# Chromosomal Location and RAPD Marker Development for Tan Spot Resistance in Hexaploid Wheat

By

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A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfilment of the Requirements

for the Degree of

Master of Science

Department of Plant Science

University of Manitoba

Winnipeg, Manitoba

ISBN 0-612-16313-X

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# Chromosomal Location and RAPD Marker Development for Tan Spot Resistance in Hexaploid Wheat

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

## ABSTRACT

Several sources of resistance to tan spot induced by *Pyrenophora tritici-repentis* Died. have been identified in hexaploid wheat (*Triticum aestivum* L.). The overall objectives of this thesis were to determine the number and chromosome location of tan spot necrosis resistance gene(s) in the cultivar Chinese Spring (CS) and to develop RAPD markers for identified gene(s). CS (resistant/insenstive) and Kenya Farmer (KF) (susceptible/sensitive) have contrasting reactions to the *P. tritici-repentis* isolate 86-124 ($nec^+chl^-$) and Ptr necrosis toxin, respectively. Analysis of $F_1$, $F_2$ and $F_2$-derived $F_3$ families from the reciprocal cross CS/KF identified a single, nuclear, recessive gene governing resistance to isolate 86-124 and Ptr necrosis toxin. Evaluation of the CS(KF) substitution series, $F_2$ monosomic analysis, nullisomic analysis, and screening of various CS nulli-tetrasomic and ditelosomic lines indicated that the identified resistance gene was located on chromosome arm 5BL. It was proposed that the necrosis resistance gene be named *tsn1*. No linkage existed between the leaf rust resistance gene *Lr18*, previously mapped to chromosome 5BL, and *tsn1*. The identification of a single resistance gene allowed the opportunity to develop a random amplified polymorphic DNA (RAPD) marker. The genomic DNA of CS and KF were screened with 420 arbitrary sequence 10-mer primers. Since a low level of genomic polymorphism (10.6 percent) was resolved with agarose gel electrophoresis, a sub-set of 74 primers was analyzed using temperature sweep

gel electrophoresis (TSGE). TSGE resolved 73 genomic polymorphisms. Two polymorphisms specific to chromosome 5B sequences were identified between CS and CS(KF 5B). RAPD analysis was conducted using a single plant from each of 65 $F_2$-derived $F_3$ families from the reciprocal crosses CS/KF and CS/CS(KF 5B). Each $F_2$-derived $F_3$ family was homogeneous for disease reaction to both the isolate 86-124 and Ptr necrosis toxin. Linkage analysis identified that the polymorphic fragments amplified by the primers UBC195 and UBC102 were 23.4 $\pm$5.7 cM and 27.4 $\pm$6.9 cM from *tsn1*, respectively. UBC195 and UBC102 were tightly linked, 4.2 $\pm$ 2.5 cM apart. Potential applications of a *P. tritici-repentis* necrosis resistance marker in wheat are discussed.

## ACKNOWLEDGEMENTS

Research is not conducted without the assistance and input of many people. First and foremost, to my supervisor, Dr. Anita Brûlé-Babel, thank-you for the opportunity, and for your guidance, support and advice.

To each member of my advisory committee; Drs. Greg Penner and Lakdhar Lamari I wish to extend my thanks for their intellectual resource and support. A special thanks must be extended to Dr. Penner for taking me into his lab. Your enthusiasm and open door were greatly appreciated.

There are many people who contributed to this project and who I would like to thank: Dr. E. Kerber, Dr. J. Kolmer, Dr. K. Armstrong and Dr. R. McIntosh for contributing experimental lines, disease inoculum, and education. To all members of the greenhouse staff, past and present, for helping care for my plants.

The members of the Molecular Breeding Lab (past and present) must also be recognized. Thanks Graham, Leslie, Joanne, Elsa, and Sung for your patience and help, especially when I was running between labs.

The members of the Plant Genetics lab deserve special recognition for all the academic support and friendship that they extended to me. Thanks Scott, Darin, Jim, Gayle, Charles, and Didz. You made my stay at Plant Science unforgettable.

To Nancy, your friendship and support throughout will never be forgotten.

To my parents, John and May, thanks for your patience and understanding. Your support is appreciated more than my words can describe.

## FORWARD

This thesis is written in manuscript style. This thesis begins with a general abstract, introduction, and literature review, followed by the presentation of two chapters of experimental research, each representing a particular research theme. The thesis concludes with a general discussion including conclusions and ideas for future research, followed by the list of references cited throughout the thesis and an appendix. The format of each experimental chapter is as follows: the abstract, introduction, materials and methods, and results and discussion on the theme of the research within the chapter. The thesis is written to conform with the requirements of the Canadian Journal of Plant Science.

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# 1. INTRODUCTION

*Pyrenophora tritici-repentis* Died. is the causal agent of tan spot (yellow leaf spot, yellow leaf blotch, eye spot), a foliar disease of wheat (*Triticum aestivum* L.) and other *gramineae* species (Hosford 1982; Tekauz et al. 1982; Krupinsky 1982, 1987, 1992). In the great plains region of North America, wheat yield losses to tan spot commonly range from three to 15 percent, but under severe epidemics may reach 50 percent (Hosford 1982; Tekauz et al. 1982). There are currently no cultivars that possess tan spot resistance registered for western Canada. Consequently, current control strategies involve cultural or chemical disease control through crop rotation, residue management, or fungicide application. These practices may adversely affect management decisions, financial stability and the environment. The incorporation of high-level genetic resistance would increase management options and minimize environmental degradation. Wheat yield losses would be reduced as the durability and degree of genetic resistance to *P. tritici-repentis* increased.

It is essential that plant breeders understand how specific resistance genes are inherited in order to efficiently introgress genetic resistance into commercial cultivars. To develop appropriate breeding strategies, the number of loci, their dominance relationships, gene interactions, and gene-linkages must be determined. Several genetic studies of the inheritance of *P. tritici-repentis* resistance have been conducted to determine the allelic and genic relationships between various sources of resistance (Nagle et al. 1982; Lee and Gough 1984;

Elias et al. 1989; Lamari and Bernier 1989c; Lamari and Bernier 1991; Sykes and Bernier 1991; Duguid 1995).

A key step of resistance gene characterization is the determination of its chromosome location. The development of several aneuploid series in wheat (Sears 1954; Law et al. 1983) have allowed scientists to assign numerous genes to specific chromosomes (McIntosh 1988). The identification of chromosome location of gene loci facilitates the development of DNA markers. Advances in molecular marker technology have provided the opportunity to tag important plant traits so that plant breeders can monitor and manipulate key traits by linkage rather than by phenotype (Gale et al. 1989).

The objectives of this thesis were; (i) to determine the number of *P. tritici-repentis* necrosis resistance gene(s) segregating in the cross between *T. aestivum* cultivars Chinese Spring (CS) and Kenya Farmer (KF), (ii) to determine the chromosome location of identified *P. tritici-repentis* necrosis resistance gene(s), (iii) to identify a random amplified polymorphic DNA (RAPD) marker for identified *P. tritici-repentis* necrosis resistance gene(s), and (iv) to evaluate linkage relationships between identified *P. tritici-repentis* necrosis resistance gene(s) and a previously mapped gene(s).

Completion of this research, in conjunction with other studies investigating the *T. aestivum*/*P. tritici-repentis* interaction, will provide the information necessary for efficient incorporation of tan spot necrosis resistance into commercial cultivars.

# 2. LITERATURE REVIEW

## 2.1 *Pyrenophora tritici-repentis* Died.

*P. tritici-repentis* is the causal agent of tan spot, a foliar fungal disease of wheat (Wiese 1987). It is grouped in the Kingdom *Fungi*, division *Eumycota*, subdivision *Ascomycotina*, class *Loculoascomycetes*, order *Pleosporales*, and family *Pleosporaceae* (Ainsworth et al. 1973). The casual organism is the ascomycete *P. tritici-repentis* Died. (syn. *P. trichostoma* (Fr.) Fckl.), anamorph: *Drechslera tritici-repentis* (Died.) Shoem. (syn. *Helminthosporium tritici-repentis* Died.). This facultative pathogen is classified as a necrotroph as it causes extensive tissue damage to the host in its parasitic phase, and it is confined largely to dead or dying host plant tissue in its non-parasitic phase.

Variation for virulence on wheat cultivars has been reported among isolates of *P. tritici-repentis*. *P. tritici-repentis* induces two distinct symptoms on susceptible wheat host plants: tan necrosis and/or chlorosis. Isolates of *P. tritici-repentis* collected on wheat in western Canada have been classified into four distinct pathotypes on the basis of their ability to induce tan necrosis and/or chlorosis on a differential set of wheat genotypes: pathotype 1 induces both tan necrosis and extensive chlorosis ($nec^+chl^+$) on susceptible host genotypes; pathotype 2 induces tan necrosis ($nec^+chl^-$) only; pathotype 3 induces extensive chlorosis ($nec^-chl^+$) only; and pathotype 4 is incapable of inducing either tan necrosis or chlorosis ($nec^-chl^-$) (Lamari and Bernier 1989b; Lamari and Bernier 1991). Identification of an isolate collected from eastern Algeria that induces only chlorosis ($nec^-chl^+$) but is

distinct from isolates previously classified within pathotype 3 has lead to the recent reclassification of isolates into races (Lamari et al. 1995). Pathotypes 1, 2, 3, and 4 are now referred to as races 1, 2, 3 and 4, respectively. The new isolate from eastern Algeria is classified as race 5. Because of the timeliness of this thesis, the term pathotype was continued to be used to define race as defined by Lamari et al. 1995.

### 2.1.1 Disease Cycle

Substantial research has been conducted investigating the disease cycle of tan spot. The life cycle of the pathogen consists of both a sexual and asexual phase. The sexual phase is characterized by the production of pseudothecia. The asexual phase is characterized by the production of conidia. Each phase has important implications in the development of the disease cycle.

*P. tritici-repentis* overwinters on infected plant residue of numerous *gramineae* species (Krupinsky 1982; Tekauz et al. 1982). Pseudothecia provide the mechanism by which the fungus survives between crops (Hosford 1972; Rees and Platz 1980). Typically, periods of high moisture (Pfender et al. 1988; Wright and Sutton 1990) and temperatures less than 15°C (Odvody et al. 1982; Summerell and Burgess 1988; Wright and Sutton 1990) are necessary for maturation of pseudothecia.

Ascospores are the primary inoculum of *P. tritici-repentis* (Hosford and Busch 1974; Hosford and Morrall 1975; Rees and Platz 1980). Periods of high

moisture are required for the production of ascospores (Pfender et al. 1988; Summerell and Burgess 1988). As ascospores are only disseminated short distances by wind (Morrall and Howard 1975; Rees and Platz 1980; Tekauz et al 1982; Rees and Platz 1992), dispersion will only result in localized epidemics (Rees 1982; Schuh 1990; Schilder and Bergstrom 1993). Cool damp weather favours disease initiation (Tekauz et al. 1982; Luz and Bergstrom 1987; Adee and Pfender 1989).

Following primary infection, conidia are produced on maturing lesions and serve as a secondary source of inoculum during wet periods of the growing season (Hosford 1982; Tekauz et al. 1982; Wiese 1987; Adee and Pfender 1989). Although a wet period has been recognized as essential for disease development, its effect on host resistance has been controversial. Wet periods, longer than 48 h, have been reported to either result in host-resistance breakdown (Hosford 1982; Hosford et al. 1987) or have no effect (Lamari and Bernier 1989a).

The major factor for conidial dispersal is wind (Morrall and Howard 1975; Platt and Morrall 1980). Platt and Morrall (1980) established that wind speeds as low as 2 m $s^{-1}$ were sufficient to result in 100 percent conidia liberation. Wind dispersal of conidia may result in epidemics within a crop and facilitates spread of the disease to other fields (Rees and Platz 1980; Raymond et al. 1985; Rees and Platz 1992).

Under optimum conditions, the fungus can produce a secondary disease cycle approximately every eight days (Riaz et al. 1991). Subsequently, the

importance of pseudothecia as a source of inoculum decreases once sporulation of the pathogen commences on the crop. The abundant conidia late in the season permit dispersal of the pathogen to other crops where the resulting infected stubble provides initial inoculum for crops the following season (Rees and Platz 1980). It is important to note that the mode of saprophytic colonization of the host appears to be independent of its response to infection (Summerell and Burgess 1988). Consequently, the use of resistant cultivars is unlikely to bring about a reduction in disease carry-over. It will however, reduce wheat yield losses due to the disease.

Shoemaker (1962) published detailed anatomical descriptions of *P. tritici-repentis* that are useful in the identification of the fungus in culture.

### 2.1.2 Distribution

*P. tritici-repentis* has been reported in all major wheat growing regions of the world (Morrall and Howard 1975; Rees and Platz 1979; Hosford 1982; Krupinsky 1982; Tekauz et al. 1982; Wiese 1987; Summerell and Burgess 1989a, 1989b; Kemp et al. 1990). Tan spot has become a significant leaf spot disease on the Canadian prairies (Tekauz 1976).

*P. tritici-repentis* overwinters on infected wheat residue (Krupinsky 1982; Tekauz et al. 1982). This has significant implications on the management systems used by western Canadian producers. Many producers have adopted reduced tillage systems to conserve soil moisture, minimize soil erosion, and limit input

costs. By conserving wheat residues near the soil surface, the favourable habitat for *P. tritici-repentis* is maintained. Consequently, inoculum levels are increasing (Platz and Rees 1989; Lamari and Bernier 1989a; Summerell and Burgess 1989a) with a concomitant increase in disease incidence (Shabeer and Bockus 1988; Schuh 1990).

### 2.1.3 Symptoms

Leaf lesions caused by *P. tritici-repentis* vary greatly, depending on the pathotype assayed and the host reaction. Symptoms caused by virulent pathotypes begin as a very small dark brown to black spot on either the top or bottom of the leaf surface (Lamari and Bernier 1989b, Wiese 1987). On lines highly resistant to *P. tritici-repentis*, no further lesion expansion is observed. As host resistance decreases, the presence of tan necrosis and/or chlorosis immediately surrounding the lesion site is observed (Lamari and Bernier 1989b). On wheat lines that are highly susceptible to *P. tritici-repentis* isolates capable of inducing tan necrosis, tan necrotic lesions continue to expand into irregular oval tan blotches up to 12 mm long (Wiese 1987). On wheat lines that are highly susceptible to *P. tritici-repentis* isolates capable of inducing extensive chlorosis, chlorotic lesions exhibit a gradual yellow discoloration, initially without collapse of large areas of the leaf (Lamari and Bernier 1989a). As lesions continue to expand, the small dark brown to black spot in the centre of the lesion which is characteristic to *P. tritici-repentis* may disappear (Wiese 1987). Adjacent lesions

may coalesce under conditions favourable for disease development and heavily infected leaves wither and die. If the spike is infected, a pinkish discoloration of the seed may develop (Vanterpool 1963) and the seeds may be somewhat smaller and wrinkled (Sharp et al. 1976; Rees et al. 1981; Rees et al. 1982).

