Project title:	Developing breeding and selection tools to reduce spoilage of soft fruit and wastage in the supply chain.
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Biomathematics and Statistics Scotland (BioSS)

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Thomas Thomson (Blairgowrie) Ltd.

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Delta-T Devices Ltd.

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Marks and Spencer Group plc (M&S)

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Redeva Ltd.

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Horticultural Development Company (HDC)

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The Scottish Government.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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GROWER SUMMARY

Headline

- The project delivered for the first time, a protocol to quantitatively measure fruit firmness as faced in the supply chain, generated a ‘fruit transcriptome’ dataset that can be mined for candidate genes associated with fruit quality, developed a raspberry fruit transcriptional microarray to study gene expression, and identified key genes controlling fruit softening for a marker assisted selection program.

Background

Fresh fruit accounts for a market of £4 billion in the UK and berries account for 18.4% of this total. Projections are for a 50% increase in sales of raspberry fruit if high quality varieties are available. Demand for UK grown fruit is increasing dramatically and at present cannot be met within the UK, so there is scope for the continued expansion in UK production. Fruit softening remains the main cause of post harvest waste and lost revenue in all soft fruit.

Fruit firmness is essential to maintain quality, enabling fruit to withstand storage time, transport across the UK and the 7 days of shelf-life demanded by supermarkets. A 1-2 days improvement in fruit shelf-life would increase the value of harvested fruit and reduce waste. The UK produces around 17 million tonnes of food waste to landfill each year with perishable fruit and vegetables forming a large part of this. Soft fruit losses has been valued at aproximately at £50 m (Sustainable Development Company) consisting of an estimated 20% of the class 1 fruit depending on the season.

There is a unique opportunity to identify the genetics of fruit softening in raspberry by using a 'Latham' (soft fruit) × 'Glen Moy' (firm fruit) reference mapping population (Graham et al., 2004), which is already an established and successful resource for Quantitative Trait Loci (QTL) mapping (identification of regions on chromosomes responsible for trait variation).

The 'Latham' × 'Glen Moy' raspberry genetic map, the gene resources previously developed and the latest molecular tools (454 mRNA-seq, microarrays, genotyping, and alternative splicing panels) were used to identify and investigate the expression of important fruit softening genes to develop robust genes/markers linked to softening to improve the speed and precision involved in the development of new cultivars with improved fruit firmness.

Key cell wall modifying enzymes have a significant impact on the degree and speed of the fruit softening process; β-galactosidase and expansin genes act early and may restrict or control the activities of other ripening-related hydrolases including polygalacturonases (PG), pectinmethylesterases (PME), endo-1,4-□β-□glucanases, xyloglucan endotransglycosylases and pectate lyases. Fruit development and subsequent softening relies on the co-ordinated temporal expression of fruit specific genes.

Environmental stresses have a significant impact on gene transcription and alternative splicing events (which may lead to a single gene coding for multiple proteins) and may have an important role in fruit softening. Studying the processes involved in gene expression will improve our understanding of the response of raspberry genotypes to the environment. New technologies are expanding our ability to study and test the role of transcription and post-transcriptional processes in many different tissues grown under different conditions. For example, 454 mRNA-sequencing allows a large population of mRNA sequences to be identified (thousands of gene sequences) in selected raspberry fruit. This will give the most complete list of genes that are expressed during fruit development.

This study will aim to identify key genetic and environmental response components involved in raspberry fruit softening and generate a more complete understanding of the fruit softening process. A relevant method of measuring softness that mimics stresses in the supply chain will be developed in collaboration with the industry partners. These phenotypic traits will be assessed in association with the development of robust DNA markers to use as selection tools for traits in new seedling selections. Markers will help accelerate the development of new raspberry and potentially other soft fruit varieties with extended shelf-life and concomitant reduction in fruit spoilage and waste in the supply chain.

Summary

This Horticulture LINK project aimed to develop robust assisted breeding and selection tools that would enable breeders to accelerate development of new fruit varieties with extended shelf-life to reduce fruit spoilage.

In order to achieve the aim of the project six objectives were established:

Objective 1. Identify map locations for softening phenotypes (QTLs) from phenotypic analyses that mimic stresses in the supply chain.

Raspberry firmness was measured quantitatively for the first time using a QTS-25 Texture Analyzer by testing ripe fruit collected from both field and polytunnel production over two years. This study validated the QTS-25 Texture Analyzer as a reliable quantitative measurement of fruit firmness that is comparable with a 'breeder score' for firmness and can be reliably used to identify chromosomal regions for trait (phenotype) information.

Fruit firmness and mass trait data was added to the existing *Rubus* genetic linkage map by QTL mapping analysis. QTS-25 compression parameter measurements for Hardness, Rigidity, Final load, and Force/Mass_N/kg, together with the mass and breeders score were significantly heritable and notable QTLs were located on linkage groups (LG) 1, 3, and 5. This allowed the regions on the seven raspberry chromosomes (or linkage groups) associated with these traits to be located and the genetic markers (genes) associated with them to be identified.

The QTS-25 Texture Analyzer was successfully used to study firmness and shelf-life characteristics of ripe fruit through the supply chain to identify significant genotypic differences in firmness during storage. Twenty-two different mapping population progeny and five different varieties (Glen Moy, Latham, Glen Ample, Octavia and Tulameen) showed a range of 'firmness' scores indicating genotypic differences; Glen Moy, Octavia and 7 mapping progeny were consistently firmer during storage for 7 days at 4^{o}C, indicating a better shelf-life.

The 'shock forces' encountered by punnets of raspberry fruit during transport was recorded for the first time using a Tinytag shock logger (Gemini Data Loggers (UK) Ltd.). Results indicated significant vertical forces (up to 8 'g') but not horizontal forces were experienced during transport from the field roads to the packhouse. A laboratory trial to mimic 'shock forces' experienced by fruit during transportation indicated that the shock treatment had a more pronounced impact on storage of a 'firm' variety (Octavia) compared to a 'soft' variety (Tulameen), indicating that as well as firmness, the size, shape and flexibility of the fruit may play an important role in potential shelf-life characteristics.

Recommendation:

- Cultivars with a 'breeder score' of firmness from 1-2 (equivalent to hardness readings >0.6 Newtons) are more likely to maintain the desired quality.

- Requirement for a more comprehensive monitoring of ‘shock forces’ on fruit during transport to predict the potential impact on shelf-life.

Objective 2. Carry out large scale sequencing of genes expressed in ripening raspberry fruit.

- A comprehensive sequence database of raspberry fruit-related genes transcribed was successfully generated using next generation sequencing technology (454 mRNA-seq) from total RNA extracted from white/red and red fruit stages of Latham and Glen Moy. The raspberry ‘fruit transcriptome’ covers over 350 Mbp and contains over 23,000 sequence contigs with more than a single sequence read.
- Candidate genes with expected roles in cell wall hydrolysis, water movement, fruit ripening and cell wall flexibility were identified in the raspberry ‘fruit transcriptome’ database.
- The ‘fruit transcriptome’ database forms the sequence base for the development and expansion of a raspberry fruit transcriptional microarray.
- Added value: This sequence database can be mined for genes involved in many different fruit quality traits and fruit development in addition to fruit softening genes.

Objective 3. Identify sequence polymorphisms in at least 8 named genes and other fruit-related genes such as MADS box genes (Obj. 2) implicated in fruit ripening/softness.

Over 20 candidate genes with expected roles in the fruit softening process were identified using in-house (*Rubus*) public sequence and the raspberry ‘fruit transcriptome’ datasets. Sequence polymorphisms (insertions/deletions [indels] or single nucleotide polymorphisms [SNP]) in these genes were validated and then used to screen the parents and 188 progeny from the ‘Latham’ x ‘Glen Moy’ mapping population.

- An indel or SNP was validated in each of the identified genes with expected roles in cell wall hydrolysis and mapped onto raspberry linkage groups.

Objective 4. Generate an updated linkage map with fruit-related genes and identify genes that co-locate with softening QTLs.

This objective associated the phenotyping data from the 'Latham' × 'Glen Moy' mapping population using the ‘breeder score’ and the QTS-25 Texture Analyzer measurements (Obj.1) with the sequence polymorphisms within selected candidate genes (Obj.3).

An updated *Rubus* linkage map was completed using the JoinMap programme for 19 different genes associated with cell wall hydrolysis/modification (Obj.3) indicating segregation of these new markers within the population. These candidate genes were distributed across all 7 *Rubus* linkage groups, with the majority located on LG 3, 5 and 7.

MapQTL mapping software and the Kruskal-Wallis statistical test indicated that the most significant markers (linked to either cell wall hydrolysis, water movement, fruit ripening and cell wall flexibility) associated with each of the softening QTLs on certain linkage groups were:

- LG1, Aquaporin;

- LG3, Pectinmethylesterase (PME),
  S-Adenosylmethionine decarboxylase (SAMDC),
  Constitutive triple response1-like protein kinase (CTR1; a negative regulator of the ethylene response pathway),
  Zinc finger protein/transcription factor (Zf/TF; similar role as MADS box genes),
  Isopentenyl pyrophosphate isomerase (IPPI),
  Aconitase.

- LG5, β-1,4 xylan hydrolase (XL).

There was one softening QTL each located on LG1 and LG5, whereas 6 QTL’s were identified on LG3.

Objective 5. Study gene expression profiles and potential alternative splicing events in key softening genes (Obj. 4).

Fruit development and subsequent softening relies on the co-ordinated temporal expression of fruit specific genes. Gene expression is the process whereby genes are transcribed into a message RNA (mRNA) and translated into functional proteins, such as enzymes, that show

in the field as phenotypic traits. Alternative splicing occurs in plants and is a process that splices together different mRNA sequences transcribed from the same gene, leading to changes in protein structure and function at the individual gene level.

Development of raspberry RT-qPCR assays

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) is the method of choice to quantify differences in individual gene expression levels between messenger RNA (mRNA) samples. It is a highly sensitive technique that requires validation at several steps to assure accurate and reliable results. This project developed high quality (gold standard) standard operating procedures (SOP) for analysing raspberry soft fruit expression.

- Developed a raspberry fruit RNA extraction procedure incorporating use of a TissueLyser (Qiagen; mixermill) for disruption and extraction by RNeasy Plant Mini Kit (Qiagen) and automated QIAcube robot system (Qiagen).
- QuantiTect® Reverse Transcription Kit (Qiagen) was selected for converting fruit extracted RNA into cDNA.
- A robust pipeline for designing and evaluating PCR assays for RT-qPCR experiments was developed:
    - an extracted RNA integrity check,
    - optimal design of PCR primers and probes for RT-qPCR assays,
    - optimal primer and probe concentrations with PCR efficiencies >80%,
    - screen of 10 reference genes for transcript normalization in *Rubus* and validated by geNorm software identified the novel Clathrin, YLS8, and TIP41 as raspberry fruit genes of choice for stable expression across all fruit stages,
- Pipeline suitable for other soft fruit species.

Gene expression profiles

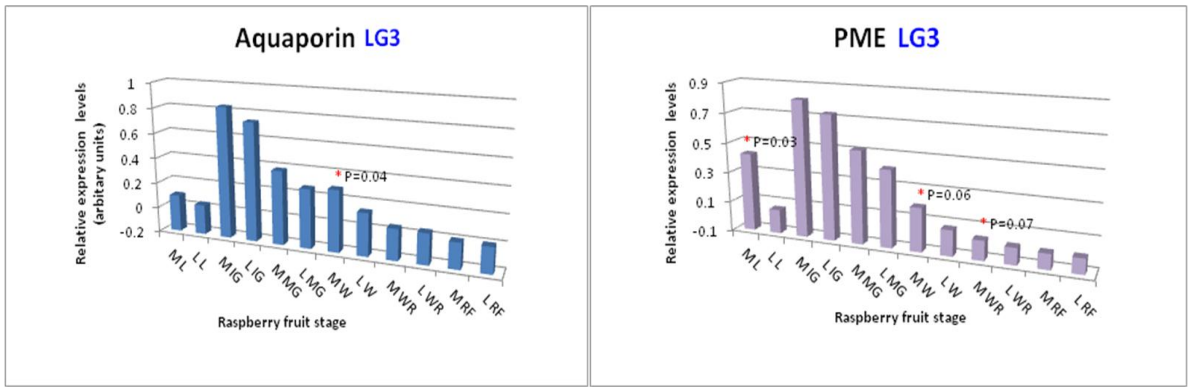
The raspberry RT-qPCR pipeline was used on different stages of raspberry fruit (Fig. A) from three biological replicates of Glen Moy, Latham, Octavia, Tulameen, Glen Ample, and several clones (one biological replicate) of the mapping population representing ‘soft’ and ‘firm’ fruit categories.

Figure A. Different stages of raspberry fruit harvested for gene expression profiles.
Key: IG, immature green; MG, mature green; W, white fruit; WR, white/red fruit; RF, ripe fruit.

The assays for 11 candidate genes, PME, XL, Aquaporin, SAMDC, polygalacturonase (PG), pectate lyase (PL), pectinmethylesterase inhibitor (PME*i*), CTR1, Zf/TF, IPPI, and Aconitase were selected for gene expression studies and the data normalized.

Raspberry fruit RT-qPCR gene expression analysis revealed:

- Relative expression levels over the fruit developmental stages indicated genotypic differences between different cultivars (Glen Moy, Latham, Octavia, Tulameen, Glen Ample) (e.g., Fig. B) and the pooled 'hard' and 'soft' category mapping population clones.
- Expression analysis, scatter plots and correlation data indicated coordinated expression between several of the candidate genes during fruit development and ripening which related to variation of fruit firmness. Strong negative relationships were detected between SAMDC and Aquaporin, SAMDC and PME, XL and Aquaporin, and between XL and PME. Strong positive relationships were also found between PME and Aquaporin, and between XL and SAMDC (Fig.B).



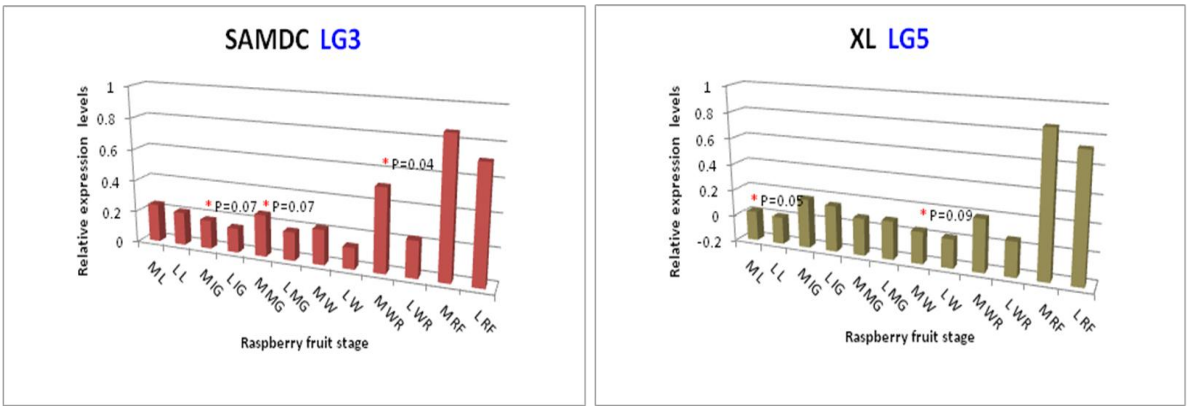


Figure B. Mean normalized relative gene expression levels obtained for selected candidate genes significantly associated with softening QTLs in different stages of fruit and leaf in Glen Moy (M) and Latham (L).
Key: L, leaf; IG, immature green; MG, mature green; W, white fruit; WR, white/red fruit; RF, ripe fruit. *P* level of significance is indicated on histograms.

PME enzymes play an important role in cell wall disassembly during fruit ripening by increasing the internal susceptibility of pectins to hydrolases. The data shows high expression of PME at the early stages of fruit development and subsequent decrease that coincides with increased levels of PME*i*, followed by increased levels of PL and then PG enzymes. Inhibition of PME activity also coincided with increased levels of SAMDC and XL. SAMDC shows a positive correlation with levels of XL, a common enzyme which hydrolysis glycosidic linkages in the most prominent structural polysaccharides fractions (cellulose and hemicellulose). Together these enzymes may help co-ordinate the processes of fruit ripening with cell wall degradation.

Fruit cells regulate their turgor pressure as well as cell wall integrity as they ripen and this requires aquaporins, which regulate water flow and turgor pressure. A positive correlation between expression levels of PME involved in cell wall integrity and an increase in aquaporins and water movement as the fruit develops and expands should allow more exposure of substrates to the actions of hydrolases.

Development of a Rubus transcriptional microarray

The large amount of sequence information produced after next generation sequencing (Obj. 2) allowed the development of a new 55k uniprobe *Rubus idaeus* fruit transcriptional microarray using the Agilent dual mode gene expression platform. This array allowed us to detect and monitor transcriptional changes throughout fruit development between different sample tissues. An initial screen of fruit from Moy and Latham identified 36,000 (65%) of the

55k probes showed an expression signal, and analysis of variance indicated that there are substantial numbers (1000s) of significant gene expression changes (up and down regulation of genes) and genotype differences within the experiment. This data set remains to be mined and gene expression levels validated.

Alternative splicing in fruit ripening genes

Alternative splicing is recognised as a key post-transcriptional process that modulates and regulates the levels of mRNA transcripts prior to translation into proteins. Varietal differences in strawberry lead to an alteration in alternative splicing in the polygalacturonase (PG) gene which is associated with variation in fruit firmness. Alternative splicing events were found in several of the raspberry fruit softening genes (Aquaporin, SAMDC, PME, PG and PL) in Latham and Glen Moy during fruit development. However, in all cases, no significant changes in alternative splicing ratios in developing raspberry fruit were detected. Nevertheless as transcription levels increased at different stages in the fruit the alternatively spliced product also increased to levels that may suggest an alternative function.

Assessment of alternative splicing identified transcription of two SAMDC alleles in Latham compared to transcription of a single allele in Moy. This indicated a doubling of SAMDC mRNA transcripts in Latham, which may translate into higher levels of translated SAMDC and contribute to reduced firmness in the softer variety Latham compared to Moy. This will be further validated as a potential marker for the ‘soft’ phenotype (Obj. 6).

Irrigation stress trial 2011-2012

Environmental conditions such as water stress are thought to exacerbate raspberry fruit softening. Over-watered and drought treated raspberry plants were evaluated against standard watering conditions in a field experiment consisting of a row of replicate pots of raspberry mapping population clones (various firmness levels) plus the parents Latham and Glen Moy under a polytunnel.

Analysis of variance revealed that there was a significant difference ($P<0.001$) in water content (% Vol) in pots between the three watering regimes monitored (standard, over-watered, and drought) demonstrating the improved reliability and consistency of the SM300 (Delta-T Devices Ltd.) sensor-based system to control irrigation *via* data loggers compared to automated irrigation.

Gene expression analysis on immature green and mature green stages collected during the three watering regimes in 2012 was performed using the established *Rubus* microarray.

This analysis resulted in the identification of many potentially important candidate genes involved in water-stress (e.g., plasma membrane proteins, major latex-like proteins, cysteine proteinases, and additional aquaporins) for future study.

Objective 6. Validate robust genetic markers and gene sequences (Obj. 5) by comparison with raspberry germplasm (and with other members of the Rosaceae and grape).

Fruit softening is a complex trait that relies on a combination and interaction between different physical and molecular processes involved in fruit ripening. A future priority is to develop the most reliable combination of markers for deployment in marker assisted breeding (MAB) for the 'soft' and 'firm' phenotype in red raspberry taking fruit resilience in transport into account.

We have established a number of key raspberry genes expressed during fruit development and identified indel and SNP variants in these genes between Latham (soft) and Glen Moy (firm). The markers accounting for the most significant impact on the phenotype will be combined and tested in additional raspberry germplasm and breeding populations to strengthen the association of these genes and markers with fruit softening.

The availability of robust markers associated with both fruit softening and fruit quality will lead to the identification of varieties that combine firmness with improved taste to all growers *via* the HDC breeding programme. The markers identified in this study will be tested in the HDC-funded raspberry breeding programme during 2013-14. This will be part of the validation process to determine the effectiveness of the markers at predicting fruit softening.

Many of the genes identified in this project may be important in the softening process for other *Rosaceous* soft fruit (and non-soft fruit species), providing added value as information on markers, genes and alternative splicing events that may be applied to other species.

Financial Benefits

- Fruit softening remains the main cause of post-harvest waste and lost revenue in all soft fruit with financial losses at the farm gate into six figures in a poor season.
- Depending on season, ~ 20% fruit is deemed unsuitable for consumption at various stages in the chain from grower farm, packaged in warehouses, on the supermarket shelf and finally in the home.
- Supermarket requirements for soft fruits vary day to day, and growers often need to store harvested fruit for an extra 24-48hrs. Fruit firmness is therefore essential to maintain quality, enabling fruit to withstand such extensions to storage time before transport across the UK.
- The reduction in perishable fruit as food waste to landfill in the UK will be significantly reduced.
- Financial savings in the retail industry alone could reach £2.5 million annually for soft fruit with additional savings at the farm.
- Increased shelf-life will further enhance the reputation of UK fruit as a high quality product.
- The majority of fruit consumed in the UK is imported and as demand for UK soft fruit outstrips supply, there is scope for the continued expansion in UK soft fruit production.

Action Points

- Markers associated with soft and firm phenotypes will be added to the raspberry breeding toolkit in the 2014 crossing season to speed up the development of new cultivars with desirable traits. These markers can also be licenced by MRS to other programmes if agreed by the consortium. These varieties once developed using marker assisted breeding will overcome the financial losses incurred by fruit softening.
- Allele mining in breeding germplasm will be carried out by the consortium breeder.
- Knowledge of the environmental impact on gene expression will enable guidelines to be developed for optimum fruit production and growers can use the information in determining watering regimes. Heat and overwatering have been identified as key causes of fruit softening and these can be considered by growers to improve the environmental impact on fruit quality.

- A web site, publications and articles based on the results will disseminate the information from the scientific partners and thus encourage future research collaborations.
- It is recommended that raspberry cultivars with a ‘breeder score’ of firmness from 1-2 (equivalent to Hardness readings >0.6 Newtons) are more likely to maintain the desired quality through the supply chain.
- This study also highlighted the need for a more comprehensive monitoring of ‘shock forces’ on fruit during transport in order to predict more accurately the potential impact on the shelf-life of fruit.

SCIENCE SECTION

Introduction

The characteristic, progressive loss of firmness associated with fruit ripening in raspberry is associated with a loss of skin strength, the separation of the drupelets from the receptacle and a breakdown of cell walls in the mesocarp (Sexton et al., 1997). Many cell wall changes are common to most ripening fruits and modifications to the polysaccharide components of cell walls are brought about by the co-ordinated, interdependent activities of many cell wall modifying enzymes that occur during fruit development and ripening (Brummell and Harpster, 2001).

