Two glycosyltransferases involved in anthocyanin modification delineated by transcriptome independent component analysis in Arabidopsis thaliana

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SUMMARY
To identify candidate genes involved in Arabidopsis flavonoid biosynthesis, we applied transcriptome coexpression analysis and independent component analyses with 1388 microarray data from publicly available databases. Two glycosyltransferases, UGT79B1 and UGT84A2 were found to cluster with anthocyanin biosynthetic genes. Anthocyanin was drastically reduced in ugt79b1 knockout mutants. Recombinant UGT79B1 protein converted cyanidin 3-O-glucoside to cyanidin 3-O-xylosyl(1→2)glucoside. UGT79B1 recognized 3-O-glucosylated anthocyanidins/flavonols and uridine diphosphate (UDP)-xylose, but not 3,5-O-diglucosylated anthocyanidins, indicating that UGT79B1 encodes anthocyanin 3-O-glucoside: 2′-O-xylosyltransferase. UGT84A2 is known to encode sinapic acid: UDP-glucosyltransferase. In ugt84a2 knockout mutants, a major sinapoylated anthocyanin was drastically reduced. A comparison of anthocyanin profiles in ugt84a knockout mutants indicated that UGT84A2 plays a major role in sinapoylation of anthocyanin, and that other UGT84As contribute the production of 1-O-sinapoylglucose to a lesser extent. These data suggest major routes from cyanidin 3-O-glucoside to the most highly modified cyanidin in the potential intricate anthocyanin modification pathways in Arabidopsis.

Keywords: xylosyltransferase, glycosyltransferase, anthocyanin, flavonoid, Arabidopsis thaliana.

INTRODUCTION
One of the goals of plant secondary metabolism research is to formulate a comprehensive understanding of gene functions in a particular synthetic pathway, including regulation and crosstalk with other metabolic processes and/or metabolites. However, the enzymes involved in secondary metabolism can be encoded by multigene families, making it difficult to determine their precise physiological functions (D’Auria and Gershenzon, 2005; Bowles et al., 2006). Completion of several plant genome sequencing projects has made cataloging a finite number of genes possible, as well as the development of multiple databases and bioresources for transcriptomes, proteomes, metabolomes and phenomes (Yonekura-Sakakibara and Saito, 2009; Mochida and Shinozaki, 2010). These ‘omics platforms also provide the tools for genome-wide approaches based on sequence similarity, transcriptomics, and correlations between transcripts and metabolites in addition to the more traditional biochemical and reverse genetics approaches (Fridman and Pichersky, 2005; Moreno-Risueno et al., 2010; Saito and Matsuda, 2010). These strategies facilitate an efficient narrowing-down of candidate genes involved in pathways of interest.
Flavonoids including the anthocyanins, flavonols, and flavones are some of the most intensely studied secondary metabolites with over 7000 known structures (Harborne and Baxter, 1999; Anderson and Markham, 2006). Many of them are important as flower pigments, UV-B protectants, signaling molecules between plants and microbes, and regulators of auxin transport (Dooner et al., 1991; Dixon and Paiva, 1995). Biosynthetic pathways leading to the flavonoid aglycones have been well-studied, and the corresponding regulatory and synthesis genes have been characterized in various plants (Davies and Schwinn, 2006; Tanaka et al., 2008). However, the pathways for sequential modification, such as glycosylation, acylation, and methylation, are still relatively unexplored, even although these modifications produce enormous chemical diversity and are essential for the stable accumulation of flavonoids.

*Arabidopsis thaliana* is one of the most studied plants for the molecular biology of flavonoid metabolism. The largest number of flavonoid-related genes including transcription factors have been identified from *Arabidopsis* because of the extensive ‘omics’-based information and bioresources available for this species. In *Arabidopsis*, flavonoid skeleton biosynthetic genes have been isolated on the basis of similarity or mutant phenotypes (Feinbaum and Ausubel, 1988; Shirley et al., 1992; Pelletier and Shirley, 1996; Pelletier et al., 1997; Kitamura et al., 2004). The genes involved in modification of flavonols and anthocyanins, flavonol 3-O-rhamnosyltransferase, flavonol 7-O-glucosyltransferase and anthocyanin sinapoyltransferase, have also been identified by genome-wide methods based on similarity (Jones et al., 2003; Fraser et al., 2007). Genes involved in flavonoid biosynthesis are in general coordinately expressed. Two genes encoding flavonoid glycosyltransferases, flavonoid 3-O-glucosyltransferase and anthocyanin 5-O-glucosyltransferase, were identified by transcriptome analysis of *A. thaliana* overexpressing *PAP1*, a transcription factor for anthocyanin biosynthesis (Tohge et al., 2005). Furthermore, two genes encoding flavonol glycosyltransferases, flavonol 7-O-rhamnosyltransferase and flavonol 3-O-arabinosyltransferase, were efficiently targeted from among over 100 candidate UGTs by transcriptome coexpression analyses using correlation coefficients which had been calculated based on publicly available transcriptome data (Yonekura-Sakakibara et al., 2007, 2008). Functional identification of six kinds of flavonoid glycosyltransferases in one plant species allowed us to expand the search for substrate specificity, regiospecificity and evolutionary processes. However, even in this highly studied species, its flavonoid structures suggest that there are as yet identified genes encoding modification enzymes.

