Molecular and cytogenetic tools for selecting and fixing disease resistance genes in wheat (*Triticum aestivum* L.) populations

BY

Colin W. Hiebert

A thesis submitted to the Faculty of Graduate Studies of

the University of Manitoba

in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Biological Sciences

University of Manitoba

Winnipeg

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Abstract

This thesis integrates classical, cytogenetic and molecular approaches to selecting disease resistance gene stacks in common wheat. Wheat leaf rust resistance (Lr) gene *Lr22a* was previously introgressed into wheat from *Aegilops tauschii* Coss and is located on chromosome 2DS. *Lr22a* was mapped with microsatellite (SSR) markers to allow stacking with other Lr genes; the closest marker was GWM296 (2.9 cM distal). Genetic size of the *Ae. tauschii* introgression was determined with SSRs and was tracked through the ancestry of various Canadian wheat varieties. Disease resistance genes are often more effective and durable when they are stacked. To investigate the use of telocentric chromosomes to increase the frequency of desirable alleles in breeding populations four populations were produced each with a different combination of disease resistance genes to either leaf rust or fusarium head blight (FHB). Each population had F_1 plants that were either double monotelodisomic (dmtd), with both resistance genes in the hemizygous state, or were dihybrid. F_3 families were produced and tested for disease resistance. The families derived from dmtd F_1 plants showed an increased frequency in disease resistance compared to the families derived from dihybrids. Testing the female and male transmissions of the four double telo combinations revealed no gametic selection against telosomes in ovules while there was reduced transmission of telosomes through pollen. Pollen competition increased the frequency of gene stacks. Nineteen of 21 monosomics were isolated in elite germplasm by screening the progeny of haploid by diploid crosses. All monosomics were crossed with a normal parent to generate telocentric chromosomes through the misdivision of the univalent. Eleven telocentrics were recovered out of a possible 38. Eight of these represented telosome pairs i.e. long and short arm telosomes

of the same chromosome. This result is significantly improbable if all centromeres are equally likely to break and be recovered. The telosome for 1BS was truncated by the loss of its satellite. This telosome formed bivalents (≈ 50%) with its normal homologue but failed to yield recombinants. Combining DNA markers and telosomes is a powerful tool for wheat breeding.

Acknowledgement

I would like to thank Dr. Julian Thomas for acting as my advisor and his mentorship. The many interesting discussions have been stimulating. I also would like to thank Dr. Brent McCallum for his keen interest and encouragement throughout my graduate studies. I also extend my thanks to the rest of my committee, Drs. Georg Hausner, Dana Schroeder and Perry Gustafson, for their helpful suggestions and support.

I would like to thank the following people from the CRC for their technical assistance: Erica Riedel, Jadwiga Budzinski, Sasanda Nilmalgoda, Lorelle Furst, Pat Set-Goh, Laura Trump, Ron Kaethler, Denis Green and the breeding crew, and Debbie Jones.

I would like to thank Dr. Daryl Somers for his mentorship over last couple of years. I would also like to thank my lab mates and the rust group for their supportive friendship and willingness to share lab space.

I would like to acknowledge Natural Sciences and Engineering Research Council of Canada (NSERC) and AAFC for their financial support.

Lastly, I would like to thank my wife Nichole. The last few years have been difficult for our family due to health concerns. Your strength and love are the reasons I was able to keep going.

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Chapter 1 - Literature Review

Wheat

Wheat (*Triticum aestivum* L.) is an important cereal crop. In Canada approximately 7.7 million hectares of wheat (excluding durum) was seeded in 2008 which accounts for 28% of the total seeded acreage (Statistics Canada 2008). Most of this wheat (92%) is seeded in the Prairie Provinces. The most common class of wheat seeded is hard red spring wheat. Hard red spring wheat accounts for 88% of spring wheat seeded and 81% of all common wheat (Statistics Canada 2008).

Wheat is an allohexaploid species containing three sub-genomes that are referred to as the A, B and D genomes. Thus, the genomic constitution of wheat is $2n = 6x = 42$, AABBDD. These three genomes were derived from related diploid progenitor species. The progenitor of the A genome was first reported as *Triticum monococcum* L. ($2n = 14$, AA; Sax 1922). However, subsequent cytological and molecular evidence showed that *T. urartu* L. was likely the A genome progenitor for tetraploid and hexaploid wheat (Chapman et al. 1976; Dvořák et al. 1988, 1993). Much evidence has been presented to show that *Aegilops tauschii* Coss. ($2n = 14$, DD) was the progenitor of the D genome (eg. McFadden and Sears 1946, Riley and Chapman 1960). The identity of the B genome progenitor has not been determined to the same level of certainty. Some evidence supports *Aegilops speltoides* L. ($2n = 14$, BB) as the progenitor of the B genome in wheat (Kimber and Riley 1963, Daud and Gustafson 1996), while others suggest that either the B genome progenitor has not yet been found or is extinct (Jauhar et al. 1991). Some intergenomic rearrangements have occurred such as a reciprocal translocation between

chromosomes 4A and 4B but the ancestral genomes are largely unchanged (Vasu et al. 2001). The three genomes of wheat were each shown to be divided into seven homœologous groups (Sears 1954). Chromosomes within a homœologous group all derive from the same ancestral chromosome and share similar genetic content and structure. However, the level of similarity is not as high as that seen in homologous chromosomes. Comparing wheat genetic maps between chromosomes within the same homœologous group can elucidate similarities and differences in the structure of homœologous wheat chromosomes. For example, the dense microsatellite (SSR) map presented by Somers et al. (2004) indicates co-linearity in many instances when primer pairs amplify SSR's across chromosomes of a homœologous group. However, apparent rearrangements are not uncommon in these maps.

Another example of the similarity between homœologous chromosomes can be seen with the red seed coat genes found on homœologous group 3 chromosomes (Sears 1944; Metzger and Silbaugh 1970). In a study that used molecular markers to map homœologous group 3 chromosomes, the red seed genes on chromosomes 3BL and 3DL were shown to be in homœologous regions, however the red seed gene on 3AL did not segregate in the population used (Nelson et al. 1995).

A third example are the gibberellin-insensitive dwarfing genes *Rht-B1b* and *Rht-D1b* that are found on homœologous chromosomes 4B and 4D respectively (Ellis et al. 2002). At both loci the allele responsible for short plant type contains a non-sense mutation that causes a truncated gene product due to the early termination of translation (Ellis et al. 2002). There are no reports of a similar gene on chromosome 4A. This could be the case because either 1) no homœologous locus is found on 4A, 2) a similar allele

exists on 4A but has not been identified or 3) this mutation has not occurred on chromosome 4A.

There are no reported instances of homœologous disease resistance genes.

Wheat breeding and plant diseases

In general, plant breeders have many breeding objectives including higher yields, improved end-use quality, short duration to maturity and disease resistance (Poehlman 1987). For Canadian wheat breeders there are many diseases to consider when incorporating resistance genes into their breeding programs. In the Canadian prairies some of the diseases found include leaf rust, stem rust, stripe rust, tan spot, septoria tritici blotch, loose smut and fusarium head blight (McCallum and Depauw 2008; Martens et al. 1988). Both wheat leaf rust (*Puccinia triticina* Eriks.) and fusarium head blight (FHB, *Fusarium graminearum* Schwabe.) are prevalent diseases in the Canadian prairies (McCallum and Seto-Goh 2003; Gilbert and Tekauz 2000). Genetic resistance or partial resistance is available for both of these pathogens as either resistance genes or quantitative trait loci (QTL) (McIntosh et al. 1995; Waldron et al. 1999; Ban and Suenaga 1998). The level and durability of genetic resistance is improved when multiple resistance genes or QTL are stacked (Kolmer 1999; Bai and Shaner 1994). Accurate gene stacking is made possible with the availability of closely linked DNA markers (Schachermayr et al. 1994; Hussien et al. 1997; Huang and Gill 2001; Somers et al. 2003). Unfortunately, as the number of loci required for disease resistance increases (i.e. the complexity of the cross has increased) there is a geometric increase in the frequency of individuals that must be discarded because only a few plants will carry all the desired

alleles (Thomas et al. 2004). This presents an additional challenge to the breeder as there are fewer lines to select for other traits if many are discarded based on the absence of the desired resistance genes or QTL. While selection of desired allele combinations using genetic tools such as molecular markers can assure retention of desired genotypes, these selection methods do not change the frequencies of these genotypes in breeding populations. Cytogenetic solutions, including telocentric chromosomes and Robertsonian translocations, have the potential to increase the frequency of desired alleles in breeding populations thereby increasing the effective size of the breeding population (Thomas et al. 2003, 2004).

Wheat leaf rust and leaf rust resistance genes

In wheat, leaf rust is caused by the basidiomycete *Puccinia triticina* Eriks. *Puccina triticina* is an obligate parasite that has a macrocyclic life cycle where *Thalictrum speciosissimum* L. is the alternate host required for sexual reproduction (Anikster et al. 1997). The sexual part of the life cycle is not an important factor in the epidemiology or the evolution of virulence in leaf rust in North America because the sexual cycle infrequently occurs naturally (Samborski 1985). The frequency of races, or virulence phenotypes, of wheat leaf rust is monitored by annual disease surveys (e.g. McCallum and Seto-Goh 2003). Virulence phenotypes are assigned to isolates of *P. triticina* based on whether the isolate is virulent or avirulent on a defined set of differential lines (Long and Kolmer 1989). These differential lines are a set of near-isogenic lines (NILs) that carry single resistance genes in a susceptible genetic background. Populations of *P. triticina* evolve by either changing the frequencies of

exisitng virulences or by developing new virulence that is most likely caused by mutation (Samborski 1985).

The *P. triticina* urediospores that infect wheat in Canada are transported by wind from Mexico via the United States infecting green crops along the way (Roelfs 1985). Thus the race structure of the inoculum received in Canada depends on the races that successfully infect wheat in the United States. Grain yield losses in the eastern Canadian prairies are generally between 5-15% depending on the resistance in the cultivars grown, the inoculum load and the environmental conditions (Samborski 1985). The losses can exceed this level if circumstances are particularly favorable. For example, in 1999 yield losses were estimated at 20% in some areas of Manitoba (McCallum et al. 2000). This was attributed to the leaf rust susceptibility of the most commonly grown cultivar, the large inoculum load and environmental conditions that favored infection.

The gene-for-gene model describes one mode of host-pathogen interactions where the interaction between resistance genes in the host and virulence genes in the pathogen determine if a compatible or incompatible interaction occurs (Flor 1956). Resistance to the pathogen (incompatible interaction), in the gene-for-gene model, occurs when a resistance gene in the host recognizes the product of an avirulence allele from the pathogen. A susceptible response (compatible interaction) occurs when there is no resistance gene in the host, there is no avirulence factor for the resistance gene to recognize, or both the resistance gene and avirulence factors are absent. Most leaf rust resistance (Lr) genes in wheat interact in this manner with *P. triticina* (Samborski and Dyck 1968, 1976; Dyck and Samborski 1970). Genes that follow this model display qualitative resistance. However, heterozygosity or homozygosity of either or both the

resistance gene in the host wheat plant and the virulence/avirulence gene in the *P. triticina* isolate may alter the outcome of the interaction (Kolmer and Dyck 1994). The interaction between wheat and *P. triticina* can also be sensitive to temperature (Dyck and Johnson 1983). The gene-for-gene model can only be demonstrated when there are isolates of the pathogen that are virulent and avirulent to lines carrying the resistance gene in question. Therefore Lr genes that produce qualitative resistance, but have no identified virulent races are likely to follow the gene-for-gene model but this cannot be demonstrated until a virulence gene is identified in the pathogen.

Two named Lr genes that confer quantitative resistance to leaf rust appear to have no race-specificity (i.e. horizontal resistance that does not appear to follow gene-for-gene model). These are *Lr34* (Dyck and Samborski 1982; Dyck 1987) and *Lr46* (Singh et al. 1998). A review of these genes is found below.

Mapping Lr genes

Until recently most Lr genes have been assigned to chromosomes and chromosome arms using cytogenetic stocks. More specifically, monosomics and telocentrics have been widely used to locate and map genes in wheat (McIntosh 1987). Monosomics are individuals that have only one homologue of a given chromosome rather than two. Telocentrics are chromosomes missing one arm and thus the missing arm can be found in the hemizygous condition or can be entirely deficient depending if there is one of two copies of the telocentric.

Monosomic analysis involves crossing a line carrying the gene of interest with each of the 21 possible monosomic lines, selecting monosomic F_1 plants followed by

observation of the segregation within each cross in the F_2 or F_3 generations (Sears 1953). Distorted (non-Mendelian) segregation occurs when the gene of interest is found in hemizygous condition in the F_1. In the case of a dominant gene, plants showing the phenotype conditioned by the gene of interest will either be disomic or monosomic and only those that are nullisomic will show the alternative phenotype. Distortion is due to a low frequency of nullisomic individuals in the progeny of self-pollinated monosomics (Sears 1953). This technique, or similar variants, has been used to locate many Lr genes (eg. Sears 1961; Dyck and Kerber 1981; Dyck et al. 1987; Hussein et al. 1997).

An alternative to monosomic mapping, called haploid deficiency mapping, has recently been reported (Thomas et al. 2001). This method involves generating an array of random aneuploid hybrids by pollinating haploid wheat ($n = 21$, ABD) with the pollen from a normal wheat plant ($2n = 42$, AABBDD). The chromosome deficiencies, which can be accompanied by duplications in whole or in part, arise from the irregular meiosis of the haploid parent. The haploid parent carries the gene of interest and the pollinator should have an alternate phenotype. Most of the hybrids will carry the gene of interest, which can be detected phenotypically or using another marker. A few of the hybrids will lack the gene of interest because of the failed transmission of the critical chromosome from the haploid to the hybrid. These critical individuals can be analyzed for chromosome deficiencies using chromosome-specific, co-dominant markers such as SSR markers. The chromosome deficiency that is common across the critical individuals will reveal which chromosome carries the gene of interest. This technique was been used to locate *Lr52* and *Lr60* (Hiebert et al. 2005, 2008) and the midge resistance gene *Sm1* (Thomas et al. 2001).

Telocentric mapping assigns the gene of interest to one arm of the chromosome and determines the linkage relationship between the gene and the centromere (Sears 1962; Sears and Sears 1978). Linkage between the gene of interest and the centromere is essentially two-point linkage and procedures have been described for calculating genetic distance using F_2 data (The and McIntosh 1975). These experiments are performed by crossing a line carrying the gene of interest with both telocentric stocks for the chromosome which also carries the gene. Monotelodisomic plants (41 chromosomes + one telosome) are selected in the F_1 and are self-pollinated to produce the F_2. The F_2 individuals are classified for the number of telocentric chromosomes and the phenotype (such as rust resistance or susceptibility). Genetic distance can be calculated from such data (The and McIntosh 1975). This method has been used to map many Lr genes (eg. Dyck and Kerber 1981; Dyck et al. 1987). In place of F_2 populations, telocentric mapping can be performed by analyzing the progeny of testcrosses (Kerber 1988). Testcross progeny can be directly classified as recombinant or parental by recording the phenotype for the trait in question and the karyotype for each individual. The recombination frequency is directly calculated by dividing the number of recombinants by the population total.

Several types of molecular markers have been used to map Lr genes. Lr genes deployed singly are expected to lose their effectiveness more rapidly than stacked combinations of genes (Dyck and Kerber 1985; McIntosh et al. 1995). Reliable markers can allow specific Lr gene combinations to be accordingly selected (Schachermayr et al. 1994; Hussien et al. 1997; Huang and Gill 2001). Hybridization-based markers, namely restriction fragment length polymorphism (RFLP), have been used to map Lr genes

(Schachermayr et al. 1994, 1995; Autrique et al. 1995; Huang and Gill 2001). Various marker systems based on the polymerase chain reaction (PCR) have been used to map Lr genes including random amplified polymorphic DNA (RAPD) (Schachermayr et al. 1994, 1995), amplified fragment length polymorphism (AFLP) (Prins et al. 2001) and microsatellite markers (SSR) (Raupp et al. 2001; Hiebert et al. 2005, 2008). RFLP markers are laborious and ill-suited for the high throughput necessary for marker-assisted breeding (Röder et al. 1998). Markers linked to Lr genes as detected by RFLP, RAPD and AFLP may be converted to sequence-tagged-site (STS) markers (Schachermayr et al. 1994, 1995, 1997; Prins et al. 2001; Huang and Gill 2001). SSR markers with a simple electrophoretic profile and STS markers are well suited for high throughput genotyping.

Function of resistance genes

In most host-pathogen systems that follow the gene-for-gene model for hypersensitive resistance responses to fungal attack, the resistance genes are receptors that recognize a product from the pathogen and trigger a defense response (Heath 2000). These resistance genes often contain a nucleotide-binding site (NBS) domain and leucine-rich repeats (LRR). Genes of this nature recognize a ligand (avirulence factor) from the pathogen and trigger a resistance response (van der Biezen and Jones 1998; Dangl and Jones 2001). At the amino terminus of the resistance gene product there is a Toll/Interleuken-1 Receptor-like (TIR) domain or a coiled-coil (CC) motif (Meyers et al. 1999; Pan et al. 2000). Thus, resistance gene products of this class can be either TIR-NBS-LRR (TNL) or CC-NBS-LRR (CNL)

To date three Lr genes that have been cloned: *Lr10* (Feuillet et al. 2003), *Lr21* (Huang et al. 2003) and *Lr1* (Cloutier et al. 2007). All three code for proteins that contain coiled coil, nucleotide-binding site and leucine rich repeat domain and are thus CNL proteins. Similarly, the wheat gene *Pm3b*, which confers resistance to the fungal pathogen causing powdery mildew (*Blumeria gramminis* f.sp. *tritici*), has been cloned and also codes for a protein with CNL domains (Yahiaoui et al. 2004). So far resistance genes with TNL-type products have not been detected in cereals, including an extensive search in rice (*Oryza sativa* L.) (Zhou et al. 2004). This lies in contrast to products of resistance genes found in Arabidopsis where TNL genes are the most common, although CNL genes are also present (Meyers et al. 2003). The Lr genes that have been cloned in wheat confer qualitative resistance characterized by necrosis surrounding the infection site and little to no sporulation of the fungus. In contrast *Lr34* and *Lr46* confer quantitative resistance which increases latent period, reduces pustule size and slows the rate of pustule development. There have been mapping efforts with quantitative Lr genes, particularly *Lr34* (Suenaga et al. 2003; Spielmeyer et al. 2005, 2008; Lagudah et al. 2006). Fine mapping has lead to the putative cloning of *Lr34* (Krattinger et al. 2008). It appears that *Lr34* falls into an entirely different gene class then *Lr1*, *Lr10* and *Lr21*.

Review of specific Lr genes used in the following research

There are five Lr genes that were used in the study described in this thesis. These are *Lr16*, *Lr22a*, *Lr34*, *Lr46* and *Lr52*. These represent a range of resistance genes from seedling resistance to quantitative adult plant resistance. These genes are reviewed below.

Lr16 is a seedling Lr gene located on wheat chromosome 2BS that was found in common wheat (Anderson 1961; Dyck and Samborski 1968; McIntosh et al. 1995). The infection type produced by *Lr16* in response to avirulent races of *P. triticina* ranges of from 1 to 3C ("C" denotes heavy chlorosis surrounding the pustule) and is often characterized by a heavy necrotic or chlorotic ring around the pustule (McIntosh et al. 1995). Unlike many Lr genes, *Lr16* shows lower infection types as temperature increases (Dyck and Johnson 1983). *Lr16* has been mapped with microsatellite markers and was the terminal locus on the genetic map of chromosome 2BS (McCartney et al. 2005). It is not uncommon for Lr genes to be either terminal loci or mapped to distal regions on genetic maps of wheat chromosomes. For example *Lr1*, *Lr52* and *Lr21* have been mapped to terminal chromosomal regions (Cloutier et al. 2007, Hiebert et al. 2005, 2008).

Several North American wheat varieties have been shown to carry *Lr16*, including AC Karma, AC Domain, AC Majestic, AC Splendor, Columbus and Grandin (Samborski and Dyck 1982; Liu and Kolmer 1997a, b; Kolmer and Liu 2002). Virulence to *Lr16* is common in the Canadian prairies (McCallum and Seto-Goh 2003). However, isolates considered to be virulent to *Lr16* often have lower infection types on the *Lr16* line compared to the susceptible check (McIntosh et al. 1995). Thus *Lr16* can still play an important role in providing Canadian germplasm with leaf rust resistance, especially considering the prevalence of *Lr16* in Canadian cultivars and breeding lines (McCartney et al. 2005). However, with increased selection for *Sm1*, a gene conferring antibiotic resistance to orange wheat blossom midge (*Sitodiplosis mosellana* Gehin), in Canadian wheat breeding programs there could be a corresponding decrease in the frequency of

Lr16 as *Sm1* and *Lr16* are normally linked in repulsion in Canadian germplasm (Thomas et al. 2005).

