

RESEARCH PAPER

# Flavour compounds in tomato fruits: identification of loci and potential pathways affecting volatile composition

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## Abstract

The unique flavour of a tomato fruit is the sum of a complex interaction among sugars, acids, and a large set of volatile compounds. While it is generally acknowledged that the flavour of commercially produced tomatoes is inferior, the biochemical and genetic complexity of the trait has made breeding for improved flavour extremely difficult. The volatiles, in particular, present a major challenge for flavour improvement, being generated from a diverse set of lipid, amino acid, and carotenoid precursors. Very few genes controlling their biosynthesis have been identified. New quantitative trait loci (QTLs) that affect the volatile emissions of red-ripe fruits are described here. A population of introgression lines derived from a cross between the cultivated tomato *Solanum lycopersicum* and its wild relative, *S. habrochaites*, was characterized over multiple seasons and locations. A total of 30 QTLs affecting the emission of one or more volatiles were mapped. The data from this mapping project, combined with previously collected data on an IL population derived from a cross between *S. lycopersicum* and *S. pennellii* populations, were used to construct a correlational database. A metabolite tree derived from these data provides new insights into the pathways for the synthesis of several of these volatiles. One QTL is a novel locus affecting fruit carotenoid content on chromosome 2. Volatile emissions from this and other lines indicate that the linear and cyclic

apocarotenoid volatiles are probably derived from separate carotenoid pools.

Key words: Apocarotenoids, flavour, metabolism, quantitative trait loci, *Solanum lycopersicum*.

## Introduction

A significant portion of the human diet is made up of fruits that provide essential vitamin micronutrients as well as fibre and antioxidants. Fruits contain thousands of ripening-associated secondary metabolites generated with different biological functions, many as yet undefined. Fruit flavour can have an important nutritional effect with good flavour promoting a higher intake. While flavour is widely viewed as deficient in most commercially produced tomato varieties, little progress has been made in the last half century toward its improvement. This lack of progress can be attributed to multiple factors. Post-harvest shipping and handling practices as well as the development of extended shelf-life varieties have greatly contributed to the decline in flavour. But a major factor has been the emphasis of breeding programmes on larger, firmer fruits. Flavour has not been generally emphasized because of its complex nature and the very large number of genes ultimately contributing to the trait.

The flavour of a particular food can be thought of as the sum of a complex interaction between taste receptors, the ortho- and retronasal olfactory systems, mouth texture,

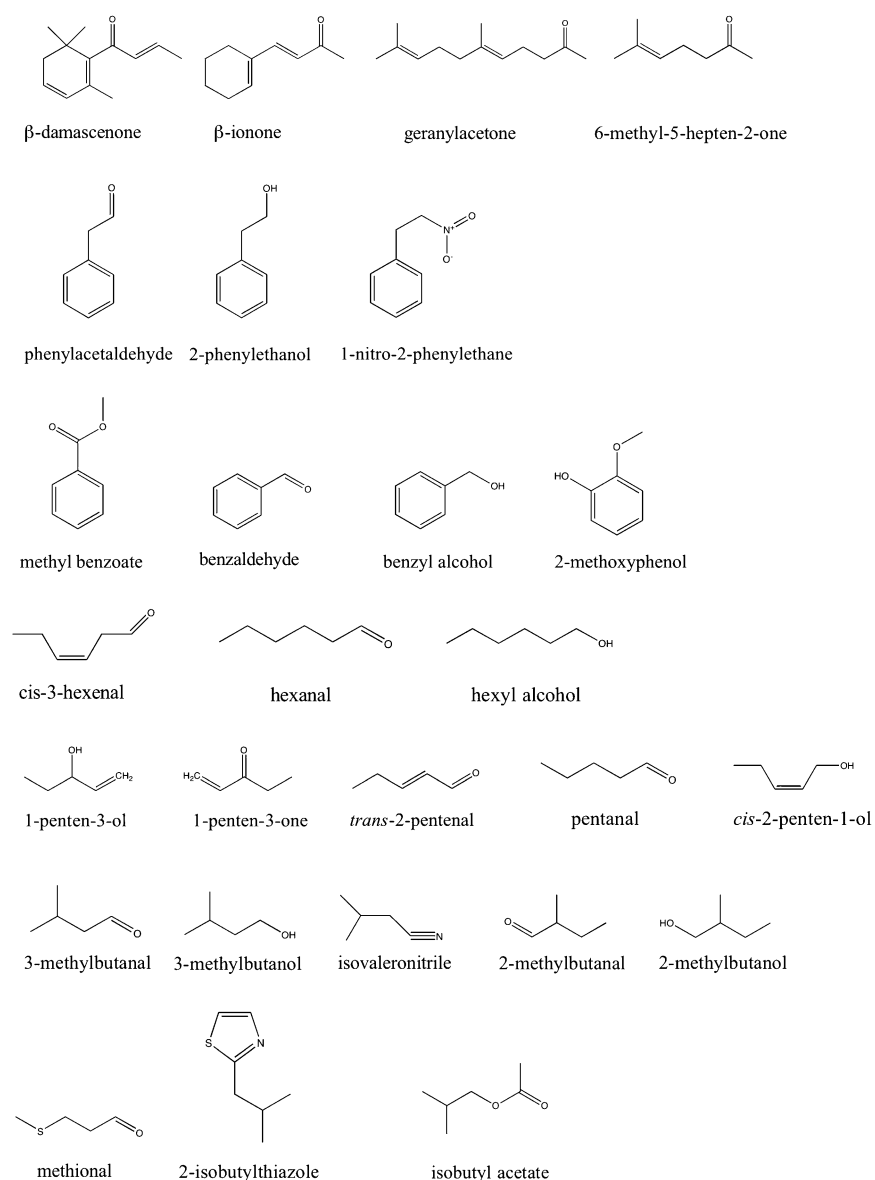
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Abbreviations: IL, introgression line; LOX, lipoxygenase; MHO, 6-methyl-5-hepten-2-one; QTL, quantitative trait locus.

and visual appearance (Shepherd, 2006). In the case of tomato fruit, the flavour chemicals have been identified (Baldwin *et al.*, 1998). Taste receptors respond to sugars (principally glucose and fructose), acids (citric, malic, and ascorbic) and glutamate. Significant quantities of sugars and acids must be present in the fruit for it to be perceived as good and different human populations have different preferences for various sugar to acid ratios. Beyond the foundation of sufficient sugars and acids, a fruit must also possess sufficient quantities of volatile chemicals that impart the distinct tomato flavour. While over 400 volatiles have been detected in tomato (Buttery and Ling, 1993), a smaller set of 15–20 are made in sufficient quantities to have an impact on human perception

(Baldwin *et al.*, 2000). With a minimum of 20–25 distinct chemicals all contributing to overall flavour, it is easy to understand the magnitude of the challenge for flavour improvement through classical breeding.

