UGT73C6 and UGT78D1, Glycosyltransferases Involved in Flavonol Glycoside Biosynthesis in Arabidopsis thaliana*

Received for publication, April 4, 2003, and in revised form, August 1, 2003
Published, JBC Papers in Press, August 4, 2003, DOI 10.1074/jbc.M303523200

Patrik Jones‡‡, Burkhard Messner*, Jun-Ichiro Nakajima‡, Anton R. Schaaffner‡, and Kazuki Saito‡‡‡

From the Department of Molecular Biology and Biotechnology, Graduate school of Pharmaceutical Sciences, Chiba University, Core Research for Evolutional Science and Technology of Japan Science and Technology Corporation (JST), Yayoi-cho 1-33, Inage-ku, Chiba 263-8522, Japan and the Institute of Biochemical Plant Pathology, GSF-National Research Center for Environment and Health, Ingolstädt Landstrasse 1, D-85764 Neuerberg, Germany

Flavonol glycosides constitute one of the most prominent plant natural product classes that accumulate in the model plant Arabidopsis thaliana. To date there are no reports of functionally characterized flavonoid glycosyltransferases in Arabidopsis, despite intensive research efforts aimed at both flavonoids and Arabidopsis. In this study, flavonol glycosyltransferases were considered in a functional genomics approach aimed at revealing genes involved in determining the flavonol-glycoside profile. Candidate glycosyltransferase-encoding genes were selected based on homology to other known flavonoid glycosyltransferases and two T-DNA knockout lines lacking flavonol-3-O-rhamnoside-7-O-rhamnosides (ugt78D1) and quercetin-3-O-rhamnoside-7-O-glucoside (ugt73C6 and ugt78D1) were identified. To confirm the in planta results, cDNAs encoding both UGT78D1 and UGT73C6 were expressed in vitro and analyzed for their qualitative substrate specificity. UGT78D1 catalyzed the transfer of rhamnose from UDP-rhamnose to the 3-OH position of quercetin and kaempferol, whereas UGT73C6 catalyzed the transfer of glucose from UDP-glucose to the 7-OH position of kaempferol-3-O-rhamnoside and quercetin-3-O-rhamnoside, respectively. The present results suggest that UGT78D1 and UGT73C6 should be classified as UDP-rhamnosylflavonol-3-O-rhamnosyltransferase and UDP-glucose:flavonol-3-O-glucosyl-7-O-glucosyltransferase, respectively.

Flavonols constitute a major class of plant natural products (PNPs) that share a common flavonoid nucleus and that accumulate in a wide range of conjugate structures. For example, over 350 different conjugate forms of a single flavonol, quercetin, have been observed to accumulate in plants to date (1). A large proportion of this structural variability is due to the attachment of one or several sugar moieties at different positions as illustrated in Fig. 1. Flavonoids, including flavonols, have recently attracted a lot of attention due to their proposed preventative role in coronary disease as a result of dietary intake (2). However, the uptake, metabolism, and biological effect of different flavonols and flavonol-conjugates vary greatly depending on the presence or absence and nature of the glycosidic structure (2–5). The ability to engineer modified flavonol glycoside accumulation profiles in crop plants is therefore an interesting proposition in an effort to improve flavonol bioavailability (5).

The enzymes involved in transferring sugars from a range of nucleoside donors onto acceptor molecules, glycosyltransferases (GTs), have been intensively studied recently (6–9). Over 110 putative family 1 GTs (UGTs) have been postulated to exist in the genomic sequence of the model plant Arabidopsis (8, 9). UGTs commonly utilize small molecular weight compounds as acceptor substrates and UDP-sugars as donors, and a large proportion of these predicted GTs are expected to act on PNPs. The functional determination of Arabidopsis UGTs in vitro is still limited to a handful of genes (10–13), whereas the in planta role of only three plant UGTs has been demonstrated to date (14–16). Furthermore, whereas several flavonoid GTs, primarily acting on anthocyanidin and anthocyanin acceptor substrates, have been characterized at a molecular level in a range of species including Petunia hybrida (14, 17), Vitus vinifera (18), and Perilla frutescens (19), none has as yet been reported from Arabidopsis. Although most anthocyanin-GTs are capable of also glycosylating flavonols, closer examination of the enzyme kinetics has revealed a strong preference for anthocyanidins (18), and there is as yet only one report that describes a flavonol-exclusive GT (20). Hence, our current knowledge regarding the synthesis of several hundred different conjugate forms of flavonols is still in its infancy.

Only recently were the structures of the major flavonoids in Arabidopsis revealed (21, 22) (Fig. 1). In an attempt to identify UGTs involved in determining the flavonol glycoside accumulation pattern of Arabidopsis, this paper reports on the in vitro and in planta characterization of two predicted UGTs (UGT78D1 and UGT73C6) that appear to be flavonol-specific in planta. The substrate specificities of both enzymes are novel with regards to sugar moieties and acceptor substrates, including the first in vitro characterization of a PNP rhamnosyltransferase (UGT78D1).

