Theor Appl Genet (2010) 121:611–627
DOI 10.1007/s00122-010-1334-5

ORIGINAL PAPER

Genetic and environmental effects influencing fruit colour and QTL analysis in raspberry

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Received: 8 December 2009 / Accepted: 26 March 2010 / Published online: 24 April 2010

Abstract Raspberry (*Rubus idaeus*) fruit colour was assessed in the Latham × Glen Moy mapping population using a colour meter and visual scores over three seasons and three environments. The colour measurements were found to be significantly associated with pigment content, have high heritability, and stable QTL were identified across environments and seasons. Anthocyanin content has previously been shown to be the major contributor to fruit colour in red raspberry. Major structural genes (F3′H, FLS, DFR, IFR, OMT and GST) and transcription factors (bZIP, bHLH and MYB) influencing flavonoid biosynthesis have been identified, mapped and shown to underlie QTL for quantitative and qualitative anthocyanin composition. Favourable alleles for the selected traits were identified for the aspects of fruit colour and partitioning of individual pigments.

Communicated by H. Nybom.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-010-1334-5) contains supplementary material, which is available to authorized users.

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Introduction

Raspberry is a high-value horticultural crop, interest in which is expanding due to the beneficial impact on human health (Ness and Powles 1997; Palace et al. 1999; Stewart et al. 2007). Quality considerations are paramount to the success of any raspberry variety, with visual traits important for initial purchase and acceptability. The main visual traits of interest to producers and buyers alike are the overall appearance of the fruit, mainly colour, but other fruit traits such as size, overall drupelet cohesion, shape and firmness are also important (Graham and Jennings 2009). The colour of fruit not only directly affects the appearance, desirability and associated freshness but is also an important determinant of flavour perception. Many attempts have been made to evaluate the role of colour cues (for review see Clydesdale 1993; Delwiche 2004) which have been shown to dramatically affect the perceptions in a variety of different foods and drinks (DuBose et al. 1980; Garber et al. 2000; Roth et al. 1988; Zellner and Durlach 2003; Zampini et al. 2007). The addition of red colouring to strawberry-flavoured sucrose solutions, for example, was found to increase the perceived sweetness (Johnson et al. 1983), and colours that are typically associated with fruit ripening may be particularly effective at modulating the sweetness perception (Maga 1974).

Colour in raspberry is a complex trait with anthocyanin content (predominantly cyanidin and pelargonidin pigments) thought to be the major contributing factor (Jennings 1988; Wang et al. 2009). Jennings and Carmichael (1980) described the genes *R*, *So* and *Xy* necessary for synthesising the sugars rhamnose, sophorose and xylose, respectively, which are required to give the array of different anthocyanin pigments observed in red raspberry, as well as a series of genes controlling pigment concentration (Jennings 1988).

The final expression of fruit colour is influenced by both co-pigments and pH. Co-pigmentation allows the formation of complex interactions between the pigments and colourless compounds which enhance the colour intensity. Several compounds may act as co-pigments including flavonoids, alkaloids, amino acids, polysaccharides, metals, organic acids, nucleotides and other anthocyanins (Castañeda-Ovando et al. 2009). In most plants, the colour of fruit and flowers results from the accumulation of anthocyanins in cell vacuoles, and as the absorption spectrum of anthocyanins depends on the pH of their environment, the observed tissue colour effectively reflects vacuolar pH (Yoshida et al. 2003).

The enzymes involved in flavonoid biosynthesis, which includes anthocyanins and flavonols, are well characterised and are shown in Fig. 1. The common precursors involved in the biosynthesis of all flavonoid classes are malonyl-CoA and *p*-coumaroyl-CoA which are condensed into chalcone intermediates via the action of chalcone synthase (Xie et al. 2004). The pathway then divides to form various flavonoid compounds (Fig. 1).

Flavonols are derived from dihydroflavonols by flavonol synthase (FLS), a soluble enzyme requiring the presence of the cofactors 2-oxoglutarate, ascorbate and ferrous iron to introduce a double bond between the C-2 and C-3 rings of dihydroflavonols (Nielsen et al. 2002). This reaction also requires molecular oxygen, so FLS is therefore referred to as a 2-oxoglutarate-dependent dioxygenase (Wellman et al. 2002). Flavonoid 3′-hydroxylase (F3′H) catalyses the stereospecific 3-*β*-hydroxylation of flavonones to produce dihydroflavonols which is an important step for flavonol, anthocyanin, catechin and proanthocyanidin biosynthesis (Jaakola et al. 2004). Dihydroflavonol 4-reductase (DFR) catalyses the conversion of dihydroflavonols to leucoanthocyanidins, which is an important step in the biosynthesis of anthocyanins, proanthocyanins and other flavonoids pertinent to plant survival.

Enzymatic *O*-methylation involves the transfer of a methyl group to a hydroxyl group on an acceptor molecule (i.e. phenylpropanoids and flavonoids) which is catalysed by *O*-methyltransferases (OMT). There have been several hundred *O*-methylated flavonoids identified in plants, and these include chalcones, flavonones, flavones, isoflavones, flavonols and anthocyanins (Ibrahim et al. 1998).

Following flavonoid synthesis in the cytoplasm, anthocyanins and proanthocyanins are transported to the vacuole, where they can be permanently stored. This transfer is facilitated by glutathione *S*-transferase (GST). GST is involved in the last genetically defined step in anthocyanin biosynthesis involving the addition of a glutathione onto

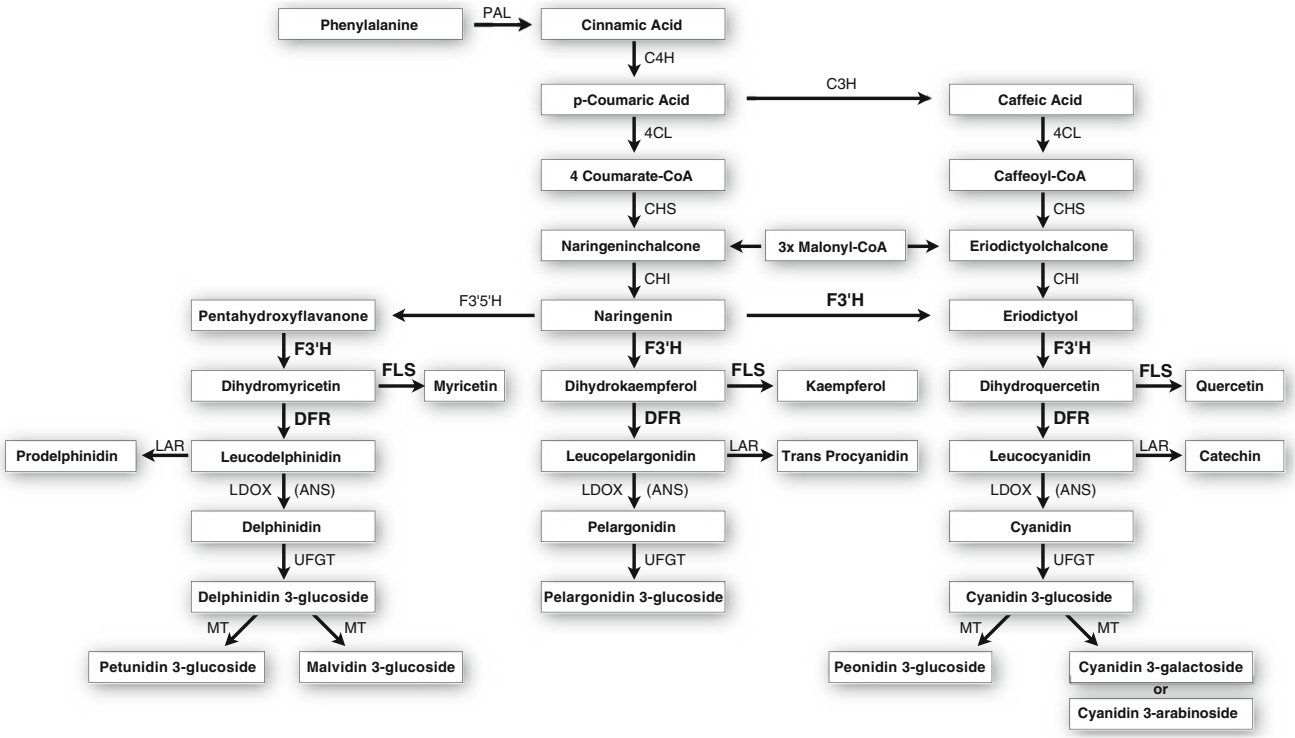

Fig. 1 Schematic representation of flavonoid biosynthesis. *PAL* phenylalanine lyase; *C4H* cinnamate 4-hydroxylase; *C3H* *p*-coumarate 3-hydroxylase; *4CL* 4-coumarate CoA ligase; *CHS* chalcone synthase; *CHI* chalcone isomerase; *F3′H* flavonoid 3′-hydroxylase; *F3′5′H* flavonoid 3′5′-hydroxylase; *FLS* flavonol synthase; *DFR* dihydroflavonol 4-reductase; *LAR* leucoanthocyanidin reductase; *LDOX* leucoanthocyanidin dioxygenase; *ANS* anthocyanin synthase; *UFGT* UDP glucose:flavonoid 3-*O*-glycosyl transferase; *MT* methyl transferase. Enzymes in bold have been mapped in this study

