Multidisciplinary approaches and the improvement of fruit quality in red raspberry (*Rubus idaeus* L.)

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Introduction The UK is a net importer of raspberries, mainly due to a restricted growing season and limited fruit shelf-life caused by natural softening and decay processes. These are often exacerbated by the ingress of diseases such as grey mould (*Botrytis cinerea*). Despite these limitations, consumers continue to demand high quality, long shelf-life fruit which is competitively priced. The soft fruit processing sector also encounters problems due to the high levels of soluble cell-wall polysaccharides and phenolics in juice and concentrate. The clarification and filtration processes required to resolve these issues are particularly expensive for products derived from raspberry fruit. The issues of improved fruit quality and storage characteristics are therefore central to the continued success of the raspberry industry.

Tomato has been a 'model' species for studies on fruit ripening related processes for many years. This reflects both its economic importance, its readily dissectible inheritance characteristics and, latterly, the ease with which it can be genetically manipulated to target the improvement of key traits using modern biotechnological approaches. Research on ripening and quality of soft fruit, such as strawberry, is gaining momentum but with other, relatively minor crops, such as raspberry, our understanding of mechanisms is rather poor. Given SCRI's successful history of raspberry breeding and the potential for adding value to fruit using modern scientific approaches and technologies, renewed emphasis on this crop is justified.

Within the Unit of Plant Biochemistry, the research effort is geared toward an understanding of the physicochemical, biochemical and molecular mechanisms underpinning the natural ripening of raspberry fruit. This will facilitate the identification of targets for fruit improvement, utilising biotechnology and traditional breeding-based approaches in a synergistic manner. The research approach encompasses the expertise of biochemists, cell wall chemists and molecular biologists, delivering high quality science which is of direct relevance to the raspberry industry.

Biochemical and molecular analysis of soft fruits such as raspberries, strawberries and blackcurrants, is intrinsically problematic. This is due to the naturally high levels of polyphenols and soluble polysaccharides and low protein levels. Despite these problems, we have developed an armoury of techniques which have been used effectively to identify numerous targets for raspberry breeders and genetic engineers. Furthermore, and in conjunction with the more fundamental aspects of raspberry ripening processes, the retardation of post-harvest spoilage and decay has been studied using modern packaging technology, specifically modified gas atmospheres and packaging films with modified permeability characteristics. Expansion of work in this area will provide benefits in the short-term, while the longer-term approaches involving gene transfer technology are established.

Genotype	Relative fruit-firmness	Specific druplet firmness (mN)	Ethene evolution (mg hr^{-1} $g\ fw^{-1}$)	Time to ripen (days)	Receptacle fresh weight(g)
Glen Clova	Soft	121^{b}	34.34^{a}	58.08^{b}	0.47^{a}
Glen Prosen	Firm	210^{a}	23.35^{b}	65.00^{a}	0.34^{b}

$^{a, b}$ denotes ANOVA categories for significant differences where P<0.05

Table 1 Relationships between genotype, rate of ripening, ethylene evolution, fruit firmness and receptacle fresh weight. Values relate to fruit parameters quantified from red-ripe fruit. Increased rates of ethylene evolution are found in softer fruit which ripen faster and have heavier receptacles than firmer fruit.

Physiology and biochemistry of raspberry fruit ripening The raspberry fruit undergoes dramatic changes in firmness during ripening, particularly during the later stages (Table 1). The rate of ripening and degree of firmness maintained in ripened fruit is genotype-dependant. For experimental, comparative purposes, two SCRI raspberry varieties, Glen Prosen and Glen Clova, have been examined in detail. Fruit of these varieties have been classified, subjectively, as firm and soft, respectively, when ripe.

Changes in fruit firmness were found to be directly related to the rate of ripening and the rate of production of the gaseous plant growth regulator, ethylene, which exerts a strong influence on ripening processes in many fruit (e.g. melon, tomato). A correlation between the rate of ethylene evolution and the size of the raspberry receptacle (plug) has been established. Furthermore, exposure of green fruit to exogenous ethylene stimulates colour (anthocyanin) development, accelerates fruit softening and enhances the activities of cell-wall modifying enzymes (Fig. 1).

