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Manipulation of flavour and aroma compound sequestration and release using a glycosyltransferase with specificity for terpene alcohols

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SUMMARY
Glycosides are an important potential source of aroma and flavour compounds for release as volatiles in flowers and fruit. The production of glycosides is catalysed by UDP-glycosyltransferases (UGTs) that mediate the transfer of an activated nucleotide sugar to acceptor aglycones. A screen of UGTs expressed in kiwifruit (Actinidia deliciosa) identified the gene AdGT4 which was highly expressed in floral tissues and whose expression increased during fruit ripening. Recombinant AdGT4 enzyme glycosylated a range of terpenes and primary alcohols found as glycosides in ripe kiwifruit. Two of the enzyme’s preferred alcohol aglycones, hexanol and (Z)-hex-3-enol, contribute strongly to the ‘grassy-green’ aroma notes of ripe kiwifruit and other fruit including tomato and olive. Transient over-expression of AdGT4 in tobacco leaves showed that enzyme was able to glycosylate geraniol and octan-3-ol in planta whilst transient expression of an RNAi construct in Actinidia eriantha fruit reduced accumulation of a range of terpene glycosides. Stable over-expression of AdGT4 in transgenic petunia resulted in increased sequestration of hexanol and other alcohols in the flowers. Transgenic tomato fruit stably over-expressing AdGT4 showed changes in both the sequestration and release of a range of alcohols including 3-methylbutanol, hexanol and geraniol. Sequestration occurred at all stages of fruit ripening. Ripe fruit sequestering high levels of glycosides were identified as having a less intense, earthier aroma in a sensory trial. These results demonstrate the importance of UGTs in sequestering key volatile compounds in planta and suggest a future approach to enhancing aromas and flavours in flowers and during fruit ripening.

Keywords: flavour, aroma, glycosyltransferase, Actinidia deliciosa, kiwifruit, fruit, glycoside, volatile.

INTRODUCTION
In plants, glycosylation typically occurs as one of the last steps in natural product biosynthesis. Glycosides can be found for all major classes of natural compounds including flavonoids (Jones et al., 2003; Frydman et al., 2004), terpenoids (Richman et al., 2005; Nagatoshi et al., 2011), carotenoids (Moraga et al., 2004), glucosinolates (Grubb et al., 2004) and cyanohydrins (Thorsoe et al., 2005; Franks et al., 2008). Glycosylation reactions are mediated by UDP-glycosyltransferases (UGTs) that catalyse the transfer of an activated nucleotide sugar (such as UDP-glucose) to acceptor aglycones to form O-, S- and N-glycosides as well as sugar esters. The addition of the sugar moiety to plant natural products changes their solubility, chemical properties, compartmentation, storage, and biological activity (Bowles et al., 2005). Biochemical and molecular characterisation of UGTs has been driven by the pharmacological and agronomic importance of many glycosides, e.g. in human health (Achnine et al., 2005; Ono et al., 2010), disease resistance (Matros and Mock, 2004), plant development (Jackson et al., 2001; Hou et al., 2004), and flower and fruit colour (Fukuchi-Mizutani et al., 2003; Montefiori et al., 2011).
Plants contain large families of UGTs with over 100 genes being described in the genomes of Arabidopsis (A. thaliana), soybean (Glycine max), rice (Oryza sativa) and grape (Vitis vinifera) (Yonekura-Sakakibara and Hana-dai, 2011). These genes have a common signature motif of ~44 amino acids that is thought to be involved in the binding of the UDP moiety of the activated sugar (Li et al., 2001). Many GTs show relatively broad substrate specificity (Hefner et al., 2002; Landmann et al., 2007); however, there are examples of UGTs with quite specific activities (Fukuchi-Mizutani et al., 2003; Jugdé et al., 2008). UGTs show high sequence divergence; however, phylogenetic analysis has established the presence of distinct groups (A–N) and families of UGT genes in plants (Ross et al., 2001). Systematic classification has facilitated the characterisation of many new activities; nevertheless, there are still large numbers of uncharacterised UGTs and functionality is difficult to ascribe through phylogenetic relatedness.

In many flowers and fruit, aroma and flavour compounds accumulate as non-volatile glycosides (Loughrin et al., 1992; Oka et al., 1999; Cabrita et al., 2006; Birtic et al., 2009; Aurore et al., 2011; Garcia et al., 2011b). Glycosidically bound compounds are often significantly more abundant than free volatile aglycones, making glycosides an important potential contributor to aroma and flavour. In flowers, the release of odiferous volatile aglycones is dependent on floral maturity, is often diurnally regulated, and co-ordinate with increased \( \beta \)-glucosidase activity (Loughrin et al., 1992; Reveni et al., 1999; Picone et al., 2004). In fruit, aglycones may be released from the sugar moiety during ripening, storage, and processing. In tomato, NON-SMOKEY GLYCOSYLTRANSFERASE1 has been shown to prevent damage-induced release of smoky aroma-associated phenylpropanoids volatiles such guaiacol, methyl salicylate and eugenol. NSGT1 was induced during fruit ripening and converted cleavable diglycosides of smoky-related volatiles into noncleavable triglycosides (Tikunov et al., 2013). SIUGT5 has also been shown to preferentially glycosylate the same substrates \textit{in vitro} and the gene mapped to chromosome I in a region containing a QTL that affected the content of guaiacol and eugenol in tomato crosses (Louveau et al., 2010). In Citrus species such as grapefruit and pummelo, accumulation of flavanone-7-O-neohesperidosides determines the bitter quality of the fruit. The 1,2 rhamnosyltransferase Cm1,2RhaT was shown \textit{in vitro} to direct regiospecific rhamnosylation of naringenin 7-O-glucoside to produce the bitter compound (Frydman et al., 2004). In strawberry FaGT2 catalyses the formation \textit{in vitro} of glucose esters of cinnamic acid and \( p \)-coumaric acid which are the precursors of the volatile flavour compounds methyl and ethyl cinnamate. Transgenic strawberry fruit that were down-regulated for expression of FaGT2 showed decreased sequestration of cinnamoyl and \( p \)-coumaroyl glucose esters. However, the effect on methyl and ethyl cinnamate emissions could not be measured as they did not normally accumulate in the fruit of the cultivar used for transformation (Lunkenbein et al., 2006).