anthocyanins such as cyanidin-3-glucoside. This glutathione "tag" allows the cyanidin molecule to be transported into the vacuole by means of a tonoplast Mg-ATP requiring glutathione pump (Alfenito et al. 1998; Lu et al. 1998; Marrs 1996).

Recently, Kassim et al. (2009) mapped QTLs for eight individual anthocyanin pigments [cyanidin-3-sophoroside (C3S), cyanidin-3-glucosylrutinoside (C3GR), cyanidin-3-glucoside (C3G), cyanidin-3-rutinoside (C3R), pelargonidin-3-sophoroside (P3S), pelargonidin-3-glucoside (P3G), pelargonidin-3-glucosylrutinoside (P3GR) and pelargonidin-3-rutinoside (P3R)] in raspberry to the same chromosome region on linkage group (LG) 1 and seven of the pigments to a region on LG 4. A chalcone synthase (PKS 1) gene mapped to LG 7 and did not underlie the anthocyanin QTLs identified. Candidate genes were identified within the QTLs, including a basic helix-loop-helix protein (bHLH) on LG 1 and a NAM/CUC2-like protein and bZIP transcription factor on LG 4 (Kassim et al. 2009).

The importance of transcription factors in anthocyanin biosynthesis was first described in maize kernels with the discovery of COLORLESS1 (C1) and RED (R) regulators (Cone et al. 1986). Since then transcription factors related to the C1 and R genes, which belong to the MYB and bHLH family, respectively, have also been shown to regulate the flavonoid accumulation pathway in other plant species (Allan et al. 2008), including apple (Espley et al. 2007) and grape (Lijavetzky et al. 2006). The transcription factors bind to specific regions in the promoters of certain structural genes in the anthocyanin pathway, leading to the co-ordinated expression of anthocyanin pigments. Five tandem repeats of a 23-bp sequence in the upstream regulatory region of the MdMYB10 gene are responsible for the elevated levels of anthocyanin biosynthesis throughout the plant. This rearrangement is present in red foliage apple plants but absent in white fleshed varieties, and is attributed to the MYB10 gene becoming an autoregulatory locus (Espley et al. 2009). In raspberry, two MYB genes underlie a QTL on LG 3 with a major impact on fruit ripening, importantly at the transition from the green to the green/red stage (Graham et al. 2009), but it is unclear at present if these are implicated in the expression of fruit colour.

Gene families like aquaporins may have a role in colour as these are water channel proteins capable of transporting water and small molecules across cellular membranes. Three main types of aquaporins are known in plants, membrane intrinsic proteins (MIPs), tonoplast intrinsic proteins (TIPs) and plasma membrane intrinsic proteins (PIPs) (Smart et al. 2001). Plant MIPs are reported to play an important role in cell division and expansion as well as water transportation in relation to environmental conditions (Oliviusson et al. 2001). Tonoplast intrinsic proteins have been shown to act as water channels expressed predominantly within storage tissues. As glucose is accompanied by the transport of water, these genes are strong candidates for quantitative differences relating to the storage and transport of sugar molecules (Martinoia et al. 2000).

The objective of this work was to understand the effect of genetics and environment on the regulation of colour in raspberry by identifying colour QTL across three fruiting seasons and three environmental locations (one open field site and two different types of protective tunnels) on the *Rubus* cv. Latham × Glen Moy genetic linkage map (Graham et al. 2009). The association between colour and individual and total anthocyanins was examined here. Potential candidate genes related to the structural or functional synthesis of flavonoid components were also mapped in order to identify any gene association. Candidate genes examined for the association with QTL included the aquaporin genes, MIP2, MIP3 and TIP, and flavonoid genes: flavonol synthase (FLS), isoflavonone reductase (IFR), dihydroflavonol reductase (DFR), flavonoid 3′-hydroxylase (F3′H), glutathione *S*-transferase (GST), *O*-methyltransferase (OMT) and transcription factors bHLH, bZIP and MYB (Fig. 1).

Materials and methods

Mapping population

The raspberry mapping population as previously described (Graham et al. 2004, 2006, 2009) consists of a full-sib family of 320 progeny derived from a cross between the European red raspberry cv. Glen Moy (large, pale red, moderately sweet fruit) and the North American red raspberry cv. Latham (small, sweet, dark red fruit). This population was planted in randomised complete block trials at three different environmental locations. The sites were an open field site at SCRI, Dundee, UK, a protected cultivation site at SCRI and a protected cultivation site on a commercial farm at Blairgowrie, UK. All trials were arranged in a randomised block design with three replicates each containing two replicated plants of 320 genotypes in the field and one plant of 188 genotypes (from the original 320 full sib family randomly selected for mapping purposes) under protection. The protected sites were covered with standard 150-μ Visqueen polythene. The tunnels had three rows per bay, 2.5 m between rows with a 2.8-m leg row. Alleyways were grassed and leg rows covered with UV-stable fabric mulch (Phormasol) to control weeds. Irrigation and fertigation were controlled by a D8 Dosatron water-powered dosing system fed through Ram Light tape under the bedding polythene (Jennings, personal communication; Thomson, personal communication).

Sample collection

The progeny arising from the 'Latham' × 'Glen Moy' cross segregate widely for a number of key characteristics, including the time to ripe fruit and fruit colour that impacted on the ability to visually select and then collect fruit at the same stage of ripeness across all progeny. For this study, it was crucial to determine as closely as possible when material from each of the progeny was ripe. An assessment of fruit ripening was therefore required across the entire season starting in the middle of May and scoring all plants two or three times a week initially, then on a daily basis as the season progressed to give a standardisation point for fruit ripening. The latest stage present on each bush was allocated a grade as follows: Stage 1, buds present; Stage 2, bud break/open flowers; Stage 3, fruit set; Stage 4, green fruit; Stage 5, green/red fruit; Stage 6, red fruit; Stage 7, over-ripe fruit. Fruit was then picked from the progeny when the majority of berries on the plant reached the red fruit stage (6).

Fruit was harvested at the same time of day and from one side of the plant to avoid any effect of sun/shade. Fruit was placed into labelled polythene bags and stored in cool boxes for transport to the cold room (4°C). All analyses were performed on the day of collection.

Only the open field location was selected for analysis in 2006 with multiple clones for each individual assessed. Samples were analysed for clone 1 and clone 2 and also repetition 1 and repetition 2 giving a total of four plants per individual.

Following the multiple clone analysis it was found for all traits analysed that while significant variations were detected between progeny, there were no significant variations between individual clones or repetitions. As a result of these findings only one clone per individual was assessed in subsequent seasons from each environmental location.

Colour analysis

Samples were prepared by placing 35 g of fresh fruit into a commercial Waring® blender (Torrington, USA) on full power (16,000×*g*) for 10 s. The number of berries represented by 35 g varied between progeny and depended on the size of the individual fruit taken from samples collected in polythene bags which ranged from 0.9 to 5.5 g per berry. The puree was mixed for a few seconds and blended for another 10 s. Fifteen grams of puree was then transferred into a sterile Petri dish which was gently shaken to provide an even surface for analysis.