Efforts by others and ourselves have been focused on identifying genes that regulate the synthesis of the most important 15–20 volatiles that positively contribute to tomato flavour (Fig. 1) (Saliba-Colombani *et al.*, 2001; Causse *et al.*, 2004; Tieman *et al.* 2006a). Recombinant introgression lines (ILs) produced from a cross between tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) and its wild relative *S. pennellii* (formerly *L. pennellii*) (Eshed and Zamir, 1995) were used. Using this population, over 30 quantitative trait loci (QTLs) were



**Fig. 1.** Structures of tomato volatiles for which a locus has been attributed in this study. The compounds are grouped by family precursor.

identified that are reproducibly altered in one or more volatiles contributing to flavour (Tieman *et al.*, 2006a). The large number of QTLs emphasizes the complexity of undertaking a molecular-assisted flavour breeding programme. To simplify the process, the QTLs were used as tools to identify the genes responsible for controlling volatile compound synthesis in two ways. First, precise knowledge of map positions will facilitate molecular cloning, particularly as the tomato genome sequence becomes available. Second, by cluster analysis of the metabolite profiles for the complete set of ILs, relationships can be determined between the various metabolites, thus identifying common pathways. This latter approach is important because, in many cases, the biosynthetic pathways have not been formally demonstrated. Rather, precursor–product relationships have been inferred based on structural considerations.

Very few genes involved in the biosynthetic pathways of tomato flavour volatiles have been identified. Although the actual QTL remains to be defined, the *malodorous* locus on *S. pennellii* IL8-2 has been used as a tool to elaborate the biosynthetic pathway of a set of phenylalanine-derived volatiles, 2-phenylacetaldehyde, 2-phenylethanol, and 1-nitro-2-phenylethane (Tadmor *et al.*, 2002; Tieman *et al.*, 2006b). The availability of an IL that greatly over-produces a set of metabolically related compounds facilitated defining the biosynthetic pathway and ultimately establishing candidate genes encoding the biosynthetic steps. The first step in this pathway is performed by a small family of aromatic amino acid decarboxylases (Tieman *et al.*, 2006b). The final step is performed by another small family of phenylacetaldehyde reductases (Tieman *et al.*, 2007). This work illustrates the value of robust ILs affecting the various flavour volatiles.

Our interest was in using QTLs for establishing the biosynthetic pathways, cloning the structural genes responsible for their synthesis, and, ultimately, identifying regulatory loci controlling the rate-limiting steps. Such loci are also valuable in assessing broader questions regarding the importance of individual volatiles in tomato flavour. ILs affected in one or a small subset of volatiles can be used for consumer taste panels. Such data provide qualitative and quantitative information for the subsequent genetic alteration of fruit composition. In order to identify the widest possible range of QTLs, a set of ILs derived from a cross between *S. lycopersicum* and a second wild relative of tomato, *S. habrochaites* (formerly *L. hirsutum*) (Monforte and Tanksley, 2000) was used. This small, green-fruited species is, like tomato, climacteric (Grumet *et al.*, 1979). During ripening, it produces a volatile profile that is distinct from that of tomato. Thirty QTLs affecting 24 different volatile compounds have been identified. Only a few of these QTLs are likely to be alleles of the previously identified *S. pennellii* QTL (Tieman *et al.*, 2006a). In addition, the metabolic profiles of the ILs

derived from crosses between *S. lycopersicum* and *S. pennellii* or *S. habrochaites* have been used to construct a metabolic tree of the important tomato flavour volatiles. This tree provides useful information about metabolic relationships and the potential limiting steps in the biosynthesis of a number of volatiles.

## Materials and methods

### Growth of plant materials

Eighty-nine introgression lines derived from a cross between *S. lycopersicum* and *S. habrochaites* (Monforte and Tanksley, 2000) and the two parents of the cross, *S. lycopersicum* cv. E6203 (LA4024) and *S. habrochaites* accession LA1777, were analysed for volatiles. The plants were grown over seven trials/seasons: spring 2003, field, Citra, FL; spring 2003, greenhouse, Gainesville, FL; autumn 2003, field, Citra, FL; spring 2004, field, Citra, FL; autumn 2004, greenhouse, Gainesville, FL; spring 2006, greenhouse, Gainesville, FL; autumn 2006, field, Live Oak, FL. Subsets of the lines were grown in the field in randomized, replicated plots with two plots of three plants per season. Field-grown plants were grown using standard commercial practices in raised plastic mulch beds. Plants were staked and supported with string. Plants were drip irrigated with tubing under the plastic mulch. For greenhouse trials, three plants of each line were grown in a randomized trial. Greenhouse-grown tomatoes were planted in three gallon pots in Metro-Mix 300 soil mix (Sun Gro Horticulture, Belleview, WA) and Osmocote fertilizer (Scotts, Marysville, OH). For the purpose of the analysis presented here, the site datasets were combined. Fruits from all plants for each line were combined and analysed as they reached the red ripe stage. Each line was harvested on multiple successive weeks within a season. Each harvest within a season was considered as a biological replication for its respective IL. Trials where volatile data for only a single harvest were available were discarded from further analysis.

### Volatile determinations

The volatile sampling and analysis was performed as described by Tieman *et al.* (2006a). Ripe tomato fruits from each plant and both replicates of each IL line were harvested and volatiles from pooled fruits were sampled on the day after harvest. Tomato fruit volatiles were sampled from chopped fruit as described by Schmelz *et al.* (2003). Briefly, chopped fruits were enclosed in glass tubes. Air filtered through a hydrocarbon trap (Agilent, Palo Alto, CA) flowed through the tubes for 1 h with collection of the volatile compounds on a Super Q column. Volatiles collected on the Super Q (Alltech, Deerfield, IL) column were eluted with methylene chloride after the addition of nonyl acetate as an internal standard. Volatiles were separated on an Agilent (Palo Alto, CA) DB-5 column and analysed on an Agilent 6890N gas chromatograph with retention times compared to known standards (Sigma Aldrich, St Louis, MO). Volatile levels were calculated as ng g<sup>-1</sup> FW h<sup>-1</sup> sampling. Identities of volatile peaks were confirmed by GC-MS as described by Schmelz *et al.* (2001).

### HPLC analysis of carotenoids

Tomato fruits were sampled from plants grown in a greenhouse. Tomatoes were tagged at the breaker stage and harvested at breaker (B), turning (T), pink (P), and red ripe (R) according to USDA guidelines. Volatile measurements were performed on three biological replicates of tomato fruit as described above. Carotenoid extraction was performed on 0.05 g of dry weight (DW) tomato

fruit according to the protocol of Fraser *et al.* (2000). Astaxanthin (Sigma A-9335) was added as an internal control prior to extraction. Compounds were separated by HPLC and identified by their UV/Vis absorbance spectra on a photodiode array detector as described in Fraser *et al.* (2000) as well as comparison to standards purified from *Escherichia coli* strains expressing carotenoid biosynthetic genes (Cunningham and Gantt, 2007).

