Kaempferol 3-O-rhamnoside-7-O-rhamnoside is an endogenous flavonol inhibitor of polar auxin transport in Arabidopsis shoots

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Summary

- Polar auxin transport (PAT) plays key roles in the regulation of plant growth and development. Flavonoids have been implicated in the inhibition of PAT. However, the active flavonoid derivative(s) involved in this process in vivo has not yet been identified. Here, we provide evidence that a specific flavonol bis-glycoside is correlated with shorter plant stature and reduced PAT.
- Specific flavonoid-biosynthetic or flavonoid-glycosylating steps were genetically blocked in Arabidopsis thaliana. The differential flavonol patterns established were analyzed by high-performance liquid chromatography (HPLC) and related to altered plant stature. PAT was monitored in stem segments using a radioactive [3H]-indole-3-acetic acid tracer.
- The flavonoid 3-O-glucosyltransferase mutant ugt78d2 exhibited a dwarf stature in addition to its altered flavonol glycoside pattern. This was accompanied by reduced PAT in ugt78d2 shoots. The ugt78d2-dependent growth defects were flavonoid dependent, as they were rescued by genetic blocking of flavonoid biosynthesis. Phenotypic and metabolic analyses of a series of mutants defective at various steps of flavonoid formation narrowed down the potentially active moiety to kaempferol 3-O-rhamnoside-7-O-rhamnoside. Moreover, the level of this compound was negatively correlated with basipetal auxin transport.
- These results indicate that kaempferol 3-O-rhamnoside-7-O-rhamnoside acts as an endogenous PAT inhibitor in Arabidopsis shoots.

Introduction

The phytohormone auxin, represented predominantly by indole-3-acetic acid (IAA), plays a crucial role in plant growth and development. Auxin needs to be transported from the sites of synthesis, mainly in the apices and young leaves, to the distal part of the plant to exert its function (Berleth et al., 2007). Auxin transporters, including ABCB proteins, AUX1/LAX family members and PIN proteins (Noh et al., 2001; Friml, 2003; Petrasek et al., 2006; Yang et al., 2006; Zažímalová et al., 2010; Peer et al., 2011), are responsible for the auxin fluxes and patterning in plants (Friml et al., 2003). Flavonoids, phenylpropanoid secondary metabolites, have been implicated in the blocking of auxin transport (Peer & Murphy, 2007). The supply of flavonoids to the shoot, which is implicated in basipetal auxin movement, is rather simple in inflorescence stems (Yonekura-Sakakibara et al., 2010b). Thus, the inflorescence stem, which is implicated in basipetal auxin movement, is the optimal organ for searching for the flavonol derivative(s) active in auxin transport modulation.

By contrast, PAT was reduced in the flavonol over-production mutant tt3 defective in dihydroflavonol reductase (Fig. 1), consistent with an inhibitory role of flavonols in PAT (Peer et al., 2004). Despite these and other substantial pieces of evidence supporting a role of flavonoids in the modulation of auxin transport (Kuhn et al., 2011; Lewis et al., 2011; Grunewald et al., 2012), neither specific flavonol aglycones nor their conjugates active in this process in vivo have been identified so far.

The difficulty in relating specific flavonoids to auxin transport modulation is, in part, a result of the complex flavonol modification in planta. Flavonol aglycones are intensively modified by UDP-dependent glycosyltransferases (UGTs), which include UGT78D1, UGT78D2, UGT78D3, UGT73C6 and UGT89C1 in the model plant Arabidopsis thaliana (Fig. 1) (Jones et al., 2003; Tohge et al., 2005; Yonekura-Sakakibara et al., 2007, 2008). The glycosides are distributed in an organ-specific manner. In contrast with the complex flavonol profile in flowers, it is rather simple in inflorescence stems (Yonekura-Sakakibara et al., 2008; Stracke et al., 2010b). Thus, the inflorescence stem, which is implicated in basipetal auxin movement, is the optimal organ for searching for the flavonol derivative(s) active in auxin transport modulation.
Here, we show that the loss of the flavonoid 3-O-glucosyltransferase UGT78D2 resulted in an altered flavonol glycoside pattern and reduced PAT in shoots, which was accompanied by a reduced plant height and increased branching. Blocking of flavonoid biosynthesis and/or glycosylation at specific steps clearly related the enhanced accumulation of kaempferol 3-O-rhamnoside-7-O-rhamnoside (k1) to the growth defects of ugt78d2. Through analyses of auxin transport in several genotypes, which contained different levels of k1, an inverse correlation between basipetal auxin transport and k1 level was identified. Therefore, we propose that k1 acts as an endogenous auxin transport inhibitor in Arabidopsis shoots.

