Mapping Spot Blotch & Common Root Rot (Causal Agent: *Bipolaris sorokiniana*) Resistance Genes in Barley

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Abstract

The fungal pathogen *Bipolaris sorokiniana* (teleomorph *Cochliobolus sativus*) causes the foliar disease spot blotch (SB) and the root disease common root rot (CRR). Spot blotch and CRR are serious disease constraints to barley production in warmer growing regions of the world, with estimated yield losses ranging from 30-70% from SB and 15-30% for CRR. Although chemical treatments may assist in controlling spot blotch infections, the most effective and environmentally sound means of control for each disease is breeding for varieties with natural resistance. In Australia, no commercially available varieties offer resistance to either SB or CRR. This study has sought to establish molecular markers that will be useful for selecting for resistance to each of these important fungal diseases.

Barley cultivars derived from the breeding line NDB112 have provided durable SB resistance in the North Dakota region of the USA for over 40 years. The robustness of this resistance had not been determined under Australian environmental conditions or with those *B. sorokiniana* pathotypes present within Australia. To elucidate the genetics of resistance, two seedling and two field trials were conducted on an ND11231-12/VB9524 (ND/VB) doubled haploid (DH) population (180 lines). A molecular map of the ND/VB population was curated in order to provide a firm basis for mapping of resistance loci. Composite interval mapping revealed that different gene combinations are effective at different stages of plant development. Seedling resistance was found to be conditioned by a major locus on the short arm of chromosome 7H and this region was validated in the related population ND11231-11/WI2875*17. A minor quantitative locus on chromosome 5HS was detected in one of the two seedling trials. However, this region requires further investigation to confirm its association to SB resistance in this population. Field resistance to SB in adult plants was found to be associated with two major quantitative trait loci (QTL) on chromosomes 7HS and 3HS; and a putative third minor QTL on chromosome 2HS. The 7H region is common between seedling and field resistance and is the most important locus for the expression of resistance at both stages of plant

development. These findings largely concur with genetic studies of this trait in two-rowed barley germplasm in North American environments.

Common root rot is a difficult disease to phenotype for, and breeding programs will benefit from the identification of molecular markers linked to resistance. Data was provided from field trials of subsets of the population over four years. Using a novel approach combining the efficiency of bulked-segregant analysis with high-throughput Diversity Arrays Technology markers (BSA-DArT), CRR resistance was found to be conditioned by three putative QTL in an unmapped Delta/Lindwall population. QTL were identified on chromosomes 2HS, 4HS, and 7HS. To validate the trait-linkage associations between the DArT markers and the CRR QTL, microsatellite (SSR) markers known to map to the regions identified by BSA-DArT were used. The 2H and 4H regions were validated using marker regression of the SSR markers in most seedling trials, whereas the 7H QTL, which is proximal to the location of the SB resistance QTL in the ND/VB population, was detected in only one seedling trial.

The QTL identified in this study offer potential to combat the foliar and root diseases causes by this fungal pathogen. The chromosomal location of QTL for SB and CRR resistance have been found to differ in the ND/VB and D/L populations, which suggests that resistance to each disease is independently inherited. Further research is required to confirm the hypothesis that it is possible to combine resistance to both diseases into a single genotype. Such allelic combinations would provide elite germplasm that would benefit barley breeding programs world-wide.

Certification of Dissertation

I certify that the ideas, experimental work, results, analyses, and conclusions reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original and has not been previously submitted for any other award, except where otherwise acknowledged.

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Signature of Candidate Date

ENDORSEMENT

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Signature of Principal Supervisor Date

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1. Introduction and Literature Review

1.1 Barley: History, Origin and Breeding

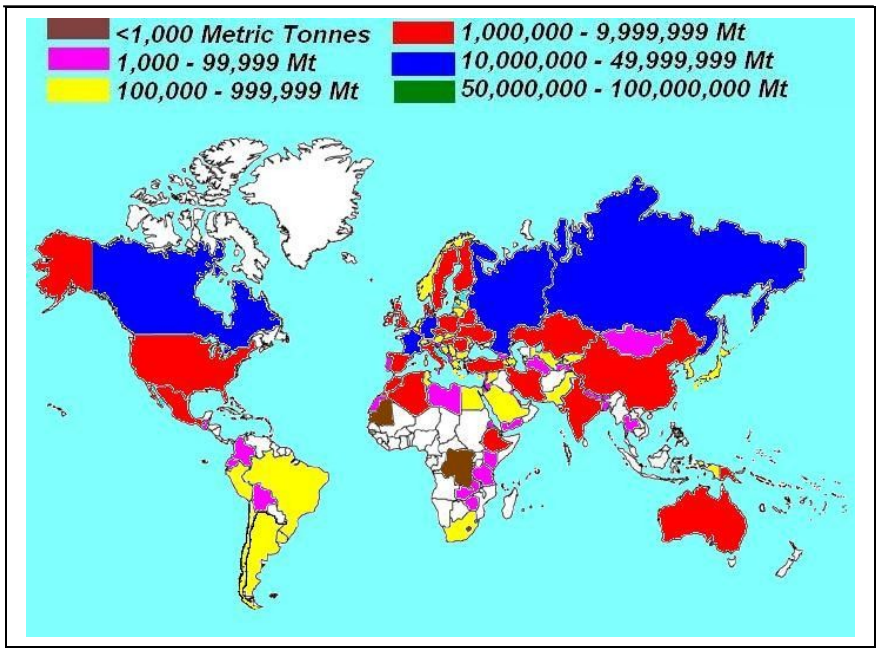
Barley (*Hordeum vulgare* L.) is the world's fourth most important cereal crop after wheat, maize, and rice, and is grown predominantly for animal feed and malt for the brewing industry. Barley is high in carbohydrates, with moderate amounts of protein, lipids, calcium and phosphorus, and small amounts of the B vitamins (Ullrich, 2002). This nutritional balance makes barley ideal for stock fodder. Barley is also used in the production of fermented beverages. Germinating barley seeds produce two enzymes, alpha-amylase and beta-amylase, which hydrolyze starch to dextrins and fermentable sugars during the commercial malting process. This sugar provides the basic ingredient for the production of beer and other alcoholic beverages (Schwarz and Horsley, 1995).

The geographical origin of barley has been the subject of much debate; however the general consensus appears to be the Nile Valley (Badr et al., 2000). Barley was one of the earliest crops domesticated and is considered one of the foundations of modern agriculture. Archeological remains suggest that barley was first domesticated in the Fertile Crescent around 10,000 years ago, along with wheat (Langridge and Barr, 2003). Barley is generally considered to be an inferior staple when compared to wheat, due to its lower level of gluten (historically referred to as poor man's bread). However, barley is the hardier of the two species, which has contributed to its continued cultivation throughout history (Zohary and Hopf, 1988).

Barley is grown over a broader environmental range than any other cereal and much of the world's barley is produced in regions with climates unfavorable for the production of other major cereals (Figure 1; Langridge and Barr, 2003). It requires less water than many other cereal crops and as a result much of the world's production is in sub-humid or semi-arid regions (Badr et al., 2000). It is also noted for its tolerance to cold. Barley is grown in Alaska, Finland, and Norway, and is the

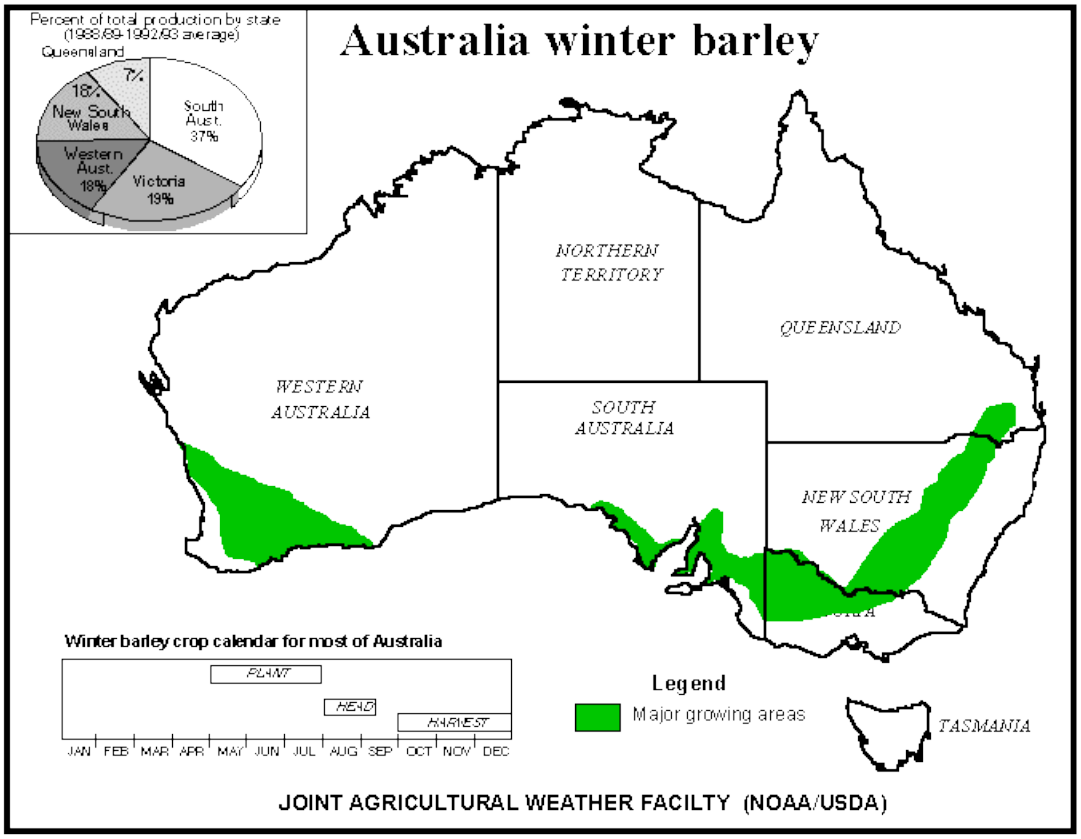
only grain that survives at these northern (above 640°) latitudes. In Tibet and Ethiopia, it is grown higher on the mountain slopes than other grains. Its wide distribution is a result of the wide genetic variation within the crop, with particular varieties adapted to specific environments (Frischbeck, 2002).

Figure 1-1. Worldwide distribution of barley showing levels of production in different regions (map taken from Gramene, 2005).



Barley is the second most widely grown cereal in Australia, occupying approximately 4 million hectares (Figure 2). Similarly, barley is the second most valuable grain crop in Australia, generating more than $900 million in revenue per year (Australian Bureau of Statistics, 2005). Australia produces a high quality 2-row spring-type barley and is the world's third largest exporter. Australia provides 32% of the world's malting barley trade and 20% of the world's feed barley trade (Barley Australia, 2005). Annual barley production in Australia is approximately 700,000 tonnes per year, of which 200,000 tonnes is consumed domestically (predominantly in the brewery industry), while the majority is exported to Asia, the Middle East, South America and Europe (Australian Bureau of Statistics, 2005).

Figure 1-2. Distribution map of major barley growing regions in Australia (map taken from Barley Australia, 2005).



Barley is a member of the Triticeae group within the family Graminaceae, and thus shares a common ancestry with rice, rye, wheat and oats (Wise, 2000). The genus *Hordeum* comprises 32 species, of which *H. vulgare* is the only one to have undergone domestication. It is derived from the wild progenitor *Hordeum spontaneum*, which continues to grow wild in the Middle East. Barley can be classified by the number of kernel rows in the head. Three forms have been cultivated; two-row barley (traditionally known as *Hordeum distichum*), four-row barley (*Hordeum tetrastichum*) and six-row barley (*Hordeum vulgare*; Badr et al., 2000).

Domesticated barley (*Hordeum vulgare)* is a true diploid with a haploid chromosome number of seven. Its genome has been estimated to consist of approximately $5.0x10^9$ base pairs (Wise, 2000). Among the wild *Hordeum* sp., there are diploid, tetraploid, and hexaploid species (Ellis, 2002).

1.2 Major Limitations on Crop Production

The worldwide production of barley has increased over the last two decades, due to advances in conventional plant breeding techniques resulting in the production of new, higher yielding and better adapted cultivars, and to improved agricultural practices. Despite this, many environmental factors continue to threaten barley production and challenge growers. Abiotic factors such as drought, excess rainfall, wind and temperature extremes significantly impact on grain yield and quality. In addition, biotic factors including weeds, pests and diseases, also contribute to a significant decrease in yield.

Diseases caused by fungal pathogens can significantly impact barley production by reducing final yield and lowering grain quality. In Australia the main foliar and root diseases affecting barley include net-from net blotch, spot form net blotch, spot blotch, powdery mildew, leaf rust, stem rust, crown rot, scald and common root rot (Vock, 1978). This review will focus specifically on the impact of two diseases, namely spot blotch and common root rot, on commercial barley production, resulting from infection with the fungal pathogen *Bipolaris sorokiniana* (Sacc. In Sorok) Shoem. (teleomorph: *Cochliobolus sativus* (Ito and Kurib. Drechsl. Ex Dastur)).

1.3 Spot Blotch and Common Root Rot

Spot blotch and common root rot are two of the most important fungal diseases adversely affecting barley yield and quality, and they constitute an increasing threat

to intensive crop growing systems worldwide (Kumar et al., 2002). Yield reductions due to spot blotch can range from 15% to 40% and result from a reduction in the number of heads, reduced seed size and fewer seeds per head (Steffenson, 1997). This disease has been particularly damaging in the upper midwest of the USA and the central provinces in Canada where yield losses in barley of up to 30% have been recorded (Steffenson, 1997). In Australia, spot blotch predominantly occurs in temperate northern New South Wales (NSW) and Queensland (QLD), where localized yield losses of up to 70% have been reported (G. Platz, personal .communication.).

Common root rot occurs throughout the world and has been particularly severe in Canada, South Asia and South Africa (Kumar et al., 2002). In Australia common root rot has been recorded in wheat and barley cropping fields in every mainland state, with yield losses of up to 15% reported in central NSW (Moore and Herridge, 1983) and Queensland (Wildermuth et al., 1992).

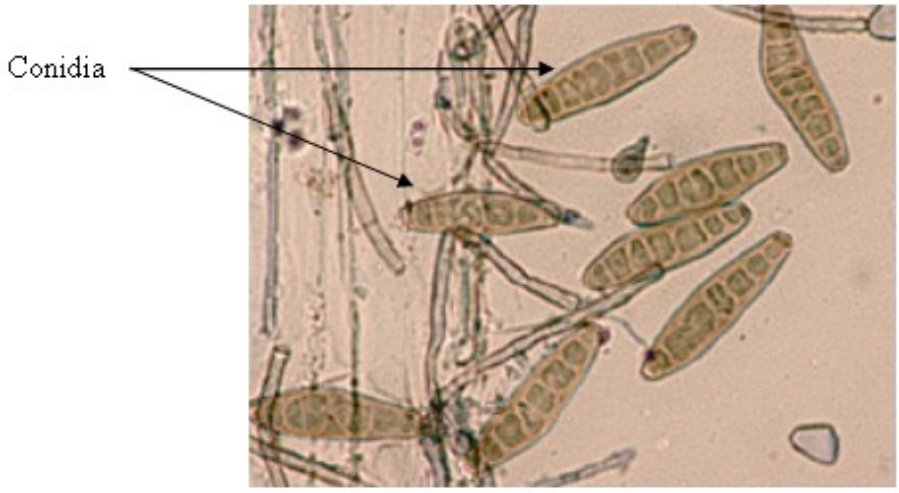
1.3.1 The Fungal Organism

Spot blotch and common root rot of barley are caused by the hemibiotrophic fungal pathogen *Bipolaris sorokiniana*, which is also the causal agent of seedling blight and head blight of wheat, barley and many grasses. *Bipolaris sorokiniana* is a cereal pathogen of increasing global concern and occurs predominantly in East India, Southeast China, Southeast Australia, Southeast Brazil, Eastern Europe, Northwest Africa and North America (Figure 3) (Kumar et al., 2002). This fungal pathogen has a wide range of hosts within wild and cultivated Poaceae and is extremely variable in pathogenicity towards barley (Steffenson, 1997). These factors significantly complicate efforts to control the disease within cereal cropping systems.

Figure 1-3. Worldwide cereal cropping systems where *Bipolaris sorokiniana* infections have been detected (adapted from Kumar et al., 2002). Highlighted regions depict distribution of disease occurrence.

Bipolaris sorokiniana is an asexual fungus belonging to the class Loculoascomycetes, order Pleosporales, family Pleosporaceae (Alcorn, 1988). The fungus reproduces by means of conidia which develop from single or clustered conidiophores (Figure 4). The conidia are curved to straight, fusiform to broadly ellipsoidal, and germinate by one germ tube from each end (bipolar germination; Sivanesan, 1987). Conidiophores are simple, erect and septate. The sexual state of the fungus (*Cochliobolus sativus* (Ito and Kurib.) has rarely been encountered in nature, where it has only been witnessed in Zambia in the presence of opposite mating types (Raemaekers, 1988).

Figure 1-4. Light microscope image showing conidia of *Bipolaris sorokiniana* (magnification = x1000; image taken from the Danish Research Centre for Organic Farming, 2005).



1.3.2 Infection and Symptoms

The fungal pathogen affects all parts of the plant and produces a variety of symptoms that can vary according to a number of factors, including host genotype and growth stage, pathogen isolate and environment. Most infections are initiated by soil-borne conidia or existing mycelium or conidia in plant residue, however inoculum can also be carried in the seed (Steffenson, 1997). Secondary conidia of *B. sorokiniana* form on infected tissue above the soil level and are dispersed by wind and splashing water, causing lesions on the leaves and stems later in the season.

Fungal infection of both leaves and roots comprises several phases: conidial germination, formation of appressoria, penetration and colonisation (Alcorn, 1988). *Bipolaris sorokiniana* produces toxins which interact with the host membranes resulting in cell death and leakage of metabolites (Kumar et al., 2002). The phytotoxins induce both chlorosis and necrosis in plant tissue (Hodges and Campbell, 1999). The three predominant phytotoxins produced by *Bipolaris sorokiniana* are: prehelminthosporol (most abundant and active component - it

weakens plant cells by inhibiting enzyme activity); helminthosporol (affects membrane permeability); and sorokinianin (inhibits barley seed germination; Kumar et al., 2002).

1.3.2.1 Spot Blotch

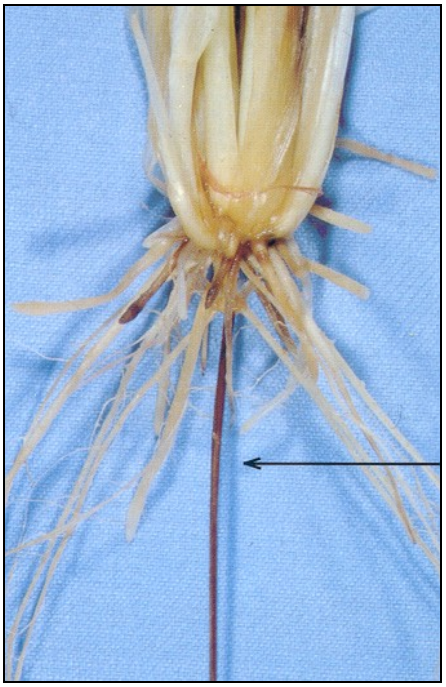
In seedlings, the spot blotch infection starts as dark brown-to-black spots on leaf sheaths that cover the young shoot and progresses from lower to upper leaves during crop development. If infection occurs early enough in the crop cycle and conditions remain favourable for disease development, complete defoliation is possible, resulting in major yield reductions (Kumar et al., 2002). On susceptible adult plants (Figure 5) lesions are round to oblong (up to 20mm) with chlorotic margins. The lesions may coalesce to form blotches that cover and kill large portions of the leaf, with severely infected leaves senescing prematurely (Steffenson, 1997).

Figure 1-5. Spot blotch symptoms on mature barley plants (photo: J. Bovill)

1.3.2.2 Common Root Rot

The common root rot infection is characterised by elongated blotches on roots, subcrown internodes, crowns, and lower leaf sheaths (Figure 6). On leaves and stems the disease causes wilting, stunting, and chlorosis (Mathre et al., 2003).

Figure 1-6. Symptoms of common root rot of barley. Diseased plants show the characteristic infection of the sub-crown internode (arrow) and poor root development. Image reproduced with permission from K. Moore (NSW DPI).



Common root rot impairs the functioning of roots and crown resulting in fewer tillers and heads, which causes a reduction in yield and lower grain quality. The disease is most severe when infection occurs in the seedling stage, however infections may occur at any stage of plant development. If infection occurs during the seedling stage plants may be killed outright. Severe root rot at other growth stages can also cause plant death. This usually occurs during heading or early grain filling stages (Mathre et al., 2003).

1.3.3 Disease Cycle and Management

The fungi survive as thick-walled, resistant conidia in soil on old barley and wheat stubble, and can remain viable in the soil for 8 to 10 years (Steffenson, 1997). New spores are produced on infected plants or on crop debris and are spread by wind, water and cultivation. Infested seeds also serve to transmit the pathogen over long distances. Due to the long viability of the conidia and the fact that many native grasses can support the pathogen, it is impossible to entirely eliminate the pathogen from a field (Kumar et al., 2002).

Different environmental conditions predispose barley plants to either the spot blotch infection or the common root rot infection. The susceptibility of barley plants to spot blotch increases around Zadoks' growth stage 56. Zadoks' scale refers to cereal growth stages, with 56 being equivalent to heading stage. High temperatures and humidity favour the outbreak of the disease, thus if weather conditions are conducive during this growth stage an epidemic may rapidly develop (Kumar et al., 2002).

For common root rot, multiple infections of root rot pathogens are often necessary for severe disease to occur, as root rotting is tolerated by the plant as long as new roots are generated. The common root rot infection causes the most damage when plants are under stress from adverse environmental conditions such as drought, flooding and high salinity (Duczek, 1993; Piccinni et al., 2000). High soil temperatures, low soil moisture and windy weather also contribute to the severity of the disease (Mathre et al., 2003; van Leur et al., 1997). Therefore, damage due to common root rot can vary widely from year to year in a given location.

Integrated strategies for controlling *B. sorokiniana* on barley include soil and residue management, chemical control, crop rotation and resistance breeding (Mehta, 1988; Steffenson, 1997). Primary inoculum in crop residue can be reduced by rotation with non-susceptible crops or by tillage practices which facilitate rapid breakdown of residue (Steffenson, 1997). Wildermuth and McNamara (1991) demonstrated that

rotation of wheat with lucerne resulted in the reduction of soil populations of *B. sorokiniana* and thus a reduction in the severity of common root rot. However, following a second crop, soil populations of *B. sorokiniana* were effectively restored to their previous levels, thus demonstrating the limited effectiveness of crop rotations in the control of common root rot.

Due to fact that the pathogen can be seed borne, the use of pathogen-free seed or fungicide-treated seed is beneficial (Steffenson, 1997). Foliar applications of fungicides can significantly reduce the level of infection, however several applications are often required to achieve adequate control (Sharma-Poudyal et al., 2005; Shefelbine et al., 1986). Thus the economics of fungicide application depends on the susceptibility of the variety and the value of the potential yield loss.

Effective control strategies for both spot blotch and common root rot need to be developed. The use of resistant cultivars offers the most economically and environmentally sound means of control and should be considered as a major component of integrated disease management (Arabi, 2005; Mehta, 1988; Williams, 2003).

1.4 The Nature of Plant Disease Resistance

Resistance can be defined as a plant's ability to impede the growth or development of a pathogen once contact has been initiated (Agrios, 1997). A plant may achieve resistance through active and/or passive defence mechanisms, involving biochemical, physiological or morphological characteristics (Ayliffe and Lagudah, 2004; Guest and Brown, 1997). Two broad resistance types have been identified in crop plants: major gene resistance and multigenic resistance (Keane and Brown, 1997).

Major gene resistance (also known as vertical resistance) is generally controlled by a single gene, referred to as an *R*-gene. These *R*-genes can be remarkably effective in

controlling disease and can confer complete resistance (Ayliffe and Lagudah, 2004). In general, *R* genes function to recognise specific "elicitor" molecules produced by the invading pathogen which results in a rapid signal cascade and an active defence response (Wise, 2000). This form of resistance is explained by the gene-for-gene concept, first proposed by Flor (1956), where for each genetic locus conditioning resistance (*R* gene) or susceptibility in a host, there is a corresponding locus in the pathogen controlling avirulence (*avr* gene) or virulence. However, each *R*-gene confers resistance to only a subset of races of the pathogen. Thus, major gene resistance is prone to breakdown as new virulent pathotypes evolve through mutation to the *avr* gene, which results in the absence of an active defence response in the host (Keane and Brown, 1997). Vertical resistance has been popular in conventional breeding programs due to the ease in which the *R* gene can be detected and transferred through cross-breeding (Wise, 2000).

Multigenic resistance (also referred to as horizontal resistance) is controlled by multiple genes, each segregating according to Mendel's laws. This type of resistance is referred to as quantitative, in that the plants that possess it show various degrees of susceptibility to the disease (Keane and Brown, 1997). Unlike major-gene resistance, individual genes contributing to horizontal resistance condition only a partial resistance. This form of resistance does not completely prevent a plant from becoming damaged, however it slows the infection process and decreases spore production, thus diminishing disease severity and spread of the pathogen to other plants. Horizontal resistance is generally effective against all races of a pathogen, and is often referred to as non-race specific resistance. When managed properly multi-gene resistance can be very effective in controlling plant diseases, due to less selection pressure in favour of specific pathotypes (Keane and Brown, 1997). Multigenic resistance is the preferred type of resistance for plant breeding programs, mainly due to its robustness over a long period of time (durable resistance) in disease prone systems. However currently little is known about the complex interactions between the multigenic host and pathogen (Agrios, 1997), and due to the

(sometimes) large number of genes involved it is much more difficult to breed varieties with this form of resistance (Wise, 2000).

1.4.1 Current Resistance Status

Variation in resistance exists in barley for both spot blotch (Steffenson et al., 1996; Wilcoxson et al., 1990) and common root rot (Kutcher et al., 1994); however, the degree of resistance in modern cultivars is insufficient (Arabi, 2005). In Australia, there are currently no commercial resistant cultivars available to growers for either disease (Platz pers. comm.; Wildermuth pers. comm.). Thus, the identification of parental stocks possessing an adequate level of resistance to spot blotch and common root rot is required (Arabi, 2005; Steffenson, 1997).

