

Modifying expression of closely related UDP-glycosyltransferases from pea and *Arabidopsis* results in altered root development and function

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Glycosyltransferases (GTs) play diverse roles in cellular metabolism by modifying the activities of structural and regulatory metabolites. Previous studies indicated that a *Pisum sativum* UDP-glycosyltransferase (PsUGT1) is essential for plant development, and suggested a role for this enzyme in the regulation of the cell division cycle. Here we report that recombinant PsUGT1 expressed in vitro exhibits activity on diverse flavonoids including kaempferol. In *Arabidopsis* expressing PsUGT1, gravity sensing is impaired, and this loss of function is corrected by exogenous addition of kaempferol. HPLC of tissue extracts of *Arabidopsis* expressing PsUGT1 revealed the accumulation of glycosides of kaempferol, but not of other related flavonoids. A search of the NCBI gene bank (<http://www.ncbi.nlm.nih.gov/>) using PsUGT1 revealed that six genes from the *Arabidopsis* AtUGT85A subfamily show similarities both in DNA and protein sequences (Woo et al. 2007). In the current study, we examined the hypothesis that one or more members of this family, like PsUGT1, is required for *Arabidopsis* development. Altered expression of AtUGT85A7, but not other AtUGT85A subfamily members, resulted in changes in life cycle, leaf morphology, auxin response, and root development, including loss of gravity sensing. The phenotypes of plants where AtUGT85A7 gene expression was suppressed, by RNAi mutagenesis, were very similar to those occurring in plants with altered expression of PsUGT1.

Introduction

Regulation of cell proliferation underlies development and maintenance of growth in multicellular organisms and as such is responsive to many external and internal perturbations. One important level of regulation involves the reversible conjugation of endogenous chemicals that regulate the cell cycle (Dixon 2004). In plants, several thousand glycosylated chemicals, including families of important signal molecules, such as flavonoids, mono-

terpenes, and plant hormones, have been identified (Dixon and Ferreira 2002). These compounds are usually glycosylated (Pflugmacher and Sandermann 1998). Although the genetic mechanisms controlling their metabolism are not well defined, glycosylation of phytochemicals is known to alter their regulatory properties by causing enhanced water solubility and lower chemical reactivity (Bowles et al. 2006, Poppenberger et al. 2003). Glycosylated compounds are thought of as transportable storage

Abbreviations – ESI-MS, electrospray ionization mass spectrometry; GTs, glycosyltransferases; MS, Murashige and Skoog; 1-NAA, 1-naphthalene acetic acid; PAT, polar auxin transport; PCR, polymerase chain reaction; PsUGT1, *Pisum sativum* UDP-glycosyltransferase; RT-PCR, reverse transcriptase-PCR; WISH, whole mount in situ hybridization.

compounds or waste/detoxification products lacking physiological activity (Gachon et al. 2005). These conjugates can be stored in vacuoles or cell walls for extended periods. In the case of phytohormones, such as auxin, cytokinin, gibberellin, abscisic acid, jasmonate and brassinolide, conjugates have been proposed to act as reversibly deactivated storage forms (Manzano et al. 2006, Martin et al. 1999). Sugar conjugation thus may be a key factor in establishment of 'homeostasis' for the regulation of physiologically active hormone levels (Szerszen et al. 1994). In other cases, sugar conjugation of hormones might accompany or introduce irreversible deactivation.

Previous studies established that expression of a meristem-localized, inducible UDP-glycosyltransferase gene [*Pisum sativum* UDP-glycosyltransferase (PsUGT1)] is needed for normal cell-cycle regulation in pea and alfalfa (Woo et al. 1999). Inhibition of endogenous PsUGT1 activity in transgenic plants by antisense mRNA expression under the control of its own promoter is lethal. However, a partial inhibition of PsUGT1 expression results in a distinct phenotype, which includes an extended cell cycle (from 15 h in wild type to 30 h in the mutant), reduced growth rate, and associated phenotypic changes during growth and development (Woo et al. 1999). The results are consistent with the hypothesis that PsUGT1 reversibly conjugates a metabolite needed for control of the cell cycle in higher plants (Woo et al. 1999). In support of this hypothesis was the observation that PsUGT1 ectopic expression or PsUGT1 antisense mRNA expression when transformed into *Arabidopsis* results in increased or decreased duration of life cycle, respectively (Woo et al. 2003, 2005). To explore the potential of the *Arabidopsis* model for examining this phenomenon in detail, we reported that the *Arabidopsis* genome contains six genes with significant homology to pea PsUGT1 (Woo et al. 2007). In this study, we present evidence indicating that among six AtUGT85A genes, AtUGT85A7 represents the *Arabidopsis* ortholog of PsUGT1.

Materials and methods

Plant materials and growth conditions

The *Arabidopsis* ecotype Columbia was grown in greenhouse soil at 22°C and 80% relative humidity with 16 h of light and at 20°C with 8 h of dark.

Expression of PsUGT1 in *Pichia pastoris* and in vitro enzyme assay for identification of flavonoids

To express PsUGT1 for the in vitro enzyme assay, a full-length PsUGT1 mRNA was cloned into a pPIC9 vector

and transformed into *P. pastoris* according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). To induce the expression of recombinant PsUGT1 in *P. pastoris*, an overnight culture was treated with 0.5% methanol for 1 day at 29°C. The secreted recombinant protein was concentrated by saturated ammonium sulfate precipitation and dialyzed in 100 mM sodium phosphate buffer (pH 7.0) at 4°C. An in vitro enzyme reaction was performed in 50 mM Tris-HCl (pH 7.9), 3 mM MgCl₂, 0.01% BSA, 1–5 μM UDP-¹⁴C-sugar, 0–200 μM cold UDP-sugar, 10–100 μM flavonoid (kaempferol, quercetin, apigenin, taxifolin and naringenin) in 50% DMSO, and the recombinant enzyme preparation in a final volume of 50 μl at 30°C for 1–2 h. The resulting enzyme reaction product was chloroform extracted to remove unincorporated UDP-¹⁴C-sugar and subsequently separated on silica TLC plate in chloroform/methanol/water (10:10:5, by volume).

Extraction of methanol-soluble compounds and HPLC analysis

To extract methanol-soluble compounds from *Arabidopsis* for HPLC analysis, fresh tissues were collected in liquid nitrogen. The frozen tissues were ground in liquid nitrogen using a mortar and pestle and were immediately transferred to HPLC-grade methanol (20 ml solvent per 1 g of tissue). The methanol-soluble components were extracted for 1 h at 4°C. After filtration (Whatman No. 1), the extract was concentrated using a vacuum evaporator at low pressure. All steps were performed as quickly as possible to avoid oxidation of the flavonoids.

