

RESEARCH PAPER

Identification of loci affecting flavour volatile emissions in tomato fruits

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Abstract

Fresh tomato fruit flavour is the sum of the interaction between sugars, acids, and a set of approximately 30 volatile compounds synthesized from a diverse set of precursors, including amino acids, lipids, and carotenoids. Some of these volatiles impart desirable qualities while others are negatively perceived. As a first step to identify the genes responsible for the synthesis of flavour-related chemicals, an attempt was made to identify loci that influence the chemical composition of ripe fruits. A genetically diverse but well-defined *Solanum pennellii* IL population was used. Because *S. pennellii* is a small green-fruited species, this population exhibits great biochemical diversity and is a rich source of genes affecting both fruit development and chemical composition. This population was used to identify multiple loci affecting the composition of chemicals related to flavour. Twenty-five loci were identified that are significantly altered in one or more of 23 different volatiles and four were altered in citric acid content. It was further shown that emissions of carotenoid-derived volatiles were directly correlated with the fruit carotenoid content. Linked molecular markers should be useful for breeding programmes aimed at improving fruit flavour. In the longer term, the genes responsible for controlling the levels of these chemicals will be important tools for understanding the complex interactions that ultimately integrate to provide the unique flavour of a tomato.

Key words: Carotenoids, fruit flavour, loci, tomato, volatiles.

Introduction

Human perception of flavour consists of a complex interaction between taste receptors in the tongue and olfactory receptors located in the nose. The contribution of smell, mediated by the olfactory system, to taste is not well understood. A necessary prerequisite to improving fresh, unprocessed foods is an understanding of the chemicals that contribute both positively and negatively to flavour. In the case of fresh tomato fruits, flavour is the sum of the interaction between sugars, acids, and approximately 400 volatile compounds (Buttery, 1993). Good tomato flavour requires minimum levels of sugars (glucose and fructose) and acids (citric and malic) that are perceived by receptors in the tongue. But the contributions of the volatile constituents perceived by the olfactory system are much less understood. Human sensitivity to volatile chemicals varies hugely and the impact of a volatile on flavour is determined by both its concentration and its odour threshold (Baldwin *et al.*, 2000). Based on these factors, a set of approximately 30 volatiles that significantly impact tomato flavour has been identified (Buttery, 1993; Buttery and Ling, 1993). Some of these volatiles impart desirable qualities while others are negatively perceived (Baldwin *et al.*, 1998).

The important tomato flavour volatiles are believed to be synthesized from a diverse set of precursors, including amino acids, lipids, and carotenoids. The structures of many of these important volatiles are shown in Fig. 1. While a few of the pathways are known, several of the pathways to the major volatiles have not been elucidated. For example,

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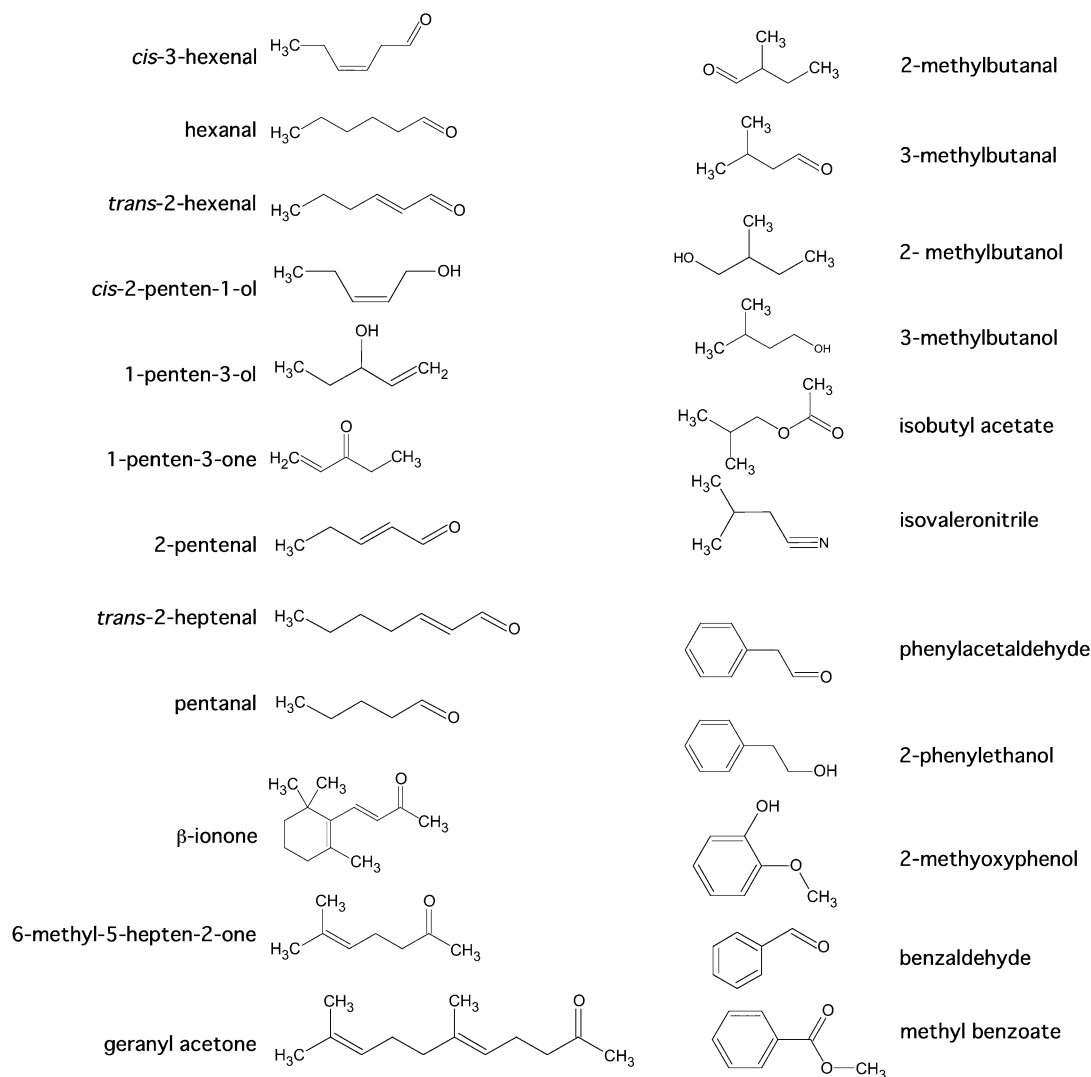


