

RESEARCH PAPER

Multi-substrate flavonol *O*-glucosyltransferases from strawberry (*Fragaria* × *ananassa*) achene and receptacle

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Abstract

In an effort to characterize fruit ripening-related genes functionally, two glucosyltransferases, *FaGT6* and *FaGT7*, were cloned from a strawberry (*Fragaria* × *ananassa*) cDNA library and the full-length open reading frames were amplified by rapid amplification of cDNA ends. *FaGT6* and *FaGT7* were expressed heterologously as fusion proteins in *Escherichia coli* and target protein was purified using affinity chromatography. Both recombinant enzymes exhibited a broad substrate tolerance *in vitro*, accepting numerous flavonoids, hydroxycoumarins, and naphthols. *FaGT6* formed 3-*O*-glucosides and minor amounts of 7-*O*-, 4'-*O*-, and 3'-*O*-monoglucosides and one diglucoside from flavonols such as quercetin. *FaGT7* converted quercetin to the 3-*O*-glucoside and 4'-*O*-glucoside and minor levels of the 7- and 3'-isomers but formed no diglucoside. Gene expression studies showed that both genes are strongly expressed in achenes of small-sized green fruits, while the expression levels were generally lower in the receptacle. Significant levels of quercetin 3-*O*-, 7-*O*-, and 4'-*O*-glucosides, kaempferol 3-*O*- and 7-*O*-glucosides, as well as isorhamnetin 7-*O*-glucoside, were identified in achenes and the receptacle. In the receptacle, the expression of both genes is negatively controlled by auxin which correlates with the ripening-related gene expression in this tissue. Salicylic acid, a known signal molecule in plant defence, induces the expression of both genes. Thus, it appears that *FaGT6* and *FaGT7* are involved in

the glucosylation of flavonols and may also participate in xenobiotic metabolism. The latter function is supported by the proven ability of strawberries to glucosylate selected unnatural substrates injected in ripe fruits. This report presents the first biochemical characterization of enzymes mainly expressed in strawberry achenes and provides the foundation of flavonoid metabolism in the seeds.

Key words: Achenes, detoxification, flavonols, *Fragaria* × *ananassa*, multisubstrate glucosyltransferase, plant defence, salicylic acid, strawberry, xenobiotics.

Introduction

Plant glycosyltransferases transfer activated sugar molecules to a broad range of endogenous and xenobiotic substrates. The enzymes exhibit a rather broad acceptor tolerance, but have been shown to display strict regioselectivity in many cases (Vogt and Jones, 2000). Glycosylation is an essential modification contributing to the complexity of secondary metabolites in plants, which is reflected in the fact that more than 300 different glycosides of quercetin have been detected so far (Jones and Vogt, 2001).

Strawberry (*Fragaria* × *ananassa*) is one of the most popular fruit crops worldwide. The so-called fruit is actually an aggregate composed of the swollen receptacle and numerous achenes (Hancock, 1999). Fruit ripening is triggered by a decline in the concentration of auxin which

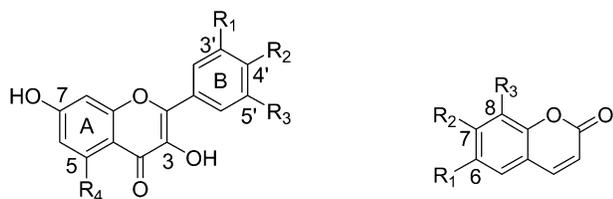
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is released by the achenes in early developmental stages (Perkins-Veazie, 1995). Quercetin and kaempferol (Fig. 1) have been reported to be the most important flavonols in strawberry fruits (Häkkinen *et al.*, 1999). The glycosylation pattern of strawberry flavonols is quite complex. Seven different glycosides of kaempferol alone have been identified. It was reported that 3-*O*-glucosides and 3-*O*-glucuronides of quercetin and kaempferol, as well as kaempferol 7-*O*-glucoside, occur in strawberry fruits (Ryan, 1971). Additionally, 3-*O*-xylosylglucuronides and minor amounts of 3,7-*O*-diglycosides were identified (Henning, 1981). Achenes and green fruits contain high amounts of total phenolics (Wang and Lin, 2000; Aaby *et al.*, 2005), but besides anthocyanin profiles no detailed study about the polyphenolic composition of the achenes has been performed so far.

Glycosyltransferases that transfer sugars to either hydroxy group 3 or 7 of flavonols in a regioselective way have been described in a number of plants including *Petunia hybrida*, *Scutellaria baicalensis*, and *Arabidopsis thaliana* (Miller *et al.*, 1999; Hirotani *et al.*, 2000; Jones *et al.*, 2003). However, some glycosyltransferases from *Allium cepa* and *Arabidopsis thaliana* show no strict regioselectivity and glucosylate the hydroxy groups 3, 7, 3', and 4', and even form diglycosides in some cases (Kramer *et al.*, 2003; Lim *et al.*, 2004). In strawberries, flavonoid 3-*O*-glucosyltransferase activity was detected, but the respective protein was not purified (Given *et al.*, 1988; Halbwrith *et al.*, 2006). Although a transferase with broad substrate tolerance glucosylating quercetin at position 7 was purified from strawberry fruit (Cheng *et al.*, 1994), little is known about the enzymes that form the complex array of glycosylated flavonoids or their spatial and temporal regulation in *Fragaria*.



Flavonol	R ₁	R ₂	R ₃	R ₄	Coumarin	R ₁	R ₂	R ₃
Galangin	H	H	H	OH	Esculetin	OH	OH	H
Fisetin	OH	OH	H	H	Daphnetin	H	OH	OH
Kaempferol	H	OH	H	OH	Scopoletin	OCH ₃	OH	H
Quercetin	OH	OH	H	OH				
Isorhamnetin	OCH ₃	OH	H	OH				
Myricetin	OH	OH	OH	OH				

Fig. 1. Flavonols and hydroxycoumarins are substrates of FaGT6 and FaGT7 *in vitro*. Glycosides of quercetin and kaempferol are the main flavonols in strawberries (Ryan, 1971; Henning, 1981; Häkkinen *et al.*, 1999) while hydroxycoumarins have not been detected in strawberry fruits.

As sedentary organisms, plants have evolved remarkable mechanisms to protect themselves from pathogens, herbivores, and xenobiotics. One strategy is the synthesis of preformed defence compounds such as cyanogenic glucosides and glucosinolates (Osborn, 1996). Additionally, plants possess a flexible detoxification system. The conjugation with glucose leads to increased water solubility and reduced toxicity of most compounds. The respective reaction products can be subsequently stored in the plant vacuole (Jones and Vogt, 2001). Several glycosyltransferases that were cloned from cell cultures of *Nicotiana tabacum* exhibited broad substrate tolerance and were induced by salicylic acid (SA). They are assumed to be involved in the detoxification of xenobiotic compounds (Taguchi *et al.*, 2001, 2003a, b). Other inducible glycosyltransferases from *Arabidopsis thaliana* were shown to form both *N*- and *O*-glucosides of 3, 4-dichloroaniline and 2,4,5-trichlorophenol (Loutre *et al.*, 2003) or to accept the *Fusarium* mycotoxin desoxynivalenol (Poppenberger *et al.*, 2003). The glycosyltransferase with the broadest substrate tolerance reported so far was found in *Rauwolfia serpentina*. The enzyme accepts a total of 45 different substrates out of 74 tested. This includes simple phenolics, phenylpropanoids, flavonoids, and hydroxycoumarins (Hefner *et al.*, 2002).

As a result of an ongoing project on strawberry glycosyltransferases, the cloning and biochemical characterization of the first two glucosyltransferases that are strongly expressed in achenes are reported here. Both enzymes show a rather broad substrate tolerance *in vitro* and glucosylate a range of endogenous and xenobiotic substrates. Gene expression studies combined with metabolite analyses suggest that both enzymes are involved in the biosynthesis of flavonols in achenes and the receptacle, while protein modelling studies revealed essential amino acids in the active sites of the enzymes.

Materials and methods

Plant material

The strawberry fruits were sampled from *Fragaria × ananassa* cv. Elsanta and Camarosa. Elsanta and Camarosa are octaploid cultivars that were grown under field conditions in Freising, Germany and Huelva, in the south-western part of Spain, respectively. Small-sized green and fully ripe red fruits were harvested and immediately frozen in liquid nitrogen and stored at -80 °C until used.

Chemicals

Except when otherwise stated, all chemicals, solvents, and reference compounds were obtained from Sigma, Aldrich, Fluka, Riedel de Haën (all Taufkirchen, Germany), Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany). Anthocyanidins were purchased from Polyphenols Laboratories (Sandnes, Norway). Quercetin 7-*O*-glucoside was a donation of Thilo Fischer (Chair for Ornamental Crops, Technical University Munich). Radiolabelled [6-³H]UDP-glucose (1 mCi ml⁻¹, 60 Ci mmol⁻¹) was obtained from American Radiolabelled Compounds (St Louis, MO, USA).