The methanol-soluble components dissolved in 50% methanol were injected onto a reversed-phase HPLC column (Supelcosil LC-18-S, 150 × 4.6 mm, 120 Å, 5 μm; Sigma-Aldrich, St Louis, MO) equilibrated in 10% water in methanol and eluted at 1 ml min⁻¹ with an increasing linear gradient of methanol/glacial acetic acid (100/2.5, v/v, buffer B) to 100% buffer B over 60 min. When using a diode array detector, the entire UV spectrum (200–400 nm) was continuously recorded for each chromatogram (scan time 1 s), and the individual absorbance traces for 254 and 360 nm were reconstructed. From these data sets, the complete UV spectrum of each peak of interest was recorded.

Electrospray ionization mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was carried out using a Perkin-Elmer Sciex (Thornhill, Canada) API III triple quadrupole mass spectrometer fitted with an Ion Spray source, tuned, and calibrated as

previously described (Chernushevich et al. 2001). Spectra were recorded in the positive ion and negative ion modes. Fractions of interest in methanol were injected (10–20 μl injection⁻¹) into a stream of the same solvent entering the ion source (10 μl min⁻¹). Spectra were produced by scanning from m/z 100–1000 (step size 0.3 Da, dwell time 2.0 ms, 6.14 s/scan, orifice voltage 90). Because of the strong responses obtained for flavonoids during negative ion ESI-MS (Fabre et al. 2001), negative ion ESI-MS was used to determine the kaempferol glycosides.

RNAi mediated downregulation of AtUGT85A7 and AtUGT85A5 in *Arabidopsis*

To downregulate AtUGT85A7 gene expression by RNAi, a 223-bp region located in the N-terminus of the AtUGT85A7-encoded protein was amplified using primers described in Table 1. This region excluded a conserved sugar-binding site (PSPG motif) located in the C-terminal domain to make sure that RNAi mutagenesis suppressed only the expression of AtUGT85A7 gene, and not any other GTs in the genome. Polymerase chain reaction (PCR) products were cloned into the pKANNIBAL vector and then transferred into the pART27 vector (Wesley et al. 2001). The resulting construct expressing CaMV35S::AtUGT7-RNAi was transformed into *Escherichia coli* HB101 and then into *Agrobacterium tumefaciens* ASE by triparental conjugation. Both wild-type Columbia and Columbia (DR5::uidA, kindly provided by Tom J. Guilfoyle, University of Missouri, Columbia, MO) were transformed with the construct. Four-week-old plants were transformed by floral dip with *A. tumefaciens* ASE as described previously (Clough and Bent 1998). Seeds harvested from transformed plants were grown on Murashige and Skoog (MS) selection plates containing kanamycin (50 μg ml⁻¹). For selection of transgenic plants, kanamycin-resistant primary plants were analyzed for the presence of the transgene by PCR. Six lines of transgenic plants were grown in a controlled environmental chamber with 16 h of light (mixed fluorescent

bulbs) at 22°C and 8 h of dark at 20°C and were carried through three to five generations before the analysis of the phenotype of AtUGT85A7-suppression mutant. Suppression of AtUGT85A7 genes was confirmed by reverse transcriptase-PCR (RT-PCR) and whole mount in situ hybridization (WISH). RNA was treated by RNase free-DNase to remove any genomic DNA contamination before RT-PCR. In addition, PCR primers used go over an intron site. Similarly, AtUGT85A5 gene expression was downregulated by RNAi with the 482-bp fragment located in the N-terminal domain of the AtUGT85A5-encoded protein.

Cloning and WISH

PCR reactions for amplifications of genomic or cDNA templates were performed with 1.25 mM dNTPs, 5 μM each of the primers, 1 \times Taq buffer and 0.5 units of Taq polymerase (Roche, Indianapolis, IN) in a volume of 20 μl . The amplification program consisted of an initial 95°C cycle for 2 min followed by 20–25 cycles of 95°C, 15 s; (Tm-5)°C, 15 s; and 65–70°C, 60–120 s and a final extension at 70°C for 6 min. Reverse transcription reactions for RT-PCR were performed using the Superscript II reverse transcriptase system following recommendations from the manufacturer (Gibco-BRL, Rockville, MD). An oligo-dT₁₈ primer was used to generate first-strand cDNAs, and gene-specific primers were used as the reverse primers in the PCR reaction (Gibco-BRL). The primer pairs used for RT-PCR are listed in Table 1. AtUBC8 was used as a positive control because it is expressed in all of the plant organs examined (Woo et al. 1994). The plasmids carrying cDNAs corresponding to the genes for AtUGT85A7 were linearized for T3 or T7 polymerase-directed RNA synthesis, and sense and antisense strands were synthesized for each by standard procedures. RNA was labeled by incorporating digoxigenin-conjugated UTP (Roche Applied Science, Indianapolis, IN). WISH and reporter gene β -glucuronidase (GUS) assays were carried out as described (Woo et al. 1999, 2007).

Table 1. List of PCR primers used for WISH and RNAi mutagenesis of AtUGT85A7 gene. AtUGT85A7-WISH primers were used to clone cDNAs into pCRII vector to generate antisense and sense probes for WISH. AtUGT85A7-RNAi primers were used to clone sequences into pKANNIBAL vector for RNAi mutagenesis. Primers for RT-PCR are listed in Table 4 (Woo et al. 2007). AtUBC8 primers were used for RT-PCR for AtUBC8 mRNAs (Genbank accession no. NC_003076) for RNA loading control

UGT85A7-WISH 5'	ATGTCTG GGTACC AAGCTT ACTGTCTCGCTCCAT TCAAAGAG
UGT85A7-WISH 3'	ATGTCTG TCTAGA CTCGAG CTTTAAAGGAGATAGACCCCTTCTC
UGT85A7-RNAi 5'	ATGTCTG GGTACC AAGCTT ACTGTCTCGCTCCAT TCAAAGAG
UGT85A7-RNAi 3'	ATGTCTG TCTAGA CTCGAG CTTTAAAGGAGATAGACCCCTTCTC
AtUBC8-5'	ATGGCTTCGAAACGGATCTTGAAGG
AtUBC8-3'	AGCCCATG GCATACTT CTGAGTCC

Assay of root gravitropic responses

To assay root gravitropic responses, surface sterilized seeds were cold treated in the dark at 4°C for 3–5 days and then germinated in constant white light at 20°C for 2–3 days. Seedlings were transferred to the plates maintaining the same orientation and allowed to grow vertically for a further 2–3 days. The plates were then turned 90° and grown for a further 2–3 days under the same growth conditions before scoring for reorientation of root growth. Similar experiments were also performed in the dark. The gravitropic response was similar in the dark-grown plants as in the light-grown plants. To rescue gravitropic responses, seedlings were grown in MS medium in plates supplemented with 10–100 μM flavonoids. Tested flavonoids include kaempferol, quercetin, apigenin, taxifolin, flavone, luteolin, catechin, naringenin, hesperetin, genistein and daidzein.