### 2.1.4 Toxin

Cytological studies have shown that hyphae of *P. tritici-repentis* grow intercellularly without penetrating the mesophyll cells, but at some point the mesophyll cells are disrupted and the cellular contents become available to the fungus (Larez et al. 1986). This is characteristic of toxin-producing microorganisms (Dushnickey 1993). These microorganisms kill plant cells in advance of colonization and then live as saprophytes on the dead tissues (Scheffer 1983). Two research groups recognized this characteristic in necrosis-inducing isolates of *P. tritici-repentis* (Tomas and Bockus 1987; Ballance et al. 1989; Lamari and Bernier 1989c; Tomas et al. 1990).

*P. tritici-repentis* has been shown to release a necrosis-inducing, host-selective, toxin in culture (Tomas and Bockus 1987; Ballance et al. 1989; Lamari and Bernier 1989c; Tomas et al. 1990) which has been designated Ptr necrosis toxin (Ballance et al. 1989) or Ptr toxin (Tomas et al. 1990). Tomas and Bockus (1987) identified a toxin capable of inducing tan necrosis after infiltration into susceptible genotypes, while no symptoms developed in resistant genotypes. Upon further purification and characterization, the toxin was found to be a protein

with a molecular weight of 14700 (Tomas et al. 1990).

Lamari and Bernier (1989c) independently isolated a similar toxin only produced by *P. tritici-repentis* isolates capable of inducing tan necrosis ($nec^+chl^-$ or $nec^+chl^+$) on susceptible genotypes. According to Lamari and Bernier (1989c) the toxin is not required for host penetration paralleling the observations of Larez et al. (1986). Upon further purification and characterization, the toxin was found to be a monomeric protein with a molecular weight of 13900 (Ballance et al. 1989). Lamari et al. (1995) confirmed the *in vivo* presence of Ptr necrosis toxin in the intercellular washing fluid from leaves infected with $nec^+$ isolates less than 72 h post-inoculation.

The mode of action of Ptr necrosis toxin was investigated by Deshpande (1993). The cell membrane appeared to be the site of action of Ptr necrosis toxin in toxin-sensitive genotypes. It was suggested that in susceptible wheat genotypes, the toxin binds to sites on the plasma membrane which alters cell membrane permeability causing cell death and necrotic lesions (Deshpande 1993). This means that toxin-insensitive wheat genotypes actively transcribe proteins or other factors that prevent the binding of Ptr necrosis toxin to the cell membrane (Deshpande 1993).

Orolaza et al. (1995) isolated a host-selective toxin from one pathotype 3 isolate of *P. tritici-repentis.* This toxin elicited extensive chlorosis on Katepwa similar to the chlorotic symptom induced by the fungus on the same wheat genotype.

### 2.1.5 Control Measures

All *T. aestivum* cultivars grown in western Canada are susceptible to *P. tritici-repentis*. Without genetic resistance, control methods necessarily rely on cultural or biological control methods. Because *P. tritici-repentis* overwinters on infected *gramineae* residues, removal of infected residues has been used to delay disease onset (Schuh 1990; Bockus and Claassen 1992). The most common methods of residue removal include burying with deep tillage, straw baling and removal, and stubble-residue burning (Hosford 1982; Tekauz et al. 1982).

Both extensive tillage and stubble-residue burning leave the soil prone to wind and water erosion and decrease soil organic matter. To minimize soil degradation, many producers are incorporating reduced tillage into their rotations. Consequently, inoculum levels are increasing (Platz and Rees 1989; Lamari and Bernier 1989a; Summerell and Burgess 1989a) with a concomitant increase in disease severity (Shabeer and Bockus 1988; Schuh 1990). Indicators of viability of the fungus include the recovery of *P. tritici-repentis* from stubble, and the production of fertile pseudothecia on stubble residues that have been weathered over a several years in various environments (Adee and Pfender 1989; Summerell and Burgess 1989a). *P. tritici-repentis* is infrequently recovered from stubble after 26 weeks of burial, 52 weeks after incorporation or 104 weeks on the soil surface. Consequently, long-term rotations away from wheat are advisable. However, as *P. tritici-repentis* is able to colonize residue of resistant genotypes, this method of control has serious limitations (Summerell and Burgess 1988).

Other cultural control methods for tan spot have been investigated. Huber et al. (1987) found that high applications of nitrogen reduced tan spot severity. Similarly, fungicides can effectively control *P. tritici-repentis*, but their use is not usually economically or environmentally justified. Dithane DG and Tilt 250E are both registered in Manitoba for control of *P. tritici-repentis* (Manitoba Agriculture 1995). The active ingredients of Dithane DG and Tilt 250E are mancozeb and propiconazole, respectively.

Several scientists have investigated the use of biocontrol agents. Use of *Limonomyces roseipellis* (Pfender 1988) and *Cochliobolus sativus* (Luz and Bergstrom 1987) have been investigated as potential biocontrol agents. Both have been shown to antagonize *P. tritici-repentis* and effectively reduce *P. tritici-repentis* populations. However, *L. roseipellis* is pathogenic to turf grass and both *L. roseipellis* and *C. sativus* are pathogenic to wheat. Therefore, it is unlikely that these organisms would be suitable biocontrol agents.

## 2.2 Host/Parasite Interaction

The *T. aestivum*/*P. tritici-repentis* interaction consists of two separate components; tan necrosis and extensive chlorosis (Lamari and Bernier 1989b). Lamari and Bernier (1989b) identified three virulent pathotypes based on the ability of isolates tested to induce either tan necrosis ($nec^+$), extensive chlorosis ($chl^+$), or both on a set of differential wheat genotypes. Lamari et al. (1995) have since identified a fourth virulent isolate that induces only chlorosis but is distinct from

isolates previously classified within pathotype 3.

Prior to the identification of tan necrosis-inducing and chlorosis-inducing components of *P. tritici-repentis*, disease resistance was scored primarily as a quantitative trait. Various quantitative rating systems were developed utilizing such measures as; percent infection and number of lesions per $cm^2$ (Nagle et al. 1982), lesion size and percent infection (Luz and Hosford 1980), an index combining lesion size, percent leaf area infected and leaf location (Raymond et al. 1985), and lesion size alone (Cox and Hosford 1987). Confusion existed on how to define host resistance and susceptibility. Further complications arose due to inconsistency in use and characterization of isolates used in each study.

Lamari and Bernier (1989a) developed a numeric five-level rating system based on lesion type that qualitatively categorized host reactions (Appendix 7.1) to the different pathotypes. Lesion types 1 and 2 describe levels of resistance. Lesion types 3, 4 and 5 describe levels of susceptibility.

To obtain full resistance to *P. tritici-repentis*, resistance to both tan necrosis and extensive chlorosis must be incorporated in *T. aestivum* cultivars. Genetic studies of host resistance can be simplified by assaying resistance to each component separately. Consequently, the inaccuracies that plagued earlier genetic studies can be avoided.

### 2.2.1 Inheritance of Host Resistance

Prior to the identification of two separate components of *P. tritici-repentis*,

few studies were conducted to determine the inheritance to tan spot in hexaploid wheat. Nagle et al. (1982) examined six hexaploid wheat crosses for resistance to isolate PyW17. Disease severity based on percent leaf area affected was used to discriminate between host resistance and susceptibility. For each cross, $F_2$ and $BC_1F_1$ progeny did not fit expected monogenic or digenic ratios. It was concluded that the inheritance of tan spot resistance was quantitatively inherited.

The realization that genetic studies could be simplified by assaying for individual components of the *T. aestivum*/*P. tritici-repentis* syndrome, enabled accurate and precise definition of qualitative resistance. Genetic studies involving the interaction between both the *P. tritici-repentis* necrosis-inducing isolate 86-124 ($nec^+chl^-$) and Ptr necrosis toxin with *T. aestivum* cultivars identified one or two genes for resistance, depending on the segregating population evaluated (Lamari and Bernier 1991; Duguid 1995). Lamari and Bernier (1991) identified a single recessive gene governing resistance to the isolate 86-124 in segregating $F_2$ populations of Glenlea/6B365 and Salamouni/Glenlea. Duguid (1995) identified two recessive genes segregating in $F_2$ and $F_2$-derived $F_3$ populations of BH1146/ST6, Katepwa/Erik, Katepwa/ST6, and Katepwa/ST15.

Genetic studies involving the interaction between *P. tritici-repentis* chlorosis-inducing isolate D308 ($nec^-chl^+$) and *T. aestivum* cultivars identified different genetic ratios depending on the cross analyzed (Duguid 1995). In crosses involving the susceptible genotype ST15 and the resistant genotypes Erik, ST6, 6B367 and 6B1043, a single dominant gene governed resistance. In the cross

BH1146/ST15, resistance was governed by two dominant genes, while resistance was governed by two recessive genes in the cross Katepwa/ST15.

## 2.3 *Triticum aestivum* L.

### 2.3.1 Genetics

Hexaploid wheat (*Triticum aestivum* L.) is an allopolyploid (2n=6x=42) of recent origin, approximately 10000 B.C. (Devos and Gale 1993). It is composed of three homoeologous genomes (A, B, and D) each derived from a distantly-related diploid species. The A genome donor is *T. monococcum*. The B genome donor is unknown, but speculated to be from the *Sitopsis* section. The D genome donor is *T. tauschii*. In order to maintain the integrity of the three chromosome sets, pairing must be very tightly controlled at meiosis to allow homologous but not homoeologous chromosomes to pair and recombine. The presence of the major homoeologous pairing gene (*Ph1*) on chromosome arm 5BL (Riley and Chapman 1958) forces polyploid wheats to behave cytologically like diploids and thereby maintain a high level of fertility and stability (Wall et al. 1971; Kimber and Sears 1987; Ji and Langridge 1990).

Wheat has a very large genome. It has a haploid nuclear DNA content of 18.1 picograms (Flavell et al. 1974). A nuclear genome of this size approximates 1.6-1.7 x $10^7$ base-pairs (Devos and Gale 1993; Moore et al. 1993). Much of the genome consists of repeated DNA sequences of varying degrees of reiteration and length. Approximately twenty percent consists of low-copy number or unique

sequences (Smith and Flavell 1975; Flavell and Smith 1976; Ranjekar et al. 1976). Approximately one percent consists of actual coding genes (May and Appels 1987).

The wheat karyotype has been well described and arm length and DNA content has been reported (Sears 1954; Furuta et al. 1988). Assuming an average chromosome map size of 300 cM, as estimated for chromosomes 5A and 2B, the total map size for all 21 chromosomes would be approximately 6300 cM (Lui and Tsunewaki 1991).

## 2.4 Gene Mapping

Only sparse genetic maps of wheat have been completed to date (Gale et al. 1989; Devos and Gale 1993). There are several reasons for this. First, genetic analysis is complicated due to the large number of linkage groups in wheat (Chao et al. 1989; Kam-Morgan and Gill 1989). Secondly, many of the recessive or null alleles are not phenotypically distinct in a polyploid as they are masked by dominant, or active alleles at homoeologous loci in the other genomes (Sorrells 1992). Thirdly, in order to map a locus, variation must exist between two parents. Without any variation, the appropriate mapping populations cannot be developed. Several researchers have encountered low levels of DNA polymorphism in wheat (He et al. 1992; Joshi and Nguyen 1993).

The occurrence of three homoeologous genomes in wheat allows tolerance to the loss or addition of chromosomes (Sears 1954; Gale et al. 1989; Devos and

Gale 1993). In no other species has the buffering ability of homoeologous genomes been taken advantage of as it has in wheat. Sears (1954) developed various aneuploid series in the cultivar Chinese Spring. Aneuploidy is defined as an increase or decrease in chromosome number that does not involve entire chromosome sets (McIntosh 1987). One of the many uses of these lines is in determining the chromosomal location of genes (McIntosh 1987; Chao et al. 1989).

### 2.4.1 Determination of Chromosomal Location of Genes

Knowledge of chromosome location of gene loci allows breeders to predict the likelihood of advantageous or disadvantageous blocks of genes being transferred together in a segregating population (Fehr 1987; Poehlman and Sleper 1995). The availability of various aneuploid lines facilitates location of genes on chromosomes and mapping gene-to-centromere distances (McIntosh 1987). Most of the gene location work has been based on the original Chinese Spring monosomic series developed by Sears (1954). Since that time, several other Chinese Spring aneuploid sets such as ditelosomics and nulli-tetrasomics, or lines derived using aneuploids such as substitution lines have become available.

#### 2.4.1.1 $F_2$ Monosomic analysis

The first aneuploid plants developed by Sears (1954) were monosomic. A monosomic line has one chromosome missing. The remaining homologue to the missing chromosome is a structurally normal chromosome (Schulz-Shaeffer 1985).

Monosomics were chiefly obtained from haploids or from plants nullisomic for chromosome 3B which have reduced chromosome pairing at meiosis (Sears 1954). $F_2$ monosomic analysis involving segregating populations is commonly used for locating genes to chromosomes (Sears 1953; Kuspira and Unrau 1957; Sears 1969; Dyck and Kerber 1971; Kerber and Dyck 1973; Sanghi and Baker 1974). Genes can be located by making all twenty-one crosses to a monosomic series in a cultivar with the contrasting phenotype. The monosomics are used as female parents because 75 percent of the functioning megaspores contain 20-chromosomes (Sears 1953). However, on the male side only a small percentage, 3 percent, of 20-chromosome male gametes function in fertilization due to differential survival between 20- and 21-chromosome gametophytes during pollen-tube growth (Sears 1953) If the gene carried by the monosomic line involved is both dominant and hemizygous-effective, direct phenotypic observations on $F_1$ monosomic progenies will enable location of the gene.

