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# Substrate specificity and promiscuity of horizontally transferred UDP-glycosyltransferases in the generalist herbivore *Tetranychus urticae*

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A B S T R A C T

Uridine diphosphate (UDP)-glycosyltransferases (UGTs) catalyze the addition of UDP-sugars to small hydrophobic molecules, turning them into more water-soluble metabolites. While their role in detoxification is well documented for vertebrates, arthropod UGTs have only recently been linked to the detoxification and sequestration of plant toxins and insecticides. The two-spotted spider mite *Tetranychus urticae* is a generalist herbivore notorious for rapidly developing resistance to insecticides and acaricides. We identified a set of eight UGT genes that were overexpressed in mites upon long-term acclimation or adaptation to a new host plant and/or in mite strains highly resistant to acaricides. Functional expression revealed that they were all catalytically active and that the majority preferred UDP-glucose as activated donor for glycosylation of model substrates. A high-throughput substrate screening of both plant secondary metabolites and pesticides revealed patterns of both substrate specificity and promiscuity. We further selected nine enzyme-substrate combinations for more comprehensive analysis and determined steady-state kinetic parameters. Among others, plant metabolites such as capsaicin and several flavonoids were shown to be glycosylated. The acaricide abamectin was also glycosylated by two UGTs and one of these was also overexpressed in an abamectin resistant strain. Our study corroborates the potential role of *T. urticae* UGTs in detoxification of both synthetic and natural xenobiotic compounds and paves the way for rapid substrate screening of arthropod UGTs.

## 1. Introduction

Glycosyltransferases catalyze the transfer of sugar moieties from activated donor molecules to an acceptor molecule and are ubiquitous across all kingdoms of life (Lairson et al., 2008). Uridine diphosphate (UDP)-glycosyltransferases (UGTs) are the largest family of glycosyltransferases (Lombard et al., 2014) and catalyze the addition of UDP-sugars to small hydrophobic molecules. Next to important roles in biosynthesis, storage and transport of secondary metabolites, UGTs are, together with glutathione-S-transferases, also well-known as phase II enzymes in the detoxification process (Jancova et al., 2010). By catalyzing the conjugation of hydrophobic compounds with UDP sugars, more hydrophilic compounds are generated, that enhance excretion (Mackenzie et al., 1997). In contrast to human UGTs, the glycosylation of small hydrophobic compounds by arthropod UGTs has been poorly studied. Only recently, biochemical and functional studies could specifically link arthropod UGTs to the detoxification and sequestration of plant allelochemicals and insecticides (Highfill et al., 2017; Krempl et al., 2016; Li et al., 2017). Krempl et al. (2016) detected glycosylated gossypol isomers in the feces of *Helicoverpa armigera* and *Heliothis virescens*, and showed that two UGTs were capable of glycosylating gossypol. Hence, they suggested that these UGTs might play a crucial role in gossypol detoxification in generalist herbivores utilizing cotton as a host plant.

The two-spotted spider mite *Tetranychus urticae* (Chelicerata: Acari: Trombidiformes), is able to feed on more than 1100 plant species which belong to more than 140 different plant families (Migeon et al., 2018). Next to being extremely polyphagous, *T. urticae* is also considered as the 'resistance champion' among arthropods, as it has the most documented instances of resistance to diverse pesticides (Van Leeuwen et al., 2010; Van Leeuwen and Dermauw, 2016). Eighty UGT genes were earlier identified in the *T. urticae* genome, similar to *Bemisia tabaci* (81 UGTs), but a substantially larger number than any other arthropod species reported so far (Ahn et al., 2014; Chen et al., 2016). They are classified

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in seven distinct families (UGT201-207) and recent lineage-specific gene expansions have been reported for the subfamilies UGT201A, UGT201B and UGT202A. Moreover, it was shown that these *T. urticae* UGT genes were very likely acquired from bacteria through horizontal gene transfer (Ahn et al., 2014; Bajda et al., 2015; Van Leeuwen and Dermauw, 2016). As a consequence, similar to bacterial UGTs, *T. urticae* UGTs do not harbor a signal peptide and a transmembrane domain (TM), which indicates that they are cytosolic enzymes (Ahn et al., 2014). This contrasts to other eukaryotes, were the N-terminal signal peptide is removed upon insertion of the UGT into the endoplasmic reticulum (ER), and a C-terminal TM domain anchors the protein to the ER (Ahn et al., 2012, 2014; Erb et al., 2009; Magdalou et al., 2010).

In this study, we identified a set of eight UGTs that were over-expressed in *T. urticae* upon long term plant acclimation/adaptation and/or resistance against certain acaricides. Next, we recombinantly expressed this set of *T. urticae* UGTs in *Escherichia coli* and determined their catalytic properties against model substrates, as well as examined their potential to conjugate an array of secondary plant metabolites and acaricides. Nine enzyme-acceptor interactions were further characterized by determining their steady state kinetic parameters and their preferred UDP-donor substrate.

## 2. Material and methods

### 2.1. T. urticae strains, chemicals and plant secondary metabolites

All *T. urticae* populations were described previously (Dermauw et al., 2013; Grbić et al., 2011; Jonckheere et al., 2016; Snoeck et al., 2018) and mass reared on their respective host plants at 26 °C ( ± 0.5 °C), 60% relative humidity (RH) and 16/8 h light/dark photoperiod. For a more detailed description of each population, see Supplementary Table 1. All chemicals, including pesticides, and plant secondary metabolites used in this study were of analytical grade. Detailed information about suppliers and purity of substrates can be found in Supplementary Table 2.

### 2.2. UGT gene expression analysis

The UGT metanalysis of existing microarray gene expression data was performed in three different batches. An analysis of A) microarrays run with RNA from adult *T. urticae* females and the 1st array design (GPL15756/Agilent-028213; Dermauw et al., 2013), B) microarrays run with RNA from deutonymph *T. urticae* females and the 1st array design (GPL15756/Agilent-028213; Demaeght et al., 2013) and C) microarrays run with RNA from adult *T. urticae* females and the 2nd array design (GPL16890/Agilent-033850; (Jonckheere et al., 2016; Khalighi et al., 2015; Pavlidi et al., 2017; Snoeck et al., 2018; Wybouw et al., 2015, 2014)). For each analysis, raw intensity data were used as input for final processing and statistical analysis in limma (version 3.30.13) of the Bioconductor framework (Smyth, 2004). Prior to differential gene expression analysis, the probe sequences were remapped to the *T. urticae* genome annotation (cDNA sequences) of August 11, 2016 using Bowtie2-2.3.4.3 and following setting "–norc -f -a" (Langmead and Salzberg, 2012). Only the probes that aligned uniquely and without mismatches (probes having "AS:i:0" in the 1st optional field of the SAM output of Bowtie2) to the annotated genome (42577 probes for analysis A and B while 39650 probes for analysis C) were incorporated in the differential expression analysis. Next, background correction was performed by the 'normexp' method, using an offset of 50 (Ritchie et al., 2007). Background-corrected data were within- and between-array normalized (global loess and Aquantile, respectively) and quality was subsequently assessed using arrayQualityMetrics (Kauffmann et al., 2009). A linear model (using "lmFit" in the limma R package) was fitted to the processed data that treated the London (analysis A and C) or LS-VL (analysis B) strain as a common reference (cy3 channel in sample GSM980545-GSM980555 for analysis A, cy3 channel in sample GSM1065002-GSM1065006 for analysis B and cy3 channel in sample GSM1214964-GSM1214967, GSM2124774-GSM2124784, GSM1679383-GSM1679385 and GSM1633888- GSM1633891 for analysis C). Empirical Bayes moderated t-statistics (using the "eBayes" function in the limma R package; Smyth, 2004) was used to determine differences in transcript expression levels in reference to the London (adult females, analysis A and C) or LS-VL (deutonymph females, analysis B) strain. UGT gene expression heatmaps were created in R (R Developement Core Team, 2015) using the relative transcript levels ($log_2$FC) and the R package gplots_3.0.1 (Warnes et al., 2009).

