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Biosynthesis of the Dihydrochalcone Sweetener Trilobatin Requires *Phloretin Glycosyltransferase 2*

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Short title

PGT2 is Required for Trilobatin Biosynthesis

One sentence summary

Genetic, biochemical and molecular characterization of *phloretin glycosyltransferase 2* reveals its importance to the production of sweet-tasting trilobatin (phloretin-4'-*O*-glucoside) in wild apple.

Keywords: apple, dihydrochalcone, glucoside, glycosyltransferase, *Malus*, phloretin,

sweetener, trilobatin

FOOTNOTES

AUTHOR CONTRIBUTIONS

Y.W., Y.Y., F.M., H.L., R.A., and P.L. designed the research; R.A. and P.L. ran the sensory evaluation; Y.W.; Q.Z., and S.T. produced the transgenic plants; E.L.G., K.G. and D.C. performed the mapping and HRM analysis; J.C. and Z.X. conducted LC-MS and HPLC analyses; C.H. did the structural analysis; Y.W., Q.Z. and L.Z. undertook the protein purification; Y.W. and Y.Y. carried out transcriptomic, HPLC, biochemical and molecular analyses; Y.W., P.L. and R.A. analyzed data and wrote the paper.

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ABSTRACT

Epidemics of obesity and type 2 diabetes drive strong consumer interest in plant-based low calorie sweeteners. Trilobatin (phloretin-4'-*O*-glucoside) is a sweetener found at high concentrations in the leaves of a range of crabapple (*Malus*) species, but not in domesticated apple (*M.* × *domestica*) leaves, which contain trilobatin's bitter positional isomer phloridzin (phloretin-2'-*O*-glucoside). Variation in trilobatin content was mapped to the *Trilobatin* locus on linkage group 7 in a segregating population developed from a cross between domesticated apples and crabapples. *Phloretin glycosyltransferase 2* (*PGT2*) was identified by activity-directed protein purification and differential gene expression analysis in samples high in trilobatin but low in phloridzin. Markers developed for *PGT2* co-segregated strictly with the *Trilobatin* locus. Biochemical analysis showed PGT2 efficiently catalyzed 4'-*O*-glycosylation of phloretin to trilobatin as well as 3-hydroxyphloretin to sieboldin (3-hydroxyphloretin-4'-*O*-glucoside). Transient expression of *MdDBR* (double bond reductase), *MdCHS* (chalcone synthase) and *PGT2* genes reconstituted the apple pathway for trilobatin production in *Nicotiana benthamiana*. Transgenic *M.* × *domestica* plants over-expressing *PGT2* produced high concentrations of trilobatin in young leaves. Transgenic plants were phenotypically normal, and no differences in disease susceptibility were observed compared to wildtype plants grown under simulated field conditions. Sensory analysis indicated that apple leaf teas from *PGT2* transgenics were readily discriminated from control leaf teas and were perceived as significantly sweeter. Identification of *PGT2* allows marker-aided selection to be developed to breed apples containing trilobatin, and for high amounts of this natural low calorie sweetener to be produced via biopharming and metabolic engineering in yeast.

INTRODUCTION

Diabetes is a major public health problem that is approaching epidemic proportions globally. The key risk factor linked with type 2 (adult-onset) diabetes is obesity, which is associated with physical inactivity and over-eating (Bray and Popkin, 2014). One response to this health crisis by the food and beverage industry has been to develop products utilizing a range of non-sugar sweeteners to reduce calorie intake. Artificial sweeteners such as aspartame, sucralose and saccharin are the most widely used (Kroger et al., 2006); however, there has been increasing consumer awareness and demand for the use of natural low calorie sweeteners. Natural compounds with intense sweetening capacity can be found in numerous chemical families, including proteins, terpenoids and flavonoids. Examples of plant-based sweeteners include steviol glycosides from stevia (*Stevia rebaudiana*) leaves, mogrosides from the juice of monkfruit (*Siraitia grosvenorii*), and glycyrrhizhin from liquorice (*Glycyrrhiza glabra*) roots (Kim and Kinghorn, 2002).

Trilobatin is a plant-based sweetener that is reported to be ~100x sweeter than sucrose (Jia et al., 2008). It is found at high concentrations in the leaves of a range of crabapple species, including *Malus trilobata*, *M. sieboldii* and *M. toringo* (Williams, 1982; Gutierrez et al., 2018b). It is not found in the domesticated apple (*M.* × *domestica*), but has been reported in low amounts in the leaves of wild grape (*Vitis)* species (Tanaka et al., 1983). Some *Lithocarpus* species also contain trilobatin and their leaves are used to prepare sweet tea in China (Sun et al., 2015). The potential utility of trilobatin as a sweetener is recognized in many food and beverage formulations e.g. (Jia et al., 2008; Walton et al., 2015); however, its usefulness is limited by its scarcity. Methods for extraction have been documented from a range of tissues (Sun, 2015; Sun and Zhang, 2015) and following biotransformation of citrus waste (Lei et al., 2018). Biosynthesis of trilobatin in yeast has also been achieved (Eichenberger et al., 2016), but efficient production has been hampered by lack of knowledge of all the enzymes in the biosynthetic pathway.

Sweet-tasting trilobatin (phloretin-4'-*O*-glucoside) and bitter-tasting phloridzin (phloretin-2'-*O*-glucoside) are positional isomers of the dihydrochalcone (DHC) phloretin, which is produced on a side branch of the phenylpropanoid pathway. The first committed step in the biosynthesis of DHCs can be catalyzed by a double bond reductase (DBR) that converts

116 *p*-coumaryl-CoA to *p*-dihydrocoumaryl-CoA (Gosch et al., 2009; Dare et al., 2013b; Ibdah et
117 al., 2014). The next step involves decarboxylative condensation and cyclisation of
118 *p*-dihydrocoumaryl-CoA and three units of malonyl-CoA by chalcone synthase (CHS) to
119 produce phloretin (Gosch et al., 2009; Yahyaa et al., 2017). The final step in the pathway
120 requires the action of UDP-glycosyltransferases (UGTs) to attach glucose at either the 2' or 4'
121 positions of the chalcone A-ring. Another apple DHC, sieboldin
122 (3-hydroxyphloretin-4'-*O*-glucoside), is also glycosylated at the 4' position either after the
123 conversion of phloretin to hydroxyphloretin or by conversion of trilobatin directly to
124 sieboldin.

125 UGTs are typically encoded by large gene families, with over 100 genes having been
126 described in Arabidopsis (Ross et al., 2001) and over 200 genes in the *M.* × *domestica*
127 genome (Caputi et al., 2012). All UGTs contain a conserved Plant Secondary Product
128 Glycosyltransferase (PSPG) motif that binds the UDP moiety of the activated sugar (Li et al.,
129 2001). Although some UGTs can utilize a broad range of acceptor substrates (Yauk et al.,
130 2014; Hsu et al., 2017), others have been shown to be highly specific (Fukuchi-Mizutani et
131 al., 2003; Jugdé et al., 2008). Systematic classification can facilitate the identification of
132 some UGT activities; however, functionality is generally difficult to ascribe through
133 phylogenetic relatedness alone. In apple, multiple UGTs have been identified that catalyze the
134 2'-*O*-glycosylation of phloretin to phloridzin: UGT88F1/*MdPGT1* (Jugdé et al., 2008),
135 UGT88F8 (Elejalde-Palmett et al., 2018), UGT88F4, UGT71K1 (Gosch et al., 2010), and
136 UGT71A15 (Gosch et al., 2012).

137 Dihydrochalcone levels have been manipulated in transgenic apples by down-regulation or
138 over-expression of UGTs and other genes in the phenylpropanoid pathway. UGT88F1
139 knockdown lines showed significantly reduced phloridzin accumulation (30% of wildtype)
140 and were severely dwarfed, with greatly reduced internode lengths and narrow lanceolate
141 leaves (Dare et al., 2017). UGT88F1 knockdown lines also showed increased resistance to
142 *Valsa* canker infection (Zhou et al., 2019). *MdCHS* knockout lines with 3-5% of wildtype
143 foliar phloridzin levels also showed a severely dwarfed growth pattern (Dare et al., 2013b).
144 Overexpression of UGT71A15 in transgenic apples increased the molar ratio of phloridzin to
145 phloretin, but did not affect plant morphology or susceptibility to the fire blight causing

bacterium *Erwinia amylovora* (Gosch et al., 2012). Overexpression of a chalcone 3'-hydroxylase in transgenic apple seedlings increased concentrations of 3-hydroxyphloridzin and reduced susceptibility to both fire blight and apple scab (Hutabarat et al., 2016).

Two apple enzymes, UGT71A15 and UGT75L17 (*MdPh-4'-OGT*), that glycosylate phloretin at the 4' position *in vitro* have been reported (Gosch et al., 2012; Yahyaa et al., 2016). However, these proteins are expressed in the leaves and fruit of domesticated apples, which do not accumulate trilobatin. To identify the glycosyltransferase leading to the production of trilobatin in planta, we focused on tissue from a range of wild apple accessions that produced trilobatin and sieboldin, and compared this with material from cultivated and wild apples producing only phloridzin. Using a range of genetic, biochemical, molecular and transgenic tools, we demonstrate that *phloretin glycosyltransferase 2* (*PGT2*) is responsible for trilobatin production in *Malus*, and that its over-expression increases perception of sweetness in apple leaf teas.

RESULTS

Candidate Glycosyltransferases Identified by Activity-directed Protein Purification

Tissues high in phloretin 4'-oGT (Ph4'-oGT) activity required for trilobatin production, but containing very low phloretin 2'-oGT (Ph2'-oGT) activity for phloridzin synthesis, were used to identify candidate Ph4'-oGTs by activity-directed protein purification. Flower petals of the crabapple hybrid 'Adams' were identified as suitable experimental material as they have low amounts of Rubisco compared with leaves, but higher Ph4'-oGT activity compared with fruit. Purification involved sequential chromatographic steps (Q-sepharose, phenyl sepharose and Superdex 75; Figure 1A–C), after which fractions with high Ph4'-oGT activity were pooled and used for further purification. In the final step, after size exclusion chromatography, four protein fractions with different Ph4'-oGT enzyme activities were analyzed by SDS-PAGE. The abundance of a single band 44–66 kDa (the size expected of a typical UGT) changed in a similar pattern (Figure 1D) to that of the Ph4'-oGT activities (Figure 1C). This band was subjected to LC-MS/MS analysis and peptides corresponding to 50 proteins were identified in the *M.* × *domestica* 'Golden Delicious' v1.0 genome assembly (Velasco et al., 2010) (Table S1). Of the five most abundant proteins, peptides corresponding to gene models MDP0000836043/MDP0000318032, encoding predicted UDP-glycosyltransferase 88A1-like proteins, were observed at highest abundance (26% of total peptides) (Table 1).

Candidate Glycosyltransferases Identified by Differential Gene Expression Analysis

A second approach using differential gene expression (DGE) analysis was used to identify candidate Ph4'-oGTs in tissues high in trilobatin but low in phloridzin. A cross was produced between the ornamental crabapple hybrid 'Radiant' (containing both trilobatin and phloridzin), and *M.* × *domestica* 'Fuji' (containing only phloridzin). The F1 progeny were separated into two phenotypes with or without trilobatin for RNA extraction and transcriptome analysis. Expression levels of 109 genes were up-regulated at least 16-fold (log2-fold change >4) in progeny producing trilobatin (Table S2). Of the five genes showing the greatest log-fold change, the expression level of the predicted UDP-glycosyltransferase 88A1-like protein MDP0000836043 exhibited the largest differential expression, and was up-regulated over 128-fold (log2-fold change >7) in plants with trilobatin compared with those without (Table

2).

