Identification and Mapping of Host Resistance Genes to Septoria Tritici Blotch of Wheat

BY

SHARLA GAIL COWLING

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Plant Science
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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MASTER'S THESIS/PRACTICUM FINAL REPORT

The undersigned certify that they have read the Master's Thesis/Practicum entitled:

Identification and Mapping of Host Resistance Genes

to Septoria Tritici Blotch of Wheat

submitted by

SHARLA GAIL COWLING

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

The Thesis/Practicum Examining Committee certifies that the thesis/practicum (and oral examination if required) is:

Approved

(Approved or Not Approved)

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Date: September 15, 2005

ACKNOWLEDGMENTS

I extend my sincerest thanks to my advisor Dr. A. L. Brûlé-Babel for her advice, guidance, and encouragement throughout the course of this project. I would also like to thank Dr. Brûlé-Babel for her continuous support of my athletic endeavors while attending the University of Manitoba.

I would like to acknowledge Dr. D. J. Somers for his assistance during the molecular mapping portion of my project and the opportunity to work in his lab at the Cereal Research Centre. In addition, thank you to all of the technicians in the lab for their assistance with a variety of tasks.

Thank-you to Drs. L. Lamari and G. Hausner for taking the time to review my thesis and their advice as members of my advisory committee.

My sincerest thanks goes out to Eppie Austria for her time, patience, and encouragement thoughout the course of my project. I would also like to thank Eppie for assisting me on weekdays and weekends when I was traveling with hockey. A special thanks to Leslie Bezte at the Cereal Research Centre for teaching me the techniques for the molecular mapping portion of my project.

I would also like to extend my gratitude to Curt McCartney for his advice and assistance with disease ratings and other aspects of my project.

Thanks to Bert Luit for his creative assistance taking the photographs found in this thesis.

Thank-you to my fellow graduate students for your encouragement and friendship. I would also like to extend a special thanks to Ian Brown, Cathy Bay, Linda Pearn and Bill Scrimgeour for their assistance in the greenhouse.

Financial support from the Natural Sciences and Engineering Research Council of Canada and the Canadian Wheat Board made this research possible.

Finally, thank you to family and friends for their support and encouragement. A special thanks goes to Brian Lai for his encouragement, understanding and support.

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ABSTRACT

Cowling, Sharla Gail. MSc., The University of Manitoba, September, 2005. Identification and Mapping of Host Resistance Genes to Septoria Leaf Blotch of Wheat.

Major Professor; Anita L. Brûlé-Babel.

Septoria tritici blotch (STB) of wheat is caused by the fungal pathogen *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz.). STB is a devastating foliar disease that occurs in all wheat producing countries worldwide. The losses in yield and quality caused by severe STB epidemics have left researchers looking to further understand the inheritance of STB resistance and pathogen variability to develop agronomically sound, STB resistant cultivars. The objective of this study was to identify molecular markers linked to the STB resistance genes in the wheat line Salamouni. Doubled haploid (DH) lines were generated from a cross between Salamouni and the susceptible cultivar Katepwa. DH lines were evaluated for their reaction to isolates MG96-36 and MG2. Lines exhibiting resistance to both isolates and those only resistant to isolate MG2 were crossed back to Katepwa to develop F_1 and F_2 generations. The F_2 populations that segregated in a 3 : 1 resistant to susceptible ratio when screened with the appropriate isolate were selfed to create F_2-derived-F_3 ($F_{2:3}$) mapping populations. Genomic DNA extracted from each $F_{2:3}$ family was analyzed with microsatellite markers (SSRs) using bulked-segregant analysis (BSA) to identify markers putatively linked with the resistance genes. Linkage analysis identified two microsatellite markers on chromosome 3BS, *Xwmc500* and *Xwmc623*,

linked to *Stb14*, the resistance gene in Salamouni that provides resistance to only isolate MG2. The above molecular markers were linked to *Stb14* at distances of 2 and 5 centimorgans (cM), respectively. *Stb13* mapped to chromosome 7BS in two separate populations. In the first population, linkage analysis indicated *Stb13* is 9 cM from the nearest microsatellite marker, *Xwmc396*. In the second population, linkage analysis indicated *Stb13* is linked to the same molecular marker, but at a distance of 7 cM. Although two single gene lines were identified that segregated for resistance to isolate MG96-36, molecular analyses confirmed both lines contained the same resistance gene, *Stb13*. Phenotypically, there is no isolate identified to distinguish the two resistance genes that provide resistance to both MG96-36 and MG2, therefore, only one of the two resistance genes controlling resistance to both isolates was identified. The microsatellite markers identified in this study should facilitate marker-assisted selection (MAS) and the pyramiding of several STB resistance loci into a single cultivar. This will provide wheat growers with a more durable, effective, and affordable source of STB resistance in the future.

FOREWARD

This thesis follows the manuscript style outlined by the Department of Plant Science, University of Manitoba. Manuscripts follow the style recommended by the Canadian Journal of Plant Science. This thesis is presented as two manucripts, each containing an introduction, materials and methods, results, and discussion sections. A general review of the literature precedes the manuscripts, and a general discussion follows the manuscripts.

CHAPTER 1

GENERAL INTRODUCTION

In the last decade, the occurrence of Septoria tritici blotch (STB) of wheat has become increasingly problematic for wheat growers in western Canada. This residue-borne leaf-spotting disease caused by the fungal pathogen *Mycosphaerella graminicola* has the potential to reduce wheat yields by 25-50% (Shipton et al. 1971; Eyal and Ziv 1974; Ziv and Eyal 1978; King et al. 1983). Although crop rotations and/or fungicide applications may control the disease, incorporation of genetic resistance would be the most economical and environmentally sound method of control (McCartney et al. 2002).

The key to incorporating STB resistance into a breeding program is a firm understanding of the underlying factors that control the inheritance of a response to the pathogen isolates (McCartney et. al. 2002). Conflicting results have made it difficult to determine the exact mechanism of the inheritance of STB resistance. Past studies have reported that one or two dominant or partially dominant genes (Rosielle and Brown 1979; Wilson 1979; Lee and Gough 1984; Wilson 1985; Somasco et. al. 1996; Brading et. al. 2002), two or three recessive genes (Rosielle and Brown 1979; Wilson 1985), or even several genes with additive and dominant effects (Van Ginkel and Scharen 1987, 1988a, 1988b; Jlibene et. al. 1994; Simón and Cordo 1998; Zhang et. al. 2001) may control resistance. Because of differences in isolates, methods of inoculation, rating scales and environmental conditions, comparisons between these various studies have been difficult (McCartney et. al. 2002). More recently, the development of a high-density

microsatellite map of wheat (Somers et al. 2004) has accelerated the mapping of several new and previously identified resistance loci. At this time 12 STB resistance loci, designated *Stb1-12*, have been mapped using primarily microsatellite markers (Arraiano et al. 2001; Brading et al. 2002; Adhikari et al. 2003; McCartney et al. 2003; Adhikari et al. 2004a; Adhikari et al. 2004b; Adhikari et al. 2004c; Chartrain 2004; Chartrain et al. 2005b; Chartrain et al. 2005c). With 1,235 mapped loci (Somers et al. 2004), microsatellite markers have become the marker system of choice in wheat because they are polymerase chain reaction (PCR) based, co-dominant, and highly polymorphic in relation to other marker systems. By identifying molecular markers closely linked to desirable traits, such as disease resistance genes, it is anticipated that marker-assisted selection (MAS) will become commonplace in many wheat breeding programs.

Understanding the extent of pathogen variation is also essential in implementing an effective breeding program. Physiological specialization in the wheat-*M. graminicola* pathosystem has been reported in various parts of the world (Saadaoui 1987; Ballantyne and Thomson 1995; Kema et al. 1996a; Kema et al. 1996c). In particular, Kema et al. (2000) discovered that avirulence in the pathogen is controlled by a single locus, which lends support for a gene-for-gene interaction operating in the wheat-*M. graminicola* pathosystem. This theory was further supported by the identification of two distinct virulence groups (group 1 and group 2) in western Canada, based on the differential reaction of the hexaploid wheat line ST6 (Grieger et al. 2005). Further studies concluded that the hexaploid wheat line Salamouni contains three, incompletely dominant resistance genes to group 2 (isolate MG2), two of which also control resistance to group 1 (isolate MG96-36).

The objectives of this study are to; (i) Determine the chromosome location of the resistance gene in Salamouni that confers resistance to only isolate MG-2, and identify closely linked microsatellite markers to facilitate MAS and (ii) Map one of the resistance genes that controls resistance to both MG96-36 and MG2, and identify microsatellite markers linked to this resistance gene that will be useful for MAS.

CHAPTER 2

LITERATURE REVIEW

2.1 Septoria tritici blotch

Septoria tritici blotch (STB) is a residue-borne leaf-spotting disease caused by the ascomycete fungus *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn (anamorph: *Septoria tritici* Rob. ex Desm.) (Sanderson 1972, 1976). STB is now one of the most devastating leaf-spotting diseases to affect wheat crops worldwide (Shipton et al. 1971; Ziv and Eyal 1978; McKendry et al. 1995). Although STB epidemics are most severe and prevalent in the United Kingdom and along the Mediterranean Coast (Eyal et al. 1987), over the last 20 years, western Canadian wheat producers have been seeing an increase in the frequency and severity of STB (Gilbert et al. 1998).

STB diminishes the economic value of wheat by decreasing yield and quality. During severe STB epidemics, yield losses of 20-50% have been reported (Shipton et al. 1971; Eyal and Ziv 1974; Ziv and Eyal 1978; King et al. 1983; Gilbert et al. 1998). Downgrades in quality are the result of shriveled kernels that are often unfit for milling (McKendry et al. 1995). STB is one of five predominant leaf-spotting diseases found in western Canada that has been increasing in prevalence and severity over the last 20 years. Other than STB, Stagonospora nodorum blotch (*Phaeosphaeria nodorum*), Septoria avenae blotch (*Phaeosphaeria avenaria*), tan spot (*Pyrenophora tritici-repentis*) and spot blotch (*Cochliobolus sativus*), alone or in combination, have been causing annual yield

losses as high as 20% in Manitoba (Tekauz et al. 1982; Gilbert and Tekauz 1993). Changing cultural practices, lack of genetic resistance, and reduced competition for susceptible tissue from other diseases such as leaf and stem rust, all seem to be contributing factors to the increase in leaf-spotting diseases in western Canada.

Environmental conditions usually dictate the type and severity of leaf-spotting diseases that develop in any given year (Pedersen and Hughes 1993; Gilbert et al. 1998). Although STB epidemics have been reported in a variety of climatic conditions, it is generally accepted that cool, wet weather favours disease development (Shipton et al. 1971; Shaner and Finney 1976; Eyal 1981; Shaner 1981; Pedersen and Hughes 1993; Gilbert et al. 1998).

2.2 Hosts of *M. graminicola*

2.2.1 Wheat – the primary host of *M. graminicola*

Triticum aestivum L. em. Thell (common bread wheat) and *T. turgidum* (L.) Thell. subsp. *durum* L. (durum wheat) are the two main types of wheat that are of prime economic value on the Canadian prairies. Both serve as hosts for *M. graminicola*.

The evolution of today's durum and common bread wheat are classic examples of how closely related species naturally combine to form a polyploid species. *T. turgidum* subsp. *durum* (2n = 4x = 28, AABB) is a tetraploid wheat containing genomes from the diploid species, *T. monococcum* (AA) and an unknown and possibly extinct B genome donor (Kimber and Sears 1987; Poehlman and Sleper 1995). *T. aestivum* (2n = 6x = 42, AABBDD) is a hexaploid species that was created when three distinct, but similar genomes were combined through natural hybridization and chromosome doubling. The

A and B genomes were derived from *T. turgidum* subsp. *durum*, while the D genome is believed to originate from a progenitor of *T. tauschii* (2n = 2x = 14, DD) (Kimber and Sears 1987; Poehlman and Sleper 1995). Although the A, B, and D genomes of hexaploid wheat are somewhat distinct, the identification of repetitive loci across homoeologous groups suggests that these three genomes originated from a common ancestor (Poehlman and Sleper 1995). For example, there are several loci conferring resistance to leaf rust located on the homoeologous group two chromosomes (Poehlman and Sleper 1995).

Wheat is a cool season crop that is cultivated in many parts of the world, but primarily between the latitudes of 30-65 ° N and 27-40° S (Ñuttonson 1955; Percival 1921). Wheat is utilized for making bread, flour, confectionary products, semolina, breakfast cereals, pasta, and animal feed. Wheat is one of the most important foodstuffs worldwide, because of its many uses, nutritional value, and storage qualities (Poehlman and Sleper 1995). In Canada alone, wheat is grown on between 10 and 11 million hectares of land each year, producing approximately 23 millions tonnes of grain (Statistics Canada 2004).

The geographic center of origin for wheat is southwestern Asia, where wheat has been cultivated for more than 10,000 years. Despite years of natural and artificial selection to improve yield, quality, plant architecture, and resistance to pests, related wild wheat species are still found in Lebanon, Syria, northern Israel and eastern Turkey (Poehlman and Sleper 1995).

2.2.2 Alternative hosts of *M. graminicola*

Although wheat is the host of prime economic importance, there are other species which may act as alternative hosts. *Agropyron* spp., *Agrostis* spp., *Brachypodium* spp., *Bromus* spp., *Dactylis* spp., *Festuca* spp., *Hordeum* spp., *Glyceria* spp., *Poa* spp., *Secale cereale*, and wild *Triticum* spp. such as *T. turgidum* var. *dicoccoides* (wild-emmer) have all been suggested as possible alternative hosts to *M. graminicola* (Eyal 1999a). Along with the above species, Brokenshire (1975) indicated *M. graminicola* had the ability to infect the less common weed species *Vulpia bromoides*. The contribution of these hosts as a potential source of primary inoculum in initiating epidemics on wheat and/or their contribution to the virulence range of *M. graminicola* is not known (Eyal 1999b).

2.3 The Pathogen

2.3.1 General biology of *M. graminicola*

STB is caused by the ascomycete *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn. The ascogenous state of *Septoria tritici* Rob. and Desm. was first identified in New Zealand by Sanderson (1972). Sexual reproduction in *M. graminicola* is controlled by a bipolar heterothallic mating system (Kema et al. 1996c) and leads to the formation of dark brown, globose pseudothecia, which typically range in size from 76-80 x 77-100μm (Sanderson 1972). Asci are bitunicate, obpyriform, 34-41 x 11-13 μm and contain eight irregularly arranged ascospores. *M. graminicola* produces two-celled, hyaline ascospores, which typically measure 10-15 x 2.5-3.5 μm (Sanderson 1972, 1976).

Before the identification of the telomorphic state (Sanderson 1972), the pathogen was thought to propagate solely through the production of asexual pycnidiospores (Eyal

et al. 1987). Pycnidia are formed directly below the stomatal aperture and are embedded in the epidermal and mesophyll leaf tissue of the host plant (Eyal et al. 1987; Cohen and Eyal 1993; Kema et al. 1996d). The light brown, elliptical pycnidia are arranged longitudinally between the veins and range from 80-150 μm in diameter (Sanderson 1976). Pycnidia contain either single-celled micropycnidiospores (30-75 μm in diameter) or two to seven celled macropycnidiospores (72-165 μm in diameter) (Harrower 1976). Both types of pycnidiospores are equally capable of infecting susceptible wheat tissue (Eyal et al. 1987).

2.3.2 Life cycle and epidemiology of *M. graminicola*

M. graminicola was first recognized as the perfect state of *S. tritici* in New Zealand (Sanderson 1972). Since 1972, the sexual state of *S. tritici* has been identified in Australia, the United Kingdom, Chile, the United States, and Canada (Harrower 1976; Madariaga 1986 as cited by Scott et al. 1988; Garcia and Marshall 1992; Hoorne 2002).

Unburied, infected crop debris from previous wheat crops is the main source of primary inoculum for STB epidemics (Shipton et al. 1971; Brown et al. 1978; Eyal et al. 1987; Shaner 1981). Moisture in the form of rain, dew or fog stimulates the release of wind-borne ascospores and rain-splashed pycnidiospores from the infested crop residue (Eyal et al. 1987). Genetic variability studies have confirmed that sexually produced, wind-blown ascospores are the main source of primary inoculum (Brown et al. 1978; Sanderson and Hampton 1978; Eyal et al. 1987; Shaw and Royle 1989; McDonald and Martinez 1990). Spores land on uninfected wheat tissue and proceed to germinate,

penetrate, and infect the leaf tissue, provided there is sufficient moisture (Eyal et al. 1987; Hess and Shaner 1987; Magboul et al. 1992; Shaner and Finney 1976).

Asexual pycnidiospores from established lesions are the main source of inoculum for secondary infection cycles that occur throughout the growing season (Weber 1922; Eyal 1971; Shaner and Finney 1976; Eyal et al. 1987; Shaw and Royle 1989, 1993). Pycnidiospores exuded from oozing pycnidia are dispersed by rain splashes and can only travel short distances (Shaner 1981). The liberation of spores from pycnidia is stimulated by rain events and high relative humidity (Gough and Lee 1985). In addition to multiple asexual cycles that occur throughout the growing season, it is now believed that multiple sexual cycles can occur within the growing season, which also contributes to the polycyclic nature of the disease (Kema et. al 1996c; Zhan et al. 1998).

After the crop is harvested, *M. graminicola* over-winters on unburied crop residue until the following spring (Shipton et al. 1971; Brown et al. 1978; Shaner 1981; Eyal et al. 1987). As mentioned previously, infected debris from the previous growing season is widely accepted as the main source of primary inoculum in the spring, however, the possibility of seed-borne infection and spores over-wintering on alternative grass hosts have also been suggested as a source of primary inoculum. Although seed infection can occur, the transmission from the infected seed to seedling has not been detected (Brokenshire 1973 as cited by Brokenshire 1975); therefore infected seed does not likely contribute to the development of STB epidemics (Shaw and Royle 1993). Infection of alternative grass hosts with STB has been reported (Brokenshire 1975), however, their ability to supply primary inoculum has not been investigated.

Environmental conditions play a critical role in the development of STB epidemics. It is generally accepted that cool, moist weather is most conducive to the development of STB in the field (Shipton et al. 1971; Shaner and Finney 1976; Eyal 1981; Shaner 1981; Pedersen and Hughes 1993; Gilbert et al. 1998). Evidence indicates that moisture is important in all stages of the infection cycle; spore liberation, dispersal, germination, penetration and symptom development (Renfro and Young 1956; Sanderson and Hampton 1978; Gough and Lee 1985). Studies in controlled environments found moist periods of 72-96h post-inoculation resulted in increased disease severity (Hess and Shaner 1987; Magboul et al. 1992). Once initial infection has occurred, high relative humidity favours lesion growth and pycnidial formation (Renfro and Young 1956). Optimal post-inoculation temperatures range from 18-25 °C in controlled environment studies. Temperatures outside this range resulted in decreased disease severity (Hess and Shaner 1987; Wainshilbaum and Lipps 1991; Magboul et al. 1992).

2.3.3 Population genetics of *M. graminicola*

Knowledge of the amount and distribution of genetic variability in the *M. graminicola* pathogen population is essential to interpreting gene flow, natural selection, or host-pathogen co-evolution in the wheat-*M. graminicola* pathosystem (McDonald and Martinez 1990). In order to gain insight into *M. graminicola* population genetics, McDonald et al. (1995, 1999) sampled several populations to determine the amount of genetic variation that exists within and among populations. Data from geographically distinct populations indicated the majority of genetic variation is distributed on a local scale, rather than a macrogeographical scale (McDonald and Martinez 1990; Boeger et al.

1993; McDonald et al. 1995; McDonald et al. 1999; Linde et al. 2002). When isolates from separate field locations were compared, 22 different genotypes were identified from 93 isolates using RFLP analysis (McDonald et al. 1995). When distinct lesions on a single leaf were examined, between two and four different genotypes were present (Boeger et al. 1993; Linde et al. 2002). In tests for variation within a single lesion, different genotypes were identified in approximately 25% of the lesions (McDonald et al. 1995). However, pycnidiospores originating from a single pycnidium consistently shared the same genotype (Boeger et al. 1993; McDonald et al. 1995). This distribution of genetic variation supports the hypothesis that asexual ascospores play a key role in establishing STB epidemics (McDonald and Martinez 1990; Boeger et al. 1993; McDonald et al. 1995; McDonald et al 1999). In general, isolates that shared the same DNA fingerprint were clustered within a field location, confirming rain-dispersed conidia only travel short distances (McDonald et al. 1995; McDonald et al. 1999).

2.3.4 Infection process of *M. graminicola*

Within two to six hours post-inoculation (hpi), germinating conidia were evident on wheat leaves at the microscopic level (Cohen and Eyal 1993; Grieger et al. 2005). Regardless of the inoculated wheat cultivar (resistant or susceptible), it was observed that between 85-90% of the conidia developed germ tubes (Cohen and Eyal 1993). Cohen and Eyal (1993) reported that germ tubes were always orientated in the direction of the stomata, which may suggest the involvement of a stomatal stimulus. However, Kema et al. (1996d) and Grieger et al. (2005) disagreed with the concept of the involvement of a stimulus, since they observed germ tubes to regularly cross the stomata or grow along

guard cells without entering the stomatal aperture. Although germination frequency was high, only 25% of germinated conidia actually penetrated the stomate (Kema et al. 1996d). Germ tubes with appressorim-like structures have been visualized near the stomatal aperture, however, penetration has also been observed in the absence of these appresorium-like structures (Cohen and Eyal 1993; Kema et al. 1996d). Penetration is generally accepted to occur through the stomata, although direct penetration may be a secondary mechanism (Cohen and Eyal 1993).