Fig. 1. Structures of volatile compounds associated with mapped loci. Compounds are grouped by the nature of the precursors.

3-methylbutanal and 3-methylbutanol are believed to be synthesized from leucine (Tressl and Drawert, 1973), but these relationships are largely based on structural considerations and empirical evidence is scarce. It is assumed that synthesis of this set of volatiles is initiated by enzymatic decarboxylation (van der Hijden and Bom, 1996), but none of the genes encoding activities essential for their synthesis or accumulation have been identified.

The flavour of fresh commercially-produced tomatoes is generally considered to be poor. The causes for the less-than-ideal quality of these tomatoes can be attributed both to the cultivars and handling of the harvested materials. Breeders have focused on traits related to production and handling, while flavour has not been a high priority. However, opportunities exist for premium quality produce and there is renewed interest in genetic markers linked to quality and, in particular, flavour. There has been significant effort to map genetic loci affecting the chemical com-

position of tomato fruits. For example, Saliba-Colombani *et al.* (2001) published a map of quantitative trait loci (QTLs) affecting various physical traits of tomato fruits, including loci affecting six volatiles. This map was generated from a cross between a large-fruited tomato and a cherry tomato. Causse *et al.* (2004) subsequently mapped QTLs of additional loci affecting sugars and organic acids. This latter study was performed on recombinant inbred lines (ILs) derived from a cross between a processing tomato, *Solanum lycopersicum* M82, and a small, green-fruited wild species, *S. pennellii* (Eshed and Zamir, 1995). Separately, a locus affecting 2-phenylacetaldehyde and 2-phenylethanol, *malodorous*, was identified from this same IL population (Tadmor *et al.*, 2002).

Although volatiles are generally acknowledged to be critical to flavour in tomato, only recently have a few of the genes encoding enzymes involved in their synthesis been identified. For example, a lipoxygenase (Chen *et al.*, 2004)

and an alcohol dehydrogenase (Speirs *et al.*, 1998) involved in the synthesis of C6 volatiles derived from linoleic and linolenic acid have been identified. Also dioxygenases involved in the synthesis of apocarotenoid volatiles have been identified (Simkin *et al.*, 2004). One approach to identifying the genes responsible for the synthesis of flavour volatiles is to exploit the natural diversity present in the germplasm in combination with genomics tools. This approach has been particularly fruitful for strawberry, where genes encoding biosynthetic enzymes for several important flavour volatiles have been identified (Aharoni *et al.*, 2000, 2004). As a first step to identify the genes responsible for the synthesis of the chemicals that contribute to tomato flavour, an attempt was made to identify loci that influence the chemical composition of ripe fruits. Although there is great diversity in flavour among cultivated tomato varieties (Baldwin *et al.*, 1991; Mayer *et al.*, 2004), the genetic make-up and the relationships between the different cultivars is rarely well defined. Therefore, this study's efforts have focused on the genetically diverse but well-defined *L. pennellii* Introgression Line (IL) population (Eshed and Zamir, 1995). This set of 75 lines is fixed such that each line contains a single introgressed portion of *S. pennellii* DNA in an otherwise fixed *S. esculentum* genome. Because *S. pennellii* is a small green-fruited species, this population exhibits great biochemical diversity and is a rich source of genes affecting both fruit development (Fridman *et al.*, 2004) and chemical composition (Causse *et al.*, 2004; Schauer *et al.*, 2005). This population was used here to identify multiple loci affecting the composition of chemicals related to flavour.

Materials and methods

Growth of plant materials and statistical analysis

Seventy-four *S. pennellii* introgression lines (Eshed and Zamir, 1995) and the *S. lycopersicum* control M82 were analysed for volatiles. All lines were grown in randomized, replicated plots in three different sites (Gainesville, Citra, and Live Oak, Florida), over the spring and the autumn of 2002 to 2004. Plants were grown using standard commercial practices in raised plastic mulched beds. Most lines were replicated over multiple years and seasons (autumn 2002: 69 lines; spring 2003: 73 lines; autumn 2003: 30 lines; spring 2004: 32 lines; autumn 2004: 15 lines). In the spring of 2003, lines were evaluated both in the field and in a greenhouse. For the purpose of the analysis presented here datasets from the two sites were combined. Fruits from all plants for each line were combined and analysed as they reached the red ripe stage. Each harvest is considered as a replication for its respective introgression lines. Trials where volatile data for a single harvest was available were discarded from further analysis.