Materials and Methods

Plant material and growth conditions

The ugt78d1 (SAIL_568_F08), ugt78d2 (SALK_049338), ugt89c1 (SALK_028793), tt4 (SALK_020583), tt7 (GK_349F05) and tt18 (SALK_028793) mutants are based on Arabidopsis thaliana (L.) Heynh., accession Columbia (Col-0). These mutant lines have been described in previous studies (Abrahams et al., 2003; Alonso et al., 2003; Jones et al., 2003; Rosso et al., 2003; Tohge et al., 2005; Yonekura-Sakakibara et al., 2007; Stracke et al., 2010b). The n3-1 (NASC_N84; Ler background) and f31 (Riken_PST16145; Nö background) mutants have different genetic backgrounds (Shirley et al., 1995; Ito et al., 2005).

tt4 ugt78d2, tt7 ugt78d2, ugt78d1 ugt78d2, ugt89c1 ugt78d2, tt18 ugt78d2 and fls1 ugt78d2 double mutants were generated by genetic crossing and confirmed by PCR genotyping. Plants were grown in a sun simulator chamber, which provides a photobiological environment very close to natural global solar radiation (Seckmeyer & Payer, 1993; Thiel et al., 1996). Plants were exposed to photosynthetically active radiation (PAR, 400–700 nm) of 200–230 μmol m⁻² s⁻¹ with a 16 h : 8 h, light : dark regime at a temperature of 22°C and a relative humidity of 70%. Similar phenotypes were observed when grown in a glasshouse with higher natural light intensities at temperatures of 20–25°C (European springtime).

A DR5::β-glucuronidase (DR5::GUS) line (Col background; Sabatini et al., 1999) was introgressed into ugt78d2; plants were grown on half-strength Murashige and Skoog (MS) medium agar plates supplemented with 1.5% sucrose for 9 d and stained for β-glucuronidase expression for 1 h at 37°C (Deruitre et al., 1999).
Genetic complementation of *ugt78d2*

A genomic fragment containing the *UGT78D2* gene was amplified from Col-0 genomic DNA using the primers 5′-GGGGAGAAAGTCTGCAGGCTCTGGTTTACAAAGCAAGTTCAG-3′ and 5′-GGACCTTTCTGACAAAGGCTGGGTAGTTTCTGAGCCGTGCAT-3′. The PCR product was cloned into vector pDONR221 using GATEWAY™ (Invitrogen, Karlsruhe, Germany) recombination, confirmed by DNA sequencing and further recombined into pBGW (Karimi et al., 2002). The resulting binary vector was used to transform *ugt78d2* knockout mutant plants by the floral dip method (Clough & Bent, 1998). Transgenic plants with a single insertion of the transgene were selected by segregation analysis for experiments.