Rapid amplification of cDNA ends (RACE)

Two partial sequences obtained from a strawberry cDNA library were identified as putative glucosyltransferases and named *FaGT6* and *FaGT7*. As both clones already contained the poly(A)-tail at the 3' end of the open reading frame (ORF), only the 5' end had to be amplified by RACE (Schaefer, 1995). RNA was extracted from strawberry fruits (*Fragaria×ananassa*) according to a protocol originally developed for banana fruit (Asif *et al.*, 2000). cDNA was generated using Superscript III (Invitrogen, Karlsruhe, Germany) and gene-specific primers (5'-GTT CAA TAT GGG TCC CAC CGG ATA CA-3' for *FaGT6* and 5'-GTT CCA CCC ACA GTG AGT CAC AAA T-3' for *FaGT7*). Following purification of the cDNA obtained with a commercial kit (Qiagen, Hilden, Germany), a new poly(A)-tail at the 5' end of the sequence was added with terminal transferase (Promega, Madison, WI, USA). The following Touchdown-PCR (Don *et al.*, 1991) was performed with Oligo(dT)18, GeneRacer 3' (both Invitrogen, Karlsruhe, Germany) and a nested gene-specific primer (5'-CTT ACC GTC GGA ACT GAG AGA CTG AA-3' for *FaGT6* and 5'-CAC TTG AGG AGC CCA ATC TCT TAT-3' for *FaGT7*). Because the first RACE did not yield the complete 5' end of the ORFs for both *FaGT6* and *FaGT7*, new primers were designed which are located closer to the 5' end and the RACE was repeated as described before. For reverse transcription, 5'-ATC ACG TAA GGC TTG GAG GTG GA AC-3' (*FaGT6*) and 5'-CCA TAG CTC CTT TCT TCA ATC TCT-3' (*FaGT7*) were used as gene-specific primers. The final Touchdown-PCR was conducted with 5'-ATC CGC AGC TCC AGA GGT GTA GAA C-3' (*FaGT6*) and 5'-CTG ACT CAT CAG GAA ACA CAG GTA-3' (*FaGT7*) as gene-specific primers and the complete coding sequences were obtained.

Construction of expression plasmids

The full-length ORFs were subcloned to the pGEM-T vector (Promega, Madison, WI, USA). For the *FaGT6* construct, 5'-TGA ATT CAT GAA GAA AGC TTC AGA GC-3' was used as forward primer and 5'-TGC GGC CGC CGA GGT TTG AAT TTG ATC G-3' as reverse primer introducing *EcoRI* and *NotI* restriction sites. The ORF of *FaGT7* was amplified with 5'-TGG ATC CAT GGC CAT GGA AAC TAA ATC ATG C-3' as forward primer and 5'-TCT CGA GTT CAA CTA AGC CTC CAA AAG CTA G-3' as reverse primer with *BamHI* and *XhoI* restriction sites. Subsequently, the ORFs were cloned to the pGEX-4T-1 expression vector (Amersham Bioscience, Freiburg, Germany). The identity of the cloned gene was confirmed by sequencing the complete insert (MWG Biotech, Ebersberg, Germany).

Heterologous protein expression

Escherichia coli BL21 (DE3) pLysS cells (Novagen, Schwalbach, Germany) were transformed with the pGEX-4T-1 expression vector containing the complete ORFs of *FaGT6* or *FaGT7*. Cultures were grown overnight at 37 °C in Luria-Bertani medium containing 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol. The next day, the OD₆₀₀ was measured and the cultures were diluted with Luria-Bertani medium containing the appropriate antibiotics to an OD₆₀₀ of 0.06 and a final volume of 800 ml. This culture was grown at 37 °C until the OD₆₀₀ reached 0.4–0.6. After cooling down the culture to 16 °C, 1 mM IPTG was added to induce protein expression. After overnight incubation at 16–18 °C, the cells were harvested by centrifugation and stored at –20 °C.

Cell lysis and protein purification

The GST-fusion proteins were affinity-purified using the GST Bind resin (Novagen) as recommended by the manufacturer. Briefly, cells were resuspended in binding buffer and sonicated three times on

ice. All buffers contained 10 mM β-mercaptoethanol and all steps were performed at 4 °C. The crude protein extract was incubated for 30 min with the GST Bind resin to bind GST fusion protein. The target protein was eluted with GST elution buffer containing glutathione and the protein concentration was determined (Bradford, 1976). The presence of recombinant protein was confirmed by SDS-PAGE and western blot using anti-GST antibodies (Sigma, Taufkirchen, Germany). As *FaGT7* could only be partially purified, the amount of *FaGT7* was quantified using the Alpha Imager 2200 Documentation and Analysis System and the Alpha Ease Software (Alpha Innotech, San Leandro, CA, USA).

Biochemical characterization

The optimization of the assay conditions was carried out with radioactively labelled UDP-glucose. The biochemical characterization was performed with McIlvaine buffer (citrate-phosphate buffer, 5 mM β-mercaptoethanol, 10% glycerol) using 0.6–0.8 µg affinity purified recombinant protein in a total volume of 200 µl. The UDP-glucose was a mixture of 1 µl 0.016 mM [6-³H]UDP-glucose (1 mCi ml⁻¹) and 99 µl unlabelled 101 mM UDP-glucose. The specific reaction conditions for the determination of substrate preferences are given in the text. Assays were incubated for 30 min and extracted with 1 ml water-saturated *n*-butanol. The radioactivity of the product was determined by liquid scintillation counting (LKB Rackbeta 1219, Wallac, Turku, Finland) following the addition of 4 ml Ultima Gold XR LSC cocktail (Perkin-Elmer, Boston, MA, USA). The kinetic constants were calculated with SigmaPlot 8.0 software (Systat Software, Erkrath, Germany) using the regression wizard and presuming a one-site saturation binding.

Identification of formed products

Assays were performed in a buffer containing 100 mM TRIS-HCl (pH 7.0), 10% glycerol, and 10 mM β-mercaptoethanol. Standard assays contained 5 mM UDP-glucose, 200 µM substrate, and crude protein extract with 15–20 µg total protein in a volume of 250 µl. Enzyme assays were incubated for 30 min at 30 °C and stopped by the addition of 200 µl 5 % HCl (anthocyanidin substrates) or 50 µl acetic acid (all other substrates). As a control, BL21 (DE3) pLysS cells were transformed with an empty pGEX-4T-1 vector and the respective protein extract was assayed under the same conditions. Furthermore, assays were conducted with heat-inactivated enzyme solution (5 min at 95 °C). Enzyme activity was monitored by detecting the reaction products with LC-UV-ESI-MSⁿ.

Metabolite analysis in the receptacle and achenes

Strawberry fruits (50 g) were harvested in the green and red ripening stages and were homogenized with 20 ml water using an Ultra Turrax (T18 basic; IKA Works Inc. Wilmington, NC, USA) followed by centrifugation (3500 g, 10 min). The supernatant was collected for metabolite analysis of the receptacle and sedimented achenes were separated from the residue. Achenes (5 g) were homogenized with 5 ml water using a mortar and pestle, centrifuged (3500 g, 10 min), and analysed by LC-UV-ESI-MSⁿ. 4-Methylumbelliferyl glucuronide was added as internal standard.

LC-UV-ESI-MSⁿ

A Bruker Daltonics esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) connected with an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a variable wavelength detector was utilized. Components were separated with a Phenomenex (Aschaffenburg, Germany) Luna C-18 column (150 mm long×2.0 mm inner diameter, particle size 5 µm) which was held at 25 °C. Enzyme assays were analysed using a linear gradient from

100% A (0.1% formic acid in water) to 100% B (acetonitrile) in 30 min with a flow rate of 0.2 ml min⁻¹. To distinguish between flavonoid glucoside isomers and for metabolite analysis in strawberry fruit extracts, the gradient went from 100% A to 40% B in 40 min and in a further 5 min to 100% B. The detection wavelength was either 360 nm for assays (520 nm for anthocyanidin substrates) or 280 nm for metabolite analysis. The electrospray ionization voltage of the capillary was set to -4000 V and the end plate to -500 V. Nitrogen was used as dry gas at a temperature of 300 °C and a flow rate of 10 l min⁻¹. The full scan mass spectra were measured in a scan range from *m/z* 50 to 800 with a scan resolution of 13 000 *m/z/s* until the ICC target reached 20 000 ms or 200 ms, whatever was achieved first. Tandem mass spectrometry was carried out using helium as collision gas (3.56×10⁻⁶ mbar) with the collision voltage set at 1 V. Spectra were acquired in the positive and negative ionization mode. Data analysis was performed using the DataAnalysis 3.1 software (Bruker Daltonics, Bremen, Germany).

Auxin treatment

The hormonal treatment was performed as described previously (Medina-Escobar *et al.*, 1997). Briefly, achenes from mid-sized green fruits were removed carefully with a scalpel blade. One set of fruits was treated with a lanolin paste containing 1 mM 1-naphthaleneacetic acid (NAA) in 1% (v/v) DMSO. The other set of deached fruits was treated with the same paste but without the synthetic auxin NAA. Fruits were harvested 96 h after treatment and immediately frozen in liquid nitrogen and stored at -80 °C.