### 2.3. Phylogenetic analysis

*T. urticae* UGT protein sequences were derived from the ORCAE genome portal while those of *Panonychus citri* and *Panonychus ulmi* were obtained from Bajda et al. (2015) and Sterck et al. (2012). *Tetranychus evansi* UGTs were identified by mining the *T. evansi* transcriptome (Villarroel et al., 2016): *T. urticae* UGT protein sequences were used as query in a tblastn search (E-value threshold of $E^{-3}$, BLAST 2.2.31 + ) against the *T. evansi* transcriptome. In cases where *T. evansi* contigs showed more than 95% identity at the nucleotide level, they were considered as allelic variants and the longest transcript was retained for further analysis using cd-hit-est with sequence identity threshold (-c) of 0.95 (Fu et al., 2012). Open reading frames (ORFs) of *T. evansi* were identified using "EMBOSS 6.6.0.0 getorf" integrated in the Mobyle portal framework (http://mobyle.pasteur.fr/). *T. evansi* contig sequences were manually corrected to contain the longest UGT encoding ORFs. Those ORFs that showed identical overlap and had the same blastx top-hit against the *T. urticae* proteome, were considered to be part of the same gene and were manually merged using BioEdit v7.2.5 (Hall, 2013). Subsequently, ORFs were once more filtered for allelic variants using cd-hit-est (sequence identity threshold (-c) 0.95) and the longest ORF was retained for further analysis (Fu et al., 2012). Finally, only *T. evansi* ORFs longer than 150 AA were retained for phylogenetic analysis. *T. urticae* UGTs were aligned with those of *T. evansi*, *P. citri* and *P. ulmi* using the online version of MAFFT 7 with the E-INS-I iterative refinement method strategy, 1000 iterations and the option "reorder" (Katoh et al., 2002). A phylogenetic analysis was performed on the Cipres web portal using RAxML v8.2.10 HPC2-XSEDE with the automatic protein model assignment algorithm using maximum likelihood criterion and 1,000 bootstrap replicates (Miller et al., 2010; Stamatakis, 2014). The LG + G protein model was selected as the best scoring model for maximum likelihood analysis. The resulting tree was midpoint rooted, visualized using MEGA7 (Tamura et al., 2013) and edited in Corel-DRAW Home & Student x7.

### 2.4. Cloning, functional expression and purification of recombinant UGTs

The cDNA sequences were amplified from the maize (*tetur05g09325, tetur01g05690 and tetur05g05050*), tomato (*tetur05g00060* and *tetur22g00270*) and cotton (*tetur04g02350*) lines and from the London (*tetur22g00440*) and the MAR-AB (*tetur02g09850*) strain (Supplementary Table 3). For cDNA preparation, total RNA of adult spider mites was extracted using RNeasy Plus Mini kit (Qiagen, USA) and reverse transcribed using Maxima first strand cDNA synthesis kit (Thermo Scientific). One microliter of the prepared cDNAs was used as the PCR template using the blunt-end Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and specific primers (Supplementary Table 4). Conditions were 98 °C for 2 min, followed by 30 cycles of 98 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s. PCR product was purified using E.Z.N.A.® Gel Extraction Kit (Omega Bio Tek) and ligated into pET100/D-TOPO (Invitrogen Life Technologies), which allows expression of recombinant protein with an N-terminal 6x His tag, following manufacturer's instructions. TOP10 competent *Escherichia coli* cells were transformed with the ligation reaction and the resulted colonies were screened by colony PCR using the cloning primers (Supplementary Table 4) for the presence of the inserts. At least 5 positive colonies were further grown on liquid cultures and the corresponding plasmids were

extracted using E.Z.N.A.® Plasmid Mini Kit I (Omega Bio Tek) and sent for sequencing (Macrogen, The Netherlands). A clone of the correct DNA sequence was selected for heterologous expression. *Escherichia coli* BL21 (DE3) STAR competent cells transformed with UGT-pET100/D-TOPO construct were grown over-night and used for the 1 to 50 inoculation of 2 L LB medium containing 100 mg/ml of ampicillin. The cultures were grown at 37 °C with shaking, until the absorbance at 595 nm reached 0.8–1. Then, the expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM. The cultures were further grown at 18 °C overnight and subsequently the cells were harvested by centrifugation at 5000*g* for 30 min. Cell pellets were re-suspended in 25 ml of sodium phosphate buffer pH 7.4 (containing 20 mM sodium phosphate, 40 mM imidazole and 500 mM NaCl). Cells were lysed by 2 rounds of freezing/thawing followed by incubation with 10 mg/ml of lysozyme (Sigma Aldrich) on ice for 30 min and sonication. Cell lysate was centrifuged at 10,000 g at 4 °C for 30 min and the supernatant was used for the purification of the recombinant enzymes. Supernatant was loaded in 0.2 ml of pre-equilibrated Ni-NTA resin (Qiagen) following manufacturer's instructions. Unbound proteins were washed with 30 ml (15 bed volumes) of 160 mM sodium phosphate buffer pH 7.4 and the recombinant enzyme was eluted using 500 mM imidazole in 160 mM sodium phosphate buffer pH 7.4. Protein eluates were applied to PD-10 desalting columns (GE Healthcare) following manufacturer's protocol to remove imidazole, proteins were eluted with 20 mM Tris-HCl, pH 7.4 and stored at −20 °C in 25 mM DDT and 40% glycerol. Protein concentration was measured by Bradford assay (Bradford, 1976). Purified protein was run on a 12% SDS-PAGE gel and subsequently a Western blot was performed using anti-His-tag primary antibodies (Bio-Rad) to verify the purity and potential proteolytic degradation of the recombinant protein. As negative controls for the downstream activity assays, fractions of non-induced constructs for *tetur05g09325* and *tetur01g05690* were purified following the procedure as described above except that IPTG was not added.

### 2.5. Activity assays and determination of specificities for model substrates

UGT activity was determined against the model substrates p-nitrophenol (Leszczynski and Dixon, 1990), 1-naphthol and 2- naphthol (Sigma-Aldrich). All reactions were performed at 25 °C in 0.1 M of sodium phosphate buffer pH 7.5 in the presence of 0.83 mM of UDP-glucose (Sigma-Aldrich), 16.7 mM MgCl2 and 0.16 mM of the model substrate. The total reaction volume was 250 µl and the incubation time 20 min. Depletion of p-nitrophenol was detected spectrophotometrically at 400 nm, and depletion of 1- and 2-naphthol substrates was quantified after fast blue staining (55.6 mM Fast Blue RR crystalline, Sigma-Aldrich) as the resulted azo-dyes were monitored at 570 nm.

In addition to depletion, formation of reaction product was also monitored for 20 min to calculate the specific activity against each model substrate. The formation of the corresponding glucoside was detected spectrophotometrically at 295 nm for p-nitrophenol, while the conjugates of 1-napthol and 2-napthol were quantified by fluorescence spectrophotometry, using excitation/emission wavelength of 287/335 nm and 283/341 nm respectively, with 1 nm slit widths. For all experiments, measurements were performed in three independent replicates in 96-well plates (Greiner Bio-One) using a Bio-Tek Synergy H1 multimode microplate reader (Bio-Tek). Reactions without recombinant enzyme were included as a negative control for non-enzymatic glycosylation.

