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RESEARCH PAPER

Feedback inhibition of the general phenylpropanoid and flavonol biosynthetic pathways upon a compromised flavonol-3-*O*-glycosylation

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Abstract

Flavonols, phenylalanine-derived secondary metabolites, have protective and regulatory functions in plants. In *Arabidopsis thaliana*, they are consecutively glycosylated at their 3-OH and 7-OH groups. UGT78D1 and UGT78D2 are the major flavonol 3-*O*-glycosyltransferases in *Arabidopsis* leaves. The *ugt78d1 ugt78d2* double mutant, which was strongly compromised in the initial 3-*O*-glycosylation, showed a severe and specific repression of flavonol biosynthesis, retaining only one-third of the wild-type level. This metabolic phenotype was associated with a repressed transcription of several flavonol biosynthetic genes including the committed step chalcone synthase [(CHS) or TRANSPARENT TESTA 4 (TT4)]. Furthermore, the committed step of the upstream, general phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), was down-regulated in its enzyme activity and in the transcription of the flavonol-related *PAL1* and *PAL2*. However, a complete blocking of flavonoid biosynthesis at CHS released PAL inhibition in a *tt4 ugt78d1 ugt78d2* line. PAL activity was even enhanced in the *flavonol synthase 1* mutant, which compromises the final formation of flavonol aglycones. The dependence of the PAL feedback inhibition on flavonols was confirmed by chemical complementation of *tt4 ugt78d1 ugt78d2* using naringenin, a downstream flavonoid intermediate, which restored the PAL repression. Although aglycones were not analytically detectable, this study provides genetic evidence for a novel, flavonol-dependent feedback inhibition of the flavonol biosynthetic pathway and PAL. It was conditioned by the compromised flavonol-3-*O*-conjugation and a decrease in flavonol content, yet dependent on a residual, flavonol synthase 1 (FLS1)-related capacity to form flavonol aglycones. Thus, this regulation would not react to a reduced metabolic flux into flavonol biosynthesis, but it might prevent the accumulation of non-glycosylated, toxic flavonols.

Key words: Feedback inhibition, flavonoids, flavonols, flavonol synthase, phenylalanine ammonia-lyase, phenylpropanoids, UDP-carbohydrate-dependent glycosyltransferase.

Introduction

The phenylalanine-derived flavonoids are important plant secondary metabolites. In *Arabidopsis thaliana*, flavonoids comprise flavonols, anthocyanins, and proanthocyanidins. Flavonols have been the subject of intense research interest

Abbreviations: CA, cinnamic acid; I, isorhamnetin; K, kaempferol; K3G, kaempferol 3-*O*-glucoside; K3R, kaempferol 3-*O*-rhamnoside; Q, quercetin; SM, sinapoyl malate; UGT, UDP-carbohydrate-dependent glycosyltransferase.

because they were shown to have diverse roles in plants such as protection from oxidative damage and UV radiation, inhibition of auxin transport, control of pollen function, and signalling to symbiotic organisms (Mo *et al.*, 1992; Li *et al.*, 1993; Peer *et al.*, 2004; Harrison, 2005; Taylor and Grotewold, 2005; Wasson *et al.*, 2006; Santelia *et al.*, 2008; Buer *et al.*, 2010). In addition, flavonols taken in as food and feed ingredients have also been implicated as being either protective, regulatory, or cytotoxic molecules in mammals (Lin and Weng, 2006).

The biosynthesis of flavonols as well as the general phenylpropanoid pathway supplying the flavonoid precursor *p*-coumaroyl-CoA have been well characterized in many plant species including *A. thaliana* (Dixon and Steele, 1999; Harborne and Williams, 2000, 2001; Winkel-Shirley, 2001) (Fig. 1). Phenylalanine ammonia-lyase (PAL) catalyses the committed step of the phenylpropanoid pathway at the link between primary and secondary metabolism. Different PAL isoforms have been proposed to have different metabolic roles in *A. thaliana*. PAL1 and PAL2 are involved in flavonoid and lignin biosynthesis, while PAL4 appears to be specific for the lignin branch (Raes *et al.*, 2003; Rohde *et al.*, 2004; Huang *et al.*, 2010). Chalcone synthase [(CHS) or TT4 (TRANSPARENT TESTA 4)] catalyses the committed step of flavonoid biosynthesis and is encoded by a single gene in *A. thaliana* (Winkel-Shirley, 2006). The *tt4* mutant is completely devoid of flavonoids, which provides a good tool for analysing the consequences of a complete loss of flavonoids in plants. Although six *Arabidopsis* flavonol synthase (FLS) isoforms were identified, only FLS1 contributed considerably to the last step of the formation of the flavonol aglycones (Owens *et al.*, 2008; Stracke *et al.*, 2009). FLS3 had a very low activity, which could be only tracked down in an *fls1* background (Preuß *et al.*, 2009).

Flavonol aglycones are further glycosylated in a complex manner in plant cells. Glycosylation is frequently involved in the biosynthesis of secondary metabolites to modify their stability, solubility, or localization, and thereby the biological properties of the conjugated molecules (Li *et al.*, 2001; Meßner *et al.*, 2003; Bowles *et al.*, 2006). In the case of the hydrophobic flavonols, glycosylation renders them more water soluble and less toxic, and it may enable flavonol transport and compartmentation (Cos *et al.*, 2001; Bowles *et al.*, 2006; Winkel-Shirley, 2006; Moreira *et al.*, 2007; Xiao *et al.*, 2009). However, the physiological significance of the complex glycosylation pattern found in plants remains unclear. Glycosylation is achieved by UDP-carbohydrate-dependent glycosyltransferases (UGTs), which transfer sugars to their aglycone substrates by the formation of a glycosidic bond (Li *et al.*, 2001). Flavonols are usually glycosylated at their 3-OH and 7-OH positions in *A. thaliana* (Veit and Pauli, 1999; Bloor and Abrahams, 2002; Jones *et al.*, 2003; Tohge *et al.*, 2005; Yonekura-Sakakibara *et al.*, 2007, 2008). Based on the *in vitro* substrate preferences of recombinant UGT proteins and the flavonol glycoside profiles of the *Arabidopsis* mutants deficient for glycosylation at these two positions, the 3-*O*-glycosylation is considered to be the first step of conjugation followed by 7-*O*-glycosylation. Five UGTs involved in flavonol glycosylation have been identified: UGT78D1, UGT78D2, UGT78D3, UGT73C6, and UGT89C1 (Fig. 1) (Jones *et al.*, 2003; Tohge *et al.*, 2005; Yonekura-Sakakibara *et al.*, 2007, 2008). In leaves, UGT78D1 and UGT78D2 are the major 3-*O*-glycosyltransferases, whereas UGT78D3 makes only a minor contribution to the overall 3-*O*-glycosylation (Jones *et al.*, 2003; Tohge *et al.*, 2005; Yonekura-Sakakibara *et al.*, 2008). UGT73C6 contributes to 7-*O*-glucosylation in leaves, but only trace amounts of flavonols are 7-*O*-glucosylated. Instead, 7-*O*-rhamnosylation catalysed by UGT89C1 is the major form of 7-*O*-conjugation (Yonekura-Sakakibara *et al.*, 2007).

The phenylpropanoid and flavonoid biosynthesis pathways are subject to multiple levels of control. They are dependent on developmental stages and tissues as well as on exogenous stimuli, in particular on irradiation, nutrient deprivation, and temperature (Kubasek *et al.*, 1992; Taylor and Grotewold, 2005; Kaffarnik *et al.*, 2006; Winkel-Shirley, 2006; Olsen *et al.*, 2009; Götz *et al.*, 2010). Both post-transcriptional modification and transcriptional controls have been implicated in these different types of regulation. Several MYB-related and bZIP-type transcription factors regulating flavonol biosnythesis have been identified, which themselves are subject to transcriptional control (Franken *et al.*, 1991; Pairoba and Walbot, 2003; Ulm *et al.*, 2004; Ramsay and Glover, 2005; Tohge *et al.*, 2005; Quattrocchio *et al.*, 2006; Winkel-Shirley, 2006; Stracke *et al.*, 2007; Dubos *et al.*, 2008; Lillo *et al.*, 2008; Olsen *et al.*, 2009; Stracke *et al.*, 2010). In particular, PAL, as the committed step into the phenylpropanoid pathway, has been shown to be metabolically regulated through negative feedback by cinnamic acid on *PAL* transcription and on enzyme activity (Lamb, 1979; Bolwell *et al.*, 1986; Mavandad *et al.*, 1990; Blount *et al.*, 2000).

