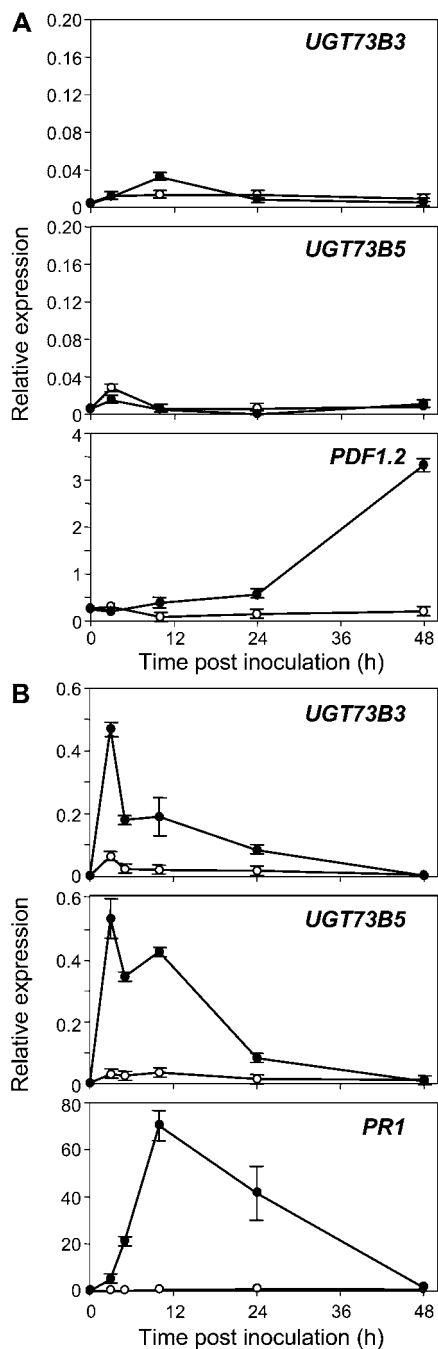


Figure 4. RT-qPCR expression profiles of *UGT* belonging to group D following MeJA and SA treatments. A, Transcript levels of *UGT73B3*, *UGT73B5*, and *PDF1.2* after MeJA treatment (black circles) compared with mock control (white circles). B, Transcript levels of *UGT73B3*, *UGT73B5*, and *PR1* after SA (black circles) and mock (white circles) infiltration. The transcript level (relative expression) is represented as a ratio of the transcript abundance of the studied gene to the transcript abundance of the *TUBα2* gene. Values correspond to the mean and SD of duplicates. SA experiment was repeated with similar results.

RNA isolated from roots, rosette leaves, stems, cauline leaves, and flowers. It revealed that *UGT* D members are weakly expressed in rosette leaves, stems, and cauline leaves, with the exception of *UGT73C6*, which exhibited a high expression in cauline leaves (Fig. 7A). *UGT73B2*, *UGT73B3*, and *UGT73B5* were abundantly expressed in roots and flowers, whereas *UGT73B4* and *UGT73D1* transcripts were only detectable in roots. *UGT73C6* was expressed in cauline leaves and flowers, and *UGT73C1*, *UGT73C2*, and *UGT73C7* were undetectable in all organs except in flowers, where low levels of transcripts were found. Microarray data mining showed that *UGT* D members were mainly expressed in roots and reproductive organs confirming our RT-qPCR analyses (Fig. 7B). *UGT73B2*, *UGT73B3*, *UGT73B4*, and *UGT73B5* were highly expressed in roots grown on agar plates containing Murashige and Skoog basal medium. Surprisingly, these genes were barely expressed in soil-grown roots, suggesting a specific induction of gene expression either under light exposure or through medium composition. Furthermore, the HR-responsive *UGT73B3*, *UGT73B5*, and *UGT73D1* were also highly expressed in developmental cell death as shown in senescent leaves (Fig. 7B).

ugt73b3* and *ugt73b5* Mutants Display Decreased Resistance to an Avirulent Strain of *P. syringae

In the Salk library (<http://signal.salk.edu/cgi-bin/tdnaexpress>; Alonso et al., 2003), we identified putative *ugt73b3* and *ugt73b5* knockout lines exhibiting a T-DNA insertion. Plants were selfed to obtain homozygous knockout lines. The T-DNA flanking sequences were amplified by PCR and sequenced to confirm the position of the insertion. The *ugt73b3* mutant harbors the T-DNA insert 29 bp downstream of the start codon, whereas T-DNA insertion is 164 bp upstream of the start codon in *ugt73b5* knockout line. As expected, no