Mapping Concentrations of Trilobatin and Candidate genes in a Segregating Population

Trilobatin levels were mapped in a segregating population developed from a cross between domesticated and wild apples ('Royal Gala' x Y3). The female parent 'Royal Gala' (*M.* × *domestica*) produced only phloridzin, whilst the male parent Y3 (derived from *M. sieboldii*) produced both trilobatin and phloridzin. Of the fifty-one plants phenotyped, 30 contained trilobatin and phloridzin, and 21 phloridzin alone. The segregation ratio (1:0.7; χ^2 = 1.59) suggested trilobatin content was segregating as a qualitative trait controlled by a single gene. Data obtained by screening leaf DNA with the International RosBREED SNP Consortium (IRSC) 8K SNP array (Chagné et al., 2012) were analyzed using JoinMap version 4.0 (van Ooijen, 2006). A single locus for control of trilobatin biosynthesis (*Trilobatin*) was identified on the lower arm of linkage group (LG)7, distal to a single nucleotide polymorphism (SNP) marker located at position 32,527,873 bp (Figure 2A) on the 'Golden Delicious' doubled-haploid genome assembly (GDDH13 v1.1) (Daccord et al., 2017). The locus was further defined using high resolution melting (HRM) SNP markers developed from two candidate *PGT* genes at 34,460,000–34,461,000 bp (Figure 2A, B) close to the base of LG7 (total length of LG7 is 36,691,129 in GDDH13 v1.1). The position mapped on LG7 was consistent with one of the three independently segregating loci for DHC content reported recently in *Malus*, using linkage and association analysis (Gutierrez et al., 2018a).

Genetic mapping by HRM marker analysis of gene model MDP0000836043, the candidate UDP-glycosyltransferase 88A1-like protein identified by activity-directed protein purification and DEG analysis, demonstrates that it co-locates with the locus identified for trilobatin production on LG7 (Figure 2A). In the 'Golden Delicious' v1.0p assembly (Velasco et al., 2010) MDP0000836043 is located at ~24,531,751 bp and corresponds to gene models MD07G1281000/1100 (located at 34,459,260 bp) in the doubled-haploid assembly GDDH13 v1.1 (Figure 2B). The second UDP-glycosyltransferase 88A1-like protein identified by activity-directed protein purification encoded by MDP0000318032 (MD07G1280800) is located ~10.3 kb away in both assemblies (Figure 2B). Four SNP variants identified in the region of these two UDP-glycosyltransferase gene models were used to develop markers for

HRM analysis (Figure S1, HRM primer sequences in Table S3). The mapping results validated the position of the MDP0000836043 and MDP0000318032 on LG7 (Figure 2A), and three of the markers (HRM1–3) showed precise concordance with presence/absence of trilobatin in the segregating progeny (Table S4). HRM1 and 3 amplified both MDP0000836043 (MD07G1281000/1100) and MDP0000318032 (MD07G1280800), whilst HRM2 was specific for MDP0000836043 (MD07G1281000/1100). HRM4 was not specific to the locus and was not used further.

Biochemical Characterization of *PGT2* and *PGT3*

Complete open reading frames (ORFs) corresponding to MDP0000836043 (hereafter termed *PGT2,* UGT88A32) were amplified from the leaves of six *Malus* accessions. The *PGT2* ORFs from five accessions synthesizing trilobatin showed 91–94% amino acid identity to the MDP0000836043 gene model from *M. domestica* 'Golden Delicious' (Figure S2). A complete ORF for *PGT2* obtained from the leaves of *M. domestica* 'Fuji' was identical to that obtained from *M. toringoides*, although *PGT2* was difficult to obtain from 'Fuji' because of very low expression levels. The complete ORF corresponding to MDP0000318032 (termed *PGT3,* UGT88A33) was also amplified from five *Malus* accessions and exhibited 97–99% identity to the MDP0000318032 gene model from 'Golden Delicious', and 85–87% identity to *PGT2* (Figure S3).

PGT2 and *PGT3* from five *Malus* accessions and *PGT1* from 'Fuji' were expressed in *Escherichia coli* and the products formed using phloretin and UDP-glucoside as substrates were determined by HPLC. All purified recombinant PGT2 enzymes produced a single peak at 7.5 min that ran at the same retention time as the trilobatin standard (Figure 3A). A representative HPLC trace for the product produced by PGT2 from *M. toringoides* is shown in Figure 3B. All purified recombinant PGT3 enzymes (Figure 3C) and PGT1 from 'Fuji' (Figure 3D) produced a peak at 6.0 min with the same retention time as phloridzin (Figure 3A), but no peak for trilobatin. No phloridzin or trilobatin were produced by the empty vector control (Figure 3E).

The substrate specificity of recombinant PGT2 from *M. toringoides* was further characterized using UDP-glucoside as the sugar donor and twelve substrates typically found

in apple or with structural homology to phloretin (Table 3). The products of each reaction were determined by LC-MS/MS. Phloretin was the best acceptor for PGT2 and base peak plots indicated that a single peak at 21.5 min was formed that co-eluted with the trilobatin standard (Figure 4A, B). PGT2 also catalyzed glycosylation of 3-OH phloretin to produce sieboldin (Figure 4C, D) with a relatively high conversion rate of ~60% (Table 3). Quercetin-3-*O*-glucoside was detected as a reaction product using quercetin (Figure S4A–F) with a lower conversion rate of 9.1% (Table 3). This result is surprising, as the 4' position of dihydrochalcones corresponds to the 7 position of quercetin, but no quercetin-7-*O*-glucoside was observed. No products were detected in reactions with the other acceptor substrates, however trace activity (1.4% compared with UDP-glucose) was detected with *PGT2* using phloretin and UDP-galactose as the activated sugar donor. Fullscan and MS⁄MS mass spectral data were used to further characterize the products of the PGT2 reactions. Phloretin (Figure 4E) was detected as its pseudo-molecular ion *m/z* 273 $[M-1]^-$), whereas trilobatin (Figure 4F), 3-OH phloretin (Figure 4G) and sieboldin (Figure 4H) were detected predominantly as the corresponding formate adducts $[M+formate])^{-1}$. MS^2 on the formate adducts identified the expected pseudo-molecular ion at *m/z* 435 and 451 $[M-1]^-$) for the trilobatin and sieboldin glucosides. MS^3 on the *m/z* 435 and 451 $[M-1]^-$) glucoside ions identified the *m/z* 273 and m⁄z 289 $[M-1]^-$) ions of the phloretin and 3-OH phloretin aglycones, respectively.

PGT2 and PGT1 enzyme activities were compared over a pH range of 4–12 and with temperatures from 15 to 60°C. The pH optimum of both enzymes was between pH 8.0–9.0 (Figure S5A, B), while the optimum temperature was ~40°C (Figure S5C, D). The K_m values of PGT2 for phloretin were 18.0 ± 6.7 μM (V_{max} = 1.85 ± 0.17 nmol·min^{-1}) and for UDP-glucose 103.6 ± 23.0 μM: (V_{max} = 2.07 ± 0.17 nmol·min^{-1}) (Figure S5E, F). These K_m values are comparable to those obtained for PGT1 for phloretin of 4.1 ± 1.2 μM (V_{max} = 1.54 ± 0.08 nmol·min^{-1}) and for UDP-glucose of 491 ± 41 μM (V_{max} = 8.84 ± 0.35 nmol·min^{-1}) under the same purification conditions (Figure S5E, F).

Expression of PGT2 and PGT3 in *Malus* spp.

The relative expression of *PGT2* and *PGT3* were determined by RT-qPCR in the leaves of nine *Malus* accessions (Figure 3). Three accessions produced predominantly trilobatin, three

both trilobatin and phloridzin, and three only phloridzin. *PGT2* was highly expressed in all six *Malus* accessions producing trilobatin; however, expression was essentially absent in the three accessions that did not synthesize trilobatin (Figure 3F). Conversely, the expression of *PGT1* was high in the six *Malus* accessions producing phloridzin (Figure 3G). Expression of *PGT3* was observed in all nine accessions, and did not correspond with the presence/absence of trilobatin or phloridzin in the samples (Figure 3H).

Structural Comparison of the Active Sites in PGT1-PGT3

To investigate the structural basis for the difference in positional specificity for glycosylation on phloretin, structural homology models were independently obtained for PGT1-PGT3 using the iTASSER server (Yang and Zhang, 2015). The models were superimposed and compared with the crystal structure of UGT72B1 bound with UDP-glucose (donor) and 2,4,5-trichlorophenol (TCP, acceptor) (Brazier-Hicks et al., 2007) (PDB entry 2VCE). Overall, all structures were very similar, with RMSDs of ~1 Å between each other (Figure S6). Sequence identities between PGT2/PGT1, PGT2/PGT3, and PGT1/PGT3 were 48, 86 and 47%, respectively. Around the predicted UDP binding site, however, the amino acid conservation between the three enzymes was much higher (>95% identity) (Table S5), consistent with the ability of these enzymes to bind the same donor molecule. Furthermore, the positions of the catalytic dyad residues in the models (His16/Asp118 in PGT2 and PGT3, His15/Asp118 in PGT1) were in excellent agreement with the crystal structure of UGT72B1. In contrast, conservation between PGT2 and PGT1 was considerably lower among the amino acids shaping the acceptor binding pocket (23% identity; 3/13 residues) (Figure 5A, B). Similarly, although less pronounced, conservation between PGT2 and PGT3 around the acceptor binding pocket dropped to 69% (9/13 residues) (Figure 5B, C).

Metabolic Engineering of Trilobatin Production in *N. benthamiana*

To reconstitute the full apple pathway for trilobatin and phloridzin production in *Nicotiana benthamiana*, *MdMyb10* and two biosynthetic genes *MdDBR* and *MdCHS* were transiently expressed together to catalyze the synthesis of phloretin substrate for glycosylation. Leaves infiltrated with *MdMyb10*, *MdDBR, MdCHS* and *PGT2* were analyzed by Dionex-HPLC and

exhibited a peak at 32 min that corresponded to the trilobatin standard (Figure 6A, B), whilst those infiltrated with *PGT1* exhibited a peak at 27.2 min corresponding to phloridzin (Figure 6C, D). Concentrations of trilobatin produced with *PGT2* and phloridzin produced with *PGT1* were similar (45 vs 87 $ug \cdot g^{-1}$ respectively; Table S6). Neither phloridzin nor trilobatin was detected in leaves inoculated with the GUS control vector (Figure 6E). Interestingly, no phloretin was detected in the reactions, suggesting that glycosylation is needed to stabilize the reactive phloretin in *N. benthamiana* leaves.

MdDBR and *MdCHS1* were required for synthesis of trilobatin in co-infiltrations with *PGT2* (Table S6). Substitution of *MdDBR* for *MdENRL3* (Dare et al., 2013a) abolished trilobatin production. The *MdMyb10* transcription factor increased trilobatin formation presumably via increased substrate flux through the phenylpropanoid pathway (Table S6). These results indicate that three *Malus* biosynthetic genes and a transcription factor are sufficient to reconstitute the pathway to trilobatin and phloridzin production in *N. benthamiana* (and likely any other plant) for biotechnological applications.

Over-expression of *PGT2* in *M.* × *domestica*

PGT2 was over-expressed in *M.* × *domestica* backgrounds GL3 and 'Royal Gala'. Fourteen transgenic GL3 lines were obtained and *PGT2* expression was significantly increased in the leaves of 4-week-old plants from eight lines (# 1, 4, 5, 6, 7, 9, 11, 14) compared with wildtype (Figure 7A). Amounts of trilobatin were significantly increased in the same eight lines + line #10 compared with wildtype, with concentrations ranging from ~5 to 11 $mg \cdot g^{-1}$ FW (Figure 7B). No significant differences were observed in phloridzin, phloretin (Figure 7B) or total content of trilobatin + phloridzin (Figure S7A) among the GL3 transgenic lines. Eleven transgenic 'Royal Gala' lines over-expressing *PGT2* were also regenerated. An initial screen of shoots in tissue culture showed that all transgenic lines contained increased concentrations of trilobatin (Figure S8A), but similar total DHC content (Figure S8B) compared with wildtypes. Further analysis of the young leaves from six of the 'Royal Gala' lines grown in a containment glasshouse confirmed these results (Figure S8C and Figure S8D).

