UNIVERSITY OF STRATHCLYDE, GLASGOW

CENTRE FOR FOOD QUALITY

STRATHCLYDE INSTITUTE FOR PHARMACY AND BIOMEDICAL SCIENCES

Marker assisted breeding for fruit quality characteristics in red raspberry (*Rubus idaeus*)

SUSAN A.H. MCCALLUM

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

November 2009

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Susan McCallum

Acknowledgements

This study would not have been possible without funding through the HortLINK project HL0170 with contributions from Horticultural Development Council, Kentish Garden, Marks and Spencer, Mylnefield Research Services, Ltd. and ReDeva as well as access to commercial raspberries from Thomas Thomson, Blairgowrie, for which I am grateful.

I would like to take this opportunity to thank my SCRI supervisor Dr Julie Graham for unwavering support, encouragement and enthusiasm throughout this project. I would also like to thank my University supervisor Dr Alistair Paterson for advice and editing of this thesis. I would like to express my gratitude to Dr Mary Woodhead and Dr Joanne Russell for valuable advice during the formation of this thesis, Dr Christine Hackett (BIOSS) for support, assistance and patience with statistical analysis and Dr Gordon McDougall and Mrs Pat Dobson for assistance with biochemical analysis.

I would also like to thank my SCRI colleagues Kay, Linzi, Sandra and Nikki for their technical assistance, humour, friendship and fruit and I am incredibly grateful to Philip Smith for the proof reading of this thesis.

Finally I would like to thank my family, Mum, Dad, Carol, Steven and James and my friends, Louise, Sharon and Ashley for their continued support, encouragement and patience throughout my higher education.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

A_{230}	Absorbance at 230 nm
A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
ACN	Acetonitrile
AcOH	Acetic acid
AFLP	Amplified fragment length polymorphism
AIX	Ampicillin, IPTG, X-gal
amp	Ampicillin
ANOVA	Analysis of variance
ANR	Anthocyanin reductase
ANS	Anthocyanin synthase
ATP	Adenosine triphosphate
AVI	Anthocyanin vacuolar inclusions
BAC	Bacterial artificial chromosome
bHLH	Basic helix loop helix
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BRNV	Black raspberry necrosis virus
ca	Circa
C3G	Cyanidin-3-glucoside
C3GR	Cyanidin-3-(2-glucosylrutinoside)
C3R	Cyanidin-3-rutinoside
C3S	Cyanidin-3-sophoroside
CCD	Charge coupled device
cDNA	Complementary DNA
CFI	Chalcone flavanone isomerase
CHI	Chalcone isomerase
CHS	Chalcone synthase
CIE	Commission Internationale de l'Eclairage

CIM	Composite interval mapping
CM	Colour meter measurements
cM	CentiMorgan
contig	Contiguous sequence
Cs	Citrate synthase
CT	Commercial polytunnel
CTAB	Hexadecyl trimethyl ammonium bromide
c.v	Cultivar
^{o}C	Degrees Celsius
DAFB	Days after full bloom
dCTP	Deoxycytidine-5'-triphosphate
DEPC	Diethyl pyrocarbonate
DFR	Dihydroflavonol 4-reductase
DHK	Dihydroflavonols
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide
DTT	Dithiothreitol
EBI	European Bioinformatics Institute
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
EMBL	European Molecular Biology Laboratory
ER	Endoplasmic reticulum
EST	Expressed sequence tag
EtBr	Ethidium bromide
EXP	Expansin
F	Field site, SCRI
F3'H	Flavonoid 3'-hydroxylase
F3'5'H	Flavonoid 3', 5'-hydroxylase
FLS	Flavonol synthase
fw	Free weight

fw	Fruit weight gene
GAE	Gallic acid equivalents
Gal-dh	Galactose dehydrogenase
GST	Glutathione S-transferase
H^+	Hydrogen ions
h_2	Heritability
Hg	Mercury
Hcl	Hydrochloric acid
Hk	Hexokinase
Hz	Hertz
IAA	Isoamyl alcohol
Inv	Invertase
IPM	Integrated pest management
IPTG	Isopropylthio-β-galactopyranoside
kb	Kilobase pairs
KW	Kruskal Wallis
l	Litre
LB	Luria Bertani
LD	Linkage disequilibrium
LDOX	Leucoanthocyanin dioxygenase
LG	Linkage group
LN_2	Liquid nitrogen
LOD	Limit of detection
LRU	Light regulatory units
M	Molar (concentration)
MAB	Marker assisted breeding
MAS	Marker assisted selection
Mbp	Mega base pairs
mdh	Malate dehydrogenase
$MgCl_2$	Magnesium chloride

$MgSO_4$	Magnesium sulphate
MIP	Membrane intrinsic protein
ml	Millilitres
MLP	Major latex like protein
mM	Millimolar
mRNA	Messenger RNA
µF	Microfarad
µg	Micrograms
µl	Microlitres
µm	Micrometre
MYB	Gene derived from *avian myeloblastosis virus*
Na_2EDTA	Ethylenediaminetetra acetic acid (disodium salt)
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	Nanograms
NI	Neutral invertase
NIP	Nodulin 26-like intrinsic protein
nm	Nanometres
oligo (dT)	Oligodeoxythymidylic acid
P3G	Pelargonidin-3-glucoside
P3GR	Pelargonidin-3-glucosylrutinoside
P3S	Pelargonidin-3-sophoroside
P3R	Pelargonidin-3-rutinoside
PAL	Phenylalanine ammonia-lyase
PEPC	Phosphoenolpyruvate carboxylase
PCC	Pearson correlation coefficient
PCR	Polymerase chain reaction
PG	Polygalacturonase
pH	Potential of Hydrogen

PIP	Plasma intrinsic protein
PME	Pectinmethylesterase
Poly $(A)^+$	Polyadenylated
PPi	Pyrophosphate
psi	Pounds per square inch
PSQ	Pyrosequencing
PT	Polytunnel, SCRI
QTL	Quantitative trait loci
R1C1	Repetition 1, Clone 1
R1C2	Repetition 1, Clone 2
R^2	Phenotypic variation
R2C1	Repetition 2, Clone 1
R2C2	Repetition 2, Clone 2
R2R3	MYB gene domain
RAPD	Random amplified polymorphic DNA
RBDV	Raspberry bushy dwarf virus
RFLP	Restriction fragment length polymorphism
RLMV	Raspberry leaf mottle virus
RLSV	Raspberry leaf spot virus
RNase	Ribonuclease
rRNA	Ribosomal RNA
RYNV	*Rubus yellow net virus*
S. cerevisiae	*Saccharomyces cerevisiae*
SCRI	Scottish Crop Research Institute
SDS	Sodium dodecyl sulphate
SIP	Small and basic intrinsic protein
SNP	Single nucleotide polymorphism
SPS	Sucrose-phosphate synthase
STP	Sucrose transporter
Subsp	Subspecies

SuSy	Sucrose synthase
Taq	*Thermus aquaticus*
TBE	Tris borate-EDTA
TCA	Tricarboxylic acid
TE	Tris-EDTA
TEMED	N,N,N',N' tetramethylethylenediamine
TIP	Tonoplast intrinsic protein
tRNA	Transfer RNA
Tris HCl	Tris (hydroxymethyl) methylamine-hydrochloric acid
TSS	Total soluble solids
UV	Ultra violet
UFGT	UDP-glucose:flavonoid 3-O-glucosyltransferase
UDP	Uridine diphosphate
var	Variety
VI	Vacuolar invertase
Vp2	Vacuolar H^+ pyrophosphatase
v/v	Volume for volume
w/v	Weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
4Cl	4-coumarate CoA ligase

Summary

Raspberry breeding is a long, slow process in this highly heterozygous out-breeder. Selections for complex traits, like fruit quality, are broad-based and few simple methodologies and resources are available. The main quality traits of interest to producers and buyers alike are the overall appearance of the raspberry, which includes not only size and colour, but overall drupelet cohesion and shape. Second to that is the overall sweetness and sourness of fruit as well as the balance and intensity of the two combined.

Knowledge of metabolic pathways involved in the uptake and regulation of sugars, organic acids and flavonoid synthesis were utilised in order to identify and map candidate genes involved in phenotypic variation. These include transporter genes; membrane intrinsic proteins (MIP 2 and MIP 3) and tonoplast intrinsic protein (TIP), and flavonoid genes; flavonol synthase (FLS), anthocyanin reductase (ANR), dihydroflavonol 4-reductase (DFR), glutathione S-transferase (GST) and flavonoid 3'-hydroxylase (F3'H). Other genes mapped in red raspberry, involved in fruit quality, include expansin (EXP) and galactose dehydrogenase (Gal-dh). A total of 75 putative QTLs were located across the raspberry genome for all traits analysed across different environments and seasons, the majority of which were subsequently linked to underlying candidate genes or markers. The large number of QTLs detected for some traits (22 for colour meter analysis) was further evidence towards phenotypes caused by a cumulative result of numerous QTLs of small effect and not by a few of large effect.

This study demonstrates the need for a multifaceted approach towards understanding and identifying components underlying quality traits in red raspberry. While seasonal effects were evident for many phenotypic traits, the potential to enhance quality by genotypic selection was clear. Once the genetic controls of traits contributing to sensory qualities have been identified, linkage of such traits to molecular markers heralds the future of plant breeding.

Chapter 1 General introduction

1.1 Red raspberry introduction

The European red raspberry belongs to the genus *Rubus*, diverse with around 500 species from diploid to dodecaploid, sub-divided into 12 subgenera (Jennings, 1988). Notable are the European red (*R. idaeus* subsp. *vulgatus* Arrhen or simply *R. idaeus*), North American red (*R. idaeus* subsp. *strigosus* Michx or *R. strigosus*) and black raspberries (*R. occidentalis* L.) (Jennings, 1988). *Rubus* belongs to the economically important Rosaceae family of perennial fruit crops with *Malus* (apple), *Pyrus* (pear), *Fragaria* (strawberry) and *Prunus* (stone fruit). The red raspberries are diploid species with seven chromosomes (2n = 2x = 14) (Pool *et al*., 1981) and genomes of ca. 275 Mb (Jennings, 1988). The term raspberry is thought to have derived from the sharp "rasping" flavour of the fruit although it may also originate from the Anglo-Saxon term "resp" which means shoots or suckers (Jennings, 1988). Indigenous to Asia Minor, raspberries were reportedly gathered wild on the foothills of Mount Ida by the people of Troy (Troas, Turkey) in the 1st Century BC with domestication recorded by Roman agriculturist Palladius in the 4th Century AD. Pliny the Elder wrote about red raspberries in 45 AD and described how the Greeks referred to the fruit as "Ida" fruit after Mount Ida (Jennings, 1988). The term "Ida" continued to be used for the fruit; Linnaeus (1707-1778) derived *Rubus idaeus* from Latin "Ruber" meaning red and "*idaeus*" thought to be inspired from Mount Ida. The Ide Mountains of Turkey may however be a more accurate origin (Jennings, 1988). Although berries were initially gathered from the wild, the plants were not cultivated for food but were used for medicinal purposes, particularly in the treatment of eye and stomach disorders (Jennings, 1988). Seeds have been found at Roman forts in Britain following archaeological excavation, indicating the spread of raspberry cultivation throughout Europe by the Romans (Jennings, 1988).

1.1.1 Raspberry cultivation

Red raspberries were cultivated by the English herbalist, Turner in 1548, and by 1629 full chapters were devoted to raspberries: John Parkinson wrote about red, white and thorn-less "raspis-berries" (Ourecky, 1975; Jennings, 1988). By the turn of the 17th Century, British land owners grew raspberries and by the 18th Century cultivation had spread throughout Europe. The first commercial sale of red raspberry nursery plants is credited to William Price of Flushing, New York in 1737 (www.pan-am-llc.com/history_raspberry.htm). Britain popularised and improved raspberries throughout the Middle Ages (ca. 4th Century to the 15th Century), producing larger, higher quality fruit, and began exporting plants to New York in 1771 (Jennings, 1988). This led to a number of commercial growers selecting clones grown in the wild and importing cultivars and seeds in order to produce higher quality crosses of their own (Darrow, 1937). Successful British cultivars of the early 19th Century include 'Red Antwerp', renowned for large fruit, thought to have been grown in 1800 and derived from this was 'Fastolff' in 1820. 'Superlative' was discovered in Dover by 1888 which was heralded as the "giant" of the 1900s (Jennings, 1988). By the early 1920s the first controlled breeding programme began for red raspberries in Britain and this was introduced at the East Malling Research Station in Kent. Breeding is the selection of individuals, based on qualities of interest, in order to combine desirable morphological, physiological or genetic traits into a single cultivar (Roach, 1985). The ancestry of red raspberry breeding is dominated by five parental cultivars; 'Pynes Royal' (1913) and 'Lloyd George' (1919) derived exclusively from *R. idaeus* var. *vulgatus* and 'Cuthbert' (1865), 'Preussen' (1919) and 'Newburgh' (1930) derived from both *R. idaeus* var. *strigosus* and *R. idaeus* var. *vulgatus* (Roach, 1985; Jennings, 1988).

While initial breeding focused on producing new raspberry varieties bearing larger berries and higher yields, significant advances arose from the cross of *R. idaeus* and *R. strigosus* cultivars. High quality fruit were produced with higher yields which were resistant to a variety of pests and diseases including root rot (*Phytophthora fragariae)*.

Today there are three commercially accepted varieties grown in Britain, that is to say three varieties that the supermarkets deem suitable in terms of size, quality and shelf life. These include 'Glen Ample' and 'Tulameen' which fruit mid season between July and August and 'Octavia' which fruits slightly later in the season at the beginning of August. With an estimated retail value of £65 million per year (2007, UK Figures) (http://www.fao.org/statistics) current varieties are insufficient to meet the requirements of growers and consumers and with concerns over environmental impact, regarding food miles and carbon emissions, and the desire to buy locally grown food, the problems with current limited varieties are exacerbated.

1.1.2 Raspberry industry

The UK raspberry industry, until recently, was traditionally a processed fruit industry which had been in decline for 20 years through competition from imports and decreases in yield due to climatic changes affecting production (frost damage, prolonged dry periods), damage caused by disease, and reduction in pesticides. In order to prevent a further decline in the UK industry, it became evident that alternative strategies must be employed. Over the last 10 years the industry has been revitalised by growing fruit under protected cultivation for the high quality fresh market. Breeding raspberries of high quality and extending the current narrow growing season would further assist market development.

1.1.3 Flowers and fruit structure

Plants have both vegetative primocanes and fruiting fructocanes which produce fruit on an annual basis from the 2nd year of growth and fruiting can exceed 15 years in favourable environments (Jennings, 1988). Excess primocanes are pruned to reduce inter cane competition for water and nutrients and to enable efficient light exposure (Jennings, 1988). Pruning of fruiting canes following harvest allows new growth and also prevents the build up of fungal inocula (Graham and Jennings, 2009). Small white/pink flowers (0.5–1.5 cm), produced in the 2nd year of growth, consist of five petals, five sepals a very short hypanthium and around 60 to 90 stamens (Figure 1.1).

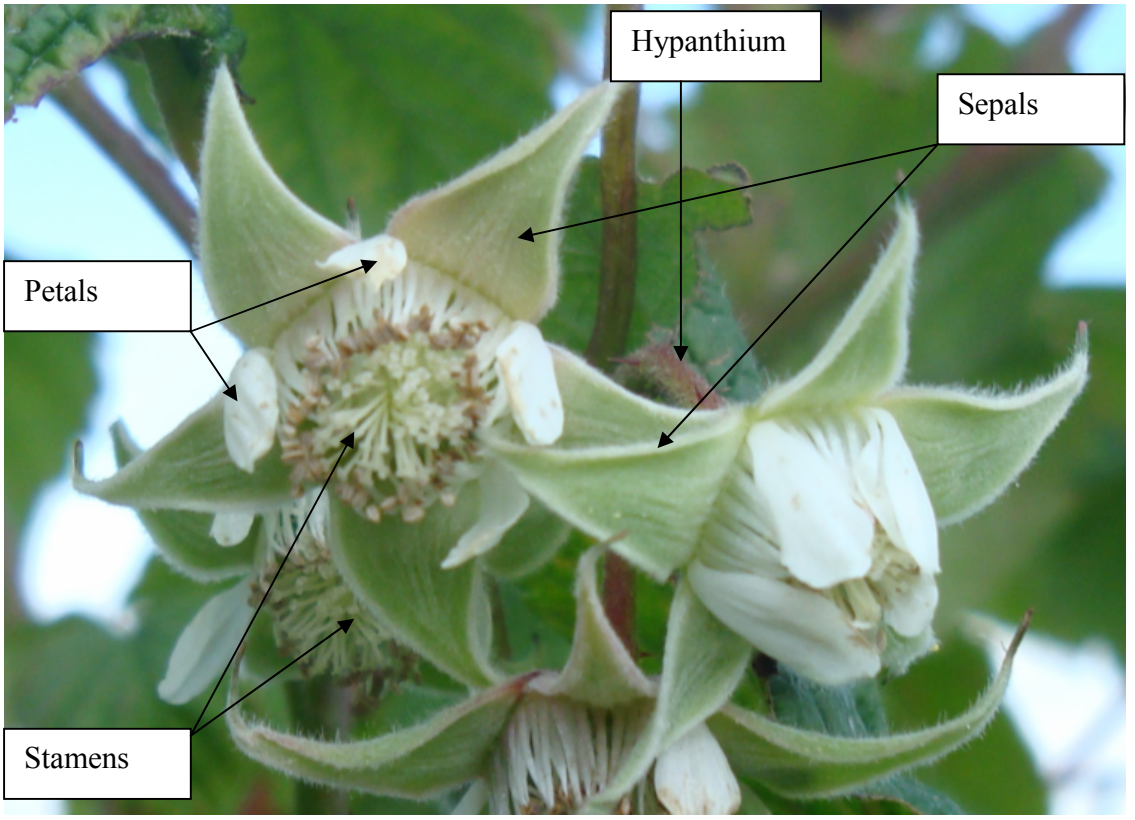


Figure 1.1. Red raspberry flower. Small white/pink flowers are produced in the 2nd year of plant growth and consist of five petals, five sepals a very short hypanthium and around 60 to 90 stamens.

Flowers produce profuse amounts (up to 33 mg) of nectar, 50% of which is sugar (Petkov, 1963). Raspberries are compound aggregate fruits which consist of an apocarpous gynoecium that contains 60 to 80 ovaries (carpels) each of which develops into a drupelet connected by minute hairs formed around a central receptacle. These epidermal hairs are unicellular linear trichomes that are the main source of drupelet cohesion (Jennings, 1988). Each drupelet consists of a fleshy mesocarp, with thin walled parenchyma cells and a hard endocarp that forms a tiny pit enclosing a single seed (Jennings, 1988). While most drupes contain only a single seed, a few may contain two (Graham and Jennings, 2009). Firmness of raspberry fruits is associated with both size and number of individual drupelets and their relative cohesion (Jennings, 1988). Pectins are the cell wall component that influences texture and function by containing turgor pressure within cells which consequently regulate the cells ability to extend and grow (Jarvis, 1984). Stewart *et al*. (2001) attributed varietal differences in pectin levels in raspberry fruit as a factor in fruit firmness promoting cell to cell adhesion.

1.1.4 Fruit development and ripening

There are four main stages involved in the development of fruit: 1) fertilisation and fruit set; 2) cell division; 3) cell expansion and 4) fruit ripening (Gillaspy *et al*., 1993). The successful completion of each stage determines the outcome of the following stages with early defects impacting on mature fruit characteristics (Causse *et al*., 2004). Raspberries ripen between 30 to 36 days after pollination with variations determined by maternal genotype and environmental conditions (Jennings, 1988). Following pollination, fruits grow rapidly for around 10 to 12 days due to cell division, and then as the embryo develops and the endocarp hardens, growth slows down for a period of 10 to 12 days. A final stage of rapid cell growth occurs due to cell enlargement that lasts for 10 to 12 days (Hill, 1958). Fruit development is a series of genetically determined biochemical and physiological processes with changes in appearance (colour), texture and flavour (aroma and taste) occurring during the terminal stage. The outcome is fruit which is desirable and edible to both consumers and seed dispersing animals (for review see Tucker, 1993; Giovannoni, 2001). Ripening involves the expansion of ovule derived tissues, loss of

chlorophyll and a progressive decrease in tissue firmness during which time metabolites involved in pigmentation and volatiles also accumulate (Kumar and Ellis, 2001).

Changes within fruit during ripening include modification of cell wall ultrastructure and texture, conversion of starch to sugars, pigment biosynthesis and accumulation of flavour volatiles (Giovannoni, 2001). Several enzymes have been identified which impact on fruit ripening and softening including polygalacturonase (PG), pectin methylesterase (PME), β galactosidase and expansins (Giovannoni, 2001; Vrebalov *et al*., 2002). Regulation involves transcription factors such as MADS box genes, primarily in the floral development of petals, carpel and stamen (Giovannoni, 2001; Vrebalov *et al*., 2002).

1.1.4.1 Cell wall structure and disassembly

The disassembly of the cell wall during fruit ripening is an accomplished modification brought about by a series of enzyme activities performed in a controlled manner (Brummell and Harpster, 2001). Degradation of pectin produces glucose and galactose (Fischer and Bennett, 1991) with xylose also released during raspberry softening (Gross and Sams, 1984). Guerrero-Prieto *et al*. (1996) reported that ripening began with β galactosidase activity initiating cell wall changes through the release of galactose side chain residues at the green stage. This was followed by cellulase and polygalacturonase activities during the green/red stage with activity declining during the red ripe stage.

1.1.4.2 Abscission of fruit and ethylene biosynthesis

Ethylene is involved in the regulation of several physiological events in fruit ripening (Tucker and Brady, 1987). Fruit differ in their utilisation of ethylene for completion of ripening: climacterics require ethylene and include banana, peach and apple; non climacterics do not, but may still respond to the hormone notably strawberry, citrus and grape (Giovannoni, 2001). In climacteric fruit an increase in respiration coincides with ethylene biosynthesis whereas no respiratory peak is present in non climacteric fruit (Vrebalov *et al*., 2002). Raspberry shows typical climacteric behaviour with ethylene

rising during early stages of ripening reaching maximum in fully ripe fruit but respiration rate decreases during ripening, characteristic of non climacteric fruit (Jennings, 1988). Ethylene is also involved in the abscission of fruit from receptacles on ripening (Sexton *et al*., 1997). Abscission zones in stipes weaken in synchrony allowing drupelets to be harvested as a single cap due to the entanglement of epidermal hairs (Iannetta *et al*., 2000).

1.2 Quality traits of interest in red raspberry

Quality attributes of ripe, red raspberries result from the accumulation of anthocyanin pigments (cyanidin glycosides) and characteristic flavour components such as those derived from phenolics (p-hydroxyphenylbutan-2-one). Kramer and Twigg (1966) described fruit quality as the conjunction of physical and chemical characteristics that confer good appearance and acceptability. The main quality traits of interest to producers and buyers alike are the overall appearance of fruit including not only size and colour but drupelet cohesion and shape, which if found to be visually attractive to the buyer, will encourage initial purchase. Equally important are the overall sweetness and sourness of fruit as well as the balance and intensity of the two combined which, if subsequently enjoyed, may lead to repeat purchases. Raspberry quality is generally described as a combination of taste, texture and aroma components: fruity, sweet and floral whilst having some acidity but without any bitterness (Harrison *et al*., 1999). Although genotype is the major determinant in fruit quality, other factors contribute to individual variation and include environmental and cultivation techniques as well as ripening season (Capocasa *et al*., 2008). In apple, key factors contributing to quality are appearance, texture and flavour, the last of which has been further broken down to include aroma and taste involving around 350 volatiles (Maarse, 1991). The balance between sugar and acid composition is deemed central to taste with malic acid identified as the principal acid and main substrate for respiration in apples which is proposed to be controlled by a single (*Ma*) gene (Liebhard *et al*., 2003). An appropriate balance is responsible for optimal flavour whereby a given sugar level is found to correspond to an optimal acid content (Malundo *et al*., 1995).

The peach vacuolar H^+ pyrophosphatase (VP2) gene regulates the establishment of an electrochemical gradient across the tonoplast which provides the proton motive force for sugar and organic acid transport by pumping protons into vacuoles. The sucrose transporters (STP) are involved in uploading sucrose from the phloem and the subsequent storage of soluble sugars in the vacuole via tonoplastic soluble STP (Etienne *et al*., 2002). Inheritance of quality traits have been studied in fruits including strawberry, peach, pear, tomato, peppers, kiwifruit, sugarcane and apricot (de Ancos *et al*., 1999; Dirlewanger *et al*., 1999; Steyn *et al*., 2004; Yates *et al*., 2004; Huh *et al*., 2001; Cheng *et al*., 2004 and Soriano *et al*., 2005. Most reports were of only moderate to low heritability for the majority of traits analysed although Cheng *et al*. (2004) found heritability traits in kiwifruit for male parents to be significant for all traits whereas female heritability was only significant for quinic, citric, ascorbic and titratable acid. Quality parameters are the end result of a number of biochemical pathways which interact at various levels and are influenced to a greater or lesser extent by environmental conditions. Understanding key steps in regulation and how variations determine the intensity of individual quality traits will facilitate targeted breeding strategies.

1.2.1 Sugar biosynthesis

The biological pathways involved in individual quality traits are extensive and any polymorphisms detected here are likely to play an important part in quality differences between individuals and indeed populations. Sugar compositions of many fruits are similar with raspberry, strawberry and kiwifruit containing high levels of fructose and glucose along with lower levels of sucrose. In addition to these, kiwifruit also contain inositol while strawberries and raspberries contain xylose (de Ancos *et al*., 1999). The lower levels of sucrose are attributed to enzymatic hydrolysis which occurs in fruit following translocation from leaves (Wang and Zheng, 2005). Fructose is sweeter than either glucose or sucrose and a higher content is a desirable trait (Liu *et al*., 2007). Of particular interest in fruit quality is the biosynthesis of sucrose, involved in plant growth and development (translocation and storage) but also implicated as a regulator of cellular

metabolism (direct or indirect gene expression) (Huber and Huber, 1996). Studies have confirmed roles for sucrose synthase (SuSy), sucrose phosphate synthase (SPS) and invertase (Inv) in the transport of sugar between the cytosol and the vacuole (Huber and Huber, 1996). SPS is active in both photosynthetic tissues and ripening fruit and the gene has been cloned from maize, spinach, potato and rice with significant quantitative differences being reported (Huber and Huber, 1996). Micallef *et al*. (1995) reported increases in both fruit number and dry fruit weight in transgenic tomato plants expressing increased SPS activity. A similar study of SuSy activity in tomato by D'Aoust *et al*. (1999) found reductions in fruit set and overall fruit production in plants with low SuSy levels. It was concluded that SuSy plays a central role in the sucrose import capacity of young fruit contributing to the subsequent fruit set and development potential of plants (D'Aoust *et al*., 1999).

In peach, Etienne *et al*. (2002) identified several candidate genes involved in sucrose unloading in both the phloem and the cytosol: sucrose transporters (STP) and invertase (Inv) and to a lesser extent hexokinase (Hk). Tonoplast intrinsic proteins (TIPs) are members of the MIP family (major intrinsic proteins) which have been shown to act as water channels expressed predominantly within storage tissues. As glucose is accompanied by the transport of water, these genes are strong candidates for quantitative differences relating to the storage and transport of sugar molecules (Martinoia *et al*., 2000). Understanding the complexities of sugar uptake, accumulation and metabolism gives a greater insight into the potential candidate genes which control these, the nature of quantitative trait loci (QTL) which underly traits and may yield associated molecular markers. The enzymatic pathways involved in the cleavage of sucrose in plants are catalysed by invertase (hydrolase) in the form of sucrose + H_20 = glucose + fructose, or sucrose synthase (glycosyltransferase) sucrose + UDP = UDP-glucose + fructose (Figure 1.2). Invertase results in the formation of glucose (instead of UDP-glucose) which produces twice as many hexoses giving invertase a greater capacity for sugar sensing than sucrose synthase (Koch, 2004).

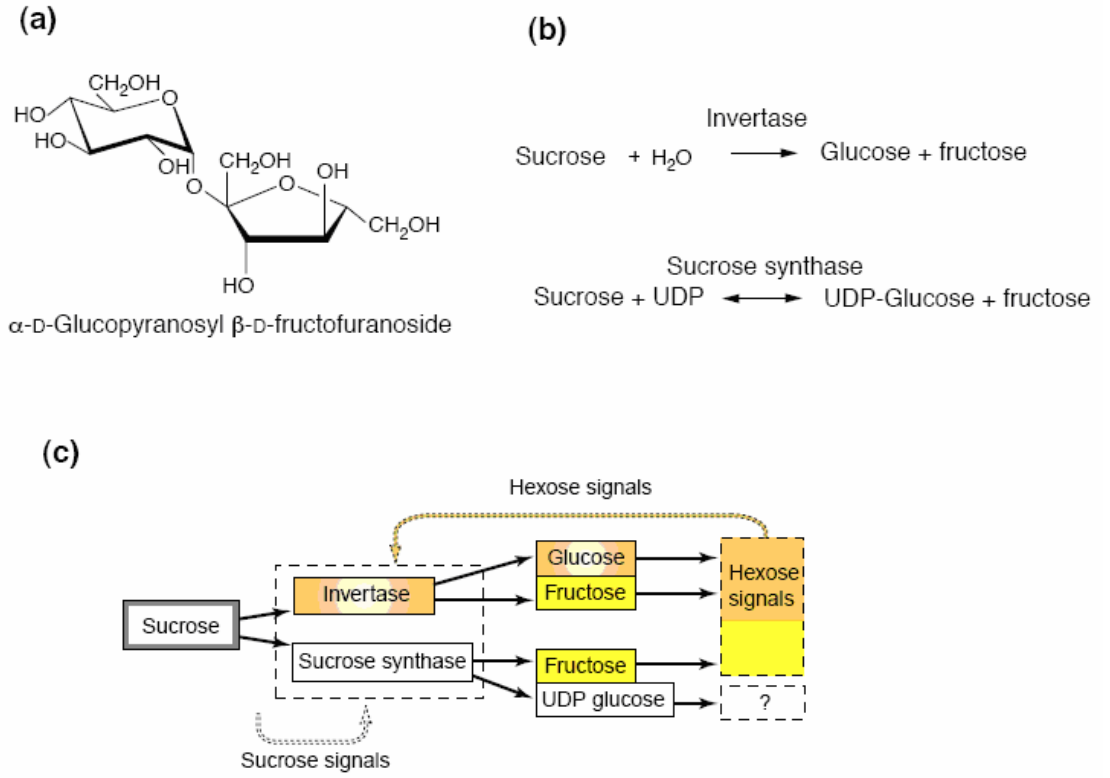


Figure 1.2. Schematic representation of sucrose sensing and cleavage in plants. a) Structural representation of the disaccharide sucrose. b) Invertase irreversibly cleaves sucrose into glucose and fructose while sucrose synthase converts sucrose into UDP-glucose and fructose in the presence of UDP. c) Different sensing mechanisms are activated by hexose, sucrose and other metabolites downstream with the invertase pathway producing twice as much substrate as the reversible sucrose synthase pathway. (Figure adapted from Sturm and Tang, 1999; Koch, 2004).

1.2.2 Organic acid biosynthesis

Fruit quality is dependent not only on total content of sugars but on a correlation between individual sugars and acids. Wu *et al*. (2003) analysed 18 genotypic variations in peach across two different environmental locations and found glucose and fructose content were consistent between locations unlike sucrose, sorbitol and the acids which were significantly different. The content of organic acids tend to be lower than sugar levels in many fruit, with acids contributing to both flavour and mouthfeel (Liu *et al*., 2007). Citric is the main acid in raspberry, present in smaller amounts are malic and succinic acids with lower contents of isocitric and fumaric acids (Wang and Zheng, 2005). Citric and fumaric acids dominate in strawberry which have lower levels of tartaric and shikimic acids (Sturm *et al*., 2003) while kiwifruit contains quinic, malic and citric acids (Richardson *et al*., 2004). The balance between individual sugar and acid compositions can be important for consumer acceptance of new cultivars. Tartaric acid is deemed to have a stronger sour taste than malic acid which itself is stronger than citric acid (de Bruyn *et al*., 1971; Wu *et al*., 2003). Liu *et al*. (2007) reported that the syntheses of organic acids are controlled by single genes with both malic and tartaric acid showing strong broad sense heritability.

High acid concentrations in fruit along with a low pH are critical for preservation of fruit and also aid in the stabilisation of ascorbic acid and anthocyanins (Wang *et al*., 2009). Several candidate genes for acid metabolism have been identified by Etienne *et al*. (2002) and include malate dehydrogenase (Mdh) and phosphoenolpyruvate carboxylase (PEPC) for malic acid synthesis while citrate synthase and the tricarboxylic acid cycle (TCA) were identified for citric acid synthesis. Titratable acidity is used as a measurement of the quantity of acid present in fruit or more accurately the total concentration of H^+ ions present free in solution and those still associated with anions while pH measures only H^+ ions which have dissociated and are free in solution (Lodish *et al.,* 1999). In fruit, titratable acid is generally high and then decreases as fruit ripens and thus may be used as a quantitative measurement to following the ripening process (Jennings, 1988).

1.2.3 Colour biosynthesis in fruit

The pigments which colour most fruit are flavonoid secondary metabolites which are composed of flavonols (colourless) and anthocyanins, polymeric phlobaphenes and proanthocyanidins (coloured) (Koes *et al*., 2005). Following flavonoid synthesis in the cytoplasm, anthocyanins and proanthocyanins are transported to the vacuole for permanent storage. This transfer is facilitated by glutathione S-transferase (GST), and the gene encoding GST has been identified in several plants including *Petunia hybrida* (AN9 gene) (Mueller *et al*., 2000) and maize (Bz2 gene) (Alfenito *et al*., 1998). Anthocyanins are vacuolar flavonoid compounds which contribute to aroma volatile formation and are responsible for the colour pigmentation in plants. Other flavonoid functions include auxin transport, male fertility and signalling during nodulation (Koes *et al*., 2005).

1.2.3.1 Polyphenol biosynthesis

Phenolic compounds are secondary metabolites which are ubiquitous in all higher plants. In berries, phenolics include flavonoids, phenolic acids, lignans, stilbenes and polymeric tannins as shown in Figure 1.3 (Puupponen-Pimiä *et al*., 2005). Phenolics occur in plant tissues as both simple substituted phenols, such as anthocyanins, or as complex polymers such as ellagitannins of high molecular weight (Puupponen-Pimiä *et al*., 2005). Phenolic compounds are key regulators of red colouration in apple while a further requirement for the biosynthesis of red pigmentation is sunlight, with fruit always shaded, e.g. inside the tree canopy, having insufficient anthocyanin content to develop red colouration (Treutter, 2001). Fruits grown in conditions of strong nitrogen fertilisation do not develop red pigmentation although shoot growth is increased (Treutter, 2001). The quality of fruit juice is also affected by the presence of phenolic compounds and this is attributed to the polymeric procyanidins (tannins). These polymers have a high affinity to macromolecules including proteins and polysaccharides and these interactions are responsible for the formation of hazes, precipitates and confer bitterness and astringency to fruit juice quality (Treutter, 2001).

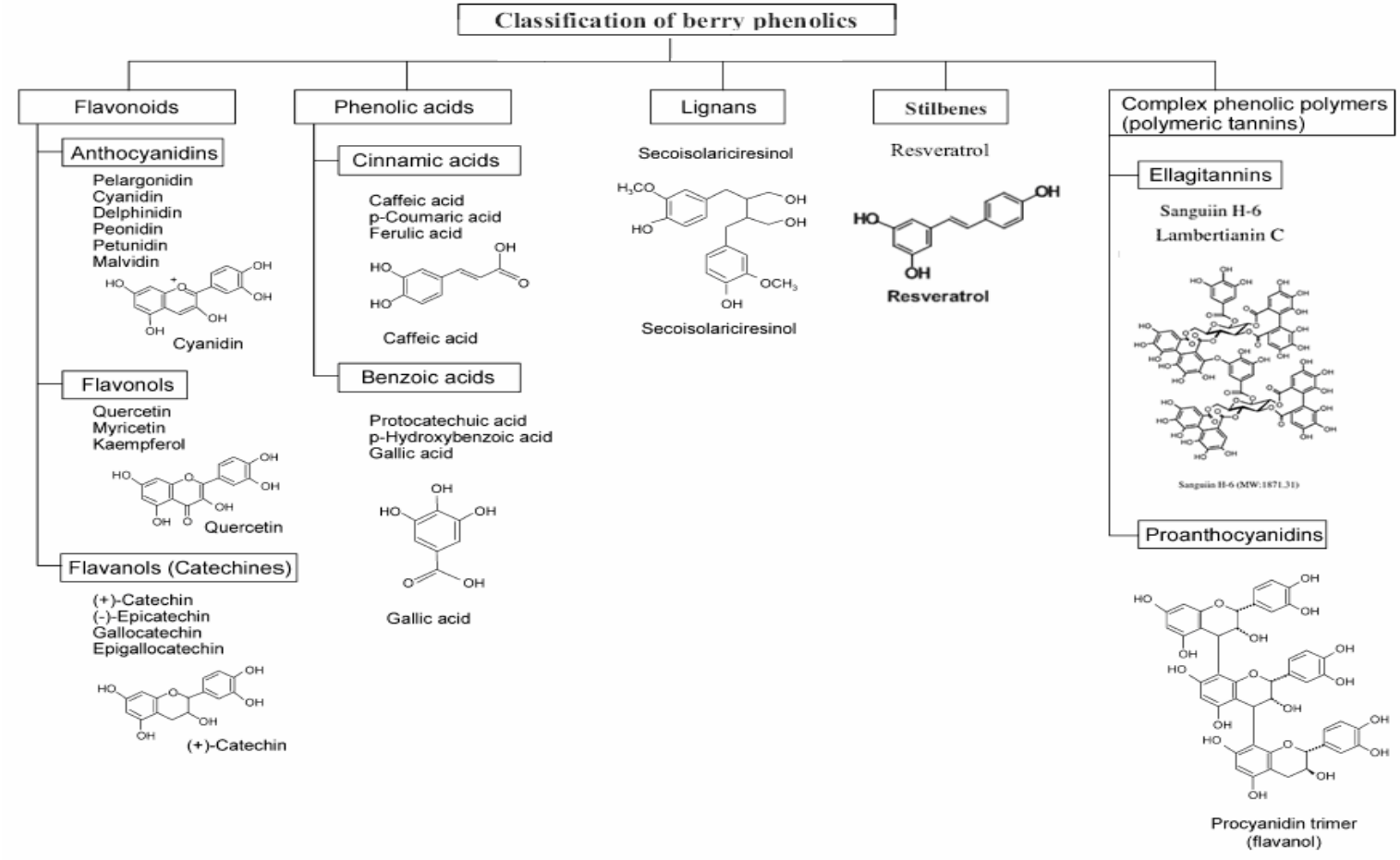
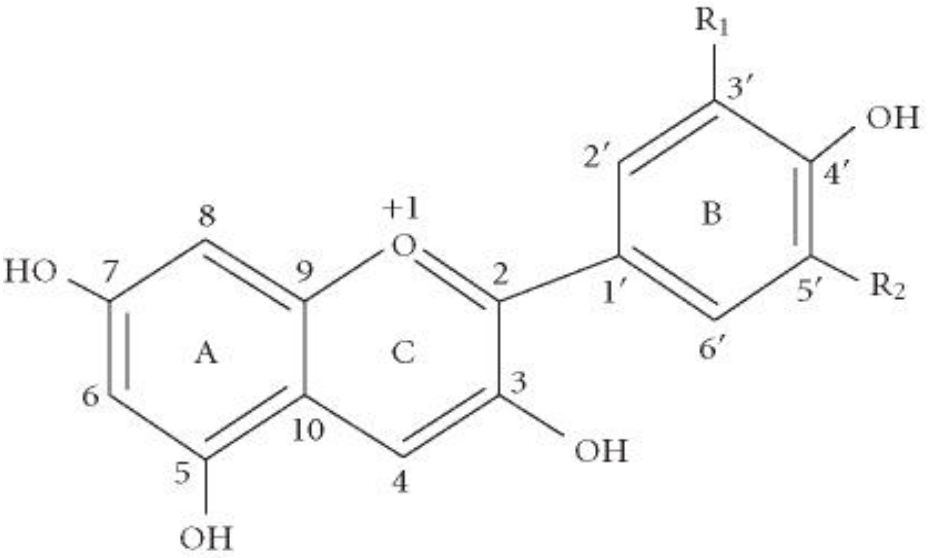


Figure 1.3. Classification of the variations in polyphenols identified in berries. Phenolic compounds include flavonoids, phenolic acids, lignans, stilbenes and polymeric tannins, diagram extracted from Puupponen-Pimiä *et al*. (2005).

1.2.3.2 Anthocyanin biosynthesis

Anthocyanins are a form of secondary metabolites found in higher plants which contribute to the red, purple, blue and violet colours found in flowers and fruit and are used by consumers to judge fruit quality (Jennings, 1988; de Pascual-Teresa and Sanchez-Ballesta, 2008). Derived from the Greek term "*Anthos"* meaning flower and "*kianos*" meaning blue, anthocyanins consist of anthocyanidins (aglycones) which are bonded to a sugar moiety (Castañeda-Ovando *et al*., 2009). The anthocyanidin complex contains a hydroxyl group at position 3 which, due to its instability, is seldom found in its free form in fruit or vegetables. The anthocyanins however have an -O-sugar group at position 3 which confer significantly greater stability (Furtado *et al*., 1993). It has been proposed that anthocyanin biosynthesis in plants involves the addition of glucose to the unstable colourless chalcone form of the 3-deoxyanthocyanidin to form the 3-monoglucoside anthocyanin (Brouillard, 1982). This results in a stabilising effect on the molecule by favouring ring closure to create the hemiacetal form that can rapidly take on other stable equilibrium forms (Brouillard, 1982).

Belonging to the flavonoid group of polyphenols, anthocyanins have $C_6C_3C_6$ skeletons and consist of an aromatic ring (A) linked to a heterocyclic ring (C) containing oxygen with a further carbon-carbon bond to a third aromatic ring (B) (Figure 1.4). Anthocyanins are glycosylated polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (flavylium) cation (Brouillard, 1982). Of the 23 different anthocyanidins most commonly identified to date only six are regularly found in fruit and vegetables; cyanidin (50%), delphinidin (12%), pelargonidin (12%), peonidin (12%), petunidin (7%) and malvidin (7%) (Castañeda-Ovando *et al*., 2009). Anthocyanidins are structurally based on the single aromatic structure of cyanidin with derivations arising as a result of the addition or subtraction of hydroxyl groups, the degree of methylation of hydroxyl groups or through the nature, number and position of sugars on the glycone as shown in Figure 1.4.



Delphinidin, $R_1 = R_2 = OH$ Pelargonidin, $R_1 = R_2 = H$

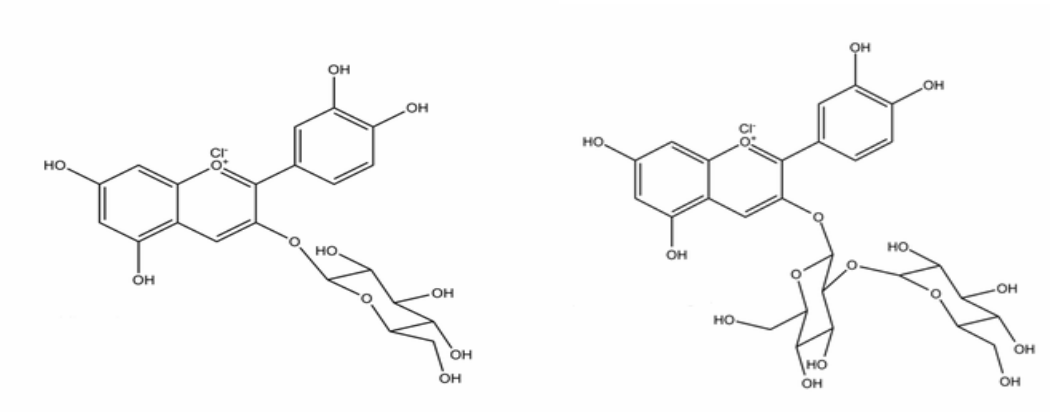
Cyanidin, $R_1 = OH, R_2 = H$ Peonidin, $R_1 = OCH_3, R_2 = H$

Malvidin, $R_1 = R_2 = OCH_3$ Petunidin, $R_1 = OCH_3, R_2 = OH$

Figure 1.4. Carbon skeleton showing the basic anthocyanin structure. The anthocyanin skeleton consist of an aromatic ring (A) linked to a heterocyclic ring containing oxygen (C) with a further carbon-carbon bond to a third aromatic ring (B).

Anthocyanins can be classified depending on the number of glycosyl units they contain and their relative position on the carbon rings. Monoglycosides comprise of a single saccharide moiety, primarily attached to the 3-hydroxyl group of the glycone. In diglycosides two monosaccharides are generally attached to the 3-hydroxyl and 5-hydroxyl groups, occasionally to 3-hydroxyl and 7-hydroxyl group, but it is also possible that two monosaccharides are attached to the 3-hydroxyl. In triglycosides the monosaccharides are attached to the anthocyanidin with two sugar groups on the 3-hydroxyl and one on either 5-hydroxyl or 7-hydroxyl group (Jennings, 1988; Wrolstad *et al*., 2005; Castañeda-Ovando *et al*., 2009). The most abundant anthocyanins in red raspberry are cyanidin 3-glucoside and cyanidin 3-sophoroside (de Ancos *et al*., 1999; Mullen *et al*., 2002a; Kassim *et al*., 2009) (Figure 1.5).

Anthocyanin synthesis is dependent on many physiological and environmental factors as well as fruit species and cultivar. Red colouration of fruit and flowers are assumed to be the most primitive form of anthocyanins found (Castellarin and Di Gaspero, 2007). In flowers, anthocyanins serve to attract pollinators, fruit skin attracts animals for seed dispersal and anthocyanins also aid plants in the protection against harmful UV rays (Holton and Cornish, 1995). Wild species of grape generate dark red coloured fruit and it was proposed that white coloured grapes were the result of a mutation within the anthocyanin biosynthetic pathway (Walker *et al*., 2007). A key gene, VvUFGT (*Vitis vinifera* UDP-Glucose 3-O-flavonoid:glucosyltransferase) that acts late in the biosynthetic pathway, is expressed at lower levels in white grapes than red (Walker *et al*., 2007). Similar VvUFGT gene sequences suggested transcription differences. Although all biosynthetic genes can be detected in fruit cultivars, it is genotype specific regulation of genes along the core pathway and at the main branching points that are thought to be responsible for quantitative variation in anthocyanin content (Castellarin and Di Gaspero, 2007). Many genes in numerous biosynthetic pathways determine colour variation. Synthesis of anthocyanins, procyanidins (tannins) and flavonols occurs via the flavonoid branch of the phenylpropanoid pathway the primary precursor of which is phenylalanine and the end product glycosides of cyanidin (Jeong *et al*., 2006).



Cyanidin 3-glucoside Cyanidin 3-sophoroside

Figure 1.5. Structure of two of the most common anthocyanins found in red raspberry. The monoglycoside, cyanidin-3-glucoside, contains one glucose molecule attached to the 3-hydroxyl position. The diglycoside, cyanidin 3-sophoroside has two glucose molecules, both of which are attached to the 3-hydroxyl position.

Intermediary steps are catalysed by many enzymes, several of which are critical in the regulation of anthocyanins (Jeong *et al*., 2006; Castellarin and Di Gaspero, 2007). Genes early in the pathway for the formation of dihydroflavonols encode phenylalanine ammonia lyase (PAL), 4-coumarate CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerise (CHI) and flavanone 3-hydroxylase (F3H). Later genes for anthocyanin formation and modification encode dihydroflavonol 4-reductase (DFR), anthocyanin reductase (ANR), flavonoid 3' and 3' 5'-hydroxylase (F3'H and F3'5'H) and glutathione S-transferase (GST) as shown in Figure 1.6 (Jeong *et al*., 2006).

In fruit cultivars genetically determined to produce red fruit, flavonoid synthesis is directed towards anthocyanin formation, and less bound procyanidin is accumulated. It is accepted that the synthesis of flavonoid end products are also regulated and this phenomenon may be explained by precursor competition theory (Ju *et al*., 1997). The precursor, dihydroquercetin for example, is sequestered by both leucocyanidin and quercetin. If internal conditions favour synthesis of anthocyanins, such as during fruit maturation, increased DFR activity will accelerate anthocyanin and bound procyanidin formation while retarding quercetin synthesis (Ju *et al*., 1997).

1.2.3.2.1 Transcription factors involved in anthocyanin biosynthesis

Genes identified in the regulation of transcription complexes involved in anthocyanin biosynthesis in grape include MYB, bHLH (basic helix loop helix) and WD40 factors (Walker *et al*., 2007). WD40 proteins, belonging to the β-propeller family, facilitate protein to protein interactions and form a surface to which similar groups, such as bHLH and MYB proteins, can interact (Denisenko *et al*., 1998; Zhang *et al*., 2003). A mutation found in two MYB alleles, in all white grape cultivars examined, suggested these genes had been inactivated and anthocyanin biosynthesis rendered incomplete (Espley *et al*., 2007). In apple, a MYB gene is associated with the regulation of structural anthocyanins and proanthocyanidin genes, in the form of two repeat R2R3 domains (Espley *et al*., 2007). In *Arabidopsis thaliana*, 126 R2R3 type MYB genes have been identified (Stracke *et al*., 2001).

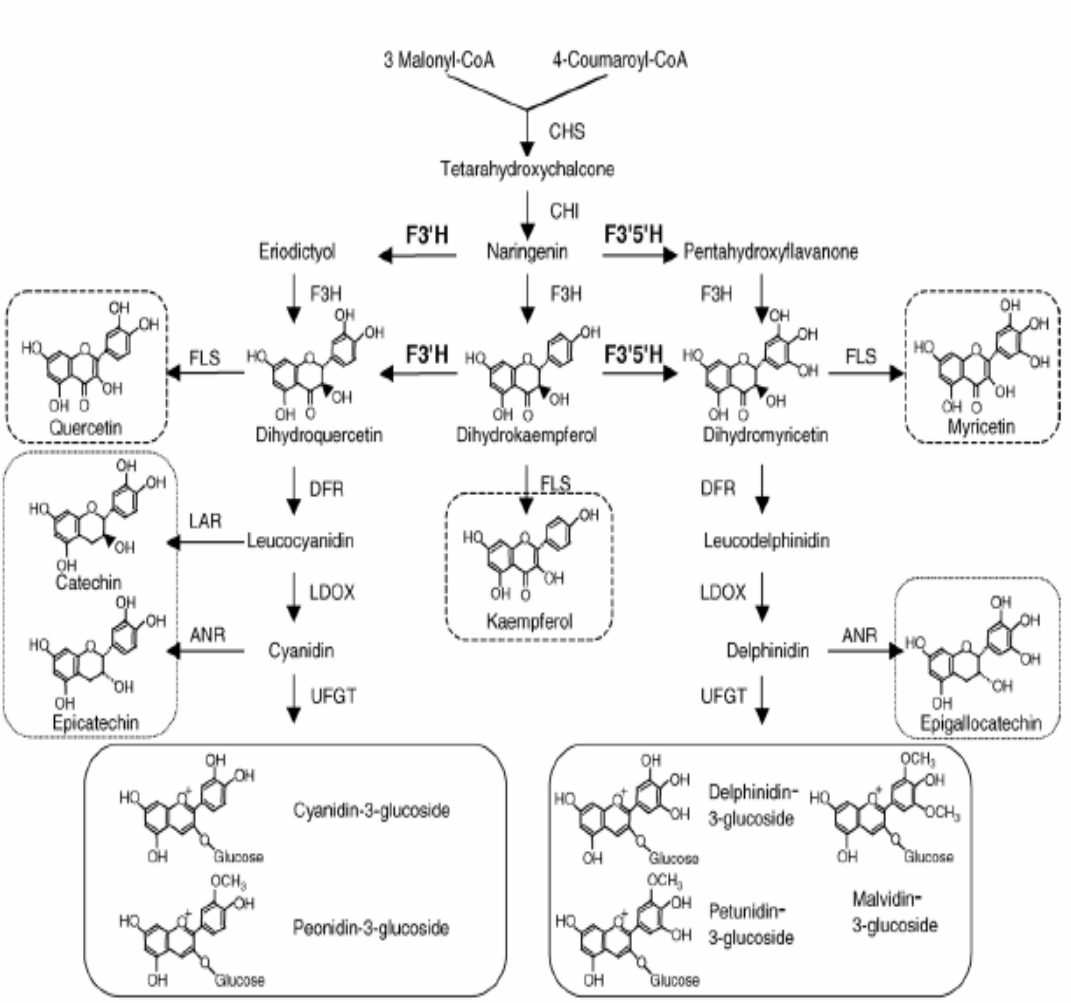


Figure 1.6. Schematic representation of enzymes and intermediates in grape flavonoid biosynthesis. Abbreviated names are as follows; CHS chalcone synthase, CHI chalcone isomerise, F3'H flavonoid 3'-hydroxylase, F3'5'H flavonoid 3'5'-hydroxylase, FLS flavonol synthase, DFR dihydroflavonol 4-reductase, LDOX leucoanthocyanin dioxygenase, UFGT UDP-glucose:flavonoid 3-O-glucosyltransferase and ANR anthocyanin reductase. Broken lines are flavonol products, dotted lines contain proanthocyanins and the unbroken lines contain the anthocyanins, diagram extracted from Jeong *et al*. (2006).

The transcription factor bHLH, involved in several biological processes in plants including anthocyanin biosynthesis, has been found to interact closely with R2R3 MYB genes in anthocyanin regulation (Quattrocchio *et al*., 1998; Espley *et al*., 2007). Recent work in apple has established that the efficient induction of anthocyanin production is dependent on the co-expression of a MYB gene, MdMYB10, and two distinct bHLH transcription factors MdbHLH3 and MdbHLH33 (Espley *et al*., 2007). Two MYB genes mapped in apple and associated with red colouration are now available for marker assisted selection and breeding (Allan *et al*., 2008) with colour differences between individuals attributed to different expression levels of anthocyanin biosynthetic genes and expression profiles (Espley *et al*., 2007). Such transcription factors bind to specific regions in the promoters of certain biosynthetic structural genes, leading to the co-ordinated production of anthocyanin pigments (Espley *et al*., 2007).

1.2.3.2.2 Co-pigmentation and anthocyanin stability

Co-pigments (such as flavonols) are often associated with anthocyanins, and have been described as central to flower colouration (Davies and Mazza, 1993; Mazza and Brouillard, 1990; Castañeda-Ovando *et al*., 2009). Co-pigmentation is thought to offer a stabilising effect on perceived pigment colouration while often inducing a blue shift in visible colour (Nielsen *et al*., 2002). Several compounds may act as co-pigments including flavonoids, alkaloids, amino acids, polysaccharides, metals, organic acids, nucleotides and other anthocyanins (Castañeda-Ovando *et al*., 2009). Co-pigmentation allows the formation of complex interactions between pigments and colourless compounds which enhance colour intensity. Although co-pigments are often colourless on their own, association with anthocyanins results in an interaction which produces a hyperchromic effect (increase in absorbance) in the visible spectra as well as a bathochromic shift (increased wavelength, Σ_{max}) along absorption spectra (Malien-Aubert *et al*., 2001). Self-association, a special case of co-pigmentation, was first identified in 1931, but not investigated in detail until 1972 (Asen *et al*., 1972). The magnitude of the co-pigment effect in solution has been shown to be dependent on several factors including the structure and amount of anthocyanin and co-pigment

molecules as well as the pH, temperature and composition of the aqueous solvent solution (Mazza and Brouillard, 1990).

1.2.4 Aquaporins and transport genes

Aquaporins are water channel proteins that transport water and small molecules across cellular membranes which may also have a role in fruit colour. Plant membrane intrinsic proteins (MIPs) play important roles in cell division, expansion and water transportation in response to environmental conditions (Oliviusson *et al*., 2001). Members of the aquaporin gene family can conduct water in the direction of an osmotic gradient in a highly efficient and selective manner (Agre *et al*., 1993). Aquaporins also increase membrane permeability of water across cell membranes. Aquaporins belong to a large super family of MIPs: tonoplast intrinsic (TIPs), plasma intrinsic (PIPs), nodulin 26-like intrinsic (NIPs) and small and basic intrinsic proteins (SIPs) (Figure 1.7; Maeshima and Ishikawa, 2008). MIPs are likely to be regulated to allow cellular control of water influx and efflux in plant responses to stress conditions and in particular dehydration (Smart *et al*., 2001). In spinach, PIP phosphorylation was detected only under conditions of high water potential (Smart *et al*., 2001). Johansson *et al*. (1996; 1998) concluded that aquaporins were dephosphorylated and inactivated under dehydration conditions to conserve cellular water, whereas Yamada *et al*. (1997) found aquaporin genes were induced by dehydration increasing osmotic permeability and facilitating water flux. As well as the conservation of water in environments of low water availability there are a number of internal processes which require water transport. These include single cell expansion and the osmoregulation and long distance transport of water and assimilate (Oliviusson *et al*., 2001). Several assimilates have been associated as transport substrates for aquaporins and these include urea, ammonia, glycerol and hydrogen peroxide via TIPs, ammonia, urea, glycerol, boron, silicon, lactate and arsenite with NIPs and carbon dioxide and urea associated with PIPS (Maeshima and Ishikawa, 2008).

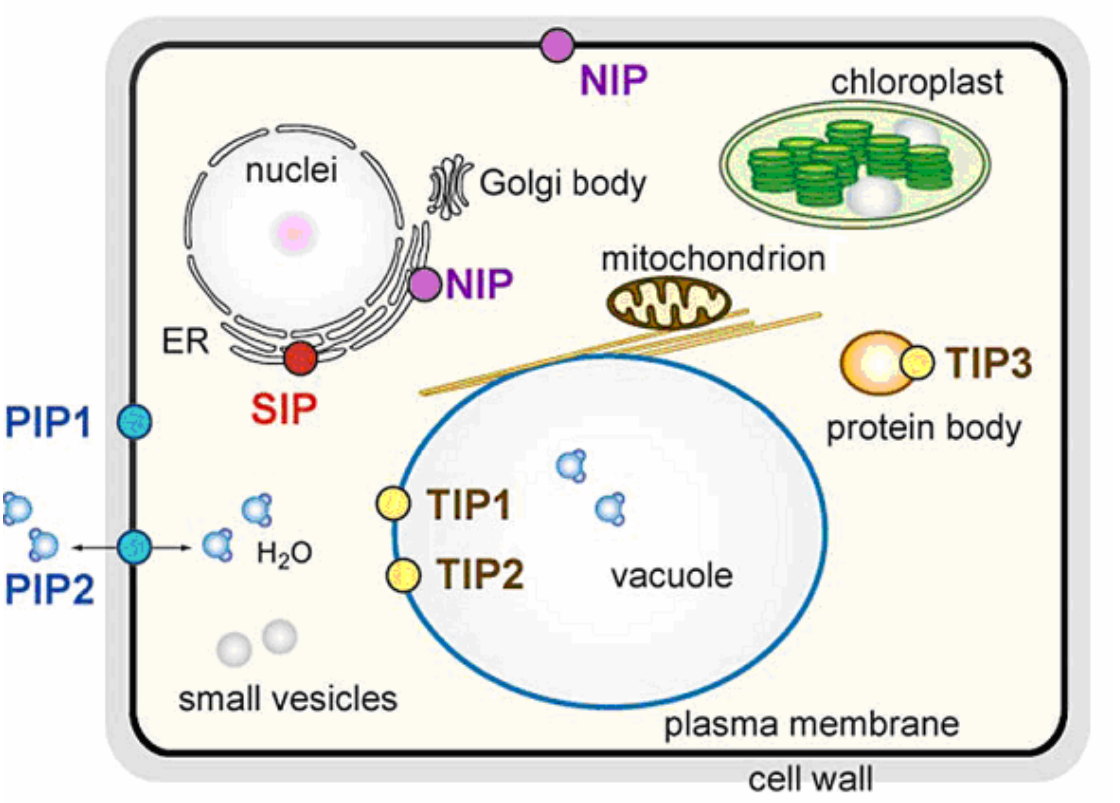


Figure 1.7. Schematic representation of plant aquaporins. Plasma intrinsic proteins (PIPs) are found localised to plasma membranes; Tonoplast intrinsic proteins (TIPs) within vacuolar and protein body membranes (including those pigmented); Small and basic intrinsic proteins (SIPs) are found in endoplasmic reticulum (ER) membranes and Nodulin 26-like intrinsic proteins (NIPs) within plasma and ER membranes (Figure extracted from Maeshima and Ishikawa, 2008).

1.2.5 Expansin and cell growth

For differentiated cells to fulfill their potential in cell shape and size, individual cell walls are remodelled and shaped by means of cell turgor pressure achieved, in part, by expansins (Rose *et al*., 1997; Cosgrove, 2000). Expansins are proteins which are secreted by cell walls during growth unlocking networks of cell wall polysaccharides to allow turgor driven cell enlargement (Figure 1.8). There have been at least 25 expansin related genes identified in *Arabidopsis thaliana* (Cosgrove, 2000) while seven have been reported in tomato (Brummell and Harpster, 2001). Plant cell walls give cells shape, strength, rigidity, bond cells and act as barriers against pathogens and consist of crystalline cellulose microfibrils embedded in a hydrophilic matrix of hemicelluloses and pectins (Cosgrove, 2000). Expansins are associated with cell growth, cosequently with the later stages of fruit ripening in tomato and strawberries delaying (when expansin is silenced) or increasing (when overexpressed) fruit ripening (Cosgrove, 2000). An increase in cell wall bound protein found in raspberry fruit has, in part, been attributed to expansin accumulation (Iannetta *et al*., 1998).

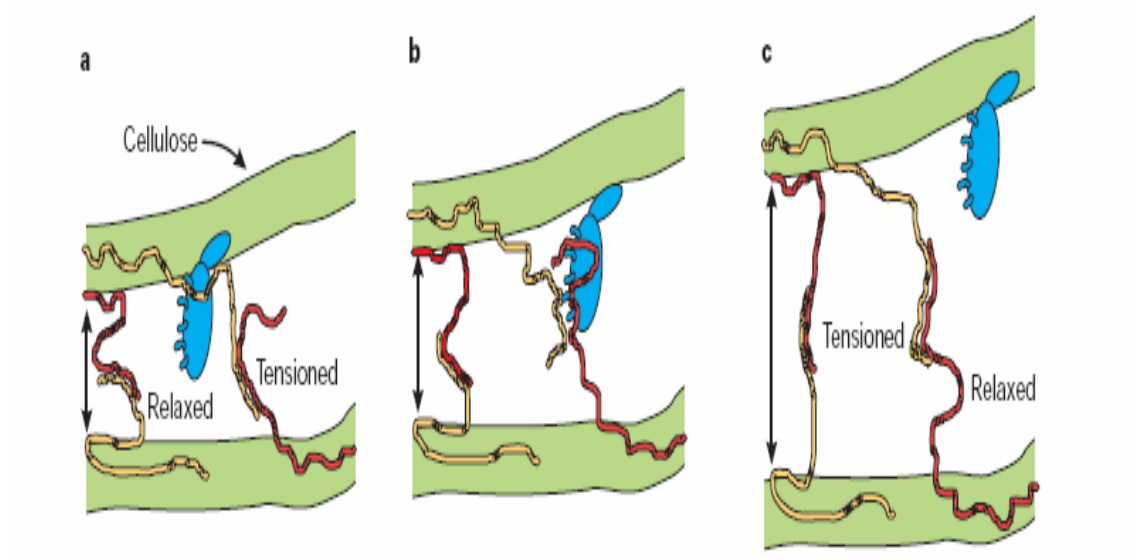


Figure 1.8. Activity of expansin on cell wall loosening. Yellow and red strands denote glycans which connect cellulose microfibrils (green) while blue graphics represent expansin proteins. Figure a) shows expansin disrupting the bond between microfibrils surface and glycans, Figure b) disrupts bonds between individual glycans and Figure c) shows expansin displacing the wall polymers due to mechanical stress arising from turgor (Figure extracted from Cosgrove, 2000).

