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Mapping QTLs for developmental traits in raspberry from bud break to ripe fruit

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Abstract Protected cropping systems have been adopted by the UK industry to improve fruit quality and extend the current season. Further manipulation of season, alongside consideration of climate change scenarios, requires an understanding of the processes controlling fruit ripening. Ripening stages were scored from May to July across different years and environments from a raspberry mapping population. Here the interest was in identifying QTLs for the overall ripening process as well as for the time to reach each stage, and principal coordinate analysis was used to summarise the ripening process. Linear interpolation was also used to estimate the time (in days) taken for each plot to reach each of the stages assessed. QTLs were identified across four chromosomes for ripening and the time to reach each stage. A MADS-box gene, Gene H and several raspberry ESTs were associated with the QTLs and markers associated with plant height have also been identified, paving the way for marker assisted selection in *Rubus idaeus*.

Introduction

In recent years, consumer demand for fresh raspberries extending beyond the main production season has increased, with high premiums being paid for fresh market raspberries. Protected cultivation techniques have been developed to improve fruit quality and also to extend the season of availability. By careful manipulation of the plant dormancy cycle, flower initiation and fruit ripening, coupled with breeding for high-quality varieties differing in season, it should be possible to produce fresh raspberries in Europe for sale across most of the year. Protected cropping and out of season production in European countries are expanding; for example, in areas of southern Spain nearly 100% of fresh market raspberries are grown under tunnels. These protected cropping systems have been adopted by the UK industry to improve fruit quality and extend the current season. In addition to the demand for fruit grown across a wide season, the changing climate is already a major consideration for soft fruit growing due to the succession of mild winters leading to poor bud break in some fruit species.

The ability to understand first and subsequently manipulate the ripening period of raspberry crops would allow targeted variety scheduling across a wide season. Raspberry breeding is a long, slow process. However, with marker assisted selection and allele mining in a range of germplasm, it should be feasible to develop suitable varieties in a shorter time scale. Understanding the key genetic control points across the ripening period is a major challenge in raspberry. Elongation of shoots starts in spring and continues until autumn when shortening days and falling temperatures cause shoot elongation to cease. The time at which dormancy begins and the intensity it attains are influenced both by environmental conditions during the growing season (Måge 1975) and the genotype of the plant. Plant age has also been shown to be a factor (Jennings 1987). The initiation of flower buds begins at a time when the canes start to acquire dormancy and development starts from the terminal bud and in axillary buds five to ten buds below the apex, and proceeds progressively down the cane with a

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second period of flower bud development occurring before bud break. Dormancy induction and release is genetically and environmentally controlled with photoperiod and temperature playing key roles in these processes as well as hormonal control (Lang 1987; Darnell et al. 2003; Olsen 2003; Serce and Hancock 2005). Bud growth regulates important aspects of plant architecture and development such as height and cane number (Horvath et al. 2003). Floral initiation is largely dependent on the developmental stage of the plant with photoreceptors and transcription factors acting on the various environmental cues (Simpson and Dean 2002). Flower development can be divided into four steps that occur in a temporal sequence. In response to environmental and endogenous signals the plant switches to reproductive growth controlled by a large group of flowering time genes (Klejnot and Lin 2004). Signals from various flowering time pathways are integrated, activating meristem identity genes that specify floral identity. The meristem identity genes activate floral organ identity genes which in turn activate organ building genes, specifying various tissue types (for reviews see Jack 2004; Tan and Swain 2006). Fruit development results in an increase in size through both cell division and expansion accompanied by changes in texture, colour and flavour. Squamosa promoter binding protein (SBP-box) genes and their putative MADS-box promoter appear to regulate fruit tissue development (Unte et al. 2003; Manning et al. 2006). Phenolic compounds signal when a fruit is ripe and ready for consumption. They are central components in phenylpropanoid metabolism that contribute to fruit pigmentation but also the formation of the building blocks for lignin polymers (Tani et al. 2007) and certain volatile components such as 4-(4-hydroxyphenyl)-butan-2-one or raspberry ketone.

This work aimed to map key trait data on fruit development in red raspberry, from bud break through to ripe fruit and to identify associated markers with potential utility in developing a range of varieties suitable for growing across a wide cropping season. The work also aimed to examine regulatory genes that may directly control the transcription of structural genes in the ripening pathway and determine if these underlie any QTLs identified. These were a MADS-box gene that was identified during a project examining gene expression during dormancy phase transition (Mazzitelli et al. 2007) and also R2R3 MYB transcription factors. In *Arabidopsis* there are 133 R2R3 MYB transcription factor-encoding genes, divided into 24 sub-groups (Stracke et al. 2001) and involved in many plant processes including anthocyanin production (Koes et al. 2005; Espley et al. 2007). Recent work in apple for example, has demonstrated that efficient induction of anthocyanin production during ripening depends upon the co-expression of both MdMYB10 and two distinct basic-helix-loop-helix (bHLH) transcription factors (MdbHLH3 and MdbHLH33; Espley

et al. 2007). A bHLH gene similar to the bHLH33 from apple has recently been mapped to chromosome 1 in raspberry and does underlie a QTL for 8 anthocyanin pigments (Kassim et al. 2009). This work will serve to map QTLs across ripening and will form the basis for examining candidate gene functions which control the whole process in raspberry from bud break to ripe fruit.

Materials and methods

Mapping population

The population, as described previously (Graham et al. 2004, 2006), consists of a full sib family generated from a cross between the European red raspberry cv. Glen Moy and the North American red raspberry cv. Latham. These varieties differ in a number of key traits including resistance to various diseases and phenological traits such as dormancy requirement and fruit development. This population (MP1) was planted in autumn 2001 at two different environmental locations, one a high health plot with pest and disease control and the second with no chemical applications. A randomised block design with three replicates and two plant plots was used at both locations. In 2004, a new trial of 188 offspring propagated from the same cross (MP1 and a further 94 lines, called population MP2) was grown under protected cultivation as mature long canes, again in a randomised block design with three replicates.

Phenotypic data collection

Ripening data was collected in 2003, 2004 and 2005 seasons from the two environmental locations on 94 individuals of MP1 and in 2006 from the three locations including the protected site from 188 individuals (MP1 and MP2). All phenotypic data were collected as visual assessments of fruit ripening by scoring the stages of development from bud break to over-ripe fruit from April/May to July each year using a 1–7 scale (1 = bud break, 2 = open flowers, 3 = fruit set, 4 = green fruit, 5 = green/red fruit, 6 = ripe and 7 = over-ripe). On some dates a single score was made for each plot; on other dates the two plants in a plot were scored separately. Different assessors each scored the entire plots. In 2005 there was an additional, more detailed assessment of fruit colour using a scale 1 = white, 2 = green, 3 = green/red, 4 = yellow, 5 = pink, 6 = red, 7 = overripe. In 2006, an additional estimate of the % open flowers was made on 13 June, for the protected trial only. Table 1 summarises the number of assessments for each trial.

