Journal of Plant Biology, August 2006, 49(4) : 309-314

Molecular Genetic Analysis of Tandemly Located Glycosyltransferase Genes, *UGT73B1*, *UGT73B2*, and *UGT73B3*, in *Arabidopsis thaliana*

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In the *Arabidopsis* genome, approximately 120 UDP-glycosyltransferases (UGTs) have been annotated. They generally catalyze the transfer of sugars to various acceptor molecules, including flavonoids. To better understand their physiological roles, we analyzed a tandemly located putative flavonoid UGT cluster comprising *UGT73B1*, *UGT73B2*, and *UGT73B3* on Chromosome IV. We then isolated four loss-of-function mutations - *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, and *ugt73b3-2*. In our expression analysis, the closely related *UGTs* exhibited tissue-specific patterns of expression that were severely altered in their respective mutant plants. For example, *UGT73B2* was up-regulated in *ugt73b1-1*, whereas *UGT73B1* was highly expressed in *ugt73b2-1*, *ugt73b3-1*, and *ugt73b3-2*. Interestingly, each recessive mutant was resistant to methyl viologen (paraquat), an herbicide thought to cause oxidative stress. Our results suggest that *UGTs* play an important role in the glycosylation pathways when responding to oxidative stress.

Keywords: *Arabidopsis*, flavonoids, glycosylation, methyl viologen, oxidative stress, UDP-glycosyltransferases

Plant secondary metabolites play an important role in diverse growth and developmental processes, including disease resistance, stress tolerance, and protection against herbivory (Markham, 1982; Stafford, 1990; Harborne, 1994). Of these, the flavonoids, which share a common 15-carbon polyphenolic skeleton, are widely found in all plant parts (Markham, 1982). These compounds have diverse biochemical roles, e.g., pigmentation of flowers and fruits, UV protection, defense against microbial pathogens, and antioxidant properties (Dixon and Steele, 1999; Harborne and Williams, 2000; Winkel-Shirley, 2001; Saslowsky et al., 2005). Their aromatic rings and substituents contribute to their diverse biological activity *in vivo*. In addition, various structural modifications, including hydroxylation, methylation, acetylation, acylation, and glycosylation, increase the diversity of their functions (Markham, 1982; Harborne and Williams, 1988; Mehrtens et al., 2005). In particular, flavonoids are usually conjugated to sugars through the action of UDP-glycosyltransferases (UGTs) (Kroon et al., 1994; Ford et al., 1998; Miller et al., 1999; Yamazaki et al., 1999, 2002; Jones et al., 2003). This glycosylation by the UGTs is thought to have a wide range of effects *in planta*, such as on solubilization, stabilization, and detoxification (Jones and Vogt, 2001; Langlois-Meurinne et al., 2005). UGTs are encoded by large multigene families, of which family 1 comprises the most members (Vogt and Jones, 2000; Ross et al., 2001; Bowles, 2002). Based on their sequence similarities, approximately 120 UGTs, classified into 14 groups (A through N), have been predicted in the *Arabidopsis* genome (Li et al., 2001; Ross et al., 2001; Paquette et al., 2003). Their natural substrates have been being identified (Jones et al., 2003; Langlois-Meurinne et al., 2005; Poppenberger et al., 2005; Kim et al., 2006a, b). For example, analysis on two loss-of-function mutations in the *UGT73C6* and *UGT78D1* genes have revealed that *Arabidopsis* UGT73C6 and UGT78D1 can be classified as flavonol-3-*O*-rhanmosyltransferase and flavonol-3-*O*-glycoside-7-*O*-glucosylransferase, respectively (Jones et al., 2003). Moreover, *UGT78D2* and *UGT75C1* encode for flavonoid 3-*O*-glucosyltransferase and anthocyanin 5-*O*-glucosyltransferase, respectively, based on *in vitro* substrate identification and targeted profiling of a loss-of-function mutation (Tohge et al., 2005). The UGT group D, which includes *UGT73B1*, *UGT73B2*, and *UGT73B3* in *Arabidopsis*, is thought to be involved in stress responses caused by exposure to salicylic acid (SA), hydrogen peroxide (H_2O_2), and microbial pathogens (Langlois-Meurinne et al., 2005). In addition, *in vitro* analysis has revealed that UGT73B1 and UGT73B2 act preferentially as kaempferol-3-*O*-glucosyltransferase and flavonoid 7-*O*-glucosyltransferase, respectively (Kim et al., 2006a, b). However, the natural substrates and biological roles of individual UGTs remain elusive.

