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Final Report

Project title: Molecular breeding for root rot resistant raspberries suitable for low input growing systems (Project code: HL0169 & SF63)

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Glossary of Abbreviations used in this report

AFLP	Amplified Fragment Length Polymorphism
BioSS	Bioinformatics and Statistics Scotland
cDNA	complementary DNA
cM	centiMorgans
Dig	Digoxigenin
DNA	deoxyribonucleic acid
dH_2O	distilled water
EPPO	European and Mediterranean plant protection organisation
EST	expressed sequence tag
EtOH	Ethanol
IAA	Isoamyl alcohol
ICM	Integrated Crop Management
ITS	internally transcribed spacer regions
LG	linkage group
PCO	principal coordinate
PCR	polymerase chain reaction
QTL	quantitative trait loci
Reps	replicates
RNA	ribonucleic acid
SD	standard deviation
SNP	single nucleotide polymorphism
SSR	simple sequence repeat

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Grower Summary

Headline

Prospects for accelerated breeding of new, root rot-resistant raspberry varieties have been improved by the identification of resistant selections. Molecular markers enabling rapid screening of germplasm for resistance and an improved genetic map which links markers to observable traits, together with validation of screening procedures for these visual traits have been developed.

Background and expected deliverables

The UK raspberry industry has come under increasing pressure by consumers to produce high quality fruit using a minimal quantity of chemical pesticides. To remain financially viable, commercial producers must achieve high yields of high quality fruit. This can be extremely difficult without the use of pesticides, particularly as the raspberry varieties currently in demand by the market are susceptible to a wide range of insect pests and fungal diseases.

Raspberry root rot, caused by *Phytophthora fragariae* var. *rubi* is currently the most economically damaging of all pests and diseases that affect raspberries in the UK. None of the commercially used varieties at present exhibit resistance, although the Canadian variety Cowichan tolerates the disease to some degree.

The industry desperately requires new commercially acceptable varieties that are resistant to *Phytophthora*. This will allow raspberries to be grown on infected sites that are otherwise ideally suited to raspberry production. It will also avoid the need to use fungicides and improve the viability of raspberry production for many fruit growers.

Breeding for root rot resistance is a major objective of the breeding programme at SCRI. However, traditional breeding is a very slow process, particularly with raspberry and other *Rubus* species and it can take up to nine years to find a seedling that has the necessary traits for a new variety.

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New methods of evaluating seedlings for *Phytophthora* resistance at an early stage in the selection process are required. New techniques have been developed that allow breeders to identify molecular markers on the genetic map of plants which can be used to identify seedlings with specific traits such as disease resistance.

The aim of this project was to identify map regions significantly associated with resistance to raspberry root rot and develop molecular markers that can be used in raspberry breeding to quickly and effectively identify selections with likely resistance to raspberry root rot. This technique would significantly reduce the time taken to breed new varieties with *Phytophthora* resistance.

Summary of the project and main conclusions

The work was undertaken using the progeny derived from a cross between the varieties Latham and Glen Moy. Latham is highly resistant to *Phytophthora* root rot and has been used frequently in breeding programmes as a source of resistance. Glen Moy is highly susceptible to the disease. The work was carried out within five different objectives.

Objective 1: Field screen for segregation of root rot resistance

Field screening was carried out to determine resistance status of each individual as inherited from the parents, therefore enabling identification of resistant and susceptible individuals within the population in two field environments.

Work under this objective provided some useful information for future breeding programmes.

- Longer field screening gives better development of disease symptoms. Four years in an infected field should be a minimum for any root rot screening in conventional breeding programmes. Alternatively shorter screening can be carried out with careful evaluation of the distribution of the data.

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- Selections with resistance from the Latham x Glen Moy cross are now available for use in future breeding programmes.

- There is a highly significant correlation between root sucker parameters and root rot resistance. This suggests that root vigour can be used as a single visual indication of disease resistance status.

- Varieties with vigorous root systems are likely to be more resistant than those with sparse or weak roots.

Objective 2: Glasshouse screen for segregation of root rot resistance

Work in this objective provided further help for scientists breeding for Phytophthora resistance:

- Glasshouse screening can be used by breeders for designation of progeny for root rot resistance but is less severe than field screening.

- This provides a quick means of testing new varieties and promising selections for the UK market.

Objective 3: Map enhancement to generate 7 linkage groups

Enhancement of the map allows DNA locations (or markers) to be identified which are linked to a desirable trait for breeding purposes. These markers can then be used to allow plant breeders to screen large populations of plants for those that have the trait of interest. The screening is based on the presence or absence of a certain gene as determined by laboratory procedures, rather than on the visual identification of the expressed trait in the plant.

The work in this objective provided:

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- A good well saturated linkage map which is available for marker assisted selection.

- This should be used by any breeding programme delivering varieties to the UK industry.

Objective 4: Correlation of data and mapping resistance loci

From this objective, the work has ensured that for further raspberry breeding programmes:

- Molecular markers are available for marker assisted selection for root rot resistance.

- Other markers can be developed as required.

Objective 5: Validation of markers

Validation is a crucial step in marker development to ensure that the marker is genuinely linked to the trait of interest. By also validating in a range of germplasm of raspberry plants from diverse genetic backgrounds allows the identification of other alleles present at that locus.

In undertaking this objective, it allows:

- Existing varieties, seedling selections and germplasm to be tested quickly for root rot resistance using molecular markers.

Financial benefits

This work has identified germplasm with resistance to root rot, developed molecular

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markers for screening promising selections for resistance and produced an enhanced map of genes associated with traits of interest for raspberry breeding.

- These developments will enhance raspberry breeding programmes, increase the potential to breed varieties resistant to Phytophthora root rot and reduce the time taken for breeders to release resistant varieties to the industry.

- More rapid release of improved raspberry varieties with resistance to Phytophthora root rot will help the industry to reduce losses to this disease and increase yields and returns per unit area of land.

- The breeding of resistant varieties will obviate the need to rely upon fungicidal drenches, thus reducing the risk of pesticide residues occurring in harvested fruit.

Action points for growers

- There are no direct action points for growers arising from this project.

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Science Section

General Introduction

The UK raspberry industry is currently faced with several major challenges in a rapidly-evolving market; one of the most serious is the growing demand for fruit grown in low input/ICM production systems, rather than the replicated pesticide/fungicide regimes that are the norm for most producers. Many of the chemicals currently available to growers for pest and disease control are under review and will be withdrawn from use in the foreseeable future. The relatively low value of most horticultural markets compared to arable, means that it is unlikely that new active molecules targeted specifically for horticulture will be forthcoming in any quantity in the future.

The main obstacle in shifting production to low input systems is a lack of cultivars resistant to some of the most damaging pathogens, notably to raspberry root rot caused by *Phytophthora fragariae* var. *rubi*. In this case there are no cultivars of commercial merit with acceptable resistance to raspberry root rot.

Raspberry root rot has had a devastating effect on many growers, rendering their plantations uneconomic and ultimately unsuitable for raspberry production. The control measures for root rot involve the use of significant quantities of fungicide. The only real long-term solution to root rot is the development of high quality resistant cultivars, which can then play a major part in the development of ICM growing systems and ultimately in the production of the residue-free fruit in demand from consumers and retailers alike.

Plant Breeding

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Breeding for root rot resistance is a major objective of breeding programmes at SCRI and elsewhere. Plant breeding however is a long slow process which involves crossing parents, known from breeders experience to be good candidates for the traits desired by end users. Rubus breeding however is not straight forward, being hampered by several genetic problems including polyploidy, pollen incompatibility and poor seedling germination. The highly heterozygous (genetically variable) nature of the germplasm requires evaluation of large seedling populations.

Breeding is based on a generation by generation improvement in breeding stock through selection and inter-mating of individuals showing promise of producing superior progeny. This translates to a strategy of crossing the best parents (best with the best) and evaluating a large number of the seedlings produced. Seedling numbers in excess of 12,000 plants are evaluated at an early stage in the glasshouse for characteristics that are easy to screen for example, spines and aphid resistance and planted in a seedling plot for further evaluation. Five years later after general field evaluation the breeder is ready to select those seedlings with potential for further examination by which time the numbers have dropped dramatically to around 100 plants. These individual seedlings are propagated by root to produce five or more plants of each for further evaluation in five plant plots for a further two years. Once selections have been made from the five plant plots, numbers are usually down to single figures. At this stage they may be propagated and put into farm trials and also put into disease plots to assess their resistance, a procedure which in itself can take over four years. After this length of time few if any seedlings will contain all the traits necessary for a new variety.
This procedure is extremely time-consuming and costly in terms of field and glasshouse resources, breeder's time and labour requirements. Breeding methods used in raspberry have changed very little over the last 40 years or so. What is required are methods for

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more accurately predicting at an early stage, what characteristics the seedlings have and especially those characteristics that are time consuming to determine before any screening and confirmation takes place. With Marker assisted breeding the time taken can be greatly reduced to approximately four years as can be compared in figure 1.

Some novel germplasm has made its way into commercial cultivars. However, with the narrowing genetic base (Graham and McNicol 1995) coupled with the increasing demands from consumers, new breeding methods are required to meet demands of the market which dictates more rapid turnover of varieties.

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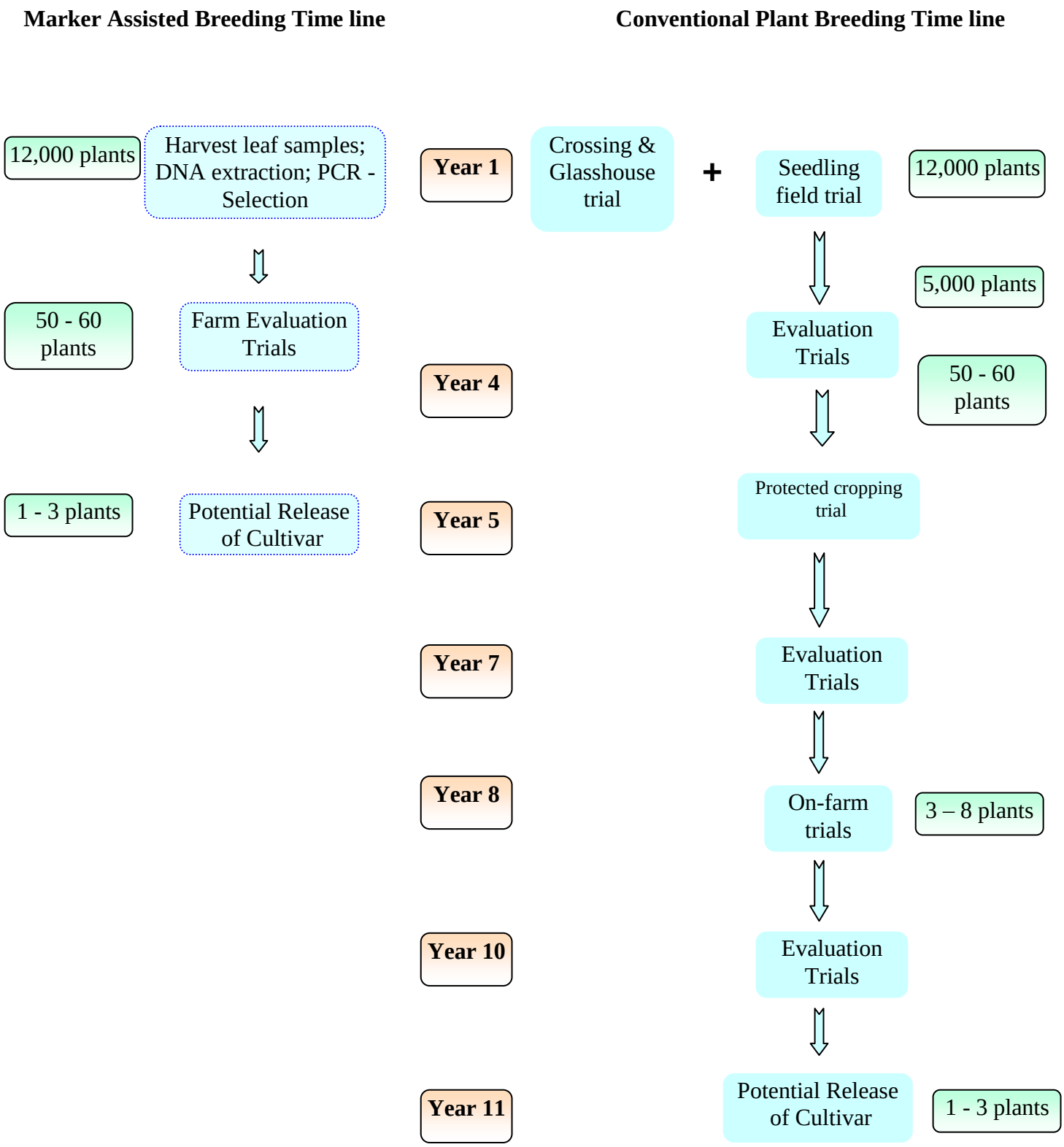

Figure 1. SCRI Raspberry breeding programme time line (courtesy of Nikki Jennings) with estimated Marker Assisted breeding time line as a comparison.

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Molecular Breeding

All traits of plants are controlled by genes, and the combination of alleles (versions) of the genes in the plant is known as the genotype. Red raspberry is diploid and therefore can have two alleles of each gene. The genotype, together with the environmental conditions in which the plant is grown, determines the phenotype (the way the plant looks and reacts to stresses etc.). The ultimate aim of plant genetics is to understand completely how genotype controls phenotype, and this information can then be transferred to plant breeders in a way they can easily access and use. Significant progress in the development of strategies for relating genotype to phenotype has been achieved with the development of markers and genetic mapping in plants and the ability to use map based gene isolation approaches. Although a large number of gene sequences are available in data bases and held privately, little is known about what these genes do and how they influence phenotype. With the availability of genetic linkage maps (which are simply a linear representation of the plant chromosomes), through field and glasshouse evaluation of progeny the location of the genes controlling traits can be determined on the map. In the first instance this provides a marker for the trait, and with more evaluation and study through, for example, DNA sequencing, large portions of DNA in the map region onto which the trait has been located, the genes themselves can be identified. For breeding purposes however the marker is sufficient to make a prediction on the likelihood of the presence of a trait of interest.

Molecular markers are DNA sequences (both known and unknown function) that are located near genes and inherited characteristics of interest (Antonius-Klemola 1999; Hokanson 2001), allowing selective breeding and identification of progeny with desired characteristics. Molecular markers have been rapidly adopted by researchers globally as

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an effective and appropriate tool for basic and applied studies addressing physiological traits. Markers are most informative when integrated into genetic linkage maps (Bradshaw et al., 1994). These molecular markers are used as tools that identify DNA polymorphisms (variations at particular points in the sequence) between DNA samples of different individuals. These polymorphisms can be of many different types from single nucleotide changes, large or small insertions and deletions or length variation in repeat sequences. All however provide information on a particular locus in the genome and importantly when that locus is known to be associated with a particular plant phenotype. An important way of linking marker loci to a particular plant phenotype is through the use of genetic linkage maps. These maps when coupled with field trials and glasshouse or laboratory experiments to measure traits of interest in the population of individuals used for map construction can then be used to relate phenotypic data to marker data on linkage maps.

For map construction, individual marker loci are genetically characterized in a segregating population (progeny from the cross of two genetically diverse parents) and the recombination rate of alleles at each pair of loci can be determined using classical linkage analysis. Loci can then be ordered into a linkage map and distance between loci can be expressed as recombination units given in centiMorgans (cM) where one cM is equal to 1% recombination. Once a sufficient number of markers have been mapped, the number of linkage groups should equal the haploid number of chromosomes. Several computer programmes are available to quickly generate a map once markers have been applied to a segregating population. In the initial phase of map creation genetically diverse parents are chosen which are known to segregate for the trait(s) of interest and depending on the biology of the crop an F1, F2 or backcross used for map construction. Once a map and segregating population have been developed attempts

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can be made to identify map locations of traits of interest. The speed and precision of crop enhancement can be improved by the development of genetic linkage maps which allow the development of diagnostic markers for polygenic traits and in the future, aid the identification of the genes behind the traits.

The developments in bioinformatics and genomics, especially in the construction, development and use of expressed sequence tag (EST) databases provide further tools to link genotype with phenotype.

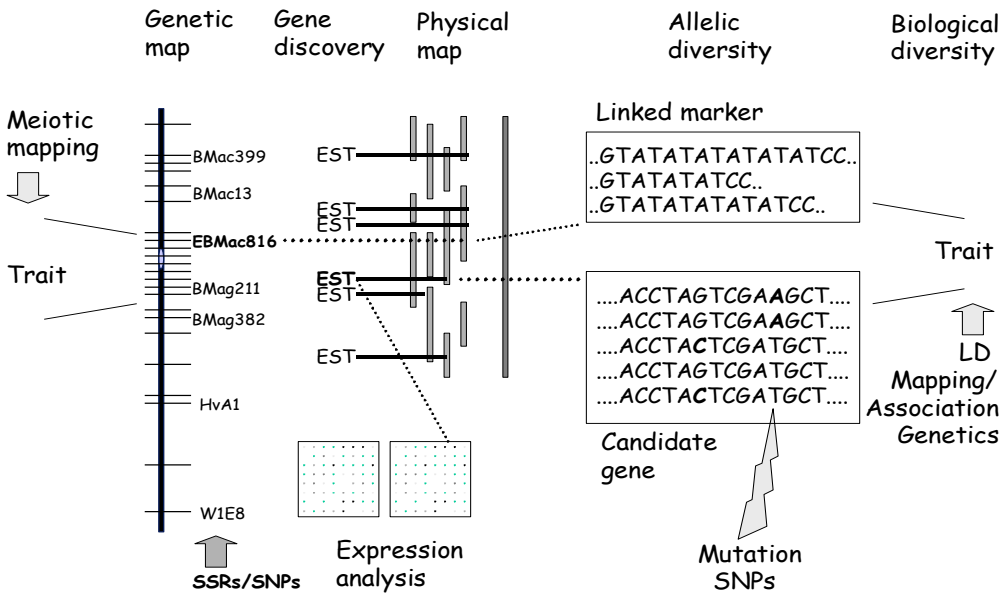

Figure 2. Integration of different approaches to understand the genes controlling a given phenotype (Graham, Ratnaparkhe and Powell 2007).

One of the most important developments in genetic mapping has been the demonstration that quantitative trait loci (QTL) can be located near DNA markers with

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very high accuracy (Bradshaw and Stettler 1994). QTLs are stretches of DNA that are closely linked to the genes that underlie the trait in question. QTLs are often associated with traits of great economic importance that are usually difficult to manipulate in plant breeding programmes. Genetic maps based on DNA markers have allowed the dissection of some quantitative traits into single component loci which contribute to the phenotypic variation for a trait (Graham et al., 2007). Thus identification of DNA markers linked to specific QTLs offers the possibility of marker-assisted selection for such agronomically important traits and high resolution mapping of QTL will allow map based cloning of the genes involved.

Red raspberry and molecular breeding

Red raspberry (*Rubus idaeus*) is a good species for the application of such techniques, being diploid (2n = 2x = 14) with a very small genome (275 Mbp). The availability of abundant genetic variation in natural and experimental populations and adaptation to a range of diverse habitats (Graham et al. 1997b; Marshall et al. 2001; Graham et al. 2003) offers researchers a rich source of germplasm to increase variation in morphology, anatomy, physiology, phenology and response to a range of biotic and abiotic stresses. This coupled with the application of more knowledge-based breeding should secure raspberry breeding in a changing climate and allow introgression of species material in a shorter timescale. An evolving genetic linkage map of raspberry is available (Graham et al. 2004, 2006). This map was constructed utilising a cross between the phenotypically diverse European red raspberry cultivar Glen Moy and the North American cultivar Latham. This cross produced a segregation population of 323 individuals. Latham was one of the first cultivars to be produced through controlled breeding in the 1930s, and is estimated to be approximately 60% genetically similar to Glen Moy (Graham and

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McNicol 1995). Latham is a hardy, spiny-brown caned plant producing small fruits and is resistant to raspberry root rot. Glen Moy was produced in 1981, has large fruits, is spine free, green-caned, susceptible to low temperature damage and has no resistance to root rot. The progeny of this cross, therefore, will segregate for root rot resistance as well as a broad number of other characteristics including pest resistance, crop architecture and fruit quality, which can be phenotyped and placed onto the linkage map. This provides, for the first time in raspberry, an unequalled resource for mapping both single gene and polygenic traits and developing diagnostic markers for the ones of premium value. In this instance, resistance to raspberry root rot will be targeted.

