Genetic Analysis of Leaf Rust Resistance Gene *Lr34* in Wheat

BY
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A ThesisSubmitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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ABDULSALAM DAKOURI

Dedicated to
“My parents”
“My wife Buthina”
“My sons Ahmad and Adam”

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LIST OF ABBREVIATIONS

ABC	ATP Binding Cassette
AM	Association Mapping
AMOVA	Analysis of Molecular Variance
APR	Adult Plant Resistance
CYP	Cytochrome P-450
EST	Expressed Sequence Tag
HC	Hexose Carrier
HIT	High Infection Type
ISBP	Insertion Site Based Polymorphism
IT	Infection Type
LD	Linake Disequillibrium
LIT	Low Infection Type
LRK	Lectin Receptor Kinase
MCMC	Markov Chain Monte Carlo
MJ	Median Joining
MP	Maximum Parsimony
NBD	Nucleotide Binding Domain
NBS-LRR	Nucleotide Binding Site and Leucine-Rich Repeat
PDR	Pleiotropic Drug Resistance
PGRC	Plant Gene Resource Canada
PIC	Ploymorphic Information Content
SNP	Single Nucleotide Polymorphism

SSR	Simple Sequence Repeat
STS	Sequence Tag Site
TE	Transposable Element
TMD	Transmembrane Domain
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WC	World Collection

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ABSTRACT

Leaf rust is a major wheat production constraint worldwide. Genetic resistance has been the method of choice to manage leaf rust in wheat producing areas. *Lr34*, an adult plant resistance gene, is considered the most important leaf rust resistance gene known to date. Of the six genes located at the *Lr34* locus on 7DS, the ABC transporter was determined to be the sole candidate for the *Lr34* function (Krattinger et al 2009). Here we developed ten novel molecular markers spanning the *Lr34* locus , including six microsatellites (cam), one insertion site-based polymorphism (caISBP), two single nucleotide polymorphisms (caSNP) and one indel marker (caIND). Analysis of these markers on five segregating populations revealed a recombination between caSNP4 and cam8 which provided further support for the identity of the ABC transporter as *Lr34*. Characterization of a large wheat germplasm using the four *Lr34* specific markers resulted in the identification of the three previously reported haplotypes. An additional novel mutation was discovered in exon 10 of the ABC transporter which permitted to define a fourth haplotype. A fifth haplotype was identified in two accessions of the germplasm. Of the ten markers developed herein, marker caIND11 is highly diagnostic, co-dominant and is recommended for marker assisted selection of *Lr34*.

A comprehensive analysis of the genetic diversity at the *Lr34* locus in a world wheat germplasm collection was done. On the basis of the *Lr34*-specific markers, the world collection was divided into five major haplotypes (H) of which H1 was consistently associated with the resistance phenotype *Lr34+*. Maximum parsimony network and other observations revealed that H4, an *Lr34-* haplotype, was probably the most ancient haplotype and H1 the most recent and that it likely arose after the advent of

hexaploid wheat. Combined analysis of the 10 molecular markers divided the major haplotypes into 118 different sub-haplotypes. Structure and clustering analyses grouped these sub-haplotypes into two main clusters and seven sub-clusters. Analysis of geographical distribution showed that H1 was at a high frequency in the Asian germplasm while H4 was more frequent in the European germplasm. *Lr34*, a gain of function mutation, was hypothesized to have originated in Asia.

The world wheat collection (WC) of 275 accessions from 42 countries was characterized for seedling and adult plant resistance (APR) genes using gene specific markers and gene postulation. In total, 14 seedling genes were determined or postulated to be present in the collection. *Lr1*, *Lr10*, *Lr3* and *Lr20* were the most highly represented genes while *Lr9*, *Lr14b*, *Lr3ka* and/or *Lr30* and *Lr26* were rare. The WC was evaluated for field resistance at three locations over three years. The rust rating in the field ranged from nearly immune or highly resistant with severity of 1% and host response of (R) to highly susceptible with severity of 84% and host response of (S). Most *Lr34* -containing accessions had maximum rust severity (MRS) of 35%, similar to accession RL6058. Many accessions showed immune to high levels of resistance under field conditions. The high levels of resistance in some accessions are the result of synergy between APR genes or between APR and seedling genes. Accessions that were immune or highly resistant should be considered potential sources of resistance for wheat breeding to improve leaf rust resistance.

FOREWORD

The thesis follows the paper style format recommended by the Plant Science Department and the Faculty of Graduate Studies of the University of Manitoba. The thesis has seven chapters: a general introduction, a literature review, three manuscripts, a general discussion and conclusion and the literature cited. Manuscripts were formatted following the guidelines of Theoretical and Applied Genetics. Each manuscript contains abstract, introduction, materials and methods, results, discussion and conclusion.

1.0 GENERAL INTRODUCTION

1.1 Wheat

Wheat is one of the most important crops worldwide representing a staple food for 35% of the world inhabitants. It provides more nourishment than any other food crop making wheat a main diet component (Orth and Shellenberger 1988). More than 17% (nearly 217 million hectares) of the world's cultivated land is planted to wheat and the total annual world production for this crop in 2010 reached 651 million tonnes (FAO statistics 2010). The six largest wheat producers in the world are the European Union, China, India, USA, Russia and Canada. With 8.3 million hectares and an annual production of ~23.2 million tonnes, Canada is the sixth largest wheat producer (FAO statistics 2010). Approximately 91% of Canadian wheat is grown in the southern areas of the Prairie Provinces of Saskatchewan (46%), Alberta (27%) and Manitoba (18%). The remaining 9% is grown in Ontario (8%) and Quebec (1%). In Canada, spring wheat is the major type with 75% of the production followed by durum wheat and winter wheat with 19% and 5%, respectively (Peterson 1965).

Bread wheat (*Triticum aestivum* L. em.Thell) is an allohexaploid (2n=6x=42) with a large genome (16,000 Mb) composed of three homoeologous sub-genomes (A, B and D) (Roder et al. 1998). Each sub-genome includes seven metacentric or submetacentric chromosomes (Akhunov et al. 2003). Hexaploid wheat is the result of two historical consecutive hybridization events involving three diploid grass species. The first event occurred between *Triticum urartu* Thum, an A genome species and *Aegilops speltoides* (Tausch) Gren or a close relative, donor of the B genome, giving rise to tetraploid wheat

(AABB, 2n=4x=28). The second hybridization event involved the tetraploid wheat and *Aegilops tauschii* Coss, the D genome donor (Akhunov et al. 2003; Dvorak et al. 2006).

1.2 Diseases of Wheat

Wheat is host to numerous pathogens that can cause biotic stresses. Wheat diseases are mainly caused by fungal pathogens, those caused by viruses and bacteria remain limited (Rajaram and Van Ginkel 1996; McIntosh 1998). Wheat disease-causing fungi are either obligate biotrophic parasites including rust, powdery mildew and smuts or facultative necrotrophic parasites such as those causing Septoria blotches, tan spot and Fusarium head blight (Singh and Rajaram 2002). Rust diseases have been most devastating to wheat production all over the world. There are three major species of rust on wheat (Saari and Prescott 1985): stripe or yellow rust caused by *Puccinia striiformis* f. sp. *tritici,* stem or black rust caused by *Puccinia graminis* f. sp. *tritici* and leaf or brown rust caused by *Puccinia triticina* (formerly known as *Puccinia recondita* f. sp. *tritici*).

1.3 Wheat leaf rust

1.3.1 The importance of leaf rust

Among the three rust species, leaf rust has been the most frequent disease in wheat-producing areas (Kolmer 2005). Leaf rust can cause significant yield losses exceeding 15% (Samborski 1985). *Puccinia triticina* is adapted to a range of environments and can co-exist with wheat wherever it is grown. The world wheat growing regions are divided into several leaf rust epidemiological zones: Mexico, Canada and USA, South Asia, West Asia, Eastern Europe and Egypt, Southern Africa, Northern Africa and Western Europe,

the Far East, Southeast Asia, South America, Australia and New Zealand (Huerta-Espino, 1992; Saari and Prescott 1985). Leaf rust-related yield losses vary between areas. In the USA, leaf rust caused yield losses worth 350 million dollars between 2000 and 2004 (Appel et al. 2009). In Mexico, yield losses were estimated at 32 million dollars from 2000 to 2003 (Singh et al. 2004a). In South Americ, a an estimated yield loss of 172 million dollars was caused by leaf rust between 1996 and 2003 (German et al. 2004), while in China it was estimated at three million tonnes annually (Huerta-Espino et al. 2011).

1.3.2 Physiological specialization of leaf rust

Physiological races are genetically pure isolates of *P. triticina* differing from one another in virulence. Caten (1987) defined a race as 'a taxon of pathogen characterized by specialization to different cultivars of one host species'. For instance, leaf rust races are distinguished from one another by their reaction on a set of host lines each carrying a single known resistance gene and thus called differential lines (McCallum et al. 2012b). Physiological specialization in *P. triticina* was initially established by Mains and Jackson (1921, 1926) who identified 12 leaf rust races on 11 differential lines. Three differentials were later removed (Johnston and Mains 1932) while the remaining eight were considered the internationally accepted standard differential set.

1.3.3 Biology of leaf rust

Puccinia triticina belongs to the order Uredinales, the family Pucciniaceae and the genus *Puccinia*. The primary host of *P. triticina* is bread wheat (*Triticum aestivum*), but leaf rust can also infect tetraploid durum wheat (*T. durum*), wild emmer (*T. dicoccoides*), domestic emmer wheat (*T. dicoccon*) and triticale (Yehuda et al. 2004). This fungus is distinguished from the other rust species by the uredinial stage (Anikster et al. 2005). Uredinia are the fruiting bodies of the rust fungi that bear urediospores. They are two mm in diameter, orange to brown and are dispersed on both the upper and the lower leaf surfaces causing chlorosis or necrosis. *Puccinia triticina* responds to similar environmental conditions with the other rust species. A minimum dew period of three hours at temperatures around 20°C are required to initiate infection and longer dew periods are more favorable to infection (Stubbs et al. 1986).

