Functional Genomics Reveals That a Compact Terpene Synthase Gene Family Can Account for Terpene Volatile Production in Apple[1][W]

Niels J. Nieuwenhuizen[2], Sol A. Green[2]*, Xiuyin Chen, Estelle J.D. Bailleul, Adam J. Matich, Mindy Y. Wang, and Ross G. Atkinson

New Zealand Institute for Plant and Food Research Limited, Private Bag 92169, Auckland, New Zealand (N.J.N., S.A.G., X.C., E.J.D.B., M.Y.W., R.G.A.); and New Zealand Institute for Plant and Food Research Limited, Private Bag 11600, Palmerston North, New Zealand (A.J.M.)

Terpenes are specialized plant metabolites that act as attractants to pollinators and as defensive compounds against pathogens and herbivores, but they also play an important role in determining the quality of horticultural food products. We show that the genome of cultivated apple (*Malus domestica*) contains 55 putative terpene synthase (TPS) genes, of which only 10 are predicted to be functional. This low number of predicted functional TPS genes compared with other plant species was supported by the identification of only eight potentially functional TPS enzymes in apple 'Royal Gala' expressed sequence tag databases, including the previously characterized apple (*E*,*E*)-α-farnesene synthase. In planta functional characterization of these TPS enzymes showed that they could account for the majority of terpene volatiles produced in cv Royal Gala, including the sesquiterpenes germacrene-D and (*E*)-β-caryophyllene, the monoterpenes linalool and α-pinene, and the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene. Relative expression analysis of the *TPS* genes indicated that floral and vegetative tissues were the primary sites of terpene production in cv Royal Gala. However, production of cv Royal Gala floral-specific terpenes and *TPS* genes was observed in the fruit of some heritage apple cultivars. Our results suggest that the apple *TPS* gene family has been shaped by a combination of ancestral and more recent genome-wide duplication events. The relatively small number of functional enzymes suggests that the remaining terpenes produced in floral and vegetative and fruit tissues are maintained under a positive selective pressure, while the small number of terpenes found in the fruit of modern cultivars may be related to commercial breeding strategies.

Terpenes (also referred to as terpenoids or isoprenoids) constitute a large class of plant natural products with highly diversified functionality. Terpenes serve as precursors for the biosynthesis of essential plant metabolites (Croteau et al., 2000), including those involved in growth regulation (GAs, abscisic acid, and strigolactones), membrane stabilization (sterols), photosynthesis (carotenoids and the phytol side chain of chlorophyll), and electron transport coenzymes (ubiquinone and plastoquinone). Primarily, however, terpenes function as specialized plant metabolites that operate as attractants to pollinators or as defensive compounds against pathogens and herbivores (Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002). Terpenes are also important in determining the quality of horticultural food products, including the taste and aroma of wine (Styger et al., 2011) and fruit crops such as *Citrus* spp. (Vora et al., 1983; Maccarone et al., 1998; Aharoni et al., 2004) and strawberry (*Fragaria* spp.; Aharoni et al., 2004).

Terpenes are derived from linear assemblages of prenyldiphosphates, including the C10 monoterpene precursor geranyl diphosphate (GDP), the C15 sesquiterpene precursor farnesyl diphosphate (FDP), and the C20 diterpene precursor geranylgeranyl diphosphate (GGDP). The catalytic conversion of these relatively simple precursors to the diverse array of terpenes seen in nature is carried out by terpene synthase (TPS) enzymes, which have the capacity to direct myriad precursor-binding conformations through subtle variations in their conserved catalytic fold (Yoshikuni et al., 2006; O'Maille et al., 2008; Miller and Allemann, 2012).

TPS gene families have been explored in Arabidopsis (*Arabidopsis thaliana*; Aubourg et al., 2002; Lange and Ghassemian, 2003), grape (*Vitis vinifera*; Martin et al., 2010), poplar (*Populus trichocarpa*; Tuskan et al., 2006), rice (*Oryza sativa*; Goff et al., 2002), and sorghum (*Sorghum bicolor*; Paterson et al., 2009) as well as most recently in cultivated tomato (*Solanum lycopersicum*; Bleeker et al., 2011; Falara et al., 2011). The identification of 44 tomato *TPS* genes concurs with the comparative genome analysis work of Chen et al.

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[2] These authors contributed equally to the article.

* Corresponding author; e-mail sol.green@plantandfood.co.nz.

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(2011), indicating that the plant *TPS* gene family is midsized, with gene numbers ranging from approximately 20 to 150. The only exception so far appears to be the moss *Physcomitrella patens*, which has a single functional *TPS* gene (Chen et al., 2011). This analysis has also extended the phylogenetics used to define the initial TPS subfamilies (TPS-a to TPS-g; Bohlmann et al., 1998; Dudareva et al., 2003; Martin et al., 2004), culminating in the merging of the TPS-e and TPS-f subgroups (comprising gymnosperm and angiosperm *ent*-kaurene genes associated with primary metabolism) and the TPS-h subgroup so far specific to the lycopod *Selaginalla moellendorffii*.

The domestic apple (*Malus domestica*), which has long been recognized by consumers for its flavor, health, and nutritional properties (Harker et al., 2003), is one of the most widely cultivated fruit species in the world's temperate regions. Apple belongs to the Rosaceae family, and while most Rosaceae have a haploid chromosome number of seven, eight, or nine, *Malus* spp., as the result of a relatively recent genome-wide duplication (GWD) event in the Pyreae, have transitioned from nine ancestral chromosomes to 17 chromosomes (Velasco et al., 2010).

Apple fruit produce more than 300 volatile organic compounds (VOCs), including alcohols, aldehyde esters, and ketones (Dimick and Hoskin, 1983; Paillard, 1990; Dixon and Hewett, 2000). Various terpenes have also been identified, although they only contribute a relatively minor component of total VOCs produced (Rapparini et al., 2001; Fuhrmann and Grosch, 2002; Hern and Dorn, 2003; Rowan et al., 2009a). The specific VOC composition in apple depends on several factors, including cultivar, climacteric ethylene production levels, maturity, and environmental conditions (Loughrin et al., 1990; Dixon and Hewett, 2000; Rapparini et al., 2001; Vallat et al., 2005). While the acyclic branched sesquiterpene (*E,E*)-α-farnesene appears to be the predominant terpene volatile associated with ripe fruit (Sutherland et al., 1977; Bengtsson et al., 2001; Hern and Dorn, 2003; Ferreira et al., 2009; Rowan et al., 2009b), various monoterpenes, cyclic sesquiterpenes, and terpene derivatives have also been identified, particularly in floral and vegetative tissues (Takabayashi et al., 1991; Bengtsson et al., 2001; Rapparini et al., 2001; Vallat and Dorn, 2005). Although many of these compounds are constitutively produced in relatively low amounts, a subset of common apple terpenes either are induced in response to insect infestation or have been observed to affect apple pest behavior directly. For example, (*E*)-β-ocimene is induced in immature apple fruit following infestation by codling moth (*Cydia pomonella*) larvae (Hern and Dorn, 2002), while (*E,E*)-α-farnesene exerts a concentration-dependent sexually dimorphic response in adult codling moth, attracting mated females at low dosages and repelling them at high dosages, while only attracting mated males at high dosages (Hern and Dorn, 1999). The relative abundance and diversity of terpenes in floral and vegetative tissues compared with ripe fruit suggests that these compounds are more likely to participate in defense and pollinator attraction roles rather than to attract seed dispersers or contribute to fruit flavor and aroma.

Despite numerous studies on apple terpene volatile production, to our knowledge the only reported apple TPS enzyme to be functionally characterized is that of the α-farnesene synthase (AFS1/MdAFS1; Rupasinghe et al., 2000; Pechous and Whitaker, 2004; Green et al., 2007). The recent availability of the draft sequence assembly of the apple genome (Velasco et al., 2010), combined with a large number of accessible apple ESTs, provided an opportunity to extend the current knowledge on the organization and functional annotation of the apple *TPS* gene family. In this study, we initially survey the volatile terpenes produced in cv Royal Gala and then use available apple genomic and EST information to identify candidate *TPS* genes for functional characterization and quantitative expression analysis. We show that only a small number of the *TPS* genes in the apple genome encode functional TPS enzymes, but these enzymes can account for the diversity of terpenes present in apple. Our results also provide insight into how this small but evolutionarily conserved family was shaped by GWD events and how the range of terpenes has been influenced by selective pressures and commercial breeding strategies.

RESULTS

Phenological and Tissue-Specific Terpene Volatile Production in cv Royal Gala

Terpene volatile production in apple 'Royal Gala' leaves, flowers, and fruit was investigated at various phenological stages by dynamic headspace trapping and gas chromatography-mass spectrometry (GC-MS). Excluding mature leaves, which showed an almost complete absence of volatile terpene production, floral and vegetative tissues produced a range of monoterpene, sesquiterpene, and homoterpene compounds (Fig. 1). Linalool, germacrene-D, and the C11 homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT], assumed to have derived from nerolidol oxidation (Donath and Boland, 1995; Boland et al., 1998), were the most prevalent nonfruit volatiles in cv Royal Gala and showed the most significant variation in their production. Specifically, germacrene-D, (*E*)-DMNT, and to a lesser extent bourbonene, (*E*)-β-caryophyllene, and linalool were the predominant vegetative terpenes, particularly in stipules. Stipules are lateral organs produced in pairs on stem nodes in association with leaves in many angiosperm species (Tyler, 1897), and in cv Royal Gala they have a leaf-like morphology. Linalool and (*E*)-DMNT were also the predominant terpenes in mature flowers and floral buds. The highest floral terpene production was seen in mature flowers, with linalool accounting for 55% to 58% of the total terpenes.