#### TomloxC mapping

*S. lycopersicum*, *S. pennellii* DNA, and DNA from 74 *S. pennellii* introgression lines (Eshed and Zamir, 1995) were amplified with TomloxC forward primer 5'-TCCGATTGGTTACGGTATGTT and TomloxC reverse primer 5'-TGTTCAACAGTCATATTGTTTCCA using a 60 °C annealing temperature. The 471 bp PCR products were digested with *Hind*III. *S. pennellii* amplification products digested with *Hind*III, whereas *S. lycopersicum* products did not.

#### Statistical analysis

The volatile level (ng g<sup>-1</sup> FW h<sup>-1</sup>) values were first log<sub>2</sub> transformed. Values of zero (undetectable) were replaced by the smallest non-zero value in the whole dataset before log<sub>2</sub> transformation. Then, for each volatile, two-way ANOVA analysis was performed to test whether there is a significant difference in the volatile level among the ILs, as well as the effect of different trials on the volatile level. The raw *P* values were corrected for multiple tests using the Benjamini and Hochberg false discovery rate (FDR) test (Benjamini and Hochberg, 1995). As expected, both line and trial have significant effects on the levels of all volatiles investigated in this study (data not shown). Then, for each volatile, each IL was compared to the control line (LA4024) using Dunett's test to determine whether the volatile level in the IL is significantly different from the control line. The resulting raw *P* values were also corrected for multiple tests using the Benjamini and Hochberg FDR test. All the statistical analyses were performed using the SAS program (SAS institute, Cary, NC). The complete dataset is provided in Supplementary Table S1 at JXB online.

The same analysis was also performed on the dataset of *S. pennellii* derived ILs described in Tieman *et al.* (2006a). All the data and analysis results are available at the Tomato Functional Genomics Database (<http://ted.bti.cornell.edu>).

#### Construction of metabolite trees

Volatile profile data (the ratio between each IL and the *S. lycopersicum* parents) of the *S. habrochaites* and *S. pennellii*-derived ILs were combined and log<sub>2</sub> transformed. The log<sub>2</sub> transformed volatile profiles were clustered hierarchically using the average linkage clustering method implemented in the BCLUST program (Zhang and Zhao, 2000). The Pearson correlation coefficient was used to measure the similarity of the profiles in the clustering. The Robustness of the clustering trees was tested using the bootstrap method implemented in BCLUST (Zhang and Zhao, 2000) with 1000 replicates. The clustering trees were drawn using the MEGA3 program (Kumar *et al.*, 2004).

## Results

#### Volatiles analysis of the *S. habrochaites* and *S. lycopersicum* parent lines

Monforte and Tanksley (2000) described a set of near isogenic lines derived from a cross between *S. lycopersicum* LA4024 and *S. habrochaites* accession LA1777. To assess

the potential of this population for the identification of volatile QTLs, emissions of the most important flavour-related volatiles from ripe fruits of LA4024 and LA1777 (Table 1) were examined. The results indicated that the volatile profiles of LA1777 are very different from those of tomato. Particularly striking are the differences in apocarotenoid volatiles. As expected, emissions of 6-methyl-5-hepten-2-one (MHO) were greatly reduced in the green-fruited LA1777. MHO is derived from oxidative cleavage of lycopene, the primary pigment in red-fruited tomatoes (Vogel *et al.*, 2008). Surprisingly, the fruits emit relatively large quantities of β-ionone and β-damascenone, two volatiles believed to be derived from cyclic carotenoid precursors. Emissions of methyl jasmonate, an important plant defence compound, are also highly elevated in *S. habrochaites* relative to *S. lycopersicum* fruits (Table 1).

#### Identification of loci altered in volatile content

Overall, the large number of differences in volatile emissions encouraged us to assess the IL population derived from the cross between *S. lycopersicum* and *S. habrochaites* (LA1777) for QTLs. A set of 89 ILs was chosen that together cover most of the tomato genome. The lines were evaluated over several seasons and locations. The portions of chromosome replacement for each line are defined by a limited number of markers (Monforte and Tanksley, 2000). Overlaps between different IL segments permit assignment of a QTL to a 'bin' defined by the overlap. In this manner, ILs with overlapping chromosome segments showing the same significant volatile changes confirm the existence of a QTL, while ILs with overlapping segments that show different volatile profiles delimit the bins. A set of approximately 40 volatiles were evaluated as previously described (Tieman *et al.*, 2006a). Using a FDR cut-off of 0.05, a total of 30 loci altered in one or more flavour volatiles was identified (Fig. 2; Table 2). Since some of the identified loci are affected in biochemically unrelated volatiles, the actual number of QTLs is probably larger than 30. QTLs having both a positive and a negative influence on volatile emissions were identified in the screens.

As would be expected, loci affecting biochemically related volatiles were identified. For example, loci that coordinately influence emissions of multiple six-carbon (C6) volatiles were identified (1B, 12C). Similarly, several instances of co-ordinated effects on five-carbon (C5) volatiles were observed (1B, 4D, 6A, 6C). The C6 volatiles are known to be synthesized from linoleic and linolenic acids via a 13-lipoxygenases pathway (Bate and Rothstein, 1998; Chen *et al.*, 2004). As has previously been observed with ILs derived from a cross between *S. lycopersicum* and *S. pennellii* (Tieman *et al.* 2006a), there are also several loci affected in emissions of multiple volatiles believed to be derived from leucine or isoleucine (1D, 4E, 5A, 7A).

**Table 1.** Volatiles emitted by ripe fruits of the two parents of the IL population, *S. habrochaites* LA1777 and *S. lycopersicum* LA4024

Volatile (ng g <sup>-1</sup> FW h <sup>-1</sup> )	<i>S. habrochaites</i> LA1777	<i>S. lycopersicum</i> LA4024	Ratio LA1777/LA4024
β-Damascenone	0.18±0.07	0.01±0.00	30.31
Methyl jasmonate	0.69±0.55	0.03±0.01	23.53
β-Ionone	0.31±0.21	0.03±0.00	12.00
2-Methylbutanal	12.53±1.54	2.56±0.42	4.90
<i>cis</i> -2-Penten-1-ol	2.68±1.31	0.64±0.09	4.15
Isobutyl acetate	11.14±4.07	2.71±0.47	4.11
Geranylacetone	10.04±4.58	2.65±0.65	3.78
Phenylacetaldehyde	1.03±0.29	0.28±0.09	3.69
2-Methylbutanol	46.96±22.95	14.99±2.99	3.13
Benzaldehyde	14.90±7.45	4.93±0.81	3.02
1-Penten-3-ol	10.78±3.61	3.64±0.28	2.96
1-Penten-3-one	2.00±0.81	0.68±0.11	2.93
Pentanal	12.78±4.42	5.07±0.36	2.52
Methylsalicylate	3.15±2.98	1.26±0.28	2.50
Methyl benzoate	4.00±2.00	1.61±0.49	2.48
<i>cis</i> -3-Hexenal	169.21±117.83	69.13±13.94	2.45
2-Methoxyphenol	2.39±1.26	1.01±0.31	2.36
<i>trans</i> -2-Hexenal	4.47±1.53	2.52±0.72	1.77
Methional	0.24±0.18	0.14±0.02	1.70
Geranial	0.37±0.28	0.23±0.07	1.65
<i>cis</i> -3-Hexen-1-ol	63.87±32.25	45.12±9.39	1.42
<i>trans</i> -2-Pentenol	0.81±0.50	1.14±0.16	0.72
Benzyl alcohol	0.27±0.15	0.43±0.11	0.63
2-Phenylethanol	0.24±0.04	0.42±0.15	0.58
Hexyl alcohol	20.02±16.24	36.08±13.02	0.56
3-Methylbutanol	15.74±6.04	34.80±5.91	0.45
Hexanal	54.12±27.38	120.18±14.58	0.45
1-Pentanol	2.21±1.42	4.98±1.07	0.44
1-Nitro-2-phenylethane	0.42±0.28	1.25±0.30	0.33
<i>trans</i> -2-Heptenal	0.20±0.10	0.84±0.23	0.24
2-Isobutylthiazole	0.48±0.29	5.05±0.75	0.09
Isovaleronitrile	0.61±0.33	6.90±1.80	0.09
6-Methyl-5-hepten-2-one	0.27±0.10	6.31±1.45	0.04

