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ORIGINAL PAPER

Environmental and seasonal influences on red raspberry flavour volatiles and identification of quantitative trait loci (QTL) and candidate genes

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Abstract Raspberry volatiles are important for perceptions of sensory quality, mould resistance and some have nutraceutical activities. Twelve raspberry character volatiles were quantified, 11 of them in fruit from two seasons, from plants from the Glen Moy × Latham mapping population growing in both open field and under cover (polytunnels). Effects of season and environment were examined for their impact on the content of α-ionone, α-ionol, β-ionone, β-damascenone, linalool, geraniol, benzyl alcohol, (Z)-3-hexenol, acetoin, acetic and hexanoic acids, whilst raspberry ketone was measured in one season. A significant variation was observed in fruit volatiles in all progeny between seasons and method of cultivation. Quantitative trait loci were determined and mapped to six of the seven linkage groups, as were candidate genes in the volatiles pathways.

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Introduction

Flavour character in fruit originates through interactions between sugars, organic acids and a subset of approximately 200 volatile compounds (Klesk et al. 2004). Raspberry fruit produce an array of volatile compounds (Robertson et al. 1995; Aprea et al. 2010) with content showing significant variations influenced by genotype, environment and season which impacts on flavour (Moore and Burrows 2002; Du and Qian 2010). Of the 230 volatiles characterised in raspberry, only 12 have been identified as of character impact (Jiang 1991; Larsen et al. 1991): α-, β-ionones, α-ionol, β-damascenone, linalool, geraniol, (Z)-3-hexenol, benzyl alcohol, acetoin, raspberry ketone, acetic and hexanoic acids (Table 1). These are produced from primary metabolites via several pathways during fruit ripening (Buttery and Ling 1993; Schaffer et al. 2007), notably from fatty acids, terpenes and carotenoid breakdown and phenylpropanoid biosynthesis (Table 1; Fig. 1). A few genes in volatile biosynthesis pathways have recently been mapped in raspberry (Woodhead et al. 2010).

Improving fruit flavour, a set of complex, multigenic traits provides unique challenges in fruit breeding. Selection for yield, size and shelf-life characteristics have had negative effects on fruit flavour, notably in tomato (Goff and Klee 2006) but also strawberries, where cultivated fruits have less flavour than wild berries due to the loss of enzymic activities during domestication (Aharoni et al. 2004). Cultivars with premium characters including improved flavour encourage greater fruit consumption with many associated health benefits to the human diet. The breeding process in raspberry is lengthy, up to 15 years from the first cross to the release of a new cultivar (Graham and Jennings 2009). A strategy that allows early selections for sensory quality in terms of flavour volatile content

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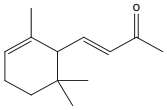
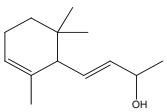
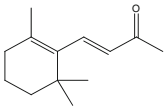
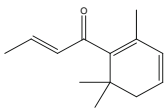
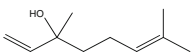
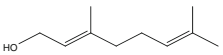
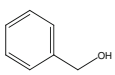
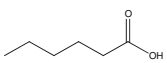
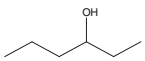
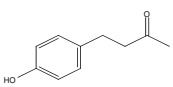
Table 1 Raspberry character volatiles (Jiang 1991; Larsen and Poll 1993)

Aroma compound	Chemical structure	Metabolic pathway	Candidate gene	Sensory descriptor
α-Ionone		Carotenoid	Phytoene synthase, PSY Carotenoid cleavage enzyme, CCD	Berry, cherry, woody, violet
α-Ionol		Carotenoid	Phytoene synthase, PSY Carotenoid cleavage enzyme, CCD	Hot tea, lemon, sweet, violet
β-Ionone		Carotenoid	Phytoene synthase, PSY Carotenoid cleavage enzyme, CCD	Almond, balsam, berry, orange, woody, rose
β-Damascenone		Carotenoid	Phytoene synthase, PSY Carotenoid cleavage enzyme, CCD	Apple, smoky, woody, nutty, citrus, rose
Linalool		Monoterpenes	1-deoxy-D-xylulose 5-phosphate synthase, DXS Linalool synthase, LIS	Lemon, floral, sweet, orange
Geraniol		Monoterpenes	1-deoxy-D-xylulose 5-phosphate synthase, DXS Geraniol synthase, GES	Apple, apricot, berry, rose, sweet
Benzyl alcohol		Fatty acids	Alcohol dehydrogenase, ADH	Berry, cherry, citrus, walnut
Acetic acid		Fatty acids		Pungent, sour, vinegar
Hexanoic acid		Fatty acids		Cheese, fatty, sour
(Z)-3-hexenol		Fatty acids	Alcohol dehydrogenase, ADH	Pungent, green, piney
Acetoin		Fatty acids	HMG-CoA reductase, Acetoin synthase	Butter, creamy
Raspberry ketone		Phenylpropanoid	Benzalacetone synthase, BAS	Raspberry, sweet

would be valuable. This requires a greater understanding of the genetic control of the pathways involved in volatile synthesis, environmental factors influencing volatile production and contributions of impact volatile compounds to sensory character in raspberry. As in grape (Câmara et al. 2004), α-, β-ionones, α-ionol and β-damascenone (C13 norisoprenoid volatiles) are important for flavour. These are formed by carotenoid breakdown (Fig. 1) (Winterhalter and Rouseff 2002) via the plastidial pathway (Hampel et al. 2007) and confer desirable "berry", "honey", "sweet" and "rose" notes to fruit (Table 1). Precursor double bonds are susceptible (Baldermann et al. 2005) to oxidation by regiospecific carotenoid cleavage dioxygenases (CCD) characterised in *Arabidopsis* (Auldridge et al. 2006), tomato (Simkin et al. 2004), grape (Mathieu et al. 2005) and peach (Brandi et al. 2011). Such volatiles contribute to differentiating varietal fruits and yield health benefits from consequent nutraceutical phytochemical intake: β-ionone, for example, is reported to act as a chemopreventative against colon cancer cells (Janakiram et al. 2008).

Raspberry monoterpene volatiles, linalool and geraniol, are synthesised from isopentenyl pyrophosphate (IPP) by