Terpene production in different floral tissues was also investigated in cv Royal Gala (Fig. 2). These results confirmed that linalool and (*E*)-DMNT were the

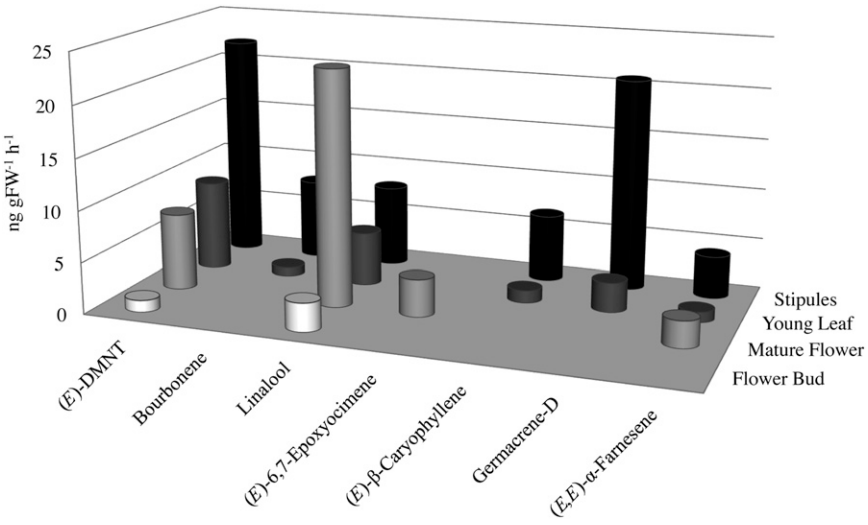

Figure 1. Floral and vegetative terpene emission in cv Royal Gala. GC-MS analysis is shown for the predominant terpene volatiles trapped from the headspace of cv Royal Gala vegetative and floral tissues. Averaged data for biologically replicated analyses are shown. Terpenes produced at a rate less than 0.5 ng g^{-1} fresh weight (FW) h^{-1} are not included. The complete data set is presented in Supplemental Table S1. The bourbonene isomer was not determined, as the retention indices for α- and β-bourbonene are very similar.

predominant floral terpenes. Although linalool was produced at relatively high concentrations in all the floral tissues investigated, the highest production of linalool (in ng g^{-1} fresh weight h^{-1}) was observed from petals and ovaries (accounting for approximately 96% and 28%–41% of the total volatile terpenes, respectively). (*E*)-DMNT, which was absent from petals, was primarily released from the ovaries and pedicles. Floral (*E*)-6,7-epoxyocimene [(5*E*)-2,3-epoxy-2,6-dimethylocta-5,7-diene] and (*E*,*E*)-α-farnesene production were also primarily associated with the ovaries. Volatile production from whole apple fruit during the approximately 150-d period from anthesis to harvest ripe (Fig. 3) showed that, with the exception of 30-d after anthesis (DAA) fruit, terpene concentrations were barely detectable (less than 0.005 ng g^{-1} fresh weight h^{-1}; Supplemental Table S3). In 30-DAA fruit, (*E*)-DMNT was the predominant terpene, accounting for approximately 89% of the total terpenes identified. (*E*,*E*)-α-Farnesene was the only terpene of any significance to be released from intact ripe fruit at 150 DAA, although only at relatively low amounts (approximately 0.046 ng g^{-1} fresh weight h^{-1}).

To investigate whether the skin of whole apple fruit was acting as a physical barrier to the release of terpenes derived from internal tissues, separate volatile analyses on skin, cortex, and seed tissues were carried out (Supplemental Table S4). The results indicated that skin, cortex, and seeds all contribute to the total pool of fruit terpenes identified in cv Royal Gala. The highest terpene production on the basis of fresh weight was observed in 60-DAA seeds, with (*E*)-β-ocimene accounting for approximately 93% of the total terpenes. There was a qualitative increase in the rate of terpene emissions in the separate fruit tissues compared with that in whole fruit, especially over the 60- to 150-DAA period. Notably, the emission rate of (*E*)-DMNT from 60-DAA skin was approximately 45-fold higher than that observed for 60-DAA whole fruit, while the

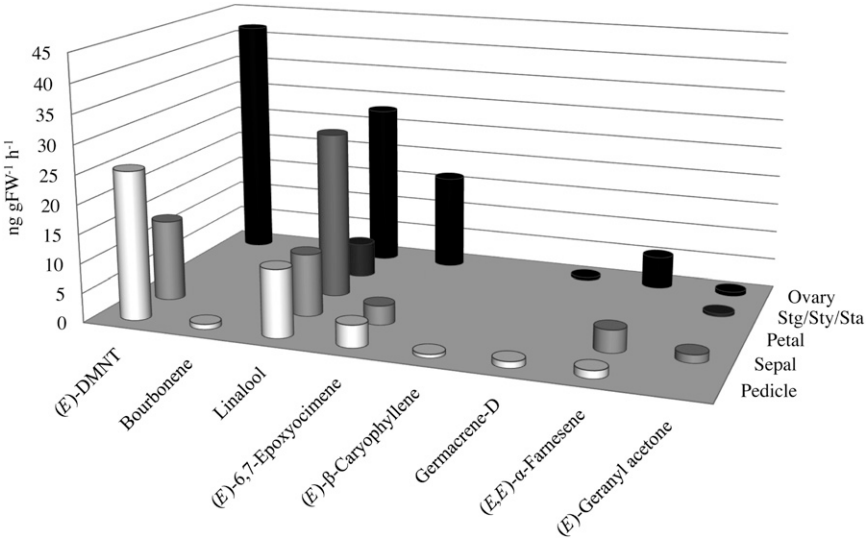

Figure 2. Volatile terpene emission in cv Royal Gala floral tissues. GC-MS analysis is shown for the predominant terpene volatiles trapped from the headspace of cv Royal Gala floral tissues. Stg/Sty/Sta, Combined sample containing stigma, style, and stamen tissue. Averaged data for biologically replicated analyses are shown. Terpenes produced at less than 0.5 ng g^{-1} fresh weight (FW) h^{-1} are not included. The complete data set is presented in Supplemental Table S2.

Figure 3. cv Royal Gala fruit at various stages of development: 30 DAA (A), 60 DAA (B), 90 DAA (C), 120 DAA (D), and 150 DAA (harvest ripe; E). Bars = 1 cm.

emission rates of (*E*,*E*)-α-farnesene and its oxidation product 6-methyl-5-hepten-2-one (Anet, 1972) in ripe fruit (150 DAA) were approximately 36-fold and between 500- and 900-fold higher, respectively, in the skin than in whole apple fruit. Although a concentration-dependent bias (in g^{-1} fresh weight) favoring the skin analyses might explain some of the difference, most of the other terpene compounds produced from the 150-DAA skin remained at low concentrations (Supplemental Table S5).

Considering the prevalence of (*E*)-DMNT in flowers, vegetative tissues, and, to a lesser extent, in unripe fruit, the absence of any detectable (*E*)-DMNT precursor nerolidol in cv Royal Gala is surprising. Although the presence of (*E*)-DMNT indicated that a nerolidol synthase was likely to be active in these tissues, the lack of nerolidol suggests that a highly efficient mechanism for converting nerolidol to DMNT is also operating. It is likely that this process will be mediated by a cytochrome P450 monooxygenase similar to that identified in Arabidopsis homoterpene biosynthesis (Lee et al., 2010).

Organization of the TPS Gene Family in the cv Golden Delicious Genome

Interrogation of the genome sequence of apple 'Golden Delicious' using previously published TPS sequences identified 55 gene models with significant homology to known TPS enzymes (Supplemental Table S6). While putative *TPS* genes were located on 11 of the 17 apple linkage groups (chromosomes), a significant proportion of *TPS* genes were found to colocate on the same chromosome region. However, given the highly heterozygous nature of the apple genome and the challenges this poses for genome sequencing and assembly (Zharkikh et al., 2008), it is difficult to determine accurately what proportion of these clustered genes are the result of gene duplications and what proportion are allelic variants (haplotypic).

A striking feature of the analysis was that only 10 genes were predicted to encode functional TPS enzymes (Table I; Supplemental Fig. S1). This prediction required a TPS open reading frame (ORF) to be of the expected size and organization (i.e. seven exons for TPS-a, TPS-b, and TPS-g and between 13 and 15 exons for TPS-e/TPS-f and TPS-c) and also that it contain structural features such as the Mg^{2+} binding [DDXX(D/E); Christianson, 2006] and (N,D)Dxx(S,T,G)xxxE (NSE/DTE) regions (Cane et al., 1996; Rynkiewicz et al., 2002) required for TPS activity. The remaining 45 genes were categorized as putative pseudogenes, as they either contained multiple deletions or encoded small TPS fragments (possessing one to two exons only), presumably due to being misspliced and/or containing premature stop codons. Three of the predicted pseudogenes also appeared to contain retrotransposon-like insertions (Supplemental Fig. S2).

On the basis of the current apple genome annotation, the TPS subgroup clustering of the cv Golden Delicious *TPS* genes (rationalized on the basis of amino acid sequence homology) showed that the *TPS-a* gene family in apple was the most expanded, with 23 or approximately 40% of the total *TPS* genes identified (Supplemental Table S6). This is consistent with other plant species, including grape, Arabidopsis, and rice (Chen et al., 2011). For the remaining TPS subgroups, six genes representing the TPS-b subgroup, five representing the TPS-c subgroup, and 12 and nine genes representing the TPS-e/TPS-f and TPS-g subgroups, respectively, were also identified. Not surprisingly, the only subgroup with no representative apple genes was the gymnosperm-specific TPS-d subgroup (Bohlmann et al., 1999).

The largest clusters of *TPS* genes occurred on chromosomes 17 and 12, with 13 and 12 genes, respectively, eight genes on chromosome 11, seven genes on chromosome 3, and smaller clusters of five genes on chromosomes 10 and four genes each on chromosomes 5 and 15. Single *TPS* genes were also identified on chromosomes 2, 6, and 7. The identification of *TPS-a* genes on homeologous chromosomes 3 and 11 and *TPS-g* genes on homeologous chromosomes 5 and 10 indicates that the expansion of these subgroups can be attributed to the recent GWD in apple (Velasco et al., 2010).

Table I. *Putative full-length TPS genes in the cv Golden Delicious genome*

Instituto Agrario San Michele all'Adige-annotated gene models (Gene Models 1) encoding potentially functional cv Golden Delicious TPS enzymes were identified following tBLASTn searches of the GDR database with known TPS sequences obtained from the nonredundant protein database at NCBI. For a detailed description of column headings, see Supplemental Table S7.

Name	Gene Model	Predicted Subgroup	Protein Length	Linkage Group	No. of Exons	RRX8W	DDXXD	NSE/DTE	CTP
MdTPS1-GD	MDP0000120176	TPS-a	574	7	7	RPX8W	DDVYD	DDxxDxxxE	No
MdTPS5-GD	MDP0000161084	TPS-a	556	12	7	KPX8W	DDVYE	DDxxExxxE	No
MdTPS15-GD	MDP0000276976	TPS-a	564	11	7	RRX8W	DDIYD	NDxxSxxxE	No
MdTPS16-GD	MDP0000295452	TPS-a	591	5	7	No	DDIYD	NDxxSxxxE	Yes
MdTPS26-GD	MDP0000297049	TPS-b	616	17	7	RRX8W	DDMYD	DDxxTxxxE	Yes
MdTPS30-GD[a]	MDP0000147908	TPS-c	810	11	15	No	GSHFRE	LSSPEHEQL	No
MdTPS46-GD	MDP0000828007	TPS-e/TPS-f	733	15	13	No	DDFFD	NDxxGxxxE	No
MdTPS50-GD	MDP0000298903	TPS-g	535	10	7	No	DDIFD	DDxxSxxxE	No
MdTPS52-GD	MDP0000398063	TPS-g	554	5	7	No	DDIFD	DDxxSxxxE	No
MdTPS54-GD	MDP0000562538	TPS-g	568	5	7	No	DDIFD	DDxxSxxxE	No

[a]Probable type II diterpene synthase, which uses a protonation-dependent catalytic mechanism (Wendt and Schulz, 1998) and does not require DDXXD or NSE/DTE motifs common to type I TPSs.