1.2.6 Fruit size and weight

Raspberries are an aggregate fruit composed of an aggregation of small drupelets (around 80 to 90 per raspberry) held together around a central core by almost invisible hairs (Jennings, 1988). Consumers often judge fruit based on a rapid visual assessment, initially rejecting those over/under ripe (based on colour) or low quality (damaged, crumbly or small fruit). Cellulase is associated with drupelet cohesiveness of raspberries and activity increases during drupelet softening and is also connected with weakening of abscission zones within drupelet stipes during fruit ripening (Sexton *et al*., 1997). Quantitative differences in cellulase activity between cultivars could account for reduced drupelet cohesion and hence smaller fruit production (Sexton *et al*., 1997). Drupelet number, size and degree of cohesion are determined by fertility of parental material influenced by genetics, chromosome number and presence of viral infections (Jennings, 1988). Fruit that lacks drupelet cohesion, resulting in a crumbling appearance when picked, was shown to be caused by a genetic defect by Jennings (1967) who initially observed a mutation present in a dominant allele identified in a heterozygote of cv. Malling Jewel and to a lesser extent cv. Latham, the gene of which he linked to a balanced lethal system. The lack of fertile pollen in individuals and retarded embryo sac development was suggested by Daubeny *et al*. (1967) to be responsible for a crumbly fruit phenotype in cv. Sumner clone (Ourecky, 1975).

A definitive value of drupelet cohesion is one which can be assessed and measured within field trials, along with the overall size of fruits, considered individually or collectively as ten berry weights. Several intrinsic processes involved in the transport of solutes across vacuolar membranes impact on fruit metabolite concentrations of fruit, making these ideal candidates for gene analysis. A single major QTL for fruit weight (*fw* 2.1) was identified in tomato chromosome 2, in close proximity to a cloned fruit weight gene *fw* 2.2 (Frary *et al*., 2000; Zygier *et al*., 2005). Although potential benefits of QTL analysis and molecular assisted breeding are clear for enhancement of individual quality traits, to date results have been disappointing in regards to overall crop improvement,

e.g. tomato soluble solid content could be enhanced but at a predicted cost of fruit yield (Tanksley *et al*., 1996; Chen *et al*., 1999; Saliba-Colombani *et al*., 2001).

1.2.7 Sensory perception of flavour

Perception of flavour is a complex combination of gustatory (sweet, salty, bitter, sour and umami taste), olfactory (sense of smell), visual, oral somatosensory (stimuli detection between oral cues and sensory receptors), auditory and nociceptive (sensory receptors which respond to pain) cues (Zampini *et al*., 2007). Enjoyment of food comes from its visual appeal, smell and taste and also its texture, sound and mouthfeel (Vickers, 1983, cited in Zampini *et al*., 2007). In strawberry, flavour volatiles consist of over 100 constituents, the majority of which are generated during ripening from non-volatile precursors such as amino acids and lipids and include acids, aldehydes, ketones, alcohol, esters, lactones, phenols, terpenes and epoxides (Aharoni *et al*., 2004). Terpenoids including C10 monoterpenes and C15 sesquiterpenes, have also been identified at varying levels involved in the flavour profiles of most if not all soft fruit analysed to date (Aharoni *et al*., 2004). In red raspberry, there have been more than 200 individual volatiles identified (Roberts and Acree, 1996) with 10 compounds highlighted as important to raspberry aroma; 4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one (α-ionone), 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-3-buten-2-one (β-ionone), (Z)-3-hexenol, (E)-3,7-dimethyl-2,6-octadien-1-ol (geraniol), 2,6-dimethyl-2,7-octadien-6-ol (linalool), phenylmethanol (benzyl alcohol), 3-hydroxybutan-2-one (acetoin), ethanoic acid (acetic), hexanoic acid and 4-(*p*-hydroxyphenyl)-2-butanone (raspberry ketone) (Larsen *et al*., 1991; Klesk *et al*., 2004).

Studies on flavour perception in tomato have linked nutritional health benefits of food to consumer preference, with essential long chain *cis*-polyunsaturated fatty acids stimulating taste responses (Goff and Klee, 2006). Sensory assessments have been made regarding the taste experience of various fruit acids (Pangborn, 1963; Liu *et al*., 2007). Wu *et al*. (2003) found malic acid to have a stronger acidity than citric acid while quinic acid was described as having a sour and bitter taste although more beneficial to health

due to its antibacterial properties. Lopez *et al*. (2007) found the sour taste of wine to be elicited by organic acid content which was influenced by pH. A study by Pangborn (1963), however, reported no relationship between pH, total acidity and relative sourness.

1.3 External influences on quality traits

Plant phenotype is controlled by three main factors: genotype, environment and their interaction (Florez *et al*., 2009). Environmental variations in fruit phenolic biosynthesis were reported in apple (Mayr *et al*., 1997) with a sixfold variation found in apple leaves of the same genotype grown at different locations (Treutter, 2001). Fertilisation of the soil in which fruit is grown can have a significant effect on the polyphenol accumulation, both positively and negatively. Nitrogen stimulates plant growth and in moderate supply, prior to fruit bloom, can favour polyphenol synthesis in grape. In excess, nitrogen is associated with a delay in fruit ripening and poor fruit colouration (Delgado *et al*., 2004) and may also interfere with metabolic routes involved in aroma and flavour volatile synthesis (Ju *et al*., 1997; Delgado *et al*., 2004). Excessive growth may also occur in high nitrogen environments and this can result in an increase in vegetative growth which will compete with fruit for sugar and pigment translocation and accumulation (Delgado *et al*., 2004). Anthocyanins and procyanidins may also appear diluted in fruit with an increased berry size (Delgado *et al*., 2004). Potassium, indicative of fruit quality, can increase colour and polyphenolic content by stimulating photosynthetic activity which favours sugar translocation in fruit (Delgado *et al*., 2004). This benefits phenolic compound synthesis during the ripening process in particular and can be related to carbohydrate accumulation in grapes. A balance between nitrogen and potassium content in soils for the production of quality fruit products is therefore required (Ju *et al*., 1997; Delgado *et al*., 2004).

1.3.1 Environmental hydrogen ion content

The colour of anthocyanins expressed in nature is pH dependent. In acidic conditions anthocyanins appear red, at neutral pH, they are violet and in basic conditions they are

blue (Yawadio and Morita, 2007). The phenotype of an organism can vary depending on the environment in which it is grown. One example is the Hydrangea flowering plant which may alter colour of the sepals produced depending on soil pH (Toyama-Kato *et al*., 2003). pH values have also been found to determine the relative nucleophilic and electrophilic character of anthocyanins and flavonols affecting their reactivity and thus colour stability (de Freitas and Mateus, 2006).

1.3.2 Light impact on flavonoid synthesis

Light has an essential role in the synthesis of polyphenol compounds. Ju *et al*. (1995a) reported on a study in which 30 apple fruits per tree were enclosed in light-excluding bags (double layered paper bags with a wax coating) 40 days after full bloom (DAFB). Half of the fruit had the bags removed 110 DAFB and the remaining 15 were removed 130 DAFB at harvesting. The latter were found to contain only trace amounts of flavonoids, low levels of chalcone synthase and no anthocyanin activity was detected. The initial fruits harvested 20 days after bag removal (110 DAFB), however, had equivalent anthocyanin content as unbagged control fruit harvested from the same tree and chalcone synthase and flavonoid synthesis levels were significant (Ju *et al*., 1995b). Chalcone synthase is the initial enzyme in the flavonoid biosynthesis pathway, responsible for the establishment of the flavonoid compound C15 skeleton. Fruit bagging completely inhibited dihydroflavonol reductase (DFR) gene expression and flavonoid accumulation (Ju *et al*., 1997). Light intensity has also been shown to have a profound effect on the anthocyanin accumulation found in apple fruit with peel exposed to sunlight containing twice as much anthocyanin as that which has been shaded throughout fruit maturation. Flavonoid accumulation and chalcone synthase activity however were not affected by light exposure (Ju *et al*., 1997). Ju *et al*. (1999) reported an increase in anthocyanins detected in apple peel following the application of metal foil to the orchard floor which enhanced light penetration into the canopies. Position of the fruit on the bush is also a factor in anthocyanin accumulation with those found in the shade and subjected to less sunlight throughout development showing a decrease in anthocyanin content compared with those exposed to sunlight on the same bush (de

Pascual-Teresa and Sanchez-Ballesta, 2008). In several apple and grape varieties, covering developing fruit after flowering was found to have little effect on the anthocyanin accumulation. This treatment did, however, reduce the individual content of delphinidin, cyanidin, petunidin and malvidin while increasing the peonidin glucosides (Gao and Cahoon, 1994; Cortell and Kennedy, 2006)

1.3.3 Temperature and flavonoid synthesis

The stability of anthocyanins can be adversely affected by temperature with degradation observed during processing and storage and this is exacerbated by ambient temperature rises (Adams, 1973; Robbins and Moore, 1990). The degradation of anthocyanins occurs as the result of losing glycosyl moieties caused by the hydrolysis of glycosidic bonds (Adams, 1973). The loss of the glycosyl moieties results in an unstable aglycone formation and a further loss of anthocyanin colour. Thermal degradation results in the appearance of brown patches within fruit which is enhanced by the presence of oxygen. It is believed that chalcone formation is the first step involved in thermal degradation (Markakis *et al*., 1957; Adams, 1973).

Low temperature, however, can have a positive effect on colouration including the formation of anthocyanins during sub-ambient storage of fresh fruit. Kalt *et al*. (1999) reported an increase in anthocyanin accumulation in strawberry and raspberry fruit which had been stored at 0^{o}C for eight days. Exposure to low temperatures can have several effects on the internal mechanism of plant cells including membrane fluidity and enzyme kinetics (Christie *et al*., 1994). This leads to other cellular processes becoming affected such as photosynthesis, respiration and cell transport (Christie *et al*., 1994). Temperature effects on fruit anthocyanin biosynthesis are thought to be dependent on the stage of ripening, particularly in response to changes in phenylalanine ammonia lyase (PAL) synthesis (Faragher, 1983). Li *et al*. (2004) recorded rapid anthocyanin accumulation in the skin of two apple varieties grown in cool conditions compared to the same varieties grown in warmer sites. Micro-sprinkler irrigation to cool apples during

warmer conditions was also found to increase the red coloration and anthocyanin content and was most effective when applied during sunset and sunrise (Iglesias *et al*., 2002).

1.4 Conventional breeding in red raspberry

Breeding methods in raspberry have remained similar over the last 40 years with little in the way of novel germplasm being introduced into commercial cultivars. As the genetic diversity of current cultivars is reduced, as a result of domestication, and retail demands for new cultivars increase, it becomes evident that new breeding strategies must be employed (Graham and Jennings, 2009). Domestication of red raspberry has seen a reduction in both morphological and genetic diversity with many modern cultivars being genetically similar (Graham and McNicol, 1995). As a general rule, breeders use a darker fruited clone as the seed parent and a lighter fruited clone as the pollen parent in order to secure seed set (Ourecky, 1975). 'Glen Moy' and 'Glen Prosen' were the first spine-free raspberries released by the Scottish Crop Research Institute in 1981. 'Glen Moy' is an early fruiting alternative to 'Glen Clova' (released in 1970) with notable improvements in fruit size and flavour. The development and introduction of new varieties can take between 8 and 15 years due to the highly heterozygous nature of this perennial fruit crop and even then screening for selected quality traits is problematic due to the limited resources breeders have available (Graham *et al*., 2004). Conventional breeding begins with an initial cross of cultivars with different genetic characteristics, which results in a large number of progeny (typically 10,000 seedlings) which require phenotypic evaluation. The characteristics discarded at the first evaluation stage are seedlings with spiny phenotypes and those susceptible to aphids. At this stage around 5000 seedlings remain which are then propagated prior to a two year field trial. The characteristics taken into account during field trials are the fruit size, shape, firmness, flavour and shelf life, after which around 50 plants remain. Further propagation, replication and field trials follow to test for characters such as resistance to pests and diseases and fruit yield compared with standard cultivars of 'Glen Ample' and 'Tulameen'. Only after rigorous field trials and cultivar evaluations is a new cultivar considered for commercial release (N. Jennings, personal communications, Figure 1.9).

Raspberry breeding programme

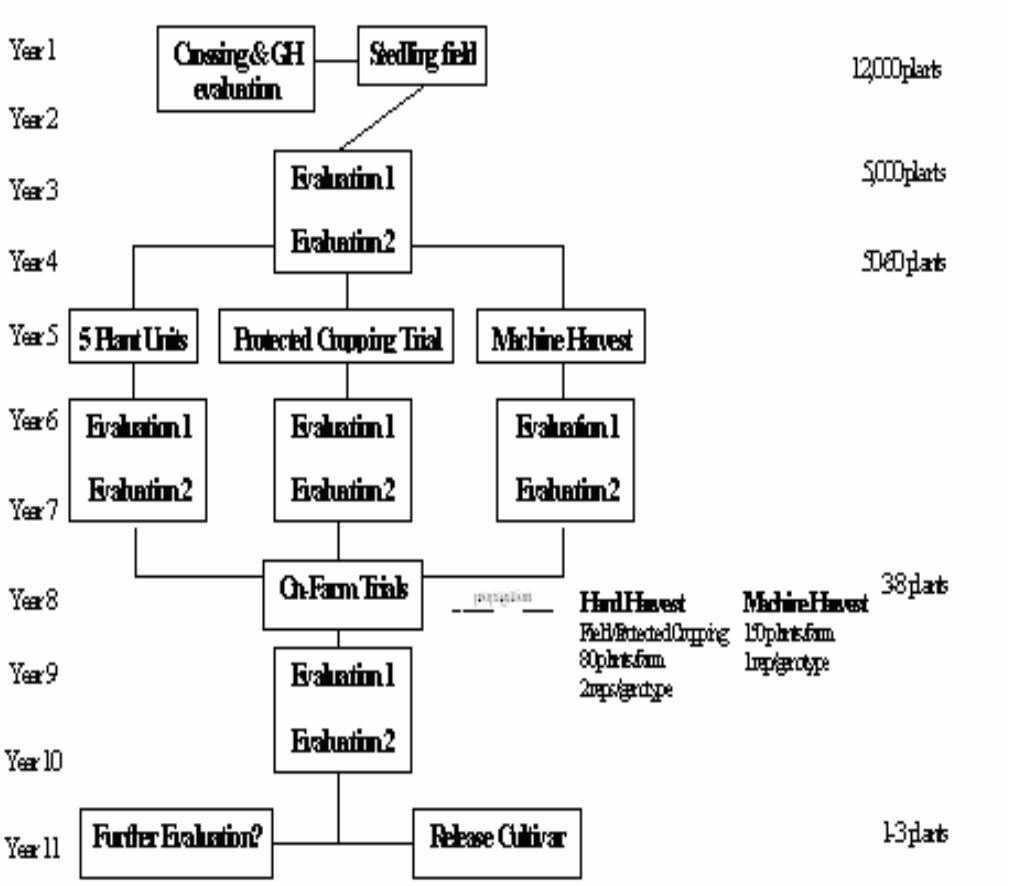


Figure 1.9. Raspberry breeding schedule as described by the Scottish Crop Research Institute. Conventional raspberry breeding begins with the crossing of a selection of parental cultivars with different genetic characteristics which results in a large number of progeny (typically 10,000 seedlings) requiring phenotypic evaluations. Around 5000 seedlings remain which are then propagated prior to a further two year field trial which often results in only 50 potential new cultivars remaining. Propagation, replication and field trials follow, reducing numbers further, before a new cultivar with superior agronomic characteristics can be considered for commercial release.

The evaluation of large populations of seedlings has brought improvements in current breeding stock. The primary objectives in raspberry breeding have remained unchanged over the years. The core objectives are high quality fruit, flavour, appearance (colour, brightness, size and shape), good yield and shelf life, increased pathogen resistance and fruit which are suitably adapted to the local environment (Ourecky, 1975; Jennings, 1988; Graham and Woodhead, 2009). Genetic resistance to many pests and diseases through plant breeding is a feasible strategy for raspberry cultivars for successful commercial production. This requires identifying resistance genes present in wild and domesticated varieties as well as key genes and chromosomal regions that underpin quality characteristics.

1.5 Marker assisted breeding in red raspberry

Marker assisted breeding strategies are a non-controversial means for developing high quality cultivars with selected agronomic traits. Conventional breeding relies on phenotypic selection that has strict limitations particularly when analysing complex physiological traits influenced by the environment (Graham *et al*., 2007). The speed and precision of breeding in red raspberry can be improved with molecular tools including genetic linkage maps and developing associations between specific traits and a candidate gene or a genetic marker. Markers can be found to occupy specific genomic positions within chromosomes which are referred to as loci (locus, singular) (Collard *et al*., 2005).

Genetic markers can be categorised depending on their association with the genes with which they are linked to and include:

- Morphological markers, which influence phenotypic traits such as fruit colour and shape.
- Biochemical markers, which are allelic variations of an enzyme (isozyme).
- Molecular (DNA) markers, which disclose actual sites of DNA variation.

1.5.1 Morphological markers

Mendelian markers are associated with a physical qualitative characteristic identified in an individual which can be assessed visually. Morphological markers can be either dominant or recessive and are often associated with an undesirable trait such as dwarfism or altered morphology. The constraints of such markers, however, include the masking of minor gene effects due to large phenotypic effects and they are also highly influenced by the environment. This makes it inappropriate to compare morphological data collected from varieties grown in different environments or seasons. Performing breeding experiments using these markers alone can also be time consuming and labour intensive as large populations are required for accurate analysis (Chawla, 2002).

1.5.2 Biochemical markers

Biochemical markers include protein differences emerging from gene expression. Isozymes are an example of a biochemical marker which have been utilised in plant breeding and these are composed of different molecular forms of the same enzyme (Chawla, 2002). Biochemical markers are not influenced by the environment and are therefore more easily transferable between cultivars grown over separate seasons or environments. Constraints, however, exist as isozymes do not exhibit high levels of polymorphism and are therefore difficult to use as markers in genetically similar cultivars. Chromatographical analysis of flavonoid components from raspberry leaf extracts (Haskell and Garrie, 1966) have been used to identify genotypic variations between cultivars but with limited success with only one variety out of seven being readily identified (Antonius-Klemola, 1999).

1.5.3 Molecular markers

Molecular markers are DNA sequences located in close proximity to genes and loci of phenotypic interest. This co-location of markers with traits allows for selective breeding and identification of progeny with desirable characteristics (Graham *et al*., 2007). The identification of polymorphisms, which may be single nucleotide variations, insertions, deletions or repeat sequence length variations, provides information on a particular

genomic locus which may subsequently associate with a particular plant phenotype. The variations in plant phenotypes related to molecular markers can then be located onto a genetic linkage map which can be screened rapidly in a molecular assisted breeding strategy. Molecular markers allow the identification and selection of genotypes combining specific quality characteristics long before the traits are phenotypically expressed (Dirlewanger *et al*., 1998). Molecular markers indicate the genomic location of specific genes, traits and alleles and offer the possibility of plant selection at a juvenile stage from an early generation, and unfavourable alleles can be eliminated or reduced (Korzun, 2002). Analysing DNA sequences by means of amplified fragment length polymorphisms (AFLP), restriction fragment length polymorphisms (RFLP) and microsatellite technology can also lead to the identification of specific short sequences (molecular markers) which associate with the trait of interest in a significant manner.

Molecular markers, developed in the 1980s, led to the characterisation and selection potential of quantitative trait loci becoming recognised as well as being integrated directly in the construction of linkage maps (Collard *et al*., 2005). RFLPs were one of the first molecular markers to be used in the construction of genetic maps and QTL identification (Botstein *et al*., 1980; Lander and Botstein, 1989). This marker system utilises restriction endonucleases which cut the DNA samples into distinct fragments. The resultant fragments are then separated through an agarose matrix according to their mass. Although RFLPs are robust, large amounts of DNA are required and it is a time consuming technique (Chawla, 2002). The introduction of polymerase chain reaction (PCR) technology revolutionised molecular genetics: markers which followed included random amplified polymorphic DNA (RAPD), microsatellites and single nucleotide polymorphisms (SNPs). All of which were found to be cheaper and safer alternatives to previous hybridisation strategies with a greater number of markers identified per unit of DNA analysed (Westman and Kresovich, 1999; Kearsey and Farquhar, 1998). It is the subsequent identification of variations within candidate genes or molecular markers inherited by individuals which make successful molecular assisted breeding a reality.

1.5.4 Genetic linkage maps

The discovery of genetic linkage was attributed to the British geneticist William Bateson in 1909 (Harper, 2005). The first linkage map produced in *Rubus* was accredited to Lewis in 1939 and 1940 (Graham and Woodhead, 2009). Formed as a result of linkage analysis based on fruit and leaf architecture, this early work has led to the elucidation of many more important genes related to both fruit quality and disease resistance (for review see Graham and Woodhead, 2009).

Specific chromosomal regions relating to both simple Mendelian traits (single gene) and quantitative traits (under multiple gene control) can be identified by means of a linkage map (Collard *et al*., 2005). This allows the frequency of inherited sequences to be visualised as an indication of the relative distance between two DNA sequences on a genetic map. The higher this recombination frequency is, the less likely the two markers or genes will be inherited together. By selectively screening for the presence of specific genes within potential breeding cultivars, the possibility of linking genotype to phenotype for selected traits becomes more of a reality. This molecular technology has been employed in several commercial species including cherry, sugarcane, apple, melon, peach and tomato in the identification of QTLs and candidate genes relating to fruit quality and size (Cristosto *et al*., 2002; Soriano *et al*., 2005; Kenis *et al*., 2008; Monforte *et al*., 2004; Dirlewanger *et al*., 1999 and Frary *et al*., 2000). To construct a genetic linkage map a segregating population must first be created by crossing genetically diverse parents which are known to segregate for the trait(s) of interest (Goodenough, 1984). The segregating population can then be scored in order to determine the recombination rate of alleles present at each locus. This allows loci to be ordered onto the linkage map and the relative distance between loci can be expressed in terms of recombination units. One percent recombination is equal to one centiMorgan (cM) map distance (Goodenough, 1984). Once sufficient markers have been mapped, linkage group numbers should equal the chromosome haploid number, which in the case of red raspberries is seven (for review see Graham *et al*., 2007). Linkage maps have been constructed in other soft fruit species including strawberry and blueberry both of which

were based on RAPD markers (Rowland and Levi, 1994; Davis and Yu, 1997; Qu and Hancock, 1997). Other maps from the Rosaceae family have also been constructed and utilised and these include *Prunus* (Dirlewanger *et al*., 1998) and *Malus* (Hemmat *et al*., 1994).

1.5.5 Quantitative trait loci

Quantitative trait loci (QTL) analysis is the study of genetic variation, location of genes and exploration of gene effects and interactions (Kearsey, 1998). Initially known as polygenes (Mather, 1949, as cited by Kearsey, 1998) molecular marker technology allowed genes controlling specific traits to be associated with regions of linkage groups or chromosomes (Kearsey, 1998). Qualitative genes that undergo Mendelian segregation generally have a recessive non-functional or very dysfunctional allele which results in a clear phenotype (Kearsey, 1998). Quantitative variation occurs as a result of allelic differences found within structural or regulatory genes that cause an alteration in gene function and hence produce a smaller phenotypic effect (Kearsey, 1998). Studies in fruit plants where continuous traits have been mapped by means of QTL analysis allowed the identification of genomic locations and genes that underlie genetic or phenotypic variation. Dirlewanger *et al*. (1999) mapped QTLs for peach quality including fruit weight, acidity and soluble solids content. Although fruit colour was correlated significantly with other quality traits no QTL was detected.

Quantitative traits controlled by multiple genes, such as disease resistance and quality (Collard *et al*., 2005), can be linked to specific regions within a genome and the availability of molecular markers and linkage maps allows the dissection of complex, polygenic traits into QTLs (Lander and Botstein, 1989; Liebhard *et al*., 2003). Several QTLs related to fruit development traits, flowering time, time to ripe fruit and fruit quality have been successfully mapped (Conner *et al*., 1998; King *et al*., 2001; Graham *et al*., 2009). One study in tomato identified 81 significant QTLs for 26 quality traits, including total soluble solids (TSS), fruit weight, colour, volatiles and sugar content (Saliba-Colombani *et al*., 2001). Three QTLs were found to control TSS content, two in

the same linkage group (chromosome 2) where QTLs for dry matter weight were located. Three QTLs for colour showed close associations with fruit weight and dimension and titratable acids (Saliba-Colombani *et al*., 2001).

1.5.6 Candidate gene identification

A candidate gene is a gene which is located on a specific chromosomal region suspected of being involved in the expression of a particular trait (de Vienne *et al*., 1999). The identification of candidate genes may result from the association of a specific phenotype or by linkage analysis to a target region of the genome. Genes may be functionally related to the trait of interest, as selected on the basis of previous functional or genetic hypothesis, where subsequent allelic polymorphisms within the gene may prove to account for individual phenotypic variability (Pflieger *et al*., 2001). The working hypothesis, for this approach, assumes that the presence of a molecular polymorphism within a candidate gene is therefore related to the phenotypic variation (Pflieger *et al*., 2001). An assumption made on the biological function of a specific gene of interest facilitates the candidate gene approach for identifying genes governing traits of agronomic importance (Pflieger *et al*., 2001). One approach to the identification of candidate genes for QTL analyses and mapping in raspberry is through the construction and sequencing of fruit expressed sequence tag (EST) libraries which represent expressed genes from various developmental stages. An alternative source is the large insert genomic library constructed in *Rubus* which consists of a (publicly available) bacterial artificial clone (BAC) library, with coverage six to seven times the genome equivalent, consisting of >15,000 clones with an average insert size of 130 kb (Hein *et al*., 2005).

1.5.6.1 Candidate gene functions

Several candidate genes and QTLs for fruit quality have been reported (Etienne *et al*., 2002; Maeshima and Ishikawa, 2008) and these include tonoplast intrinsic proteins (TIP), vacuolar H^+ pyrophosphatase (VP2) and sucrose transporters (STP). The candidate gene approach is thus a strategy for the study of quality traits in fruit,

emerging from a complex combination of biological and genetic factors, the full nature of which remains unclear (Dirlewanger *et al*., 1999). Etienne *et al*. (2002) isolated ESTs for 12 candidate genes involved in phloem uploading of sucrose, sugar and organic acid metabolism, solute accumulation into the vacuole, and cell expansion. All 12 genes were mapped onto four peach linkage groups. The candidate genes involved in trait variation may be either structural or regulatory in the expression of genes found in biological pathways which regulate and influence fruit quality.

1.5.6.2 Structural and regulatory genes

The products of structural genes are enzymes in metabolic pathways regulated under negative control, transcribed until "turned off" by a regulatory protein (repressor), and/or positive control whereby expression must be "turned on" by a regulatory protein (activator) (Busby, 1999). Activator proteins act by binding to the chromosome effecting an increase in the transcription of an adjacent gene, repressor proteins by binding to an operator or promoter preventing RNA polymerase from transcribing RNA (Busby, 1999). Relevant structural genes in raspberries include chalcone synthase (CHS), 4-coumarate CoA ligase (4CL), chalcone isomerase (CHI), flavonoid 3'-hydroxylase (F3'H) and dihydroflavonol 4-reductase (DFR) (Holton and Cornish, 1995; Gong *et al*., 1997).

A plethora of regulatory genes have been recognised in the regulation of traits such as pigment synthesis showing a complex network of genes are linked to seemingly unrelated pathways such as hair development (Koes *et al*., 2005). The regulation of the flavonoid pathway is primarily at the level of transcription of structural genes that encode enzymes for each subsequent step. Two families of regulatory proteins, bHLH (regulating pigment formation) and MYB (chalcone synthase expression) are conserved in anthocyanin regulation in all plants to date (Koes *et al*., 2005; Takos *et al*., 2006). bHLH proteins contain overlapping regulatory targets (Zhang *et al*., 2003; Hartmann *et al*., 2005; Takos *et al*., 2006) whereas MYB proteins provide specificity for individual gene activation (Takos *et al*., 2006). Knowledge of the physiological pathways involved

in the uptake and regulation of sugars and organic acids, as well as colour, is an important area of research as in order to improve fruit quality the underlying control processes must first be identified.

1.6 Pests and diseases of red raspberry

The development of new raspberry cultivars is a long and challenging process with breeders faced with increasing demands from consumers to produce high quality aromatic fruits while growers require pest and disease resistant varieties capable of utilising the extended growing season. Many pests and pathogens can affect red raspberries and these include viruses, fungal diseases, aphids and beetles (reviewed by Gordon *et al*., 2006a). Viruses are a particularly serious problem as an infected plant is unlikely to recover and future propagules will also be infected (Ourecky, 1975).

1.6.1 Phytophthora fragariae

A significant proportion of British raspberries are lost to disease each year with outbreaks of *Phytophthora fragariae* (root rot) proving particularly devastating in susceptible crops (Pattison and Samuelian, 2002). *Phytophthora* is a soil borne fungus which can produce viable resting spores which remain infective within the soil for many years following initial infection (Daubeny, 2002). Considerable attention has focused upon identifying markers associated with resistance with an ultimate goal of identifying the actual genes involved (Valois *et al*., 1996; Daubeny, 2002; Kempler *et al*., 2005; Pattison and Samuelian, 2007) while BAC clones spanning a particular QTL of interest relating to root rot resistance are currently being sequenced (J. Graham, personal communication). Current root rot resistant cultivars lack acceptable quality and or yield but through molecular assisted breeding techniques, genetic resistance, found in cultivars derived from *R. idaeus* var. *strigosus* and to a lesser extent *R. idaeus* var. *vulgatus,* could be combined with desirable quality traits (Graham *et al*., 2007). Genetic resistance through breeding offers a feasible method for control, alhough ensuring planting material is free from the disease is also very important as it is unlikely that the

pathogen will be found in soil which raspberries have not been previously grown (Graham and Jennings, 2009).

1.6.2 Amphorophora idaei

Aphids (*Amphorophora idaei*) are particularly damaging arthropod pests affecting raspberries as these feed directly on susceptible cultivars and act as vectors for the transmission of viruses (Gordon *et al*., 2006a; 2006b) including: Raspberry leaf mottle virus (RLMV), Raspberry leaf spot virus (RLSV), Black raspberry necrosis virus (BRNV) and *Rubus* yellow net virus (RYNV) (Sargent *et al*., 2007). Although varieties can be bred which confer resistance to raspberry aphids, reducing the requirement for pesticide use, it has been found over the years that such resistance genes have been broken down by aphid evolution (Sargent *et al*., 2007). The identification of new sources of resistance from both wild and domesticated cultivars must therefore remain ongoing.

1.6.3 Raspberry bushy dwarf virus

Raspberry bushy dwarf virus (RBDV) is a pollen borne virus which infects susceptible plants reducing fruit yield and causing crumbly unattractive fruit phenotypes (Murant *et al*., 1974). While resistant varieties exist, these are often found to exhibit inferior agronomic traits. Foliar symptoms may also be present in plants containing RBDV which range from no obvious symptoms to bright yellow chlorosis and in some cases stunted plant growth (Murant *et al*., 1974). Plants pollinated by infected pollen are often found to produce crumbly fruit, many with a large proportion of aborted drupelets.

1.6.4 Botrytis cinerea

Fungal infections in raspberry result in severe fruit loss both mid season and post harvest, particularly when environmental conditions are wet and warm. Infections by *Botrytis cinerea* are difficult to control due to the multiple sites of infection and, as yet, no strongly resistant cultivars are available (Graham and Woodhead, 2009). Studies have progressed, however, in the identification and mapping of resistance to raspberry cane

botrytis, which has been mapped in association with Gene H (Graham *et al*., 2006; Graham and Jennings, 2009).

1.6.5 Byturus tomentosus

Another major pest is the raspberry beetle (*Byturus tomentosus*) which causes damage in two distinct forms. Firstly adult beetles feed on buds and flowers during early summer and secondly larvae are deposited on developing fruit drupelets causing contaminated and discoloured fruit (Griffiths *et al*., 2000). Although a greater understanding of the genetics controlling specific disease resistance is now developing, the mechanics behind fruit quality, which is perceived as the primary factor behind successful market production, still remains relatively difficult to accurately predict.

1.7 Health attributes from fresh fruit

Raspberry is a high value horticultural crop, interest in which is expanding due to benefits associated with human health. A growing body of evidence has suggested a positive correlation between diets which are rich in fruit and vegetables and a reduction in chronic diseases such as cardiovascular disease, breast cancer (Ingram *et al*., 1997), prostate cancer and immune dysfunctions (Giovanucci *et al*., 1995). Beneficial effects are attributed to the content of vitamins, minerals and secondary phytochemicals such as carotenoids, anthocyanins, flavonoids and other phenolic compounds (Luthria *et al*., 2006). Initially consumed for medicinal purposes (Jennings, 1988), interest is focused once more on the health benefits of raspberries with the identification of antioxidants conferring protective qualities in a variety of human ailments including cardiovascular disease and epithelial cancers (Deighton *et al*., 2000; Moyer *et al*., 2002). Anthocyanins have pharmaceutical effects in humans specifically inhibiting proliferation of colon and breast carcinoma cells (Olsson *et al*., 2004), while demonstrating excellent anti-inflammatory and antimicrobial properties (Wang *et al*., 1999; Liu *et al*., 2002). Regular consumption of raspberry anthocyanins is also believed to improve cognitive brain functions and age-related degeneration of eye-sight (Liu *et al*., 2002; Stone *et al*., 2007). Kahnau (1976) estimated American diets had an intake of approximately 215 mg/day of

anthocyanins in the summer which fell to 180 mg/day during the winter. Wu *et al*. (2006) estimated the actual intake of anthocyanins in Americans to be a rather modest 12 mg/day while Chun *et al*. (2007) estimated the intake to be 3 mg/day. In 1996 a study of Italians suggested anthocyanin consumption varied between 25 and 215 mg/day, the largest intake of which was from red wine (Bridle and Timberlake, 1996). This phenomenon has also been reported in the French population and was thus termed the French Paradox (Kaur and Kapoor, 2001). Finland however has an average diet containing 82 mg/day of anthocyanins and this is attributed to government promotion for a diet rich in fruit and berries (Heinonen, 2001).

While incontrovertible evidence suggests an array of health benefits from the consumption of fruit and vegetables, it remains unclear whether these are from anthocyanins alone or as a result of synergistic interactions between a number of phenolic compounds (Konczak and Zhang, 2004). Questions remain on the bioavailability of anthocyanins following consumption, with many reports observing less than 1% detectable in human plasma and urine (Matsumoto *et al*., 2001; McGhie and Walton, 2007). While health protective qualities of anthocyanin *in vitro* are certainly clear and encouraging, the biological activity displayed *in vivo* remains somewhat inconclusive.

1.7.1 Antioxidants

Anthocyanins have powerful antioxidant action whereby single electrons or hydrogen atoms can be donated to reactive free radicals produced by UV light or as a result of metabolic processes. Raspberries are known to have a high free radical scavenging capacity and contain several compounds which elicit protective properties *in vitro* and *in vivo*. Most notable are the ellagitannins, which on hydrolysis release ellagic acid, reported to contain antiviral activity as well as conferring protection against colon, lung and oesophageal cancers (Mullen *et al*., 2002a). The ellagitannin lambertianin C and sanguiin H6 are also responsible for the vasodilation of aortic vessels in rabbit (Mullen *et al*., 2002a; 2002b). Key free radicals, highly unstable molecules often derived from

oxygen, include superoxide (O_2^-), peroxyl (ROO^-) and hydroperoxyl (HOO^-) (Kaur and Kapoor, 2001). Free radicals are destructive in the body, removing electrons required to regain their own stability thus rendering the "donor" compound a free radical (Kaur and Kapoor, 2001). Antioxidants, however, can neutralise free radicals by donation of one electron, as these compounds remain stable in either form (Kaur and Kapoor, 2001). This protective antioxidant action can be transferred to humans following consumption of anthocyanin rich food such as raspberries (Deighton *et al*., 2000). Antioxidants found in fruit extracts can inhibit blood oxidation of low density lipoproteins preventing plaque formation within arteries reducing the incidence of heart attacks (Verlangieri *et al*., 1985 as cited by Wang and Lin, 2000). Antioxidants are also important food preservatives reducing food oxidation, retarding deterioration, rancidity and food discolouration (Kaur and Kapoor, 2001).

1.7.2 Ascorbate biosynthesis

Ascorbic acid (vitamin C) is an important compound in plants with three main roles: acting as an antioxidant in plants, preventing oxidative stress and plant development and hormone signalling (Stevens *et al*., 2007). Fry (1998) suggesed that ascorbic acid may also be involved in the loosening of cell walls during cell expansion and fruit ripening. The ascorbic acid biosynthetic pathway (Figure 1.10) has many essential enzymes characterised in *Arabidopsis* (Conklin *et al*., 2000), strawberry (Agius *et al*., 2003) and tomato (Stevens *et al*., 2007) including L-galactose dehydrogenase that acts by catalyzing the oxidation of L-galactose to L-galactono-1, 4-lactone the penultimate step in ascorbate synthesis in plants (Wheeler *et al*., 1998; Laing *et al*., 2004).

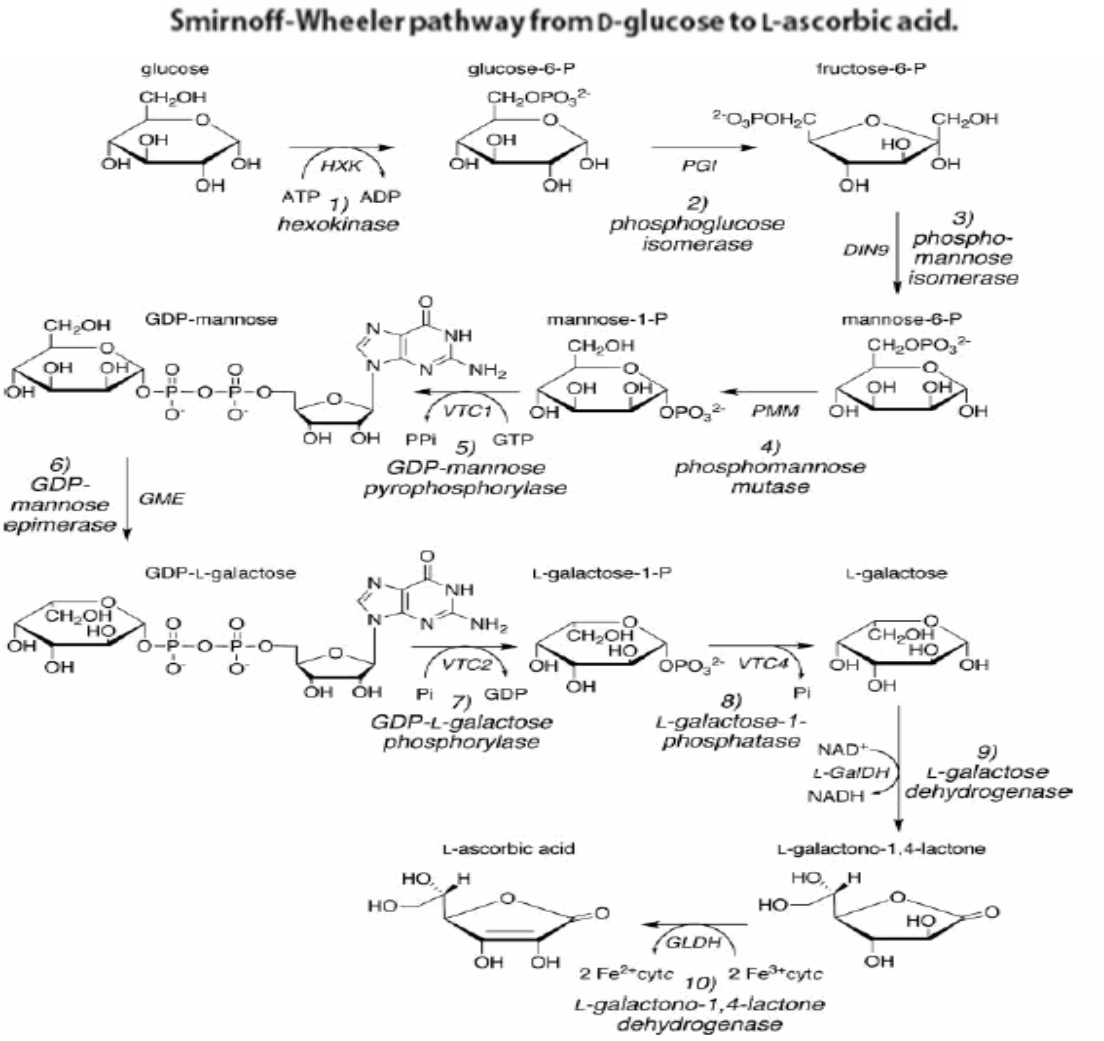


Figure 1.10. Schematic representation of ascorbic acid synthesis. There are six steps involved in the initial conversion of glucose to GDP-D-mannose and GDP-L-galactose which are the precursors for protein glycosylation and cell wall biosynthesis. Following glucose conversion to GDP-mannose the first committed step for L-ascorbic acid synthesis is the conversion of GDP-L-galactose to L-galactose-1-P. The L-galactose, which is produced from L-galactose-1-P, is then oxidised by the action of L-galactose dehydrogenase to produce L-galactono-1, 4-lactone which is then converted to L-ascorbic acid by the action of L-galactono-1,4-lactone dehydrogenase (Figure extracted from Linster *et al*., 2007).

1.8 Aims of this study

In soft fruit, quality has been shown to be a complex and highly regulated series of changes influenced by both internal and external factors. In order to sustain the development of high quality fresh raspberry production in the UK it is evident that new approaches must be adopted.

The purpose of this study was to contribute to a marker assisted breeding programme for the production of new, high quality raspberry cultivars, which can utilise British growing seasons and strategies. This was achieved by developing a number of different approaches aimed at phenotypic analysis of a segregating population over three environmental sites and seasons to define genotypic and environmental influences (Chapter 3).

Understanding the biochemical pathways determining key fruit characteristics, as well as transcription factors that regulate gene expression, will allow a candidate gene approach to be applied to trait mapping. This strategy will lead to the identification of markers, chromosomal loci and ultimately the genes responsible for a large percentage of trait phenotypic variation providing a basis for molecular assisted breeding programmes (Chapter 4).

The collection of replicated trait data will allow information to be linked with genetic markers, candidate genes and regions of quantitative trait loci on the evolving raspberry genetic linkage map for quality to ensure new varieties have specific sensory characters and quality parameters. The resultant genes or markers underlying QTLs will then undergo validation in a wider genetic background in order to confirm their stability and hence transferability prior to utilising this information in a marker assisted breeding strategy (Chapter 5).

Chapter 2 Materials and methods

2.1 Materials and methods for phenotypic analysis (Chapter 3)

2.1.1 Mapping population

The mapping population consists of a full-sib family of 320 progeny derived from a cross between the European cv. Glen Moy and the North American cv. Latham. This population was planted in randomised complete block trials consisting of one open field site and two protected sites. The open field site at SCRI, Dundee, UK, planted in 2002 consisted of three replicates and two clones of each plant, a protected cultivation site at SCRI, planted in 2005 with three replicates of each plant and a protected cultivation site on a commercial farm, T. Thomson Ltd, Blairgowrie, UK, planted in 2006 with one replicate of each plant. This gave a total of 10 plants replicated per individual genotype across all three sites analysed. For the purpose of phenotypic and genotypic analysis, 188 individuals (from the original 320 full-sib family) used in the construction of a genetic linkage map by Graham *et al*. (2004; 2006; 2009), were used in this study.

2.1.1.1 SCRI open field

Covering approximately 0.60 hectares, the open field site at SCRI, comprised 18 rows with 120 plants per row. The alleyways between rows were grassed with a distance of 2.5 metres between each row. Fertigation and irrigation were carried out as necessary depending on seasonal requirements.

2.1.1.2 SCRI protected site

One hectare of Haygrove SMART series polytunnels were constructed at SCRI in 2005 to evaluate germplasm under a protected cropping system. The 100 m × 100 m Spanish-style, multi-bay tunnel consisted of 13 bays, spanning 7.8 m, built on 2 m legs. Tunnels were covered with standard 150 μm Visqueen polythene. Raspberry tunnels had three rows per bay, 2.5 m between rows with a 2.8 m leg row. Alleyways were grassed and leg rows covered with UV-stable fabric mulch (Phormasol) to control weeds. The plants were covered once the fruit reached the green/white stage of development (early to mid

June) and remained so until the end of harvesting. Tunnels were vented by raising the polythene sides as required, normally when the temperature outside reached 25°C. Irrigation and fertigation was controlled by D8 Dosatron water powered dosing system which was fed through Ram light tape under the bedding polythene. Irrigation was monitored and advised weekly by AgriTech (AgriTech Solutions Ltd), using a neutron probe. A 4 m high Paraweb windbreak was erected on the west side of the tunnels to protect the structure from wind damage (N. Jennings, personal communication).

2.1.1.3 Commercial protected site

Several hectares of Visqueen luminance series polytunnels were constructed in Blairgowrie, Perthshire in 1997 for the commercial production of soft fruit. The 100 m x 100 m structures were Spanish-style multi-bay tunnels with thirteen bays, each spanning 7.0 m wide. Raspberry tunnels were covered with standard 150 μm Visqueen polythene. Raspberry tunnels had three rows per bay, 2.15 m between rows with a 2.7 m leg alley. The fields were covered at the start of flowering (late May to early June) and remained so until the end of harvesting. Tunnels were vented as required, normally when the temperature outside reached 25°C. Irrigation and fertigation was fed through Ram 20 tape under the bedding polythene with 1.6 L/min drippers situated at 50 cm spacing. Tunnels were watered for 1 hour at a time with anything from 1 to 8 applications per week depending on seasonal requirements (P. Thomson, personal communication).

2.1.2 Sample collection

The progeny arising from the Latham × Glen Moy cross segregate widely for a number of key characteristics, including time to ripe fruit and fruit colour, that impact on the ability to visually select and collect fruit at the same stage of ripeness across all progeny (Table 2.1). Fruit were harvested at the same time of day and from the same side of the plant to avoid any effect of sun/shade. Fruit were picked when the majority of berries on the plant reached the red fruit of stage 6 (Section 2.1.3). Fruit were placed into labelled polythene bags (self seal, 152 mm by 229 mm) and stored on ice for transport to the cold room (4°C) prior to analysis which was carried out on fresh fruit.

Table 2.1. Segregating characteristics of the mapping population parents.

Latham	**Glen Moy**
North American	European (Scottish)
Derived from *R.strigosus*	Derived from *R. idaeus*
Released in the 1930s	Released in the 1980s
Small fruited	Large thimble fruit
Sweet, aromatic	Juicy but less sweet
Dark red colour	Pale red colour
Round glossy berry	Dullish large drupelets
Firm, hard	Fleshy and collapsing
Good shelf life	Short shelf life
Late ripening	Early ripening
Root rot resistant	Root rot susceptible
Canes not hairy (hh)	Canes hairy (Hh)
Susceptible to cane diseases	Resistant to cane diseases
Hardy	Not hardy

2.1.3 Ripening stages

It was crucial to determine as closely as possible when material from each of the progeny was ripe. An assessment of fruit ripening was therefore required across the entire season starting in the middle of May and scoring all plants two or three times a week initially, then on a daily basis as the season progressed to give a standardisation point for fruit ripening. Ripening stages of each plant were scored individually based on a visual assessment whereby the latest stage present on each bush was allocated a grade as follows:

- Stage 1 - buds present
- Stage 2 - bud break/open flowers
- Stage 3 - fruit set
- Stage 4 - green fruit
- Stage 5 - green/red fruit
- Stage 6 - red fruit
- Stage 7 - over ripe fruit

2.1.4 Colour meter analysis

Colour was assessed using a Minolta Chroma meter CR-110, (Minolta Camera Co, Osaka, Japan). This allowed each sample to be measured in terms of chromaticity under the separate formats of Yxy, (CIE, 1931 Yxy International Commission of Illumination, Vienna), L*a*b* (CIE 1976 Hunters Lab International Commission of Illumination, Vienna) and ΔE by analysing the reflectance ratio between the emitted light from the measuring heads PXA lamp (8 mm diameter) and the light reflected from the samples (Minolta operating manual). The Yxy measurements were used to assess the brightness of samples as represented by Y (illuminance and reflection), the saturation of colour denoted by x (colour intensity/chroma) and the wavelength of individual colour

represented by y (red, yellow, green and blue). The L*a*b* analysed individual colour composition with L* measuring brightness to darkness (Black 0, White 100), a* green to red spectrum (-a = greenness, +a = red) and b* blue to yellow spectrum (-b = blueness, +b = yellow) as shown in Figure 2.1 (Almela *et al*., 1995).

ΔE is a measurement of colour deviation which represents the total colour difference within samples assuming that colour is three dimensional. ΔE was measured on the basis of the following equation (Figure 2.2) where colour was calculated as the square root of the sum of the squares of the three components (L*a*b*) representing sample coordinate differences compared with standard white tile with a range of 1.0 to 100% reflectance.

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$$

Figure 2.2. Equation showing the colour calculation of colour deviation (ΔE). This equation calculates ΔE as the square root of the sum of the squares of the three colour co-ordinates (L*a*b*) which represents the difference in colour deviation measured between individual sample co-ordinates (brightness, redness and yellowness) and the white tile standard used as a blank.

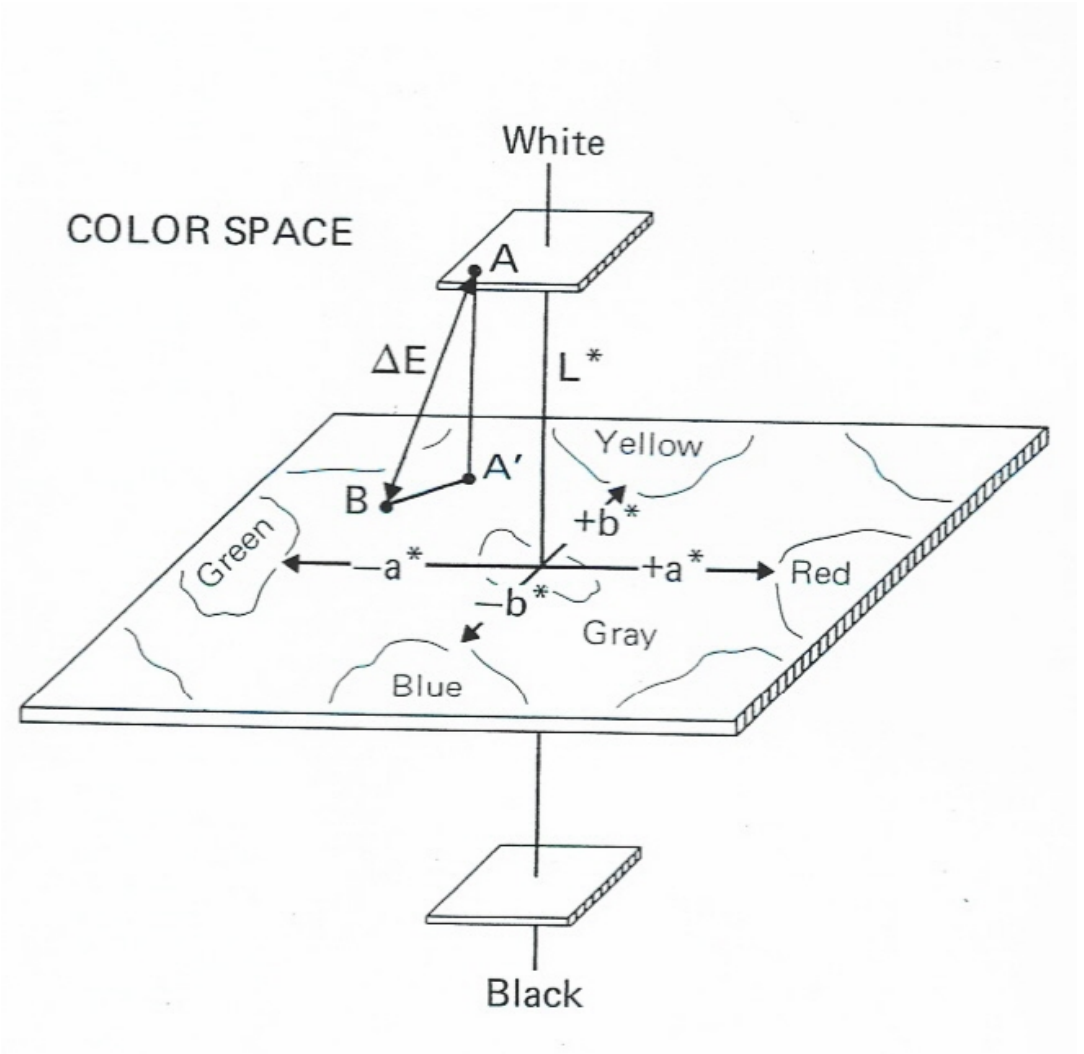


Figure 2.1. Colour space and deviation as analysed by L*a*b* and ΔE (Figure extracted from Minolta Chroma meter manual). Distance, as expressed by L*a*b* measures, represents approximate equal visual distances as perceived by human sensitivity to colour with L* representing sample brightness, while a* and b* measure the chromaticity co-ordinates of the red-green and yellow-blue spectrum respectively.

Due to the variation found between the samples in terms of their consistencies, careful preparation of fruit was necessary to prevent inaccurate measurements being recorded. This was achieved by carrying out extensive testing prior to the commencement of sample analysis in 2006 and involved direct comparisons between blenders and sample weights used.

2.1.4.1 Fruit homogenization tests

Two different blenders were used to homogenize fruit. Different quantities of fresh fruit (25 g, 30 g and 35 g) were processed using: 1) a hand held domestic blender and 2) a table top commercial Waring® blender (Model 38BL41, Torrington, USA). This found the Waring® blender to produce the most consistent, smooth results for all varieties tested whereas the hand held blender failed to produce a smooth result for drier fruit with lumps collecting within the blender's bulbous guard. Testing also found that while 25 g of fresh fruit produced sufficient sample for colour analysis, 35 g created a more consistent homogeneous puree. Based on these results, 35 g of each sample was analysed using the commercial blender for all three seasons as follows.

2.1.4.2 Preparation of samples for colour meter analysis

Thirty five grams of fresh fruit was weighed and placed into the two-speed laboratory Waring® blender and blended on full power (16,000 × g) for 10 seconds. The puree was then stirred for a few seconds and blended for another 10 seconds. Fifteen grams of puree was transferred into a sterile Petri dish (9 cm diameter) and gently shaken to provide an even surface for analysis (in 2006 10 g was also analysed to allow for subsequent statistical analysis to be carried out measuring any reflectance differences obtained from a reduced sample thickness). The Chroma meter was then set up as per the operating manual and blanked using the accompanying white tile (101974) with a reflectance of Y: 87.4, x: 0.308 and y: 0.315 and samples measured as described for Yxy, L*a*b* and ΔE (Section 2.1.4).

2.1.4.3 Visual colour analysis of samples

As well as the chromaticity of samples being analysed for colour, a visual assessment was also carried out while fruit were still on the bush for all samples in 2007 and 2008. The perceived colour of fruit was scored on a scale of 1 to 5 as follows:

1= pale pink

2= pale pink/red

3= mid red

4= mid red/dark red

5= dark red

2.1.4.4 Extraction of raspberry juice

Following colour meter analysis in 2008, 15 ml Sterilin centrifuge tubes (Barloworld Scientific, UK) were labelled and filled with blended raspberry puree and frozen, prior to individual biochemical analysis. Raspberry juice was then extracted from the thawed puree into 2 ml labelled microfuge tubes using the following procedure. (Chemicals were purchased from Sigma-Aldrich or BDH laboratories unless otherwise stated.)

Five millilitres of thawed puree was removed from the original centrifuge tube and transferred to a new 15 ml centrifuge tube before 5 ml of 4% (v/v) acetic acid in acetonitrile (AcOH in ACN) was added. The tubes were centrifuged at 1,000 × g for 3 minutes in an Eppendorf 58190R centrifuge (Eppendorf, Germany) and four 2 ml aliquots of 50% (w/v) juice were obtained.

2.1.4.5 Total phenolic content

The total phenol content of each sample was estimated from a standard curve of gallic acid (gallic acid equivalent, GAE) using the Folin-Ciocalteu method as described by Deighton *et al*. (2000). Twenty microlitres of extracted juice was transferred into 1.5 ml microfuge tubes and 980 µl of deionised water was added to produce 1% (v/v) juice for

analysis. Two hundred and fifty microlitres of each 1% juice sample was then transferred into disposable cuvettes in replicates of three and 250 µl of 50% Folin-Ciocalteu reagent was added before the samples were left for 3 minutes. After this time 500 µl of filtered saturated sodium carbonate solution (Fluka Chemicals, Switzerland) was added to each sample, mixed by gentle pipetting and left for 1 hour. Samples were then read at a wavelength of 750 nm, using an Ultraspec 2100 pro spectrophotometer (Amersham Biosciences), and the results recorded in triplicate. The mean of the three readings was calculated and from this the mg GAE per 100 g fresh weight (fw) phenol content recorded.

2.1.4.6 Total anthocyanin content

The total anthocyanin content of each sample was measured using the pH differential method of Cheng and Breen (1991) of $A= [(A510 - A700)_{pH1.0} - (A510 - A700)_{pH4.5}]$ which relates to the molar extinction coefficient for cyanidin 3-glucoside (29,600). For each sample 40 µl of extracted juice was transferred into disposable 1.6 ml semi micro cuvettes (Greiner, Bio-one) in triplicate for a buffer of pH 1.0 (0.2 M HCl/KCl) and a further triplicate for a buffer of pH 4.5 (0.1 M sodium acetate). The samples were then read using an Ultraspec 2100 pro spectrophotometer (Amersham Biosciences) at wavelengths of 510 nm and 700 nm and the results were recorded for the ratio of each buffer in triplicate. The averages for the 3 readings per buffer were then obtained and from this the mg per 100 g fw calculated and recorded.

2.1.4.7 Measurement of sample pH

Sample pH was measured on the initial 15 ml centrifuge tubes containing the remainder of the blended fruit puree (Section 2.1.4.4). Samples were collected from the freezer and allowed to thaw at room temperature while the pH meter was calibrated for standard buffers of pH 7.0 and 4.0. The 15 ml tubes were centrifuged at 1,000 × g for 90 seconds in order to obtain sufficient juice for pH testing using an H1 8521 pH meter (Hanna, Bedfordshire, UK). The samples were then analysed in duplicate and the mean measurement recorded.

2.1.5 Total soluble solids analysis

Brix testing is a measure of the total soluble solids (TSS) in a given weight of plant extract (Liebhard *et al*., 2003). Refractometry results are recorded as ^{o}Brix compared to a concentration of sugar content in grams per 100 ml of juice. This is assumed correct at a temperature of 20^{o}C whereby 1^{o} Brix is equal to 18 g per litre of sugar (Liebhard *et al*., 2003). Due to the presence of other substances, for example salts, minerals and proteins, the ^{o}Brix represents the total concentration of all soluble solids within each sample analysed and is reported accurate from 0.0 to 53.0^{o} (Pocket Pal-1 instruction manual, Tokyo, Japan). Two berries from each sample were randomly selected and placed into a cut piece of muslin before two or three drops of juice were squeezed onto the pocket refractometer prism (Atago PAL-1, Tokyo, Japan). The samples were then analysed in duplicate and the average reading recorded. The prism was cleaned using deionised water between samples and blanked using deionised water every 10 samples.

2.1.6 Sample weight

Ten berries from each sample were randomly selected from the individual bags of ripe fruit (Section 2.1.2) and weighed into a sterile Petri dish in order to give an assessment of individual fruit size per plant sample (ten berry weight).

2.2 Materials and methods for genotypic analysis (Chapter 4)

2.2.1 Mapping population

The mapping population as described in Section 2.1.1 consisted of 188 progeny used in the construction of a raspberry genetic linkage map (Graham *et al*., 2004; 2006; 2009) and for subsequent marker and candidate gene analysis.

2.2.2 DNA extraction from mapping population

DNA was extracted from young raspberry leaves collected from two *Rubus* subspecies, *Rubus idaeus* (cv. Glen Moy) and *Rubus strigosus* (cv. Latham) along with the 188 progeny derived from a cross between the two parents, using a CTAB/chloroform method as described by Graham *et al*. (2003). All mortars and pestles were washed and

autoclaved prior to extraction. The extraction buffer was prepared using 2% (w/v) hexadecyl trimethyl ammonium bromide (CTAB), 100 mM Tris-HCl (pH 8.0), 1.4 M sodium chloride, 20 mM Na_2EDTA (pH 8.0), 0.1% (w/v) dithiothreitol (DTT) adjusted to 500 ml using distilled water. For each sample, 1 g of leaf material, collected fresh and stored on ice or previously collected and stored at -80^{o}C, was ground in a pre-cooled mortar and pestle with liquid nitrogen. Towards the end of grinding, samples were left to stand at room temperature to ensure excess liquid nitrogen volatised, without allowing samples to thaw, and 5 ml of 2% CTAB extraction buffer was added and samples transferred to a sterile 15 ml centrifuge tube. The samples were incubated at 65^{o}C for 30 minutes followed by the addition of 7.5 ml chloroform-isoamyl alcohol (24:1). The samples were agitated for 15 minutes followed by centrifugation for 10 minutes at 2,000 × g. The aqueous layer was filtered through sterile muslin and an equal volume of ice cold propan-2-ol was added slowly, mixed and incubated at room temperature for 15 minutes to allow DNA precipitation. The DNA was pelleted by centrifuging the mixture for 20 minutes at 2,000 × g. The supernatant was discarded and the DNA dried by inverting the tube over tissue. The pellet was then resuspended in 1 ml of TE buffer (1 M tris, pH 7.5, 500 mM EDTA, pH 8.0) and 10 µl RNase was added to the DNA. The samples were then incubated at 37^{o}C for 1 hour and stored at -80^{o}C prior to use.

2.2.3 Candidate gene analysis

Candidate genes identified through literature and database searching and thought to be involved in quality traits, such as those found within sugar, acid or colour pathways, were analysed in raspberry (Chapter 4, Section 4.2.1 and 4.2.2). Primer sequences and amplification conditions were replicated from initial sources in order to screen for the presence of these genes in raspberry. This involved analysing the primer sequences used in other species, against the sequences reported for each gene, following insertion of the corresponding accession number, into NCBI 'BLAST' database (National Centre for Biotechnology Information, Basic Local Alignment Search Tool) and identifying the primer sequences reported (Altschul *et al*., 1990). BLAST aligns two sequences by identifying and scoring regions of identity between two sequences. Similarity scores are

then assigned depending on amino acid matches, likelihood of mismatches and relative abundance of amino acids present. For the final production of an optimal alignment between sequences (pairwise alignment) the position of the query search is compared with each position of the database sequence and a quality match is scored. The database sequence results are then displayed in ascending order with the lowest p-value as applied in Karlin-Altschul Sum statistics, (Karlin and Altschul, 1993) depicting the highest level of identity.

2.2.4 Polymerase chain reaction

Polymerase chain reaction (PCR) begins with the denaturation of templates and any other previously synthesised products to release the single stranded DNA followed by a reduction in temperature. This allows the primers to scan the genomic template in order to find the corresponding target sequence permitting the primers to anneal to the template (Sambrook and Russell, 2001). The temperature is then increased during the extension stage, which allows DNA synthesis to occur in the presence of *Taq* polymerase which is derived from the thermophilic bacterium *Thermus aquaticus* (Saiki *et al*., 1988). DNA samples of the parents 'Glen Moy' and 'Latham' along with 6 individuals were amplified with each of the primers obtained using the following PCR programme, performed on a Perkin Elmer 9700 Thermal Cycler (Applied Biosystems). A typical 25 µl reaction contained 25 ng template DNA, 1.0 µM forward and reverse primer, 0.2 mM dNTPs, 1× PCR buffer (containing 10 mm Tris-HCl, 1.5 mm $MgCl_2$, 50 mm KCl, pH 8.3) and 0.1 unit *Taq* polymerase (Roche, Switzerland). Two PCR profiles were commonly used.

2.2.4.1 SSR programme

Reactions were incubated at 94^{o}C for 5 minutes to allow denaturation of the template DNA: 35 amplification cycles were then carried out as follows; denature at 94^{o}C for 1 minute, anneal at 57^{o}C for 1 minute, extend at 72^{o}C for 1 minute, and incubate further at 72^{o}C for 8 minutes before holding at 4^{o}C. The extension time was varied depending on the product size expected.