A recessive allele in the monosomic parent may be located by examination of $F_2$ populations derived from selected monosomic $F_1$ plants. All $F_1$ hybrids will have the dominant phenotype. For 20 of the $F_2$ monosomic families the usual 3 dominant: 1 recessive phenotypic ratio will be obtained, whereas for the critical monosomic family $F_2$ ratios will deviate significantly from a 3 dominant: 1 recessive phenotypic ratio.

### 2.4.1.2 Substitution line analysis

Substitution lines can be used in the identification of chromosome location of genes of interest (Unrau et al. 1956; Kuspira and Unrau 1957; Law et al. 1987; McIntosh 1987). A substitution series consists of 21 different lines, each having one complete chromosome pair substituted from another variety (Unrau 1958). The purpose of substitution analysis is to identify the effect of the substituted chromosome on the phenotype of the recipient line (Yen and Baenzinger 1992). Identification of the critical chromosome only occurs if the substituted variety expresses a different allele than does the recipient variety. Substitution lines are true-breeding, but discrepancies can arise during development. Consequently, substitution series development should include duplicate lines and use genetic or cytogenetic markers (Person 1956) to maintain and verify the chromosome integrity of each substitution line.

Discrepancies in substitution line designation can arise if univalent shift occurred (Person 1956), if background variation existed in the original monosomic series (Endo and Gill 1984), if an insufficient number of back-crosses has been conducted (Berke et al. 1992), or if record-keeping errors have occurred. Singh and McIntosh (1984) found that CS(Ciano 3B) failed to carry *Sr2* which was known to be located on chromosome 3B. On the other hand, *Sr2* was present in CS(Ciano 5B), presumably due to chance transfer during back-crossing. Endo and Gill (1984) observed heteromorphic banding patterns in each of the common wheat cultivars Cheyenne, Wichita, and Chinese Spring used in the development of

substitution lines. Adding to the heterogeneity is that monosomic and the substitution lines are usually back-cross derived. Hence, no matter how many back-crosses are performed, there are always chances for factors other than those on the target chromosome to be transmitted from the donor cultivar (McIntosh 1987; Yen and Baenzinger 1992). For example, a sixth back-cross monosomic will continue to be heterozygous for approximately one percent of the genes by which it differed from the donor parent. Because each line of a substitution series is independently developed, there will be 20 opportunities for a nominated gene being present in one non-critical substitution line. Berke et al. (1992) observed significant differences for grain yield, seed weight, grain test weight, and anthesis date between duplicated substitution lines generally involving six back-crosses. Results of analysis of varietal substitution lines should not be considered conclusive evidence of the location of the gene because such back-cross lines are prone to incomplete genotype restoration (McIntosh 1987; Koebner et al. 1988; Yen and Baenzinger 1992). To identify if any deviation occurred during the development of a substitution line, duplicate lines should be run. Any deviation between the lines would suggest that deviations had occurred in the substituted chromosomes.

#### 2.4.1.3 Nullisomic analysis

The development of the original monosomic series by Sears (1954) allowed the development of other aneuploid series that could be used to determine gene

location. The most common are ditelosomics and nulli-tetrasomics. A ditelosomic line in hexaploid wheat is one that has two homologous telocentric chromosomes in addition to 20 normal chromosome pairs (Schulz-Schaeffer 1985). Compensating nulli-tetrasomics consist of the deletion of one chromosome pair and the subsequent compensation by an additional chromosome of the homoeologous series (Sears 1966). Ditelosomics and nulli-tetrasomics allow gene localization by expression of the nullisomic condition of the missing arm. Because ditelosomics breed true, they are also useful in checking for univalent shift in the development and maintenance of monosomic and substitution lines through the back-cross procedure (Person 1956; McIntosh 1987). Numerous loci have been located through the use of nulli-tetrasomic and ditelosomic lines (Benito and Perez de la Vega 1979; Koebner and Sheperd 1982; Chenicek and Hart 1987).

Nullisomic analysis, where the progeny of a selfed monosomic segregates into nullisomic, monosomic, and disomic plants, is based on the identification of nullisomic chromosomes (Sears 1953). Only nullisomics of the critical chromosome will have the non-parental phenotype.

## 2.5 DNA Markers

DNA-based genetic diagnostics are expected to play an increasingly important role in plant breeding (Rafalski et al. 1991). Previous to DNA-based diagnostics, protein and isozyme electrophoresis were commonly used in many crops, including *T. aestivum* (McMillin et al. 1986). The major limitation of these

techniques is insufficient polymorphism among closely related cultivars. Because proteins are the products of gene expression, they may vary in different tissues, at different developmental stages, and different environments (Beckman and Soller 1983). The ability to select nucleotide sequences closely linked to the trait of interest avoids many of the problems inherent for direct phenotypic selection. In addition, the number of loci that can be sampled using morphological and isozyme markers is much lower than that which can be sampled by DNA-based markers.

Phenotypic selection is advantageous in that recognition of the trait marks its absolute presence in that plant. However, if the trait under selection shows a high level of genotype by environmental interaction, accurate selection may be impossible. Phenotypic selection may also require expensive assays, involve complicated procedures, or have a limited window of opportunity for assessment. DNA-based markers solve many of these problems. DNA markers are not affected by genotype by environment interactions, as only the nucleotide sequence is being scored. Since DNA sequence is conserved within an organism, the type or age of tissue analyzed should not be a concern. A single DNA extraction usually allows as many traits to be scored as markers are available. Only a limited amount of tissue is required, therefore destructive sampling of the plant is eliminated.

The two main types of DNA markers are restriction fragment length polymorphisms (RFLPs) or the polymerase chain reaction (PCR)-based marker, random amplified polymorphic DNA (RAPDs).

### 2.5.1 RFLP Analysis

RFLPs are developed by the use of restriction endonucleases which cleave DNA molecules at specific sites and the detection of specific restriction fragments using Southern blots (Botstein et al. 1980; Beckman and Soller 1983). RFLPs are ideal markers for determining linkage for several reasons. RFLPs are co-dominant markers. This enables the identification of heterozygous genotypes, increasing the precision of linkage determination. Multiple allelic forms of the RFLP can be developed which facilitates selection.

However, RFLP analysis has several disadvantages which limit its effectiveness as a selection tool in breeding programs. RFLP analysis is technically difficult and expensive to perform. The DNA required must be of high quality and relatively large amounts are needed for each assay. Potentially hazardous radioisotopes are required to visualize results. Furthermore, informative probes are of necessity low-copy gDNA or cDNA clones. Consequently, the number of RFLP sites within the genome which can be screened may be limited. Consequently, the development and use of RFLP markers in wheat has been slowed because of limited polymorphism (Chao et al. 1989; Kam-Morgan and Gill 1989). Since polymorphism in hexaploid wheat is relatively rare for any given pair of lines, a large pool of clones will be required (Gale et al. 1989).

Many plant breeding laboratories are not equipped with the expertise or facilities necessary to handle radioisotopes, therefore, the use of RFLPs in breeding programs has been limited. Plant breeder's are looking towards DNA-

marker technologies that are cheaper, less technologically difficult, more amenable to large through-put and that do not use radioisotopes. Advances in PCR technology (Saiki et al. 1988) has provided selection techniques that address many of the limitations of RFLPs.

### 2.5.2 RAPD Analysis

Random amplified polymorphic DNA (RAPD) analysis is a PCR-based (Saiki et al. 1988; Yu et al. 1993) strategy to amplify short arbitrary stretches of DNA (100 to 4500 bp) (Rafalski et al. 1991; Quiros et al. 1991; Vierling and Nguyen 1992) from a target genome (Welsh and McClelland 1990; Williams et al. 1990; Caetano-Anolles et al. 1991). PCR amplification is a cyclical process that involves using a thermostable DNA polymerase directed by a short, usually 10-mer, oligonucleotide of arbitrary sequence to generate a characteristic spectrum of amplification products. The amplification products are usually resolved using agarose gels and visualized by the fluorescence of intercalated ethidium bromide under ultra-violet light. RAPD analysis has been applied in plants for construction of genetic maps (Quiros et al. 1991; Reiter et al. 1992), estimation of genetic relationships (He et al. 1992; Vierling and Nguyen 1992; Joshi and Nguyen 1993; Wilkie et al. 1993), tagging disease resistance traits (Barua et al. 1993; Penner et al. 1993b; Penner et al. 1993c; Timmerman et al. 1995), identification of cultivars (Demeke et al., 1993), identification of fungal isolates (Guthrie et al. 1992), and determination of parentage (Dweikat et al. 1993).

The application of the RAPD technique as a selection tool has several advantages in a plant breeding program (Williams et al. 1990). The use of arbitrary-sequence primers theoretically allows the whole genome to be surveyed. Typically, the PCR reaction does not require large quantities of high quality DNA. Simple DNA extraction procedures (Edwards et al. 1991) that do not require phenol and chloroform allow greater through-put of samples. Visualization of amplification fragments involves the intercalation of ethidium bromide and its fluorescence under ultra-violet light, thus avoiding the hazards associated with radioisotopes. Finally, only knowledge of the primer sequence and amplification product size is needed to define the RAPD fragment (Williams et al. 1991). Consequently, no exchange of samples is needed to reproduce the results.

However, the application of RAPD analysis has several disadvantages that limit its acceptance as a selection tool in a breeding program. A RAPD fragment can only identify the allele from which it was developed. Consequently, heterozygous individuals cannot be identified. Analysis of simulated and experimental data suggest that $F_2$ populations are not suitable for *de novo* construction of genetic maps consisting only of dominant markers (Williams and Rafalski, unpublished results cited in Williams et al. 1991). Plant breeder's have expressed concern about the reliability and reproducibility of RAPD markers both within, and among laboratories, and among different experimental crosses (Penner et al. 1993a; Kleinhofs et al. 1993). The drawbacks of RAPD fragments are that they are sensitive to reaction conditions, in particular, $Mg^{2+}$ concentration and

template-primer ratios (Kesseli et al. 1992), and thermocycling conditions (Penner et al. 1993a). Penner et al. (1993a) suggested the need to establish standard conditions so that RAPD results could be shared among laboratories.

There will always be a question whether RAPD bands derive from the same locus in different varieties (Devos and Gale 1992). Cross-hybridization of the product of interest with bands of similar molecular weight in the varietal RAPD profiles will provide strong evidence for allelism, but since both homologous and homoeologous sequences will generate positive signals in hexaploid wheat, segregation analysis will be needed to confirm the identity of RAPD bands (Devos and Gale 1992).

Various strategies for enriching DNA sources have been devised to facilitate the development of RAPD markers. Recombinant inbred lines (Reiter et al. 1992) and doubled haploid populations (Barua et al. 1993) are mapping populations which are most amenable to analysis. Other strategies for enriching the populations for the target trait include bulk segregant analysis (Michelmore et al. 1991), identifying DNA intervals from any segregating population (Giovannoni et al. 1991), and utilizing near-isogenic lines (Young et al. 1988).

The inability to detect adequate levels of DNA polymorphism in self-pollinating species such as wheat continues to be a problem. The complexity of patterns obtained with denaturant-gradient-gel-electrophoresis (DGGE) and temperature sweep gel electrophoresis (TSGE) suggests that a large number of different amplification fragments are contained within a single band resolved by

agarose gel electrophoresis (Dweikat et al. 1993; Penner and Betze 1994). Polyacrylamide gel electrophoresis incorporating a chemical denaturation gradient (He et al. 1992; Dweikat et al. 1993) or an increasing temperature gradient (Penner and Betze 1994) allowed separation of amplification fragments based both on fragment size and nucleotide sequence. Both methods significantly increased the ability to identify polymorphisms in cereals.

In order for RAPDs to be successfully utilized in a breeding program, the cost involved with each PCR reaction must be kept to a minimum. RAPD analysis has the potential for automation, integration, and standardization (Caetano-Anolles et al. 1991). These include automated DNA extraction, robotic manipulation of pre-amplification steps, PCR amplification, and post amplification analysis.

# 3. Location of a *Pyrenophora tritici-repentis* Died. Necrosis Resistance Gene to 5BL of *Triticum aestivum* L. cultivar Chinese Spring.

## 3.1 Abstract

Several sources of high-level resistance to tan spot caused by *Pyrenophora tritici-repentis* Died. have been identified in hexaploid wheat (*Triticum aestivum* L.). This study was conducted to determine the number and chromosome location of gene(s) in the cultivar Chinese Spring (CS) that confers resistance to a tan necrosis-inducing isolate ($nec^+chl^-$) of *P. tritici-repentis*, 86-124, and insensitivity to Ptr necrosis toxin. Reciprocal crosses were made between CS (resistant/insensitive) and Kenya Farmer (KF) (susceptible/sensitive). Analysis of the CS/KF $F_1$ and $F_2$ populations and $F_2$-derived $F_3$ families identified a single, nuclear, recessive gene governing resistance to isolate 86-124 and Ptr necrosis toxin. Evaluation of the CS(KF) substitution series, $F_2$ monosomic analysis, and screening of a series of 19 CS compensating nulli-tetrasomic and two ditelosomic lines (2AS and 5BL) indicated that the resistance gene was located on chromosome arm 5BL. No linkage was detected between *Lr*18 and the tan spot necrosis resistance gene on chromosome arm 5BL. It is proposed that the gene for resistance to the tan necrosis-inducing isolate 86-124 ($nec^+chl^-$) of *P. tritici-repentis* and Ptr necrosis toxin be named *tsn1*.

## 3.2 Introduction

Tan spot is a foliar disease of wheat caused by the ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoem. Tan spot has been identified throughout the major wheat growing regions of the world (Morrall and Howard 1975; Rees and Platz 1979; Hosford 1982; Krupinsky 1982; Wiese 1987; Kemp et al. 1990). Disease infection during the growing season may significantly reduce wheat yields (Tekauz et al. 1982; Hosford 1982) and grain grades (Vanterpool 1963; Sharp et al. 1976).

*P. tritici-repentis* overwinters on infected residue of wheat and other *gramineae* species (Krupinsky 1982). Increasing use of soil conservation practices has resulted in an increase of inoculum levels (Platz and Rees 1989) and a concomitant increase in disease severity (Schuh 1990). In 1982, severe seedling infections were observed for the first time in western Canada (Tekauz et al. 1982). There are currently no resistant cultivars registered for western Canada. Therefore, the significance of this pathogen is expected to increase.