Identification of cv Royal Gala TPSs in EST Databases

To identify expressed TPSs in apple, BLAST searches of National Center for Biotechnology Information (NCBI) GenBank EST databases and an in-house *Malus* EST database (Newcomb et al., 2006), which now includes approximately 1.2M cv Royal Gala Next Generation Sequences (R.D. Crowhurst, unpublished data), were carried out. In addition to the previously characterized apple α-farnesene synthase (now termed *MdAFS-RG1*; accession no. AY787633) and a putative cv Royal Gala *ent*-kaurene synthase (accession no. JQ281521), six new full-length TPS ORFs were identified (Supplemental Table S7). A partial TPS (termed MdCDS-RG1) encoding a putative copalyl diphosphate (CDP) synthase gene (TPS-c subgroup) was also identified with significant homology to the predicted Golden Delicious TPS-c gene *MdTPS30-GD*. However, the predicted ORF of MdCDS-RG1 appeared to be missing approximately 80 N-terminal amino acids. Although closer examination of the cv Golden Delicious gene models identified another partial *CDS* gene (*MdTPS33-GD*) that included the missing N-terminal amino acids, we have so far been unable to amplify a full-length CDP synthase from cv Royal Gala. Confirmation of the TPS subgroup placement for the cv Royal Gala and Golden Delicious TPS ORFs was also obtained using phylogenetic analysis versus selected nonapple TPS sequences (Fig. 4). This analysis also confirmed that none of the cv Royal Gala TPS enzymes clustered within the TPS-c subgroup, which is specific to CDP synthases.

The proportion of pseudogenes to *TPS* genes predicted to encode functional enzymes in apple (45:10) is higher than expected, considering that plants with comparably sized *TPS* gene families, including Arabidopsis (Aubourg et al., 2002) and tomato (Falara et al., 2011), possess mostly functional or potentially functional enzymes. However, the eight functional and/or potentially functional *TPS* genes identified in cv Royal Gala is in agreement with the number predicted from the in silico cv Golden Delicious genome analysis. Given the relatively high degree of amino acid sequence and structural conservation in plant TPS enzymes (Aubourg et al., 2002), we are confident that the majority of full-length apple TPS enzymes have now been identified.

Functional Characterization of Full-Length TPSs from cv Royal Gala

To investigate their likely in planta activity, the seven full-length TPS ORFs from cv Royal Gala that encoded monoterpene synthase and sesquiterpene synthase enzymes likely to be associated with terpene volatile production were initially expressed in tobacco (*Nicotiana benthamiana*) leaves and analyzed for terpene production. On the basis that *ent*-kaurene was not identified as a volatile terpene in any of the cv Royal Gala tissues and that *ent*-kaurene synthases, along with CDP synthases, have a primary metabolic role in GA biosynthesis (Yamaguchi et al., 1996; Hedden and Thomas, 2012), MdEKS-RG-1 was not characterized in planta.

For the TPS enzymes associated with volatile terpene emission in cv Royal Gala, four sesquiterpene synthase enzymes (Fig. 5) and three monoterpene synthase enzymes (Fig. 6) were identified. Consistent with previous experiments conducted in vitro where (*E*,*E*)-α-farnesene was the major product, *MdAFS-RG1* produced (*E*,*E*)-α-farnesene (95.5%) and (*Z*,*E*)-α-farnesene (4.5%) in planta (Fig. 5A). Two cyclic sesquiterpene synthases were also identified: MdCAR-RG1 (Fig. 5B), which synthesized (*E*)-β-caryophyllene (96.3%) with smaller amounts of α-caryophyllene (3.7%), and MdGDS-RG1 (Fig. 5C), which synthesized germacrene-D (85.3%) with smaller quantities of (*E*)-β-caryophyllene (14.7%). MdNES-RG1 produced a small peak of (*E*)-nerolidol (Fig. 5D) and is likely to be the source of floral and leaf-derived DMNT in cv Royal Gala resulting from the breakdown of (*E*)-nerolidol.

For the monoterpene synthase enzymes, MdPIN/CAM-RG1(Fig. 6A) was the only multiproduct monoterpene synthase enzyme, synthesizing camphene (48.5%)

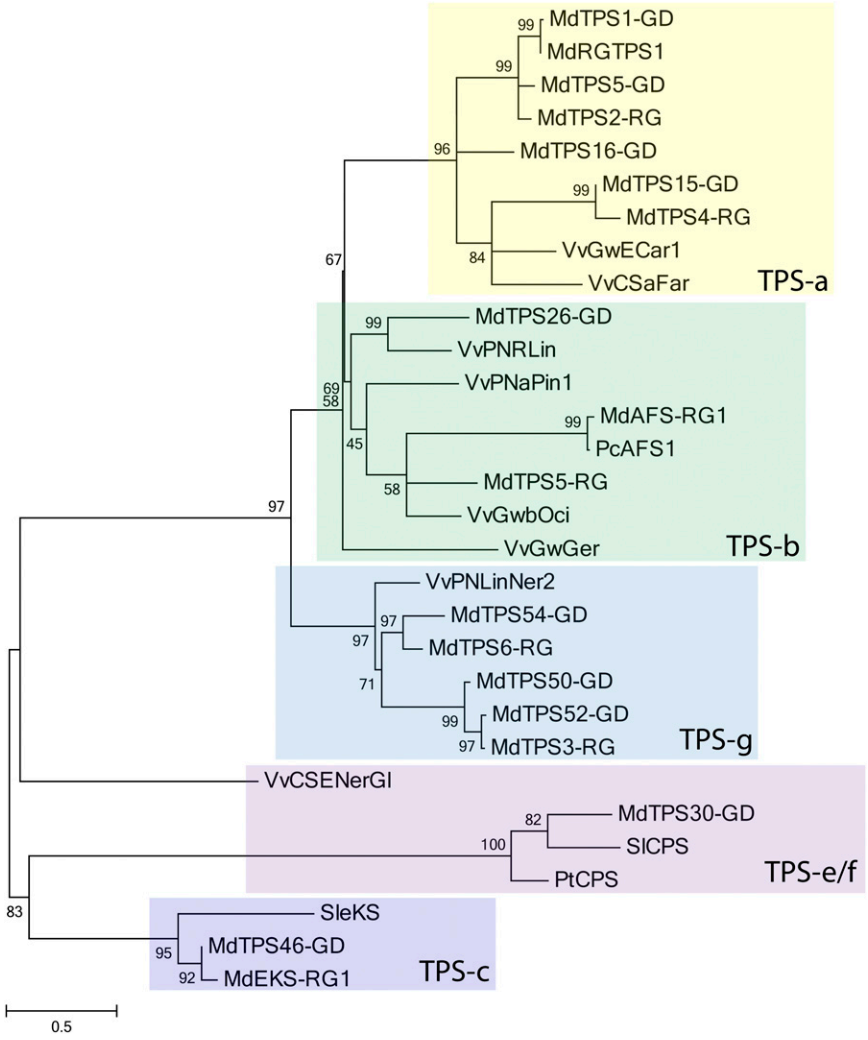

Figure 4. Phylogeny of the apple TPS family. Maximum likelihood analysis is shown for the predicted full-length TPSs from cv Royal Gala (RG) and cv Golden Delicious (GD) apple with selected full-length grape, poplar, pear, and tomato TPSs. Bootstrap values are shown as a percentage of 100 replicates. The scale bar represents 0.5 substitutions per site. Abbreviations for previously identified and/or functionally annotated TPS enzymes, including GenBank accession numbers in parentheses, are as follows: grape VvGwECar1 = (*E*)-β-caryophyllene synthase (ADR74192), VvCSaFar = (*E,E*)-α-farnesene synthase (ADR74198), VvPNRLin = (*3R*)-linalool synthase (ADR74209), VvPNaPin1 = pinene synthase (ADR74202), VvGwbOci = (*E*)-β-ocimene synthase (ADR74204), VvGwGer = geraniol synthase (ADR74217), VvPNLinNer2 = (*3S*)-linalool/(*E*)-nerolidol synthase (ADR74211), and VvCSENerGl = (*E*)-nerolidol/(*E,E*)-geranyl linalool synthase (ADR74219); apple MdRGAFS1 = (*E,E*)-α-farnesene synthase (AAX19772) and MdRGEKS = *ent*-kaurene synthase (AFG18184); pear PcAFS1 = (*E,E*)-α-farnesene synthase (AAT70237); tomato SleKS = *ent*-kaurene synthase (AEP82778) and SlCPS = CDP synthase (BAA84918); poplar PtCPS = CDP synthase (EEE81383).

and α-pinene (43.8%) as its major products, with smaller amounts of β-pinene (4.8%), limonene (1.5%), and β-myrcene (1.3%); MdOCS-RG1 (Fig. 6B) and MdLIS-RG1 (Fig. 6C) produced (*E*)-β-ocimene and linalool, respectively, as single products.

No significant amounts of terpenes were identified in vector-only control inoculations, indicating that the terpenes identified in the tobacco leaf analyses were the results of the transiently expressed apple TPS enzymes.

For additional confirmation of cv Royal Gala TPS function, the above enzymes (with the exception of the previously characterized MdAFS-RG1) were also overexpressed in *Escherichia coli* and the volatiles were solvent extracted and analyzed by GC-MS. Active recombinant proteins were obtained for all the apple TPS enzymes except MdNES-RG1. In agreement with the tobacco transient expression analysis, the recombinant monoterpene synthase enzyme activity results (Supplemental Fig. S3) showed that linalool and (*E*)-β-ocimene were produced as single products from MdLIS-RG1 and MdOCS-RG1, respectively, while α-pinene (32%) and camphene (26.3%) were the predominant products from MdPIN/CAM-RG. The only notable difference was the identification of several hydroxylated terpenes and terpene derivatives in the MdPIN/CAM-RG1 solvent extractions that were not seen in the headspace analysis, a likely consequence of their decreased volatility. These included borneol (17.4%), a pinene derivative tentatively assigned as 2-pinanol (4.1%), and a probable sabinene derivative, possibly sabinene hydrate (4.1%).