The volatile components of green-fleshed ‘Hayward’ kiwifruit (Actinidia deliciosa) have been well characterised (reviewed in Garcia et al., 2011a) with the major components being the esters methyl and ethyl butanoate and the C-6 aldehydes and alcohols (Z)- and (E)-hex-2-enal, hexanal, (Z)- and (E)-hex-3-enol, and methyl benzoate. The C-6 compounds are responsible for the fresh, grassy-green notes perceived by consumers when eating kiwifruit (Wang et al., 2011). In comparison, there is relatively little information on the glycosidically bound volatile components of kiwifruit. Young and Paterson (1995) identified 29 glycosides in ‘Hayward’ juice, whilst Garcia et al. (2013) identified 95 glycosides in extracts of ripe ‘Hayward’ fruit. The major compounds identified included terpenoids and C-6 alcohols as well as 3-methylbutanol, benzyl alcohol and \( \beta \)-phenylethanol (Table S1).

In this study we describe the isolation and \textit{in vitro} biochemical characterisation of AdGT4, a glycosyltransferase that utilises compounds that contribute to the aroma of ripe green-fleshed kiwifruit. Transient expression studies using tobacco leaf and \textit{A. eriantha} fruit and analysis of stable transgenic petunia and tomato plants are used to demonstrate that the enzyme sequesters ‘grassy-green,’ terpene and other volatiles \textit{in planta}. Our results in tomato demonstrate that manipulating expression of GTs can affect the balance of volatile compound sequestration and release thereby affecting fruit flavour and aroma.

**RESULTS**

**Identification of putative glycosyltransferases from ripe kiwifruit**

Four contigs with homology to known UGT sequences were identified by BLAST searches from a library of expressed sequence tags derived from \textit{A. deliciosa} ‘Hayward’ ripe fruit (Crowhurst et al., 2008). Full-length cDNAs from each contig were sequenced and designated \textit{AdGT1-4} (\textit{Actinidia deliciosa} glycosyltransferase 1-4). A framework phylogenetic tree was constructed with representative members of an Arabidopsis UGT phylogenetic tree described by Ross et al. (2001). This framework tree indicated that \textit{AdGT1-4} were placed in four different families, \textit{AdGT1} in UGT72, \textit{AdGT2} in UGT89, \textit{AdGT3} in UGT88 and \textit{AdGT4} in UGT85 (Figure 1). All four kiwifruit UGTs showed highest amino acid identity to predicted UGT proteins in the \textit{Vitis vinifera} genome (57–75%). Homology to functionally characterised proteins was lower, with \textit{AdGT1} showing 46% amino acid identity to UGT72E2 from \textit{Arabidopsis thaliana} capable of glycosylating coniferyl and sinapyl.
alcohols and aldehydes (Lim et al., 2005). AdGT2 showed 50% identity to UGT89A2 from A. thaliana with specificity for 3,4- and 2,5- dihydroxybenzoic acid (Lim et al., 2002) and AdGT3 showed 63–66% identity to several UGTs with specificity to flavonoids, flavones and coumarin (Tian et al., 2006; Witte et al., 2009; Kim et al., 2010). AdGT4 (99598) showed highest identity (72%) to the iridoid-specific UGT85A24 from Gardenia jasminoides (Nagatoshi et al., 2011) and 64% identity to UGT85A19 a cyanohydrin mandelonitrile GT from Prunus dulcis (Franks et al., 2008).

More distantly AdGT4 showed homology to the cyanohydrin UGT85B1 from Sorghum bicolor (Hansen et al., 2003) and UGT85C2 from Stevia rebaudiana involved in the production of diterpene steviol glycosides (Richman et al., 2005). All four kiwifruit ORFs encoded proteins of between 52 and 54 kDa and contained the PSPG motif of 44 amino acids found in most plant UGTs. An alignment of AdGT1-4 with other biochemically characterised UGT enzymes is shown in Figure S1.

Each AdUGT1-4 ORF was expressed as an N-terminal His<sub>6</sub>-tagged recombinant protein in Escherichia coli. Partially purified proteins were tested for their ability to glucosylate a pool of substrates containing geraniol, linalool and octan-3-ol or benzyl alcohol, 2-phenylethanol and hexanol. Only AdGT4 (designated ActdUGT85A38 according to the systematic GT nomenclature of Ross et al., 2001) showed significant GT activity towards the substrate pools and was purified by Ni<sup>2+</sup> affinity chromatography for further biochemical analysis.

Enzymatic activity of recombinant AdGT4 enzyme

Purification of recombinant AdGT4 protein was indicated in SDS-PAGE and confirmed by western analysis using a His<sub>6</sub>-specific monoclonal antibody (Figure S2). AdGT4 activity was characterised initially by deconvoluting the original two substrate pools (described above) using UDP-glucose as the sugar donor. AdGT4 showed significant activity towards geraniol, hexanol and octan-3-ol, weak activity towards 2-phenylethanol and negligible activity towards benzyl alcohol and linalool. AdGT4 activity was then tested on a further 25 substrates selected from a range of chemical classes (Table 1). AdGT4 showed activity against a range of alcohols in particular nerol (the cis isomer of geraniol), α-terpineol, and (Z)-hex-3-enol. Low but detectable activity (>5%) was measured towards other alcohols including 2-furylmethanol, Furaneol<sup>10</sup> and (E)-hex-2-enol. Negligible activity was observed towards aromatic compounds such as resorcinol and hydroquinone and flavonoids such as quercetin and naringenin. Negligible activity was also shown for several other alcohols, e.g. butanol, and pentan-2-ol that have been reported in A. deliciosa fruit volatile headspace and solvent extraction analyses (Crowhurst et al., 2008; Wang et al., 2011).
Table 1 Relative activity of purified recombinant AdGT4 enzyme