SA treatment of strawberry fruits

One millilitre of a sterile solution of 0.75 mM SA or sterile water (control) was carefully injected into white-stage fruit still attached to the plants using a hypodermic syringe with a needle gauge. Fruits were harvested at 4, 6, and 8 h, immediately frozen in liquid nitrogen, and stored at -80 °C until RNA isolation.

SA treatment of strawberry cell cultures

To initiate callus cultures, young leaves from micropropagated strawberry plants were removed and cut into strips (3–4 mm wide) with a sterile scalpel and forceps. These explants were transferred to Petri dishes on the surface of solid medium (Murashige and Skoog, 1962), supplemented with 2.5 mg 2,4-dichlorophenoxyacetic acid, with the lower epidermis downward. The dishes were sealed with parafilm and incubated at 25 °C in a growth chamber under diffuse light. When sufficient callus was developed from the explants (4–6 weeks), small pieces (0.3–0.5 cm diameter) were excised and transferred to fresh medium and cultured as before. In this way, callus stocks were maintained indefinitely by sub-culturing to fresh medium at monthly intervals. To initiate cell suspension cultures, 1 g of callus was excised, disaggregated, and transferred to 30 ml of liquid medium in a 100 ml Erlenmeyer flask. These cultures were incubated at 25 °C at 100 rpm under diffuse light. The cell cultures were maintained by sub-culturing 1 g of filtered cells in fresh liquid medium every 15 d. For the SA treatment, 0.5 l of cellular culture refreshed for 5 d was divided in two Erlenmeyer flasks. To 0.25 l of cellular culture, 0.75 mM SA was added, while the same volume of sterile distilled water was added to the other 0.25 l used as control. Both SA and control cellular cultures were incubated at 25 °C at 100 rpm under diffuse light. Cells were harvested by filtering after 4 h and 8 h, immediately frozen in liquid nitrogen, and stored at -80 °C until RNA isolation.

Quantitative PCR (qPCR)

Total RNA was isolated from a pool of six or seven strawberry fruits as described before (Asif *et al.*, 2000). Subsequently, RNA

was treated with DNase I (Amersham Bioscience, Barcelona, Spain) to remove any DNA contamination prior to cDNA synthesis. Reverse transcription was carried out using 1 µg of total RNA and the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) as recommended by the manufacturer. qPCR analysis was performed using the iCycler system (Bio-Rad) as described previously (Benítez-Burraco *et al.*, 2003; Raab *et al.*, 2006). Specific primers for *FaGT6* (5'-CGC AGC GCA TCA GGT TCG TC-3' and 5'-GAT CCG CAG CTC CAG AGG TGT AGA-3') and *FaGT7* (5'-CTC GCG GCC ACA GCA TAC-3' and 5'-GTC GGC ACT TTC GCA ATC TT-3') were designed. The results were normalized with an interspacer 26S-18S RNA gene used as the housekeeping gene (5'-ACC GTT GAT TCG CAC AAT TGG TCA TCG-3' and 5'-TAC TGC GGG TCG GCA ATC GGA CG-3'). PCR reactions contained 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of the respective primers, 2.5 µl SYBR Green I (diluted 1:15000), 2 µl of cDNA, and 0.5 U of Taq polymerase (Biotools, Madrid, Spain) in a total volume of 25 µl. The thermal cycling conditions were as follows: 2 min at 94 °C, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Subsequently, melting point analysis and agarose gel electrophoresis were applied to confirm that the desired amplicon had been generated. In qPCR analysis, quantification is based on the threshold cycle (*C_t*) when the fluorescence is first detectable above background level. The *C_t*-values are inversely proportional to the logarithm of the initial copy number. Each reverse transcription was repeated at least once and every PCR was performed at least in triplicate. All results were normalized in relation to the housekeeping gene. Relative expression values were calculated according to Livak and Schmittgen (2001), normalizing the results to a calibrator (receptacle from full-sized green fruits or untreated control).

Glucosylation of xenobiotics in planta

Ripe strawberry fruits were injected with 10 mM 3-hydroxycoumarin, 2-naphthol (both dissolved in 10% ethanol), or 3-hydroxyflavone (as alkaline aqueous solution) using a syringe with a needle (0.4×20 mm; Braun, Melsungen, Germany). In an independent experiment, 10 mM kaempferol and 2-naphthol were separately injected into fruits and in combination. As controls, the solvents without substrates were used. The fruits were harvested after 1 d and freeze-dried in a lyophilizer (ALPHA 1-4; Martin-Christ, Osterode, Germany). Fifty milligrams of the fine powder were extracted with 250 µl methanol. The solvent was removed in a rotary vacuum concentrator (RVC 2-18; Martin-Christ) and the extract was redissolved in 35 µl water and analysed by LC-UV-ESI-MSⁿ.

Protein modelling

DeepView (<http://www.expasy.org/spdbv/>) software was used for visualization of the amino acids in the protein models (Guex and Peitsch, 1997).

Results

RACE and phylogenetic analysis

FaGT6 and *FaGT7* were identified in a strawberry (*Fragaria×ananassa*) cDNA library and annotated as glycosyltransferases due to their sequence similarities to other known enzymes. To obtain the full-length sequences, RACE was successfully applied to amplify the unknown 5' end of the ORF (Schaefer, 1995). The resulting ORF of *FaGT6* is 1440 bp long and codes for

a protein with 479 amino acids. FaGT7 is a protein of 487 amino acids and is encoded by 1464 bp.

Phylogenetic analysis (Fig. 2) was carried out with the full-length protein sequences of FaGT6, FaGT7, and five other glycosyltransferases from strawberry (FaGT1–5; Griesser *et al.*, 2008). FaGT6 shows highest homology to FaGT3, another uncharacterized glycosyltransferase from strawberry. Similarities were also found to several multi-substrate glycosyltransferases from *Nicotiana tabacum* acting on flavonols, hydroxycoumarins, naphthols, and hydroxycinnamic acids (Taguchi *et al.*, 2001, 2003a, b) and to an enzyme from *Dorotheanthus bellidiformis* which accepts the betacyanin betanidin, but also anthocyanidins and flavonols (Vogt *et al.*, 1997; Vogt, 2002). UGT71D1 and UGT71C2 from *Arabidopsis thaliana* glucosylate flavonols and hydroxycoumarins *in vitro* (Lim *et al.*, 2003, 2004).

FaGT7 belongs to a cluster of several inducible glycosyltransferases including two enzymes from *Nicotiana*

tabacum accepting flavonols, hydroxycoumarins, hydroxycinnamic and hydroxybenzoic acids *in vitro* (Horvath and Chua, 1996; Fraissinet-Tachet *et al.*, 1998; Vogt *et al.*, 1999), and glycosyltransferases from *Scutellaria baicalensis* (Hirotani *et al.*, 2000) and *Dianthus caryophyllus* (Ogata *et al.*, 2004). The betanidin 5-glycosyltransferase from *D. bellidiformis* shows activity with certain flavonols, too (Vogt *et al.*, 1999). UGT73B2, UGT73B4, and UGT73B5 from *A. thaliana* accept quercetin and hydroxycoumarins *in vitro* (Lim *et al.*, 2003, 2004).

Although FaGT6 and FaGT7 are grouped in two different clusters, they can be assigned as putative multi-substrate glycosyltransferases based on their phylogenetic classification.

Enzymatic activity of the recombinant protein

The full-length ORFs of *FaGT6* and *FaGT7* were cloned in the expression vector pGEX-4T-1 and the recombinant enzymes were expressed as GST-fusion proteins in