2.2.4.2 Touchdown programme

Reactions were incubated at 94°C for 5 minutes followed by: denature at 94°C for 30 seconds, anneal at 65°C for 30 seconds, and extend at 72°C for 30 seconds. This temperature was then decreased by 1°C for 6 subsequent cycles after which, 28 cycles followed: denature at 94°C for 30 seconds, anneal at 58°C for 30 seconds, extend at 72°C for 30 seconds and incubate further at 72°C for 7 minutes before holding at 4°C.

2.2.5 Primer re-design

For the candidate genes which failed to amplify in raspberries, primers were re-designed. This involved importing the known accession number or gene name of interest into the NCBI database. The gene sequences obtained were then imported into the NCBI BLAST database where similarities between sequences could be identified and statistically analysed for significant alignment. The six most similarly aligned sequences were imported into ClustalW, a sequence analysis tool, available through European Molecular Biology Laboratory, European Bioinformatics Institute (EMBL-EBI) which allowed the alignment of divergent sequences to be directly compared to find the most conserved regions (Appendix 2.1). Selected sequence areas were then analysed further using Primer3 software (Rozen and Skaletsky, 2000) for optimal primer design. In order to account for any degenerative sites present within and between plant species, primers were also designed with (Operon) degenerate base pairs (wobbles). Following the re-designing of new primers (Appendix 2.2) further amplification were performed on each primer pair using the parental DNA and six progeny (Section 2.2.4).

2.2.6 Electrophoresis agarose gel visualisation

DNA fragments obtained following PCR amplification were separated on the basis of their size or molecular weight by electrophoresis through an agarose gel matrix. Agarose gels were prepared as 1.0% or 1.5% (w/v) depending on requirement and expected fragment size. Gels were prepared using Hi pure low EEO agarose (Biogene) and 1× Tris borate EDTA buffer (TBE) (10.8 g/l Tris base, 5.5 g/l boric acid, 930 mg/l Na_2EDTA, $2H_2O$, pH 8.0). Fragment sizes were estimated by running 5 µl of either 1 kb

ladder (Invitrogen, Carlsbad, USA) or 100 bp (Promega, www.promega.com) ladder, depending on fragment size expected, alongside the PCR product.

Loading dye (0.04% bromophenol blue, 0.04% xylene cyanol FF, 6% glycerol) was added to each sample prior to analysis to add density to the sample to ensure it entered into the well, while allowing visual monitoring during gel migration. Ethidium bromide (EtBr) (0.25 µl/ml) was added to the gel to allow UV visualisation (302 nm) through intercalation with DNA molecules causing fluorescence. Electrophoresis was performed using a Consort Flowgen E455 (Sittingbourne, UK) powerpack set at between 80 and 120 volts depending on the number of samples/size of gel analysed. For the candidate genes which were successfully amplified 15 µl of each PCR product was analysed on a 1.5% (w/v) agarose gel to allow the individual bands to be extracted from the gel using a sterile scalpel blade in preparation for DNA sequencing.

2.2.7 MinElute gel extraction protocol using a microcentrifuge

DNA was extracted from each of the candidate genes embedded within the agarose gel matrix following the manufacturer's guidelines (Qiagen MinElute Handbook, 2006). Each gel slice was individually weighed in a microfuge tube before 3 volumes of buffer QG (as supplied in the kit) was added. The tube was incubated in a water bath at 50^{o}C for 10 minutes in order to completely dissolve the agarose gel and the sample was vortexed every 3 minutes to aid mixing. The colour of the sample and hence the pH was then checked to ensure an efficient pH of < 7.5 was maintained before one volume of high grade isopropanol (Fisher Scientific) was added to the tube and mixed by inversion. The sample was then transferred into a MinElute column and the DNA allowed to bind to the membrane following centrifugation at 10,000 × g for 1 minute. The flow through was discarded, 500 µl of buffer QG added and the sample further centrifuged for 1 minute. The membrane was washed following the addition of 750 µl of buffer PE (supplied in the kit), left to stand at room temperature for 1 minute before centrifugation for 1 minute at 10,000 × g. The flow through was again discarded and the columns centrifuged for another 1 minute at 10,000 × g to remove any residual ethanol present in

the buffer PE. The MinElute column was then transferred to a sterile microfuge tube before 12 µl of sterile distilled water was added directly onto the membrane. The sample was then allowed to stand for 1 minute prior to a final 1 minute centrifugation at 10,000 × g to elute the bound DNA.

2.2.8 Determination of DNA concentration

Nanodrop™ (Thermo Science, Wilmington, USA) is a spectrophotometer linked to a computer which measures nucleic acid concentrations. The sensitivity range for DNA is between 2 ng and 3700 ng per 1 µl (Labtech International, 2006). DNA has an absorption peak at 260 nm with an A_{260}/A_{280} ratio of 2.0 and purified DNA can then be estimated assuming that an A_{260} of 1.0 is equivalent to 50 µg of DNA per 1 ml (Dadd, 1996). Each sample was quantified in turn using the Nanodrop™ which had been previously blanked using sterile distilled water. Each sample (1 µl) was added to the detection surface of the spectrophotometer and the DNA concentration and wavelength recorded. Each sample was then diluted appropriately, depending on the PCR product size, using distilled water prior to sequence analysis.

2.2.8.1 Sequencing protocol

A minimum of 5 µl of sample was required per sequencing reaction and each sample was sequenced in both the forward and reverse direction. Twelve microlitres of DNA were sent to the SCRI Sequencing Service for each sample along with 6 µl of 10 mM forward and reverse primers, used for initial amplification, in order to allow sufficient quantities for analysis using an ABI 3730 DNA sequence analyser (Applied Biosystems).

2.2.9 DNA cloning protocol

For the candidate genes which produced multiple bands or mixed sequences on gel electrophoresis, it was necessary to clone these samples to obtain a single gene product.

2.2.9.1 Clean up using Qiagen MinElute PCR purification kit

PCR products from the selected candidate genes were purified for subsequent cloning and sequence analysis following standard Qiagen MinElute instructions. Five volumes of buffer PB (provided in purification kit) was added to one volume of each of the PCR products in turn and mixed by gently vortexing. Each sample was then transferred into a MinElute column and collection tube in order to bind the DNA, following centrifugation at 10,000 × g for 1 minute. The flow through was discarded and the DNA washed by adding 750 µl of buffer PE (provided in kit) and centrifuged for 1 minute. Again the flow through was discarded and residual ethanol was removed by centrifuging at 10,000 × g for a further 1 minute. The MinElute column was then transferred to a clean microfuge tube and the DNA eluted from the membrane following the addition of 12 µl of buffer EB (provided in kit). The column was allowed to stand for 1 minute before eluting the DNA by centrifuging at 10,000 × g for 1 minute. All 12 µl of eluted DNA from each sample was then analysed on a 1.5% agarose gel before the bands were extracted and purified (Section 2.2.7).

2.2.9.2 Ligation reaction using pGEM® - T Easy vector

PCR products from Section 2.2.9.1, were cloned into the T/A vector pGEM® -T Easy (Promega.com), an efficient cloning vector for PCR products with 'A' overhangs. The pGEM® -T Easy vector contains the RNA polymerase promoters T7 and SP6 which flank a multiple cloning region within the α peptide coding region found in the enzyme β-galactosidase. As a result insertional inactivation of the α peptide allows a visual identification of recombinant clones via screening of colonies present on appropriate indicator plates (Promega.com). The presence of multiple restriction sites within the multiple cloning region of pGEM®-T Easy vector allows the inserts to be released following digestion using a single restriction digest. The pGEM®-T Easy vector itself contains recognition sites for restriction enzymes including *Eco*RI, *Bst*ZI and *Not*I leading to 3 single enzyme digestions, which can result in insert release (Promega.com). The pGEM®-T Easy vector and control insert DNA tubes were centrifuged for 10 seconds prior to use in order to collect the content at the bottom of the tubes. Ligation

reactions were set up in labelled microfuge tubes (Table 2.2) for a standard reaction (for 7 samples), positive control (× 1) and background control (× 1), taking care to ensure the 2× ligation buffer was vortexed vigorously before each use. The contents of each tube were then mixed by gently pipetting each sample, before incubating for 48 hours at 4^{o}C as per the manufacturer's instructions in order to obtain the maximum number of transformants.

Table 2.2. Preparation of ligation reactions used for gene cloning.

REAGENT	STANDARD	POSITIVE	BACKGROUND
2X rapid ligation buffer	5 µl	5 µl	5 µl
pGEM® -T Easy vector	1 µl	1 µl	1 µl
PCR Product	3 µl	NIL	NIL
Control insert DNA	NIL	2 µl	NIL
T4 DNA ligase (3 Weiss units/µl)	1 µl	1 µl	1 µl
Deionised water (to final volume of 10 µl)	NIL	1 µl	3 µl

2.2.9.3 Transformation of samples using pGEM® - T Easy vector ligation reactions

The tubes containing the ligation reactions were centrifuged at 16,000 × g for 60 seconds in order to collect the contents at the bottom of each tube before 2 µl of each reaction were transferred into sterile 1.5 ml microfuge tubes containing chemically competent DH5α cells (Invitrogen) stored on ice. Each of the tubes were mixed by gently flicking before incubating on ice for 30 minutes. The cells were heat shocked for 45 seconds in a water bath at exactly 42°C and replaced on ice for a further 10 minutes and then 80 µl of SOC medium (2% Bactotryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, adjusted to pH 7.0 using NaOH) stored at room temperature was added to each of the tubes giving a total volume of 100 µl. All tubes were then incubated at 37°C with shaking at 25 Hz for 1 hour in an orbital incubator SI 50 (Stuart Scientific).

Plates (LB/AIX), 0.5 ml ampicillin (100 µg/ml), 1.6 ml isopropylthio-β-galactopyranoside (IPTG, 32 µg/ml) and 1.6 ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal, 32 µg/ml), were inoculated by transferring 100 µl of transformed sample onto the surface of each of the labelled Petri dishes in turn including 1 background control and 1 positive control. Each sample was spread aseptically over the surface of the plates, using approximately 8 sterile glass beads, and then incubated upside down at 37°C overnight. Plates were examined for colony number and appearance (in the form of blue/white colony development).

2.2.9.4 Miniprep colony isolation

Five hundred milliliters of LB (1% Bactotryptone, 0.5% Bacto yeast extract, 1% NaCl) medium was inoculated with 500 µl of 100 mg/ml ampicillin and mixed gently before 1 ml was added to each individual well of a sterile 96 deep well plate. One single white transformant from each plate was added using a yellow pipette tip (8 colonies transferred per sample). The plates were covered with gas permeable adhesive seal (AbGene) and placed in an orbital incubator SI 50 (Stuart Scientific) at 37°C overnight with shaking at 25 Hz (~ 24 hours).

2.2.9.5 Large scale plasmid minipreparation

Plasmid DNA was isolated using an alkaline lysis protocol. The deep 96 well plate (2.2.9.4) was spun at 700 × g for 5 minutes before discarding the supernatant and inverting the plate over paper towel to remove residual media. The pellets were resuspended following the addition of 80 µl of solution I (30 mM glucose, 15 mM Tris-HCl, 30 mM Na_2EDTA pH 8.0, RNase A, 60 µg/ml) to each well and mixed by vortexing to ensure complete resuspension (increasing the osmotic pressure outside of the cells, due to the presence of glucose, while the EDTA protects the exposed DNA from degradative enzymes). This was followed by the addition of 80 µl of freshly prepared solution II (0.2 M NaOH, 1.0% SDS) to each well. The plate was vortexed for 1 minute then left at room temperature for no more than 2 minutes in order to rupture the cells and solubilise the cellular proteins without causing DNA lysis following NaOH denaturation. Eighty microlitres of solution III (3.6 M Potassium acetate) was then added to each well and the plate vortexed for 1 minute, providing a neutral pH which favours DNA renaturation. The *E. coli* debris was trapped within the cell precipitate while allowing the DNA to remain in solution. Binding solution (160 µl) of 8 M guanidine hydrochloride was added to each well of a multiscreen binding MAFBNOB plate (Millipore) placed on the pump manifold.

A multiscreen clearing plate (MANANLY, Millipore) was placed on top of the manifold containing the multiscreen binding plate. The bacterial lysate was mixed by pipetting up and down twice before 130 µl of this solution was transferred to each well of the clearing plate. A 10 mm Hg vacuum from a Millipore pump was applied to the manifold for 3 minutes to draw the lysate through to the binding plate. The clearing plate was discarded and the solutions in the wells of the binding plate were mixed by pipetting three times. An inverted plate lid was then placed onto the manifold base and the binding plate placed on top before full vacuum of around 20 mm Hg was applied for 1 minute and the waste discarded. The addition of 200 µl of 70% (v/v) laboratory grade ethanol to each well followed and the plate vacuumed on full (20 mm Hg) for 1 minute. This removed any remaining salt and SDS detergent. Again the waste was discarded and

this wash step repeated. The plates were then allowed to dry by assembling the Millipore alignment frames appropriately and spinning the plate at 700 × g for 10 minutes. The plate was allowed to sit at room temperature for a further 10 minutes to ensure no residual ethanol remained. Plasmid DNA was eluted following transfer of the binding plate to a new microtitre storage plate and 75 µl of sterile distilled water was added to each well before a final 5 minute centrifugation at 700 × g. Three microlitres of a selection of plasmid DNA were then analysed on a 1% (w/v) agarose gel in order to assess DNA quality prior to sequencing.

2.2.9.5.1 BigDye® terminator cycle sequencing

A 1/16th sequencing reaction was prepared for the plasmid reactions using BigDye® cycle sequencing v 3.1 (Applied Biosystems, Foster City, USA) master mix: (0.5 µl fluorescent BigDye®, 1.88 µl dilution buffer (400 mM Tris-HCl pH 9.0, 10 mM $MgCl_2$), 1 µl Universal M13 forward and reverse primers (M13F 5' GTA AAA CGA CGG CCA GT 3') and (M13R 5' CAG GAA ACA GCT ATG AC 3') (10 µM) and 3.62 µl sterile distilled water). The master mix (7 µl) was transferred into each well of a non skirted PCR plate and 3 µl of plasmid DNA (Section 2.2.9.5) was added to individual wells and amplified using a sequencing plus programme; a denaturing step at 96^{o}C for 10 seconds, 40 cycles of 50^{o}C for 5 seconds, 60^{o}C for 4 minutes followed by a hold at 4^{o}C.

2.2.9.5.2 Purification and analysis of DNA sequencing reactions

The plasmid sequencing reactions (2.2.9.5.1) were purified for sequencing. The 96 well plate was briefly spun to ensure samples were at the bottom of the wells before 2.5 µl of EDTA was added to the bottom of each well. Thirty microlitres of 100% ethanol was added to each well before the plate was sealed with a PCR lid, vortexed and incubated at room temperature for 15 minutes. The plate was then spun at 3,000 × g for 30 minutes, the supernatant drained and the plate spun upside down on tissue for 10 seconds at 200 × g. Pellets were washed twice following the addition of 150 µl of 100% ethanol and centrifuged at 4^{o}C for 15 minutes. Finally the cover was removed and the plate centrifuged upside down at 200 × g for 1 minute. The DNA was analysed on an ABI

3730 DNA sequence analyser (Applied Biosystems) at the SCRI Sequencing and Genotyping Service. Sequencing data was analysed manually using Sequencher 4.5, 4.8 (DNA Codes Corporation) or BioEdit 5.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to identify sequence polymorphisms present.

2.2.10 Single nucleotide polymorphism detection

The two main genetic variations reported to cause differences between individuals are microsatellite tandem repeats of single sequences and single nucleotide polymorphisms. Single nucleotide polymorphisms are the most commonly found variations across a genome and are reported as occurring every 1000 nucleotides in the human genome (Wang *et al*., 1998). These polymorphisms can often be used to directly detect individual alleles which are responsible for variations found for a trait of interest (Nakitandwe *et al*., 2007). Where SNPs were identified in candidate genes, primers were designed for Pyrosequencing® assays using PSQ Assay design software 1.0 (Qiagen, Germany). The Pyrosequencing® assay was performed on all 188 individuals of the mapping population plus both parents, for each of the identified polymorphisms according to the manufacturers protocol (Qiagen) using a PSQ96MA Pyrosequencing® instrument (Qiagen).

2.2.10.1 Pyrosequence® analysis

A Pyrosequence® reaction involves a number of enzymatic steps. The first step releases pyrophosphate (PPi) following incorporation of a nucleotide to the template DNA or RNA sample. The PPi is then converted to ATP by ATP sulfurylase which is the catalyst for light generation of oxidized luciferin by luciferase. The remaining unincorporated nucleotides are degraded by apyrase and the cascade is repeated with the introduction of a new nucleotide. This sequence is repeated until the full template has been sequenced (Ronaghi, 2001; Fakhrai-Rad *et al*., 2002). In solid phase Pyrosequencing® the ATP sulfurylase used was from the yeast *Saccharomyces cerevisiae* while the luciferase was derived from the American firefly *Photinus pyralis*. The light generated following

nucleotide incorporation is around 560 nm in wavelength and is identified by a charge coupled device camera (CCD) (Ronaghi, 2001).

2.2.10.2 Sample preparation for Pyrosequence® analysis

The PCR reaction differs from normal amplification protocols in so far as one of the PCR primer pairs is covalently bound to a Biotin tag. This allows the DNA strand to be immobilised onto a streptavidin Sepharose high performance bead while the complementary strand is released producing two single strands of targeted DNA. Qiagen (PSQ) assay design software 1.0 (Qiagen, Germany) was used to develop appropriate primers for subsequent Pyrosequence® analysis. The identified sequence of interest was transferred into the software sequence editor with the single nucleotide polymorphism clearly identified, for example G/A. For insertion or deletions, the sample is identified within closed brackets, [GA]. From here the PSQ programme will analyse the sequence and identify the most appropriate primers to amplify the target SNP. This results in 1 forward and 1 reverse primer, either of which may be selected for biotinylation, and a sequencing primer which will specify the subsequent dispensation order of nucleotides for genotypic analysis. The nucleotides are added to the DNA sequence complementary to the selected template and are designed to include both alleles specific to each SNP to be analysed (Alderborn *et al*., 2000).

2.2.10.3 Pyrosequencing® protocol

The PCR template was prepared as per normal amplification specifications as follows:
A typical 25 µl reaction contained 25 ng template DNA, 1.0 µM forward and reverse primers, 0.2 mM dNTPs and 0.1 units *Taq* polymerase (Roche, Switzerland). Reactions were incubated at 94^{o}C for 5 minutes to allow denaturation of the template DNA. Amplifications were then carried out for 40 cycles as follows: 94 ^{o}C for 45 seconds, 57 ^{o}C for 45 seconds, 72 ^{o}C for 1 minute, and incubated at 72^{o}C for 8 minutes before holding at 4^{o}C. The extension time was varied depending on the product size expected. Five microlitres of PCR samples were analysed on a 1.5% (w/v) agarose gel (see Section 2.2.6), in order to confirm appropriate product amplification.

Each PCR plate required a final volume of 80 µl prior to Pyrosequence® analysis and this consisted of the addition of Sepharose beads (3.0 µl), molecular biology grade (MBG) water (20.0 µl) and binding buffer (Qiagen) (37.0 µl) to the 20 µl PCR product.

The plate was agitated at 10,000 × g on an Eppendorf thermomixer comfort for 15 minutes at 22°C while the Pyrosequencing® low plate was prepared with the following 45 µl volume per sample: 0.15 µl sequencing primer, designed specifically to analyse the SNP of interest, 3.50 µl annealing buffer (Qiagen) and 1.35 µl MBG water. Five microlitres of this mix was transferred into each well of the Pyrosequencing® low plate along with a further 40 µl of annealing buffer and this was placed into the Pyrosequencing® sample preparation station. The three reservoirs of the sample preparation station were filled with: reservoir 1) 70% (v/v) ethanol, reservoir 2) 0.2 M NaOH and reservoir 3) 1× wash buffer (Qiagen). The Pyrosequencing® software was then prepared by importing the relevant SNP information from the PSQ Assay design software. This information was used to prepare the reagent cartridge with appropriate reagents for each run including substrate (luciferine, and adenosine 5'-phosphosulfate), enzyme (single stranded DNA binding protein, luciferase, DNA polymerase, apyrase and sulfurylase) and dNTP's (A, C, G, and T). At this stage the thermomixer comfort was stopped and the sample plate containg the Pyrosequencing® PCR reaction placed onto the preparation station and the suction pump activated. The handset was placed into the wells of the plate and gently massaged for 10 seconds in order to attach the immobilised beads onto the filter tips while the excess liquid was discarded before being placed into each reservoir in turn for 5 seconds. The pump was switched off and the handset placed into the Pyrosequencing® low plate and the beads released from the filter probes by crudely shaking for 30 seconds. The plate was then incubated at 80°C on a PCR machine for 3 minutes and then brought back to room temperature before being placed onto the Pyrosequencing® machine along with the cartridge, containing sufficient reagent for full plate analysis. The run was then initiated on the computer with sequence results obtained in the form of Pyrogram® traces which were analysed using PSQ 2.1 software (Qiagen).

2.2.11 Gene prediction following single nucleotide polymorphism detection

Gene prediction software was utilised in order to examine the potential effect each of the SNPs could have on the candidate gene of interest. The gene sequences obtained (Section 2.2.8.1 and 2.2.9.5) were imported into gene software FGENESH (www.softberry.com) twice, to account for each allele identified, and the resultant amino acid chains were compared.

2.3 Materials and methods for EST library utilisation

Expressed sequence tag (EST) libraries, constructed from the roots of Latham (*R. strigosus*) constructed in 2007 from young potted plants grown in glasshouses at the SCRI, Dundee (I. Tierney, personal communications) and both endodormant and paradormant raspberry buds cultivated from rootstocks grown in unheated glasshouses at the SCRI, Dundee from cv. Glen Ample (Mazzitelli *et al*., 2007), were explored for potential genes identified within biological pathways involved in fruit colour development**.**

2.3.1 EST library construction

In order to enhance the sequence information available in red raspberry it was necessary to construct further EST libraries containing transcribed gene sequences in order to establish whether polymorphisms could be identified between alleles and between parents which may underlie candidate genes involved in fruit quality.

2.3.2 Plant material for library construction

Fruit samples were collected from the mapping parents, 'Glen Moy' and 'Latham', for two distinct ripening stages, green red expanding fruit and red ripe fruit. Fruit were harvested at the appropriate stage from the same side of the plant located under protected covering at SCRI in 2007 and stored immediately in liquid nitrogen prior to transfer to -80^{o}C storage.

2.3.3 cDNA library preparation

In order to identify and characterise genes which are specifically expressed in raspberry fruit, it was necessary to isolate mRNA from fruit tissues to produce complementary DNA (cDNA) copies in the form of a cDNA library. All mortars, pestles and accessory glassware were cleaned using RNase away (Ambion) and autoclaved prior to RNA extraction and sterile tips and plasticware used. Solutions were prepared fresh and autoclaved where necessary while gloves were worn at all times and changed frequently in order to prevent RNase contamination of samples.

2.3.4 Purification of total RNA from raspberry fruit

RNA was extracted from parents 'Glen Moy' and 'Latham' for the formation of EST libraries using the following Qiagen RNeasy plant minikit protocol, with modifications. Buffer RLT (Guanidine thiocyanate solution, provided with kit) was prepared fresh prior to each 100 mg purification by adding 4.5 µl of β mercaptoethanol per 450 µl buffer RLT. RNA isolation aid (Ambion) was also added to the RLT buffer to enhance RNA purification with 45 µl used per 100 mg of tissue extract. Approximately 500 mg of frozen fruit drupelets were weighed and placed into an RNase-free mortar, covered with liquid nitrogen (LN_2) and ground thoroughly. Tissue powder and LN_2 were decanted into a 50 ml centrifuge tube without allowing the tissue to thaw and once the liquid nitrogen had volatised, buffer RLT was added. The extract was vortexed vigorously before the lysate was transferred to 5 separate QIAshredder columns placed in a 2 ml collection tube. The tubes were centrifuged at 10,000 × g for 2 minutes before the supernatant from the 5 tubes were transferred to a sterile 15 ml microcentrifuge tube without disturbing the cell debris pellet. The addition of 0.5 volume ethanol followed and the solution was mixed immediately by pipetting. The sample was then transferred into an RNeasy spin column and 2 ml collection tube and centrifuged at 8,000 × g for 15 seconds. The flow through was discarded before 700 µl of buffer RW1 (provided in kit) was applied and tubes centrifuged for 15 seconds to wash the column membrane. The flow through was again discarded and 500 µl of buffer RPE (provided in kit) was added followed by an additional wash step and centrifuged for 15 seconds. The flow through was discarded

and the wash repeated followed by centrifugation for 2 minutes to ensure no ethanol was carried through. The spin column was transferred to a new 2 ml collection tube and centrifuged at 10,000 × g for 1 minute to prevent the carry over of buffer RPE or residual flow through. The RNeasy spin column was then placed carefully into a sterile 1.5 ml microfuge tube and 50 µl of RNase free water was added directly onto the column membrane. The RNA was eluted by centrifuging the tube for 1 minute at 8,000 × g. Six microlitres of RNA was transferred to a new microfuge tube for subsequent Nanodrop™ and agarose gel analysis while the remaining RNA was stored at -80^{o}C. Once sufficient total RNA had been isolated from each parent and each stage (approximately 100 µg per library), the samples were combined and cleaned up as per the following Qiagen protocol.

2.3.5 RNA cleanup protocol using Qiagen RNeasy mini kit

A combined total of 300 µl RNA for each library was transferred to a 15 ml centrifuge tube and 1050 µl of buffer RLT containing 10.5 µl of β-mercaptoethanol was added and mixed well. The RNA solution was then diluted by adding 750 µl of 100% ethanol and mixed by pipetting. The sample was transferred (700 µl at a time) to an RNeasy spin column inside a 2 ml collection tube and centrifuged for 15 seconds at 8,000 × g. The flow through was discarded between each 700 µl sample transfer and 500 µl buffer RPE was then added. The sample was centrifuged for 15 seconds in order to wash the column membrane before a further 500 µl of buffer RPE was added. The sample was centrifuged for 2 minutes to prevent ethanol carryover and the column transferred to a new 2 ml collection tube before further centrifugation at 10,000 × g for 1 minute to eliminate residual flow through. The spin column was then placed into a sterile microfuge tube and 45 µl of RNase free water was added directly to the membrane to elute the purified RNA. The sample was then centrifuged for 1 minute at 8,000 × g. This step was then repeated with a further 45 µl of RNase free water to give a total eluted sample of 90 µl. Purified RNA (2 µl) was analysed by spectrophotometer (2.2.8) in order to assess the concentration and absorption ratios prior to DNase treatment.

2.3.6 DNase treatment of total RNA

In order to remove any contaminating DNA which may be present, the samples were treated with DNA-free (Ambion). Using the Ambion protocol, 10 µl (0.1 volume) of 10× DNase 1 buffer and 1 µl of recombinant DNase 1 buffer (rDNase) was added to 88 µl of eluted RNA. This was mixed gently before incubating at 37^{o}C for 30 minutes. The DNase inactivation reagent was resuspended by gently flicking the tube and 10 µl (0.1 volume) of the slurry added to the sample. This was incubated at room temperature for 1 minute with regular flicking of the tube to mix. The sample was centrifuged at 10,000 × g for 1 minute 30 seconds in order to pellet the DNase inactivation reagent, allowing the supernatant containing RNA to be transferred to a fresh microfuge tube. This step was repeated as necessary to remove supernatant. The samples (2 µl) were then quantified via Nanodrop™ leaving 106 µl.

2.3.7 mRNA isolation

The isolation of poly $(A)^{+}$ RNA required for construction of cDNA libraries was obtained using Dynabead mRNA purification technology (Invitrogen). Total RNA comprises only 2 to 5% of Poly $(A)^{+}$ RNA which can be enriched prior to downstream cDNA synthesis. Dynabead purification utilises magnetic separation by binding poly $(A)^{+}$ to oligo (dT) which is covalently bound to the surface of the magnetic Dynabeads. Following a series of wash steps most other RNA species are removed allowing mRNA to be released in the final RNase elution step. Poly $(A)^{+}$ RNA was isolated using Dynabeads following the manufacturer's protocol. Approximately 100 µg total RNA in 100 µl was desirable at this stage. The RNA was heated for 10 minutes in a water bath at 65^{o}C, to disrupt any secondary structures and the samples placed on ice. Meanwhile the Dynabeads were resuspended by gently flicking the tube and 200 µl transferred to a sterile RNase free microfuge tube. The tube was then placed into the Dynal magnet for 30 seconds and the resultant supernatant was removed. The Dynabeads were then removed from the magnet and resuspended in 100 µl of 2× binding buffer (supplied with kit). The tube was placed back into the magnet for 30

seconds and the buffer removed. A further 100 µl of 2× binding buffer was then added to the beads followed by 100 µl of denatured RNA.

The Dynabead/RNA solution was mixed at ambient temperature in an orbital incubator SI 50 (Stuart Scientific) with shaking for 5 minutes to allow annealing of the poly $(A)^+$ tail of the mRNA with the oligo (dT) on the beads. The supernatant was then removed and discarded and the beads washed twice with 200 µl of washing buffer (supplied with kit) ensuring all supernatant was removed at each step. The tube was removed from the magnet and 20 µl of elution buffer (10 mM Tris-HCl, provided with the Dynabead purification kit) was added before incubating at 65^{o}C for 5 minutes. The tube was then placed immediately into the magnet and the supernatant containing mRNA was removed and transferred into an RNase free tube. A further 2 µl was removed for Nanodrop™ analysis leaving a total of 18.0 µl for first strand cDNA synthesis.

2.3.8 First strand cDNA synthesis

The mRNA from Section 2.3.7 was converted into first strand cDNA following the Superscript™ choice protocol (Figure 2.3). For optimal results 9 µl mRNA was added to a sterile 1.5 ml microfuge tube containing 2 µl of *Not*I primer adapter. The tube was heated in a water bath at 70^{o}C for 10 minutes before quick chilling on ice. The contents were briefly centrifuged before adding the first strand components: 5× first strand buffer (4 µl), 0.1 mM DTT (2 µl), 10 mM dNTP mix (1 µl), RNase free water (1 µl). The contents were mixed by gently vortexing before collecting the mixture via brief centrifugation. The tube was then incubated at 37^{o}C for 2 minutes in order to allow temperature equilibration of the mixture. Then 1 µl of Superscript ^{TM} II RT was added to the tube. The samples were incubated at 37^{o}C for 1 hour and then the reaction was terminated by placing the tube on ice.

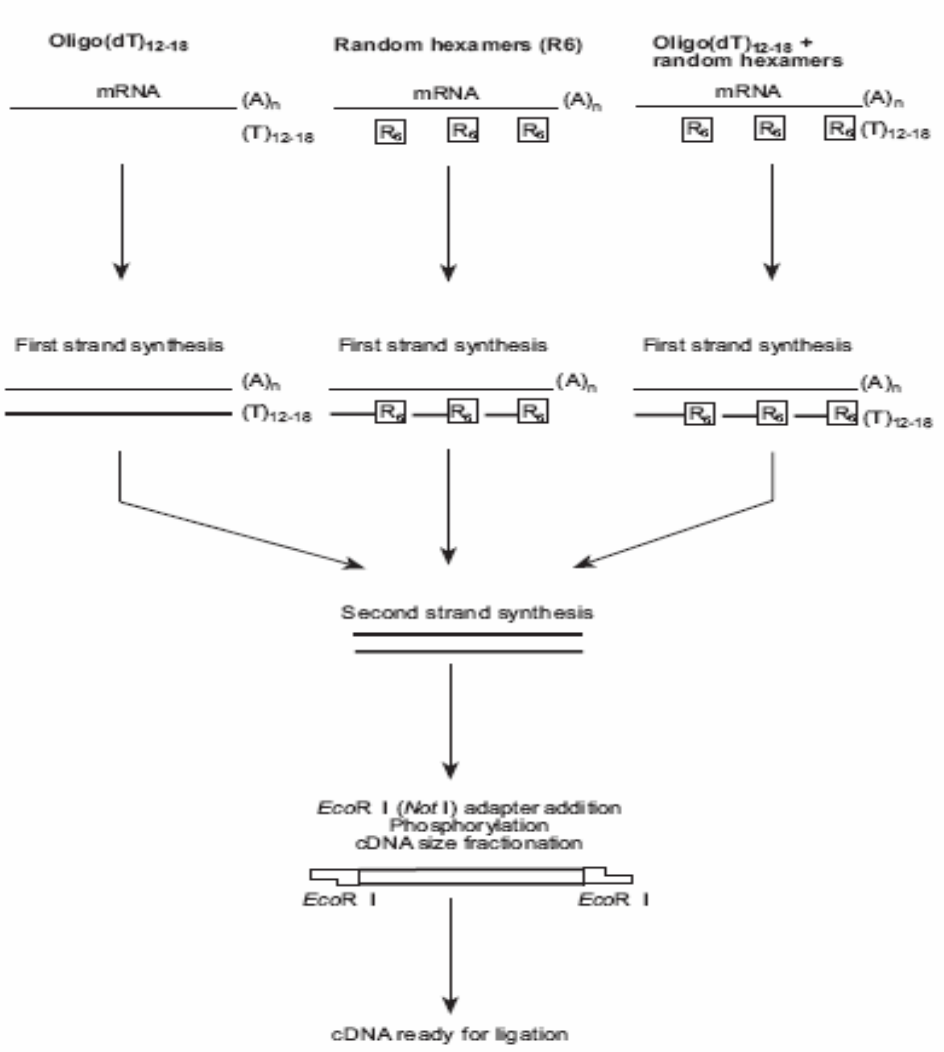


Figure 2.3. Superscript™ choice systems schematic representation of the cDNA construction stages (Figure extracted from Invitrogen Superscript™ instruction manual). The major steps involved in constructing a random cDNA library include the isolation of mRNA from total RNA followed by first and second strand cDNA synthesis. The addition of primer adapters are then required for efficient cDNA cloning and ligation.

2.3.9 Second strand cDNA synthesis

The following components were added to the 20 µl of first strand cDNA on ice:
DEPC treated water (91 µl), 5× second strand buffer (30 µl), 10 mM dNTP mix (3 µl) *E. coli* DNA ligase (10 units/µl) (1 µl), *E. coli* DNA polymerase I (10 units/µl) (4 µl) and *E. coli* RNase H (2 units/µl) (1 µl) to give a total volume of 150 µl. The mixture was vortexed gently and the reaction incubated at 16^{o}C for 2 hours. Finally 2 µl (10 units) of T4 DNA polymerase was added and the mixture was heated at 75^{o}C for 10 minutes before being placed back on ice. This allowed subsequent ligation of adapters by blunt ending the termini of the cDNA. The resultant 152 µl of cDNA produced from second strand synthesis was purified as described in Section 2.2.9.1.

2.3.10 SalI adapter ligation

Efficient cDNA cloning requires the addition of primer adapters such as *Not*I and *Sal*I to allow directional cloning into the vector pSportI. Using the *Sal*I adaptors from the Invitrogen kit the following reaction was set up: second strand cDNA (10 µl), 5× T4 DNA ligase buffer (4 µl), *Sal*I Adaptor (2.5 µl) T4 DNA ligase (1 unit/µl) (1.0 µl), sterile distilled water (2.5 µl) for a total volume of 20 µl. The reaction was incubated overnight at 16^{o}C in a water bath before being placed into a 65^{o}C water bath for 10 minutes and the reaction terminated by placing on ice. The 20 µl reaction was purified using Qiagen MinElute PCR purification kit outlined in 2.2.9.1.

2.3.11 NotI Digestion

The digestion with *Not*I removes a portion of the primer adapter located at the 3' end of the cDNA. The 10 µl of purified adaptor ligated cDNA (Section 2.3.10) was digested in the following reaction: the adapter ligated cDNA (10 µl) and 10× React 3 buffer (2 µl) was mixed together before the addition of the enzyme *Not*I (2 µl) and sterile distilled water (6 µl) to give a total volume of 20 µl. The reaction was then incubated at 37^{o}C for 2 hours before the temperature was raised to 65^{o}C for 20 minutes and the reaction then terminated by placing on ice. The full reaction was then size fractionated through a 1% (w/v) low melting point agarose gel (Sigma-Aldrich), containing 0.5 µg/ml of EtBr, for

approximately 20 minutes. A gel slice containing cDNA larger than 500 bp was excised using a sterile scalpel and the cDNA purified using the Qiagen MinElute gel extraction kit (Section 2.2.7).

2.3.12 Ligation of cDNA to the pSport1 vector

Using the pre-cut (*Sal*I – *Not*I) vector from Invitrogen the digested and size selected cDNA was directionally ligated into the pSport I vector as follows: cDNA (4 µl) 5× ligase buffer (2 µl), 50 ng pSport I, pre-cut (1 µl), T4 Ligase (0.5 µl) and sterile distilled water (2.5 µl) to give a total reaction volume of 10 µl. The reaction was incubated overnight at 4^{o}C then stored at -80^{o}C prior to transformation into *E. coli*.

2.3.13 Transformation of cDNA

Electrocompetent *E. coli* host cells (ElectoMAX DH10B, Invitrogen) were removed from -80^{o}C storage and 20 µl aliquots transferred to sterile microfuge tubes. One microlitre of ligation reaction was added to each 20 µl aliquot (3 for 'Glen Moy' and 3 for 'Latham' for each stage) on ice before the 21 µl were transferred into chilled 1 mm electrode gap cuvettes. The samples were electroporated in turn at 1.8 KV with a 25 µF capacitance resistance (Gene Pulser, BioRad) and 1 ml of SOC media added immediately before the samples were transferred to a 15 ml centrifuge tube and incubated for 1 hour at 37^{o}C with moderate shaking. Following incubation 20 µl, 50 µl and 100 µl of each sample were aseptically plated onto plates containing LBAmp/X-gal/IPTG (100 µg/ml ampicillin, 40 µg/ml X-gal and 20 µg/ml IPTG) media and incubated overnight at 37^{o}C to allow calculation of colony forming units. The remainder of the transformed cDNA was stored at 4^{o}C until required.

2.3.14 Insert check of transformed cDNA

A selection of white colonies (eight samples) were individually selected from each 50 µl transformed plate (Section 2.3.13) and placed into separate wells of a PCR plate containing 10 µl sterile distilled water. After 30 minutes 1 µl of each sample was removed as template for a standard 25 µl PCR reaction using M13 universal forward and

reverse primers (Section 2.2.9.5.1). A 25 µl reaction contained 25 ng template DNA, 1.0 µM forward and reverse primers, 0.2 mM dNTPs and 0.1 units *Taq* polymerase**.** PCR conditions were: denature at 94^{o}C for 1 minute, anneal at 57^{o}C for 1 minute, extend at 72^{o}C for 1 minute, and a final extension at 72^{o}C for 8 minutes before holding at 4^{o}C. The PCR products were visualised by electrophoresis of 5 µl of each sample on a 1% (w/v) agarose/TBE gel (Section 2.2.6).

2.3.15 Large scale library preparation

LBCarb/X-gal/IPTG (100 µg/ml carbenicillin, 40 µg/ml X-gal and 20 µg/ml IPTG) Q-plates (22.2 cm by 22.2 cm) were collected and allowed to dry for around 3 hours in a laminar flow cabinet, turning each plate every hour. Appropriate volumes of each sample were aseptically transferred to individual Q-plates to give a density of between 2000 to 3000 colonies before being incubated at 37^{o}C overnight. Once the colonies had grown to a sufficient size the blue/white separation was enhanced by transferring the plates to 4^{o}C for 2 hours. The white colonies were then picked into 384 well plates containing prepared LB freezing media (Section 2.3.16) using the automated Q-Bot.

2.3.16 Genetix Q-Bot colony selection

The Q-Bot (Genetix, Hampshire, United Kingdom) consists of an imaging system which is capable of scanning and analyzing a complete plate in order to distinguish between colonies. Linked to a computer with a robotic arm and picking head attachment the Q-Bot scans and selects only white colonies present on each plate based on pre-set criteria. The Q-Bot was set up under standard conditions with pin head checks, picking height and camera alignment set. The three baths were filled to allow the cleaning of the pin head with 70% (v/v) ethanol, sterile distilled water and 70% (v/v) ethanol. The plastic base was used instead of the metal base for plates to sit on in order to allow more light through, aiding colony selection. Colour parameters were set to ensure high quality white colonies were selected with no blue traces. A 384 well plate for each library was prepared for individual colony selection as follows. The sterilised manifold was attached

to the Q-filler and a 500 ml bottle containing prepared LB freezing media (20 g/l Bacto tryptone, 10 g/l Bacto yeast extract, 10 g/l NaCl, 36mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM Na_3 citrate, 0.4 mM $MgSO_4$, 6.8 mM $(NH_4)_2SO_4$, 4.4% glycerol) and ampicillin (100 ng/µl) was connected. Seventy microlitres of prepared media were transferred into 384 well plates which were then placed into the Q-Bot hotel ready for colony selection. The colonies were allowed to grow overnight at 37^oC before covering with PCR adhesive foil seal (Thermo Scientific) preventing the risk of contamination and these were stored prior to library construction at -80^oC.

2.3.17 EST library construction

2.3.17.1 Overnight cell growth

The EST libraries produced for stage 2 (green/red) fruit and stage 3 (red ripe) fruit for cvs. Glen Moy and Latham (Section 2.3.16) were removed from -80^oC storage and thawed at room temperature. Two times LB media (20 g/l Bactotryptone, 10 g/l Bacto yeast extract and 10 g/l NaCl) was prepared with the addition of ampicillin 100 mg/ml (500 µl in 500 mls): 1 ml of this solution was then added to each well of a 96 deep well plate for a total of 4 plates (384 colonies) for each of the libraries produced (16 plates in total). To each well, 5 µl of cDNA was added using a multichannel pipette in the laminar flow cabinet. Each plate was covered with a gas permeable adhesive seal and incubated overnight at 37^oC with shaking. Large scale plasmid minipreparations, (Millipore) were then carried out on all of the 96 well plates (Section 2.2.9.5) and the integrity of the plasmid DNA analysed by electrophoresis of 8 samples per plate (Section 2.2.6) using 3.5 µl of plasmid DNA on a 1% agarose gel. cDNA clones were then sequenced using M13 forward primer in order to sequence the 5' end of the cDNA insert, as described in Section 2.2.9.5.1.

2.3.17.2 cDNA sequencing clean up using Genetix Genclean

The cDNA was then prepared for sequencing by following the Genetix Genclean protocol (Bioexpress, Kaysville, USA). The foil wrapped plate was removed from the fridge and the buffer eluted by centrifuging on top of a wash plate for 5 minutes at

1,000 × g. To each well 30 µl of distilled water was added before centrifuging for a further 5 minutes. The plate was placed on top of a collection plate and the 10 µl sequencing reaction was transferred slowly to the centre of each well. The plate was centrifuged for 5 minutes and the elution containing the sequencing reaction sent for sequence analysis on an ABI 3730 DNA sequence analyser (Applied Biosystems) at the SCRI Sequencing and Genotyping Service.

2.3.17.3 cDNA sequence analysis

cDNA sequences were quality scored using Phred Software (http://www.phrap.com) before undergoing analysis using NCBI (http://www.ncbi.nlm.nih.gov) databases for the identification of similar sequences using BLAST algorithms (Woodhead *et al*., 2008).

Manual examinations of sequences were performed to identify consistent polymorphisms (Section 2.2.9.5.2) and primers were designed to these sequences using Primer3 (Section 2.2.5).

2.3.17.3.1 Sample preparation for genotyping

PCRs were performed (Section 2.2.4.1) on the 188 individuals of the mapping population, for a selection of polymorphisms identified within cDNA library contigs, using fluorescently labelled 5' end primers (FAM or HEX). Successful amplification was confirmed by gel electrophoresis (Section 2.2.6) and products were diluted as required before submission for genotyping. For each sample, 60 µl of sterile distilled water was added to each PCR well and 2 µl of this diluted sample was transferred to a new PCR plate. A mixture containing 4 µl of ROX™ (Applied Biosystems) and 495 µl of HI DI™ formamide (Applied Biosystems) was prepared and 8 µl of this was added to each of the PCR wells. The samples were subsequently sent for genotypic analysis on an ABI 3730 DNA sequence analyser (Applied Biosystems) to the SCRI Sequencing and Genotyping Service.

2.4 Materials and methods for validation of quality QTLs (Chapter 5)

2.4.1 Plant material

Twelve individual raspberry cultivars were selected for QTL validation based on phenotypic characteristics. The primary characteristic of interest was colour with individuals selected including red, purple, black and yellow raspberry samples (Table 2.3).

2.4.2 DNA extraction

DNA was extracted from young expanding leaves from the 12 samples obtained from plants grown in pots in SCRI glasshouses which were maintained at 20^{o}C (16/8hrs day/night) as described in Section 2.2.2.

2.4.3 Candidate genes selected for QTL validation

The candidate genes listed in Table 2.4 were utilised for validation of QTLs previously detected in the 'Glen Moy' × 'Latham' population. The genes were selected on the basis of their location within the QTL region of interest (Chapter 5) and genotypic and sequence analysis carried out in order to fully assess polymorphisms within a wider genetic background (Sections 2.4.3.1 and 2.4.4).

Table 2.3. Raspberry cultivars selected for QTL validation

Germplasm used for QTL comparisons	
Blacknight	Black raspberry
Blacksatin	Black raspberry
Glen Coe	Purple fruit
Williamette Spinefree	Darkest red raspberry
Chilliwack	Very dark red raspberry
Malling jewel	Dark red fruit
Prestige	Yellow receptacles, red fruit
Rose de cote d'or	Very aromatic , red fruit
Glen Ample	Quality, flavour and size, bright red
Tulameen	Flavour, quality and glossy, red fruit
Glen Shee	Very pale red fruit
Golden Queen	Yellow Fruit

Table 2.4. Candidate genes selected for QTL validation and method of analysis

Genotypic Analysis	**Sequence Analysis**	**Pyrosequence® Analysis**
basic helix loop helix (bHLH)	Tonoplast intrinsic protein	Tonoplast intrinsic protein
MYB	Membrane intrinsic protein 3	Membrane intrinsic protein 2
118b	Flavonol synthase	Flavonol synthase
		Expansin
		Anthocyanin reductase
		Dihydroflavonol 4-reductase

2.4.3.1 Polymerase chain reaction

Ten microlitre PCR reactions were performed for all candidate genes selected for analysis of diverse germplasm, via sequencing or genotyping, using the primer pairs as per Appendix 2.3a and 2.3b. A typical 10 µl reaction contained 25 ng DNA, 1.0 µM each primer, 0.2 mM dNTPs, 1× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.0) and 1 unit *Taq* DNA polymerase (Roche). For samples analysed via Pyrosequence® analysis a 25 µl PCR reaction was performed as per Section 2.2.10.3. The primers used for QTL validation were the same primers utilised for mapping populations which resulted in initial QTL association on the linkage map. The PCR reactions were carried out using a Perkin Elmer 9700 Thermal Cycler (Applied Biosystems) using the touchdown protocol described in Section 2.2.4.2. Products were analysed by electrophoresis as described in Section 2.2.6.

2.4.4 Preparation of samples for sequence clean up and analysis

Exo Sap-it® (USB Corporation, Ohio, USA) is a convenient technique for the preparation of PCR products for direct sequencing. Following PCR amplification, Exo Sap-it® was added to the amplified product where the enzyme Exonuclease I degrades single-stranded primers and shrimp alkaline phosphatase dephosphorylates the remaining dNTPs from the PCR mixture. Two microlitres of Exo Sap-it® was added to 5 µl of PCR product for subsequent sequence analysis. Samples were incubated at 37°C for 15 minutes, 80°C for 15 minutes followed by a 4°C hold. After this incubation the primers used for analysis were diluted 1 in 10 to give 10 mM concentrations and all samples were sent for sequence analysis using an ABI Prism 3730 DNA analyser (Applied Biosystems). Samples which were quantified by genotypic analysis were prepared as detailed in Section 2.3.17.3.1, and samples for Pyrosequence® analysis were prepared as described in Section 2.2.10.3. All samples were then examined for the presence of polymorphisms, as previously identified within the mapping parents, to assess the transferability of markers identified within a segregating population to a wider genetic pool.

Chapter 3 Phenotypic analysis and correlations

3.1 Introduction

The term phenotype is derived from the Greek term "*phainein*" which means to appear, and denotes particular characteristics expressed in an individual (Wolfe *et al*., 2007). Phenotypic traits expressed in plants are controlled by genes and the combination of alleles or variations within the genes are classified as the plant's genotype. Any effect exerted on the phenotype of an organism by the actions of the environment cannot affect the underlying genotype (Andrade, 2006). Organisms which contain the same genotype will not necessarily look or behave in the same manner, as many phenotypic aspects are modified by developmental or environmental conditions. Likewise not all organisms which look the same will have the same genotype (Kole and Abbott, 2008).

3.1.1 Phenotypic traits of visual importance

Quality considerations are paramount to the success of any raspberry variety, with visual traits important for initial purchase and acceptability. Colour, shape and size are the most obvious attributes involved in fruit quality as these are the first characteristics observed by potential consumers. It is these visual traits which consumers relate to acceptability of fresh goods while making assumptions relating to fruit maturity and quality. If fruit appears too dark it is often regarded as being over ripe whereas too light a fruit may be regarded as under ripe (Traveset and Willson, 1998). The development and introduction of new raspberry varieties can take between 8 and 15 years due to the highly heterozygous nature of this perennial fruit crop and even then selected quality traits would be difficult to screen for due to the limited resources breeders have available today (Graham *et al*., 2004). Conventional breeding relies on phenotypic selection of desirable traits, along with the elimination of undesirable ones, through successive generations. This requires each generation to be tested in relation to phenotypic characteristics and processing traits which in perennial crops is a slow, demanding and expensive process.

3.1.1.1 Fruit colour

Colour of fruit not only directly affects appearance, desirability and associated freshness but is also an important determinant of flavour and acceptability (Bridle and Timberlake, 1996). Many factors contribute to colour in raspberries including anthocyanin content, enzymatic reactions, pH, ascorbic and organic acids content (de Ancos *et al*., 1999). Many attempts have been made to evaluate the role of colour cues (for review see, Clydesdale, 1993; Delwiche, 2004) which have been shown to influence perceptions in a variety of food and drinks (DuBose *et al*., 1980; Garber *et al*., 2000; Roth *et al*., 1988; Zellner and Durlach, 2003; Zampini *et al*., 2007). Adding red colouring to strawberry flavoured sucrose solutions, for example, increased perceived sweetness (Johnson *et al*., 1983). Du Bose *et al*. (1980) reported sensory assessors were less accurate in identifying fruit flavour in drinks when colour was masked with only 20% correct compared with 100% unmasked. It was also found that 40% of participants identified a cherry flavoured beverage as orange when so coloured (Du Bose *et al*., 1980; Zampini *et al*., 2007). Colours typically associated with fruit ripening may also be particularly effective at modulating sweetness perceptions (Maga, 1974).

3.1.1.2 Fruit Size

Size is a visually important trait for consumer acceptance of fresh fruit. In tomato, fruit size has been improved in many cultivars since domestication from wild progenitors (Causse *et al*., 2004). Although this trait is found to be highly heritable, it is thought that several separate genes, many of which may be influenced by the environment, may determine this trait in tomato (Causse *et al*., 2004). Developmental studies have subsequently indicated size as a function of the number of cells present within the ovary prior to fertilisation, the number of successful fertilisations, the number of cell divisions within developing fertilised fruit and the extent of cell enlargement following fertilisation (Bohner and Bangerth, 1988; Gillaspy *et al*., 1993; Lippman and Tanksley, 2001).

In strawberry a negative correlation was identified between fruit size and the majority of nutritional quality parameters such as soluble solids and titratable acidity (Capocasa *et al*., 2008).

3.1.1.3 Total soluble solids content

Total soluble solids and acidity of fruit are important factors in flavour quality. The soft fruit industry uses the total soluble solid content, in the form of ^{o}Brix, as a measure of quality to make decisions on individual varieties prior to purchasing fruit from growers. A berry with high sugar content and high acidity produce good berry flavour whereas low sugar and high acid form tart fruit, while low sugar and low acid result in a bland tasteless fruit (Wang *et al*., 2009). In table grape it is advantageous to have a cultivar with a high ratio of tartaric to malic acid as this improves wine's stability. In terms of palatability, however, a lower ratio of tartaric to malic acid is desirable as this improves flavour and mouthfeel (Liu *et al*., 2007). As with most food products the organoleptic quality of grapes is described as being dependent on the content and composition of both sugars and acids and these traits should be considered paramount for the selection of new cultivars (Liu *et al*., 2007). Studies by Liu *et al.* (2007) concluded that parental sugar content in grape has no significant bearing on the sugar content of the progeny. The same is not true for acid content which is highly heritable and parents with high tartaric acid should be selected in order to obtain this trait within progeny. While many breeding programmes have succeeded in improving the overall sugar and acid content (^{o}Brix) in tomatoes this most often occurs with a decrease in fruit size (Chen *et al*., 1999). Lecomte *et al*. (2004) could offer no physiological explanation for the relationship between ^{o}Brix and weight but proposed that the increase in fruit size may be causing a dilution of ^{o}Brix content.

3.1.2 Protected cultivation practices

Many growers have recorded significant increases in fresh fruit production since the introduction of protected cultivation. This can be improved upon even further with the introduction of new varieties of high quality cultivars capable of sustained production

throughout the fruiting season while utilising a protected cultivation environment (Zhao *et al*., 2007). In some areas in Southern Spain 100% of fresh market grown raspberries are produced under tunnels. These changes in agronomic practice can have an effect on many aspects of plant growth from seasonality and quality to a shift in pressures exerted by pests and pathogens (for review see Graham and Woodhead, 2009). The production of melons, an important commercial crop in Italy, is influenced strongly by external weather conditions but availability can be expanded by the utilisation of protected cultivation (Ferrante *et al*., 2008). The changing climate experienced in the UK and throughout Europe is already a major consideration for future soft fruit production with mild winters leading to poor bud break in several soft fruit species (Graham *et al*., 2008). High quality raspberry cultivars, which can adapt to the challenges faced by their environment, are fundamental for the future expansion of the UK raspberry industry.

3.1.3 Marker assisted breeding

Marker assisted breeding (MAB) is the application of molecular markers, which have shown association (linkage) to genes involved in desirable traits, for selection in potential new cultivars as opposed to plant selection on the basis of phenotypic behaviour alone (Kellerhals *et al*., 2000). As demands for new cultivars with improved organoleptic traits increase, fruit breeders require alternative strategies to improve efficiency and reduce time for new varieties to be produced (Kenis *et al*., 2008). MAB may take the form of presence or absence markers which may be used as a substitute for, or concurrently with, phenotypic selection which may aid efficiency and reliability when compared with conventional breeding (Mohan *et al*., 1997). These markers can be utilised at the seedling stage of new cultivar development which would allow selection of individuals with favourable combinations of multiple fruit quality traits (Kenis *et al*., 2008). *Rubus* is a highly heterozygous species which requires large numbers of progeny to be phenotypically screened in order to identify potentially promising genotypes (Jennings, 1988). The deployment of marker assisted breeding strategies could therefore yield substantial economic benefits to the producer, while producing high quality, fresh fruit with recognised health, economic and environmental benefits to the consumer.

In order to develop MAB strategies for premium sensory characteristics in raspberry, it was necessary to evaluate a population which segregate for traits of interest across a variety of environmental conditions. In this chapter, a segregating population was assessed to evaluate a range of phenotypic quality traits for subsequent genotypic analysis including the identification of QTL regions which may contain genetic markers or candidate genes which can be screened as part of a MAB strategy. This raspberry population was replicated over three environments with analyses performed over three seasons to account for any environmental influence on phenotypic traits of interest.

3.2 Results

3.2.1 Fruiting season one, July 2006

3.2.1.1 Plant material for phenotypic data collection in 2006

The mapping population used for analysis consists of a cross between the European red raspberry cv. Glen Moy and the North American red raspberry cv. Latham as described in Section 2.1.1. The phenotypically diverse parents can be seen in Figure 3.1. Season one analysis was carried out on fruit samples from one location only, the field site at SCRI, planted in the summer of 2002. One location was selected to enable a high volume of samples to be analysed, with multiple clones for each individual assessed. This was done to establish experimental parameters and assess variability for subsequent seasonal analysis. Approximately 150 individual progeny were chosen from the 188 used for mapping (Section 2.1.1) and samples were analysed for clone 1 and clone 2 and also repetition 1 and repetition 2 giving a total of four plants per individual.

Ripe fruit was collected from all samples as described in Section 2.1.2 from 7th of July 2006 until 26th July 2006.

Figure 3.1. The phenotypically diverse parents used in the construction of a segregating mapping population. The European cv. Glen Moy with large, thimble shaped, pale red fruit (left) and the North American cv. Latham which produces small, round, dark red fruit (right).

3.2.1.2 Seasonal Data for 2006

Seasonal analysis carried out in 2006 by the meteorological department at the SCRI, Dundee, recorded temperature increases throughout the year which were higher than the long term averages for all months except March, which at 6.8^{o}C was the lowest temperature recorded in 10 years. The most significant months were June, July and September which recorded the highest temperatures on SCRI records, Table 3.1 (SCRI meteorological records, 2006). The total sun hours recorded in 2006 were particularly high from April through to August with a mean of 206 hours of sunshine per month.

3.2.1.3 Colour meter analysis

A chroma meter (Minolta, CR-110) was used to analyse sample colour for the detection of variations between progeny as detailed in Section 2.1.4. The Yxy measurements were used to assess the sample brightness and the L*a*b* analysed individual colour composition. A further analysis, ΔE, is a measurement of colour deviation which represents the total colour difference within samples, as calculated in the equation in Section 2.1.4.

3.2.1.3.1 Analysis of variance

Analyses of variance (ANOVA) were performed to estimate mean sample response from each of the repetitions and clones analysed in 2006 in order to test for differences which may be present between sample means within one environmental location. This analyses measures the distribution (spread) contained within a data set to allow the information from different environments/genotypes to be combined in a single analysis. While this analyses was carried out for all phenotypic traits analysed in 2006 it was found that within a single environment, regardless of clone or repetition location, only sample genotype contributed significantly to the variations detected ($p < 0.001$).

Table 3.1. Seasonal analysis showing temperature measurements across season 1.

Monthly weather means and totals for 2006							
Month	Total rain mm	Days rain >0.2mm	Days rain >0.1mm	Total sun hours	Mean soil 20°C	Mean air max °C	Mean air min °C
January	23.2	13	7	30.2	3.3	7.0	0.9
February	24.4	12	7	73.3	3.0	7.1	0.7
March	118.5	20	19	77.8	3.2	6.8	0.8
April	6.2	8	2	212.5	6.9	12.4	2.7
May	68.2	16	12	215.2	10.6	14.7	5.5
June	41.0	9	5	187.4	15.6	19.4	9.6
July	34.0	9	6	217.0	18.4	22.5	12.3
August	54.6	10	6	199.6	16.1	20.4	10.8
September	88.3	19	11	125.8	14.3	19.0	11.1
October	109.8	17	14	79.6	11.0	14.6	8.2
November	72.5	19	13	80.3	6.0	10.6	3.3
December	69.0	16	12	60.5	4.1	7.9	1.8

3.2.1.3.2 Colour meter correlations

Although Yxy and L*a*b* measured different elements of colour attributes, statistical analysis suggested that in terms of significance and correlations, they were analysing the same parameters. The scatter plot matrix (Figure 3.2), plotted using Genstat 10 for Windows, shows the correlation between colour measurements Yxy and L*a*b* for one clone analysed in 2006. In order to establish the strength of association between two random variables, the oldest and most common method utilises the Pearson correlation coefficient (PCC) (Daly *et al*., 1995). The PCC is described as a quantity which is denoted by the term 'r' with values recorded between +1 and -1. The absolute value of 'r' is the measurement of the strength of variable association and the further the 'r' value is from 0 the stronger the variable relationship is. If the bivariate data is plotted on a graph and results in a straight line with a positive slope then the PCC shows the value of +1. If however, the data has a straight line with a negative slope the PCC takes the value of -1. If no straight line is plotted and the PCC is 0 then the two variables are regarded as uncorrelated. Correlations were recorded for each of the colour meter measurements and significant linear relationships were identified between Y and L* (r = 0.973), x and a* (r = 0.969) and y and b* (r = 0.869). Two samples which are identical would show a correlation of r = 1.0, results of >0.3 are significant for this sample size, and the closer two samples are to 1.0 the higher their significance. This confirmed that the measurements Yxy and L*a*b* were measuring the same aspects of colour but on different scales.

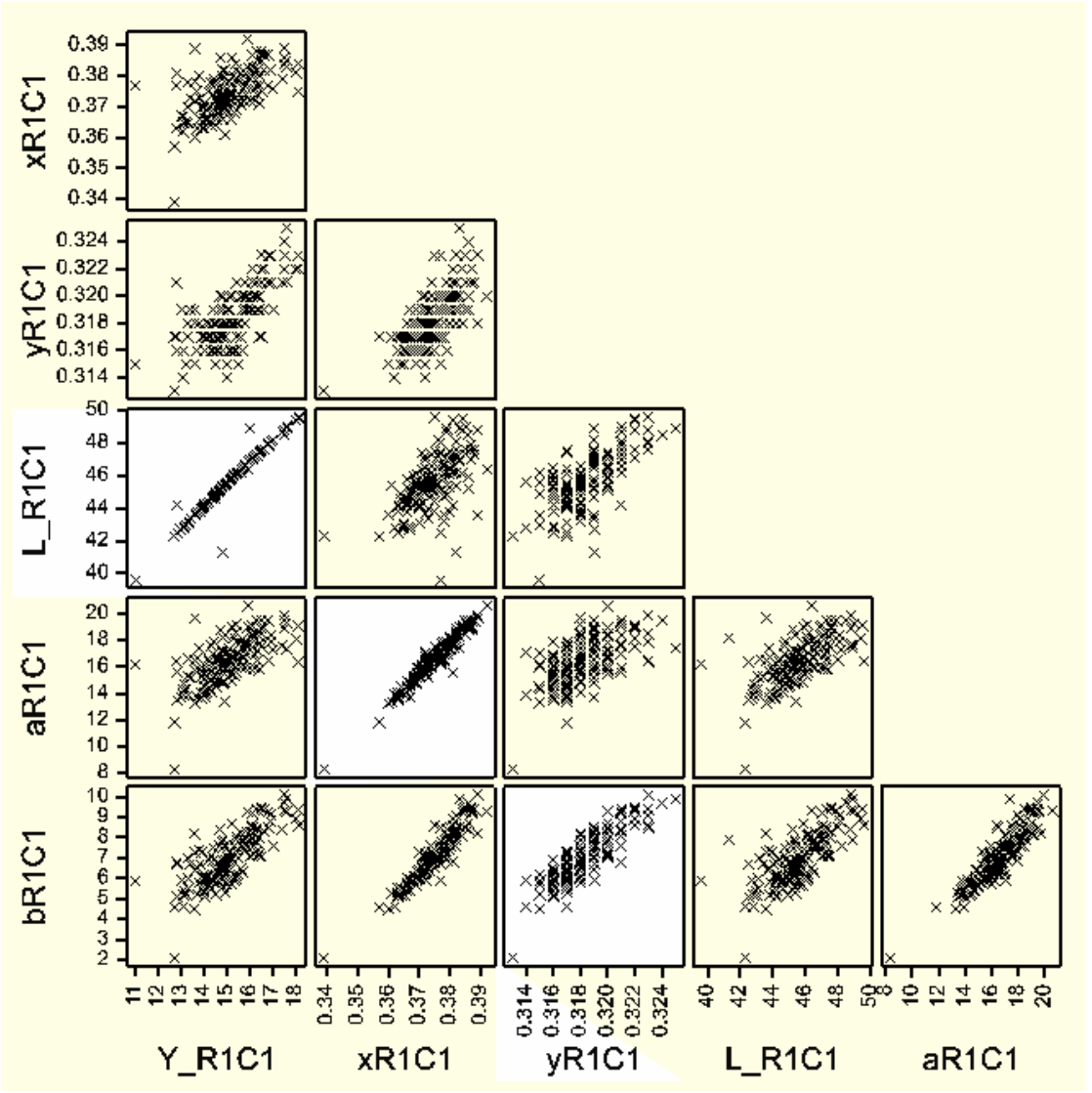


Figure 3.2. Scatter plot matrix showing the linear relationship between Yxy and L*a*b* in 2006 for repetition 1, clone 1 (R1C1) from the field site at SCRI. The axis show the individual colour measurements; reflection (Y), intensity (x), wavelength (y), brightness (L*), green/red (a*) and blue/yellow colour spectrum (b*). The white highlighted boxes show the linear relationship detected between the colour parameters Y and L*, x and a* and y and b* although positive relationships can be seen between all colour meter measurements.