Here, an *Arabidopsis ugt78d1 ugt78d2* double mutant was characterized that strongly compromised initial 3-*O*-glycosylation of flavonols. However, instead of an accumulation of flavonol aglycones or alternative conjugations by UGT78D3 or other unknown 3-*O*-glycosyltransferase in this line, the biosynthesis of flavonols was strongly repressed. Other branches of the phenylpropanoid pathway were not or only marginally affected. This reduced biosynthesis of flavonols was correlated with a repression of both flavonol biosynthetic genes and *PAL* genes, and of PAL enzyme activity. Further genetic and molecular analyses suggested a novel feedback repression of PAL that was directly related to the strongly compromised initial flavonol-3-*O*-conjugation, yet dependent on the capacity for flavonol aglycone formation.

Materials and methods

Plant lines and growth conditions

Mutant lines were originally obtained from NASC and ABRC stock centres and have been described elsewhere (Jones *et al.*, 2003; Tohge *et al.*, 2005; Yonekura-Sakakibara *et al.*, 2007, 2008; Bashandy *et al.*, 2009; Stracke *et al.*, 2009). *ugt78d1* (SAIL_568F08), *ugt78d2* (SALK_049338), *ugt73c6* (SAIL_525H07), *ugt89c1* (SALK_071113), and *tt4* (SALK_020583) mutants have the Columbia wild-type (Col-0) background, while *fls1* (RIKEN_PST16145) has been

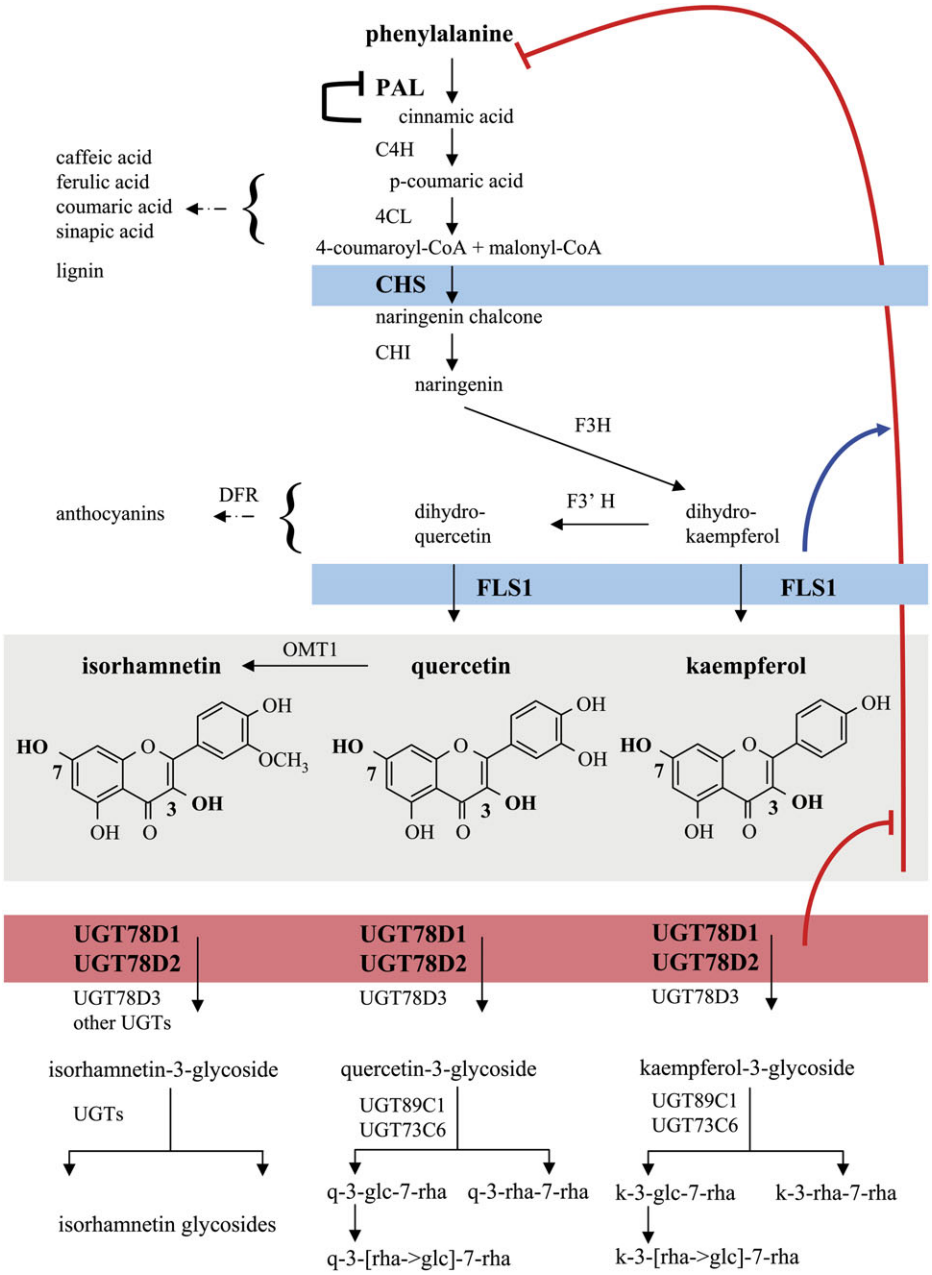

Fig. 1. Phenylpropanoid and flavonol biosynthesis pathways in *Arabidopsis thaliana* including the FLS1-dependent feedback inhibition. The pathways leading from the central precursor phenylalanine to flavonols as well as to other phenylpropanoids such as anthocyanins, sinapic acid derivatives, and lignin are displayed (Winkel-Shirley, 2001; Tohge *et al*., 2005; Yonekura-Sakakibara *et al*., 2007). Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonol synthase (FLS1), *O*-methyltransferase (OMT1), dihydroflavonol 4-reductase (DFR), flavonol 3-*O*-rhamnosyltransferase (UGT78D1), flavonoid 3-*O*-glucosyltransferase (UGT78D2), flavonol 3-*O*-arabinosyltransferase (UGT78D3), flavonol 7-*O*-rhamnosyltransferase (UGT89C1), and flavonol 7-*O*-glucosyltransferase (UGT73C6). Known (cinnamic acid) and the newly discovered metabolic feedback inhibitors (flavonols) are indicated. The inhibition (red) of PAL (and downstream flavonoid-specific genes) is provoked by a compromised 3-*O*-glycosylation in the *ugt78d1 ugt78d2* double mutant. This regulation was shown to depend on (blue) the function of CHS or FLS1, which are necessary to generate the flavonol aglycones. k-3-glc-7-rha, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (f1); k-3-[rha→glc]-7-rha, kaempferol-3-*O*-[rhamnosyl (1→2 glucoside)]-7-*O*-rhamnoside (f2); k-3-rha-7-rha, kaempferol 3-*O*-rhamnoside-7-*O*-rhamnoside (f3). The structurally equivalent quercetin (q) glycosides are abbreviated in an analogous way.

generated in the Nössen accession. Double and triple mutant combinations were verified by PCR-based genotyping of the knockout insertions. Overexpression lines were established in Col-0 using vectors pAlligator2 and pK2GW7 (Karimi *et al*., 2002; Bensmihen *et al*., 2004) to constitutively express UGT78D1 and UGT78D2, respectively. Plants were grown in a growth chamber at 22°C employing a 16/8 h photoperiod and 100–120 µmol m^{-2} s^{-1} irradiance. Plants were usually grown on soil, except for flavonol

quantification in roots and for β-glucuronidase (GUS) reporter analyses, for which plants were cultured on half-strength Murashige and Skoog medium (M5524, Sigma GmbH, Germany) containing 1.5% sucrose and 0.6% phyto-agar.