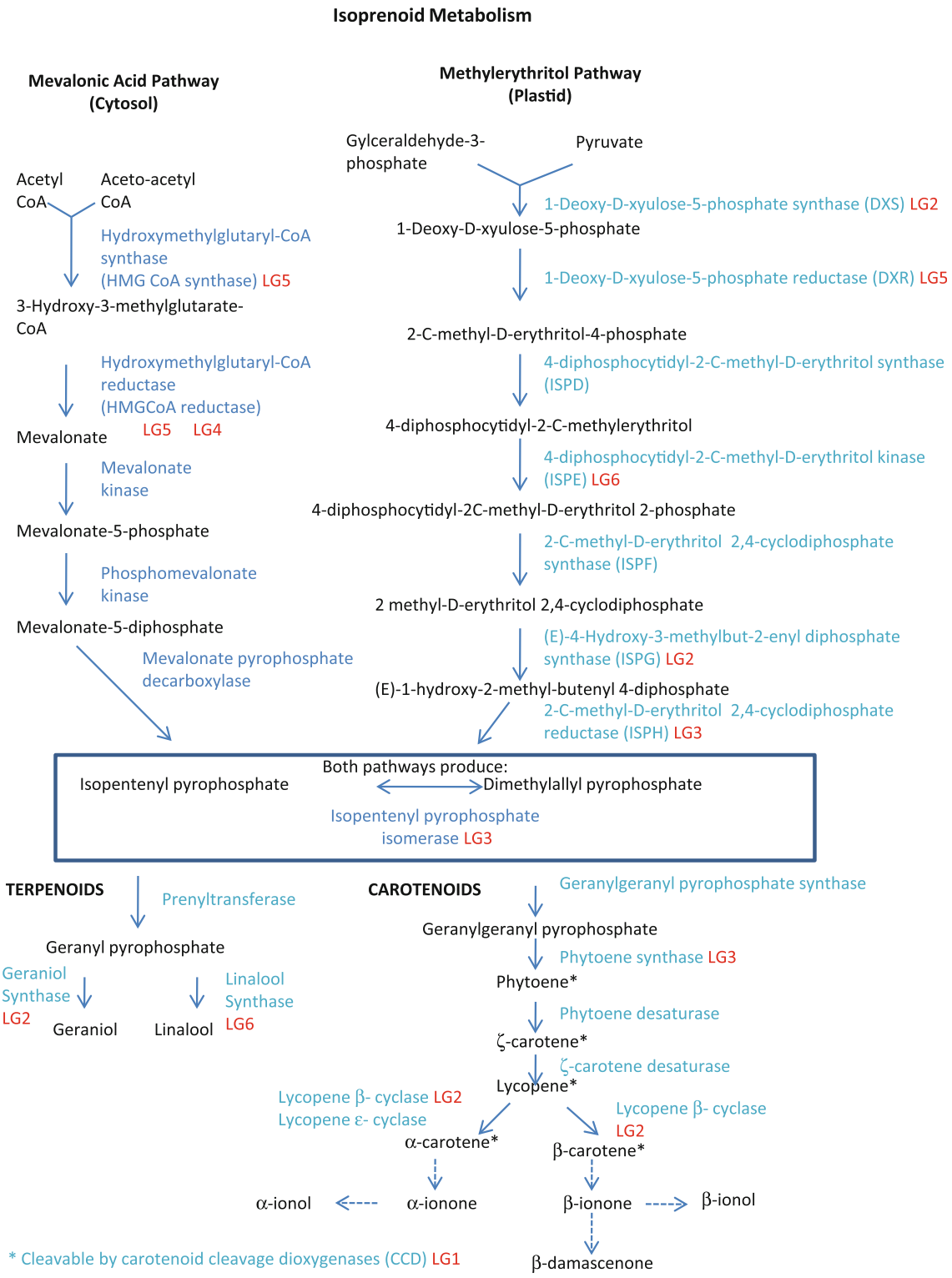


Fig. 1 Isoprenoid metabolism

monoterpene synthases (Fig. 1) (Hampel et al. 2007). Such compounds can also be synthesised and accumulated in roots and rhizomes (Bos et al. 2002; Chen et al. 2004). These volatiles can be detected at a distance from a plant, serving as attractants or deterrents for pollinators or herbivores (Dudareva and Pichersky 2000; Pichersky and

Gershenzon 2002), acting also against plant pathogens. Volatile release is thought to be developmentally regulated (Dudareva et al. 2003; Arimura et al. 2004). Geraniol is a natural antioxidant reported to inhibit polyamine biosynthesis in human colon cancer cells (Carnesecchi et al. 2001) and suppress pancreatic tumour growth (Burke et al. 1997).

Raspberry fruit accumulate the phenylpropanoid *p*-hydroxyphenylbutan-2-one (raspberry ketone) with content correlated with those of anthocyanins and soluble solids—Brix (Borejsza-Wysocki and Hrazdina 1994). Although only a small proportion of total volatiles (Borejsza-Wysocki et al. 1992), it has been reported to be a key determinant of raspberry flavour (Hradzina 2006). Raspberry ketone is produced via a two-stage reaction utilising 4-coumaroyl-CoA and malonyl-CoA (Borejsza-Wysocki and Hrazdina 1996) with rate-limiting steps catalysed by benzalacetone synthase, a bifunctional polyketide synthase producing both 4-hydroxybenzalacetone and naringenin chalcone. This is encoded by the raspberry gene, *Ri*PKS4 (Zheng and Hrazdina 2008), whereas that encoding the enzyme for conversion of *p*-hydroxyacetone to raspberry ketone (*p*-hydroxybutanone) has yet to be identified.

Certain volatiles produced by fatty acid metabolism are important: (Z)-3-hexenol, benzyl alcohol, acetoin, acetic and hexanoic acids. Ethylene and two specific enzymes alcohol acyltransferase (AAT) and alcohol dehydrogenase (ADH) are also implicated in fruit ripening and flavour profiles (Speirs et al. 1998).

Fruit flavour volatile contents are generally continuous traits which are found to display a normal pattern of distribution which may be controlled by several genes of small effect or one or two genes conferring a large effect, or a combination of both. Previous reports of flavour volatile quantitative trait loci (QTL) include strawberry (Aharoni et al. 2004), tomato (Tieman et al. 2006), melon (Katzir et al. 2008), grape (Sevini et al. 2004; Duchêne et al. 2009; Battilana et al. 2009) and apple (Zini et al. 2005; Dunemann et al. 2009). In tomato, a QTL for six important volatiles seems responsible for the key cherry characters in larger progeny fruit and an allele *malodorous* correlated with a QTL for undesirable flavour notes (Fernie et al. 2006). This area has been reviewed by Klee (2010).

This work set out to characterise fruit contents of 12 flavour volatiles in the 'Latham' × 'Glen Moy' reference red raspberry mapping population, examining seasonal and environmental variations, mapping QTL and candidate genes and identification of associations with key loci. This marks the first step towards understanding the effects of genetics, season and environment on the production of these compounds in raspberry fruit.

Materials and methods

Fruit samples

Fruits from a full-sib family from a red raspberry cross, European cv. Glen Moy × North American cv. Latham, were utilised (Graham et al. 2004). 'Latham' yields small, firm, dark glossy fruit, both sweet and aromatic, late in the season, whereas 'Glen Moy' produces large, pale, soft berries of very sweet flavour character earlier in the season. The entire segregating population of 330 individuals and both parents were planted at two field locations, one open and one under cover (polytunnel, PT) (McCallum et al. 2010), in Invergowrie, Dundee, UK in randomised complete block trials with three replicates and two plant plots at both locations. Fruits from a subset of 188 progeny from two replicate field plants (parents and progeny) were hand harvested in 2006 and 2007 and also from replicate plants grown under polytunnel in 2007 when the bush was fully ripe. Berries, harvested at a similar time of day and from the same side of the plant, were immediately stored at −20 °C.

Volatiles extraction (HS/SPME) and analysis (GC-FID)

Frozen raspberries were thawed overnight at 4 °C, homogenised with a glass rod, 2.5 g weighed and volumes made up to 5 ml with distilled water. The puree, in a 70 °C jacketed (30 ml) vial, was equilibrated for 15 min with agitation. A PDMS/DVB phase on an SPME fibre (Supelco, Sigma-Aldrich Ltd., Poole, Dorset, UK) was exposed to berry headspaces for 15 min then desorbed into a splitless injector port (230 °C) of a Carlo Erba Mega GC with a flame ionisation detector (FID) at 250 °C for 5 min. Volatiles were resolved on a DB-wax WCOT column [30 m × 0.25 mm i.d. cross-linked poly(ethyleneglycol), 0.25 μm film thickness (J&W Scientific, Milton Keynes, UK)] with mobile phase (helium) at 70 kPa. The temperature gradient program was 5 min at 70 °C, 70–115 °C at 12 °C min^{-1}, and 115–240 °C at 10 °C min^{-1}, terminated by 3 min hold at 240 °C. Samples were analysed in duplicate.