The height of the tallest cane on each plant was measured in 2005 and 2006 to examine the relationship between height and bud development.

Table 1 Summary of ripening scores in 2003–2006

Trial	Date of first score	Date of last score	No. of assessment dates	No. of lines assessed
2006: field	19 May	6 July	11	188
2006: protected	15 May	7 July	15 + 1	188
2005: field	10 May	23 July	10 + 5	94
2004: field	18 May	7 July	5	94
2003: field	21 April	24 July	13	94

In 2005, there were ten assessments comparable to other years and five more detailed colour scorings. In the 2006 protected trial, there was an additional assessment of the % open flowers

Candidate gene information

MYB genes

Five accessions encoding R2R3 MYB genes (AF470080, X52201, DQ267900, DQ074463 and AF336282) were aligned using ClustalW to determine conserved regions of sequence. Primers (Table 2) were then designed to the apple sequence (DQ074463) as primers from apples may be more successful for amplification in raspberry than other members of the Rosaceae (D. Sargent, personal communication). Amplification of the *Rubus* R2R3MYB sequences was performed using Glen Moy and Latham DNA and apple DNA as a control (25 ng DNA, 1.0 μM primer, 0.2 mM dNTPs, 0.1 units Taq polymerase (Roche)) and amplification was performed on a GeneAmp 9700 PCR System Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using a protocol of 2 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°, followed by 7 min at 72°C. Products were analysed on a 1.5% agarose gel and bands from Glen Moy and Latham samples at approximately 900 bp were excised, purified using the Qiagen MinElute gel extraction kit according to the manufacturer's instructions, cloned into pGEM-T Easy (Promega) and transformed into DH5α competent cells (Invitrogen). Plasmid DNA was subsequently isolated from transformants using the MiniSpin kit from Qiagen and sequenced using the universal M13 forward and reverse primers (5′-GTAAAACGACGGCCAG and 5′ CAGGAAACAGCTATGAC) in conjunction with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Conditions were 25 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. Sequences were determined using a 3730 DNA Analyzer (Applied Biosystems) and data were analysed manually using Sequencher 4.5 (DNA Codes Corporation). The *R. idaeus* R2R3 MYB gene identified from Glen Moy was used to prepare a probe to screen the raspberry BAC library (Hein et al. 2005). Ten putative positive clones were identified and BAC DNA was prepared using the Sigma PhasePrep kit according to the manufacturer's instructions. From PCR and Southern analysis one clone, Ri9O22, was taken forward and sequenced using the primers designed for the original MYB PCR and the BigDye® Terminator v3.1 Cycle Sequencing Kit. Cycling conditions were: 95°C for 5 min, followed by 80 cycles of 95°C for 30 s, 50°C for 10 s and 60°C for 4 min on a GeneAmp 9700 PCR System Thermal Cycler. Sequences were analysed using a 3730 DNA Analyzer and data were analysed manually using Sequencher 4.5. Further primers were designed to walk along the MYB gene region (Table 2).

Direct sequencing of the Ri9O22 BAC clone identified an (AG)*n* SSR motif and primers were designed to amplify this region. A size polymorphism between the parents was identified which was subsequently amplified and analysed in the entire mapping population using a touchdown PCR as described by Woodhead et al. (2003) to provide a map location for this gene and the corresponding BAC.

MADS box gene

Primers were designed to a raspberry MADs box gene (Accession No. EE284413; designated *Ri*MADS_01) cloned from endodormant buds from cv. Glen Ample. This

Table 2 The primers used to amplify, sequence and map *R. idaeus* R2R3 MYB and MADS-box genes in the Glen Moy and Latham population

Primer name	Left primer sequence (5′–3′)	Right primer sequence (5′–3′)
*Ri*MYB	AGGAGTCCTTGTTGTTCAAAGGA	TAATTCTTGATTTCATTGTCTGTTCGG
Bac_9O22_SSR01	AGCCATCCTACTGGTTGTGG	CCATAGCTGTCCATGCTCCT
*Ri*MADS_01	CGATCTGTTGGCGGAAACG	ATTCAGCCCTTCTAGATCCTCACC
*Ri*MADS_01_seq	CGTTCAACTGCCTGT	

gene, which encodes an short vegetative phase (SVP)-type MADS box transcription factor, exhibited a declining transcript profile during dormancy release in *R. idaeus* (Mazzitelli et al. 2007). Primers were designed to amplify the corresponding region in cv. Glen Moy and Latham (Table 2) and the products were sequenced as described above to identify polymorphisms. A SNP was identified and primers were designed for a Pyrosequencing® assay using the PSQ Assay Design software 1.0 (Biotage, Uppsala, Sweden). The Pyrosequencing® assay was performed on all 188 individuals of the mapping population according to the manufacturer's protocol (Biotage) using a PSQ96MA Pyrosequencing® instrument (Biotage). Sequence results were obtained in the form of pyrograms and analyzed using PSQ96MA 2.1 software (Biotage).

Linkage analysis

An updated marker map was calculated as described in Graham et al. (2004, 2006) using JoinMap 3 (Van Ooijen and Voorrips 2001), based on markers that were scored in both mapping populations. Markers were tested for heterogeneity (using the chi-square test in JoinMap) between the two mapping populations, and any markers with significant heterogeneity were excluded. Markers were partitioned into linkage groups using a lod of 4.0. Map distances between markers are derived using the Kosambi mapping function.

QTL analysis

A detailed analysis of the ripening data was carried out for the 2006 clean trial and the protected trial of 188 offspring (MP1 and MP2). Markers identified in this population as associated with the stages of ripening were then validated using data from MP1 scored in 2003–2005.

There are many approaches to analysing repeated observations of this type. Here the interest was in the overall ripening process and identifying QTLs that control this process. However, it is not possible to observe this process continuously, but only on a finite number of occasions. Therefore an approach was used that combined the scores on different dates into an overall summary, using principal coordinates to extract the principal sources of variation in ripening across the mapping population. For each location, a matrix of similarities between the 2006 ripening profiles for each plot was calculated using the city-block metric. Principal coordinates were then derived from the similarity matrix. Correlations between the principal coordinates and the original scores were examined to see how these were related. Analysis of variance of the first five principal coordinates was used to estimate the genetic and environmental components of variance, and to predict the mean principal coordinate

scores for each genotype. The genotype means were then used as phenotypic traits for QTL mapping.

An advantage of using the principal coordinates is that they are uncorrelated, and so can be interpreted independently. However, they are not easy to interpret in terms of the stages of development. Linear interpolation was therefore used to estimate the time (in days) taken for each plot to reach each of stages 2 (open flowers) to 6 (ripe fruit). As for the principal coordinates, analysis of variance was used to estimate the genotypic means for QTL mapping.