EXPERIMENTAL PROCEDURES

Biochemicals and General Methods—All biochemicals were obtained from Sigma, Roche Applied Science, Wako, or Kanto and were all of analytical or higher grade. SDS-PAGE was performed using high Tris linear 10% SDS-polyacrylamide gels (23), and polypeptides were visualized by staining with Coomassie Brilliant Blue (R-250). Protein concentrations were estimated using bovine serum albumin as a reference
Arabidopsis thaliana Flavonol-glycosyltransferases

Building—A. thaliana (ecotype Columbia) T-DNA mutant lines Garlic_525_H07.b.1a.Lb3Fa and Garlic_568_F08.b.1a.Lb3Fa, prepared according to Ref. 27 were kindly provided by Syngenta Research and Technology (San Diego, CA). Analyses (28) of the two mutant lines according to Ref. 27 were kindly provided by Syngenta Research and Technology (Kisarazu, Japan) and sequenced to confirm the identity. The control cDNA derived from pAW109 RNA was amplified according to Ref. 31 with an additional DNase treatment to remove traces of genomic DNA. After spectrophotometric OD measurements, equal amounts were reverse transcribed by Superscript II (GIBCO) according to manufacturer's instructions. Qualitative PCR reactions were performed using primers 78D1GF 5’-CACCGTTCTCGAGCAAGACCA and 78D1GR 5’-TCAATTATGGCATGTTGCTAGTTGCAT, UGT78D1 was amplified using first strand cDNA from leaves as template and primers 78D1GF 5’-GCTGAATGTCTTCTCTCAGTTCA and 78D1GR 5’-ATAGTCACCTGACTCACTCGAC for detection of UGT78D1 transcripts (32 cycles, annealing at 55 °C). Control reactions employed TUBF 5’-CCTGATACTCTGCTCTTTGG and TUBR 5’-CTGAATCCTCCATCTGGCAT for detection of β-tubulin transcript.

Enzymatic Assays—Activity was determined by filtration through Amicon ULTRAFREE-MC 5,000 nominal molecular weight filter devices (Millipore) and the intensity of dominant ions as recorded by LC-MS as described above for metabolite analysis. Nevertheless, where appropriate mono- and di-glycoside standards were not available, such comparisons remain approximate at best.

Results

Choice of Candidate Flavonol GTs—Arabidopsis leaves accumulate kaempferol glycosylated at the hydroxyl-group of the 3-position (multiple sugars) and 7-position of the flavonol nucleus (Fig. 1) in the absence of UV-light, as reported in Refs. 21 and 22. The results from the present paper suggest that quercetin-glycosides with similar structures to that of the kaempferol-glycosides also accumulate in flowers of wild type Arabidopsis (as described under “Identification of Missing Flavonol Glycosides”). The only report of a flavonoid-7-

Plant growth—A. thaliana (ecotype Columbia) T-DNA mutant lines Garlic_525_H07.b.1a.Lb3Fa and Garlic_568_F08.b.1a.Lb3Fa, prepared according to Ref. 27 were kindly provided by Syngenta Research and Technology (San Diego, CA). Analyses (28) of the two mutant lines predicted T-DNA insertions in the putative genes At2g6790 (UGT73C6) and At1g30530 (UGT78D1), respectively, as revealed by BLAST searches using the predicted amino acid sequences encoded by UGT78D1 and UGT73C6 (Syngenta Research and Technology). BASTA (UGT73C6) and At1g30530 (UGT78D1), respectively, as revealed by BLAST searches using the predicted amino acid sequences encoded by UGT78D1 and UGT73C6 (Syngenta Research and Technology). Analyses (28) of the two mutant lines verified by sequence analysis. UGT73C6 was amplified using AVS321229 as template and primers 73C6GF 5’-AAGAGATCCATTCGCTTCCAAA-AACACACGAAACC and 73C6GR 5’-AAGTCCGATCCAAATATGTCGAC-GTGTCGATGTTGCAT, UGT78D1 was amplified using first strand cDNA from leaves as template and primers 78D1GF 5’-GCTGAATGTCTTCTCTCAGTTCA and 78D1GR 5’-ATAGTCACCTGACTCACTCGAC for detection of UGT78D1 transcripts (32 cycles, annealing at 55 °C). Control reactions employed TUBF 5’-CCTGATACTCTGCTCTTTGG and TUBR 5’-CTGAATCCTCCATCTGGCAT for detection of β-tubulin transcript.