The in vitro results for the two active recombinant sesquiterpene synthase enzymes, MdGDS-RG1 and MdCAR-RG1 (Supplemental Fig. S4), were also very similar to those obtained for the transient expression analysis. MdGDS-RG1 predominantly synthesized germacrene-D (77.7%) with smaller amounts of a number of additional compounds, including (*E*)-β-caryophyllene (5.9%) and a germacrene-D derivative, possibly germacrene-D-4-ol (5.8%). MdCAR-RG1 predominantly synthesized (*E*)-β-caryophyllene (91.4%) with smaller amounts of α-caryophyllene (3.6%), α-copaene (2.7%), and germacrene-D (2.3%). Notably,

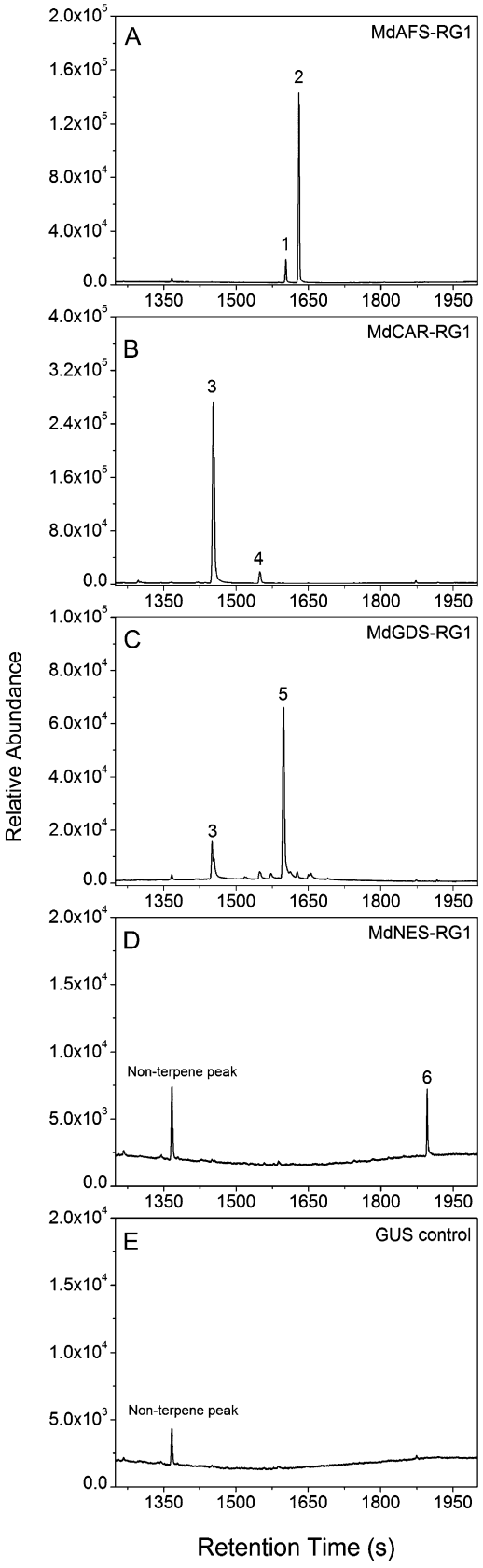

Figure 5. In planta analysis of cv Royal Gala sesquiterpene synthase enzyme activity. Full-length sesquiterpene synthase enzymes were cloned into the pHEX2 plant transformation vector and transiently expressed in tobacco leaves. Selected ion (*m/z* 93) headspace GC-MS profiles are as follows. A, MdAFS-RG1: peak 1, (*Z,E*)-α-farnesene; peak 2, (*E,E*)-α-farnesene. B, MdCAR-RG1: peak 3, (*E*)-β-caryophyllene; peak 4, α-caryophyllene. C, MdGDS-RG1: peak 3, (*E*)-β-caryophyllene; peak 5, germacrene-D. D, MdNES-RG1: peak 6, (*E*)-nerolidol. E, GUS: empty vector control. Products were confirmed by comparison with authentic standards and/or GC-MS retention indices.

the recombinant linalool synthase (MdLIS-RG1) was also shown to produce nerolidol in the presence of FDP, suggesting that this enzyme could also potentially contribute to nerolidol production in cv Royal Gala. None of the remaining apple TPS enzymes showed any bifunctional FDP/GDP substrate specificity in vitro.

Enantioselective GC-MS analysis was also carried out to establish the main terpene enantiomers produced in cv Royal Gala flowers, stipules, and young leaves and also from the recombinant TPS enzymes. Monoterpene enantiomeric analysis (Supplemental Fig. S5) indicated that MdLIS-RG1 exclusively synthesized the (*S*)-(+)-linalool enantiomer in vitro, while the predominant enantiomers produced for MdPIN/CAM-RG1 in vitro were (*R*)-(+)-α-pinene, (*R*)-(+)-β-pinene, and (*R*)-(+)-limonene. There was good correlation between the pinene and limonene enantiomers produced from MdPIN/CAN-RG1 and those identified in the cv Royal Gala flowers, young leaves, and stipules (Supplemental Fig. S5, C–E). However, these tissues produced a mix of (*S*)-(+)-linalool and (*S*)-(−)-linalool in varying ratios, suggesting the presence of a second linalool synthase in apple capable of producing the (*S*)-(−)-linalool enantiomer. Sesquiterpene enantiomeric analysis (Supplemental Fig. S6) indicated that (−)-germacrene-D was produced in cv Royal Gala tissues and by the MdGDS-RG1 and MdCAR-RG1 enzymes. However, due to the lack of an appropriate enantiomeric standard, it was also not possible to determine the enantiomeric specificity of (*E*)-β-caryophyllene in cv Royal Gala. (*S*)-(*E*)-Nerolidol was produced by the bifunctional MdLIS-RG1 enzyme. As with the previous cv Royal Gala headspace volatile analyses, nerolidol was not identified in any of the solvent extracts, so further comparisons were not possible.

Together, these results indicate that the above full-length *TPS* complementary DNAs (cDNAs) can account for the majority of the terpenes produced in cv Royal Gala. The only disparities were the absence of TPS enzymes capable of synthesizing (*S*)-(−)-linalool and bourbonene. It is likely that bourbonene, which is most abundant in the stipules and to a lesser extent in young leaves (Fig. 1), is derived from an as yet unidentified sesquiterpene synthase that is either specific to these particular tissues in cv Royal Gala or is induced under specific conditions.

The identification of seven functional and one potentially functional (i.e. *MdEKS-RG1*) *TPS* genes in cv Royal Gala supports the cv Golden Delicious in silico genome

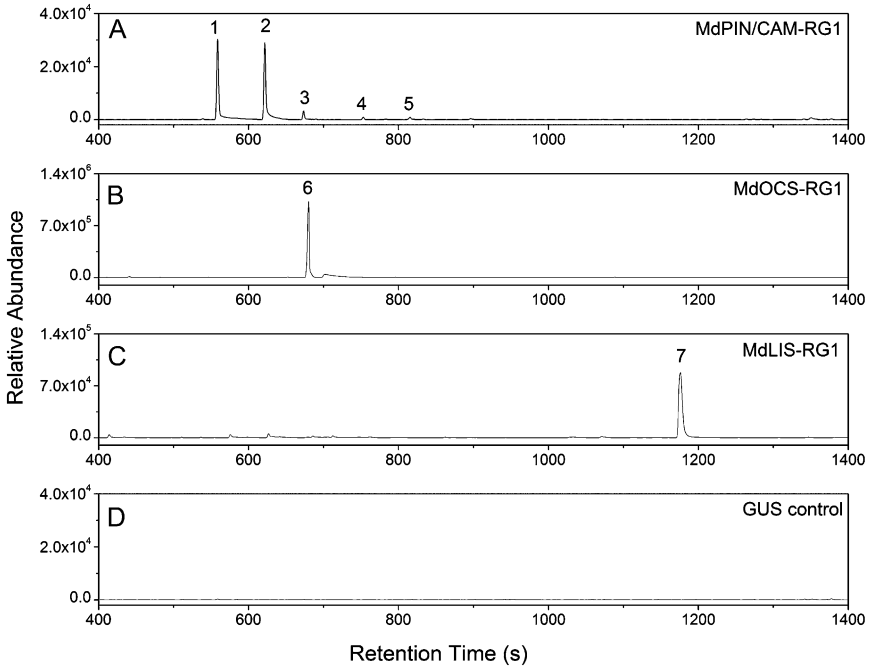

Figure 6. In planta analysis of cv Royal Gala monoterpene synthase enzyme activity. Full-length monoterpene synthase enzymes were cloned into the pHEX2 plant transformation vector and transiently expressed in tobacco leaves. Selected ion (*m/z* 93) headspace GC-MS profiles are as follows. A, MdPIN/CAM-RG1: peak 1, α-pinene; peak 2, camphene; peak 3, β-pinene; peak 4, β-myrcene; peak 5, limonene. B, MdOCS-RG1: peak 6, (*E*)-β-ocimene. C, MdLIS-RG1: peak 7, linalool. D, GUS: empty vector control. Products were confirmed by comparison with authentic standards and/or GC-MS retention indices.

findings, while the above in planta data correlate with the qualitative range of terpenes identified in cv Royal Gala and also in other apple cultivars (Takabayashi et al., 1991; Bengtsson et al., 2001; Rapparini et al., 2001; Vallat and Dorn, 2005). Taken together, these findings support our contention that the number of *TPS* genes encoding functional enzymes in apple is small compared with that in other plant species (Chen et al., 2011).

TPS Gene Expression in cv Royal Gala

Relative gene expression analysis was conducted on all genes encoding functional TPS enzymes and the two putative cv Royal Gala *di-TPS* genes (*MdEKS-RG1* and *MdCDS-RG1*) in floral and vegetative tissues (Fig. 7) and fruit (Fig. 8). *MdLIS-RG1* was the most highly expressed gene in flowers, leaves, and stipules. In ovaries and pedicels of cv Royal Gala flowers, high rates of *MdLIS-RG1* mRNA accumulation correlated with high linalool production (Fig. 2). However, comparatively low levels of *MdLIS-RG1* were observed in petals, which are one of the highest linalool-producing tissues in cv Royal Gala (Fig. 2). The expression of an (*S*)-(−)-linalool synthase in petals might account for this discrepancy. Elevated rates of *MdCAR-RG1* and *MdGDS-RG1* mRNA accumulation were also observed in stipules, which were the primary sources of (*E*)-β-caryophyllene and germacrene-D production in cv Royal Gala. Similarly, the higher accumulation of *MdNES-RG1* transcript in pedicels correlated with the comparatively high amounts of DMNT (Fig. 2) produced in this tissue. For the *di-TPS* genes, *MdEKS-RG1* transcript was highest in flower buds while *MdCDS-RG1* was predominantly expressed in the stamens.

In fruit, mRNA levels for the different *TPS* genes (Fig. 8) were generally lower than those for the equivalent genes in floral and vegetative tissues. The linalool and nerolidol synthase-encoding genes (*MdLIS-RG1* and *MdNES-RG1*, respectively) were the most highly expressed *TPS* genes in fruit. Expression of *MdLIS-RG1* and *MdNES-RG1* was primarily confined to the skin and cortex of young fruit (30 DAA), although a small peak of *MdNES-RG1* expression was also observed in 90-DAA skin (Fig. 8). A slight increase in (*E,E*)-α-farnesene synthase (*MdAFS1-RG1*) transcript was also observed between 120 and 150 DAA, as was a small peak of (*E*)-β-ocimene synthase (*MdOCS-RG1*) expression at 60 DAA. The fruit *di-TPS* expression analysis showed that *MdEKS-RG1* transcript levels peaked at 30 and 90 DAA in the cortex and, with the exception of a peak in seed expression at 150 DAA, remained relatively constant during fruit maturation in both seeds and skin. A peak of *MdCDS-RG1* (putative CDP synthase) expression was also observed in 60-DAA seeds, while a gradual increase in *MdCDS-RG1* cortex expression between 90 and 150 DAA was also apparent. The lack of detectable *ent*-kaurene in any of the GC-MS headspace volatile analyses indicates that *ent*-kaurene flux is predominantly toward GA biosynthesis in apple.

The constitutive low levels of TPS expression in apple are in agreement with the low volatile production from whole apple fruit (Supplemental Table S3). However, increased linalool and nerolidol production in 30-DAA fruit, ocimene production in 60-DAA seeds, and (*E,E*)-α-farnesene production in harvest-ripe fruit (Fig. 2) all correlate with increases in expression levels of the equivalent *TPS* genes.

