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Reciprocal Regulation of *Arabidopsis* UGT78D2 and BANYULS Is Critical for Regulation of the Metabolic Flux of Anthocyanidins to Condensed Tannins in Developing Seed Coats

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Anthocyanins are major color pigments in plants. Their biosynthetic pathways are well established, and the majority of these biosynthetic enzymes have been identified in model plants such as *Arabidopsis*, maize, and petunia. One exception in *Arabidopsis* is UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT). This enzyme is known as Bronze-1 (Bz1) in maize, where it converts anthocyanidins to anthocyanins. Phylogenetic sequence analysis of the *Arabidopsis thaliana* UDP-glycosyltransferase (UGT) family previously indicated that UGT78D1, UGT78D2, and UGT78D3 cluster together with UF3GTs from other species. Here, we report that *UGT78D2* encodes a cytosolic UGT that is functionally consistent with maize Bz-1. Biochemically, UGT78D2 catalyzes the glucosylation of both flavonols and anthocyanidins at the 3-OH position. A T-DNA-inserted *ugt78d2* mutant accumulates very little anthocyanin and lacks 3-O-glucosylated quercetin. Expression analysis indicated that *UGT78D2*, in opposite to *BANYULS*, is highly expressed in anthocyanin-accumulating seedlings but repressed in condensed tannin-accumulating seed coats. This suggests that the reciprocal regulation of these two genes is important in directing the metabolic flux to either anthocyanins or condensed tannins. Consistent with this, the ectopic expression of UGT78D2 produces purple-colored seed coats due to the accumulation of anthocyanins. Taken together, our data indicate that *UGT78D2* encodes an enzyme equivalent to maize Bz1, and that the reciprocal regulation of UGT78D2 and BANYULS is critical for the regulation of metabolic flux of anthocyanidins in *Arabidopsis*.

Keywords: anthocyanin, *Arabidopsis* flavonoid, metabolic flux, UF3GT, UGT78D2

Anthocyanins are major color pigments in plants. Their biosynthetic pathways are well established, and the majority of these biosynthetic enzymes have been identified in model plants such as *Arabidopsis*, maize, and petunia (Holton and Cornish, 1995; Dixon and Steele, 1999; Winkel-Shirley, 2001; Springob et al., 2003). In the pathway, one coumaroyl-CoA and three malonyl-CoA molecules are condensed to chalcone by chalcone synthase (CHS). Chalcones are then converted to dihydrokaempferol by the actions of chalcone isomerase (CHI) and flavanone hydroxylase (F3H). Dihydrokaempferol can be further hydroxylated to dihydroquercetin or dihydromyricetin by flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′,5′-hydroxylase (F3′5′H), respectively. These three dihydroflavonols can be converted to corresponding colorless flavonols by flavonol synthase (FLS) or to colored anthocyanidins by the actions of dihydroflavonol 4-reductase (DFR) and leucoanthocyanidin dioxygenase (LDOX). Finally, anthocyanidins are 3-O-glycosylated by UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT; corresponding to maize *Bronze-1*) (Rhoades, 1952). They are further modified by several enzymes to form various anthocyanins. Anthocyanins are glutathionated and transported into the vacuoles for storage. UF3GT has not yet been identified in *Arabidopsis*.

The characterization of various anthocyanins and their biosynthetic enzymes from different plants has shown that the types of anthocyanins produced in a specific species are determined not only by the repertoire of biosynthetic genes expressed but also by the biochemical characteristics of their gene products (Tanaka et al., 1998; Mol et al., 1999; Forkmann and Martens, 2001; Zufall and Rausher, 2003, 2004). The inherent lack of blue pigments (delphinins) in carnations and roses and the subsequent engineering of blue-colored flowers by introducing the petunia *F3′5′H* gene is a good example of how the combination of biosynthetic genes determines the types of

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anthocyanins produced in a species (Holton, 1995, 1996; Holton and Tanaka, 1999). Differing substrate specificities of various dihydroflavonol 4-reductases (DFRs) reveal that biochemical characteristics of the expressed gene products can also determine the types of anthocyanins produced in some species (Gerats et al., 1982; Forkmann and Ruhnau, 1987; Beld et al., 1989; Johnson et al., 1999; Zufall and Rausher, 2004). For example, DFRs from petunia and cymbidium cannot use dihydrokaempferol as a substrate, therefore precluding these species from producing pelargonin-based brick-red pigments. However, transgenic petunias with such pigments have been engineered through the introduction of *DFR*s from other species (Meyer et al., 1987; Elomaa et al., 1995; Johnson et al., 2001). These studies illustrate the necessity of characterizing anthocyanin biosynthetic enzymes, not only to improve our understanding of anthocyanin biosynthetic pathways in different plant species, but also to facilitate metabolic engineering that uses enzymes with desirable biochemical properties.

The isolation and characterization of anthocyanin mutants has allowed researchers to elucidate the function of various biosynthetic genes. In *Arabidopsis*, mutants have been isolated based primarily on their seed coat color (Shirley et al., 1992, 1995; Albert et al., 1997; Devic et al., 1999; Schoenbohm et al., 2000; Debeaujon et al., 2001; Kitamura et al., 2004). The brown hue of its seed coat is conferred by condensed tannins, which are synthesized from anthocyanidins mainly by an anthocyanidin reductase called BANYULS (BAN) (Xie et al., 2003, 2004). In other species, such as *Desmodium uncinatum*, these condensed tannins are also synthesized from leucoanthocyanidins by leucoanthocyanidin reductase (LAR) (Tanner et al., 2003). Analysis of *transparent testa* (yellow seed coat) mutants (*tt*) and *tannin deficient seed* mutants (*tds*) has enabled the identification and characterization of most core biosynthetic mutants in *Arabidopsis*. However, the *tt* phenotype cannot be used to isolate the *uf3gt* mutant because synthesis of condensed tannins occurs regardless of UF3GT activity. Thus, no previous mutant screening has identified the gene for *Arabidopsis* UF3GT.

Although several UF3GT-encoding cDNA clones have been isolated through *in vitro* biochemical assays (Tanaka et al., 1996; Gong et al., 1997; Ford et al., 1998; Yamazaki et al., 2002), the only *uf3gt* mutant gained so far has been the maize *bronze-1* (*bz1*) mutant. The isolation of other *uf3gt* mutants, especially from dicot species, will help elucidate the function of UF3GT in plants. Because the entire *Arabidopsis* genome sequence is now known, and a large collection of mutants is available, this model dicot plant seems a good choice for the isolation of a new *uf3gt* mutant. Previously, phylogenic sequence analysis of the *Arabidopsis* UDP-glycosyltransferases (UGTs) indicated that three UGTs (UGT78D1, UGT78D2, and UGT78D3) belonging to UGT Group F are clustered with UF3GTs from other species (Li et al., 2001; Ross et al., 2001). Here we analyzed the biochemical activity of both recombinant UGT78D2 and a T-DNA inserted mutant to determine if *UGT78D2* encodes *Arabidopsis* UF3GT. We also studied its functioning in developing seed coats using an ectopic expression lines with regard to the accumulation of anthocyanins.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana plants were reared in a growth room at 22 to 24°C and under a 16-h photoperiod to study their general development and to harvest their seed. A T-DNA inserted *ugt78d2* mutant was obtained from the *Arabidopsis* Stock Center (Salk_049338) (Alonso et al., 2003). The insertion of T-DNA in *UGT78D2* (At5g17050) was confirmed by amplifying a *UGT78D2* or *UGT78D2-T-DNA* hybrid fragment using a primer set (5′-ACACACCCGTTGCTTCGT GTT-3′,5′-CGGAGGTTGGTACGGAAGTGA-3′,5′-TGG TTCACGTAGTGGGCCATCG-3′). All plants in these experiments were of the Col-0 ecotype.