Colour was assessed using a Minolta Chroma meter CR-100/CR-110 (Minolta Camera Co., Osaka, Japan). This allowed each sample to be measured in terms of chromaticity under two separate formats. Overall sample brightness as described by *Yxy* (where Y = illuminance and reflection, x = colour intensity/chroma and y = wavelength of individual colour) and the individual colour composition as described by $L^*a^*b^*$ (where L^* represents brightness–darkness, a^* represents green–red spectrum and b^* represents the blue–yellow spectrum (Almela et al. 1995) were assessed. The Chroma meter was set out as described by the manufacturer and blanked using the accompanying white tile (101974) with a reflectance of Y 87.4, x 0.308 and y 0.315, and results were recorded for *Yxy*, $L^*a^*b^*$ and ΔE (a measurement of colour deviation representing the total colour difference within the samples).

Visual colour analysis

The colour of the fruit on the bush was also assessed visually using a scale from 1 to 5, with 1 pale pink, 2 pale pink/red, 3 mid-red, 4 mid-red/dark-red and 5 dark-red. Colour was assessed on individual plants once the majority of the fruits were deemed ripe having reached stage 6 (red fruit) and were easily removed from their receptacle. This was assessed at the same time of day and from the same side of the bush.

Extraction of raspberry juice

Following colour meter analysis in 2008, blended raspberry puree was frozen prior to individual biochemical analysis. Raspberry juice was extracted from the thawed puree using 5 ml of fruit puree and 5 ml of 4% (v/v) acetic acid in acetonitrile (AcOH in ACN). The tubes were then centrifuged at 1,000×*g* for 3 min in an Eppendorf 58190R centrifuge (Eppendorf, Germany), and four 2-ml aliquots of 50% (w/v) juice were obtained.

Total anthocyanin pigments

To assess total anthocyanin content per sample, 40 µl of the extracted juice (50% w/v) was transferred into disposable 1.6-ml semi-micro cuvettes (Greiner, Bio-one) in replicates of three for a buffer of pH 1.0 (0.2 M HCl/KCl) and a further three replicates for a buffer of pH 4.5 (0.1 M sodium acetate). The samples were then analysed using an Ultraspec 2100 prospectrophotometer (Amersham Biosciences) at wavelengths of 510 and 700 nm, and the results were recorded for the ratio of each buffer in triplicate. The mean for the three reads per buffer were obtained and used to calculate the anthocyanin content (mg per 100 g fresh weight) according to the pH differential method of Cheng and Breen (1991) ($A = [(\text{A510} - \text{A700})_{\text{pH1.0}} - (\text{A510} - \text{A700})_{\text{pH4.5}}]$) which relates to the molar extinction coefficient for cyanidin-3-glucoside (29,600).

Individual anthocyanin pigments

Data were available on the concentration of eight individual anthocyanin pigments from fruit from 188 of the progeny from the same cross in the same seasons from the open field and the polytunnels in 2007 (Kassim et al. 2009). These data were re-examined for the comparison with the colour and total anthocyanin measures obtained in this study.

Ripening scores

The analysis of the ripening data of the same 188 progeny from the open field trial has been presented by Graham et al. (2009), who used principal coordinates to summarise the ripening scores through the season. The principal coordinates' scores were re-examined for correlations with the mean colour measurements of the 188 progeny from the current study.

Cloning of raspberry candidate gene and marker sequences for mapping

Potential candidate genes influencing the fruit quality QTLs in peach (Etienne et al. 2002) and other members of the Rosaceae or from the SCRI *Rubus* in house database were identified (Tables 1, 2). Primers were designed using Primer 3 software (Rozen and Skaletsky 2000) and used in raspberry on the 'Glen Moy' and 'Latham' parents plus six progeny to determine PCR success. A typical 25 µl reaction contained 25 ng template DNA, 1.0 µM primer, 0.2 mM dNTPs, 0.1 units Taq polymerase (Roche). PCR was performed using a Perkin Elmer 9700 Thermal Cycler as follows: 5 min at 95°C, then 30 s at 95°C, 30 s at 57°C and 45 s at 72°C for 40 cycles followed by 10 min at 72°C before PCR products were visualised on a 1.5% agarose gel. PCR products were cloned into pGEMT-Easy (Promega) followed by transformation into electrocompetent DH5α cells (Invitrogen) following the manufacturer's instructions. Single transformed colonies were identified, and the plasmid DNA subsequently sequenced as described below.

For the genes of interest for which no amplification with the raspberry DNA was obtained, new primers were designed using the peach sequence accession and using the BLAST algorithm at NCBI to identify similar sequences in *Prunus*, *Fragaria* and *Pyrus*. Sequences were aligned using CLUSTALW to identify conserved regions, and primers subsequently designed using Primer 3 (Rozen and Skaletsky 2000). Products were subsequently cloned into pGEMT-Easy (Promega) and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with M13 forward and reverse primers (5′-GTAAAACGACGGCCAG and 5′-CAGGAAACAGCTATGAC, respectively) using 25 sequencing cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min on a GeneAmp 9700 PCR System Thermal Cycler (Applied Biosystems). Sequences were analysed using a 3730 DNA Analyzer (Applied Biosystems). Sequence data were analysed manually using Sequencher 4.5 (DNA Codes Corporation) to identify sequence polymorphisms, and sequences were searched against the non-redundant nucleotide databases at NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul et al. 1990) to confirm whether they were the desired products.

Single nucleotide polymorphisms (SNPs) were identified, and the primers were designed for a Pyrosequencing® assay (Table 1) using the PSQ Assay Design software 1.0 (Qiagen, Germany). The Pyrosequencing® assay was performed on all 188 individuals of the mapping population according to the manufacturer's protocol (Qiagen) using a PSQ96MA Pyrosequencing® instrument (Qiagen). Sequence results were obtained in the form of Pyrograms® and analysed using PSQ96MA 2.1 software (Qiagen).

BAC Ri29G13 was identified as part of a project examining regions associated with ripening traits on LG 2 (Woodhead et al., personal communication) from the *Rubus* BAC library (Hein et al. 2005). BAC DNA was prepared and end-sequenced as described in Graham et al. (2009). Sequence data were analysed as described above, and the primers were designed to the SSR present in the sequence. The SSR was amplified, analysed and scored in the mapping population as described in Woodhead et al. (2008).

Statistical analyses

Heritability

The colour measurements from the mapping population of 188 progeny were analysed using the statistical programme Genstat 10 for Windows (Genstat 2007) to determine the correlations among the measurements and their broad sense heritability. Because of the different replication, the 2006 field trial was analysed separately from the 2007 and 2008 trials to determine heritability. In 2006, the heritability H^2 of the progeny means was estimated as

$$H^2 = a\sigma_G^2 / \left(a\sigma_G^2 + b\sigma_p^2 + \sigma_e^2\right)$$

where σ_G^2, σ_p^2 and σ_e^2 are the (additive, dominance and epistatic) variance components for genotypes, plots and clones within plots, $a \leq 4$ (number of replicates × number of clones within a plot) and $b \leq 2$ (number of clones within a plot). In 2007 and 2008, a single replicate of each genotype was available from each site in each year. In this case the heritability was estimated as

Table 1 Potential candidate genes and markers impacting on fruit colour, the primer sequences designed to amplify and map products in the Latham × Glen Moy population and the linkage group genes mapped