Volatile determinations

Volatile level ($\text{ng g}^{-1} \text{FW h}^{-1}$) values were analysed with a mixed-model analysis of variance (ANOVA), using PROC MIXED in SAS (SAS Institute, Cary, NC). Each volatile was analysed individually in the model $y_{ijkl} = \mu + L_i + S_j + (L \times S)_{ij} + T_k + (S \times T)_{jk} + \epsilon_{ijkl}$, where y_{ijkl} is the l th observation of the i th introgression line, collected in the j th season and k th year. Variation associated with each introgression

line (L_i , $df=74$), season (S_j , $df=1$) and interactions were treated as fixed effects, while year (T_k) was considered as random. F -tests were carried out to identify the effects of season and season \times line interactions on each volatile. For identification of introgression lines that contributed to volatile variation, pairwise t -tests were done, comparing the level of each volatile between individual introgression lines and the control line M82. Residuals of the ANOVA were visually inspected using JMP (SAS Institute, Cary, NC) to confirm that consistency of error variances and normality of error terms were obtained.

Ripe tomato fruit from each plant from both replicates of each IL line were harvested and volatiles from pooled fruits were collected on the day after harvest. Carotenoid mutants and the corresponding controls were grown in the greenhouse and tomato fruit volatiles collected immediately after harvest. Tomato fruit volatiles were collected from chopped fruit with nonyl acetate as an internal standard as described by Schmelz *et al.* (2003). Briefly, chopped fruit was 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 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 $\text{ng g}^{-1} \text{FW h}^{-1}$ collection. Identities of volatile peaks were confirmed by GCMS as described by Schmelz *et al.* (2001).

Citric acid determination

Pooled tomato fruit were homogenized in a blender for 30 s and frozen at -80°C until analysis. Samples were thawed, centrifuged at $16\,000\text{ g}$ for 5 min. The supernatant was analysed for citric and malic acid content using a citric acid and malic acid analysis kits (R-Biopharm, Marshall, MI) according to the manufacturer's instructions.

Results

Volatile emissions from fruits of the IL parents

Previously Schauer *et al.* (2005) showed that the wild relatives of tomato exhibit significant variance from tomato in non-volatile chemical composition of leaves and fruits. They identified a number of biochemical differences between the two parents of the IL population, LA3475 (M82) and LA0716. In order to assess the potential utility of the IL population as a source of genes influencing the volatile constituents of flavour, a subset of volatiles emitted from ripe fruits of the two parents, M82 and LA0716 was evaluated. The results, shown in Table 1, indicated that the wild species does have significantly different emissions than M82 for volatiles derived from lipids (*trans*-2-hexenal, *cis*-3-henen-1-ol), carotenoids (geranyl acetone, 6-methyl-5-hepten-2-one), and amino acids (benzaldehyde, 2-phenylethanol).

The patterns of volatile emissions in the *S. lycopersicum* parent, M82, were further examined during fruit development. Emissions of a set of 23 volatiles were measured from the immature green to the light red (Stage 5) stages.

Table 1. Emissions of selected volatiles from ripe fruits of *S. lycopersicum* (M82) and *S. pennellii* (LA0716)Emitted volatiles were collected as described in the Materials and methods and quantified by GC-MS. Values are ng g⁻¹ FW h⁻¹.

| | e-2-hexenal | z-3-hexen-1-ol | benzaldehyde | 6-methyl-5-heptene-2-one | 2-phenylethanol | geranyl acetone |
|--------|-------------|----------------|--------------|--------------------------|-----------------|-----------------|
| M82 | 68.04 | 44.49 | 1.34 | 15.16 | 1.07 | 4.83 |
| LA0716 | 156.19 | 20.66 | 5.09 | 5.76 | 24.79 | 0.73 |

Emissions of most of the volatiles increased during ripening with 18 of the 23 having maximal emissions at either turning or light red (Fig. 2).

Identification of loci affecting chemical composition

All of the lines were grown in randomized, replicated plots in two different sites. Subsets of the most interesting lines were grown over three additional seasons. The effect of the autumn and spring seasons on the level of each volatile across all lines, and for each line specifically, was evaluated using analysis of variance (ANOVA). A significant season effect should be apparent if the level of a volatile is different across all introgression lines, in the autumn and spring seasons. On the other hand, when analysing the season effect on each line individually, it was examined whether specific lines have higher levels of one volatile in one of the two seasons. These latter effects are referred hereafter as season×line interactions.

For nine volatiles, a significant season×line interaction could be detected: methylsalicylate, 3-methylbutanol, phenylacetaldehyde, 2-phenylethanol, 1-penten-3-one, 2-methylbutanol, *trans*-2-pentenal, *trans*-2-heptenal, and 2-methoxyphenol (Table 1S of the supplementary material can be found at JXB online). For these nine volatiles, measurements made from spring and autumn materials were compared within each of the 74 introgression lines (pairwise *t*-test, Table 2S of the supplementary material can be found at JXB online). For 24 introgression lines, significant pairwise differences ($P < 0.01$) were detected in the level of up to four volatiles, in the spring and autumn. In summary, 24 introgression lines show variable levels of volatiles in the autumn and spring seasons. With a few exceptions, volatile levels in the autumn were lower than in the spring. Volatiles with levels significantly different between the autumn and spring across all lines have not been identified (Table 3S of the supplementary material can be found at JXB online). Lack of a detectable seasonal effect may be due to low statistical power to detect significant differences between seasons, because of the high variance within seasons.