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative activity (%)</th>
<th>Compound</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geraniol</td>
<td>100</td>
<td>Quercetin dihydride</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Nerol</td>
<td>47.6 ± 10.2</td>
<td>Propanol</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Octan-3-ol</td>
<td>28.2 ± 2.6</td>
<td>Hydroquinone</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Hexanol</td>
<td>25.8 ± 1.5</td>
<td>Linanolol</td>
<td>2.5 ± 0.1</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>22.9 ± 1.1</td>
<td>Resorcinol</td>
<td>2.4 ± 0.1</td>
</tr>
<tr>
<td>(Z)-Hex-3-enol</td>
<td>11.9 ± 0.4</td>
<td>3-Methylbut-3-enol</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>2-Phenylethanol</td>
<td>7.9 ± 0.1</td>
<td>cis-Linalool oxide</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>(E)-Hex-2-enol</td>
<td>7.2 ± 1.0</td>
<td>Propan-2-ol</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Furanol&lt;sup&gt;[9]&lt;/sup&gt;</td>
<td>6.1 ± 0.2</td>
<td>Butanol</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>2-Furylmethanol</td>
<td>5.0 ± 0.3</td>
<td>Cyanidin chloride</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Butan-2-ol</td>
<td>4.3 ± 0.1</td>
<td>Naringenin</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>3-Methylbutanol</td>
<td>4.3 ± 0.5</td>
<td>Chlorogenic acid</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Pentan-2-ol</td>
<td>4.3 ± 0.1</td>
<td>Caffeic acid</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>2-Methylbutanol</td>
<td>3.9 ± 0.2</td>
<td>Menthol</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>3.3 ± 0.4</td>
<td>Pyrogallol</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

UGT activity towards geraniol is set at 100%. Data are presented as mean ± standard error of the mean (SEM) (n = 3).

Kinetic parameters of recombinant AdGT4 in vitro

AdGT4 activity towards geraniol and UDP-glucose was tested over a pH range of 5.0–10.0. The enzyme showed highest activity at pH 7.5 with activity decreasing to 50% at pH 7.0 and 9.0 but still more than 20% at pH 5.0 and 10.0 (Figure S3a). The enzyme showed similar activity overall a broad temperature range from 20–40°C, but negligible activity at 50°C (Figure S3b). Enzyme activity was not strictly dependent on the presence of monovalent (Na<sup>+</sup>, K<sup>+</sup>) or divalent cations (Mg<sup>2+</sup>, Mn<sup>2+</sup>), but activity increased 1.7-fold in the presence of 0.1–0.5 mM Mg<sup>2+</sup> and 1.5-fold in the presence of 10–20 μM Mn<sup>2+</sup> (Figure S3c,d).

Kinetic parameters were determined for AdGT4 with respect to geraniol, octan-3-ol, hexanol and (Z)-hex-3-enol. The enzyme had similar apparent Km values for all four substrates (57–117 μM), however the catalytic efficiency towards geraniol was 6.17-fold higher compared with that of the two C-6 alcohols and octan-3-ol (Table 2). This result suggests that geraniol is the preferred sugar acceptor of the four substrates tested. The apparent Km for UDP-glucose was 44.7 ± 15.2 μM.

LC-MS analysis of reaction products

Products of 16 h reactions between AdGT4 + UDP-glucose and two substrates, geraniol and octan-3-ol were analysed by LC-MS. Base peak plots indicated that a single glucosylated product was formed with a retention time of 44.6 min with geraniol (Figure 2a) and a product with a retention time of 42.2 min with octan-3-ol (Figure 2c). Full scan and MS/MS mass spectral data were used to further characterise the AdGT4 enzyme reaction products. Geraniol-glucoside was detected predominantly as the corresponding

Table 2 Kinetic properties of purified recombinant AdGT4 enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>k&lt;sub&gt;c&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (sec&lt;sup&gt;-1&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;c&lt;/sub&gt; (sec&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geraniol</td>
<td>11.05 ± 0.42</td>
<td>76.2 ± 11.1</td>
<td>14 500</td>
</tr>
<tr>
<td>Octan-3-ol</td>
<td>0.75 ± 0.07</td>
<td>66.8 ± 16.2</td>
<td>1130</td>
</tr>
<tr>
<td>Hexanol</td>
<td>2.72 ± 0.18</td>
<td>116.9 ± 26.1</td>
<td>2330</td>
</tr>
<tr>
<td>(Z)-Hex-3-enol</td>
<td>0.49 ± 0.04</td>
<td>57.0 ± 20.0</td>
<td>860</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>6.93 ± 0.74</td>
<td>44.7 ± 15.2</td>
<td>15 550</td>
</tr>
</tbody>
</table>

Substrate concentrations were varied from 5 mM to 2 μM with a fixed UDP-glucose concentration of 100.27 μM [3H]-UDP-glucose: 0.27 μM + UDP-glucose: 100 μM and 500 ng of protein. The K<sub>m</sub> for UDP-glucose was determined by varying the concentration of UDP-glucose from 300 to 0.27 μM in the presence of geraniol (500 μM) and (Z)-Hex-3-enol (0.27 μM). Data are presented as mean ± standard error (SE) (n = 3).

formate adduct, m/z 361 [M + formate]<sup>-1</sup> in full scan mode. MS<sup>2</sup> on the formate adducts identified the expected pseudo-molecular ion at m/z 315 for the geraniol-glucoside (Figure 2e). Octan-3-ol-glucoside was also detected as the corresponding formate adduct (m/z 337) in full scan mode and gave the expected pseudo-molecular ion at m/z 291 for the octan-3-ol-glucoside in MS<sup>2</sup> (Figure 2g).