The most sustainable method of minimizing the effect of tan spot is to incorporate high-level genetic resistance into commercial cultivars. To develop appropriate breeding strategies, the number of loci, their dominance relationships, gene interactions, and gene-linkages must be identified. In addition, knowledge of the chromosome location of important gene loci would allow breeders to predict the likelihood of advantageous or disadvantageous blocks of genes being transferred together in a segregating population (Fehr 1987; Poehlman and Sleper

1995). Substitution analysis (Unrau et al. 1956; Kuspira and Unrau 1957; Law et al. 1987; McIntosh 1987), $F_2$ monosomic analysis (Sears 1953; Kuspira and Unrau 1957; Sears 1969; Dyck and Kerber 1971; Kerber and Dyck 1973; Sanghi and Baker 1974), and evaluation of the nullisomic condition of various aneuploid lines (Benito and Perez de la Vega 1979; Koebner and Sheperd 1982; Chenicek and Hart 1987) can be used to identify the chromosome location of genes of interest.

*P. tritici-repentis* induces two distinct symptoms on susceptible wheat host plants: tan necrosis and/or chlorosis. Isolates of *P. tritici-repentis* collected on wheat in western Canada have been classified into four distinct pathotypes on the basis of their ability to induce tan necrosis and/or chlorosis on a differential set of wheat genotypes: pathotype 1 induces both tan necrosis and extensive chlorosis ($nec^+chl^+$) on susceptible host genotypes; pathotype 2 induces tan necrosis ($nec^+chl^-$) only; pathotype 3 induces extensive chlorosis ($nec^-chl^+$) only; and pathotype 4 is incapable of inducing either tan necrosis or chlorosis ($nec^-chl^-$) (Lamari and Bernier 1989b; Lamari and Bernier 1991). Identification of an isolate collected from eastern Algeria that induces only chlorosis ($nec^-chl^+$) but is distinct from isolates previously classified within pathotype 3 has lead to the recent reclassification of isolates into races (Lamari et al. 1995). Pathotypes 1, 2, 3, and 4 are now referred to as races 1, 2, 3 and 4, respectively. The new isolate from eastern Algeria is classified as race 5.

Genetic studies involving the interaction between the necrosis-inducing isolate 86-124 ($nec^+chl^-$) and hexaploid wheat cultivars have identified one or two

recessive genes for necrosis resistance, depending on the segregating population evaluated. Lamari and Bernier (1991) identified a single recessive gene segregating in $F_2$ populations of Glenlea/6B365 and Salamouni/Glenlea. Duguid (1995) suggested one or two recessive genes conferred resistance in $F_2$ and $F_2$-derived $F_3$ populations of BH1146/ST6, Katepwa/Erik, Katepwa/ST6, and Katepwa/ST15.

Ballance et al. (1989) purified and characterized a host-specific necrosis-inducing toxin that is only produced by necrosis-inducing isolates ($nec^+chl^+$ or $nec^+chl^-$). This toxin, termed Ptr necrosis toxin, induces tan necrosis on wheat cultivars that are susceptible to necrosis-inducing isolates (Lamari and Bernier 1989c).

The objectives of this study were (i) to determine the number of *P. tritici-repentis* tan necrosis resistance gene(s) possessed by the hexaploid wheat cultivar Chinese Spring (CS), (ii) to determine their chromosome location, and (iii) to evaluate linkage relationships between identified tan spot necrosis resistance gene(s) and a previously mapped locus.

## 3.3 Materials and Methods

### 3.3.1 Host Materials

Cultivars and lines used in this study were chosen based on their reaction to the *P. tritici-repentis* necrosis-inducing isolate 86-124 ($nec^+chl^-$), Ptr necrosis toxin, and to *Puccinia recondita* f.sp. *tritici* race 1. Host materials included:

(i) *T. aestivum* cultivars CS and Kenya Farmer (KF).

(ii) The complete CS(KF) substitution series supplied by Dr. R.A. McIntosh (University of Sydney Plant Breeding Institute, Cobbitty, N.S.W., Australia).

(iii) CS monosomic 5B (CS mono 5B) and CS monosomic 7D (CS mono 7D) supplied by Dr. E. Kerber (Agriculture and Agri-Food Canada, Winnipeg, MB).

(iv) The complete CS nulli-tetrasomic series with the exceptions of nulli-2A and nulli-5B supplied by Dr. K. Armstrong (Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa, ON).

(v) Various CS ditelosomics supplied by Dr. E. Kerber (Agriculture and Agri-Food Canada, Winnipeg, MB).

(vi) *P. recondita* f.sp. *tritici* isogenic line RL 6009 supplied by Dr. J. Kolmer (Agriculture and Agri-Food Canada, Winnipeg, MB).

All populations developed were forced to self-fertilize by covering individual spikes with glassine bags prior to anthesis. All spikes were harvested individually. The $F_2$-derived $F_3$ seed from the same plant was bulked.

### 3.3.2 Gene Number Study

The resistant cultivar, CS, was reciprocally crossed with the susceptible cultivar, KF. $F_1$, $F_2$, and $F_2$-derived $F_3$ families were generated. All material was evaluated for its reaction to the *P. tritici-repentis* necrosis-inducing isolate 86-124

($nec^{+}chl^{-}$) and Ptr necrosis toxin. Specific procedure described in 3.3.8.

### 3.3.3 Substitution Analysis

All 21 lines of the CS(KF) substitution series were evaluated for their reaction to the isolate 86-124 and Ptr necrosis toxin. Critical substitution lines were reciprocally crossed with CS to determine the number of tan necrosis resistance genes that reside on each chromosome. $F_1$, $F_2$, and $F_2$-derived $F_3$ families were developed for each cross. All material was evaluated with the isolate 86-124 and Ptr necrosis toxin.

### 3.3.4 $F_2$ Monosomic Analysis

$F_2$ monosomic analysis was conducted to verify the designation of critical lines identified by the substitution analysis. The following crosses were performed; (i) CS mono 5B/CS(KF 5B) and (ii) CS mono 7D/CS(KF 7D). The chromosome number of $F_1$ plants was determined cytologically by examining mitotic preparations of root-tip cells. Monosomic $F_1$ plants were selected and selfed to produce the $F_2$ generation. The $F_1$ plants and $F_2$ progenies were evaluated for their response to the isolate 86-124 and Ptr necrosis toxin. Four $F_2$ plants of the cross CS mono 7D/CS(KF 7D) were examined cytologically for aneuploidy. $F_2$ populations segregating for chromosome number would verify that the population had been developed from a $F_1$ monosomic plant.

### 3.3.5 Aneuploid Screening

To verify the $F_2$ monosomic analysis and the substitution analysis results, ditelosomic 2AS, ditelosomic 5BL, and the complete series of CS compensating nulli-tetrasomics, with the exceptions of lines nullisomic for chromosome 5B and chromosome 2A, were evaluated for their reaction to isolate 86-124 and/or Ptr necrosis toxin.

### 3.3.6 Nullisomic Analysis

Nullisomic analysis was conducted using CS mono 5B and CS mono 7D. Monosomic plants of both aneuploid lines were selfed to develop populations segregating for chromosome number. Disease reaction of individual seedlings from each population were evaluated by screening with Ptr necrosis toxin. All Ptr necrosis toxin sensitive seedlings and a sample of approximately 10 insensitive seedlings were cytologically examined to determine chromosome number.

### 3.3.7 Cytological Examination

The chromosome number of plants was determined by examining mitotic cells in root tip squashes. Root tips were collected from potted plants at the four to six leaf stage. Root-tips were harvested and placed immediately into pre-chilled (0 to 1°C) distilled water for 20 h. Root-tips were then blotted to remove surface water and placed in Farmer's solution (3:1, 95% ethanol: glacial acetic acid) for a minimum of 48 h at 4°C. Immediately prior to examination, root-tips were

hydrolysed in 1 N HCl for eight min at 60°C. Root-tips were stained with aceto-carmine for at least one h before examination. The root apical meristem was dissected onto a microscope slide in a drop of aceto-carmine, heated and squashed. The chromosome number was determined from at least two cells per root.

### 3.3.8 Inoculation procedure - *P. tritici-repentis* (86-124)

#### 3.3.8.1 Inoculum production

The inoculum of isolate 86-124 (obtained from Dr. L. Lamari, University of Manitoba, Winnipeg, MB) was produced as described by Lamari and Bernier (1989a). Infected leaf samples were placed in a Petri plate containing wet filter paper to maintain high humidity. The plates were incubated for 24 h at room temperature and a constant light intensity (61 μmol $m^{-2}$ $s^{-1}$) to promote conidiophore production. The plates were incubated 24 h in the dark at 15°C to produce conidia. Single conidia were transferred to V8-PDA medium (150 ml V8-juice, 10 g Difco potato-dextrose agar, 3 g $CaCO_3$, 10 g of Bacto agar, 850 ml of distilled water) and incubated at 20°C in the dark until the colony reached 3-4 cm in diameter. These cultures were then stored at 4°C and used as stock cultures for inoculum production.

The inoculum was produced on V8-PDA media. Small plugs, 0.5 cm in diameter, were transferred singly from the stock culture to 9 cm Petri plates, containing approximately 30 ml of V8-PDA media. The cultures were then

incubated at 20°C under continuous darkness to prevent conidiophore formation during mycelial growth. When the cultures were 3 to 4 cm in diameter, they were flooded with sterile distilled water and the mycelium flattened with the bottom of a sterile test tube. After the water was decanted, the cultures were subjected to a regime of 18-24 h of light (61 μmol $m^{-2}$ $s^{-1}$) at room temperature to induce formation of conidiophores, followed by 18-24 h of dark at 15°C to induce formation of conidia.

The conidia were harvested as described by Lamari and Bernier (1989a). The culture plates were flooded with sterile distilled water and the conidia were dislodged with a wire loop. One additional water rinse was made to suspend and recover the conidia that had settled. The spore concentration was measured with a haemocytometer (Hausser Scientific 3720, Blue Bell, Pa) and adjusted with sterile distilled water to approximately 3000 conidia $ml^{-1}$. Ten drops of Tween 20 (polyoxyethylene sorbitan monolaurate) were added per litre of conidial suspension to reduce surface tension.

Purified Ptr necrosis toxin was produced from cultures of 86-124 and processed as described by Ballance et al. (1989) and Lamari and Bernier (1989c).

### 3.3.8.2 Inoculation procedure

Ten plants of each cultivar; Erik (resistant to isolate 86-124/insensitive to Ptr necrosis toxin) and Katepwa (susceptible to isolate 86-124/sensitive to Ptr necrosis toxin) and the appropriate parental populations were included as checks

for every 50 plants of any population tested for response to isolate 86-124 or Ptr necrosis toxin.

Seedlings were inoculated with a conidial suspension of the isolate 86-124 as described by Lamari and Bernier (1989b). A DeVilbis sprayer, operated at a pressure of approximately 67 KPa, was used to spray the conidial suspension onto seedlings at the two-leaf stage until run-off. After inoculation the seedlings were placed in a growth room with a 22°/17°C (day/night) temperature regime and a 16 h photoperiod. During the initial 24 h after inoculation, the seedlings were kept at 100 percent relative humidity to insure continuous leaf wetness. This was accomplished by covering the plants with a polyethylene tent and maintaining the humidity level with an ultra-sonic humidifier.

Three to four days after inoculation, the third leaf of all plants was infiltrated with approximately 30 µl of purified Ptr necrosis toxin at a concentration of $1x10^{-3}$ mg $ml^{-1}$ using a Hagborg Device (Hagborg 1970) as described by Lamari and Bernier (1989c).

### 3.3.8.3 Disease rating

Inoculated plants were rated for disease reaction seven to eight days after inoculation. The five-level numerical system based on lesion type described by Lamari and Bernier (1989a) was used to categorize host reactions to isolate 86-124 (Appendix 7.1). The lesion type on the second leaf was rated. Plants with lesion types of 1 or 2 were classed as resistant, while plants with lesion types of

3, 4 or 5 were classed as susceptible. At the same time, plants were rated for the presence (sensitive) or absence (insensitive) of a necrotic lesion at the site of Ptr necrosis toxin infiltration.

### 3.3.9 Linkage with *Lr18*

To initiate this study, CS, KF, CS(KF 5B), and *Lr18* isogenic line RL 6009 were evaluated for their response to Ptr necrosis toxin and to *P. recondita* f.sp. *tritici* race 1. Reciprocal crosses were conducted between CS(KF 5B) and RL 6009. $F_2$-derived $F_3$ families were developed and evaluated for their response to the isolate 86-124 and/or Ptr necrosis toxin. $F_2$-derived $F_3$ families were also evaluated for their response to *P. recondita* f.sp. *tritici* race 1.

#### 3.3.9.1 Inoculation procedure - *P. recondita* f.sp. *tritici* race 1

$F_2$-derived $F_3$ families were planted in a soil bed (2 soil: 1 sand: 1 peat) in a greenhouse. Eighteen to 23 plants per family were sown in 23 cm long rows 13 cm apart. Temperatures were maintained between 15 and 18°C with a photoperiod of 18 h day/ 6 h night. Supplemental light was provided by High-bar sodium lights (30 $\mu$mol $m^{-2}$ $s^{-1}$). Urediospores of *P. recondita* f. sp. *tritici* race 1 were obtained from Dr. J. Kolmer (Agriculture and Agri-Food Canada, Winnipeg, MB). Plants were inoculated when the first leaf was fully expanded. Twenty h prior to inoculation, the rust inoculum ampoule was opened from its vacuum seal and kept at 4°C to allow the urediospores to rehydrate. Immediately prior to

inoculation, the seedlings were misted with a solution of distilled water and Tween 20 (ten drops of Tween 20 per litre of distilled water) to increase contact between the leaf surface and the fungal spore. The seedlings were then dusted with a mixture of talcum and inoculum using a 'Speed-Spray' double-bellows bulb inoculator. Once the seedlings were inoculated, they were covered with polyethylene plastic to create a humidity chamber. Three ultra-sonic humidifiers were used to maintain high relative humidity levels for 20 h. Thirteen days after inoculation, disease reaction of individual $F_2$-derived $F_3$ plants were rated according to Stakman et al. (1962) (Appendix 7.2). Plants with infection types 0, ;, 1 or 2 were classified as resistant, while those with infection types 3 or 4 were classified as susceptible.