For semi-quantitative RT-PCR, total RNA was extracted from roots, leaves, and stems in florences according to Ref. 30, and from siliques according to Ref. 31 with an additional DNase treatment to remove traces of genomic DNA. After spectrophotometric OD measurements, different RNA samples were equalized according to the ethidium bromide fluorescence encompassing the region of 25 % to 18 % S RNA bands after gel electrophoresis. Equal amounts were reverse transcribed by Superscript II (Invitrogen). For all genes analyzed the number of cycles was selected from the phase of linear amplification. The artificial control transcript pAW109 was added prior to reverse transcription and used for normalization (32). Primers 78D1TRF 5’-AAACAGATGCTCTTCTTG, 78D1TRR 5’- CATACTCCCTCTAGCAGTCAT, 73C6TRF 5’- AGGATGGAGTAGAGCAACAGGA, 73C6TRR 5’- CAACTATTGAGACTGTCGAGTTG, TUBF, and TUBR were added for amplification of UGT78D1, UGT73C6, and β-tubulin cDNA fragments, respectively. The control cDNA derived from pAW109 RNA was amplified according to manufacturer's instructions (Applied Biosystems, Darmstadt, Germany).

Metabolite Analysis—Low molecular weight metabolites were extracted using 3 µl of extraction solvent (33) per mg of plant material and analyzed by liquid chromatography (LC) mass spectrometry (MS) as described previously (33) with the following modifications: A TSK-GEI ODS-80TM column (4.6 mm × 150 mm) (TOSOH) was employed for LC separation using buffer A (0.3% acetic acid) and buffer B (acetonitrile) and the following elution profile (0 min, 100% A; 3 min, 100% A; 30 min, 70%; 40 min, 0% A; 48 min, 100% A; 52 min, 100% A) using linear gradients in between the time points. All retention-times refer to absorbance at 340 nm.

Synthesis and Purification of Recombinant Proteins—The full-length EST clone AVS21229 (UGT73C6) was obtained from Kazusa DNA Institute (Kisarazu, Japan) and sequenced to confirm the identity. The full-length coding sequences were amplified by PCR, cloned into pGEX-6p2 (Amersham Biosciences) after appropriate restriction digests, and
O-glycosyltransferase to date is that of a baicalein-7-O- glucosyltransferase from Scutellaria baicalensis (accession number AF165148) (20). Our in silico approach toward finding candidates of novel Arabidopsis flavonol-GTs started with the identification of homologues to these known plant flavonoid-GTs for which evidence of expression was available as ESTs. The two strongest groups of Arabidopsis homologues to the S. baicalensis 7-GT included the UGT73B and UGT73C families, whereas the UGT78D family was the strongest candidate to the P. hybrida 3-GT (Table I). For this study UGT78D1 and UGT773C6 were chosen based on the availability of both ESTs and candidate mutant lines.

Identification of ugt78D1 and ugt73C6 Insertion Mutant Lines—A search of the Syngenta Arabidopsis T-DNA insertion collection (28) identified two candidate mutant lines for UGT73C6 and UGT78D1 genes (see “Experimental procedures”). The exact location of the T-DNA insertion in these candidate mutant lines was determined by sequencing PCR products obtained from genomic DNA using insert-specific and gene-specific primers. In both cases the T-DNA insertions were located within the open reading frames of the UGT genes (Fig. 2A). Qualitative reverse transcript PCR was then performed using RNA extracted from flowers or leaves of mutant lines and wild type Arabidopsis plants, confirming the absence of transcripts for both ugt73C6 and ugt78D1 mutant lines, respectively (Fig. 2B).

Identification of Missing Flavonol Glycosides—The main flavonoids in leaves of unstressed Arabidopsis (Columbia var.) wild type, grown in a plant growth room void of sunlight, are kaempferol-glycosides, with a spurious and variable presence of anthocyanins at low concentrations (Fig. 3A). The ugt78D1 mutant could easily be spotted by an absence of the flavonoid that accumulates to the highest concentration in leaves of unstressed Arabidopsis, previously identified as kaempferol-3-O-rhamnosome-7-O-rhamnoside (Fig. 3B, 32.8 min, dominant ions m/z 579.2 (M + H+), 433.1 (M + H+ − rhamnose), 287.4 (M + H+ − rhamnose − rhamnose) (21, 22). An equal scale mass spectra comparison, at 32.8 min, between wild type and ugt78D1 mutant leaf extracts clearly showed that no kaempferol-di-rhamnosome accumulated at all (Fig. 3, C compared with inset C2). The concentration of the other two main kaempferol-glycosides in ugt78D1 mutant leaves were greater compared with wild type lines.