To provide a better understanding of UGT functions in *Arabidopsis*, we examined a cluster of tandemly located putative *UGTs* - *UGT73B1*, *UGT73B2*, and *UGT73B3* - that are highly homologous in their amino acid sequences (Li et al., 2001). These *UGTs* are expressed in a tissue-specific manner, with *UGT73B1* exhibiting the highest transcript levels in all tissues. This study also involved the isolation and characterization of loss-of-function mutations in *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, and *ugt73b3-2* to investigate their roles in oxidative stress caused by the herbicide methyl viologen (paraquat).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All plants used here were *Arabidopsis thaliana* ecotype Columbia (Col-0) and its derivatives. Seeds were surface-sterilized in 5% sodium hypochlorite and 0.15% Tween 20 for 3 min, rinsed in distilled water, and placed on MS plates (1X Murashige-Skoog salts; 0.5 mM MES, pH 5.7; 1% sucrose; and 0.8% agar). They were cold-treated at 4°C for 3 d, and then were grown vertically as described in the Arabidopsis Biological Resources Center manual (ABRC; http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm).

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Abbreviations: MV, methyl viologen; RT-qPCR, quantitative reverse transcriptase polymerase chain reaction; UGTs, UDP-glycosyltransferases.

Isolation of T-DNA Insertional Mutants

From database searches (SIGNAL; http://signal.salk.edu), we identified T-DNA insertional mutations in *UGT73B1* (At4g34138), *UGT73B2* (At4g34135), and *UGT73B3* (At4g-34131). To determine whether individual mutants were homozygous for no T-DNA insertion, heterozygous for the T-DNA insertion, or homozygous for the T-DNA insertion, we designed a couple of primers specific for the T-DNA left border (LB1 5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′), as well as gene-specific primers for *UGT73B1-1* (F1 5′-GCCATGAAAGGGAAGAGCAAG-3′, R1 5′-TCGCTTTTCAAA-TTTTGGTTTCA-3′, nested F2 5′-CCGGAGTGTTACGGTT-TAGGC-3′), *UGT73B2-1* (F1 5′-TCTGTTGGGTGACTCCAT-GAT-3′, R1 5′-CATCCTTCTGGTAACCCCAGC-3′, nested R2 5′-TGGTGAGGATTGTGGATTTGG-3′), *UGT73B3*-1 (F1 5′-GGGAAGAACACAACATGGAGC-3′, R1 5′-CCTGTAGGAG-CGGAGCAATTC-3′, nested R2 5′-TCTTGTCGTGTTGAAT-GATGTGG-3′), and *UGT73B3-2* (F1 5′-TTGGAGTTGAGAG-GTGTGGTGA-3′, nested F2 5′-CGATCTAAACAGCTTCA-TGGAAGAG-3′, R1 5′-TAAGCGGTGAGGGAGGTTTTG-3′). Using a pair of gene-specific primers for each *UGT* and the T-DNA border primers, we performed PCR-based genotyping analyses for the T-DNA insertional mutations. Each PCR product was sequenced to confirm the positioning of the insertions. Homozygous mutant plants were, in turn, backcrossed to wild-type (Col-0) plants, and plants homozygous for the T-DNA insertion were identified in the F_2 generation.

