Activity based profiling of a physiologic aglycone library reveals sugar acceptor promiscuity of family 1 UDP-glucosyltransferases from *Vitis vinifera*.

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One sentence summary:

A novel method based on an aglycone library was developed for the targeted analysis of acceptor molecules of UDP-glycosyltransferases.

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Monoterpenoids serve various biological functions and accumulate in grapes (Vitis vinifera) where a major fraction occurs as non-volatile glycosides. We have screened the V. vinifera genome for sequences with similarity to terpene uridine diphosphate glycosyltransferases (UGTs) from Arabidopsis thaliana. A ripening related expression pattern was shown for three candidates by spatial and temporal expression analyses in five grape varieties. Transcript accumulation correlated with the production of monoterpenyl β-D-glucosides in grape exocarp during ripening and was low in vegetative tissue. Targeted functional screening of the recombinant UGTs for their biological substrates was performed by activity based metabolite profiling (ABMP) employing a physiologic library of aglycones build from glycosides isolated from grapes. This approach led to the identification of two UDP-glucose:monoterpenol β-D-glucosyltransferases. Whereas VvGT14a glucosylated geraniol, R,S-citronellol, and nerol with similar efficiency, the three allelic forms VvGT15a-c preferred geraniol over nerol. Kinetic resolution of R,S-citronellol and R,S-linalool was shown for VvGT15a and VvGT14a, respectively. ABMP revealed geraniol as major biological substrate but also disclosed that these UGTs may add to the production of further glycoconjugate in planta. ABMP of aglycone libraries provides a versatile tool to uncover novel biologically relevant substrates of small molecule glycosyltransferases that often show broad sugar acceptor promiscuity.

Plant secondary metabolites are frequently decorated with glucose that is transferred from uridine diphosphoglucose by a so called family 1 uridine diphosphate-glucosyltransferases (UGTs) (Caputi et al., 2012). Since the glycosylation process can be envisaged as a simple nucleophilic displacement reaction of S_N2 type, the product is a β-glucoside. A remarkable large array of different small molecules is glucosylated in planta including terpenoids, alkaloids, cyanohydrins and glucosinolates as well as flavonoids, isoflavonoids, anthocyanidins and phenylpropanoids. In Vitis vinifera alone, more than 200 different glucosides have been identified and there is a special interest in those glucoconjugates, which can contribute to wine flavor after the hydrolytic release of volatiles during the biotechnological vinification sequence leading from grape to aged wine (Wirth et al., 2001). These flavorless glucoconjugates accumulate in grape berries during maturation and can be grouped according to their linked aglycones into monoterpenoids, C13-norisoprenoids, aliphatic alcohols, and shikimate-derived benzoids and phenylpropanoids (Sefton et al., 1994). It still remains an open question how this large number of structurally different flavor precursors is actually glucosylated in vivo because the in vitro activity of single UGTs show large differences in the individual range of acceptors. Some UGTs are considered highly specific with respect to substrate-, regio- and stereospecificity, whereas others glucosylate a broad range of acceptors (Osmani et al., 2009). The latter phenomenon is called promiscuity and could decisively contribute to the immense structural variations of small plant secondary metabolites regarding their glucosylation pattern (Schwab, 2003). Biochemical characterization of the substrate specificity of the UGTs is therefore a major challenge that scientists face when approaching the study of the actual biological function of these enzymes whose number of available sequences is rapidly increasing as a result of the EST and genome sequencing programs. The potential broad substrate preference of many UGTs requires a wide range of substrates to be tested, which is often impeded by a biased collection of acceptor molecules. This problem has been recently alleviated by the application of the so called activity based metabolic profiling (ABMP) approach (Duckworth and Aldrich, 2010). ABMP allows unbiased discovery of enzymatic activities encoded by genes of unknown function, and applies chromatographic techniques to analyze the impact of a recombinant enzyme on the homologous cellular extract as the most physiologic chemical library of potential substrates and products (de Carvalho et al., 2010). Notably, it provides a synthesis and label-free approach that circumvents the unavailability of a complete set of relevant substrate molecules. We have adapted this powerful technique to probe the substrate specificity of heterologously expressed UGTs whose sequences were obtained from the Vitis vinifera genome database. A grape berry aglycone library was prepared by isolation and subsequent enzymatic hydrolysis of V. vinifera glycoconjugates. Incubation of the liberated aglycones with the recombinant UGTs yielded glucosides that were separated from the unreacted free aglycones by extraction and were identified by complementary GC-MS and LC-MS techniques. This approach allowed the identification of UDP-glucose:monoterpenol β-D-glucosyltransferases that are characterized by the glucosylation of a rather broad monoterpenol spectrum and complemented with considerable activities
towards aliphatic alcohols and benzoic compounds. Gene expression analyses in combination with metabolite analysis confirmed their biological function.

RESULTS

Expression analysis – temporal and spatial

The most prominent terpene compounds occurring generally and in high concentrations in aroma related grapes (e.g. Muscat FR 90) and wines are geraniol, nerol, citronellol, α-terpineol, and linalool (Figure 1). To identify UGT genes that are likely to contribute to the glucosylation of these terpenols during grape ripening we screened the Vitis vinifera cv. Pinot noir genome for sequences with substantial identity to monoterpene UGTs found in Arabidopsis thaliana (Caputi et al., 2008, 2010). Seven putative VvGT genes (VvGT14: VIT_18s0001g06060, VvGT15: VIT_06s0004g05780, VvGT16: VIT_03s0017g01130, VvGT17: VIT_18s0001g05950, VvGT18: VIT_03s0180g00280, VvGT19: VIT_18s0001g05910, and VvGT20: VIT_05s0062g00430) were selected (Figure 2; for genome location refer to supplemental data Figure S1) and their transcript levels analyzed using GeXP profiling in two cultivars in three different vegetative tissues (Figure 3 and supplemental data Figure S2) and in grape berry exocarp of five cultivars at different developmental stages (Figure 4 and supplemental data Figure S3). In non-berry tissue (inflorescence, leaf and root) VvGT14 and 15 showed the lowest relative transcript levels of the seven putative UGT genes but displayed a ripening related expression pattern in berry skins similar to VvGT16. Significant amount of VvGT14-16 mRNA was found in berry exocarp at late stages of berry ripening (Figure 4). Notably, their transcript levels differed considerably between varieties. VvGT14 was expressed primarily in the two surveyed clones of White Riesling, VvGT15 in Muscat and VvGT16 in the two clones of Gewurztraminer. In contrast, VvGT17-20 transcripts accumulated to comparatively high levels in inflorescence, leaf tissue and immature green berries (supplemental data Figure S2), but their amount decreased after veraison (the onset of ripening; (supplemental data Figure S3). The expression patterns of VvGT17-20 were very similar in all analysed cultivars (supplemental data Figure S3). Expression profiling was also performed for berry skins of Muscat FR 90 in two subsequent years. In 2011, in comparison to 2012, similar relative transcript levels of VvGT14-15 were reached, but slightly later (2-3 weeks; supplemental data Figure S4). The same is true for the ripening related parameters such as sugar content and pH value. However, this effect was not observed for VvGT17-20 as their relative expression levels are negligible at the start of ripening (10-12 weeks post flowering; supplementary data Figure S4). Thus, VvGT14-16 appear to play an important role in grape berry ripening as their expression levels peak after veraison and they are barely expressed in other tissues, except VvGT16.