To identify more candidate genes involved in flavonoid biosynthesis, we used independent component analysis (ICA) in addition to transcriptome coexpression analyses using correlation coefficients. ICA, a form of unsupervised algorithm, has been used as an effective analytical tool for microarray gene expression data (Kong et al., 2008). Originally ICA was developed as a method for multi-channel signal processing to separate mixed signals into their different sources. In the gene expression context, ICA has been applied to extract and characterize the informative features of biological signals from microarray data on the assumption that the level of gene expression is determined by a linear combination of some independent components corresponding to biological signals. Precise gene clustering and classification was achieved by ICA on yeast microarray data during sporulation (Hori et al., 2001) and during the cell replication cycle (Liebermeister, 2002). ICA can be also used for screening genes involved in oncogenesis and in Alzheimer’s disease (Chiappetta et al., 2004; Saidi et al., 2004; Frigyesi et al., 2006; Kong et al., 2009). The genes involved in the biosynthesis of anthocyanins were categorized by ICA into two clusters for anthocyanin skeleton biosynthesis and modification. Two glycosyltransferases, UGT79B1 and UGT84A2, were predicted to be anthocyanin biosynthetic genes by both transcriptome coexpression analyses using correlation coefficients and ICA, and by ICA only, respectively. Analyses of anthocyanin profiles in knockout mutants and recombinant protein assays demonstrated that UGT79B1 encodes anthocyanin 3-O-glucoside: 2”-O-xylosyltransferase and that, of the four UGT84As, UGT84A2 plays the major role in sinapoylation of anthocyanins. When considered in the context of the previously known genes in anthocyanin biosynthetic pathway, we have now assembled a ‘roadmap’ for the major anthocyanin modification routes in *Arabidopsis*.

**RESULTS**

**Anthocyanin UGT candidates deduced by independent component analysis and transcriptome coexpression analysis**

Previously, we conducted transcriptome coexpression analyses using correlation coefficients to identify all of the flavonoid-related genes and the relationship between flavonoid synthesis and other metabolic pathways. This examination of all *Arabidopsis* genes was performed using known 24 genes encoding flavonoid biosynthetic enzymes and transcription factors as query sequences (Yonekura-Sakakibara et al., 2008). Twenty-four genes had two or more positive correlation ($r > 0.525$) with known flavonoid pathway-related genes. Over 100 genes also showed some correlation with one of the known flavonoid pathway-related genes. To shed new light on the transcriptome data and to identify previously undetected genes in the flavonoid biosynthetic pathway, ICA of a total of 1877 genes, including the flavonoid biosynthetic genes and all genes annotated in AraCyc, was performed on 1388 microarray data with ATTED-II (http://atted.jp/ver.3) (Obayashi et al., 2009) using

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the fastICA algorithm (Hyvärinen and Oja, 1997) (Figure S1, Data S1). A hierarchical cluster analysis of gene signature matrix \( S \) based on eight independent components (ICs) indicates that the genes involved in the biosynthesis of anthocyanins and flavonols form distinct clusters with a few exceptions. Anthocyanin biosynthetic genes form a cluster with two sub-clusters, one of which contains dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS) and glutathione-S-transferase (GST), and is involved in anthocyanin skeleton biosynthesis (sub-cluster 2, Figure 1a). The other contains anthocyanin 5-O-glucosyltransferase (At4g14090), anthocyanin 3-O-glucoside: 6'-O-p-coumaroyltransferase (At1g03495), anthocyanin 5-O-glucoside: 6'-O-malonyltransferase (At3g19550), and the anthocyanin-specific MYB transcription factors PAP1 (At1g56650) and PAP2 (At1g66390) (sub-cluster 1; Figure 1A). These data suggest that ICA can be used to classify genes into biologically meaningful clusters. In addition to known anthocyanin-related genes, UGT79B1 (At5g54060) and a 2-oxoacid dehydrogenase family protein (At5g55070) also belong to the anthocyanin modification sub-cluster. UGT84A2 (At3g21560) is part of the anthocyanin biosynthesis sub-cluster. Of these three candidate genes, UGT79B1 showed high correlation (Yonekura-Sakakibara et al., 2008) and similar expression profile with known anthocyanin-specific biosynthetic genes (Solfanelli et al., 2006; Kusano et al., 2011). However, genes encoding 2-oxoacid dehydrogenase family protein and UGT84A2 did not correlate significantly with the anthocyanin-specific biosynthetic genes. By transcriptome coexpression analyses using correlation coefficients, UGT84A2 had relatively higher correlation coefficients with flavonol-specific biosynthetic genes (At3g55120, chalcone isomerase, \( r = 0.521 \); At3g51240, flavanone 3-hydroxylase, \( r = 0.515 \); At1g06000, flavonol 7-O-rhamnosyltransferase, \( r = 0.488 \); At5g08640, flavonol synthase, \( r = 0.425 \); At5g13930, chalcone synthase, \( r = 0.424 \); ATTED-II all data ver. 3) than with anthocyanin-specific genes (At5g42800, DFR, \( r = 0.387 \); At5g17220, GST, \( r = 0.378 \); At4g22880, ANS, \( r = 0.369 \); ATTED-II all data ver. 3) although the slight but reproducible induction of UGT84A2 by PAP1 over-expression suggests that UGT84A2 may be more distantly regulated by PAP1 (Tohge et al., 2005).