Gene name	Raspberry accession	Primers for cloning	Primers for mapping	Polymorphism	Linkage group
MIP2 small molecule transporter	GQ916537	L CTCGCAAGGTTTCACTGCCGAGGGCAG	L GAGCAAATGAGTTGGCAGATGG	SNP[a]	2
		R ACTTCTAGCAGGGTTGATACCAGTTCC	R ATCTCAGCCGCCAATCCAG		
			S TGAGTTGGCAGATGG		
MIP3 small molecule transporter	GQ916538	L ATGATCTTYRTBCTTGTYTACTGCAC	Cloning primers	Indel	2
		R TCCCADGCYTTSTSWTBGTT			
RaspTIP small molecule transporter	GQ916536	L AACCGGTGGATTGGTAAGG	L TAGCTGCTTTCTCCCTGTCAACC	SNP[a]	2
			R ATGTTTCCGGCGGACCTC		
		R GAAGGAGACTGCTGGGTTCA	S GGTGTTAGTGTATGGAACG		
bes_29G13R (SSR)	GQ902037		L TCATCGTCAAATCTGCAAAAA	SSR	2
			R CGCAAATGTCAGAGCTGTTT		
Flavonol synthase	GT029981	L AGGTGAACAGGTGGAGTTGG	L GGTCTTGAGCAACGGCAAGT	SNP[a]	4
		R TGAAGACCATCATCGAATGC	RS TTTGCGGTATCGGTATTCAGCAT		
			GTGGCGGCGCAATGA		
Glutathione *S*-transferase ERub-161	GT029978	L CTATGCTTGGCTTGGTCACA	Cloning primers	Indel	4
		R TTTTGCCCCTTAAAATGCAG			
Dihydroflavonol 4-reductase DFR	GT029979	L ATGCGAAACAACTTGCATTT	L AAACTCAAACCCAGTCTCCAACAA	SNP[a]	4
		R GCTACGATTCACGACATTGC	R GAATTGGTGTACGTGTGTACAGGT		
			S CCTTTGTCAAGTTCTCCTC		
Flavonoid 3′-hydroxylase (F3′H)	GT029980	L TGATGAAGCTTTATAAGCATGTGAGG	L GGTCTTGAGCAACGGCAAGT	SNP[a]	5
		R GGGTCCACTCTCTTGGTGAA	R TTTGCGGTATCGGTATTCAGCAT		
			S GTGGCGGCGCAATGA		
ERubLR_SQ13.4_D09 (CAD)	GT128440	L TTTGCCACTGAAATTGAGAATC	Cloning primers	SNP[a]	4
		R CATGTCCATTATACTAAAGGGTTCAA			

[a] SNP polymorphism mapped by Pyrosequencing®

Table 2 Details of previously mapped genes associated with ripening QTLs in red raspberry

Gene name	Putative function	Associated trait	Primer sequences	Linkage group	Reference
bHLH	bHLH	Anthocyanin pathway	AAAGTGCCTTCTGCTGCATT CCGTTTGCTAATGCTCTTCC	1	Kassim et al. (2009)
RiMYB	MYB	Ripening	CCCAATTGTCAGTACGTTGG CAAGTTTTCTTCTTGAACGACCA	3	Graham et al. (2009)
Bac_9O22_SSR01	MYB	Ripening	AGCCATCCTACTGGTTGTGG CCATAGCTGTCCATGCTCCT	3	Graham et al. (2009)
FruitE8	*O*-Methyltransferase	Ripening	CATAACCTCCAGTGGTTCC TGATGATGTCGATGCAGGTT	3	Graham et al. (2004)
FRUITE4	bZIP	Anthocyanin pathway	TCAAGGCTCCTGCTTTAACC CGCAGAGTTGGAGACATGAA	4	Graham et al. (2004)
ERubLR_SQ07_2_H02	Isoflavone reductase	Flavonol pathway	TGGCAATCAACCACTCTGTG CAAACTGACAAACGCTCTTCC	4	Woodhead et al. (2008)
RubendoSQ004_N23	GAST like	Ripening	CACTGCAAGGTGTCGTTTGT ATAGCTCCGGCAATCCATC	4	Woodhead et al. (2008)

$$H^2 = c\sigma_G^2/\left(c\sigma_G^2 + d\sigma_{GS}^2 + e\sigma_{GY}^2 + \sigma_{GSY}^2\right)$$

where σ_G^2, σ_{GS}^2, σ_{GY}^2 and σ_{GSY}^2 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).

Linkage map construction

JoinMap 3.0 (Van Ooijen and Voorrips 2001) was used in the construction of the linkage map. Details of the map construction are given in Graham et al. (2009).

QTL analysis

The QTL mapping was carried out using the MapQTL 5 software (Van Ooijen 2004). A Kruskal–Wallis test was used as a preliminary test to identify the regions of the genome linked to each of the colour measurements and the total anthocyanin content (TA) across seasons and sites and to explore whether alleles from one or both parents were contributing. Interval mapping was then carried out using MapQTL. If the Kruskal–Wallis analysis indicated that the phenotype was affected by alleles from both the parents, the trait was analysed using a four-mean QTL model. If alleles from only one parent were affecting the trait, the marker data were recoded so that MapQTL fitted a two-mean model.

A mixed model analysis was used to test for consistency of the QTL effects on the colour measurements across the three sites in years 2007 and 2008 and the open field site in 2006, giving a total of seven environments. Following the approach of Malosetti et al. (2008), different models were compared for the matrix of genetic covariances across environments. These allowed the same or different variances in the different environments, and no genetic correlation, uniform genetic correlation or varying genetic correlation between environments. The best model for each trait was identified using the Bayesian Information Criterion (BIC) (Schwarz 1978) as a uniform genetic correlation together with different variances in the different environments. A marker close to each QTL and with fewest missing values was identified, and environment, marker and their interaction were fitted as fixed effects and their significance evaluated using an F statistic (Kenward and Roger 1997).

The total anthocyanin content (TA) (mg/100 g fresh weight) measured in 2008 was not directly comparable to measures of total anthocyanins (based on the sum of the eight individual pigments (g/ml) measured in 2007 (Kassim et al. 2009). In order to use an analysis over environments, the TA in each environment was standardised to mean zero, variance one before the mixed model analysis.

Kassim et al. (2009) analysed eight individual anthocyanin compounds and identified two key QTL regions affecting these, especially the cyanidin compounds, the first close to bHLH on LG 1 and the second close to bZIP on LG 4. The mixed model approach was used to explore these effects over environments and to see how these related to the colour measurements.

Results

Colour analysis

Colour measurements were made for each year and site using the *Yxy* and the *L*a*b** approaches. The progeny means, standard error of the means and range are shown for

Table 3 Progeny values and means for measures of colour and associated traits across seasons and environments

Season	Colour meter measures							Total anthocyanins
	Y	*x*	*y*	*L**	*a**	*b**	Δ*E*	
Field 2006								
Mean ± SEM	15.0 ± 0.1	0.374 ± 0.001	0.318 ± 0.000	45.6 ± 0.1	16.4 ± 0.1	7.0 ± 0.1	52.3 ± 0.1	_
Min–Max	12.5–18.0	0.349–0.393	0.315–0.325	42.0–49.4	10.2–20.4	3.5–10.5	49.3–54.7	_
Field 2007								
Mean ± SEM	14.3 ± 0.1	0.379 ± 0.001	0.321 ± 0.000	44.6 ± 0.1	16.7 ± 0.2	8.0 ± 0.1	53.7 ± 0.1	_
Min–Max	11.6–18.7	0.361–0.404	0.317–0.327	40.6–50.3	12.0–22.7	5.2–12.4	50.8–57.4	_
Polytunnel 2007								
Mean ± SEM	14.2 ± 0.1	0.383 ± 0.001	0322 ± 0.000	44.5 ± 0.2	17.1 ± 0.2	8.5 ± 0.1	54.0 ± 0.2	_
Min–Max	10.7–20.6	0.356–0.424	0.317–0.329	39.0–52.5	10.8–24.1	4.7–12.8	48.0–61.1	_
Commercial 2007								
Mean ± SEM	14.3 ± 0.1	0.377 ± 0.001	0.321 ± 0.000	44.6 ± 0.2	16.2 ± 0.1	7.8 ± 0.1	53.5 ± 0.1	_
Min–Max	12.1–17.9	0.356–0.395	0.317–0.329	41.4–49.3	11.2–20.5	4.6–11.0	49.7–55.6	_
Field 2008								
Mean ± SEM	15.8 ± 0.1	0.383 ± 0.001	0.319 ± 0.000	46.7 ± 0.1	18.6 ± 0.2	8.3 ± 0.1	52.5 ± 0.1	71.2 ± 2.4
Min–Max	12.9–21.7	0.364–0.410	0.315–0.326	42.6–53.6	14.1–25.6	5.4–12.4	47.5–57.5	14.6–142.1
Polytunnel 2008								
Mean ± SEM	15.8 ± 0.1	0.384 ± 0.001	0.320 ± 0.000	46.6 ± 0.2	18.8 ± 0.2	8.5 ± 0.1	52.7 ± 0.1	82.1 ± 2.7
Min–Max	11.6–19.1	0.366–0.415	0.316–0.326	40.5–50.7	14.6–24.1	5.4–11.8	19.5–60.0	27.4–177.7
Commercial 2008								
Mean ± SEM	16.0 ± 0.1	0.389 ± 0.001	0.321 ± 0.000	46.9 ± 0.2	19.4 ± 0.1	9.1 ± 0.1	52.6 ± 0.2	63.4 ± 2.1
Min–Max	13.4–19.2	0.371–0.401	0.315–0.326	43.3–50.9	15.9–22.1	6.4–12.6	41.3–56.9	24.2–120.4

– not assessed

each combination of year and site in Table 3. The correlations of these, Δ*E* and visual colour with total and individual anthocyanins, and the first two principal coordinates (PCO1, PCO2) of the ripening scores (Graham et al. 2009) in the 2006 field trial (based on the offspring means for the mapping population) are shown in Table 4. The correlations for the other years and sites are similar (not shown). Although all the measures from the colour meter assessed different parameters, all measurements are highly correlated ($P < 0.001$), especially *Y* and *L*, *x*, *a* and *b*, and *y* and *b*. They are also significantly correlated ($P < 0.001$) with the TA and with anthocyanins C3S (the most abundant) and C3G.