As an illustration of the practical utility of genetic linkage maps in raspberry, a study on *Gene H* is discussed briefly (fig 3). *Gene H,* the gene responsible for cane pubescence, had been reported for some time to be associated with resistance to cane botrytis and spur blight as well as susceptibility to rust and cane spot. With the availability of the raspberry genetic linkage map, it was possible to map *Gene H,* and to also map resistance to the four diseases with reported association. This work confirmed the association between *Gene H* and cane botrytis and spur blight but not with rust and cane spot (Graham et al., 2006).

With the demonstration of the linkage between *Gene H* and resistance to cane botrytis and spur blight, the gene itself can be used as a marker to predict resistance status for these two important diseases (Graham et al., 2006). The map has confirmed for two diseases, the close association with *Gene H*. This information has been transferred to breeders and cane morphology as determined by alleles for *Gene H*, provides an

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accurate prediction of disease resistance.

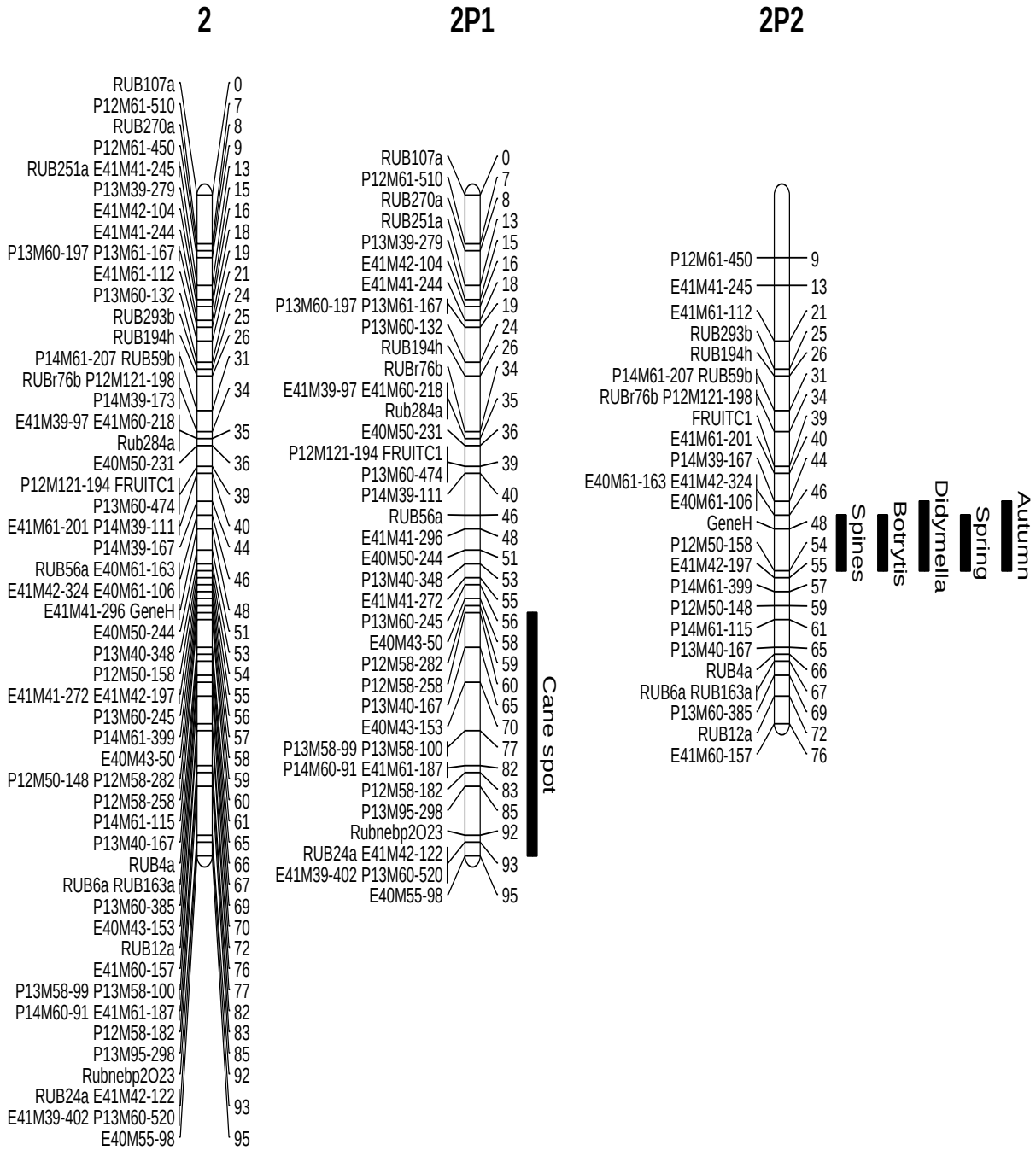

Fig. 3 Linkage group 2 of the raspberry map. *Gene H* and map locations of spur blight and cane botrytis resistance shown.

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Raspberry root rot

Raspberry root rot, caused by *Phytophthora fragariae* var. *rubi,* continues to be one of the most serious diseases of raspberry (Harrison et al., 1998). Root rot diseases have always been a problem in North America but were not regarded as a problem in Europe until the 1980s when *Phytophthora* root rot emerged as a major problem of raspberry with outbreaks in the U.K. (Duncan et al., 1987), Scandinavia and Germany (See Muller et al., 1986). Raspberry root rot became a serious problem throughout temperate Australia during the unusually wet years of 1994–1996 with *Phytophthora fragariae* var. *rubi* (Wilcox et al., 1993) identified as the major causal agent. This disease is now the most destructive disease of raspberries. Although named as root rot, all parts of the plant below or at ground level can be infected, including roots, root buds before emergence, crowns and the bases of canes (primocane and fruiting cane). On primocanes, the lesions can spread above soil level for up to 20 cm. Affected canes die in the first year of growth or their buds fail to emerge at the start of the second growing season. Alternatively, emerged laterals wilt and die at any time from emergence until late in fruiting.

The almost simultaneous outbreaks of a new disease across Europe in traditional raspberry-growing areas (raspberries have been grown in Tayside, Scotland for more than a century) suggested that the disease had spread through the propagation network and had been distributed to farms in new planting material. Introduction of new and highly susceptible cultivars was a major factor in disease spread. Local spread of the pathogen in water running down slopes from one field into another also contributed to

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the problem.

Diagnosing root rot

The prevention of new outbreaks must become the underpinning philosophy in control strategies for root rot. Ensuring that the planting material is free of disease is the most effective strategy. The pathogen is unlikely to be present widely in soil where raspberries have never been grown previously, and if present it would be in amounts so low and in such a form (oospores) as to render any soil test inaccurate and unreliable. Testing planting material, in particular roots, where *Phytophthora fragariae* var. *rubi* is present in an active form and in amounts higher than could ever be recovered from old decaying pieces of roots in soil, is the correct approach (Duncan and Cooke 2002).

The difficulty of unequivocally diagnosing *Phytophthora fragariae* var. *rubi* was resolved by resorting to a PCR diagnostic devised for red core of strawberry *Phytophthora fragariae* var. *fragariae* (Duncan et al., 2000). The test is based on selective amplification of a target DNA located between the internally transcribed spacer regions (ITS1 and ITS2) of the genomic ribosomal RNA gene repeat (rDNA). The target is present in large copy number in eukaryotes and is usually different enough among *Phytophthora* spp. to be a good way of identifying and distinguishing them (Cooke et al., 2000). The sequences of this region of DNA in *Phytophthora fragariae* var. *rubi* and *Phytophthora fragariae* var. *fragariae* are identical and the same test can therefore be used for both pathogen varieties. With a soil bait test and PCR diagnostic it is now possible to detect infection in propagation stocks and to prevent new root rot outbreaks on land that has never before grown raspberries.

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Chemical control and management

The negligible area of raspberry crop production within the overall agro industry means that no fungicide would be developed by the agrochemical industry on the basis of its specific potential to control raspberry root rot. Fungicides developed for similar diseases of other more important crops can be tested with 'off-label' approval for use on raspberries. Since 1980, the search for new and better fungicides has never ceased. Metalaxy1 + copper nitrate (Ridomil Plus) was introduced after the first surge of root rot outbreaks in the mid-1980s. Trials at SCRI led to off-label registration promoted by the East of Scotland College of Agriculture (now the Scottish Agricultural College) (Duncan and Kennedy 1987). However, almost from the time it received approval (Heiberg 1995; Maloney et al., 1993; Wilcox et al., 1999), growers complained that the level of control was not consistent and attempts to find a more effective replacement chemical continued uninterrupted.

Oxadixyl+mancozeb (Recoil) emerged as the replacement for metalaxyl+copper nitrate. Small and large-scale field trials in the early 1990s proved its efficacy. It was quickly adopted as the industry's fungicide of choice. Despite some complaints about lack of control when disease pressure is high, it remained in wide use throughout the UK until 2002 when Recoil was withdrawn. Fluazinam (Shirlan) has emerged through the usual route as the best of a newer generation of materials with anti-oomycete activity. Its efficacy was proved in a large-scale field trial held on a commercial site in Scotland. A specific off-label approval (SOLA) for control of raspberry root rot using metalaxyl-M was given in the UK (2002). Phosphonate anion salts have been used in Australia since 1985 to control phytophthora root rot (Guest et al., 1995). The complex mode of action of the phosphonate anion mitigates against selection for resistant pathogens and the product is

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environmentally benign (Guest and Grant 1991).

Planting raspberries on ridges (hilling) is now standard practice worldwide to improve drainage and aeration, and in some soils, improves plant growth and fruit yields (Heiberg 1995; Heiberg 1999; Maloney et al., 1993; Wilcox et al., 1999). Gypsum (calcium sulphate) is used as a soil amendment to improve calcium availability and thereby improve soil structure without altering pH. Calcium has also been implicated in the regulation of the life cycle of several *Phytophthora* species (Jackson and Hardham 1996; von Broembsen and Deacon 1996; Xu and Morris 1998) and has improved field resistance of avocados to *Phytophthora* (Duvenhage et al., 1992).

An integrated control programme involving clean planting material, fungicides and host resistance are undoubtedly the most effective ways of preventing new outbreaks and controlling the severity of existing outbreaks of raspberry root rot. Controlling the health of planting material is beyond the scope of individual growers but they should ensure that their material is sound and comes from certification schemes that meet EPPO plant propagation standards. The other elements such as choice of cultivar (in discussion with end users), ridging, mulching polythene, probably in combination with trickle irrigation, are options for growers. Other elements of the control strategy remain to be included in management regimes (e.g. biocontrol agents and chemicals for improving soil structure or aeration) but it seems doubtful if any of these would give long-term and significant improvements throughout the normal life of a raspberry plantation. It seems likely that this potent disease will be managed most effectively in the future by enhanced host resistance.

Genetic based resistance

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Screening cultivars of red and other raspberries and wild Rubus species have identified potential sources of resistance. 'Latham' and 'Winkler's Sämling' were identified early as having significant disease resistance, and species material such as *R.strigosus* (in the pedigree of Latham) and *R. ursinus* have been identified (Barritt et al., 1979). It has been speculated that these two sources are the same or genetically very similar. It has also been noted that the resistance in Latham has never broken down. The raspberry-blackberry hybrid cv. Tayberry, also demonstrates immunity to this disease (Duncan et al., 1987). However, none of the commercially important raspberry cultivars in Europe seem to have useful levels of resistance (Scherer and Riedel 1990).

Genetic resistance through plant breeding offers a feasible and effective method of control, but because of the difficulty in developing an accurate screening system and the time involved in the selection of resistant seedlings from the screen and then combining resistance with other desirable traits, e.g. fruit size and quality, breeding has not yet had the anticipated impact in commercial production. More research on the genetic basis of resistance and breeding is required as well as improved selection strategies. Future breeding plans with respect to root rot resistance are underpinned by the development of molecular maps (Graham et al., 2004, 2006) and the development of molecular markers linked to resistance to improve and accelerate selection efficiencies (Graham and Smith 2002). The identification and utilisation of resistance from Latham would provide a genetic based strategy for control. Tackling the impact of root rot (figure 4) through a molecular breeding approach would offer a tool for a quick screen for the likelihood of resistance. The variety Latham derived from *R. strigosus* is one of the few sources of resistance and if resistance loci can be determined a marker assisted breeding approach can be used to more quickly introgress genetic based resistance into commercial varieties.

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Figure 4. Aerial photograph of a raspberry field affected by *Phytophthora fragariae* var. *rubi.* Plant death due to the disease is clear

It may also be prudent to collate information from breeding programmes worldwide on the germplasm available both breeding lines and species material to determine how much resistance exists within the gene pool and how different other resistances are to Latham derived resistance. This will become possible with identification of map regions significantly associated with resistance.

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Project Aims

The aim of this project was to develop genetic tools, namely molecular markers, that could be used in raspberry breeding to quickly and effectively identify selections with likely resistance to raspberry root rot caused by *Phytophthora fragariae* var *rubi*.

To achieve this, the project utilised a cross between the root rot resistant cultivar 'Latham' and the susceptible cultivar 'Glen Moy'. Progeny from this cross segregate for root rot resistance and if the resistance status of each individual can be determined through field and/or glasshouse trials this data on disease segregation can be used to identify regions on the genetic linkage map important for resistance.

Based on the mapping information, molecular tools (markers) can be developed and integrated into breeding programmes to allow the early selection of raspberry cultivars resistant to raspberry root rot without the need for field based screening.

In breeding programmes screening for disease resistance including that of raspberry root rot is often carried out in glasshouse trials involving controlled inoculations. Debate among raspberry breeders and pathologists on the value of this screening has continued over a number of years in regard to its efficiency. A secondary output from this project was to determine how data from field and glasshouse trials correlate, as a large number of identical clones from each of the progeny could be produced for replicated experiments.

Resistant cultivars would contribute to the sustainability of the UK fruit industry producing

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crops suitable for low input growing systems, while providing a source of high quality red raspberry fruit. The availability of root rot-resistant cultivars would therefore benefit the industry through:

- Increased plantation life
- Reduced fungicide costs
- Reduced crop loss
- Increased options for siting of plantations

In order to achieve the aim of the project seven objectives were set for the project.

1. Set up glasshouse and field-based screens of the Moy x Latham mapping population for root rot resistance to allow the status of each individual to be determined.
2. Identify molecular markers linked to root rot resistance by mapping the phenotypic data for resistance from both glasshouse and field screens onto the existing raspberry genetic linkage map at SCRI using the Joinmap and MapQTL computer programmes.
3. Enhance the existing map with new SSRs and EST-SSRs.
4. Validate the putative marker(s) in segregating progeny from SCRI and develop a diagnostic system for root rot resistance.
5. Examine a range of historical germplasm from SCRI and HRI with different genetic backgrounds and with known resistance or susceptibility status for the presence of alleles identified to be associated with root rot resistance.
6. Identify material with resistance from the mapping population for incorporation into the breeding programme and evaluate other traits of commercial merit within the mapping population.
7. Assess the value of red raspberry as a model for deployment of molecular breeding strategies into horticulture.

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These have been organised into five main objectives for ease of presentation for the final report as in table 1.

Table 1. Objectives as listed in report against Objectives as presented in Report

Project Objectives		**Objectives as listed in Report**	
Objective 1	Set up glasshouse and field-based screens of the Moy x Latham mapping population for root rot resistance to allow the status of each individual to be determined	Objectives 1 & 2	Objective 1. Field-based screens of the Moy x Latham mapping population for root rot resistance to allow the status of each individual to be determined Objective 2. Glasshouse -based screens of the Moy x Latham mapping population for root rot resistance to allow the status of each individual to be determined
Objective 2	Identify molecular markers linked to root rot resistance by mapping the phenotypic data for resistance from both glasshouse and field screens onto the existing raspberry genetic linkage map at SCRI using the Joinmap and MapQTL computer programmes	Objectives 1, 2 & 4	Objective 1. Field Screen for segregation of root rot resistance Objective 2. Glasshouse screen for segregation of root rot resistance Objective 4. Correlation of data and mapping resistance loci
Objective 3	Enhance the existing map with new SSRs and EST-SSRs	Objective 3	Objective 3 Enhance the existing map with new SSRs and EST-SSRs

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Project Objectives		**Objectives as listed in Report**	
Objective 4	Validate the putative marker(s) in segregating progeny from SCRI and develop a diagnostic system for root rot resistance	Objective 5	Objective 5. Validation of markers
Objective 5	Examine a range of historical germplasm from SCRI and HRI with different genetic backgrounds and with known resistance or susceptibility status for the presence of alleles identified to be associated with root rot resistance	Objective 5	Objective 5. Validation of markers
Objective 6	Identify material with resistance from the mapping population for incorporation into the breeding programme and evaluate other traits of commercial merit within the mapping population	Objective 4	Objective 4. Correlation of data and mapping resistance loci
Objective 7	Assess the value of red raspberry as a model for deployment of molecular breeding strategies into horticulture	Objective 5	Objective 5. Validation of markers

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Objective 1

Field-based Screening of the Moy x Latham mapping population for root rot resistance to allow the status of each individual to be determined

Introduction

Before any mapping work can be carried out to identify map locations and markers linked to root rot, the resistance status of each of the progeny from a cross segregating for root rot resistance is required. This resistance data can then be linked to molecular data generated from the same progeny, to identify markers closely associated with disease resistance and/or susceptibility.

No screening system can be 100% accurate due to a number of complex and interacting factors. These include escape of susceptible progeny due to patchy or low disease pressure, irrigation, slope of land, soil structural factors and seasonal variations such as temperature, wind direction, rainfall etc. To date the best protocol for root rot screening remains planting the material on field plots known to be heavily infested with the disease.

Root rot is a serious disease which affects the root and therefore ultimately whole plant performance. It has been suggested that root vigour may actually be a major contributing factor for resistance to root borne stresses such as fungal pathogens and root feeding pests. In previous research on strawberry we found strawberry cultivars with vigorous root systems could tolerate high levels of vine weevil larvae compared to varieties with less vigorous root systems (Gordon pers. Comm; Graham et al., 2002). Root vigour was also investigated in the field trials.

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Materials and methods

Plant Material

To allow the identification of the resistance status of the progeny from the Glen Moy x Latham cross, the parents and the segregating population were cloned by the propagation of root material. Mother plants from the Glen Moy x Latham cross were maintained for propagation in a gauze house. The mother cane was cut from the pot and re-planted, and the root material remaining in the pot was chilled for six weeks. After this time the root from each mother plant was put in trays with compost and placed in a warm glasshouse. Plants growing from the root material served as a source for the establishment of replicated field trials. Once plants were established and grown they were placed outside under rain shelters to acclimatise. Plants were then maintained for two seasons as long canes before use as planting stock.

Root rot screening

Two field trials were set up, both located at SCRI and these were planted in three replications (replicates) per site, with two plants of each progeny planted together in each replicate (6 clones per progeny at both sites). These were planted in spring 2003; one year before screening was initiated. The two trial sites consist of one uninfected field, and the other a site heavily infested with *Phytophthora fragariae* var *rubi*. The diseased site was known to be infected by root rot (SCRI farm records) and further infection of this site was carried out by spreading and rotovating known infected topsoil from another site, in an attempt to evenly distribute the fungus. A decision was taken to water the infected site on a daily basis using a tape irrigation system from June until

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September to increase the spread of infection throughout the field, an example of which as can be seen in figure 1.1.