Raspberry ketone quantification

Quantification of raspberry ketone was based on a method modified from Borejsza-Wysocki et al. (1992) and Hamid (1996), and was only carried out on 2007 polytunnel fruit. Frozen berries (ca. 50 g), were thawed overnight at 4 °C, and weighed into a Jelly Bag (Ref no. 3810, Lakeland Ltd., UK) and pressed with a screw winepress, and the juice filtered (coffee filter) into a 20-ml glass beaker. Sterile

distilled water (1× volume) and filter aid (150 g fresh fruit, Hyflo SuperCel medium; Sigma-Aldrich, MO, USA) were added before juice was decanted into 30 ml Oak Ridge centrifuge tubes (Nalgene Thermo Fisher Scientific Inc., NY, USA), and centrifuged at ambient temperature for 20 min at 10,000×*g*. Supernatant was decanted into a 20-ml glass beaker, sealed with parafilm and raspberry ketone collected immediately by a non-polar solid phase extraction (SPE) (Bond Elut) C18 SPE 500 mg 3 ml cartridge (Varian Inc., CA, USA) method. To condition the bonded phase, solvents were passed through successively 3 ml methanol and 10 ml sterile distilled water under 17 kPa pressure, discarding eluent. Raspberry juice (3 ml) was followed by 10 ml of water to elute sugars and acids, discarding eluent. Raspberry ketone was eluted with 10 ml of pentane:dichloromethane (1:1; w/v), dried with 100 mg Na_2SO_4 and the mixture dried by rotary evaporation. Raspberry ketone was re-suspended with 1.5 ml HPLC mobile phase (30 % acetonitrile, 30 mM KH_2PO_4, pH 6.2) centrifuged in 1.5 ml microfuge tubes at 14,000×*g* for 15 min and supernatant aliquoted into HPLC autosampler vials and stored at −20 °C. All extractions were done in duplicate.

Quantification by HPLC of raspberry ketone was effected with mobile phase, at 1.0 ml min^{-1} and a reversed phase C18 static phase (Waters Bondapack C18 column, 10 µm, 46 × 150 mm, Waters Corp., MA, USA), with UV detection at 289 nm, operated at 25 °C for both calibration and quantification in extracts (20 µl aliquots).

Identification of flavour volatiles

Authentic standards were purchased from Sigma-Aldrich Ltd (Poole, UK). Fruit pulp samples were spiked with authentic standards for metabolite confirmation during fibre phase exposure. Standard curves were prepared using compounds in methanol.

Candidate gene identification and genotyping

Rubus transcriptome (454) database (Woodhead M, Cardle L, Hedley P, Bayer M and Graham J, personal communication) and the existing *Rubus* EST database (Woodhead et al. 2008) were mined for genes of interest. Primers were designed to these genes using Primer 3 (Rozen and Skaletsky 2000) (Table 5). Products were amplified in the parents and progeny as previously described (Woodhead et al. 2010) and sequence polymorphisms determined using Sequencher 4.9. Size polymorphisms were amplified using the Qiagen Multiplex PCR plus kit according to the manufacturer's instructions or as described previously (Graham et al. 2009; Woodhead et al. 2010), and analysed on the ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA) using ROX500 (Applied Biosystems) as an internal size standard. Single nucleotide polymorphisms (SNPs) were identified and mapped either by Sanger sequencing or by Pyrosequencing as previously described (Graham et al. 2009; Woodhead et al. 2008, 2010).

Statistical and mapping analyses

Data were analysed in Genstat 12 for Windows. Volatile data was log transformed before analyses were carried out. Heritability was estimated as $h^2 = c\sigma^2_G/(c\sigma^2_G + d\sigma^2_{GS} + e\sigma^2_{GY} + \sigma^2_{GSY})$, where σ^2_G, σ^2_{GS}, σ^2_{GY} and σ^2_{GSY} are the variance components for genotypes, genotype × site interaction, genotype × year interaction and genotype × site × year interaction, $c \leq 6$ (number of sites × number of years), $d \leq 3$ (number of sites) and $e \leq 2$ (number of years).

Markers were added to the raspberry linkage map version as in Graham et al. (2011) using Joinmap 3.0 (Van Ooijen and Voorrips 2001). Volatile data from 188 progeny and parents were analysed using Genstat v12 to examine variation across progeny, seasons and environments. A Kruskal–Wallis test was used as a preliminary test to identify regions of the genome linked to the volatile data, to explore whether alleles from one or both parents were contributing. Interval mapping was then carried out using MapQTL 5 software (van Ooijen 2004) a small permutation test of 200 permutations was carried out for each volatile under each treatment to determine the LOD threshold at 90 and 95 % significance.

Results

Fruit volatiles contents

Twelve character volatiles were quantified in raspberry fruit from the 'Latham' × 'Glen Moy' mapping population including four norisoprenoids, two monoterpenes, two organic acids, two alcohols and two ketones (Table 1). In most progeny, the norisoprenoid β-damascenone was the most abundant volatile followed by α- and β-ionones. In contrast, benzyl alcohol, acetic acid and acetoin were minor fractions (Table 2). As expected, most volatiles—except for benzyl alcohol, acetoin, acetic and hexanoic acids—were present in higher concentrations in the more aromatic parent, 'Latham'. The concentration ratios, 'Latham' to 'Glen Moy' (L/GM), of five volatiles were more than double: (Z)-3-hexenol = 6; α-ionol = 3; geraniol = 2.8; β-ionone = 2.8 and α-ionone = 2.3.

Significant seasonal variation ($p < 0.001$) was observed between field fruit for all volatiles except β-damascenone and acetoin. The warmer 2006 summer yielded higher

Table 2 Range of volatile contents (min–max) in 188 mapping progeny (μg/ml)

Volatile sample	Season					
	Field 2006 progeny		Field 2007 progeny		Polytunnel 2007 progeny	
	Mean ± SEM	Min–max	Mean ± SEM	Min–max	Mean ± SEM	Min–max
β-Damascenone	72.29 ± 4.02	0–463.8	87.10 ± 6.82	0–872.10	64.88 ± 4.71	3.17–368.8
β-Ionone	13.65 ± 0.53*	0.46–47.88	9.83 ± 0.44	0.61–44.33	6.43 ± 0.36*	0.25–33.02
α-Ionone	7.31 ± 0.19*	1.65–17.16	3.86 ± 0.14	0.79–13.82	5.16 ± 0.24*	0.22–21.61
α-Ionol	2.24 ± 0.09*	0.16–7.28	1.83 ± 0.09	0.24–11.68	5.96 ± 0.30*	0.16–29.97
Linalool	4.72 ± 0.32*	0.67–22.26	2.90 ± 0.17	0.33–14.52	4.39 ± 0.20*	0.90–29.28
Geraniol	2.64 ± 0.08*	0.68–8.87	1.82 ± 0.07	0.44–10.74	3.68 ± 0.14*	0.11–15.46
(Z)-3-hexenol	22.35 ± 0.34*	0.71–28.15	9.06 ± 0.29	0.63–28.18	16.70 ± 0.67*	0.07–54.03
Acetic acid	1.39 ± 0.06*	0.06–8.26	0.64 ± 0.05	0.01–4.05	0.72 ± 0.03	0.01–3.54
Hexanoic acid	6.54 ± 0.35*	0.89–41.68	7.97 ± 0.30	1.71–22.88	7.04 ± 0.34	0.28–30.84
Acetoin	1.02 ± 0.05	0.09–4.74	1.03 ± 0.04	0.10–4.79	0.80 ± 0.05*	0.02–4.95
Benzyl alcohol	0.59 ± 0.03*	0.15–2.18	1.07 ± 0.04	0.09–3.16	2.67 ± 0.11*	0–9.94

* $p < 0.001$ for 2006/2007 field data shown in 2006 column and for 2007 field/2007 polytunnel data in polytunnel column

contents for most volatiles except β-damascenone, benzyl alcohol and hexanoic acid whilst acetoin remained constant (Table 2). Correlations between individual volatiles in 2006 and 2007 fruit were highly significant for most of the more abundant volatiles (Table 3). In 2007, a significant variation was observed between polytunnel and field fruit for most volatiles except β-damascenone, hexanoic and acetic acid (Table 2), whereas correlations across the environments were rarely significant (Table 3). Seven volatiles were more abundant in polytunnel berries but both β-damascenone and β-ionone were lower.