P3G and C3R are also correlated with some of the colour measurements, with $0.001 < P < 0.05$. The first principal coordinate of the ripening scores from the 2006 open field trial (PCO1, which represents an overall summary of the variability of the speed of the ripening process; Graham et al. 2009) is significantly correlated ($P = 0.005$) with measurement *x*, and also with measurements *a* and *b* ($0.01 < P < 0.05$), but there are no significant correlations of the colour meter measurements with the second principal coordinate (PCO2) which is a measure of the rate of ripening in later stages (Graham et al. 2009). The visual score of colour is most significantly correlated with measurement *y* ($P < 0.001$) and also with PCO2 ($P < 0.001$), but has lower correlations with the other colour measurements. Because of the high correlations, we restrict attention here to the *Y*, *x* and *y* measurements from the colour meter. Table 5 shows the heritabilities for the *Y*, *x* and *y* measurements for the 2006 trial and for the 2007–2008 combined trials and for the visual scores from 2007 to 2008.

Candidate genes

All candidate genes of interest selected for analysis in raspberry, based on their potential influence on fruit colour (Tables 1, 2), were mapped by either length or single nucleotide polymorphism as previously described (Fig. 2).

QTL analysis

A Kruskal–Wallis test was used for a preliminary identification of areas of the genome affecting the colour and other measurements, using a significance level $P < 0.001$. This identified a region close to marker RaspTIPSNP on LG 2 as associated with colour meter measurements,

Table 4 Correlations of the colour measurements with total and individual anthocyanins and principal coordinates of ripening scores from the 2006 field trial, based on the genotype means

Y								
x	0.74***							
y	0.73***	0.83***						
L	0.99***	0.74***	0.72***					
a	0.80***	0.97***	0.73***	0.80***				
b	0.82***	0.97***	0.93***	0.82***	0.93***			
ΔE	–0.91***	–0.42***	–0.51***	–0.92***	–0.51***	–0.55***		
Visual	–0.31*	–0.32**	–0.53***	–0.30*	–0.23	–0.41**	0.26*	
TA	–0.60***	–0.54***	–0.53***	–0.60***	–0.56***	–0.58***	0.52***	0.36**
C3S	–0.47***	–0.42***	–0.37***	–0.47***	–0.45***	–0.44***	0.40***	0.18
C3G	–0.44***	–0.50***	–0.39***	–0.44***	–0.52***	–0.48***	0.33***	0.42***
C3GR	–0.12	–0.03	–0.16	–0.12	–0.01	–0.10	0.14	0.04
C3R	–0.17*	–0.11	–0.19*	–0.18*	–0.10	–0.16	0.17*	0.16
P3S	0.07	0.04	0.04	0.07	0.05	0.05	–0.08	–0.09
P3G	–0.16	–0.23**	–0.13	–0.17*	–0.25**	–0.20*	0.11	–0.02
P3GR	–0.05	–0.01	–0.05	–0.06	–0.01	–0.05	0.05	–0.23
P3R	0.01	–0.02	–0.04	0.01	0.00	–0.02	–0.02	–0.06
PCO1	0.06	0.24**	0.15	0.06	0.21*	0.19*	0.06	0.01
PCO2	0.05	–0.13	–0.11	0.05	–0.08	–0.12	–0.16	0.43***
	Y	*x*	*y*	*L*	*a*	*b*	ΔE	Visual

The significance of the correlations is shown as *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

Table 5 Variance components for colour meter measurements

	2006	2007–2008				
Trait	h^2 (%)	σ^2_G	σ^2_{GS}	σ^2_{GY}	σ^2_{GSY}	h^2 (%)
Y	86.3	0.820	0.000	0.000	1.056	76.8
x	83.4	2.2×10^{-5}	2.4×10^{-6}	5.7×10^{-7}	4.8×10^{-5}	65.4
y	88.8	2.0×10^{-6}	1.7×10^{-8}	0.00	2.1×10^{-6}	79.6
Visual	–	0.420	0.003	0.007	0.641	73.2

especially *Y*, visual scores and TA for most combinations of sites and years. Markers segregating in 'Latham' only showed highly significant associations, while those segregating in 'Glen Moy' only were not significant, but there was evidence from markers segregating in both parents, particularly the < ab × cd > marker bes_Ri29G13R, of a contribution from the 'Glen Moy' alleles. A region close to marker P13M40-85 on LG 6 was also identified across all sites, and seasons as significantly associated with measurement *y* and the visual colour scores, and occasionally related to other traits. In this region, the markers are mostly heterozygous in 'Latham' and homozygous in 'Glen Moy'.

Other regions that were identified across sites were on LG 4, either close to the FLS marker (segregating in both parents) or near the RiCAD marker (from 'Latham'). There were also significant effects on LG 3 close to marker Rub2a1. For a few traits, the Kruskal–Wallis test indicated associations with the alleles of both parents, but for most traits the alleles from 'Glen Moy' showed more significant differences. Two further areas of LG 3 were significant for a small number of traits.

A permutation test with 1,000 permutations was used to establish 95 and 99% significance levels for QTL interval mapping as a lod score of 4.3 and 5.1, respectively, for a four-mean model and a lod score of 2.8 and 3.5 for a two-mean model. Figure 2 shows the locations and lod scores of the significant QTLs. Usually there was some indication that these regions affected further traits, but the lod score did not reach the threshold of the permutation test.

Mixed model analysis

The following markers were identified as close to the QTLs for use in mixed model analysis: bes_Ri29G13R, a marker segregating in both parents at 105 cM on LG 2; P13M39_195R, a marker from 'Latham' at 12 cM on LG