RNA Isolation and Real-Time RT-qPCR Analysis

Total RNA was isolated from various plant tissues, using an RNeasy Plant Mini kit (Qiagen, USA). Roots were harvested from 3-week-old plants grown vertically on MS plates, and whole seedlings were harvested at 7 d old from MS plates. Flower buds at several stages were collected from mature *Arabidopsis* plants (4 to 6 weeks old). Other tissue types (rosette leaves, cauline leaves, and stems) were obtained from 4-week-old plants grown in soil. After RNA extraction, we treated the samples with RNase-Free DNase I to remove potential contamination from genomic DNA (Promega, USA). The quality of the isolated RNA was checked by both gel electrophoresis and spectrophotometry. Reverse transcription using the RNA samples from different tissues and mutants was carried out with Superscript II RNase H - reverse transcriptase (Invitrogen, USA), according to the manufacturer's instructions. The synthesized cDNAs were used as templates in real-time quantitative RT-PCR (RT-qPCR). For that, a pair of gene-specific primers was designed to amplify approximately 110 to 130 bp of PCR product unique to each gene (Table 1). RT-qPCR analyses were performed with the Rotor-Gene 3000 (Corbett, Australia), using a SYBR Green Master Mix (Qiagen) according to the manufacturer's instructions. For the internal reference, 18S rRNA was used (Table 1). PCR reactions for all genes were performed as follows: 95°C for 15 min; 40 cycles at 95°C 10 s, 60°C 15 s, 72°C 20 s. Each reaction was duplicated, and expression of the *UGTs* in each mutant was analyzed in three separate biological replicates. The comparative quantitation method was employed to evaluate quantitative expression of the *UGTs*, and relative expression levels of 18S rRNA were used to normalize all the data.

Methyl Viologen (MV) Treatments and Measurement of Root Lengths and Chlorophyll Contents

For MV treatment, mutants and wild-type (WT) plants were grown in 1X MS plates containing 0, 0.5, 1.0, or 1.5 μM of methyl viologen (paraquat; Aldrich, USA). After 10 d, root lengths of the mutant and WT seedlings were measured via Scion Image for Windows vers. Beta 4.0.2 (Scion, USA). Chlorophyll contents were determined spectrophotometrically at 664 and 648 nm, after fresh plant materials were extracted with 95% ethanol for 20 min in an 80°C water bath, as previously described (Lichtenthaler, 1987). Standard deviations (± SD) of triplicates were calculated using the EXCEL program (Microsoft, USA).

Table 1. Primer sequences used in the RT-qPCR experiments. (AGI, *Arabidopsis* Genome Initiative; F, forward primer; R, reverse primer)

Gene	AGI Code	Primer Pairs
UGT73B1	At4g34138	F: 5′-AAGGAGTTAGCAGAAATGGCGA-3′ R: 5′-TACCTTCTCTTTTTGCAGTTTAAC-3′
UGT73B2	At4g34135	F: 5′-AGAGGCGGAGACGGGCAAA-3′ R: 5′-TGAACTAAACTCTTCCATGAAGC-3′
UGT73B3	At4g34131	F: 5′-AAGCGGTGAGGGAGGTGTTG-3′ R: 5′-CGAGGTAAACTCTTCTATGAAG-3′
18S rRNA	-	F: 5′-ACTGCTCTGCTCCACCTTCC-3′ R: 5′-TATTCAGAGCGTAGGCCTGC-3′

RESULTS AND DISCUSSION

Identification of Tandemly Located *UGT73B1, UGT73B2,* and *UGT73B3* Genes

In the *Arabidopsis* genome, approximately 120 UDP-glycosyltransferases (UGTs) have been predicted and classified into 14 distinct groups (Li et al., 2001; Ross et al., 2001; Bowles, 2002). Extensive effort has been made to characterize their physiological roles and substrate specificity (Jones et al., 2003; Langlois-Meurinne et al., 2005; Lee et al., 2005; Poppenberger et al., 2005; Kim et al., 2006a, b). Through a combination of metabolite-profiling and *in vitro* characterization of individual enzymes, *UGT73B1*, *UGT73B2*, *UGT73C6*, and *UGT78D1* have been proven likely to encode flavonoid UGTs (Jones et al., 2003; Kim et al., 2006a, b). However, the biological function and substrate specificity of most UGTs are yet undetermined.