Norisoprenoids (α-, β-ionones, β-damascenone and α-ionol) were ca. four times more abundant than monoterpenes and fatty acid metabolites indicating red raspberry character flavour volatiles were largely derived from apocarotenoid compounds in this population.

Table 3 Correlations between years, based on the genotype means, and broad sense heritability over field sites only and all three environments after log transformation (‡ is $0.05 < p < 0.1$)

Volatile sample	Season			Heritability	
	Field 2006–2007	Field 2006 polytunnel	Field 2007 polytunnel	Field only (%)	3 data sets (%)
β-Damascenone	0.34**	0.20‡	0.05 ns	18.7	13.4
β-Ionone	0.32**	0.26*	0.40***	17.4	21.8
α-Ionone	0.38***	0.40***	0.21‡	36.4	29.6
α-Ionol	0.39***	0.02 ns	0.13 ns	33.4	13.8
Linalool	0.57***	−0.17 ns	−0.13 ns	50.7	11.2
Geraniol	0.22*	0.37***	0.27*	16.5	21.8
(Z)-3-hexenol	0.23 ns	0.13 ns	0.14 ns	16.3	6.5
Acetic acid	0.05 ns	−0.09 ns	0.04 ns	0.0	3.8
Hexanoic acid	0.28‡	0.27‡	−0.02 ns	26.2	14.9
Acetoin	0.25‡	0.08 ns	−0.07 ns	11.7	6.4
Benzyl alcohol	0.12 ns	0.04 ns	−0.15 ns	4.9	0.0

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ‡ $p < 0.1$

Mean concentrations lay towards the lower end of ranges for all volatiles and each exhibited a continuous variation in progeny, typical for a polygenic inheritance. An example for raspberry ketone content is shown in Fig. 2 and the other volatiles are shown in Supplementary Fig. 1. This was indicative of a skewed distribution that would be better analysed after a log transformation [log (trait + 0.001) due to zeros]. Several genotypes had zero values for all of the least four volatile traits: hexenol, acetoin, and acetic and hexanoic acids. These were very influential, especially on the correlations, and were replaced with missing values. Correlations and broad sense heritability over all three environments were calculated (Table 3) as were correlations between volatiles (Table 4). In general, more significant correlations were identified between field fruit data and heritability was also greater for most volatiles from field to field. Several volatiles were significantly correlated across all three treatments. These include β-damascenone with α-, β-ionones, all three derived from lycopene, geraniol with α-ionol, β-ionone and hexanoic acid, and α-ionone with β-ionone. Other correlations between volatiles were only significant under certain treatments. Heritabilities of volatiles also varied from field to field across the two seasons or from field to polytunnel within a season.

Mapping of candidate genes and QTL of raspberry volatiles

An evolving genetic linkage map from this reference population was available (Graham et al. 2004, 2006, 2009,

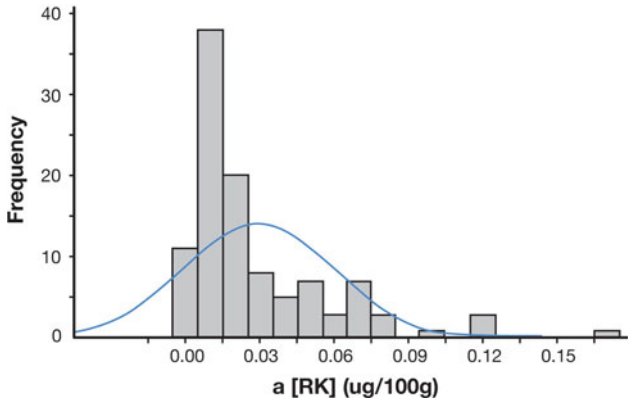


Fig. 2 Amount of raspberry ketone quantified in progeny (ug/100 g fruit) grown under polytunnel conditions in 2007

2011; Kassim et al. 2009; McCallum et al. 2010; Woodhead et al. 2010, 2012). Genes related to volatile regulation were identified and were mapped when polymorphism was detected. These genes were distributed across all seven *Rubus* linkage groups (LG) (Fig. 2; Table 5). Six of the seven linkage groups had regions significantly associated with one or more volatiles (Fig. 3). Significance of volatile QTL varied from year to year and from field to polytunnel after interval mapping, though in general the same markers were identified by KW analysis for each data set although there were exceptions. QTL are shown in Fig. 3 with LOD scores identified as significant at the 95 or 90 % level. A number of overlapping QTL for different volatiles were identified. Candidate genes known to be involved in isoprenoid metabolism are underlined on the linkage groups.

Carotenoid metabolism

α-Ionol

Two loci on two linkage groups LG 3 and LG 5 were identified as having regions significantly associated with α-ionol (Fig. 3). On LG 3, a QTL was identified for 2006 and 2007 field fruit with phytoene synthase (454Contig2985_Physyn) and an ethylene biosynthesis regulator CTR1 gene (454CL6403C1_CTR1) as mid-point. On LG 5, a QTL was identified again for 2006 and 2007 field fruit with 454C4050HMG (HMG-CoA reductase) as most significant.

α-Ionone

Two QTL were identified on LG 1 with P13M60-117 as the most significant marker for 2006 field and 2007 polytunnel fruit (Fig. 3). On LG 3, overlapping QTL were identified with Ri*β*Gal1 (*β*-galactosidase) as the most significant marker for 2006 field and 454CL6403Contig1_CTR1/454Contig2985_Physyn for 2007 polytunnel fruit.

β-Ionone

Three QTL were identified for *β*-ionone on LGs 3, 5 and 6 (Fig. 3). On LG 3, overlapping QTL were identified with (Ri*β*Gal1) the mid-point for polytunnel fruit and RiMYB, a transcription factor, for field fruit in 2007 but no significant markers were identified in this region for 2006. On LG 5, QTL for field fruit from both 2006 and 2007 seasons were identified with ERubLRSQ13.1_F09HMGS (HMG-CoA synthase) as most significant marker and 454CL4590C1_DXR (a deoxy xylulose phosphate reductase) for 2007 fruit. On LG 6, overlapping QTL were identified from both field and polytunnel fruit in 2007. Here, markers in the BAC region Ri38J3 (Graham et al. 2011) were highly significant.

β-Damascenone

Three map regions were identified for *β*-damascenone. These were on LGs 1, 3 and 5. On LG 1, E40M43-93 was identified as most significant from field fruit in both 2006 and 2007. On LG 3, in 2006, a large number of highly significant markers were identified at this locus with the QTL hard to isolate, though RiMYB is shown representative of the mid-point. A similar region was identified from 2007 polytunnel data with RiMYB as most significant. On LG 5, the region from 454C4050_HMG to 454CL4590C1_DXR was highly significant with ERubLR_SQ13.1F09HMGS, the most significant marker in the region in 2007 from field fruit. A similar region was identified in this case with 454C4050_HMG as most significant in 2006.