Metabolite profiling

To correlate the expression profiles of putative UGTs with terpenyl glucoside concentration we performed metabolite analysis in five cultivars during grape ripening (Table 1). Solid phase extraction was used to isolate free (non-glycosylated) and glycosylated monoterpenes from grape skins (exocarp) of various grapevine cultivars (Gunata et al., 1988; Mateo and Jiménez, 2000). Since grape skins (exocarp) accumulate the majority of terpene metabolites detected in grape berries they were separated from the flesh and extracted (Wilson et al., 1986). The main monoterpenes of grapes (geraniol, nerol, linalool and citronellol) were quantified by GC-MS analysis whereas their non-volatile monoterpenyl glucosides were determined by a stable isotope dilution analysis (SIDA) method using HPLC-MS/MS. Isotopically labelled internal standards were chemically synthesized. Grape berries of the V. vinifera cultivars differed not only in their amounts of total terpenes, but also in their terpene profiles at different developmental stages (Table 1). Monoterpenols (free and glucosidically bound) were hardly detected (less than 0.25 mg/kg grape skins) in grape exocarp of Gewurztraminer FR 46-107, probably due to impaired monoterpenol biosynthesis of this clone. Gewurztraminer 11-18 Gm and Muscat a petits grains blanc FR 90 skins accumulated significant levels of geraniol, citronellol and nerol derivatives (up to 5.5 mg/kg grape skins) and displayed a heterogenous spectrum of monoterpenes at every stage of ripening. Both Riesling clones produced smaller amounts of the metabolites that were mainly observed at weeks 15-17. In general, the highest concentration of free and bound terpenols was found in the late stages of ripening in all investigated cultivars, whereupon geraniol and its β-D-glucoside were the predominant terpene metabolites. The ratios of the amount of free to glucosidically bound forms of individual monoterpenes varied considerably at week 15 and 17 post flowering. These values provide a first indication of variable UGT activity in different varieties and/or differential preference of the UGTs for their monoterpenyl substrates. Notably, the evolution of monoterpenyl β-D-glucosides in grape exocarp of the Riesling clones (Table 1) correlated well with the expression pattern of VvGT14 in the same tissue (Figure 4). While significant transcript levels were
only detected at week 11 post flowering remarkable levels of the glucosides were not found until week 13. In contrast, the time course of VvGT15 mRNA levels in Muscat FR 90 coincided with the terpenyl glucoside concentrations in the same clone as considerable amounts of transcripts and glucosides were found throughout weeks 6 to 17 post flowering. At the very late stages of ripening (weeks 15 to 17) expression of VvGT16 increased strongly in Gewurztraminer 11-18 Gm, a variety that produced a high concentration of geranyl β-D-glucosides.

**Heterologous expression of VvGT14, VvGT15 and VvGT16 and enzymatic activity**

The alleles of VvGT14a-c, 15a-c and 16 were isolated from V. vinifera cultivars and cloned in the expression vector pGEX-4T-1. The recombinant proteins were expressed in *Escherichia coli* with an N-terminal GST-tag, affinity purified and verified by SDS-PAGE and Western Blot using GST-specific antibody (supplementary data Figure S5-S6). Enzyme activity studies were performed with UDP-[^14C]glucose and various putative substrates (terpenols, flavonoids and different mono-alcohols) that are known to be glycosidically bound and present in grapes (Ford and Hoj, 1998; Gunata et al., 1988; Sefton et al., 1996; Voirin et al., 1990; Wirth et al., 2001). Recombinant VvGT14a, VvGT15a-c and VvGT16 converted several of the tested substrates (Figure 5). VvGT14a preferred geraniol and citronellol but also efficiently (> 80% relative activity) glucosylated nerol, hexanol and octanol. Additionally, VvGT14a showed catalytic activity towards further monoterpenes (terpineol, 8-hydroxylinanol, linanol), short-chain mono-alcohols (3-methyl-2-butanol, 3-methyl-3-butanol, cis 3- and trans 2-hexanol), benzyl alcohol, phenylethanol, eugenol, farnesol, mandelonitrile, and furaneol. The tested anthocyanidins and flavonoids (cyanidin, pelargonodin, quercetin, and kaempferol,) were not converted at all (< 1%). The three active proteins VvGT15a-c showed a more limited substrate spectrum and glucosylated primarily geraniol, citronellol, nerol, octanol, and hexanol. VvGT15a and c were also able to use 8-hydroxylinanol and trans 2-hexanol as acceptor molecules and VvGT15a had low activity for farnesol. Other tested substrates were not converted. VvGT16 showed highest activity towards benzyl alcohol, geraniol and hexanol (> 80% relative activity). Additionally, the protein transformed the terpenoids citronellol and nerol as well as phenylethanol, 3-methyl-2-butanol, trans 2-hexanol and cis 3-hexanol. The formation of monoterpenyl glucosides was confirmed by HPLC-MS/MS analysis in comparison with chemically synthesized glucosides (Figure 6). The retention times and fragmentation patterns of the reference material and products formed by VvGT14a, 15a and 16 were identical and in accordance with the proposed fragmentation mechanism (Domon and Costello, 1988; Cole et al., 1989; Salles et al., 1991). Besides, selected glucosides of the transformed terpenoids were visualized by radio-TLC (supplementary data Figure S7 and S8). The extracted radioactivity of the enzyme assays consisted exclusively of the terpenyl mono-glucosides, except when 8-hydroxylinanol was used. It seemed that this monoterpen diol is glucosylated at both hydroxyl groups as two spots, presumably the mono- and diglucoside appeared on the radio-TLC plate. The two allelic enzymes VvGT14b and 14c were unable to glycosylate any of the tested substrates. The alignment of the three VvGT14 alleles showed that VvGT14c has an internal deletion of 21 amino acids at position 165 (supplementary data Figure S9), while VvGT14a and b differed in a single position (P391L).

**Enantioselectivity of VvGT14a, VvGT15a-c and VvGT16**

Grape berries accumulate free and bound S-citronellol and S-linalool, albeit in lower levels than nerol and geraniol (Table 1, Luan et al., 2004). To elucidate the enantiomeric preference of VvGT14a and VvGT15a, racemic citronellol was used as substrate and racemic linalool was transformed by VvGT14a. Chiral phase GC-MS analysis of liberated citronellol after acid hydrolysis of citronellyl β-D-glucoside demonstrated no enanto-discrimination by VvGT14a and 15a if the reaction mixture is incubated for a prolonged time (24 h; Figure 7). Nevertheless, VvGT15a-c and VvGT14a preferred S-over R-citronellol (1/0.4 and 1/0.8, respectively) when choosing short incubation times for kinetic assays (supplementary data Figure S10A,C) whereas VvGT16 transformed R- and S-citronellol with the same efficiency in radiochemical assays (supplementary data Figure S10B). Accordingly, the kinetic data for VvGT15a-c were calculated for S-citronellol (Table 2). Furthermore, liberated linalool, after enzymatic hydrolysis of linaloyl β-D-glucoside (formed by VvGT14a), showed a slight enrichment of the R-enantiomer (Figure 7). It is important to mention that the hydrolysis of “racemic” (1:1 diastereomeric mixture) linaloyl β-D-glucoside by AR 2000 revealed no enanto-discrimination by the action of this enzyme, which confirmed the results of previous works (Gunata et al., 1990; Lücker et al., 2001). Thus, VvGT14a and VvGT15a-c show low enantioselectivity towards the S-enantiomer of citronellol and VvGT14a towards R-linalool during short-term assays. The enantiomers of racemic
Biochemical characterization of VvGT14a, VvGT15a-c and VvGT16

The assay conditions were optimized for the conversion of geraniol to determine the kinetic constants of the active enzymes. The highest activity of VvGT14a, 15a-c and 16 was found in Tris-HCl buffer (pH 8.5, 7.5 and 8.5, respectively) at 30 °C. The product formation of VvGT14a (0.2 µg purified enzyme) was linear for at least 90 minutes. KM and kcat values were obtained for geraniol, citronellol, nerol, terpineol, 8-hydroxylinalool, and linalool with a constant UDP-glucose level (108 µM) and for UDP-glucose with a fixed geraniol concentration (100 µM; Table 2). The kinetic parameters were determined from a hyperbolic Michaelis-Menten saturation curves. Due to the low conversion rates of 8-hydroxylinalool, terpineol and linalool, the amount of purified enzyme was increased (2, 2, and 10 µg protein, respectively). Interestingly, the KM and kcat value of VvGT14a for citronellol, geraniol, nerol and UDP-glucose was quite similar (KM 9-10 µM; kcat 0.02 sec⁻¹; kcat/KM 2.0-2.6 sec⁻¹ mM⁻¹), while the kinetic data for 8-hydroxylinalool, terpineol and linalool explained the significantly lower enzyme activity towards these substrates. The kinetic data of VvGT15a-c (0.5 or 1 µg purified enzyme) were maintained for geraniol, S-citronellol, nerol and 8-hydroxylinalool with a fixed UDP-glucose amount (833 µM) and for UDP-glucose with a constant geraniol concentration (1.25 mM; Table 2). The formation of geranyl, neryl, and citronellyl ß-D-glucoside was linear for at least 10 minutes (VvGT15c) and 20 minutes (VvGT15a and b), but was extended for 8-hydroxylinaloyl ß-D-glucoside up to 30 min (VvGT15c) and 60 min (VvGT15a and b). The kinetic data confirmed the high enzymatic activity of the VvGT15 alleles towards geraniol and UDP-glucose. The KM and kcat value for S-citronellol and nerol was similar, whereas 8-hydroxylinalool was a poor substrate. Furthermore, the data illustrated that VGT15c is superior to VvGT15a and b. Kinetics of VvGT16 were calculated for geraniol, citronellol and nerol (Table 2). Product formation of VvGT16 (5 µg purified protein) was linear for at least 4 hours. KM and kcat values were obtained for the monoterpenes with a constant UDP-glucose level (512.5 µM) and for UDP-glucose with a fixed geraniol concentration (1.25 mM). VvGT16 exhibited the lowest enzymatic activity towards the substrates of the tested UGTs.