Puccinia triticina is a heteroecious basidiomycete fungus with a lifecycle that includes five spore stages. To complete its life cycle, it needs two hosts: a primary (telial/uredinal) host (usually wheat) and an alternative (pycnial/aecial) host (*Thalictrum speciosissimun* or *Isopyrum fumaroids*). The fungus survives the summer as teliospores on mature wheat and residues (Anikster et al. 1986). Under favorable conditions, teliospores germinate giving rise to basidia. Mature basidiospores are expelled from basidia and carried by wind to the alternative host. Basidiospores directly infect the epidermal cells leading to the development of yellow-orange pycnial pustules on both leaf surfaces (Allen 1932) which subsequently give rise to aecial pustules containing aeciospores that are wind-blown and may land on wheat, the primary host (Gold et al. 1979). The aeciospores germinate and penetrate the stomata resulting in the formation of

asexual urediniospores, which germinate and asexually produce more spores that are in turn wind-blown to other plants, fields or longer distances where they can perpetuate the asexual infection cycles on wheat (Allen 1926). Populations of *P. triticina* are extremely diverse for virulence races. Annually, up to 70 different races in the USA (Kolmer and Ordonez 2007), 30-50 races in France (Goyeau et al. 2006), and 10-15 races in Australia (Park 1996) are regularly observed in surveys.

1.3.4 Genetic management of leaf rust

Genetic resistance is one of the best strategies for preventing yield losses caused by *P. triticina*. Mains et al. (1926) first indicated that the wheat varieties Malakof and Webster, each possessed a gene conferring resistance against *P. triticina*, genes that were later named *Lr1* and *Lr2*, respectively. To date, Leaf rust resistance genes named *Lr1* to *Lr68* have been characterized in bread wheat, durum wheat and several diploid wheat species. Many of these genes have been incorporated into the hexaploid wheat germplasm (McIntosh et al. 2007). Most of the aforementioned genes are seedling genes conferring race-specific resistance characterized by a hypersensitive response (HR) or programmed cell death. Many of these genes have now been overcome by new races of the leaf rust pathogen (McIntosh et al. 1995).

Beside seedling resistance genes, several *Lr* genes are effective at the adult plant stage and in general are characterized as slow rusting. Genes *Lr12*, *Lr13* and *Lr34* (Dyck et al. 1966), *Lr22a* (Rowland and Kerber 1974), *Lr46* (Singh et al. 1998), *Lr67* (Hiebert et al. 2010; Herrera-Foessel et al. 2011) and more recently *Lr68* (Herrera-Foessel et al. 2012) are all adult plant resistance (APR) genes. *Lr12* and *Lr13*, similar to seedling

resistance genes, are race-specific APR genes triggering a hypersensitive response (Bender et al. 2000). *Lr34*, *Lr46*, *Lr67* and *Lr68* are race non-specific and provide partial or slow rusting resistance under field conditions (Rowland and Kerber 1974; Caldwell 1968). A list of all known leaf rust resistance genes including seedling and APR, their origins and chromosomal locations can be found in McCallum et al. (2012b).

A typical APR reaction is characterized by a longer latent period, a lower level of receptivity, smaller uredinia sizes and an overall lower pustule density (Ohm and Shaner, 1976; Das et al. 1993). Slow rusting can be measured in the field using the area under disease progress curve (AUDPC) and the maximum disease severity (MDS) (Wilcoxson 1981). The heritability of partial resistance ranged from moderate to high (0.5-0.9) indicating that this type of resistance has selection potential in breeding for leaf rust resistance (Lee and Shaner 1985; Bjarko and Line 1988; Broers and Jacobs 1989; Jacobs and Broers 1989). APR genes are in general more durable than seedling genes and they interact in an additive manner when pyramided, leading to very high levels of resistance or near immunity (Singh et al. 2000; Lillemo et al. 2011). Genes such as *Lr34*, *Lr46* and *Lr67* were also found to provide resistance to other wheat diseases such as stripe rust and powdery mildew caused by *Blumeria graminis* making them ideal candidates for strategic breeding for multi-disease resistance (Lillemo et al. 2008; Herrera-Foessel et al. 2011). Functional alleles of the few APR genes known to date are widely distributed in bread wheat germplasm (Singh et al. 2000).

1.4 The rationale and scope of the research

Effective at the adult plant stage, *Lr34* has been the most important gene in the history of plant breeding for resistance to leaf rust in wheat. Its race non-specificity, durability and pleiotropy have made it a key gene in breeding programs not only for leaf rust but also for other diseases including yellow (McIntosh 1992; Singh 1992b) and stem rusts (Dyck 1987), powdery mildew (Spielmeyer et al. 2005) and barley yellow dwarf virus (Singh 1993a). Because of its adult plant effectiveness and quantitative inheritance, precise detection for efficient incorporation of *Lr34* in adapted germplasm using classical plant breeding was difficult. Use of closely linked molecular markers has been the best alternative to facilitate the incorporation of economically important genes in well-adapted plant germplasm. This is especially true in the case of *Lr34* which was recently cloned (Krattinger et al. 2009). The availability of *Lr34* sequence information provides a valuable tool to develop allele specific markers such as SNPs and indels targeting polymorphic sites. With the help of these types of markers, tracking the introgression of *Lr34* in well adapted germplasm and pyramiding it with other seedling and APR genes would be facilitated. Molecular characterization of germplasm using molecular markers has many implications. Haplotyping a specific gene provides the data needed to study the evolution of the target gene and to determine its functional units. Investigation of the genetic diversity of a crop is also paramount for its genetic improvement.

The present study had the following objectives: (i) the development of *Lr34*-specific or closely linked molecular markers and fine mapping of the region (ii) structural and functional characterization of the *Lr34* gene using locus specific markers; (iii) haplotype diversity and the evolutionary history of the *Lr34* locus and, (iv),

understanding the role of *Lr34* and its interaction with other rust genes in providing resistance to leaf rust under field conditions.

2.0 LITERATURE REVIEW

2.1 The significance of the *Lr34* gene

Lr34 has received credit for being the most important leaf rust resistance gene for managing leaf rust. This gene was reported to have a great effect on yield loss reduction. Singh and Huerta-Espino (1997) observed up to 84% reduction in yield loss in lines with *Lr34* as compared to lines without. The importance of this gene comes from its race-non specificity, durability, synergistic and pleiotropic effects. *Lr34* provides the same level of resistance to all leaf rust isolates (Sambroski 1985). In Canada, *Lr34* has provided durable resistance for more than 50 years with no records of virulence of *P. triticina* (McCallum et al. 2007). Under field conditions, the effect of *Lr34* differs from year to year and is normally observed as a mixture of infection types ranging from resistant to susceptible pustules (Dyck et al. 1994).

The synergistic interactions between *Lr34* and other leaf rust resistance genes have been reported in several studies. Sawhney (1992) reported interaction between *Lr34*, *Lr12* and *Lr27+Lr31*. The last two are seedling genes and were found to be complementary, that is, the function of either gene relies upon the presence of the other gene. Five genotypes with five gene combinations were compared (Sawhney 1992); Thatcher+*Lr12*, Hope with *Lr27*, Shortim with *Lr27+Lr31*, Chinese Spring with *Lr34*, *Lr12* and *Lr31*, and Chinese Spring with *Lr34*, *Lr12*, *Lr27+Lr31*. The results showed that the highest level of field resistance, rated as trace (very resistant), was found in Chinese Spring with gene combination *Lr34*, *Lr12*, *Lr27+Lr31* as compared to moderate resistance with a rating of 20MS in Chinese Spring with *Lr34*, *Lr12* and *Lr31* while the

susceptible phenotypes of Thatcher+*Lr12*, Shortim and Hope had rust readings of 70S, 70S and 50S, respectively. Thus Sawhney (1992) suggested that the high level of resistance was the result of a synergy between *Lr34*, *Lr12* and *Lr27+Lr31*. Resistance to leaf rust was also enhanced when *Lr34* was combined with *Lr12* or *Lr13*, suggesting that these combinations are probably major sources of durable resistance (Roelfs 1988). Also, the gene combination *Lr34* and *Lr37* resulted in a near-immune response to leaf rust that indicated a synergy between these two genes (Kloppers and Pretorius 1997). High levels of resistance to leaf rust were also observed in cultivars containing gene combinations *Lr34+Lr17* and *Lr34+Lr18* (German and Kolmer 1992).