For our co-segregation analysis, F2 seeds from a cross between the *ugt78d2* mutant and Col-0 were spotted on agar plates containing 3% sucrose and no MS salts. After plating, the seeds were illuminated with white light (110 μmol m^{-2} s^{-1}) for 6 h to induce germination. They were then kept for 3 d in far-red light (3.2 μmol m^{-2} s^{-1}). In all, 92 out of 363 seeds lacked dark coloring. To determine if the non-pigmented seedlings were all homozygous *ugt78d2* mutants, the pooled genomic DNAs of pigmented and non-pigmented seedlings were used to amplify with either a *UGT78D2* gene-specific primer set (5′-GATCGAATTCATGACCAAACCCTCCGACCCA-3′,5′-GATCCTCGAGAATAATGTTTACAACTGCATC-3′) or a UGT78D2-T-DNA hybrid fragment-specific primer set (5′-ACACACCCGTTGCTTCGTGTT-3′,5-CGGAGGTTG GTACGGAAGTGA-3′,5′-TGGTTCACGTAGTGGGCC ATC G-3′).

For the transgenic *Arabidopsis* over-expressing UGT78D2 under the control of the CAMV 35S promoter, UGT78D2 was cloned into pCAMBIA1303 and transformed into *Arabidopsis* (Col-0). Five independent transgenic lines were established; two were used for further analysis.

Seedlings, trichomes, and seeds were observed under microscopes (SZX7 and BX51; Olympus, Japan) and pictures were taken by a digital camera connected to the microscope (DP70; Olympus). For the staining of condensed tannins, seeds were immersed in DMACA (dimethylaminocinnamaldehyde) solution (2% [w/v] DMACA in 3 M HCl/ 50% methanol) for 3 d at 4°C.

Sequence and Expression Analysis of UGT78D2

Amino acid sequences of eight UF3GT from other species and one rhamnosyltransferase from *Petunia* were aligned with UGT78D1, UGT78D2, and UGT78D3 by the CLUSTALX program. A phylogenetic tree was generated by the neighbor joining method, using bootstrap number 1000. The gene accession numbers included: *Petunia* 3RT (CAA50377), *Gentiana* UF3GT (UFOG_GENTR), *Perilla* UF3GT (BAA19659), *Forsythia* UF3GT (AAD21086), *Ipomea* UF3GT (AAB86473), *Petunia* UF3GT (BAA89008), *Vitis* UF3GT (AAB81682), UGT78D1 (AAL07161, At1g30530), UGT78D2 (AAM91139, At5g17050), UGT78D3 (AAM65712, At5g17030), Bronze-1 (CAA31856), and Barley UFGT (CAA33729).

Expression of UGT78D2 and the anthocyanin biosynthetic genes was determined by RT-PCR. For tissue-specific analyses, four different organs (root, stem, leaf, and flower) were sampled from 25-d-old, soil-grown *Arabidopsis*, and placed in liquid nitrogen. To monitor the expression of UGT78D2 in monochromatic light, seeds were spotted on filter papers laid on MS-agar plates containing 1% sucrose, then imbibed for 3 d at 4°C in dark, and treated with white light (110 μmol m^{-2} s^{-1}) for 6 h to induce germination. Afterward, the plates were kept for 3 d either in the dark or in red (660 nm) or far-red (730 nm) LED chambers (Vision, Korea) before samples were placed in liquid nitrogen. For the expression of anthocyanin biosynthetic genes, both wild-type and mutant seeds were plated and germination was induced as described above. Two-day-old far-red light-grown seedling samples were placed in liquid nitrogen. All seedlings and organs were ground with a pestle and mortar. RNAs were purified with an RNeasy Miniprep Kit (Qiagen, Germany).

RT-PCR was done with 2 μg of total RNA. DNase-treated RNA samples were reverse-transcribed with M-MLV reverse transcriptase and Oligo$(dT)_{15}$, according to the manufacturer's protocol (Promega, USA). They were equalized by either ubiquitin (5′-GATCTTTGCCGGAAAACAATTGCAGGATGGT-3′,5′-CGACTTGTCATTAGAAAGAAAGACATAACAGG-3′) or S18 (5′-CCAGCGATCGTTTATTGCTT-3′,5′-AGTCTTTCCTCTGCGACCAG-3′). Primers for this expression analysis included: UGT78D2 (At5g17050, 5′-GTTCTTCTGGTTCGCGGCTG-3′,5′-GAACCAGAAGATCTCTTCTC-3′), CHS (At5g13930, 5′-GTCGTCTTCTGCACTACCTC-3′,5′-CACCATCCTTAGCTGACTTC-3′), CHI (At3g55120, 5′-ATGTCTTCATCCAACGCC-3′,5′-GAAGATCTCCAAGAACTT-3′), F3H (At3g51240, 5′-ATGGCTCCAGGAACTTTG-3′,5′-TGGTCCATATCGACGCAT-3′), F3′H (At5g07990, 5′-ACGGTCAAGATCAAAAGC-3′, 5′-TGCTAACGGGTCGGACC-3′), FLS (At5g08640, 5′-ATGGAGGTCGAAAGAGTC-3′,5′-TAACCCTAATCCATCCGA-3′), DFR (At5g42800, 5′-ATGGTTAGTCAGAAAGAGAC-3′, 5′-CATTCCATTCACTGTCGG-3′), LDOX (At4g22880, 5′-ATGGTTGCGGTTGAAAGA-3′, 5′-TCTAGACGGTCAGGCTCT-3′), MYB75 (At1g56650, 5′-GAGTCTAGAAAAAATGGAGGGTTCGTCCAAAGG-3′, 5′-GAGGGATCCAACTAATCAAATTTCACAGTCTCTCC-3′), MYB90 (At1g66390, 5′-GCAAATGGCATCAAGTTCCT-3′, 5′-TACCAACCAAGACGGCTTTT-3′), and HY5 (At5g11260, 5′-ACGTGAATTCATGCAGGAACAAGCGACTAGC-3′,5′-TGCACTCGAGAAGGCTTGCATCAGCATTAG A-3′).

Subcellular Localization of UGT78D2

To assay for subcellular localization, UGT78D2 was amplified using the primer set of 5′-GATCTCTAGAATGACCAAACCCTCCGACCCA-3′,5′-GATCTGATCACCAATAATGTTTACAACTGCATC-3′, then cloned into pBI222-GFP to make the *UGT78D2: GFP* construct. As a control, CHS was amplified with a primer set (5′-GATCCTCGAGATGGTGATGGCTGGTGCTTCT-3′,5′-GATCGGATCCAAGAGAGGAACGCTGTGCAAGAC-3′) and cloned into the same vector to produce the *CHS:GFP* construct. The *NbGyrA:GFP* construct, described by Cho et al. (2004), was kindly provided by Dr. Hye Sun Cho; the *H^+-ATPase:GFP* construct, described by Lee et al. (2001) was kindly provided by Dr. Inhwan Hwang.

Protoplasts were prepared according to the method of Jin et al. (2001). Various GFP-fusion constructs were introduced into protoplasts as described by Kang et al. (1998). The transformed protoplasts were incubated overnight in dim light. Localization of

UGT:GFP and other GFP fusion proteins was determined with a confocal microscope (FV10-ASW; Olympus). For the GFP fluorescence, a 488 nm-laser was used for the excitation, and detection covered a spectral range from 500 to 530 nm. For the chlorophyll autofluorescence, a 633-nm laser was used for the excitation; a BA650IF filter for detection.

In Vitro Biochemical Assay

For our *in vitro* biochemical assay, UGT78D2 was amplified with a primer set (5'-GATCGAATTCATGAC CAAACCCTCCGACCCA-3',5'-GATCCTCGAGAATAA TGTTTACAACTGCATC-3') and cloned into pGEX4T-1. The subsequent recombinant GST-UGT78D2 proteins were purified using glutathione-sepharose 4B beads (Pharmacia Biotech, USA) from *Escherichia coli* strain BL21 (DE3). The reaction mixture (200 μL), which contained the recombinant GST-UGT78D2 protein (2 μg) and a reaction buffer [50 mM Tris-HCl (pH 7.0), 14 mM 2-mercaptoethanol, 5 mM UDP-glucose, and 1 mM of substrates], was incubated for 30 min at 30°C. This reaction was stopped by adding 20 μL of trichloroacetic acid (240 mg mL^{-1}) for the flavonols or 50 μL of methanol containing 1% HCl for the anthocyanins. Afterward, the reaction mixtures were frozen in liquid nitrogen and stored at -20°C prior to HPLC analysis.