Figure 1.1. Aerial-view of the infected site replicates 4 and 5 being watered by the tape irrigation system 2005.

Data were collected on visual symptoms of root rot damage from April to October on a daily/weekly basis over three seasons (2004, 2005 and 2006). In the first year (2004) a scale of 1 (yes) or 0 (no) was recorded for evidence of disease symptoms due to the short period of time for symptom development. In 2005 and 2006 a 1 to 5 scale was utilised as symptom production was more developed, this breeder scoring system is statistically more functional. This scale is described in Table 1.1.

Table 1.1. The breeders' root rot scoring system and the description for the collection

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and analysis of data in 2005 and 2006

Scale	Description of symptoms
1	Very healthy no disease symptoms
2	Shoot tips affected
3	Clear signs of root rot on canes
4	All canes & buds seriously affected, canes would pull out if slight pressure applied
5	Dead

Other plant characteristics

In order to investigate root viability in relation to root rot resistance, the density and the spread of root suckers from the mother plants were recorded on a 0-5 scale (0 being no production) under field conditions at both locations. For density scores, 0 = no root suckers, 1 = 1-4 suckers, 2 = 5-8 suckers, 3 = 9-20, 4= 21- 40 and 5 = >40 suckers. For root sucker spread, distance of root suckers from the mother plant was estimated; a score of 1 being suckers up to 10 cm from the mother plant and 5 being suckers at 1 m or greater from the mother plant.

Other phenotypic data were collected on plant height and cane number at both the clean and root rot sites.

Data were analysed using Genstat version 7, with assistance from Bioinformatics and Statistics Scotland (BioSS).

Results

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Data were collected in 2004 mainly to confirm that root rot infection was occurring and that Glen Moy was becoming infected. Data analysis from 2004 on a scale of 1 (yes) and 0 (no) to the presence or absence of disease symptoms confirmed that Glen Moy and Latham were behaving as expected (tables 1.2), with significant differences in the response to root rot observed.

Table 1.2 Mean responses of the parents Latham and Glen Moy in 2004 on a 0 or 1 scale

Mean disease score (SD)	
Glen Moy	Latham
0.67 (0.18)	0 (0)

These results on disease symptom development were confirmed in 2005 and 2006 with the parents behaving as predicted. In 2005 and 2006 clear signs of difference in progeny response to root rot disease could be differentiated on a growers scale of 1 to 5 with the individuals showing a spread across the disease scores from 1-5 (Fig 1.3 and 1.4). The clear distribution of mean root rot scores (1-5 scale) for 330 individuals in 2005 and 2006 (figs 1.3&1.4) demonstrated that significant differences exist across the progeny in disease scores and therefore resistance status an example of which is given in figure 1.2. Disease scores were collected on each of the progeny eight times across the season in both 2005 and 2006. Mean disease scores were calculated for each progeny by replicate, and also overall. A final season mean score (last score) was also calculated for each progeny.

Table 1.3 Mean overall response and Standard deviation (SD) of the parents Latham

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and Glen Moy in 2005 and 2006 on a 1-5 scale.

Mean disease score (SD)			
	Glen Moy	Latham	Progeny Mean
2005	4.62 (0.51)	1	2.5 (0.7)
2006	4.62 (0.74)	1 (0.35)	3.0 (0.6)

Table 1.3 shows the mean progeny disease scores in 2006 are significantly higher than 2005 ($p<0.001$) and this trend can be clearly seen in figure 1.3 and 1.4.

Figure 1.2. Differences between the 2 plant plots of progeny clones at the root rot site. Clones on the right are almost dead with those on the left still healthy.

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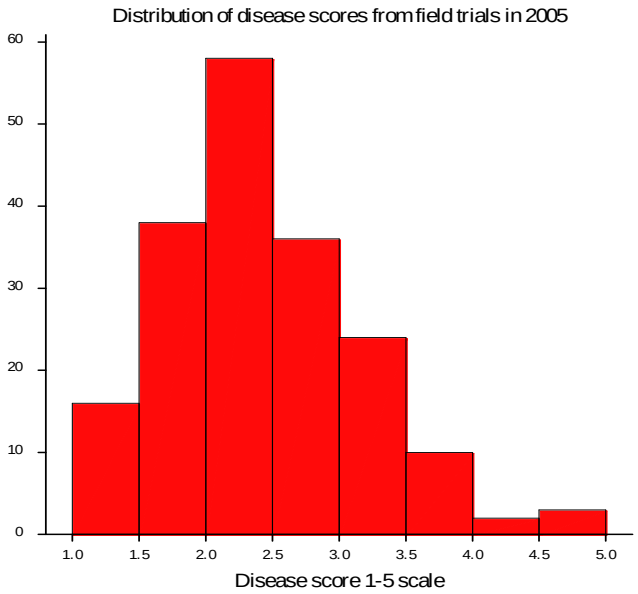

Figure. 1.3 Distribution of disease scores across the progeny in 2005

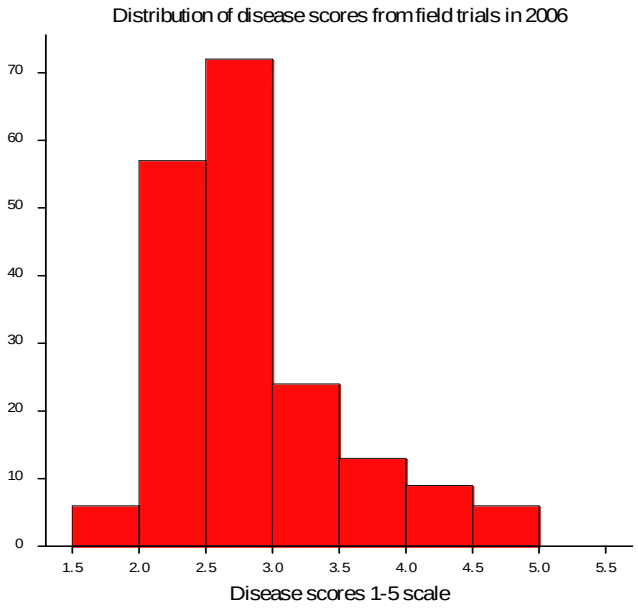

Figure. 1.4 Distribution of disease scores across the progeny in 2006

Data analysis showed some difference between replicates (replicates) with regard to fungal severity. For example replicate 4 was more heavily infested than replicate 5 with damage scores for replicate 4 higher than replicate 5 in both 2005 and 2006. Information on disease spread within and between replicates is crucial for mapping purposes to

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avoid mis-identification of QTLs and this detailed analysis is discussed further in Objective 4.

Root and other plant characteristics

Measurements of other plant characteristics from the population at the clean and root rot sites in 2005 and 2006 demonstrated that there were significant differences in plant growth in terms of cane height, cane number and root sucker parameters (spawn density and diameter) between the clean and root rot infested sites (Table 1.4). This suggests the fungal disease has a serious impact on the plants (table 1.4). Figures 1.5 and 1.6 illustrate the difference in growth at the two sites.

Table 1.4. Mean plant characteristics (SD) at the clean and root rot sites 2003-2005. (managed – canes cut out to reduce density of the rows)

	Clean 2004	**Root rot 2004**	**Clean 2005**	**Root rot 2005**	**Clean 2006**	**Root rot 2006**
Height cm.	136.0 (2.9)	111.4 (2.4)	136.1 (3.5)	111.2 (3.5)	185.7 (4.0)	153.7 (3.9)
Cane number	3.5 (0.21)	2.5 (0.12)	7.9 (0.21)	4.7 (0.31)	managed	managed
Root sucker diameter	3.30 (0.12)	2.85 (0.09)	3.25 (0.06)	2.26 (0.05)	2.64 (0.03)	1.78 (0.04)
Root sucker density	2.12 (0.06)	2.09 (0.09)	2.84 (0.05)	2.63 (0.05)	2.24 (0.05)	1.3 (0.03)
Root rot score	NA	NA	1 (0.0)	3.09 (0.04)	1 (0.0)	3.45 (0.04)

All individual trait comparisons show significant differences between the clean and root rot sites in each year ($p<0.001$) except for root sucker density 2004 and 2005. Plants at the clean site were significantly larger, had more canes, and the root suckers spread further from the mother plant, and in 2006 root density was also significantly different.

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Figure 1.5. Glen Moy x Latham population at the root rot infected site.

Figure 1.6. Glen Moy x Latham population at the clean site.

Correlations of Root sucker (spawn) Density and Diameter with Root Rot Scores from the field

A correlation matrix was prepared for the root rot scores from the infected field data collected in 2005 and 2006 and the spawn scores from both the clean (uninfected) site and the root rot site. The results produced illustrated that all correlations were highly

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significant ($p<0.001$) and that spawn density is positively correlated with spawn diameter and negatively correlated with root rot score (fig 1.7), indicating that the larger the spawn density and diameter the “healthier” or more resistant that the plant is to *Phytophthora fragariae* var. *rubi*,, and that the smaller the spawn density and diameter the more susceptible the plant is to *Phytophthora fragariae* var. *rubi*. This greater root vigour is not merely a reflection of disease effect on the plants, as this correlation is maintained when the final disease scores were examined beside the spawn density and diameter scores from the clean site, which effectively demonstrated the plants potential for spawn growth.

Table 1.5 Mean root density and diameter (SD) at clean site from plants designated as resistant or susceptible based on final disease scores.

	Root Density	**Root Diameter**
Resistant	2.9 (0.5)	3.3 (0.4)
Susceptible	2.6 (0.4)	3.0 (0.4)

Sig. 0.004

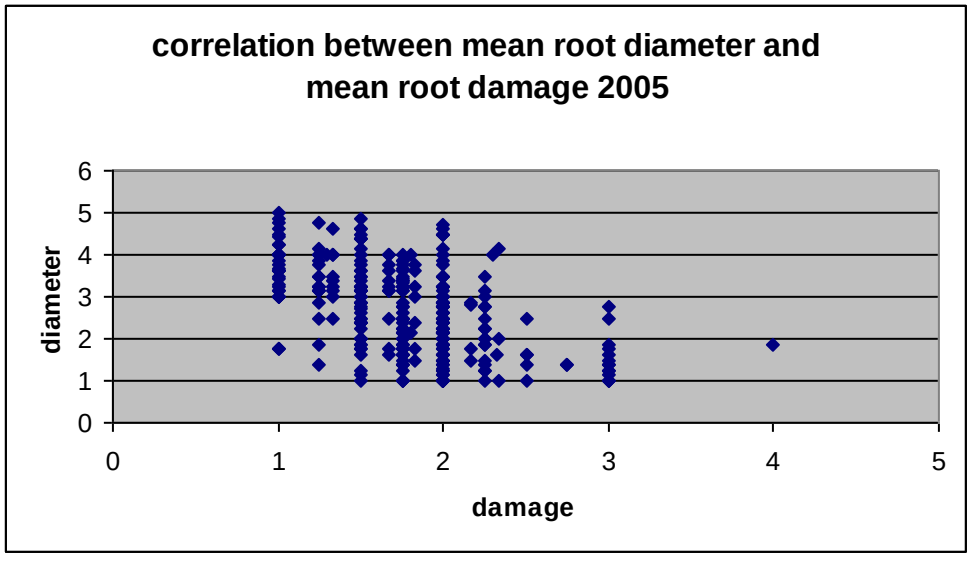

Figure 1.7. Correlation between root damage and root density from infected site in 2005

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Discussion

Root rot is clearly a devastating disease in red raspberry. This disease affects all aspects of plant growth from plant height, cane number and root sucker characteristics. The field results show that field screening of this nature is an effective way of differentiating progeny based on their disease resistance. Glen Moy and Latham as controls behaved absolutely as expected. What is interesting is the spread of mean disease scores in both 2005 and 2006 (fig 1.3 and 1.4). If an arbitrary score of 3 (as the mid point) is chosen for the cut off for disease resistance, then more progeny are assigned to the resistant category than in the susceptible category and this is particularly so for 2005. It should be noted that root rot resistance is a continuous trait and this cut off is somewhat artificial, but this may suggest the field screen is not stringent enough or there are some, (though probably a small proportion) of escapes. Alternatively the threshold value for resistance should be set lower.

In terms of actual numbers of progeny 75% of progeny had mean scores of 3.1 or less in 2005 with the 50% threshold at a mean score of 2.5. By 2006 75% of progeny had scores of 3.4 or less and the 50% threshold had mean scores of 2.9 or less. Longer screening times clearly give a better distribution of progeny disease scores across the 1-5 scale.

The field experiment has achieved its objective and produced replicated data on each of the progeny with regard to their resistance status and their root sucker parameters. This will allow mapping to proceed (Objective 4) to identify map locations with markers closely associated with root rot resistance and root sucker parameters

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The demonstration of a highly significant correlation between the root sucker parameters of density and diameter and root rot resistance is potentially of great value to breeders as it provides an easy visual screen for germplasm with some level of resistance/tolerance to root rot.

Outputs from Objective 1

1. Data on resistance status of progeny for mapping and marker development.
2. Recommendation to have longer field screening over 3 years (4 years at site) which gives a better distribution of disease symptoms across the scale and should be a minimum for any root rot screening or alternatively shorter screening can be carried out with careful evaluation of the distribution of the data.
3. Data on selections showing root rot resistance for transfer to consortium breeding programmes.
4. Demonstration of a highly significant correlation between root sucker parameters and root rot resistance suggesting this can be used as an indication of disease resistance status. Indication that cultivars with vigorous root growth are more likely to resist/tolerate root rot.

The outputs from Objective 1 fulfil Milestone 2 and 5; Objective 1 of the original project proposal.

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Objective 2

Glasshouse-based screens of the Moy x Latham mapping population for root rot resistance to allow the status of each individual to be determined

Introduction

Glasshouse experiments are often used to determine the resistance status of seedlings against pest and diseases. How the glasshouse results relate to plant performance under field conditions is often unclear, and for root rot in particular this has been the cause of much discussion. The Glen Moy x Latham mapping population serves as a source of replicated material which can be used in both field experiments and glasshouse screening to determine the correlation between plant responses under both conditions. Screening under glasshouse conditions can be more easily standardised in terms of inoculums, temperature etc. but cannot completely replicate field conditions. What is important is to determine how well results from the glasshouse correlate with field screening and whether ultimately the map locations identified would be the same.

Materials and methods

Plant Material

The parents and the segregating population produced from the Glen Moy x Latham cross were cloned by the propagation of root material. The mother cane was cut from the pot and re-planted, and the root material remaining in the pot was chilled for six weeks. After this time the root from each mother plant was put in trays with compost and placed in a warm glasshouse. Plants were placed in individual four inch diameter pots and twelve week old plants served as a source for inoculation.

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Due to the number of plants and the replication required (totalling approx. 5,000 plants), the glasshouse screening was carried out over the three year duration of the project in five rounds, and was completed in June 2006. This has allowed the entire population and the sub set mapping population to be screening against *Phytophthora fragariae* var. *rubi* within the glasshouse. For each round approximately 80 progeny were cloned to provide nine plants for inoculation and three plants as non-inoculated controls (12 clones per progeny). The trial was set up in a randomised block design with four blocks, one of which remained un-inoculated. Each block had three clones per progeny. Glen Moy and Latham were included in each round.

Fungal material

Fungal cultures of *Phytophthora* were stored at 4^{o}C on oatmeal agar slopes. For inoculum production, these were inoculated onto French Bean Agar (FBA) plates, 3 inoculants per plate, and then incubated for 7-10 days at 20^{o}C where sufficient growth coverage of the plate was seen. Two types of fungi were used as follows; *Phytophthora fragariae* var. *rub*i, Codes: SCRP333, FVR11, IMI 355974, CBS 967.95; isolated in Scotland in 1985 from a single zoospore; raspberry host (Latin binomial *Rubus idaeus*); known as race 3; and *Phytophthora fragariae* var. *rubi* , Code: SCRP324, FVR 67; isolated in 1991; raspberry host (Latin binomial *Rubus idaeus*); known as race 1.

Figure 2.1 illustrates the two types of *Phytophthora fragariae* var. *rubi* used in this experiment.

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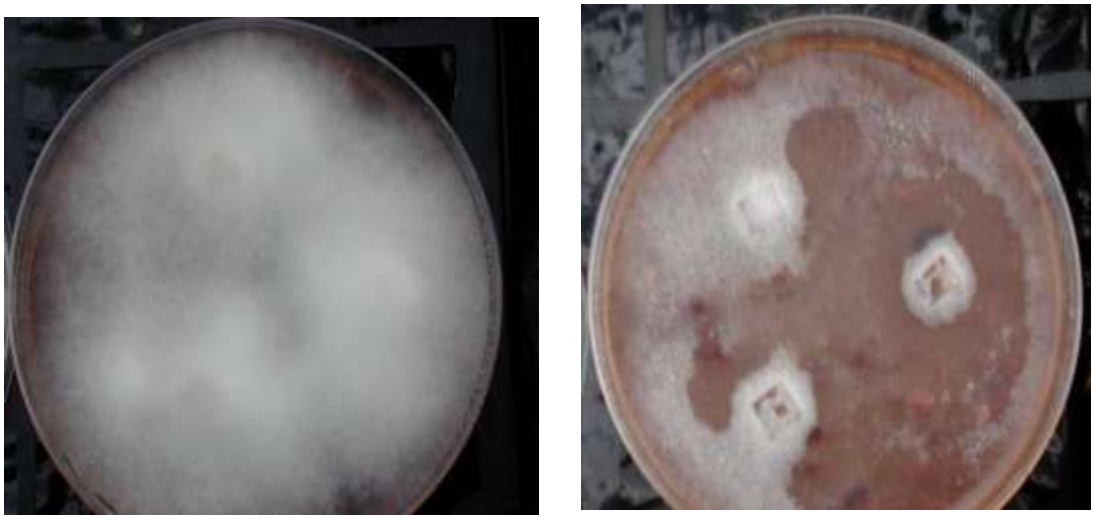

Figure 2.1. The two types of *Phytophthora fragariae* var *.rubi* (race 1 and 3) used in the glasshouse experiment.

Each pot containing a plant was inoculated with two mycelial plugs (7 mm in diameter) from two actively growing mycelium of *Phytophthora fragariae* var. *rubi* (race 1 & 3), maintained at 10-15^{o}C, and watered twice a day to create a disease producing environment. Inoculations were at the four corners of each pot (4 plugs in total; 2 from each fungus. Plants were maintained in a cool glasshouse (max. day temperature 12^{o}C) and over-watered for the duration of the experiment (figs.2.2 & 2.3).

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Figure 2.2. Glasshouse screening experiment (Round 5) March 2006 at week 1.

Figure 2.3. Glasshouse from round 1 after 8 week exposure to *Phytophthora fragariae*

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Eight weeks after inoculation, the plants were assessed for root rot symptoms. The roots of the plants were assessed for amount of root and symptoms of root rot. The scale used for root amount was on a scale of 1 to 5, where 1 had virtually no roots and 5 was extremely dense and “pot bound”. The scale used for root rot was on a scale of 0 to 5 where 0 represented no signs of rot and 5 was extreme rot leading to death of the plant.

Figure 2.3 illustrates the scoring system with roots of Latham (left hand side) and Glen Moy after inoculation. Latham roots appear vigorous and receive a root density score of 4 in this case, and a disease score of 1. Glen Moy on the other hand in this example receives a density score of 1 and a damage score of 5.

Figure 2.4. The roots of Latham (left) and Glen Moy after 8 weeks inoculation.

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Results

The glasshouse screening for disease symptoms was carried out across the three years due to the size of the population (330) with replication (3 plants per progeny for non-inoculated and 9 plants per progeny for inoculation (330 x 12)). Additionally Latham and Glen Moy were included in each glasshouse trial carried out. The distribution of disease scores across all four trials are shown in Figure 2.5. As can be seen the progeny display a continuous variation in scores across the scale.