Hyphal colonization of the sub-stomatal cavities occurred within 12-24 hpi in susceptible cultivars (Kema et al. 1996d; Duncan and Howard 2000; Grieger et al. 2005). Between 48 hpi to eight days post inoculation (dpi), hyphae continued to fill the substomatal regions and then spread intercellularly throughout the mesophyll tissue until cell death occurred at 10-12 dpi. At that time, organization of pycnidia was apparent and hyphae were observed between and below the epidermal cells (Cohen and Eyal 1993). In incompatible interactions, fungal colonization in the substomatal cavity was normally apparent three dpi, however, colonization remained restricted as time progressed when compared to susceptible lines (Grieger et al. 2005). Within four to ten dpi, fluorescing materials were observed in the mesophyll cells surrounding infection sites of resistant lines, which is an expression of incompatibility in wheat rust (Tiburzy and Reisener 1990) and oat powdery mildew (Carver et al. 1991). Little is known about the exact mechanism that restricts pathogen growth and reproduction in resistant cultivars in the wheat-*M. graminicola* pathosystem (Cohen and Eyal 1993; Grieger et al. 2005).

2.3.5 Physiological specialization of *M. graminicola*

Speculation of whether physiological specialization occurs in the wheat-*M. graminicola* pathosystem has been debated for several years. Initial studies failed to detect the existence of "true" physiological races, because results indicated disease severity ratings were similar for all cultures on each of the infected cultivars (Arsenijević 1965 as cited by Eyal et al. 1973; S'Jacob 1968 as cited by Eyal et al. 1973). Therefore *M. graminicola* was classified as a pathogen with "aggressive races" that could vary in virulence, but did not interact differentially with the host. Eyal et al. (1973) was the first to report physiological specialization in the wheat-*M. graminicola* pathosystem. Since 1973, several authors from around the globe have confirmed that cultures of *M. graminicola* have the ability to interact differentially within a single host plant (Eyal et al. 1985; Saadaoui 1987; Ahmed et al. 1995; Ballantyne and Thomson 1995; Kema et al. 1996a; Grieger et al. 2005).

Specialization at the host species level (bread wheat vs. durum wheat) was documented when bread wheat derived isolates were almost exclusively virulent on bread wheat cultivars and durum wheat isolates were almost entirely confined to durum wheat cultivars (Eyal et al. 1973; Saadaoui 1987; Ballantyne and Thomson 1995; Kema et al. 1996a). In western Canada, specialization has also been identified within a single host plant. Grieger et al. (2005) identified two distinct *M. graminicola* virulence groups (group 1 and group 2) based on the differential reaction of the hexaploid wheat line ST6. These two virulence groups also reacted differentially on the tetraploid wheat lines Coulter and 4B1149 (McCartney et al. 2002). Coulter exhibited resistance to both group 1 and 2, while 4B1149 was susceptible.

If *M. graminicola* possesses specific interactions with its hosts, there are implications for breeders wishing to incorporate genetic resistance into new and existing wheat lines (Cowger et al. 2000). The presence of physiological specialization implies that when a resistant cultivar is widely deployed over large geographic area, selection pressure on the pathogen population will operate to overcome that resistance. New biotypes virulent on the resistant cultivar have a selective advantage, resulting in an eventual breakdown of resistance (Browning and Frey 1969). Although rare, such breakdowns in resistance have been reported. The resistance in the cvs. Florence-Aurore and Etit 38 was overcome once they were widely grown in Israel (Eyal et al. 1973). In addition, resistance has been overcome in the cvs. Heron and Robin in Australia (Ballantyne and Thomson 1995) and cv. Gene in Oregon, which contains the *Stb4* gene (Cowger et al. 2000).

2.4 Cultural practices affecting Septoria tritici blotch

Foliar applied fungicide treatments have proven effective in protecting against yield losses traditionally associated with severe STB epidemics (Entz et al. 1990). These fungicides are designed to protect the flag and penultimate leaves, which are primarily responsible for supplying carbohydrates during grain filling. Under high levels of disease, Entz et al. (1990), found that a single application of the systemic fungicide Tilt (propiconazole) reduced the level of leaf diseases and increased yield and kernel weight in wheat. Whether or not fungicide applications are economical depends on yield potential, vulnerability of the wheat cultivar to Septoria, history of previous wheat crops in that location, tillage practices, and weather conditions (Eyal 1981). The effect of

systemic fungicides applied as seed treatments have been shown on several occasions to delay the development of STB up to the fifth leaf stage (Dinoor 1977; Eyal 1981; Brown 1984; Shtienberg 1992). However, treated seed was ultimately not found to affect the incidence and severity of STB later on in the season, as the effects of the seed treatment had dissipated. Therefore no significant yield advantage was gained with the application of a systemic seed treatment prior to planting (Eyal 1981; Brown 1984; Shtienberg 1992).

In the past, several tillage passes were used as a mechanism to bury crop residue from the previous cropping season. The burial of crop residue limits pathogen survival between subsequent crops and serves to minimize the amount of initial inoculum present in the following growing season (Sutton and Vyn 1990; Bailey and Duczek 1996). Over the last two decades, reduced tillage practices have become increasingly popular among producers on the Canadian Prairies. The practice of leaving some or all crop residue on the soil surface not only promotes soil moisture conservation through reduced evaporation and better infiltration, but also helps to protect valuable topsoil from wind and water erosion (Bailey and Duczek 1996). However, since zero or minimum tillage practices have been widely adopted, there has been an increase in the severity and incidence of residue-borne leaf-spotting diseases in wheat (Gilbert and Woods 2001). This is because unburied crop residue serves as an ideal environment for many pathogens to overwinter. During the next growing season, the unburied crop residue has the potential to be a primary contributor of initial inoculum in the subsequent growing season (Bailey and Duczek 1996). Although conservation tillage practices are generally believed to favor the establishment of residue-borne diseases, contradictory findings by several researchers in Manitoba, Saskatchewan, and Ontario have reported higher levels

of *M. graminicola* in conventional till fields compared to minimum or zero-till fields (Sutton and Vyn 1990; Bailey and Duczek 1996; Gilbert and Woods 2001). It is believed that *M. graminicola* is less competitive than other leaf-spotting pathogens such as *Pyrenophora tritici-repentis*, which causes tan-spot in wheat. Because high disturbance systems typically decrease pathogen densities non-specifically, a niche becomes available for the less competitive *M. graminicola* (Sutton and Vyn 1990; Bailey and Duczek 1996; Gilbert and Woods 2001). These findings also imply that stubble-borne inoculum cannot always be destroyed (Shaner and Buechley 1995) or that infections may be initiated by ascospores from other fields. In addition, the increased incidence of STB on the Canadian Prairies may also be attributed to environmental conditions that favour *M. graminicola* over other pathogens.

Crop rotations are another cultural practice employed by western Canadian producers to limit the severity and incidence of diseases. By increasing the amount of time between susceptible hosts, infected debris has a greater opportunity to decompose, thereby limiting the amount of initial inoculum present in the field. One and two year breaks from wheat were found to decrease inoculum densities of various leaf-spotting pathogens, including *M. graminicola* (Sutton and Vyn 1990; Pedersen and Hughes 1992). However, in Saskatchewan, the appearance of STB after a two-year rotation of non-host crops indicates the Septoria complex can survive for at least two years given a suitable environment (Pedersen and Hughes 1992). This unexpected occurrence was likely due to the substantial decrease in other types of leaf-spotting pathogens, which eventually allowed the spores of *M. graminicola* a competitive advantage on young host tissue (Sutton and Vyn 1990; Bailey and Duczek 1996; Gilbert and Woods 2001). Since most

producers grow wheat on average once every three years on the same piece of land (Statistics Canada 2004), crop rotations alone are unlikely to control STB.

Interest in using biological methods to control plant diseases has increased in recent years (Nolan and Cooke 2000). *Pseudomonas spp.* is one biological organism that has been effective in suppressing the development of *M. graminicola* in controlled environment studies (Levy and Eyal 1988; Flaishman et al. 1990; Flaishman et al. 1996). The suppressive characteristics of *Pseudomonas putida* are mediated by the production of diffusible antibiotics, siderophores, and HCN (Flaishman et al. 1996). Pre-treatment of wheat leaves with *Drechslera teres*, the causal agent of barley net blotch, resulted in a significant reduction in diseases caused by *Stagonospora nodorum* and *M. graminicola*, compared to plants pre-treated with water (Nolan and Cooke 2000). However, the level of control achieved was dependent on the wheat cultivar. The exact mechanism(s) responsible for the suppressive qualities exhibited by *D. teres* have not been examined in detail. There are currently no biological control agents registered for the control of *M. graminicola* in Canada.

Although supplemental nitrogen applications are often needed to maintain high levels of productivity in today's intensive cropping systems, the application of nitrogen is a cultural method that has influenced the incidence of foliar wheat diseases (Boquet and Johnson 1987; Howard et al. 1994). Howard et al. (1994) found there was an increase in severity of leaf rust, powdery mildew, Septoria tritici blotch, and Stagonospora nodorum blotch of wheat with the addition of nitrogen fertilizer. One possible explanation for this occurrence is that nitrogen stimulates plant growth, and the resulting increase in canopy density creates a hospitable environment for leaf-spotting pathogens. A dense canopy

slows drying of leaves and plant parts following a precipitation event by limiting the amount of airflow through the canopy. Abundant leaf tissue also shades the underlying soil from the sun, which promotes a cool, moist environment within the crop. The longer free water and high relative humidity conditions exist within the canopy, the more favorable the environment for the germination, infection and colonization of leaf-spotting pathogens.

Incorporating host resistance to *M. graminicola* would be the most economical and environmentally sound method of control. In order for this to become a reality, resistant germplasm must be identified. In the last five years, 12 STB resistance genes have been mapped using molecular markers (Table 2.1). However, in order for these sources to be utilized effectively, more research is needed regarding the types of resistance available, their mode of action, and inheritance patterns (Eyal et al. 1987).

2.5 Disease rating scales

Leaf necrosis and pycnidial density are the two main parameters used to evaluate disease severity resulting from infection by *M. graminicola*. Leaf necrosis is a visible symptom that indicates that tissue death has occurred (Cohen and Eyal 1993). Necrosis caused by *M. graminicola* may be the result of growth and colonization of the pathogen, production of a fungal toxin or a diffuse host response (McCartney 2002). Pycnidial formation is the direct result of successful infection, colonization, and reproduction of *M. graminicola* in the host. These two disease parameters have been used alone, or together, in order to classify resistant and susceptible host responses. Unpublished data by Kema et al. (1996a) suggests that discrepancies between necrosis and pycnidial formation

Table 2.1. Source, map location, closest molecular markers and references of the twelve named genes for resistance against the Septoria tritici blotch pathogen, *Mycosphaerella graminicola*, in wheat.

Gene	Original Source	Chromosomal location	Nearest molecular marker and distance from gene (cM)	Reference(s)
Stb1	Bulgaria 88	5BL	$G7_{1200}$[b](0.68), $H19_{520}$[b](1.4), *Xbarc74*[a](2.8)	Rillo and Caldwell 1966; Adhikari et al. 2004a
Stb2	Veranopolis	3BS	*Xgwm533.1*[a](0.9), *Xgwm493*[a](3.7)	Wilson 1979; Adhikari et al. 2004b
Stb3	Israel 493	6DS	*Xgdm132*[a](3.0)	Wilson 1979; Adhikari et al. 2004b
Stb4	Tadorna	7DS	E-AGG/M-CAT10[c](4.0), *Xgwm111*[a](0.7)	Somasco et al. 1996; Adhikari et al. 2004c
Stb5	Synthetic 6x	7DS	*Xgwm44*[a](7.0)	Arraiano et al. 2001
Stb6	Flame, Hereward	3AS	*Xgwm389*[a](2.0)	Brading et al. 2002
Stb7	Estanzuela Federal	4AL	*Xwmc313*[a](0.5), *Xwmc219*[a](1.0)	McCartney et al. 2003
Stb8	Synthetic W7984	7BL	*Xgwm146*[a](3.5), *Xgwm577*[a](5.3), *Eco*RI-ACG/*Mse*I-CAG5[c](5.3)	Adhikari et al. 2003
Stb9	n/a	n/a	n/a	Chartrain 2004 as cited by Chartrain et al. 2005b
Stb10	Kavkaz-K4500 L.6.A.4	1DL	*Xgwm848*[a](42.0)[d]	Chartrain et al. 2005b
Stb11	TE 9111	1BS	*Xbarc008*[a](38.0)[d]	Chartrain et al. 2005c
Stb12	Kavkaz-K4500 L.6.A.4	4AL	*Xwmc219*[a](16.0)[d]	Chartrain et al. 2005b

[a] Microsatellite marker (SSR)
[b] Random amplified polymorphic DNA marker (RAPD)
[c] Amplified fragment length polymorphism marker (AFLP)
[d] *Stb10*, *Stb11*, *Stb12* were mapped using QTL analysis. The number in brackets following the closest associated molecular marker represents the QTL interval identified in cM.

during cluster analysis implies these two symptoms are under different genetic control. In several instances, certain plants exhibited extensive leaf necrosis with no or few pycnidia. Histological studies of these phenotypes indicated low levels of pathogen colonization. These observations, along with the smaller standard error of the mean for pycnidia, lends support that pycnidial density may be the most reliable disease parameter to classify compatible and incompatible reaction types (Kema et al. 1996a).

Both qualitative and quantitative disease rating scales have been used to assess STB symptoms. The most common quantitative disease rating scale uses percentage leaf area with lesions bearing pycnidia to examine disease severity (Yechilevich-Auster et al. 1983; Jlibene et al. 1994; Camacho-Casas et al. 1995; Kema et al 1996a; Simón and Cordo 1998; Rubiales et al. 2000; Arraiano et al. 2001; Rubiales et al. 2001; Brading et al. 2002; Adhikari et al. 2003; Adhikari et al. 2004a; Adhikari et al. 2004b; Adhikari et al. 2004c; Chartrain et al. 2005b, 2005c). To more accurately define the percentage leaf area containing pycnidia, Eyal and Brown (1976) developed a diagrammatic scale estimating pycnidial coverage of *M. graminicola* on wheat leaves. This scale was used in several studies to estimate pycnidial coverage on four to six upper leaves during the dough development stage (Zadoks growth stage 86) (Danon et al. 1982; Baltazar 1990; Danon and Eyal 1990). Another study determined STB disease scores using a quantitative scale ranging from 0 (none) to 10 (80 to 90% leaf area infected), based on a visually estimated percentage leaf area of necrotic lesions bearing pycnidia (Somasco et al. 1996). Percent necrotic tissue of the total leaf area has also been visually assessed to evaluate STB disease severity (Van Ginkel and Scharen 1987, 1988a, 1988b; Simón et al. 2001; Kema et al. 1996a). Less common methods to evaluate STB disease

symptoms include calculating area under the disease progress curve (Ahmed et al. 1995) and monitoring incubation, latent and pycnidial maturation periods (Simón and Cordo 1998).

Rosielle (1972) was the first to develop a well-defined qualitative STB assessment scale (appendix 1). This scale with six basic classes (0-5) incorporates hypersensitive flecking, coalescence of lesions, and pycnidial density. However, this scale does not incorporate the relationship between necrosis and pycnidial density. In several instances, it was noted that certain varieties showed only light pycnidial formation, but extensive leaf necrosis. These reaction types were dealt with by placing an 'X' after the numerical classification (0-5). One downfall of this scale is that it fails to differentiate between chlorotic and necrotic lesions. This is important because chlorosis is often associated with a resistant response (Eyal 1973), whereas necrosis is often linked with a susceptible reaction type (Kema et al. 1996a). In order to better accommodate expanding chlorotic lesions typical of heterozygous individuals, McCartney et al. (2002) slightly modified Rosielle's (1972) original scale. When using this modified scale, reaction types 0-3 are considered to be resistant, whereas reaction types 4 and 5 are classified as susceptible.

Tolerance is the ability of a host plant to allow pathogen growth and reproduction with little effect on yield performance (Caldwell et al. 1958). Because plants expressing tolerance exhibit phenotypic symptoms comparable to susceptible plants (Caldwell et al. 1958), the above disease rating scales are of little use. Therefore, replicated trials are used to compare yield components of infected and protected plants in order to gain an

appreciation of the level of tolerance expressed (Ziv and Eyal 1976; Ziv et al. 1981; McKendry and Henke 1994b).

2.6 Genetics of host resistance and tolerance

2.6.1 Resistant sources

Host resistance is the ability of a host plant to slow the growth and/or development of the pathogen (Parlevliet 1979). Resistant germplasm has been identified in several bread (spring and winter growth habit) and durum wheat breeding lines and cultivars (Rosielle 1972; Eyal et al. 1983; Eyal et al. 1985; Mann et al. 1985; Kema et al. 1996a; Kema et al. 1996b; Arraiano et al. 1999; Gilchrist et al. 1999; Brown et al. 2001; Eriksen et al. 2003; Chartrain et al. 2004a, 2004b, 2005a). Resistant germplasm has been identified in Brazilian, Chinese, Argentine, and Russian sources, with the Brazilian and Chinese types being the most effective (Mann et al. 1985; Dubin and Rajaram 1996). Although early studies identified high levels of resistance, it was believed that resistance to *M. graminicola* was genetically associated with late maturity and tall plant stature (Tavella 1978; Rosielle and Brown 1979; Eyal 1981; Danon et al. 1982). Attempts to recover high levels of resistance in early maturing, high-yielding semidwarfs was met with difficulty (Dubin and Rajaram 1996). It was believed that the genes responsible for resistance were linked with the genes responsible for tall plant stature and/or late maturity. However, these observations may have been confounded by the architectural differences that exist between tall and semidwarf cultivars. Vertical disease progression (secondary infection cycles) from the lower to upper leaves is ultimately affected by the distance between consecutive leaves. Because semidwarf cultivars (70-90 cm) tend to

have less space between adjacent leaves compared to their tall counterparts, rain splashed pycnidiospores are more likely to be transferred to newly emerging tissue. This results in a more rapid spread of disease upward, which affects upper plant parts responsible for grain filling. Tall cultivars generally have more distance between adjacent leaves, which may slow vertical disease progression. This may have resulted in tall cultivars expressing fewer symptoms, which could have mistakenly been interpreted as resistance (Eyal 1981; Eyal et al. 1987). In addition, Danon et al. (1982) suggested that failures to incorporate resistance to *M. graminicola* from tall, late maturing germplasm to short, early maturing wheat, was partly due to strong selection in early generations for short plant stature, earliness, milling quality etc., followed by selection for resistance to *M. graminicola* in much later generations, which resulted in lines lacking desirable resistance levels. With the recent interest in trait identification, the once believed linkage between STB resistance genes and those responsible for tall plant stature can also be examined in terms of chromosome location. To date, the primary genes used to control plant stature, *Rht1* (4BS), *Rht2* (4DS), *Rht3* (4BS), and *Rht8* (2DL) (GrainGenes 2005) are not associated with any of the 12 mapped STB resistance genes (Table 2.1).

Studies conducted by Arama et al. (1999) concluded that heading date and resistance to STB were not genetically associated, despite previous reports (Eyal 1981). Since these initial obstacles were encountered, high-yielding, early maturing, semidwarf lines with high resistance have been developed by CIMMYT (International Maize and Wheat Improvement Centre). Examples of these include Milan (French source), Corydon (Brazilian source), Catbird (Chinese source), and Bobwhite (Russian source) (Dubin and Rajaram 1996).

The numerous wild relatives of hexaploid wheat are potentially another gene pool that harbours STB resistance (Rosielle 1972; Yechilevich-Auster et al. 1983). In the past, wild relatives have provided wheat breeders with valuable resistant germplasm, which has led to improved pest resistance in cultivated wheat (McKendry and Henke 1994a). Until recently, the use of wild relatives in breeding for STB resistance has not been extensively explored. Resistance has been detected in *T. monoccocum* (AA), *T. turgidum* (wild emmer – AABB), and *T. Tauschii* (DD) (Yechilevich-Auster et al. 1983; McKendry and Henke 1994a). Five years ago, CIMMYT announced the registration of ten synthetic hexaploid wheat lines, with resistance derived from the wild progenitors *T. turgidum/Aegilops tauschii* (Mujeeb-Kazi et al. 2000). Both *Stb5* and *Stb8* have been identified and mapped using synthetic hexaploid wheat lines (Arraiano et al. 2001; Simón et al. 2001). The resistance in Synthetic 6x, generated from a cross between *T. dicoccoides* and *A. squarrosa*, has been identified as *Stb5*, which is located on chromosome 7D. *Stb8* is located on chromosome 7B and was derived from the synthetic W7984 (Adhikari et al. 2003).

Although resistance has been identified, studies have indicated that certain resistant effects may be expressed at varying growth stages. This phenomenon is observed in the wheat-rust pathosystem. Some resistance genes are specifically expressed in the adult or seedling stage only, while others are expressed during all growth stages (Knott 1989). Whether or not genes that control resistance to *M. graminicola* also act in this manner has not been thoroughly investigated (Kema and Van Silfhout 1997). Studies conducted by Kema and Van Silfhout (1997) supported the expression of resistance genes at the seedling stage, but not at the adult plant stage. Similar

observations were made on the winter wheat cultivars Maris Nimrod and Maris Huntsman (Brokenshire 1976). Conversely, two quantitative trait loci (QTLs) identified in adult plants on chromosomes 2B and 7B, were not detected at the seedling stage (Eriksen et al. 2003). Other researchers have found that growth stage at the time of inoculation had no effect on disease severity (Eyal et al. 1973; Wainshilbaum and Lipps 1991; McKendry and Henke 1994b). It is likely that expression of resistance at different growth stages will vary with the resistant source, therefore, screening for STB resistance should be conducted at seedling and adult stages in promising breeding material to ensure acceptable resistance levels (Brokenshire 1976).

2.6.2 Qualitative resistance and mapped STB resistance genes

Mackie (1929) was the first to report qualitatively inherited STB resistance, controlled by a single recessive gene. Since then, simply inherited resistance to STB has been found to be controlled by one, two, or three dominant, or incompletely dominant genes, or two or three recessive genes (Narvaez and Caldwell 1957; Rosielle and Brown 1979; Lee and Gough 1984; Gough and Smith 1985; Wilson 1985; Potts and Hughes 1987; May and Lagudah 1992; McCartney et al. 2002). Although several resistance sources were identified in the mid-seventies, the resistance genes were not given symbols until Wilson (1985).

