The genetics of leaf rust resistance in the durably resistant wheat cultivar 'Toropi'

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ACKNOWLEDGEMENTS

This thesis could not have been completed without the support of many people. I would like to sincerely thank all that have contributed to this thesis.

First of all, I would like to thank my supervisors Dr. Anita Brûlé-Babel and Dr. Brent McCallum for accepting me and giving me the opportunity to work with their groups. I appreciated all their help, teachings, support, supervision and trusting throughout my research.

I am grateful to my committee members Dr. Sylvie Cloutier and Dr. Georg Hausner for their time, advice and discussions.

I also would like to thank Dr. Colin Hiebert, Mr. Steve Shorter, OR Melhoramento de Sementes and Biotrigo for their collaboration. Dr. Hiebert was an important participant of this thesis for guiding me during the development of the genetic maps, supporting the research with laboratory material, use of his lab facilities and for his important suggestions and discussions. I am thankful to Mr. Shorter for the leaf and stripe rust ratings in New Zealand. OR Melhoramento de Sementes e Biotrigo were extremely important to have the populations analyzed in Brazil, principally Camilla Turra and Rodrigo Oliboni for rating the plants, and Dr. Andre Rosa for seeding the material. I also would like to thank Fazenda Mutuca and Cabanha Butiá for allowing my material to be grown in their farms. I thank Dr. Ron DePauw for believing in me, for his friendship and help.

I have no words to thank all the technicians of CRC that helped me during this

project. I would especially like to thank Pat Seto Goh, Mira Popovic and Allison Brown for their constant attention, help and friendship. I appreciate the help of Erica Riedel, Lorelle Furst, Sasanda Nilmalgoda, and the people from double haploid, rust and breeding groups.

I also want to thank the staff, professors and students of the Plant Science Department.

The financial support provided by the Willy-Wiebe Graduate Student Fellowship, the Faculty of Graduate Studies, the Natural Sciences and Engineering Research Council of Canada and the Agri-Food Research and Development Initiative is gratefully acknowledged.

I would like to give my special thanks to my family and friends. My parents Amarilis and Ottoni for constant collaboration, trusting, ratings in Brazil, discussions, teachings and principally love. My mother was a special person during all this process. I thank my sister Maria for all her attention and friendship.

I thank my husband Murilo for his love, collaboration, incentive, understanding and support.

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ABSTRACT

Rosa, Silvia Barcellos. Ph.D., The University of Manitoba, 2012. The genetics of leaf rust resistance in the durably resistant wheat cultivar 'Toropi'. Major Professors: Dr. Anita Brûlé-Babel and Dr. Brent McCallum.

Wheat is infected by leaf rust disease (*Puccinia triticina* Eriks.) almost everywhere it is cultivated. An important characteristic of this disease is the dynamic nature of *P. triticina* populations, which affects the effective life span of genes conferring leaf rust resistance (*Lr* genes). Genetic resistance is recommended as the best approach to control rust by its relatively low cost and environmental-friendly character. Genetic diversity and combinations of *Lr* genes should be used to achieve durable resistance. An effective strategy is to combine genes conferring partial resistance and minor effects. Toropi, a Brazilian wheat cultivar, has maintained leaf rust resistance since its release in 1965, suggesting that it is a good candidate for durable resistance. Two recessive complementary adult plant genes were previously described in Toropi. The objective of this study was to characterize and map the sources of resistance present in Toropi. Double haploid (DH) populations developed by crossing the leaf rust susceptible cultivar Thatcher with Toropi were analysed in Canada (Glenlea – 2010 and Portage La Prairie – 2011), New Zealand (Lincoln – 2010 and 2011) and in Brazil (Parana – 2011), and in greenhouse inoculation experiments. The leaf rust reactions indicated the presence of at least four leaf rust resistance genes in Toropi: one seedling gene and three adult plant genes. The seedling resistance gene, responsible for immune response in New Zealand, was mapped on chromosome 3D of wheat using simple sequence repeat (SSR) markers.

QTL analyses identified a QTL associated with leaf rust resistance (*QLr.crc-5AL.1*) on chromosome 5AL, which overlapped with a QTL for stripe rust (*QStr.crc-5AL.1*) in the same population. This gene, designated *Trp1,* is believed to be one of the two adult plant complementary partial resistance genes. The position of the *Trp-2* is not confirmed yet. One minor race specific adult plant gene, temporarily designated *Trp-3,* was mapped on 4BL chromosome. Individually, the *Lr* genes in Toropi have minor effects against leaf rust, except for *Trp-Se*, which conditioned immunity in New Zealand. However, when the Toropi *Lr* genes were combined an almost immune response resulted. Toropi had very good leaf rust resistance in South and North America, and in New Zealand. The molecular markers identified during this project could facilitate the incorporation of the Toropi genes in new cultivars, helping to achieve more diverse and durable wheat.

FOREWORD

This thesis has been written in manuscript style following the guidelines established by the Department of Plant Science at the University of Manitoba. A general introduction and literature review precedes the four manuscripts that comprise the main part of the thesis. Each manuscript consists of an abstract, introduction, material and methods, results and discussion. The first manuscript "Double artificial inoculation of *Puccinia triticina* in the study of wheat leaf rust resistance" was submitted to Plant Disease Journal as a special report. The second manuscript "Seedling leaf rust resistance derived from wheat cultivar Toropi: identification, characterization and genetic mapping of *Trp-Se*" was submitted to Theoretical and Applied Genetics Journal. The manuscripts were formatted following the Theoretical and Applied Genetics Journal. After the manuscripts, a general discussion and conclusions are presented, followed by literature cited and appendices.

1.0. INTRODUCTION

Wheat is one of the most important crops in the world. It is one of the major crops grown in Canada, which produced 23 million tonnes in 2010 (FAOSTAT 2012). Abiotic and biotic stresses challenge wheat production annually (Gustafson et al. 2009). Rust diseases are major constraints to wheat production in many regions throughout the world. Three rusts are able to attack wheat, leaf rust (*Puccinia triticina* Eriks.), stem rust (*P. graminis* Pers. f. sp. *tritici*) and stripe rust (*P. striiformis* Westend f. sp. *tritici*). Leaf rust tends to cause less damage than the other two rusts, but it is the most common and widely distributed wheat rust (Kolmer et al. 2009a). *P. triticina* affects yield because of premature defoliation of plants (Knott 1989) and alteration of photosynthesis, causing a reduction in the number of kernels per spike and kernel weight.

Improving resistance to rust fungi is one of the major tasks of wheat breeders worldwide. The most environmental-friendly and low cost method of controlling leaf rust is to use resistant cultivars. More than 60 genes conferring leaf rust resistance (*Lr* genes) have been described (Herrera-Foessel et al. 2012; McCallum et al. 2012a; McIntosh et al. 2011). Most leaf rust resistance genes are race specific and effective during all of the host life cycle, being called seedling genes. Seedling resistance is usually manifested by hypersensitive resistance response (Bolton et al. 2008). Leaf rust resistance conditioned by adult plant genes can be expressed only at adult plant stage. Some adult plant resistance genes are characterized by conferring partial resistance, which is not associated with a rapid hypersensitive response, but with a slow-rusting development. Slow-rusting genes result in fewer and smaller uredinia and longer latent periods (Knott 1989; Lagudah

et al. 2009). The partial resistance genes condition longstanding effectiveness. Three adult plant partial resistance genes have been identified: *Lr34*, *Lr46* and *Lr67* (Dyck 1977; Dyck et al. 1966; Herrera-Foessel et al. 2011; Hiebert et al. 2010; Krattinger et al. 2009; Rosewarne et al. 2006; William et al. 2006). *Lr68* is another gene conferring partial adult plant resistance that was described recently (Herrera-Foessel et al. 2012).

P. triticina populations are highly diverse. On an annual basis, an average of 35 races have been identified in Canada, while around 60 have been identified in US and South America (Huerta-Espino et al. 2011; Kolmer et al. 2011b; McCallum et al. 2011). The diversity of the *P. triticina* populations results principally from mutations in existing populations or migration from other areas. New genetic variation in the fungi occurs continuously and the deployment of a single resistance gene in a large wheat area leads to the selection and perpetuation of mutants or existing variants at low frequency. Virulence shifts in the *P. triticina* population can cause rapid loss of race specific gene effectiveness (Huerta-Espino et al. 2011).

Despite the fact that more than 60 *Lr* genes have been described, wheat breeders rely on relatively few genes. Loss of effectiveness, genetic drag, absence of diagnostic molecular markers, and non-adapted backgrounds make the deployment of some *Lr* genes difficult. Identification of new sources of resistance is important.

The best approaches to achieve durable resistance and avoid the evolution of virulent *P. triticina* races are to diversify crop production, utilize different combination of genes, and use partial resistance genes as the basis of leaf rust resistance (Singh et al. 2011b).

Toropi (Frontana 1971.37/Quaderna A//Petiblanco 8), a Brazilian cultivar, has maintained its leaf rust resistance since it was released in 1965, and cultivated for 15 years. Barcellos et al. (2000) reported two complementary leaf rust recessive genes in Toropi in the field in Brazil. Crosses between Toropi and near isogenic line Thatcher-*Lr34* indicated the presence of a dominant and two recessive adult plant resistance genes when artificially inoculated with race TCB-TD in the field in Mexico. As the F_2 population of this cross segregated for susceptibility, the presence of *Lr34* in Toropi was excluded. In this same study, two recessive genes for leaf tip necrosis (LTN) were identified, and necrosis was only visualized when both genes occurred together in an F_2 line, independently of leaf rust response. The genes from Toropi do not have an official designation and are being called *Trp-1* and *Trp-2*. The absence of *Lr13* in Toropi was confirmed by postulation and by the evaluation of hybrid necrosis (Zoldan 1998). The presence of *Lr34* and *Lr13* in Toropi was analysed because these genes are present in Frontana, which is in the Toropi pedigree. In Canada, F_2 families of backcross populations between Toropi and Thatcher fit a one gene segregation ratio, but different levels of resistance in the field indicated the presence of at least two genes conditioning adult plant leaf rust resistance (Kolmer and Liu 1997). The two leaf rust recessive genes of Toropi were previously localized on chromosomes 1A and 4D using monosomic analyses (Brammer et al. 1998). Amplified fragment length polymorphism (AFLP) markers associated with these two *Trp* genes were described, *XPcg/Mac 3* (25 cM from *Trp-1*) and *XPcg/Mac 6* (24.4 cM from *Trp-2*), and simple sequence repeat (SSR) markers positioned gene *Trp-1* on chromosome 1AS and gene *Trp-2* on chromosome 4DS (Da Silva 2002).

The main objective of this project was the identification, characterization and mapping of new sources of leaf rust resistance that can effectively be incorporated by breeding programs to improve resistance of wheat cultivars in Canada and other regions of the world. The steps to achieve the objective are described below:

1. Evaluation and characterization of leaf rust reaction of Toropi at seedling and adult plant stage against different *P. triticina* races in greenhouse and in the field.

2. Postulation of seedling resistance genes present in Toropi.

3. Development of double haploid and backcross populations crossing Toropi and Thatcher to determine the inheritance of Toropi resistance. Evaluation of these populations in different locations and years: Canada, Brazil and New Zealand.

4. Mapping the leaf rust resistance genes of Toropi using SSR and single nucleotide polymorphisms (SNP) markers.

2.0. LITERATURE REVIEW

2.1. Wheat

Wheat is one of the most important food crops and is a major dietary component worldwide (Gustafson et al. 2009). World production of wheat was 650 million tonnes in 2010; after sugar cane (1680 million tonnes), maize (844 million tonnes), and rice (672 x million tonnes) (FAOSTAT 2012). Wheat production is widespread globally from 67^{o} N to 45^{o} S latitude (Gustafson et al. 2009). The world's main wheat producing countries in 2010 were China, India, United States of America, Russian Federation and France (FAOSTAT 2012). Canada produced 26 million tonnes of wheat in 2009 and 23 million tonnes in 2010 (FAOSTAT 2012), representing 5% of the world's wheat. Approximately 80% of Canada's wheat production is exported (DePauw and Hunt, 2001). In 2009, Canada was in the 6th position of wheat-production countries, but in 2010 it became the 8th major producer, losing positions to Germany and Pakistan (FAOSTAT 2012).

Species of *Triticum* genera present different ploidy levels with the basic chromosome number of n = 1x = 7. Wild and cultivated species include diploid (2n = 2x =14), tetraploid (2n = 4x = 28, *T. turgidum* L. var. durum with BBAA genomes) and hexaploid species (2n = 6x = 42, *Triticum aestivum* L. with BBAADD genome). The diploid genomes are highly conserved with similar gene order along the seven pairs of chromosomes and are considered homoeologous in nature. It has been estimated that the formation of the various wheat species began approximately 10,000 years ago. *Triticum turgidum* received the A genome from *T. urartu* as a male donor (Dvorak et al. 1993). The donor of the B genome was identified as an ancestor of *Aegilops speltoides*, but there

are some controversies. The B genome is the largest of the wheat genomes, and the large degree of changes at the DNA level makes it difficult to establish its donor. *Triticum aestivum* (BBAADD) was derived from the hybridization of *Triticum turgidum*, as the female, and *Triticum tauschii* spp. *strangulata*, which was the ancestral donor of D genome. *Triticum turgidum* L., or durum wheat, and *Triticum aestivum* L., or bread wheat, are the major wheat species grown around the world (Gustafson et al. 2009).

Crop domestication started 10,000 – 20,000 years ago in the Fertile Crescent of the Near East, in Central America, and in Southern China. Wheat domestication was based primarily on selection for non-shattering, free-threshing characteristics and for higher grain yield (Gustafson et al. 2009). Further wheat improvement has combined other traits such as end-use quality, disease resistance, and abiotic stress tolerance. Global efforts have increased wheat yield to match the population growth demand, however severe under nourishment and hunger in poor countries exist due to poor distribution, lack of resources and infrastructure, and political instability. In order to meet world wheat demand due to population growth and increased meat consumption, it has been estimated that world wheat production will need to increase by 66% by 2040. Limitations to increased wheat production are land, environmental pressures and genetic diversity. Loss of genetic diversity has been happening since the domestication of wheat, but one important approach to meet increasing wheat demand will be the manipulation of secondary and tertiary gene pools for new sources of genetic diversity (Gustafson et al. 2009).

2.2. Wheat rust

Wheat rusts have been threatening the sustainable production of wheat for thousands of years, and still are responsible for frequent yield losses in wheat. Three rusts are able to attack wheat, leaf rust (*Puccinia triticina* Eriks.), stem rust (*P. graminis* f. sp. *tritici*) and stripe rust (*P. striiformis* f. sp. *tritici*). They differ in morphology and optimal environmental conditions for growth. Leaf rust is the most common and widespread rust of wheat worldwide; stripe rust is an important disease where wheat is grown in cold temperatures and has been increasing in prevalence even in warm conditions; while stem rust is considered to be the most destructive disease among the rusts. All three rusts occur worldwide, however their incidence is dependent on climate, *Puccinia* populations, and on the degree of resistance of the predominant cultivars (Knott 1989; Kolmer et al. 2009a).

Optimum environmental conditions for infection and sporulation of the rust pathogens are specific for each rust species, but all three wheat rusts require free moisture for spore germination and infection. Leaf and stem rust development require similar temperature conditions, while stripe rust infection is more efficient in colder temperatures. Temperature affects the disease by having effects on the pathogen and the host. The effectiveness of many rust resistance genes is dependent on temperature. For example, high temperatures reduce the effectiveness of *Lr34*, but increase *Lr13* resistance (McIntosh et al. 1995). The temperature sensitivity of genes for resistance in wheat to *P. triticina* is also dependent on the host background (Dyck and Johnson 1983).

Cultural methods, chemical control and/or genetic resistance can control rust infection. Removing volunteer plants should reduce sources of inoculum, while planting early can allow plant maturation before serious infection occurs in places where rust inoculum normally arrives late in the growing season, as is the case in Canada (rust pathway coming from Texas – US – and arriving in Canada from June to August). Several fungicides are available to control rust, but they increase input costs, can cause environmental contamination and pathogens have become resistant to them. The most effective method (economically and environmental-friendly) to control rust is to develop resistant cultivars (Knott 1989).

Between 1910 and 1920, Stakman and his coworkers discovered the presence of a number of forms of wheat rusts with differences in virulence and established the concept of physiologic races (Stakman and Levine 1922). The origin of pathogenic variability is sexual recombination, somatic hybridization (Wang and McCallum 2009) or mutation. In many parts of the world, the alternate host which supports the sexual phase of *P. triticina* is not present, or it is resistant to wheat leaf rust, reducing the importance of sexual recombination on pathogenic variability (Bolton et al. 2008; Knott 1989).

2.3. Life cycle of wheat rusts

The life cycle of the three rust species is similar. Rust pathogens are heteroecious and macrocyclic fungi, having five spore stages. The asexual stages occur on wheat and related grasses and the sexual stages on alternate hosts, which are different depending on the rust species. The sexual stages of leaf rust occur on *Thalictrum speciosissimum* or

Isopyrum fumaroides; barberry (*Berberis vulgaris L.*) and *Mahonia spp.* are the alternate host of stem rust; and barberry was also recently identified as the alternate host of stripe rust (Jin et al. 2010; Knott 1989; Kolmer et al. 2009a; Lagudah et al. 2006). The alternate host is not present in a number of countries where wheat rust is present, and it may be resistant to the rust pathogens; therefore, in some regions, the rust pathogens survive entirely in the asexual stage (Bolton et al. 2008).

Uredinia are the pustules formed in the primary host (*e.g.* wheat) during its growing season. Depending on the level of plant resistance, uredinia can be surrounded by chlorosis or necrosis. Urediniospores, which are dikaryotic, are carried by the wind over meters or thousands of kilometers. Wind is frequently the dispersal mechanism of urediniospores, but insects, animals and humans can also spread them. After a urediniospore falls onto a plant, germination happens depending on presence of free water, the temperature and light conditions. Germination induces the growth of the germ tube. When the germ tube reaches a stoma, an appressorium is produced over the stomatal opening (*P. striiformis* does not produce an appressorium), and a vesicle develops in the substomatal cavity. Then, hyphae growing from the vesicle give rise to haustoria inside host cells. After haustorial formation, infecting hyphae produced by the haustorial mother cells grow and come into contact with additional host cells. The mycelium growing in susceptible leaf tissue further differentiates to form uredinia that rupture the epidermis and produce urediniospores. Sporulation begins in seven to 14 days after infection, depending on temperature (Bolton et al. 2008; Knott 1989; Kolmer et al. 2009a; Lagudah et al. 2006).

At the end of the growing season, the urediniospore production ceases and black teliospores are produced. The teliospore is dikaryotic and two-celled, and is the survival spore stage for the rust. In many locations these are the overwinter stage of rusts, but they can also be used to survive hot dry summer conditions. After overwintering, each cell of a teliospore may germinate to produce a basidium. Meiosis at this stage results in four haploid cells, which are called basidiospores. These spores are forced through the air to reach an alternate host. The germinated basidiospores penetrate directly into epidermal cells, resulting in the formation of a pycnia on the upper leaf surface. Pycnia are of two mating types, '+' or '-'. Pycnidiospores in a viscous liquid (honeydew) are carried from one pycnia to another by insects, rain or leaf contact. After mating of pycnidiospores of opposite types, aeciums are formed on the underside of the alternate host leaf. Aeciospores, dikaryotic, are forcibly discharged from the aecium and may be carried by wind to nearby wheat fields. The infection by the aeciospores develops into uredinia, completing the life cycle (Bolton et al. 2008; Knott 1989; Kolmer et al. 2009a; Lagudah et al. 2006).

2.4. Wheat leaf Rust

Wheat leaf rust, also called brown rust, is caused by *P. triticina* Eriks. In the past, it was designated *Uredo rubigo-vera* (DC) (1815); *P. rubigo-vera* Eriksson (1899); *P. recondite* Rob. ex Desm. f. sp. *tritici* (1966); and currently is described as *Puccinia triticina* Eriks (Bolton et al. 2008).

Leaf rust is distributed all around the world; it is found almost everywhere wheat is grown. Primary hosts of *P. triticina* are common wheat (*T. aestivum* L.), durum wheat (*T. turgidum* L. var. durum), cultivated emmer wheat (*T. dicocc*um), wild emmer wheat (*T. dicoccoides*), *Aegilops speltoides*, goatgrass (*Ae. cylindrical*), and triticale (*X Triticosecale*) (Bolton et al. 2008; Roelfs 1989). The sexual stage occurs on the alternate host *Thalictrum speciosissimum* or *Isopyrum fumaroides* (Jackson and Mains 1921; Saari et al. 1968).

Leaf rust infection is characterized by the presence of small, round, orange-red pustules, up to 1.5 mm in diameter, present primarily on leaf blades. The pustules are larger and more predominant on the upper than the bottom leaf surface (Knott 1989; Lagudah et al. 2006).

The infection process requires free moisture for at least eight hours with temperatures around 20 ^{o}C, but infection can occur from 2 to 30 ^{o}C; however, longer periods of dew are required at lower temperatures (Kolmer et al. 2009a).

Leaf rust tends to cause less damage than stem rust, but it is the most common and widely distributed wheat rust (Kolmer et al. 2009a). *P. triticina* affects yield by causing premature defoliation of plants (Knott 1989) and altering photosynthesis, which leads to a reduction in the number of kernels per spike and kernel weight (Kolmer et al. 2009a; Lagudah et al. 2006). Losses are dependent on the growth stage of the plants when infection occurs. Early infections cause higher damage. Defoliation occuring at the jointing stage may cause yield losses as high as 95%, while defoliation in the dough stage may lead to losses of only 10% (Kolmer et al. 2009a).

Windblown urediniospores and urediniospores that overwinter on alternate hosts or volunteer plants are the sources of leaf rust inoculum (Kolmer et al. 2009a). The alternate hosts are rarely present to complete the sexual cycle. Also, many species of alternative hosts are resistant to infection by *P. triticina* (Bolton et al. 2008; Lagudah et al. 2006). Although the frequency of sexual cycle is low, the genetic variability of *P. triticina* population detected annually is high. On an annual basis, 50 to 70 phenotypes of leaf rust are identified in the United States (Kolmer et al. 2007; Kolmer et al. 2008a) and 30 to 50 races are found in bread wheat in France (Goyeau et al. 2006). About 100 different virulence combinations were identified during 2002 and 2004 in the Southern Cone of America (German et al. 2007). Forty virulence phenotypes of *P. triticina* were described in Canada in 2005 (McCallum and Seto-Goh 2008), 31 were identified in 2006 (McCallum and Seto-Goh 2009) and 46 in 2008 (McCallum et al. 2011).

2.5. Leaf rust resistance genes

To date, 65 leaf rust resistance genes (*Lr*) have been described in common hexaploid wheat, tetraploid durum wheat and diploid wild wheat species (Herrera-Foessel et al. 2012; Lagudah et al. 2006; McIntosh et al. 2011).

Most leaf rust resistance genes are race specific and effective from the seedling stage throughout the life of the plant; therefore these genes are designated seedling resistance genes. Seedling resistance is usually manifested by a hypersensitive response of rapid host cell death in the infection region, limiting the expansion of biotrophic pathogens in the host tissue (Lagudah et al. 2006; Lowe et al. 2011). Virulence shifts in

the *P. triticina* population can cause rapid loss of effectiveness of race specific genes. Generally, race specific genes provide clear levels of protection and are designated as major genes. Three major resistance genes in wheat have been cloned: *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003) and *Lr21* (Huang et al. 2003). The three genes display a common NBS-LRR structure (nucleotide binding site and leucine rich repeat domain), but the genes do not have similarities at the DNA or protein levels (Cloutier et al. 2007; Lowe et al. 2011). Virulence has evolved to each of these genes (McCallum et al. 2011).

Another category of leaf rust resistance is called adult plant resistance in which resistance genes are not expressed during the complete life cycle of the host but rather the expression is generally stronger after the development of the flag leaf. Some adult plant resistance genes are characterized by conferring partial resistance, which is not associated with a rapid hypersensitive response, but with a slow-rusting development. Slow-rusting genes are characterized by conferring fewer and smaller uredinia with longer latent periods (Knott 1989; Lagudah et al. 2009). The partial resistance genes present long standing effectiveness. Three adult plant partial resistance genes have been described: *Lr34*, *Lr46* and *Lr67*. These genes condition a non-specific response to leaf rust races and are also associated with resistance to yellow rust, stem rust and powdery mildew (Lagudah 2011). Therefore, these three genes are not just race non-specific, but also species non-specific (Herrera-Foessel et al. 2011; Hiebert et al. 2010; Kolmer et al. 2009a; Krattinger et al. 2009; Lowe et al. 2011; Rosewarne et al. 2006; William et al. 2006). *Lr68* is another gene conferring partial adult plant resistance that was described recently (Herrera-Foessel et al. 2012). The pleiotropic leaf rust, stripe rust, powdery mildew resistance gene *Lr34/Yr18/Pm38* encodes a protein with homology to ATP-

binding cassette (ABC) transporters, which are involved in the transport of a wide variety of substances across membranes (Krattinger et al. 2009). The locus is also associated with tolerance to stem rust (Dyck 1993a) and Barley Yellow Dwarf Virus, *Bdv1* (Singh 1993). Transgenic plants containing the *Lr34* sequence expressed comparable leaf rust resistance as the endogenous *Lr34* gene in flag leaves and at seedling stage under cold temperature (4 to 10 ^{o}C) (Risk et al. 2012). *Lr34* is temperature sensitive and is less effective at high temperature (McIntosh et al. 1995). Because alone *Lr34* is not capable of ensuring high level of protection against leaf rust in some regions, it is better deployed in combination with other *Lr* genes. In Hungary, the leaf rust infection of the near isogenic line Thatcher-*Lr34* was between 40 MS (moderate susceptible) and 60 S (susceptible), confirming the importance of combining with other genes (Vida et al. 2009).