1.5 Selecting for Resistance

Conventional breeding involves the ability to identify plants containing the desired gene combinations for the trait of interest, the generation of genetic variation through hybridisation, and the identification of superior recombinants from the pool of genetic variation (Lamkey and Lee, 1993). Conventional plant breeding selection methods are based on plant performance characteristics (morphological markers), such as height, seed size and colour. These traditional selection methods are time consuming, often involving many generations, and are very dependent on environmental conditions (Francia et al., 2005; Korzun, 2002).

1.5.1 Phenotypic Screening Methods for Spot Blotch Resistance

Resistance in barley to spot blotch is often assessed at the seedling stage in the greenhouse and at the adult plant stage in the field. When screening the disease reactions of barley lines for breeding purposes it is important to have a rating scale that describes the full spectrum of possible infection responses encountered. Fletch

and Steffenson (1999) developed a comprehensive infection response rating scale based on the lesion type polymorphisms of several thousand barley lines responding to a diverse collection of fungal isolates. The 1-9 rating scale proposed by Fletch and Steffenson (1999) is based on the presence of necrosis and chlorosis and the relative size of spot blotch lesions observed on the second leaves of barley seedlings. These infection responses (IRs) are classified into three general categories of low (IRs 1 to 3), intermediate (IRs 4 and 5) and high (IRs 6 to 9) host-parasite compatibility. Low IRs show small necrotic lesions with little or no marginal chlorosis. Intermediate IRs consist of medium-sized necrotic lesions with a distinct chlorotic margin, while high IRs show large necrotic lesions with distinct chlorotic margins and expanding diffuse chlorosis. In addition to the seedling infection response scale, Fletch and Steffenson (1999) devised a four class adult infection response scale (R=Resistant, MR=Moderately Resistant, MS=Moderately Susceptible, and S=Susceptible) based on the type and relative size of lesions present on the leaves.

1.5.2 Phenotypic Screening Methods for Common Root Rot Resistance

The severity of common root rot can be assessed by the degree of necrosis of the sub-crown internode (Tinline et al., 1975). Plants are separated into six categories:

1 = no lesions;
2 = 1 -2 lesions covering <10% of the sub-crown internode;
3 = lesions covering 10-25% of the sub-crown internode;
4 = lesions covering 25-50% of the sub-crown internode;
5 = lesions covering 50-99% of the sub-crown internode; &
6 = lesions covering 100% of the sub-crown internode.

Disease incidence is determined from the number of plants in categories 2 to 6, and disease severity is calculated from the formula;

$$\text{Disease severity} = (2N_1 + 5N_2 + 10N_3) \times 100/10(N_1 + N_2 + N_3)$$

Where N_1 is number of plants in categories 2 and 3; N_2 is the number in plants in category 4; and N_3 is the number of plants in category 5 and 6. This system for measuring common root rot severity has been widely used in various studies examining the common root rot infection (Ledingham, 1970; Tinline et al., 1975; 1988; Wildermuth, 1986; 1997).

This traditional screening method to assess common root rot severity in barley genotypes is somewhat imprecise, difficult to apply, and prone to inconsistencies among observers (Tinline et al., 1975). Due to these limitations, Arabi and Jawhar (2001) developed an in vitro quantification method to determine the infection response of barley to common root rot. Arabi and Jawhar (2001) proposed that quantification of disease severity be based on the percentage of germinated infected pieces of sub-crown internodes cultured on potato dextrose agar media.

These phenotypically based screening methods have limitations due to the effect of environmental conditions on phenotypic expression (Francia et al., 2005). In addition, the expression of traits such as disease resistance often cannot be accurately measured by simple field observation. As a result of these limitations, molecular marker technology has been adopted by plant breeders to improve the selection strategies in breeding programs.

1.5.3 Molecular Markers

Genetic markers act as “flags” to reveal genetic differences (polymorphisms) between individuals or species. Genetic markers may be either located within the genes themselves, referred to as perfect markers (Paterson, 1996b), or are located in close proximity or “linked” to a gene controlling a trait (Falconer and Mackay, 1996). All genetic markers occupy a specific locus on a chromosome and most

markers do not affect the phenotypic expression of an individual (Collard et al., 2005).

There are 3 major types of genetic markers: morphological; biochemical; and molecular. Morphological (classical) markers are phenotypic markers or traits, such as flower colour, growth form or seed size (Collard et al., 2005). These markers formed the basis of selection in traditional breeding programs (Korzun, 2002). Biochemical markers are those which reveal polymorphisms between individuals based on their chemical characteristics, for example different molecular forms or isozymes of an enzyme (Arus and Moreno-Gonzalez, 1993). The major disadvantage of morphological and biochemical markers are that they are often limited in number and may be strongly influenced by the environment or the developmental stage of a plant (Chelkowski et al., 2003; Winter and Kahl, 1995).

Molecular markers reveal sites of variation in DNA and arise from different classes of DNA mutations (point mutations, insertions or deletions) or errors in replications of tandem DNA (Collard et al., 2005). Molecular markers can be divided into 3 classes, based on their method of detection: hybridization-based; polymerase chain reaction (PCR) based and DNA sequence based (Gupta and Roy, 2002). Unlike biochemical and morphological markers, molecular markers eliminate the influence of environment on gene expression and are potentially unlimited in number. In addition, many molecular markers are selectively neutral because they are usually located in non-coding regions of a genome (Simpson, 1999). Due to these advantages and their abundance they are the most widely used marker type in genomic research.

1.5.3.1 Linkage Maps

One of the major uses of molecular markers in agricultural research is the construction of linkage (genetic) maps by analysing the co-segregation of markers and phenotypes in defined populations (Korzun, 2002). The construction of linkage-

maps is based on the principle that markers and genes that are situated close together on a chromosome will be transmitted from parent to progeny during chromosomal recombination more frequently than markers that are located further apart (Collard et al., 2005). The analyses of marker segregation in a segregating population indicate the relative genetic distances between markers on a chromosome; and are thus referred to as genetic maps. There are three main steps of linkage map construction: the production of a segregating plant population in which the parents differ by one or more traits of interest; the identification of markers that reveal differences (polymorphisms) between the parents in the mapping population; and finally, the analysis of marker segregation and linkage within a population to locate regions of a chromosome containing genes linked to a trait of interest. These regions of a chromosome are referred to as Quantitative Trait Loci (QTL) and are regions of the genome that contribute to the phenotypic variation of a quantitative trait (Doerge, 2002). QTL mapping is a highly effective approach for studying polygenic (quantitative) forms of disease resistance (Young, 1996).

1.5.3.2 Mapping Populations

Linkage map construction based on recombination requires a population derived from sexual reproduction, where the parents differ for one or more traits of interest (Francia et al., 2005). The size of the mapping population generally ranges from 50 to 250 individuals (Mohan et al., 1997), however larger population sizes are generally required for high-resolution mapping (Babu et al., 2004; Collard et al., 2005; Young, 1996). If the mapping population is to be used for subsequent quantitative trait loci (QTL) analysis, then the segregating progeny must be phenotypically evaluated.

Several different population types can be utilised for linkage map construction. These include: backcross; F_2; recombinant inbred (RI); and doubled haploid. F_2 populations, (derived from F_1 hybrids) and backcross populations (derived from crossing an F_1 hybrid to one of the parents), are the simplest types of mapping

populations developed for self pollinating species. They require only a short time to produce and are relatively easy to construct (Collard et al., 2005). Recombinant inbred lines are constructed from inbreeding individual F_2 lines over several generations and consist of a series of homozygous lines. Each line contains a unique combination of chromosomal segments from the parents. The major disadvantage of RI lines is they usually take 6 to 8 generations to produce (Mohan et al., 1997).

The production of doubled haploid lines (DH) is a biotechnological approach that has been used to produce homozygous breeding lines and varieties (Palmer and Keller, 2005). DH lines are generated by doubling the chromosome set of a monoploid (haploid) plant or tissues, either spontaneously or by chemical means such as colchicine treatment (Kammholz et al., 1996). Monoploids and their derived DH lines are expected to show the (1:1) ratio of segregation over all loci without the interference of dominant alleles. The production of a DH population is only possible in species amenable to tissue culture, such as rice, barley, and wheat (Collard et al., 2005). In wheat, DH populations are produced by gynogenesis of the embryonic sack. In barley, DH populations are produced by androgenesis, where microspores are used as target tissues (Palmer and Keller, 2005).

1.5.3.3 Identification of Polymorphisms and Marker Systems

The second step in linkage map construction is the identification of molecular markers that reveal genetic differences (polymorphisms) between the parents of the mapping population. Numerous DNA-based genetic marker analysis methods have been developed over the last two decades, including restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) (microsatellites) and single nucleotide polymorphisms (SNPs). A detailed description of all of these marker types is beyond the scope of this review, however the relative advantages and disadvantages of these marker systems are summarised

in Table 1. Of the marker types listed in Table 1, SSRs, Diversity array technology markers (DArTs), and SNPs are most relevant to this project, and these will be discussed in more detail.

Table 1-1. Comparison of common marker types used in cereal breeding (adapted from Korzun, 2003).

Marker Type	RFLP	RAPD	AFLP	SSR	DArT*	SNP
DNA required (μg)	10	0.02	0.5-1.0	0.05	0.05	0.05
PCR-based	No	Yes	Yes	Yes	No	Yes
Number of loci	1-3	1-50	20-100	1-3	300-600	1
Ease of use	Difficult	Easy	Easy	Easy	-#	Easy
Reproducibility	High	Unreliable	High	High	High	High
Cost per analysis	High	Low	Moderate	Low	Low#	High

*DArT appraisals are based upon personal experience and discussion with colleagues. #DArT's are a commercial marker (Triticarte Pty, Ltd) and their overall cost is low if consumables and man hours are included in a cost per data point analysis.

1.5.3.3.1 Simple Sequence Repeats

Simple sequence repeats (SSRs) are a class of marker that relies on the high rate of polymorphism observed at microsatellite loci (Korzun, 2003). These are tandem repeats of two or more bases that are widespread in eukaryotic genomes. Variation in the number of repeats is observed by developing locus-specific primers that anneal to sequences flanking the repeat region, and use of the polymerase chain reaction (PCR) to amplify the intervening DNA fragments (Kearsey and Luo, 2004). The major advantages of microsatellites are their ease of use, low cost of analysis and their ability to detect genetic differences even among closely related individuals (Korzun, 2003). The first two advantages are critical for the widespread use of DNA markers in large scale breeding programs. The third advantage is of vital importance in modern plant breeding programs, where crosses are often made between elite parental lines that are genetically quite similar.

1.5.3.3.2 Expressed Sequence Tags

Expressed sequence tags are short sequences of the genome obtained from the analysis of complementary DNA (cDNA) from mRNA, and have been instrumental in gene discovery and gene sequence determination (Rudd, 2003). EST-SSRs are molecular markers derived from ESTs. SSR-EST markers are functional, in that they may assist the role of genetic markers by assaying variation in known functional genes. Their other advantage is that their development cost is very low due to the abundance of EST sequence information in public databases.

1.5.3.3.3 Single Nucleotide Polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence differs between members of a species. SNPs can occur in both coding (gene) and noncoding regions of the genome and are the most common feature underlying genetic variation within species. This form of genetic variation can be screened by means of a wide range of technologies, usually based on primer extension or on the ligation of oligonucleotide ends (Wittenberg et al., 2005). The development of SNP scoring technologies has led to an increase in throughput capacity, however this method requires the DNA sequences of sets of loci for both parents of a cross (Korzun, 2003). In addition, it is rarely cost-effective to perform SNP discovery for marker assisted selection in crop breeding, as a large number of markers are required for the identification of QTLs (Wittenberg et al., 2005).

1.5.3.3.4 Diversity Array Technology

Due to the limitations of existing marker technologies, hybridisation-based methods using nucleic acids immobilised on solid-state surfaces have been developed. Diversity Array Technology (DArT) is a new DNA hybridisation-based genotyping

technology that allows whole genome scanning using a microarray platform. DArT uses an array of individualised clones from a genomic representation prepared from amplified restriction fragments. Labelled genomic representations of individuals to be genotyped are hybridised to the array. Polymorphisms are scored based on the presence/absence of hybridisation to individual array elements. This allows high-throughput screening of hundreds of molecular markers simultaneously and is especially suited to genome-wide analysis. Thus, DArT can detect and type DNA variation at several hundred genomic loci in parallel (Wenzl et al., 2004). In addition, DArT markers require low quantities of DNA per sample, and are low cost. The polymorphisms detected by DArT include single nucleotide polymorphisms, insertion-deletions and methylation changes (Wenzl et al., 2004; Wittenberg et al., 2005).

1.5.3.4 Bulked Segregant Analysis

Bulked segregant analysis (BSA), developed by Michelmore et al. (1991), is a method to rapidly identify markers in specific regions of a genome. BSA involves comparing two pooled DNA samples of individuals from a segregating mapping population, originating from a single cross. Within each pool (or bulk) of DNA, individuals have identical genomic regions for the target locus, but random genotypes at loci unlinked to the selected region (Michelmore et al., 1991). In this way, pools of DNA from plants that differ for a particular trait, such as disease resistance or susceptibility, can be analysed to identify polymorphic markers associated with the trait. This method saves both time and money, in that polymorphic markers can be identified using only a small number of samples (two parents and two bulks).

1.5.3.5 Marker Genotyping

Once polymorphic markers have been identified by screening across the parents, then the entire mapping population is screened, referred to as marker genotyping. Thus, DNA must be extracted from each individual in the mapping population and the parents (Collard et al., 2005). Generally, the markers will segregate in a Mendelian fashion, allowing parental and recombinant lines to be genotyped (Paterson, 1996a).

1.5.3.6 Linkage Analysis of Markers

The final step in the construction of a linkage map is to analyse the linkage of markers to determine whether an association exists between the markers and a quantitative trait. The linkage analysis of markers is usually performed by computer programs, such as MapManager QTX (Manly et al., 2001). Linkage between markers is usually expressed in terms of a logarithm of odds (LOD) score, where a LOD score of 3 between two markers indicates that linkage is 1000 times more likely than no linkage. Linked markers are grouped together and represent either an entire chromosome or a chromosomal segment (Collard et al., 2005).

It should be noted that distance on a linkage map is not linearly related to the physical distance between markers on a chromosome, as it is measured in terms of the frequency of recombination between the genetic markers (Paterson, 1996a). Recombination frequency is not linearly related to the frequency of crossing over during meiosis (Hartl and Jones, 2001). Thus, mapping functions (including the Haldane and Kosambi function) are required to convert recombination fractions into centimorgans (cM) of genetic distance.

QTL analysis aims to detect an association between genetic markers and the genomic region controlling the phenotypic expression of a quantitative trait. Markers are used to partition the mapping population into different genotypic classes

according to their genotype at a particular marker locus. It is then determined mathematically whether a significant difference exists between the phenotypic means of the classes (Tanksley, 1993); where a significant difference indicates that the marker is linked to the trait of interest (Collard et al., 2005). Common methods used to detect QTL are: single marker analysis; simple interval mapping (SIM) (Lander and Botstein, 1989); and composite interval mapping (CIM) (Zeng, 1993).

Single marker analysis employs statistical methods, such as analysis of variance (ANOVA), t-test and linear regression, to detect QTL associated with a single marker. A single-factor ANOVA measures the probability that a QTL is present at the same chromosomal location as the marker, and is the quickest way to establish whether a significant association exists between a marker and the expression of a phenotypic trait (Collard et al., 2005), by. The results of this statistical inference are presented as a *P*-value, where a *P*-value of 0.01 indicates a 1% probability that these results would have been obtained in the absence of a marker-trait association. The lower the *P*-value, the higher the probability that a QTL truly exists in the region of the marker (Falconer and Mackay, 1996). Linear regression is the more commonly used statistical method as it provides the coefficient of determination (R^2). The R^2 value for a marker is considered to be the percentage of total phenotypic variance of a trait that is explained by the marker. Single marker analysis does not require a complete linkage map; however QTL locations are detected only in terms of the nearest marker and, therefore, are imprecisely estimated (Falconer and Mackay, 1996). In addition, the size of the QTL effect is confounded with distance of the QTL from the nearest marker (Francia et al., 2005).

Simple interval mapping (SIM; Lander and Botstein, 1989) requires a linkage map, however unlike single marker analysis, SIM analyses intervals between linked markers simultaneously, thus compensating for recombination between the markers and the QTL (Collard et al., 2005; Falconer and Mackay, 1996).

The basis of composite interval mapping (CIM) is an interval test that attempts to separate and isolate individual QTL effects by combining interval mapping with multiple regressions. It controls for genetic variation in other regions of the genome, thus reducing background “noise” that can affect QTL detection (Zeng, 1993). To control background variation, the analysis software incorporates "cofactors" into the model, which are a set of markers that are significantly associated with the trait and may be located anywhere in the genome. They are typically identified by forward or backward stepwise regression, with user input to determine the number of cofactors and other characteristics of the analysis. CIM is more precise and effective at mapping QTLs compared with SIM (Collard et al., 2005).

The results of SIM and CIM are represented by either a likelihood ratio statistic (LRS), or a logarithmic of odds (LOD) score. LRS and LOD profiles reveal the most likely position of a QTL (highest LOD value) in relation to the linkage map (Collard et al., 2005; Falconer and Mackay, 1996). These profiles are usually represented in graphical form, with the test statistic on the *y* axis and markers comprising linkage groups on the *x* axis. The most likely QTL position is considered to be the point where the peak LOD score occurs (Falconer and Mackay, 1996), where the peak must exceed a threshold significance level before the QTL can be referred to as statistically significant. Significant thresholds can be determined by carrying out between 500 and 1000 permutation tests to eliminate false positive marker-trait associations.

There are a number of factors which limit QTL detection. Firstly, only QTLs with large phenotypic effects will be detected. Thus, depending on the size of the mapping population, small effect QTLs may fall below the threshold of detection. The second factor is the size of the mapping population. Populations must be relatively large in order to detect minor QTLs against the background of environmental variation in phenotypic expression. Therefore, the larger the population the greater the chance large and small QTLs will be detected. Thirdly, linked QTLs may be recognized as only a single QTL. Combinations of these factors

generally contribute to the underestimation of the number of QTLs controlling traits (Asins, 2002).

1.5.4 Spot Blotch Resistance in Barley

Genetic information on this plant-pathogen interaction and the mechanism of resistance in barley is scarce (Arabi, 2005). In early research, Griffee (1925; cited in Bilgic et al., 2005) reported that three unlinked spot blotch resistance genes conferred field resistance in the cultivar Svanhals. In an inheritance study, Wilcoxson et al. (1990) reported that resistance to spot blotch in barley cultivars was controlled by one or two genes. More recent research has utilized molecular marker technology and QTL analysis to build upon the correlations of spot blotch resistance with morphological characters and inheritance reported in earlier studies.

Using molecular marker technology, Steffenson et al. (1996) reported that seedling resistance to spot blotch was monogenetically inherited and was governed by a single gene (*Rcs5*) on the short arm of chromosome 7H in the Steptoe/Morex DH population. In this study, Steffenson et al. (1996) also found that adult plant resistance was conferred by 2 QTLs; one major QTL on chromosome 1H explaining 62% of the phenotypic variance and a second minor QTL on chromosome 7H, explaining 9% of the phenotypic variance. The 1H QTL has been successfully incorporated into six-rowed malting barley cultivars, originally derived from the breeding line NDB112, and is one of the most successful examples of resistance breeding in the United States (Steffenson et al., 1996).

Recent reports by Bilgic et al. (2005) have shown that the 1H gene is completely suppressed when introgressed into other genetic backgrounds (H/M and D/M). In this study, Bilgic et al. (2005) identified three QTLs conferring adult plant resistance in a Dicktoo/Morex DH population: one on the short arm of chromosome 7H at or near the *Rcs5* gene explaining 20 % of the phenotypic variance; a second on the short arm of chromosome 3H explaining 36% of the phenotypic variance; and a third

on the long arm of chromosome 3H explaining 11% of the phenotypic variance. In addition, Bilgic et al. (2005) identified three QTLs conferring seedling resistance in the Dicktoo/Morex DH population; one near *Rcs5* on chromosome 7H explaining 30%, a second near the centromere of chromosome 7H explaining 9%, and a third on chromosome 3H explaining 19% of the phenotypic variance.

1.5.5 Common Root Rot Resistance in Barley

Relatively few studies have been conducted on the genetics of resistance to common root rot in barley. Of the few studies conducted it has been determined that resistance to common root rot is quantitative. However, the exact mechanism of resistance is unknown (Kutcher et al., 1994). Kutcher et al. (1994) examined the association between 2 morphological markers (glossy sheath 4 (*gs4*) and orange lemma (*o*)) and common root rot reaction. Results of this study found that the morphological markers gs*4* and *o* were found to be associated with the allele for common root rot susceptibility, while the RAPD marker UBC198, closely linked to gs*4* and *o*, was associated with the allele for resistance. Kutcher et al. (1994) concluded that the genes conditioning common root rot reaction were located on chromosome 6 and that the RAPD marker UBC198 would be useful in the selection of common root rot resistant germplasm.

In a further study, Kutcher et al. (1996) investigated the heritability of common root rot resistance in two resistant-by-susceptible barley crosses, Fr962-77 x Deuce and Virden x Ellice. The heritability of common root rot resistance of cross Fr962-77 x Deuce ranged from 56 to 85% and heritability for cross Virden x Ellice ranged from 53 to 78%. The continuous nature of the distributions of mean disease reactions of progeny lines, and the estimation of the number of genes conditioning resistance in this study indicated that the inheritance of common root rot resistance was quantitative in nature. Kutcher et al. (1996) also examined the relationship between common root rot resistance and spot blotch resistance. Their results suggested that the genes controlling common root rot were not the same as those controlling spot

blotch reaction. This finding confirmed work conducted by Clark (1966), who found that all progeny from a interspecific cross of barley cultivars were susceptible to spot blotch, but displayed various infection responses to common root rot infection.

1.6 Marker Assisted Selection

Major efforts have been devoted to the incorporation of genetic resistance in cereal breeding programs to offset yield losses caused by fungal pathogens (Wise, 2000). Marker assisted selection (MAS) utilises molecular marker technology to identify regions of a genome that are strongly associated with highly desirable traits, such as disease resistance, to assist selection strategies in cereal breeding programs. The ability to select desirable individuals based on their genotypic configuration, thereby eliminating the need for phenotypic testing, is an extremely powerful application of DNA markers and QTL mapping (Young, 1996). MAS allows for early generation phenotypic selection, and enables quicker and often more reliable identification of those plants containing genes of agronomic importance (Wise, 2000). In addition, MAS can allow a faster response to a breakdown in resistance, rapid introgression of multiple genes from diverse germplasm, gene pyramiding, and selection of rare recombinants between tightly linked resistance genes (Michelmore, 2003).

1.6.1 MAS Applications

The major applications of molecular markers in most breeding programs have been in backcross breeding where loci are tracked to eliminate specific genetic defects in elite germplasm, for the introgression of recessive traits, and for the selection of lines with a genomic structure similar to the recurrent parent (Langridge and Chalmers, 2005). Markers have also improved strategies for gene deployment and enhanced the understanding of the genetic control of complex traits (Francia et al., 2005).

In general, marker based breeding systems depend on four main factors (Babu et al., 2004; Francia et al., 2005):

1. a genetic map with an adequate number of uniformly spaced polymorphic markers to accurately locate QTLs or major genes;

2. close linkage between the QTL of interest and adjacent markers;

3. adequate recombination between the markers and the rest of the genome; and

4. an ability to analyse a large number of individuals from a segregating population in a time and cost effective manner.

The success of MAS depends on the location of the marker with respect to the gene controlling a quantitative trait. Markers located within the gene of interest are the most sought after but these usually require the target gene to be cloned (Francia et al., 2005). Generally, markers are not located within the target gene and tightly linked flanking markers are required to accurately locate the QTL controlling a trait of interest. Markers located closely on either side of the QTL minimise the chance of double recombination events between the QTL and both flanking markers (Doerge, 2002).

Cost-effective marker assisted selection is also essential for plant breeding programs to be effective in the development of disease resistant, high yielding cultivars (Wise, 2000). The cost of employing MAS rather than conventional techniques varies considerably between studies and should be considered on a case by case basis (Babu et al., 2004; Collard et al., 2005; Dreher et al., 2003). In some cases phenotypic screening and selection is cheaper than marker assisted selection. However, if phenotypic evaluation is time consuming and laborious, then the utilisation of markers may be the preferred method of selection. The method of

selection ultimately depends on the inheritance of the trait, method of phenotypic assessment and the cost of the required resources (Collard et al., 2005).

The identification of a marker linked to a trait of importance is only the first step in the application of a marker into a practical breeding program. Marker validation is a key element. In general, the validation process establishes the value of the marker in lines where various opportunities for recombination have occurred and where the marker may have been separated from the trait of interest (Sharp et al., 2001). The validation process involves the testing of an allele for its effect in genetic backgrounds other than the original mapping population, thus establishing whether the marker can be used in routine screening of MAS (Langridge et al., 2001). For markers to be most effective for MAS, they should detect the trait of interest in populations derived from different parental genotypes (Francia et al., 2005).

1.6.2 MAS Constraints

Although molecular markers have been successfully associated with QTLs, in some cases these associations have proved to be of limited usefulness in practical breeding programs. A number of constraints have imposed significant limitations on the efficient utilization of QTL mapping in MAS, including: the identification of major QTLs controlling quantitative traits; uncertainty in the position of small effect QTLs; deficiencies in QTL analysis resulting in the underestimation or overestimation of the number and effect of QTL; and, a lack of validation of QTL-marker associations in populations of different genetic backgrounds (Babu et al., 2004; Francia et al., 2005; Holland, 2004).