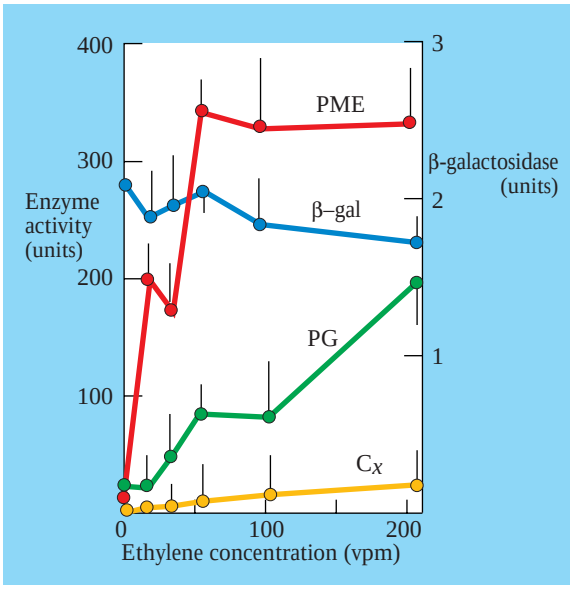

Figure 1 Effect of ethylene on the activities of cell wall hydrolases. Ethylene applied to green fruit, enzymes measured after 48h exposure. Bars indicate S.E.M.s.

Therefore, ethylene plays an important role in the ripening of raspberry fruit.

The natural ripening of raspberry fruit is also associated with changes in the activities of cell wall modifying enzymes. The activities of polygalacturonase (PG), pectin methylesterase (PME), cellulase (Cx; endo-β-1,4-glucanase) and β-galactosidase (β-gal) increase during ripening and appear to contribute to the soft fruit character of ripe raspberries (Fig. 2). The data suggests that PME may be important in determining the onset of softening whilst in the later, and post-harvest stages, PG and Cx activities appear to play more major roles in regulating firmness and texture. As described later in this article, molecular approaches are confirming hypotheses generated from the comparative biochemistry.

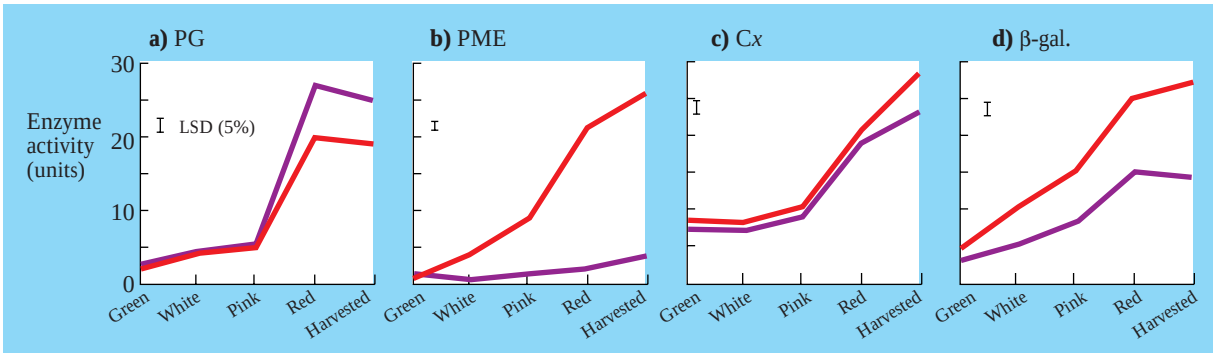

Figure 2 Activities of PG (a), PME (b), C*x*(c) and β-gal (d) in ripening druplets of Glen Prosen (red) and Glen Clova (purple).