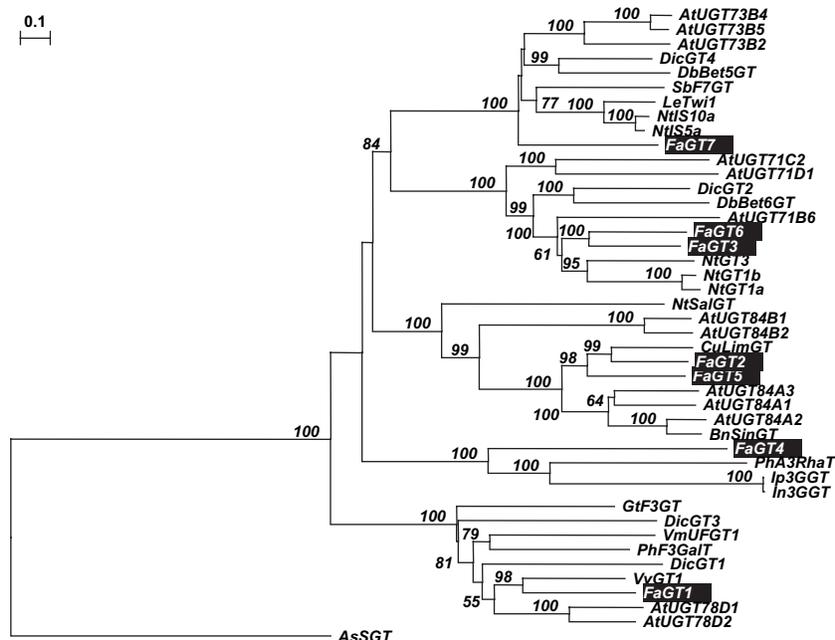


Fig. 2. Phylogenetic analysis of selected plant secondary product glycosyltransferases. The neighbor-joining tree was calculated with the Treecon software package (Van de Peer and De Wachter, 1994). Distance calculation was performed with Poisson correction and insertions/deletions were not taken into account. The tree was rooted using a sterol glycosyltransferase from *Avena sativa* (AsSGT) as an outgroup. Branch lengths indicate the number of substitutions per site. Bootstrap analysis was performed with 100 replicates and only values above 50% are shown. GenBank accession numbers and sources for the respective protein sequences are: AtUGT73B4, AAD17393 (*A. thaliana*); AtUGT73B5, AAD17392 (*A. thaliana*); AtUGT73B2, AAR01231 (*A. thaliana*); DicGT4, BAD52006 (*Dianthus caryophyllus*); DbBet5GT, CAB56231 (*Dorotheanthus bellidiformis*); SbF7G, BAA83484 (*Scutellaria baicalensis*); Letwi1, CAA59450 (*Lycopersicon esculentum*); NtS10a, AAB36653 (*N. tabacum*); FaGT7, ABB 92749 (*F. × ananassa*); AtUGT71C2, AAC35238 (*A. thaliana*); AtUGT71D1, AAC35239 (*A. thaliana*); DicGT2, BAD52004 (*D. caryophyllus*); DbBet6GT, AAL57240 (*D. bellidiformis*); AtUGT71B6, BAB02840 (*A. thaliana*); FaGT6, ABB92748 (*F. × ananassa*); FaGT3, AAU09444 (*F. × ananassa*); NiGT3, BAB88934 (*N. tabacum*); NiGT1b, BAB60721 (*N. tabacum*); NiGT1a, BAB60720 (*N. tabacum*); NtSalGT, AAF61647 (*N. tabacum*); AtUGT84B1, AAB87119 (*A. thaliana*); AtUGT84B2, AAB36652 (*N. tabacum*); CuLimGT, BAA93039 (*Citrus unshiu*); FaGT2, AAU09443 (*F. × ananassa*); FaGT5, ABB92747 (*F. × ananassa*); AtUGT84A3, CAB10327 (*A. thaliana*); AtUGT84A1, CAB10326 (*A. thaliana*); AtUGT84A2, BAB02351 (*A. thaliana*); BnSinGT, AAF98390 (*Brassica napus*); FaGT4, AAU09445 (*F. × ananassa*); PhA3RhaT, CAA81057 (*Petunia hybrida*); Ip3GGT, BAD95882 (*Ipomea purpurea*); In3GGT, BAD95881 (*I. nil*); GtF3GT, BAA12737 (*Gentiana triflora*); DicGT3, BAD52005 (*D. caryophyllus*); VmUFGT1, BAA36972 (*Vigna mungo*); PhF3GalT, AAD55985 (*P. hybrida*); DicGT1, BAD52003 (*D. caryophyllus*); VvGT1, AAB81682 (*Vitis vinifera*); FaGT1, AAU09442 (*F. × ananassa*); AtUGT78D1, NP_564357 (*A. thaliana*); AtUGT78D2, NP_197207 (*A. thaliana*); AsSGT, CAB06081 (*A. sativa*).

E. coli. FaGT6 and FaGT7 were affinity-purified using an immobilized reduced glutathione resin. The presence of target protein was confirmed by SDS-PAGE and western blot using antibodies against the GST domain at the N-terminus of the fusion enzyme (data not shown). Although GST-fusion proteins may not necessarily share all biochemical properties of the native enzymes, the use of recombinant proteins is widely accepted as a valuable tool to study plant secondary product glucosyltransferases (Vogt and Jones, 2000).

Initial activity tests were performed with [$6\text{-}^3\text{H}$]UDP-glucose and various flavonols. FaGT6 readily showed activity with most flavonols tested (3-hydroxyflavone, 3,7-dihydroxyflavone, galangin, kaempferol, quercetin, myricetin, isorhamnetin, and morin), but not with 5-hydroxyflavone and 7-hydroxyflavone (Fig. 3A). Flavonones (taxifolin and naringenin) and some anthocyanidins (malvidin, peonidin, and petunidin) were also accepted, while delphinidin, cyanidin, and pelargonidin could not serve as substrates. Additionally, FaGT6 glucosylated several hydroxycoumarins (3-, 6-, and 7-hydroxycoumarin, esculetin, daphnetin, and scopoletin) as well as 1-naphthol and 2-naphthol. Other substrates such as coumarin, 4-hydroxycoumarin, 7-methoxycoumarin, catechin, epicatechin, *p*-coumaric acid, 4-hydroxybenzoic acid, and 4-hydroxy-2,5-dimethyl-3-(2*H*)-furanon were not con-

verted. UDP-glucuronic acid and UDP-galactose were not accepted as donors with quercetin as acceptor.

FaGT7 showed a similar substrate tolerance and accepted all flavonols tested (3-hydroxyflavone, 3,7-dihydroxyflavone, galangin, kaempferol, quercetin, myricetin, and isorhamnetin) as well as certain flavones (7-hydroxyflavone, chrysin), and flavanones (taxifolin), while anthocyanidins (pelargonidin and malvidin), catechin, naringenin, apigenin, and 5-hydroxyflavone were not accepted (Fig. 3B). Selected hydroxycoumarins (3- and 7-hydroxycoumarin, esculetin, daphnetin, and scopoletin) as well as 1-naphthol and 2-naphthol were also converted. However, 4-hydroxycoumarin, 6-hydroxycoumarin, and 7-methoxycoumarin could not serve as substrates. No activity could be detected with several hydroxybenzoic and hydroxycinnamic acids and the important flavour compound 4-hydroxy-2,5-dimethyl-3-(2*H*)-furanon. UDP-glucuronic acid and UDP-galactose were not substrates when assayed with quercetin and isorhamnetin.

A comparison of the substrate preferences of the two glucosyltransferases reveals that galangin, isorhamnetin, and 3-hydroxycoumarin are the preferred acceptors in both cases. FaGT6 also showed high activity with 3-hydroxyflavone and elevated activities with 2-naphthol and scopoletin. Both glycosyltransferases exhibited a rather low relative activity with the abundant natural

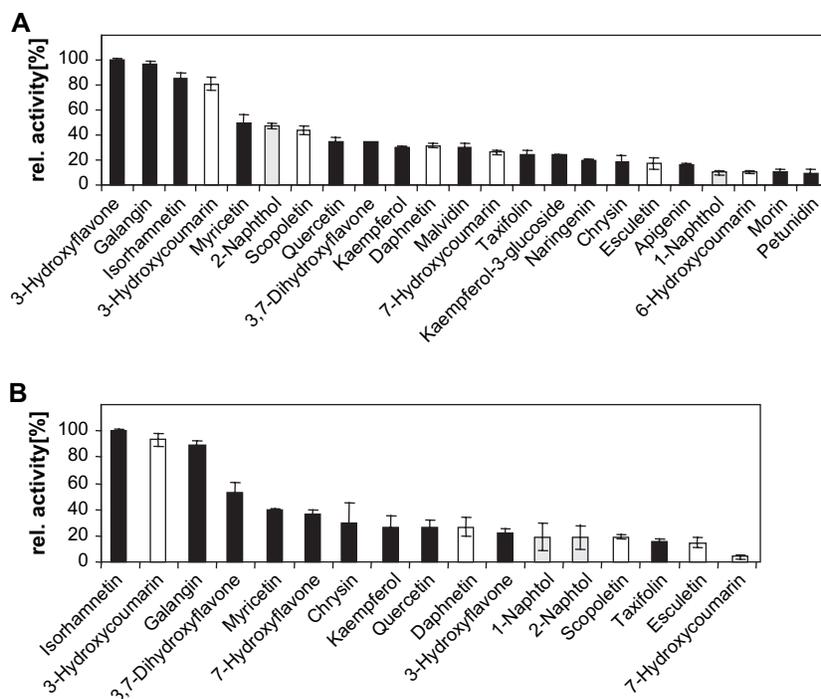


Fig. 3. Substrate preference of the recombinant enzymes. Enzymatic activity was measured with liquid scintillation counting testing a range of flavonoids (black columns), hydroxycoumarins (open columns), and naphthols (shaded columns) with radioactive donor substrate [$6\text{-}^3\text{H}$]UDP-glucose as described in Materials and methods. FaGT6 (A) reactions were performed with 30 min incubation time in McIlvaine buffer (pH 7.5) at 30 °C. Relative activities refer to the best substrate 3-hydroxyflavone (100%). FaGT7 (B) reactions were performed with 30 min incubation time in McIlvaine buffer (pH 6.5) at 35 °C. Relative activities refer to the best substrate isorhamnetin (100%).

substrates quercetin and kaempferol. In accordance with the phylogenetic assignment, FaGT6 and FaGT7 are multi-substrate glucosyltransferases accepting a broad range of natural and xenobiotic substrates *in vitro* and show high activity with substrates that have not been detected in strawberry fruits until now.