The relative expression of *PGT1*, *MdCHS* and *PGT3* were also analyzed by RT-qPCR in the GL3 transgenic *PGT2* over-expression lines. The expression levels of *PGT1* (Figure S7B)

and *MdCHS* (Figure S7C) were not significantly altered in the 14 transgenic apple lines. Interestingly, the relative expression of *PGT3* in all 13/14 transgenic GL3 lines decreased significantly (Figure S7D). Strongest suppression was observed in lines expressing *PGT2* and trilobatin at the lowest levels, suggesting co-suppression of the endogenous *PGT3* gene by the introduced *PGT2* transgene.

Physiology of *PGT2* Over-expression Lines Grown under Simulated Field Conditions

Manipulation of dihydrochalcone levels in some transgenic apple lines has been shown to severely affect plant morphology (Dare et al., 2013b; Dare et al., 2017; Zhou et al., 2019). Multiple seedlings of three *PGT2* over-expression lines (#'s 1, #5 and #14) and matching wildtypes were assessed for changes in morphology at eighteen months of age when grown under simulated field conditions. No significant differences were observed in plant height (Figure 8A, B), total number of branches per tree (Figure 8C), leaf morphology (Figure 8D) or fresh weight per leaf (Figure 8E).

Changes in dihydrochalcone levels have also been implicated in pathogen susceptibility in some transgenic apple lines (Hutabarat et al., 2016; Zhou et al., 2019), but not in others (Gosch et al., 2012). *PGT2* over-expression lines and matching wildtypes grown under simulated field conditions were subject to natural pathogen infection; no disease or pest management schemes were imposed on the plants. Under these conditions, no differences in disease susceptibility were observed between the *PGT2* transgenics and wildtypes. The most common infection on all plants was by powdery mildew (Figure 8F), but no significant differences in powdery mildew infection rate were observed (Figure 8G).

Sensory Evaluation of Apple Leaf Teas from *PGT2* Transgenic Plants

Sensory analysis was used to investigate the impact of *PGT2* over-expression on the taste of apple leaf tea. Leaves were harvested from 4-month-old wildtype GL3 plants and two transgenic lines (#'s 1, 9). After drying, the phloridzin contents in the wildtype and transgenic leaves were similar (~150 $mg \cdot g^{-1}$ DW). The transgenic lines also contained trilobatin (~100 $mg \cdot g^{-1}$ DW), whilst wildtype contained none (Figure 9A). After steeping, ~27% of the phloridzin and 16% of the trilobatin was extracted into the tea (Figure 9A).

In triangle tests, panelists were clearly able to distinguish the flavor of tea produced from the transgenic *PGT2* leaves compared with tea produced from wildtype (p <0.01, 38 correct observations out of n = 70). To determine the basis for this discrimination, panelists were then asked to rate the sweetness of each sample on a scale from 1–10. The average sweetness of the two transgenic lines was rated significantly (p <0.05, n = 23) higher at 4.8 and 4.6 respectively, compared with that of wildtype, rated at 3.2.

DISCUSSION

Glycosyltransferases are encoded by large gene families, and identifying enzymes with specific activities based on homology is difficult. Two enzymes capable of 4'-*O*-glycosylation of phloretin in vitro have been reported (Gosch et al., 2012; Yahyaa et al., 2016), but these genes are expressed in tissues that produce only phloridzin. In this study, we used multiple approaches to show that *PGT2* is responsible for production of trilobatin in apple. The genetic locus for trilobatin production co-located with the *PGT2* gene, and HRM markers developed to *PGT2* segregated strictly with trilobatin production. Molecular and biochemical analysis demonstrated that *PGT2* was only expressed in accessions where trilobatin (or sieboldin) was produced and that the enzyme showed Ph4'-oGT activity in vitro. Finally, over-expression of *PGT2* in domesticated apple confirmed that *PGT2* leads to the production of trilobatin in planta.

The modeling results for PGT1-PGT3 showed strong amino acid conservation between the three enzymes around the predicted UDP binding site, a feature common to the UGT superfamily (Li et al., 2001). Although both are members of the UGT88 family, PGT2 (UGT88A32) and PGT1 (UGT88F1) showed large variations in the amino acid composition of their respective acceptor binding pockets, which is consistent with these two enzymes having different activities and generating different products. More surprisingly, PGT2 and PGT3 (UGT88A32), which are putative paralogs and share high amino acid identity, also show strong differences in overall activity and specificity. In the case of PGT3, the 3D model analysis suggests that the low enzymatic activity may be due to one (or to a combination) of the four amino acids differing with PGT2 in the acceptor binding pocket. Among these, the substitution at position 145 of an Asn in PGT2 for a bulkier Phe in PGT3 may restrict the size of the pocket and impair the binding of the acceptor. However, site-directed mutagenesis and/or crystallographic work is required to confirm this hypothesis and to further understand the Ph2'-oGT activity of PGT3, compared with the Ph4'-oGT activity of PGT2.

UGTs with the ability to glycosylate phloretin have been described in *M. domestica* from multiple UGT families (Jugdé et al., 2008; Gosch et al., 2010; Yahyaa et al., 2016; Zhou et al., 2017; Elejalde-Palmett et al., 2018). However, the majority, including the *PGT2* and *PGT3*, belong to the UGT88 family (for a phylogeny see Figure S9). The absence of trilobatin

biosynthesis in *M.* × *domestica* is not due to mutations in the *PGT2* gene, as a complete ORF was amplified from 'Fuji' and shown to be identical to the *PGT2* ORF from *M. toringoides* (a species producing trilobatin). Instead, DGE and RT-qPCR analyses indicated that the mutation was at the transcriptional level, with *M.* × *domestica* expressing *PGT2* at very low levels. *M.* × *domestica* and all other accessions tested do express the putative *PGT3* paralog located within ~10 kb of *PGT2*. However, despite high homology, *PGT3* does not exhibit Ph4'-oGT activity, but weak Ph2'-oGT activity. The *Pyrus* (pear) genome (Linsmith et al., 2019) contains a close homolog of the *PGT3* gene (XP_009368718.2), but not *PGT2*, suggesting that the *PGT2* gene may have evolved recently only in the *Malus* lineage, or have been lost from the *Pyrus* lineage. The absence of trilobatin production in *M.* × *domestica* does not appear to be driven by domestication, as a number of wild apple species (e.g. *M. baccata*; Figure 3F) produce only phloridzin and lack expression of *PGT2*. Most wild *Malus* species that express *PGT2* accumulate sieboldin, or trilobatin in combination with phloridzin. Only one species expressing *PGT2*, *M. trilobata*, accumulates trilobatin alone.

Manipulation of dihydrochalcone levels in transgenic apples has been associated with changes in plant physiology, notably in plant morphology (Dare et al., 2017), and susceptibility to pathogen infection (Gosch et al., 2012; Hutabarat et al., 2016; Zhou et al., 2019). Transgenic *PGT2* over-expressing lines appeared phenotypically normal, and no differences in disease susceptibility were observed between the *PGT2* transgenics and wildtypes grown under simulated field conditions. In transgenic apple seedlings over-expressing chalcone 3'-hydroxylase, total DHC content was not altered, but reduced susceptibility to both fire blight and apple scab was associated with an increase (up to 11.5%) in 3-hydroxyphloridzin accumulation (Hutabarat et al., 2016). In the *PGT2* transgenics, total DHC content was similarly not altered, but trilobatin accumulation (up to 38%) did not alter disease susceptibility. This difference may suggest that sieboldin is more effective in promoting disease resistance. However, in Hutabarat et al., (2016) pathogen sensitivity assays were performed using artificial shoot inoculations and it was not reported if increased sieboldin concentrations altered susceptibility under field conditions. Conversely, our observations do not preclude the possibility that disease susceptibility in the *PGT2* transgenics

will change as the plants age, or that the plants were not exposed to pathogens sensitive to trilobatin under simulated field conditions.

Sensory analysis of apple leaf teas made from transgenic plants over-expressing *PGT2* demonstrated that they could be clearly distinguished from teas made from wildtype apple leaves. The concentrations of trilobatin extracted into tea (Figure 9A, ~150 $mg \cdot L^{-1}$) were above the sweetness detection threshold reported for trilobatin (3–200 $mg \cdot L^{-1}$) (Jia et al., 2008). The perception of increased sweetness in the transgenic leaf teas was consistent with increased production of trilobatin and not a decrease in concentrations of bitter-tasting phloridzin. However, a more detailed analysis by trained panelists would be required to understand the sensory properties fully. An iso-sweetness comparison test between trilobatin purified from leaves of the crabapple hybrid 'Adams' and sucrose indicated that trilobatin was ~35-fold sweeter than sucrose (Figure 9B). This number is slightly lower than figures reported previously (Jia et al., 2008), which may relate to the purity of the trilobatin tested, the delivery system, or variation in panelist sensitivity to sucrose or trilobatin.

Identification of the Ph4'-oGT for trilobatin production will allow us to investigate whether *PGT2* over-expression in the fruit affects consumer perception of sweetness. Transgenic 'Royal Gala' plants over-expressing the *PGT2* gene have been produced and these contain both trilobatin and phloridzin in the leaves. This result indicates that *PGT2* is reasonably competitive with *PGT1* for the pool of phloretin substrate available in leaves and that *PGT2* should be competitive with *PGT1* for the smaller pool of phloretin produced in fruit. This hypothesis will be tested when the transgenic 'Royal Gala' plants reach maturity in several years' time. An alternative approach to increasing trilobatin production in *M.* × *domestica* would be to use molecular markers to accelerate the introgression of *PGT2* into elite breeding material. However, for this approach to be successful, parental material in which *PGT2* is well-expressed in fruit (rather than leaves and flowers) needs to be identified.

Identification of the Ph4'-oGT for trilobatin production and reconstitution of the apple pathway to trilobatin and phloridzin production in *N. benthamiana* may also allow high amounts of trilobatin to be produced via biotechnological means, such as biopharming and metabolic engineering in yeast. The utility of this approach has already been demonstrated for *PGT1* in yeast (Eichenberger et al., 2016), but not in planta. The ability to produce large

quantities of trilobatin would allow it to be tested not only as a natural sweetener in the food and beverage industry, but also for its potential health benefits (Fan et al., 2015; Xiao et al., 2017).

MATERIALS AND METHODS

Plant Material

Trilobatin production was mapped in an F1 seedling population between 'Royal Gala' and Y3 grown in a greenhouse at PFR, Auckland, New Zealand. Y3 is derived from the crabapple hybrid 'Aotea' x *M.* × *domestica* 'M9'. 'Aotea' is an open-pollinated *M. sieboldii* (which produces sieboldin) selection. *M. trilobata* and 'Aotea' were grown at the PFR research orchard in Havelock North, New Zealand.

M. micromalus 'Makino' and the F1 population for differential gene expression analysis between the crabapple hybrid 'Radiant' and *M.* × *domestica* 'Fuji' were grown at the Luochuan Apple Experimental Station, Northwest A&F University, Shaanxi, China. All other material was grown in an experimental orchard at Northwest A&F University, Yangling, Shaanxi, China. All trees were grown on their own roots and managed using standard horticultural growth practices and management for disease and pest control.

For physiological experiments on transgenic lines, GL3 and wildtype plants were grown in a plastic tunnel house in a randomized layout at Northwest A&F University. To meet transgenic containment restrictions, plants were grown in pots (height 22 cm, diameter 32 cm). To simulate field conditions, plants were grown under ambient temperature and environmental conditions. As the tunnel house was open ended, plants were subject to natural pathogen infection. No disease or pest management schemes were imposed.

