Research Article

Environmental and seasonal influences on red raspberry anthocyanin antioxidant contents and identification of quantitative traits loci (QTL)

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Consumption of raspberries promotes human health through intake of pharmaceutically active antioxidants, including cyanidin and pelargonidin anthocyanins; products of flavonoid metabolism and also pigments conferring colour to fruit. Raspberry anthocyanin contents could be enhanced for nutritional health and quality benefits utilising DNA polymorphisms in modern marker assisted breeding. The objective was to elucidate factors determing anthocyanin production in these fruits. HPLC quantified eight anthocyanin cyanidin and pelargonidin glycosides: -3-sophoroside, -3-glucoside, -3-rutinoside and -3-glucosylrutinoside across two seasons and two environments in progeny from a cross between two *Rubus* subspecies, *Rubus idaeus* (cv. Glen Moy) × *Rubus strigosus* (cv. Latham). Significant seasonal variation was detected across pigments less for different growing environments within seasons. Eight antioxidants mapped to the same chromosome region on linkage group (LG) 1, across both years and from fruits grown in field and under protected cultivation. Seven antioxidants also mapped to a region on LG 4 across years and for both growing sites. A chalcone synthase (PKS 1) gene sequence mapped to LG 7 but did not underlie the anthocyanin quantitative traits loci (QTL) identified. Other candidate genes including basic-helix-loop-helix (bHLH), NAM/CUC2-like protein and bZIP transcription factor underlying the mapped anthocyanins were identified.

Keywords: bHLH / bZIP / Marker assisted breeding / NAM / PKS1 (CHS)

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1 Introduction

Berries can be differentiated from other phenolics containing fruit and vegetables in having high concentrations of anthocyanins, with strong antioxidant capacities, up to 4 times greater than nonberry fruits, 10 times more than vegetables and 40 times that of cereals [1]. However, raspberries which are highly valued by North European and North American consumers have relatively modest contents. Such compounds, present at up to 500 mg 100 g/FW in berries are also pigments imparting colorations to raspberries thus a desirable dietary source of anthocyanins, as nonberry anthocyanins are typically at less than 100 mg 100 g/FW [2]. Pharmaceutical effects of anthocyanins have been reported in humans such as inhibition of malignant cell growth, specifically proliferation of colon and breast carcinoma cells [3], and excellent anti-inflammatory and antimicrobial properties [4]. Pure anthocyanidins inhibited colon carcinoma proliferation by 50% in the range of 35 – 213 μM [5]. Plant phenolics seem to have multiple mechanisms of action in combating cancer including angiogenesis denying tumours vascular supply required for proliferation, inhibiting DNA synthesis, inducing apoptosis, and cellular differentiation inhibiting cancer progression [5, 6]. Regular consumption of raspberry anthocyanins is also reported to improve cognitive brain functions, age-related degeneration of eyesight and influence cardiovascular disease [7].

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Abbreviations: CHS, chalcone synthase; **C3G,** cyanidin-3-glucoside; **C3GR,** cyanidin-3-glucosylrutinoside; **C3R,** cyanidin-3-rutinoside; **C3S,** cyanidin-3-sophoroside; **LG,** linkage group; **QTL,** quantitative traits loci; **P3G,** pelargonidin-3-glucoside; **P3R,** pelargonidin-3-rutinoside; **P3S,** pelargonidin-3-sophoroside