Aegilops tauschii Coss. has been a source of disease resistance genes (eg. Rowland and Kerber 1974) and other genetic resources for common wheat, such as microsatellite markers (Pestova et al. 2000), because *Ae. tauschii* is a close relative (progenitor) of common wheat. The first two Lr genes introgressed from *Ae. tauschii* into wheat were *Lr21* and *Lr22a*, which are located on chromosomes 1D and 2D respectively (Rowland and Kerber 1974). These genes were transferred into common wheat by crossing *Ae. tauschii* ($2n = 2x = 14$, DD) accessions with Tetra-Canthatch ($2n = 2x = 28$, AABB) to generate a synthetic hexaploid that could be readily crossed with common wheat. To date three additional named wheat Lr genes that have also originated from *Ae. tauschii*. They are *Lr32* (Kerber 1987), *Lr41* (Cox et al. 1994; Singh et al. 2004) and *Lr42* (Cox et al. 1994). Of these five genes *Lr22a* is the only one classified as an adult-plant resistance (APR) gene. Inoculating at different plant growth stages has revealed that the resistance conferred by *Lr22a* is activated at the four leaf stage (Pretorius et al. 1987). Plants inoculated at the four leaf stage show the same resistant infection type on the fourth leaf as that found on flag leaves and higher infection types on the lower leaves while plants inoculated at earlier plant growth stages show a susceptible response on all leaves (Pretorius et al.1987). Furthermore, *Lr22a* is the only Lr gene discovered in *Ae. tauschii* that has an allele, *Lr22b*, found in common wheat that also confers resistance to *P. triticina* (Dyck 1979).

Unlike some APR genes, such as *Lr34*, that provide quantitative type resistance, *Lr22a* confers resistance that is a strong qualitative resistance similar to that found in

seedling genes similar to adult-plant genes *Lr12* and *Lr13* (McIntosh et al. 1995). To date there is no reported virulence to *Lr22a* (Park and McIntosh 1994; McCallum and Set-Goh 2005; Kolmer et al. 2005). Despite conferring broad-spectrum effective resistance, *Lr22a* has not been widely deployed commercially. In Canada there have been three registered wheat varieties, AC Minto, 5500HR and 5600HR, that carry *Lr22a* (Kolmer 1997; Hiebert et al. 2007), however none have accounted for substantial seeded acreage (McCalluma and Depauw 2008; Canadian Wheat Board, http://www.cwb.ca).

Lr52 was isolated from accession V336 of the Watkin's wheat collection (Dyck and Jedel 1989). In the original report of this gene there was no genetic evidence provided to demonstrate its genetic novelty and so it was given the temporary designation of *LrW*. *LrW* was recently assigned to chromosome 5BS using cytogenetic and molecular mapping techniques (Hiebert et al. 2005). As no other named Lr genes are carried on 5BS, *LrW* was deemed unique from other Lr genes and was renamed *Lr52* (Hiebert et al. 2005). Lines carrying *Lr52* display low infection types (; to 1+) when inoculated with an array of *P. triticina* isolates and no virulence to *Lr52* has been reported in North America (Dyck and Jedel 1989; Hiebert et al. 2005). Although this effective resistance gene was discovered in Canada approximately 20 years ago there are no reports of Canadian wheat cultivars that carry *Lr52*.

Lr34 was the first Lr gene discovered that confers slow rusting resistance (quantitative resistance) to *P. triticina* (Dyck and Samborski 1982; Dyck 1987). *Lr34* is known as an adult plant resistance (APR) gene; however, there are some reports of resistant phenotypes at the seedling stage in certain genetic backgrounds or under specific growth conditions (Dyck and Samborski 1982; Singh and Gupta 1991; Singh and Gupta

1992). As an APR gene *Lr34* increases latent period and decreases uredia size and receptivity (Singh and Huerta-Espino 2003). The resistant infection types of several seedling Lr genes are improved in the presence of *Lr34* even at the seedling stage (German and Kolmer 1992). *Lr34* is completely linked to *Yr18*, a gene that confers resistance to stripe rust (*Puccinia striiformis* Westend. f. sp. *tritici*) and additional resistance to powdery mildew and may represent a pleiotropic gene (Singh 1992a; Spielmeyer et al. 2005). Furthermore, *Lr34*, or a closely associated gene, is a nonsupressor of some stem rust resistance (Sr) genes (Kerber and Aung 1999). These Sr genes fail to express when *Lr34* is absent. There is also a strong association between leaf tip necrosis and the resistant allele of *Lr34* (Singh 1992b) which has been used as a selection tool. To develop closely linked molecular markers to *Lr34* several mapping projects have been conducted. These efforts to map *Lr34* began with QTL analysis (Suenaga et al. 2003). With accurately phenotyped populations where *Lr34* was only source of resistance, it was mapped as a single Mendelian factor (Spielmeyer et al. 2005; Lagudah et al. 2006). Recently a fine mapping study of *Lr34* revealed markers on chromosome 7DS that were tightly linked to the gene (0.13 cM) (Spielmeyer et al. 2008). Fine mapping has facilitated the putative cloning of *Lr34* (Krattinger et al. 2008).

The second Lr gene to confer slow-rusting resistance to *P. triticina* is *Lr46* (Singh et al. 1998). The nature of the resistance conferred by *Lr46* is similar to *Lr34* and is characterized by increased latent period and decreased uredia size and receptivity (Martinez et al. 2001). Also, like *Lr34*, the *Lr46* locus has been shown to reduce infection from *P. stiiformis* (stripe rust). The stripe rust resistance conferred by this 1BL locus has been named *Yr29* which is either tightly linked to *Lr46* or a single resistance

locus has pleiotropic effects (Singh et al. 2001). *Lr46* was isolated from the wheat cultivar Pavon 76 and was located to chromosome 1B using monosomic analysis (Singh et al. 1998). Singh et al. (1998) also report that the slow-rusting resistance of Pavon 76 has remained effective since the release of Pavon 76 in 1976. Mapping *Lr46* with molecular markers as either a single gene or as a QTL has placed *Lr46* at the distal end of short arm on chromosome 1B (William et al. 2003; Suenaga et al. 2003).

Fusarium head blight

Fusarium head blight (FHB) is a disease that is found globally in cereals, including wheat (Gilbert and Tekauz 2000). Several species of Fusarium can be isolated from fusarium-damaged kernels (FDK) such as *F. avenaceum* (Corda; Fr.) Sacc., *F. culmorum* (W.G. Smith) and others, however, *F. graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) is the principal casual agent of FHB on wheat in Canada (Clear and Patrick 2000). *Fusarium graminearum* is an acomycete that produce ascospores (sexual spores) in perithecia and macroconidia (asexual spores) from mycelia. The fungus persists and over-winters as a saprophyte in fields on crop residues and ascospore and macroconidial production coincides with anthesis of cereal crops (Markell and Francl 2003). Thus crop residue is the primary source of inoculum (Sutton 1982; Martens et al. 1988). Dispersion of ascospores and macroconidia is via wind and rain splashing respectively with subsequent infection occurring on flowering wheat spikes (Fernando et al. 2000). Infection is favored when flowering spikelets are contacted by spores in humid conditions and the disease progression is optimal when the plant surfaces are wet and the temperature range is 20-30°C (Sutton 1982). *Fusarium graminearum*

penetrates the host through stomata in softer tissues such as anthers and developing caryopses and also through the stomata of the paleas and lemmas (Boshoff et al. 1996). The germ tubes are unable to penetrate the hard, waxy surfaces of some floral structures such as glumes (Pritsch et al. 2000). Symptoms of the disease include premature bleaching of florets, dark spots (perithecia) on the florets, pink mycelia growth at the margins of the glumes, a darkened rachis and seeds that are shriveled and bleached (Sutton 1982; Martens et al. 1988). An additional effect of FHB is the accumulation of mycotoxins (most commonly with *F. graminearum* deoxynivalenol or DON) in the grain (Snijders 1990). Mycotoxins render the grain unsuitable for food or feed (Gilbert and Tekauz 2000). Thus, FHB affects grain yield, seed germination, grade and end use quality due to the release of proteases and accumulation of DON (Wong et al. 1992).

Resistance to FHB

Cultural methods intended to reduce inoculum loads and fungicidal control may not effectively eliminate or even substantially control FHB (Bai and Shaner 1994). This is partly due to the ability of *F. graminearum* to incite severe FHB even under low inoculum loads if conditions are sufficiently moist (McMullen et al. 1997). Thus, introgressing FHB resistance into elite germplasm has become a priority for wheat breeders (Waldron et al. 1999).

Resistance to FHB is quantitative and shows a continuous distribution (Bai and Shaner 1994). The resistance of wheat to FHB has been classified into different types of resistance which are type I: resistance to inititial infection of the fungus, type II: resistance to the spread of the fungus with the spike, type III: resistance to kernel

infection, type IV: tolerance to disease (disease has a reduced impact on yield loss) and type V: resistance to DON accumulation in the grain (Schroeder and Christensen 1963; Mesterházy 1995). Efforts to genetically map FHB resistance has focused on type I and particularly on type II resistance (Waldron et al. 1999; Buerstmayr et al. 2002; Somers et al. 2003).

The Chinese wheat cultivar Sumai 3 has good resistance to FHB and has been the subject of many genetic studies (eg. Waldron et al. 1999; Buerstmayr et al. 2002; Cuthbert et al. 2006, 2007). Other sources of FHB resistance include Ning, Wanshuibai, Frontana, Wuhan 1 and Nyubai (Zhou et al. 2002; Buerstmayr et al 2002; Somers et al. 2003; Steiner et al. 2004; Han et al. 2005). These sources have mostly been examined for type II resistance (resistance to disease spread), however type I (resistance to initial infection) and type IV (resistance to DON accumulation) resistances have been studied to a lesser extent. The genotype by environment variance component of FHB resistance is generally high (Campbell and Lipps 1998). This complicates genetic and mapping studies involving FHB resistance.

Until recently, FHB resistance has been mapped as quantitative trait loci (QTL). QTL have been identified on wheat chromosomes 2A (Waldron et al. 1999), 3A (Steiner et al. 2004), 3B (Waldron et al. 1999; Bai et al. 1999), 5A (Buerstmayr et al. 2002; Somers et al. 2003; Steiner et al. 2004), 6B (Anderson et al. 2001), 2D (Somers et al. 2003) and 4B (Somers et al. 2003). These have been largely mapped as type II resistance. The QTL on chromosome 3B, or more specifically 3BS, has been mapped repeatedly (Waldron et al. 1999; Bai et al. 1999; Anderson et al. 2001; Buerstmayr et al. 2002). In these studies the effect of the QTL on 3BS explained between 25-60% of

variance associated with FHB resistance. Buerstmayr et al. (2003) indentified QTL from Sumai 3 on chromosomes 3B and 5A. The evidence suggested that the QTL on 3B was largely conferring type II resistance and the QTL on 5A conferred type I resistance. Fine mapping of the Sumai 3 FHB resistance QTL on chromosome 3BS using sequence tagged sites (STS) indentified a narrow interval containing a major gene conferring the FHB resistance (Lui et al. 2006; Cuthbert et al. 2006). The gene was named *Fhb1* and has been marked with an STS marker that has reported genetic distances as little as 0.2 cM depending on the population (Cuthbert et al. 2006). The resistance conferred by the QTL on 5A may not be as strong as that from the 3B QTL however the resistance from 5A is still significant and useful (Buerstmayr et al. 2003). The second named FHB resistance gene, *Fhb2*, came from fine mapping the 6B resistance QTL derived from Sumai 3 (Cuthbert et al. 2007).

In a recent study where resistance to DON accumulation was examined it was found that the monogenic resistance mapped to same genetic region on chromosome 3BS that had previously been identified as a type II resistance QTL (Lemmens et al. 2005). The authors found that infected kernels in resistant lines had detoxified DON by converting DON to DON-3-O-glucoside. Previously a QTL for resistance to DON accumulation had been reported to coincide with the QTL for type II resistance on 3BS (Somers et al. 2003). Further QTL for DON resistance were identified on chromosomes 2D and 5A. These QTL corresponded with QTL for height and FHB resistance respectively (Somers 2003).

Breeding for FHB resistance can be assisted using DNA markers. One factor that makes selecting FHB resistance difficult is the high environmental influence on FHB

infection and resistance. This can result in low heritability, making marker-assisted selection (MAS) an important tool for targeted breeding efforts (van Sanford et al. 2001). Complex crossing and MAS strategies have been implemented to generate adapted germplasm with good FHB resistance, desired agronomic performance and acceptable end use quality characteristics (Somers et al. 2003). Thomas et al. (2004) suggested a cytogenetic approach for fixing FHB resistance in breeding populations. This strategy employs a Robertsonian translocation that will pair with two chromosomes, each carrying FHB resistance QTL on the arms that are not paired with the translocated chromosome. This places the chromosome arms carrying the QTL in the hemizygous condition thus gametes receiving the standard (non-translocated) chromosomes must have the QTL. As a result of the predominant meiotic configuration of the trivalent (the translocation paired with the two standard chromosomes) there is quasi-linkage between the QTL on the different chromosomes because the meiotic configuration promotes the co-segregation of these chromosomes to the same pole. Coupled to the favorable non-Mendelian segregation of the chromosomes carrying the QTL (i.e. they do not assort independently) is the increased success of male gametes carrying the standard chromosomes, and thus both QTL, compared to pollen carrying the translocation. This results in rapid fixation of the QTL in the population without selection for FHB resistance. One negative effect of this method is the large linkage block associated with the hemizygous chromosome arms.

Haploid wheat

Haploid wheat ($n = 3x = 21$, ABD), also referred to as a polyhaploid, can be generated in different ways. In some early research, wheat haploids were produced

inadvertently as a result of random parthenogenesis (Gaines and Aase 1926; Person 1955). Intergeneric crosses have also produced haploids, although the objective of these experiments was to study intergeneric hybrids, not to generate haploid wheat (Sears 1939; Riley and Chapman 1957). Recent techniques for producing haploids include chromosome elimination in interspecific crosses, anther culture, and microspore culture.

Certain intergeneric crosses with wheat, where wheat is the female, generate wheat haploids by chromosome elimination (Barclay 1975; Laurie and Bennett 1988). Intergeneric pollination results in a fertilized embryo with a haploid chromosome set from each parent. Chromosomes are eliminated, for example in wheat by maize (*Zea mays* L.) crosses, because of abnormal mitotic behavior of paternal chromosomes. Normal maize chromosome constrictions at the centromere and secondary constrictions are less visible or absent. These centromeres fail to function normally, have a low affinity for wheat spindle, and tend to lie away from the metaphase plate. Consequently the number of maize chromosomes per cell decreases with each cell division until none remain. Most wheat embryos have lost all maize chromosomes after the third cycle of cell division (Laurie and Bennett 1989). Embryo rescue is required to recover high frequencies of haploids due to early embryo and endosperm abortion (Laurie and Bennett 1988, 1989). The number of embryos produced by wheat by maize crosses can be increased by treating wheat florets with synthetic hormones such as 2,4-dichlorophenoxyacetic acid (2,4-D). This results in an increased number of pollen tubes that reach the micropyle (Wedzony and Lammeren 1996).

Several different pollinators have been used to produce wheat haploids by chromosome elimination including *Hordeum bulbosum* L. (Barclay 1975), *Zea mays* L.

(Laurie and Bennett 1988), *Tripsacum dactyloides* (Li et al. 1996), and rye (*Secale cereale* L.) (Sears 1939). Tetraploid *H. bulbosum* was thought to be a promising pollinator for haploid wheat production (Barclay 1975). However two genes that restrict crossability between wheat and rye, *Kr1* (Riley and Chapman 1967) and *Kr2* (Sitch et al. 1985), have been shown to cause incompatibility between genotypes of wheat and *H. bulbosum* (Snape et al. 1979; Falk and Kasha 1981). In addition, some genetic control in *H. bulbosum* also affects crossability, but this is mostly due to ploidy level (Sitch and Snape 1986). Diploid *H. bulbosum* is less a less effective pollinator of wheat than tetraploid *H. bulbosum* (Barclay 1975; Sitch and Snape 1986). It was shown in durum wheat (*Triticum turgidum* L.) that germination of *H. bulbosum* pollen was constant across durum genotypes, however inhibition of pollen tube growth occurred in the ovaries (O'Donoughue and Bennett 1994).

Producing haploid wheat by crossing with maize is advantageous because maize pollen is insensitive to *Kr1* and *Kr2* (Laurie and Bennett 1987, 1988). Thus, the success rate of haploid wheat production is more consistent across wheat genotypes when pollinating with maize as compared to *H. bulbosum* (O'Donoughue and Bennett 1994). However genotypes of wheat and maize do result in some variation in success rate. Genotype of durum wheat influences ovary development, embryo and plant formation, opposed to maize, where genotype only influences embryo formation (Cherkaoui et al. 2000). Similarly, in hexaploid wheat the maize genotype influenced embryo formation (Verma et al. 1999).

Crossing wheat with *Tripsacum dactyloides* results in a higher rate of embryo formation compared to crossing with maize, and displays similar insensitivity to *Kr*

genes. However, the haploid wheat plants recovered from such crosses frequently have *T. dactyloides* chromosomes that were not eliminated (Li et al. 1996).

Two methods of androgenesis can be used to recover haploid plants, anther culture and microspore culture. Anther culture is a process where microspores become embryogenic while in the anther. Uninucleate microspores, that are starch-free, develop into embryo-like structures from calli that progress through the normal embryo stages including the globular, heart, and torpedo stages (Nitsch and Nitsch 1969). A drawback of anther culture is that many plantlets recovered are albino. The ability of wheat genotypes to generate green plants is under the additive polygenic control of nuclear genes (Zhou and Konzak 1992). This could be partially overcome by increasing the number of plantlets produced, which is made possible by using modified culture media (Zhou and Konzak 1989). Similar to male certation in monosomic wheat, gametic selection favoring 21 chromosome microspores occurs in anther culture (DeBuyser et al. 1989).

Microspore culture is similar to anther culture in that microspores are become embryonic at the uninucleate stage. However unlike anther culture, microspore culture isolates individual microspores that produce pseudoembryos from microspores with fibrillar cytoplasm and avoid callus formation. This technique has been refined to the point where up to 5500 green plants can be produced from a single wheat spike (Liu et al. 2002). Microspore culture is advantageous because of the nearly 4-fold increase in green plant recovery compared to anther culture (Holme et al. 1999). The time needed to produce haploid wheat by chromosome elimination or by androgenesis is approximately the same. However androgenesis is more cost effective on a per plant basis (Snape et al.

1986). Comparisons of genetic maps developed from doubled haploids arising from chromosome elimination (female meiosis) and anther culture (male meiosis) revealed significant differences in genetic distances between RFLP markers used to generate the map (Wang et al. 1995).

Cytogenetic stocks in wheat

In wheat there are numerous stocks that carrying chromosomal aberrations ranging from small deletions to the nullisomic/tetrasomic series (see Table 1 for definitions). The uses of these stocks are numerous, including locating genes to a chromosome, mapping genes relative to the centromere, accurate physical gene mapping and demonstrating the homœologous relationship between wheat chromosomes (see below). Haploid wheat plants ($n = 3x = 21$, ABD) were the starting point from many of these aneuploid stocks (Sears 1939). The first report of a haploid wheat plant occurred in 1926 (Gaines and Aase 1926).

Haploid wheat plants show male sterility but some of the female gametes are viable and will set seed if pollinated with pollen from a normal plant. Meiosis in haploids is irregular thus the viable female gametes often contain aberrations such as chromosome deletions, chromosome additions and translocations (Sears 1939). Sears (1939) suggested two principle routes for the origin of monosomes in the progeny of pollinated haploids. One likely route is the random meiotic elimination of one or more univalents from a partially restituted egg sac. If such an ovule is pollinated the normal result is single or multiple monosomic of standard (untranslocated) karyotype. However,

Table 1 – Definitions of cytogenetic stocks of wheat.

Cytogenetic stock	Definition
Disomic	Both members of a homologous pair are present.
Haploid	Only half the number of chromosomes are present with each homologue represented once.
Monosomic	Only one member of a homologous pair is present.
Nullisomic	No members of a homologous pair is present.
Telosomic	A chromosome that is missing one arm.
Monotelodisomic	One member of a homologous pair is a normal chromosome and the other member is a telocentric.
Ditelosomic	Both members of the homologous pair are telocentric chromosomes that are missing the same arm.
Double monotelodisomic	Two different homologous pairs are both in the monotelodisomic condition.
Tetrasomic	Four copies of a given homologue.
Nullisomic/tetrasomic	When one homologous pair is missing (nullisomic) and a different homologue is in the tetrasomic condition. Most of the maintained stocks carry nullisomic/tetrasomic combinations from the same homoeologous group.
Deletion	Part of a chromosome is absent.
Isochromosome	A chromosome with the same chromosome on each side of the centromere.

monosomy can also arise from disjunction of the mostly open bivalents that are observed in haploid meiosis (Gaines and Aase 1926; Person 1955; Riley and Chapman 1957; Kimber and Riley 1963; Jauhar et al. 1991; Thomas et al. 1997), when subsequent first metaphase disjunction of all or most of the univalents is followed by restitution of the second division. Recovering such ovules by pollination should give rise to monosomic-trisomic duplications and deficiencies. Since bivalents present in the original haploid meiocyte are usually intergenomic (Jauhar et al. 1991, 1999), and usually involve homœologues, the resulting duplication-deficiencies are expected to show homœologous compensation. Isolated terminal markers from otherwise deficient chromosomes have been detected in the progeny of haploids, presumably translocated to a homœologue by non-homologous crossover (Thomas et al. 2001; Hiebert et al. 2008).