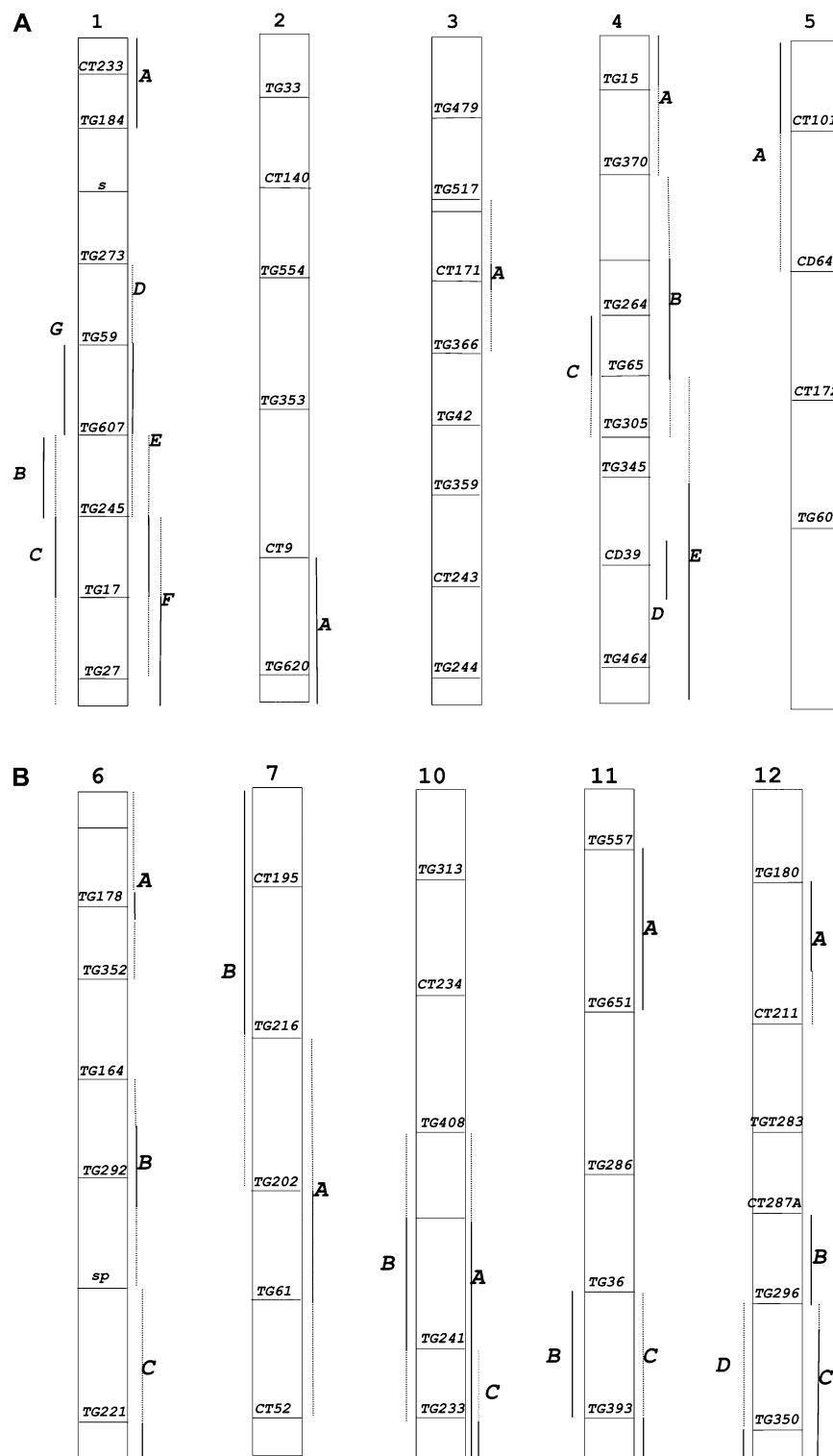
While the pathway(s) for synthesis of 2-methylbutanal, 2-methylbutanol, 3-methylbutanal, and 3-methylbutanol have not been established in plants, structural considerations support leucine and isoleucine precursors.

One previously undescribed QTL localized on chromosome 2 (2A) of ILs 3922 and 3923 was associated with a greatly reduced accumulation of carotenoids and their volatile apocarotenoid derivatives (Fig. 3). Fruits containing the *S. habrochaites* 2A locus accumulated ripening-associated lycopene much slower than the controls and never turned fully red. Carotenoid analysis revealed that the fruits are deficient in lycopene and its linear carotenoid precursors (Fig. 3C). While the gene encoding vegetative phytoene synthase, *PSY2*, is located on chromosome 2, the two ILs have the wild-type *S. lycopersicum* *PSY2* gene (data not shown). Further, the *PSY1* gene, encoding the enzyme responsible for fruit lycopene production, is localized on chromosome 3. It has previously been shown that the carotenoid content of a fruit is directly correlated with apocarotenoid emissions (Lewinsohn *et al.*, 2005; Tieman *et al.*, 2006a). For the ILs 3922 and 3923, much lower emissions of geranylacetone and MHO are the consequence of significantly lower levels of lycopene and its more saturated precursors (Fig. 3B, C). Unexpectedly,

levels of β-carotene and its corresponding apocarotenoid volatile β-ionone were lower, but not significantly reduced (see the Supplementary data at *JXB* online). A significant pool of β-carotene exists in fruits prior to ripening as part of the chloroplast photosynthetic apparatus (Fraser *et al.*, 1994) and this may be the source of the β-ionone.