Biochemical characterization

The assay conditions for FaGT6 were optimized using the endogenous substrate quercetin and [6-³H]UDP-glucose. FaGT6 showed maximum activity at 35 °C and pH 7.5–8.0 in McIlvaine buffer, and product formation was linear for at least 30 min. The optimum reaction conditions for FaGT7 were determined with 3-hydroxycoumarin. The highest activity was monitored in McIlvaine buffer (pH 6.5) at 45 °C, and product formation was linear for at least 60 min.

Kinetic constants were determined for UDP-glucose and 3-hydroxyflavone (FaGT6) or isorhamnetin (FaGT7). Different concentrations of the flavonol substrate were assayed keeping the level of UDP-glucose constant and vice versa. The apparent K_m and V_{max} values were obtained from the hyperbolic Michaelis–Menten saturation curves. The K_m and V_{max} values for 3-hydroxyflavone were determined to be 5.3 μ M and 1.4 nkat mg⁻¹, respectively. As sugar donor for FaGT6, UDP-glucose showed a K_m of 2.0 mM and a V_{max} of 0.5 nkat mg⁻¹. As acceptor substrate for FaGT7, isorhamnetin exhibited K_m and V_{max} values of 3.5 μ M and 2.8 nkat mg⁻¹, respectively. For UDP-glucose, a K_m of 0.6 mM and a V_{max} of 2.7 nkat mg⁻¹ were determined.

Identification of reaction products

The substrate screening indicated already that FaGT6 and FaGT7 probably act on the hydroxyl group at position 3 of flavonols. Thus, the products formed from isorhamnetin, kaempferol, and quercetin with UDP-glucose as the donor were analysed by LC-UV-ESI-MSⁿ. The ion traces indicated that besides one major product, two or three additional monoglucosides were produced by FaGT6 and FaGT7 while diglucosides were only formed by FaGT6 (Fig. 4A–C). Using authentic reference compounds, the major product formed from quercetin was identified as the 3-*O*-glucoside while the minor products were assigned as 7-*O*-, 3'-*O*-, and 4'-*O*-glucoside (Fig. 4C), according to the published elution order of the isomers (Lim *et al.*, 2004; Shao *et al.*, 2005) and their mass spectra (Ablajan *et al.*, 2006). The fragmentation behaviour of flavonoid mono-*O*-glucosides can be used to distinguish between the different regioisomers. The relative abundance of the radical aglycone ion [Y-H][•] (compared with [Y]⁻) shows a close correlation with the glycosylation site. The product ion spectra of 3-*O*- and 7-*O*-glucosylated isomers of quercetin (kaempferol and isorhamnetin) show clear differences in the abundances of the fragment ions *m/z*

300 and 301 (*m/z* 284 and 285 as well as *m/z* 314 and 315), respectively (Fig. 4D–F). Similar to quercetin, the 3-*O*-glucoside constituted the major product when kaempferol and isorhamnetin were used as substrates for FaGT6, while the 7-*O* and 4'-*O*-glucosides of the flavonols were only produced in small amounts. The product ion spectra of the diglucosides revealed that both glucose moieties are located at different hydroxy groups of the flavonols. Only in this case, the daughter ions *m/z* 477, 447, and 463 can be detected, because a covalent linkage between two sugar molecules would not be cleaved effectively under the LC-UV-ESI-MS² conditions applied (Fig. 4D–F insets; Giusti *et al.*, 1999; Ablajan *et al.*, 2006).

FaGT7 catalysed the formation of two major and two minor quercetin-monoglucosides as revealed by LC-UV-ESI-MSⁿ (Fig. 4C). The 3-*O*- and 4'-*O*-glucosides were produced at almost equal amounts in addition to low levels of the 7-*O*- and 3'-*O*-isomers. Diglucosides were not detected. When kaempferol was used as the substrate the abundance of the 4'-*O*-glucoside was much lower and the 3'-*O*-glucoside could not be formed due to the missing 3'-hydroxyl group in kaempferol. The 4'-*O*-glucoside of isorhamnetin was only produced in trace amounts because the bulky 3'-methoxy group in the *ortho* position probably inhibits the attachment of a glucose molecule at the adjacent hydroxyl group. Instead, large amounts of the 3-*O*-glucoside were formed in addition to minor levels of the 7-*O*-isomer.

Metabolite profiling

Levels of the different flavonol (quercetin, kaempferol, and isorhamnetin) glucoside isomers were quantified by LC-UV-ESI-MS² by means of the internal standard 4-methyl umbelliferyl glucuronide in extracts obtained from strawberry achenes and receptacles of small-sized green and ripe red fruits (Table 1). Kaempferol 3-*O*- and 7-*O*-, quercetin 3-*O*- and 4'-*O*-, and isorhamnetin 7-*O*-glucosides were detected in the achenes and receptacle of both ripening stages. In general, almost all potential products of the glucosyltransferases were found in the two tissues. Levels of 3-*O*-glucosides increased throughout ripening. Kaempferol 3-*O*-glucoside constituted the major flavonol glucoside in red achenes while almost equal amounts of kaempferol- and quercetin 3-*O*-glucoside were isolated from the red receptacle. Isorhamnetin occurred mainly as 7-*O*-glucoside.

Gene expression in achenes and receptacle

Transcript levels of *FaGT6* and *FaGT7* were determined by qPCR. RNA was extracted from the receptacle and achenes of small-sized green and red fruits. Following reverse transcription, the respective mRNA levels were quantified using sequence-specific primers. Transcript levels of *FaGT6* are higher in achenes than in the receptacle of

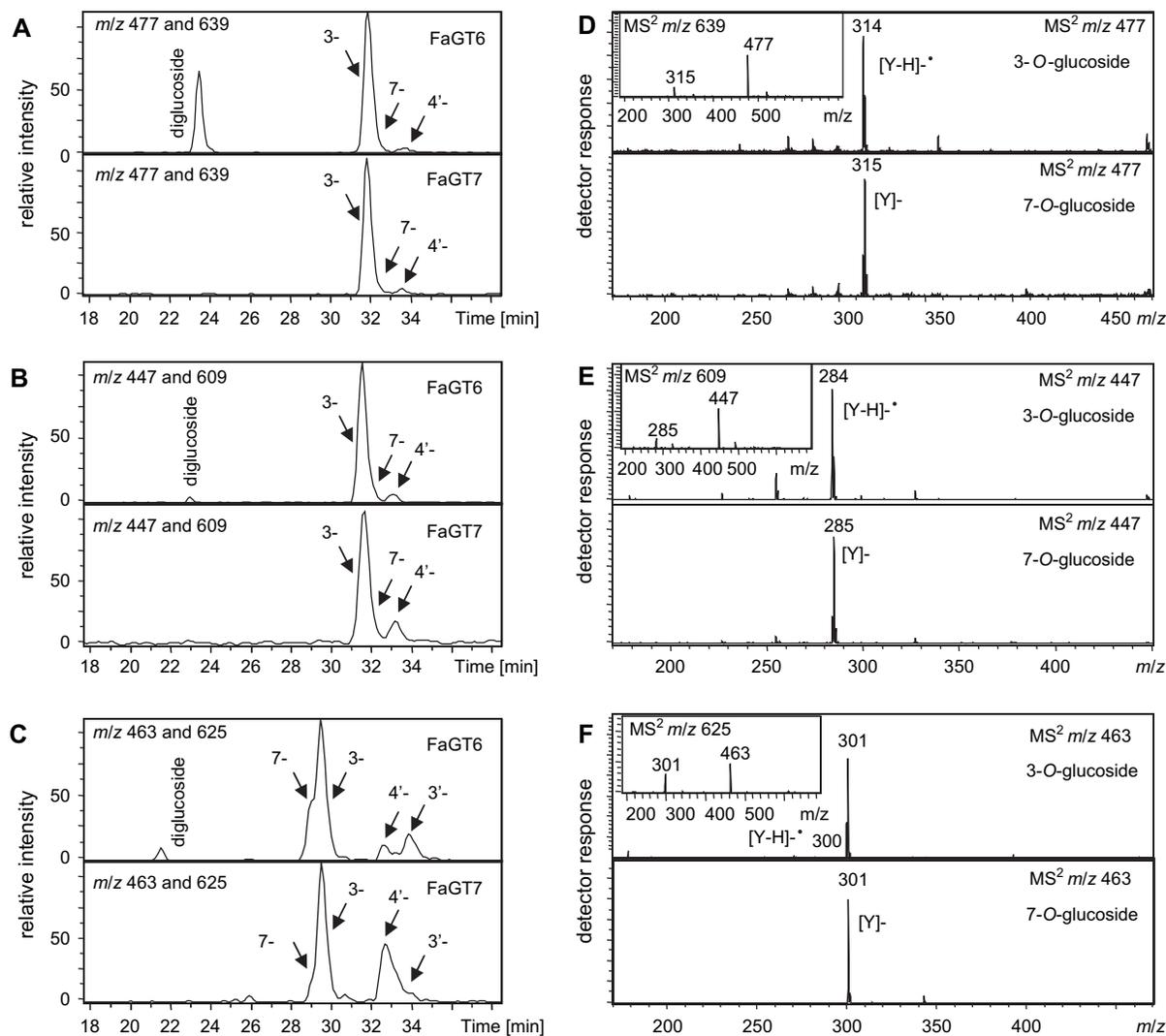


Fig. 4. Identification of enzymatically formed products. Isorhamnetin (A), kaempferol (B), and quercetin (C) were incubated with UDP-glucose and recombinant FaGT6 (top) or FaGT7 (bottom) and subsequently analysed by LC-UV-ESI-MSⁿ. Products were tentatively identified by means of authentic references or by comparison with published data (Giusti *et al.*, 1999; Ablajan *et al.*, 2006). Coeluting 3-*O* (top) and 7-*O*-glucosides (bottom) of isorhamnetin (D), kaempferol (E), and quercetin (F) were clearly distinguishable by their product ion spectra (MS²) according to Ablajan *et al.* (2006). The MS² spectra of additional compounds formed by FaGT6 from isorhamnetin (inset in D), kaempferol (inset in E), and quercetin (inset in F) demonstrated the loss of two glucose molecules that are attached to different hydroxyl groups of the flavonols. They were tentatively identified as diglucoside, probably 3,7-*O*-diglucosides (Ablajan *et al.*, 2006).