For naringenin feeding experiments, seeds were surface sterilized and planted on half-strength MS agar medium supplemented with 1% sucrose; seedlings were allowed to grow for 12 d with an 11 h photoperiod. Then seedlings were transferred to 250 ml beakers containing half-strength MS medium with 1% sucrose and 100 μM naringenin (Roth, Karlsruhe, Germany). After a 45 h incubation under continuous light with gentle shaking, the plantlets were rinsed with water and the rosettes were harvested for further analyses.

Histochemical GUS assay

Genomic fragments upstream of the start codon of both *UGT78D* isoforms (–1 bp to –1469 bp for *UGT78D1* or to –1481 bp for *UGT78D2*) were amplified from Col-0 genomic DNA by PCR and introduced into vector pBGWFS7 (Karimi *et al.*, 2002), forming $UGT78D_{pro}$:*GUS-GFP* fusions, which were transformed into Col-0 plants. Histochemical analysis of the GUS reporter gene was performed as described before (Deruère *et al.*, 1999).

Extraction of flavonol metabolites for HPLC analysis

Different plant materials were collected for flavonol extraction. Extracts were prepared by incubating ground (in liquid N_2) plant material with methanol (1 ml per 100 mg) for 1 h with moderate rotation at 4°C. Except for the naringenin feeding experiment, naringenin was used as internal standard for flavonol quantification. The extracts were then clarified by centrifugation at 14 000 *g* for 10 min. One-third volume of distilled water was added to the supernatant, and centrifugation as above removed chlorophyll and lipids. For flavonol aglycone analysis, 100 μl of cleared extract was hydrolysed by adding an equal volume of 2 N HCl and incubating at 70°C for 40 min. Then 100 μl of methanol was added to prevent the precipitation of aglycones. Samples were centrifuged at 14 000 rpm for 15 min. A total of 20 μl of the cleared extract was analysed by high-pressure liquid chromatography (HPLC).

HPLC analysis of flavonols and sinapate esters

Samples were analysed using a reverse phase HPLC system at a flow rate of 1 ml min^{-1} with a 45 min linear gradient starting at 100% solution A to 100% B; 100% B was maintained for 5 min. Solvent A was 2% formic acid with 0.1% ammonium formate, and solvent B was 88% methanol with 0.1% ammonium formate. According to previous work, absorbance at 280 nm was used for detection (Turunen *et al.*, 1999). The flavonol aglycones and sinapate esters were identified by the diode array spectra and retention time in comparison with authentic standards. In addition to the diode array spectra, the published flavonol glycoside patterns of the *ugt78d1* and *ugt78d2* single mutants were used for flavonol glycoside identification (Jones *et al.*, 2003; Tohge *et al.*, 2005).

Quantitative RT-PCR

Total RNA was isolated with Trizol Reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. The first-strand cDNAs were synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Data were collected in accordance with the 7500 real-time PCR system (Applied Biosystems). The experiments were repeated three times with independently cultured plant materials. *S16* (At5g18380) and *TUB9* (At4g20890) were used as reference genes; their stability was tested using geNorm and shown to be suitable as reference genes under these experimental condition (Vandesompele *et al.*, 2002). The gene expression was compared between genotypes by the paired *t*-test. The paired testing takes into account the dependency of the data by the experimental design. The mean difference of the $\log_{10}$ intensities, which is identical to the log fold change, is tested against zero. In addition, *t*-test-based 95% confidence intervals (CIs) were calculated for the mean difference of the log intensities. The results were back-transformed by taking the power to base 10 to obtain the fold change and a 95% CI for the fold change. For all calculations, the R software was employed (R-Development-Core-Team, 2009).

The absolute expression levels of the *PAL* and *4CL* isoforms analysed were different in Col-0 leaves. The C_t values of *PAL1*, *PAL2*, *PAL3*, *PAL4*, *4CL1*, *4CL2*, and *4CL3* were ~25, 30, 34, 35, 32, 39, and 32 cycles, respectively, under the quantitative RT-PCR conditions used, indicating low (≥30) or undetectable transcript levels (≥34) for some isoforms.

Photometric determination of anthocyanins

Photometric determination of anthocyanins was performed according to a method published previously (Mehrtens *et al.*, 2005). Anthocyanins of 3-week-old *Arabidopsis* leaves were extracted at 4°C for 1 h with moderate shaking after adding 250 μl of acidic methanol (1% HCl, w/v) to 50 mg of plant material, which had been homogenized on ice. The homogenate was clarified by centrifugation (14 000 rpm at room temperature for 5 min). Anthocyanins were quantified based on the absorption at 530 nm and 657 nm using $Q_{anthocyanins}=(A_{530}-0.25\times A_{657})\times M^{-1}$, where $Q_{anthocyanins}$ is a corrected absorption value linearly correlated with the amount of anthocyanins, A_{530} and A_{657} are the absorptions at the indicated wavelengths, and M is the mass of the plant material used for extraction.

PAL activity assay

The assay method was modified from Olsen *et al.* (2008). Approximately 100 mg of fresh plant material was thoroughly homogenized with 3 ml of extraction buffer (containing 100 mM TRIS-HCl, pH 8.8, 12 mM 2-mercaptoethanol) using a mortar and pestle on ice. After centrifugation at 14 000 *g* for 10 min at 4 °C the supernatant was desalted using a Sephadex G25 column. The assays were performed at 37°C for 2 h in a mixture containing 500 μl of protein extract, 50 μl of 100 mM L-phenylalanine, and 450 μl of 100 mM TRIS-HCl (pH 8.8). The reaction was terminated by adding 50 μl of 5 M HCl and centrifuged at 14 000 *g* for 15 min. Control reactions were run without adding L-phenylalanine. The UV absorbance was recorded at 290 nm. Assays were performed in five technical repeats in each of the three independent experiments.

LC-ESI-MS^n metabolite analysis

A 100 mg aliquot of freeze-dried *Arabidopsis* leaf powder was extracted with 500 μl of methanol containing 0.1 μg μl^{-1} 4-methylumbelliferyl-β-D-glucuronide as an internal standard. Methanol was removed in a rotary vacuum concentrator and the extract was re-dissolved in 35 μl of water for analysis by liquid chromatography/electrospray ionization multistage mass spectrometry (LC-ESI-MS^n). Metabolites were identified by their retention times, mass spectra, and product ion spectra in comparison with the data determined for authentic reference materials. Relative metabolite quantification was performed using the DataAnalysis 3.1 and QuantAnalysis 1.5 software (Bruker Daltonics, Bremen, Germany) normalizing all results to the internal standard.

Statistics

Statistical analyses of quantitative RT-PCR data were performed by the paired *t*-test using the R software package (R-Development-Core-Team, 2009). Other statistical analyses were performed using SAS 9.1 (SAS/STAT User's Guide, Version 9.1, Cary NC, USA).

Results

Strongly reduced total flavonol content in leaves of the ugt78d1 ugt78d2 *double mutant*

Three major flavonol glycosides, kaempferol 3-*O*-rhamnoside-7-*O*-rhamnoside (f1), kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (f2), and kaempferol-3-*O*-[rhamnosyl (1→2 glucoside)]-7-*O*-rhamnoside (f3), were present in leaves of *A. thaliana* accession Columbia grown under non-stressed conditions (Bloor and Abrahams, 2002) (Fig. 2A). In *ugt78d1* single mutant leaves, f1 was not detectable, whereas f2 and f3 accumulated to higher levels (Jones *et al.*, 2003). However, in *ugt78d2* single mutant leaves, f2 and f3 were strongly decreased, while f1 was increased (Tohge *et al.*, 2005) (Fig. 2A). To quantify the total flavonol content in these *ugt* mutants, methanolic leaf extracts were hydrolysed and the released flavonol aglycones were analysed by HPLC. Although the flavonol glycoside pattern was altered in the *ugt78d1* and *ugt78d2* single mutants, both mutants maintained wild-type, total flavonol content (Fig. 2C). These observations suggest that UGT78D1 and UGT78D2 compete for flavonol aglycones as substrates, and the loss of flavonol 3-*O*-glycosylation in either single mutant is fully compensated by the remaining active flavonol 3-*O*-UGTs. However, the flavonol glycoside profile in leaves of the *ugt78d1 ugt78d2* double mutant plants was severely altered; f2 and f3 were strongly reduced and f1 was not detectable. Furthermore, neither other forms of flavonol derivatives nor any flavonol aglycones were detected (Fig. 2A; data not shown). LC-MS analysis could also not reveal any flavonol aglycones (see Supplementary Table S1 available at *JXB* online). However, in contrast to the *ugt* single mutants, the total flavonol content was reduced; the *ugt78d1 ugt78d2* double mutant contained less than one-third of the wild-type flavonol level. Kaempferol and quercetin contents were decreased to a greater extent, with only 21% and 18% of the wild-type content, respectively (Fig. 2B, C). However, isorhamnetin, a low abundant flavonol in wild-type plants, accumulated ~3.5-fold in the *ugt78d1 ugt78d2* double mutant (Fig. 2B, C). LC-MS analyses detected the strongly increased level of a compound corresponding to the molecular mass of an isorhamnetin-glucoside-rhamnoside, which may at least partly account for the increased isorhamnetin level in *ugt78d1 ugt78d2* (see Supplementary Table S1). Enhanced isorhamnetin glucosides had also been described for the