Sears (1939, 1944) was able to isolate a complete series of monosomics in Chinese Spring from the progeny of haploids and individuals nullisomic for chromosome 3B. Individuals nullisomic for 3B were partially asynaptic and monosomy and trisomy were found regularly in their progeny. Monosomic stocks are useful for assigning genes to chromosomes (Sears 1953). The nullisomic individuals that can be recovered from monosomics have been used to compare the phenotypic effects of different deficiencies (Sears 1939, 1944).

Univalents can misdivide during meiosis giving rise to isochromosomes (a chromosome with the same arm on both sides of the centromere) and telocentric chromosomes (chromosomes with one arm deleted) (Sears 1952a; Morris et al. 1977). The frequency univalent misdivision has been calculated by observing pollen mother cell (PMC) in anaphase I and anaphase II. Most of the data collected has been for

homœologous group 5 chromosomes, especially chromosome 5A (Sears 1952a; Morris et al. 1977). The frequency that isochromosomes and telocentrics were recoved is less than expected based on the frequency of univalent misdividion (Sears 1952a). Sears (1952a) speculates that the products of anaphase II misdivision are usually lost which would approximately account for the discrepancy between observed apparent univalent misdivision and recovery of the products of misdivision. Although the data available is relatively limited, the frequency of misdivision is variable (2% to 41%) and significant difference have been observed between different chromosomes within the same variety and between the same chromosome across different varieties (Sears 1952a; Morris et al. 1977). All of the possible 42 telocentric chromosomes have been isolated (Sears 1978). Of these, 41 were isolated in Chinese spring and telo-7DL was isolated in Canthatch (Sears and Sears 1978). Some of the uses of telocentric chromosomes include indentifying monosomics and preventing univalent shift, indentifying trisomes and translocations, assigning genes to chromosome arms and determining linkage to the centromere, determining cytological characters such as arm length and determining relatedness of chromosomes by studying non-homologous pairing (Sears 1962; Sears and Sears 1978).

There are 42 nullisomic/tetrasomic (nulli/tetra) combinations in wheat that are compensating. This compensation, called homœologous compensation, demonstrated that are 3 homœologous genomes in wheat (Sears1952b; 1966). This means that abnormalities caused by nullisomy for one chromosome can be offset by tetrasomy of a homœologous chromosome. Other nulli-tetra combinations do not show such compensation (Sears 1966). Nulli-tetra combinations were developed by crossing

monosomic or nullisomic plants with trisomic or tetrasomic plants (Sears 1966). While the demonstration of homœologous groups was perhaps the most important contribution, the nulli/tetra series is still a valuable tool in modern genetics. For example, the nulli-tetra series was used to anchor microsatellite marker linkage groups to chromosomes (Röder et al. 1998).

More recently a series has been developed that contain chromosome deletions of varying size (Endo and Gill 1996). When a particular chromosome from *Aegilops cylindrical* L. is found in Chinese Spring in the monosomic condition an array of terminal deletions are produced in the gametes (Endo 1988). Similarly, alien chromosome additions from *A. truncialis* can also generate deletions (Endo and Gill 1996). Using the above mechanisms approximately 300 lines were developed that are homozygous for different deletions and, in some cases, multiple deletions (Endo and Gill 1996). The extent of the deletions was determined using C-banding. These deletion stocks represent a powerful tool for physical mapping in wheat.

Chapter 2 -Microsatellite mapping of adult-plant leaf rust resistance gene *Lr22a* in wheat

As published in: Hiebert CW, Thomas JB, Somers DJ, McCallum BD, Fox SL (2007) Microsatellite mapping of adult-plant leaf rust resistance gene *Lr22a* in wheat. Theor Appl Genet 115:877-884.

Summary

This study was conducted to identify microsatellite markers (SSR) linked to the adult-plant leaf rust resistance gene *Lr22a* and examine their cross-applicability for marker-assisted selection in different genetic backgrounds. *Lr22a* was previously introgressed from *Aegilops tauschii* Coss. to wheat (*Triticum aestivum* L.) and located to chromosome 2DS. Comparing SSR alleles from the donor of *Lr22a* to two backcross lines and their recurrent parents showed that between two and five SSR markers were co-introgressed with *Lr22a* and the size range of the *Ae. tauschii* introgression was 9-20 cM. An F_2 population from the cross of 98B34-T4B x 98B26-N1C01 confirmed linkage between the introgressed markers and *Lr22a* on chromosome 2DS. The closest marker, GWM296, was 2.9 cM from *Lr22a*. One hundred and eighteen cultivars and breeding lines of different geographical origins were tested with GWM296. In total 14 alleles were amplified, however only those lines predicted or known to carry *Lr22a* had the unique *Ae. tauschii* allele at GWM296 with fragments of 121 bp and 131 bp. Thus, GWM296 is useful for selecting *Lr22a* in diverse genetic backgrounds. Genotypes carrying *Lr22a* showed strong resistance to leaf rust in the field from 2002 to 2006. *Lr22a* is an ideal candidate to be included in a stack of leaf rust resistance genes because

of its strong adult-plant resistance, low frequency of commercial deployment, and the availability of a unique marker.

Introduction

Leaf rust (*Puccinia triticina* Eriks.) of wheat (*Triticum aestivum* L.) is a widespread foliar disease that causes significant reductions in grain yield and quality (Samborski 1985). Host genetic resistance is a desirable and proven method of leaf rust control. However, deployment of single race-specific genes allows the pathogen to evolve and accumulate new virulence (Dyck and Kerber 1985). This creates the need to identify new effective genes and can create an ongoing cycle of breeding, deployment and subsequent erosion of resistance.

Over 50 leaf rust resistance (Lr) genes have been identified in wheat. Of these, approximately half were introgressed from related species (McIntosh et al. 1995). Five named Lr genes have been introgressed into common wheat from *Aegilops tauschii* Coss.; these are *Lr21* (Rowland and Kerber 1974), *Lr32* (Kerber 1987), *Lr41* (Cox et al. 1994; Singh et al. 2004), *Lr42* (Cox et al. 1994), and the adult-plant resistance (APR) gene *Lr22a* (Rowland and Kerber 1974). It should be noted that while *Lr22a* is expressed only at the adult-plant stage, the degree of resistance conferred is comparable to highly resistant seedling Lr genes in contrast to the slow-rusting type APR conferred by genes like *Lr34*.

In the search for improved genetic solutions for leaf rust resistance it is widely believed that combinations or stacks of multiple Lr genes would confer a more durable resistance than the same genes deployed individually. Such gene stacks might include race non-specific adult plant resistance genes (e.g. *Lr34*), "undefeated" genes for which

no virulence has been detected such as *Lr22a*, and partially "defeated" genes, for which virulence already exists in the pathogen population (e.g. *Lr16*). One advantage of creating gene stacks is the synergistic interaction of leaf rust resistance genes which often confers a higher level of resistance than would be expected from the level of resistance demonstrated by the genes in isolation (Samborski and Dyck 1982). The resistance genes *Lr34* (German and Kolmer 1992) and *Lr13* (Kolmer 1992) were both demonstrated to enhance the level of resistance synergistically when in combination with other Lr genes. Complex stacks of Lr genes can be difficult to construct using phenotypic selection. For example the Canadian cultivar Pasqua carries five Lr genes including *Lr11*, *Lr13*, *Lr14b*, *Lr30* and *Lr34*, but failed to retain *Lr16* and *Lr22a* from the parental cross (Dyck 1993a). With suitable markers a stack of seven Lr genes could have been stabilized in this cross.

Close molecular markers for all Lr genes can help in the assembly and dissection of complex gene stacks in any cross. *Lr22a* has been physically mapped to chromosome 2DS using telocentric mapping (Rowland and Kerber 1974). In this paper we report both the genetic location and the identification of a closely linked, unique molecular marker allele for *Lr22a* that was retained from *Ae. tauschii*. The application of this marker in different genetic backgrounds was examined on a broad range of wheat germplasm.

Materials and Methods

Plant material and populations

Lr22a was previously transferred to a synthetic hexaploid RL5404 by crossing tetra-Canthatch ($2n$=$2x$=28, AABB) with *Aegilops tauschii* var. *strangulata* RL5271 ($2n$=14, DD; Dyck and Kerber 1970, Rowland and Kerber 1974). This was followed by six backcrosses with Thatcher to produce the line RL6044 (Thatcher*7//tetra-

Canthactch/RL5271) which was then backcrossed with AC Domain to produce the leaf rust resistant line 98B34-T4B (AC Domain*6/RL6044) with *Lr22a*. RL5404 was also crossed with Neepawa (Neepawa*6/RL5404) to create the *Lr22a*-carrying near-isogenic line (NIL) RL4495.

An F_2 population that segregated for *Lr22a* was created by crossing 98B34-T4B with the leaf rust susceptible line 98B26-N1C01 (AC Domain*2/Sumai3//Grandin*2/AC Domain).

Thatcher NILs carrying single Lr genes (*Lr11*, *Lr13*, *Lr16*, *Lr22a* and *Lr34*), cultivars carrying *Lr22a* (AC Minto) and cultivars believed to carry *Lr22a* (5500HR and 5600HR) were used in field tests to evaluate leaf rust resistance.

Disease rating

Segregation of *Lr22a* was followed in the 98B34-T4B x 98B26-N1C01 F_2 population by inoculating flag leaves with *Puccinia triticina* isolate TJBJ-77-2 (Long and Kolmer 1989; McCallum and Seto-Goh 2003) following the procedures of McCallum and Seto-Goh (2003). Plants were grown with 16 hours supplemental lighting in a greenhouse at approximately 20°C. Twelve days after inoculation plants were classified as resistant (infection types ; to 1), moderately resistant (infection types 1+ to 2) and susceptible (infection types 3 to 4) as described by Stakman et al. (1962). Parents of the F_2 population were tested for leaf rust resistance at the seedling and adult stage.

Thatcher NILs and cultivars carrying *Lr22a* were grown in field plots at Glenlea, MB, Canada from 2003 to 2006 and 2002 to 2006 respectively to observe Lr gene effectiveness. Plants were infected with natural leaf rust inoculum and rows of susceptible wheat throughout the nurseries were artificially inoculated with a mixture of

virulence phenotypes found in western Canada during the previous year. Adult-plants were rated for leaf rust based on a modified Cobb scale for incidence (Peterson *et al.* 1948) and severity was scored as R = resistant (flecks and small uredinia with necrosis), MR = moderately resistant (large necrotic flecks and large uredinia), M = moderate (mixture of uredinia sizes), MS = moderately susceptible (moderate to large uredinia with chlorosis), and S = susceptible (large uredinia without chlorosis or necrosis).

Molecular Mapping

DNA was extracted from lyophilized leaf tissue using a modified CTAB extraction (Kleinhofs et al. 1993). PCR was performed with a cycle of 2 min. 94°C, then 1 min. 95°C, 1 min 50°C - 60°C, and 50 sec. 73°C for 30 cycles, followed by 5 min. 73°C, using the following conditions: PCR buffer 1x, dNTPs 0.2mM each, $MgCl_2$ 1.5 mM, primers 10 pmol each, *Taq* DNA polymerase 1 U, and approximately 50 ng genomic DNA. PCR products were separated on 5% denaturing polyacrylamide gels in TBE buffer (89 mM tris, 89 mM boric acid, 20 mM EDTA) at 85 W for 2 hours, and stained with silver (Promega, Madison, WI, USA). *Lr22a* has been previously located on chromosome 2DS (Rowland and Kerber 1974). Microsatellite marker (SSR) alleles located on chromosome 2DS were compared between RL5271, RL5404, RL6044, Thatcher, 98B34-T4B, and AC Domain. Polymorphic markers were tested for linkage to *Lr22a* using 68 of the susceptible individuals from the F_2 population of 98B34-T4B x 98B26-N1C01. Susceptible F_2 plants were used for mapping because their genotypes at the *Lr22a* locus were known. These susceptible individuals were selected for pustule uniformity to avoid assigning homozygous susceptible genotypes at the *Lr22* locus to heterozygous individuals that would falsely appear to be recombinants. The location of

linked SSR alleles was confirmed using nulli/tetra and ditelocentric cytogenetic stocks (Sears 1966; Sears and Sears 1978).

SSR markers on chromosome 2DS were compared between RL5271, RL5404, RL6044, Thatcher, 98B34-T4B, AC Domain, RL4495, Neepawa, BW63, AC Minto, 5500HR, and 5600HR to determine which markers were co-introgressed with *Lr22a* from *Ae. tauschii* into Canadian wheat.

New SSR markers were added to the Agriculture and Agri-Food Canada Cereal Research Centre (AAFC-CRC) International Triticeae Mapping Initiative (ITMI) genetic map (Opata85/Synthetic W-7984; Somers et al. unpublished, ITMI-CRC). A newly developed Opata85/Synthetic doubled haploid (DH) population was tested to confirm the marker order on 2DS found in the ITMI-CRC population. The ITMI-CRC map was used to align the *Lr22a* linkage group with the genetic map of 2DS. Genetic distances were calculated using the Kosambi mapping function (Kosambi 1944). All maps were constructed using MapMaker version 3.0 (Lander et al. 1987).

To asses the potential of GMW296, the closest marker to *Lr22a*, as a selection tool in varying genetic backgrounds 118 different cultivars and breeding lines were surveyed (Table 3). Alleles from these genotypes were compared to the allele amplified from RL5271, the *Ae. tauschii* donor of *Lr22a*.

Results

Fourteen polymorphic microsatellite markers on chromosome 2DS were evaluated in Thatcher, AC Domain, the *Aegilops tauschii* donor of *Lr22a* (RL5271), the original synthetic (RL5404 = Tetra-Canthatch/RL5271) and two backcross lines (RL6044 = Thatcher*7/RL5404 and 98B34-T4B = AC Domain*6/RL6044). Microsatellite alleles

from GWM296 and GWM455 were introgressed from RL5271 via RL5404 to these two resistant backcross lines (Table 2; Figure 1). Thus GWM296 and GWM455 alleles from RL5271 have remained associated with *Lr22a* through a minimum of 13 cycles of recombination in common wheat.

The same 14 SSR markers were used to assess the introgression of *Lr22a* into RL4495 (Neepawa*6/RL5404) and its derivatives which included BW63, AC Minto, 5500HR and 5600HR. RL4495 retained *Ae. tauschii* alleles from GWM455, GWM296, GWM261, WMC25, and WMC503, and transmitted them to its derivatives (Table 2). Two other introgression sizes were detected in RL4495, one with GWM455, GWM296, and GWM261, and one with GWM455 and GWM296. Since these introgressions are smaller than the one found in BW63 they cannot be in the line of descent to AC Minto, 5500HR and 5600HR as these cultivars have retained the largest introgression found in RL4495.

In the F_2 of 98B34-T4B x 98B26-N1C01, 88 plants were resistant, 216 plants had a moderately resistant infection type and 102 plants were susceptible. These numbers fit both a 3:1 ratio (resistant plus moderate resistant : susceptible; $\chi^2_{3:1} = 0.02$, $p = 0.87$), and a 1:2:1 ratio (resistant : moderate resistant : susceptible; $\chi^2_{1:2:1} = 2.64$, $p = 0.27$). As adult plants 98B34-T4B was resistant to *P. triticina* and 98B26-N1C01 was susceptible, while as seedlings both lines were susceptible. This confirms that the F_2 population segregated for the APR conferred by *Lr22a*. The *Ae. tauschii* alleles of GWM296 and GWM455 (both present in 98B34-T4B) were both polymorphic with respect to 98B26-N1C01. Based on 68 susceptible F_2 plants representing 136 gametes, GWM296 was 2.9 cM and GWM455 was 4.4 cM from the *Lr22a* locus (Figure 2). Testing with nulli-2D/tetra-2B,

Table 2 – SSR alleles on chromosome 2DS introgressed along with *Lr22a* from *Ae. tauschii* into common wheat.

	Alleles on 2DS[b]									
Locus[a]	RL5271[c]	RL5404[c]	RL4495[c]	BW63[c]	AC Minto	RL6044[c]	98B34-T4B[c]	Neepawa	Thatcher	AC Domain
BARC124	T	T	A	A	A	A	A	A	A	A
WMC111	T	T	A	A	A	A	A	A	A	A
GWM210	T	T	A	A	A	A	A	A	A	A
GWM455	T	T	T	T	T	T	T	A	A	A
GWM296	T	T	T	T	T	T	T	A	A	A
Lr22a	T	T	T	T	T	T	T	A	A	A
GWM261	T	T	T	T	T	A	A	A	A	A
WMC025	T	T	T	T	T	A	A	A	A	A
WMC503	T	T	T	T	T	A	A	A	A	A
WMC112	T	T	A	A	A	A	A	A	A	A
WMC470	T	T	A	A	A	A	A	A	A	A
GWM484	T	T	A	A	A	A	A	A	A	A
WMC453	T	T	A_1	A_1	A_2	A_1	A_2	A_1	A_1	A_2
GWM102	T	T	A_2	A_2	A_1	A_2	A_2	A_2	A_2	A_2
GWM515	T	T	A	A	A	A	A	A	A	A

[a] GWM markers are from Röder et al. (1998); WMC markers are from Somers et al. (2004); BARC markers are from Song et al. (2005).

[b] T = *Ae. tauschii* allele; A = *T. aestivum* allele; subscript numbers differentiate between alleles within the same species.

[c] RL5271 = *Ae. tauschii* donor of *Lr22a*; RL5404 = synthetic hexaploid from tetra-Canthatch/RL5271; RL4495 = Neepawa*6/RL5404; BW63 is derived from RL4495 and is the donor of *Lr22a* in all currently registered Canadian wheat cultivars; RL6044 = Thatcher*6/RL5404; 98B34-T4B = AC Domain*6/RL6044. Two other introgressions were detected in RL4495 but are not shown because they lack the full introgression transferred to AC Minto via BW63.

nulli-2A/tetra-2B, ditelo 2DS, and ditelo 2DL confirmed that the locus of GWM296 shown to be linked to the APR of *Lr22a* was on chromosome 2DS (Figure 1).

A minor conflict exists between our proposed order for markers close to *Lr22a* and those published elsewhere (Raupp et al. 2001). The marker order was clarified by re-genotyping lines recombinant in the region and adding additional markers to the ITMI-CRC map. An alternate DH population from the ITMI cross was also used to make a comparative map. The DH map agreed with the ITMI-CRC map (data not shown). Based on these results, we concluded that the marker order on 2DS is as shown (Table 2; Figure 2) with the GWM296 locus 2.9 cM distal to *Lr22a*.

One hundred and five North American wheat cultivars and breeding lines, four Asian lines, eight European cultivars and one South American wheat cultivar were surveyed for alleles of GWM296 (Table 3). In total 14 alleles were detected, with the most common being that found in Thatcher (Figure 1). Most Canadian hard-red spring wheat cultivars are derived from Thatcher (McCallum and DePauw 2008). In this collection of wheat, the allele from *Ae. tauschii* RL5271 was present in those lines and cultivars that were expected to carry *Lr22a* and was absent from all others (Table 3). BW63 was heterogeneous for GWM296 with half the individuals tested carrying the RL5271 allele. Two products were amplified from RL5271 that were 131 bp and 121 bp in size (Figure 1). The size range of all other chromosome 2DS alleles found were between 167 bp and 135 bp.

Cultivars expected to carry *Lr22a*, including AC Minto, 5500HR, and 5600HR, showed strong resistance to *P. triticina* in the field from 2002-2006 (Table 4). Furthermore, no isolates of *P. triticina* that are virulent to *Lr22a* have been found in

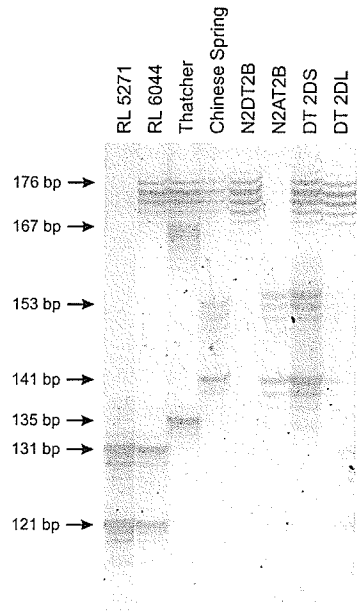

Figure 1 – Alleles of GWM296 found in RL5271 (*Lr22a* donor), RL6044 (Thatcher*6/RL5271; carries *Lr22a*), Thatcher (recurrent wheat parent), Chinese Spring, nulli-2D/tetra-2B, nulli-2A/tetra-2B, ditelo 2DS, and ditelo 2DL on a silver-stained polyacrylamide gel. The allele associated with *Lr22a* has DNA fragments that are 131 bp and 121 bp in size. A common chromosome 2DS allele in the North American wheat lines tested has the 167 and 135 bp fragments. The 176 bp fragment is from the chromosome 2A locus.