While all measurements were analysed and recorded, only one set of measurements are referred to from here on and those relate to Yxy assuming Y = L*, x = a* (x also shows a positive linear relationship with b*) and y = b*.

3.2.1.3.3 Colour variation in different weights analysed

In order to ensure consistent colour meter analysis, careful preparation of samples were required as detailed in Section 2.1.4.2. This involved testing samples using different blenders, fresh fruit weight and puree sample weight. Once reproducible results were obtained a summary of statistics for each individual measurement (Yxy) for both 15 g and 10 g was carried out and resulted in no significant difference being recorded for the separate measurements across two field replicates. Correlations for the two weight differences show all clones and repetitions are highly similar with figures of r = 0.942, 0.926, 0.951 and 0.907 for each of the repetitions and clones analysed ($p < 0.001$).

It can be seen from the box plot in Figure 3.3 that measurements obtained for each of the clones and repetitions analysed were similar for both 10 g and 15 g weights. As a result only 15 g samples were analysed in subsequent seasons. Although differences in sample reflection (Y measurements) between individual progeny were recorded within the SCRI field site ($p < 0.001$), the measurements for the separate clones and repetitions remained consistent with no significant differences attributed to individual plant location ($p = 0.537$).

The Y values (sample reflection/brightness) recorded across the repetitions and clones ranged from a minimum of 11.8 (R1C2) to a maximum of 18.7 (R2C1) with a mean across the progeny of 15.1 for 10 g fruit samples. In 15 g fruit the minimum was 11.0 (R1C1) and the maximum was 18.7 (R2C1) with a mean measurement of 15.1.

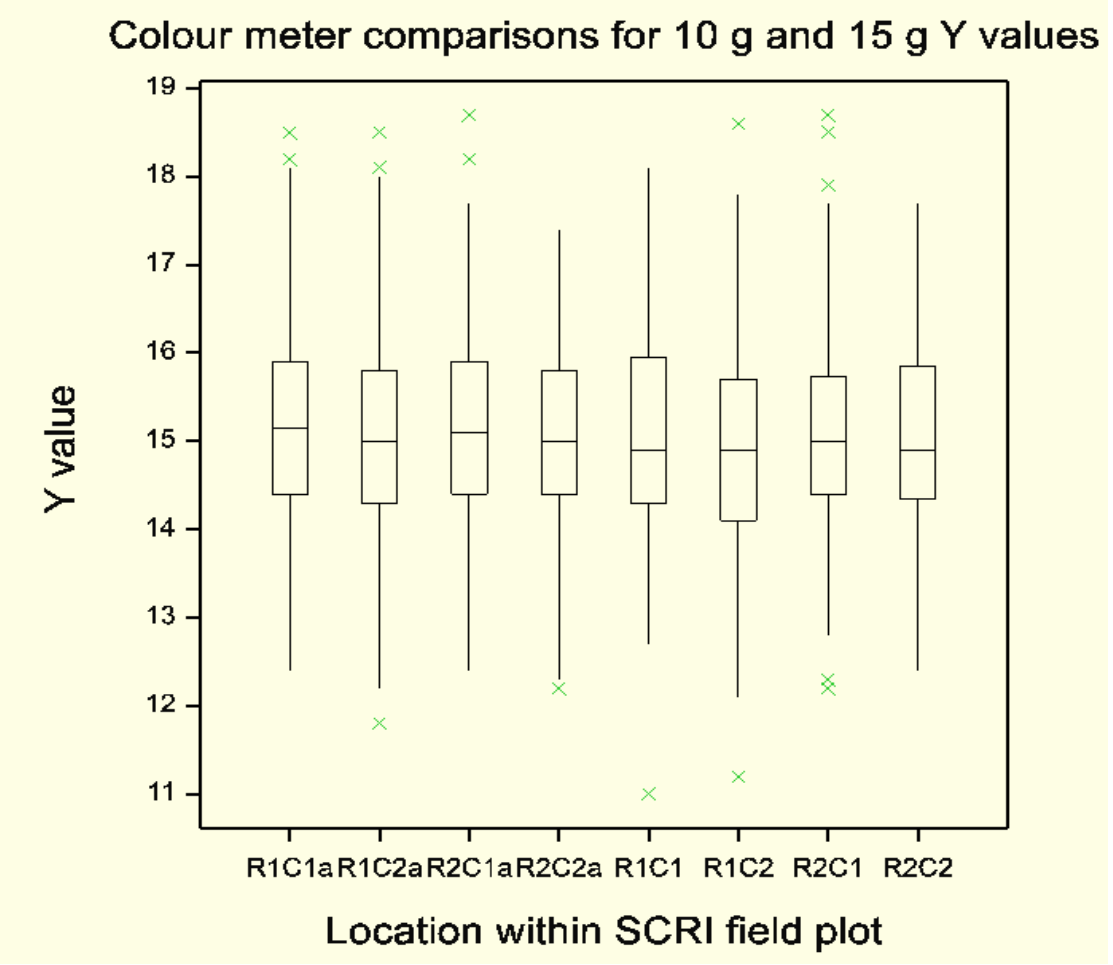


Figure 3.3. Colour meter measurements for fruit brightness (Y) for two weights, 10 g and 15 g analysed across four individual clones from the field site at the SCRI in 2006. Samples annotated with an 'a' (first four measurements) are 10 g weight, samples without an 'a' are 15 g weight (last four measurements). The horizontal bar in the middle of the individual boxes denote the median of samples, the upper hinge indicates the 75th percentile or upper quartile range, and the lower hinge the 25th percentile or lower quartile range while the range in the middle of two quartiles is the inter-quartile range. The whiskers protruding from either end of the box signify the minimum and maximum values while the samples denoted by the green x are values outwith the 1.5 times maximum range covered by the whiskers and are indicative of potential outliers.

3.2.1.4 Analysis of total soluble solids

Brix is a measurement of the total soluble solids (TSS) contained in a fruit sample. Named after the German inventor A.F.W. Brix in the 19th Century degrees Brix (^{o}Brix) initially analysed the specific gravity of a liquid to assess sample quality (Shachman, 2004). The density of the liquid, in relation to pure water, was then calculated assuming that 1 g of sugar is present per 100 g of juice. As technology has progressed and the specific gravity of sucrose solutions is known, the soluble solids content is now measured using a refractometer (Shachman, 2004). Degrees Brix is equal to grams per 100 ml juice at 20^{o}C therefore 1 ^{o}Brix corresponds to ca. 18 g/l sugar (Liebhard *et al*., 2003).

Statistical analysis of ^{o}Brix juice measurements recorded highly significant differences between individual progeny ($p < 0.001$) but no significant differences were recorded between clones or replicates (Figure 3.4).

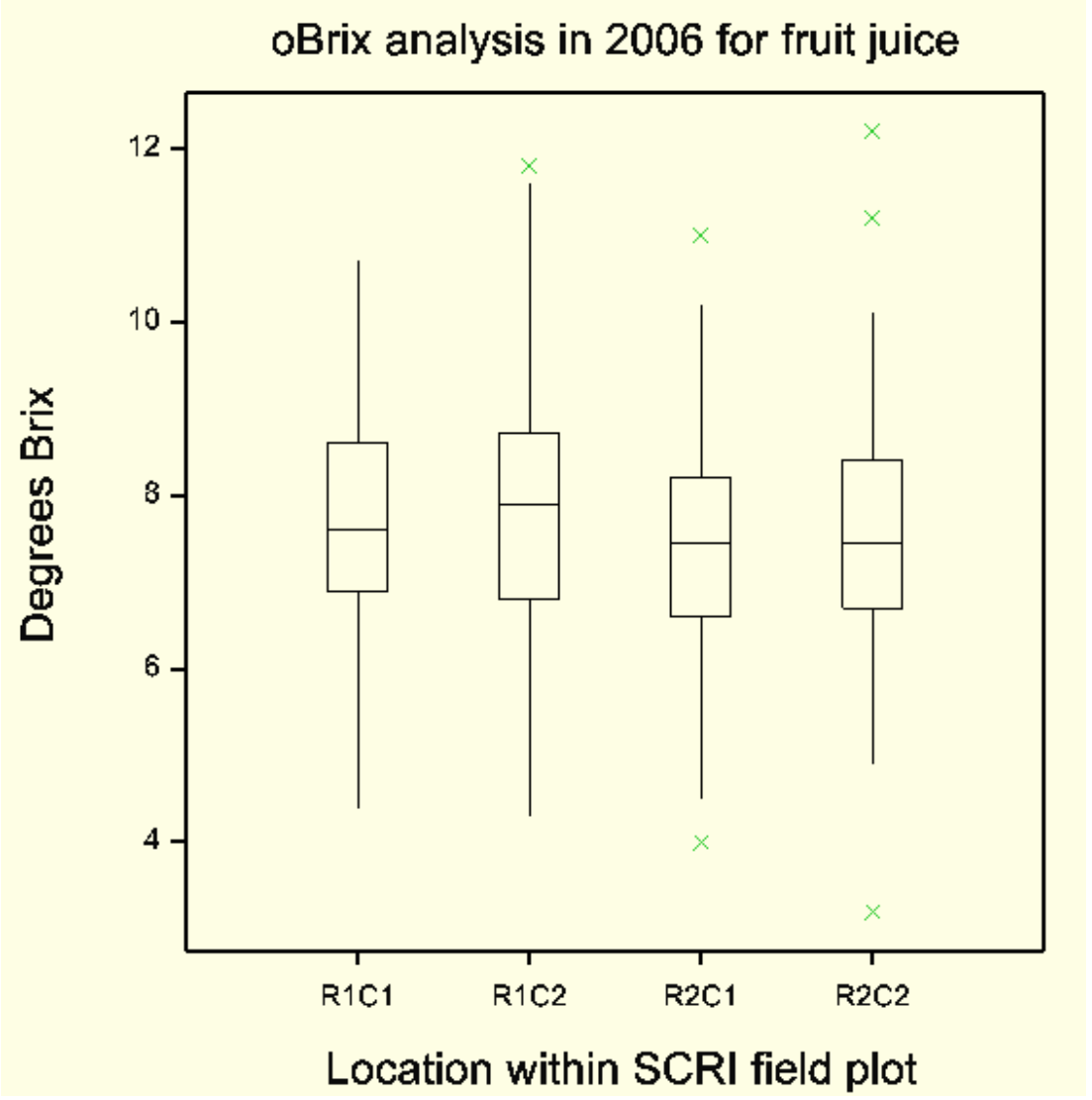


Figure 3.4. ^{o}Brix values obtained for sample fruit juice from the field site in 2006. The median (middle horizontal bar), minimum (lower horizontal bar) and maximum (upper horizontal bar) values for total soluble solids for samples analysed across all four clones show minimal variation due to the field location of individual samples as reflected in the similar box plot pattern obtained for each clone. The four clones analysed were from R1C1, repetition 1 clone 1, R1C2, repetition 1 clone 2, R2C1, repetition 2 clone 1 and R2C2, repetition 2 clone 2. The box plot can be interpreted as described in Figure 3.3.

3.2.1.4.1 Comparison of ^{o}Brix measurements between sample juice and puree

As each sample was subsequently blended for colour analysis, a few drops of pureed sample was also analysed in order to allow a direct comparison to be carried out between the soluble solid content of both raspberry juice and blended puree. Although slight differences were recorded for the ^{o}Brix measurements depending on the source of sample analysed, squeezed juice or blended puree, the differences were consistent for corresponding samples and correlations were recorded across all clones (Figure 3.5). This box plot shows comparable results for individual samples obtained across all four clones in 2006 regardless of the source of sample material analysed. In terms of quality analysis carried out by the soft fruit industry, it is standard practice for ^{o}Brix to be tested on fruit juice. As no significant differences were recorded between the two preparations ($p = 0.103$) only fruit juice samples were analysed in subsequent seasons.

Fruit juice samples produced measurements in the range of 3.2 to 12.2 with a mean of 7.6. For blended samples the minimum range was 3.8 with a maximum of 10.4 and a mean result of 7.2.

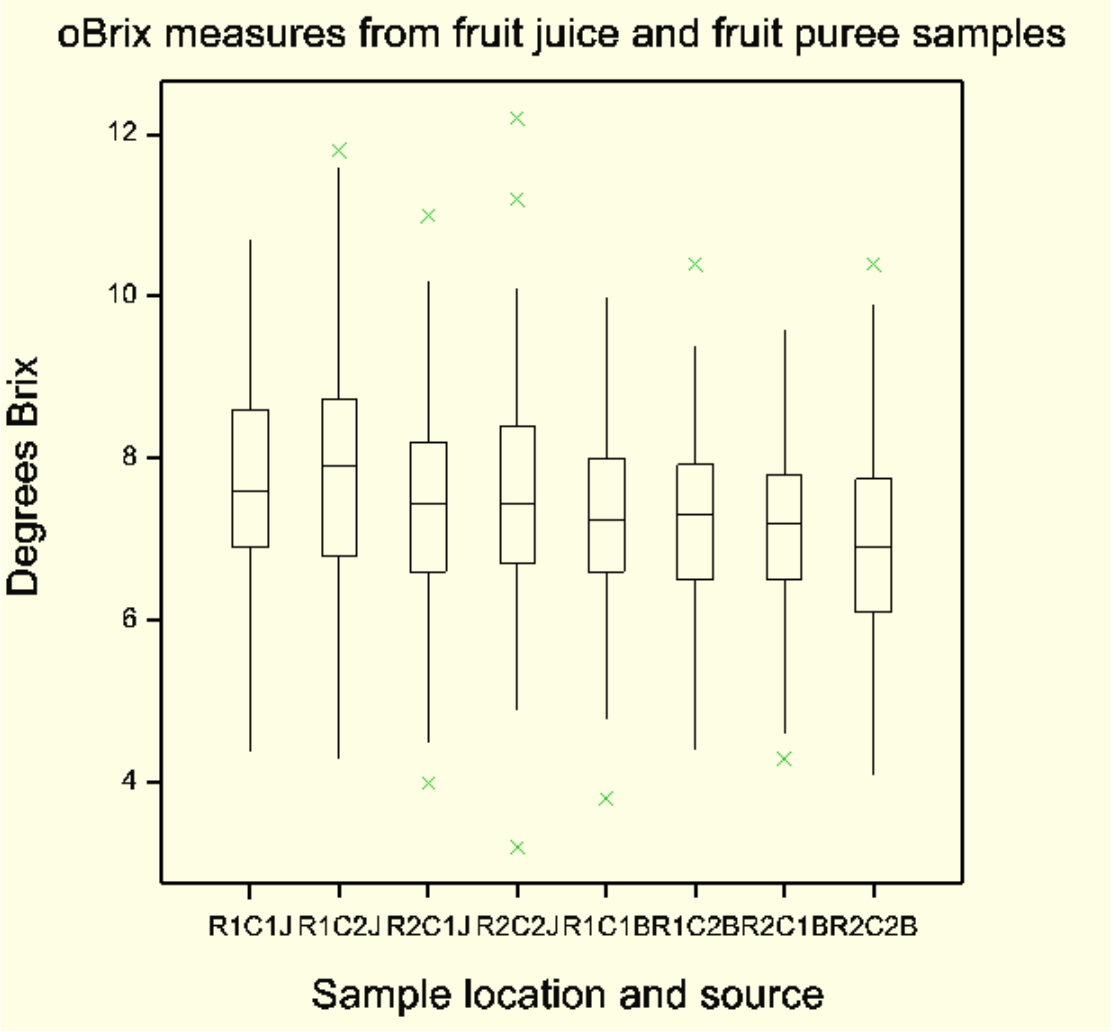


Figure 3.5. ^{o}Brix measurements obtained from fruit juice and fruit puree samples from the field site in 2006. Fruit juice samples are annotated with a 'J' (first four samples), while blended puree samples are identified with a 'B' (last four samples). The plots obtained for each of the clones, regardless of sample source, remained consistent with minimal variation recorded. The four clones analysed for each sample source were R1C1, repetition 1 clone 1, R1C2, repetition 1 clone 2, R2C1, repetition 2 clone 1 and R2C2, repetition 2 clone 2. The box plot can be interpreted as described in Figure 3.3.

3.2.1.5 Ten berry weight

Ten berries were selected at random from labelled polythene bags collected from each individual (Section 2.1.2) giving an indication of fruit size per plant. Statistical analysis of sample weight found significant differences in measurements between individual progeny ($p < 0.001$) with consistent results being recorded between clones and repetitions. It can be seen in the box plot (Figure 3.6) that sample weights across all four plant's from the field site remained consistent with a mean of 20 g per 10 berries selected. The variability of progeny weights ranged from 8.7 g to 37.0 g for 10 individual berries sampled from any one plant.

Following multiple clone analysis carried out in 2006, it was found for all traits analysed, colour meter, ^{o}Brix and ten berry weights that while significant variations were detected between progeny, there were no significant variations between individual clones or repetitions. As a result of these findings only one clone per individual was assessed in subsequent seasons from each environmental location.

Figure 3.6. Ten berry weight measurements recorded from the field site in 2006. The variations in sample weight detected in one season, for the analysed progeny, recorded a consistent pattern across all four clones as shown in the box and whisker plots generated for each of the clones analysed. The box plot can be interpreted in full as described in Figure 3.3. The four clones analysed per individual were from R1C1, repetition 1 clone 1, R1C2, repetition 1 clone 2, R2C1, repetition 2 clone 1 and R2C2, repetition 2 clone 2.

3.2.2 Fruiting season two, July 2007

Full analysis was carried out across three environmental locations based on results obtained from season one. The addition of visual colour analysis was initiated in season two in order to assess any relationship between colour meter analysis and perceived visual colour observation.

Analyses of variance (ANOVA) were performed in 2007 and 2008 with genotype, site and years as fixed effects. For all analyses carried out, significance levels were set at $p < 0.05$ and were performed using Genstat 10 for windows. Due to the inability to replicate seasons, the samples analysed in 2007 and 2008 underwent regression analysis in order to assess the variability expressed by the two independent seasons.

3.2.2.1 Plant material for phenotypic data collection in 2007

Season two analysis consisted of three environmental locations. The open field at SCRI, Dundee (Section 2.1.1.1), as assessed in season one, a protected site at SCRI, Dundee, (Section 2.1.1.2) and a protected site on a commercial farm, Blairgowrie, Perthshire, (Section 2.1.1.3) which can be seen in Figure 3.7. For season two, fruit was picked across the three sites once the majority of fruit reached stage 6, red ripe (Section 2.1.3). This was achieved by visiting each site up to three times a week until all progeny had reached the appropriate stage for selection. The mapping population of 188 individuals were analysed, where available, across each site consisting of one clone and one repetition.

Ripe fruit was collected from the protected site at Blairgowrie from 2nd July to 16th July, from the field site at SCRI from 3rd July to 17th July and from the protected site at SCRI from 11th July to 27th July 2007.

Figure 3.7. Three environmental locations analysed in 2007. Open field SCRI, Dundee (top), polytunnel, SCRI, Dundee (middle), polytunnel, Blairgowrie, Perthshire (bottom).

3.2.2.2 Seasonal Data for 2007

Although 2007 recorded higher average maximum temperatures than the long term averages for most of the year it also recorded a higher annual rainfall. June and July of 2007 recorded almost twice the level of expected rainfall with 104.6 mm and 117.3 mm respectively, while April was especially dry with only 11.4 mm of rainfall which was a quarter of the long term average rainfall for this month as recorded in Table 3.2 (SCRI meteorological records, 2007). The average sunshine hours recorded from April to August 2007 had a mean of 163 hours which was considerably lower than the equivalent months in 2006 (average 206 hours).

Table 3.2. Seasonal analysis showing temperature measurements across season 2.

Monthly weather means and totals for 2007							
Month	Total rain mm	Days rain >0.2mm	Days rain >0.1mm	Total sun hours	Mean soil 20°C	Mean air max °C	Mean air min °C
January	71.0	16	11	56.5	11.8	9.0	2.4
February	91.1	17	14	64.5	12.6	8.8	2.4
March	38.2	11	7	131.1	15.9	10.6	3.0
April	11.4	6	4	194.4	15.7	15.3	5.6
May	58.1	15	8	205.2	15.5	14.3	6.0
June	104.6	12	10	90.3	12.0	16.0	10.0
July	117.3	22	18	153.7	11.1	18.5	10.4
August	76.2	13	8	170.3	5.5	19.0	10.0
September	19.7	9	4	149.2	0.0	17.5	8.3
October	25.6	6	3	117.9	0.0	14.5	6.0
November	89.6	13	12	59.2	0.0	10.6	4.2
December	38.7	13	9	25.8	0.0	6.8	-0.3

3.2.2.3 Colour meter analysis

Summary statistics were calculated for each individual measurement (Yxy) for 15 g fruit samples measured in 2007 as detailed in Section 3.2.1.3. In season 2, the mapping population, where available, was analysed across the three environmental locations in order to assess sample variability.

Colour brightness, denoted by the Y measurement, showed little variation across the three environmental sites ($p = 0.799$) in one season as seen in the box plot in Figure 3.8. The median sample brightness (Y) across all sites was consistent within one season with only slight differences recorded in the minimum (SCRI protected site) and the maximum (SCRI protected site). It can be seen that sample brightness was consistent within any one season regardless of the environmental location.

Figure 3.8. Colour meter analysis of sample brightness (Y) in 2007 across three environmental sites. Field_Y represents SCRI field site, Poly_Y were SCRI protected samples and Comm_Y were the commercial protected samples. The horizontal bar in the middle of the box represents the median of samples, the upper hinge indicates the upper quartile range, and the lower hinge is the lower quartile range while the range of the middle two quartiles is the inter-quartile range. The whiskers protruding from either end of the box signify the minimum and maximum values while the outlying samples, denoted by the green x, are values outwith the 1.5 times maximum range covered by the whiskers. The red x illustrates an isolated individual which recorded an extreme value which was higher than all other samples analysed.

3.2.2.3.1 Visual colour analysis

In order to ascertain the relationship between colour meter analysis and perceived colour, a visual assessment was carried out on individual fruit samples while still on the bush. The fruit was assessed on a 1 to 5 scale as described in Section 2.1.4.3. In order to maintain consistency the fruit was analysed from the same side of the bush at the same time of day to reduce any sun/shade errors. As the fruit were assessed across the ripening season, plants were harvested only when around 80% of the bush had reached the red ripe stage (Section 2.1.3) and providing fruit detached from the receptacle with ease. From this it was assumed that fruit samples which differed in colour intensity were as a result of genotypic variation and not a variation in ripeness. The visual colour results obtained across the three environments were compared in a histograph which can be seen in Figure 3.9.

The samples from the open field show a higher number of individuals being scored as lighter in colour (scores 1 and 2) compared with those found under polytunnel. The protected site at SCRI shows a consistent number of samples being rated as darker in colour (scores 4 and 5). A similar trend was also found for the commercial samples.

Parameters measured by the colour meter were examined for correlations with the visual score made on the fruit. Visual colour analysis is highly negatively correlated ($p < 0.001$) with all three colour meter results with the most significant correlation identified between visual colour and sample brightness/reflection (Y) ($r = -0.638$). The lower the visual score (lighter coloured fruit), the higher the Y measurement. Likewise the darker the fruit the lower the Y measurement. This is consistent with the fact that the deeper red the fruit samples are the less light they transmit and hence record lower Y (and L*) measurements (Cliff *et al*., 2007).

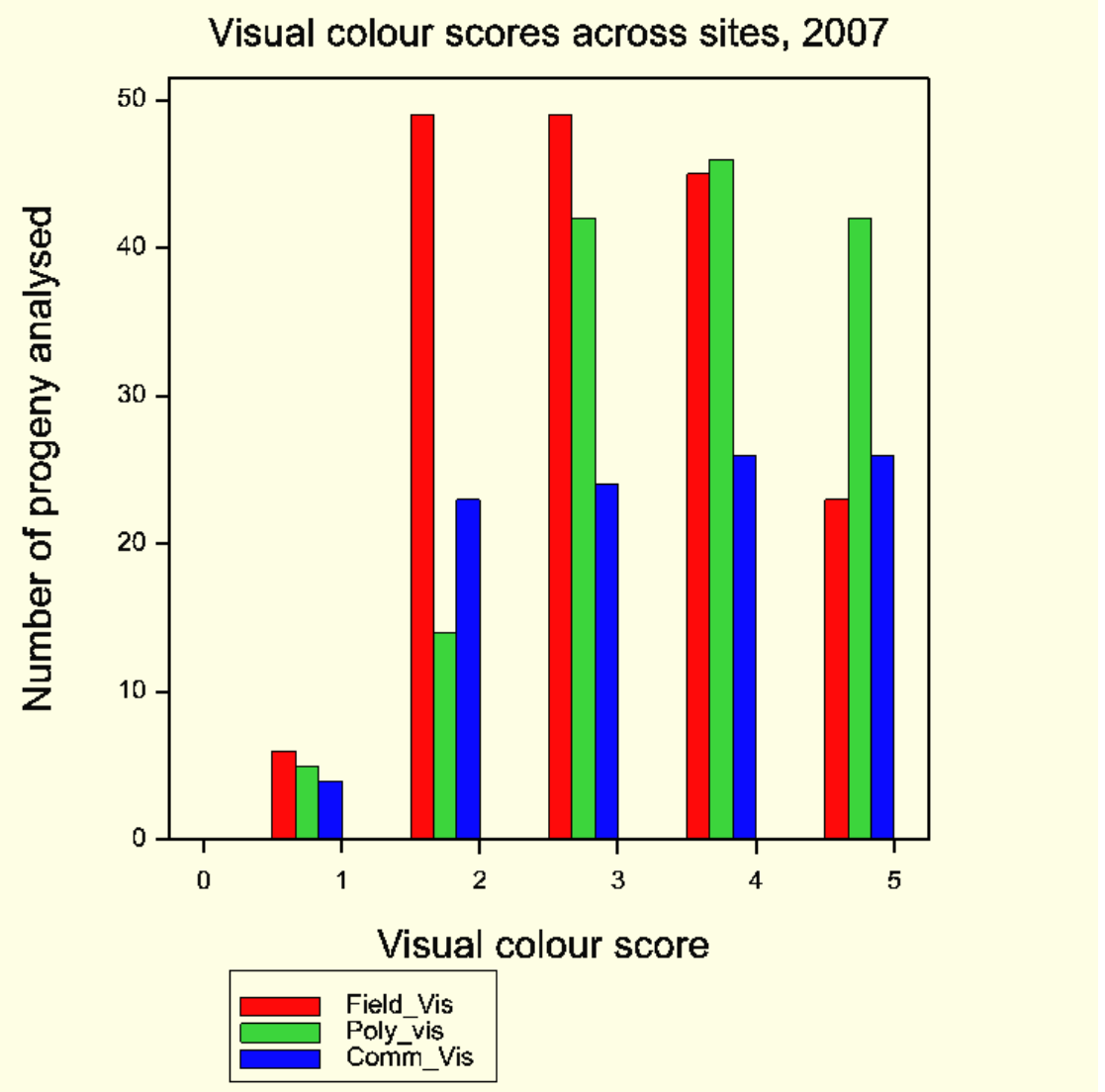


Figure 3.9. Histograph showing the distribution of visual colour analysis for the three environmental locations in 2007. The fruit samples were analysed from plants while still on the bush, from the same side of the plant at the same time of day, where possible. Fruit ripening was assessed daily to ensure fruit was collected when the majority had reached the red ripe stage. Red bars correspond to SCRI field samples (Field_Vis), green bars correspond to SCRI protected samples (Poly_vis) and the blue bars the commercial protected samples (Comm_Vis). 1 = pale pink, 2 = pale pink/red, 3 = mid red, 4 = mid red/dark red and 5 = dark red.

3.2.2.4 Analysis of total soluble solids

The total soluble solids, (^{o}Brix), for the mapping population were analysed for the three environmental locations in season 2, 2007 as described in Section 3.2.1.4. The ^{o}Brix measurements recorded across the three sites showed consistently higher results for all fruit grown under protected cultivation regardless of the polytunnels location (Figure 3.10). ANOVA identified environmental site to be significantly associated with variations in soluble solid content ($p < 0.001$).

Figure 3.10. °Brix measurements for three environmental sites in 2007. Field_Brix represents SCRI field site, Poly_Brix SCRI protected site and Comm_Brix the commercial protected site. The box plot generated for the field samples can be seen to record significantly lower results for the majority of samples analysed while the two protected sites show similar results for most samples as shown in the box plots generated. The box plot can be interpreted in full as described in Figure 3.8.

3.2.2.5 Ten berry weight

The average weight for ten random fruit samples were recorded as detailed in Section 3.2.1.5.

It can be seen from initial Genstat 10 analysis that the fruit grown under protection, especially those grown at the SCRI, were consistently larger than the equivalent grown in the field (Figure 3.11). An example of the size differences can be seen between one of the parents 'Glen Moy' grown in the SCRI field and under protected cover in Figure 3.12. ANOVA recorded significance values of $p < 0.001$ for environmental site and ten berry weight measurements confirming site as a significant factor for fruit weight variation identified within a season.

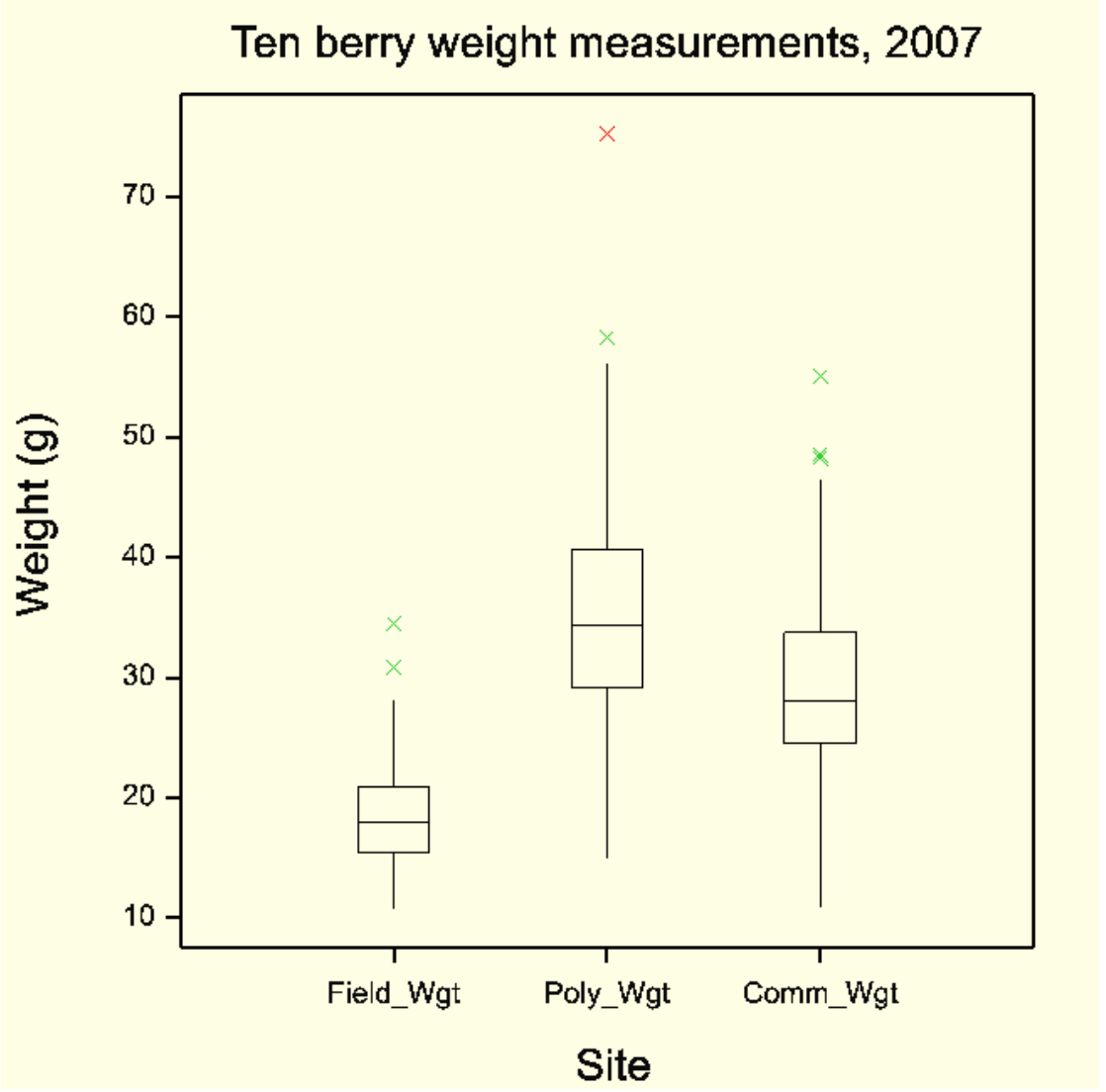


Figure 3.11. Ten berry weight measurements for three environments in 2007. Field_Wgt represents measures for ten berry weights from the SCRI field site, Poly_Wgt SCRI protected site and Comm_Wgt the commercial protected site. The variations recorded for field samples were considerably reduced, as can be seen in the small box generated for Field_Wgt compared with the corresponding samples grown under protection, both of which can be seen to record higher median values than field grown samples. The box plot can be interpreted fully as previously described in Figure 3.8.

Figure 3.12. Glen Moy pictured from SCRI polytunnel site (left) and SCRI field site (right) from 2007. Ten fruit samples were selected from one of the parental cultivars, Glen Moy, from plants grown under protected cultivation (left picture) and from plants grown in the field (right picture) for visual comparison of fruit size obtained between environments.

3.2.3 Fruiting season three, July 2008

Full analysis was carried out across the three environments based on results obtained from seasons one and two. The addition of biochemical analysis of fruit colour was initiated in season three in order to further understand the components controlling this trait.

3.2.3.1 Plant material for phenotypic data collection in 2008

The mapping population used for season one and season two was once again utilised for season three analysis. As described in Section 3.2.2.1, all three environments were visited in 2008 and fruit collected for comparative analysis. Ripe fruit was collected from the protected site at Blairgowrie from 2nd July to 16th July, from the field site at SCRI from 4th July to 22nd July and from the protected site at SCRI from 9th July to 21st July 2008.

3.2.3.2 Seasonal data for 2008

The weather in 2008 was similiar to that of 2007 with the exception of 2008 having slightly more rainfall (781 mm in total compared to 742 mm in 2007). There were however significantly more sunshine hours in June of 2008 (160.1 hours) compared with only 90 hours in June of 2007. The average sun hours from April to August 2008, however, recorded only 145 hours (Table 3.3) compared with 163 in 2007 and 206 in 2006.

Table 3.3. Seasonal analysis showing temperature measurements across season 3.

Monthly weather means and totals for 2008							
Month	Total rain mm	Days rain >0.2mm	Days rain >0.1mm	Total sun hours	Mean soil 20°C	Mean air max °C	Mean air min °C
January	154.3	22	18	40.7	2.3	7.5	1.1
February	26.4	12	7	86.2	2.7	8.9	1.5
March	41.7	12	11	156.7	4.2	9.1	1.3
April	69.1	20	13	156.5	6.8	11.4	3.5
May	18.7	12	6	170.8	10.6	15.7	8.0
June	56.5	17	13	166.1	13.7	18.0	8.7
July	104.5	15	13	130.8	15.7	19.0	12.1
August	141.5	20	18	100.2	15.0	18.5	12.1
September	46.1	14	9	94.5	12.0	16.1	8.9
October	50.0	19	11	120.9	8.6	12.2	4.5
November	24.6	13	9	88.1	5.1	9.4	2.3
December	47.6	14	10	46.0	3.3	6.7	0.6

3.2.3.3 Colour meter analysis

Samples were analysed for colour meter measurements across the three sites as detailed in Section 3.2.1.3. The box plot (Figure 3.13) shows the similarities in sample brightness (Y) obtained for the mapping population across the three environmental locations in 2008. It can be seen that sample brightness and reflective values remain consistent within one season regardless of the environmental location of the fruit analysed with little variation detected between sites ($p = 0.412$).

Figure 3.13. Colour meter analysis of sample brightness (Y) in 2008 across three environmental sites. The horizontal bar in the middle of the inner boxes represent the median of samples, the upper hinge indicates the upper quartile range, and the lower hinge the lower quartile range. The whiskers protruding from either end of the boxes signify the minimum and maximum values while the outlying samples, denoted by the green x, are values outwith the 1.5 times maximum range covered by the whiskers. The red x indicates an isolated individual which recorded an extremely high value for sample brightness (Y).

3.2.3.3.1 Visual colour analysis

Visual colour analysis was carried out on fruit in 2008 as described in Section 3.2.2.3.1. A further visual analysis was also carried out on fruit which had been blended for colour meter analysis. This was assessed using the same 1 to 5 visual scale to ascertain if perceived colour changed once the cell walls have been disrupted.

As was found in 2007, the whole fruit samples from the open field in 2008 showed a consistently higher number of individuals being scored as lighter in colour (scores 1 and 2) compared with those found under polytunnel. For the blended fruit, however, the pattern appears to be more evenly distributed (Figure 3.14).

As with visual analysis on whole fruit, blended fruit analysis found linear negative relationships with colour meter measurements, Yxy which equates to darker visual fruit (rated as a 5) showing consistently reduced colour meter Y values.

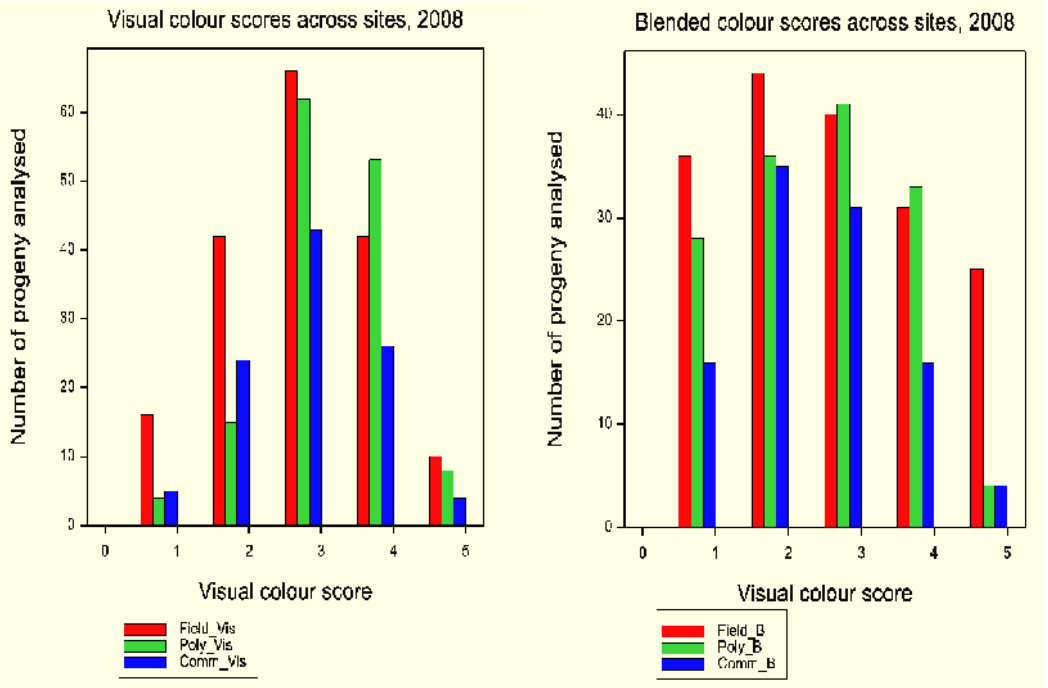


Figure 3.14. Distribution of visual colour analysis for whole fruit and blended samples across the three locations in 2008. The whole fruit samples (left side) were analysed from fruit while still on the bush, from the same side of the plant at the same time of day, where possible. The blended samples (right side) were analysed following mechanical blending of 35 g of fruit collected from the same side of the plant at the same time of day, where possible. Red bars correspond to SCRI field samples (Field_Vis), green bars correspond to SCRI protected samples (Poly_vis) and the blue bars the commercial protected samples (Comm_Vis). 1 = pale pink, 2 = pale pink/red, 3 = mid red, 4 = mid red/dark red and 5 = dark red.

3.2.3.3.2 Biochemical analysis of colour

Initial studies from seasons one and two suggested colour was a particularly important (and complex) factor involved in both perceived quality (visual acceptability) and actual measurable quality parameters (sample brightness). As a result additional compositional analyses were carried out on all samples across all sites in season three.

3.2.3.3.2.1 Total phenol content

The total phenol content of each sample was estimated from a standard curve of gallic acid using the Folin-Ciocalteu method as described in Section 2.1.4.5. The phenol ranges detected across the three environmental sites varied considerably with the two sites at SCRI recording similar values, field 67.3 to 375.0 mg per 100 g fresh weight (fw), and protected SCRI site 62.5 to 314.1 mg per 100 g fw, whereas the protected site in Blairgowrie recorded lower values of between 28.2 to 157.3 mg per 100 g fw (Figure 3.15).

The ANOVA for total phenol content found both site and genotype had the most significant effect ($p < 0.001$) across samples analysed. Site by genotype interactions, however, were not found to be significant ($p = 0.500$) with half the variation attributed equally to both site and genotype.

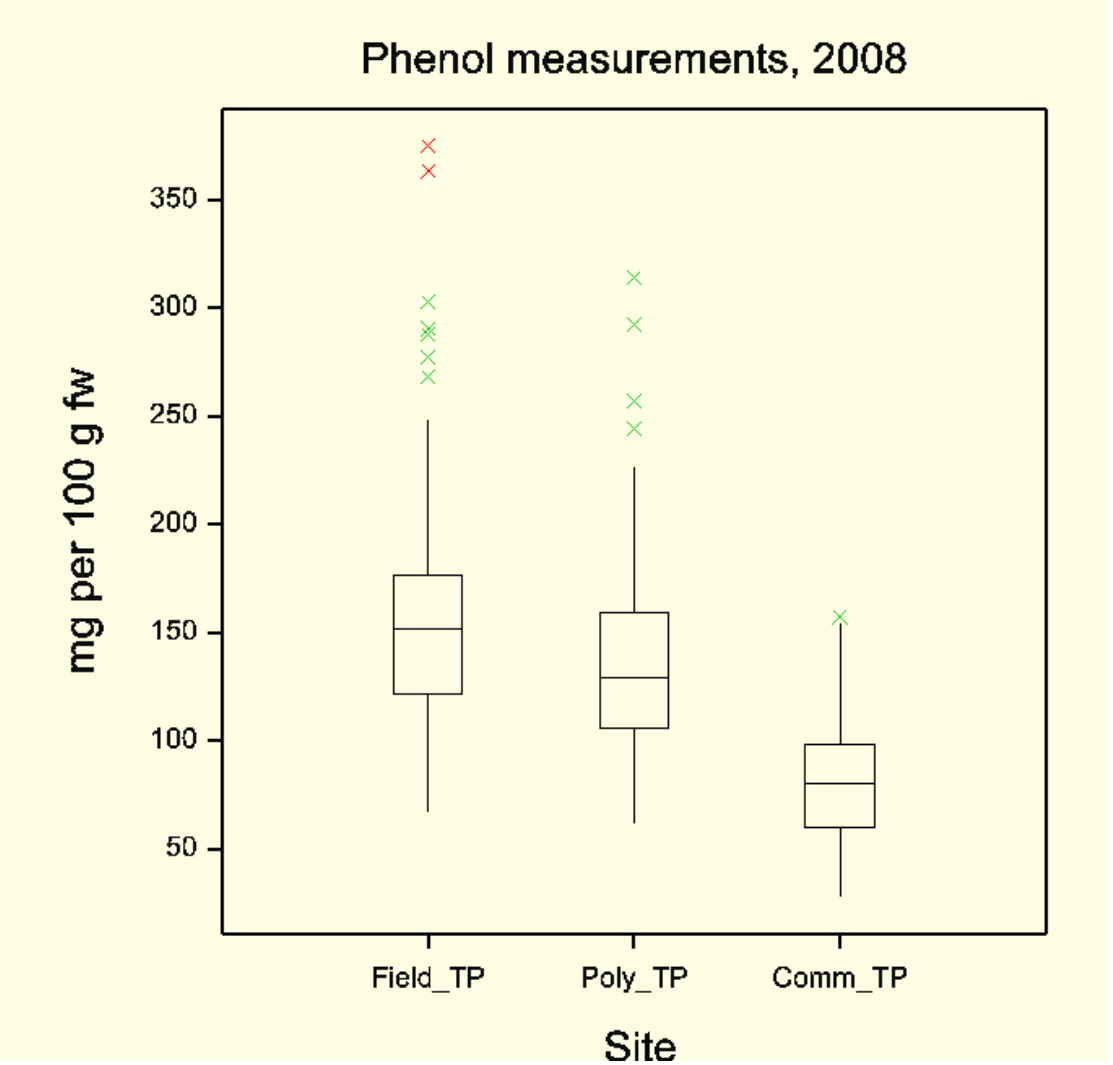


Figure 3.15. Total phenol content recorded across the three sites in one season. TP represents total phenol measurements and were recorded as mg per 100 g free weight of gallic acid equivalents (GAE). Field_TP were samples from SCRI field site, Poly_TP SCRI protected samples and Comm_TP were samples from the commercial protected sites. The samples analysed from the commercial site recorded lower total phenol median content as well as lower variation, which is reflected in the smaller box plot generated for Comm_TP, compared with the equivalent samples analysed from the SCRI. The boxplot can be interpreted as described in Figure 3.13.

A significant negative correlation is present between total phenols and colour meter y in the field (r = -0.407) and in the protected site at SCRI (r = -0.341) but no correlation was seen with the commercial site (r = -0.154). Phenol values and pH measurements were seen to be significant in the protected site at SCRI (r = 0.363) but not in the other sites. A positive correlation was also detected between blended visual score and total phenol, particularly in commercial samples (r = 0.363) and to a lesser extent the SCRI field (r = 0.203) and protected site (r = 0.254). This correlation was not however detected between visual analysis of whole fruit and phenol content (SCRI open field, r = 0.091, protected r = 0.171 and commercial r = 0.071).

3.2.3.3.2.2 Total anthocyanin content

The total anthocyanin content of each sample was measured using the method of Cheng and Breen (1991) as described in Section 2.1.4.6. It can be seen from the box plot (Figure 3.16) that total anthocyanin content varied significantly across the three environmental locations within one season ($p < 0.001$). While both sites at the SCRI recorded consistently high values for the majority of individuals analysed the commercial site showed considerably lower measurements across all individuals. The anthocyanin ranges detected across the three sites were, field site at SCRI 14.6 to 142.1 mg, the protected SCRI site 27.4 to 177.7 mg and the protected Blairgowrie site 24.2 to 120.4 mg (all per 100 g fw). Although 2008 experienced, on average, less sunshine hours per month than 2007, the lower air and soil mean temperatures in 2008 may have favoured anthocyanin synthesis in fruit. The most significant relationships between total anthocyanin content and measureable colour analysis can be seen between colour meter Y and anthocyanins (Figure 3.17) (r = -0.737) and visual colour and anthocyanins (r = 0.630 for whole fruit and r = 0.780 for blended fruit). The darker the fruit is visually correlates significantly with the higher total anthocyanin values found in fruit samples. These correlations were consistent across all three environmental sites.

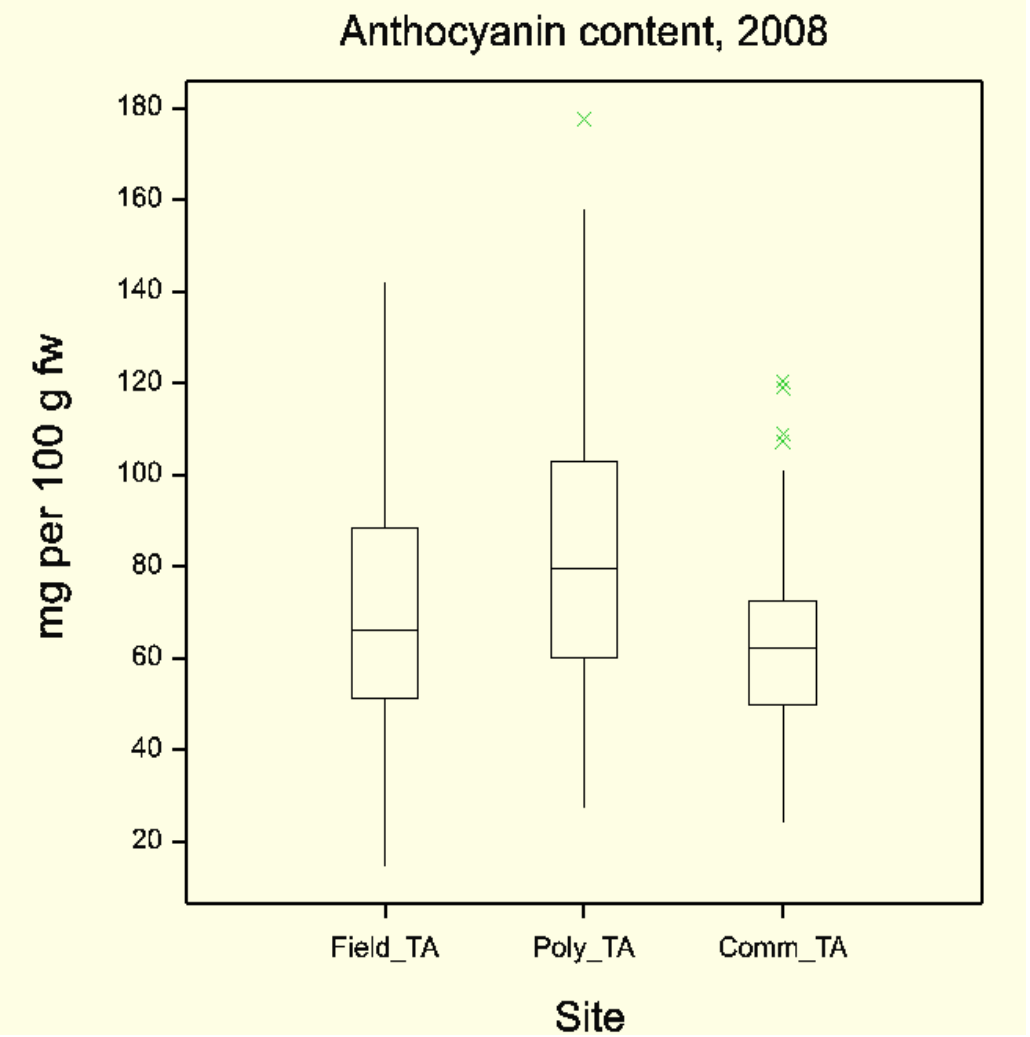


Figure 3.16. Total anthocyanin content for the three environmental sites in 2008. TA represents total anthocyanin measurements and was recorded as mg per 100 g free weight of cyanidin 3-glucoside equivalents. Field_TA were samples from SCRI field site, Poly_TA were SCRI protected samples and Comm_TA were samples from the commercial protected sites. The samples analysed from the commercial site recorded lower total anthocyanin variation compared with the equivalent samples analysed from the SCRI, as shown in the smaller box plot generated for Comm_TA. The boxplot can be interpreted as described in Figure 3.13.

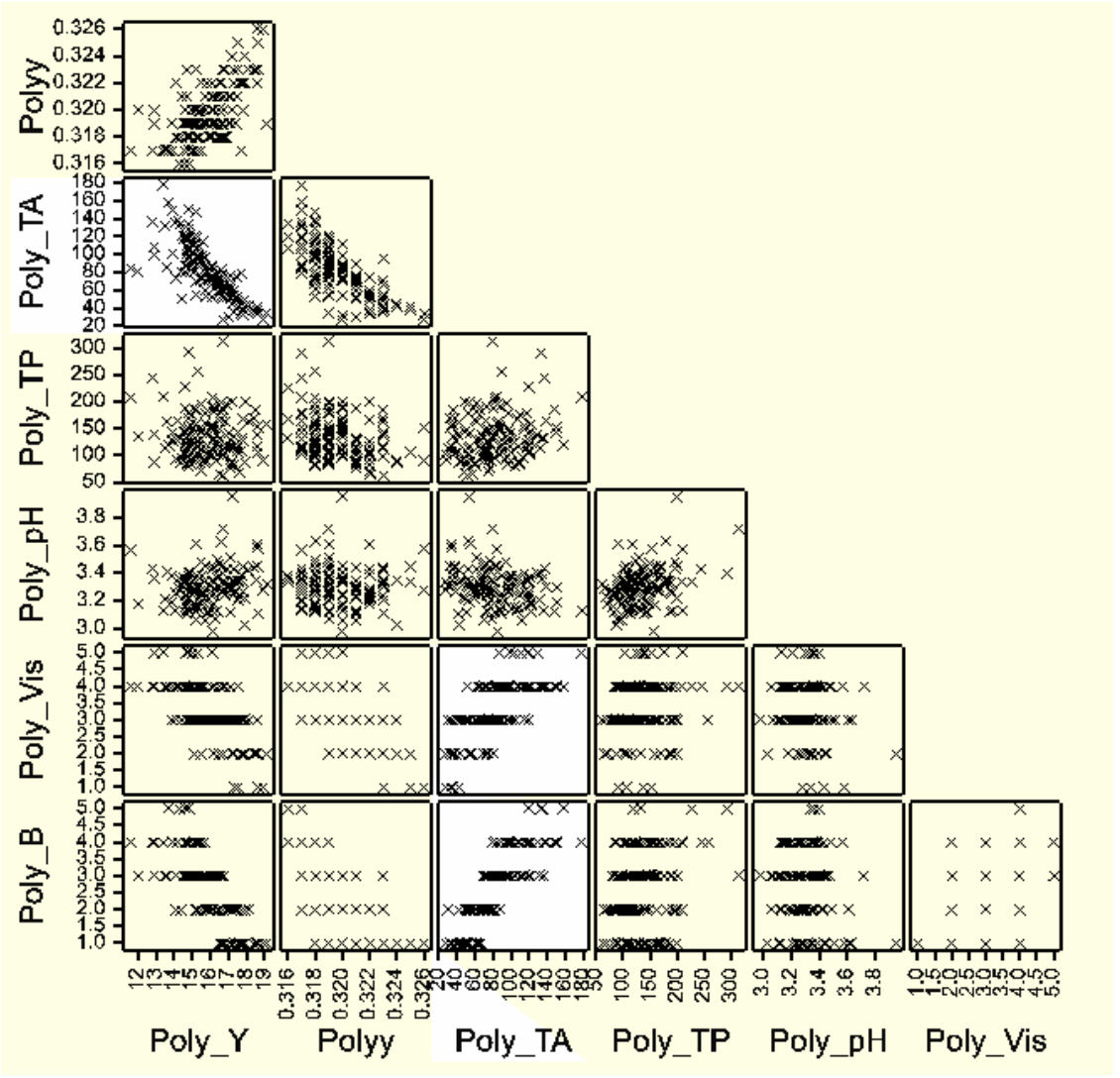


Figure 3.17. Scatterplot matrix of total anthocyanin correlations. This matrix shows the associations detected between measures for colour meter brightness (Y) and chroma (y), total anthocyanins (TA), total phenolics (TP), sample pH (pH), visual colour of whole fruit (Vis) and blended fruit (B). Significant correlations are highlighted in white boxes between total anthocyanin content (TA) and colour measurements (Y) as shown in the top left white box and between TA and visual colour for both blended fruit (Poly_B) and whole fruit (Poly_Vis) as highlighted in the bottom white boxes.

The ANOVA for total anthocyanin content found both site and genotype had the most significant effect ($p < 0.001$). Site by genotype interactions were not found to be significant ($p = 0.500$) which was also found for total phenol content.

3.2.3.3.2.3 Hydrogen ion concentration

The pH was measured on fruit juice samples across all three sites in 2008 as described in Section 2.1.4.7. The pH ranges detected across the three sites were from 2.98 to 3.95 both extremes detected in SCRI polytunnel samples (Figure 3.18). The pH of fruit samples were found to be significantly correlated across all three sites for ^{o}Brix measurements only. This showed a negative correlation (the higher the pH, the lower the ^{o}Brix) of r = -0.422, -0.356 and -0.289 for the SCRI field, SCRI protected and commercial sites respectively. For the field site only, a negative correlation was identified between pH measurement and ten berry weight (r = -0.347) and pH and colour meter ΔE (r = -0.309). For the SCRI protected site, a correlation was present between pH and phenols (r = 0.363) and within the commercial fruit pH was negatively correlated with colour meter x (r = -0.398).

The ANOVA for sample pH found site had the most significant effect ($p < 0.001$) while genotype was not as significant with a p value of 0.008.

Figure 3.18. Variation of pH measurements recorded across the three sites in one season. Field_pH were samples from SCRI field site, Poly_pH SCRI protected samples and Comm_pH were samples from the commercial protected sites. Variations in the box plots generated can be seen between sites with lower average values recorded for samples grown from the Commercial site. The box plot can be interpreted as described in Figure 3.13.

3.2.3.4 Analysis of total soluble solids

The total soluble solids (^{o}Brix) were recorded for the three environmental locations as detailed in Section 3.2.1.4. The box plot (Figure 3.19) highlights the variation found between sites for total soluble solid content of individuals. Both protected sites were found to measure consistently higher values than the field site for soluble solid content within one season with ANOVA recording significant associations between environment ($p < 0.001$).

Figure 3.19. Variation of °Brix measurements recorded across the three sites in one season. FieldBrx were samples from SCRI field site, PolyBrix SCRI protected samples and CommBrx samples from the commercial protected sites. The variations in °Brix detected across the sites were found to be similar although median sample values were found to be higher for samples grown under protection. The box plot can be interpreted as described in Figure 3.13.

3.2.3.5 Ten berry weight

The average weight of fruit samples were recorded in 2008 as described in Section 3.2.1.5. The box plot (Figure 3.20) highlights the variation found between sites for ten berry weight measurements with both protected sites measuring consistently higher weights than the field site within one season. ANOVA recorded significance values of $p < 0.001$ for the effect of environmental site on ten berry weight measurements.

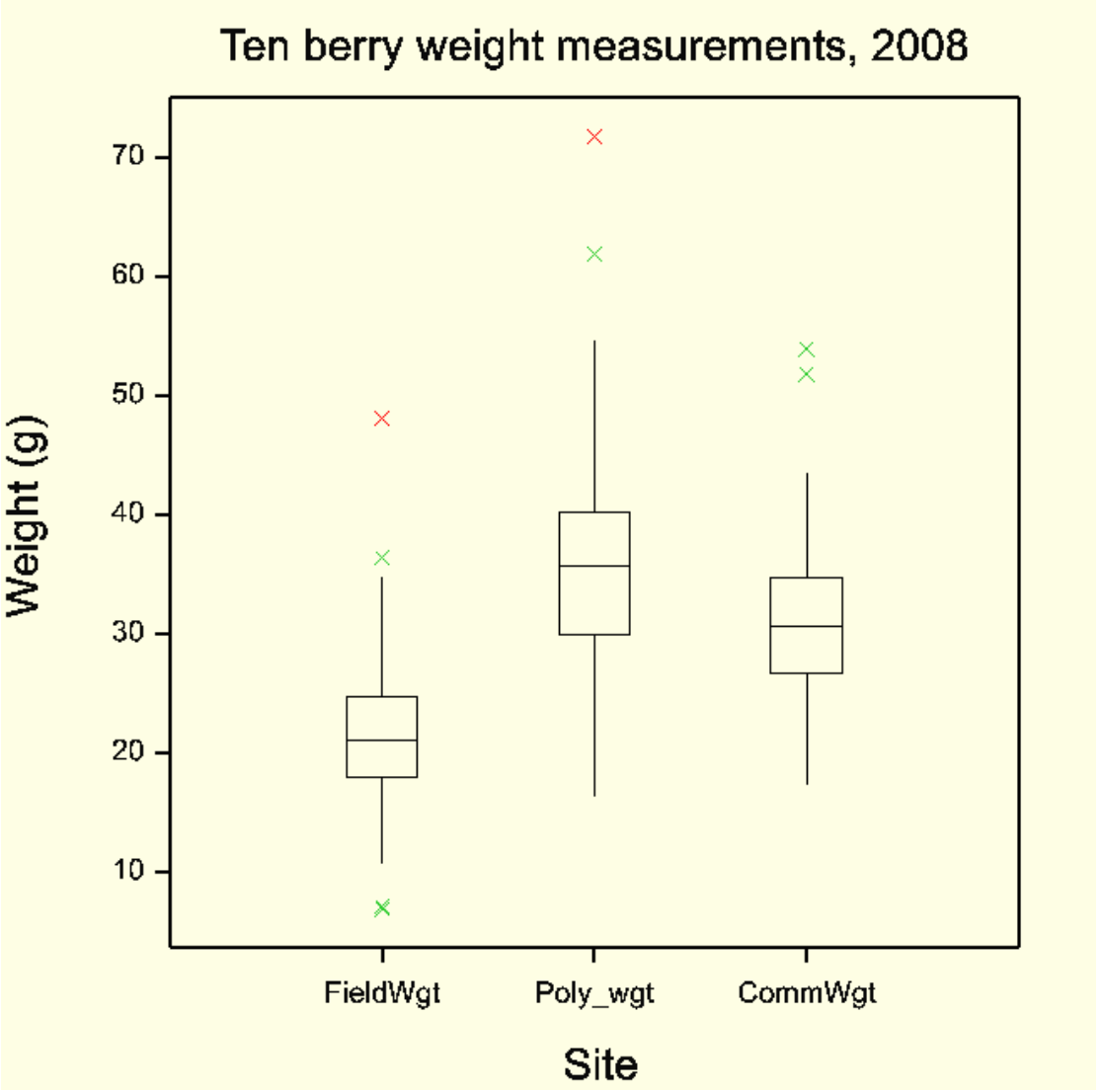


Figure 3.20. Variation of ten berry weights across the three sites in one season. FieldWgt were samples from SCRI field site, Poly_wgt SCRI protected samples and CommWgt were samples from the commercial protected sites. The box plot generated from field samples shows a lower median value and lower overall variation compared with samples grown under protected cultivation. The box plot can be interpreted as described in Figure 3.13.

3.2.4 Correlations across three seasons

3.2.4.1 Seasonal variation

The three seasons analysed in this study varied considerably (Table 3.4). It can be seen from these results that a typical Scottish growing season can be highly variable and often unpredictable for growing soft fruit, particularly in an open field environment.

The first season, 2006, averaged a high number of sunshine hours (208) in the months leading to fruit harvest in July and with this came a high average maximum air temperature of 17.3^{o}C. 2006 also experienced the lowest average minimum air temperature of 2.7^{o}C in April, as well as a monthly low mean of 7.5^{o}C from April through to July and only 11 days average rain fall per month of 2 mm and above.

Season two, 2007, averaged slightly less sunshine hours (161) than the previous season prior to fruit harvest while experiencing a mean minimum air temperature of 8.0^{o}C, which was similar to that in 2006. The mean soil temperature, at 20 cm, however, was found to be higher at 13.6^{o}C than 2006 which was 12.9^{o}C.

Season three, 2008, experienced the lowest sunshine hours of the three seasons (156) recorded between April and July. Both minimum and maximum air temperature were similar across all seasons but 2008 averaged a lower mean soil temperature at 11.9^{o}C and a higher average rainfall of 16 days above 2 mm per month.

Table 3.4. Seasonal analysis showing temperature variations across three seasons.