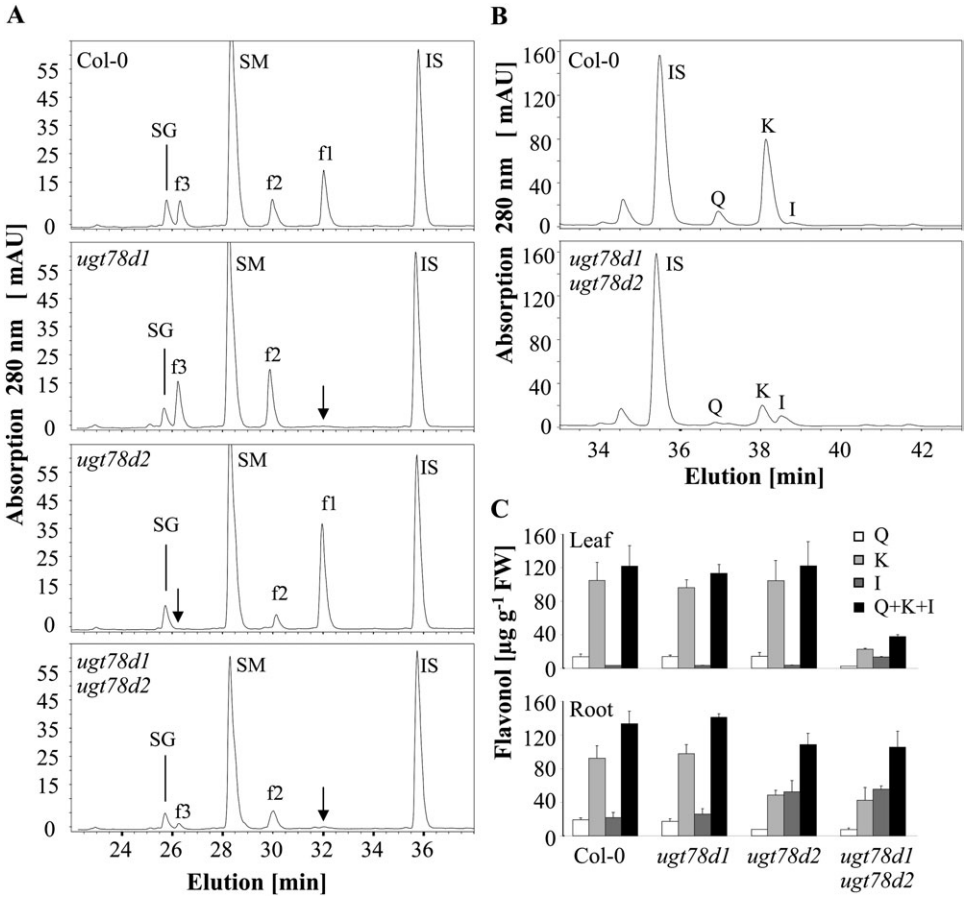

Fig. 2. Determination of flavonols from Col-0, *ugt78d1*, *ugt78d2*, and *ugt78d1 ugt78d2* by HPLC analyses. (A) Representative HPLC diagrams of the flavonol glycoside profiles from leaves of 3-week-old plants. IS, internal standard (naringenin); SM, sinapoyl malate; SG, sinapoyl glucose; f1, f2, and f3 are as described in the legend of Fig. 1. Arrows indicate missing or repressed glycosides. (B) Representative HPLC diagrams of flavonol aglycone profiles from leaf extracts after acid hydrolysis. Q, quercetin; K, kaempferol; I, isorhamnetin. (C) Total flavonol aglycone quantification after acid hydrolysis of the methanolic extracts from leaves and roots. Means ±SD determined from three independent experiments are shown.

ugt78d2 single mutant, indicating the existence of an unknown 3-*O*-glucosyltransferase (Yonekura-Sakakibara *et al.*, 2008).

In summary, the loss of the major flavonol 3-*O*-glycosylation resulted in a strong reduction of total flavonol content. On the other hand, enhanced 3-*O*-glycosylation in UGT78D1 or UGT78D2 overexpression lines did not raise total flavonol levels (see Supplementary Fig. S1 at *JXB* online).

The expression pattern of UGT78D1 *and* UGT78D2 *matches organ-specific reduction in flavonols*

The reduction of total flavonol content was organ specific and different in inflorescences, stems, and leaves from that in roots. Similar to leaves, the total flavonol content was strongly reduced in both inflorescences and stems of *ugt78d1 ugt78d2* plants as compared with the wild-type counterpart (data not shown). In roots, however, the total flavonol content of the double mutant was maintained at higher levels similar to the *ugt78d2* single mutant (Fig. 2C).

The expression pattern of both *UGT78D1* and *UGT78D2* positively correlated with the organ-dependent reduction in total flavonol content in the *ugt* double mutant. Two-week-old transgenic lines expressing the GUS reporter gene under the control of either the *UGT78D1* or *UGT78D2* promoter indicated that both genes were well expressed at the basal part of the young leaves (Fig. 3A, B). The expression of *UGT78D2* was also observed along the central vein of the leaves as well as in the cotyledons (Fig. 3B). In 3-week-old plants, the expression of both *UGT78D1* and *UGT78D2* was lower compared with that of 2-week-old plants and predominantly at the basal part of leaf petioles (Fig. 3C, D). The expression of both *UGT78D1* and *UGT78D2* was very low in roots (data not shown; Jones *et al.*, 2003). These findings were further corroborated by publicly available microarray data using the *Arabidopsis* eFP Browser (Schmid *et al.*, 2005; Winter *et al.*, 2007) (Fig. 3E).

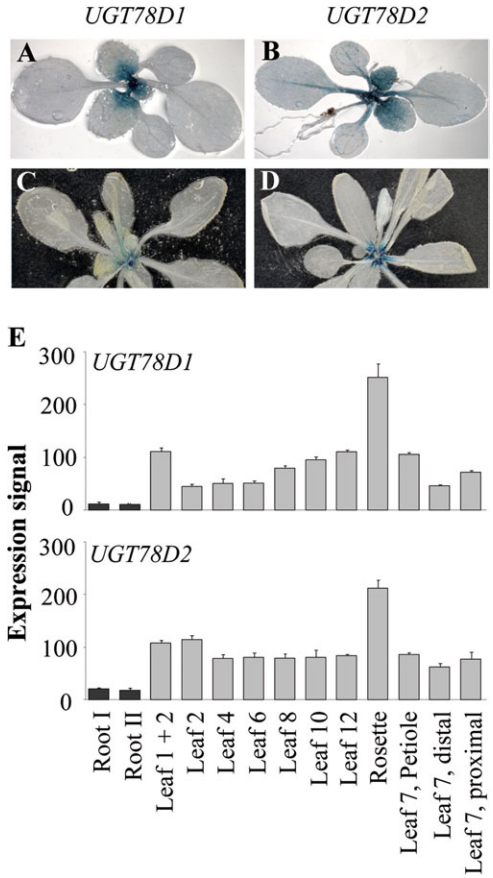

Fig. 3. Expression pattern of *UGT78D1* and *UGT78D2* indicated by staining transgenic lines harbouring promoter–GUS fusions. (A, B) Two-week-old plants. (C, D) Three-week-old plants. (E) Expression values of both *UGT78D1* and *UGT78D2* genes obtained using the *Arabidopsis* eFP Browser (bar.utoronto.ca) (Schmid *et al.*, 2005; Winter *et al.*, 2007) in roots, defined rosette leaves, complete rosettes, as well distal and proximal halves of leaf 7.