Based on their sequence similarities, we have now identified a group of putative flavonoid UGT candidates in *Arabidopsis*. Of these, we selected for further study three tandemly located genes: *UGT73B1* (At4g34138), *UGT73B2* (At4g34135), and *UGT73B3* (At4g34131). These were chosen because previous *in vitro* analysis demonstrated that *UGT73B1* and *UGT73B2* encode for UDP-dependent glycosyltransferase and preferentially transfer a glucose group to the flavonoid 7-OH group and 3-OH group, respectively (Kim et al., 2006a, b). In our analysis, these three *UGTs* were highly homologous at the nucleotide and amino acid sequence levels (Fig. 1). For instance, *UGT73B1* and *UGTB73B2* exhibited 72.1% identity at the nucleotide level and 66.7% at the amino acid level, while *UGT73B2* and *UGTB73B3* showed 88.7% identity at the former and 84.2% at the latter. In addition, our sequence analysis revealed that *UGT73B1*, *UGTB73B2*, and *UGTB73B3* had a UDP-glycosyltransferase signature motif, comprising 45 consensus amino acid residues on the C-terminal regions (Fig. 1). These have

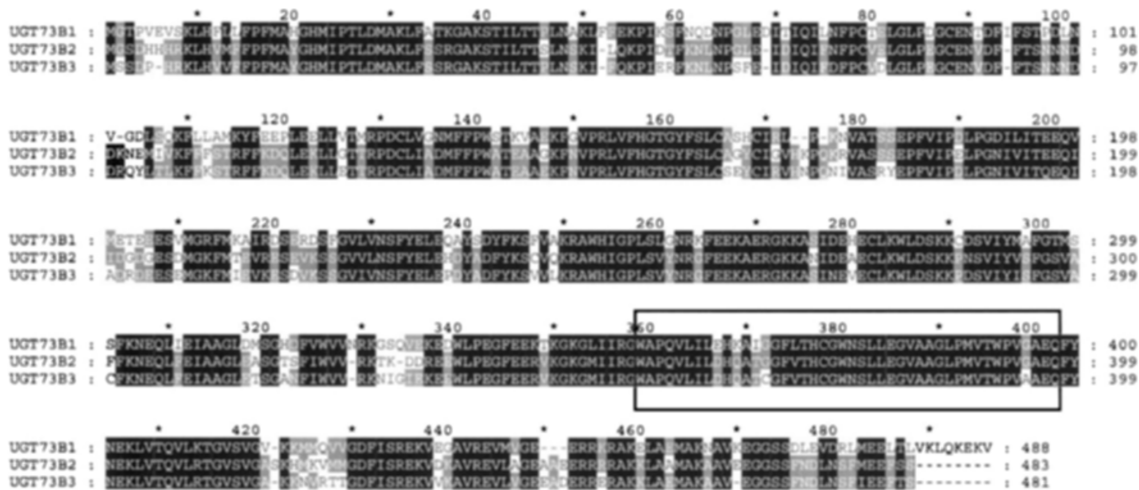

Figure 1. Comparison of deduced amino acid sequences for *UGT73B1*, *UGT73B2*, and *UGT73B3*. Identical amino acids are illustrated as white letters on black background; amino acids with weak (<50%) similarity are indicated as white letters on gray background. Boxed residues indicate consensus UDP-glycosyltransferase signature motif. Deduced amino acid sequences were aligned by ClustalX (vers. 1.83; Thompson et al., 1997), then adjusted manually.

been suggested as essential for binding the UDP moiety of activated sugar donors (Mackenzie et al., 1997). Interestingly, these *UGTs* were located as a cluster in tandem on Chromosome IV, confirming the fact that segmental duplication is common in the *Arabidopsis* genome (AGI, 2000; Li et al., 2001).