Figure 3 shows the map positions of loci altered in volatile content. The overlap generated by different ILs permits the assignment of a locus to one of 107 regions, or bins (Eshed and Zamir, 1995). In a few cases, lines were not available. For loci affecting volatile composition as well as citric acid, a cutoff of $P < 0.05$ was used. Where possible, the focus was on loci that showed co-ordinated effects on

volatiles that were presumed to be related. For example, loci altered in multiple C5 or C6 volatiles that are assumed to be lipid-derived were identified. Using the above criteria, 25 loci were identified that were significantly altered in one or more of 23 different volatiles and four that were altered in citric acid content. With the exception of certain apocarotenoid volatiles associated with the *r*, *beta*, and *del* loci, the identified loci produced more of the metabolite than M82. The differences for each volatile are expressed as a percentage of M82 control in Table 2. Most of these loci are altered in multiple, metabolically-related volatile compounds. For example, there are seven loci that are altered in multiple C5 volatiles. In at least one instance, co-ordinated regulation of volatiles was observed that strongly supports a common pathway; increased emissions of isovaleronitrile were always associated with increases in one or more of the leucine and isoleucine-derived volatiles, for example. This correlation strongly suggests that isovaleronitrile is derived from one of these amino acids.

Evaluation of the citric and malic acid contents of the ILs indicated much greater variability from season to season than was observed with volatile composition. It was possible to identify four loci (chromosomes 5, 8, 9, 10) that are significantly higher in citric acid content. It was not possible to identify any loci consistently and significantly altered in malic acid content.

Characterization of carotenoid-derived volatiles

Several important volatiles are believed to be derived from carotenoids. It has previously been shown that a carotenoid cleavage dioxygenase, *LeCCD1*, can cleave multiple linear and cyclic carotenoids at the 9,10 position, generating geranylacetone, pseudoionone, and β -ionone. Antisense reduction of the tomato genes led to ~50% reduced emissions of geranylacetone and β -ionone, confirming roles of these enzymes. However, a reduction rather than a loss in emissions suggests that other factors are important for generating these apocarotenoid volatiles. Indeed, no QTL for any of these volatiles was associated with the map positions of *LeCCD1A* or *LeCCD1B* in the IL population (data not shown). In order to determine whether carotenoid composition is a determinant in the emission of apocarotenoid volatiles, the volatile emissions of several mutants altered in carotenoid biosynthetic genes were examined (Table 3). The *yellow flesh* (*r*) mutation results in loss of phytoene synthase function, greatly reducing ripening-associated carotenoid synthesis (Fray and Grierson, 1993).