#### Metabolite cluster analysis

Despite the importance of the volatiles analysed in the present work to tomato flavour as well as to the flavour of many other fruits and vegetables, the genes controlling the synthesis of many of them have not been identified. In some instances, the pathways for synthesis are not fully elaborated. Cluster analysis of metabolites can be a valuable way to gain insights into pathways for synthesis (Tikunov *et al.*, 2005; Morreel *et al.*, 2006). Volatiles derived from the same precursors should group together. In theory, clustering can also be informative about the rate-limiting steps in synthesis. In order to gain further insights into the particular set of volatiles analysed here, the data set used in the mapping was combined with the previously generated data set from ILs derived from a cross between *S. lycopersicum* and *S. pennellii* (Tieman *et al.*, 2006a). This approach allowed us to cluster the data

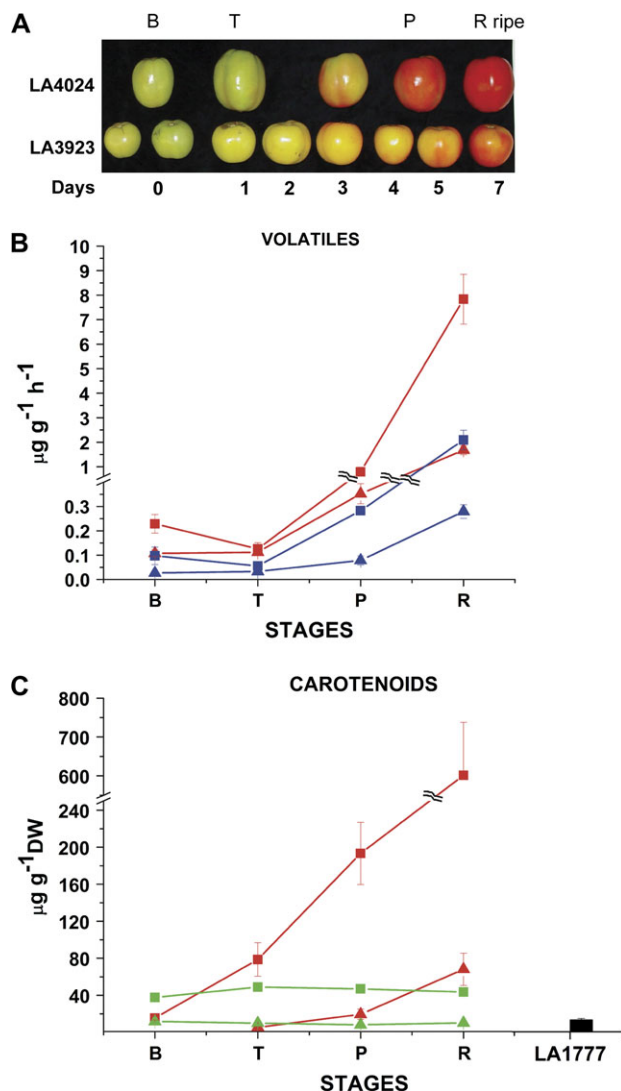


**Fig. 2.** Map locations of QTLs with altered volatile emissions. The labels refer to loci identified in Table 2. The solid lines indicate the space between markers that are present in an IL with a given QTL. Dashed lines indicate the distance to the next adjacent marker absent from the relevant line(s). No QTLs were identified on chromosomes 8 and 9.

**Table 2.** QTLs affected in volatile emissions identified in the present study

The loci refer to map positions as indicated in Fig. 2. The ratio of emissions of the IL relative to the *S. lycopersicum* parent is indicated for each volatile. All values are significant at  $P < 0.01$  unless indicated with an asterisk for which the value is significant at  $P < 0.02$ .