small-sized green and red fruits (Fig. 5A). The expression in achenes does not change in these two developmental stages while the expression in the receptacle is ripening-related. *FaGT7* shows higher expression in achenes than in the receptacle of small-sized green fruits (Fig. 5B). In the receptacle, the expression is ripening-related and transcript levels in red fruits reach a level comparable to achenes of small-sized green fruit. *FaGT6* and *FaGT7* show similar relative expression levels in the receptacle. In conclusion, both glucosyltransferases display an achene-related gene expression different from other known strawberry glucosyltransferases (Lunkenbein *et al.*, 2006; Griesser *et al.*, 2008).

Hormonal control of developmental expression

In early developmental stages, auxin is synthesized by the achenes and subsequently transported to the receptacle (Perkins-Veazie, 1995). It has been reported that a range of strawberry ripening-related genes are negatively regulated by auxin (Medina-Escobar *et al.*, 1997; Manning, 1998; Aharoni *et al.*, 2002; Lunkenbein *et al.*, 2006; Raab *et al.*, 2006; Griesser *et al.*, 2008). Because expression levels of *FaGT6* and *FaGT7* increase during fruit ripening in the receptacle, the influence of auxin on the respective transcript levels was studied. Mid-sized green fruits were carefully de-achened and gene expression was analysed by qPCR after 5 d. Additionally, de-achened fruits were

treated with a lanolin paste containing NAA, a synthetic auxin. Gene expression of both glucosyltransferases is induced after removing the achenes (Fig. 5C, D). Furthermore, the induction of *FaGT6* and *FaGT7* can be repressed by the subsequent application of a synthetic auxin. This indicates that the expression of both genes in the receptacle is indeed under the control of auxin.

SA-induced gene expression

To determine whether *FaGT6* and *FaGT7* could play a role in plant defence responses, their gene expression

Table 1. Quantification of flavonol glucosides in *Fragaria × ananassa* cv. *Elsanta* achenes and receptacle of different ripening stages

Values are expressed as mg-equiv. kg⁻¹ 4-methyl umbelliferyl glucuronide. Shown are the mean values of duplicates which differed by <20%.

	Achene		Receptacle	
	Green	Red	Green	Red
Kaempferol 3- <i>O</i> -glucoside	8.3	82.8	2.2	15.5
Kaempferol 7- <i>O</i> -glucoside	4.8	10.4	0.3	6.9
Kaempferol 4'- <i>O</i> -glucoside	<0.1	<0.1	0.2	<0.1
Quercetin 3- <i>O</i> -glucoside	3.0	9.8	0.5	15.2
Quercetin 7- <i>O</i> -glucoside	1.0	2.4	0.2	<0.1
Quercetin 4'- <i>O</i> -glucoside	14.2	7.3	0.3	3.4
Isorhamnetin 3- <i>O</i> -glucoside	<0.1	<0.1	0.6	3.1
Isorhamnetin 7- <i>O</i> -glucoside	7.2	8.0	1.3	0.4
Isorhamnetin 4'- <i>O</i> -glucoside	<0.1	<0.1	<0.1	0.2

was studied by qPCR in strawberry cell cultures and strawberry fruits treated with SA. Treatment of strawberry cells with SA induced the accumulation of *FaGT6* and *FaGT7* transcripts by a factor of 5–6 already 4 h after treatment. Transcripts remained at this high level until 6 h and then declined to negative values (–5 to –4) in relation to a housekeeping gene (Fig. S1A, B in Supplementary data available at *JXB* online). Furthermore, in strawberry fruits injected with SA, the levels of both transcripts increased by a factor of 5–6 as soon as 4 h after injection, remained stable until 6 h, and fell below control levels until 8 h (Fig. S1C, D in Supplementary data available at *JXB* online). *FaGT6* and *FaGT7* genes show a similar expression profile both in SA-treated cells and SA-injected fruits, suggesting that these genes are co-regulated under these conditions.

Glucosylation of xenobiotics in planta

Both *FaGT6* and *FaGT7* were able to glucosylate a number of exogenous substrates *in vitro*. To test if the enzymes could exhibit the same functionality *in vivo*, selected substrates were injected into ripe fruits. 3-Hydroxyflavone and 3-hydroxycoumarin are both natural compounds which do not occur in strawberry fruits, while 2-naphthol is a synthetic chemical. One day after injection, the fruits were harvested, extracted with methanol, and analysed by LC-UV-ESI-MS². Fruits injected with solvents served as controls. The substrates and their glucosylated products were not detected in any of the controls. By contrast, in all fruits injected with one of the

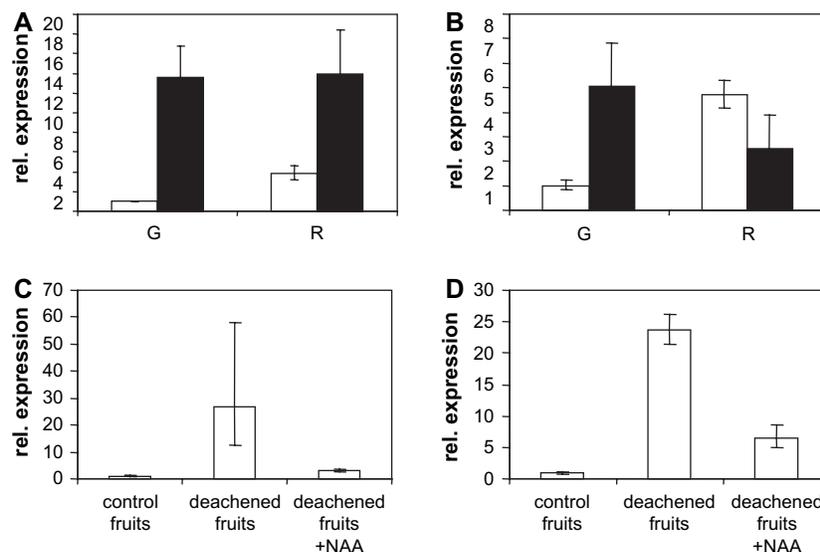


Fig. 5. Gene expression of *FaGT6* (A) and *FaGT7* (B) in achenes (black columns) and the receptacle (open columns) of small-sized green (G) and red (R) fruits. Transcript levels were analysed by qPCR as described in Materials and methods. Gene expression is shown as relative expression normalized with receptacle tissue from small-sized green fruit (G). Hormonal control of *FaGT6* (C) and *FaGT7* (D) gene expression. The achenes were carefully removed at mid-sized green stage and the fruits were harvested after 5 d. Additionally, deached green fruits were treated with a lanolin paste containing the synthetic auxin NAA. Gene expression was analysed by qPCR as described in Materials and methods and the data were normalized against untreated strawberries with the achenes still attached to the fruit.

selected substrates, the respective glucoside could be detected and its formation was confirmed by product ion experiments (Fig. S2 in Supplementary data available at *JXB* online). Thus, it could be shown that strawberry fruit has the ability to glucosylate selected exogenously applied substrates of FaGT6 and FaGT7 *in vivo*. To demonstrate that xenobiotics and natural substrates compete for glucosylation, equal amounts of kaempferol and 2-naphthol were injected into strawberry fruits separately and in combination. Quantification of the levels of produced glucosides showed that lower amounts of 2-naphthol glucoside and especially kaempferol glucosides were produced when both substrates were offered at once, indicating a competition of the substrates for the glucosyltransferases (Fig. S2 in Supplementary data available at *JXB* online).