The modifications to the polysaccharide cell wall architecture in fruit are brought about by the co-ordinated, interdependent activities of several cell wall modifying enzymes, some of which (e.g., β-galactosidase and ripening related expansin) may act early in the ripening process but also restrict or control the activities of other ripening-related enzymes such as polygalacturonases (PG), pectinmethylesterases (PME), endo-1,4-β-glucanases (Sexton et al., 1997; Ianetta et al., 2000), xyloglucan endotransglycosylases and pectate lyases (Jimenez-Bermudez et al., 2002; Santiago-Doménech et al. 2008) which are necessary for the fruit softening process (Brummell & Harpster, 2001). In a study of fruit from firm (Glen Prosen) and soft (Glen Clova) raspberry cultivars, it was established that the cell walls from softer fruit contained less ordered cellulose and less pectin than firmer fruit (Stewart et al., 2001). However, it is not clear whether there is a difference between the activities of specific cell wall hydrolases or if there is a different mechanism affecting the degree of cell wall modification in soft and firm raspberry varieties.

There is a unique opportunity to identify the genetics of fruit softening by using the 'Latham' × 'Glen Moy' mapping population, which is already an established and successful resource for Quantitative Trait Loci (QTL) mapping (identification of regions on chromosomes responsible for trait variation) for linking phenotype (plant traits) to genotype (plant genes). The progeny from this cross also segregate for fruit firmness/softness and so this population provides an excellent opportunity to map this quantitative trait on the evolving raspberry genetic linkage map.

A new generation of genomic tools (454 mRNA-seq, microarrays, genotyping, and alternative splicing panels) now exist to identify genes that are expressed in raspberry fruit. This work will identify key genetic and environmental response components involved in

raspberry fruit softening and generate a more complete understanding of the fruit softening process. Phenotypic traits will be assessed in association with the development of robust DNA markers that can be used as selection tools for breeders to assist accuracy of trait selection in new seedling selections. This will accelerate the development of new raspberry cultivars with extended shelf-life and concomitant reduction in fruit spoilage and waste in the supply chain.

The work is reported on the basis of six separate Objectives:

- Objective 1. Identify map locations for softening phenotypes (QTLs) from phenotypic analyses that mimic stresses in the supply chain.
- Objective 2. Carry out large scale sequencing of genes expressed in ripening raspberry fruit.
- Objective 3. Identify sequence polymorphisms in 8 named genes and other fruit-related genes such as MADS box genes (Obj. 2) implicated in fruit ripening/softness.
- Objective 4. Generate an updated linkage map with fruit-related genes and identify genes that co-locate with softening QTLs.
- Objective 5. Study gene expression profiles and potential alternative splicing events in key softening genes (Obj. 4).
- Objective 6. Validate robust genetic markers and gene sequences (Obj. 5) by comparison with raspberry germplasm (and with other members of the Rosaceae and grape).

Objective 1. Identify map locations for softening phenotypes (QTLs) from phenotypic analyses that mimic stresses in the supply chain.

Introduction

Quantitative trait loci (QTL) analysis is the study of genetic variation, location of genes and exploration of gene effects and interactions. QTLs are regions of DNA which correspond to a continuous phenotypic trait within a population, displaying a normal pattern of distribution which may be controlled by several genes of small effect or one or two genes conferring a large effect. Plant breeding aims to develop cultivars which fit specific environment and production practices and high yielding products whether for food or further processing. Understanding the genetic control of physiological traits and the linkage of these physiological characteristics to molecular markers on chromosomes, and ultimately the alleles(s) underlying the preferred phenotype of a trait is the future of plant breeding.

For map construction, individual marker loci are genetically scored in a segregating population and the recombination rate of alleles at loci can be determined using classical linkage analysis. Loci can then be ordered into a set of linkage maps (groups) and distance between loci in a linkage group (chromosome) can be expressed as recombination units, centiMorgans (cM), where one cM is equal to 1% recombination. Red raspberry (*Rubus idaeus*) is a good species for the application of such techniques, being diploid (2n = 2x = 14 chromosomes) with a very small genome (275 Mbp). All traits (characteristics) are controlled by genes and the combination of alleles (versions) of the genes in the plant is known as the genotype. Red raspberry is diploid and therefore can have two alleles of each gene. The ability to link the trait data to the linkage map and to genes involved in fruit ripening will allow the identification of QTL and subsequent association with candidate genes. Recent raspberry genetics research at The James Hutton Institute (JHI) has focused on the development of mapping populations under different environments, markers and a genetic linkage map that form the basis for linking phenotype to genotype, providing a tool to understand how traits are controlled and where variation exists at the gene level. The developments were based on a widely segregating cross between 'Latham' × 'Glen Moy' and have led to the development of an evolving genetic linkage map.

Materials and methods

1.1 Develop screening protocols for fruit firmness and shelf life and assess material from the 'Latham' × 'Glen Moy' cross.

The raspberry mapping population, as previously described (Graham *et al*., 2004; 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). In many experiments, use of a 188 progeny subset simplifies analyses.

Fruit softness was evaluated using two procedures. Firstly, fruit was assessed by the previously validated subjective ‘breeder score’ of firmness on the bush on a 1 - 4 (Firm – Soft) scale for the 188 progeny from the ‘Latham’ x ‘Glen Moy’ cross replicated in triplicate at an open field site and polytunnel site at JHI. Secondly, ripe fruit (15 berries) were collected in plastic punnets from one and two replicates from each of the field and polytunnel sites, respectively. The weight (mass) of 10 berries per progeny was measured and the softness was determined on 6-8 individual berries using 16 various compression parameter measurements (trait data) with a QTS-25 Texture Analyzer.

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 regions of the genome linked to each trait. Interval mapping and Restricted MQM Mapping were then carried out using MapQTL.

1.2 Understand stresses in the supply chain and how they relate to firmness scores.

(i) Storage of mapping population fruit

Preliminary trials were carried out with raspberries purchased from a supermarket and clones harvested at JHI in order to ascertain an appropriate length of storage time in the cold store (4^{o}C) for monitoring firmness and shelf-life characteristics. The main study used 22 different mapping progeny with a range of 'firmness' scores (high, medium, soft) plus both parents (Latham and Glen Moy). Ripe fruit was picked from three replicates on the same day and placed into labeled 40ml plastic punnets (single layer; identical numbers of fruit per rep) and temporarily stored in cold boxes until transport to a cold room (4^{o}C). Fruit was collected until two cold boxes were full to capacity with punnets. After collection in the polytunnel, the fruit was transported by van in the cold box for a period of 25 minutes *via* a set route (4 miles; 6.4 km) to simulate the transport process from a commercial grower's field to a packhouse, and hence, introduce impact damage effects on the raspberries. The 'hardness' of fruit (six individuals) for each progeny was measured in triplicate using the QTS-25 Texture Analyzer on the day of harvest (0 days), 2 days, 4 days, and after 7 days storage. Restricted Maximum Likelihood (REML; Mixed Modelling) was used to analyze the data sets.

(ii) Storage of commercial cultivars

Ripe fruit was also randomly picked from several plants each of cultivars Glen Ample, Octavia, and Tulameen at a commercial farm and placed into labeled 40ml plastic punnets (containing pre-weighed Whatman paper) to form a single layer. Four replicate samples were taken for each cultivar and punnets were temporarily stored in cold boxes. On return to the laboratory, the Whatman papers were carefully removed from each punnet and re-weighed to determine the amount of 'juice bleed' after transport from the field to the laboratory. The 'hardness' of fruit (six individuals) for each commercial variety was measured using the QTS-25 Texture Analyzer on the day of harvest (0 days), and then monitored after 4 and 7 days storage at 4^{o}C to determine the shelf-life characteristics.

The shock forces encountered by punnets of fruit during transport in a transit van were also recorded by using a Tinytag shock logger (Gemini Data Loggers (UK) Ltd.) at the same commercial farm. A punnet containing the shock logger was placed into a tray of punnets of freshly picked fruit from the field for transportation back to the packhouse to measure both vertical and horizontal forces.

(iii) Shock treatment and storage of commercial cultivars

Ripe fruit was randomly picked from several plants each of a firm (Octavia) and soft (Tulameen) variety and placed into labelled 40ml plastic punnets to form a single layer. Four replicate samples were taken for each cultivar and punnets were temporarily stored in cold boxes. Two replicate punnets of each variety were dropped from a height of 10 cm (x20 repetitions) in order to mimic 'shock forces' experienced by fruit during transportation. The remaining two replicate punnets served as 'non-shock' treatment controls. The 'hardness' of fruit (six individuals) for each variety was measured immediately after treatment using the QTS-25 Texture Analyzer on the day of harvest (0 days), and then monitored after 4 and 7 days storage at 4^{o}C to determine the shelf-life characteristics. Previous measurements using the Tinytag shock logger indicated that a drop height of 10 cm would represent excessive shock forces (up to 75 'g') and should therefore offer greater discrimination during shelf-life assessments.

Results & Discussion

1.1 Develop screening protocols for fruit firmness and shelf life and assess material from the 'Latham' × 'Glen Moy' cross.

Analysis of variance indicated there were significant differences (genetic variation) among the 'Latham' × 'Glen Moy' mapping progeny for:

- the breeder's score (field and polytunnel) of firmness (P<0.001),
- combined field and polytunnel data for the analyzer traits Hardness (Newtons, N; P=0.005), Rigidity (N, P=0.052), Final load (N, P=0.005), and Force/Mass_N/kg (P< 0.001) using mass as a covariate.
- The mass (10 berry weight) value also varied significantly between the progeny (P<0.001).

This analysis indicated that these measurements corresponded to continuous phenotypic traits within the mapping population displaying a normal pattern of distribution.

The trait data collected was assigned to the *Rubus* genetic linkage map using MapQTL software:

- Calculations for Hardness, Rigidity, Final load, and Force/Mass_N/kg, together with the mass and breeder's score were therefore significantly more heritable and QTLs are located on linkage group (LG) 3.
- QTLs for mass are also located on LG1 and LG5, and the QTL for breeder's score of firmness from field grown fruit is located on LG1.

- The most significant markers associated with each of these QTLs are shown in Table 1. Two candidate softening genes, pectinmethylesterase (PME) and β-1,4 xylan hydrolase (XL1) were significantly associated with the QTLs for breeder's score (polytunnel data) and mass (Table 1).

Although the remaining measurements of the QTS-25 Texture Analyzer were not significantly heritable in the mapping population, these traits were consistently associated with the same group of markers presented in Table 1, further confirming their association. Thus, both the breeder's score and some of the Texture Analyzer data can be used to identify soft and firm phenotypes within the 'Latham' × 'Glen Moy' mapping population and can be used to identify chromosomal regions responsible for trait variation.

Table 1. Key markers significantly associated with softening QTLs on the *Rubus* genetic linkage map.

Trait	QTL Location	Associated Markers	Sequence Identification
Mass	LG1	crumbly2009	Not identified
	LG1	**ERubLR_SQ10.2_H07**	Aquaporin
	LG1	RiPME	Pectinmethylesterase
	LG3	**SAMDC**	S-Adenosylmethionine decarboxylase
	LG3	ERubLRSQ05.3_D11	Allene oxide cyclase
	LG3	**C3991_PME**	Pectinmethylesterase
	LG5	**RUBENDOSQ07P15_XL1**	β-1,4 xylan hydrolase
	LG5	RUB289a	*Rubus* microsatellite DNA
Breeder's Score: Field	LG1	crumbly2009	Not identified
	LG1	**ERubLR_SQ10.2_H07**	Aquaporin
	LG3	Rub120a	*Rubus* microsatellite DNA-no hits
	LG3	**SAMDC**	S-Adenosylmethionine decarboxylase
Polytunnel	LG1	crumbly2009	Not identified
	LG3	Rub120a	*Rubus* microsatellite DNA
	LG3	**SAMDC**	S-Adenosylmethionine decarboxylase
	LG3	ERubLRSQ05.3_D11	Allene oxide cyclase
	LG3	**C3991_PME**	Pectinmethylesterase
	LG5	**RUBENDOSQ07P15_XL1**	β-1,4 xylan hydrolase
QTS-25 TA: Hardness	LG3	RUB22a	*Rubus* microsatellite DNA-no

Trait	QTL Location	Associated Markers	Sequence Identification
			hits
	LG5	RiM019	Microsatellite DNA
Force_Mass	LG3	E41M31-153	AFLP marker
	LG3	RUB22a	*Rubus* microsatellite DNA
	LG5	RiM019	Microsatellite DNA
Final_load	LG3	RUB22a	*Rubus* microsatellite DNA
	LG3	E41M31-153	AFLP marker
	LG5	RiM019	Microsatellite DNA
Rigidity	LG3	P13M39-195R	AFLP marker
	LG5	**RUBENDOSQ07P15_XL1**	β-1,4 xylan hydrolase
	LG5	RUB289a	*Rubus* microsatellite DNA
	LG7	**Expan**	Expansin

1.2 Understand stresses in the supply chain and how they relate to firmness scores.

(i) Storage of mapping population fruit

REML analysis demonstrated that all the compression parameters of hardness, rigidity, and final load measured were highly correlated, so subsequent analysis of samples is reported only for the hardness parameter.

- REML analysis indicated that there were significant differences between several of the progeny when the overall mean hardness values were compared (Table 2; significant when mean value > x1.96 SED).

- When Glen Moy (hard) was compared to Latham (soft) and all 22 progeny, there was a significant difference in all cases except for R42, R73 and R252 (Table 2). Thus, these three clones showed a similarity to Glen Moy in terms of hardness and potential shelf-life properties.

- Mean hardness values for most clones were not significantly different from the parent Latham, except for R42, R73, R252, as well as additional clones R104, R171, R241, and R184.

- When predicted means of hardness for the interaction between Day_Cultivar were compared, the parent Glen Moy and clones R42, R73, R241, and R252 were significantly firmer than all other clones on the first day of measurement (Day 0) as well as after two and four days storage, and although hardness values were significantly reduced after seven days, they were still significantly higher compared

to the majority of the other progeny (Fig.1). This trend was also observed for clones R171 and R184 except on the first day of measurement.

Table 2. Predicted means of hardness measurement for all cultivars

Cultivar	**Latham**	**Moy**	R1	R13	**R42**	R56	R61	R62
BS	0.42664	0.72511	0.49414	0.41791	0.73612	0.41343	0.45244	0.48853
Cultivar	R66	**R73**	R88	R93	R102	**R104**	R114	R136
BS	0.49054	0.70811	0.48313	0.50901	0.41734	0.53543	0.38434	0.38974
Cultivar	**R171**	**R184**	R214	R234	**R241**	R248	**R252**	R254
BS	0.61012	0.58933	0.45532	0.47262	0.57151	0.39403	0.70781	0.43704

BS = Breeder Score 2009.

Scale: 1=Hard; 2=Medium Hard; 3=Medium Soft; 4=Soft

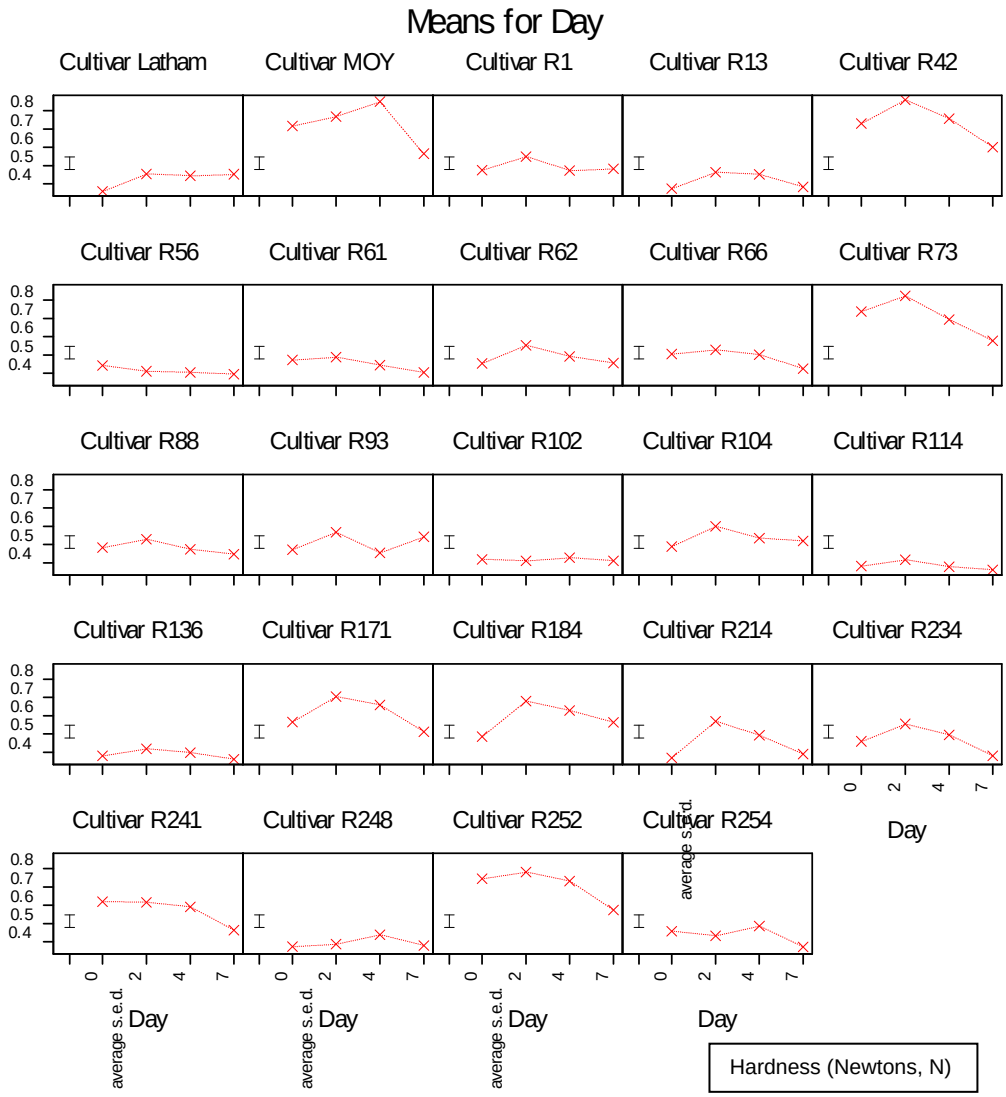


Figure 1. Mean hardness values for all progeny during storage over 0, 2, 4 and 7 days at 4^{o}C.

(ii) Storage of commercial cultivars

- ANOVA revealed that the 'juice bleed' was significantly greater for the known 'soft variety' Tulameen compared to Glen Ample or Octavia (P<001).

- The Tinytag logger indicated that significant vertical forces but not horizontal forces were experienced during the transport of punnets from the field roads to the packhouse and forces as high as 8 'g' were recorded (Fig. 2).

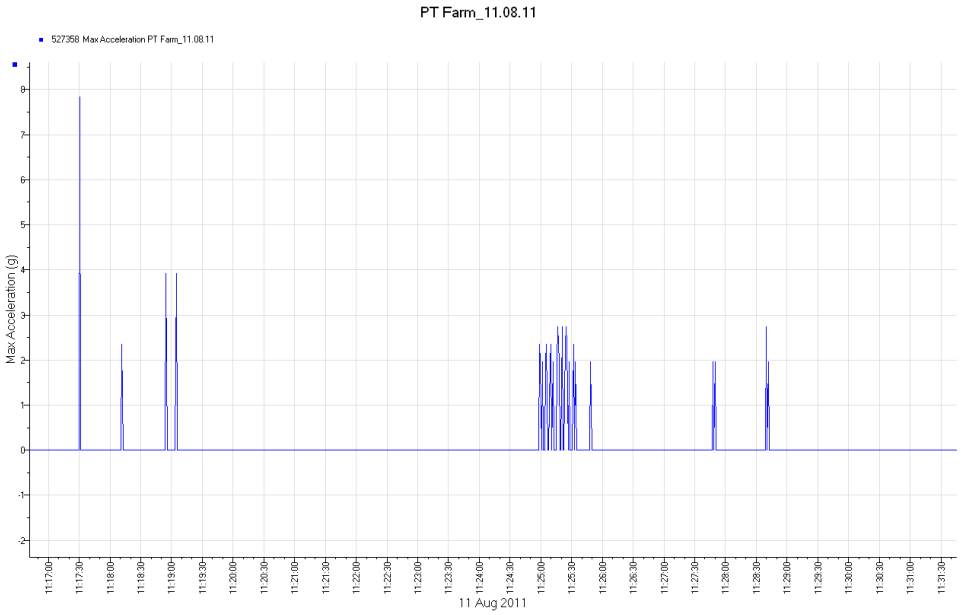


Figure 2. The vertical shock forces encountered by punnets of raspberry fruit during transport from the field to the packhouse at PT Farm as recorded by a Tinytag shock logger.

The analysis of variance of the Texture Analyzer's measurements for hardness, rigidity and final load on the collected fruit for storage revealed high correlations between these traits ($P<0.001$) and indicated a significant interaction between day and cultivar. As the results for all the measured traits were similar, findings for hardness need only be discussed.

- Octavia was consistently firmer ($P<0.001$) than Tulameen and Glen Ample on both the day of harvest and during storage over 7 days at 4^{o}C (Fig. 3).

- For Tulameen, there is a significant decrease in hardness between days 0 (harvest) and 4 ($P=0.006$), but not between days 4 and 7, whereas Octavia and Glen Ample showed no significant decrease between days 0 and 4, but a significant decrease between 4 and 7 days of storage ($P=0.006$).

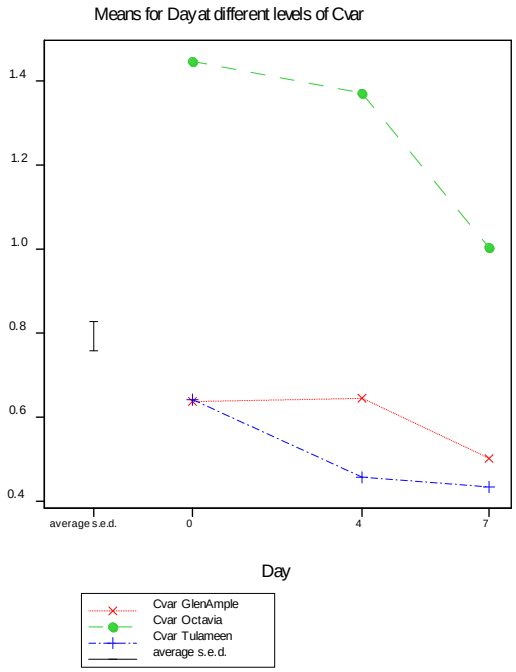


Figure 3. Mean 'hardness' levels as measured by texture analyzer of fruit collected from plants of Glen Ample, Octavia, and Tulameen and stored at 4^{o}C over 7 days.

- The QTS-25 measurements also indicated that the mean diameter width of fruit on day 7 was significantly less than on days 0 and 4 (P<001), and Octavia was a significantly wider fruit than Glen Ample, which in turn was wider than Tulameen (P<001) (Fig. 4).

- Because of these differences, ANOVAs were re-run using diameter as a covariate. After this adjustment, the significant day effects in the hardness trait were removed for Glen Ample and Tulameen, but were still significant for Octavia (P<0.001).

The results indicated that changes in firmness were proportional to changes in diameter width in Glen Ample and Tulameen but not Octavia fruit, and that biological considerations were important since there was an obvious difference between cultivars.