Chemicals

Trilobatin was purified from the crabapple hybrid 'Adams' (Xiao et al., 2017). Sieboldin, 3-OH phloretin and quercetin glycosides were purchased from PlantMetaChem (www.PlantMetaChem.com) and cyanidin from Extrasynthese (www.extrasynthese.com). All other chemicals, including phloridzin and phloretin, were obtained from Sigma Aldrich (sigmaaldrich.com).

Mapping the *Trilobatin* Locus

Leaf tissue from seedlings in the 'Royal Gala' x Y3 population were harvested and weighed before snap-freezing in liquid nitrogen. Phenolics were extracted from 100–250 mg

of leaf tissue as described by Dare et al., (2017) and polyphenols quantified by Dionex-HPLC on an Ultimate 3000 system (Dionex, Sunnyvale, CA, USA) equipped with a diode array detector at 280 nm as described by André et al., (2012). Seedling DNA was extracted using the DNAeasy Plant Mini Kit (Qiagen) and genotypes determined using the IRSC 8K SNP array (Chagné et al., 2012). The SNP array data were analyzed using the Genotyping Module of the GenomeStudio Data Analysis Software (Illumina). The genetic map was constructed using JoinMap version 4.0 and the position of the *Trilobatin* locus on LG7 of Y3 was identified after conversion of SNP physical coordinates in the 'Golden Delicious' v1.0 genome assembly (upon which the IRSC 8K SNP array was designed) to the latest version of the apple reference genome (GDDH13 v1.1). The position of *PGT2* was then defined using HRM primers designed within the *PGT2* candidate genes (Figure 1, Table S3) and PCR conditions as in Chagné et al., (2008).

Activity-directed Protein Purification

A detailed protocol for activity-directed purification of Ph4'-oGT activity from apple flower petals is given in the legend to Table S1. Purified protein fractions were separated on 12% (w/v) SDS-PAGE gels and visualized by Coomassie Blue R-250 staining. Target bands were cut and digested in gel with trypsin according to the method of Gao et al., (2017). The peptide mixture was then loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap 100, 100 µm x 2 cm, nanoViper C18) connected to the C18-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 µm inner diameter, 3 µm resin) in buffer A (0.1% (v/v) formic acid) and separated with a linear gradient of buffer B (84% (v/v) acetonitrile and 0.1% (v/v) formic acid) at a flow rate of 300 $nL \cdot min^{-1}$ controlled by IntelliFlow technology. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Scientific) for 60 min, and the mass spectrometer was operated in positive ion mode. Details of MS/MS spectra analysis are given in the legend to Table S1.

Differential Gene Expression Analysis

The F1 population developed from a cross between the crabapple hybrid 'Radiant' and *M.*

× *domestica* 'Fuji' was screened for trilobatin and phloridzin by HPLC. Eighty-one plants containing trilobatin + phloridzin (T+P) and 81 plants containing only phloridzin (P) were identified. One expanding leaf was collected from each seedling (~20 cm tall) and three pooled replicate samples for T+P and P were prepared (each replicate containing leaves from 27 plants). Total RNA was extracted from frozen ground powder using Trizol Reagent (Life Technologies) following the manufacturer's instructions and checked for RNA integrity on an Agilent Bioanalyzer 2100. Sequencing libraries were generated from 3 μg RNA per sample using NEBNext Ultra RNA Library Prep Kit for Illumina (Thermo Fisher) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. RNA was sequenced by Novogene (Beijing, China) using the Illumina HiSeq4000 platform. Details of sequence alignment and differential gene expression analysis are given in the legend to Table S2.

RT-qPCR Analysis

Total RNA was extracted from young leaves as described by Malnoy et al., (2001). First-strand cDNA was synthesized from 1 μg of total RNA using the PrimeScript RT Reagent Kit (Takara, Dalian, China), according to the manufacturer's instructions. RT-qPCR was performed with a Bio-Rad CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) using the TB Green Premix Ex Taq (Takara, Dalian, China). *MdACTIN* was used as the reference gene. The relative expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Three biological replicates each with three technical repeats were used for RT-qPCR analysis. Gene-specific primers are listed in Table S3.

Biochemical Characterization of *PGT1–3* in *E. coli*

The ORFs of *PGT1–3* were amplified using primers in Table S3 and ligated into pET28a(+) using the One Step Cloning Kit (www.vazyme.com). Recombinant proteins were expressed in *E. coli* BL21 (DE3) cells with 0.5 mM isopropyl-1-thio-β-galactopyranoside (IPTG) at 16°C for 24 h at 80 rpm. Purification of recombinant proteins was performed using Ni-NTA agarose (Millipore). Eluted fractions were used for determining enzyme activity and for SDS-PAGE analysis (Figure S10). Active fractions were concentrated using Vivaspin 2 concentrators

(Sartorius, Germany).

GT activity assays were performed in triplicate in 200 μL reactions containing 50 mM Tris-HCl (pH 9.0), 1 mM DTT, 0.5 mM phloretin, 0.5 mM UDP-glucose, and 30–80 ng enzyme. Reaction mixtures were incubated for 10 min at 40°C and reactions stopped by adding 40 μL of 1 M HCl. NaOH (1 M) was used to adjust the pH to neutral for HPLC analysis of the products at 280 nm.

HPLC and LC-MS/MS Analysis of Phenolic Compounds

Leaf tissue (500 mg) for HPLC was snap frozen in liquid nitrogen, and extracted with 1.5 mL of a solution containing 50% (v/v) methanol and 2% (v/v) formic acid at 0–4°C. The homogenate was centrifuged at 10,000 g for 10 min, and the supernatant used for HPLC after filtering with a 0.45 μM syringe (Li et al., 2013). Polyphenols were quantified on a LC-20A liquid chromatograph equipped with a diode array detector (Shimadzu Corporation, Tokyo, Japan) at 280 nm as described previously (Zhang et al., 2018).

For LC-MS/MS analysis, scaled up reactions were performed containing approximately 10 μg enzyme, 10 μM substrate and UDP-glucose at a final concentration of 250 μM. Reactions were performed in triplicate, and stopped after 1 h by addition of 10 μL of 10% (v/v) glacial acetic acid and blown down to dryness under a gentle stream of N_2. The reaction products were reconstituted in 100 μL 5:95 (v/v) acetonitrile: water + 0.1% (v/v) formic acid, and then a 10 μL aliquot taken which was diluted 10-fold in the same solvent. LC-MS/MS employed an LTQ linear ion trap mass spectrometer fitted with an ESI interface (Thermo Scientific) coupled to an Ultimate 3000 UHPLC and PDA detector (Dionex).

Phenolic compound separation was achieved using a Hypersil Gold aQ 1.9 μ (Thermo Scientific), 150 × 2.1 mm analytical column maintained at 45°C. Solvents were (A) water + 0.1% (v/v) formic acid and (B) acetonitrile + 0.1% (v/v) formic acid and the flow rate was 200 $\mu L \cdot min^{-1}$. The initial mobile phase, 5% B/95% A, was held for 2 min then ramped linearly to 15% B at 10 min, held for 3.75 min, before ramping linearly to 25% B at 18 min, 33% B at 25 min, 50% B at 28 min, 100 % B between 29 and 32 min before resetting to the original conditions. The sample injection volume was 2 μL. MS data were acquired in the negative mode using a data-dependent LC/MS^3 method. This method isolates and fragments the most

intense parent ion to give MS^2 data, then isolates and fragments the most intense daughter ion (MS^3 data). The ESI voltage, capillary temperature, sheath gas pressure and sweep gas were set at −10 V, 275°C, 35 psi and 5 psi, respectively.

Molecular Modeling

The sequences for PGT1–3 were independently submitted to the iTASSER server (Yang and Zhang, 2015). C-scores of the best models used for structural analysis were -0.38, 0.94 and 1.52 for PGT1, 2 and 3, respectively. Superimposition, structural analysis and figures were performed using the PyMOL Molecular Graphics System, Version 2.0 (Schrödinger, LLC).

Transient Expression in *N. benthamiana*

PGT2 was amplified from *M. trilobata*, pHEX2-*MdCHS* and *MdDBR* (Yahyaa et al., 2016) from 'Royal Gala' using the primers in Table S3. Genes were cloned into pHEX2 to generate the binary vectors pHEX2-PGT2, pHEX2-CHS and pHEX2-DBR respectively. Construction of pHEX2-Myb10, pGreen0029-ENRL3, pBIN61-p19 (containing the suppressor of gene silencing p19) and the control construct pHEX2-GUS have been reported previously (Espley et al., 2007; Dare et al., 2013a; Nieuwenhuizen et al., 2013). All constructs were electroporated in *Agrobacterium tumefaciens* strain GV3101. Freshly grown cultures were mixed in equal ratio and infiltrated into *Nicotiana benthamiana* leaves as described in Hellens et al., (2005). After 7 d, leaves were harvested and phenolic compounds extracted for Dionex-HPLC analysis.

Generation of Transgenic Apple Plants

The coding region of *PGT2* was amplified from *M. toringoides* using the primers in Table S3 and cloned into pCAMBIA2300 using the One Step Cloning Kit (www.vazyme.com). The PGT2:pCAMBIA plasmid was then transformed into *Agrobacterium tumefaciens* (strain GV3101) cells. Transgenic GL3 apple plants were generated by *Agrobacterium*-mediated transformation according to Dai et al.; (2013) and Sun et al.; (2018). Transgenic 'Royal Gala' plants were transformed with pHEX2-PGT2 and plants regenerated as described previously

(Yao et al., 1995; Yao et al., 2013).

Sensory Panel Analysis

Apple leaves from wildtype and two *PGT2* transgenic GL3 lines were washed with water and dried at room temperature. Leaves were held at 200°C for 1 min to inactivate enzymes, then dried at 80°C in an oven for 60 min. Apple leaf tea was made using 5 g of dried leaves with the ratio of leaves:water being 1:100 (g:mL). Water at ~80°C was added to the leaves for 15 min, then all leaves were removed to stop further extraction. The tea was then kept at 50°C in water bath for sensory analysis. The sensory panel consisted of 23 individuals and included 14 females and 9 males (all 20–30 years of age). Participation was voluntary and all participants gave their written consent prior to participation in the study. For the triangle tests, participants were given three trays, each tray had three cups (2 mL tea in each cup) with transgenic and wildtype leaf tea in a random design, either two transgenic and one wildtype or two wildtype and one transgenic. Participants were asked to sequentially taste the three samples on each tray and select which sample was different. To assess the relative sweetness of wildtype *vs* transgenic apple leaf teas, two samples (one transgenic and one wildtype) were presented and the 23 panelists were asked to score the two samples on a sweetness scale from 1 to 10. For all the tasting tests, participants kept the samples in their mouths for 1–2 seconds, then spat them out into a waste container. Participants rinsed their mouths between samples with water and a dry biscuit was provided between each sample set.

Five participants with high acuity for trilobatin in the triangle test were selected to perform the iso-sweetness comparison test between trilobatin and sucrose. Each participant was given one trilobatin solution and eight sucrose solutions at different concentrations to taste. Solutions were prepared as described above for the apple leaf teas. The trilobatin solutions were presented at 12.3, 18.5, 27.8 and 41.7 mg per 100 mL, while the sucrose solutions were presented at 296.3, 444.4, 592.6, 666.7, 888.9, 1000, 1333.3 and 2000 mg per 100 mL.

ACCESSION NUMBERS

Nucleotide sequences for genes characterized as part of this study were deposited in GenBank and received the accession numbers MN38099-MN381012.

SUPPLEMENTAL DATA

The following supplemental materials are available.

Supplemental Figure S1: HRM profiles of the four SNP markers located at the *Trilobatin* locus.

Supplemental Figure S2. Amino acid alignment of *PGT2* sequences from *Malus* accessions.