QTL mapping was carried out using the MapQTL 5 software (Van Ooijen 2004). The Kruskal–Wallis test implemented in MapQTL was used as a preliminary test to identify regions of the genome that were linked to each of the first five principal coordinates, and whether the phenotype was affected by alleles from one parent or both. A small permutation test was carried out using the statistical programme Genstat 10 for Windows (Genstat 2007) to establish appropriate thresholds for the Kruskal–Wallis test. Interval mapping was then carried out using MapQTL. If the Kruskal–Wallis analysis indicated that the phenotype was affected by alleles from both parents, the trait was analysed using a four-mean QTL model. If alleles from only one parent were affecting the trait, the marker data was recoded so that MapQTL fitted a two-mean model.

Stepwise regression of trait values on marker genotypes was used to explore more complicated models. The principal coordinate analysis, analysis of variance, regression analysis and linear interpolation were carried out using the Genstat 10 programme.

Results

Linkage map

The updated linkage map (Fig. 1) has 243 markers and a total length of 843 cM. As for the map presented in Graham et al. (2006), six linkage groups from the two parents can be paired by markers segregating in both parents, but there remain two linkage groups, one from each parent, that cannot be linked to each other or to any of the other groups.

The *Ri*MYB sequence and the corresponding BAC clone BAC9022SSR 01 (Accession No. FJ527833) mapped to chromosome 3 at 69 and 67 cM respectively, and the RiMADS_01 box gene mapped to chromosome 5 at 5 cM.

Analysis of height of the tallest cane

A Kruskal–Wallis analysis of the 2006 height measurements identified two regions where markers from Latham

Fig. 1 Linkage map of Glen Moy × Latham showing one-lod confidence intervals (to the *right* of the chromosome) for QTL locations for developmental traits in 2006. *F* before a trait name indicates the field trial, *P* protected trial. *Black bars* show QTLs segregating in Latham only, diagonally *filled bars* show QTLs from Glen Moy only and *unfilled bars* show QTLs with segregating alleles from both parents. Bold loci are involved in the interaction for height. *Underlined* loci were tested in the validation analysis
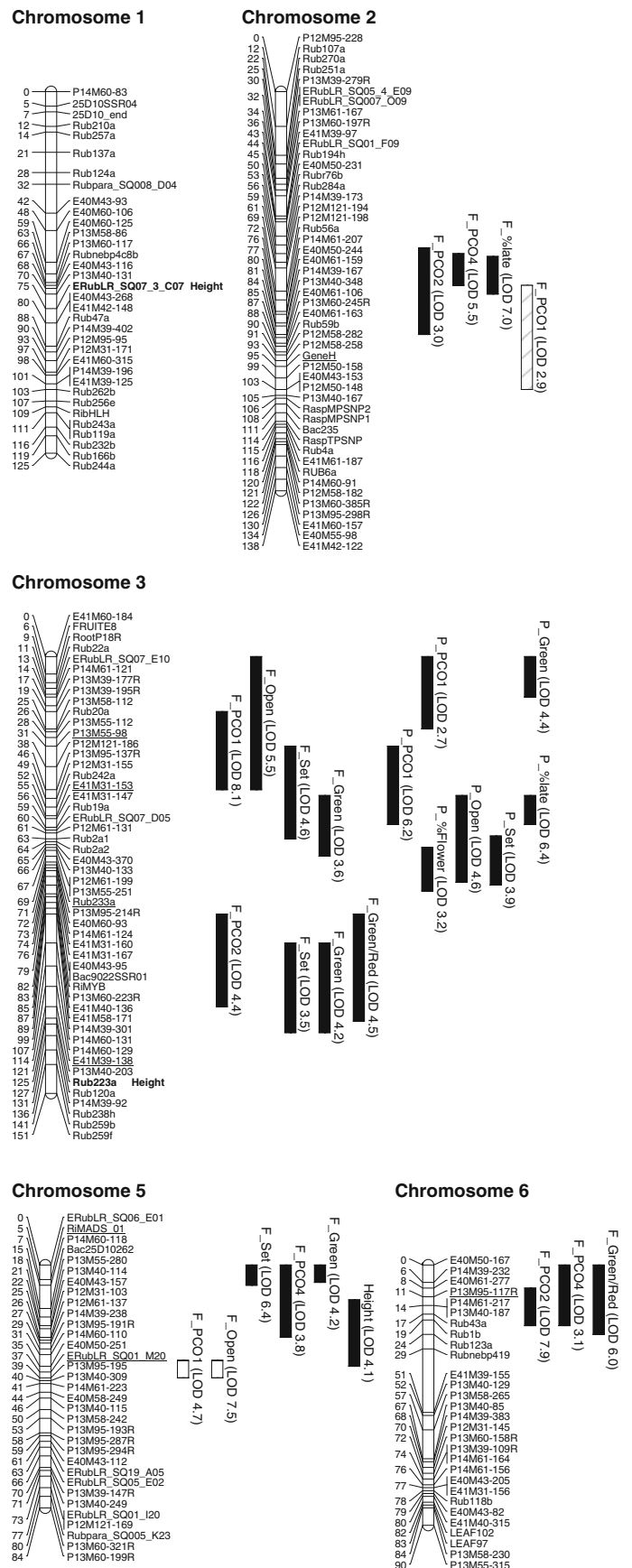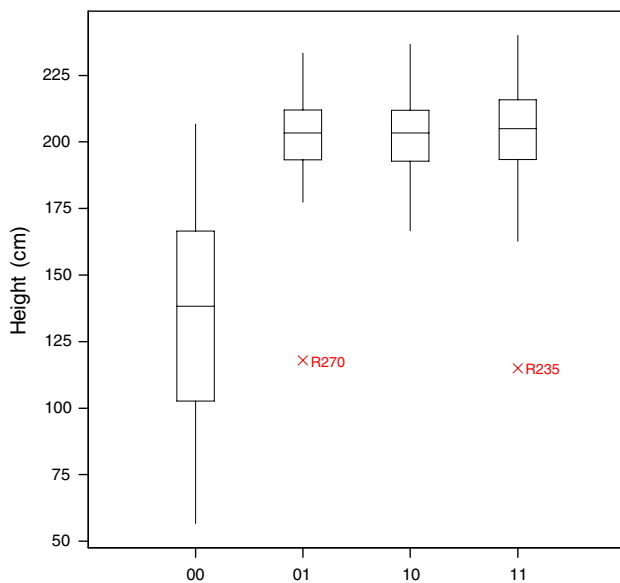
Fig. 2 Boxplots of the distribution of height data in the Glen Moy × Latham mapping population in the 2006 field trial, according to the genotypes at markers RUB223a and ERubLR_SQ07_3_C07 (*0* absence of band, *1* presence). The *horizontal band* in the centre of each box represents the median and the *vertical lines* at the end of each box represent the upper and lower quartiles. The whiskers extend to the smallest and largest trait values, provided these are within the inner "fences", which are 1.5 times the inter-quartile range beyond the quartiles. Points beyond these fences are outliers are shown as a *cross* (genotypes R270 and R235)