1.7 Project Goals

The line ND11231, originating from North Dakota, was introduced into Australia as a source of low grain protein and a doubled haploid (DH) population (ND11231-12/ VB9524) was developed to study this trait (Emebiri et al., 2005). When the parents were found to differ markedly in their response to the spot blotch infection by the *Bipolaris* pathotype present within Australia (with ND11231-12 resistant to spot blotch and VB9524 susceptible; Platz, unpublished results), the population presented an opportunity to study the genetics and mechanism of seedling and field spot blotch resistance under Australian environmental conditions. This project aims to identify molecular markers linked to seedling and field resistance in the barley DH population ND11231-12/VB9524, under Australian environmental conditions and using the *Bipolaris* isolate SB61.

The genetics of resistance to common root rot are poorly understood. This project also aims to identify molecular markers for common root rot in the RI barley population Delta/Lindwall. The information generated from this research will assist cereal breeding programs to select and maintain resistant barley cultivars to offset yield losses currently caused by these cereal diseases.

The specific goals of this project are:

- To assess the spot blotch reaction of individual DH lines in the population ND11231-12/VB9524 (ND/VB) in replicated seedling and field trials;
- To curate a map of the ND/VB DH population (provided by Emebiri et al., 2005) to provide a firm basis for mapping QTL for resistance to spot blotch;
- To conduct a robust QTL analysis, using a variety of software packages, to identify molecular markers linked to QTL for resistance to spot blotch;
- To conduct seedling and field trials of a related population and validate any major QTL that may be identified;

- To apply BSA-DArT technology to rapidly identify loci for CRR resistance in an unmapped Delta/Lindwall population and confirm these regions with SSR markers; and
- To assess whether the same genomic regions confer resistance to both SB and CRR.

1.7.1 Project Significance

Resistance breeding has been identified as the most efficient and environmentally sound means of control for SB and CRR in barley. Some of the most successful applications of MAS to plant breeding have been the introgression of resistance genes into elite breeding germpalsm. The generation of robust markers for spot blotch and common root rot resistance will greatly assist the selection strategies of barley breeding programs aiming to produce commercially available resistant cultivars in Australia. The production of resistant cultivars will providing greater protection to the barley farming community against the yield losses currently caused by these fungal diseases.

Six-rowed malting cultivars bred in upper mid-west of the United States from the breeding line NDB112 have provided durable spot blotch resistance in the region for over 4 decades (Bilgic et al., 2005; Steffenson et al., 1996). This project will test the robustness of this resistance to spot blotch under Australian environmental conditions and the *Bipolaris* pathotype present within Australia. This research will provide important information for both domestic and international barley breeding programs wishing to breed for resistance to these cereal diseases.

The genetics of resistance to spot blotch in barley, particularly in Australia, is not well understood. This research will expand upon current knowledge of the genetics of resistance and will also test the usefulness of molecular markers for selecting superior genotypes within barley breeding programs. In addition, this project will

seek to compare the genetics of spot blotch resistance with common root rot resistance in different barley populations.

2. Phenotypic Assessments for Spot Blotch Resistance

2.1 General Introduction

The foliar disease spot blotch, caused by the fungal pathogen *Bipolaris sorokiniana*, represents a serious disease constraint to barley growing regions worldwide, with estimated yield losses ranging from 15 to 70% (Kumar et al., 2002; Platz, personal communication 2005). Current Australian commercial cultivars do not possess adequate levels of resistance to this disease and major yield reductions have occurred under optimal environmental conditions, particularly in northern NSW and SE Queensland (Wildermuth et al., 1992). The use of resistant cultivars offers the most effective means of established control for this disease. The identification of resistant parental lines and the introgression of resistance into elite germplasm are therefore considered a priority within Australian barley breeding programs.

This form of integrated management relies on accurate phenotypic screening methods for resistance. Phenotypic screening for spot blotch resistance within a barley population is often conducted at the seedling stage in the greenhouse and at the adult plant stage in the field. A comprehensive rating scale to assess the infection responses (IRs) of barley cultivars to the spot blotch disease at the seedling stage of plant development has been proposed by Fletch and Steffenson (1999). The rating scale was developed based on the evaluation of a large and diverse set of host accessions and pathogen isolations, to encompass the full range of lesion type variation observed over a 9 year period (Fletch and Steffenson, 1999). The 1-9 rating scale is based on the relative size of lesions and presence of necrosis and chlorosis observed on barley seedlings infected with isolates of *C. sativus*.

The infection responses of progeny within a segregating population can be broadly classified into qualitative categories of either resistant or susceptible genotypes. The resulting frequency ratio can be examined for Mendelian inheritance patterns, to

indicate the number of genes controlling a trait (Steffenson et al., 1996; Bilgic et al., 2005). Using a similar method, Steffenson et al. (1996) identified a single gene (denoted the *Rcs5* gene) controlling seedling spot blotch resistance in the DH Steptoe/Morex (S/M) population. Based on the phenotypic analysis of spot blotch glasshouse trials, Steffenson qualitatively separated individual lines into general categories of resistant and susceptible genotypes based on lesion size and type. On the basis of this classification scheme the population segregated 76:74 (X^2=0.03, p=0.87) for resistance: susceptibility, indicating the presence of a single resistance gene. From this analysis Steffenson concluded that seedling resistance to spot blotch was monogenetically inherited in this genetic background. In the same study, Steffenson (1996) investigated the number of genes conferring adult plant resistance to the spot blotch infection in the S/M population. On the basis of chi-square analysis of the segregating progeny, Steffenson et al. (1996) indicated that adult plant resistance was controlled by more than one gene.

Prediction of the response of progeny to the spot blotch infection is assisted by knowledge of the heritability of the trait. Heritability is the proportion of phenotypic variation in a population that is attributed to the genetic variation among individuals (Falconer and Mackay, 1996). Heritability analysis estimates the relative contributions of genetic and non-genetic factors to the total phenotypic variation in a population trial. Relatively few studies have been conducted on the heritability of spot blotch infection response. Kutcher et al. (1994) reported heritability estimates of 43 and 61% for spot blotch resistance in the cultivar cross Fr926-77/Deuce and heritability estimate of 73 and 78% in the cultivar cross Virden/Ellice. This suggests that the heritability of spot blotch resistance is moderate to high.

The purpose of this chapter is to conduct extensive phenotypic assessments of the ND11231-12/VB9525 (ND/VB) population to the spot blotch disease. The specific aims are; firstly to screen DH lines of the ND/VB population for a spot blotch infection response in the glasshouse and the field; secondly, perform chi-square analysis of the segregating progeny to determine if it followed a single

gene/Mendelian inheritance structure; thirdly, estimate the heritability of spot blotch resistance in this population, and finally examine the progression of the spot blotch infection in the field over a six week period.

2.2 Materials and Methods

2.2.1 Plant Materials

The doubled haploid (DH) population ND11231-12/VB9524 consists of 180 lines and was developed by Emebiri et al. (2005) to investigate and identify regions of the barley genome that influence variations in grain protein concentration. ND11231-12, which shows a high level of resistance to spot blotch, originated from breeding programs at the North Dakota University, Fargo, USA. ND11231-12 is a narrow leafed sister line of the cultivar Logan (pedigree ND7085/ND4994-15/ND7556) which was released from the North Dakota Agricultural Experiment Station in 1995. This cultivar has low protein content and is grown to a limited extent in south central North Dakota. However, it was not selected for malting in the United States by the American Malting Barley Association because the low protein trait was not consistently expressed in the field (J. D. Franckowiak, pers.comm.) The susceptible line VB9524 was developed by the Department of Primary Industries, Victoria, Australia, and is an advanced selection from a cross of Arapiles with Franklin (Emebiri et al., 2005). Seed from this DH population was kindly supplied by the Department of Primary Industries, Victoria, Australia. The barley reference lines Gilbert, NDB112, Skiff, Stirling, Tallon, WPG8412-9-2-1 and the wheat line Hartog, were also included in all trials to verify the purity and virulence of the pathotype.

2.2.2 Fungal Preparation and Inoculation

The fungal isolate SB61 culture was supplied by Greg Platz, Senior Barley Pathologist at the Queensland Department of Primary Industries and Fisheries

(QDPI&F) Hermitage Research Station. The SB61 pathotype originated from a field epidemic in Kaputar barley at Monto in Queensland in 2001 and is used in all their routine screening for spot blotch resistance (G. Platz, pers. comm.) The inoculum was sub-cultured on starch-nitrate agar plates and actively grown for 2 weeks. Five ml aliquots of medium containing 5 drops of Tween 20 and 100 ml of milli-Q water were added to the fungal cultures and the conidia were gently dislodged with a camel hair brush. The fluid was strained off into a beaker and the plate was rinsed into the beaker with distilled water. The beaker was then placed on an electronic stirrer to assist in keeping the conidia in suspension. A drop of the suspension was added to a Neubauer counter (haemocytometer) using a pasteur pipette and the number of conidia per ml were determined. The average of 12 counts was calculated, and the conidial suspension was diluted to a final concentration of 10,000 conidia per ml.

2.2.3 Seedling Screening for Spot Blotch Resistance

Two independent seedling trials were carried out at the Hermitage Research Station, Qld in 2005 and 2006. A completely randomized design with 2 replicates was employed for both seedling trials. Seven-to-ten seeds of each line were sown at three evenly spaced sites around the circumference of 10 cm sterile pots containing 1:1 scrub soil and mushroom compost. The pots were placed in the glasshouse in water-filled trays and fertilized weekly. After approximately 15 days, when the second leaf was fully expanded, the seedlings were inoculated with the conidial suspension using a Passche air brush at 220 KPa**.** Plants were placed on the bench as per bench design and were inoculated by spraying from all four sides of the bench using a constant movement, distance and angle. The plants were placed immediately into a dew chamber as per bench design under 14 hrs dark: 10 hrs light at 22°C. The plants were then transferred to a growth room (25/15°C, 12 hrs light, 60% relative humidity (RH).

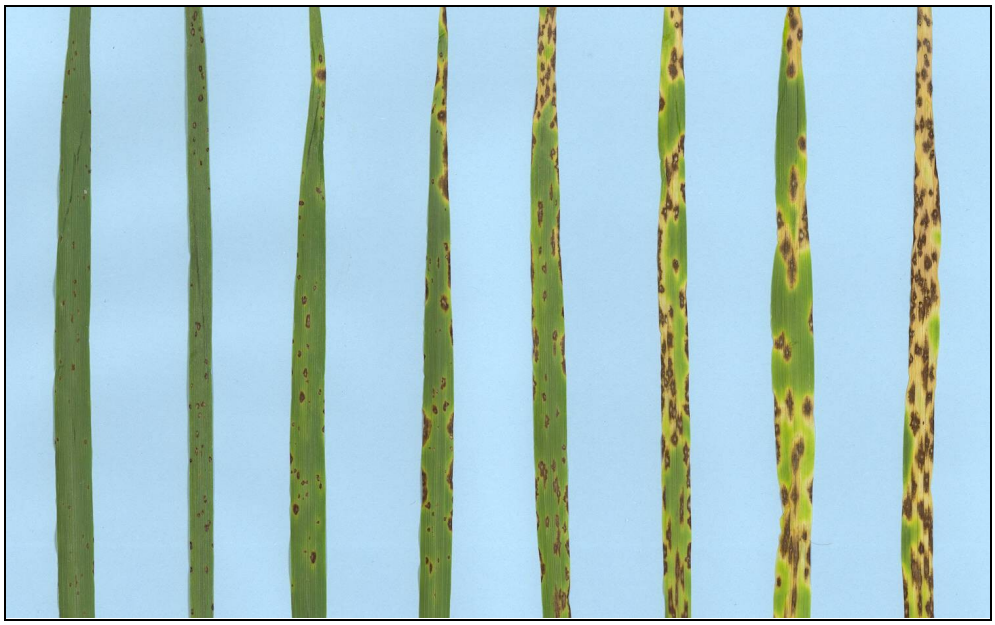
2.2.3.1 Assessment of Infection Response

Seedlings were rated 13 days post-inoculation, based on a 0-9 scale similar to that of Fletch and Steffenson (1999). Figure 7 illustrates the range of infection responses observed in the seedling trials. No individuals performed better than a rating of 2 in the trials.

The scale is explained as follows:

- 2 - minute round or oblong necrotic lesions (0.3 mm length, 0.3 mm width), without chlorosis;
- 3 - small round or oblong necrotic lesions (0.7 mm length, 0.5 mm width), without chlorosis;
- 4 - round or oblong necrotic lesions (0.8-1.3 mm long and 0.5-0.7 mm wide), with very slight marginal chlorosis;
- 5 - ovate necrotic lesions with distinct but restricted chlorotic margins;
- 6 - elliptical to elongated elliptical necrotic lesions with distinct chlorotic margins and minimal expanding diffuse chlorosis
- 7 - elongated necrotic lesions (4-6 mm in length and 1.4 -2.0 mm in width), with diffuse chlorosis
- 8 - elongated necrotic lesions with extended diffuse chlorosis; and
- 9 – large lesions with extended diffuse chlorosis adjoining adjacent lesions.

Figure 2-7. Infection rating scale of seedling infection responses to the spot blotch disease.



2.2.4

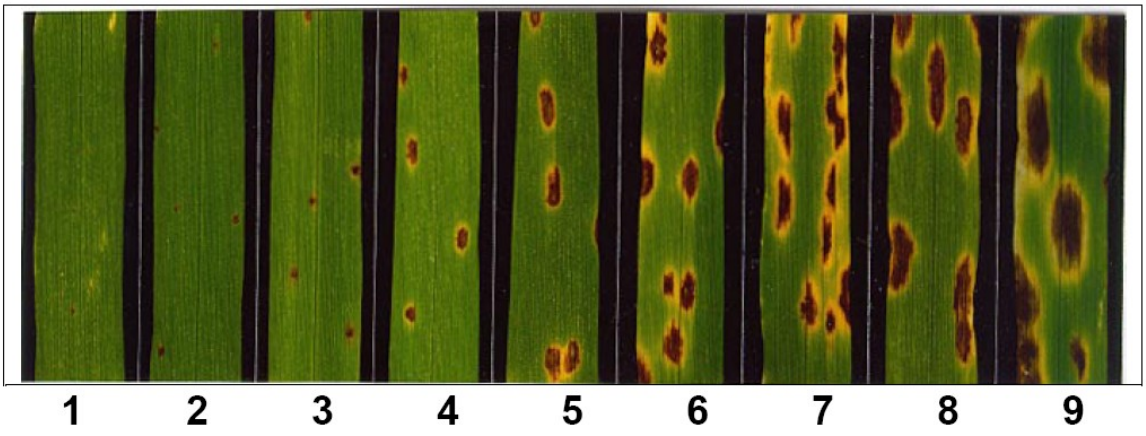
The experimental design for the field trials was completely randomized with 2 replicates. Field trials were conducted at Redlands Research Station, QDPI&F, in 2005 and 2006. The 2005 trial was overcome by the powdery mildew disease, caused by the fungal pathogen *Erysiphe graminis hordei*, and thus prevented an accurate phenotypic assessment of the spot blotch disease. Data from a previous phenotypic trail conducted at Redlands in 2004 was kindly supplied by Greg Platz. To prevent a powdery mildew epidemic in the 2006 trial, plants were sprayed with Triad-125 (Farmoz, St Leonards NSW 2065). The seed supplied for the 2006 seedling and field trials was dressed with the flutriafol, Vincit. The seed of each line was individually washed to remove excess seed dressing as follows: seed was placed in 250 ml Schott bottles, washed with 100 ml of de-ionised water (by shaking manually for 60 seconds), drained, and rinsed again, before being dried and stored for use in field trials.

Two replicates of each line were sown as hill plots of 10-20 seeds between spreader rows of Tallon (a very susceptible barley line planted around paired rows to increase disease development). Rows were spaced 80 cm from the spreader rows, with 75 cm between row spacing. Hill plots were spaced 0.54 cm apart. Spreader rows were sown on 16/6/06 (4 rows 25 cm apart @ 90 KG/Ha). Spreader rows were artificially inoculated with conidia suspension at Zadoks growth stage 32 (Z32 = stem elongation stage) and an epidemic promoted by regular sprinkler irrigation.

2.2.4.1 Assessment of Infection Response

Assessment of infection response was determined at approximately growth stage Z75 on a 1-9 scale (Fletch and Steffenson, 1999; Figure 8), similar to the rating scale for the seedling trial. Plots were scored 2 times in the 2004 trial and 3 times in the 2006 trial. The first rating was taken after anthesis (flowering), then at 2 week intervals during the grain fill period.

Figure 2-8. Infection rating scale for field screening for spot blotch (scale adapted from Fletch and Steffenson, 1999)



2.2.5 Seedling and Field Trial Data Analysis

Frequency histograms of host infection responses were generated in Microsoft Excel. Raw phenotypic data was screened for normality and homoscadiscity and analysed using SPSS for windows version 14.0. Chi-square analysis was conducted by separating progeny into two general categories (resistant : susceptible) at the seedling and adult stage of plant development, based on their mean infection response. Genotypes were broadly classified as resistant with an infection response rating of 2-5 and those with an infection response of 6-9 were considered susceptible, based on criterion previously described by Steffenson et al. (1996). The chi-square tests were applied for an expected ratio of 1:1 with one degree of freedom.

Analysis of variance for infection response of seedling and adult phenotypic data was conducted in SPSS for windows using univariate linear analysis, where genotype and replication were treated as fixed factors (i.e. the doubled haploid progeny from the ND/VB cross were homozygous and therefore without genetic variation within progeny lines, thus were considered fixed). Estimates of variance components were computed by equating mean squares to their expectations (Table 2). The genetic component of variance was estimated as: $\sigma^2_g = (M3-M1)/r$.

Table 2-2. Variance components used to calculate heritability for reaction to the spot blotch disease.

Source of variation	df	Mean Square	Variance expectation
Genotype	g-1	M3	$\sigma_{gr}^2 + r\sigma_g^2$
Replication	r-1	M2	$\sigma_{gr}^2 + g\sigma^2 r$
Error	(g-1) (r-1)	M1	$\sigma_{gr}^2 + \sigma^2_e$

Broad sense heritability of spot blotch resistance based on entry mean was calculated by the following formula;

$$H^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e/r)$$

Where: σ^2_g is the genetic variance among DH lines, σ^2_e is the error variance and r is the number of replications (Kutcher et al, 1994; Rahman et al., 2001).

2.3 Results

2.3.1 Statistical Analysis of Phenotypic Trials

The infection responses of parents and progeny were consistent between the replicates within the experimental trials, and a mean of the replicates was used in the analysis of the data. Summary statistics of the phenotypic trials (Table 2-2) revealed that the mean infection response was lower at the seedling stage of plant development. Further more, the seedling data displayed a greater range of infection responses to spot blotch (from highly susceptible to highly resistant) when compared with the range of infection responses observed under field conditions. Thus, the field trials exhibit a higher minimum infection response and lower maximum infection response than those expressed under glasshouse conditions and are considerably more negatively skewed towards susceptibility.

Table 2-3. Means, standard deviations and skewness for spot blotch phenotypic trials

Trial	N	Minimum	Maximum	Mean	Std. Dev.	Skewness
Seed 2005	180	2	9	6.07	1.74	-0.276
Seed 2006	149	2	9	5.85	2.14	-0.305
Field 2004	180	3.5	8.5	6.62	1.08	-0.580
Field 2006	180	3.8	8.3	6.46	1.01	-0.766
Mean of Seed Trials	180	2	9	5.98	1.81	-0.385
Mean of Field Trials	180	3.7	8.4	6.35	0.96	-0.692

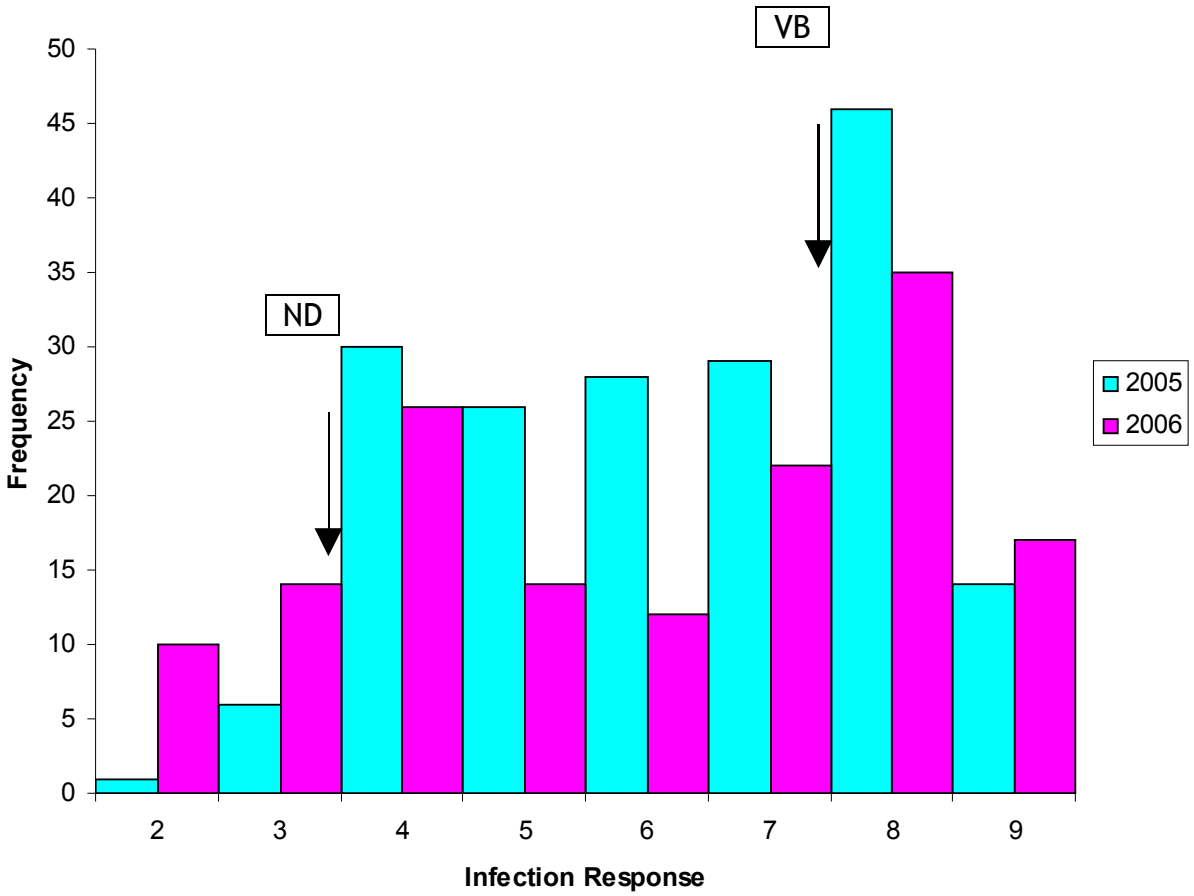
A large standard deviation was observed in the 2006 seedling trail. Thirty-two (32) missing lines were also recorded in the 2006 seedling trial, due to poor germination rate of the treated seed. A large degree of physiological damage was noted on these plants prior to inoculation and screening, which may have confounded the results of this trial.

The phenotypic data was screened for normality and homeoscadicity (equal variances). Both of these assumptions were invalid. Since transformed data, using a log transformation, still failed the normality test, raw data values were used in the analysis.

2.3.1.1 Seedling Trials

In both independent seedling trials the ND11231-12 (ND) parent exhibited a low infection response (resistant) and the VB9524 (VB) parent displayed a susceptible infection response to the spot blotch disease (Figure 2-3). The frequency of seedling infection response tended towards a bimodal distribution (resistant or susceptible), however many lines were considered intermediate in their response to the SB infection. A number of transgressive segregate lines were observed (lines which were more susceptible or more resistant than the parents).

Figure 2-9. Frequency of infection responses to the spot blotch disease at the seedling stage. Arrows represent mean parental scores (ND11231-12 = ND; VB9524 = VB).



2.3.1.2 Field Trials

The frequency distribution of the infection responses of progeny to the spot blotch infection displayed continuous variation under field conditions (Figure 2-4), indicating that resistance may be quantitative (conditioned by a number of genes) at the adult stage of plant development. Progeny of the segregating population were negatively skewed towards the susceptible parent VB, as most genotypes displayed an intermediate-to-susceptible reaction to spot blotch infection.

Figure 2-10. Frequency of infection responses to the spot blotch disease under field conditions. Arrows represent mean parental scores (ND11231-12 = ND; VB9524 = VB).

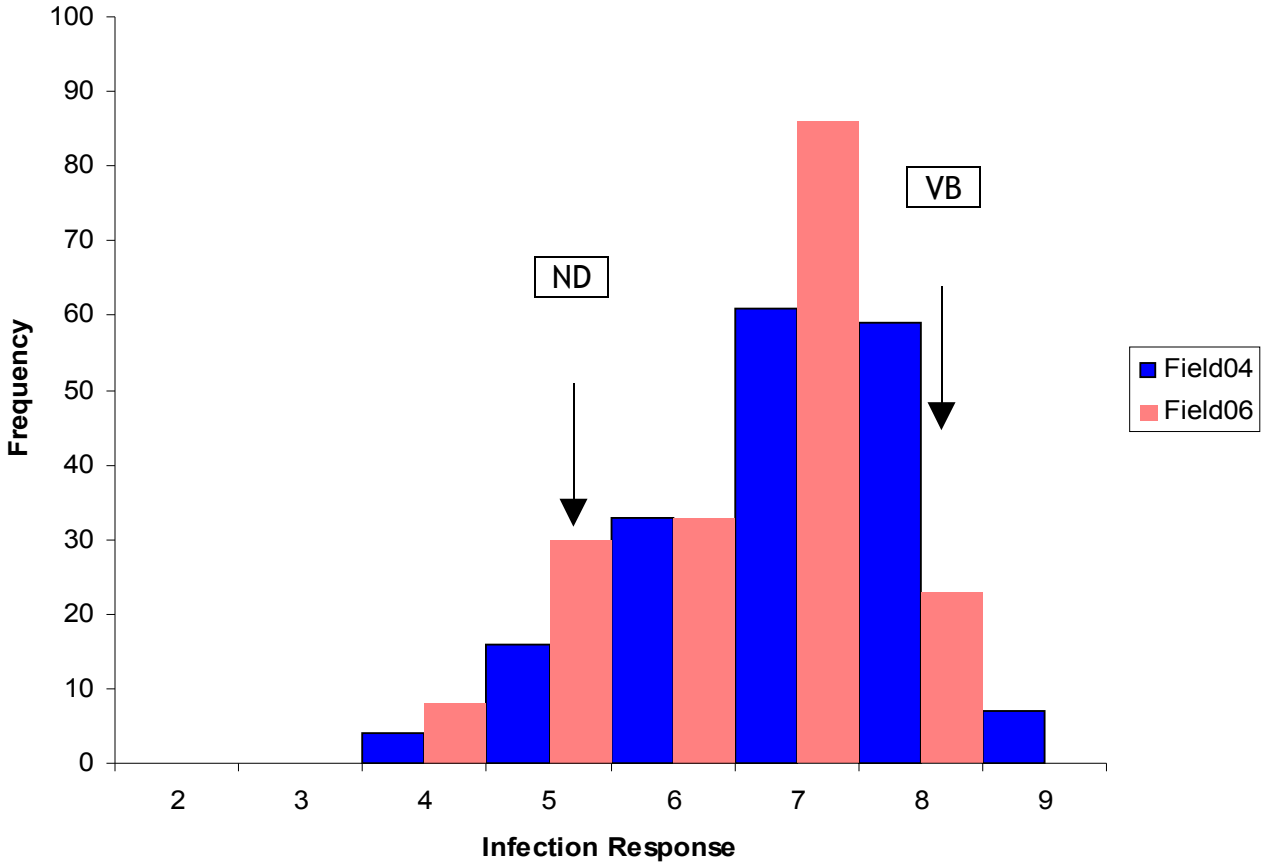


Table 2-4. Pearson's correlation between independent seedling and field trials.