Flowers of wild type Arabidopsis also accumulate quercetin-glycosides, in addition to kaempferol-glycosides, under the same growth conditions (Fig. 3D), although their structures are yet to be characterized. A tentative assignment of the main putative quercetin-glycosides based on mass spectra and comparisons with the already characterized kaempferol-glycosides includes: quercetin-glucoside-rhamnose (26.1 min, dominant ions m/z 633.2 (M + Na+), 465.1 (M + H+ − rhamnose), 303.4 (M + H+ − rhamnose − glucose)), quercetin-glucosyl-rhamnoside-rhamnoside (26.3 min, dominant ions m/z 779.3 (M + Na+), 611.1 (M + H+ − rhamnose), 449.1 (M + H+ − rhamnose − glucose)), quercetin-glucosyl-rhamnoside-rhamnoside (28.6 min, dominant ions m/z 633.2 (M + Na+), 449.1 (M + H+ − glucose), 303.4 (M + H+ − glucose − rhamnose)), and quercetin-rhamnoside-rhamnoside (30.6 min, dominant ions m/z 617.2 (M + Na+), 449.1 (M + H+ − rhamnose), 303.4 (M + H+ − 2 × rhamnose)). The relative order of elution, MS spectra and quantity of the three main quercetin-glycosides suggest that they share the same conjugate-structures as the three main kaempferol-glycosides. Two putative quercetin-glycosides (26.1 min and 30.6 min) were missing in flowers of ugt78D1 mutants (Fig. 3, D versus F) in addition to kaempferol-3-O-rhamnoside-7-O-rhamnoside. The former peak (26.1 min) was also missing in ugt73C6 mutants (Fig. 3E).

Expression of UGT78D1 and UGT773C6 in Arabidopsis Tissues—Because the metabolic phenotypes of UGT78D1 and UGT78D1 mutant lines were identified in leaves and flowers, the expression of both genes was examined in different Arabidopsis wild type tissues. Using semi-quantitative RT-PCR and normalization via an externally added artificial reference transcript, the expression of both Arabidopsis flavonoid glycosyltransferases was analyzed in roots, rosette leaves, stems, inflorescences, and immature siliques (see “Experimental Procedures”). UGT78D1 was expressed at similar levels in leaves, flowers, siliques, and stems, whereas UGT73C6 was essentially restricted to leaves and flowers (Fig. 2C). Both genes were detectable only at very low levels in roots. As an internal control, β-tubulin expression was shown to be ubiquitously transcribed.

In Vitro Characterization of Recombinant UGT78D1 and UGT73C6—Recombinant UGT78D1 and UGT73C6 were expressed in Escherichia coli strains AD494 as glutathionine-S-transferase fusions. Affinity purification revealed homogenous preparations of the expected molecular mass (UGT73C6 80 kDa, UGT78D1 75 kDa) after SDS-PAGE (data not shown). However, the affinity-purified enzyme preparations rapidly lost activity and further assays were therefore conducted with crude extracts containing the respective recombinant GTs and compared with crude extracts prepared from bacteria harboring only the empty vector. The in planta data suggested that UGT78D1 encodes a flavonol-3-O-GT with UDP-rhamnose as the preferred donor nucleoside. However, UDP-rhamnose is not commercially available and was therefore synthesized using UDP-glucose as precursor and crude desalted plant extracts as a source of enzymatic activity as described previously (35, 36). The presence of UDP-rhamnose was monitored by LC-MS using separation by hydrophilic-interaction chromatography (37).

Crude tobacco leaf extracts were prepared and initial experiments indicated that thorough desalting of the crude extract was absolutely essential for detectable UDP-rhamnose synthesis (data not shown). A product with the expected mass and UV spectrum of UDP-rhamnose, eluting shortly before the residual UDP-glucose peak, could be observed (Fig. 4).

In vitro confirmation of the substrate specificity predicted by in planta mutant line analysis was first attempted, although
**Arabidopsis thaliana Flavonol-glycosyltransferases**

**Fig. 2. Characterization of mutant lines.** A, position of T-DNA insertions in UGT78D1 and UGT73C6 derived from the sequencing of genomic DNA isolated from mutant lines (see “Experimental Procedures”). B, qualitative RT-PCR of UGT78D1 and UGT73C6 transcripts in homozygous mutant lines and wild type plants. PCR reactions were conducted using specific primers as indicated on the side of the gel pictures (see “Experimental Procedures”). C, expression of UGT78D1 and UGT73C6 in Arabidopsis tissues. Semi-quantitative RT-PCR, normalized using an externally added artificial reference transcript pAW109 (32) was conducted using primers specific for UGT78D1, UGT73C6, and β-tubulin. Each of the three gel pictures includes parallel gene-specific PCR reactions (upper) and artificial transcript reactions (pAW, lower). The figures above each lane quote the ethidium bromide intensity of two independent analyses normalized by the pAW109 RT-PCR product intensity and related to the expression in leaf tissue (set at 1.00). L, leaf; r, root; F, flower; Si, silique; St, stem.