were significantly associated with height ($P < 0.001$). The most significant markers were RUB223a, a genomic SSR marker on chromosome 3, and an AFLP marker, P13M95–191R, on chromosome 5 (Fig. 1). However, stepwise regression found a highly significant epistatic interaction between RUB223a and another marker, ERubLR_SQ07_3_C07 (an EST-SSR with homology to an unknown rice protein (Woodhead et al. 2008) on chromosome 1, which comes from Glen Moy. Together these two markers explained 49.2% of the variation in the height data. Figure 2 shows boxplots (Tukey 1977) of the height distribution for the four genotype classes. Apart from two outliers (individuals R270 and R235), the separation into long and short canes is very clear. Lines without these two markers (designated 00) have canes that are an average of 66 cm (SE 5.4) shorter than individuals with either (designated 01 or 10) or both (designated 11) of these markers (Fig. 2). Marker P13M95–191R from the Latham parent on chromosome 5 does not interact with the other markers for height, but shows a significant main effect when added to the stepwise model ($P = 0.002$). The presence of this marker is associated with a decrease in height of 13 cm (SE 4.1). The height data from 2005 is very highly correlated with that from 2006 ($r = 0.98$) and shows similar marker effects (data not shown).

Analysis of ripening data from 2006 field trial

The first five principal coordinates (PCO1–PCO5) derived from a matrix of city-block similarities among the plots explained 34.6, 15.8, 8.0, 6.6 and 5.6% of the variation in the matrix respectively. Further principal coordinates explained less of the variation and were not investigated. The first scores were all made on 19 May 2006 when all scores were equal to one (i.e. bud break stage). These scores were uncorrelated with the principal coordinates. PCO1 had negative correlations with all the scores from 30 May onwards, especially the earliest ones. PCO1 could therefore be interpreted as an overall summary of the variability of the speed of the ripening process, so that a large negative score for PCO1 indicated a genotype that ripened quickly, especially in the early stages. PCO2 had large positive correlations with the late June and early July scores and negative correlations with the late May scores, and so a positive score could be interpreted as slow development in May but a rapid ripening in late June and early July. PCO4 and PCO5 had similar interpretations to PCO2, but comparing different times: PCO4 contrasted the ripening in mid June with that in early July and PCO5 contrasted the ripening in mid June and late June. PCO3 appeared to identify a discrepancy among three scores made on 30 May, contrasting one with the two others.

Analysis of variance of these five components showed that PCO1, PCO2, PCO4 and PCO5 had significant differences between the genotypes ($P < 0.001$), and the heritabilities were estimated as 70.6, 62.0, 51.0 and 49.9% respectively. For PCO3, the significance of the genotype effect was $P = 0.025$ and the estimated heritability was 17.3%.

Based on a small permutation test with 400 permutations, thresholds for the Kruskal–Wallis (KW) statistic with 1 *df* of 13.0 and 13.8 were selected to correspond to a genome-wide significance of $P = 0.1$ and 0.05 respectively. For PCO1, the only significant markers at this level were on chromosome 3 with marker P13M55–112 (from Latham), at 27.9 cM, having the largest KW statistic of 31.5. However the maximum was not well-defined, with markers from Latham showing significant association by the permutation test for locations from 6 to 69 cM. In a regression analysis, marker P13M55–112 explained 16.7% of the variance in PCO1. A stepwise regression was performed to see if further markers were significant after adjustment for this marker. This regression selected ERubLR_SQ01_M20 (an EST with similarity to a red fruit EST from strawberry) segregating in a 1:2:1 ratio on chromosome 5 and Gene H, a gene for hairy canes from Glen Moy on chromosome 2, which were both significant with $P < 0.001$. Together these explained 36.2% of the variance. There were no significant interactions among these markers.

For PCO2, the most significant markers were P14M61–217 on chromosome 6 with KW = 38.8 and E41M39–138 on chromosome 3 with KW = 20.5, both from Latham. A stepwise regression also selected RUB56a on chromosome 2 ($P < 0.001$). Together these explained 30.6% of the variance. For PCO4, the most significant markers were P14M39–173 on chromosome 2 (close to RUB56a), with KW = 20.7, *Ri*MADS_01 on chromosome 5 (with KW = 16.8) and P13M95–117R on chromosome 6 (KW = 15.0). A stepwise regression also selected P14M61–124 on chromosome 1 ($P < 0.001$). Again, all these markers originate from Latham. PCO3 and PCO5 had no markers where the KW statistic was above the threshold of the permutation test with $P < 0.05$.

With the exception of marker ERubLR_SQ01_M20 the markers most closely associated with each trait were heterozygous in one parent, usually Latham, and homozygous in the other. Therefore a QTL interval mapping model with two QTL genotypes was used. Figure 1 shows the maximum lod score and the one-lod confidence intervals for the locations of each QTL. A more general (four genotype) model was used to estimate a confidence interval for the QTLs affecting PCO1 close to ERubLR_SQ01_M20.

As the principal coordinates were not easy to interpret in terms of the effects of the marker genotypes on the ripening schedules, an alternative analysis used the estimated time to reach each ripening stage 2–6. There were significant differences among the lines of the mapping population ($P < 0.001$) for the time to reach each stage, and the heritabilities were high, between 46.8 and 68.4%. As some plots did not reach stage 6 (red raspberries) by the final scoring on 6 July 2006, a summary variable, lateprop, was calculated as the proportion that ripened later than this. These variables were analysed as for the principal coordinates, and Fig. 1 shows the one-lod confidence intervals for the QTLs. In most cases these overlap with the QTLs from the analysis of the principal coordinates, with QTLs for the times to the early stages (open flowers and fruit set) overlapping with QTLs for PCO1 (on chromosomes 3 and 5), and QTLs for later stages of fruit ripening overlapping with PCO2 and PCO4 (on chromosomes 2, 3, 5 and 6). The sizes of the estimated QTL effects are discussed below for the data from all years together.

Analysis of ripening data from 2006 protected trial

The scoring of the protected trial started at a similar time to the field trial, on 15 May 2006. The first five principal coordinates (PCO1–PCO5) explained 44.0, 10.2, 8.8, 6.1 and 4.2% of the variance in the similarity matrix respectively. Analysis of variance of these components showed that PCO1, PCO2 and PCO3 had significant differences between the genotypes ($P < 0.001$, $P = 0.030$ and $P = 0.003$

respectively), and the heritabilities were estimated as 46.6, 29.1 and 32.0% respectively. There were no significant differences between the genotypes for PCO4 and PCO5. As for the field trial, PCO1 had significant negative correlations with all the individual scores and could be interpreted as an overall measure of the speed of ripening. PCO2 contrasted the ripeness in late May with that in mid-June, while PCO3 contrasted the ripeness in late May with the final scores in early July.