Flavonol biosynthetic genes fall into two sub-clusters, but there is no clear separation between skeleton biosynthesis and modification (Figure 1B). Genes encoding peroxisomal 3-keto-acyl-CoA thiolase (At5g48880), pyrophosphorylase (At2g18230), arogenate dehydratase (At1g08250), cinna-moyl-CoA reductase like protein (At4g30470) and chorismate mutase (At3g29200) were found in the flavonoid cluster. Of these five candidate genes, only At5g48880 showed high correlation with known flavonoid-related genes (Yonekura-Sakakibara et al., 2008). Thus, ICA identified additional candidate genes that are different from those tagged by transcriptome coexpression analyses based on simple correlation coefficients. We focused on the anthocyanin-related candidate genes, UGT79B1 and UGT84A2, because ICA based on eight ICs is apparently more suitable for analysis of the anthocyanin pathway.

**UGT79B1 belongs to the subfamily of UGTs catalyzing glycosylation at the sugar moiety of flavonoid glycosides**

The flavonoid UGT phylogenetic tree indicates that UGT79B1 belongs to a cluster in which UGTs transfer a...
glycosyl group to a sugar moiety of flavonoid glycosides (Figure 2). UGT79B1 has some amino acid sequence identity with IpA3G2″GlCT (48%), AcA3Ga2″XylT (48%), Ph3G2″RhaT (37%), BpA3G2″GlCAT (28%), and CmF7G2″RhaT (27%), all of which are known to catalyze glycosyl transfer to a sugar moiety of flavonoid glycosides (GTT) (Bar-Peled et al., 1991; Brugliera et al., 1994; Kroon et al., 1994; Morita et al., 2005; Sawada et al., 2005; Montefiori et al., 2011). The high correlation coefficients with anthocyanin biosynthetic genes, the anthocyanin substituted patterns found in Arabidopsis (Bloom and Abrahams, 2002) and the primary sequence similarity of UGT79B1 to related genes suggest that UGT79B1 encodes anthocyanin 3-O-glucoside: 2″-O-xylanosyltransferase.

Analysis of ugt79b1 transposon mutants indicates that UGT79B1 encodes anthocyanin 3-O-glucoside: 2″-O-xylanosyltransferase

Homozgyotes of two independent ugt79b1 Arabidopsis transposon insertion lines (Kuromori et al., 2004; Ito et al., 2005), Ds53-4592-1 and Ds54-1263-1, were isolated and designated as ugt79b1-1 and ugt79b1-2, respectively (Figure 3). The transposon was inserted into the exon of UGT79B1 of both mutants but at the positions +557 (ugt79b1-1), and between +61 and +86 base pairs (ugt79b1-2) (Figure 3A). No transcripts of UGT79B1 were detected by reverse-transcription polymerase chain reaction (RT-PCR) in homozgyotes of either line (Figure 3B). Seedlings of
wild-type and Ds parental lines of *ugt79b1-1* and *ugt79b1-2* accumulated anthocyanins when grown on 12% sucrose (Suc)-containing media. Homozygous insertion lines, *ugt79b1-1* and *ugt79b1-2*, grown on 12% Suc-containing media lacked the purple-coloration phenotype (Figure 3C).

The anthocyanin profiles of wild-type, Ds parental lines, *ugt79b1-1* and *ugt79b1-2* were analyzed by high performance liquid chromatography (HPLC)/photodiode array (PDA)/electrospray ionization mass spectrometry (ESI-MS) (Figure 4B). The major anthocyanin A11 was detected in wild-type and Ds parental lines, and A5, A8, A9 and A10 were also detected as minor anthocyanins (Figure 4A). While A5, A8, A9, A10 and A11 were not detected in insertion lines, *ugt79b1-1* and *ugt79b1-2*, total anthocyanin content was reduced to ca. 33% of wild-type. Instead, a compound which has an m/z value corresponding with cyanidin 3-O-(6‴-O-p-coumaroylglucoside)-5-O-(6‴-O-malonylglucoside) (m/z 843, RT 26.09 min) was detected as a major peak in addition to cyanidin 3-O-glucoside (m/z 449, RT 16.07 min). A peak with an m/z value corresponding to cyanidin 3-O-6‴-O-p-coumaroylglucoside (m/z 595, RT 29.26 min) was also found. *ugt79b1-1* plants were transformed with UGT79B1 cDNA under the control of cauliflower mosaic virus (CaMV) 35S promoter to complement the *ugt79b1-1* mutation. Independent transgenic lines had essentially the same anthocyanin composition as wild-type and Ds parental plants.

**In vitro characterization of recombinant UGT79B1**

Recombinant UGT79B1 protein was expressed in *Escherichia coli* as a His-tag fused protein and purified. The His-UGT79B1 protein catalyzed the conversion of cyanidin 3-O-glucoside to a single product, cyanidin 3-O-xilosyl(1→2)glucoside (Figure 5) as confirmed by retention time and MS/MS spectra. The His-tag alone as a negative control did not catalyze conversion to the 2‴-O-xiloside. Thus, UGT79B1 can be defined as an anthocyanin 3-O-glucoside: 2‴-O-xilosyltransferase.

The specificity of UGT79B1 as a sugar acceptor was also examined. UGT79B1 had significant activity with the 3-O-glucoside derivatives of anthocyanidins and flavonols (Table 1). Interestingly, UGT79B1 recognizes cyanidin/kempferol 3-O-rhamnosyl(1→6)glucoside but not cyanidin 3,5-O-diglucoside or kaempferol 3-O-glucoside-7-O-rhamnoside although the latter compounds appear to have more free space around the glucosyl moiety at the C-3 position.
UGT79B1 can utilize flavonol 3-O-glucosides as substrates, but no flavonol 3-O-xylosylglucosides were detected in Arabidopsis seedlings grown in 12% Suc-containing medium.