*Arabidopsis* does not appear to accumulate any flavonol-mono-glycosides and the sequence of addition of the carbohydrate moieties was not known. Recombinant UGT78D1 catalyzed the synthesis of one major product (34.7 min, dominant ions m/z 433.3 (M + H+), m/z 287.5 (M + H+ – rhamnose)), using quercetin as an acceptor substrate and a UDP-rhamnose preparation as donor, with the expected mass profile and retention time of quercetin-3-O-rhamnoside (quercetrin) (Fig. 5A). The commercial quercitrin standard also contained a compound with the expected mass spectrum and UV-profile of an independently synthesized kaempferol-3-O-rhamnoside (37.1 min, Fig. 5B). The elution-time, UV-spectra, and mass-spectra were identical to that of an independently synthesized quercetin-3-O-rhamnoside-7-O-glucoside standard. Recombinant UGT73C6 produced very low amounts of several products, using the aglycons quercetin and kaempferol as acceptor substrates and UDP-glucose as donor substrate, with the expected mass profile of kaempferol- and quercetin-digluco-

sides (Table II). However, only one major product was synthesized using quercitrin as the acceptor substrate (26.5 min, dominant ions 610.9 (M + H+), 465.0 (M + H+ – rhamnose), 303.4 (M + H+ – rhamnose / glucose)). This product had the expected mass spectrum, UV spectrum, and retention time of the quercetin-rhamnoside-glucoside that is missing in flowers of both *ugt78D1* and *ugt73C6* mutant plants (Fig. 5B, inset B1). The elution-time, UV-spectra, and mass-spectra were identical to that of an independently synthesized quercetin-3-O-rhamnoside-7-O-glucoside standard. A minor product with the expected mass spectrum of a kaempferol-rhamnoside-glucoside (28.2 min, dominant ions 595.0 (M + H+), 449.0 (M + H+ – rhamnose), 287.4 (M + H+ – rhamnose – glucose)) could also be observed (Fig. 5B, inset B2). The same product was also

---

*B. Messner, A. Schäffner, W. Heller, and N. Hertkorn, unpublished data.*
observed when an independently synthesized kaempferol-3-O-rhamnoside standard was employed as acceptor substrate. UGT73C6 displayed more than 10-fold higher specific activity toward quercetin-3-O-rhamnoside compared with kaempferol and quercetin, indicating that glycosides, as opposed to aglycons, may be the preferred substrates.

To complement the in vitro qualitative substrate specificity of recombinant UGT73C6 and UGT78D1, a small range of other potential acceptor substrates were also tested (Table II). Both GTs appeared specific for flavonoids or flavonol-glycosides (UGT73C6) and flavonols (UGT73C6, UGT78D1) although other substrates that were not tested also may act as acceptor substrates. The positional specificity of UGT73C6 was confirmed using a range of hydroxyflavones that contain only a single hydroxyl group in one of four positions. Although 6- and 7-hydroxyflavone were glucosylated by recombinant UGT73C6, neither 3- nor 5-hydroxyflavone were accepted (Table II).

**DISCUSSION**

The Positional Specificity of UGT78D1 and UGT73C6—The kaempferol-glycoside missing in ugt78D1 mutant leaves is the only previously characterized kaempferol-glycoside that carries a rhamnose-moiety at the 3-position (21, 22). The availability of commercial and independently synthesized and verified standards for both the acceptor substrates and end-products of the UGT78D1 catalyzed reactions, unambiguously determined it as a flavonol-3-O-rhamnosyltransferase, despite an absence of flavonol-monoglycosides in Arabidopsis. The dual involvement of both UGT73C6 and UGT78D1 in the synthesis of the quercetin-rhamnoside-glucoside at 26.1 min (Fig. 3, D–F) suggests that the rhamnose-moiety of the UGT73C6 product is attached to the 3-position. Homology matching of predicted amino acid sequences (Table I) and in vitro hydroxyflavone specificity studies (Table II) point to the positional specificity of...
UGT73C6 as a 6-, 7-glucosyltransferase. No flavonols with a hydroxyl-group at the 6-position have yet been described from Arabidopsis, and the only so far characterized flavonol glycosides all carry sugar moieties at either the 3- or 7-position. An LC-MS comparison between the recombinant UGT73C6 product and an independently synthesized quercetin-3-O-rhamnoside-7-O-glucoside standard supported the positional specificity of UGT73C6 in planta as a flavonol-3-O-glycoside-7-O-glucosyltransferase, although a varied range of complex glycosides can be synthesized under non-native conditions in vitro (Table II). The combination of mutant phenotypes and in vitro analyses using recombinant proteins allowed the unequivocal identification of the first two GTs involved in flavonol glycosylation in Arabidopsis. Confirmation of the activity of the respective recombinant proteins in vitro suggests that the observed phenotypes of the mutant lines are due solely to the inactivation of each respective gene.

A combination of the present results together with previously identified flavonol-glycoside structures (21, 22) allows us to draw a most plausible flavonoid glycosylation scheme that predicts the involvement of at least five GTs in flavonol biosynthesis in Arabidopsis (Fig. 6). The preference displayed by UGT73C6 for glycosides compared with flavonol-aglycons, and the change in the glycoside accumulation pattern of the UGT78D1 mutant, indicate that 7-O-glucosylation, and possibly also 7-O-rhamnosylation, could occur after 3-O-glycosylation.