Fruit firmness is affected by several factors, for example, firmness may change due to altered hydrostatic pressure (turgor) within fruit cells, membrane damage and dehydration. Reasons for the change in diameter width could be attributed to the reduction in the cell turgor pressure of fruit as a result of transport and the subsequent softening process during storage together with dehydration and 'juice bleed' and hence, a reduced fruit circumference, and such events were less prominent in Octavia.

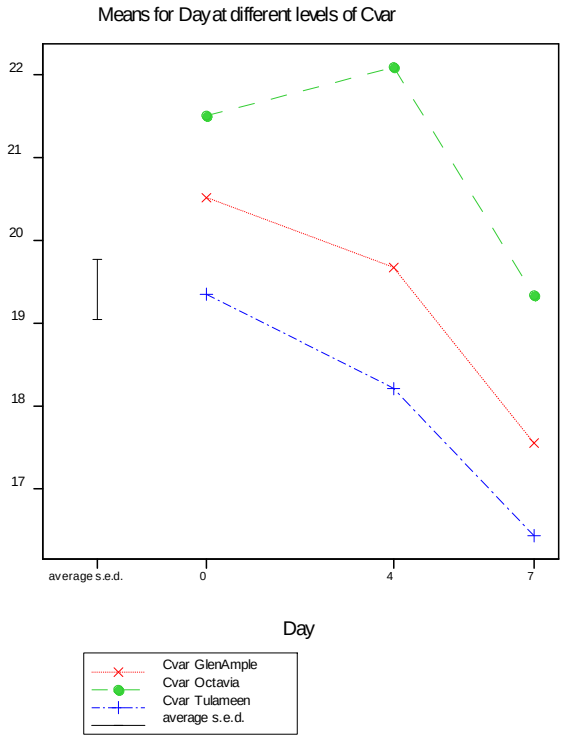


Figure 4. Mean diameter width as measured by texture analyzer of fruit collected from plants of Glen Ample, Octavia, and Tulameen and stored at 4^{o}C over 7 days.

(iii) Shock treatment and storage of fruit

- The analysis of variance indicated that all the measured traits were again very similar (P<0.001) so findings for hardness need only be discussed. Octavia was again significantly firmer (P<0.001) than Tulameen in the controls ('non-shock') on the day of harvest and during storage for 7 days.

- However, significant differences were recorded between the cultivars after the initial 'shock treatment' (Fig. 5). The hardness of Octavia was significantly lower (P=0.003) immediately after the 'shock treatment' on day 0 and throughout storage compared to the control, and the hardness significantly decreased further after storage for 4 days but not between 4 and 7 days. For the control samples of Octavia, a significant decrease in hardness between days 0 and 4 was recorded (P=0.043), but not between 4 and 7 days of storage.

- In Tulameen, which started at a significantly lower hardness, the 'shock treatment' made no significant difference in hardness at day 0 or had a significant effect during storage after 4 and 7 days compared to the control, although there was a significant decrease in hardness between days 0 and 4 but not between 4 and 7 days (Fig. 5).

In contrast, for Tulameen without 'shock treatment' there was no significant change in hardness during storage, but otherwise the hardness decreased.

It is clear that the extreme 'shock treatment' in this experiment had a greater effect on the shelf-life characteristics of Octavia compared to Tulameen, resulting in 'hardness' values of Octavia after 'shock treatment' comparable to those recorded for Tulameen after 4 and 7 days storage.

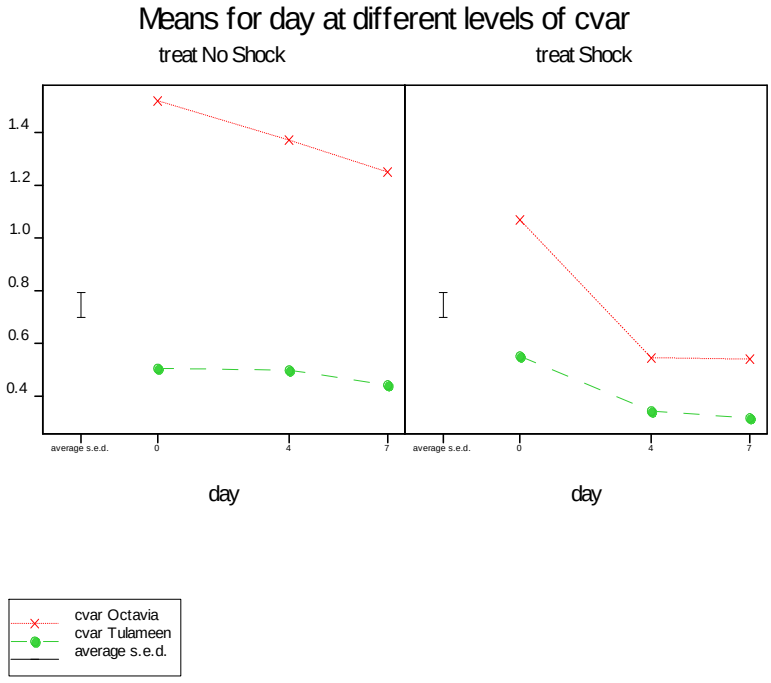


Figure 5. Mean 'hardness' levels as measured by texture analyzer of fruit collected from Octavia and Tulameen with and without 'shock treatment' and subsequent storage at 4^{o}C over 7 days.

- The sample diameter width in both varieties also significantly decreased during days 0 and 4 and between 4 and 7 days ($P<0.001$), and the 'shock treatment' resulted in fruit with a significantly reduced width ($P=0.002$) during storage confirming the previous results. When diameter width was used as a covariate in the analyses of these hardness traits the level of significances dropped considerably as observed for the previous storage trial, indicating that the size of the fruit had an effect on the hardness.

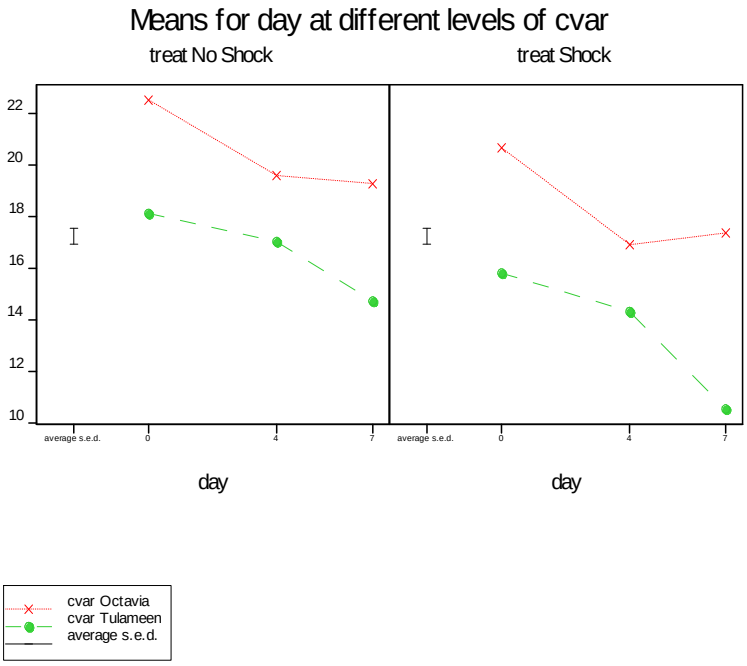


Figure 6. Mean diameter width as measured by texture analyzer of fruit collected from Octavia and Tulameen with and without 'shock treatment' and subsequent storage at 4^{o}C over 7 days.

These storage trials have indicated that Octavia is clearly a more firm and wider variety than Tulameen. However, the extreme shock treatment had a more pronounced impact on this cultivar perhaps due the size and shape of the fruit leading to greater cell wall damage and loss of turgor, whereas, the softer fruit of Tulameen on this occasion was more adept at cushioning the effects of the 'shock treatment' in contrast.

Objective 2. Carry out large scale sequencing of genes expressed in ripening raspberry fruit.

Introduction

The 454 mRNA-seq technology is a high throughput next generation sequencing procedure that will allow a large population of mRNA sequences to be identified in selected raspberry fruit. This will give the most complete list of genes that are switched on in these tissues, and is of further value because the transcript profile will be applicable to many aspects of fruit development. In this case, important fruit softening genes such as transcription factors which are involved in switching genes on and off, and cell wall hydrolases can be readily identified and selected for further analysis.

Materials and methods

Total RNA was extracted using a modified RNeasy method (Qiagen) from white/red (pre-ripe) and red (ripe) fruit of both parents Glen Moy and Latham. Poly(A)$^+$ RNA (mRNA) was isolated from the total RNA using Dynabeads (Invitrogen) and used to synthesise complementary DNA (cDNA) using the SMARTer PCR cDNA Synthesis kit (Clontech) according to the manufacturer's instructions. The cDNA, which represents stable copies of the genes being expressed in the fruit, was sent to the Genomics Facility at the University of York for 454 sequencing, a new technology that reads hundreds of thousands of sequences in a single sequencing run. The samples were run using a Titanium plate on a GS FLX system (Roche) and image analysis and base calling was done using the GS FLX system software version 2.0 (Roche).

Results & Discussion

The 454 data was used to create an extensive sequence database of genes expressed in raspberry fruit (described as a 'fruit transcriptome') that can be compared to the publicly available genomes of grape, peach, and *Arabidopsis* and subsequently mined for genes known to be involved in many fruit quality traits as well as softening. For the first time, a raspberry fruit transcriptome was successfully generated using next generation sequencing technology. Over 1.3 million sequences were generated across the four samples with similar size distributions (400-500 bases), and an assembly using MIRA software was created to produce 63,811 contigs from 936,487 reads (Table 3).

Table 3. The numbers of reads and total length of sequence obtained from each 454 run after quality trimming during sequence assembly.

Parent Sample*	Number of Reads	Total Length (MBases)
White/Red Moy	788562	225.9
Red Moy	136595	41.2
White/Red Latham 1	23821	7.2
Red Latham 1	7653	2.3
White/Red Latham 2	189938	55.8
Red Latham 2	179795	53.9
Total	1326364	386.3

*A repeat run was carried out for both Latham samples, and the first run and second runs are designated by 1 and 2, respectively.

Abundant genes that may have roles in the fruit softening process and linked to cell wall hydrolysis and flexibility, water movement, fruit ripening were identified from this dataset. Creation of libraries from Glen Moy and Latham allowed the identification of putative sequence polymorphisms for future validation (see below) and mapping. In addition, this

454 dataset was utilized to design microarray experiments to investigate different ripening stages in fruit by designing a *Rubus* RNA chip of candidate genes.

Objective 3. Identify sequence polymorphisms in 8 named genes and other fruit-related genes such as MADS box genes (Obj. 2) implicated in fruit ripening/softness.

Introduction

Molecular markers are DNA sequences (both known and unknown in structure) located near genes and inherited traits of interest, allowing selective breeding and identification of progeny with specific desired characteristics. Molecular markers have been rapidly adopted by researchers globally as an effective and appropriate tool for basic and applied studies addressing physiological traits. Such molecular markers are used as tools that identify polymorphisms between chromosomal DNA of different individuals. These polymorphisms can differ and include single nucleotide changes, large or small insertions and deletions, and length variation in repeat sequences. All of these provide information on alleles at that particular locus in the genome and importantly when that locus is associated with a particular plant phenotype. A key way of linking marker loci to a particular plant phenotype is through use of genetic linkage maps. Such maps when coupled with field trails, glasshouse or laboratory experiments to quantify traits of interest on the population of individuals used for map development can be used to relate phenotypes to markers.

Materials and methods

Candidate genes with expected roles in the fruit softening process were identified using in-house (*Rubus*) and public sequence databases together with 454 datasets, and were used to identify sequence polymorphisms (insertions/deletions [indels] or single nucleotide polymorphisms [SNP]) in Glen Moy and Latham. The procedure involved the PCR amplification of a portion of the candidate gene, sequencing of products, and sequence alignments (ClustalW2) to identify any polymorphisms between the parents and in 6-12 selected progeny to confirm the polymorphism segregated and therefore could be mapped.

The 188 progeny from the ‘Latham’ x ‘Glen Moy’ cross and both parents were subsequently PCR amplified for each candidate gene selected and Sanger sequencing or Pyrosequencing procedures were used to identify the SNPs, whereas indels were identified using a standard genotyping protocol and an ABI 3730 capillary sequencer.

Results & Discussion

- Data for 20 different genes associated with cell wall hydrolysis were mapped onto the JHI *Rubus* genetic linkage map using the JoinMap programme and their locations are shown in Table 4.

- These candidate genes were distributed across all 7 *Rubus* linkage groups, with the majority located on LG 3, 5 and 7.

Table 4. Status of mapping of candidate genes implicated in cell wall disassembly and fruit firmness.

Candidate gene	**Identifier**	**'Latham' x 'Glen Moy' linkage map group (LG)**
Pectinmethylesterase (PME)	ERubLR_SQ9.2_C12	LG 1
Polygalacturonase (PG)	ERubLR_SQ13.4_H10	LG 5
Cellulase2 (Cel2)	RiCellulase	LG 5
β-xylosidase	ERubLR_SQ01F_D13	LG 7
Xyloglucan endotransglycosylase (XET)	ERubLR_SQ5_3_H01	LG 7
Expansin4 (Exp4)	RiExpansin	LG 7
Pectate lyase (PL)	RiPL	LG 7
β-galactosidase1 (β-gal1)	CL1386Contig1 (454)	LG 3
β-galactosidase2 (β-gal2)	CL2657Contig1 (454)	LG 2
β-galactosidase3 (β-gal3)	CL5915Contig1 (454)	LG 6
Expansin1 (Exp1)	ERubLR_SQ13.2_E09	LG 3
Expansin2 (Exp2)	CL6813Contig1 (454)	LG 3
Arabinofuranosidase (Arab)	CL6475Contig1 (454)	LG 6
Cellulase1 (Cel1)	CL5428Contig1 (454)	LG 1
Pectinmethylesterase (PME)	CL3991Contig1 (454)	LG 3
Polygalacturonase (PG)	ras3_PG_Moy_Contig 29 & 60 (454)	LG 4
β-1,4 xylan hydrolase (XL)	M13_RUBENDOSQ07P15 (Bud library)	LG 5
Pectate lyase (PL)	C8580 (454)	LG 3
Pectinmethylesterase inhibitor	C8186 (454)	LG 3

ERubLR – indicates sequences held in the database derived from Latham root cDNA library from HortLink HL0170.

Objective 4. Generate an updated linkage map with fruit-related genes and identify genes that co-locate with softening QTLs.

Introduction

Plant breeding has advanced through an understanding of the principles of heredity, with Mendelian genetics forming one basis. Importantly an understanding of biometrical genetics which applies to traits showing continuous variation and controlled by more than a single gene, has allowed the manipulation of quantitative traits. Plant breeding aims to develop cultivars which fit specific environment and production practices and high yielding products.

Progress in any breeding programme is based on the available amount of genetic variability and the effectiveness of the selection and evaluation of the trait in question. Phenotypic selection has limitations especially when interest is focused on more complex physiological traits. A more accurate way of selection would be to screen at the genetic level DNA markers linked to the gene(s) or quantitative trait loci (QTLs) underlying desirable alleles. A prerequisite for genotypic selection is the establishment of associations between traits of interest and genetic markers. Understanding the genetic control of physiological traits and the linkage of these physiological characteristics to molecular markers on chromosomes, and ultimately the alleles(s) underlying the preferred phenotype of a trait is the future of plant breeding.

A key way of linking marker loci to a particular plant phenotype is through use of genetic linkage maps. For map construction, individual marker loci are genetically scored in a segregating population and the recombination rate of alleles at loci can be determined using classical linkage analysis. Loci can then be ordered into a set of linkage maps (groups) and once a sufficient number of markers have been mapped, the number of linkage groups should equal the haploid number of chromosomes. Once a map and segregating population have been developed it can be possible to identify map locations (loci) for traits of interest.

Results & Discussion

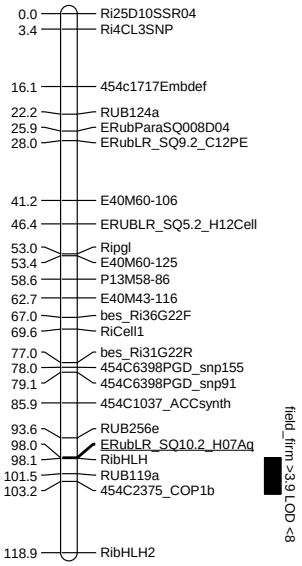
This objective was completed by successfully phenotyping the 'Latham' × 'Glen Moy' mapping population using the ‘breeder score’ and the QTS-25 Texture Analyzer measurements (Obj.1) together with the identification of sequence polymorphisms within selected candidate genes (Obj.3) and SNPs detected from the 454 datasets using protocols developed by the bioinformatics group at JHI (Obj. 2).

- An updated linkage map was completed using the JoinMap programme for the 20 different genes associated with cell wall hydrolysis (Obj.3) indicating segregation of these new markers within the population.

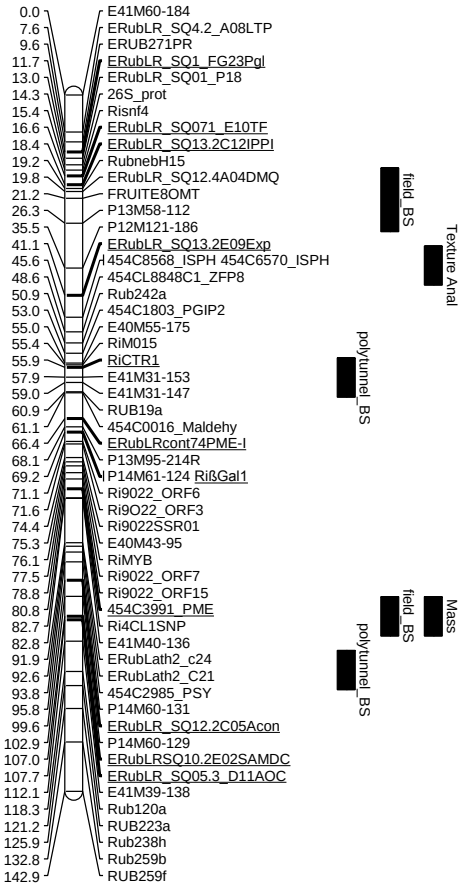
Mapping and the Kruskal-Wallis test indicated that the most significant markers associated with each of the softening QTLs on certain linkage groups were (see Fig. 7):

- LG1, Aquaporin (ERubLR_SQ10.2_H07);
- LG3, Pectinmethylesterase (PME; 454C3991), S-Adenosylmethionine decarboxylase (SAMDC; ERubLR_SQ10.2_E02SAMDC), Constitutive triple response1-like protein kinase (CTR1; a negative regulator of the ethylene response pathway; RiCTR1), Zinc finger protein/transcription factor (Zf/TF; similar role as MADS box genes; ERubLR_SQ071_E10TF), Isopentenyl pyrophosphate isomerase (IPPI; ERubLR_SQ13.2_C12) and Aconitase (ERubLR_SQ12.2_C05Aco);
- LG5, β-1,4 xylan hydrolase (XL1; RUBENDOSQ07_P15).
- There was one softening QTL each located on LG1 and LG5, whereas 5 QTLs were identified on LG3 (Fig. 7).

LG1



LG3



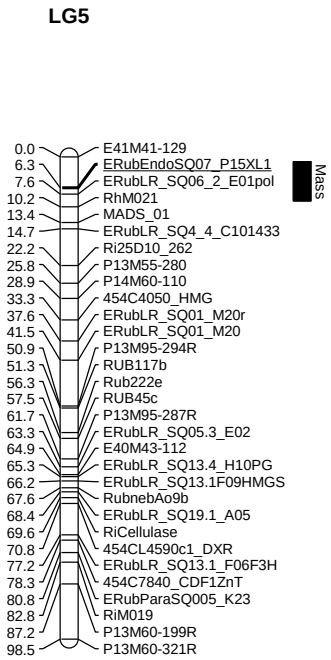


Figure 7. Location of softening QTLs mapped onto *Rubus* linkage groups 1, 3 and 5 and the associated DNA markers significantly associated are underlined.

Objective 5. Study gene expression profiles and potential alternative splicing events in key softening genes (Obj. 4).

Introduction

Fruit development and subsequent softening relies on the co-ordinated temporal expression of fruit specific genes. Gene expression is the process whereby genes are transcribed into a message RNA (mRNA) and translated into functional proteins, such as enzymes, that show as phenotypic traits. Genes are switched on through the activation of their promoter regions in response to developmental and environmental cues and the mRNAs that are made are subjected to further processing.

Protein coding sequences of eukaryotic genes are interrupted by non-coding sequences that must be spliced out from transcribed mRNAs to allow gene expression to occur. Many of these processes can have a profound effect on the final functional activity of the gene. Alternative splicing occurs in all higher eukaryotes and is a process that splices together different mRNA sequences transcribed from the same gene, leading to changes in protein structure and function at the individual gene level (Black, 2003). Recently developed panels that measure changes in alternative splicing in around 300 genes in Arabidopsis showed that significant alterations in the ratios of the alternative splicing events occur in response to plant development and in response to environmental changes. These will have a significant impact on plant development and biological adaptation of the plant to its surrounding

environment (Simpson et al., 2008 and unpublished). Strawberry polygalacturonase genes are subject to alternative splicing and may contribute to the selection of firm strawberry varieties (Villareal,et al., 2008).

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) is the method of choice to quantify differences in gene expression levels between messenger RNA (mRNA) samples. RT-qPCR is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in a real-time format. It is a highly sensitive technique that requires validation at several steps to assure accurate results.

Although it has become the method of choice for the quantification of RNA, there are several concerns that have a direct impact on the reliability of the assay. Principally, these are that (i) results depend on template quantity, quality and optimal PCR assay design, (ii) the reverse transcription reaction is not standardized, hence can be very variable, and (iii) data analysis is highly subjective and, if carried out inappropriately, confuses the actual results obtained. Consequently, it is essential to minimize variability and maximize reproducibility by quality-assessing every component of the RT-qPCR assay and adhering to common guidelines for data analysis. This clear need for standardization of gene expression measurements led to Bustin et al. 2009 establishing the *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments (MIQE) guidelines to ensure that published articles with RT-qPCR data are accurate and reproducible. Consequently, we focused on quality control throughout the guidelines described in this publication, so as to make it applicable to the development of standard operating procedures for soft fruit.

This objective was also carried out to validate transcription and post-transcriptional processing of genes identified within the QTLs (Obj. 4). However, as a result of the successful sequencing of the fruit transcriptome, this led us to further develop a new raspberry fruit transcriptional microarray that allowed us to monitor transcriptional changes throughout fruit development. DNA microarray technology provides a powerful way to investigate differential gene expression. The method is fluorescence based and allows the simultaneous and quantitative analysis of gene expression for a large set of genes. Environmental conditions, specifically water and temperature stress, are thought to exacerbate raspberry fruit softening. Water stresses, over-watered and drought treatments were evaluated against standard in a field experiment consisting of replicates of raspberry mapping population clones plus the parents Latham and Moy.