The sugar donor specificity of UGT79B1 was examined with UDP-xylose, UDP-glucose, UDP-arabinose, UDP-glucose, UDP-rhamnose, UDP-galactose and UDP-glucuronic acid as donors and cyanidin 3-O-glucoside as acceptor (Table 1). No UGT activity was detected for UDP-sugars other than UDP-xylose, indicating that UGT79B1 is highly specific to UDP-xylose.

### Table 1 Substrate specificity of UGT79B1 from Arabidopsis thaliana

| Sugar acceptor | Relative activity (%) |
|----------------|-----------------------|
| Cyanidin 3-O-glucoside | 100.0 ± 27.5 |
| Cyanidin 3-O-rhamnoside | ND |
| Cyanidin 3-O-rhamnosyl(1 → 6)glucoside | 48.1 ± 10.7 |
| Cyanidin 3,5-O-diglucoside | ND |
| Cyanidin 3-O-(6″-O-p-coumaroylglucoside)-5-O-glucoside | ND |
| Cyanidin 3-O-(6″-O-p-coumaroylglucoside)-5-O-(6′-O-malonylglucoside) | ND |
| Delphinidin 3-O-glucoside | 28.3 ± 0.8 |
| Pelargonidin 3-O-glucoside | 48.1 ± 8.8 |
| Kaempferol 3-O-glucoside | 285.4 ± 34.0 |
| Quercetin 3-O-glucoside | 121.6 ± 16.7 |

| Sugar donor | Relative activity (%) |
|-------------|-----------------------|
| UDP-xylose | 100.0 ± 27.5 |
| UDP-arabinose | ND |
| UDP-galactose | ND |
| UDP-glucose | ND |
| UDP-glucuronic acid | ND |
| UDP-rhamnose | ND |

ND, not detected.

aThe reactions were performed with UDP-xylose as the sugar donor.
bThe reactions were performed with cyanidin 3-O-glucoside as the sugar acceptor.

Phylogenetic analyses of the UGTs from six genome-sequenced plants

To assess the origin of GGTs from a broader perspective, we conducted phylogenetic analyses of the UGTs from six genome-sequenced plants (Physcomitrella patens, Selaginella moellendorffii, Populus trichocarpa, Oryza sativa, Arabidopsis thaliana and A. lyrata) in addition to known flavonoid GGTs. Plant UGTs fell into 24 orthologous groups (OGs) that contained genes derived from a common ancestor of these six species (Yonekura-Sakakibara and Hanada, 2011). All known flavonoid GGTs belong to an orthologous group (OG8), suggesting that flavonoid GGTs are derived from a common ancestral gene (Figure 6). Unfortunately, the functions of other UGTs in this orthologous group have remained elusive. However, a furofuran lignan GGT (UGT94D1), which glucosylates at the 6′-hydroxyl group of (+)-sesaminol 2-O-glucoside, have been isolated from Sesamum indicum (Noguchi et al., 2008). UGT94D1 cannot utilize flavonoid glycosides as substrates, but belongs to the same UGT94 family with BpA3G2′GlcAT.
This finding suggests that GGT function including a recognition mechanism for the hydroxyl group of the sugar moiety was established before the divergence of UGT94s, UGT91s and UGT79s and only then acquired the ability to specify substrates (sugar acceptors and sugar donors) and regiospecificity. Functional identification of other UGTs in OG8 will provide useful information for UGT evolution in terms of acquiring substrate specificity.

UGT84A2 supplies sinapoylgalactose for anthocyanin modification

By the hierarchical clustering of genes based on ICA, UGT84A2 was localized to sub-cluster 2, which is involved in anthocyanin skeleton biosynthesis. It has been reported that UGT84A2 encodes UDP-glucose: sinapic acid glucosyltransferase required for the biosynthesis of 1-O-sinapoylgalactose (Lim et al., 2001; Sinlapadech et al., 2007). 1-O-sinapoylgalactose is utilized as an acyl donor by serine carboxypeptidase-like acyltransferases including malate sinapoyltransferase and choline sinapoyltransferase (Lehfeldt et al., 2000; Shirley et al., 2001). In *ugt84a2* knockout mutants, sinapoylmalate content was slightly decreased and other sinapoylated/feruloylated compound contents were altered compared to wild-type (Sinlapadech et al., 2007; Meissner et al., 2008). Anthocyanin sinapoyltransferase (SAT, AT2g23000) also belong to serine carboxypeptidase-like acyltransferases, suggesting that 1-O-sinapoylgalactose produced by UGT84A2 may be a major source of sinapoyl groups to anthocyanin sinapoyltransferase. However, there is no direct evidence for a relationship between anthocyanin biosynthesis and UGT84A2 in * planta*.

Analysis of *ugt84a* mutants supports the predominant involvement of UGT84A2 in anthocyanin acylation

A homozygote of an ectotype Nossen transposon insertion line, Ds11-5836-1, was isolated and designated as *ugt84a2-1*. The G-edge of the transposon was inserted into the exon of the mutant at position +266 (Figure 7A), creating a null mutation (Figure 7B). When grown on 12% Suc-containing medium, *ugt84a2-1* seedling pigment phenotype was the same as the Ds parental and wild-type lines (Figure 7C).