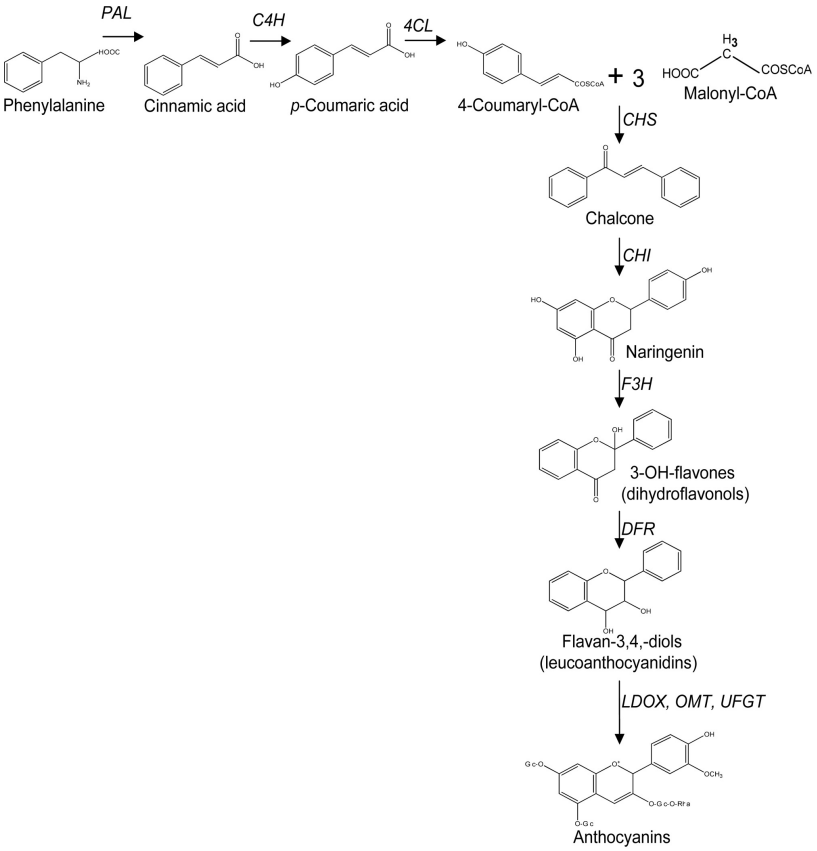


Figure 1. Biosynthesis of anthocyanins.

Anthocyanins are synthesized from precursors from two biosynthetic pathways (Fig. 1): shikimate, producing phenylalanine; and that generating malonyl-CoA. These two precursors are linked by chalcone synthase (CHS) *via* a polyketide folding mechanism, to form an intermediate chalcone, a substrate for chalcone isomerase (CHI) generating prototype pigment naringenin, subsequently oxidized by a series of enzymes such as flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), then reduced by dihydroflavonol-4-reductase (DFR) to leucoanthocyanidins, and finally converted to anthocyanidins by leucoanthocyanidin dioxygenase (LDOX). Unstable anthocyanidins are coupled to a small range of sugars by activities such as UDP-glucose/flavonoid 3-*O*-glucosyltransferase (UFGT) and *O*-methyltransferase (OMT) to yield relatively stable anthocyanins [8]. Anthocyanin cyanidin, with B ring 3′,4′-dihydroxy substituents and conjugation between A and B rings, have antioxidant potentials four times that of a vitamin E analogue [9]. Red raspberries typically contain 23 – 59 mg total anthocyanin 100 g/FW fruit [10] and cyanidins are more abundant, than pelargonidins [11]. Compared to certain other fruits raspberry anthocyanin levels are low but if genes underlying production were determined marker assisted breeding strategies could be deployed to enhance antioxidant contents.

Anthocyanin biosynthesis is influenced by factors including light exposure [12, 13], temperature [14], phytohormones [14, 15], sugar [15] and concentration of certain ions [16]. Most enzymes involved in anthocyanin biosynthesis and their encoding genes have been characterized in plants including maize, *Arabidopsis*, petunia and snapdragon: also many regulatory genes directly controlling transcription of structural genes have been identified [17]. In the related species apple, efficient anthocyanins production depends upon co-expression of both myb and two distinct basic-helix-loop-helix (bHLH) transcription factors [18]. In raspberry, key anthocyanin polyketide synthase genes have been allocated enzymic activities [19]: PKS genes 1 and 5 as CHS; PKS 4 as benzalacetone synthase, important for synthesis of the impact compound raspberry ketone [20, 21]. Genes for anthocyanin biosynthesis and fruit colouration have been mapped to specific linkage groups (LGs) related in *Rosaceae* strawberry [22] and apple [23].

The aim was to examine variation in anthocyanin contents in a segregating red raspberry population from a cross between two strongly differentiated parents [24, 25]. Pigments were quantified in two fruiting seasons and under two environmental conditions to clarify genetic and environmental influences on control of anthocyanin synthesis in this fruit. Anthocyanin data were then analysed for marker associations on the genetic linkage map and quantitative traits loci (QTL) identified. As a first step to identifying more clearly genes underlying these traits, two potential candidate genes were mapped, one a structural gene, PKS1,

Table 1. Accession numbers of sequences used for primer design and primer sequences used to clone and map candidate genes in raspberry Glen Moy X Latham