Reverse-phase HPLC analysis was performed on a C18 column (Luna 5 μ, 4.6×150 mm; Torrance, USA), with a linear gradient of 10 to 50% acetonitrile in water containing 0.5% trifluoroacetic acid at a 0.5 ml min^{-1} flow rate (Model LC-2000 with a PU2080 pump and MD-2010 Diode Array Detector; JASCO, Japan). Flavonoids were detected at either 340 nm for flavonols or 520 nm for anthocyanidins and anthocyanins. Flavonoid substrates and standards were purchased from the Indofine Chemical (Belle Mead, USA).

Flavonoids in Plants

To quantify anthocyanins, seeds were spotted on MS-agar plates containing 1% sucrose. Following imbibition, they were illuminated with white light to induce germination, then grown in far-red light (3.2 μmol m^{-2} s^{-1}). On specified days after their transfer into the far-red chamber, 50 seedlings each were collected and their anthocyanins extracted overnight at 4°C in 300 μL of an extraction buffer (MeOH, 1% HCl). Anthocyanin content was determined by calculating A_{530}-0.33A_{657}, using 5 replicates. Flavonoid profiles for the wild-type and *ugt78d2* mutant were determined by HPLC, as described above.

To determine the structure of flavonols, ^{1}H-, ^{13}C-, and 2D-NMR spectra were taken on an AS400 spectrometer (Varian-Inova, USA), operating at 400 or 100 MHz, followed by GCMS (Jeol-JMS700 Instrument, USA).

RESULTS

UGT78D2 Encodes a Cytosolic UDP-Glycosyltransferase that Phylogenetically Clusters with the UDP-Glucose:Flavonoid 3-O-Glucosyltransferases

The *Arabidopsis* genome contains 107 UGTs, classified into 14 groups (Li et al., 2001; Ross et al., 2001). Our phylogenetic analysis revealed that three closely related *Arabidopsis* UGTs (UGT78D1, UGT78D2, and UGT78D3) in Group F are clustered with UF3GTs from other species (Fig. 1A). This suggests that at least one of these UGT family members is a UDP-glucose:flavonoid 3-O-glucosyltransferase (UF3GT). A previous functional analysis indicated that UGT78D1 acts as a flavonol 3-O-rhamnosyltransferase, not as a UF3GT (Jones et al., 2003), whereas UGT78D2 catalyzes the glucosylation of two flavonols (kaempferol and quercetin) at the 3-OH position (Lim et al., 2004). Based on those reports, we selected a variety of biochemical and functional analyses to test whether *UGT78D2* encodes a UF3GT.

If the *Arabidopsis UGT78D2* encodes a UF3GT, it is likely that its expression pattern would resemble that of other anthocyanin biosynthetic genes. We first compared the tissue-specific expression of *UGT78D2* to those of genes encoding chalcone synthase (CHS) and flavonoid 3'-hydroxylase (F3'H). Here, *CHS* and *F3'H* showed high expression levels in flowers and lower but obvious expression in leaves and stems (Fig. 1B). Similarly, *UGT78D2* was expressed ubiquitously, but at higher levels in leaves and flowers. Because the expression of anthocyanin biosynthetic genes, e.g., *CHS* and *F3'H*, is highly induced by blue (450 nm), red (670 nm), and far-red (730 nm) lights, we next tested whether *UGT8D2* expression was induced by monochromatic light. *CHS*, *F3'H* and *UGT78D2* were all highly induced by red and far-red light (Fig. 1C), further indicating that *UGT78D2* and the known anthocyanin biosynthetic genes have overlapping expression patterns.

Although the *Arabidopsis* gene annotation database predicts that UGT78D2 is localized in the chloroplasts

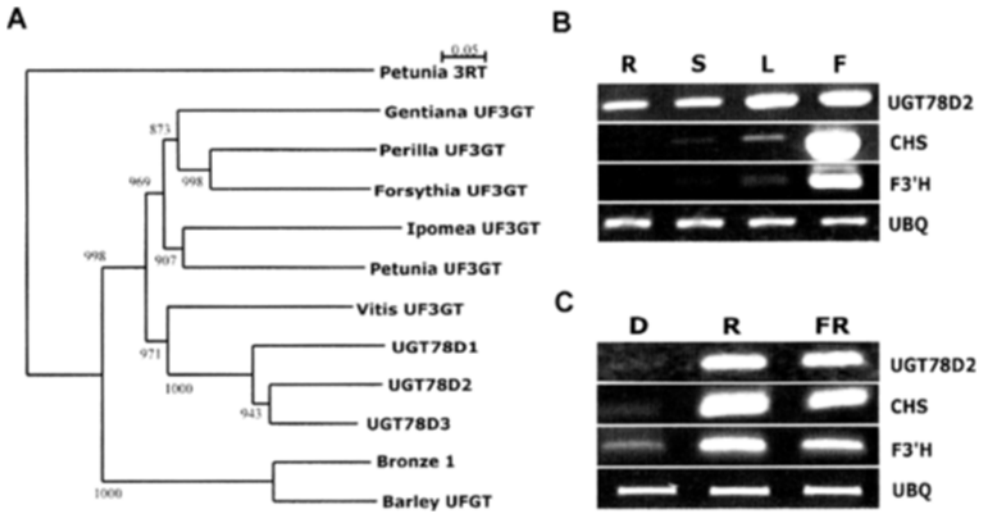

Figure 1. *UGT78D2* clusters with UF3GTs in phylogenetic analysis, and is expressed similarly to *CHS* (chalcone synthase) and *F3'H* (flavonoid 3'-hydroxylase). **(A)** Phylogenetic tree of various known UF3GTs and three *Arabidopsis* UGTs (UGT78D1, UGT78D2, and UGT78D3) belonging to Group F. Numbers are bootstrap values of 1000 replicates. Amino acid sequences used for tree construction are indicated in Methods section. **(B)** Expression patterns of *UGT78D2*, *CHS*, and *F3'H* in various plant organs. UBQ indicates ubiquitin gene, used as control in RT-PCR. R, root; L, leaf; S, stem; F, flower. **(C)** Expression of *UGT78D2*, *CHS*, and *F3'H* under monochromatic light conditions. D, dark; R, red light; FR, far-red light.

(www.arabidopsis.org), other computer-assisted predictions have suggested that it is found in the cytosol (Ross et al., 2001). Because a UF3GT should be located in the latter, where anthocyanins are synthesized, we experimentally determined the subcellular localization of UGT78D2. A *UGT78D2:GFP* expression vector and various control constructs were introduced into *Arabidopsis* protoplasts. GFP fluorescence was observed by confocal microscopy. Both UGT78D2:GFP and CHS:GFP were localized in the cytosol, whereas NbGyrA:GFP (chloroplast control) overlapped with chlorophyll autofluorescence (Fig. 2); H^+-ATPase:GFP (membrane control) showed the typical circular shape. These results indicate that, contrary to the annotation data, UGT78D2 is localized in the cytosol rather than the chloroplasts.

UGT78D2 Glucosylates Both Flavonols and Anthocyanidins *in Vitro*

The biochemical function of UF3GT is glucosylation of both flavonols and anthocyanidins at the 3-OH position. Lim et al. (2004) previously reported that UGT78D2 could glucosylate flavonols at the 3-OH position *in vitro*. Therefore, we investigated whether UGT78D2 could also glucosylate anthocyanidins *in vitro*. Recombinant UGT78D2 was expressed and purified as a GST-fusion protein. For the biochemical reaction, we used kaempferol and quercetin as flavonol substrates and pelargonidin and cyanidin as anthocyanidin substrates. After the reaction, the products were analyzed by HPLC. The addition of GST alone did not convert the two flavonol substrates to their corresponding glucosylated forms (Fig. 3B). In contrast, GST-UGT78D2 glucosylated both kaempferol and quercetin (Fig. 3C and D). Similarly, GST-UGT78D2 glucosylated both pelargonidin and cyanidin (Fig. 3G and H). These results indicate that UGT78D2 can glucosylate both flavonols and anthocyanidins, and thus has biochemical activity consistent with that of UF3GT.

