Grower Summary

SF 106 (HL 0195)

Developing breeding and selection tools to reduce spoilage of soft fruit and wastage in the supply chain.

Final 2013

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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.
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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).

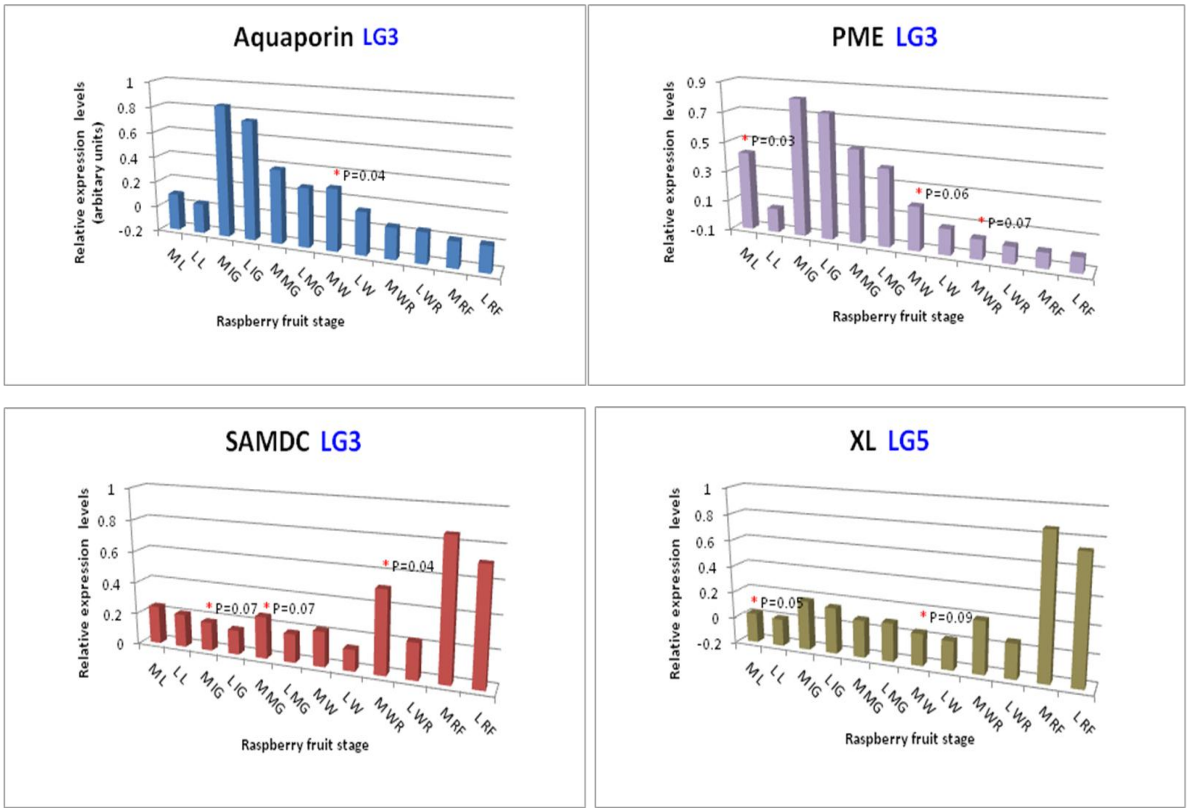

Figure B. Mean normalized relative gene expression levels obtained for selected candidate genes significantly associated with softening QTLs in different stages of fruit and leaf in Glen Moy (M) and Latham (L).

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.