Substrate Specificity of UGT73C6 and UGT78D1—Although limited in breadth, a comparison between the in vitro substrate specificity profile of UGT73C6 and UGT78D1 indicated a
broader substrate specificity for the former enzyme. However, the impact of changes in the expression of both of the responsible genes have very precise effects on the final PNP profile, suggesting that in vitro studies alone do not always reflect fully the biological role in planta. Undoubtedly, the final glycosidic profile in any given plant is also determined by the availability of precursors as well as competition, compartmentation, and enzyme substrate specificity and kinetics. For example, isoflavones do not naturally accumulate in Arabidopsis (40), although UGT73C6 is capable of glucosylating genistein. The importance of the substrate specificity of the GT complement for stable accumulation of certain PNPs has been highlighted recently by attempts to introduce novel PNP biosynthetic pathways into Arabidopsis (41). Although a part of the entire PNP-GT complement of a given plant may display cross-specificity (40), it is clear that the entire complement (41) and the cross-specificity thereof is limited and that this is important for changes in the accumulation of PNPs as a result of altered expression of specific GTs.

UGT78D1 is only the fourth example of a family 1 GT that utilizes a nucleoside other than UDP-glucose as donor substrate, apart from Refs. 14, 17, 20, and 42. The data presented here suggests that a modification of the flavonol-glycoside profile in Arabidopsis is feasible due to an absence of complementing cross-specificities by other GTs, at least with regards to UGT73C6 and UGT78D1. Following the conclusions of Olthof et al. (5), the removal of all rhamnosyltransferases, presumably resulting in an accumulation of only flavonol-glucosides in Arabidopsis thaliana Flavonol-glycosyltransferases

FIG. 5. In vitro confirmation of the substrate specificity of recombinant UGT78D1 and UGT73C6, analyzed by reverse-phase LC-MS and monitored by absorbance at 340 nm. A, incubation with recombinant UGT78D1, a UDP-rhamnose preparation and quercetin produced a compound with the expected retention-time, UV spectrum and mass spectrum (inset A1) of a commercial standard of quercetin-3-O-rhamnoside (Sigma). B, incubation with recombinant UGT73C6, UDP-glucose, and quercetin-3-O-rhamnoside produced a major product (26.5 min) with the expected mass spectrum of a quercetin-rhamnoside-glucoside (inset B1) and a minor product with the expected mass spectrum of a kaempferol-rhamnoside-glucoside (inset B2). Note that the employed commercial standard of quercetin-3-O-rhamnoside also contains a compound with the expected mass-spectrum of a kaempferol-rhamnoside (37.1 min).
**CONCLUSION**

Flavonol glycosides are major PNP s in *A. thaliana* and the brassicaceae. Here, two GTs involved in flavonol-glycoside formation in *Arabidopsis* have been identified for the first time. Nevertheless, several others still remain unidentified. From the postulated glycoside biosynthetic scheme (Fig. 6) we can predict at least a flavonol-3-O-glucosyltransferase, a flavonol-3-O-glycoside-7-O-rhamnosyltransferase, and a flavonol-7-O-glycoside-3-O-(glycoside-2'-rhamnosyltransferase), if they display as strict *in planta* specificity as UGT78D1 and UGT73C6. The results from this paper and that of (15, 16) suggest that the glycosidic profile is strongly influenced by the substrate specificity and kinetics of the entire GT complement of any given plant as well as by the presence of appropriate precursors. Therefore, *in vitro* characterizations may not always fully reflect what occurs *in planta*, emphasizing the importance of the dual approach employed by (15, 16) and the present paper. The manipulation of plant flavonol-glycoside profiles has been demonstrated and the identification of mutant lines, such as those presented in this paper, indicate that modifications to the natural plant product accumulating profile of plants can be altered by changes to single genes.

**Acknowledgements**—We thank Werner Heller for helpful discussions and the donation of kaempferol-3-O-rhamnoside. We thank Syngenta Biopharmaceuticals for kindly donating materials. We thank the Plant Biotechnology Institute, CBG, and the SYNTEX Foundation for generous access to facilities.

**TABLE II**

Qualitative substrate specificity of recombinant UGT78D1 and UGT73C6 as analyzed by LC-MS

| acceptor                           | recombinant UGT73C6 |           | recombinant UGT78D1 |           |
|------------------------------------|---------------------|-----------|---------------------|-----------|
|                                   | activity            | retention time (dominant ions) | major product | activity            | retention time (dominant ions) | major product |
| kaempferol                         |                     |           |                     |           |
| quercetin                          |                      |           |                     |           |
| apigenin                           |                      |           |                     |           |
| genistein                          |                      |           |                     |           |
| cyanidin                           |                      |           |                     |           |
| kaempferol-3-O-glucoside           |                      |           |                     |           |
| quercetin-3-O-rhamnoside           |                      |           |                     |           |
| cyanidin-3-O-glycoside             |                      |           |                     |           |
| apigenin-7-O-glucoside             |                      |           |                     |           |
| 3-hydroxyflavone                   |                      |           |                     |           |
| 5-hydroxyflavone                   |                      |           |                     |           |
| 6-hydroxyflavone                   |                      |           |                     |           |
| 7-hydroxyflavone                   |                      |           |                     |           |
| kaempferol-7-O-neohesperidoside    |                      |           |                     |           |
| chrysin                            |                      |           |                     |           |
| p-hydroxybenzoic acid              |                      |           |                     |           |