Tissue-specific Expression of *UGT73B1*, *UGT73B2*, and *UGT73B3*

The tissue-specific expression patterns of *UGT73B1*, *UGT73B2*, and *UGT73B3* were investigated by RT-qPCR, using RNA samples from various tissues (whole seedlings, roots, rosette and cauline leaves, stems, and flowers). Expression was relatively higher for *UGT73B1* than for the others (Fig. 2A). The lowest level of transcript was detected with *UGT73B3*, which suggests that this gene is either rarely expressed in the tissues tested or is expressed only in very specific cells or at certain developmental stages. Interestingly, *UGT73B1* and *UGT73B2* exhibited similar expression patterns in the tissues sampled, even though higher similarity in our pair-wise sequence comparisons had been found between *UGT73B2* and *UGT73B3* (Fig. 1). To substantiate our expression analysis, we compared the RT-qPCR results to the microarray analysis from the AtGenExpress consortium (http://www.weigelworld.org/resources/microarray/AtGenExpress) (Fig. 2A, B). Even though differences in patterns were minor between these two types of analyses, overall expression patterns were similar when these independent platforms were utilized, providing strong validation of the tissue-specific expression patterns of the *UGTs* (Fig. 2).

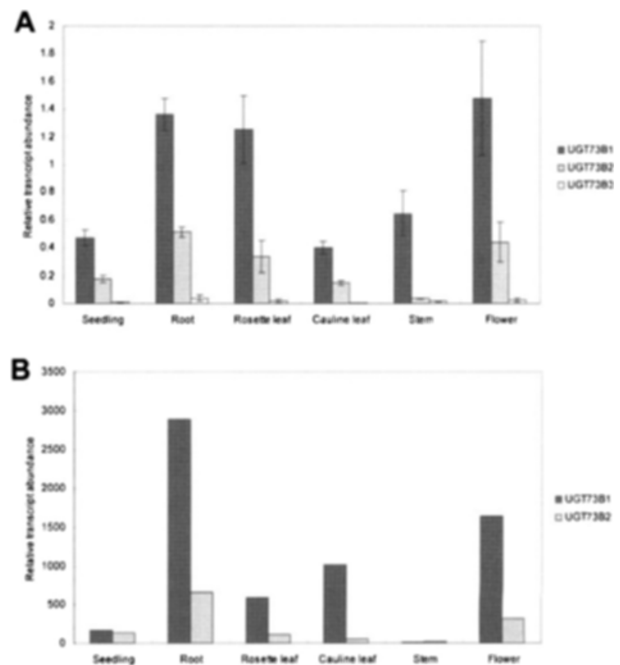

Figure 2. Tissue-specific expression of *UGT73B1*, *UGT73B2*, and *UGT73B3*. (**A**) RT-qPCR for samples of whole seedlings, roots, rosette leaves, cauline leaves, stems, and flowers. Standard deviations (± SD) of triplicates are indicated. (**B**) Microarray results for *UGT73B1* and *UGT73B2* from AtGenExpress consortium.

Identification of Loss-of-function Mutations in *UGT73B1*, *UGT73B2*, and *UGT73B3*

To better understand the physiological roles of UGTs *in planta*, we have begun to isolate loss-of-function mutations in *UGT73B1*, *UGT73B2*, and *UGT73B3*. Using database searches, we identified four T-DNA insertion lines (Fig. 3A). To verify these insertions, approximately 20 plants from each line were genotyped by a PCR-based method. The resulting PCR products were sequenced to verify the precise positioning of the insertions. For *UG73B1*, the T-DNA insertion was placed at 139 b upstream of the ATG start codon (named *ugt73b1-1*), while that of *UGT73B2* was located 115 b upstream of the ATG start codon (named *ugt73b2-1*) (Fig. 3A). For *UGT73B3*, two alleles were isolated, of which *ugt73b3-1* harbored the T-DNA insertion at 278 b upstream of the ATG start codon, whereas, for *ugt73b3-2*, the insertion was located 38 b downstream of the ATG start codon (Fig. 3A). No obvious phenotypes of these mutants were visible under standard growth conditions, suggesting that the highly homologous *UGTs* play a redundant role in the glyco-

