7-Rhamnosylated flavonols modulate auxin homeostasis

7-Rhamnosylated Flavonols Modulate Homeostasis of the Plant Hormone Auxin and Affect Plant Development

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ABSTRACT

Flavonols are a group of secondary metabolites that affect diverse cellular processes. They are considered putative negative regulators of the transport of the phytohormone auxin, by which they influence auxin distribution and concomitantly take part in the control of plant organ development. Flavonols are accumulating in a large number of glycosidic forms. Whether these have distinct functions and diverse cellular targets is not well understood. The roll1-2 mutant of Arabidopsis thaliana is characterized by a modified flavonol glycosylation profile that is inducing changes in auxin transport and growth defects in shoot tissues. To determine whether specific flavonol glycosides are responsible for these phenotypes, a suppressor screen was performed on the roll1-2 mutant, resulting in the identification of an allelic series of UGT89C1, a gene encoding a flavonol 7-O-rhamnosyltransferase. A detailed analysis revealed that interfering with flavonol rhamnosylation increases the concentration of auxin precursors and auxin metabolites, while auxin transport is not affected. This finding provides an additional level of complexity to the possible ways by which flavonols influence auxin distribution and suggests that flavonol glycosides play an important role in regulating plant development.

INTRODUCTION

Flavonoids are secondary metabolites produced via the phenylpropanoid pathway (1) and serve different functions such as protection from UV irradiation (2), plant-microbe interaction (3), regulation of levels of reactive oxygen species (4), inhibition of cell cycle, and control of cell growth (5,6). In addition, they are also thought to influence transcriptional and signaling processes (7-9). A major role of flavonols, a subgroup of flavonoids, appears to be the modification of auxin-related processes due to their property of modulating auxin transport (10-14). In some tissues, auxin is transported from cell to cell in a polarized fashion, operated mainly via auxin transporters of the ABCB, AUX1/LAX, and PIN families (15,16). Flavonoids bind to and inhibit the auxin transport proteins ABCB1 and ABCB19 (17) and interfere with the interaction of these two proteins with the immunophilin-like protein, FKBP42/TWISTED DWARF1 (18). Recent data show that the PINOID kinase, which influences polar localization of PIN proteins (19) and ABCB transport activity, is a likely target of flavonol action (20). The accumulation of flavonols is altered in the pin2 mutant, the agravitropic phenotype of which can be partially complemented by exogenous flavonols due to their ability to modify expression of PIN genes (21-23). Hence, flavonols and auxin are able to mutually influence each other, confirming a functional interdependence in their biological activity.

Flavonoid biosynthesis is well characterized and a number of mutants affected in the enzymes committed to the different steps have been identified in Arabidopsis thaliana (1) (Fig. 1). These lines frequently show a pale-yellow seed coat due to the absence of proanthocyanidins and were thus named transparent testa (tt) mutants (24). Flavonols are produced from dihydroflavonols via the activity of the FLAVONOL SYNTHASE, and thus represent a side branch of the flavonoid biosynthesis pathway. In Arabidopsis, the flavonols kaempferol, quercetin, and isorhamnetin are glycosylated by one or several sugars, in Arabidopsis mainly Glc, Rha, and rarely Ara, at the C3 and C7 position of the flavonol backbone (25,26) through the function of UDP-dependent glycosyltransferases (UGTs), some of which have been identified (8,26-29). The biological relevance of flavonol glycosylation remains controversial. In vitro experiments show activity of flavonol aglycones, suggesting that these are the compounds active in modulating polar auxin transport (10,17,18,20). However, the identification of mutant phenotypes induced by changes in the flavonol glycosylation profile suggests function for at least some flavonol glycosides (30,31).

Of the major auxin (indole-3-acetic acid; IAA) produced by plants, only a minor fraction of around 1% exists in this active form. Auxin can be conjugated to amino acids and/or sugars (mainly Glc in Arabidopsis) for storage in an inactive form or as an initial step for degradation (32,33). Indole-3-acetyl-alanine (IAA-Ala) and indole-3-acetyl-leucine (IAA-Leu) are readily hydrolysable resulting in active free IAA, whereas indole-3-acetyl-aspartate (IAA-Asp) and indole-3-acetyl-glutamate (IAA-Glu) remain largely stable, suggesting that the dynamics of the reversible (de-
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...conjugation are decisive in regulating the pool of active auxin. Auxin homeostasis can be affected by mutations in loci involved in auxin (de-)conjugation, and multiple mutations in these loci can induce auxin-related phenotypes ([34] and references therein). In Arabidopsis, the most abundant degradation products of auxin are produced via oxidation, resulting predominantly in oxIAA (2-oxindole-3-acetic acid) and oxIAA-Glc (2-oxindole-3-acetyl-glucose, glucosyl-ester of oxIAA) ([35], [36]). This oxidation contributes to the formation of auxin gradients ([37]) that are crucial for auxin-driven organ development ([38]-[41]). oxIAA levels are increased in the flavonoid-deficient tt4 mutant but decreased in the flavonol over-accumulator tt3 ([42]). This correlation suggests that the anti-oxidant activity of flavonols ([4]) influences auxin degradation.

The roll-2 (repressor of lrx1) mutant is affected in one of three RHM genes, RHM1, encoding RHAMNOSE SYNTHASE which converts UDP-Glc to UDP-Rha ([43]). The roll-2 mutant was identified in a screen for alterations in cell wall structures and shows modifications in the Rha-rich cell wall component pectin ([43]). In addition, roll-2 also shows changes in the flavonol glycosylation profile, mainly a reduction in the degree of rhamnosylation, while the overall quantity of flavonols is not affected ([30], [44]). This confirms the importance of RHM1, which is co-expressed with genes important for flavonol biosynthesis ([26]), for flavonol glycosylation. roll-2 seedlings are characterized by shorter roots and root hairs than in the wild type. The roll-2 seedling shoot develops deformed trichomes, and hypostatic cotyledons with brick-like shaped pavement cells, while the wild type develops regular trichomes and epinastic cotyledons with puzzle-shaped jigsaw-like pavement cells ([30], [43]). There is no obvious growth defect detectable in roll-2 adult plants, presumably due to the functional redundancy among the three RHM genes ([45]).