2.6. Leaf rust in Canada

In North America, all three rusts are present every year, but losses are prevented principally by cultivation of resistant cultivars and fungicide applications. In Rouen, France, growing of barberry (*Berberis vulgaris* L.) was prohibited in 1660. In 1916, Canada and the US suffered a severe stem rust epidemic, causing an estimated loss of 8 million tonnes of wheat. In 1918, an eradication program to eliminate barberry in North America was started and reduced the frequency of stem rust epidemics similar to the experience in France. The last major rust epidemic in Canada and the US occurred in 1954, when both leaf and stem rust infections were severe, causing losses of more than

500 million dollars (Knott 1989). Since that time there have been leaf rust epidemics but not stem rust epidemics.

Resistance to rust is a constant major focus in wheat breeding programs. Rust reaction is an important factor in the decision to register wheat cultivars in Canada (DePauw and Hunt 2001; McCallum and DePauw 2008). The Western provinces of Canada, Manitoba, Saskatchewan and Alberta, cover more than 90% of the total Canadian small grain cereal production (McCallum et al. 2012b). The first significant stem rust resistant cultivar in Canada was Thatcher (1939 – 1967). Thatcher was effective in controlling stem rust until the 1950s epidemics caused by race 15B. After these epidemics, Thatcher was replaced by Selkirk. Since the release of cultivar Selkirk in 1954, stem rust in Canada has been effectively controlled (McCallum et al. 2007). Durable resistance to stem rust has been attributed to the use of effective resistance genes in combinations of two or more genes (Kolmer et al. 1991; McCallum et al. 2007).

In contrast, leaf rust is a constant threat to wheat production in Canada due to constant pathogen population shifts. Leaf rust occurs in all wheat-growing regions, but is more prevalent in Manitoba, Saskatchewan and Southern Ontario. The primary leaf rust resistance genes present in Canadian cultivars have been *Lr1, Lr2a, Lr10, Lr11, Lr12, Lr13, Lr14a, Lr14b, Lr16, Lr21, Lr22a, Lr30* and *Lr34* (McCallum and DePauw 2008). Leaf rust genes that remain effective in Canada despite use in cultivars are *Lr34* and *Lr22a*; and promising resistance genes include *Lr52, Lr60, Lr18, Lr35, Lr46* and *Lr67* (Herrera-Foessel et al. 2011; Hiebert et al. 2010; McCallum et al. 2007). Although there is virulence on both *Lr16* and *Lr21*, they remain useful in gene combinations.

Stripe rust is an annual problem in Southern Alberta and since 2000 it was also found in the central Canadian Prairies and Southern Ontario (McCallum et al. 2007; McCallum and Seto-Goh 2004).

Field surveys are conducted annually for stem and leaf rust in US and Canada since 1926 and 1931 (Johnson 1956), respectively, to monitor the development of new races and to analyze the prevalent races in each region. This can help breeders and farmers select the best cultivars and sources of resistance. To determine the predominant virulence, leaf rust isolates are inoculated onto a differential host set composed of near isogenic lines of *Lr* genes in Thatcher. Infection types on the first 12 *Lr* genes of the differential set are used to designate the isolate according to the North American virulence phenotype nomenclature (Long and Kolmer 1989). The leaf rust differential set in Canada and US contains the following genes: set 1 – *Lr1, Lr2a, Lr2c, Lr3*; set 2 – *Lr9, Lr16, Lr24, Lr26;* set 3 – *Lr3ka, Lr11, Lr17, Lr30*. Supplemental differentials are added to the Canadian differential set to provide additional virulence information: set 4 – *LrB, Lr10, Lr14a and Lr18* (McCallum and Seto-Goh 2003). Extra *Lr* genes are analysed in each country to get better virulence information in relation to the resistance gene(s) prevalent in commercial cultivars (Table 2.1).

Leaf rust is the most common and widespread disease of wheat in US. *Puccinia triticina* is able to overwinter in the Southern US, from the Gulf Coast of Texas and Georgia and the Atlantic coast of South and North Carolina to Oklahoma and Kansas. Many cultivars grown in this region are susceptible to leaf rust, allowing the establishment of a large population of *P. triticina* in US annually by February. Southerly

winds carry urediniospores from the Southern region to the Southeastern States, Ohio Valley, and Southern Great Plains; where the infections are widespread by mid May. By the end of May, leaf rust infections have started in the Northern Great Plains (Minnesota and South and North Dakota) (Kolmer et al. 2007). In Canada, typically leaf rust reaches a significant level during June, causing epidemics in the Canadian Prairies in late June to early August (McCallum et al. 2007). The movement of *P. triticina* urediniospores from the Southern to Northern US, ending in Canada, is recognized as the North America rust pathway. Therefore, it is important to know the predominant races in US as they are the prevalent races to reach Canada. USDA-ARS Cereal Disease Laboratory in St. Paul, MN, US provides updated information about the progress and spread of wheat leaf rust in US during the wheat growing season.

Similar predominant virulence leaf rust races are often found in the Great Plains regions of the US and the Western Canada Prairies (Manitoba, Saskatchewan); however differences in prevalent rust races have been reported in Ontario, Québec and in Prince Edward Island (McCallum and Seto-Goh 2004, 2008, 2009) (Table 2.2). Differences between the *P. triticina* population in different Canadian regions were also reported in the genetic background analyzed by random amplified polymorphic DNA (RAPD) (Kolmer et al. 1995), amplified fragment length polymorphism (AFLP) (Kolmer 2001) and expressed sequence tag derived simple sequence repeat (EST-SSR) markers (Wang et al. 2010).

Table 2.1. Differential set used in US, Canada and Brazil to determine the virulence phenotype of *P. triticina* isolates.

		Lr genes			
Set 1	Brazil	*Lr1*	*Lr2a*	*Lr2c*	*Lr3*
	US	*Lr1*	*Lr2a*	*Lr2c*	*Lr3*
	Canada	*Lr1*	*Lr2a*	*Lr2c*	*Lr3*
Set 2	Brazil	*Lr9*	*Lr16*	*Lr24*	*Lr26*
	US	*Lr9*	*Lr16*	*Lr24*	*Lr26*
	Canada	*Lr9*	*Lr16*	*Lr24*	*Lr26*
Set 3	Brazil	*Lr3ka*	*Lr11*	*Lr17*	*Lr30*
	US	*Lr3ka*	*Lr11*	*Lr17*	*Lr30*
	Canada	*Lr3ka*	*Lr11*	*Lr17*	*Lr30*
Set 4	Brazil	*Lr10*	*Lr18*	*Lr21*	*Lr23*
	US	*LrB*	*Lr10*	*Lr14a*	*Lr18*
	Canada	*LrB*	*Lr10*	*Lr14a*	*Lr18*
Set 5	Brazil	*Lr14a*	*Lr14b*	*Lr27 + Lr31*	*Lr20*
	US	*Lr21*	*Lr28*	*Lr39/Lr41*	
Extra differential lines	Brazil	*Lr3bg, ORL 4002*[a]			
	Canada	*Lr1 + LrCen, Lr2b, Lr3bg, Lr14b, Lr15, Lr19, Lr20, Lr21, Lr23, Lr25, Lr28, Lr29, Lr32*			

[a]ORL 4002 possesses a gene that was not identified yet.
References: Kolmer et al. (2011b); McCallum and Seto-Goh (2003); Dr. Barcellos, personal communication.

According to Wang et al. (2010), the virulence diversity of *P. triticina* has decreased in Manitoba and Saskatchewan over time due to selection of predominant races. Only a few races constitute more than 50% of the races in 2000 and 2001 (MBDS race) and over 70% of the total in 2006 and 2007 (TDBG and TDBJ). On the other hand, the *P. triticina* population has been maintained at a relatively constant level of diversity in Quebec and Ontario.

Ordonez et al. (2010) reported a high degree of similarity for SSR genotypes between leaf rust isolates from South and North America, concluding that leaf rust

isolates in both places probably have a common origin and suggesting the movement of spores from Mexico to South America and North America.

The predominant leaf rust races in the US, Canada and Brazil were compared (Table 2.2). The three countries adopted the North American nomenclature to designate the leaf rust races, however the genes present in the differential set vary from one country to the other (Table 2.1). The first three sets of the differential are the same for the three countries, but each country has chosen different additional genes to be analyzed to provide a better distinction among the isolates. The first three sets originate the first three letters that designate the race according to its virulence (Table 2.3). Letter B means avirulence to four genes of the set, the virulence increase toward the alphabet, ending with letter T, which indicates virulence to the four genes of the set (Long and Kolmer 1989). For example, an isolate with avirulence to *Lr1*, *Lr2a, Lr2c* and *Lr3* (genes that compose the first set of differential set) receives the designation B for the first set. If the same race is avirulent to *Lr9, Lr16, Lr24*, but virulent to *Lr26* (set 2), the race receives the designation C for this set, resulting in a race BC-. If the isolate is virulent to all genes of the third set (*Lr3ka, Lr11, Lr17* and *Lr30*), this set receives letter T and the race is designated BCT.

Table 2.2. Predominant races of *Puccinia triticina* on wheat in United States, Canada and Brazil.

Country	Year	Location	*Puccinia triticina* race (frequency of the isolate)[a]
Brazil	Before 1983	Not specified	**NBB** **LCB**
	2007	Rio Grande do Sul, Paraná, São Paulo	**MFK**-MT + ORL4002 V[b] (53%) **MDP**-MR (17%) **MFK**-MT (14%) **MDP**-MR (9%) **TFK**-CS (3%) **MFP**-CT (2%) **MCR**-RS (2%)
	2008	Not specified	**MFK**-MT + ORL4002 V (predominant)
	2009	Not specified	**MDT**-MR + ORL4002 V (30%) **MFT**-MT + ORL4002 V (17.5%) **MDP**-MR + ORL4002 V (12.5%) **MDR**-MR + ORL4002 V (7.5%) **TP(T)-**HT (5%) **TF(P)-**MT, TFT-MT (5%) **MDT**-MT (5%) **TDT**-MR (5%)
Canada	2007	British Columbia, Alberta	**MBD**S
		Manitoba, Saskatchewan	**TDB**G (61%) **TDBJ** (15.2%)
		Ontario	**MLD**S (36.4%) **TDB**G (21.2%) **TDBJ** (15.2%)
		Quebec	**TDBJ** **TDB**G **MFD**S
		Prince Edward Island	**MCN**S **FFD**S **MHN**Q **MHN**S
United States	2007	Considering all wheat regions	**TDB**GH (18.1%) **MFP**SC (13.9%) **MLD**SD (12.2%) **TDBJ**G (10.7%) **TDBJ**H (4.8%) **TDB**GG (4.7%) **MFGJ**H (3.7%) **TFBJ**G (3.5%) **MCD**SB (3%)

[a] Nomenclature follows the genes present in the differential set of each country. The first three sets are the same for the three countries, they are in bold. [b] V means virulence to ORL4002 gene.
References: Barcellos (2009); Kolmer et al. (2009b); McCallum et al. (2010).

The predominant races in Brazil in 2007, 2008 and 2009 (Barcellos 2009; Kolmer et al. 2008b) were compared with the predominant races in Canada (McCallum et al. 2010) and US (Kolmer et al. 2009b) in 2007 (Table 2.2). Comparing the virulence of the races by the first three sets only, it was possible to determine that US and Canada present similar virulence, as the prevalent race TDB in Manitoba and Saskatchewan (principal Canadian wheat region) was also one of the highest in US. Race MLD showed predominance in Ontario in 2007 and corresponded to 12.2% of the US races. In comparison, prevalent Brazilian races were all different from US and Canadian races.

Table 2.3. North American nomenclature to designate the leaf rust races based on four gene differential set (Knott 1989).

Nomenclature	**Four gene differential set Infection type**			
B	L[a]	L	L	L
C	L	L	L	H[b]
D	L	L	H	L
F	L	L	H	H
G	L	H	L	L
H	L	H	L	H
J	L	H	H	L
K	L	H	H	H
L	H	L	L	L
M	H	L	L	H
N	H	L	H	L
P	H	L	H	H
Q	H	H	L	L
R	H	H	L	H
S	H	H	H	L
T	H	H	H	H

[a]L = Low Infection Type (0 to 2 infection type)
[b]H = High Infection Type (3 to 4 infection type)

2.7. Breeding for leaf rust resistance

Producing cultivars with effective and durable leaf rust resistance is a challenge for wheat breeding programs. A new cultivar takes a long time to be released and new races are identified each year. A good approach to achieve durable resistance is to pyramid multiple *Lr* genes. Combination of genes may confer a more durable and effective resistance than the same genes in isolation by a synergistic interaction among leaf rust resistance genes (Hiebert et al. 2007; Samborski and Dyck 1982). Pyramiding of genes can include race non-specific adult plant resistance genes, genes for which no virulence has been identified, and genes for which virulence has evolved (Hiebert et al. 2007). The ability of the pathogen to overcome multiple genes is theoretically more difficult than overcoming only one single major resistance gene (Boyd 2006; McDonald and Linde 2002). Some examples of effective combinations of *Lr* genes are *Lr34* with *Lr13, Lr16, Lr17* and/or *Lr18* (German and Kolmer 1992). Combinations of *Lr13* with *Lr2a, Lr3ka, Lr11, Lr17, Lr18* or *Lr21* showed enhanced resistance relative to the single effect of either gene at adult plant stage (Kolmer 1992).

Gene pyramiding is very difficult through traditional phenotypic-based evaluation when genes with similar infection types or pleiotrophic genes that mask the effect of other genes are combined (Khan et al. 2005). A principle of host-pathogen genetics is when more than one interacting gene is involved, the reaction will be as low as, or lower than, the reaction level produced by the most effective resistant gene (McIntosh et al. 1995). This principle is used in the resistance gene postulation method to hypothesize which genes confer resistance for a target line. Gene postulation utilizes the gene-for-gene

specificity between host resistance genes and pathogen avirulence genes. The presence of genes is evaluated by comparing the leaf rust reaction of the target line with the reaction of plants with a known resistance gene against pathogen races with different avirulence gene combinations. Resistance gene postulation is a rapid method to identify possible genes conferring resistance, but it is not able to evaluate the presence of genes when the resistance is given for more than one gene, the interaction of genes, or for race non-specific resistance genes (Knott 1989; McCartney et al. 2005).

Presence of phenotypic markers linked with *Lr* genes is a tool to evaluate resistance genes of the target line. Leaf tip necrosis (LTN) associated with the *Lr34* gene is a useful trait to identify the presence of this gene in wheat lines. However, the presence of LTN is not exclusively for *Lr34*. The gene *Lr46* is also associated with the LTN trait (Rosewarne et al. 2006), and LTN is also associated with other wheat disease resistance, *e.g.* resistance to spot blotch caused by *Bipolaris sorokiniana* (Joshi et al. 2004). The absence of LTN does not exclude the presence of *Lr34* or *Lr46* because its expression is variable in different backgrounds and environmental conditions (Lagudah 2011; Lagudah et al. 2009). Another phenotypic marker used to identify leaf rust resistance genes is hybrid necrosis linked with *Lr13* (Singh and Rajaram 1991). In this case, presence of *Lr13* is identified by hybrid necrosis in progeny of a cross between the target line and durum wheat.

Because phenotypic characterization of some resistance genes could be complicated by minor genes, environmental conditions or genetic background, diagnostic molecular markers could facilitate the incorporation and combination of resistance genes in new

cultivars. The use of polymerase chain reaction (PCR)-based markers for the identification of leaf rust resistance genes can reduce time, effort and expenses to select for resistance and to combine genes (Dadkhodaie et al. 2011; McCallum et al. 2012b; Miedaner and Korzun 2012). Table 2.4 demonstrates molecular markers linked to *Lr* genes that could be used in breeding programs. The SSR or microsatellite markers are the most prevalent molecular markers being utilized to map rust resistance genes. The high abundance of polymorphism, reproducibility, low cost, general co-dominance, automation (Meksem and Kahl 2005), and the presence of a high-density SSR consensus map for bread wheat helps to localize the position of target genes using SSR markers (Somers et al. 2004). Single-nucleotide polymorphism (SNP) and diversity arrays technology (DArT) markers are being used more frequently, principally for genome wide screening. SNP (Allen et al. 2011) and DArT markers (http://www.triticarte.com.au/default.html) are array-based high-throughput low cost markers, allowing these markers to be used to genotype lines in a breeding program for a price as low as US$1 per line (Gupta et al. 2010).

Table 2.4. Molecular markers linked with leaf rust resistance genes. Origin, type of gene, chromosome position, name of the marker and distance to the marker are described.

***Lr* gene**	**Origin**	**Type**	**Chromosome position**	**Markers[a]**	**Distance[a]**	**DNA marker reference[b]**
Lr1	*T. aestivum*	Seedling	5DL	*RGA567-5*	0	Cloutier et al. (2007)
				WR003, WR001	0, 0.2 cM	Qiu et al. (2007)
Lr2a	*T. aestivum*	Seedling	2DS			
Lr2b	*T. aestivum*	Seedling	2DS			
Lr2c	*T. aestivum*	Seedling	2DS			
Lr3a	*T. aestivum*	Seedling	6BL	$UBC840_{540}$	6 cM	Khan et al. (2005)
Lr3bg	*T. aestivum*	Seedling	6BL			
Lr3ka	*T. aestivum*	Seedling	6BL			
Lr9	*Ae. umbellulata*	Seedling	6BL	$SCS5_{550}$	0.8 cM	Gupta et al. (2005)
				LR9-Res-2F-T, LR9-Sus -2F-C		Vida et al. (2009)
Lr10	*T. aestivum*	Seedling	1AS			Feuillet et al. (2003)
Lr11	*T. aestivum*	Seedling	2A			
Lr12	*T. aestivum*	Adult	4BS			
Lr13	*T. aestivum*	Adult	2BS			
Lr14a	T. turgidum	Seedling	7BL	*gwm344*	1 cM	Herrera-Foessel et al. (2008a)
Lr14b	*T. aestivum*	Seedling	7BL			
Lr15	*T. aestivum*	Seedling	2DS			
Lr16	*T. aestivum*	Seedling	2BS	*wmc764*	< 10 cM	McCartney et al. (2005)
Lr17a	*T. aestivum*	Seedling	2AS	*gwm614 / wmc407*	0.7 / 2.5 cM	Bremenkamp-Barrett et al. (2008)
				gwm636	4.0 cM	Zhang et al. (2008)
Lr17b	*T. aestivum*	Seedling	2AS			
Lr18	*T. timopheevi*	Seedling	5BL			
Lr19	*Thinopyrum ponticum*	Seedling	7DL	$STSLr19_{130}$	Inside alien segment	Prins et al. (2001)
Lr20	*T. aestivum*	Seedling	7AL	*STS638*	7.1 cM	Khan et al. (2005); Neu et al. (2002)
Lr21 (=Lr40)	*T. tauschii*	Seedling	1DS	*ksud14*	0.2 cM	Huang and Gill (2001)
Lr22a	*T. tauschii*	Adult	2DS	*gwm296*	2.9 cM	Hiebert et al. (2007)
Lr22b	*T. aestivum*	Adult	2DS			
Lr23	*T. turgidum*	Seedling	2BS			
Lr24	*Thinopyrum ponticum*	Seedling	3DL	*ASTS212*		Zhang et al. (2011)

***Lr* gene**	**Origin**	**Type**	**Chromosome position**	**Markers[a]**	**Distance[a]**	**DNA marker reference[b]**
Lr25	*Secale cereale*	Seedling	4BL	*gwm251 / gwm538*	3.8 / 3.8 cM	Singh et al. (2012)
Lr26	*Secale cereale*	Seedling	1BL	*Iag95*	1.7 cM	Mago et al. (2005)
Lr27	*T. aestivum*	Seedling (+ *Lr31*)	3BS	*DOX_1 / RKO_1*	Interval of 0.07 cM	Mago et al. (2011)
Lr28	*Ae. speltoides*	Seedling	4AL	*wmc313*	5 cM	Bipinraj et al. (2011)
				$SCS421_{570}$	15.3 cM	Bipinraj et al. (2011); Cherukuri et al. (2005)
Lr29	*Thinopyrum ponticum*	Seedling	7DS			
Lr30	*T. aestivum*	Seedling	4BL			
Lr31 (=Lr12)	*T. aestivum*	Seedling (+ *Lr27*) Adult	4BS			
Lr32	*T. tauschii*	Seedling	3DS	*wmc43, barc135*	0.6	Thomas et al. (2010)
Lr33	*T. aestivum*	Seedling	1BL			
Lr34	*T. aestivum*	Adult	7DL	*csLV34*	0.4 cM	Kolmer et al. (2008b); Lagudah et al. (2006)
				cssfr5, cssfr6	Inside the coding gene	Lagudah et al. (2009)
				caIND11, caISBP1	Inside the coding gene	Dakouri et al. (2010); McCallum et al. (2012b)
Lr35	*Aegilops speltoides*	Seedling	2B	*BCD260F1/35R2*	0 cM	Seyfarth et al. (1999)
Lr36	*Aegilops speltoides*	Seedling	6BS			
Lr37	*T. ventricosum*	Seedling	2AS	*URIC–LN2* followed by DpnII digestion	Inside 2NS-2AS translocation	Helguera et al. (2003)
Lr38	*Agropyron intermedium*	Seedling	6DL	*wmc773, barc273*	6.1, 7.9 cM	Mebrate et al. (2008)
Lr39 (= Lr41)	*T. tauschii*	Seedling	2DS	*gdm35, gwm210*	1.9, 10.7 cM	Raupp et al. (2001); Singh et al. (2004)
Lr42	*T. tauschii*	Seedling	1DS	*wmc432*	0.8 cM	Sun et al. (2010)
Lr44	*T. spelta*	Seedling	1BL			
Lr45	*Secale cereale*	Seedling	2AS			
Lr46	*T. aestivum*	Adult	1BL	*xsts1bl2 / xsts1bl9 / xsts1bl17*	2.2 / 0 / 2.2 cM	Mateos-Hernandez et al. (2006);

***Lr* gene**	**Origin**	**Type**	**Chromosome position**	**Markers[a]**	**Distance[a]**	**DNA marker reference[b]**
						Rosewarne et al. (2006)
Lr47	*Triticum speltoides*	Seedling	7AS	*CAPS Xabc465*		Helguera et al. (2000)
Lr48	*T. aestivum*	Adult	2BS	*gwm429 / barc7*	6.1 / 7.3	Bansal et al. (2008b)
			2BL	*wmc175 / wmc332*	10.3 / 2.5 cM	Singh et al. (2011a)
Lr49	*T. aestivum*	Adult	4BL	*barc163 / wmc349*	8.1 / 10.1 cM	Bansal et al. (2008b)
Lr50	*Triticum timopheevii*	Adult	2BL	*gwm382 / gdm87*	6.7 / 9.4 cM	Brown-Guedira et al. (2003)
Lr51	T. speltoides	Seedling	1BL	*S30-13L and AGA7-759R* plus digestion with PstI or BamHI		Helguera et al. (2005)
Lr52	*T. aestivum*	Seedling	5BS	*wmc149 / gwm234, Xtxw200*	11.3 / 7.2, 3.6 cM	Hiebert et al. (2005); Tar et al. (2008)
				gwm234 / cfb309	10.2 / 12.9 cM	Bansal et al. (2011)
Lr53	*T. dicoccoides*	Seedling	6BS	*cfd1 / gwm508*	1.1 / 4.5 cM	Dadkhodaie et al. (2011)
Lr54	*A. kotschyi*	Seedling	2DL			
Lr55	*Elymus trachycaulis*	Seedling	1B			
Lr56	*Ae. sharonensis*	Seedling	6AL	*gwm427*		Marais et al. (2010a)
Lr57	*Ae. geniculate*	Seedling	5DS	*FBB276* and *GSP*		Kuraparthy et al. (2007a)
Lr58	*Ae. triuncialis*	Seedling	2BL	*XksuH16, XksuF11, Xbg123, cfd50*		Kuraparthy et al. (2007b)
Lr59	*Ae. peregrina*	Seedling	1AL			
Lr60	*T. aestivum*	Seedling	1DS	*barc149*	8.4 cM	Hiebert et al. (2008)
Lr61	*T. turgidum*	Seedling	6BS	*P81/M70$_{269}$, P87/M75$_{131}$*	2.2 cM	Herrera-Foessel et al. (2008b)
Lr62	*Ae. neglecta*	Seedling	6AS-L			Marais et al. (2009)
Lr63	*T. monococcum*	Seedling	3AS	*barc57, barc321*	2.9 cM	Kolmer et al. (2010)
Lr64	*T. dicoccoides*	Seedling	6AL			
Lr65		Seedling	2AS	*barc124, barc212, gwm614*	2 cM	Mohler et al. (2012)

***Lr* gene**	**Origin**	**Type**	**Chromosome position**	**Markers[a]**	**Distance[a]**	**DNA marker reference[b]**
Lr66	Ae. speltoides	Seedling	3A	*S13-R16*	Close	Marais et al. (2010b)
Lr67	*T. aestivum*	Adult	4DL	*gwm165, gwm192*	0.4 cM	Herrera-Foessel et al. (2011); Hiebert et al. (2010)
Lr68	*T. aestivum*	Adult	7BL	*Psy1-1 / gwm146*	0.5 / 0.6 cM	Herrera-Foessel et al. (2012)
LrWo (Lr52?)	*T. turgidum*	Seedling	5BS	*gwm234 / wPt-1420*	7.2 / 20.3 cM	Singh et al. (2010)

[a]In the marker and distance "/" means that the markers are flanking the gene, and "," means that there is no recombination among them.
[b]Gene references and more information can be found in McIntosh et al. (1995) and at http://www.ars.usda.gov/Main/docs.htm?docid=10342.