Analysis of leaf morphology in *Arabidopsis* and alfalfa

For comparative analysis of leaf morphology in *Arabidopsis* and alfalfa, fully expanded leaves were collected randomly from more than 100 *Arabidopsis* plants and 10 alfalfa plants. The ratio of length to width was determined by measuring the longest and widest places in the leaf. Average length and width of 30 leaves was determined, and the standard deviation was calculated.

Analysis of life cycle in *Arabidopsis*

Analysis of life cycle in *Arabidopsis* was done as described previously (Woo et al. 2003). Transgenic seeds (e.g. 80–100 seeds each) in soil in flats (27 \times 54 cm) were cold treated for 5 days and germinated. Plants were grown in a growth chamber (16-h photoperiod, 22°C and 8-h dark period, 20°C at 80% relative humidity). Time of bolting and leaf senescence was determined visually when >95% of the plants showed morphological changes. These experiments were performed three times.

Treatment with 1-NAA to *Arabidopsis* seedlings for DR5::uidA analysis

DR5::uidA analysis of seedlings treated with 1-naphthalene acetic acid (1-NAA) was carried out after seeds were germinated in medium composed of MS minimal organics (Sigma-Aldrich) containing various concentrations of 1-NAA (10^{-10} M to 10^{-6} M) and solidified with 1% (w/v) agar (type E; Sigma-Aldrich). The sucrose content of the medium was 0.3% (w/v). After 5 days of cold treatment in the dark, the seedlings were grown in a growth chamber (22°C, 80% relative humidity, and 16-h light period;

Convicon, Winnipeg, Manitoba, Canada) for 7–10 days before histochemical GUS-staining analysis.

Results

Recombinant PsUGT1 expressed in *P. pastoris* glycosylates flavonoids in vitro

As a preliminary qualitative screen for substrate specificity, PsUGT1 was expressed in *P. pastoris* and assayed for glycosylation using UDP- ^{14}C labeled sugars (Fig. 1). When expressed in *P. pastoris*, recombinant PsUGT1 glycosylated flavonoids including kaempferol, quercetin, apigenin and taxifolin (Fig. 1, lanes 1, 2, 5, 6, respectively), but not naringenin (not shown). Both UDP- ^{14}C -glucuronic acid and UDP- ^{14}C -glucose (not shown) served as sugar donors for flavonoid glycosylation. Phytohormones including IAA (Fig. 1, lane 3) and cytokinin (Fig. 1, lane 4) did not serve as substrates for PsUGT1 in vitro. Other researchers have shown a lack of specificity in glycosylation by plant glycosyltransferases (GTs) expressed in vitro (Hansen et al. 2003, Taguchi et al. 2003).

Accumulation of kaempferol diglucuronides in *Arabidopsis* expressing PsUGT1

To examine the hypothesis that PsUGT1 glycosylates one or more flavonoids in vivo, HPLC was used to compare

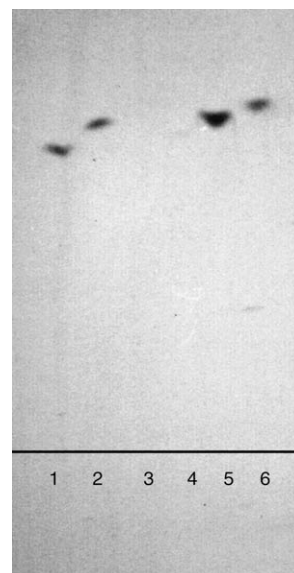


Fig. 1. Flavonoid glycosylation by recombinant PsUGT1. In vitro reaction products were separated on silica TLC plate. 1 = kaempferol- ^{14}C -glucuronide, 2 = quercetin- ^{14}C -glucuronide, 3 = IAA (no glycosylation), 4 = cytokinin (no glycosylation), 5 = apigenin- ^{14}C -glucuronide, 6 = taxifolin- ^{14}C -glucuronide.

flavonoid glycoside profiles in wild-type *Arabidopsis* with lines expressing PsUGT1 sense (PsUGT1-OE) or antisense mRNA (PsUGT1-AS). Profiles of metabolites from both lines were separated using reversed-phase HPLC and detected at UV₂₅₄ (Fig. 2A–C) or UV₃₆₀ (Fig. 2D–F). When compared with wild-type profiles (Fig. 2A, D), a single new peak was detected in *Arabidopsis* expressing PsUGT1 (Fig. 2B, E, arrow). This peak was absent in HPLC profiles of wild type (Fig. 2A, D) and PsUGT1-AS mutants (Fig. 2C, F). UV spectrum and mass spectrometry analysis revealed that this peak represents kaempferol diglucuronides with UV max at 266 and 347 nm, and m/z 655 (Fig. 2G, H). Other peaks which are altered in their sizes may represent quantitative differences in other products. The reason for this is not known at this time, but accumulation of kaempferol diglucuronides may be responsible for this alteration.

Kaempferol restoration of gravitropic response in *Arabidopsis* expressing PsUGT1

If an excess of kaempferol glycosylation underlies phenotypic changes associated with PsUGT1 expression in *Arabidopsis*, then exogenous addition of kaempferol might be predicted to alter short-term phenotypic effects. In previous studies, PsUGT1-OE roots were found to show altered root morphology and response to exogenous auxin (Woo et al. 2003). Further analysis revealed that the roots of these plants do not respond to gravity (Fig. 3A–D). When plants germinated and was grown on agar plates oriented vertically, wild-type roots grew downward (Fig. 3A, arrows), whereas roots of plants expressing PsUGT1 failed to reorient their growth (Fig. 3B). Hypocotyls of the PsUGT1-OE mutants still reoriented in response to gravity (not shown). Root growth in *Arabidopsis* expressing PsUGT1 antisense mRNA was poor, and these plants also failed to respond to gravity, but not as poorly as the PsUGT1-OE mutants (Fig. 3C).

The flavonoids kaempferol, quercetin, apigenin, taxifolin, flavone, luteolin, catechin, naringenin, hesperetin, genistein and daidzein were individually added to growth medium of *Arabidopsis* seedlings. At high concentrations (100 μ M), all tested flavonoids inhibited root growth and the gravitropic response (not shown), but at 10–25 μ M, there was no visible effect on wild-type *Arabidopsis*, which grew normally and responded to gravity (Fig. 3E). Root growth in lines expressing PsUGT1 antisense mRNA was inhibited at 10–25 μ M kaempferol, but not the other metabolites (Fig. 3G). In contrast, kaempferol at 10–25 μ M, but not the other tested metabolites, restored the gravitropic response in *Arabidopsis* expressing PsUGT1 (Fig. 3F).