Monoterpene metabolism

Linalool

LGs 1 and 6 were identified as significantly associated with linalool (Fig. 3). In 2007, from both field and polytunnel fruit, a QTL on LG 1 was identified with P13M60-117 as the mid-point, however, this region was not identified in 2006. A QTL on LG 6 had a large number of highly significant markers with 454CL4788_linalool (a linalool synthase) as the most significant marker in all three data sets.

Geraniol

Geraniol mapped to two QTL, one on LG 2 and the other on LG 5. On LG 2, RiTerpSynth was the most significantly associated marker in 2007 polytunnel fruit. On LG 5, a

Table 4 Significant correlations in flavour volatiles (Pearson value) based on the genotype means (on log-transformed scale) for the mapping population

Volatile compound	Linalool	β-Damascenone	Geraniol	α-Ionone	Benzyl alcohol	α-Ionol	β-Ionone	Acetic acid	(Z)-3-hexenol	Acetoin
β-Damascenone	0.43***									
	0.19*									
	–									
Geraniol	0.49***	0.25**								
	0.41***	0.27**								
	–	–								
α-Ionone	–0.19*	0.37***	–							
	–	0.31***	0.25**							
	–	0.21*	–							
Benzyl alcohol	–	–	0.40***	–						
	0.17*	0.24**	0.35***	0.28***						
	–	0.22*	–	–						
α-Ionol	–	–	0.64***	–	0.62***					
	0.24**	0.28***	0.47***	0.26**	0.49***					
	–	0.22*	0.21*	0.50***	–					
β-Ionone	–	0.23**	0.63***	0.22**	0.41***	0.65***				
	0.35***	0.26**	0.24**	0.38***	0.26**	–				
	–0.19*	0.26*	0.26**	0.66***	–	0.53***				
Acetic acid	–	–	–	–	–	–	−0.23**			
	–	–	–	–	–	–	–			
	–	–0.27**	–	–	–	–	–			
(Z)-3-hexenol	–	–	–	–	–	–	0.19*	–		
	–	–	–	–	–	–	–	0.30**		
	–	–0.24*	–	–	–	–	–	–		
Acetoin	0.24**	–	–	–	–	–	–	0.33***	0.47***	
	0.39***	–	0.29**	–	–	–	–	–	–	
	–	0.21*	0.27**	–	–	–	–	–	–	
Hexanoic acid	0.45***	–	0.43***	−0.26**	–	0.32***	0.22*	–	–	–
	0.25*	0.22*	0.20*	–	–	0.24*	–	0.36***	0.23*	0.29**
	–	–	0.23*	–	0.44***	–	–	0.37***	–	–

$N = 147$ for 2006 (not involving acetic onwards, $N = 122$ with acetic), $N = 138$ for 2007 (not involving acetic onwards, $N = 97$ with acetic), $N = 116$ for 2007 PT (not involving acetic onwards, $N = 96$ with acetic)

*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

region was identified with (ERubLR_SQ13.1_F09_HMGS) most significantly associated only in 2006 data.

Fatty acids

Benzyl alcohol

Overlapping QTL on LG 3 in 2006 field and 2007 polytunnel fruit were identified with a negative regulator of ethylene biosynthesis 454CL6403Contig1_CTR1/phytoene synthase gene (454Contig2985_Physyn) being most significant for 2006 field data and Rub233a being the most highly significant for 2007 polytunnel fruit.

Acetic acid

A single QTL was identified on LG 7 only from 2007 polytunnel fruit with RiPKS5 (a polyketide synthase) as the most significant marker in the region.

Hexanoic acid

Two QTL on LGs 2 and 3 were identified but only in 2007 polytunnel fruit. On LG 2, marker MIP2_SNP, encoding a membrane intrinsic protein was the most significant marker. On LG 3, marker ERubLRcont74 PME-I (a pectin methylesterase inhibitor) was identified as significant.

Table 5 Red raspberry markers derived from expressed sequence tags (ERubLR) or fruit transcriptome databases (454) for volatile and related pathways of impact on volatile production and the primer sequences for mapping

Marker	Pathway	Left (L), right (R) and sequencing (S) primer sequences (5′–3′)	Linkage group
ERubLR_SQ5.3_D11 (AOC)	Jasmonate	GAAGGAGTGTACGGGCATGT	3
Allene oxide cyclase		AAAACCAAATCGGTAAAGCTGA	
454Contig2893_COI1	Jasmonate	TGATTGACGATTGAAGGAACC	1
Coronatine insensitive 1		GAGCTCTTAGCTCGGTCACG	
454CL3440Contig1_CTR1	Ethylene	[Btn]GAAGCGTGTAGTTCGATCTCCT	3
Constitutive triple response 1		GGCGGGTTTAAGGGATTCTA	
		TGTGACGCAGCAATA	
454Contig1037_ACC	Ethylene	CATTCCTCCATTGACAACTCA	1
synthase		TGAACTCCTCCACTCCTTCG	
ACC synthase			
454Contig3060_DXS	Volatiles	GGGTTTCGCATCTTCTCCTT	2
1-deoxy-D-xylulose 5-phosphate synthase		TCAAGTTGAGAGCCATCCTG	
454CL4590c1_DXR	Volatiles	GGCACCATGACAGGAGTTCT	5
1-deoxy-D-xylulose 5-phosphate reductase		CTCATGCCAAGACAGGGATT	
454C8568_ISPH	Volatiles	CAATGCTCCTCATGCCTTTT	3
2-C-methyl-D-erythritol 2,4-cyclodiphosphate reductase		CCTACAAGGGTGATGCCAAG	
454CL3132c1_ISPE	Volatiles	TGCCTACAGGAGCAGGACTT	6
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase		CAAAGGGTACTGGTGGAGGA	
454C3592_ISPG	Volatiles	CCCTCACAAAATCCATGCTT	2
(E)-4-Hydroxy-3-methylbut-2-enyl diphosphate synthase		TCAGTCCCAAAATCCAGAGC	
454Contig4050_HMG-CoA reductase	Volatiles	CGAACCAAACCAAACGAAAC	5
Hydroxymethylglutaryl-CoA reductase		ACCATGATCTTGGAGCAAACC	
454Contig2985_PSY	Volatiles	AGAGCTGGCCTAACCTCACA	3
Phytoene synthase		GGCATTCTCTTTGGGATTCA	
454Contig1027_Lycopene ε-cyclase	Volatiles	GCAAGAATTTCAAGGAAAGCAT	6
		CAACATTGGTAGCCGTCAAA	
454c199C1_Lycopene B cyclase	Volatiles	TTGGACCGTCCTTACGGTAG	2
		AGCCATGATTCCTTGGCTTA	
454Contig1149_CCD	Volatiles	TCTCTAAATTTCTCAACTTCTTCACC	1
Carotenoid cleavage dioxygenase		ACGTGGCTCCTTTCTTCAAA	
454CL4788Contig1_	Volatiles	AACGGCTTGGCATTGACTAC	6
Linalool synthase		CTTGAACATCCCGTTGCTTT	
454Contig2575_LOX	Volatiles	GATAAGGCTGCAGTGGAAGC	7
Lipoxygenase		TCAGAATCCCCTGACCAATC	

Hexenol

Two non-overlapping QTL were identified on LG 3 for 2006 field and 2007 polytunnel fruit with markers ERubLRSQ12.4_A04_DMQ (demethoxy ubiquinone) and RiMYB, respectively. No significant markers were identified for 2007 field fruit. A further QTL on LG 6 was identified only for 2007 polytunnel fruit with markers in BAC Ri38J3 region (Graham et al. 2011) as highly significant.