For PCO1, a Kruskal–Wallis test identified only markers on chromosome 3 as significant using a threshold of $P < 0.05$ from a permutation test. The most significant marker was E41M31–153, at 54.6 cM, with KW = 27.2, but, as for the field trial, markers were significant over a range of locations from 6 to 89 cM. Marker E41M31–153 explained 17.8% of the variance in a regression analysis. A stepwise regression selected a further marker, also on chromosome 3, P13M39–195R at 18.6 cM, as significant with $P < 0.001$. Together these explained 24.4% of the variance. There was no significant interaction between these markers. No markers were significant for PCO2 or PCO3 using the threshold for the permutation test.

Times to reach each ripening stage were estimated as before. There were significant differences among the genotypes ($P < 0.05$) for the times to reach open flower (stage 2) to green/red fruit (stage 5), but the heritabilities were lower than for the field trial, typically close to 30%. There were highly significant differences ($P < 0.001$) among the genotypes for the proportion that ripened later than the final scoring (lateprop). As for PCO1, the only significant associations were with markers on chromosome 3. The time to reach open flowers (stage 2) and set fruit (stage 3), and the % open flowers on 13 June were most highly associated with marker RUB233a, at 69.1 cM, with KW = 27.2, 19.4 and 17.8 respectively. No markers were significant, using a permutation test, for the times to reach stages 4 or 5 but marker ERubLR_SQ01_P18 [similar to a gene encoding a pathogenesis-related protein from *Prunus persica* (Woodhead et al. 2008)] at 9.1 cM on chromosome 3 was significant for the time to reach red fruit (stage 6), with KW = 14.2. The proportion ripening after the last scoring was significantly associated with marker E41M31–153 at 54.6 cM on chromosome 3, with KW = 23.5. Stepwise regression did not identify further significant markers for any of these traits.

All the above markers come from the Latham parent, and were mapped with a two-mean QTL model as described above. Figure 1 shows one-lod confidence intervals for the QTL locations.

The timing and distribution of ripening in the field and protected trial is shown in Fig. 3 which demonstrates the effect of environment on the overall ripening process with polytunnel grown plants setting more fruit earlier than

Fig. 3 Distribution of the estimated days to reach each ripening stage for the Glen Moy × Latham mapping population. 2006 field data is in *white*, and polytunnel data is in *black*. Some lines did not reach the final ripening stages: these are shown as a time of 60 days
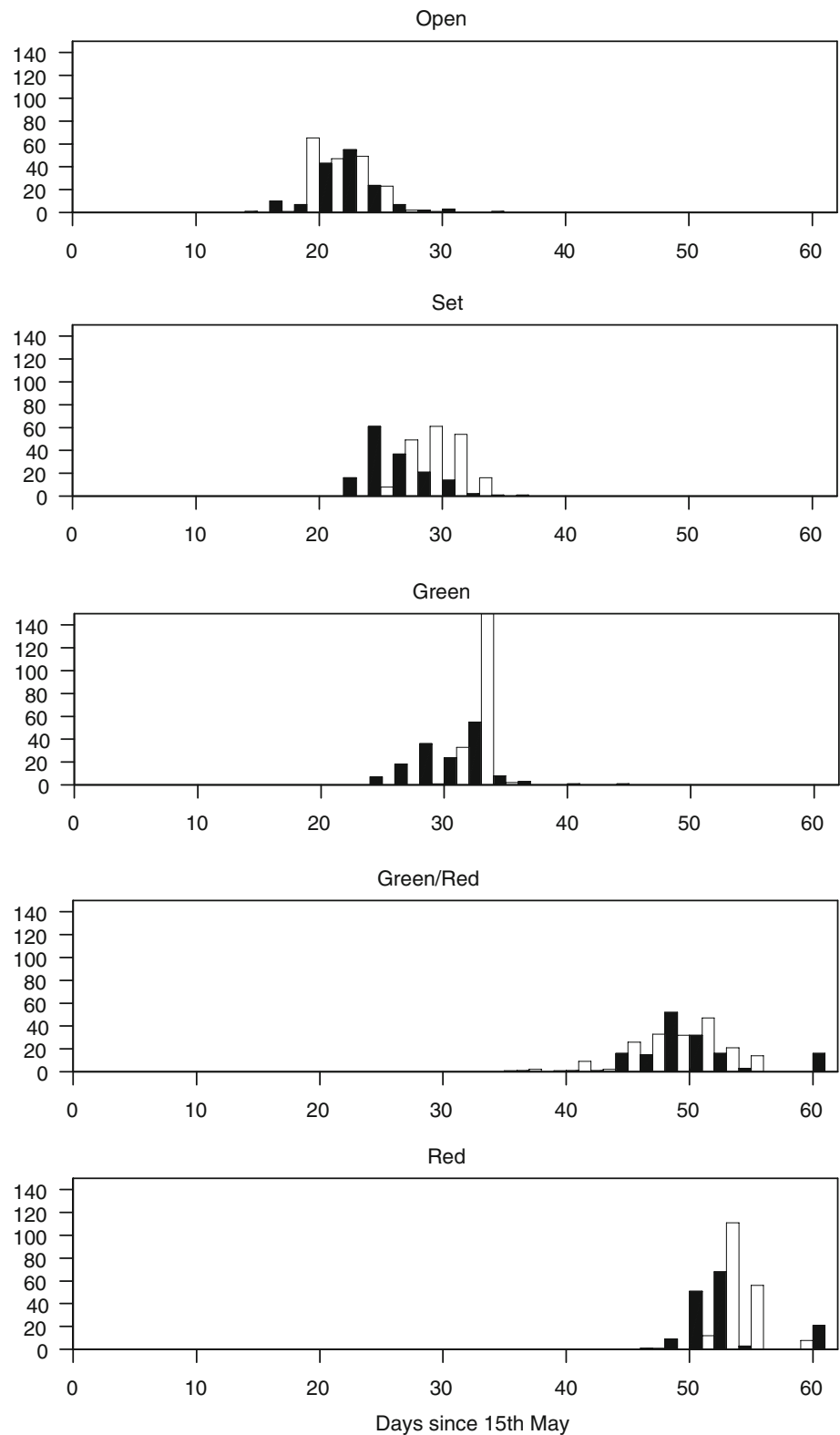
plants in field plots. Although fruit reach the green stage earlier under protection than in the field, more of the field grown plants reach the green fruit stage together. However, progression to the green/red fruit stage takes place over a

longer timescale in a field situation than in the polytunnel, but in both environments the time to reach red fruit is concentrated over a much shorter time period, with protected fruit ripening earlier. By 60 days some fruit in both trials

had not reached the red fruit stage and this appeared to be more pronounced in the polytunnel conditions.