TPS gene expression was also investigated in cv Royal Gala root tissue (Supplemental Fig. S7) and

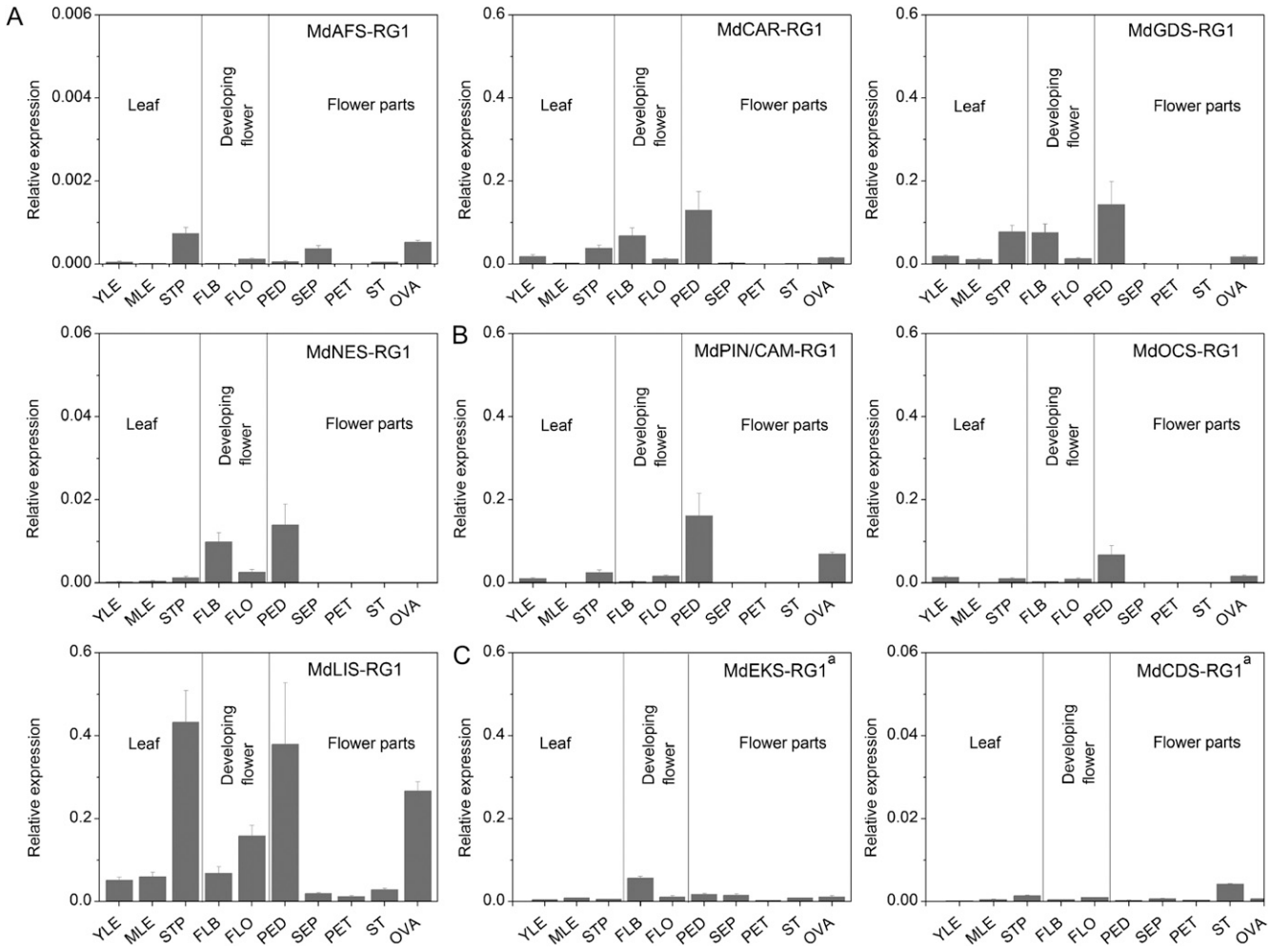

Figure 7. TPS gene expression in cv Royal Gala flowers and leaves. Expression profiles are shown for sesquiterpene (A), monoterpene (B), and diterpene (C) synthases in different flower and leaf tissues. [a]*MdEKS-RG1* and *MdCDS-RG1* have not been functionally verified but are predicted to encode the respective cv Royal Gala *ent*-kaurene and CDP synthases. YLE, Young leaf; MLE, mature leaf; STP, stipule; FLB, flower bud; FLO, fully opened flower; PED, pedicel; SEP, sepal; PET, petal; ST, stigma, style, and stamen; OVA, ovary. The data were analyzed using the target-reference ratio calculated with the LightCycler 480 software, enabling a comparison of the level of expression of different gene family members compared with the *EF1α* reference gene considered stable and unchanging in the different tissues during development. The specific primers used for each gene are detailed in Supplemental Table S7. Data are presented as means ± SE ($n = 4$).

showed that the pinene/camphene synthase (*MdPIN/CAM-RG1*) gene was the most highly expressed. Lower levels of expression were also observed for the linalool synthase (*MdLIS-RG1*), the (*E,E*)-α-farnesene synthase (*MdAFS-RG1*), and the putative *ent*-kaurene synthase (*MdEKS-RG1*).

TPS Expression in the Fruit of Heritage Apple Cultivars

In the ripe fruit of many different apple cultivars including cv Royal Gala, terpene production appears to be restricted to the sesquiterpene α-farnesene (Sutherland et al., 1977; Bengtsson et al., 2001; Hern and Dorn, 2003; Ferreira et al., 2009; Rowan et al., 2009a). The expression of terpene genes typically found in apple flowers and leaves might be expected to introduce new flavor notes into fruit. Five heritage varieties with unusual spicy, nutty, or tangy flavor notes were screened for *TPS* gene expression and terpene volatile production. Volatile production was assessed in skin from these varieties and cv Royal Gala using solvent extraction to maximize the chances of detecting low-level terpene alcohol production.

GC-MS analysis (Table II) showed that cv Royal Gala was a comparatively low terpene producer compared with the five heritage cultivars. Interestingly, in cv Royal Gala, solvent extraction analysis showed that (*E,E*)-farnesol was the predominant accumulated terpene (approximately 84% of total terpenes) and not (*E,E*)-α-farnesene, which only accounted for approximately 9% of the total terpenes. In the other cultivars, (*E,E*)-α-farnesene was by far the most prevalent terpene,

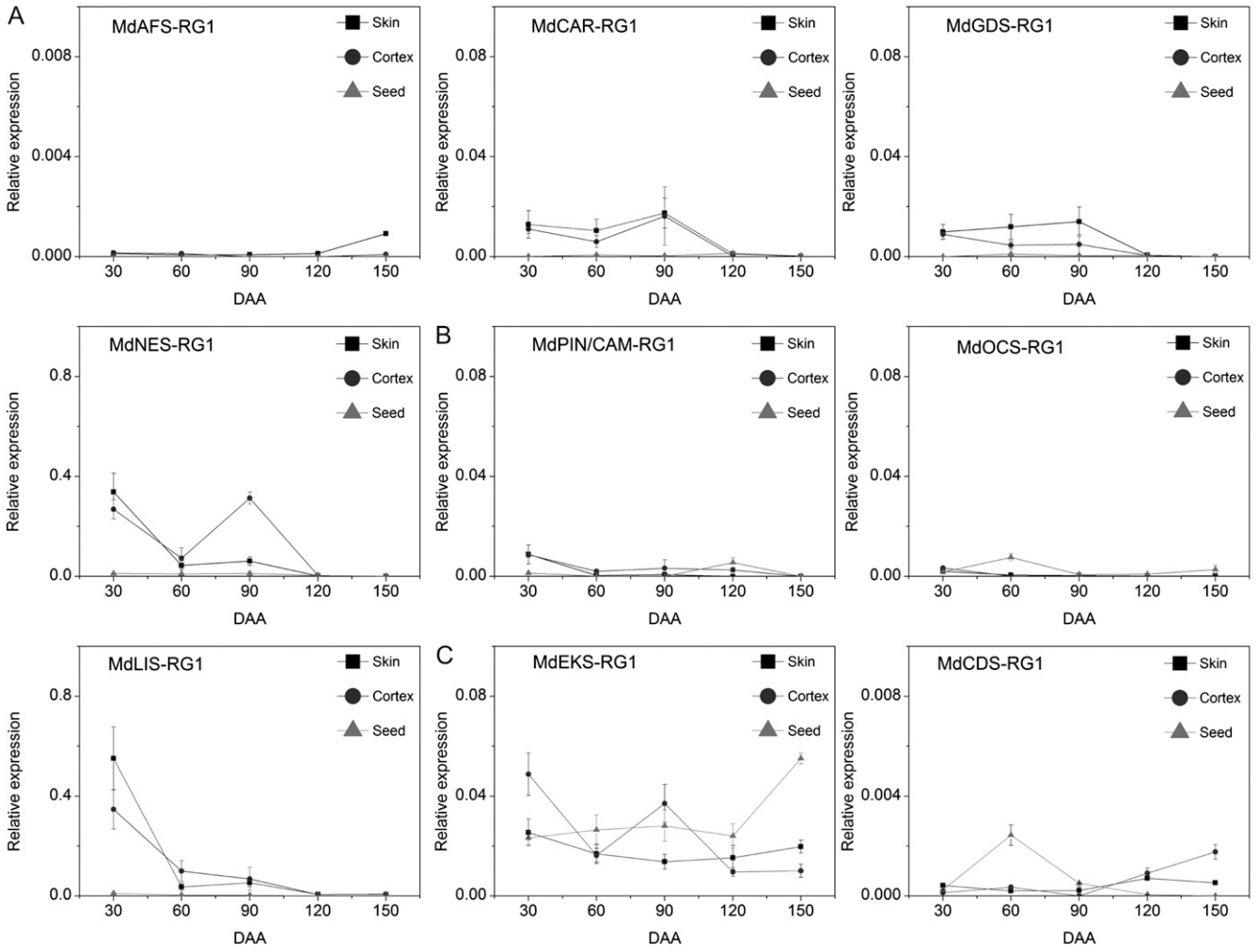

Figure 8. TPS gene expression in cv Royal Gala fruit tissues. Gene expression profiles are shown for sesquiterpene (A), monoterpene (B), and diterpene (C) synthases in different tissues during apple fruit development. Gene expression data analysis was carried out as for Figure 7. Data are presented as means ± SE ($n = 4$).

with concentrations ranging from 220 to 7,500 ng g^{-1} fresh weight. The relative amounts of *AFS* mRNA accumulation (Fig. 9) correlated well with (*E*,*E*)-α-farnesene production in the heritage apple cultivars. Production of other terpenes in the heritage varieties was generally low, but seven terpenes not detected in the cv Royal Gala control were present, including limonene, which was found in all five heritage varieties. The detection of terpineol in the cv Royal Gala solvent extractions was a little surprising given that it was not detected in any of the fruit headspace analyses (Supplemental Tables S3 and S4). However, the decreased volatility of this terpene alcohol means that it is more likely to be found in the solvent extract than in the headspace. It is also likely that terpineol is a product of one of the existing TPS enzymes, possibly the α-pinene/camphene synthase MdPIN/CAM-RG1.