Description	Accession no. of sequence used for primer design	Primer name	Sequence (5′-3k)
bHLH	ABB84474.1	bHLHF	AGCTATGCAATTTTCTGGTCATT
		bHLHR	CCTCCATTCTTAAACTGCAGAACT
		bHLH_IntL	TCAGCATACTGAGCATTGCAT
		bHLH_IntR	GCAAAACTTCCCTTTTTCCA
		bHLH_mapL	AAAGTGCCTTCTGCTGCATT
		bHLH_mapR	CCGTTTGCTAATGCTCTTCC
CHS	AF292367	PKS1F	CGCTTCTCAACCCTTGTTTC
		PKS1R	CGATCGAATCACCCTTCTGT
	AF292368	PKS2F	ACAGATCACATATGGTGACCGTCG ATG
		PKS2R	CAA ATT ACT GAG GGG ATC CCC CAA GTG AAC
p-Coumarylacetone synthase	AF292369	PKS3F	GGC CCT CTC CAG TAG TCT TG
		PKS3R	TCC GTG TGG TAC TGT TGC TC
BAC_Ri29M05		Ri29M05_S1[a)]	AGCTAGGTTGATCGTTGCAT
		Ri29M05_S2	TGCCATTGCCATTGTAACAT
		Ri29M05_S5	CCCATGCAATCCTTGAAATA
		Ri29M05_F2	GCTTGGCCGACAAGACTATC
		Ri29M05_mapL	GACCACATTGGAGACAAATAATCA

a) Primer used for mapping with Ri29M05_mapL.

and second a transcription factor, bHLH and other gene associations identified.

2 Materials and methods

2.1 Fruit samples

A full-sib family from a cross between the European red raspberry cv. Glen Moy and the North American red raspberry cv. Latham, estimated at *ca*. 60% similar was utilized [24, 25]. Latham, a cultivar from controlled breeding in the 1930s, produces small, firm, dark glossy fruited late in the season with a sweet aromatic taste. Glen Moy, released in 1981, in contrast, is large-fruited, pale, soft berry with a very sweet flavour character. The entire segregating population of 330 individuals and both parents were planted at two field locations with one open and one under protection (polytunnel), in randomised complete block trials with three replicates and two plant plots at both locations. Replicate open field grown samples of parents and progeny were hand harvested in 2006 and 2007 and in 2007 from fruit grown under protected cultivation. Berries were harvested at the same time of day and from the same side of the plant and immediately stored at -20°C.

2.2 Anthocyanin extractions

Frozen raspberries (4 g aliquots) were thawed for 60 min at 4°C. Methanol containing 1% HCl (3 mL) was added and fruit were homogenized using a glass rod. Tubes were centrifuged at $4000 \times g$ at 4°C for 30 min to separate juice from solids. Supernatants were transferred to 1.5 mL microcentrifuge tubes and stored at -80°C. Prior to analysis, juice was centrifuged at $13\,000 \times g$ for 30 min to sediment impurities and 0.8 mL was pipetted into 1 mL amber glass vials (Waters Associates, Milford, MA), covered and placed in an auto sampler tray at ambient temperature.

2.3 HPLC analysis

Anthocyanins in 20 µL juice were separated using a 150×4.6 mm (internal diameter id) 5 µm Novapac C18 column fitted with a 20×4.6 mm (id) Novapac C18 guard column (Waters Associates, Milford, MA). The mobile phase (0.8 mL/min) was 1% v/v aqueous formic acid developed by a gradient of 8 – 18% ACN over 30 min. Anthocyanins were detected at 520 nm and data collected and processed using Waters Millenium Manager 32 software.

2.4 Identification of anthocyanin pigments

Authentic standard solutions (0.03 mg/mL) were prepared using cyanidin-3-glucoside, cyanidin-3-rutinoside and pelargonidin-3-glucoside (Extrasynthase, Lyon, France). Other anthocyanins were identified from relative retention factors based on a previous report [26].

2.5 Cloning of raspberry candidate gene sequences for mapping

An alignment using bHLH sequences from Arabidopsis (NM_148067, NM_105042, NM_117050) and apple (DQ266451) was perfomed using ClustalW and primers were designed (Table 1) to the apple cDNA sequence using

Primer3 (Rozen, S., Skaletsky, H. J., Primer3, 1998, Code available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). bHLH products were amplified by PCR in the parents, Glen Moy and Latham. A typical 20 μL reaction contained 25 ng template DNA, 1.0 μM primer, 0.2 mM dNTPs, 0.1 units Taq polymerase (Roche). PCR was performed on a PerkinElmer 9700 Thermal Cycler as follows: 5 min at 95°C, then 30 s at 95°C, 30 s at 55°C and 45 s at 72°C for 30 cycles followed by 10 min at 72°C. 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). Internal primers were subsequently designed (Table 1) to complete the sequencing of the products and identify sequence polymorphisms suitable for mapping.