The AdGT4 enzyme was then incubated for 16 h with geraniol and octan-3-ol in the presence of two additional activated sugar donors, UDP-galactose and UDP-xylose. Two peaks of similar intensity were observed in the base peak plots for geraniol + UDP-galactose at 44.1 and 44.6 min (Figure 2b). Full scan and MS/MS mass spectral data indicated that both peaks were consistent with a geraniol-galactoside (Figure 2f). The peak at 44.1 min most likely corresponds to geraniol-galactoside, as the peak at 44.6 min has the same retention time as geraniol-glucoside in MS<sup>2</sup> (Figure 2g).

AdGT4 acts primarily as a glucosyltransferase with weak but detectable galactosyltransferase activity.

Expression analysis of AdGT4 in kiwifruit

The tissue-specific expression of AdGT4 mRNA was determined by quantitative RT-PCR in young leaf, vegetative bud, mature fully-open flower and ripe fruit. Expression was highest in flower tissue, but was high in bud and...
ripe fruit (Figure 3a). By analysing floral parts dissected from mature fully-open flowers, the high levels of AdGT4 expression in flowers appeared to be localised to sepal and stamen but not petal and pistil (Figure 3b). During ‘Hayward’ fruit ripening, expression of AdGT4 increased progressively as fruit softened from 100 N firmness (at harvest) to 20 N. A slight decrease in expression was observed as fruit achieved eating firmness (8 N) when they produce endogenous climacteric ethylene (Figure 3c). In kiwifruit, free volatile release is strongly linked to the production of climacteric ethylene (Atkinson et al., 2011). AdGT4 expression appears to be developmentally-regulated during ripening, and not ethylene-regulated as has been reported for flavour-related genes in kiwifruit involved in ester production, e.g. AATs (Günther et al., 2011).

**Transient over-expression of AdGT4 in tobacco**

Transient over-expression in tobacco leaves was used to investigate the glycoside products produced by AdGT4 in planta. Leaves were infiltrated with pART27-AdGT4 or a control pHEx2-GUS construct in the presence/absence of the volatile aglycones geraniol, hexanol, (Z)-hex-3-enol, octan-3-ol and 2-phenylethanol. Glycosides were purified, treated with β-glucosidase and volatiles released were extracted into solvent for GC-MS analysis. Extremely low levels of glycosides were extracted from tobacco plants that were not co-infiltrated with volatile aglycones and no glycosylated hexanol was observed in either AdGT4 infiltrated or control plants. Elevated glycoside levels were observed in both AdGT4 infiltrated and control plants infiltrated with each volatile aglycone, particularly (Z)-hex-3-enol and 2-phenylethanol, suggesting the presence of native tobacco GT enzymes capable of glycosylating these substrates in planta. However, significantly higher levels of octan-3-ol (40-fold) and geraniol (2.5-fold) glycosides were observed in AdGT4 infiltrated plants (Figure 4).

**Stable over-expression of AdGT4 in petunia flowers**

Stable transgenic petunia plants were generated to investigate glycosylation by AdGT4 in flowers. Glycosides were purified, treated with β-glucosidase and the released volatiles analysed by GC-MS. Significantly reduced levels of volatile terpene alcohol glycosides were observed in fruit inoculated with the pTKO27S-AdGT4 RNAi construct compared to the pHEx2-GUS control (Figure 5a). At the individual compound level there were significant decreases in geraniol, α-terpineol and carveol (Figure 5b, all compounds listed in Table S3) that contribute to the flavour of ripe kiwifruit.
showed significant increases in glycosides for hexanol, 3-methylbutanol and 2-butyloctanol. Lines T3157 and T3152 also showed significant differences in glycosylated 2-phenylethanol and octanol (Figure 6b). Levels of glycosides for most other alcohols including geraniol, decanol and benzyl alcohol (all compounds listed in Table S4) also increased in the transgenic lines, but not at $P < 0.05$ significance. Lines T3157 and T3152 also showed significant increases in total aldehydes arising from the glycoside fraction, with this variation being almost entirely due to an increase in benzaldehyde.

**Stable over-expression of AdGT4 in tomato fruit**

**Glycoside analysis.** Six homozygous transgenic tomato lines were generated (T518, T519, T528–T530 and T586) to investigate how AdGT4 over-expression affects sequestration and release of flavour and aroma compounds in red ripe fruit compared to a wildtype 'MicroTom' line. All six transgenic lines showed a significant increase (5–8-fold) in total extractable volatile-alcohol glycosides (Figure 7a). In contrast to petunia flowers, no difference in total aldehydes was observed in the glycoside fractions (Figure 7a). At the individual compound level, nearly all volatile-alcohol glycosides were more abundant in the transgenic lines than the control (all compounds listed in Table S5). 3-Methylbutanol was the most abundant volatile-alcohol glycoside detected in the control line and this compound was 10–30-fold more abundant as a glycoside in the AdGT4 over-expressing lines, with lines T519, T529 and T530 showing significant differences ($P < 0.05$). Hexanol, butanol, and geraniol glycosides were significantly more abundant in three to four of the transgenic lines (Figure 7b). Lines T529 and T530 showed the largest number of volatile-alcohol glycosides with elevated levels compared with the control which is consistent with the higher level of AdGT4 transgene expression in these two transgenic lines (Figure S4). Despite changes in glycoside accumulation, fruit from all six transgenic lines were physiologically similar to the control for colour, firmness and crop load (Figure S5a–e). However, a significant increase in soluble solids content (3.5 to ~5°Bx) was observed in all lines (Figure S5f).
Free volatiles and solvent extractions. Free volatiles released from red ripe fruit of the six transgenic AdGT4 lines were measured by dynamic headspace trapping and GC-MS analysis (all compounds listed in Table S6). Individual volatiles were categorised into alcohol, aldehyde, acid + ester, terpene and ‘other’ (ketone, sulfur, thiazole and nitrile, furan and hydrocarbon) categories. Total free volatile-alcohols were significantly decreased (2–9-fold) in all six transgenic tomato lines. No significant difference in total free volatile aldehydes, acids + esters, terpenes or ‘other’ compounds released was observed, with the exception of an increase in total free volatile acids + esters in line T528 (Figure 8a).