The short root phenotype of the roll-2 mutant is most likely induced by the changes in pectin structures ([43]). By contrast, the aberrant shoot phenotype of the roll-2 mutant is connected to the altered flavonol composition. Mutations affecting steps in flavonoid biosynthesis (Fig. 1) upstream of flavonols such as tt4 or tt6, mutations in the FLAVONOL SYNTHASE (FLS1), or in a positive regulator of flavonol biosynthesis encoded by MYB111 all suppress roll-2, resulting in wild type-like shoot development while root development is comparable to the roll-2 single mutant ([30], [44]). A mutation in TT7, blocking quercetin accumulation, or any step further downstream in the pathway has no effect on the roll-2 shoot phenotype ([30], [44]). Together, these findings suggest that the roll-2 phenotype is induced by the accumulation of flavonol glycosides that interfere with proper plant development and that kaempferols are sufficient to induce this defect. Yin et al. ([31]) have shown that the dwarf growth phenotype of the flavonoid 3-O-glucosyltransferase mutant ugt78d2 correlates with the overaccumulation of 3-O-7-O-rhamnosylated kaempferol (K-R-3-R-7) and interfering with K-R-3-R-7 biosynthesis suppresses the growth defect of the ugt78d2 mutant. By contrast, the flavonol species inducing the roll-2 phenotype is most likely not K-R-3-R-7, since this compound is present in lower amounts than in the wild type. Thus, it is likely that several flavonol glycosides can have an effect on plant development. Both roll-2 and ugt78d2 show changes in auxin concentration or transport activity. For roll-2, it was shown that auxin transport is reverted to wild-type levels when blocking flavonol accumulation ([44]), thus indicating that flavonols do interfere with auxin distribution.

Aiming at the identification of flavonol glycosides that induce the roll-2 phenotype, EMS-induced suppressor mutants of roll-2 were selected for specific changes in flavonol glycosylation. Several alleles of the 7-O-rhamnosyltransferase locus UGT89C1 were identified. The auxin transport activity in roll-2 is not changed by a ugt89c1 mutation, but the levels of auxin conjugates and catabolites are strongly increased in the ugt89c1 mutant background. This indicates that flavonols affect not only auxin transport but also auxin turnover, and in this way modify auxin homeostasis.

**EXPERIMENTAL PROCEDURES**

*Plant material, growth conditions, EMS mutagenesis and mutant screen*

All lines described in this study are in the Col-0 genetic background. The roll-2 allele and fls1-3 allele used in this study are described elsewhere ([43], [44]). For all analyses described, the ugt89c1-3
nonsense allele was used. Seeds were surface sterilized with 1% sodium hypochlorite, 0.03% Triton X-100, plated on half-strength MS medium containing 0.6% phytagel, 2% sucrose, 100 µg/mL myo-inositol, stratified for 4 d at 4°C and grown in a vertical orientation in a 16 h light, 8 h dark cycle at 22°C. For propagation and crossings, 10 d-old plants were transferred to soil and grown in a 16 h light, 8 h dark cycle at 22°C and irradiated with white light (Biolux, Osram). The EMS screen was performed as described (44) and 75’000 M2 seedlings were screened for a suppressed roll-2 mutant phenotype. All ugt89c1 alleles were backcrossed at least three times to Col-0 and roll-2 prior to analysis. Plant transformation was done as described (43) and transgenic plants were selected with BASTA (10 µg/mL).

DNA constructs, plant transformation and molecular markers

For the UGT89C1 complementation construct, the UGT89C1 genomic clone was PCR-amplified with the primers F7RT_PC 5’-GGCCGCGCAGACTACAGTTTGCCCTAACCAG-3’ and F7RT_R3C 5’-TGAACCCGCTGTGATGATGATC-3’, encompassing 1.5 kb promoter, 1.3 kb CDS, and 0.35 kb terminator sequence. The resulting fragment (UGT89C1:UGT89C1) was cloned into pGEM-T easy (Promega) for sequencing. For the GFP fusion construct, a Bam HI site was introduced into UGT89C1:UGT89C1 clone by PCR 3’ of the ATG (N-terminal fusion). PCR was performed with the primers F7RT_R4_NGFP 5’-GGATCCCCATGATGTTTTTTTCTTTC-3’ and F7RT_F6_NGFP 5’-GGATCCACACAAACAAACAAACAGGAACGC-3’. A previously produced GFP construct flanked by Bam HI sites (46) was cloned into this Bam HI site, resulting in UGT89C1:GFP-UGT89C1. This construct was cloned into the binary vector pBART (47) by Not I.

The molecular marker for the roll-1-2 and fls1-3 mutations are described elsewhere (43,44). For detection of ugt89c1-3, a PCR was performed using the primers F7RT_F4 5’-TGATGCTTTTCTATTAAGTCCAT-3’ and F7RT_R5_CGFP 5’-GGATCCAAACACATCTCTGCAACGAG-3’.

The PCR product was cut by Apa I (cuts wild type) and run on a 1.5% agarose gel. For detection of ugt89c1-11, PCR was performed using the primers F7RT_F4 5’-TGATGCTTTTCTATTAAGTCCAT-3’ and F7RT_R5_CGFP 5’-GGATCCAAACACATCTCTGCAACGAG-3’. PCR was performed at 55°C annealing temperature, 1.5 mm MgCl2, 1 min elongation time and with 35 cycles. The PCR product was cut by Bgl II (cuts mutant) and run on a 1.5% agarose gel. All other ugt89c1 alleles were confirmed by sequencing.