Earlier studies utilized diallel analysis, as well as specifically designed mathematical formulas (Burton 1951; Lawrence and Frey 1976) to estimate the number of loci controlling STB resistance in a variety of sources (Danon et al. 1982; Danon and Eyal 1990). Disease severity as measured by pycnidial density was found to be

controlled by relatively few genes (one or two) in the wheat cvs. Colotana, Fortaleza-1, Polk/Waldron, Sheridan, Titan, Bezostaya 1, and Oasis (Danon et al. 1982). Similar to previous studies, Danon and Eyal (1990) evaluated a number of resistant sources using diallel analysis, but utilized two distinct tester isolates (ISR398A1 and ISR8036) to evaluate disease symptoms. Although general combining ability (GCA) was the major source of variation, dominance effects were also present. When the same resistance sources were evaluated using formulas to estimate the number of genetic factors segregating in the F_2 generation (Burton 1951; Falconer 1981), it was found that only one or two genes were controlling resistance in Aurora, Kavkaz, Bezostaya 1, Trakia, Colotana and Klein Titan (Danon and Eyal 1990). In addition, differences in disease severity created by the two *M. graminicola* isolates and the small number of loci estimated in Aurora, Kavkaz, Bezostaya 1, and Trakia suggested a gene-for-gene relationship in the wheat-*M. graminicola* pathosystem (Danon and Eyal 1990).

Today, with the power of molecular markers, 12 distinct STB resistance genes (*Stb1-12*) have been identified and assigned chromosome locations using a variety of molecular markers. Details for each mapped resistance gene including original source, chromosome location, closest molecular markers, and references can be found in Table 2.1.

Inheritance studies have concluded that *Stb1* (Rillo and Caldwell 1966; Wilson 1985; Shaner and Buechley 1989; Adhikari et al. 2004a), *Stb2* (Rosielle and Brown 1979; Wilson 1979, 1985; Adhikari et al. 2004b), and *Stb3* (Wilson 1979, 1985; Adhikari et al. 2004b) are simply inherited, dominant resistance genes. *Stb1* was identified in the winter wheat cultivar Bulgaria 88 (Rillo and Caldwell 1966) and was incorporated into

the US wheat cultivars Oasis and Sullivan. Until recently, *Stb1* was considered a durable source of STB resistance in the United States (Jackson et al. 2000). Cytogenetic analysis and molecular markers were both used to assign *Stb2* to chromosome 3BS (Adhikari et al. 2004b). Upon further analysis, the *Stb2* gene appeared to be part of a resistance gene cluster on 3BS. This cluster contains resistance genes for stem rust (*Sr2*) (Spielmeyer et al. 2003), leaf rust (*Lr27*) (Faris et al. 1999), and stripe rust (*Yr30*) (Suenaga et al. 2003), in addition to a gene that codes for phenylalanine ammonia lyase, which is believed to be involved in plant defense responses (Faris et al. 1999). This area is also known to contain quantitative trait loci (QTL) for Fusarium head blight (Buerstmayr et al. 2003, Guo et al. 2003) and Stagonospora nodorum glume blotch (Schnurbusch et al. 2003). The STB resistance gene *Stb3* was first identified in the wheat cultivar Israel 493 (Wilson 1985) and was later mapped to chromosome 6D (Adhikari et al. 2004b). Although there are other resistance genes present on 6D, *Stb3* does not appear to be part of a larger resistance gene cluster. However, all of the inheritance studies used to identify these three genes were conducted in the field and defined *M. graminicola* isolates were not used to induce infection, therefore, defining isolate-specificity of these resistance genes could be examined in the future.

The *Stb4* and *Stb5* resistance genes have both been mapped near the centromere on chromosome 7D (Arraiano et al. 2001; Adhikari et al. 2004c). *Stb4* is an incompletely dominant resistance gene that was first identified in the wheat cultivar Tadinia using the Californian isolate CA30 (Somasco et al. 1996). Until recently, this gene provided Californian wheat crops with protection against *M. graminicola* (Jackson et al. 2000). The *T. tauschii* derived *Stb5*, provides resistance to several *M. graminicola* isolates from

Argentina, Portugal, and the Netherlands (Arraiano et al. 2001; Simón et al. 2001). The *Stb5* resistance gene is also located near the centromere on chromosome 7D (Arraiano et al. 2001, Simón et al. 2001). The microsatellite marker *Xgwm44* and a gene responsible producing a red coleoptile (*Rc3*) in plants are both approximately 7cM from the *Stb5* gene (Arraiano et al. 2001). *Stb6* is an incompletely dominant resistance gene that recognizes the avirulence locus in isolate IPO323 (Kema et al. 2000; Brading et al. 2002). This demonstrated isolate-cultivar specificity implies that a gene-for-gene relationship exists in the wheat-*M. graminicola* pathosystem (Kema et al. 2000). The wheat cultivars Flame, Shafir, Vivant, Hereward, NSL92-5719, Bezostaya 1, Amigo, Arian, Armada, Atlas 66, Blé Seigle, Bulgaria 88, Chinese Spring, Gene, Heines Kolben, Israel 493, Kavkaz, Kavkaz-K4500 L.6.A.4 (KK), Poros, Tadinia and Veranopolis are all believed to carry *Stb6*, a gene allelic to it, or a gene closely linked to it (Brading et al. 2002; Chartrain et al. 2005a). The *Stb7* locus was identified by McCartney et al. (2002) in the wheat line ST6, a selection of the cultivar Estanzuela Federal. *Stb7* is an incompletely dominant, isolate-specific resistance gene that confers resistance to isolate MG-2 (McCartney et al. 2002), one of two distinct virulence groups identified in western Canada (Grieger et al. 2005). The STB resistance loci present in the synthetic hexaploid wheat W7984 is designated *Stb8* and maps to the long arm of chromosome 7B (Adhikari et al. 2003).

Kavkaz-K4500 L.6.A.4 (KK) is believed to have at least five isolate-specific resistance genes including *Stb6, Stb7, Stb10* and *Stb12*. A fifth, unmapped gene is also believed to be present and confers resistance to isolate IPO323. The *Stb10* locus provides resistance to isolates IPO94269 and ISR8036. *Stb12* (Chartrain et al. 2005b) is closely linked to

microsatellite marker *Xwmc313*, also used to identify *Stb7* (McCartney et al. 2003). *Stb10* provides resistance to the Dutch and Israeli isolates IPO94269 and ISR8036, respectively. The Portuguese wheat breeding line TE 9111 is one of the most resistant lines in Europe and is believed to combine isolate non-specific, partial resistance with several isolate-specific resistances. TE 9111 contains the STB resistance gene *Stb11* that provides resistance to isolate IPO90012. TE 9111 may also contain *Stb6* and *Stb7* (Chartrain et al. 2005c).

In addition to sources where resistance genes have been mapped, other sources have been identified. Single dominant (Narvaez and Caldwell 1957; Rosielle 1972; Rosielle and Brown 1979; Lee and Gough 1984; Gough and Smith 1985; Wilson 1985; Potts and Hughes 1987; May and Lagudah 1992), incompletely dominant (Narvaez and Caldwell 1957; Wilson 1985; McCartney et al. 2002) or recessive genes (Rosielle and Brown 1979; Wilson 1985; Potts and Hughes 1987) were identified in Lerma 52, P14, IAS-20, Carifen 12, Vilmorin, Canrock 2, Gala, IRN643, K4500-4, Pavon 'S', French Peace, Bunyip, and ST6. Two dominant (Wilson 1985) or incompletely dominant (Narvaez and Caldwell 1957; McCartney et al. 2002) genes are believed to be present in Nabob, Malta Yellow, PF70216, M1696, and Coulter. Three incompletely dominant resistance genes are present in the hexaploid wheat line Salamouni (McCartney et al. 2002). Two or three recessive genes confer resistance in the cultivar Seabreeze (Rosielle and Brown 1979; Wilson 1985) and Bobwhite 'S' (Gilchrist 1994). Single dominant resistance genes derived from *T. tauschii* have been recovered in the synthetic hexaploid wheat lines AUS3999, AUS10741, AUS22445, and AUS22452 (May and Lagudah

1992). Although the mode of inheritance has not been investigated, isolate-specific resistance has also been detected in several winter and spring wheat lines and cultivars (Brown et al. 2001; Chartrain et al. 2004a, 2004b, 2005a). Several of these sources are believed to contain *Stb6*, or a gene allelic or closely linked to it.

2.6.3 Quantitative resistance

Resistance that is quantitatively inherited is considered to be polygenic, incomplete (Jlibene et al. 1994; Simón and Cordo 1998; Zhang et al. 2001) and isolate non-specific (Chartrain et al. 2004), which enables it to be more durable than the simply inherited, qualitative type of resistance (Parlevliet 1979). Several studies have reported that STB resistance is a quantitative trait. In order to determine the mode of inheritance of STB resistance, diallel and generation mean analyses have been used to provide estimates of the additive, dominance, and epistatic gene effects contributing to variation in disease responses. Studies by Jlibene et al. (1994) and Zhang et al. (2000) found that additive effects were of primary importance in the genetic control of resistance in the wheat lines Ias20*5/H567.71, RPB709.71/Coc, Thornbird, KS94U338, Jagger, KS91W005-1-4, KS91W0935-29-1. Van Ginkel and Sharen's (1987, 1988a, 1988b) studies involving STB resistance in durum wheat also indicated the importance of additive gene action, however significant dominant effects also contributed to the variation in disease severity. Similar analysis of the resistant wheat line II50-18/VGDWf/3/PMF indicated that additive and non-additive gene action contributed to the expression of disease resistance, however, dominance and epistatic effects were the most important (Camacho-Casas et al. 1995).

Simón and Cordo (1998) used diallel analysis to investigate the four components that contribute to partial resistance: incubation period, latent period, pycnidial maturation period and pycnidial coverage. This study concluded that incubation period was inherited independently of pycnidial maturation period and pycnidial coverage, however, the other resistance components were correlated, which may imply similar genetic control. Once again additive effects explained the majority of the genetic variation observed for all components (Simón and Cordo1998).

High levels of quantitative resistance were detected in diverse cultivars and breeding lines from Brazil, Portugal, France, Switzerland, the Czech Republic, Germany, the USA, the UK and the Netherlands when challenged with six distinct isolates (Brown et al. 2001). This same study identified Cappelle Desprez, an important germplasm source of modern European cultivars, as one of the most valuable sources of quantitative resistance (Brown et al. 2001).

Now that detailed molecular maps have been developed in wheat, it is possible to detect quantitative trait loci responsible for STB resistance using molecular markers. A mapping population comprised of 65 RILs (W7984 x Opata 85) was analyzed separately for resistance to STB at seedling and adult plant stages with two Argentinean isolates (IPO 92067 and IPO93014) (Simón et al. 2004). At the seedling stage, three QTL loci were discovered on the short arms of chromosomes 1D, 2D, and 6B. All three loci were detected with both isolates. At the adult stage, two isolate-specific loci were detected on the long arms of chromosomes 3D and 7B (Simón et al. 2004). A QTL for partial resistance to STB was identified on chromosome 6B in Riband, a wheat cultivar previously thought to be susceptible (Chartrain et al. 2004a). Eriksen et al. (2003) also

identified QTLs on chromosomes 2D, 3A, 6B, 2B and 7B, in addition to a major gene for resistance on 3A in the resistant parent Senat. Because different microsatellite markers were used, the QTLs identified on 6B by Eriksen and Chartrain et al. (2004a), cannot be compared. The cultivars Arina and Milan have also been noted to have specific resistance, as well as high levels of partial resistance (Chartrain et al. 2004a, 2002b).

2.6.4 Tolerance

Tolerance is the ability of a host plant to maintain yield performance in the presence of disease symptoms similar in severity to a susceptible host (Caldwell et al. 1958). Because a tolerant host supports growth and reproduction of the pathogen, there is no selection pressure on the pathogen, making tolerance more durable than true resistance (Caldwell et al.1958; Schafer 1971). Varying levels of tolerance to *M. graminicola* have been identified in the wheat cultivars Miriam (Ziv and Eyal 1976), Pike, Cardinal and HybriTech Pacer (McKendry and Henke 1994b). The mechanisms underlying tolerant reactions are not well understood. Studies conducted by Zuckerman et al. (1997) concluded that the tolerance of Miriam was due to a relatively high CO_2 fixation rate per unit of infected tissue throughout the grain filling process. It is believed that the increase in photosynthetic efficiency compensated for the loss of photosynthesizing leaf area caused by *M. graminicola* (Zuckerman et al. 1997). In theory, tolerance is a promising tool in the battle against STB, however, difficulties in the detection and transmission of tolerance given its probable quantitative nature has limited its use in breeding programs (Schafer 1971; McKendry and Henke 1994b).

2.7 Resistance gene localization

2.7.1 Molecular markers

One of the most promising molecular tools for locating genes of interest and improving selection efficiency in crop species are molecular markers. The most widely recognized molecular markers are restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs) and microsatellites or simple sequence repeats (SSRs) (Gupta et al. 1999). Although each type of marker has its advantages and disadvantages, the type of marker system used is largely dependent on the crop species, convenience, cost, and intended application (Gupta et al. 1999).

RFLP analysis involves digesting genomic DNA with a restriction endonuclease to create numerous DNA fragments (Poehlman and Sleper 1995). Once the DNA has been digested, the fragments are separated using gel electrophoresis and then the fragment of interest is detected by using a defined, labeled DNA probe. Polymorphisms are detected as a result of mutations, insertions, or deletions that can potentially create or remove restriction sites specific to the particular restriction enzyme. Although RFLPs are a co-dominant maker system, which means that homozygotes and heterozygotes can be distinguished in the population, their use in wheat is limited because of low levels of detectable polymorphism (Gupta et al. 1999). In addition, RFLP analysis is time-consuming and labour-intensive and is therefore not practical for large-scale studies (Gupta et al. 1999).

RAPDs are a polymerase chain reaction (PCR) based marker system, which makes them highly amenable for large scale screening procedures required in breeding

programs. This marker system uses PCR to amplify random segments of genomic DNA using single primers of arbitrary nucleotide sequence (Williams et al. 1990). The primer hybridizes at several locations throughout the genome. When two hybridization events occur in close proximity to one another, the DNA segment between the two primers is amplified. The PCR products are then separated using electrophoresis so polymorphisms can be detected. Polymorphisms result when there is a difference in specific nucleotide sequence information (Williams et al. 1990). Although RAPD analysis requires only small amounts of DNA and are relatively inexpensive to use, they are a dominant marker system, which means that homozygotes and heterozygotes cannot be distinguished in a mapping population. This limits the amount of information provided by any single primer. In addition to the dominant nature of RAPDs and their inconsistent reproducibility, the low levels of polymorphism detected and complexity of the wheat genome make it a less than ideal marker system for developing a highly informative wheat genetic map (Devos and Gale 1992; Gupta et al. 1999).

AFLP analysis is a technique for DNA fingerprinting developed by Vos et al. (1995). AFLP loci are detected using selective PCR amplification of genomic DNA fragments that were generated from a restriction enzyme digestion. Once the genomic DNA is digested with restriction endonucleases, end-specific, oligonucleotide adapters are ligated to the fragments. These adapters, along with the restriction site sequence are used as primer sites for non-specific PCR amplification. Once enough product has been amplified, sequence specific primers containing between one and three arbitrary nucleotides are used to selectively amplify DNA. The amplified fragments are then separated electrophoretically and viewed using either radioactive or fluorescent labeling

(Vos et al. 1995). Although AFLPs are a dominant marker system, they are able to generate DNA fingerprints regardless of the origin or the complexity of the genome of interest. In addition, AFLPs require no prior sequence knowledge and generate genetic information about a large number of loci in a single PCR reaction. Although high levels of polymorphism are detected in wheat using AFLP analysis, this method is expensive and labour intensive (Mohan et al. 1997; Gupta et al. 1999).

SSRs or microsatellite markers consist of short tandem repeats of usually 2-4 base pairs (Röder et al. 1998; Pestova et al. 2000). The unique sequences flanking the repeat region are used to develop forward and reverse primer pairs, which allow for amplification of the microsatellite using PCR. The PCR products are then separated using electrophoresis. Microsatellite markers are extremely useful in a number of species because of their locus specificity and co-dominant nature. For bread wheat in particular, microsatellites detect higher levels of polymorphism than any other marker system available today (Röder et al. 1998; Pestova et al. 2000). In many cases, these markers are also chromosome specific, which is a useful feature when working with the hexaploid bread wheat genome. Although microsatellite markers are initially expensive to develop, they are relatively inexpensive to utilize once primer pairs are established. More recently, a detailed microsatellite consensus map was published by Somers et al. (2004), which gives a good estimation of marker positions from four genetic maps. This feature, along with the co-dominant and highly polymorphic nature of microsatellite markers makes it an ideal marker system for trait identification and marker-assisted selection (MAS) in bread wheat.

2.7.2 Mapping populations

Developing a mapping population segregating for the trait of interest is one of the first steps in successfully locating and mapping any type of gene. The most commonly used mapping populations are F_2, backcross, doubled haploid (DH), and recombinant inbred lines (RILs). The type of mapping population chosen is largely dependent on the cost, time and labour commitment involved, as well as the characteristics of the molecular marker.

Although F_2 and backcross populations can be created with a small investment of time and resources, a large proportion of the population is heterozygous at any given locus, which limits the amount of information a dominant marker system can provide. However, a co-dominant marker system can exploit the high level of heterozygosity characteristic of F_2 and backcross populations by identifying heterozygous individuals. DH and RIL mapping populations have the advantage of being homozygous at all loci, which allows dominant and co-dominant marker systems to be equally informative. In addition, phenotyping traits that are not completely dominant are facilitated in DH and RIL populations because there are no heterozygous individuals. Developing DH populations is practical when working with a species that has an efficient method of DH production (Fedak et al. 1997). Despite the labour intensive nature of DH development, these populations are a popular choice because they reach homozygosity after only one generation. RILs on the other hand are less labour intensive to produce, but require multiple generations of selfing to achieve the same level of homozygosity. The several generations of selfing result in multiple meiotic events occurring, which ultimately leads

to a greater probability of recombination (Burr et al. 1988). Therefore, RIL mapping populations generally create genetic maps of higher resolution than similar sized DH populations. Because of their homozygous nature, both types of populations can be maintained via selfing. This enables offspring to be created which are identical to the original parent. In this regard, DH and RIL populations are considered to be "immortal", because genotypically identical individuals can be tested for an unlimited number of different traits in multiple different environments. This allows for a more accurate assessment of the genetic component of variance for quantitative traits, because a single genotype is represented by a line, rather than a single individual (Burr et al. 1988).

Either near-isogenic lines (NILs) or bulked segregant analysis can be used to identify DNA markers that are potentially linked to the trait of interest (Michelmore et al. 1991; Young et al. 1988). NILs are developed by backcrossing a line containing the gene of interest (donor parent) to a suitable re-current parent. After several backcrosses to the re-current parent and selection based on the phenotype of the trait of interest, the genome of the resulting progeny will almost be exclusively derived from the recurrent parent, with the exception of the trait of interest and a section of donor parent DNA flanking the trait. In this way, DNA markers linked to the trait of interest can be rapidly identified using pairs of NILs that differ in the presence or absence of the target gene and the small region of flanking DNA (Young et al. 1988).

When segregating populations from a single cross are available, bulked segregant analysis (BSA) is a strategy that can efficiently detect molecular markers putatively linked to the gene of interest. BSA involves comparing two bulked DNA samples from the segregating population with each bulk carrying the contrasting allele for the trait of

interest (e.g., resistant and susceptible to a particular disease). Each bulk contains individuals that are identical for the trait of interest, but are arbitrary for all other genes. When molecular markers across the genome are used to screen the two bulks, those markers that are polymorphic between the bulks will be linked to the phenotypic trait used to create the contrasting bulks. Once a general location has been identified, more markers in that region can be screened across the entire segregating population in order to construct a genetic map including the gene of interest (Michelmore et al. 1991).

2.8 Marker-assisted selection (MAS)

MAS utilizes molecular markers closely linked to the trait of interest to indirectly select individuals in segregating populations carrying the desirable trait. In order for MAS to be successfully incorporated into a breeding program, there needs to be an efficient method of screening large breeding populations, the technique must be highly reproducible, economical and easy to use, and markers should co-segregate or be closely linked to the desired trait (<1cM) to decrease the possibility of selecting recombinant individuals (Gupta et al. 1999). MAS holds the most promise in assessing traits that are easily influenced by the environment, have low heritability, or are difficult and/or expensive to evaluate. Because molecular markers are independent of the environment and can be evaluated at all stages of plant growth, MAS is a valuable tool when evaluating traits such as resistance to pathogens, tolerance to biotic and abiotic stresses, certain quality parameters, and quantitative traits (Young 1996; Mohan et al. 1997; Gupta et al. 1999).

In addition to selecting desirable characteristics, the idea of using MAS in backcross breeding for the purpose of gene introgression has also been proposed (Lee 1995). MAS would be first used to aid in the selection of a desirable gene(s) from a donor parent. Once individuals with the desired donor DNA are selected, MAS can be used to reduce linkage drag of the donor parent DNA near the desired trait and select regions of the recurrent parent genome unlinked to the introgressed region (Lee 1995). The main goal of this would be to reduce the number of backcross generations required to recover the converted recurrent parent (Lee 1995). Although in theory, utilizing MAS in backcross breeding sounds promising, its actual usefulness depends on a number of factors such as selection intensity in different generations, the intensity of linkage between markers and the target gene(s), and the genetic relationship between the donor and recurrent parent (Lee 1995).