### 3.3.10 Statistical Analysis

Tests for homogeneity were conducted to determine which segregating populations could be pooled (Little and Hills 1977). Chi square analyses were used to determine goodness-of-fit of the data to the proposed models for gene number (Strickberger 1985). Segregation analysis, tests of the significance of linkage, and the estimation of the recombination frequencies between linked loci in the $F_2$-derived $F_3$ families were performed using JOINMAP (Stam 1993).

### 3.4 Results and Discussion

Characterization of a *P. tritici-repentis* necrosis resistance gene in CS was accomplished by screening all experimental material with the isolate 86-124 ($nec^{+}chl^{-}$) and/or the host-specific Ptr necrosis toxin. CS and the check cultivar, Erik, were highly resistant to the isolate 86-124 with reaction types of 1 or 2 and insensitive to Ptr necrosis toxin. KF and the check cultivar, Katepwa, were highly susceptible to the isolate 86-124 with a reaction type of 5 and sensitive to Ptr necrosis toxin. Since the toxin used in this study was produced from isolate 86-124, it is assumed that genes that confer resistance to 86-124 also confer resistance to Ptr necrosis toxin. Analysis of $F_1$ plants, and $F_2$, and $F_2$-derived $F_3$ families from the reciprocal cross CS/KF indicated that resistance to the isolate 86-124 and Ptr necrosis toxin was conditioned by a single, nuclear, recessive gene (Table 3.1).

Substitution analysis was conducted to determine which chromosome carried the resistance locus. Analysis of eight to ten plants of each CS/KF substitution line suggested that chromosomes 5B and 7D conferred different levels of resistance in CS to the isolate 86-124 and Ptr necrosis toxin (Table 3.2). CS(KF 5B) amd CS(KF 7D) produced reaction types of 5 and 3 or 4, respectively. Both substitution lines were sensitive to Ptr necrosis toxin. All other CS(KF) substitution lines were resistant to isolate 86-124, producing a reaction type of 1 or 2, and were insensitive to Ptr necrosis toxin (Table 3.2)

The reciprocal crosses CS/CS(KF 5B) and CS/CS(KF 7D) indicated that the

**Table 3.1.** Response to isolate 86-124 and Ptr necrosis toxin of $F_1$, $F_2$ and $F_2$-derived $F_3$ families from three crosses: CS/KF, CS/CS(KF 5B) and CS/CS(KF 7D)

| | No. of $F_1$ plants | | No. of $F_2$ plants | | $F_2$ | No. of $F_2$-derived $F_3$ families | | | $F_3$ |
|---|---|---|---|---|---|---|---|---|---|
| Cross | S[a] | R[b] | S | R | P(3:1)[c] | HS[d] | Seg.[e] | HR[f] | P(1:2:1)[c] |
| CS/KF | 5 | 0 | 119 | 37 | 0.78 | 19 | 48 | 19 | 0.65 |
| CS/CS(KF 5B) | 6 | 0 | 143 | 58 | 0.24 | 13 | 24 | 14 | 0.96 |
| CS/CS(KF 7D) | 4 | 0 | 78 | 26 | 0.92 | 8 | 25 | 15 | 0.46 |

[a] S = Susceptible to 86-124 and sensitive to Ptr necrosis toxin.

[b] R = Resistant to 86-124 and insensitive to Ptr necrosis toxin.

[c] Chi square probability values greater than 0.05 indicate that the data did not differ significantly from the expected ratio.

[d] HS = Homozygous susceptible to 86-124 and homozygous sensitive to Ptr necrosis toxin.

[e] Seg. = Segregating for response to 86-124 and Ptr necrosis toxin.

[f] HR = Homozygous resistant to 86-124 and homozygous insensitive to Ptr necrosis toxin.

resistance on each chromosome was conditioned by a single, nuclear, recessive gene (Table 3.1). These results were unexpected as they did not correlate with the initial finding that only a single, recessive gene governs resistance to the isolate 86-124 in the cross CS/KF (Table 3.1). Therefore $F_2$ monosomic analysis of the CS mono 5B/CS(KF 5B) and CS mono 7D/CS(KF 7D) were required to verify the designations of CS(KF 5B) and CS(KF 7D).

$F_2$ monosomic analysis conclusively identified a tan spot necrosis resistance gene on chromosome 5B of CS. Ten monosomic $F_1$ plants from the cross CS mono 5B/CS(KF 5B) and eight monosomic $F_1$ plants from the cross CS mono 7D/CS(KF 7D) were self-fertilized to develop the $F_2$ generations. Monosomic $F_1$ plants from both crosses were susceptible to Ptr necrosis toxin. The $F_2$ progenies from the cross CS mono 5B/CS(KF 5B) were all susceptible to the isolate 86-124 and Ptr necrosis toxin (combined ratio: 199:0) indicating that a resistance gene resides on chromosome 5B of CS (Table 3.3). The $F_2$ progeny from each of eight selfed monosomic CS mono 7D/CS(KF 7D) $F_1$ plants segregated 3 susceptible/insensitive : 1 resistant/insensitive (combined ratio: 121:36 $P>0.9$). Cytological examination of four CS mono 7D/CS(KF 7D) $F_2$ plants identified aneuploids, confirming that they had been properly derived from a monosomic $F_1$. Therefore, a tan spot necrosis resistance gene does not lie on chromosome 7D of CS.

There are several possible explanations why the substitution analysis identified a resistance gene on chromosome 7D of CS yet could not be verified

**Table 3.2. Response of the CS(KF) substitution lines to *P. tritici-repentis* isolate 86-124 and Ptr necrosis toxin**

| Substitution Line | No. Plants Screened | Reaction Type[1] | Ptr Necrosis Toxin[2] | Disease Classification |
|---|---|---|---|---|
| CS | 10 | 1 - 2 | - | resistant |
| KF | 10 | 5 | + | susceptible |
| 1A | 10 | 1 - 2 | - | resistant |
| 2A | 10 | 1 - 2 | - | resistant |
| 3A | 10 | 1 - 2 | - | resistant |
| 4A | 10 | 1 - 2 | - | resistant |
| 5A | 9 | 1 - 2 | - | resistant |
| 6A | 9 | 1 - 2 | - | resistant |
| 7A | 9 | 1 - 2 | - | resistant |
| 1B | 10 | 2 | - | resistant |
| 2B | 10 | 1 - 2 | - | resistant |
| 3B | 10 | 1 - 2 | - | resistant |
| 4B | 10 | 2 | - | resistant |
| 5B | 10 | 5 | + | susceptible |
| 6B | 10 | 1 - 2 | - | resistant |
| 7B | 10 | 1 - 2 | - | resistant |
| 1D | 10 | 1 - 2 | - | resistant |
| 2D | 8 | 1 - 2 | - | resistant |
| 3D | 9 | 1 - 2 | - | resistant |
| 4D | 10 | 1 - 2 | - | resistant |
| 5D | 8 | 2 | - | resistant |
| 6D | 8 | 1 - 2 | - | resistant |
| 7D | 10 | 3 - 4 | + | susceptible |

[1] Lesion types 1 and 2 denote resistance; lesion types 3, 4, and 5 denote susceptibility.

[2] - = host insensitivity to Ptr necrosis toxin; + = host sensitivity to Ptr necrosis toxin

**Table 3.3. Segregation analysis for disease reaction in monosomic $F_2$ progenies from crosses CS mono 5B/CS(KF 5B) and crosses of CS mono 7D/CS(KF 7D). $F_2$ progenies of crosses of CS mono 7D/CS(KF 7D) have been confirmed to segregate for chromosome number**

| Cross[1] | $F_1$ | $F_2$ | | Ratio | |
|---|---|---|---|---|---|
| | | Susc. | Res. | susc : res | *P* |
| CS mono 5B/CS(KF 5B) (1) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (2) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (3) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (4) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (5) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (6) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (7) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (8) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (9) | susc. | 20 | 0 | 1:0 | 1.0 |
| CS mono 5B/CS(KF 5B) (10) | susc. | 19 | 0 | 1:0 | 1.0 |
| CS mono 7D/CS(KF 7D) (1) | susc. | 11 | 8 | 3:1 | 0.16 |
| CS mono 7D/CS(KF 7D) (2) | susc. | 16 | 3 | 3:1 | 0.47 |
| CS mono 7D/CS(KF 7D) (3) | susc. | 16 | 4 | 3:1 | 0.79 |
| CS mono 7D/CS(KF 7D) (4) | susc. | 16 | 4 | 3:1 | 0.79 |
| CS mono 7D/CS(KF 7D) (5) | susc. | 15 | 5 | 3:1 | 1.00 |
| CS mono 7D/CS(KF 7D) (6) | susc. | 15 | 4 | 3:1 | 0.81 |
| CS mono 7D/CS(KF 7D) (7) | susc. | 16 | 4 | 3:1 | 0.79 |
| CS mono 7D/CS(KF 7D) (8) | susc. | 16 | 4 | 3:1 | 0.79 |

[1] CS monosomic 5B and CS monosomic 7D = resistant to 86-124 and insensitive to Ptr necrosis toxin.

CS(KF 5B) and CS(KF 7D) = susceptible to isolate 86-124 and sensitive to Ptr necrosis toxin.

**Table 3.4. Reaction of CS nulli-tetrasomics and ditelosomics to isolate 86-124 and/or Ptr necrosis toxin**

| Aneuploid Line | Reaction Type | Ptr Necrosis Toxin | Disease Rating |
|---|---|---|---|
| CS | 1 to 2 (20)[1] | - (40)[2] | resistant |
| N1A-T1B | 1 to 2 (6) | - (10) | resistant |
| Ditelo 2AS | 1 to 2 (6) | - (6) | resistant |
| N3A-T3B | 2 (6) | - (8) | resistant |
| N4A-T4D | 1 to 2 (5) | - (7) | resistant |
| N5A-T5B | 1 to 2 (6) | - (10) | resistant |
| N6A-T6B | 1 to 2 (6) | - (9) | resistant |
| N7A-T7B | 2 (5) | - (9) | resistant |
| N1B-T1D | 2 (6) | - (10) | resistant |
| N2B-T2D | 1 to 2 (6) | - (10) | resistant |
| N3B-T3D | 1 (5) | - (9) | resistant |
| N4B-T4D | 1 (1) | - (6) | resistant |
| Ditelo 5BL | 2 (6) | - (9) | resistant |
| N6B-T6D | 1 to 2 (2) | - (4) | resistant |
| N7B-T7D | 2 (4) | - (7) | resistant |
| N1D-T1A | 1 (5) | - (9) | resistant |
| N2D-T2A | 1 (6) | - (10) | resistant |
| N3D-T3A | 2 (6) | - (10) | resistant |
| N4D-T4A | 1 to 2 (6) | - (10) | resistant |
| N5D-T5A | n/a (0) | - (3) | resistant |
| N6D-T6A | 1 to 2 (5) | - (7) | resistant |
| N7D-T7A | 1 to 2 (5) | - (10) | resistant |

[1] The value in parenthesis indicates the number of plants evaluated for reaction to isolate 86-124. These plants were also screened with Ptr necrosis toxin and the results are included in the Ptr necrosis toxin column.

[2] The value in parenthesis indicates the total number of plants evaluated for reaction to Ptr necrosis toxin.

through $F_2$ monosomic analysis. Discrepancies in substitution line designation have been attributed to univalent shifts (Person 1956), background variation in the original monosomic line (Endo and Gill 1984), insufficient number of back-crosses (Berke et al. 1992), or if human error occurred during either the development or subsequent maintenance of the substitution series. Since the reaction of CS(KF 7D) to 86-124 (lesion type 3 or 4) is different from that of KF (lesion type 5), it is possible that contamination of seed stocks may have occurred. Further work would be required to identify the reasons for the discrepancy.

Homozygous null alleles for *P. tritici-repentis* necrosis resistance should result in susceptibility of CS to the isolate 86-124 and Ptr necrosis toxin. Therefore, screening various ditelosomics and nulli-tetrasomics should identify chromosomes carrying resistance genes to necrosis-inducing isolates. Screening of the available CS nulli-tetrasomics and ditelosomics, did not identify any susceptible lines (Table 3.4). This infers that the resistance gene in CS must either lie on chromosome arm 5BL or chromosome arm 2AS as these were the only chromosome arms for which disease reaction was not evaluated.

The progeny of selfed CS monosomic lines for the critical chromosome carrying the necrosis resistance gene should segregate for disease resistance (Sears 1953). Since other evidence indicated that a gene for resistance was located on either 5B or 7D, CS mono 5B plants and CS mono 7D plants were selfed and the progeny evaluated for response to Ptr necrosis toxin. While the progeny from selfed CS monosomic 5B plants segregated for disease resistance,

progeny from selfed CS monosomic 7D plants did not. The progeny from one selfed CS monosomic 5B plant was examined cytologically to determine if susceptible plants were nullisomic. Of 100 plants screened with Ptr necrosis toxin, seven were susceptible to Ptr necrosis toxin. All seven plants were confirmed nullisomics (Table 3.5). Twelve resistant plants were examined cytologically, revealing nine monosomic and three disomic plants (Table 3.5).