Microscopic analysis

Phenotypic screening for and analysis of ugt89c1 roll-2 was performed using a binocular. GFP fluorescence was photographed using a Leica DM6000 stereomicroscope. Gel prints of epidermal cells were produced following an established protocol (48) and observed by differential interference contrast microscopy using a Leica DMR microscope.

Flavonol content analysis

The analysis of the flavonol accumulation profile was done as described (44). Seedlings were grown in a vertical orientation for 6 d as described. One hundred intact seedlings were cut in the hypocotyl region, and roots and shoots were pooled separately, frozen in liquid nitrogen, and lyophilized to determine the dry weight. The dried material was incubated in 500 µL of 80% methanol overnight at 4°C and subsequently extracted by vigorous vortexing. After pelleting the cell debris by centrifugation, the supernatant was transferred to a fresh tube and evaporated in a Speed-Vac centrifuge, with the temperature being limited to a maximum of 42°C. After evaporation, the pellet was resuspended in 100 µL of fresh 80% methanol and used for analysis. HPLC-ESI-MS and MS/MS experiments were performed on an Acquity UPLC (Waters) connected to a Bruker maXis high-resolution quadrupole time-of-flight mass spectrometer (Bruker Daltonics). An Acquity BEH C18 HPLC column (1.7 µm, 2.1x100 mm fitted with a 2x2 mm guard column) was used with a gradient of solvent A (H2O, 0.1 % (v/v) HCOOH) and solvent B (CH3CN, 0.1 % (v/v) HCOOH), at 0.45
mL flow rate and with a linear gradient from 5 to 95 % B within 30 min.

The mass spectrometer was operated in the negative electrospray ionization mode. MS acquisitions were performed in the full scan mode in the mass range from m/z 50 to 2'000 at 25'000 resolution (full width at half maximum) and 2 scans per second. The MS instrument was optimized for maximum signal intensities of Quercitrine at m/z 447. Masses were calibrated with a 2 mM solution of sodium formate between m/z 180 and 1472 prior to analysis. The lock mass signal of hexakis [1H,1H,2H-perfluoroethoxy]phosphazine at m/z 556.00195 was further used as lock mass during the HPLC run. Flavonols were identified by the molecular mass determined by MS and by interpretation of fragments obtained by MS/MS, by comparison of both fragmentation patterns and retention times with previous analyses (30), and the absence in flavonol-less rol1-2 fls1-3 mutants (44). Purification of each flavonol glycoside in sufficient quantities for producing standard curves was not possible. Therefore, the content of each individual flavonol was expressed as peak area of the corresponding high resolution extracted ion chromatogram. The total flavonol content was calculated by summing up all flavonol peak areas, divided by the weight of plant material used for extraction. Either the total or the individual flavonol contents were compared between different experiments.

**Auxin transport experiments and quantification of derivatives**

Arabidopsis mesophyll protoplasts were prepared as described (49) from rosette leaves of plants grown on soil under 100 µM m² sec⁻¹ white light, 8 h light, 16 h dark cycle at 22°C. Intact protoplasts were isolated as described (50) and loaded by incubation with 4-[^3]H-1-naphthalene acetic acid (25 Ci mmol⁻¹; American Radiolabeled Chemicals) on ice. External radioactivity was removed by separating protoplasts using a 50–30–5 % percoll gradient. Samples were incubated at 25°C and efflux halted by silicon oil centrifugation. Effluxed radioactivity was determined by scintillation counting of aqueous phases and is presented as the relative efflux of the initial efflux (efflux prior to incubation), which was set to zero. Efflux experiments were performed with three to five independent protoplast preparations with four replicas for each time point. Basipetal (shoot-ward) auxin transport was measured according to (51). Radioactive auxin ([^3]H-l-IAA) was applied to the root tips of 15 seedlings (n=4), 5-10 mm segments were cut after 18h and counted. Segments in the wild type Col were set as 100% and others calculated accordingly.

Measuring the concentration of auxin, auxin conjugates, and auxin degradation products was done as follows: The determination of IAA and its metabolites included extraction and purification [according to (52)] followed by quantitation using LC-MS/MS [as described in (53)]. Briefly, a sample (about 100 mg fresh weight) was homogenized in liquid nitrogen. The homogenate was supplied with 500 µl of cold extraction buffer (methanol/water/formic acid, 15/10/5, v/v/v, -20°C) and with a mixture of stable-isotope-labelled internal standards: ^13^C₅-l-IAA (Cambridge Isotope Laboratories, MA, USA), ^2^H₀-[^15]N₁-IAA-Asp, ^2^H₀-[^15]N₁-IAA-Glu, ^2^H₂-OxIAA (OIChemml, Olomouc, Czech Republic): 10 pmol each. After incubation for 30min at -20°C, the extract was centrifuged at 28000 x g (centrifuge Eppendorf 5430 R, Hamburg, Germany) and pellet was re-extracted once. Pooled supernatants were evaporated in vacuum concentrator (Alpha RVC, Christ, Osterode am Harz, Germany) and then, a sample residue was dissolved into 0.1 M formic acid and applied to mixed mode reversed phase – cation exchange SPE column (Oasis-MCX, Waters, MA, USA). Auxin and its metabolites were eluted with methanol. Eluate was evaporated to dryness in vacuum concentrator and dissolved into 30 µl of 15 % acetonitrile. An aliquot was analysed on HPLC (Ultimate 3000, Dionex, CA, USA) coupled to hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, MA, USA) set in selected reaction monitoring mode. Quantification of hormones was done using isotope dilution method with multilevel calibration curves (r²>0.99). Data processing was carried out with Analyst 1.5 software (Applied Biosystems).