Analyses of flavonols

For flavonol extraction, the lower 2.5-cm stem segment of 4-wk-old plants was harvested and immediately frozen in liquid nitrogen. The plant material was thoroughly ground in liquid nitrogen using a mortar and pestle, and further homogenized after the addition of 1 ml of methanol per 100 mg of fresh material in a Douncer on ice. The suspension was incubated for 1 h with moderate rotation at 4°C. The extracts were then clarified by centrifugation at 14,000 *g* for 10 min with a table-top centrifuge at 4°C. One-third volume of distilled water was added to the supernatant, vortexed and centrifuged as above. The cleared extract was analyzed using a reverse-phase high-performance liquid chromatography (HPLC) system (Gemini C18 Phenyl, 5 μm, 150 × 4.6 mm; Phenomenex, Aschaffenburg, Germany). Solvent A was 1% acetic acid and solvent B consisted of 89% methanol with 1% acetic acid. The elution gradient program followed a linear gradient from 20% solvent B to 100% solvent B (75 min) at room temperature. The quantification of k1 was referred to a kaempferol aglycone authentic standard (Carl Roth, Karlsruhe, Germany).

Inflorescence stem gravitropic response assay

Arabidopsis plants were grown on commercial soil substrate (Einheitserde; Einheitserde- und Humuswerke, Sinntal-Altengronau, Germany) for 4 wk in a phyto chamber (light intensity, 120 μmol m⁻² s⁻¹; 16 h:8 h, light:dark cycle; relative humidity, 60%; 23°C). Plants were adapted in darkness for 2 h before gravitropic stimulation. Gravitropic stimulation was begun by turning plants by 90° at 23°C in darkness. Except for the 5 min needed for the measurement of the bending angles at each time point, the whole gravitropic response assay was conducted in darkness.

Examination of parenchyma cell size of inflorescence stems

Seven-week-old inflorescence stems were fixed in ethanol : glycerol : water (36:1:10, v/v/v), hand sectioned along the longitudinal axis, stained with astral blue and safranin, and examined under a microscope in bright field mode. Cell length was measured using Cell^P Professional Imaging Software (Olympus Europe, Hamburg, Germany).

**PAT assay**

The auxin transport assays were performed as described previously with slight modifications (Okada et al., 1991). Segments (length, 2.5 cm) from the basal part of the primary inflorescence stem were inserted into 30 μl of 1 × MS medium (pH 5.5) with 10.5 μM [³H]-IAA (460 kBq ml⁻¹). Both basipetal and acropetal auxin transport were assayed. One end of the stem fragment (apical end for basipetal auxin transport and basal end for acropetal auxin transport) was dipped into the assay mixture. After 6 h of incubation at room temperature in the dark in a closed vial, a 5-mm segment was cut off from the non-submerged end and measured by scintillation counting. For the inhibition assay, 10 μM of *N*-1-naphthylphthalamic acid (NPA) was added. The measurements without NPA treatment were compared between each genotype by a paired *t*-test.

**IAA quantification**

Col-0 and *ugt78d2* plants were grown for 28 d before the inflorescence stems were harvested. The whole inflorescence stem was cut into three segments of equal length, namely the apex, middle and basal segment. The extraction and quantification of free IAA were carried out as described previously, with the endogenous IAA determined by isotope dilution followed by liquid chromatography-mass spectrometry (Dobrev *et al.*, 2005; Dobrev & Vankova, 2012).

**Results**

**Shoot architecture of *ugt78d2* plants is altered**

A loss-of-function mutant of the flavonoid 3-O-glucosyltransferase UGT78D2 showed a pronounced shoot phenotype (Fig. 2). *ugt78d2* mutant plants exhibited a significantly shorter stature soon after bolting (Fig. 2). At a later growth stage, the mutants developed more branches with an obvious loss of apical dominance (Supporting Information Table S1). To elucidate the mechanism underlying this dwarfism, the cellular architectures of inflorescence stems were examined. In *ugt78d2*, the parenchymal cell length was reduced significantly when compared with the wild-type. In contrast with stems, other organs were not obviously affected. The lengths of hypocotyls and siliques of *ugt78d2* were similar to those of the wild-type (Table S1). Young seedlings and root systems developed in the same way in both genotypes (Fig. S1) Transformation of *ugt78d2* with a genomic fragment covering the complete *UGT78D2* gene rescued plants from growth disturbances and restored the wild-type phenotype (Fig. S2). Thus, the lesion in *UGT78D2* was responsible for the observed shoot growth defects.
The *ugt78d2* growth phenotype is flavonol dependent