UGT78D2 Is Necessary for the Synthesis of Anthocyanins

We next used a T-DNA inserted mutant to investigate the *in planta* role of UGT78D2 in anthocyanin biosynthesis. T-DNA inserted at the second exon of *UGT8D2* (Fig. 4A) disrupted gene expression (Fig. 4B). To determine the role of UGT78D2, we grew seedlings under continuous far-red light for 4 d on agar plates containing 3% sucrose without MS salts. Under these conditions, anthocyanins are easily visualized due to the lack of chlorophyll biosynthesis. Wild-type (WT) seedlings showed dark purple coloring in their upper hypocotyls and cotyledons, indicating the accumulation of anthocyanins in those regions (Fig. 4C). In contrast, the *ugt78d2* mutant seedlings

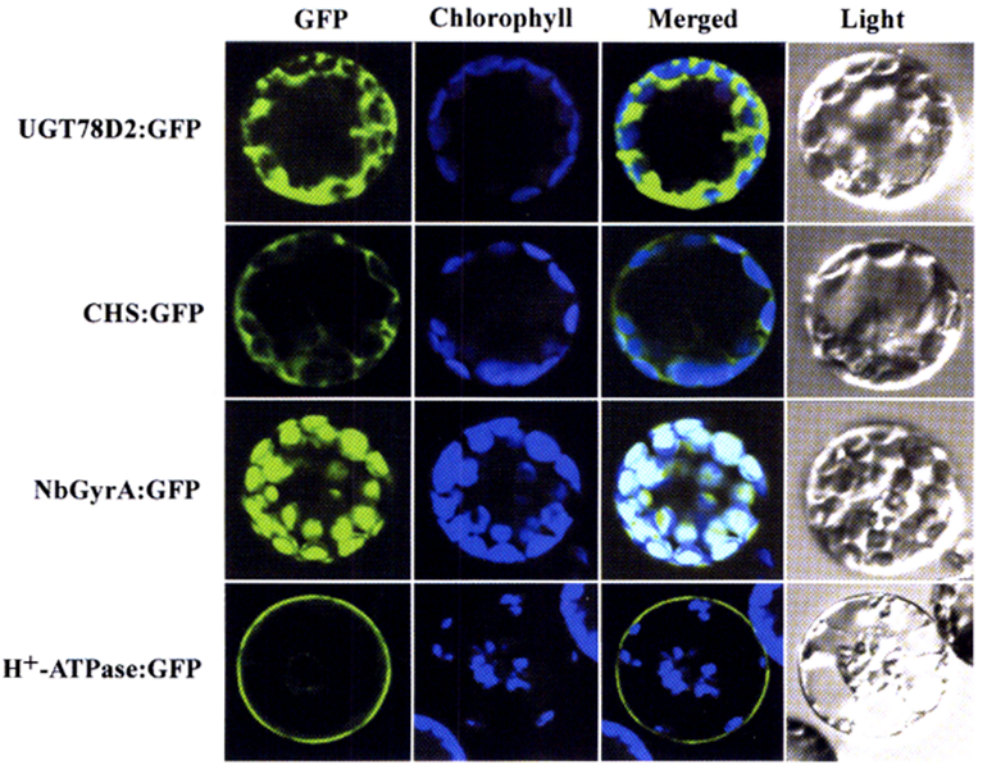

Figure 2. UGT78D2 is localized in cytosol. GFP fluorescence was detected by confocal microscopy in *Arabidopsis* protoplasts transiently expressing UGT78D2:GFP or CHS:GFP. Chloroplast-localized NbGyrA:GFP and membrane-localized H^+-ATPase:GFP were used as localization controls. GFP, GFP fluorescence; Chlorophyll, chlorophyll autofluorescence; Merged, merged pictures of GFP fluorescence and chlorophyll autofluorescence; Light, optical images taken by Differential Interference Contrast microscopy.

showed either yellow or very mild purple colors in these regions. Because anthocyanin biosynthesis is correlated with seedling stage, this difference could be due to a change in the rate of development (Kubasek et al., 1992, 1998). To exclude this possibility, we quantified the amount of anthocyanins present at various days after germination in mutant and WT seedlings, and found that the mutants showed highly decreased anthocyanin accumulation, regardless of seedling stage (Fig. 4C). Therefore, these results suggest that UGT78D2 is required for anthocyanin synthesis in *Arabidopsis* seedlings.

Using this same T-DNA insertion line, we next performed co-segregation analysis to confirm that the mutant phenotype was due to the disruption of *UGT78D2*. After crossing mutant and WT plants, we self-crossed the resulting F1 plants to generate F2 seeds. The F2 seedlings were grown under far-red light, and examined for whether the phenotype co-segregated with the T-DNA insertion. Out of 363 seedlings, 92 were yellow and 272 were purple (Fig. 4D). To examine whether all those yellow seedlings were homozygous *ugt78d2* mutants, we pooled them by color, purified genomic DNA from the pooled samples, and performed PCR to test for the T-DNA insert. Both the WT and the *UGT78D2-T-DNA* hybrid fragments were amplified from the purple seedlings, while the yellow seedlings yielded only the *UGT78D2-T-DNA* hybrid fragment but no WT *UGT78D2* fragment. This indicated that all yellow seedlings were of the homozygous *ugt78d2* mutant, thereby demonstrating that the yellow-seedling phenotype co-segregated with the homozygous T-DNA insertions.

We also observed defective anthocyanin synthesis in the mutants at other developmental stages. For example, the *ugt78d2* plants grew and flowered comparable to the WT. However, at the flowering stage, the basal portion of their stems was darkly pigmented in the WT plants, but only mildly pigmented in the mutants (Fig. 4E), indicating that UGT78D2 is also necessary for the synthesis of anthocyanins at this stage.

Our observation of lighter pigmentation in the mutants was somewhat surprising because anthocya-