Materials and methods

(i) RNA Quality Control Steps

It is critical that an efficient RNA extraction method is used to produce high purity and high integrity RNA for reliable and reproducible results.

- Various manual protocols employing mortar and pestle or homogenizer/tissue lyser devices for tissue disruption, reagents (TRI Reagent®, Sigma) and commercial kits (Qiagen) for extracting total RNA from raspberry ripe fruit were evaluated following the manufacturers guidelines.

- Two brands of reverse transcription enzyme kits were evaluated for converting RNA (standard concentration of 1 µg) into cDNA:
  a. Ready-To-Go You-Prime First-Strand Beads (GE Healthcare), contains single-dose reaction beads containing FPLC™*pure* M-MuLV Reverse Transcriptase, RNase Inhibitor and Nucleotides, followed by addition the addition of *Oligo(dT)$_{12\text{-}18}$ Primer (Invitrogen)* which hybridizes to the poly(A) tail of mRNA.
  b. QuantiTect® Reverse Transcription Kit (Qiagen), includes a step with gDNA Wipeout buffer for a final elimination of any contaminating genomic DNA, Quantiscript Reverse Transcriptase (mixture of Omniscript and Sensiscript reverse enzymes), RNase Inhibitor and Nucleotides, a blend of oligo-dT and random primers for high cDNA yields.

- The concentration and purity of all RNA samples was routinely estimated by spectrophotometry (A_{260}/A_{280} ratio of 1.8-2.0 indicates good quality RNA devoid of protein and phenol contamination).

- The integrity of RNA samples was visualized by agarose gel electrophoresis and objectively evaluated using the automated Agilent 2100 Bioanalyzer which reports an RNA integrity indicator value (RIN) ranging from 1 (degraded) to 10 (intact).

Different stages of raspberry fruit (immature green, mature green, white, white/red, and ripe fruit) were harvested from three biological replicates of Glen Moy, Latham, Octavia, Tulameen, Glen Ample, and several clones (one biological replicate) of the mapping population representing ‘soft’ and ‘firm’ fruit categories (Fig. 8), and total RNA was extracted following the optimized protocol and evaluated according to the aforementioned criteria.

Figure 8. Different stages of raspberry fruit harvested from three biological replicates of Glen Moy and Latham in 2010 and the total RNA extracted.

Key: IG, immature green; MG, mature green; W, white fruit; WR, white/red fruit; RF, ripe fruit.

(ii) Candidate and Reference Genes Selected for Expression Studies

The assays for 11 candidate genes (pectinmethylesterase (PME); β-1,4 xylan hydrolase (XL); Aquaporin; S-Adenosylmethionine decarboxylase (SAMDC); polygalacturonase, PG; pectate lyase, PL; pectinmethylesterase inhibitor, PME*i*); constitutive triple response1-like protein kinase, CTR1; zinc finger protein/transcription factor, Zf/TF; isopentenyl pyrophosphate isomerase, IPPI; and Aconitase) were selected for gene expression studies based on their involvement in fruit cell wall hydrolysis/modification and/or significant association with softening QTLs.

- Full length candidate gene sequences were initially searched using several sequenced BAC library clones and the *Rubus* 454 dataset, and this was successful for both Aquaporin and SAMDC. Contig sequences for the remaining genes were also assembled from the 454 dataset to construct a longer consensus but incomplete gene sequence.

- Ten reference genes (GADPH, UBC, PP2A, SAND, Ubiquitin transferase, UBC9, YLS8, TIP41-like, Clathrin, and 'Unknown gene') for transcript normalization in *Rubus* were selected from the literature (Czechowski et al. 2005) and gene sequences were retrieved from the 454 dataset.

(iii) Design of PCR Primers/Probes for RT-qPCR

PCR primers and probes for real-time RT-qPCR were subsequently designed for all candidate and reference genes using the UPL Design Centre software (Roche) to ensure common thermal cycling parameters and a series of rigorous *in silico* specificity checks were performed before purchase:

a. blastn search of primer sequences against the *Rubus* database and the 454 dataset for other potential homologous sequences which may cause cross-reactions;
b. the occurrence of SNPs or indels in annealing regions of primers which may hamper efficient annealing of the primer or prevent amplification of a variant allele; and
c. amplicon screens for potential secondary structures (hairpins) that may prevent efficient PCR amplification were checked by the UNAfold software package for nucleic acid folding and hybridization prediction_ (http://mfold.rna.albany.edu/?q=unafold-man-pages).

- A new primer or assay was re-designed in the event that the aforementioned criteria checks were not met.

After *in silico* assay evaluation, the primers were empirically validated by doing an actual PCR experiment and inspection by gel electrophoresis for single products of the correct size.

(iv) RT-qPCR Validation

The next step was to determine the optimal primer and probe concentrations, reaction efficiency of an assay, and validate the suitability of reference genes for normalization in real-time qPCR. Optimal RT-qPCR conditions were determined for each assay by varying the primer (150, 300, and 900nM) concentrations and comparing the calculated Cq (quantification cycle) values. After the optimal primer combination was obtained, the probe concentration was optimised by testing three different concentrations (100, 150, and 200nM). A primer/probe combination was considered to be optimal when the amplification produced the lowest Cq value.

- All assays were optimal using equimolar concentrations of forward and reverse primers at 900 nM and there was no significant difference in varying the probe concentration, so 100 nM was used throughout.

The efficiency of each PCR assay was determined from the slope value derived from the standard curve linear regression equation using the formula, Efficiency (E) = $[10^{(-1/S)}]$-1, where efficiencies between 80% and 110% are considered acceptable. A pooled sample of cDNA was first prepared by mixing 2µl of cDNA from all stages of fruit including leaf obtained from all 3 biological replicates of Glen Moy and Latham, and a 5-fold dilution series

was prepared over 6 points (10ng/µl to 3.2 pg/µl). Each dilution was run in triplicate for each primer set.

(v) Reference Gene Validation

Reference gene controls are used to normalize the gene expression data by correcting for differences in quantities of cDNA used as template. A perfect reference gene is one that does not exhibit changes in expression between samples from various experimental conditions or time points. In this case, GADPH, UBC9, Clathrin, YLS8, TIP4 were initially evaluated as they covered the expression level range of the 11 candidate genes and cDNA from all stages of fruit including leaf obtained from 3 biological reps of Glen Moy and Latham was tested in a real-time RT-qPCR format (2 technical reps).

The Cq values were transformed to relative gene expression quantities (not normalized) by the comparative Cq method incorporating PCR assay efficiencies and subsequently imported into the geNorm program and genes were ranked according to their expression stability. This software indicates how many reference genes are optimally required to remove most of the technical variation and produces a normalization factor (NF) value for each reference gene per sample tested.

(vi) Real-time RT-qPCR conditions

RT-qPCR was performed in MicroAmp® Fast Optical 96-Well Reaction Plates (Applied Biosystems) using the 7500 Fast Real-Time PCR System (Applied Biosystems). Real-time qPCR reactions involved the addition of 2.0 µl of cDNA to a FastStart TaqMan® Probe Master (Rox) Mix containing 100 nM of probe, and 900 nM each of forward and reverse primers, and molecular biology grade water to a final volume of 25µl. The generic two step cycling protocol (95^{o}C/15s + 60^{o}C/1.00m) over 45 cycles was used to amplify target gene sequences.

(vii) Development of an Agilent Rubus microarray

The large increase in raspberry transcriptome sequence assembled (Obj. 2) permitted the development of a comprehensive *Rubus idaeus* microarray at JHI using the Agilent dual mode gene expression platform to globally measure changes in gene expression as fruit develops and softens.

- This custom gene chip was designed from a unigene set (transcripts from the same transcription locus) assembled from existing sequence resources at JHI, comprising

transcript sequences isolated from a range of *Rubus* tissues, developmental stages and conditions, including developing fruit and buds.

- This set was composed of sequences originating from four sources: (i) Roche 454 transcripts (52,263); (ii) Illumina GAII transcripts (118,275); (iii) Sanger Expressed Sequence Tags (4,360) and; (iv) BAC coding sequences (1,425).
- In total, 176,323 sequences were assembled using CAP3 Sequence Assembly software, generating 41,155 contigs and 22,098 singletons.
- All sequences were BLASTx searched against known plant polypeptide sequences to identify the top protein homologues which, along with the presence of a polyA or polyT tract, enabled determination of predicted orientation for 55,920 unigenes.
- A total of 55,708 oligonucleotide probes (60mers) were designed and utilised for generation of a custom Agilent microarray in an 8 x 60k format.

Firstly, two arrays (8 x 60k format) were run to determine the relative abundance of nucleic acid sequences in the various fruit developmental stages (immature green, mature green, white, white/red, and ripe fruit) collected from 3 biological replicates of Glen Moy and Latham to identify 1000s of additional candidate genes potentially involved in the softening process. In order to focus on a reduced number of important candidate softening genes, an additional array was run for several progeny of the 'Latham' × 'Glen Moy' mapping population representing ‘soft’ and ‘firm’ fruit categories. Mature green and white/red fruit stages were tested since previous analysis indicated high variation in gene expression levels between Moy and Latham. A pooling strategy was used to prepare RNA samples (at a constant concentration) from the two fruit stages to produce two replicates each consisting of 4 different clones (8 in total) representing both hard and soft fruit mapping population clones.

(viii) Irrigation stress trial 2011-2012

In order to assess the effects of water stress on fruit softening, an irrigation trial was set up under a polytunnel over two seasons (2011-2012) consisting of pots of six mapping population progeny plus Moy and Latham. SM300 sensors were used to monitor water levels in the growth media and control irrigation *via* data loggers and automated irrigation to provide standard (~60% water content), over-watered (~80%) and drought (~20%) treatments.

Raspberries established in 9 litre pots in 2010 were transferred to one row under a polytunnel and arranged in a randomised block design in January 2011 (Appendix I). Three irrigation hoses were installed along the entire length of the plot to independently control

watering rates. Drippers were installed in the relevant hose above pots to provide a random distribution. The SM300 water sensors (Delta-T Devices Ltd.) were installed in 1 x Latham and 1 x Glen Moy pots per watering treatment replicates to monitor levels in the growth media. The data collected triggers irrigation when water content goes above or below prescribed limits. During crop establishment, all pots will receive the same water treatment so that fruit establishment is not biased. Water stresses will be applied prior to sampling (2-4 days) of a particular fruit stage.

For the second season 2012, the plants were overwintered under glasshouse conditions, transplanted from 9 Litre into 15 Litre pots containing vine weevil biological control agent (Met 52; Fargro Ltd.) and fertilizer, and transferred into a cold store (4^{o}C) without light to induce dormancy for 7 weeks. Pots were transferred to one row under a polytunnel in June 2012, and the SM300 sensors were again installed into each pot of Latham and Glen Moy pots per treatment block to monitor levels in the growth media and control irrigation.

(ix) Assessment of alternative splicing in raspberry fruit ripening genes.

Alternative splicing is recognised as a key post-transcriptional process that modulates and regulates the levels of mRNA transcripts prior to translation. It has previously been shown that varietal differences in strawberry leads to an alteration in alternative splicing in the polygalacturonase (PG) gene and is associated with variation in fruit firmness. We investigated whether alternative splicing exists in several of the raspberry fruit ripening genes previously shown to be transcribed in developing fruit.

- Gene structures were determined for 7 genes (Aquaporin, SAMDC, PME, PG, PL, XL, and PMEi), and in all cases with the exception of an inhibitor of pectin methyl esterase (PMEi), the selected raspberry genes were predicted to contain an exon (coding sequence) and intron (non-coding sequence) structure.

- In two cases (Aquaporin and SAMDC), it was possible to directly determine gene structure by comparing the 454 RNAseq generated sequence contigs with available raspberry BAC cloned genomic sequence. The remaining cDNA sequences were compared to available strawberry genomic sequence and high sequence identity was found between strawberry and raspberry in these genes. Maintenance of intron position in gene sequence orthologues is well known which permitted the putative positioning of intron sequences in the raspberry fruit ripening genes.

Primers were designed across introns to identify possible alternative splicing events and high resolution RT-PCR was performed on developing fruit (immature green, mature green, white, white/red and ripe) collected from 3 biological replicates of Glen Moy and Latham.

Results & Discussion

In an effort to produce consistent and high quality ('gold standard') data from RT-qPCR studies, the *m*inimum *i*nformation for publication of *q*uantitative real-time PCR *e*xperiments (MIQE) guidelines (Bustin et al. 2009) were followed. This rigorous pipeline involved evaluating methods for total RNA extraction and cDNA synthesis from 5 different raspberry fruit stages, routinely checking RNA integrity by gel electrophoresis or Agilent 2100 Bioanalyzer, optimal design of PCR primers and probes in RT-qPCR involving *in silico* specificity checks, optimal primer and probe concentrations with PCR efficiencies >80%, and the suitability of 10 reference genes for transcript normalization in *Rubus* by validation using geNorm software.

The end result was the development of a reliable raspberry fruit RNA extraction procedure which incorporated the use of a TissueLyser (Qiagen) for disruption and purification using the RNeasy Plant Mini Kit (Qiagen) and the automated QIAcube robot system (Fig. 9). The optimised protocol was as follows: two frozen fruit samples per stage were extracted in duplicate by transferring samples into previously labeled and frozen 30ml wide-mouth HDPE bottles (Nalge Nunc) containing six 9.0mm chrome steel ball bearings. Two bottles were attached to a Mixer Mill 301 (Retsch) and the fruit was disrupted for 1 min or 30s intervals at a maximum frequency of 30 Hz with intermittent freezing of samples in liquid N_2 until a fine powder was produced (usually 2-3 pulses required). A 120mg of the milled powder extracted for each fruit stage was then purified following the manufacturers guidelines for the RNeasy Plant Mini Kit (Qiagen) and QIAcube robot system. Samples were either kept frozen in liquid N_2 or in a -80^{o}C freezer and at no time were allowed to thaw during the procedure.

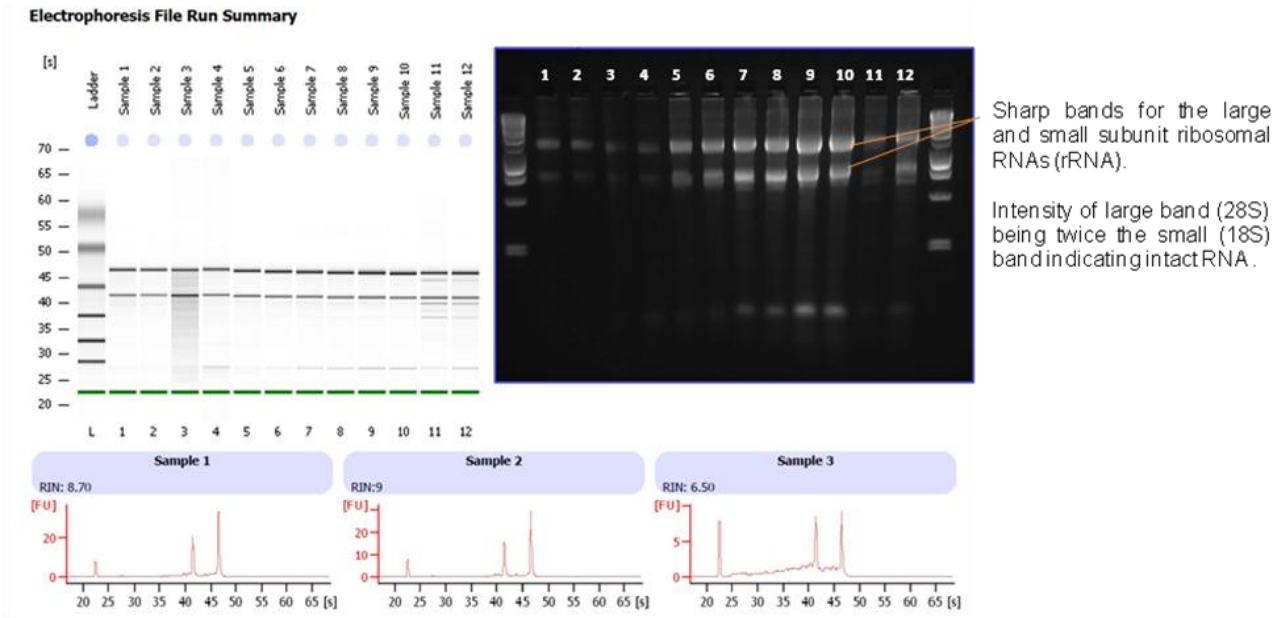
Ambion® Plant RNA Isolation Aid (0.1 volume, 45 µl) was also added to the Qiagen kit extraction buffer (450 µl per sample) for more efficient removal of common contaminants of plant RNA preparations, such as polysaccharides and polyphenolics. The Ambion® DNA-*free*™ Kit was also used following the manufacturer's instructions with the purified eluate (50 µl) from the QIAcube to completely remove any residual contaminating gDNA from the RNA preparations.

Figure 9. Workflow of the developed raspberry fruit RNA extraction procedure.

- The Quantitect® Reverse Transcription (Qiagen) kit produced the most consistent and highest yields compared to the Ready-To-Go You-Prime First-Strand Beads Kit (GE Healthcare) based on the PCR amplification of the resulting cDNA samples for several candidate genes.

- In all cases, the RNA extracted from each fruit stage met the quality control standards, intact and sharp bands for the large (28S) and small (18S) subunit ribosomal RNAs (rRNA) were evident, A_{260}/A_{280} ratios >1.8, and RIN values were usually >8.0 (Fig. 10).

RNA integrity output from the Agilent 2100 Bioanalyzer and corresponding agarose gel of samples tested



KEY: 1-2, ripe fruit; 3-4, white/red; 4-6, white fruit; 7-8, mature green; 9-10, immature green; 11-12, leaf samples.
RIN values shown on output for samples 1-3.

Figure 10. RNA integrity output from the Agilent 2100 Bioanalyzer and corresponding agarose gel (2%) of some raspberry fruit samples tested.

- A robust pipeline for designing and evaluating PCR assays for RT-qPCR experiments was also developed. All sets of designed primers successfully amplified target from cDNA by conventional PCR and a real-time RT-qPCR format to produce a single band of the correct size from various fruit samples (Fig. 11). All standard curves for each PCR assay had r^2 values >0.990 (high linearity) and efficiencies, E >80%.

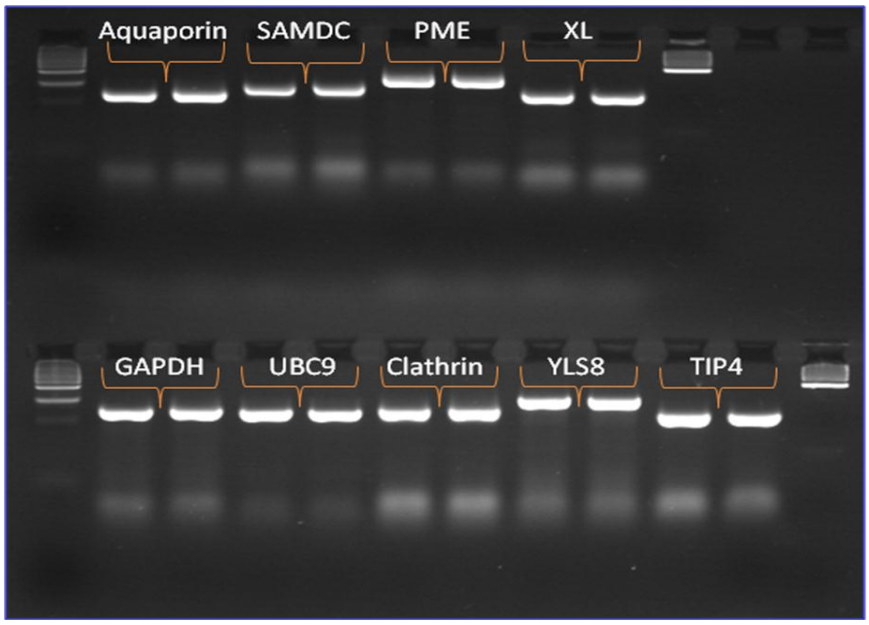


Figure 11. Agarose gel (3%) showing single PCR products amplified from several examples of candidate (upper lanes) and reference gene (lower lanes) assays indicated.

- The geNorm software indicated that the novel reference genes Clathrin, YLS8, and TIP41 were the most suitable for accurate normalization with fruit samples, although all 5 genes met the criteria standards. This validation also demonstrated that the novel reference genes (Clathrin, YLS8, and TIP4) performed better than the traditional ('housekeeping') genes (GADPH and UBC9) selected for RT-qPCR normalization as highlighted by Czechowski et al. 2005. This is the first report of a systematic validation of reference genes for gene expression studies in *Rubus*.

Normalized Gene Expression Results

The assays of all the 11 candidate genes were subsequently tested with the same cDNA samples and the data normalized before analysis of variance was used to determine any significant differences in gene expression levels between Glen Moy and Latham and different stages of fruit.

- Some significant differences in gene expression levels were found at particular stages of fruit development, and in every case, expression levels were greater in Glen Moy than Latham (Table 5).

Table 5. Significant differences in levels of gene expression between Moy and Latham

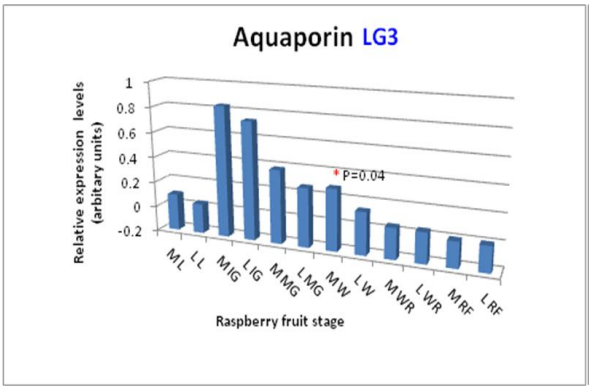
Candidate Gene	Sample	Result	Level of Significance
Aquaporin	White fruit	Moy > Latham	P=0.04
SAMDC	Immature green	Moy > Latham	P=0.07
SAMDC	Mature green	Moy > Latham	P=0.07
SAMDC	White/red	Moy > Latham	P=0.04
PME	Leaf	Moy > Latham	P=0.03
PME	White fruit	Moy > Latham	P=0.06
PME	White/red	Moy > Latham	P=0.07
XL	Leaf	Moy > Latham	P=0.05
XL	White fruit	Moy > Latham	P=0.09
Pectate lyase	Leaf	Moy > Latham	P=0.002
Pectate lyase	Immature green	Moy > Latham	P=0.039
Pectate lyase	Ripe fruit	Moy > Latham	P=0.097
CTR1	White fruit	Moy > Latham	P=0.032
CTR1	Ripe fruit	Moy > Latham	P=0.048
Zf/TF	Ripe fruit	Moy > Latham	P=0.006
Aconitase	Mature green	Moy > Latham	P=0.094
Aconitase	White fruit	Moy > Latham	P=0.065
Aconitase	Ripe fruit	Moy > Latham	P=0.092

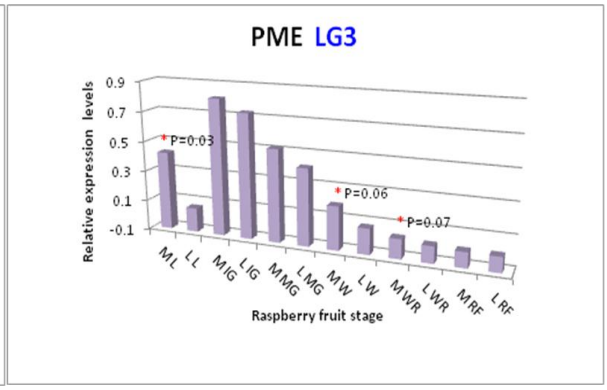
Note: Data analyzed by ANOVA, and the means were compared by the LSD test at a significance level of 0.05 and 0.10.