**Accession numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers:
RESULTS

Altered flavonol glycosylation suppresses the rol1-2 shoot growth defect

The rol1-2 shoot phenotype is characterized by hyponaustic cotyledons, brick-shaped adaxial pavement cells of cotyledons, and deformed trichomes (Fig. 2A). Mutations in a glycosyltransferase involved in producing the rol1-2 phenotype-inducing flavonol(s) would be expected to revert the rol1-2 phenotype and act as suppressor(s) of the rol1-2 phenotype. So, to identify possible rol1-2 phenotype-inducing flavonols, rol1-2 seeds were mutagenized with ethylmethane sulfonate. After mutagenesis, the seeds were propagated, and seedlings of the M2 generation were screened for a wild-type shoot phenotype that is characterized by epinastic cotyledons with puzzle-shaped pavement cells, and upright, branched trichomes (Fig. 2A). Identified lines were propagated and confirmed in the M3 generation. Positive lines were backcrossed with rol1-2 and only those with an F2 segregation pattern indicating recessive suppressor mutations were further analyzed. Several lines were identified that suppressed the rol1-2 phenotype, yet showed accumulation of flavonols to levels comparable to the wild type. Among these lines, only one group exhibited the lack of one specific type of flavonols, namely those rhamnosylated at the C7 position. Based on this finding, the previously identified flavonol 7-rhamnosyltransferase gene UGT89C1 (29) was identified as a potential candidate suppressor gene and sequencing of the UGT89C1 locus At1g06000 indeed led to the identification of a mutation in this gene in all these mutants. In total, 9 different ugt89c1-alleles were identified. Since two T-DNA insertion alleles of ugt89c1 were already described, the newly identified EMS alleles were numbered ugt89c1-3 to ugt89c1-11. In total, six missense and three nonsense mutations were identified (Table I). The ugt89c1-3 and ugt89c1-6 alleles are affected in the same codon, but the resulting nonsense mutations originate from two different mutation events. Hence, ugt89c1-3 and ugt89c1-6 lines represent two independent alleles with the same effect at the protein level. All mutant ugt89c1 alleles identified in this study lead to a comparable suppression of the rol1-2 shoot phenotype, resulting in seedlings with epinastic cotyledons, jigsaw puzzle-shaped pavement cells, and wild type-like trichomes (Fig. 2A).

ugt89c1 mutants specifically lack 7-rhamnosylated flavonols

A detailed analysis of the flavonol content of rol1-2 ugt89c1 mutants was performed by HPLC-MS. Individual flavonols were identified by comparing the data with previous ones where flavonols were identified by HPLC-MS/MS, correlation with reference compounds, and the absence in a flavonol-deficient flavonol synthase1 (fls1) mutant background (30, 44). For these and all subsequent analyses, the ugt89c1-3 nonsense allele was used. The quantification of flavonol accumulation using the sum of areas of all identified flavonol-peaks in HPLC-MS analyses confirmed that the overall flavonol content of the rol1-2 ugt89c1-3 line is comparable to the wild type or the rol1-2 mutant (Fig. 3A). As a negative control, the rol1-2 fls1-3 double mutant was included which shows a strongly reduced flavonol content due to a lesion in the FLAVONOL SYNTHASE 1 gene (44). The quantification of individual flavonol peaks confirmed the absence of all identifiable 7-O-rhamnosylated flavonols in rol1-2 ugt89c1 mutants. Other flavonol glycoside derivatives were still present, some at levels higher than in the wild type or rol1-2 single mutant, as shown for the kaempferol-3-O-glucoside (K-G-3) (Fig. 3B). Flavonols of root and shoot tissue are affected, confirming the ubiquitous importance of UGT89C1 for flavonol 7-O-rhamnosylation. These data indicate a lack of genetic redundancy in Arabidopsis with respect to 7-O-rhamnosylation of flavonols. Hence, UGT89C1 appears to be the only enzyme which is capable of catalyzing this reaction to a significant extent. The lack of 7-O-rhamnosylated flavonols in rol1-2 ugt89c1-3 suggests an important role of these compounds in the induction of the rol1-2 shoot phenotype.
Different protein domains are affected in the ugt89c1 alleles

Analysis of the UGT89C1 protein sequence was chosen as a strategy to elucidate the effect of the different mutations on the secondary and tertiary protein structure. UGT89C1 was compared to the flavonol-specific UGTs VvGT1 from Vitis vinifera and Mt71G1 of Medicago truncatula whose secondary and tertiary structures are known (54,55). VvGT1 and Mt71G1 accept flavonol substrates and therefore were selected for sequence and structural comparison. Previous analysis of four plant UDP-dependent glycosyltransferases revealed a low overall conservation on the protein sequence level, but a high conservation on the level of secondary and tertiary protein structure (56). The alignment (Fig. 4) shows only one short region of 45 amino acids, the PSPG domain that is relatively well conserved and important for binding of the UDP-sugar substrate. All three nonsense alleles and the missense allele ugt89c1-9, introducing an Asp356Asn substitution, affect residues in this PSPG domain. Mutational analysis has shown that the Asp residue affected in ugt89c1-9 is essential for function of VvGT1 (55). Of the remaining 5 alleles, four alter the N1- or C1-loops (ugt89c1-5, -10 and ugt89c1-7, -11, respectively) that are involved in forming the sugar donor binding pocket (56).

Altogether, protein sequence analysis revealed that a vast majority of mutations that revert the rol1-2 phenotype are directly related to sugar donor binding on the UGT89C1 enzyme.