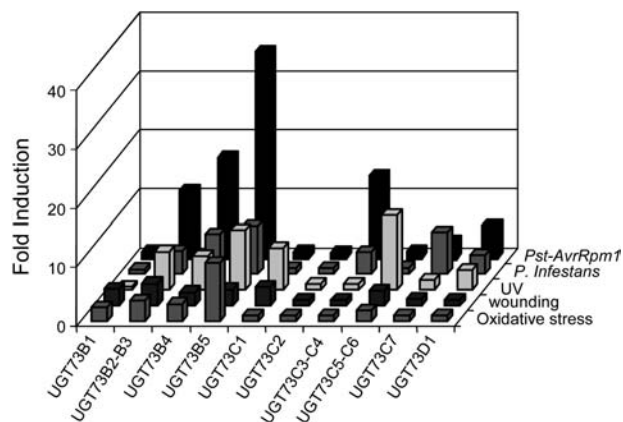


Figure 5. Stress induction of *UGT* D revealed by microarray data mining. Induction of *UGT*, expressed in fold induction, was evaluated 6 h after infection with *Pst* (*Pst-AvrRpm1/Pst* DC3000), inoculation with *P. infestans* (*P. infestans*/water), and UV-B stress or 12 h after wounding and oxidative stress. Data were obtained from the AtGenExpress microarray datasets. Values correspond to the mean of triplicates.

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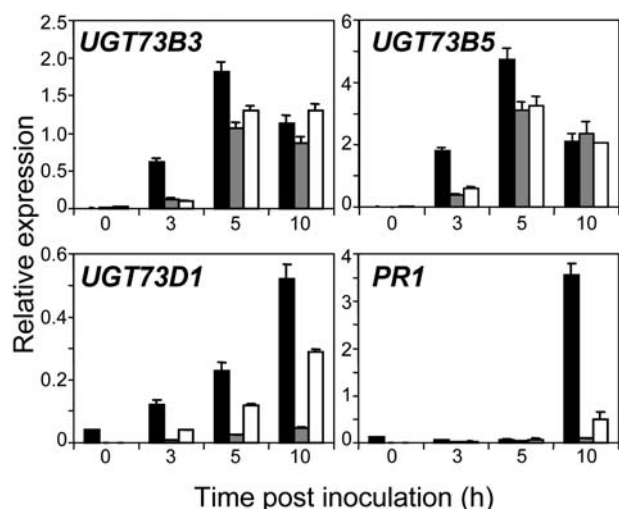


Figure 6. RT-qPCR expression profiles of *UGT73B3*, *UGT73B5*, *UGT73D1*, and *PR1* genes in wild-type plants and signaling-pathway mutants after *Pst* challenge. Transcript levels were quantified in wild-type plants (black bars), *NahG* plants (gray bars), and *npr1-1* mutants (white bars) after *Pst-AvrRpm1* inoculation. The transcript level (relative expression) is represented as a ratio of the transcript abundance of the studied gene to the transcript abundance of the *TUBα2* gene. Values correspond to the mean and sd of duplicates. Experiment was repeated with similar results.

induction of the mutated gene could be detected in both mutant lines following *Pst-AvrRpm1* challenge (Fig. 8A).

Resistance to pathogens was investigated using inoculation with *Pst*. After inoculation with a medium titer (10^5 colony forming units [cfu] mL⁻¹) of the virulent *Pst* DC3000 strain, *ugt73b3* and *ugt73b5* mutants developed disease symptoms similar to those observed in wild-type plants (data not shown) and supported the same bacterial growth as in wild-type plants, 24 and 72 h after *Pst* DC3000 challenge (Fig. 8B). After inoculation with the avirulent *Pst-AvrRpm1* strain, bacterial growth did not increase in wild-type plants. Conversely, bacterial population dramatically increased in *ugt73b3* and *ugt73b5* mutants, indicating an almost complete loss of resistance in these two genotypes.