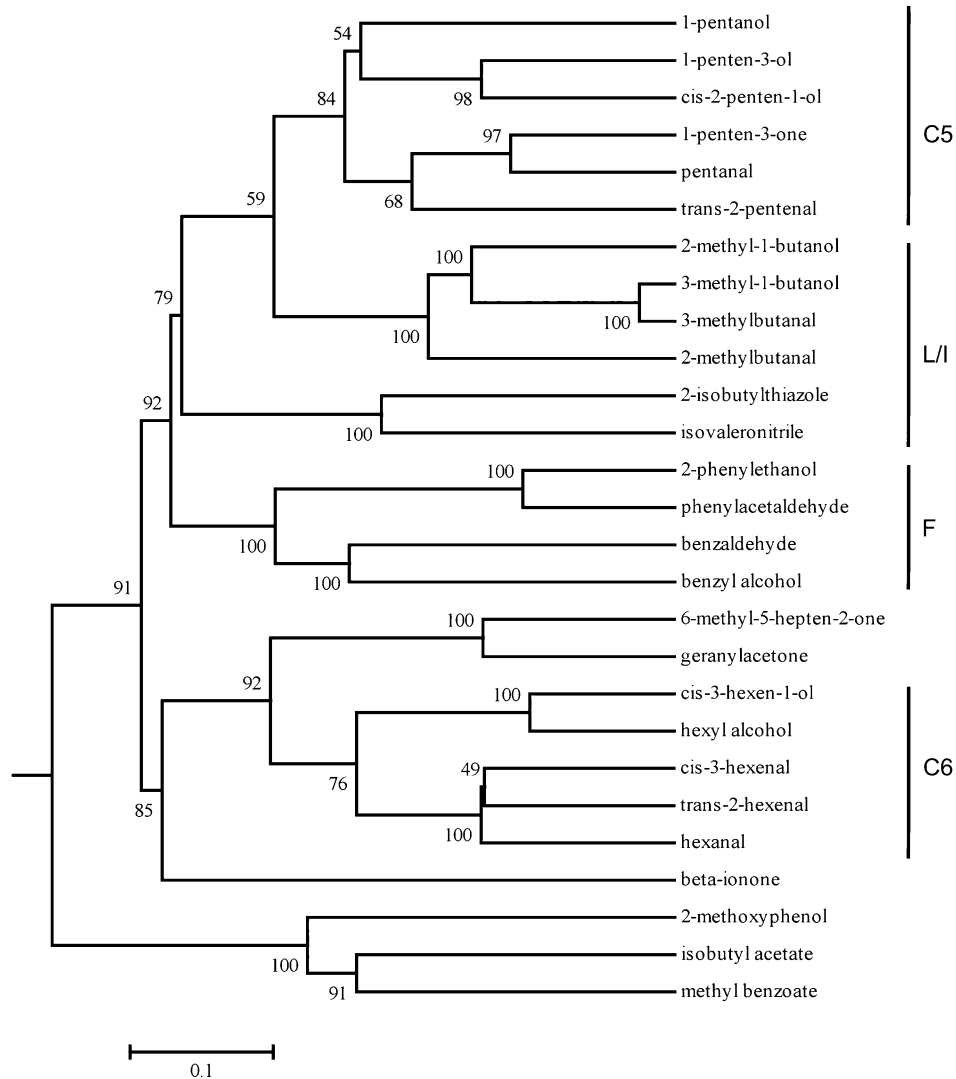
Locus	Volatile	Line	Ratio (IL/LA4024)
1A	<i>cis</i> -3-Hexenal	LA3920	0.19
1B	1-Penten-3-ol	LA3995	0.62
	<i>cis</i> -2-Penten-1-ol	LA3995	0.40
	Hexyl alcohol	LA3917	0.18
	Hexanal	LA3917	0.20
	<i>cis</i> -3-Hexen-1-ol	LA3995	0.47
	<i>cis</i> -3-Hexenal	LA3995	0.24
1C	Isobutyl acetate	LA3916	11.54
1D	3-Methylbutanol	LA3917	0.15
	2-Methylbutanol	LA3913	0.17
	Isovaleronitrile	LA3917	0.11
1E	2-Phenylethanol	LA3916	11.70
	Methylbenzoate	LA3915	4.30
1F	2-Methoxyphenol	LA3998	14.80
1G	Methional	LA3917	0.26
2A	Geranylacetone	LA3922	0.05
	6-Methyl-5-hepten-2-one	LA3923	0.05
3A	<i>cis</i> -3-Hexenal	LA3944	0.25
4A	2-Isobutylthiazole	LA4000	0.14
4B	2-Phenylethanol	LA3932	0.17
4C	3-Methylbutanol	LA3932	0.20
4D	1-Penten-3-one	LA3935	2.18
	1-Penten-3-ol	LA3937	1.61
	1-Pentanol	LA3935	1.72
	Pentanal	LA3937	1.75
4E	3-Methylbutanal	LA4007	2.70
	3-Methylbutanol	LA4007	3.38
5A	2-Phenylethanol	LA3980	5.10
	Isobutyl acetate	LA3938	3.14
	2-Methylbutanol	LA3938	4.60
	Isovaleronitrile	LA3980	5.25
	2-Methylbutanal	LA3980	3.63
	3-Methylbutanal	LA3980	2.31
	2-Isobutylthiazole	LA3980	3.00
	Benzyl alcohol	LA3980	3.62
6A	1-Penten-3-ol	LA3945	2.41
	1-Penten-3-one	LA4009	1.95*
6B	<i>cis</i> -3-Hexenal	LA3944	0.25
6C	1-Penten-3-one	LA4008	3.00
	1-Penten-3-ol	LA4008	2.00
7A	2-Methylbutanal	LA3950	0.22
	3-Methylbutanal	LA3950	0.17
	<i>trans</i> -2-Pentanal	LA3950	0.04
7B	2-Methylbutanal	LA4009	0.42
10A	Benzaldehyde	LA3965	3.91
	Benzyl alcohol	LA3965	6.30
10B	Isovaleronitrile	LA3965	3.96
	Methional	LA3965	2.92
10C	1-Nitro-2-phenylethane	LA3946	0.22
11A	Benzaldehyde	LA4003	0.32
11B	2-Methoxyphenol	LA3995	32.63
11C	Benzaldehyde	LA3964	3.32
	Benzyl alcohol	LA3965	6.28
	Isovaleronitrile	LA3965	3.96
12A	1-Penten-3-one	LA4002	2.01
12B	3-Methylbutanal	LA3969	2.40
12C	Hexanal	LA3995	0.30
	<i>cis</i> -3-Hexenal	LA3995	0.24
	<i>cis</i> -3-Hexen-1-ol	LA3999	0.36
12D	Isobutyl acetate	LA3998	23.30



**Fig. 3.** Carotenoids and apocarotenoid volatiles produced by LA3923. (A) Developmental series of fruits from control (LA4024, squares) and LA3923 (triangles) harvested at breaker (0) and subsequent days indicated below (see Materials and methods). (B) Emissions of 6-methyl-5-hepten-2-one (MHO) (red) and geranylacetone (blue) from fruits at the indicated stages of ripening. (C) Quantification of lycopene (red) and  $\beta$ -carotene (green) from the same fruits as for (B). LA1777 is the *S. habrochaites* parent. Note the broken ordinate scales for values on the graphs shown in (B) and (C). Graphs show values  $\pm$ SE.

from 74 ILs derived from a cross between *S. lycopersicum* and *S. pennellii*, 89 ILs derived from a cross between *S. lycopersicum* and *S. habrochaites*, as well as the four parents grown over 4 years at three different locations. This large number of data points from two IL populations and their parents yielded the tree shown in Fig. 4. The correlation matrix of the volatile profiles as well as a heatmap of the matrix are provided as supplementary information in Table S2 and Fig. S1 at *JXB* online.

Validation of the approach is provided by certain predictions. All of the C5 volatiles cluster together, as do the C6 volatiles. Within the C5 and C6 volatile clusters,



**Fig. 4.** Volatiles tree. The relationships among the various volatiles in this study was determined by clustering of the data derived from 74 ILs derived from a cross between *S. lycopersicum* and *S. pennellii*, 89 ILs derived from a cross between *S. lycopersicum* and *S. habrochaites*, as well as the four parents of the two populations. The method for derivation of the tree is described in Materials and methods. Numbers are bootstrap values for each branch. C5, five-carbon volatiles; C6, six-carbon volatiles; L/I, leucine or isoleucine-derived volatiles; F, phenylalanine-derived volatiles.

there is a clear separation of the aldehydes from the alcohols. Synthesis of the C5 and C6 volatiles is likely to be initiated by the action of a lipoxygenase or hydroperoxide lyase. A lipoxygenase gene has been shown to be responsible for the production of C6 volatiles in tomato fruit (Chen *et al.*, 2004). A marker that is polymorphic between *S. lycopersicum* and *S. pennellii* was used to determine if *TomloxC* maps to one of the loci identified with altered C6 or C5 volatiles. By screening the ILs derived from a cross between *S. lycopersicum* and *S. pennellii* (Eshed and Zamir, 1995) for the presence or absence of that polymorphism, it was possible to map *TomloxC* to the top of chromosome 1 (bin 1A). This is the same region associated with much lower levels of *cis*-3-hexenal in *S. habrochaites* QTL 1A (Fig. 2; Table 1). These data suggest that identifying loci associated with

altered volatile levels can be used as a tool to identify the genes responsible for these traits.

Similar clustering of most, though not all, of the phenylalanine-derived volatiles is observed. For example, it has been shown that 2-phenylethanol is derived from phenylacetaldehyde by the action of a reductase enzyme (Tieman *et al.*, 2007). These two compounds have a bootstrap value of 100 (Fig. 4).