Flavonoids are probable candidates responsible for the *ugt78d2* phenotype, as UGT78D2 has been characterized as a flavonoid 3-O-glucosyltransferase and the *ugt78d2* mutant exhibits an altered flavonoid pattern (Lee *et al.*, 2005; Tohge *et al.*, 2005). Moreover, transformation of *ugt78d2* with the *UGT78D2* gene did not only re-establish the wild-type growth phenotype, but also restored its flavonol pattern (Fig. 3a). A flavonoid-deficient *tt4 ugt78d2* double mutant was generated to further check this effect. The growth defects of *ugt78d2* were indeed eliminated in *tt4 ugt78d2*, indicating that they were flavonoid dependent. Moreover, this observation in the flavonoid-deficient background indicated that the growth defects were not related to the repression of specific flavonol-3-O-glucoside derivatives (k2, k3, q2 and q3) in *ugt78d2* (Figs 2, 3a). In addition to flavonols, UGT78D2 also recognizes anthocyanins as substrates (Lee *et al.*, 2005; Tohge *et al.*, 2005). To test the possible involvement of anthocyanins in the development of the *ugt78d2* growth phenotype, an anthocyanin-deficient *tt18 ugt78d2* double mutant was generated. However, loss of the anthocyanin synthase in the *tt18 ugt78d2* line did not rescue the growth defects of *ugt78d2* (Figs 1, 2). The flavonol glycoside pattern of *tt18 ugt78d2* was similar to that of *ugt78d2* (Fig. 3a). By contrast, the introgression of *fs1* (accession Nö) into *ugt78d2*, which specifically blocks the biosynthesis of the flavonol moiety, restored wild-type-like growth (Fig. S3). The impact of flavonols was further supported by the slightly repressed growth of the *tt3* mutant in comparison with its genetic background Ler; the loss of dihydroflavonol reductase in the *tt3* mutant leads to enhanced k1 levels (Fig. S4; Peer *et al.*, 2001). Collectively, these observations indicated that the *ugt78d2* growth phenotype was flavonol dependent and that anthocyanins were not responsible for the *ugt78d2* growth defects.

The *ugt78d2* phenotype is correlated with kaempferol 3-O-rhamnoside-7-O-rhamnoside

To identify the flavonols responsible for the *ugt78d2* phenotype, a genetic approach using mutants defective in flavonol biosynthetic and/or conjugating steps was employed. In concert with published data (Yonekura-Sakakibara *et al.*, 2008; Stracke *et al.*, 2010b), six flavonol glycosides were detected in wild-type stems. Three kaempferol glycosides (k1–k3) were the most abundant...
flavonols, whereas three quercetin glycosides (q1–q3) were present at much lower levels (Fig. 3a). No flavonol aglycones were detected in the wild-type and ugt78d2, which is in accordance with previous analyses of the single 3-O-glycosylation mutants ugt78d1 and ugt78d2 (Tohge et al., 2007; Yin et al., 2012) (Fig. 1). Furthermore, even the double mutant ugt78d1 ugt78d2, which blocks the major enzymes performing the initial 3-O-glycosylation of the flavonol moiety, did not accumulate aglycones (Yin et al., 2012). In accordance with the loss of 3-O-glucosyltransferase activity in ugt78d2, the 3-O-glucoside derivatives (k2, k3, q2 and q3) were strongly reduced, whereas the 3-O-rhamnoside derivatives (k1 and q1) were elevated, when compared with the wild-type counterpart (Fig. 3a). Therefore, both k1 and q1 were positively correlated with the ugt78d2 growth defects.