	Ara	Xyl	Man	Gal	Glc	UA	%Me
Glen Prosen							
Green	2.9 (0.1)	24.1 (0.3)	0.7 (0.1)	2.7 (0.1)	1.3 (0.1)	28.0 (1.3)	41 (6)
White	3.0 (0.1)	24.0 (0.2)	0.6 (0.1)	2.7 (0.1)	1.4 (0.2)	22.0 (1.1)	35 (4)
Red	2.1 (0.2)	24.6 (0.3)	0.6 (0.1)	2.1 (0.2)	1.4 (0.1)	9.7 (0.8)	14 (3)
Glen Clova							
Green	2.2 (0.1)	23.2 (1.0)	0.5 (0.1)	1.7 (0.3)	0.6 (0.2)	26.8 (1.4)	39 (5)
White	2.2 (0.1)	23.5 (0.8)	0.5 (0.1)	1.2 (0.2)	0.7 (0.2)	21.6 (1.5)	31 (6)
Red	2.1 (0.1)	26.6 (0.4)	0.4 (0.1)	1.1 (0.1)	0.5 (0.1)	5.5 (1.1)	7 (4)

Figures in parenthesis are the standard errors. Non-cellulosic neutral monosaccharide and uronic acid contents are expressed as mg/100mg cell wall.
The non-cellulosic neutral monosaccharide contents are the mean of triplicates whilst the uronic acid contents and % methyl esterification are the mean of five replicates.

Table 2 The neutral sugar, uronic acid and methyl esterification content of Glen Prosen and Glen Clova raspberries at the green, white and red stages.

The cell wall chemistry of ripening fruit Physicochemical analysis of isolated fruit cell walls has also produced a broad agreement with results of biochemical studies. The most significant changes accompany the progression of fruit from yellow to red ripening stages. A large reduction in the cell wall constituents, uronic acid and associated methyl ester, account for the majority of these changes. There are also small but significant reductions in the levels of residual arabinose and galactose (Table 2). These are derived from pectic arabinogalactans. This agrees with the extensive pectin solubilisation and demethylation reported for other fruit and concurs with the elevated activities of enzymes such as PG and PME during raspberry ripening.

This change in pectin structure is reflected in both the FT-IR and NMR spectra (Fig. 3). Both genotypes, Glen Clova and Glen Prosen, show similar reductions in the FT-IR absorbance at ~1660-1600 and 1440 cm^{-1}, the regions associated with pectic acid/anion and ester, respectively. In the NMR spectra, the ester resonance at 172 ppm is reduced in the red-ripe stage