	Year/Month	April	May	June	July	Average
Mean air maximum temperature (^{o}C)	2006	12.4	14.7	19.4	22.5	**17.3**
	2007	15.3	14.3	16.0	18.5	**16.0**
	2008	11.4	15.7	18.0	19.0	**16.0**
Mean air minimum temperature (^{o}C)	2006	2.7	5.5	9.6	12.3	**7.5**
	2007	5.6	6.0	10.0	10.4	**8.0**
	2008	3.5	8.0	8.7	12.1	**8.1**
Mean soil temperature at 20cm (^{o}C)	2006	6.9	10.6	15.6	18.4	**12.9**
	2007	15.7	15.5	12.0	11.1	**13.6**
	2008	6.8	10.6	13.7	15.7	**11.7**
Sunshine (hours)	2006	213	215	187	217	**208**
	2007	194	205	90	154	**161**
	2008	157	171	166	131	**156**
Rainfall above 0.2 mm (days)	2006	8	16	9	9	**11**
	2007	6	15	12	22	**14**
	2008	20	12	17	15	**16**

3.2.4.2 Colour meter variations

Colour measurements were collected over three years and across all sites using a colour meter (Section 2.1.4.2) and visual assessment (Section 2.1.4.3). Analyses of variance showed fruit colour across all three sites varied significantly from year to year but remained generally consistent across the three sites in one season (Table 3.5). The variation detected in colour between progeny were attributed primarily to genotypic and year effects ($p < 0.001$).

Visual colour scores were also found to be significantly correlated with readings of the colour meter ($p < 0.001$), both of which were based on berry brightness.

Table 3.5. Analysis of variance for colour meter Y.

Source	**d.f**	**s.s**	**m.s**	**Probability**
Year	1	533.17	533.17	< 0.001
Site	2	2.52	1.26	0.305
Genotype	183	873.84	4.78	< 0.001
Year:Site	2	1.32	0.66	0.535
Year:Genotype	178	175.72	0.99	0.672
Site:Genotype	250	253.59	1.01	0.672
Residual	224	235.75	1.052	

Although variations between the three seasons were evident, within one season the Y measurement remained consistent (Figure 3.21). The Y value was significantly higher for 2006 than 2007 but across sites in 2007 no significant differences exist. In 2008, however, all three sites showed a higher average Y measurement than 2006 and 2007 but again this rise was consistent across the three sites. In each year analysed, the same individual (r202) was found to have the highest value for colour brightness (Y). A similar pattern was observed across the three years for samples recording the lowest sample brightness (r270).

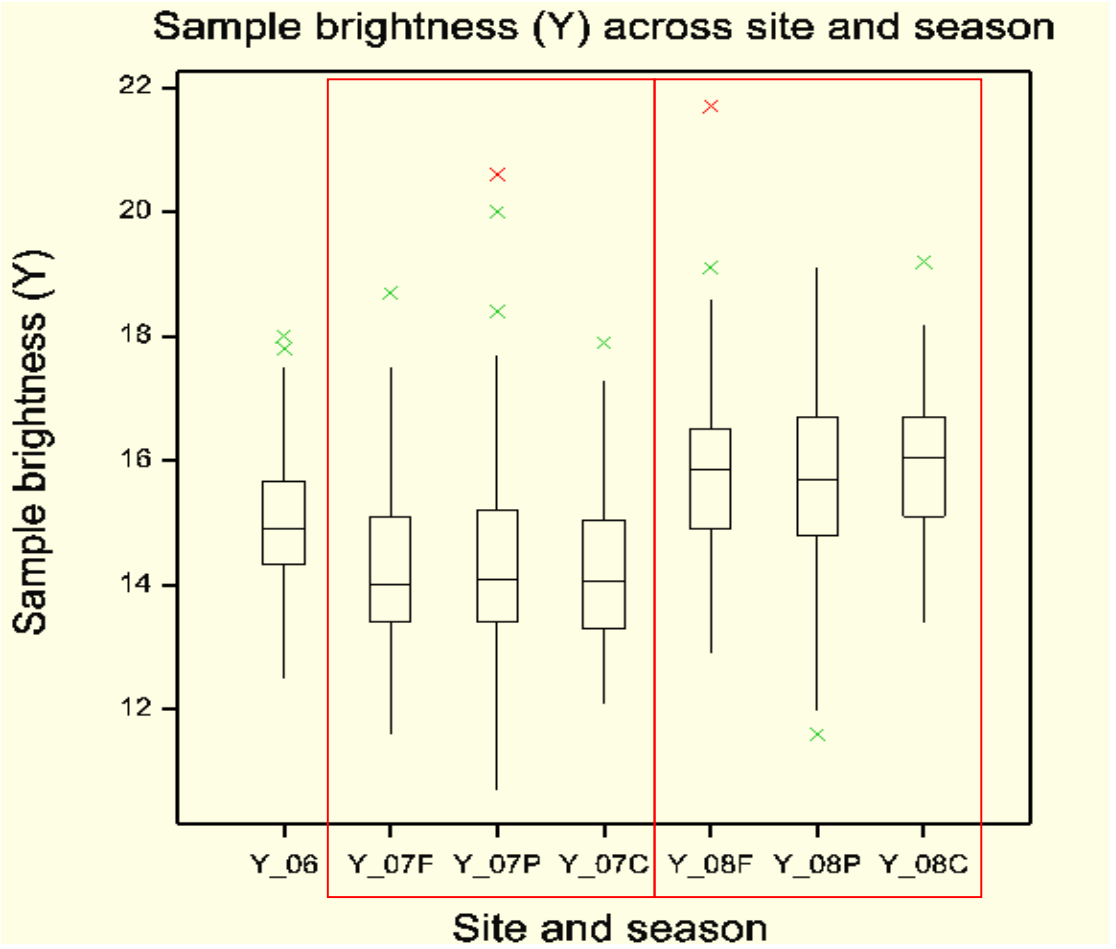


Figure 3.21. Sample brightness (Y values) recorded across the three seasons and sites. The figure has been segregated to allow a visual comparison to be made between the three seasons. Y_06 were the SCRI field samples in 2006, Y_07F were field samples from 2007, Y_07P were SCRI protected samples, 2007, Y_07C were commercial protected samples, 2007, Y_08F were field samples, 2008, Y_08P were SCRI protected samples, 2008, and Y_08C were commercial protected samples, 2008. The horizontal bar in the middle of the box is the sample median, the upper hinge indicates the upper quartile range, and the lower hinge is the lower quartile range. The whiskers protruding from either end of the box signify the minimum and maximum values while the outlying samples, denoted by the green x, are values outwith the 1.5 times maximum range covered by the whiskers, the red x are samples with extreme values.

3.2.4.3 Biochemical correlations

For anthocyanin measurements, there were several individual samples which were identified as containing the highest total anthocyanin (TA) values for one environmental location. Some individuals were subsequently found to have the highest contents in at least one other location, if not all three. The same pattern emerged for samples found to contain the lowest TA values. Individuals which were identified as having the highest TA content were recorded as having the lowest brightness (Y). This was to be expected however, as linear negative correlations had previously been identified between total anthocyanin content and sample brightness, where fruit identified as containing the higher anthocyanin content were found to be the darker in colour (Section 3.2.3.3.2.2).

Total anthocyanin content shows signs of environmental influence with the two locations at the SCRI, field and protected sites, showing comparable levels of anthocyanin values and distribution. The protected commercial site, however, shows significantly lower values for almost all individuals. This was not expected as all other correlations with anthocyanin and visual colour/colour meter analysis were consistent across the three sites, with higher anthocyanin individuals scoring lower for sample brightness, and although this was reflected in the commercial samples, the Y values recorded were comparable across sites (SCRI field 15.8, protected 15.8, commercial 16.0) but the respective anthocyanin mean values were considerably lower SCRI field 71.1, protected 82.4 and commercial 58.0 mg (all mg/100 g fw). Total anthocyanin content has been found to correlate significantly with total phenolics in small fruit (Moyer *et al*., 2002) although antioxidant capacity has been found to correlate more accurately with total phenolics than total anthocyanins. Moyer *et al*. (2002) also found fruit size correlated significantly with anthocyanin content.

During this study, however, a correlation was found in the SCRI open field and the SCRI polytunnel between phenol content and anthocyanin content and no correlation was detected in any of the sites between fruit weight and anthocyanin content.

A positive correlation has also been reported between anthocyanins and sugar content in fruit (Kliewer, 1977; Crisosto *et al*., 2002). It has been postulated that this correlation is in response to light exposure with anthocyanin accumulation found to commence at the onset of sugar accumulation in grapes (Boss *et al*., 1996). This study however considers sugar content only as a factor of total soluble solid content and as such a correlation was detected between ^{o}Brix and anthocyanin content within the commercial site only. pH, however, has been found to correlate consistently with total soluble solids (^{o}Brix), for all three sites, with high pH measurements recording lower ^{o}Brix readings in corresponding samples. Additional correlations were also identified between pH and individual colour meter measurements in individual locations as described in Section 3.2.3.3.2.3.

3.2.4.4 Total soluble solids

The differences in total soluble solids between progeny is highlighted in the box plot (Figure 3.22), where samples can be seen to range from a low 3.5^{o} up to almost 12^{o} (both extremes recorded from the SCRI protected site). The differences between sites were also quite noticeable with the field site recording a significant decrease in ^{o}Brix for the 2007 season compared with 2006. This did improve slightly in 2008 which noted improvements in mean results for all three sites. This may be due to the increased rainfall experienced in 2007 which may have hampered sugar uptake, storage and metabolism. The protected sites however were not so adversely affected by the weather and had a larger ^{o}Brix mean value comparable to field season 2006 which was a relatively dry, warm and bright summer. ANOVA found year, site and genotype were all significant for the variations detected in ^{o}Brix samples ($p < 0.001$). The ^{o}Brix for almost all individuals were found to increase consistently across both seasons when fruit were grown under protected cultivation.

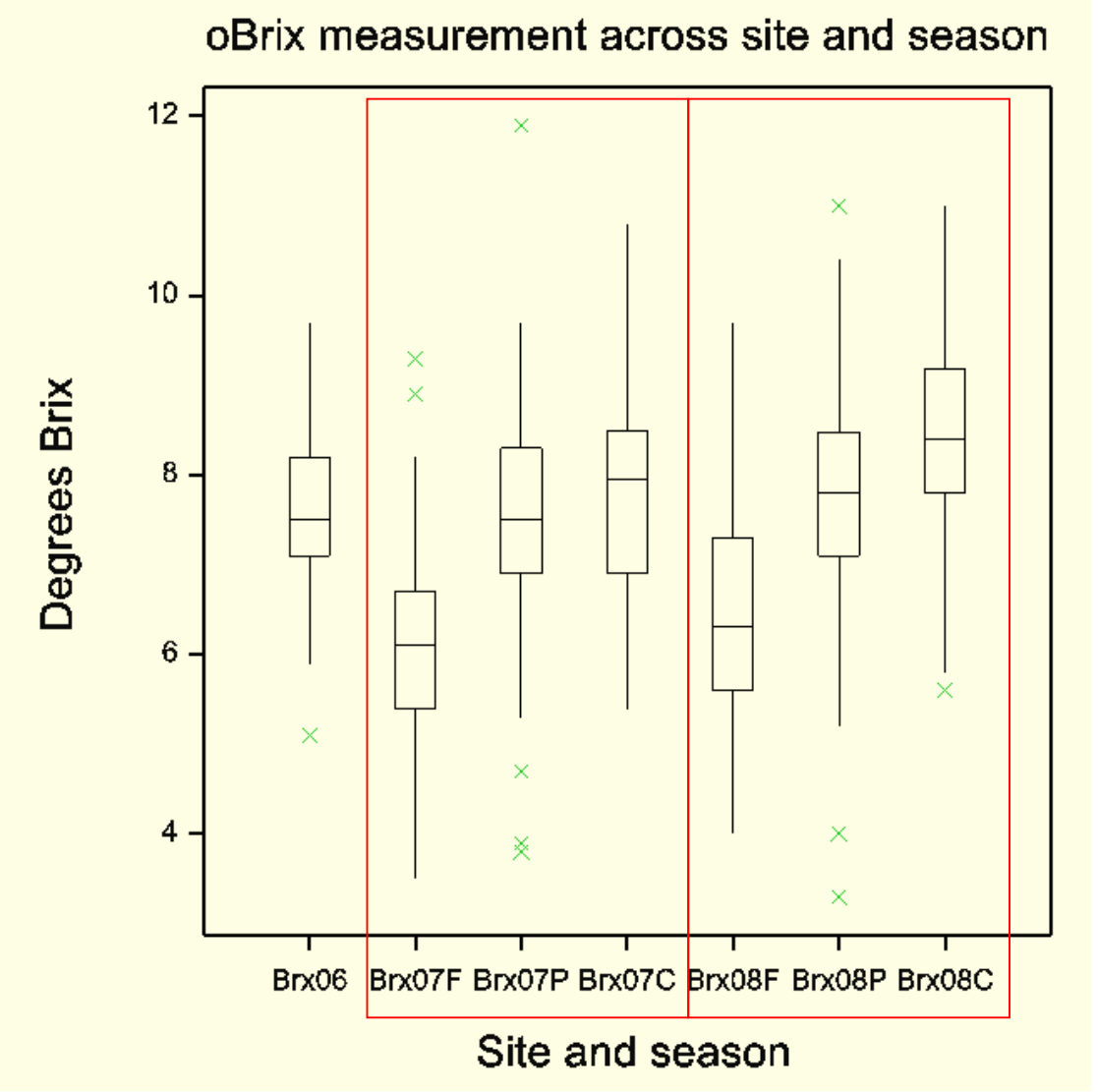


Figure 3.22. ^{o}Brix measurements obtained across sites and seasons. ^{o}Brix for the mapping population for season 2006 field (Brx06), season 2007, SCRI field (Brx07F), SCRI Polytunnel (Brx07P) and Commercial protected site (Brx07C) and season 2008, SCRI field (Brx08F), SCRI Polytunnel (Brx08P) and Commercial site (Brx08C). The figure has been segregated to allow a visual comparison to be made between the three seasons. The boxplot can be interpreted as described in Figure 3.21.

Semi-trained sensory panels recruited for a study carried out at Strathclyde University assessed raspberry fruit in terms of overall sweetness, sourness and flavour intensity. Additional compositional analysis was also carried out to assess sugar and acid content within progeny to establish correlations which may exist between sensory scoring of traits and mean biochemical values (D. Zait, personal communication). On analysing sensory results, in conjunction with phenotypic measurements obtained from this study, linear correlations were identified, from the samples analysed, between fruit sweetness and ^{o}Brix (field r = 0.491, SCRI protected 0.560 and commercial 0.232), fruit sourness and ten berry weight (open field r = 0.277, protected 0.227 and commercial 0.164) and flavour intensity and ^{o}Brix (field r = 0.311, protected 0.549 and commercial 0.265). While correlations were found between the three sites it can be seen that this correlation is slightly lower for fruit grown under protection on the commercial farm.

Analysis of total sugar content in fruit samples showed correlations within the field samples between ^{o}Brix (r = -0.491) and ten berry weight (r = -0.734) with individual mean fructose and glucose contents found to correlate in the same manner (data not shown). Correlations were not however, detected between citric, malic or total acid values and any of the phenotypic measurements analysed in this study (data not shown).

Negative correlations have also been found between scoring of flavour descriptors, such as sweetness and sourness, and fruit weight. It has been suggested by Causse *et al*. (2002) that this may be the result of a dilution effect whereby small fruit have a higher content of sugars and acids compared to a larger fruit which contain comparative soluble solid content.

3.2.4.5 Ten berry weight

Comparative analysis was carried out for the ten berry weight measurements found for all three seasons with significant differences in overall weight being noted for almost all progeny grown under protected cultivation. Weight varied from site to site and also from year to year. ANOVA found year, site and genotype were significant for the variations

detected in ten berry weight ($p < 0.001$) while site by genotype interactions were also found to be significant ($p < 0.001$) for sample weight. Year by genotype interactions were not significant ($p = 0.011$).

Many individual samples under protection showed an increase in size of up to three and four times compared with those in the field, while results between seasons for the field showed little change in the three years analysed. It can be seen clearly from the box plot (Figure 3.23) that the ten berry weights for all individuals across seasons were consistently higher when fruit were grown under protected cultivation.

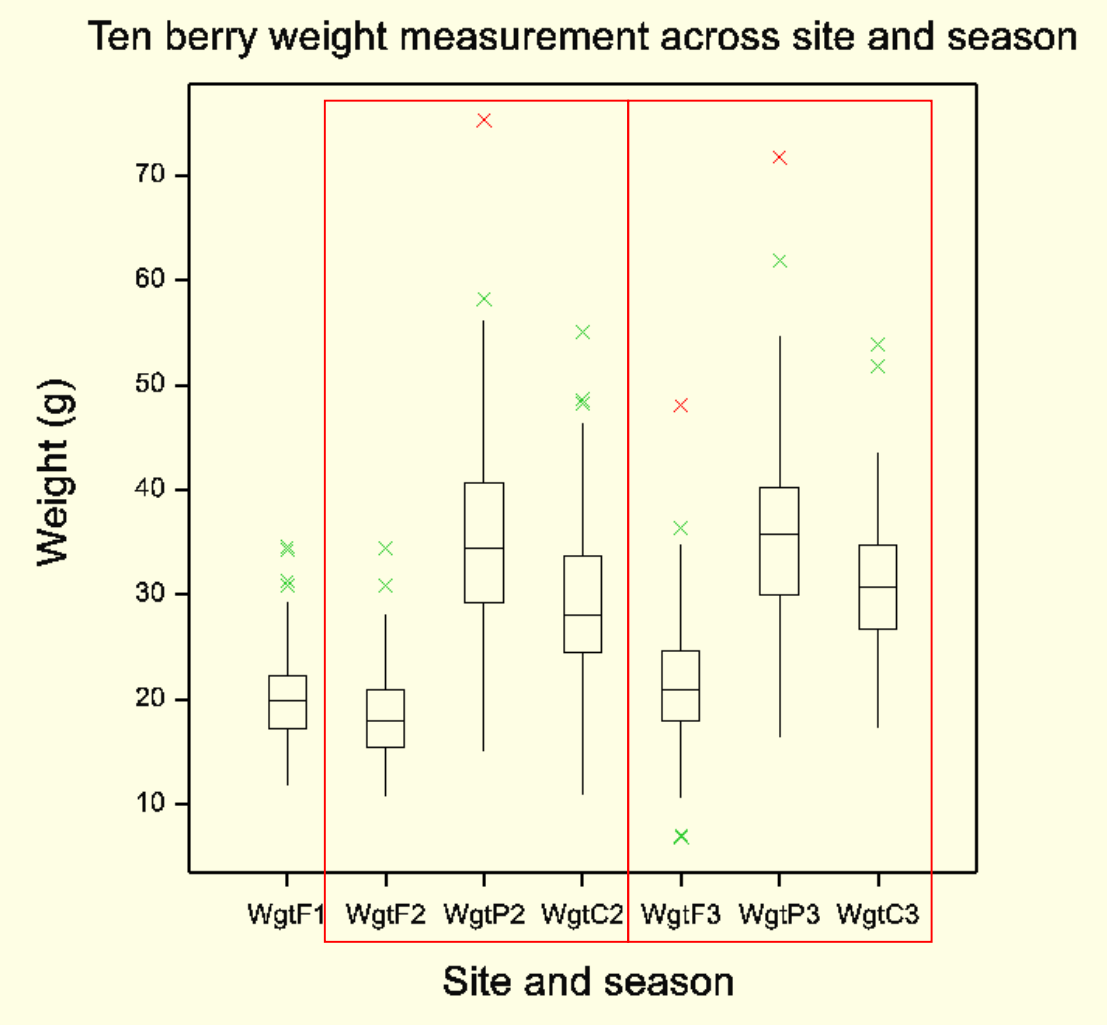


Figure 3.23. Ten berry weights for the mapping population across sites and season. WgtF1 were the SCRI field samples in 2006, WgtF2 were field samples in 2007, WgtP2 were SCRI protected samples, 2007, WgtC2 were commercial protected samples, 2007, WgtF3 were field samples, 2008, WgtP3 were SCRI protected samples, 2008 and WgtC3 were commercial protected samples, 2008. The figure has been segregated to allow a visual comparison to be made between the three seasons. The box plot can be interpreted as described in Figure 3.22.

3.3 Discussion

There have been several key findings identified as a result of the phenotypic analysis carried out across sites and seasons for all traits of interest, including fruit colour, composition and weight. There were no significant differences for any of the traits analysed in one season between the different repetitions or clones analysed within one site. Similarly for ^{o}Brix analysis there were no significant differences found regarding the type of sample analysed, squeezed juice or blended puree ($p = 0.003$). These findings were important in order to allow a larger number of samples to be analysed in subsequent years when three sites were selected.

While the three seasons analysed in this study differed regarding total sunshine hours, temperature and rainfall experienced, only colour and ^{o}Brix were found to be significantly affected. Site, however, was found to contribute significantly to trait measurements with ^{o}Brix and ten berry weights found to increase consistently when fruit were grown under protected tunnels. Biochemical measurements were also found to be affected by site with total phenols, total anthocyanins and pH all found to decrease slightly in fruit which were grown under the commercial tunnel unlike SCRI field and protected fruit which showed similar higher average measurements.

3.3.1 Biochemical influence on fruit quality

Phenol compounds found in fruit have been highlighted as important factors concerning both inner and outer quality attributes. Inner quality relates to beneficial effects on human health following flavonoid intake while outer quality relates specifically to attractive colouration of fruit derived from phenolic compounds (Treutter, 2001). Total phenol content has previously been reported to vary significantly according to genotype (Anttonen and Karjalainen, 2005) with late harvesting cultivars containing higher phenol content than early fruit (de Ancos *et al*., 1999). This study found total phenol content, on average, to be similar to total anthocyanin content, whereas it would be expected to find phenol values to be proportionally higher than anthocyanins as total phenolics encompass all flavonoid (anthocyanidins and flavonols) and non-flavonoid (lignans and

stilbene) compounds. In strawberry, for example, 44% of phenolic compounds were anthocyanins (Puupponen- Pimiä *et al*., 2005) whereas Moyer *et al*. (2002) estimated 34% of total phenolics in blueberry, blackcurrant and blackberry were anthocyanins. It has been reported however, that some phenolic compounds remain undetected following some extraction procedures, such as the Folin-Ciocalteu method. Such undetected phenolics are likely to consist of hydroxycinnamic acids (involved in cell wall stiffening) and proanthocyanidins (Treutter, 2001). Kähkönen *et al*. (2001) reported significant variations in total phenolics extracted from berries, including red raspberries, depending on the extraction method used.

The colour of raspberry fruit has been described as the most important characteristic for the preservation of a fresh appearance and this is often related to individual anthocyanin composition (de Ancos *et al*., 1999). Hours of sunshine, soil composition and humidity are all factors which were described by de Pascual-Teresa and Sanchez-Ballesta (2008) as affecting the anthocyanin composition of fruit. Other factors including fruit variety, maturity and environment were also attributed to anthocyanin composition of fruit (de Ancos *et al*., 1999). Recently Kassim *et al*. (2009) identified and quantified eight individual anthocyanin pigments (cyanidin-3-sophoroside (C3S), cyanidin-3-(2-glucosylrutinoside) (C3GR), cyanidin-3-glucoside (C3G), cyanidin-3-rutinoside (C3R), pelargonidin-3-sophoroside (P3S), pelargonidin-3-glucoside (P3G), pelargonidin-3-glucosylrutinoside (P3GR) and pelargonidin-3-rutinoside (P3R) across two seasons and two environments in progeny derived from ‘Glen Moy’ × ‘Latham’.

Kassim *et al*. (2009) found significant seasonal variation in individual anthocyanin pigments across experimental years ($p = 0.001$), but the variation was not as significant between environments in one season ($p = 0.07$). Highly significant variations within individual anthocyanins were identified between progeny and between 2006 and 2007 field measurements ($p < 0.001$), which were attributed to the hotter, drier, sunnier conditions of 2006. There were however, only slight differences recorded in 2007 between the field and polytunnels samples at SCRI with slightly lower levels recorded

for C3S, C3GR, C3G, C3R, P3R and P3S when grown under protection (Kassim *et al*., 2009).

While total anthocyanins were found to have comparable values between the SCRI field site and the SCRI protected site, in both 2007 and 2008, the commercial protected fruit were found to contain significantly lower anthocyanins (Figure 3.16). It is unlikely that this is due to the polytunnels covering as in terms of size, thickness and UV transmission, both polythene tunnels were comparable (Section 2.1.1). Differences do exist however regarding the polytunnel locations as well as when fruit is covered in the season, with the commercial site, on average a week or two earlier than the SCRI and also irrigation and fertigation was varied between sites. Temperatures measured in Dundee, by the SCRI meteorological department, give an indication as to the external conditions experienced by fruit in the field, but the actual temperature and humidity experienced by the fruit under polytunnels is unknown. The actual soil composition across all three locations is also unknown with differences in several components such as potassium and nitrogen likely to cause varying degrees of stress on the plants, any of which may affect anthocyanin biosynthesis.

While the study by Kassim *et al*. (2009) did not seek any correlation between individual anthocyanin measurements and colour, associations were made between individual pigments and transcriptional regulation. de Ancos *et al*. (1999) found individual anthocyanin pigments, in a selection of raspberry cultivars, did not correlate with objective colour analysis although cultivars with the highest total anthocyanin content, 'Ceva' and 'Rubi' were found to record the lowest L* values, lowest pH and highest ^{o}Brix. Giovanelli and Buratti (2009), however, found darker skinned wild blueberries (as denoted by the lower L* values) contained higher levels of total anthocyanins, a similar trend was reported in cranberries and grapes (Ozgen *et al*., 2002; Cliff *et al*., 2006). Likewise, in this thesis, raspberry colour and total anthocyanins have been found to correlate significantly. Colour is often used to judge quality in berry fruits and visual colour analysis, which is simple and non-destructive, can be used for identifying

samples which are likely to contain a higher total anthocyanin content (Gonçalves *et al*., 2007).

Colorimeters, such as the Minolta, chroma meter used in this study, measure light in terms of a tristimulus colour space which relates to human vision and are thus restricted to the visible light region (Abbott, 1999). Pigments such as chlorophyll, carotenoids and anthocyanins absorb light energy through chemical bonds within the visible wavelength region (380 to 770 nm) while fats, proteins, carbohydrates and water have absorption bands within the near infrared range (770 to 2500 nm) (Abbott, 1999).

Organic acid composition of fruit is regarded as a component of fruit quality, a determinant of fruit ripening and also a factor contributing to fruit colour (de Ancos *et al*., 1999). Greater acidity would be expected to enhance the colour strength of anthocyanins, which in nature sees red anthocyanins at a low pH. Anthocyanins are sensitive to changes in pH with a low pH favouring flavylium cations which are red; an increase in pH sees blue quinoidal forms as well as the appearance of colourless pseudobases and chalcones (de Pascual-Teresa and Sanchez-Ballesta, 2008). Anthocyanins contain highly conjugated chromophores which are affected by changes within pH values they are exposed to. Such pH changes result in an alteration in the conjugation of the anthocyanin double bonds which affect wavelengths of light transmitted and absorbed (Hajji *et al*., 1997). The pH differences recorded in this study, however, were small (from 2.98 to 3.95) and did not result in any obvious changes in the colour recorded.

3.3.2 Environmental and seasonal influence on fruit quality

Low temperatures before harvest or during storage have been found to favour anthocyanin synthesis in apple, pear, grape and cranberry (Kliewer, 1970; Curry, 1997; Steyn *et al*., 2004). It has been found that a deficiency in soil nitrogen favours anthocyanin biosynthesis in both vegetative tissue and fruit (Steyn *et al*., 2002). An increase in nitrogen has been found to reduce anthocyanin in grape and apple (Kliewer,

1977) as well as reducing the perceived colour intensity in fruit through the increase in chlorophyll stimulation (Marsh *et al*., 1996; Reay *et al*., 1998). Water drought increases secondary metabolism and in particular, flavonoid and anthocyanin biosynthesis, which are thought to be initiated as part of the plants response to stress. Anthocyanin concentration was found to increase in grapes following a period of water deficit early after the onset of ripening (Castellarin *et al*., 2007). Even transient exposure to water deficits before the onset of ripening may have profound and prolonged effects on ripening, particularly relating to berry size (Castellarin *et al*., 2007). A study by Kamsteeg *et al*. (1979) also found soil which was subjected to physiological stresses such as excessive water, resulting in water logged soil can enhance anthocyanin formation.

For some quality traits analysed, such as total soluble solids (^{o}Brix), it was apparent that variations due to season and location exist and this was particularly evident when fruit were grown in the field site. For fruit grown under protection the seasonal variation was found to be reduced. This appears to be in line with breeder observations of raspberry varieties grown under both field and tunnel, whereby the protected environment appears to minimize genotypic effects which were found to influence plant behavior in a field setting (N. Jennings, personal communication). Significant differences were recorded in a study for ^{o}Brix values in apples over two separate seasons. Kenis *et al*. (2008) concluded that differences were due to environmental conditions or interactions with other genetic factors and not due to genetic control alone. Total soluble solids, is one of the most extensively studied traits in tomato, due to the economical importance of fruit ^{o}Brix. Significant crop improvements for ^{o}Brix have remained somewhat elusive due to the polygenic nature of this trait with environmental influence and pleiotropic relationships with traits such as size, yield and weight affecting any successful crop improvements (Fridman *et al*., 2002). The negative correlation between fruit weight and total soluble solids is one which has been found in numerous studies including apple and tomato (Saliba-Colombani *et al*., 2001; Tanksley *et al*., 1996; Bernacchi *et al*., 1998;

Chen *et al*., 1999). This was not found in this study, instead no correlation, positive or negative, were detected in any year or location between fruit weight and ^{o}Brix.

Traits such as ten berry weights, however, displayed variations according to location with the varying seasons having little impact overall. Fruit grown under protection were all significantly bigger than the corresponding field grown samples and this was consistent regardless of season (Figure 3.23). These variations highlight the importance of constructing field based experiments for phenotypic analysis over a series of sites and seasons in order to obtain accurate results which allow genotypic and environmental influences to be determined.

In this study it has been shown that with appropriate polytunnel selection in terms of field location, polythene covering and irrigation practices, it may be possible, not only to extend the growing season in soft fruit, but to produce fruit superior in size to field grown fruit without compromising flavour and colour. Techniques which utilise different cultivars with different fruiting seasons or sequential planting of cold stored plants in conjunction with growing fruit under protection in the form of polytunnels, allow the production of domestic raspberries both earlier and later in the season which limit the need for expensive and often lower quality imported fruit. Polytunnels were first introduced to British farming in 1993 and have extended the growing season of raspberries from eight weeks in June and July to around sixteen weeks from May to August. This has allowed fresh British berries to dominate the market place which was previously dominated by imported fruit (Britishsummerfruits.co.uk) The combined area of glasshouse and plastic structures in Scotland has increased by 64% in only four years to give a total of 1,278,884 m^2 recorded in 2007. The main fruit grown under protection were strawberries and raspberries and these have increased from 248,039 m^2 in 2003 to 548,533 m^2 in 2007, an increase in 121% (SASA PC 2007). Although concern has been raised in many communities regarding the construction of polytunnels it is reported that only 0.01% of UK agricultural land is actually under polytunnels, the majority of which are then removed at the end of each growing season (Britishsummerfruits.co.uk).

For all traits analysed, regardless of site or season, progeny were identified which showed considerably higher (and lower) values than either of the parents (Appendix 3.1). The mean sample values, however, were found to be within parental measurements except for colour meter measurements in 2006 with Yxy and L* a* b* all recording higher average values than the parents. Strawberry offspring resulting from several crosses have also been found to contain genotypes which produce fruits with far higher nutritional values than parents. This phenomenon of transgressive segregation, whereby the offspring exceed the parental values for a specific trait, can create new breeding perspectives for the release of new cultivars with improved sensory and nutritional qualities (Capocasa *et al*., 2008). Phenotypic traits can subsequently be associated with underlying molecular markers for MAB programmes. In order to develop MAB in plants, it is necessary to identify regions along the chromosome with genes for these major quality traits. The findings from this chapter have been important in advancing understanding of red raspberry quality and how these traits interact. While consistent correlations were not found for all phenotypic traits assessed, data will be analysed to identify quantitative trait loci (QTL) for raspberry colour, size and flavour attributes, across sites and seasons on the *Rubus* genetic linkage map (Graham *et al*., 2004; 2006). This will be carried out in order to further understand the genetic and environmental controls underlying quality and flavonoid synthesis in red raspberry fruit.

Chapter 4 Identification of candidate genes related to fruit quality

4.1 Introduction

Candidate gene approaches have been used to establish associations between genes involved in relevant metabolic pathways and quantitative trait loci (QTL). Knowledge of metabolic pathways involved in the uptake and regulation of sugars and organic acids as well as pigment synthesis and accumulation in fruit can be utilised to identify primary candidate genes. Alternative genes may include transporters and transcription factors important in the development of fruit quality. This is a key area of research, as to improve fruit quality it is necessary to identify and characterise control processes, and determine key polymorphic alleles. By analysing a population segregating for the defined traits of interest, QTL can be determined and positioned onto existing linkage maps.

4.1.1 Sources of putative candidate genes

The long term commercial aim of research carried out on fruit quality today is to improve characteristics related to colour changes, flavour, texture and nutritional value. Fruit firmness has implications not only related to mouthfeel and sensory acceptance but for shelf life and processing characteristics. A greater understanding of the biochemical processes, genes and interactions contributing to fruit quality remains a major goal for this area of research. With *Prunus* currently ahead of *Rubus* in terms of providing a model for Rosaceous species, examining candidate genes characterised in *Prunus persica* for sugar and acid content (Etienne *et al*., 2002), was a step towards identifying homology between species and possibly molecular markers for quality traits.

4.1.1.1 Homology as a source of candidate genes

If the sequence information for a specific gene of interest is unavailable then sequence information from related species can be utilised to deduce consensus motifs for degenerate primer design (Pflieger *et al*., 2001). The value of analysing and comparing data from species can be seen with the identification of major genes and QTLs located

within the equivalent genomic regions across diverse progenies and species (Varshney and Tuberosa, 2008). Leister *et al*. (1996) developed a strategy of resistance gene analogues (RGA) to map resistance gene clusters in potato which has since been applied in peach (Bliss *et al*., 2002) and apricot (Soriano *et al*., 2005). This suggests that the conservation of gene sequence, order and distribution (i.e synteny) can aid in the development and identification of gene action and variation between individuals. Several studies have confirmed this conservation of genes with structural genes involved in carotenoid biosynthesis in pepper (*Capsicum*) showing comparative positioning on linkage maps in tomato and potato (Thorup *et al*., 2000). Similar comparative studies and mapping have also been carried out between perennial ryegrass (*Lolium perenne* L.) (Cogan *et al*., 2006) and tomato (*Solanum lycopersicum*) (Tanksley and Hewitt, 1988).

4.1.1.2 Database mining as a source of candidate genes

Expressed sequence tag (EST) libraries hold great potential for the identification of putative candidate genes involved in specific tissues, developmental stages or stress responses invoked in plants through differential screening approaches (Pflieger *et al*., 2001). Horn *et al*. (2005) initiated the development of a peach (*Prunus persica*) EST database for identifying and cloning genes important for fruit and tree development. This resulted in 9,984 ESTs with an average read length of 502 base pairs. From 3,500 identified singletons, 2,533 were found to be unique reads of which 1,708 had significant sequence matches using Genbank databases (Horn *et al*., 2005). Overall the number of putative unique genes comprises some 3,842 individuals (Horn *et al*., 2005). A high proportion of these genes have been functionally identified in peach with molecular, biological and physiological processes represented (Horn *et al*., 2005). In tomato there have been more than twenty three EST libraries constructed and sequenced to create in excess of 30,000 unigenes for candidate gene mining (Causse *et al*., 2004). A recent database search carried out using NCBI search engine "Entrez" produced 62,751,407 EST results. Fruit ESTs contributed 779,804 hits which were further broken down to yield 335,971 for *Malus*, 101,791 for *Prunus*, 54,771 *Fragaria* and 3132 *Rubus* EST sequences. 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. One such example is 454 mRNA sequencing which allows a large population of mRNA sequences to be identified (thousands of gene sequences) in fruit (Ronaghi, 2001). If this was applied to give the most complete list of genes that are expressed in these tissues, it can be of further value because the transcript profile will be applicable to many aspects of fruit development. If SNPs can be identified between genes from different accessions these can be used to map a large number of sequences.

4.1.1.3 Biological pathways

If the pathways underlying phenotypic trait expression are known, then it may be possible to identify, isolate and characterise the individual enzymes involved in order to find genes responsible for trait variation. Genes associated with individual biosynthetic pathways are obvious candidates for trait variation. The colour of fruit is a major determinant for the overall acceptance of new cultivars and is often used as a visual measure to determine fruit quality and maturity (Maga, 1974). Due to the complexity of this trait a vast amount of research has led to the identification and characterisation of many genes involved in the numerous biosynthetic pathways which may regulate colour variation. An example of genes analysed include dihydroflavonol 4-reductase (DFR) (Ju *et al*., 1997; Gollop *et al*., 2002), phenylalanine ammonia-lyase (PAL) (Ju *et al*., 1995a), chalcone synthase (CHS) (Ju *et al*., 1995b; Zheng and Hrazdina, 2008), glutathione S-transferase (GST) (Alfenito *et al*., 1998; Lu *et al*., 1998), flavonoid 3'-hydroxylase (F3'H) (Castellarin and Di Gaspero, 2007) and flavonol synthase (FLS) (Davies *et al*., 2003). Several studies have focused on the effect of over-expression of individual genes in transgenic plants with many novel flower colours generated. For example, Tanaka *et al*. (1995) found the over-expression of DFR in Petunia produced novel flower pigmentation. Ueyama *et al*. (2002) showed overexpression of F3'H resulted in a redder flower colour in transgenic *Torenia hybrida* due to the abundance of 3'-hydroxylated anthocyanins. Over expression of F3'5'H however, was shown to produce purple to violet flowers due to the increase in 3'5'-hydroxylated anthocyanins (Fukui *et al*., 2003). Shimada *et al*. (1999) reported a dramatic shift in Petunia flower colour from pink to

magenta when F3'5'H was preferentially expressed (reviewed by de Pascual-Teresa and Sanchez-Ballesta, 2008). Further changes in flower pigmentation have also been obtained through the suppression of individual genes with flower pigment in Petunia being hindered following CHS suppression (Van der Krol *et al*., 1988). The co-suppression of CHS and DFR were also found to generate white and white/blue flowers in transgenic *Torenia* varieties (Suzuki *et al*., 2000).

FLS has been linked with a higher affinity to dihydroquercetin than dihydrokaempferol or dihydromyricetin in Petunia (Suzuki *et al*., 2000). This trend was also found in a study by Jeong *et al*. (2006) in grape with the major flavonol identified as quercetin. Wellman *et al*. (2002) found in citrus, however, that a recombinant FLS showed a greater affinity to dihydrokaempferol compared with dihydroquercetin, although quercetin conjugates showed highest accumulation, which suggested that this may indicate the expression of more than one FLS polypeptide. In *Arabidopsis* all enzymes central to flavonoid biosynthesis, were encoded by single copy genes with the exception of FLS which was found to be encoded by six genes (Winkel-Shirley, 2001). FLS and DFR utilise the same dihydroflavonol substrates. DFR is not expressed in high levels until late in flower development so under normal conditions these enzymes will not compete as FLS will utilise dihydroflavonols available prior to the onset of DFR synthesis (Nielsen *et al*., 2002).

This chapter aimed to utilise various strategies to isolate and amplify individual genes in red raspberry. Techniques included homology searches, data base mining and fruit EST library construction and mining to identify genes involved in pathways related to fruit quality and in particular colour. Once genes were identified and characterised for the mapping population, results can be analysed for addition onto the *Rubus* linkage map and associations between candidate genes and QTL regions, identified through phenotypic evaluation (Chapter 3) can then be utilised in the establishment of MAB strategies.

4.2 Results

4.2.1 Candidate genes identified in Rubus through homology sources

Candidate genes relating to fruit quality, identified in literature searches in homologous species (Table 4.1), were tested in raspberry to ascertain which genes, if any, showed close consensus across species. From this study 21 genes were analysed from homologous searches which resulted in five being mapped. Five further genes were sequenced but no polymorphisms were detected while seven were amplified via PCR but yielded poor or very short sequences. Three failed to amplify using initial primers or following primer re-design while one gene (VP2) showed no conserved regions preventing successful primers being re-designed.

Primer sequences obtained were amplified for the mapping parents, plus six progeny (Sections 2.2.3 and 2.2.4). Of the primer sequences analysed, five genes related to quality have been successfully amplified, resulting in a clear product being obtained. MIP 2, MIP 3, TIP and expansin genes identified in peach by Etienne *et al*. (2002) in a Texas × Earlygold mapping population and L-galactose dehydrogenase (Gal-dh) amplified by Laing *et al*. (2004) in kiwifruit (*Actinidia deliciosa*). Expansin and MIP 3 initially failed to amplify in raspberry and primers were redesigned using conserved regions from homologous species (*Prunus* AB047518, AF297522 and *Fragaria* AF226701 for expansin) and (*Prunus* AB218716, *Pyrus* AB058680 and *Mimosa* AB206100 for MIP 3) to include degenerate wobbles (Operon.com) for base pairs where necessary (Section 2.2.5).

Table 4.1. Candidate gene status in *Rubus* from homologous species.

Homologous candidate	Species	Genbank accession	Results
Expansin (EXP)	*Prunus*	AF367459	Mapped to LG 7
Tonoplast intrinsic protein (TIP)	*Prunus*	AF367456	Mapped to LG 2
Membrane intrinsic protein 2 (MIP 2)	*Prunus*	AF367458	Mapped to LG 2
Membrane intrinsic protein 3 (MIP 3)	*Prunus*	AF367460	Mapped to LG 2
Galactose dehydrogenase (Gal-dh)	*Actinidia*	AY176585	Mapped to LG 4
Hexokinase 1 (HK1)	*Prunus*	AF367451	Sequenced, no SNP
Hexokinase 2 (HK2)	*Prunus*	AF367452	Sequenced, no SNP
Sucrose transporter 2 (STP2)	*Prunus*	AF367455	Sequenced, no SNP
Vacuolar invertase (VI)	*Arabidopsis*	AT1G12240	Sequenced, no SNP
Acid invertase (Ail)	*Prunus*	AF367453	Sequenced, no SNP
Sucrose transporter 1 (STP1)	*Prunus*	AF367454	Amplified, poor sequence
Sucrose synthase 1 (Sus1)	*Prunus*	AF367450	Amplified, poor sequence
Vacuolar H^+ pyrophosphatase (Vp1)	*Prunus*	AF367446	Amplified, poor sequence
Membrane intrinsic protein 1 (MIP 1)	*Prunus*	AF367457	Amplified, poor sequence
Neutral invertase (NI)	*Arabidopsis*	AT3G05820	Amplified, poor sequence
Invertase (Inv)	*Arabidopsis*	AT3G06500	Amplified, poor sequence.
Fruit weight 2.2 (fw2.2)	*Solanum*	AY097189	Amplified, poor sequence
Malate dehydrogenase (Mdh1)	*Prunus*	AF367442	Redesigned, no amplification
Isocitrate dehydrogenase (Icdh)	*Prunus*	AF367443	Redesigned, no amplification
Citrate synthase 1 (Cs1)	*Prunus*	AF367444	Redesigned, no amplification
Vacuolar H^+ pyrophosphatase 2 (Vp2)	*Prunus*	AF367447	No amplification or conserved regions

4.2.2 Candidate genes identified in Rubus through database mining

Rubus EST libraries which were available for database mining (Section 2.3) were explored for potential genes identified within pathways involved in fruit colour development. As a result five putative candidate genes were identified, (dihydroflavonol 4-reductase (DFR), anthocyanidin reductase (ANR), flavonoid 3'-hydroxylase (F3'H), flavonol synthase (FLS) and glutathione S-transferase (GST). BLAST search confirmed their involvement in the development of colour in other plants' including strawberries, grape and rose hybrids. The genes selected were isolated and amplified, in both parents and six progeny, (Section 2.2.4) once primers had been designed using areas of conserved sequences (Section 2.2.5). Following successful amplification all five candidate genes were sequenced (Section 2.2.8.1) and the sequences aligned and screened for polymorphisms within and between samples.

4.2.3 Candidate genes identified through the construction of an EST Library

In order to enhance the sequence database currently available in *Rubus* a cDNA library was constructed from 'Glen Moy' and 'Latham' samples for two distinct developmental stages: green/red and red ripe fruit (Section 2.3.1).

4.2.3.1 RNA extraction and cDNA synthesis

With an absorbance ratio of around 2.0 (A_{260}/A_{280}) for all RNA obtained, and two intact ribosomal RNA bands visualised (Figure 4.1), it was assumed that contamination was minimal. The average yield of total RNA for each stage of fruit analysed was typically 120 to 440 ng per gram fresh weight (Table 4.2). Following isolation and purification, samples were prepared for EST library construction (Section 2.3.1). cDNA inserts of between 500 and 1000 bp were obtained and all clones were utilised in the final stages of library construction prior to sequence analysis.

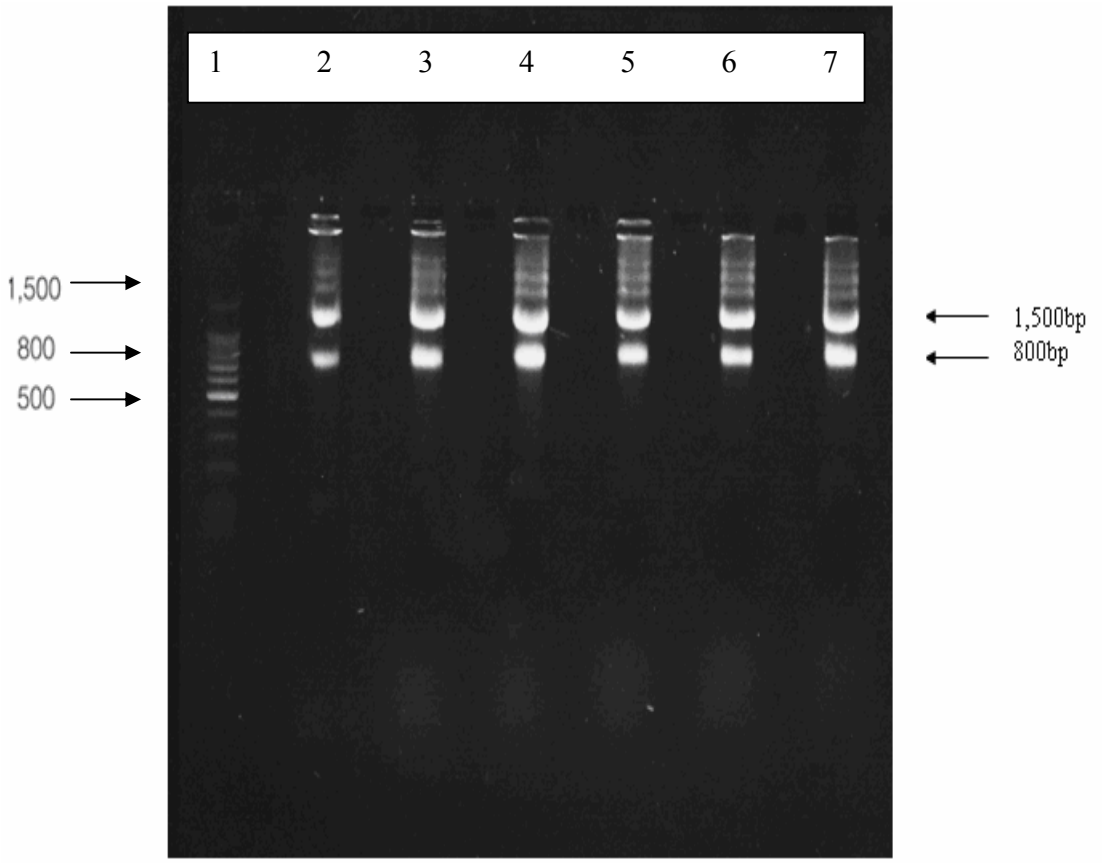


Figure 4.1. A 1.5% (w/v) agarose gel of RNA isolated from fruit. The average yield of total RNA was typically 362 ng per gram (fw). Two intact ribosomal RNA bands indicate that isolated RNA was not degraded. The top band represents the 28S rRNA large subunit (1,500 bp) the second main band the smaller 18S rRNA subunit (800 bp). Lane 1 contained 100bp DNA ladder (Promega), lane 2, 3 and 4 'Glen Moy' red fruit RNA samples and lane 5, 6 and 7 'Latham' red fruit RNA samples.

Table 4.2. RNA yield for a selection of samples from each fruit library constructed.

Parent	Stage	Total RNA (0.4 g fruit) (ng/µl)	A_{260}/A_{280}
Latham	Green/Red	396.7	2.03
Latham	Green/Red	439.8	2.04
Latham	Green/Red	481.2	2.04
Latham	Green/Red	413.1	2.06
Moy	Green/Red	330.2	2.05
Moy	Green/Red	321.1	2.07
Moy	Green/Red	331.2	2.06
Moy	Green/Red	300.9	2.07
Latham	Red	410.8	2.08
Latham	Red	333.4	2.09
Latham	Red	399.5	1.91
Latham	Red	253.8	2.11
Moy	Red	376.2	2.07
Moy	Red	428.1	2.00
Moy	Red	317.3	2.03
Moy	Red	257.3	2.05

4.2.4 Approaches to mapping candidate genes

Expansin and MIP 2 were successfully amplified in raspberry but subsequent gel visualisation found faint surrounding bands so these bands were excised, purified and cloned (Section 2.2.9). TIP and Gal-dh genes amplified from the original primers produced a single clear product and were extracted and purified from agarose gel (Section 2.2.7) and sequenced (Section 2.2.8.1).

4.2.4.1 Size polymorphism

Following amplification with re-designed primers in both parents and six progeny, MIP 3 produced a visible PCR product, which contained a size polymorphism. This could be scored directly from a 1.5% agarose gel (Figure 4.2) and was carried out for the full mapping population based on the presence of either one ('Glen Moy') or two bands ('Latham') following gel electrophoresis.

4.2.4.2 Single nucleotide polymorphism

Sequence results obtained from all candidate genes amplified were analysed by comparing base pair sequences between parents for each of the candidate genes, in the forward and reverse complement, using the software BioEdit 5.0.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and subsequently Sequencher 4.8 for the assembly and analysis of sequence contigs (Table 4.3). Polymorphisms were detected for each of the genes analysed, an example of which can be seen in Figure 4.3 for dihydroflavonol 4-reductase (DFR).

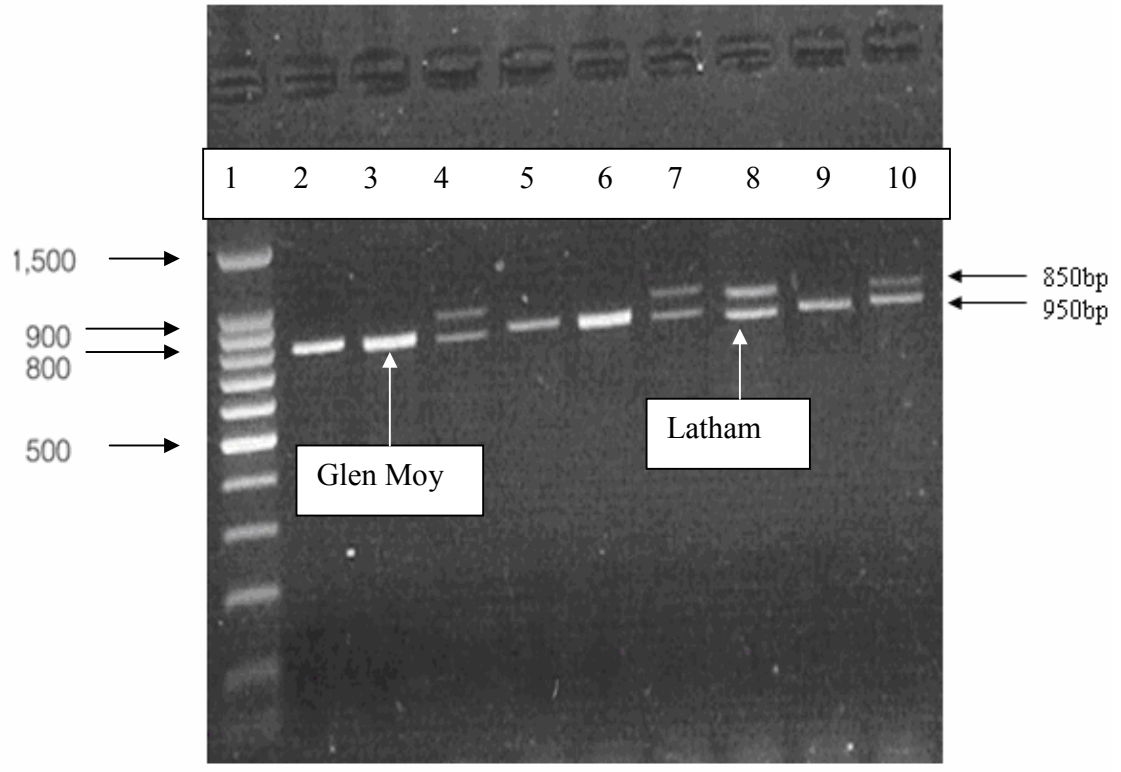


Figure 4.2. A 1.5% agarose gel showing PCR products obtained for the candidate gene membrane intrinsic protein (MIP) 3. The re-designed MIP 3 primers, containing Operon wobbles, produced an amplified PCR product of 850 bp. The 100 bp DNA ladder, on the left hand side, shows the approximate fragment sizes obtained. 'Glen Moy' was found to contain one 850 bp band while 'Latham' has one 850 bp and one 950 bp band. Lane 1 contained 100bp DNA ladder (Promega), lane 2, 4, 5, 6, 7, 9 and 10 contained examples from progeny RNA samples, lane 3 'Glen Moy' RNA samples and lane 8 'Latham' RNA samples.

Table 4.3. Sequence length and SNPs identified in the ten candidate gene sequences in *R. idaeus*.

Candidate gene	Sequence obtained (bp)	Putative SNP's identified	Gene source
Expansin	430	2	Etienne *et al*., 2002
MIP 2	350	3	Etienne *et al*., 2002
MIP 3	340	3	Etienne *et al*., 2002
TIP	300	2	Etienne *et al*., 2002
Gal-dh	350	2	Laing *et al*., *2004*
ANR	600	3	*Rubus* database
DFR	280	4	*Rubus* database
GST	450	3	*Rubus* database
FLS	500	4	*Rubus* database
F3'H	580	3	*Rubus* database

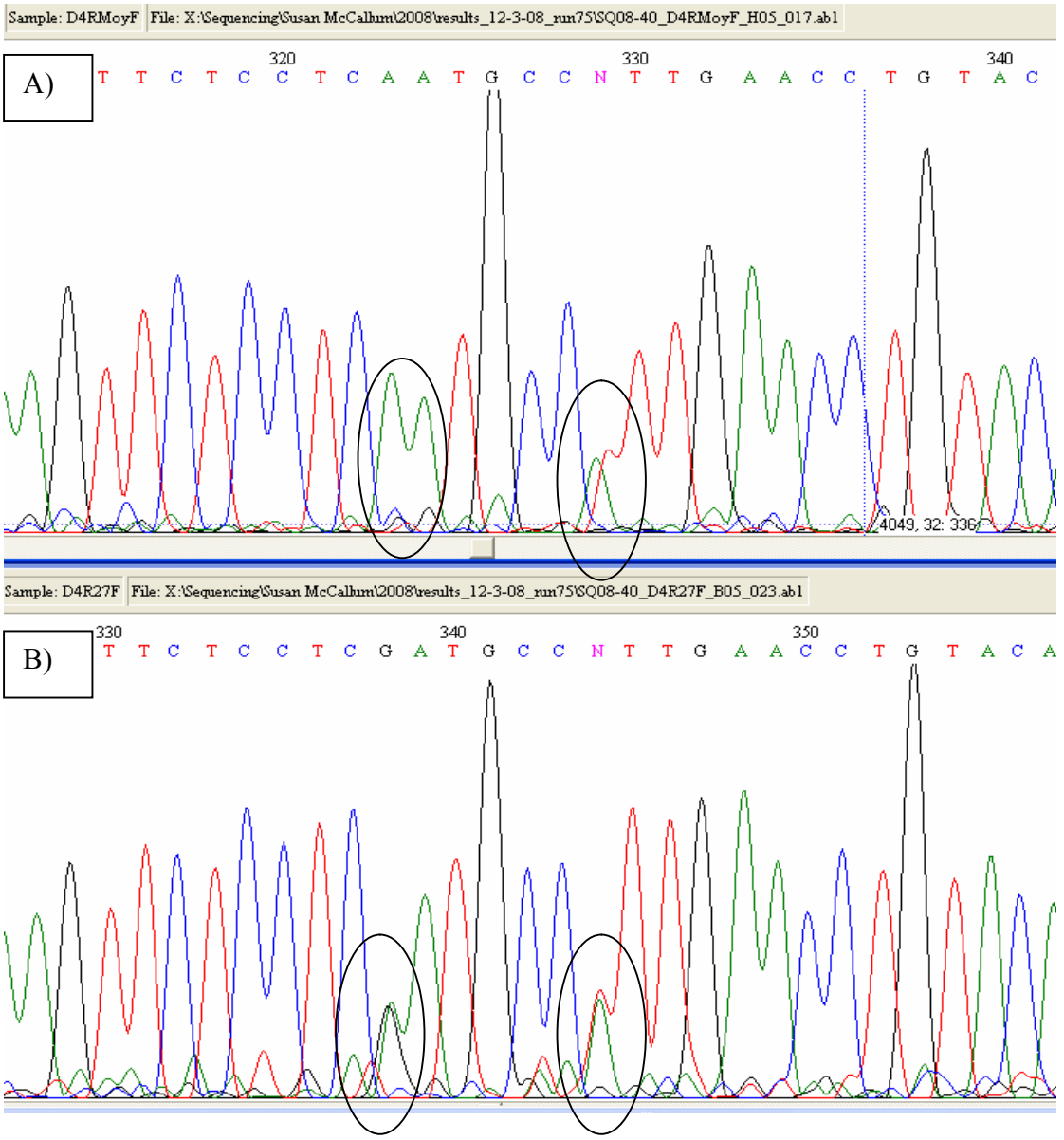


Figure 4.3. Chromatograph obtained from BioEdit 5.0.9 for the candidate gene dihydroflavonol 4-reductase (DFR). This chromatograph shows the two polymorphisms identified between one of the parents, cv. Glen Moy (trace, A) containing an A/A and T/A and progeny 'r27' (trace, B) with alleles A/G and T/A.

4.2.4.3 Sequence results from EST fruit libraries

Sequences were analysed using Sequencher 4.8 and trimmed, where necessary, to ensure high quality sequence reads. Vector derived sequences were removed from each sample and DNA sequences were quality scored using the Phred software (http://www.phrap.com) and then searched against the non-redundant nucleotide databases at NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul *et al*., 1990). Several contiguous sequences (contigs) were found to contain polymorphisms either between parental alleles or between parents themselves, for several genes of interest, allowing primers to be designed to analyse the full mapping population.

This resulted in sequence data from around 1000 clones added to the SCRI *Rubus* sequence database which are now available for EST mining. Analysis of these clones resulted in the formation of > 100 contigs and the identification of ca. 50 SSRs and almost 900 positive BLAST queries. Several potentially interesting sequences were identified with significant homology to known genes in raspberry and other fruit impacting on fruit ripening, berry development and other quality traits (Table 4.4). The sequences obtained from isolated cDNA clones were submitted to a peptide sequence database via version 2.2.18 of the BLAST programme. A selection of genes identified from the EST libraries were examined for the presence of single nucleotide polymorphisms for subsequent analysis. The genes selected were from contig 16, 20, 21, 22 and 24 as consistent polymorphisms were identified which allowed primers to be designed for amplification across the full mapping population. Following sequence analysis, each gene was characterised in the mapping population by means of genotypic analysis (Section 2.3.17.3.1) for addition onto the *Rubus* linkage map (Chapter 5).

Table 4.4. Examples of genes of interest identified from EST sequences from cv. Latham (ERubLath) and cv. Glen Moy (ERubMoy).

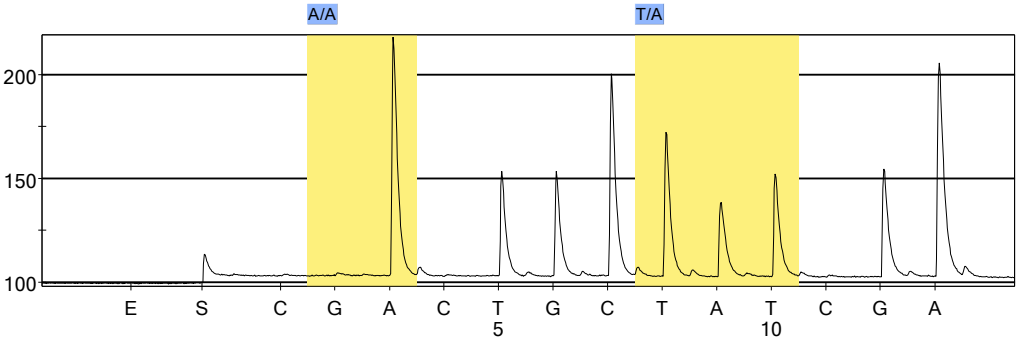
NAME	HOMOLOGY
GREEN/ RED LIBRARY	
ERubLath2_SQ01_ERubLath2_SQ01_L05_L05_011	Dehydrin implicated in bud burst
ERubLath2_SQ01_ERubLath2_SQ01_K14_K14_027	Agamous (MADS box)
Contig 12	Senescence associated
Contig 13	Trehalase
Contig 9	D-allose transporter
Contig 6	Purple acid phosphatase
Contig 16	Unknown protein
Contig 20	Hypothetical protein
Contig 21	Hypothetical protein
Contig 22	Steroid receptor/signal transduction
Contig 24	Hypothetical protein
RED RIPE LIBRARY	
ERubMoy3_SQ01_ERubMoy3_SQ01_M11_M11_	Metallothione-like
Contig 5	Major latex like
ERubMoy3_SQ01_ERubMoy3_SQ01_B07_B07_	Major latex like
ERubMoy3_SQ01_ERubMoy3_SQ01_D05_D05_	Tomato high throughput cDNA sequences (HTC) in leaf
ERubMoy3_SQ01_ERubMoy3_SQ01_P05_P05_009	Hypothetical protein *Vitis vinifera*
ERubLath3_SQ01_ERubLath3_SQ01_F19_F19_	Predicted protein *Vitis vinifera*
ERubLath3_SQ01_ERubLath3_SQ01_C15_C15_	Mitochondrial F1 ATP synthase
ERubLath3_SQ01_ERubLath3_SQ01_D13_D13_	Mitochondrial F1 ATP synthase
ERubLath3_SQ01_ERubLath3_SQ01_P19_P19_	Drought stress

4.2.5 Pyrosequence® analysis of candidate genes

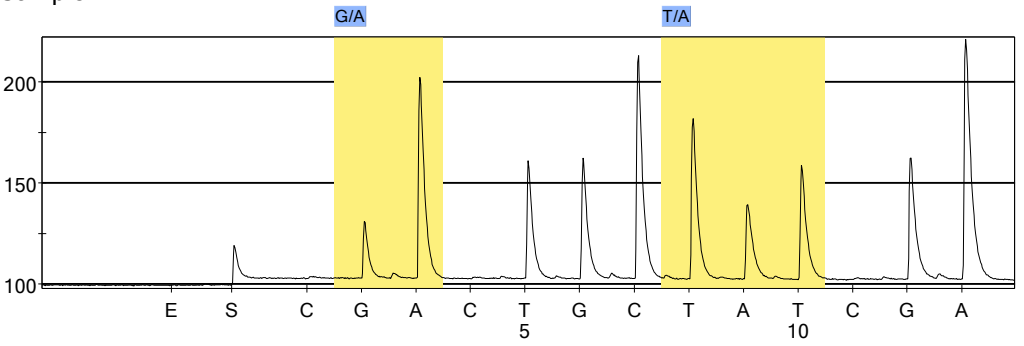
Once polymorphisms were identified from either homology or database searches, either within parental alleles or between parents, it was necessary to use the mapping population to determine how the SNP segregated within a population. The method favoured in this study was Pyrosequence® technology which is a fast, reliable, robust technique which has been used successfully in previous genotyping studies (Alderborn *et al*., 2000; Ronaghi, 2001). Following sequence analysis of MIP 2, a short sequence differing between 'Glen Moy' alleles was found to contain two SNPs separated by only seven base pairs. As both polymorphisms were clear, a Pyrosequencing® assay was designed which allowed both polymorphisms to be analysed within the same sequencing reaction. Results for MIP 2 were reported for each of the SNPs separately, MIP 2 SNP1 and MIP 2 SNP2. Likewise for dihydroflavonol 4-reductase (DFR) two SNPs were identified in a contig containing one of the parents, cv. Glen Moy and one of the progeny 'r27'. The SNPs were only six base pairs apart so a Pyrosequencing® assay was designed which incorporated both polymorphisms for sequence analysis. Results for DFR were reported for each of the SNPs separately, DFR 1 and DFR 2 (Figure 4.4).