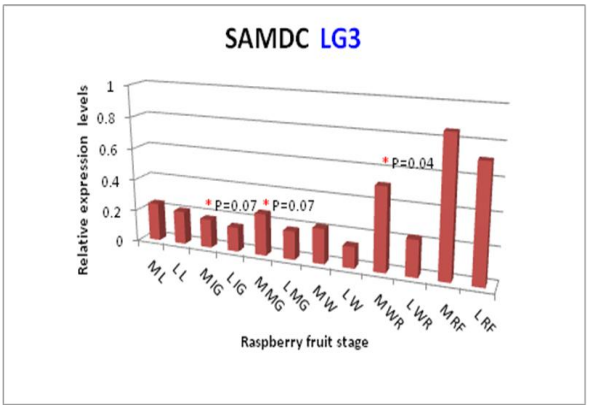
When the data is represented in the form of histograms (Fig. 12), there are several tends evident:

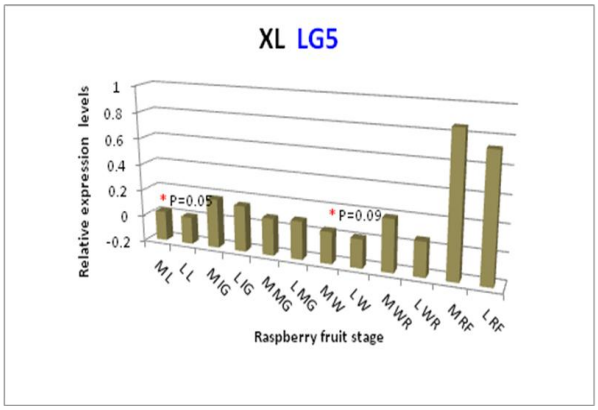
- The mean relative expression levels for Aquaporin and PME show a similar steady rate decline during ripening from immature green to the ripe fruit stage.
- The relative expression levels for SAMDC show the opposite trend of a steady increase up to the ripe fruit stage. In XL, there is a decrease in expression from the immature green stage to white fruit, an increase in white/red fruit, followed by a significant rise in ripe fruit (Fig. 12).
- The mean relative expression levels for PG are only significantly detectable at the white/red and ripe fruit stages in Latham and Moy, whereas PL levels show a steady rate increase during ripening from immature green and peak at the white and white/red stages.

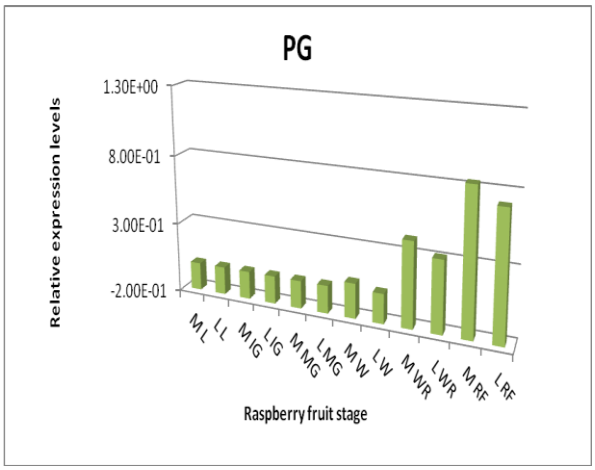
- The relative expression levels for PME*i* are only significantly detectable at the white/red and ripe fruit stages (Fig. 12), the opposite trend of PME levels in the same tissue.
- Both the expression levels for CTR1 and IPPI show a steady increase in expression from the immature green stage and peak at the white/red fruit stage, and then show a significant reduction at the ripe stage (Fig. 12).
- The relative expression levels for the Zf/TF gene show a high steady state of expression throughout the various fruit developmental stages culminating in a significant reduction at the ripe stage in Latham (Fig. 12).
- Levels of Aconitase decrease after the immature green stage through to the white stage, then continue to rise through the white/red to ripe fruit stage in Glen Moy, in contrast to a significant reduction in expression at the ripe stage in Latham after a significant increase at the white/red stage (Fig. 12).
- In every case, the results for Moy and Latham follow the same patterns of change in relative gene expression levels at each fruit stage and in the majority of cases, expression levels were greater in Glen Moy than Latham.

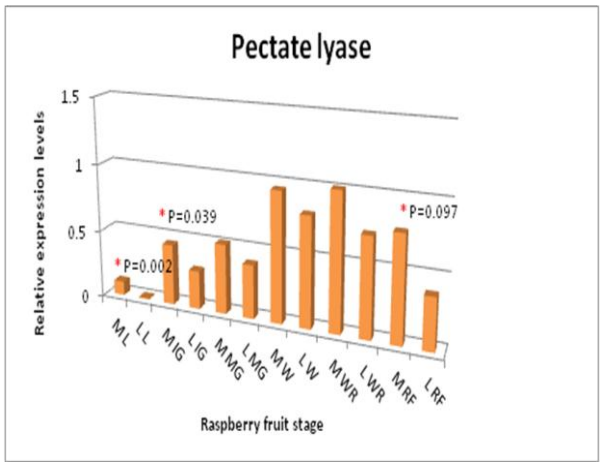


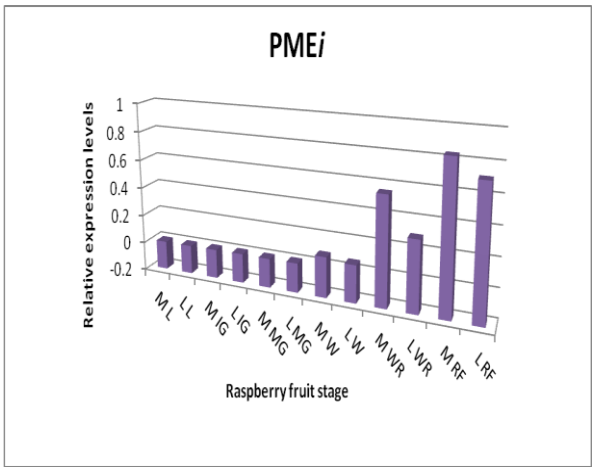


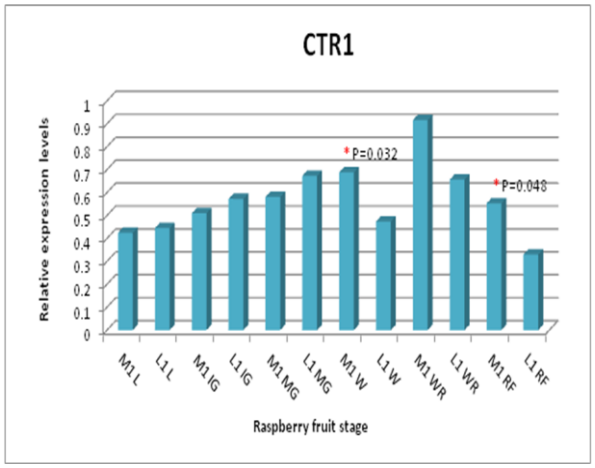


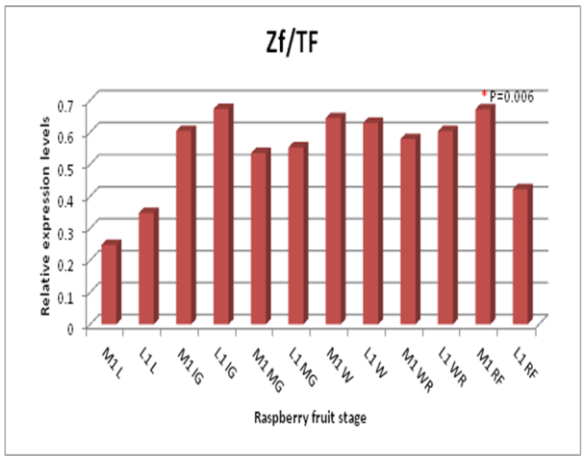


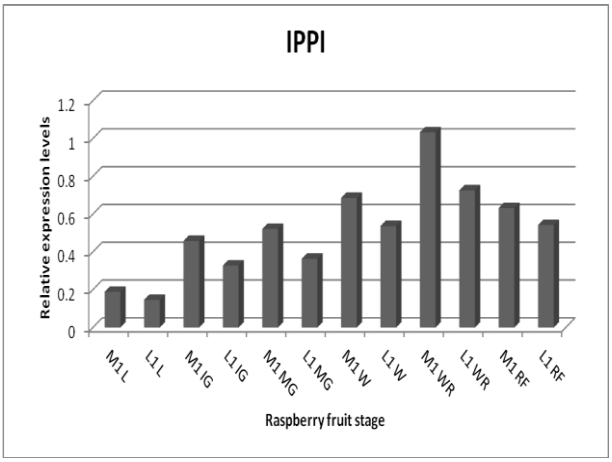












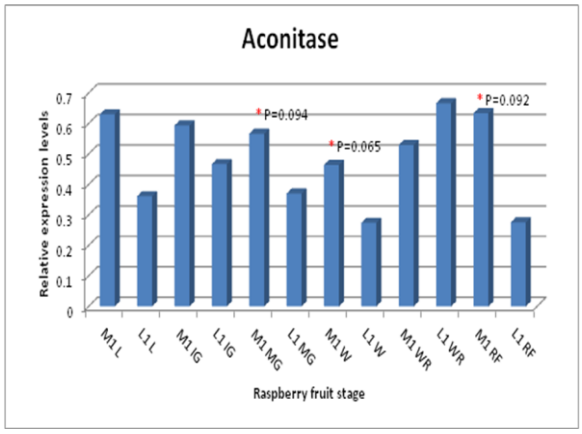


Figure 12. Mean normalized relative gene expression levels obtained for those candidate genes most significantly associated with softening QTLs in different stages of fruit and leaf in Glen Moy (M) and Latham (L).
Key: L, leaf; IG, immature green; MG, mature green; W, white fruit; WR, white/red fruit; RF, ripe fruit. *P* level of significance is indicated on histograms.

The normalized data was converted to a log10 scale and further differences are highlighted and correlations were evident with a scatter plot showing the cultivar response for each stage of fruit (not including leaf) and candidate gene expression levels (Fig. 13):

- Strong negative relationships between SAMDC and Aquaporin (r=-0.78), between SAMDC and PME (r=-0.73), between XL and Aquaporin (r=-0.62), and between XL and PME (r=-0.54).

- Very strong positive relationships between PME and Aquaporin (r=0.97), and between XL and SAMDC (r=0.80).

- Strong negative relationships between PG and Aquaporin (r=-0.86), between PG and PME (r=-0.91), and a positive relationship between PG and SAMDC (r=0.62).

- Slight positive correlation of PL expression with PG levels (r=0.41).

- Very strong negative relationships between PME*i* expression and Aquaporin (r=-0.87), PME*i* and PME (r=-0.91), strong positive relationships between PME*i* and PG (r=0.98), PME*i* and SAMDC (r=0.69), and weaker positive relationships between PME*i* and PL (r=0.44), PME*i* and XL (r=0.40).

- Positive correlation of Zf/TF expression levels with PL levels (r=0.41).

- Positive relationships between IPPI and PL (r=0.78), IPPI and PME*i* (r=0.61), IPPI and PG (r=0.56), and IPPI and CTR1 (r= 0.40).

- Positive correlation between Aconitase expression levels and CTR1 levels (r=0.45).

These positive and negative correlations are driven by stage of fruit, and the relationships within each stage are also usually positive (Fig. 13).

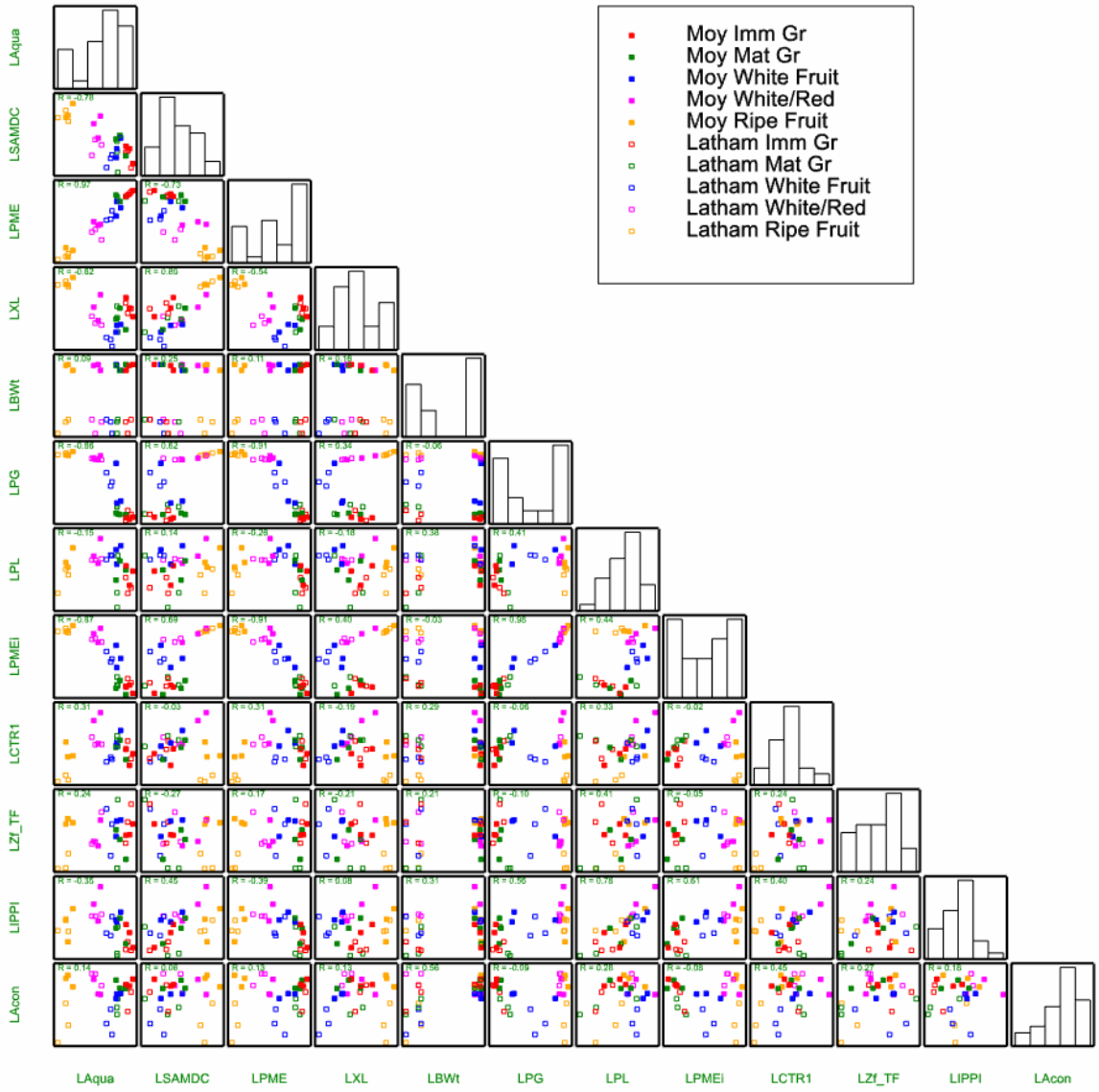


Figure 13. Scatter plots (Log10 scale) categorized by stage of fruit and normalized expression levels of all 11 candidate genes in Glen Moy and Latham.

LBWt = Log Berry Weight.

Note: The correlation coefficient r-values (see Appendix II), a measure of the strength and direction of the linear relationship between two candidate genes (variables) are given in each square.

In order to complete the analyses of expression data, repeated measures ANOVA with log transformation was used to determine the overall significant difference in expression levels among pooled datasets for all candidate genes between Glen Moy and Latham (Table 6).

Table 6. Overall significant difference in levels of candidate gene expression between Glen Moy and Latham for combined datasets of all fruit stages

Candidate Gene	Level of Significance:	
	Cultivar (Cv)	Cv_Fruit Stage Interaction
Aquaporin	***P=0.053****	0.447
SAMDC	0.217	***P=0.10****
PME	***P=0.062****	0.243
XL	0.295	0.161
PG	0.291	0.570
PL	0.169	0.537
PMEi	0.703	0.381
CTR1	***P=0.089****	***P=0.014****
Zf/TF	0.461	0.195
IPPI	0.210	0.706
Aconitase	***P=0.027****	***P=0.024****

Note: Data analyzed by repeated measures ANOVA with log transformation, and the means were compared by the LSD test at *P* significance levels of 0.05* or 0.10*.

- Significant differences in combined gene expression levels between cultivars (Glen Moy and Latham) and/or cultivar by fruit stage interaction were found for Aquaporin, SAMDC, PME, CTR1 and Aconitase (Table 6).

Line plots showing log transformed combined relative expression levels per candidate gene over time (fruit stage) further highlight significant differences between Glen Moy and Latham during fruit development and aid in the selection of genes for further study (Appendix III).

- The line graphs for PG, PMEi, Zf/TF, and in particular, CTR1 and Aconitase show departure from parallel behaviour (lines cross-over) over time (stage), indicating an interaction between cultivar and fruit developmental stage implying that there is differences between genotypes at different stages.

In order to confirm the previous findings with Glen Moy and Latham and increase our understanding of the important fruit softening controls, RNA was also extracted from the same stages of fruit collected from three commercial raspberry varieties (three biological replicates each Glen Ample, Octavia, Tulameen) and several clones (one biological replicate) of the ‘Latham’ x ‘Glen Moy’ mapping population representing ‘soft’ and ‘hard’ fruit categories to carry out the same gene expression studies. A pooled sample consisting of 3 different progeny each was created to produce 3 biological replicates (9 clones in total) for ‘hard’ and ‘soft’ mapping clone categories. Total RNA was extracted, cDNA synthesised and evaluated according to aforementioned criteria.

PCR primers and the suitability of reference genes (GADPH, UBC9, Clathrin, YLS8, TIP4) for normalization in RT-qPCR were again validated with these new cDNA samples.

- The geNorm software indicated that Clathrin, YLS8, and TIP4 were the most suitable for accurate normalization with these new cDNA samples in agreement with the cDNA samples derived from Glen Moy and Latham.

The cDNA from the three commercial varieties and pooled mapping population samples were subsequently tested with primers of seven candidate gene assays (Aquaporin, SAMDC, PME, CTR1, Zf/TF, Aconitase, and PL). These assays were selected on the basis of showing the most significant variation in relative gene expression levels between Glen Moy and Latham.

- Some significant differences in candidate gene expression levels were found at particular stages of fruit development in the three commercial varieties of raspberry and pooled categories of 'hard' and 'soft' mapping population progeny (Table 7).

Table 7. Significant differences in levels of candidate gene expression between three commercial varieties of raspberry and pooled categories of 'hard' and 'soft' mapping population progeny.

Candidate Gene	Sample	Result	Level of Significance
Aquaporin	Mature green	Tul < Oct = GA	P=0.073
Aquaporin	White/red	Tul > Oct = GA	P=0.055
PL	Mature green	Oct < Tul = GA	P=0.020
PL	White fruit	Tul > Oct = GA	P=0.010
PL	White fruit	Hp > Sp	P=0.010
PL	White/red	Tul > Oct = GA	P=0.010
CTR1	Mature green	Tul > Oct = GA	P=0.078
Zf/TF	Immature green	GA < Oct = Tul	P=0.057
Zf/TF	Mature green	GA > Tul = Oct	P=0.051
Zf/TF	White fruit	GA > Tul	P=0.044
Zf/TF	White/red	GA < Oct = Tul	P=0.053
Zf/TF	Ripe fruit	Hp > Sp	P=0.039
Aconitase	White fruit	Oct > Tul = GA	P=0.025
Aconitase	White fruit	Sp > Hp	P=0.039
Aconitase	White/red	Tul < Oct = GA	P=0.060
Aconitase	White/red	Sp > Hp	P=0.037

Note: Data analyzed by ANOVA, and the means were compared by the LSD test at a *P* significance level of 0.05 and 0.10.

Key: Oct, Octavia; Tul, Tulameen; GA, Glen Ample; Hp and Sp represent 'hard' and 'soft' mapping population progeny, respectively.

When the data is represented in the form of histograms (Fig. 14), similar trends of gene expression during fruit development as found with Glen Moy and Latham (Fig. 12) were evident as well as several notable exceptions.

- The mean relative expression levels for Aquaporin and PME show a similar steady rate decline during ripening from immature green to the ripe fruit stage.

  The highest level of Aquaporin expression was detected at the mature green stage in Glen Ample in contrast to the immature green stage in Octavia and Tulameen (including Moy and Latham), and higher levels of Aquaporin and PME were detected from the white to ripe fruit stages in the 'soft' mapping population clones compared to the 'hard' category.

- The relative expression levels for SAMDC show the opposite trend of a steady increase up to the ripe fruit stage.

  The highest SAMDC level was recorded at the ripe fruit stage in Tulameen (soft cultivar), and higher levels were recorded throughout fruit development in the 'soft' mapping population clones.

- Cultivar differences were evident in the expression levels for CTR1.

  The highest levels were detected at the immature green stage for Octavia, mature green stage for Tulameen, and the white/red stage for Glen Ample. Levels of CTR1 expression were higher in the 'soft' mapping population clones apart from at the white/red stage of development.

- The Zf/TF gene showed a high steady state of expression throughout the early developmental stages in the commercial cultivars and mapping population clones, culminating in a reduction at the white /red and ripe fruit stages.
  The highest levels of the Zf/TF gene were detected at the mature green stage in Glen Ample.