Volatile-alcohols and aldehydes in red ripe fruit were also extracted into solvent and analysed by GC-MS (all compounds listed in Table S7). Levels of total volatile-alcohols extracted into solvent were low (0.1 µg g⁻¹) from both control and transgenic AdGT4 lines (Figure 8b). Significantly reduced levels were observed in three of the transgenic tomato lines. Extraction of volatile aldehydes into solvent was much higher (40 µg g⁻¹), but levels were comparable between control and all transgenic lines (Figure 8b).

Glycosides in development. Glycoside production was assessed during three additional stages of fruit development (green, breaker, and pink) using transgenic lines T529 and T530 that showed the most consistent changes in both free volatile and glycoside production in red ripe fruit. Significant differences in total extractable volatile-alcohol glycosides were observed in all stages of development for line T529 (Figure 9) and three of the four stages for T530. The most abundant compound contributing to the difference in the alcohol glycoside pool was...
3-methylbutanol; however, the majority of alcohol glycosides observed in the control were sequestered at higher levels in both T529 and T530 (all compounds listed in Table S8). No significant difference in total aldehydes was observed in the glycoside fractions. Variability between samples was high for both control and T529. This was related to variation in the levels of (E)-hex-2-enal that was only found at breaker and pink stages in the control and T529 samples but not at any stage in T530.

Sensory analysis. A sensory panel investigated the impact of AdGT4 over-expression on ripe tomato fruit aroma. In triangle tests, panelists were clearly able to distinguish the aroma of transgenic T530 fruit from control fruit, with 27 correct answers out of a total of 47 tests ($P < 0.001$), run over three independent sessions. Panelists perceived the AdGT4 over-expressing fruit as having a significantly more ‘earthy’ aroma, and to be globally less ‘intense’ than control fruit (Table 3). The sensory analysis suggested also that T530 tomatoes were less floral, less sweet and less fruity, but without harboring a significant difference.

DISCUSSION

Using a molecular and biochemical screen we have isolated and characterised AdGT4, a ripening-related GT from kiwifruit with closest homology to Group 85A GTs. Recombinant AdGT4 enzyme showed a broad specificity, accepting many primary and secondary alcohols as substrates, but not phenolic substrates (e.g. resorcinol, hydroquinone). AdGT4 preferentially glycosylated geraniol, nerol, the C-6 alcohols hexanol, (Z)-hex-3-enol and (E)-hex-2-enol, and octan-3-ol. UGT85B1 from Sorghum bicolor shows a similar broad specificity but with a preference for...
Statistical analysis was performed using a Data are the average occurrence of each descriptor level.

dent harvests). Statistical analysis as per Figure 6. *Different at the 0.05 level.

A. deliciosa glycosides were observed. This suggests that in ripe A. deliciosa fruit remains an open question.

In both transgenic petunia flowers and tomato fruit, stable over-expression of AdGT4 led to a significant increase in the total pool of volatile-alcohols that were sequestered. Significant increases in glycosylated hexanol and 3-methylbutanol were observed in both tomato and petunia, whilst increased glycosylation of other volatile-alcohols was specific to each system, e.g. octanol and decanol in petunia versus butanol, 6-methyl-5-hepten-2-ol and geraniol in tomato. These differences most likely relate to substrate availability, e.g. geraniol and 6-methyl-5-hepten-2-ol are reported as free volatile-alcohols in tomato fruit, e.g. (Ortiz-Serrano and Gil, 2007), but not in petunia flowers, e.g. (Verdonk et al., 2005). Differences in substrate availability are also likely to contribute to the changing glycoside profiles evident during fruit development. For example, 3-methylbutanol glycosides increased ~7-fold in control fruit as fruit ripened from green to red. An increase in 3-methylbutanol glycosides also occurred during ripening of the AdGT4 over-expressing lines, but to an even greater extent (80–140-fold; Table S8).

Aldehydes are not directly glycosylated by UGTs as they lack a hydroxyl group. However, the presence of aldehydes in glycosidic fractions is widely reported (Birtic et al., 2008; Ortiz-Serrano and Gil, 2010). The ratio of alcohols to aldehydes in the glycosidic fraction can vary widely depending on the extraction conditions, as can been seen in Table S1 where benzyl alcohol:benzaldehyde and (Z)-hex-3-enol: (E)-hex-2-enal ratios varied markedly. In this study, a significant difference in total aldehydes was observed in the glycoside fractions of petunia flowers over-expressing AdGT4 but not from tomato fruit which might be due to the glycoside digestion with almond β-glucosidase versus Rapidase.