An unexpected result from the clustering concerns the accumulation of the linear and cyclized apocarotenoid volatiles. Synthesis of these two classes of apocarotenoid volatiles is not highly correlated. The linear apocarotenoids geranylacetone and MHO clustered together with a bootstrap value of 100, whereas  $\beta$ -ionone is separated from these two volatiles on the metabolic tree. Although  $\beta$ -damascenone was not included in the earlier study of

ILs derived from the cross between *S. lycopersicum* and *S. pennellii* (Tieman *et al.*, 2006a), it was included in the analysis of ILs derived from the cross between *S. lycopersicum* and *S. habrochaites*. The pathway for synthesis of this apocarotenoid has not been determined, but our data indicate that it is probably derived from  $\beta$ -carotene since it clustered with  $\beta$ -ionone, having a bootstrap value of 100 (data not shown). The results suggest that there are probably significant differences in the two pools of carotenoids. This conclusion is further bolstered by the differential effects on apocarotenoid volatiles produced by the 3922 and 3923 lines (Fig. 3).

The clustering predicts that 2-isobutylthiazole and isovaleronitrile should be synthesized from leucine (Fig. 4). While this has not been formally shown, such a relationship makes structural sense. The data also show an unexpected relationship between 2-methoxyphenol and methylbenzoate. Methylbenzoate does not cluster with benzaldehyde or benzyl alcohol, other compounds derived from benzoic acid, suggesting that the methyltransferase(s) responsible for its synthesis is limiting.

## Discussion

The approach of using wild relatives of crop species as a source of valuable genes is well established. For example, in tomato, the sexually compatible wild relatives have been used as a source of disease resistance (Rick and Chetelat, 1995). ILs derived from a cross between *S. lycopersicum* and *S. pennellii* are a rich source of QTLs affecting fruit volatile content (Tieman *et al.*, 2006a) as well as a wide range of primary metabolites (Schauer *et al.*, 2006). *S. habrochaites* is another wild relative of tomato that produces extremely high levels of ethylene during climacteric ripening (Grumet *et al.*, 1979). The ripe fruits are pale green with a purple stripe, lacking significant quantities of lycopene. Monforte and Tanksley (2000) described a set of near isogenic lines derived from a cross between *S. lycopersicum* and *S. habrochaites* accession LA1777. In the present work, the IL population derived from this cross was examined for volatile content, allowing us to identify at least 30 loci affecting the emission of one or more volatiles. Using the biochemical data generated in the analysis of both populations, it was possible to derive a metabolic tree for the major flavour-associated tomato volatiles. The associations derived from clustering of the 167 lines permitted relationships among the compounds to be defined. These relationships are predictive of both commonalities of precursors and of limiting steps in the pathways for their synthesis.

In several instances, the *S. habrochaites*-derived QTLs identified in this study localize to similar positions as previously mapped QTLs derived from a cross between two tomato cultivars (Saliba-Colombani *et al.*, 2001) or the IL population derived from a cross between *S.*

*lycopersicum* and *S. pennellii* (Tieman *et al.*, 2006a). These potentially common loci are listed in Table 3. It must be noted that each of the three studies analysed slightly different subsets of volatiles. Further, the rather large segments defined by some of the ILs do not exclude multiple loci affecting the same volatiles. By examining the patterns of cosegregation between the different volatiles as well as the relationships shown on the metabolic tree (Fig. 4), we can begin to extract information about the various metabolic pathways.

### Apocarotenoid volatiles

A QTL on chromosome 2, with greatly reduced emissions of two apocarotenoid volatiles, geranylacetone and MHO, was identified. These reduced emissions are a consequence of the significantly lower levels of linear carotenoid substrates present in ripening LA3922 and LA3923 fruits. There are also lower levels of  $\beta$ -carotene and its corresponding apocarotenoid,  $\beta$ -ionone, in these fruits although the reductions are not statistically significant. All of these volatiles are produced by the oxidative cleavage of double bonds present in the backbones of the various carotenoids. It has previously been shown that the carotenoid cleavage dioxygenases, CCD1A and CCD1B, are at least partially responsible for the synthesis of the apocarotenoid volatiles (Simkin *et al.*, 2004). The CCD1 enzymes have broad substrate specificity, cleaving multiple linear and cyclic carotenoids at either the 5,6 or 9,10 double bond positions (Vogel *et al.*, 2008). Interestingly, the apocarotenoid volatiles are not emitted until relatively late in fruit development (Tieman *et al.*, 2006a) and the carotenoid content of the fruit determines which apocarotenoids are synthesized (Lewinsohn *et al.*, 2005; Tieman *et al.*, 2006a). Further, *LeCCD1A* and *LeCCD1B* have been mapped and neither gene corresponds to an apocarotenoid QTL (data not shown). CCD1 enzymes lack plastid targeting signals and are cytoplasmic (Auldridge *et al.*, 2006). Since expression of the *CCD1* genes greatly precedes volatile emissions (Simkin *et al.*, 2004), it is possible that the

**Table 3.** QTLs from the present study with possible overlap to previously identified QTLs altered in volatile emissions (Saliba-Colombani *et al.*, 2001; Tieman *et al.*, 2006a)

QTL	Affected volatiles	Reference
1A	<i>cis</i> -3-Hexenal	Saliba-Colombani
1B	<i>cis</i> -2-Penten-1-ol	Tieman
1F	2-Methoxyphenol	Tieman
4A	Isobutylthiazole	Saliba-Colombani
4D	Pentanal, 1-penten-3-one	Tieman
4E	3-Methylbutanal, 3-Methylbutanol	Tieman
5A	2-Methylbutanol, 3-Methylbutanal, isovaleronitrile	Tieman
7A	3-Methylbutanal	Tieman
10B	Isovaleronitrile	Tieman

limiting step in apocarotenoid synthesis is the access of the enzymes to the substrates, possibly during or subsequent to conversion of chloroplasts to chromoplasts. Notably, the apocarotenoids cluster with the C6 volatiles in the metabolic tree (Fig. 4). It has been proposed that the synthesis of the C6 volatiles is limited by the availability of non-esterified fatty acids to the chloroplast-localized 13-lipoxygenase (Chen *et al.*, 2004). The chloroplast to chromoplast transition and disruption of the thylakoid membranes that bring 13-lipoxygenase in contact with its fatty acid substrates may also permit access of CCD enzymes to their carotenoid substrates.

The differential effects on the emissions of geranylacetone and MHO versus  $\beta$ -ionone and  $\beta$ -damascenone in LA3922 and LA3923 suggest that these two sets of volatiles could be derived from different pools of carotenoids. This conclusion is supported by the clustering of MHO and geranylacetone and their separation from  $\beta$ -ionone and  $\beta$ -damascenone (Fig. 4). Since the same enzyme(s) are possibly involved in the synthesis of both linear and cyclic apocarotenoids (Simkin *et al.*, 2004; Vogel *et al.*, 2008), the data strongly suggest that they are derived from separate substrate pools of carotenoid precursors. Geranylacetone can only be derived from the 9,10 bond cleavage of more saturated linear carotenoid precursors of lycopene. These precursors do not normally accumulate to significant levels in tomato fruits (Fraser *et al.*, 1994).  $\beta$ -ionone, in contrast, is derived from 9,10 and/or 9',10' bond cleavage of  $\beta$ -carotene (Vogel *et al.*, 2008).

