Chapter 8
QTL Mapping and Marker Assisted Breeding in *Rubus* spp.

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8.1 Background

Recent developments in genetics and genomics have advanced research in all crops including soft fruit species. Molecular markers which detect genome-wide variability in both protein coding and non-coding regions have enabled genetic mapping studies to move beyond linkages between simple morphological traits (Jennings 1967a, 1988; Ourecky 1975; Crane and Lawrence 1931; Keep 1968) to linkage maps containing numerous genetic markers which can be utilised in marker assisted breeding. Until recently, mapping in blackberry and other *Rubus* species has lagged behind that of red raspberry due to their more complex genetic make-up and lesser economic importance.

The marker techniques developed for linkage mapping include amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), and Single nucleotide polymorphisms (SNPs) (Antonius-Klemola 1999; Hokanson 2001; Graham et al. 2002; Stafne et al. 2005; Woodhead et al. 2008, 2010; Castillo et al. 2010; Dossett et al. 2012; Bassil et al. 2014). Utilising a range of markers as they have been developed has led to the generation of linkage maps which provide a framework for increased efficiency of selection, where markers linked to the gene(s) or quantitative trait loci (QTL) underlying the trait(s) of interest can be developed further for use in breeding programmes, as well as a research tool for assisting in the understanding of the genetic control of desirable phenotypes.

The first marker-based genetic map of raspberry was developed by Graham et al. (2004) utilizing SSR and AFLP markers in a full sibling population of 'Latham' x 'Glen Moy'. SSR markers were developed from both genomic and cDNA libraries from the cultivar 'Glen Moy', and AFLP markers further saturated the map.

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The initial map consisted of nine linkage groups (LGs) with 273 markers covering 789 cM of map distance and has subsequently been enhanced in a number of QTL mapping studies (Graham et al. 2006, 2009, 2011, 2014; Woodhead et al. 2010, 2013; Kassim et al. 2009; McCallum et al. 2010; Paterson et al. 2013; Simpson et al. 2017). Aside from the 'Latham' x 'Glen Moy' population, a number of other linkage maps in red raspberry have been generated. A 'Latham' x 'Titan' population (Pattison et al. 2007) was used to construct a map based on AFLP, RAPD, and uncharacterized resistant gene analog polymorphism (RGAP) markers. Sargent et al. (2007) generated a map from a 'Malling Jewel' x 'Malling Orion' cross using AFLP and SSR markers. Ward et al. (2013) used Genotyping by Sequencing (GBS) to produce highly saturated maps for a *R. idaeus* pseudo-testcross progeny. GBS has also been applied to the 'Latham' x 'Glen Moy' population (Hackett et al. submitted.) to greatly enhance marker saturation. Castro et al. (2013) published the first genetic map of a primocane-fruiting and thornless tetraploid blackberry (*Rubus* subgenus *Rubus* Watson). Bushakra et al. (2015) constructed the first linkage map of black raspberry (*Rubus occidentalis*) using single-nucleotide polymorphism and simple sequence repeat markers representing seven linkage groups. This research group also created a new genetic mapping population from a cross between black and red raspberry. They performed comparative genomic mapping by using BLAST analysis of 131 markers from the black and red raspberry linkage map, with genomic sequence of strawberry, apple and peach. Over two-thirds of the markers showed a near perfect match with strawberry linkage groups and each of the seven *Rubus* LGs were aligned to each of the seven strawberry chromosomes supporting high synteny between *Rubus* and *Fragaria*. Synteny was reduced in apple and peach with *Rubus* LGs aligning with parts of the apple and peach chromosomes (Bushakra et al. 2012). Breeding and genetic studies are already greatly benefiting from these marker based linkage maps, which have allowed the linking of traits to chromosomal loci and in some cases, even the genes responsible for trait variation. In light of climate change and other legislative constraints on production, there is an increasing need for both conventionally valued traits like quality but also traits that will improve the sustainability and resilience of these crops by reducing the need for pesticides and other chemical inputs; managing water requirements and other climatic responses and responding to the desire by consumers for locally grown food. Many pests and pathogens can affect red raspberries including viruses, fungal diseases, aphids and beetles. Viruses are a particularly serious problem as an infected plant is unlikely to recover and future propagules will also be infected eg. RBDV (Ourecky 1975).

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. At the same time growers require pest and disease resistant varieties capable of utilising the extended growing season and with resilience traits adapted to changing climatic conditions. Breeding is discussed in Chap. 2 of this volume. In this chapter we discuss where QTL mapping has been applied towards the understanding and improvement of *Rubus* crops for a range of traits to meet the various challenges in production.

8.2 QTL Studies for Pest and Disease Resistance

Increasing pressure from governments, end-users and consumers to reduce chemical inputs has meant that the incorporation of resistance/tolerance traits into new cultivars is a key factor in ensuring future sustainability. The soft fruit industry faces particularly serious challenges, as it relies on a small number of cultivars which are generally bred for fruit quality. Together with a steady decline in the number of available chemicals for pest and disease control, there is a strong possibility that within 10–15 years the fruit industry will have to operate in an environment with few or no conventional pesticides and fungicides. In order to survive and retain viability, the soft fruit industry therefore needs a renewal of approaches that select either specific resistance genes, and/or physical or structural characteristics linked to resistance. An overall decline in plant defense in domesticated crops has been linked to changes in gross plant morphology, reduced variation in plant phenology and modification of secondary plant metabolites (Chen et al. 2015). Plant defensive traits could be exploited more widely as part of any crop protection strategy. Studies on the cost/benefits of fungicide/pesticides vs resistant varieties identified a x 4 return on investment in chemical controls but this increased to a 10–12 times return on resistance breeding with the added advantage of reducing environmental impacts and application costs (Pimentel et al. 1992, 1997; Morris and Heisey 2003).