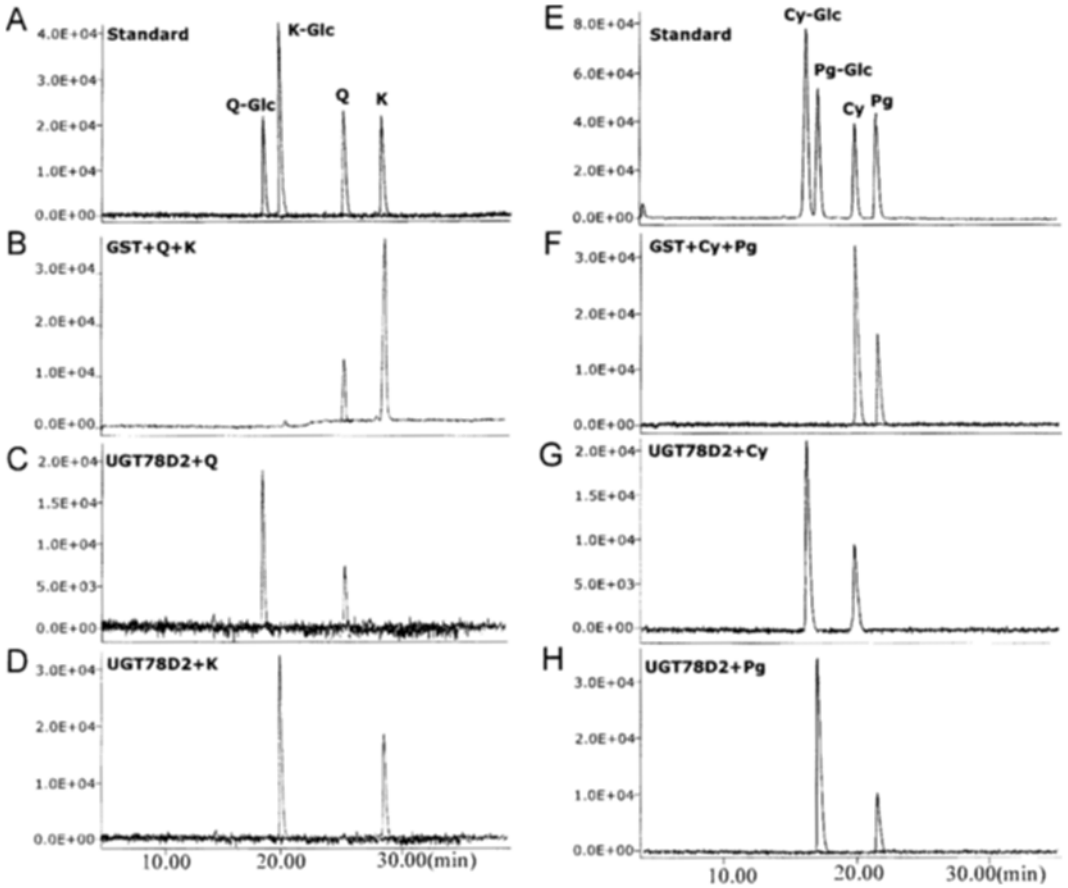

Figure 3. UGT78D2 glucosylates both flavonols and anthocyanidins at 3-OH position. **(A)** HPLC profile of flavonols and flavonol 3-O-glucosides. K, kaempferol; Q, quercetin; K-Glc, kaempferol 3-O-glucoside; Q-Glc, quercetin 3-O-glucoside. **(B)** GST alone did not convert kaempferol or quercetin to their glucoside forms. **(C)** Conversion of kaempferol to kaempferol 3-O-glucoside by recombinant UGT78D2. **(D)** Conversion of quercetin to quercetin 3-O-glucoside by UGT78D2. **(E)** HPLC profile of anthocyanidins and anthocyanidin 3-O-glucosides. Pg, pelargonidin; Cy, cyanidin; Pg-Glc, pelargonidin 3-O-glucoside; Cy-Glc, cyanidin 3-O-glucoside. **(F)** GST alone did not convert pelargonidin or cyanidin to their glucoside forms. **(G)** Conversion of pelargonidin to pelargonidin 3-O-glucoside by recombinant UGT78D2. **(H)** Conversion of cyanidin to cyanidin 3-O-glucoside by recombinant UGT78D2.

nidins (the precursors to anthocyanins) are also colored. This seems to suggest that anthocyanidin accumulation might be impaired in the mutants, perhaps because of the instability of aglycosylated anthocyanidins (Brouillard, 1982) or decreased expression of upstream anthocyanin biosynthetic genes. We first performed RT-PCR to determine whether this lower accumulation of colored flavonoids (anthocyanidins) was due to lower expression of upstream anthocyanin biosynthetic genes in the *ugt78d2* mutant. Here, all tested upstream genes were expressed at comparable levels in the WT and mutant plants (Fig. 5), as were three regulatory genes, *MYB75*, *MYB90*, and *HY5* (Oyama et al., 1997; Ang et al., 1998; Borevitz et al., 2000). These results indicate that the lower accumulation of colored flavonoid compounds in the *ugt78d2* mutant was not due to decreased expression of those upstream anthocyanin biosynthetic genes.

Arabidopsis seed coat color is conferred by condensed tannins, which are synthesized from anthocyanidins mainly by the anthocyanidin reductase encoded by *BANYULS* (*BAN*) (Albert et al., 1997; Devic et al., 1999; Xie et al., 2003). In other species, leucoanthocyanidin reductase (LAR) also synthesizes condensed tannins from leucoanthocyanidins (Tanner et al., 2003). Because the anthocyanin biosynthetic genes upstream of anthocyanidin synthesis were expressed normally in our *ugt78d2* mutant, it was likely that condensed tannins were synthesized in the mutant plants. As expected, both WT and *ugt78d2* mutant seeds had brown coats, while chalcone synthase mutant (*tt4*) seeds had yellow coats

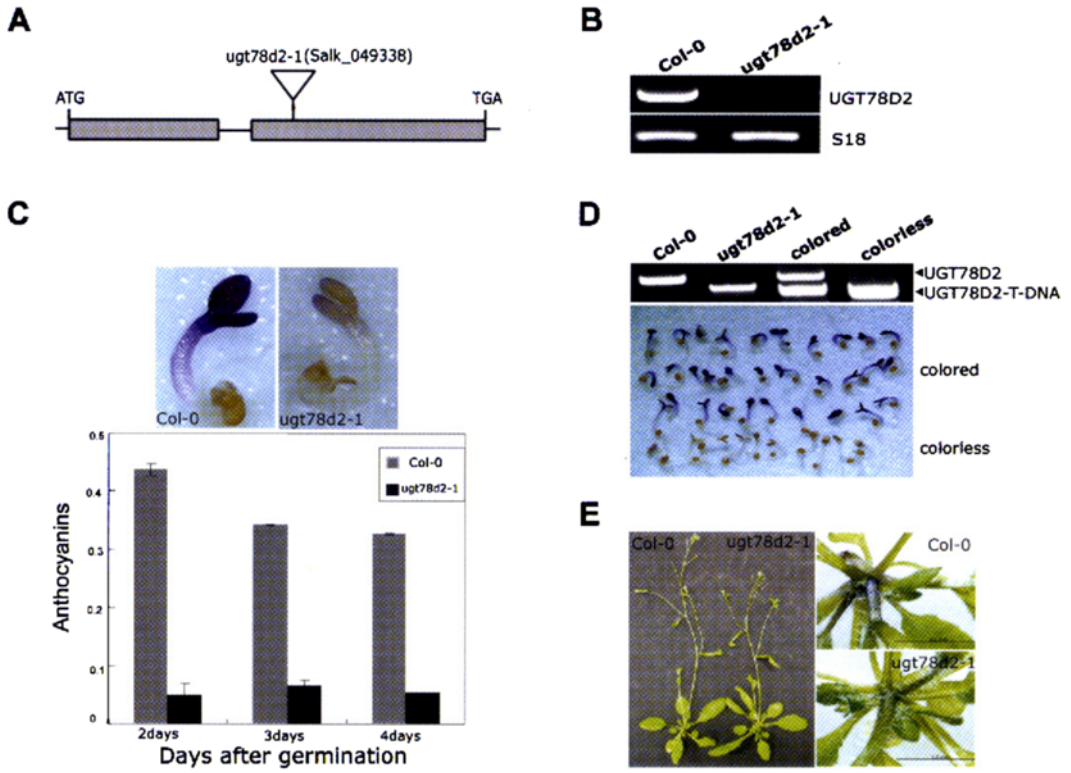

Figure 4. The *ugt78d2* mutant does not synthesize anthocyanins. **(A)** T-DNA insertion into *UGT78D2* gene. Boxes and lines represent exons and introns, respectively. **(B)** RT-PCR data showing that full-length *UGT78D2* gene is not expressed in *ugt78d2-1* mutants. **(C)** Decreased accumulation of anthocyanins in *ugt78d2-1* mutant. Error bars represent ± SD. **(D)** Co-segregation of decreased anthocyanin synthesis and homozygous T-DNA insertion in *UGT78D2* gene. Representative F2 seedlings accumulating different amounts of anthocyanins are shown. Homozygosity of T-DNA insertion was determined by amplification of UGT78D2 and/or UGT78D2-T-DNA hybrid fragments from genomic DNA of pooled colored and colorless seedlings. **(E)** Decreased accumulation of anthocyanins in adult plants. Left panel shows that *ugt78d2-1* mutant grows and flowers similarly to wild type. Right panel shows that mutant accumulates lower levels of anthocyanins at base of each inflorescence.

(Fig. 6). Staining of the condensed tannins by dimethylaminocinnamaldehyde (DMACA) also showed that both the WT and *ugt78d2* mutant seeds became darkened while the *tt4* mutant seeds did not. Though it was difficult to quantify the level of condensed tannins in this assay, these results clearly indicate that condensed tannins are synthesized in *ugt78d2* mutant plants, suggesting that anthocyanidins, substrates of BAN, are synthesized in the mutant. Moreover, the lighter pigmentation of the *ugt78d2* mutant is likely due to the instability of aglycosylated anthocyanidins.