Protein modelling

Although FaGT6 and FaGT7 display similar substrate donor and acceptor preference and comparable kinetic data, they show low amino acid identities and can be clearly distinguished by the products they form (Fig. 6). To understand the structural basis for the different product tolerance of FaGT6 and FaGT7, their amino acid sequences and the sequences of VvGT1 from grape and UGT71G1 from *Medicago truncatula* were aligned. The 3D structures of the latter enzymes have already been elucidated and these proteins also glucosylate kaempferol and quercetin. VvGT1 forms regioselectively only 3-*O*-glucosides, while UGT71G1 represents a multi-product enzyme (Shao *et al.*, 2005; Offen *et al.*, 2006). Conserved amino acids identified by the ClustalX program in the four sequences were visualized by DeepView (<http://www.expasy.org/spdbv/>) in the Michaelis complex of VvGT1 (Guex and Peitsch 1997; Offen *et al.*, 2006; Fig. S3 in Supplementary data available at *JXB* online). Although kaempferol is used as glucose acceptor by all four glucosyltransferases, only the catalytically active His23 and Asp128 as well as Phe130 and Leu156 are conserved in the flavonol binding pocket. By contrast, the amino acids assumed to be responsible for UDP-glucose binding are mainly residues of the UGT signature PSPG motif (residues 370–413) and are well conserved, e.g. Trp370, Ala371, Gln373, His388, Trp391, Asn392, Ser393, Glu396, and Gln413 (Shao *et al.*, 2005; Offen *et al.*, 2006). Next, the amino acids that lie within 5 Å from kaempferol in the Michaelis complex of VvGT1 were visualized (Fig. S4 in Supplementary data available at *JXB* online). To compare the binding pockets of the four glucosyltransferases, the amino acids in close vicinity of kaempferol in the VvGT1 model were substituted by amino acids that were aligned in the same positions of FaGT6 and FaGT7 according to the ClustalX alignment. Although this simple procedure does not exactly describe the 3D structures of the active sites, the models obtained

demonstrate significant differences for FaGT6 and FaGT7 (Fig. S4 in Supplementary data available at *JXB* online). The binding pockets in FaGT7 and VvGT1 show several consensuses while FaGT6 groups with MtUGT71G1. In FaGT7 and VvGT1, kaempferol stands in a narrow, deep pocket built by five non-polar amino acids (Phe18, Phe130, Phe210, Leu214, and Phe410). The general base His23 responsible for the deprotonation of the flavonol and the acceptor UDP-glucose are located on the bottom of the channel. Hydrogen bonds between 4'-OH of the flavonol and His159 and Gln198 (VvGT1) as well as Gln198 (FaGT7) further stabilize the transition states. Comparison with the models of FaGT6 and MtUGT71G1 shows that only Phe130 is conserved in these enzymes. Phe410 which forms π -stacking interactions with the A ring of the flavonol in VvGT1 and FaGT7 is replaced by Tyr410 that swings over the channel due to its polar character and enlarges the binding pocket. The rigid non-polar channel in the 3-*O*-glucosyltransferases formed by one Leu and four Phe residues becomes wider and more polar in FaGT6 and MtUGT71G1 due to substitutions with short-chain and polar amino acids and may explain the ability of FaGT6 to form diglucosides.

Discussion

Biochemical characterization

Plant secondary product glycosyltransferases have been reported to exhibit a rather strict regioselectivity towards the position of the sugar attachment (Vogt and Jones, 2000). FaGT6 and FaGT7 accepted a number of substrates and formed multiple products when assayed with quercetin, kaempferol, and isorhamnetin, which are potential substrates that occur naturally in strawberry. While FaGT6 produced mainly quercetin 3- and 7-*O*-glucoside, FaGT7 converted quercetin to almost equal amounts of quercetin 3-*O*-glucoside and quercetin 4'-*O*-glucoside in addition to small amounts of the 7-*O*- and 3'-*O*-isomers. The monoglucoside patterns of FaGT6 and FaGT7 with kaempferol as well as with isorhamnetin were almost indistinguishable because the ratios of the levels of 3-*O*- and 4'-*O*-glucoside were equal. Glycosyltransferases from *Allium cepa* and *Arabidopsis thaliana* show a comparable regioselectivity forming several monoglucosides and diglucosides of flavonol substrates but their expression pattern and regulation have not been investigated (Kramer *et al.*, 2003; Lim *et al.*, 2004). More recently, it was reported that the hydroxylation pattern in ring B of the acceptor molecule can influence product specificity. UGT73A4 from *Beta vulgaris* accepts the positions 4' and 7 of flavonols. If a hydroxy group is present at position 3' (e.g. quercetin), 4'-*O*-glucosides are preferentially formed. If the hydroxy group is missing (e.g. kaempferol), the enzyme produced 7-*O*-glucosides

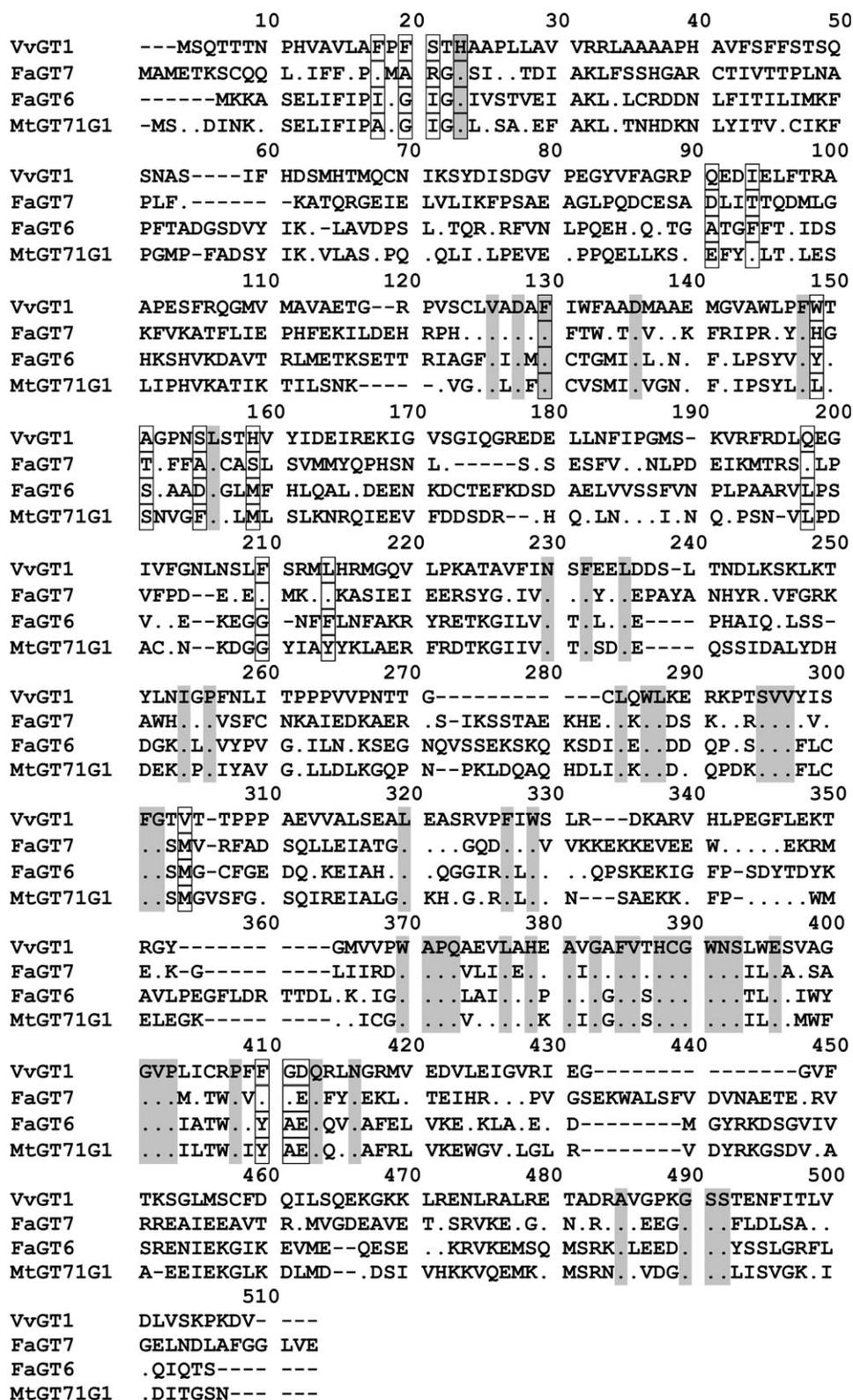


Fig. 6. Sequence alignment of quercetin and kaempferol *O*-glucosyltransferases, including two already crystallized proteins from *Vitis vinifera* (VvGT1) and *Medicago truncatula* (UGT71G1) as well as *Fragaria x ananassa* GT6 (FaGT6), and *Fragaria x ananassa* GT7 (FaGT7). The alignment was performed using ClustalX (Thompson *et al.*, 1997). Conserved amino acids are shaded, amino acids within 5 Å to kaempferol in the protein model of VvGT are boxed.

(Isayenkova *et al.*, 2006). Likewise FaGT7 formed less kaempferol-4'-*O*-glucoside than quercetin-4'-*O*-glucoside. RUGT-5, the first recombinant glycosyltransferase from *Oryza sativa* showed a similar behaviour. The presence of hydroxy group 3' led to the formation of 4'-*O*-glucosides, but its absence directed the reaction towards the formation of 3-*O*-glucosides (Ko *et al.*, 2006). However, in the case of FaGT6, such an effect was not observed.

FaGT6 and FaGT7 also accepted a series of secondary metabolites not occurring in strawberry fruits and unnatural substrates such as 2-naphthol. This indicates that these glycosyltransferases may not only function in flavonol biosynthesis, but perhaps play a role in xenobiotic metabolism as well. Surprisingly, FaGT7 exhibited a relatively high temperature optimum (45 °C). A similar effect was observed when a multifunctional glycosyltransferase from *Rauwolfia serpentina* was characterized showing maximum activity at 50 °C (Hefner *et al.*, 2002). This could indicate that these enzymes maintain their activity even under stress conditions, as required for enzymes involved in plant protection.