### 2.6. Treatment with β-glucosidase

To further ensure that model substrates were glycosylated by *T. urticae* UGTs, a treatment with β-glucosidase (Sigma-Aldrich) was conducted after incubation of the model substrates p-nitrophenol and 1-naphthol with the UGTs tetur05g09325 and tetur01g05690, respectively. UGT reactions were performed as described above (Section 2.5) and upon incubation of 20 min, the reaction mixture was boiled for 2 min to inactivate the UGT enzymes. Subsequently, 1 mg of β-glucosidase (Sigma-Aldrich) was added and incubation was continued for 1 h. Negative control reaction mixtures without recombinant enzymes were included and measurements were performed in 9 replicates. Model substrate concentrations were measured spectrophotometrically (Section 2.5) before and after incubation with β-glucosidase. R and the R package ggplot2_2.2.1 were used to plot the results.

**Table 1**
**Specific activities of recombinant *T. urticae* UGT enzymes for model substrates.**

Enzyme characteristics were measured at 25 °C and calculated as ΔOD/min/mg for the model substrate p-nitrophenol and ΔRFU/min/mg for the model substrates 1-naphthol and 2-naphthol.

| enzyme | p-nitrophenol (ΔOD/min/mg) | 1-naphthol (ΔRFU/min/mg)*$10^5$ | 2-naphthol (ΔRFU/min/mg)*$10^5$ |
|---|---|---|---|
| tetur05g09325 | 18.92 ± 4.40 | 4 ± 0.12 | 0.15 ± 0.02 |
| tetur01g05690 | 9.48 ± 1.23 | 350 ± 70 | 27 ± 2.00 |
| tetur02g09850 | 8.30 ± 0.99 | 100 ± 10 | 20 ± 6.45 |
| tetur05g05050 | 0.01 ± 0.00 | n.d. | 0.005 ± 0.001 |
| tetur04g02350 | 0.02 ± 0.00 | 0.40 ± 0.05 | 0.02 ± 0.01 |
| tetur22g00270 | 5.78 ± 0.88 | 120 ± 17 | 21 ± 8.00 |
| tetur22g00440 | 1.25 ± 0.42 | 5 ± 0.47 | 0.64 ± 0.08 |
| tetur05g00060 | 0.04 ± 0.01 | 0.02 ± 0.001 | 0.04 ± 0.008 |

*n.d.: not detected.

### 2.7. UDP-sugar preference

Ultra-pure UDP-glucose, UDP-glucuronic acid, UDP-galactose, UDP-N-acetylgalactosamine (UDP-GlcNAc) and UDP-N-acetylglycosamine (UDP-galNAc) were purchased from the Promega corporation and were tested as potential UDP-sugar substrates for all functionally expressed enzymes by using either p-nitrophenol or 1-naphthol depending on the model substrate preference of each enzyme (Table 1). Model substrate depletion was monitored spectrophotometrically after 20 min of incubation as described above (Section 2.5). Measurements were performed in three independent replicates. Results were plotted in R with the R package ggplot2_2.2.1.

### 2.8. UGT incubation and UDP-glo glycosyltransferase assay

Incubation of 50 µM substrate, 0.1 µg enzyme, 400 µM UDP-sugar, in a total reaction volume of 125 µl containing 0.1 M sodium phosphate buffer (pH 7.5) and 16.7 mM MgCl2 at 25 °C for 1 h was performed. Enzyme reactions were stopped by the addition of the UDP Detection Reagent of the UDP-Glo™ glycosyltransferase assay (Promega), and detection of free-UDP was performed as described below. Negative controls used for the calculations consisted of all reaction components except the substrates, all reaction components except the UDP-sugar and all reaction components except the enzyme. In addition, another incubation set-up was tested for two enzymes with a relatively lower activity (tetur04g02350 and tetur05g05050) similar to the former one, except for the amount of enzyme, which was elevated to 1 µg.

The formation of free-UDP by the glycosyltransferase reaction was quantified by using the UDP-Glo™ Glycosylotransferase assay (Promega). This assay detects the UDP release by converting free-UDP to ATP which results in the generation of light in a luciferase reaction. Following the manufacturer's protocol, each glycosyltransferase reaction was combined in a ratio of 1:1 (25 µL:25 µL) with the UDP-Glo™ Detection Reagent (three technical replicates) in independent wells of a white, flat bottom 96-(chimney)-well lumitrac medium binding assay plate (Greiner) and was allowed to incubate at room temperature for 1 h. Subsequently, luminescence was measured in Relative Luminescence Units (RLU) with a Bio-Tek Synergy H1 multimode microplate reader (Bio-Tek) in triplicates. For absolute quantification, a

UDP standard curve was determined (0–25 μM UDP) and plotted with SigmaPlot 12.0 software (Fig. S1). The range of measurements was determined to be in the linear range of detection.

### 2.9. Substrate screening

The following potential substrates were tested in the substrate screening experiment with UDP-glucose as activated donor; 7-hydroxyflavone, abamectin, acequinocyl, atropine, azadirachtin, bifenazate, bifenthrin, caffeic acid, caffeine, capsaicin, catechol, chlorfenapyr, chlorogenic acid, chrysin, clofentezine, coumestrol, cyenopyrafen, cyflumetofen, DIMBOA, dopamine, eriodictyol, fenpyroximate, gossypol, hesperetin, hexythiazox, jasmonic acid, kaempferol, L-3,4-dihydroxyphenylalanine, l-canavanine, MBOA, methanol, naringenin, nicotinic acid, profenofos, pyflubumide, pyridaben, quercetin, rutin hydrate, salicylic acid, scopoletin, spirodiclofen and vanillin (Supplementary Table 5). All substrates were dissolved in methanol and glycosylation was quantified by using the UDP-Glo™ Glycosylotransferase assay (Promega) as described above. Plots were created in R (R Developement Core Team, 2015) using R package ggplot2_2.2.1.

### 2.10. Kinetic studies of specific enzymes-substrate combinations (9) and UDP-sugar preference

Based on the substrate screening (indirect measurement of glycosylation by quantification of released free-UDP), steady-state kinetic parameters were determined for the UDP-glucose conjugation reaction of nine enzyme-acceptor combinations: tetur02g09850 – (capsaicin, kaempferol, abamectin), tetur22g00270 – (capsaicin, kaempferol), tetur22g00440 – (kaempferol), tetur04g02350 – (DIMBOA, kaempferol) and tetur05g00060 – (abamectin). Initial velocities were determined by using a constant concentration of UDP-glucose (400 μM) while acceptors were used in the concentration range of 0–400 μM, using UDP-Glo assay protocol and the reaction set-up described above. The Michaelis-Menten or Hill equation was fitted to the obtained data to define $K_m$ and $V_{max}$ parameters using OriginLab (OriginLab Corporation) (see Table 2 for the type of equation that was fitted). Subsequently, ultra-pure UDP-glucose, UDP-glucuronic acid, UDP-galactose, UDP-N-acetylgalactosamine and UDP-N-acetylglycosamine (Promega) were tested as sugar donor using acceptor substrates in a final concentration of 200 μM. A Wilcoxon rank sum test (pairwise comparison – $p < 0.05$) was performed in R using the R package pgirmess (version 1.6.9) and the data was plotted using the R package gplots_3.0.1 (Warnes et al., 2009; Giraudoux et al., 2018).

### 2.11. Image processing

CorelDRAW Home & Student × 7 was used for processing of images.