UGT78D2 Is Required for Synthesis of Quercetin-3-O-Glucoside *in planta*

Our biochemical analyses indicated that UGT78D2 can glucosylate both flavonols and anthocyanidins. Because *Arabidopsis* contains various flavonoids that are modified by compounds such as glucose, rhamnose, and coumarate (Veit and Pauli, 1999; Bloor and Abrahams, 2002; Jones et al., 2003), we determined the role of UGT78D2 in flavonoid biosynthesis in planta by HPLC analysis. When WT samples were examined at 520 nm (where anthocyanin signals are detected), we observed two major peaks (19.53 min and 20.24 min) and some small peaks (Fig. 7A). However, in the mutant, the two major peaks were greatly diminished, consistent with the lower accumulation of anthocyanins detected by spectroscopy (Fig. 4C). When the WT samples were measured at 340 nm (where flavonols signals are detected), we observed three major peaks (14.89 min, 16.24 min, and 17.18 min) and a few small peaks (Fig. 7B). In the mutant, we were unable to detect one of those major peaks (at 16.24 min). This missing flavonol in the mutant was isolated from the WT seedlings and identified as quercetin 3-O-β-D-glucopyranoside-7-

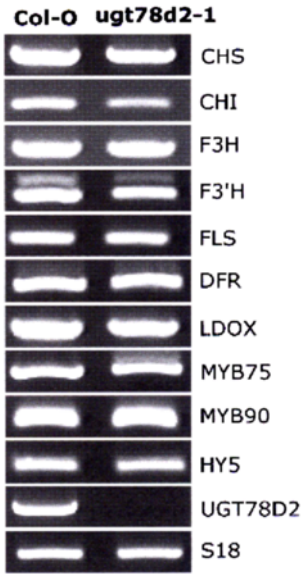

Figure 5. Anthocyanin biosynthetic genes upstream of anthocyanidin synthesis are expressed comparably in wild-type and *ugt78d2* mutant plants. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3' hydroxylase; DFR, dihydroflavonol reductase; LDOX, leucoanthocyanidin dioxygenase.

O-α-L-rhamnopyranoside (Fig. 7C). Assignments were made on the basis of ^{1}H-, ^{13}C-, and 2D-NMR spectra (Table 1) and GCMS ($[M]^{+}$ m/z 610); its structure was confirmed by published ^{1}H-NMR data. These observations indicated that UGT78D2 is necessary not only for the synthesis of anthocyanins, but also for the *in vivo* synthesis of a 3-O-glucosylated quercetin.

Expression of UGT78D2 Redirects the Metabolic Flux of Anthocyanidins to Anthocyanins in Developing *Arabidopsis* Seed Coats

The *Arabidopsis* seed coat is a very unusual organ in terms of its anthocyanin biosynthesis. Although opaque during its early stages of development, the seed coat then turns green and finally browns as the seed matures. This brown coloring is caused by the accumulation of condensed tannins without the concomitant synthesis of anthocyanins. Because anthocyanidins are the precursors of both anthocyanins and condensed tannins, the lack of anthocyanin biosynthesis in the developing seed coats suggests that anthocyanidins are mainly used for the synthesis of condensed tannins. To investigate whether this lack of anthocyanin biosynthesis is related to the expression of UGT78D2, we separated developing seeds from their siliques and determined the expression of various anthocyanin biosynthetic genes. For comparison, we also evaluated the expression of various anthocyanin biosynthetic genes in far–red–irradiated, anthocyanin-accumulating seedlings. Here, all anthocyanin biosynthetic genes, except *BAN* and *UGT78D2*, were expressed at comparably high levels in both samples (Fig. 8A). This seemed to indicate that anthocyanidins are synthesized equally well in both types of seedlings, and that the metabolic flux toward production of condensed tannins or anthocyanins is regulated by the expression of *BAN* and *UGT78D2*. In the far–red–irradiated seedlings, which accumulated mainly anthocyanins, *UGT78D2* was highly expressed but *BAN* was repressed. In contrast, *UGT78D2* was repressed and *BAN* was highly expressed in the developing seed coats. Unlike *UGT78D2*, *UGT78D1* (encoding flavonol 3-O-rhamnosyltransferase) was expressed at comparably high levels in both samples. These results indicate that the absence or lower accumulation of anthocyanins in the developing seed coat is at least partly due to repression of *UGT78D2* coupled with up-regulation of *BAN*.

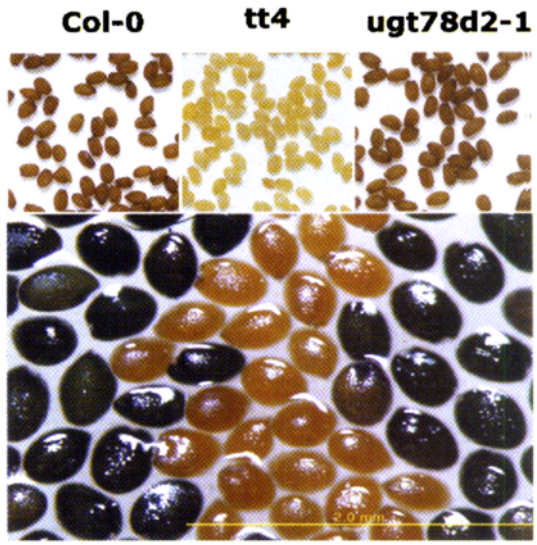

Figure 6. Condensed tannins are synthesized in *ugt78d2* mutant. Unlike *tt4*, the *ugt78d2-1* mutant has brown seed coats (upper panels) that can be stained by DMACA (lower panel).

To determine whether increased expression of *UGT78D2* in the developing seed coat could redirect the metabolic flux toward production of anthocyanins, we generated transgenic *Arabidopsis* strains expressing *UGT78D2* under the control of the CAMV 35S promoter. We found that ectopic expression did not significantly change overall levels of pigmentation in either leaves or flowers, although the petioles on

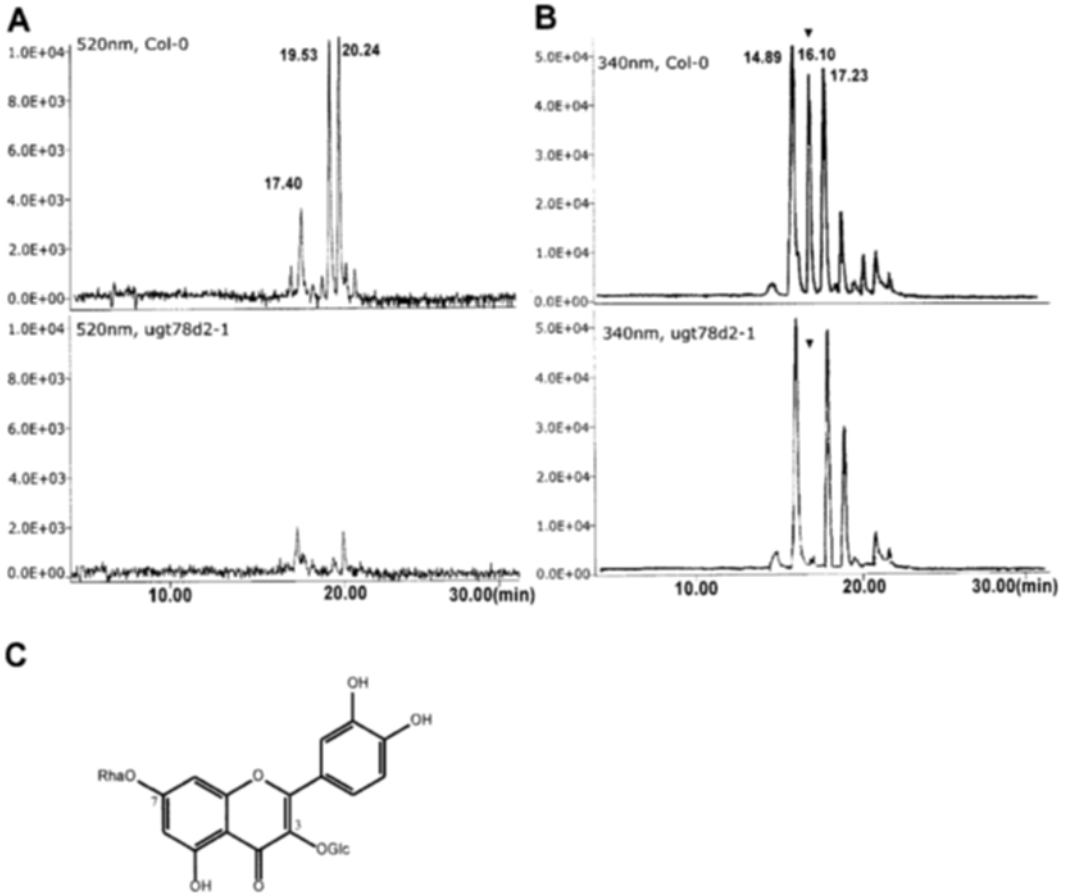

Figure 7. The *ugt78d2* mutant has altered flavonoid profiles. **(A)** HPLC profiles of anthocyanins detected at 520 nm. Upper panel shows anthocyanin profile of wild-type plant; lower panel, the anthocyanin profile of *ugt78d2-1* mutant. **(B)** HPLC profiles detected at 340 nm. Upper panel shows profile of wild-type plant; lower panel, the profile of *ugt78d2-1* mutant. **(C)** Chemical structure of quercetin 3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside, a missing flavonol at 16.24 min in mutant.