Lr34 was found to have pleiotropic (i.e., one gene affects more than one trait) effects on, or tight linkage with, leaf tip necrosis (*Ltn1*), stripe rust (*Yr18*) and powdery mildew *(Pm38*) adult plant resistance (APR) genes. Segregating population analyses revealed the single gene segregation of *Lr34* and *Ltn1*, hence concluding pleiotropism of *Lr34* and *Ltn1* (Dyck 1991; Singh 1992a). Furthermore, perfect co-segregation was also observed between *Lr34* and *Yr18* (McIntosh 1992; Singh 1992b). All lines carrying *Lr34* exhibited adult plant resistance to stripe rust. Segregation analysis of F_2 and F_3 populations revealed no segregation of *Lr34* lines for susceptibility to stripe and leaf rust which indicated that the same gene was responsible for both phenotypes. Moreover, Spielmeyer et al. (2005) reported that *Lr34* genotypes also showed field resistance to *Blumeria graminis* (DC) EO Speer *f. sp. tritici*, the causal agent of powdery mildew of wheat. They suggested co-segregation of *Lr34* with a powdery mildew APR gene later named *Pm38*. Further, it was reported that *Lr34* also enhanced resistance to stem rust (Dyck 1987) and barley yellow dwarf virus (BYDV) (Singh 1993a). Dyck (1987)

observed that the Thatcher-*Lr34* near isogenic line RL6058 showed enhanced resistance to stem rust as compared to Thatcher. He also reported that chromosome 7D carries suppressor genes that inhibit the expression of stem rust resistance genes in the wheat line Canthatch. From these two pieces of information, he concluded that *Lr34* is probably a non-suppressing allele of the inhibitor gene(s) or is tightly linked to it. Segregating populations resulting from crosses between the susceptible cultivar Jupateco 73S and the tolerant cultivars Jupateco 73R and Condor, were analysed for resistance to leaf rust, stripe rust and BYDV. The results showed that lines that were tolerant to BYDV were also resistant to leaf and stripe rusts leading to the conclusion that resistance to the three diseases is conferred by a pleiotropic single gene or by genes that are tightly linked (Singh 1993a). To date, no other tightly linked genes have been identified for any of these other diseases and evidence for a single pleiotropic gene model remains the most likely explanation.

2.2 Distribution of *Lr34*

Lr34 was initially found in the wheat accession PI58548 (Dyck 1977) and later appeared to be present in a number of hexaploid wheat accessions (Shang et al. 1986; Singh 1993b). Though described as an APR gene (Dyck et al. 1966), *Lr34* can also be detected at the seedling stage under a certain cold temperature regime (Dyck and Samborski 1982). The occurrence of *Lr34* in world-wide wheat germplasm, including cultivars, landraces, hexaploid wheat sub-species, diploid wheat with the D genome and polyploid *Aegilops* species were studied using the molecular marker *csLV34* which is closely linked to *Lr34* was studied (Kolmer et al. 2008). The marker produces two alleles; *csLV34-a* and

csLV34-b associated, although not perfectly, with the absence and presence of *Lr34*, respectively.

Within the North American germplasm, most winter type cultivars analysed, including old and current soft and hard red winter wheat (SRWW& HRWW), possessed the *csLV34-a* allele, i.e., they do not have *Lr34*. On the other hand a high frequency of the *csLV34-b* allele was observed within spring type varieties including hard red spring wheats (HRSW). The presence of the *csLV34-b* allele in HRSW cultivars dates back to the mid 1960s after the release of the Frontana-derived spring cultivar "Chris", the first spring wheat in the USA showing resistance to leaf rust. Varieties with the *csLV34-b* allele remained common until the 1990s while most of the USA cultivars produced from 1994 through 1998 possessed the non-resistance associated allele *csLV34-a*.The *csLV34-b* allele reappeared in cultivars released after 1999 and is commonly found in current germplasm (Kolmer et al. 2008). A high frequency of *Lr34* was observed in CIMMYT germplasm (Singh and Rajaram 1991). The presence or absence of *Lr34* in CIMMYT wheat accessions was examined using the morphological marker *Ltn1*. All cultivars showing the *Ltn1* phenotype possessed the *csLV34-b* allele while cultivars without *Ltn1* had the *csLV34-a* allele (Kolmer et al. 2008). The majority (79/80) of Western European winter wheat cultivars tested by Kolmer et al. (2008) possessed the allele *csLV34-a* suggesting that *Lr34* has not been widely exploited in this wheat class in Europe. In the Australian wheat germplasm, *Lr34* was found at a high frequency in the south-eastern states (about 50%) while it was rare in Western and South Australia (Kolmer et al. 2008).

Within landraces collected from 29 countries, *csLV34-b* was present at a low frequency compared to its frequency in cultivated wheats. The *csLV34-b* allele was

frequent in Chinese genotypes including Chinese Spring which was one of the earliest sources of *Lr34* (Kolmer et al. 2008). Analysis of 50 accessions of *Ae. tauschii* and a set of other *Aegilops* polyploids containing a D genome, revealed the exclusive presence of *csLV34-a*. This data suggested that *csLV34-b* possibly arose only following the advent of hexaploid wheat (Kolmer et al. 2008). Analysis of 231 cultivars and 422 landraces of Chinese wheat at the *csLV34* locus (Wen-Xiong et al. 2008) revealed that in contrast to cultivars which had a very low *csLV34-b* allele frequency of ~6%, the landraces had a very high frequency of ~85%.

2.3. *Lr34*-based resistance mechanisms

Lr34 does not provide resistance in a gene-for-gene manner, i.e., the resistance phenotype is not visualized by clear chlorosis or necrosis (Dyck 1977). The resistance mechanism of *Lr34* is characterized by five components including (i) a longer incubation period, (ii) a longer latent period, (iii) a lower spore density, (iv) small uredinia sizes (Drijepondt and Pretorius 1989) and (v) minimal initial rust development (Singh et al. 2007). All of these components contribute to reduce the amount of inoculum, lowering the disease pressure and subsequently reducing yield losses by delaying the occurrence of epidemics. Previous studies on wheat and barley using histological examination reported that partial resistance was associated with poor haustorium development and minimum growth rates of *P. triticina*. Failure in haustorium development was related to early abortion of fungal germination tubes caused by the formation of papillae or cell wall appositions (Niks 1986; Jacobs 1989; Rubiales and Niks 1995). Partial resistance components conferred by *Lr34* were found to be affected by temperature. A longer latent period, restriction in

pustule sizes and reduction in uredinia density were observed on line RL6058 at temperatures ranging from 13-17°C as compared to 26-30°C (Drijepondt and Pretorius 1989).

2.4 Map-based cloning (MBC) of *Lr34*

The MBC approach is a several step process that starts by locating the gene of interest on a chromosome, usually at an approximate location flanked by relatively distant markers and ends with the isolation and the functional analysis of the isolated gene. Due to the significance of *Lr34*, substantial efforts were devoted to isolate this gene (Krattinger et al. 2009). Map-based or positional cloning strategy relies on the genetic to physical distance, the size of the population, the ability to develop a fine map and to phenotype accurately (Peters et al. 2003). The approach has been widely used to isolate genes of economic importance from different crop plants. In wheat these included, leaf rust resistance genes *Lr34* (Krattinger et al. 2009), *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003), *Lr21* (Huang et al. 2003), and the powdery mildew resistance gene *Pm3b* (Yahiaoui et al. 2004). Altough for the cloning of *Lr34*, the MBC strategy was complemented by the use of mutants (Krattinger et al. 2009).

The large genome size of common wheat, its hexaploid nature and the high content of repetitive sequences pose challenges to the development of molecular markers targeted to specific regions. In spite of these complications, good progress has been made, and all seven chromosome groups have been mapped with a reasonable level of saturation (Somers et al. 2004; Röder et al. 1998). MBC has also capitalized on the availability of arrayed bacterial artificial chromosome (BAC) libraries of the wheat

diploid ancestors in particular with respect to *Ae. tauschii*, the donor of the D genome (Luo et al. 2003) and the hexaploid wheat BAC libraries including high-density filters and PCR screenable BAC pools (Nilmagoda et al. 2003).

Lr34 was first mapped to the short arm of chromosome 7D of the hexaploid wheat genome (Dyck 1987) and was later genetically located in a 3.6 cM interval between microsatellite markers gwm1220 and gwm295 (Spielmeyer et al. 2005). BAC clones from rice and *Ae. tauschii* orthologous to *Lr34* in wheat were further analysed for potential markers and the closely linked molecular marker swm10 was developed (Bossolini et al. 2006). Intensive RFLP analyses utilizing wheat expression sequence tag (EST) derived clones as probes on a set of wheat genetic materials were performed. An EST clone that consistently produced an RFLP pattern that discriminated *Lr34* lines was converted to a codominant sequence tag site (STS) biallelic marker named *csLV34* which was mapped within 0.4 cM from *Lr34* (Lagudah et al. 2006). The genetic and physical distance between *Lr34* and its flanking markers were further narrowed down using interstitial deletion mutants carrying the *Lr34* region and, the SSR marker csLVMS1 was thus developed (Spielmeyer et al. 2008). This marker mapped only 0.13 cM from *Lr34* and was located within 3 kb of a pectate lyase-like gene, the putative orthologous gene isolated from *Ae. tauschii* BAC clones (Spielmeyer et al. 2008). Further work was conducted to saturate the *Lr34* locus exploiting the sequence information from the orthologous region of *Brachypodium* (*B. distachyon*) which is highly homologous to wheat and *Ae. tauschii* (Krattinger et al. 2008). Two flanking molecular markers namely csLVE17 and SWSNP3 were developed defining a 0.15 cM interval for *Lr34*.

Locus-specific PCR and RFLP probes derived from an *Ae. tauschii* BAC contig known to physically cover the flanking interval between csLVE17 and SWSNP3 were used to analyse BAC libraries from Chinese Spring and Glenlea known to possess *Lr34*. Four BAC clones from Chinese Spring covering the flanking regions were identified and entirely sequenced. The sequence information indicated that the flanking region between markers csLVE17 and SWSNP3 comprised a gene rich island containing six genes and two pseudogenes surrounded by repetitive elements (Krattinger et al. 2008; 2009). The six genes were a hexose carrier (HC) and ATP-binding cassette (ABC) transporter, two cytochrome P450 (CYP-1 and CYP-2) and two lectin receptor kinases (LRK-1 and LRK-2).