Phenylpropanoid metabolism

Raspberry ketone

Two QTL were identified on LG 2 and LG 3 for 2007 PT data. On LG 2, all markers from 454C2657_βGal2 to ERubLR_SQ12.1_B12CAF were highly significant at this locus with the mid-point of the QTL at MIP2_SNP. On LG 3, a QTL was identified with 454C3991_PME as the most significant marker in the region.

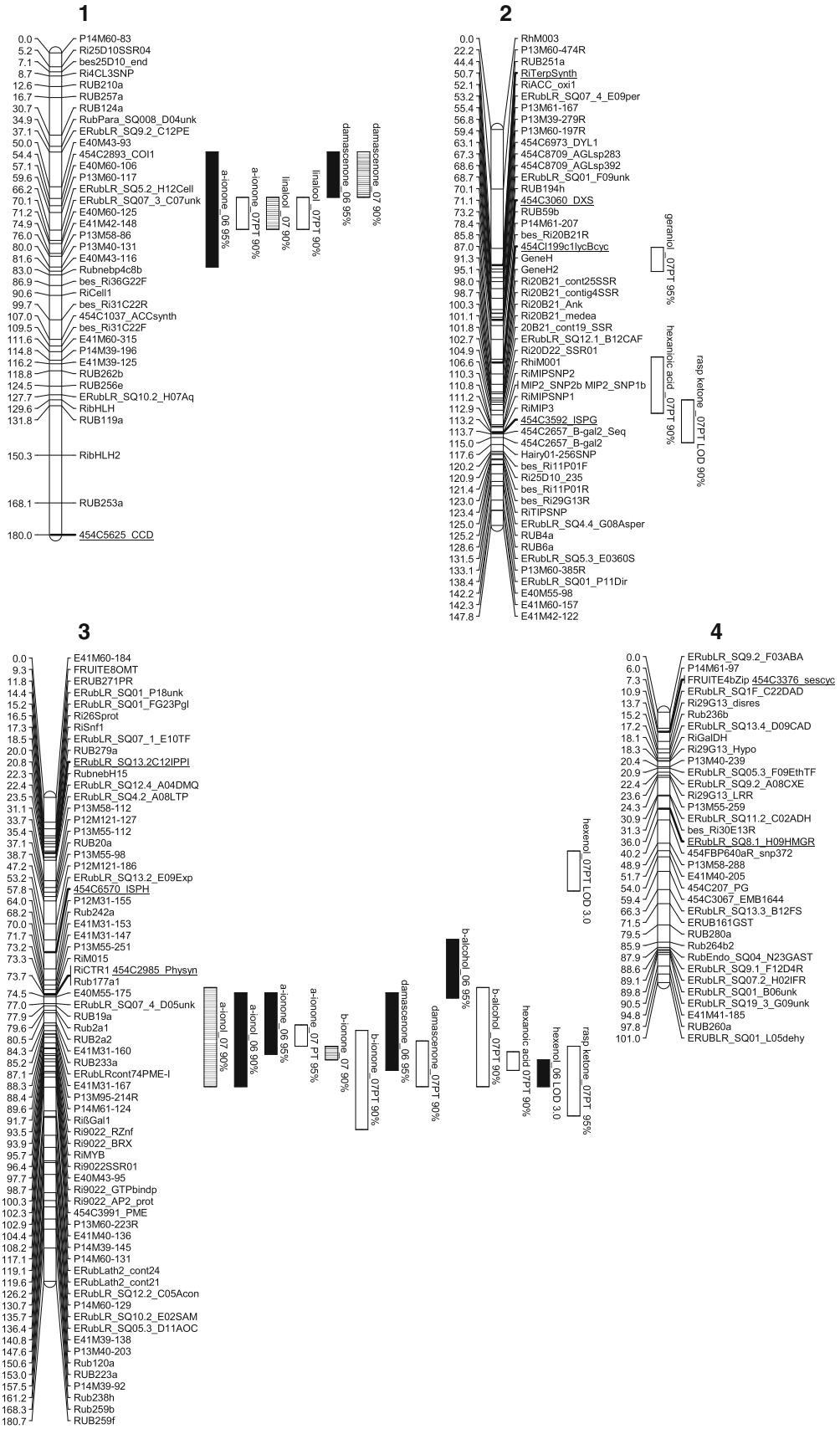


Fig. 3 Linkage groups with QTL associated with raspberry volatiles. Candidate genes are *underlined*