TPS gene expression analysis suggested that a number of the *TPS* genes expressed in cv Royal Gala flowers and leaves were expressed in the heritage apple cultivar fruit. In particular, nerolidol synthase expression was elevated in all five varieties, and the germacrene-D and (*E*)-β-caryophyllene synthases were elevated in cv King David. However, there was not a strong correlation between the expression of a particular *TPS* gene and an elevation in the corresponding terpene volatile. This suggests that other factors including substrate availability may also be important for determining the volatiles produced. Nevertheless, the data indicate the potential for screening germplasm to identify cultivars expressing novel terpenes in the fruit.

DISCUSSION

Volatile Terpene Production in cv Royal Gala Appears To Be Predominantly Defense Associated

Terpene production in cv Royal Gala is primarily associated with mature flowers and vegetative tissues, while fruit terpenes are typically produced at low

Table II. *Terpene volatiles in five heritage apple cultivars*

Terpene volatiles from five heritage apple cultivars and cv Royal Gala were sampled at approximately 150 DAA by solvent extraction. Compound identification was as described in Table I with the following additions: Additional authentic standards were obtained from: *Aldrich, **Synthesis, ***Givaudan. References for retention indices are listed in the footnotes.

Compound	Retention Time	Retention Index	cv Royal Gala	cv King David	cv Wilmont Russet	cv Belle Bonne	cv Adam's Permain	cv Merton Russet
Limonene	6.59	1,234[a]	ND	0.7	0.8	0.9	1.2	0.8
6-Methyl-5-hepten-2-one*	10.1	1,348[b]	11	35	100	27	24	14
Bourbonene[i]	14.35	1,496[c]	ND	ND	ND	2.4	11	ND
Menthyl acetate**	15.53	1,541[d]	ND	33	ND	ND	ND	ND
(*E*)-β-Caryophyllene	16.12	1,618[e]	ND	ND	ND	3	1.4	ND
(*E*)-β-Farnesene***	18.09	1,674[a]	1.6	2.8	1.5	2.2	12	ND
α-Terpineol*	19.63	1,720[f]	17	0.3	5.7	6.0	7.6	8.6
Germacrene-D	18.66	1,722[a]	ND	ND	ND	9.9	9.9	ND
(*Z,E*)-α-Farnesene	19.41	1,727[d]	2.8	47	39	45	61	2.9
(*E,E*)-α-Farnesene	19.88	1,749[g]	47	2,600	2,700	1,400	7,500	220
(*E*)-Geranyl acetone	22.04	1,840[h]	ND	18	21	15	21	ND
(*E*)-Nerolidol*	25.87	2,054[a]	ND	8.6	ND	7.2	16	ND
(*E,E*)-Farnesol*	31.44	2,371[a]	430	900	11	390	440	45
Total terpenes (ng g^{-1} fresh weight)			509	3,645	2,879	1,909	8,105	291

[a]Choi (2003). [b]Başer et al. (2009). [c]Bendiabdellah et al. (2012). [d]Davies (1990). [e]Högnadóttir and Rouseff (2003). [f]Culleré et al. (2004). [g]Werkhoff et al. (1998). [h]http://www.flavornet.org/d_kovats_c20m.html. [i]Retention time and distinctive MS (retention index in this sample is 1,498; the compound runs just before pentadecane).

concentrations. The ovaries were the primary sites of floral terpene production, rather than petals as seen in other plants, including *Antirrhinum majus* (Dudareva et al., 2003), *Clarkia breweri* (Pichersky et al., 1994), and *Actinidia arguta* (Chen et al., 2010). Linalool and (*E*)-DMNT were the predominant floral terpenes. Although linalool has also been identified as the major floral terpene in other apple cultivars (Rapparini et al., 2001), it appears to be largely sequestered as a nonvolatile glycoside in apple leaves (Wei et al., 2004); hence, its production rate in cv Royal Gala leaves is likely to be higher than indicated in our analyses (Fig. 1).

(*E*)-DMNT is a common constitutively produced floral terpene (Kaiser, 1991; Azuma et al., 1997) and is also induced in response to herbivore damage of foliage in gymnosperms (Su et al., 2009) and in many monocot and dicot angiosperm plant species (Bouwmeester et al., 1999; Arimura et al., 2000, 2008; Degenhardt and Gershenzon, 2000; Kant et al., 2004; Lee et al., 2010). However, (*E*)-DMNT is not generally detected from undamaged or mechanically damaged foliage. It is interesting, therefore, that the primary source of DMNT production in cv Royal Gala is stipules rather than flowers (Fig. 1). Although very little is known about the function of these organs in plants, a possible role in protecting differentiating leaf and axillary bud tissues against pests, pathogens, and/or extreme heat or cold conditions has been put forward (Kumar et al., 2012). Moreover, stipule-derived production of DMNT and germacrene-D, which have also been shown to deter insect herbivores (Arimura et al., 2004; Ghirardo et al., 2012), would support an insect defense-associated role for these leaf-like structures in apple.

Although petal-associated linalool production in cv Royal Gala points to a role in pollinator attraction, especially considering that apples rely on cross pollination by insects (Thomson and Goodell, 2001; Katoh et al., 2002), the remaining floral terpenes, which are released from nonpetal tissues (particularly ovaries and pedicels; Fig. 2), are likely to have different ecological roles. For example, as proposed for floral terpenes in Arabidopsis (Chen et al., 2003), an important role for the non-petal-associated floral terpenes in apple and other plants, including grapevine (Martin et al., 2009) and *Magnolia* spp. (Lee and Chappell, 2008), could be to provide protection for the reproductive organs, and more specifically, the germ line cells, against oxidative stress and pathogenic microbes. Terpenes, including β-myrcene, (*E*)-β-ocimene, linalool, and α-caryophyllene, have been shown to react readily with reactive oxygen species (Calogirou et al., 1999; Loreto and Velikova, 2001), while a variety of monoterpenes and sesquiterpenes are also reported to have antimicrobial activity (Cowan, 1999).

Although terpene production in apple fruit was generally low, several features were noteworthy. For example, (*E,E*)-α-farnesene, which is the terpene most associated with ripe apple fruit (Sutherland et al., 1977; Bengtsson et al., 2001; Hern and Dorn, 2003; Ferreira et al., 2009; Rowan et al., 2009a), was only produced at very low concentrations in undamaged harvest-ripe (150-DAA) fruit in our analysis. (*E,E*)-α-Farnesene has previously been shown to increase significantly in ripe apple following exposure to exogenous ethylene (Johnston et al., 2009), with the greatest increase in (*E,E*)-α-farnesene production (approximately 0.1–1.3 ng g^{-1} fresh weight h^{-1}) occurring as ethylene concentrations were increased from

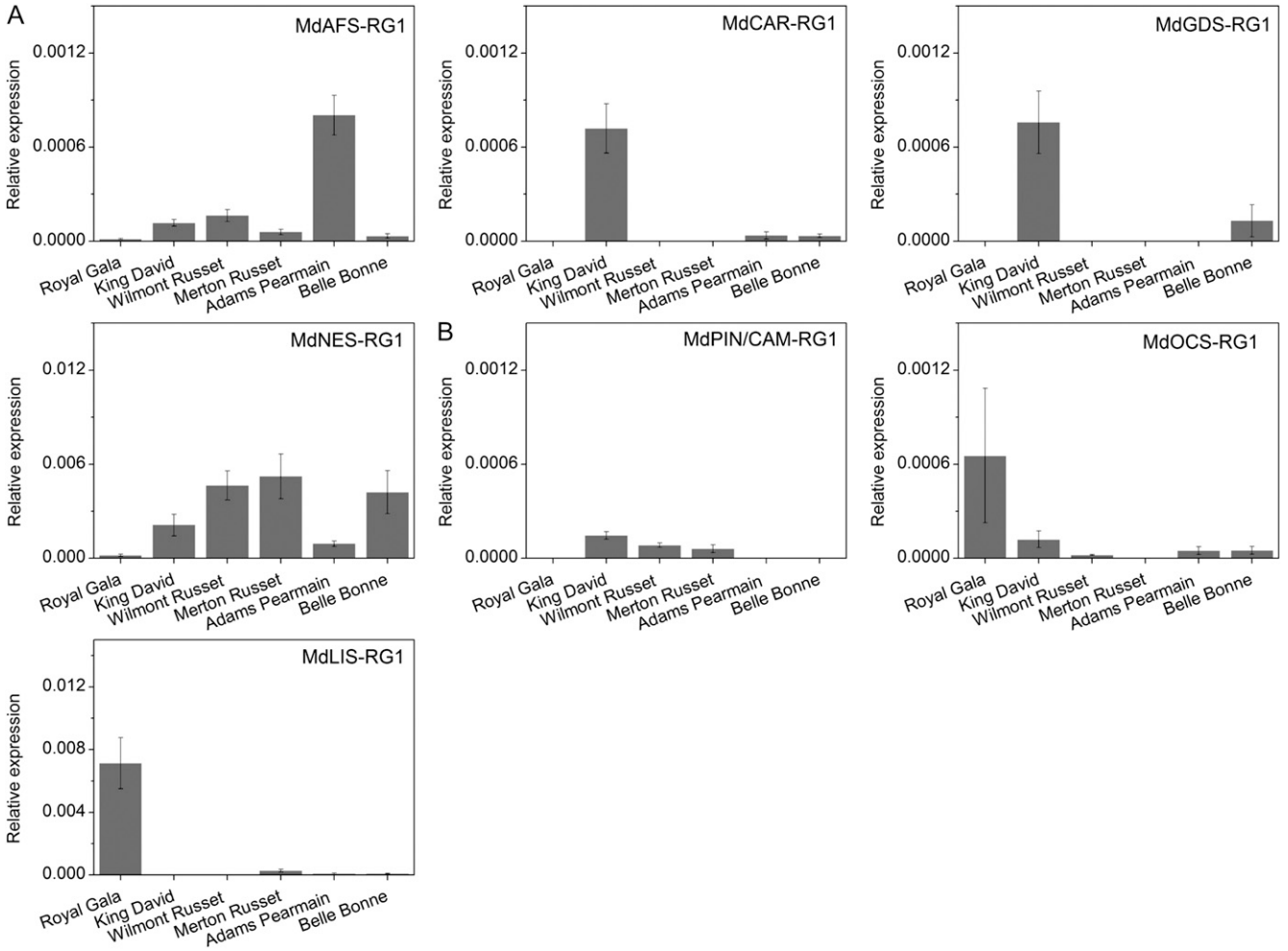

Figure 9. TPS gene expression in the fruit of five heritage apple cultivars. Fruit were picked at approximately 150 DAA, and gene expression data analysis was carried out using the same conditions and primers as described in Figure 5. The expression of each TPS gene in cv Royal Gala fruit at 150 DAA from Figure 8 has been included for comparison. Data are presented as means ± SE ($n = 4$).