A tt7 ugt78d2 double mutant was generated in order to further distinguish whether the increases in k1 and/or q1 levels were related to the ugt78d2 growth phenotype. The conversion of kaempferol to quercetin is blocked in tt7 (Fig. 1). As expected, tt7 ugt78d2 was devoid of q1 and accompanied by an even larger amount of k1 than in ugt78d2 (Fig. 3b). tt7 ugt78d2 was more severely dwarfed than ugt78d2, indicating that a further enhanced k1 level led to a more pronounced phenotype (Fig. 2). These observations suggested that the development of the ugt78d2 growth defects was independent of quercetin derivatives, and that k1 was sufficient to induce the ugt78d2 phenotype.

k1 contains 3-O-rhamnosyl and 7-O-rhamnosyl residues (Fig. 1b). To further substantiate that k1 was responsible for the observed ugt78d2 phenotype, additional mutants were employed to selectively inhibit the attachment of 3-O- or 7-O-rhamnosyl residues to the k1 backbone. The attachment of these residues to the k1 backbone is catalyzed by UGT78D1 and UGT89C1, respectively (Jones et al., 2003; Yonekura-Sakakibara et al.,

Fig. 3 Flavonol glycoside pattern in Arabidopsis thaliana mutants affecting flavonoid biosynthesis and conjugation. (a) Flavonols extracted from stems (lower region of 2.5 cm) of the wild-type and flavonoid metabolism-related mutants. The scale of all representative high-performance liquid chromatograms is identical. Peaks u1–u8 represent unknown flavonoids. SM, sinapoyl malate. (b) Quantification of k1 (Fig. 1b) in the wild-type and flavonoid metabolism-related mutants. The means of k1/C6 ± SD from four biological samples (a pool of three to five individual stem segments for one sample) are shown. k1 was quantified using kaempferol aglycone as a standard. Letters above the error bars represent a significant difference between genotypes by a paired t-test (P < 0.01).
Basipetal auxin transport is reduced in ugt78d2 plants

The dwarf phenotype of ugt78d2 resembled that of the abcb1 abcb19 double mutant, which was greatly impaired in auxin transport (Noh et al., 2001). As flavonols have been postulated to be endogenous auxin transport inhibitors, we reasoned that the increase in k1 suppressed auxin transport in inflorescence stems, resulting in the growth defects. Therefore, polar transport of [3H]IAA was measured in inflorescence stem segments. NPA-sensitive, basipetal auxin transport in ugt78d2 was reduced significantly to 45% of the wild-type level, whereas acropetal auxin transport remained unchanged (Figs 4a, S5). Importantly, auxin transport activity further decreased to 25% of the wild-type level in the tt7 ugt78d2 double mutant, which accumulated even greater amounts of k1 (Figs 3b, 4a). There was a dose-dependent, linear relationship between the k1 level and PAT, when comparing the wild-type, ugt78d2, tt7 and tt7 ugt78d2 (Fig. 4b). The regulation did not affect the transcript level of genes encoding major, putative auxin export proteins, as mRNA levels of PIN1, PIN3, PIN7, ABCB1 and ABCB19 were not significantly different in wild-type and ugt78d2 stems (Fig. S6).

The altered PAT also raised the question of whether there was a general difference in auxin responsiveness. However, several auxin-inducible marker genes were up-regulated by exogenous IAA application in the same manner in ugt78d2 and the wild-type (Fig. S7).

Free IAA level is reduced in ugt78d2 inflorescence stems

To examine whether the reduced auxin transport influenced the concentration of free IAA in ugt78d2 stems, the free IAA contents of three consecutive stem segments of ugt78d2 and wild-type plants were compared. The endogenous levels of free IAA in the apical inflorescence segments were not different from those of the wild-type (P > 0.05), whereas the middle and basal stem segments of ugt78d2 contained lower steady-state IAA levels than the wild-type counterparts (P < 0.05; Table 1). This suggested that the decreased IAA content in the lower parts of the inflorescence stem of ugt78d2 was caused by the reduced basipetal transport of auxin from sites of its biosynthesis in the apex.