	Seed 2005	Seed 2006	Field 2004	Field 2006
Seed 2005	1			
Seed 2006	0.755**	1		
Field 2004	0.632**	0.674**	1	
Field 2006	0.542**	0.500**	0.664**	1

** Correlation significant at the 0.01 level (2 tailed)

All correlations between the independent seedling trials and field trials (Table 2-3) were significant at the 0.01 level of significance. A good correlation was observed between the independent seedling trials, despite the limitations associated with the trial conducted in 2006. Lower correlations were observed between the seedling trials and the field trial conducted in 2006.

Table 2-5. The segregation ratios of spot blotch resistant and susceptible progeny and broad-sense heritability at the seedling and adult plant stages (ratio values of resistant: susceptible progeny represent the mean of the replicated trials).

	Number of resistant:susceptible progeny	X^2	*P* value	H^2
Seedling stage	65:101	7.807	0.0052	0.87
Adult Stage	27:119	82.689	<0.0001	0.83

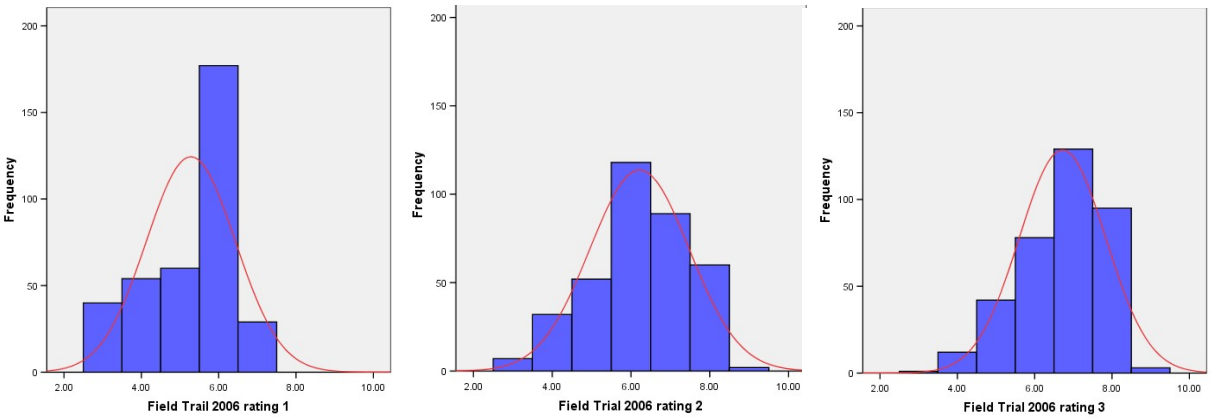
Chi-square analysis of the ratio of mean resistant and susceptible progeny at seedling and adult plant stages (Table 2-4) were not consistent with a single gene ratio. The segregation pattern at the seedling stage differed statistically significnat ($p = 0.0052$) from the expected 1:1 gene ratio if a single gene was conditioning resistance. The segregation pattern at the adult stage deviated with high significance (p-value of <0.0001) from the expected 1:1 gene ratio for a single gene trait. Further statistical analysis in the form of an independent samples t-test revealed that there is a significant difference between the mean infection response of seedling trials and field trials when variances are assumed to be unequal. This suggests that the mechanism for spot blotch resistance at the adult stage of plant development differs from the mechanism of resistance at the seedling stage of plant development.

Heritability estimates for spot blotch resistance, based on variance component analysis, revealed that the genetic component of variance was very large, with

heritability on a genotype mean basis ranging from 0.83 for adult to 0.87 for seedling resistance.

An examination of the progression of the spot blotch infection in the field over a 6 week period (Figure 2-5) revealed that the disease was quite virulent in the field environment at Redlands Bay. The frequency distribution of infection responses of progeny in the first rating (taken after anthesis) indicated that the lines predominantly exhibited an intermediate infection response; with a number of lines displaying resistance under field conditions and few lines displaying susceptibility to the spot blotch infection. However, by the third rating the distribution shifted to susceptibility; with very few lines displaying resistance to the spot blotch infection and most lines displaying a high intermediate-to-susceptible infection response.

Figure 2-11. Progression of the spot blotch infection in the field at two week intervals (rating 1-3). The red line represents the normal distribution curve.



2.4 Discussion

In all independent seedling and field trials ND11231-12 displayed resistance to spot blotch infection. The line ND11231-12 is a sister line of cultivar Logan, which originated from breeding programs in North Dakota and is known to carry good resistance to spot blotch. This resistance to the spot blotch infection can be traced back through ND7556 to Norbett which is presumed to carry the resistance of NDB112 (J. Franckowiak, personal communication, 2007). The NDB112 resistance has remained completely effective in the upper Midwest of the USA for over 40 years and is considered durable (Wilcoxson et al., 1990; Bilgic et al., 2006). Results of the phenotypic trials indicate that the resistance of NDB112 is also expressed under Australian environmental conditions to the Australian pathotype of *C. sativus* (SB61) employed in these trials. In a study examining the virulence variability of 35 *C. sativus* isolates from eastern and southern Australia (N. Knight, unpublished results), it was found that the North Dakotan lines ND11231-12 and ND B112 were completely resistant to all isolates tested. These results provide further evidence that the resistance of ND11231-12 will provide a valuable SB resistance source in Australia.

Chi-square analysis of the segregating progeny indicated that more than one gene conferred seedling resistance in the ND/VB population. The segregation ratio of resistant-to-susceptible progeny deviated statistically significant ($p = 0.0052$) from the 1:1 gene ratio expected if a single gene was conditioning resistance within this genetic background. In a comprehensive analysis of spot blotch seedling infection data from four DH populations, Bilgic et al. (2005), found that the segregation pattern of progeny within three populations (Steptoe/Morex, Harrington/Morex, and Harrington/TR306) were consistent with a single gene ratio. However, the progeny from the Dicktoo/Morex DH population did not fit a single gene ratio ($p<0.01$). The results of the current study provide further evidence that seedling resistance may be conferred by more than one locus in certain genetic backgrounds.

The continuous frequency distribution of the infection responses of genotypes in the field indicates that adult plant resistance is quantitative. This is supported by a goodness of fit test. Chi-square analysis of the segregating population indicated that adult plant resistance was highly significantly different ($p>0.0001$) from the 1:1 gene segregation ratio, suggesting that adult plant resistance is conditioned by a number of loci. These results are consistent with other studies. Bilgic et al. (2005) also found that the segregation pattern of progeny in the Dicktoo/Morex population was not consistent with a single gene ratio ($p=0.01$) and thus concluded that spot blotch resistance was controlled by more than one gene. Steffenson et al. (1996) reported that adult plant resistance was conditioned by 2 genes in the Steptoe/Morex population. Joshi et al. (2004) reported that adult plant resistance to spot blotch in spring wheat is controlled by three additive genes. Kutcher et al. (1994) also reported that resistance to spot blotch was quantitative.

The progression of the spot blotch disease over a six week period suggests that few lines are considered resistant to spot blotch under conducive environmental conditions (high temperature and humidity) late in the growing season. Lines that were identified as resistant based upon earlier phenotypic assessment often displayed a susceptible reaction to the disease late in the adult plant growing season (during grain fill). This suggests that the severity of the disease increases with plant maturation and this has important implications for phenotypic screening criteria (Joshi et al., 2004). In order to identify lines with suitable resistance plants must be screened at an appropriate time in the growing season, or screened a number of times. Joshi et al. (2004) recommended screening when the susceptible parent reaches maximum severity, prior to the late milk stage of plant growth. This might also suggest that these resistances are differentially expressed during plant development and may be less effective late in the plant's life cycle. Alternatively it might indicate a change in the growth of the pathogen on leaves that are beginning to senescence.

The phenotypic assessment of the ND/VB DH population appears to suggest that resistance is quantitative at both stages of plant development in the ND/VB population. To further elucidate the exact genetic mechanism of spot blotch resistance in the ND/VB population, a QTL mapping approach was undertaken to identify the number and position of QTL which condition resistance at both the seedling and adult stages of plant development.

3. Molecular Investigation of Spot Blotch Resistance

3.1 General Introduction

DNA marker technology is being increasingly used by plant breeding programs to improve their selection efficiency (Collard et al., 2005). This is because the use of molecular markers has the potential to increase the precision and efficiency of selection methods by largely eliminating the need of intensive phenotypic assessments, and thus significantly reduce the screening costs and time taken to develop new varieties.

The advent of DNA marker technology has also increased our understanding of the genetic basis of agronomical important traits, such as disease resistance. Such traits can be controlled by a single major gene or a multiple number of major and minor genes. Major gene, or race-specific resistance, such as that of powdery mildew of barley, is easily incorporated into plant breeding programs; however this form of resistance is prone to break down over a short period of time as new pathotypes evolve. For diseases such as net form of net blotch (Raman et al., 2003), resistance is controlled by a number of minor genes and is referred to as quantitative or non-race specific resistance. This form of resistance is the preferred type of resistance for plant breeding (due to its durability) but is often more challenging to identify and incorporate into cereal breeding programs.

The genetic basis of spot blotch resistance in barley is currently poorly understood. There are no reports on the molecular genetics of resistance to spot blotch in Australia; however a few studiers have been conducted in the USA. Steffenson et al. (1996), working with a Steptoe/Morex DH population, observed that seedling resistance to spot blotch was monogenetically inherited and was governed by a gene (*Rcs5*) on the short arm of chromosome 7H. This gene has also been identified in the DH population Harrington/Morex (Bilgic et al., 2005), and work is currently being conducted in the USA to clone this gene. A study by Bilgic et al., (2006) also

revealed that at the seedling stage of plant development, different pathotypes of *C. sativus* invoked a single gene response in the Calicuchima-sib/Bowman-BC DH population. Resistance to Pathotype 1 was conferred by a single QTL on chromosome 4H and resistance to Pathotype 2 was conditioned by a single gene on chromosome 1H, designated the *Rcs6* gene.

The indication of a single gene responsible for spot blotch resistance has been challenged in other studies. A further report by Bilgic et al. (2005, refer to table 2, p1244), comparing the differential expression of spot blotch resistance in four different DH populations, reported that in three of the populations tested, seedling resistance was conditioned by a number of genes. Three regions controlled the expression of seedling resistance to spot blotch in the Dicktoo/Morex DH population. A major QTL was identified near *Rcs5* on chromosome 7H explaining 30% of the phenotypic variance. Two minor QTLs were identified; one near the centromere of chromosome 7H (inherited by the susceptible parent and explaining 9%) and one on the short arm of chromosome 3H explaining 19% of the phenotypic variation. In the Steptoe/Morex and the Harrington/TR306 populations two genes conditioned resistance. In these populations the *Rcs5* gene (7HS) was again linked to seedling resistance, and minor QTLs were also detected on chromosomes 3HL and 7HL, respectively. This study demonstrates that different mechanisms condition seedling resistance to spot blotch in different genetic backgrounds, highlighting the complex nature of the plant-pathogen interaction and the expression of disease resistance.

Multigenic disease resistance has previously been reported for spot blotch at the adult stage of plant development. In the study conducted by Steffenson et al. in 1996, adult plant resistance in the Steptoe/Morex population was conditioned by two QTL. The largest QTL effect was detected on chromosome 1H, explaining 62% of the phenotypic variance, and a second QTL of minor effect was detected on chromosome 7H, explaining 9% of the phenotypic variation. Bilgic et al. (2005) further reported that adult plant resistance to spot blotch was also multigenic in the

Dicktoo/Morex population. Three QTLs were identified, one on the short arm of chromosome 3H explaining 36% of the phenotypic variation, a second on the long arm of chromosome 3H explaining 11% of the phenotypic variance, and a third on chromosome 7H (at or near *Rcs5*) explaining 20% of the phenotypic variance. Despite this, a single gene evoked adult plant resistance in the related Harrington/Morex population.

It is apparent that there are conflicting reports regarding the nature of resistance to spot blotch in the glasshouse and the field. The phenotypic assessment of the spot blotch disease in the DH population ND/VB suggests that both seedling and adult plant resistance is controlled by a number of genes in this population. To confirm this observation and further elucidate the exact genetic mechanism of spot blotch resistance, a molecular investigation of the ND/VB DH population was undertaken to identify the genomic regions (QTL) associated with spot blotch resistance at both the seedling and adult stages of plant development.

The specific aims of the work reported in this chapter are:

1) To enhance the ND/VB map already available (Emebiri et al., 2005) by applying appropriate map curation techniques (Lehmensiek et al., 2005) and increasing marker densities in regions of interest;

2) To identify QTL conferring spot blotch resistance at the seedling and adult plant stages of development; and

3) To validate the QTL identified for seedling and field resistance in a related population.

3.2 Materials and Methods

3.2.1 Linkage Mapping of the ND/VB Population

A framework molecular map of the DH population VB/ND was originally produced by Emebiri et al. (2005). This map was constructed with 270 markers: 197 AFLPs; 23 RFLPs; 43 SSRs; 6 SSR derived ESTs and 1 RAPD marker (Emebiri et al., 2005). After Emebiri et al. (2005) removed redundant markers (i.e. those markers that co-segregated with others), 211 markers were left, covering 2016.0 cM of the genome with an average interval length of 9.6 cM.

3.2.2 Map Curation

Weaknesses in previously published genetic maps, particularly the order of markers and overall map distances, have led to the development of software with advanced algorithms for the linkage analysis of markers. Recently specific guidelines to improve the efficiency and accuracy of genetic map construction have been published (Lehmensiek et al., 2005). Map curation of the ND/VB linkage map was undertaken to improve the quality of the original ND/VB linkage map.

Detailed genotypic data for the 270 markers was provided by Lavinius Emebiri (Department of Primary Industries, Victoria). Linkage analysis of markers was initially carried out manually within MapManager QTXb20 (Manly et al., 2001) using the "links report" function, and the order of markers was confirmed using the program RECORD (Van Os et al., 2005). Following RECORD analysis, double crossovers within a flanking marker distance of less than 25 cM were considered as genotyping errors and the corresponding score was changed to a missing score. Redundant loci were not included in the QTL analysis.

3.2.3 QTL Analysis

Composite interval mapping was undertaken using Windows QTL Cartographer version 2.0 (Wang et al., 2001-2004), employing model 6 with a 10 cm window and 2 cm walk speed. One thousand (1000) permutation tests at 2 cM intervals were conducted to determine significance thresholds for QTL detection.

3.2.4 Epistatic Interactions Between QTL

All identified QTL were examined for the presence of digenic epistatic effects using the program QTLNetwork 2.0 (Yang et al., 2008). QTL Network is a recently developed software package for detection of main effect QTL, QTL x QTL (i.e. epistatic) interactions, and QTL x environment effects.

3.2.5 Fine Mapping with SSRs

The molecular map ND/VB consisted of a high density of AFLPs markers in some regions of the genome. Genomic regions deemed important for the expression of resistance through QTL analysis were screened with a total of 21 SSR and SSR-EST markers. Candidate SSR markers were selected from published barley consensus maps (Wenzl et al., 2006). Primers were synthesized by Invitrogen (Mount Waverley, Victoria, Australia). Thermal cycling conditions for SSR markers were as recommended by GrainGenes. SSR markers were initially screened across both parents and polymorphic markers were mapped to the entire population using MapManager QTXb20. Marker regression was undertaken to determine the marker-trait association.

3.2.6 DNA Extraction and Quantification

Approximately 200 mg of collected leaf material were placed into 96-well extraction plates with five ball bearings and shredded using a QIAGEN DNA shredder. DNA extractions were performed with the Wizard Genomic DNA extraction kit (Promega) as per the manufacturer's instructions. DNA concentration was determined by agrose gel electrophoresis and adjusted to a final concentration of 10 ng/μL . DNA samples were amplified by Polymerase Chain Reaction (PCR). PCR was conducted in a 10 μL reaction containing: 500 nM of each primer; 1.5 mM $MgCl_2$; 200 μM of each dNTP; 1 x PCR buffer; and 0.5 U *Taq* DNA polymerase (Bioline). Thermocylcing was carried out in Biometra TGradient machines, with an initial 5 min 94˚C denaturation step, followed by 30-45 cycles of: 94˚C for 30 s or 1 min; 50-60˚C (depending on the annealing temperature) for 30 s or 1 min; and 72˚C for 30 s or 1 min.

Electrophoresis of PCR product was performed on Bio-Rad Sequi-Gen GT Sequencing Cells. A gel mix (composed of 15 mL of 40% acrylamide/bis-acrylamide (29:1; Astral Scientific), 15 mL of 40% (w/v) urea, and 6 mL of 10 x TBE (890 mM Tris, 890 mM boric acid, and 20 mM EDTA) was prepared and MilliQ water was added to a final volume of 60 mL. 600 μL of ammonium persulfate (10% w/v) and 60 μL of TEMED was added to the gel mix. The gel mixture was poured between two glass plates (spaced 0.4 mm apart). One of the plates had previously been treated with bind silane and the other with Rainex. Five μL of PCR product was loaded on to the gel and the gel was run at 60W for 1.5 hours. Visualisation of DNA was carried out according to the silver staining procedure previously described by Sourdille et al. (1998). Briefly, the gel was fixed for 10 minutes in 7.5% glacial acetic acid, which was followed by 3 x 2 minute rinses with MilliQ water. The gel was stained for 30 min in a solution containing 0.1% silver nitrate and 0.05% formaldehyde. After a quick rinse (approximately 10 seconds), development was achieved by adding a solution of 3% sodium carbonate, 0.05% formaldehyde, and 2 mg/L sodium thiosulphate. The developing reaction

was stopped by the addition of 7.5% glacial acetic acid. The gel was then rinsed in MilliQ water, allowed to dry, visually scored and scanned for preservation of the image.

3.2.7 Validation Analysis

The QTL identified for seedling and field spot blotch resistance in the DH population ND11231*12/VB9534 were validated using the population ND11231*11/W12875*17 (84 DH lines). ND11231*11 has the same pedigree as Logan, released by the North Dakota Agricultural Experimental Station in 1995, and is known to have good spot blotch resistance (J. Franckowiak, personal communication, 2006). This population was phenotyped for spot blotch resistance at the seedling stage in the glasshouse and the adult stage in the field (as per methods in Chapter 2). Flanking markers identified by QTL analysis of the ND/VB population were screened across the parents to determine if these markers were also polymorphic in the validation population. Marker regression was undertaken to determine the marker-trait association and to examine whether the QTL conferring spot blotch resistance in the ND/VB population were expressed in this related population.

3.3 Results

3.3.1 Map Curation

Emebiri et al. (2005) originally produced a genetic linkage map of the ND/VB population to identify regions of the barley genome that influence variations in grain protein concentration. In order to review the ordering of markers in this map following inclusion of extra markers, the recently developed software package RECORD was employed, which orders markers by minimizing the total number of recombination events (Van Os et al., 2005). The linkage analysis of markers using the program RECORD altered the marker order of the original map on chromosomes 2H, 3H, 5H and 7H (Figure 3-1). In some cases the re-ordering of markers significantly decreased the length of the chromosome (i.e. 6H). However, in other cases the chromosomal length was marginally increased. In these instances, this was due to the increase in the number of markers in the curated map compared to the original map. The final map was composed of 244 markers, as opposed to the original map of 211 markers. A small group of five markers were unlinked to any other marker and therefore were excluded from the map.

The process of removing double-crossovers between markers less than 25 cM apart resulted in substantial decreases in the map distances in the original map (Table 3-1). In the original map the total length of the map was 2016 cM, with an average chromosomal length of 288 cM. Through the map curation process, a total of 597 double cross-overs were removed from the map. This resulted in the reduction in the overall map distance to 1275.5 cM, with an average chromosome length of 182.2 cM. In addition 23 redundant loci were also removed.

Figure 3-12. Revised genetic linkage map of the doubled haploid population ND11231*12/VB9524.

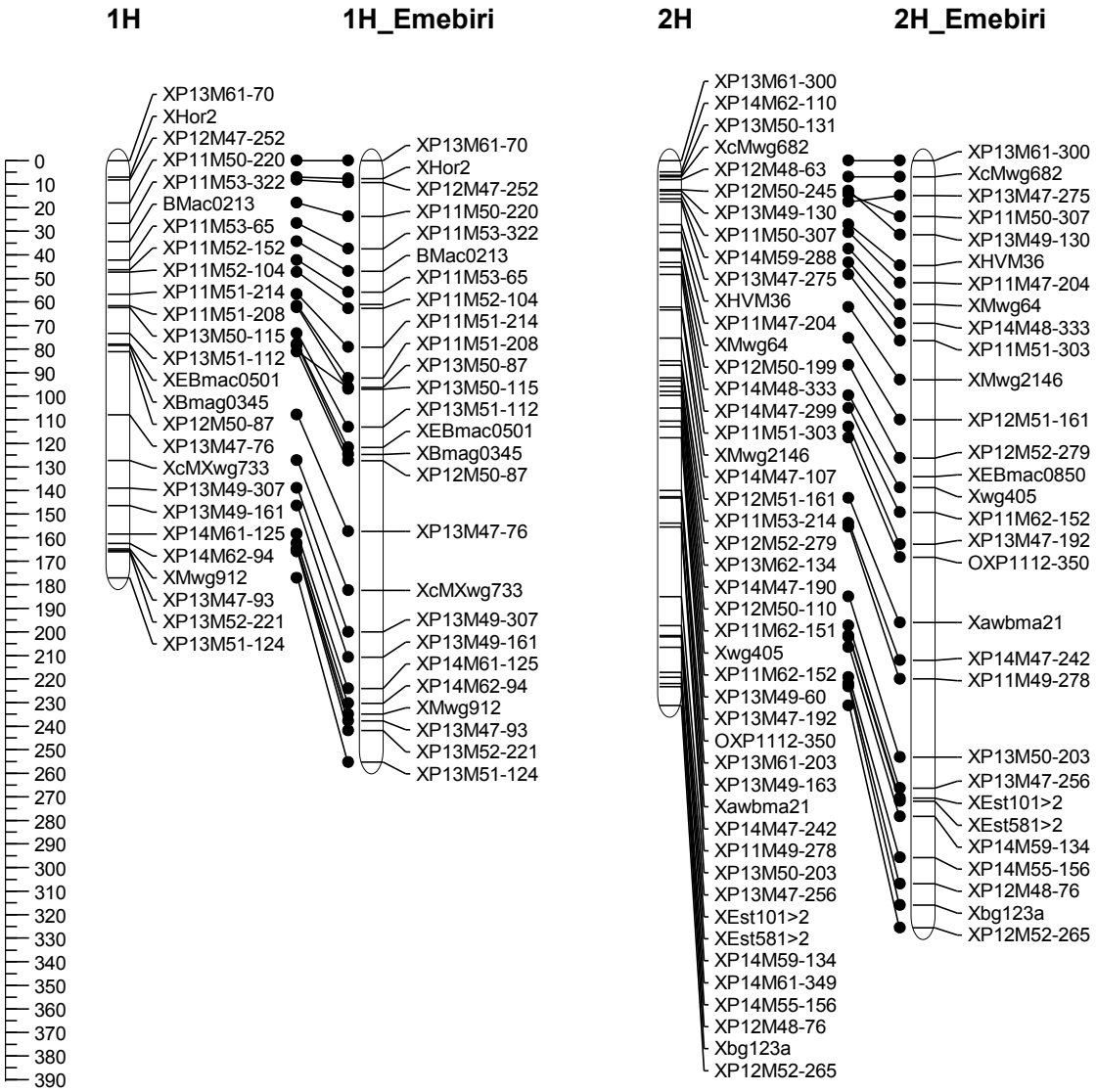


Figure 3-1. (Continued)