## 3. Results

### 3.1. Selection of glycosyltransferases

*T. urticae* UGTs were selected based on their expression profile in acaricide resistant strains or in mite lines acclimatized or adapted to a challenging host (Fig. 1, Fig. S2, Supplementary Table 6). Next to gene expression data, a phylogenetic analysis was used to select UGT genes that belonged to diverse UGT clades/subfamilies, including those of lineage-specific subfamilies (Fig. 2). *Tetur05g00060* and *tetur22g00270* were selected based on their high expression in the *T. urticae* line adapted to tomato. *Tetur01g05690, tetur05g05050* and *tetur05g09325* were highly expressed in a mite population acclimatized to maize. *Tetur04g02350* was highly expressed in a cotton acclimatized population. Finally, *tetur02g09850* and *tetur22g00440* were chosen because of their high expression in acaricide resistant strains MAR-AB, JP-R and MR-VP, and TU008R, respectively.

### 3.2. Cloning, heterologous expression and purification of T. urticae UGTs

Coding sequences of selected UGTs were successfully cloned into the pET100/D-TOPO expression vector and inspection of cloned sequences did not reveal any sequencing errors. IPTG induction of expression resulted in good levels of protein production. Although the majority of the expressed protein was found in the insoluble fraction, the remaining yield in the soluble fraction was sufficient to allow efficient metal affinity purification of recombinant enzymes. The overall amount of recombinant UGTs ranged from 4 to 10 mg derived from 2 L bacterial cultures. All UGTs were successfully purified close to homogeneity, as verified by obtaining a main band of the expected size after both SDS-PAGE as well as a Western-blot with anti-His-tag primary antibodies (Fig. S3, panel A and B).

### 3.3. Kinetic properties of recombinant T. urticae UGTs

The recombinant UGTs were assayed towards the model substrates p-nitrophenol, 1-naphthol and 2-naphthol to investigate whether they exhibited UDP glycosyltransferase activity (Table 1). All recombinant UGTs were capable of conjugating at least one of the model substrates. Nevertheless, for all model substrates tested, tetur05g05050, tetur05g00060 and tetur04g02350 showed low activity compared to the other recombinant UGTs. Incubation with a recombinant glutathione S-transferase, GSTd05, did not result in glycosylation of the model

**Table 2**
**Steady-state kinetic parameters of recombinant *T. urticae* UGT enzymes for the conjugation of UDP-glucose to selected substrates.**
Results were determined by varying the concentration of substrates (1.5–200 μM) at fixed concentration of UDP-glucose (400 μM). All values are means ± SD of three independent experiments.

| enzyme | substrate | equation | kinetic parameters | | | |
|---|---|---|---|---|---|---|
| | | | $K_m$ | $V_{max}$ | $k_{cat}$ | $k_{cat}/K_m$ |
| | | | (μM) | (nmol UDP $min^{-1}$ $ml^{-1}$) | ($min^{-1}$) | ($\mu M^{-1}$ $min_{-1}$) |
| tetur02g09850 | capsaicin | Michaelis-Menten | 6.20 ± 0.54 | 0.22 ± 0.008 | 14.90 | 2.40 |
| | kaempferol | Michaelis-Menten | 34.45 ± 6.17 | 0.44 ± 0.02 | 29.81 | 0.86 |
| | abamectin | Hills (n = 2.97 ± 0.29) | 1.83 ± 0.06 | 1.83 ± 0.05 | 12.40 | 6.77 |
| tetur22g00270 | capsaicin | Michaelis-Menten | 690.53 ± 180.77 | 0.90 ± 0.18 | 59.62 | 0.08 |
| | kaempferol | Michaelis-Menten | 31.69 ± 7.18 | 0.33 ± 0.02 | 21.86 | 0.68 |
| tetur22g00440 | kaempferol | Hills (n = 2.12 ± 0.23) | 14.95 ± 2.36 | 0.33 ± 0.04 | 21.98 | 1.47 |
| tetur04g02350 | DIMBOA | Hills (n = 1.59 ± 0.08) | 128.46 ± 18.28 | 0.13 ± 0.01 | 0.85 | 0.006 |
| | kaempferol | Hills (n = 2.15 ± 0.11) | 48.55 ± 1.38 | 0.26 ± 0.005 | 1.71 | 0.03 |
| tetur05g00060 | abamectin | Hills (n = 2.20 ± 0.07) | 7.91 ± 0.13 | 0.96 ± 0.009 | 63.84 | 0.87 |

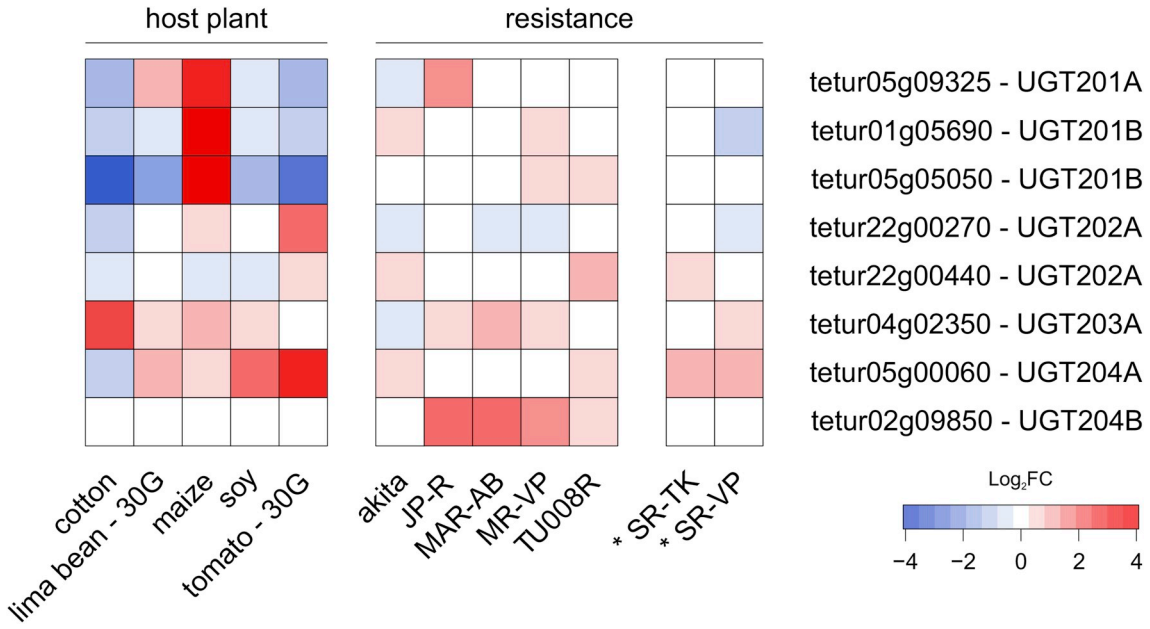


**Fig. 1. Expression heatmap of a selection of *T. urticae* UGTs.**
Expression heatmap of the recombinant UGTs in adult (or deutonymph*) *T. urticae* females adapted to tomato and lima bean (≥30 generations), long-term acclimatized to soy, cotton and maize (≥5 generations) and resistant against acaricides (Supplementary Table 1). The $\log_2$ transformed fold changes are relative to adult *T. urticae* females of the susceptible London strain or susceptible deutonymph *T. urticae* females of the LS-VL strain.

substrates (Fig. S4, panel A and B), strongly indicating that the above measured activities are the result of recombinant *T. urticae* UGT enzymes and not from *E. coli* background proteins. Elutions from non-induced constructs of tetur01g05690 and tetur05g09325 were also included as a control and resulted in a relatively low glycosylation of the model substrates compared to the induced constructs. A sign of leakage in expression which increased when the elution volume was increased from 12.5 to 25 µl (Fig. S4, panel A and B). Finally, as beta-glucosidase is known to remove the glucose group of a glycosylated model substrate, we added this enzyme after incubation of model substrates with tetur01g05690 and tetur05g09325. The amount of non-glycosylated model substrate increased after incubation with beta-glucosidase in comparison to the amount of non-glycosylated model substrate before incubation with beta-glucosidase (Fig. S4, panel C).