All polymorphisms identified following sequence analysis were transferred into Pyrosequencing® assays to analyse the genotype of the mapping population (Section 2.2.10.3). Pyrosequence® results were converted prior to mapping analysis, which required individuals to be scored. Heterozygotes were *ab* while homozygotes were either *aa* or *bb*. (Table 4.5). This was also carried out on the size polymorphism seen in MIP 3, whereby samples containing one band become *aa* and those with two bands *ab*.

(A) DFR SNP1, 2
Sample: MOY



(B) DFR SNP1, 2
Sample: LATH



(C) DFR SNP1, 2

Sample: 222

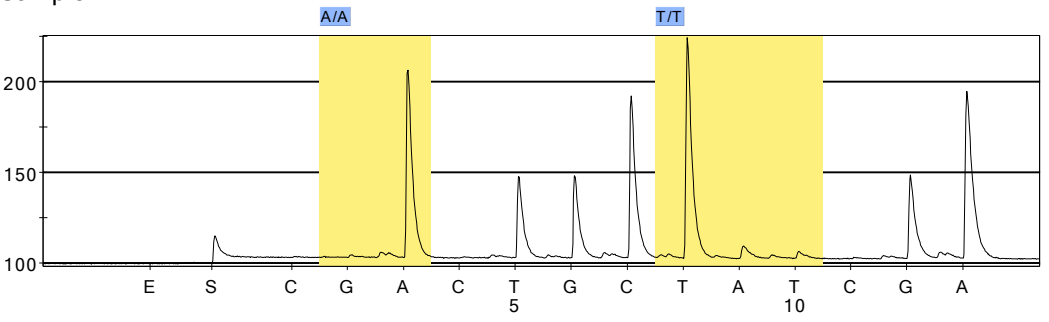


Figure 4.4. Pyrogram® traces for the parents (A) Glen Moy (B) Latham and (C) one of the progeny (r222) for the candidate gene dihydroflavonol 4-reductase (DFR). 'Glen Moy' was found to contain an A/A and T/A for the SNP sequence analysed while 'Latham' revealed G/A and T/A. The progeny were found to segregate 1:1 either G/A or A/A for the first SNP and 1:2:1 T/T, T/A or A/A for the second SNP, an example of which can be seen in trace (C) for the individual 'r222 containing an A/A and T/T genotype.

Table 4.5. Mapping results for candidate genes.

Candidate Gene	Source of identity	Parent 1 Latham	Parent 2 Glen Moy	Mapping population	Linkage Group
MIP 3	Etienne *et al*., 2002	ab	aa	ab = 84 aa = 87 Ratio 1:1	2
TIP	Etienne *et al*., 2002	ab	aa	ab = 93 aa = 87 Ratio 1:1	2
MIP 2 SNP 1	Etienne *et al*., 2002	ab	ab	ab = 98 aa = 40 bb = 48 Ratio 1:2:1	2
MIP 2 SNP 2	Etienne *et al*., 2002	ab	aa	ab = 95 aa = 48 bb = 43 Ratio 1:2:1	2
Gal-dh	Laing *et al*., 2004	aa	ab	ab = 73 aa = 107 Ratio 1:1	4
EXP	Etienne *et al*., 2002	ab	aa	ab = 104 aa = 80 Ratio 1:1	7
ANR	"In house" *Rubus* database	ab	ab	ab = 94 aa = 43 ab = 46 Ratio 1:2:1	2
FLS	"In house" *Rubus* database	ab	ab	ab = 83 aa = 40 bb = 40 Ratio 1:2:1	4
DFR SNP 1	"In house" *Rubus* database	ab	aa	ab = 83 aa = 99 Ratio 1:1	4
DFR SNP 2	"In house" *Rubus* database	ab	ab	ab = 88 aa = 50 bb = 45 Ratio 1:2:1	4
GST	"In house" *Rubus* database	ab	aa	ab = 65 bb = 77 Ratio 1:1	4
F 3' H	"In house" *Rubus* database	ab	aa	ab = 77 aa = 76 Ratio 1:1	5

4.2.6 Gene prediction of amino acid substitutions

The partial gene sequences obtained for each of the candidate genes analysed were transferred into gene prediction software FGENESH (Section 2.2.11). This allowed regions which may encode proteins to be predicted through the detection of coding regions within the sequence which will imply gene structure. As a result four genes (EXP, DFR, FLS, MIP 2) were found to contain an amino acid change caused by the identified SNP, four genes produced no reliable prediction (Gal-dh, F3'H, GST, ANR), one gene (TIP) showed no amino acid change while MIP 3 (size polymorphism) was not analysed. An example of the potential effect a polymorphism can have on a candidate gene is shown in Figure 4.5 for MIP 2. The parents are homozygous G/A in both SNP positions which results in a 1:2:1 segregation within the mapping population. For the partial sequence analysed, it was found that a G in SNP position 1 resulted in a polypeptide chain of 98, whereas an A in SNP position 1 resulted in a truncated polypeptide of only 60 amino acids. Full gene sequencing would be necessary, however, to determine whether these are functional differences between the two alleles which may influence phenotypic traits.

A) MIP 2

SNP1 G

Predicted protein(s):
>FGENESH:[mRNA] 1 1 exon (s) 20 - 316 297 bp, chain -
ATGTATTTGCTAAACAATATGACAGGAGGAGGGATAATGGTTATGGGTGCTTACAGGAA
CGTGGCAGTCTCTGGCATTTCTCTTAGGGTCAGTGGCAGAGAAGACAGTGTAGACAAGA
ACAAAGGTACCGATGATCTCAGCCGCCAATCCAGTGCCCTTGCTGTACCCATCTGCCAA
CTCATTTGCTCCACCACCATACTTGGTGTACAATGTCTTCTGGAAGGCCTTGACCAACC
CTACACCAATGATTGCACCCAAAGACTGTGCTACTATGTACAGCACTGCCCTCGGCAGT
GA

>FGENESH: 1 1 exon (s) 20 - 316 **98** aa, chain -
MYLLNNMTGGGIMVMGAYRNVAVSGISLRVSGREDSVDKNKGTDDLSRQSSALAVPICQ
LICSTTILGVQCLLEGLDQPYTNDCTQRLCYYVQHCPRQ

B) MIP 2

SNP1 A

Predicted protein(s):
>FGENESH:[mRNA] 1 1 exon (s) 81 - 262 180 bp, chain +
TTGGTCAAGGCCTTCCAGAAGACATTGTACACCAAGTATGGTGGTGGAGCAAATGAGTT
GGCAGATGGATACAGCGAGGGCACTGGATTGGCGGCTGAGATCATCGGTACCTTTGTTC
TTGTCTACACTGTCTTCTCTGCCACTGACCCTAAGAGAAATGCCAGAGACTGCCACGTT
CCT

>FGENESH: 1 1 exon (s) 81 - 262 **60** aa, chain +
LVKAFQKTLYTKYGGGANELADGYSEGTGLAAEIIGTFVLVYTVFSATDPKRNARDCHV
P

Figure 4.5. Prediction of potential genes formed from the polymorphisms found in MIP 2 using the gene prediction software FGENESH. Sequence A) shows the predicted protein obtained from MIP 2 in progeny containing a 'G' base in the identified SNP region. This results in a polypeptide chain (in bold) of 98 amino acids. Sequence B) shows an individual with an 'A' base in the same SNP region which results in a truncated chain of only 60 amino acids.

4.3 Discussion

The results obtained in this chapter have allowed the identification and mapping of a number of key genes involved in the regulation and transport of metabolites central to fruit quality. The construction of an EST library for different fruit stages is central for further identification and characterisation of candidate genes and transcription factors involved in metabolic pathways.

To construct a high quality EST library it was necessary to isolate high quality RNA from appropriate source material. The high acidity of fruit extract along with the associated high levels of polysaccharides and phenolic compounds often creates low quality contaminated samples which may also be degraded by the presence of RNases (Romani *et al*., 1975; Woodhead *et al*., 1997). No functional homologs may indicate novel raspberry genes or clones with insufficient coding sequence to accurately assign a function. Raspberry ESTs were assigned putative functions determined by the closest homologs identified from NCBI databases. Many other sequences and contigs were identified with homology to known candidate genes of interest in other species, many of which have important implications in fruit ripening and development (Woodhead *et al*., 2008).

Candidate gene analysis as an approach for assessing phenotypic variations among individuals is not a novel strategy. Work has been focusing on the potential exploitation of candidate genes for the characterisation of disease heritability in humans (Takahashi *et al*., 1992) and animal diseases as human models (Gorin *et al*., 1993) since the early nineties. In plant genomics, candidate genes have been explored for an understanding of many agronomic traits involved in both quality and disease resistance since the mid nineties (Byrne and McMullen, 1996).

Once candidate genes have been isolated and analysed for the mapping population, the dataset can be transferred and positioned onto the *Rubus* linkage map. This allows candidate genes to be associated with previously identified chromosomal regions linked

to phenotypic traits. If a candidate gene is found to underlie particular QTLs which are associated with a phenotypic trait of interest, then this is further evidence in support of the candidate gene which may account for individual phenotypic variability. Several candidate genes of interest have now been isolated in red raspberry as a result of utilising homologous species and current databases throughout this study which is a major step towards marker assisted breeding strategies.

4.3.1 Aquaporin water channel proteins

Aquaporins, such as membrane and tonoplast intrinsic proteins, offer an alternative route to the lipid bilayer for water flux by means of water channels. Aquaporins are highly efficient and selective in their ability to conduct water in the direction of an osmotic gradient (Agre *et al*., 1993) while facilitating an increase in water permeability of cell membranes through the formation of water specific pores (Smart *et al*., 2001).

4.3.2 Expansin

Tissue softening is found to accompany ripening of many fruit species while often initiating the process of irreversible deterioration. Expansins are plant cell wall proteins expressed in growing organs which have been proposed to disrupt hydrogen bonds found within the cell wall polymer matrix. The resultant cell wall disassembly is a key ripening associated metabolic event in fruit which determines not only timing but extent of fruit softening and ultimately the rate of fruit deterioration (Civello *et al*., 1999).

4.3.3 Galactose dehydrogenase

Due to a deficiencies found in the enzyme catalysing L-gulono-1,4-lactone dehydrogenase in humans, primates and some other small mammals, fresh fruit and vegetables remain the principal source of vitamin C (ascorbic acid). This vitamin has several advantageous properties in both man, acting as an antioxidant and enzyme co-factor, and in plants, as an antioxidant and preventing oxidative stress. As one of the last enzymes in the defined biosynthetic pathway, galactose dehydrogenase plays a vital role in ascorbic acid synthesis (Stevens *et al*., 2007).

4.3.4 Anthocyanin reductase

Anthocyanin reductase catalyses the NADPH-dependent reduction of anthocyanidins, such as cyanidin and delphinidin, to the 2,3-cis-flavan-3-ols epicatechin and epigallocatechin (Xie *et al*., 2003). Anthocyanin synthase (ANS) is responsible for the conversion of leucoanthocyanidins to anthocyanidins which is the first step in anthocyanin biosynthesis. Leucoanthocyanidin reductase (LAR) is the branching point for subsequent condensed tannin synthesis of 2,3-trans-flavan-3-ols, such as catechin, through the conversion of leucoanthocyanidins (Xie *et al*., 2003).

4.3.5 Dihydroflavonol 4-reductase

Dihydroflavonol 4-reductase (DFR) is an enzyme involved in the conversion, by a process of reduction, of dihydroflavonols to leucoanthocyanidins. An example of this is the conversion of dihydrokaempferol (DHK) to leucopelargonidin which is the precursor for pelargonidin production. In plants, pelargonidin is implicated in a change of flower colour from pale pink to brick red and accounts for around 12% of the edible elements of plants (de Pascual-Teresa and Sanchez-Ballesta, 2008). This is an important process in the biosynthesis of anthocyanins, proanthocyanins and other flavonoids pertinent to plant survival.

4.3.6 Flavonoid 3'-hydroxylase

Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H) are genes belonging to the cytochrome P450 superfamily involved in the hydroxylation of flavonoid B rings (Xie *et al*., 2003). These rings are involved in determining flower colour controlling the ratio of anthocyanins present (Xie *et al*., 2003; de Pascual-Teresa and Sanchez-Ballesta, 2008). F3'H initiates the synthesis of 3'-hydroxylated anthocyanins such as cyanidin whereas F3'5'H is involved in the synthesis of 3'5'-hydroxylated anthocyanins such as delphinidin (de Pascual-Teresa and Sanchez-Ballesta, 2008). Pelargonidins are reported to produce red to orange colouring, cyanidin red to violet and delpinidin a violet to blue colour (Xie *et al*., 2003). F3'H and F3'5'H are also associated with the biosynthetic pathway of flavonol synthesis (quercetin and

myricetin) and flavan-3-ols (catechin, epicatechin and epigallocatechin) in grape (Jeong *et al*., 2006).

4.3.7 Flavonol synthase

The synthesis of flavonols such as kaempferol, quercetin and myricetin from dihydroflavonols are achieved by the action of flavonol synthase (FLS) (Nielsen *et al*., 2002). Flavonols are derived from dihydroflavonols forming an alternative branch of the flavonoid pathway. This branching effect is facilitated by the action of flavonol synthase which is a soluble enzyme requiring the presence of the cofactors 2-oxoglutarate, ascorbate and ferrous iron to introduce a double bond between the C-2 and C-3 rings of dihydroflavonols (Nielsen *et al*., 2002). This reaction requires molecular oxygen and FLS is therefore referred to as a 2-oxoglutarate dependent dioxygenase (Wellman *et al*., 2002).

4.3.8 Glutathione S-transferase

Following synthesis of anthocyanins in the cytoplasm, the molecules are transported to the vacuole where they bind with a protein matrix forming anthocyanin vacuolar inclusions (AVIs) (Zhang *et al*., 2004). This transfer is facilitated by glutathione S-transferase (GST). GST is involved in the last defined step in anthocyanin biosynthesis and involves the addition of a glutathione onto cyanidin 3-glucoside. This glutathione "tag" allows the cyanidin molecule to be transported into the vacuole by means of a tonoplast Mg ATP GS-X pump (Alfenito *et al*., 1998; Lu *et al*., 1998; Marrs *et al*., 1995).

4.3.9 Hypothetical proteins

A hypothetical protein is a protein whose existence has been predicted following a bioinformatics search which has identified a large open reading frame (but no evidence as to its function), therefore the sequence is unassigned but could potentially encode a protein.

4.3.10 Major latex-like proteins

Major latex-like proteins (MLP) have been identified in several plant species including strawberry, peach, melon, cucumber, soybean, *Arabidopsis* and were first identified in opium poppy latex (Lytle *et al*., 2009). Although the functions of MLPs remain unknown, they have been associated with the development of fruit and flowers and also in pathogen defence responses in plants (Lytle *et al*., 2009).

4.3.11 Metallothioneins

Metallothioneins are small proteins, with a coding region of around 80 amino acids, which contain a high proportion of Cys motifs. Found in a variety of organisms, and often in fruit, metallothioneins are involved in the detoxification of heavy metals including cadmium, copper and zinc (Moisyadi and Stiles, 1995).

The findings from this chapter have highlighted the importance of using various plant sources for the isolation of candidate genes in target species. While some gene sequences were found to be highly conserved across species others required alternative methods for isolation. The results from the fruit EST libraries identified a large number of polymorphisms within individual samples which suggests that employing 454 mRNA sequence technology will yield a great deal of informative sequence data in raspberry. Genes which were successfully isolated in this Chapter will be used to enhance the current *Rubus* linkage map which is a further step towards developing marker assisted breeding strategies.

Chapter 5 Quantitative trait loci mapping

5.1 Introduction

Many traits of agronomic importance, such as disease resistance and organoleptic quality, exhibit a quantitative inheritance. This often results in phenotypic inheritance which differs markedly in its expression amongst individuals (Pflieger *et al*., 2001). This is often found as a result of multiple gene involvement, many of which can be influenced by the environment (Pflieger *et al*., 2001). It is the identification of molecular markers which allow quantitative traits, such as quality, to be dissected into discrete regions of quantitative trait loci (QTLs) (Causse *et al*., 2004). The existence of such QTLs within chromosomal regions reveals the presence of at least one polymorphic locus segregating in this location and it is this which imparts trait variation (Causse *et al*., 2004). A high proportion of natural variation found within plant species can be attributed to minor genetic changes occurring within many individual genes. In order to explore this genetic variation and identify genes involved, a method commonly deployed is quantitative trait loci analysis (Kearsey, 1998). Annual environmental variations are also likely to involve a large number of genes as well as varied physiological mechanisms involved in fruit quality traits. This could therefore lead to the detection of different QTLs for the same trait identified in different years (Fanizza *et al*., 2005).

5.1.1 Quantitative trait loci mapping studies

Quantitative trait loci are regions of DNA which correspond to a continuous phenotypic trait within a population, while 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 (Collard *et al*., 2005). The identification of QTLs can then progress to DNA sequencing to find genes responsible for specific variations in phenotype. Gene information can then be inserted into databases for direct comparisons to genes of known function for a greater understanding of genotype:phenotype relationships. QTL mapping is aimed at the identification of loci which are responsible for the variation seen in complex quantitative traits. By identifying the loci responsible for improved quality traits it may

be possible to bring favourable alleles together to produce an elite cultivar (O'Borevitz and Chory, 2004).

One of the most studied factors for QTL analysis in economically important crops is total soluble solids (TSS). This trait however has remained somewhat elusive due to its polygenic nature as well as evidence suggesting a pleiotropic relationship between TSS and fruit yield, size and overall weight (Fridman *et al*., 2002). Yates *et al*. (2004) found fruit yield decreased while soluble solids increased in tomato while Monforte *et al*. (2004) identified a different relationship between the two traits in melon. This led to the discovery that many genes identified in tomato correlate with those in melon allowing candidate genes identified in one plant to be transferred across to another (Monforte *et al*., 2004; King *et al*., 2001). QTLs contributing to fruit size and shape were also found to be similarly localised within tomato and peppers indicating these QTLs may be orthologous. Comparative maps have so far been developed between tomato and potato, tomato and pepper and tomato and aubergine allowing the development of synteny between genomes (Cheng *et al*., 2004).

Other recent studies looked for repeatable QTLs involved in sucrose accumulation between two different environments over two years in sugarcane. This study found TSS to be moderately heritable despite previous breeding techniques showing limited improvements (Aitken *et al*., 2006). One explanation for this may be that the QTL for TSS has unfavourable pleiotropic effects on other traits under selection (Aitken *et al*., 2006). Dirlewanger *et al*. (1999) identified the organic acid, sugar content and composition of fruit to be the main contributing factors to perceived quality in peach. Using the candidate gene strategy this study looked at QTLs associated with peach quality by measuring factors involved in overall development such as blooming date, maturity and sugar/acid contents over two years. QTLs were found for all agronomic characters except skin colour while QTLs for pH and sugar content were co-located as found in tomato (Dirlewanger *et al*., 1999). This led to eight putative candidate genes being mapped for peach, (two of which had a previously known function) the remaining

genes were selected from differential cDNA screened at early growth stages (Dirlewanger *et al*., 1999).

5.1.2 Functional candidate gene analysis

A potential strategy for the identification of genes which underlie a QTL region is the functional candidate gene approach (de Vienne *et al*., 1999). This method is based on the "a priori" selection of genes, which are suspected of being functionally related to the trait of interest. If a correlation is identified between a trait and a putative candidate gene and a subsequent allelic polymorphism is discovered within this gene then this provides a strong argument in favour of the candidate gene. If no polymorphism is present, however, then the candidate gene can be ruled out of involvement in trait variation (Etienne *et al*., 2002). This approach has been successful in the identification of several candidate genes including sucrose accumulation in tomato (Klann *et al*., 1996) and carotenoid accumulation in red pepper (Huh *et al*., 2001).

5.1.3 Linkage maps

Genetic linkage is the inheritance of genetic loci or alleles found on the same chromosome which are located in close proximity to each other which, as a result, stay together during meiosis. The first genetic linkage map was constructed in 1915 by Thomas Hunt Morgan (Goodenough, 1984). Morgan proposed that genes which are located in close proximity to one another on a chromosome are most likely to assort with each other on a regular basis (close linkage) compared with those which are located further apart (Watson *et al*., 2004). It was reasoned therefore that genes could be located according to their relative position to one another on a chromosome in the form of a genetic map (Watson *et al*., 2004). This was carried out using the number of recombination events as a measure of proposed distance between genes. One centimorgan (cM) is defined as the distance between genes in which one product of meiosis in one hundred is recombinant (Goodenough, 1984).

The production of linkage maps can facilitate the development of molecular markers with the identification of genes which underlie phenotypic traits. These differences in phenotypic traits may be identified in several ways including the appearance of enzymatic activities or variations in restriction fragment length (Mohan *et al*., 1997).

Once the appropriate number of markers have been identified segregating in a desired mapping population, a linkage map can be constructed by first grouping the markers into appropriate linkage groups. The number of linkage groups will ideally equal the number of haploid chromosomes (Graham *et al*., 2008). Once a comprehensive linkage map has been developed identifying molecular markers across the chromosomes, these can be used to examine homology which may exist between other Rosaceous crops as well as looking for synteny between the position/ordering of markers identified on anchored physical maps.

5.1.4 Genetic Markers

Genetic markers are specific DNA sequences which represent genetic differences which are present between organisms or species. While they do not represent individual genes they are indicative of their presence. These markers do not necessarily affect the phenotype of individuals but are linked to the genes which control the trait (Paterson, 1996). Molecular markers are the most abundantly found and used type of marker and these vary in format from point mutations to insertions, deletions and tandem repeat errors and include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs) amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs; microsatellites), and single nucleotide polymorphisms (SNPs) (Paterson, 1996). While morphological and biochemical markers are found to be influenced by environmental and developmental factors, molecular markers are not (Collard *et al*., 2005). Molecular markers and resultant linkage maps are useful tools for the identification of genes underlying both quantitative and qualitative traits (Tanksley *et al*., 1989). Candidate genes which underlie these QTLs can then be

proposed from an understanding of the biochemical or developmental pathways which are known to affect the trait of interest (Pot *et al*., 2006).

5.1.5 Quantitative trait loci validation

Genetic markers are a common means for enhancing the selection of new cultivars with specific quantitative traits. It has been recognised, however, that quantitative trait loci (QTL) must first be validated across a wider genetic background before marker assisted selection (MAS) can commence (Knoll and Ejeta, 2008). It was suggested by Dudley (1993) that there are three important factors regarding whether a QTL marker identified within one population can be selected for in an unrelated population; firstly the presence of marker QTL segregation, which may be in the form of a polymorphism within the QTL or marker of choice, secondly the linkage phase of the marker and QTL of interest should be identified (coupling or repulsion) and thirdly possible epistatic interactions between the QTL and other loci must be explored (Dudley, 1993).

It would be wrong to assume that marker-QTL linkage will still be present in different genetic populations or environments, particularly when the phenotypic trait is of a complex nature such as quality (Reyna and Sneller, 2001). QTL mapping experiments must therefore be validated over several years and/or environments in order to be transferrable across populations (Kenis *et al*., 2008). Such QTL validation involves testing marker effectiveness in the determination of a targeted phenotype in unrelated populations or in individuals from a wider genetic background (Collard *et al*., 2005).
Therefore, if markers are to be considered useful in a breeding programme they must reveal polymorphisms across a range of populations (Langridge *et al*., 2001).

5.1.6 Genetic Diversity

In tomato, as with many other self pollinating crops, a reduced level of genetic variation has been identified in elite cultivars in comparison to their wild relatives. As a result Tanksley and McCouch (1997) proposed that wild germplasm containing novel alleles could be introduced into elite cultivars to increase genetic variation and hence crop

quality. This has proven successful in tomato where Frary *et al*. (2002) introduced selected DNA inserts from wild to elite species resulting in an increase in soluble solid yield, colour, viscosity and earlier fruit ripening. As a result, QTLs for soluble solids were found to be located on different introgressions in two separate wild species indicating the possibility of combining such QTLs via *cis* recombination into an elite variety (Yates *et al*., 2004). This involved constructing overlapping introgressions for portions of chromosome 4 from two wild tomato species which led to the identification of multiple non-allelic loci responsible for soluble solids and fruit weight content in individuals as well as shape and epidermal reticulation co-localised within a single chromosomal region (Yates *et al*., 2004). Similar studies in raspberries found a reduction in both morphological and genetic diversity between cultivars, as a result of domestication, leaving wild germplasm as a potential reserve for genetic diversity (Graham *et al*., 2002). This similarity in modern cultivars raises concerns as a lack of genetic diversity can lead to inbreeding depression as well as an increased susceptibility to external stresses (Graham *et al*., 2003; 2007). The introduction of new genes from a different background can reverse the deleterious effects encountered through inbreeding which, following the identification of QTLs linked to desirable traits can be combined in order to produce an elite cultivar targeted towards the high-value fresh market. This chapter aimed to enhance the current *Rubus* linkage map with the addition of candidate genes and ESTs identified in Chapter 4. QTL mapping of phenotypic traits analysed in Chapter 3 will then allow chromosomal regions to be associated with markers and genes which is a major step towards MAB.

5.2 Results

5.2.1 Linkage map construction

A linkage map from two diverse raspberry cultivars, the European cv. Glen Moy and the North American cv. Latham (ca. 60% similarity; Graham and McNicol, 1995) produced an initial map with SSRs, derived from genomic and cDNA libraries and AFLP markers (Graham *et al*., 2004), while an enhanced map used additional EST-SSR and gene markers (Graham *et al*., 2006). This linkage map has been further updated following the

addition of candidate genes and ESTs derived from this project, using JoinMap 3 (Van Ooijen and Voorrips, 2001). Markers were tested for heterogeneity (using the chi-squared test in JoinMap), and markers with significant heterogeneity were excluded. Markers were partitioned into linkage groups using a LOD from 10.0 to 4.0 (Table 5.1). Map distances between markers were derived using the Kosambi mapping function to convert recombination units into genetic distances (Kosambi, 1944).

5.2.2 Candidate gene mapping

The identification and isolation of putative candidate genes served to enhance the current *Rubus* linkage map while facilitating the potential of marker assisted breeding strategies. Candidate genes (identified in Chapter 4) were added to the current genetic linkage map in order to identify any trait association which may exist between genes and phenotypic variation (Table 5.2). The mean chi-squared values, obtained from JoinMap 3.0, were used as an indication of the goodness of fit of regression mapping to the pairwise estimates of recombination frequencies for all markers and candidate genes obtained. The results for all linkage groups were within a range of 0.152 to 4.180 indicating good support for the ordering of markers within groups.

Table 5.1. Raspberry genetic linkage map based on 283 markers mapped after round 1 and 2 of Joinmap 3.0 analysis.

Linkage group	cM	AFLP	SSR	EST-SSR	Bac-SSR	Candidate genes	Mean marker distance
1	171.0	13	10	4	4	3	5.03 cM
2	148.6	30	8	6	7	6	2.61 cM
3	158.7	35	16	6	2	2	2.60 cM
4	148.9	13	11	4	1	5	4.38 cM
5	106.0	26	10	6	1	3	2.30 cM
6	105.9	25	6	1	4	0	2.94 cM
7	81.7	4	2	6	1	2	5.45 cM

Table 5.2. Raspberry candidate gene and QTL mapping

Candidate Gene	**Function**	**LG**	**Traits with significant association to gene**
Tonoplast Intrinsic Protein (TIP)	Transporter	2	Colour Brix Total anthocyanins
Membrane Intrinsic Protein (MIP 1/2)	Transporter	2	Colour Weight
Membrane Intrinsic Protein (MIP 3)	Transporter	2	Colour Weight
Galactose Dehydrogenase (Gal-dh)	Acid biosynthesis	4	Weight
Flavonol synthase	Flavanol pathway	4	Colour Total anthocyanins
Glutathione S-transferase	Transportation of flavonoid compounds	4	Colour Total anthocyanins
Dihydroflavanol 4-reductase	Anthocyanin pathway	4	Colour Total Anthocyanin
Flavonoid 3'Hydroxylase F3'H	Flavonoid biosynthesis	5	Weight Brix Total phenol
Expansin (EXP)	Cell expansion	7	pH
Contig 16	Unknown	6	Colour
Contig 21	Hypothetical	3	Colour
Contig 24	Hypothetical	3	Colour

5.2.3 QTL analysis

QTL mapping was carried out using the MapQTL 5 software (Van Ooijen, 2004). A Kruskal-Wallis test was used as a preliminary assessment to identify regions of the genome linked to each of the phenotypic measurements across seasons and site (Chapter 3) and whether the phenotype was affected by alleles from one parent or from both. A permutation test with 1000 permutations was used to establish 95% and 99% significance levels for QTL interval mapping (IM), with a LOD score of 4.3 and 5.1 respectively, selected for a four-mean QTL model. If the Kruskal-Wallis analysis indicated that the phenotype was affected by alleles from only one parent, the trait can be analysed using a two-mean QTL model, although for this analysis a four-mean QTL model was adopted for all traits.

For the purpose of QTL illustration in this thesis, the bars representing individual QTLs have been aligned directly opposite markers which underlie the trait. This was carried out as many of the linkage groups (LG) are saturated with markers making individual marker associations difficult to determine when a large number of QTLs are presented. All seven linkage groups, or chromosomes, were identified as having significant associations with raspberry fruit quality traits.

5.2.3.1 Linkage group 1

Measures for ten berry weight mapped to LG 1 for all three environments in 2007 and 2008 only. The most significant markers for fruit weight include bes_Ri31G22R (81.8 cM) and bHLH (107.5 cM) accounting for 21.4% and 13.4% variation respectively. QTLs were also detected for pH in the field (mid point Rub262b, 95.8 cM) and the commercial (Ri4CL3SNP, 3.4 cM) fruit only. One QTL was recorded for total phenol measurements from the SCRI protected tunnel with a mid point at Rub262b accounting for 15% trait variation (Figure 5.1).

1

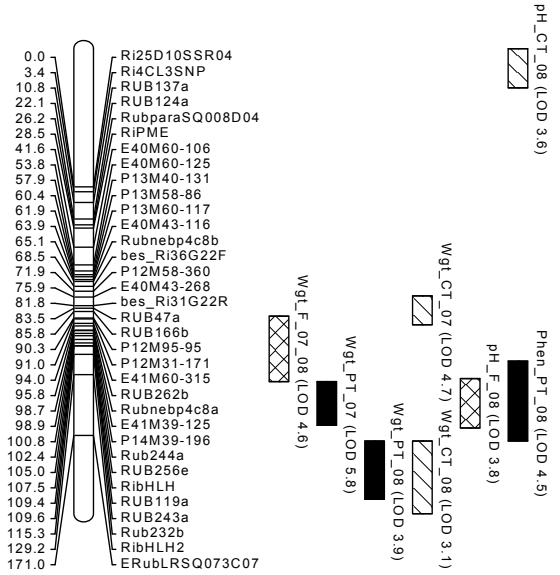


Figure 5.1. QTL regions on linkage group 1. Check boxes represent SCRI field (F), filled boxes SCRI protected (PT) and striped boxes represent commercial protected sites (CT). Each trait is identified with an abbreviation (i.e wgt: weight, Phen: phenol) followed by the site (F, PT or CM) and the year (06, 07 or 08).

5.2.3.2 Linkage group 2

All measures for colour, whether visual or instrumental, mapped to LG 2 and were stable from year to year and site to site. Candidate genes have been identified for certain colour QTL. The mid-point of a range of QTLs on LG 2 were the raspberry aquaporin, TIP at 99 cM (transporter) but other transport genes have also been identified in this region including two MIPs (86.3 to 88.0 cM). This major QTL explained around 21% of the variation and was found across all sites and seasons.

A Kruskal-Wallis test was used for preliminary identification of areas of the genome affecting colour and other measurements, using a significance level $p < 0.001$. This identified a region close to marker bes_Ri29G13R (97.7 cM) and Rub6a (101.9 cM) on LG 2, containing aquaporins (MIP and TIP), as associated with colour meter measurements, visual scores and total anthocyanin (TA) for most combinations of sites and years (Figure 5.2). Markers from both parents were significant in this region ($p < 0.001$).

Table 5.3 shows the results obtained for a selection of traits in response to marker Ri29G13R. Individuals with an *a* allele were found to contain higher values of total anthocyanin, total phenol, ^{o}Brix, ten berry weight and visual colour measurements, while displaying lower values for colour meter measurements Yxy. Individuals with a *b* allele showed high colour meter measurements and lower visual and total anthocyanin results while the *d* and *c* alleles were found to display intermediate results.

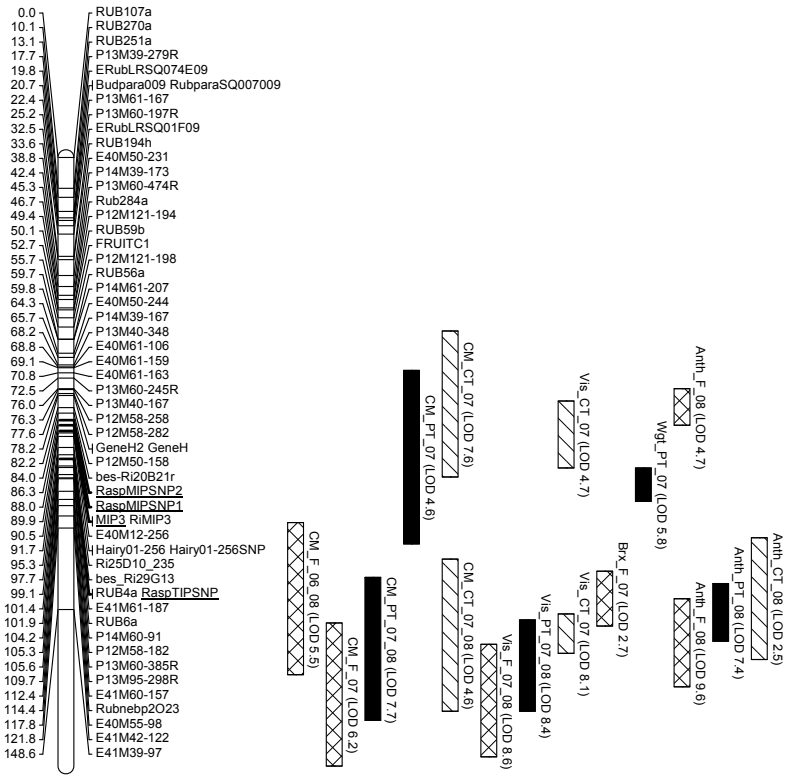


Figure 5.2. QTL regions on linkage group 2. Check boxes represent SCRI field (F), filled boxes SCRI protected (PT) and striped boxes represent commercial protected sites (CT). Each trait is identified as follows: CM, colour meter; Vis, visual colour; Brx, ^{o}Brix; Anth, total anthocyanin and wgt, ten berry weight.

Table 5.3. Potential marker from LG 2 for marker assisted breeding. The preferential allele combination for each trait is highlighted in bold. (Results displayed were obtained from the SCRI polytunnel, 2008 and were reflected across environments).

Marker Ri29G13R (97.7 cM)

Trait measurement	Allele combination				
	ac	**ad**	**bc**	**bd**	**Std Dev**
Y	**15.2**	15.7	15.8	16.7	0.6
X	**0.383**	0.384	0.385	0.386	0.002
Y	**0.319**	0.320	0.320	0.321	0.001
ΔE	**53.2**	52.8	52.8	51.9	0.6
Visual colour	**3.7**	3.3	3.2	2.7	0.4
Anthocyanins	**94.0**	92.4	77.6	58.7	16.4
Total phenol	**137.8**	133.3	137.4	132.1	2.9
^{o}Brix	**7.8**	7.5	7.8	7.6	0.2
Ten berry weight	**37.4**	34.4	33.1	34.8	1.8

5.2.3.3 Linkage group 3

Several markers across linkage group 3 were significant ($p < 0.001$), particularly for fruit harvested in 2006 from the field ($p < 0.0001$), but four separate QTL clusters were identified. The first QTL cluster, for colour, at the top of the LG had a mid point at Leaf86 (32.6 cM) accounting for 17% of the variation. The next QTL for colour, ^{o}Brix and total anthocyanins had a midpoint at Rub2a1, an SSR (63.2 cM) with a variation of between 15% and 23%. Rub2a1 was found to segregate in both parents and was putatively identified as an aminotransferase.

A further QTL for colour, total anthocyanins and total phenols, contained a MYB (82.5 cM) and a 4CoA ligase gene (Ri4CL1SNP, 83.6 cM) and accounts for 15% variation (Figure 5.3). Two MYB genes were mapped together on LG 3 associated with ripening in raspberry, particularly in the transition from green to red fruit (Graham *et al*., 2009). MYB genes are known to control phenylpropanoid metabolism including anthocyanin accumulation in fruit but also have important roles in many other aspects of plant development including trichome development, signal transduction, disease resistance and cell division (Koes *et al*., 1994). Rub2a1 was significant ($p < 0.001$) for Yxy and TA and for the visual colour score ($p = 0.014$). Allele combinations of *ac* and *bc* for this marker had significantly higher values for colour meter values (Yxy) and were significantly lower for visual colour, total anthocyanins and total phenol than the *ab* and *bd* combinations. This indicates that it is the 'Glen Moy' allele which is most important here (Table 5.4).

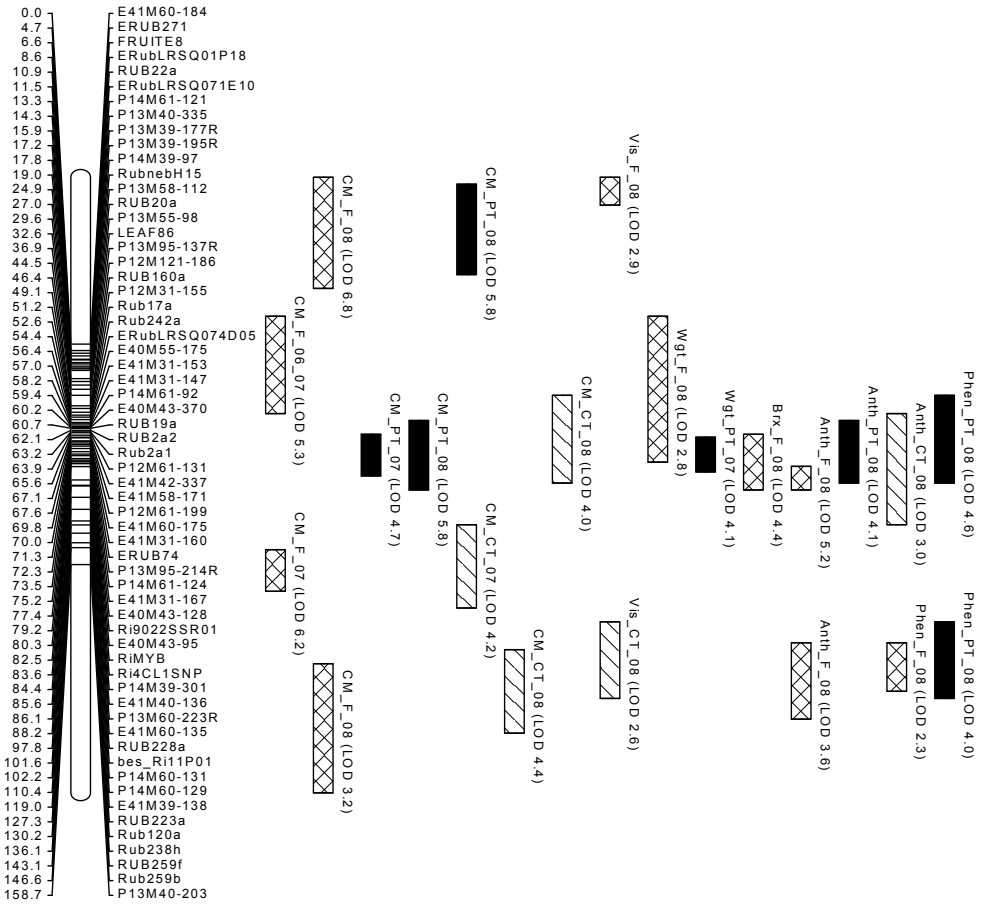


Figure 5.3. QTL regions on linkage group 3. Check boxes represent SCRI field (F), filled boxes SCRI protected (PT) and striped boxes represent commercial protected sites (CT). Each trait is identified as in Figure 5.2.

Table 5.4. Potential marker from LG 3 for marker assisted breeding. The preferential allele combination for each trait is highlighted in bold. (Results displayed were obtained from the SCRI polytunnel, 2008 and were reflected across environments).

Marker Rub2a1 (63.2 cM)

Trait measurement	Allele combination				
	ac	**ad**	**bc**	**bd**	**Std Dev**
Y	16.2	**15.5**	16.6	15.6	0.5
X	0.390	**0.382**	0.388	0.381	0.004
Y	0.320	**0.319**	0.321	0.319	0.001
ΔE	52.9	**52.8**	52.3	52.7	0.3
Visual colour	3.2	**3.5**	3.1	3.3	0.2
Anthocyanins	76.8	**91.0**	63.3	86.8	12.3
Total phenol	120.6	**155.7**	115.2	132.3	18.0
^{o}Brix	**7.9**	7.8	7.5	7.5	0.2
Ten berry weight	36.1	36.7	**37.3**	32.0	2.4

5.2.3.4 Linkage group 4

LG 4 contained two discrete QTL clusters which were significant for 2007 and 2008 only (Figure 5.4). The first region accounts for around 12% variation and contains three genes involved in anthocyanin biosynthesis, flavonol synthase (RiFS), dihydroflavonol 4-reductase (RiDFR1 and 2) and glutathione S-transferase (ERUB161). FLS is a key enzyme in flavonoid biosynthesis regulating the balance between anthocyanin and flavonol synthesis with mutant *fls1* seedlings in *Arabidopsis* showing enhanced anthocyanin levels (Stracke *et al*., 2009).

Marker FLS (*ab* × *ab* marker at 73.1 cM) on LG 4 was significant for colour meter analysis (particularly sample brightness, Y), the visual score and TA ($p < 0.001$). The *bb* genotype had significantly lower values of Y, x and y and significantly higher visual scores and TA than the *aa* genotype, while *ab* was intermediate. Similiarly, for dihydroflavonol 4-reductase (RiD4R2, 64.6 cM) the *bb* genotype was found to confer lower Yxy values and higher total anthocyanins while the *aa* genotype displayed higher total phenol, ^{o}Brix and ten berry weight measurements (Table 5.5). The second QTL region, significant for visual colour analysis in fruit from the SCRI field and protected tunnel in 2007 only, contained an EST-SSR, FruitE4 (110 cM), encoding a bZIP transcription factor (Kassim *et al*., 2009). A single discrete QTL was also found on LG 4 for fruit weight under protected covering at SCRI which has a mid point at RaspGalDHSNP at 114.8 cM (galactose dehydrogenase) accounting for 10% variation. A number of genes in the organic acid (galactose dehydrogenase), anthocyanin and flavonol pathways (flavonol synthase and dihydroflavonol 4-reductase) were identified on LG 4 as well as a gene involved in flavonoid transportation (glutathione S-transferase). bZIP transcription factors can be expressed constitutively or tissue specifically and regulate diverse processes including photomorphogenesis and hormone signalling (Holm *et al*., 2002). Precise roles for the transcription factors underlying antioxidant QTL remain unclear but roles in light or hormone signalling cannot be ruled out.

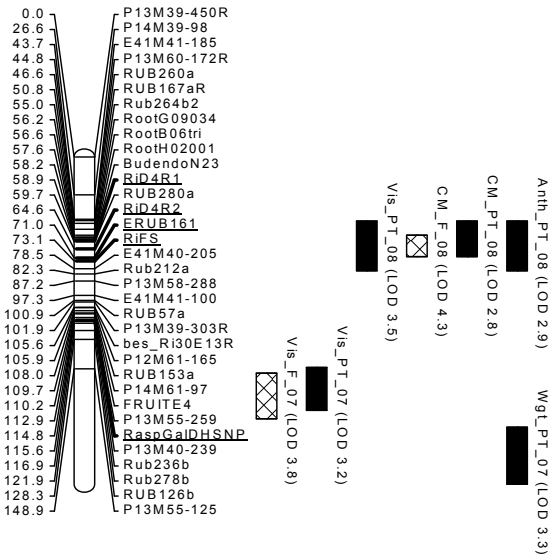


Figure 5.4. QTL regions on linkage group 4. Check boxes represent SCRI field (F), filled boxes SCRI protected (PT) and striped boxes represent commercial protected sites (CT). Each trait is identified as in Figure 5.2.

Table 5.5. Potential markers from LG 4 for marker assisted breeding. The preferential allele combination for each trait is highlighted in bold. (Results displayed were obtained from the SCRI polytunnel, 2008 and were reflected across environments).

Candidate gene, Flavonol synthase (RiFLS at 73.1 cM)

Trait measurement	Allele combination			
	aa	**ab**	**bb**	**Std Dev**
Y	16.2	15.7	**15.5**	0.4
X	0.388	0.384	**0.382**	0.003
Y	0.321	0.320	**0.319**	0.001
ΔE	52.6	52.8	**52.8**	0.1
Visual colour	3.1	3.2	**3.7**	0.3
Anthocyanins	68.9	82.0	**94.5**	12.8
Total phenol	134.2	**141.5**	127.6	7.0
^{o}Brix	**7.8**	7.6	7.5	0.2
Ten berry weight	**36.4**	34.1	34.6	1.2

Candidate gene, dihydroflavonol 4-reductase (RiD4R2 at 64.6 cM)

Trait measurement	Allele combination			
	aa	**ab**	**bb**	**Std Dev**
Y	16.1	15.7	**15.6**	0.3
X	0.386	0.385	**0.381**	0.002
Y	0.320	0.320	**0.319**	0.001
ΔE	52.6	52.9	**52.6**	0.2
Visual colour	3.3	3.3	**3.4**	0.1
Anthocyanins	73.7	82.9	**89.2**	7.8
Total phenol	**142.7**	137.4	129.0	6.9
^{o}Brix	**7.87**	7.59	7.70	0.1
Ten berry weight	**36.9**	35.7	33.8	1.6

5.2.3.5 Linkage group 5

Three significant QTL regions were found on LG 5 related, predominantly, to fruit weight and total soluble solids (^{o}Brix) with many clusters showing stability across both site and season. The first QTL region had a mid point of RubnebAo9b (28.5 cM) and included QTLs for weight, ^{o}Brix, pH and total phenol content in a chromosomal region in close proximity to the candidate genes flavonoid 3'-hydroxylase (F3'H) and polygalacturonase (RiPG). The second main QTL cluster for weight and ^{o}Brix, spans around 15cM and contains an AFLP marker P13M60-110 (65.3 cM) as the mid-point (Figure 5.5) accounting for 15% variation for each individual QTL. The final QTL cluster for weight and ^{o}Brix, contains the MADS-01 at 82.7 cM (MADS-Box) transcription factor, involved in flavonol biosynthesis, reproductive, leaf and root development (Vrebalov *et al*., 2002).

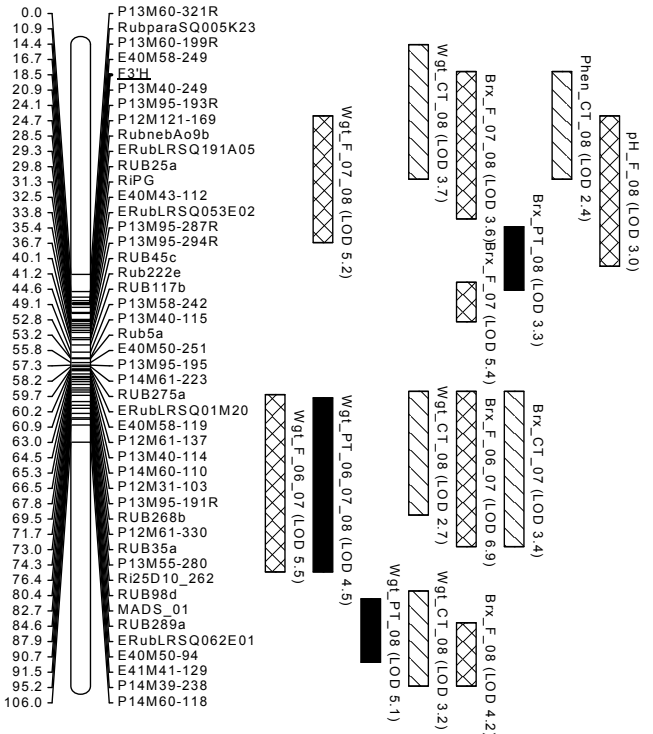


Figure 5.5. QTL regions on linkage group 5. Check boxes represent SCRI field (F), filled boxes SCRI protected (PT) and striped boxes represent commercial protected sites (CT). Each trait is identified as in Figure 5.2.

5.2.3.6 Linkage group 6

LG 6 contained a scattering of discrete QTLs for colour meter and visual colour measurements only. In particular colour measurement y which identifies individual colour wavelengths was found to be significant across sites and seasons. The genomic SSR, Rub118b (8.3 cM) was found to be the most significant marker on LG 6 with 14% trait variation explained (Figure 5.6).

Although the dominant AFLP markers are most significant around the QTL regions on LG 6 there were two significant co-dominant EST-SSRs, Leaf 97 (2.8 cM) and Leaf 102 (13.2 cM). Leaf 102 is an *ab* × *aa* marker with no (current) identified homology. Leaf 97 has been identified as having significant homology ($p = 0.017$) with a cyclin 2b protein (Graham *et al*., 2004).

6

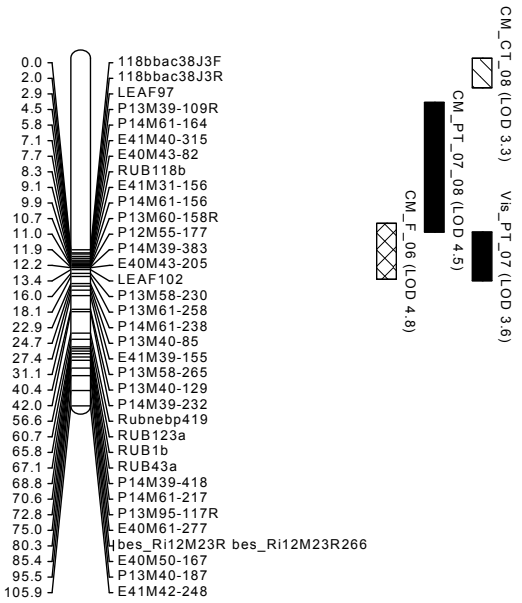


Figure 5.6. QTL regions on linkage group 6. Check boxes represent SCRI field (F), filled boxes SCRI protected (PT) and striped boxes represent commercial protected sites (CT). Each trait is identified as in Figure 5.2.

5.2.3.7 Linkage group 7

LG 7 contained three QTLs which were detected in 2008 only. Although the QTLs detected showed a lower threshold LOD than all others reported, both KW and IM indicated that these markers were involved in trait variation. One QTL, located at the top of the LG, for pH in fruit grown at the SCRI protected site had an expansin gene (13.2 cM) underlying the trait which was responsible for 8% variation. At the lower end of the LG (67.6 cM) a QTL for SCRI protected and field grown fruit was detected for ^{o}Brix and colour meter analysis in a region spanning 16 cM. This area accounted for around 8% of trait variation and contained a number of interesting markers (Figure 5.7).

RootH01001 (70.4 cM) showed significant homology to xyloglucan endotransglycosylase (Woodhead *et al*., 2008) which is a major component of the primary cell wall (Eckardt, 2004). RiPL is a pectate lyase gene which catalyses the cleavage of de-esterified pectin which is a major component of the primary cell wall and also acts in the activation of plant defence systems (Marĩn-Rodrĩguez *et al*., 2002; J. Graham, personal communications). Finally a PKS5 gene (78.8 cM), which is a member of the aromatic polyketide synthase (PKS) family, identified as a chalcone synthase gene, underlies this QTL. With at least 11 different PKS genes identified in raspberry to date, these genes carry out a number of functions, for example acting as precursors for flavonoid synthesis and aroma volatiles (Zheng and Hrazdina, 2008).

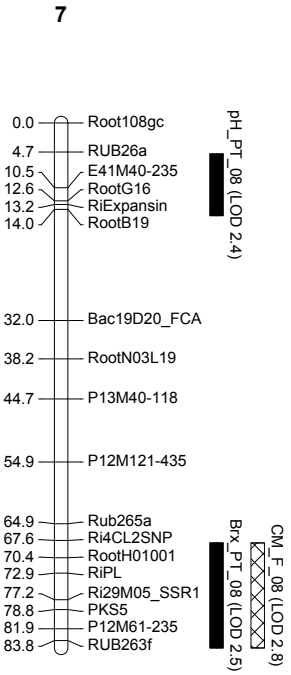


Figure 5.7. QTL regions on linkage group 7. Check boxes represent SCRI field (F), filled boxes SCRI protected (PT) and striped boxes represent commercial protected sites (CT). Each trait is identified as in Figure 5.2.

5.2.4 QTL heritability

Trait heritability is the expression of the proportion of total phenotypic variation obtained within a sample which has been contributed by genetic variance (Goodenough, 1984). Heritability can be expressed as either broad sense, which reflects all possible genetic contributions to phenotypic variance, or narrow sense, which quantifies only the proportion of phenotypic variation which is additive in nature and therefore more informative regarding variation within a specific population. Therefore if a trait is found to have a narrow sense heritability of 1 (100%) then this trait is expressed without environmental influence. Similarly if a trait shows a heritability of 0.5 (50%) this intimates a trait which is expressed as a result of both environmental and genotypic factors (Goodenough, 1984).

The colour measurements from the mapping population of 188 progeny were analysed using the statistical programme Genstat 10 for Windows (Genstat, 2007) to determine correlations among measurements and heritability. As 2006 analysed one open field site only, heritability was estimated from 2007 and 2008 trials.

In 2007 and 2008, a single replicate of each genotype was available from each site in each year. Heritability was estimated as:

$$h^2 = a\sigma_G^2 / (a\sigma_G^2 + b\sigma_{GS}^2 + c\sigma_{GY}^2 + \sigma_{GSY}^2)$$

where σ_G^2, σ_{GS}^2, σ_{GY}^2 and σ_{GSY}^2 are the variance components for genotypes, genotype × site interaction, genotype × year interaction and genotype × site × year interaction, $a \leq 6$ (number of sites × number of years), $b \leq 3$ (number of sites) and $c \leq 2$ (number of years).

Heritabilities (h^2) were found to be between 65.4 and 79.6% across the two years analysed (Table 5.6) which indicates the ability to improve traits in an appropriate marker assisted breeding strategy.

Table 5.6. Heritability estimates for fruit grown in 2007 and 2008

	2007 and 2008 Heritability estimates				
Trait	σ^2_G	σ^2_{GS}	σ^2_{GY}	σ^2_{GSY}	h^2
Y	0.820	0.000	0.000	1.056	76.8%
x	2.2×10^{-5}	2.4×10^{-6}	5.7×10^{-7}	4.8×10^{-5}	65.4%
y	2.0×10^{-6}	1.7×10^{-8}	0.00	2.1×10^{-6}	79.6%
Visual	0.420	0.003	0.007	0.641	73.2%

5.2.5 QTL validation

A selection of candidate genes underlying QTLs of phenotypic interest were validated by examining the link between polymorphisms found in a segregating population with those in a wider gene pool. Ten individual genes/markers were selected for validation (Section 2.4.3) and from this 14 previously identified SNPs, present in cv. Latham, were analysed in 12 individual cultivars. Polymorphisms were detected in all named cultivars, selected on the basis of their colour or quality attributes, for the candidate genes identified in the mapping parents and these ranged from only one SNP detected in cv. Blacknight to eight in cv. Prestige and cv. Glen Shee (Table 5.7). Although it would be possible to identify further genetic differences within the candidate genes studied and between cultivars, for the purpose of this study only the previously identified SNPs from cv. Latham were analysed. An example of one SNP detected in flavonol synthase can be seen in Figure 5.8 for the parents as well as the 12 individual cultivars.

The phenotypic consequence found in individuals, as a result of a polymorphism, will depend on where the SNP is located. A SNP in the coding region of a gene, for example, may cause an alteration in protein function or structure (Syvänen, 2001). Likewise a SNP within a promoter region can affect transcription factor binding and hence gene expression (Abraham and Kroeger, 1999). Most SNPs are found within non-coding regions of the genome, the majority of which have no direct effect on individual phenotypes, they do however, make useful markers for subsequent population, linkage disequilibrium and evolutionary studies as well as roles in MAB strategies (Syvänen, 2001). While gene prediction software identified potential amino acid changes in polypeptides in four of the candidate genes mapped, it was impossible to accurately predict SNP location and hence phenotypic consequence in partial gene sequences.

Table 5.7. Single nucleotide polymorphisms identified in a range of raspberry germplasm for a selection of candidate genes

Cultivar	Reason for interest	TIP	MIP 2	MIP 3	EXP	FLS	ANR	DFR	bHLH	MYB	118b
Blacknight	Black raspberry								1		
Blacksatin	Black raspberry		1				1			1	
Glen Coe	Purple fruit		2			1		1	1		
Willamette Spinefree	Darkest red fruit									1	
Chilliwack	Very dark red								2	1	
Malling Jewel	Dark red fruit					2			1		
Latham	Dark red fruit	1	2	1	1	2	1	2	2	1	1
Glen Ample	Quality, flavour and large fruit			1			1			1	1
Tulameen	Flavour, quality and glossy fruit			1					1	1	
Glen Moy	Quality, pale red fruit		2			1	1	1	1	1	
Rose d'cote d'or	Very aromatic					2	1		1		
Prestige	Yellow receptacles		2	2				1	1	1	1
Glen Shee	Very pale red			1		2	1	1	1	1	1
Golden Queen	Yellow fruit		2			2				1	1

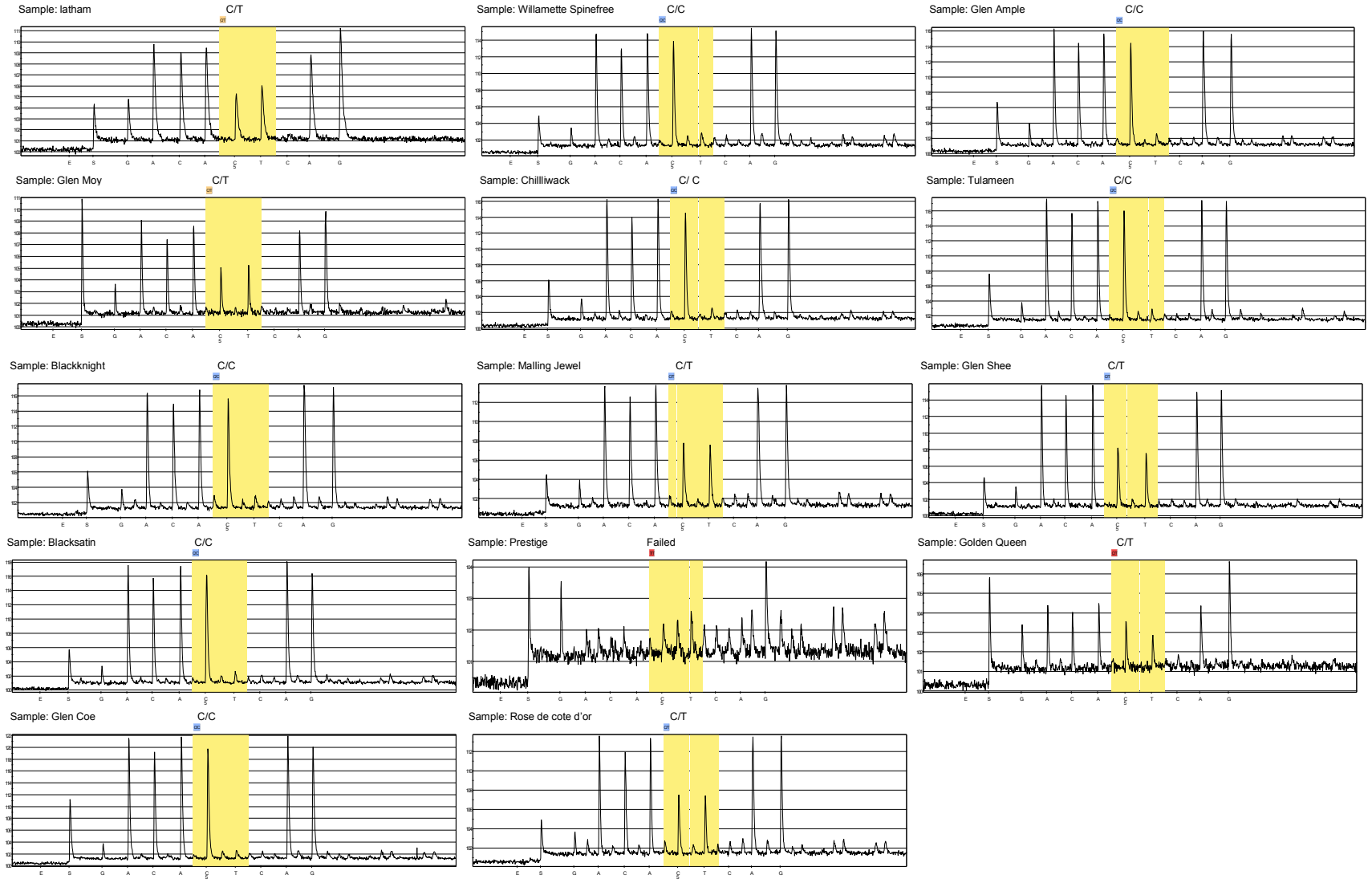


Figure 5.8. Single nucleotide polymorphisms detected in flavonol synthase for the mapping parents and a selection of raspberry germplasm. The SNP previously identified and mapped within the segregating mapping population was analysed across a diverse range of raspberry germplasm in order to confirm the presence of polymorphisms in new populations.

5.3 Discussion

A total of 75 putative QTLs were detected for all of the traits analysed. For each attribute assessed, between 4 (for pH) and 22 (colour meter) QTLs were discovered. Phenotypic variations explained by the QTL (R^2) were estimated within the MapQTL software and ranged from 32% for total anthocyanins to 8% for ten berry weight which was the lowest limit of detection included. Eight QTLs showed an R^2 of less than 10%, 55 QTLs between 10% and 20%, 9 QTLs had an R^2 of 20% to 30% and the remaining 3 QTLs had an R^2 above 30%. It is likely that these latter QTLs, for colour meter and visual measurements in fruit grown under protection, may be considered as containing major genes and include TIP and RUB6a on chromosome 2.

Several clusters of QTLs were identified mainly located on chromosomes 2, 3 and 5. These clusters may be indicative of either a fortuitous linkage or due to the segregation of a unique QTL which may control two traits as a result of a causal relationship between traits or related metabolisms. These co-located areas can be compared with the original correlations identified in Chapter 3 as two related traits would be expected to share common QTLs. The results of this Chapter can be used for marker assisted selection in order to transfer the favourable quality characteristics inherited from cv. Glen Moy into elite lines with darker colouring (higher anthocyanin content) and root rot resistance obtained from cv. Latham. As a result of the number of QTLs detected, many of which showed strong phenotypic effects, genetic progress is expected.

Improving fruit quality in red raspberry is one of the major goals facing soft fruit breeders across the world. Meeting consumer demand regarding fresh fruit quality and environmental concerns as well as superior fruit which are resistant to many pests and diseases would facilitate a quantum leap in raspberry genetics. Gene function, regulation and expression can be identified through the mapping and sequencing of plant genomes (Ribaut and Hoisington, 1998). The construction of high resolution linkage maps is paving the way for molecular markers which can identify and tag desirable genes. Composite interval mapping (CIM) allows the data collected across different

environments and seasons to become integrated into joint analysis which can establish 'stable' QTLs for detailed study. CIM can also be combined with linkage mapping to identify QTL regions across the genome which could further elucidate coupled QTLs (favourable alleles from the same parental lines) and those in repulsion (favourable alleles from different parental lines) for MAB programmes (Ribaut and Hoisington, 1998).