QTL validation from trials in 2003–2005

As fewer lines were scored for ripening in 2003–2005, this data was used to validate the regions identified as associated with ripening from the larger 2006 trials. A marker regression approach was taken, selecting markers that were close to the peak of the QTL location and that were present for as many lines as possible to be used as test markers. The time to reach each ripening stage was estimated as above, and the line means were regressed on each selected marker in turn. In 2003, the time to reach open flowers (stage 2) differed significantly among the lines ($P = 0.003$), and the time to reach stages of fruit set, green/red and red fruit (stage 3, 5 and 6) were significant ($P < 0.001$). The time to reach green fruit, stage 4, was not significantly different among the genotypes. It seems likely that this represents a scarcity of observations during that period of fruit development rather than a difference in the genetic control. For 2004, the time to reach each of stages 2–5 was significantly different among the genotypes with $P < 0.001$, while for stage 6 (red fruit) the significance was $P = 0.002$. For 2005, a more detailed scoring of fruit colour was undertaken. There were significant differences ($P < 0.001$) in the time to reach each stage.

The markers identified as significant for the 2006 field or protected trials (Tables 3, 4) were generally significant in the earlier trials, and had effects in the same direction. Marker P12M121–194 on chromosome 2 was associated with a significantly earlier red stage in the 2006 field and protected trial, and there was weak evidence for this in 2003 ($0.05 < P < 0.1$). There was also weak evidence ($0.05 < P < 0.1$) of an effect of this marker on the variable lateprop in 2005, with fewer lines with the marker failing to reach the red stage. Gene H was significantly associated with later development in 2003 and 2005, mainly at the fruit setting and green stages. The *Ri*MADS_01 box gene on chromosome 5 was associated with later opening of flowers in 2006 and 2005, but earlier opening in 2004 and in the protected trial, although evidence for these effects was weaker ($P < 0.05$ and $0.05 < P < 0.1$ respectively). This was the only instance where the direction of the effect differed. ERubLR_SQ01_M20, a marker on chromosome 5 that segregated in a 1:2:1 ratio, was significantly associated with fruit colour development in 2005, with the bb homozygote earliest and the heterozygote intermediate. There was weak evidence ($0.05 < P < 0.1$) for an effect on the % open flowers in the 2006 protected trial. Marker P13M95-117R on chromosome 6 was associated with earlier development, especially the green and green/red stages, in 2003 and 2005 as well as in the 2006 field and protected trials.

The markers identified on chromosome 3 had effects with the same sign for each trait. In general more than one marker was significant for each trait. More complicated models, with more than one marker and possibly interactions between them, explained higher proportions of the variance. However, it was difficult to select a model that was broadly applicable for different years and stages. An inspection of the joint frequencies of the marker alleles showed that there were 37 lines with the genotype P13M55_98 absent, E41M31_153 absent, RUB223a present and E41M39_138 absent. This haplotype, denoted 0010, is derived from one homologous chromosome of Latham, without recombination over this range (or possibly with a double recombination between two of the markers). This was the most common haplotype among the offspring; other common types were the other non-recombinant haplotype 1101 (27 lines) and a recombinant type, 1100 (29 lines). The four markers spanned a region that includes the locations of *Ri*MYB and Bac9022SSR01, but these were not as good predictors as the selected markers.

Haplotype 0010 was found to be consistently associated with early development. The last columns of Table 3 shows the effect of an indicator variable (equal to 1 for this haplotype, 0 otherwise) and the % variance explained. This was usually as good as the most significant marker, and often better. In the 2006 field and protected trials, and also in 2003, lines with this haplotype typically had open flowers 1.5 to 2 days before the other haplotypes and this persisted through fruit set and green fruit. In 2004 and 2005, the difference between lines with this haplotype and others was greater.

Discussion

This analysis has allowed the stages of fruit ripening and cane height to be associated with regions on the raspberry genetic linkage map. The identified markers were significant across years and across different environments. Both field and polytunnel populations have generally similar results, although heritabilities are lower in the polytunnel population. This is in line with breeder observations of raspberry and blackberry varieties grown under both field and tunnel, where the protected environment appears to minimize genotype effects that influence plant behavior in a field setting (N. Jennings, personal communication). Clear differences in the timing of stages of fruit development and ripening under the two environments were determined with time to fruit set, green fruit and red fruit being earlier under protection (Fig. 3).

The ripening process was assessed by repeated ordinal scores for each plot, and so an interval mapping approach to locate QTLs for the scores on each date in turn would

Table 3 Effects of markers on chromosome 3. The final two columns show the effect and % trait variance explained by one haplotype (P13M55_98 absent, E41M31_153 absent, RUB233a present and E41M39_138 absent)

	P13M55–98	R^2	E41M31–153	R^2	Rub233a	R^2	E41M39–138	R^2	I_{Hap}	R^2
2006, F, open	1.6 (.33)***	10.3	1.1 (.34)**	5.2	−1.1 (.34)**	5.2	NS	–	−1.8 (.42)***	9.4
2006, F, set	0.9 (.26)***	6.2	1.0 (.26)***	8.0	−1.1 (.26)***	8.2	1.0 (.26)***	6.3	−1.7 (.31)***	13.5
2006, F, green	0.4 (.13)**	3.9	0.4 (.12)**	5.2	−0.5 (.12)***	7.8	0.5 (.13)***	7.4	−0.7 (.15)***	10.4
2006, F, green/red	1.2 (.51)*	2.5	1.8 (.53)***	5.6	−1.6 (.43)**	4.0	2.6 (.53)***	11.0	−2.7 (.64)***	9.2
2006, F, red	0.4 (.17)*	2.4	0.4 (.18)*	2.1	−0.3 (.18)‡	1.0	NS	–	−0.4 (.22)*	1.8
2006, P, open	1.6 (.46)***	6.6	1.7 (.46)***	7.8	−2.2 (.45)***	13.8	NS	–	−2.1 (.56)***	8.4
2006, P, set	1.0 (.40)*	3.1	1.5 (.39)***	8.3	−1.8 (.39)***	12.0	NS	–	−1.9 (.48)***	9.2
2006, P, green	1.1 (.47)*	3.1	1.3 (.47)**	4.4	−1.5 (.47)**	5.6	NS	–	−1.8 (.58)**	5.8
2006, P, green/red	1.1 (.46)*	3.5	1.5 (.46)***	7.2	−1.0 (.48)*	2.3	NS	–	−2.1 (.53)***	10.4
2006, P, red	0.6 (.20)**	5.7	0.5 (.21)*	4.2	NS	–	NS	–	−0.7 (.24)**	6.0
2005, open	NS	–	0.2 (.09)‡	2.9	−0.2 (.09)*	5.7	NS	–	−0.3 (.12)*	6.0
2005, set	NS	–	0.3 (.16)‡	2.3	−0.4 (.16)*	5.1	NS	–	−0.5 (.20)*	5.7
2005, green	8.9 (2.43)***	12.2	9.5 (2.37)***	13.8	−7.4 (2.47)**	8.1	NS	–	−10.3 (2.94)***	11.3
2005, green/red	3.5 (1.09)**	9.2	3.9 (1.05)***	12.1	−3.5 (1.07)**	9.5	NS	–	−5.6 (1.25)***	17.7
2005, yellow	4.8 (.84)***	26.2	4.8 (.83)***	26.2	−3.8 (.89)***	15.6	NS	–	−5.9 (1.01)***	27.5
2005, pink	5.0 (.81)***	28.8	4.7 (.82)***	25.7	−2.6 (.88)***	14.3	NS	–	−6.1 (.98)***	30.1
2005, red	4.7 (.76)***	29.5	3.8 (.79)***	19.8	−2.9 (.85)***	10.6	NS	–	−6.0 (.90)***	33.5
2004, open	NS	–	NS	–	NS	–	2.0 (.94)*	3.6	−2.2 (1.18)‡	2.8
2004, set	6.6 (1.8)***	12.6	4.5 (1.8)*	5.4	−3.3 (1.83)‡	2.3	3.2 (1.87)‡	2.2	−5.7 (2.23)*	5.9
2004, green	5.1 (1.2)***	15.1	3.4 (1.3)**	6.4	−2.2 (1.29)‡	2.0	NS	–	−6.8 (1.47)***	18.7
2004, green/red	1.7 (.43)***	14.4	1.2 (.44)**	6.6	−0.9 (.45)‡	2.7	NS	–	−2.0 (.53)***	13.8
2004, red	0.8 (.23)**	10.1	0.5 (.24)*	4.4	NS	–	NS	–	−0.9 (.28)**	8.6
2003, open	1.4 (.69)*	3.4	NS	–	−1.5 (.68)*	3.8	1.6 (.69)*	4.7	−2.6 (.81)**	9.6
2003, set	1.4 (.63)*	3.9	1.4 (.62)*	4.6	−1.9 (.61)	9.0	1.3 (.64)*	3.6	−2.7 (.72)***	13.4
2003, green	NS	–	NS	–	NS	–	NS	–	−1.0 (.50)‡	3.1
2003, green/red	NS	–	NS	–	−1.5 (.78)‡	2.8	NS	–	−2.1 (.92)*	4.7
2003, red	1.8 (.86)*	3.6	NS	–	NS	–	NS	–	−2.7 (1.01)**	6.7
2006, F, lateprop	.12 (.040)**	4.4	.11 (.040)**	3.5	−.08 (.041)‡	1.4	.10 (.042)*	2.9	−.17 (.050)***	5.8
2006, P, lateprop	.22 (.057)***	8.7	.31 (.053)***	18.0	−.24 (.057)***	10.4	.12 (.062)*	2.0	−.34 (.066)***	15.7
2005, lateprop	.21 (.053)***	13.4	.18 (.054)**	9.8	−.15 (.055)**	6.5	NS	–	−.28 (.064)***	17.7
2006, P, open %	−7.5 (3.13)*	3.1	−8.8 (3.10)**	4.5	12.7 (3.04)***	10.1	NS	–	14.7 (3.55)***	10.5