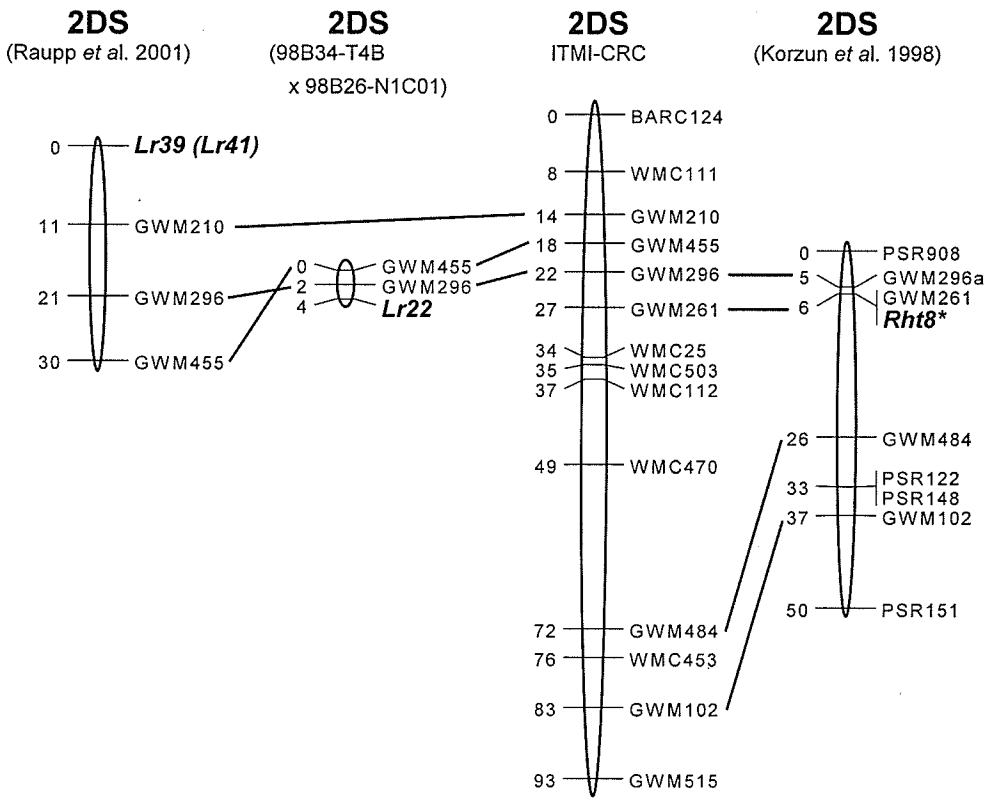

Figure 2 – Alignment of the *Lr22a* and ITMI-CRC maps with two previously published maps of chromosome 2DS. The map of 98B34-T4B/98B26-N1C01 was based on 68 F_2 plants while the ITMI-CRC map was derived from 68 recombinant inbred lines from the cross of Opata85/W-7984 (W-7984 = *Ae. tauscii*/Altar84 durum; this cross is commonly called Opata/Synthetic). The map of Raupp et al. (2001) reports the mapping of *Lr39*, however Singh et al. (2004) found that *Lr39* = *Lr41*.

Table 3 – Cultivars[a] from different geographical origins screened with GWM296, the closest marker to *Lr22a*, to survey allele diversity and cross-applicability.

Ae. tauschii allele	Other alleles[b]		
North America	*North America*	CDC Teal	Superb
5500HR	5601HR	Columbus	Thatcher
5600HR	AC Barrie	Grandin	*South America*
AC Minto	AC Bellatrix	Invader	Frontana
BW63[c]	AC Cadillac	Kanata	*Asia*
	AC Cora	Katepwa	Chinese Spring
	AC Domain	Lancer	Nyu Bai
	AC Elsa	Laura	Wuhan-1
	AC Intrepid	McClintock	Sumai-3
	AC Majestic	McKenzie	*Europe*
	AC Readymade	Neepawa	Alcedo
	AC Splendor	Norstar	Alidos
	AC Tempest	Park	Altos
	Alikat	Pasqua	Bezostaya
	Alsen	Prodigy	Kontrast
	CDC Falcon	Roblin	Milan 13
	CDC Kestrel	Snowbird	Welford
	CDC Osprey	Somerset	Zentos

[a] Only named cultivars are listed. An additional 65 North American breeding lines were tested. Six of these breeding lines carried the *Ae. tauschii* allele. Five of these had AC Minto as a parent while the sixth line was 98B34-T4B (see Materials and Methods).

[b] Thirteen alleles of GWM296 were found in addition to the allele introgressed from *Ae. tauschii*.

[c] BW63 was not a released cultivar, however it was an important Lr gene source in Canadian wheat breeding programs. The allele scores for BW63 indicate it was heterogeneous for the GWM296 marker and presumably for *Lr22a*.

Canadian virulence surveys between 2002 and 2005 (McCallum and Seto-Goh 2005; McCallum and Seto-Goh unpublished data; Table 4). The Thatcher NILs tested in the field showed that *Lr22a* provided good resistance singly, while *Lr34* showed moderate resistance, *Lr16* provided moderate to poor resistance, and *Lr11* and *Lr13* showed no improved resistance compared to Thatcher (Table 4).

Discussion

Wheat SSR maps reveal that coverage by polymorphic markers is non-uniform (Somers et al. 2004). Of the three genomes of common wheat, the D genome was added most recently and has the shortest evolutionary life span at the hexaploid level. Therefore, the degree of polymorphism is lowest for the D genome chromosomes. By contrast, *Ae. tauschii* is an older, polymorphic, broadly distributed diploid that has contributed the D genome to many polyploids in the Triticeae. These differences in polymorphism were evident in this study as all 14 SSR markers tested on chromosome 2DS were polymorphic between the *Ae. tauschii* accession RL5271 and the two recurrent parents, Thatcher and AC Domain. Thatcher and AC Domain were monomorphic for 13 of the 14 SSR markers (Table 2).

In this study SSR markers previously mapped to 2DS were compared between backcross lines and the recurrent parents in order to identify candidate markers that might be linked to *Lr22a*. Out of the 14 loci investigated, two polymorphic markers were transferred through thirteen rounds of backcrossing and both were then shown to be closely linked to the target gene. This screening strategy was successful because of the high marker density and high degree of polymorphism between chromosome 2DS of *Ae. tauschii* and the corresponding chromosome of common wheat.

Table 4 – Virulence of *P. triticina* on *Lr22a* and to selected cultivars and NILs in Canada.

Year	No. of isolates[a]	Virulence on *Lr22a*	Field reaction to *P. triticina*[e]								
			AC Minto[b]	5600 HR[c]	5500 HR[c]	Tc[d]	Tc-*Lr11*[d]	Tc-*Lr13*[d]	Tc-*Lr16*[d]	Tc-*Lr22a*[d]	Tc-*Lr34*[d]
2002	71	0	TR R	TR R	10 RMR	80 S	-	-	-	-	-
2003	29	0	TR R	0 R	0 R	80 S	70 S	80 S	65 M	7 RMR	25 M
2004	58	0	3 MR	0 R	15 MR	80 S	80 S	80 S	60 M	19 MR	20 M
2005	81	0	15 RMR	5 RMR	5 RMR	80 S	80 S	80 S	70 MS	20 M	40 M
2006	-	-	0 R	0 R	0 R	80 S	80 S	80 S	50 M	10 RMR	40 M

[a] Survey of Canadian *P. triticina* isolates for 2002 is from McCallum and Seto-Goh (2005) and 2003 to 2005 is from McCallum and Seto-Goh (unpublished data).

[b] AC Minto is reported to have *Lr11*, *Lr13* and *Lr22a* (Kolmer 1992).

[c] 5600 HR and 5500 HR are derivatives of AC Minto that carry the SSR allele linked to *Lr22a*. Our evidence suggests that these cultivars carry *Lr11*, *Lr16* and *Lr22a*. It is also possible that these two carry *Lr34*.

[d] Tc = Thatcher, Tc-*Lr11* = RL6053, Tc-*Lr13* = RL4031, Tc-*Lr16* = RL6005, Tc-*Lr22a* = RL6044, Tc-*Lr34* = RL6058.

[e] Field ratings are based on a modified Cobb scale for incidence (Peterson *et al.* 1948) and severity was scored as R = resistant (flecks and small uredinia with necrosis), MR = moderately resistant (large necrotic flecks and large uredinia), M = moderate (mixture of uredinia sizes), MS = moderately susceptible (moderate to large uredinia with chlorosis), and S = susceptible (large uredinia without chlorosis or necrosis), TR = a trace amount of visible infection.

Based on recombination in the F_2 population, the minimum size of the segment introgressed into RL6044 and 98B34-T4B was 4.4 cM and the probable size was 9 cM (assuming the crossovers occurred at the midpoint of the flanking intervals; Figure 2). In contrast, the *Lr22a* introgression from RL5271, via RL5405, into RL4495 included up to five SSR markers. The largest introgression represents a minimum of 17 cM and a probable size of 20 cM as estimated from the ITMI-CRC map (Table 2, Figure 2). This larger introgression has persisted from RL4495 into three Canadian cultivars, AC Minto, 5500HR, and 5600HR.

Three-point linkage values observed in the F_2 population (98B34-T4B/98B26-N1C01) confirmed that *Lr22a*, GWM296 and GWM455 are closely linked (Figure 2). In a study of durable leaf rust resistance in a Swiss winter wheat QTL analysis revealed a significant narrow QTL close to the GWM296 locus (Schnurbusch et al. 2004). Schnurbusch et al. (2004) speculated that this QTL on 2DS could represent *Lr22* and our data supports this hypothesis. GWM455 was previously reported to be polymorphic between Thatcher and RL6044 in an effort to exclude *Lr22a* as an allele of other *Lr* genes introgressed from *Ae, tauschii*, but linkage experiments between *Lr22a* and GWM455 were not performed (Raupp et al. 2001).

Other genes of interest are found near the *Lr22* locus on chromosome 2DS. *Lr41* (*Lr41=Lr39*; Singh et al. 2004) is distal to GWM210 (Raupp et al. 2001) whereas *Lr22a* is proximal. The order of loci near *Lr22* is inconsistent between our map (ITMI-CRC) and the map of Raupp et al. (2001). After re-genotyping lines recombinant on 2DS, the marker order as presented in the ITMI-CRC map is the most likely (Figure 2). However, the marker orders in the ITMI-CRC map and the map presented by Raupp et al. (2001)

both place *Lr22a* and *Lr41* on opposite sides of GWM210 (Figure 2). Both introgressions of *Lr22a* into wheat, RL6044 and RL4495, retained SSR markers proximal of GWM210. Furthermore, *Lr41* is a seedling resistance gene and *Lr22a* is an adult plant resistance gene. McIntosh et al. (1995) recorded no instances of an adult-plant resistance gene with an allele that confers seedling resistance for any of the three rusts of wheat. In the particular case of *Lr22* there are two alleles that have been reported and both provide adult-plant resistance (Rowland and Kerber 1974; Dyck 1979). This data agrees with the conclusion of Raupp et al. (2001) that *Lr41* is not allelic to *Lr22a.* The dwarfing gene *Rht8* is closely linked with GWM261 (Korzun et al. 1998) and is proximal to the *Lr22* locus (Figure 2).

Even though the D genome of wheat has reduced polymorphism 14 alleles for GWM296 were identified on 2DS. Although both of the 2DS alleles illustrated in Figure 1 have two fragments, other genotypes tested exhibited a single fragment. For example, the 2DS allele of GWM296 found in AC Splendor has a single fragment of 167 bp while Superb has fragments of 149 bp and 137 bp (not shown). Thus heterozygotes may have two, three or four fragments from chromosome 2DS while homozygotes may have one or two. Therefore caution is required when classifying an individual's genotype without prior knowledge of parental haplotypes and the degree of homozygosity. It should be noted that GWM296 also amplified a locus on chromosome 2A; however this larger fragment (176 ± 4 bp) did not overlap with the 2DS fragments in any of the genotypes tested. Despite the allele diversity of GWM296 on chromosome 2D the allele that was co-introgressed with *Lr22a* from *Ae. tauschii* was not found in any common wheat lines tested except for those believed to carry *Lr22a* (Table 3). The uniqueness of the

GWM296 allele, ease of scoring, and its close association with *Lr22a* provides a means to include *Lr22a* in complex gene stacks and to classify existing lines for the distribution of *Lr22a* provisionally.

In the survey of wheat breeding lines and cultivars GWM296 was useful in tracking the transmission of *Lr22a* through the genealogy of Canadian wheat. BW63 is an important source of leaf rust resistance in the pedigree of several Canadian bread wheat varieties; containing *Lr11*, *Lr14b*, *Lr22a*, *Lr30* and *Lr34* (Dyck 1993a). The data indicated that BW63 was heterogeneous for the presence of *Lr22a* and this could explain why AC Minto (Columbus/BW63//Katepwa/BW552; Townley-Smith et al. 1993) inherited *Lr22a* from BW63 while Pasqua (BW63*2/Columbus) did not (Dyck 1993a). Since BW63 would have demonstrated a high degree of adult-plant resistance it would have been difficult to track the incorporation *Lr22a* during the development of Pasqua. All of the Canadian cultivars (Table 3) and breeding lines tested that inherited the RL5271 allele of GWM296, and presumably *Lr22a,* were derived from AC Minto except for 98B34-T4B (AC Domain*6/3/Thatcher*7//tetra-Canthatch/*Ae. tauschii* RL5271).

In Canada *Lr22a* is deployed in AC Minto (registered in 1991) and putatively in 5500HR (2000) and 5600HR (1999). There are no reports of US cultivars that carry *Lr22a* (JA Kolmer, personal communication). The Canadian cultivars that carry *Lr22a* have only occupied small percentages (0.13% - 0.95%) of the wheat production area in Canada from 1998 to 2006 (Canadian Wheat Board, http://www.cwb.ca). Thus *Lr22a* has had relatively low exposure to *P. triticina* in Canada and the US. The detection of an ineffective race-specific "*b*" allele of *Lr22* (Dyck 1979) implies that *Lr22a* will also

prove to be race specific. The absence of virulence on *Lr22a* could be explained in part by its lack of exposure to *P. triticina*.

AC Minto carries *Lr11*, *Lr13* and *Lr22a* (Kolmer 1997). Presense of *Lr22a* in AC Minto is confirmed by presense of the five *Ae. tauschii* alleles found in BW63 (Table 2). Each year AC Minto showed field resistance that was better than the *Lr22a* NIL, indicating that *Lr13* and/or *Lr11* had the ability to interact synergistically with *Lr22a* even though they were ineffective independently (Table 4). *Lr13* is known to interact synergistically with some other Lr genes (Kolmer 1992), however no data has been reported on the potential interaction between *Lr13* and *Lr22a.* Cultivars 5600HR (AC Minto//Columbus/Roblin) and 5500HR (AC Minto/3/MN72506/Columbus//RL4473) both received the *Ae. tauschii* alleles found in BW63, and presumably *Lr22a*, via AC Minto (Table 2). In addition to *Lr22a* 5600HR and 5500HR may also carry *Lr11*, *Lr13*, and *Lr16*, and *Lr11* and *Lr16* respectively based on pedigree and their reactions to different *P. triticina* virulence phenotypes (Samborski and Dyck 1982; Dyck 1993b; B. McCallum unpublished). In the field all three Canadian cultivars that carry *Lr22a* showed leaf rust resistance that is better than the individual genetic components of their resistance (Table 4). Although *Lr22a* appeared to be the key component of the leaf rust resistance in these cultivars, the undesirable scenario where *Lr22a* is deployed as a single resistance gene has not occurred.

It would be desirable to stack *Lr22a* with other broadly effective resistance genes such as *Lr21* (Huang and Gill 2001; Huang et al. 2003) or *Lr34* (Lagudah et al. 2006) that have been characterized with reliable DNA markers. While the markers and mapping we have described can facilitate the stacking of *Lr22a* with *Lr21*, in fact markers are not

needed to stack these two genes together. *Lr21*, or other seedling Lr genes, could be detected in the presence of *Lr22a* with seedling tests while *Lr22a* could be fixed by top crossing or back crossing with one or more "single gene" lines. Markers may prove more beneficial in complex crosses with several Lr genes segregating.

The low level of deployment in commercial cultivars and the lack of reported virulence to *Lr22a* (Table 4; Park and McIntosh 1994; McCallum and Seto-Goh 2005; Kolmer et al. 2005) make it a good candidate for use in Lr gene stacks. While various strategies can be used to include *Lr22a* in complex gene combinations the marker reported here will facilitate gene stacking without the need to backcross. With careful management *Lr22a* could be widely deployed while maintaining its status as a broad-spectrum and effective Lr gene.

Chapter 3 – Stacking pairs of disease resistance genes in wheat populations using telocentric chromosomes

Summary

Two fungal diseases of wheat that are important in the Canadian prairies are fusarium head blight (FHB) and leaf rust. Host genetic resistance to these diseases is more effective and durable when resistance genes or quantitative trait loci (QTL) are found in stacks. This study investigates the effect of placing pairs of genes in the hemizygous condition using telocentric chromosomes to facilitate gene stacking. Four resistance gene combinations were analyzed in four populations. These combinations were *Lr16/Lr34*, *Lr22a/Lr52* and *Lr34/Lr46* for resistance to leaf rust and a combination of FHB resistance genes *Fhb1* and QTL Qfhs.ifa-5A. Each of these gene combinations were involved in a crossing and selection scheme that produced F_1 plants that were either dihybrid or double monotelodisomic. For each resistance gene combination F_3 families were produced for phenotypic testing. The *Lr16/Lr34* and *Lr22a/Lr52* populations showed a sharp increase in leaf rust resistance amongst families derived from double monotelodisomics. A smaller increased resistance found in the *Lr34/Lr46* and FHB populations. This was caused by additional, uncharacterized resistance in the *Lr34/Lr46* population and the relatively low heritability of FHB resistance. The four telosome combinations (2BL/7DL, 2DL/5BL, 1BS/7DL and 3BL/5AL) were tested for the male and female transmission of hemizygous chromosome arms using reciprocal testcrosses. Male transmission frequency of the telosomes was about 0.25 due to pollen competition while the average female transmission was about 0.5. For F_7 plants derived from double monotelodisomics the predicted frequency of individuals homozygous for both resistance

genes was about 0.5 compared to 0.25 for plants derived from dihybrids. However, this prediction is likely an underestimate as ditelosomic or double ditelosomic individuals would be less fit thus adding an element of zygotic selection on top of gamete selection. We conclude that telocentric chromosomes are a useful breeding tool for rapidly fixing gene stacks.

Introduction

Wheat (*Triticum aestivum* L.) is a cereal crop that is economically important in Canada. Breeders have many objectives including yield, time to maturity, end use quality and disease resistance (McCallum and Depauw 2008; Poehlman 1987). There are many diseases that wheat breeders must consider in their programs. In Canada some diseases of wheat are fusarium head blight (FHB), leaf rust, stripe rust, stem rust, septoria tritici and tan spot (Martens et al. 1988). In the eastern prairies both leaf rust and FHB are the diseases currently of most concern.

Leaf rust, caused by *Puccinia triticina* Eriks., is responsible for an annual yield losses of 5-15% in western Canada (Samborski 1985). Losses will exceed this level when high inoculum loads, favourable environmental conditions for the pathogen and poor resistance in popular cultivars are found (McCallum and Seto-Goh 2003). With the recent naming of *Lr60*, there are approximately 50 named leaf rust resistance (Lr) genes to date (McIntosh et al. 1995). Annual disease surveys reveal which of these Lr genes are most useful for wheat breeders to incorporate into their programs.

In Canada the major pathogen present in FHB is *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) in Canada (Clear and Patrick 2000). FHB affects grain yield, seed germination, grade and end use quality (Wong et al.