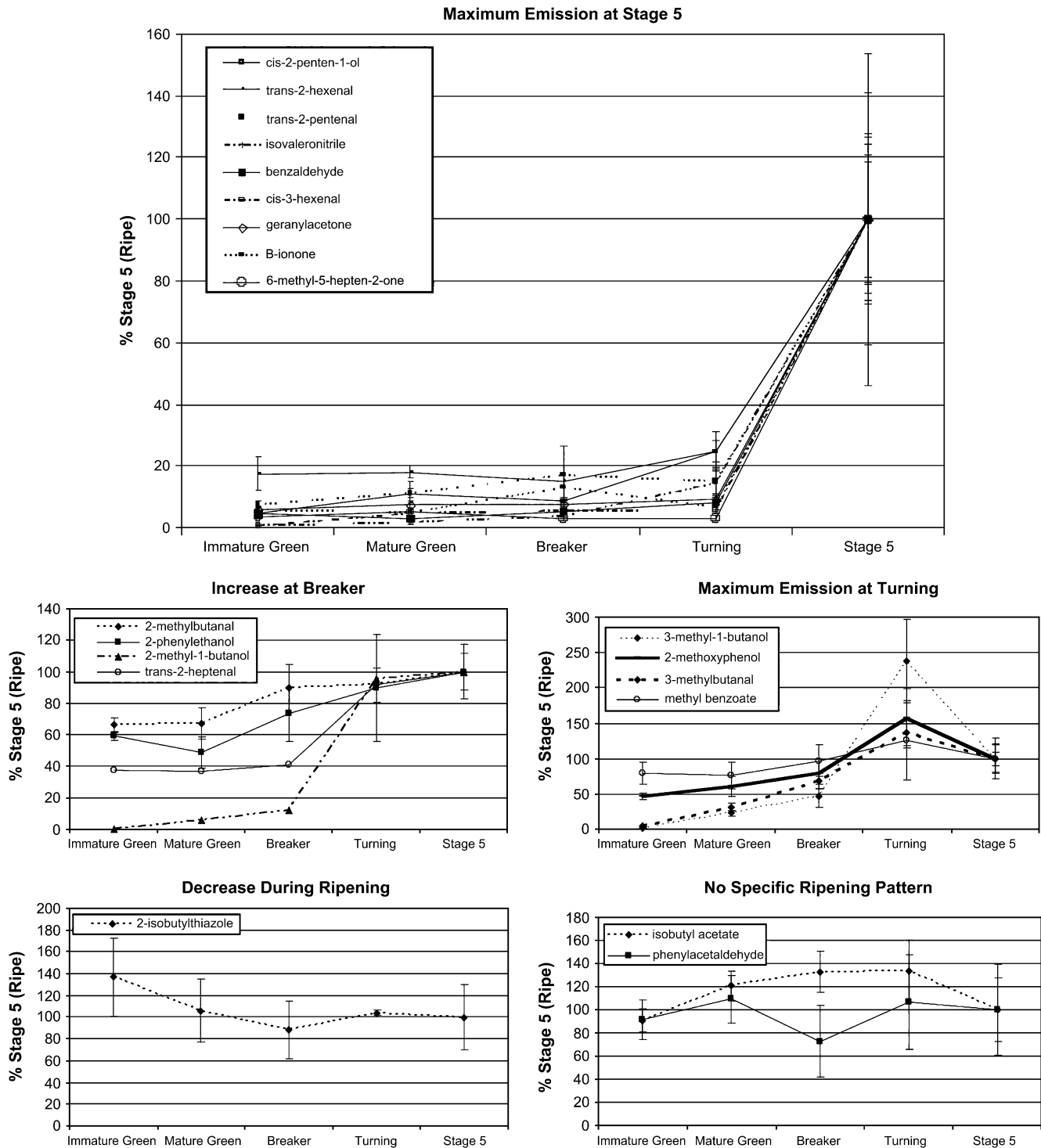


Fig. 2. Volatile emissions during tomato fruit development. Emissions of the 23 volatiles associated with mapped loci were collected and measured as described in the Materials and methods.

As would be expected, *r* is greatly reduced in emissions of all apocarotenoid volatiles (Table 3). Similar results were obtained with the *sherry* (*sh*) mutant. The molecular basis of *sh* has not to our knowledge been reported. Two mutants, *B* and *old gold* (*og*) are affected in opposite ways in

accumulation of β -carotene and lycopene. *B* is a dominant gene that increases fruit β -carotene content at the expense of lycopene while *og* is deficient in β -carotene and accumulates higher levels of lycopene (Ronen *et al.*, 2000). The apocarotenoid volatile β -ionone is produced