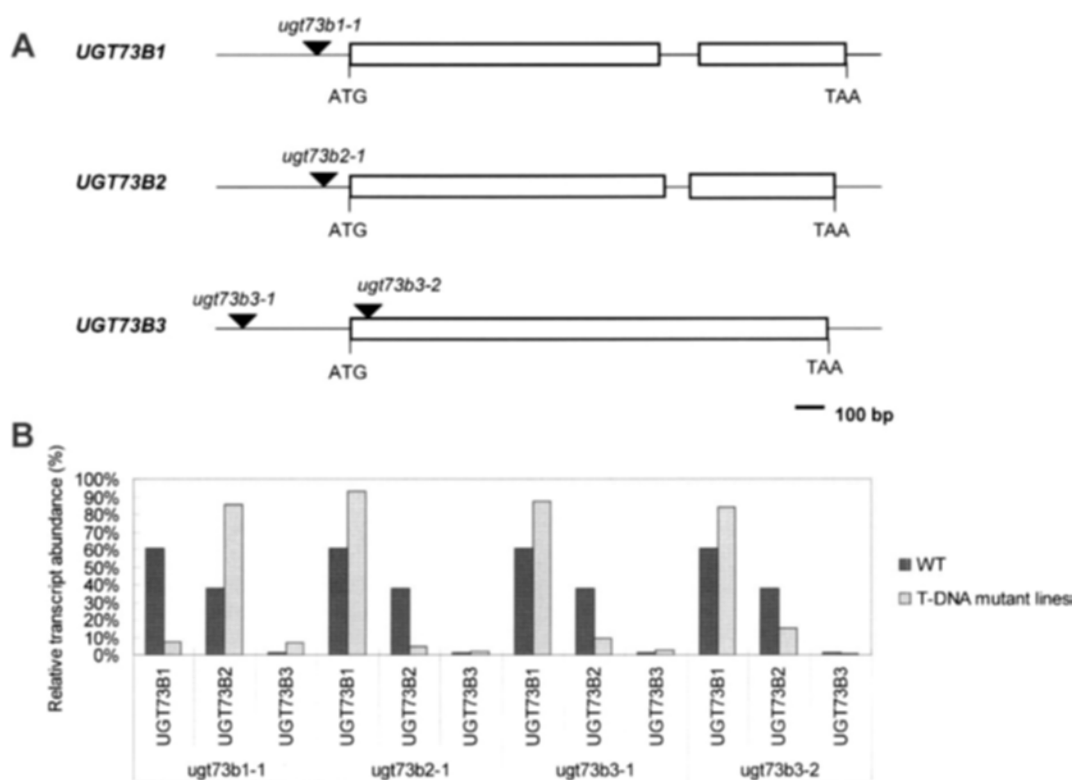

Figure 3. Expression analysis of *UGT73B1*, *UGT73B2*, and *UGT73B3*. (**A**) Loss-of-function mutations with arrow heads indicating T-DNA insertions. Lines represent non-coding regions of respective genes; boxes indicate exons. (**B**) Comparative analysis of *UGT73B1*, *UGT73B2*, and *UGT73B3* expression in *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, *ugt73b3-2*, and wild-type. RT-qPCR results were normalized using 18S rRNA as internal reference.

sylation pathways.

UGT expression was altered in each mutant line (Fig. 3B). As expected, that of *UGT73B1* was reduced in *ugt73b1-1*, compared with the wild-type (Fig. 3B). Interestingly, expression of *UGT73B2* and *UGT73B3* in *ugt73b1-1* was higher than in the WT (Fig. 3B). Similarly, expression of *UGT73B2* was severely reduced, and *UGT73B1* expression in *ugt73b2-1* was up-regulated compared with the WT (Fig. 3C). In *ugt73b3-1* and *ugt73b3-2*, *UGT73B3* expression was almost undetectable, while that of *UGT73B2* was also down-regulated. In contrast, *UGT73B1* expression was up-regulated in *ugt73b3-1* and *ugt73b3-2*, compared with the WT (Fig. 3B). The T-DNA insertion in *ugt73b3-1* was located in the promoter region, whereas T-DNA in *ugt73b3-2* was inserted in the first exon, 38 b downstream from the ATG start codon, suggesting that *ugt73b3-2* is a null allele (Fig. 3A, B). In general, high sequence conservation within an individual group of UGTs is assumed to be associated with similar glycosylation pathways (Ford et al., 1998; Martin et al., 1999; Vogt and Jones, 2000; Jackson et al., 2001; Lim et al., 2001; Ross et al., 2001). However, our analysis showed that expression of *UGT73B2* was up-regulated in *ugt73b1-1* while that of *UGT73B1* was up-regulated in *ugt73b2-1*, *ugt73b3-1*, and *ugt73b3-2* (Fig. 3B). Such discrete alterations in the *UGT* expression patterns for *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, and *ugt73b3-2* suggest that these *UGTs* function in similar glycosylation pathways.