Supplemental Figure S3. Amino acid alignment of *PGT3* sequences from *Malus* accessions.

Supplemental Figure S4: LC-MS/MS analysis of reactions containing *PGT2*, quercetin and UDP-glucose.

Supplemental Figure S5: Biochemical properties of recombinant *PGT2* and *PGT1.*

Supplemental Figure S6: 3D-superimposition of PGT1, 2 and 3 models with the UGT71B1 crystal structure.

Supplemental Figure S7: HPLC and RT-qPCR analysis of transgenic GL3 plants over-expressing *PGT2.*

Supplemental Figure S8: HPLC analysis of transgenic 'Royal Gala' plants over-expressing *PGT2*.

Supplemental Figure S9: Phylogeny of UDP-glycosyltransferases.

Supplemental Figure S10: SDS-PAGE of recombinant PGT2 and PGT1 proteins..

Supplemental Table S1: Proteins identified after activity-directed purification of Ph4'-oGT activity.

Supplemental Table S2: Differential gene expression analysis in tissues high in trilobatin but low in phloridzin.

Supplemental Table S3: Primer sequences for HRM analysis, RT-qPCR and cloning**.**

Supplemental Table S4: Phenotype-to-genotype comparisons for individuals used to construct the genetic map and in HRM assays.

Supplemental Table S5: Amino acids surrounding the donor and acceptor binding sites in PGT1–3, identified from the respective 3D models.

Supplemental Table S6: Transient production of dihydrochalcones in *N. benthamiana.*

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682 **Table 1: Proteins Identified after Activity-directed Purification**

Protein	Description	iBAQ R1	iBAQ R2
MDP0000836043/ MDP0000318032	*M.* × *domestica* UDP-glycosyltransferase 88A1-like	3786800000	3146000000
MDP0000155691	*M.* × *domestica* pentatricopeptide repeat-containing protein At4g14190, chloroplastic	96169000	33639000
MDP0000267350	*M.* × *domestica* monodehydroascorbate reductase-like	85148000	273050000
MDP0000705244	*M.* × *domestica* UDP-glycosyltransferase 76B1-like	75047000	628300
MDP0000234480	*M.* × *domestica* transaldolase-like	31272000	246460000

683

684 Abundant proteins identified by LC-MS/MS analysis of bands isolated after activity-directed
685 purification of Ph4'-oGT activity from flowers of the crabapple hybrid 'Adams' (containing
686 trilobatin and not phloridzin). IBAQ = sum of all peptide intensities divided by the number of
687 observable peptides of a protein. The analysis was performed twice and the most abundant
688 proteins found in both analyses are given as R1 and R2.

689

690 **Table 2: Differentially Expressed Genes.**

Gene	Description	log2-fold change
MDP0000836043	*M.* × *domestica* UDP-glycosyltransferase 88A1-like (LOC103410306), mRNA	7.54
MDP0000204525	*M.* × *domestica* cinnamoyl-CoA reductase 1 (LOC103427062), mRNA	6.89
MDP0000206483	*M.* × *domestica* cytokinin hydroxylase-like (LOC114826167), mRNA	6.77
MDP0000219066	*M.* × *domestica* cytochrome P450 CYP72A219-like (LOC103427349), mRNA	6.68
MDP0000737403	*M.* × *domestica* probable mannitol dehydrogenase (LOC103446373), mRNA	6.63

691

692 The five most differentially expressed genes identified after transcriptome analysis of pooled
693 leaf samples of an F1 population between the crabapple hybrid 'Radiant' (containing both
694 trilobatin and phloridzin) and *M.* × *domestica* 'Fuji' (containing only phloridzin).

695

Table 3. Substrate Specificity of Recombinant PGT2 Cloned from *M. toringoides*.

Substrate	Product	Conversion (%)
phloretin	trilobatin	100.0±6.1
3-OH phloretin	sieboldin	58.7±2.3
quercetin	quercetin 3-*O*-glucoside	9.1±0.1
phloridzin	Nd	0
trilobatin	Nd	0
sieboldin	Nd	0
naringenin	Nd	0
cyanidin	Nd	0
caffeic acid	Nd	0
4-coumaric acid	Nd	0
neohesperidin	Nd	0
chlorogenic acid	Nd	0
boiled protein	Nd	0

The products of reactions using UDP-glucoside as the sugar donor and the twelve substrates shown were determined by LC-MS/MS. Conversion % is the amount of product formed relative to the conversion of phloretin to trilobatin which was set at 100%. nd = no products detected. All reactions were performed in triplicate.

FIGURE LEGENDS

Figure 1. Activity-directed Purification of Ph4'-oGT Activity from Flowers of the Crabapple Hybrid 'Adams'.

Active fractions are shown as dark gray bars. (A) Purification by Q-sepharose chromatography. (B) Purification by phenyl sepharose chromatography using pooled fractions from Q-sepharose. (C) Purification by Superdex 75 chromatography using pooled fractions from phenyl sepharose. Protein concentration (280 nm), enzyme activity, pooled fractions and NaCl or $(NH_4)_2SO_4$ gradient in the elution buffer are indicated. (D) SDS-PAGE analysis of the four active fractions after purification by Superdex 75 chromatography are shown in lanes 1–4. M = Premixed Broad protein marker (Takara, Dalian, China). Arrow indicates the band sent for LC-MS/MS analysis.

Figure 2. Genetic Mapping of Trilobatin production in a 'Royal Gala' x Y3 Segregating Population.

(A) The *Trilobatin* locus was mapped near the base of LG7 of Y3 using the IRSC 8K SNP array (Chagné et al., 2012). Genetic locations in centiMorgan (cM) are shown on the left and physical location in base pairs on the right (based on the 'Golden Delicious' doubled-haploid assembly GDDH13 v1.1). The physical locations of three HRM-SNP markers (Figure S1; Table S3) are indicated. (B) The genomic region of the *Trilobatin* locus in the 'Golden Delicious' v1.0p assembly (top) and the doubled-haploid assembly GDDH13 v1.1 (bottom). The physical positions of two UDP-glucosyltransferase genes identified at the locus in each assembly are shown below the gene model. The black arrow corresponds to *PGT2*, the speckled arrow to *PGT3* and the gray arrow to MD07G1280900 (annotated as a suppressor of auxin resistance). N.B. *PGT2* in the doubled-haploid assembly was incorrectly annotated as two truncated gene models MD07G1281000 and MD07G1281100. N.B. HRM1 and 3 amplified on both genes.

Figure 3. Biochemical and Expression Analysis of *PGT1–3*.

(A) Authentic standards of P = phloridzin, T = trilobatin and Pt = phloretin compared with the products formed by recombinant PGT2 from *M. toringoides* (B), PGT3 from *M. sieboldii* (C),

PGT1 from *M.* × *domestica* 'Fuji' (D) and an empty vector control (E) in the presence phloretin and UDP-glucose. Experiments were performed in triplicate and a single representative trace is shown. Expression of *PGT2* (F), *PGT3* (G) and *PGT1* (H) were analyzed by RT-qPCR using gene-specific primers (Table S3) in three *Malus* accessions containing only trilobatin (black bars), three containing trilobatin and phloridzin (white bars), and three containing only phloridzin (gray bars). *MdACTIN* was used as the reference gene. RG = 'Royal Gala'. Data are means (± SE) of three biological replicates from young leaves. Expression is presented relative to *M.* × *domestica* 'Fuji' in (F) and (G) and to *M. toringoides* in (H) (values set as 1).

Figure 4. LC-MS/MS Analysis of Products Formed by *PGT2*.

Base peak plots: (A) mixed standard of phloretin (Pt) and trilobatin (T); (B) *PGT2* + phloretin + UDP-glucose; (C) mixed standard of 3-OH phloretin (3Pt) + sieboldin (S); (D) *PGT2* + 3-OH phloretin + UDP-glucose; Mass spectra for reaction products and standards: (E) fullscan, MS^2 and MS^3 data for phloretin; (F) fullscan, MS^2 and MS^3 data for trilobatin; (G) fullscan, MS^2 and MS^3 data for 3-OH phloretin; and H) fullscan, MS^2 and MS^3 data for sieboldin.

Figure 5. Structural Comparison of the Acceptor Binding Pockets of PGT1–PGT3 Models.

The models were constructed by the iTASSER server. PGT1 (A) and PGT3 (C) residues labelled in red are different from PGT2 (B). The TCP (2,4,5-trichlorophenol) acceptor (shown in gray) from the UGT72B1 co-crystal structure (PDB 2VCE) is superimposed onto the PGT models to highlight the approximate position of the acceptor binding pocket.

Figure 6. Engineering of Trilobatin and Phloridzin Production in *N. benthamiana*.

Nicotiana benthamiana leaves were infiltrated with *Agrobacterium* suspensions containing pHEX2_PGT2, pHEX2_PGT1 or the negative control pHEX2_GUS (each in combination with pHEX2_MdMyb10, pHEX2_MdCHS, pHEX2_MdDBR + pBIN61-p19). Production of trilobatin and phloridzin were analyzed by Dionex-HPLC 7 d post-infiltration. Experiments

were performed in triplicate and a single representative trace is shown. (A) pHEX2_PGT2; (B) trilobatin [T] standard; (C) pHEX2_PGT1; (D) phloridzin [P] standard; (E) negative control pHEX2_GUS.

Figure 7. *PGT2* Expression Levels and Dihydrochalcone Content in Transgenic GL3 Apple Lines.

(A) Relative expression of *PGT2* in fourteen transgenic GL3 lines (#) was determined by RT-qPCR using RNA extracted from young leaves. Expression was corrected against *MdACTIN* and is given relative to the wildtype (WT) GL3 control (value set at 1). Primers and product sizes are given in Table S3. (B) Phenolic compounds were extracted from young leaves and individual DHC content determined by HPLC. Data in panels A and B are presented as mean ± SE, n = 3 biological replicates. Statistical analysis was performed in GraphPad Prism: one-way ANOVA using Dunnett's Multiple Comparison Test *vs* WT. No significant differences in phloridzin or phloretin content were observed. Significantly higher *PGT2* expression and trilobatin content *vs* control are shown at P<0.001 = ***, P<0.01 = **, P<0.05 = *, ns = not significant.

Figure 8. Physiology of *PGT2* Transgenic Lines Grown Under Simulated Field Conditions.

(A) Phenotype at age 18 months of wildtype (WT) and three *PGT2* transgenic lines (#1, #5 and #14); (B) plant height, n = 3 trees per line; (C) the total number of branches per tree, n = 5 trees per line; (D) leaf phenotype; (E) fresh weight per leaf, n = 5 trees per line, 3 leaves per tree; (F) powdery mildew infection phenotype in the young leaves of *PGT2* line #1; and (G) powdery mildew infection rate, n = 5 trees per line, 3 leaves per tree. Data are presented as mean ± SE. Statistical analysis was performed in GraphPad Prism: one-way ANOVA using Dunnett's Multiple Comparison Test *vs* WT. No significant differences in plant height, branch number, leaf weight, or infection rate were observed.

Figure 9. Analysis of Apple Leaf Teas and Trilobatin Iso-sweetness.

(A) Phenolic compounds were extracted from dried leaf material and apple leaf tea prepared

from wildtype (WT) apple and two transgenic GL3 lines over-expressing *PGT2* (#1, #9). Individual phloridzin (P) and trilobatin (T) content was determined by HPLC. Data are presented as mean ± SE, n ≥ **7** for dried leaf material (DM) and n = 3 for apple leaf teas (LT). Statistical analysis was performed in GraphPad Prism: one-way ANOVA using Dunnett's Multiple Comparison Test *vs* WT. Significantly higher than WT at P<0.001 = ***. (B) Iso-sweetness comparison test between trilobatin and sucrose. Each participant was given one trilobatin solution and eight sucrose solutions at different concentrations to taste. Iso-sweetness was established as 35.2 ± 1.66 (R^2 = 0.98). Data presented are mean ± SE, n = 5 participants.