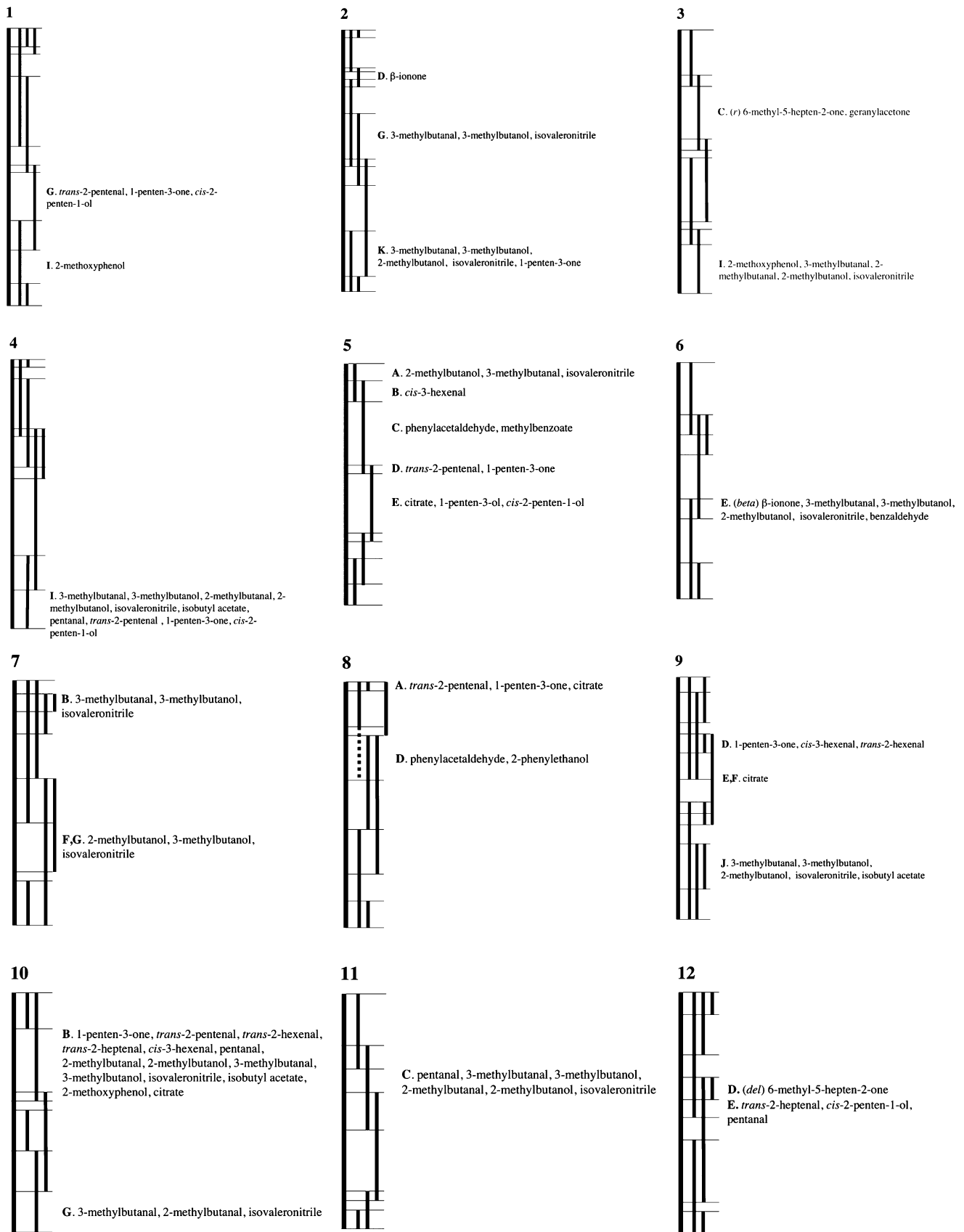


Fig. 3. Map positions of flavour-related QTLs. Each chromosome is divided into bins as defined by Eshed and Zamir (1995). Positions of introgressed *S. pennellii* segments are indicated for each chromosome with the appropriate bin designation.

Table 2. Average volatile levels associated with each QTL

Values are expressed as percentage of a M82 value. All values are significant at $P < 0.01$ unless indicated by an asterisk; for those marked with an asterisk, the values are significant at $P < 0.05$.

| Volatile | Bin | % of M82 |
|----------------------------|------|----------|
| 1-penten-3-one | 1G | 420 |
| 2-methoxyphenol | 1G | 16035 |
| <i>cis</i> -2-penten-1-ol* | 1G | 215 |
| <i>trans</i> -2-pentenal | 1G | 297 |
| β -ionone | 2D | 4402 |
| 3-methyl-1-butanol* | 2F | 310 |
| 3-methylbutanal | 2F | 227 |
| isovaleronitrile | 2F | 300 |
| 1-penten-3-one | 2K | 337 |
| 2-methyl-1-butanol* | 2K | 272 |
| 3-methyl-1-butanol | 2K | 616 |
| 3-methylbutanal* | 2K | 415 |
| isovaleronitrile | 2K | 170 |
| 6-methyl-5-hepten-2-one* | 3C | 8 |
| geranylacetone* | 3C | 8 |
| 2-methoxyphenol | 3I | 16948 |
| 2-methyl-1-butanol | 3I | 231 |
| 2-methylbutanal* | 3I | 150 |
| 3-methylbutanal* | 3I | 158 |
| isovaleronitrile | 3I | 227 |
| 1-penten-3-one | 4I | 494 |
| 2-methyl-1-butanol | 4I | 408 |
| 2-methylbutanal* | 4I | 185 |
| 3-methyl-1-butanol | 4I | 528 |
| 3-methylbutanal | 4I | 273 |
| <i>cis</i> -2-penten-1-ol* | 4I | 218 |
| isobutyl acetate | 4I | 407 |
| isovaleronitrile | 4I | 343 |
| pentanal* | 4I | 227 |
| <i>trans</i> -2-pentenal | 4I | 394 |
| 2-methyl-1-butanol* | 5A | 229 |
| 3-methylbutanal | 5A | 214 |
| isovaleronitrile | 5A | 440 |
| <i>cis</i> -3-hexenal | 5B | 236 |
| methylbenzoate | 5C | 537 |
| phenylacetaldehyde | 5C | 1030 |
| 1-penten-3-one* | 5D | 278 |
| <i>trans</i> -2-pentenal* | 5D | 217 |
| 1-penten-3-ol* | 5E | 179 |
| <i>cis</i> -2-penten-1-ol | 5E | 248 |
| 2-methyl-1-butanol | 6E | 296 |
| 3-methyl-1-butanol | 6E | 456 |
| 3-methylbutanal | 6E | 328 |
| benzaldehyde | 6E | 811 |
| β -ionone* | 6E | 598 |
| isovaleronitrile | 6E | 524 |
| 3-methyl-1-butanol | 7B | 364 |
| 3-methylbutanal | 7B | 270 |
| isovaleronitrile | 7B | 372 |
| 2-methyl-1-butanol | 7F/G | 366 |
| 3-methyl-1-butanol* | 7F/G | 367 |
| isovaleronitrile | 7F/G | 355 |
| 1-penten-3-one | 8A | 335 |
| <i>trans</i> -2-pentenal | 8A | 255 |
| 2-phenylethanol | 8D | 12953 |
| phenylacetaldehyde | 8D | 4590 |
| 1-penten-3-one* | 9D | 291 |
| 2-methyl-1-butanol | 9D | 284 |
| 3-methyl-1-butanol | 9D | 507 |
| 3-methylbutanal* | 9D | 326 |
| <i>cis</i> -3-hexenal | 9D | 187 |
| isovaleronitrile* | 9D | 478 |
| <i>trans</i> -2-hexenal | 9D | 212 |
| 1-penten-3-one | 10B | 550 |
| 2-methoxyphenol* | 10B | 5849 |