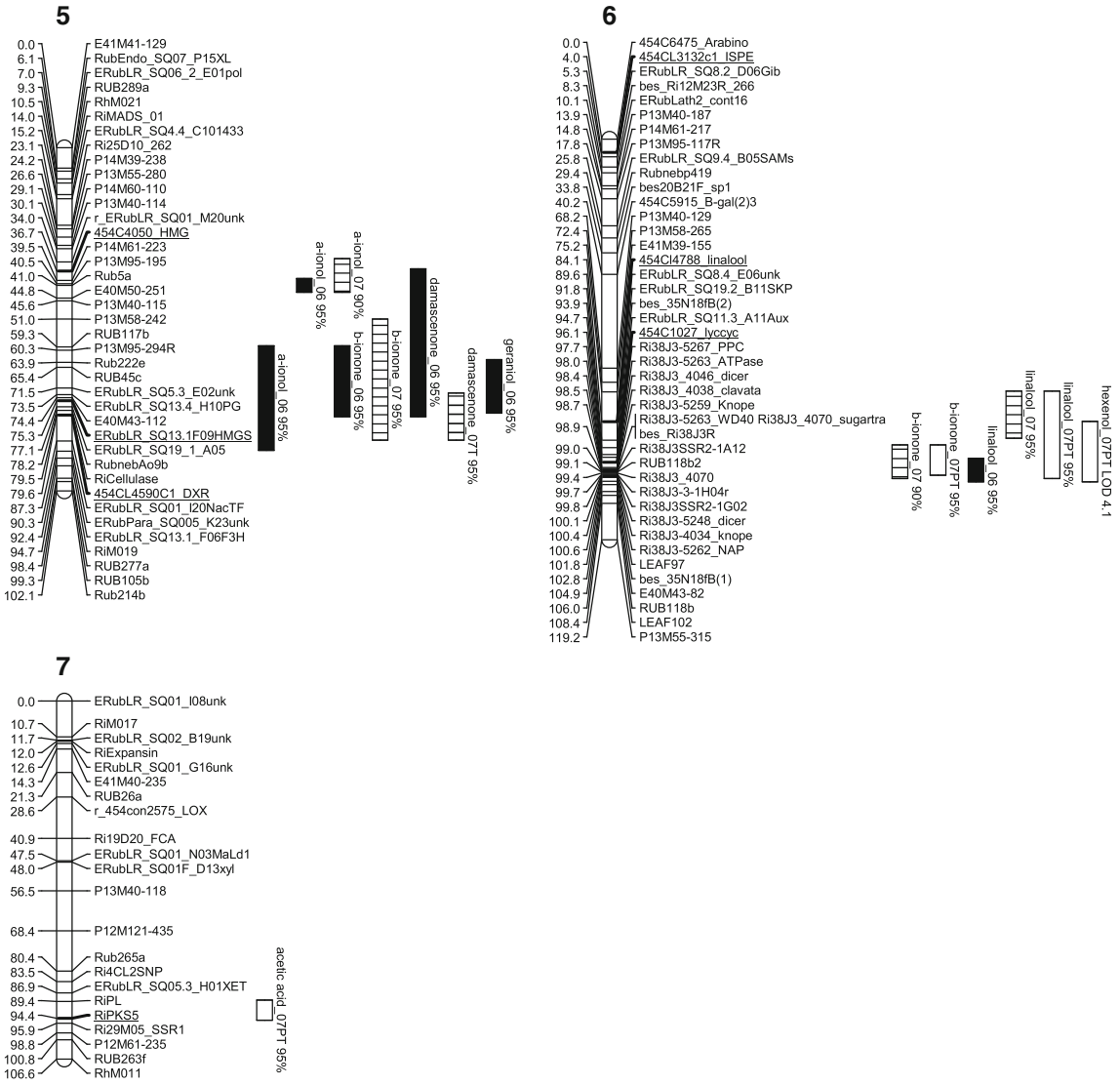


Fig. 3 continued

Discussion

Both season (2006/2007) and environment (field/polytunnel) significantly influenced raspberry fruit content of flavour active volatiles. Nine volatiles, excluding β-damascenone, hexanoic acid and benzyl alcohol, were more abundant in the favourable 2006 season. In 2007, contents of β-damascenone and β-ionone, the major volatiles represented, and hexanoic acid and acetoin were lower in polytunnel fruit, possibly through lower light levels or higher temperatures, though the other volatiles were more abundant. Contents of major volatiles were highly correlated across seasons but generally less so across environments. Protected (polytunnel) cultivation therefore significantly impacts fruit content of flavour volatiles. The impact of environment and season on sensory characters in this mapping population fruit is the focus of another study (Zait, personal communication).

Examination of fruit volatiles across 14 raspberry cultivars also showed clear genotype and seasonal variation, with fruit from a cooler season having higher contents (Aprea et al. 2010). Such differences may be a function of environment and adapted germplasm as humidity also has an effect. Jennings (1988) suggested fruit were more aromatic in warm, dry areas than in mild and humid regions. Grape linalool and geraniol contents from mapping populations also showed significant differences in successive years (Duchêne et al. 2009).

Carotenoid biosynthesis is a UV-light harvesting process (von Lintig et al. 1997) which would suggest that apocarotenoid volatiles should be more abundant in 2006 fruit and in that from the field in 2007 as was the overall pattern observed. However, as in grape, the positive effect of sunlight was not reflected in increases in β-damascenone content (Marais et al. 1991). In this study this difference

was only observed between 2007 field and polytunnel fruit, with the reduction in the latter suggesting an impact of temperature on components regulating this biosynthetic pathway. Cultivation in polytunnels reduced contents of certain phenolic compounds (anthocyanins) in this raspberry population (Kassim et al. 2009), also reported in strawberry (Josuttis et al. 2010). In this study, fewer correlations between volatiles were significant in polytunnel fruit. Field to polytunnel correlations were also less clear than between seasons in field fruit.

Carotenoids

The C13-norisoprenoid volatiles, thought to be derived enzymatically from specific carotenoids, β-carotene, neoxanthin and zeaxanthin (Winterhalter and Rouseff 2002; Lutz-Wahl et al. 1993), were found to be the most abundant flavour active volatiles (Fig. 1) in this study. This is consistent with the previous work which found raspberry aroma, quantified by relative aroma values, to be determined to a large extent (60–90 %) by fruit contents of α- and β-ionones (Larsen et al. 1991). Greater understanding of the genetics behind the biosynthesis of these volatiles may prove useful for future breeding programmes. Green raspberries contain β-carotene and lutein as chloroplastic photoprotective compounds providing a pool of carotenoid precursors for ionones, whereas α-carotene biosynthesis later in ripening may provide a basis for further apocarotenoid production (Beekwilder et al. 2008). In all three volatiles data sets (field 2006; field and polytunnel 2007), significant positive correlations were detected between α- and β-ionone, derived from a common precursor (lycopene), and between β-ionone and β-damascenone, suggesting as predicted that β-ionone is in part derived from β-damascenone (Table 4). However, other biosynthetic pathways have also been proposed for β-damascenone (Fig. 1), involving the breakdown of neoxanthin to grasshopper ketone which is further transformed and modified into β-damascenone (Winterhalter and Rouseff 2002; Lutz-Wahl et al. 1993). The presence of multiple pathways of β-damascenone biosynthesis may contribute to a higher content in many progeny compared to the other apocarotenoid volatiles, α-, β-ionones and α-ionol. Alternatively, it could indicate that this is a terminal point in the carotenoid breakdown pathway in raspberry fruit.

In raspberry, overlapping QTL for multiple carotenoid-derived compounds suggests common control points in the biosynthetic pathways. The cytosolic MAP pathway at the HMG-CoA synthase gene (Fig. 1) on LG 5 producing IPP/dimethylallyl diphosphate (DMAPP) feeding into carotenoid metabolism appears important in regulating the synthesis of β-damascenone, geraniol, and α-and β-ionones, whereas HMG-CoA reductase is underlying α-ionol content. Regulation may also occur within the carotenoid pathway, for example, the phytoene synthase gene (454Contig2985_PSY), and the proximity of a gene in the ethylene regulatory pathway (454CL6403Contig1_CTR1) at the locus on LG 3 may suggest a functional relationship. The phytoene synthase gene underlies QTL for β-damascenone, α-ionone, and α-ionol, though interestingly, not β-ionone which is possibly regulated by ripening genes, RiMYB or RiβGal depending on cultivation. Unique QTL for β-ionone on LG 6 and α-ionol on LG 5 suggest other points of regulation. Candidate genes have not yet been identified on LG 6 but a lycopene ε-cyclase gene (454C1027-lyccyc) is proximal to the QTL for β-ionone (Fig. 3). In tomato, this gene influences flux of precursors into α- and β-ionone biosynthesis (Alba et al. 2005; Tieman et al. 2006) and it may have a similar role in raspberry. In each of the present three volatiles data sets, α-ionone was less abundant than β-ionone and showed high heritability across all three environments (Table 3). The availability of QTL data associated with highly significant markers represents a step towards identifying robust tools for germplasm identification for these important carotenoid volatiles.

Monoterpenes

Two pathways of isoprenoid metabolism have been identified in plants (reviewed by Rodríguez-Concepción 2010) (Fig. 1). Both of these pathways, the cytosolic mevalonic acid (MVA) pathway and the plastid methylerythritol 4-phosphate (MEP) pathway, produce IPP and DMAPP which are transformed into monoterpenes geraniol and linalool by their respective synthases. In grape, a 1-deoxy-D-xylulose 5-phosphate synthase (DXS) gene from the MEP pathway co-localises with a major QTL for linalool, geraniol and neriol (Battilana et al. 2009; Duchêne et al. 2009), suggesting the MEP pathway is the major route to monoterpene production. In raspberry, QTL for these monoterpenes are located on different linkage groups: geraniol on LGs 2 and 5 and linalool on LGs 1 and 6 (Fig. 4). Relevant candidate genes underlying the geraniol QTL on LG 2 are RiTerpSynth encoding a terpene, possibly a geraniol synthase, and on LG 5, HMG-CoA synthase and HMG-CoA reductase (LG 5), both enzymes in the MVA pathway (Fig. 1). In contrast, linalool QTL co-localise on LG 6 with a linalool synthase and with an anonymous AFLP marker on LG 1. Hampel et al. (2007) showed that (S)-linalool biosynthesis occurs via the cytosolic MVA pathway (Fig. 1) but did not examine that of geraniol. Co-localisation of the geraniol QTL with the MVA pathway genes may suggest that this provides precursors for geraniol and may also act as regulatory control

points for geraniol, but not linalool, synthesis. The two monoterpenes were highly correlated.