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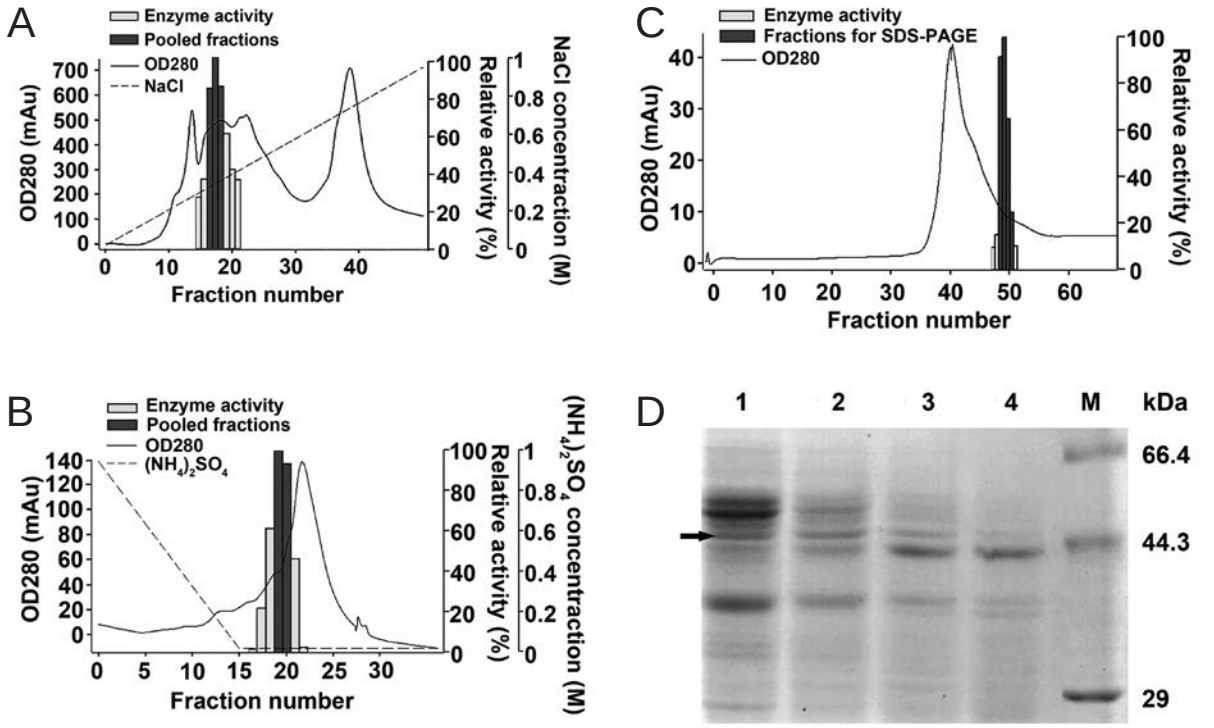

Figure 1. Activity-directed Purification of Ph4'-oGT Activity from Flowers of the Crabapple Hybrid 'Adams'.

Active fractions are shown as dark grey bars. (A) Purification by Q-sepharose chromatography. (B) Purification by phenyl sepharose chromatography using pooled fractions from Q-sepharose. (C) Purification by Superdex 75 chromatography using pooled fractions from phenyl sepharose. Protein concentration (280 nm), enzyme activity, pooled fractions and NaCl or $(NH_4)_2SO_4$ gradient in the elution buffer are indicated. (D) SDS-PAGE analysis of the four active fractions after purification by Superdex 75 chromatography are shown in lanes 1–4. M = Premixed Broad protein marker (Takara, Dalian, China). Arrow indicates the band sent for LC-MS/MS analysis.

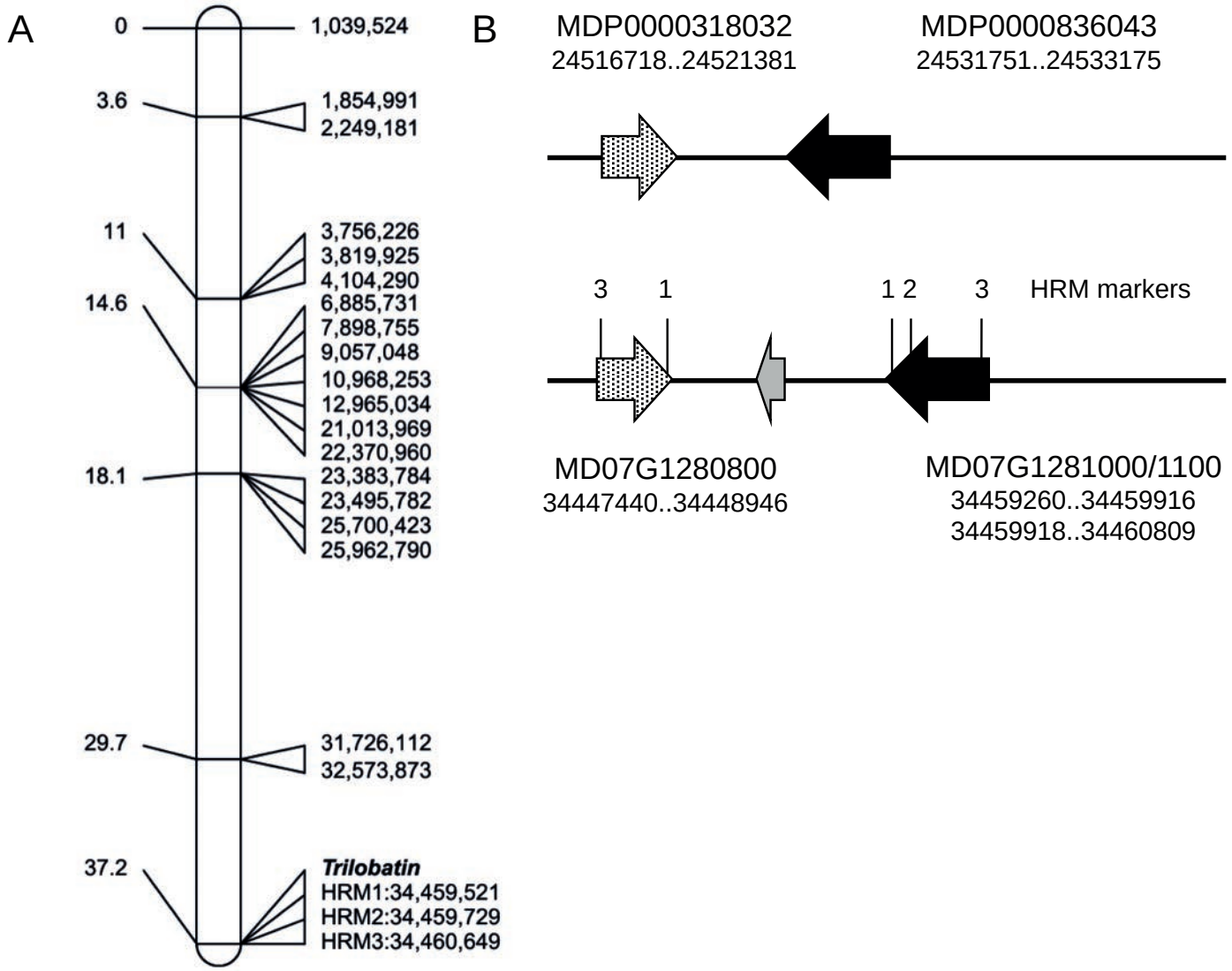

Figure 2. Genetic Mapping of Trilobatin production in a 'Royal Gala' x Y3 Segregating Population.

(A) The *Trilobatin* locus was mapped near the base of LG7 of Y3 using the IRSC 8K SNP array (Chagné et al., 2012). Genetic locations in centiMorgan (cM) are shown on the left and physical location in base pairs on the right (based on the 'Golden Delicious' doubled-haploid assembly GDDH13 v1.1). The physical locations of three HRM-SNP markers (Figure S1; Table S3) are indicated. (B) The genomic region of the *Trilobatin* locus in the 'Golden Delicious' v1.0p assembly (top) and the doubled-haploid assembly GDDH13 v1.1 (bottom). The physical positions of two UDP-glucosyltransferase genes identified at the locus in each assembly are shown below the gene model. The black arrow corresponds to *PGT2*,the speckled arrow to *PGT3* and the gray arrow to MD07G1280900 (annotated as a suppressor of auxin resistance). N.B. *PGT2* in the doubled-haploid assembly was incorrectly annotated as two truncated gene models MD07G1281000 and MD07G1281100. N.B. HRM1 and 3 amplified on both genes.

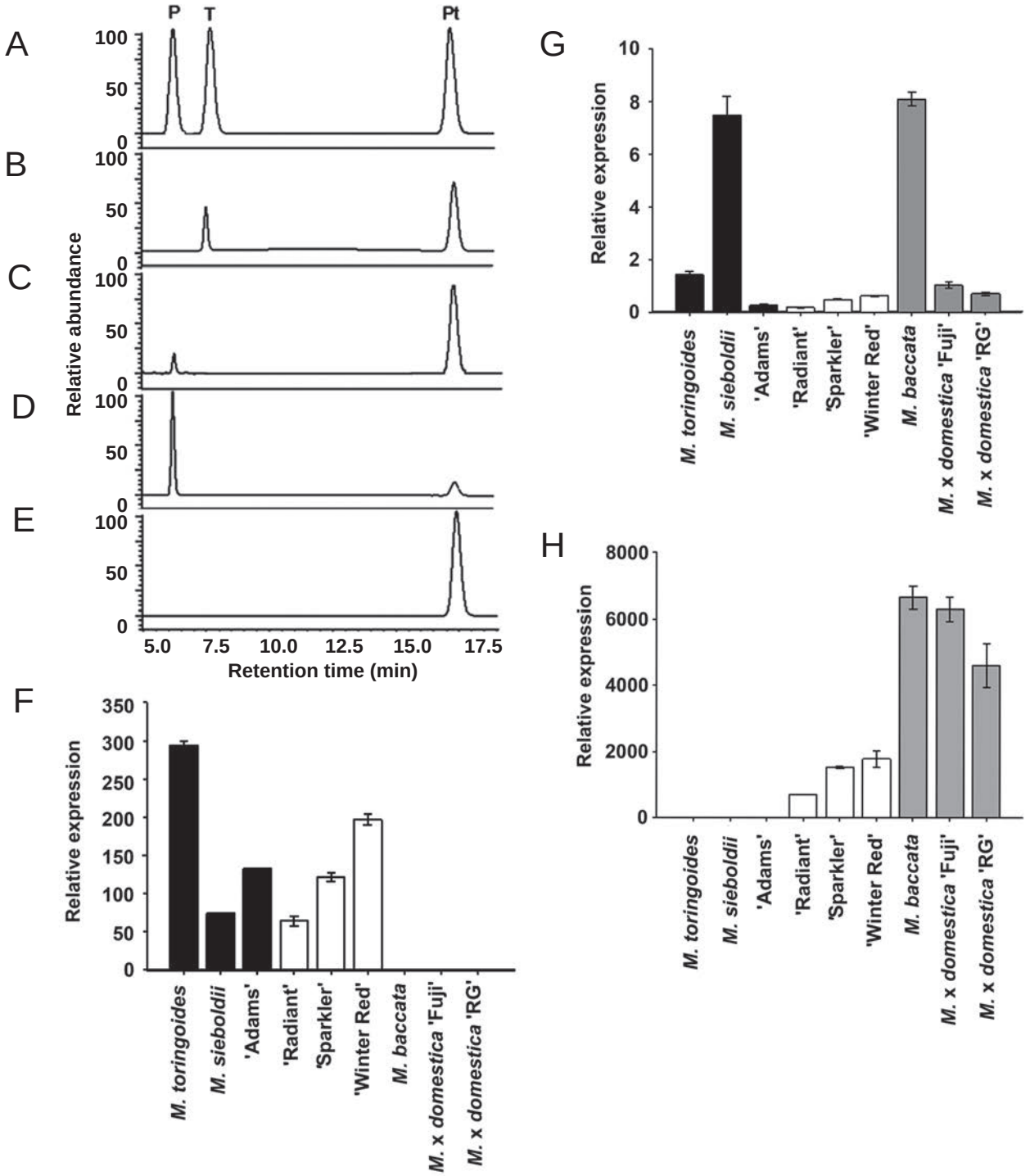

Figure 3. Biochemical and Expression Analysis of *PGT1–3*.
(A) Authentic standards of P = phloridzin, T = trilobatin and Pt = phloretin compared with the products formed by recombinant PGT2 from *M. toringoides* (B), PGT3 from *M. sieboldii* (C), PGT1 from *M.* × *domestica* 'Fuji' (D) and an empty vector control (E) in the presence phloretin and UDP-glucose. Experiments were performed in triplicate and a single representative trace is shown. Expression of *PGT2* (F), *PGT3* (G) and *PGT1* (H) were analyzed by RT-qPCR using gene-specific primers (Table S3) in three *Malus* accessions containing only trilobatin (black bars), three containing trilobatin and phloridzin (white bars), and three containing only phloridzin (gray bars). *MdACTIN* was used as the reference gene. RG = 'Royal Gala'. Data are means (± SE) of three biological replicates from young leaves. Expression is presented relative to *M.* × *domestica* 'Fuji' in (F) and (G) and to *M. toringoides* in (H) (values set as 1).