DISCUSSION

By transferring sugars to a wide range of secondary metabolites, UGTs increase the stability and solubility of aglycones and therefore modify their bioactivity (Lim and Bowles, 2004). In Arabidopsis, 120 UGT genes have been recognized (Paquette et al., 2003) and classified into 14 groups on the basis of their sequence similarity (Ross et al., 2001). Even if UGT substrate specificities have been extensively studied in vitro (Lim et al., 2001, 2002), little functional information is available for the majority of the genes in this family. To

extend our knowledge of the physiological role of UGTs in plant-pathogen interactions, we first analyzed the stress-responsive expression of 11 members belonging to the group D, combining RT-qPCR analyses and data mining of public microarray datasets.

Previous studies based on northern- or classical-PCR analyses have revealed that group D UGTs are induced under stress conditions in Arabidopsis and other plant species (Horvath and Chua, 1996; Fraissinet-Tachet et al., 1998; O'Donnell et al., 1998; Asai et al., 2000; Mazel and Levine, 2002; Nishimura et al., 2003; Poppenberger et al., 2003). Although these studies highlight the stress inducibility of UGT D genes, they were limited by a lack of specificity and did not allow the detection of lowly expressed genes. Therefore, we used RT-qPCR, a highly sensitive and specific method to accurately measure transcript levels of UGTs (Gachon et al., 2004b). We first examined the pathogen-responsive expression of some UGTs of group D in time-course experiments after inoculation with the *Pst-AvrRpm1* avirulent bacteria (Figs. 1 and 2). Importantly, these genes were not induced during the compatible interaction of Arabidopsis with the virulent *Pst*, suggesting that these UGTs play a role in resistance and that induction is dependent on the onset of the HR. Our results clearly indicate that some UGTs of group D exhibit all the transcriptional features of HR-inducible genes, like glutathione-S-transferase (*GST*) genes (Lieberherr et al., 2003). In addition to pathogen signals, eight UGTs were responsive to the defense-associated signal SA and three of them were also responsive to H₂O₂ (Fig. 4). Furthermore, besides RT-qPCR analysis, mining of microarray data revealed that UGT expression was induced after wounding, *B. cinerea* and *P. infestans* inoculation, and during UV, ozone, and oxidative stresses (Fig. 5). Altogether, these results point to a pathogen- and stress-responsive expression of most UGTs belonging to group D.

It is also clear that UGTs of group D are differentially regulated after inoculation with *Pst-AvrRpm1* or after SA and H₂O₂ treatments. *UGT73B3* and *UGT73B5* share 77.9% similarity at the amino acid level and exhibit the same pattern of expression after pathogen challenge and signaling-molecule application (Fig. 3). However, *UGT73B1*, which shows a comparable level of sequence divergence with *UGT73B3* and *UGT73B5*, does not respond to any treatment (Fig. 3). Moreover, *UGT73C3* and *UGT73C2*, which exhibit 89.5% similarity, present totally different expression profiles. *UGT73C3* is induced after *Pst-AvrRpm1* infection and SA treatment in Arabidopsis leaves, whereas *UGT73C2* expression is barely detectable. On the contrary, *UGT73C7* and *UGT73D1*, which have 64.7% similarity within their coding sequences, present similar transcript profiles after *Pst-AvrRpm1* inoculation and SA application (Fig. 3). Despite numerous studies aimed at deciphering the modalities of expression divergence between duplicated genes (Haberer et al., 2004), our data are in line with the idea that at present, no predictive conclusion can be drawn on the regulation