1992). The fungus persists over winter as a saprophyte on crop residue. Ascospores, and to a lesser extent macroconidia, are released and infect wheat heads in early anthesis (Sutton 1982; Markell and Francl 2003; Del Ponte et al. 2003). Infected heads show bleached florets, a darkened rachis and peduncles, and pink mycelia at the edge of the glumes and seeds that are shrivelled and bleached (Sutton 1982; Martens et al. 1988). The fungus also releases mycotoxins into the seed that makes the grain unsuitable for human consumption or feed (Snijders 1990; Gilbert and Tekauz 2000). Resistance to FHB has been identified and mapped in wheat (Bai and Shaner 1994; Waldron et al. 1999; Buerstmayr et al. 2002; Somers et al. 2003). Most of the resistance genes that have been identified derive from Japanese, Chinese or Brazilian cultivars. Though several quantitative trait loci (QTL) for FHB have been indentified, the QTL on chromosomes 3B, 5A and 6B have been studied the most (eg. Waldron et al. 1999; Bai et al. 1999; Anderson et al. 2001; Buerstmayr et al. 2002, 2003; Somers et al. 2003). Recently two of the QTL (3BS and 6BS) were mapped as Mendelian genes and were re-named *Fhb1* (3BS) and *Fhb2* (6BS) (Lui et al. 2006; Cuthbert et al. 2006, 2007).

For both FHB and leaf rust, resistance is improved when multiple resistance genes or QTL are stacked (Bai and Shaner 1994; Kolmer 1999). Availability of closely linked DNA markers allows the stacking of several resistance genes (Schachermayr et al. 1994; Hussien et al. 1997; Huang and Gill 2001; Somers et al. 2003). While markers provide a means to select desired allele combinations in breeding populations the breeder is still limited by the low frequency at which these combinations are recovered. Telocentric chromosomes and Robertsonian translocations have been proposed as tools for increasing the frequency of desired alleles in breeding populations (Thomas et al. 2003, 2004). In

the case of telocentric chromosomes, the wheat midge resistance gene *Sm1* was shown to increase in frequency without selection when it was placed in the hemizygous condition in the F_1 (Thomas et al. 2003). The increase in frequency is caused by the competitive advantage of pollen and zygotes carrying the standard chromosome versus the telosome. Pairs of genes can be stacked when a Robertsonian translocation is deficient for the chromosome arms carrying the genes of interest (Thomas et al. 2004). This places both resistance genes in the hemizygous condition. The trivalent formed by the translocation and the two standard chromosomes carrying the desired alleles is most often (>90%) found in a "V" configuration. This causes co-migration of both standard chromosomes to the same pole during meiosis. Thus, the pollen with 21 chromosomes, which have higher male transmission than pollen carrying the translocation, will carry both of the desired alleles (Thomas et al. 2004). Both of these cytogenetic approaches increase the frequency of the desired allele(s) in the population with each generation; thus more material can be kept for selection on other criteria.

Cytogenetic stocks have been shown to provide a mechanism to increase the frequency of desired pest resistance genes in wheat breeding populations (Thomas et al. 2003, 2004). To date only a single telocentric chromosome has been used to fix a monogenic trait (Thomas et al. 2003). The purpose of this study is to investigate the effectiveness of using pairs of telocentric chromosomes to increase the frequency of gene stacks for leaf rust and FHB resistance in wheat populations.

Materials and Methods

Plant material

Seven different telocentrics were used to produce four populations. These were 1BS (A2743/Key 190//97B64-K15C3), 2BL (M16/Superb//AC Cadillac), 2DL (ditelo Chinese Spring developed by E.R. Sears), 3BL (M16/Superb//AC Cadillac), 5AL (M16/Superb//AC Cadillac/3/Superb), 5BL (RL6107/AC Foremost//Superb/3/BW346), 7DL (ditelo in Canthatch developed by E.R. Kerber). It should be noted that telo 1BS was an abnormal telosome that had the short arm satellite deleted. Six lines with different Lr genes were used as sources of leaf rust resistance for three of the four populations. These were *Lr16* (99B60-EJ2L), *Lr22a* (98B34 = AC Domain*6/RL6044), *Lr34* (RL6058 and Glenlea), *Lr46* (CG203 = Parula; obtained from Dr. Ravi Singh), and *Lr52* (RL6107). One line, CF121 (Remus/CM-82036; obtained from Dr. Herman Buerstmayr), carried FHB resistance genes *Fhb1* and Qfhs.ifa-5A on chromosomes 3BS and 5AS respectively.

DNA extraction, PCR conditions and electrophoresis

DNA was extracted from macerated lyophilized leaf tissue or from seeds that were cut in half, with the half containing the embryo retained for planting, and the other half crushed into a fine powder for extraction. The DNA was extracted using the hexadecyltrimethyl-ammonium bromide (CTAB) method (Kleinhofs et al. 1993) except that phenol was not used. DNA was suspended in 0.1X TE buffer (0.1 mM tris, 0.01 mM EDTA) that contained RNAse (10μg/ml).

All PCR reactions were performed under the following conditions: PCR buffer 1x, dNTPs 0.2mM each, $MgCl_2$ 1.5 mM, primers 10 pmol each, Taq DNA polymerase 1 U, and approximately 50 ng genomic DNA; 2 min. 94°C, then 1 min. 95°C, 1 min 50-60°C, and 50 sec. 73°C for 30 cycles, followed by 5 min. 73°C. PCR products were run

on 5% denaturing polyacrylamide gels in TBE buffer (0.089M tris, 0.089M boric acid, 0.050M EDTA) at 85 W for 1.5-2 hours, and visualized by silver staining (Promega, Madison, WI, USA; manufacturers protocol).

Populations

Four populations were produced to study the following gene stacks: *Lr16* and *Lr34*, *Lr22a* and *Lr52*, *Lr34* and *Lr46*, and *Fhb1* and Qfhs.ifa-5A. Each of these populations were generated in pairs from two classes of F_1 plants, those that carried both genes in the hemizygous condition ($2n = 40 + t + t$) and those that carried both genes in the heterozygous condition ($2n = 42$). All of the plants used to generate the populations in this study were grown in growth cabinets with 16 hours of light at 18-20°C and 8 hours of dark at 16-18°C.

The various crossing schemes used to generate the dihybrid and double monotelodisomic F_1 plants are outlined in Figure 3. The markers used for selection in each population are shown in Table 5. All of the karyotypes were confirmed by cytological examination of pollen mother cells (PMC) in metaphase I of meiosis from fixed anthers (6 95% ethanol : 3 chlorofrom : 1 glacial acetic acid) that were stained with aceto-carmine. The selected dihybrid and double monotelodisomic F_1 progeny for each population were used to make $F_{2:3}$ families for disease evaluation.

Inoculations with *P. triticina* were performed by suspending urediospores in light mineral oil (Bayol 55, Imperial Oil Canada, Toronto, ON) and spraying the suspension on the leaves of seedlings at the two leaf stage or on the flag leaves for adult plant testing. Plants were placed near a fan for 15 minutes to speed oil evaporation and were moved

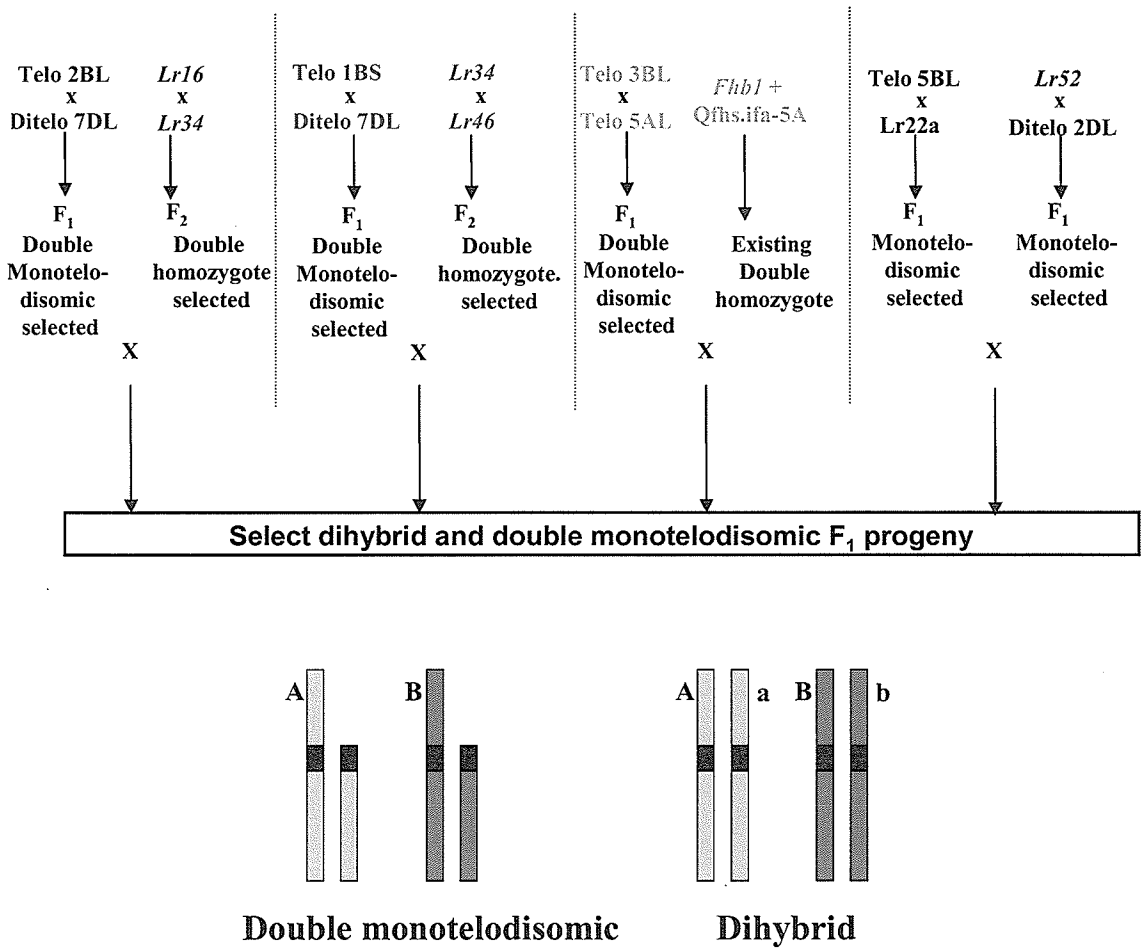

Figure 3 – Crossing schemes used to generate dihybrid and double monotelodisomic F_1 plants for each of the four populations. The chromosome locations of the resistance genes used are: *Lr16* (2BS), *Lr34* (7DS), *Lr46* (1BL), *Fhb1* (3BS), Qfhs.ifa-5A (5AS), *Lr22a* (2DS) and *Lr52* (5BS). Included is a schematic figure of two genes in the heterozygous condition as found in a dihybrid plant compared to two genes found in the hemizygous condition as found in a double monotelodisomic plant.

Table 5 – Markers used to select resistance genes and chromosome arms in each of the four populations.

Population	Gene or arm	Marker[a]	Reference
Lr16/Lr34	*Lr16*	WMC764 & phenotype	McCartney et al. 2005
	Lr34	GWM295 & GWM1220	Spielmeyer et al. 2005
	2BS	WCM764	
	7DS	GWM295	
Lr22a/Lr52	*Lr22a*	GWM296 & phenotype	Hiebert et al. 2007
	Lr52	Phenotype	
	2DS	GWM296	
	5BS	GWM443	
Lr34/Lr46	*Lr34*	GWM295 & GWM1220	Spielmeyer et al. 2005
	Lr46	WMC44 & BARC80	Suenaga et al. 2003
	1BL	GWM140	
	7DS	GWM295	
FHB	3BS	GWM493	
	5AS	GWM304	

[a] GWM markers are from Röder et al. (1998); WMC markers are from Somers et al. (2004); BARC markers are from Song et al. (2005).

into a dew chamber for approximately 15 hours. After incubation in the dew chamber the plants were moved to growth cabinets under the conditions described above. Twelve days after inoculation the seedlings were rated for their infection type using the scale described in McIntosh et al. (1995).

Evaluation of F_3 families

F_3 families from the *Lr16/Lr34* population and the *Lr34/Lr46* population each had 2 replicates, including susceptible and resistant checks, in a complete randomized block planted at Portage la Prairie, Manitoba on May 28, 2007. Spreader rows of leaf rust susceptible cultivars were seeded within the experimental plots and surrounding the experimental plots. The plots were sown in 0.62 metre rows. The proposed second location in 2007 was unsuitable for seeding, thus a second planting of an additional two replicates was planted at Portage la Prairie on June 1, 2008. Spreader rows were inoculated with a proportional and representative mixture of *P. triticina* races found in the Canadian prairies in 2006. Inoculations were performed by suspending urediospores in a light mineral oil and spraying the suspension onto the spreader row plants. The inoculations occurred on a day before anticipated evening dew shortly after the spreader row plants had begun to tiller. The plots were rated for incidence of leaf rust approximately at the onset of anthesis and when the susceptible checks showed good infection and symptoms.

The *Lr22a/Lr52* population had limited amounts of seed thus a combination of field and indoor evaluation was performed. Two replicates of complete randomized blocks were seeded in 0.62 metre rows at Portage la Prairie, MB on June 1, 2007. Inoculations with *P. triticina* and rating were as described above. For the indoor

evaluation, F_2 seedlings were inoculated with *P. triticina* at the two leaf stage. After rating the disease response, clean leaf tissue was used to extract DNA and individuals were tested with GWM296 to predict the presence of *Lr22a*. DNA extraction, PCR and electrophoresis were performed as described above. Ten individuals from F_3 families were grown and inoculated with *P. triticina* as seedlings. Any families that were entirely susceptible at the seedling stage and were predicted to not carry *Lr22a* were tested for their leaf rust reaction at the adult plant stage. As an additional check some families that were susceptible as the seedling stage, but were predicted to carry *Lr22a* were also inoculated at the adult plant stage.

The FHB population was seeded in two reps in a complete randomized block design at two locations, Portage la Prairie, MB and Carman, MB on May 28, 2007 and June 6, 2007 respectively. In Portage la Prairie the rows were 0.62 metres long and in Carman the rows were 1.5 metres long. In Carman the inoculations were performed by spraying 50 mL of a conidial suspension (5×10^4 spores per mL of distilled water) per row when the row was at 50% anthesis and again two days later. Plots were mist irrigated following inoculation. In Portage la Prairie FHB inoculations were performed by spreading corn kernels infected with *F. graminearium* when the plots were at stage 37-38 of the Zadocks growth scale (when the flag leaf emerges). The field was irrigated to promote perithecial development and subsequent infection of the plots with ascospores. Plots were rated 18 to 21 days after initial inoculation in Carman and after the checks showed clear symptoms in Portage la Prairie. An FHB index was determined by multiplying the percentage of infected heads (incidence) by the average percentage of infected spikelets in heads showing infection (severity).

For all populations comparisons were made between families derived from dihybrids and from double montelodisomics by calculating the mean and variance of each group. Significance was established using t-tests and F-tests respectively.

Transmission of hemizygous chromosome arms

Individuals that were double monotelodisomic were testcrossed as male and female to find the frequency of male and female transmission of hemizygous chromosome arms. Individuals that were double monotelodisomic for telo 3BL and 5AL were crossed with CF121. All other double monotelodisomic individuals (2BL/7DL, 2DL/5BL and 1BS/7DL) were crossed with AC Elsa. To track the transmission of hemizygous chromosomes arms progeny from each testcross was tested with one SSR per hemizygous arm by using DNA extracted from macerated half-seeds.

Results

Evaluation of F_3 families

The F_2 from the *Lr16/Lr34* population, 94 derived from double monotelodisomic F_1 plants and 94 derived from dihybrid F_1 plants, were inoculated as seedlings with *P. triticina*. Of the 94 progeny from the double monotelodisomic 92 were resistant to *P. triticina* and two plants were stunted and died. The two stunted plants appeared to have a susceptible reaction however the leaves were too narrow to accurately assess the phenotype. The F_2 derived from disomic F_1 plants followed a 3:1 resistant to susceptible ratio (63 resistant : 31 susceptible; $p = 0.07$) for a single gene (*Lr16*) as *Lr34* is not detected in seedlings.

The distribution of average leaf rust incidence across four observations showed that F_3 families derived from double hemizygous F_1 plants ($n = 85$) was less than the F_3

Table 6 – The means, variance and significance tests for the disease ratings in each of the four populations. In the *Lr16/Lr34, Lr22a/Lr52* and *Lr34/Lr46* populations the mean measures the average leaf rust incidence and the in the FHB population the mean measures the average FHB index.

Population	F_1 karyotype[1]	Mean	t-test p value	Variance	F statistic p value
Lr16/Lr34	dmtd	3.912	<0.01	8.398	<0.01
	disomic	19.830		88.339	
Lr22a/Lr52	dmtd	3.864	<0.01	4.249	<0.01
	disomic	9.193		90.652	
Lr34/Lr46	dmtd	3.405	0.125	2.099	<0.01
	disomic	4.310		40.534	
FHB	dmtd	0.099	<0.01	0.006	0.226
	disomic	0.152		0.008	

[1] dmtd – double monotelodisomic

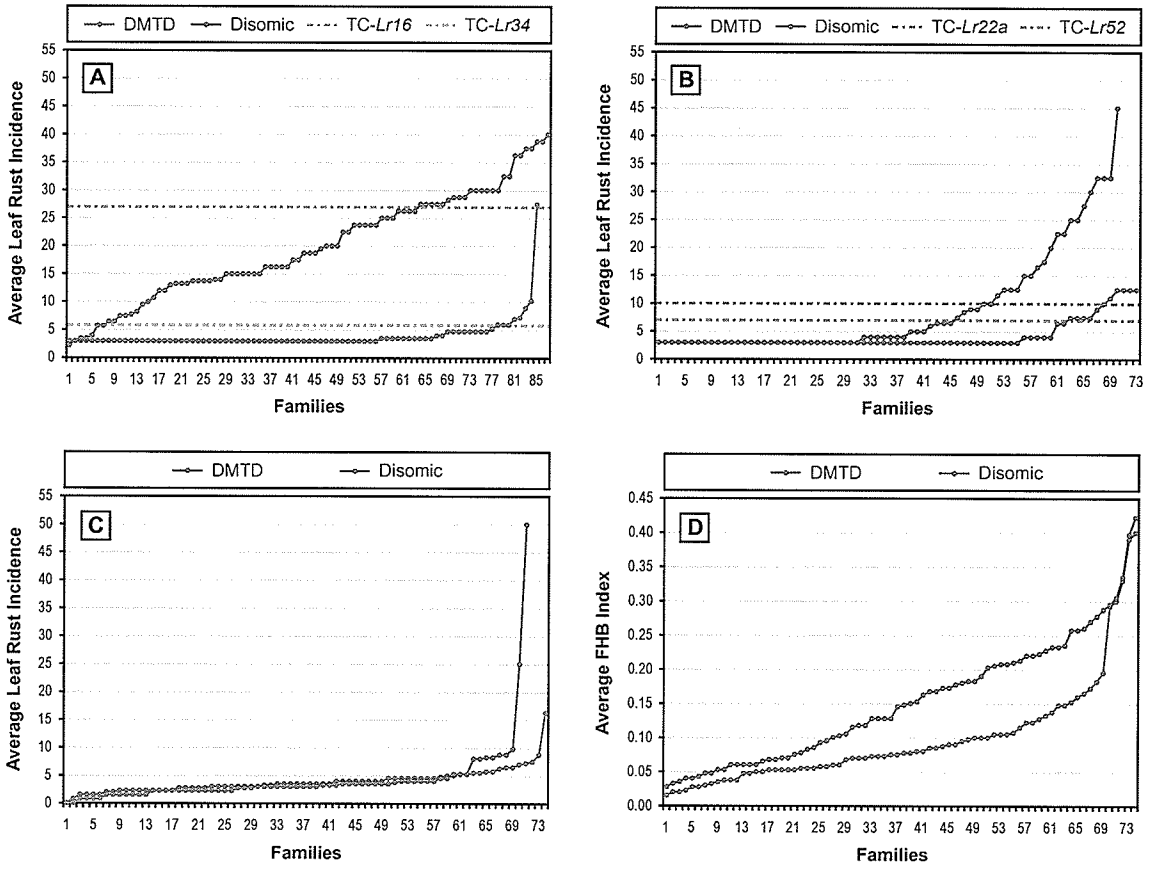

Figure 4 - Distribution of F_3 families sorted by average disease rating for each population. The families derived from double monotelodisomic (dmtd) F_1 plants were plotted separately from the families derived from disomic F_1 plants. **A** – F_3 families from the *Lr16/Lr34* population. The average ratings for Tc-*Lr16* and Tc-*Lr34* were plotted as a dotted line for an approximate threshold the each gene singly. **B** - F_3 families from the *Lr22a/Lr52* population. The average ratings for Tc-*Lr22a* and Tc-*Lr52* were plotted as a dotted line for an approximate threshold the each gene singly. **C** – F_3 families from the *Lr34/Lr46* population. **D** - F_3 families from the *Fhb1* and Qfhs.ifa-5A population

families derived from dihybrid F_1 plants (n = 87) (Figure 4a). The mean incidence and variance for rust severity (0-100%) of the double hemizygous-derived families was 3.912 and 8.398, respectively, compared to 19.830 and 88.339 for families derived from dihybrids (Table 6). Thus, families derived from double hemizygotes had much lower infection and were more uniform.