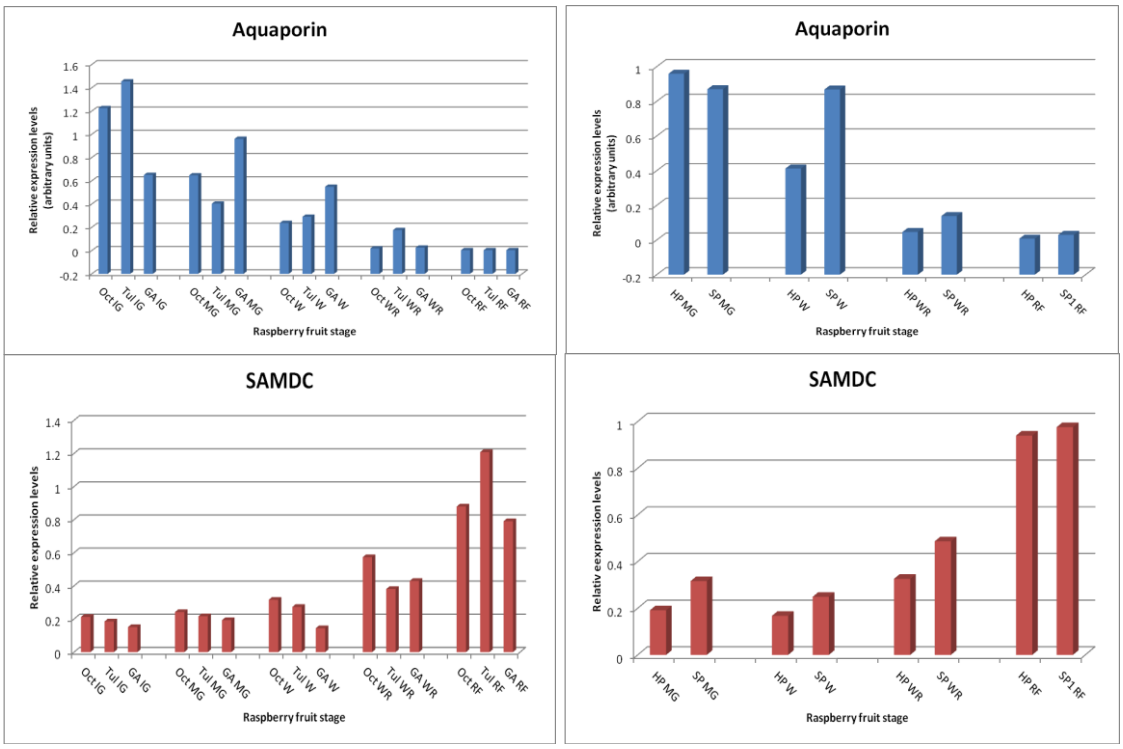
- Levels of Aconitase expression in the commercial cultivars are high at the early developmental stages, lowest at the white to white/red fruit stages, and begin to rise

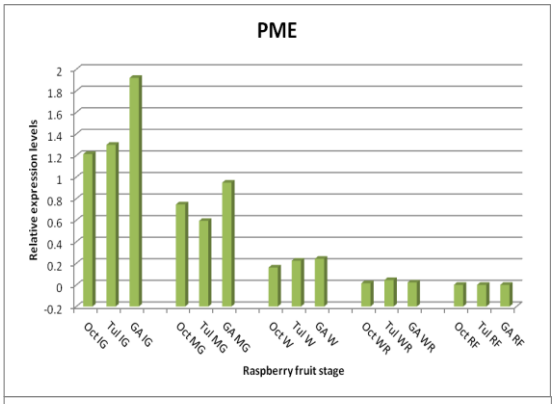
again at the ripe fruit stage. The same pattern of Aconitase expression was detected in both ‘hard’ and ‘soft’ mapping population clones, and levels were higher throughout in the soft progeny.

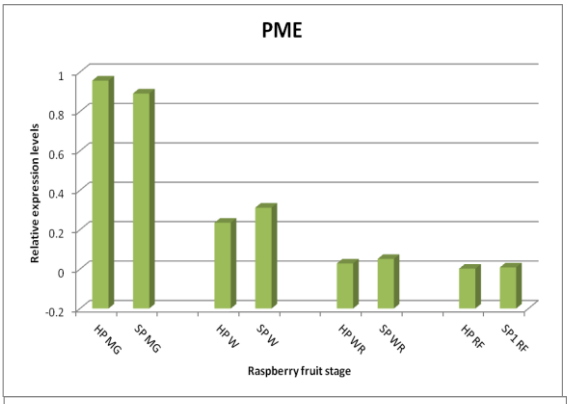
- The relative expression levels for PL in the commercial cultivars and the mapping population clones both show an increase during early fruit development, levels peak at the white to white/red stage, and then decline at the ripe stage (similar to Moy and Latham).

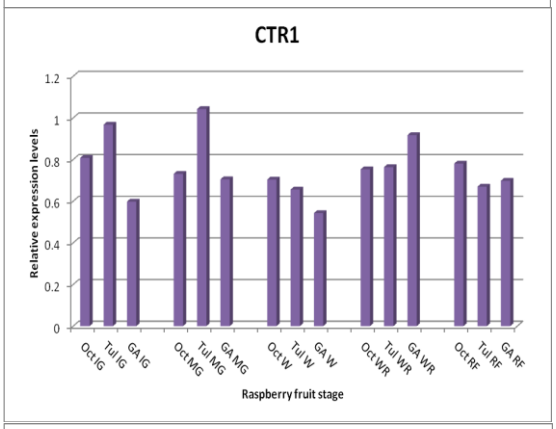
  Significantly higher levels of PL were detected at the white and white/red stages in Tulameen; PL levels were generally higher throughout fruit development in the ‘hard’ mapping population clones.

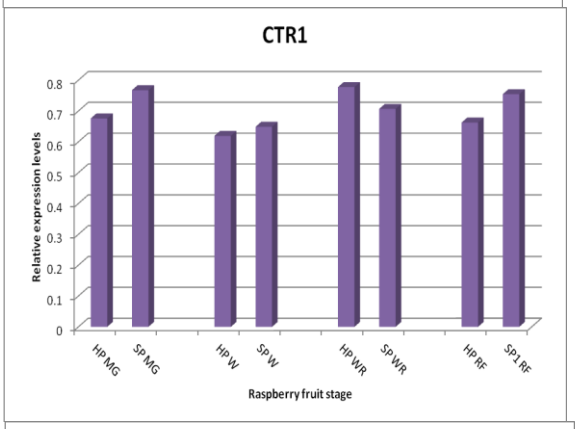
The relative expression levels of the tested candidate genes have indicated that in some cases genotypic differences exist between the different cultivars (Glen Moy, Latham, Octavia, Tulameen, Glen Ample) and the pooled ‘hard’ and ‘soft’ category mapping population clones. This lends further weight to the identification of suitable candidate genes for potential use in a marker assisted selection program.

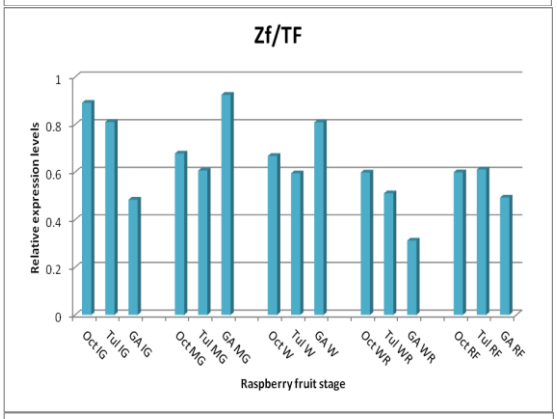


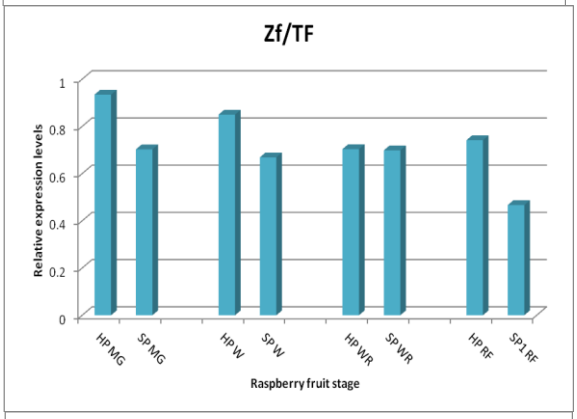


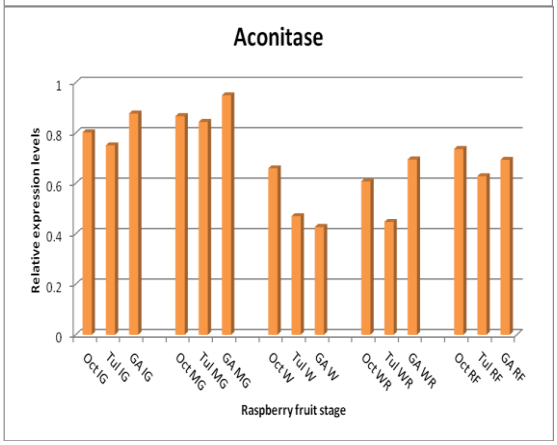


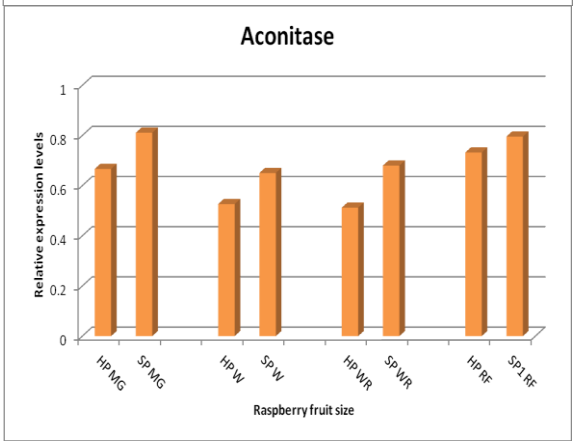












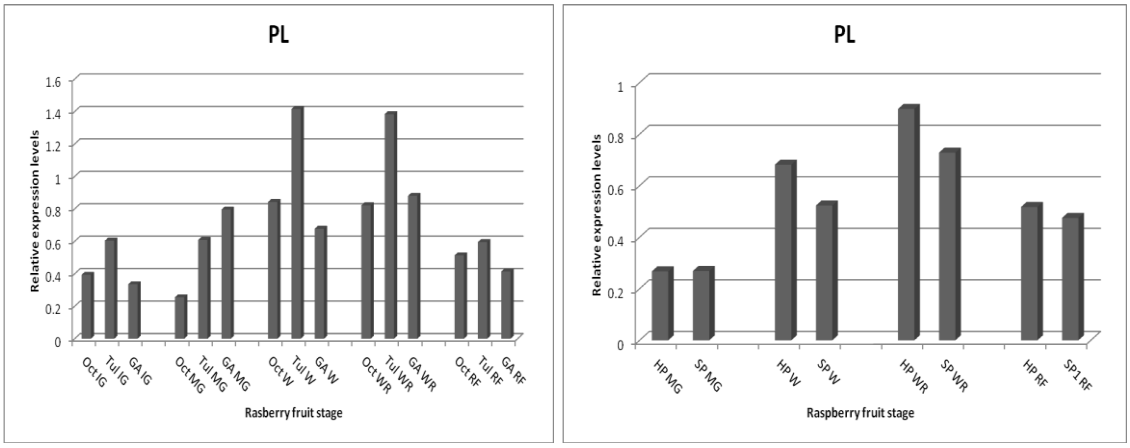


Figure 14. Mean normalized relative gene expression levels obtained for candidate genes tested in three commercial varieties and pooled categories of 'hard' and 'soft' mapping population progeny.
Key: Oct, Octavia; Tul, Tulameen; GA, Glen Ample; HP and SP represent 'hard' and 'soft' mapping population progeny, respectively.
IG, immature green; MG, mature green; W, white fruit; WR, white/red fruit; RF, ripe fruit.

This normalized data was again converted to a log10 scale and further differences are highlighted and similar correlations were evident as determined with Moy and Latham with a scatter plot showing the commercial cultivar/mapping progeny response for each stage of fruit and candidate gene expression levels (Figs. 15 and 16).

- Strong negative relationships between SAMDC and Aquaporin for commercial varieties (r=-0.88) and 'hard' and 'soft' mapping population progeny (-0.77), between SAMDC and PME (r=-0.87 and -0.79, respectively).

- Very strong positive relationships between PME and Aquaporin for commercial varieties (r=0.98) and 'hard' and 'soft' mapping population progeny (r=0.96).

- Slight negative correlation of PL expression with Aquaporin (r=-0.40) and PME levels (r=-0.48) in the hard' and 'soft' mapping population progeny.

- Positive correlation of Zf/TF expression levels with Aquaporin levels (r=0.46) for the commercial varieties; and positive correlation between Zf/TF levels and Aquaporin levels (r=0.47) and PME (r=0.40), and negative correlation with SAMDC levels (r=-053) in the hard' and 'soft' mapping population progeny.

- Negative correlations between Aconitase expression levels and PL levels for commercial varieties (r=-0.45), Aconitase with PL (r=-0.47) and Zf/TF (r=-0.43) in the 'hard' and 'soft' mapping population progeny, and positive correlation between Aconitase expression levels and CTR1 levels for commercial varieties (r=0.42).

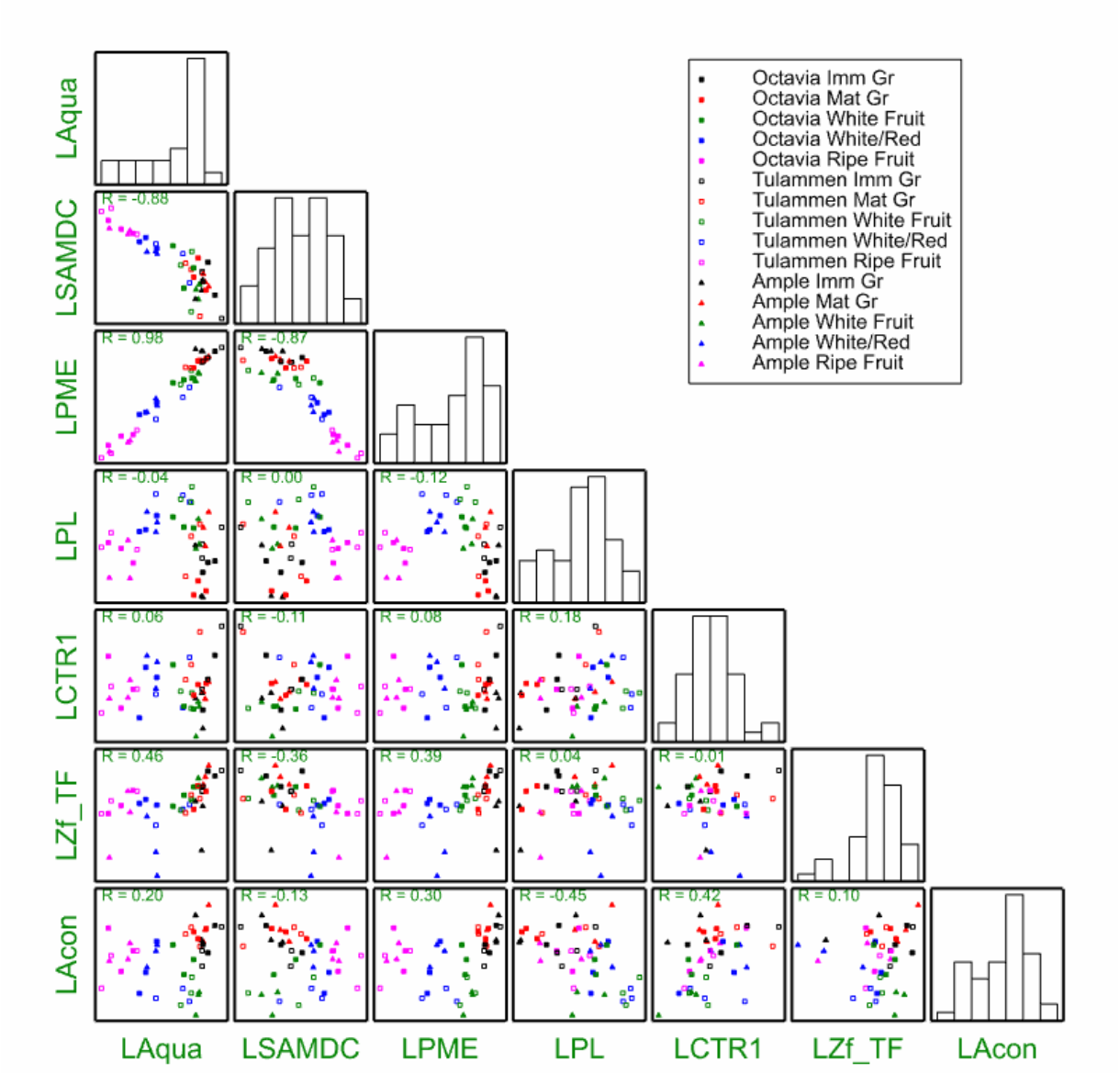


Figure 15. Scatter plots (Log10 scale) categorized by stage of fruit and normalized expression levels of 7 candidate genes for three commercial varieties of raspberry (Octavia, Tulammen, Glen Ample).

Note: The correlation coefficient r-values (see Appendix II), a measure of the strength and direction of the linear relationship between two candidate genes (variables) are given in each square.

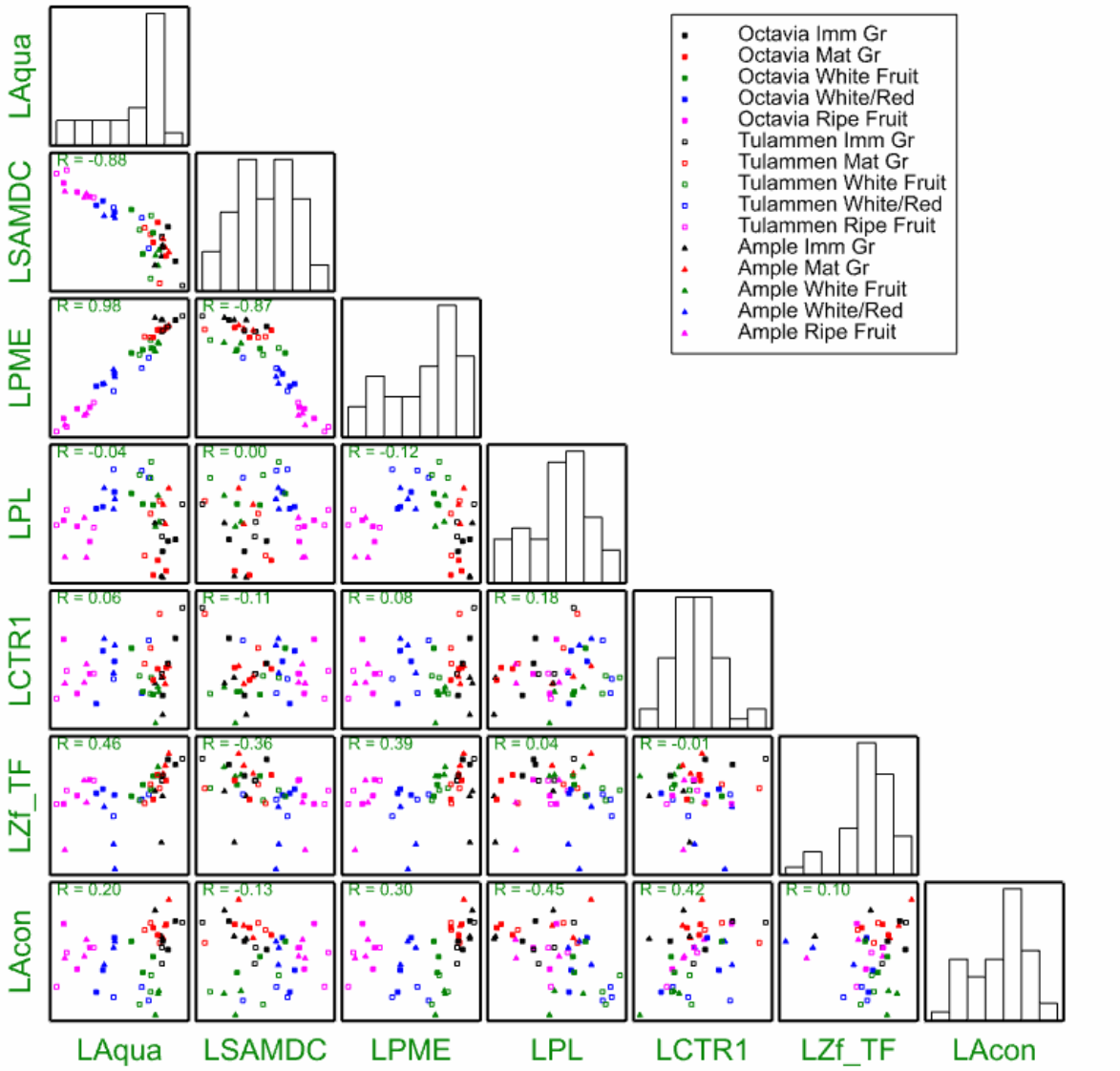


Figure 16. Scatter plots (Log10 scale) categorized by stage of fruit and normalized expression levels of 7 candidate genes for pooled categories of 'hard' and 'soft' mapping population progeny.

Note: The correlation coefficient r-values (see Appendix I), a measure of the strength and direction of the linear relationship between two candidate genes (variables) are given in each square.

This correlation data further indicates a coordinated expression between several of the candidate genes during fruit development and ripening whose differential expression could be related with the variation of fruit firmness. Several factors contribute to the overall fruit texture and firmness (e.g. turgor pressure, cell shape, mechanical properties of cell walls, and the strength and extension of adhesion areas between neighbouring cells). However,

the cell wall disassembly and the reduction of cell adhesion, resulting from dissolution of the middle lamella, are the main factors that cause fruit softening, and at the molecular level, the largest changes in the cell wall occur in the pectin fraction. PME enzymes have been considered to play an important role in cell wall disassembly during fruit ripening by increasing the *in vivo* susceptibility of pectins to hydrolases.

- This data also confirms the same sequence of events during fruit softening in several cultivars of raspberry, whereby there is a high expression of PME at the early stages of fruit development, a subsequent decrease that coincides with increased levels of PME*i*, followed by increased levels of PL and then PG enzymes to break down the accumulation of pectin substrates.

- The inhibition of PME activity coincides with the increased activities of other genes such as SAMDC and XL. Ethylene is a plant hormone that has a central role in raspberry fruit ripening and abscission. SAMDC is a key enzyme in the formation of polyamines and competes with the ethylene biosynthesis pathway gene ACC synthase for the substrate S-adenosyl methionine. SAMDC shows a positive correlation with levels of XL, a common enzyme which hydrolysis glycosidic linkages in the most prominent structural polysaccharides fractions (cellulose and hemicellulose). Together these enzymes may help co-ordinate the process of fruit ripening with the cell wall degradation process.

- It is interesting to note that the highest expression levels of SAMDC occur during the ripe fruit stage as expected, and this coincides with a significant reduction in levels of the ethylene repressor gene CTR1, indicating a deactivation event in response to ethylene signals (Figs 12 and 14).

- Fruit cells regulate their turgor pressure as well as cell wall integrity as they ripen and this will involve the aquaporins, integral membrane proteins which regulate the flow of water and hence turgor pressure. A positive correlation between expression levels of PME involved in cell wall integrity and an increase in aquaporins and subsequent water movement as the fruit develops should allow more exposure of substrates to the actions of hydrolases during alterations in cell wall integrity.

- We can speculate that the remaining candidate genes studied (Zf/TF, IPPI and Aconitase) all play key roles during fruit ripening due to their significant association

with softening QTLs and relationship with the other studied genes, playing roles in either the control of gene expression (Zf/TF) or to contributing to flavour and quality of fruit (IPPI and Aconitase).