### 3.4. Sugar selectivity of the recombinant UGTs

The model substrates p-nitrophenol and 1-naphthol were used to determine the sugar selectivity of the functionally expressed UGTs, depending on the acceptor preference of each enzyme (Table 1). Most enzymes were able to use multiple UDP-sugars as activated donor although five out of eight enzymes clearly preferred UDP-glucose (Fig. 3). For those UGTs that showed the lowest activity against any of the model substrates, tetur04g02350, tetur05g05050 and tetur05g00060, the preferred UDP-sugar could not be clearly determined. Interestingly, tetur22g0440 could only use UDP-glucose as an activated donor molecule. Based on the sugar selectivity experiment, UDP-glucose was chosen as UDP-sugar for the subsequent experiments (Section 3.5 and 3.6).

### 3.5. Substrate/acceptor specificity

The substrate promiscuity of selected *T. urticae* UGT enzymes was evaluated towards a diverse set of 44 substrates (3 model substrates, 27 plant secondary metabolites and 14 acaricides) using the UDP-Glo™ glycosyltransferase assay (Promega) as a quick qualitative screening method. Methanol was used as a substrate solvent for all assays. Assays with tetur05g09325 caused very high (RLU) background values in the negative control (solvent without substrate), and this issue could not be overcome by using a different solvent (aceton, acetonitrile, DMSO or ethanol). Hence, this recombinant *T. urticae* UGT was excluded from the substrate screening.

Some common features emerged regarding substrate specificity of the recombinant UGTs, at least for the parameters of the set-up of this high-throughput screening (Fig. 4, Supplementary Table 7). (1) Three UGTs (tetur02g09850, tetur22g00270 and tetur22g00440) could glycosylate multiple substrates of the flavonoid class of secondary plant metabolites. The flavonoids included in our screening were: 7-hydroxyflavone, chrysin, coumestrol, eriodictyol, hesperitin, kaempferol, naringenin, quercetin and rutin hydrate. (2) The latter three UGTs and tetur02g09850 in particular also glycosylated a broad spectrum of acaricides. (3) In contrast with the broad spectra of the earlier mentioned UGTs, the other recombinant UGT enzymes had a narrower substrate spectrum. Tetur05g00060 glycosylated three substrates (capsaicin, abamectin and cyenopyrafen) while tetur04g02350 and tetur05g05050 showed no activity towards any of the substrates.

Specific enzyme-substrate combinations of potential interest included: (1) tetur01g05690 and the substrate vanillin, which resulted in the highest amount of released free-UDP (~glycosylation) of all combinations tested (2) next to flavonoids, tetur02g09850 and tetur22g00270 also glycosylated capsaicin (3) the plant secondary metabolite DIMBOA was not glycosylated by any of the recombinant enzymes at the default parameters of our screening assay. However, upon augmentation of the enzyme amount from 0.1 µg to 1 µg for two enzymes with a relatively lower activity (Table 1), both DIMBOA and kaempferol were glycosylated by tetur04g02350 (Fig. S5).

### 3.6. Enzymatical characterization of the recombinant UGTs

Based on the results of the high-throughput screening, a subset of enzyme-acceptor combinations was further enzymatically characterized by determination of the steady-state kinetic parameters and presented in Table 2. Tetur02g09850 and abamectin had the highest affinity ($K_m$) of all enzyme-substrate combinations tested ($K_m$ 1.83 ± 0.06 µM). Additionally, tetur02g09850 had a high affinity for capsaicin ($K_m$ 6.20 ± 0.54 µM). Abamectin and tetur05g00060 also resulted in a

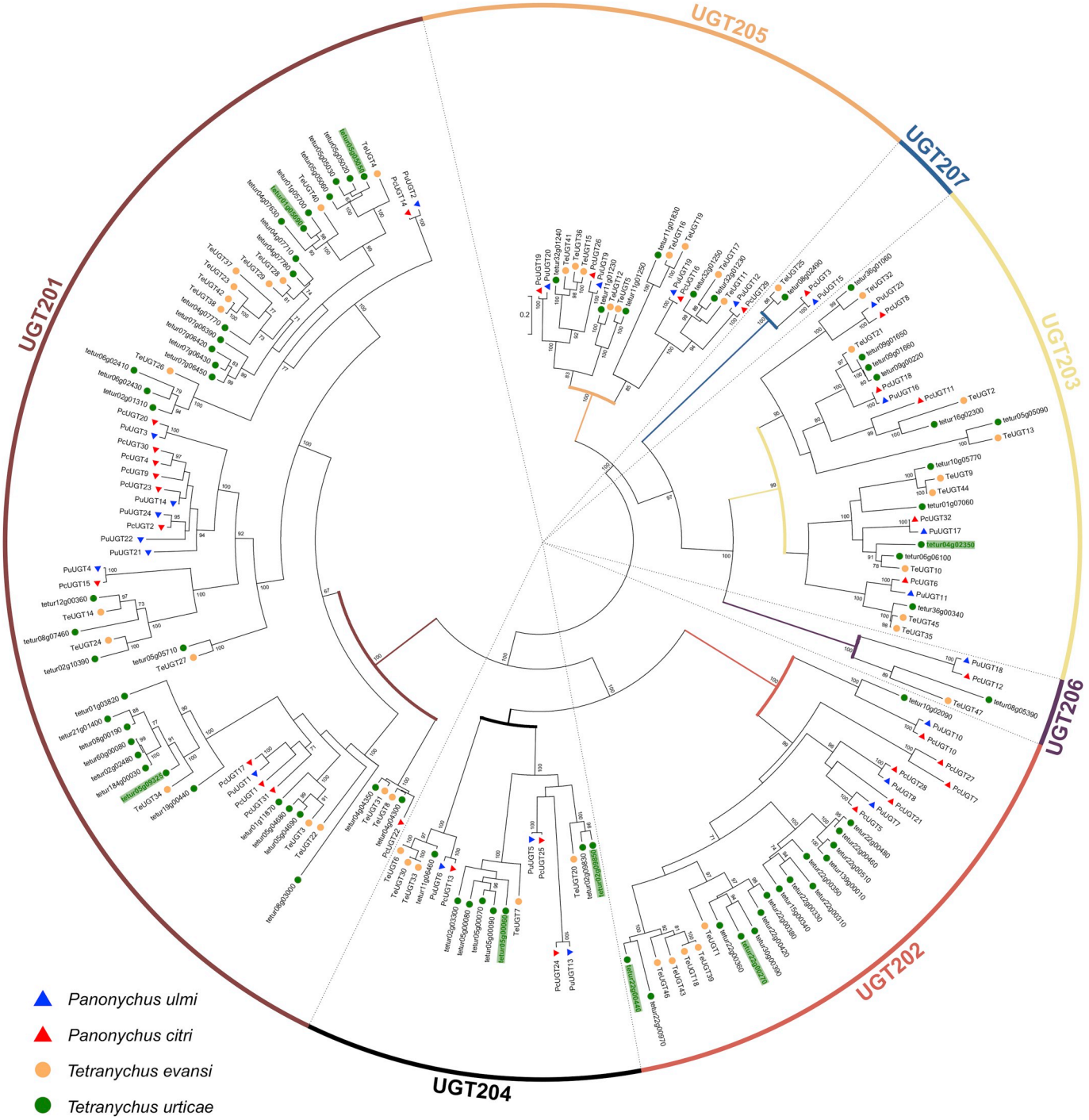


**Fig. 2. Phylogenetic analysis of UGTs.**
Maximum likelihood phylogenetic analysis of the UGTs of *P. citri*, *P. ulmi*, *T. evansi* and *T. urticae*. UGT families (UGT201-UGT207) are labelled in the phylogenetic tree. Functionally expressed enzymes are highlighted in green. Only bootstrap values higher than or equal to 65 are shown. The scale bar represents 0.2 amino acid substitutions per site. Information and accession numbers of the used UGT sequences can be found in Supplementary Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