A BAC library has been constructed in *Rubus* using Glen Moy DNA [27]. This has been screened with probes for CHS and 30 clones were Southern positive (I. Hein, pers. comm.). BAC DNA was prepared from these clones using the Sigma Phase Prep BAC DNA kit according to manufacturer's instructions. PCRs were performed using three sets of PKS primers (Table 3) designed to PKS sequences from *R. idaeus* cv. Royalty using Primer3. Three BAC clones were PCR positive and ~10 μg BAC DNA was partially digested with Sau3A1 and cloned into pUC19. Recombinant clones were screened by colony hybridization on grid membranes with DIG-labelled (Roche) PKS 1 probe. Plasmid DNA of the positive clones was prepared using WizardPreps (Promega) and sequenced as described above with the universal M13 forward and reverse primers, primers for the PKS genes and internal primers were designed to fill gaps in the sequences using Primer3 software (Table 1). DNA sequences were analysed using Sequencher 4.5 (DNA Codes Corporation) to identify sequence polymorphisms.

Fluorescently end-labelled primers were designed (Table 1) to amplify the polymorphic gene regions for PKS and bHLH and PCRs were performed as described above on the 188 individuals from the Glen Moy X Latham progeny. Samples were prepared for analysis on the ABI3730 and ROX350 (Applied Biosystems) was used as an internal size standard. Data were analysed using Genemapper software (Applied Biosystems).

2.6 Statistical and mapping analysis

Anthocyanin data from the 188 individuals and parents were analysed using Genstat (10th Edn.) to examine variation across pigments, progeny, seasons and sites. Size polymorphisms in the bHLH and PKS1 genes were scored in the 188 individuals and added to the genetic linkage map of Graham *et al*. [25] using Joinmap v3. LGs were initially separated at a LOD score of 10.0 and map distances were calculated using the Kosambi mapping function. QTL mapping was carried out using the MapQTL 5 software [28]. A Kruskal – Wallis test was used as a preliminary test to identify regions of the genome linked to each of the eight anthocyanins. Interval mapping and Restricted MQM mapping were then carried out using MapQTL.

Table 2. Parental values for anthocyanin content in 2007 fruits under polytunnel cultivation.

Parental pigment	Latham (μg/mL)	Glen Moy (μg/mL)
C3S	302.1	206.6
C3GR	106.5	37.5
C3G	41.6	37.1
C3R	33.4	11.6
P3S	0.0	1.2
P3GR	0.0	1.9
P3G	2.4	0.0
P3S	0.0	0.9
Total	486.0	296.8

3 Results

3.1 Anthocyanin measurements

Eight anthocyanins, cyanidin-3-sophoroside (C3S), cyanidin-3-glucoside (C3G), cyanidin-3-glucosylrutinoside (C3GR), cyanidin-3-rutinoside (C3R), pelargonidin-3-sophoroside (P3S), pelargonidin-3-glucoside (P3G), pelargonidin-3-glucosylrutinoside (P3GR) and pelargonidin-3-rutinoside (P3R) were detected in parents (Table 2) and progeny from the Glen Moy X Latham population (Table 3). In each of the progeny C3S was most abundant followed by C3GR. In contrast, the pelargonidin anthocyanins were a minor fraction comprising less than 2% of the total anthocyanin content in all progeny (mean composition). One-way ANOVA confirmed that highly significant differences ($p < 0.001$) in each of the eight individual and total anthocyanins exist between progeny. Certain progeny had compositions far from the mean values (Table 3). No significant difference was found between replicate samples for any of the pigments measured.

Significant seasonal variation exists for six out of eight anthocyanins between 2006 and 2007: C3G, C3R, P3S, P3GR, P3G and P3R (all with $p < 0.001$) (Fig. 2). Meteorological data (Table 4) identified the 2006 growing season as hotter, drier and with more sunshine hours than 2007, and was reflected in the higher total fruit anthocyanin contents observed in 2006 than in 2007.

In 2007, fruit were grown under two conditions, field and polytunnel, and although there were variations in the levels

Table 3. Range of anthocyanin contents (min – max) (μg/mL) in 188 progeny in 2006 and 2007 and under different cultivation conditions