Gravitropism of the inflorescence stem is delayed in ugt78d2 plants

The reduction of PAT in the inflorescence stems of ugt78d2 could also affect tropic responses. Therefore, gravitropism was analyzed in the mutant. Four-week-old plants were laid down horizontally in the dark to exert a gravistimulus, which would lead to an upward bending of the inflorescence stems. The bending angle of the inflorescence stem was monitored at different time points after the gravistimulus. ugt78d2 exhibited a strongly delayed gravitropic response, indicating that the repressed auxin transport was correlated with a reduced gravitropism in ugt78d2 inflorescence stems (Fig. 5).

Discussion

Over the past decade, strong evidence, both in vitro and in vivo, has accumulated implicating flavonols in plant growth regulation and auxin transport modulation (Jacobs & Rubery, 1988; Buer & Muday, 2004; Peer et al., 2004; Ringli et al., 2008; Santelia

Table 1 Free indole-3-acetic acid (IAA) quantification in Arabidopsis thaliana inflorescence stem segments

|                | Apical segment | Middle segment | Basal segment |
|----------------|---------------|---------------|--------------|
| Wild-type      | 207 ± 50      | 253 ± 23      | 247 ± 27     |
| ugt78d2        | 194 ± 17      | 118 ± 14*     | 142 ± 18*    |

Free IAA was measured in three consecutive stem segments (pmol g⁻¹ FW). The mean values ± SE of three independent biological samples (one sample is a pool of four to six individual stem segments) are displayed. Statistical analyses were performed by a paired t-test for uneven variance for each part of the stem between the two genotypes (*, P < 0.05).
et al., 2008; Kuhn et al., 2011; Lewis et al., 2011; Grunewald et al., 2012; Buer et al., 2013). However, the physiologically active flavonol derivative(s) could not be pinpointed unequivocally. In vivo feeding studies were hampered by the possible modification and metabolism of the exogenously added compounds. Similarly, genetic studies affecting either flavonol biosynthesis or flavonol conjugation did not generate effects that could be traced back to a single flavonol moiety (Buer & Muday, 2004; Peer et al., 2004; Ringli et al., 2008; Buer et al., 2013). Nevertheless, the genetic identification of the kaempferol derivative k1 as an endogenous inhibitor of PAT in this work is in agreement with previous studies. These have indicated activity associated with exogenously applied flavonols that could be metabolized to k1 (Jacobs & Rubery, 1988; Mathesius et al., 1998). Furthermore, a release from PAT suppression in flavonol-free tt4 was observed, whereas PAT is further repressed in tt3, which exhibits an enhanced flavonol content at the expense of anthocyanins, and, importantly, in tt7, which shows specifically increased kaempferol glycoside levels at the expense of quercetins (Buer & Muday, 2004; Peer et al., 2004). Furthermore, the formation of k1 is dependent on the 3-O-rhamnosyltransferase UGT78D1, which is relatively abundant at the shoot apex in comparison with other tissues and almost absent from roots in agreement with a low k1 level in roots (Jones et al., 2003) (Figs S1, S8). Thus, the expression pattern of UGT78D1 correlates with an in situ activity of k1 in modulating auxin transport and affecting the shoot phenotype.

Nevertheless, the identification of k1 as an active compound does not exclude an impact of other flavonol derivatives on auxin fluxes. Flavonol biosynthesis is under developmental and organ-specific regulation by MYB and WRKY transcription factors (Stracke et al., 2007, 2010b; Grunewald et al., 2012).