A decrease in the total pool of solvent extracted volatile-alcohols was measured by GC-MS in three tomato lines over-expressing AdGT4, whilst the pool of free volatile-alcohols decreased in all lines. No significant change in the

cyanohydrin substrates not found in kiwifruit (Hansen et al., 2003). The $K_m$ value for AdGT4 towards geraniol (76 μM) is lower than that reported for other UGT85 family members towards terpenoids e.g. 610 μM for UGT85A24 towards 7-deoxynoririnogenet (Nagatoshii et al., 2011) and 140 μM for UGT85B1 towards geraniol (Hansen et al., 2003), but higher than those reported towards flavanols e.g. ~3 μM for UGT85H2 towards quercetin and kaempferol (Li et al., 2007). LC-MS analysis indicated AdGT4 was primarily a glucosyltransferase with weak but detectable galactosyltransferase activity. In transient assays using tobacco leaves, AdGT4 was able to glycosylate exogenous octan-3-ol and geraniol substrates thereby showing AdGT4 was an active GT in planta. However, analysis using this system was complicated by glycosylation via endogenous GTs in the leaves using (Z)-hex-3-enol and 2-phenylethanol. Transient down-regulation of AdGT4 in unripe A. eriantha fruit indicated that the enzyme primarily glycosylates terpene alcohols in kiwifruit as significant reductions in geraniol, α-terpineol and carveol glycosides were observed. This suggests that in ripe A. deliciosa fruit, AdGT4 will glycosylate α-terpineol and especially geraniol which is found at 10-fold higher levels

![Figure 9. Total extractable glycosides of volatile-alcohols and aldehydes that are sequestered during tomato fruit ripening. Glycosides were isolated from tomato transgenic lines T529 and T530 and a wildtype ‘WT’ control during tomato ripening stages green (grn), breaker (brk), pink (pkn) and red ripe. Glycosides were treated with β-glucosidase and the volatiles released extracted into solvent for GC-MS analysis. Data are presented as mean ± standard error of the mean (SEM) (n = 3 independent harvests). Statistical analysis as per Figure 6. *Different at the 0.05 level.](image)

Table 3 Sensory comparison of ripe transgenic T530 and control tomato fruit

<table>
<thead>
<tr>
<th>Descriptor</th>
<th>Intense</th>
<th>Green</th>
<th>Floral</th>
<th>Sweet</th>
<th>Fruity</th>
<th>Earthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>T530</td>
<td>0.8 ± 0.5a</td>
<td>4.0 ± 0.6</td>
<td>1.5 ± 0.5</td>
<td>0.8 ± 0.3</td>
<td>2.8 ± 0.6</td>
<td>9.0 ± 0.7a</td>
</tr>
<tr>
<td>Control</td>
<td>3.2 ± 0.8</td>
<td>3.8 ± 0.7</td>
<td>2.6 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>4.4 ± 1.1</td>
<td>5.6 ± 0.2</td>
</tr>
</tbody>
</table>

Data are the average occurrence of each descriptor ± standard error of the mean (SEM) (n = 3 independent sessions with 16 panelists). Statistical analysis was performed using a t-test.

*Different at the 0.05 level.
pool of free volatile aldehydes, acids – esters, or terpenes was observed. The sensory panel analysis of line T530 versus control fruit highlighted that the changes in free volatiles were obvious to detect by sniffing, as the statistical significance of the triangle test was very strong (P < 0.001). The descriptive sensory analysis suggests that the reduction in free volatile-alcohols leads to fruit that are perceived as having less overall aroma intensity and that are earthier than control fruit. Hexanol, (Z)-hex-3-enol, 6-methyl-5-hepten-2-ol, and geraniol are all noted as having grassy-green/fruity notes, whilst 3-methylbutanol and butanol have fusel/alcoholic/banana notes (http://www.thegoodscentscompany.com/search.html). Our hypothesis is that the increased sequestration of these compounds with fruity/floral notes leads to the perception of a fruit that is ‘earthier.’ Compounds such as 2-isobutylthiazole and 3-methylbutanal, which are noted as having musty/earthy aromas in tomato (Baldwin et al., 2008), were slightly reduced in the T530 line (Table S6). Our hypothesis is also consistent with sensory analysis of ripe tomato fruit with elevated ADH activity. These transgenic lines had higher levels of C-6 alcohols such as hexanal and (Z)-hex-3-enol and were identified as having a more intense ‘ripe fruit’ flavor (Speirs et al., 1998).

In conclusion, we have demonstrated that AdGT4 can glycosylate a range of terpenes and C-6 alcohols in vitro and four systems (including kiwifruit) in planta. Our results in tomato indicate that over-expression of AdGT4 influences volatile compound release which has a measurable effect on sensory perception of fruit aroma. This work further suggests that glycosyltransferases with specificity for key odour-active compounds are good candidate genes for manipulating levels of different aromas and flavours in flowers and fruit by transgenic or conventional breeding techniques.

EXPERIMENTAL PROCEDURES

Plant material

Actinidia delicosa Lindl. var. delicosa (A. Chev.) C.F. Liang et A.R. Ferguson ‘Hayward’ samples were obtained from the PFR orchard in Te Puke, NZ. Outer pericarp tissue was sampled from fruit when immature (firmness 100 N), mature but unripe (80 N), during the rapid period of fruit softening (50 N or 20 N), and when eating ripe (8–10 N, producing endogenous ethylene, 1.03 μmol kg⁻¹ sec⁻¹). Fruit firmness was determined with an Effegi penetrometer (7.9 mm diameter head). Endogenous ethylene production was measured by flame ionisation chromatography (Atkinson et al., 2011). Whole flowers were harvested at noon and immediately dissected into flower part samples (Nieuwenhuizen et al., 2009). All tissues were snap frozen in liquid nitrogen immediately after collection and stored at -80°C.

Sequence analysis

A ‘Hayward’ ripe fruit library containing ~10300 ESTs (Crowhurst et al., 2008) was BLAST-searched for sequences with homology to known GTs. Homologous contigs (expect value <exp⁻²⁰) were identified and the most 5’ EST was selected for full-length sequencing. For phylogenetic analysis amino acid sequences were initially aligned using CLUSTALX (version 1.8) then manually edited. Phylogenetic trees were constructed using PHYLIP, analysed using BOOTSTRAP N-J TREE and visualised in TREEVIEW (v.1.6.6). http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.