Flavonoid biosynthetic genes are transcriptionally down-regulated in ugt78d1 ugt78d2

To examine whether the reduced flavonol content in leaves was accompanied by reduced transcript levels of the flavonoid biosynthetic genes, their mRNA abundance was assessed by quantitative RT-PCR in both *ugt78d1 ugt78d2* double mutant and wild-type plants. In *ugt78d1 ugt78d2*, the transcripts of *CHS*, *F3'H*, *FLS1*, and *DFR* were significantly lowered to ~50% in comparison with the wild-type counterpart (Fig. 4). However, the transcripts of *CHI* and *F3H* were not clearly different from wild-type levels. Furthermore, genes in the general phenylpropanoid pathway upstream of the flavonoid branch including *PAL1*, *PAL2*, *PAL3*, *PAL4*, *4CL1*, *4CL2*, *4CL3*, and *C4H* were analysed. *PAL1* and *4CL3* transcripts were significantly reduced to almost half in the double mutant compared with the wild type, whereas the reduction of *C4H* was less pronounced and *4CL1* mRNA levels were not altered. *PAL2* showed only a tendency for repression, but it was more variable and also expressed to a lower extent than *PAL1* in the wild type (Fig. 4). Eventually, the transcription of *PAL3*, *PAL4*, and *4CL2* in leaves was too low to allow an unambiguous evaluation in both the wild type and the mutant (Materials and methods). Thus, the committed steps of the phenylpropanoid pathway (*PAL1*) and of flavonoid biosynthesis (*CHS*) along with several intermediate steps were suppressed at the transcriptional level in *ugt78d1 ugt78d2* to about one-half of wild-type expression. Neither *PAL1* (and *PAL2*) nor *CHS* transcript levels were affected in either one of the single *ugt* mutants (see Supplementary Fig. S2 at *JXB* online).

Transcriptional control of biosynthetic genes

Several families of transcription factors, in particular R2R3-MYB, bHLH (basic helix–loop–helix), and WD40 proteins, as well as bZIP, WRKY, and MADS-box proteins interact and establish transcriptional networks to control the general phenylpropanoid and flavonoid biosynthetic genes (Borevitz *et al.*, 2000; Zimmermann *et al.*, 2004; Ramsay and Glover, 2005; Tohge *et al.*, 2005; Quattrocchio *et al.*, 2006; Stracke *et al.*, 2007, 2010; Dubos *et al.*, 2008; Hichri *et al.*, 2011). Among them, *Arabidopsis* MYB11, MYB12,

MYB111, or MYBL2 are flavonol-specific activators or repressors which are able to regulate flavonol biosynthesis independently from other cofactors (Czemmel *et al.*, 2009). Since many of these transcription factors are controlled at the transcriptional level, several genes were analysed for deregulation in the *ugt* double mutant. However, neither *MYB111*, *MYB12*, *MYBL2*, *PAP1/MYB75*, *PAP2/MYB90*, nor *HY5* (*bZIP*) transcript levels were significantly changed in parallel with the observed repression of the flavonoid biosynthetic genes (Supplementary Fig. S3 at *JXB* online). *PAP2* was even induced, thus seemingly counteracting the suppression of the flavonoid branch.

Recently, miRNA156 was shown to affect the anthocyanin/flavonol ratio by targeting SPL9, a regulator of flavonoid-related transcriptional complexes (Gou *et al.*, 2011). RNA interference could be also involved in the repression of flavonol biosynthesis by directly affecting the related genes. However, none of the repressed biosynthetic genes was listed as a confirmed or predicted target of known microRNAs (miRNAs) in *A. thaliana* (mpss.udel.edu/at/target.php). Furthermore, a blast search for known *A. thaliana* miRNAs derived from miRBase (www.mirbase.org) did not reveal any significant, high scoring hits. An independent search for homologous regions (20-mers allowing up to three mismatches) only identified imperfect overlaps within at most four genes (Y. Wang and G. Haberer, personal communication). Thus, a coordinated regulation of the repression based on direct RNA interference targeting the biosynthetic genes is unlikely.

Reduced PAL enzyme activity in ugt78d1 ugt78d2

The decreased transcript levels of *PAL1* in *ugt78d1 ugt78d2* prompted examination of whether PAL activity was also reduced. PAL activity was assayed in total protein extracts from *ugt78d1*, *ugt78d2*, *ugt78d1 ugt78d2*, and wild-type leaves. Both *ugt78d1* and *ugt78d2* single mutants retained wild-type levels of PAL activity, whereas the *ugt78d1 ugt78d2* double mutant showed a significant reduction to ~60% (Fig. 5). However, the endogenous levels of the PAL substrate phenylalanine were not changed in the double mutant compared with the wild type (Supplementary Fig. S4 at *JXB* online).

The influence of reduced PAL activity on non-flavonol branches of phenylpropanoid metabolism

In addition to the flavonol pathway, the repression of PAL activity in *ugt78d1 ugt78d2* might also affect other phenylpropanoid-dependent branches; that is, the biosynthesis of anthocyanins, sinapate esters, and lignin. In another scenario affecting a single branch of the phenylpropanoid pathway, suppression of lignin biosynthesis has led to metabolic overflow and enhanced flavonoid production (Besseau *et al.*, 2007).

ugt78d1 accumulated essentially wild-type levels of total anthocyanins, while anthocyanin accumulation was already strongly suppressed in the *ugt78d2* single mutant without any inhibitory effect on PAL expression (Figs 5, 6A) (Tohge *et al.*, 2005). Since *ugt78d1 ugt78d2* had low amounts of anthocyanin comparable with *ugt78d2* (Fig. 6A), the repression of PAL did not further affect this downstream branch. However, *DFR* transcription was down-regulated in *ugt78d1 ugt78d2* (Fig. 4).

Sinapate esters, particularly sinapoyl malate, are abundantly present in *Arabidopsis* leaves. The double mutant, in contrast to the single mutants, had a slightly, yet significantly, reduced sinapoyl malate content compared with the wild type (Fig. 6B). Sinapoyl glucose was also slightly reduced in the *ugt* double mutant (data not shown).

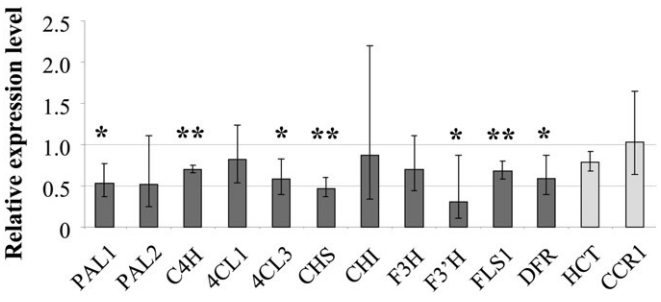

Fig. 4. Transcription of genes involved in phenylpropanoid and flavonoid biosynthetic pathways. Quantitative RT-PCR analyses were used to assess transcript levels of the indicated genes in the *ugt78d1 ugt78d2* double mutant relative to Col-0 (Supplementary Table S2 at *JXB* online). Data were obtained from three independent experiments. Expression levels were normalized using *S16* and *TUB9* transcripts. Fold changes with 95% CIs are displayed. Asterisks indicate significance of the comparison with wild-type plants, $^{*}P < 0.05$; $^{**}P < 0.01$. The lower mean value of *PAL2* indicated a tendency for repression of this gene which is expressed only to a low extent (P=0.0658).