**Fig. 6.** A proposed biosynthetic scheme based on the structures of previously identified flavonol-glycosides (21, 22), knowledge regarding plant flavonoid-GTs (6, 7, 18), and results from the present study. Enzyme annotations are in gray. Products are in black. Underlined products are missing in *ugt78D1* mutant lines. flav, flavonol; GT, glycosyltransferase; RT, rhamnosyltransferase; gly, glycoside; glc, glucoside; rha, rhamnoside.

**Table II.** Qualitative substrate specificity of recombinant UGT78D1 and UGT73C6 as analyzed by LC-MS.

| acceptor                           | recombinant UGT73C6 |           | recombinant UGT78D1 |           |
|------------------------------------|---------------------|-----------|---------------------|-----------|
|                                   | activity            | retention time (dominant ions) | major product | activity            | retention time (dominant ions) | major product |
| kaempferol                         |                     |           |                     |           |
| quercetin                          |                      |           |                     |           |
| apigenin                           |                      |           |                     |           |
| genistein                          |                      |           |                     |           |
| cyanidin                           |                      |           |                     |           |
| kaempferol-3-O-glucoside           |                      |           |                     |           |
| quercetin-3-O-rhamnoside           |                      |           |                     |           |
| cyanidin-3-O-glycoside             |                      |           |                     |           |
| apigenin-7-O-glucoside             |                      |           |                     |           |
| 3-hydroxyflavone                   |                      |           |                     |           |
| 5-hydroxyflavone                   |                      |           |                     |           |
| 6-hydroxyflavone                   |                      |           |                     |           |
| 7-hydroxyflavone                   |                      |           |                     |           |
| kaempferol-7-O-neohesperidoside    |                      |           |                     |           |
| chrysin                            |                      |           |                     |           |
| p-hydroxybenzoic acid              |                      |           |                     |           |

**Fig. 6.** A proposed biosynthetic scheme based on the structures of previously identified flavonol-glycosides (21, 22), knowledge regarding plant flavonoid-GTs (6, 7, 18), and results from the present study. Enzyme annotations are in gray. Products are in black. Underlined products are missing in *ugt78D1* mutant lines. flav, flavonol; GT, glycosyltransferase; RT, rhamnosyltransferase; gly, glycoside; glc, glucoside; rha, rhamnoside.
Science and Technology for access to T-DNA lines, Jin Tatsuzaki and Birgit Geist for helpful assistance, and Mami Yamazaki for help with obtaining T-DNA mutant lines. We also thank the Kazusa DNA Institute for providing EST clones.