The anthocyanins of wild-type, Ds parental line Ds11 and *ugt84a2-1* were analyzed by HPLC/PDA/ESI-MS (Figure 8). In wild-type and Ds parental lines, A11 was detected as a major anthocyanin (>50% of total anthocyanins), and A5, A8, A9 and A10 were detected as minor peaks (5–20% of total anthocyanins). In *ugt84a2-1*, A11 made up only about 20% of the total anthocyanins and A5 accounted for 40–50%, although there is no significant change in total anthocyanin contents. The A11 levels of *ugt84a2* knockout mutants were reduced to approximately 25% of wild-type. Compared with the impact on other sinapoylated compounds such as sinapoylmalate and sinapoylcholine (60–70% of wild-type) (Sinlapadech et al., 2007; Meissner et al., 2008), the effect on...
A11 content was more severe. Other sinapoylated anthocyanins (A9 and A10) were largely unaffected. To determine if the changes in anthocyanin composition can be ascribed to the UGT84A2 mutation, ugt84a2-1 plants were transformed with UGT84A2 cDNA under the control of CaMV 35S promoter. Independent complemented UGT84A2 transgenic lines had substantially the same anthocyanin composition as wild-type and Ds parental plants (Figure 8A). These data indicate that 1-O-sinapoylglucose produced by UGT84A2 is a significant source of sinapoyl moieties for anthocyanins, and that the limited supply of 1-O-sinapoylglucose affects anthocyanin composition, reducing the content of sinapoylated anthocyanin, A11.

UGT84A2 and closely-related UGTs (UGT84A1 and UGT84A3) have significant UDP-glucose: sinapic acid glucosyltransferase activity, but the specific activities of both UGT84A1 and UGT84A3 for sinapic acid are nearly half that of UGT84A2 (Lim et al., 2001). UGT84A2 showed the highest correlation coefficients with UGT84A1 \( (r = 0.403, \text{ATTED-II, ver.3}) \) among UGT84As \( (UGT84A3, r = 0.077, UGT84A4, r = 0.144) \). To investigate the contribution of other UGT84As to anthocyanin composition, we also isolated a homozygous T-DNA insertion mutant of UGT84A2 with a Col-0 background, brt1-1 (ugt84a2-2), because ugt84a1-1 has a Col-0 background. When grown on 12% Suc-containing media, ugt84a1-1 seedlings showed a purple-color phenotype that was indistinguishable from wild-type (Col-0) and brt1-1 (Figure 7C). We analyzed the anthocyanin profiles of ugt84a1-1 and brt1-1 (ugt84a2-2) (Figure 8B). The brt1-1 (ugt84a2-2) mutant in a Col-0 background had a similar anthocyanin profile to ugt84a2-1. However, no significant change of the anthocyanin profile in ugt84a1-1 was observed compared with wild-type (Col-0). These data indicate that UGT84A2 is a major supplier of 1-O-sinapoylglucose for anthocyanin modification. The predominance of A11 in ugt84a2 knockout mutants from different backgrounds suggests that other UGT84As also contribute the production of 1-O-sinapoylglucose, but to a much lesser extent.

DISCUSSION

Flavonoid UGTs which glycosylate the sugar moiety attached to flavonoid aglycones

The functional identification of UGT79B1 allowed us to compare flavonoid GGTs that glycosylate the sugar moiety attached to flavonoid aglycones. Generally, flavonoid UGTs form a unique cluster based on their regiospecificity for sugar acceptors (i.e. the glycosylation position of sugar acceptors). Furthermore, in the case of UGT, which glycosylates at the C-3 position of flavonoids (3GT), the phylogenetic tree indicates that the function of 3GT was established before the divergence of monocots and dicots, and the specificity of sugar donors was afterward (Figure 2). However, no such systematic phylogenetic trace was found in the GGTs. IpA3G2‘GlicT (UGT79G16) is distant from Ph3G6‘RhaT although they are from the same order (Solanales). Anthocyanin 3-0-galactoside: 2”-O-xylosyltransferase from kiwifruit (Actinidia chinensis, AcA3Ga2‘XylT), recognizes cyanidin 3-0-glucoside and UDP-xylose like
UGT79B1 does. However, AcA3Ga2"XylT had higher similarity with IpA3G2"GlcT, which uses UDP-glucose as a sugar donor (63%), than with UGT79B1 (48%). Plant UGTs have a carboxyl-terminal consensus sequence of 44 amino acid residues termed the plant secondary product glycosyltransferase (PSPG) box (Mackenzie et al., 1997; Paquette et al., 2009). The PSPG box is thought to be involved in binding to the UDP moiety of the sugar nucleotide (Mackenzie et al., 1997). The PSPG box of UGT79B1 showed higher sequence identity with that of IpA3G2"GlcT (68%) than with that of AcA3Ga2"XylT (59%), although both UGT79B1 and AcA3Ga2"XylT recognize UDP-xylose.

Phylogenetic comparisons of flavonoid GGTs, including predicted common ancestral UGTs at nodes 7–10 suggest possible conserved amino acid residues involved in recognizing UDP-xylose and anthocyanin 3-O-glucosides (Figure S2). Ancestral sequences for four nodes (node 7 to node 10) were inferred by a maximum likelihood method (PAML: Phylogenetic Analysis by Maximum Likelihood, ver. 4.3). We assumed that the occurrence of enzymatic divergence was due to amino acid replacements, and ancestral GGT(s) obtained the ability to recognize UDP-xylose at the branch of node 7 to node 8, and was preserved in the two branches (node 8 to node 9, and node 9 to AcA3Ga2"XylT), but was lost at node 9 to IpA3G2"GlcT. Following this simple assumption, amino acid residues involved in recognition of UDP-xylose and anthocyanin 3-O-glucosides are expected to be conserved in all divergences except for two branches (node 10 to BpA3G2"GlcAT and node 7 to PhA3G6"RhaT). The multiple alignment of flavonoid GGTs shows that most amino acid residues conserved in flavonoid GGTs are common to other known flavonoid UGTs, and Met16 is clearly specific to flavonoid GGTs (Figure S2). In general, UGTs belong to the GT-B fold with two Rossmann-like domains (Coutinho et al., 2003), and the crystal structures of plant UGTs (grape VvGT1, UGT71G1 and UGT85H2 from Medicago truncatula) have been determined (Shao et al., 2005; Offen et al., 2006; Li et al., 2007). Protein modeling and site-directed mutagenesis of BpA3G2"GlcAT suggest that N123 and D152 are key residues for recognition of cyanidin 3-O-glucoside (Osmani et al., 2008). However, the residues are not conserved in other flavonoid GGTs. Crystallization of UGT79B1 would be required for precise determination of the amino acid residues involved in substrate recognition because of low sequence identity between plant UGTs.
Anthocyanin modification pathway forms metabolic grids in Arabidopsis