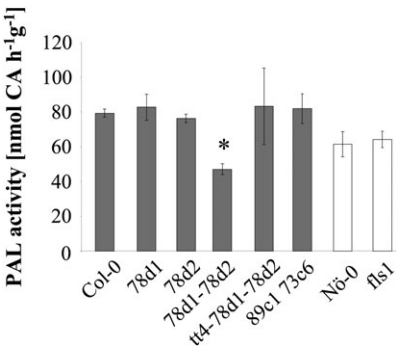

Fig. 5. Quantification of PAL activity of *ugt78d1*, *ugt78d2*, *ugt78d1 ugt78d2*, *tt4 ugt78d1 ugt78d2*, *ugt89c1 ugt73c6*, *fls1*, and wild-type (Col-0, Nö-0) plants. Crude protein extracts from leaves of 3-week-old plants were assayed for PAL activity. CA, cinnamic acid. The mean values ±SD of three independent biological samples (pools of 4–6 plants) are displayed. Col-0-based (dark) and Nö-0-derived (white bars) lines are indicated. $^{*}P < 0.05$ (based on multiple comparisons of different mutants with the corresponding wild type using Dunnett's *t*-tests). The experiment was repeated three times with similar results.

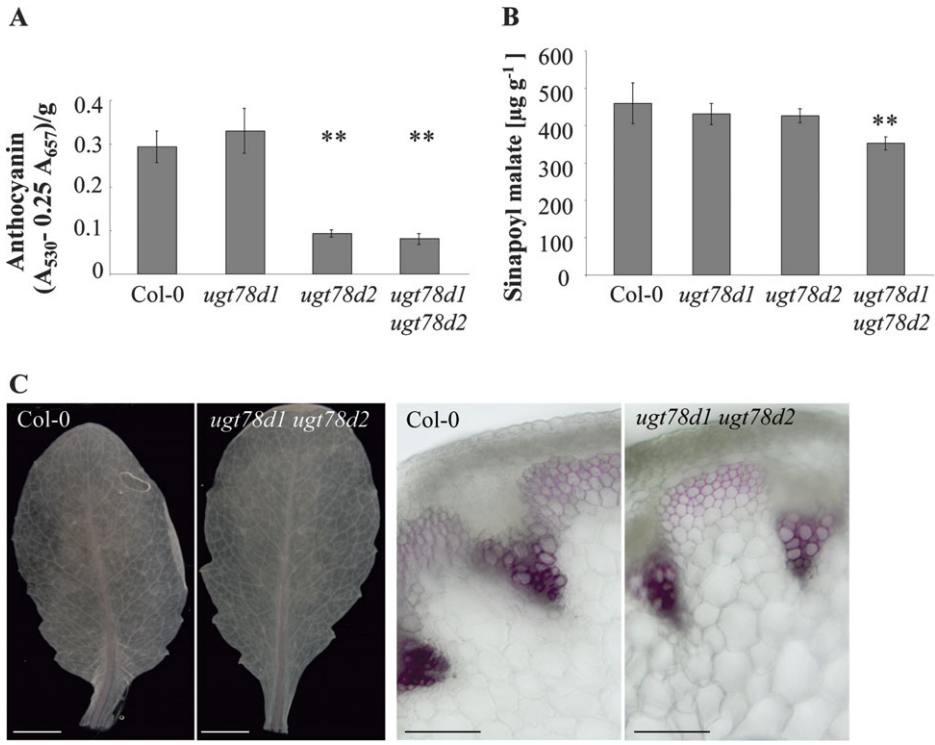

Fig. 6. Phenylpropanoid compounds of *ugt78d1 ugt78d2* and Col-0 plants. (A) Photometric determination of the anthocyanin content in acidic methanolic leaf extracts of 3-week-old *Arabidopsis* plants: A_{530}, absorption at 530 nm; A_{657}, absorption at 657 nm. The mean values ±SD of three independent biological samples (pools of 4–6 plants) are displayed. $^{**}P < 0.01$; paired *t*-tests. (B) Quantification of sinapoyl malate. The mean values ±SD of six independent biological samples (pools of 4–6 plants) are displayed. $^{**}P < 0.01$; paired *t*-tests. This experiment was repeated three times with similar results. (C) Phloroglucinol-HCl-stained rosette leaves (left, bar=3 mm) from 3-week-old plants and hand cross-sections of inflorescence stems (directly below the third internode) (right, bar=100 μm) from 5-week-old plants.

To test whether the lignin biosynthetic branch was affected, two key genes involved in lignin biosynthesis were analysed by quantitative RT-PCR. The single *HCT* (hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase) and the isoform *CCR1* (cinnamoyl-CoA reductase Class I) are important in initiating the biosynthesis of monolignol precursors (and of other cinnamic acid derivatives) (*HCT*) and catalysing the first step of the monolignol-specific pathway (*CCR1*) in *A. thaliana* (Jones *et al.*, 2001; Lavergeat *et al.*, 2001; Raes *et al.*, 2003; Besseau *et al.*, 2007). Both *HCT* and *CCR1* transcripts were not significantly affected in *ugt78d1 ugt78d2* leaves (Fig. 4). In addition, changes in upstream steps contributing to lignin biosynthesis such as *4CL1*, *4CL2*, *PAL3*, and *PAL4* (Raes *et al.*, 2003) did not indicate transcriptional up-regulation (see above; Fig. 4). Thus, the biosynthesis of lignin precursors was essentially not altered at the transcriptional level. Furthermore, total lignin was examined histochemically using phloroglucinol-HCl staining of leaves and stems. The total flavonol content in inflorescence stems of *ugt78d1 ugt78d2* was also reduced to a similar extent to that in leaves (data not shown). However, lignin deposits in both leaves and stems of *ugt78d1 ugt78d2* were similar to wild-type levels (Fig. 6*C*). Finally, metabolites associated with the lignin biosynthesis pathway were assessed by LC-MS analysis. No immediate monolignol precursor was detected in the leaf extracts. Nevertheless, LC-MS analysis showed that the *ugt78d1 ugt78d2* double mutant accumulated wild-type levels of the monolignol precursor-related derivatives *p*-coumaroyl-glucoside, *p*-coumaroyl-glucose-ester, and caffeoyl-glucose-ester (Supplementary Table S1 at *JXB* online). Thus, these results indicated that lignin biosynthesis was not significantly affected in the *ugt* double mutant.

In summary, these analyses demonstrated that there were at most marginal effects on non-flavonol-specific branches of phenylpropanoid biosyntheses in *ugt78d1 ugt78d2*; notably, there were no compensatory up-regulations.

The reduction of PAL expression of ugt78d1 ugt78d2 *is dependent on flavonol aglycone formation*

To examine whether flavonoids themselves could play a role in the flavonol-specific reduction in PAL expression, the *tt4 ugt78d1 ugt78d2* triple mutant was employed. The triple mutant *tt4 ugt78d1 ugt78d2* was blocked at the committed step into flavonoid biosynthesis in the *ugt* double mutant background (Fig. 1). In contrast to the *ugt78d1 ugt78d2* line, *tt4 ugt78d1 ugt78d2* as well as the parent *tt4* mutant had wild-type PAL activity (Fig. 5; data not shown) and the transcripts of both *PAL1* and *PAL2* even showed a tendency towards being up-regulated in the triple mutant (Supplementary Fig. S5 at *JXB* online). Thus, the suppression of PAL expression in *ugt78d1 ugt78d2* was released either by the loss of flavonoids or by the loss of CHS activity. To distinguish these

two possibilities, a chemical complementation approach was employed. The feeding of naringenin—an intermediate downstream of CHS/TT4, but upstream of FLS—to *tt4 ugt78d1 ugt78d2* could re-implement the inhibition of PAL expression. Naringenin feeding led to the repression of PAL activity and to the transcriptional down-regulation of *PAL1* similar to the extent observed for the glycosyltransferase double mutant (Fig. 7). The transcription of *PAL2* which was expressed to a much lower extent was not significantly affected under this condition (Fig. 7B). Flavonols had been formed after administration of naringenin, confirming the uptake and processing of the compound by *tt4 ugt78d1 ugt78d2* (Supplementary Fig. S6 at *JXB* online). Thus, the repression of PAL in the 3-*O*-glycosylation-compromised *ugt78d1 ugt78d2* double mutant was flavonoid-dependent and related to naringenin or downstream naringenin-dependent steps. To narrow down the key regulatory step further, additional mutants defective in reactions either directly upstream or downstream of 3-*O*-glycosylation were examined for effects on PAL expression.