Many rust resistance genes are no longer effective. The cultivation of a large area with a single resistance gene results in selection of virulent races, overcoming the resistance. Adult plant genes conferring partial resistance, and gene pyramiding provide a good approach to preserve or lengthen the efficacy of rust genes (Dadkhodaie et al. 2011; Ingala et al. 2012; Kolmer et al. 2009b; Lagudah 2011). Incorporation of *Lr34*, *Lr46* or *Lr67* has the advantage of not only conferring durable leaf rust resistance, but also resistance to other diseases such as stripe rust and powdery mildew (Lagudah 2011; McCallum et al. 2012b). Singh et al. (2011b) recommended the combination of four to five minor genes to achieve "near-immunity" and durable resistance. The International Maize and Wheat Improvement Center (CIMMYT) started to breed for resistance using minor genes in 1970s. Lines with 20 to 30% rust severity with susceptible infection types were selected in the early phase. To date, CIMMYT has released wheat lines with good rust resistance.

Major genes have provided significant economic benefits to wheat growers all around the world over many years (Lowe et al. 2011). *Sr31* and *Sr24* are stem rust resistance genes responsible for controlling this disease in all continents. Even though they have been overcome by Ug-99 and its variant races in Africa (Pretorius et al. 2000), these genes are still very important in other regions, keeping stem rust absent from the wheat fields. In Western Canada, the leaf rust resistance of the current commercial cultivars is partially conferred by the major seedling gene *Lr21* (Thomas et al. 2010), which after many years of effectiveness (available for breeding since 1970s) (Huang et al. 2003; Rowland and Kerber 1974) was overcome by shifts in the *P. triticina* population in 2011 (Dr. B. McCallum, personal communication).

Durable resistance to wheat rust is needed (Ayliffe et al. 2008). Diversification of leaf rust resistance is extremely important to prevent other threats like the Ug-99, which threatens 25% of the world's wheat crop. Over 90% of wheat cultivars in the proposed migration route are susceptible to this race (Ayliffe et al. 2008). Ug-99 is a potentially devastating wheat rust because two genes, *Sr24* and *Sr31*, for which Ug-99 and variants have virulence, were the primary genes used globally to provide stem rust resistance. Durable resistance helps assure economic security and food accessibility throughout the world (Stuthman et al. 2007).

The best strategy for durable crop protection is to diversify disease resistance. Even though more than 60 *Lr* genes have being described; many of them have not been released in commercial cultivars. One reason is the genetic drag that accompanies some genes, principally genes derived from alien species. For example, *Lr19*, derived from

Thinopyrum ponticum, confers excellent resistance, but it is not widely used because it is associated with yellow flour colour (McIntosh et al. 1995). The gene *Lr62* is associated with a large translocation derived from *Ae. neglecta* composed by the short arm and a small part of the long arm of chromosome 6A, requiring reduction of the translocation before it can be deployed in wheat cultivars (Marais et al. 2009). Another reason that limits the deployment of certain genes is the background where the gene is present, *i.e.* breeders prefer to incorporate genes present in elite cultivars. Efficient genetic marker strategies can help the effective incorporation of "novel" genes (Thomas et al. 2010).

The identification of new sources of genes is dependent on characterization of leaf rust reactions and localization of the gene in the wheat genome. Bulked segregant analysis (BSA) and quantitative trait loci (QTL) analysis could be used to genetically map the target gene in the wheat genome, depending on the qualitative or quantitative nature of the trait. BSA involves the comparison of two bulks of lines with opposite phenotypes, in this case a bulk of resistant and a bulk of susceptible plants derived from a bi-parental cross (Hiebert et al. 2012; Michelmore et al. 1991). Linkage of the trait and marker is characterized by the presence of a specific allele to a determinate marker in all or most of the plants from the resistant bulk and absence of the allele in the susceptible bulk. Recombination between the trait and the marker gives the genetic distance between the marker and the gene, measured in centiMorgans (cM) - length of chromosome over which crossing over occurs with one percent frequency.

Analysis of QTL is the classical approach to identify the loci involved with complex polygenic traits. A large number of individuals from a segregating population

are screened with markers distributed throughout the genome, followed by statistical analysis to identify regions in the genome associated with the trait (Meksem and Kahl 2005). The mapping of the genes *Lr48, Lr49* (Bansal et al. 2008b), *Lr14a* (Herrera-Foessel et al. 2008a), *Lr61* (Herrera-Foessel et al. 2008b) are examples of resistance genes mapped using BSA. *Lr68* was mapped on 7BL after QTL analyses performed using an Avocet-YrA/Parula population (Herrera-Foessel et al. 2012). Only a few QTL with high effects have been successfully transferred into elite wheat (Miedaner and Korzun 2012).

3.0. DOUBLE ARTIFICIAL INOCULATION OF *Puccinia triticina* IN THE STUDY OF WHEAT LEAF RUST RESISTANCE

3.1. Abstract

Artificial inoculation is required for most studies of cereal rust disease. The techniques used to inoculate *Puccinia triticina* Eriks., the causal agent of wheat leaf rust, have been successfully employed for decades without many alterations. Field experiments are often exposed to natural as well as artificial infection. Greenhouse experiments are usually limited by space, particularly if inoculations are conducted on adult plants. Multiple genes present in a cultivar can be differentiated by inoculating different races. This is usually done by inoculating one set of plants with one race and a second set of plants with a second race. A technique was developed to inoculate two or more *P. triticina* races on a single plant. The difference between rust inoculations with a single race and the double inoculation technique is that the plants are subjected to two cycles of inoculation. Tillers were split up and covered with vinyl cylinders to protect them from inoculum spray when they were not being inoculated. The double inoculation technique allowed perfect differentiation between resistant and susceptible rust reactions on a single plant, saving time, space, and seed. This technique also proved to be a good method to study quantitative resistance at the adult plant stage.

3.2. Introduction

Most plant pathology investigations require infection of the plants using artificial inoculation. Wheat leaf rust artificial inoculation is applied for several purposes, including determining the virulence of the rust pathogen population, increasing and conserving the fungi, studying pathogen/host interactions, and determining the effects of host resistance genes.

The basic procedure for cereal rust inoculation using urediniospore inoculum has been used for decades (Bushnell and Roelfs 1984; Knott 1989; McIntosh et al. 1995; Roelfs et al. 1992), however, slight modifications have been applied depending upon the species of rust and the host being studied. Dusting, brushing, injection, and spraying are four rust inoculation methods that can be used depending on the following factors: the number of plants to be inoculated, the objective of the inoculation, the amount of inoculum available, acceptable contamination risk, labour, and the availability of dew or wet periods during the incubation period (Roelfs et al. 1992).

Artificial inoculation is extremely important when studies are done on host resistance. Genetic studies are often performed outdoors because the large population sizes required are difficult to accommodate in the greenhouse. One disadvantage of outdoor experiments for genetic studies is the lack of control over the rust races infecting the plants, since naturally occurring inoculum can also infect the plants. When multiple race specific resistance genes are involved, mixtures of races in the natural and applied inoculum make it more difficult to identify individual resistance genes. In the greenhouse,

rust races can be controlled; however only one race is normally applied to a single plant, and space is limited for growing large numbers of adult plants.

Few studies have been conducted applying multiple rust races to the same plants in wheat. Browder (1972) designed an inoculator to apply urediospores of four different isolates of *P. triticina* Eriks. in adjacent patches in the same leaf. This technique was used to develop wheat lines with a combination of seedling race specific genes and to test F_2 segregating host populations.

The interactions of *Erysiphe graminis* and *Septoria nodorum* on wheat were studied by inoculating the pathogens on the same plant within a one-week interval (Weber et al. 1994). A multiple inoculation technique was performed to evaluate the resistance of barley seedlings to *Rhynchosporium secalis*, *Puccinia hordei* and *Erysiphe graminis hordei* (Kilpatrick et al. 1981). In this study, *R. secalis* and *P. hordei* were inoculated on day 11 and 12 after planting, respectively, followed by moist incubation after each inoculation. *E. graminis hordei* was inoculated on day 19. Successful evaluation of the three diseases was achieved with this multiple inoculation technique on a single plant.

The purpose of this study was to develop an efficient and effective method to inoculate single adult plants with two different *Puccinia triticina* races, thereby saving time, space, and seed. The two separate host/pathogen interactions on the same plant must be independent so that inoculation with one race does not confound the interpretation of the inoculation with the second race.

3.3. Material and Methods

Plants of Thatcher and its *Lr37* near isogenic line RL6081 (Tc-*Lr37:* Thatcher*8/VPM) were grown in pots in a growth cabinet. The temperature was kept at 18 ^{o}C and 16 ^{o}C during the day and night, respectively, with a 16h:8h (light:dark) photoperiod. Adult plants of Thatcher and Tc-*Lr37* were divided for inoculation according to the following categories: A) plants inoculated with race "A": isolate 136-1-3 FBM (avirulent to *Lr37*); B) plants inoculated with race "B": 09-167-1 TDBJ (virulent to *Lr37*); C) plants double inoculated with race "A" and then "B". Two experiments were performed. In the first experiments, two plants of Thatcher and Tc-*Lr37* were submitted to a single inoculation, while five plants of each were double inoculated. In the second experiment, five plants of Thatcher and Tc-*Lr37* were used in single and double inoculations. The nomenclature of the *P. triticina* races follows the current North American virulence phenotype designation (Long and Kolmer 1989). The avirulent race FBM was used first in the inoculation cycle. Plants having young, but completely developed, flag leaves were inoculated using a light mineral oil (Bayol 35, Imperial Oil Canada, Toronto, Ont.) as the carrier for the urediniospores when the inoculum was sprayed onto the leaves using an atomizer. The double inoculation was based on the inoculation of specific tillers in the first inoculation, while covering tillers that were to be inoculated in the second cycle of inoculation. Clear vinyl cylinders (8 to 12 cm of diameter, 90 cm of height) were used to isolate and protect the tillers not inoculated with race "A". The cylinders were opened at both ends. It was important to make sure that the flag leaves to be inoculated with the second isolate were completely protected by the cylinders. Depending on the total number of tillers in the plant, two or three tillers to be

inoculated were tagged together. A tag with fabric cord was used to assemble the tillers and to indicate the inoculum received. The flag leaves were directly inoculated avoiding any contact with the top of the open cylinder. The plants were allowed to dry for at least one hour before they were moved to an incubation chamber set at 100% humidity and an ambient temperature of 17 ^{o}C for approximately 17 hr. After the incubation period, the plants were moved to light, and the cylinders were removed after the leaves had completely dried. The interval between incubation and the second cycle of inoculation was 24 hr. That is, 24 hr after incubation, plants were subjected to the second cycle of inoculation, now with race "B", using the same protocol that was used for the first inoculation. This time, tillers already inoculated in the first cycle were covered with cylinders and the remaining tillers were inoculated with the second isolate (race "B"). Figure 3.1 is a diagram of the double inoculation method. The inoculation of additional Thatcher and Tc-*Lr37* plants with a single race was conducted at the same time as the double inoculation, however these plants were subjected to just one inoculation cycle. Leaf rust infection types were evaluated 14 d after inoculation. The infection type was evaluated using a rating scale of 0 to 4 (Long and Kolmer 1989).

The double race inoculation method was used to test progeny populations from the cross of the leaf rust resistant cultivar Toropi and the susceptible cultivar Thatcher. A double haploid (DH) population consisting of 463 lines was evaluated using the double inoculation technique. A BC_1F_1 population of 282 single plants was also tested. The plants from each of these populations were double inoculated with races BBBD (an avirulent race) in the first inoculation cycle and TJBJ (a virulent race) in the second inoculation cycle.

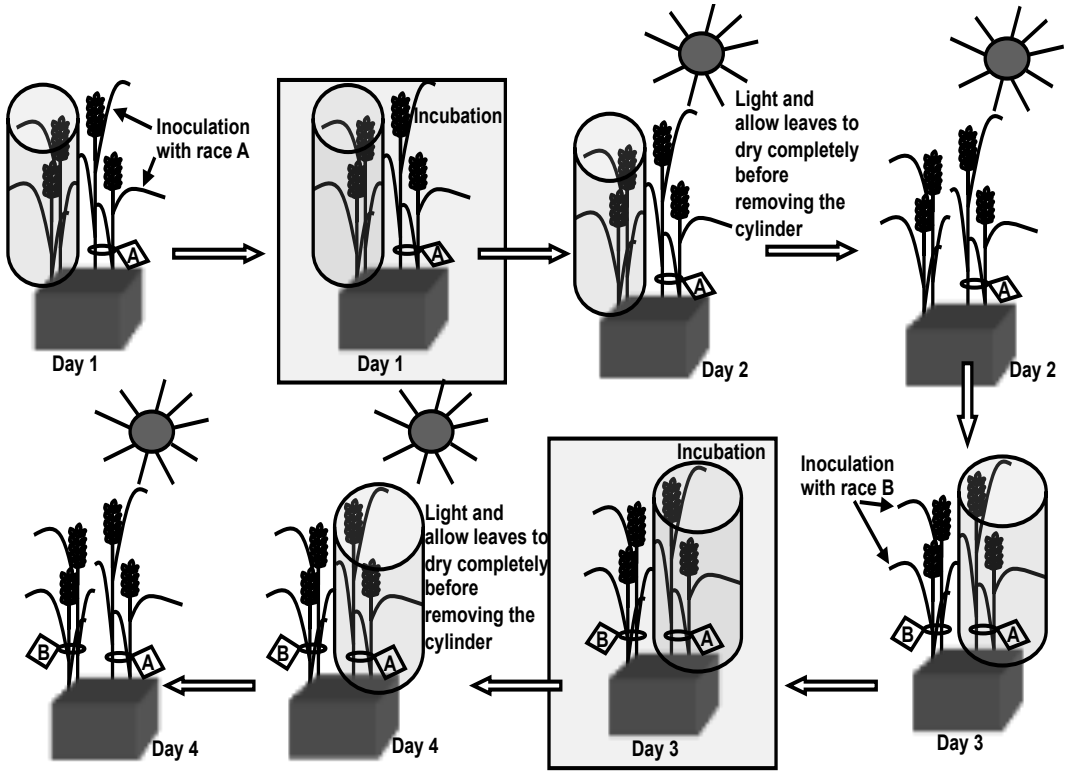

Figure 3.1. Steps of the double inoculation technique. During the first cycle of inoculation, tillers of the plant were separated into two groups; half of them were covered with a vinyl cylinder. The half not covered was inoculated with race "A". The plant was moved to a dew chamber for at least 17 hours in the dark. The next day, the plants were left to dry under light. After the leaves had completely dried the cylinders were removed and the plant was returned to the growth chamber. For the second cycle of inoculation, 24 hours after the first incubation, the tillers that were previously inoculated were covered with a vinyl cylinder, while the other half of the tillers were then inoculated with race "B". Incubation and drying periods were performed as in the first cycle of inoculation.

3.4. Results

When Thatcher was infected with any of the races used (BBBD, FBM, TDBJ or TJBJ), the reactions were susceptible, resulting in type '3' pustules. When Tc-*Lr37* was infected with race TDBJ it also produced susceptible type '3' pustules. However, Tc-*Lr37* was resistant to FBM resulting in a fleck (';') to '1' type resistant pustules (Table 3.1, Figure 3.2). The infected leaves from the plants that had been double inoculated showed the same infection type as the flag leaves from the same line inoculated singly with the same race (Table 3.1). Flag leaves on different tillers of Thatcher plants that were double inoculated with FBM and TDBJ were uniformly susceptible. On plants of Tc-*Lr37* that were double inoculated, the flag leaves on tillers inoculated with FBM were resistant while those inoculated with TDBJ were susceptible. The results were consistent in the five plants tested. This method allowed perfect differentiation of the effects of the two different *P. triticina* races on different tillers of a single plant (Figure 3.2).

Table 3.1. Comparison of single and double artificial inoculation on Thatcher and the near isogenic line Thatcher-*Lr37*. Two *P. triticina* Eriks. races were used: FBM and TDBJ. Two experiments were conducted. The double inoculation was performed on five plants of Thatcher or Thatcher-*Lr37*, while the single inoculation was tested on two plants of each line in the first experiment and five plants in the second experiment.

	P. triticina races	
	FBM	TDBJ
Single inoculation		
Thatcher	3	3
Thatcher-*Lr37*	; to 1	3
Double inoculation		
Thatcher	3	3
Thatcher-*Lr37*	; to 1	3

The reaction of the DH and BC_1F_1 plants from the cross Toropi/Thatcher with BBBD and TJBJ *P. triticina* races were perfectly distinguishable using the double inoculation on a single plant. In the BC_1F_1 population, 155 plants were resistant to BBBD (';' or '; to 1' reaction) and susceptible to TJBJ ('3' reaction). The predominant reaction within this population to TJBJ was susceptibility. The results confirmed field evaluations and previous studies on these populations using traditional inoculation with a single race on each adult plant (Rosa et al. 2012). A few contaminating pustules were observed but these did not cause problems in determining the rust reaction. A low level of contaminating pustules was also observed in the plants inoculated with a single race, which indicates that contamination could have resulted from the original inoculum or stray urediniospores in the air during the inoculation process.

3.5. Discussion

Genetic studies of quantitative resistance are usually limited to outdoor experiments as large population sizes are normally required. Since it is not possible to prevent natural inoculum from infecting the plants in the field, experimental results could be confounded by the effects of many races infecting wheat plants simultaneously. Indoor experiments combined with outdoor nurseries could save time, and improve the study of specific genes, for example, race specific genes that are not effective in the field.

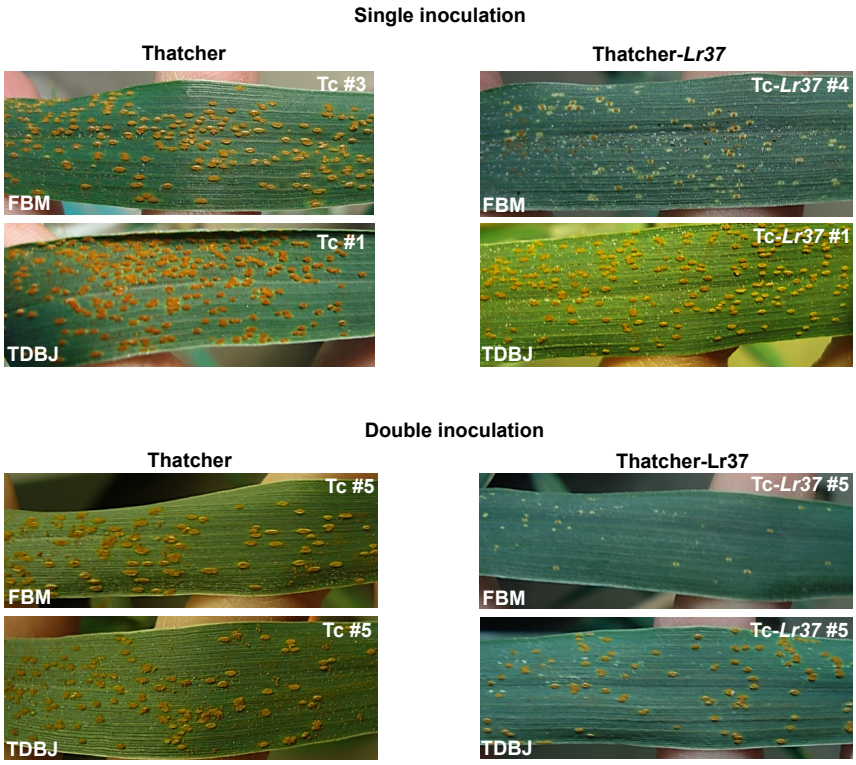

Figure 3.2. Pictures of Thatcher and the near isogenic line Thatcher-*Lr37* inoculated with FBM and TDBJ *P. triticina* races as single or double artificial inoculation.

The double inoculation method described in this paper proved to be an efficient and effective technique to save time, space, and seed, doubling the data per analyzed plant. In populations such as a BC_1F_1 or F_2 where only one plant represents each line, the double inoculation method could provide the leaf rust reactions of the line against two or more races, which would not be possible using a single inoculation. In this way, the double inoculation technique can help in interpreting the genetic segregation ratios of multiple resistance genes.

Few susceptible pustules (one to six) were observed in tillers of resistant DH and BC_1F_1 plants inoculated with BBBD and TJBJ. The presence of these odd pustules could be caused by contamination from a virulent race, or contaminants in the isolate used to inoculate that tiller; *i.e.* the isolate was not pure; or could be the result of stray urediniospores in the air during inoculation. Contamination was not observed in the experiment involving Thatcher and Tc-*Lr37*.

Utilization of two races in a single plant did not seem to affect the reaction of the host to either of the races. No differences in the leaf rust reaction were found comparing Tc-*Lr37* under single inoculation versus those under double inoculation. A multiple inoculation technique using three pathogens that cause scald, leaf rust, and powdery mildew was performed in barley (Kilpatrick et al. 1981). The disease reaction for each disease was the same as with single inoculations with each of the pathogens. On the other hand, interaction with *Erysiphe graminis* and *Septoria nodurum* in wheat resulted in a significant reduction in the final accumulation of *E. graminis* in the presence of *S. nodorum* (Weber et al. 1994). Inoculation with two to approximately 40 different *Puccinia spp*. isolates is currently a method used in field tests to study rust reactions in different countries including Australia (Bansal et al. 2008a), Mexico (Herrera-Foessel et al. 2011), United States (Kolmer et al. 2011a), Canada (Hiebert et al. 2010; McCallum and Seto-Goh 2010; Thomas et al. 2010), and Italy (Maccaferri et al. 2008). Studies have been conducted mixing different species of *Puccinia*, as well as different isolates of the same species. In natural conditions in the field, the plants could interact with different species of *Puccinia*, even with different races of the same species.

The multiple inoculation techniques developed by Kilpatrick (1981) and by Weber (1994) were based on inoculating different pathogens on a single plant. In the case of Kilpatrick (1981), the plant as a whole, without separating leaves or tillers, could be inoculated because they were observing different pathogens with distinct reactions. In our experiments, tillers had to be separated to be able to differentiate reactions from different races.

The technique of Browder (1972) was useful in testing several rust races on a single leaf by using an inoculator composed of four pads, in which each pad was moistened with inoculum from a different rust race. The inoculator was then squeezed onto the seedling leaf. The plants were moistened with water and oil before and after pressing the inoculator onto the leaves. According to the author, special handling was not necessary before incubating the plants. Browder's (1972) multiple inoculation technique was not tested on adult plants and it also required the construction of a specialized inoculator. Although no contamination was reported in his publication, it is possible that the incubation dew could carry urediniospores from one race patch to the other; and according to the author, high infection densities made some infection types difficult to evaluate. Some leaf rust resistance genes in wheat are characterized by different resistance intensities along the length of the leaf. For example, the presence of *Lr34* results in susceptible pustules at the base of the leaf diminishing in size and number towards the leaf tip, which is free of pustules (Krattinger et al. 2009). Therefore, the inoculation of more than one race in separate patches of the same leaf could be misinterpreted. This does not happen with the technique developed in this study as it is based on different races inoculated along the entire flag leaf on separate tillers. One

disadvantage of the double inoculation technique used in this study is the time spent preparing the plants to be inoculated, as it is necessary to separate and cover the protected tillers with vinyl cylinders; and then moving the pots to the inoculation and incubation rooms more than once. However, over all, time is saved in reducing the number of pots to be seeded and grown to the adult stage.

3.6. Conclusion

The double inoculation technique proved to be an efficient way to double the data obtained per plant. Our approach in developing the double inoculation was to maximize the information from a single plant. It may be possible to extend this approach to inoculations with three or more races by isolating the tillers and inoculating separately as we have done for two races in the current study. It is a good approach to study quantitative resistance when it is possible to differentiate genes depending on the rust race applied. This technique could be modified to study the effect of different *Puccinia* species on one plant and avoid the overlap of rusts. It is worth investigating the effectiveness of the double inoculation when aiming to investigate more than one plant disease.

4.0. SEEDLING LEAF RUST RESISTANCE DERIVED FROM WHEAT CULTIVAR TOROPI: IDENTIFICATION, CHARACTERIZATION AND GENETIC MAPPING OF *Trp-Se*

4.1. Abstract

Leaf rust is one of the most important diseases of wheat. *Puccinia triticina* Eriks., the causal agent, decreases grain yield and is present almost everywhere wheat is cultivated. Breeding for leaf rust resistance is challenging because resistance genes are overcome by frequent virulence shifts in the *P. triticina* population. The aim of this study was to identify, characterize and map a seedling resistance gene in the Brazilian cultivar Toropi. This study was based on a double haploid population created by crossing Toropi-6.3 and Thatcher. The expression of this gene ranged from necrotic flecks to small sporulating pustules when plants with this gene were inoculated with avirulent *P. triticina* races. Single gene lines carrying this gene were susceptible in Canada and Brazil, but were immune in New Zealand. In combination with other adult plant resistance genes in Toropi, this seedling gene improved the resistance to almost immunity even where the gene was not effective singly. The seedling resistance gene in Toropi-6.3 was mapped on the 3D chromosome inside a linkage block composed by 22 microsatellite markers. According to the results from gene postulation and confirmation by genetic mapping, this could be a novel leaf rust gene and was temporarily designated *Trp-Se*. The *Trp-Se* gene could be an important source of resistance to leaf rust when combined with other *Lr* genes, and in locations such as New Zealand where virulence is rare or absent in the *P. triticina* population.