Expression in Escherichia coli

The complete ORFs of AdGT1-4 were amplified using gene-specific primers (Table S9) into pET30A(+) (Novagen, http://www.novagen.com/). DNA from individual clones was sequence verified against the original ORF, then transformed into BL21-Codon-Plus RIL cells. For recombinant protein isolation, 30 ml cultures in LB broth containing 50 μg ml⁻¹ kanamycin were grown at 37°C, 300 rpm, for 4 h until they reached an OD₆₀₀ of ~0.6. Cultures were induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration 1 mM and grown for a further 72 h at 16°C. Cells were harvested and then lysed with 2 ml protein extraction buffer (8%PER in phosphate buffer) following manufacturer’s instructions. Recombinant protein obtained at this point is referred to as ‘partially purified’. Purified N-terminal His₆-tagged AdGT4 protein was obtained using His SpinTrap columns (GE Healthcare Life Sciences, www.ge lifesciences.com) following manufacturer’s instructions. Protein concentrations were measured using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., www.nanodrop.com).

Glucosyltransferase activity assays

Standard UGT activity assays were performed in 50 μl reactions containing 10 μl of 5× UGT assay buffer (50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol), 1 ng recombinant protein, 0.2 mM substrate and 0.27 μM [³H]-UDP-glucose (uridine diphospho-D-[6-³H]glucose, 13.6 Ci mmol⁻¹; GE Healthcare). Reactions were performed at 30°C for 30 min and terminated using 10 μl of 2 N HCl. Reaction mixtures were extracted with 100 μl of ethyl acetate, and 20 μl of organic phase was added to 1 ml of non-aqueous scintillation fluid and analysed by liquid scintillation counting (Tri-Carb 2900TR; PerkinElmer, www.perkinelmer.com). Boiled protein or empty vector protein as a negative control was treated in parallel with all enzyme activity reactions. Reactions were shown to be linear with respect to enzyme concentration and time under standard reaction conditions.

LC-MS analysis

Scaled-up reactions contained 100 ng enzyme, 100 μM substrate and 250 μM UDP-glycoside (UDP-glucose, UDP-galactose; Sigma-Aldrich, www.sigmaaldrich.com; or UDP-xyllose, Carbosource Services). For Figure 2, reactions were incubated at 30°C for 16 h and stopped with 20 μl glacial acetic acid. Three reactions for each substrate:UDP-glycoside combination were pooled for LC-MS analysis. LC-MS employed an LTQ linear ion trap mass spectrometer fitted with an ESI interface coupled to an Etten MDLC (GE Healthcare Life Sciences). Compound separation was achieved as described in Jugué et al. (2008). Sample injection volume was 10 μl. MS data was acquired in the negative mode using a data-dependent LC-MS² method (Jugué et al., 2008). Authentic geraniol and octan-3-ol standards were obtained from Sigma-Aldrich.

For the LC-MS time course analysis of glycosides, scaled-up reactions were incubated for 10 min, 1 h or 16 h and analysed as described in Table S2.
Quantitative real-time PCR

RNA from kiwifruit was isolated according to Chang et al. (1993) and from tomato using NucleoSpin RNA Plant columns (Macherey-Nagel). cDNA was synthesised as described in Nieuwenhuizen et al. (2009). qRT-PCR gene expression analysis was performed on a LightCycler 480 platform using SYBR Green master mix. Results were analysed using the LightCycler 480 software (Roche Applied Science, www.roche.com). Amplification conditions included an initial denaturation step of 95°C for 5 min followed by 45 cycles of 95°C for 10 sec, 60°C for 10 sec and 72°C for 12 sec. Fluorescence was measured at the end of each annealing step followed by a melting curve analysis with continual fluorescence acquisition from 65 to 95°C to check for single product amplification. Expression was calculated relative to the control kiwifruit gene EF1α. qRT-PCR primer sequences are shown in Table S9 together with predicted product sizes.

Binary vectors and plant transformation

The over-expression construct pART27-AdGT4 (CaMV35S:AdGT4:nos) contained the full-length AdGT4 ORF in the binary vector pART27 (Gleave, 1992). The first 610 bp of AdGT4 (excluding the conserved PSPG motif) was used to produce the RNAi hairpin construct pTKO27S-AdGT4 (CaMV35S:AdGT4:act2) in the binary vector pTKO2 (Snowden et al., 2005). The control construct pHK2-GUS (CaMV 35S:GUS reporter gene:nos) is as reported in Nieuwenhuizen et al. (2009).

For transient expression, all constructs were electroporated into Agrobacterium strain GV3101. Transient experiments in tobacco leaves were performed as described in Hellens et al. (2006). Seven days after inoculation, leaves were infiltrated with a 100 μM solution of volatile aglycone (except geraniol 10 μM, as it was toxic at 100 μM). After 4 h the leaves were detached and frozen at −80°C for glycoside extraction. Transient experiments using unripe A. eriantha fruit were performed as described in Montefiori et al. (2011). Three fruit for each treatment were injected and then stored for 4 days at 25°C.

Transgenic petunia (Petunia hybrida) ‘V26’ plants were generated according to Jorgensen et al. (1996) using pART27-AdGT4 in Agrobacterium strain LBA4404 and rooted in the presence of kanamycin (100 μg ml⁻¹). The presence of the AdGT4 transgene was confirmed in transgenic plants using primers YKGG1 and YKGG1R (Table S9). Transgenic tomato (Solanum lycopersicum Mill.) ‘MicroTom’ plants were regenerated according to Wang et al. (2005) and rooted in the presence of kanamycin (50 μg ml⁻¹). Plants were self-pollinated to produce homozygous seed lines. Transgenic and control plants were grown under containment greenhouse conditions with a minimum maximum temperature range of 20–30°C and a minimum of 12 h light. Fully-open petunia flowers with mature pollen were harvested at 10:30 am. Tomato fruit were harvested according to the standard ripening scale (DellaPenna et al., 1986). For glycoside analysis and solvent extraction, pools of at least 10 petunia flowers and 15 tomato fruit were collected from multiple plants for each time point and frozen at −80°C.