One of the most promising prospects of MAS is that it will greatly facilitate the pyramiding of resistance genes into a single cultivar. From a traditional breeding standpoint, incorporating several resistance genes to a single pathogen requires the availability of defined tester isolates to identify each resistance gene (Flor 1971). However, in some cases, defined isolates do not exist or have not been discovered, which severely limits the efficiency and practicality of pyramiding resistance genes. Even if tester isolates exist, it is time consuming to test a single host genotype with each of the isolates required to confirm the presence of a particular resistance gene. MAS has the ability to overcome these limitations. As long as each resistance gene is tightly associated with a molecular marker, an unlimited number of resistance genes to an unlimited number of pathogens can be monitored in a single genotype (Mohan et al.

1997). In addition, MAS allows breeders to select resistant individuals very quickly in early generations, which will reduce the amount of breeding material carried through to the next generation.

CHAPTER 3

IDENTIFICATION AND MAPPING OF *STB14*, AN ISOLATE-SPECIFIC RESISTANCE GENE TO ISOLATE MG2 (GROUP 2) OF SEPTORIA TRITICI BLOTCH OF WHEAT

3.1 Abstract

Septoria tritici blotch of wheat (STB), caused by the pathogen *Mycosphaerella graminicola*, is a serious foliar disease of wheat that compromises both yield and quality. New resistance sources and knowledge of the genetics of resistance are essential to incorporating durable resistance into new wheat lines. Previous inheritance studies identified that the hexaploid wheat line Salamouni contains three incompletely dominant resistance genes to isolate MG2, two of which also provide resistance to isolate MG96-36. The objective of this study was to identify and map the resistance gene that provides resistance to only isolate MG2. Doubled haploid (DH) lines generated from the resistant/susceptible Salamouni/Katepwa cross were evaluated for resistance to both isolates. Three DH lines resistant to isolate MG2 and susceptible to isolate MG96-36 were crossed back to Katepwa to generate F_1 populations. The F_1 population derived from DH line 98S08A*09, was selfed to generate a F_2 population and F_2-derived-F_3 ($F_{2:3}$) families. F_2 screening confirmed the 98S08A*09 population contained a single gene segregating for resistance to isolate MG2. $F_{2:3}$ families were screened to identify homozygous resistant, segregating, and homozygous susceptible families. Bulk

segregant analysis (BSA) identified markers on chromosome 3B that were potentially linked to the gene of interest. Additional polymorphic markers on 3B were used to create a large linkage group, which included the isolate-specific resistance gene, now designated *Stb14*. The microsatellite markers *Xwmc500* and *Xwmc623* were linked to *Stb14* at distances of 2 and 5 centimorgans (cM), respectively. This isolate-specific resistance gene is located near a cluster of resistance genes on 3BS, which provide resistance to a variety of other pathogens, in addition to a previously mapped *Stb2* resistance gene. The microsatellite markers identified in this study may be useful for marker-assisted selection (MAS).

Keywords: *Triticum aestivum, isolate-specific disease resistance, linkage analysis, microsatellite markers, marker-assisted selection*

3.2 Introduction

Septoria tritici blotch (STB) is a devastating foliar disease of wheat (*Triticum aestivum* L. em. Thell) caused by the ascomycete fungus *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn (anamorph: *Septoria tritici* Rob. ex Desm.) (Sanderson 1972, 1976). The severity of this residue-borne leaf spotting disease has increased worldwide over the last two decades. It is generally accepted that this trend is in part due to changing cultural practices such as reduced tillage, lack of proper crop rotations, and the cultivation of susceptible cultivars (Eyal et al. 1987). Frequent rainfall, periods of high relative humidity, and moderate temperatures favor the development of STB epidemics (Eyal 1981; Eyal et. al 1987; Magboul et al. 1992; Shaner and Finney 1976; Wainshilbaum and Lipps 1991). Severe epidemics have been reported to cause yield

losses as high as 50% (Eyal and Ziv 1974; Ziv and Eyal 1978; King et al. 1983). In addition to yield losses, grain quality is also compromised by shriveled kernels that are unfit for milling (McKendry et al. 1995). STB can be effectively controlled with systemic fungicides; however, it is often uneconomical to do so (Eyal 1981; Entz et al. 1990). Although alternative cultural control methods exist, such as crop rotation and tillage, they have not proven to be effective in providing consistent control from year to year (Sutton and Vyn 1990; Pedersen and Hughes 1992; Bailey and Duczek 1996; Gilbert and Woods 2001). Therefore, the most practical and cost-effective solution to prevent STB epidemics would be to provide producers with agronomically sound, STB resistant cultivars (Eyal et al. 1987; Cowger et al. 2000; McCartney et al. 2002).

An understanding of the mode of inheritance and map locations of STB resistance genes is the first step in designing efficient breeding strategies for the development of resistant cultivars (Adhikari et al. 2004b). Genetic resistance to STB has been reported to be controlled by one, two, or three dominant or partially dominant genes (Narvaez and Caldwell 1957; Rosielle and Brown 1978; Wilson 1979; Lee and Gough 1984; Somasco et al. 1996; McCartney et al. 2002), two or three recessive genes (Rosielle and Brown 1978; Wilson 1985), or several genes with additive and dominant effects (Van Ginkel and Scharen 1987, 1988a, 1988b; Jlibene et al. 1994; Simón and Cordo 1998; Chartrain et al. 2004a). The polymorphic nature of wheat microsatellite markers have enabled researchers to map 12 STB resistance genes (*Stb1-12*) to precise chromosome locations (Table 2.1).

Stb1 is a dominant resistance gene that was first identified in Bulgaria 88 (Rillo and Caldwell 1966; Wilson 1985; Shaner and Buechley 1989) and was mapped to

chromosome 5BL (Adhikari et al. 2004a). The wheat cultivar Veranopolis is the original source of resistance conditioned by *Stb2* (Wilson 1979). Molecular mapping indicated *Stb2* was positioned on chromosome 3BS (Adhikari et al. 2004b). The STB resistance gene *Stb3* was first identified in Israel 493 and was later mapped to chromosome 6D (Adhikari et al. 2004b). *Stb4* and *Stb5* have both been mapped near the centromere on chromosome 7D (Arraiano et al. 2001; Adhikari et al. 2004c). *Stb4* was identified in the wheat cultivar Tadinia (Somasco et al. 1996), while a synthetic derived from a hybrid of *T. dicoccoides* and *T. tauschii* (Synthetic 6x) was used to identify *Stb5* (Adhikari et al. 2004c). The resistance gene present in the cultivar Flame was assigned a position on chromosome 3AS and was designated *Stb6* (Brading et al. 2002). *Stb7* was mapped to the distal end of chromosome 4AL in the wheat line ST6, a selection of the cultivar Estanzuela Federal (McCartney et al. 2003). The STB resistance locus present in the synthetic hexaploid wheat W7984 was designated *Stb8* and mapped to the long arm of chromosome 7B (Adhikari et al. 2003). Chartrain (2004) as cited in Chartrain et al. (2005b) identified *Stb9*. *Stb10* and *Stb12* were both identified in Kavkaz-K4500 L.6.A.4 (KK) and mapped to chromosomes 1D and 4A, respectively (Chartrain et al. 2005b). The *Stb11* gene was identified in the Portuguese breeding line TE 9111 and is positioned on chromosome 1BS (Chartrain et al. 2005c). In several cases, these resistance genes are tightly linked with a molecular marker(s) (Table 2.1), which will facilitate their incorporation into new cultivars via marker-assisted selection (MAS).

A firm understanding of pathogen variation is key when attempting to develop resistant cultivars. For many years there was a debate as to whether true physiological specialization was present in the wheat-*M. graminicola* pathosystem. This was partly due

to the fact that earlier inheritance studies were often conducted in the field and isolate mixtures were used to evaluate disease responses. If any type of physiological specialization existed, it would be difficult to detect, because of the numerous isolates used, and the possibility of natural infection. However, more recent studies have utilized single isolates to examine resistance and pathogen variability. This has led several researchers to confirm that cultures of *M. graminicola* have the ability to interact differentially within a single host plant (Eyal et al. 1985; Saadaoui 1987; Ahmed et al. 1995; Ballantyne and Thomson 1995; Kema et al. 1996a; Grieger et al. 2005). The majority of the mapped STB resistance genes have been identified using single isolates and have demonstrated isolate-specificity, which is characteristic of a gene-for-gene interaction. The possibility of the gene-for-gene mechanism operating in the wheat-*M. graminicola* pathosystem is further supported by the discovery that avirulence in the Dutch isolate IPO323, is controlled by a single locus (Kema et al. 2000). In western Canada, two distinct *M. graminicola* virulence groups have been identified (group 1 and group 2) based on the differential reaction of the hexaploid wheat line ST6 (Grieger et al. 2005).

Inheritance studies conducted by McCartney et al. (2002) identified three incompletely dominant resistance genes in the hexaploid wheat line Salamouni, a highly resistant landrace from Lebanon. Three of these resistance genes confer resistance to isolate MG2 (group 2). Two of these resistance genes also control resistance to isolate MG96-36 (group 1). The objective of this study is to determine the chromosome location of the STB resistance gene in Salamouni that confers resistance to only isolate MG2 and to identify microsatellite markers closely linked to the resistance gene.

3.3 Materials and Methods

3.3.1 Population development

McCartney et al. (2002) identified three independently segregating STB resistance genes in Salamouni, a highly resistant landrace from Lebanon. Reciprocal crosses were made between Salamouni and the highly susceptible cultivar Katepwa. DH lines were generated from the F_1 hybrids using the maize hybridization/embryo rescue method (Fedak et al. 1997). Thirty-seven DH lines were inoculated, as described in section 3.3.2, with isolates MG96-36 (group 1) and MG2 (group 2) and resulting disease reactions were recorded. Three DH lines (98S08A*09, 98S08A*10 and 98S05B*04) exhibiting resistance to isolate MG2 and susceptibility to isolate MG96-36 were crossed back to Katepwa to generate approximately 200 F_1 seeds per cross. Ten F_1 plants were grown from each cross and were evaluated for reaction with MG2 and self-pollinated to produce the F_2 generation. Seventy-five F_2 plants generated from the DH line 98S08A*09 were evaluated with MG2 and subsequently selfed to produce $F_{2:3}$ families. Because the 98S08A*09 F_2 segregation ratio was characteristic of a single gene segregating (3 : 1 resistant to susceptible), the 98S08A*10 and 98S05B*04 F_2 populations were not evaluated. Seventy-five $F_{2:3}$ families derived from the DH line 98S08A*09 were screened with isolate MG2 (group 2) to validate F_2 phenotypes. Twenty $F_{2:3}$ families of 20 individuals were also inoculated with isolate MG96-36 (group 1) to ensure susceptibility.

Seeds were germinated in the dark prior to planting (2 days 4°C, 2 days room temperature) to promote uniform germination. Depending on the amount of seed

required for the next generation, one (F_1), two (F_2) or five seeds ($F_{2:3}$) were planted per 15-cm-diameter clay pot containing a 1 : 1 : 1 or 2 : 1 : 1 soil mix (soil/sand/peat). Plants were grown prior to inoculation in a growth room set at 21/16°C (day/night) with a 16-h photoperiod (250μE $m^{-2}s^{-1}$). All plants were fertilized with 20-20-20 fertilizer (3.75 ml/L) and watered as required. Throughout population generation, glassine bags were placed over spikes to prevent cross contamination.

3.3.2 Inoculation

Single spore isolates of *M. graminicola* representative of group 1 (MG96-36) and group 2 (MG2) were used to evaluate the DH lines. These were the same isolates used by McCartney et al. (2002). Infected leaf tissue from Manitoba wheat fields was the original source of the cultures (Grieger et al. 2005). MG2 was used to evaluate the F_1, F_2, and $F_{2:3}$ families. Each culture was derived from a single, sporulating pycnidium, which was transferred to a yeast malt agar plate containing 0.25 % chloramphenicol (YMA^+): 4g Difco malt extract (Difco Laboratories, Detroit), 4 g of sucrose (Fisher Scientific, Fair Lawn, NJ), 15 g of Difco agar, 250 mg of chloramphenicol (Sigma Chemical, St. Louis) and 1000 ml of distilled water (Eyal et al. 1987). After seven days in the dark at 20°C, single conidiospores were isolated using a dissecting microscope to ensure a pure culture (Grieger et al. 2005). Cultures were incubated under fluorescent lights for seven days at room temperature. Conidia were harvested by flooding cultures with sterile distilled water and suspending the spores using a wire loop. The suspension was filtered through three layers of cheesecloth and subsequently quantified using a hemacytometer. The

conidial suspension was adjusted to 10^7 spores/ml and one drop of Tween 20 (polyoxyethylene sorbitan monolaurate) was added per 50 ml of inoculum as a surfactant.

Seedlings at the three-leaf stage were inoculated with the spore suspension until run-off using a DeVilbiss-type sprayer. Plants were allowed to dry and then placed in a misting chamber where continuous leaf wetness was achieved using two ultrasonic humidifiers. After an incubation period of 72 h, seedlings were transferred to a growth cabinet set at 21/19°C (day/night) with a 16-h photoperiod (390μE $m^{-2}s^{-1}$) and relative humidity between 70 and 80%.

A total of ten F_1 seedlings were inoculated with MG2. The 98S08A*09 F_2 population of 75 individuals was evaluated during a single inoculation. During inoculations of the $F_{2:3}$ families, only 20 families of 20 individuals each could be challenged during a single inoculation due to space limitations. Although different families of a single population were screened during separate inoculations, all individuals of a family were inoculated as a unit.

Along with the DH parental line and corresponding F_1 individuals, four to ten plants of Salamouni, Katepwa, Erik, and ST6 were always included with each inoculated set to provide symptom comparisons and facilitate disease ratings. The wheat cultivar Erik served as an additional susceptible check for both isolates, while the differential reaction of ST6 was used to confirm isolate identity. ST6 is susceptible to isolate MG96-36, but is resistant to isolate MG2.

3.3.3 Disease assessment

Plants were assessed for their reaction to *M. graminicola* 17 days after inoculation (dai). Reactions were classified using a qualitative evaluation scale originally developed by Rosielle (1972) and modified by McCartney et al. (2002) (Fig. 3.1). This scale integrates the amount of necrotic tissue and pycnidial density to assign a particular reaction type. Previous authors have indicated that both of these symptoms are informative about the host's response to the pathogen (Rillo and Caldwell 1966; Eyal et al. 1987; Saadaoui 1987; Ballantyne and Thomson 1995; Kema et al. 1996a; Somasco et al. 1996).

Reaction types 0-3 were considered to be resistant, while 4 and 5 were considered to be susceptible. The intermediate "3" reaction was considered to be resistant, primarily because growth and reproduction of the pathogen was restricted (McCartney et al. 2002). In addition, chlorotic lesions characterized the intermediate reaction, which in other pathosystems such as the wheat-*Puccinia graminis* f. sp. *tritici* system, the presence of chlorosis indicates a hypersensitive or resistant reaction (Roelfs and Martens 1988). In order to verify individual F_2 plant ratings, $F_{2:3}$ families were scored as either homozygous resistant, segregating, or homozygous susceptible based on the reactions of 20 seedlings per family. Data were tested for goodness of fit to specific genetic ratios using Chi-Square analysis. Yates correction factor was used where appropriate (Strickberger 1985).

3.3.4 DNA extraction

Leaf tissue was collected 18 dai from the youngest, non-inoculated leaf of the $F_{2:3}$ families. Equal amounts of leaf tissue from each individual of a particular family were pooled to reconstitute the genotype of the F_2 plant. Tissue was lyophilized for 72h and

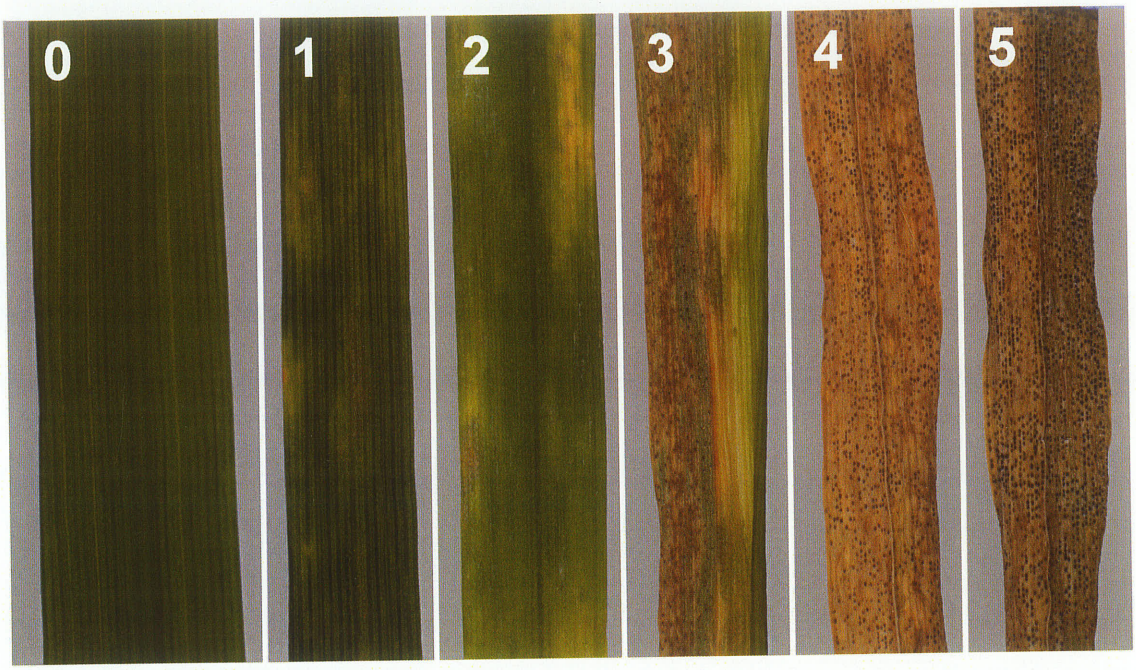

Fig. 3.1. Modified Rosielle scale. **0** = immune, characterized by an absence of pycnidial formation, an occasional hypersensitive fleck, or no visible symptoms; **1** = highly resistant with hypersensitive flecking; **2** = resistant with small chlorotic or necrotic lesions, typically no pycnidial formation; **3** = intermediate, characterized by coalescence of chlorotic or necrotic lesions normally evident towards the leaf tips and to a lesser extent elsewhere on the leaf blade, very light pycnidial formation; **4** = susceptible with moderate pycnidial formation, coalesced necrotic lesions; **5** = very susceptible with large, abundant pycnidia, necrotic lesions, extensively coalesced.

then stored at -20°C. Tissue from each family was placed in a 15 ml falcon tube (Fisher Scientific, Pittsburgh, Pa.) with four 3-mm tungsten carbide beads (Qiagen, Missassauga, ON). Tissue was ground to a fine powder by shaking in a paint shaker for 10 min. Approximately 0.15 g of ground tissue was transferred to 1.2 ml collection tubes (Qiagen, Missassauga, ON) containing 200 μl of glass beads. Tissue was further ground by shaking in a paint shaker for 3-4 min. DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Mississauga, ON) and quantified using Hoechst 33258 stain.

3.3.5 Bulked segregant analysis

Bulked segregant analysis (BSA) was used to identify putatively linked markers to the STB resistance gene (Michelmore et al. 1991) contained in the 98S08A*09 population. The resistant DNA bulk was prepared by pooling equal concentrations of DNA from nine homozygous resistant $F_{2:3}$ families and the susceptible DNA bulk was prepared by pooling equal concentrations of DNA from ten homozygous susceptible $F_{2:3}$ families. Initially, 48 primer pairs located in areas of previously mapped STB resistance genes were used to initiate BSA (appendix 2). In total, 98 primer pairs were used to screen the two bulks, along with the resistant DH (98S08A*09) and susceptible parent (Katepwa) in an attempt to identify potentially linked makers to the gene of interest (Michelmore et al. 1991). These markers, along with their chromosome location are listed in appendix 2.

3.3.6 PCR amplification

PCR reactions were performed in 10-μl volumes and contained 24 ng of template DNA, 1U of Taq DNA polymerase (Gibco/BRL, Mississauga, ON), 1X PCR buffer (Applied Biosystems, Foster City, Calif.), 1.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 0.2 pmols of forward primer, 2.0 pmols of reverse primer, and 1.8 pmols of 6-FAM/HEX/NED-labelled M13 primer (5'- 3' CACGACGTTGTAAAACGAC; Applied Biosystems, Foster City, CA). GWM, GDM, and WMC primer sequences were obtained from Röder et al. (1998), Pestova et al. (2000) and Gupta et al. (2002), respectively. The CFA and CFD primer sequences were provided by Dr. P. Sourdille (INRA), while the BARC marker sequences were obtained from Song et al. (2002). All forward microsatellite primers were modified to contain a 5', 19-nucleotide M13 tail (Schuelke 2000). The reaction mixture was denatured at 94°C for 2 min, followed by 30 cycles of 95°C for 1 min, 51/61°C for 50 sec, 73°C for 1 min, with a final extension step of 73°C for 5 min. PCR products were resolved with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) with GeneScan-500 software and GeneScan-500 ROX as an internal size standard (Applied Biosystems, Foster City, CA). Genographer version 1.6.0 (http://hordeum.oscs.montana.edu/genographer) was used to convert chromatograms to gel images.

3.3.7 Linkage analysis

Three markers showing potential linkage to the resistance gene through BSA were identified on chromosome 3B. Eventually all 53 microsatellite markers from the wheat microsatellite consensus map located on the short arm of chromosome 3B (Somers et al. 2004), were screened on the bulks and the parents. Eleven of these markers were

polymorphic between the parents. These were subsequently screened on the entire mapping population. Chromosome locations of these makers were previously determined in other mapping populations (Röder et al. 1998; Pestova et al. 2000; Chalmers et al. 2001; Gupta et al. 2002; Song et al. 2002; Somers et al. 2004). Phenotypic data from 68 $F_{2:3}$ families and genotypic data from eight microsatellite markers were used to create a linkage map of chromosome 3B.