4.2. Introduction

Leaf rust, caused by *Puccinia triticina* Eriks., is one of the most important diseases of wheat globally. The disease affects 90% of the total wheat area, causing up to 50% yield loss in epidemic conditions (Dubin and Brennan 2009b). Deployment of effective leaf rust (*Lr*) resistance genes is the best approach to control leaf rust, reducing costs to farmers and is also environmentally friendly. While more than 60 *Lr* genes have been formally designated (FAOSTAT 2012; McIntosh et al. 2011), identification of new sources of resistance is an ongoing task. Most leaf rust resistance genes confer hypersensitive seedling resistance, acting through gene-for-gene recognition, which can be quickly overcome by genetic variation and recombination in *P. triticina* populations. In some regions, the effective life span of wheat cultivars has been reduced to one to three years because of the breakdown of leaf rust resistance (German et al. 2007). The rapid evolution of virulence in *P. triticina* makes farmers rely on fungicides to manage leaf rust, but *P. triticina* can also evolve tolerance to these fungicides (Arduim 2009).

Durable resistance is a major target of breeding programs. Incorporation of slow-rusting adult plant resistance genes is one way to produce cultivars with durable resistance. To date, only three *Lr* genes have been described with this type of resistance: *Lr34* (Dyck 1977; Dyck et al. 1966; Krattinger et al. 2009), *Lr46* (William et al. 2006) and *Lr67* (Herrera-Foessel et al. 2011; Hiebert et al. 2010). *Lr68* was recently reported to confer slow-rusting resistance (Herrera-Foessel et al. 2012). The effect of partial resistance genes can be improved by combining them with other *Lr* genes. The field resistance conferred by many of the seedling resistance genes became more effective

when combined with *Lr34*, particularly the combination of *Lr16* with *Lr34* (German and Kolmer 1992). Improvement of resistance was also described when combining *Lr34* with *Lr13* or *Lr37* (Kloppers and Pretorius 1997). Gene pyramiding is a good strategy to develop more durable cultivars (Nelson 1978). The Canadian cultivar "Pasqua", with five *Lr* genes – *Lr11, Lrl3, Lr14b, Lr30* and *Lr34* (Dyck 1993b) – is an example of a gene pyramid cultivar with good resistance to leaf rust (McCallum et al. 2007).

Gene pyramiding is complicated by traditional phenotypic-based selection when breeders try to combine genes with similar infection types or epistatic genes that mask the presence of other genes (Khan et al. 2005). A principle of host-pathogen genetics is that when more than one interacting gene is involved, the reaction will be as low as, or lower than, the reaction level produced by the most effective resistance gene (McIntosh et al. 1995). This principle is used to postulate which genes confer resistance to a target line. Gene postulation utilizes gene-for-gene specificity between host resistance genes and pathogen avirulence genes (Flor 1942). The presence of genes is evaluated by comparing the leaf rust reaction of the target line with those near isogenic lines when inoculated with a diverse set of pathogen isolates with different avirulence gene combinations. Resistance gene postulation is a rapid method to identify possible genes conferring resistance, but it is difficult to evaluate the presence of genes when resistance is the result of more than one gene, the interaction of genes, or non-specific resistance genes (Knott 1989; McCartney et al. 2005). Efficient genetic marker strategies could facilitate pyramiding resistance genes, with the advantage of independence of the plant stage of gene expression, environment and gene interactions (McCartney et al. 2005; Thomas et al. 2010; Vida et al. 2009).

Toropi (Frontana 1971.37/Quaderna A//Petiblanco 8) is a Brazilian cultivar released in 1965, which was widely cultivated for over 15 years, while maintaining its leaf rust resistance for more than 40 years (Brammer et al. 2000). Two recessive complementary adult plant genes have been described in Toropi (Barcellos et al. 2000), but seedling resistance has never previously been reported (Barcellos et al. 2000; Liu and Kolmer 1998).

This work describes the identification, characterization and mapping of a seedling gene present in the cultivar Toropi. The gene was temporarily designated *Trp-Se.* This designation will be used throughout.

4.3. Materials and Methods

4.3.1. Plant material

Five leaf rust resistant lines (255644, 255693, 255725, 255764 and 255777) were selected at the F_6 generation from the cross between the wheat cultivar Toropi line 6 (Toropi-6) and IAC13-Lorena 2 (Ciano 67/IAS 51 – leaf rust susceptible cultivar to all known Brazilian *P. triticina* races) (Barcellos et al. 2000). The five lines were chosen because each expressed a different leaf rust reaction pattern. A second source of Toropi, which originated from seed increases in Uruguay (Toropi-Ur), was also tested (kindly provided by Dr. Sylvia German, INIA, Uruguay).

One plant each of Thatcher and Toropi-6.3 were crossed (Thatcher/Toropi-6.3). Three F_1 plants were used to generate 219 doubled haploid (DH) lines. The maize

pollination method was used to create the DH population following the technique described by Thomas et al. (2010).

4.3.2. Leaf rust resistance testing

Toropi-6, Toropi-Ur, IAC13-Lorena, the five leaf rust resistant lines, Thatcher, and 28 Thatcher near-isogenic lines (NILs; developed by the late Dr. Peter Dyck, Agriculture and Agri-Food Canada, Canada) were inoculated at the seedling stage with urediniospores of leaf rust (*Puccinia triticina* Eriks.), following the methods described by McCallum and Seto-Goh (2003). About six plants of each cultivar or line at the two leaf stage were inoculated. After 12 days, the phenotype was scored and the leaf rust reaction compared with NILs of *Lr1, Lr2a, Lr2b, Lr2c, Lr3, Lr3bg, Lr3ka, Lr9, Lr10, Lr11, Lr16, Lr17, Lr14a, Lr14b, Lr15, Lr18, Lr19, Lr20, Lr21, Lr23, Lr24, Lr25, Lr26, Lr28, Lr29, Lr30, Lr32 and LrB*. Based on the North American nomenclature (Long and Kolmer 1989), the following Canadian races were used: 15 BBBD, 96-14-1 CCDS, 161-1-1 FBDJ, 04-63-1 KBBJ, 12-3 MBDS, 128-1 MBRJ, 08-8-1 MDNS, 74-2 MGBJ, 06-11-1 TBBG, 06-1-1 TDBG, 05-10-1 TDBG, 77-2 TJBJ and 05-4-2 TPBG. The number in the race name indicates the isolate culture. Toropi-6 was heterogeneous for resistance at the seedling stage; therefore plants with the most resistant reaction were used to postulate the seedling gene present in Toropi.

Race BBBD was used to inoculate the Thatcher/Toropi-6.3 DH population in the greenhouse. The DH population was also tested under field conditions in Canada (Glenlea – 2010; Portage La Prairie - 2011), Brazil (Mutuca, Parana – 2011) and New Zealand

(Lincoln, Canterbury – 2010). In New Zealand, the leaf rust epidemic originated from natural inoculum. Spreader rows of susceptible lines in Canada and Brazil were inoculated with a mixture of local races by spraying light mineral oil (Bayol 35, Imperial Oil Canada, Toronto, Ont.) with urediniospores on leaves at early tillering to initiate the leaf rust epidemic. Plots were rated at anthesis when the susceptible checks (Thatcher) showed good infection and symptoms.

Greenhouse and field data were compared to interpret the reaction of the seedling gene in the field. In the greenhouse, the leaf rust reaction was classified according to Long and Kolmer (1989), using a 0 to 4 scale. Resistant infection types were ";" (hypersensitive flecks), "1" (small uredinia with necrosis), and "2" (small to medium uredinia with chlorosis). Susceptible reaction was given to plants with infection type "3" (medium uredinia without chlorosis or necrosis) or "4" (large uredinia without chlorosis or necrosis) (McCallum and Seto-Goh 2003). An extra class, designated "X", for heterogeneous or mesothetic reaction was also considered resistant (Knott 1989). In field trials in Canada and Brazil, adult plants were rated based on a modified Cobb scale (Peterson et al. 1948), which is based on the severity and infection type of the disease on the flag leaf (Knott 1989; McIntosh et al. 1995). In the field in New Zealand, a 0 to10 scale was used to rate the plants, where each unit increase was equivalent to another 10% of leaf area affected in the entire plant.

4.3.3. Inheritance analyses

Chi-square tests were used to evaluate the goodness-of-fit of observed and expected segregation ratios of both DH populations grown in the greenhouse.

4.3.4. Mapping

Leaf tissue was collected and freeze-dried from a single seedling of each DH line and six seedlings for the parental lines. DNA was extracted from each plant using a modified ammonium acetate extraction (Chao and Somers 2012). Polymerase chain reaction (PCR) of simple sequence repeat (SSR) markers and fragment analyses using an Applied Biosystems ABI 3100 genetic analyzer (Applied Biosystems, Streetville, ON, Canada) were performed as described by Somers et al. (2004). Data were first converted to a gel-like image, using Genographer version 2.1.4 (Benham et al. 1999), and Genescan 500-LIZ or 500-ROX (Applied Biosystems, Foster City, California) as internal molecular weight standards for the ABI 3100. All fragment sizes included 19 bp of the M13 fluorescent tag (Schuelke 2000).

Multiple bulked segregation analysis (MBSA) was used to localize the gene within the wheat genome (Ghazvini et al. 2012; Hiebert et al. 2012). A set of 423 SSR markers was used to screen the parental lines and 14 “mini-bulks” that each consisted of four independent DH lines. Ten bulks were composed of four resistant DH lines at the seedling stage and four bulks contained four susceptible DH lines. Linkage was confirmed by testing the markers with the full DH population. After removing lines that

were not true doubled haploids (e.g.. outcrosses) or did not have a consistent reaction in the field and greenhouse experiments, 188 DH lines were used to develop a genetic map of the region carrying *Trp-Se*. Polymorphic markers used to map *Trp-Se* are described in Appendix 1. Linkage analyses was conducted using Mapdisto software (Lorieux 2012) using the Kosambi (1944) mapping function and automatic commands. Default settings of Mapmaker were used for LOD threshold of 3.0 and for maximum recombination frequency of 0.3.

4.4. Results

4.4.1. Gene Postulation

The inoculation of Toropi-6 with 13 *P. triticina* races showed that it has seedling resistance (Table 4.1, Appendix 2), which has not been previously identified. Toropi was heterogeneous for this resistance, since Toropi-Ur did not have seedling resistance to any of the races tested. To test the extent of potential heterogeneity in Toropi-6, 58 additional Toropi-6 plants were inoculated at the two leaf stage with BBBD race. The reaction ";" or "; to 1", typical of the resistance found in seedlings of Toropi-6, was expressed in 37 of the 58 plants. IAC13-Lorena, the five leaf rust resistant lines derived from the cross Toropi-6/IAC13, and Thatcher were also inoculated with the 13 races. The data showed that the resistant lines and the assumed susceptible parent IAC13-Lorena also expressed resistance at the seedling stage (Table 4.1).

Table 4.1. Seedling leaf rust reaction of Toropi-6, Toropi-Uruguay (Toropi-Ur), IAC13-Lorena, Thatcher, five resistant lines derived from the cross Toropi-6/IAC13-Lorena (255644, 255693, 255674, 2556764, 255777) and near isogenic lines Thatcher-*Lr24* and Thatcher-*Lr32*. About six plants of each cultivar or line were inoculated in the greenhouse against different Canadian races. The name of the races follow the North American nomenclature (Long and Kolmer 1989) and the isolation number is indicated.

Leaf rust races	Toropi-6	Toropi Ur	IAC13-Lorena	255644	255693	255725	255764	255777	Tc-*Lr24*	Tc-*Lr32*	Thatcher
BBBD	H[a]	S[b]	S	R[c]	R	R	R	R	R	R	S
CCDS 96-14-1	H	S	R	R	R	R	R	R	R	R	S
FBDJ 161-1	R	S	H	R	R	R	R	R	R	R	S
KBBJ 04-63-1	R	S	S	n[d]	n	n	n	n	R	R	S
MBDS 12-3	H	S	S	S	S	S	H	S	R	R	S
MBRJ 128-1	R	S	S	S	S	S	R	S	R	n	S
MDNS 08-8-1	H	S	S	S	S	S	S	S	S	R	S
MGBJ 74-2	R	S	S	S	S	S	R	S	R	n	S
TBBG-L 06-11-1	R	S	R	n	n	n	n	n	R	R	S
TDBG 06-1-1	S	S	S	S	S	S	S	S	S	n	S
TDBG 05-10-1	H	S	H	S	S	S	S	H	S	R	S
TJBJ 77-2	S	S	S	S	S	S	S	S	S	n	S
TPBG 05-4-2	R	S	R	n	n	n	n	n	R	R	S

[a]H: Heterogeneous infection type (R and S plants) – light gray
[b]S: Susceptible plants (infection type "3" to "4")
[c]R: resistant plants (infection type ";" to "2") – dark gray
[d]n: indicates that the line was not tested to the race.
The five resistant lines were derived from a population developed by Barcellos et al. (2000).

To identify the genes conferring the resistance to Toropi-6, gene postulation was performed by comparing the seedling reaction of the cultivar with the leaf rust reaction of

28 *Lr* genes in near isogenic lines of Thatcher. Following the principle that when more than one gene is present, the reaction expressed is from the gene with higher resistance (McIntosh et al. 1995), the presence of any of the 28 *Lr* genes was excluded from Toropi-6 with the exceptions of *Lr14b* and *Lr15*. However, using avirulent races, the leaf rust infection type of the Thatcher NILs with either *Lr14b* or *Lr15* was higher than Toropi-6, which is ";" or "; to 1", indicating that if either of these genes are present in Toropi-6, another gene should be responsible for the superior resistance of Toropi at the seedling stage.

4.4.2. Inheritance of leaf rust resistance of Toropi-6.3 at the seedling stage

Twelve F_1 plants from the cross Thatcher/Toropi-6.3, including the F_1 plants used to develop the DH population, were inoculated at the seedling stage with BBBD race. All plants expressed ";" or "; to 1" reaction. This indicated that the gene in Toropi that expresses the ";" or "; to 1" reaction at seedling stage is a dominant gene.

When the Thatcher/Toropi-6.3 DH population was inoculated at the seedling stage with BBBD, two types of resistant responses were observed, ";" or "; to 1" reaction, characteristic of the Toropi seedling gene, and a mesothetic or X reaction. Chi-square test indicated the presence of one resistance gene plus three complementary resistance genes that acted together as a second source of resistance in Toropi at the seedling stage (Table 4.2). The first of these resistance genes is *Trp-Se*. Heterogeneity tests were performed to check the homogeneity between the DH lines derived from the three F_1 plants

(Heterogeneity of 1.785, 0.571 and 1.661 for each of the three experiments), allowing their data to be pooled (Table 4.2).

Table 4.2. Segregation ratios for seedling resistance of Thatcher/Toropi-6.3 double haploid population inoculated with race BBBD of *Puccinia triticina* in the greenhouse, Cereal Research Center, Winnipeg, Canada. The results comprised the average of three experiments.

			Average of three experiments (standard deviation)
Observed	R[a]		114.0 (7.93)
	S[b]		93.3 (8.33)
	Total		207.3 (12.01)
3 complementary + 1 gene			
Expected	R	9	116.63
	S	7	90.71
Chi-square			0.135
Probability			0.713

[a]R: resistant infection type: "; to 1" or "X" reaction; [b]S: susceptible infection type: "3" or "4" reaction.

4.4.3. Phenotypic reaction

Trp-Se conditions a ";" or "; to 1" reaction to all avirulent races of *P. triticina* at seedling stages in greenhouse (Figure 4.1, Appendix 2). Eleven races showed avirulence to this gene: BBBD, 96-14-1 CCDS, 161-1 FBDJ, 04-63-1 KBBJ, 12-3 MBDS, 128-1 MBRJ, 08-8-1 MDNS, 74-2 MGBJ, 06-11-1 TBBG, 05-10-1 TDBG and 05-4-2 TPBG. However, two races were virulent to seedlings in the greenhouse: 06-1-1 TDBG and 77-2 TJBJ.

In the field, *Trp-Se* was not effective singly in Canada or Brazil. However, DH lines that carried *Trp-Se* in combination with the adult plant resistance genes present in Toropi showed improved resistance compared to lines with only the adult plant resistance genes

in both locations. Among all Thatcher/Toropi-6.3 DH lines tested in New Zealand, 84 lines carried the seedling *Trp-Se* gene. All lines which had the seedling gene showed a resistant reaction, including 75 lines that showed the "0" reaction type. The susceptible check Thatcher had a "9" reaction type (0 to 10 scale). Therefore, all lines having the seedling gene showed a resistant, near immune, reaction to *P. triticina* races in New Zealand. Virulence to this gene must be absent or in low frequency in New Zealand, whereas virulent races were common in Canada and Brazil. In the Thatcher/Toropi-6.3 DH population, that has *Trp-Se* gene, there were more fully resistant lines compared to moderately resistant lines, while the opposite was true in the Thatcher/Toropi-6.4 DH population, where *Trp-Se* is absent. The ratio of number of resistant lines versus moderate lines in Canada (Glenlea – 2010) were 0.29 to Thatcher/Toropi-6.4 (absence of *Trp-Se* gene) and 1.44 Thatcher/Toropi-6.3 (presence of *Trp-Se*), indicating that even in locations where *Trp-Se* is not effective on its own it can increase the resistance when in combination with other effective genes. A similar effect of *Trp-Se* enhancing the level of resistance in lines with *Trp-1* and *Trp-2* was also found in all other locations where we tested the DH Toropi populations.

Figure 4.1. Pictures of leaves of Toropi-6.3 and Thatcher at seedling stage inoculated with BBBD race in the greenhouse. (A) Toropi-6.3: the leaf rust reaction is typical of the presence of *Trp-Se*; (B) Thatcher: susceptible to BBBD race.

4.4.4. Molecular mapping of Trp-Se

MBSA showed a strong association between *Trp-Se* and two markers on chromosome 3D, *barc71* (Figure 4.2) and *barc274*. Both markers were screened with 188 DH lines, confirming the linkage of *Trp-Se* on 3D.

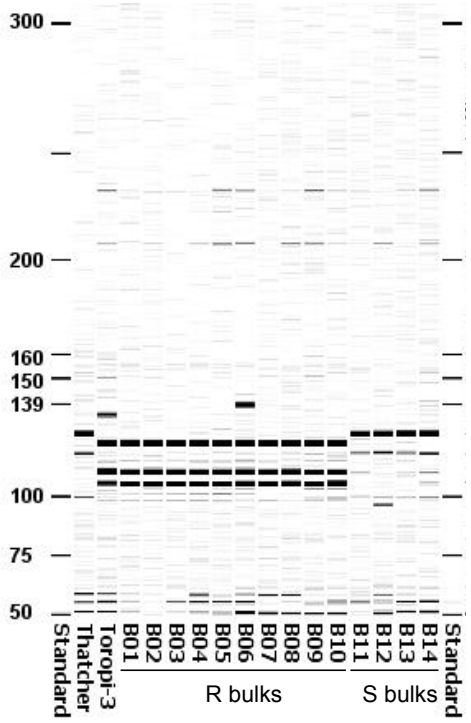

Figure 4.2. Multiple bulked segregation analysis (MBSA) performed to localize the seedling gene of Toropi-6.3 - *Trp-Se*. Co-dominant *barc71* marker showed to be associated with Toropi seedling gene. The picture is representing the gel-like image developed by Genographer version 2.1.4 with 500-ROX as internal molecular weight standards for the ABI 3100. All fragment sizes include 19bp of the M13 fluorescent labeled primer. R bulks means bulks of four resistant plants at the seedling stage; S bulks are bulks of four susceptible plants.

The genetic map of *Trp-Se* was constructed with 28 polymorphic markers on chromosome 3D with 188 DH lines (Figure 4.3; Appendix 1). Three SSR markers previously mapped on the terminal position of the short arm of chromosome 3D (Somers et al. 2004) were not associated with the seedling gene: *cfd51*, *cfd79* and *wmc43*. Twenty-two SSR markers formed a linkage block composed of 17 dominant (all SSR markers null for Toropi-6.3) and five co-dominant markers. The block was defined by SSR markers covering about 60 cM on a modified version of the consensus map described by Somers et al. (2004). The following SSR markers were co-dominant markers inside the block: *wmc492*, *gwm383*, *barc71*, *gpw114* and *gpw307*. Three SSR markers inside the linkage block were monomorphic between the parents: *barc323*, *gpw5094* and *wmc552*. No recombination occurred between *Trp-Se* and the linkage block (Figure 4.3). All distal markers on the long arm of this chromosome were tested but polymorphic markers outside the linkage block at the terminal location were not found.

4.5. Discussion

The wheat cultivar Toropi was found to have seedling leaf rust resistance, in addition to its previously reported adult plant resistance genes (Barcellos et al. 2000). The seedling leaf rust gene, *Trp-Se*, induces a "; to 1" reaction to avirulent races. Virulence was found for *Trp-Se* in Canada and in Brazil, but the presence of *Trp-Se* resulted in an immune reaction to leaf rust in New Zealand.

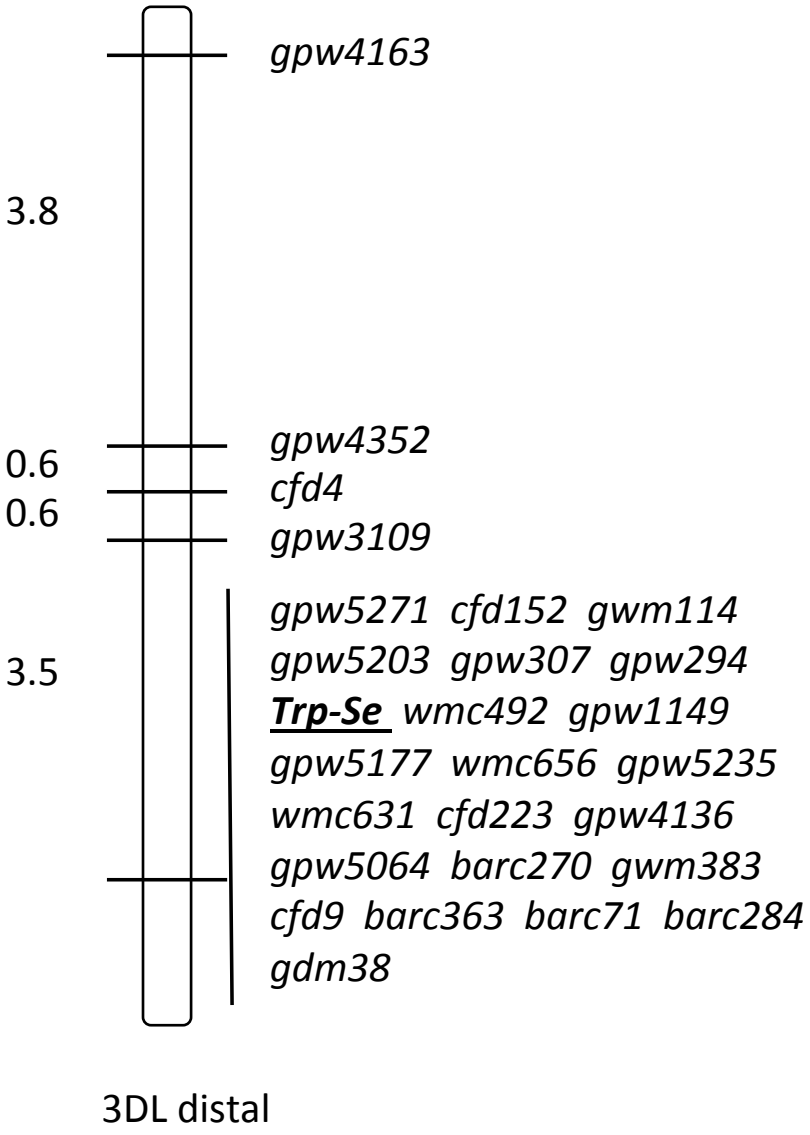

Figure 4.3. Genetic map of *Trp-Se* on chromosome 3D. The map was based on 188 DH lines derived from the cross between Thatcher and Toropi-6.3. Map distances are in centiMorgans.

Chi-square analyses of Toropi-6 at seedling stage indicated the presence of *Trp-Se* plus three complementary genes (Table 4.2). Besides the "; to 1" leaf rust reaction found in the Thatcher/Toropi-6.3 DH population, we also found lines with mesothetic reaction, indicating the presence of extra genes in Toropi. The mapping of adult plant genes in Toropi-6 indicated that a race specific adult plant gene, temporarily designated *Trp-3*, was expressed at seedling stage in the presence of other genes, conferring mesothetic reaction (see chapter 6), explaining the rare segregation ratio Toropi-6.3 at seedling stage. The hypothesis is that the three complementary genes indicated by chi-square test are *Trp-3* combined with the adult plant genes *Trp-1* and *Trp-2*.