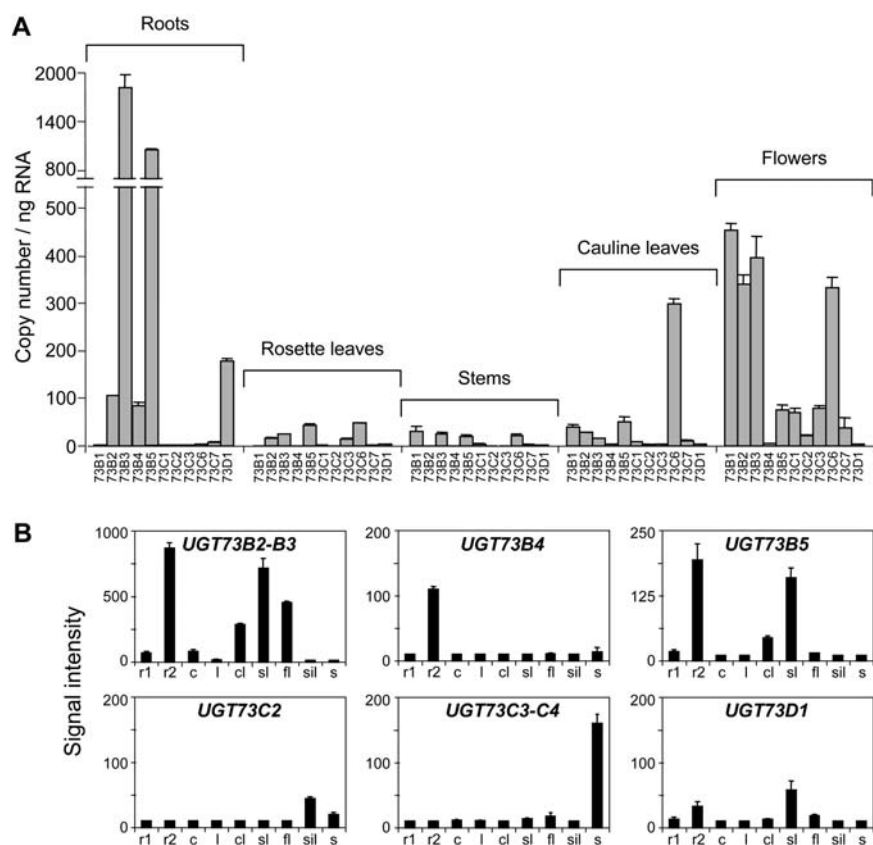


Figure 7. Expression of *UGT D* genes in Arabidopsis tissues. A, RT-qPCR analysis of *UGT* expression in roots, rosette leaves, stems, cauline leaves, and flowers. The transcript level of each gene is expressed in absolute copy number per ng total RNA. Values correspond to the mean and sd of duplicates. B, Expression of *UGT D* genes in soil-grown roots (r1), Murashige and Skoog-grown roots (r2), cotyledons (c), leaves (l), cauline leaves (cl), senescent leaves (sl), flowers (fl), siliques (sil), and seeds (s). Gene expression level, obtained from the AtGenExpress microarray datasets, are expressed in an arbitrary unit. Values correspond to mean and sd of triplicates.

of paralogue genes from the comparison of their primary sequence (Duan and Schuler, 2005). As a result, a systematic study of differential regulation within a gene family is required to identify candidates involved in an inducible response and to point out possible redundant genes that would be able to complement a mutation. Among the 11 genes investigated through the RT-qPCR and data mining analyses, *UGT73B3* and *UGT73B5* appear to be highly responsive to pathogens and therefore are good candidates for playing an effective role in defense responses.

Importantly, their kinetics of expression resemble those of early induced genes such as *GSTs* (Lieberherr et al., 2003). Some *UGTs* of group D are up-regulated as soon as 3 h after challenge with *Pst-AvrRpm1*, whereas the late-induced *PR1* gene is peaking at 24 h after inoculation (Fig. 2). Early induced genes serve a function of stress adaptation, intercellular communication, and transcriptional regulation of late genes to coordinate long-term biological responses (Abel and Theologis, 1996). Consistent with this role, the activation of the early induced genes does not require de novo protein synthesis (Horvath and Chua, 1996; Horvath et al., 1998; Uquillas et al., 2004). This early induction of *UGTs* may indicate a role in stress or defense responses to regulate the activities and subcellular localization of substrates, which may preexist prior to pathogen infection, and the biosynthesis of which does not necessitate induction of biosynthetic pathways.