Season site	2006 Open field (OF)			2007 Open field (OF)			2007 Polytunnel (PT)		
Pigment	Min	Max	Mean SEM	Min	Max	Mean SEM	Min	Max	Mean SEM
C3S	59.2	716.7	248.9 ± 11.45	39.2	624.6	228.85 ± 8.70	46.1	586.1	205.9 ± 9.50
C3GR	0	250.9	90.38 ± 6.32	0	492.6	96.75 ± 6.85	0	300.3	83.62 ± 5.93
C3G	0	612.6	88.95 ± 5.60	0	217.4	39.26 ± 3.05	0	204.4	48.65 ± 3.02
C3R	0	231.4	6.29 ± 1.11	0	223.1	5.01 ± 0.47	0	128.6	4.48 ± 0.62
P3S	0	66.29	45.71 ± 3.89	0	71.65	29.11 ± 2.35	0	29.5	30.97 ± 2.45
P3GR	0	43.32	5.10 ± 0.78	0	13.01	2.33 ± 0.14	0	19.54	2.66 ± 0.28
P3G	0	108.5	6.29 ± 0.61	0	28.76	1.89 ± 0.20	0	7.28	1.76 ± 0.14
P3R	0	63.16	5.51 ± 1.00	0	13.26	2.44 ± 0.15	0	12.79	1.86 ± 0.20
Total	160.9	1001.6	385.3 ± 19.63	83.09	975.2	362.25 ± 14.76	78.4	823.0	280.5 ± 15.39

Table 4. Weather conditions in 2006 and 2007 for Dundee (UK)

	Year/ month	May	June	July	August
Mean max	2006	14.9	19.5	22.8	20.6
temp (°C)	2007	14.6	16.8	19.6	19.4
Sunshine	2006	173.1	229.7	253.3	137.9
(hours)	2007	182.2	142.3	160.7	159.1
Rainfall above	2006	13	13	14	21
0.2 mm (days)	2007	28	23	24	23

of the eight anthocyanins the significance was low (Fig. 2). However, the slight variations across the individual pigments (C3S ($p = 0.07$), C3GR ($p = 0.07$); C3G ($p = 0.05$), C3R ($p = 0.07$); and P3R ($p = 0.01$) and P3S ($p = 0.01$)) resulted in the total content of these pigments being lower for fruit grown under polytunnel cultivation.

There are significant correlations between individual pigments in the data which are maintained across seasons and environments. The level of C3S across progeny is significantly correlated with C3G and P3G ($p = 0.001$). C3GR is significantly correlated with C3R ($p = 0.001$) and negatively correlated with cyanidin-3-glucoside ($p = 0.001$).

3.2 Mapping QTL for anthocyanin pigments

Identification of mapped loci was carried out using the linkage map and LG numbering of Graham *et al.* [25]. Potential candidate genes were added to the map as described above, after identification of length polymorphisms between the parental alleles. KW analysis identified ten highly significant markers association on LG 1 for all four cyanidin pigments in both seasons and across environments (Fig. 3). All four pelargonidin pigments also demonstrated marker associations on LG 1 across seasons and environments however significance was lower than for cyanidin pigments ($p = 0.01 – 0.005$ depending on pigment and year). Interval mapping identified bHLH and Rub119a, a genomic SSR marker with high level of sequence similarity BlastX E value = 4e-25 identity = 72/189 (38%), positives = 101/189 (53%), Gaps = 37/189 (19%) to a NAM/NAC like protein from grape as the most significant markers for the cyanidin and pelargonidin pigments explaining 35 – 60% of variation depending on pigment and season.

All four cyanidin pigments mapped to LG 4 (Fig. 3) across years and seasons although for 2007 field data the significance for 3 out of the 4 pigments increased to $p < 0.001$. All four pelargonidin pigments mapped to the same region of LG 4 in 2006 ($p < 0.001$) but only P3G still remained significant ($p < 0.001$) in 2007. KW analysis identified 6 – 20 (depending on pigment) highly significant markers and the most significant marker here identified by interval mapping was FruitE4, an EST-SSR [25] derived from a ripe fruit library with similarity to a bZIP transcription factor [29] and explains between 10 and 40% of the variation, again depending on year and pigment.

3.3 Candidate genes

bHLH genes are transcription factors involved in many processes, including anthocyanin biosynthesis in plants. bHLH gene fragments were amplified from the Glen Moy and Latham parents. The sequences from Moy (1112 bp) and one Latham allele (872 bp) show greatest homology to the bHLH33 sequence from apple (Acc. no. ABB84474.1). The second Latham allele (1059 bp) contains a 50 bp insertion absent in the other Latham allele and this size polymorphism allowed the bHLH gene to be mapped to LG 1 in the Moy X Latham population.