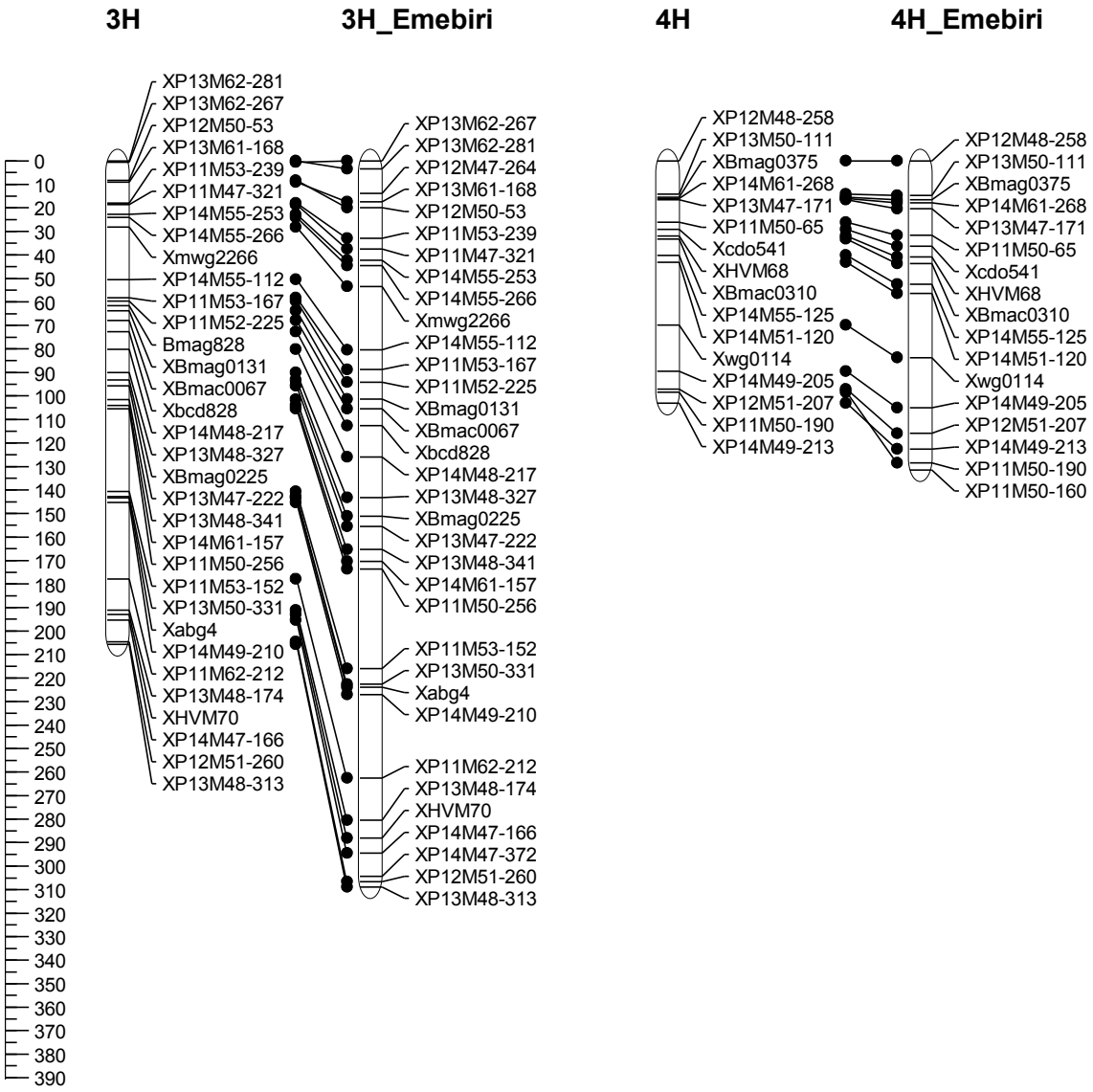


Figure 3-1. (Continued)

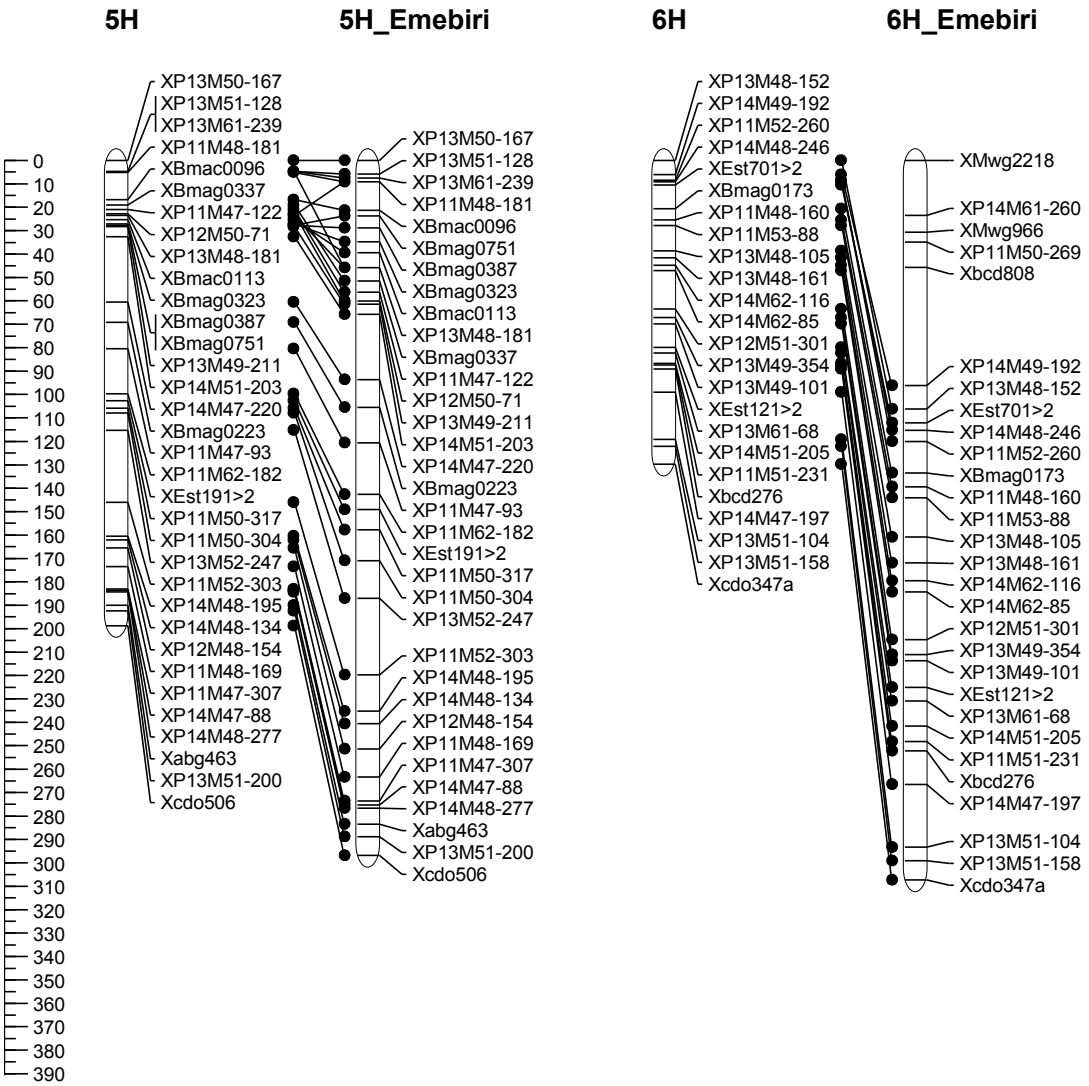


Figure 3-1. (Continued)

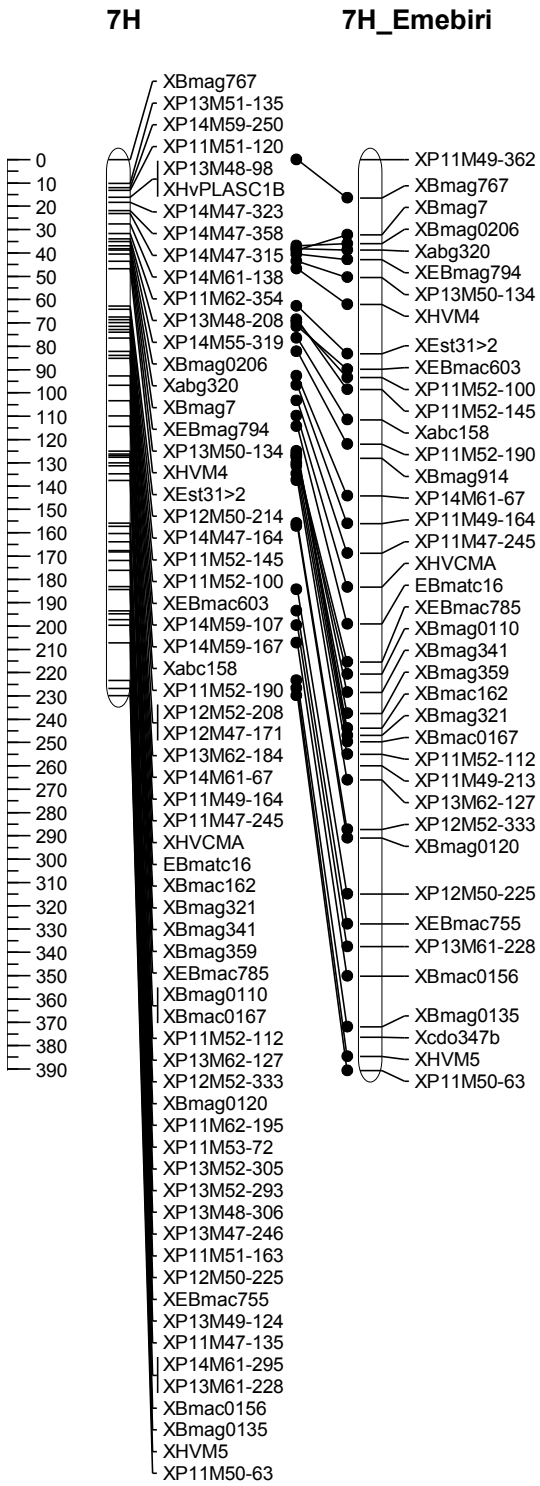


Table 3-6. Summary of map curation, comparing the original map by Emebiri et al. (2005) with the curated map.

Chromosome	Emebiri et al. (2005) (cM)	Record (cM)	No.2X's	No.red.loci	Distance (cM)
1H	255.3	177.1	66	1	177.1
2H	325.6	351.9	105	1	231.3
3H	308.9	311.9	119	3	205.7
4H	131.4	131.7	15	1	103.1
5H	296.8	295.9	56	0	198.7
6H	307.3	211.9	55	0	129.8
7H	390.7	494.8	181	17	229.8

No.2X's=Number of double crossovers removed

3.3.2 QTL Identification and Analysis

The critical likelihood ratio statistic values (LRS) for QTL detection, based upon permutation tests, indicated that QTL above a LRS threshold of 6.7 were suggestive; QTL above the LRS threshold of 12.8 were significant, and QTL above the threshold of 19.6 were considered as highly significant in conferring resistance to spot blotch. QTL analysis using CIM of the spot blotch seedling and field trials revealed that resistance at both stages of plant development is controlled by a number of loci.

3.3.2.1 Seedling Resistance

QTL analysis of the seedling trials revealed that seedling resistance to spot blotch is conditioned by a number of loci (Figure 3-2, Table 3-2). The most significant QTL detected, located on the short arm of chromosome 7H, was identified in both seedling trials and explained approximately 62% of the phenotypic variation for disease severity. This QTL was contributed by the ND parent and the marker XEst3 1>2 had an LRS of 145.23 in the 2005 seedling trial and an LRS of 194.45 in the

2006 trial. The high LRS values for this QTL are indicative of a major effect of this region in conditioning seedling resistance in this population. This QTL is flanked by the SSR marker hmv4 and RFLP marker abc158 and is highly associated with the SSR markers Bmag794 and Bmag603.

Other minor suggestive and significant QTL were detected from analysis of seedling resistance data. In the 2005 seedling trial, a suggestive QTL contributed by the resistant parent was detected on the short arm of chromosome 1H, with an LRS of 8.62 and explaining 1.52% of the phenotypic variance. Other QTL for seedling resistance were detected on chromosome 5H, contributed by both the resistant parent ND and the susceptible parent VB, but these QTL were not consistent across both trials. A suggestive QTL contributed by the susceptible parent in 2005 was detected on the short arm of chromosome 5H, with an LRS of 8.34 and explaining approximately 2% of the phenotypic variance. A peak was detected on the end of chromosome 5H for the 2006 trial in the same region as the QTL identified in the 2005 trial, but it fell just below the suggestive threshold for QTL detection. The most significant QTL located on chromosome 5H was contributed by the susceptible parent in the 2006 trial, with a LRS of 16.55 and explaining approximately 3% of the phenotypic variance.

A region near the centromere of chromosome 5H, contributed by the ND parent, was suggestive in both the 2005 and 2006 seedling trials. In 2005 marker XP11M47-93 had the highest association with resistance, with an LRS of 9.76 and explaining almost 3% of the phenotypic variance. In the 2006 trial marker Bmag223 had an LRS of 11.64 and explained over 2% of the phenotypic variance for disease severity.

3.3.2.2 Adult Plant Resistance

Composite interval mapping (CIM) of the field trials identified numerous QTLs conferring adult plant resistance to spot blotch (Figure 3-2, Table 3-2). Two highly

significant QTLs were consistently identified in both trials for field resistance on chromosome 7H and 3H, contributed by the resistant parent ND.

The 7H QTL identified for field resistance mapped to the same region as the seedling resistance QTL on 7H and is highly associated with the SSR markers Bmag794 and Bmag603. This QTL was highly significant, with an LRS of 157.3 and explaining approximately 45% of the phenotypic variance in 2004 and an LRS of 119.3 and explaining approximately 42% of the phenotypic variance for disease severity in 2006. A QTL on the short arm of chromosome 3H was also highly significant for resistance in both field trials. The 3H region had an LRS ranging from 56.66 in 2006 to 73.94 in 2004 and explained approximately 15% of the phenotypic variance for disease severity in each trial. This QTL is associated with the AFLP marker XP13M61-168.

Other minor QTLs, contributed by both the resistant and susceptible parent, were identified on chromosomes 1H, 2H, 4H, 5H, 6H, and 7H. However, these results were not consistent across both trials. Of the ND-inherited QTL, a suggestive QTL on chromosome 1H was detected in the 2006 trial, with an LRS 8.62 and explaining 1.52% of the phenotypic variance; and a suggestive QTL on the distal end of chromosome 2H was detected in 2004 with LRS of 9.32 and explaining 1.71% of the phenotypic variance. Another suggestive QTL was detected on chromosome 6H in 2004, with an LRS 9.96 and explaining almost 1.86 % of the phenotypic variance for disease severity. A minor suggestive QTL was detected on the end of the long arm of chromosome 7H in 2006, also inherited by the resistant parent, with marker Bmac156 having an LRS of 10 and explaining 2.93% of the phenotypic variance.

Other minor significant and suggestive QTLs were identified on chromosome 2H, 4H and 5H, inherited from the susceptible parent VB. A significant QTL was detected on chromosome 2H in 2004 and one suggestive QTL was detected on chromosome 2H in 2006. The most significant of the QTL identified on 2H was located on the short arm of the chromosome with marker XP11M47-204 having an

LRS 17.08 and explaining over 4% of the phenotypic variation for disease severity. A large region on chromosome 4H was suggestive for field resistance in both trials, also inherited from the susceptible parent VB. However, the peak of the QTL for 2004 was in a different region of the chromosome than the peak for the 2006 trial. A minor QTL on chromosome 5H was significant in 2006, with an LRS of approximately 17.89 and explaining 4.03 % of the phenotypic variance for disease severity. This QTL however was not detected in the 2004 field trial.

Figure 3-13. QTLs identified by composite interval mapping of seedling and field trials using the program QTL Cartographer. Horizontal red, purple and blue lines represent thresholds for suggestive, significant, and highly significant QTL respectively. The additive effect [a(H1)] of the QTL are also shown – a positive additive effect indicates that the QTL was inherited from the VB parent; a negative effect indicates that the QTL is inherited from the ND parent.

a) Chromosome 1H

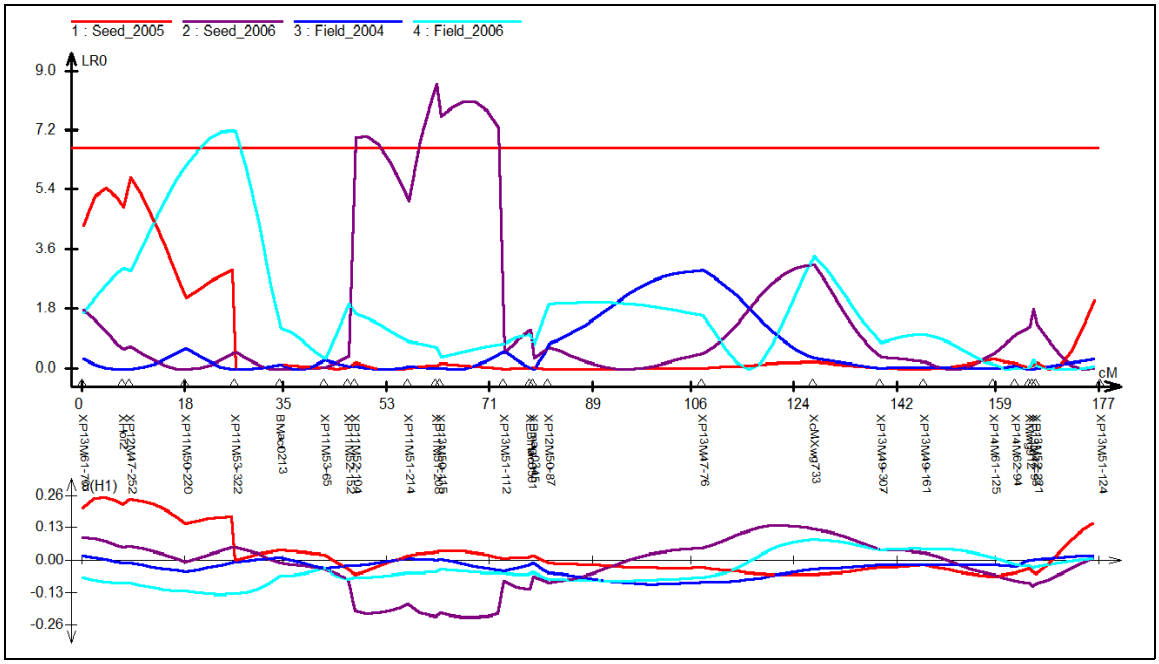
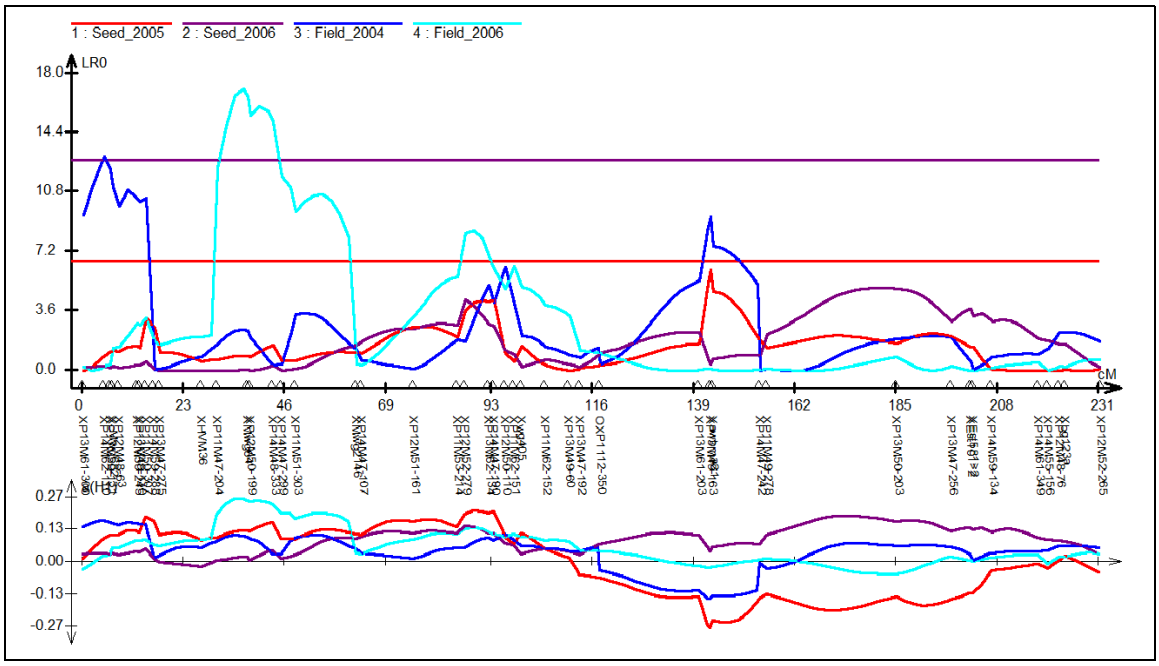


Figure 3-2. (Continued)

b) Chromosome 2H



c) Chromosome 3H

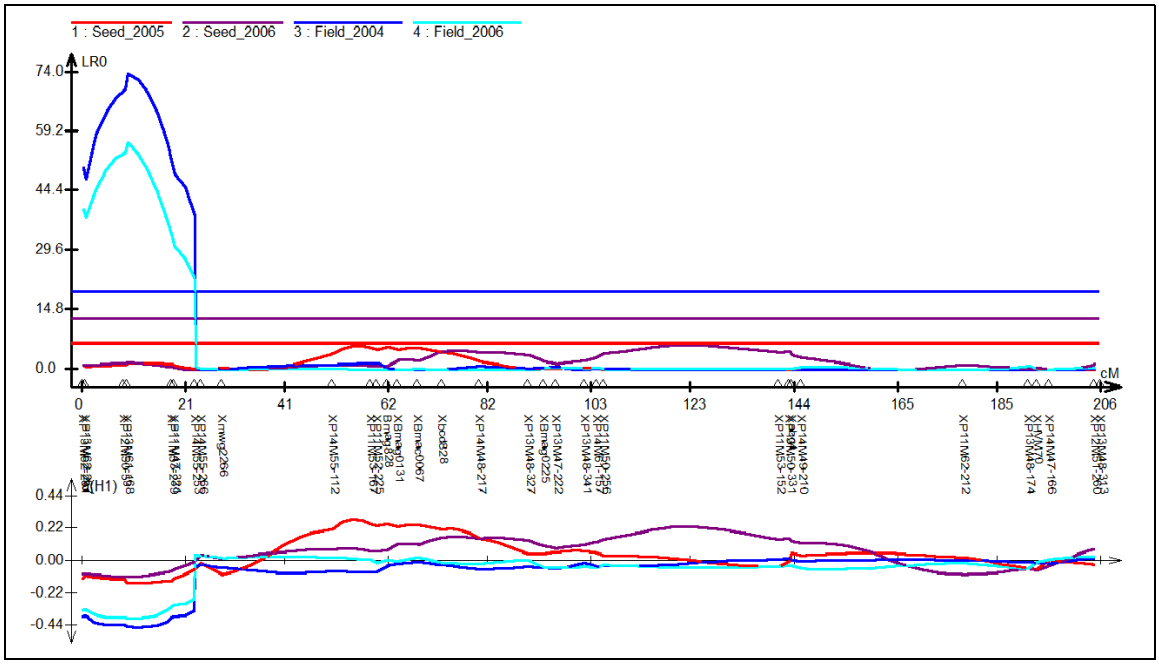
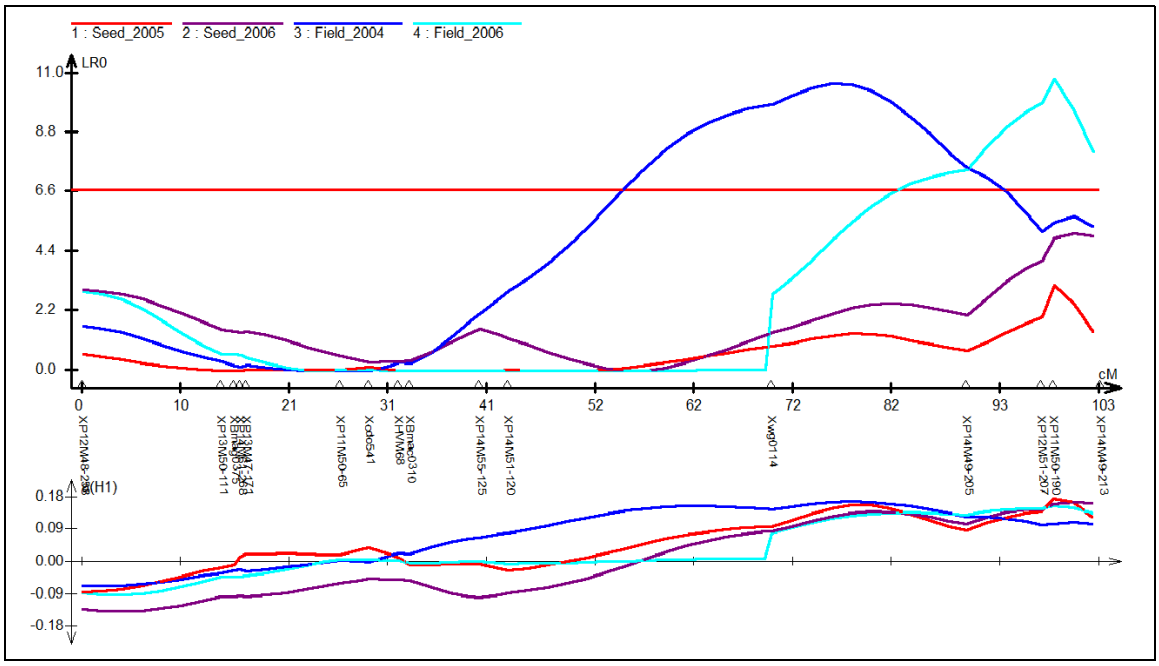