Most group D *UGTs* were responsive to SA whereas none of them was induced after exposure to MeJA, suggesting a jasmonate-independent and SA-dependent induction of these *UGTs*. Likewise, the wound-inducible tomato *UGT Tomato wound induced 1*, which belongs to group D, was previously shown to be induced by SA but not by MeJA (O'Donnell et al., 1998). Transduction of the SA signal requires the function of the regulatory protein NPR1 to activate *PR* gene expression and systemic acquired resistance (Dong, 2004). Interestingly, activation of SA early induced genes like *GST6* and *IS10a*, a tobacco GT, was shown to be NPR1 independent (Horvath and Chua, 1996; Uquillas et al., 2004). *UGT73D1* was not induced in *NahG* plants after *Pst-AvrRpm1* inoculation. However, transcripts of this gene were still found in *npr1-1* mutants at 5 and 10 h after pathogen challenge, suggesting a SA-dependent, NPR1-independent induction, which is the feature of some SA early induced genes (Uquillas et al., 2004). On the contrary, although *UGT73B3* and *UGT73B5* were hardly expressed in *NahG* plants at 3 h after *Pst-AvrRpm1* inoculation, their transcript levels were similar in wild-type plants, *NahG* transformants, and *npr1-1* mutants at 5 and 10 h after inoculation (Fig. 6). This suggests an SA dependence in the early times of infection and the contribution of other unknown signal(s) later. Such a modular regulation of stress-inducible genes by different transduction pathways has already been shown for *GST* induction (Lieberherr et al., 2003).

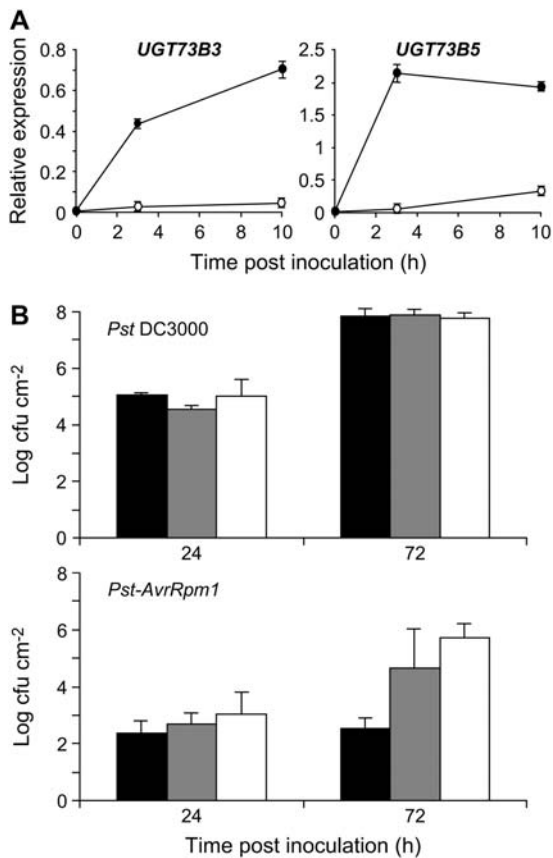


Figure 8. A, RT-qPCR expression profiles of *UGT* genes in wild-type plants and *ugt* mutants after *Pst-AvrRpm1* challenge. Transcript levels were quantified for *UGT73B3* and *UGT73B5* in wild-type plants (black circles) and in *ugt73b3* mutant and *ugt73b5* mutant, respectively (white circles). Values correspond to the mean and SD of duplicates. B, Bacterial growth of *Pst* in Arabidopsis leaves of wild-type plants and *ugt* D mutants. Leaves were infiltrated with 10^5 cfu mL⁻¹ *Pst* DC3000 or *Pst-AvrRpm1* and harvested 24 and 72 h after inoculation. The bacterial population (log cfu cm⁻²) was quantified in wild-type plants (black bars), *ugt73b3* (gray bars), and *ugt73b5* (white bars) mutants. The graph represents the mean and SD of three replicates. In the incompatible interaction, the bacterial amount in mutant plants was significantly higher than that of the wild type at 72 h post inoculation, according to one-tailed Student's *t* test (*P* value = 0.03 and 0.0004 for *ugt73b3* and *ugt73b5*, respectively). Experiment was repeated with similar results.

UGT73B3 and *UGT73B5* were responsive to H₂O₂, whereas *UGT73D1* was not (Fig. 3). ROS and notably H₂O₂ function as signaling molecules in plants and control various processes, including pathogen defense and programmed cell death (Apel and Hirt, 2004). Given their responsiveness to H₂O₂ and oxidative stresses, *UGT73B3* and *UGT73B5* induction might be SA dependent at 3 h after *Pst-AvrRpm1* challenge and ROS dependent at later times.

Our data further provide direct evidence of the involvement of *UGTs* during the HR of Arabidopsis to pathogens. Thus, *ugt73b3* and *ugt73b5* mutants both exhibited a loss of resistance to *Pst-AvrRpm1* (Fig. 8).