Linkage analysis was conducted between the *P. tritici-repentis* necrosis resistance gene and the *P. recondita* f.sp. *tritici* resistance gene *Lr18* (McIntosh 1983) both located on chromosome 5BL. CS(KF 5B) and RL 6009 produce contrasting reactions to *P. tritici-repentis* and *P. recondita* f.sp. *tritici*. CS(KF 5B) is sensitive to Ptr necrosis toxin and susceptible to *P. recondita* f.sp. *tritici* race 1 with an rust infection type of 4. RL 6009 is insensitive to Ptr necrosis toxin and resistant to *P. recondita* f.sp. *tritici* race 1 with an rust infection type of 1 or 2. 206 $F_2$-derived $F_3$ families generated from the reciprocal cross CS(KF 5B)/RL 6009 were screened with *P. recondita* f.sp. *tritici* race 1, identifying 43 homozygous susceptible families, 106 that were segregating, and 57 that were homozygous resistant. This fit a 1:2:1 ratio (P=0.35) indicating that a single gene conferred resistance to *P. recondita* f.sp. *tritici* race 1 (Table 3.6). When the same families were tested for response to Ptr necrosis toxin, 52 families were homozygous sensitive, 116 were segregating, and 38 were homozygous insensitive. This fit a 1:2:1 ratio (P=0.08) indicating that a single gene conferred resistance to Ptr necrosis toxin (Table 3.6). When results were combined, the population

**Table 3.5. Chromosome number and Ptr necrosis toxin sensitivity of progeny from a selfed CS mono 5B plant**

| Plant Number | Chromosome Number | No. of Cells Identified | Ptr Toxin Reaction |
|---|---|---|---|
| A1 | 2n=41 | 5 | - |
| A2 | 2n=41 | 5 | - |
| A3 | 2n=41 | 2 | - |
| A4 | 2n=41 | 4 | - |
| A5 | 2n=42 | 2 | - |
| A6 | 2n=41 | 3 | - |
| A7 | 2n=42 | 3 | - |
| A8 | 2n=42 | 4 | - |
| A9 | 2n=41 | 2 | - |
| A23 | 2n=41 | 7 | - |
| A44 | 2n=41 | 3 | - |
| A12 | 2n=41 | 7 | - |
| A19 | 2n=40 | 4 | + |
| A10 | 2n=40 | 5 | + |
| A33 | 2n=40 | 8 | + |
| A34 | 2n=40 | 2 | + |
| A40 | 2n=40 | 3 | + |
| A15 | 2n=40 | 3 | + |
| A57 | 2n=40 | 3 | + |

**Table 3.6. Chi-square goodness-of-fit test for one gene segregating in the $F_2$-derived $F_3$ generation for *P. recondita* f.sp. *tritici resistance* (*Lr18*) and for resistance to Ptr necrosis toxin**

| | $F_2$ derived $F_3$ Families | | | | |
|---|---|---|---|---|---|
| Gene | Susc. | Seg. | Res. | $X^2$ (1:2:1) | P |
| *Lr18* | 43 | 106 | 57 | 2.1 | 0.35 |
| *tsn1* | 52 | 116 | 38 | 5.1 | 0.08 |

segregated into a 9 Ptr sensitive/Lr resistant : 3 Ptr sensitive/Lr susceptible : 3 Ptr insensitive/Lr resistant : and 1 Ptr insensitive/Lr susceptible genetic ratio (chi-square=6.40, P=0.10) (Table 3.7) suggesting independent segregation of the two gene loci. Analysis of the results with JOINMAP (Stam 1993) revealed no linkage between the gene loci. Therefore, even though both loci are mapped to the same chromosome arm, they must be more than 50 map units apart.

Three virulent pathotypes of *P. tritici-repentis* have been identified among isolates collected from western Canada (Lamari and Bernier 1989b). This study only investigated resistance to the necrosis-inducing component of *P. tritici-repentis* as represented by the isolate 86-124 ($nec^+chl^-$). Consequently, to develop durable, high-level resistance to the pathogen, resistance to the chlorosis-inducing component must also be addressed.

The results obtained reiterated the observation of Yen and Baenzinger (1992) that the identification of chromosome location of gene loci should not rely solely on the results of substitution analysis. $F_2$ monosomic analysis and/or screening of various aneuploid lines should also be conducted to substantiate results obtained. Even though chromosome 2AS was not evaluated for response to the disease, the summation of the results obtained indicated high level resistance to the *P. tritici-repentis* necrosis-inducing isolate 86-124 and the Ptr necrosis toxin is governed by a single, nuclear, recessive gene located on chromosome arm 5BL. This resistance gene was not linked to the leaf rust resistance gene, *Lr18*, which has been previously mapped to chromosome arm

**Table 3.7. Summary of the phenotypic classifications observed of the 206 $F_2$-derived $F_3$ families of the reciprocal cross CS(KF 5B)/RL 6009**

| Genotype[1] | Observed (no.) | Predicted (no.)[2] | Chi-square |
|---|---|---|---|
| *Tsn1_Lr18_* | 105 | 115.8 | 1.02 |
| *Tsn1_lr18lr18* | 49 | 38.6 | 2.78 |
| *tsn1tsn1Lr18_* | 44 | 38.6 | 0.75 |
| *tsn1tsn1lr18lr18* | 8 | 12.9 | 1.85 |
| total | 206 | 205.9 | 6.40(0.10) |

[1] *Tsn1_Lr18_* = sensitive to Ptr necrosis toxin, resistant to Race 1

*Tsn1_lr18lr18* = sensitive to Ptr necrosis toxin, susceptible to Race 1

*tsn1tsn1Lr18_* = insensitive to Ptr necrosis toxin, resistant to Race 1

*tsn1tsn1lr18lr18* = insensitive to Ptr necrosis toxin, susceptible to Race 1

[2] Number predicted based on the following ratio: 9 *Tsn1_Lr18_* : 3 *Tsn1_lr18lr18* :3 *tsn1tsn1Lr18_* : 1*tsn1tsn1lr18lr18*.

[3] Values in parenthesis indicates the chi square probability. Chi square probability values greater than 0.05 indicate that the data did not differ significantly from the expected ratio.

5BL (McIntosh 1983).

This study represents the first localization of a *P. tritici-repentis* resistance gene to a specific wheat chromosome. It is proposed that this gene be named *tsn1* according to the recommended rules for gene symbolization in wheat (McIntosh 1988).

# 4. A Random Amplified Polymorphic DNA (RAPD) Linked to a Tan Spot (*Pyrenophora tritici-repentis* Died.) Necrosis Resistance Gene on 5BL of Chinese Spring (*Triticum aestivum* L.)

## 4.1 Abstract

A recessive gene (*tsn1*) for resistance to tan spot, induced by *Pyrenophora tritici-repentis* Died. isolate 86-124 ($nec^{+}chl^{-}$), has been identified on chromosome arm 5BL of the hexaploid wheat (*Triticum aestivum* L.) cultivar Chinese Spring (CS). This study was conducted to identify a random amplified polymorphic DNA (RAPD) marker for this gene. The genomic DNA of CS (resistant) and Kenya Farmer (KF) (susceptible) were screened with 420 arbitrary sequence 10-mer primers. Since low levels of genomic polymorphism (10.6 percent) were resolved with agarose gel electrophoresis, a sub-set of 74 primers was analyzed using temperature sweep gel electrophoresis (TSGE). TSGE resolved 73 genomic polymorphisms. Two polymorphisms specific for chromosome 5B sequences were identified using CS and CS(KF 5B). Linkage analysis was conducted using a single plant from each of 65 $F_2$-derived $F_3$ families from the reciprocal crosses CS/KF and CS/CS(KF 5B). Each $F_2$-derived $F_3$ family was homogeneous for disease reaction to both the isolate 86-124 and Ptr necrosis toxin. Linkage analysis identified that UBC195 and UBC102 were 23.4 $\pm$ 5.7 cM and 27.4 $\pm$ 6.9 cM from the resistance gene, respectively. UBC195 and UBC102 were tightly linked, 4.2 $\pm$ 2.5 cM to each other. Potential applications of a *P. tritici-repentis* necrosis resistance marker in wheat are discussed.

## 4.2 Introduction

Tan spot is a foliar fungal disease of wheat caused by the ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs., anamorph *Drechslera tritici-repentis* (Died.) Shoem. which has world-wide distribution (Morrall and Howard 1975; Rees and Platz 1979; Hosford 1982; Krupinsky 1982; Wiese 1987; Kemp et al. 1990). *P. tritici repentis* overwinters and grows saprophytically on infected residue of wheat and other *gramineae* species (Krupinsky 1982). Increasing use of conservation tillage systems that retain crop residues on the soil surface has resulted in an increase of inoculum levels (Platz and Rees 1989) and a subsequent increase in disease severity (Schuh 1990). Disease infection during the growing season may result in significantly reduced wheat yields (Tekauz et al. 1982) and grain grades (Vanterpool 1963; Sharp et al. 1976).

Most commercial cultivars of hexaploid wheat grown in western Canada are susceptible to tan spot. In 1982, severe infections at the seedling stage were observed for the first time in western Canada (Tekauz et al. 1982). Use of reduced tillage systems is expected to increase in the future. Therefore, until resistant cultivars are developed, the incidence of tan spot is expected to increase.

*P. tritici-repentis* induces two distinct symptoms on susceptible wheat host plants: tan necrosis and/or chlorosis. Isolates of *P. tritici-repentis* collected on wheat in western Canada have been classified into four distinct pathotypes on the basis of their ability to induce tan necrosis and/or chlorosis on a differential set of wheat genotypes: pathotype 1 induces both tan necrosis and extensive chlorosis

($nec^+chl^+$) on susceptible host genotypes; pathotype 2 induces tan necrosis ($nec^+chl^-$) only; pathotype 3 induces extensive chlorosis ($nec^-chl^+$) only; and pathotype 4 is incapable of inducing either tan necrosis or chlorosis ($nec^-chl^-$) (Lamari and Bernier 1989b; Lamari and Bernier 1991). Identification of an isolate collected from eastern Algeria that induces only chlorosis ($nec^-chl^+$) but is distinct from isolates previously classified within pathotype 3 has lead to the recent reclassification of isolates into races (Lamari et al. 1995). Pathotypes 1, 2, 3, and 4 are now referred to as races 1, 2, 3 and 4, respectively. The new isolate from eastern Algeria is classified as race 5.

Genetic studies involving the interaction between the isolate 86-124 and hexaploid wheat cultivars have identified one to two recessive genes for necrosis resistance, depending on the segregating population evaluated. Lamari and Bernier (1991) identified a single recessive gene segregating in $F_2$ populations of Glenlea/6B365 and Salamouni/Glenlea. Duguid (1995) suggested that one or two recessive genes confer resistance in segregating $F_2$ and $F_2$-derived $F_3$ populations of BH1146/ST6, Katepwa/Erik, Katepwa/ST6, and Katepwa/ST15. A single, nuclear, recessive resistance gene on chromosome arm 5BL of CS was identified previously (Chapter 3).

Ballance et al. (1989) purified and characterized a host-selective necrosis-inducing toxin that is only produced by *P. tritici-repentis* isolates capable of inducing tan necrosis ($nec^+chl^+$ or $nec^+chl^-$). This toxin, termed Ptr necrosis toxin, induces tan necrosis on wheat genotypes that are susceptible to tan necrosis

(Lamari and Bernier 1989c).

Williams et al. (1990) demonstrated the use of single arbitrary primers to amplify DNA utilizing the polymerase chain reaction (PCR). This technique, termed random amplified polymorphic DNA (RAPD) analysis, can be used to identify polymorphisms useful in the development of genetic markers (Martin et al. 1991; Paran et al. 1991; Miklas et al. 1993; Penner et al. 1993b; Penner et al. 1993c; Timmerman et al. 1995). These studies utilized RAPD analysis in combination with agarose gel electrophoresis. However, for studies involving hexaploid wheat, the frequency of polymorphisms detected with this approach was too low to allow identification of markers in a reasonable time frame (Devos and Gale 1993). Penner and Betze (1994) described a denaturing acrylamide electrophoresis system termed temperature sweep gel electrophoresis (TSGE) that incorporated an increasing temperature gradient as an alternative method to agarose gels for increasing the resolution of randomly amplified polymorphic DNA.

A RAPD marker would have several applications in a plant breeding program. First, it could be used to facilitate introgression of resistance genes into adapted genotypes by back-crossing. Secondly, it could be used to differentiate between resistance genes with similar expressions. Finally, a tightly linked marker may be used in marker assisted selection.

The objective of this study was to identify a random amplified polymorphic DNA (RAPD) marker for the *P. tritici-repentis* necrosis resistance gene, *tsn1*, previously identified on chromosome arm 5BL of Chinese Spring (CS).

## 4.3 Materials and Methods

### 4.3.1 Plant Populations

Cultivars and lines used in this study were chosen based on their reaction to the *P. tritici-repentis* necrosis-inducing isolate 86-124 ($nec^+chl^-$) and to Ptr necrosis toxin. Reciprocal crosses were conducted between (i) CS (resistant/insensitive) and KF (susceptible/sensitive), and (ii) CS and CS(KF 5B) (susceptible/sensitive). CS(KF 5B) was obtained from Dr. R.A. McIntosh (University of Sydney, Plant Breeding Institute, Cobbitty, Cobbitty Road, N.S.W., Australia). Material was advanced to the $F_2$-derived $F_3$ generation by selfing individual plants. $F_2$-derived $F_3$ families were evaluated for response to isolate 86-124 and reaction to Ptr necrosis toxin. A single plant from each of 65 $F_2$-derived $F_3$ families homogeneous for disease reaction was used to estimate linkage.

### 4.3.2 Inoculum Production

Inoculum of the isolate 86-124 (obtained from Dr. L. Lamari, University of Manitoba, Winnipeg, MB.) was produced as described by Lamari and Bernier (1989a) (See section 3.3.8.1).

### 4.3.3 Inoculation Procedure

All the plant material was inoculated with a conidial suspension of the isolate 86-124 and injected with Ptr necrosis toxin, as described by Lamari and Bernier (1989b) (See section 3.3.8.2). Ptr necrosis toxin was produced from the

isolate 86-124 (Ballance et al. 1989). A Hagborg device (Hagborg 1970) was used to infiltrate a single leaf of each $F_2$ derived-$F_3$ seedling with approximately 30 µl of purified Ptr necrosis toxin at a concentration of 1 x $10^{-3}$ mg $ml^{-1}$.

### 4.3.4 Disease Rating

All material was rated for disease reaction using the five-level numerical scale describing lesion types (Lamari and Bernier 1989a) (Appendix 7.1) and Ptr necrosis toxin sensitivity (Lamari and Bernier 1989c) (See section 3.3.8.3)

### 4.3.5 RAPD Analysis

Initially genomic DNA of CS and KF were screened using RAPD analysis (Williams et al. 1990) to identify genomic polymorphisms. Once a polymorphism was identified, CS(KF 5B) was included to determine its specificity to chromosome 5B. Genomic DNA was extracted using the method of McCouch et al. (1988). In this study 420 primers were screened. UBC primers 1 through 300 were obtained from Dr. J. Carlson (University of British Columbia, Vancouver, Canada). Primer sets OPC 1-14; OPD 1-18; OPE 1-18; OPI 1-18; OPJ 1-18; OPN 1-20; OPO 1-12, 13, 16 were obtained from Operon Technologies, Inc. (Alameda, Calf., USA). All primers were 10-mers of arbitrary sequence with no palindromes and a G/C content of between 50 and 80 percent. PCR reactions were performed in a volume of 25 µl over-layered with 20 µl of light mineral oil (Fisher). Each reaction consisted of 1X *Taq* activity buffer (Promega Biotech), 0.80 units *Taq* polymerase

(Promega Biotech), 800 μM total deoxyribonucleoside-5'-triphosphates (dNTPs) (200 μM each) (Pharmacia), 40 pmol primer, and 35 ng genomic DNA. Only one primer and one genomic DNA sample were added to any single reaction.