transgenic plants were slightly darker. However, we observed a dramatic pigmentation difference in the developing seed coats from transgenic plants versus controls, with the former showed the purple pigmentation characteristic of anthocyanin accumulation (Fig. 8). This purple coloring was lighter in young siliques and became darker in older siliques. However, dry seeds of transgenic *Arabidopsis* were only marginally darker than those of the WT. Anthocyanins accumulated in the innermost cell layer, where condensed tannins normally are accumulated (Fig. 8C). Therefore, these results indicate that over-expression of *UGT78D2* redirects the metabolic flux toward anthocyanin production in *Arabidopsis* seed coats.

DISCUSSION

We have now identified *UGT78D2* as the *Arabidopsis* equivalent of maize *Bronze-1*, and have located it in the cytosol. Our biochemical analyses revealed that recombinant UGT78D2 glucosylates both flavonols and anthocyanidins at the 3-OH position. Consistent with this, the HPLC and spectroscopy analyses of our *ugt78d2* mutant showed that it lacks 3-O-glucosylated quercetin and the majority of anthocyanins. These results agree with those that indicate UGT78D2 is a UF3GT in *Arabidopsis* (Tohge et al., 2005). In addition, we showed that UGT78D2 is repressed in the developing testae and that its increased expression is sufficient to redirect metabolic flux toward anthocyanin production in developing seed coats. Taken together, our results demonstrate that UGT78D2 is a UF3GT required for the synthesis of anthocyanin and 3-O-glucosylated flavonol. Furthermore, the lack of its activity coupled with the higher activity of anthocyanin reductase is critical for the synthesis of condensed tannins rather than antho-

Table 1. ^{1}H-, ^{13}C-NMR data[a] of quercetin 3-O-β-D-glucopyranoside-7-O-α-L rhamnopyranoside in CD_3OD.

Position	α_C	α_H (mult, *J* Hz)	HMBC
2	59.26		
3	135.62		
4	179.41		
5	162.61		
6	100.49	6.45 (d 2.0)	C-5, C-7, C-8, C-10
7	163.38		
8	95.38	6.74 (d 2.0)	C-6, C-7, C-9, C-10
9	157.78		
10	107.26		
1'	122.76		
2'	117.42	7.71 (d 2.0)	C-2, C-3', C-4', C-6'
3'	145.78		
4'	149.86		
5'	115.94	6.86 (d 8.4)	C-1', C-3', C-4', C-6'
6'	123.23	7.61 (dd 8.4, 2.0)	C-2, C-2', C-4'
G1	103.81	5.32 (d 7.2)	C-3
G2	75.68	3.48 (m)	C-G1, C-G3, C-G4
G3	78.01	3.42 (m)	C-G2, C-G4
G4	71.20	3.33 (m)	C-G3, C-G5
G5	78.38	3.22 (m)	C-G4
G6	62.50	3.71 (dd 11.6, 2.0)	C-G4
		3.56 (dd 11.6, 5.6)	C-G5
R1	99.77	5.56 (d 1.6)	C-7, C-R2
R2	71.63	4.02 (m)	C-R3, C-R4
R3	72.04	3.82 (dd 9.6, 3.6)	C-R2, C-R4
R4	73.55	3.47 (m)	C-R3, C-R5
R5	71.20	3.58 (m)	C-R4
R6	18.13	1.24 (d 6.4)	C-R4, C-R5

[a]Assignments made on the basis of COSY, HSQC, and HMBC experiments.

cyanins in the developing seed coat.

UGT78D2, Functionally Equivalent to Maize Bronze-1, Limits the Synthesis of Anthocyanins in the Developing Seed Coat

Although biochemical assays have identified UF3GTs in many species, our *ugt78d2* mutant is only the second published *uf3gt* mutant. The first, maize *bronze-1* (*bz1*) (Ralston et al., 1988; Tanaka et al., 1996; Gong et al., 1997; Ford et al., 1998; Yamazaki et al., 2002), displays a light brown coloring in its aleurone layer, in contrast to a purple pigmentation in the WT. Our generation and characterization of an *Arabidopsis uf3gt* mutant will facilitate further investigations into the roles of UF3GT in dicot plants.

The lighter pigmentation found in mutant seedlings and adult plants is likely due to the instability of aglucosylated anthocyanidins (Brouillard, 1982). A previous chemical characterization showed that anthocyanidins are unstable at a pH >4.0, but may be stabilized by the glucosylation-based conversion to anthocyanins. This explains why the coloration of anthocyanidins was not observed in our mutants, as these molecules are unstable unless converted to anthocyanins by UF3GT. Characterizations of maize *bronze-2* (*bz2*), petunia *an9*, and *Arabidopsis tt19* mutants have indicated that glutathionation and subsequent transportation of anthocyanins into the vacuoles are also necessary for the further stabilization of anthocyanins (Marrs et al., 1995; Alfenito et al., 1998; Kitamura et al., 2004). Because the GST encoded by *Bronze-2* (*Bz2*) was shown to glutathionate anthocyanins but not anthocyanidins, this decreased glutathionation might also have played a role in the lower accumulation of colored flavonoids in our *ugt78d2* mutant.

A previous report showed that the three major *Arabidopsis* anthocyanins detected in the leaves and stems are 3-O-glucosylated forms (cyanidin 3-O-[2-O-(2-O-(sinapoyl)-xylosyl)-6-O-(4-O-glucosyl)-p-coumaroyl)) glucoside], cyanidin 3-O-[2-O-(xylosyl)-6-O-(4-O-(glucosyl)-p-coumaroyl)] glucoside], and cyanidin 3-O-[2-O-(2-O-(sinapoyl)-xylosyl)-6-O-(p-coumaroyl) glucoside]) (Bloor and Abrahams, 2002). Our HPLC analysis revealed two major peaks (19.53 min and 20.24 min) and a few small peaks detectable at 520 nm under experimental conditions (Fig. 7A). Although it is unclear that the anthocyanins produced in our far-red-irradiated seedlings are identical to the three major anthocyanins previously detected in the leaf and stem, the anthocyanins found in our *Arabidopsis* seedlings are likely to be 3-O-glucosylated forms. Thus, our observation of undetectable or highly reduced anthocyanin peaks in the *ugt78d2* mutant suggests that UF3GT activity is mainly provided by UGT78D2 in *Arabidopsis*. However, because the mutant is likely to be a null mutant, its synthesis of only small amounts of anthocyanins suggests that other minor UF3GTs are present in *Arabidopsis* seedlings. Because UGT78D3 is closely related to UGT78D2, it will be interesting to determine whether UGT78D3 provides minor UF3GT activity. Beyond UGT Group F, a previous report has indicated that UGT73B5 and UGT73B4 efficiently glucosylates quercetin at 3-OH position *in vitro*. Therefore, it is possible that UGTs belonging to groups other than F may also contribute to the synthesis of remaining anthocya-