Altered AtUGT85A7 expression results in altered root development and function

These results supported previous observations suggesting that altered PsUGT1 expression in legumes and *Arabidopsis* may modulate the activity of a metabolite that plays a critical role in root development and function. If so, then identification of a functional ortholog of the PsUGT1 gene in *Arabidopsis* may provide experimental tools for a detailed structure–function analysis to define this previously unknown level of plant cell-cycle regulation. To examine the hypothesis that an *Arabidopsis* ortholog of PsUGT1 is required for normal development, the PsUGT1 sequence was used to identify a related family of six *Arabidopsis* genes (Woo et al. 2007). A high degree of sequence similarity among the AtUGT85A gene family members precluded the use of simple sequence comparison to identify which, if any, was most likely to be functionally equivalent to PsUGT1. Instead, comparative phenotype analysis was used to examine predictions of the hypothesis that one or more of the AtUGT gene family members encode an enzyme whose expression is required for normal root development and function. T-DNA insertion mutant lines for AtUGT85A1 (SALK_085809.55.45.x), AtUGT85A3 (SALK_070258), AtUGT85A2 (SALK_045078) and AtUGT85A4 (SALK_046500, SALK_078716, CS821695) were obtained from the ‘*Arabidopsis* Biological Resource Center’ at the Ohio State University.

AtUGT85A7-suppression mutant lines were generated by RNAi mutagenesis, and six lines segregating for a single insertion were selected by DNA blot analysis. Previously, we reported the expression of all six AtUGT85A genes in wild-type *Arabidopsis* (figures 3 and 4 in Woo et al. 2007). All selected lines showed suppression of target genes confirmed by RT–PCR (Fig. 4A, B). AtUGT85A3 and A4 genes were not expressed in wild-type leaves (Fig. 4C, A3, A4), but a low-level expression of these genes was detected in leaves of AtUGT85A7-suppression mutant (Fig. 4D, A3, A4).

AtUGT85A5-suppression mutant line was also generated by RNAi mutagenesis. However, lines expressing the AtUGT85A5-RNAi construct initially germinated, but died within several days.

Four T-DNA insertion lines (i.e. AtUGT85A1, 2, 3 and 4) and AtUGT85A7-suppression mutant line were screened for changes in root growth, development and gravity response as markers for phenotypes previously shown to be altered in pea, alfalfa and *Arabidopsis* with altered PsUGT1 expression. In four lines (AtUGT85A1, 2, 3 and 4), no phenotypes were found to be distinct from wild type. Root growth, development and gravity response were normal (not shown). In contrast, root

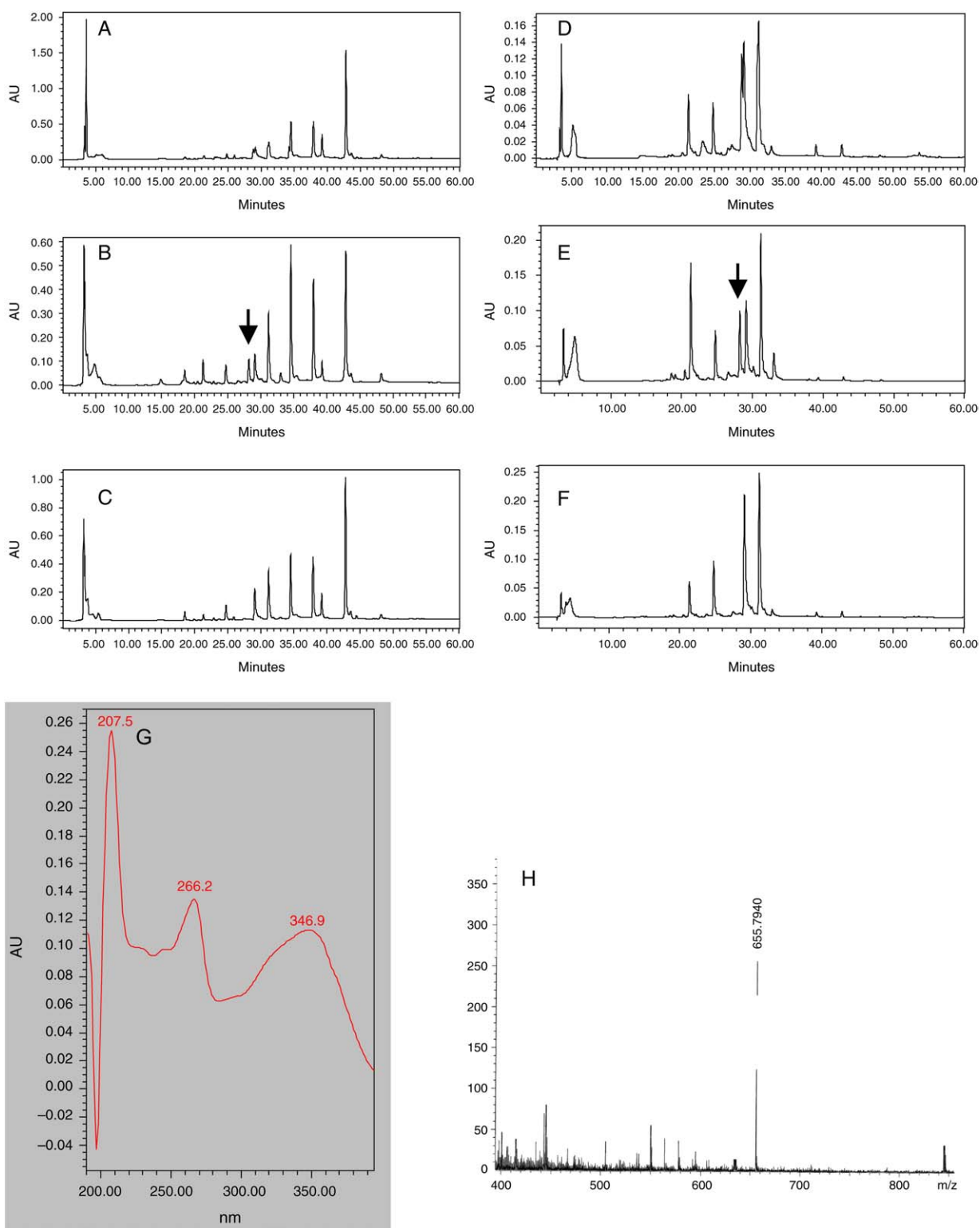


Fig. 2. Accumulation of kaempferol diglucuronides in *Arabidopsis* expressing PsUGT1. (A–C) profiles of tissue extracts separated by reversed-phase HPLC and detected at UV_{254nm}, (D–F) profiles of tissue extracts separated by reversed-phase HPLC and detected at UV_{360nm}, (A, D) wild-type seedlings, (B, E) PsUGT1-OE mutant seedlings, (C, F) PsUGT1-AS mutant seedlings, (G) UV spectrum of peak I showing kaempferol diglucuronides, and (H) MS analysis of peak I, kaempferol diglucuronides (m/z 655). I, arrow indicates accumulation of kaempferol diglucuronides.