Identification of the natural substrates of VvGT14a, VvGT15a-c and VvGT16

Activity based metabolite profiling (ABMP) allows unbiased discovery of enzymatic activities encoded by genes of unknown function. This approach applies chromatographic methods to analyze the effects of a recombinant enzyme on the homologous cellular extract as a physiologic library of potential substrates and products (De Carvalho et al., 2010; Duckworth and Aldrich, 2010). We adapted this method to reveal the natural substrates of VvGT14a, VvGT15a-c and VvGT16 (Figure 8). In brief, glycosides were isolated by solid phase extraction from grape skins (Gewurztraminer 11-18 Gm) and blooms (Muscat FR90) as they showed highest expression levels of the target genes. An aglycone library of the two tissues was obtained by enzymatic hydrolysis of the glycosides followed by liquid-liquid extraction of the released alcohols and acids. This physiologic library which contained potential natural substrates of UGTs was screened with recombinant VvGTs and either radiochemically labeled or unlabeled UDP-glucose. The formed glycosides were separated by thin layer chromatography (TLC) and visualized by radiodetection whereas identification could be achieved by LC-MS/MS analysis and, after hydrolysis, by GC-MS and LC-MS (Figure 8). Initially, the aglycone extracts were incubated with the purified recombinant enzymes (VvGT14a, 15a, and 16) using radiolabeled UDP-[14C] glucose. Formed products were extracted; radioactivity was quantified by liquid scintillation counting and analyzed by radio-TLC (Figure 9). Screening of the aglycone library obtained from grape skins by VvGT14a and 15a yielded products that showed identical chromatographic properties as geranyl ß-D-glucoside. Enzymatic hydrolysis of the glucosides formed by VvGT14a liberated a substantial amount of geraniol but also remarkable quantities of citronellol, nerol, benzyl alcohol, phenylethanol, and linalool (Figure 9). A similar result was gained with VvGT15a. VvGT16 did not form a visible product neither by using the berry nor from the bloom extracts. The extract from bloom was only tested with VvGT16. Thus, the aglycone library clearly enabled the detection and identification of the natural substrates of VvGT14a and VvGT15a and confirmed the role of these enzymes during grape berry ripening.
DISCUSSION

Small molecule glycosyltransferases transfer carbohydrates to a wide range of acceptors, from antibiotics, lipids, hormones and secondary metabolites to toxins and anthropogenic chemicals (Bowles et al., 2006). Recent progress in genome sequencing has allowed an assessment of the extent of the UGT multigene family in plants. UGT proteins can be easily identified by a signature motif in their primary sequence that is thought to be involved in the binding to the UDP moiety of the sugar nucleotide (Yonekura-Sakakibara and Hanada, 2011). Numerous in vitro studies have demonstrated that a single UGT gene product can glycosylate multiple structurally diverse substrates whereas multiple UGTs can also glycosylate the same substrate. Thus, in cells, substrate availability can be a determining factor of the product spectrum, and redundancy pinpoints to sophisticated gene regulation mechanisms. Although glycosylation of plant hormones, phenylpropanoids, flavonoids, betalains, and coumarins by recombinant UGTs has been frequently described, enzymatic transfer to monoterpenols has been observed quite rarely. Geraniol, nerol and citronellol have been identified as promiscuous substrates of the cyanohydrin UGT from Sorghum bicolor (Hansen et al., 2003) and were among the terpene alcohols that were glycosylated by a group of 27 UGTs from A. thaliana (Caputi et al., 2008, 2010). A BLAST search for similar sequences in the V. vinifera genome yielded candidate genes that were analyzed in detail in our study as it has been assumed that UGTs discriminate compounds as substrate in a lineage-specific manner (Yonekura-Sakakibara and Hanada, 2011). Since enzymes involved in secondary metabolism display broad substrate tolerance (in this context tolerance describes the property of the enzyme much better than specificity as specificity relates to a very limited number of substrates), forward and reverse genetic approaches are frequently applied to reveal in planta functions of genes. However, in non-model plant such as grapes these techniques are not yet very well established. Thus, alternative methods are needed. In this study we propose a novel approach employing a physiologic library of aglycones to identify the natural substrates and function of UGTs by activity based metabolite profiling (ABMP).

Correlation of metabolite analysis and expression analysis of putative VvGTs

Only a few odor impact compounds have been identified in wines. Among these are terpenes that significantly contribute to floral and fruity characters to white wines (Marais, 1983). It has been suggested that monoterpane biosynthesis occurs during grape ripening, starting from berry set (Wilson et al., 1984) whereas our results revealed a large varietal variation in the content of terpenes and their glycosidic derivatives (Table 1). Gewurztraminer 11-18 Gm and Muscat FR 90, two genotypes that are rich in monoterpenes, also accumulated high levels of the glucosides that can also be detected at early stages of berry set (six weeks post flowering). Besides, the ratio of free to glycosylated terpenes differed for the varieties and sampling dates indicating differential glycosylation activities during berry ripening in the diverse genotypes. As terpenoids and their glucosides were accumulating in berry skins after veraison, the strong expression of VvGT14, VvGT15 and VvGT16 during late stages of berry ripening suggested an important role during this period and led to in-depth analysis of these genes (Figure 4). Notably, VvGT14 transcript levels detected in both White Riesling clones correlated well with the late formation of monoterpenyl glucosides in the same varieties (Table 1 and Figure 4) whereas expression level of VvGT15 in Muscat FR 90 showed a similar time course pattern as the amounts of the glucosides. In Muscat FR 90 transcription of VvGT15 and production of terpenyl glucosides started already at 6 to 9 weeks post flowering and remained at a high level. Besides, in Gewurztraminer 11-18 Gm, VvGT15 expression peaked at 9 weeks post flowering and then decreased continuously although monoterpenol derivatives accumulated to high levels in grape skin. However, compensation of VvGT15 activity by VvGT16 was assumed as VvGT16 expression levels even exceeded the range of GeXP quantification at the late stages of ripening. Based on the positive correlation of monoterpenyl glucoside evolution and transcript analysis VvGT14, 15 and 16 were chosen for further analysis.

Biochemical characterization of VvGT14a-c, VvGT15a-c and VvGT16

One of three allelic forms of VvGT14 catalyzed the transfer of glucose to alcohols that have been shown to be glycosylated in grape skins (Wirth et al., 2001). Two alleles (b,c) were inactive. VvGT14a converted a broad range of tested substrates among them were monoterpenoids, short-chain aliphatic and aromatic alcohols while flavonoids were not converted. Up to now, only flavonoid and (hydroxycinnamic acid glycosyltransferases have been characterized in grapes (Ono et al. 2010;
and VvGT15a-c readily formed radiolabeled products when incubated with an aglycone library (Offen et al., 2006; January et al., 2009; Kather et al., 2012). The $K_M$ and $k_{cat}$ values of VvGT14a for geraniol (9 $\mu$M; 0.02 sec$^{-1}$), citronellol (9 $\mu$M; 0.02 sec$^{-1}$), nerol (10 $\mu$M; 0.02 sec$^{-1}$) and UDP-glucose (16 $\mu$M; 0.03 sec$^{-1}$) were alike (Table 2) and resembled the kinetic data of VvGT1 (Offen et al., 2006) and CuUGT2 (Catharanthus roseus) (Masada et al., 2007) for their natural substrates quercetin (31 $\mu$M; 0.075 sec$^{-1}$) and curcumin (43.9 $\mu$M; 0.0165 sec$^{-1}$), respectively. The data were also similar to those of VvGT5 (5.6 $\mu$M; 7.16 sec$^{-1}$), VvGT6 (9.24 $\mu$M; 0.76) and UGT71G1 (57 $\mu$M; 0.0175 sec$^{-1}$) from Medicago truncatula (He et al., 2006) for the conversion of quercetin (Ono et al., 2010). Hence, the specificity constants ($k_{cat}/K_M$) identified the monoterpenol as most probable in vivo substrates of VvGT14a. However, the Michaelis constant for terpineol, 8-hydroxylinalool and linalool were 3 to 5-fold higher while the turnover numbers were 10 to 100-fold lower than for geraniol, nerol and citronellol. Thus, the enzyme efficiency values made it unlikely that they are converted by VvGT14a, in planta.