Using coding sequence based molecular markers, three genes including one of the lectin receptor kinases, the ABC transporter and the hexose carrier were found to co-segregate with *Lr34*. Analysis of the sequence differences in the coding regions of the three co-segregating genes from three parental pairs with and without *Lr34*, revealed sequence variation only within the ABC transporter gene (Krattinger et al. 2009). Sequence analysis of mutant lines selected for loss of function of *Lr34* also displayed genetic polymorphism for lines carrying mutations solely in the ABC transporter gene (Krattinger et al. 2009). The sequence variations within the mutants included putative alternate splice site mutations, amino acid substitutions, frame shifts and premature stop codons. These analyses identified the ABC transporter as the sole *Lr34* candidate.

2.5 Functional analysis of *Lr34*

Wheat leaf rust resistance genes *Lr1, Lr10* and *Lr21* encodes proteins with nucleotide binding sites and leucine-rich repeats (NBS-LRR) while *Lr34* encodes an ATP-binding cassette (ABC) transporter of the pleotropic drug resistance (PDR) subfamily (Krattinger et al. 2009). Full length ABC transporters have a conserved structure including two cytosolic nucleotide binding domains (NBDs) and two hydrophobic transmembrane domains (TMDs). These four domains appear on a single polypeptide in the NBD1-TMD1-NBD2-TMD2 direction forming a translocation pathway for substrate movement through biological membranes (Jasinski et al. 2003). The full size monomeric ABCG transporter subfamily may be derived from a single duplication of an ancestral half size dimeric ABCG transporter comprising one NBD and one TMD (Crouzet et al. 2006). The ABCG transporters are thought to transport a broad range of structurally and functionally different biomolecules (Rea 2007). Fifteen and 23 full size ABCG transporters have been identified in the genomes of *Arabidopsis* and rice respectively (Crouzet et al. 2006) and 60 are predicted in hexaploid wheat (Krattinger et al. 2011).

ABC transporter genes are ubiquitous in plant genomes, however, few have been characterized and the physiological role(s) of most remain(s) non elucidated (Sanchez-Fernandez et al. 2001; Martinoia et al. 2002). Nevertheless, putative functions have been ascribed to ABC transporters including cross resistance to toxic compounds such as herbicides (Dudler and Hertig 1992). No ABC-PDR genes have been characterized in either *Arabidopsis* or rice. NpABC1, isolated from wild tobacco, localizes in the plasma membrane and expresses in the leaf epidermis (Jasinski et al. 2001). Its expression is triggered by an antifungal diterpene called sclareol secreted at the leaf surface of

Nicotiana species. Expression activation of the gene enabled cells to exudate a marked sclareol analog. NpABC1was thus proposed to be involved in the production of secondary metabolites involved in plant defense mechanisms.

The actual mechanism by which *Lr34* confers resistance to leaf rust remains unknown. Krattinger et al. (2009) proposed two possible scenarios. First, resistance is the result of senescence-like processes and second, similarly to *PEN3*, a pleiotropic drug-resistance protein in *Arabidopsis*, *Lr34* is directly involved in resistance through exporting molecules essential (or detrimental) for fungal growth. If the latter is true, *Lr34* and *PEN3* may have similar roles in plant defense through non-host and durable resistance to a plant disease. Gene expression analysis of leaves collected from *Lr34* lines at 3 and 7 days after inoculation revealed a list of overexpressed genes including genes encoding defense and stress-related proteins, secondary metabolite enzymes, transcriptional regulators and cellular-signalling proteins (Bolton et al. 2008). Up-regulated genes playing key roles in carbon flux through the tricarboxylic cycle seemed to be indicative of the energy required for the activation of the *Lr34* pathways. However, upregulation of these metabolic pathways was not maintained at the same level seven days after inoculation which may explain the failure of *Lr34* to completely suppress the pathogen growth while increasing the latent period (Bolton et al. 2008). The possible variation in *Lr34*-mediated resistance was studied using *Lr34* transgenic and non-transformed wheat lines (Risk et al. 2012). Similar levels of resistance were observed in all lines with *Lr34*. Similarly to native *Lr34*, transgene-based *Lr34* showed resistance at the seedling stage following a cold regime, in flag leaves at the adult plant stage and displayed the typical leaf tip necrosis associated with *Lr34*.

2.6. Molecular characterization of *Lr34*

DNA sequence analysis at the *Lr34* locus derived from four genotypes, namely the hexaploid wheats Chinese Spring, Glenlea and Renan, and the diploid species *Ae. tauschii* was performed (Wicker et al. 2009). The first two genotypes have *Lr34* while the latter two do not. High sequence similarity was found between Chinese Spring and Glenlea as compared to Renan and *Ae. tauschii* which had higher homology with one another. No major insertions or deletions were identified between Chinese Spring and Glenlea. Only a single nucleotide substitution and 30 indels were detected indicating a divergence time of approximately 700 years ago. In contrast, two major differences were found between these two genotypes and Renan. The first one was a major insertion/deletion of 9.8 kb affecting parts of an *RLC_Angela* retrotransposon and its flanking region. This sequence was present in Chinese Spring and Glenlea but not in Renan. The second major difference is a 20 bp indel in Renan. Additionally, nine SNPs were detected between Renan and Chinese Spring which translates into an estimated divergence time of 6,339 years ago. A total of six indels between Renan and Chinese Spring and two between Renan and Glenlea were found.

Sequence polymorphism was further investigated within the ABC transporter gene derived from two hexaploid genotypes with and without *Lr34*, namely Chinese Spring and Renan (Krattinger et al. 2009). The ABC transporter has a nucleotide sequence of 11,805 bp comprising 24 exons. Three mutations were observed within the ABC transporter sequence of these two genotypes. Two single nucleotide polymorphisms (SNPs) in intron 4 and exon 12 and a 3-bp indel in exon 11 differentiated the *Lr34* alleles

of Chinese Spring and Renan and formed the basis of the first two *Lr34* haplotypes described (Krattinger et al. 2009). The Chinese Spring ABC transporter protein lacks the phenylalanine residue present in Renan and associated with the 3-bp indel in exon 11 and also harbors a histidine residue caused by the 'C' SNP in exon 12 (as opposed to a tyrosine in Renan as a result of the 'T' SNP in that exon). The two mutations in exon 11 and 12 alter the structure of the first transmembrane domain linking the two nucleotide binding domains, and thus may modify the substrate specificity of the transporter (Krattinger et al. 2009).

Taken together, the three mutations defined two haplotypes. Haplotype 1 (A/N/C) found in Chinese Spring (*Lr34*+) has the 'A' SNP in intron 4, lacks the 3-bp indel in exon 11 (Null) and has the 'C' SNP in exon 12. Haplotype 2 exemplified by Renan (*Lr34*-) can similarly be defined as T/TTC/T. Haplotypes 1 and 2 were also observed in a larger collection of hexaploid wheat thus concluding a single origin of *Lr34* (Krattinger et al. 2009). Characterization of a world wheat collection of 33 cultivars by Lagudah et al. (2009) revealed the presence of a third haplotype: A/TTC/T (Lagudah et al. 2009). This third haplotype displayed the 'A' SNP in intron 4 characteristic of *Lr34*+ haplotype 1 lines but was formed in *Lr34*- lines. Two additional mutations in a landrace of haplotype 3 would have led to the development of the functional allele of *Lr34* (Lagudah et al. 2009). Additionally, a G/T point mutation in the exon 22 of the ABC transporter that results in a premature stop codon was detected in winter wheat cultivar Jagger (Lagudah, et al. 2009). This mutation was associated with leaf rust susceptibility in a segregating biparental population from a cross between Jagger and the cultivar 2174, suggesting that the premature stop codon constituted a loss of function mutation (Cao et al. 2010).

Investigation for the presence of *Lr34* homoeologous and orthologous genes was conducted (Krattinger et al. 2011). Two homoeologous genes to *Lr34*-D were detected on chromosome 7A (*Lr34*-B) and 4A (*Lr34*-A), this latter homoeolog has been functionally disrupted by the insertion of repetitive sequences. Protein sequences *Lr34*-D and *Lr34*-B showed 97% identity. Also, genes orthologous to the *Lr34*- alleles of hexaploid wheat were identified in two grass species including rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*). No orthologous genes were detected in maize (*Zea mays*), *Brachypodium* (*Brachypodium distachyon*) and barley (*Hordeum vulgare*).