The indoor evaluation of F_2 individuals and F_3 families showed Mendelian segregation for *Lr22a* and *Lr52* in plants derived from disomic F_1 plants and skewed segregation in plants derived from double monotelodisomic F_1 plants (Table 7). The number of individuals that carried neither resistance gene corresponded to expected values for both disomic and double monotelodisomic derived individuals. The phenotypes (rust reaction) of the F_3 families matched expected phenotypes based on F_2 seedling inoculations (*Lr52*) and molecular marker data (*Lr22a*). In each of the seedling susceptible families GWM296 accurately predicted the presence or absence of *Lr22a* as confirmed by adult plant inoculations with *P. triticina*. None of the progeny derived from double monotelodisomic F_1's entirely lacked both *Lr22a* and *Lr52*. However four F_2 plants derived from dihybrid F_1's, and their F_3 progenies, were homozygous for the susceptible allele at both loci. This value closely matches the expected value of 5.2, or 1/16 of the population. The F_3 families from these four susceptible plants showed a high incidence of leaf rust in the field trial. As expected, the F_2 progeny from double monotelodisomic plants showed only the presence or absence of the GWM296 allele closely linked to *Lr22a* (i.e. no heterozygotes) while F_2 progeny from disomic plants were homozygous for either allele or heterozygous and fit the expected 1:2:1 ratio ($p = 0.28$).

Table 7 – Frequency of resistance alleles found in F_2 plants segregating for *Lr22a* and *Lr52* that were derived from either dihybrid F_1 plants or double monotelodisomic F_1 plants that were tested in growth cabinets. *Lr22a* was tracked using a closely linked microsatellite marker and classified as having *Lr22a* or the susceptible allele and *Lr52* was tracked by recording infection types at the seedling stage following inoculation with *P. triticina*.

	Predicted F_2 phenotype based on GWM296			Seedling resistance (*Lr52*) in F_2			Plants susceptible at both loci	
F_1 source	Res	Sus	$\chi^2_{3:1}$	Res	Sus	$\chi^2_{3:1}$	Expected	Observed
dihybrid	64	19	p = 0.66	65	18	p = 0.49	5.2	4
dmtd[a]	78[b]	5[c]	p = 0.00007	77	6	p = 0.0002	0.4	0

[a] dmtd – double monotelodisomic.

[b] No heterozygotes were observed in this class of progeny.

[c] These individuals were null for the microsatellite.

In replicated field testing of the *Lr22a/Lr52* population the mean and the variance for the disomic-derived F_3 families (n = 70), 9.193 and 90.652 respectively, were higher than 3.864 and 4.249 found for families derived from double monotelodisomic plants (n = 73) (Table 6). The distribution of the average incidence of leaf rust illustrates the lower and more uniform resistance in families derived from double monotelodisomic plants (Figure 4b).

The level of leaf rust resistance in the dihybrid-derived F_3 families of the *Lr34/Lr46* population was unexpectedly high with only a few susceptible families. Consequently the overall distribution of leaf rust incidence across F_3 families was similar for families derived from dihybrid F_1 plants (n = 71) and families derived from double monotelodisomic F_1 plants (n = 74) (Figure 4c). The average leaf rust incidences between these two classes of F_3 families were not significantly different, though the mean of the F_3 families derived from dihybrid F_1 plants was slightly higher due to the increased level of susceptibility in the right hand tail (Figure 4c, Table 6). While the variances between the two classes were significantly different (Table 6), this difference is the result of only two highly susceptible families derived from the dihybrid (Figure 4c). Therefore, apart from the right hand tail, resistance of these two groups can be considered similar.

The distribution of average FHB index shows that F_3 families derived from double monotelodisomic plants (n = 74) had less FHB than families derived from dihybrids (n = 74) (Figure 4d). The population means, which were significantly different, confirm the graphical trend that the resistance found in F_3 families from double monotelodisomic F_1's was better than that found in families derived from dihybrids

Table 8 – Transmission frequencies of hemizygous chromosome arms and gamete class frequencies as determined by reciprocal testcrosses.

		Testcross transmission				Gamete classes[1]			
Combination		Arm	Freq.	Arm	Freq.	S/S	T/T	S/T	T/S
2BS/7DS	Male	2BS	0.71	7DS	0.76	49	4	19	24
	Female	2BS	0.52	7DS	0.47	20	19	30	27
2DS/5BS	Male	2DS	0.79	5BS	0.78	62	3	18	13
	Female	2DS	0.53	5BS	0.54	30	23	21	22
1BL/7DS	Male	1BL	0.85	7DS	0.73	62	7	19	7
	Female	1BL	0.47	7DS	0.55	55	47	46	65
3BS/5AS	Male	3BS	0.71	5AS	0.75	87	7	36	43
	Female	3BS	0.41	5AS	0.49	31	46	38	51

[1] S/S = gametes with all standard chromosomes, T/T = gametes with both telosomes, S/T = gametes carrying the second telosome listed in the combination, T/S = gametes carrying the first telosome listed in the combination.

(Table 6). Variances of the two sources of F_3 families were not significantly different (Table 6).

Transmission of hemizygous chromosome arms

Hemizygous chromosome arms were transmitted at higher frequencies through male gametes than through female gametes as determined by reciprocal testcrosses (Table 8). The transmission of hemizygous chromosome arms through pollen deviated significantly from a 1:1 ratio in all cases and the deviation always favoured the presence of the hemizygous arm. In contrast, the female transmission of the hemizygous arm did not deviate significantly from a 1:1 ratio with the exception of 3BS, which was transmitted less often than gametes that were null for 3BS (Table 8). When tested with χ^2 contingency testing the gametic classes was independent (i.e. one telosome did not alter the transmission of the other). Using these transmission values (Table 8) a model was constructed to predict the frequency of homozygosity up to F_7 for two genes found in the hemizygous condition in the F_1 (i.e. F_1 plants that are double monotelodisomic) compared to the expected frequency of fixation in plants arising from dihybrids (Figure 5). The expected fixation frequency by F_7 for two genes using telosome combinations of 2BL/7DL, 2DL/5BL, 1BS/7DL and 3BL/5AL are 0.52, 0.65, 0.62 and 0.46 respectively. This is substantially higher than the value of 0.242 expected in a standard population (i.e. standard karyotype). It should be noted that this model assumes equal viability and fertility of ditelo classes compared to disomic classes (i.e. no other zygotic selection). Thus, these models are based solely on gametic selection and likely underestimate the actual rate of fixing two genes that are hemizygous in the F_1.

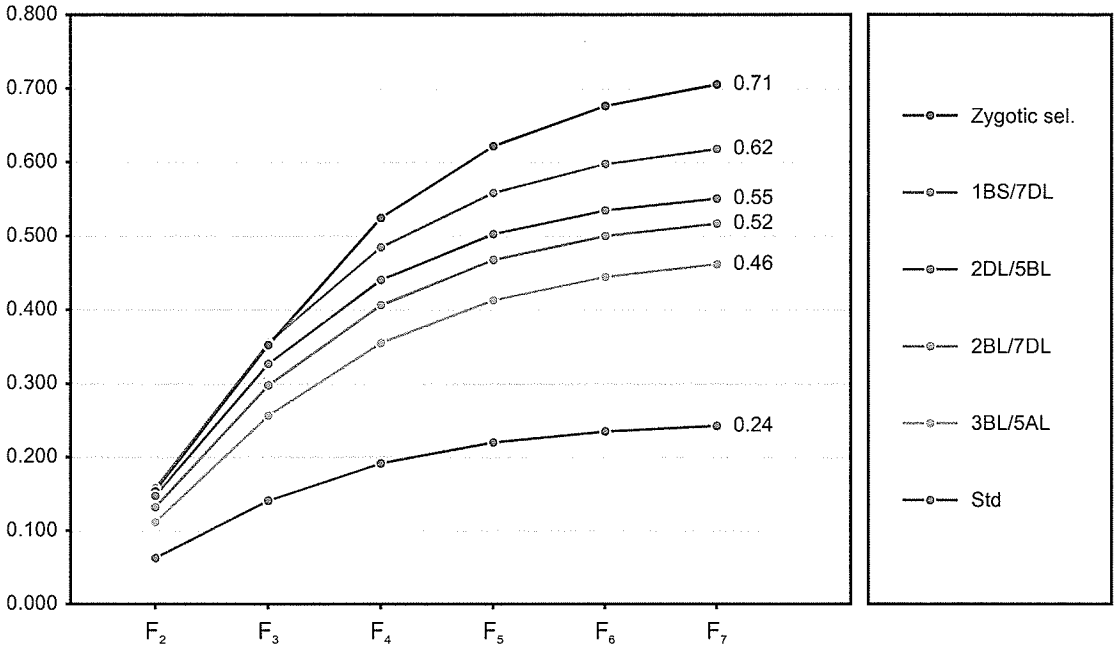

Figure 5 - Predicted effects of gamete selection on the frequency of individuals homozygous for two resistance genes for each telosome combination. The expected fixation rate of a normal population is shown for comparison (Std). An example of gametic and zygotic selection is shown using 2BL/7DL with 100% selection against ditelo 2BL progeny in each generation (Zygotic sel.). Predictions are based on female and male transmission rates determined from testcrosses and then extrapolated for seven generations of self pollination.

Discussion

Thomas et al. (2003) demonstrated that telocentric chromosomes can be employed to rapidly fix monogenic traits without phenotypic selection. Similarly, the data presented here shows that pairs of telocentric chromosomes (i.e. double monotelodisomic) can greatly increase the frequency of two-gene stacks in wheat populations. In both cases the competitive advantage of pollen carrying 21 standard chromosomes over pollen with chromosomal deficiencies is the mechanism that drives this increased frequency of desired alleles. With the availability of DNA markers that are conducive to high throughput formats (eg. Somers et al. 2004) molecular breeding approaches have become a common method for seeking genetic solutions to problems such as complex disease resistance gene stacks (Somers et al. 2003). However, DNA markers only allow desired genotypes to be identified and do not change the frequency at which these genotypes occur. In contrast, implementing cytogenetic tools, as shown here and previously (Thomas et al. 2003, 2004), changes the frequency of progeny carrying the desired alleles. This allows more breeding material to be retained for selection based on other criteria and can increase the number of objectives in a cross.

Using cytogenetic stocks as a breeding tool also has some drawbacks. The biggest challenge in the use of telocentric chromosomes is the large linkage blocks represented in the hemizygous chromosome arm. This may be of concern when traits are introgressed from poorly adapted germplasm and results in linkage drag. The solutions are either to incorporate genes of interest into elite germplasm and minimize the introgression size before crossing with telosomic parents or to use a three-way crossing scheme that would result in variability in the hemizygous chromosome arm in the

population. If heterozygous parents are used as the donor parent and a double monotelodisomic plant is the other parent, as would be the case in a three-way cross, only $1/16^{th}$ of the progeny would carry both alleles of interest plus the two telocentric chromosomes. Thus, there is a trade-off in early generation selection in favour of high frequencies of the desired alleles in later generations.

While it is unknown exactly how the pairs Lr genes used in this study interact, it appears the phenotypic distributions for the *Lr16/Lr34* and *Lr22a/Lr52* populations matched the expected outcomes. Individually *Lr16* and *Lr34* showed an average incidence of 27.5 and 6.25 respectively while the resistant parent in the cross, which carried both genes, had an average incidence of 3. About $1/16^{th}$ of the families derived from dihybrid F_1 plants had an average leaf rust incidence that matched resistance found in the resistant parent. The distribution of these families follows what is expected with one gene showing a higher incidence of leaf rust and the other showing a low incidence (Figure 4a). In contrast the *Lr16/Lr34* families derived from double monotelodisomic F_1 plants predominantly showed low incidences that match the resistant parent. Given the expected gamete transmission rates, based on testcross transmission values (Table 8), and the possibility of zygotic selection favouring standard chromosomes (see below), the distribution of leaf rust incidence amongst these families agrees with a model of few susceptible plants because of the low frequency of ditelosomic plants.

Similarly, the distribution of leaf rust incidence followed what was expected in the *Lr22a/Lr52* population. In this case both *Lr22a* and *Lr52* produced low incidences individually, 10 and 7, respectively. Thus, a higher number of families derived from dihybrid F_1 plants should show relatively low incidence values compared to the

Lr16/Lr34 population and this was observed (Figure 4b). The families derived from double monotelodisomic F_1 plants showed the expected improved frequency of highly resistant families (Figure 4).

Unexpected results were observed in the *Lr34/Lr46* population. There was little difference between the families derived from dihybrids and those derived from double monotelodisomic plants (Figure 4c). Two families that were derived from dihybrid F_1 plants showed a high rust severity which is approximately the frequency that would be expected if three Lr genes were segregating in the population. Parula, the donor of *Lr46* in this population, may carry two or three additional Lr genes (Ravi Singh, personal communication). However, the locations, identities and effectiveness for these genes are not known. Three dihybrid F_1 plants used to generate the $F_{2:3}$ families, thus it is likely that all of these F_1 plants carried additional resistance beyond *Lr34* and *Lr46*. This could explain the remarkable level of resistance found in this population.

In the population segregating for FHB resistance there was a significant difference in average FHB index between families derived from dihybrids and those derived for double monotelodisomics (Figure 4d, Table 6). The contrast between dihybrid-derived families and double monotelodisomic-derived families was not as striking for this population compared to the *Lr16/Lr34* and *Lr22a/Lr52* populations. This reflects the relatively low heritability of FHB resistance (van Sanford et al. 2001) rather than a failure of gamete competition to increase the frequency of resistance genes in the population.

The fixation rate of hemizygous genes was predicted using only estimates of gamete selection (Figures 5). This likely underestimates the rate at which pairs of genes will become fixed because there is the potential for zygotic selection. For example, in

Figure 6 – Photograph of a plant ditelosomic for chromosome 2BL. The other plants in the pot were seeded at the same time and were at the boot stage at the time of this photograph. This plant did not continue to develop beyond this stage.

this study plants that were ditelosomic for 2BL were stunted, and died prematurely without seed set (Figure 6). This phenomenon has been observed in Canadian germplasm previously (Thomas et al. 2003). Zygotic selection against ditelosomics would further increase the frequency of desired alleles. For example, if all zygotic types in the *Lr16/Lr34* population that carried ditelo 2BL were eliminated the frequency of individual homozygous for *Lr16* and *Lr34* would increase to 71% from 52% when only gamete selection is considered (Figure 5). Sears and Sears (1978) found that seven ditelosomics were sterile in Chinese Spring while those that were fertile or partially fertile had an average seed set reduction of 68%. Thus, the reduced fitness of ditelosomic plants favours the selection of individuals carrying both genes of interest in either the homozygous or hemizygous condition and would increase the rate at which genes become homozygous.

Previously, male transmission of hemizygous chromosome arms in monotelodisomic plants had an average frequency of about 75% while female transmission was about 50% (Dyck et al. 1987; Kerber 1988; Thomas et al. 2003). In this study the average male transmission was 76.1% and the female transmission was 49.8%. One hemizygous arm, 3BS, was transmitted through female gametes at frequency that was significantly less than 50% (Table 8). The reason for this low transmission rate could either be sampling error, female gamete selection or pairing failure of the telosome. Cytological observation revealed no increase in pairing failure. Gamete competition favours a standard karyotype (Sears 1944; Thomas et al. 2003), thus it is unlikely gamete selection occurred. It is probable that the observed deviation happened by chance.

Employing a non-reciprocal Robertsonian translocation as a method for fixing pairs of genes has been demonstrated (Thomas et al. 2004). In this scenario, the translocation will pair with the chromosomes carrying the genes of interest; however the chromosome arms that carry the genes of interest will remain unpaired and are in the hemizygous condition. The predominant meiotic configuration of such an association is a "V", thus the two standard chromosomes tend to co-segregate resulting in "quasi-linkage" between the two hemizygous arms (Thomas et al. 2004). This is in contrast to the independent assortment of the hemizygous chromosome arms observed in double monotelodisomic plants. In addition, pollen carrying a standard karyotype is more competitive than pollen carrying 19 standard chromosomes plus the translocation. As a result the pair of genes will be homozygous in 90% of the individuals by F_6 (Thomas et al. 2004). Thus Robertsonian translocations are more efficient than using pairs of telosomes as done in the present study. However, translocations are difficult to produce and producing all of the possible combinations would be an onerous task. If a series of telosomes is available it would be comparatively easy to create any given combination.

The use of pairs of telosomes to place genes of interest in the hemizygous condition (i.e. double monotelodisomic plants) is an efficient way to increase the frequency of desired alleles in wheat breeding populations. As most cytogenetic stocks are found in Chinese Spring (Sears 1966, 1939; Sears and Sears 1978; Endo and Gill 1996), which is poorly adapted for Canadian production, it would be worthwhile to generate cytogenetic stocks, namely monosomes and telosomes, in adapted germplasm. While this method has drawbacks, such as large linkage blocks, with well planned crossing regimes this method will increase the frequency of desired alleles thereby

increase the effective size of the breeding population. Furthermore, the power of this method can be increased if useful gene combinations are constructed for a given chromosome arm. For example, the wheat midge resistance gene *Sm1* and *Lr16* are both on chromosome 2BS (Thomas et al. 2003). If the resistance genes were found on the same hemizygous chromosome arm than an even higher number of desired alleles could be easily fixed. Bundling favourable alleles potentially makes this method a powerful tool for wheat breeders.

Chapter 4 – Isolating monosomic and telocentric stocks in elite wheat germplasm

Summary

Wheat cytogenetic stocks, particularly monosomic and telosomic lines have been mostly used for genetic studies. Recently telocentric chromosomes were identified as a tool for increasing the frequency of desirable alleles in wheat breeding populations. Most wheat cytogenetic stocks are present in an poorly adapted background. Herein we assess the feasibility of generating *de novo* a set of monosomic lines in elite germplasm and then use the monosomics to produce telosomes. Monosomic plants were isolated from the progeny of haploid ($n = 21$) by diploid ($2n = 42$) crosses by screening the progeny with chromosome specific simple sequence repeats and confirming monosomy by cytology. Nineteen of a possible 21 monosomics were isolated; the exceptions were 4D and 6D. All 19 monosomics were crossed as females and the progeny screened for the presence of telosomes. Eleven (5AS, 5AL, 7AS, 1BS, 1BL, 2BL, 3BS, 3BL, 5BS, 5BL and 2DS) of the 38 possible telosomes were isolated including a truncated telosome for 1BS. Despite the frequent bivalent pairing, attempts to restore the telosome were unsuccessful. Producing a set of monosomic lines is feasible; however recovery of telosomes was more or less difficult and was influenced by particular centromeres. A significant tendency for telosomes to be recovered in pairs (long and short arms obtained) show that some centromeres are more prone to breakage and recovery than others.

Introduction

Wheat (*Triticum aestivum* L.), an important cereal crop, is a hexaploid species with three homœologous genomes ($2n = 6x = 42$, AABBDD). Wheat cytogenetic stocks

were first reported in 1939 (Sears 1939) and have been a valuable tool for genetic studies. The types of available wheat cytogenetic stocks include monosomic stocks (Sears 1939, 1944), nullisomic/tetrasomics (Sears 1966), telosomics (Sears and Sears 1978) and more recently the deletion stocks (Endo and Gill 1996). The most extreme example of a cytogenetic stock is tetra-Canthatch ($2n = 4x = 28$, AABB) which contains only the A and B genomes of common wheat (Kerber 1964). Some of the uses of these stocks include locating a gene to a chromosome (Sears 1953), locating a gene to a chromosome arm and finding the linkage relationship between a gene and the centromere (Sears 1962; Sears and Sears 1978), demonstrating the relationship between homœologous chromosomes (Sears 1952b, 1966) and physical mapping of genes and DNA markers (Endo and Gill 1996). More recently telocentric chromosomes have been shown to be a useful breeding tool (Thomas et al. 2003). In this instance genes found in the hemizygous condition will increase in frequency in breeding populations to near fixation without phenotypic selection because of the competitive advantage of pollen carrying 21 normal chromosomes compared to pollen carrying a telocentric chromosome.

Most of the described aneuploid stocks have been isolated in Chinese Spring. There are no reports of cytogenetic stocks isolated in genetic backgrounds that are well adapted for North American wheat breeding programs. Cytogenetic stocks in Chinese Spring are not suitable for direct use in North American breeding programs and material produced from genetic studies involving these cytogenetic stocks cannot be easily incorporated into the breeding stream.

In wheat monosomics were mostly isolated from the progeny of haploids (Sears 1939, 1944). Haploid wheat is male sterile but a proportion of female gametes are viable.

Thus pollinating haploid plants with pollen from a normal diploid plant will produce some seed. Given the abnormal meiosis of haploids, i.e. no homologous pairing and a few heterologous bivalents, most female gametes that are successfully fertilized and set seed will contribute karyotype abnormalities including monosomy, trisomy and translocations to their progeny (Sears 1939). This makes the progeny of haploids a key source for aneuploid stocks.