Sources of resistance to many pests and diseases have been identified in diverse *Rubus* species and exploited in conventional cross-breeding (Keep et al. 1977; Jennings 1988; Knight 1991; Williamson and Jennings 1996). However, germplasm bearing single resistance genes when planted over extensive areas can in many cases, depending on the mode of action, eventually be overcome by the rapid evolution of new biotypes of pests or virulent races of pathogenic fungi. New types of host resistance are therefore required to sustain plant protection (Birch et al. 2002; Jones 2002). Although studies frequently focus on individual traits, plant defense is more likely to involve a suite of traits, and there is surprisingly little evidence for trade-offs in investment between multiple defenses (Koricheva et al. 2004). Widespread use of tunnels and covers in soft fruit growing systems in recent years enables more use of natural enemies against pests and development of resistant cultivars are a key factor in the success of such integrated systems (Birch et al. 2011). However, increasingly specific demands by multiple retailers and processors mean that any advances in the resistance status of new cultivars must maintain the harvested quality of the crop. Marker assisted breeding can therefore assist in the development of resistant varieties through identification and subsequent transfer of linked markers of resistance/tolerance traits into breeding programmes. A number of major pests and diseases affect *Rubus*, some sporadically while others have serious longer-term impacts on cultivation. In *Rubus* control of root rot, weevils, mites, aphids, cane diseases and viruses consistently require applications of pesticides. Pest and diseases of raspberry in Europe have been extensively reviewed in Gordon et al. (2006) and Jennings and Dolan (2014). Emerging pests such as spotted wing Drosophila (SWD) (*Drosophila suzukii*) with a wide host range

including cane and bush crops will also require strategies for control, although so far there are few indications of robust sources of resistance.

8.2.1 Raspberry Root Rot

Root rot, caused by *Phytophthora rubi,* continues to be one of the most serious and destructive diseases of raspberry (See Seemüller et al. 1986; Duncan et al. 1987; Harrison et al. 1998) and poses a significant threat at present to the industry's survival in Northern Europe. There are no effective control strategies nor any commercially accepted resistant varieties, and uptake of new high-yielding but highly susceptible cultivars has been a major factor in the spread of the disease within Europe. Traditionally the lifetime of a raspberry plantation was >15 years, but raspberry root rot has greatly diminished this, with some growers now treating raspberry as a long cane annual crop whilst others assume a maximum lifespan of up to 6 years due to disease pressure. Large parts of the Northern European raspberry industry are now growing in pots with coir as a result of *P. rubi* infestation in available soils. Root vigour has been shown to have an impact on the ability of the plant to resist *Phytophthora* root rot infection (Graham et al. 2011). The demonstration of a highly significant correlation between the root sucker parameters and root rot resistance is of great value to breeders as it provides a simple visual screen for identifying germplasm with some level of resistance/tolerance to *Phytophthora* root rot (Graham et al. 2011). QTL for root vigour on LG3 and LG6 co-locate with resistance and can be used alongside markers identified.

In terms of QTL mapping, screening cultivars of red and other raspberries and wild *Rubus* species have identified a few potential sources of resistance including cvs. Latham and Winkler's Sämling and species material such as *R. strigosus, R. occidentalis* and *R. ursinus* (Barritt et al. 1979; Jennings 1988), though the basis of resistance remains unknown. 'Latham' is one of the few sources of root rot resistance that has remained durable, suggesting a complex basis of resistance, possibly several minor genes or more structural traits compared to R gene resistance, offering a feasible and effective long-term method of control. Recently, markers linked to a resistance QTL have been developed using a segregating population derived from a cross between a North American red raspberry 'Latham' and the European red raspberry 'Glen Moy' with good fruit quality characteristics but susceptibility to root rot (Graham et al. 2011). These have been utilised in the industry-funded UK raspberry breeding consortium, and resistant progeny are currently under selection for quality traits (Jennings, pers. com.) (Fig. 8.1). At present, the mechanism of resistance presented by 'Latham' is unknown but it may involve complex interactions between disease resistance processes and plant root development (Graham et al. 2011). Pattison et al. (2007) combined generational means analysis with molecular markers and QTL analysis to map resistance to *Phytophthora* root rot in a BC_1 population of NY00-34 ('Titan' x 'Latham') x 'Titan'. Separate genetic linkage maps of NY00-34 and 'Titan' were developed using RAPD, AFLP and resistance gene analog polymorphisms (RGAP) and analysed for QTL associated with various parameters of

Fig. 8.1 Selections with (left of post) and without (right of post) the root rot resistance marker on an infected site

root rot resistance assayed in a hydroponic system (Pattison et al. 2004). Regions on LG1, 5 and 7 were associated in multiple parameters of the resistance response. Bulked segregant analysis (BSA) corroborated this conclusion by identifying markers from these regions associated with bulked samples of resistant and susceptible genotypes. Generational means analysis suggests two major genes controlling resistance, possibly corresponding to the two regions on each parental linkage map associated with resistance. Genetic resistance through breeding offers a feasible method for control, although 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).

Given the parallel developments in genomic and transcriptomic technologies that are applicable to both raspberry and the *Phytophthora* pathogen, work is currently in progress to identify the genes that play a significant role in this interaction, determine the key resistance/susceptibility components and elucidate the mechanisms of resistance in 'Latham' (Graham et al., unpublished data). Comparison of the 'Glen Moy' and 'Latham' genome sequences is underway to identify any differences within QTL regions (Milne et al., unpublished data).