FINE-MAPPING OF THE LEAF RUST *LR34* LOCUS IN *TRITICUM AESTIVUM* (L.) AND CHARACTERIZATION OF LARGE GERMPLASM COLLECTIONS SUPPORT THE ABC TRANSPORTER AS ESSENTIAL FOR GENE FUNCTION

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3.0 FINE-MAPPING OF THE LEAF RUST *LR34* LOCUS IN *TRITICUM AESTIVUM* (L.) AND CHARACTERIZATION OF LARGE GERMPLASM COLLECTIONS SUPPORT THE ABC TRANSPORTER AS ESSENTIAL FOR GENE FUNCTION

3.1 Abstract

Leaf rust resistance gene *Lr34* is likely the most important leaf rust gene characterized to date. It has been characterized as an adult plant resistance gene, is known to enhance the resistance of other leaf rust resistance genes and to condition resistance to a number of other diseases. Located on chromosome 7D, this gene was identified to be one of six co-located genes of which, an ABC transporter was shown to be the only valid candidate. Ten new molecular markers were developed spanning the *Lr34* locus, including six novel microsatellite markers (cam), one insertion site based polymorphism marker (caISBP), two single nucleotide polymorphisms (caSNP), and one gene specific marker (caIND). Using these new markers and others that were previously published, a comparative fine-map of the locus was constructed from five segregating populations representing 1,742 lines. Identification of a susceptible line with a recombination in the 4.9 Kb interval between caSNP4 located in the ABC transporter gene and cam8 located just upstream of this gene provided further evidence to support the identity of the ABC transporter as *Lr34* by ruling out four of the adjacent genes. Originally, three mutations forming two haplotypes had been described for the ABC transporter gene. A third combination of the three mutations and an additional rare mutation in exon 22 were subsequently described. We identified an additional novel mutation in exon 10 that would cause a frameshift and is likely non-functional. This mutation was only found in *Lr34*- lines and constituted a novel molecular haplotype. Characterization of a germplasm collection of 700 *Triticum*

aestivum lines permitted us to gain an understanding of the frequency of the ABC haplotypes characterized to date and their distribution in germplasm from around the world. In addition to the four haplotypes previously described, a fifth haplotype was found in two of the 700 lines from the germplasm collection. These lines displayed the deletion in indel 11 characteristic of *Lr34+* lines but are likely susceptible to leaf rust. Mapping and haplotyping data suggest that, of all the markers described herein, marker caIND11 is the best diagnostic marker for marker-assisted-selection of *Lr34* because it is co-dominant, robust and with the exception of 2/700 lines, it is highly diagnostic. Other markers are also described to provide alternatives for laboratories with different technologies.

3.2 Introduction

Leaf rust caused by the fungal pathogen *Puccinia triticina* is an important disease of wheat, a staple food for 50% of the world population. Leaf rust is the most frequent of the rust diseases (Kolmer 2005), causing significant yield loss reaching 5-15% on susceptible wheat varieties (Samborski 1985). Genetic resistance has been the strategy of choice to manage leaf rust (McCallum et al. 2007). To date, leaf rust resistance genes named *Lr1* to *Lr68* have been characterized in bread wheat, durum wheat and several diploid wheat species. Many of these genes are race-specific-resistance genes and they have been overcome by the occurence of new races of the leaf rust pathogen (Samborski 1985; McCallum et al. 2007). However, *Lr34*, an adult plant resistance gene (Dyck et al. 1966), confers similar levels of resistance to all races of leaf rust (Spielmeyer et al. 2005). *Lr34* has provided effective field resistance to leaf rust since the 1970s in Canada

(McCallum et al. 2007). Besides providing resistance to leaf rust, *Lr34* enhances the level of resistance conditioned by other leaf rust resistance genes (German and Kolmer 1992), and is associated with leaf tip necrosis (*Ltn*) (Singh 1992b), later renamed *Ltn1* to distinguish it from other Ltn phenotypes (Rosewarne et al. 2006). *Lr34* enhances resistance to stem rust (*Puccinia graminis*) (Dyck 1992) and barley yellow dwarf virus (Singh 1993a). It co-segregates with *Yr18* conferring resistance to stripe rust (*Puccinia striiformis*) and was shown to have a positive interaction with *Pm38* conferring resistance to powdery mildew (*Blumeria graminis*) (Spielmeyer et al. 2005). *Lr34* is durable and its pleiotropic gene action renders it the most important wheat resistance gene characterized to date.

Using QTL analysis, *Lr34* was first mapped to the short arm of chromosome 7D (Dyck 1987). In the last few years, substantial efforts have been dedicated to map-based cloning of *Lr34* which was facilitated by the development of closely linked polymorphic markers combined with the development of interstitial deletion mutant lines (Spielmeyer et al. 2008; Krattinger et al. 2009). Spielmeyer et al. (2005) first positioned *Lr34* between two microsatellite markers, namely gwm1220 and gwm295. Chromosome walking efforts led to the development of the closely linked microsatellite marker swm10 (Bossolini et al. 2006). An RFLP marker converted to a PCR-based marker (csLV34) was developed almost at the same time (Lagudah et al. 2006). This marker has been used extensively to postulate the presence or absence of *Lr34* because of its ease of use, the simple allele patterns produced and the relatively close linkage to *Lr34*. Marker csLV34 was used to characterize *Lr34* in Australian wheat cultivars (Singh et al. 2007) and in a world-wide collection (Kolmer et al. 2008) but it was not diagnostic in some genetic

backgrounds, including broadly used Canadian wheat germplasm (McCallum et al. 2008; Lagudah et al. 2009). Using interstitial deletion mutants, Spielmeyer et al. (2008) were able to position microsatellite marker csLVMS1 only 0.13 cM from *Lr34* and assessed the position of gwm1220 to be flanking with an estimated distance of 0.4 cM. More recently, Krattinger et al. (2009) reported the development of markers csLVE17 and csLVA1/SWSNP3 positioning *Lr34* in a 0.15 cM interval that contained six predicted genes, namely a hexose carrier (HC), an ABC transporter (ABC), two cytochrome P-450s (CYP-1, CYP-2), and two lectin receptor kinases (LRK-1, LRK-2), as well as two pseudogenes.

Krattinger et al. (2009) identified the ABC transporter gene as the sole candidate for *Lr34*. While they did not demonstrate the gene function by complementation, analysis of mutants exclusively pointed to the ABC transporter gene as *Lr34*. The 11,805-bp nucleotide genomic sequence of this ABC transporter comprised 24 exons and was predicted to encode a protein of 1,401 amino acids. Krattinger et al. (2009) discovered three mutations within the ABC transporter sequence. Two single nucleotide polymorphisms (SNPs) in intron 4 and exon 12 and one 3-bp indel in exon 11 were observed between the resistant cultivar Chinese Spring (*Lr34*+) and susceptible cultivar Renan (*Lr34*-). Based on their assessment of 27 wheat accessions of diverse origins, the three polymorphisms were reported to constitute only two haplotypes, namely A/Del/C and T/TTC/T for *Lr34*+ and *Lr34*- lines, respectively. Based on this observation, they hypothesized that *Lr34* had a single origin. When a more diverse germplasm collection was assayed, Lagudah et al. (2009) identified a third *Lr34* haplotype, i.e., A/TTC/T for which no lines were found to carry the reciprocal allele. Hence, they concluded that

mutation rather than recombination gave rise to this haplotype. Lagudah et al. (2009) also identified a fourth mutation in the ABC transporter gene, i.e., a G/T SNP in exon 22 in cultivar 'Jagger'. This mutation is predicted to result in a premature stop codon, be non-functional, and likely explain why Jagger has the *Lr34+* haplotype A/Del/C but does not exhibit *Lr34* field resistance. The frequency of this mutation is still unknown.

Here, we report on the development of several novel markers spanning the *Lr34* locus including dominant and co-dominant markers characterizing the various alleles of the ABC transporter. These markers were assessed on two large germplasm collections consisting of 700 *Triticum aestivum* lines, and two novel haplotypes are reported. The novel markers were used to screen more than 1,700 recombinants from five populations that were extensively characterized for leaf rust resistance, thereby permitting fine-mapping of *Lr34* and providing further evidence to support the identity of the ABC transporter gene as *Lr34*. A novel mutation in exon 10 of the ABC transporter is also described.

3.3 Materials and methods

3.3.1 Plant materials

Two different collections of germplasm were used as sources for our genetic materials. A world collection (WC), representing germplasm from various geographical areas of the world, comprised 337 hexaploid wheat (*T. aestivum*) accessions. The second collection (AM) included mostly North American germplasm (cultivars and breeding lines) and some historical germplasm totalling 363 accessions. To assess the 7D specificity of markers, Chinese Spring (CS) and its 21 nulli-tetrasomic lines (CSNT) were used. A

subset of 10 parental lines was used simultaneously to assess potential marker polymorphism. Additionally, 5 segregating populations derived from three crosses and totalling 1,742 segregating lines were used to fine map the *Lr34* locus (Table 3.1). All populations were F_1-derived (Radovanovic and Cloutier 2003; McCartney et al. 2005, 2006; Huang et al. 2006). Populations were grown in inoculated field leaf rust nurseries at Glenlea, Manitoba and Portage La Prairie, Manitoba, during the years 2005-2008. A mixture of *P. triticina* virulence phenotypes, representative of those found in Western Canada, was used to inoculate rows of susceptible wheat lines, which were interspersed among the test lines and served as an inoculum source. Flag leaves on each progeny line were scored at maturity for leaf rust severity using a modified Cobb scale (Peterson et al. 1948). Each population was phenotyped from two to four times in randomized trials with two replications per year and location to ensure accurate and reliable rust phenotyping.

Table 3.1 Description of the five populations used to fine map the *Lr34* locus

Cross	**Name**	**Type**	**No. lines**	***Lr34-***	***Lr34+***
AC Karma×87E03-S2B1[1]	98C18-DH	DH	372	AC Karma	87E03-S2B1[2]
AC Karma×87E03-S2B1[1]	98C18-SSD	RIL	372	AC Karma	87E03-S2B1[2]
AC Domain×RL4452[3]	93E54[4]	DH	271	AC Domain	RL4452[4]
Thatcher×RL6058-886C	03B10A	DH	217	Thatcher-885J	RL6058-886C
Thatcher×RL6058-886A	03B10B	DH	510	Thatcher-885B	RL6058-886A

[1] Radovanovic and Cloutier 2003; Huang et al. 2006
[2] 87E03-S2B1 = Glenlea*7/C7932 = White Glenlea
[3] RL4452 = Glenlea*6/Kitt = Short Glenlea
[4] McCartney et al. 2005, 2006

3.3.2 Genomic DNA extraction

Plants were grown in a greenhouse to the 3-4 leaf stage. A total of 100 mg of leaf tissue was collected from each line, immediately frozen in liquid nitrogen and then lyophilized in a freeze dryer for approximately 48 h. The freeze dried tissues were ground to a fine powder using a tissuelyser (Qiagen, Newtown City, USA). DNA extraction was performed using the DNeasy® 96 Plant kit as per manufacturer's instruction (Qiagen). The genomic DNA samples were quantified by fluorometry and diluted to 15 ng/µl.