UGT89C1 expression profile

The rol1-2 cell growth phenotype in cotyledons is characterized by brick-shaped pavement cells on the adaxial side. Previous studies revealed that the flavonol synthase FLS1 is expressed asymmetrically on the adaxial side (44), providing an explanation for the limitation of the flavonol-related cell growth defect to this side of the cotyledons. To investigate the localization of UGT89C1, a GFP-UGT89C1 construct under the control of the UGT89C1 promoter was produced and transformed into Arabidopsis. Complementation of the rol1-2 ugt89c1-3 mutant was used to assess the impact of GFP on UGT89C1 protein activity. In the T2 generation, transgenic plants developed the typical rol1-2 shoot growth phenotype (Fig. 2B), confirming complementation and thus activity of the GFP-UGT89C1 fusion protein. To assess protein localization, the UGT89C1:GFP-UGT89C1 construct was transformed into wild-type Arabidopsis and GFP fluorescence was analyzed in seedlings of the T2 generation of several independent transgenic lines. GFP fluorescence could be detected on the adaxial side of cotyledons and emerging leaves (Fig. 5A, C) comparable to the previously reported pattern observed in FLS1: GFP-FLS1 (Fig. 5B) and RHMI: RHMI-GFP (RHMI is identical to ROL1) transgenic lines (44). Trichomes also showed a strong GFP fluorescence (Fig. 5G). In roots, GFP fluorescence was predominant in columella cells and in the late elongation/early differentiation zone, correlating with zones of high auxin concentrations. Subcellularly, GFP-UGT89C1 localizes to the cytoplasm and the nucleus (Fig. 5D-F). The expression pattern of GFP-UGT89C1 is similar to FLS1 (44) confirming the previous finding that UGT89C1 is coexpressed with other genes coding for enzymes of the flavonoid biosynthesis pathway (29).

Auxin distribution but not auxin transport is modified by the ugt89c1 mutations

Flavonols have been shown to modify auxin-related processes (13,14). rol1-2 mutant seedlings indeed show an altered auxin distribution and transport activity, as determined by auxin concentration measurements and transport assays (30,44). To determine whether ugt89c1 functions as a suppressor of rol1-2 by influencing auxin distribution, auxin export was measured in the wild type, rol1-2, and rol1-2 ugt89c1 mutant lines. Due to the strong growth defect of rol1-2 mutant seedlings which might interfere with interpretation of auxin transport in a whole-seedling assay, rosette leaf mesophyll protoplasts were used following an established protocol (44). In a first step, flavonols were extracted from wild-type, rol1-2, and rol1-2 ugt89c1 protoplasts to confirm that the mutations have an effect on flavonol accumulation in this cell type. Comparable to flavonols of seedlings, flavonols of rol1-2 protoplasts revealed a glycosylation profile that is distinct from wild-type protoplasts in that they accumulate less rhamnosylated and more glucosylated flavonols [Fig. 6A; (30)]. rol1-2 ugt89c1 protoplasts did not accumulate 7-O-rhamnosylated flavonols. Hence,
protoplasts of the different genotypes showed the expected flavonol glycosylation profile, making them suitable to investigate the effect of the two mutations on auxin transport. Auxin export rates were determined in protoplasts using the synthetic auxin, 1-NA, and revealed that the rol1-2 mutant shows a modified transport efficiency compared to the wild type. rol1-2 ugt89c1-3 double mutant protoplasts, however, show a transport rate that is not different from the one of rol1-2 (Fig. 6B). Hence, mutations in ugt89c1 suppress the rol1-2 phenotype by an auxin transport-independent manner. This is particularly interesting since the flavonol-less rol1-2 fls1 mutant suppresses rol1-2 by changing auxin transport back to wild-type levels (44). Hence, the mode of action of fls1 and ugt89c1 is different. In order to sustain cellular auxin transport data and to back up measurements performed with synthetic auxin 1-NA, we measured basipetal (shoot-ward) IAA transport in roots. Comparable to 1-NAA transport in protoplasts, root basipetal transport of IAA revealed to be changed in rol1-2 compared to the wild type. The rol1-2 ugt89c1 double mutant shows transport activity similar to rol1-2, again indicating that ugt89c1 does not modify the rol1-2 induced alteration in auxin transport (Fig. 6C). To investigate whether ugt89c1 changes auxin levels in a different way, concentrations of free auxin and auxin-related metabolites were determined in wild type, rol1-2, and rol1-2 ugt89c1 mutant seedlings. Here, two additional lines were included: the ugt89c1 single mutant to confirm effects found for rol1-2 ugt89c1; and the rol1-2 fls1-3 double mutant to obtain indications whether ugt89c1 and fls1-3 indeed suppress rol1-2 by different means. The levels of the prevailing auxin of Arabidopsis, IAA, was subject to variation between several biologically independent experiments. As a consequence, the increase in auxin concentration in rol1-2 compared to the wild type was not as pronounced as previously reported (30) and did not fulfill the criteria of statistical significance (t-test, P<0.05). The reason for the observed variation is not clear at this point. Quantification of the auxin precursor indole-3-acetonitrile (IAN) and auxin conjugates and degradation products, on the other hand, gave very consistent results: ugt89c1 shows increased accumulation of IAN as well as of auxin conjugates and degradation intermediates such as IAA-Glc or oxIAA-Glc (Fig. 7). This effect of ugt89c1 is not only observed in the context of the rol1-2 mutation that causes aberrant accumulation of flavonols, but also in the ugt89c1 single mutant compared to the wild type, suggesting that the accumulation of auxin precursor and derivatives is genuinely affected by flavonol glycosides.

**DISCUSSION**

Flavonoids are a group of phenylpropanoic secondary metabolites known to influence cellular processes. They are of interest as nutrition additives due to their health-promoting effect (57). Yet, the knowledge on the effects of flavonoids on cellular processes in either animals or plants is still limited. The Arabidopsis rol1-2 shoot phenotype, characterized by defects in cell growth and development, is induced by flavonols, a subgroup of flavonoids, (30,44) and thus represents a model to characterize the active flavonol(s) and their mode of action.