In Arabidopsis, it has been estimated that the anthocyanin modification pathway forms a metabolic grid as proposed for *Perilla frutescens* and *Gentiana triflora* (Yamazaki et al., 1999; Fukuchi-Mizutani et al., 2003; Fraser et al., 2007). Enzymatic characterization of anthocyanin xylosyltransferase and of the anthocyanin profile in *ugt84a2*, together with previous reports, provides a clue as to the likely major routes of anthocyanin modification (Figure 9).

UGT79B1 recognizes cyanidin 3-O-glucoside and cyanidin 3-O-rhamnosyl(1 → 6)glucoside as substrates, but not cyanidin 3,5-O-diglucoside. Other UGTs which catalyze glycosylation at the sugar moiety of flavonoid glycosides also show considerable activity toward anthocyanin 3-O-glucosides, but no or negligible activity toward anthocyanin 3,5-O-diglucosides (Morita et al., 2005; Sawada et al., 2005). Arabidopsis anthocyanin coumaroyltransferase, which transfers a coumaroyl moiety to the C-6’ position of cyanidin 3-O-glucoside prefers cyanidin 3-O-glucoside, but has negligible activity for cyanidin 3,5-O-diglucosides (Luo et al., 2007). These data imply that xylosylation and coumaroylation at C-2’ and C-6’ of cyanidin 3-O-glucoside, respectively, occur prior to glucosylation at the C-5 position. It has been reported that acylation with a coumaroyl moiety is effective for anthocyanin stability (Luo et al., 2007). On the other hand, acylated anthocyanin 3-O-glucosides are unstable, but are the best substrates for anthocyanin 3-O-glucoside 2’-O-xylosyltransferase in *Matthiola incana* R.Br (Teusch, 1986). Unfortunately, acylated anthocyanin 3-O-glucosides are not commercially available. The substantial anthocyanin reduction observed in *ugt79b1* mutants suggests that 2’-O-xylosylation is crucial for stable accumulation of anthocyanin in Arabidopsis.

In *ugt84a2* knockout mutants, A5 accumulated as a major anthocyanin instead of A11. The content of sinapoylated anthocyanin A9 and A10, and the non-sinapoylated anthocyanin A8 showed no significant change. This finding suggests that anthocyanin A11 may be mainly produced from A5 via A9. Interestingly, in an *sng1-5* mutant that lacks anthocyanin sinapoyltransferase, A8 and A5 accumulated as major anthocyanins (Fraser et al., 2007). The differences in accumulated anthocyanins in these mutants suggest that the affinity of A5 for anthocyanin sinapoyltransferase may be higher than for anthocyanin coumaroyltransferase. Anthocyanin sinapoyltransferase may hold A5 in mutants and thus inhibit further modification.

Independent component analysis provides a different perspective on microarray data analysis by IC numbers

The hierarchical clustering of a gene signature matrix with a total of 1877 metabolism-related genes based on eight ICs formed clear clusters of genes involved in the biosynthesis of anthocyanins and flavonols, which are slightly different from those formed by transcriptome coexpression analysis. Among the algorithms with various numbers of ICs we applied, the closest anthocyanin/flavonol clusters were observed based on eight ICs. Fukushima et al. proposed that a small number of samples (~20) are enough to find coexpression linkage (Fukushima et al., 2008). Kinoshita and Obayashi examined principal component analysis (PCA) for identifying the major factors of gene expression correlation, and found the contribution of the first 10 principal components (PCs) to be enough to describe 80% of the variation within the 1388 samples of ATTED-II (Kinoshita and Obayashi, 2009).

Interestingly, the cluster that flavonoid 3-O-glucosyltransferase (Fd3GlcT; UGT78D3, At5g17050) belongs to changes depending on the number of ICs. For example, Fd3GlcT falls into the flavonol cluster based on 8 ICs, but into the anthocyanidin modification sub-cluster when based on 10 ICs (data not shown). Further, using only simple correlation coefficients, Fd3GlcT, which can recognize both flavonols and anthocyanidins as substrates, localizes to a flavonol gene cluster, but not to an anthocyanin group (Yonekura-Sakakibara et al., 2007). In addition, UGT84A2 also plays an important role for other sinapoyltransferases, but no
correlation with other sinapoyltransferase genes was found in this study. These data suggest that transcriptome analyses using ICA should specify optimum IC numbers for each metabolic pathway. Additionally, bi-functional genes may become apparent by fine adjustment of IC number. For example, eight ICs may be most suitable for analysis of anthocyanin pathway, as demonstrated by the dual role of flavonoid 3-O-glucosyltransferase. Recently, novel acyl-glucose-dependent anthocyanin glucosyltransferases, which belong to glycoside hydrolyase family 1, but not UGT, were isolated from carnation (Dianthus caryophyllus) and delphinium (Delphinium grandiflorum) (Matsuba et al., 2010). The application of ICA in various plant species thus might also be useful for finding 'unexpected' genes.