0.1 to 10 μg mL^{-1}. Ethylene concentrations in 150-DAA cv Royal Gala fruit occur at approximately 1 μg mL^{-1} (Johnston et al., 2002); hence, the low (*E*,*E*)-α-farnesene emission we observed in 150-DAA whole fruit (approximately 0.046 ng g^{-1} fresh weight h^{-1}; Supplemental Table S3) are consistent with internal ethylene below 1 μg mL^{-1}.

(*E*,*E*)-α-Farnesene also appears to be induced in ripe apple skin in response to mechanical wounding, in this case during sample preparation to peel the skin (Supplemental Table S3). Similar induction of (*E*)-DMNT was also noted in 60-DAA skin. The peak of (*E*)-β-ocimene production observed in maturing apple seeds (60 DAA; Supplemental Table S2), however, does not appear to be produced in response to wounding. Instead, it may be related to increased plastidial 2-C-methyl-D-erythritol 4-phosphate pathway flux (responsible for both GDP and GGDP isoprenoid precursors) toward GA biosynthesis, which is 15 to 500 times higher in apple seeds than in leaves and shoots (Luckwill, 1974). Although previous studies have shown that GDP and GGDP levels can be modulated in planta by the GDP synthase small subunit, leading to decreased GGDP-derived metabolites (including GA) and increased monoterpene production (Orlova et al., 2009), the (*E*)-β-ocimene peak in apple seeds coincides with maximal GA biosynthesis at 9 weeks (i.e. approximately 60 DAA; Luckwill et al., 1969). The concomitant increase in GDP- and GGDP-derived metabolites, namely (*E*)-β-ocimene and GA, respectively, suggests that GDP synthase small subunit-directed effects are either absent or suppressed in cv Royal Gala seeds.

On the basis that GAs are biosynthesized from GGDP via CDP and *ent*-kaurene (Yamaguchi, 2008; Hedden and Thomas, 2012), the peak of *MdCDS-RG1* seed expression at 60 DAA (Fig. 8) also supports the assumption that this gene encodes the functional CDP synthase in cv Royal Gala.

The Apple TPS Gene Family

The genome duplication events that have shaped the overall organization of the apple genome (Velasco et al., 2010) also appear to have significantly affected the organization and diversity of the apple *TPS* gene family. However, unlike grape, where duplication events have culminated in a *TPS* gene family encoding a large number of functionally diversified enzymes (Martin et al., 2010), our in silico analysis of the apple 'Golden Delicious' genome identified only 10 potentially functional TPS enzymes. Importantly, our in silico findings are also strongly supported by the identification and functional annotation of an equivalent number of cv Royal Gala TPS enzymes. This equivalency not only gives us confidence that we have identified the majority of TPS enzymes in apple but also indicates that the preservation of TPS enzymes since the GWD is of physiological importance.

It is possible that the high proportion of *TPS* pseudogenes in the cv Golden Delicious genome compared with other plant species (Chen et al., 2011) arose after the ancestral duplication events in the *Malus* genome (Velasco et al., 2010), allowing for the acquisition of deleterious mutations in one of the duplicated genes (Walsh, 1995; Lynch and Conery, 2003). Alternatively, some of the pseudogenes may have arisen from retrotransposition. Evidence for this process can be seen in a number of the predicted *TPS* gene models that contain retrotransposon-like insertions (Supplemental Fig. S2). Specifically, the expansion of the TPS-a subgroup in apple appears to have been driven by a combination of the recent GWD and more recent gene duplication events. The GWD-associated gene duplications are inferred from the identification of equivalent *TPS-a* genes on homeologous chromosomes 3 and 11, while more recent duplications are inferred from an additional *TPS-a* gene cluster located on chromosome 12, with the corresponding absence of equivalent genes on the homeologous chromosome 4 (Supplemental Table S6). Although it is conceivable that a *TPS-a* gene cluster on chromosome 4 may have been lost after the GWD, the complete absence of *TPS*-like genes anywhere on this chromosome suggests that this is unlikely. Gene duplications occurring after the GWD could also account for the expansion of the *TPS-e/TPS-f* family. Gene clusters for this family are primarily located on the nonhomeologous chromosomes 15 and 17, while the approximately 50:50 split of *TPS-g* genes on homeologous chromosomes 5 and 10 (Supplemental Table S6) is again indicative of a GWD-associated expansion.

TPS Regulation and Conservation

It has previously been shown that volatile production in apple fruit is primarily associated with ripening and that ethylene modulates the biosynthesis of pathways leading to ester, phenylpropanoid, and sesquiterpene [specifically (*E*,*E*)-α-farnesene] production by up-regulating the expression of end-point genes in these pathways (Defilippi et al., 2005; Schaffer et al., 2007). Our work supports the notion that in cv Royal Gala, only (*E*,*E*)-α-farnesene production is ethylene responsive and that terpene metabolism is predominantly associated with unripe fruit in the absence of climacteric ethylene production (Reid et al., 1973; Lay-Yee et al., 1990; Volz et al., 2003; Schaffer et al., 2007). The higher amounts of monoterpenes in early fruit development are more likely to be related to the transition of flowers (which have comparatively high TPS expression) to fruit. Although cv Royal Gala ripe fruit produce relatively small amounts of terpenes compared with other volatiles such as esters (Souleyre et al., 2005), the conservation and phenological variation in terpene production indicate that there is selective pressure to maintain functional TPS enzymes in cv Royal Gala fruit. It is also reasonable to assume that the conservation of fruit terpene production is directly related to the specialized "fitness" roles to which they contribute. Specifically, the primary function of terpenes in flowers appears to relate to both the attraction of pollinators and the protection of reproductive structures. In immature fruit, this function appears to transition to a defense role against herbivores and pathogens, while changes in volatile compounds that occur during fruit maturation, in combination with changes in texture, taste, and color, are necessary to attract legitimate seed-dispersal agents (Jensen, 1976; Herrera, 1982; Sallabanks and Courtney, 1992). The conservation of (*E*,*E*)-α-farnesene in particular points to a role in the attraction of frugivorous animals to apple fruit as they transition from harvest ripe (approximately 150 DAA) to fully ripe.

The Effect of Commercial Breeding Strategies on Terpene Production

The presence of additional terpenes in the five heritage apple varieties tested compared with cv Royal Gala suggests that modern breeding strategies might be reducing the range of terpenes in ripe fruit. In apple, modern breeding strategies have placed the emphasis on the selection of sweet, dessert varieties with superior texture and "fruity" ester flavor/aroma notes (Volz et al., 2004). This focus would select against the terpenes associated with nutty/aromatic notes in the heritage varieties and consequently reduce the range of aromas/flavors in modern apple cultivars such as cv Royal Gala. Alternatively, the lack of terpenes could be linked to producing apples with longer storage qualities, which can be associated with reduced climacteric ethylene production. The effects of breeding practices on fruit flavor have previously been observed in modern tomato varieties, which have desirable production traits, including yield and uniform ripening, but appear to have been negatively affected in terms of fruit quality traits, particularly fruit flavor (Klee, 2010; Powell et al., 2012).

The conservation of (*E*,*E*)-α-farnesene production in ripe fruit suggests that (unlike other terpenes) there must be a specific selective pressure to maintain the

production of this simple acyclic sesquiterpene in apple fruit. The amino acid sequence identity of functional and potentially functional (*E*,*E*)-α-farnesene synthase-encoding genes in different apple and pear (*Pyrus communis*) cultivars (Supplemental Fig. S8), combined with the fact that only a single predicted apple (*E*,*E*)-α-farnesene synthase-like gene model (MdTPS25-GD; Supplemental Table S6) was identified, supports this assumption.

In conclusion, this work brings together, to our knowledge for the first time, an overall picture of the organization and expression of the apple *TPS* gene family and provides a reference point to initiate future studies into the evolution of TPS enzymes in other important Rosaceous plant species. This work also highlights the different biological roles these compounds are likely to fulfill in apple and will help with the breeding of new apple varieties with novel and interesting flavors.

MATERIALS AND METHODS

Headspace Volatile Trapping

Apple (*Malus domestica* 'Royal Gala') flowers, flower parts (approximately 5 g), fruit parts (approximately 5 g), and leaves were harvested and placed in 50-mL Quickfit tubes, and volatiles were trapped for 4 h in direct thermal desorption tubes (ATAS GL International) packed with 80 mg of Chromosorb 105 absorbent (Shimadzu) using purified air at a flow rate of 25 mL min^{-1}. For the separate analyses of whole fruit harvested at various stages of maturity, five cv Royal Gala apples were placed in 5-L wide-necked sampling vessels that were each attached to a gas line and a sorbent cartridge containing 100 mg of Chromosorb 105. The headspace in the flask was allowed to equilibrate at 23°C for 1 h, after which the headspace was purged with air (25 mL min^{-1}, 15 min).

Solvent Extraction of Fruit Terpene Volatiles

Solvent extractions were carried out on pooled samples (approximately 5 g) of apple skin taken from 10 apples harvested at approximately 150 DAA from heritage varieties grown under research orchard conditions. Tissue was ground to a fine powder in liquid N_2 in 50-mL Quickfit conical flasks and extracted twice with 10 mL of diethyl ether for 30 min with gentle shaking. The two extractions were combined and stored overnight at −20°C. The following day, the upper solvent layer was carefully removed from the lower frozen water layer using a glass pipette and reduced to 2 mL under a gentle stream of nitrogen. The concentrated extract was then dried by passage through a column of anhydrous $MgSO_4$.

GC-MS Analysis

Headspace volatiles were desorbed directly from the direct thermal desorption tubes with a temperature ramp of 45°C to 175°C at 16°C s^{-1} and cryofocused on the front of the capillary column by a liquid nitrogen-cooled cryogenic trap at −110°C. After cryofocusing, the trap temperature was ramped to 175°C at 50°C min^{-1} (Optic 3 thermal desorption system; ATAS GL). A 15:1 split was employed while the volatiles were transferred into the capillary column at a column flow of 1 mL min^{-1}. The GC oven ramp was 35°C for 2 min, 3°C min^{-1} to 60°C, 5°C min^{-1} to 100°C, 8°C min^{-1} to 170°C, 10°C min^{-1} to 200°C, and then held for 13 min. GC separations were on a 30-m × 0.25-mm (i.d.) × 0.25-μm film thickness DB-Wax (J&W Scientific) capillary column in an HP6890 GC apparatus (Agilent Technologies) with helium as the carrier gas. The GC device was coupled to a time of flight-mass spectrometer (Leco Pegasus III). The ion source temperature was kept at 200°C, and ionization energy of 70 eV was used for electron-impact ionization. The detector voltage was 1,700 V, and ion spectra from 33 to 320 atomic mass units were collected with a data acquisition rate of 20 Hz. The total ion chromatograms were processed using the LECO ChromaTOF software.