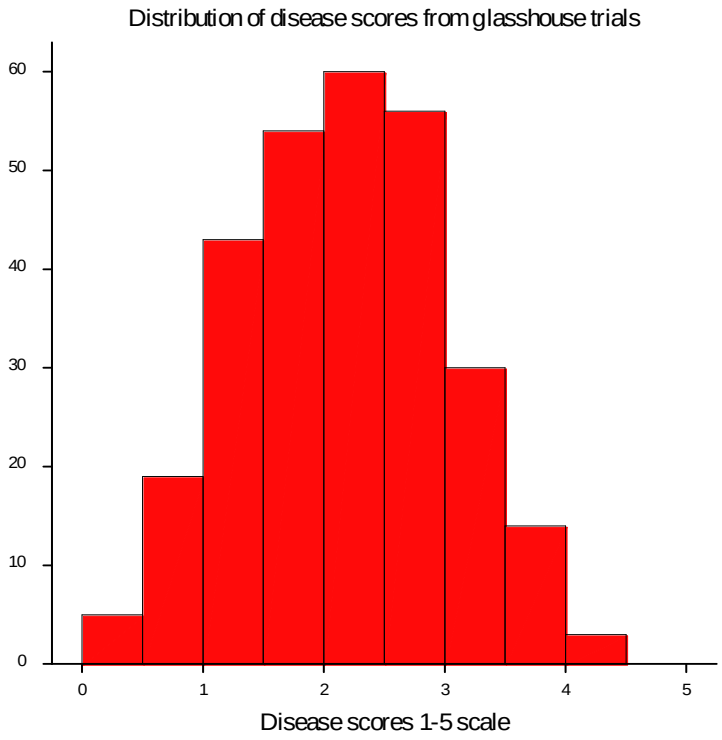

Figure 2.5. Distribution of glasshouse disease scores (0-5 scale).

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Data analysis across the five glasshouse experiments demonstrated significant differences between the means for the inoculated and control plants as would be expected.

No significant differences were detected between benches or dates for untreated control plants for root damage.

For inoculated plants, differences in disease severity were observed, with glasshouse trial 2 showing the most serious root rot symptoms. No significant differences were recorded between trials 1, 3, 4 and 5 for root damage (Table 2.1). Trial 2 plants also had a lower mean root density scores than traits 1, 3, 4 and 5. There are no significant differences between the densities for trials 1, 3, 4 and 5.

Table 2.1: Mean root damage of Glen Moy & Latham inoculated plants from data collected over five glasshouse trials.

Mean Root Damage Across 5 Trials						**Overall Mean**
Trial no.	*1*	*2*	*3*	*4*	*5*	
Latham	0.933	1.013	0.833	1.033	1.000	0.96
Moy	3.353	4.215	3.167	3.636	3.880	3.65

SED 0.11

The difference in disease severity within a trial across replicates was also examined (Table 2.2). Here replicate 2 has a slightly lower damage score than the other 2 inoculated replicates. The overall progeny mean damage score and root density score is given in Table 2.3.

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Table 2.2: Mean Root Damage of Glen Moy & Latham from data collected over four replicates (3 inoculated and 1 non-inoculated) within a glasshouse trial.

Mean Root Damage Across Replicates Within A Trial					**Overall Mean**
Replicate	*1* **(non-inoculated)**	*2*	*3*	*4*	
Latham	**0.300**	0.867	0.923	1.250	1.013
Moy	**1.100**	3.800	4.429	4.417	4.215

SED 0.17

An overall progeny mean was also calculated as shown in table 2.3.

Table 2.3 Overall progeny means from glasshouse screening across all trials

	Damage	**Root Density**
Progeny	2.42 (0.4)	3.92 (0.37)

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Discussion

Glasshouse trials, as with the field trials, could successfully differentiate the progeny on the basis of resistance against root rot. Like the field trials the data were normally distributed but skewed towards the lower end of the scale with 75% of the progeny having a mean score of 2.8 or less and 50% of progeny having a mean score of 2.2 or less. This suggests that glasshouse screening is less stringent than field screening and any designation of resistance at an arbitrary category of 3 will lead to a significantly higher proportion of escapes than field screening. Unlike in the field where root damage and root parameters were significantly correlated, little correlation existed between damage and root density glasshouse scores. This may be a reflection of the glasshouse system being less stringent and also the shorter timescale over which this is carried out.

Outputs from objective 2

1 Data on resistance status of progeny for mapping and marker development.
2 Demonstration that glasshouse screening is less stringent than field screening and care must be taken in determining where on the scale, progeny are designated as resistant.

The Outputs from objective 2 fulfil Milestones 1 and 4 from Objective 1 of the project proposal.

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Objective 3

Enhance the existing map with new SSRs and EST-SSRs

- Task 3.1 Development of new co-dominant markers derived from a root cDNA library and a nebulised genomic DNA library.
- Task 3.2 The addition of 94 new progeny onto the linkage map.
- Task 3.3 Assessment of cross transferability of primers from other Rosaceae.

Introduction

A genetic linkage map allows phenotypic traits to be linked to genetic markers. Generally the more markers on the map the more accurately the traits can be linked to those markers. At the start of the project the raspberry genetic linkage map had nine linkage groups. Given that raspberry has seven chromosomes the intention was to develop other markers to saturate the map further and reduce the number of linkage groups to match the number of chromosomes. Additionally after discussion with Bioinformatics and Statistics Scotland (BioSS) it was decided to further enhance the map by adding segregation data from another 94 progeny from the Glen Moy x Latham population thus allowing the identification of minor as well as major QTLs for root rot. A third objective was to examine cross transferability of markers from other *Rosaceae* mainly peach. After discussions with the peach community (B. Abbott pers. comm.) it was clear they had identified a resistance gene cluster on one of the peach linkage groups which they may be willing to share, depending on some form of material transfer agreement being signed. As a first step in utilising this, the transferability of markers between the species had to be examined to determine if the linkage maps could be easily related.

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Task 3.1 Development of new co-dominant markers derived from a root cDNA library

Introduction

The development of further co-dominant markers provides a resource for further map enhancement and ultimately allows the dominant Amplified Fragment Length Polymorphism (AFLP) markers to be removed from the map. By developing markers through the screening and sequencing of expressed sequence tag (EST) libraries also generates sequence information on potentially useful genes.

Materials and methods

Root cDNA library construction and SSR screening

RNA Extraction from Latham Root Tissue

The Latham raspberry plants were grown in high sand content to soil ratio with additional liquid feed. After approximately 4 weeks the Latham plants were carefully removed from the sand and soil mix to prevent damage to the roots. The roots were cut from the plant and washed twice for two minutes in sterile H_2O to remove all sand and soil before being snap frozen in liquid nitrogen. Standard procedures were used to eliminate RNA degradation by RNases. Approximately 5g of frozen root material was used. The roots consisted of white and dark brown roots and it was found after a few attempts at RNA extraction, that the white roots gave a higher RNA yield.

Total RNA was extracted as follows;

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Five grams of frozen root material was ground into a fine powder with a small amount of sand using a mortar and pestle and placed in a 50ml tube. Lysis buffer (10mls) (Lysis buffer (2%SDS, 50mM EDTA, 300mM Tris pH 8.0, 1% Mercaptoethanol), 2.5mls ethanol and 1.1 ml of 5M potassium acetate were added to the ground root material and vortexed for one minute. Chloroform/IAA (13.6 mls) was added and further vortexed for one minute before being centrifuged at 20,000g for 10 minutes at 5^{o}C. The upper aqueous layer was removed and placed into a separate tube with 13mls of phenol and then vortexed for one minute prior to centrifugation at 20,000g for 20 minutes at 5^{o}C. The upper layer was then removed and one third of this volume of 12M lithium chloride was added and gently shaken. Following mixing the solution was then stored overnight at -20^{o}C. This mixture was then centrifuged at 20,000g for 90 minutes at 5^{o}C and the supernatant removed. The tubes were placed on tissue upside down to dry the pellet for one hour. The dry pellet was then washed in 1ml 70% EtOH and centrifuged for 5 minutes at maximum speed in a sterile eppendorf tube. This wash step was repeated twice. The supernatant was removed, the pellet dried and then resuspended in 100µl sterile dH_2O. The RNA was quantified using a Nanodrop spectrophotometer and analysed on an 1% wv agarose gel (0.5g agarose, 5xTBE, DEPC water) to determine the integrity of the RNA. The RNA was stored at -80^{o}C until required.

cDNA Library Construction

DNase treatment is necessary to remove any potential DNA contamination of the RNA sample and the DNA-*free*^{TM} kit was used for this purpose (following manufacturers

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instructions (Ambion)). mRNA isolation was carried out using a Dynabead mRNA Extraction Kit (Dynal Biotech ASA, Oslo, Norway) and quantified using the Nanodrop spectrophotometer.

Synthesis of first strand cDNA template from the mRNA was carried out using the "Ready-to-Go™ You-Prime First-Strand Beads" (Amersham Pharmacia Biosciences) using the *Not*1 primer-adaptor from the superscript plasmid (Invitrogen) [5′- pGAC TAG TTC TAG ATC GCG AGC GGC CGC $CC(T)_{15}$-3′]. cDNA library construction was carried out with the Superscript™ Plasmid System with Gateway® Technology for cDNA Synthesis and Cloning (Invitrogen, Carlsbad, CA), where an overview is given in Figure 3.1.

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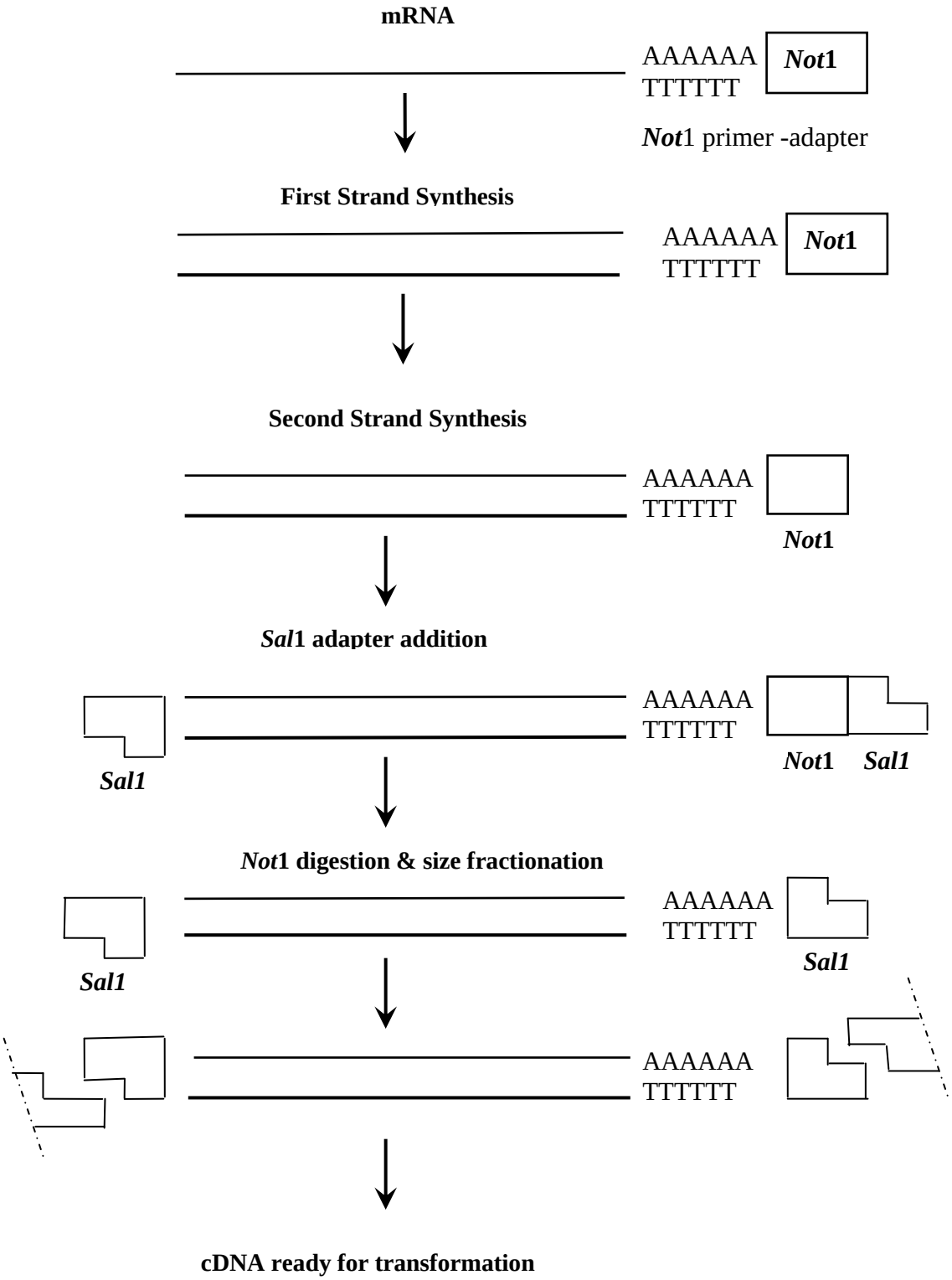

Figure 3.1 Overview of the Superscript™ Plasmid System procedure

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Second Strand cDNA synthesis was catalysed by *E. coli* DNA polymerase 1 in combination with RNase H and DNA ligase. T4 DNA polymerase was added to the second strand reaction mixture to ensure that the termini of the cDNA were blunt. *Sal*1 adaptors were ligated onto the blunt ends of the cDNA. The *Not*1/*Sal*1 fragments were directionally cloned into the *Not*1/*Sal*1 sites of the pSport1 vector. The plasmids were then transformed into *E.coli* electrocompetent DH10B cells (Invitrogen, Carlsbad, CA) for subsequent amplification of the cDNA library. These transformed *E.coli* cells were grown overnight on LBamp/X-gal/IPTG plates at 37^{o}C. Single transformed colonies were identified and picked into 19 x 384-well plates using robotics (Q-Bot, Genetix). Copies of the cDNA library were produced and stored at -80^{o}C.

Preparation of Filters for Digoxigenin (DIG) hybridisation

The Latham cDNA library was then "spotted" onto nylon filters (Genetix) using the Q-Bot and these filters were incubated at 37^{o}C overnight on LB agar with 100µgml^{-1} of ampicillin. The colony DNA was fixed to the filter by standard chemical treatments prior to being cross-linked by exposure to ultra violet light for 18 seconds.
The filters were transferred to preheated wash solution at 68^{o}C and washed for 1-3hours. The wash solution was changed twice during this time. The filters were placed on blotting paper and vigorously rubbed with a tissue soaked in wash solution to remove cell debris prior to storage in sealed plastic bags and frozen until required.

Probing/hybridisation of Filters with DIG labelled SSRs

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Digoxigenin (Dig) labelled DNA probes were used to detect target nucleic acids after hybridisation by enzyme linked immunoassay using an anti-body conjugate (anti-digoxygenin alkaline phosphatase). A subsequent enzyme catalysed colour reaction with 5, Bromo 4, chloro 3, indolyl phosphatase (BCIP) and nitroblue tetrazolium salt (NBT) produces an insoluble blue precipitate which allows hybridised molecules to be viewed. DIG labelled SSR probes (AC, AG, CTT, CCT, AGG, AAG, GCT, CAT) were synthesised by MWG Biotech (Munich). After the hybridization procedure the probed filters and NBT/BCIP buffered solution were sealed in a plastic bag. This was then placed at 37^{o}C in an incubator overnight for the reaction to proceed.

SSR identification, primer design and testing using the DIG hybridisation method

The positive hits identified by the DIG labelled probes were sequenced and assembled as described in the following section on sequencing of plasmids**.**

Primers flanking simple sequence repeats (SSR) regions were tested on the parents and ten randomly chosen progeny from the mapping population, for polymorphism using PCR. For each SSR positively identified as showing polymorphism, one primer of each primer pair was fluorescently end-labelled and used for genotyping the mapping population using the ABI 3730 automated sequences. Allele sizes were determined using GENESCAN software programme (Applied Biosystems) and GeneScan-350 (Tamra), as an internal size standard.

Sequencing of the cDNA Root Library

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The cDNA library was sequenced in order to discover further SSRs and ESTs of potential interest. The plasmids were prepared as follows prior to sequencing.

Preparation of Plasmids for Sequencing

From the 384 well plates aliquots (5µls) of bacterial culture were used to inoculate into 4x96 deep 1ml plates that contained 2x Luria-Bertani broth (20g/L tryptone, 10g/L yeast extract, 10g/L NaCl) and 100µgml^{-1} of ampicillin using the BIOMEK for culture transfer. These were the grown over night in an orbital incubator at 37^{o}C. The bacterial cells were harvested by centrifugation at 3000rpm for 5 minutes and plasmids prepared using the Multiscreen Plasmid Minipreparation system (Millipore).

Sequencing of plasmids

Plasmid DNA was sequenced using M13 reverse primer (5'-GGAAACAGCTATGACCATG-3') and Big Dye Terminator version 3.1 chemistry (Applied Biosystems), and analysed on ABI 3730 capillary sequencer. Sequences were quality scored using the PHRED (Ewing *et al*, 1998) base calling program. Vector and host contamination were identified and masked using a sequence comparison program Cross_match (Gordon *et al.*, 1998). Following vector trimming, which identifies the longest non-masked sequence, a further round of trimming removed low quality bases at both ends of a read where low quality is defined as those bases having a Phred score less than 13. The library file was then assembled using the contig assembly program CAP3 (Huang and Madan, 1999). The data generated for the library file was then searched against the non-redundant nucleotide databases at NCBI (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm (Altschul *et al*, 1990).

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SSR identification and testing

SSRs were identified using the SPUTNIK program and oligonucleotide primer pairs were then designed to the flanking sequences of the SSR regions using Primer 3 software (Rozen & Skaletsky 2000). Primer pairs were tested on their ability to amplify microsatellite loci on Latham, Glen Moy and ten selected progeny using PCR and initially analysed on 2% agarose. PCRs were performed on a PE Applied Biosystems 9700 thermocycler using 2 µl of 10ng DNA, 0.5 µM of both reverse and forward primers, 200 µM dNTPs, 2 µl 10x *Taq* buffer and 0.5 U *Taq* polymerse (Roche). The PCR programme used was as follows: 95^{o}C (5 min), 35 cycles of 94^{o}C (1min)/ 55^{o}C (1min)/ 72^{o}C (1min), followed by 72^{o}C for 8 minutes.

The samples were prepared after carrying out PCRs using a labelled primer with Hex or Fam for detection of SSR polymorphism of the successful primers with the use of the ABI3730 capillary sequencer. An aliquot of 1µl PCR was placed into a new 96 well plate and a mix containing 6.96 µl of Hi-Di formamide and 0.04 µl Rox added.

These were then analysed using Genemapper v3.7 (Applied Biosystems) software for allele determination.

Results

The Latham Root cDNA library consisted of 19 x 384 well plates, equalling 7296 potential EST clones. A proportion of the library was sequenced (table 3.1) and searched against the nucleotide databases at NCBI using the BLAST algorithm. The

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results demonstrated a degree of homology for other ESTs in the database, including many from the *Rosaceae* family. Examples are given in Table 3.2, where homology for genes of interest such as defensin protein 1 is evident. The remainder of the sequence data is provided in a database.

Table 3.1 Summary of sequencing results from the root EST library

Total No. ESTs Sequenced	4608
No. Sequences >200 bp	3415
No. Singletons	1438
No. Contigs	503
Total No. Sequences containing SSRs	153
Total No. SSRs >12 bp	335

SSR development from the Latham root cDNA library is given in tables 3.3 and 3.4. Table 3.3 lists the primers developed tested for polymorphism and for which mapping was attempted. Table 3.4 lists more recently developed SSRs which have been tested for polymorphism and are now available for mapping.

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Table 3.2. Examples of the homology of the Latham Root cDNA library when BLAST search carried out.