Notably, a pathway for synthesis of  $\beta$ -damascenone has not been established, although this compound has been postulated to be generated from neoxanthin, with grasshopper ketone as an intermediate (Skouroumounis *et al.*, 1993). The cluster analysis strongly supports  $\beta$ -carotene as the precursor, although there must be subsequent modifications.

#### Leucine and isoleucine-derived volatiles

On the basis of structural considerations, it is assumed that 3-methylbutanal and 3-methylbutanol are derived from leucine while 2-methylbutanal and 2-methylbutanol are derived from isoleucine. The precise pathways for their synthesis and the responsible enzymes have not been identified in plants but a pathway has been described in yeast (Dickinson *et al.*, 2000, 2003). This proposed pathway begins with the action of branched chain aminotransferases (BCATs) that remove the amino groups from the respective amino acids. Subsequently, there is a decarboxylation to produce the aldehydes and a reduction to form the alcohols. The tight linkage within the metabolite clustering of the two aldehydes and the two alcohols indicates co-ordinate synthesis (Fig. 4). In *Arabidopsis thaliana*, AtBCAT1 has been shown to catalyse the deamination of all three branched chain amino acids (leucine, isoleucine, and valine) (Schuster and Binder, 2005).

Multiple QTLs affect the emission of the volatiles derived from branched chain amino acids. Seven out of the 30 QTLs identified here are affected in one or more of these volatiles. There are six BCAT enzymes in *Arabidopsis*, although nothing is known about their relative contributions to anabolic versus catabolic functions. It is also possible that amino acid pools may contribute to the regulation of volatile synthesis. Several QTLs affecting fruit leucine and isoleucine pools were identified within the IL population derived from a cross between *S. lycopersicum* and *S. pennellii* (Schauer *et al.*, 2006) and these could influence the rates of volatile synthesis. A QTL for higher leucine and isoleucine levels was found at the bottom of chromosome 4. This locus was associated with higher levels of 3-methylbutanol and 3-methylbutanal in the IL populations derived from crosses of both *S. pennellii* and *S. habrochaites* with *S. lycopersicum*. Conversely, a QTL for lower isoleucine levels is located at the top of chromosome 5, but higher levels of 2-methylbutanol, 3-methylbutanal, and isovaleronitrile were observed in both IL populations. (Table 3; Schauer *et al.*, 2006) It is possible that multiple loci for the isoleucine and leucine-derived volatiles are the result of altered substrate levels or altered biosynthetic enzyme activities.

#### C5 and C6 volatiles

Several loci that are affected in C5 or C6 volatiles were identified. A locus on chromosome 1 (1B) was associated with lower levels of both C5 and C6 alcohols and C6 aldehydes (Table 2; Fig. 2). Interestingly, a locus in the same region of chromosome 1 in the IL population derived from a cross between *S. lycopersicum* and *S. pennellii* was associated with higher levels of C5 alcohols and aldehydes.

The metabolite clustering of all C6 volatiles in a single branch is consistent with the results of Chen *et al.* (2004), who observed that antisense reduction of one isoform of the 13-lipoxygenase, TomloxC, resulted in almost complete loss of multiple C6 volatiles in tomato fruits. These authors did not report on synthesis of C5 volatiles. TomloxC maps to bin 1A in the *S. pennellii* population, a region associated with lower levels of *cis*-3-hexenal (Tables 2, 3). These data further suggest that TomloxC is responsible for the formation of C6 volatiles in tomato fruit.

The metabolite clustering results indicate that the C5 volatiles are all co-ordinately synthesized. Although the biosynthetic pathway of the C5 volatiles has not been elucidated, it has been shown that 1-penten-3-ol was directly generated from a so-called C<sub>5</sub>-13-cleavage activity of lipoxygenase(s) in soybean (Salch *et al.*, 1995; Fisher *et al.*, 2003). The combined tree does not closely cluster C5 and C6 volatiles, suggesting that 13-lipoxygenase alone cannot be the limiting factor for their synthesis.

The C5 aldehydes and C5 alcohols are separated within the C5 branch of the metabolite tree. The C6 alcohols also cluster separately from the C6 aldehydes. These results are

consistent with a single reductase/alcohol dehydrogenase enzyme acting on multiple aldehydes. Speirs *et al.* (1998) have reported that antisense knockdown of a single gene, *ADH2*, influenced the aldehyde to alcohol ratios of both hexanol and *cis*-3-hexenol. These results are also consistent with reductase activity being limiting for the aldehyde to alcohol conversion in both instances.

### Phenolics

2-methoxyphenol, also known as guaiacol, is a compound that imparts a distinct flavour, frequently associated with off-odours. Tikunov *et al.* (2005) have reported that 2-methoxyphenol and methylbenzoate were closely associated with other phenylpropanoids such as salicylaldehyde, eugenol, methyl salicylate, and ethyl salicylate. This metabolite cluster analysis clearly separates 2-methoxyphenol and methyl benzoate from other phenylpropanoids. These data suggest that methylation of the phenylpropanoids is separately regulated from the synthesis of the substrates. If methylation is also limiting for the synthesis of 2-methoxyphenol, the results would implicate catechol as the immediate precursor to 2-methoxyphenol. It should be noted that there is no obvious relationship with a third volatile, isobutyl acetate, which is also part of this same cluster.

Although its biosynthetic pathway has not been determined in tomato, researchers have shown that *O*-methyltransferases are capable of methylating catechol and related polyphenolics *in vitro* (Pellegrini *et al.*, 1993; Maury *et al.*, 1999; Lavid *et al.*, 2002). These phenolics have been shown to be important defence compounds against pathogens (Chet *et al.*, 1978) and insects (Duffey and Isman, 1981) and transcription of the *O*-methyltransferase genes is frequently up-regulated by exposure to pathogens (Pellegrini *et al.*, 1993; Lavid *et al.*, 2002). Similarly, methylbenzoate has been shown to be synthesized by a benzoic acid/salicylic acid carboxyl methyltransferase (BSMT) from benzoate in several plant organisms (Effmert *et al.*, 2005). However, the enzymes responsible for synthesis of methylbenzoate are believed to be quite distinct from the catechol-*O*-methyltransferases.

By examining an IL population derived from a cross between *S. lycopersicum* and *S. habrochaites*, multiple loci involved in the altered production of flavour volatiles have been identified. The QTLs presented in this paper can be used as a tool to identify genes involved in the production of flavour volatiles. Along with the previously published data describing volatile analysis of an IL population derived from a cross between *S. lycopersicum* and *S. pennellii* (Tieman *et al.*, 2006a), these data were used to construct the metabolite tree. This tree can be used to determine relationships and possible common biosynthetic pathways within groups of volatiles. Mapping of TomLoxC to a locus altered in *cis*-3-hexenal further demonstrates the use of QTL mapping for the identification of pathways to important flavour volatiles.

### Supplementary data

Supplementary materials for this manuscript can be found at *JXB* online. The combined values for all volatiles and lines can be found in Supplementary Fig. S1 and in Supplementary Tables S1 and S2.

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