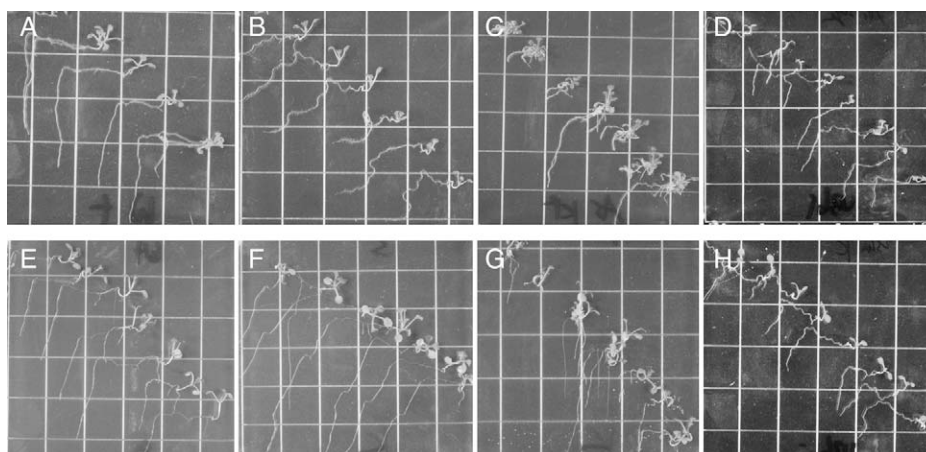


Fig. 3. Altered response to gravity in response to altered expression of glycosyltransferase-encoding genes, and rescue of altered response by exogenous kaempferol. (A) Wild-type roots responded to gravity. (B) Roots of PsUGT1-OE did not respond to gravity and formed wavy, curly roots. (C) Root growth of PsUGT1-AS was poor and did not respond to gravity. (D) In the AtUGT85A7-RNAi-suppression mutant, root growth was poor and the gravitropic response was incomplete. (E) No visible inhibition of the gravitropic response by 25 μ M kaempferol was observed in wild-type seedlings. (F) Treatment of 25 μ M kaempferol rescued gravitropic responses in the PsUGT1-OE mutant. (G) Treatment of 25 μ M kaempferol inhibited root growth partially in PsUGT1-AS mutant. (H) Treatment of 25 μ M kaempferol inhibited root growth partially in AtUGT85A7-RNAi suppression mutant.

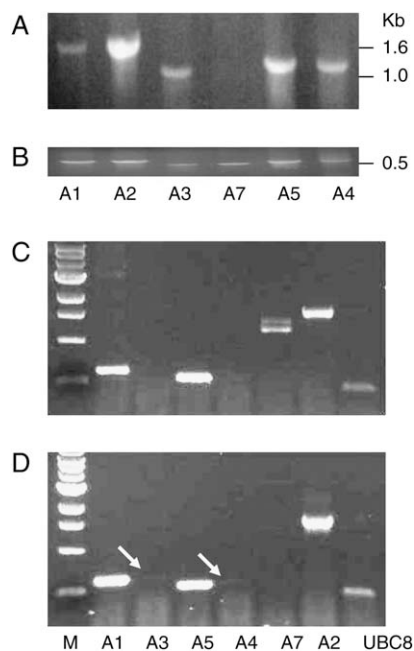


Fig. 4. AtUGT85A subfamily member gene expression in AtUGT85A7-suppression mutants. (A) RT-PCR of RNAs from 6-day-old seedlings of AtUGT85A7-suppression mutants. (B) RT-PCR of AtUBC8 mRNA showing equal loading of RNA in (A). (C) RT-PCR of RNAs from wild-type leaves. (D) RT-PCR of RNAs from AtUGT85A7-suppression mutant leaves. Arrows indicate expression of AtUGT85A3 and AtUGT85A4 mRNAs in AtUGT85A7-suppression mutants. Expression of these genes was not detected in wild-type leaves (C) (Woo et al. 2007). M, DNA size marker; A1, AtUGT85A1; A3, AtUGT85A3; A5, AtUGT85A5; 4, AtUGT85A4; A7, AtUGT85A7; A2, AtUGT85A2; UBC8, AtUBC8 as an RNA loading control. (A, B) Full length mRNAs are amplified; (C, D), PCR primers from internal sequences were used.

growth, development and gravity response of the AtUGT85A7-suppression mutant line were markedly distinct from wild type (Fig. 3D). Treatment with 10–25 μ M kaempferol showed inhibitory effects on root growth in AtUGT85A7-RNAi-suppression mutants (Fig. 3H).

The AtUGT85A7 gene effects were examined in detail, with respect to quantitative phenotypic changes previously found to be associated with altered expression of PsUGT1. These phenotypes include changes in auxin responsiveness, leaf and root development, and duration of life cycle.

Altered auxin levels and *Arabidopsis* development in AtUGT85A7 mutant

In previous studies, alfalfa expressing PsUGT1 antisense mRNA exhibited altered auxin distribution in correlation with delayed leaf and root development (Woo et al. 2003). Similarly, leaf and root development in the AtUGT85A7-suppression mutant line was delayed (Tables 2 and 3). The auxin-responsive promoter DR5::*uidA* was used to confirm that *Arabidopsis* expressing PsUGT1 exhibits altered auxin levels (Woo et al. 2003). The same approach was used here to document that similar changes occur when AtUGT85A7 expression is suppressed. In wild-type Columbia (DR5::*uidA*), 5 days after germination, expression of DR5::*uidA* was localized to the leaf vein and leaf periphery including tip (Fig. 5A), leaf primordia (Fig. 5B) and root apex (Fig. 5C). In the AtUGT85A7-suppression mutant, DR5::*uidA* expression was markedly reduced in leaves (Fig. 5D) and leaf primordia (Fig. 5E) and root tips (Fig. 5F). These data

Table 2. Life cycle comparison between wild type, PsUGT1-OE mutant and AtUGT85A7 mutant *Arabidopsis*. Bolting, leaf senescence and completion of senescence were determined visually. The date of bolting was set as the time when >95% of the population of 80–100 plants distributed among four replicate rectangular flats (27 × 54 cm) started to bolt. The onset of senescence was designated when >95% of the population exhibited >50% chlorosis in leaves, as previously described (Woo et al. 2003). Completion of senescence was designated based on 100% chlorosis in leaves. The experiment was repeated three times, with the same results

	Bolting (days)	Leaf senescence started (days)	Leaf senescence completed (days)
WT	24	39	51
PsUGT1-OE	20	29	38
AtUGT85A7	28	45	60

are consistent with the hypothesis that, as with reduced PsUGT1 expression in alfalfa (Woo et al. 1999), leaf and root development inhibition in plants with reduced AtUGT85A7 expression is correlated with changes in endogenous auxin distribution.