The sequence comparison of the active VvGT14a allele with the two inactive alleles showed one point mutation at position 391 (P391L) in VvGT14b and a deletion of 21 aa at position 165 in VvGT14c rendering them inactive (supplementary data Figure S9). Proline in position 391 is located in the PSPG motif plant secondary product glycosyltransferase (Hughes and Hughes, 1994) and is quite conserved among UGTs (Osmani et al., 2009). The exchange of proline to leucine in VvGT14b leads to an inactive enzyme and has not been reported, yet.

The allelic forms of VvGT15a-c showed a similar substrate spectrum (Figure 5). Remarkably, these alleles had a distinct preference for the monoterpenes geraniol, citronellol and nerol as comparison with the accepted substrates of VvGT14a clearly demonstrated. The kinetics identified geraniol as superior substrate (Table 2). The turnover numbers of VvGT15a-c for the glucosylation activity of geraniol (0.12, 0.1, and 0.17 sec$^{-1}$, respectively) were 3 to 6-fold higher and the Michaelis constants (63, 81, and 43 $\mu$M, respectively) about 2-fold greater than the $k_{cat}$ and $K_M$ values for nerol and S-citronellol. This resulted in a highest enzyme specificity constant of VvGT15a-c for geraniol and confirmed the data of the substrate screening (Figure 5) whereas allele c was the most effective ($k_{cat}/K_M$ 3.9 sec$^{-1}$ mM$^{-1}$). All three alleles showed a 30 to 40-fold lower turnover number for 8-hydroxylinalool compared to geraniol, although the $K_M$ value was similar to the ones obtained for S-citronellol and nerol.

Notably, the glucosylation of monoterpenes by UGT85B1, the cyano hydrins (mandelonitrile) GT from Sorghum bicolor, can be seen as promiscuous activity (Hansen et al., 2003). However, the $K_M$ data of VvGT14a and VvGT14c were 1.6 to 125-fold lower and the $k_{cat}/K_M$ values up to 78 times higher than the data obtained for the glucosylation of terpenoids by UGT85B1. Mandelonitrile was only a poor substrate of VvGT14a and was not accepted at all by VvGT15a-c.

VvGT16 glucosylated monoterpenols, some short-chained and aromatic alcohols, albeit with low efficiency (Figure 5, Table 2). Interestingly, benzyl alcohol, an alcohol which has been frequently detected in hydrolysates of glycosides from grape, showed the highest relative activity (Gunata et al., 1988). However, the low $k_{cat}/K_M$ values argue against a role of VvGT16 in the glucosylation of these compounds in planta.

**In planta substrates of VvGT14a and VvGT15a-c**

More than 200 volatiles have been identified in grape berries and most of them are glycosylated (Selton et al., 1998). Furthermore, about 240 putative UGT genes have been annotated in the Vitis genome (Jaillot et al., 2007). Since substrate promiscuity is a frequently observed feature of UGTs unambiguous identification of their *in vivo* substrates becomes a challenge. Screening of VvGT14a, 15a-c and 16 with a number of alcohols that have been shown to be glycosylated in grapes revealed broad substrate tolerance also for the UGTs isolated from *V. vinifera*. However, the clear correlation of spatial and temporal transcript accumulation of VvGT14 and VvGT15 in the Riesling clones and Muscat FR 90, respectively with monoterpenyl glucoside evolution provided first evidence for the biological function of the encoded proteins. Detailed biochemical analyses and structural confirmation of the products by LC-MS analysis further substantiated the hypothesis of the identification of the first monoterpenyl glucosyltransferases from *V. vinifera*.

However, ultimate proof was delivered by application of activity based metabolite profiling (ABMP) of a physiologic aglycone library (Figure 8) which has several advantages. An aglycone library can be prepared from any tissue that shows high UGT transcript levels. The library contains the natural substrates in high concentration as they are enriched during the preparation of the extract and can be easily screened with recombinant UGT enzymes and various labeled and unlabeled UDP-sugars.

Formation of products can be rapidly quantified by LSC and confirmed by TLC (Figure 8). Identification of glycosides by LC-MS and NMR analyses is greatly facilitated as levels of impurities are very low. Finally, the structure of the carbohydrate moiety is already known from the used UDP-sugar. VvGT14a and VvGT15a-c readily formed radiolabeled products when incubated with an aglycone library.
obtained from grape skins of Gewurztraminer 11-18 Gm (Figure 9A). Geranyl and minor amounts of
citronellyl and neryl glucoside (as their free alcohols after hydrolysis) were identified as products of
VvGT14a and VvGT15a-c (Figure 9B). In contrast, VvGT16 did not form a glucosylated product
although expression level of VvGT16 and amounts of monoterpenyl glucosides were extraordinary
high in berry skins in the late stages of ripening and blooms (Gewurztraminer 11-18 Gm). However,
additional UDP-sugars, besides UDP-glucose, were not tested as putative donor molecules and the
possible formation of di- and triglycosides of one aglycone was not taken into account (Gunata et al.,
1988; Voirin et al., 1990; Janvary et al., 2009). We conclude that by using alternative UDP-sugars for
the screening of the libraries, novel glucosides can be formed and identified which also occur in
nature. In addition, combination of different UDP-sugars with a diversity of recombinant UGTs would
also yield aglycones attached to di- and triglycosides. Last but not least, the method can be applied in
high-throughput screens. However, insufficient release of aglycones by acidic and enzymatic
hydrolysis and instability of liberated aglycones are two drawbacks which have to be kept in mind.
Moreover, the obtained glycosidic extract, which is used for ABMP, contains glycosides from various
cellular compartments like cytosol and vacuole. Because it is known that glycosyltranferases are
usually located in the cytosol, it cannot be ruled out that in an in vivo scenario some aglycones and
glycosyltransferases do not come into contact in intact cells. Nevertheless, ABMP of aglycone libraries
has great potential to unravel the physiologic substrates of a wide range of UGTs and thus to clarify
their biological roles.

Interestingly, VvGT14a and VvGT15a-c also displayed high level of activity toward aliphatic alcohols
such as octanol, hexanol, and hexenols (Figure 5). Grape leaves and berries contain appreciable
amounts of glycosides derived from these alcohols (Wirth et al. 2001). However, functional screening
of an aglycone library produced from glycosides isolated from grape exocarp did not reveal the
aliphatic alcohols as natural substrates (Figure 9), probably due to their low levels in this tissue. The
amounts of glycoconjugates derived from aliphatic alcohols are much higher in leaves from V. vinifera
cultivars (Wirth et al., 2001). Thus, we assume that VvGT14a and VvGT15a-c might contribute, at
least to a certain extent, to the formation of hexyl, hexenyl, and octyl glucoside in leaves. The
hypothesis will be tested in future studies by functional screening of an aglycone library produced from
leaves of V. vinifera.

**Enantiomeric discrimination**

Free and glycosylated citronellol and linalool occur predominantly in S-configuration in grapes
whereas their free and glycosylated forms exhibit similar enantiomeric excesses (Luan et al., 2005).
While VvGT14a and VvGT15a-c preferentially glucosylated S-citronellol in short time assays
enantiomolecivity of these enzymes for citronellol was not observe in long term studies (Figure 7;
supplementary data Figure S10). This effect is characteristic for studies on the kinetic resolution of
racemates (Strauss et al., 1999). The reaction slows down at 50% conversion, when the fast reacting
enantiomer is almost consumed and only the slow reacting counterpart is gradually transformed. Thus,
the enantiomeric excess of the product peaks at 50% transformation and then decreases slowly but
steadily. In contrast, VvGT14a preferred R- over S-linalool even in long term assays. The slight
preference for R-linalool explained the previously observed enrichment of this enantiomer in the
glycosidically bound fraction of linalool in Morio Muskat and Muscat Ottone berries relative to the free
fraction (Luan et al., 2004). Hence, the moderate enantiomolecivities of VvGT14a and 15a-c were in
accordance with their proposed biological role as the availability of highly enriched S-citronellol and S-
linalool mainly determined the diastereomeric ratio of the glucosidic product.