relatively good $K_m$ and the highest turnover number ($k_{cat}$) observed of all enzyme-substrate combinations ($K_m$ 7.91 ± 0.13 μM and $k_{cat}$ 63.84 $min^{-1}$). Tetur22g00440 had the highest affinity of all four enzymes that were characterized with kaempferol ($K_m$ 14.95 ± 2.36 μM). Finally, tetur04g02350 had a rather low affinity for DIMBOA (128.46 ± 18.28 μM).

Additionally, sugar selectivity of the recombinant UGTs was re-determined with the substrates from the selected subset of enzyme-acceptor combinations, instead of with the preferred model substrates as in Section 3.4. Out of the UDP-sugars tested (UDP-glucose, UDP-glucuronic acid, UDP-galactose, UDP-galNAc and UDP-GlcNAc), UDP-glucose was the preferred activated sugar donor for all enzyme-substrate combinations (Fig. S6).

## 4. Discussion

In arthropods, cytochrome P450 monooxygenases (P450s), glutathione-S-transferases (GSTs) and carboxyl/cholinesterases (CCEs) are

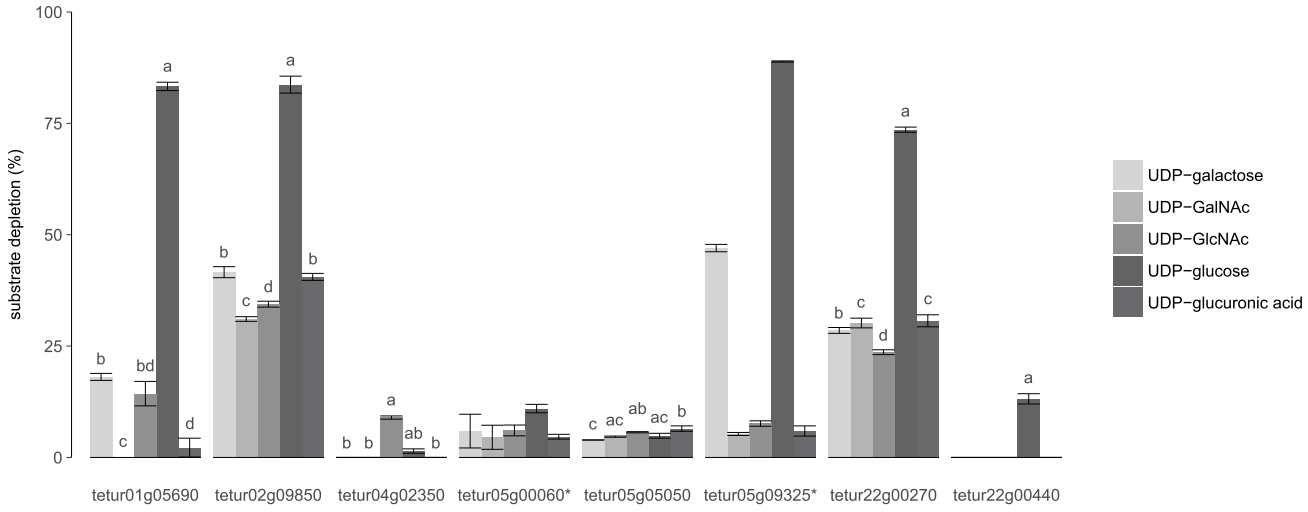


**Fig. 3. Sugar-preference for model substrates**.
Model substrate depletion after incubation with the respective recombinant *T. urticae* UGT enzyme and the activated donors (UDP-galactose, UDP-GalNAc, UDP-GlcNAc, UDP-glucose and UDP-glucuronic acid). The model substrate p-nitrophenol or 1-napthol was used depending on the model substrate preference of the enzyme (Table 1). Error bars represent the standard deviation of the calculated mean of three to four independent replicates. Statistical differences were analyzed using the Wilcoxon rank sum test (pairwise comparison), and are indicated as different letters ($p < 0.05$). Two enzymes were marked with an asterisk (*) since they only had three replicates, which was insufficient to detect statistical differences.

well-known players in the detoxification process of noxious compounds (Bajda et al., 2017; Després et al., 2007; Feyereisen et al., 2015; Li et al., 2007; Pavlidi et al., 2018). UGTs, like GSTs, act in phase II of the detoxification process and catalyze the glycosylation of compounds, making them more water soluble and resulting in more rapid excretion. However, in contrast to human UGTs, the role of arthropod UGTs in detoxification has been overlooked for many years. Recently, a number of UGT genes were found to be overexpressed in insecticide resistant and host plant acclimatized/adapted populations (Faucon et al., 2015; Kaplanoglu et al., 2017; Li et al., 2018, 2017; Snoeck et al., 2018; Tian et al., 2018) and the role of UGTs in detoxification of pesticides was investigated using inhibitors such as sulfinpyrazone and 5-nitrouracil (Li et al., 2017; Pan et al., 2018; Tian et al., 2018; Wang et al., 2018). In addition, glycosylated plant toxins were detected in either feces or after incubation with homogenates of arthropods as UGT enzyme source (Ahn et al., 2011; Kojima et al., 2010; Krempl et al., 2016; Maag et al., 2014; Sasai et al., 2009; Wouters et al., 2014) and in some cases specific insect UGTs could be linked with detoxification of toxic compounds (Highfill et al., 2017; Krempl et al., 2016; Li et al., 2017). In the genome of the spider mite *T. urticae*, 80 UGT genes were identified. Although their pattern of diversification as well as the observed plasticity of gene expression strongly suggests an important role in detoxification (Ahn et al., 2014; Snoeck et al., 2018), specific associations between *T. urticae* UGTs and the glycosylation of a certain compound are scarce. At present only one UGT (UGT201D3, corresponding to tetur04g02350) from the red morph of *T. urticae* has been partially characterized and was shown to be inhibited by the acaricide abamectin (Wang et al., 2018). In this study, we functionally expressed and characterized eight UGT genes that were highly expressed in *T. urticae* populations acclimatized/adapted to host plants or resistant to acaricides and that belonged to diverse UGT subfamilies, including lineage-specific UGT expansions (Fig. 2). In contrast to both insect and vertebrate UGTs, which are anchored in the endoplasmic reticulum, *T. urticae* UGTs are, like bacterial UGTs, cytosolic enzymes (Ahn et al., 2014). Hence, *T. urticae* UGTs could be readily functionally expressed in *E. coli* and all showed catalytic activity against at least one of the tested model substrates (p-nitrophenol, 1-napthol and 2-napthol, Table 1). Nevertheless, this does not rule out that eukaryotic expression systems (such as insect cells) could generate proteins with different properties. Except for tetur22g00440, recombinant *T. urticae* UGT enzymes were able to use multiple UDP-sugars for glycosylation. Interestingly, all recombinant *T. urticae* UGTs could use UDP-glucose, with five out of eight enzymes having a distinct preference for UDP-glucose (Fig. 3). Hence, UDP-glucose was used as activated donor for all further glycosylation experiments. Biochemical studies in insects have shown that insect UGT enzymes also typically use UDP-glucose as the main activated donor for glycosylation (Ahn et al., 2012). Likewise, plant UGTs typically use UDP-glucose next to other donors such as UDP-rhamnose, UDP-arabinose, UDP-galactose, UDP-xylose, and UDP-glucuronic acid (Bowles et al., 2006; Kim et al., 2013). In contrast, vertebrate UGTs mainly utilize UDP-glucuronic acid (Bock, 2003).