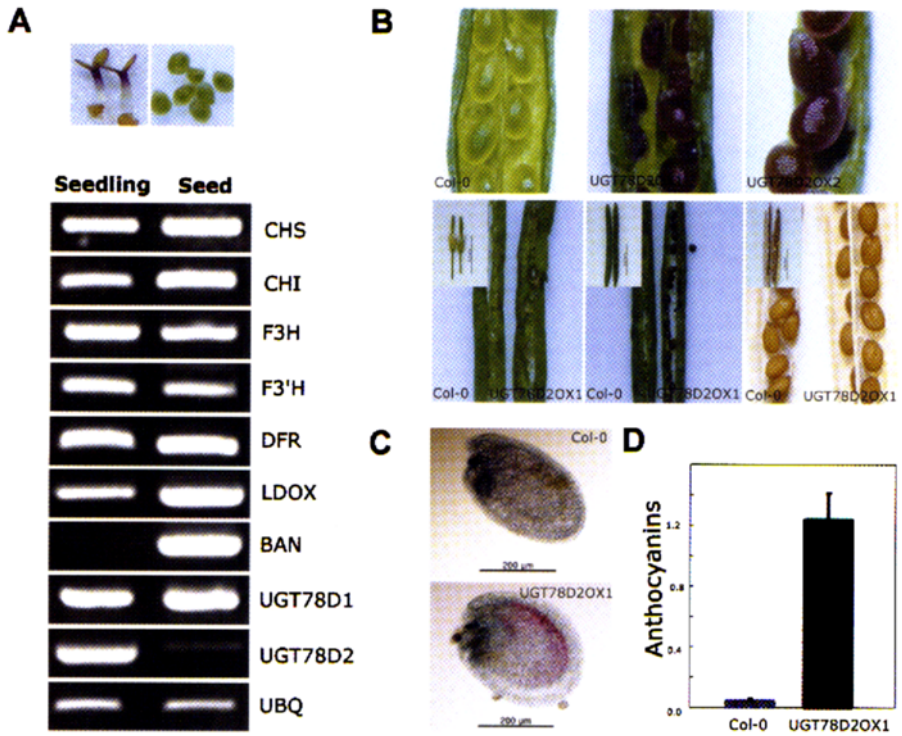

Figure 8. UGT78D2 limits synthesis of anthocyanins in developing seed coats. **(A)** RT-PCR expression analysis of various anthocyanin biosynthetic genes. Upper panels show pigmentation of seedlings and seeds used for analysis. **(B)** Accumulation of anthocyanins in developing seed coats of transgenic *Arabidopsis* expressing *UGT78D2* under control of CAMV35S promoter (UGT78D2OX1, UGT78D2OX2). **(C)** Accumulation of anthocyanins in endothelium of transgenic seeds. **(D)** Quantification of anthocyanins extracted from developing seeds.

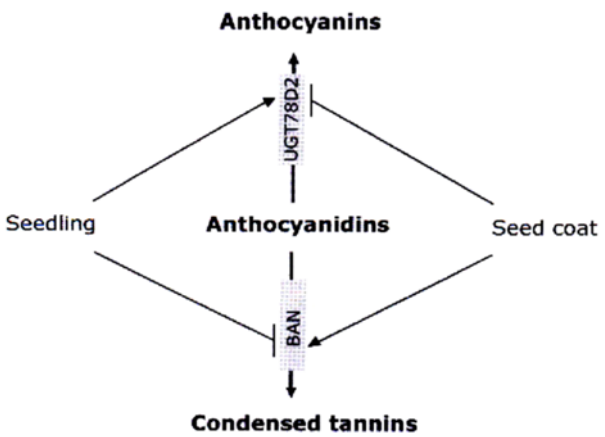

Figure 9. Schematic diagram of anthocyanidin metabolic flux in seedlings and developing seed coats. Our results indicate that reciprocal regulation of *UGT78D2* and *BAN* determines metabolic flux toward production of anthocyanins in seedlings and condensed tannins in developing seed coats.

nins in the *ugt78d2* mutant (Lim et al., 2004).

Our analyses further indicated that a 3-O-glucosylated flavonol is also missing or highly reduced in *ugt78d2* mutant plants (Fig. 7B). *Arabidopsis* contains flavonols that are modified in many different compounds, including via glucosylation and rhamnosylation (Veit and Pauli, 1999; Jones et al., 2003). Here, HPLC analysis showed that the compounds detected at 340 nm in *Arabidopsis* seedlings comprised three major peaks (14.89 min, 16.24 min, and 17.18 min) and a few small peaks. In contrast, one major peak (16.24 min) and a few small peaks were undetectable in the mutant plants. Structure analysis indicated that the missing major peak (16.24 min) is a 3-O-glucosylated form (quercetin 3-O-β-D-glucopyranoside-7-O-α-L-rhamnopyranoside). We were unable to determine the structures of the flavonols corresponding to the missing small peaks in the mutant. Because the *F3'H* gene is highly expressed under far-red irradiation (Fig. 1C), it is likely that the peaks corresponding to the faster-migrating quercetin glucosides are higher than the peaks corresponding to the slower-moving kaempferol glucosides. Considering that UGT78D2 can glucosylate both kaempferol and quercetin, we might speculate that one of those small peaks abolished in the mutant plants may correspond to 3-O-

glucosylated kaempferol.

Interestingly, our HPLC profile of flavonols is much simpler than those reported previously (Veit and Pauli, 1999; Jones et al., 2003; Tohge et al., 2005). Our profiles were obtained from far-red light-grown seedling samples, whereas those other were developed from mainly white light-grown leaves, a difference that might reflect the various flavonols synthesized in those different tissues. Further analysis to determine which genes are differentially expressed in these two leaf types will be useful for identifying the enzymes responsible for the modification of flavonols.

We showed that the activity of UF3GT plays a critical role in organ pigmentation in *Arabidopsis*. The substrates of UF3GT are aglycosylated anthocyanidins, which also serve as the substrates of anthocyanin reductase. Thus, anthocyanidins form a branching point that can lead to the synthesis of either anthocyanins or condensed tannins. Anthocyanidins are mainly converted to condensed tannins in the *Arabidopsis* seed coat, whereas they are primarily converted to anthocyanins in seedlings. Our results demonstrate that this regulation of metabolic flux toward the production of condensed tannins or anthocyanins in different organs is likely due to the reciprocal regulation of *BAN* and *UGT78D2*. The latter was highly expressed in anthocyanin-accumulating seedlings, but was repressed in condensed tannin-accumulating seed coats (Fig. 8B). In contrast, *BAN* was repressed in seedlings, but was highly expressed in seed coats. The functional significance of this reciprocal regulation is manifested by the purple coloring and anthocyanin accumulation in the seed coats of *ban* mutants (Albert et al., 1997) and our *UGT78D2*-overexpressing lines (Fig. 8B). Taken together, these results suggest that the reciprocal regulation of *UGT78D2* and *BAN* likely plays a key role in determining whether an organ directs metabolic flux toward the production of anthocyanins or condensed tannins in *Arabidopsis* (Fig. 9).

UGT78D1 and UGT78D2, Two Closely Related Glycosyltransferases, Have Different Substrate and UDP-Sugar Specificities

UGT78D1 and UGT78D2 are 71% identical at the amino acid sequence level. These two proteins are phylogenetically clustered together with other known UF3GTs. Consistent with this clustering, our analysis of UGT78D2 clearly indicated that it catalyzes glucosylation of both flavonols and anthocyanidins at the 3-OH position. In contrast, UGT78D1 rhamnosylates its substrates (Jones et al., 2003), a function not associated with the UF3GTs clustered nearby in the phylogenetic analysis. Interestingly, the functional difference between UGT78D1 and UGT78D2 is not restricted to their UDP-sugar specificities. Our biochemical analysis also showed that the latter could glucosylate both flavonols and anthocyanidins, while a previous report indicated that the former could rhamnosylate flavonols but not anthocyanidins (Jones et al., 2003). Thus, these two very closely related glycosyltransferases differ in both their substrate and UDP-sugar specificities.

Close inspection of the two amino acid sequences has revealed differences in several amino acids within the UDPGT motif thought to bind UDP-sugar (Hundle et al., 1992; Kapitonov and Yu, 1999). The 381st residue of UGT78D2 is conserved among known UF3GTs, and has proven important for UDP-sugar specificity in UDP-galactose:cyanidin 3-O-galactosyltransferase (Kubo et al., 2004). However, the conserved glutamine at this residue is replaced by asparagine in UGT78D1. Because the mutation of a single amino acid or a few amino acids is capable of altering the substrate specificity of an enzyme, it will be interesting to investigate whether this residue, alone or in conjunction with other amino acid substitutions, allows UGT78D1 to function as a flavonol rhamnosyltransferase rather than as a flavonoid glucosyltransferase.

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