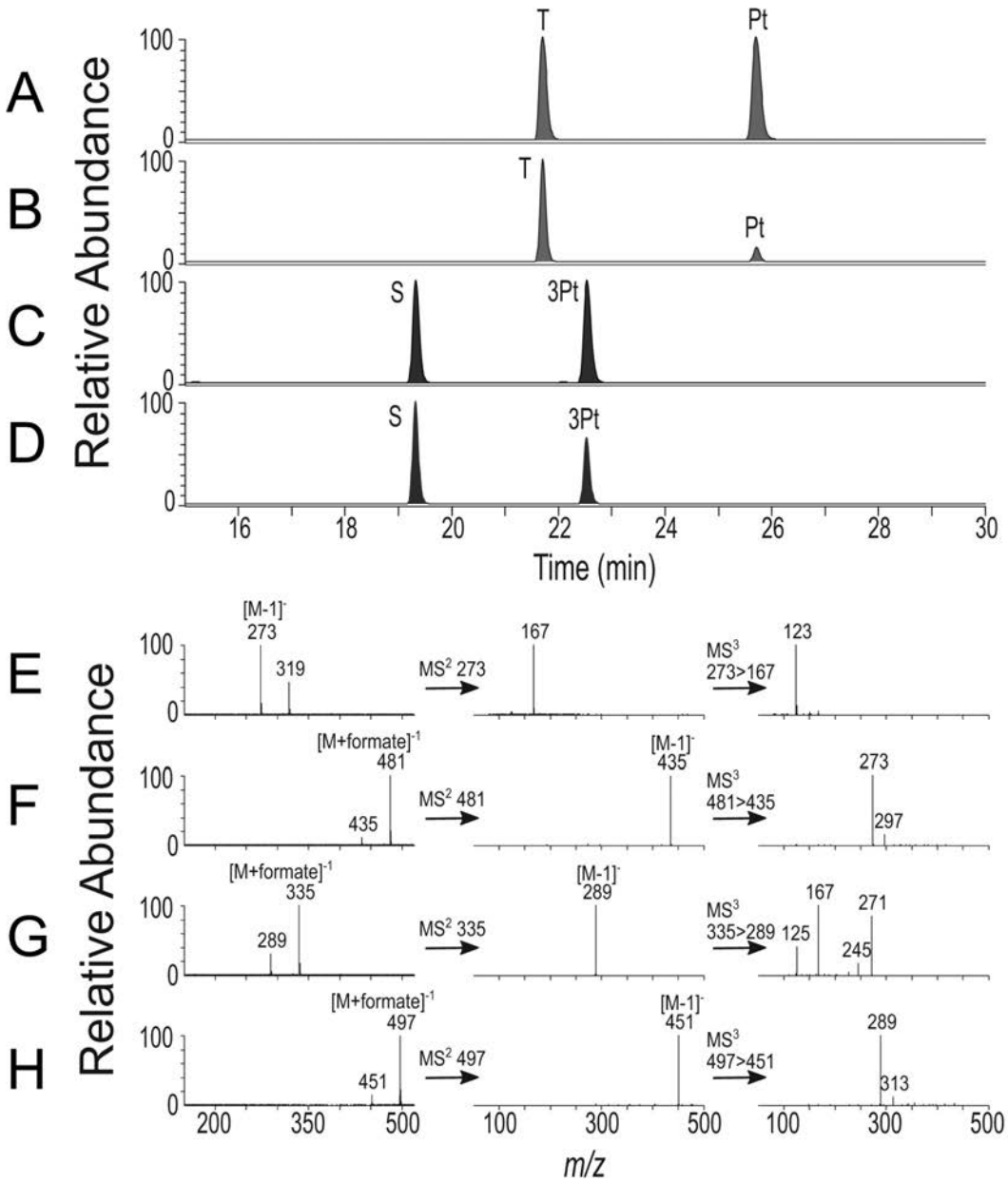

Figure 4. LC-MS/MS Analysis of Products Formed by *PGT2*.

Base peak plots: (A) mixed standard of phloretin (Pt) and trilobatin (T); (B) *PGT2* + phloretin + UDP-glucose; (C) mixed standard of 3-OH phloretin (3Pt) + sieboldin (S); (D) *PGT2* + 3-OH phloretin + UDP-glucose; Mass spectra for reaction products and standards: (E) fullscan, MS^2 and MS^3 data for phloretin; (F) fullscan, MS^2 and MS^3 data for trilobatin; (G) fullscan, MS^2 and MS^3 data for 3-OH phloretin; and H) fullscan, MS^2 and MS^3 data for sieboldin.

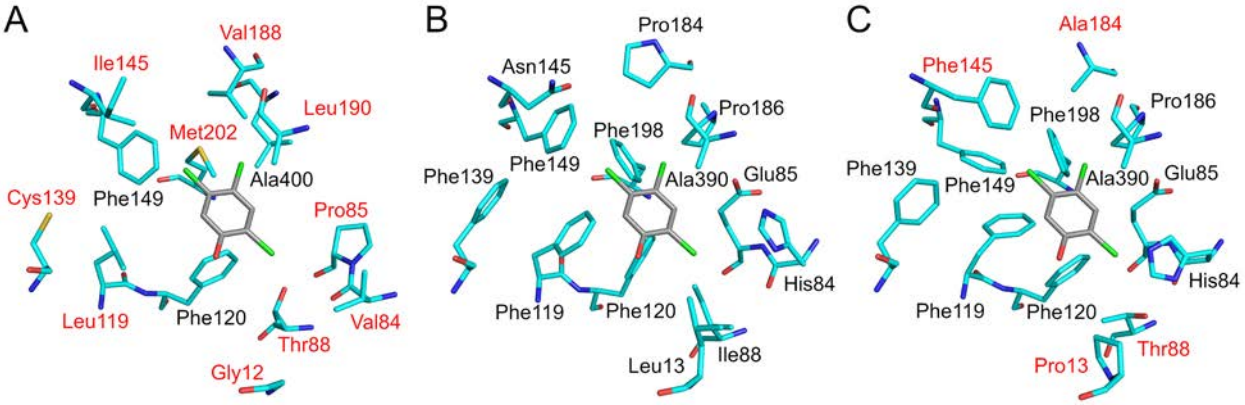

Figure 5. Structural Comparison of the Acceptor Binding Pockets of PGT1–PGT3 Models. The models were constructed by the iTASSER server. PGT1 (A) and PGT3 (C) residues labelled in red are different from PGT2 (B). The TCP (2,4,5-trichlorophenol) acceptor (shown in gray) from the UGT72B1 co-crystal structure (PDB 2VCE) is superimposed onto the PGT models to highlight the approximate position of the acceptor binding pocket.

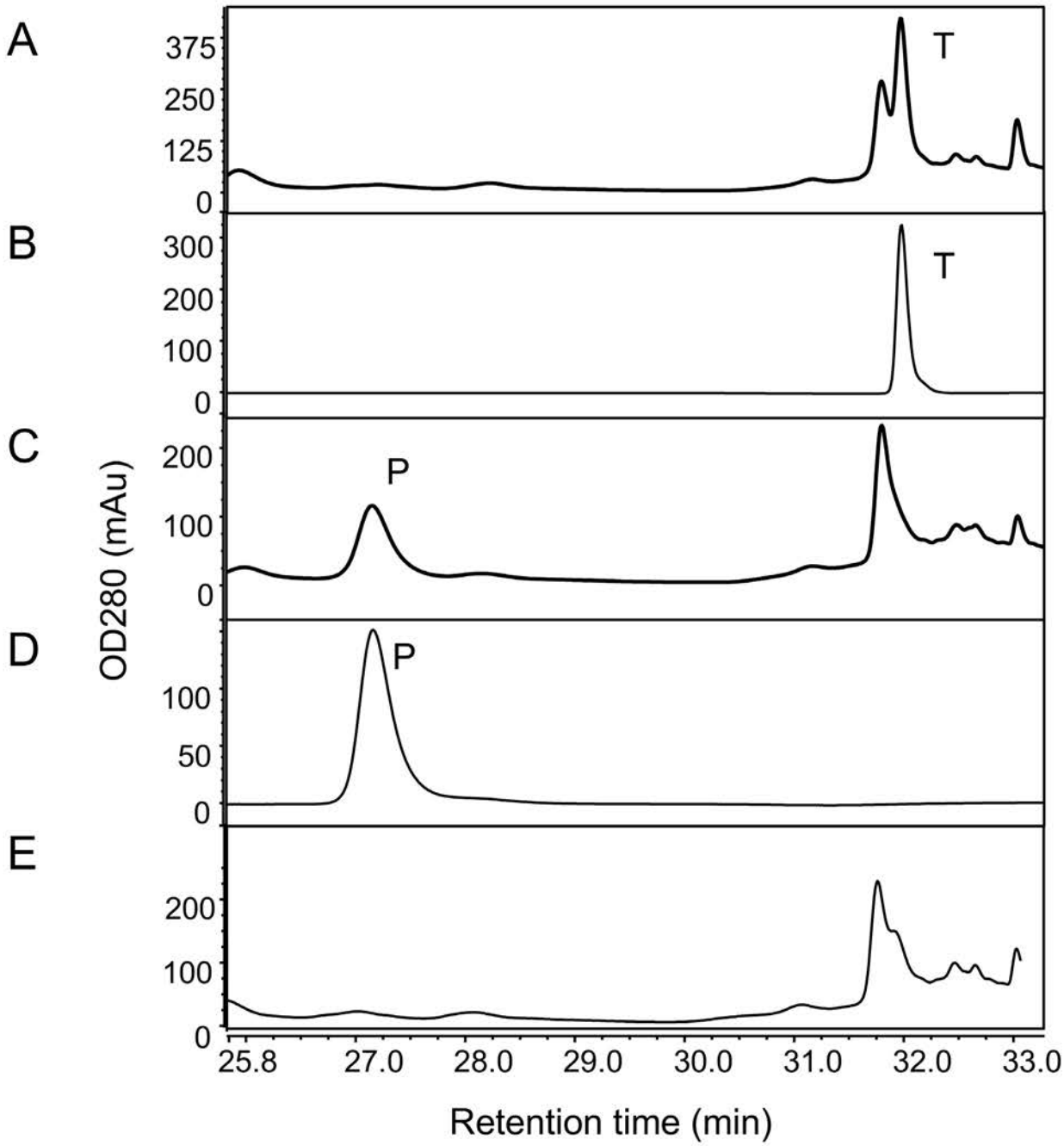

Figure 6. **Engineering of Trilobatin and Phloridzin Production in Tobacco.**

Nicotiana benthamiana leaves were infiltrated with *Agrobacterium* suspensions containing pHEX2_PGT2, pHEX2_PGT1 or the negative control pHEX2_GUS (each in combination with pHEX2_MdMyb10, pHEX2_MdCHS, pHEX2_MdDBR + pBIN61-p19). Production of trilobatin and phloridzin were analyzed by Dionex-HPLC 7 d post-infiltration. Experiments were performed in triplicate and a single representative trace is shown. (A) pHEX2_PGT2; (B) trilobatin [T] standard; (C) pHEX2_PGT1; (D) phloridzin [P] standard; (E) negative control pHEX2_GUS.