Telocentric chromosomes are generally produced from the misdivision of univalents during anaphase I of meiosis (Sears 1952a; Morris et al. 1977). Thus, a monosomic series is required for producing telosomic stocks. The set of telosomic stocks was found in Chinese Spring with the exception of telo-7DL which was found in Canthatch (Sears and Sears 1978).

Haploids of Canadian varieties and genetic stocks (near iso-genic lines) have been shown to readily produce aneuploid progeny, including monosomy, when pollinated by plants with a normal karyotype (Thomas et al. 2001, Hiebert et al. 2005; Hiebert et al. 2008). One of these monosomic stocks was used to generate a telocentric chromosome through the misdivision of the univalent (Thomas et al. 2003). The purpose of this study was to evaluate the feasibility of isolating all monosomics from the progeny of adapted (i.e. registered Canadian cultivars or advanced breeding lines) haploids and subsequently producing telocentric chromosome stocks with sufficient merit for use in a breeding program.

Materials and Methods

Producing and crossing haploids

Haploid wheat plants ($1n = 3x = 21$, ABD) were produced by chromosome elimination using a maize pollination method (Laurie and Bennett 1988, 1989; Thomas et al. 1997). One day prior to the anticipated onset of anthesis the primary and secondary florets of each spikelet were emasculated and the remaining florets were removed from the spike. The next day florets were pollinated with fresh maize (*Zea mays* L.) pollen. Heads were sprayed with a fine mist of dicamba (100ppm) to the point of runoff at both one and two days post-pollination. Caryopses were removed from heads sixteen days after pollination and embryos were excised in a sterile environment. Embryos were cultured on media (11g agar, 40g sucrose, and 2.75g Gamborg's B5 in 1 litre) in small glass bottles and subjected to 4 days of cold treatment at 4°C in the dark, 3 days of dark treatment at room temperature (approximately 20°C), and then were placed under lights (14 hrs light, 10 hrs dark) at 20°C. Plantlets with roots and shoots at least 2 cm in length were transplanted into three-inch pots containing soilless mix and grown in a growth cabinet with 16 hours of light at 17°C, and 8 hours dark at 16°C. When plants were at the three-leaf stage they were transplanted into larger pots with a soil mix. The cultivars and lines used to make haploids included AC Domain, Intrepid, AC Elsa, AC Barrie, Splendor, 96 M11, RL6107 and RL6172.

The florets of haploid plants were clipped to expose the stigmata and two to four heads were pollinated at a time in glassine bags. Pollinators were prepared by clipping detached heads in anthesis thereby stimulating further anther emergence and pollen shed. The heads used for pollinating were twirled rapidly in glassine bags to distribute freshly shed pollen. Haploids of RL6107and RL6172 were pollinated with the Canadian wheat cultivar AC Foremost as part of genetic studies involving two novel leaf rust resistance

genes (Hiebert et al. 2005; Hiebert et al. 2008). All other haploids were pollinated with the Canadian wheat cultivar Superb. Seed from the crosses were harvested at maturity.

DNA Extraction and PCR

DNA was extracted from macerated lyophilized leaf tissue or from seeds that were cut in half, with the half containing the embryo retained for planting, and and the other half crushed into a fine powder. The DNA was extracted using the hexadecyltrimethyl-ammonium bromide (CTAB) method described by Kleinhofs et al. (1993) except that phenol was omitted. DNA was suspended in 0.1X TE buffer (0.1 mM tris, 0.01 mM EDTA) that contained RNAse (10μg/ml).

All PCR reactions were performed under the following conditions: PCR buffer 1x, dNTPs 0.2mM each, $MgCl_2$ 1.5 mM, primers 10 pmol each, Taq DNA polymerase 1 U, and approximately 50 ng genomic DNA; 2 min. 94°C, then 1 min. 95°C, 1 min 50-60°C, and 50 sec. 73°C for 30 cycles, followed by 5 min. 73°C. PCR products were run on 5% denaturing polyacrylamide gels in TBE buffer (0.089M tris, 0.089M boric acid, 0.050M EDTA) at 85 W for 1.5-2 hours, and visualized by silver staining (Promega, Madison, WI, USA), following the manufacturers protocol.

Isolating monosomics

Parental genoytpes were screened for polymorphisms with microsatellite markers (simple sequence repeats; SSR). The progeny of the haploid plants were tested for apparent chromosome deficiencies using proximally located microsatellite markers (Table 9) with DNA collected from half-seeds or young leaves. Most of the progeny were tested using DNA extracted from half-seeds. The number of individuals tested for a given chromosome varied from 96 to 512 depending on the rate of apparent monosomy

Table 9 – List of microsatellite (SSR) markers used to screen or monosomes and telosomes for each chromosome.

	SSR markers used for screening[a]		
		Telosome screening	
Chromosome	Monosome screening	Short arm	Long arm
1A	WMC278	WMC24	BARC17
2A	WMC296	WMC522	GWM294
3A	BARC67	BARC67	CFA2193
4A	GWM601	WMC680	WMC232
5A	GWM595	GWM205	GWM595
6A	WMC417	BARC146	WMC524
7A	WMC17	GWM635	WMC809
1B	WMC694	GWM413	GWM140
2B	GWM257	GWM257	GWM120
3B	GWM493	GWM533	GWM340
4B	GWM149	WMC710	GWM149
5B	GWM159	WMC376	WMC235
6B	GWM132	GWM389	GWM340
7B	WMC76	GWM537	WMC517
1D	GWM642	WMC339	GWM337
2D	GWM445	GWM102	GWM301
3D	WMC492	GWM161	BARC71
4D	WMC622	-	-
5D	WMC215	GWM190	WMC765
6D	WMC469	-	-
7D	GWM295	GWM295	WMC14

[a] GWM markers are from Röder et al. (1998); WMC markers are from Somers et al. (2004); BARC markers are from Song et al. (2005)

detected. Once two or three putative monosomic individuals were identified the search for additional monosomic lines for that chromosome would cease. Half-seeds of putative monosomic plants were planted in soilless mix and placed in growth chambers set for 16 hours of light at 18°C and 8 hours of dark at 16°C.

To confirm monosomy immature spikes were harvested from tillers when located just below the penultimate leaf. Spikes were fixed (9 95% ethanol : 6 chloroform : 1 glacial acetic acid) for 24 hours at -20°C and stored in 70% ethanol at -20°C, changing the ethanol once a day for three to four days. Anthers were macerated in acetocarmine to liberate pollen mother cells (PMC) and stain the chromatin. PMC preparations were warmed on a hot plate and gently squashed to spread the chromosomes. Cells in metaphase I of meiosis were analyzed to confirm the presence of univalents.

The population derived from 96M11 haploids (and pollinated with Superb) was used to locate the midge resistance gene *Sm1* by analyzing hybrids deficient for DNA markers linked to *Sm1* (Thomas et al. 2001). Similarly, in the populations where RL6107 and RL6172 were used as the haploid parents (and pollinated with AC Foremost) only hybrids missing *Lr52* and *Lr60* respectively were tested for chromosome deficiencies. The procedure for identifying chromosome deficiencies was the same as described above.

Isolating telocentrics

To generate telocentric stocks plants carrying chromosomes in the monosomic condition were emasculated and pollinated as described above using AC Cadillac, BW346 (RL 4802//BW 174*2/Clark) or 97B64-K15C3 (McKenzie*3//BW174*2/Clark) as the pollinator. At least five spikes were crossed per monosomic. All monosomic plants were produced as described above. Some monosomic plants were crossed with

AC Cadillac immediately after being identified while others were selected from the progeny of self-pollinated monosomic plants. Monosomic progeny from self-pollinated plants were selected by analyzing root tip smears. Root tips were collected from seeds germinated *in vitro* and placed immediately in vials filled with water stored in an ice-filled container for pre-treatment. After 24 hours the root tips were transferred into fresh vials containing fixative (3:1 95% ethanol : glacial acetic acid) and stored at 4°C for at least three days. Fixed root tips were hydrolysed in 1N hydrochloric acid for seven minutes at 60°C and then transferred to Feulgen stain (12 mM Basic Fuchsin, 68 mM sodium metabisulfite, 130 mM hydrochloric acid) for at least 20 minutes but less than 90 minutes. Stained root tips were spread and squashed in acetocarmine under gentle heat. A minimum of six cells were counted per sample. Monosomic plants were confirmed by analyzing PMC in metaphase I as described above. Seed from the $2n$-1 x $2n$ crosses was harvested at maturity.

Hybrid seed was planted in soilless mix and grown in growth cabinets with 16 hours of light at 18°C, and 8 hours dark at 16°C. Tissue was collected from the first leaf and lyophilized. DNA was extracted from leaf tissue as described above. Some or all of the hybrids involving plants monosomic for 1B, 2B, 2D, 5B or 7D were tested using DNA extracted from half-seeds. For each population two SSR markers (Table 9), one per chromosome arm of the monosome was tested on the critical chromosome to screen for chromosome arm deletions (i.e. telocentric chromosomes). Plants with putative arm deletions were selected and their PMC analyzed as described above. The female transmission of the monosomic chromosome was also recorded for each population.

Results

Isolating monosomics

The quality of the seed produced in the haploid by diploid crosses was variable. Some seed was plump and of normal size compared to the seeds produced by normal ($2n$) plants in ideal growth cabinet conditions. About half of the seed produced was at least somewhat shrivelled. The population size for crosses with 96M11, RL6107 and RL6172 were 48, 417 and 488 respectively. For the remainder of the haploid-derived hybrids there were a total of 512 individuals available to analyze.

Out of the 21 possible wheat monosomics 19 were isolated and confirmed by cytological analysis (Figure 7) and the subsequent transmission of SSR markers located distally on opposite chromosome arms that showed quasi-linkage. The only two monosomics not successfully isolated were 4D and 6D. While a few individuals screened with SSR markers showed putative monosomics for chromosomes 4D and 6D none of these half-seeds germinated. The minimum number of individuals screened for monosomy was 96 and maximum was 512. In most cases confirmed monosomic individuals could be found within the first 192 individuals screened.

Isolating telocentrics

From the 19 monosomic stocks that were isolated it is possible to recover a maximum of 38 telocentric chromosomes. In total there were 11 telocentric chromosomes identified and recovered, and one isochromosome from a total of 2552 screened progeny (Table 10, Figure 8). The telosomes recovered include 5AS, 5AL, 7AS, 1BS, 1BL, 2BL, 3BS, 3BL, 5BS, 5BL and 2DS. Recovery of telocentric chromosomes was variable. For example the population derived from mono 5B produced

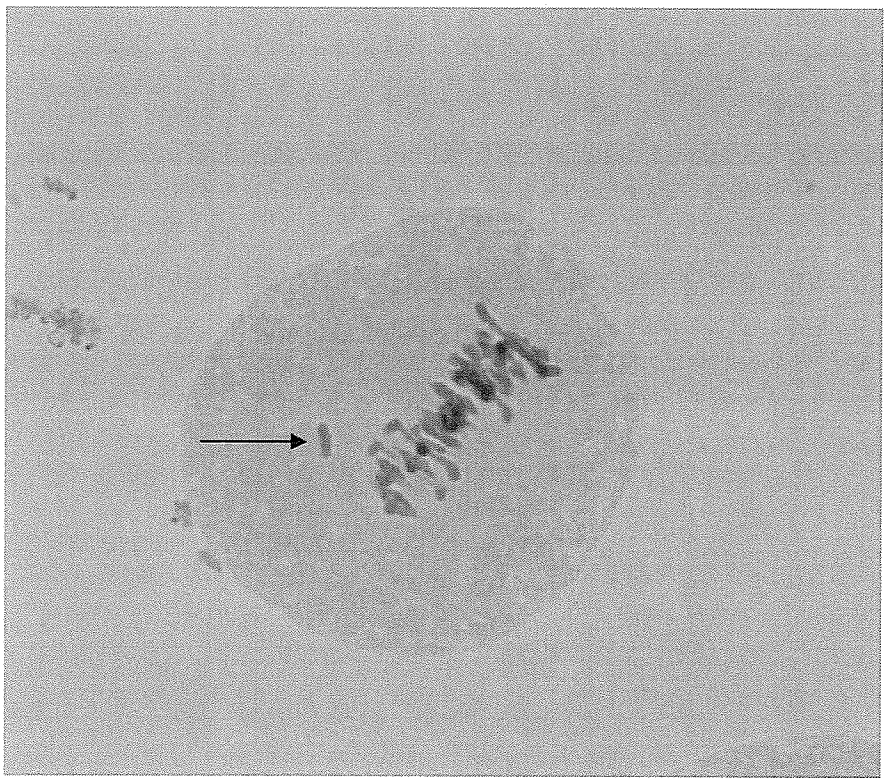

Figure 7 – An example of a univalent in a pollen mother cell in metaphase I that was used to confirm monosomy. The univalent is indicated with the arrow. This particular plant was monosomic for chromosome 2D.

Table 10 – The female monosome transmission in number of gametes and percentage and the telosomes isolated for each chromosome.

	Female gamete transmission[a]							
Mono	n = 21		n = 20		Total	Telos found[b]	Monosome donor	Pollinator
1A	16	24%	50	76%	66	none	Superb	AC Cadillac
2A	41	50%	41	50%	82	none	Superb	AC Cadillac
3A	7	12%	51	88%	58	none	Superb	AC Cadillac
4A	48	41%	69	59%	117	none	Superb	AC Cadillac
5A	18	12%	134	88%	152	both	AC Cadillac	Superb
6A	37	54%	32	46%	69	none	Superb	AC Cadillac
7A	14	18%	64	82%	78	short	Superb	AC Cadillac
1B	53	26%	153	74%	206	both	Superb	97B64-K15C3
2B	16	23%	55	77%	71	none	Superb	AC Cadillac
	24	34%	46	66%	70	long	Superb	AC Cadillac
3B	20	13%	139	87%	159	both	Superb	AC Cadillac
4B	60	36%	108	64%	168	none	Superb	AC Cadillac
5B	55	65%	29	35%	84	both	Superb	BW346
6B	8	10%	74	90%	82	iso	Superb	AC Cadillac
7B	14	20%	57	80%	71	none	Superb	AC Cadillac
1D	76	43%	100	57%	176	none	Superb	AC Cadillac
2D	31	22%	112	78%	143	none	97B64-K15C3	AC Cadillac
	50	19%	219	81%	269	short	Superb	97B64-K15C3
3D	6	8%	67	92%	73		Superb	AC Cadillac
4D	-	-	-	-	-	-	-	-
5D	54	36%	98	64%	152	none	Superb	AC Cadillac
6D	-	-	-	-	-	-	-	-
7D	44	32%	93	68%	137	none	Superb	BW346
	33	48%	36	52%	69	none	BW346	AC Cadillac

[a] Transmission of telosomes was not included in the sample for the transmission of monosomes.

[b] short = telosome carrying the short arm was isolated; long = telosome with long arm was isolated; both = both possible telosomes were isolated; iso = no telosomes were isolated but an isochromosome was isolated.

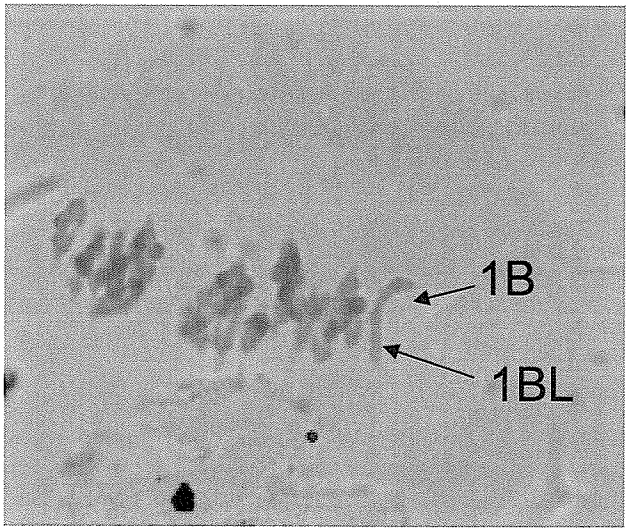

Figure 8 – An example of a heteromorphic bivalent in a pollen mother cell in metaphase I that was used to confirm the presence of a telosome. The members of the heteromorphic bivalent are labelled as 1B, a standard chromosome, and 1BL, a telocentric chromosome that is missing the short arm.

3 telosomes in a population size of 84 (3.6%). In contrast 206 individuals were screened for chromosome 7D and no telocentric chromosomes were recovered (Table 10).

For telo 1BS a truncated short arm was recovered, which will be referred to as 1BSt, where the short arm satellite was deleted in addition to the entire long arm. The evidence verifying the identity of this very short chromosome includes the absence of long arm SSR markers, the presence of a short arm SSR marker and the appearance of only three satellites in the root tip preparations of plants carrying this truncated chromosome (i.e. plants that were monotelodisomic). A large sample of PMC from three plants was analyzed to study the pairing frequency of 1BSt (Figure 9). Out of the 362 PMC studied 1BSt was part of a heteromorphic bivalent 160 times for a frequency of 44% (Table 11). The pairing frequency found in plant #1 and plant #2 was similar but the third plant had a higher rate of pairing. In a cross with plant #3 as the female, that carries one copy of each chromosome 1BSt and standard chromosome 1B, 50 progeny that were monomorphic for SSR markers on 1BL (i.e. plants that would be monosomic or monotelodisomic) were studied cytologically in order to find recombinant telocentric chromosomes that carried a normal short arm (i.e. a standard 1BS telocentric). For plant #3 the pairing rate of 1BSt with 1B was 52% (Table 11) and the frequency of individuals negative for SSR markers on 1BL was 54% (121/226). About half of the individuals that failed to receive 1BL from the Plant #3 can be accounted for by gametes carrying 1BSt because of pairing and disjunction of 1BSt and 1B during meiosis. Out of the 50 progeny studied cytologically there were 34 that carried 1BSt (68%) and 16 that were monosomic for 1B. Therefore 1BSt was occasionally included in gametes even when it failed to pair. Given the above pairing frequency of 1BSt, approximately 25% of the 50 progeny

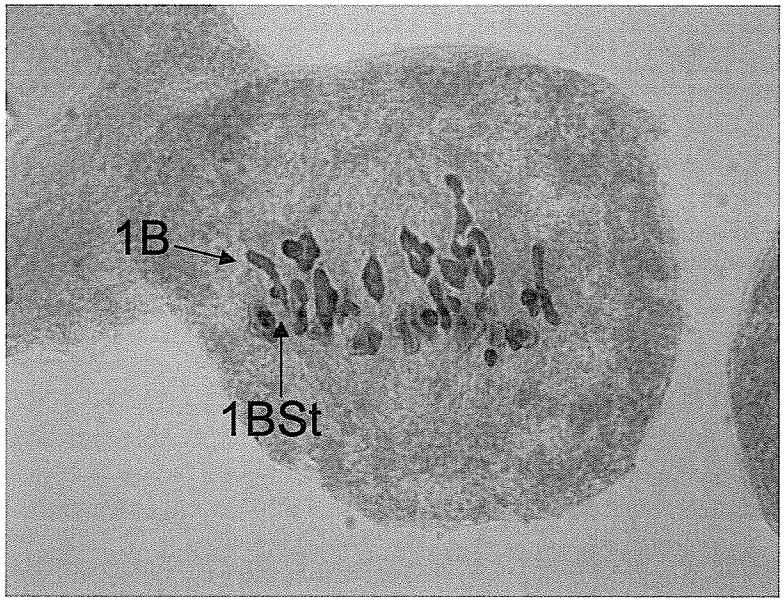

Figure 9 – A pollen mother cell in metaphase I where a heteromorphic bivalent was formed between standard chromosome 1B and the truncated telosome 1BSt, which is missing the long arm and the satellite on the short arm.

Table 11 – The pairing frequency of the truncated 1BS telosome (1BSt) with the standard 1B chromosome in three different plants.

	Paired		Unpaired		Total
	Number	%	Number	%	
Plant 1	18	35%	33	65%	51
Plant 2	49	37%	83	63%	132
Plant 3	93	52%	86	48%	179
TOTAL	160	44%	202	56%	362

studied should carry a recombinant 1BS telocentric chromosome; however none were recovered.

Monosomic transmission

The female transmission of monosomic chromosomes (i.e. gametes with 21 chromosomes) was variable depending on which chromosome was in the monosomic condition. The range of monosome transmission through female gametes was 8% to 65% (Table 10). Out of the 19 monosomics tested only four, 2A, 6A, 5B and 7D, showed a transmission frequency of approximately 50% or greater. In most cases female transmission of monosomes was less than 50%. The average female transmission, including those monosomes with two populations (2B, 2D and 7D), was 31%. In the cases where more than one population was produced for a given monosome both similar female transmission (eg. 2D) and variable female transmission (eg. 7D) was observed (Table 10).