**Flavonol glycosides influence plant development**

The study presented here identifies 7-O-rhamnosylated flavonols as being important for inducing the growth defects in rol1-2. The enzyme UGT89C1, conjugating rhamnose onto flavonols (29), is encoded by a single gene in Arabidopsis and mutations in UGT89C1 were identified as suppressors of the rol1-2 phenotype. In seedling shoots, UGT89C1 expression is limited to the adaxial side of cotyledons, emerging leaves, and trichomes. The same protein accumulation pattern has been found for ROL1/RHM1 and FLS1 (44), which explains the asymmetry in the flavonol-induced growth phenotype that is limited in cotyledons to the adaxial side. The colocalization is also in line with previous findings that genes involved in flavonoid biosynthesis (including FLS1, RHM1, and UGT89C1) are co-regulated (26). The data suggest that flavonol glycosides are the active flavonol species and that 7-O-rhamnosylated flavonols are involved in inducing the rol1-2 phenotype. Evidence for an active role of glycosylated flavonols in plant development was also provided by the analysis of the 3-O-glucosyltransferase mutant ugt78d2 which shows a dwarfed growth phenotype. This mutant contains increased levels of 3-O-7-O-rhamnosylated flavonols and mutating either the 3-O- or 7-O-rhamnosyltransferases ugt78d1 or ugt89c1.
respectively, suppresses the dwarfed phenotype of ugt78d2. Hence, 3-O-7-O-rhamnosylated flavonols appear to induce the growth defects developed by the ugt78d2 mutant (31). Both the ugt78d2 and rol1-2 mutant phenotypes are also found in combination with the tt7 mutation (30,31) that prevents synthesis of quercetin and causes over-accumulation of kaempferol (Fig. 1). Thus, accumulation of the flavonol kaempferol appears to be sufficient to interfere with proper plant development. Similarly, the tt7 single mutant was reported to have increased inhibition of auxin transport, again suggesting that kaempferol and its glycosylated forms are particularly active flavonols (13). The mechanism of flavonol-induced growth defects, however, is not the same in rol1-2 and ugt72d2. First, in contrast to ugt78d2, there is a reduction of 3-O-7-O-rhamnosylated flavonols in rol1-2 compared to the wild type (30). Second, while introducing the ugt89c1 mutation suppresses both ugt78d2 and rol1-2, the introduction of the 3-O-rhamnosyl transferase mutation ugt78d1 does only suppress ugt72d2 but not rol1-2 (30,31). This raises the question as to how suppression of the ugt78d2 and rol1-2 mutant phenotypes is induced. While the mechanism of suppression of the ugt78d2 mutant by ugt78d1 and ugt89c1 has not yet been investigated (31), the work presented here suggests that ugt89c1 suppresses the rol1-2 phenotype by modulating levels of the auxin precursor IAN and auxin metabolites rather than auxin transport.

Flavonols influence auxin homeostasis in several ways

The suppression of rol1-2 by tt4, tt6, and fls1 (30,44) fit the picture of flavonols being negative regulators of auxin transport (11). Indeed, interfering with flavonol biosynthesis in rol1-2 by an fls1 mutation reverts the modified auxin transport to wild-type levels and suppresses the rol1-2 phenotype. In contrast to the fls1 mutation, however, ugt89c1 does not seem to interfere with auxin transport. Compared to the wild type, the rol1-2 mutant shows an increase in auxin efflux in the protoplast assay but a decrease in auxin transport in the root basipetal transport. This discrepancy can be explained by the very different experimental setup in the assays. In the protoplast assay, primarily auxin efflux is measured from a non-polarized, cellular system after loading with radioactive auxin. In contrast, the root basipetal transport assay is performed on the entire root, so that the resulting value comes from the complex auxin transport machinery present in the root tissues. Yet and importantly, the absence of any effect of the additional ugt89c1 mutation is found in both systems. This unexpected finding can be explained by different flavonol species having different functions in terms of regulating auxin levels. While some interfere with auxin transport, others seem to influence auxin metabolism. Kaempferols are important for the development of the ugt78d2 and rol1-2 phenotypes or for pollen development in maize (6,30,31), whereas other reports point at quercetin as an important flavonol (58,59). In the rol1-2 fls1 double mutant, all flavonols are absent, resulting in wild type-like auxin transport, whereas a defect in 7-O-rhamnosylation only interferes with auxin metabolism but not transport. An indication for multiple modes of action of flavonols was obtained also from analyzing the rol1-2 shoot phenotype in more detail. In seedlings, a correlation between increased auxin levels and growth defects was observed. Yet, phenocopying the increased auxin levels in wild-type seedlings by exposing them to the auxin-efflux inhibitor 1-naphthylphthalamic acid (NPA) resulted in hyponastic cotyledons comparable to the rol1-2 shoot phenotype, whereas the defects in pavement cell shape and trichome development were not observed (30). Hence, negatively regulating auxin transport is likely not the only way by which flavonols in the rol1-2 mutant interfere with plant development. The results presented here suggest that UGT89C1 influences the activity of auxin biosynthesis and its conjugation/degradation. While oxIAA and oxIAA-Glc are irreversible degradation intermediates, IAA-amino acid derivatives can mostly be converted back to active, free auxin (32-34,60). Interfering with the activity of amidohydrolases that convert IAA-amino acid conjugates to free IAA affects free auxin levels and induces auxin-related phenotypes (61,62). A mutation in ugt89c1 causes an increase in the level of auxin precursor and auxin metabolites, the only exception being oxIAA accumulation in rol1-2 ugt89c1 that is comparable to rol1-2. Both low-abundant (such as IAA-Glu or IAA-Glc) as well as highly abundant (such as the auxin precursor IAN or the auxin degradation product oxIAA-Glc) auxin derivatives are affected to a similar extent. However, the low-abundant
IAA-derivatives are reversible conjugates that contribute to the pool of all IAA metabolites only very little (<1%). The accumulation of the precursor IAN in the ugt89c1 and ugt89c1 rol1-2 mutants suggests that conversion of this molecule to free IAA is inhibited. On the other hand, according to the levels of oxIAA-Glc, irreversible oxidative degradation is increased in those mutants. Thus, UGT89C1 seems to influence the production as well as degradation of auxin. Considering the large amount of the precursor IAN in the entire pool of auxin-related metabolites, the regulation of the last step of auxin biosynthesis appears to be a main target process of the activity of UGT89C1. At this point, it is not clear why these changes in the pool of auxin and its derivatives are not reflected in a clear change in the level of free auxin. Several repetitions of auxin measurements gave somewhat varying results with an increase in roll-2 compared to the wild type while roll-2 ugt89c1 reduced auxin levels again. Yet, the differences were not several-fold, did not fulfill the criteria of statistical significance, and therefore cannot be considered in the interpretation. Such variations may reflect fine-tuning of levels of free auxin as the only auxin-active form in relation to subtle variations in developmental stages of plant material used to analyze auxin content in biologically independent samples. It can be assumed that the level of free auxin is under strict control and may be modified only temporarily and locally by control mechanisms that are functional in the absence of UGT89C1. Blocking flavonol biosynthesis in the roll-2 fls1-3 mutant has comparatively little effect on the auxin derivatives. In three out of five auxin-related compounds, the changes induced by fls1-3 are opposite of those induced by ugt89c1, i.e. oxIAA-Glc is reduced in roll-2 fls1-3 compared to roll-2, but increased in roll-2 ugt89c1. This data indicates again that changes in the flavonol-glycosylation pattern do not have the same effect on auxin levels as blocking altogether flavonol synthesis. Presumably, the balance between different glycosylated forms of flavonols including 7-O-rhamnosylated flavonols is critical for auxin homeostasis and, thus, for proper plant development. The mechanism by which flavonols influence auxin conjugation and catabolism remains to be uncovered. The level of oxIAA has been shown to be increased in the flavonol-deficient tt4 mutant and reduced in the flavonol over-accumulator tt3, an observation that is attributed to the ROS-scavenging activity of flavonols (9,42). However, mutations in ugt89c1 do not affect the overall accumulation of flavonols. This suggests that different flavonol glycosides might have different ROS scavenging activities, possibly because of distinct subcellular localization. Alternatively, ugt89c1 mutations might not influence auxin oxidation via modulated ROS-scavenging activity. It has been shown that changing flavonol glycosylation affects gene expression (8) and thereby, genes involved in auxin metabolism might also be affected. Formally, it cannot be excluded that UGT89C1 has another biochemical activity in addition to its function as a rhamnosyltransferase. Yet, UGT89C1 appears to recruit specifically Rha as the substrate and flavonols as acceptor for the glycosyltransferase reaction and its gene expression is tightly linked with other genes involved in flavonol biosynthesis (26,29) making such a scenario less likely.