3.3.3 Molecular marker development

The hexaploid wheat cultivar 'Glenlea' BAC library (Nilmalgoda et al. 2003) was screened with microsatellite marker swm10 (Bossolini et al. 2006) and 5 BAC clones were identified, fingerprinted and BAC-end sequenced. Chromosome walking steps were repeated with BAC end markers and a contig of several overlapping clones was obtained from which 4 BAC clones spanning 275,208 nucleotides were sequenced (Appendix I). Putative microsatellites were predicted as previously described (Cloutier et al. 2009) using the default settings of the MISA software (Thiel et al. 2003).

Genomic sequences from the *Lr34* locus of Chinese Spring (FJ436983), Glenlea (FJ436984), Renan (FJ436985), and *Aegilops tauschii* (FJ436986) (Wicker et al. 2009, Krattinger et al. 2009) were used to develop additional microsatellite, single nucleotide polymorphism (SNP), insertion site based polymorphism (ISBP) and gene-specific markers. For microsatellite development, the ~250 Kb sequence defined by these four BAC clones were individually analysed as described above. The genomic sequence information was further mined for ISBP by DNA sequence alignments. Dominant

markers were developed to assess the SNP located in intron 4 (caSNP4) and exon 12 (caSNP12) of the ABC transporter sequence. A co-dominant marker was developed to flank the 3-nucleotide indel of exon 11 (caIND11).

3.3.4 Marker specificity and polymorphism assessment

The designed primers were assessed on CSNT lines and a small collection of 10 parental wheat lines to verify 7D specificity and potential polymorphism. Markers polymorphic between parents were mapped onto 1 to 5 segregating populations (Table 3.1). Parents 87E03-S2B1 and RL4452 are known as white and short Glenlea, respectively. They were derived by backcrossing using Glenlea as the recurrent parent and both carry the Glenlea *Lr34* locus.

All markers, primer sequences, PCR conditions, resolution methods and observed allele sizes are reported in Table 3.2. For microsatellite markers resolved on the ABI3130xl (Applied Biosystems, Foster City, CA), three separate reactions were resolved simultaneously. They were amplified independently with one of three M13 labelled fluorochromes (FAM, HEX, or NED) as described in Schuelke (2000). Aliquots of 2 μL from each reaction were pooled and diluted with 24 μL of water. A 2 μL aliquot of the combined diluted products was subsequently transferred to an ABI compatible plate. A total of 4 μL of Hi-Di formamide/GenScan Rox500 marker in a 3.9:0.1 ratio was added prior to denaturation and resolution on an ABI3130xl (Applied Biosystems). The ABI files were subsequently converted into a gel-like image using a modified version of Genographer (Benham et al. 1999, modified by Travis Banks and available at http://sourceforge.net/projects/genographer/) and sized using the peak size information.

Table 3.2 List of primers used as markers and for characterizing the *Lr34* locus

Marker name	Primer sequence[1]	PCR conditions	Resolution method	observed allele sizes[2]
cam1	F: TGGCATGAGAAGAAAGCGTA		ABI3130x1	338-361 (11 alleles)
	R: CCGCTACCGCAGTAATGTCT			
cam2	F: GACAACAAACAGGACCTGGCACT		ABI3130x1	179/N
	R: CACCCAAAGAGAAGGAACCA			
cam8	F: GCTGGATCTCAACCTCCTGA	Initial denaturation: 93˚C, 2 min	ABI3130x1	207/N
	R: CAGGTCACAGATGTGGATGG	Denaturation: 94˚C, 1 min		
cam11	F: CCAGGGTGCATCCCAAGTA	Annealing: 58˚C, 50 sec	ABI3130x1	298-303/N (7 alleles)
	R: ACCGAAAGTGTTTTGGAGTG	Extention: 72˚C, 1 min		
cam16	F: AACAATGAATACCCTAGCAGAGC	Cycles: 30	ABI3130x1	308-314 (7 alleles)
	R: CGAACGCGTGGTTGTCAT	Final extension: 72˚C, 10 min		
cam23	F: CGGCCCTGAAAATCGTACT	End: 15˚C constant	ABI3130x1	373/383/385/389
	R: CATGTATGGTGCGTGCTTGT			
caIND11	F: GTCTCCCAATCTGCATGCTC		ABI3130x1	394/397
	R: TACCTCCCAAAAGCCAGTTG			
caSNP4	F: GCGTTTCTGTCACCAGAAGT	Initial denaturation: 94˚C, 5 min	1.5% agarose gel	390/N
	R: AATAAACTCGCGCCTCTTGA	Denaturation: 94˚C, 1 min		

Marker name	Primer sequence[1]	PCR conditions	Resolution method	observed allele sizes[2]
caSNP12	F: TCCCCAGTTTAACCATCCTG	Annealing: 65˚C, 30 sec	1.5% agarose gel	234/N
	R: CATTCAGTCACCTCGCAGC	Extention: 72˚C, 1 min		
		Cycles: 35		
		Final extension: 72˚C, 10 min		
		End: 15˚C constant		
caISBP1	F: CATATCGAGCTTGCCAAACG		1.5% agarose gel	391/509
	F: TCAGCCACACAATGTTCCAT	as SNP4 and 12 but annealing at 60˚C		
	R: CGTGAGCACAGAGAAAACCA			
SEQ-SNP4	F: TAGCCAAAGAGCCAAACTTA[3,4]		ABI3130x1 - seq	604
	R: TGATCGCCTAGACGCCTACT[3,4]			
SEQ-IND10/	F: CATATGCCCAGAAAGAAAAG[3,4]	as ISBP1	ABI3130x1 - seq	1159
	R: AAATCCCCAGTTTAACCATCCT[3,4]			
IND11/SNP12	F: CATATGCAATACCAGCTTCA[4]			
	R: GCTGCATCAAGGAGTAAATC[4]			

[1] Forward primer of markers resolved on the ABI3130xl were tailed at the 5' end with sequence CACGACGTTGTAAAACGAC

[2] Size as estimated by Genographer and including the tail sequence

[3] Primers used for amplificaton of a portion of the 7D ABC transporter gene identified as *Lr34*

[4] Primers used to sequence the PCR amplicons flanking SNP4 or IND10/IND11/SNP12

3.3.5 Fine mapping of *Lr34* locus markers

Novel microsatellite markers (cam1, cam2, cam8, cam11, cam16, cam23), ISBP marker (caISBP1), SNP markers (caSNP4, caSNP12), and co-dominant gene-specific marker (caIND11) as well as other previously described markers located near *Lr34* (gwm1220, Spielmeyer et al. 2005; swm10, Bossolini et al. 2006; csLV34, Lagudah et al. 2006; csLVMS1, Spielmeyer et al. 2008) were assessed on one to five of the segregating populations characterized for *Lr34* depending on polymorphism (Tables 3.1 and 3.2). The genetic maps were constructed with MAPMAKER/Exp version 3.0b (Lander et al. 1987). The Kosambi mapping function (Kosambi 1944) was used to convert recombination fractions into centiMorgans (cM) as map distances.

3.3.6 Germplasm characterization of the *Lr34* locus

ABC transporter specific markers caSNP4, caSNP12 and caIND11 were assessed on 700 wheat lines using genomic DNA from the two germplasm collections (WC and AM) following PCR and resolution methods outlined in Table 3.2.

3.3.7 Sequencing of the ABC transporter

Gene-specific primers (Table 3.2) were designed to flank the three mutations (SNP4, SNP12 and IND11) previously identified in the ABC transporter gene (Krattinger et al. 2009). One primer pair amplified a 604 bp fragment flanking the SNP in intron 4 and a second primer pair amplified an 1159 bp fragment flanking both SNP12 and IND11 mutations (Table 3.2). Single band amplification and chromosome 7D specificity of the

primers were determined using the CSNT lines as described above. Genomic DNA from a subset of 172 lines from the two germplasm collections were PCR amplified in triplicate (3 × 10 μL) for each of the two primer pairs. The PCR amplicons from the triplicate reactions were combined and purified using MultiScreen-384 plates (Millipore, Billerica, MA) and resuspended in 30 μL of water. A 2 μL aliquot was used to sequence the purified amplicon using BigDye V3.0 in 6 μL reactions as previously described (Huang and Cloutier 2008). Primers used for amplification were also used for sequencing. For the 1159 bp fragment flanking SNP12 and IND11, two additional internal primers (Table 3.2) were used thereby generating 4 overlapping sequences ensuring that at least one sequence from both strands was generated for each of the mutation points. Sequencing reactions were resolved on an ABI3130xl. The sequences were processed using PHRED (Ewing et al. 1998) and assembled using CAP3 (Huang and Madan 1999) as implemented in SOOMOS (T. Banks, unpublished). The assembly was visualized in Jalview (Waterhouse et al. 2009) and SNPs and indels were recorded.