Rubus Latham Root cDNA library address	**BLAST search type**			**Database similarity**
ERubLR_SQ01_F_A06	**BLASTN**	4e-16	98/115 (85%)	gb|AY742294.1| Malus x domestica zinc finger protein-like mRNA, complete sequence
ERubLR_SQ01_F_A13	**BLASTN**	2e-19	67/72 (93%)	emb|X67957.1|AMUBIQMRP A.majus mRNA for ubiquitin (partial)
ERubLR_SQ01_F_A16	**DBEST**	e-106	390/449 (86%)	gb|CO817650.1| FA_SEa0014D07r Fragaria x ananassa 'Strawberry Festival' cultivar 24h after treatment with salicylic acid Fragaria x ananassa cDNA clone FA_SEa0014D07r, mRNA sequence.
ERubLR_SQ01_F_A18	**DBEST**	3e-56	171/190 (90%)	gb|BQ641018.1| EST754 almond cDNA library Prunus dulcis cDNA 5' similar to putative gamma tonoplast intrinsic protein (TIP), mRNA sequence.
ERubLR_SQ01_F_B02	**BLASTN**	7e-07	37/39 (94%)	gb|AY078426.1| Prunus persica defensin protein 1 (DFN1) mRNA, complete cds
ERubLR_SQ01_F_B05	**DBEST**	1e-05	34/36 (94%)	gb|CV051972.1| EST 11420 Half-Ripe Apricot Fruit Lambda Zap II Library Prunus armeniaca cDNA clone bge014l18q 5', mRNA sequence.
ERubLR_SQ01_F_B06	**DBEST**	7e-41	177/207 (85%)	gb|BI977827.1| mC12 Old Blush petal SMART library Rosa chinensis cDNA 5', mRNA sequence.
ERubLR_SQ01_F_B10	**BLASTN**	2e-30	101/111 (90%)	gb|AF317062.1|AF317062 Prunus persica abscisic stress ripening-like protein mRNA, complete cds

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Table 3.3. SSR development from Latham root cDNA

SSR name	Replicateeat	F Primers	R Primer	Expected size	Product size (bp) of alleles of Latham/ Glen Moy	Mapped
Root1BO6	(AGCG)4	CCTCTACACCACCCCATCAG	CGTCATCGTCATCTCTCTCG	200	190/198:198	Y
RootIFO9	(GCA)5-(CAAAA)2	GGCATACCCAAGACGTTCTC	GTCTTTGGTGGTGCTTGAGG	210	208/214:208	Y
Root1G16	(TC)8	GCACCCTAATCTCCATGACC	CCGCTGTAGTTCCTGTAGGC	206	198/200:200	Y
Root1I08	(GC)7	GCTTCAGGAAGCTCGATCAC	TCACCTAAGCACCTAATTAAGGAAG	202	198/200:200	Y
Root1I20	(TA)9	TCTTTTGCGGTGGCTACAAG	CAACCCGAAGTCTACAACAGC	221	217/221:221	Y
Root1M12	(TCA)4	AGGCAAGTAGACCTCACATCC	CCCAAGGAGCACAAGAGG	160	160: 152/160	N
Root1M20	(ATA)5	TTACGAACACCCATTAATTTAAGTC	AATCCTGAGACCGACGAGTG	235	234/242:234.242	Y
Root1N03	(AT)5	GATTCAAATCCAGTAGACCAGTACC	CATTGAGACCCACCTCTTGG	235	230/232:230	Y
Root1P18	(TG)7-(TAGC)3	CCACTTTATTTGATTTATTCCATCC	ACGGACAAAAGTGGGTATGC	197	198/202/202	Y
Root2B23	(AG)5	CGTACTGGGTTTTCTTCCTTG	GCTACTCCAGCAGCAAGCAG	158	217/267	N
Root2B19	(GC)7	CTCCGCAGACATTCCTTCTC	GCTTCAGGAAGCTCGATCAC	220	218/220:218	N
Root2E14	(CTAG)3	CTAACCTTGCCATGCAGCTC	CCAGTAGCAGCTAGAACAGCAC	231	227/231	N
Root2M13a	(AAG)4	CACAAGAGCTGGGGAGATTC	CCATGTTCCCAACTGATCG	134	134	N

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Table 3.4. SSR development from Latham root cDNA library

SSR name	Repeat motif	F Primers	R Primer	Expected product size (bp)	Polymorphic /monomorphic
ERubLRSQ05_2_B10_039	(AAAAG)2	TTGGACTTTGTGCTTAGGAG	AAATTCTTCAAAACGCAATC	179	m
ERubLRSQ05_3_E02_004	(GGT)4	GTCACACAAGGCTACCAAG	ATTGAACTGGTCAACAATGC	204	p
ERubLRSQ05_3_H01_001	(TA)5	CTATTGCAAGGATACCAAGC	GTTGCAACATGACAATTCC	182	p
ERubLRSQ05_4_E01_004	(GAA)5	GATGTCTCCACTACCCAAAG	TTGGACACTTGTACTGCTTG	209	p
ERubLRSQ05_4_E09_036 – ggc & gga - complex	(GGA)6-(GGC)5	TCAGCTCCCAACCTATTTAC	CTCCTCGCCTCTATCGTTAC	162	p
ERubLRSQ06_2_C11_046	(TGA)4	CACTCGACTGCCAAGAAC	GACTTAACCCTCAGTTGCTG	173	p
ERubLRSQ06_2_E01_004	(AT)6	GCAGGAGTTGGACGAGTAG	TTTCCAGATCAAACAAGACC	197	p
ERubLRSQ06_3_G01_002 agct	(AGCT)3	GATAGACACGGACAAAAGTG	CGATGAAGTAGTTTATTCGAG	150	m
ERubLRSQ07_1_E10_036	(GAA)6	GAGGAGAAGATTGTGAATCG	ACACACCTCCCAGACATAAC	249	p
ERubLRSQ07_1_F06_019	(TTTA)3	TTGATCCTAACAAGCCAATC	TTAACCATCAAGGGAAAATG	233	m
ERubLRSQ07_2_C08_030	(CTT)6	GACGAGAAGTTAAGGGTGTG	GATTCATCTTCCTCGTCTTC	218	m
ERubLRSQ	(GAAG)	GCTGGAAGACATA	GCCAAGTCCAAA	238	p

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SSR name	Repeat motif	F Primers	R Primer	Expected product size (bp)	Polymorphic /monomorphic
07_2_D07_029	3	GCCAAGC	CAATGTCC		
ERubLRSQ07_2_G02_002	(TTCT)3	GCTCAGCCAACCCAGTAATC	TGGATCGAATTACTTCCTTTCAT	234	m
ERubLRSQ07_2_H02_001	(CTAG)4	TGGCAATCAACCACTCTGTG	CAAACTGACAAACGCTCTTCC	238	p
ERubLRSQ07_3_C07_030	(ACC)4	ATGGCTTGTAGGTTTCACTC	CATTTGCTCAAACGATTATG	247	p
ERubLRSQ07_3_D06_021	(CCG)4	GAGGAGTACATGGCTCTCTG	CAGTTTGAATTTCGGTCTTG	235	m
ERubLRSQ07_3_F05_019 17	(CTAG)3	CTCGTACAAGAACTCGAACC	TTAAGCATTTCACGTACTCC	197	p
ERubLRSQ07_4_D05_021 tcta	(TCTA)3	AAGGATGGATCACTTTGTTG	CTCACAAGACACGTACAAGG	235	p
ERubLRSQ07_4_D05_021 agc	(AGC)7	CTTCTTTCCAACCGATTTC	ACGAATTGATTTCATCAACC	249	p
ERubLRSQ07_4_E09_036	(CTAG)4	CACTAGGTCGATCAAGAAGC	CTGCCATAGAAACAAACGAC	182	p
ERubLRSQ19_1_A05_024	(GAA)11	GTTTGCTTCCTTTCGTAGTC	TATACTAATGGCCACCTTGG	219	p
ERubLRSQ19_2_B12_047	(GAA)4	CAGTTCTACTCTTGGCAGTG	ACAGTCTTCTCGGACACATC	156	p
ERubLRSQ19_2_E04_012	(TG)5	TGTCCTGCTGTCTGTCCAAG	TGAAGACCATCATCGAATGC	161	m
ERubLRSQ19_3_C01_	(TGGC)3	TATCGAGGACTTGGAGAAGC	AACACTGCTACTAGGCAAGC	163	m

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SSR name	Repeat motif	F Primers	R Primer	Expected product size (bp)	Polymorphic /monomorphic
006					
ERubLRSQ19_3_C10_038	(ACC)4	TGACTAATGGCTTCAAAGATG	AGCCTTATTCAATGTGGAAG	178	m
ERubLRSQ19_3_G09_034	(GCTC)4	GTTCGTCATCGTCATCTCTC	AGAAAACCAAACCCCTCTAC	216	p

Discussion

From the Dig screening and sequencing 45 new gene tagged SSR markers were developed. These provide an excellent resource for map enhancement with possible utility in other *Rosaceae*. Other SSRs are available in the library for mapping. The generation of a root EST library also offers great opportunity for mapping a range of candidate genes for various traits of commercial value and an exploitation plan for utilising this resource is required.

Task 3.2 Map enhancement by the addition of a further 94 progeny

Introduction

To increase the ability to detect QTLs on the linkage map a further 94 progeny were added to the map.

Materials and methods

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Generation of AFLPs

Total genomic DNA from the parents (Glen Moy and Latham) and an additional 94 progeny not represented on the map was extracted using a 2% CTAB method (Graham et al 2003). The AFLP template was prepared with *Pst*I/*Mse*I and *Eco*RI/*Mse*I combinations according to AFLP Analysis Systems II (Life Technologies) using 100ng template DNA digested with *Eco*RI, *Pst*I and *Mse*I. Pre-amplification reactions were carried out using E00, P00 and M00 primers. Pre-amplification reactions were performed with 'core' primers E00, 5'GACTGCGTACCAATTC or P00 5' GACTGCGTACATCCAG and M00 5' GATGAGTCCTGAGTAA. Selective amplification was performed using primers with 2, 3 or 4 base extensions (*Pst*I- Preamp primer plus AC, AG, AT ; *EcoR*I-Preamp primer plus AGC, AGG ; and *MseI*-Preamp primer plus AAA, AGA, AGC, AGT, ATA, CAT, CGA, CGT, CTC, CTG, AAAA and ACGG) resulting in a total of 17 P*st*1/M*se*1 and 14 E*co*/M*se* primer combinations.

SSR mapping

The SSRs previously described (Graham et al., 2004; 2006) for map development, were used on the additional 94 progeny to enhance the *Rubus* map. PCR reactions were done on 20 ng DNA from 94 progeny and the two parents in 25 µl reaction with 2 µM of each primer, 200 µM of each nucleotide, 1.5 mM of $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Roche) per reaction, in a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for 25 cycles with denaturation at 94^{o}C for 45 s, annealing at 59^{o}C for 45 s and extension at 72^{o}C for 1 min, with a final extension step of 5 min at 72^{o}C. For mapping, the 5' primers were fluorescently labelled with HEX, FAM or TET and PCR products were prepared according to Macaulay et al. (2001) for analysis on the ABI

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Prism 377. Allele sizes were determined using GENESCAN software programme (Applied Biosystems) and GeneScan-350 (Tamra), as an internal size standard.

Results

All markers on the existing map (Table 3.5) and the new markers in table 3.3 above were applied to a further 94 individuals chosen at random. The two maps were merged by BioSS and good agreement of the marker locations was found across the map. Only linkage group 1 showed some discrepancy in marker positioning, and these data are being checked at present before a final order is agreed.

Table 3.5. Number of markers on enhanced map with additional 94 progeny

Marker type	**No. mapped**
EST-SSRs	17
SSRs	94
AFLPs	350
SNPs (candidate genes)	10

Discussion

An enhanced map with more markers and more progeny increases the likelihood of mapping both major and minor QTL for marker assisted selection and this is discussed further in objective 4. An enhanced map satisfies BioSS requirement for mapping QTL loci.

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Task 3.3 Assessment of cross transferability of primers from other Rosaceae

Introduction

In order to determine how easy data can be transferred across species in the *Rosaceae* a number of primer sequences were identified across the genus.

Materials and methods

Primers were designed from the general database publicly available on NCBI and GDR website (http://www.bioinfo.wsu.edu/gdr/). Table 3.6 summarises the primers designed to sequences from mapped ESTs in the first linkage group only on the general the Prunus linkage map. These primers were tested on Glen Moy and Latham.
An additional 12 pairs of primers were designed to SSRs of Prunus, Rosa and Rubus (Table 3.7) in the NCBI database, using Primer3. These sequences in the database were chosen as they had known regions of microsatellites.

Results

Table 3.6 List of primers designed to sequences from mapped ESTs in first linkage group of the Prunus linkage map in the peach database at GDR, Clemson and are on the NCBI database.

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EST Prunus (NCBI)	F Primers	R Primer			Product amplified
PP_LEa0009A14f	AACCATCAGGGTATGGCAAG	GCACCCTGCATAAATCTTCC	Arabidopsis thaliana	hypothetical protein	yes
PP_LEa0013K20f	ACAAAGTCTTCGGCCAGTTTG	CACTGTCCTGATCTGGTTG	Oryza sativa	putative rna binding protein	yes
PP_LEa0003M21f	ACCATTCAGGCGTGGATAAC	TGTTCAAGAAAGCGACTTGG	Oryza sativa (japonica cultivar-group)	putative mannan endo-1,4-beta-mannosidase	no
PP_LEa0003L17f	AGTAGCTGACCGGTTGATCG	ATAAGCACCGGCACTCATTC	Arabidopsis thaliana	unknown protein	yes
PP_LEa0026O13f	AGTCCTGTATGGTGGGCATC	ACAGGCGCAAAATGGATAAG	Arabidopsis thaliana	similar to mlo proteins from h. vulgare	yes
PP_LEa0010I06f	ATGCAAGCTCATGCATTGTC	TGCGAACGGATATTTGTGAG	Arabidopsis thaliana	prl1 protein	yes
PP_LEa0003A21f	CAAGCTTCTCTGGGATGGAC	TGGGATTCCGGATAAGTCAG	Glycine max	putative resistance protein	yes
PP_LEa0007M11f	CTCTTCGTCCAGCTCCAAAC	GGGAGCAGACGTAGAAATCG	Vitis vinifera	putative ripening-related p-450 enzyme	yes
PP_LEa0013C10f	GAATGGAAGGGCCTCCTTAG	AAATAGCCTGCACCGAACC	Arabidopsis thaliana	dna topoisomerase like- protein	yes
PP_LEa0025D04f	GAATGTTCGACGTGATGCTG	GGAACTCCAGCACTGAAAGC	Arabidopsis thaliana	ring finger - like protein	yes
PP_LEa0008B15f	GAGAGCAGTTCCGGCAATAG	ACTCCATCTCCAACCACAGC	unknown	unknown	yes
PP_LEa0003G23f	GAGATGCTTCACCCCTTACG	TCTGATGCTGAGACCCTTCC	Arabidopsis thaliana	expressed protein	yes
PP_LEa0007E22f	TCACATGATTGGAAGGCAAC	CATTCCAGGCAGGCTTAGAG	Danio rerio	makorin ring zinc-finger protein 1	yes
PP_LEa0013A14f	TGGAAGTTGCAGCATTCTTG	TCAGGGCAACGAAAAGTAGC	Arabidopsis thaliana	unknown protein	no

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PP_LEa0011H02f	TGTCAGATTGGTTTGGATGG	GCTGTACAGTCGCAGCAGTC	Arabidopsis thaliana	expressed protein	yes
PP_LEa0009C17f	TGTCATTGGCGATGTTCAAG	TACCAACAAAGGCCTCATCC	Arabidopsis thaliana	expressed protein	no

Table 3.7 Summary of primer pairs designed to SSRs of Prunus, Rosa and Rubus from the public NCBI database

Species	Sequence Name	F Primer	R Primer	Expected product size	Product in Moy & Latham
R. alceifolius	AF205117	GATGTGTGGGTGTGTATCTGC	CCTGGATATGTTTACCCTGACC	179	yes
	AF205115	TTTGAGGCCGAATATCAAGC	CAAGAGCATAGCAACTTTGTTCC	245	yes
	AF205116	ATGCAATAATTGGTATGCTTGG	GCAAAAGTGAAATGGTTCAGG	209	yes
	AF205118	GGAAGAAGAGAGGAGTATGAAAGC	CGACCGGACGTATATATTTTGC	398	yes
	AF261693	CGGCAATCTTTGCTTACTCC	TTGAAAGGCTTGAAGAACTCG	176	yes
	AF261694	GAGGGGCACTTTCGTCATAC	CGACTTTGAAAACCGACAGC	208	yes
	AF261695a	GTGTTGTTGATCCTCCCAGAG	ATCGCAAGAAACATGCAAGC	226	yes
	AF261695b	CAGAGATATCATTTGGTGTTTGG	ATCGCAAGAAACATGCAAGC	210	yes
	AF261696	CCTTAGTTTTTCCGGATTGG	AAGGCTTGAATGGAAAATTGG	235	yes
Rosa roxburghii	AY583611	ATTGAGGCTTCCAGCTAACG	CGTCAATTTGAGCATTGTTGC	243	no
Prunus persica	AY599223	AGGAACTCCACCAAACAAG	TTTTCCATGAGTTCCCAAGC	379	no
Fragaria spp *	RGA_s1&as1	GGTGGGGTTGGGAAGACAACG	CAACGCTAGTGGCAATCC	510	yes

Discussion

Information on the likely transferability of other sequences across the genus suggests good transferability across species. From the *Prunus* Linkage group 1, 13 out of the 16 primers tested generated a product in Glen Moy and Latham. From a further 12

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sequences randomly generated from the data base 10 generated a product. This generated real opportunities for exploiting the vast resources and knowledge available in peach.

Outputs from objective 3

1 New primers for map enhancement

2 Database of DNA sequences many of known function

3 Revised map with 2 x 94 progeny

4 Knowledge of transferability of *Rosaceae* sequences for comparative mapping and utilisation of data from other *Rosaceae*.

5 Transfer of knowledge of candidate genes and database resources into HLO170.

The outputs form Objective 3 fulfils and greatly exceeds those of Milestone 3 and 6; Objective 3.

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Objective 4

Data analysis and mapping of resistance loci; Analyse all data for mapping and identify QTL(s) and markers associated with root rot resistance.

Introduction

To determine the map location of any trait on a genetic linkage map, robust phenotypic data must be available from a population segregating for the trait of interest. This project aimed to identify map location(s) and then markers linked to root rot resistance to allow the development of a marker assisted breeding protocol for resistance to this serious raspberry fungal disease. This required a method, or methods for determining how resistant or susceptible each of the progeny was to root rot. Two methods were chosen, 1) a field based screen of the type used by plant breeders and 2) a glasshouse screen used by breeders and pathologists studying *Phytophthora fragariae* var *rubi*. Both systems generated data on all the progeny from the replicated field and glasshouse screenings.

No data were available at the outset of this project which provided any evidence of how results from disease screening in glasshouse pot tests correlate with field resistance. By carrying out screening of the replicated Glen Moy x Latham population under both conditions we hoped to determine the value of glasshouse pot tests in raspberry breeding and also to allow mapping of the best data available at the end of the screening phase of the project.

Materials and Methods

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Field and glasshouse trials were carried out as described in Objectives 1 and 2 and map enhancement described in objective 3.

All mapping was carried out by BioSS.

Results

Correlation of disease data from glasshouse and field experiments

The data for the glasshouse was analysed with the field data to determine if there was any correlation between the glasshouse trial, and the results taken from the field in 2005 and 2006 for disease resistance. There are highly significant correlations between 2005 and 2006 field data ($p<0.001$) with a correlation coefficient of 0.98 (fig 4.1). No significant correlations exist between glasshouse damage scores and field damage scores (correlation coefficient of 0.07 for 2005 vs. glasshouse and 0.1 for 2006 vs. glasshouse). Correlations exist between inoculated glasshouse root density and field trial root rot score for 2006 and between inoculated glasshouse root density score and damage score, however the significance is very low and the relationship is not very strong. The means of field damage scores in 2005 and 2006 are significantly different as are the mean of glasshouse damage scores ($p<0.001$).