**CONCLUSION**

In plants, UGTs represent a large gene family whereas the individual gene products show substrate
promiscuity especially if they are related to secondary metabolism. In this study we devise an activity
based metabolite profiling method for targeted functional screening of small molecule UGTs which
allowed the rapid identification of in vivo substrates by using aglycone libraries prepared from different
tissues. The approach has the potential to be broadly applied as it is suitable for HT screens.
ABMP of an aglycone library obtained from grape berry led to the identification of two
glycosyltransferase genes that are involved in the metabolism of monoterpenol in vivo. Spatial and
temporal gene expression analysis in combination with metabolite and chiral phase analysis revealed
that enzyme specificity and substrate availability dictate the formation of monoterpenol glucosides
during grape ripening. Furthermore, it appears that different UGTs such as VvGT14 and 15a-c are
major players for monoterpenyl glucoside formation in different varieties such as the Riesling clones
and Muscat FR90, respectively.
MATERIALS AND METHODS

Plant Material

Vitis vinifera grapevines of cultivars Gewurztraminer 11-18 Gm, Gewurztraminer FR 46-107, White Riesling 239-34 Gm, White Riesling 24-196 Gm and Muscat a petit grains blancs FR 90 were grown in the Geisenheim research center vineyard at Geisenheim, Germany, during vintages 2011 and 2012. Grape berries, leaves, inflorescences and roots were collected. Sampling was conducted for a total of six dates between 6 and 17 weeks after bloom in 2011 including berries from pea-size to harvest ripeness. Muscat a petit grains blancs FR 90 was additionally sampled in 2012 every two weeks from week 4 to week 18 after bloom (Figure 1). After veraison (the onset of ripening) 100 berries were collected for the determination of ripening related parameters like sugar content. For terpenoid analysis 250 g of berries were stored at -20 °C, while three replicates consisting of ten berries were peeled and skins immediately frozen in liquid nitrogen for subsequent RNA extraction. Roots were obtained from scions of White Riesling 239-34 Gm and Gewurztraminer 11-18 Gm grown in the greenhouse. Leaves were sampled from the same cultivars at the approximate age of one, three and five weeks. In addition, inflorescences four and two weeks before flowering and at full bloom were collected in 2012. Samples were immediately frozen in liquid nitrogen and stored at -20°C until work-up. Samples were extracted within 6 months of storage at -20°C.

Chemicals

Except when otherwise stated, all chemicals, solvents, and reference compounds were purchased from Sigma-Aldrich (Steinheim, Germany), Fluka (Steinheim, Germany) and Roth (Karlsruhe, Germany). UDP-[14C]glucose (300 mCi/mmol, 0.1 mCi/mL) was obtained from American Radiolabelled Compounds (St Louis, MO, USA). (R.S)-3,7-dimethyl-1,6-octadien-3-ol (linalool) and (E)-3,7-dimethyl-2,6-octadien-1-ol (geraniol) were obtained from Roth (Karlsruhe, Germany). (R,S)-3,7-dimethyl-6-octen-1-ol (citronellol) and pure (R)-(+)-ß-citronellol were purchased from Sigma Aldrich (Steinheim, Germany). (Z)-3,7-dimethyl-2,6-octadien-1-ol (nerol) was purchased from Alfa Aesar (Karlsruhe, Germany). (R,S)-3,7-dimethyl-6-octenyl ß-D-glucopyranoside (citronellyl ß-D-glucoside), (Z)-3,7-dimethyl-2,6-octadienyl ß-D-glucopyranoside (neryl ß-D-glucoside) and (E)-3,7-dimethyl-2,6-octadienyl ß-D-glucopyranoside (geranyl ß-D-glucoside) were synthesized according to the Koenigs-Knorr-procedure (Paulsen et al., 1985). (R,S)-3,7-dimethyl-1,6-octadienyl ß-D-glucopyranoside (linaloyl ß-D-glucoside), as less reactive tertiary alcohol, was synthesized according a modified Koenigs-Knorr-procedure, using another catalyst (Hattori et al., 2004). Deuterium labeled 1,1-[2H2]-citronellyl ß-D-glucoside was prepared as described (Hill et al., 1993; Wüst et al., 1998). Spectral data of the synthesized compounds were in all cases in good agreement with the data given (Konda et al., 1997; Paulsen et al., 1985; Salles et al., 1991).

Sample preparation for metabolite analysis

Grapes berries were peeled and 10 g (fresh weight) of the grape skins were taken for one analysis. In case of root, leaf and inflorescence, 4 g plant material was taken per analysis. The material was frozen in liquid nitrogen, ground and extracted with a mixture of phosphate buffer (0.1 M, pH 7) and 13% ethanol for 24 h under nitrogen with exclusion of light (Jesús Ibarz et al., 2006). 2-Octanol was used as internal standard for the determination of free monoterpenes. For the determination of monoterpenyl ß-D-glucosides, stable isotope dilution analysis (SIDA) was applied, using [2H2]-citronellyl ß-D-glucoside as a labeled, internal standard. The concentration of the internal standards was adapted for the variety, the tissue and the ripening stage of the plant material. 2-Octanol was added in a range of 0.3 to 6.8 mg/kg plant material, [2H2]-citronellyl ß-D-glucoside in a range of 0.1 to 3.5 mg/kg plant material. To purify the sample, Carrez reagents (Merck Millipore, Darmstadt, Germany) were added (1 mL each) and the sample was then centrifuged at 14500 rpm for 20 min at 5 °C. The supernatant was taken for subsequent solid phase extraction (SPE) to isolate and separate free monoterpenes from glycosidically bound monoterpenes. Therefore, a 200 mg Lichrolut EN column (Merck, Darmstadt, Germany) was conditioned as described (Piñeiro et al., 2004). Free monoterpenes were eluted with dichloromethane and glycosidically bound monoterpenes with methanol. For GC-MS detection, the dichloromethane fractions were dried with Na2SO4, concentrated using nitrogen to 200 µL and analyzed. For HPLC-MS/MS detection, the methanolic fractions were concentrated under reduced pressure and the residues were dissolved in water/acetonitrile (7/3; v:v). The samples were analyzed by LC-MS/MS.

Nucleic acid extraction
For total RNA extraction plant material was ground to a fine powder in liquid nitrogen using mortar and pestle. One g of the powder was used for RNA-extraction with the CTAB method following an established protocol (Zeng and Yang, 2002, adapted by Reid et al. 2006) to meet the requirements of different grape tissues. Remaining genomic DNA was digested by DNase I and cleaned up with the High Pure RNA Isolation kit (Roche, Mannheim, Germany).

Transcription analysis

Transcription analysis was performed using the Genome Lab GeXP Genetic Analysis System (Beckman Coulter, Krefeld, Germany), a multiplex quantitative gene expression analysis system. The gene expression patterns of eight VvGT genes and five reference genes (VvActin, VvAP47, VvPP2A, VvSAND, and VvTIP41) were analyzed simultaneously from one sample of total RNA. Reverse transcription was carried out with the GenomeLab™ GeXP Start kit (Beckman Coulter) following the manufacturer’s instructions. As an internal control gene KAN RNA was co-reverse transcribed and subsequently amplified together with the reference genes and genes of interest. The gene specific primers for reverse transcription are chimeric, providing a 19 nt universal tag for the binding of universal reverse primers in the subsequent PCR reaction. The final concentration of the primers ranged from 0.1 nM to 100 nM to compensate for the different transcription levels of the analyzed genes (Supplemental data Table S1). Multiplex PCR reactions were conducted with Thermo-Start DNA Polymerase (Thermo Fisher Scientific, Dreieich, Germany). Each reaction contained 9.3 µL of reverse transcription products as template and 10.7 µL of a PCR reaction mix including gene specific forward primers providing an 18 nt universal tag (Supplemental data Table S1). The universal forward primer is labeled with a fluorescent dye for detection during subsequent capillary electrophoresis. Primer pairs were designed to yield PCR products ranging from 119 bp to 374 bp and differing in size by at least 8 bp. Of each PCR product, 4 µL were separated by capillary electrophoresis using the GenomeLab Genetic Analysis System (Beckman Coulter). Individual standard curves for each gene in the multiplex were performed with serial two-fold dilutions ranging from 3.91 ng to 500 ng of an RNA mixture from all samples. Raw data were analyzed using the Fragment Analysis tool. The fragment data of the standard curves and samples were then normalized to the peak area of KAN RNA with the Express Analysis tool. Subsequently, the relative signal level of each sample replicate was interpolated from the standard curve. The data was further normalized to the geometric mean of the five reference genes with Quant tool. All software for GeXP data analysis was purchased from Beckman Coulter.