Estimated effects of the selected markers on the times (in days) to reach each ripening stage (open, set, green, red/green, red) in the Glen Moy × Latham mapping population. For the 2006 trial, *F* field trial and *P* protected trial. In 2005 and 2006 the proportion not reaching the red stage is shown (lateprop). '2006 P open%' is the extra assessment of the % open flowers in the 2006 polytunnel trial (on 13 June). The marker effect is the mean difference between the genotypes with and without the allele giving rise to a band, with the standard error in *brackets*

***, **, *, ‡ indicate effects that are significant with $P < 0.001$, 0.01, 0.05 and 0.1 respectively. *NS* not significant i.e. $P > 0.1$. R^2 = % trait variance explained

For the 1:2:1 marker ERubLR_SQ01_M20, the difference between the homozygotes is shown; the heterozygote had an intermediate value

be inappropriate for the ordinal nature of the scores. An analysis of the separate scores also would have an uncertain experimentwise type-I error because of the dependence between successive scores. Our main interest was in the main features of the ripening process and their genetic control, and therefore QTLs for the ripening process were located using two complementary summary statistics derived from the scores. The first approach was based on a principal coordinate analysis of the similarities of the plots. The ordinal scores were converted to a similarity matrix using a city-block measure, which used the ordered nature of the scale. The principal coordinates represented the variation in the similarity matrix in a small number of coordinates, which were tested for significant genetic variation. The fact that the principal coordinates are uncorrelated makes them easier to interpret than the original scores. A similar approach was used by Weller et al. (1996).

Table 4 Effects of markers not on chromosome 3

Marker	Trait	Effect (SE)	R^2
P12M121-194, chr 2	2006, F, red	−0.4 (.18)**	3.5
	2006, P, red	−0.5 (.21)*	2.8
	2003, red	−1.6 (.90)‡	2.4
	2006, F, lateprop	−0.22 (.038)***	15.5
	2005, lateprop	−0.10 (.059)‡	2.3
Gene H, chr 2	2006, F, open	1.0 (.34)**	4.3
	2006, F, set	0.6 (.26)*	2.1
	2006, F, green	0.4 (.13)**	3.9
	2006, F, green/red	1.8 (.53)***	5.2
	2006, P, set	1.0 (.40)*	3.2
	2006, P, green	1.0 (.47)*	2.2
	2005, set	0.4 (.16)*	5.1
	2003, set	1.6 (.62)	5.6
	2003, green	1.2 (.39)**	8.9
	2003, green/red	1.5 (.77)‡	3.0
	2003, red	1.5 (.88)‡	2.2
	2005, lateprop	0.13 (.056)*	4.3
*Ri*MADS_01, chr 5	2006, F, open	1.28 (.34)***	6.7
	2006, F, set	1.25 (.26)***	11.4
	2006, F, green	0.50 (.13)***	7.8
	2006, F, green/red	1.22 (.55)*	2.1
	2006, P, open	−0.89 (.48)‡	1.6
	2005, open	0.2 (.09)*	3.9
	2004, set	−3.8 (1.84)*	3.6
ERubLR_SQ01_M20, chr 5	2006, F, open	−2.8 (.48)***	17.5
	2006, F, set	−1.5 (.39)**	7.6
	2006, F, green	−0.6 (.20)**	5.7
	2005, green/red	−3.8 (1.56)*	5.1
	2005, yellow	−3.7 (1.28)**	10.9
	2005, pink	−3.4 (1.27)**	10.5
	2005, red	−2.8 (1.22)*	5.3
	2005, lateprop	−.20 (.078)*	11.9
	2006, P, open %	10.4 (4.91)‡	2.5
P13M95 − 117R, chr 6	2006, F, set	−0.8 (.26)**	4.6
	2006, F, green	−0.4 (.13)**	4.1
	2006, F, green/red	−2.5 (.52)***	10.6
	2006, P, green/red	−1.0 (.46)*	3.0
	2005, open	−0.3 (.09)**	7.5
	2005, set	−0.5 (.16)**	10.1
	2005, green	−7.19 (2.47)**	7.5
	2005, green/red	−2.6 (1.10)*	4.8
	2003, set	−1.3 (.63)*	3.7
	2003, green	−1.1 (.40)**	6.4
	2003, green/red	−4.0 (.67)***	27.7

See Table 3

This approach was useful for locating QTLs, but it was hard to interpret the QTL effects in terms of the original ripening stages. Therefore the second approach was based on interpolating the time of each plot to reach each stage of ripening. These variables were correlated, but provided a natural interpretation of the QTL effects in terms of the difference due to the presence/absence of a marker in the number of days to reach a given ripening stage.