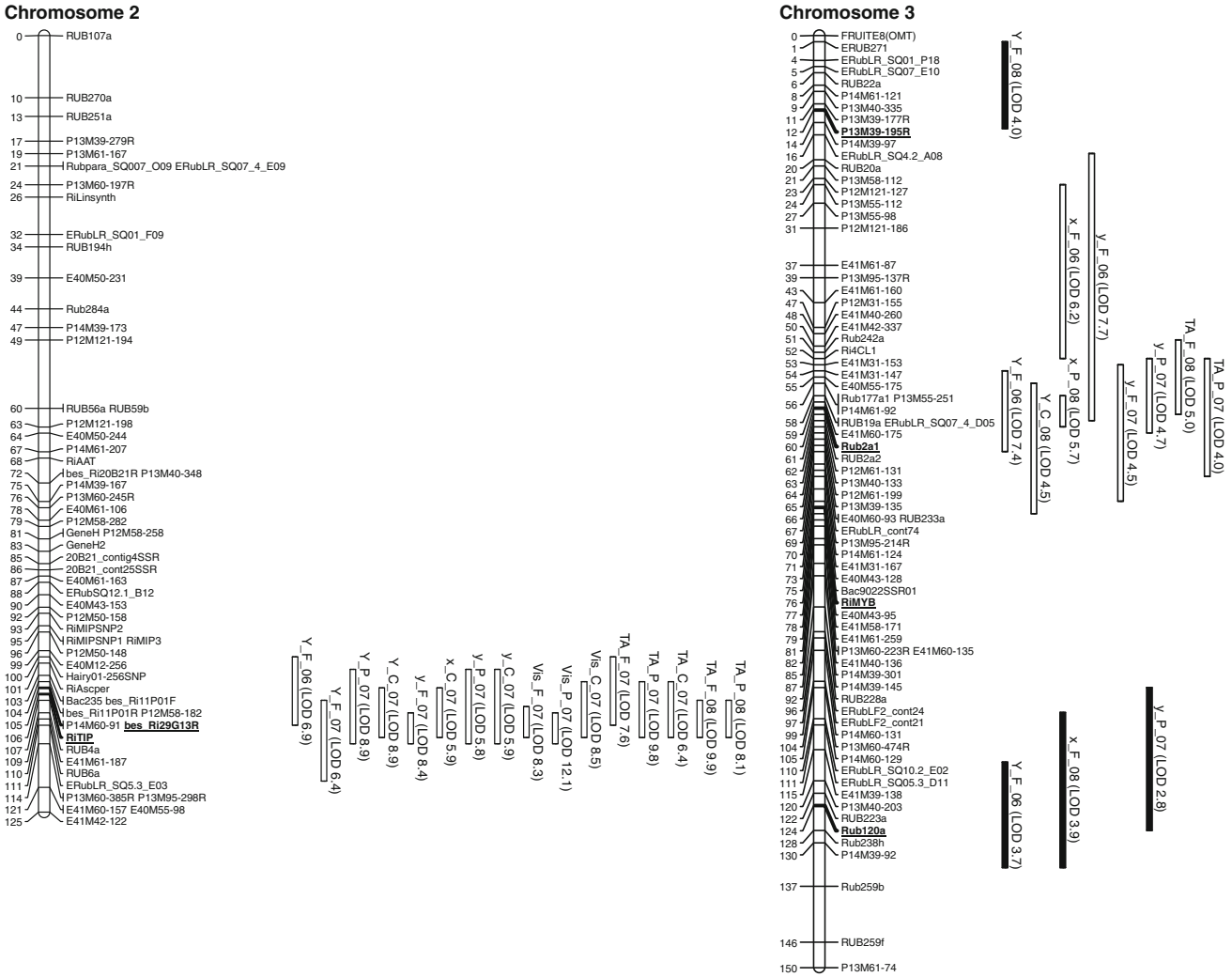

Fig. 2 Linkage groups with candidate genes and QTL associated with colour. Linkage map of the regions associated with QTLs for colour, showing one-lod confidence intervals (to the right of the chromosome) for QTL locations for developmental traits. *F* field trial; *P* protected trial at SCRI; *C* commercial trial. *Black bars* show QTLs segregating in Latham only, and *unfilled bars* show QTLs with segregating alleles from both parents

3; Rub2a1, a marker segregating in both parents at 60 cM on LG 3; Rub120a, a marker from 'Latham' at 124 cM on LG 3; P13M40_85, a marker from 'Latham' at 70 cM on LG 6; RiFLS, an ab × ab marker at 26 cM on LG 4; RiCAD, a marker from 'Latham' at 69 cM on LG 4. Table 6a–g shows the marker genotype means for each trait and marker over the environments. For most traits and markers, there were no significant interactions between the marker and the environment. When interactions were significant, the ranking of the marker genotypes were usually unaffected. The most significant interaction involved marker P13M39_195R on LG 3, which showed a significant interaction ($P < 0.001$) with environment for measurement *Y*. The presence of the marker was associated with a significantly lower value of *Y* at all sites in 2008 but only in the polytunnel in 2007.

Mixed model analysis of individual anthocyanins

Kassim et al. (2009) reported QTLs affecting the cyanidin anthocyanin compounds close to loci bHLH on LG 1 and FRUITE4 on LG 4, but no QTLs for colour or total anthocyanin content were found in these regions.

Table 7 shows the mean levels of the cyanidin compounds associated with the genotypes at these two loci, both of which are heterozygous in 'Latham' and homozygous in 'Glen Moy'. The b allele of bHLH is associated with decreased levels of C3S and C3G but increased levels of C3GR and C3R. The difference in the total cyanidins is smaller than for the individual compounds. Similarly the b allele of FRUITE4 is associated with increased levels of C3S and C3R but decreased levels of C3GR and C3G. A mixed model containing bHLH and FRUITE4 and their

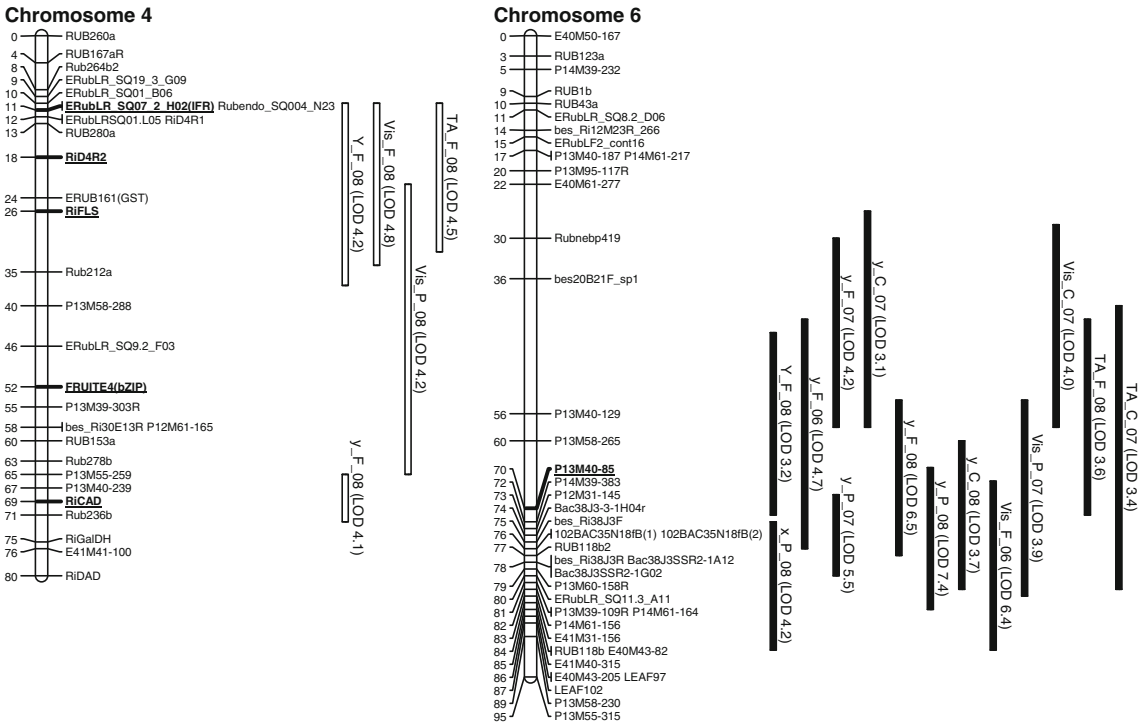

Fig. 2 continued

interactions with each of the six markers above, in addition to the marker and environment effects, was used to search for further QTLs. As only a small set of markers are being considered, we report all effects and interactions with $P < 0.05$.

For C3S, the most abundant of the individual anthocyanins, there is a significant effect of bes_Ri29G13R on LG 2 ($P < 0.001$), as shown in Table 6a. There are also effects of P13M40_85 ($P = 0.003$), RiFLS ($P = 0.019$), RiCAD ($P = 0.021$), Rub2a1 ($P = 0.034$) and Rub120a ($P = 0.013$), but P13M39_195R is not significant. In each case, the effect has the same sign as for the TA, as would be expected. For the other cyanidin compounds, the main effects are either non-significant or only weakly significant. There is a significant effect of RiFLS ($P = 0.043$) on C3GR, and of P13M39_195R and RiCAD on C3R ($P = 0.014$ and 0.016, respectively).

There are also some significant interactions with the previously detected markers bHLH and FRUITE4. For C3G, there is a significant interaction ($P < 0.001$) between bHLH and Rub120a. There is also a significant interaction ($P = 0.004$) between bHLH and P13M39_195R. In each case, there is no significant effect of the marker when the bHLH genotype is ab (low C3G), but a significant effect of the marker when the bHLH genotype is aa. For C3GR and C3R, there are significant interactions ($P = 0.002$ and $P = 0.007$, respectively) between FRUITE4 and P13M40_85. In each case, there is no significant effect of P13M40_85 when the FRUITE4 genotype is ab (low cyanidins), but a significant effect of P13M40_85 when FRUITE4 is aa. None of these terms interacts with environment, indicating a consistent effect in the different environments. Trait means for these interaction terms are shown in the Supplementary Table S1.