JoinMap^R version 3.0 (van Ooijen and Voorrips, 2001) was used to create linkage groups and a genetic-linkage map of chromosome 3B. Map distances were converted to centimorgans (cM) using the Kosambi function (Kosambi 1944). Linkage maps were generated using an LOD threshold of 2.0.

3.4 Results

3.4.1 Disease reactions of the DH lines, F_1's, F_2's, and $F_{2:3}$ families

Previous inheritance studies conducted by McCartney et al. (2002) indicated that Salamouni contains three independent resistance genes to isolate MG2, two of which also control resistance to isolate MG96-36. DH lines generated from the F_1 hybrids of the reciprocal cross between Salamouni and the susceptible cultivar Katepwa were evaluated with isolates MG2 and MG96-36. The observed disease reactions were 30 : 3 : 4 (resistant to MG96-36 and MG2 : susceptible to MG96-36 and resistant to MG2 : susceptible to MG96-36 and MG2), which fit the expected 6 : 1 : 1 segregation ratio (X^2 = 0.84, P = 0.66), confirming that Salamouni contains three independent resistance genes. Three DH lines were susceptible to MG96-36 and resistant to MG2, indicating they contained the single resistance gene responsible for the differential reaction. The DH

parents 98S08A*09, 98S08A*10 and 98S05B*04 scored a "2" or "3" reaction type according to the disease rating scale described previously, when challenged with isolate MG2. When inoculated with isolate MG96-36, these lines exhibited coalesced necrotic lesions with moderate pycnidial formation, a typical susceptible reaction according to the disease assessment scale modified by McCartney et al. (2002) (Fig.3.1). The reaction of the DH parent 98S08A*09 to isolates MG96-36 and MG2 is shown in Fig. 3.2.

Ten F_1 seedlings from 98S08A*09 and 98S05B*04 showed an intermediate reaction type characterized by coalesced chlorotic lesions and few pycnidia when inoculated with isolate MG2. The F_1 progeny derived from 98S08A*09 and 98SO5B*04 were either slightly less resistant or equally resistant to the corresponding DH parent. However, the F_1 individuals from the 98SO8A*10 demonstrated a poor intermediate reaction type, therefore an F_2 population was not evaluated. A F_2 population of 75 individuals derived from 98S08A*09 was evaluated with isolate MG2. The F_2 population segregated in a 3 : 1 (resistant/susceptible) ratio characteristic of a single gene segregating for resistance to MG2 (Table 3.1). Based on these results, the 98S05B*04 F_2 population was not evaluated.

The segregation ratio of the corresponding 98S08A*09 $F_{2:3}$ families also fit a 1 : 2 : 1 (homozygous resistant : segregating : homozygous susceptible) ratio (Table 3.1), which was consistent with a single, incompletely dominant resistance gene segregating for resistance to isolate MG2. However, due to the incompletely dominant nature of this resistance gene, it was sometimes difficult to classify particular families. In order to avoid inaccuracy during linkage analysis, the phenotypic data from seven families were scored as missing data. To confirm that 98S08A*09 contained only the gene that

Fig. 3.2. Differential reaction of the doubled haploid (DH) line, 98S08A*09 used to map *Stb14*. **A** = Resistant reaction type "3" of DH line 98S08A*09 when inoculated with isolate MG2. **B** = Susceptible reaction type "4" of DH line 98S08A*09 when inoculated with isolate MG96-36.

Table 3.1. Segregation of the F_2 and $F_{2:3}$ generations derived from the cross 98S08A*09/Katepwa[b] for reaction to isolate MG2 of *Mycosphaerella graminicola*.

Cross	Observed F_2 (R : S)	Ratio tested in F_2 (R : S)	F_2 X^2 (P)[a]	Observed $F_{2:3}$ (HR : SEG : HS)	Ratio tested in $F_{2:3}$	$F_{2:3}$ X^2 (P)[a]
98S08A* 09/Katepwa	58 : 17	3 : 1	0.21 (0.64)	14 : 47 : 14	1 : 2 : 1	4.81 (0.09)

[a] X^2 corrected with Yates correction factor where appropriate. A fit to the expected segregation ratio is accepted if $P > 0.05$
[b] 98S08A*09 is a doubled haploid derived from Katepwa/Salamouni

controls resistance to isolate MG2, 20 $F_{2:3}$ families of 20 individuals were evaluated with isolate MG96-36 (group 1). All individuals inoculated were susceptible.

3.4.2 Marker and linkage analysis

A total of 98 primer pairs were used to conduct BSA on a selection of homozygous resistant and susceptible individuals chosen from the 98S08A*09 mapping population. The microsatellite markers *Xwmc500*, *Xcfd79*, *Xwmc754*, *Xwmc11* and *Xwmc489* detected potential linkage with the gene conferring resistance to isolate MG2. However, upon further analysis, only markers *Xwmc500*, *Xcfd79*, and *Xwmc754* demonstrated potential linkage with the resistance gene. All markers putatively linked with the resistance locus mapped to the short arm of chromosome 3B, as determined by the wheat microsatellite consensus map (Somers et al. 2004). The entire mapping population was screened with *Xwmc500*, *Xcfd79*, and *Xwmc754* in addition to other markers on 3BS that demonstrated polymorphism between the parents (Table 3.2).

Linkage analysis of all scoreable markers on 3BS and phenotypic data from 68 individuals in the 98S08A*09 mapping population generated a 39 cM linkage group, including the gene of interest, now designated *Stb14* (Fig. 3.3). Marker location and order was consistent with the wheat microsatellite consensus map published by Somers et al. (2004). The closest microsatellite marker was *Xwmc500* and it was linked to *Stb14* at a distance of 2 cM. *Xwmc500* amplified a 184 bp allele in the DH (resistant) parent and a 186bp allele in Katepwa (susceptible parent). A flanking marker, *Xwmc623*, was identified and was linked to *Stb14* at a distance of 5 cM. This marker also amplified polymorphic alleles in the resistant (143 bp) and susceptible parent (133 bp). The *Stb2*

Table 3.2. PCR primer sequences and annealing temperatures of the SSR markers used to map *Stb14*

Marker	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Annealing Temperature (°C)
BARC75[a]	AGGGTTACAGTTTGCTCTTTTAC	CCCGACGACCTATCTATACTTCTCTA	51
BARC87[a]	GCTCACCGGGCATTGGGATCA	GCGATGACGAGATAAAGGTGGAGAAC	51
CFD79[b]	TCTGGTTCTTGGGAGGAAGA	CATCCAACAATTTGCCCAT	61
GWM389[c]	ATCATGTCGATCTCCTTGACG	TGCCATGCACATTAGCAGAT	61
GWM533[c]	AAGGCGAATCAAACGGAATA	GTTGCTTTAGGGGAAAAGCC	61
WMC500[d]	ATAGCATGTTGGAACAGAGCAC	CTTAGATGCAACTCTATGCGGT	61
WMC632[d]	GTTTGATTGGTCGTTCCTGGTC	AACAGCGAATGGAGGGCTTTAG	61
WMC754[d]	ATCCACATGAACCTCAACTTATGG	GGCATTGTTGTTGTACTGCAGTC	61

[a]Source: USDA-ARS

[b]Source: INRA

[c]Source: Röder et al. 1998

[b]Source: Agrogene

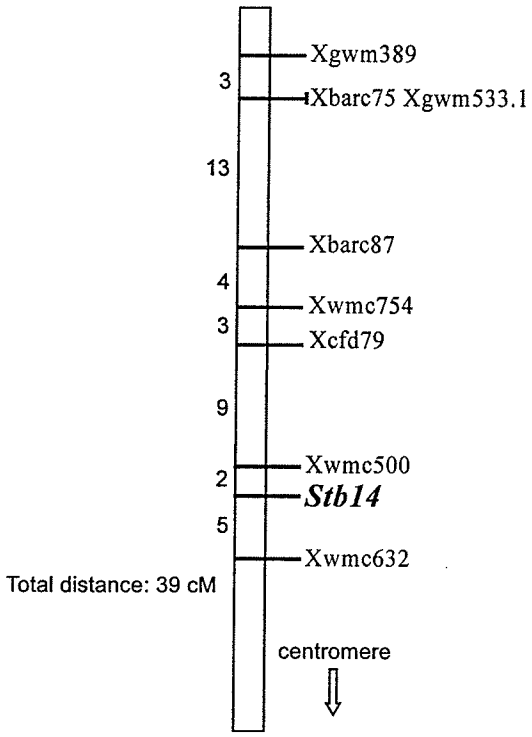

Fig. 3.3. Linkage map displaying the microsatellite loci and the *Stb14* gene on the short arm of chromosome 3B. *Stb14* provides resistance to isolate MG2 of *Mycosphaerella graminicola*. Markers were mapped using 68 $F_{2:3}$ families developed from the cross between DH line 98S08A*09/Katepwa. Distances are reported in Kosambi centimorgans.

locus, which was originally identified in Veranopolis (Table 2.1), has also been mapped to the short arm of chromosome 3BS (Adhikari et al. 2004b). *Stb2* is tightly linked with the microsatellite markers *Xgwm533.1*, *Xgwm389*, and *Xgwm493*. Although markers *Xgwm533.1* and *Xgwm389* were polymorphic between the DH line and Katepwa, no polymorphism was detected between the bulks of the 98S08A*09 mapping population. Although these two markers were used to generate a linkage map of chromosome 3B, the *Stb14* gene identified in the DH line 98S08A*09 was not linked to either of these markers. This indicated that the *Stb14* resistance locus is distinct from the *Stb2* locus.

3.5 Discussion

The mapping population derived from the DH line 98S08A*09 was used to map one of three resistance genes present in the highly resistant hexaploid wheat line, Salamouni. *Stb14* was mapped to chromosome 3BS using microsatellite markers and is the 14th STB resistance locus to be given a map location. This single resistance gene confers resistance to isolate MG2, belonging to one of two distinct virulence groups identified in western Canada (Grieger et al. 2005). Linkage analysis identified that *Stb14* was situated in between the microsatellite loci *Xwmc500* and *Xwmc623* at distances of 2 cM and 5 cM, respectively. Previous mapping studies determined that *Stb2* is also present on 3BS (Adhikari et al. 2004b), however its mapped location is distal to that of *Stb14* by approximately 30 cM, as estimated from the wheat microsatellite consensus map (Somers et al. 2004). *Stb2* is linked to the microsatellite markers *Xgwm533.1* and *Xgwm493* at distances of 0.9 and 3.7 cM, respectively (Adhikari et al. 2004b). The map

location of *Stb14* suggests it is close, but not part of a previously mapped resistance gene cluster (Adhikari et al. 2004b). This genomic region on 3BS appears to be abundant in resistance loci for a variety of different fungal pathogens. For example, the *Sr2* locus for adult resistance to stem rust has been mapped to chromosome 3BS using the microsatellite markers *Xgwm493*, *Xgwm533.1* and *Xgwm389* (Spielmeyer et al. 2003), all of which are also linked to the *Stb2* gene. Genes providing resistance against leaf rust (*Lr27*) (Faris et al. 1999) and yellow rust (*Yr30*) (Suenaga et al. 2003) are also on 3BS and are believed to be a part of the same resistance gene cluster. In addition to qualitative characters, major QTLs for Fusarium head blight (Buerstmayer et al. 2003; Guo et al. 2003), and Stagonospora nodorum glume blotch (Schnurbusch et al. 2003) are also within this genomic region. A gene that encodes for phenylalanine ammonia lyase, a substance believed to be involved in defense responses, has been identified near the region of *Stb2* (Faris et al. 1999). Because this region is rich in resistance genes to a wide variety of fungal pathogens, it will be an area of interest to exploit in terms of increasing disease resistance in wheat. A similar type of resistance gene cluster, which includes *Stb4* and *Stb5*, in addition to several other resistance genes to pests and pathogens, has been reported near the centromere on chromosome 7D (Adhikari et al. 2004c). The presence of multiple disease resistance loci with closely linked markers will facilitate large-scale sequencing of these areas in hopes of elucidating the physical arrangement of these clusters and the detailed mechanisms under which these genes operate (Michelmore 2000).

Twelve STB resistance genes (*Stb1-12*) have been identified and mapped to specific chromosome locations as mentioned previously. The original source,

chromosome location and their most closely linked molecular markers are summarized in Table 2.1. Until recently, all STB resistance genes have been mapped to a distinct chromosome, with the exceptions of *Stb4* and *Stb5*, which are both on 7D, and *Stb7* and *Stb12*, which are both located at the distal end of chromosome 4AL. This study however, has placed *Stb14* on chromosome 3B, along with *Stb2*. It is also believed that *Stb13* (as discussed in Chapter 4) shares chromosome 7B with *Stb8*. Especially in the cases of the *Stb4* and *Stb5*, *Stb7* and *Stb12*, and perhaps the *Stb2* and *Stb14* loci, where mapping has placed the resistance genes in close proximity to one another, it may be wise to conduct allelism tests to ensure that these genes are in fact different from one another. This type of test would involve making a cross between the two resistant sources and evaluating the F_2 populations with each of the isolates used to identify the resistance gene. If susceptible individuals are detected with either isolate, the resistance genes are distinct from one another. However, it is important when conducting allelism tests to ensure that the population size is large enough to allow for identification of two distinct, but closely linked genes. Large populations must be developed in order to account for the lower recombination frequency between closely linked genes (Brûlé-Babel, Personnel Communication).

The majority of the STB resistance genes have been identified using microsatellite markers. Microsatellites are usually the marker system of choice in wheat because they have proved to detect high levels of polymorphism, are co-dominant in nature, are PCR based, and a detailed high-density consensus map including 1,235 loci has been recently published (Somers et al. 2004). In addition, many of the microsatellite markers are specific and amplify only a single locus from one of the three wheat genomes

(Röder et al. 1998; Pestova et al. 2000). These attributes make microsatellite markers a valuable tool in the future advances towards utilizing MAS in wheat breeding programs.

The recent interest in characterizing and mapping STB resistance genes will provide several benefits to wheat breeders incorporating STB resistance into new breeding lines. The fact that *M. graminicola* has very specific temperature and moisture requirements to allow for maximal differentiation between resistant and susceptible genotypes (Shaner and Finney 1976; Eyal 1981; Eyal et al. 1987; Wainshilbaum and Lipps 1991; Magaboul et al. 1992; Gilbert et al. 1998), can often hinder selection in the field. In addition, the difficulty of scoring reaction types in segregating populations can be made easier with MAS and can eliminate the need to perform time consuming progeny tests in order to confirm phenotypic data (Brading et al. 2002; McCartney et al. 2002). Another factor interfering with accurate genotype identification is the simple fact that other foliar pathogens can co-exist with *M. graminicola*, which makes identifying disease symptoms attributable to *M. graminicola* alone, difficult to distinguish (Gilbert et al. 1998). *M. graminicola* has also been documented to interact with other wheat pathogens such as *Blumeria graminis* f. sp. *tritici*. This interaction has been reported to allow *M. graminicola* to sporulate on normally resistant cultivars (Brokenshire 1974). MAS can help to overcome these barriers because selection is based on genotype rather than phenotype. In addition, molecular markers are not influenced by the environment and can be evaluated at all stages of plant growth (Gupta et al. 1999).

One of the most promising applications of MAS will be pyramiding resistance genes into a single host genotype. This technology will be especially useful for the introgression of multiple resistance genes to a variety of pests and pathogens, where it is

not possible to phenotype all of the different reactions on the same plant. For STB in particular, the availability of closely linked markers will allow for the incorporation of STB resistance genes that cannot be distinguished phenotypically, for example, *Stb7* and *Stb14*, which both provide resistance to isolate MG2. Inheritance studies conducted by McCartney et al. (2002) suggested the resistance genes in Salamouni (*Stb14*) and ST6 (*Stb7*), which both provide resistance to MG2 and not MG96-36, were not allelic. This study confirms their independence as each resistance gene has been assigned a distinct chromosome location.

In summary, *Stb14* provides specific resistance to the western Canadian isolate MG2. *Stb14* was mapped to chromosome 3BS and is flanked by the microsatellite markers *Xwmc500* and *Xwmc623* at distances of 2 and 5cM, respectively.

CHAPTER 4

IDENTIFICATION AND MAPPING OF *STB13*, A ISOLATE-SPECIFIC RESISTANCE GENE TO ISOLATE MG96-36 (GROUP 1) OF SEPTORIA TRITICI BLOTCH OF WHEAT

4.1 Abstract

Septoria tritici blotch, caused by *Mycosphaerella graminicola*, is a foliar disease that affects wheat crops worldwide. The hexaploid wheat line, Salamouni, was previously identified to have three incompletely dominant resistance genes to isolate MG2. Two of these resistance genes also control resistance to isolate MG96-36. The objective of this study is to isolate one of the two resistance genes in Salamouni that confers resistance to isolate MG96-36 and to identify its chromosome location using microsatellite markers. Doubled haploid (DH) lines from the resistant/susceptible Salamouni/Katepwa cross were evaluated for resistance to both isolates. Thirteen DH lines resistant to isolates MG2 and MG96-36 were crossed back to Katepwa to generate F_1 and F_2 generations. F_2 screening identified which crosses contained a single gene segregating for reaction to isolate MG96-36. These F_2 populations were selfed to generate F_2-derived-F_3 ($F_{2:3}$) families. $F_{2:3}$ families were screened to identify homozygous resistant, segregating, and homozygous susceptible families. Bulk segregant analysis (BSA) identified markers on chromosome 7B that were potentially linked to the gene of interest. An additional selection of polymorphic markers on 7B

were used to create a large linkage group, which included the isolate-specific resistance gene, now designated *Stb13*. *Stb13* was identified in two separate mapping populations and was found to be linked to the microsatellite marker *Xwmc396* in both instances, at distances of 9 and 7 centimorgans (cM), respectively. Unfortunately, no markers flanked *Stb13*. Because *Stb13* is not that closely linked with *Xwmc396*, it may be necessary to investigate alternative molecular markers, which may reside closer to *Stb13*, such that marker-assisted selection (MAS) can be used to select for this isolate-specific gene.

Keywords: *Triticum aestivum, isolate-specific disease resistance, linkage analysis, microsatellite markers*

4.2 Introduction

Septoria tritici blotch (STB) is a serious foliar disease that affects bread and durum wheat (*Triticum aestivum* L. em. Thell and *T. turgidum* subsp. *durum*) crops worldwide (Eyal et al. 1981; King et al. 1983; Eyal et al. 1987). STB is caused by the ascomycete fungus *Mycosphaerella graminicola* (Fuckel) J. Schröt. in Cohn (anamorph: *Septoria tritici* Roberge in Desmaz.). Frequent rainfall and moderate temperatures generally favour the development of STB epidemics (Shaner and Finney 1976; Eyal 1981; Eyal et. al 1987; Wainshilbaum and Lipps 1991; Magboul et al. 1992). Severe epidemics compromise wheat quality (McKendry et al. 1995) and yield, with losses as high as 25 to 50% being reported (Eyal and Ziv 1974; Ziv and Eyal 1978; King et al. 1983). Although chemical and cultural methods can be implemented to control STB epidemics, the incorporation of host resistance would be the most economical and

environmentally sound method of control (Eyal et al. 1987; Cowger et al. 2000; McCartney et al. 2002).

Understanding the mode of inheritance of STB resistance is the first step in designing an effective breeding program against this disease. Genetic resistance to STB has been reported to be conditioned by one, two or three dominant or partially dominant genes (Narvaez et al. 1957; Wilson 1979; Rosielle and Brown 1978; Lee and Gough 1984; Somasco et al. 1996; McCartney et al. 2002), two or three recessive genes (Rosielle and Brown 1978; Wilson 1985) or several genes with additive and dominant effects (Van Ginkel and Scharen 1987, 1988a, 1988b; Jlibene et al. 1994; Simón and Cordo 1998; Chartrain et al. 2004a). More recently, the growing abundance of DNA markers characterized in the wheat genome has greatly facilitated the mapping of 12 STB resistance genes (*Stb1-12*). *Stb1* was mapped to chromosome 5BL (Adhikari et al. 2004a); *Stb2* and *Stb3* were assigned to chromosomes 3BS and 6DS, respectively (Adhikari et al. 2004b); *Stb4* and *Stb5* were both positioned near the centromere on chromosome 7D (Arraiano et al. 2001; Adhikari et al. 2004a); *Stb6* was identified in the resistant cultivar Flame and was later mapped to chromosome 3AS (Brading et al. 2002); *Stb7* and *Stb12* have both been positioned at the distal end of chromosome 4AL (McCartney et al. 2003; Chartrain et al. 2005b); *Stb8* was mapped by Adhikari et al. 2003 to the long arm of chromosome 7B; *Stb9* was identified by Chartrain (2004) as cited by Chartrain et al. (2005b); the *Stb10* locus was identified in Kavkaz-4500 L.6.A.4 and was assigned to chromosome 1D (Chartrain et al. 2005b), and the *Stb11* locus mapped to chromosome 1BS (Chartrain et al. 2005c). This information will allow wheat breeders to utilize MAS in incorporating STB resistance into new wheat cultivars.

Understanding the extent of pathogen variation is also an important component of implementing an effective breeding program. Physiological specialization of the pathogen has been reported on several occasions (Saadaoui 1987; Ballantyne and Thomson 1995; Kema et al. 1996a; Kema et al. 1996c; Grieger et al. 2005). Isolate-specific resistance of wheat to the STB pathogen, consistent with a gene-for-gene relationship, has been identified with *Stb4* (Somasco et al. 1996), *Stb5* (Arraiano et al. 2001), *Stb6* (Brading et al. 2001), *Stb7* (McCartney et al. 2002), *Stb10*, *Stb11,* and *Stb12* (Chartrain 2005b, 2005c). In addition, Kema et al. (2000) reported that avirulence in *M. graminicola* was controlled by a single locus, which is also consistent with a gene-for-gene interaction. Based on the differential reaction of the hexaploid wheat line ST6, two distinct virulence groups (group 1 and group 2) of *M. graminicola* have been identified in western Canada (Grieger et al. 2005).