Table 3. Comparison of phenotypes in UGT mutants

PsUGT1 suppression mutants of alfalfa	
PsUGT1-AS	
Changes in leaf, flower and root morphology (Woo et al. 1999)	
Increased life cycle	
Root growth is poor	
Changes in auxin distribution in stem cuttings (Woo et al. 2003)	
Cell cycle delayed	
PsUGT1 suppression mutant of <i>Arabidopsis</i>	
PsUGT1-AS	
Increased life cycle (Woo et al. 2005)	
Root growth is poor	
Loss of root gravitropic response	
Kaempferol did not rescue root gravitropic response	
AtUGT85A suppression mutants of <i>Arabidopsis</i>	
AtUGT85A7-RNAi	
Changes in leaf morphology	
Increased life cycle	
Root growth is poor	
Loss of root gravitropic response	
Kaempferol did not rescue root gravitropic response	
AtUGT85A3-T-DNA	
No distinct phenotypic changes	
AtUGT85A2-T-DNA	
No distinct phenotypic changes	
AtUGT85A4-T-DNA	
No distinct phenotypic changes	
AtUGT85A5-RNAi	
Lethal	
PsUGT1 overexpression mutant of <i>Arabidopsis</i>	
PsUGT1-OE	
Shorter life cycle (Woo et al. 2003)	
Loss of root gravitropic response	
Kaempferol rescued root gravitropic response	

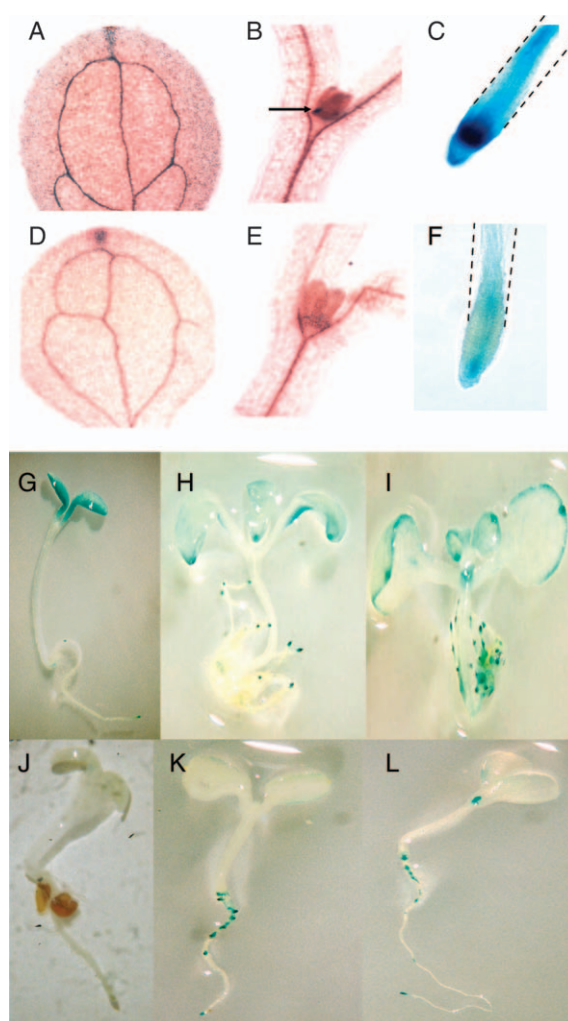


Fig. 5. Auxin-responsive DR5::uidA reporter gene expression used to illustrate altered endogenous (A–F) and exogenous (G–L) auxin responsiveness in wild-type Columbia (A–C, G–I) compared with AtUGT85A7-suppression mutant (D–F, J–L). Blue color illustrates a positive reaction. Expression of DR5::uidA in wild-type Columbia is localized in veins (A), leaf primordia (B, arrow), and root tip (dotted line indicates root outline) (C). Reduced DR5::uidA expression in veins (D), leaf primordia (E) and root tips (dotted line indicates root outline) (F) of AtUGT85A7 mutant. Localized DR5::uidA expression in leaf periphery, lateral root initials, and root tips of wild-type Columbia seedlings germinated for 7 days on MS medium without 1-NAA (G); or with 10^{-8} M 1-NAA (H); or 10^{-7} M 1-NAA (I). Expression of DR5::uidA in AtUGT85A7 mutant in leaf periphery, lateral root initials, and root tips of seedlings germinated on MS medium without 1-NAA treatment (J); or with 10^{-8} M 1-NAA (K); or 10^{-7} M 1-NAA (L). Chlorophyll was removed by methanol for better observation of staining.

Dosage-dependent changes in exogenous auxin responsiveness in AtUGT85A7-suppression mutant

The hypothesis that response to auxin is changed in plants with altered AtUGT85A7 expression was further explored using DR5::uidA as a reporter to document

dosage-dependent changes in responses to exogenous NAA. Wild-type (Fig. 5G-I) and AtUGT85A7-suppression mutant (Fig. 5J-L) seedlings were germinated on MS medium with NAA at 10^{-7} M to 10^{-8} M to examine whether the capacity to respond to exogenous auxin parallels the observed changes in response to endogenous auxin. In wild-type seedlings without NAA, extensive expression of DR5::uidA was observed both at the leaf edges and in root tips (Fig. 5G). Similar patterns were observed in AtUGT85A7-suppression mutants, but the intensity of expression was much reduced compared to wild-type seedling (Fig. 5J). Treatment with 10^{-8} to 10^{-7} M NAA accelerated the rate of leaf growth and enhanced lateral root initiation in wild type (Fig. 5H, I) and AtUGT85A7-suppression mutant seedlings (Fig. 5K, L). However, leaf growth and lateral root development were reduced in AtUGT85A7-suppression mutant seedlings (Fig. 5K, L) compared with wild-type seedlings (Fig. 5H, I). At 10^{-6} M NAA, root growth was inhibited both in wild-type and AtUGT85A7-suppression mutant seedlings (not shown).

Altered leaf morphology in AtUGT85A7-suppression mutant

The ratio of length to width was 2.12 ± 0.06 and 1.54 ± 0.07 in wild-type and antisense mutant alfalfa, respectively (Fig. 6A) (Woo et al. 1999). Thus, in transgenic alfalfa expressing PsUGT1 antisense mRNA, leaflets were shorter and rounded, in contrast to the long oval shape of wild-type leaflets (Woo et al. 1999). Similar results occurred in *Arabidopsis* expressing AtUGT85A7 antisense mRNA. In contrast to the long oval shape of wild-type leaves (Fig. 6B), suppression of AtUGT85A7 expression resulted in a shorter leaf shape with rounded outer tips (Fig. 6C). The ratio of length to width was 2.12 ± 0.06 and 1.78 ± 0.04 in wild-type and AtUGT85A7-suppression mutant *Arabidopsis*, respectively (Fig. 6A).