8.2.2 Raspberry Aphids

Aphids are a major problem on *Rubus* crops, with a number of aphid species reported, four of which (*Amphorophora idaei*, *A. agathonica*, *Aphis idaei* and *A. rubicola*) are known to cause economic damage mainly through their role as virus

vectors, leading to virus build up over several years in long-lived soil based plantations. In the UK, the A_1 gene identified in the cultivar 'Baumforth A' by Knight et al. (1959), has been incorporated into many of the resistant cultivars released in Europe in the early 1970s but a virulent A_1-breaking biotype exists (Birch et al. 1994; Jones et al. 2000). Other resistance genes, designated A_2 to A_7, were identified by Knight et al. (1960) in the North American cultivar 'Chief', and further genes were identified in *Rubus idaeus* subsp. *strigosus*. Each of these genes was found to provide resistance against specific biotypes of *A. idaei* (Briggs 1965). As resistance-breaking biotypes against A_1 have emerged, a further resistance gene effective against *A. idaei*, designated A_{10}, identified in the black raspberry (*Rubus occidentalis* L.) cultivar 'Cumberland' (Keep and Knight 1967), has become widely used in breeding (Keep 1989; Birch et al. 2011). An A_{10} resistance-breaking biotype has emerged on 'Autumn Bliss' which carries A_{10} but not on 'Malling Leo' which carries both A_1 and A_{10} (Sargent et al. 2007). As a result, breeders in the UK are now combining A_{10} with other aphid resistance genes to increase durability, and it is hoped that developments in marker technology will facilitate this gene pyramiding strategy to bring aphid-resistant cultivars to the market in a shorter time scale. This is especially important as the use of aphid-resistant cultivars forms a significant part of Integrated Crop Management strategies in the UK (Mitchell et al. 2010; Birch et al. 2011). Sargent et al. (2007) mapped the A_1 locus conferring aphid resistance to LG3 in a population of 'Malling Jewel' x 'Malling Orion'. In black raspberry (Bushakra et al. 2015) a locus for aphid resistance, Ag_4, has also been mapped. In the Pacific Northwest the current standard commercial cultivar is highly susceptible to the aphid *Amphorophora agathonica* Hottes, a vector for the Raspberry mosaic virus complex. Sources of aphid resistance have been identified in wild germplasm and used to develop mapping populations to study the inheritance of these valuable traits (Dossett and Finn 2010). A mechanism located in the phloem for resistance to *A. agathonica* has been suggested by Lightle et al. (2012). The development of maps of progenies carrying resistance genes and the identification of molecular markers linked to these genes will thus provide a key tool in differentiating reported genes, identifying their presence in modern hybrid material and in managing strategies for pyramiding.

8.2.3 Raspberry Cane Diseases

Cane disease resistance is increasing in importance due to the impact on yield of lateral shoot loss due to spur blight (caused by *Didymella applanata*) and cane *Botrytis*. Field screening using either natural infection or a simple wound inoculation method (Jennings and Williamson 1982) remains the best method for analysis of resistance status, as glasshouse inoculations do not result in characteristic disease symptoms. However, breeders with limited resources can rarely include a primary screen for these diseases. These two pathogens occupy the same ecological niche on

raspberry canes and it has been shown that a common resistance operates against them (Williamson and Jennings 1986), although the genetic control has yet to be determined. In attempts to control cane diseases, it has been known for some time that the presence of cane hairs in red raspberry (*Rubus idaeus* subsp. *vulgatus* Arrhen.) is associated with resistance to *Botrytis* and spur blight (Knight and Keep 1958; Jennings 1982; Jennings and Brydon 1989) and this was confirmed by linkage mapping (Graham et al. 2006). This effect may be due to linkage with major resistance genes or minor gene complexes that independently contribute to the resistance or susceptibilities of the six diseases affected or that the gene itself is responsible through pleiotrophic effects on each of the resistances (Williamson and Jennings 1992). Alternatively, it may be that cane hairiness affects the ability of fungi to adhere to and infect tissues (Jennings 1962). It may be that gene *H* acts early in development to affect several cell characteristics. For example, resistance to *Botrytis* and spur blight is highest in immature tissues, and it is possible that gene *H* increases resistance by delaying cell maturity. This hypothesis is supported by an effect of gene *H* on timing of fruit ripening (Graham et al. 2009). In a recent study (McKenzie et al. 2015), an attempt was made to explain the role of gene *H* by characterising gene content in this region. This study identified PDF2/GLABROUS2 as gene *H* and also provided an insight into the effects the region has on disease resistance through identification of a number of candidate genes that may suggest genetic resistance is a possibility. A mechanism for the delay to ripening was also hypothesized through the identification of a DIVIA like *Myb* transcription factor that could be controlling both trichomes and flowering time. *WEREWOLF*, a regulator of root hair pattern, has also been shown to be a post-transcriptional regulator of *FT*, a key floral regulator (Seo et al. 2011). There may therefore be more than one effect of gene *H*.

Cane spot or anthracnose (*Elsinoe veneta*) can develop in most raspberry tissues but it is most recognisable in the second year canes where it produces deep lesions that can lead to vascular damage, and therefore reduce yields. The resistance to this pathogen has been associated to the presence of hairy cane *H* in European red raspberry but not so in North-American cultivars (Graham et al. 2006). Genetic control of the trait has not yet been firmly established although Graham et al. (2006) identified two QTL in LG2 and LG4 of the 'Latham' x 'Glen Moy' progeny associated with response to the disease.