The importance of *Trp-Se* in Toropi is principally in combination with other *Lr* genes. However, it could be an effective source of resistance in locations such as New Zealand where virulence is rare or non-existent. Our experiments indicated that the presence of *Trp-Se* enhanced the resistance conditioned by the adult plant genes in Toropi to near-immunity. Synergistic interaction among leaf rust resistance genes is well documented. Even partially "defeated" genes, such as *Trp-Se* for which virulence already exists, retain the ability to improve the resistance to leaf rust when in combination. The combinations of *Lr16* and *Lr34* (German and Kolmer 1992) is an example of the effective synergic interaction using "defeated" genes (McCallum et al. 2007). The slow-rusting resistance genes *Lr34*, *Lr46* and *Lr67* are characterized as having partial resistance, with few susceptible pustules on the leaves. This characteristic is very useful to the durability of the gene, but some farmers still prefer to have a disease-free crop. Pyramiding of resistance genes could be deployed to increase the resistance level of these genes to near immunity.

Gene pyramiding could also increase the durability of qualitative *Lr* genes. The emergence of virulence in the *P. triticina* population is related to the widespread deployment of a single gene, which leads to rapid selection for virulence to this gene in the population. Therefore, genes used in combination delay the virulence selection, keeping the resistance gene effective for a longer time.

Toropi was heterogeneous for the presence of the *Trp-Se* seedling resistance. Our experiments were based on the Toropi variant that originated from a selection made by Dr. A. Barcellos, who designated the line as Toropi-6 (Barcellos et al. 2000). A source of Toropi from Uruguay was also tested, but did not have this seedling leaf rust resistance. However, the leaf rust reactions of Toropi-6 and Toropi-Ur were similar in the field. This was the first time that seedling resistance in the Brazilian cultivar Toropi was described. Liu and Kolmer (1998) evaluated Toropi at the seedling stage with different Canadian races, but did not find resistance. The source of Toropi used in their experiment was from Uruguay, which in our experiments also did not express resistance at the seedling stage. Barcellos et al. (2000) could not identify seedling rust resistance in Toropi-6 likely because the *P. triticina* races used to test Toropi-6 in Brazil were all virulent to the gene. Toropi-6 was tested in 2008 with six different Brazilian races, and it was susceptible at the seedling stage to all of these races (Dr. Barcellos, personal communication).

Trp-Se was localized on wheat chromosome 3D inside a linkage block of 22 SSR markers. It was not possible to identify any SSR markers that mapped distal to the linkage block. Linkage blocks are usually characteristics of alien chromosome translocations or large chromosome mutations. The predominance of null alleles related to Toropi-6.3 in

the markers inside the linkage block is another indication that the *Trp-Se* was derived from an alien translocation (Appendix 1) (Gupta et al. 2006b). No recombination was found inside the linkage block, which corresponds to about 60 cM in the modified consensus map of Somers et al. (2004). There are no alien species included in the pedigree of Toropi, but it is known that Toropi was derived from old European cultivars, which may have been submitted to interspecific crosses. The linkage block is composed of mostly dominant and some co-dominant markers. The SSR markers *gwm114*, *barc71* and *gwm383* are clear co-dominant markers inside the linkage block that could be used in marker assisted selection.

Two *Lr* seedling genes have been described on chromosome 3D. *Lr32*, derived from *Aegilops tauschii*, was mapped on the short arm of the 3D wheat chromosome (Thomas et al. 2010). According to the authors, *Lr32* is flanked by the SSR markers *wmc43* (proximal) and *barc128* and *cfd79* (distal). The *wmc43* marker was not linked with the *Trp-Se*, nor *cfd79; barc128* was not tested. The other gene on 3D is *Lr24* (Gupta et al. 2006a). It is derived from *Agropyrum elongatum* and provides seedling resistance. Gene postulation gave an indication that the seedling gene in Toropi was not *Lr24* (Table 4.1, Appendix 2). Inoculation with 08-8-1 MDNS and 05-10-1 TDBG races resulted in susceptibility to the near isogenic line Tc-*Lr24*, while Toropi-6 expressed "; to 1" reaction. Experiments are being performed to confirm that the seedling gene of Toropi-6.3 (*Trp-Se*) is definitely not *Lr24*.

In conclusion, Toropi carries seedling resistance conferred by *Trp-Se* on chromosome 3D. Molecular markers *barc71*, *gwm114* and/or *gwm383* could be used

effectively in marker assisted selection to integrate this gene in new cultivars. The deployment of *Trp-Se* should be done in combination with other *Lr* genes, as virulence to it was already identified, and because it is able in combination with other genes to improve the resistance to almost immunity. *Trp-Se* was highly effective in New Zealand, conferring immunity against leaf rust.

5.0. INHERITANCE OF LEAF RUST RESISTANCE IN THE BRAZILIAN WHEAT CULTIVAR TOROPI

5.1. Abstract

Puccinia triticina Eriks. affects wheat production worldwide by causing leaf rust disease. The best management of the disease is the deployment of genetic resistant cultivars, principally containing durable resistance. It is difficult to breed for leaf rust resistant cultivars that remain resistant over time due to a high frequency of virulence shifts in the *P. triticina* population. To achieve durable leaf rust resistant cultivars the best approach is to stack resistance genes. Race non-specific genes should be used as the basis to control the disease combined with other genes. The Brazilian cultivar Toropi has demonstrated durable leaf rust resistance in South America. It was previously found to have two complementary race non-specific, adult plant resistance genes *Trp-1* and *Trp-2*. The leaf rust resistance of Toropi was studied by analyzing double haploid populations made by crossing with the susceptible cultivar Thatcher. Toropi expressed nearly immune resistance in field trials in Canada, Brazil and New Zealand. Based on field and greenhouse testing, the resistance of Toropi is conferred by at least four genes; a seedling gene, *Trp-Se,* two adult plant resistance genes, *Trp-1* and *Trp-2*, which acted in a complementary manner in Canada and Brazil, but conditioned resistance individually in New Zealand, and a minor race specific adult plant resistance gene, temporarily designated *Trp-3*.

5.2. Introduction

Leaf rust disease is a frequent threat to wheat farmers. Although the frequency and severity of leaf rust have been reduced by the use of resistance genes, the yield losses in endemic areas average 15 to 20% in developing countries (Dubin and Brennan 2009a) and 10% in developed countries (Huerta-Espino et al. 2011). Favorable environmental conditions, cultivation of susceptible cultivars, and high disease pressure from *Puccinia triticina* Eriks., the causal agent of wheat leaf rust, can lead to higher wheat yield losses, and lower wheat quality. Application of foliar fungicides can reduce the damage caused by leaf rust. In South America and China, fungicides are the principal control method for leaf rust. If a fungicide is not applied, potential yield losses can exceed 50% (Huerta-Espino et al. 2011).

To date, more than 60 leaf rust resistance genes (*Lr*) have been named (McIntosh et al. 2011), however breeders worldwide rely on relatively few genes in their programs. Many *Lr* genes are from wild species (McIntosh et al. 1995). Linkage drag from introgression of large chromosomal regions from wild species can lead to incorporation of undesirable traits associated with the *Lr* genes (Knott 1980). Other *Lr* genes have lost their effectiveness to evolving *P. triticina* races (Kolmer and Anderson 2011; Kolmer et al. 2008a; Kolmer et al. 2009b; Kolmer et al. 2011b; McCallum and Seto-Goh 2003, 2004, 2008, 2009; McCallum et al. 2010; McCallum et al. 2011). Gene combinations are used to increase the effective longevity of resistance genes, and consequently the acceptance of resistant wheat cultivars. Molecular markers linked to the *Lr* genes are important in facilitating the incorporation of multiple resistance genes in a cultivar (Gupta

et al. 2010). Therefore, the use of *Lr* genes is limited due to the effectiveness of the gene, the absence of tightly linked or gene specific molecular markers, the presence of linked deleterious genes, the incorporation into elite cultivars, and durability of the gene over time in production. The combination of four to five minor genes with partial resistance can result in a nearly immune durable wheat cultivars (Singh et al. 2011b). Adult plant resistance (APR) genes, particularly the genes conferring partial resistance in a race non-specific manner to one or multiple rust diseases, should be deployed in combination with other *Lr* genes as the basis of leaf rust resistance in wheat cultivars (Lagudah 2011). *Lr34, Lr46, Lr67* are the three genes described that confer slow-rusting race non-specific APR (Herrera-Foessel et al. 2011; Hiebert et al. 2010; Krattinger et al. 2009; Rosewarne et al. 2006; William et al. 2006). *Lr68* was recently described conferring partial resistance to leaf rust (Herrera-Foessel et al. 2012).

Toropi (Frontana 1971.37/Quaderna A//Petiblanco 8) is a Brazilian cultivar released in 1965, which was widely cultivated for over 15 years, while maintaining its leaf rust resistance for more than 40 years. Toropi resistance is a combination of seedling and adult plant resistance (Barcellos et al. 2000; Liu and Kolmer 1998; Rosa et al. 2012). Our group has described the presence of a seedling resistance gene *Trp-Se* in Toropi, conferring a high level of resistance to some *P. triticina* races (see chapter 4). Barcellos et al. (2000) identified the presence of two complementary recessive APR genes in Toropi (temporary called *Trp-1* and *Trp-2*), which were estimated to be race non-specific APR genes conferring durable resistance.

In the present study, the inheritance of leaf rust resistance in Toropi was deciphered using greenhouse data and field results from Canada, Brazil and New Zealand.

5.3. Materials and Methods

5.3.1. Plant materials

A selection of Toropi (Toropi-6) was crossed with the Brazilian leaf rust susceptible line IAC13-Lorena (Ciano 67/IAS 51) (Barcellos et al., 2000), to generate segregating F_2 populations. From the segregating population, five resistant lines at the F_6 generation were selected based on their different phenotypic expression of rust resistance (255644, 255693, 255724, 255764 and 255777) (Barcellos et al. 2000). Toropi-6 was used throughout this study.

To analyze the inheritance of leaf rust resistance in Toropi, Toropi-6 was crossed with the susceptible cultivar Thatcher. Since Toropi-6 was heterogeneous for seedling leaf rust reaction (see chapter 4), two Toropi parental lines Toropi-6.3 and Toropi-6.4 were crossed with Thatcher, producing two double haploid (DH) populations from the Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 F_1 plants. The seedling gene *Trp-Se* was present in the Thatcher/Toropi-6.3 population, but not in the Thatcher/Toropi-6.4 population. Three F_1 plants were used to generate each DH population, which were composed of 217 and 246 DH lines, respectively, for the Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 populations. The wheat/maize hybridization and embryo rescue method was used to create the DH population (Thomas et al. 2010).

Backcross populations (BC) were also developed using Thatcher and Toropi-6.3 as recurrent parents. Plants of Toropi-6.3 expressing ";" resistance at the seedling stage were used in the backcrosses. A total of 277 BC_1F_2 families of Thatcher//Thatcher/Toropi-6.3 and 258 BC_1F_2 families of Toropi-6//Thatcher/Toropi-6.3 were analysed in the field.

5.3.2. *Leaf rust evaluation*

In the greenhouse, Toropi-6, IAC13-Lorena, the five resistant lines from the cross of Toropi-6/IAC13-Lorena (255644, 255693, 255724, 255764 and 255777) and Thatcher were inoculated at seedling and adult stage following the procedures described by McCallum and Seto-Goh (2003). Six Canadian *P. triticina* races were inoculated at both plant stages: BBBD, TDBG, TJBJ, MGBJ, MBDS and MBRJ. The designations of the races is according to the North American nomenclature (Long and Kolmer 1989).

The Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 DH populations were inoculated at seedling and adult stage with *P. triticina*. Three experiments in which BBBD was inoculated onto the plants at the seedling stage were performed. The three experiments differed in the adult plant inoculation method. In the first experiment, BBBD was inoculated onto one plant of each line and TJBJ was inoculated onto a second plant of each line. In the second experiment one tiller on each plant was inoculated with BBBD while the rest of the tillers were separately inoculated with TJBJ (see chapter 3). The inoculations were isolated from each other so that the effects of each race could be observed on the same plant without confounding effects. In the third experiment, the lines were only inoculated with BBBD at the adult plant stage.

In the greenhouse, the leaf rust reaction was classified according to Long and Kolmer (1989), using a 0 to 4 scale. Resistant infection types were considered ";" (hypersensitive flecks), "1" (small uredinia with necrosis), and "2" (small to medium uredinia with chlorosis). Susceptible reaction was given to plants with infection type "3" (medium uredinia without chlorosis or necrosis) and "4" (large uredinia without chlorosis or necrosis) (McCallum and Seto-Goh 2003). An additional class, designated "X", for heterogeneous or mesothetic reaction was also considered resistant (Knott 1989).

Field experiments for leaf rust using the DH populations were performed in Canada, New Zealand and Brazil. In Canada, nurseries to test the DH populations were located at Glenlea in 2010 and Portage La Prairie in 2011 and 2012, where irrigation was present. The resistant lines derived from Toropi-6/IAC13-Lorena and the parents were tested in Glenlea in 2009 and in Portage La Prairie in 2011. In New Zealand (Lincoln, Canterbury), the DH populations were analyzed in the 2010-2011 and 2011-2012 field seasons with natural inoculum. In Brazil, artificial inoculation was used to test the DH population at Mutuca farm (Parana) in 2011. The backcross populations were analyzed in Portage La Prairie in 2010, where the BC_1F_2 families were classified as segregating or uniformly resistance or susceptible to leaf rust. Thatcher and Toropi-6 were analysed in all locations. A randomized complete block design composed of two replicates was used in each field location. The nurseries were organized in small rows of 60 cm in Canada and 1 m in New Zealand, and in hills in Brazil. Spreader rows of susceptible lines were used to increase and spread the inoculum. In Canada and in Brazil, spreader rows were added in regular intervals of five lines. A selected line of Morocco with better powdery mildew resistance was used as the spreader in Brazil. In Canada, the spreader rows were

composed of a mixture of leaf rust susceptible Morocco, Little Club and Thatcher. In New Zealand, the entire nursery area was surrounded by a susceptible stripe rust cultivar (Tiretea), but there were no leaf rust spreaders in a 50 m distance.

In field trials, adult plants were rated based on a modified Cobb scale (Peterson et al. 1948), which is based on the severity and infection type of the disease on the leaf (Knott 1989; McIntosh et al. 1995). In the 2010-2011 field season in New Zealand, a 0 to10 scale was used to rate the plants, where each unit increase was equivalent to another 10% of leaf area affected in the entire plant.

5.3.3. Inheritance analyses

Chi-square tests were used to evaluate the goodness-of-fit of observed and expected segregation ratios of DH and BC populations grown in the greenhouse and in the field.

5.4. Results

5.4.1. Toropi leaf rust response

In the greenhouse, the five resistant lines from the cross Toropi-6/IAC13-Lorena, the parents and Thatcher were inoculated at seedling and adult stage with six *P. triticina* races (Table 5.1). The leaf rust reaction of the lines and cultivars indicated that Toropi-6 has both seedling and adult plant resistance. The seedling resistance of Toropi-6 was conferred by *Trp-Se* (see chapter 4). Toropi-6 and the resistant lines derived from Toropi-

6/IAC13-Lorena were all resistant at the adult plant stage to all six races, with the exception of 255777. This line showed adult plant race specific resistance, being resistant only at adult stage to TDBG and MBRJ, but susceptible to the TJBJ, MGBJ and MBDS races. Line 255777 had a mixture of susceptible and resistant pustules, being defined by "3 ;" or "; 3" depending on the predominance of the pustules. The adult plant resistance gene in 255777 was given the temporary designation *Trp-3*. IAC13-Lorena was susceptible to all Brazilian races that were tested, but it had some seedling and adult resistance to the Canadian races used in our tests. The adult plant resistance of IAC13-Lorena was indicated by susceptibility at the seedling stage and resistance at the adult stage to BBBD, TJBJ, MBDS and MBRJ; and it was characterized by a "3 ;" reaction. Although line 255777 and IAC13-Lorena expressed similar adult plant leaf rust reactions, 255777 was resistant to TDBG, whereas IAC13-Lorena was susceptible. Toropi-6 was also resistant to TDBG, indicating that the source of 255777 resistance was derived from Toropi-6, not IAC13-Lorena.

Table 5.1. Leaf rust reaction at seedling and adult plant stage of Toropi-6, IAC13-Lorena, Thatcher and five resistant lines derived from the cross Toropi-6/IAC13-Lorena. The experiment was performed three times under greenhouse conditions using one plant of each line or cultivar to be inoculated at seedling and adult stage to each *Puccinia triticina* race.

	Plant Stage	Toropi-6	IAC13-Lorena	Thatcher	255644	255693	255725	255764	255777
BBBD	Seedling	H[a]	S	S[b]	R[c]	R	R	R	R
	Adult	R	R	S	R	R	R	R	R
TDBG	Seedling	S	S	S	S	S	S	S	S
	Adult	R	S	S	R	R	R	R	R
TJBJ	Seedling	S	S	S	S	S	S	S	S
	Adult	R	R	S	R	R	R	R	S
MGBJ	Seedling	R	S	S	S	S	S	R	S
	Adult	R	S	S	R	R	R	R	S
MBDS	Seedling	H	S	S	S	S	S	H	S
	Adult	R	R	S	R	R	R	R	S
MBRJ	Seedling	R	S	S	S	S	S	R	S
	Adult	R	R	S	R	R	R	R	R

[a]H: Heterogeneous – mixture of resistance and susceptible reaction
[b]S: Susceptible reaction
[c]R: Resistant reaction
The resistant lines derived from the cross Toropi-6/IAC13-Lorena were developed by Barcellos et al. (2000).

Toropi-6 was tested under field conditions in Canada for three years, one year in Brazil and two years in New Zealand. The leaf rust reaction in Canada was between "0" (immune reaction) and "10 M" (10 percent of the flag leaf covered with a mixture of moderately resistant and moderately susceptible pustules). Immune reaction "0" was found for Toropi-6 in New Zealand; and not more than "5 R" (5 percent of the leaf covered with resistant pustules) in Brazil. Few moderately susceptible and moderately resistant pustules distributed throughout the flag leaf characterized the leaf rust phenotypic reaction of Toropi-6 in the greenhouse and in the field. The resistant lines

derived from Toropi-6/IAC13-Lorena were tested in Canada during two years. The leaf rust reaction of the 255644, 255693, 255725 and 255764 were between "0" and "10 MR", while 255777 had "80 MS" leaf rust infection in 2009, and "50 MRMS" in 2011. The leaf rust pressure was relatively low in the Canadian field trials in 2011.

The greenhouse and field experiments indicated that line 255777 has a minor adult plant gene with race specific resistance, *Trp-3*. Toropi-6 also has seedling resistance (*Trp-Se*) and partial adult plant resistance, probably conferred by race non-specific genes *Trp-1* and *Trp-2*.

5.4.2. Genetic analyses

The inheritance of leaf rust resistance in Toropi-6 was analyzed based on the Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 DH populations. In the greenhouse (Table 5.2), the avirulent race BBBD indicated the presence of three complementary genes plus one non-complementary gene in the Thatcher/Toropi-6.4 DH population at the adult stage. The Thatcher/Toropi-6.3 DH population, which has the *Trp-Se* seedling gene, fitted the expected 5R:3S ratio ($p = 0.459$) for two complementary genes plus one non-complementary gene. The TJBJ race, which is avirulent to only the *Trp-1* and *Trp-2* genes and virulent to both *Trp-Se* and *Trp-3*, indicated the presence of two complementary genes in both populations as predicted.

Table 5.2. Segregation for leaf rust resistance of Toropi-6 double haploid populations Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 to race BBBD and TJBJ of *Puccinia triticina* at the adult plant stage in greenhouse tests at Winnipeg, Canada. The BBBD race is avirulent to all leaf rust genes of Toropi, while TJBJ is avirulent to *Trp-1* and *Trp-2* genes and virulent to *Trp-Se* and *Trp-3*. The expected ratios are based on the predicted leaf rust genes in effect for each population/race combination.

Population	Race	Number of genes	Leaf rust reaction	Expected ratio	Expected	Observed	Chi-square	Probability	Effective genes
Thatcher/Toropi-6.3	BBBD	2 complementary + 1	R[a]	5	133.75	128	0.550	0.459	*Trp-1*, *Trp-2*, *Trp-Se*
			S[b]	3	80.25	86			
			Total			214			
	TJBJ	2 complementary	R	1	48	54	0.840	0.359	*Trp-1*, *Trp-2*
			S	3	144	138			
			Total			192			
Thatcher/Toropi-6.4	BBBD	3 complementary + 1	R	9	134.44	130	0.264	0.608	*Trp-1*, *Trp-2*, *Trp-3*, extra gene
			S	7	104.56	109			
			Total			239			
	TJBJ	2 complementary	R	1	50	38	3.527	0.060	*Trp-1*, *Trp-2*
			S	3	150	162			
			Total			200			

[a]R: Resistant reaction; [b]S: Susceptible reaction.

In the field, the DH populations were analyzed in Canada, Brazil and New Zealand (Table 5.3). Segregation ratios for two complementary genes were the best fit in Brazil and Canada – Glenlea 2010 for both DH populations, indicating that the *Trp-1* and *Trp-2* genes were the only effective genes in these locations. In Canada – Portage La Prairie 2011 and in New Zealand 2011-2012, the Thatcher/Toropi-6.4 showed the presence of two complementary plus one gene, while three non-complementary genes were identified in New Zealand in the 2010-2011 season. In these field trials, the *Trp-3* gene appeared to be effective in addition to the *Trp-1* and *Trp-2* genes. The Thatcher/Toropi-6.3 fitted two complementary genes plus one gene in New Zealand 2011-2012 season, these were probably *Trp-1*, *Trp-2* and *Trp-Se* genes, because *Trp-Se* conferred complete resistance in

New Zealand. Besides the presence of these three genes, the Thatcher/Toropi-6.3 population showed an additional gene in Canada – Portage La Prairie 2011 and in New Zealand 2010-2011 season. As was previously stated, 2011 in Canada was a year with low leaf rust infection. This could have affected the segregation results. The fact that this additional resistance gene was found in only two of the field environments could indicate that it is a partially effective gene, which is capable of conditioning resistance under low rust pressure, but not under more severe epidemics.

The backcross population using Toropi-6 as recurrent parent did not segregate, indicating that there are genes in effect in this cultivar that are common in all Toropi-6 plants used in the crossings. The backcross population Thatcher//Thatcher/Toropi-6.3 fitted three complementary genes plus one gene in Portage La Prairie in 2010 ($p = 0.481$). Therefore, the double haploid and the backcross populations developed using Toropi-6.3 expressed the same number of genes in Portage La Prairie in different years.

Table 5.3. Segregation for leaf rust resistance of Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 double haploid populations in the field at New Zealand, Brazil and Canada. The results were based on the average of two replicates.

Thatcher/Toropi-6.3 DH Population								
Location and Year	Number of genes	Leaf rust reaction	Expected ratio	Expected	Observed	Chi-square	Probability	Effective genes
Canada - Glenlea 2010	2 complementary	R[a]	1	51	58	2.359	0.125	*Trp-1, Trp-2*
		S[b]	3	153	164			
		Total			222			
Canada - Portage La Praire 2011	3 complementary + 1	R	9	115.88	108	1.073	0.300	*Trp-1, Trp-2, Trp-Se,* additional gene
		S	7	90.13	98			
		Total			206			
Brazil 2011	2 complementary	R	1	24	32	3.125	0.077	*Trp-1, Trp-2*
		S	3	72	64			
		Total			96			
New Zealand 2010-2011	4 non-complementary	R	15	155.63	154	0.130	0.718	*Trp-1, Trp-2, Trp-Se,* additional gene
		S	1	10.38	12			
		Total			166			
New Zealand 2011-2012	2 complementary + 1	R	5	99.38	99	0.000	0.984	*Trp-1, Trp-2, Trp-Se*
		S	3	59.63	60			
		Total			159			
Thatcher/Toropi-6.4 DH Population								
Location	Number of genes	Leaf rust reaction	Expected ratio	Expected	Observed	Chi-square	Probability	Effective genes
Canada - Glenlea 2010	2 complementary	R	1	55.5	58	0.096	0.757	*Trp-1, Trp-2*
		S	3	166.5	164			
		Total			222			
Canada - Portage La Prairie 2011	2 complementary + 1	R	5	143.75	131	2.784	0.095	*Trp-1, Trp-2, Trp-3*
		S	3	86.25	99			
		Total			230			
Brazil 2011	2 complementary	R	1	25.5	31	1.307	0.253	*Trp-1, Trp-2*
		S	3	76.5	71			
		Total			102			
New Zealand 2010-2011	3 non-complementary	R	7	149.63	147	0.241	0.623	*Trp-1, Trp-2, Trp-3*
		S	1	21.38	24			
		Total			171			
New Zealand 2011-2012	2 complementary + 1	R	5	85	88	0.196	0.658	*Trp-1, Trp-2, Trp-3*
		S	3	51	48			
		Total			136			

[a]R: Resistance reaction; [b]S: Susceptible reaction.

5.5. Discussion

This study demonstrated the complex nature of leaf rust resistance in the Brazilian cultivar Toropi. The inheritance of leaf rust resistance was analyzed following two DH populations Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 in the greenhouse and in the field. The summary of the data indicated that Toropi has a seedling gene, *Trp-Se* (see chapter 4), two partially complementary adult plant genes, *Trp-1* and *Trp-2*, and an APR race specific gene, *Trp-3*. An additional gene was identified in two field locations, but was not effective in most trials.