Previous inheritance studies conducted by McCartney et al. (2002) revealed that the hexaploid wheat line Salamouni contains three incompletely dominant resistance genes to isolate MG2 (group 2). Two of these resistance genes also control resistance to isolate MG96-36 (group 1). The objective of this study is to determine the chromosomal location of one of the STB resistance genes in Salamouni that confers resistance to both isolates MG96-36 and MG2, and to identify microsatellite markers closely linked to the resistance gene.

4.3 Materials and Methods

4.3.1 Population development

McCartney et al. (2002) identified three independently segregating STB resistance genes in Salamouni, a highly resistant landrace from Lebanon. Reciprocal crosses were made between Salamouni and the highly susceptible cultivar Katepwa. DH lines were generated from the F_1 hybrids using the maize hybridization/embryo rescue method (Fedak et al. 1997). Thirty-seven DH lines were inoculated, as described in section 4.3.2, with isolates MG96-36 (group 1) and MG2 (group 2) and resulting disease reactions were recorded. Thirteen DH lines exhibiting resistance to isolates MG2 and MG96-36 were crossed back to Katepwa to generate approximately 200 F_1 seeds per cross. Ten F_1 plants were grown from each cross, evaluated for reaction with MG96-36 and self-pollinated to produce the corresponding F_2 generation. Due to time and space limitations, only eight of the 13 corresponding F_2 populations, comprised of 80 individuals per cross, were evaluated with MG96-36. F_2 populations segregating in a 15 : 1 resistant to susceptible ratio, characteristic of two genes segregating for resistance to isolate MG96-36, were not advanced to the next generation. F_2 populations segregating in a 3 : 1 resistant to susceptible ratio, characteristic of a single gene segregating for resistance to isolate MG96-36, were subsequently selfed to produce $F_{2:3}$ families. Approximately 80 families per cross were screened with isolate MG96-36 to validate F_2 phenotypes. The 79 $F_{2:3}$ families originating from DH line 98S08C*03 were also evaluated with isolate MG2 to confirm that the single gene present also conferred resistance to isolate MG2.

Seeds were germinated in the dark prior to planting (2 days 4°C, 2 days room temperature) to promote uniform germination. Depending on the amount of seed required for the next generation, one (F_1), two (F_2) or five seeds ($F_{2:3}$) were planted per 15-cm-diameter clay pot containing a 1 : 1 : 1 or 2 : 1 : 1 soil mix (soil/sand/peat). Plants

were grown prior to inoculation in a growth room set at 21/16°C (day/night) with a 16-h photoperiod (250μE $m^{-2}s^{-1}$). All plants were fertilized with 20-20-20 fertilizer (3.75 ml/L) and watered as required. Throughout population generation, glassine bags were placed over spikes to prevent cross contamination.

4.3.2 Inoculation

Single spore isolates of *M. graminicola* representative of group 1 (MG96-36) and group 2 (MG2) were used to evaluate the DH lines. These were the same isolates used by McCartney et al. (2002). Infected leaf tissue from Manitoba wheat fields was the original source of the cultures (Grieger et al. 2005). MG96-36 was used to evaluate the F_1, F_2, and $F_{2:3}$ families. Each culture was derived from a single, sporulating pycnidium, which was transferred to a yeast malt agar plate containing 0.25 % chloramphenicol (YMA^+): 4g Difco malt extract (Difco Laboratories, Detroit), 4 g of sucrose (Fisher Scientific, Fair Lawn, NJ), 15 g of Difco agar, 250 mg of chloramphenicol (Sigma Chemical, St. Louis) and 1000 ml of distilled water (Eyal et al. 1987). After seven days in the dark at 20°C, single conidiospores were isolated using a dissecting microscope to ensure a pure culture (Grieger et al. 2005). Cultures were incubated under fluorescent lights for seven days at room temperature. Conidia were harvested by flooding cultures with sterile distilled water and suspending the spores using a wire loop. The suspension was filtered through three layers of cheesecloth and subsequently quantified using a hemacytometer. The conidial suspension was adjusted to 10^7 spores/ml and one drop of Tween 20 (polyoxyethylene sorbitan monolaurate) was added per 50 ml of inoculum as a surfactant.

Seedlings at the three-leaf stage were inoculated with the spore suspension until run-off using a DeVilbiss-type sprayer. Plants were allowed to dry and were then placed in a misting chamber where continuous leaf wetness was achieved using two ultrasonic humidifiers. After an incubation period of 72 h, seedlings were transferred to a growth cabinet set at 21/19°C (day/night) with a 16-h photoperiod (390μE $m^{-2}s^{-1}$) and relative humidity between 70 and 80%.

Along with the DH parental line and corresponding F_1 individuals, four to ten plants of Salamouni, Katepwa, Erik, and ST6 were always included with each inoculated set to provide symptom comparisons and facilitate disease ratings. The wheat cultivar Erik served as an additional susceptible check for both isolates, while the differential reaction of ST6 was used to confirm isolate identity. ST6 is susceptible to isolate MG96-36, but is resistant to isolate MG2.

A total of ten F_1 seedlings were inoculated with MG96-36. Entire F_2 populations of approximately 80 individuals were evaluated during a single inoculation. During inoculations of the $F_{2:3}$ families, only 20 families of 20 individuals each could be challenged during a single inoculation due to space limitations. Although different families of a single population were screened during separate inoculations, all individuals of a family were inoculated as a unit.

4.3.3 Disease assessment

Plants were assessed for their reaction to *M. graminicola* 17 days after inoculation (dai). Reactions were classified using a qualitative evaluation scale originally developed by Rosielle (1972) and modified by McCartney et al. (2002) (Fig. 3.1). This scale

integrates the amount of necrotic tissue and pycnidial density to assign a particular reaction type. Previous authors have indicated that both of these symptoms are informative about the host's response to the pathogen (Rillo and Caldwell 1966; Eyal et al. 1987; Saadaoui 1987; Ballantyne and Thomson 1995; Kema et al. 1996a; Somasco et al. 1996).

Reaction types 0-3 were considered to be resistant, while 4 and 5 were considered to be susceptible. The intermediate "3" reaction was considered to be resistant, primarily because growth and reproduction of the pathogen was restricted (McCartney et al. 2002). In addition, chlorotic lesions characterized the intermediate reaction, which in other pathosystems such as the wheat-*Puccinia graminis* f. sp. *tritici* system, the presence of chlorosis indicates a hypersensitive or resistant reaction (Roelfs and Martens 1988). In order to verify individual F_2 plant ratings, $F_{2:3}$ families were scored as either homozygous resistant, segregating, or homozygous susceptible based on the reactions of 20 seedlings per family. Data were tested for goodness of fit to specific genetic ratios using Chi-Square analysis. Yates correction factor was used where appropriate (Strickberger 1985).

4.3.4 DNA extraction

Leaf tissue was collected 18 dai from the youngest, non-inoculated leaf of the $F_{2:3}$ families. Equal amounts of leaf tissue from each individual of a particular family were pooled to reconstitute the genotype of the F_2. Tissue was lyophilized for 72h and then stored at -20°C. Tissue from each family was placed in a 15 ml falcon tube (Fisher Scientific, Pittsburgh, Pa.) with four 3-mm tungsten carbide beads (Qiagen, Missassauga, ON). Tissue was ground to a fine powder by shaking in a paint shaker for 10 min.

Approximately 0.15 g of ground tissue was transferred to 1.2 ml collection tubes (Qiagen, Missassauga, ON) containing 200 μl of glass beads. Tissue was further ground by shaking in a paint shaker for 3 - 4 min. DNA was extracted with the DNeasy 96 Plant Kit (Qiagen, Mississauga, ON) and quantified using Hoechst 33258 stain.

4.3.5 Bulked segregant analysis

Bulked segregant analysis (BSA) was used to identify putatively linked markers to the STB resistance gene (Michelmore et al. 1991) contained in the 98S08C*03 population. The resistant DNA bulk was prepared by pooling equal concentrations of DNA from ten homozygous resistant $F_{2:3}$ families and the susceptible DNA bulk was prepared by pooling equal concentrations of DNA from eight homozygous susceptible $F_{2:3}$ families. Primers for BSA were chosen using the wheat microsatellite consensus map published by Somers et al. (2004). Forty-six primer pairs located near previously mapped STB resistance genes were used to direct a targeted evaluation. When no linkage was detected, a much larger screening utilizing markers located throughout the wheat genome was pursued. Primers were chosen approximately every 10-15 cM to ensure adequate genome coverage (Somers et al. 2004). In total, 445 primer pairs were used to screen the two bulks, along with the resistant DH (98S08C*03) and susceptible parent (Katepwa) in an attempt to identify potentially linked makers to the gene of interest (Michelmore et al. 1991). These markers, along with their chromosome location(s) are listed in appendix 2.

Once the resistance gene was identified in 98S08C*03, markers identified to be linked to the 98S08C*03 gene were used to conduct BSA on the 98S05B*01 population,

as described above (appendix 2). If markers identified polymorphism between the two bulks, additional primers were chosen to investigate the surrounding area. In total, 210 primer pairs were evaluated on the bulks and the parents from the 98SO5B*01 population.

4.3.6 PCR amplification

PCR reactions were performed in 10-μl volumes and contained 24 ng of template DNA, 1U of Taq DNA polymerase (Gibco/BRL, Mississauga, ON), 1X PCR buffer (Applied Biosystems, Foster City, Calif.), 1.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 0.2 pmols of forward primer, 2.0 pmols of reverse primer, and 1.8 pmols of 6-FAM/HEX/NED-labelled M13 primer (5'- 3' CACGACGTTGTAAAACGAC; Applied Biosystems, Foster City, CA). GWM, GDM, and WMC primer sequences were obtained from Röder et al. (1998), Pestova et al. (2000) and Gupta et al. (2002), respectively. The CFA and CFD primer sequences were provided by Dr. P. Sourdille (INRA), while the BARC marker sequences were obtained from Song et al. (2002). All forward microsatellite primers were modified to contain a 5', 19-nucleotide M13 tail (Schuelke 2000). The reaction mixture was denatured at 94°C for 2 min, followed by 30 cycles of 95°C for 1 min, 51/61°C for 50 sec, 73°C for 1 min, with a final extension step of 73°C for 5 min. PCR products were resolved with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) with GeneScan-500 software and GeneScan-500 ROX as an internal size standard (Applied Biosystems, Foster City, CA). Genographer version 1.6.0 (http://hordeum.oscs.montana.edu/genographer) was used to convert chromatograms to gel images.

4.3.7 Linkage analysis

Seven markers showing potential linkage to the resistance gene present in 98S08C*03 were identified on chromosome 7B using BSA. Eventually all 68 microsatellite markers on 7B from the wheat genome consensus map (Somers et al. 2004) were screened on the bulks and the parents (appendix 2). Forty-four of these markers were polymorphic between the parents and subsequently screened on the entire mapping population. Chromosomal locations of these makers were previously determined in other mapping populations (Röder et al. 1998; Pestova et al. 2000; Chalmers et al. 2001; Gupta et al. 2002; Song et al. 2002; Somers et al. 2004). Phenotypic data from 79 $F_{2:3}$ families and genotypic data from 20 microsatellite markers was used to create a linkage map of chromosome 7B.

A selection of markers used to create the linkage map of chromosome 7B in the 98S08C*03 population were evaluated on the 98S05B*01 population to determine whether the resistance genes were the same (appendix 2). Phenotypic data from 71 98S05B*01 $F_{2:3}$ families and genotypic data from 23 microsatellite markers were used to create a second linkage map of chromosome 7B.

JoinMap^R version 3.0 (van Ooijen and Voorrips, 2001) was used to create linkage groups and a genetic-linkage map of chromosome 7B. Map distances were converted to centimorgans (cM) using the Kosambi function (Kosambi 1944). Linkage maps were generated using an LOD threshold of 2.0.

4.4 Results

4.4.1 Disease reactions of the DH lines, F_1's, F_2's, and $F_{2:3}$ families

Previous inheritance studies conducted by McCartney et al. (2002) indicated that Salamouni contains three independent resistance genes to isolate MG2, two of which also control resistance to isolate MG96-36. DH lines generated from the F_1 hybrids of the reciprocal cross between Salamouni and the susceptible cultivar Katepwa were evaluated with isolates MG2 and MG96-36. The observed disease reactions were 30 : 3 : 4 (resistant to MG96-36 and MG2 : susceptible to MG96-36 and resistant to MG2 : susceptible to MG96-36 and MG2), which fit the expected 6 : 1 : 1 segregation ratio (X^2 = 0.84, P = 0.66), confirming that Salamouni contains three independent resistance genes, two of which provide resistance to isolate MG96-36. At this time there is no defined tester isolate to phenotypically distinguish the two resistance genes in Salamouni, which both control resistance to isolate MG96-36. Thirty DH lines exhibited the resistant reaction types to both virulence groups. This indicated that these DH lines contained one of the following combinations of resistance genes: (i) All three resistance genes, (ii) Any combination of two resistance genes, or (iii) One of the two resistance genes which confer resistance to both virulence groups.

Resistant reaction types of the 30 DH lines ranged from the near immune response, similar to that of the resistant parent Salamouni, to a "3" reaction type, according to the disease rating scale described previously (McCartney et al. 2002) (Fig. 3.1). In an attempt to isolate each resistance gene providing resistance to group 1, 13 DH lines of varying resistance levels were chosen to cross back to Katepwa to generate F_1 populations.

Ten F_1 seedlings from each line were evaluated with MG96-36 and exhibited resistant reactions slightly less resistant than the DH parent. Due to time constraints, only

eight of the 13 original DH lines were evaluated in the F_2 generation. Five of these eight F_2 populations demonstrated a 3:1 resistant to susceptible ratio, which indicated the presence of a single gene segregating for resistance to group 1 (Table 4.1). However, upon creating and screening $F_{2:3}$ families, only two (98S08C*03 and 98S05B*01) of the five corresponding $F_{2:3}$ families exhibited a typical 1 : 2 : 1 (homozygous resistant : segregating : homozygous susceptible) ratio, characteristic of single gene segregation (Table 4.1). The resistant reaction types of 98S08C*03 and 98S05B*01 to isolate MG96-36 are illustrated in Fig. 4.1. Difficulties in classifying the intermediate reaction type as either resistant or susceptible in the F_2 generation is responsible for these indiscrepencies and supports the need to perform progeny tests. However, despite progeny testing, the intermediate reaction sometimes made it difficult to classify particular families in the 98S05B*01 population. In order to avoid inaccuracy during linkage analysis, the phenotypic data from 11 families were scored as missing data. The F_2 populations demonstrating a 15 : 1 resistant to susceptible ratio, characteristic of two genes segregating for resistance to isolate MG96-36, were not advanced to the next generation (Table 4.1).

4.4.2 Marker and linkage analysis

The 98S08C*03 population was subjected to BSA using a total of 445 microsatellite primer pairs (appendix 2). Markers *Xgwm46*, *Xgwm333*, *Xwmc218*, *Xwmc364*, *Xwmc426*, *Xwmc475*, and *Xwmc758* were putatively linked with the resistance locus, now designated *Stb13*. The resistant DH allele only amplified in the resistant bulk and the Katepwa allele only amplified in the susceptible bulk. All of the above markers

Table 4.1. Segregation of F_2 and $F_{2:3}$ generations to isolate MG96-36 of *Mycosphaerella graminicola*. The crosses were derived from doubled haploid lines resistant to isolate MG96-36 crossed back to Katepwa.

Cross	Observed F_2 (R : S)	Ratio tested in F_2 (R : S)	F_2 X^2 (P)[a]	Observed $F_{2:3}$ (HR : SEG : HS)	Ratio tested in $F_{2:3}$	$F_{2:3}$ X^2 (P)[a]
98S08C*03/Katepwa	59 : 20	3 : 1	0.004 (0.95)	18 : 46 : 15	1 : 2 : 1	2.37 (0.33)
98S05B*01/Katepwa	63 : 19	3 : 1	0.065 (0.70)	29 : 36 : 17	1 : 2 : 1	4.73 (0.09)
98S05B*03/Katepwa	57 : 21	3 : 1	0.06 (0.69)	43 : 27 : 8	7 : 8 : 1[b]	5.00 (0.08)
98S05C*13/Katepwa	61 : 21	3 : 1	0.06 (0.69)	32 : 32 : 4	7 : 8 : 1[b]	0.30 (0.86)
98S08A*17/Katepwa	66 : 14	3 : 1	2.02 (0.12)	40 : 34 : 6	7 : 8 : 1[b]	1.81 (0.40)
98S05C*21/Katepwa	74 : 5	15 : 1	0.04 (0.98)			
98S05C*22/Katepwa	75 : 6	15 : 1	0.04 (0.98)			
98S08D*20/Katepwa	73 : 8	15 : 1	1.25 (0.18)			

[a] X^2 corrected with Yates correction factor where appropriate. A fit to the expected segregation ratio is accepted if $P > 0.05$

[b] Although the F_2 generation segregrated in what appeared to be a 3 : 1 resistant to susceptible ratio, progeny tests gave results typical of two genes segregating, therefore a 7 : 8 : 1 ratio was tested in the $F_{2:3}$ families which would correspond to a 15 : 1 resistant susceptible ratio in the F_2 generation

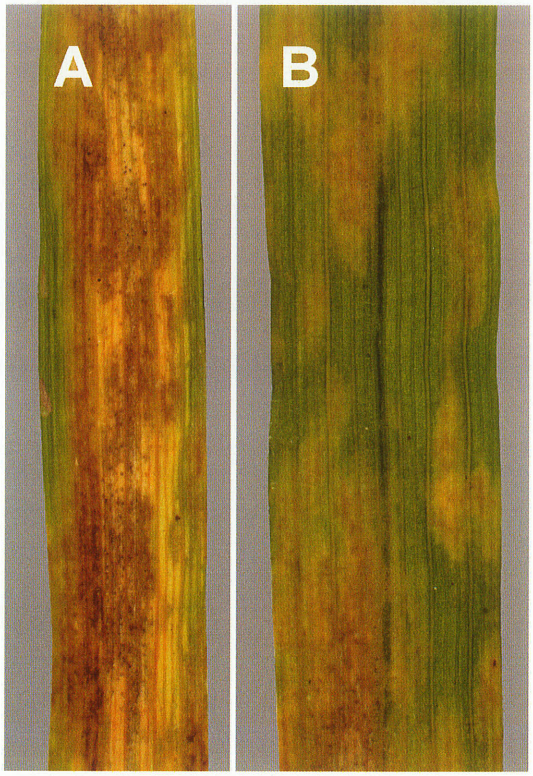

Fig. 4.1. Resistant reactions of the two doubled haploid (DH) lines used to identify *Stb13*, when inoculated with isolate MG96-36. **A** = Resistant reaction type "3" of DH line 98S08C*03. **B** = Resistant reaction type "2" of DH line 98S05B*01.

mapped to chromosome 7B (Somers et al. 2004). Marker order and location were consistent with the wheat microsatellite consensus map (Somers et al. 2004). The entire mapping population was screened with the above markers, in addition to other microsatellite markers on 7B that were polymorphic between the parents. Linkage analysis using the 98S08C*03 population indicated that the microsatellite locus *Xwmc396* was linked to *Stb13* at a distance of 9 cM. *Xwmc396* amplified a 173 bp allele specific to the DH parent and a 155 bp allele representative of the susceptible parent, Katepwa. Linkage analysis of all scoreable markers on 7B and phenotypic data from 79 $F_{2:3}$ families generated a 42 cM linkage group (including the *Stb13* gene) (Fig. 4.2) consistent with the wheat microsatellite consensus map published by Somers et al. (2004). Primer sequences for the microsatellite markers used to generate the linkage maps of 7B are listed in Table 4.2.

Before commencing BSA on the 98S05B*01 population, markers closest to *Stb13* were evaluated on the entire population. This was done to identify if the 98S05B*01 population contained *Stb13* or the second gene that provides resistance to isolate MG96-36 (group 1). Initial results indicated the 98S05B*01 population also contained *Stb13*, and not the second resistance gene. BSA was used to identify polymorphic markers in order to generate a second linkage map of chromosome 7B, including *Stb13*. Genotypic data from 23 polymorphic markers and phenotypic data from 71 individuals was used to create an 82 cM linkage group (including the *Stb13* gene) (Fig 4.2). Linkage analysis indicated that *Stb13* was most closely linked to the microsatellite marker *Xwmc396* at a distance of 7 cM, consistent with the data generated from the 98S08C*03 population.