From the three PKSs positive BAC clones identified, work focused on one clone (Ri29M05). This BAC contains a PKS gene with an SSR (AT_n repeat) in the promoter region. The SSR was polymorphic and was used for mapping purposes. Sequence analysis of a Ri29M05 subclone positive for the PKS 1 probe showed this region to be 3101 bp in length, containing 1193 bp upstream of the putative ATG start codon, one intron, (between 1374 and 1755 bp)

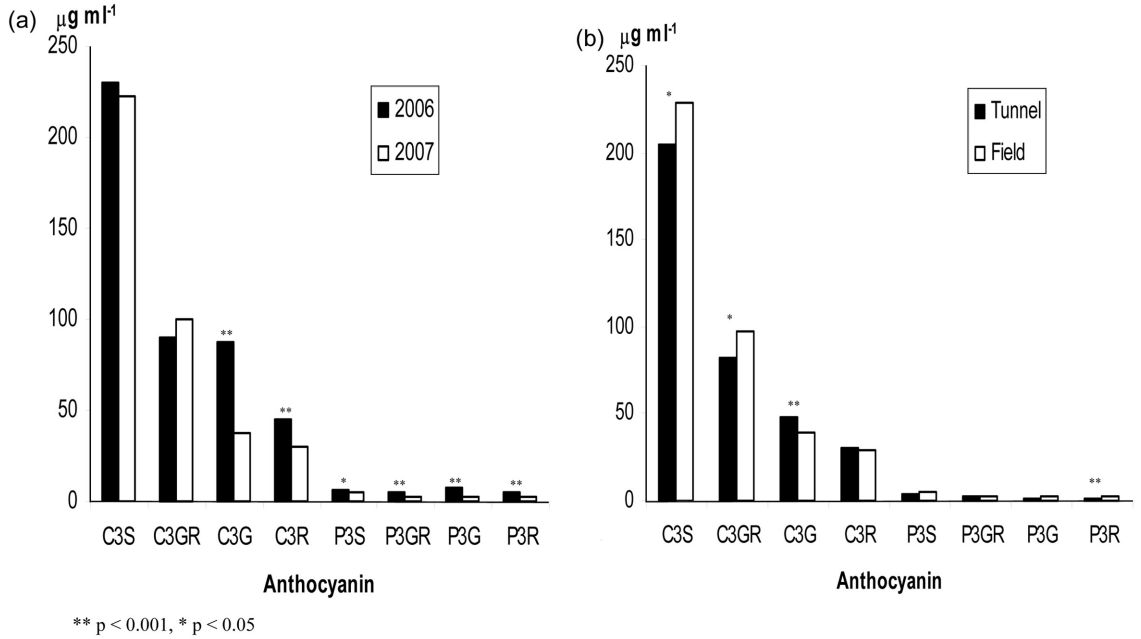


Figure 2. (a) Seasonal effect on open field fruits 2006 *versus* 2007 and (b) environmental effect in 2007 fruits under different cultivation method, mean values of anthocyanin antioxidants.

and a stop codon located at 2749 bp. Database searches using BLASTX [30] suggest it is likely to encode a PKS1 homologue, being identical to Accession no. AF292367 from cv. Royalty (Table 1). This PKS1 gene, which is a CHS, mapped to LG 7.