A total of 35 PCR cycles were performed. The first cycle consisted of a two min 94°C melting segment, a 30 sec 36°C annealing segment, and a one min 72°C extension segment. Following the first cycle, 34 additional cycles were performed, with each cycle consisting of a five sec 94°C melting segment, a 30 sec 36°C annealing segment, and a one min 72°C extension segment. Following the final cycle, transcripts were completed with a 10 min 72°C segment followed by incubation at 4°C. An aliquot of five μl of 6X loading buffer (1.2 M sucrose, 3.7 x $10^{-3}$ M bromophenol blue dye, 1.2 mM EDTA [Ethylenediamine-tetraacetic acid]) was added to each reaction. If samples could not be electrophoresed immediately they were stored at -20°C. A Gibco-BRL Horizon 20.25 gel electrophoresis apparatus was used to perform agarose gel electrophoresis. Electrophoresis was performed using 1.6% or 1.8% (w/v) agarose gels with a 1X TAE (TRIS [Tris(hydroxymethyl)-aminomethane]/sodium acetate/EDTA) buffer for 270 V x h (constant voltage). Ethidium bromide-stained gels (0.5 μg $ml^{-1}$) were photographed on an UV transilluminator.

Due to the low frequency of polymorphism detected using agarose electrophoresis, a subset of 74 random primers previously evaluated using agarose gel electrophoresis were evaluated using the TSGE technique reported by Penner and Betze (1994). A Bio-rad Protean II gel electrophoresis apparatus and a

Hoeffer gel electrophoresis apparatus were used to perform TSGE. Gels of 0.75 mm thickness were composed of 8% acrylamide (37.5:1 acrylamide:bis-acrylamide), 3.5 M urea, 20% (v/v) deionized formamide, 1X TAE buffer, 0.05% (w/v) ammonium persulfate and 0.1% (v/v) TEMED (N,N,N',N'-Tetramethylethylene-diamine). Following electrophoresis, gels were cooled to room temperature, then stained for 10 min in a dilute ethidium bromide stain solution (0.5 μg $ml^{-1}$). The gels were then rinsed in water for several minutes and photographed on an UV transilluminator.

### 4.3.6 Marker Development

Arbitrary-sequence primers that amplified chromosome 5B sequences were examined for linkage to the necrosis resistance gene (*tsn1*). Homozygous $F_2$-derived $F_3$ families were identified by screening 17-25 plants from each family for reaction to the isolate 86-124 and/or Ptr necrosis toxin. Only families in which all plants exhibited the same reaction were used for RAPD analysis. DNA was extracted from one plant from each homozygous $F_2$-derived $F_3$ family.

DNA was extracted using the method of Edwards et al. (1991). One μl of sample was added to each PCR reaction. CS, KF, and CS(KF 5B) were used as controls. Segregation analysis, tests of the significance of linkage, determination of LOD values, and the estimation of the recombination frequencies between linked loci in the $F_2$-derived $F_3$ families were performed using the JOINMAP computer program of Stam (1993). The map distances in centiMorgans (cM) were

calculated using the Kosambi mapping function (Kosambi 1944).

Length of the polymorphic fragment amplified by UBC195 was determined by excising the fragment from TSGE gels. The excised fragment was placed in 1X TE (TRIS/EDTA) buffer overnight. The fragment was reamplified utilizing 1 µl of the TE solution. The reamplified fragment was then run on a 1.6% agarose gel with lambda DNA marker as a reference for size.

## 4.4 Results and Discussion

CS is highly resistant to the isolate 86-124 with a reaction type of 1 or 2 and is insensitive to Ptr necrosis toxin. KF and CS(KF 5B) are both highly susceptible to the isolate 86-124 with a reaction type of 5 and both are sensitive to Ptr necrosis toxin. Analysis of $F_1$, $F_2$ and $F_2$-derived $F_3$ families indicated that a single, nuclear, recessive gene governs resistance to the isolate 86-124 and Ptr necrosis toxin in reciprocal crosses of CS/KF and CS/CS(KF 5B) (Chapter 3.). From the CS/KF reciprocal cross, 19 $F_2$-derived $F_3$ families were identified as homozygous susceptible and 19 $F_2$-derived $F_3$ families were identified as homozygous resistant. From the CS/CS(KF 5B) reciprocal cross, 13 $F_2$-derived $F_3$ families were identified as homozygous susceptible and 14 $F_2$-derived $F_3$ families were identified as homozygous resistant. A single plant from each of these $F_2$-derived $F_3$ families was used in the linkage analysis.

In order to identify a RAPD marker for the *P. tritici-repentis* necrosis resistance gene, random primers were screened on the genomic DNA of CS, KF,

and CS(KF 5B) (Table 4.1). Of the 420 primers screened, 208 successfully amplified both CS and KF. Other researchers have encountered similar levels of amplification when applying RAPD analysis to cereals (Shin et al. 1990; Joshi and Nguyen 1993).

A total of 830 fragments were amplified by the 208 primers. Twenty-two primers amplified polymorphic fragments between CS and KF, however, none were specific to chromosome 5B. The level of genomic polymorphism (ratio of the number of polymorphisms identified/total number of primers successfully amplified) was 10.6 percent. The homoeologous nature of wheat's three sub-genomes could explain how amplified bands could possible co-migrate when resolved using 1.6% agarose gels. The low levels of polymorphism resolved by agarose gel electrophoresis necessitated the adoption of TSGE (Penner and Betze 1994). The incorporation of an increasing temperature gradient throughout the gel run resulted in separation of amplified fragments based both on size and nucleotide sequence variation.

A sub-set of 74 primers was chosen from the 208 primers that successfully amplified. The criterion for primer selection was ability to amplify discrete fragments with low levels of background. TSGE significantly increased the resolution of polymorphisms between CS and KF. Typically, there was a three-fold increase in the number of amplification fragments resolved with TSGE as compared to agarose gel electrophoresis. This suggested that many different fragments co-migrate as single bands on agarose gels (Figure 4.1). This

observation was similar to those of Dweikat et al. (1993) and Penner and Betze (1994). Of the 74 primers evaluated, 73 resulted in amplification products, identifying 73 genomic polymorphisms (Table 4.1). Two polymorphisms were specific to chromosome 5B sequences. Primers UBC102 (5' GGT GGG GAC T 3') and UBC195 (5' GAT CTC AGC G 3') amplified polymorphic fragments specific to chromosome 5B (Figure 4.2). Without the higher resolution capability of TSGE, marker identification in hexaploid wheat would be laborious. Linkage analysis was conducted on single plants of homozygous resistant and susceptible $F_2$-derived $F_3$ families. This methodology avoided the inherent problems associated with estimating linkage distances between a dominant marker and a recessive gene in an $F_2$ population (Williams and Rafalski, unpublished results cited in Williams et al. 1991). Both polymorphic fragments were shown to be linked in coupling to the necrosis resistance gene in Chinese Spring (Figure 4.2). The diagnostic fragment amplified by UBC195 was approximately 160 base-pairs in length and mapped 23.4 $\pm$ 5.7 cM (LOD 4.1) from the resistance locus. The diagnostic fragment amplified by UBC102 mapped 27.4 $\pm$6.9 cM (LOD 1.7) from the resistance locus. Fragments amplified by UBC102 and UBC195 mapped 4.2 $\pm$2.5 cM (LOD 12.8) apart. While the LOD for the UBC102 fragment was below standard cutoff levels, it was retained because of its known chromosome location. The summary of the phenotypic classifications of the single plants from each $F_2$-derived $F_3$ family is given in Table 4.2. Linkage data for UBC195 was based on at least two (two to four) separate amplifications. Linkage data for UBC102 was based on one to three

**Table 4.1. Comparison between agarose gel electrophoresis and TSGE for polymorphism identification**

| | Electrophoretic Method | |
|---|---|---|
| | Agarose | TSGE |
| No. of primers amplified | 208 | 73 |
| No. of loci amplified | ~830 | ~880 |
| No. of genomic polymorphisms | 22 | 73 |
| No. of 5B-specific polymorphisms | 0 | 2 |

separate amplifications. Fewer replications in scoring UBC102 was due to the difficulty in accurately resolving the polymorphic fragment and a closely associated monomorphic fragment. Errors in identifying the polymorphic fragment would have contributed to the low LOD value for UBC102. Moreover, the ease with which the polymorphism amplified by UBC195 could be identified, obviated the need for another, less tightly linked marker.

The results obtained are similar to those of other researchers utilizing RAPD technology for the identification of markers in wheat. Devos and Gale (1993) suggested that RAPD analysis would not be applicable to wheat due to the lack of polymorphism as revealed by agarose gel electrophoresis. TSGE is more laborious than agarose gel electrophoresis, hence it will be necessary to sequence polymorphic fragments and develop an amplicon that identifies the specific locus using a defined pair of oligonucleotide primers (Michelmore et al. 1992; Paran and Michelmore 1992). A second alternative would be the identification of the diagnostic fragment using dot-blot analysis (Penner et al. 1995). Only then would marker-assisted selection be simple and efficient enough to be routinely used in a plant breeding program.

The Ptr necrosis toxin was easier to apply as a selection tool than was RAPD analysis. However, due to the dominant nature of necrosis susceptibility, the toxin alone cannot be used to identify heterozygous loci. The identification of two diagnostic RAPD markers linked in coupling with the *P. tritici-repentis* necrosis resistance gene (*tsn1*) would facilitate the identification of plants that have at least

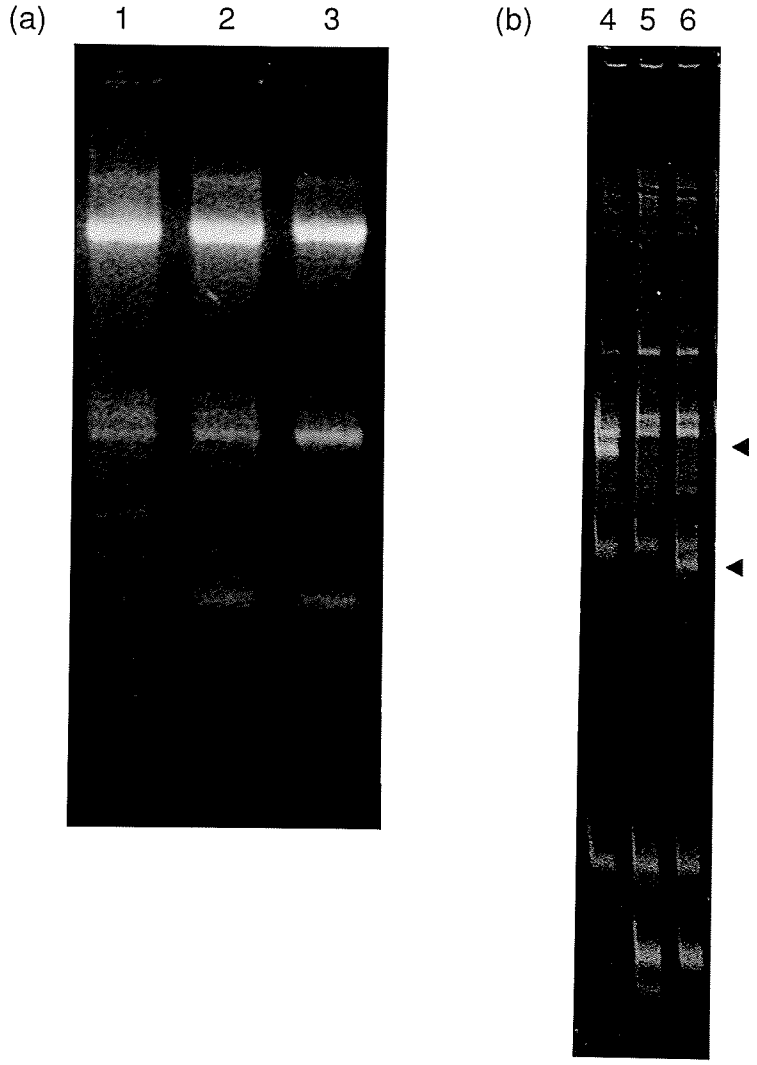


**Figure 4.1.** Comparison of the electrophoretic separation of RAPD fragments amplified by UBC195 using 1.6% (w/v) agarose gels and TSGE. PCR reaction resolved on a (a) 1.6% (w/v) agarose gel (figure is enlarged 200%), (b) TSGE. Lanes 1 and 4 KF, lanes 2 and 5 CS(KF 5B), lanes 3 and 6 CS. Arrows indicate polymorphisms.

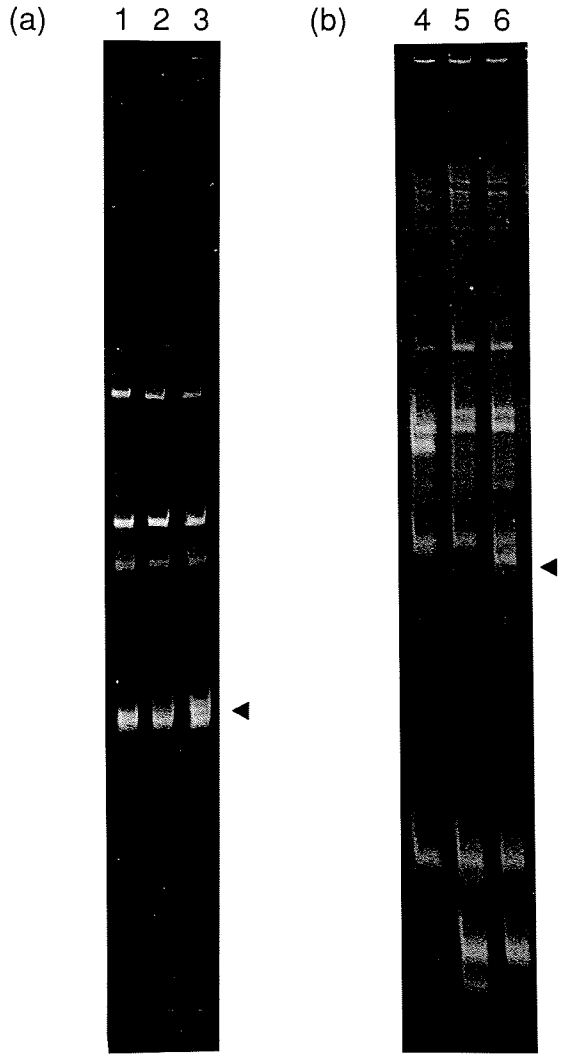


**Figure 4.2.** Electrophoretic separation using TSGE of RAPD fragments amplified by primers (a) UBC102 and (b) UBC195 from CS, KF, and CS(KF 5B) genomic DNA. Lanes 1 and 4 KF, lanes 2 and 5 CS(KF 5B), lanes 3 and 6 CS. The arrows indicate chromosome 5B specific polymorphisms.