However, both mutants were not fully susceptible to *Pst-AvrRpm1*, due to either the presence of other effectors of disease resistance or additional *UGT* genes partially complementing *UGT73B3* and *UGT73B5*. One question that still remains to be answered is which role *UGT73B3* and *UGT73B5* could play in this process. Since they are also induced during senescence and after challenge with different pathogens, we can assume that these genes are associated to the cell death process occurring during senescence and HR. Moreover, their responsiveness to H₂O₂ and paraquat oxidative stress suggests that they may also participate in the maintenance of cellular redox homeostasis. As GSTs, which might use stress metabolites as substrates (Edwards et al., 2000) and were thought to be antioxidant proteins against H₂O₂ produced during the HR (Levine et al., 1994), *UGT73B3* and *UGT73B5* might contribute to redox homeostasis. In line with this hypothesis, the expression profiles of *UGT73B3* and *UGT73B5* display striking similarities with the closely related TOGT gene from tobacco. TOGT-mediated glucosylation was shown to be required for scopoletin accumulation in cells surrounding TMV lesions, where this compound plays a role in ROS buffering (Chong et al., 2002). Finally, *UGT73B3* and *UGT73B5* transcripts were mainly detected in reproductive organs in the absence of stress (Fig. 7). This suggests that these *UGTs* could be involved in different tissues both in defense responses and in development. The next step in the determination of *UGT73B3* and *UGT73B5* function is the identification of their substrates in planta. Scopoletin and its glucoside scopolin have recently been identified in Arabidopsis (Rohde et al., 2004), but they are not detected in leaves inoculated with *Pst-AvrRpm1* (F. Bellvert and P. Saindrean, unpublished data). Therefore, it is less than probable that the function of *UGT73B3* and *UGT73B5* during the HR is linked to scopoletin glucosylation. Transcripts of *UGT73B3* and *UGT73B5* were found in Murashige and Skoog-grown roots, whereas they were not expressed in soil-grown roots, suggesting a light induction of these genes (Fig. 7). Interestingly, in Arabidopsis roots, light was recently shown to induce phenylpropanoid metabolism, accumulation of flavonoid glucosides, and high levels of coniferin and syringin (coniferyl and sinapyl-4-O-glycosides; Hemm et al., 2004). The identification of *UGT73B3* and *UGT73B5* endogenous substrates using metabolic profiling is currently under progress with a special interest in phenylpropanoid and flavonoid compounds. Our working hypothesis is that *UGT73B3* and *UGT73B5* might contribute to redox homeostasis through the glucosylation of a still-unknown metabolite that would fulfill in Arabidopsis the role of scopoletin in tobacco. To the best of our knowledge, this would represent the first example of closely related enzymes displaying a conserved function and a conserved expression profile but having evolved a new substrate specificity between species.

MATERIALS AND METHODS

Biological Materials and Plant Treatments

Arabidopsis thaliana ecotype Columbia (Col-0) was used throughout this study. The transgenic line harboring the *NahG* gene was obtained from R. Dietrich (Syngenta). The Col-0 mutant *npr1-1* was provided by X. Dong (Duke University). T-DNA insertion lines *ugt73b3* and *ugt73b5* were obtained from the Salk institute (SALK_097487 and SALK_078055, respectively). Plants were grown in soil in individual pots under an 8 h/16 h light/dark photoperiod at $150 \mu\text{E cm}^{-2} \text{s}^{-1}$ of light intensity, at 20°C and 75% relative humidity. The virulent strain *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) and the avirulent strain *Pst* DC3000 carrying the avirulence gene *AvrRpm1* (*Pst-AvrRpm1*) were obtained from J. Glazebrook (University of Minnesota). Six- to 7-week-old plants were used for *Pst* inoculations or signaling-molecule treatments. Half leaves were infiltrated using a 1-mL syringe without a needle. Bacteria were grown over night at 28°C on King B liquid medium containing the appropriate antibiotics (kanamycin, $25 \mu\text{g mL}^{-1}$; and rifampycin, $50 \mu\text{g mL}^{-1}$). They were collected by centrifugation, resuspended in water, and quantified using a spectrophotometer. Plants were infiltrated with *Pst* DC3000 or *Pst-AvrRpm1* at the concentration of 10^7cfu mL^{-1} ($A_{600} = 0.01$). For signaling-molecule treatments, mature leaves were infiltrated with SA (1 mM in K_2HPO_4 5 mM pH 6.5) or H_2O_2 (5 mM). Control plants were infiltrated with K_2HPO_4 5 mM and water, respectively. For MeJA treatment, plants were kept in closed 12-L chambers with $10 \mu\text{L}$ MeJA. For each time point, one leaf of six different treated plants was harvested. The resultant six leaves were pooled and frozen in liquid nitrogen. For tissue-specific expression, roots were obtained from 4-week-old plants grown on Murashige and Skoog medium. Rosette leaves were harvested from 4-week-old plants grown in soil under an 8 h/16 h light/dark photoperiod. Plants were then transferred under a 16 h/8 h light/dark photoperiod and cauline leaves, stems, and flowers were harvested after 2 to 3 weeks.