Development of an Agilent Rubus microarray

DNA microarray technology was used to identify genes that determine cultivar differences in fruit-firmness by comparing the expression profiles of raspberry cultivars (Glen Moy and Latham) that differ substantially for this trait. For this purpose, a *Rubus idaeus* microarray was developed at JHI using the Agilent dual mode gene expression platform. These arrays were utilised to analyse high-throughput gene expression in a 2-channel format by detection of different fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the same fruit stages (immature green, mature green, white fruit, white/red fruit, ripe fruit) (Fig. 17).

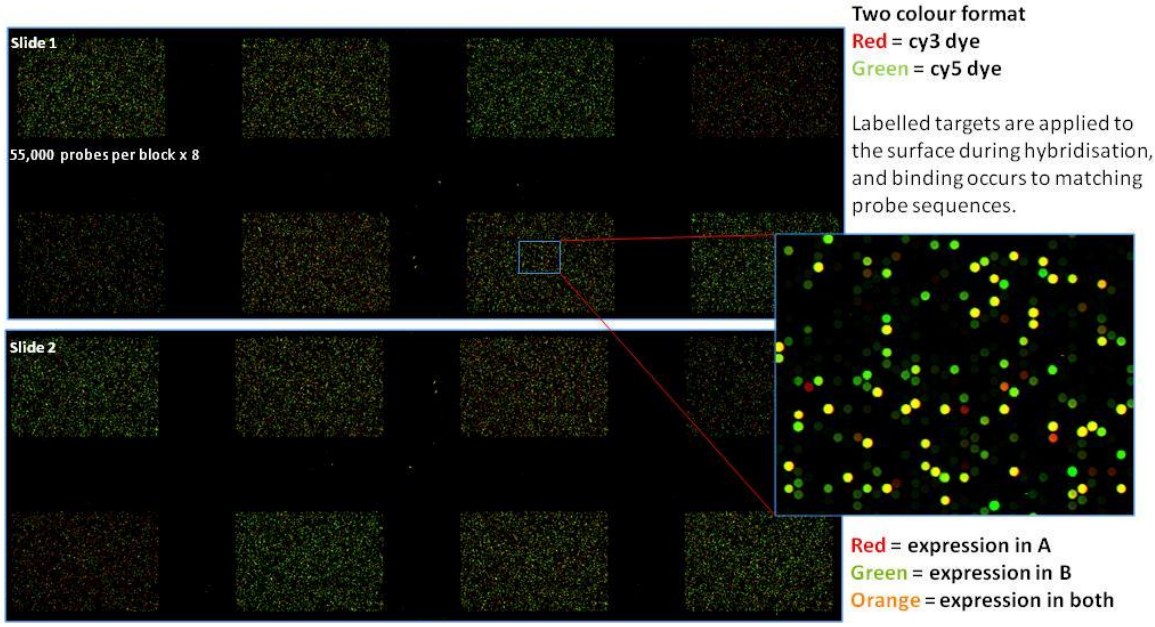


Figure 17. Close up view of the two developed *Rubus idaeus* microarray slides
Note: Level of activity = spot intensity; Relative gene activity (A : B) = colour.

- The array data was annotated by using the top NCBI BLASTx hits for each of the 55,708 unigene probes designed, and around ~36,000 (65%) showed an expression signal within the experiment (Fig. 18).

- Two-way analysis of variance indicated that there are substantial numbers of significant gene expression changes:

~20,000 on the basis of fruit stage, ~8,000 on the basis of genotype, and ~700 have interaction between stage & genotype.

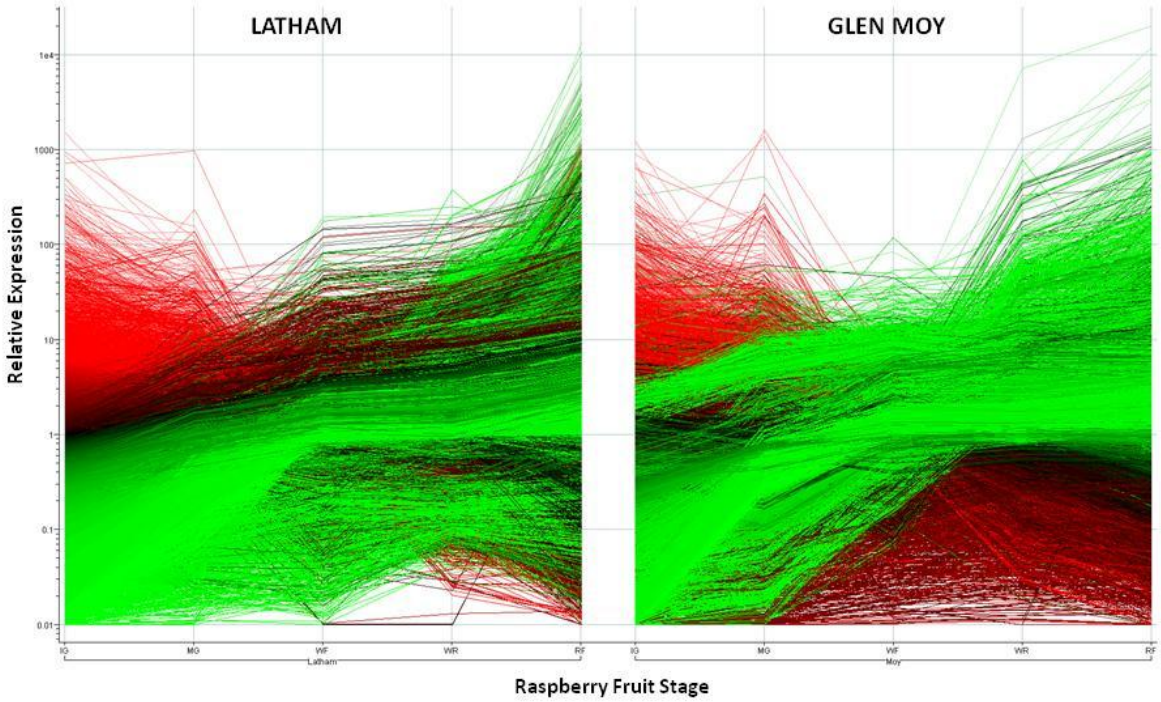


Figure 18. Graphical view of all the reliable expression profiles across each fruit stage in Glen Moy and Latham from the total array data set.

- Probes were clustered using a gene tree and Pearson correlation to generate a heatmap and a list of potentially important fruit softening candidate genes were identified. The list includes genes encoding major latex-like proteins, transferases, transcription factors, proteinases, and a pectinmethyesterase inhibitor.

- As a quality control check, the expression data obtained for each candidate gene tested in the real-time RT-qPCR format was also cross-referenced to the data for the equivalent genes used as probes from the array dataset, and importantly analysis revealed the same expression profiles throughout the different fruit stages in Latham and Glen Moy (see Figs 12 and 14).

Irrigation stress trial 2011-2012

Plants did not yield sufficient fruit for a statistical meaningful data set in season 2011 but it was still possible for the irrigation setup to be successfully implemented to control water stress levels in pots to the desired levels using SM300 sensors and automated ‘timer’ irrigation equipment.

The irrigation experiment was repeated in 2012 using the same system and clones as used previously in 2011. Immature green and mature green fruit stages were sampled from as many replicate pots as possible prior to switching on treatments to act as a control (all standard), and the same stages were collected after water treatments were initiated. Unfortunately due to high winds the polythene had to be removed from the tunnel starting the week (25.09.12) it was planned to sample white, white/red, and ripe fruit stages simultaneously from the plants. Although the polythene was put back up 3 days later, the plants (including fruit) had suffered wind damage so it was not possible to collect these developmental stages. When the water contents in the pots had stabilised, water treatments were turned on and immature green and mature green fruit stages were again sampled to serve as a comparison following wind damage stresses.

- Analysis of variance revealed that there was a significant difference (P<0.001) in water content (% Vol) in pots between the three watering regimes monitored (Fig. 19), but no overall difference between Moy and Latham in terms of water retention properties, or during the different water treatments and/or sampling dates (Fig. 20).

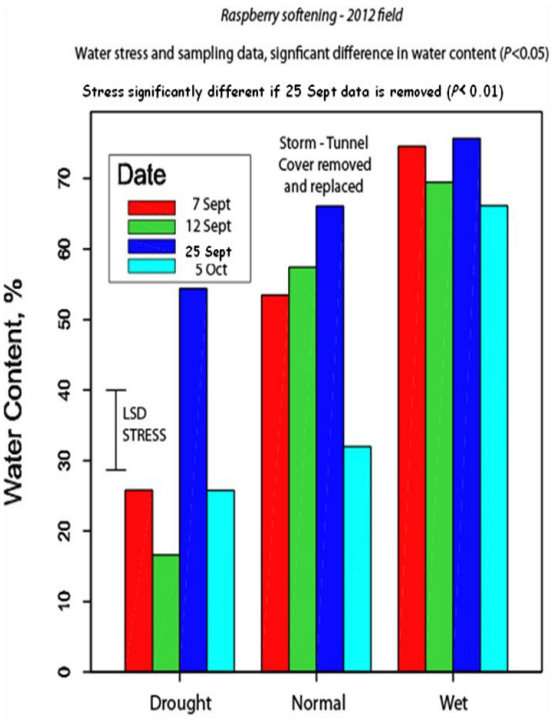


Figure 19. Water content levels (% Vol) recorded in pots during sensor-based irrigation for three different watering regimes over four sample dates in 2012.

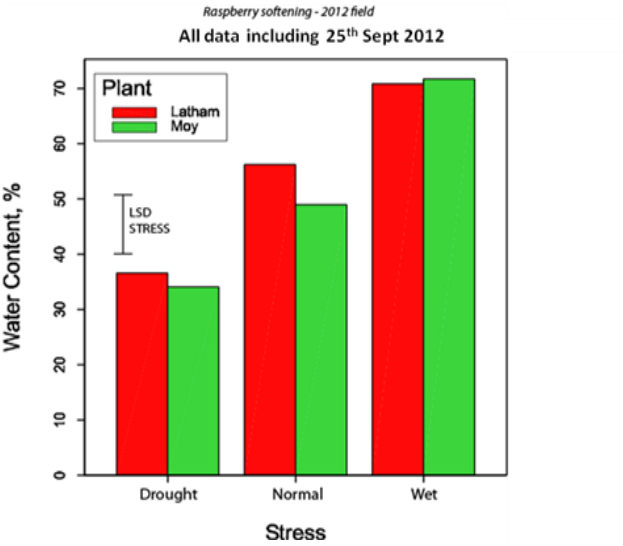


Figure 20. Water content levels (% Vol) recorded in all pots of Glen Moy and Latham during sensor-based irrigation for three different watering regimes and all sample dates in 2012.

RNA was extracted from immature and mature green fruit stages collected from all clones during the three watering regimes at each sampling date, and gene expression analysis was performed using the established *Rubus* microarray to identify potentially important candidate water-stress related proteins. Although the expression variation between replicate samples of each clone prevented accurate statistical analyses, the array successfully resulted in the identification of many potentially important candidate genes involved in water-stress (e.g., plasma membrane proteins, major latex-like proteins, cysteine proteinases, and additional aquaporins) for future study.

Assessment of alternative splicing in raspberry fruit ripening genes

- We found evidence of alternative splicing in Aquaporin, SAMDC, PME, PG and pectate lyase (PL), particularly so in the Aquaporin and PG genes (Fig. 21).

- Transcription levels followed closely the expression levels identified previously in developing fruit stages during RT-qPCR expression studies (Figs 12 and 14).

Regulation of alternative splicing is identified by alteration of alternative splicing ratios between different tissues.

- In all cases, no significant changes in alternative splicing ratios in developing raspberry fruit were detected. Nevertheless as transcription levels increased at

different stages in the fruit the alternatively spliced product also increased to levels that may suggest an alternative function.

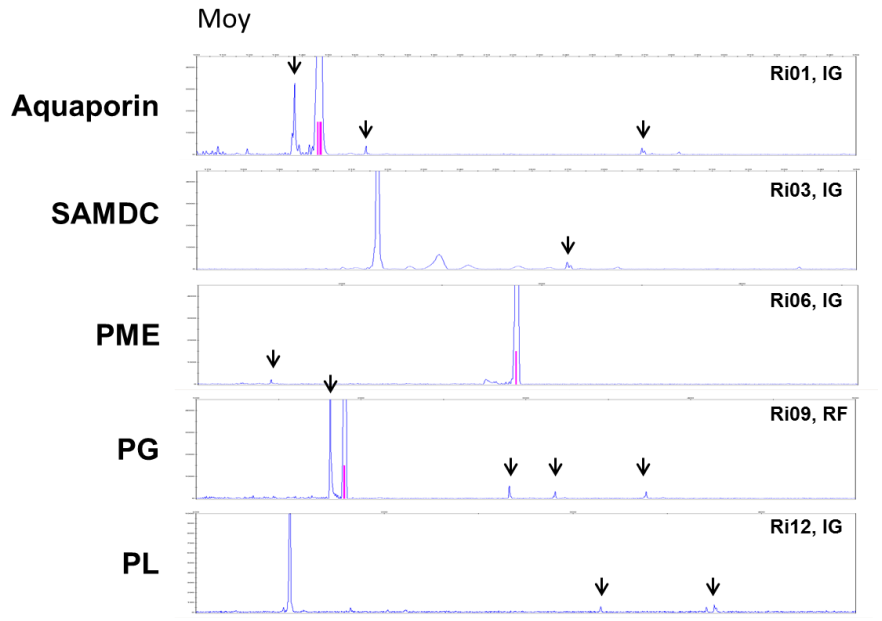


Figure 21. Evidence of alternative splicing in raspberry fruit ripening genes. Arrows indicate the position of putative alternative splicing events. Aquaporin and PG show alternative peaks that represent a significant proportion (5% and 11%, respectively) of the total transcript. Large peaks lacking arrows are the transcript peak sizes expected for fully spliced mRNAs.
Key: Ri numbers are primer pair numbers; IG, Immature green fruit; RF, ripe fruit.

Allelic variation detected in the raspberry SAMDC gene

High resolution RT-PCR analysis of SAMDC identified the transcription of two alleles in the variety Latham (soft) that varied by 3 nucleotides (nt), while Moy (firm) showed only transcription of a single allele (Fig 22). Transcription of these alleles was consistent throughout the five fruit development stages. SAMDC is part of a small multigene family that functions in a key biosynthetic pathways that produces polyamines and ethylene, which has a significant role in fruit ripening. Interestingly, both alleles are transcribed equally with a potential doubling of the amount of translated SAMDC in the softer variety Latham compared to Moy, and hence will be further validated as a potential marker for the 'soft' phenotype (Obj. 6).

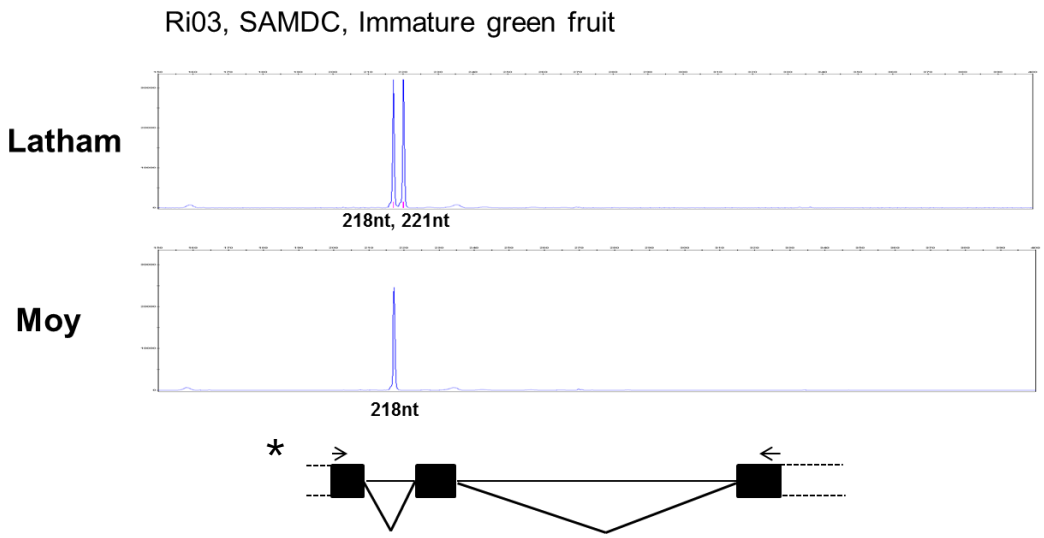


Figure 22. Allelic variation in the raspberry SAMDC gene. High resolution RT-PCR analysis of SAMDC across exons1 to 3 in raspberry varieties Latham and Glen Moy.
Each peak represents a separate transcript. The expected size was 218 bp and the allelic variation shows an increase of 3 nt.

*A schematic diagram of the predicted gene structure of SAMDC across exons 1 to 3 for Moy is drawn to scale. Black boxes are the exon sequences covered by the RT-PCR analysis, introns are indicated as lines between boxes. Expected splicing events are shown.

Objective 6. Validate robust genetic markers and gene sequences (Obj. 5) by comparison with raspberry germplasm (and with other members of the Rosaceae and grape).

Introduction

Raspberry is a high value horticultural crop, interest in which is expanding due to the beneficial impact on human health. 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.

Conventional plant breeding in woody perennials like red raspberry, a member of the Rosaceae, is a long and expensive process (Graham and Jennings, 2009). However advances in molecular genetics, in particular the development of molecular markers, linkage maps, and QTL that can be employed within traditional plant breeding programmes have

the potential to greatly speed up this process. This project aimed to develop markers linked to the key quality attribute of firmness.

Materials and methods

The candidate genes Aquaporin, Aconitase, CTR1, SAMDC, and XL (Obj. 5) were initially selected for use in a marker assisted selection program due to the significant association to softening QTLs ($P<0.001$) and QTL interval mapping analysis by MapQTL indicated that the LOD (logarithm of the odds) scores were greater than >3.0. The role of these genes throughout fruit development was validated by detecting significant differences in expression levels among different raspberry genotypes.

The original PCR primers used for mapping these genes onto the Rubus linkage map were initially tested on DNA from ten different commercial cultivars (Autumn Bliss, Autumn Treasure, Glen Ample, Tulameen, Octavia, Glen Lyon, Glen Doll, Meeker, Malling Delight, Loch Fyne) plus Glen Moy and Latham.

Results & Discussion

The majority of cultivars produced single PCR products for each assay (Fig. 23), these were purified and sequenced to identify and confirm the appropriate polymorphic marker.

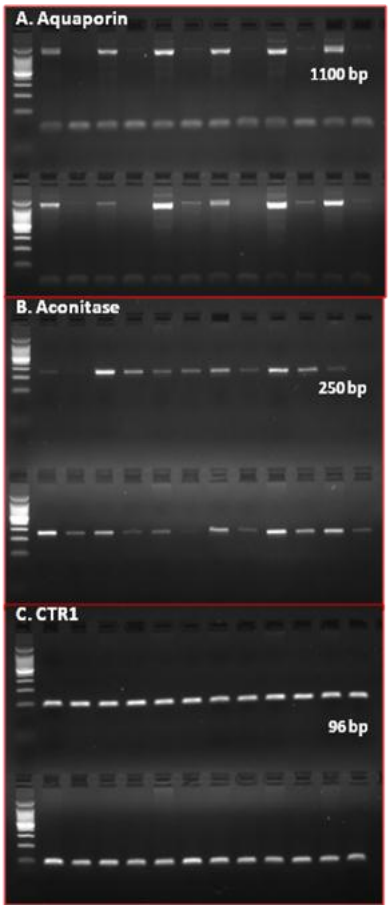


Figure 23. PCR amplification of specific products of several candidate genes (A. Aquaporin), B. Aconitase, and C. CTR1) in different commercial raspberry cultivars.
Lanes show the same sequence of samples tested in duplicate (neat and 1/10 diluted gDNA) for each assay: Upper lanes, 100bp marker; Latham, Glen Moy, Octavia, Glen Ample, Tulameen, Autumn Bliss.
Lower lanes, 100bp marker; Glen Lyon, Glen Doll, Meeker, Malling Delight, Loch Fyne, Autumn Treasure.

Genotyping for the indel marker (size polymorphism) used for mapping Aquaporin, Aconitase (example shown in Fig. 24), and SAMDC has shown association with a 'firm' or 'soft' fruit phenotype. For example, the indel marker (size polymorphism) used for mapping Aconitase was detected in the 'firm' varieties Autumn Bliss (heterozygous), Malling Delight (homozygous) as well as Latham (heterozygous) (Fig. 24) and further validation will proceed by testing additional cultivars and genotyping. The sequences for both Latham and Autumn Bliss were mixed after the insertion due to the presence of both alleles. The SNP markers used for mapping the CTR1 and XL genes are being tested in a wider range of genotypes for SNP association with phenotype.

These markers and possibly other candidate genes already studied (see Obj. 5.) and others identified from microarray analysis (Obj. 5) will be taken forward into breeding populations for confirmation for marker assisted selection programs.

Position of size marker (indel) in Aconitase gene

Figure 24. Alignment of the portion of sequence containing the polymorphic markers for the Aconitase gene. Arrow indicates position of polymorphic marker.

*= identical bases within a column of the aligned portion of gene sequence.

Note: ERubLR_SQ12.2_C05_022 is the original Latham root sequence used to map the gene, and the corresponding sequence retrieved from the recent 454 dataset is Contig4266.

Development of a marker assisted toolkit for the softening trait is now possible through the rigorous characterization of candidate genes significantly associated with softening QTLs. DNA markers for screening commercially acceptable traits in new selections will greatly improve the accuracy of breeding and will reduce the time involved in the development of new cultivars by at least 3 years per cross.

General Discussion

This Horticulture LINK project aimed to develop robust assisted breeding and selection tools that would enable breeders to accelerate development of new fruit varieties with extended shelf-life to reduce fruit spoilage. To achieve this aim, and identify the genetics of fruit softening, the project utilized a 'Latham' × 'Glen Moy' mapping population together with the latest generation of genomic tools (454 mRNA-seq, genotyping, microarrays, and alternative splicing panels).

Molecular tools were used to generate an updated linkage map for *Rubus* and increased our understanding of the timing of expression of some of the key candidate genes involved in the softening process. Variation in these genes in different cultivars and mapping populations lead to variation in fruit softening and provided several candidate DNA markers for targeted breeding of new fruit cultivars within the UK industry. Red raspberry was used as a model to study softening in the *Rosace*ae but the results will be valuable to other soft fruits including strawberry.

Understanding the genetic control of major soft fruit traits in raspberry was advanced through the identification of QTLs, large scale sequencing in both targetted chromosome regions and in expressed sequences (454 mRNA-seq) to provide a 'fruit transcriptome', development of a comprehensive *Rubus idaeus* microarray, and validation of candidate gene markers by expression analysis (RT-qPCR). This has resulted in databases of genomic and expressed sequence information from different stages of fruit and a *Rubus* microarray for research which can be used for selective breeding to increase accuracy of variety development by selecting markers rather than phenotypes.

Raspberry firmness was measured quantitatively for the first time using a QTS-25 Texture Analyzer by testing ripe fruit collected from both field and polytunnel production over two years. This study validated the QTS-25 Texture Analyzer as a reliable quantitative measurement of fruit firmness that is comparable with a 'breeder score' for firmness and can be reliably used to identify chromosomal regions for trait information. Objective1 was successfully completed as the trait data collected was assigned to the *Rubus* genetic linkage map using MapQTL software.