Terpenes were identified using the following reference compounds: α-pinene, linalool, β-myrcene, and α-terpineol (Aldrich), limonene (BDH), β-pinene (K&K Laboratories), (*E*)-β-caryophyllene (Koch-Light and Givaudan), (*E*)-β-farnesene (Givaudan), and germacrene-D (RC Treatt). (*E*,*E*)-α-Farnesene and (*Z*,*E*)-α-farnesene were obtained by pentane extraction of Granny Smith apple skin (Anet, 1970). Other compounds for which we did not have authentic standards were identified by the ChromTOF software (version 2.3; Pegasus; Leco Australia) using the National Institute of Standards and Technology (version 2.0d, 2005) mass-spectral database, in combination with comparison of analyte retention indices with those of a series of straight-chain hydrocarbon standards (C8–C23, 0.005 μL mL^{-1} for each hydrocarbon). Peaks were selected and integrated manually using the molecular ion and/or specific diagnostic ions of each compound. The terpenes were quantified by measuring mass-to-charge ratio (*m/z*) 93 peak areas against an average response factor for the *m/z* 93 peak areas of 1,8-cineole (eucalyptol; 0.0366 μL mL^{-1}), linalool (0.0263 μL mL^{-1}), and β-caryophyllene (0.03 μL mL^{-1}), contained in an external standard. Calibration curves determined that the GC-MS system gave a linear response, with respect to analyte concentration, over the range of concentrations for which they were measured in the headspace and in the solvent extracts.

GC-MS analysis of solvent extraction samples from the heritage apple fruit was performed on an Agilent 6890N GC device coupled to a Waters GCT time of flight-mass spectrometer with an electron-impact energy of 70 eV and a scan time of 0.4 s. One-microliter splitless (30-s) injections were made at 220°C onto a 20-m × 0.18-mm (i.d.) × 0.18-μm film thickness DB-Wax (J&W Scientific) capillary column with a helium flow of 0.9 mL min^{-1}. The oven temperature program was 1 min at 35°C, 5°C min^{-1} to 230°C, and hold for 5 min.

Terpene quantitation standards for the heritage apple fruit analysis were as follows: linalool, β-myrcene, α-terpineol, 6-methyl-5-hepten-2-one, (*E*)-nerolidol, and (*E*,*E*)-farnesol (Aldrich), limonene (also for menthyl acetate; BDH), β-caryophyllene (Koch-Light), and germacrene-D (also for α- and β-farnesene; RC Treatt).

Enantioselective GC-MS

A 1-μL splitless sample injection (30 s) was made onto a 30-m × 0.25-mm (i.d.) × 0.25-μm film thickness β-Dex 325 (Supelco) enantioselective capillary column. The phase on this column was 25% 2,3-di-*O*-methyl-6-*O*-tertbutyldimethylsilyl-β-cyclodextrin in SPB-20 (poly-[20% dimethylsiloxane/80% dimethylsiloxane]). The flow rate of helium was 0.9 mL min^{-1}, and the injection port was at 220°C. The oven temperature program was 1 min at 35°C, increasing by 5°C min^{-1} to 230°C, and hold for 5 min.

(+/−)-Germacrene-D [mainly the (−)-enantiomer] was obtained from goldenrod (*Solidago canadensis*) plants, and the enantiomers were assigned as described previously (Nieuwenhuizen et al., 2009). (*S*)-(*E*)-Nerolidol was obtained from neroli essential oil (Neroli; *Citrus aurantium*; Lotus Essential Oils). The essential oil (0.25 mL) was partially purified by flash chromatography on silica gel (pentane:dichloromethane:methanol, 100:99:1), and (*E*)-nerolidol was identified by GC-MS comparison with (*E*/*Z*)-nerolidol (Aldrich). Neroli contains exclusively (*E*)-nerolidol and predominantly (*S*)-(*E*)-nerolidol (Mondello et al., 2002). Enantioselective GC-MS of the partially purified neroli essential oil determined it to be 14:86 (*R*):(*S*)-(*E*)-nerolidol. (*S*)-(−)-Limonene and (*R*)-(+)-limonene, *rac*-linalool, and (+)- and (−)-α-pinene were obtained from Aldrich, and (+)- and (−)-β-pinene and (*R*)-(−)-linalool were from Fluka.

Identification of cv Golden Delicious and Royal Gala TPS Genes

The release of the draft apple 'Golden Delicious' genome from the Genome Database for Rosaceae (GDR; http://www.rosaceae.org/) facilitated the screening of the Instituto Agrario San Michele all'Adige-annotated gene models (Gene Models 1) with known TPS sequences obtained from the nonredundant protein database at NCBI. The previously identified TPS sequences were used as query sequences for tBLASTn searches of the GDR database. The genomic data presented here are based on the genome assembly as annotated on July 30, 2012, and no additional curation of putative cv Golden Delicious *TPS* gene models was carried out.

Putative full-length cv Royal Gala TPS ORFs were identified from BLAST searches (tBLASTn and BLASTp) of an in-house EST database (primarily containing cv Royal Gala sequences; Newcomb et al., 2006) and nonredundant

protein, nucleotide, and apple-specific EST sequence databases available at NCBI. EST sequences from the in-house and publicly accessible EST databases were derived from a wide variety of apple tissues, including flower, vegetative, and root tissues collected at various developmental stages, as well as from tissues subjected to conditions that would be expected to induce terpene production.

For simplification, the "MDP" (for *Malus domestica* protein) gene model annotations are given corresponding MdTPS numbers for TPS-specific gene models and, where relevant, a full-length cDNA identifier is given to previously predicted full-length TPS cDNAs and functionally characterized TPS enzymes. In accordance with the grape (*Vitis vinifera*) *TPS* gene annotation (Martin et al., 2010), this identifier includes a "GD" or "RG" notation to differentiate between TPS enzymes identified or cloned from the cv Golden Delicious and Royal Gala apple cultivars, respectively. For example, the previously characterized cv Royal Gala (*E,E*)-α-farnesene synthase (MdAFS1) will now be termed MdAFS-RG1.

Sequence Analysis

Multiple amino acid sequence alignments of *TPS* genes were performed with ClustalX (Thompson et al., 1997) using default parameters and were manually adjusted in GeneDoc (www.nrbsc.org/gfx/genedoc/). Prediction of the protein ORF was carried out using the EMBOSS (http://www.ch.embnet.org/EMBOSS/index.html) translation tool Transeq (Rice et al., 2000). The bioinformatics tool ChloroP (Emanuelsson et al., 1999), available at http://www.cbs.dtu.dk/services/, was used to predict the intracellular targeting of full-length TPS ORFs.

The evolutionary history was inferred by using the maximum likelihood method based on the Dayhoff matrix-based model (Schwarz and Dayhoff, 1979). The tree with the highest log likelihood is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was less than 100, or less than one-quarter of the total number of sites, the maximum parsimony method was used; otherwise, the BIONJ method (Gascuel, 1997) with the Maximum Composite Likelihood matrix (Tamura et al., 2004) was used. A discrete γ-distribution was used to model evolutionary rate differences among sites (five categories; +G, parameter = 5.1574). The analysis involved 30 TPS amino acid sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2007).

Transient Expression in Tobacco Leaves

In planta transient expression in tobacco (*Nicotiana benthamiana*) leaves was carried out as described previously (Nieuwenhuizen et al., 2009; Green et al., 2012a) utilizing the *Agrobacterium tumefaciens* strain GV3101. For the construction of cv Royal Gala TPS plant transformation vectors, full-length TPS ORFs were amplified by PCR, using the primers listed in Supplemental Table S8, and cloned into either pENTR/D-TOPO (Invitrogen) or pDONR221. The resulting clones were transferred by Gateway LR reactions as recommended by the manufacturer (Invitrogen) into the binary destination vector pHEX2 (Hellens et al., 2005) that contains the cauliflower mosaic virus 35S promoter and octopine synthase terminator.

In Vitro Analysis of Recombinant TPS Enzymes

TPS genes were cloned into pET200/D-TOP0 or pET300/NT-DEST (Invitrogen) for bacterial overexpression according to the manufacturer's recommendations. Details of the primers are given in Supplemental Table S8. Recombinant TPS enzymes were expressed under autoinducible conditions and purified by immobilized affinity metal chromatography according to previous methods (Green et al., 2007, 2012b). Solvent extraction assays for terpene identification and enantiomeric determinations were carried out as described previously (Green et al., 2012b). Terpene reference compounds used for the in vitro analysis were as for the headspace analysis but also included α-copaene and α-caryophyllene (Givaudan) and borneol (Aldrich).

Real-Time Gene Expression Analysis

RNA was extracted from apple flowers, flower parts, fruit, and leaves according to Nieuwenhuizen et al. (2009) and treated with 10 units of DNaseI (Roche Applied Science) before cDNA synthesis. First-strand cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions and diluted 50-fold before use. Relative quantitative real-time gene expression analyses of targets and the reference gene *ELONGATION FACTOR1α* (*EF1α*) were performed (four technical replicates) on a LightCycler 480 platform using the LightCycler 480 SYBR Green master mix, and the results were analyzed using the LightCycler 480 software (Roche). To determine the level of expression, the differences (Δ) between the threshold cycle (Ct) or crossing points (CP) were measured (i.e. $-\Delta$Cp method) according to Montefiori et al. (2011), enabling a comparison of the level of expression of multiple target genes normalized to a common reference gene considered stable and unchanging in the different samples. The program was as follows: 5 min at 95°C; 40 cycles of 10 s at 95°C, 10 s at 60°C, and 20 s at 72°C; followed by melting curve analysis: 95°C for 5 s, 65°C for 60 s, then ramping at 0.18°C s^{-1} to 95°C. Primers for real-time gene expression analysis were designed using Vector NTI software (Invitrogen) based on a melting temperature of 60°C and amplicon length of 70 to 120 bp and are listed in Supplemental Table S9.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers JX848729, JX848730, JX848731, JX848732, X848733, and JX848734.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Genomic, predicted cDNA, and protein sequences for the functional cv Royal Gala sesquiterpene synthase and monoterpene synthases and the putatively functional diterpene synthases.

Supplemental Figure S2. TPSs in the cv Golden Delicious genome with predicted retrotransposon insertions.

Supplemental Figure S3. In vitro analysis of cv Royal Gala monoterpene synthase enzyme activity.

Supplemental Figure S4. In vitro analysis of cv Royal Gala sesquiterpene synthase enzyme activity.

Supplemental Figure S5. Monoterpene enantiomeric determinations.

Supplemental Figure S6. Sesquiterpene enantiomeric determinations.

Supplemental Figure S7. TPS gene expression in cv Royal Gala roots.

Supplemental Figure S8. Alignment of apple and pear (*E,E*)-α-farnesene synthases.

Supplemental Table S1. Volatile terpenes released from cv Royal Gala leaves and flowers.

Supplemental Table S2. Volatile terpene production from cv Royal Gala floral tissues.

Supplemental Table S3. Volatile terpenes released from whole cv Royal Gala fruit.

Supplemental Table S4. Phenological terpene emission in cv Royal Gala fruit tissues.

Supplemental Table S5. Fold difference in skin and whole apple fruit terpene production at 150 DAA.

Supplemental Table S6. Tabulated information on the 55 TPS gene models identified in the cv Golden Delicious genome.

Supplemental Table S7. Full-length TPSs in cv Royal Gala EST collections.

Supplemental Table S8. TPS gene-specific cloning primers.

Supplemental Table S9. Gene-specific TPS primers used for expression analysis.

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