Comparative Sequencing

The reference genome of PN40024 (Jaillon et al., 2007) was used to design gene specific primers in the untranslated regions of the three putative VvGT genes, VvGT14, VvGT15, and VvGT16 using the tool Primer-BLAST (Ye et al., 2012). Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany, supplemental data Table S2). The cDNA–synthesis was performed with the SuperScript® III First-Strand Synthesis SuperMix (Life Technologies, Darmstadt, Germany) following the manufacturer’s instructions. The template for cDNA-synthesis was total RNA, extracted from samples with high transcript-levels of the concerned VvGT gene, as determined by gene expression analysis. The cDNA was used as template in the following PCR-reaction. PCR was performed with Phusion DNA Polymerase (Thermo Fisher Scientific, Dreieich, Germany) using high-fidelity (HF)-buffer and the following thermal cycling conditions: 98 °C for 30 s followed by 32 cycles consisting of 98 °C for 5 s, 60 °C for 5 s and 72 °C for 30 s and a final elongation step of 72 °C for 1 min. PCR products were gel purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). A-tailing of purified PCR-products was performed with Taq DNA Polymerase (Thermo Fisher Scientific). A-tailed PCR-products were ligated into pGEM-T Easy vector (Promega, Madison, USA) and cloned in One Shot TOP10 Chemically Competent E.coli (Life Technologies, Darmstadt, Germany). Plasmids were isolated with the PureYield Plasmid Miniprep System (Promega, Madison, USA) and sequenced with the vector specific primers M13 uni (-21) and M13 rev (-29) on an ABI 3730 capillary sequencer (StarSEQ, Mainz, Germany). Raw data was edited with the FinchTV software (Geospiza, Seattle, USA). Sequences were assembled with SeqMan and aligned with MegAlign (DNASTAR, Madison, USA).

Construction of expression plasmids

The full-length ORFs of the VvGT sequences were subcloned into the pGEM-Teasy vector (Promega, Madison, WI, USA). All genes were amplified with primer introducing BamHI und NotI restriction sites. Subsequently, the genes were cloned in frame with the N-terminal GST-tag into the pGEX-4T-1.
fraction, which was obtained after SPE, was enzymatically hydrolysed. Therefore, the dried sample isolation of glycosides was carried out as described above (see metabolite analysis). The methanolic inflorescences of Gewurztraminer Gm 11-18 were ground to a fine powder in liquid nitrogen. The Preparation of aglycone extracts 50 g (fresh weight) grape skins or 4 g (fresh weight) for the preparation of aglycone libraries. The reaction was stopped by adding 1 µL 24% trichloroacetic acid and glucosides were extracted with 500 µL water-saturated 1-butanol. The organic phase was mixed with 2 mL Pro Flow P+ cocktail (Meridian Biotechnologies Ltd., Epsom, UK) and radioactivity was determined by liquid scintillation counting (LSC, Tri-Carb 2800TR, Perkin Elmer, Waltham MA, USA). Additionally, negative controls without substrate were performed. After determining the optimal conditions for the best substrate of each gene, the substrate screening was repeated under these conditions. The kinetic data were determined with increasing concentrations of the substrates (VvGT14a: citronellol, geraniol, 8-hydroxylinalool, linalool, nerol and terpineol; VvGT15a-c: S-citronellol, geraniol, 8-hydroxylinalool and nerol; VvGT16: citronellol, geraniol and nerol) from 1 µM to 100 µM (VvGT14a and VvGT15a-c) or 50 µM to 500µM (VvGT16) and a fixed UDP-glucose concentration of 108 µM (100 µM unlabeled UDP-glucose and 8 µM UDP-[^14C] glucose; VvGT14a), 833 µM (825 µM unlabeled UDP-glucose and 8 µM UDP-[^14C] glucose; VvGT15a-c) or 512.5 µM (500 µM unlabeled UDP-glucose and 12.5 µM UDP-[^14C] glucose; VvGT16). The total volume was 40 µL and 0.2 µg (VvGT14a), 0.5 µg (VvGT15a-c) or 5 µg (VvGT16) of purified protein. The measurements were performed under the following conditions. The assays were carried out at 30 °C for 1.5 h using a Tris-HCl buffer (100 mM, 10 mM 2-mercaptoethanol, pH 8.5 for VvGT14a and VvGT16). The assay of VvGT15a-c was performed at 30 °C using a Tris-HCl buffer (100 mM, 10 mM 2-mercaptoethanol, pH 7.5) and 10 min (VvGT15c) or 30 min (VvGT15a-b) of incubation for the best substrates. The amount of the purified enzyme and the incubation time were adapted depending on the counting sensibility. The reaction was stopped by adding 1 µL 24% trichloroacetic acid and glucosides were extracted with 100 µL ethyl acetate. Radioactivity was determined by LSC. To determine the kinetic data of UDP-glucose, the value of geraniol was fixed (1.25 mM for VvGT15a-c and VvGT16; 0.1 mM for VvGT14a) and UDP-[^14C] glucose was mixed with non-radiolabeled UDP-glucose to obtain concentrations ranging from 5 µM to 100 µM (VvGT14a and VvGT15a-c) or 25 µM to 500 µM (VvGT16). The Km- and vmax-values were calculated from Lineweaver-Burk plots, Hanes-Woolf plots and non-linear fitting of the experimental data. 

Activity assay and kinetics

In the initial screening, each reaction mixture (200 µL in total) contained Tris-HCl buffer (100 mM, pH 8, 10 mM 2-mercaptoethanol), 37 pmol UDP-[^14C]glucose (0.01 µCi, Biotrend, Köln, Germany), substrate (50 µL of a 1 mg/mL stock solution) and purified protein (0.5-0.8 µg/µL). The reaction mixture was incubated at 30 °C for 18.5 hours. The assays were stopped by adding 1 µL 24% trichloroacetic acid and extracted with 500 µL water-saturated 1-butanol. The organic phase was mixed with 2 mL Pro Flow P+ cocktail (Meridian Biotechnologies Ltd., Epsom, UK) and radioactivity was determined by liquid scintillation counting (LSC, Tri-Carb 2800TR, Perkin Elmer, Waltham MA, USA). Additionally, negative controls without substrate were performed. After determining the optimal conditions for the best substrate of each gene, the substrate screening was repeated under these conditions. The kinetic data were determined with increasing concentrations of the substrates (VvGT14a: citronellol, geraniol, 8-hydroxylinalool, linalool, nerol and terpineol; VvGT15a-c: S-citronellol, geraniol, 8-hydroxylinalool and nerol; VvGT16: citronellol, geraniol and nerol) from 1 µM to 100 µM (VvGT14a and VvGT15a-c) or 50 µM to 500µM (VvGT16) and a fixed UDP-glucose concentration of 108 µM (100 µM unlabeled UDP-glucose and 8 µM UDP-[^14C] glucose; VvGT14a), 833 µM (825 µM unlabeled UDP-glucose and 8 µM UDP-[^14C] glucose; VvGT15a-c) or 512.5 µM (500 µM unlabeled UDP-glucose and 12.5 µM UDP-[^14C] glucose; VvGT16). The total volume was 40 µL and 0.2 µg (VvGT14a), 0.5 µg (VvGT15a-c) or 5 µg (VvGT16) of purified protein. The measurements were performed under the following conditions. The assays were carried out at 30 °C for 1.5 h using a Tris-HCl buffer (100 mM, 10 mM 2-mercaptoethanol, pH 8.5 for VvGT14a and VvGT16). The assay of VvGT15a-c was performed at 30 °C using a Tris-HCl buffer (100 mM, 10 mM 2-mercaptoethanol, pH 7.5) and 10 min (VvGT15c) or 30 min (VvGT15a-b) of incubation for the best substrates. The amount of the purified enzyme and the incubation time were adapted depending on the counting sensibility. The reaction was stopped by adding 1 µL 24% trichloroacetic acid and glucosides were extracted with 100 µL ethyl acetate. Radioactivity was determined by LSC. To determine the kinetic data of UDP-glucose, the value of geraniol was fixed (1.25 mM for VvGT15a-c and VvGT16; 0.1 mM for VvGT14a) and UDP-[^14C] glucose was mixed with non-radiolabeled UDP-glucose to obtain concentrations ranging from 5 µM to 100 µM (VvGT14a and VvGT15a-c) or 25 µM to 500 µM (VvGT16). The Km- and vmax-values were calculated from Lineweaver-Burk plots, Hanes-Woolf plots and non-linear fitting of the experimental data. 