**Table 4.2 Summary of the phenotypic classifications of $F_2$-derived $F_3$ families (RR = homozygous susceptible, rr = homozygous resistant, +_ = polymorphic amplicon present, -- = polymorphic amplicon absent)**

| Phenotype | UBC102[3] | UBC195 |
|---|---|---|
| RR+_[2] | 11 | 10 |
| RR--[1] | 21 | 24 |
| rr+_[1] | 18 | 21 |
| rr--[2] | 12 | 5 |
| total | 62 | 65 |

[1] Parental phenotypes
[2] Recombinant phenotypes
[3] 3 of 65 lines were not scored with UBC102

one resistance allele. This would minimize the number of generations of self-fertilization and progeny testing that would be required when conventionally back-crossing the recessive gene into adapted cultivars.

Several genetic studies (Lamari and Bernier 1991; Duguid 1995) identified either one or two recessive genes governing resistance to the isolate 86-124. UBC195 could be used to determine if any of these resistance genes are the same as *tsn1* identified on chromosome arm 5BL of CS (Chapter 3). Finally, because UBC195 and UBC102 are specific for chromosome arm 5BL sequences, they may be useful in linkage studies and selection for other traits located on 5BL.

## 5. GENERAL DISCUSSION

The ultimate aim of genetic analysis is the understanding of how different characters are inherited. This knowledge permits an overall assessment of the probable effects of selection on any generation or population. Genetic analysis of the inheritance of resistance to tan spot requires that plants exhibiting resistance and susceptibility be accurately, and precisely, scored. The development of an effective inoculation procedure (Lamari and Bernier 1989a), identification of three virulent pathotypes of *P. tritici-repentis* common to western Canada (Lamari and Bernier 1989b), isolation of a host-specific toxin from necrosis-inducing isolates (Ballance et al. 1989) and development of a five-level qualitative resistance scale (Lamari and Bernier 1989a) have enabled accurate and consistent differentiation between resistant and susceptible genotypes.

Genetic ratios obtained in $F_1$, $F_2$, and $F_2$-derived $F_3$ generations of the cross CS/KF identified a single, recessive, nuclear gene in CS that conferred high-level resistance to the isolate 86-124. The simple inheritance of this trait will facilitate introgression of high level genetic resistance into susceptible wheat genotypes.

Substitution analysis indicated that CS chromosomes, 5B and 7D, each carry a resistance gene to the isolate 86-124 and Ptr necrosis toxin. However, data from CS/KF indicated that only one gene was segregating for resistance. Past experience has shown that the use of substitution lines, alone, for genetic analysis has been unreliable. Various explanations have been put forward including univalent shifts (Person 1956), background variation in the original

monosomic line (Endo and Gill 1984), insufficient back-crosses (Berke et al. 1992) and human error. Even though the parental seed used in this study was developed from a single plant, it is not known whether this material is genetically identical to the lines used to develop the aneuploid series. As a result, confirmation of the substitution analysis results using other methods was required.

$F_2$ monosomic analysis was conducted to determine if the chromosome designation of CS(KF 5B) and CS(KF 7D) was correct. The CS monosomics used in this study were originally obtained from the Plant Breeding Institute, Cambridge, England. They have been used extensively by Dr. E. Kerber (Agriculture and Agri-Food Canada, Winnipeg, MB) therefore, the confidence in their chromosome designation is high. $F_2$ monosomic analysis indicated that only CS(KF 5B) was designated correctly. This fits the observed results from the CS/KF cross that only one gene segregates for resistance between CS and KF.

The progeny of selfed CS mono 5B and 7D plants indicated that a resistance gene was carried on chromosome 5B only. The identification of susceptible plants (2n=40) among the progeny of selfed CS mono 5B plants indicated that the necrosis resistance gene (*tsn1*) resided on that chromosome.

Analysis of CS nulli-tetrasomics (obtained from Dr. K. Armstrong, Plant Research Centre, Agriculture and Agri-Food Canada, Ottawa, ON) and CS ditelosomics (obtained from Dr. E. Kerber) inferred that the single necrosis resistance gene to be located on chromosome arm 5BL. All 42 chromosome arms of CS were screened with the exception of chromosome arms 5BL and 2AS. No

line was susceptible to the isolate 86-124 or Ptr necrosis toxin. Although chromosome arm 2AS was not evaluated for resistance, the combined results from the cross CS/KF, the $F_2$ monosomic analysis, the nullisomic analysis, and the screening of various nulli-tetrasomics and ditelosomics strongly suggest that the single resistance gene to be located on chromosome arm 5BL. This study provides evidence of the need to use several different populations to identify and verify gene location.

Having identified chromosome arm 5BL as carrying a resistance gene to the isolate 86-124, further characterization of the chromosome location was conducted by determination of its linkage to the leaf rust resistance gene, *Lr18*. As previously mentioned, the characterization of disease resistance genes necessitates that plants exhibiting resistance and susceptibility be accurately and precisely differentiated. Often genotype by environment interactions are significant enough that disease reaction based on single plants is unreliable. Therefore, material must often be advanced to the $F_3$ generation or higher so that disease reaction can be evaluated based on family reaction. Analysis of $F_2$-derived $F_3$ families of the cross CS(KF 5B)/RL 6009 indicated that *Lr18* and *tsn1* were not linked. To precisely locate *tsn1* on chromosome arm 5BL, linkage should be determined to other mapped loci assigned to chromosome 5B.

Phenotypic selection is often laborious, complicated, and/or expensive. The development of a RAPD marker for *tsn1* would facilitate the selection of heterozygous back-cross progeny, reducing the time required for selfing back-cross

progeny to identify heterozygous plants. A closely linked marker would also be useful in determining if genes identified in other genetic studies (Lamari and Bernier 1991; Duguid 1995) are identical to *tsn1*. However, the opportunity for direct selection for resistance to the isolate 86-124 within segregating populations will be limited. Ptr necrosis toxin screening is a cheap and simple selection method that accurately differentiates between resistant and susceptible plants. Also, Ptr necrosis toxin insensitivity is completely correlated with resistance to the isolate 86-124 and could be applied to all other populations segregating for resistance to the isolate 86-124. However, a closely linked marker could be multiplexed with other RAPD markers to screen for various traits concurrently, which greatly increases selection efficiency.

RAPD analysis combined with TSGE identified two primers, UBC195 and UBC102 linked in coupling 23.4 $\pm$ 5.7 cM and 27.4 $\pm$ 6.9 cM from *tsn1*, respectively. The polymorphism amplified by UBC195 was much easier to identify than the polymorphism identified by UBC102. Because both markers map to the same position, PCR-amplification conditions were not optimized for UBC102. However, the utility of pursuing condition optimization for UBC102 may lie in the fact that it may be applicable in the cross CS(KF 5B)/RL 6009. Also, analysis of the linkage data suggests that UBC102 may lie closer to *tsn1* than UBC195 due to high numbers of double cross-overs that are required to explain some of the data. However, the most probable explanation for this observation is that errors occurred when interpreting electrophoretic results. Once amplification conditions

for UBC102 have been optimized, the exact location of the RAPD may be determined.

Several points need to be discussed concerning the application of RAPD analysis to hexaploid wheat based on the results obtained. Moderate levels (49.5 percent) of PCR-amplification success were observed. This is most probably due to a single standard amplification protocol used throughout the study. Several researchers (Williams et al. 1991; Tingey et al. 1992) have shown that most primers can be amplified when amplification conditions are optimized. However, *a priori* consensus was that screening larger numbers of primers would be more efficient than optimizing conditions for individual primers. The majority of primers that supported amplification, did so in both CS and KF, and usually occurred in the same experiment. This observation would indicate that experimental technique was not problematic. Similar levels of amplification success were observed by Shin et al. (1990) in barley and by Joshi and Nguyen (1993) in durum wheat.

Standard RAPD analysis usually combines PCR-amplification of DNA and the separation of amplified fragments using agarose gel electrophoresis. Only 10.6 percent of successfully amplified primers identified a polymorphic locus between CS and KF when utilizing this method. However, the resolution of similar amplification products using TSGE, increased fragment resolution approximately three-fold, with a concomitant increase in the identification of polymorphic loci. However, TSGE is much more laborious than agarose gel electrophoresis and it is not feasible to be used in marker-assisted selection. Therefore, linked RAPD

fragments should be sequenced and specific primers developed (Michelmore et al. 1992; Paran and Michelmore 1992). This should also increase the number of segregating populations to which the marker can be applied. However, it appears that the conversion of RAPDs to specific amplicons may be difficult in wheat due to the homoeologous relationships among the genomes (Penner et al. 1995). Dot blot hybridization of PCR products with labelled diagnostic fragments could circumvent these problems (Penner et al. 1995). The decrease in resources would facilitate its use in marker-assisted selection.

Because RAPDs are dominant in nature, heterozygous individuals cannot be identified. A RAPD marker can only identify the allele from which it was developed. Therefore, linkage analysis was conducted on single plants of homozygous $F_2$-derived $F_3$ families for resistance to the isolate 86-124 and Ptr necrosis toxin. This avoided the problem of trying to map a dominant RAPD marker coupled to a recessive gene in the $F_2$ generation. Mapping efficiency is increased when using $F_2$-derived $F_3$ plants as the genotype of the resistance locus is known and recombination events can be identified.

A common criticism of RAPD analysis is that markers are usually restricted to the population which they were developed. This was also observed in this study. The fragment amplified by UBC195 in CS could not be reproduced in RL 6009. The lack of cross-applicability means that another RAPD fragment linked to *tsn1* will have to be generated specifically for the CS(KF 5B)/RL 6009 cross. Unsuccessful attempts were made to determine if UBC102 could be applied to the

same population. Amplification patterns obtained with UBC102 could not be repeated on agarose gels. Several explanations including that the DNA *Taq* polymerase activity had decreased or that the primer had degraded could explain the results.

An advantage of RAPD analysis is that it does not require high quality or large quantities of DNA. This enables quick and simple DNA extraction protocols (Edwards et al. 1991) to be utilized, facilitating the application of this technology to marker-assisted selection. However it was observed that wheat DNA extracted by the method of Edwards et al. (1991) rapidly degraded when stored at 4°C. This necessitated that leaf tissue be collected, the DNA extracted, and RAPD analysis be performed within 48 h. Although high quality DNA has been touted as not essential to PCR-amplification success, it was observed that amplification of DNA was most successful and repeatable if DNA was extracted from young, vigorously growing tissue.

Although the linkage between *tsn1* and UBC195 or UBC102 is loose, the identification of two RAPD primers that amplify chromosome 5B sequences is of great value when developing a linkage map that can be applied to map-based selection. As this study was undertaken with no knowledge of primers that were specific to chromosome 5B, 460 random primers were analyzed. However, once a saturated linkage map exists in wheat, the inefficiencies of random primer selection can be eliminated. Then, once a trait has been identified to a specific chromosome, a sample of markers approximately 20 cM apart could be selected

and tested for linkage to the trait of interest. When linked markers are identified, closer markers can be tested until the precise location of the trait has been identified. Herein lies the great utility of saturated linkage maps and their application towards map-based selection strategies.

Both conventional and aneuploid methods were used to characterize a single, recessive, nuclear resistance gene, *tsn1*, to the *P. tritici-repentis* necrosis-inducing isolate 86-124 to chromosome arm 5BL in CS. This study reiterated the fact that gene location studies should not be based on individual methods. The more chromosome location studies that are conducted utilizing different populations, the stronger the final conclusions will be. Continued research should be conducted to explain the incorrect designation of CS(KF 7D). RAPD analysis combined with TSGE identified two fragments, amplified by UBC102 and UBC195, loosely linked in coupling to *tsn1*. The development of a saturated linkage map in wheat will facilitate mapping of genetic loci in the future.

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## 7. APPENDIX

**Appendix 7.1.** Description of lesion types in response to Pathotype 2 isolates of the pathogen *Pyrenophora tritici-repentis* after Lamari and Bernier (1989a)

| Lesion Type | Description |
|---|---|
| 1 | Resistant: small dark brown to black spots without any surrounding tan necrosis |
| 2 | Moderately resistant: small dark brown to black spots with very little tan necrosis |
| 3 | Moderately susceptible: small dark brown to black spots completely surrounded by a distinct tan necrotic ring; lesions generally not coalescing |
| 4 | Susceptible: small dark brown or black spots completely surrounded with tan necrotic zones; some of the lesions coalescing |
| 5 | Highly susceptible: the dark brown or black centres may or may not be distinguishable; most lesions consist of coalescing chlorotic or tan necrotic zones. |

Adapted from Lamari and Bernier, 1989a

**Appendix 7.2.** Description of infection types rated in the greenhouse in response to the pathogen *Puccinia recondita* f.sp. *tritici* after Stakman et al. (1962).

| Infection Type | Description |
|---|---|
| 0 | Immune: no rust pustules, no macroscopic symptoms |
| ; | Nearly immune: no rust pustules, occasional small flecks of dead tissue |
| 1 | Very resistant: extremely small rust pustules surrounded by necrotic tissue |
| 2 | Moderately resistant: small to medium pustules with necrotic ring, green island |
| 3 | Moderately susceptible: medium sized elongated pustules without necrotic tissue |
| 4 | Very susceptible: large pustules, elongated and erumpent without necrotic tissue |
| X | Mesothetic: variable symptoms, often including all the above types on one leaf |