3.4 Results

3.4.1 *Lr34* locus molecular marker development and physical location

A total of 21 and 24 putative microsatellite markers were identified by MISA from the ~275 Kb and ~250 Kb sequences contiguous to the swm10 marker and the ABC transporter gene, respectively. A total of 31 primer pairs were designed from these 45 putative microsatellites and tested on the CSNT lines for 7D specificity. Six of the 31 primer pairs were 7D specific and polymorphic. Microsatellite markers cam1 and cam2 were developed from the swm10 contiguous sequence (Appendix I) and cam8, cam11,

cam16, and cam23 were derived from the Chinese Spring, Renan, Glenlea, and *A. tauschii* sequences containing the ABC transporter gene. Primer sequences, amplification conditions, resolution methods and observed allele sizes for all novel microsatellite and other markers used in this study are listed in Table 3.2. Figure 3.1A illustrates the 7D specificity and polymorphism of one of the polymorphic microsatellites, namely cam23. Primers were designed for 11 potential ISBPs and were similarly tested. Marker caISBP1 was developed as a co-dominant marker using a 3 primer combination PCR (Fig. 3.1B).

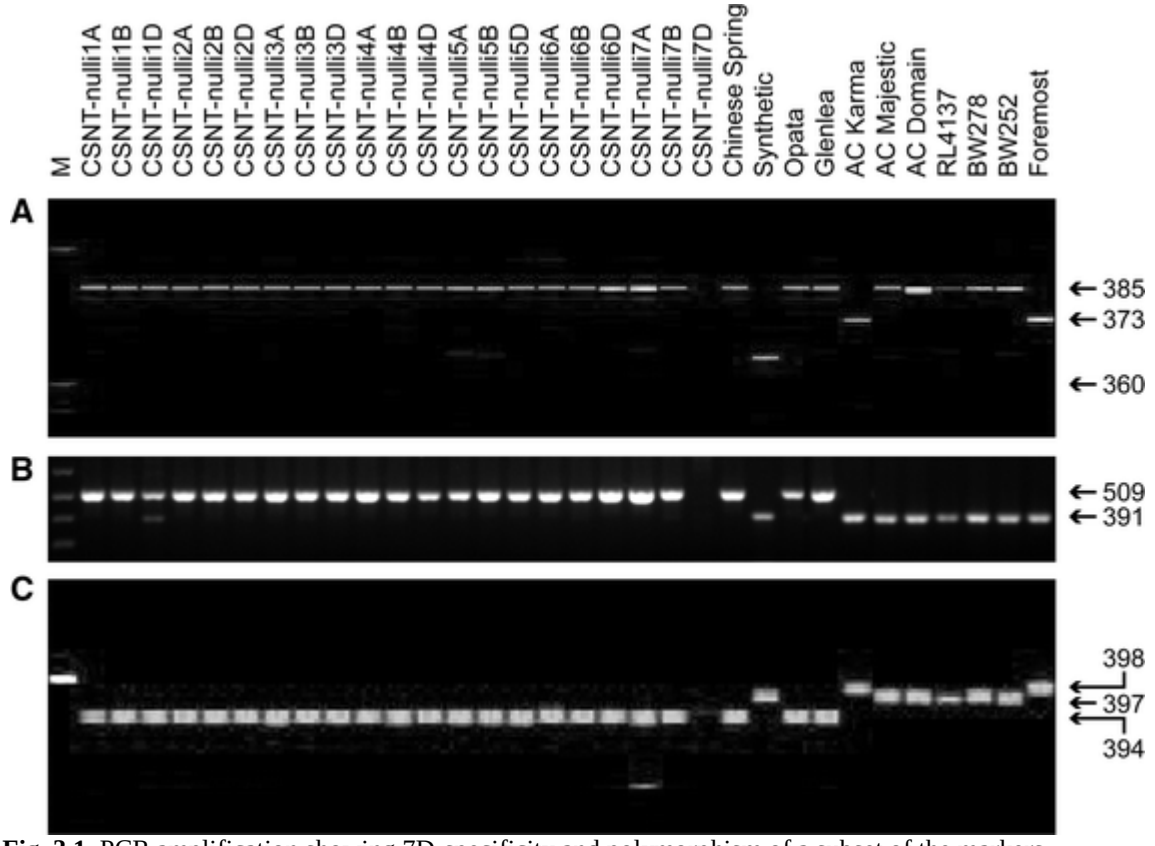

Fig. 3.1 PCR amplification showing 7D-specificity and polymorphism of a subset of the markers developed herein. A. cam23; B. caISBP1; and C. caIND11. Chinese Spring nulli-tetrasomic lines and a subset of 10 parental lines were used and are labelled at the top. M is for marker GenScan Rox-500 (Applied Biosystems) or 1KB plus DNA ladder (Invitrogen). Fragment sizes are indicated on the right.

Microsatellite markers cam8, cam11, cam16, cam23, and marker caISBP1 further saturate the *Lr34* locus as previously defined to be located between markers csLVA1/SWSNP3 and csLVE17 (Fig. 3.2). Marker cam8 is located just upstream of the ABC transporter gene. In fact, marker cam8, cam11, and caISBP1 are all positioned between the ABC transporter and the first cytochrome P-450 (CYP-1). Marker cam16 is located between CYP-1 and LRK-1 while cam23 was distal to CYP-2 (Fig. 3.2). These five novel markers will be useful in dissecting the role of the six genes found at the *Lr34* locus thus defined by the 0.15cM csLVA1/SWSNP3-csLVE17 interval (Krattinger et al. 2009).

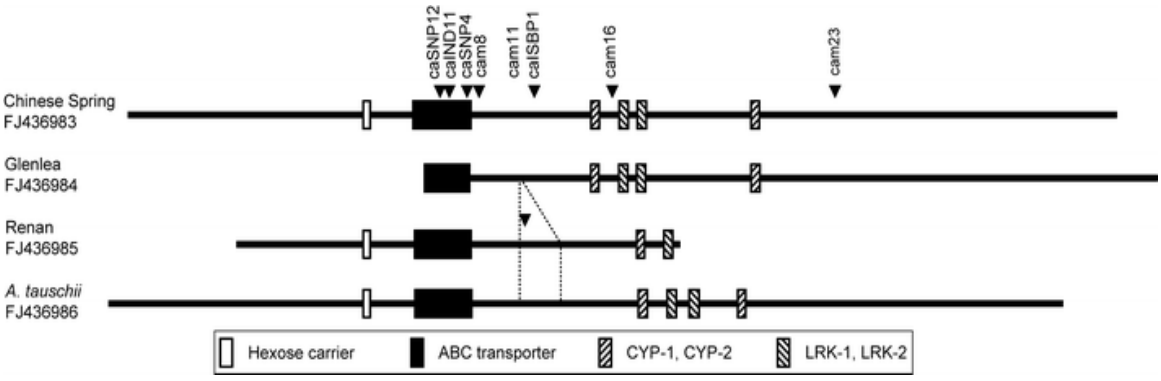

Fig. 3.2 Physical map of the *Lr34* locus BAC clones previously sequenced showing the positions of the novel markers in reference to the hexose carrier, the ABC transporter, the two cytochrome P-450s, and the two lectin receptor kinases. Filled triangles indicate marker position

3.4.2 ABC transporter gene specific SNP and indel-based markers

Two dominant markers specific for *Lr34* were developed to target the SNPs located in intron 4 (caSNP4) and in exon 12 (caSNP12) of the ABC transporter gene using primers with a selective 3' end nucleotide and stringent PCR conditions (Table 3.2). Also an *Lr34*-specific co-dominant marker was developed to flank the 3 nucleotide indel of exon 11 (Fig. 3.1C). The 394 bp fragment represented lines with a TTC deletion (coding for a

phenylalanine residue) in exon 11, characteristic of *Lr34+* lines. The most common alternate allele (*Lr34*- associated) was 397 bp in size. However, some lines, e.g., AC Karma and Foremost (Fig. 3.1C, lane 26 and 32) produced a 398 bp amplicon, indicating the presence of an additional nucleotide in these amplicons (see below). Physical location of these three markers within the ABC transporter sequence is illustrated (Fig. 3.2).

3.4.3 Sequencing the ABC transporter polymorphisms

To confirm the accuracy of the caSNP4 dominant marker, a subset of 172 lines from the two germplasm collections were amplified with the 7D specific SEQ-SNP4 primer pair and the 604 bp fragments were sequenced. Sequencing confirmed the A/T SNP4 results obtained with the dominant caSNP4 marker. Similarly, the same subset of lines was amplified with primer pair SEQ-IND10/IND11/SNP12 and the 1159 bp amplicons were sequenced in both orientations with a total of 4 primers (Table 3.2). Sequencing confirmed the C/T SNP12 results obtained with the dominant caSNP12 marker and the three nucleotide TTC indel of exon 11 obtained with the co-dominant marker caIND11. A new mutation in exon10 was also revealed. Lines that amplified the 398 bp amplicon with the caIND11 primer pair had an additional A in exon 10. This frameshift mutation would cause amino acid changes in the last 26 residues of this exon. This A/- mutation was termed IND10. Cultivar Invader was one of the 71 accessions with the A indel in exon 10. This haplotype could be traced back in its pedigree to accessions Sinton, CT-262, and Lee (Appendix II) confirming that it is stably inherited.

3.4.4 Fine mapping of the *Lr34* locus

All novel markers developed herein (cam1, cam2, cam8, cam11, cam16, cam23, caISBP1, caSNP4, caSNP12, caIND11) and markers previously described (gwm1220, csLVMS1, swm10, and csLV34) were mapped in one to five segregating populations phenotypically characterized for *Lr34* (Table 3.1 and Fig. 3.3). The gwm1220/csLV34 interval spanned 1.8 to 6 cM and included 5 to 12 of the 14 markers depending on the population.