Preparation of aglycone libraries

For the preparation of aglycone extracts 50 g (fresh weight) grape skins or 4 g (fresh weight) inflorescences of Gewurztraminer Gm 11-18 were ground to a fine powder in liquid nitrogen. The isolation of glycosides was carried out as described above (see metabolite analysis). The methanolic fraction, which was obtained after SPE, was enzymatically hydrolysed. Therefore, the dried sample
was dissolved in citric-acid buffer (0.1 M, pH 4), 50 mg AR 2000 (DSM Food Specialties Beverage Ingredients, Delft, Netherlands) was added and incubated for 24 h with exclusion of light. The liberated aglycones were extracted by 20 mL methyl-tert-butyl ether and the organic phase was reduced to 1000 µL using a gentle stream of nitrogen.

Activity based profiling using a physiologic aglycone library

Aliquots of this aglycone extract were incubated with UDP-glucose and various VvGT-enzymes. Optimum conditions at 30 °C for 24 hours were applied. Each solution contained 100 µL purified enzyme, 100-150 µL Tris-HCl buffer (100 mM, pH7.5 or 8.5, 10 mM 2-mercaptoethanol), 37 pmol UDP-[14C]glucose (0.01 µCi) and 50-100 µL extract (dissolved in methyl-tert-butylether). The buffer was mixed with the extract and the organic solvent was gently vaporized with nitrogen. The missing volume was adjusted with buffer before the enzyme was added. The reaction was stopped by adding 1 µL 24% trichloroacetic acid and extracted with 500 µL ethyl acetate. After termination of the reaction free aglycones, which were not converted by VvGT14 and VvGT15, were measured via SPME-(Solid Phase Microextraction)-GC-MS. These residual, free aglycones were completely removed by extraction with dichloromethane. Enzymatically formed glucosides were extracted by ethyl acetate, which was removed under nitrogen. The residue was dissolved in methanol and analyzed by LC-MS/MS to detect the generated monoterpenol glucosides. Enzymatic hydrolysis of the glucosides was performed using AR 2000 and 2 mL citric-acid-buffer. After hydrolysis, a 100 µL aliquot was used to detect volatile aglycones via SPME-GC-MS. The remaining solution was extracted with methyl-tert-butyl ether and reduced under nitrogen to approximately 200 µL. One µL were measured by GC-MS via liquid injection.

Enantioselectivity of VvGT14a, VvGT15a-c

To determine the enantioselectivity of VvGT14 and VvGT15 the enantiomeric ratio of glucosidically bound citronellol and linalool was determined by enantioselective GC-MS. Following the incubation, residual citronellol and linalool were completely removed by extraction with dichloromethane. Citronellyl β-D-glucoside which remained in the aqueous phase was hydrolyzed by HCl (2 mL, 0.1 M, pH 1) for one hour at 100 °C to release citronellol (Skouroumounis and Sefton, 2000). In case of linalyl β-D-glucoside an enzymatic hydrolysis (AR 2000, citric acid buffer pH 4, 24 h) was applied due to the instability of linalool in acid solutions (Williams et al., 1982). Hydrolysis of a synthetic 1:1 mixture of R- and S-linalyl β-D-glucoside revealed that AR 2000 does not discriminate between the two diastereomeric glucosides. After hydrolysis, citronellol and linalool were analyzed by SPME-GC-MS as described above.

HPLC-MS/MS analysis

For HPLC-MS/MS analysis of monoterpenyl β-D-glucosides a Shimadzu LC20AD HPLC system coupled to an API 2000 (Applied Biosystems, AB Sciex, Framingham, USA) triple-quadrupol-MS was used. Data acquisition was performed using Analyst software version 1.6.1. (Applied Biosystems, AB Sciex, Framingham, USA). The column (Phenomenex Gemini-NX 5u C18, 250 x 3 mm, Aschaffenburg, Germany) was eluted with a linear gradient starting at water/acetonitrile (7/3; v:v) containing 0.2% ammonia till 12 min to water/acetonitrile (4/6; v:v; 0.2% ammonia) at 18 min. The column temperature was maintained at 40 °C. The mass spectrometer was operated in ESI-MRM negative ion mode. Nitrogen was used as curtain (setting 20), nebulizing and collision gas (collision energy was -20 eV). Monoterpenyl β-D-glucosides were identified by the following, characteristic MRM transitions (LinGlc: m/z 315→161(Glu), 315→113(Glu); NerGlc: m/z 315→119(Glu), 315→113(Glu); GerGlc: m/z 315→119(Glu), 315→113(Glu); CitrGlc: m/z 317→101(Glu), 317→161(Glu)) (Cole et al., 1989; Domon and Costello, 1988; Salles et al., 1991; supplemental data Table S3).

GC-MS analysis

GC-MS analysis was performed with a Varian GC-450 coupled to a Varian MS-240 ion-trap employing a Phenomenex Zebron ZB-WAXplus column (30 m x 0.25 mm x 0.25 µm, Aschaffenburg, Germany). Helium flow rate was 1 mL/min. The analysis was carried out in split mode (liquid injections) or splitless mode (SPME measurements) with 220 °C injector temperature. Transfer line temperature was 230 °C. EI (electron impact ionization)-MS spectra were recorded from m/z 40 to 300 (ionization energy 70 eV; trap temperature 170 °C). The oven temperature program was 60 °C (3 min), 10 °C/min up to 250 °C (5 min). In case of SPME measurements, liberated aglycones were isolated for 10 min at 60°C using a fiber coated with a 85 µm film of polyacrylate (SUPELCO, Bellefonte, USA). After
extraction the SPME fiber was desorbed for 10 min at 250°C in the injection port of the GC-MS system and the column oven program was carried out as described above. Enantioselective GC-MS analysis was performed with a Varian GC-450 coupled to a Varian MS-240 ion-trap. The column was a DiAcß (heptakis-(2,3-di-O-acetyl-6-O-tert.-butyldimethylsilyl)-ß-cyclodextrin), 26 m x 0.32 mm i.d. with a 0.1 μm film. Helium was used as carrier gas at a flow rate of 1 mL/min, injector temperature was 250 °C with a split ratio of 1/100 (liquid injections), or splitless (SPME measurement). The oven temperature program was 70 °C (3 min), 0.5 °C/min to 130 °C 20 °C/min up to 200 °C (3 min). Transfer line temperature was 230 °C. GC-MS measurements were recorded in full scan mode. Selected ion monitoring (SIM) mode was used for quantification.

Radio-TLC analysis

Assays containing UDP-[14C]glucose and aglycone libraries or substrates were performed as described above and subsequently extracted with 500 µL ethyl acetate. The organic solvent was vaporized and the pellet was re-suspended in 10 µL methanol and was applied on Silica Gel 60 F254 plates (Merck, Darmstadt, Germany). The dried plates were developed in a solvent system chloroform:acetic acid:water (50/45/5, v:v:v). Plates were dried and analyzed by digital autoradiograph (digital autoradiograph, EG&G Berthold, Wildbad, Germany).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Supplemental Figure S1. Location of putative UGT-genes
Supplemental Figure S2. Gene expression analysis of VvGTs by GeXP in non-berry tissues.
Supplemental Figure S3. Gene expression analysis of VvGTs by GeXP during berry development.
Supplemental Figure S4. Gene expression analysis of VvGTs by GeXP during two consecutive years.
Supplemental Figure S5. SDS-PAGE and Western blot analysis VvGT14 and 16.
Supplemental Figure S6. SDS-PAGE and Western blot analysis VvGT15.
Supplemental Figure S7. Radio-TLC analysis of products formed by VvGT14 and 16.
Supplemental Figure S8. Radio-TLC analysis of products formed by VvGT15.
Supplemental Figure S9. Multiple protein sequence alignment of the three variants of VvGT14.
Supplemental Figure S10. Enantiomeric discrimination of VvGT enzymes.
Supplemental Table S1. Gene specific primers used for GeXP.
Supplemental Table S2. Primers used in PCR for subsequent cloning and sequencing.
Supplemental Table S3. Gene specific primers used for GeXP.
Supplemental Table S4. Multiple reaction monitoring (MRM) transitions and excitation voltage of the ESI-MS/MS method.