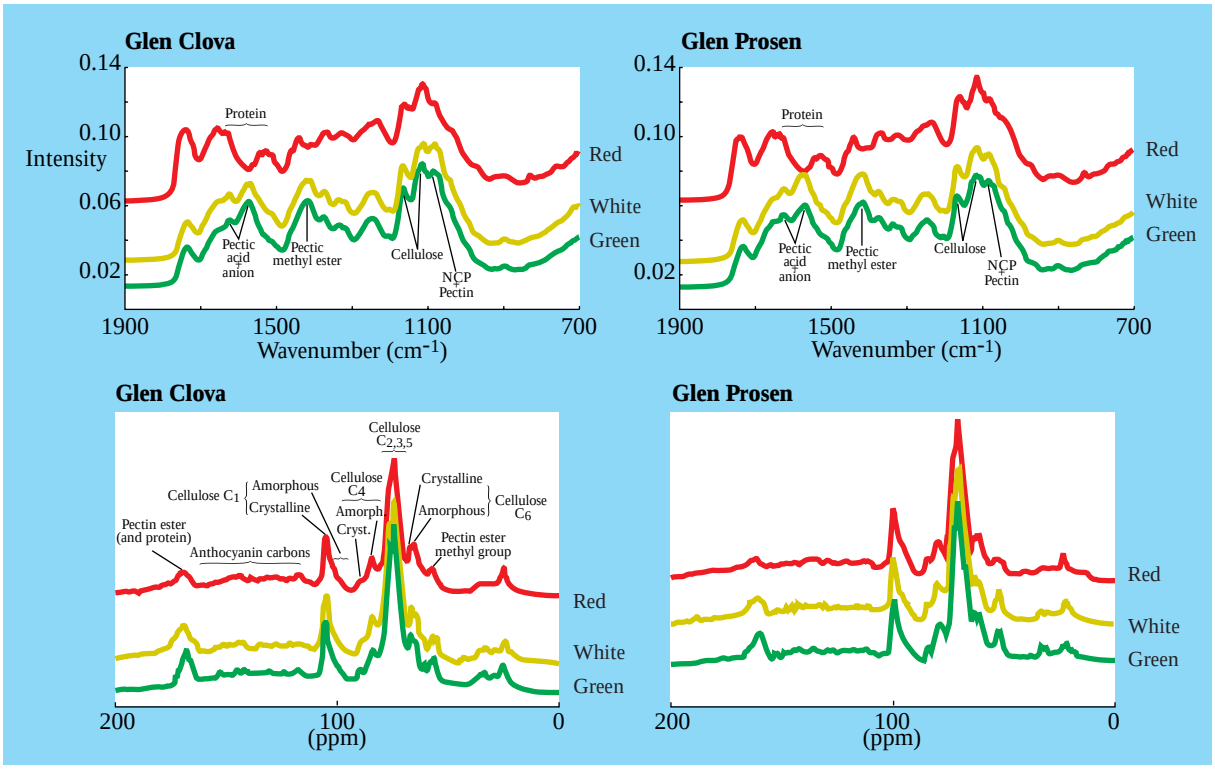

Figure 3 The DRIFT (upper) and solid-state NMR (lower) spectra of Glen Clova and Glen Prosen cell walls showing reductions in pectin methyl ester and cellulose crystallinity and increased protein accretion accompanying ripening.

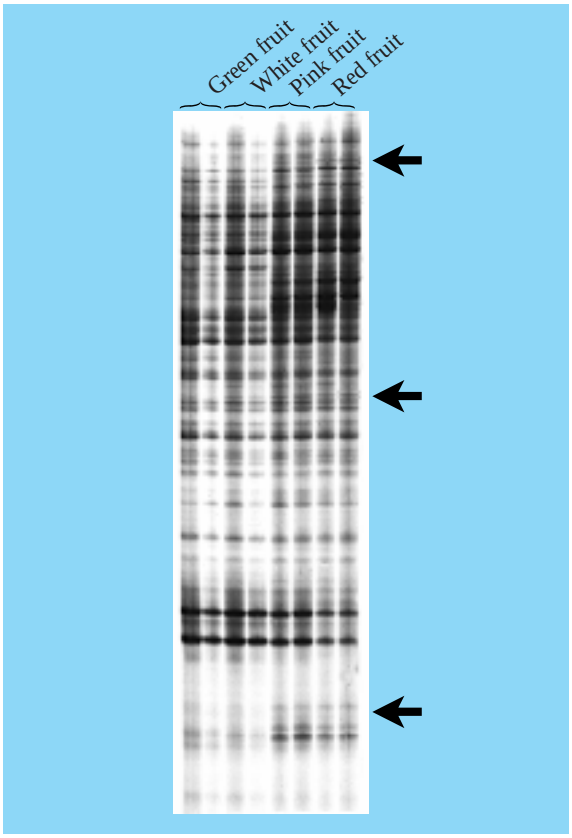

Figure 4 Differential display gel. Arrows indicate genes up-regulated in the ripening raspberry fruit.

but not as much as the measured level of methyl ester content would suggest. This is due to the presence of increased level of protein in the red-ripe stages. The protein (amide) carbonyl resonance (175 ppm) is close to that of the methyl ester (172 ppm), producing an apparently inconsistent reduction in the latter.

Definitive evidence for increased protein levels is seen in the FT-IR spectra. At the red-ripe stage, both genotypes have significant absorbances at 1650 and 1550 cm^{-1}, corresponding to the protein C=O and N-H absorbances respectively. These may be due to interstitial proteins, such as expansins, or to adsorbed cell wall hydrolases, such as cellulase. Cellulase levels increase enormously during the latter stages of ripening and the spectra show definitive evidence of cellulose breakdown. In the NMR spectra, the principal cellulose $C_{2,3,5}$ resonances, at ~73 ppm, have collapsed to a single peak in the red-ripe stage, especially in Glen Clova. Also, there are net increases in the amorphous cellulose resonances. Significantly, the cellulose absorbances in the FT-IR spectra are greater and sharper in the spectrum of Glen Prosen. This suggests that macromolecular cellulose breakdown has