A high-throughput substrate screening was performed to examine the substrate breadth of *T. urticae* UGTs by testing a diverse array of 44 substrates, comprising both plant metabolites and acaricides. To our knowledge, solely Luque et al. (2002) performed a similar experiment for a *Bombyx mori* UGT. While in the latter study, the amount of conjugated radio-labelled sugar was quantified after thin layer chromatography (TLC), we measured the release of free-UDP upon glycosylation spectrophotometrically, making a high-throughput set-up more feasible. In our substrate screening assay, detected free-UDP values ranged from 0.1 to 40 μM. In several cases, none or a very low level of free-UDP was detected. However, this does not necessarily imply that glycosylation is absent or very low as evidenced by enzyme activity measurements with model substrates. For example, tetur01g05690, tetur02g09850 and tetur22g00270 showed low enzyme activity towards the model substrate p-nitrophenol (Table 1), but only for one of these UGTs (tetur22g00270) free-UDP was detected in the substrate screening assay (Supplementary Table 7). Likewise, only for those UGTs that had a relatively high specific activity for the model substrates 1-napthol and 2-napthol, free-UDP was detected in the screening assay, suggesting that our screening assay is rather conservative. The low amount of conjugation in the high-throughput screening could be a consequence of substrate inhibition when acceptor concentrations are higher than optimal (Chaplin and Bucke, 1990), or UDP-glucose might not be the preferred activated donor for a specific UGT-acceptor combination (Fig. 3). In general, the parameters of our UGT screening assay were not optimal for all enzyme-substrate combinations included in the screening. However, this cannot be expected since we tested 44 different potential acceptors against seven recombinant enzymes. Hence, the magnitude of released UDP (μM) should not be interpreted/used as

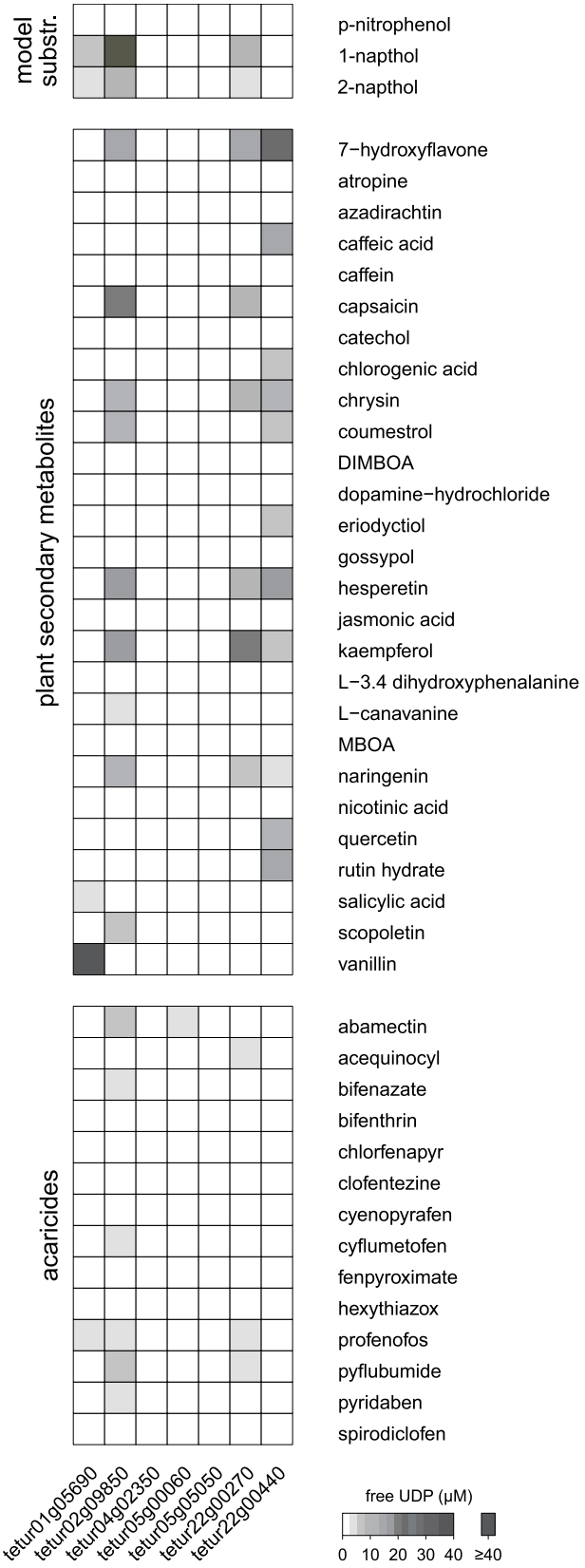


**Fig. 4. Substrate screening assay.**
Endpoint measurement of the release of free-UDP (μM) after incubation of the respective recombinant *T. urticae* UGT enzyme (0.1 μg) with 44 substrates (3 model substrates, 27 plant secondary metabolites and 14 acaricides) and UDP-glucose (Table S6). Release of free-UDP is directly linked with the activity of glycosyltransferases.

an absolute predictor of affinities for enzyme-substrate combinations. Although tetur02g09850 was able to glycosylate capsaicin > kaempferol > abamectin in the screening assay, 20.1 μM, 16.5 μM and 6.7 μM (out of 50 μM) respectively, enzyme characterization showed that tetur02g09850 had the highest affinity for abamectin (Table 2). Nevertheless, all acceptor-enzyme combinations that were selected based on the screening assay for detailed kinetic studies showed affinities in the micromolar range. Hence, the substrate screening assay showed its value as a high-throughput method to test potential substrates for glycosylation.

Flavonoids are one of the major classes of plant secondary metabolites and are widely distributed in the plant kingdom. Most of them are present in the form of a glycoside under natural conditions (Bohm, 1998) and the majority of functions of flavonoids result from their strong anti-oxidative properties. They participate in plant protection against both biotic (herbivores and pathogens) and abiotic (UV, radiation and heat) stress (Dakora and Phillips, 1996; Mierziak et al., 2014). Furthermore, they are able to influence the behavior, growth and development of insects (Falcone Ferreyra et al., 2012; Mierziak et al., 2014; War et al., 2012). Nine flavonoids (7-hydroxyflavone, chrysin, coumestrol, eriodictyol, hesperitin, kaempferol, naringenin, quercetin and rutin hydrate) were included in our screening assay and all were glycosylated by at least one of the following recombinant *T. urticae* UGTs (tetur02g09850, tetur22g00270 and tetur22g00440) (Fig. 4). In arthropods, two *B. mori* UGTs were shown to glycosylate quercetin and/or naringenin (Daimon et al., 2010; Luque et al., 2002), but broad flavonoid substrate spectra like for *T. urticae* UGTs were not reported before. Nevertheless, such spectra have previously been described for certain plant, microbial and human UGTs (Hyung Ko et al., 2006; Jones et al., 2003; Kim et al., 2007; Modolo et al., 2007; Su et al., 2017; Xie et al., 2007). Kaempferol was selected for further detailed kinetic analysis since it is omnipresent in plants and fruits (Robards and Antolovich, 1997) and because this flavonoid was glycosylated by the afore mentioned UGT enzymes. From the characterized enzymes, tetur22g00440 had the highest affinity and catalytic efficiency for kaempferol (Table 2).