Figure 3-2. (Continued)

d) Chromosome 4H



e) Chromosome 5H

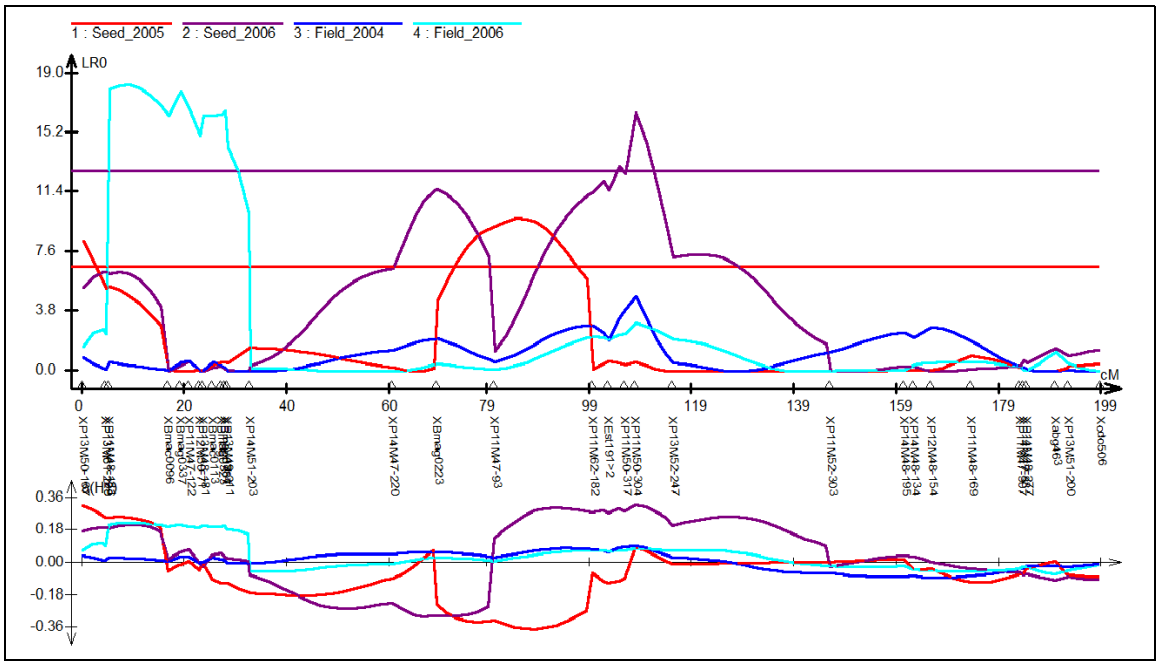
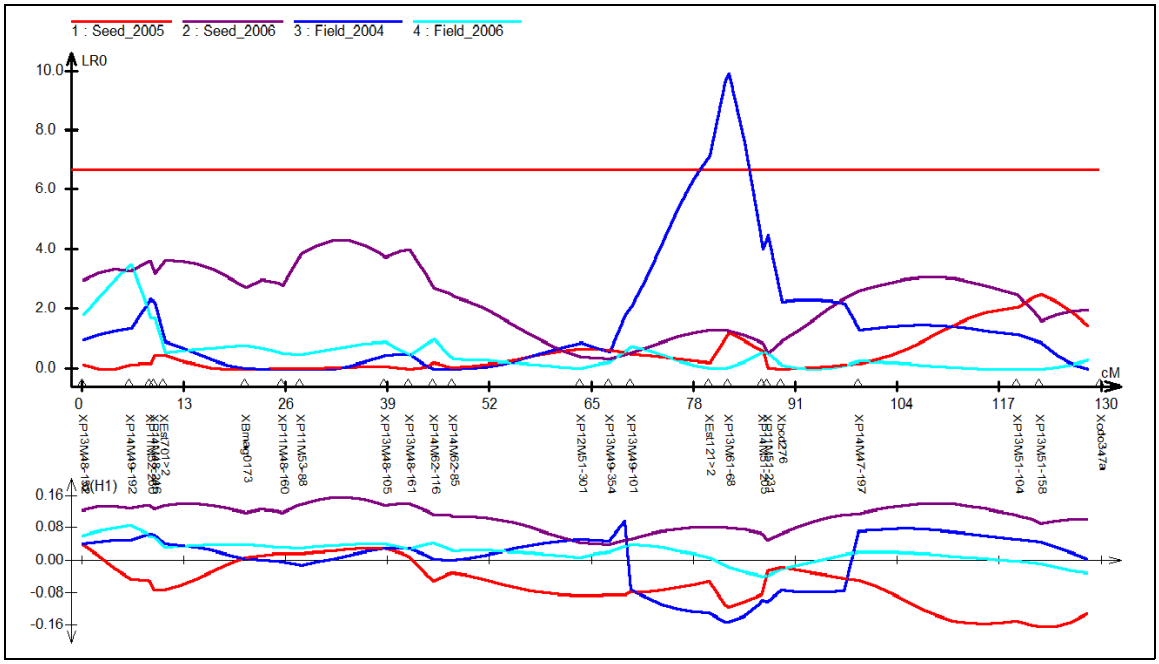


Figure 3-2. (Continued)

f) Chromosome 6H



g) Chromosome 7H

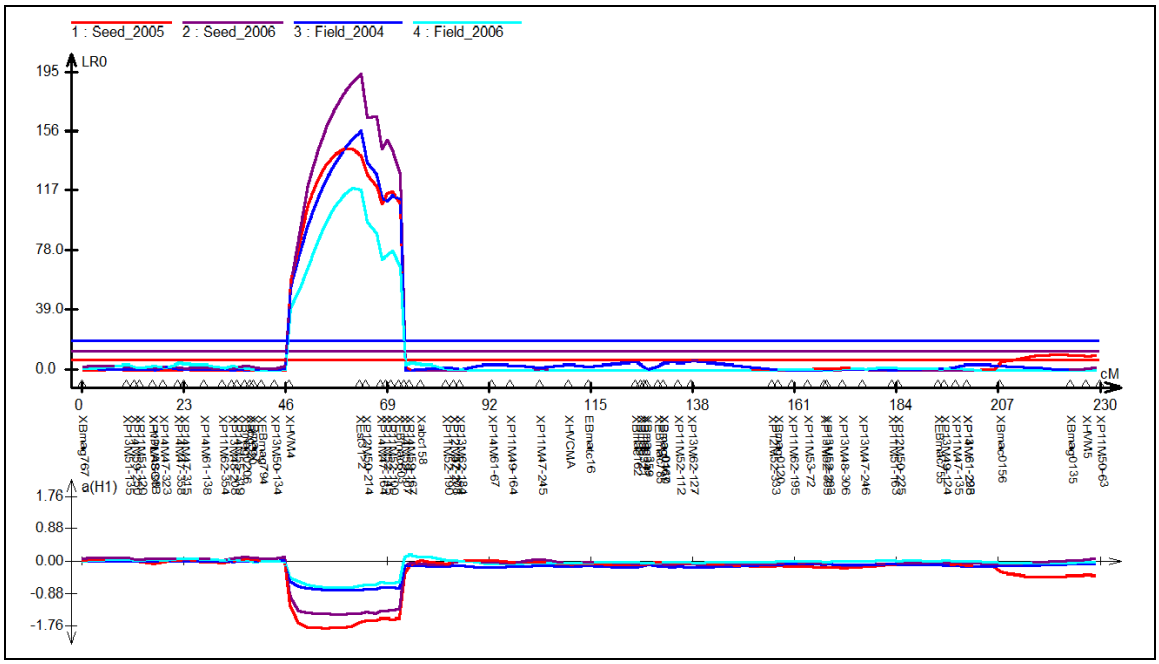


Table 3-7. Summary of QTL identified using QTL Cartographer for spot blotch resistance seedling and field resistance in DH population ND11231-12/VB9534

		SEEDLING RESISTANCE								FIELD RESISTANCE							
		2005				2006				2004				2006			
Chr.	Marker	LRS	%	Sig.	Par.	LRS	%	Sig.	Par.	LRS	%	Sig.	Par.	LRS	%	Sig.	Par.
1H	XP11M53-322													7.22	1.54	S	ND
	XP11M51-208					8.62	1.52	S	ND								
2H	XP14M62-110									12.99	2.41	SIG	VB				
	XP11M47-204													17.08	4.09	SIG	VB
	XP12M52-279													8.5	1.91	S	VB
	XP13M49-163									9.32	1.71	S	ND				
3H	XP13M61-168									73.94	16.22	HS	ND	56.66	14.28	HS	ND
4H	XP14M49-205									10.62	2.19	S	VB				
	XP11M47-190													10.81	2.39	S	VB
5H	XP13M50-167	8.34	1.93	S	VB												
	XP11M47-122													17.89	4.03	SIG	VB
	Bmag223					11.64	2.24	S	ND								
	XP11M47-93	9.76	2.71	S	ND												
	XP22M50-304					16.55	3.03	SIG	VB								
6H	XP13M61-68									9.96	1.86	S	ND				
7H	XEst3 1>2	145.2	62.44	HS	ND	194.45	61.29	HS	ND	157.33	44.85	HS	ND	119.3	42.16	HS	ND
	Bmac156									10.06	2.93	S	ND				

LRS = Likelihood ratio statistic
% = Percent of phenotypic variation explained
Sig. = Significance thresholds for QTL (S=Suggestive, SIG=Significant, HS=Highly Significant)
Par. = Contributing Parent
Red = highly significant QTL from ND Blue = Significant QTL from VB

3.3.3 Epistatic Interactions between QTL

All identified QTL were examined for the presence of digenic epistatic effects using the program QTL Network. No episatic interactions between the QTL identified for seedling and field resistance were detected by the program.

The program QTL network also identifies main effect QTL (Table 3-3), and was used to provide a comparison with the results obtained using QTL Cartographer. QTLNetwork identified a single major QTL on chromosome 7H for seedling resistance, explaining approximately 60% of the phenotypic variance for disease severity. Three QTL were identified for field resistance; a major QTL on chromosome 7H, in the same interval as the seedling QTL, and a major QTL on chromosome 3H, both contributed by the resistant parent, together explained approximately 43% of the phenotypic variance. A third minor QTL on chromosome 2H contributed by the susceptible parent, explained only 2.71% of the phenotypic variance for disease severity. The values for the % of phenotypic variance explained by the marker are lower than those reported by QTL Cartographer and only the QTL identified as significant by CIM were identified using QTL Network.

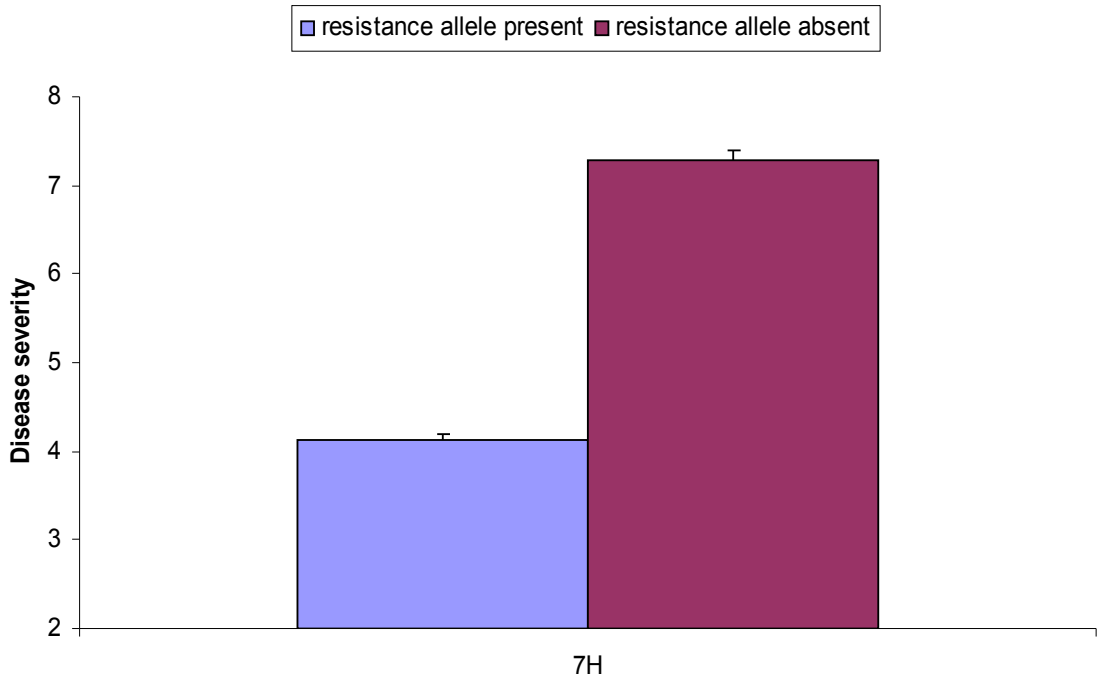
Table 3-8. QTL identified for spot blotch resistance using QTL Network program.

			Seedling Resistance		Field Resistance	
CH.	Marker-Interval	Parent	% P.V.E	P-value	% P.V.E	P-value
7H	XEst31>2 – XP12M50-214	ND	60.46	0.00000	27.31	0.00000
3H	XP13M61-168 – XP11M53-239	ND	-	-	15.35	0.00000
2H	XP13M47-275 – HVM36	VB	-	-	2.71	0.00000

% P.V.E. = Percent of phenotypic variance explained
CH. = Chromosome

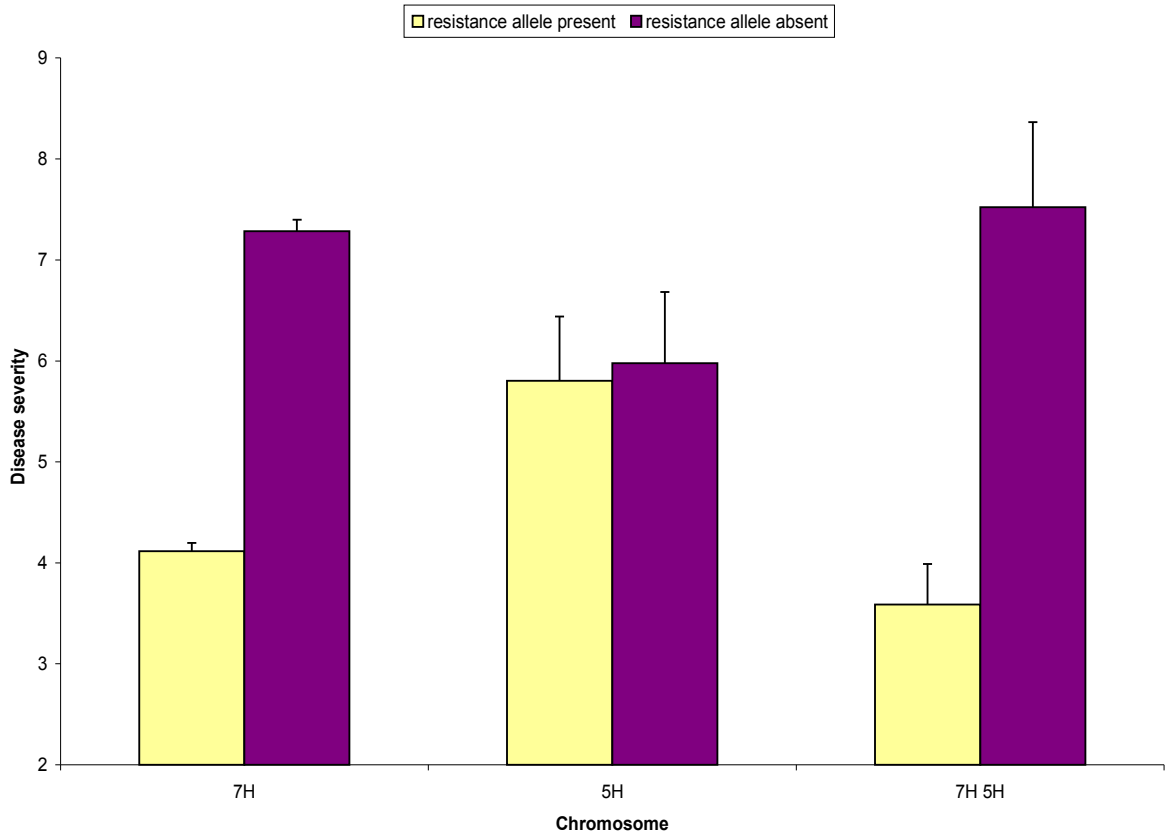
Further investigation of the mean infection response of the doubled-haploid lines at the seedling stage, with the resistance parent allele on chromosome 7H present and absent (Figure 3-3), indicated that lines with this allele present had a mean disease severity of 4.12 and those lines with the susceptible parent allele had a mean disease severity of 7.28. A student t-test revealed that this difference is significant at the 0.001 level of significance.

Figure 3-14. Mean disease severity of seedlings of doubled-haploid lines with the 7H QTL present and absent.



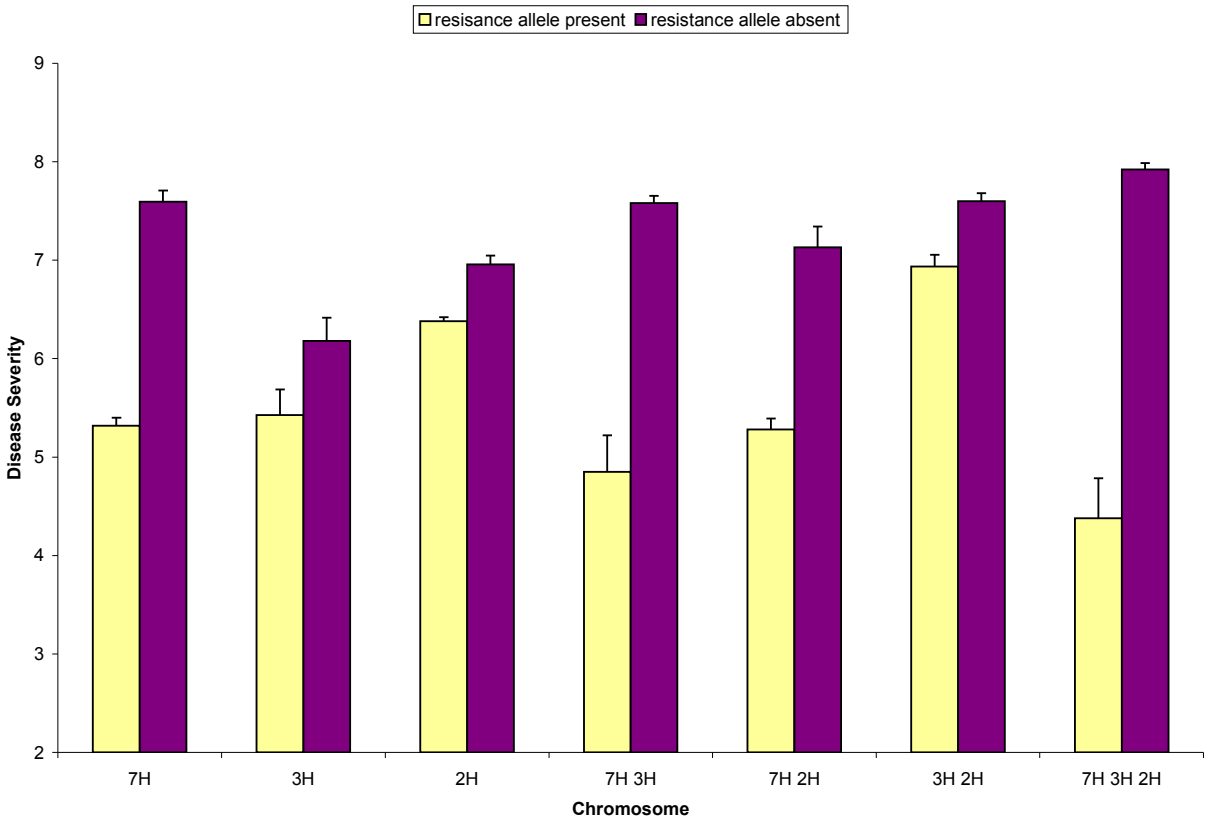
QTL Cartographer identified another putative minor QTL for seedling resistance on chromosome 5H in 2006, inherited from the susceptible parent VB. Further investigation of the mean disease severity of lines with combinations of the 7H and 5H resistance alleles in 2006 (Figure 3-5) revealed that lines with both the 7H and 5H QTL had a mean disease severity significantly lower than lines that contained only the 7H resistance allele

Figure 3-15. Mean disease severity of doubled-haploid lines with combination of alleles for seedling resistance.



The mean disease severity of doubled-haploid lines with combinations of resistance alleles for field resistance (Figure 3-4) revealed that lines containing the 7H, 3H and 2H QTL performed on average better than those lines that contained only the 7H and 3H QTL.

Figure 3-16. Mean disease severity of doubled-haploid lines with combination of alleles for field resistance.



3.3.4 Fine Mapping of Chromosome 3H

The region on chromosome 3H responsible for the expression of field spot blotch resistance contained many AFLP and no SSR markers. Due to the difficulties of using AFLP markers for routine screening (see section 1.5.3.3.), a total of 21 SSR and EST markers from published barley consensus maps were screened across the ND and VB

parents in order to map the region with largely co-dominant PCR-based markers. Of the markers screened, very low levels of polymorphism were observed. The SSR marker Bmag828 mapped to the short arm of 3H, however the marker trait association was low due to the distance between the marker and the QTL.

3.3.5 Validation Analysis

The major 7H QTL identified in the expression of both seedling and field resistance to spot blotch is highly associated with the SSR markers Bmag794 and Bmag603. These markers identified in ND/VB were mapped on the DH population ND11231*11/W12875*17 (ND/W) to determine if the regions identified by QTL analysis are also expressed for spot blotch resistance in a related population. Results of the validation analysis indicated that the 7H QTL also conferred both seedling and adult plant resistance to spot blotch in the ND/W DH population. The seedling resistance QTL on chromosome 7H, explained by marker Bmag794, had a LRS of 70.9 and explained 62% of the phenotypic variation for disease severity. The field resistance QTL on chromosome 7H gave an LRS of 7.8 and explained 11% of the phenotypic variation for disease severity. The LRS values were lower in the validation population than the mapping population.

The region on 3H, though deemed important for field resistance, could not be validated due to the low level of polymorphism observed in this region (see Section 3.3.4 above). The AFLP marker XP13M61-168 might be useful for validating this region. However, due to the low reproducibility of AFLP markers and the cost involved in screening a single marker across the population it was deemed to be beyond the scope of this research project.

3.4 Discussion

3.4.1 Map Curation

Map curation has been shown to significantly improve the quality of previously published genetic maps. Lehmensiek et al. (2005) have shown that map curation, through the reordering of markers and editing marker data for double crossovers, improves map resolution and the magnitude of the marker-trait association; both of which have significant impacts on QTL detection. In the case of the original ND/VB map (Emebiri et al., 2005), the addition of further SSR markers, the reordering of markers using the program RECORD and the removal of double crossovers, greatly improved the quality of the genetic map and significantly decreased the overall map distance and average chromosome length.

Marker order has been shown to substantially impact on QTL detection of quantitative traits (Collard et al., 2005). The program RECORD orders markers by minimising recombination events and is capable of dealing with large datasets for the construction of high density genetic linkage maps (Van Os et al., 2005). Significant decreases in the overall map distances in the ND/VB map supported the efficacy of this approach.

In addition to marker order, genotypic errors can significantly effect linkage map construction and overall map distances (Collard et al., 2005; Lehmensiek et al., 2005). Hackett and Broadfoot (2003) have shown, through the analysis of simulation data, that non-random typing errors resulted in large increases in map length and missing values had less of an impact on map distances. The removal of suspected genotyping errors by changing double crossovers between markers less that 25 cM apart to a missing score also had a significant impact on chromosomal length.

3.4.2 QTL Analysis

The programs QTL Cartographer and QTL Network were used to identify and examine the genomic regions associated with the expression of spot blotch resistance in the DH ND/VB population under both seedling and field conditions. QTL Cartographer combines interval mapping and multiple regressions to identify main effect QTL, and where QTL by environment interaction can be inferred by the presence and absence of a QTL across replicated trials. QTL Network employs a mixed linear model to identify main (additive) effect QTL, epistatic (non-additive) effect QTL, and QTL by environment interactions. Both programs consistently identified main effect QTL. However, while QTLNetwork failed to detect any of the minor QTL identified by QTL Cartographer it did consistently identify the major QTL. The minor QTL detected by QTL Cartographer suggests that QTL Cartographer is more sensitive to loci of small effect.. However, QTL Network appears to be more consistent across years and provide a smaller marker interval. This result has important implications for the investigation of quantitative traits, where the expression of resistance may involve several minor loci which are prone to significant environmental variation. It may be necessary to use multiple programs to confidently identify consistent minor QTL across different environments.

3.4.2.1 Identification of Seedling Resistance QTL

QTL analysis using QTL Cartographer identified a number of regions associated with the expression of seedling resistance in the ND/VB population. A highly significant QTL on chromosome 7H, a significant QTL on chromosome 5H and a number of suggestive QTL on chromosomes 1H and 5H were detected for seedling resistance. However, these minor, suggestive 1H and 5H QTL were not consistently identified in both trials. This inconsistency in QTL effect across trials highlights the complex nature of disease resistance, in that significant phenotypic variation is exhibited by cultivars under different environmental conditions. The highly significant QTL detected on chromosome 7H, contributed by the resistant parent ND was the most consistent across

seedling trials and is the most important region associated with the expression of seedling spot blotch resistance in this population.

The examination of consensus maps revealed that this 7H QTL appears to be in the same region as the *Rcs5* gene, previously reported by Steffenson et al. (1996). The *Rcs5* gene was significant in the expression of seedling resistance in North Dakota in three DH populations derived from the resistant source Morex, crossed with the six-rowed cultivars Steptoe and Harrington and the two-rowed cultivar Dicktoo. The comprehensive genetic analysis of these populations revealed that the region on 7H was the major locus associated with the expression of seedling spot blotch resistance, accounting for between 30% and 75% of the phenotypic variance for disease severity in these populations (Bilgic et al., 2005).

There is little doubt that the region on chromosome 7H is the major locus responsible for the expression of seedling resistance in the ND/VB population. However, the overall distribution and chi-square analysis of seedling infection response data did not fit a single gene ratio (see Chapter 2). In addition, the observation of transgressive segregates in the seedling trials may be due to natural small variation, or may also infer that the susceptible parent VB9524 possesses one or more minor spot blotch resistance genes. QTL Analysis using the program QTL Cartographer identified a significant QTL, inherited from the susceptible parent, on chromosome 5H in 2006. Further investigation of the mean disease severity of lines with combinations of the 7H and 5H resistance alleles in 2006 (Figure 3-5) revealed that lines with both the 7H and 5H QTL had a mean disease severity significantly lower than lines that contained only the 7H resistance allele. The identification of an additional seedling resistance allele in this population may be important for providing long term durable seedling resistance under Australian conditions and *Bipolaris* pathotypes. The putative correlation of the region on 5H with seedling spot blotch resistance needs to be examined further in this population and in lines related to VB9524, to determine if a significant association exists with this region and seedling spot blotch resistance.

3.4.2.2 Identification of Field Resistance QTL

Composite interval mapping of the field trials, using both the program QTL Cartographer and QTL Network revealed that adult plant resistance to the spot blotch disease is polygenic. Two highly significant QTL detected on chromosomes 3H and 7H, contributed by the resistant parent, were consistently detected across trials and appear to be the most important genomic regions associated with the expression of field spot blotch resistance in this population.