Yellow rust (*Phagmidium rubi-idaei*) increased its prevalence with cultivation under tunnels of susceptible cultivars e.g. 'Glen Ample' and 'Tulameen'. Recently, however, this has not been in evidence, though the reasons why are unclear (Jennings, pers. com.). A major resistance gene (*Yr*) from 'Latham' was identified by Anthony et al. (1986), and Graham et al. (2006) postulated this gene to be located on LG3 of the 'Latham' x 'Glen Moy' progeny. The inheritance of complete and incomplete resistance to rust in a half diallel cross including 'Boyne' was studied, which derives complete resistance from 'Latham' (Anthony et al. 1986). Here crosses of 'Boyne' to susceptible varieties all segregated for complete resistance and it was proposed

that ‘Boyne’ was heterozygous for a single resistance gene, designated *Yr*, which was derived from ‘Latham’. Graham et al. (2006) proposed that ‘Latham’ is also heterozygous for *Yr*, and that this lies on LG3. Anthony et al. (1986) also found variation in the degree of susceptibility among offspring of ‘Boyne’ without complete resistance, and concluded ‘Boyne’ to also be a source of incomplete resistance. In the ‘Latham’ x ‘Glen Moy’ cross, there is some evidence, although not highly significant, for a gene on LG5, also from ‘Latham’, affecting the susceptibility of the offspring that do not carry the ‘resistant’ allele on LG3. This area on LG5 is also implicated in spur blight/botrytis resistance. There was no evidence, however, of gene *H* being related to incomplete resistance in this cross. None of the offspring in this cross were as susceptible to rust as the ‘Glen Moy’ parent suggesting another resistance gene, for which ‘Latham’ is homozygous.

8.2.4 Virus Resistance

Attempts to identify markers for viral resistance genes have been carried out for raspberry leaf spot and raspberry vein chlorosis. Field screening measured symptom production of these two viruses in two different environments. Significant linkages to mapped markers and resistance loci were found on LG2 and 7 of the ‘Latham’ x ‘Glen Moy’ map (Raluca et al. 2006).

8.2.5 Physical Resistance Traits

As an alternative to resistance gene based control, plants with certain physical and or structural characteristics may be able to resist attack by exploiting morphological structures or biomechanical characteristics that interfere with pest/pathogen movement, host recognition, feeding or reproduction on or in the plant (Hanley et al. 2007; Moles and Westoby 2000). These features could make the plant less attractive visually, or present formidable physical barriers to pests and diseases. Plant traits that may confer resistance/tolerance include structural traits (e.g. trichomes, spinescence, waxy cuticles, sclerophylly, and granular minerals), gross morphology (architecture and plant size), life history (flowering time, growth rate) and secondary metabolites that include a wide range of chemical classes. Allelochemicals such as glucosinolates, tannins and terpenoids can be effective in insect deterrence, as anti-feeding or toxic compounds, and as precursors to structural defense traits (Bennett and Wallsgrove 1994). Plant vigor has also been linked to tolerance in some plants (Price 1991). Breeding for physical resistance traits in crops has not been capitalized upon, despite the potential advantages of this

approach compared to chemical resistance traits such as anti-feedants and toxins. Part of the reason for the under-exploitation of physical resistance traits in crops is that the genetic basis and heritability of these traits is poorly characterized and any associations of particular traits with pest and disease resistances have yet to be determined.

As described above for *Botrytis* and spur blight, the presence of cane hairs in red raspberry is associated with resistance. This may be through the hairs providing a physical barrier, or could be through a developmental response making the plant more or less susceptible. Regardless of the mechanism, cane hairs are easy to select for in crossing programmes (Graham et al. 2006) and provide a strategy for protection against these cane diseases.

Cane splitting in raspberry is another example of a physical trait that affects pest and disease burden. Cane splitting is a normal feature of raspberry growth and severe splitting can lead to plant infestation by cane midge (*Resseliella theobaldi* Barnes), followed by fungal infection by many pathogens leading to a disease complex called cane blight, with losses in yield of up to 50% if left untreated (Jennings 1988). Raspberry genotypes have been shown to differ considerably in the degree of cane splitting, and regions of the raspberry genome that are associated with cane splitting have been identified (Woodhead et al. 2013). A correlation between cane splitting and cane height has also been shown, with shorter genotypes exhibiting less cane splitting than longer ones. Loci accounting for 49% of height variation have been identified (Graham et al. 2009; Woodhead et al. 2013) thus indirectly allowing breeding for genotypes with reduced propensity to splitting.

Other plant physical traits with potential to affect pest resistance in *Rubus* (leaf trichomes, leaf density, cane density, bush density, overall density, lateral length and lateral numbers) have been investigated in raspberry to determine the heritability and therefore breeding potential of these traits. These traits had anecdotal evidence for an effect against insect pests (Jennings, pers. com.). This study showed the incidence of spider mites was positively associated with all the density traits assessed both in field and under polytunnel conditions. Thus, increases in plant mass supported a greater increase in observable mite damage. From other observations on field trials in raspberry, spider mites appear to prefer the middle of the plant, possibly to gain protection (Jennings, pers. com.). A number of factors might contribute here including microclimate effects, leaf quality differences and natural enemy evasion. For aphids, there was a positive association between aphid presence and leaf trichome density. This might be due to the hairs offering protection from predation. Analysis of heritability and QTLs for these physical traits identified candidate chromosome regions and associated markers that could be targeted for understanding the genetic control of these traits (Graham et al. 2014), leading to markers with utility for breeding.