The *fls1* mutant severely blocks flavonol biosynthesis at its final step (i.e. the formation of the aglycone moiety) and is almost devoid of flavonols (Fig. 1) (Stracke *et al.*, 2009). However, PAL activity was not reduced, and transcription of *PAL1* and *PAL2* was even enhanced in the *fls1* background (Fig. 5; Supplementary Fig. S5 at *JXB* online).

Flavonol 3-*O*-glycosylation is followed by 7-*O*-glycosylation. To examine whether a compromised flavonol 7-*O*-glycosylation would also lead to a reduction in flavonol content and a concomitant repression of PAL activity, the *ugt89c1 ugt73c6* double mutant was generated (Fig. 1). The flavonol glycoside profile of *ugt89c1 ugt73c6* leaves indicated a strongly suppressed 7-*O*-conjugation. The contents of the three abundant, 7-*O*-conjugated wild-type flavonol glycosides (f1, f2, and f3) were strongly decreased, whereas two flavonol 3-*O*-monoglycosides, kaempferol 3-*O*-glucoside and kaempferol 3-*O*-rhamnoside, accumulated (Fig. 8A). Yet, *ugt89c1 ugt73c6* contained wild-type levels of total flavonols and had unchanged PAL enzyme activity (Figs 5, 8B).

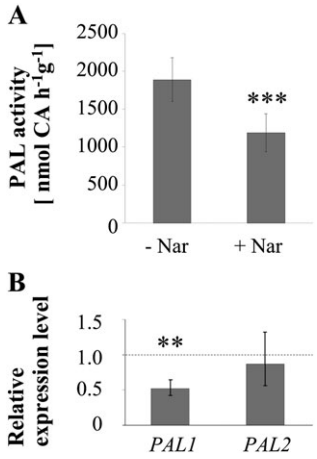

Fig. 7. Chemical complementation of *tt4 ugt78d1 ugt78d2* with naringenin. (A) PAL activity of *tt4 ugt78d1 ugt78d2* with and without feeding of naringenin. The mean values ±SD of six independent biological samples (pools of 4–6 plants) are displayed. CA, cinnamic acid. ***$P < 0.001$; paired *t*-tests. The experiment was repeated twice with similar results. (B) Expression of *PAL1* and *PAL2* genes in the *tt4 ugt78d1 ugt78d2* triple mutant after naringenin feeding relative to the non-fed triple mutant by quantitative RT-PCR analysis. Fold changes with 95% CIs are displayed. Asterisks indicate significance of the difference from non-fed mutant plants: **$P < 0.01$. Data obtained from six biological sample samples (pools of 4–5 seedlings) are shown. The experiment was repeated twice with similar results.

Discussion

Inhibition of flavonol biosynthesis upon a compromised 3-O-glycosylation

Flavonol biosynthesis is under developmental and environmental control in accordance with the diverse regulatory and protective functions of these secondary metabolites (Rohde *et al.*, 2004; Winkel-Shirley, 2006; Hichri *et al.*, 2011). Since flavonols constitute only one, usually minor, branch of phenylpropanoid-derived metabolites, their synthesis is also intensely regulated and integrated with other parts of the phenylpropanoid pathway. Flavonoid biosynthesis is regulated at the transcriptional level by ternary complexes comprising MYB, bHLH, and WD40 proteins, while MYB proteins that act independent of other cofactors specifically control the synthesis of flavonols. However, these transcription factors are subject to transcriptional control themselves (Baudry *et al.*, 2004; Stracke *et al.*, 2007, 2010; Hichri *et al.*, 2011). A block in the lignin-directed branch led to a metabolic redirection and the accumulation of flavonoids (Besseau *et al.*, 2007).

Here it is shown that a strongly compromised 3-*O*-glycosylation of flavonols resulted in a repression of flavonol biosynthesis itself, with ~30% of residual total flavonols, which was specifically associated with the repression of transcripts of flavonol biosynthetic genes as well as the flavonol-related *PAL* genes and PAL enzyme activity of the general phenylpropanoid pathway (see below). However, it was crucial to observe that the repression of PAL was completely released upon blocking the committed step (*tt4*/*chs*) or the very last step of flavonol aglycone biosynthesis (*fls1*).

Specificity of inhibition of the flavonol branch

Flavonol biosynthesis is mostly dependent on PAL1 and PAL2; *pal1 pal2* double mutants are almost devoid of flavonoids; that is, both anthocyanins and flavonols (Rohde *et al.*, 2004; Huang *et al.*, 2010). The *pal1 pal2* mutant still contained ~50% of PAL enzyme activity in leaves due to the activity of other isoforms (Rohde *et al.*, 2004). The reduced flavonol biosynthesis in the *ugt* double mutant was specifically down-regulating the flavonol-related *PAL1* and *PAL2* transcription by ~50% associated with a reduction in PAL activity by ~40%. However, in comparison with the double *pal1 pal2* knockout situation, this seemingly moderate reduction in the

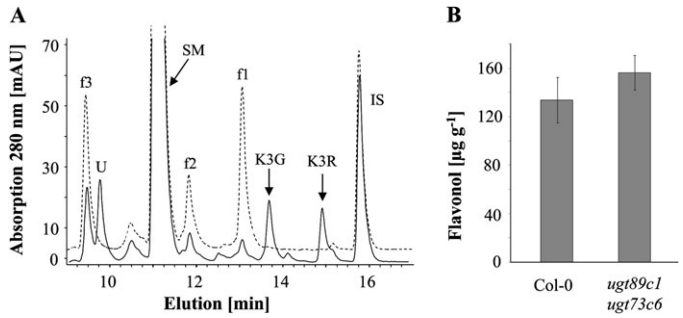

Fig. 8. Flavonol determination in *ugt89c1 ugt73c6* and Col-0 leaves. (A) Representative HPLC diagrams of *ugt89c1 ugt73c6* (solid line) and Col-0 (dashed line). Flavonols were extracted from the leaves of 3-week-old plants. IS, internal standard (naringenin); SM, sinapoyl malate; U, unknown peak; K3G, kaempferol 3-*O*-glucoside; K3R, kaempferol 3-*O*-rhamnoside. The identities of K3G and K3R were determined through comparison with authentic standards by HPLC analysis and then confirmed by LC-MS analysis (Supplementary Fig. S7 at *JXB* online). (B) Total flavonol aglycone quantification after acid hydrolysis of the leaf extracts. Each bar represents the mean ±SD as determined in three independent experiments. Abbreviations: CA, cinnamic acid; I, isorhamnetin; K, kaempferol; K3G, kaempferol 3-*O*-glucoside; K3R, kaempferol 3-*O*-rhamnoside; Q, quercetin; SM, sinapoyl malate; UGT, UDP-carbohydrate-dependent glycosyltransferase.

ugt78d1 ugt78d2 line is likely to represent a major and specific suppression of flavonol-related PAL activity.

Similar to PAL, four active *4CL* genes have been identified in the genome of *A. thaliana* within the general phenylpropanoid pathway. The isoforms 4CL1 and 4CL2 are considered to be important for lignin biosynthesis, whereas 4CL3 is involved in flavonoid biosynthesis (Ehlting *et al.*, 1999; Raes *et al.*, 2003; Costa *et al.*, 2005). Consistent with a specific repression of flavonoids, only the transcription of the flavonol-related *4CL3* was down-regulated in *ugt78d1 ugt78d2* (Fig. 4).

Furthermore, the *ugt78d1 ugt78d2*-dependent suppression of the flavonol branch did not strongly affect other branches of the general phenylpropanoid pathway. In particular, lignin biosynthesis appeared not to be affected. This was in contrast to the reverse situation provoked by a repression of lignin biosynthesis. In this scenario, a re-direction of the metabolic flux and an increased production of flavonoids have been found (Besseau *et al.*, 2007). These differing consequences from repressing lignin versus flavonoid biosynthesis may well be due to the fact that the metabolic flux into the lignin branch is far higher than into flavonoids. The specificity of flavonol-related PAL suppression is also corroborated by the finding that the concentration of phenylalanine was not changed in the *ugt* double mutant. In contrast, in a *pal1* or *pal2* knockout line, it was enhanced 3-fold (Rohde *et al.*, 2004).