The 7H QTL is the main locus involved in the expression of field resistance and mapped to the same region as the major seedling resistance QTL, in the region of the *Rsc5* gene (Steffenson et al., 1996). This gene has been reported for the expression of field spot blotch resistance in other diverse DH populations in North Dakota, however the level of resistance it conferred varied markedly in the different populations and at the different ontogenetic stages (Bilgic et al., 2005; Steffenson et al., 1996). The identification of the *Rcs5* region in this study reveals that this locus is also providing significant spot blotch field resistance under Australian environmental conditions, with the *Bipolaris* pathotype that was used as inoculum. This finding has important implications for current Australian cereal breeding programs wishing to incorporate significant spot blotch resistance into Australian germplasm.

A region on the telomeric end of the short arm of chromosome 3H is also important in the expression of field spot blotch resistance, accounting for approximately 15% of the phenotypic variation for disease severity. It appears that this region on 3H has previously been reported for spot blotch resistance in the cultivar Bowman (based upon the postulated chromosomal position), where it accounted for 9% phenotypic variation for disease severity (Bilgic et al., 2006). A region on 3HS also been reported in the study involving different DH populations derived from the resistant cultivar Morex. A QTL was also detected on the telometric end of 3HS for field resistance in the Steptoe/Morex DH population and accounted for 6% of the phenotypic variation for disease severity. A region on 3HS was detected in the Dictoo/Morex population, where it accounted for

36% of the phenotypic variation, (Bilgic et al., 2005). However, due to a lack of common markers it is impossible to determine if the same region is being expressed in these different populations.

QTL Network and QTL Cartographer also identified a minor QTL on chromosome 2H for field resistance, contributed by the susceptible parent VB. The detection of a resistant QTL from the susceptible parent (although not detected in both trials) is consistent with the observation of transgressive segregants in the field trials (see Chapter 2). The 2H QTL is in a similar region to a QTL identified for adult plant resistance in the Steptoe/Morex population, where it accounted for 4% in the phenotypic variation in disease severity (inherited from Steptoe) (Bilgic et al., 2005). Further investigation of the infection response of lines which carry the 7H, 3H and 2H QTL (see Figure 3-4) revealed that lines with all three QTL had a significantly lower disease severity than those lines which carry only the 7H and 3H QTL. The association of this 2H QTL with resistance needs to be investigated further in other crosses to VB9524 and related lines, to confirm its association with spot blotch resistance under multiple environments.

Other minor QTL were also detected for field resistance. A significant minor QTL detected on chromosome 5H, also inherited from the susceptible parent, was only detected in the 2006 trial using QTL Cartographer. A number of minor suggestive QTL for field resistance were inherited from the ND parent, located on chromosomes 1H, 2H, 6H and 7H; however they were not consistently detected in each year. It may be that the expression of these QTL is conferred by other biotic or abiotic factors, and are only expressed under certain environmental conditions. Further investigation is required to determine their potential usefulness for breeding programs.

3.4.3 Validation of ND11231-12 derived QTL

Molecular markers for use in MAS are most effective for breeding programs if they can consistently identify trait-linked loci within diverse populations. Therefore, it is important to validate the QTL within other genetic backgrounds. The major regions

identified in this study involved in the expression of SB resistance are located on chromosome 3H and 7H, inherited from the resistant ND parent. The validation of the 7H QTL (*Rcs5*) for seedling resistance and field resistance was undertaken using a DH population derived from the sister line ND11231*11 and using the SSR marker Ebmac603. Results indicate that the 7H region is also expressed for seedling and field resistance in this genetic background. The SSR marker EBmac603 was consistently associated with the region and predicted a resistant phenotype. Thus, the results of the validation analysis confirm that the *Rcs5* gene is providing broad-based resistance in the glasshouse and the field within different genetic backgrounds. This result enhances the potential of this resistance QTL being used for marker assisted selection within Australian barley breeding programs.

The LRS values for the 7H QTL were significantly lower in this validation population, but the percentage of phenotypic variance explained by the region was comparable across both populations. This may be possibly due to the smaller population size (84 lines) used in the analysis of the validation population. Other studies have reported that population size significantly impacts on the number of QTL detected as well as the magnitude of the marker-trait association (Beavis, 1994).

The 3H QTL identified in this study is deemed very important in the expression of field resistance. However, it could not be validated in the ND11231-11 population due to the very low level of polymorphism observed in the region of the QTL in this population. Polymorphisms in this region need to be sought using other approaches, such as high resolution melting analysis (Lehmensiek et al., 2008), to identify single nucleotide polymorphisms (SNPs) between individuals. Alternatively, bioinformatics approaches based on syntenous regions in other cereal genomes such as rice may provide other polymorphic loci. This was beyond the scope of the present study but is considered to be an important future direction of this research.

In summary, spot blotch resistance in the ND/VB population is conferred by a different, but overlapping set of genetic loci at the different ontogenetic stages of plant

development. In this population, seedling resistance is controlled by a significant major QTL on chromosome 7H, and possibly a minor QTL on chromosome 5H, whereas field resistance is quantitative and controlled by two major QTL on chromosome 7H and 3H, and possibly a third minor QTL on chromosome 2H. The next chapter will examine whether these same regions confer common root rot (CRR) resistance, a root disease also caused by the *Bipolaris sorokinina* fungal pathogen. CRR will be examined in the recombinant inbred (RI) Delta/Lindwall population, employing bulked segregant analysis (BSA) and Diversity Array Technology to rapidly identify regions linked to resistance.

4. Common Root Rot Resistance

4.1 General Introduction

The fungal pathogen *Bipolaris sorokiniana* (teleomorph; *Cochliobolus sativus*), is the causal agent of not only the barley foliar disease spot blotch (SB) but also the root disease common root rot (CRR). CRR is a serious disease constraint in the dry temperate cereal growing regions of the world, and has been particularly damaging in Canada, South Asia and Africa (Kumar et al., 2002). In Australia, CRR has been reported in wheat and barley cropping fields in every mainland state, with estimated yield losses of up to 15% reported in QLD and NSW (Wildermuth et al., 1992).

This disease is considered to pose a significant threat to cereal growing regions across Australia and resistance breeding has been identified as the best method to control the disease (Kumar et al., 2002) As a result, current barley breeding programs aim to produce elite cultivars which exhibit significant resistance to the CRR infection. Phenotypic screening for CRR resistance is time consuming, expensive, and prone to significant environmental variation. Due to these limitations, molecular marker technologies combined with conventional breeding methods may provide a more reliable means of identifying resistant progeny and of increasing the efficiency of selection in cereal breeding programs.

The specific aim of the work in this chapter was to identify the genomic regions involved in the expression of CRR resistance in the Delta/Lindwall RI population. Field trials of this population indicated that CRR resistance segregated across this population (G. Wildermuth, pers. comm.). Since a genetic map for this population was not available, a novel bulked segregate analysis approach was taken which successfully employed DArT markers to identify chromosomal regions which segregated with resistance. These linkages have been largely validated by targeted mapping of the QTL regions. Results indicate that QTLs contributing resistance to SB and CRR are located in different regions of the genome.

4.2 Materials and Methods

4.2.1 Plant Materials

Common root rot resistance was examined in the unmapped recombinant inbred (RI) population Delta/Lindwall. Phenotypic data from CRR field trials conducted in 2002, 2003, 2004, and 2005 was supplied by Dr Graham Wildermuth, from the Queensland Department of Primary Industries and Fisheries (QDPI&F).

The system for measuring common root rot severity has been detailed in numerous studies examining common root rot infection (Ledingham, 1970; Tinline et al., 1975; Tinline et al., 1988; Wildermuth, 1986; Wildermuth et al., 1997). Briefly, the severity of common root rot is assessed by the degree of necrosis of the sub-crown internode (Tinline et al., 1975). Plants are separated into six categories: 1, no lesions; 2, 1 -2 lesions covering <10%; 3, 10-25%; 4, 25-50%; 5, 50-99%; 6, 100% of the sub-crown internode covered by lesions. Disease incidence is calculated from the number of plants in categories 2 to 6, and disease severity is calculated from the formula;

$$\text{Disease severity} = (2N_1 + 5N_2 + 10N_3) \times 100/10(N_1 + N_2 + N_3)$$

Where N_1 is number of plants in categories 2 and 3; N_2 is the number in plants in category 4; and N_3 is the number of plants in category 5 and 6.

4.2.2 Bulked Segregant Analysis and Diversity Array Technology

In the absence of a genetic linkage map for this population, BSA and DArT technology were employed to rapidly identify putative regions associated with CRR resistance. Sixteen lines from the population were selected for BSA based on the phenotypic data from CRR field trials conducted in 2002, 2003 and 2004 (the 2005 data was not available prior to conducting BSA; Table 4-1).

Table 4-9. Lines selected for BSA from the Delta/Lindwall population. Disease Severity, taken as a percentage of Timgalen, represents the average rating from trials in 2002, 2003 and 2004 (2005 data was not available to use for constructing the bulks).

Infection Response	Genotype	Disease Severity
Resistant	1. 256	51.53
	2. 022	52.49
	3. 165	52.71
	4. 144	54.69
	5. 233	55.30
	6. 159	55.49
	7. 138	55.57
	8. 024	56.53
Susceptible	1. 011	88.49
	2. 267	89.94
	3. 287	90.71
	4. 193	91.64
	5. 016	102.61
	6. 054	117.44
	7. 032	122.84
	8. 230	122.98

DNA from the 8 resistant and 8 susceptible lines was extracted as per previously described methods (see Chapter 3) and DNA concentration determined by agarose gel electrophoresis. Equal amounts of DNA from each line (see Table 4-1) were pooled respectively for BSA. To reveal genomic regions associated with resistance, bulked DNA and parental DNA were screened commercially by Triticarte Pty. Ltd. using Diversity Array Technology (DArT), a hybridization-based marker technology (see Section 1.5.3.3.3).

4.2.3 Identification of Regions Associated with Common Root Rot Resistance

DArT data was analysed by examining the hybridisation intensity of parental and bulked DNA to individual DArT Markers. Markers were selected based on the contrast in hybridisation intensity of both the parents and the bulks using an arbitrary threshold of >1.0. Markers were first sorted according to this threshold based on the parental data, and then markers were sorted based on the difference in hybridisation intensity between the bulks. Markers with the largest difference in hybridisation intensity between the bulks were considered to have the highest probability of association with CRR resistance.

Further investigation of DArT data was carried out by examining individual DArT markers with a normalised difference in hybridisation intensity between the parents equal to or greater than 1.0 (the normalized difference is equal to the difference between the bulks divided by the difference between the parents). The regions with the highest peak (greatest normalised difference) are considered to have the greatest association with the trait. Markers with a normalised difference greater than 100 were considered to warrant further investigation.

4.2.4 Confirmation of Regions with SSR Markers

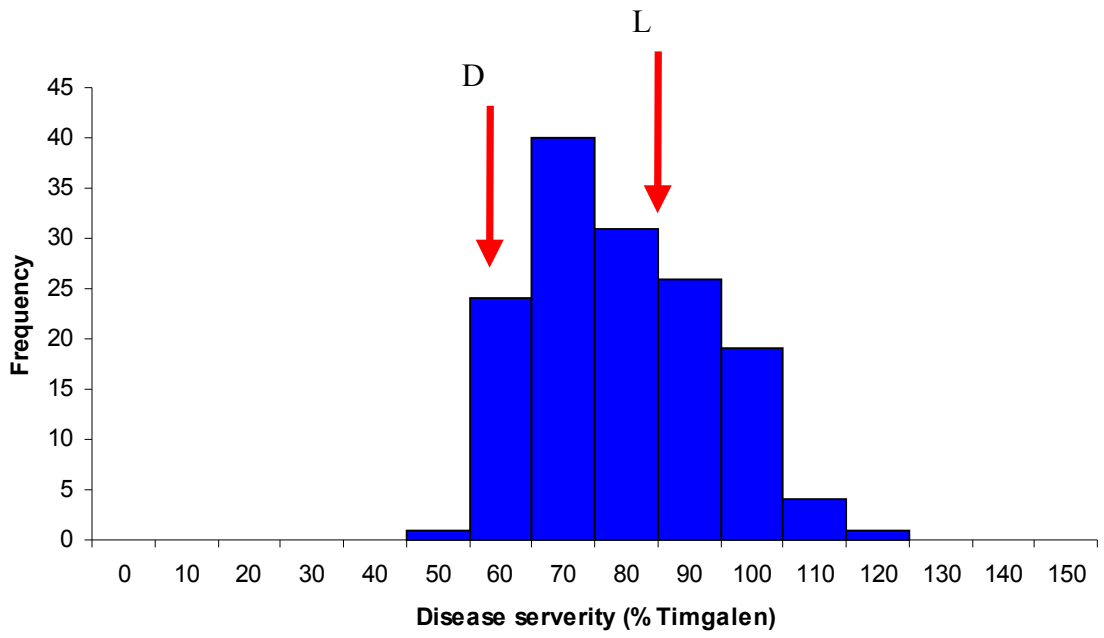
The confirmation of the chromosomal regions suggested by DArT analysis to be putative resistance QTL, was undertaken using SSR markers known to map to these regions. Candidate SSR markers were selected from a barley consensus map (Wenzl et al., 2006) based on their distribution along the barley chromosome. Primers were synthesized by Invitrogen (Mount Waverley, Victoria, Australia) and thermal cycling conditions were obtained from GrainGenes. SSR markers were initially screened across both parents and polymorphic markers were mapped to a subset of the population (85 lines) using MapManager QTXb20 (Manly et al., 2001). Marker regression was undertaken to determine the marker-trait association.

4.3 Results

4.3.1 Response to Infection

The frequency of the average adult infection responses to CRR over three growing seasons (Figure 4-1) displays a continuous distribution, indicative of a quantitative trait. In each field trial the parental line Delta displayed partial resistance to the CRR infection.

Figure 4-17. Frequency of average adult infection responses to CRR. Disease severity is expressed as a percentage of the susceptible cultivar Timgalen. Arrows indicate average parental scores (D= Delta, L=Lindwall)



4.3.2 Identification of Genomic Regions Associated with Common Root Rot Resistance

Eighteen DArT markers were identified as having a greater than 1.0 difference in the hybridisation intensity between the parents and the resistant and susceptible bulks (Table 4-2). By using this method of analysis three genomic regions were identified as having an association with the expression of CRR resistance. Two major regions were located on chromosomes 2H and 4H, and a third minor region was located on chromosome 7H. A large region on chromosome 4H appears to be the most significantly associated with CRR resistance. This region on 4H was identified as having a group of closely linked DArT markers with a contrast in hybridisation intensity between the parents and the bulks greater than 3.0, with the largest difference between the bulks (3.58) detected at marker bPb-3045. In comparison, the region on chromosome 2H only revealed one DArT marker (bPb-6052) with a difference of this magnitude (3.76). The region on 7H is considered minor, as only two DArT markers had a contrast in the hybridisation intensity between the bulks of >1.5.

Table 4-10. DArT anaysis of bulked DNA

Marker Name	Chromosome position	P-value	Diff.Parents	Diff. Bulks
bPb-6052	2HS	81.82	1.30	3.76
bPb-4148	2HS	84.48	1.73	1.46
bPb-3608	2HS	78.14	1.43	1.15
bPb-0485	2HS	92.26	1.58	1.40
bPb-2501	2HS	84.94	3.22	1.72
bPb-3190	2HS	86.33	2.99	1.57
bPb-7906	2HS	88.00	3.14	1.65
bPb-8750	2HS	78.48	2.17	1.13
bPb-4333	4HS	98.39	2.92	3.07
bPb-3045	4HS	99.28	3.60	3.58
bPb-3684	4HS	99.27	3.48	3.33
bPb-0513	4HS	98.76	3.61	3.28
bPb-0130	4HS	98.84	3.62	3.16
bPb-0365	4HS	97.71	3.56	3.07
bPb-2427	4HS	99.42	2.73	2.31
bPb-9504	4HS	93.62	2.83	1.99
bPb-9202	7HS	88.15	1.73	1.90
bPb-4674	7HS	74.01	1.08	1.50

P-value = marker quality parameter

Further investigation of the DArT data was undertaken by graphing all markers with a normalised difference greater than 1.0 (Figure 4-2). The normalised difference graphs confirmed the results of the previous analysis of DArT data and again identified the 3 regions on chromosome 2HS, 4HL and 7HS (Figure 4-2). The normalised difference of these regions was greater than 100, and was therefore considered to indicate a putative association with resistance. Results of this analysis indicate that the region on 2H may have a greater association with resistance than the region on 4H. Although 4H had a greater number of markers with a large normalised difference, the region on 2HS had the highest peak. In addition, by using this method and examining the normalised difference, the region on 7H appears to be more significant than indicated by the previous analysis.

Figure 4-18. Normalised difference graphs of DArT-BSA genome scan identifying regions associated with CRR resistance in the Delta/Lindwall RI population. A normalised difference (plotted on y-axes) >100 is considered significant.

a. Chromosome 1H

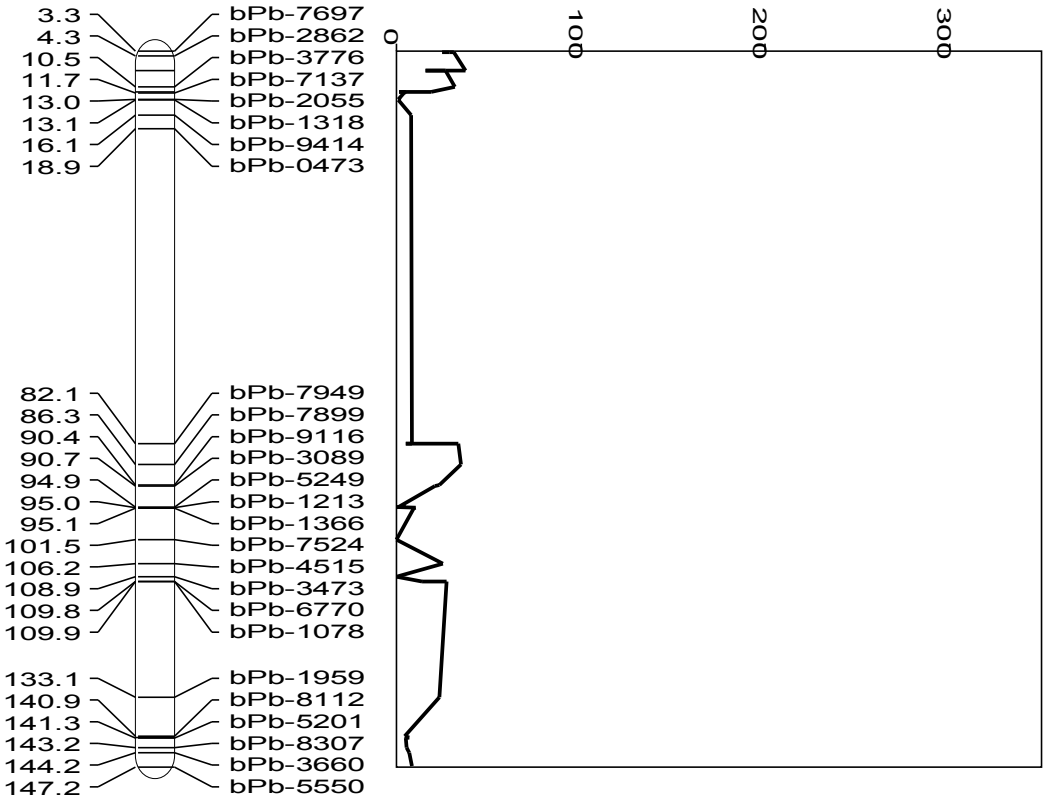
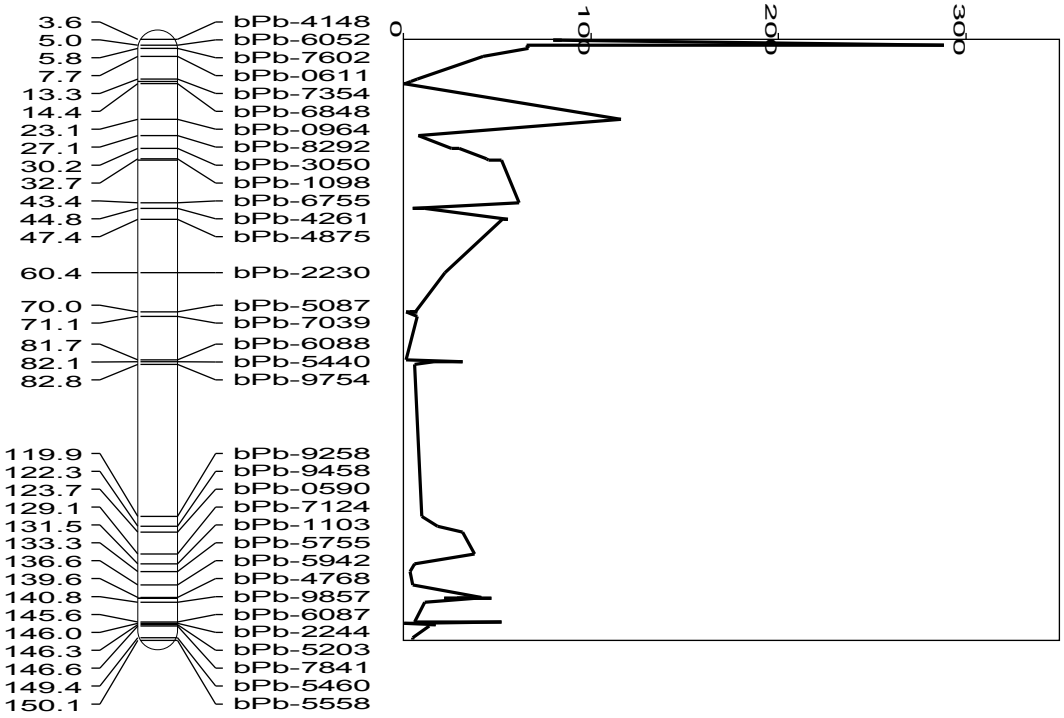


Figure 4-2. (Continued)

b. Chromosome 2H



c. Chromosome 3H

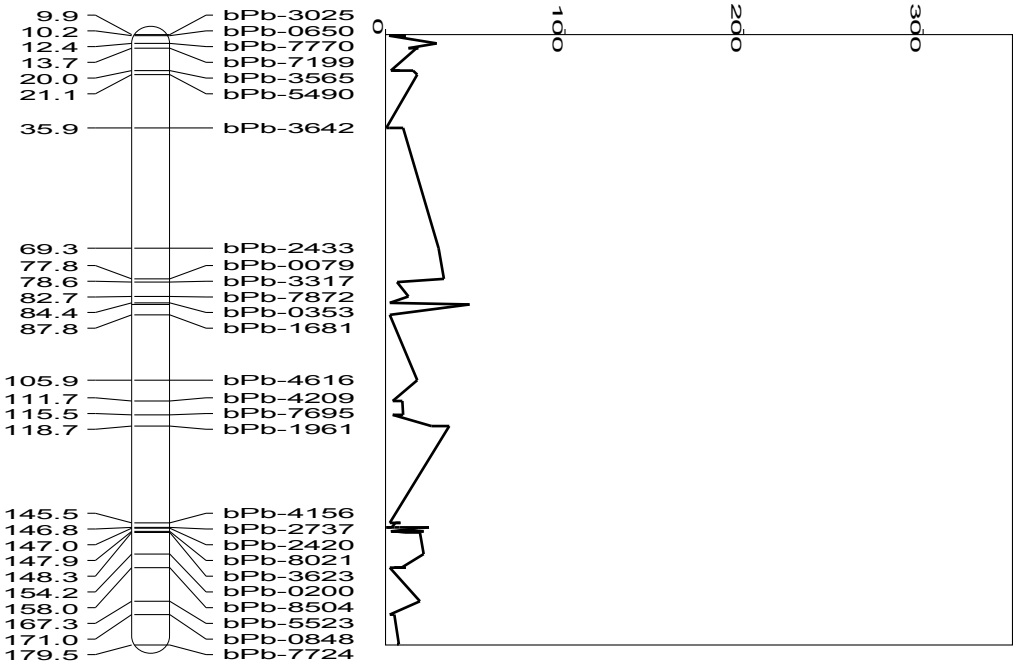
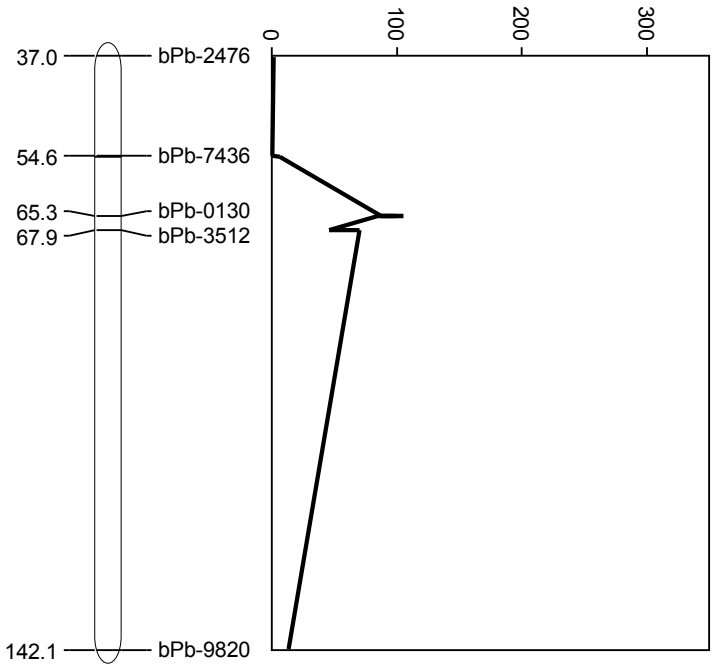