8.3 QTL Studies for Quality Characteristics

Fruits from *Rubus* species are highly valued for their flavour and nutritive qualities, and this is the major factor behind the success of a variety. Flavour, appearance and shelf life are the main attributes of fresh market quality and are essential for repeat purchase of fruit by consumers. Flavour can be broken down into multiple descriptors for taste, texture and other sensory characteristics. Good, acceptable flavour in raspberry is fruity, sweet and floral with some acidity but no bitterness (Harrison et al. 1999). Colour, brightness, size and shape contribute to the appearance and are crucial to appeal to consumers. A dark colour can be perceived as overripe by fresh market retailers. Large fruit size is an attractive characteristic to both consumers and producers, in the latter case as it is more cost-effective to pick.

8.3.1 Flavour

To enable determination of what can be improved through both conventional and marker assisted breeding, an understanding of what drives flavour perception and how this is influenced by season and environment is required. In raspberries two main flavour attributes are sweetness and sourness (Harrison et al. 1999) and perception varies with season and environment, where flavour is considered 'good' or 'bad' depending on weather conditions (Jennings, pers. com.). Little work has been carried out in raspberry to correlate sensory evaluations with composition. A study by Zait (2010) aimed to understand the association between sugars and acids and sweetness and sourness perceptions. Here the work suggests that sugar levels are controlling flavor with acid level important when sugars are lower. Data from Paterson et al. (2013) on volatiles content, and from Kassim et al. (2009) on anthocyanin content from the 'Latham' x 'Glen Moy' population was utilized in a recent study to develop a preliminary flavour model. This model found that sweetness, sourness and flavor intensity traits were not adequately explained by singular contributions of either sugars or acids content, but through synergistic relationships between all flavour metabolites. Seasonal and environmental variability made it difficult to identify tight QTL, but this is not surprising as sugars and acids are central to metabolism. A number of overlapping QTL were identified on LG2, 3, 4 and 5. 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 QTL which underlie the traits and the potential for associated molecular markers.

In the volatile study of Paterson et al. (2013), 12 raspberry character volatiles were quantified. Effects of season and environment were examined for their impact on the content of alpha-ionone, alpha-ionol, beta-ionone, beta-damascenone, linalool, geraniol, benzyl alcohol, (Z)-3-hexenol, acetoin, acetic and hexanoic acids, whilst raspberry ketone was measured in one season. A significant variation was observed in fruit volatiles in all progeny between seasons and method of cultivation. QTL were determined and mapped to six of the seven linkage groups, as were candidate genes in the volatiles pathways including phytoene synthase, CTR1, HMG CoA reductase and HMG CoA synthase, β-galactosidase, linalool synthase and terpene synthase.

8.3.2 Colour

Colour in raspberry is a complex trait with anthocyanin content (predominantly cyanidin and pelargonidin pigments) thought to be the major contributing factor (Jennings 1988; Wang et al. 2009). Jennings and Carmichael (1980) described the genes *R*, *So* and *Xy* necessary for synthesising the sugars rhamnose, sophorose and xylose respectively, which are required to give the array of different anthocyanin pigments observed in red raspberry, as well as a series of genes controlling pigment concentration (Jennings 1988). The final expression of fruit colour is influenced by both co-pigments and pH. Co-pigmentation allows the formation of complex interactions between pigments and colourless compounds which enhance colour intensity. 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). In most plants, the colour of fruit and flowers results from the accumulation of anthocyanins in cell vacuoles and as the absorption spectrum of anthocyanins depends on the pH of their environment, the observed tissue colour effectively reflects vacuolar pH (Yoshida et al. 2003).

Raspberry fruit colour was assessed in the ‘Latham’ x ‘Glen Moy’ mapping population by McCallum et al. (2010), and colour measurements were significantly associated with pigment content. Measures of individual anthocyanins mapped to the bHLH gene on LG1 and a bZIP gene on LG4 (Kassim et al. 2009; Bushakra et al. 2013) whereas colour and total anthocyanins mapped to overlapping QTL on LG2, LG3, LG4 and LG6 (McCallum et al. 2010). Major structural genes (F3’H, FLS, DFR, IFR, OMT, GST) and transcription factors (bZIP, bHLH, MYB) influencing flavonoid biosynthesis were shown to underlie the relevant QTL. Favourable alleles were identified for aspects of fruit colour and partitioning of individual pigments. Molina-Bravo et al. (2014) examined colour in a modified backcross between (*R. parvifolius* x ‘Tulameen’) and ‘Qualicum’. Unlike in the study of McCallum et al. (2010) where the pigments are mainly cyanidin based, fruit from this wider cross also contained pelargonidin pigments, presumably from

the *R. parvifolius* parent. Here around a quarter of the population exhibited yellow fruits. Two QTL for berry colour were identified on LG1 and LG5 (relating to LG6 and LG5 of the map used in McCallum et al. (2010).

8.3.3 Health Traits

In terms of the value to health, epidemiological studies have suggested the efficacy of compounds found at high concentrations in berries for the prevention of a number of chronic diseases. Studies are now aimed at understanding the mechanisms of action of specific groups of phytochemicals (eg. Marinova and Ribarova 2007). For a review of the literature on the potential health benefits of berry fruits see McDougall and Stewart (2012).