A fruit size QTL identified in tomato (*fw*2.2) was one of the first to be tagged in plants and was reported to account for between 30 to 47% of the phenotypic variation. This QTL was later cloned and the gene was found located within 1.6 cM of the original QTL peak (Frary *et al*., 2000). Other QTLs have since been found linked to genes situated close to the original peak and these include the flowering time gene *Cry2* found in *Arabidopsis* which was 0.8 cM from the QTL peak and *Xmwg79* associated with protein content of wheat which was 0.2 cM from the QTL peak (Price, 2006). These studies show that candidate genes can be accurately identified for specific traits by looking at the genes found located around the QTL position to within 0 to 1.9 cM either side of the proposed major QTL peak and between 0 and 3 cM of a small QTL peak. These genes can then be individually tested for candidacy in regards to the presence of allelic diversity or polymorphisms associated with the trait of interest (Price, 2006).

All candidate genes present within a QTL region will absorb the QTL peak when tested against it statistically, so they are not always conclusive as to their involvement, it will depend how many genes are subsequently identified within the region. Progress in any breeding programme is based on the amount of genetic variability available and the effectiveness of the selection and evaluation of the trait in question. Once the genetic control of traits contributing to sensory qualities has been identified, linkage of such traits to molecular markers heralds the future of plant breeding (Graham and Jennings, 2009). A more accurate way of assessing genetic variability and trait selection would be at the genetic level. Markers can assess allele diversity across a range of germplasm, or markers can be examined for linkage to the trait or QTL underlying more complex traits.

A prerequisite for genotypic selection is the establishment of associations between the trait of interest and genetic markers which requires the development of markers and linkage maps to allow this to proceed effectively. In order to facilitate successful QTL mapping in a species, it is necessary to have a high density genetic map of even marker coverage as well as good phenotypic data derived from the same population. High heritability estimates have been found for colour meter values and visual colour analysis, both of which were related to total anthocyanins content in red raspberry, paving the way for improvements to fruit colour and anthocyanin content through plant breeding. Following the identification of several important allelic combinations in genes and markers associated with phenotypic traits of interest (Section 5.2.3), the ability to improve fruit traits through the utilisation of molecular tools becomes a reality.

Due to the complexity of fruit quality it was not expected to identify a specific marker or gene within the selected genetic pool, used for QTL validation, which conveys complete trait variation. Instead this study aimed to identify polymorphisms within an unrelated population in which to confirm the transferability of marker assisted selection. As the majority of major QTLs identified were present across environments and seasons, within a segregating population, the next phase in validation involved analysis within a new population. As this was not feasible within the scope of this project a smaller subset of raspberry germplasm were selected in which to test a selection of genetic markers and genes deemed significant. This resulted in a number of polymorphisms identified for all of the markers tested and showed the narrow genetic base found in domesticated raspberries. This restricted genetic diversity is of great concern for future *Rubus* breeding, particularly in the development of new high quality fruit which is resistant to a variety of pests and diseases. The gene base can, however, be increased following the introduction of wild raspberry material and the utilisation of more species-like cultivars such as ‘Latham’.

This study has highlighted the high levels of heterozygosity found in ‘Latham’ and ‘Glen Moy’ cultivars while paving the way for the development of new genetic crosses

which are based on both segregating characteristics within individuals and independent levels of polymorphisms. The level of transferability of the studied markers may prove useful for future population genetics and diversity studies in raspberry and indeed related species as well as their proposed use in a raspberry breeding programme. As the *Rubus* linkage map and resultant QTLs become closely associated with functional EST-SSRs and candidate genes it becomes possible to utilise these in the identification of BACs which can then be used to link genetic and physical maps for *Rubus*.

Chapter 6 Discussion, conclusions and future direction

6.1 Summary of findings

Strategies which can improve identification, selection and monitoring of desirable plant characteristics in breeding, are critical to secure improvements in the genetic resources of food and agriculture. Marker assisted selection is a prime example of an emerging technology utilising DNA sequences associated, through linkage mapping, to genes which affect a phenotype. Markers can then be used to track individual genes, genomic sequences or alleles known to affect specific phenotypic traits (Ruane and Sonnino, 2007).

The purpose of this thesis was to gain an insight into the underlying genetic and environmental factors relating to red raspberry quality, and hence identify potential markers useful for molecular assisted breeding strategies. This study demonstrates the need for a multivariate approach towards understanding and identifying components underlying quality traits in red raspberry. While seasonal effects were evident for many phenotypic traits analysed, the potential to enhance quality by genotypic selection was clear.

Shewfelt (1999) suggested quality can mean different things depending on where in the distribution chain it is being assessed and by whom i.e. the stakeholders. There is a complex relationship between product oriented quality characteristics, inherent attributes which can be readily quantified, while consumer oriented quality is defined as consumer satisfaction, which is less tangible, can be difficult to quantify but more relevant to market success (Shewfelt, 1999). Although Abbott (1999) described quality in terms of instrumental and sensory measurements, in practice these do not combine to provide a robust indication of overall consumer acceptance. Researchers prefer instrumental measurements for quality attributes over sensory evaluations (and dislike consumer testing), for research purposes, as these are found to reduce variation, are more precise and provide a common language for researchers (Abbott, 1999; Shewfelt, 1999).

Marketers and multiple retailers, however, take a more complex view often focusing on fault aversion.

Work presented in Chapter 3 described the evaluation of a phenotypically diverse mapping population to establish variations in fruit quality traits desirable to growers, multiple retailers and consumers, although these may differ and possibly conflict. There were several key findings identified through phenotypic analysis with particular emphasis on the effect of site and season on trait variation. Importantly, for the experimental work, initial findings showed no significant quantitative differences for any of the traits within a season between fruit of repetitions or clones from a single site, allowing more progeny to be analysed across sites and season. Similiarly ^{o}Brix analysis showed no significant differences regarding fruit treatment: squeezed juice or blended puree. On the other hand colour co-ordinate systems (Yxy and L*a*b*) differ in the colour space, symmetry and points used to define colour and there are issues with respect to correlations between colour differences as perceived by the human eye (Abbott, 1999).

Analyses have shown both environment and season can affect quality characteristics and thus QTLs detected for individual traits. Seasonal differences were apparent regarding total sunshine hours, temperature and rainfall experienced in any given year, but it was found that primarily colour and ^{o}Brix were affected (Figure 3.21 and 3.22). While the field site produced brightly coloured fruit with high ^{o}Brix content during 2006, which was a particularly good summer, this was not found in the following two seasons which were not as favourable. The protected fruit, however, were not as adversely affected and produced fruit which were comparable to a more favourable summer. Environment contributed to quality parameters, in particular ^{o}Brix and ten berry weights, which were found to increase consistently in polytunnel fruits. This is likely to be a result of the fruit being protected from the unpredictable, maritime Scottish climate by being under polytunnels allowing fruit to develop in a warmer environment with less external stress during development and growth. Biochemical measurements were also found to be

affected by environment with total phenols, anthocyanins and pH measurements found to decrease in fruit grown under the commercial tunnel in Blairgowrie compared with the field and protected sites at the SCRI. Although genetically regulated, significant environmental effects were found for secondary metabolites between locations. This was most likely related to light, which has been reported previously in relation to anthocyanin synthesis with greater light exposure yielding higher anthocyanin content (Ju *et al*., 1995a). Soil conditions have also been found to influence plant phenol composition with a deficit in soil moisture resulting in reduced phenylalanine ammonia lyase (PAL) activity and hence lower phenolic content (Anttonen and Karjalainen, 2005). Several studies suggest the genotype of plants may be a greater influence on the phenolic variation of fruits with seasonal effects playing a secondary role (Howard *et al*., 2003; Anttonen and Karjalainen, 2005). In this study, however, with analyses performed over one year only, it was found that both genotype and site played an important role in the total phenolic variation detected.

Phenolic compounds, central to fruit colouration, have been credited with several defence functions in plants, including protection from harmful UV rays and both herbivory and mechanical wounding, with environmental factors such as light, temperature and humidity contributing to effective synthesis. Internal factors such as genetic differences, nutrients and hormones will also affect phenolic biosynthesis (Kähkönen *et al*., 2001). Studies of the phenolic content found in two cultivars of tomato grown under polytunnels that either transmitted ambient solar UV radiation from 290 to 400 nm (designated +UV) or blocked UV below 380 nm (-UV) demonstrated that those receiving the UV wavelengths contained 20% more phenolic compounds (Luthria *et al*., 2006), while other important antioxidant components, quality and flavour may also be affected. This will be influenced not only by the choice of polytunnel material but also tunnel agronomic environment and therefore warrants further investigation.

Quality parameters are the result of multiple complex biochemical pathways which interact at various levels and are influenced to a greater or lesser extent by

environmental conditions. Understanding key steps in, and the regulation of, pathways involved in the development of quality traits facilitates targeted breeding strategies. The successful mapping of candidate genes which are known to influence biochemical pathways can provide steps for identifying genes responsible for pathway-related natural variation (De Jong *et al*., 2004).

Genes mapped in this study (Chapter 4) include those encoding transporter genes: membrane intrinsic proteins (MIP 2 and MIP 3) and tonoplast intrinsic protein (TIP) on LG 2; flavonoid synthetic enzymes; anthocyanin reductase (ANR) on LG 2; flavonol synthase (FLS), glutathione S-transferase (GST) and dihydroflavonol 4-reductase (DFR) on LG 4, and flavonoid 3'-hydroxylase (F3'H) on LG 5. Other genes mapped include those encoding expansin (EXP) on LG 7 and galactose dehydrogenase (Gal-dh) on LG 4. Etienne *et al*. (2002) found that transporters TIP, MIP 2 and MIP 3 clustered together on LG 6 of the peach Ferjalou Jalousia × Fantasia linkage map showing close association between MIP 2 and QTLs for fresh weight and total soluble solids. A similiar association was also found in raspberry on LG 2 with additional QTL regions for total anthocyanins and fruit colour. Aquaporins, such as MIPs and TIPs, transport water and small molecules across cellular membranes with an impact on quality traits, especially fruit colour (Maeshima and Ishikawa, 2008).

As discussed in Chapter 3, the most significant statistical correlations detected between traits were for berry colour (instrumental and visual) and total anthocyanin content which detected several overlapping QTL regions. ^{o}Brix was found to correlate with total and individual sugar content but not with ten berry weights, although again overlapping QTLs were detected. This was in line with reports by Tanksley *et al*. (1996); Bernacchi *et al*. (1998); Chen *et al*. (1999); Fridman *et al*. (2002) and Saliba-Colombani *et al*. (2001), all finding correlations between ^{o}Brix and ten berry weights. It is unsurprising therefore to find underlying QTL regions which are shared by these phenotypic traits. pH showed separate QTL regions for each of the three environments, with SCRI field

and Blairgowrie, commercial fruit displaying regions on LG 1 while that of SCRI polytunnel was linked to LG 7 (Figures 6.1a; 6.1b).

In Chapter 5, a total of 75 putative QTLs were located across the raspberry genome for all traits across different environments and seasons, the majority of which were subsequently linked to underlying candidate genes or markers. The large number of QTLs detected for some traits (22 for colour meter analysis) was further evidence towards phenotypes caused by the cumulative influence of numerous QTLs of small effect and not by a few of large effect, a finding which has been reported in other QTL studies analysing fruit and flower characteristics (Saliba-Colombani *et al*., 2001; Causse *et al*., 2002; Yates *et al*., 2004 and Buckler *et al*., 2009). Overlapping QTL regions for environments and seasons are thought indicative of a stable genetic architecture for several of the traits analysed (Buckler *et al*., 2009).

Phenotypic variations (R^2) explained by these QTLs ranged from 32% for total anthocyanins to 8% for ten berry weight. Eight QTLs showed an R^2 of <10%, 55 between 10% and 20%, 9 had an R^2 of 20 to 30% and 3 QTLs had an R^2 >30%. Several clusters of QTLs, for colour and total anthocyanins and for ^{o}Brix and ten berry weights, were determined on chromosomes 2, 3 and 5. Such clusters may be indicative of a fortuitous linkage or due to the segregation of a unique QTL which may control two traits as a result of a causal relationship between traits or related metabolisms. These co-located areas can be compared with the original correlations identified in Chapter 3 as two related traits would be expected to share common QTLs. Following QTL mapping of traits it could be seen that all of the candidate genes were associated with QTL regions. The transporter genes (LG 2) were linked to fruit colour, ^{o}Brix, total anthocyanin and ten berry weight, but expansin (LG 7) was associated with pH for the SCRI protected site only. The most interesting association however was on LG 4 which showed three key candidate genes involved in anthocyanin biosynthesis, flavonol synthase, glutathione S-transferase and dihydroflavonol 4-reductase on the same region as colour and total anthocyanin content.

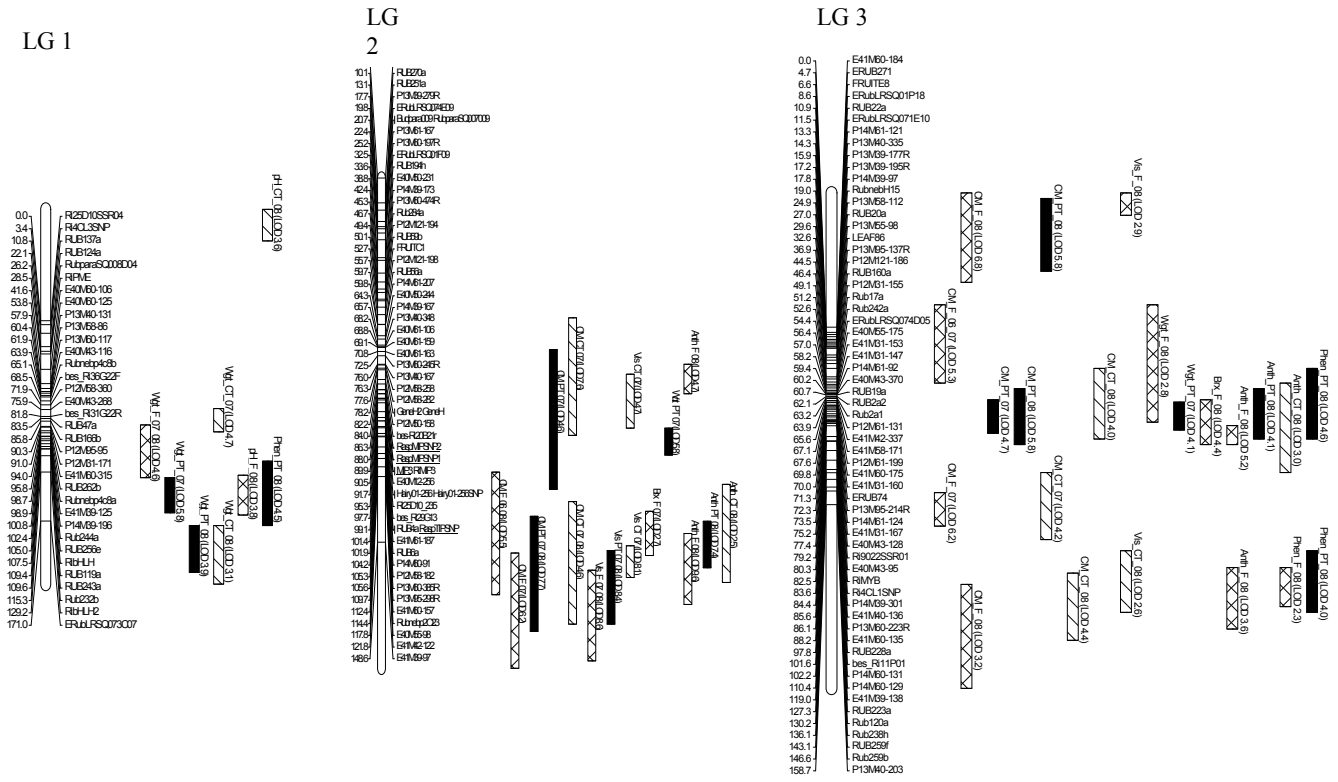


Figure 6.1a. Linkage groups displaying quantitative trait loci for raspberry traits. Linkage groups 1, 2 and 3 of the 'Glen Moy' × 'Latham' population showing the QTL clusters obtained for the phenotypic traits relating to fruit quality. QTL can be interpreted as per Figure 5.1 and 5.2.

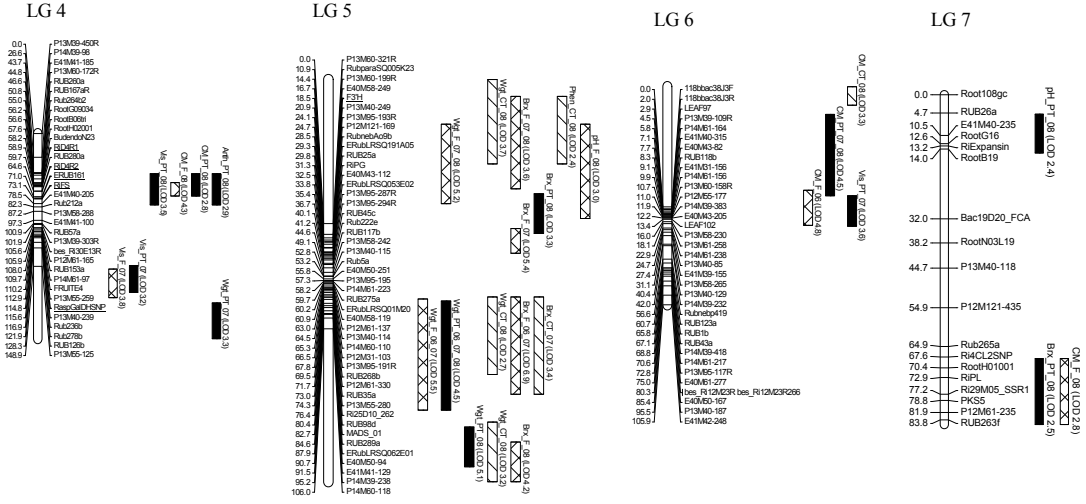


Figure 6.1b. Linkage groups displaying quantitative trait loci for raspberry traits. Linkage groups 4, 5, 6 and 7 of the 'Glen Moy' × 'Latham' population showing the QTL clusters obtained for the phenotypic traits relating to fruit quality. QTL can be interpreted as per Figure 5.1 and 5.2.

In fruit cultivars genetically determined to produce red fruit, flavonoid synthesis is directed towards anthocyanin formation, and less bound procyanidin is accumulated. If internal tissue development favours synthesis of anthocyanins, such as during fruit maturation, increased DFR activity will accelerate anthocyanin and bound procyanidin formation (Figure 1.6). FLS gene product will utilise dihydroflavonols which are available prior to the onset of DFR synthesis as this gene is not highly expressed until late in flower development so under normal conditions these enzymes would not compete for precursors (Ju *et al*., 1997).

In grape (*Vitis*), flavonol content normally increases during fruit ripening with a flavonol synthase gene expressed during flowering and fruit ripening. However, if fruit were shaded, the flavonol content of grape skin and the expression of the FLS gene and its putative transcriptional regulator, MYB12, would be greatly reduced (Downey *et al*., 2004; Matus *et al*., 2008). In *Arabidopsis*, Hartmann *et al*. (2005) demonstrated that four genes in the flavonoid pathway, chalcone synthase (CHS), chalcone flavanone isomerase (CFI), flavanoid 3-hydroxylase (F3'H) and flavonol synthase (FLS) were co-ordinately expressed in response to light and contained light regulatory units (LRUs) in the promoter regions. In flavonoid biosynthesis, FLS is a key enzyme regulating the balance between anthocyanin and flavonol synthesis and mutant *fls1* seedlings in *Arabidopsis* show enhanced anthocyanin levels (Stracke *et al*., 2009). Based on structural analysis of grape DFR, it has been suggested that flavonols could act as inhibitors of DFR activity on dihydroflavonols (Trabelsi *et al*., 2008) promoting this balance between anthocyanin and flavonol compounds.

Examination of candidate genes is one strategy for validating QTLs and this study has identified ten candidate genes which are significantly associated with trait variation in raspberry fruit. From a total of 75 QTLs mapped in this study, eight were located on LG 1, five of which were for ten berry weight alone. On LG 1 three transcription factors (which control gene expression) have previously been identified which show significant associations: bHLH (RibHLH, 107.5 cM), NAM/NAC (RUB119a, 109.4 cM) and a

WD40 (Ri25D10SSR04, 0.0 cM) (Kassim *et al*., 2009; Woodhead *et al*., unpublished) particularly in the production of individual anthocyanin pigments. In raspberry, the bHLH gene (RibHLH) was found to show similarity with MdbHLH33 from apple with close association with anthocyanin production on LG 1 (Kassim *et al*., 2009). While Kassim *et al*. (2009) found bHLH underlying QTLs for eight individual anthocyanin pigments analysed, this was not found for total anthocyanin content. Instead only ten berry weight measurements were found associated with both bHLH and NAM/NAC. Genes with the NAC domain (NAC family genes) are plant-specific transcriptional regulators which are expressed in various tissues and at various developmental stages which include defence and abiotic stress, flowering and secondary cell wall biosynthesis (reviewed by Jin and Martin, 1999). The strong association of this putative transcription factor with production of individual cyanidin and pelargonidin pigments is interesting and therefore warrants further investigation (Kassim *et al*., 2009).

On LG 2, 16 specific QTL regions were detected for traits including colour and total anthocyanins. A range of transporters have also been identified with highly significant association with trait variation, particularly colour and total anthocyanin content.

On LG 3, a number of QTLs (22 in total) and genes involved in anthocyanin and flavonol biosynthesis as well as MYB genes involved in transcriptional control have been identified from this study and others (Graham *et al*., 2009). MYB genes are known to control phenylpropanoid metabolism including anthocyanin accumulation in fruit but also have important roles in many other aspects of plant development including trichome development, signal transduction, disease resistance and cell division (Mounet *et al*., 2009). Several MYB genes that generally interact closely with basic helix-loop-helix (bHLH) proteins have been placed on the *Rubus* linkage map (Graham *et al*., 2009) and underlie total anthocyanin and phenol QTLs in this present study.

Seven QTLs were detected on LG 4 for colour, total anthocyanins and ten berry weights. A number of genes in the acid, anthocyanin and flavonol pathways have been identified

on LG 4 as well as a gene involved in flavonoid transportation. A bZIP transcription factor (FruitE4, 110.2 cM) was also identified. These transcription factors are expressed constitutively or tissue specifically and regulate diverse processes such as photomorphogenesis and light signalling stress and hormone signalling (Holm *et al*., 2002).

In LG 5 there are 15 QTLs, (7 for ten berry weight and 6 for ^{o}Brix) and two interesting genes: a MADS-box transcription factor and a gene involved in flavonol biosynthesis (F3'H). MADS-box genes are known to regulate the transition from vegetative to reproductive development as well as the specification of flower meristem and flower organ identity (Giovannoni, 2001; 2004; Vrebalov *et al*., 2002). In addition, MADS-box genes also regulate fruit, pollen, seed, embryo, leaf and root development (Vrebalov *et al*., 2002).

On LG 6 four QTLs have been detected for instrumental colour measurements.

On LG 7 only three QTLs were detected with the expansin gene, mapped in this study, weakly associated with pH for the SCRI protected site only and a pectate lyase and polyketide synthase associated with ^{o}Brix and colour (unpublished data).

Although the potential to utilise molecular markers for the improvement of quality traits and breeding strategies is by no means new research (Sax, 1923; Thoday, 1961), the implementation in practice has been limited by a lack of knowledge regarding QTL stability (Tanksley, 1983; Kenis *et al*., 2008). The potential benefits from QTL studies have been tempered with the realisation that individual QTL experiments require validation over a number of years, sites, generations or genetic backgrounds (Kenis *et al*., 2008). The initiation of molecular assisted breeding (MAB) for monogenic traits, however, has been readily implemented for a number of traits including red pigmentation of apple skin associated with the dominant gene *Rf* (Wilcox and Angelo, 1936, cited in Janick, 1992), fruit acidity in apples related to the *Ma* gene (Nybom,

1959, cited in Janick, 1992) and resistance to fungal infection from *Cladosporium fulvum* in tomato through the *Cf*-9 gene (Hammond-Kosack and Jones, 1997). While a number of studies have identified QTLs for fruit quality traits, including pH, fresh weight and total soluble solids in peach (Dirlewanger *et al*., 1999), melon (Monforte *et al*., 2004), tomato (Saliba-Colombani *et al*., 2001) and apple (Liebhard *et al*., 2003), it is necessary to compare QTL positions within different populations, prior to the development of practical breeding tools. While polymorphisms can be identified from genetic markers, it may be necessary to identify marker associations with QTLs experimentally in a new population (Knoll and Ejeta, 2008). Other factors which may influence QTL association in a novel population include the interaction of other loci which may mask QTL expression to varying degrees (Knoll and Ejeta, 2008).

Many of the QTLs identified in this study have been detected across a number of environments and seasons with colour proving to be particularly robust over seasons on LG 2 and across environments on LG 3 (Figure 6.1a; 6.1b). Total anthocyanins were also found co-located on an area of LG 3 for all three sites with marker Rub2a1 (63.2 cM) significant for both total anthocyanin and fruit colour (Section 5.2.3.3).

Further validation was also carried out on a number of raspberries from a wider genetic background which resulted in a range of polymorphisms being detected across samples for each of the markers tested. In one experiment MYB (LG 3) was found to show polymorphism in 8 out of 12 plants (Table 5.7). This study has highlighted the restricted genetic diversity currently present within domesticated raspberries while paving the way for the development of new genetic crosses derived from individuals found to be polymorphic for the trait(s) of interest.

This study demonstrates the need for a multifaceted approach towards understanding and identifying components underlying quality traits in red raspberry. While seasonal effects were evident for many phenotypic traits, the potential to enhance quality by genotypic selection was clear. However for the purpose of mapping quality traits for subsequent

marker assisted breeding strategies in red raspberry, it was necessary to identify polymorphisms within genes without speculating on potential effects on gene function.

Successful marker assisted breeding strategies require the identification of chromosomal regions for each trait of interest. Having identified markers within or surrounding genes of phenotypic interest, it has become possible to select marker variants (alleles) that yield the favourable variation of the gene of interest. Superior alleles, which have been identified among genomes within targeted genes, can then be used as DNA markers for the development of efficient screening strategies. The data presented here has facilitated the identification and mapping of candidate genes related to quality traits through the development of a saturated robust genetic linkage map.

6.2 Future work

Future studies focused on the regulation and function of individual candidate genes identified here could yield greater insights into factors underlying fruit quality metabolites and interactions with the environment. As pathways and enzymes involved in fruit quality become better understood the requirements for new technology follows. Examples of molecular advances which can be utilised to follow from this project include association mapping and metabolomic studies.

6.2.1 Association mapping

As molecular technology and genome programmes continue to advance, a wealth of information is becoming available in the form of high throughput sequencing and genotyping for the identification of thousands of polymorphisms (Varshney *et al*., 2009). Loci involved in the inheritance of complex traits may be identified through association mapping which allows gene localisation by linkage disequilibrium (LD) studies, without the need for gene cloning (Morton, 2005). This involves markers identified with significant allele-frequency differences between individuals displaying the phenotype of interest and an unrelated control population. Once a statistical association has been made between a genotype at a particular locus and a phenotype, this can often be considered as

evidence of close physical linkage between a marker and a phenotype (Pritchard *et al*., 2000). LD studies centre on the relationship between two alleles which are found to arise more often than would be expected by chance in an individual or population. This indicates that such alleles are physically located in close proximity to one another on the chromosome and are thus separated infrequently due to recombination (Morton, 2005). Such an approach can be applied to a range of accessions, which are not closely related, to infer relationships between phenotypic variation and genetic polymorphisms within any given population. Once LD maps have been constructed, these can be utilised independently or in combination with linkage analysis studies allowing further elucidation of plant genomes while facilitating marker-assisted screening and map-based cloning of genes underlying complex traits (Gupta *et al*., 2005).

6.2.2 Metabolomics

Plant metabolites are products of multiple cellular regulatory processes and as such levels can be regarded as the biological response influenced by genetic or environmental change (Fiehn, 2002). Forward genetics involves the identification of natural (or indeed laboratory induced) mutants or dichotomous accessions with phenotypes of interest, such as sugar metabolism or pigment formation. Several ripening mutants (*rin*, *Cnr*, *hp*1 and *hp*2) with clear biological functions have been found to affect signal transduction proteins, including transcription factors (such as MADS box genes) or proteins involved in light signalling (Mounet *et al*., 2009). Such mutants can be studied to identify and characterise individual phenotypes which represent nucleotide sequence changes within genes of interest in independent biosynthetic pathways or complete developmental processes (Fiehn, 2002; Mounet *et al*., 2009). In reverse genetics a cloned gene or isolated protein is used to determine likely phenotypic effect on an individual (Fiehn, 2002). Studies using reverse genomic approaches have shown alterations in metabolism or composition can affect fruit development. An alteration in ethylene-dependent signal transduction, for example, has considerable effect on fruit ripening including on colour, aroma and soluble solids content (Mounet *et al*., 2009). Such studies are imperative for the functional elucidation of genes with various strategies for the systemic perturbation

of gene expression now widely available. With large-scale sequencing and genotyping projects generating tens of thousands of gene reads, the subsequent elucidation of gene function becomes a priority. This allows correlation of genes with gene products that serve towards a greater understanding of the chemical complexity of the metabolome and ultimately, with increased biological knowledge, the potential for plant improvement follows (Sumner *et al*., 2003).

References

Abbott, JA, (1999). Quality measurements of fruits and vegetables. *Postharvest Biology and Technology*. **15**: 207-225

Abraham, LJ and Kroeger, KM, (1999). Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease. *Journal of Leukocyte Biology*. **66**: 562-566

Adams, JB, (1973). Thermal degradation of anthocyanins with particular reference to the 3-glycosides of cyanidin. I. In acidified aqueous solution at 100^{o}C. *Journal of the Science of Food and Agriculture*. **24**: 747-762

Agius, F, González-Lamothe, R, Caballero, JL, Muñoz-Blanco, J, Botella, MA and Valpuesta, V, (2003). Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nature Biotechnology*. **21**: 177-181

Agre, P, Sasaki, S and Chrispeels, MJ, (1993). Aquaporins: a family of water channel proteins. *The American Journal of Physiology*. *Renal Physiology*. **265**: 461

Aharoni, A, Giri, AP, Verstappen, FWA, Bertea, CM, Sevenier, R, Sun, Z, Jongsma, MA, Schwab, W and Bouwmeester, HJ, (2004). Gain and loss of fruit flavor compounds produced by wild and cultivated strawberry species. *The Plant Cell*. **16**: 3110-3131

Aitken, KS, Jackson, PA and McIntyre CL, (2006). Quantitative trait loci identified for sugar related traits in a sugarcane (*Saccharum* spp.) cultivar × *Saccharum officinarum* population. *Theoretical and Applied Genetics*. **112**: 1306-1317

Alderborn, A, Kristofferson, A and Hammerling, U, (2000). Determination of single-nucleotide polymorphisms by real-time pyrophosphate DNA sequencing. *Genome Research*. **10**: 1249-1258

Alfenito, MR, Souer, E, Goodman, CD, Buell, R, Mol, J, Koes R and Walbot, V, (1998). Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferases. *The Plant Cell*. **10**: 1135-1149

Allan, AC, Hellens, PR and Laing WA, (2008). MYB transcription factors that colour our fruit. *Trends in Plant Science*. **13**: 99-102

Almela, L, Javaloy, S, Fernández-López, JA and López-Roca, JM, (1995). Comparison between the tristimulus measurements Yxy and L* a* b* to evaluate the colour of young red wines. *Food Chemistry*. **53**: 321-327

Altschul, SF, Gish, W, Miller, W, Myers, EW and Lipman, D, (1990). Basic and local alignment search tool. *Journal of Molecular Biology*. **215**: 403-410

Andrade, E, (2006). The Interrelations between genotype/phenotype/environment: A semiotic contribution to the Evo:Devo debate. *Journal of Agricultural and Food Chemistry*. **50**: 2926-30

Antonius-Klemola, K, (1999). Molecular markers in *Rubus* (Rosaceae) research and breeding. *Journal of Horticultural Science and Biotechnology*. **74**: 149-160

Anttonen, MJ and Karjalainen, RO, (2005). Environmental and genetic variation of phenolic compounds in red raspberry. *Journal of Food Composition and Analysis*. **18**: 759-769

Asen, S, Stewart, RN and Norris, KH, (1972). Co-pigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry*. **11**: 1139-1144.

Bernacchi, D, Beck-Bunn, T, Eshed, Y, Lopez, J, Petiared, V, Uhlig, J, Zamir, D and Tanksley, S, (1998). Advanced backcross QTL analysis in tomato. Identification of QTLs for traits of agronomic importance from *Lycopersicon hirsutum*. *Theoretical and Applied Genetics*. **97**: 381-397

Bliss, FA, Arulsekar, S, Foolad, MR, Becerra, V, Gillen, AM, Warburton, ML, Dandekar, AM, Kocsisne, GM and Mydin, KK, (2002). An expanded genetic linkage map of *Prunus* based on an interspecific cross between almond and peach. *Genome*. **45**: 520-529

Bohner, J and Bangerth, F, (1988). Effects of fruit set sequence and defoliation on cell number, cell size and hormone levels of tomato fruits (*Lycopersicon esculentum* Mill.) within a truss. *Plant Growth Regulation*. **7**: 141-151

Boss, PK, Davies, C and Robinson, SP, (1996). Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. *Plant Physiology*. **111**: 1059-1066

Botstein, D, White, RL, Skolnick, M and Davis, RW, (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*. **32**: 314-331

Bridle, P and Timberlake, C, (1996). Anthocyanins as natural food colours - selected aspects. *Food Chemistry*. **58**: 103-109

Brouillard, R, (1982). Chemical structure of anthocyanins. In: Anthocyanins as Food Colors. Markakis P (Ed.), pp 1-40. Academic Press, New York

Brummell, DA and Harpster, MH, (2001). Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Molecular Biology*. **47**: 311-340

Buckler, ES, Holland, JB, Bradbury, PJ, Acharya, CB, Brown, PJ, Browne, C, Ersoz, E, Flint-Garcia, S, Garcia, A, Glaubitz, JC, Goodman, MM, Harjes, C, Guill, K, Kroon, DE, Larsson, S, Lepak, NK, Li, H, Mitchell, SE, Pressoir, G, Peiffer, JA, Rosas, MO, Rocheford, TR, Romay, MC, Romero, S, Salvo, S, Villeda, HS, da Silva, HS, Sun, Q, Tian, F, Upadyayula, N, Ware, D, Yates, H, Yu, J, Zhang, Z, Kresovicg, S and McMullen, MD, (2009). The genetic architecture of maize flowering time. *Science*. **325**: 714-718

Busby, AJW, (1999). Repressors and activators. In: Prokaryotic gene expression. Baumberg, S (Ed.), University Press, Oxford

Byrne, PF and McMullen, MD, (1996). Defining genes for agricultural traits: QTL analysis and the candidate gene approach. *Probe*. **7**: 24-27

Capocasa, F, Scalzo, J, Mezzetti, B and Battino, M, (2008). Combining quality and antioxidant attributes in the strawberry: The role of the genotype. *Food Chemistry*. **111**: 872-878

Castañeda-Ovando, A, Pacheco-Hernández, Ma.dL Páez-Hernández, Ma.E, Rodríguez, JA and Galán-Vidal, CA, (2009). Chemical studies of anthocyanins: A review. *Food Chemistry*. **113**: 859-871

Castellarin, SD and Di Gaspero, G, (2007). Transcriptional control of anthocyanin biosynthetic genes in extreme phenotypes for berry pigmentation of naturally occurring grapevines. *Plant Biology*. **7**: 46-49

Castellarin, SD, Matthews, MA, Di Gaspero, G and Gambetta, GA, (2007). Water deficits accelerate ripening and induce changes in gene expression regulating flavonoid biosynthesis in grape berries. *Planta*. **227**: 101-112

Causse, M, Saliba-Colombani, V, Lecompte, L, Duffé, P, Rousselle, P and Buret, M, (2002). QTl analysis of fruit quality in fresh market tomato: a few chromosome regions control the variation of sensory and instrumental traits. *Journal of Experimental Botany*. **53**: 2089-2098

Causse, M, Duffé, P, Gomez, MC, Buret, M, Damidaux, R, Zamir, D, Gur, A, Chevalier, C, Lemaire-Chamley M and Rothan C, (2004). A genetic map of candidate genes and QTLs involved in tomato fruit size and composition. *Journal of Experimental Botany*. **403**: 1671-1685

Chawla, HS, (2002). Introduction to Plant Biotechnology, 2nd Edition. Science Publishers Inc, New Hampshire, USA

Chen, FQ, Foolad, MR, Hyman, J, St Clair, DA and Beeleman, RB, (1999). Mapping QTLs for lycopene and other fruit traits in a *Lycopersicon esculentum* – L. *pimpinellifolium* cross and comparison of QTLs across tomato species. *Molecular Breeding*. **5**: 283-299

Cheng, GW and Breen, PJ, (1991). Activity of phenylalanine ammonia-lyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. *Journal of the American Society for Horticultural Science*. **116**: 865-869.

Cheng, CH, Seal, AG, Boldingh, HL, Marsh, KB, MacRae, EA, Murphy, SJ and Ferguson, AR, (2004). Inheritance of taste characters and fruit size and number in a diploid *Actinidia chinensis* (kiwifruit) population. *Euphytica*. **138**: 185-195

Christie, PJ, Alfenito, MR and Walbot, V, (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta*. **194**: 541-549

Chun, OK, Chung, SJ and Song, WO, (2007). Estimated dietary flavonoid intake and major food sources of U.S. adults. *The Journal of Nutrition*. **137**: 1244-1252

Civello, PM, Powell, ALT, Sabehat, A and Bennett, AB, (1999). An expansin gene expressed in ripening strawberry fruit. *Plant Physiology*. **121**: 1273-1279

Cliff, MA, King, MC and Schlosser, J, (2006). Anthocyanin, phenolic composition, colour measurement and sensory analysis of BC commercial red wines. *Food Research International*. **40**: 92-100

Clydesdale, F, (1993). Color as a factor in food choice. *Critical Reviews in Food Science and Nutrition*. **33**: 83-101

Cogan, N, Ponting, R, Vecchies, A, Drayton, M, George, J, Dracatos, P, Dobrowolski, M, Sawbridge, T, Smith, K, Spangenberg, G and Forster, J, (2006) Gene-associated single nucleotide polymorphism discovery in perennial ryegrass (*Lolium perenne* L.). *Molecular Genetics and Genomics*. **276**: 101-112

Collard, BCY, Jahufer, MZZ, Brouwer, JB and Pang, ECK, (2005) An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica*. **142**: 169-196

Conklin, PL, Saracco, SA, Norris, SR and Last, RL, (2000). Identification of ascorbic acid-deficient *Arabidopsis thaliana* mutants. *Genetics*. **154**: 847-856

Conner, JP, Brown, SK and Weeden, NF, (1998). Molecular marker analysis of quantitative traits for growth and development in juvenile apple trees. *Theoretical and Applied Genetics*. **96**: 1027-1035

Cortell, JM and Kennedy, JA, (2006). Effect of shading on accumulation of flavonoid compounds in (*Vitis vinifera* L.) Pinot noir fruit and extraction in a model system**.** *Journal of Agricultural and Food Chemistry*. **54**: 8510-8520

Cosgrove, DJ, (2000). Loosening of plant cell walls by expansins. *Nature*. **407**: 321-326

Crisosto, CH, Crisosto, GM and Ritenour, MA, (2002). Testing the reliability of skin color as an indicator of quality for early season 'Brooks' (*Prunus avium* L.) cherry. *Postharvest Biology and Technology*. **24**: 147-154

Curry, EA, (1997). Temperatures for optimal anthocyanin acculmulation in apple tissue. *Journal of Horticultural Science*. **72**: 723-729

Dadd, A, (1996). Working efficiently with GeneQuant® II DNA/RNA calculator. *Science Tools from Pharmacia Biotechology*. **1**: 20

Daly, F, Hand, DJ, Jones, MC, Lunn, AD and McConway, KJ, (1995). Elements of Statistics. Addison-Wesley, Chichester

D'Aoust, M, Yelle, S and Nguyen-Quoc, B, (1999) Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. *The Plant Cell*. **11**: 2407-2418

Darrow, GM, (1937). Blackberry and Raspberry improvement. In: Yearbook of the United States Department of Agriculture, pp. 496-533.

Daubeny, HA, (2002). Raspberry breeding in the 21st Century. *Acta Horticulturae*. **585**: 69-72

Daubeny, HA, Crandall, PC and Eaton, GW, (1967). Crumbliness in the red raspberry with special reference to the 'Sumner' variety. *Journal of the American Society for Horticultural Science*. **91**: 224-230

Davies, AJ and Mazza, G, (1993). Copigmentation of simple and acylated anthocyanins with colorless phenolic compounds. *Journal of Agricultural and Food Chemistry*. **41**: 716-720

Davies, KM, Schwinn, KE, Deroles, SC, Manson, DG, Lewis DH, Boor, SJ and Bradley, JM, (2003). Enhancing anthocyanin production by altering competition for substrate between flavonol synthase and dihydroflavonols 4-reductase. *Euphytica*. **131**: 259-268

Davis, TM and Yu, H, (1997). A linkage map of the diploid strawberry, *Fragaria vesca*. *Journal of Heredity*. **88**: 215-221

de Ancos, B, Gonzalez, E and Cano, MP, (1999). Differentiation of raspberry varieties according to anthocyanin composition. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*. **208**: 33-38

de Bruyn, JW, Garretsen, F and Kooistra, E, (1971). Variation in taste and chemical composition of the tomato (*Lycopersicon esculentum* Mill.). *Euphytica*. **20**: 214-227

de Freitas, V and Mateus, N, (2006). Chemical transformations of anthocyanins yielding a variety of colours (Review). *Environmental Chemistry letters*. **4**:175-183

de Jong, WS, Eannetta, NT, De Jong, DM and Bodis, M, (2004). Candidate gene analysis of anthocyanin pigmentation loci in the *Solanaceae*. *Theoretical and Applied Genetics*. **108**: 423-432

de Pascual-Teresa, S and Sanchez-Ballesta, MT, (2008). Anthocyanins: from plant to health. *Phytochemistry Reviews*. **7**: 281-299

de Vienne, D, Leonardi, A, Damerval, C and Zivy, M, (1999). Genetics of proteome variation as a tool for QTL characterization. Application to drought-stress responses in maize. *Journal of Experimental Botany*. **50**: 303-309

Deighton, N, Brennan, R, Finn, C and Davies, H, (2000). Antioxidant properties of domesticated and wild *Rubus* species. *Journal of the Science of Food and Agriculture*. **80**: 1307-1313

Delgado, R, Martín, P, del Álamo, M and González, M, (2004). Changes in the phenolic composition of grape berries during ripening in relation to vineyard nitrogen and potassium fertilisation rates. *Journal of the Science of Food and Agriculture*. **84**: 623-630

Delwiche, J, (2004). The impact of perceptual interactions on perceived flavour. *Food Quality and Preference*. **15**: 137-146

Deng, C and Davis, TM, (2001). Molecular identification of the yellow fruit color (*c*) locus in diploid strawberry: a candidate gene approach. *Theoretical and Applied Genetics*. **103**: 316-322

Denisenko, O, Shnyreva, M, Suzuki, H and Bomsztyk, K, (1998). Point mutations in the WD40 domain of Eed block its interaction with Ezh2. *Molecular and Cellular Biology*. **18**: 5634-5642

Dirlewanger, E, Pronier, V, Parvery, C, Rothan, C, Guye, A and Monet, R, (1998). Genetic linkage map of peach [*Prunus persica* (L.) Batsch] using morphological and molecular markers. *Theoretical and Applied Genetics*. **97**: 888-895

Dirlewanger, E, Moing, A, Rotham, C, Svanella, L, Pronier, V, Guye, A, Plomion, C and Monet, R, (1999). Mapping QTLs controlling fruit quality in peach (*Prunus persica* (L.) Batsch). *Theoretical and Applied Genetics*. **98**: 18-31

Downey, MO, Harvey, JS and Robinson, SP, (2004). The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. *Australian Journal of Grape and Wine Research*. **10**: 55-73

DuBose, C, Cardello, A and Maller, O, (1980). Effects of colourants and flavorants on identification, perceived flavour intensity, and hedonic quality of fruit-flavoured beverages and cake. *Journal of Food Science*. **45**: 1393-1399

Dudley, JW, (1993). Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. *Crop Science*. **33**: 660-668

Eckardt, NA, (2004). Inside the matrix: crystal structure of a xyloglucan endotransglycosylase. *Plant Cell Wall*. **16**: 792-793.

Espley, R, Hellens, R, Putterill, J, Stevenson, D, Kutty-Amma, S and Allan, A, (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *The Plant Journal*. **49**: 414-427

Etienne, C, Rothan, C, Moing, A, Plomion, C, Bodenes, C, Svanella-Dumas, L, Cosson, P, Pronier, V, Monet, R and Dirlewanger, E, (2002). Candidate genes and QTLs for sugar and organic acid content in peach [*Prunus persica* (L.) Batsch]. *Theoretical and Applied Genetics*. **105**: 145-159

Fakhrai-Rad, H, Pourmand, N and Ronaghi, M, (2002). Pyrosequencing: An accurate detection platform for single nucleotide polymorphisms. *Human Mutation*. **19**: 479-485

Fanizza, G, Lamaj, F, Costanitini, L, Chaabane, R and Grando, MS, (2005). QTL analysis of fruit yield components in table grapes (*Vitis vinifera*). *Theoretical and Applied Genetics*. **111**: 658-664

Faragher, JD, (1983). Temperature regulation of anthocyanin accumulation in apple skin. *Journal of Experimental Botany*. **34**: 1291-1298

Ferrante, A, Spinardi, A, Maggiore, T, Testoni, A and Gallina, PM, (2008). Effect of nitrogen fertilisation levels on melon fruit quality at the harvest time and during storage. *Journal of the Science of Food and Agriculture*. **88**: 707-713

Fiehn, O, (2002). Metabolomics – the link between genotypes and phenotypes. *Plant Molecular Biology*. **48**: 155-171

Fischer, R and Bennett, A, (1991). Role of cell wall hydrolases in fruit ripening. *Annual Reviews in Plant Physiology*. **42**: 675-703

Florez, A, Pujolà, M, Valero, J, Centelles, E, Almirall, A and Casañas, C, (2009). Genetic and environmental effects on chemical composition related to sensory traits in common beans (*Phaseolus vulgaris* L.). *Food Chemistry*. **113**: 950-956

Frary, A, Nesbitt, TC, Grandillo, S, Knapp, E, Cong, B, Liu, J, Meller, J, Elber, R, Alpert, KB and Tanksley, SD, (2000). *fw*2.2: a quantitative trait locus key to the evolution of tomato fruit size. *Science*. **289**: 85-88

Frary, A, Doganlar, S, Frampton, A, Fulton, TM, Uhlig, J, Yates, HE and Tanksley, SD, (2002). Fine mapping of quantitative trait loci for improved fruit characteristics from *Lycopersicon chmielewskii* chromosome 1. *Genome*. **46**: 235-243

Fridman, E, Lui, YS, Carmel-Goren, L, Gur, A, Shoresh, M, Pleban, T, Eshed, Y and Zamir, D, (2002). Two tightly linked QTLs modify tomato sugar content via different physiological pathways. *Molecular Genetics and Genomics*. **266**: 821-826

Fry, SC, (1998). Oxidative scission of plant cell wall polysaccharides by Ascorbate-induced hydroxyl radicals. *Biochemical Journal*. **332**: 507-515

Fukui, Y, Tanaka, Y, Kusumi, T, Iwashita, T and Nomoto, K, (2003). A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavonoid 3', 5'-hydroxylase gene. *Phytochemistry*. **63**: 15-23

Furtado, P, Figueiredo, P, Chaves das Neves, H and Pina, F, (1993). Photochemical and thermal degradation of anthocyanins. *Journal of Photochemistry and Photobiology A: Chemistry*. **75**: 113-118.

Gao, Y and Cahoon, GA, (1994). Cluster shading effects on fruit quality, fruit skin color, and anthocyanin content and composition in Reliance (*Vitis* hybrid). *Vitis*. **33**: 205-209

Garber, L, Hyatt, E and Starr, R, (2000). The effects of food colour on perceived flavour. *Journal of Marketing Theory and Practice*. **8**: 59-72

Genstat, (2007). Genstat for Windows release 10.2. VSN International Ltd, Hemel Hempstead, Hertfordshire

Gillaspy, G, Ben-David, H and Gruissem, W, (1993). Fruits: A developmental perspective. *The Plant Cell*. **5**: 1439-1451

Giovanelli, G and Buratti, S, (2009). Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. *Food Chemistry*. **112**: 903-908

Giovannoni, JJ, (2001). Molecular biology of fruit maturation and ripening. Annual Reviews in Plant Physiology. *Plant Molecular Biology*. **52**: 725-749

Giovannoni, JJ, (2004). Genetic regulation of fruit development and ripening. *The Plant Cell*. **16**: 170-180

Giovanucci, E, Asherio, A and Willett, WC, (1995). Intake of carotenoids and retinol in relation to the risk of prostate cancer. *Journal of National Cancer Institute*. **87**: 1767-1776

Goff, SA and Klee, HJ, (2006). Plant volatile compounds: Sensory cues for health and nutritional value? *Science*. **311**: 815-819

Gollop, R, Even, S, Colova-Tsolova, V and Perl, A, (2002). Expression of the grape dihydroflavonols reductase gene and analysis of its promoter region. *Journal of Experimental Botany*. **53**: 1397-1409

Gonçalves, B, Paula-Saliva, A, Moutinho-Pereira, J, Bacelar, E, Rosa, E and Meyer, AS, (2007). Effect of ripeness and postharvest storage on the evolution of color and anthocyanins in cherries (*Prunus avium* L.). *Food Chemistry*. **103**: 976-984

Gong, Z, Yamazaki, M, Sugiyama, M, Tanaka, Y and Saito, K, (1997). Cloning and molecular analysis of structural genes involved in anthocyanin biosynthesis and

expressed in a forma-specific manner in *Perilla frutescens*. *Plant Molecular Biology*. **35**: 915-927

Goodenough, U, (1984). Genetics, Third edition. Washington University Press, Philadelphia, USA

Gordon, SC, Williamson, B and Graham, J, (2006a). Current and future control for major arthropod and fungal diseases of red raspberry (*Rubus idaeus*) in Europe. In: Crops - Growth, Quality and Biotechnology, Dris, R (Ed.). WFL Publisher, Helsinki, Finland ISBN-952-91-8601-0

Gordon, SC, Birch, ANE, Brennan, R, Graham, J, Barker, H, Dolan, A and Jennings, SN, (2006b). Predicted developments in cane and bush fruit pest and disease management in Europe, with special reference to high health planting stock, genomics and plant breeding. Cost 863 Euroberry, Zagreb, Croatia, 19-22 April 2006.

Gorin, M, Snyder, S, To, A, Narfstrom, K and Curtis, R, (1993). The cat RDS transcript: candidate gene analysis and phylogenetic sequence analysis. *Mammalian Genome*. **4**: 544-548

Graham, J and McNicol, RJ, (1995). An examination of the ability of RAPD markers to determine the relationship within and between *Rubus* species. *Theoretical and Applied Genetics*. **90**: 1128-1132

Graham, J and Jennings, N, (2009). Raspberry breeding. In: Breeding Plantation Tree crops, Temperate Species. Mohan Jain, S and Priyadarshan, PM (Eds). Springer, New York, USA

Graham, J and Woodhead, M, (2009). The genomics of *Rubus*. In: Plant Genetics/Genomics Volume 6, Genetics and Genomics of Rosaceae. Folta, KM. and Gardiner, SG (Eds). Springer, New York, USA.

Graham, J, Marshall, B and Squire, GR, (2003). Genetic differentiation over a spatial environmental gradient in wild *Rubus ideaus* populations. *New Phytologist*. **157**: 667-675.

Graham, J, Hein, I and Powell, W, (2007). Raspberry. In: Genome Series Vol.4. Fruit Crops. C. Kole (Ed). Springer, Berlin, Germany

Graham, J, Ratnaparkhe, M and Powell, W, (2008). Molecular mapping and breeding of physiological traits. In: Principles and Practices of Plant Molecular Mapping and Breeding. Kole, C. and Abbott, AG. (Eds), Chapter 7 pp. 217-241. Science Publishers Inc, New Hampshire, USA.

Graham, J, Smith, K, Woodhead, M and Russell, J, (2002). Development and use of simple sequence repeat SSR markers in *Rubus* species. *Molecular Ecology Notes*. **2**: 250-252

Graham, J, Smith, K, MacKenzie, K, Jorgensen, L, Hackett, CA and Powell, W, (2004). The construction of a genetic linkage map of red raspberry (*Rubus ideaus* subsp. *ideaus*) based on AFLPs' genomic-SSR and EST-SSR markers. *Theoretical and Applied Genetics*. **109**: 740-749

Graham, J, Smith, K, Tierney, I, MacKenzie, K and Hackett, CA, (2006). Mapping gene H controlling cane pubescence in raspberry and its association with resistance to cane botrytis and spur blight, rust and cane spot. *Theoretical and Applied Genetics*. **112**: 818-831

Graham, J, Hackett, CA, Smith, K, Woodhead, M, Hein, I and McCallum, S, (2009). Mapping QTLs for developmental traits in raspberry from bud break to ripe fruit. *Theoretical and Applied Genetics*. **118**: 1143-1155

Griffiths, DW, Robertson, GW, Shepherd, T, Birch, ANE, Gordon, SC and Woodford, JAT, (2000). A comparison of the composition of epicuticular wax from red raspberry (*Rubus idaeus* L.) and hawthorn (*Crataegus monogyna* Jacq.) flowers. *Phytochemistry*. **55**: 111-116

Gross, K and Sams, C, (1984). Changes in cell wall neutral sugar composition during fruit ripening: a species survey. *Phytochemistry*. **23**: 2457-2461

Guerrero-Prieto, V, Biles, C, Wall, M and Berghage, R, (1996). Cell wall degrading enzyme activity in ripening red raspberry fruit. *Subtropical Plant Science*. **48**: 1-4

Gupta, PK, Rustgi, S and Kulwal, PL, (2005). Linkage disequilibrium and association studies in higher plants: Present status and future prospects. *Plant Molecular Biology*. **57**: 461-485

Hajji, HE, Dangles O, Figuerredo, P and Brouillare, R, (1997). 3'-(β-D-Glucopyranosyloxy) flavylium ions: Synthesis and investigation of their properties in aqueous solution. Hydrogen bonding as a means of colour variation. *Helvetica Chimica Acta*. **80**: 398-413

Hammond-Kosack, KE and Jones, JDG, (1997). Plant disease resistance genes. Annual Review of Plant Physiology. *Plant Molecular Biology*. **48**: 575-607

Harborne, JB and Williams, CA, (1998). Anthocyanins and other flavonoids. *Natural Product Reports*. **15**: 631-652.

Harper, PS, (2005). William Bateson, human genetics and medicine. *Human Genetics*. **118**: 141-151

Harrison, R, Muir, D and Hunter, A, (1999). Genotypic effects on sensory profiles of drinks made from juice of red raspberries (*Rubus idaeus* L.). *Food Research International*. **31**: 303-309

Hartmann, U, Sagasser, M, Mehrtens, F, Stracke, R and Weisshaar, B, (2005). Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, bZIP, and bHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. *Plant Molecular Biology*. **57**: 155-171

Haskell, G and Garrie, JB, (1966). Fingerprinting raspberry cultivars by empirical paper chromatography. *Journal of the Science of Food and Agriculture*. **17**: 189-192

Hein, I, Williamson, S, Russell, J and Powell, W, (2005). Isolation of high molecular weight DNA suitable for BAC library construction from woody perennial soft-fruit species. *Biotechniques*. **38**: 69-71

Heinonen, M, (2001). Anthocyanins as dietary antioxidant, in Third International Conference on Natural Antioxidants and Anticarcinogens in Food, Health and Disease (NAHD), June 6–9, 2001. Salonen, JT and Voutilainen, S, (Eds). Helsinki, Finland.

Hemmat, M, Weeden, NF, Manganaris, AG and Lawson, DM, (1994). Molecular marker linkage map for apple. *Journal of Heredity*. **85**: 4-11

Hill, RG, (1958). Fruit development of the red raspberry and its relation to nitrogen treatment. Ohio Agricultural Experimental Station Research Bulletin No: 803

Holm, M, Ma, LG, Qu, LJ and Deng, XW, (2002). Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in *Arabidopsis*. *Genes and Development*. **16**: 1247-1259

Holton, TA and Cornish, EC, (1995). Genetics and biochemistry of anthocyanin biosynthesis. *The Plant Cell.* **7**: 1071-1083

Horn, R, Lecouls, A, Callahan, A, Dandekar, A, Garay, L, McCord, P, Howad, W, Chan, H, Verde I, Main, D, Jung, S, Georgi, L, Forrest, S, Mook, J, Zhebentyayeva, T, Yu, Y, Ran Kim, H, Jesudurai, C, Sosinski, B, Arus, P, Baird, V, Parifitt, D, Reighard, G, Scorza, R, Tomkins, J, Wing, R and Abbott, AG, (2005). Candidate gene database and transcript map for peach, a model species for fruit trees. *Theoretical and Applied Genetics*. **110**: 1419-1428.

Howard, LR, Clark, JR and Brownmiller, C, (2003). Antioxidant capacity and phenolic content in blueberries as affected by genotype and growing season. *Journal of the Science of Food and Agriculture*. **83**: 1238-1247.

Huber, SC and Huber, JL, (1996). Role and regulation of sucrose-phosphate synthase in higher plants. *Plant Molecular Biology*. **47**: 431-444

Huh, JH, Kang, BC, Nahm, SH, Kim, S, Ha, KS, Lee, MH and Kim, BD, (2001). A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum* spp.). *Theoretical and Applied Genetics*. **102**: 524-530

Iannetta, PPM, Jones, CS, Stewart, D, Taylor, MA, McNicol, RJ and Davies HV, (1998). Multidisciplinary approaches and the improvement of fruit quality in red raspberry (*Rubus idaeus*). Scottish Crop Research Institute Annual Report. pp. 99-103

Iannetta, PPM, Wyman, M, Neelam, A, Jones, C, Taylor, M, Davies, HV and Sexton R, (2000). A causal role for ethylene and endo-beta-1,4-glucanase in the abscission of red-raspberry (*Rubus idaeus*) druplets. *Physiologia Plantarum*. **110**: 535-543

Iglesias, I, Salvia, J, Torguet, L and Cabŭs, C, (2002). Orchard cooling with overtree microsprinkler irrigation to improve fruit colour and quality of 'Topred Delicious' apples. *Scientia Horticulturae*. **93**: 39-51

Ingram, D, Sanders, K, Kolybaba, M and Lopez, D, (1997). Case–control study of phyto-oestrogens and breast cancer. *Lancet*. **350**: 990-994

Janick, J, (1992). Plant Breeding Reviews, Volume 9. John Wiley and Sons. USA

Jarvis, M, (1984). Structure and properties of pectin gels in plant cell walls. *Plant, Cell and Environment*. **7**: 153-164

Jennings, DL, (1967). Observations on some instances of partial sterility in red raspberry cultivars. *Horticultural Research*. **7**: 116-122

Jennings, DL, (1988). Raspberries and Blackberries: Their Breeding, Diseases and Growth. Academic Press, London

Jeong, ST, Goto-Yamamoto, N, Hashizume, K and Esaka, M, (2006). Expression of the flavonoid 3'-hydroxylase and flavonoid 3',5'-hydroxylase genes and flavonoid composition in grape (*Vitis vinifera*). *Plant Science*. **170**: 61-69

Jin, H, and Martin, C, (1999). Multifunctionality and diversity within the plant *MYB*-gene family. *Plant Molecular Biology*. **41**: 577-585

Johansson, I, (1998). Water transport activity of the plasma membrane aquaporin

PM28A is regulated by phosphorylation. *The Plant Cell*. **10**: 451-459

Johansson, I, Larsson, C, Ek, B and Kjellbom, P, (1996). The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca21 and apoplastic water potential. *The Plant Cell*. **8**: 1181-1191

Johnson, J, Dzendolet, E and Clydesdale, F, (1983). Psychophysical relationships between sweetness and redness in strawberry-drinks. *Journal of Food Protection*. **46**: 21-25

Ju, Z, Liu, C and Yuan, Y, (1995a). Activities of chalcone synthase and UDPGal:flavonoid-3-O-glycosyltransferase in relation to Anthocyanin synthesis in apple. *Scientia Horticulturae*. **63**: 175-185

Ju, Z, Duana, Y and Zub, Z, (1999). Effects of covering the orchard floor with reflecting films on pigment accumulation and fruit coloration in 'Fuji' apples. *Scientia Horticulturae*. **82**: 47-56

Ju, Z, Yuan, Y, Liou, C and Xin, S, (1995b). Relationships among phenylalanine ammonia-lyase activity, simple phenol concentrations and anthocyanin accumulation in apple. *Scientia Horticulturae*. **61**: 215-226

Ju, Z, Yuan, Y, Liu, C, Wang, Y and Tian, X, (1997). Dihydroflavonol reductase activity and anthocyanin accumulation in 'Delicious', 'Golden Delicious' and 'Indo' apples. *Scientia Horticulturae*. **70**: 31-43

Kähkönen, MP, Hopia, AI and Heinonen, M, (2001). Berry phenolics and their antioxidant activity. *Journal of Agricultural and Food Chemistry*. **49**: 4976-4082

Kahnau, J, (1976). The flavonoids: A class of semi-essential food components: Their role in human nutrition. *World Review of Nutrition and Diet*. **24**: 117

Kalt, W, Forney, CF, Martin, A and Prior, RL, (1999). Antioxidant capacity, vitamin C, phenolics, and anthocyanins after fresh storage of small fruits. *Journal of Agricultural and Food Chemistry*. **47**: 4638-4644

Kamsteeg, J, Van Brederode, J and Van Nigtevecht, G, (1979). Genetics of anthocyanin formation in petals of the red campion (*Silene dioica* (L.) Clairv.). *Genetica*. **51**: 5-13

Kaponen, JM, Happonen, AM, Mattila, PH and Törrönen, AR, (2007). Contents in anthocyanins and ellagitannins in selected foods consumed in Finland. *Journal of Agricultural and Food Chemistry*. **55**: 1612-1619

Karlin, S and Altschul, SF, (1993). Applications and statistics for multiple high-scoring segments in molecular sequences. *Proceeding of the National Academy of Sciences, USA*. **90**: 5873-5877

Kassim, A, Poette, J, Paterson, A, Zait, D, McCallum, S, Woodhead, M, Smith, K, Hackett, CA and Graham, J, (2009). Environmental and seasonal influences on red raspberry anthocyanin antioxidant contents and identification of QTL. *Molecular Nutrition and Food Research*. **53**: 625-634

Kaur, C and Kapoor, HC, (2001). Antioxidants in fruit and vegetables – the millennium's health. *International Journal of Food Science and Technology*. **36**: 703-725

Kearsey, MJ, (1998). The principles of QTL analysis (a minimal mathematics approach). *Journal of Experimental Botany*. **49**: 1619-1623

Kearsey, MJ and Farquar, AGL, (1998). QTL analysis in plants; where are we now? *Heredity*. **80**: 137-142

Kellerhals, M, Dolega, E, Dilworth, E, Koller, B and Gessler, C, (2000). Advances in marker-assisted apple breeding. *Acta Horticulturae*. **583**: 535-540

Kempler, C, Daubeny, H and Harding, B, (2005). 'Esquimalt' red raspberry. *HortScience*. **40**: 2192-2194

Kenis, K, Keulemans, J and Davey, MW, (2008). Identification and stability of QTLs for fruit quality traits in apple. *Tree Genetics and Genomes*. **4**: 647-661

King, GJ, Lynn, JR, Dover, CJ, Evans, KM and Seymour, GB, (2001). Resolution of quantitative trait loci for mechanical measures accounting for genetic variation in fruit texture of apple (*Malus pumila* Mill.) *Theoretical and Applied Genetics*. **102**: 1227-1235

Klann, EM, Hall, B and Bennett, AB, (1996). Antisense acid invertase (TW7) gene alters soluble sugar composition and size in transgenic tomato fruit'. *Plant Physiology*. **11**: 1321-1330

Klesk, K, Qian, M and Martin, RR, (2004). Aroma extract dilution analysis of cv. Meeker (*Rubus idaeus* L.) red raspberries from Oregon and Washington. *Journal of Agricultural and Food Chemistry*. **52**: 5155-5161

Kliewer, WM, (1970). Effect of day temperature and light intensity on colouration of *Vitis vinifera* L. grapes. *Journal of the American Society of Horticultural Science*. **95**: 693-697

Kliewer, WM, (1977). Influence of temperature, solar radiation and nitrogen on colouration and composition of Emperor grapes. *American Journal of Enology and Viticulture*. **24**: 17-26

Knoll, J and Ejeta, G, (2008). Marker-assisted selection for early-season cold tolerance in sorghum: QTL validation across populations and environments. *Theoretical and Applied Genetics*. **116**: 541-553

Koch, K, (2004). Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology*. **7**: 235-246

Koes, RE, Quattrocchio, F and Mol, JNM, (1994). The flavonoid biosynthetic pathway in plants: function and evolution. *BioEssays*. **16**: 123-132

Koes, RE, Verweij, W and Quattrocchio, F, (2005). Flavonoids: a colourful model for the regulation and evolution of biochemical pathways. *Trends in Plant Science*. **10**: 236-242

Kole, C and Abbott, AG, (2008). Principles and Practices of Plant Genomics. Volume 1. Genome Mapping. C. Enfield, (Ed.). Science Publishers, South Carolina, USA

Konczak, I and Zhang, W, (2004). Anthocyanins - more than nature's colours. *Journal of Biomedicine and Biotechnology*. **5**: 239-240

Korzun, V, (2002). Molecular markers and their applications in cereals breeding. *Cell Molecular Biology Letters*. **7**: 811-820

Kosambi, DD, (1944). The estimation of map distances from recombination values. *Annals of Eugenics*. **12**: 172–175

Kramer, A and Twigg, BA, (1966). Fundamentals of Quality Control for the Food Industry. AVI Publishing Co. Westport, Conn., USA

Kumar, A and Ellis, BE, (2001). The phenylalanine ammonia-lyase gene family in raspberry. Structure, expression, and evolution. *Plant Physiology*. **127**: 230-239

Laing, WA, Frearson, N, Bulley, S and MacRae, E, (2004). Kiwifruit L-galactose dehydrogenase: molecular, biochemical and physiological aspects of the enzyme. *Functional Plant Biology*. **31**: 1015-1025

Lander, ES and Botstein, D, (1989). Mapping Mendelian factors underlying Quantitative traits using RFLP linkage maps. *Genetics*. **121**: 185-199

Langridge, P, Lagudah, E, Holton, T, Appels, R, Sharp, P and Chalmers, K, (2001). Trends in genetic and genome analyses in wheat: A review. *Australian Journal of Agricultural Research*. **52**: 1043-1077

Larsen, M, Poll, L, Callesen, O and Lewis, M, (1991). Relations between the content of aroma compounds and the sensory evaluation of 10 raspberry varieties (*Rubus idaeus* L.) *Acta Agriculturae Scandinavica*. **41**: 447-454

Lecomte, L, Saliba-Colombani, V, Gautier, A, Gomez-Jimenez, MC, Duffé, P, Buret, M and Causse, M, (2004). Fine mapping of QTLs of chromosome 2 affecting the fruit architecture and composition of tomato. *Molecular Breeding*. **13**: 1-14

Leister, D, Ballvora, A, Salamini, F and Gebhardt, C, (1996). A PCR-based approach for isolating pathogen resistance genes from potato with potential for wide application in plants. *Nature Genetics*. **14**: 421-429.