EXPERIMENTAL PROCEDURES

Plant materials

A. thaliana accession Columbia-0 (Lehle Seeds), or accession Nossen (Fedoroff and Smith, 1993) were used as wild-types in this study. The mutant brt1-1 (ugt84a2-2) was described previously (Sinlapadech et al., 2007). The Arabidopsis transposon-tagged lines Ds35-5492-1 and Ds54-1263-1 for UGT79B1 (ugt79b1-1 and ugt79b1-2, respectively), and Ds11-9386-1 for UGT84A2 (ugt84a2-1) were obtained from RIKEN Bioresource Center. The T-DNA-insertion mutant GABI_765F10 for UGT84A1 (ugt84a1-f) was obtained from the Arabidopsis Biological Resource Center. Homozygous knockout lines were screened by PCR using specific primers for UGT79B1, UGT84A1, UGT84A2, Ds transposon and T-DNA: UGT79B1f, UGT79B1r, UGT84A1f, UGT84A1r, UGT84A2f, UGT84A2r, Ds5-2a, Ds3-2a, o8409, o3144 (see Table S1). PCR products were sequenced to determine the exact insertion points.

For analyses of anthocyanin accumulation, plants were cultured on one-half-strength MS-agar medium containing 1% sucrose (Valvekens et al., 1988) in a growth chamber at 22°C with 16 h light and dark cycles for 14 days with a light intensity of 40 μmol of photons m⁻² s⁻¹, then transferred on one-half-strength MS-agar medium containing 1% sucrose for 3 days with a light intensity of 80 μmol of photons m⁻² s⁻¹. Plants were harvested, immediately frozen with liquid nitrogen, and stored at –80°C until use. At least three biological replicates were used for anthocyanin analysis.

Chemicals

Chemicals of the highest grade commercially available were used unless specifically noted. Flavonoid standards were purchased from Extrasynthese and Analyticon. UDP-α-arabinose and UDP-α-L-arabinose were purchased from CarboSource Services (supported in part by National Science Foundation-Plant Cell Wall Biosynthesis Research Network grant 0090281).

Independent component analyses

ICA is based on the assumption that a given gene expression level is determined by a linear combination of some independent components corresponding to biological signals. Assuming that an expression data matrix could be denoted as an \( m \times n \) matrix \( X \) with rows and columns representing \( m \) genes and \( n \) samples, respectively, and could be considered to be a linear combination of ICs (i.e. \( m \times k \) gene signature matrix \( S \), we can describe \( X = SA \) where \( A \) denotes a latent mixing matrix \( (k \times n) \) latent vectors of the gene expression data) (Figure S1). Here we assumed \( k \) ICs. Rows of \( S \) (i.e. ICs) are statistically independent from each other in ICA. ICA was carried out using fastICA algorithm, which is based on a fixed point algorithm for seeking a maximum of non-Gaussian properties of the components (Hyvärinen and Oja, 1997), with statistical R package ‘fastICA’. Pre-processed expression data, ‘GeneExp_v3’ file, from ATTED-II website (http://atted.jp/) consisting of 1388 Affymetrix ATH1 GeneChips were used for the analyses. These data were originally from TAIR AtGenExpress and were normalized by robust multichip average (Irizarry et al., 2003). For simplicity, we selected genes associated with AraCyc metabolic pathways using flat file, ftp://ftp.arabidopsis.org/Pathways/OLD/aracyc_dump.20091014. The array element mappings of Affymetrix probe set identifiers to AGI locus from TAIR dated 29 July 2009 (affy_AT1H1, array elements-2009-7-28.txt) was used. The resulting matrix size is 1388 samples \( \times 1877 \) genes. After applying the fastICA algorithm with \( k \) components to be extracted (in this study, \( k = 8 \)), the data were subjected to hierarchical cluster analysis (HCA) with the ICs using correlation (uncentered) and average linkage methods. The HCA was performed by CLUSTER 3.0 (de Hoon et al., 2004) and was visualized by JAVA TREEVIEW (http://jtreeview.sourceforge.net).

Phylogenetic analysis

UGT protein sequences were aligned by CLUSTALW implemented in MEGA4 (version 4.02; http://www.megasoftware.net/) (Tamura et al., 2007). A phylogenetic tree was constructed with the aligned UGT protein sequences by MEGA4 using the neighbor-joining method (Saitou and Nei, 1987) with the following parameters: Poisson correction, complete deletion, and bootstrap (1000 replicates, random seed = 64236). The alignment data are available in the Supplementary material online (Data S2).

Anthocyanin profiling by HPLC/PDA/ESI–MS

Anthocyanin extraction was carried out in triplicate as described previously (Tohge et al., 2005). For anthocyanin profiling, Agilent HPLC 1100 series and Agilent single quadrupole LC-MS 8120 series (Agilent Technologies Inc., http://www.home.agilent.com/) were used with an Atlantis® T3 column (3 μm, 150 mm, 5 μm, Waters) at a flow rate of 0.5 ml min⁻¹ at 30°C. Anthocyanins were separated with solvent A (10% acetonitrile, 0.1% trifluoroacetic acid in water) and solvent B (90% acetonitrile, 0.1% trifluoroacetic acid in water) using an elution gradient (0 min, 0% B; 40 min, 40% B; 40.1 min, 100% B; 45 min 100% B; 45.1 min, 0% B; 50 min, 0%). PDA was used for the detection of UV-visible absorption in the range of 200–600 nm. A mass analyzer was used for the detection of anthocyanin glycosides [M]⁺, and the peak of fragment ions in a positive ion scanning mode with the following setting: drying gas temperature, 350°C with drying gas flow of 12 L/min; capillary voltage, 4.0 kV; nebulizer pressure, 35 psig; fragmentor, 90 V; detection mode, scan (m/z 100–1400).