The two complementary genes in Toropi-6 (*Trp-1* and *Trp-2*) were first described by Barcellos et al. (2000). In their study, it was shown that these genes were complementary in Brazil, but acted independently in Mexico, and they both were recessive genes. The F_1 plant analysis at the adult stage with TJBJ race, avirulent to *Trp-1* and *Trp-2* and virulent to *Trp-Se* and *Trp-3* gene, confirmed that the genes were recessive. In this experiment, all F_1 plants were susceptible to TJBJ, while further studies indicated that some DH lines were resistant to the same race. Results from Brazil and in Canada – Glenlea 2010 indicated the presence of two complementary effective genes, confirming the Barcellos et al. (2000) results. Therefore, *Trp-1* and *Trp-2* genes are the only effective genes in these two locations. *Trp-1* and *Trp-2* were effective in all locations tested to date, supporting the idea that these two genes have an important role in controlling leaf rust in different regions of the world.

The *Trp-Se* seedling gene conditioned an immune response in New Zealand, but was not effective in the field in Brazil. However, it was able to improve the resistance of

other Toropi genes when in combination with other genes leading to an almost immune leaf rust response (see chapter 4).

The *Trp-3* gene phenotype is characterized by a mixture of susceptible and resistant pustules, "3 ;" reaction, similar to a minor gene. In the presence of other genes (probably *Trp-1* and *Trp-2*), it expresses a mesothetic reaction at the seedling stage, confirming the findings that the seedling resistance of Toropi-6 is conferred by one gene (*Trp-Se*) plus three complementary genes (see chapter 4). *Trp-3* conditioned an intermediate level of resistance in New Zealand, intermediate to susceptible in Canada, and susceptible in Brazil.

The segregation in the 2010-2011 season in New Zealand indicated independent expression of *Trp-1* and *Trp-2* in the Toropi DH populations. *Trp-1* and *Trp-2* were also independently expressed in Mexico (Barcellos et al. 2000). The *Trp-Se* and *Trp-3* genes were not effective in all locations, and an extra gene was expressed in the greenhouse, Canada – Portage La Prairie (DH and BC_1F_2 populations) and in New Zealand 2010-2011. Accordingly, the complementary nature of the *Trp-1* and *Trp-2* genes, and the effect of the *Trp-Se*, *Trp-3* and the extra Toropi gene appeared to be modified by the environment or by the pathogen. In the case of the *Trp-Se,* virulent races have been identified in Brazil. The line ORL4002 (Onix*6/Toropi-6), which is thought to contain *Trp-Se*, is being used in the differential set to differentiate the *Puccinia triticina* race B55 from the B55-ORL4002 virulent race (B55 race corresponds to MDR-MR, MDK-MR, MDT-MR, MFK-MT, MFT-MT in the North American nomenclature). The ORL4002 line was resistant to B55, but became susceptible to the new variant of B55 (designated

B55-ORL4002 virulence), which was first identified in 2007. Molecular marker analyses confirmed the presence of *Trp-Se* in ORL4002 (data not shown). Since 2008, the B55-ORL4002 virulence or B55-*Trp-Se* virulence was the most predominant leaf rust race in Brazil (Barcellos 2009). In our analysis *Trp-Se* was not effective in Brazil, which would make sense if this gene has been overcome by the evolution of virulence in the pathogen.

The effectiveness of *Trp-3* and the additional Toropi gene could be dependent on temperature, growth conditions or inoculum pressure. Both genes were expressed in the field in Canada – Portage La Prairie 2011, but not in Canada – Glenlea 2010. The inoculum pressure in Canada in 2011 was relatively low, which could have facilitated the identification of minor genes. The plants in Glenlea in 2010 were clearly stressed because of a flood that affected the region in the beginning of the growing season. The expression of some leaf rust genes could be modified according to temperature. For example, *Lr34* is less effective in high temperature conditions, while the resistance derived from *Lr13* becomes better in the same conditions (McIntosh et al. 1995). The dependence on environment conditions of the complementary effect of *Trp-1* and *Trp-2* gene and the expression of the other Toropi genes is still a speculation; further studies should be performed to confirm the hypothesis.

The two race non-specific genes in Toropi (*Trp-1* and *Trp-2*) are the most important genes in this cultivar, however, it is the combination of all genes in Toropi that makes its leaf rust resistance so durable and effective. The future aim is to locate and map the APR genes in Toropi. To date, Toropi demonstrated a high level of durable resistance in South

America, North America and in New Zealand, indicating that it is a good source of leaf rust resistance.

6.0. MAPPING ADULT PLANT GENES RESPONSIBLE FOR THE DURABLE LEAF RUST RESISTANCE OF THE WHEAT CULTIVAR TOROPI

6.1. Abstract

Leaf rust, caused by *Puccinia triticina* Eriks., is a disease of wheat that affects its production almost everywhere wheat is cultivated. The best economically and environmental-friendly approach to control this disease is through genetic resistance. *P. triticina* is genetically diverse due to constant evolution and migration of the fungus making it difficult to develop and maintain resistance in wheat cultivars. Combinations of genes involving partial resistance and diversity of resistance among cultivars are recommended to achieve durability. Toropi is a Brazilian cultivar, which has maintained its leaf rust resistance for more than 40 years. At least four genes conferring leaf rust resistance (*Lr* genes) were described in this cultivar. Previously a seedling resistance gene in Toropi, *Trp-Se*, was mapped to chromosome 3D. In this study, quantitative trait locus (QTL) analyses and bulked segregant analyses were used to locate the genes *Trp-1*, *Trp-2* and *Trp-3,* conferring adult plant resistance to Toropi. Doubled haploid population developed from a cross between Toropi-6.4 and the susceptible cultivar Thatcher were used to map the genes. Leaf rust nurseries were conducted in Canada, Brazil and New Zealand. Stripe rust was also evaluated in New Zealand. *Trp-1* and *Trp-2* are partially complementary and race non-specific genes and were previously assigned to chromosomes 1A and 4D, respectively, whereas *Trp-3* is race specific and has not previously been mapped. However, our results indicated significant QTL (*QLr.crc-5AL.1* and *QStr.crc-5AL.1*) on 5AL conferring leaf rust and stripe rust resistance. The resistance

gene on chromosome 5AL, *Trp-1*, is a novel source of resistance as there is no *Lr* gene described in this location. A second adult plant race non-specific resistance gene in Toropi, *Trp-2*, was not assigned to a chromosome although regions on chromosomes 2B, 5D and 7A are possible locations. The *Trp-3* gene was mapped to chromosome 4BL. The leaf rust resistance derived from Toropi could be an important contribution to durable resistance as Toropi expressed almost an immune response to leaf rust in different parts of the world.

6.2. Introduction

Wheat is one of the most important crops, and its production is affected worldwide by rust infections. Three rusts are able to attack wheat, leaf rust (*Puccinia triticina* Eriks.), stem rust (*P. graminis* f. sp. *tritici*) and stripe rust (*P. striiformis* f. sp. *tritici*). Leaf rust tends to cause less damage than the other two rusts, but it is the most common and widely distributed wheat rust (Kolmer et al. 2009a). The most environmentally friendly and cheapest method of controlling leaf rust is the use of resistant cultivars.

Over 60 genes conferring leaf rust resistance (*Lr* genes) have been described (Herrera-Foessel et al. 2012; McIntosh et al. 2011). Most leaf rust resistance genes are seedling genes that are race specific and effective during the whole host life cycle. Seedling resistance genes commonly confer major resistance to leaf rust (Lagudah 2011) and are generally manifested by a hypersensitive response (Bolton et al. 2008). Genes that are expressed only at the post-seedling stage are called adult plant resistance (APR) genes. Some APR genes are characterized by conferring partial resistance, which is not

associated with a rapid hypersensitive response, but with slow-rust development. Slow-rusting genes confer fewer and smaller uredinia and have longer latent periods (Knott 1989; Lagudah et al. 2009). The partial resistance genes condition longstanding effectiveness. Four adult plant partial resistance genes have been identified: *Lr34*, *Lr46*, *Lr67* and *Lr68* (Herrera-Foessel et al. 2011; Herrera-Foessel et al. 2012; Hiebert et al. 2010; Krattinger et al. 2009; Rosewarne et al. 2006; William et al. 2006).

P. triticina evolves constantly and the deployment of a single resistance gene in a large wheat growing area leads to the selection and perpetuation of mutants or existing variants at low frequency (German et al. 2007; Huerta-Espino et al. 2011). Virulence shifts in the *P. triticina* population can cause rapid loss of race specific gene effectiveness (Huerta-Espino et al. 2011). The best approaches to achieve durable resistance, and to avoid evolution of virulent *P. triticina* populations, are by employing diverse resistance sources and gene combinations, and using partial resistance genes as the basis of leaf rust resistance (Singh et al. 2011b).

Toropi (Frontana 1971.37/Quaderna A//Petiblanco 8) is a Brazilian cultivar that was released in 1965 and maintained its leaf rust resistance with 15 years of extensive cultivation. Barcellos et al. (2000) determined the presence of two complementary recessive leaf rust genes in Toropi in the field in Brazil. Crosses between Toropi and the near isogenic line Thatcher-*Lr34* indicated the presence of one dominant (*Lr34*) and two recessive (*Trp-1* and *Trp-2*) adult plant resistance genes in the field in Mexico. The presence of *Lr34* and *Lr13* in Toropi was excluded (Barcellos et al. 2000; Zoldan 1998).

Toropi exhibited good leaf rust resistance in South America, North America and in New Zealand, indicating that it has an important source of leaf rust resistance (see chapter 5).

Double haploid (DH) and backcross populations developed by crossing Toropi and Thatcher indicated the presence of at least four genes conferring leaf rust resistance to Toropi. These include one race specific seedling gene (*Trp-Se*), two race non-specific complementary adult plant genes (*Trp-1* and *Trp-2*) and a race specific adult plant gene (*Trp-3*) (see chapter 4 and 5). To date, the race specific seedling gene, *Trp-Se*, was mapped on chromosome 3D (see chapter 4). The purpose of this study was to map the locations of *Trp-1*, *Trp-2* and *Trp-3*.

The present study describes the location of two APR genes (*Trp-1* and *Trp-3*) in a DH population of Toropi and Thatcher using quantitative trait (QTL) analyses and bulked analysis segregation. Leaf rust reaction was analysed in Canada, Brazil and New Zealand.

6.3. Materials and Methods

6.3.1. Development of a mapping population

A selection of Toropi (Toropi-6.4) was crossed with the susceptible cultivar Thatcher to develop a DH population: Thatcher/Toropi-6.4. Three F_1 plants were used to generate the population, which was composed of 246 DH lines. The maize/wheat hybridization followed by embryo rescue was the method used to create the DH population (Thomas et al. 2010).

6.3.2. Rust evaluation

In the greenhouse, Thatcher, Toropi-6 and the Thatcher/Toropi-6.4 DH population were inoculated at the seedling and adult stages with *P. triticina* following the procedures described by McCallum and Seto-Goh (2003). Three experiments were performed. In all three experiments, BBBD was inoculated onto the plants at the seedling stage and the plants were rated for their seedling response. The same plants were subsequently re-inoculated at the adult plant stage. The three experiments differed in the adult plant inoculation method. In the first experiment, BBBD was inoculated onto one plant of each line and TJBJ was inoculated onto a second plant of each line. In the second experiment one tiller on each plant was inoculated with BBBD while the rest of the tillers were separately inoculated with TJBJ (see chapter 3). The inoculations were isolated from each other so that the effects of each race could be observed on the same plant without confounding effects. In the third experiment the lines were only inoculated with BBBD at the adult plant stage. The race designations are according to the North American nomenclature (Long and Kolmer 1989).

In the greenhouse, the leaf rust reaction was classified according to Long and Kolmer (1989), using a 0 to 4 scale. Resistant infection types are considered ";" (hypersensitive flecks), "1" (small uredinia with necrosis), and "2" (small to medium uredinia with chlorosis). Susceptible reaction was given to plants with infection type "3" (medium uredinia without chlorosis or necrosis) and "4" (large uredinia without chlorosis or necrosis) (McCallum and Seto-Goh 2003). An extra class, designated "X", for mesothetic reaction was also considered resistant (Knott 1989).

Field experiments were performed in Canada, New Zealand and Brazil to analyse leaf rust reactions (see chapter 5). In Canada, nurseries were located at Glenlea in 2010, and at Portage La Prairie in 2011, where artificial inoculation and irrigation were applied. In New Zealand (Lincoln), the DH population was analyzed in the 2010-2011 and 2011-2012 field seasons with natural inoculum. In Brazil, artificial inoculation was used to test the DH population at Mutuca farm, Parana, in 2011. Stripe rust was analysed in New Zealand (Lincoln) in both field seasons.

In field trials, adult plants were rated based on a modified Cobb scale (Peterson et al. 1948), which is based on the severity and infection type of the disease on the leaf (Knott 1989; McIntosh et al. 1995). The exception was in New Zealand, during the 2010-2011 season, when a 0 to10 scale was used to rate the plants. In this scale, each unit increase was equivalent to another 10% of leaf area affected in the entire plant.

6.3.3. Molecular mapping and QTL analyses

6.3.3.1. Trp-1 and *Trp2*

Wheat leaf tissue was collected from seedlings after rust evaluation in greenhouse. Leaves that emerged after inoculation with *P. triticina* were collected and freeze-dried from each DH line and the respective parents. DNA was extracted using a modified ammonium acetate extraction (Chao and Somers 2012) for simple sequence repeat (SSR) marker amplification. The Qiagen DNeasy 96 Plant Kit (Qiagen, Mississauga, Ontario)

was used to extract DNA for single nucleotide polymorphisms (SNP). Analyses of SNP markers was performed by Kbioscience (UK) (Allen et al. 2011).

Fragment analyses of SSR marker were done using Applied Biosystems ABI 3100 genetic analyzer (Applied Biosystems, Streetville, ON, Canada) and were performed as described by Somers et al. (2004). Data were first converted to a gel-like image, using Genographer version 2.1.4 (Benham et al. 1999), and Genescan 500-LIZ or 500-ROX (Applied Biosystems, Foster City, California) as the internal molecular weight standards for the ABI 3100. All fragment sizes include 19 bp of the M13 fluorescent tag (Schuelke 2000).

Mapdisto software was used to construct the linkage maps (Lorieux 2012) using the default settings for LOD threshold of 3.0 and for maximum recombination frequency of 0.3, the Kosambi (1944) mapping function and automatic commands.

QTL analyses were performed using the computer program Windows QTL Cartographer (V2.5) (Wang et al. 2011). A composite interval-regression mapping (CIM) was performed to evaluate marker intervals putatively associated with leaf rust and stripe rust resistance in the Thatcher/Toropi-6.4 DH population. CIM control parameters used were the standard model with the control marker number of five, window size of 10 cM and the forward regression method. Walk speed of 1 cM was used. One thousand permutations with a significance level of 0.05 determined the LOD threshold of 3.1 in this population for disease severity measured at all locations, except for Glenlea, in which a LOD threshold of 4.0 was used. The LOD threshold for leaf rust infection type was 3.2,

with the exception of the greenhouse test with BBBD (3.1), New Zealand 2011 (3.1) and Glenlea (11.0).

QTL analyses were performed considering leaf and stripe rust severity (0 to 100 percentage of the flag leaf covered with rust) and leaf rust infection type, using the following conversion: a) Resistant = 1; b) Moderately resistant = 2; c) Moderately susceptible = 3; d) Susceptible = 4. The leaf rust scores considered were the average of two replicates in the field locations and the average of three experiments with BBBD and two experiments with TJBJ race inoculations in greenhouse. The locations were considered singly and combined, when the arithmetic mean of each DH line was calculated using the severity scores of all locations. Lsmeans scores could not be estimated to some DH lines, therefore arithmetic means were used. A total of 184 lines of the DH population were randomly selected to perform the QTL analyses. The total number of lines was chosen due to optimization of DNA amplification and capillary electrophoresis analyses.

6.3.3.2. Trp-3

Multiple bulked segregant analysis (MBSA) was used to localize the race specific APR gene – *Trp-3* – within the wheat genome (Hiebert et al. 2012). A set of 423 SSR markers was tested in 16 bulks. Three plants of Thatcher and three selfed progenies of Toropi-6.4 composed each parental bulk. A bulk of three selfed progeny plants from the Toropi-6.3 (with the *Trp-Se* gene) was also analyzed. Thirteen different bulks were made up of four different resistant DH lines each. These lines were selected because they were

resistant to BBBD but susceptible to TJBJ at the adult plant stage, indicating the presence of the *Trp-3* gene. The position of *Trp-3* was further refined by assessing 16 SSR and eight SNP markers on 126 Thatcher/Toropi-6.4 DH lines. SSR markers used to construct a genetic map of the *Trp-3* gene are described in Appendix 5.

Linkage analyses was conducted by Mapmaker version 3 (Lander et al. 1987) using the Kosambi (1944) mapping function.

6.4. Results

6.4.1. Identification of QTL: Trp-1 and Trp-2

Linkage maps developed for the Thatcher/Toropi-6.4 DH population to perform QTL analysis consisted of 230 SSR and 164 SNP markers covering the wheat genome with an average distance of 8.84 cM between markers (Appendix 3 and 4.1 to 4.4).

QTL from five genomic regions were associated with leaf rust severity in the DH population Thatcher/Toropi-6.4 field experiments (Table 6.1, Figure 6.1). When the infection reaction type was used, QTL at similar locations as the QTL for severity were also identified. All QTL associated with reduced disease were derived from the Toropi 6.4 parent.

An important QTL (*QLr.crc-5AL.1*) was identified on 5AL between *gpw7007* and *cfa2163*, covering and interval of 40.3 cM (Table 6.1, Figure 6.1). This QTL was significant in Canada in two locations, Glenlea in 2010 and Portage La Prairie in 2011,

explaining 39 and 22% of the phenotypic variation for leaf rust severity, respectively. A QTL in the same region as *QLr.crc-5AL.1* was identified in New Zealand in 2010 for infection type and also when the data was combined as a mean of all locations, confirming the importance of this locus. The *QLr.crc-5AL.1* was also identified for leaf rust severity in New Zealand in 2010, but it was not significant (LOD 2.9, threshold 3.1). The same marker interval of *QLr.crc-5AL.1* was significant for stripe rust severity in New Zealand in 2010, named *QStr.crc-5AL.1*, which explained approximately 13% of phenotypic variation. However, different QTL on 5A were identified when the mean stripe rust scores were considered (Figure 6.1).

Five QTL on chromosome 4BL were significant in New Zealand 2010 and in the greenhouse with BBBD inoculation. The 4BL QTL explained only a small amount of phenotypic variation in the field in New Zealand (around 7%), but showed a stronger effect in the greenhouse (43 to 85%) (Table 6.1, Figure 6.1).

The remaining QTL were located on wheat chromosomes 2B, 5D and 7AL (Table 6.1, Figure 6.1). The QTL on 2B and 5D were derived from the mean of leaf rust severity scores in all locations, while the QTL on 7AL was identified only in New Zealand 2011.

Table 6.1. Quantitative trait loci (QTL) detected by composite interval mapping significantly associated with disease severity of leaf rust and stripe rust and type of reaction of leaf rust in the Thatcher/Toropi 6.4 double haploid population. Experiments were conducted in Canada, Brazil and New Zealand. The disease severity and infection type data were the average of two replicates considering field experiments and average of three experiments considering BBBD inoculation in greenhouse. A total of 184 lines of the DH population were randomly selected to perform the QTL analyses.

QTL	Marker interval[a]	Location	Additive effect	R^2 value	LOD
Leaf rust					
Disease severity					
QLr.crc-2BS.1	*B12471*	New Zealand 2011	16.55	0.22	10.9
QLr.crc-2BS.2	*B09972 - B10318*	New Zealand 2011	15.89	0.20	8.9
QLr.crc-2B.3	*B04637 - B01128*	Mean all locations	6.30	0.16	7.1
QLr.crc-4BL.1	*barc353*	New Zealand 2010	0.72	0.08	4.1
QLr.crc-4BL.2	*gpw5010*	New Zealand 2010	0.71	0.07	3.8
QLr.crc-5AL.1	*gpw7007 - cfa2163*	Glenlea 2010	14.99	0.39	23
		Portage 2011	8.78	0.22	9.4
	gpw2243 - cfa2163	Mean all locations	5.80	0.15	8.5
QLr.crc-5D.1	*B00763 - gpw4457*	Mean all locations	4.57	0.09	4.6
QLr.crc-7AL	*gpw2338 - gpw4109*	Portage 2011	4.93	4.93	3.9
Infection type					
QLr.crc-2BS.1	*B12471*	New Zealand 2011	0.54	0.12	6
QLr.crc-2BS.2	*B09972 - B10318*	New Zealand 2011	0.53	0.12	5.1
QLr.crc-2BL.1	*cfd73 - gpw7506*	New Zealand 2010	0.35	0.07	3.8
QLr.crc-4BL.3	*B09915*	BBBD Greenhouse	0.76	0.43	24.5
QLr.crc-4BL.4	*gpw4079*	BBBD Greenhouse	0.95	0.66	43.7
QLr.crc-4BL.5	*cfd39 - barc114*	BBBD Greenhouse	1.10	0.85	56.9
QLr.crc-5AL.1	*gpw7007 - cfa2163*	Glenlea 2010	0.75	0.32	13.9
		Portage 2011	0.70	0.31	11.8
	gpw7007 - B10702	New Zealand 2010	0.39	0.09	4.9
Stripe rust					
Severity					
QStr.crc-5AL.1	*gpw7007 - gpw2243*	New Zealand 2010	0.762	0.128	6.2
QStr.crc-5AL.2	*B10702*	New Zealand 2010	0.765	0.131	6.4
QStr.crc-5AL.3	*B05311 - barc330*	Mean all locations	5.42	0.048	3.6
QStr.crc-5AL.4	*wmc524 - wE101_02*	Mean all locations	11.70	0.23	14.0

[a.] Presence of only one marker indicates the peak of the QTL.

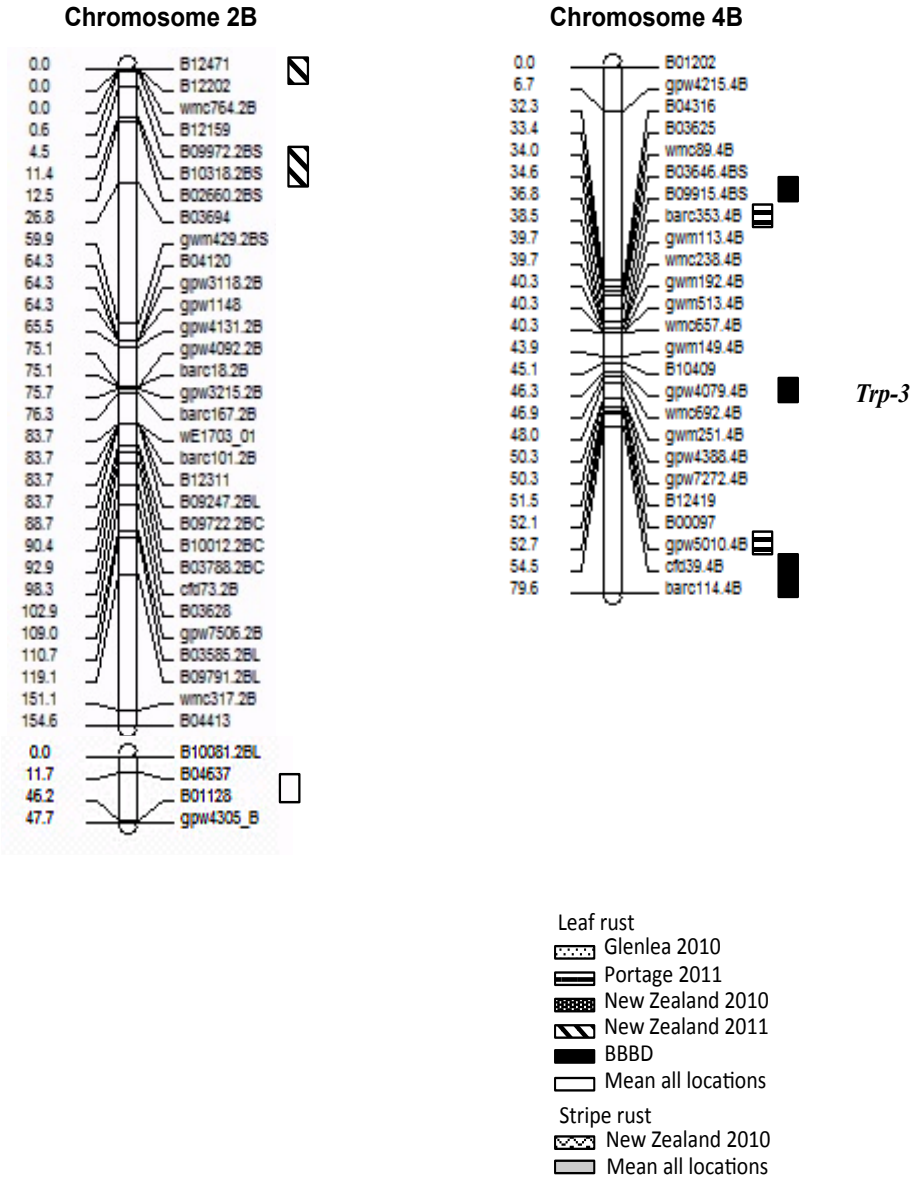

Figure 6.1. Linkage maps of chromosomes 2B, 4B, 5A, 5D and 7A developed using 184 lines of the Thatcher/Toropi-6.4 double haploid population and locations of significant quantitative trait loci (QTL) associate with leaf rust severity, infection type and stripe rust severity identified by composite interval mapping. The QTL were derived from disease severity analyses, except for QTL of New Zealand 2010 on 5AL and BBBD, which were derived from type of infection. It is indicated the position of the adult plant resistance genes of Toropi, *Trp-1* and *Trp-3*. *Trp-1* gene could confer leaf and stripe rust resistance according to the QTL analyses.