Gene expression studies

FaGT6 showed higher expression values in achenes than in the receptacle of small-sized green and ripe red fruits. In microarray experiments, 441 sequences were identified showing a different expression in achenes and the receptacle. Surprisingly, 259 genes (59%) were predominantly expressed in achenes (Aharoni and O'Connell, 2002). However, up to now mainly receptacle- and ripening-related strawberry genes were identified and characterized (Medina-Escobar *et al.*, 1997; Lunkenbein *et al.*, 2006; Raab *et al.*, 2006; Griesser *et al.*, 2008). Genes showing higher expression in achenes were not heterologously expressed and further characterized (Medina-Escobar *et al.*, 1998; Blanco-Portales *et al.*, 2004). Both *FaGT6* and *FaGT7* displayed a different expression pattern in achenes and the receptacle. This complies with earlier assumptions that gene expression is regulated differently in these tissues (Aharoni and O'Connell, 2002). Transcription of *FaGT6* and *FaGT7* in the receptacle is under the negative control of auxin, as it has been shown for several ripening-related genes (Medina-Escobar *et al.*, 1997; Manning, 1998; Lunkenbein *et al.*, 2006; Raab *et al.*, 2006; Griesser *et al.*, 2008).

Additionally, gene expression of both glycosyltransferases can be induced by SA, an important signal molecule in plant defence (Reymond and Farmer, 1998). Many glycosyltransferases including UGT73B3 and UGT73B5 from *Arabidopsis thaliana* were induced in an SA-dependent way after infection with *Pseudomonas syringae* pv. *tomato* (Langlois-Meurinne *et al.*, 2005). UGT73C5 which glucosylates the *Fusarium* mycotoxin desoxynivalenol is induced by SA as well (Poppenberger

et al., 2003). Furthermore, several multifunctional glycosyltransferases from *Nicotiana tabacum* that are putatively involved in plant defence are induced by SA (Taguchi *et al.*, 2001). Similarly FaGT6 and FaGT7 might be part of a defence mechanism.

Function of FaGT6 and FaGT7 in planta

Although quercetin and kaempferol are moderate substrates for FaGT6 and FaGT7 *in vitro*, it is reasonable to propose that both enzymes are involved, at least in part, in the glucosylation of phenolics in achenes and the receptacle (Table 1). The gene expression patterns correlate with high amounts of phenolic compounds in achenes (Aaby *et al.*, 2005) and fruits (Wang and Lin, 2000). It was shown that *FaGT6* and *FaGT7* gene expression in the receptacle (Fig. 5A, B) correlates with higher levels of kaempferol, quercetin, and isorhamnetin 3-*O*-glucosides determined in the red receptacle compared with the green stage (Table 1). Achenes generally accumulate the same flavonoid glucosides as the receptacle. Interestingly mainly isorhamnetin 7-*O*-glucosid was detected, although the 3-*O*-glucoside was the major product formed by FaGT6 and FaGT7. Significant levels of the 3-isomer were only determined in the receptacle of the late-ripening stage consistent with the increased transcript levels. The metabolite analyses combined with gene expression data point towards a possible role of both glycosyltransferases in flavonol biosynthesis in the strawberry receptacle and achene during ripening. This is supported by the fact that it was possible to identify for the first time quercetin 4'-*O*-glucoside in strawberry achenes and receptacles. It is noteworthy to mention that it was the product specificity of FaGT7 and its high expression in achenes which led to the identification of this novel strawberry flavonoid.

Achenes of strawberry accumulate a series of storage and defence compounds to ensure survival of the seeds for prolonged periods. In microarray experiments a large set of achene-related genes was identified belonging to a group of stress-associated genes (Aharoni and O'Connell, 2002). Accordingly, a heat-shock protein was reported to be mainly expressed in strawberry achenes (Medina-Escobar *et al.*, 1998). Furthermore, transcripts of a lipid-transfer protein with a putative function in plant defence were localized in strawberry achenes using *in situ* hybridization (Yubero-Serrano *et al.*, 2003). It was assumed that some of the stress-associated genes from achenes code for proteins involved in xenobiotic metabolism. This included a putative glutathione-S-transferase with the ability to conjugate xenobiotic substances in a phase-II reaction. However, a glycosyltransferase was not identified in this experiment (Aharoni and O'Connell, 2002). FaGT6 and FaGT7 are multifunctional glycosyltransferases that are mainly expressed in strawberry achenes and are induced by SA. This indicates that they

may take over an additional role in the detoxification of xenobiotics, contributing to the defence capacity of strawberry achenes. A role for FaGT6 and FaGT7 in the glucosylation of xenobiotics was supported by the capability of strawberry fruits to glucosylate several unnatural compounds including 2-naphthol when injected in ripe fruits.

Similar to FaGT6 and FaGT7 there are several glycosyltransferases that act on endogenous and exogenous substrates at the same time. The best-studied enzymes were isolated from *Arabidopsis thaliana*. *In vitro*, UGT73C5 glucosylated the flavonol quercetin (Lim *et al.*, 2004) and the mycotoxin deoxynivalenol. Plants with constitutively overexpressed UGT73C5 showed increased tolerance against deoxynivalenol, but the concentrations of flavonols were not analysed (Poppenberger *et al.*, 2003). UGT72B1 glucosylated the xenobiotic compounds 2,4,5-trichlorophenol (TCP) and 3,4-dichloroanilin (DCA) (Loutre *et al.*, 2003). T-DNA insertion lines with disrupted UGT72B1 showed strongly reduced conjugating activity towards DCA and TCP while activities towards natural phenolics were not affected (Brazier-Hicks and Edwards, 2005). In another experiment, it has been shown that endogenous substrates can inhibit the conversion of xenobiotics (Meßner *et al.*, 2003). Similarly, we have shown that the xenobiotic compound 2-naphthol and natural kaempferol compete for glucosylation. Together this indicates that the detoxification of xenobiotics is probably a side-reaction of glycosyltransferases involved in secondary metabolism rather than the task of specialized plant defence enzymes.

Protein modelling

To investigate the active sites of FaGT6 and FaGT7, the Michaelis complex of the recently crystallized VvGT1 was used (Offen *et al.*, 2006) with the kaempferol acceptor and the non-transferable donor UDP-2-fluoroglucose as template and the positions of the conserved amino acids of FaGT6, FaGT7, and MtUGT71G1 compared (Shao *et al.*, 2005) in the 3D structure. The red grape enzyme VvGT1 is responsible for the formation of anthocyanins but also accepts a number of flavonols and transfers the hexose to the 3-*O* position (Offen *et al.*, 2006). MtUGT71G1 is a triterpene glucosyltransferase that glucosylates certain isoflavones and the flavonol quercetin with higher efficiency than triterpenes (Achnine *et al.*, 2005). UDP-glucose is almost completely buried in a long, narrow channel within the C-terminal domain and interacts through residues in the highly conserved PSPG motif (residues 370–413) (Fig. S3 in Supplementary data available at *JXB* online). In contrast to the numerous conserved amino acids in the UDP-glucose recognition site of the four flavonol glucosyltransferases, there is an apparent lack of consensus for amino acids except for Phe130 and

Leu156 in the acceptor binding site (Fig. S3 in Supplementary data available at *JXB* online). A closer look at the amino acids in the vicinity of the acceptor molecule showed that FaGT6 is more closely related to MtUGT71G1 while FaGT7 groups with VvGT1, although the *Fragaria* proteins exhibit similar catalytic activities (Fig. S4 in Supplementary data available at *JXB* online). Because FaGT6 produced higher levels of quercetin-3'-*O*-glucoside than FaGT7 and the enzyme from *M. truncatula* glucosylated quercetin primarily at the 3'-*O*-position, it is conceivable that their active sites show some similarities. Secondly, it has been suggested that the acceptor binding pocket of MtUGT71G1 is much longer and larger than quercetin, and quercetin fits in easily with any of its hydroxyl groups which may explain the formation of multiple products. Such a large pocket would also account for the formation of diglucosides, which has been shown in the case of FaGT6. These findings nicely confirm the hypotheses that glucosyltransferases are two-domain enzymes that were produced during evolution, with one domain recognizing similar donors and another adapting to acceptors (Shao *et al.*, 2005).

The first multi-substrate, multi-product glucosyltransferases that are strongly expressed in strawberry achenes have been isolated and characterized. Metabolite profiling suggested that they are involved in the glucosylation of flavonols in achenes and the receptacle but they probably also function in the metabolism of xenobiotics. As glucosides are no longer regarded as useless end products but rather serve as precursors for diverse biosynthetic pathways and have important physiological roles, the interest in their metabolism has increased in recent years. The data reported in this study provide a basis for the exploration of their formation and their functions in achenes and the receptacle during strawberry fruit development.

Supplementary data

Gene expression data, mass spectra of glucosylated xenobiotics, and protein models are presented in the Supplementary Figs S1–S4.

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