RNA Extraction and cDNA Preparation

Total RNA were extracted from ground, frozen material using Extract All mix (Eurobio) according to the manufacturer's instructions. Samples were treated with the RNase-free DNase I Amplification Grade (Invitrogen Life Technologies) for 15 min. The reaction was stopped by a phenol-chloroform extraction (Sambrook et al., 1989). Total RNA were loaded on a 0.8% agarose gel to check their integrity, and the amount of total RNA was determined spectrophotometrically. Five micrograms of total RNA were reverse transcribed using the SuperScript II First Strand synthesis system for reverse transcription-PCR kit (Invitrogen) according to the manufacturer's instructions.

RT-qPCR

Nucleotide sequences were aligned to identify divergent regions between the most closely related genes. In this region, position of the primers were determined so that the size of the PCR product ranges between 50 and 200 bp. Oligo 4.0 software (Rychlik and Rhoads, 1989) and Primer Express 1.0 software (Applied Biosystems) were used to evaluate primer dimer formation and to estimate their melting temperature ($59^\circ\text{C} \pm 1^\circ\text{C}$). A PCR reaction was then simulated in silico using Amplify 1.2 software (Engels, 1993) to test primers amplification on gene of interest and most closely related gene sequences. The sequences of all primers used are summarized in Table II.

Twenty-five-microliter reactions were prepared by mixing $10 \mu\text{L}$ samples with $12.5 \mu\text{L}$ Sybr Green Mastermix (Eurogentec), $1 \mu\text{L}$ of each primer (final concentration 300 nM), and $0.5 \mu\text{L}$ water. RT-PCR reactions were run on a GeneAmp 5700 device (Applied Biosystems). After 2 min at 50°C followed by a 10-min denaturation step at 95°C , samples were run for 40 cycles of 15 s at 95°C and 1 min at 60°C . After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 60°C to 95°C . Data were analyzed with the GeneAmp 5700 SDS software (Applied Biosystems). Despite RNA treatment with DNase I before cDNA synthesis, contamination by genomic DNA was checked in each sample using primers annealing on *Ask* alpha gene intron (Table II; Charrier et al., 2002). Serial dilutions of genomic DNA extracted with the DNeasy plant maxi kit (Qiagen) were used to trace a calibration curve, which was used to deduct the amount of cDNA for each target gene. Samples were run in duplicates. Experiments were repeated twice independently except for H_2O_2 infiltration, MeJA treatment, and tissue-specific expression. To standardize the data, the amount

of target gene was normalized over the abundance of the constitutive *TUBa2* gene except for the organ-specific expression analysis.