Accordingly, the active compounds could also be dependent on the developmental stage and/or on the organ. WRKY23 has been revealed to be part of a feedback loop of auxin to repress its own transport in roots, as it was induced by auxin and enhanced the biosynthesis of flavonols, in particular of quercetins. Thus, quercetins have been suggested to be active agents in roots (Grunewald et al., 2012; Buer et al., 2013). This finding was supported by the analysis of the quercetin-less tt7, which indicated that no kaempferol derivatives, but rather quercetins, were involved in the suppression of basipetal auxin flux in roots (Lewis et al., 2011; Grunewald et al., 2012). However, Buer et al. (2013) also found a repressed root PAT in both quercetin-accumulating tt3 and quercetin-deficient tt7. Nevertheless, these data and the very low k1 level in roots, as well as the lack of obvious ugt78d2-related phenotypes in hypocotyls and roots, including a similar auxin accumulation in mutant and wild-type root tips monitored by a DR5::β-glucuronidase reporter line, strongly support the notion that different flavonol compounds may affect PAT in root and shoot (Figs S1, S9; Table S1).

Suppression of rhamnose biosynthesis in rol1-2 strongly altered the flavonol profile and induced, for example, hyponastic growth of cotyledons and aberrant leaf cell development. This was related to enhanced auxin accumulation and a repressed auxin (non-IAA) efflux from mesophyll protoplasts (Ringli et al., 2008; Kuhn et al., 2011). As rol1-2 growth defects were rescued in tt4 rol1, but retained in tt7 rol1, they were attributed to kaempferols. However, k1 was not the causal metabolite for this particular phenotype, as rol1 ugt78d1 lacking k1 retained the rol1 cotyledon phenotype (Ringli et al., 2008).

Several possible mechanisms have been reported on how flavonols might affect auxin transport. Flavonol biosynthetic mutants led to an altered expression, subcellular localization and perhaps modified dynamics in the plasma membrane of some PIN proteins, which could have been caused by a direct or indirect impact of flavonols on these proteins (Peer et al., 2004; Santelia et al., 2008). At the transcriptional level, there was no difference in mRNA abundance of several PIN as well as ABCB1 and ABCB19 genes in ugt78d2 and wild-type plants (Fig. S6). ABCB1 and ABCB19 are ABC transporters which primarily function in the long-distance auxin transport streams and the movement of auxin out of apical tissues (Bandyopadhyay et al., 2007). Therefore, these ABCB transporters are potential targets of k1 in planta. Indeed, ABCB1/19 proteins are able to bind the quercetin aglycone (Murphy et al., 2002), and inhibition of ABCB1 auxin transport activity by the quercetin aglycone has been demonstrated in Arabidopsis protoplasts (Geisler et al., 2005). These observations are in agreement with previous in vitro experiments showing that flavonol aglycones can compete with the synthetic auxin transport inhibitor NPA for binding to isolated microsomal vesicles, although these results may not necessarily reflect an in vivo relevance (Jacobs & Rubery, 1988; Murphy et al., 2002). Nevertheless, these studies indicate that flavonol derivatives, including k1, could interact directly with and/or inhibit auxin transport proteins.

Despite these in vitro studies, a possible involvement of flavonol aglycones in auxin transport inhibition in vivo and, in Fig. 5 Gravitropic response of wild-type and ugt78d2 inflorescence stems. Four-week-old Arabidopsis thaliana plants were laid down from an upright to a horizontal position in darkness. The curvature of the primary inflorescence stems was measured at different time points to assess the gravitropic response. The angles refer to a horizontal positioning of the tip of the inflorescence (0°) and a fully upright positioning of the tangential approximation of the bent tip (90°). Wild-type, blue line; ugt78d2, red line. Means and SE are shown (n = 15).
particular, in the ugt78d2 growth phenotype appears to be unlikely. This notion is based on two lines of evidence: first, to the best of our knowledge, so far only two of numerous studies have detected flavonol aglycones in Arabidopsis by either HPLC (Peet et al., 2001) or mass spectrometry (Buer et al., 2013); however, the latter authors also discussed inconsistencies with respect to the occurrence and absence of aglycones in different it mutants. Although flavonol aglycones exist at least as biosynthetic intermediates, only minute amounts of the hydrophobic molecules might be tolerated in living cells, and therefore free flavonols are not consistently detected; however, this would not preclude a regulatory role (Yin et al., 2012). The important second line of evidence stems from loss-of-function mutants affecting the two major UGTs in Arabidopsis, UGT78D1 and UGT78D2, which perform the initial 3-O-flavonol glycosylation (Fig. 1). The lack of either UGT is compensated by the remaining enzyme, and results in a shift of the flavonol glycoside pattern with no aglycones being detected (this work; Jones et al., 2003; Yonekura-Sakakibara et al., 2008; Yin et al., 2012). The ugt78d1 ugt78d2 double mutant strongly enhances the chance of flavonol aglycone accumulation, yet no free flavonols were detected (Yin et al., 2012) and, more importantly, the ugt78d2-dependent growth retardation was reversed in the double mutant (Fig. 2).