Work towards understanding the genetic control of health-related compounds has been initiated in *Rubus* using a metabolomics approach to identify bioactive compounds in the 'Latham' x 'Glen Moy' under two different environments (Stewart et al. 2007). As a greater understanding of the relative importance and bioavailability of the different antioxidant compounds is achieved, it may become possible to develop and identify those raspberry genotypes with enhanced health-promoting properties from breeding programs (Beekwilder et al. 2005). Preliminary metabolic profiling showed that the fruit polyphenolic profiles divided into two gross groups segregating on the basis of relative levels of cyanidin-3-sophoroside and cyanidin-3-rutinoside, compounds implicated as conferring human health benefits. From the 'Latham' x 'Glen Moy' mapping population, data was collected on anthocyanin content across seasons and under different environments (Kassim et al. 2009). High performance liquid chromatography (HPLC) was used to quantify eight major anthocyanins, cyanidins, and pelargonidin glycosides: -3-sophoroside, -3-glucoside, -3-rutinoside and -3-glucosylrutinoside. All eight mapped to the same chromosome region on LG1 of the map of Graham et al. (2006), across both years and from fruits grown in the field and under protected cultivation. Seven antioxidants also mapped to a region on LG4 across years and for both field and protected sites. Candidate genes including *bHLH* (Espley et al. 2007), *NAM/CUC2* (Ooka et al. 2003) like protein and *bZIP* transcription factor (Holm et al. 2002; Mallappa et al. 2006) underlying the mapped anthocyanins were identified. In another study on red and black raspberry Bushakra et al. (2013) used ultra and high-performance liquid chromatography (UHPLC and HPLC) on two *Rubus* mapping populations to explore the presence of associations between concentrations of five anthocyanins in fruit and genotype. In total, 27 QTL were identified on the *Rubus* linkage maps, four of which were associated with molecular markers designed from transcription factors and three of which are associated with molecular markers designed from anthocyanin biosynthetic pathway candidate genes. Using the 'Latham' x 'Glen Moy' mapping population, total phenol content (TPC) and total anthocyanin content (TAC) in ripe fruit was examined over five seasons under two environments (Dobson

et al. 2012) to examine variability. Corresponding measurements of antioxidant capacity (e.g. ferric reducing antioxidant capacity (FRAP) and trolox equivalent antioxidant capacity (TEAC)) were also carried out. TPC was highly correlated with TEAC and FRAP over the entire dataset. The subset of anthocyanin content was genotype-dependent and also correlated with TPC though the proportion of anthocyanin compounds contributing to total phenolic pool varied from progeny to progeny. QTL were identified on LG2, 3, 5 and 6. The QTL that influence TPC but not TAC are of particular interest to boost the antioxidant capacity of raspberry fruits, which is often related to their bio-activities.

8.3.4 Crumbly Fruit

In terms of fruit quality (as well as yield) the disorder known as 'crumbly' fruit has become a serious problem in the raspberry industry. In 'crumbly' fruit drupelets are generally reduced in number but greatly enlarged or, in the case of small reductions, cohere imperfectly so fruit readily crumbles when picked (Daubeny et al. 1967; Jennings 1988). There have been a number of causes suggested for the crumbly condition including infection with certain viruses (Jennings 1988; Murant et al. 1973; Daubeny et al. 1978). A genetic cause has been demonstrated where the crumbly phenotype arises from virus-tested mother plants (Jennings 1988). The cultivar 'Latham' can show a crumbly phenotype and this is thought to be due to mutation of the dominant allele at a heterozygous gene locus causing plants to become homozygous for a deleterious recessive gene (Jennings 1967b). From a 'Latham' self, Jennings (1967b) demonstrated that seedlings obtained could be classified into three groups: normal, crumbly and sterile. Studies have also shown that extensive tissue culturing of plants may increase the emergence of the condition (Jennings pers. com.). Additionally, environmental factors appear to play an important role with variations in the extent of crumbliness apparent from year to year (Dolan, pers. com.). A study on the 'Latham' x 'Glen Moy' population examined the occurrence of crumbly fruit over a 6 year period, in both open field and under polytunnel. This highlighted that seasonal, environmental and genetic factors all influence the condition. Two QTL that are important for the genetic control of the condition were located on LG1 and 3 (Graham et al. 2015). Contrary to the suggestion by Jennings (1967a) that crumbly fruit was related to the gene *H* region, no genetic association with this region on LG2 could be identified with the crumbly fruit syndrome. However there was an association with ripening, with the longer the fruit takes to fruit set and reach green fruit stage, the more likely it is to be crumbly. This may explain the association hypothesized by Jennings as the *Hh* genotype of gene *H* is associated with a slowing down of ripening across all stages from open flowers to the green/red stage compared to the *hh* genotype (Graham et al. 2009).

8.3.5 *Fruit Size*

Large size is an attractive characteristic to consumers and producers as it is both cost effective to pick and visually appealing. Uniformity of fruit size is also visually appealing to potential customers and when combined with regular shape can encourage sales (Graham and Jennings 2009). 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. Raspberry genotypes show a wide variation in fruit size. This range may be as a result of differences in cell number or cell volume. Genetic differences, season and environmental conditions as well as crop management practices have an effect on fruit size (Cheng and Breen 1992). Mutation of a major gene designated L_1 was identified in a large-fruited 'Malling Jewel' mutant which resulted in an increase in both drupelet number and size. However, the gene itself however proved to be unstable with the gene mutating back to its normal sized form (Jennings 1988). 'Glen Moy' was one of the early cultivars selected by breeders for its large fruit size which was attributed to both drupe size and number (Jennings 1988). Gene families like aquaporins may have a role in fruit size as these are water channel proteins capable of transporting water and small molecules across cellular membranes. Three main types of aquaporins are known in plants, membrane intrinsic proteins (MIPs), tonoplast intrinsic proteins (TIPs) and plasma membrane intrinsic proteins (PIPs) (Smart et al. 2001). Plant MIPs are reported to play an important role in cell division and expansion as well as water transportation in relation to environmental conditions (Oliviusson et al. 2001). Expansins are associated with cell growth, consequently with the later stages of fruit ripening in tomato and strawberries delaying or increasing fruit ripening (Cosgrove 2000).