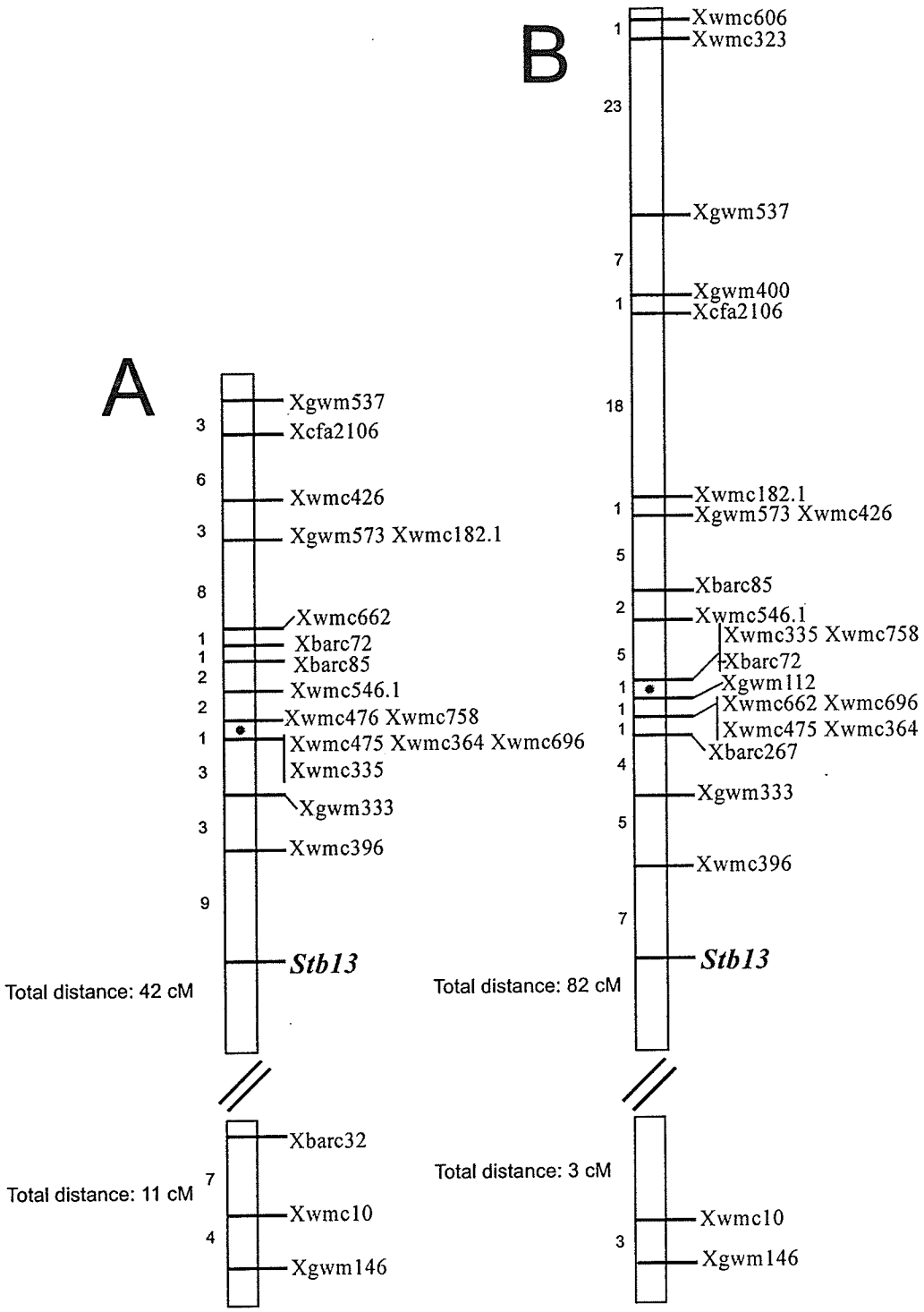

Fig. 4.2. **A)** Linkage map displaying the microsatellite loci and the *Stb13* gene on chromosome 7B. *Stb13* provides resistance to isolates MG96-36 and MG2 of *Mycosphaerella graminicola*. Markers were mapped using 79 $F_{2:3}$ families developed from the cross between DH line 98S08C*03/Katepwa. **B)** Linkage map displaying the microsatellite loci and the *Stb13* gene on chromosome 7B. Markers were mapped using 71 $F_{2:3}$ families developed from a cross between DH line 90S05B*01/Katepwa. The • symbol gives an approximation of the centromere based on the wheat microsatellite consensus map (Somers et al. 2004). Distances are reported in Kosambi centimorgans.

Table 4.2. PCR primer sequences and annealing temperatures of the SSR markers used to map *Stb13*

Marker	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Annealing Temperature (°C)
BARC32[a]	GCGTGAATCCGGAAACCCAATCTGTG	TGGAGAACCTTCGCATTGTGTCATTA	51
BARC72[a]	CGTCCTCCCCCTCTCAATCTACTCTC	CGTCCCTCCATCGTCTCATCA	51
BARC85[a]	GCGAACGCTGCCCGGAGGAATCA	GCGTCGCAGATGAGATGGTGGAGCAAT	61
BARC267[a]	GCGTGCTTTTTATTTTTGTGGACATCTT	GCGAATAATTGGTGGGTGAAACA	51
CFA2106[b]	GCTGCTAAGTGCTCATGGTG	TGAAACAGGGGAATCAGAGG	61
GWM112[c]	CTAAACACGACAGCGGTGG	GATATGTGAGCAGCGGTCAG	61
GWM146[c]	CCAAAAAAACTGCCTGCATG	CTCTGGCATTGCTCCTTGG	61
GWM333[c]	GCCCGGTCATGTAAAACG	TTTCAGTTTGCGTTAAGCTTTG	61
GWM400[d]	GTGCTGCCACCACTTGC	TGTAGGCACTGCTTGGGAG	61
GWM537[c]	ACATAATGCTTCCTGTGCACC	GCCACTTTTGTGTCGTTCCT	61
GWM573[d]	AAGAGATAACATGCAAGAAA	TTCAAATATGTGGGAACTAC	51
WMC10[d]	GATCCGTTCTGAGGTGAGTT	GGCAGCACCCTCTATTGTCT	61
WMC182[d]	GTATCTCACGAGCATAACACAA	GAAAGTGTATGGATCATTAGGC	61
WMC323[d]	ACATGATTGTGGAGGATGAGGG	TCAAGAGGCAGACATGTGTTCG	61
WMC335[d]	TGCGGAGTAGTTCTTCCCCC	ACATCTTGGTGAGATGCCCT	61

Marker	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Annealing Temperature (°C)
WMC364[d]	ATCACAATGCTGGCCCTAAAAC	CAGTGCCAAAATGTCGAAAGTC	61
WMC396[d]	TGCACTGTTTTACCTTCACGGA	CAAAGCAAGAACCAGAGCCACT	51
WMC426[d]	GACGATCGTTTCTCCTACTTTA	ACTACACAAATGACTGCTGCTA	61
WMC475[d]	AACACATTTTCTGTCTTTCGCC	TGTAGTTATGCCCAACCTTTCC	61
WMC546[d]	CGGCTAAAATCGTACACTACACA	CTCACTTGCACGATTTCCCTAT	61
WMC606 [d]	CCGATGAACAGACTCGACAAGG	GGCTTCGGCCAGTAGTACAGGA	61
WMC662[d]	AGTGGAGCCATGGTACTGATTT	TGTGTACTATTCCCGTCGGTCT	61
WMC696[d]	ACCCGAGAGAGATTAGGGCTTG	CACTCGCAGCCTCTCTTCTACC	61
WMC758[d]	TAGGGGAGGCGACGGAG	GTTGCTGGAGAGTGGATTGC	61

[a]Source: USDA-ARS

[b]Source: INRA

[c]Source: Röder et al. 1998

[b]Source: Agrogene

The STB resistance gene, *Stb8*, was recently mapped to the long arm of chromosome 7B. Two flanking microsatellite markers *Xgwm146* and *Xgwm577* were linked at distances of 3.5 and 5.3 cM, respectively (Adhikari et al. 2003). To ensure that *Stb13* was distinct from *Stb8*, *Xgwm146* and *Xgwm577*, along with other microsatellite markers in the vicinity were screened during BSA. Markers in the area of *Stb8* were monomorphic on the bulks used to evaluate the 98S08C*03 and 98S05B*01 populations, which indicated a linkage relationship does not exist between *Stb13* and these markers. However, *Xwmc10*, *Xbarc32* and *Xgwm146* exhibited polymorphism between the parents and were screened on the mapping population. A linkage group including these three markers was generated in the vicinity described for *Stb8* (Fig. 4.2). Linkage analysis confirmed that they were not part of the *Stb13* linkage group, which indicated that *Stb8* and *Stb13* are distinct from one another.

4.5 Discussion

Stb13 was one of three resistance genes identified in the resistant wheat line Salamouni. The 7BL map location of *Stb13* determined in this study suggests that *Stb13* is distinct from *Stb8*, also located on chromosome 7BL (Adhikari et al. 2003). *Stb13* confers resistance to isolate MG96-36 and MG2 (McCartney et al. 2002), representative isolates of virulence groups 1 and 2, which were identified in western Canada (Grieger et al. 2005).

Eriksen et al. (2003) identified a QTL associated with the microsatellite marker *Xwmc517* on chromosome 7BL (Grain Genes 2005). Although this QTL has been identified approximately 30 cM from the map location of *Stb13*, future studies are needed

to investigate whether or not this QTL and *Stb13* are the same, allelic, closely linked, or are separate resistance sources. A cross between the two resistant sources (Senat x Salamouni) and the subsequent screening of a large F_2 population with isolates MG96-36 and IPO323 should identify if *Stb13* and the QTL detected by Eriksen et al. (2003) are in fact the same or different.

In addition to *Stb8*, there are several other disease resistance genes located on chromosome 7BL. These include resistance genes to powdery mildew (*pm5*), stem rust (*Sr17*), and leaf rust (*Lr14a*). (McIntosh et al. 1998). According to map locations published in GrainGenes (2005) and the microsatellite consensus map (Somers et al. 2004), these resistance genes appear to be located approximately 55 cM from the centromere. *Stb13* mapped within 15 - 20 cM from the centromere, according to the wheat microsatellite consensus map (Somers et al. 2004). Based on this information, *Stb13* does not appear to be associated with these other resistance genes.

In addition to *Stb13*, 12 other STB resistance loci have been identified and assigned chromosome locations using molecular markers. *Stb1*, originally identified in the winter wheat cultivar Bulgaria 88 (Rillo and Caldwell 1966), and transferred to the soft, red winter cultivars Oasis and Sullivan (Patterson et al. 1975) has proven to be a durable source of resistance in the STB prone regions of the United States (Shaner and Finney 1982; Shaner and Buechley 1989). Adhikari et al. (2004a), recently mapped *Stb1* to chromosome 5BL (Adhikari et al. 2004a). *Stb2* and *Stb3* were mapped to chromosome 3BS and 6DS, respectively, using microsatellite markers (Adhikari et al. 2004b). Until recently, *Stb4*, the single, dominant resistance gene present in the spring wheat cultivar Tadinia was effectively used to control STB epidemics in California (Somasco et al.

1996). Although *Stb4* is no longer effective in areas of the United States, it was mapped near the centromere on chromosome 7D (Adhikari et al. 2004c). Interestingly enough, *Stb5* was also mapped to a location near the centromere on 7D (Arraiano et al. 2001). Both *Stb6* (Brading et al. 2002) and *Stb7* (McCartney et al. 2002) have been identified to exhibit isolate-specific interactions and have been mapped to chromosomes 3AS and 4AL, respectively. As stated previously, *Stb8* was mapped to the long arm of chromosome 7B using AFLP and microsatellite markers (Adhikari et al. 2003). *Stb9* was identified by Chartrain (2004) as cited by Chartrain et al. (2005b). Both *Stb10* and *Stb12* were identified in Kavkaz-K4500 L.6.A.4 (KK) and were mapped to chromosomes 1D and 4A, respectively (Chartrain et al. 2005b). Microsatellite markers were also used to identify *Stb11* in the Portuguese wheat breeding line TE9111 (Chartrain et al. 2005c).

Salamouni still contains one unmapped resistance gene that controls resistance to both virulence groups (McCartney et al. 2002). Inheritance studies conducted by McCartney et al. (2002) have indicated that the three resistance genes in Salamouni are unlinked to *Stb7*. This has been supported by the subsequent mapping of two of the three resistance genes in Salamouni (*Stb13* and *Stb14*), however, the location of the third resistance gene and its relationship with the other STB resistance loci is unknown.

Homoeologous loci found on the A, B, and D genomes of bread wheat (*T. aestivum* L. em. Thell), suggest that all three genomes likely originated from a common ancestor. This phenomenon has been illustrated with the presence of leaf rust resistance genes located on all three homoelogous group two chromosomes (2A: *Lr11,Lr17*; 2B: *Lr13, Lr 16, Lr 23, Lr 35*; 2D: *Lr15, Lr 22, Lr 22b*) (Poehlman and Sleper 1995; McIntosh et al. 1998). This theory gains further support with the mapping of *Stb13*.

Although other STB resistance loci have mapped to various areas of the genome, the relationship between *Stb4, Stb5,* and *Stb13* is unique. These three resistance loci appear to be part of a homoeologous area of the group seven chromosomes. As stated previously, *Stb13* maps near the centromere on 7B, while *Stb4* and *Stb5* map near the centromere on chromosome 7D (Arraiano et al. 2001; Adhikari et al. 2004c). This similarity of loci between homoeologous groups supports the theory that the A, B, and D genomes of bread wheat (*T. aestivum* L. em. Thell) share a common ancestor.

With the development of a detailed microsatellite map of wheat (Röder et al. 1998; Pestova et al. 2000; Gupta et al. 2002; Song et al. 2002; Somers et al. 2004), the effectiveness and practicality of using MAS in addition to conventional wheat breeding methods is becoming a reality. A high-resolution linkage map increases the probability of finding a high quality, closely linked, polymorphic marker in the area of interest (Somers et al. 2004). Incorporating MAS into a conventional breeding program can overcome several barriers when breeding for STB resistance.

M. graminicola requires very specific climatic conditions to allow for differentiation between resistant and susceptible genotypes (Shaner and Finney 1976; Eyal 1981; Eyal et al. 1987; Wainshilbaum and Lipps 1991; Magaboul et al. 1992; Gilbert et al. 1998). Although it is not difficult to create an ideal environment for the pathogen in the greenhouse, achieving similar results in the field can be challenging depending on temperature and moisture conditions. Even under ideal conditions, scoring reaction types in segregating populations can be difficult, especially when working with an incompletely dominant resistance gene. Although progeny tests can assist in clarifying phenotypic data, they are time consuming and labour intensive (Brading et al.

2002; McCartney et al. 2002). In addition, other foliar pathogens can co-exist with *M. graminicola*, which may lead to inaccurate disease data (Gilbert et al. 1998). Brokenshire (1974) reported that *M. graminicola* could interact with other wheat pathogens. For example, plants normally resistant to *M. graminicola* would allow the pathogen to grow and sporulate in the presence of *Blumeria graminis* f. sp. *tritici*. MAS can overcome these barriers because selection is based on genotype rather than phenotype, the trait of interest is not influenced by the environment, and can be evaluated at all stages of plant growth (Young 1996; Gupta et al. 1999).

Breeding for STB resistance is also complicated by the long latent period of the pathogen. Having a tightly linked marker to the resistance gene could speed up the selection process by allowing for rapid identification of resistant plants in early generations (McCartney et al. 2002; Adhikari et al. 2004a). In addition, pyramiding resistance genes into new cultivars could be facilitated with MAS. The technology is especially helpful when multiple resistance genes cannot be distinguished phenotypically (Young 1996; Gupta et al. 1999), as is most often the case in the wheat-*M. graminicola* pathosystem, because of the lack of defined tester isolates (Adhikari et al. 2004a). Pyramiding resistance genes to multiple pathogens is also simplified with MAS when screening for one resistance gene interferes with the ability to screen for the others in the same plant (Young et al. 1996). MAS is a powerful tool for wheat breeders, which will allow them to meet the demands of wheat growers more efficiently.

Because *Stb13* is not tightly linked with *Xwmc396*, it may be necessary to investigate populations segregating for the *Stb13* gene with other types of molecular markers, to possibly locate a more tightly linked and/or a flanking marker. If this attempt

is successful, these markers could then be converted to PCR-based markers that are convenient for large-scale screening. This would increase the practicality of selecting for *Stb13* in a MAS breeding program. The identification and mapping of STB resistance genes will allow wheat breeders to develop STB resistant cultivars that will provide wheat producers with durable and affordable protection against *M. graminicola*.

CHAPTER 5

GENERAL DISCUSSION

The inheritance of STB resistance is a subject that has been examined in detail by several researchers. This study supports the inheritance studies conducted by McCartney et al. (2002), which concluded that the resistance in Salamouni, a hexaploid wheat line, is conditioned by three incompletely dominant resistance genes. This is consistent with other reports of qualitative resistance in hexaploid wheat (Rosielle and Brown 1979; Wilson 1979; Lee and Gough 1984; Somasco et al. 1996; Arraiano et al. 2001; Brading et al. 2002). The three resistance genes in Salamouni were identified using two distinct pathogen isolates, MG96-36 and MG2, found in western Canada (Grieger et al. 2005). Using these isolates, McCartney et al. (2002) determined that all three resistance genes in Salamouni confer resistance to isolate MG2, while two of the three genes also provide resistance to isolate MG96-36. This type of isolate-specific resistance supports the theory that a gene-for-gene interaction is operational in the wheat-*M. graminicola* pathosystem (Brading et al. 2002, McCartney et al. 2002). The recent discovery that avirulence in *M. graminicola* is controlled by a single locus also supports the presence of a gene-for-gene interaction (Kema et al. 2000).

Ideally it would be beneficial to identify each resistant source using a defined tester isolate. If this was accomplished, a set of near-isogenic lines (NILs) could be developed as a diagnostic tool for identifying future resistance genes and monitoring pathogen variability. Although this may be possible in the future, the recent development

of a high-density microsatellite map of wheat (Somers et al. 2004) has encouraged an alternative, more technologically advanced system for monitoring resistance genes.

There are several reasons why microsatellites are the marker system of choice for molecular mapping in wheat. Not only are microsatellite markers co-dominant and highly polymorphic, they are PCR based which makes them highly amenable to screening large numbers of samples, which is essential if they are to be useful in a molecular breeding program. Microsatellite markers are also highly specific and often only amplify a single locus from one of the three wheat genomes (Röder et al. 1998; Pestova et al. 2000). In addition, a high-density microsatellite consensus map of bread wheat with 1,235 loci, accompanied by an allele database containing the parent allele sizes for each marker mapped, will allow users to predict allele sizes in new breeding populations and encourage the development of molecular breeding strategies (Somers et al. 2004).

This study utilized microsatellite markers to locate and map two of the three resistance genes in Salamouni. *Stb14* is the resistance gene that provides resistance to only isolate MG2 and was identified in the doubled haploid (DH) line 98S08A*09. This isolate-specific resistance gene was assigned to chromosome 3BS and is linked to the microsatellite loci *Xwmc500* and *Xwmc623* at distances of 2 and 5 cM, respectively. *Stb13* was identified in the DH lines 98S08C*03 and 98S05B*01, and is one of two genes in Salamouni that provide resistance to both isolates MG96-36 and MG2. The STB resistance gene, *Stb13*, was mapped to the short arm of chromosome 7B in both populations and was linked to the microsatellite locus *Xwmc396* at distances of 9 and 7 cM.

At this time there is no *M. graminicola* isolate to differentiate between the two resistance genes in Salamouni that provide resistance to both western Canadian isolates. Therefore, there remains a single, unmapped resistance gene in Salamouni that controls resistance to both MG96-36 and MG2. However, with two mapped genes, it may be possible to screen the remaining DH lines with microsatellite primer pairs, keeping in mind the markers identified to be linked to *Stb13* and *Stb14*. This may assist in the identification of a DH line(s), which may potentially contain the third resistance gene. These select lines could then be moved forward to create mapping populations in an attempt to identify and map the third resistance gene present in Salamouni. An alternative option would be to create mapping populations from the F_2 populations previously identified to contain both of the resistance genes controlling resistance to isolate MG96-36 based on their 15 : 1 resistant to susceptible segregation ratio. If a large enough F_2 population can be created with corresponding $F_{2:3}$ families, it may be possible to identify individuals carrying the *Xwmc396* Katepwa (susceptible) allele. This would create a sub-set of individuals, which are homozygous susceptible at the *Stb13* locus, but are segregating at the unmapped locus. Once enough of these individuals are identified, BSA can be performed using this sub-set to identify putatively linked markers to this second gene in Salamouni conferring resistance to isolate MG96-36.

The actual chromosome locations of the STB resistance genes mapped in this study addressed some interesting issues with regard to the wheat genome. The *Stb14* resistance gene appears to be located approximately 30 cM (Somers et al. 2004) from a much larger resistance gene cluster present on chromosome 3BS. The *Stb2* resistance locus, originally identified in the resistant cultivar Veranopolis, has also been mapped to

chromosome 3B, although distal to *Stb14* (Adhikari et al. 2004b). However, in this same region, the *Sr2* locus for stem rust resistance was identified and is linked to *Xgwm533.1* (Spielmeyer et al. 2003), the same microsatellite locus used to map *Stb2* (Adhikari et al. 2004b). Also in this area are genes for leaf rust resistance (*Lr27*) (Faris et al. 1999), yellow or stripe rust resistance (*Yr30*) (Suenaga et al. 2003) and phenylalanine ammonia lyase, an enzyme believed to be involved in defense responses (Faris et al. 1999). In addition to qualitative characters, major QTLs for Fusarium head blight (Buerstmayer et al. 2003; Guo et al. 2003) and Stagonospora nodorum glume blotch (Schnurbusch et al. 2003) have also been identified in this cluster. A QTL for karnal bunt resistance has also been identified on 3BS, but is believed to be separate from the resistance gene cluster, as it is positioned closer to the centromere (Nelson et al. 1998).

The exact reasons for the occurrence of resistance gene clusters have not been clarified to any great extent (Dickinson et al 1993). However, there are possible theories that attempt to explain their existence; (i) Duplication and subsequent divergence of a progenitor resistance gene; (ii) Unequal recombination at intergenic regions between family members, which creates additional copy number variability within the population; and (iii) Recombination at highly conserved regions in intragenic regions may allow for the formation of novel gene combinations (Ronald 1998). Although none of these theories has been thoroughly investigated, resistance gene clusters are often located near the telomere or centromere (Michelmore 2000). This phenomenon is clearly demonstrated even with the previously mapped STB resistance genes. For example, *Stb4* and *Stb5* appear to be part of a larger resistance gene cluster near the centromere on chromosome 7B (Adhikari et al. 2004c). In addition, the resistance gene cluster

discussed previously, containing *Stb2*, is located near the telomere on chromosome 3BS (Adhikari et al. 2004b). There are also similar examples in lettuce, barley and tomato (Michelmore 2000).

To find out more about the resistance genes that make up these clusters, map-based cloning of these areas is a future prospect (Michelmore 2000). Map-based cloning involves locating closely linked molecular marker(s) and then chromosome walking to the gene of interest. Subsequent cloning and sequencing of these genes will hopefully reveal the detailed genetic organization of these resistance gene clusters and the genetic mechanisms involved in generating new resistance specificities (Michelmore 2000).