Tetur22g00440 was also able to glycosylate chlorogenic acid, which contributes to the physiological defenses of maize. Other major players in the plant defense of maize are protein inhibitors, the flavonoid maysin and benzoxazinoids (BXs) (Meihls et al., 2012). BXs are synthesized constitutively and stored as an inactive form (BX-glucosides) in the plant cell. Upon tissue disruption, unstable aglycone BXs are released which are highly reactive and toxic to a wide range of arthropod herbivores including *T. urticae* (Bui et al., 2018; Morant et al., 2008; Niemeyer, 2009; Wouters et al., 2016). DIMBOA is the more prevalent BX in maize (Niemeyer, 2009), and a relatively slow, perhaps non-enzymatic breakdown of DIMBOA results in the formation of MBOA, another toxic BX (Grambow et al., 1986). Glycosylation of DIMBOA has been detected for *Mythimna separate*, *Spodoptera exigua, S. littoralis* and *S. frugiperda* (Glauser et al., 2011; Sasai et al., 2009; Wouters et al., 2014) and glycosylation of MBOA for *S. littoralis* and *S. frugiperda* (Maag et al., 2014). Although six of the recombinant UGT enzymes were upregulated in *T. urticae* upon long-term acclimation to maize (Fig. 1), none of them glycosylated DIMBOA or MBOA at the default parameters of the screening. Since two of our enzymes had shown lower activity against the model substrates (Table 1), all substrates were additionally screened after incubation with a ten-times higher amount of both enzymes. This resulted in glycosylation of DIMBOA by tetur04g02350, but both the affinity and turnover rate were rather low (Table 2). Additionally, tetur04g02350 was only marginally upregulated after long-term acclimation to maize ($log_2$FC 1.33). Hence, no strong conclusions can be made considering the involvement of tetur04g02350 in host-plant acclimation to maize.

The plant secondary metabolite capsaicin was glycosylated upon incubation with four recombinant UGTs: tetur01g05690, tetur05g00060, tetur02g09850 and tetur22g00270 (Supplementary

Table 7). Capsaicin (capsaicinoids) is found solely in hot peppers (*Capsicum* spp.) and is known to deter oviposition (Cowles et al., 1989), inhibit feeding (Hori et al., 2011) and delay larval growth (Weissenberg et al., 1986) in insects. Glycosylation has been linked with the detoxification of capsaicin by the Lepidopteran species *Helicoverpa armigera*, *H. zea* and *H. assulta*, resulting in capsaicin glucoside, a more water-soluble compound than its aglycone and subsequently easier to excrete (Ahn et al., 2011). The enzyme characteristics of tetur02g09850 and tetur22g00270 in combination with capsaicin were determined since they resulted in a higher amount of glycosylation in the substrate screening assay. In contrast to tetur22g00270, tetur02g09850 had a strong affinity for capsaicin (Table 2) and although this might suggest a functional role in adaption, a more dedicated study is needed to draw any further conclusions.

The highest amount of glycosylation detected in the substrate screening assay resulted from the incubation of tetur01g05690 with vanillin (Fig. 4). Tetur02g09850 and tetur22g00270 were able to glycosylate vanillin too but at a lower rate under the default parameters of the screening assay. Vanillin is one of the most widely used flavors and aromas worldwide. Since multiple UGTs from different plants possess a high in vitro catalytic activity towards vanillin (Hansen et al., 2009; Jones, 1998; Jones et al., 1999; Song et al., 2016), vanillin has been suggested to be an ubiquitous metabolite that is easily converted by a number of plant UGTs (Song et al., 2016). This statement seems not to be constrained to the plant kingdom as nine human UGTs were able to glucuronidate vanillin (Yu et al., 2013) and in arthropods, a recombinant UGT of *Bombyx mori* was able to glycosylate vanillin (Luque et al., 2002). However, in the scope of this study, detoxification of toxic compounds by *T. urticae* UGTs, we decided to not analyze the latter enzyme-substrate combination in depth.

Tetur04g02350 was the highest upregulated UGT after long-term host plant acclimation on cotton (Fig. S2), and the most highly overexpressed UGT of all comparisons made in this study (Fig. 1). The major defense compound of cotton is the toxic sesquiterpene dimer, gossypol (Dodou, 2005), which was partially metabolized by UGTs via glycosylation in *Helicoverpa armigera* and *Heliothis* virescens (Krempl et al., 2016). However, gossypol was not glycosylated by tetur04g02350 or any of the other recombinant *T. urticae* UGTs in this study.

Next to plant secondary metabolites, we also tested whether our set of recombinant *T. urticae* UGTs could glycosylate a selection of pesticides. The acetylcholinesterase inhibitor profenofos was glycosylated by tetur01g05690, tetur02g09850 and tetur22g0270. Profenofos is just like pyraclofos an organophosphate (Mode of Action group (MoA) 1b (IRAC, 2017)), and previously it was shown that glycosylation plays a role in pyraclofos resistance in *Musca domestica* (Lee et al., 2006). Besides profenofos, tetur02g09850 glycosylated abamectin, bifenazate, cyflumetofen, pyflubumide and pyridaben. In contrast, tetur05g00060 only glycosylated abamectin. The chloride channel activator abamectin (MoA 6 (IRAC, 2017)), has been and is still widely used to control *T. urticae* and other phytophagous mite species and field resistance has been reported (Brown et al., 2017; Ferreira et al., 2015; Ilias et al., 2017; Memarizadeh et al., 2013; Riga et al., 2014). Glutamate-gated chloride channel target-site mutations have been associated with abamectin resistance in *T. urticae* (Dermauw et al., 2012; Kwon et al., 2010; Liu et al., 2014; Mermans et al., 2017; Wolstenholme and Rogers, 2005) as well as metabolic resistance by a P450 mono-oxygenase (CYP392A16), capable of metabolizing abamectin (Riga et al., 2014). Most recently, UGT201D3 (corresponding to *T. urticae* tetur04g02350, UGT 201 family) was specifically linked to abamectin resistance (Wang et al., 2018). In the latter study, the inhibition of 1-napthol glycosylation was inhibited by abamectin ($K_i$ = 9.9 ± 6.2 μmoL/l), but the glycosylated product was not detected. Here, we performed detailed kinetic analysis for tetur02g09850 and tetur05g00060 with abamectin and both recombinant enzymes were able to glycosylate the acaricide. Tetur02g09850 had the highest affinity and tetur05g00060 the highest turnover number. Tetur02g09850 was also overexpressed ($log_2$FC 2.20) in the abamectin resistant strain MAR-AB (Dermauw et al., 2013) (Fig. 1). Noteworthy, tetur02g09850 and tetur05g00060 both cluster in the UGT204 family, in contrast to UGT201D3 of Wang et al., (2018) (UGT 201 family, Fig. 2). In conclusion, these results suggest a potential contribution of glycosylation to abamectin resistance in *T. urticae*.

## 5. Conclusion

Eight *T. urticae* UGTs were functionally expressed and all could use UDP-glucose as activated donor for the glycosylation of model substrates. A high-throughput substrate screening comprising both toxic plant metabolites and pesticides led to the selection of nine enzyme-substrate combinations which were further enzymatically characterized. Strong affinities of the recombinant enzymes with both plant secondary metabolites as wells as an acaricide corroborate the potential role of *T. urticae* UGTs in detoxification.

## Declarations of interests

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.04.010.

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