Multiple control of PAL activity

PAL, the committed step of the general phenylpropanoid pathway, is subject to multiple levels of control at both transcription and enzyme activity. Importantly, at least in *A. thaliana*, a high correlation between transcription and enzyme activity has been found (Olsen *et al.*, 2008). There is strong evidence that the flavonol biosynthetic enzymes and the upstream enzymes of the general phenylpropanoid pathway are organized as metabolons (Winkel, 2004). Thereby, the specific association of different PAL isoforms could be controlled, which might also depend on post-translational modifications of PALs such as phosphorylation (Allwood *et al.*, 2002).

In addition to widely studied transcriptional networks in response to diverse stimuli such as light, UV-B irradiation, temperature, or nutrient deprivation (Ulm *et al.*, 2004; Quattrocchio *et al.*, 2006; Olsen *et al.*, 2009), the phenylpropanoid biosynthetic pathway is known to be metabolically regulated by phenylpropanoid intermediates. The transcription of *CHS* is inhibited by cinnamic acid, but stimulated by *p*-coumaric acid (Loake *et al.*, 1991, 1992). PAL activity and transcription of *PAL* genes have been shown to be negatively regulated by its product trans-cinnamic acid either via exogenous application of the compound or via blocking the downstream, cinnamic acid-metabolizing C4H activity (Lamb, 1979; Bolwell *et al.*, 1986; Mavandad *et al.*, 1990; Blount *et al.*, 2000) (Fig. 1). Isolated tomato PAL isoforms were differentially sensitive *in vitro* to various phenylpropanoid compounds including cinnamic acid, suggesting an allosteric inhibition (Sarma *et al.*, 1998). However, a further mechanistic explanation for these feedback inhibitions, in particular concerning the transcriptional suppression, has not yet been found.

This work provides genetic evidence for an additional, novel feedback regulation, which is not dependent on these known control mechanisms to repress PAL activity and flavonol biosynthesis. Suppression of flavonol biosynthesis provoked by a compromised 3-*O*-glycosylation led to the repression of flavonol biosynthesis along with the inhibition of flavonol biosynthetic genes including *PAL1* (*PAL2*). However, a complete or almost complete block of flavonol biosynthesis in *tt4* or *fls1* mutant backgrounds released this suppression, although it eliminated the metabolite flux into the flavonol branch even more drastically than the *ugt* double mutant. Thus, these data suggest that the formation of free flavonol aglycones is required for this feedback inhibition (Fig. 1). Currently, a detailed mechanism for this regulation cannot be provided. However, in the case of the hydrophobic flavonol aglycones, two major, non-exclusive scenarios can be envisaged.

First, the proposed flavonol metabolon could be directly influenced *in situ* through binding of nascent, non-glycosylated flavonols to PAL or other components, which could transmit the repression by direct inhibition or by inducing secondary suppressive modifications. Such a scenario would be favoured as soon as the subsequent glycosylation is compromised, as evidenced in the *ugt* double mutant. Only minute, non-accumulating amounts of flavonol aglycones would be required, which could escape detection. In addition, the specificity of the regulation confining the repression basically to the flavonol branch and flavonol-related PAL

activity could be achieved in this model due to the *in situ* interaction. At the protein level, flavonols had been shown to inhibit PAL activity only in an unspecific manner or only weakly at rather high aglycone concentrations (Sato and Sankawa, 1983; Sarma *et al.*, 1998). Therefore, these *in vitro* studies may not directly translate to the situation of the metabolon *in planta*, but support such a possible interaction. Since *PAL* repression and in particular *pal1* knockout have been demonstrated to lead to a suppression of *CHS* and *DFR* transcription (Rohde *et al.*, 2004), the flavonol-dependent repression of PAL activity could promote a correlated down-regulation of the transcription of these genes, with *PAL* being a major coordinating node through an as yet unknown link to transcription.

Secondly, since flavonols have been also localized to the nucleus (Saslowsky *et al.*, 2005), the transcriptional repressions could also be dependent on this property. Flavonols could interact with and block known transcription factors. Such a scenario could also be mediated by a dual function of flavonol-related enzymes binding to flavonols *in situ* and subsequently being translocated to the nucleus. Indeed, flavonol biosynthetic enzymes, specifically CHS and CHI, had been detected in the nucleus (Saslowsky *et al.*, 2005). Thereby, the flavonol biosynthetic enzymes could regulate nuclear transcription, for example through interactions with the canonical flavonol-specific transcription factors. There are precedents for such nuclear functions of metabolic enzymes, such as the signalling dependent on hexokinase 1 in plants or on lactate dehydrogenase A and glyceraldehyde-3-phosphate dehydrogenase in mammalian cells (Kim and Dang, 2005; Cho *et al.*, 2006).

Interestingly, the regulation of transcriptional activators such as PAP1, MYB111, and HY5 as well as the repressor MYBL2 was not found in the *ugt* double mutant as has been shown in other cases, where their genes were themselves regulated at the transcriptional level resulting in a concomitant up- or down-regulation of flavonol biosynthesis (Ulm *et al.*, 2004; Ramsay and Glover, 2005; Tohge *et al.*, 2005; Quattrocchio *et al.*, 2006; Stracke *et al.*, 2007, 2010; Olsen *et al.*, 2009). Another, alternative regulation involving small RNAs appeared to be unlikely as well, since none of the regulated genes is a target of a known *Arabidopsis* miRNA nor do these genes share extended homologous regions among themselves, which could lead to a coordinated suppression of PAL and flavonol-related genes through RNA interference.

Physiological significance of the flavonol-dependent feedback inhibition

Flavonols are known to bind to proteins including histones or DNA polymerases and thus have the potential to interfere with gene regulation (Solimani, 1997; Mizushina *et al.*, 2003; Ramadass *et al.*, 2003; Peer *et al.*, 2004). Such metabolite–protein interactions could be part of the mechanism of feedback control (see above), but they also provide an explanation for the potentially detrimental effects of the hydrophobic flavonol aglycones through (un)specific interactions with cellular proteins. Therefore, the accumulation of aglycones upon specifically blocking their initial 3-*O*-glycosylation is to be prevented by the flavonol-dependent feedback inhibition. This situation resembles a recently described regulation of chlorophyll biosynthesis, which also averts the accumulation of detrimental products. Repression of the final biosynthetic step catalysed by chlorophyll synthase, which conjugates the chlorophyllide core molecule through esterification with phytol, did not lead to the accumulation of precursors. Instead, analogous to the situation found here upon blocking flavonol conjugation, a specific repression of upstream enzyme activities in the chlorophyll branch as well as the committed step of biosynthesis of the porphyrin ring was found. This was also accompanied by a repression of several transcripts encoding these enzymes (Shalygo *et al.*, 2009).

In conclusion, the flavonol-dependent feedback inhibition may serve as a means to avoid the accumulation of hydrophobic and potentially toxic flavonol aglycones upon a compromised flavonol-3-*O*-glycosylation. Genetic evidence is provided that residual flavonol aglycone formation is required for this feedback inhibition of the general phenylpropanoid pathway. In contrast, a complete block of flavonol biosynthesis and thus elimination of the metabolic flux does not lead to a repression of the flavonol biosynthetic capacity.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Total flavonol content in *UGT78D1* and *UGT78D2* overexpression lines in the Col-0 background.

Figure S2. Relative quantification of *PAL1*, *PAL2*, and *CHS* transcripts in the *ugt78d1* and *ugt78d2* single mutants compared with the wild type.

Figure S3. Transcript levels of transcription factor genes in the *ugt* double mutant versus the wild type.

Figure S4. Phenylalanine content in wild-type Col-0 and the *ugt* double mutant.

Figure S5. Transcription of *PAL1* and *PAL2* genes in *fls1* and *tt4 ugt78d1 ugt78d2* mutants relative to the wild type.

Figure S6. Flavonols detected in *tt4 ugt78d1 ugt78d2* plantlets grown in liquid culture after naringenin feeding.

Figure S7. Confirmation of kaempferol 3-*O*-monoglucoside and kaempferol 3-*O*-monorhamnoside by LC-MS analysis.

Table S1. Additional phenylpropanoid metabolites detected in the *ugt78d1 ugt78d2* double mutant and Col-0 leaves by LC-MS analysis.

Table S2. Primers used for real-time quantitative RT-PCR.

Acknowledgements

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