REFERENCES

1. Harborne, J. B., and Baxter, H. (eds) (1999) The Handbook of Natural Flavonoids, Vol. 1, pp. 297–549, John Wiley & Sons, Chichester, UK
2. Klecz, P., Kumpulainen, J., Jarvinen, R., Rissanen, H., Heliovaara, M., Reunanen, A., and Halkola, T. (2002) Am. J. Clin. Nutr. 76, 565–568
3. Holman, C. H., Bispian, N. C. P., van Gameren, Y., Cnossen, E. P. J., and Bijsman, M. N. C. P. (2000) Phytochemistry 59, 386–393
4. Jones, P. J. M., and Plaquet, S. (2003) J. Biol. Chem. 278, 10291–10296
5. Jones, P. J. M., Moller, B. L., and Bak, S. (2003) Phytochemistry 62, 399–413
6. Lim, E. K., Doucet, C. J., Li, Y., Elias, L., Worrall, D., Spencer, S. P., Ross, J., Parr, A., Jackson, R. G., Ashford, D. A., and Bowles, D. J. (2001) J. Biol. Chem. 276, 6285–6288
7. Lim, E. K., Li, Y., Purr, A., Jackson, R. G., Ashford, D. A., and Bowles, D. J. (2001) J. Biol. Chem. 276, 4344–4349
8. Lim, E. K., Li, Y., Kowalczyk, M., Sandberg, G., Hoggett, J., Ashford, D. A., and Bowles, D. J. (2001) J. Biol. Chem. 276, 4350–4356
9. Lim, E. K., Lémus, C. J., Li, Y., Elias, L., Worrall, D., Spencer, S. P., Ross, J., and Bowles, D. J. (2002) J. Biol. Chem. 277, 586–592
10. Lim, E. K., Louw, C., and Strack, D. (2000) Plant Physiol. 121, 883–886
11. Lim, E. K., Louw, C., and Strack, D. (2000) Plant J. 9, 69–80
12. Lim, E. K., Louw, C., and Strack, D. (2000) Plant Physiol. 121, 883–886
13. Lim, E. K., Louw, C., and Strack, D. (2000) Plant J. 9, 69–80
14. Lim, E. K., Louw, C., and Strack, D. (2000) Plant J. 9, 69–80
15. Chong, J., Altz, B., Schmitt, C., Beffa, R., Fritig, B., and Saindrenan, P. (2002) Plant Cell 14, 1107–1117
16. Chong, J., Altz, B., Schmitt, C., Beffa, R., Fritig, B., and Saindrenan, P. (2002) Plant Cell 14, 1107–1117
17. Yamazaki, M., Yamagishi, E., Gong, Z., Fukuchi-Mizutani, M., Fukui, Y., Tanaka, Y., Kusumi, T., Yamaguchi, M., and Saito, K. (2002) Plant Mol. Biol. 48, 401–411
18. Ford, C. M., Boss, P. K., and Hig, P. B. (1998) J. Biol. Chem. 273, 9224–9233
19. Yamazaki, M., Gong, Z., Fukuchi-Mizutani, M., Fukui, Y., Tanaka, Y., Kusumi, T., and Saito, K. (1999) J. Biol. Chem. 274, 7406–7411
20. Miller, K. D., Guyon, V., Evans, J. N. S., Shuttleworth, W. A., and Taylor, L. P. (1999) J. Biol. Chem. 274, 34011–34019
21. Veit, M., and Pauli, G. (1999) J. Nat. Prod. 62, 1301–1303
22. Bloor, S. J., and Abrahams, S. (2002) Phytochemistry 62, 343–346
23. Bloor, S. J., and Abrahams, S. (2002) Phytochemistry 62, 343–346
24. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
25. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
26. Mackenzie, P. I., Owens, I. S., Burchell, B., Bock, K. W., Bairoch, A., Belanger, F., Fournier-Gigleux, S., Green, M., Hum, D. W., Iyagani, T., Lank, D., Louie, P., Magdalou, J., Chowdhury, J. R., Ritter, J. K., Schachter, H., Teply, T. R., Tipton, K. F., and Nebert, D. W. (1997) Pharmacogenet. 7, 255–269
27. McElver, J., Tafrir, I., Auz, G., Rogers, R., Ashby, C., Smith, K., Thomas, C., Schetter, A., Zhou, Q., Cushman, M. A., Tossberg, J., Nickle, T., Levin, J. Z., Law, M., Meinke, D., and Patton, D. (2001) Genetics 159, 1751–1763
28. Session, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacawaden, J., Ko, C., Clarke, J. D., Cotton, D., Bullis, D., Snell, J., Miguel, T., Hutchinson, D., Kimerly, B., Mitzel, T., Katagiri, F., Glazebrook, J., Law, M., and Goff, S. A. (2003) Plant Cell 14, 2985–2994
29. Gibeaut, D. M., Hulet, J., Cramer, G. R., and Seemann, J. R. (1997) Plant Physiol. 115, 317–319
30. Richards, K. D., Schott, E. J., Sharma, Y. K., Davis, K. R., and Gardner, R. (1998) Plant Physiol. 116, 499–418
31. Chang, S., Puryear, J., and Cairney, J. (1993) Plant Mol. Biol. Rep. 11
32. Weig, A., DeSartere, C., and Chibespe, M. J. (1997) Plant Physiol. 114, 1347–1357
33. Yamazaki, M., Nakajima, J. I., Yamanashi, M., Sugiyama, M., Makita, Y., Springbo, K., Awaushama, M., and Saito, K. (2003) Phytochemistry 62, 987–995
34. Jones, P. R., Manabe, T., Awaushama, M., and Saito, K. (2003) J. Biol. Chem. 278, 10291–10296
35. Kamsteeg, J., van Brederode, J., Kuipers-Tierie, E., and van Nigtevecht, G. (1979) Anal. Biochem. 9, 457–464
36. Bar-Peled, M., Levinsohn, E., and Gressel, J. (1991) J. Biol. Chem. 266, 34953–34959
37. Tolstikov, V. V., and Fiehn, O. (2002) Anal. Biochem. 301, 298–307
38. Hirata, M., Kuroda, R., Suzuki, H., and Yoshikawa, T. (2000) Plant Cell 12, 1006–1013
39. Deleted in proof
40. Liu, C. J., Blount, J. W., Steele, C. L., and Dixon, R. A. (2002) Sci. U. S. A. 99, 14578–14583
41. Tattersall, D. B., Bak, S., Jones, P. B., Olsen, C. E., Nielsen, J. K., Hansen, M. L., Hoj, P. B., and Moller, B. L. (2001) Science 293, 1826–1828
42. Martin, R. C., Machteld, C. M., and Mok, D. W. S. (1999) Plant Physiol. 120, 553–557