4 Discussion

4.1 Anthocyanin contents

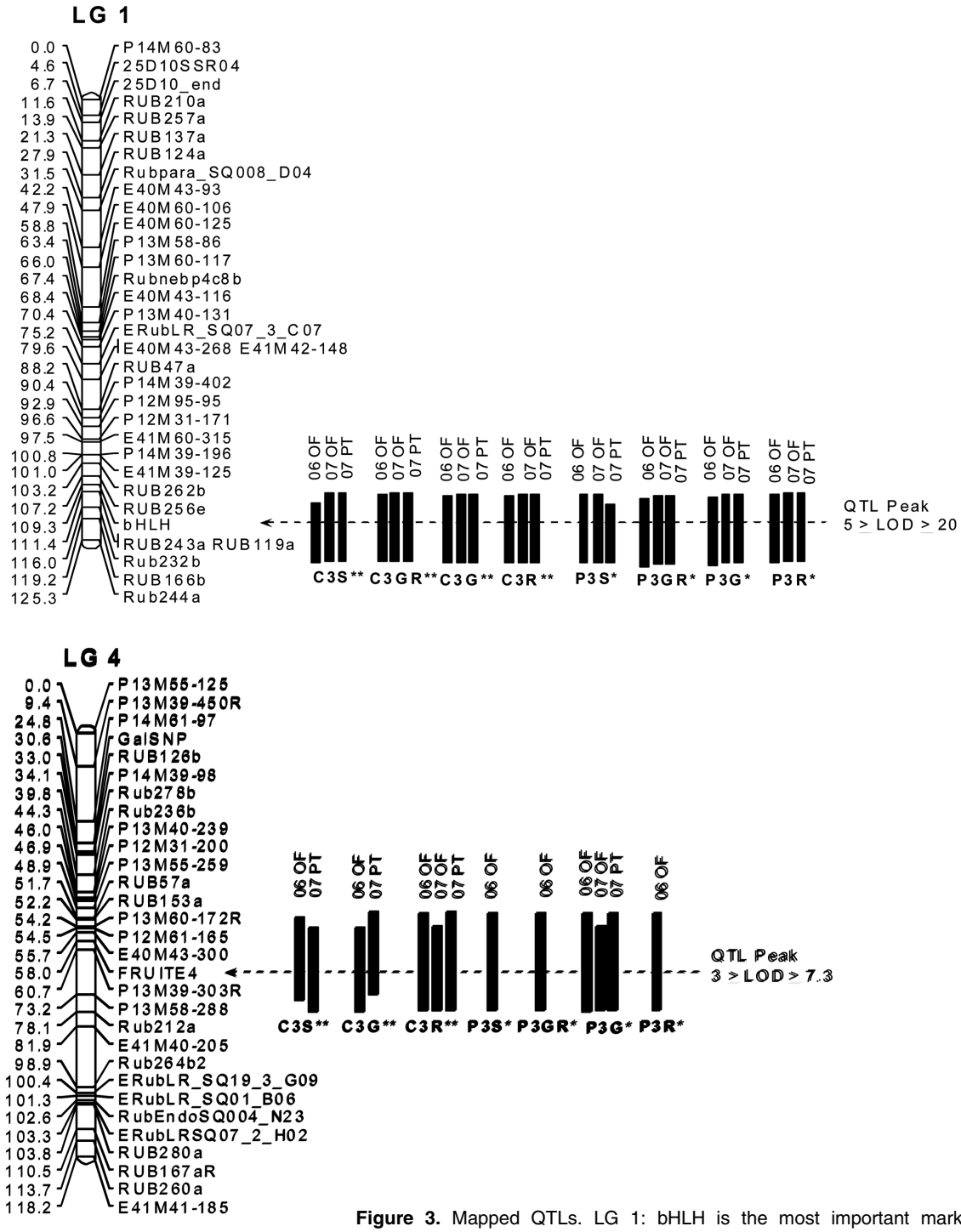
Eight anthocyanin antioxidants [26] in berries of progeny from crossing two cultivars of disparate *R. idaeus* subspecies: *idaeus* (Moy) and *strigosus* (Latham) – showed a wide range of contents with parental values between progeny extremes. Jiang [31] in a study of 18 raspberry varieties and selections grown at SCRI over three seasons in the late 1980s concluded C3S accounted for 47.9 – 78.7% of anthocyanin antioxidants which here accounted for just over 50% total, stable across years and sites. Jiang [31] recorded C3GR (2.0 – 34.1%) next most abundant anthocyanin: similar to this study and again not changing significantly across years and sites. Here, C3G contents were similar to C3GR in 2006, but dropped by almost 50% in 2007 under both field and polytunnel conditions. The minor cyanidin pigment C3R accounted for around 9% total with little change across years and sites.

Pelargonidins formed 5% anthocyanins in 2006, just over 2% in 2007 at both sites. Jiang [31] reported pelargonidins accounted for < 2% pigments with only P3S and P3G showing significant seasonal variation. Here, 3 out of 4 pelargonidins showed significant seasonal (2006 *vs*. 2007) effects with P3S less 2007 but significance was low.

Contents of individual anthocyanins varied significantly across progeny but not between replicates. Although anthocyanin production is genetically regulated [19], significant seasonal effects on content between 2006 and 2007 were observed, possibly related to light, reported to play a crucial role in stimulating anthocyanin synthesis [13].

Light dosage (UV-factor) may explain most differences in anthocyanin contents observed between the two seasons and open and protected cultivation. Two major cyanidins, C3G and C3R were significantly more abundant in 2006 (Fig. 2) with more daylight hours (Table 4). Cyanidin glucosides are UV-absorbing anthocyanins [32] and there is a report of a rapid stimulation in anthocyanin biosynthesis in potato subjected to a higher dosage of light [13].

Minor differences were also apparent between berries from field and protected cultivation, though individual pigment differences across sites were of low significance, as were changes in proportion of each pigment. Overall however there was a greater total pigment content when field was compared with protected fruit. Plants grown under polytunnel cultivation experienced warmer conditions, but less light thus a drop in total berry anthocyanins was not unexpected. Most pelargonidin glucoside contents were not influenced by protected cultivation.