Table 2. Continued

| Volatile | Bin | % of M82 |
|---------------------------|-----|----------|
| 2-methyl-1-butanol | 10B | 586 |
| 2-methylbutyaldehyde* | 10B | 157 |
| 3-methyl-1-butanol | 10B | 480 |
| 3-methylbutanal | 10B | 197 |
| <i>cis</i> -3-hexenal | 10B | 193 |
| isobutyl acetate | 10B | 263 |
| isovaleronitrile | 10B | 317 |
| pentanal* | 10B | 195 |
| <i>trans</i> -2-heptenal | 10B | 242 |
| <i>trans</i> -2-hexenal | 10B | 241 |
| <i>trans</i> -2-pentenal | 10B | 363 |
| 2-methylbutyaldehyde | 10G | 215 |
| 3-methylbutanal | 10G | 214 |
| isovaleronitrile | 10G | 305 |
| 2-methyl-1-butanol | 11C | 241 |
| 2-methylbutyaldehyde | 11C | 176 |
| 3-methyl-1-butanol | 11C | 325 |
| 3-methylbutanal | 11C | 248 |
| isovaleronitrile | 11C | 284 |
| 6-methyl-5-hepten-2-one* | 12D | 38 |
| <i>cis</i> -2-penten-1-ol | 12E | 232 |
| pentanal | 12E | 241 |
| <i>trans</i> -2-heptenal | 12E | 194 |

by oxidative cleavage of β -carotene (Simkin *et al.*, 2004). If carotenoid content is a determinant of apocarotenoid volatile emissions, it would be predicted that *B* should have significantly higher emissions of β -ionone while *og* should have lower β -ionone emissions. This is indeed the case (Table 2), confirming the precursor-product relationship of β -carotene and β -ionone. The *og* mutant also had higher emissions of pseudoionone, the product of CCD1 cleavage of lycopene, although the difference was not significant at $P < 0.05$. The *delta* (*del*) mutant has higher expression than the wild type of lycopene ϵ -cyclase (Ronen *et al.*, 1999), resulting in much higher than normal levels of α -carotene. Volatile emissions from *del* contained high levels of α -ionone, a volatile normally produced in very low quantities, and somewhat lower levels of β -ionone.

These results are largely consistent with those we obtained from the ILs. The IL population contains *B*, *del*, and *r*, all of which have altered colour due to changes in ripening-associated carotenoid accumulation. It is interesting that the LA0716 *r*, although very much reduced in 6-methyl-5-hepten-2-one and geranyl acetone emissions, was not significantly reduced in β -ionone emissions. This may be the result of an interaction between the mutant locus and the M82 genotype. Recently, a similar analysis with an overlapping set of carotenoid mutants has been published (Lewinsohn *et al.*, 2005). Their results, together with ours, indicate that a major determinant of apocarotenoid volatiles is the level of the precursor carotenoid. Since several of these apocarotenoid volatiles are considered to be major contributors to tomato flavour, it would be interesting to compare the flavour qualities of appropriate isogenic lines with altered apocarotenoid volatiles.

Table 3. The effects of carotenoid loci on volatile emissions

The genetic backgrounds for each mutant are indicated in parentheses. All values are expressed relative to the appropriate control cultivar. * $P < 0.05$; ** $P < 0.01$.

| Locus | Volatile | % of Control |
|-------------------------|-------------------------|--------------|
| <i>r</i> (Ailsa Craig) | Geranylacetone | 5.1* |
| | Citral | 24* |
| | 6-methyl-5-hepten-2-one | 2.8* |
| | β -ionone | 8.8* |
| <i>sh</i> (Pearson) | Geranylacetone | 6.6** |
| | Citral | 5.6** |
| | 6-methyl-5-hepten-2-one | 1.2 |
| | β -ionone | 37.9 |
| β (Rutgers) | Geranylacetone | 164.2 |
| | Citral | 73 |
| | 6-methyl-5-hepten-2-one | 60.7 |
| | β -ionone | 2173* |
| <i>del</i> (Rutgers) | Geranylacetone | 53.6 |
| | Citral | 70.5 |
| | 6-methyl-5-hepten-2-one | 58.7 |
| | β -ionone | 45 |
| <i>og</i> (Ailsa Craig) | α -ionone | 684.2* |
| | Geranylacetone | 109.2 |
| | Citral | 84 |
| | Pseudoionone | 221.2 |
| | 6-methyl-5-hepten-2-one | 82.5 |
| | β -ionone | 17.4* |

Discussion

Identification of loci altered in volatile emissions

The goal of this research programme has been to use a combination of genomic and targeted metabolite approaches to identify genes controlling synthesis of the important tomato flavour volatiles. As a first step toward that goal, genetic loci with increased volatile emissions relative to the *S. lycopersicum* parent, M82, have been identified. In order rigorously to test the population, volatile emissions were measured over multiple seasons. In general, the absolute levels of volatile emissions varied substantially from season to season. However, when values were expressed relative to the parental control, M82, the variation between seasons was not significant, despite three different growing locations and the different seasons (June harvest versus November harvest).

Although 25 different loci were identified, this number is likely to be an underestimate of the number of QTLs affecting volatile emissions in this population. For the most part the focus was on loci that influenced multiple related volatiles. For example, while there were loci that were significantly different for single C5 volatiles, the choice was made to include only those affected in two or more of the five C5 volatiles. Since many of the related volatiles have similar taste properties, these loci are likely to have significant effects on the taste of tomatoes.