The QTS-25 Texture Analyzer was also successfully used to study firmness and shelf-life characteristics of ripe fruit collected from different raspberry cultivars and mapping population progeny over 7 days storage at 4^{o}C. Twenty-two different mapping progeny and

five different varieties (Glen Moy, Latham, Glen Ample, Octavia and Tulameen) showed a range of 'firmness' scores indicating genotypic differences; Glen Moy, Octavia and 7 mapping progeny were consistently firmer during storage at 4^{o}C indicating a better shelf-life.

The 'shock forces' encountered by punnets of raspberry fruit during transport recorded by a Tinytag shock logger indicated that significant vertical forces (up to 8 'g') but not horizontal forces were experienced during transport from the field roads to the packhouse. A laboratory trial to mimic 'shock forces' experienced by fruit during transportation indicated that the shock treatment had a more pronounced impact on storage of a 'firm' variety (Octavia) compared to a 'soft' variety (Tulameen), indicating that as well as firmness, the size, shape and flexibility of the fruit may play an important role in potential shelf-life characteristics.

These results further validated the usefulness of the QTS-25 Texture Analyzer for quantifying firmness in raspberry during transit through the supply chain and storage, and it is recommended that cultivars with a 'breeder score' of firmness from 1-2 (equivalent to Hardness readings >0.6 Newtons) are more likely to maintain the desired quality. This study also highlighted the need for more comprehensive monitoring of 'shock forces' on fruit during transport in order to predict more accurately the potential impact on the shelf-life of fruit.

For the first time a raspberry 'fruit transcriptome' was successfully generated using next generation sequencing technology (454 mRNA-seq) from total RNA extracted from white/red and red fruit stages of Latham and Glen Moy. Candidate genes with expected roles in cell wall hydrolysis, water movement, fruit ripening and cell wall flexibility were subsequently identified using 454 datasets, together with in-house (*Rubus*) and public sequence databases.

Data for 20 different genes associated with fruit softening were successfully mapped onto the JHI *Rubus* genetic linkage map using the JoinMap programme to produce an updated version and successfully complete objectives 3 and 4. These candidate genes were distributed across all 7 *Rubus* linkage groups, with the majority located on LG 3, 5 and 7. Mapping analysis indicated that the most significant markers associated with each of the softening QTLs were: Pectinmethylesterase (PME), β-1,4 xylan hydrolase (XL1), Aquaporin, S-Adenosylmethionine decarboxylase (SAMDC), Constitutive triple response1-like protein kinase (CTR1; a negative regulator of the ethylene response pathway), Zinc finger

protein/transcription factor (Zf/TF; similar role as MADS box genes), Isopentenyl pyrophosphate isomerase (IPPI) and Aconitase.

In an effort to produce consistent and high quality data from RT-qPCR studies, the *m*inimum *i*nformation for publication of *q*uantitative real-time PCR *e*xperiments (MIQE) guidelines (Bustin et al. 2009) were followed in this study. The end result was the development of a reliable raspberry fruit RNA extraction procedure which incorporated the use of a TissueLyser (Qiagen) for disruption and purification using the RNeasy Plant Mini Kit (Qiagen) and the automated QIAcube robot system, and a robust pipeline for designing and evaluating PCR assays for RT-qPCR experiments. The geNorm software indicated that the novel reference genes Clathrin, YLS8, and TIP41 were the most suitable for accurate normalization with fruit samples, and this is the first report of a systematic validation of 10 reference genes for gene expression studies in *Rubus*.

Gene expression studies with 11 candidate genes (PME, PMEi, PG, PL, XL, Aquaporin, SAMDC, CTR1, Zf/TF, IPPI, and Aconitase) implicated in fruit softening were carried out with five different stages of raspberry fruit harvested from Glen Moy, Latham, Octavia, Tulameen, Glen Ample, and several clones of the mapping population representing 'soft' and 'firm' fruit categories. Although similar trends of gene expression during fruit development were evident, analysis of variance highlighted significant differences ($P<0.05$) in gene expression levels between the 'soft' and 'firm' genotypes at some different fruit stages. For example, relative expression levels for Aquaporin and PME showed a similar steady rate decline during ripening from immature green to the ripe fruit stage, whereas the levels for SAMDC, PG and PMEi showed the opposite trend of a steady increase up to the ripe fruit stage. Repeated measures ANOVA for each combined gene expression datasets per genotype also indicated a strong interaction ($P<0.05$) between cultivar and fruit developmental stage for the genes PG, PMEi, Zf/TF, and in particular, CTR1 and Aconitase, further implying differences between genotypes at different stages.

Correlation data indicated a coordinated expression between several of the candidate genes during fruit development and ripening whose differential expression could be related with the variation of fruit firmness. PME enzymes play an important role in cell wall disassembly during fruit ripening by increasing the *in vivo* susceptibility of pectins to hydrolases. The data confirmed the same sequence of events during fruit softening in raspberry, whereby there is a high expression of PME at the early stages of fruit development, a subsequent decrease that coincides with increased levels of PME*i*, followed by increased levels of PL and then PG enzymes to break down the accumulation of pectin

substrates. The inhibition of PME activity also coincided with the increased levels of SAMDC and XL. SAMDC shows a positive correlation with levels of XL, a common enzyme which hydrolysis glycosidic linkages in the most prominent structural polysaccharides fractions (cellulose and hemicellulose). Together these enzymes may help co-ordinate the process of fruit ripening with the cell wall degradation process.

Fruit cells regulate their turgor pressure as well as cell wall integrity as they ripen and this requires aquaporins, which regulate water flow and hence turgor pressure. A positive correlation between expression levels of PME involved in cell wall integrity and an increase in aquaporins and water movement as the fruit develops should allow more exposure of substrates to the actions of hydrolases. The RNA extraction and RT-qPCR assay quality control pipeline was successfully carried out to validate transcription and post-transcriptional processing of candidate genes identified within the softening QTLs and therefore complete objective 5.

The large increase in raspberry transcriptome sequence assembled permitted the development of a comprehensive *Rubus idaeus* microarray using the Agilent dual mode gene expression platform to globally measure changes in gene expression as fruit develops and softens. Two arrays (8 x 60k format) were initially run to determine the relative abundance of nucleic acid sequences in the various fruit developmental stages (immature green, mature green, white, white/red, and ripe fruit) collected from 3 biological replicates of Glen Moy and Latham. Around ~36,000 (65%) of the 55k unigene probes designed for the array showed an expression signal, and analysis of variance indicated that there are substantial numbers (1000s) of significant gene expression changes within the experiment.

In order to focus on a reduced number of important candidate softening genes, an additional array was run by pooling RNA from clones of the 'Latham' × 'Glen Moy' mapping population representing ‘soft’ and ‘firm’ fruit categories. A total of 158 array probes showed a significant difference in expression (P<0.005) between the ‘firm’ and ‘soft’ clones from the mature green and white/red fruit stages tested. As well as including some of the previously tested genes, such as Aquaporins, PME, SAMDC, this array allowed us to identify other potential candidate genes as markers and included major latex-like proteins, transferases, more transcription factors, proteinases, and another pectinmethyesterase inhibitor.

This project has developed a number of important expression tools that will be used in future analysis of raspberry. The 454 mRNA sequencing produced a comprehensive database of fruit-related gene sequences (‘fruit transcriptome’) that can be mined for genes

known to be involved in other fruit quality traits in addition to softening. We further developed a *Rubus* microarray that will be utilised in many experiments to follow changes in raspberry gene expression in response to changing conditions, stages of development and in specific tissues. Reliable methods for validation and gene expression studies using RT-qPCR is an essential protocol development that will be used widely for studying expression of genes involved in quality traits (e.g., aroma, flavour, biotic and abiotic resistance) in fruit.

An irrigation trial was successfully set up to assess the effects of water stress on fruit softening under a polytunnel consisting of pots of six mapping population progeny plus Glen Moy and Latham. Analysis of variance revealed that there was a significant difference ($P<0.001$) in water content (% Vol) in pots between the three watering regimes monitored (standard, over-watered, and drought) demonstrating the reliability and consistency of the SM300 sensor-based system. Gene expression analysis on immature green and mature green stages collected during the three watering regimes in 2012 was performed using the established *Rubus* microarray. This analysis successfully resulted in the identification of many potentially important candidate genes involved in water-stress (e.g., plasma membrane proteins, major latex-like proteins, cysteine proteinases, and additional aquaporins).

Alternative splicing is a key area of functional genomics that needs to be consistently addressed during gene expression studies and this is the first time that it has been systematically studied in a crop species with its role in the expression of biologically important genes involved in fruit development. PG has previously been shown to show varietal differences in splicing in strawberry and associated with fruit firmness. We found alternative splicing in 5 of 7 genes studied, including PG, in Latham and Glen Moy during fruit development, but, found that although the transcription levels changed throughout fruit development alternative splicing patterns were maintained. These alternative splicing events occur in important regions of the gene and it will be interesting in future to determine if environmental conditions (e.g., temperature and water stresses) lead to changes in splicing and regulation of fruit development under different environmental stresses. The identification of key alternatively spliced genes that have a role in fruit firmness will allow screening of raspberry germplasm from the breeding gene pool that have mutations that disrupt splicing, leading to changes in functional proteins.

The assessment of alternative splicing in raspberry fruit identified the transcription of two alleles for SAMDC in Latham compared to transcription of a single allele in Moy, indicating a doubling of SAMDC mRNA transcripts in Latham, which may be translated into higher levels

of translated SAMDC in the softer variety Latham compared to Moy. Latham is heterozygous for many genes and it will be important to determine the role variable alleles have on the levels of functional gene expression and in this case fruit softening. This result is being tested further across different genotypes to determine an association of the two alleles with softer fruit.

Fruit softening is a complex trait that relies on a combination and interaction between different physical and molecular processes involved in fruit ripening. A future priority will be to develop the most reliable combination of markers for deployment in marker assisted breeding (MAB) for the 'soft' and 'firm' phenotype in red raspberry taking fruit resilience in transport into account. We have established a number of key raspberry genes expressed during fruit development and identified indel and SNP variants in these genes between Latham (soft) and Glen Moy (firm). The markers accounting for the most significant impact on the phenotype will be combined and tested in additional raspberry germplasm and breeding populations to strengthen the association of these genes and markers with fruit softening.

Development of a marker assisted toolkit for the softening trait is now possible through the rigorous characterisation of candidate genes significantly associated with softening QTLs. DNA markers for screening commercially acceptable traits in new selections will greatly improve the accuracy of breeding and will reduce the time involved in the development of new cultivars by at least 3 years per cross. The availability of robust markers associated with both fruit softening and fruit quality will lead to the identification of varieties that combine firmness with improved taste to all growers via the HDC breeding programme. The markers will be tested in the HDC-funded raspberry breeding programme during 2013-14. This will be part of the validation process as the effectiveness for the markers at predicting fruit softening will be monitored. The markers developed here, along with markers for root rot and fruit quality developed previously under LINK research, will lead to an unrivalled suite of raspberry markers available for targeted breeding of new fruit cultivars available to the UK industry through the raspberry breeding consortium.

Robust marker assisted/gene breeding tools will be developed from the selected markers, genes, QTLs, gene expression and knowledge found in this proposal. These tools, linked to fruit softening, will identify and improve available germplasm through advanced breeding strategies, improve longevity of fruit quality with a reduction in agricultural, retail and household waste to landfill. In addition, financial savings in the retail industry alone could reach £2.5 million annually for soft fruit with additional savings at the farm. Increased shelf-

life will also further enhance the reputation of UK fruit as a high quality product. Many of the genes identified may be important in the softening process for other *Rosaceous* soft fruit (and non-soft fruit species), providing added value as information on markers, genes and alternative splicing events that may be applied to other species.

Conclusions

To achieve the aim of developing robust assisted breeding tools for accelerated variety development and identify the genetics of fruit softening, the project utilized a 'Latham' × 'Glen Moy' mapping population together with the latest generation of molecular tools (454 mRNA-seq, genotyping, microarrays, and alternative splicing panels).

- This study validated the QTS-25 Texture Analyzer as a reliable quantitative measurement of fruit firmness for the first time and is comparable with a ‘breeder score’ and can be reliably used to identify chromosomal regions for trait (QTL) information.

- The QTS-25 Texture Analyzer can be successfully used to study firmness and shelf-life characteristics of ripe fruit through the supply chain and identify significant genotypic differences in firmness during storage for 7 days at 4^{o}C.

- The vertical ‘shock forces encountered by punnets of raspberry fruit during transport from the field roads can be significant. A laboratory trial to mimic ‘shock forces’ indicated that the shock treatment had a more pronounced impact on storage of a ‘firm’ variety compared to a ‘soft’ variety, indicating that as well as firmness, the size, shape and flexibility of the fruit may play an important role in potential shelf-life characteristics.

- It is recommended that raspberry cultivars with a ‘breeder score’ of firmness from 1-2 (equivalent to Hardness readings >0.6 Newtons) are more likely to maintain the desired quality through the supply chain.

- This study also highlighted the need for a more comprehensive monitoring of ‘shock forces’ on fruit during transport, particularly on field roads at farms, in order to predict more accurately the potential impact on the shelf-life of fruit.

This project has developed a number of important expression tools that will be used in future analysis of raspberry and other soft fruits.

- The 454 mRNA next generation sequencing produced a comprehensive database of fruit-related gene sequences ('fruit transcriptome') for the first time that can be mined for genes known to be involved in other fruit quality traits in addition to softening.

- Many 100s of candidate genes of a high abundance with expected roles in cell wall hydrolysis, water movement, fruit ripening and cell wall flexibility were identified using the 454 datasets.

- Candidate genes were distributed across all 7 *Rubus* linkage groups, with the majority located on LG 3, 5 and 7.

- We further developed a *Rubus idaeus* microarray using an Agilent dual mode gene expression platform that can be utilised in many experiments to follow changes in raspberry gene expression in response to changing conditions, stages of development and in specific tissues.

- The array can identify many cases of gene expression levels decreasing and increasing throughout fruit development and obvious genotypic differences.

- Reliable methods for validation and gene expression studies using real time RT-qPCR is an essential protocol development. The development of a reliable raspberry fruit RNA extraction procedure and a robust pipeline for designing and evaluating RT-qPCR assays can be used widely applied for studying expression of genes involved in many quality traits (e.g., aroma, flavour, biotic and abiotic resistance) in soft fruit.

Molecular tools were used to generate an updated linkage map for *Rubus* and increased our understanding of the timing of expression of some of the key candidate genes involved in the softening process throughout different raspberry genotypes.

- Mapping analysis indicated that the most significant markers associated with the softening QTLs were: Pectinmethylesterase (PME), β-1,4 xylan hydrolase (XL1),

Aquaporin, S-Adenosylmethionine decarboxylase (SAMDC), Constitutive triple response1-like protein kinase (CTR1; a negative regulator of the ethylene response pathway), Zinc finger protein/transcription factor (Zf/TF; similar role as MADS box genes), Isopentenyl pyrophosphate isomerase (IPPI) and Aconitase.

- Correlation data indicated a coordinated expression between several of the candidate genes during fruit development and ripening whose differential expression could be related with the variation of fruit firmness.

- The irrigation trial set up with pots to assess the effects of water stress on fruit softening demonstrated the reliability and consistency of the SM300 sensor-based system to monitor water levels in the growth media and control irrigation via data loggers to provide standard, over-watered and drought treatments.

- *Rubus* microarray expression analysis on immature green and mature green fruit stages collected during the three watering regimes successfully resulted in the identification of many potentially important candidate genes involved in water-stress (e.g., plasma membrane proteins, major latex-like proteins, cysteine proteinases, and additional aquaporins).

- Alternative splicing events do occur in candidate softening genes studied in Latham and Glen Moy during fruit development, but although the transcription levels changed throughout fruit development alternative splicing patterns were maintained.

- The identification of key alternatively spliced genes that have a role in fruit firmness will allow screening of raspberry germplasm from the breeding gene pool that have mutations that disrupt splicing, leading to changes in functional proteins.

- The assessment of alternative splicing identified the transcription of two alleles for SAMDC in Latham compared to transcription of a single allele in Moy, indicating a doubling of SAMDC mRNA transcripts in Latham, which may be translated into higher levels of translated SAMDC and contribute to a reduced firmness in the softer variety Latham compared to Moy.

Fruit softening is a complex trait that relies on a combination and interaction between different physical and molecular processes involved in fruit ripening. A future priority will be

to develop the most reliable combination of markers for deployment in marker assisted breeding (MAB) for the ‘soft’ and ‘firm’ phenotype in red raspberry taking fruit resilience in transport into account.

- We have established a number of key raspberry genes expressed during fruit development and identified indel and SNP variants in these genes between Latham (soft) and Glen Moy (firm).

- The DNA markers accounting for the most significant impact on the softening phenotype will be combined and tested in additional raspberry germplasm and breeding populations to strengthen the association of these genes and markers with fruit softening to fully complete objective 6.

- Through this project the development of a marker assisted toolkit for the softening trait is now possible through the rigorous characterisation of identified candidate genes significantly associated with softening QTLs.

- Many of the genes identified may be important in the softening process for other *Rosaceous* soft fruit (and non-soft fruit species), providing added value as information on markers, genes and alternative splicing events that may be applied to other species.

Knowledge and Technology Transfer

Industry Flyer:

D. W. Cullen, M. Woodhead, H. A. Ross, C. G. Simpson, P. D. Hallett, C. A. Hackett & J. Graham (2011). Developing breeding and selection tools to reduce spoilage of soft fruit and wastage in the supply chain. Horticulture LINK (HL0195).

Presentations:

Graham, J. (2010). Understanding the genetics of raspberry fruit quality for improved consumer appeal. Annual Report of the Scottish Crop Research Institute, p28-29.

Paul Hallett (2010). Fruit for the Future event, SCRI, UK.

Danny W Cullen (2011). Fruit for the Future event, The James Hutton Institute, UK.

Danny W Cullen (2012). Fruit for the Future event, The James Hutton Institute, UK.

Danny W Cullen (2012). SSCR/Bulrush Soft Fruit Winter Meeting, Dundee, UK.

Danny W Cullen (2013). Fruit Quality in Red Raspberry (W308). Plant & Animal Genome (PAG) XXI Conference, San Diego, CA, USA, Jan 12-16 2013.

A poster was displayed at the following meetings:
D.W. Cullen, M. Woodhead, H. A. Ross, C. G. Simpson, P. D. Hallett, C. A. Hackett & J. Graham (2012). Developing Breeding and Selection Tools to Reduce Spoilage of Soft Fruit and Wastage in the Supply Chain. Abstracts of the International Conference Molecular Mapping & Marker Assisted Selection, Vienna, Austria, February 8-11 2012, N10.

D.W. Cullen, M. Woodhead, H. A. Ross, C. G. Simpson, P. D. Hallett, C. A. Hackett & J. Graham 2012. Developing Breeding and Selection Tools to Reduce Spoilage of Soft Fruit and Wastage in the Supply Chain. Bulrush Horticulture Limited and Scottish Society for Crop Research Information Day/Winter Meeting, Landmark Hotel, Dundee, Wednesday 15th February 2012.

D.W. Cullen, M. Woodhead, H. A. Ross, C. G. Simpson, P. D. Hallett, C. A. Hackett & J. Graham (2012). Developing Breeding and Selection Tools to Reduce Spoilage of Soft Fruit and Wastage in the Supply Chain. Autumn Growers' Meeting and Technical Conference, Berry Gardens Growers Ltd., Telford Hotel & Golf Resort, Great Hay Drive, Sutton Heights, Telford, Shropshire TF7 4DT, November 14-15th 2012.

Publications:
HDC Soft Fruit Review 2010/11.

HDC Soft Fruit Review 2011/12.

K. A. Oduse (2010). An Investigation into the Fruit Firmness Properties of some Progeny and Cultivars of Red Raspberry (*Rubus idaeus*). MSc in Food Science & Microbiology, University of Strathclyde.

K. A. Oduse & D. Cullen (2012). An Investigation into the Fruit Firmness Properties of some Progeny and Cultivars of Red Raspberry (*Rubus idaeus*). *IOSR Journal of Environmental Science, Toxicology and Food Technology,* **1**: 04-12.

D. W. Cullen (2012). Final LINK programme (Horticulture) Report. *Developing breeding and selection tools to reduce spoilage of soft fruit and wastage in the supply chain* **HL0195.**

D. W. Cullen, S. McCallum, A. Paterson, P. Hedley, C. Simpson. L. Milne. J. Graham (2013). Fruit Quality in Red Raspberry (W308). Abstracts of the International Conference Plant & Animal Genome (PAG) XXI, San Diego, CA, USA, January 12-16 2013, p. 163.

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Appendices

Appendix I. Randomised block design layout of raspberry clones in pots to one row under a polytunnel for irrigation stress trial 20011-2012.

Block	Stress	Clone
1	3	R254
1	3	R104
1	3	R234
1	3	R248
1	3	R184
1	3	LATHAM
1	3	R7
1	3	GLEN MOY
1	1	R254
1	1	R248
1	1	R7
1	1	GLEN MOY
1	1	LATHAM
1	1	R234
1	1	R184
1	1	R104
1	2	R254
1	2	R7
1	2	R248
1	2	LATHAM
1	2	R104
1	2	R184
1	2	R234
1	2	GLEN MOY
2	1	R234
2	1	GLEN MOY
2	1	R254
2	1	R184
2	1	LATHAM
2	1	R7
2	1	R248
2	1	R104
2	3	R248
2	3	R104
2	3	R234
2	3	R184
2	3	R254
2	3	GLEN MOY
2	3	R7
2	3	LATHAM
2	2	GLEN MOY
2	2	R254
2	2	R7
2	2	R234
2	2	R184
2	2	LATHAM
2	2	R248
2	2	R104
3	2	LATHAM
3	2	R184
3	2	R254
3	2	R104
3	2	R234
3	2	R7
3	2	R248
3	2	GLEN MOY
3	1	R248
3	1	R234
3	1	R184
3	1	R254
3	1	GLEN MOY
3	1	R7
3	1	LATHAM
3	1	R104
3	3	GLEN MOY
3	3	R254
3	3	R104
3	3	LATHAM
3	3	R7
3	3	R184
3	3	R234
3	3	R248

Key, Stress: 1= Normal; 3= Drought; 2= Overwatering.

Appendix II. Definition of the correlation coefficient (*r*) value.

The correlation coefficient (*r*) always takes a value between -1 and 1, with 1 or -1 indicating perfect correlation (all points would lie along a straight line in this case). A positive correlation indicates a positive association between the variables (increasing values in one variable correspond to increasing values in the other variable), while a negative correlation indicates a negative association between the variables (increasing values is one variable correspond to decreasing values in the other variable). A correlation value close to 0 indicates no association between the variables. A correlation greater than 0.8 is generally described as strong, whereas a correlation less than 0.5 is generally described as weak.

Appendix III. Line plots showing log transformed combined relative expression levels per candidate gene over time (fruit stage) in Glen Moy and Latham.