An increase in cell wall bound protein found in raspberry fruit has, in part, been attributed to expansin accumulation (Iannetta 1998). 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 as are other ripening associated genes such as auxins and ethylene regulators.

Recent work has been carried out in the 'Latham' x 'Glen Moy' population which segregates for fruit size (McCallum, pers. com.). Candidate genes for auxin, ethylene and specific size regulatory genes (fruit weight) and transcription factors were identified. Initial statistical analysis found one marker on LG3, which explained 23% trait variation in the mapping population, also contributed to 14.4% of variation in fruit size seen in two out of three unselected families assessed. A further SSR marker on LG1, contributing to 15% variation in the mapping population, contributed to 6.6% size variation across the same populations with an additive effect of 19.1% trait variation. Further work on these markers and putative candidate genes involved in fruit developmental processes are underway in a range of available germplasm and potential breeding parents. The transport of solutes across vacuolar membranes impacts on fruit metabolite concentrations, making the genes involved ideal candidates for gene analysis. In a *Rubus parvifolius* x 'Tulameen' cross one QTL was identified for fruit size (Molina-Bravo et al. 2014). A single major QTL for fruit

weight (*fw* 2.1) was identified in tomato in close proximity to a cloned fruit weight gene *fw* 2.2 (Frary et al. 2000; Zygier et al. 2005).

8.4 Resilience to Environmental Change

Climate change is demonstrably impacting agricultural and horticultural production at local, national and global scales (e.g. Mackay et al. 2011; Huang et al. 2015; Innes et al. 2015) and these impacts are also apparent in soft fruit. Continuous and reliable production of high quality fruit is critical for the profitability and this is threatened by changing temperatures. There is now evidence that flowering time has shifted in response to changes in climate (Fitter and Fitter 2002; Amano et al. 2010). Many plant species are unable to respond to the florally inductive cues until they have reached a certain developmental stage, i.e. they have what is known as a juvenile phase. This is true for raspberry, which only responds to the prolonged period of cold (vernalization) after a certain stage of development. Crop resilience and adaptation is therefore essential for future sustainability of all crops and must be considered as a factor for breeding. Understanding the key genetic control points across development is a major challenge in *Rubus* breeding for both resilient variety development and season extension.

8.4.1 Dormancy

The time at which dormancy begins and the intensity it attains and subsequent transition to flowering are regulated by multiple environmental and physiological cues (Fornara et al. 2010; Pin and Nilson 2012; Song et al. 2012) and need to be fully understood in perennial crops. A major plant trait has to have the ability to tolerate fluctuating winter temperatures. Raspberry like other perennial crops has adapted by having high chilling requirements, however as winter temperatures increase, evidence of disruption to development is evident with irregular and unexpected timings of bud break (Jennings, pers. com.). Activity of CONSTANS (*CO*) a key component in leaves of the photoperiodic pathway accumulates in long day conditions and activates transcription of FLOWERING LOCUS T (*FT*) (Simon et al. 2015) which interacts with *bZIP* transcription factors (Abe et al. 2005; Cao et al. 2015) activating a cascade of downstream genes leading to flowering. In terms of temperature regulation, the MADS box FLOWERING LOCUS C (*FLC*) is central. *FLC* and short vegetative phase proteins (*SVP*) form a complex to represses flowering until the plant is exposed to the appropriate level of cold. In raspberry RiMADS_01 was identified as a potential candidate affecting vernalization through QTL mapping. This gene is similar to *SVP* modulating the timing of the developmental transition to flowering phase in response to temperature (Lee et al. 2007). In a colder season RiMADS_01 was associated with earlier flowering. SPL was also identified in

raspberry on LG5 associated with a QTL for floral transition. Gene *H*, previously associated with cane morphology described above (Graham et al. 2006) was shown to be associated with a slowing down of ripening across all stages (Graham et al. 2009) and sequencing the gene *H* region (McKenzie et al. 2015) identified a DIVIA like Myb transcription factor (Werewolf) shown to be a post-transcriptional regulator of *FT* (Seo et al. 2011). Molina-Bravo et al. (2014) examined the progeny from a cross (*Rubus parvifolius* x 'Tulameen') x' Qualicum' for chilling requirement determined by measuring bud break in chilled cuttings. Four regions were associated with chilling requirement, and were mostly consistent across the 3 years of evaluation. This population is of interest to breeders in a time of climate change as a donor of higher chilling requirements (allowing germplasm to withstand fluctuations in winter temperature) and also as a donor of heat tolerance.

8.4.2 Heat Tolerance

In addition to a lack of winter chill affecting dormancy and bud break, high summer temperatures are also affecting raspberry cultivation. Gotame et al. (2014) carried out a study aimed at increasing our knowledge of temperature stress on raspberry cultivars with a view to mapping genes implicated in response to elevated temperatures. A range of cultivars were examined for the effects of high temperature stress on gene expression profiles at the flower initiation stage using a custom *Rubus* microarray (James Hutton Institute). An elevation of temperature (>10 °C) altered the expression of 40 genes (38 were down- and two up-regulated). Down-regulated genes included those encoding major latex-like protein (MLPs), plasma membrane proteins (PMPs), cysteine proteins and other stress-related proteins. A number of PMP candidate genes were located on the 'Latham' x 'Glen Moy' map.