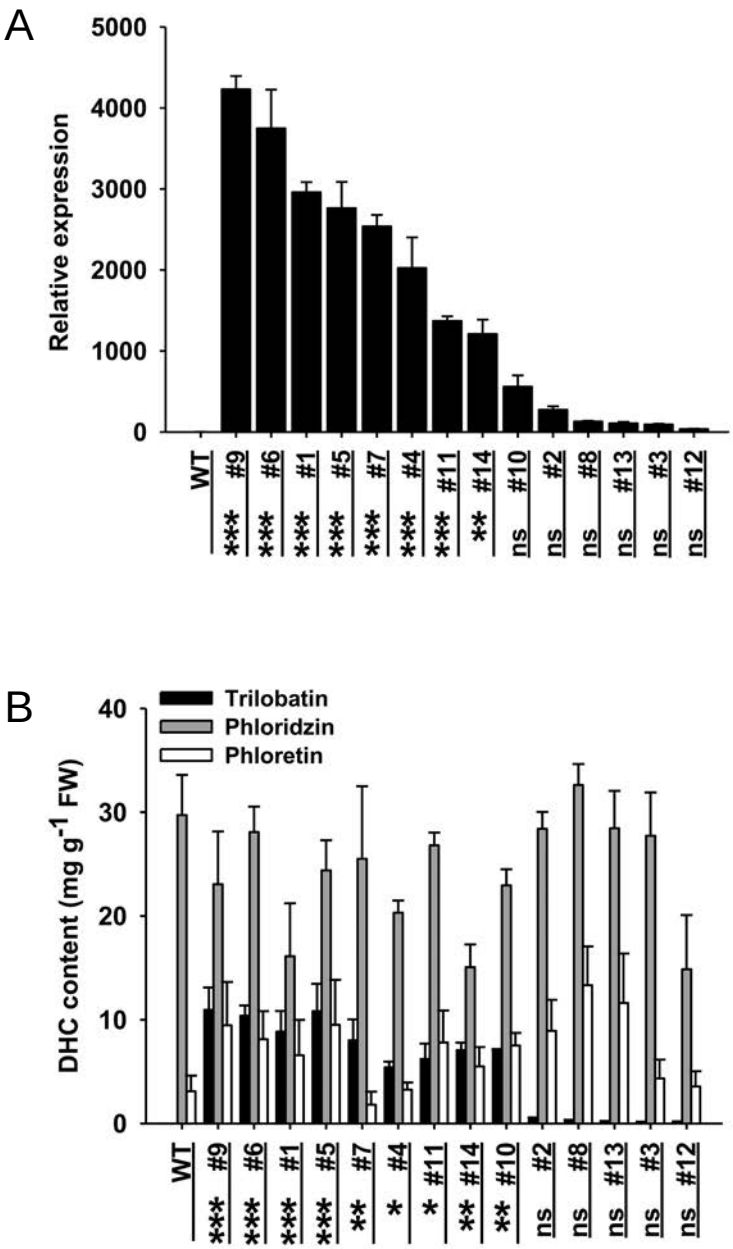

Figure 7. *PGT2* Expression Levels and Dihydrochalcone Content in Transgenic GL3 Apple Lines.

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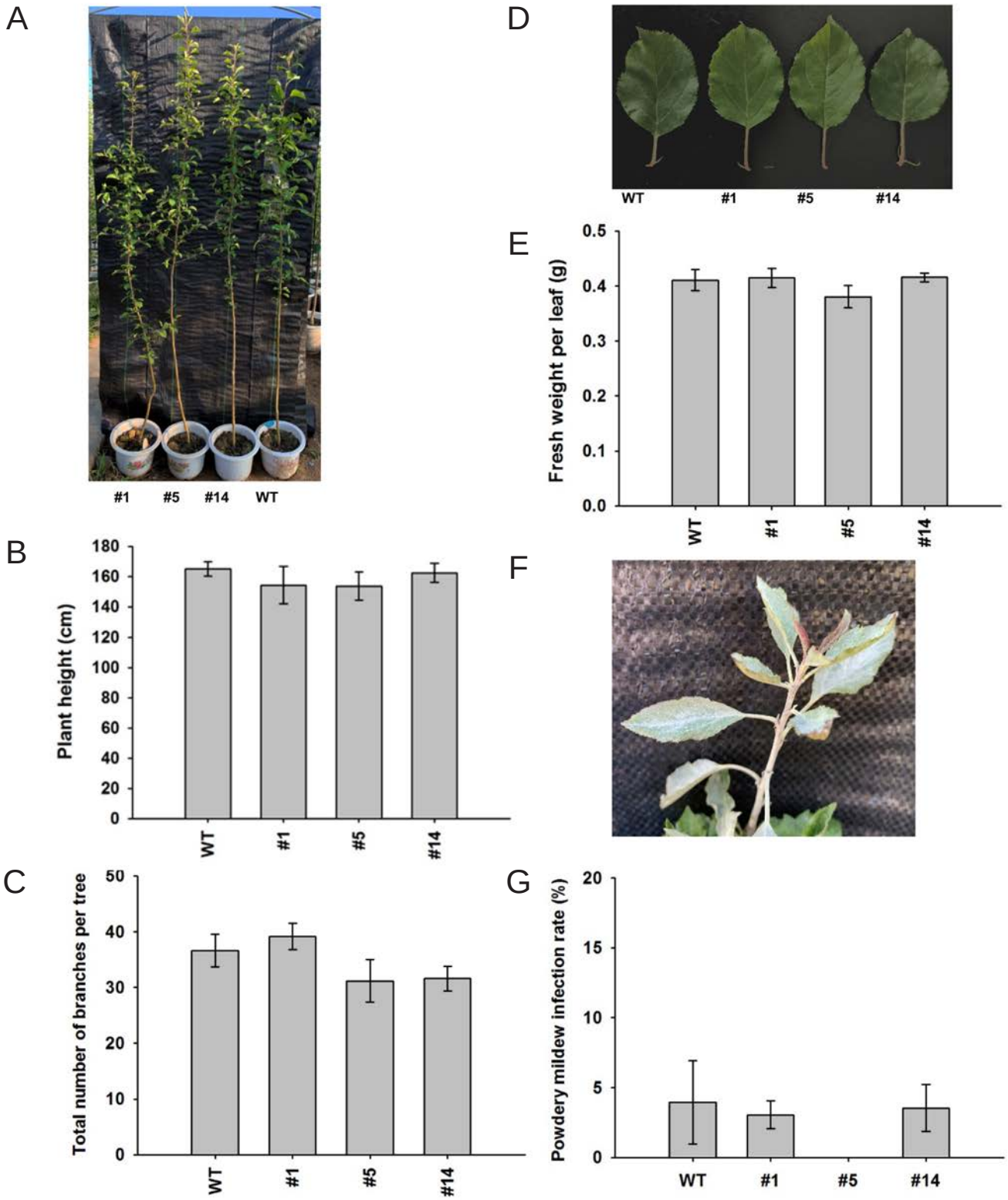

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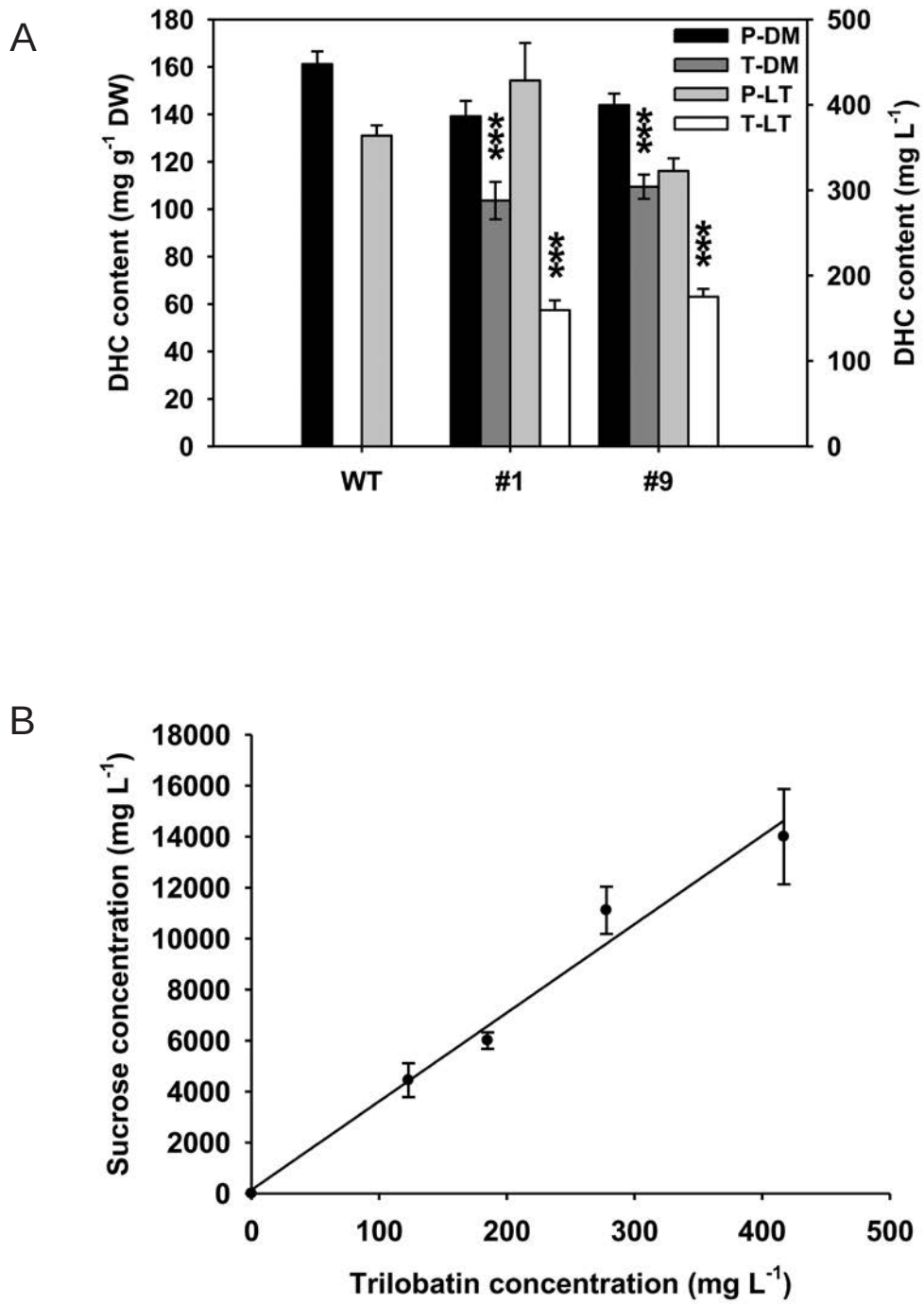

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