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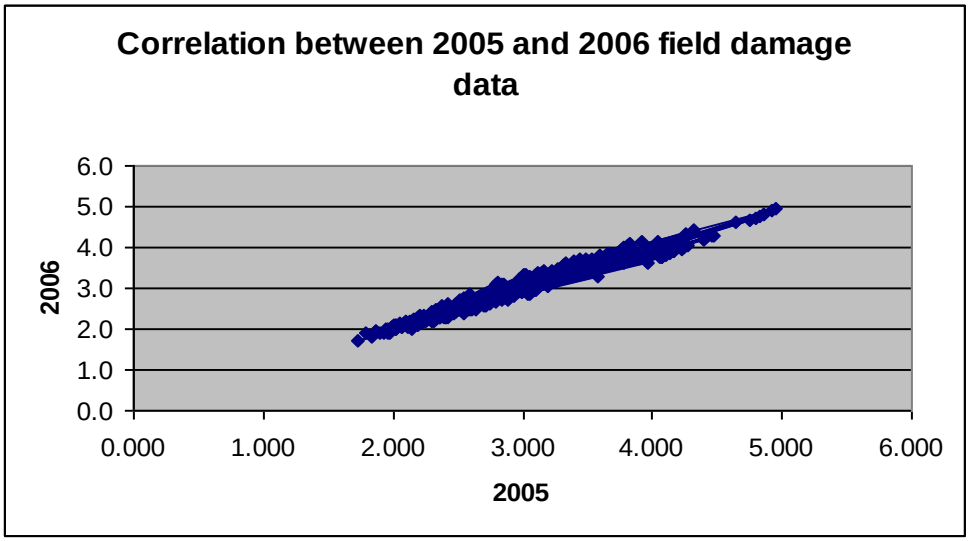

Figure 4.1. Correlation between field overall mean disease damage data from field trials in 2005 and 2006.

Table 4.2 Mean disease scores and standard deviation for Glen Moy, Latham and progeny from glasshouse and field results.

	Field 2005	**Field 2006**	**Glasshouse**
Latham	1	1 (0.35)	0.96 (0.2)
Glen Moy	4.62 (0.51)	4.62 (0.74)	3.65 (0.28)
Progeny Mean	2.5 (0.7)	2.9 (0.6)	2.42 (0.4)

Field data analysis

It was possible to map the data based on the overall progeny mean either across the scoring season or using the mean final disease score. If there were any differences in the data however for example with regard to disease pressure this may affect the accuracy of mapping. From the aerial photograph taken in 2005 (Fig 4.5), replicate 4 appeared more severely affected than the adjacent replicate, replicate 5 and particularly so in the top half of this replicate. It was therefore decided to further explore the data to

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determine any differences in the field trial. Data were explored for a replicate effect and then for a row effect. A Mann-Whitney U test was used to test for differences between the two replicates, replicate 4 and replicate 5. There is evidence to support a significant difference between the plots ($P<0.01$).

Table 4.3 Comparison of progeny means across replicates in 2005 and 2006

	2005 replicate 4	**2005 replicate 5**	**2006 replicate 4**	**2006 replicate 5**
Progeny mean	2.5	2.2	3.1	2.8

Histograms of the mean root rot scores for pairs of plants from the field trials for the two replicates in 2005 and 2006 were produced as in figures 4.2 and 4.3. These illustrate the difference between replicates and the data shows a peak of scores in the region of 5, particularly so in the 2005 trial for replicate 4.

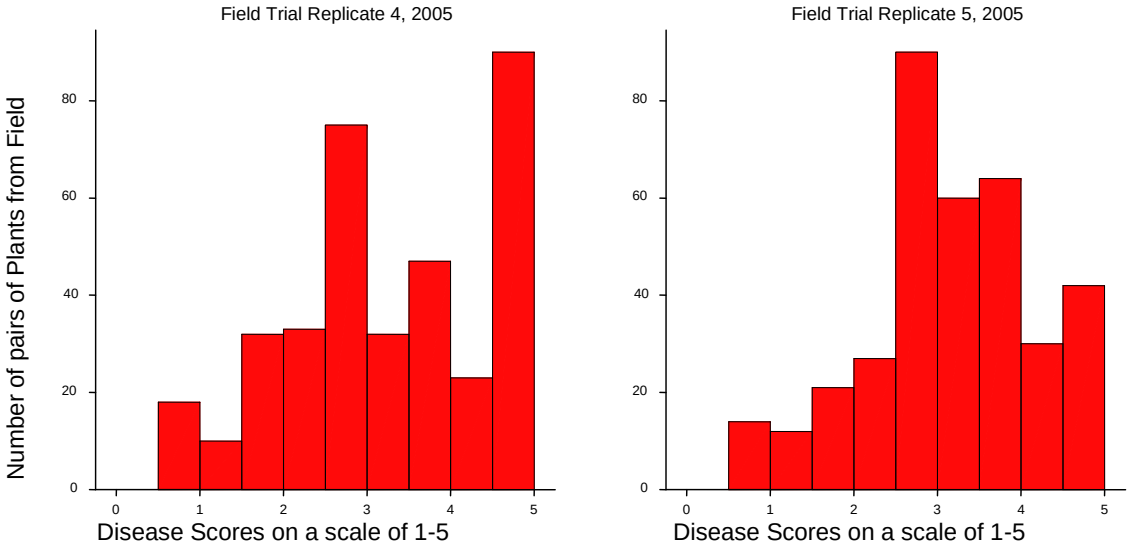

Figure 4.2. Comparison of disease score spread (1-5) (x-axis) in 2005 from replicate 4 and replicate 5.

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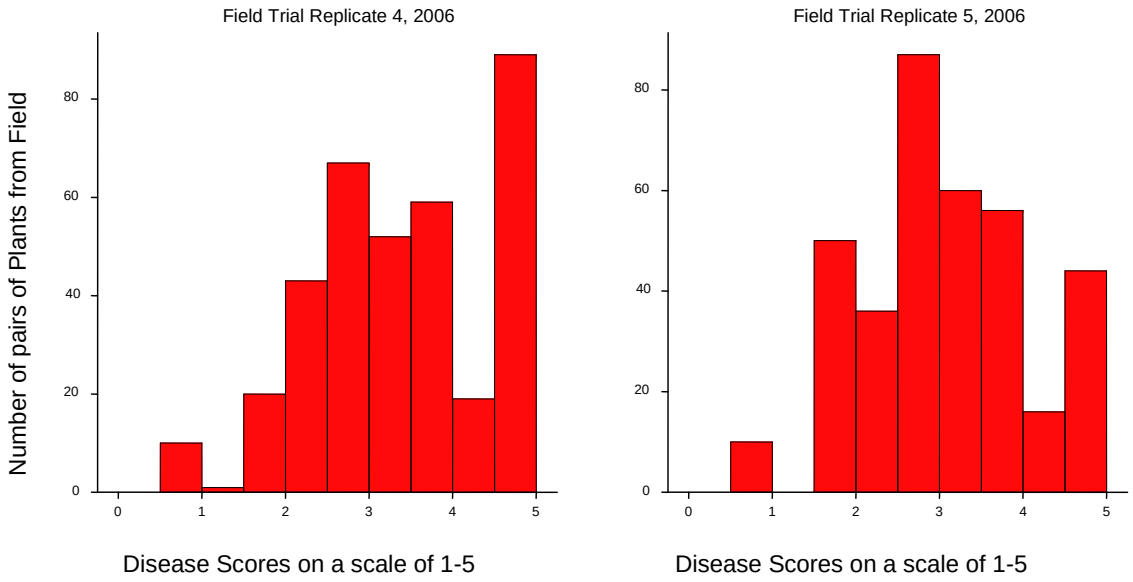

Figure 4.3. Comparison of disease score spread (1-5) (x-axis) in 2006 from replicate 4 and replicate 5.

The row effects could also be seen on the aerial photograph (fig 4.5), which again need to be taken into account for mapping purposes. Replicate 4 and then replicate 5 in both the 2005 and 2006 season are shown.

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Fig 4.5 Difference in disease pressure across replicates and rows can be seen from aerial photograph. Replicate 4 is on the right side and replicate 5 the left side. The slope of the field allows water to run into replicate 4.

The boxplot analysis was performed to look at row effect and disease load throughout the infected field as in figure 4.5. This is done by examining the mean root rot score for pairs of plants in each row in each replicate in 2005 and 2006. Due to the random design of the experiment the progeny are not planted in the same order in the field within the different replicates, so some care must be taken in interpreting the data across the replicates.

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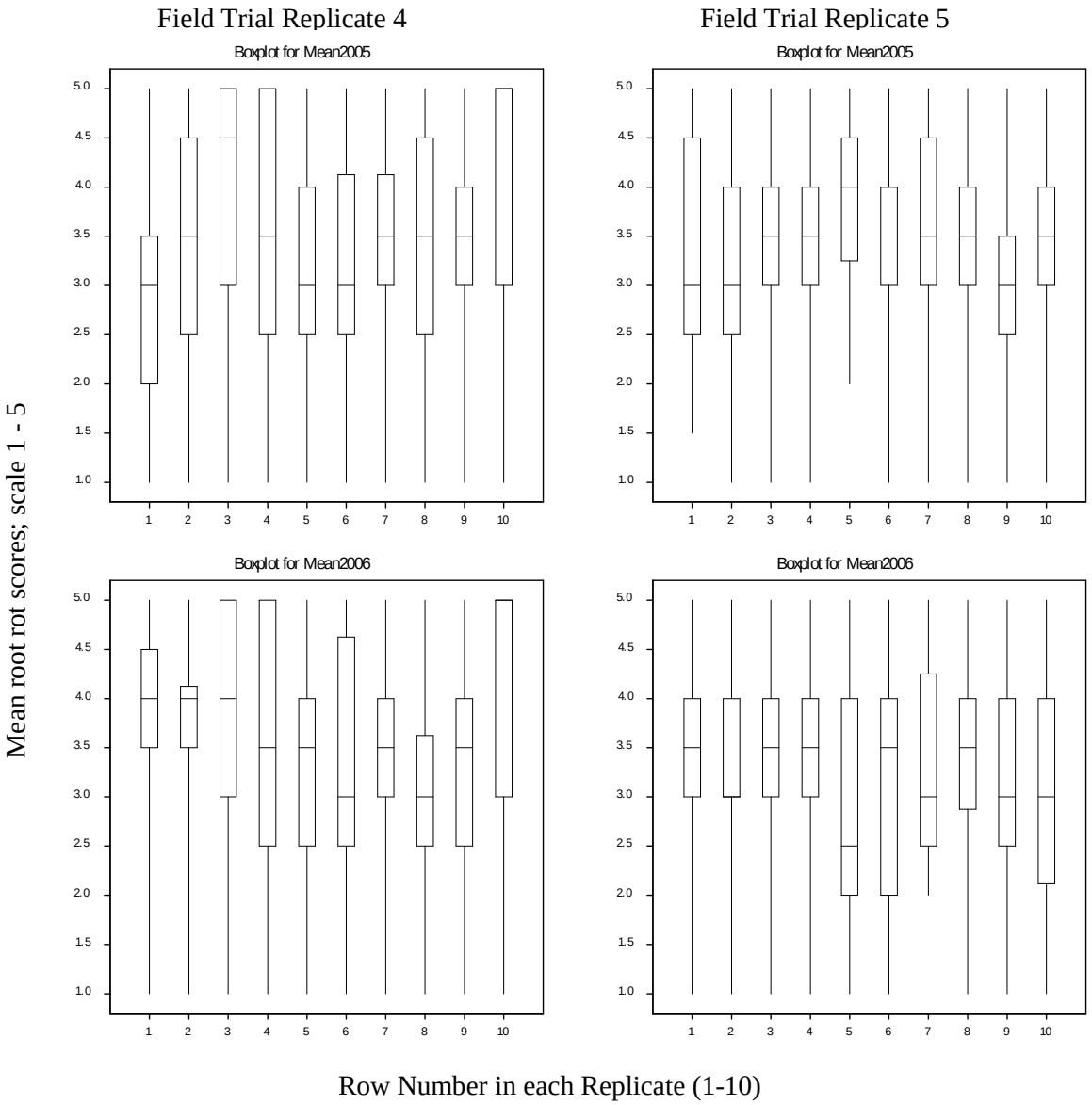

Figure 4.6. Boxplots of mean root rot score for pairs of plants in each replicate for 2005 & 2006.

The range of row means are from 2.986 to 3.539 (2006 replicate 5), 3.027 to 3.847 (2005 replicate 5), 2.932 to 4.212 (2005 replicate 4), and 3.257 to 4.192 (2006 replicate 4). The medians range from 3 to 5 for Replicate 4 in both years and 3 to 4 for Replicate 5 in 2005 and 2.5 to 3.5 for Replicate 5 in 2006. All rows contain plants with the full range of root rot scores.

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The effect of 'Row' is shown in the figure below. The average infection status of plants in each row is plotted for all data, for plants in Replicate 4 and plants in Replicate 5. The infection status of plants in Replicate 5 for low row numbers is higher than for high row numbers. Possibly, this is due to the low numbered rows being adjacent to the high numbered rows of Replicate 4 which has a higher overall infection level.

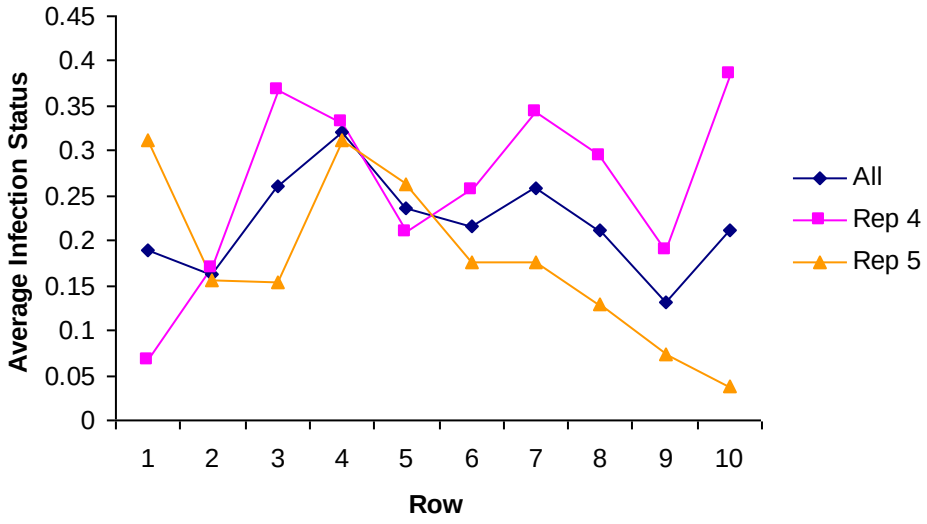

Figure 4.7 Average infection status of plants in each row (1-10)

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Data analysis and mapping Linkage Map construction and QTL analysis

Once all the data were collected and analysed, QTL analysis was carried out by BioSS using MapQTL. The Kosambi function was used to convert recombination units into genetic distance as has been already carried out for the present maps (Graham et al 2004, 2006), and a Kruskal-Wallis analysis was carried out to detect markers with significant linkage to the phenotypic data collected for disease resistance and susceptibility. Interval mapping was then conducted on the basis of the Kruskal-Wallis analysis after phase determination of the markers.

Using all the field root rot scores from 2005 and 2006, a similarity matrix were calculated for the field plots, using a city-block metric (i.e. scores of 1 and 2 are more similar than scores of 1 and 3).

Principal coordinates were calculated from the similarity matrix. The first principal coordinate explained 43.4% of the trait variation. The next four principal coordinates explained 6.7, 5.2, 4.3 and 3.3 of the trait variation respectively. Analysis of variance of these five principal coordinates, excluding the parental lines Glen Moy and Latham, showed that there were significant differences between the offspring for coordinates PCO1 and PCO3. Plots of PCO1 against spatial position in the field showed a strong spatial effect, especially in replicate 4. This could be modeled by fitting a linear effect of bed number.

Heritabilities were calculated after removing the spatial effect as h^2 = 33% for PCO1 and 20% for PCO3.

Analysis of variance was carried out on the root rot data at the infected site and the spawn density and diameter data (root parameters) from the uninfected and the infected site to identify genetic and environmental variance and it is shown that there is a major genetic effect with a very low environmental effect as in table 4.4.

Table 4.4 Genetic and environmental variances for root rot and spawn density and

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diameter

	Genetic variance	Environmental variance	Genetic x environmental variance
Root Rot	77%	9%	14%
Root parameters	75.6%	0.04%	24%

To obtain an estimate of trait scores for QTL mapping, the spatial effect was removed by fitting a model Replicate*Bed. The residuals were averaged to give a mean for each accession.

Mean of PCO1 for root rot after spatial adjustment for the full population

Mean for Latham (resistant) = -1.52

Mean for Glen Moy (susceptible) = 1.62

Analysis of variance was used to identify the markers most closely linked to PCO1 and PCO3 in the full mapping population. Markers were also tested to see whether there was evidence of a difference between the original and second populations of 94 lines. The markers listed here all show no significant differences between the two populations. The markers most closely linked to PCO1 are found on linkage groups 6 at an SSR marker Leaf 97 and 3 at an AFLP marker P14M60-131. There was no evidence of any interaction between these. Analysis of PCO3 also indicates the same region of linkage group 3.

Analysis of variance was also used to search for markers associated with differences in the mean glasshouse score. Again, the same region on linkage group 6 was identified. Linkage group 3 was also identified but in the case of glasshouse data the most significant addition from linkage group 3 is E41M31_153, which is well separated from P14M60_131 with a recombination frequency of 0.38.

Analysis of the first principal coordinate for the density and diameter scores demonstrated that for root density, both Leaf 97 (on Linkage Group 6) and E41M31_153

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(on Linkage Group 3) showed significant effects and for diameter some evidence for a weak effect of the loci on linkage group 6. However we note that while the ***ab*** genotype at Leaf 97 is associated with higher density and higher root rot resistance in field and glasshouse, the ***ab*** genotype at E41M31_153 is associated with higher glasshouse resistance but lower root density. It may be that analysis of other components of density will be helpful to understand this pattern.

Before correction of the field data, an area on linkage group I in the Moy parent P2– that has known susceptibility, at RUB243a was identified, but after correction for replicate and row effects and based on glasshouse data the QTL on linkage group 1 was no longer significant.

The trait data was mapped on the original map, on the map from the new 94 progeny developed in this project (Objective 3), and on the combined 2 x 94 map all of which identified the same QTLs.