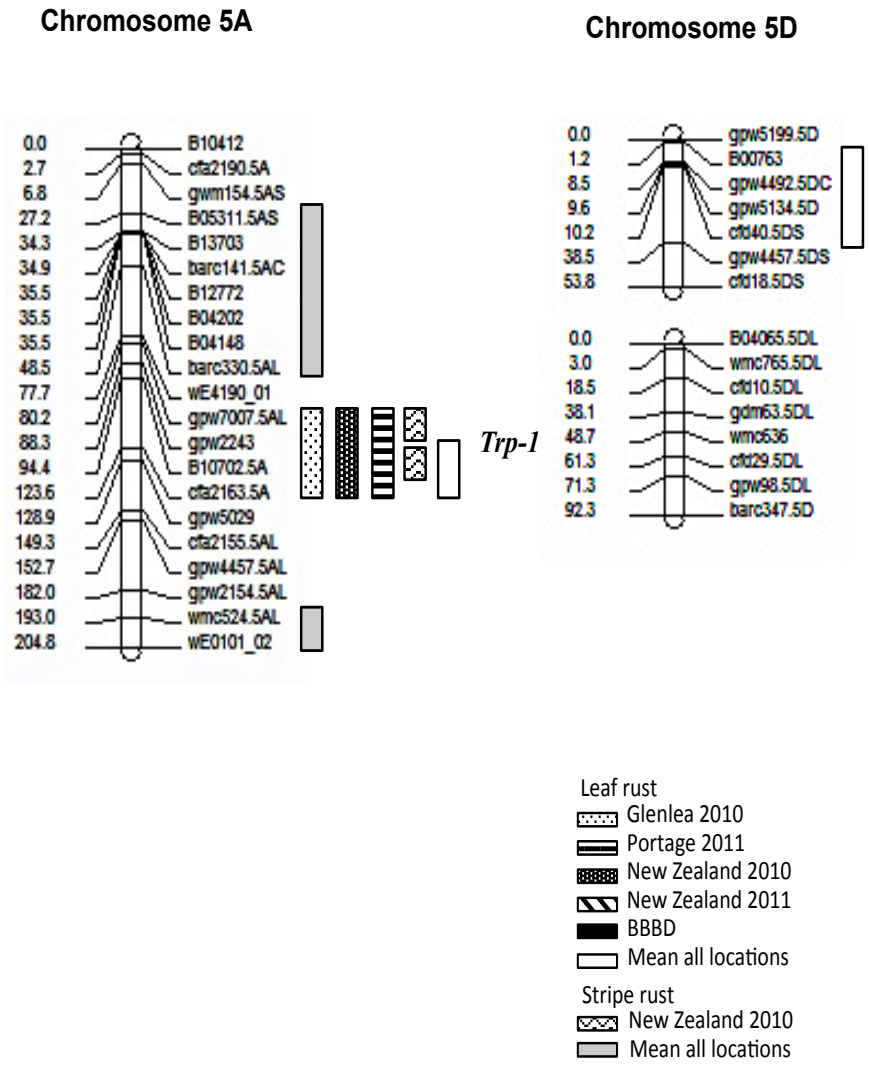

Figure 6.1. Continuation.

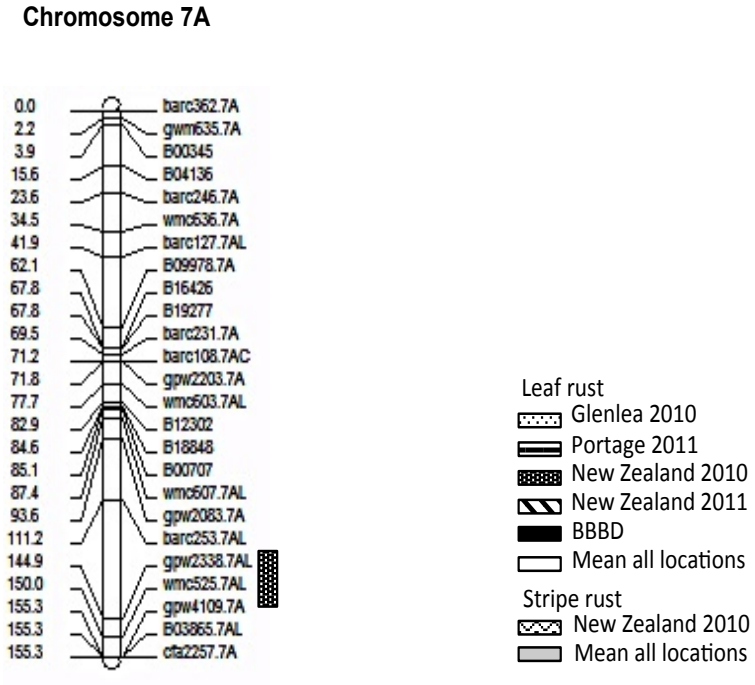

Figure 6.1. Continuation.

6.4.2. Genetic mapping of the race specific adult plant gene Trp-3

A genetic map of the race specific resistance gene expressed at the adult plant stage in Toropi-6.4 was created using 126 lines of the Thatcher/Toropi-6.4 DH population. The lines containing the *Trp-3* gene were identified based in the comparison of the leaf rust effect of the lines inoculated with BBBD, which was avirulent ("3 ;" infection type), and TJBJ, which was virulent to the gene. Field results were also used. BBBD is avirulent to all of the Toropi genes, while TJBJ is only avirulent to *Trp-1* and *Trp-2* genes but virulent

to *Trp-3* and *Trp-Se*. Lines with *Trp-3* were resistant at the adult plant stage to BBBD, but susceptible to TJBJ.

After the MBSA positioned the *Trp-3* on 4BL chromosome, 16 SSR markers on this chromosome were tested for linkage with *Trp-3*. From the 187 SNPs, eight markers were located in the 4BL linkage group. The genetic map of the *Trp-3* gene is presented in Figure 6.3. The closest markers from the gene were SSR *gpw4079* and SNP *BS00010409*, both at 0.8 cM distal from the gene. The closest proximal marker was *gwm149* at 2.4 cM. The total size of the map including the SSR and SNP markers was 61.4 cM (Figure 6.2).

In comparing the seedling and adult plant reactions of the Thatcher/Toropi-6.4 DH population to race BBBD it was found that *Trp-3* was also partially responsible for a mesothetic reaction expressed at the seedling stage. This trait was independently mapped to the same chromosomal location (4BL) as *Trp-3*, and the Toropi allele pattern was identical. However, not all DH lines that expressed *Trp-3* at the adult plant stage also expressed the mesothetic reaction at seedling stage. This indicated that the seedling expression of *Trp-3* was dependent on the presence of other genes, probably *Trp-1* and *Trp-2*.

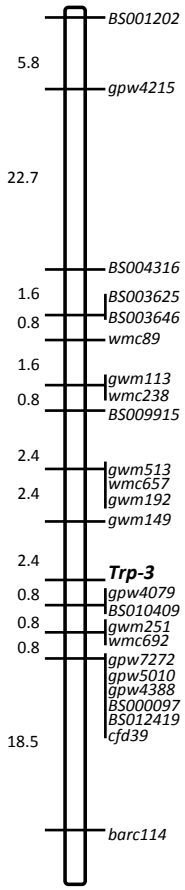

Figure 6.2. Genetic map of the Toropi race specific adult plant leaf rust resistance gene *Trp-3* on chromosome 4BL. The map was based on 126 double haploid lines derived from the cross Thatcher/Toropi-6.4. The map includes simple sequence repeat and single nucleotide polymorphism markers. Map distances are in centiMorgans.

6.5. Discussion

The leaf rust resistance of the Brazilian cultivar Toropi is conferred by race specific and race non-specific resistance genes. A seedling resistance *Lr* gene was already described in this cultivar: *Trp-Se* which was mapped on 3D (see chapter 4). According to the segregation of DH populations Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4, at least four genes are responsible for the leaf rust resistance of Toropi-6 (see chapter 5). The differences between these two populations are the presence of *Trp-Se* in Thatcher/Toropi-6.3 and its absence on Thatcher/Toropi-6.4 population. Different field testing locations fitted different genetic models for rust resistance (see chapter 5). In this study, the Thatcher/Toropi-6.4 DH population was analysed.

An important QTL observed in this study was present on 5AL (*QLr.crc-5AL.1*). This QTL was significant in Canada in two locations, Glenlea in 2010 and Portage La Prairie in 2011. In New Zealand in 2010, the QTL was significant for infection type and it was present, but not significant, for disease severity (LOD 2.9, threshold 3.1). The best model of segregation for the Thatcher/Toropi-6.4 population in Glenlea was two complementary genes, while Portage and New Zealand showed the presence of two complementary plus one gene and three non-complementary genes, respectively (see chapter 5). The hypothesis is that the two complementary genes in effect in Glenlea in 2010 were *Trp-1* and *Trp-2*, described by Barcellos et al. (2000). Following this hypothesis, 5AL should be the position of one of these genes, arbitrarily assigned as *Trp-1*. This locus was also identified using the mean of leaf rust severity in all locations indicating that this is an important QTL in different regions of the world. There are no

leaf rust resistance genes described on 5AL (McIntosh et al. 2011; McIntosh et al. 1995), indicating that this is a novel source of leaf rust resistance. The leaf rust gene on 5AL is an adult plant gene conferring partial resistance.

A stripe rust QTL was located in the same marker interval of *QLr.crc-5AL.1* in the Thatcher/Toropi-6.4 population in New Zealand in 2010, designated *QStr.crc-5AL.1*. The mean of stripe rust severity scores from both years of nurseries in New Zealand positioned the stripe rust QTL proximal and distal from the *QStr.crc-5AL.1*. Therefore, this QTL on 5AL could confer leaf and stripe rust resistance, but further studies are necessary to confirm. The 5AL locus is responsible for resistance to both diseases and could be compared with other partial adult plant leaf rust resistance genes that confer pleiotropic effect to stripe rust or are linked to genes conferring this resistance: *Lr34*, *Lr46* and *Lr67* (Herrera-Foessel et al. 2011; Hiebert et al. 2010; Krattinger et al. 2009; Rosewarne et al. 2006; William et al. 2006).

One explanation for the absence of *QLr.crc-5AL.1* in Brazil and New Zealand 2011 and the identification of different QTL in different locations is the minor effect of the QTL. Identification of minor genes are complicated by their relatively small effects and their strong interactions (Singh et al. 2011b). The resistance conferred by minor genes are often characterized by partial and slow-rusting, which means the presence of susceptible pustules and rust increase as the plant matures. Therefore, if not analysed at the appropriate time for maximum symptom differentiation, the reaction of a minor gene could be rated as a susceptible reaction. In Brazil, environmental stresses could have affected the identification of the leaf rust QTL. The wheat growing area in Brazil is

characterized by short daylength, as wheat is cultivated during the winter. Thatcher, which was one parent in the DH population, is photoperiod sensitive; this could have confounded the leaf rust rating as photoperiod sensitivity was also segregating in this cross. Slow maturity was observed in many DH lines. Disease pressure was high for many diseases in Brazil (Parana) in 2011, for example fusarium head blight, tan spot and powdery mildew (it was necessary to spray the plants with a fungicide specific to powdery mildew two weeks before leaf rust inoculation). Another explanation for the different QTL identified is differences in *P. triticina* races in each location. This could result in different loci being associated with leaf rust resistance depending on the virulence/avirulence pattern of the prevalent races.

In the greenhouse, the Thatcher/Toropi-6.4 population segregated for two complementary genes when the lines were inoculated with TJBJ and three complementary plus one gene with BBBD race (see chapter 5). No significant QTL were identified analysing the infection types of the lines inoculated with TJBJ, while three QTL on 4BL chromosome were significant for BBBD inoculation. The QTL on 4BL overlapped the region where *Trp-3* gene was mapped, between *gwm149* and *gpw4079,* which is exactly the peak of the *QLr.crc-4BL.4*. TJBJ was virulent to the *Trp-3* gene (see chapter 5), so QTL for this race were not expected on chromosome 4BL. QTL on 4BL were also identified in New Zealand in 2010. *Trp-3* is a minor adult plant gene, which confers a mixture of resistance and susceptible reactions in the greenhouse ("3 ;" reaction) and in the field against avirulent races. The resistant line 255777 derived from the cross Toropi-6/IAC13-Lorena, developed in Brazil, was demonstrated to have the *Trp-3* gene based on leaf rust reaction against Canadian races (see chapter 5). The leaf

rust reaction of line 255777 in the field in Canada varied from 80 MS in 2009 and 50 MRMS in 2011. Because *Trp-3* confers a minor resistance to leaf rust, it is difficult to observe it in the field in Canada, but it may be more effective in New Zealand, as was the case for *Trp-Se*.

The molecular markers associated with *Trp-3* were present in the Thatcher/Toropi-6.4 population and were absent in the Thatcher/Toropi-6.3 DH population (Appendix 6), indicating that Toropi is heterogeneous for the presence of *Trp-3*.

There are two other *Lr* genes, *Lr25* and *Lr49*, on the same chromosome arm (4BL) as the *Trp-3* gene. *Lr25* is a seedling gene transferred from rye (*Secale cereale* L.) (Singh et al. 2012), indicating that it is not the same as *Trp-3*. *Lr49*, derived from the wheat cultivar VL404, is an adult plant gene characterized by the mesothetic reaction (Bansal et al. 2008a). *Lr49* plants (VL404 cultivar) showed different allele size when compared with Toropi-6.4 (*Trp-3* gene positive) for *gpw4079* and *gpw7272* SSR markers and had the same allele as Thatcher (suggesting the susceptible allele) for *gpw4388* and *gpw5010* SSR markers (Appendix 7). An allelism test is in progress to determine if *Lr49* and *Trp-3* are indeed different genes. In case they are different genes, the proximity of *Lr49* and *Trp-3* indicates that they could be combined in the same line, being a useful source for breeding.

The locations of *Trp-1* and *Trp-2* genes were determined previously on 1AL and 4DL chromosomes by monosomic analyses and genetic mapping (Brammer et al. 1998; Da Silva 2002). The QTL analyses and bulked segregant analysis did not indicate leaf rust resistance associated with either 1AL or 4DL. The molecular markers previously described as being associated with the genes were located at 25 cM from *Trp-1* and 24.4

cM from *Trp-2* (Da Silva 2002). The genetic distances were large to consider linkage between the markers and the genes. However, it is possible that they identified different genes than those localized during this study.

In conclusion, we identified the location of two loci associated with leaf rust resistance of Toropi located on wheat chromosomes 5AL and 4BL. The QTL on 5AL (*Trp-1*) conferred leaf and stripe rust resistance, being designated *QLr.crc-5AL.1* and *QStr.crc-5AL.1*. The location of *Trp-2* has not yet been determined, however potential locations indicated by this study were chromosomes 2B, 5D or 7A. The 4BL QTL overlapped the region where *Trp-3* gene was mapped. The combination of leaf rust resistance genes in Toropi is responsible for the high level of durable resistance in this cultivar. The deployment of these genes in future cultivars should also be in combination to maintain their durability and effectiveness.

7.0. GENERAL DISCUSSION AND CONCLUSION

Wheat leaf rust, caused by the fungus *P. triticina* Eriks., is the most common rust disease of wheat, reducing average annual wheat yield by 10% (McCallum et al. 2007) as a consequence of premature defoliation of plants (Knott 1989) and photosynthesis alteration (Kolmer et al. 2009a; Lagudah et al. 2006).

Despite more than 60 *Lr* genes identified to date (Herrera-Foessel et al. 2012; McIntosh et al. 2011), the short-lived effectiveness of the majority of them has reduced the number of genes available to be deployed in wheat. It is a common consensus among breeders and pathologists that the best way to achieve durable rust resistance is the deployment of *Lr* genes in combination (Dadkhodaie et al. 2011; Huerta-Espino et al. 2011; Ingala et al. 2012; Kolmer et al. 2009b; Lagudah 2011; Singh et al. 2011b). Incorporation of adult plant genes conferring partial resistance – *Lr34*, *Lr46, Lr67* or *Lr68* – has the advantage of not only conferring durable leaf rust resistance, but also resistance to other diseases such as stripe rust and powdery mildew (Herrera-Foessel et al. 2011; Herrera-Foessel et al. 2012; Hiebert et al. 2010; Krattinger et al. 2009; Lagudah 2011; McCallum et al. 2012b; Rosewarne et al. 2006; William et al. 2006). Singh et al. (2011b) recommended the combination of four to five minor genes to achieve "near-immunity" and durable disease resistance.

In the present study, the leaf rust resistance of cultivar Toropi was evaluated because it has demonstrated durable leaf rust resistance. Toropi was released in 1965 and kept effective resistance even after being intensively cultivated under conditions of high leaf rust epidemics for 15 years. Two recessive genes have been described in Toropi,

however the locations of the genes were not clear (Barcellos et al. 2000; Brammer et al. 1998; Da Silva 2002). The objective of this study was to better characterize the leaf rust resistance of Toropi and map the genes to allow easier incorporation of the genes in future cultivars.

The complex resistance of Toropi required a variety of approaches to better understand it. Different kinds of populations were developed and analyzed: backcross populations using Thatcher and Toropi as the recurrent parent, and double haploid and selfed (F_2 and F_3 generations) populations. Throughout the study, it was observed that Toropi was not homozygous for leaf rust resistance, which resulted in backcross and selfed populations with different combinations of the Toropi genes.

Toropi-6, derived from a selection in Brazil (Barcellos et al. 2000) and used throughout this study, expressed seedling resistance to some Canadian races. However, Toropi-6 was heterogeneous for seedling resistance, showing leaf rust infection types that varied from resistant to susceptible. Three seedling reactions were observed: a) resistant (“;” or “; to 1”); b) moderately resistant (mesothetic reaction); and c) susceptible (“3” to “4”). Two DH populations were developed that were differentiated by the presence of the seedling resistance, which was segregating in the Thatcher/Toropi-6.3 population but not in the Thatcher/Toropi-6.4 DH population.

Chi-square tests indicated that the seedling resistance was conferred by the combination of one leaf rust gene with three complementary genes in the Thatcher/Toropi-6.3 DH population. This population was used to map the seedling gene in Toropi-6.3 using multiple bulked segregant analysis (Hiebert et al. 2012). The gene

was located on 3D chromosome inside a linkage block consisting of 22 SSR markers. This gene was temporarily designated *Trp-Se*. The *Trp-Se* gene confers ";" or "; to 1" resistance to avirulent races. It was not effective in the field in Canada, or Brazil, however, it was responsible for an immune response in New Zealand. Despite the lack of effectiveness in the field in Canada and Brazil when deployed alone, the *Trp-Se* increased the resistance of Toropi lines to almost immunity when combined with adult plant resistance of Toropi.

Analysing the leaf rust resistant lines derived from the cross between Toropi-6 and IAC13-Lorena (Barcellos et al. 2000), it was observed that the lines were resistant to all Canadian races at adult plant stage, except the resistant line 255777, that showed adult plant race specific resistance derived from Toropi. This observation indicated the presence of adult plant race non-specific and race specific resistance genes in Toropi. Line 255777 was resistant to BBBD, but susceptible to race TJBJ. To evaluate the adult plant race specific gene in isolation from the other adult plant genes, a method was developed to inoculate the same plant with two *P. triticina* races. This method was develop by testing Thatcher-*Lr37* near isogenic lines using avirulent and virulent races, and results allowed perfect distinguishability between the reactions. Therefore, the DH lines were tested at adult plant stage with BBBD, avirulent to all adult plant genes in Toropi, and with TJBJ, virulent to *Trp-3* and *Trp-Se*, but avirulent to *Trp-1* and *Trp-2*. The use of both races combined with the infection types allowed the determination of the DH lines with the adult plant race specific gene. The double artificial inoculation is important when saving time, space, and seeds are necessary and to double the results

when analyzing BC_1F_1 or F_2 generations, when clones are not present. This technique is broadly applicable to other leaf rust genetic investigations.

The inheritance of Toropi was analyzed in the two DH populations Thatcher/Toropi-6.3 and Thatcher/Toropi-6.4 in the greenhouse and in the field. The summary of the data indicated that Toropi has a seedling gene, *Trp-Se*, two partially complementary genes, *Trp-1* and *Trp-2*, and an APR race specific gene, *Trp-3* gene. An additional gene was identified in two field locations, but was not effective in most trials. The inheritance fitted different segregation models depending on the location. This could be explained principally by the *P. triticina* population diversity among the environments and other environmental factors. The plants were submitted to stress conditions in Glenlea and in Brazil. In Glenlea – 2010, the plants were covered by water for few days due to flooding in the region. In Brazil, short day length and aluminum toxicity are two facts that affected plant development. Chi-square tests indicated the presence of two complementary genes in Brazil and Glenlea, therefore it is possible that minor genes as *Trp-3* were not perceived because of plant stresses. The leaf rust resistance of Toropi was analyzed in different locations to test the broad efficacy of Toropi genes in completely different environments, Canada, New Zealand and Brazil. According to Barcellos et al. (2000), the *Trp-1* and *Trp-2* genes were the only effective genes in Toropi in Brazil, so testing the DH lines there provided an opportunity to isolate these genes from other Toropi genes.

The genetic mapping of *Trp-1* and *Trp-2* genes was complicated by the partially complementary effect of the genes, which means that the effect of each gene is dependent

on the presence of both genes, and by the absence of virulent races to the genes. It was not possible to locate the genes by multiple bulked segregant analysis, which made it necessary to perform QTL analyses. Linkage groups were constructed using 230 SSR and 164 SNP markers covering the wheat genome. The most important QTL identified associated with leaf rust resistance derived from Toropi-6.4 were located on 5AL and 4BL chromosomes. The 4BL QTL overlapped the region where *Trp-3* was mapped. The QTL identified on 5AL (marker interval between *gpw7007* and *cfa2163*) was denominated *QLr.crc-5AL.1,* and was significant in both Canadian locations where the population was tested, in one year in New Zealand and when using mean leaf rust severity of all locations. The same marker interval identified a significant QTL associated with stripe rust in New Zealand in 2010, *QStr.crc-5AL.1*. Further studies are necessary to confirm the QTL and to reduce the marker interval, but this QTL, arbitrarily designated *Trp-1*, could be an important source of resistance as it may confer leaf and stripe rust resistance.

The determination of the presence or absence of the adult race specific gene *Trp-3* in the DH lines allowed this gene to be considered as having simple Mendelian inheritance, therefore multiple bulked segregation (Ghazvini et al. 2012; Hiebert et al. 2012) was used for the identification of its chromosomal location. The race specific adult plant gene was temporarily designated *Trp-3*. The *Trp-3* gene was mapped on the chromosome 4BL flanked by *gpw4079* (0.8 cM distal) and by *gwm149* (2.4 cM proximal) using 126 DH lines derived from the cross Thatcher/Toropi-6.4. The *Trp-3* gene phenotype is characterized by mixture of susceptible and resistant pustules, “3 ;” reaction, similar to a minor gene. In the presence of other genes (probably *Trp-1* and *Trp-2*), it

expressed a mesothetic reaction at seedling stage. *Trp-3* conditioned an intermediate level of resistance in New Zealand, intermediate to susceptible in Canada, and a susceptible reaction in Brazil. An allelism test is being performed to elucidate whether *Trp-3* differs from *Lr49*, which is also located on 4BL.

This study demonstrated the complex nature of leaf rust resistance in the Brazilian cultivar Toropi, which is conferred by at least four resistance genes. Two leaf rust resistance genes of Toropi were characterized and mapped: *Trp-Se* on 3D, and *Trp-3* on 4BL. The *QLr.crc-5AL.1* and *QStr.crc-5AL.1* were identified at the same region of 5AL chromosome associated with leaf and stripe rust resistance of Toropi, which was designated *Trp-1*. The location of *Trp-2* has yet to be definitely proven but this study indicates it could be on chromosome 2B, 5D or 7A. Additional SSR markers are being analyzed on 5AL to reduce the marker interval of the *QLr.crc-5AL.1* and a field nursery is being organized to confirm the association of this QTL with leaf rust in Canada. These results will facilitate the comparison of the DH lines with *Trp-3* and *QLr.crc-5AL.1* to determine the relationship between both genes.

The leaf rust resistance conferred by *Trp-1* (*QLr.crc-5AL.1*) and *Trp-3* are both characterized as adult plant resistance with minor effects. The near immunity resistance of Toropi visualized in different parts of the world is a result of the combination of the minor effects conferred by *Trp-1, Trp-2* and *Trp-3* plus *Trp-Se* and other Toropi genes. The Toropi genes could be incorporated in future cultivars to increase the resistance and durability of wheat against leaf and stripe rust.

8.0. LITERATURE CITED

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APPENDICES

Appendix 1. Marker name, allele size, primer sequence and annealing temperature of simple sequence repeat (SSR) markers used to construct the *Trp-Se* genetic map.