8.5 Yield

High fruit yields have been shown to be associated with cane architecture traits, particularly lateral length, which alone accounted for 82% of the yield variation (Sønsteby et al. 2009). Stephens et al. (2012) reported a positive genetic correlation between cane diameter and total yield. In terms of QTL mapping, lateral length along with a range of other architectural traits (leaf trichomes, leaf density, cane density, bush density, overall density and lateral numbers) described above have been investigated in raspberry and candidate chromosome regions identified and associated markers that could be targeted for understanding the genetic control of these traits (Graham et al. 2014) leading to markers for breeding. Currently these are being investigated for any association with yield in a range of breeding populations at James Hutton Limited (Jennings unpublished data).

8.6 Further Development of Mapping Technology in *Rubus*

QTL mapping has proved an accurate methodology for identification of loci linked to traits of interest. Large scale sequencing has revolutionised our ability to sequence and assemble genomes of a wide range of crop species and gives the opportunity to develop novel markers and identify important genes. In *Rubus*, a whole genome assembly was established for black raspberry (*R. occidentalis)* consisting of 2226 scaffolds spanning an estimated 83% of the genome, which was further assembled into seven pseudo-chromosomes (VanBuren et al. 2016; Jibran et al. 2018). The black raspberry genome is largely collinear with the strawberry genome and has strong identity with red raspberry where 87% of the selected red raspberry genetic markers match the position of the black raspberry markers on the physical map (VanBuren et al. 2016). With the addition of an assembled red raspberry draft genome from 'Glen Moy' (Hackett et al. accepted), these genomes provide a physical genomic framework to compare between these related species, link the genetic maps to the physical genome and promote the development of high throughput, large scale mapping techniques. At its simplest, established markers are placed on corresponding genomic sequences of the *Rosaceae* and determine syntenic blocks of sequence (Bushakra et al. 2012; VanBuren et al. 2016). Candidate genes found in genomic regions identified by markers and linked to important traits can be selected by an understanding of the trait, variable expression of the genes in that region and comparing protein coding regions. For example, raspberry fruit softening is an important agronomical trait that involves a complex interaction of plant cell processes including cell wall solubility and water transport. QTL mapping followed by selection and expression analysis of genes that underlie these QTLs across different fruit stages identified candidate cell wall degrading and water movement genes that showed variability, in the timing of gene expression throughout fruit development (Simpson et al. 2017).

Recent efforts in the development of higher resolution Genotyping by Sequencing (GBS) maps in combination with genome sequencing have increased the utility and accuracy by which traits can be located and linked to underlying genes. A GBS map from a 'Heritage' x 'Tulameen' mapping population identified nearly 7000 SNP markers spanning all seven raspberry linkage groups. A second red raspberry GBS map of the 'Latham' x 'Glen Moy' population was aligned with the draft genome sequence of 'Glen Moy' and identified over 2000 high confidence SNPs. These confirmed previously established QTLs for fruit ripening and identified additional QTLs and underlying candidate genes (Hackett et al. submitted). The increase in marker saturation and availability of genomic sequences will help the development of genome-wide association study (GWAS) projects. These projects use SNP genotyping over a broad range of wild species, cultivars and breeding populations that are phenotyped for multiple selected traits. Raspberry has abundant natural and experimental populations that show adaptation to a range of habitats and variability to a range of traits that may be used to generate defined GWAS populations. GWAS may give an increase in allelic diversity and improve resolution, but it remains to be seen whether raspberry genetic heterogeneity and marker density is sufficient to identify loci or genes by association of markers with traits.

8.7 Other Omics Technologies

Gene expression and transcriptomic approaches provide large data sets that enable identification of important biochemical pathways and regulatory genes. Linkage analysis of quantitative genome-wide gene expression data from both microarray and large scale transcriptome sequencing and also from metabolic data has been combined in melon to identify quality trait genes (Galpaz et al. 2018; Giovannoni and Katzir, 2018). Expression QTL (eQTL) mapping has not been reported in *Rubus,* but the tools and some data are already available to utilise and develop expression patterns to identify genes that may be co-regulated. The first microarray experiment in *Rubus* was conducted to investigate bud dormancy phase transition (Mazzitelli et al. 2007). Over 220 clones exhibited up or down-regulation during the endodormancy – paradormancy transition. The results indicated that water and cell wall reorganization and sugar metabolism were key components of bud dormancy release. Transcription factors, including a *SVP*-type MADS box transcription factor, and hormone-induced genes were also identified, potentially indicating signalling molecules that may be required to release these buds from dormancy. Advancement in sequencing technology has also enabled large scale sequencing of transcriptomes that can be utilised in multiple ways. A red raspberry 'fruit transcriptome' comprising a database of 55,920 unigenes has been established and mapped to the genome scaffolds of 'Glen Moy' (Milne, personal communication). The unigene set is derived from various transcript sequences isolated from a range of raspberry tissues, developmental stages, including developing fruit and buds and also different conditions. Sequences originated from 454 and llumina transcript sequencing, Sanger Expressed Sequence Tags and BAC coding sequences. A total of 55,708 oligonucleotide probes were designed for generation of a custom Agilent microarray JHI_Ri_60k_v1 (Graham, pers.com.). Subsequent microarray experiments have investigated the effect of high temperature stress on total gene expression profiles in the annual-fruiting raspberry (*R. idaeus* L.) 'Autumn Bliss', 'Autumn Treasure', 'Erika', and 'Polka' using a customised *Rubus* microarray (Gotame et al. 2014) and examined gene expression in the development of crumbly and normal fruit to examine those within identified QTLs (Graham et al. 2015).

8.8 Conclusion

Significant developments have been achieved in *Rubus* in terms of QTL mapping and other associated developments such as genome scaffolds and microarrays. This is greatly assisting breeding practices and with the developments in high throughput phenotyping where image data can be utilized in QTL mapping, the ability to dissect more complex traits, particularly for environmental resilience should become a reality in the next few years.

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