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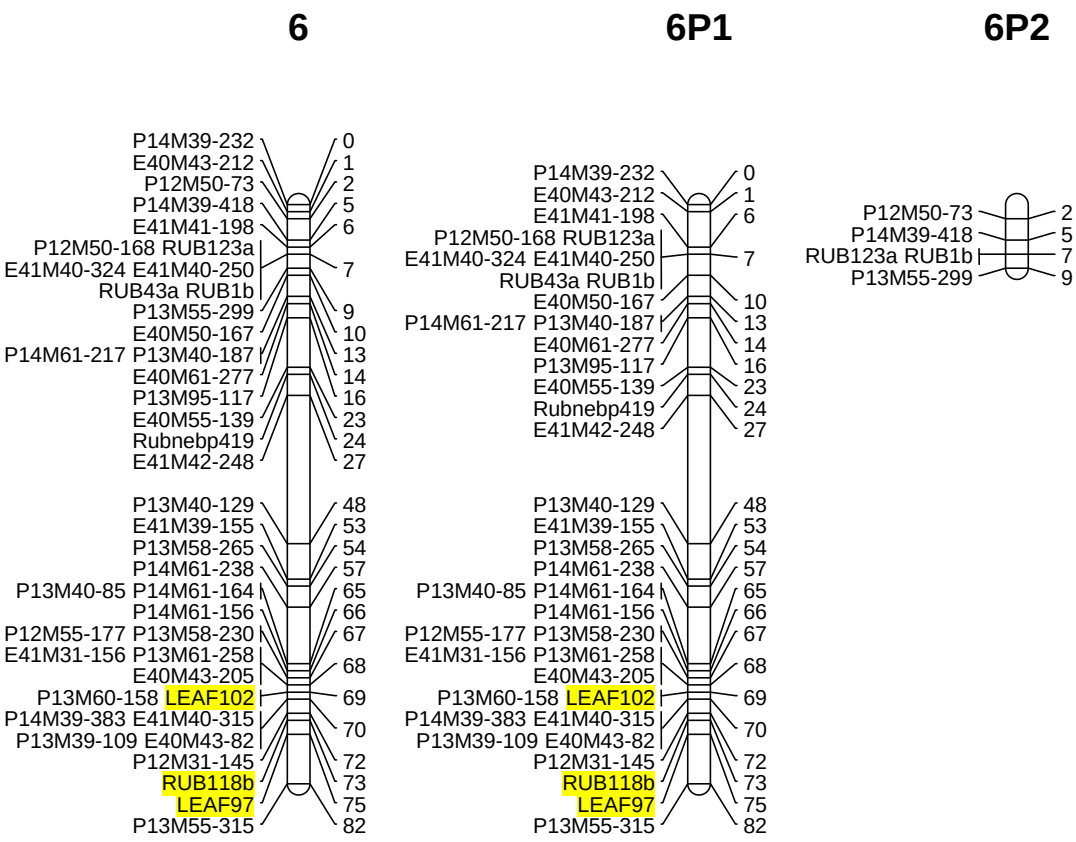

Figure 4.8: Linkage group 6 with the QTL located from 65cM along map as listed in table 4.5. Co-dominant markers within the QTL are highlighted.

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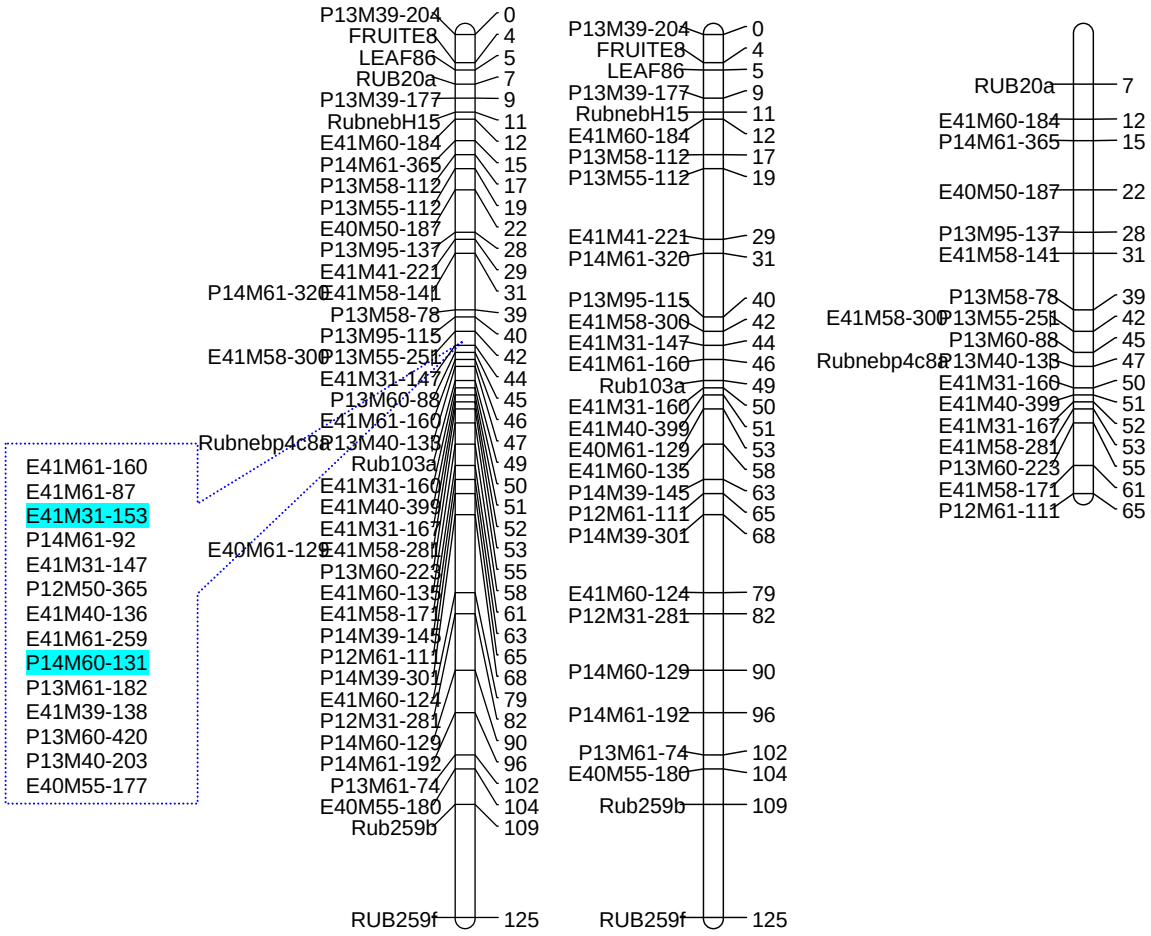

Figure 4.9 Linkage group 3 with markers around 42 cM significantly associated with trait.

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Table 4.5: QTL on linkage group 6

LG 6 cM	Marker locus	Marker type	Significance
65	P13M40-85	AFLP	0.0001
65	P14M61-164	AFLP	0.0001
66	P14M61-156	AFLP	0.0001
67	P12M55-177	AFLP	0.0001
67	P13M58-230	AFLP	0.0003
68	P13M61-258	AFLP	0.0001
68	E41M31-156	AFLP	0.0001
68	E40M43-205	AFLP	0.0002
69	P13M60-158	AFLP	0.0003
69	LEAF102	EST-SSR	0.0003
70	P14M39-383	AFLP	0.0008
70	P13M55-315	AFLP	0.0002
70	P13M39-109	AFLP	0.0001
70	E40M43-82	AFLP	0.0001
72	P12M31-145	AFLP	0.0005
73	RUB118b	SSR	0.0003
75	LEAF97	EST-SSR	0.0001
92	E41M40-315	AFLP	0.0004

The identified QTL from Linkage group 6 was examined across a range of the progeny where the resistance status was determined from the disease scores from the field and glasshouse. Care was taken in designating individuals as resistant or susceptible taking into account the distribution of disease scores identified in objectives 1 and 2 and that a score of 3 is not an appropriate cut off point. Fig 4.10 (in the appendix) demonstrates the pattern of alleles across the linkage group. There are a group of individuals (designated as intermediate and coloured green in Fig 4.10) where it is hard to determine from the field and glasshouse screening what their resistance status is. Based on the pattern of markers across the QTL their status has been determined. A final small group need to be confirmed as their status is unclear and the marker data may represent recombination or more likely poor assignment of allele status. Data on resistance and other plant characteristics have been compiled and the 20 most resistant progeny are listed in table 4.6 along with other plant characteristics.

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Table 4.6: Characteristics of 20 most root rot resistant selections

Progeny code	Resistance to Cane diseases	Spawn characteristics	Spines	Colour	Fruit Size	Firmness
R1	Resistant	vigorous	Dense	Dark red	Small	Crumbly
R7	Susceptible	vigorous	Dense	Mid red.	Large	Soft
R15	Susceptible	vigorous	Dense	Mid red	Med	Med
R16	Susceptible	vigorous	Dense	Mid red	Med	Med
R19	Susceptible	vigorous	Dense	Dark red	Large	Soft
R44	Susceptible	vigorous	Moderate	Dark red	Med	Soft, slightly crumbly
R66	Resistant	vigorous	Dense	Mid red	Med	Med
R85	Susceptible	moderate	Sparse	Dark red	Large	Soft
R94	Susceptible	vigorous	Dense	Mid red		Med
R96	Resistant	moderate	Moderate	Mid red	Large	Firm
R105	Susceptible	vigorous	Sparse	Dark red	Med	Firm, slightly crumbly
R118	Susceptible	vigorous	Moderate	Mid red	Med	Firm, slightly crumbly
R131	Resistant	vigorous	Dense	Mid red	Med	Med
R140	Susceptible	vigorous	Moderate	Mid red	Large	Soft
R141	Susceptible	vigorous	Sparse		Med	Med
R148	Susceptible	vigorous	Moderate	Mid red	Large	Firm
R149	Susceptible	vigorous	Sparse	Mid red	Large	Firm
R178	Resistant	vigorous	Moderate	Dark red	Med	Firm
R193	Susceptible	vigorous	Moderate	Pale red	Med	Soft slightly crumbly
R195	Resistant	vigorous	Moderate	Mid red	Med	Soft
R210	Resistant	vigorous	Dense	Mid red	Large	Soft slightly crumbly
R218	Susceptible	vigorous	Moderate	Mid red	Med	Med
R221	Resistant	vigorous	Moderate	Mid red	Med	Med
R261	Resistant	vigorous	Dense	Mid red	Med	Med

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Discussion

Mapping of the data generated in objectives 1 and 2 from field and glasshouse trials has identified two linkage groups and map regions to be significantly associated with the trait. A QTL on linkage group 6 and markers on linkage group 3 have been identified. PCO 1 explains over 43% of the variation and maps to linkage group 6 and linkage group 3 and PCO 3 explains 5% of the variation and maps to linkage group 3. Mapping was carried out on the original map, the new map generated in objective 3, and on the new combined map of 2 x 94 with the additional markers developed in objective 3. All identify the same QTLs. What is interesting and suggests this data is of real value, and worth having in the breeding programme, is the fact that although no correlation exists between field and glasshouse data, the map locations for disease scoring data on linkage group 6 are identical and linkage group 3 is also indicated as significant though with a different marker identified as the most significant. This suggests a gene(s) of real value in these map locations. Examination of a range of germplasm with known resistance across linkage group 6 was carried out as it has a number of co-dominant markers and the most significantly associated SSR marker Leaf 97, will help in determining the value of this region in the breeding programme.

The fact that root parameters and disease resistance map to the same QTLs, raises the question of whether Latham based resistance is based on actual resistance genes, or are the result of a morphological trait. It is very difficult to separate these parameters. If what is actually present in Latham is a tolerance due to root morphology, this may explain why the resistance in Latham has never broken down. Future work could be carried out to identify DNA sequences in the raspberry bacterial artificial chromosome library (BAC clones) containing markers in this region and to sequence these clones and look at gene content in this region.

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Outputs from objective 4

1. QTL significantly associated with root rot resistance on linkage group 6.
2. Markers associated with trait on linkage group 3.
3. Co-dominant markers from linkage group 6 significantly associated with the trait for marker assisted selection: Rub 118b, Leaf 102 and Leaf 97.
4. Other markers on linkage group 3 can be used in combination with those on linkage group 6
5. Co-localisation on the QTL for root rot resistance and root vigour traits.
6. Probes for BAC library screening to determine gene content along the QTL.
7. Table of most resistant accessions and other plant characteristics.

The outputs from Objective 4 fulfil Milestones 7; Objective 2 and Objective 6.

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Objective 5

Validate markers by assessing in a second population; and examination of allele status of markers identified as being associated with root rot resistance in a range of resistant and susceptible germplasm.

- Task 5.1 Mapping of resistance loci in another population from a second Glen Moy x Latham cross

- Task 5.2 Examine the diversity of alleles from the root rot QTL in a range of resistant and susceptible germplasm.

Introduction

In order to validate the linkage of markers to root rot resistance, two strategies were employed. The first strategy was to examine another small population of 100 individuals from a previous Glen Moy x Latham cross which had been grown in a second root rot infected site at SCRI, and examine the allele status of SSR markers from across the linkage map assuming no prior knowledge of where the root rot QTL(s) would map.

The second strategy was to bring together a range of germplasm which was reported to be either potentially resistant or susceptible to root rot, and on identification of a map location(s) linked to root rot, examine the allele status of identified markers across the QTL.

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Task 5.1 Examine mapping of resistance loci in another population from a second Glen Moy x Latham cross

Introduction

Determining the association of markers with a trait can be carried out using an approach known as bulk segregant analysis. This involved bulking DNA from all individuals that share a trait in common, in this case root rot. The theory is that by bulking DNA across different progeny that only have the particular trait of interest in common, they will vary for the status of other characteristics. To identify markers linked to the resistance locus bulks of DNA samples can be constructed from resistant, moderately resistant and susceptible genotypes based on field screening for resistance. These bulked DNA samples can be screened with the SSRs in order to develop a marker linked to root rot resistance (Yang et al 1997).

Materials and Methods

A previously developed Glen Moy x Latham population had been replicated by root propagation to provide three clones of each progeny, and planted in a randomised block design in a root rot infected site at SCRI. The status of each individual at the site was evaluated over 5 years. Field trials were scored across two seasons after 4 (2003) and 5 (2004) years in the field. Mean disease scores were determined for each of the 100 progeny from the field trial and each progeny was designated a resistance status depending on mean disease score (Table 5.1). Based on these scores, a bulk segregant analysis approach was used to screen for markers linked to disease status.
Young leaves were collected at the start of the field trial and DNA extracted using a 2%

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CTAB method (Graham et al. 2003). 200 ng DNA from 10 progeny from each category was bulked and SSR markers from the map were applied to the bulks. Six bulks were created two for each status. PCR reactions were done using co-dominant markers from across the linkage map (Table 5.2) on 20 ng DNA from the bulks in 25 µl reaction with 2 µM of each primer, 200 µM of each nucleotide, 1.5 mM of $MgCl_2$ and 0.5 U *Taq* DNA polymerase (Roche) per reaction, in a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for 25 cycles with denaturation at 94^{o}C for 45 s, annealing at 59^{o}C for 45 s and extension at 72^{o}C for 1 min, with a final extension step of 5 min at 72^{o}C. For mapping, the 5' primers were fluorescently labelled with HEX, FAM or TET and PCR products were preplicateared according to Macaulay et al. (2001) for analysis on the ABI Prism 377. Allele sizes were determined using GENESCAN software programme (Applied Biosystems) and GeneScan-350 (Tamra), as an internal size standard.

Table 5.1 Designated resistance status of progeny based on mean field disease score

Susceptible	Moderate	Resistant
3.6-5.0	2.6-3.5	1.0-2.5

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Table 5.2 Number of SSRs tested on bulks from across the linkage groups

Linkage group	Number SSRs
1	13
2	19
3	9
4	14
5	15
6	7
7	4

Results

Eighty one SSR markers were applied to the bulks, of which only two were identified as showing a pattern of allele distribution related to disease status (table 5.3). One of these alleles 118b is within the QTL identified in objective 4 and the other is also on the same linkage group.

Table 5.3: SSR markers showing a pattern of distribution across bulks related to root rot resistance.

	Resistant	Susceptible	Moderate	Latham	Glen Moy	Linkage Gp.
1b	211/231	205/237	205/237 211/205 211/231	211/237	205/231	6
118b	112/138	104/138	104/138 112/138	105/112	138	6

It should be noted that Leaf 97 and Leaf 102 being newer markers were not examined on the bulks but their association is currently being tested. The progeny designated as moderate clearly have haplotypes that occur both in the resistant or the susceptible categories.

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Discussion

The bulk segregant analysis approach identified two markers 118b and 1b out of the eighty one markers tested as significantly associated with the root rot trait. These markers both occur on linkage group 6, where the QTL has been identified. This second mapping population through a bulk segregant approach provides strong evidence to support the location of the QTL identified in objective 4. The results also emphasise the difficulty in assigning progeny to a resistance category, with the moderate group representing haplotypes from both resistant and susceptible bulks.

Task 5.2 Examine allele diversity in a range of resistant and susceptible germplasm

Introduction

By examining allele diversity across a QTL in a wider pool of germplasm, an idea of allele diversity can be determined. Association of allelic diversity with the trait of interest can also reveal other alleles at that locus of value in breeding.

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Materials and methods

Rubus leaf material was obtained from 76 accessions from SCRI, ADAS, British Columbia and EMR for allele identification. These were split into 66 susceptible and 10 resistant. DNA extraction of this was carried out as above and the DNA checked for quality on a 1.5% agarose gel. Genotyping was carried out on those accessions yielding high quality DNA as described above, using labelled primers for three co-dominant marker loci identified as being significantly associated with root rot resistance (Objective 4) Leaf 102, Rub118b and Leaf 97 (table 5.3). A selection of the Glen Moy x Latham progeny was also included in the validation to confirm initial allele identity and linkage with resistance status.

Table 5.3 QTL associated with root rot resistance

QTL associated with root rot resistance on Linkage group 6.		
LG6	marker	significance
65cM	P13M40-85	0.0001
65cM	P14M61-164	0.0001
66cM	P14M61-156	0.0001
67cM	P12M55-177	0.0001
67cM	P13M58-230	0.0003
68cM	P13M61-258	0.0001
68cM	E41M31-156	0.0001
68cM	E40M43-205	0.0002
69cM	P13M60-158	0.0003
69cM	LEAF102	0.0003
70cM	P14M39-383	0.0008
70cM	P13M55-315	0.0002
70cM	P13M39-109	0.0001
70cM	E40M43-82	0.0001
72cM	P12M31-145	0.0005
73cM	RUB118b	0.0003
75cM	LEAF97	0.0001
82cM	E41M40-315	0.0004

Results

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Leaf samples were sent from four different sources and of these, 40 yielded DNA of sufficient quality to use for examination of allele diversity across the identified QTL. Only four additional alleles were identified in the germplasm available for analysis (Table 5.5) and of these, three were from species accessions genetically distinct from the commercial raspberry gene pool. Within the additional material available for analysis only 6 were designated as resistant and 33 as susceptible. A further 20 accessions were available however the DNA quality was poor and only yielded preliminary results.

Latham clearly has three alleles distinct from Glen Moy and of these the 118b- 110 allele and the leaf 97- 217 allele have not been identified in susceptible germplasm. The leaf 102- 235 allele was found in 3 susceptible varieties.

Table 5.5 Haplotypes found across germplasm

	Leaf 97	**118b**	**Leaf 102**
Latham	204, 217	104,110	228,235
Glen Moy	204, 204	135, 135	228, 228
Across Accessions	204, 217, 217,217 204,204 **212,212**	104,110, 135,135 **80, 80** 110, 110 135, 135	228,235, 228, 228 235, 235 **223,**228 **240, 240**

Alleles out-with Glen Moy and Latham are in bold.