Li, XJ, Hou, JH, Zhang, GL, Liu, RS, Yang, YG, Hu, YX and Lin, JX, (2004). Comparison of anthocyanin accumulation and morpho-anatomical features in apple skin during color formation at two habitats. *Scientia Horticulturae*. **99**: 41-53

Liebhard, R, Kellerhals, M, Pfammatter, W, Jertmini, M and Gessler, C, (2003). Mapping quantitative physiological traits in apple (*Malus* × *domestica* Borkh.). *Plant Molecular Biology*. **52**: 511-526

Linster, CL, Gomez, TA, Christensen, KC, Adler, LN, Young, BD, Brenner, C and Clarke, SG, (2007). *Arabidopsis* VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. *Journal of Biology and Chemistry*. **282**: 18879-18885

Lippman, Z and Tanksley, SD, (2001). Dissecting the genetic pathway to extreme fruit size in tomato using a cross between the small-fruited wild species *Lycopersicon pimpinellifolium* and L. *esculentum* var. giant heirloom. *Genetics*. **158**: 413-422

Liu, M, Qi Li, Q, Weber, C, Yong Lee, C, Brown, J and Liu, RH, (2002). Antioxidant and antiproliferative activities of raspberry. *Journal of Agricultural and Food Chemistry*. **50**: 2926-2933

Liu, H, Wu, B, Fan, P, Xu, H and Li, S, (2007). Inheritance of sugars and acids in berries of grape (*Vitis vinifera* L.). *Euphytica*. **153**: 99-107

Lodish, H, Berk, A, Zipursky, S, Matsudaira, P, Baltimore, D and Darnell, J, (1999). Molecular Cell Biology Fourth edition. WH. Freeman and Company. New York, USA

Lopez, R, Mateo-Vivaracho, L, Cacho, J and Ferreira, V, (2007). Optimization and validation of a taste dilution analysis to characterize wine taste. *Journal of Food Science*. **72**: 345-351

Lu, ZX, Sosiniski, B, Reighard, G, Baird, WV and Abbott, AG, (1998). Construction of a genetic linkage map and identification of AFLP markers for resistance to root-knot nematodes in peach rootstocks. *Genome*. **41**: 199-207

Luthria, DL, Mukhopadhyay, S and Krizek, DT, (2006). Content of total phenolics and phenolic acids in tomato fruits as influenced by cultivar and solar UV radiation. *Journal of Food Composition and Analysis*. **19**: 771-777

Lytle, B, Song, J, de la Cruz, N, Peterson, F, Johnson, K, Bingman, C, Phillips, Jr. G and Volkman, B, (2009). Structures of two *Arabidopsis thaliana* major latex proteins represent novel Helix-Grip folds. Proteins: Structure, Function and Bioinformatics, Published [Online] http://www3.interscience.wiley.com/cgibin/fulltext/121675900/PDFSTART

Maarse, H, (1991). Volatile Compounds in Foods and Beverages, 6th edition, pp. 361. Marcel Dekker Inc., New York

Maeshima, M and Ishikawa, F, (2008). ER membrane aquaporins in plants. *European Journal of Physiology*. **456**: 709-716

Maga, J, (1974). Influence of color on taste thresholds. *Chemical Senses and Flavour*. **1**: 115-119

Malien-Aubert, C, Dangles, O and Amiot, M, (2001). Colour stability of commercial anthocyanin-based extracts in relation to the phenolic composition. Protective effects by intra- and intermolecular copigmentation. *Journal of Agricultural and Food Chemistry*. **49**: 170-176

Malundo, TMM, Shewfelt, RL and Scott, JW, (1995). Flavor quality of fresh-market tomato (*Lycopersicon esculentum* Mill.) as affected by sugar and acid levels. *Postharvest Biology and Technology*. **6**: 103-110

Marĩn-Rodrĩguez, MC, Orchard, J and Seymour, GB, (2002). Pectate lyases, cell wall degradation and fruit softening. *Journal of Experimental Botany*. **53**: 2115-2119.

Markakis, P, Livingston, GE and Fellers, RC, (1957). Quantitative aspects of strawberry pigment degradation. *Food Research*. **22**: 117-130

Marrs, KA, Alfenito, MR, Lloyd, AM and Walbot, V, (1995). A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene Bronze-2. *Nature*. **375**: 397-400

Marsh, KB, Volz, RK and Reay, P, (1996). Fruit colour, leaf nitrogen level, and tree vigour in "Fuji" apples. *New Zealand Journal of Crop Horticulture*. **24**: 393-399

Martinoia, E, Massonneau, A and Frangne, N, (2000). Transport processes of solutes across the vacuolar membrane of higher plants. *Plant Cell Physiology*. **41**: 1175-1186

Mather, K, (1949). Biometrical Genetics. 1st edition. Methuen, London

Matsumoto, H, Inaba, H, Kishi, M, Tominaga, S, Hirayama, M and Tsunda, T, (2001). Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. *Journal of Agricultual and Food Chemistry*. **49**: 1546-1551

Matus, JT, Aquea, F and Arce-Johnson, P, (2008). Analysis of the grape *MYB R2R3* subfamily reveals expanded wine quality-related clades and conserved gene structure organization across *Vitis* and *Arabidopsis* genomes. *BMC Plant Biology*. **8**: 83

Mayr, U, Michalek, S, Treutter, D and Feucht, W, (1997). Phenolic compounds of apple and their relationship to scab resistance. *Journal of Phytopathology*. **145**: 69-75

Mazza, G and Brouillard, R, (1990).The mechanism of co-pigmentation of anthocyanins in aqueous solutions. *Phytochemistry*. **29**: 1097-1102.

Mazzitelli, L, Hancock, RD, Haupt, S, Walker, PG, Pont, SDA, McNicol, J, Cardle, L, Morris, J, Viola, R, Brennan, R, Hedley, PE and Taylor, MA, (2007). Co-ordinated gene expression during phases of dormancy release in raspberry (*Rubus idaeus* L.) buds. *Journal of Experimental Botany*. **58**: 1035-1045

McGhie, TK and Walton, MC, (2007). The bioavailability and absorption of anthocyanins: Towards a better understanding. *Molecular Nutrition and Food Research*. **51**: 702-713

Micallef, BJ, Haskin, KA, Vanderveer, PJ, Roth, KS, Shewmaker, CK and Sharkey, TD, (1995). Altered photosynthesis, flowering and fruiting in transgenic tomato plants that have an increased capacity for sucrose synthesis. *Planta*. **196**: 327-334

Mohan, M, Nair, S, Bhagwat, A, Krishna, TG, Yano, M, Bhatia, CR and Sasaki, T, (1997). Genome mapping, molecular markers and marker-assisted selection in crop plants. *Molecular Breeding*. **3**: 87-103

Moisyadi, S and Stiles, JI, (1995). A cDNA Encoding a metallothionein 1-like protein from coffee leaves (*Coffea arabica*). *Plant Physiology*. **107**: 295-296

Monforte, AJ, Oliver, M, Gonzalo, MJ, Alvarez, JM, Dolcet-Sanjuan, R and Arus, P, (2004). Identification of quantitative trait loci involved in fruit quality traits in melon (*Cucumis melo* L.). *Theoretical and Applied Genetics*. **108**: 750-758

Morton, NE, (2005). Linkage disequilibrium maps and association mapping. *The Journal of Clinical Investigation*. **115**: 1425-1430

Mounet, F, Moing, A, Garcia, V, Petit, J, Maucourt, M, Deborde, C, Bernillon, S, Le Gall, G, Colquhoun, I, Defernez, M, Giraudel, JL, Rolin, D, Rothan, C and Lemaire-Chamley, M, (2009). Gene and metabolite regulatory network analysis of early developing fruit tissues highlights new candidate genes for the control of tomato fruit composition and development. *Plant Physiology*. **149**: 1505-1528

Moyer, RA, Hummer, KE, Finn, CE, Frei, B and Wrolstad, RE, (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: *Vaccinium*, *Rubus*, and *Ribes*. *Journal of Agricultural and Food Chemistry*. **50**: 519-525.

Mueller, L, Goodman, C, Silady, R and Walbot, V, (2000). AN9, a Petunia glutathione S-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. *Plant Physiology*. **123**: 1561-1570

Mullen, W, Lean, MEJ and Crozier, A, (2002a). Rapid characterization of anthocyanins in red raspberry fruit by high-performance liquid chromatography coupled to single quadrupole mass spectrometry. *Journal of Chromatography A*. **966**: 63-70

Mullen, W, McGinn, J, Lean, M, MacLean, M, Gardner, P, Duthie, G, Yokota, T and Crozier, A, (2002b). Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *Journal of Agricultural and Food Chemistry*. **50**: 5191-5196

Murant, AF, Chambers, J and Jones, AT, (1974). Spread of raspberry bushy dwarf virus by pollination, its association with crumbly fruit, and problems of control. *Annals of Applied Biology*. **77**: 271-281

Nakitandwe, J, Trognitz, F and Trognitz, B, (2007). Reliable allele detection using SNP-based PCR primers containing Locken nucleic acid: application in genetic mapping. *Plant Methods*. **3**: 2. doi: 10.1186/1746-4811-3-2

Nielsen, K, Deroles, SC, Markham, KR, Bradley, MJ, Podivinsky, E and Manson, D, (2002). Antisense flavonol synthase alters copigmentation and flower color in lisianthus. *Molecular Breeding*. **9**: 217-229

Nguyen-Quoc, B and Foyer, CH, (2001). A role for 'futile cycles' involving invertase and sucrose metabolism of tomato fruit. *Journal of Experimental Botany*. **52**: 881-889

Nybom, N, (1959). On the inheritance of acidity in cultivated apples. *Heriditas*. **45**: 332-350

O'Borevitz, J and Chory, J, (2004). Genomics tools for QTL analysis and gene discovery. *Current Opinion in Plant Biology*. **7**: 132-136

Oliviusson, P, Salaj, J and Hakman, I, (2001). Expression pattern of transcripts encoding water channel-like proteins in Norway spruce (*Picea abies*). *Plant Molecular Biology*. **46**: 289-299

Olsson, ME, Gustavsson, KE, Andersson, S, Nilsson, Y and Duan, RD, (2004). Inhibition of cancer cell proliferation *in vitro* by fruit and berry extracts and correlations with antioxidant levels. *Journal of Agricultural and Food Chemistry*. **52**: 7264-7271

Ourecky, DK, (1975). Brambles In: Advances in fruit breeding. Janick J and Moore JN (Eds). Purdue University Press, West Lafayette, USA.

Ozgen, M, Smith, JD and Palta, JD, (2002). Ripening stage has a dramatic influence on cranberry fruit postharvest shelf life: physiological and anatomical explanations. *Postharvest Biology and Technology*. **24**: 291-299.

Pangborn, RM, (1963). Relative taste of selected sugars and organic acids. *Journal of Food Science*. **28**: 726-733

Paterson, AH, (1996). Mapping genes responsible for differences in phenotype, In: Genome Mapping in Plants, pp. 41-54. RG. Landes Company, San Diego, California, Academic Press; Austin, Texas

Pattison, J and Samuelian, S, (2007). Inheritance of *Phytophthora* root rot resistance in red raspberry determined by generation means and molecular linkage analysis. *Theoretical and Applied Genetics*. **115:** 225-236

Petkov, B, (1963). A study of the melliferous properties of the cultured raspberry. *Selskostopanska Nauk*. **2**: 201-207

Pflieger, S, Lefebvre, V and Causse, M, (2001). The candidate gene approach in plant genetics: a review. *Molecular Breeding*. **7**: 275-291

Pool, RA, Ingram, R, Abbot, RJ, Jennings, DL and Topham, PB, (1981). Karyotype variation in *Rubus* with special reference to *R. idaeus* L. and *R. coreanus* Miquel. *Cytologia*. **46**: 125-132

Pot, D, Rodriques, JC, Rozenberg, P, Chantre, G, Tibbits, J, Cahalan, C, Pichavant, F and Plomion, C, (2006). QTLs and candidate genes for wood properties in maritime pine (*Pinus pinaster* Ait.). *Tree Genetics and Genomics*. **2**: 10-24

Price, AH, (2006). Believe it or not, QTLs are accurate. *Trends in Plant Science*. **5**: 213-216

Pritchard, JK, Stephens, M, Rosenburgh, NA and Donnelly, P, (2000). Association mapping in structural populations. *The American Journal of Human Genetics*. **67**: 170-181

Puupponen-Pimiä, R, Nohynek, L, Alakomi, H and Oksman-Caldentey, K, (2005). Bioactive berry compounds - novel tools against human pathogens. *Applied Microbiology and Biotechnology*. **65**: 8-18

Qu, LP and Hancock, JF, (1997). Randomly amplified polymorphic DNA (RAPD) based genetic linkage map of blueberry derived from an interspecific cross between diploid *Vaccinium darrowi* and tetraploid *V. corymbosum*. *Journal of the American Society of Horticultural Science*. **122**: 69-73

Quattrocchio, F, Wing, J, van der Woude, K, Mol, J and Koes, R, (1998). Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. *The Plant Journal*. **13**: 475-488

Reay, PF, Fletcher, RH and Thomas, VJ, (1998). Chlorophyll, carotenoids and anthocyanin concentrations in the skin of "Gala" apples during maturation and the influence of foliar applications of nitrogen and magnesium. *Journal of the Science of Food and Agriculture*. **76**: 63-71

Reyna, N and Sneller, CH, (2001). Evaluation of marker-assisted introgression of yield QTL alleles into adapted soybean. *Crop Science*. **41**: 1317-1321

Ribaut, JM and Hoisington, D, (1998). Marker-assisted selection: new tools and strategies. *Trends in Plant Science*. **3**: 236-239

Richardson, AC, Marsh, KB, Boldingh, HL, Pickering, AH, Bulley, SM, Frearson, NJ, Ferguson, AR, Thornber, SE, Bolitho, KM and Macrae, EA, (2004). High growing temperatures reduce fruit carbohydrate and vitamin C in kiwifruit. *Plant, Cell and Environment*. **27**: 423-435

Roach FA, (1985). Cultivated fruits of Britain: Their origin and history. Blackwell. Oxford

Robbins, J and Moore, MPP, (1990). Color change in fresh red raspberry fruit stored at 0, 4.5, or 20^{o}C. *HortScience*. **25**: 1623-1624

Roberts, DD and Acree, TE, (1996). Effects of heating and cream addition on fresh raspberry aroma using a retronasal aroma stimulator and gas chromatography olfactometry. *Journal of Agriculture and Food Chemistry*. **44**: 3919-3925

Romani, RJ, Sproule, BV, Mettler, IJ and Tuskes, SE, (1975). Extraction and purification of tRNA from fruit tissues. *Phytochemistry*. **14**: 2563-2567

Ronaghi, M, (2001). Pyrosequencing sheds light on DNA sequencing. *Genome Research*. **11**: 3-11

Rose, JKC, Lee, HH and Bennett, AB, (1997). Expression of a divergent expansin gene is fruit-specific and ripening-regulated. *Proceedings of the National Academy of Sciences, USA*. **94**: 5955-5960.

Roth, H, Radle, L, Gifford, S and Clydesdale, F, (1988). Psychophysical relationships between perceived sweetness and color in lemon-lime flavoured drinks. *Journal of Food Science*. **53**: 1116-1162

Rowland, LJ and Levi, A, (1994). RAPD-based genetic linkage map of blueberry derived from a cross between diploid species (*Vaccinium darrowi* and *V. elliottii*). *Theoretical and Applied Genetics*. **87**: 863-868

Rozen, S and Skaletsky, HJ, (2000). Primer3 on the WWW for general users and for biologists programmers. *Methods in Molecular Biology*. **132**: 356-386

Ruane, J and Sonnino, A, (2007). Marker-assisted selection as a tool for genetic improvement of crops, livestock, forestry and fish in developing countries: an overview of the issues. In: Marker Assisted Selection, Current Status and Future Perspectives in Crops, Livestock, Forestry and Fish. Guimarães, E, Ruane, B, Sonnino, A and Dargie, J (Eds). Journal of Food and Agriculture Organisation of the United Nations.

Saiki, RK, Gelfand, DH, Stoffel, S, Scharf, SJ, Higuchi, R, Horn, GT, Mullis, KB and Erlich, HA, (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. **239**: 487-491

Saliba-Colombani, V, Causse, M, Langlois, D, Philouze, J and Buret, M, (2001). Genetic analysis of organoleptic quality in fresh market tomato. Mapping QTLs for physical and chemical traits. *Theoretical and Applied Genetics*. **102**: 259-272

Sambrook, J and Russell, DW, (2001). Molecular Cloning- a Laboratory Manual, 3rd edition. Cold Spring Harbor. New York, USA.

Sargent, DJ, Fernandez-Fernandez, F, Rys, A, Knight, VH, Simpson, DW and Tobutt, KR, (2007). Mapping of A1 conferring resistance to the aphid *Amphorophora idaei* and dw (dwarfing habit) in red raspberry (*Rubus idaeus* L.) using AFLP and microsatellite markers. *BMC Plant Biology*. **7**:15. doi:10.1186/1471-2229-7-15

Sax, K, (1923). The association of size differences with seed coat pattern and pigmentation in *Phaseolus vulgaris*. *Genetics*. **8**: 552-560

Sexton, R, Palmer, JM, Whyte, NA and Littlejohns, S, (1997). Cellulase, fruit softening and abscission in red raspberry *Rubus idaeus* L. cv. Glen Clova. *Annals of Botany*. **80**: 371-376

Shachman, MC, (2004). The Soft Drinks Companion, A technical Handbook for the Beverage Industry. CRC Press LLC. Florida, USA.

Shewfelt, RL, (1999). What is quality? *Postharvest Biology and Technology*. **15**: 197-200

Shimada, Y, Nakano-Shimada, R, Ohbayashi, M, Okinaka, Y, Kiyokawa, S and Kikuchi, Y, (1999). Expression of chimeric P450 genes encoding flavonoid 3',5'-hydroxylase in transgenic tobacco and petunia plants. *FEBS Letters*. **461**: 241-245

Smart, L, Moskal, W, Cameron, K and Bennett, A, (2001). MIP genes are down-regulated under drought stress in *Nicotiana glauca*. *Plant Cell Physiology*. **42**: 686-693

Soriano, JM, Vilanova, S, Romero, C, Llácer, G and Badenes, ML, (2005). Characterization and mapping of NBS-LRR resistance gene analogs in apricot (*Prunus armeniaca* L.). *Theoretical and Applied Genetics*. **110**: 980-989

Stevens, R, Buret, M, Duffé, P, Garchery, C, Baldet, P, Rothan, C and Causse, M, (2007). Candidate genes and quantitative trait loci affecting fruit ascorbic acid content in three tomato populations. *Plant Physiology*. **143**: 1943-1953

Stewart, D, Iannetta, PPM and Davies, HV, (2001). Ripening-related changes in raspberry cell wall composition and structure. *Phytochemistry*. **56**: 423-428

Steyn, WJ, Wand, SJE, Holcroft, DM and Jacobs, G, (2002). Anthocyanins in vegetative tissues: a proposed unified function in phytoprotection. *New Phytologist*. **155**: 349-361

Steyn, WJ, Holcroft, DM, Wand, SJE and Jacobs, G, (2004). Anthocyanin degradation in detached pome fruit with reference to preharvest red color loss and pigmentation

patterns of blushed and fully red pears. *Journal of the American Society of Horticultural Science*. **129**: 13-19

Stone, ZS, Yasmin, T, Bagchi, M, Chatterjee, A, Joe, AV and Bagchi, D, (2007). Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition and Food Research*. **51**: 756-683

Stracke, R, Werber, M and Weisshaar, B, (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. *Current Opinion in Plant Biology*. **4**: 447-456

Stracke, R, De Vos, RCH, Bartelniewoehner, L, Ishihara, H, Sagasser, M, Martens, S and Weisshaar, B, (2009). Metabolomic and genetic analyses of flavonol synthesis in *Arabidopsis thaliana* support the *in vivo* involvement of leucoanthocyanidin dioxygenase. *Planta*. **229**: 427-445

Sturm, A and Tang, GQ, (1999). The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science*. **4**: 401-407

Sturm K, Koron, D and Stampar, F, (2003). The composition of fruit of different strawberry varieties depending on maturity stage. *Food Chemistry*. **83**: 417-422

Sumner, LW, Mendes, P and Dixon, RA, (2003). Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry*. **62**: 817-836

Suzuki, KI, Xue, HM, Tanaka, Y, Fukui, Y, Fukuchi-Mizutani, M, Murakami, Y, Katsumoto, Y, Tsuda, S and Kusumi, T, (2000). Flower color modifications of *Torenia hybrida* by co-suppression of anthocyanin biosynthesis genes. *Molecular Breeding*. **6**: 239-246

Syvänen, AC, (2001). Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nature Reviews Genetics*. **2**: 930-942

Takahashi, E, Takeda, O, Himoro, M, Nanao, K, Takada, G and Hayasaka, K, (1992). Localisation of PMP-22 gene (candidate gene for the Charcot-Marie-Tooth disease A1) to band 17p11.2 by direct R-banding fluorescence *in situ* hybridization. *Japanese Journal of Human Genetics*. **37**: 303-306

Takos, AM, Jaffē, FW, Jacob, SR, Bogs, J, Robinson, SP and Walker, AR, (2006). Light induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant Physiology*. **142**: 1216-1232

Tanaka, Y, Fukui, Y, Fukuchi-Mizutani, M, Holton, TA, Higgins, E and Kusumi, T, (1995). Molecular cloning and characterization of *Rosa hybrida* dihydroflavonol 4-reductase gene. *Plant Cell Physiology*. **36**: 1023-1031

Tanksley, SD, (1983). Molecular markers in plant breeding. *Plant Molecular Biology*. **1**: 3-8

Tanksley, SD and Hewitt, J, (1988). Use of molecular markers in breeding for soluble solids content in tomato - a re-examination. *Theoretical and Applied Genetics*. **75**: 811-823

Tanksley, SD and McCouch, S, (1997). Seed banks and molecular maps: unlocking genetic potential from the wild. *Science*. **22**: 1063-1066

Tanksley, SD, Young, ND, Paterson, AH and Bonierbale, M, (1989). RFLP mapping in plant breeding: New tools for an old science. *Biotechnology*. **7**: 257-264

Tanksley, SD, Grandillo, S, Fulton, TM, Zamir, D, Eshed, Y, Petiard, V, Lopez, J and Beck-Bunn, T, (1996). Advanced backcross QTL analysis in a cross between an elite processing line of tomato and its wild relative *L. pimpinellifolium*. *Theoretical and Applied Genetics*. **92**: 213-224

Thoday, JM, (1961). Location of polygenes. *Nature*. **191**: 368-370

Thorup, TA, Tanyolac, B, Livingstone, KD, Popovsky, S, Paran, I and Jahn, M, (2000). Candidate gene analysis of organ pigmentation loci in the Solanaceae. *Proceedings of the National Academy of Sciences, USA*. **97**: 11192-11197

Toyama-Kato, Y, Yoshida, K, Fujimori, E, Haraguchi, H, Shimizu, Y and Kondo, T, (2003). Analysis of metal elements of Hydrangea sepals at various growing stages by ICP-AES. *Biochemical Engineering Journal*. **14**: 237-241

Trabelsi, N, Petit, P, Manigand, C, Langlois d'Estaintot, B, Granier, T, Chaudière, J and Gallois, B, (2008). Structural evidence for the inhibition of grape dihydroflavonol 4-reductase by flavonols. *Acta Crystallographica*. **64**: 883-891

Traveset, A and Willson, MF, (1998). Ecology of the fruit-colour polymorphism in *Rubus spectabilis*. *Evolutionary Ecology*. **12**: 331-345

Treutter, D, (2001). Biosynthesis of phenolic compounds and its regulation in apple. *Plant Growth Regulation*. **34**: 71-89

Tucker, GA, (1993). Fruit ripening. In: Biochemistry of Fruit Ripening. Seymour, GB, Taylor, JE and Tucker, GA (Eds.). Academic Press, London pp. 1-51

Tucker, GA and Brady, CJ, (1987). Silver ions interrupt tomato fruit ripening. *Journal of Plant Physiology*. **127**: 287-299

Ueyama, Y, Suzuki, KI, Fukuchi-Mizutani, M, Fukui, Y, Miyazaki, K, Ohkawa, H, Kusumi, T and Tanaka, Y, (2002). Molecular and biochemical characterization of torenia flavonoid 3'-hydroxylase and flavone synthase II and modification of flower color by modulating the expression of these genes. *Plant Science*. **163**: 253-263

Valois, D, Fayad, K, Barasubiye, T, Garon, M, Dèry, C, Brzezinski, R and Beaulieu, C, (1996). Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi,* the causal agent of raspberry root rot. *Applied and Environmental Microbiology*. **62**: 1630-1635

Van der Krol, AR, Lenting, PE, Veenstra, J, Van der Meer, IM, Koes, RE, Gerats, AGM, Mol, JNM and Stuitle, AR, (1988). An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature*. **333**: 866-869

Van Ooijen, JW, (2004). MapQTL® 5, software for the mapping of quantitative trait loci in experimental populations. Kyazma BV. Wageningen, the Netherlands

Van Ooijen, JW and Voorrips, RE, (2001). JoinMap® 3.0, software for the calculation of genetic linkage maps. Plant Research International, Wageningen, the Netherlands

Varshney, RK and Tuberosa, R, (2008). Genomics-Assisted Crop Improvement: Vol 2: Genomics Application in Crops. Springer Publication, the Netherlands

Varshney, RK, Nayak, SN, May, GD and Jackson, SA, (2009). Next-generation sequencing and their implications for crop genetics and breeding. *Trends in Biotechnology*. **27**: 522-530

Verlangieri, AJ, Kapeghian, JC, el-Dean, S and Bush, M, (1985). Fruit and vegetable consumption and cardiovascular mortality. *Medical Hypothesis*. **16**: 7-15

Vickers, ZM, (1983). Pleasantness of food sounds. *Journal of Food Science*. **48**: 783-786.

Voorrips, VE, (2002). MapChart: Software for the graphical presentation of linkage maps and QTLs. *Journal of Heredity*. **93**: 77-78

Vrebalov, J, Ruezinsky, D, Padmanabhan, V, White, R, Medrano, D, Drake, R, Schuch, W and Giovannoni, J, (2002). A MADS-Box gene necessary for fruit ripening at the tomato ripening-inhibitor (Rin) Locus. *Science*. **296**: 343-346

Walker, A, Lee, E, Bogs, J, McDavid, D, Thomas, M and Robinson, S, (2007). White grapes arose through the mutation of two similar and adjacent regulatory genes. *The Plant Journal*. **49**: 772-785

Wang, SY and Lin, H, (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry*. **48**: 140-146

Wang, SY and Zheng, W, (2005). Preharvest application of methyl jasmonate increases fruit quality and antioxidant capacity in raspberries. *International Journal of Food Science and Technology*. **40**: 187-195

Wang, SY, Chen, C and Wang, CY, (2009). The influence of light and maturity on fruit quality and flavonoid content of red raspberries. *Food Chemistry*. **112**: 676-684

Wang, DG, Fan, JB, Siao, CJ, Berno, A, Young, P, Sapolsky, R, Ghandour, G, Perkins, N, Winchester, E, Spencer, J, Kruglyak, L, Stein, L, Hsie, L, Topaloglou, T, Hubbell, E, Robinson, E, Mittmann, M, Morris, MS, Shen, NP, Kilburn, D, Rioux, J, Nusbaum, C, Rozen, S, Hudson, TJ, Lipshutz, R, Chee, M and Lander, ES, (1998). Large-scale

identification, mapping and genotyping of single-nucleotide polymorphisms in the human genome. *Science*. **280**: 1077-1082.

Wang, H, Muraleedharan, GN, Strasburg, GM, Chang, YC, Booren, AM, Gray, JI and DeWitt, DL, (1999). Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. *Journal of Natural Products*. **62**: 294-296

Watson, JD, Baker, TA, Bell, SP, Gann, A, Levine, M and Losick, R, (2004). Molecular Biology of the Gene. Benjamin Cummings, Cold Spring Harbor, New York, USA.

Wellman, F, Lukačin, R, Moriguchi, T, Britsch, L, Schilitz, E and Matern, U, (2002). Functional expression and mutational analysis of flavonol synthase from *Citrus unshiu*. *European Journal of Biochemistry*. **269**: 4143-4142

Westman, AL and Kresovich, S, (1999). Simple sequence repeat (SSR)-based marker variation in *Brassica nigra* genebank accessions and weed populations. *Euphytica*. **109**: 85-92

Wheeler, GL, Jones, MA and Smirnoff, N, (1998). The biosynthetic pathway of vitamin C in higher plants. *Nature*. **393**: 365-369

Wilcox, AN and Angelo, E, (1936). Apple breeding studies in fruit color. *Proceedings of the American Society of Horticultural Science*. **33**: 108-113

Winkel-Shirley, B, (2001). Flavonoid biosynthesis. A colourful model for genetics, biochemistry, cell biology and biotechnology. *Plant Physiology*. **126**: 485-493

Wolfe, SL, Russell, PJ and Hertz, PE, (2007). Biology: The Dynamic Science. Gencage Learning, Belmont, USA.

Woodhead, M, Taylor, MA, Davies, HV, Brennan, RM and McNicol, RJ, (1997). Isolation of RNA from blackcurrant (*Ribes nigrum* L.) fruit. *Molecular Biotechnology*. **7**: 1-4

Woodhead, M, McCallum, S, Smith, K, Cardle, L, Mazzitelli, L and Graham, J, (2008). Identification, characterisation and mapping of simple sequence repeat (SSR) markers from raspberry root and bud ESTs. *Molecular Breeding*. **22**: 555-563

Wrolstad, RE, Durst, RW and Lee, J, (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science and Technology*. **16**: 423-428

Wu, B, Quilot, B, Kervella, J, Genard, M and Li, S, (2003). Analysis of genotypic variation of sugar and acid contents in peaches and nectarines through the principal component analysis. *Euphytica*. **132**: 375-384

Wu, XL, Beecher, GR, Holden, JM, Haytowitz, DB, Gebhardt, SE and Prior, RL, (2006). Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *Journal of Agricultural and Food Chemistry*. **54**: 4069-4075

Xie, D, Sharma, S, Paiva, N, Ferreira, D and Dixon, R, (2003). Role of anthocyanidin reductase, encoded by *BANYULS,* in plant flavonoid biosynthesis. *Science*. **299**: 396-399

Yamada, S, Komori, T, Myers, PN, Kuwata, S, Kubo, T and Imaseki, I, (1997). Expression of plasma membrane water channel genes under water stress in *Nicotiana excelsior*. *Plant Cell Physiology*. **38**: 1226-1231

Yates, H, Frary, A, Doganlar, S, Frampton, A, Eannetta, NT, Unlig, J and Tanksley, SD, (2004). Comparative fine mapping of fruit quality QTLs on chromosome 4 introgressions derived from two wild tomato species. *Euphytica*. **135**: 283-296

Yawadio, R and Morita, N, (2007). Color enhancing effect of carboxylic acids on anthocyanins. *Food Chemistry*. **105**: 421-427

Zampini, M, Sanabria, D, Phillips, N and Spence, C, (2007). The multisensory perception of flavour: Assessing the influence of color cues on flavour discrimination responses. *Food Quality and Preference*. **18**: 975-984

Zellner, D and Durlach, P, (2003). Effect of color on expected and experienced refreshment, intensity, and liking ratings. *American Journal of Psychology*. **116**: 633-647

Zhang, F, Gonzalez, A, Zhao, M, Payne, CT and Lloyd, A, (2003). A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. *Development*. **130**: 4859-4869

Zhang, Z, Xuequn, P, Yang, C, Ji, Z and Jiang, Y, (2004). Purification and structural analysis of anthocyanins from litchi pericarp. *Food Chemistry*. **84**: 601-604

Zhao, YH, Li, XL, Jiang, ZS, Wang, CJ and Yang, FL, (2007). Organic acid metabolism in nectarine fruit development under protected cultivation. *Chinese Journal of Eco-Agriculture*. **15**: 87-89.

Zheng, D and Hrazdina, G, (2008). Molecular and biochemical characterization of benzalacetone synthase and chalcone synthase genes and their proteins from raspberry (*Rubus idaeus* L.). *Archives of Biochemistry and Biophysics*. **470**: 139-145

Zygier, S, Chaim, AB, Efrati, A, Kaluzky, G, Borovsky, Y and Paran, I, (2005). QTLs mapping for fruit size and shape in chromosomes 2 and 4 in pepper and a comparison of the pepper QTL map with that of tomato. *Theoretical and Applied Genetics*. **111**: 437-445

Appendix 2.1. Clustal results for expansin showing first six comparative results. The underlined example shows peach expansin derived from Etienne *et al*. (2002) with primer pairs highlighted in bold.

Expansin ClustalW

```
CLUSTAL W (1.83) multiple sequence alignment

AB047518        AGGGGTACGGGACAAATACTGCAGCACTGAGCACTGCTCTGTTCAACAATGGCTTGGGCT 284
AF297522        AGGGGTATGGGACAAATACTGCAGCACTGAGCACTGCTCTGTTCAACAATGGCTTGGGCT 300
AF448467        AGGGGTATGGGACAAATACTGCAGCACTGAGCACTGCTCTGTTCAACAATGGCTTGGGCT 286
AF367459        AGGGGTACGGGACAAATACTGCAGCACTGAGCACTGCTCTGTTCAACAATGGCTTGGGCT 112
DQ183068        AGGGGTATGGAACAAACACTGCTGCTCTGAGCACTGCTCTGTTCAACAATGGGTTGGGGT 242
AF226701        AGGGGTATGGAACAAACACCGCTGCGCTGAGCACTGCTCTGTTCAACAATGGGTTGGGGT 97
                ******* ** ***** ** ** ** ************************** ***** *

AB047518        GTGGTTCTTGCTATGAGATTAGGTGTGTAAGTGACCCCAAATGGTGCTTGCCTGGCGCCA 344
AF297522        GTGGTTCTTGCTATGAGATTAGGTGTGTAAATGACCCCAAATGGTGCTTGCCTGGTGCCA 360
AF448467        GTGGTTCTTGCTATGAGATTAGGTGTGTAAATGACCCCAAATGGTGCTTGCCTGGCGCCA 346
AF367459        GTGGTTCTTGCTATGAGATTAGGTGTGTAAGTGACCCCAAATGGTGCTTGCCTGGCGCCA 172
DQ183068        GTGGTTCTTGCTATGAAATTAGGTGTGTGAATGACCCAAAATGGTGCCTGCCTGGCTCCA 302
AF226701        GTGGTTCTTGCTATGAAATTAGGTGTGTGAATGACCCAAAATGGTGCCTGCCTGGCTCCA 157
                *************** *********** * ****** ********* *******  ***

AB047518        TTGTGGTCACAGCCACCAATTTCTGCCCACCAAACAATGCCCTTCCTAACAATGCTGGGG 404
AF297522        TTGTGGTCACAGCCACCAATTTCTGCCCACCAAACAATGCCCTTCCTAACAATGCTGGGG 420
AF448467        TTGTGGTCACAGCCACTAATTTCTGCCCACCAAACAATGCCCTTCCTAACAATGCTGGGG 406
AF367459        TTGTGGTCACAGCCACCAATTTCTGCCCACCAAACAATGCCCTTCCTAACAATGCTGGGG 232
DQ183068        TTGTGGTCACAGCCACTAATTTCTGCCCTCCAAACAATGCCCTCCCTAACAATGCTGGGG 362
AF226701        TTGTGGTCACAGCCACTAATTTCTGCCCTCCAAACAATGCCCTCCCTAACAATGCTGGGG 217
                *************** *********** ************** ***************

AB047518        GCTGGTGCAATCCTCCTCAGCACCACTTTGACCTCTCTCAGCCTGTCTTCCAGCACATTG 464
AF297522        GCTGGTGCAATCCTCCTCAGCACCACTTTGACCTCTCTCAGCCTGTCTTCCAGCACATTG 480
AF448467        GCTGGTGCAATCCTCCTCAGCACCACTTTGACCTCTCTCAGCCTGTCTTCCAGCACATTG 466
AF367459        GCTGGTGCAATCCTCCTCAGCACCACTTTGACCTCTCTCAGCCTGTCTTCCAGCACATTG 292
DQ183068        GATGGTGCAACCCTCCCCAGCACCACTTTGACCTCTCTCAGCCTGTATTCCAGCACATTG 422
AF226701        GATGGTGCAACCCTCCCCAGCACCACTTTGACCTCTCTCAGCCTGTATTCCAGCACATTG 277
                * ******** ***** ***************************** *************

AB047518        CTCAGTACAAAGCTGGTGTTGTGCCTGTTGCTTACAGAAGGGTACCTTGCAGGAGAAGGG 524
AF297522        CTCAGTACAAAGCTGGTGTTGTGCCTGTTGCTTACAGAAGGGTACCTTGCAGGAGGAGGG 540
AF448467        CTCAGTACAAAGCTGGTGTTGTGCCTGTTGCTTACAGAAGGGTACCTTGCAGGAGAAGGG 526
AF367459        CTCAGTACAAAGCTGGTGTTGTGCCTGTTGCTTACAGAAGGGTACCTTGCAGGAGAAGGG 352
DQ183068        CTCAATACAAAGCTGGAGTTGTCCCTGTTTCTTACAGAAGGGTACCCTGCAGAAGAAGGG 482
AF226701        CTCAATACAAAGCTGGAGTTGTCCCTGTTTCTTACAGAAGGGTACCCTGCAGAAGAAGGG 337
                **** *********** ***** ****** ********** ***** ** ****

AB047518        GAGGTATCAGATTCACCATCAATGGGCACTCCTACTTCAACCTGGTCCTCATCACAAACG 584
AF297522        GAGGTATCAGATTCACCATCAATGGGCACTCCTACTTCAACCTGGTCCTCATCACAAACG 600
AF448467        GAGGTATCAGATTCACCATCAATGGGCACTCCTACTTCAACCTGGTTCTCATCACAAACG 586
AF367459        GAGGTATCAGATTCACCATCAATGGGCACTCCTACTTCAACCTGGTCCTCATCACAAACG 412
DQ183068        GAGGCATCAGATTCACCGTCAACGGACACTCCTACTTCAACCTGGTTTTAATCACAAACG 542
AF226701        GAGGCATCAGATTCACCATCAATGGACACTCCTACTTCAACCTGGTTTTGATCACAAACG 397
                **** ************ **** ** ********************  * **********

AB047518        TTGGTGGTGCCGGTGATGTGCACTCTGTTTCAGTCAAAGGGTCCAGGACTGGTTGGCAAG 644
AF297522        TTGGTGGTGCCGGTGATGTGCACTCTGTTTCAGTCAAAGGGTCCAGGACTGGTTGGCAAG 660
AF448467        TTGGTGGTGCCGGTGATGTGCACTCTGTTTCAGTCAAAGGGTCCAGGACTGGTTGGCAAG 646
AF367459        TTGGTGGTGCCGGTGATGTGCACTCTGTTTCAGTCAAAGGGTCCAGGACTGGTTGGCAAG 472
DQ183068        TTGGTGGTGCTGGTGATGTGCACTCTGTTTCAGTCAAAGGGTCCAAAACCGGTTGGCAAG 602
AF226701        TTGGTGGTGCTGGTGATGTGCACTCTGTTTCAGTCAAAGGGTCCAAAACCGGTTGGCAAG 457
                ********** ********************************  ** **********
```

Appendix 2.2. Redesigned primers ordered using Operon degenerative bases.

Candidate gene	**Primer 3 sequence**	**Primer sequence with degenerative bases**
AtpvA1Lg_For	ATGCTTCAGACTTGGCCTGTA	ATGCTTCARRCHTGGCCYGTA
AtpvA1Lg_Rev	CGCATCATCCAAACAGACTTGTA	CGCATCATCCARACRGACTTGTA
MIP 3 For	ATGATCTTTGTCCTTGTTTACTGCA	ATGATCTTYRTBCTTGTYTACTGCA
MIP 3 rev	TCCCAGCCTTGTCATTGTT	TCCCADGCYTTSTSWTBTT
Icdh1_for	ATGGACGGAGATGAAATGAC	ATGGAYGGRGATGAAATGAC
Icdh1_rev	GCCACAGCTTCAATGAACTCTTC	GCCACRGCWTCAATRAAYTCYTC
VP1_for	GCTGCTGATGTTGGTGCTGA	GCTGCTGATGTTGGKGCWGA
VP_1 rev	ATGTTGAGTGATGGGCCTGAGGTG	ATGTTGAGHGAYGGSCCWGARGTG
Mdh1F	CCTCTTCTTAAAGGTGTTGTTGCTA	CCHCTTCTTAARGGTGTTGTTGCTA
Mdh1R	AGCCCTTGAACAATCTTCCACT	AGHYCTTGAACWATBKWCCAYT
CS1F	CCTATTACAGCACATCCAATGA	CCTRTHWCDGCWCATCCRATGA
CS1R	CAAGAGCCCGGTCCCATA	CAAGAGCYCKRTCCCATA
Ai1F	GATCCCCTCCTCCTCCATT	GATCCMYTMYTMYTBSTT
Ai1R	ATTCCCACATGCCCGTACC	AYTCCCACATDCCMGTACC
EXPF	AATGGCTTGGGCTGTGGTT	AATGGSTTGGGSTGTGGTT

EXPR	TGGACCCTTTGACTGAAACAGA	TGGACCCTTTGACTGAAACAGA
Suc Synth F	ACTGGTGGCCAGGTTGTTTA	ACYGGTGGCCAGRTKGTKTA
Suc Synth R	CAGCTGTAAATTGGGATGAGA	TCAGCWGTRAAYTGGSAGAGA
HK Combo F	GGCAGCAAGCTCAAGATG	GGCAGCAAGCTCAAGATG
HK Combo F	GATAACCATCTCTCCTGATTTAGGT	GATRACCATYTCTCCWGATTTAGG
Fw 2.2 F	TGAGTACACGGTGGAAAAGAGAAC	TGAGTACACGGTGGAAAAGARAAC
Fw 2.2 R	AACTTTATTTTAGAAAACGAAGCA	AACTTTATTTTAGAAAACGAAGCA

Appendix 2.3a. Primer sequences used for PCR amplification of allele diversity germplasm.

Gene	Forward Primer	Reverse Primer
TIP	AACCGGTGGATTGGTAAG	GAAGGAGACTGCTGGGTTCA
MIP 2	CTCGCAAGGTTTCACTGCCGAGGGCA	ACTTCTAGCAGGGTTGATACCAGTTCC
MIP 3	ATGATCTTYRTBCTTGTYTACTGCAC	TCCCADGCYTTSTSWTBGTT
EXP	AATGGSTTGGGSTGTGGTT	TGGACCCTTTGACTGAAACAGA
Gal-dh	GAGGGACATTGTCGGAGAAA	ACCTCTCCAGGCTCTCATCA
FLS	AGGTGAACAGGTGGAGTTGG	TGAAGACCATCATCGAAT GC
DFR	ATGCGAAACAACTTGCATTT	GCTACGATTCACGACATTGC
GST	CTATGCTTGGCTTGGTCACA	TTTTGCCCCTTAAAATGCAG
F3'H	TGATGAAGCTTTATAAGCATGTGAGG	GGGTCCACTCTCTTGGTGAA
ANR	ACTAGCTGAGAAGACAGCTTGGAA	TCTCCAAACTCAGTGGGGACTT
bHLH	(6-Fam)AAAGTGCCTTCTGCTGCATT	CCGTTTGCTAATGCTCTTCC
Myb	(6-Fam)CCCAATTGTCAGTAC GTTGG	ATTCTTGATTTCATTGTCTGTTCG
118b	(5-Hex)CCGCAAAACAAAAGGTCAAG	GGATTCTTGCCAAAGTCGAA

Appendix 2.3b. Primer sequences for Pyrosequence® analysis

Gene	Forward Primer	Reverse Primer	Sequencing Primer
TIP	TAGCTGCTTTCTCCC TGTCAACC	ATGTTTCCGGCGGACC TC	GGTGTTAGTGTATGGA ACG
Mip 2	GAGCAAATGAGTTGGC AGATGG	ATCTCAGCCGCCAAT CCAG	TGAGTTGGCAGATGG
Exp 4	GCCATTGATGGTGAAT CTGAT	CACTGATTTTTTGTGG GTCTTCTT	CCTTCTGCTACAGGGT
Gal-dh	TGCCGAGAGAGTGACT AAGAGCAT	CATGGCATTGCAATAT ATCAACG	AATCTAGCTGCAACCT TT
FLS	GGTCTTGAGCAACGGC AAGT	TTTGCGGTATCGGTAT TCAGCAT	GTGGCGGCGCAATGA
ANR	TTGCACCTTCTGATTTC TTACCT	CCAGATATCCCCAGCA GTATTG	CAGTATTGGCCTCGC
DFR	AAACTCAAACCCAGTC TCCAACAA	GAATTGGTGTACGTGT GTACAGGT	CCTTTGTCAAGTTCTCC TC
F3'H	TCAGTCGTGAGCCCA TCAG	TGAGCTTGGGCTTGCG TA T	AGTCGTGAGCCCATC

Appendix 3.1a. Phenotypic measurements for mapping population plus parents

Season	Samples		°Brix	Ten berry weight	Y	x	y	ΔE
Field 2006	Progeny	Mean±SEM	7.6±0.05	20.3±0.21	15.0±0.1	0.374±0.001	0.318±0.000	52.3±0.1
		Min-Max	3.2-12.2	8.7-37.0	12.5-18.0	0.349-0.393	0.315-0.325	49.3-54.7
Field 2007	Progeny	Mean±SEM	6.1±0.1	18.6±0.3	14.3±0.1	0.379±0.001	0.321±0.000	53.7±0.1
		Min-Max	3.5-9.3	10.6-34.5	11.6-18.7	0.361-0.404	0.317-0.327	50.8-57.4
Polytunnel 2007	Progeny	Mean±SEM	7.5±0.1	34.7±0.8	14.2±0.1	0.383±0.001	0322±0.000	54.0±0.2
		Min-Max	3.8-11.9	15.0-75.3	10.7-20.6	0.356-0.424	0.317-0.329	48.0-61.1
Commercial 2007	Progeny	Mean±SEM	7.9±0.1	29.3±0.8	14.3±0.1	0.377±0.001	0.321±0.000	53.5±0.1
		Min-Max	5.4-10.8	11.0-55.1	12.1-17.9	0.356-0.395	0.317-0.329	49.7-55.6
Field 2008	Progeny	Mean±SEM	6.4±0.1	21.4±0.4	15.8±0.1	0.383±0.001	0.319±0.000	52.5±0.1
		Min-Max	4.0-9.7	6.9-48.1	12.9-21.7	0.364-0.410	0.315-0.326	47.5-57.5
Polytunnel 2008	Progeny	Mean±SEM	7.7±0.1	35.6±0.7	15.8±0.1	0.384±0.001	0.320±0.000	52.7±0.1
		Min-Max	3.3-11.0	16.4-71.8	11.6-19.1	0.366-0.415	0.316-0.326	19.5-60.0
Commercial 2008	Progeny	Mean±SEM	8.4±0.1	31.1±0.7	16.0±0.1	0.389±0.001	0.321±0.000	52.6±0.2
		Min-Max	5.6-11.0	17.4-53.9	13.4-19.2	0.371-0.401	0.315-0.326	41.3-56.9

Season	Parents		°Brix	Ten berry weight	Y	x	y	ΔE
Field 2006	G. Moy	Mean±SEM	8.2	23.0	14.9	0.370	0.318	51.7
	Latham	Min-Max	7.4	13.8	13.1	0.376	0.318	54.8
Field 2007	G. Moy	Mean±SEM	8.2	34.3	15.9	0.385	0.321	52.3
	Latham	Min-Max	*	*	*	*	*	*
Polytunnel 2007	G. Moy	Mean±SEM	7.0	48.5	16.0	0.378	0.322	51.5
	Latham	Min-Max	5.2	28.7	15.0	0.386	0.322	53.3
Commercial 2007	G. Moy	Mean±SEM	7.4	31.6	15.7	0.390	0.323	52.9
	Latham	Min-Max	*	*	*	*	*	*
Field 2008	G. Moy	Mean±SEM	9.1	31.5	16.8	0.381	0.320	51.1
	Latham	Min-Max	*	*	*	*	*	*
Polytunnel 2008	G. Moy	Mean±SEM	6.4	46.6	17.4	0.389	0.319	51.8
	Latham	Min-Max	8.8	27.9	14.7	0.380	0.318	53.6
Commercial 2008	G. Moy	Mean±SEM	*	*	*	*	*	*
	Latham	Min-Max	9.1	38.8	15.0	0.382	0.319	53.3

Appendix 3.1b. Biochemical measurements for mapping population plus parents

Season	Samples		Total phenolics	Total anthocyanins	pH
Field 2008	Progeny	Mean±SEM	158.3±4.5	71.2±2.4	3.42±0.01
		Min-Max	67.3-375.0	14.6-142.1	3.13-3.85
Polytunnel 2008	Progeny	Mean±SEM	136.2±3.8	82.1±2.7	3.31±0.01
		Min-Max	62.5-314.1	27.4-177.7	2.98-3.95
Commercial 2008	Progeny	Mean±SEM	80.1±2.9	63.4±2.1	3.28±0.01
		Min-Max	28.2-157.3	24.2-120.4	3.06-3.60

Season	Parent		Total phenolics	Total anthocyanins	pH
Field 2008	G. Moy	Mean±SEM	144.7	58.6	3.41
	Latham	Min-Max	*	*	*
Polytunnel 2008	G. Moy	Mean±SEM	121.2	75.4	3.27
	Latham	Min-Max	123.6	121.6	3.18
Commercial 2008	G. Moy	Mean±SEM	*	*	*
	Latham	Min-Max	43.8	100.9	3.16

Publications

I.) **McCallum**, **S**, Woodhead, M, Hackett, CA, Kassim, A, Paterson, A and Graham, J, (2009). Genetic and environmental effects influencing fruit colour. In preperation

II.) Kassim, A, Poette, J, Paterson, A, Zait, D, **McCallum**, **S**, Woodhead, M, Smith, K, Hackett, C and Graham, J, (2009). Environmental and seasonal influences on red raspberry anthocyanin antioxidant contents and identification of QTL. *Molecular Nutrition and Food Research*. **53**: 625-634

III.) Graham, J, Hackett, CA, Smith, K, Woodhead, M, Hein, I, MacKenzie, K and **McCallum, S**, (2009). Mapping QTL for developmental traits in raspberry from bud break to ripe fruit. *Theoretical and Applied Genetics*. **118**: 1143-1155

IV.) Woodhead, M, **McCallum, S**, Smith, K, Cardle, L, Mazzitelli, L and Graham, J, (2008). Identification, characterisation and mapping of simple sequence repeat (SSR) markers from raspberry root and bud ESTs. *Molecular Breeding*. **22**: 555-563.

V.) Zait, D, Kassim, A, Graham, J, **McCallum**, **S** and Paterson, A, (2008). Progress in marker assisted breeding in red raspberry for character (abstract only). Plant and Animal Genomes XVI Conference, San Diego, CA, USA, 239

VI.) Kassim, A, Zait, D, Vandroux, M, **McCallum**, **S**, Graham, J, Hackett, C and Paterson, A, (2008). Genomic studies in raspberry flavour: Volatiles and QTL in progeny of a cross between Scottish and American parents. 12th Weurman Symposium, Interlaken.

I.) In preparation

Genetic and environmental effects influencing fruit colour.

Susan McCallum[1], Mary Woodhead[1], Christine A. Hackett[2], Angzzas Kassim[3], Alistair Paterson[3] and Julie Graham[1]

[1]Genome Dynamics Programme, SCRI, Invergowrie,Dundee DD2 5DA, UK
[2]Biomathematics and Statistics Scotland, SCRI, Invergowrie, Dundee DD2 5DA, UK
[3] Centre for Food Quality, University of Strathclyde, George Street, Glasgow G1 1XW

Abstract

Raspberry (*Rubus idaeus*) fruit colour was assessed in the 'Latham' × 'Glen Moy' mapping population using a colour meter and visual scores over three seasons and three environments. The colour measurements were found to be significantly associated with pigment content, have high heritability, and stable QTL were identified across environments and seasons. Anthocyanin content has previously been shown to be the major contributor to fruit colour in red raspberry. Major structural genes (F3'H, FLS, DFR, IFR, OMT, GST) and transcription factors (bZIP, bHLH, MYB) influencing flavonoid biosynthesis have been identified, mapped and shown to underlie QTL for quantitative and qualitative anthocyanin composition. Favourable alleles for selected traits were identified for aspects of fruit colour and partitioning of individual pigments.

II.) Research Article
Molecular Nutrition and Research

Environmental and seasonal influences on red raspberry anthocyanin antioxidant contents and identification of QTL

Angzzas Kassim, Julie Poette, Alistair Paterson, Dzeti Zait, **Susan McCallum**, Mary Woodhead, Kay Smith, Christine Hackett and Julie Graham

Abstract
Consumption of raspberries promotes human health through the intake of pharmaceutically-active antioxidants, including cyanidin and pelargonidin anthocyanins. These are products of flavonoid metabolism and also pigments that confer colour to fruit. Breeders may wish to influence fruit anthocyanin content for nutritional health and quality benefits through modern breeding utilising DNA polymorphisms: marker assisted breeding (MAB). High performance liquid chromatography (HPLC) quantified eight anthocyanins cyanidin and pelargonidin glycosides: -3-sophoroside, -3-glucoside, -3- rutinoside and -3-glucosylrutinoside across two seasons and two environments in progeny from a cross between two Rubus subspecies, *Rubus idaeus* (cv. Glen Moy) x *Rubus strigosus* (cv. Latham). Significant seasonal variation was detected across the pigments but less so for the different growing environments within a season. The eight antioxidants mapped to the same chromosome region on linkage group (LG) 1, across both years and from fruits grown in the field and under protected cultivation. Seven antioxidants also mapped to a region on LG 4 across years and for both field and protected sites. A chalcone synthase (PKS 1) gene sequence mapped to LG 7 but did not underlie the anthocyanin QTLs identified. However other candidate genes including bHLH, NAM/CUC2 like protein and bZIP transcription factor underlying the mapped anthocyanins were identified.

III.) Research Article
Theoretical and Applied Genetics

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

Julie Graham, Christine A. Hackett, Kay Smith, Mary Woodhead, Ingo Hein and **Susan McCallum**

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

IV.) Research Article
Molecular Breeding

Identification, characterisation and mapping of simple sequence repeat (SSR) markers from raspberry root and bud ESTs

M. Woodhead, **S. McCallum**, K. Smith, L. Cardle, L. Mazzitelli and J. Graham

Abstract
Raspberry breeding is a long, slow process in this highly heterozygous out-breeder. Selections for complex traits like fruit quality are broad-based and few simple methodologies and resources are available for glasshouse and field screening for key pest and disease resistances. Additionally, the timescale for selection of favourable agronomic traits requires data from different seasons and environmental locations before any breeder selection can proceed to finished cultivar. Genetic linkage mapping offers the possibility of a more knowledge-based approach to breeding through linking favourable traits to markers and candidate genes on genetic linkage maps. To further increase the usefulness of existing maps, a set of 25 polymorphic SSRs derived from expressed sequences (EST-SSRs) have been developed in red raspberry (*Rubus idaeus*). Two different types of expressed sequences were targeted. One type was derived from a root cDNA library as a first step in assessing sequences which may be involved in root vigour and root rot disease resistance and the second type were ESTs from a gene discovery project examining bud dormancy release and seasonality. The SSRs detect between 2 and 4 alleles per locus and were assigned to linkage groups on the existing 'Glen Moy' × 'Latham' map following genotyping of 188 progeny and examined for association with previously mapped QTL. The loci were also tested on a diverse range of Rubus species to determine transferability and usefulness for germplasm diversity studies and the introgression of favourable alleles.

V.) Plant and Animal Genomes XVI Conference
January 12-16th 2008
Town and Country Convention Centre
San Diego, California

Progress in marker assisted breeding in red raspberry for flavour character.

Dzeti Zait[1], Angzzas Kassim[1], Julie Graham[2], **Susan McCallum**[2] and Alistair Paterson[1]

[1] SIPBS Royal College, University of Strathclyde, Glasgow G1 1XW, Scotland, U.K.
[2] SCRI Genetics and Breeding Group, Invergowrie, Dundee, DD2 5DA, Scotland, U.K.

Abstract:
Red raspberry (*R. idaeus*) is a profitable temperate soft-fruit crop (1). Increased consumption would benefit consumer health (2). However current retail purchase may be prejudiced by inconsistent quality. Consumers assess berries visually at purchase and subsequently on flavour character, and together these determine repeat purchase. Identification of quantitative trait loci (QTLs) associated with development of berry sweetness and flavour intensity would yield markers for favourable alleles and a tool kit for marker assisted breeding. An early fruiting Glen Moy was crossed with late cropping, North American Latham and progeny canes were established on an open field and two locations of covered sites. Trained assessors scored berries for sweetness, sourness and flavour intensity; sugars and organic acid contents were quantified by HPLC. QTL were mapped on to a revised genetic linkage map for red raspberry (3). Appearance was studied by quantifying pigment anthocyanins, instrumental measurement and visual colour scoring and 10-berry weight. Preliminary data showed correlation between 3 raspberry flavour attributes was preserved over two seasons, indicating genetic control is greater than environmental influence on flavour development. Single nucleotide polymorphisms (SNPs) differentiating parental genomes were identified through PCR of candidate genes with primers designed on the basis of sequence data from other Rosaceae members.

(1) http://faostat.fao.org/
(2) Ross et al. (2007). Antiproliferative activity is predominantly associated with ellagitannins in raspberry extracts. *Phytochemistry*. **68**: 218-228
(3) Graham et al. (2004). Construction of a genetic linkage map of red raspberry (*Rubus idaeus* subsp. *idaeus*) based on AFLPs, genomic-SSR and EST-SSR markers. *Theoretical and Applied Gentics*. **109**: 740-749

VI.) 12th Weurman Symposium
July 1-4th 2008
Interlaken, Switzerland

Genomic studies in raspberry flavour: Volatiles and QTL in progeny of a cross between Scottish and American parents.

Angzzas Kassim[1], Dzeti Zait[1], Magali Vandroux[1], **Susan McCallum**[2], Julie Graham[2], Christine Hackett[2], Alistair Paterson[1]

[1] Centre for Food Quality, SIPBS Royal College, University of Strathclyde, 204 George Street Glasgow G1 1XW, Scotland, U.K.
[2] Genetics of Soft Fruit, Scottish Crops Research Institute, Invergowrie, Dundee, DD2 5DA, Scotland, U.K.

Abstract:
The red raspberry, a diploid member of the rose family, has a small genome and seven well-established genetic linkage groups correlated with chromosomes. It forms an ideal model system for studying genetic factors encoding fruit character and quality. This perennial fruit crop, which has relatively long periods of juvenility, yields berries that consist of multiple drupelets filled with mono- and disaccharides, organic acids, anthocyanin pigments and flavour volatiles. All metabolites contribute to character but gross traits such as berry size and appearance are also important for fruit quality. Intensities of character attributes are inherited on the basis of genotype with seasonal and environmental influences. However it is difficult to identify genes influencing such quantitative traits in breeding lines because individual effects are obscured by segregation of other genes and non-genetic effects. Genes can be identified, if linked to physical genetic markers, as quantitative trait loci (QTL). A cross was effected between two raspberries differing in a range of phenotypes: the recent European cultivar Glen Moy and older North American Latham. Key metabolites for traits of sweetness and sourness and flavour were quantified in fruit from >150 replicate progeny grown in three different environments: two (exposed and tunnel) at one site, the third (tunnel) at a second remote site. Traits were analysed for significant linkages to mapped markers using specialised software (MapQTL). Metabolites from polyketide synthase, carotenoid cleavage and other volatiles pathways were quantified and linkage to segregating chromosomal DNA markers sought. Similar approaches were adopted for sugars (glucose and fructose) and organic (citric and malic) acids that influence taste. The key strategy is identification of single nucleotide polymorphisms (SNPs) in parental genomes from DNA sequencing studies and establishment of linkage to phenotypes. One component is identification of candidate genes from related peach, and model plants grape vine and *Arabidopsis* such as carotenoid dioxygenase and HMG-CoA reductase. An aim of research is to facilitate marker-assisted approaches in which fruit breeders can rapidly screen large numbers of seedling progeny for DNA markers linked to flavour interesting traits. A second is basic understanding of how fruit quality is determined by genetic and environmental factors.