There were several examples where multiple loci affected sets of related volatiles. For example, 12 loci that quantitatively affect the emission of leucine- and/or iso-

leucine-derived volatiles were identified. There are several ways in which a locus could affect entire pathways. Such loci may encode transcription factors that co-ordinately regulate genes. They may also encode enzymes that catalyse limiting steps in single pathways. Alternatively, they may influence the precursor or intermediate pools. It will be interesting to determine whether any of these loci are altered in the non-volatile precursors of the corresponding volatiles. The identification of multiple loci altered in the same sets of volatiles will be particularly useful in identification of the genes encoding the biosynthetic enzymes using genomics tools. A further benefit of identifying multiple loci altered in related volatiles is in determining the metabolic origins of compounds of unknown origin. For example, the pathway for synthesis of isovaleronitrile has not been determined. That it is always co-ordinately regulated with 3-methylbutanal and 3-methylbutanol strongly suggests that it is derived from the pathway to synthesize these two leucine-derived volatiles.

One locus in bin B on the top of chromosome 10 was significantly higher in a large number of chemically unrelated volatiles. Fruits of IL 10-1 are not visibly different from those of M82 and any obvious differences in fruit ripening or maturity that would explain such differences cannot be detected. This locus will be particularly interesting to characterize further.

Carotenoids and apocarotenoid volatiles

In general, there was good agreement between the reported values of carotenoids in the colour mutants and the apocarotenoid volatiles that are predicted to be derived from them. For example, the volatiles that are predicted to be derived from the linear carotenoids (geranylacetone, pseudoionone, citral, and MHO) are all greatly reduced in *r*, the phytoene synthase loss-of-function mutant. These volatiles are not, however, completely absent in the *r* mutant. For example, geranyl acetone must be derived from a carotenoid earlier than lycopene in the synthetic pathway. The pre-lycopene carotenoids do not accumulate to substantial levels in ripe tomato fruits (Ronen *et al.*, 1999; Fraser *et al.*, 2000). Geranylacetone, pseudoionone, citral, and MHO synthesis in ripe *r* fruits indicates that there must be continued low level synthesis of carotenoids. Work by Fraser *et al.* (1999) has shown that there is phytoene synthase-2 expression in ripening fruits.

Similar results to those of the *r* mutant were obtained with the *sh* mutant. Although the molecular basis for this mutant has not been reported, it does map to chromosome 10. Another mutant deficient in many carotenoids, *tangerine*, also maps to chromosome 10 (Isaacson *et al.*, 2002) and could potentially be allelic to *sh*. Since *sh* is on chromosome 10, it cannot be defective in phytoene synthase-1, which maps to chromosome 3. That these mutants are not significantly reduced in β -ionone emissions

likely indicates that the β -carotene substrate for β -ionone synthesis comes from pools synthesized prior to ripening.

The data presented in Fig. 2 indicate that the apocarotenoid volatiles (geranyl acetone, pseudoionone, and β -ionone) do not accumulate to maximal levels until fruits are close to fully ripe. The conclusion that emissions of carotenoid-derived volatiles are associated most closely with carotenoid content suggests that it will be challenging specifically to alter the emissions of these tomato apocarotenoid volatiles without significantly affecting the nutrient composition of the fruits. It is interesting to note that the only enzymes known to be involved directly in generating apocarotenoid volatiles, LeCCD1A and LeCCD1B, map to positions that were not associated with QTLs affecting these volatiles (chromosomes 11 and 1, respectively). While carotenoids accumulate predominantly inside plastids, the CCD1 dioxygenases are cytoplasmic (Simkin *et al.*, 2004). The peak apocarotenoid volatile emissions late in ripening strongly suggest that the availability of substrate to the enzymes determines the timing of volatile emissions. As fruits ripen and plastids differentiate into chromoplasts (Thelander *et al.*, 1986), a change in availability of carotenoids associated with the structural changes of the organelle probably permits synthesis of these important apocarotenoids.

Identification of loci altered in acid content

Four loci with a significantly higher citric acid content were identified. No loci with consistently altered malic acid were identified. In general, there was much higher variation in acid content across seasons, suggesting that acids are more susceptible to environmental influences than are volatiles. Previously, Causse *et al.* (2004) mapped seven loci with altered citric acid and five with altered malic acid contents. Of the four citric acid loci that were identified in this study, three possibly correspond to loci that were observed in the previous study while one (8-A) does not. Of the four identified by Causse *et al.* (2004) that were not identified by this laboratory, these data were consistent with their results for one locus but not at the $P < 0.05$ level (7-B) while no apparent difference from control was observed with the other three (4-F, 8-E, 10-E). Previously, Fulton *et al.* (2002) have also reported QTL affected in citric acid. The map positions of two of these loci correspond to QTL identified here and may be the same (8-A and 9-E,F). Causse *et al.* (2004) have reported on QTLs affecting brix. When they compared results from France and Israel, there was an overlapping but not identical set of loci identified between the two sites. A similar story emerges when comparing these acid data in the USA to those obtained in France by Causse *et al.* (2004). Taken together, the results underscore the challenges of profiling some metabolites, especially in field conditions. Clearly certain metabolites must be assessed over numerous seasons before the effects of

QTLs can be determined. Nonetheless, it is encouraging that there is substantial overlap in the identified loci.

In conclusion, a number of QTLs have been identified that reproducibly alter the composition of volatile and non-volatile chemicals that contribute to overall fruit flavour. These QTLs affect volatiles that are both positive and negative contributors to tomato flavour. In the short term, linked molecular markers should be useful for breeding programmes aimed at improving fruit flavour. In the longer term, genes responsible for controlling levels of these important chemicals will be important tools for understanding the complex interactions that ultimately integrate to provide the unique flavour of a tomato.

Supplementary data

Supplemental materials for this manuscript can be found at JXB online.

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