Evaluation of T-DNA and transposon insertion mutants

For complementation tests, the full-length UGT79B1 coding region was amplified by PCR using the primers UGT79B1-GWf and UGT79B1-GWf (Table S1). A full-length cDNA clone of UGT79B1 (pda08060) was obtained from the RIKEN Bioresource Center Arabidopsis full-length cDNA collection (Seki et al., 1998, 2002). The full-length UGT84A2 was amplified by PCR using the primers UGT84A2-GWf and UGT84A2-GWf (Table S1). Amplified fragments were cloned into the pENTR/D-TOPO vector (Invitrogen, http://www.invitrogen.com/) as an entry vector and sequenced to confirm the absence of PCR errors. pB2GW7 was used as a destination vector and the LR reactions for the binary vector pKYs390 for UGT79B1 and pKYs399 for UGT84A2 were catalyzed by the Gateway LR clonase.
enzyme mix (Invitrogen). Transformation into Agrobacterium and Arabidopsis, and the selection of transformants were carried out as described previously (Yonekura-Sakakibara et al., 2007).

For analyses of anthocyanin accumulation, plants were cultured on one-half-strength MS-agar medium containing 1% sucrose (Valvekens et al., 1988) in a growth chamber at 22°C with 16 h/8 h light and dark cycles for 14 days with a light intensity of 40 μmol of photons m⁻² s⁻¹, then transferred to one-half-strength MS-agar medium containing 12% sucrose for 3 days with a light intensity of 80 μmol of photons m⁻² s⁻¹. Plants were harvested, immediately frozen with liquid nitrogen, and stored at −80°C until use. At least three biological replicates were used for anthocyanin analysis.

General molecular procedures

The molecular procedures used were as described previously (Yonekura-Sakakibara et al., 2004) unless otherwise specified. RT-PCR was performed as described previously (Yonekura-Sakakibara et al., 2004) with primers UGT779B1-RTf and UGT779B1-RTr for UGT779B1, UGT84A2-RTf and UGT84A2-RTr for UGT84A2 and TUBf and TUBr for tubulin (GenBank Accession number AK117431) (Table S1).

Production of recombinant UGT79B1 protein and glycosyltransferase assays

Full-length UGT779B1 was amplified by PCR with the primers UGT79B1-1Ff and UGT79B1-1Fr to construct a protein expression vector (Table S1). The PCR product was cloned into pCDFinf using an In-Fusion Advantage PCR cloning kit (Clontech, http://www.clontech.com/). The nucleotide sequence of the resultant plasmid, pKYS398, was confirmed as above. Escherichia coli strain KRX (Promega, http://www.promega.com/) was used as a host for expression of recombinant UGT779B1 protein. Transformed cells were grown at 37°C until A₆₀₀ reached 0.5. After the addition of 20% (w/v) rhamnose to a final concentration of 0.1% (w/v), cells were cultured at 18°C for 24 h. The cells were collected, and the protein was purified as a His fusion according to the manufacturer’s instructions.

The standard enzyme assay reaction mixture (final volume, 50 μl) consisted of 50 mM HEPES-KOH, pH 7.5, 150 μM flavonoid substrates, and 500 μM UDP-sugar. For enzyme assays with anthocyanins as substrates, β-mercaptoethanol was added to a final concentration of 5 mM. The mixture was preincubated at 30°C for 2 min, and the reaction was started by the addition of enzyme. Reactions were stopped after 0, 4, 8, 12, or 30 min of incubation at 30°C by the addition of 50 μl ice-cold 0.5% (v/v) trifluoroacetic acid/MeOH for flavonols or 50 μl ice-cold 1.0% (v/v) HCl/MeOH for anthocyanidins and anthocyanins. Supernatants were recovered by centrifugation at 12,000 g for 3 min. Flavonoids in the resultant solution were analyzed using a Shimadzu HPLC system with a Unison UK-C18 column (2.0 × 150 mm, 3 μm, Imtakt corporation, http://www.imtaktusa.com/) at a flow rate of 0.2 ml/min at 35°C. Compounds were separated with a linear eluting gradient with solvent A (0.5% trifluoroacetic acid in water) and solvent B (0.5% trifluoroacetic acid in acetonitrile) set according to the following profile: 0 min, 20% B; 5 min, 20% B; 10 min, 22% B; 10.1 min, 100% B; 15 min, 100% B; 15.1 min, 20% B; 20 min, 20% B. PDA was used for the detection of UV-visible absorption in the range of 200–600 nm.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. A schematic ICA model of gene expression data as a strategy for gene discovery.

Figure S2. Phylogenetic tree and multiple alignment of flavonoid UGTs catalyzing glycosyl transfer to a sugar moiety of flavonoid glycosides.

Table S1. Primers used in this study.

Data S1. Complete hierarchical clustering data of a gene signature matrix with 1877 metabolism-related genes based on 9 ICS. Java Treeview (http://treeview.sourceforge.net) is required for visualization.

Data S2. The alignment used for construction of the phylogenetic tree shown in Figure 2.

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