Altered life cycle in AtUGT85A7-suppression mutant

In wild-type plants, the onset of bolting and leaf senescence, respectively, occurred at 24 and 39 days after germination, and leaf senescence was complete by 51 days (Table 2). By contrast, the onset of bolting and leaf senescence occurred at 20 and 29 days after germination, and leaf senescence was complete by 38 days in PsUGT1-OE mutant (Table 2; Woo et al. 2003). Suppression of AtUGT85A7 expression by RNAi mutagenesis resulted in an increase in the life cycle (Table 2). In AtUGT85A7-suppression mutant, bolting time was

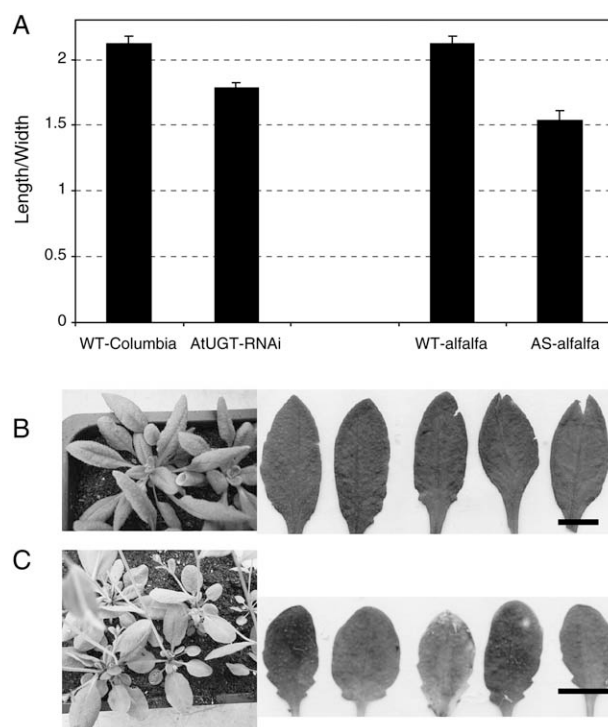


Fig. 6. Similar effects on leaf morphology in transgenic plants of *Arabidopsis* and alfalfa with altered UGT gene expression. (A) Ratio of length to width in leaves of *Arabidopsis* or alfalfa with reduced expression AtUGT85A7 or PsUGT1, respectively. WT, wild-type Columbia; AtUGT85A7, AtUGT85A7 mutant of *Arabidopsis*; WT-alfalfa, wild-type alfalfa; AS-alfalfa, alfalfa expressing CaMV::PsUGT1 antisense mRNA (Woo et al. 1999). (B) Wild-type *Arabidopsis*. (C) AtUGT85A7 mutant. Bars = 1.3 cm.

increased to 28 days, leaf senescence started in 45 days and leaf senescence was complete in 60 days.

Discussion

Manipulation of PsUGT1 expression has remarkably similar effects across dicotylenous species; these effects include changes in leaf morphology, periodicity of the cell cycle and time required to complete the life cycle (Woo et al. 1999, 2003, 2004, 2005, 2007). In previous papers, we reported that the introduction of CaMV35S::PsUGT1 antisense mRNA into alfalfa is correlated with a change in the cell cycle, a reduced rate of growth, inhibition of leaf maturation and sterility of regenerated plants (Woo et al. 1999). Alfalfa expressing CaMV35S::PsUGT1 antisense mRNA also exhibits delayed initiation of DNA synthesis during cell division, and reduced nodulation in response to inoculation with *Sinorhizobium meliloti* (Woo et al. 2004). The current data show that suppression of AtUGT85A7 expression in *Arabidopsis* resulted in phenotypes very similar to those

in alfalfa, including impaired root development and gravitropic response, altered leaf morphology and delayed life cycle (Woo et al. 1999, 2003). The fact that suppression of UGT expression changes auxin responsiveness, as measured by the DR5::*uidA* reporter system, may help to explain the observations. In general, auxin is synthesized locally, in shoot apices, leaf primordia and developing seeds (Ljung et al. 2001, Normanly et al. 1991), and moves primarily from shoot apex to roots by polar auxin transport (PAT). PAT occurs in a cell-to-cell manner and has a strictly unidirectional character, mainly running from the apex towards the base of the plant. PAT is also essential for the transduction of the gravity signal because PAT inhibitors (e.g. NPA) completely abolish the gravitropic response in shoots and roots.

Several models are consistent with the observed effects of altered UGT expression in transgenic plants, and with the direct effects of flavonoids on root function. First, the removal of flavonoid aglycones in UGT ectopic expression mutants could influence regulatory enzymes such as kinases and phosphatases, which play key roles in cell cycle regulation. In mammals, flavonoids can directly inhibit the activity of cellular regulatory proteins including tyrosine kinases, which are critical in the second messenger cascade controlling the activity of other proteins (Kuo et al. 1994). Flavonoids affect invasion and proliferation of tumor cells through direct or indirect control of the cell cycle (Anderson and Garner 1998, Bracke et al. 1994, Deschner et al. 1991, Kandaswami et al. 1991, Miksicek 1993, So et al. 1997). Although the details of their functions in plants are not known, flavonoids can act as internal physiological regulators or chemical messengers at relatively low levels. Flavonoids such as kaempferol function as auxin transport inhibitors (Jacobs and Rubery 1988). Monohydroxy B-ring flavonoids are involved in the degradation of the plant growth hormone, indole acetic acid, whereas dihydroxy B-ring flavonoids inhibit IAA-degrading-activity (Furuya et al. 1962, Galston 1969). Presumably, the site of action of these flavonoids is in the cytoplasm near the site of synthesis.

Conclusions

We have now confirmed that PsUGT1 is required for plant development, and our data suggest that kaempferol may be one target substrate playing a role in the observed effects of PsUGT1 expression. GTs from various sources, when expressed in vitro, can exhibit broad substrate specificity that may or may not reflect their normal target metabolites (Bowles et al. 2006, Gachon et al. 2005, Hansen et al. 2003, Hirotani et al. 2000, Kramer et al. 2003, Nagashima et al. 2000, Taguchi et al. 2003). The

identification and characterization of the PsUGT1 ortholog in *Arabidopsis* will provide tools to characterize how this level of cellular regulation may functionally operate with regard to substrate specificity in diverse tissues, and how the glycosylation process controls cell cycle, morphology and function.