Oxidative Stress by MV Treatments

Flavonoids are found in all plant tissues, including the leaves, roots, stems, pollen, flowers, and seeds (Markham, 1982; Bravo, 1998; Ross and Kasum, 2002). Each of flavonoids has diverse, beneficial biochemical and antioxidant effects (Liang et al., 1997). *In vitro* analysis has previously demonstrated that UGT73B1 and UGT73B2 are UDP-dependent glycosyltransferases and that they preferentially transfer a glucose group to the flavonoid 7-OH group and 3-OH group, respectively (Kim et al., 2006a, b). In addition, *UGT73B2*, *UGT73B3*, and *UGT73B5* can be induced by various biotic and abiotic stresses, e.g., pathogen infection, salicylic acid, methyl jasmonate, and oxidative stress caused by treatments with hydrogen peroxide (H_2O_2) or O_2^- (Asai et al., 2000; Mazel and Levine, 2002; Nishimura et al., 2003; Langlois-Meurinne et al., 2005). Here, we investigated the effects of oxidative stress caused by MV. *Arabidopsis* wild-type and mutant plants were grown in the presence of various MV concentrations. All mutants were relatively resistant to these treatments, whereas growth of the WT was drastically inhibited at 1 μM and 1.5 μM MV (Fig. 4A, B). Furthermore, chlorophyll *a* contents in mutants exposed to 1.0 μM MV were 3- to 6-fold higher than those of the WT (Fig. 4C). At 1.5 μM MV, chlorophyll *a* contents were 25- to 55-fold higher in the mutants. In addition, chlorophyll *b* contents were greatly reduced in the WT as the MV concentration increased (Fig. 4D). Flavonoids act as antioxidants in scavenging superoxide and peroxyl radicals (Yuting et al., 1990; Zhou and Zheng, 1991; Cotelle et al., 1992; Hanasaki et al., 1994; Jovanovic et al., 1994). In particular, the aglycones of flavonoids have been suggested as strong antioxidants compared with the flavonoid glycosides (Rice-Evans et al., 1996; Lee, 2004), because the free hydroxyl group