** $p < 0.0001$, * $p < 0.01$

Figure 3. Mapped QTLs. LG 1: bHLH is the most important marker followed by RUB119a, NAM/CuC2 like protein. LG4-FruitE4 is the most important marker which is also a bZIP.

Studies of two cultivars of tomatoes grown under polytunnels comprised of materials that either transmitted ambient solar UV radiation from 290 to 400 nm or blocked UV wavelengths below 380 nm showed the former contained 20% more phenolic compounds [33]. Although individual pigment levels may not differ significantly between open and protected cultivation, other important antioxidant components conferring flavour and volatiles may.

4.2 Candidate genes and QTL for anthocyanin antioxidants

Secondary metabolism marks the start of specific stages of development, *e.g* anthocyanin accumulation during fruit ripening. In apple, genes involved in anthocyanin synthesis are coordinately induced during fruit development [12], suggesting these genes are regulated by one or a small number of regulatory proteins [28].

Expression of genes of the anthocyanin biosynthetic pathway is regulated by transcription factors conserved in higher plants [34]. Several classes of protein appear involved; R2R3 MYB factors, which interact closely with basic helix-loop-helix (bHLH) factors [18] in determining anthocyanin production [35]. Regulation appears specific for structural genes acting either early or late in the pathway [36]. In petunia and maize kernels, a protein containing WD40 domains is known to form complexes with MYB and bHLH factors [37].

Here, several transcription factors, bHLH, FruitE4 encoding a basic leucine zipper (bZIP) transcription factor and Rub119 encoding a NAM (no apical meristem) like transcription factor appear to underlie the major QTL identified for all eight and seven antioxidants, respectively and in both seasons and at both sites. In apples anthocyanin biosynthesis is correlated with co-expression of two bHLH genes (MdbHLH3 and MdbHLH33) and a MYB10 gene although levels of bHLH transcripts levels were not positively regulated by MYB10 [18]. Several MYB genes located on the *Rubus* linkage map (Woodhead and Graham, data not shown) do not underlie anthocyanin QTL presented here. In Arabidopsis, over-expression of PAP1 (production of anthocyanin pigment 1) appears to upregulate its bHLH partner [38] and level of expression of MYB transcription factor was suggested as determining apple anthocyanin production [18].

In grape, studies of a single locus encoding two very similar MYB-related transcription factor genes, VvmybA1 and VvmybA2 located on a single BAC: show either can regulate berry colour [39]. A retrotransposon insertion into VvmybA1 gave a loss of anthocyanin production and white grape cultivars [40]. Additional sequence VvmybA1 gene polymorphisms are also strongly associated with red or pink fruited grape accessions, suggesting that variation in a single transcriptional regulator generated a series of alleles strongly associated with grape colour variation [35].

In raspberry, a bHLH gene very similar to MdbHLH33 from apple appears closely associated with anthocyanin production on LG 1 (Fig. 3). bHLH and WD40 genes are known to be involved in other plant processes including vacuolar pH [41]. As other fruit quality traits have been examined in this raspberry mapping population, the association of these and other genes, on fruit acidity and observed fruit colour will be explored further.

Also on LG 1 is an SSR marker, Rub119a, that encodes a putative protein similar to transcription factors containing a NAM/NAC domain. Genes with the NAC domain (NAC family genes) are plant-specific transcriptional regulators expressed at various developmental stages and in various tissues including in defence and abiotic stress responses, flowering and secondary wall biosynthesis [42]. The strong association of this putative transcription factor with production of cyanidin and pelargonidin pigments is interesting and will also be investigated further.

bZIP transcription factors like FruitE4 on LG 4 (Fig. 3) are expressed constitutively or tissue specifically and regulate diverse processes such as photomorphogenesis and light signalling [43, 44], stress and hormone signalling [29]. Precise roles of transcription factors underlying antioxidant QTL are as yet unclear but roles in light or hormone signalling cannot be ruled out.

Data presented here locate QTL for the major anthocyanins on the *Rubus* genetic linkage map and several candidate genes and markers associated with the QTL. In light of recent work in other *Rosaceae* species [18] it is not surprising that transcription factors underlie such QTL. Although the environment in which raspberry grows impacts on the total fruit content of anthocyanin (especially cyanidin) antioxidants that can be produced there is also a genetic component. As raspberry breeding programmes move towards using marker assisted breeding strategies, genes and markers identified here will serve as tools to begin identifying closer associations between genotypes and antioxidants contents with the objective of increasing nutritional value of these berry fruits.

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The authors have declared no conflict of interest.

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