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References

- Anderson JJB, Garner SC (1998) Phytoestrogens and bone. In: Adlercreutz H (ed) Bailliere's Clinical Endocrinology and Metabolism, Phytoestrogens. Bailliere Tindall, London, pp 543–557
- Bowles D, Lim E-K, Poppenberger B, Vaistij E (2006) Glycosyltransferases of lipophilic small molecules. *Annu Rev Plant Biol* 57: 567–597
- Bracke AK, Bruyneel E, Vermeulen SJ, Vennekens K, Van Marck V, Mafreel MM (1994) Citrus flavonoid effect on tumor invasion and metastasis. *Food Technol* 47: 121–124
- Chernushevich IV, Loboda AV, Thomson BA (2001) An introduction to quadrupole-time-of-flight mass spectrometry. *J Mass Spectrom* 36: 849–865
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16: 735–743
- Deschner EE, Ruperto J, Wong G, Newmark HL (1991) Quercetin and rutin as inhibitors of azoxymethanol-induced colonic neoplasia. *Carcinogenesis* 12: 1193–1196
- Dixon RA (2004) Phytoestrogens. *Annu Rev Plant Biol* 55: 225–261
- Dixon RA, Ferreira D (2002) Genistein. *Phytochemistry* 60: 205–211
- Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J (2001) Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry. *J Am Soc Mass Spectrom* 12: 707–715
- Furuya M, Galston AW, Stowe BB (1962) Isolation from peas of cofactors and inhibitors of indole-3-acetic acid oxidase. *Nature* 193: 456–457
- Gachon CMM, Langlois-Meurinne M, Saindrenan P (2005) Plant secondary metabolism glycosyltransferases: the

- emerging functional analysis. *Trends Plant Sci* 10: 542–549
- Galston AW (1969) Flavonoids and photomorphogenesis in peas. In: Harborne JB, Swain T (eds) *Perspectives in Phytochemistry*. Academic Press, New York, pp 193–204
- Hansen KS, Kristensen C, Tattersall DB, Jones PR, Olsen CE, Bak S, Møller BL (2003) The *in vitro* substrate regio-specificity of recombinant UGT85B1, the cyanohydrin glucosyltransferase from *Sorghum bicolor*. *Phytochemistry* 64: 143–151
- Hirota M, Kuroda R, Suzuki H, Yoshikawa T (2000) Cloning and expression of UDP-glucose: flavonoid 7-O-glucosyltransferase from hairy root cultures of *Scutellaria baicalensis*. *Planta* 210: 1006–1013
- Jacobs M, Rubery PH (1988) Naturally occurring auxin transport regulators. *Science* 24: 346–349
- Kandaswami C, Perkins E, Soloniuk DS, Drzewiecki G, Middleton E Jr (1991) Antiproliferative effects of citrus flavonoids on a human squamous cell carcinoma *in vitro*. *Cancer Lett* 56: 147–152
- Kramer CM, Prata RTN, Willits MG, Luca VD, Steffens JC, Graser G (2003) Cloning and regiospecificity studies of two flavonoid glucosyltransferases from *Allium cepa*. *Phytochemistry* 64: 1069–1076
- Kuo M-L, Lin J-K, Huang T-S, Yang N-C (1994) Reversion of the transformed phenotypes of v-H-ras NIH3T3 cells by flavonoids through attenuating the content of phosphotyrosine. *Cancer Lett* 87: 91–97
- Ljung K, Ostin A, Lioussane L, Sandberg G (2001) Developmental regulation of indole-3-acetic acid turnover in Scots pine seedlings. *Plant Physiol* 125: 464–475
- Manzano D, Busquets A, Closa M, Hoyerova K, Schaller H, Kaminek M, Arro M, Ferrer A (2006) Overexpression of farnesyl diphosphate synthase in *Arabidopsis* mitochondria triggers light-dependent lesion formation and alters cytokinin homeostasis. *Plant Mol Biol* 61: 195–213
- Martin RC, Mok MC, Mok DW (1999) A gene encoding the cytokinin enzyme zeatin O-xylosyltransferase of *Phaseolus vulgaris*. *Plant Physiol* 120: 553–558
- Miksicek RJ (1993) Commonly occurring plant flavonoids have estrogenic activity. *Mol Pharmacol* 44: 37–43
- Nagashima S, Hirota M, Yoshikawa T (2000) Purification and characterization of UDP-glucuronate: baicalein 7-O-glucuronosyltransferase from *Scutellaria baicalensis* Georgi. cell suspension cultures. *Phytochemistry* 53: 533–538
- Normanly J, Cohen JD, Fink GR (1991) *Arabidopsis thaliana* auxotrophs reveal a tryptophan-independent biosynthetic pathway for indole-3-acetic acid. *Proc Natl Acad Sci USA* 90: 10355–10359
- Pflugmacher S, Sandermann H (1998) Taxonomic distribution of plant glucosyltransferases acting on xenobiotics. *Phytochemistry* 49: 507–511
- Poppenberger B, Berthiller F, Lucyshyn D, Sieberer T, Schuhmacher R, Krska R, Kuchler K, Glössl J, Luschnig C, Adam G (2003) Detoxification of the Fusarium mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *J Biol Chem* 278: 47905–47914
- So FV, Guthrie N, Chambers AF, Carroll KK (1997) Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. *Cancer Lett* 112: 127–133
- Szerszen JB, Szczyglowski K, Bandurski RS (1994) *laglu*, a gene from *Zea mays* involved in conjugation of growth hormone indole-3-acetic acid. *Science* 265: 1699–1701
- Taguchi G, Ubukata T, Hayashida N, Yamamoto H, Okazaki M (2003) Cloning and characterization of a glucosyltransferase that reacts on 7-hydroxyl group of flavonol and 3-hydroxyl group of coumarin from tobacco cells. *Arch Biochem Biophys* 420: 95–102
- Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh, SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* 27: 581–590
- Woo HH, Brigham LA, Hawes MC (1994) Primary structure of the mRNA encoding a 16.5-kDa ubiquitin-conjugating enzyme of *Pisum sativum*. *Gene* 148: 369–370
- Woo HH, Orbach M, Hirsch AM, Hawes MC (1999) Meristem-localized inducible expression of a UDP-glucosyltransferase gene is essential for growth and development in pea and alfalfa. *Plant Cell* 11: 2303–2316
- Woo HH, Faull KF, Hirsch AM, Hawes MC (2003) Altered life cycle in *Arabidopsis thaliana* plants expressing PsUGT1, a UDP-glucuronosyltransferase encoding gene from *Pisum sativum*. *Plant Physiol* 133: 538–548
- Woo HH, Hirsch AM, Hawes MC (2004) Altered susceptibility to infection by bacteria and fungi in alfalfa roots with altered cell cycle. *Plant Cell Rep* 22: 967–973
- Woo HH, Jeong BR, Hawes MC (2005) Flavonoids: cell cycle regulations and biotechnology. *Biotechnol Lett* 27: 365–374
- Woo HH, Jeong BB, Hirsch AM, Hawes MC (2007) Characterization of *Arabidopsis* AtUGT85A and AtGUS gene families and their expression in rapidly dividing tissues. *Genomics*, in press