Microarray Data Mining

Microarray data were obtained from the publicly available dataset released by the AtGenExpress consortium (<http://web.uni-frankfurt.de/fb15/botanik>). These data were generated using the Affymetrix ATH1 GeneChip probe array (Redman et al., 2004). The probes corresponding to *UGT73B1*, *UGT73B2-B3*, *UGT73B4*, *UGT73B5*, *UGT73C1*, *UGT73C2*, *UGT73C3-C4*, *UGT73C5-C6*, *UGT73C7*, *UGT73D1*, *UGT73D2*, *UGT73D3*, *UGT73D4*, *UGT73D5*, *UGT73D6*, *UGT73D7*, *UGT73D8*, *UGT73D9*, *UGT73D10*, *UGT73D11*, *UGT73D12*, *UGT73D13*, *UGT73D14*, *UGT73D15*, *UGT73D16*, *UGT73D17*, *UGT73D18*, *UGT73D19*, *UGT73D20*, *UGT73D21*, *UGT73D22*, *UGT73D23*, *UGT73D24*, *UGT73D25*, *UGT73D26*, *UGT73D27*, *UGT73D28*, *UGT73D29*, *UGT73D30*, *UGT73D31*, *UGT73D32*, *UGT73D33*, *UGT73D34*, *UGT73D35*, *UGT73D36*, *UGT73D37*, *UGT73D38*, *UGT73D39*, *UGT73D40*, *UGT73D41*, *UGT73D42*, *UGT73D43*, *UGT73D44*, *UGT73D45*, *UGT73D46*, *UGT73D47*, *UGT73D48*, *UGT73D49*, *UGT73D50*, *UGT73D51*, *UGT73D52*, *UGT73D53*, *UGT73D54*, *UGT73D55*, *UGT73D56*, *UGT73D57*, *UGT73D58*, *UGT73D59*, *UGT73D60*, *UGT73D61*, *UGT73D62*, *UGT73D63*, *UGT73D64*, *UGT73D65*, *UGT73D66*, *UGT73D67*, *UGT73D68*, *UGT73D69*, *UGT73D70*, *UGT73D71*, *UGT73D72*, *UGT73D73*, *UGT73D74*, *UGT73D75*, *UGT73D76*, *UGT73D77*, *UGT73D78*, *UGT73D79*, *UGT73D80*, *UGT73D81*, *UGT73D82*.

ugt D Mutant Characterization

Salk T-DNA insertion lines in the Col-0 background were screened in silico for *ugt* D mutants. Two lines (SALK_097487 and SALK_078055) were identified to carry a T-DNA insertion at the *UGT73B3* and *UGT73B5* loci, respectively. The position of the T-DNA insert was confirmed by PCR and DNA sequencing using primers LB (5'-GGACCGCTTGCTGCAACT-3') and B3F (5'-CCACATCATCAACACGACAAG-3') for *ugt73b3*, and LB and B5R (5'-GACGGTTCTTCTGCTGGATTGG-3') for *ugt73b5*. Homozygous plants were identified by PCR, by amplification of the mutant allele using primers LB and B3F for *ugt73b3*, and LB and B5R for *ugt73b5*, and by the absence of amplification of the wild-type allele using primers B3F and B3R (5'-GATTTCGAACTCGGATTCAGG-3') for *ugt73b3* and B5F (5'-GTCTTCTCAACGTGCACACG-3') and B5R for *ugt73b5*.

Determination of Bacterial Growth in Plants

Wild-type plants and *ugt73b3* and *ugt73b5* mutants were inoculated with a medium titer (10^5cfu mL^{-1}) of *Pst* DC3000 or *Pst-AvrRpm1*. Whole leaves of 6- to 7-week-old plants were infiltrated using a 1-mL syringe without a needle. Leaf discs (0.5cm^2 each) were harvested from inoculated leaves at 24 and 72 h after infiltration. For each time point, three samples were made by pooling two leaf discs from different treated plants. Bacterial growth was assessed by homogenizing leaf discs in $400 \mu\text{L}$ of water, plating appropriate dilutions on solid King B medium containing Kanamycin and Rifampicin and quantifying colony numbers after 2 to 3 d. Statistical analyses of the differences between two means of log-transformed data were performed according to one-tailed Student's *t* test.

Note Added in Proof

Poppenberger et al. (2005) recently reported that *UGT73C5* glucosylates brassinosteroids (Poppenberger B, Fujjioka S, Soeno K, George GL, Vaistij FE, Hiranuma S, Seto H, Takatsuto S, Adam G, Yoshida S, et al [2005] The *UGT73C5* of *Arabidopsis thaliana* glucosylates brassinosteroids. Proc Natl Acad Sci USA 102: 15253–15258).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At4g34138 (*UGT73B1*), At4g34135 (*UGT73B2*), At4g34131 (*UGT73B3*), At2g15490 (*UGT73B4*), At2g15480 (*UGT73B5*), At2g36750 (*UGT73C1*), At2g36760 (*UGT73C2*), At2g36780 (*UGT73C3*), At2g36770 (*UGT73C4*), At2g36800 (*UGT73C5*), At2g36790 (*UGT73C6*), At3g53160 (*UGT73C7*), and At3g53150 (*UGT73D1*).

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