Together, the findings of this study reveal the effect of flavonol glycosides on auxin metabolic turnover. In addition to the known effect of flavonols on auxin transport, this represents a new mechanism for flavonols to influence auxin homeostasis.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

BMK, SE, RB, PD, and MG performed experiments; LB, EZ, and CR designed experiments; CR designed the project and wrote the paper.
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**FOOTNOTES**

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5 The abbreviations used are: K-G-3, kaempferol-3-O-glucoside; K-R-7, kaempferol-7-O-rhamnoside; K-R-3-R-7, kaempferol-3-O-7-O-rhamnoside; K-G-3-R-7, kaempferol-3-O-glucoside-7-O-rhamnoside; K-neoh-3-R-7, kaempferol-3-O-neohesperidoside-7-O-rhamnoside; Q-R-3-R-7, quercetin-3-O-7-O-rhamnoside; I-G-3-R-7, isorhamnetin-3-O-glucoside-7-O-rhamnoside; G, glucose; R, rhamnose; IAA, indole-3-acetic acid; IAA-Glc, IAA-glucose; IAA-Asp, IAA-aspartate; IAA-Glu, IAA-glutamate; oxIAA 2-oxindole-3-acetic acid; oxIAA-Glc, oxIAA-glucose; IAN, indole-3-acetonitrile.
7-Rhamnosylated flavonols modulate auxin homeostasis

FIGURE LEGENDS

FIGURE 1. Overview on flavonoid biosynthesis. The phenylpropanoid pathway leads to the synthesis of flavonoids. These encompass a number of different compounds, not all of which are indicated in this scheme. Enzymes leading to the synthesis of flavonols (kaempferol, quercetin, isorhamnetin) are indicated. TT (TRANSPARENT TESTA) proteins were identified based on the pale brown phenotype of seeds of corresponding mutant lines. FLS1: FLAVONOL SYNTHASE 1, the predominant FLS protein in Arabidopsis. UGTs: UDP-dependent glycosyltransferases needed for the glycosylation of flavonols.

FIGURE 2. The roll-2 mutant shoot cell growth phenotype is suppressed by mutations in UGT89C1. (A) The roll-2 mutant shows hyponastic bending of cotyledons, distorted trichomes, and brick-like shaped adaxial pavement cells whereas the wild type (Col) shows epinastic bending of cotyledons, normal shaped trichomes, and a jigsaw puzzle-like structure of adaxial pavement cells. All ugt89c1 alleles identified in this study suppress the roll-2 shoot phenotype, i.e. roll-2 ugt89c1 double mutants develop comparable to the wild type. Pictures of the roll-2 ugt89c1-3 line are shown which are representative for all other ugt89c1 lines. (B) The UGT89C1: GFP-UGT89C1 construct complements the ugt89c1 mutation, hence encodes a functional UGT89C1 protein, resulting in a roll-2 mutant phenotype. Bars = 1mm for shoot (A upper panels and B); 0.2 mm for trichomes (A middle panels); 40 µm for pavement cells (A lower panels).