Extraction and hydrolysis of volatile glycosides

Frozen tobacco leaf (1–2 g), petunia flower (~2–3 g), and tomato fruit (~8 g) material were ground, mixed with 20–35 ml water, and centrifuged (2000 g for 15 min). The supernatant was collected and 35 μl of 1 M n-heptyl-D-glucopyranoside (Calbiochem, www.merckmillipore.com) added as internal control. The supernatant was loaded onto a 15 ml Amberlite XAD-2 column (prepared according to manufacturer’s instructions, Supelco), washed with 50 ml water followed by 40 ml diethyl ether/pentane (1:1) to remove non-glycosylated compounds. The glycosylated compounds were eluted using 20–50 ml methanol and dried.

Glycosidic eluates were resuspended in 2 ml of deglycosylation buffer (4.2 g citric acid, 2.84 g Na₂HPO₄ in 100 ml, pH 5) and extracted 3× with 2 ml of diethyl ether/pentane to remove any non-glycosylated compounds. Petunia glycosides were hydrolysed using aldehyde β-glucosidase (2 mg) and the volatile products trapped onto SPME fibres coated with 65 μM polydimethylsiloxane-divinylbenezene (Supelco, www.sigmaaldrich.com). Volatile collection was for 24 h at 37°C in a water bath with gentle rocking. Tomato and tobacco glycosides were digested with Rapidase AR2000 (4 mg; DSM Food Specialities, www.dsm.com). Reactions were overlaid with 100 μl diethyl ether/pentane and incubated for 16 h at 37°C. Reactions were then extracted 2× with 0.8 ml diethyl ether/pentane and water removed by passage through a Na₂SO₄ column.

Headspace collection and solvent extraction of tomato volatiles

Headspace volatiles were collected by sealing 15 fruit without sepalas in 100 ml sampling jars for 20 min to equilibrate without flow and then purging the headspace with dry purified air at a flow rate of 25 ml min⁻¹ for 1 h. Volatiles were trapped onto direct thermal desorption liners (ATAS GL) packed with 80 mg of 60–80 mesh Chromosorb 105 adsorbent (Shimadzu).

For solvent extraction, fruit (20 g) were blended in 22% CaCl₂ (20 ml) and mixed with 40 ml diethyl ether/pentane (1:1) for 30 min at room temperature. The pulp was re-extracted with 20 ml diethyl ether/pentane for 10 min at room temperature, the combined extracts passed through a Na₂SO₄ column to remove water then stored at −20°C.

GC-MS analysis

GC-MS analysis of solvent extracts was performed on an Agilent 6890N GC coupled to a Waters GCT time of flight-mass spectrometer, as described previously (Nieuwenhuizen et al., 2013). The oven temperature program was 1 min at 35°C, 5°C min⁻¹ to 230°C, and hold for 5 min. Headspace volatiles were desorbed directly from the direct thermal desorption tubes and cryo-focused as described in the legend to Table S8.

Volatiles compounds were identified by comparing their mass spectra and retention data with those of a series of authentic standards, supplemented with the NIST 98 and Wiley 7 mass spectral libraries. Amounts of each chemical were semi-quantified with an average detector response factor based on a range of standards. Concentrations of glycoside volatiles were also corrected for recovery of the internal standard (n-heptyl-D-glucopyranoside).

Sensory panel analysis

The panel consisted of 16 individuals and included 10 men and 6 women. Participation was voluntary and all participants gave their written consent prior to participation in the study. Although panelists knew the general purpose of the experiment, none was aware of the exact nature of the samples. Red ripe tomato fruit without sepalas (30 ± 1 g) from the control and a representative AdGT4 over-expressing line (T530, showing a 40-fold increase in total volatile-alcohol glycosides and nine-fold decrease in total free volatile-alcohols) were placed in individual wine glasses. There was no visual difference between samples. A watch glass was placed over each glass before and after sampling so
headspace volatiles could accumulate within the glass. For the triangle tests, three samples were presented with a random code to the panelist: two the same, and one different. Each panelist was asked to smell in each glass and identify which sample was different. For the descriptive analysis panelists were asked to choose from a set of five aroma descriptors: intense, green, floral, sweet, fruity and earthy. The presented results are the average occurrence for each descriptor. The experiment was repeated three times with independent sets of samples presented to each panelist. Significance was analysed using probability tables developed for sensory analysis (Lawless and Heymann, 2010).

GenBank accession numbers

AdGT1 (KF954941); AdGT2 (KF954942); AdGT3 (KF954943); AdGT4 (KF954944).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

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

Figure S1. Alignment of kiwifruit UGTs with homologous UGTs from other species.

Figure S2. AdGT4 protein purification and western analysis.

Figure S3. Effect of pH, temperature and salts on the activity of AdGT4.

Figure S4. Relative transgene expression in tomato lines over-expressing AdGT4.

Figure S5. Colour, firmness, crop load and soluble solids measurements of tomato lines over-expressing AdGT4.

Table S1. Kiwifruit glycosides identified in ‘Hayward’ kiwifruit juice and fruit.

Table S2. LC-MS time course analysis of glycosides from in vitro reaction mixtures.

Table S3. Individual glycosides from transient down-regulation of AdGT4 in kiwi fruit.

Table S4. Individual glycosides from petunia flowers over-expressing AdGT4.

Table S5. Individual glycosides from red ripe tomato fruit over-expressing AdGT4.

Table S6. Individual head-space volatiles from red ripe tomato fruit over-expressing AdGT4.

Table S7. Individual solvent-extracted volatiles from red ripe tomato fruit over-expressing AdGT4.

Table S8. Individual glycosides from a developmental series of red ripe tomato fruit over-expressing AdGT4.

Table S9. Oligonucleotide primers.

REFERENCES