Figure 4-2. (Continued)

d. Chromosome 4H



e. Chromosome 5H

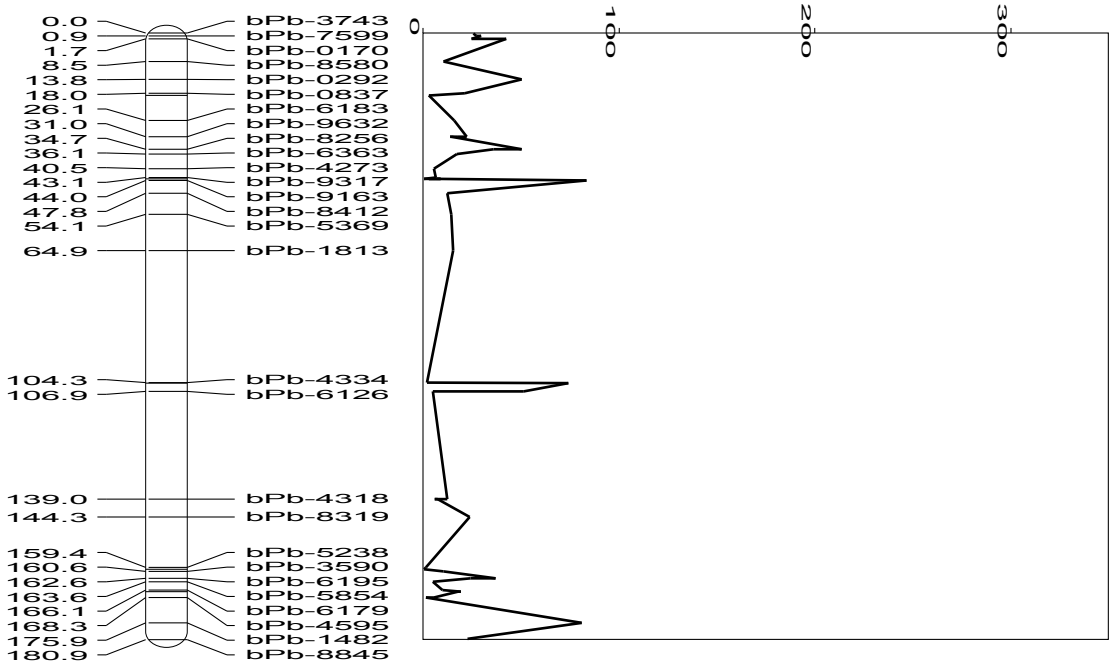
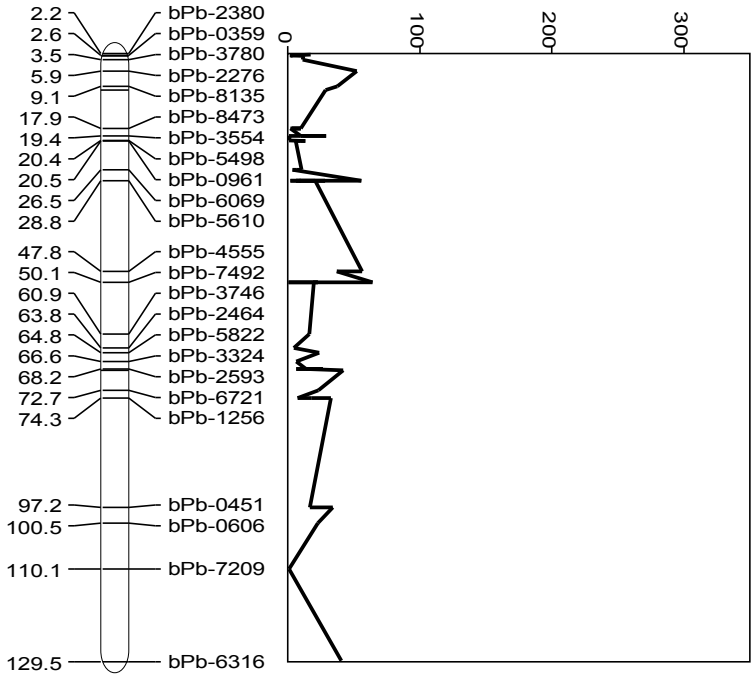
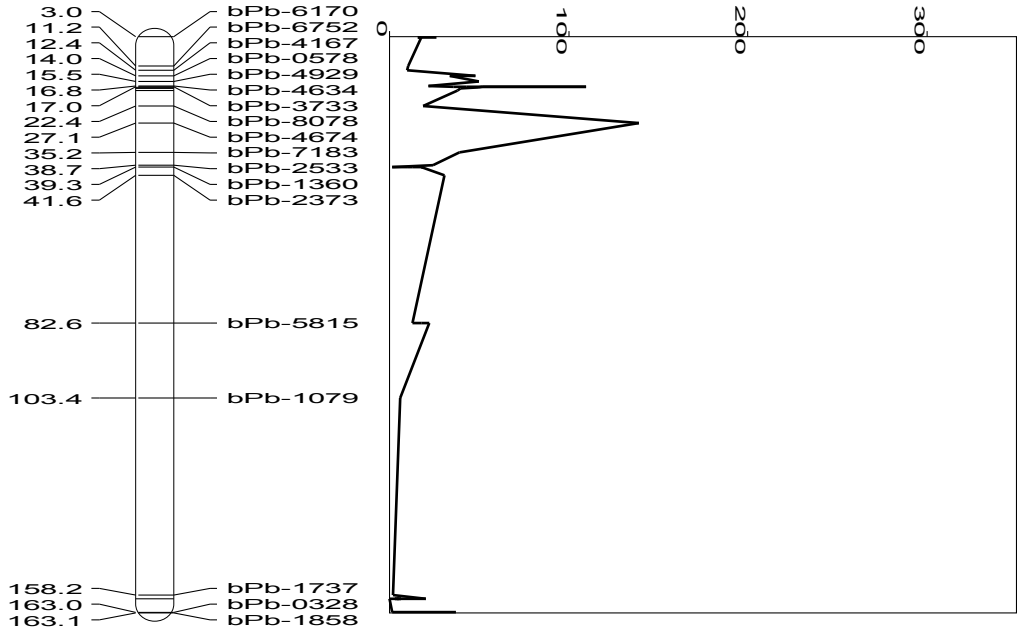


Figure 4-2. (Continued)

f. Chromosome 6H



g. Chromosome 7H



4.3.3 Confirmation of Regions with SSR Markers

Marker regression of selected SSR markers polymorphic in the Delta/Lindwall population confirmed the regions previously identified by DArT analysis of the bulks. Three putative QTL were identified; two QTL located on chromosome 4HS and chromosome 2HS, and one on chromosome 7HS (Table 4-3). The minor region detected on 7HS is in the same region as the 7H QTL identified for seedling and field spot blotch resistance in the ND11231-12/VB9524 DH population.

Table 4-11. Marker regression of SSR markers confirming regions identified by BSA and DArT analysis.

		2002		2003		2004		2005		Ave.	
Ch.	**Marker**	**LRS**	**%**	**LRS**	**%**	**LRS**	**%**	**LRS**	**%**	**LRS**	**%**
2HS	abg707	-	-	15.3	59	6.7	31	-	-	2.9	3
4HS	Ebmac906	-	-	2.3	14	15.9	65	9.3	12	8.6	11
7HS	Ebmac603	-	-	-	-	2.8	16	-	-	-	-

LRS = Likelihood Ratio Statistic
% = Percent of the phenotypic variance explained

Although these regions were detected by marker regression, they were not consistently detected in each year. The 4H was detected in three of the four trials, but the LRS values and the percent of phenotypic variance explained by the marker varied significantly across each year. The highest association was detected in 2004; however this region was only suggestive in 2005 and very minor in 2003. The region on chromosome 2H was only significant in the 2003 trial and was suggestive in 2005. The minor 7H region was only detected in the 2004 trial. The inconsistency in these results may indicate that CRR resistance is prone to significant environmental variation.

4.4 Discussion

This study combined the method of BSA with DArT technology to rapidly identify genomic regions associated with the expression of CRR resistance in the unmapped Delta/Lindwall RI population. At the time it was conducted, there were no published studies which had used DArT data in this manner. More recently, the use of DArT arrays for performing BSA has been outlined by Triticarte (Wenzl et al., 2007). Results of this investigation demonstrate that this technology will provide an efficient tool for the preliminary investigation of quantitative traits, to rapidly identify genomic regions linked to phenotypic characters in unmapped populations. This technique also has the advantage of low cost which arises from the requirement for only a very few number of samples (parental lines and the population bulks) to be analysed compared to a full population screen.

Bulked segregant analysis (BSA), originally developed by Michelmore et al. (1991), is a widely used method to rapidly identify molecular markers linked to quantitative traits (Wenzl et al., 2007). The method involves pooling DNA (bulks) from two distinctive phenotypic classes that differ for the trait of interest and then screening markers to identify those which distinguish the bulks. This method is useful and it can dramatically reduce the number of marker assays required to identify regions linked to the expression of phenotypic traits.

DArT, a hybridization diversity array technology, allows multiplexed, high-throughput screening of hundreds of markers in a single assay, and thus reduces genotyping effort. It also has the advantage of low cost and high accuracy and can be developed for any plant species, irrespective of their ploidy level (Wenzl et al., 2007).

DArT-BSA is emerging as a new technology, with only one other report detailing its application and suitability as a methodology to rapidly identify genetic loci that influence phenotypic characters (Wenzl et al., 2007). In this report it was demonstrated

that the relative hybridization contrast between bulks using DArT analysis accurately reflected the between bulk difference in the frequency of the mPub allele, a morphological marker located on chromosome 3H of the barley DH population Steptoe/ Morex. It was concluded that combining these two technologies provided a platform for the rapid quantitative analysis of the allele-frequency estimates in plants and can identify genetic loci that influence phenotypic traits with at least 5 cM accuracy (Wenzl, et al., 2007).

Using DArT-BSA technology three putative regions were identified for CRR resistance in the Delta/Lindwall RI population. Two significant regions were detected on chromosomes 4HS and 2HS, and a third minor region on chromosome 7HS. The region on chromosome 7HS appears to be in a similar region to the 7H QTL identified for seedling and field spot blotch resistance in the DH population ND11231-12/VB9524. To confirm these regions, marker regression of selected SSR markers were mapped to a subset of the population (85 lines). These regions were found to have an association with CRR resistance, confirming the results of the DArT-BSA analysis. However, the percent of the phenotypic variation explained by the markers seem to be exaggerated in effect. This may be due to what is referred to as the "Beavis effect", where the actual phenotypic variation explained by a QTL can be overestimated for populations under 100 individuals (Beavis, 1994). These regions need to be further investigated in the entire population to confirm their association with resistance, and may possibly involve the construction of a skeletal map with a high density of markers in the regions identified in this study. In addition, for breeding purposes these regions also need to be validated in a related population, to determine if they are also expressed within other genetic backgrounds. Given the small number of individuals used to construct the bulks, it may be possible that other minor QTL were not detected in this study.

The results of this investigation indicate that despite the common region on chromosome 7H (this region was only very minor in effect for CRR resistance and was only detected in one trial), it appears that different genomic regions confer CRR resistance compared to those which confer SB resistance in the populations used in this study. This may be

due to different resistance response mechanisms within different genetic backgrounds or that different *B. sorokiniana* subgroups show different host tissue specificity for pathogenicity. This has been supported by other research conducted by Knight (2007), who examined different *B. sorokiniana* isolates for their abilities to cause spot blotch infections or CRR infections on barley using a differential set of fifteen barley cultivars and three other cereal species. Results of cluster analysis of amplified fragment length polymorphisms (AFLPs) indicated that they were genetically distinct, and that isolates collected from spot blotch infections typically clustered apart from isolates collected from common root rot infections.

In summary, this is the first known report identifying genomic regions associated with CRR resistance in barley. Results of this investigation found that DArT-BSA did provide a good platform for rapid identification of genetic loci involved in the expression of CRR resistance. Secondly, it was found that CRR resistance is quantitative and is conferred by two significant loci on chromosome 2H and 4H, and possibly a third minor loci on chromosome 7H. Finally, it appears that CRR resistance and SB resistance are genetically distinct and independent characters in the populations used in this study. Further research is required to confirm this hypothesis and should be conducted using the same population to screen for both diseases and a singe marker system.

5. General Discussion

The purpose of this research project was to identify the genomic regions associated with spot blotch and common root rot resistance in barley. Molecular marker technology and a QTL mapping approach were employed to identify QTL for SB resistance, while BSA-DArT analysis was undertaken to rapidly identify regions linked to CRR resistance. This is the first known report of QTL for SB and CRR resistance in Australia and this research will provide important information for current Australian and international barley breeding programs wishing to incorporate significant SB and CRR resistance into their elite germplasm.

The specific research outcomes of this project are:

- The completion of two seedling trials and two field trials of the DH population ND11231-12/VB9524 for spot blotch resistance;
- Curation of the original ND/VB genetic linkage map produced by Emebiri et al, 2005;
- Identification of QTL for spot blotch resistance at the seedling stage and in the field using the software packages QTL Cartographer and QTL Network;
- Validation of the major ND derived resistance QTL (*Rcs5* gene) for seedling and field resistance in the related population ND11231-11/ WI2875*17; and
- Application of BSA-DArT technology to rapidly identify loci for CRR resistance in the unmapped population Delta/Lindwall, and confirmation of these regions with SSR markers; and
- That spot blotch and common root rot resistance appear to be inherited independently in the populations used in this study.

The purpose of this final chapter is to:

1) Outline the main findings of this investigation and compare these findings with previous reports detailing SB and CRR resistance;

2) Discuss the potential contribution of these findings to current barley breeding programs in Australia; and

3) Identify future directions for this research.

5.1 Spot Blotch Resistance

5.1.1 The ND11231-12 - derived resistance QTL

5.1.1.1 The 7H resistance QTL

A region on chromosome 7HS was found to be the major locus involved in both the expression of field and seedling spot blotch resistance in the two-rowed ND11231-12/VB9524 DH population. This region on chromosome 7H, contributed by the ND parent, has been shown through the examination of barley consensus maps to be in the same region as the *Rcs5* gene. The *Rcs5* gene was previously identified and named in the Steptoe/Morex DH population (Steffenson et al., 1996) and can be traced back to the original six-rowed source of NDB112 (J. D. Franckowiak, pers.comm., Wilcoxson, 1990). In the Steptoe/Morex population, *Rcs5* was the major source of seedling spot blotch resistance. However, in field trials *Rcs5* was only a minor contributor of field resistance (LOD = 2.7) in this population.

In other studies of diverse six-rowed and two-rowed populations derived from the resistant cultivar Morex, the *Rcs5* region contributed universally to both seedling and adult plant resistance. However, the level of resistance it conferred varied markedly in the different populations and at the different ontogenic stages (Bilgic et al., 2005). These observations highlight the potential effects of genetic background, environment and growth stage on the expression of this resistance.

Results of this investigation indicate that the *Rcs5* gene is the major locus involved in the expression of seedling and field spot blotch resistance in the North Dakotan line ND11231-12 and the related cultivar ND1123-11, under Australian environmental conditions, in response to the *Bipolaris* isolate (SB61) used in the inoculum. The identification of the *Rcs5* gene in this study has important implications for current Australian barley breeding programs wishing to incorporate significant SB resistance into their breeding germplasm. In this study, QTL analysis identified two PCR-based flanking markers linked to the *Rcs5* region in these populations, namely SSR markers Bmag794 and EBmac603. The utilization of these molecular markers for MAS will increase the efficiency of selection methods to identify progeny with broad based resistance to spot blotch. This in turn will potentially decrease the time taken to release a resistant commercial cultivar and alleviate the threat posed by this disease to growers in Australia.

Currently, work is being undertaken to identify the mode of action and the genetic sequence of *Rcs5* (Drader et al., 2007). In the report by Drader et al. (2007) five genes were predicted within the sequence contig covering the *Rcs5* region: a lipase-like gene, an FBOX domain, an expressed gene with unknown homology, a ribosomal-like protein, and a p450-monooxygenase. These authors have suggested that the p450-monooxygenase gene may be important in detoxifying infected tissue, since *C. sativus* is known to produce several toxic compounds that may play an important role in infection or pathogenesis (Kumar et al., 2005). The five candidate genes have been hybridized to an arrayed cDNA library and are currently being analyzed for expression and gene structure between resistant and susceptible cultivars to further elucidate their role in resistance (Drader et al., 2007). This work has the potential to lead to the development of a perfect marker for the *Rcs5* gene and an understanding of gene action.

5.1.1.2 The 3H QTL for Field Resistance

In addition to chromosome 7HS, a region on the short arm of chromosome 3H, also inherited from ND11231-12, has a significant effect on the expression of field spot blotch resistance. Based upon consensus maps (Karakousis et al., 2003; Wenzl et al., 2007) this region appears to be the same as that originally identified in the 2-rowed feed cultivar Bowman (based on their postulated chromosomal location) (Bilgic et al., 2006). Bowman is a close relative of the ND1123-12 line used in this study, through the parent ND4994-15 (J. D. Franckowiak, pers.comm. Emebiri et al., 2005). At the time of its release in 1984, Bowman was considered moderately resistant to spot blotch. However, this resistance was short lived due to the appearance of a new *C. sativus* pathotype (pathotype 2) in North Dakotan breeding nurseries in 1990 (Bilgic et al., 2006). To elucidate the genetic mechanism of this differential infection response, a study was conducted examining the genetics of resistance to pathotype 1 and 2 of *C. sativus* in the DH Calicuchima-sib/Bowman-BC mapping population (Bilgic et al., 2006). It was found that Bowman carried resistance alleles to pathotype 1 on chromosome 2H (accounting for 21% of the phenotypic variation) and chromosome 3H (where it explained 32% of the phenotypic variance for disease severity), however it was very susceptible to the pathotype 2 of *C. sativus*. The authors concluded that two-rowed cultivars require more broad range resistance to spot blotch to prevent losses and remain commercially competitive in the region (Bilgic et al., 2006).

A region on chromosome 3H QTL has also been identified in both the D/M and S/M populations, where it accounted for 36% and 6% of the phenotypic variation respectively (Bilgic et al., 2005). Based upon consensus maps (Karakousis et al., 2003; Wenzl et al., 2007) the S/M QTL appears to be located in the same chromosomal region as the QTL identified in the ND11231-12/VB9524 population. Due to a lack of flanking markers in common between maps, it could not be absolutely confirmed that the D/M QTL is in the

same region. So while it is likely that a common gene confers field resistance to SB in all three populations, further studies are needed to support this hypothesis.

The 3H region in the ND11231-12/VB9524 population is linked to the AFLP marker XP13M61-168. Due to the limitations of AFLP markers for routine screening, an objective of this study was to map co-dominant PCR-based markers to this region of the QTL. A total of 18 markers were screened for polymorphisms, with little success. Of the 18 markers screened, only 1 marker (Bmag828) was polymorphic, indicating that a low level of polymorphism exists in this chromosomal region of this population. The low level of polymorphism on chromosome 3H may be a particular characteristic of the ND/VB population, or it may be due to the conservation of specific regulatory genes across different populations. Other reports have also indicated that the 3H chromosome is a site of low polymorphism between individuals (Hoffman & Dahleen, 2002). The rice genome has been sequenced and provides a platform for the study of other graminaceous species. By examining the recognised syntenous relationship between chromosome 3H of barley and chromosome 1 of rice (Smilde et al., 2001), it may be possible to predict the functional properties of the genes in that region. Chromosome 1 of rice contains genes for cellular metabolism and signal transduction (Sasaki et al., 2002), which, if conserved, could account for the low level of polymorphism detected in the 3HS region in the ND/VB population.

Due to the lack of detectable polymorphisms in the 3HS region, other more sensitive methods may be required to identify a marker more closely linked to the QTL. While synteny between the grasses has been shown to be a potential source of new markers (Varshney et al., 2005), these EST markers tend to show low levels of polymorphism in gel based assays (Eujayl et al., 2002). Novel technologies such as high resolution melting analysis (HRM; Lehmensiek et al., 2008), which detects SNPs in genetic markers, may provide a means to detect potential polymorphisms between the ND and VB parent on 3HS. Such strategies will be important to increase genetic gain in breeding programs utilizing marker-assisted selection for this trait. A combination of

bioinformatics approaches and HRM analysis may provide other polymorphic loci in this region of chromosome 3HS.

5.1.2 The VB9524 - derived resistance QTL

A number of significant minor resistance QTLs were detected for field and seedling resistance, contributed by the VB9524 (susceptible) parent. A seedling resistance QTL was detected on chromosome 5H; however this QTL was only detected in the 2006 trial. A field resistance QTL was also detected at a different position on chromosome 5H in the 2006 trial, but was absent in the 2004 trial. This result may indicate that both these regions are environment-specific QTL and further investigation is required to confirm their association with resistance. A region on chromosome 2H was significant in both the 2004 and 2006 field trials using QTL Cartographer, and was also detected by the software QTL network. This indicates that this region, inherited from the VB parent, also contributes to the expression of field resistance in this population. Individuals in this population which carry the resistance alleles from both the resistant and susceptible parents will be candidates for further germplasm development, and may play an important role in providing long-term durable SB resistance in Australia.

5.1.3 Other resistance alleles in the ND11231-12/VB9524 population

This study has shown that the ND11231-12/VB9524 population offers a promising source of spot blotch resistance in Australian conditions. Furthermore, the ND/VB population has been characterized for resistance to other barley diseases (Emebiri et al., 2005), and malting quality characteristics (Emebiri et al., 2004). A study by Emebiri (2005) identified major QTL in the ND/VB DH population for resistance to powdery mildew (*Blumeria graminis* f.sp. *hordei*) on chromosome 1H, net form net blotch (*Pyrenophora teres* f. *teres)* on chromosome 6H, and stem rust (*Puccinia graminis* f.sp. *tritici*) on chromosome 7H. A collaborative study should now be undertaken to identify those individuals in this population which carry these multiple resistance alleles. Such

individuals may provide elite germplasm for barley breeding programs both nationally and internationally.

5.2 Common Root Rot Resistance

In this study, putative QTL for CRR field resistance were identified on chromosomes 2H, 4H and 7H in the Delta/Lindwall RI population. These QTL were detected by a novel BSA-DArT approach to rapidly identify regions linked to CRR resistance in the absence of a genetic linkage map for this population. The regions identified by BSA-DArT analysis were confirmed using marker regression between single SSR markers and these regions on 85 lines of the population, using phenotypic data from field trials conducted in 2002, 2003, 2004 and 2005.

Results of this analysis revealed that the region on chromosome 4H was consistently identified in each trial, with a maximum LRS of 15.9, explaining 65% of the phenotypic variation for disease severity in 2004. However this region was only minor in effect in the 2003 trial, with an LRS of 2.3. The region on chromosome 2HS was detected in 2003 (LRS = 15.3, 59%) and 2004 (LRS = 6.7, 31%), but not in 2005. The region on chromosome 7HS was only very minor in effect in 2004 (LRS = 2.8, 16%). The inconsistencies in these results suggest that CRR is controlled by a number of minor QTL which are prone to significant environmental variation. This highlights the variation in the expression of resistance, indicating that markers will be useful for selecting for resistance to this disease.

This is the first known report of QTL for CRR resistance in barley and suggests that BSA-DArT analysis may provide a useful efficient tool for the preliminary investigation of quantitative traits in unmapped populations. Further detailed investigations of the regions identified by this approach are required, to confirm their association with CRR resistance. There are plans to produce a DArT-based map for this population to assist in this investigation.

A further objective of this investigation was to determine if regions that confer resistance to SB also confer resistance to CRR. Both diseases are caused by the same fungal pathogen. However, a debate continues as to whether the same genetic factors condition resistance to both diseases. The SB and CRR resistance QTL identified in this study are, with one exception, in different regions of the barley genome and appear to be inherited independently of one another. This is supported by other research examining the interrelationship between SB and CRR in barley, where none of the genotypes tested were found to carry resistance to both diseases (Arabi et al., 2006). However, in this case linkage in repulsion could not be excluded. On the basis of the chromosomal locations of the resistance alleles for SB and CRR identified in this study, it appears to be possible to combine high levels of resistance to both these diseases in a single genotype using available QTL. This would permit the development of SB and CRR resistant commercial cultivars using marker-assisted selection and would significantly reduce the threat these diseases pose to barley growers world-wide.

5.3 Future Directions

A number of future directions have been identified in this research which will further assist in the development of effective breeding strategies for SB and CRR resistance.

- Identify further polymorphisms in the region of the QTL on chromosome 3H for adult plant resistance to SB and identify a PCR-based marker linked to the QTL, possibly by employing HRM Analysis of selected SSR derived EST markers;

- Validate the region on 3H in the validation population ND11231-11/WI2875*17 using PCR-based markers;

- Further investigate minor seedling and field resistance QTL inherited from the VB9524 parent in another VB9524 derived DH population, in particular the

region on chromosome 5H for seedling SB resistance and the region on 2H for field SB resistance;

- Confirm QTL for CRR resistance in the entire Delta/Lindwall population by constructing a DArT-based map, which may also reveal additional QTL not detected by BSA;

- Validate CRR findings in a related DH population, derived from the resistant source Delta, in particular the regions identified on chromosomes 2H and 4H;

- Screen the ND11231-12/VB9524 population for resistance to CRR to determine whether the allele on 7HS responsible for SB resistance also contributes to resistance against CRR;

- Develop elite breeding germplasm combining both SB and CRR resistance with other disease resistance alleles present within the ND11231-12/VB9524 DH population; and

- Develop Near-isogenic lines to study the expression of SB and CRR resistance in uniform genetic backgrounds. In particular the development of a differential set of near-isogenic lines containing different sources of resistance to each disease is required to screen field collections of Australian isolates of *B. sorokiniana* in order to establish the range of fungal pathotypes present in Australia.

5.4 Conclusions

In conclusion this research met its proposed objectives of identifying QTL linked to SB and CRR resistance in barley. The ND11231-12 line from North Dakota carries major SB field resistance alleles on chromosomes 3H and 7H, and a major SB seedling resistance allele on chromosome 7H. This study has shown that the ND11231-12 line

provides a good source of spot blotch resistance under Australian environmental conditions, and the Australian *Bipolaris* isolate used in the inoculum. QTL linked to CRR resistance were detected on chromosome 2H, 4H and 7H in the Delta/Lindwall RI population; indicating that resistance to CRR appears to be independently inherited from SB resistance. This research will benefit current Australian and international barley breeding programs and will assist in the selection of superior genotypes which carry a high degree of resistance to these fungal diseases.

6. References