FIGURE 3. Content of total flavonols and of 7-O-rhamnosylated flavonols. (A) Total flavonol accumulation is comparable in the wild type, roll-2, and roll-2 ugt89c1-3. Values of roll-2 fis1-3, which fail to accumulate flavonols, serve as negative control. (B) The roll-2 ugt89c1-3 line shows a loss of 7-rhamnosylated flavonols, whereas e.g. 3-O-glucosylated flavonols are still present and accumulate both in the shoot (upper graph) and the root (lower graph). Only a sub-selection of flavonols are shown. For quantification, the area under each peak of the HPLC elution profile was used as an arbitrary unit. K = kaempferol; Q = quercetin; I = isorhamnetin; G = glucose; R = rhamnose.

FIGURE 4. Structural alignment of primary sequences of different UGTs with UGT89C1. The crystal structure of VvGT1 (Vitis vinifera) and Mt71G1 (Medicago truncatula) was already solved and different domains are marked with colors. α-helices are highlighted in blue, β-strands in light green. The structurally important loops are labeled. Nonsense mutations identified in this screen are marked in beige, missense mutations in dark green. The dashed line indicates the PSPG motif important for UDP-sugar donor specificity. Residues reported to be involved in acceptor pocket formation are highlighted in grey. Asterisks mark identity, colons high similarities, single dots lower similarities, and blank positions no similarities between the amino acids in the compared sequences. Adopted from (56).

FIGURE 5. Localization of GFP-UGT89C1. In cotyledon of 6-d-old seedlings, fluorescence induced by UGT89C1: GFP-UGT89C1 (A) is restricted to the adaxial side, very similar to seedlings containing FLS1:FLS1-GFP (B). (C) Bright field image of a cotyledon corresponding to those shown in (A) and (B). In roots, fluorescence of GFP-UGT89C1 was observed in the cortex and vascular cell layers of the differentiation zone. On the subcellular level, a fraction of the fusion protein localizes to the nucleus (arrow) (D). In root tips, fluorescence occurred around the meristematic and later elongation zone (E). Detailed analysis of root tips revealed GFP-UGT89C1 expression in columella and lateral root cap cells (F). GFP-UGT89C1 induced fluorescence in trichomes (G). Bars = 1 mm (A – C); 15 µm (D and F); 30 µm (E); 100 µm (G).
FIGURE 6. Auxin transport in *roll*-2 plants is not affected by *ugt89c1*. (A) Flavonol content analysis of protoplasts revealed the same effects of *roll*-2 and *ugt89c1* mutations as in intact plants. The area under each peak of the HPLC elution profile was used to determine flavonol content. (B) Auxin (1-NAA) export from protoplasts is affected in *roll*-2 compared to the wild type (Col) but not altered by the *ugt89c1* mutation. (C) Compared to the wild type (Col), *roll*-2 and *roll*-2 *ugt89c1* show similar, reduced basipetal IAA transport in roots. Asterisks indicate statistically significant differences (t-test, P<0.05).

FIGURE 7. *ugt89c1* affects levels of auxin precursor and metabolites. Quantification of the auxin conjugates indole-3-acetyl-glucose (IAA-Glc) and indole-3-acetyl-glutamate (IAA-Glu), the auxin precursor indole-3-acetonitrile (IAN), and the degradation products 2-oxindole-3-acetic acid (oxIAA) and 2-oxindole-3-acetyl-glucose (oxIAA-Glc) is shown for wild type (Col), *roll*-2 and *ugt89c1* single mutants, and *roll*-2 *ugt89c1* and *roll*-2 *fls*-1-3 double mutants. Statistically significant differences (t-test) with P<0.05 and P<0.01 are indicated with one and two asterisks, respectively.

Table I: List of *ugt89c1* alleles identified in this screen.

| allele name | mutation       |
|-------------|----------------|
| *ugt89c1*-3 | Stop 314*      |
| *ugt89c1*-4 | Gly123Arg      |
| *ugt89c1*-5 | Ser19Phe       |
| *ugt89c1*-6 | Stop 314*      |
| *ugt89c1*-7 | Gly249Val      |
| *ugt89c1*-8 | Stop335        |
| *ugt89c1*-9 | Asp356Asn      |
| *ugt89c1*-10| Thr4Ile        |
| *ugt89c1*-11| Arg253Trp      |

* The mutations producing the stop codon are not identical.
Figure 1

3 Malonyl-CoA → Coumaroyl-CoA

TT4 → Tetrahydroxychalcone

TT5 → Naringenin

TT6 → Dihydrokaempferol

TT7 → Dihydroquercetin

TT3 → Cyanidin

Naringenin → Flavonols

Kaempferol → Kaempf’ glycosides

Quercetin → Querc’ glycosides

Isorhamnetin → Isorham’ glycosides

Anthocyanin derivatives
Figure 2

A

rol1-2  Col  rol1-2 ugt89c1

B

rol1-2  rol1-2 ugt89c1  rol1-2 ugt89c1

UGT89C1::GFP-UGT89C1
Figure 3

A

B

root total flavonoid content / mg DW

shoot flavonoid content / mg DW
Figure 6

A

B

C

flavonol content

K-G-3-R-7
K-R-3-R-7
K-R-3

rol1-2
rol1-2 ugt89c1

rol1-2
rol1-2 ugt89c1

0 2.5 5 7.5 10
[min]

NAA export

(% of initial export)

root basipetal transport
of IAA (% of Col)

Col
rol1-2
rol1-2 ugt89c1

*
Figure 7

**IAN**

![IAN Graph]

**IAA-Glu**

![IAA-Glu Graph]

**IAA-Glc**

![IAA-Glc Graph]

**oxIAA-Glc**

![oxIAA-Glc Graph]

**oxIAA**

![oxIAA Graph]