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Table 5.6 alleles at 3 marker loci across the QTL on linkage group 6

Linkage Group 6	69cM		73cM		75cM	
	leaf 102	leaf 102	118b	118b	leaf 97	leaf 97
Rubus coreanus	240	240	110	110	217	217
Cumberland	228	235	110	135	204	217
Autumn Bliss	228	235	110	135	217	217
Autumn Bliss (2)	228	235	110	135	204	217
R4A1	228	235	110	135	204	217
R1	228	235	110	135	204	217
R105	228	235	110	135	204	217
R105	228	235	110	135	204	217
R11	228	235	110	135	204	217
R110	228	235	110	135	204	217
R172	228	235	110	135	204	217
R118	228	235	110	135	204	217
R127	228	235	110	135	204	217
R148	228	235	110	135	204	217
R149	228	235	110	135	204	217
R168	228	235	110	135	204	217
R140	228	235	110	135	204	217
R181	228	235	110	135	204	217
R178	228	235	110	135	204	217
R184	228	235	110	135	204	217
R19	228	235	110	135	204	217
R193	228	235	110	135	204	217
R195	228	235	110	135	204	217
R210	228	235	110	135	204	217
R212	228	235	110	135	204	217
R228	228	235	110	135	204	217
R216	228	235	110	135	204	217
R237	228	235	110	135	204	217
R238	228	235	110	135	204	217
R279	228	235	110	135	204	217
R280	228	235	110	135	204	217
R43	228	235	110	135	204	217
Glen Clova	228	235	110	135	204	217
R. idaeus strigosus	228	235	104	110	204	217
Latham	228	235	104	110	204	217
EM6592/11	223	228	104	110	204	217
Preusen	228	235	104	104	204	217
Gaia	228	235	110	135	204	204
R. lasiostylus	235	235	110	110	204	204
Malling Jewel	235	235	110	110	204	204
Nagrada	235	235	104	104	204	204
Rubus niveus	235	235	80	80	212	212
Glen Prosen	228	235	104	104	204	204
8844l3	228	228	104	104	204	204

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Linkage Group 6	69cM		73cM		75cM	
Glen Coe	228	228	104	104	204	204
Medway	228	228	104	104	204	204
90-19-34	228	228	104	104	204	204
99111B-2	228	228	104	104	204	204
Woodborough	228	228	104	104	204	204
R2D7B-2	228	228	104	104	204	204
9759RD-1	228	228	104	104	204	204
9759RD-1	228	228	104	104	204	204
Woodside	228	228	104	104	204	204
Woodborough nursery samples	228	228	104	104	204	204
99111B2	228	228	104	104	204	204
Woodsia	228	228	104	104	204	204
Malahat	228	228	104	104	204	204
BC89-34-41(Saanich)	228	235	104	104	204	204
Nootka	223	235	104	104	204	204
Meeker	223	228	104	104	204	204
Coho	223	228	104	104	204	204
ESQUIMALT	223	228	104	104	204	204
EM6487/74	223	228	104	104	204	204
Cowichan	223	228	135	135	204	204
00123A-7	228	228	135	135	204	204
BC90-8-20	228	228	135	135	204	204
Baumforth B	228	228	135	135	204	204
Kitsilano	228	228	135	135	204	204
Burnetholm	228	228	135	135	204	204
Wei-Rula	228	228	104	135	204	204
9759RD-1	228	228	104	135	204	204
Autumn Cascade	228	228	104	135	204	204
8844L-3	228	228	104	135	204	204
R214	228	228	104	135	204	204
9759RD-1	228	228	104	135	204	204
R146	228	228	104	135	204	204
R167	228	228	104	135	204	204
R171	228	228	104	135	204	204
R182	228	228	104	135	204	204
R252	228	228	104	135	204	204
R258	228	228	104	135	204	204
R202	228	228	104	135	204	204
R235	228	228	104	135	204	204
R222	228	228	104	135	204	204
R40	228	228	104	135	204	204
R42	228	228	104	135	204	204
R9	228	228	104	135	204	204
R12	228	228	104	135	204	204
R14	228	228	104	135	204	204
R62	228	228	104	135	204	204

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Linkage Group 6	69cM		73cM		75cM	
R72	228	228	104	135	204	204
R89	228	228	104	135	204	204
R77	228	228	104	135	204	204
R45	228	228	104	135	204	204
R27	228	228	104	135	204	204
R201	228	228	104	135	204	204
R76	228	228	104	135	204	204
R126	228	228	104	135	204	204
Brown=susceptible						
Green=resistant						

Discussion

Although only a small number of samples were available for validation the results are encouraging for marker assisted selection. Alleles 110 and 217 were never found in the susceptible germplasm and allele 235 only occurs in two susceptible accessions. It would be ideal if further accessions which are clearly resistant can be tested for haplotype along the region.

The results also highlight the limited diversity within the raspberry breeding pool as well as the utility for molecular markers in fingerprinting germplasm. It can be seen from table 5.6 that the two accessions of Autumn Bliss are clearly different.

Outputs from objective 5

1 Confirmation of map location from a second Glen Moy x Latham population.

2 Identification of alleles which only occur in resistant germplasm.

The outputs from Objective 5 fulfil Milestones 8: Objective 5, Milestone 9; Objective 2, 4 and 5 and Milestone 10; Objective 4 and Milestone 11; Objective 7.

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General Discussion

Over the last three years data collected from the field experiments have led to the identification of an area on linkage group 6 and a region on linkage group 3 where genes involved in the plants ability to resist root rot are known. Interestingly and potentially of great value in breeding, a link has been established between the plants ability to produce root suckers (density and spread of roots overlap in a QTL location) and its ability to be resistant/susceptible to root rot. Markers across the identified QTL on LG 6 were examined in a range of germplasm to explore allele diversity.

Further saturation of the genetic linkage map with SSRs, EST-SSRs and AFLP markers, including an additional 94 progeny was carried out to enhance the map. The linkage map revised through this project together with the root EST library provides an excellent genetic framework for qualitative and quantitative trait analysis not only in Rubus but also within the Rosaceae.

The aim of having molecular markers for commercially important genes such as disease resistance is now a reality and will highly compliment the classical strategy of plant breeding by marker-assisted selection, reducing the time span in developing new and better varieties of raspberry. Such plant improvement has always relied upon the sole efforts of breeders in evaluating and selecting the right combination of alleles, which requires the manipulation of many genes just for a small improvement of a simple characteristic. However, with marker assisted selection complex traits can be simplified into component genetic units which will provide breeders with new breeding tools and to have a more efficient breeding programme.

The project has delivered on all the stated objectives and discussions have occurred

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with the MRS breeder and are taking place with BioSS on how these techniques are to be incorporated into the breeding programme.

A large volume of data has been collected on a variety of traits in raspberry, and all of this information can also be passed on to the breeder for germplasm selection.

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Exploitation Section

Background

Obtaining high yielding, high quality raspberry plants with fruit of market acceptability incorporating resistance to raspberry root rot (*Phytophthora fragariae* var. *rubi*) is a high priority for UK raspberry growers, breeders and researchers. Many raspberry growers are already unable to use land contaminated with root rot. Development of raspberry root rot resistant raspberry plants would enable growers to utilise their land fully for raspberry production and also not suffer massive plant, yield and profit loss. Dissemination of the results from this project which add to this objective needs to be done quickly and efficiently.

The markers developed in this project can be used in a marker assisted breeding programme to reduce the timescale required to obtain raspberry root rot resistant plants compared to that of conventional breeding programmes currently in use today. The consortium breeding programme based at SCRI, (Angus Soft Fruit, Berry World, British Summer Fruits, Hargreaves Plants, Highland Fruit Stocks, Horticultural Development Council, Kentish Garden, Meiosis, Mylnefield Research Services, SEERAD, Trade Solutions and Scottish Society for Crop Research) which has used traditional methods for screening for root rot resistance will now use the markers developed in this LINK project allowing the breeding of new raspberry varieties to be targeted, focused and deliver material of known root rot resistance status up to six years quicker. There will also be closer interaction between breeders and molecular biologists now that we have a diagnostic tool and the breeding programme will be altered by training the breeder to allow the inclusion of the laboratory procedures to screen for root rot resistant plants prior to the standard methods of phenotype selection in breeding. Root rot resistance is top priority for all breeders worldwide and is the main priority of this project.

This project has delivered resistant germplasm (from the original cross of the resistant root rot raspberry cultivar ‘Latham’ and the susceptible cultivar ‘Glen Moy’) , identified markers for marker assisted selection for root rot resistance in raspberry and further developed screening methods in both glasshouse and field environments for identifying resistant germplasm. Through detailed phenotyping assessments an association has

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also been demonstrated between root viability and resistance.

The partners that have been identified with an interest in the results of this project are raspberry (and rosaceous plant) researchers, breeders, producer organisations and growers. The interactive nature of the raspberry sector means that already there are consortiums which bring together all of the three mentioned partners which will be referred to as the 'industrial partners'.

Researchers

The area of Research & Development include prospective future research projects that will be undertaken to further advance and exploit the knowledge gained from this project.

Breeders

Timescale of breeding programmes will be greatly reduced by the inclusion of marker assisted breeding within programmes.

Growers

The results from this current work will be disseminated to UK raspberry growers via the HDC. This will ensure that the relevant information is transferred to the raspberry industry rapidly. However, this project will not deliver fully to the industry until there are advanced raspberry selections of suitable marketing quality with identified root rot resistance available for mass production.

Industrial Partners

The use of the markers for marker assisted breeding is a technological advance in raspberry breeding programmes and will fall under the Industry section and will be developed for the commercial advantage of the industrial partners involved in the consortium.

The Exploitation Plan

Growers

- The UK raspberry growers will be provided with a formal communication from

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HDC in the form of an article in HDC News in the next six months as regards to the identification and availability of markers for selection of root rot resistance material identified in this project.

- This formal communication (in bullet point above) will also cover the association between the root vigour of raspberries and root rot disease severity, identified in this project.

- The growers already have the access to material generated from the SCRI raspberry breeding programme through HDC and the Scottish Raspberry Breeding Consortium. The markers to screen for root rot resistance have been made available to raspberry breeders at SCRI and will be used to routinely screen crosses in the programme from 2007.

Breeders

The research carried out at SCRI has enabled the identification of root rot resistance markers for marker assisted breeding programmes for root rot resistance in raspberries and in addition identified plant material resistant to raspberry root rot that can be used as a genetic resource for the industry in future breeding programmes. This will lead to more rapid and efficient breeding programmes leading to resistant plants being available to growers.

- The marker information is confidential to the consortium and will be disseminated to SCRI raspberry breeders via MRS with the consent of HDC.

- Other interested UK industrial breeders can send their plant material to SCRI for testing to determine if their plants have the resistant trait or not, at full cost recovery. Non-UK breeders will have to wait until 2009 before they can send material to be screened using the genetic markers for root rot resistance.

- Germplasm, as parental material, can be made available to any other international raspberry breeding programme in exchange for other genetic resources or at a cost and this will be managed via MRS and SCRI.

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- The original root rot resistant plant material at SCRI will be incorporated in the ongoing raspberry breeding programme at SCRI with the purpose of producing root rot resistant progeny that are highly marketable within the fruit industry.

- Many other highly important plant trait data has been gathered during the course of this project and will be incorporated within the Scottish raspberry breeding programme.

- The application of markers and genetic resources will be made available to the raspberry breeding programme at East Malling via MRS/SCRI. MRS/SCRI will screen germplasm with the markers on behalf of EMR. East Malling will only pay for consumable costs associated with this service but not technology whereas other breeders will have to pay cost of service and IP cost.

- Future resistant varieties from the raspberry breeding programme at SCRI will be commercialised and used for further breeding programmes.

- Additionally other areas of rosaceous plant research can benefit from the knowledge gained from this project. Blackberry, strawberry and rose breeders can benefit greatly. For example roses also suffer from root diseases and they also have a diverse root suckering architecture, so they may be able to use the information on root vigour versus disease susceptibility in their breeding programmes. International blackberry and UK rose and strawberry breeders will be forward the relevant information.

Research & Development

Future research and development is also planned from the results of this project. Details of how it is planned to take the project results forward are detailed below.

- Experience gained in this project has been invaluable in supporting the new

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Horticultural LINK project HL0170 which sets out to identify markers useful in selecting fruit quality traits in strawberry.

- Future research plans are to develop the best combination of markers and then deploy marker assisted breeding (MAB) for root rot resistance. Discussions with BioSS, based at SCRI, on the best markers to take forward are proceeding. The closest marker on Linkage Group 6 is an EST based marker and requires no further development. Two other SSR based markers are also available on Linkage Group 6 without the need for further development. These can be used straight away. If Linkage Group 3 is to be included in a MAB approach, the AFLPs identified will need to be isolated and sequenced and a PCR based marker developed for ease of use. This will be carried out under SEERAD Work Package 1.3.2. in 2008.

- Further investigation of Linkage Group 6 will be carried out as detailed sequence information is available within this region which can be used to probe the BAC library and sequence the clones identified. Currently, the BAC library comprises over 15,000 clones with an average insert size of approximately 130 kb (6-7 genome equivalent). This will give information on the gene content and function of sequences in this region as well as providing information on the extent of Linkage Disequilibrium in raspberry. Resistance genes and genes for root development will be sought in this region. This will also be carried out under SEERAD Work Package 1.3.2 in 2008.

- Linkage group 3 is also proving very interesting with QTLs for a large number of traits locating here (J. Graham, preliminary data). This may represent regions with transcription factors which control a number of characteristics and further studies here would increase our knowledge of gene function and organisation in this region. QTLs for various traits span almost the complete linkage group and therefore the data on BAC screening across the QTL on Linkage Group 6 will assist in the development of a strategy for analysis of Linkage Group 3. Other traits mapping to this region include those of interest to HortLINK project HL0170 and this will be exploited here.

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- Work on comparative mapping with *Prunus* will continue. The initial results on transferability of a group of markers from *Prunus* Linkage Group 1 were encouraging and efforts to map these will be carried out and funding will be sought from Marie Curie or the Leverhulme Trust to continue work in this area. Applications to these two funding sources will be written in 2008/2009.

- It is also known that root runners in strawberries are due to a major genetic effect and as they are diploid the mapping information gained from this project maybe be transferable and used to increase the knowledge within strawberries. It would appear that root architecture research maybe of some value in root disease susceptibility and is an area for further development. There is a possibility for collaboration in this area of research within the Rosaceae community.

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Publications and conference proceedings resulting from the project – Technology Transfer

Presentations related to HL0169

Work presented at Fruit for the Future, SCRI, July 15th 2004
Work presented at Fruit for the Future, SCRI, July 2005
Work presented at Fruit for the Future, SCRI, July 2006
SSCR annual meeting 2005
SCRI open day 2005

Conferences

Rubus and Ribes Conference, Chile (December 2005)
Horticulture LINK conference (Feb 2006)
Rosaceae genomics conference, New Zealand (March 2006)
Genome Dynamics Seminar May 2006-11-21
Highlands & Islands Growers Conference November 2006-11-21
Visitor from HortResearch, New Zealand November 2006

Written information

Link leaflet; “New genetic techniques to produce root rot resistant raspberry varieties”
Article - HDC news.
Ashford Soft Fruit Conference (November 2005)
Article published in Grower 2006

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Objectives / Milestones

1. Milestones April 2004 – March 2005

Milestone	Objectives	Duration	Proposed Date	Actual Date
1	**Glasshouse Screening**			**Complete /Ongoing**
1.1	Material from the mapping population will be propagated under the appropriate conditions and to the optimal stage for glasshouse screening		June 2005	Completed
1.2	Fungal inoculum will be preplicateared for the glasshouse screen	5 weeks for bulking of cultures	October 2005	Completed
1.3	First glasshouse screen will be established in the Autumn by inoculating plants under the appropriate conditions with fungal plugs	12 weeks	November 2004	Completed
1.4	Data on plant health, growth, root mass and disease symptoms will be collected	6 weeks	January 2005	Completed
1.5	Data will be entered into Excel and analysed using Genstat 6, for significant differences in all plant growth factors		March 2005	Completed
2	**Field Screening**			
2.1	Field infestation plots will be maintained	All year round	All year round	Completed
2.2	Data on plant health, growth, viability etc. will be collected on a daily-weekly basis during and at the end of the growing season	12 weeks	June – September 2005	Completed

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Milestone	Objectives	Duration	Proposed Date	Actual Date
2.3	Data will be entered into Excel and analysed using Genstat 6			Completed
3	**Enhance map with co-dominant markers**			
3.1	Screen and sequence from genomic libraries	12 months		Completed
3.2	Sequence from cDNA libraries	12 months		Completed
3.3	Design and test primers to any SSRs identified on mapping parents	12 months		Completed
3.4	Examine new SSRs on mapping population	12 months		Completed
3.5	Place new co-dominant markers on the linkage map using Joinmap	12 months		Completed

Milestones April 2005 – March 2006

Milestones	Objectives Year 2		Target Date	Status
4	**Objective 1**	**Glasshouse Screening**		
4.1		Material from the mapping population will be propagated under the appropriate conditions and to the optimal stage for glasshouse screening	March 2006	Completed
4.2		Fungal inoculum will be preplicateared for the glasshouse screen	March 2006	Completed
4.3		First glasshouse screen will be established in the Autumn by inoculating plants under the	March 2006	Completed

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Milestones	Objectives Year 2		Target Date	Status
		appropriate conditions with fungal plugs		
4.4		Data on plant health, growth, root mass and disease symptoms will be collected	March 2006	Completed
4.5		Data will be entered into Excel and analysed using Genstat 6, for significant differences in all plant growth factors	March 2006	Completed
5	**Objective 1**	**Field Screening**		
5.1		Field infestation plots will be maintained	March 2006	Completed
5.2		Data on plant health, growth, viability etc. will be collected on a daily-weekly basis during and at the end of the growing season	March 2006	Completed
5.3		Data will be entered into Excel and analysed using Genstat 6	March 2006	Completed
6	**Objective 3**	**Enhance map with co-dominant markers**		
6.1		Screen and sequence from genomic libraries	March 2006	Completed
6.2		Sequence from cDNA libraries	March 2006	Completed
6.3		Design and test primers to any SSRs identified on mapping parents	March 2006	Completed
6.4		Examine new SSRs on mapping population	March 2006	Completed
6.5		Place new co-dominant markers on the linkage map using Joinmap	March 2006	Completed
7	**Objective 2**	**Place phenotypic data collected**		

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Milestones	Objectives Year 2		Target Date	Status
		in Milestones 1, 2 ,4 and 5 onto existing map.		
7.1		Material from the two years glasshouse and field trials will be correlated.	March 2006	Completed
7.2		Data will be mapped using Joinmap and MapQTL	March 2006	Completed
7.3		Identify markers with close linkages to resistant phenotype	March 2006	Completed

Milestones April 2006 – March 2007

Milestones	Objectives Year 3			
8	**Objective 1**	**Glasshouse Screening**		
8.1		Propagate material for glasshouse trial.	March 2006	Completed
8.2		Fungal inoculum will be prepared for the glasshouse screen	March 2006	Completed
8.3		Final glasshouse screen will be established in the Spring by inoculating plants under the appropriate conditions with fungal plugs. This will complete the screening of the entire population as well as a second screen of the mapping population.	May 2006	Completed
9	**Objective 2**	**Field Screening**		
9.1		Data on plant health, growth, viability etc. will be collected on a	Sept 2006	Completed

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Milestones	Objectives Year 3			
		daily-weekly basis during and at the end of the growing season		
	Objective 3	**Data Analyses and mapping**		
10.1		Analyse and map phenotypic data from glasshouse experiments from 2004, 2005 and 2006.	Sept 2006	Completed
10.2		Analyse and map phenotypic data from field experiments from 2004, 2005 and 2006 and correlate with glasshouse data.	Jan 2007	Completed
10.3		Identify diagnostic marker(s)	March 2007	
11	**Objective 4**	**Establish relevant collections of material for allele identification**		
11.1		Obtain material from SCRI, MRS and EMR	Sept 2006	Completed
11.2		DNA extraction from material	Feb 2007	Ongoing
11.3		Identify allele status of markers linked to resistance.	Feb 2007	Ongoing
12	**Objective 5**	**Enhance Map with further 94 individuals**		
12.1		Complete SSRs on linkage group I and VI on new 94 individuals	May 2006	Completed
12.2		Complete AFLPs on linkage group I and VI on new 94 individuals	July 2006	Completed
12.3		Complete mapping of EST-SSRs	July 2006	Completed
12.4		Map outstanding SSR data	Dec 2006	Complete
12.5		Sequence root ESTs	Dec 2006	Complete
13	**Objective 6**	**Validate marker in other populations**		

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Milestones	Objectives Year 3			
13.1		Examine marker locations in other germplasm	March 2007	Complete
13.2		Exploitation		Ongoing

	Secondary Milestones			
	Maintain mapping population in appropriate clean field plots, as glasshouse plants and in vitro			Completed
	Collect data from mapping population on other more commercial characteristics, as well as general plant data, e.g. Spawn density, cane size, phenological traits, pest and disease resistance (apart from root rot)			Completed
	Presentations to industry etc.			Completed

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