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REFERENCES

Hughes J, Hughes MA (1994) Multiple secondary plant product UDP-glucose glucosyltransferase genes expressed in cassava (Manihot esculenta Crantz) cotyledons. DNA Seq 5: 41-49
Masada S, Terasaka K, Mizukami H (2007) A single amino acid in the PSPG-box plays an important role in the catalytic function of CaUGT2 (Curcumin glucosyltransferase), a group D family 1 glucosyltransferase from Catharanthus roseus. FEBS Lett 581: 2605-2610


Osmani SA, Bak S, Moller BL (2009) Substrate specificity of plant UDP-dependent glycosyltransferases predicted from crystal structures and homology modeling. Phytochemistry 70: 325-347


Figure 1. Grapes of Muscat FR 90 (A) and major monoterpenols found in grapes of *Vitis vinifera* (B). Pictures were taken every two weeks between week four (4) and week 18 (18) after flowering. Pictured width is 10 cm.

Figure 2. Phylogenetic tree of GT protein sequences. Protein sequences from *Arabidopsis thaliana* (AtUGT) with known glucosyltransferase activity towards terpenes. VvGT14-20 were investigated in this study. GT subgroup assignment is shown in the boxes.

Figure 3. Gene expression analysis of VvGT7s by GeXP in non-berry tissues. The relative expression was quantified in Gewurztraminer 11-18 Gm (black bars) and White Riesling 239-34 Gm (grey bars). Sampled tissues: Inflorescences four weeks (I1) and two weeks (I2) before flowering and at full bloom (I3), leaves at the age of approximately one week (L1), three weeks (L2) and five weeks (L3) and roots (R). The mean values ±SD of three independent experiments are shown, o.o.r. out of range.

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Figure 5. Relative specific activity (%) of VvGT14a (A), VvGT15a-c (B), and VvGT16 (C) protein from *Vitis vinifera* towards putative substrates as determined by radiochemical analysis with UDP-[14C]glucose. The relative activities refer to the highest level of extractable radioactivity which was measured for the conversion of geraniol (100 %) in case of VvGT14a and VvGT15a-c (order of the columns 15c, 15b, 15a) and benzyl alcohol (100 %) in the case of VvGT16. Data for two biological and two technical replicates are shown. Black and white bars represent monoterpenoids and non-monomerpenoids, respectively.

Figure 6. Detection of monoterpenyl β-D-glucosides as products of VvGT14a, VvGT15a and VvGT16 by HPLC-ESI-MS/MS. HPLC-MS/MS analysis of citronellyl β-D-glucoside (A), geranyl β-D-glucoside (B), neryl β-D-glucoside (C), linaloyl β-D-glucoside (D) formed by VvGT14a, VvGT15a and VvGT16 and a mixture of synthesized monoterpenyl β-D-glucosides (E); Chromatograms display an overlay of single product measurements, traces show the total ion current of the characteristic transitions (refer to Methods) Gaussian smoothing was partly applied. The poor peak shapes of geranyl glucoside (B) and neryl glucoside (C) are probably caused by overloading of the chromatographic column.

Figure 7. Enantioselectivity of VvGT14a and VvGT15a determined by chiral phase SPME-GC-MS analysis of citronellol and linalool. A) Racemic mixture of R,S-citronellol was used as substrate for VvGT14a and VvGT15a, enantiomerically pure R-citronellol was used as reference; B, C) racemic citronellol is released by acid catalyzed hydrolysis of citronellyl-β-D-glucoside formed by VvGT14a and VvGT15a. Signals labelled with „x“ are hydrolysis by-products. Chromatograms are shown in SIM mode by using the characteristic ion traces m/z 69,81 and123 for citronellol. D) Racemic mixture of R,S-linalool was used as substrate for VvGT14a; E) Enantiomerically pure R- linalool was used as reference material; F) A slight preference for the R-linalool is revealed after enzymatic hydrolysis with AR 2000. Chromatograms are shown in SIM mode (m/z 71, 93).

Figure 8. Targeted functional screening of small molecule glucosyltransferases by means of aglycone libraries prepared from different plant tissues.

Figure 9. Functional screening of VvGT14a, 15a and 16. Radio-TLC analysis (A) of products formed by VvGT14a (1,2,3), VvGT15a (4,5,6) and VvGT16 (7,8,9) after incubation with the aglycone library obtained from grape berries of Gewurztraminer 11-18 Gm (1,4,7); Positive control: geraniol (2,5,8); negative control: no acceptor molecule (3,6); UDP-[14C]-glucose (9; approximately: 3000 dpm). The plates were analyzed by digital autoradiograph. The products formed from citronellol, geraniol and nerol were verified by LC-MS analysis. GC-MS analysis (B, total ion chromatogram) of volatiles that were enzymatically released from glucosides which were formed by incubation of an aglycone library obtained from grape berries of Gewurztraminer 11-18 Gm with UDP-glucose and VvGT14 (gray) or heat-inactivated VvGT14a (black) as control 1: linalool; 2: citronellol; 3: nerol; 4: geraniol; 5: benzyl alcohol; 6: phenylethanol. For response factors of volatiles (0.52 – 1.90) towards geraniol refer to supplemental data Table S4. Thus, even when response factors are taken into account, geraniol is the major product that is released.
Table 1. Amounts of free monoterpenes and monoterpene-ß-D-glucosides in grape skins during grape ripening

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<tr>
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<td>0.05 ±0.03</td>
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<td>n.d.</td>
<td>n.d.</td>
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<td>0.51±0.02</td>
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<td>1.00±0.21</td>
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<td>0.04±0.01</td>
<td>0.12±0.05</td>
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Plant material was prepared and analyzed as described in Methods. Grapes were collected during grape ripening at the indicated weeks post flowering. n.d.: not detected, -: not determined. Amounts are listed in mg/kg grape skins. n=2. (taken from Bönisch et al., 2014; supplemental data Table S6).
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<th>Substrate</th>
<th>$K_M$ [µM]</th>
<th>$k_{cat}$ [sec$^{-1}$]</th>
<th>$k_{cat}/K_M$ [sec$^{-1}$ mM$^{-1}$]</th>
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Figure 1. Grapes of Muscat FR 90 (A) and major monoterpenols found in grapes of *Vitis vinifera* (B). Pictures were taken every two weeks between week four (4) and week 18 (18) after flowering. Pictured width is 10 cm.
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Figure 7. Enantioselectivity of VvGT14a and VvGT15a determined by chiral phase SPME-GC-MS analysis of citronellol and linalool. A) Racemic mixture of R,S-citronellol was used as substrate for VvGT14a and VvGT15a, enantiomerically pure R-citronellol was used as reference; B, C) racemic citronellol is released by acid catalyzed hydrolysis of citronellyl-ß-D-glucoside formed by VvGT14a and VvGT15a. Signals labelled with "x" are hydrolysis by-products. Chromatograms are shown in SIM mode by using the characteristic ion traces m/z 69,81 and123 for citronellol. D) Racemic mixture of R,S-linalool was used as substrate for VvGT14a; E) Enantiomerically pure R- linalool was used as reference material; F) A slight preference for the R–linalool is revealed after enzymatic hydrolysis with AR 2000. Chromatograms are shown in SIM mode (m/z 71, 93).
**Figure 8.** Targeted functional screening of small molecule glucosyltransferases by means of aglycone libraries prepared from different plant tissues.
Figure 9. Functional screening of VvGT14a, 15a and 16. Radio-TLC analysis (A) of products formed by VvGT14a (1,2,3), VvGT15a (4,5,6) and VvGT16 (7,8,9) after incubation with the aglycone library obtained from grape berries of Gewurztraminer 11-18 Gm (1,4,7); Positive control: geraniol (2,5,8); negative control: no acceptor molecule (3,6); UDP [14C]-glucose (9; approximately: 3000 dpm). The plates were analyzed by digital autoradiograph. The products formed from citronellol, geraniol and nerol were verified by LC-MS analysis. GC-MS analysis (B, total ion chromatogram) of volatiles that were enzymatically released from glucosides which were formed by incubation of an aglycone library obtained from grape berries of Gewurztraminer 11-18 Gm with UDP-glucose and VvGT14 (gray) or heat-inactivated VvGT14a (black) as control 1: linalool; 2: citronellol; 3: nerol; 4: geraniol; 5: benzyl alcohol; 6: phenylethanol. For response factors of volatiles (0.52 – 1.90) towards geraniol refer to supplemental data Table S4. Thus, even when response factors are taken into account, geraniol is the major product that is released.