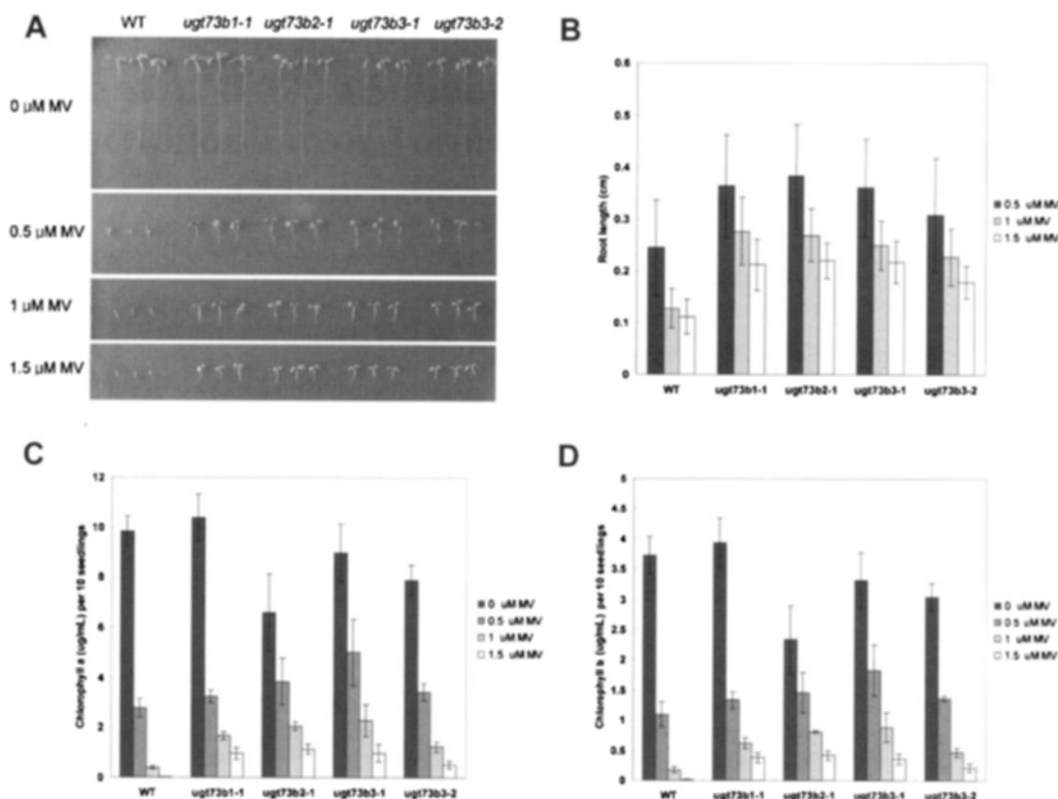

Figure 4. Effects of methyl viologen on phenotypes of *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, *ugt73b3-2*, and wild-type. (**A**) Phenotypes of 10-day-old light-grown seedlings following treatment with MV at 0, 0.5, 1.0, or 1.5 μM. (**B**) Effects of MV on root growth of *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, *ugt73b3-2*, and wild-type. (**C**) Analysis of chlorophyll *a* contents in *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, *ugt73b3-2*, and wild-type plants treated with different MV concentrations. (**D**) Analysis of chlorophyll *b* contents in *ugt73b1-1*, *ugt73b2-1*, *ugt73b3-1*, *ugt73b3-2*, and wild-type plants treated with different MV concentrations. Error bars represent standard deviations (± SD) of triplicates.

(-OH) of the aglycones plays a crucial role in scavenging free radicals, such as O_2^-, O_2, and H_2O_2 (Rice-Evans et al., 1996).

Overall, the loss-of-function mutations in *UGT73B1*, *UGT73B2* or *UGT73B3* exhibited MV-resistant phenotypes, compared with the WT, even though altered gene expression was observed in the various mutants. Based on previous *in vitro* analysis, the UGTs appear to have a substrate preference (Kim et al., 2006a, b), which implies that each UGT acts only on the subset of specific substrates in a pool of flavonoids. Considering the biochemical characteristics of flavonoid aglycones and the discrete alterations in *UGT* expression, it appears that a dynamic change in the pool of flavonoid aglycones occurs during glycosylation by the UGTs. Thus, loss-of-function mutations in any of those UGTs still exhibit inhibited growth, albeit with a less severe degree of susceptibility to MV, as demonstrated by the glycosylation of aglycones that continued with other members of UGTs *in planta*.

In summary, we have shown that a cluster of highly homologous UGTs exhibits tissue-specific expression patterns. Moreover, differential expression in the mutant plants and stress tolerance to MV treatment suggests that these *UGTs* play an important role in similar glycosylation pathways when responding to oxidative stress.

ACKNOWLEDGMENTS

For their helpful suggestions and discussions, we thank members of our laboratory, especially Dong Gwan Kim and Mi-Hyun Lee for their critical reading of the manuscript; and Jeong Ho Kim, Jung-Ok Heo, Nan-Ie Yu, In A Kim, and Ji Hye Yoon for their technical assistance and plant maintenance. This research was supported by grants from the Crop Functional Genomics Program (CG1123), the BioGreen 21 Program, and the second stage of the BK21 program, Korea. CE Lim was supported by a grant from the Korea Research Foundation (KRF2004-F00019).

Received June 5, 2006; accepted June 30, 2006.

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