Discussion

Considerable fruit colour variation exists in different raspberry varieties, and this influences consumer perception of ripeness; light coloured types may not be regarded as ripe, whereas very dark types may be considered over-ripe, and this impacts on the appeal of the fruit. Although the genetics of the anthocyanin pathway is well documented, the regulatory controls of both quantitative and qualitative variations of fruit anthocyanin content are less advanced (Castellarin and Di-Gaspero 2007). This is an issue for fruit breeding which is a lengthy and costly process (Graham and Jennings 2009), especially in woody perennials. Any improvement, particularly the development of molecular markers for identifying genotypes with improved fruit colour attributes, would be useful.

Table 6 QTL means for each marker genotype from the over-environments analysis

	ac	ad	bc	bd	SED	Sig
(a) Marker bes_Ri29G13R on LG 2						
Y	14.6	14.7	15.1	15.8	0.20	<0.001
x	0.3793	0.3804	0.3811	0.3835	0.00118	<0.001
y	0.3199	0.3199	0.3203	0.3212	0.00033	<0.001
Visual	3.68	3.53	3.18	2.70	0.151	<0.001
TA	0.29	0.34	−0.09	−0.56	0.160	<0.001
C3S	262.9	278.1	238.4	167.0	19.15	<0.001

	aa	ab	SED	Sig
(b) Marker P13M40_85 on LG 6				
Y	15.3	14.8	0.15	0.003
x	0.3825	0.3797	0.00082	<0.001
y	0.3210	0.3196	0.00022	<0.001
Visual	2.99	3.61	0.109	<0.001
TA	−0.24	0.25	0.121	<0.001
C3S	211.5	264.1	14.61	0.003

	aa	ab	bb	SED	Sig
(c) Marker RiFLS on LG 4					
Y	15.5	15.0	14.6	0.20	<0.001
x	0.3832	0.3809	0.3795	0.00111	0.024
y	0.3208	0.3204	0.3198	0.00032	0.026
Visual	2.92	3.25	3.66	0.149	<0.001
TA	−0.39	0.06	0.28	0.157	<0.001
C3S	200.2	237.5	256.6	22.32	0.019
C3GR	62.5	78.1	89.8	10.58	0.043

	aa	ab	SED	Sig
(d) Marker RiCAD on LG 4				
Y	14.9	15.2	0.152	NS
x	0.3798	0.3828	0.00080	<0.001
y	0.3199	0.3208	0.00023	<0.001
Visual	3.46	3.12	0.119	0.005
TA	0.82	−0.82	0.124	NS
C3S	273.5	178.0	21.04	0.021
C3R	31.5	23.6	5.14	0.016

	ac	ad	bc	bd	SED	Sig
(e) Marker Rub2a1 on LG 3						
Y	15.4	14.9	15.5	14.7	0.21	<0.001
x	0.3842	0.3796	0.3831	0.3790	0.00120	<0.001
y	0.3208	0.3198	0.3211	0.3200	0.00034	<0.001
Visual	3.17	3.53	2.98	3.38	0.175	0.014
TA	−0.31	0.27	−0.45	0.24	0.175	<0.001
C3S	215.9	251.2	209.1	259.2	23.51	0.034

Table 6 continued

	aa	ab	SED	Sig
(f) Marker Rub120a on LG 3				
Y	14.7	15.3	0.155	<0.001
x	0.3793	0.3826	0.00089	<0.001
y	0.3198	0.3207	0.00024	<0.001
Visual	3.47	3.21	0.119	0.008
TA	0.22	−0.14	0.125	0.004
C3S	259.8	221.1	15.08	0.013

	aa	ab	SED	Sig
(g) Marker P13M39_195R on LG 3				
Y[a]	16.2	15.4	0.189	<0.001
x	0.3816	0.3801	0.00080	0.020
y	0.3206	0.3200	0.00024	0.009
Visual	3.22	3.43	0.117	0.027
TA	−0.11	0.17	0.121	0.017
C3R	26.8	32.8	3.60	0.014

Total anthocyanins (TA) is also over all environments, but standardised to mean zero, variance one at each site

SED average standard error of difference; *Sig* significance of the marker

[a] This is the significance of the interaction with environment and the means for 2008

Table 7 QTL means from the over-environments analysis (2006 and 2007) of the individual anthocyanins

	aa	ab	SED	Sig
(a) Marker bHLH on LG 1				
C3S	290.7	184.7	14.12	<0.001
C3GR	33.7	124.2	7.56	<0.001
C3G	77.0	46.8	5.51	<0.001
C3R	19.3	42.2	3.55	<0.001
Total cyanidins	420.7	405.2		
(b) Marker bZIP (FRUITE4) on LG 4				
C3S	218.4	257.3	15.84	0.007
C3GR	97.3	62.3	9.90	0.001
C3G	69.9	49.1	5.7	0.018
C3R	41.6	20.2	3.73	<0.001
Total cyanidins	427.2	388.9		

The current lack of knowledge surrounding the genetic control of anthocyanin synthesis and colour development impedes cultivar improvement by preventing the deployment of molecular tools such as molecular markers early in the breeding process. Providing breeders with tools for a more efficient breeding programme allows the early

Table 8 Weather conditions in 2006, 2007 and 2008 for Dundee, UK

	Year	Month			
		May	June	July	August
Mean air max temperature (°C)	2006	14.7	19.4	22.5	20.4
	2007	14.3	16.0	18.5	19.0
	2008	15.7	18.0	19.0	18.5
Mean soil temperature measured at 20 cm	2006	10.6	15.6	18.4	16.1
	2007	15.5	12.0	11.1	5.5
	2008	10.6	13.7	15.7	15.0
Sunshine (h)	2006	215	187	217	200
	2007	205	90	154	170
	2008	171	166	131	100
Rainfall above 0.2 mm (days)	2006	16	9	9	10
	2007	15	12	22	13
	2008	12	17	15	20

selection of seedlings with desirable fruit colour/anthocyanin content while also allowing the early elimination of seedlings which do not meet the required trait characteristics.

The first step towards this goal is in measuring fruit colour in a segregating population to identify relevant QTL and locate associated genes and markers to gain an insight into the key controlling factors.

Measuring fruit colour in a widely segregating population required careful selection of fruit from each progeny at a time when they were individually deemed as ripe. Detailed ripening data were available for this population, analysis of which has been presented previously (Graham et al. 2009). Colour in raspberry fruit varied significantly across progeny and from season to season but not significantly across the different environments. There are several colour coordinate systems available in which colour can be described, the most common of which include the CIE (Commision Internationale de l'Eclairage) *L*a*b** and *Yxy* measures.

Colour is measured by colorimeters based on the colour receptors of the human eye (red, green, blue and any combination of those) to allow the automatic conversion along several colour coordinate systems (Abbott 1999). All measures of colour (visual and colour meter) were significantly correlated, and these also correlated significantly with total anthocyanin content. Comparisons of total anthocyanin content with individual pigments confirms that total anthocyanin levels, and therefore fruit colour, are predominantly due to the cyanidin pigments in raspberry.

Total anthocyanin content shows the signs of environmental influence with the two locations at the SCRI, field and protected sites, showing comparable levels of anthocyanin values and distribution. The protected commercial site, however, shows significantly lower values for almost all individuals (Table 3). This was not expected as all other correlations with anthocyanin and visual colour/colour meter analysis were consistent across the three sites, with higher anthocyanin individuals scoring lower for sample brightness, and although this was reflected in the commercial samples, the *Y* values recorded were comparable across sites (SCRI field 15.8, protected 15.8, commercial 16.0), but the respective anthocyanin mean values were considerably lower SCRI field 71.1, protected 82.4 and commercial 58.0 mg (all mg/100 g fw).

Seasonal variations in fruit colour were observed, and may be attributed to varying proportions of individual pigments. For example, in 2006 the most significant correlations with fruit colour were with total anthocyanin content and for the individual pigments only correlations with C3S and C3G were highly significant. In 2007, although the levels of C3S and C3G declined, they remained significantly correlated with colour, and in this poorer season, P3GR also demonstrated a significant correlation with colour. Higher pigment levels in 2006 may be due to the greater number of sunshine hours experienced during this growing season (Table 8). Overlapping QTL for total anthocyanins and fruit colour mapped to LG 2, LG 3, LG 4 and LG 6 observed in this study indicate that the traits are complex and are not purely related to ripening because for the most part, the QTL identified here do not overlap with those for ripening.