Discussion

The availability of chromosome specific markers in wheat (Röder et al. 1998; Somers et al. 2004) has simplified screening for monosomics and identifying the monosome. Previous studies have shown that aneuploids can be readily recovered from the progeny of *n* x 2*n* crosses in wheat (Sears 1939, 1944; Thomas et al. 2001; Hiebert et al. 2005; Hiebert et al. 2008). The present study demonstrated that an array of monosomics can be recovered from a reasonable number of progeny. Two monosomics, 4D and 6D, were not isolated in this study. However a few progeny (half-seeds) screened had SSR profiles consistent with monosomy for these two but these particular half-seeds did not germinate. There does not appear to be any specific selection against monosomy

of chromosomes 4D and 6D. Rather this reflects on the poor quality of some of the seeds and possibly a negative effect that cutting may have on the viability of half-seeds. Therefore it appears that haploid-derived aneuploidy is a practical method for isolating a series of monosomics in a chosen genetic background.

The alternative to producing a *de novo* monosomic series in a desired genetic background is to backcross the monosomic condition from a previously established monosomic series. In order to adequately restore most of the genome from the recurrent parent there must be six or seven backcrosses made. These crosses must be made with all 21 monosomic stocks in the series to produce the new series. This process could be assisted by making crosses with alternating elite genotypes and selecting monosomics after each cross using SSR markers. This would allow rapid identification of monosomic progeny, preserve identity of the monosomic by eliminating the chance of selecting progeny where monosomic shift has occurred and ensure that all monosomic lines would be represented. However, a haploid-derived monosomic series should take less time to produce and no genetic component would be retained from the donor.

In the first complete set of wheat monosomes, most of the monosomics were isolated from the progeny of haploids (Sears 1939, 1944). However some of the monosomics were isolated from the selfed progeny of a plants nullisomic for chromosome 3B. Plants nullisomic for chromosome 3B are partially asynaptic which increases the frequency of univalents (Sears 1944). In contrast, all of the monosomics identified in this study were directly derived from deficient female gametes from haploid plants. Haploid plants are readily produced using current techniques. At the time, Sears (1939) found two haploid plants from 105 seeds planted from a cross between wheat and

rye (*Secale cereale*) and no protocols had been developed to readily produce haploids. Taken together with DNA technology it is feasible to generate haploids, produce hybrids from the haploids and rapidly screen the hybrids to identify monosomy using current techniques. However, the genotype of the haploid may affect the frequency of seed set and chromosome deficiencies (Hiebert et al. 2005). These factors can change the efficiency of producing a monosomic set in a particular genetic background.

Using DNA extracted from half-seeds for screening progeny provides some advantages such as saving greenhouse or growth cabinet space. Furthermore, the best quality tissue samples for cytological examination are generally found in young root tips and early tillers. Screening a population with SSR markers and planting only a relatively small number of selected individuals allow the best possible tissues to be available for cytological observations. One point to consider is that the quality and viability of the seeds produced in the n x $2n$ crosses is not equal to seeds produced by normal plants ($2n$) as observed in this study. Therefore, as demonstrated in this study, critical individuals may be lost by a failure of shrivelled seeds to germinate. Screening DNA collected from seedlings ensures that only viable hybrids are selected. The advantages and disadvantages of using leaf tissue versus half-seeds as the source of DNA in such studies should be considered and depends on factors such as resource availability and throughput capacity.

Telocentric chromosomes were recovered at a low rate in this study as only 11 of a possible 38 telosomes were isolated. In populations where telosomes were recovered the frequency of telosome recovery was about 2% which is slightly lower than previously observed rates of telosome recovery (Sears 1952a; Sears and Sears 1978). Sears and

Sears (1978) identified telocentric chromosomes from the progeny of self-pollinated monosomic plants; this would allow telosomes to be generated and produced in both male and female gametes. In contrast, testcross progeny were analyzed in the present study where telocentrics could only be produced and transmitted via female gametes. Eleven of the 42 telocentrics reported by Sears and Sears (1978) were isolated by a two step process. First, plants carrying an isochromosome were identified and then telocentric chromosomes were isolated from their progeny.

Misdivision of univalents has been reportedly observed in PMC that are in anaphase I of meiosis. The reported rate of misdivision ranges from 2% to 40% (Sears 1952a; Morris et al. 1977). This implies that telocentric chromosomes and isochromosomes should be recovered at high rates. Unfortunately no systematic study has been conducted to compare the rate of univalent misdivision to the rate of telosome recovery. Furthermore, apparent misdivision of univalents cannot be substantiated as chromosome breaks. It is possible that the rate of actual misdivision (i.e. chromosome breakage) is lower than the apparent rate indicated by cytology. The low rates of telosome recovery reported by Sears and Sears (1978) compared to the relatively high rates of reported univalent misdivision (Sears 1952a; Morris et al. 1977) suggests that the cytologically based estimates of univalent misdivision are too high.

Sears and Sears (1978) speculate that most of the telocentrics derived from selfed monosomic plants were transmitted through the male gametes. However, no data was presented to support this hypothesis. In this study the monosomic parent was crossed as the female. This strategy was chosen because female gametes with abnormal gametes are readily transmitted while successful male gametes preferentially have a normal karyotype

because of pollen certation. This is true for whole chromosome deficiencies (Sears 1944) and telocentric chromosomes (Thomas et al. 2003). Testcross progeny were favoured over the progeny of self-pollinated monosomics to allow identification of potential telosomes using DNA markers.

In a detailed study of group 5 univalents, where there were 19 different univalent donors, the frequency of misdivision varied significantly between chromosomes and the genotype of the monosome (Morris et al. 1977). Similarly, in an effort to recover telo 5AL no telocentrics were recovered when the monosome was from Superb (Julian Thomas, personal communication) but telos 5AL and 5AS were quickly isolated when the monosome came from AC Cadillac. Perhaps the low rate of telosome recovery in this study can be attributed to genetic factors in Superb. It should be noted that for chromosomes 5A, 1B, 3B and 5B both possible telosomes were recovered (Table 10). If chromosome breakage and recovery was random this would be an unusual outcome (Figure 10). Since it is unlikely that this outcome is random it appears that particular centromeres are more likely to break and/or remain functional after breaking to allow recovery of the telosome. This is consistent with the findings of Morris et al. (1977).

The recovery of the truncated telo 1BS (1BSt) was not expected. The loss of the short arm satellite in addition to the loss of the entire long arm is not easily explained. The meiotic behaviour of this truncated telosome corresponded to its proposed structure. When pairing occurred between 1B and 1BSt heteromorphic bivalents were observed in metaphase I where the aberrant half of the bivalent was very small (Figure 9). When pairing did not occur there were two univalents, a large one and a very small one (Figure 11). The average pairing rate of the truncated telo 1BS with the standard 1B

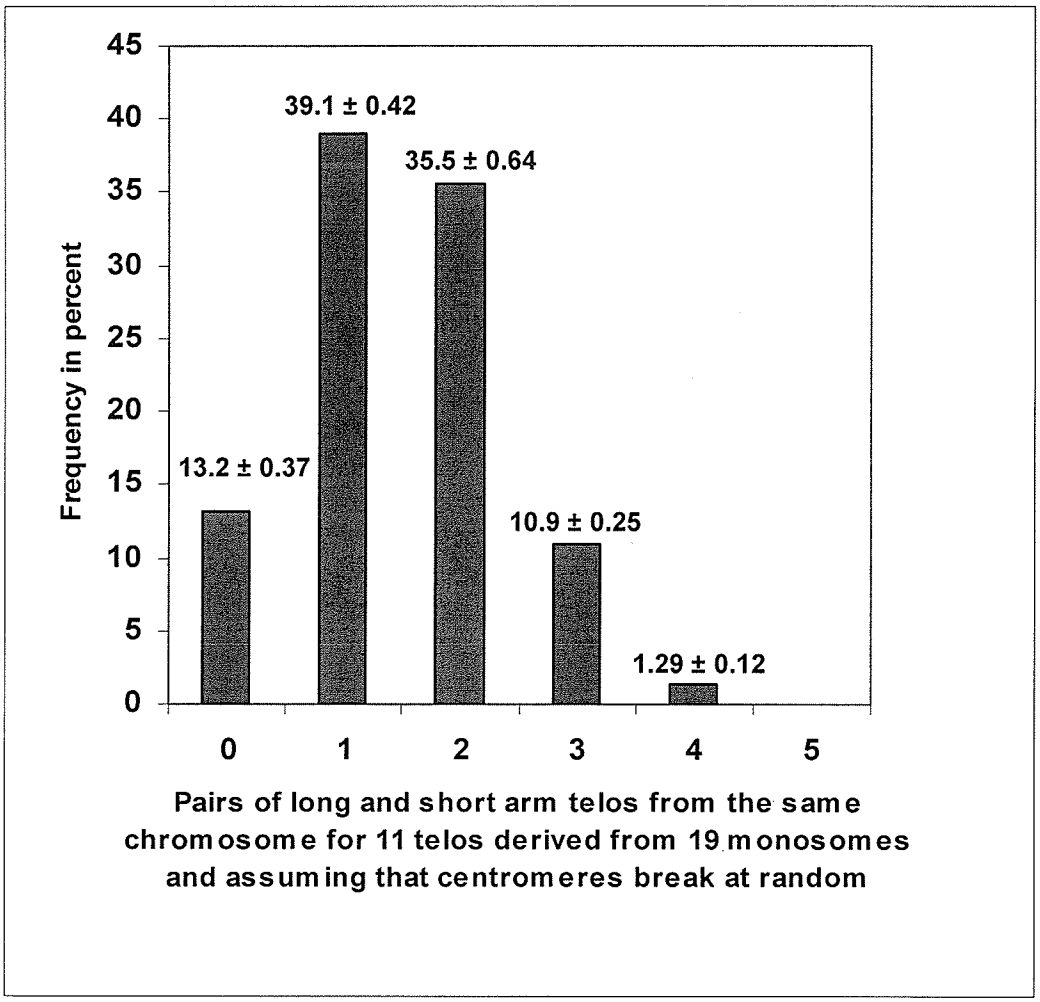

Figure 10 – The expected frequency of telosome pairs if chromosome breakage and recovery is random. The simulation was run 10,080 times.

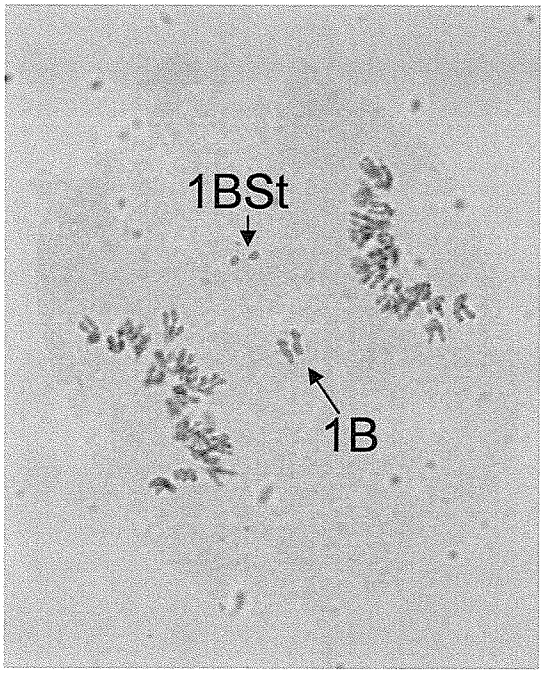

Figure 11 – In this pollen mother cell chromosome 1BSt and 1B failed to pair and were found as univalents. This cell, in late anaphase I, shows both the division of univalents in anaphase I and the large disparity in size between 1B and 1BSt.

chromosome was 44%. This reduction in pairing frequency is not surprising as distal chromosomal regions are important in organizing and initiating pairing (Corredor et al. 2007). With a deleted terminal region it follows that pairing would be affected. It is expected that 1B-1BSt pairing, with apparent chiasma, should allow a normal telo 1BS to be recovered as it is generally accepted that one crossover event is associated with each chiasma (Fu and Sears 1973). However, from a reasonable sample size no recombinant telosomes were recovered. It appears that, in this instance, chiasmata or chiasmata-like structures were formed without crossover. Plants carrying 1B and a truncated telo 1BL (missing 23% of the long arm) have also shown a reduction in pairing and a lack of recombination between the standard chromosome and the telosome with a terminal deletion (Jones et al. 2002). This suggests that the appearance of chiasma may not be invariably diagnostic of crossing over.

The female transmission of monosomes in this study varied between 8% and 65% with mean transmission of 31% (Table 10). Although there is no comparable complete data set in other literature, Sears (1944) reports on the female transmission for three monosomes which were 19%, 26% and 30% for an average of 25%. Those values are similar to the transmission frequencies observed in the present study. It is unknown if the genetic background or environment play a role in the transmission of different monosomes.

The use of aneuploids in genetic studies such as monosomic analysis (Sears 1953) and telocentric mapping (Sears 1962; Sears and Sears 1978) is well documented (eg. The and McIntosh 1975, Dyck and Kerber 1981). Recently telocentric chromosomes and Robertsonian translocations have been shown to be useful breeding tools (Thomas et al.

2003, 2004). Individuals with 41 standard chromosomes and one telocentric can produce gametes with either 21 standard chromosomes or 20 standard chromosomes and a telocentric chromosome. Similarly, individuals with 40 standard chromosomes and a Robertsonian translocation will generally produce gametes with 21 standard chromosomes or 19 standard chromosomes and the translocation. In the examples provided by Thomas et al. (2003, 2004) desired allele(s) are found in the hemizygous condition in the F_1 generation. In subsequent generations pollen competition will favour gametes with a standard karyotype (i.e. 21 standard chromosomes) causing a corresponding increase in the frequency of the desired allele(s) without phenotypic selection. For practical application of these tools the cytogenetic stocks must exist in suitable genetic background. Most wheat cytogenetic stocks are found in Chinese Spring (Sears 1939, 1944, 1966; Sears and Sears 1978; Endo and Gill 1996) which is not adapted for Canadian cultivation. Therefore, the capability to generate cytogenetic stocks in a chosen genetic background makes cytogenetic stocks a viable tool for wheat breeders.

In this study, monosomics were readily recovered from the progeny of haploids. This confirms their previous use as a reliable source of aneuploidy (Sears 1939). Sears (1939) used haploids of Chinese Spring to isolate aneuploids and here we used eight different lines, though some were highly related. Thus it appears that it should be possible to generate a set of monosomic lines in any chosen germplasm. The production of telocentric chromosomes may be sensitive to the genotype of the monosome or perhaps the entire genetic background of the monosomic individual used. In this study, few telocentric chromosomes were isolated indicating that isolation of a complete

telosomic set in a particular genetic background may be a large undertaking. Previous evidence suggests that monosome origin is a significant factor for univalent misdivision (Sears 1952a; Morris 1977). It may be advisable to test a few genotypes of monosomes with a small number of different chromosomes to determine the best genotype for recovering telocentric chromosomes which should be particularly useful if only a few select telosomes are desired. Identifying genotypes more amenable to univalent misdivision would improve the efficiency of telosome production and make these cytogenetic stocks more readily available in elite germplasm. Furthermore, telosomes were shown to be a useful tool for fixing traits in wheat breeding populations (Thomas et al. 2003; Chapter 3). Thus, targeting specific telosomes by crossing monosomes of diverse elite origin would be a good strategy.

Producing cytogenetic stocks in adapted genotypes would offer wheat breeders an additional tool to integrate into breeding programs. Furthermore, material produced from genetic studies that use cytogenetic stocks in an elite genetic background could be more readily incorporated into breeding programs. This has the potential to expedite the introduction of novel traits or alleles into new cultivars.

Chapter 5 - General Discussion and Conclusion

The development of DNA markers has allowed genes of interest to be mapped and marked by a given marker type such as microsatellites (SSR). DNA markers that are closely linked to important genes or QTL allow complex genotypes to be assembled and selected. For example, Somers et al. (2003) report a complex crossing and selection scheme designed to stack FHB resistance QTL in elite germplasm. Markers can be used to select gene combinations that could not be accurately identified by phenotypic observation. In this study *Lr22a* and *Lr52* were stacked by using a combination of phenotypic selection for *Lr52* and marker-assisted selection for *Lr22a*. When seedlings were inoculated with *Puccinia triticina, Lr52* could be detected because it is a seedling resistance gene (Dyck and Jedel 1989; Hiebert et al. 2005) where as *Lr22a* confers resistance at the adult plant stage (Rowland and Kerber 1974). If plants carrying *Lr52* are inoculated at the adult-plant stage it cannot be determined if both *Lr52* and *Lr22a* are present based on phenotype. Thus, to accurately assemble a gene stack a combination of phenotypic selection at the seedling stage for *Lr52* and marker-assisted selection for *Lr22a* can be employed.

Despite the resolving power of DNA markers to select specific genotypes, selection is limited by the frequency of desired genotypes present in the population and population size. Manipulating desired alleles into the hemizygous condition using telocentric chromosomes will fix these alleles in breeding populations. When two telocentric chromosomes are used the two hemizygous arms will be fixed as a stack over several generations by a combination of gametic and zygotic selection.

In order to employ telocentric chromosomes as a breeding tool it is advantageous use marker-assisted selection (MAS) to select hybrids that carry both the desired alleles *and* the telocentric chromosomes. This allows hybrids to be rapidly screened and improves the efficiency of this method. In some cases MAS must be used as phenotypic selection of certain gene stacks may not be currently possible. For example, this would be the case for leaf rust genes when there are no rust isolates with differential reactions and the genes show no reliable difference in infection type. In large scale selection scenarios, hemizygous chromosome arms can be identified more rapidly by MAS than by cytology.

The requirements and costs for using telocentric chromosomes to fix genes include generating the desired cytogenetic stocks, maintaining the cytogenetic stocks in elite genetic backgrounds, MAS, cytogenetic expertise and discarding a large proportion of F_1 plants. Of these the largest drawback lies in the number of F_1 plants that must be discarded. In the case where a dihybrid donor as male is crossed with a double monotelodisomic as female, only $1/16^{th}$ of the progeny will carry both of the desired alleles and both telosomes. Thus, from 200 hybrids only about 12 plants would be retained. This represents a small number of gametes and would cause a genetic bottleneck. Therefore to sample a reasonable number of gametes, say 50 individuals, about 800 hybrids would need to produced and screened. In comparison $1/4^{th}$ of the hybrids from a conventional cross would be retained making the difference in retention rate a factor of four. Likewise, if the stack donor was homozygous for both loci then the number of hybrids retained would increase four-fold in either case.

There is a clear resource investment increase to select the desired double hemizygous plants in the first generation. The benefit is that the desired gene stack will appear in almost all of the lines in later generations because of gametic and zygotic selection. Telocentrics could also be used to fix traits that are difficult, expensive or risky (e.g. requiring exotic pest isolates) to screen phenotypically. Furthermore, other objectives of the cross can be under increased selection intensity or an additional objective can be added to the cross because of the increased effective size of the population. If desired alleles from genes on the same chromosome arm can be placed in coupling it would result in satisfying multiple objectives simultaneously. For example, the short arm of chromosome 2D carries *Lr22a*, *Lr41* and *Rht8* in relatively small genetic interval (Rowland and Kerber 1974; Raupp et al. 2001; Korzun et al. 1998). Thus, telo 2DL could be used to fix all three loci.

The purpose of this study was to integrate genetic tools including MAS, phenotypic selection and cytological screening to produced populations where pairs of disease resistance genes were found in the hemizygous condition using telocentric chromosomes. We observed an increase in disease resistance in F_3 families that were derived from double monotelodisomic F_1 plants. This agreed with the predictions made based on the gamete transmissions where standard chromosomes have a higher transmission frequency than telosomes thus the frequency of resistance genes should increase. Thus, telocentrics effectively stack chromosome arms in wheat populations.

The final objective of this study was to assess the production of cytogenetic stocks in elite germplasm. Isolating monosomics proved relatively easy, however producing telocentric chromosomes was more difficult and appears dependent on the centromere of

a given chromosome. Producing a complete telocentric series appears impractical for the purposes of plant breeding. An approach that identifies key traits and targets the generation of the appropriate telosomes would be more effective. Producing the desired telosomes by crossing monosomes of diverse elite origin is recommended to improve the chance of telosome recovery.

More research is needed to investigate the production of cytogenetic stocks. For instance, it is unknown why some chromosomes produce telosomes more readily. These differences may reflect a chromosomes susceptibility to break in meiosis when found as a monosome and/or the ability of the centromere to function properly after breaking. A better understanding of the underlying reason for chromosome differences and differences between monosomes of different origin could improve the efficiency of producing telosomes.

The production of a truncated 1BS telosome (Chapter 4) can not be easily explained. Furthermore, the absence of recombinant products, despite bivalent pairing and apparent chiasma formation, with a standard 1B chromosome is surprising. Testing another large sample of progeny from monotelodisomic plant carrying the truncated 1BS telosome could confirm this observation. With more meiotic pairing data it would be possible to accurately predict the expected frequency of recombinants. If the lack of recombination persists in such an experiment this would demonstrate that chiasma-like structures can form in the absence of crossing over which would oppose the accepted view that each chiasma corresponds to a cross over event (Fu and Sears 1973).

Chapter 6 - References

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