	Allele size		Primer sequence		Annealing Temperature (^{0}C)
SSR marker[a]	Thatcher	Toropi-6	Forward Primer Sequence	Reverse Primer Sequence	
barc71	105	101, 89, 85	GCGCTTGTTCCTCACCTG CTCATA	GCGTATATTCTCTCGTCT TCTTGTTGGTT	51
barc270	244	null	GCGCATTGTGACAGGTG AAC	GGAGGGAGTACTTGGTT ATTAGGGT	51
barc284	246	null	GCGTCAGAAATGCAAGA AAAATAGG	GCGGAAGAAAAGGACG AAGACAAG	51
barc363	293	null	GCACGCTAGAAAAGAA GTCATAAAAACA	GGTGTCTGTAGAAACGC CCATAA	51
cfd4	252	276	TGCTCCGTCTCCGAGTA GAT	GGGAAGGAGAGATGGG AAAC	61
cfd9	196	null	TTGCACGCACCTAAACT CTG	CAAGTGTGAGCGTCGG	61
cfd51	265	276	GGAGGCTTCTCTATGGG AGG	TGCATCTTATCCTGTGCA GC	61
cfd152	290	null	TGGAAGTCTGGAACCAC TCC	GCAACCAGACCACACTC TCA	61
cfd223	171	null	AAGAGCTACAATGACCA GCAGA	GCAGTGTATGTCAGGAG AAGCA	61
gdm38	135	null	CAAAATGAAGCATGAAG AGG	CAGCACATAGCTTTGGT CTT	61
gpw294	112	null	TAATACCCTCCTTCCCCA CC	ACAACGTGCGAGTCACC ATA	61
gpw307	142	151	ACCCGGCCTCTGTATGG TTA	TGTTCATTGCCACAGTTT GC	61
gpw1149	135	null	CATGTCAAAGCACCAGC AGA	CTTTGGCGCTGAAGTAA AGG	61
gpw3109	212	219	CAAAAAAAGGAAGAAC TCATGG	TGTTAAGTTCAAGACCC CAGTG	61
gpw4136	240	null	ACGGGTATCGTGGAATT GAA	CCAGATTGTAACGCCCT TTC	61
gpw4163	403	389	TGGCAATGGAGGTAATG ACA	TGGTGCCCTAAAATTGG TTT	61
gpw4352	266	263	AGTCCTCCACCTGTTGC G	ACATAGATGACCAACGC CG	61
gpw5064	352	null	CATTCTTTTTGAAAGGTC TGGC	CCCCTTTGATGTCCCCTT AT	61
gpw5177	358	null	CCGGTTTTCTTTGTTTGC AT	AGTGCTACAGATCCGCC G	61
gpw5203	244	null	CAACTTATGGTCGTCGC TCA	TTCTTGTTTGCAGTACAG GGC	61
gpw5235	287	null	TTTCACAGACGAAGCCC TG	CAACCTAAAAACTCCTT GCCC	61
gpw5271	237	null	ATACATACCCAATGCAG AAGCA	GAAGGAACGAATCAGG AAACA	61
gwm114	115	111	ACAAACAGAAAATCAA AACCCG	ATCCATCGCCATTGGAG TG	61
gwm383	187	155	ACGCCAGTTGATCCGTA AAC	GACATCAATAACCGTGG ATGG	61
wmc43	324	320	TAGCTCAACCACCACCC TACTG	ACTTCAACATCCAAACT GACCG	61
wmc492	152	154	AGGATCAGAATAGTGCT ACCC	ATCCCGTGATCAGAATA GTGT	61
wmc631	99	null	TTGCTCGCCCACCTTCTA CC	GGAAACCATGCGCTTCA CAC	61
wmc656	175	null	AAGTAGGCGAGCGTTGT	TTTCCCTGGCGAGATG	61

[a]Information about the SSR markers followed Somers et al. (2004) with some modifications.

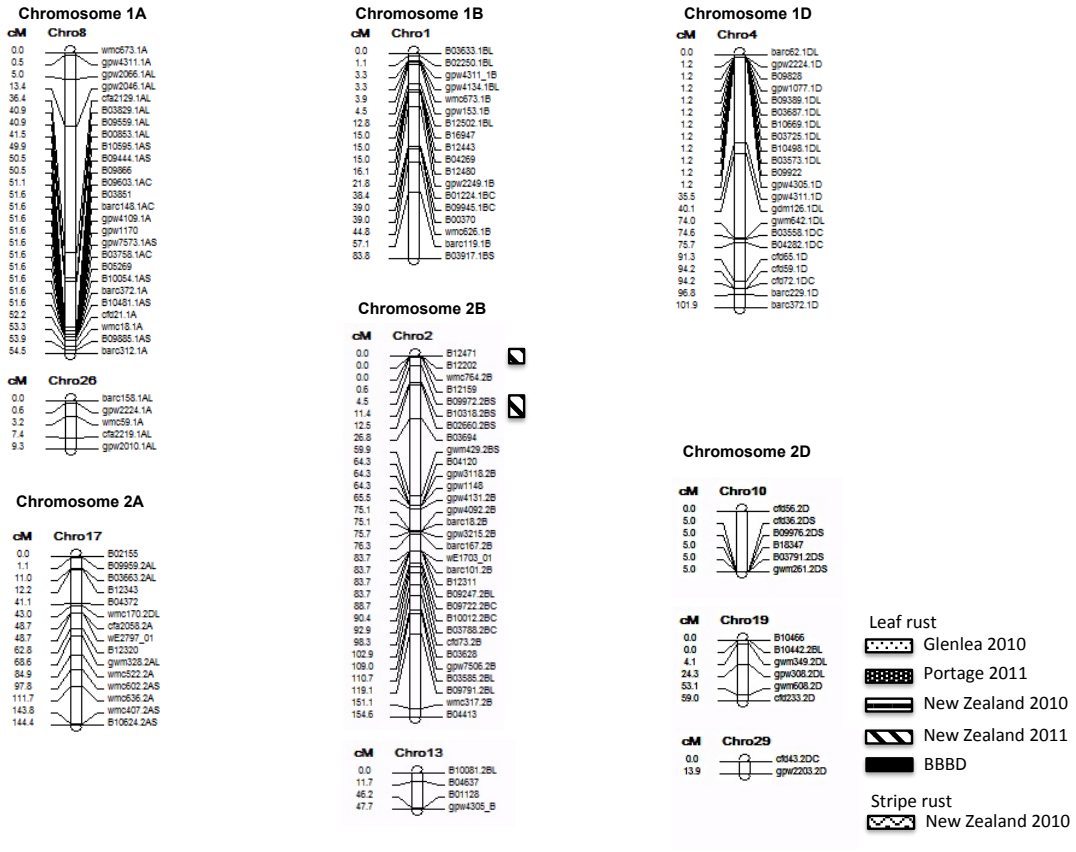
Appendix 2. Leaf rust reaction of Toropi-6, Toropi Uruguay (Ur), IAC13-Lorena, resistant lines derived from the cross Toropi-6/IAC13-Lorena, Tc-*Lr24*, Tc-*Lr32* and Thatcher. About six plants of each cultivar or line were inoculated in the greenhouse against different *P. triticina* races. The name of the races follow the North American nomenclature (Long and Kolmer 1989) and the isolation number is indicated. The leaf rust reaction was classified according to Long and Kolmer (1989), including "-" and "+" to indicate smaller or bigger pustules than the traditional classification.

Leaf rust races	**Toropi-6**	**Toropi Ur**	**IAC13-Lorena**	**255644**	**255693**	**255725**	**255764**	**255777**	**Tc-*Lr24***	**Tc-*Lr32***	**Thatcher**
BBBD	; / ; to 3 / 3[a]	3^- 3	3^- to 3^+	;	;	;	;	;	; 1^- (f)[b]	; to 1^-	3^- 3
CCDS 96-14-1	; 1(f) / 3	3^- 3	; to 3^-	; 3(f)	; 3(f)	; 3(f)	; 3(f)	; 3(f)	; 3^- (f)	; to 2	3^- 3
FBDJ 161-1	; 1^- / ; 2	3	; / 3	;	;	;	;	;	; 1^{--}	; to 2^{--}	3 4
KBBJ 04-63-1	; to 2^{--}	3^- 3	3^- 3	n[c]	n	n	n	n	; to 1	; to 1^+	3^- to 4
MBDS 12-3	0 / 1^+ / 3	3 4	3	3	3	3	;(f) / 3	3	;(f)	; to 1	3^- 3
MBRJ 128-1	;	3- to 4	3	3	3^- 3	2^+ to 3(f)	;	3^- 3	;	n	3 3^+
MDNS 08-8-1	; to 1^+ / 3	3	3	3	3	3	3	3	3^- 3	; to 1^+	3 4
MGBJ 74-2	; to 2^+	3 4	3^- 3	3^- 3	3	3	;	3	; 1^-	n	3^- to 3^+
TBBG-L 06-11-1	; to 2	3^- to 4	; to 3	n	n	n	n	n	; 1^{--}	; to 1	3^- to 4
TDBG 06-1-1	3^+ 4	3^+ 4	3^- to 4	3^+ 4	3^+ 4	3^+ 4	3^+ 4	3^+ 4	3 4	n	3^+ 4
TDBG 05-10-1	; to 1 / 3	3^- 3	; to 3 / 3	3 3	3 3	3 3	3 3	; to 3 / 3	3	; 1^-	3^- to 4
TJBJ 77-2	3^{--} 3^-	4	3^- to 3^+	3^- to 4	3 3^+	3^- to 4	3 to 4	3 to 4	3	n	3 4
TPBG 05-4-2	; to 2^+	3^- to 3^+	; to 2	n	n	n	n	n	2 to 3	; to 1	3^- 3

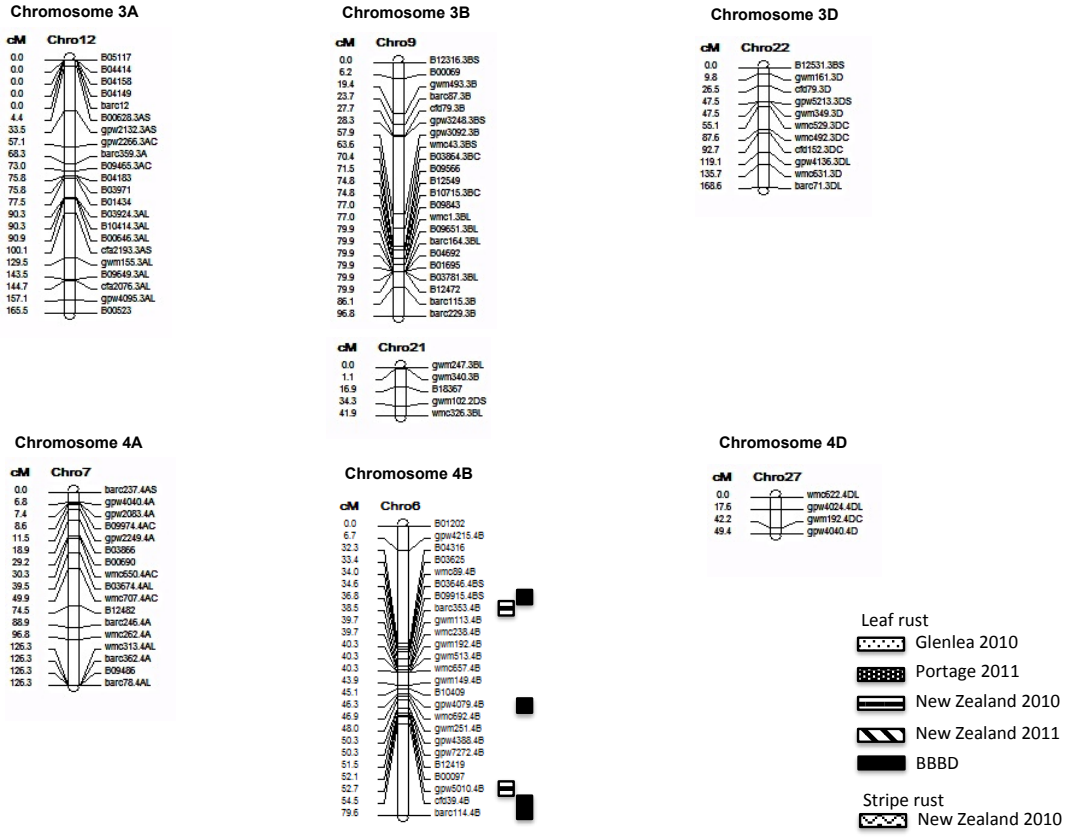
[a] Presence of "/" indicates the presence of plants with different leaf rust reaction to the specific race.
[b] "f" means few pustules of the determined type.
[c] "n" indicates that the line was not tested to the race.

Appendix 3. Linkage groups developed for the double haploid population Thatcher/Toropi-6.4 using 184 lines. Simple sequence repeat (SSR) and single nucleotide polymorphisms (SNP) markers were analysed. The genetic distance between markers is in centiMorgans (cM).

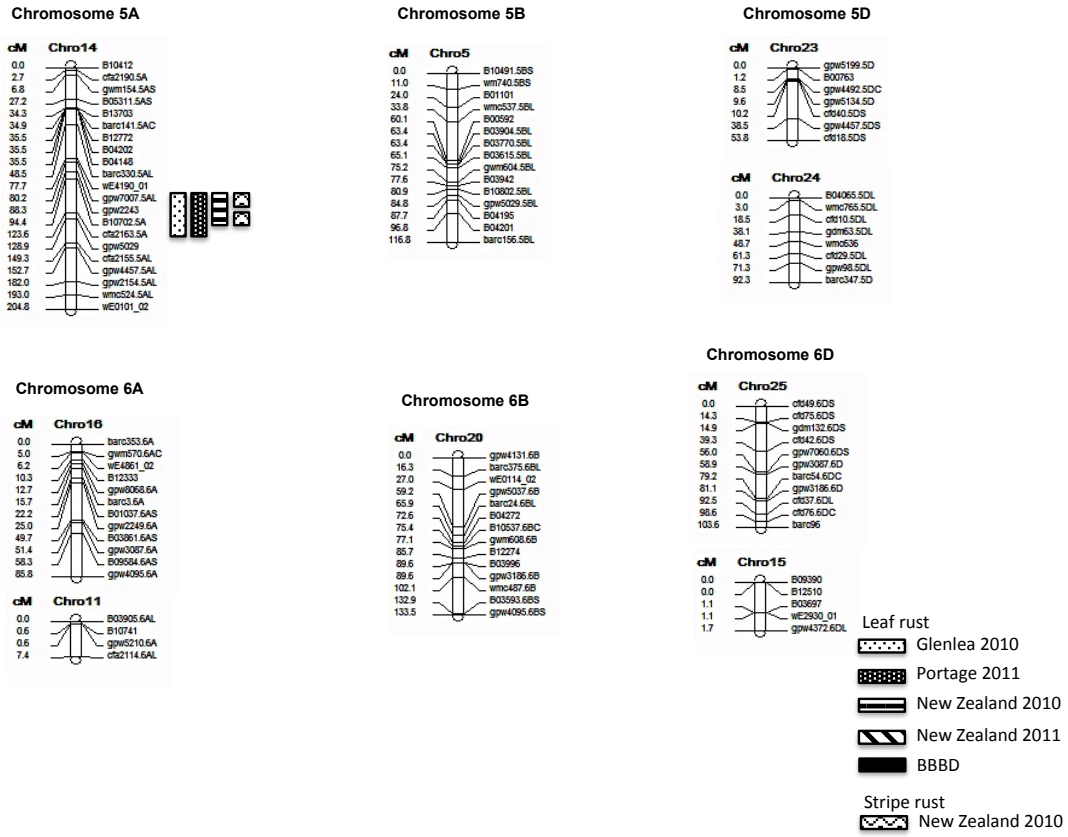
Chromosome	Linkage group	Map size (cM)	SSR	SNP	Distance (cM)	
					Average	Min. - Max.
1A	8	54.32	13	13	2.18	0 - 23.0
	26	9.36	5	0	2.33	0.6 - 4.2
1B	1	83.7	7	11	4.92	0 - 26.7
1D	4	101.82	12	10	4.85	0 - 34.3
2A	17	144.47	7	8	10.31	0 - 32.1
2B	2	154.59	13	18	5.15	0 - 33.1
	13	47.74	1	3	15.9	1.5 - 34.5
2D	10	5.05	3	3	1.0	0 - 5.0
	19	58.95	4	2	11.8	0 - 28.8
	29	13.91	2	0	13.9	13.9
3A	12	165.51	8	14	7.88	0 - 29.4
3B	9	96.7	10	12	4.61	0 - 29.6
	21	41.91	4	1	10.48	1.1 - 17.4
3D	22	168.64	10	1	16.86	0 - 32.9
4A	7	126.11	11	6	7.89	0 - 29.5
4B	6	79.61	17	8	3.31	0 - 25.6
4D	27	49.42	4	0	16.47	7.2 - 24.6
5A	14	204.61	12	9	10.24	0 - 29.3
5B	5	116.87	5	10	8.34	0 - 26.3
5D	23	53.84	6	1	8.97	0.6 - 28.3
	24	92.31	7	1	13.19	3.0 - 21.0
6A	11	7.4	2	2	2.47	0 - 6.8
	16	85.86	7	5	7.8	1.2 - 27.5
6B	20	133.38	8	6	10.27	0 - 32.2
6D	15	1.66	1	4	0.425	0 - 1.1
	25	103.54	11	0	10.36	0.6 - 24.4
7A	18	155.24	16	9	6.47	0 - 33.7
7B	3	140.35	14	7	7.02	0.6 - 27.1
7D	28	43.55	4	0	14.5	4.6 - 27.3
	30	88.04	6	0	17.62	5.2 - 30.3
Total		2628.46	230	164	8.84	0 - 34.5
			394			



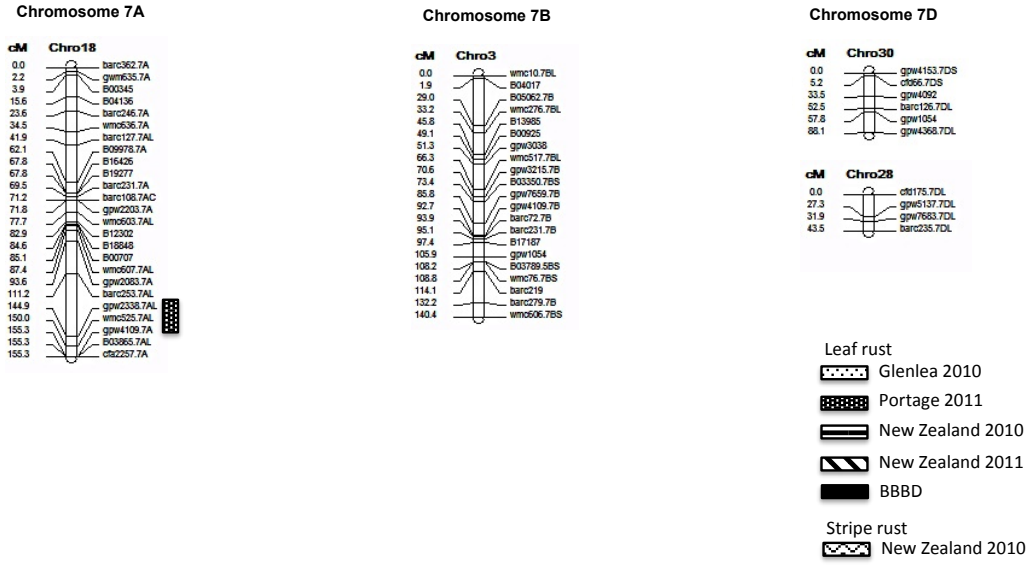
Appendix 4.1. Linkage map SNP and SSR markers on chromosomes 1A, 1B, 1D, 2A, 2B and 2D developed using 184 lines of the Thatcher/Toropi-6.4 double haploid population and locations of significant quantitative trait loci (QTL) associate with leaf rust severity, infection type and stripe rust severity identified by composite interval mapping. The QTL are derived from disease severity analyses, except for QTL of New Zealand 2010 on 5AL and BBBD, which were derived from type of infection.



Appendix 4.2. Linkage map SNP and SSR markers on chromosomes 3A, 3B, 3D, 4A, 4B and 4D developed using 184 lines of the Thatcher/Toropi-6.4 double haploid population and locations of significant quantitative trait loci (QTL) associate with leaf rust severity, infection type and stripe rust severity identified by composite interval mapping. The QTL are derived from disease severity analyses, except for QTL of New Zealand 2010 on 5AL and BBBD, which were derived from type of infection.



Appendix 4.3. Linkage map of SNP and SSR markers on chromosomes 5A, 5B, 5D, 6A, 6B and 6D developed using 184 lines of the Thatcher/Toropi-6.4 double haploid population and locations of significant quantitative trait loci (QTL) associate with leaf rust severity, infection type and stripe rust severity identified by composite interval mapping. The QTL are derived from disease severity analyses, except for QTL of New Zealand 2010 on 5AL and BBBD, which were derived from type of infection.



Appendix 4.4. Linkage map of SNP and SSR markers on chromosomes 7A, 7B and 7D developed using 184 lines of the Thatcher/Toropi 6.4 double haploid population and locations of significant quantitative trait loci (QTL) associate with leaf rust severity, infection type and stripe rust severity identified by composite interval mapping. The QTL are derived from disease severity analyses, except for QTL of New Zealand 2010 on 5AL and BBBD, which were derived from type of infection.

Appendix 5. Primer sequences, annealing temperature, and allele sizes of simple sequence repeat (SSR) markers used to construct a genetic map of the *Trp-3* gene in the Thatcher/Toropi-6.4 double haploid population. The allele sizes include 19 bp of the M13 fluorescent tag.

SSR markers[a]	Primer sequence		Annealing temperature (^{0}C)	Thatcher allele (-) (bp)	Toropi-6.4 allele (+) (bp)
	Forward	Reverse			
barc114	GGGTATCACGCATT GTAAAATCCGAAA	GCTGACTTCCGCAAAT GCCATTCGCTGAT	50	150	168
cfd39	CCACAGCTACATC ATCTTTCCTT	CAAAGTTTGAACAGC AGCCA	61	210	204
gwm113	ATTCGAGGTTAGG AGGAAGAGG	GAGGGTCGGCCTATA AGACC	61	171	168
gwm149	CATTGTTTTCTGCC TCTAGCC	CTAGCATCGAACCTGA ACAAG	61	175	185
gwm192	GGTTTTCTTTCAGA TTGCGC	CGTTGTCTAATCTTGC CTTGC	61	224	218
gwm251	CAACTGGTTGCTAC ACAAGCA	GGGATGTCTGTTCCAT CTTAG	61	128	125
gwm513	ATCCGTAGCACCTA CTGGTCA	GGTCTGTTCATGCCAC ATTG	61	166	168
gpw4079	AGGTTTGAACTAA GCCCGGT	AAATGTGATGGACTTA CCACCC	61	316	309
gpw4388	GTCGGGTTCCTATA CAAAGGG	TATTCCAAAGTTGCCA TCCC	61	269	261
gpw5010	CATCATAAGCGCA ATAGCCA	CAAACAACAAGCGGG TCACA	61	467	388
gpw7272	CGACAAGCGGGTT AGAGA	CTGGCTGAACCTGCTC C	61	208	199
wmc89	ATGTCCACGTGCTA GGGAGGTA	TTGCCTCCCAAGACGA AATAAC	61	198	194
wmc238	TCTTCCTGCTTACC CAAACACA	TACTGGGGGATCGTG GATGACA	61	243	252
wmc657	CGGGCTGCGGGGG TAT	CGGTTGGGTCATTTGT CTCA	61	132	153
wmc692	TTATCTTGATCCGA GCGA	ATGTGATTAGTCCTAA GGTCTCTCT	61	125/132	123/134

[a] Modified consensus map of Somers et al. (2004).

Appendix 6. Relationship between Thatcher/Toropi-6.3 double haploid population and simple sequence repeat (SSR) markers linked with *Trp-3* gene on Thatcher/Toropi-6.4 population. Toropi-6.3 showed a different allele size than Thatcher (A) and Toropi-6.4 (H), being denominated B allele. The data indicated no linkage between phenotypic and genotypic results for *Trp-3* gene, indicating absence of this gene in Thatcher/Toropi-6.4 population.

Thatcher/Toropi-6.3 double haploid population				
Lines predicted **absence of *Trp-3*** by phenotype				
	gpw4079	*gpw7272*	*gpw4388*	*gpw5010*
Allele A (Thatcher)	22	22	20	46
Allele H (Toropi-6.4)	0	0	1 (A + H)	0
Allele B (Toropi-6.3)	24	24	24	0
Lines predicted **presence of Trp-3** by phenotype				
	gpw4079	*gpw7272*	*gpw4388*	*gpw5010*
Allele A (Thatcher)	1	1	1	2
Allele H (Toropi-6.4)	0	0	0	0
Allele B (Toropi-6.3)	1	1	1	0
Presence of *Trp-3* was not possible to confirm by phenotype				
	gpw4079	*gpw7272*	*gpw4388*	*gpw5010*
Allele A (Thatcher)	70	71	72	143
Allele H (Toropi-6.4)	1	1	1	1
Allele B (Toropi-6.3)	75	74	74	0

Appendix 7. Molecular marker analyses comparing allele sizes of Toropi-6.4 (*Trp-3* gene positive), VL404 (*Lr49* positive) and Thatcher (susceptible) to four simple sequence repeat (SSR) markers linked to the *Trp-3* gene. The allele sizes include 19 base pairs of the M13 fluorescent tag. Dark grey indicates the susceptible allele, while light grey is the Toropi-6.4 (*Trp-3* positive) allele. Three plants of VL404-*Lr49* and Thatcher were analysed, while five plants of Toropi-6.4 were scored.

	Allele Size (base pairs)			
	gpw4079	*gpw5010*	*gpw7272*	*gpw4388*
Toropi-6.4	309	388	199	261
VL404-*Lr49*	303	467	205	269
Thatcher	316	467	208	269