The second phenomenon of the wheat genome that is addressed in this study is the homoeology between the A, B, and D genomes of common bread wheat. In several instances it has been noted that the three chromosomes within the ABD homoeologous group frequently contain common loci for a particular character (Poehlman and Sleper 1995). One well-known example is the leaf rust resistance genes located on the three homoeologous group two chromosomes. As far as STB resistance loci are concerned, *Stb4* and *Stb5* are located near the centromere on chromosome 7D (Adhikari et al. 2004c), and the newly mapped *Stb13* is located near the centromere on 7B. The identification of repetitive loci across homoeologous groups suggests that the three distinct wheat genomes (A, B, and D) possibly originated from a common ancestor (Poehlman and Sleper 1996). Once again, cloning and sequencing of these resistance genes may provide more information about their origin and gradual evolution.

With the recent advent of characterizing STB resistance loci with molecular markers, there are now four cases where two different resistance genes have been mapped

to the same chromosome. *Stb4* and *Stb5* have both been mapped near the centromere of chromosome 7D (Arraiano et al. 2001; Adhikari et al. 2004c). *Stb7* and *Stb12* have both been mapped to the distal end of 4AL (McCartney et al. 2003; Chartrain et al. 2005b). Although *Stb13* and *Stb8* have both been mapped to 7B, they are separated by more than 80 cM (Adhikari et al. 2003; Somers et al. 2004). However, there has also been a QTL identified on chromosome 7BL associated with the microsatellite marker *Xwmc517*, which is approximately 25 cM (Somers et al. 2004) from *Stb13*. This study mapped *Stb14* to a location on chromosome 3BS, within approximately 30 cM (Somers et al. 2004) of the previously mapped *Stb2* (Adhikari et al. 2004b). If the independence of these genes is questionable because of their close proximity to one another, it may be necessary to conduct allelism tests to determine if the resistance genes are the same, allelic, or closely linked. Allelism tests involve making a resistant x resistant cross to generate large F_2 populations. These populations are then screened separately with the isolates used to identify each resistance gene. If the population segregates for susceptibility when challenged with one of the isolates, the genes are distinct. However, caution must be taken when conducting allelism tests to ensure that population sizes are large enough to account for the possibility of two distinct, but closely linked genes (Brûlé-Babel, Personnel Communication).

The evaluation of disease reactions in future studies may be facilitated with the development of DH or recombinant inbred lines (RILs) (McCartney et al. 2002). These types of populations are especially useful when studying incompletely dominant resistance genes, as found in Salamouni, because heterozygotes demonstrate an intermediate reaction type, which is often difficult to classify. However, because DH and

RIL lines are homozygous at all loci, there would never be heterozygous individuals to phenotype. Although progeny tests can usually accurately classify individuals, they are incredibly labour-intensive and require abundant growth space (Brading et al. 2002; McCartney et al. 2002). In addition, because individuals within DH and RIL lines are genetically identical, multiple plants of each line can be evaluated to ensure an accurate classification (McCartney et al. 2002).

The results of this thesis support the usefulness of trait identification using microsatellite markers. The resistance gene in Salamouni conferring resistance to only isolate MG2, *Stb14*, was mapped to chromosome 3BS and was found to be linked with *Xwmc500* and *Xwmc623* at distances of 2 and 5 cM, respectively. *Stb13*, the resistance gene in Salamouni providing resistance to both isolates MG96-36 and MG2, was linked to microsatellite marker *Xwmc396* at a distance of 9 cM in the 98S08C*03 population and 7 cM in the 98S05B*01 population. Because *Stb13* is not that closely linked to *Xwmc396*, it may be necessary to investigate other types of molecular markers, such as RFLPs, which may be more closely linked to the gene. If this were the case, conversion to a PCR based marker would be warranted to facilitate large-scale screening.

Whatever method used, molecular or conventional, the main goal of this project is to provide breeders with more information about STB resistance, which will hopefully lead to the development of durable STB resistant cultivars for western Canadian wheat producers.

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APPENDICES

Appendix 1. Disease rating scale developed by Rosielle (1972)

0 = immune – no pycnidial formation, no visible symptoms or an occasional hypersensitive fleck.

1 = highly resistant – no, or only an occasional isolated pycnidium formed, particularly in older tissue, hypersensitive flecking in younger leaf tissue.

2 = resistant – very light pycnidial formation, some coalescement of lesions, mainly towards the leaf tip and in older tissue.

3 = intermediate – light pycnidial formation, coalescement of lesions normally evident towards the leaf tips and elsewhere on the leaf blade.

4 = susceptible – moderate pycnidial formation, lesions much coalesced.

5 = very susceptible – large, abundant pycnidia, lesions extensively coalesced.

An 'X' following the Arabic numeral rating indicated extensive leaf necrosis. The Arabic numeral rating for such host genotypes correspond to pycnidial formation as above.

Appendix 2. Primers used for bulked segregant analysis

Primer name	Chromosome location(s)
BARC10[ab]	2B/4B/5A
BARC101[a]	2B
BARC111[a]	7D
BARC119[a]	1A/1D
BARC121[a]	7A/7D
BARC123[abcd]	7B
BARC124[a]	2A/2B/2D
BARC126[abcd]	7D
BARC127[a]	6B/7A
BARC13[a]	2B
BARC133[abcd]	3B
BARC134[a]	6B
BARC138[a]	4A
BARC143[a]	5D
BARC146[a]	6A/6B
BARC147[ac]	3B
BARC148[a]	1A/1D
BARC149[a]	1D
BARC151[a]	5A/7A
BARC159[a]	2B/2D
BARC164[a]	3B
BARC165[a]	5A
BARC167[a]	2B
BARC168[a]	2D
BARC17[a]	1A
BARC170[a]	4A
BARC172[a]	7D
BARC173[abcd]	3B/6D

Primer name	Chromosome location(s)
BARC174 [a]	7A
BARC175 [a]	6D
BARC176 [ab]	7B
BARC178 [a]	6B
BARC18 [a]	2B
BARC180 [abcd]	3B/5A
BARC181 [a]	1B
BARC182 [ab]	7B
BARC 196 [a]	6D
BARC200 [a]	2B
BARC204 [a]	6D
BARC206 [a]	3B/4A/6A
BARC23 [a]	6A/7A
BARC232 [ab]	5A/5B/5D
BARC24 [a]	6B
BARC267 [ab]	7B
BARC3 [a]	6A
BARC32 [ab]	7B
BARC37 [a]	6A
BARC4 [a]	5B
BARC45 [abcd]	2B/3A
BARC5 [a]	2A/5D/7D
BARC54 [a]	6D
BARC59 [a]	2D/5B
BARC62 [a]	1D
BARC67 [a]	3A
BARC68 [c]	3B/3D/4B
BARC70 [a]	4A/7A/7D
BARC71 [a]	3D
BARC72 [ab]	7B

Primer name	Chromosome location(s)
BARC73 [c]	3B
BARC74 [abcd]	5B
BARC75 [abcd]	3B
BARC76 [a]	2A/6B/7D
BARC77 [a]	3B
BARC78 [a]	4A
BARC8 [c]	1B
BARC80 [a]	1B
BARC84 [a]	3B
BARC85 [ab]	7B
BARC87 [ac]	3B/7D
BARC89 [abcd]	5B
BARC92 [c]	3B
BARC95 [ab]	7B
BARC96 [a]	6D
CFA2028 [a]	7A
CFA2040 [ab]	7A/7B/7D
CFA2049 [a]	7A
CFA2076 [a]	3A
CFA2104 [ac]	5A/5D
CFA2106 [ab]	7B
CFA2110 [a]	6B
CFA2129 [a]	1A/1B/1D
CFA2141 [ab]	5A/5D
CFA2147.1 [a]	1B/1D
CFA2163 [a]	5A
CFA2185 [a]	5A
CFA2190 [ac]	5A
CFA2193 [a]	3A
CFA2219 [a]	1A

Primer name	Chromosome location(s)
CFA2226 [a]	1A
CFA2250 [a]	5A
CFA2256 [a]	4A
CFA2257 [a]	7A
CFD1 [abcd]	6B/6D
CFD10 [a]	5D
CFD116 [ab]	2D
CFD13 [abcd]	6B/6D
CFD132 [a]	6D
CFD135 [abcd]	6D
CFD168 [a]	2A/2D
CFD175 [a]	7D
CFD18 [ab]	5D
CFD183 [a]	5D
CFD188 [a]	6D
CFD19 [ab]	1D/5D/6D
CFD190 [ab]	6A/6D
CFD193 [ab]	2D/3A/3D/4D/7A/7D
CFD2 [a]	1B/2D/3A/3D/4A/4B/5A/5B/5D/7D
CFD20 [ab]	5B/7A
CFD21 [abcd]	1D/7D
CFD22 [ab]	1A/4B/7B
CFD233 [a]	2D
CFD242 [a]	7A
CFD25 [a]	7D
CFD257 [a]	4A
CFD28 [c]	3B
CFD29 [a]	5D
CFD30 [a]	1A/4A/7D
CFD36 [a]	2A/2D

Primer name	Chromosome location(s)
CFD39 [a]	4B/4D/5A
CFD4 [c]	3B/3D
CFD41 [a]	7D
CFD42 [abcd]	6D
CFD46 [abcd]	7D
CFD48 [a]	1B/1D
CFD49 [a]	6D
CFD5 [a]	5B
CFD56 [a]	2D
CFD57 [a]	5D
CFD59 [a]	1A/1B/1D
CFD6 [c]	2A/3B/7A
CFD60 [a]	5B
CFD61 [ab]	1D
CFD63 [a]	1D
CFD65 [a]	1B/1D/2D
CFD66 [a]	7D
CFD71 [a]	4A/4D
CFD73 [a]	2B/2D
CFD75 [abcd]	6D
CFD79 [abcd]	3A/3B/3D
CFD8 [a]	5D
CFD84 [a]	4D
CFD86 [ab]	2A/5B/5D
CFD88 [a]	4A
CFD92 [a]	1D
GDM126 [a]	1D
GDM132 [abcd]	6D
GDM28 [a]	1B
GDM33 [a]	1A/1B/1D

Primer name	Chromosome location(s)
GDM36 [c]	1B
GDM72 [a]	3D
GWM10 [ab]	2A/7A
GWM107 [a]	3B/4B/6B
GWM108 [a]	3B
GWM111 [abcd]	7D
GWM112 [ab]	4B/7B
GWM124 [a]	1B
GWM126 [a]	5A
GWM130 [a]	7D
GWM131 [a]	1B/3B/7B
GWM133 [abcd]	1B/3A/4D/5B/6B/6D
GWM135 [ab]	1A
GWM136 [a]	1A
GWM140 [ab]	1B
GWM146 [abcd]	7B
GWM148 [a]	2B
GWM149 [a]	4B
GWM154 [ab]	5A
GWM157 [ab]	2D
GWM16 [ab]	2B/5D/7B
GWM160 [a]	4A
GWM161 [a]	3D
GWM162 [a]	3A/4A
GWM165 [a]	4A/4B/4D
GWM169 [a]	6A
GWM182 [a]	5D
GWM183 [a]	3D
GWM190 [a]	5D
GWM192 [a]	4B/4D

Primer name	Chromosome location(s)
GWM193 [a]	6B
GWM194 [a]	4D
GWM2 [abcd]	3A/3D
GWM213 [abcd]	4D/5B/7B
GWM232 [a]	1D
GWM233 [a]	7A
GWM251 [a]	4B
GWM260 [a]	7A
GWM264 [ac]	1B/3B
GWM271 [ab]	2B/5B/5D
GWM273 [a]	1B/6B
GWM274 [abc]	1B/3B/5B/7B
GWM284 [c]	3B
GWM285 [c]	3B
GWM291 [a]	5A
GWM293 [ab]	5A
GWM294 [a]	2A
GWM295 [abcd]	7D
GWM297 [ab]	7B
GWM299 [a]	3B
GWM301 [a]	2D
GWM302 [ab]	7B
GWM304 [ab]	5A
GWM311 [ab]	2A/6B
GWM312 [ab]	2A
GWM32 [a]	3A
GWM33 [a]	1A/1B/1D
GWM332 [ab]	7A
GWM333 [abc]	7B
GWM334 [a]	6A

Primer name	Chromosome location(s)
GWM335 [abcd]	5B
GWM337 [a]	1D
GWM344 [abcd]	7B
GWM350 [ab]	7A/7D
GWM356 [a]	2A
GWM359 [a]	2A
GWM368 [a]	4B
GWM369 [abcd]	3A
GWM37 [ab]	7D
GWM371 [abcd]	5B
GWM372 [ab]	2A
GWM376 [c]	3B
GWM383 [ab]	3D
GWM388 [a]	2B
GWM389 [abcd]	3BS
GWM397 [a]	4A
GWM4 [a]	3A/3B/4A/7A
GWM400 [ab]	7B
GWM408 [ab]	5B
GWM413 [ac]	1B
GWM428 [a]	7D
GWM43 [ab]	7B
GWM44 [abcd]	7D/4A
GWM443 [a]	5A/5B
GWM445 [a]	2A
GWM456 [a]	3D
GWM46 [ab]	7B
GWM469 [a]	5D/6D
GWM473 [ab]	2A/7D
GWM493 [abcd]	3B

Primer name	Chromosome location(s)
GWM494 [a]	1B/3A/4A
GWM497 [a]	1A/2A/3A/3D/5B
GWM518 [a]	6B
GWM526 [a]	2B
GWM533 [abcd]	3B
GWM537 [ab]	7B
GWM538 [a]	4B
GWM539 [ab]	2D
GWM565 [a]	4A/5D
GWM566 [c]	3B
GWM569 [ab]	7B
GWM573 [ab]	7A/7B
GWM577 [abcd]	7B
GWM6 [a]	4B
GWM601 [a]	4A
GWM608 [a]	1B/1D/2D/4D/6B
GWM610 [a]	4A
GWM611 [abcd]	7B
GWM613 [a]	6B
GWM614 [a]	2A/2B
GWM617 [ab]	5A/6A
GWM624 [a]	4D
GWM635 [a]	7A/7D
GWM637 [a]	4A
GWM639 [a]	5A/5B/5D
GWM644 [abc]	3B/6B/7B
GWM67 [abcd]	5B
GWM68 [ab]	5B/7B
GWM705 [a]	6B
GWM72 [c]	3B

Primer name	Chromosome location(s)
GWM95 [a]	2A
GWM96 [ab]	5A
WMC1 [c]	3B
WMC10 [ab]	7B
WMC11 [abcd]	1A/3A/3D
WMC110 [a]	5A
WMC112 [a]	2D
WMC121 [a]	7D
WMC125 [a]	4B
WMC139 [a]	7A
WMC144 [ab]	2D
WMC149 [a]	2A/2B/5B
WMC15 [a]	4A
WMC160 [a]	5B
WMC161 [a]	4A/5D
WMC166 [b]	7B/7D
WMC167 [a]	2D
WMC173 [a]	3A/4A
WMC177 [a]	2A
WMC179 [ab]	2B/6A/6B/7A
WMC18 [ab]	2D
WMC181 [a]	2A/2D
WMC182 [ab]	3B/4D/6A/6B/7A/7D
WMC201 [a]	6A
WMC216 [a]	1B/1D
WMC218 [ab]	7B
WMC219 [abcd]	4A
WMC231 [abc]	3B
WMC232 [a]	4A
WMC233 [a]	5D

Primer name	Chromosome location(s)
WMC238 [a]	4B
WMC24 [a]	1A
WMC25 [a]	2B/2D
WMC254 [a]	4B/6A
WMC256 [ab]	6A
WMC258 [a]	4A/5B
WMC262 [a]	4A
WMC264 [a]	3A/5D
WMC273 [ab]	7B/7D
WMC276 [ab]	7B
WMC283 [a]	4A/7A
WMC285 [a]	4D
WMC289 [a]	5B/5D
WMC296 [ab]	2A
WMC307 [a]	3B
WMC311 [ab]	7B
WMC312 [a]	1A
WMC313 [abcd]	4A
WMC323 [a]	7B
WMC332 [a]	2B
WMC335 [a]	7B
WMC336 [a]	1A/1D
WMC339 [a]	1D
WMC349 [a]	4B
WMC357 [a]	5D
WMC364 [ab]	7B
WMC366 [c]	3B
WMC376 [a]	5B
WMC382 [a]	2A/2B
WMC388 [ab]	5A/7A

Primer name	Chromosome location(s)
WMC396 [abc]	2B/3B/4A/5B/7B
WMC397 [a]	6B
WMC399 [a]	4D
WMC405 [abcd]	1D/5B/5D/7A/7D
WMC406 [c]	1B
WMC407 [ab]	2A
WMC413 [a]	4B
WMC415 [a]	5A/5B
WMC416 [a]	1B
WMC417 [a]	6A/6B
WMC419 [a]	1B/4B/6B
WMC420 [a]	4A
WMC422 [a]	7A
WMC426 [ab]	7B
WMC428 [a]	3A
WMC43 [ac]	3B/3D
WMC430 [abcd]	3B/5B
WMC432 [a]	1D
WMC435 [abcd]	3D/5B/7B
WMC438 [abcd]	7D
WMC44 [a]	1B
WMC446 [ac]	3B/4A/5A
WMC453 [ab]	2A/2D
WMC457 [a]	4D
WMC468 [a]	4A
WMC469 [a]	1A/6D
WMC470 [a]	2D
WMC471 [ab]	3B/7B
WMC473 [a]	4D/6B/7D
WMC475 [ab]	5A/7B

Primer name	Chromosome location(s)
WMC476 [ab]	7B
WMC477 [ab]	2B
WMC48 [a]	4B
WMC487 [a]	6B
WMC489 [c]	1D/2B/3A/4D/5A/7D
WMC491 [a]	4A/4B
WMC492 [a]	3D/5A
WMC497 [abcd]	4A/7A
WMC500 [abcd]	2B/3B/4A/5B/7B
WMC503 [ab]	2D
WMC505 [c]	3A/3B/3D
WMC506 [a]	7D
WMC508 [a]	5B
WMC51 [ac]	1A/1B/2B/3B/7B
WMC513 [a]	4A
WMC516 [a]	4A
WMC517 [ab]	7B
WMC522 [a]	2A
WMC524 [a]	5A
WMC525 [a]	7A
WMC526 [ab]	7B
WMC527 [a]	3A/3B
WMC532 [abcd]	3A
WMC533 [abc]	3B/3D
WMC537 [a]	5B
WMC540 [abc]	3B/7B
WMC544 [c]	3B
WMC546 [ab]	4B/7B
WMC549 [ab]	3D
WMC552 [a]	3D

Primer name	Chromosome location(s)
WMC553 [a]	6A
WMC557 [ab]	7B
WMC559 [a]	3A
WMC580 [a]	6A
WMC581 [ab]	7B
WMC59 [a]	1A/6A
WMC594 [a]	3A
WMC597 [ac]	1B/2B/3B/4A
WMC601 [a]	2D
WMC602 [a]	2A/2B
WMC603 [ac]	7A
WMC606 [ab]	7B/7D
WMC607 [ab]	7A
WMC609 [a]	1D
WMC611 [a]	1A/1B
WMC612 [c]	3B
WMC613 [ab]	7B
WMC615 [c]	3B
WMC617 [a]	4A/4B/4D
WMC621 [a]	6A
WMC622 [a]	4D
WMC623 [c]	3B
WMC625 [abc]	3B
WMC630 [ab]	1A/2D/5A/5D/7D
WMC631 [a]	1B/3D
WMC632 [a]	2A/3B
WMC634 [ab]	7D
WMC646 [a]	7A/7D
WMC65 [a]	7A
WMC650 [a]	4A

Primer name	Chromosome location(s)
WMC653 [ab]	3B/7B/7D
WMC658 [a]	2A
WMC662 [ab]	7B
WMC667 [a]	2A
WMC674 [c]	3B/3D
WMC675 [c]	3B
WMC679 [c]	3B/4B
WMC680 [a]	4A
WMC687 [a]	3B
WMC693 [c]	3B
WMC694 [a]	1B
WMC695 [c]	3B3A/3B/4B/7A
WMC696 [ab]	7B
WMC698 [a]	4A/7D
WMC70 [abd]	7B
WMC707 [a]	4A
WMC710 [a]	4B
WMC713 [a]	5A
WMC718 [a]	4A
WMC722 [a]	4A
WMC723 [ab]	7B
WMC726 [a]	6B
WMC727 [ab]	5A
WMC73 [ab]	5B
WMC737 [a]	6B
WMC740 [a]	5B
WMC751 [c]	3B
WMC753 [ab]	6A/6D
WMC754 [c]	3B
WMC757 [a]	4A

Primer name	Chromosome location(s)
WMC758 [ab]	7B
WMC76 [ab]	7B
WMC760 [a]	4A
WMC762 [c]	3B
WMC764 [a]	2B
WMC765 [a]	5D
WMC773 [a]	5B/6D
WMC776 [a]	4A
WMC777 [ac]	3B
WMC78 [abc]	3B
WMC783 [a]	5B
WMC786 [ab]	6A/6B/6D
WMC79 [a]	6B
WMC790 [a]	7A
WMC792 [ab]	2A/7B
WMC798 [a]	1B
WMC805 [a]	5A/5D
WMC807 [ab]	6A
WMC808 [ac]	3B
WMC809 [a]	7A
WMC815 [c]	3B
WMC818 [a]	1A/1B
WMC824 [a]	7D
WMC826 [ab]	1A/4B/7A
WMC827 [ab]	2A/3B/7D
WMC83 [ab]	7A
WMC89 [a]	4A/4B/4D
WMC94 [a]	7D
WMC96 [a]	3A/4A/5D
WMC99 [a]	5B

[a] Primers used for BSA with the 98S08C*03 bulks
[b] Primers used for BSA with the 98S05B*01 bulks
[c] Primers used for BSA with the 98S08A*09 bulks
[d] Primers used in the preliminary targeted screen