QTLs for the ripening stages were identified across four chromosomes 2, 3, 5 and 6. Each of the groups had markers that had a significant effect at various ripening stages. On chromosome 2 the presence of the AFLP marker P12M121-194 (segregating in Latham) is associated with a shorter time to red fruit, while the *Hh* genotype of Gene H (segregating in Glen Moy) was associated with a slowing down of ripening across all stages from open flowers to the green/red stage compared to the *hh* genotype. This is interesting as Gene H is known to determine cane pubescence (genotype *HH* (lethal) or *Hh*), the recessive allele of which gives glabrous canes (genotype *hh*). Hairs and spines are both outgrowths of epidermal cells and their early development is inter-related (Peitersen 1921). It would therefore seem likely that Gene H acts early in development and affects several cell characteristics. Raspberry progeny with fine hairs (pubescent canes) (*Hh*) have been shown to be associated with resistance to cane botrytis (*Botrytis cinerea* Pers:Fr) and spur blight (*Didymella applanata* (Niessl) Sacc.) as well as spines (Graham et al. 2006). Resistance to *B. cinerea* and *D. applanata* is highest in immature tissues and Williamson and Jennings (1992) have postulated that the gene increases resistance by delaying cell maturity. However, this delay may reduce resistance to yellow rust (*Phragmidium rubi-idaei* (D.C.) Karst. Syn *P. imitans* Arth.) and cane spot (E*lsinoe veneta (*Borkh.*)* Jenkins) as these fungi only invade immature tissues. This postulated delay appears to have an impact on ripening. This region around Gene H in raspberry is the focus of current research efforts due to its importance in many traits of interest.

Chromosome 3 had a strong effect on ripening with a broad range of significant markers identified suggesting more than one QTL was involved. One haplotype of the Latham parent was identified as consistently early. A QTL for height was also identified on chromosome 3, but this lay outside the region of markers significantly associated with ripening. A number of ESTs and candidate genes fall within the region of the mapped QTLs on chromosome 3. Several ESTs were associated with PCO1 and with the early stages of fruit development from open flowers to green fruit and include ERubLR_SQ07_4_D05, which is similar to a cDNA of unknown function from apple leaf, ERubLR_SQ07_1_E10 (similar to a petal EST from *Rosa*) and ERubLR_SQ01_P18 (similar to *PpAz89* a pathogenesis-related (PR) EST from *Prunus* which is up-regulated during flowering, fruit ripening and during senescence in flowers, fruit and leaves (Ruperti et al. 2002)). ERubLR_SQ01_P18 is also significantly associated with time to reach ripe fruit.

Also associated with the QTL are the *Ri*MYB gene and a BAC clone, Bac9022_SSR01. BAC Ri9O22 contains a MYB gene which was most similar to MdMYB11 from *Malus* x *domestica* (Accession No. AAZ20431). R2R3 MYB genes are known to control phenylpropanoid metabolism including anthocyanin accumulation in fruit (Allan et al. 2008; Espley et al. 2007) but also have important roles in many other aspects of plant development including trichome development, signal transduction, disease resistance and cell division (reviewed by Jin and Martin 1999).

Chromosome 5 had two separate markers that significantly affected the rate of ripening, ERubLR_SQ01_M20 (which segregates in both parents), an EST associated with flower development and a MADS box gene, *Ri*MADS_01 (which segregates in Latham). These genes are known to regulate the transition from vegetative to reproductive development (Danilevskaya et al. 2008) as well as the specification of flower meristem and flower organ identity (see review by Soltis et al. 2007). In addition, MADS-box genes regulate fruit (Vrebalov et al. 2002), pollen (Verelst et al. 2007) seed (Wang et al. 2008), embryo (Colombo et al. 2008), leaf and root development (Tapia-Lopez et al. 2008). The raspberry *Ri*MADS_01 gene was most similar to dam6 (Accession No. ABJ96360; BlastX: 51% identity; E value: 8e–28) from *Prunus persica*, one of six MADS box genes located in a cluster in a 132 kb region and thought to be responsible for regulating growth cessation and terminal bud formation in peach in response to dormancy-inducing conditions (Bielenberg et al. 2008). The gene is also similar to short vegetative phase (SVP) MADS box genes which act to negatively control the expression of the *FLOWERING LOCUS T* (*FT*) modulating the timing of the developmental transition to flowering phase in response to temperature (Lee et al. 2007). This has implications for modulating flowering time in response to changing environments. No functional genes or ESTs have been mapped into the ripening QTL on chromosome 6.

This work has provided an insight into ripening and the regions of the genome likely to be important in determining variability. Some candidate genes have been identified and others will be investigated for co-locating with identified QTLs. Growing conditions have a large effect on the ripening process in raspberry, including anthocyanin pigments (Kassim et al. 2009) and considerable UK raspberry production is now under protective cultivation to provide fruit of premium quality with reduced pesticide inputs. This method of production yields raspberry fruit 10% larger than fruit grown in the field (S. McCallum, personal communication) an effect also seen in apples grown under protected conditions and which may be due to better irrigation (Atkinson et al. 1998). Protected cultivation also has the effect of minimizing genetic variation across different genotypes. For example the heritability of the overall ripen-

ing process (as assessed by PCO1) in a field environment is 70% but in a polytunnel this is reduced to 40%.

The work has also allowed the identification of genetic markers associated with early or late bud break and short or long ripening periods as well as marker combinations associated with short and tall raspberry progeny. Cane height was mapped to chromosome 3 and chromosome 5 with another region on chromosome 1 having an epistatic interaction with chromosome 3. Markers have been identified with significant effect on height and these will be validated and if successful deployed in the raspberry breeding programme in the future. Identifying shorter caned genotypes in the early stages of selection programmes is desirable due to the difficulty in harvesting fruit from taller plants and the greater management input required to control plant height.

Environmental changes are predicted to occur over the coming years and the availability of markers for the ripening process will allow us to identify germplasm that can respond to these changes. Wild raspberry germplasm from different environments is known to be phenotypically distinct from cultivated material and these differences are maintained under a common environment (Marshall et al. 2001). Ripening in wild raspberry germplasm occurs later than cultivated germplasm with ripening time associated with altitude (Graham et al. 1997, 2003). The availability of germplasm differing significantly in ripening period will allow us to examine key genes/markers in a wide gene pool. As more functional (EST) rather than anonymous markers are added to the genetic linkage map and further raspberry BAC clones are identified in the regions of interest, our knowledge of the genes involved in the processes affecting key developmental traits in raspberry will improve, leading to the development of robust markers for future breeding priorities.

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