This observation also confirms that the harvesting strategy used in here distinguished between progeny that were genuinely dark-fruited and ripe and those that were over-ripe and thus dark in colour.

Whereas measures of individual anthocyanins mapped to the bHLH gene on LG 1 and a bZIP gene on LG 4 (Kassim et al. 2009), colour and total anthocyanins mapped to overlapping QTL on LG 2, LG 3, LG 4 (not overlapping with QTL for individual anthocyanin pigments) and LG 6.

Ripening and colour mapped to a complex area on LG 3, but three discrete regions associated with colour were identified.

In Arabidopsis, Hartmann et al. (2005) demonstrated that four genes in the flavonoid pathway, chalcone synthase (CHS), chalcone flavanone isomerase (CFI), flavanone 3-hydroxylase (F3H) and FLS were co-ordinately expressed in response to light and contained light regulatory units (LRUs) in their promoters. Thus, the expression of key anthocyanin biosynthetic genes likely requires specific transcription factors which can modulate the pathway in response to various stimuli including light. The QTL on LG 6 has a midpoint at P13M40-85 which also shows an interaction with marker FRUITE4 encoding a bZIP transcription factor on LG 4 (Kassim et al. 2009). These genes are expressed constitutively or tissue specifically and regulate diverse processes such as photomorphogenesis and light signalling (Holm et al. 2002; Mallappa et al. 2006), stress and hormone signalling (Nijhawan et al. 2008), and FRUITE4 has previously been identified as the most significant marker in a QTL for individual anthocyanin pigments (Kassim et al. 2009). Here, we have shown that depending on the allele combination of bZIP, increases in C3S and C3G in 2006 are significant, possibly in preference to C3GR and C3R. Similarly the allele combination of bHLH, previously identified as the most significant marker in the other pigment QTL on LG 1 (Kassim et al. 2009), influenced the levels of C3S and C3G (decrease) and increases levels of C3GR and C3R, in an interaction with SSR marker 120a on LG 3.

From the bHLH gene fragments amplified in 'Glen Moy' (aa) and 'Latham' (ab), both alleles are predicted to encode two identical exons but the b allele present in 'Latham' contains a 50-bp deletion within the intron (Woodhead, unpublished). Sequencing of full length genes would be necessary to determine whether there are functional differences between the two alleles that may influence the different quantities of C3G and C3S observed in the mapping population (Table 7f). The raspberry bHLH gene is very similar to MdbHLH33 from apple (Kassim et al. 2009), in which efficient anthocyanin production depends upon the co-expression of both MYB and two distinct basic-helix-loop-helix (bHLH) transcription factors (Espley et al. 2007). Two MYB genes have been mapped together on LG 3 and are associated with ripening in raspberry, being important in the transition from green to red fruit (Graham et al. 2009) and lie within the QTL for colour but are not the most significant markers. The MYB genes are approximately 15 cM away from Rub2a1 on LG 3 (which has sequence homology to a gene encoding an aminotransferase) which does have a significant association with colour and with strong influence from the 'Glen Moy' alleles. Although we cannot rule out that there may be other MYB transcription factors closer to Rub2a1 that we have yet to discover, the control of anthocyanin content may be more complex. In grape, studies of the cumulative expression of four MYB-type genes, including the MybA gene that controls the activation of the UFGT gene (Kobayashi et al. 2004), were not sufficient to explain the quantitative variation in anthocyanin content observed (Castellarin and Di-Gaspero 2007).

While the transcription factors have been clearly identified as important in colour and thus in anthocyanin production, a cluster of structural genes on LG 4 have also been identified as significant. The QTL on LG 4 has the FLS as the most significant marker associated with fruit colour, but a number of other genes of interest in the context of colour and ripening lie within this region including GST (which is significant for colour meter measure *x* and visual colour in 2008 under protected cultivation), DFR and a GAST-like protein (Rubendo_SQ004_N23), as well as a number of ESTs (Woodhead et al. 2008) with similarity to ripening ESTs from other Rosaceous species. These genes are probably physically very close together (within 10 cM) as mapping of the two SNPs identified within the DFR has demonstrated (unreported data).

The FLS is a key enzyme in flavonoid biosynthesis regulating the balance between anthocyanin and flavonol synthesis. Based on the structural analysis of grape DFR, it has been suggested that flavonols could act as inhibitors of DFR activity towards dihydroflavonols (Trabelsi et al. 2008) which may be how the balance between anthocyanin and flavonol compounds is achieved. Flavonols have been described as one of the most important single factors influencing colouration (Davies and Mazza 1993; Mazza and Brouillard 1990; Castañeda-Ovando et al. 2009), and this is supported by data here identifying the FLS gene as the most significantly associated marker on LG 4 which may be due to its effect as a co-pigment and have a role in UV protection.

This work also mapped F3′H to a region on LG 5 again with no association to colour or anthocyanin levels. In grape, most of the phenotypic variation in anthocyanin content, anthocyanin composition, colour intensity and colour hue of grapes at berry maturity was found to be directly dependent on the changes in transcript levels of the global set of anthocyanin genes (F3H, F3′H, F3′5′H, UFGT, GST and OMT) (Castellarin and Di-Gaspero 2007). The main control point for quantitative anthocyanin variation was shown to be at the point of UFGT (Boss et al. 1996), but variation in anthocyanin composition occurs further up the pathway at the level of the flavonoid hydroxylases and then below UFGT at the *O*-methyltransferases (Castellarin and Di-Gaspero 2007). An OMT gene on LG 3 was also identified in this study as significant. This gene adds a methyl group to the cyanidin backbone.

On LG 2, the QTL for colour scores and total anthocyanin measures was identified in a region containing the aquaporins, genes that transport water and small molecules (Maeshima and Ishikawa 2008). The most significant marker is a BAC end sequence (bes_Ri29G13R) that maps between the MIP and TIP genes. Different allele combinations of bes_Ri29G13R were found to significantly alter the amounts of total anthocyanin, which was largely due to the effect on the major cyanidin pigment C3S (Table 7a). At present, it is not possible to speculate on the function of the gene encoded by the bes_29G13R maker, which shows significant homology to a hypothetical protein from grape, but future BAC sequencing will provide further information. This region on LG 2 is of considerable interest as it also contains Gene H (Graham et al. 2006) which is associated with delayed ripening (Graham et al. 2009), and BAC clones spanning this region are under investigation (Woodhead, personal communication).

More difficult to explain is the association identified with CAD on LG 4 with the *y* colour score in 2008 field-grown fruit only. CAD genes are mostly expressed in the vascular tissue of fruit and achenes in strawberry and grape (as well as in lignin/suberin biosynthesis). There is also a suggestion that they may be implicated in the production of cinnamyl alcohol derivatives involved in fruit flavour and aroma (Grimplet et al. 2007).

While pH and total phenolics were also measured across all three environments in 2008, no correlations were identified between these and colour measurements obtained in this study. Individual QTL regions were identified for these traits, but were not found to overlap any of the colour QTLs identified (data not shown).

Heritabilities for colour were extremely high across the years. High heritability estimates have previously been reported for anthocyanins, antioxidant activity and total phenolics content in red raspberry and grapevine (Connor et al. 2005a, b; Liang et al. 2009) paving the way for the improvements to fruit colour through plant breeding, and this is supported by the identification of robust QTLs across environments and season.

Furthermore the identification of bZIP (FRUITE4), bHLH, and a number of structural genes within QTLs, as well as identification of interaction between bZIP and bHLH and other QTL, supports the role of these genes in fruit colour determination.

Future work will focus on further characterisation of LG 2 which has the most significant effect on fruit colour.

Acknowledgments The authors would like to acknowledge Dr. Gordon McDougall and Mrs. Pat Dobson for advice on biochemical analysis, Ian Pitkethly for graphics and SCRI Sequencing and Genotyping service. This work was funded by the Scottish Government under the Horticulture LINK scheme, and the authors would like to acknowledge the help and support from David Cole.

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