Phenylpropanoid metabolism

The phenylpropanoid pathway is well characterised and produces an array of highly modified compounds required for various plant physiological functions and environmental adaptations. In raspberry fruit, this includes the flavonoid pathway for the synthesis of anthocyanins (Jennings 1988) and, also of interest here, raspberry ketone (Borejsza-Wysocki and Hrazdina 1994). This volatile is considered to be synthesised through the action of the *Ri*PKS4 and another, unidentified gene (Zheng and Hrazdina 2008). Neither of the two PKS genes, mapped to LG 7 in this population are of PKS4 type (Kassim et al. 2009; Woodhead et al. 2010) which may underlie the raspberry ketone QTL. However, an R2R3 MYB transcription factor (*ODO1*) has been shown to be a key regulator of volatile benzenoids in *Petunia hybrida* floral aroma (Verdonk et al. 2005), and an *Ri*MYB gene associated with fruit ripening (Graham et al. 2009) co-locates with the raspberry ketone QTL on LG 3. In addition, ethylene may have a role in regulating early steps in the phenylpropanoid pathway (reviewed by Singh et al. 2010) through the action of the CTR1 gene (454CL6403Contig1_CTR1). With measurements only for 2007 fruit, the heritability of this volatile could not be determined. However, this represents the first step in determining loci associated with raspberry ketone and may yield markers that can be deployed for map-based cloning to unravel the regulation of this trait.

Ripening is a complex, tightly regulated process, the result of many co-ordinated biochemical and physiological changes resulting in major changes to fruit flavour, texture and colour. Major genomic regions across six linkage groups were identified as involved in fruit volatile production in raspberry. A number of QTL were co-localised, others dispersed across linkage groups which are not surprising considering these volatiles are products of multiple and complex pathways. Clustering of QTL may be either through tightly linked multiple loci or of a single locus with pleiotropic effects such as transcription factors or enzymes that catalyse limiting steps in biosynthetic pathways (Tieman et al. 2006). In tomato, QTL for aroma volatiles derived from single pathways are clustered: LG 1 with two volatiles from fatty acids and LG 9 with two phenolic compounds (Saliba-Colombani et al. 2001). Co-localisation of 7 of the 12 QTL mapped volatiles with lycopene and fruit colour (Saliba-Colombani et al. 2001) suggested that pigmented carotenoid precursors were accountable for volatile production. In raspberry, fruit colour is largely attributed to flavonoid compounds rather than carotenoids and QTL for fruit colour (McCallum et al. 2010) as well as individual and total anthocyanins contents (Kassim et al. 2009) have been mapped. On LG 1, LG 4 and LG 6 such QTL overlap with certain QTL for volatiles contents suggesting commonalities.

Regions of the raspberry genome associated with fruit ripening (Graham et al. 2009) co-localise with QTL for specific volatiles (Fig. 3). These include geraniol on LG 2, and most QTL on LG 3, LG 5 and LG 6 (Fig. 3). Whilst little is known about the genes underlying raspberry ripening QTL (Graham et al. 2009), a possible role for the *Ri*MADS_01 box gene on LG 5 could be predicted. In tomato, MADS box and SPB box transcription factors play an important role in fruit development, ripening and quality attributes (reviewed by Barry and Giovannoni 2007).

Fruit ripening is also modulated by phytohormones. Genes involved in ethylene synthesis, signalling and response are also important in many fruit including raspberry (Ianetta et al. 1999), the related apple (Lay-Lee et al. 1990) as well as tomato (Grierson and Tucker 1983). In apple and mango, aroma volatiles synthesis increases with ripening and is associated with ethylene production (Song and Bangerth 1996; Lalel et al. 2003). Using a microarray approach, Schaffer et al. (2007) showed that ethylene often regulates the first and, in each case, the last steps of volatile biosynthetic pathways in apple. In tomato, ethylene has been shown to influence multiple steps in carotenoid synthesis (Alba et al. 2005). Several genes involved in the ethylene pathway have been mapped in raspberry (Woodhead et al. 2010, 2012) and a CTR1 gene (454CL6403Contig1_CTR1), a negative regulator of ethylene biosynthesis (reviewed by Kendrick and Chang 2008), is associated with multiple volatile QTL including α-ionone, benzyl alcohol and α-ionol (Fig. 3). CTR1 may be or lie close to, a key control point in these pathways. Other plant hormones are implicated in the fruit ripening, including jasmonic acid which can modulate the process (e.g. Sheng et al. 2000; Mukkun and Singh 2009), including aroma and ethylene production (Kondo et al. 2004). The coronatine insensitive gene, 454C2893_COI1 involved in jasmonate perception is within QTL for α-ionone, linalool and β-damascenone on LG 1, possibly indicating a role for this hormone in regulation of aroma production in raspberry.

Raspberry volatile production appears to be significantly influenced by environmental as well as complex genetic factors and this work provides a basis from which to proceed towards identifying the important variables contributing to desirable flavour/aroma characters at the genetic level. The demonstration that the concentrations of volatiles change across seasons and environments, coupled with the shift or loss of location of QTL across seasons and environments, highlights the complex regulatory nature of volatile regulation.

The differences in QTL may be providing an insight into how regulation changes with season and environmental factors. At least 12 overlapping individual volatile QTL were identified compared to eight non-overlapping QTL. Linalool was the only volatile where co-locating QTL were identified across all three data sets. Generally, two data set QTL overlap. Seven overlapping QTL were identified for 06 field/07 polytunnel, five from 06 field/07 field and four from 07 field/07 polytunnel. The seasons were very different and from observations, the fruit under polytunnel in the poor 2007 season was similar to that in the favourable field 2006 which may explain the 06 field/07 polytunnel QTL correlations. Field to field seasonal correlations were also highly heritable explaining the 06/07 field relationships. Fewer 07 field/07 polytunnel correlations were identified indicating the environment as a crucial regulator in volatile biosynthesis.

Meteorological data (Kassim et al. 2009) identified the 2006 growing season as hotter and drier with more sunshine hours than 2007. The QTL for β-damascenone on LG 3 with candidate 454C2985/RiCTR1 was not identified in field fruit in 2007 nor was the QTL on LG 5 with ERubLR_SQ13.1F09HMGS/454C4050_HMG. In contrast, the QTL on LG 1 with underlying 454C2893_COI1 involved in jasmonate perception was only identified in field fruit in both seasons. The difference in traits from field to polytunnel has previously been reported in raspberry with anthocyanin pigments (Kassim et al. 2009) and colour (McCallum et al. 2010). Berry contents of sugars and organic acids, the non-volatile metabolites, may relate to preference within a crop, however, the volatile components are thought to determine identities (Mathieu et al. 2009) and intention to consume. Epidemiological evidence suggests that intake of berry phytochemicals brings significant health benefits such as reductions in risks of cancers and increased antioxidants (Shualev et al. 2008). As fruit flavour is influenced by many contributing factors including genetic variation, climatic and soil conditions as well as the degree of ripeness the challenge remains to generate relevant information for flavour characters with correlation between sensory scoring and compositional data. Work is ongoing (Zait, personal communication) to model the relationships between sensory data and composition and this knowledge is essential for future targeted breeding. Once the key flavour determinants have been identified, marker-assisted breeding may then provide a strategy for selective reduction in undesirable progeny from favourable breeding crosses bringing together flavour preferences with associated health benefits if and when these become proven. Validation in other smaller populations (around 80–100 individuals) for favourable alleles would be carried out followed by testing on selected germplasm collections to confirm associations before release to breeding programmes.

Six of the seven linkage groups presented here (named as in Graham et al. 2004) have recently been associated with *Fragaria* linkage groups FLGVII, FLGIII, FLGVI, FLGII, FLGV and FLG1, respectively, with LG 7 not currently associated (Bushakra et al. 2012) allowing access with the diploid strawberry genome sequence which will be useful in future gene identification within QTL.

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