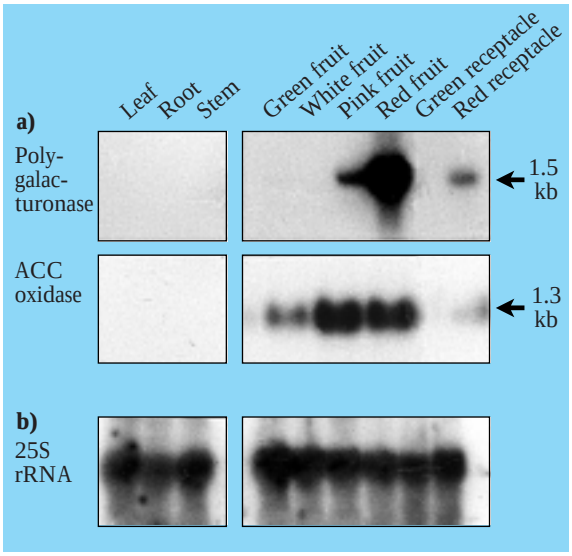

Figure 5 a) RNA blot analysis of two differentially expressed genes from raspberry fruit - polygalacturonase and ACC oxidase. Expression is shown to be up-regulated in the ripening fruit and receptacle (or plug from red fruit. b) To ensure even loading and transfer of RNA, the membrane was re-hybridised with a 25S ribosomal probe.

been more extensive in Glen Clova, the genotype classified as producing softer fruit.

Molecular approaches to raspberry ripening Studies of the molecular aspects of raspberry ripening have been initiated, with the ultimate aim of improving raspberry fruit quality via a transgenic approach. Initially, technical problems in the isolation of pure, intact messenger RNA (mRNA) from raspberry fruit had to be overcome. Raspberry fruit extracts are strongly acidic and contain high levels of RNases and polysaccharides. Nevertheless, new methods were developed to overcome these obstacles, enabling the construction of good quality cDNA libraries. Our approach is to isolate genes, up-regulated in ripening fruit, as these are good candidates for having important rôles in this complex.

Three techniques for isolating differentially-expressed genes have been applied to raspberry fruit. As well as conventional plus-minus screening, two RNA fingerprinting techniques have been employed. These PCR-based methodologies enable the rapid analysis of many transcribed genes (Fig. 4). Using these techniques, we have isolated clones representing over 30 genes that are differentially expressed during raspberry fruit ripening. Expression of two of these genes is shown in Figure 5. In agreement with the parallel

biochemical and physicochemical studies, some of these encode for enzymes that are involved in cell wall degradation. Genes isolated include those encoding polygalacturonase (PG; Fig. 5) and pectin methylesterase (PME). In the fruit of other species, most notably tomato, these activities have been down-regulated using antisense and co-suppression technology in transgenic plants. This has led to the production of fruit with improved shelf-life and processing characteristics. Another gene that has been isolated from raspberry is that encoding a putative ethylene-forming enzyme, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (Fig. 5). As has been shown biochemically, ethylene is an important trigger for ripening processes in raspberry. With fruit of several species (for example melon and tomato), down-regulation of ACC oxidase can delay ripening significantly, resulting in a longer shelf-life. Genes we have isolated that encode for PG, PME and ACC oxidase are clearly now high priority targets for genetic manipulation in raspberry fruit.

The functions of some of the other genes isolated from ripening fruit are less obvious at present, but their study may provide an insight into the complex matrix of processes that constitute ripening. Genes that fall into this category include those encoding a latex-like protein, similar to the latex of opium poppy, which seals wound sites and stores secondary metabolites. Similar sequences are expressed at high levels in the ripening fruit of melon and pepper, suggesting a common ripening-related function, which may protect the ripe fruit against attack by pathogens. Another example is a gene encoding a metallothionein-like protein. Similar genes are expressed in the fruit of many species, including kiwi, papaya, apple, blackcurrant and banana. Again, a common ripening-related function is implied. Metallothioneins are heavy-metal binding proteins, responsible for metal ion homeostasis and implicated in protection against oxidative stress. We are currently characterising the raspberry-fruit metallothionein at the biochemical level.

Our gene isolation programme has also provided us with the tools to isolate raspberry fruit-specific promoters which will be necessary to regulate the expression of genes in a tissue- and temporal-specific manner. The production of transgenic fruit with improved quality, storage and processing characteristics is now the immediate goal.

Acknowledgements

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