Flavonols have also been shown to exert their function by modulating the interaction between TWD1, a regulatory protein, and ABCB1 transporters, thus suggesting another possible scenario (Bailly et al., 2008). Furthermore, it has been reported that PINOID modulates ABCB1-mediated auxin transport through its kinase activity, and that this effect is reversed by direct quercetin binding, that is, the flavonol acts as an endogenous kinase inhibitor to modulate PAT (Henrichs et al., 2012). In summary, these mechanistic studies on auxin transport inhibition allow for both different active flavonol moieties and multiple target proteins. The discovery of k1 as an active molecule in planta may serve as a valuable tool to obtain further insight into the detailed mechanism of flavonol-dependent auxin transport inhibition.

Apart from the genetic designation of k1, the dose-dependent PAT repression by k1 further supports its identification as an endogenous PAT inhibitor in Arabidopsis shoots (Fig. 4). Interestingly, there is also almost the same quantitative relationship in ugt78d2 relative to the wild-type between the concentrations of k1 (c. two-fold higher in the mutant), endogenous auxin (c. two-fold lower in the lower florescence stem segments of mutant plants) and basipetal auxin transport (c. two-fold lower in the basal part of the florescence stem of the mutant) (Figs 3b, 4a; Table 1). A further increase in k1 in it7 ugt78d2 resulted in more severe growth defects. However, the complete lack of k1 (in it4, ugt78d1, ugt89c1) did not result in a higher stature of the Arabidopsis thaliana plants, suggesting a threshold for k1-dependent growth repression or an independent limitation of growth of these mutants.

In plants, a minor increase in k1 levels would only lead to moderate PAT inhibition, which might not lead to obvious phenotypic changes (as seen in the shoots of abcb1 and abcb19 single mutants; Noh et al., 2001). However, under harsh natural growth conditions, including high light, UV-B irradiation, extreme temperatures and drought, k1 as well as other flavonol glycosides could be strongly increased (Hannah et al., 2006; Rausher, 2006; Caldwell et al., 2007; Korn et al., 2008; Götz et al., 2010; Stracke et al., 2010a). Thus, regulation of flavonol glycoside levels may constitute a means to adjust plant growth and stature by environmental factors and in ecological contexts.

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**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Morphology and flavonol content of young seedlings.

**Fig. S2** Genetic complementation of the *ugt78d2*-dependent shoot growth phenotype.

**Fig. S3** Growth phenotype and k1 levels of *flt1 ugt78d2*.

**Fig. S4** Growth phenotype and k1 levels of *tt3-1* and Ler.

**Fig. S5** Acropetal auxin transport activity in inflorescence stems of wild-type and *ugt78d2*.

**Fig. S6** Expression of auxin transporter genes in *ugt78d2* stem segments relative to wild-type.

**Fig. S7** Auxin-responsive gene expression in *ugt78d2* and wild-type.

**Fig. S8** Analysis of *UGT78D1* gene expression in plant organs.

**Fig. S9** Expression of auxin reporter *DR5::GUS* in wild-type and *ugt78d2* roots.

**Table S1** Phenotypic analysis of Col-0 and *ugt78d2* plants

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