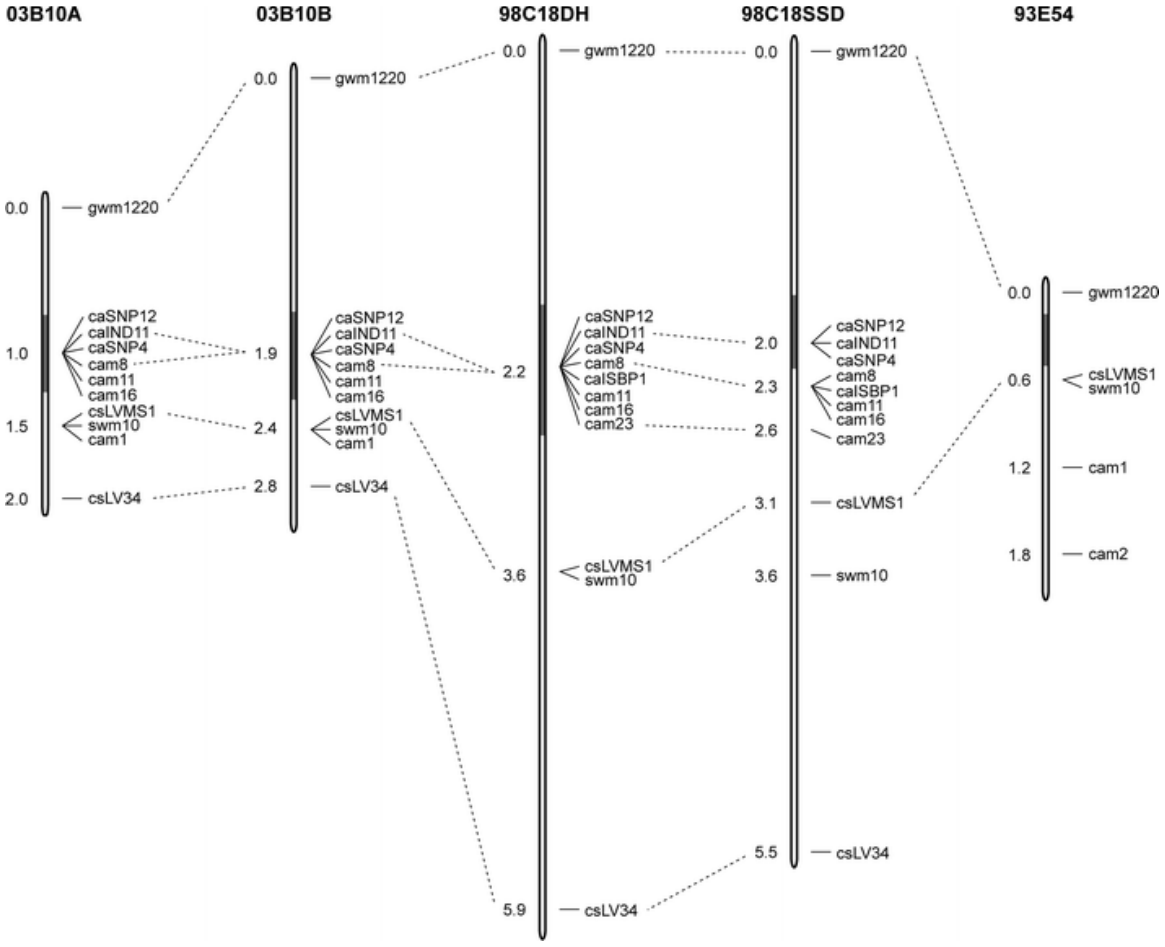

Fig. 3.3 Comparative fine mapping of the *Lr34* locus using five segregating populations shows co-segregation of *Lr34* with all three ABC transporter markers (caSNP4, caIND11, and caSNP12). A 0.3 cM interval between the ABC transporter markers and cam8 in population 98C18SSD is illustrated. Filled sections illustrate the location of *Lr34* in each population.

Twenty-three of 1,742 segregating lines had a recombination between marker gwm1220 and csLVMS1 (Fig. 3.4). Line 98C18SSD-K16 displayed an *Lr34+* phenotype and the ABC transporter *Lr34+* genotype, namely caSNP4 A, caIND11 394 (Del), and caSNP12 C, but had the *Lr34-* genotype for cam8 (null), caISBP1 (391), cam11 (299), cam16 (313) and cam23 (385). The recombination in this line physically mapped to the 4.9 Kb interval between cam8 and caSNP4, thereby providing evidence to eliminate the two cytochrome P450s and the two kinases as being essential for the functioning of *Lr34*.

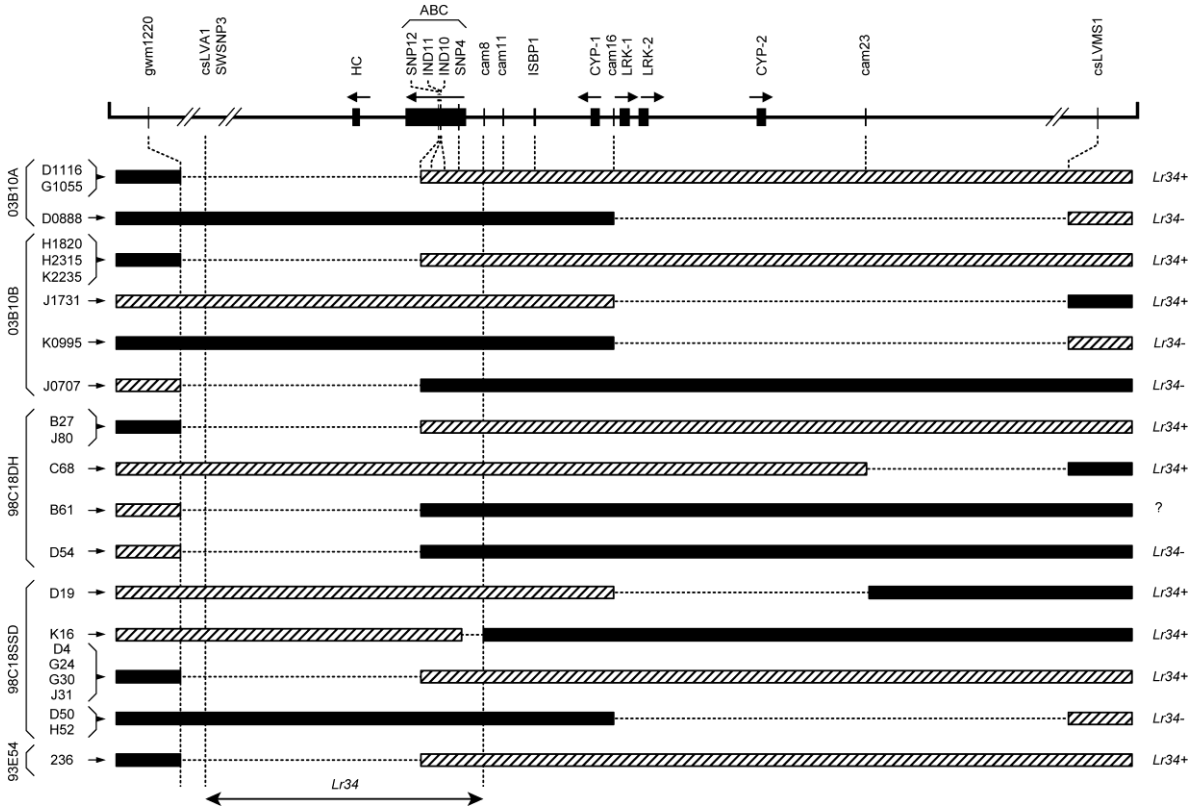

Fig. 3.4 Schematic representation of the 23 gwm1220-csLVMS1 recombinant lines from the five segregating populations (Table 3.1) showing the narrower *Lr34* locus defined to be between csLVA1/SWSNP3 and cam8. Solid bars represent *Lr34-* alleles and hatched bars *Lr34+*. Arrows indicated gene orientation.

3.4.5 Germplasm characterization

We characterized 700 accessions of hexaploid wheat from the WC and AM collections for the ABC-specific markers caSNP4, caSNP12 and caIND11. A subset of 172 lines including all the lines with a putative novel haplotype and all the lines that produced a 398 bp IND11 amplicon were sequenced over SNP4 and IND10/IND11/SNP12. Incomplete data, i.e., missing marker data for one or more markers was obtained for 47 of the 700 lines. The remaining 653 lines were characterized for all four polymorphisms. They partitioned into five different haplotypes (Table 3.3). Haplotypes 1 and 2 represent the two haplotypes originally described by Krattinger et al. (2009). Haplotype 1 is the only haplotype resulting in a *Lr34+* phenotype and was found in 29.5% and 21.9% of the AM and WC collections, respectively. Haplotype 2 (T/N/TTC/T) is associated with the *Lr34-* phenotype and is the most common haplotype representing 55.4% of all accessions from the two collections combined. Haplotype 3, recently described by Lagudah et al. (2009), was present in 7.4% of all accessions. Haplotypes 4 and 5 were novel. Haplotype 4 possessed the additional A in exon 10 and was found only in *Lr34-* lines with the T/TTC/T haplotype for SNP4/IND11/SNP12. Haplotype 5 was rare as it was found in only two lines. Varieties ‘Odess kaja 13’ and ‘Koktunkulskaja 332’ were obtained from the Plant Gene Resource Centre of Canada and had been donated by the Russian Federation. They originated from Ukraine and Kazakhstan respectively, and were considered susceptible to leaf rust (http://pgrc3.agr.gc.ca/search_grinca-recherche_rirgc_e.html).

Table 3.3 Haplotypes of the ABC transporter for SNP4, IND10, IND11, and SNP12 determined in two hexaploid wheat collections

Haplotype	SNP4	IND10	IND11	SNP12	No of lines in the AM collection	No of lines in the WC collection	Total	Phenotype Association
1	A	N	N	C	104	66	170	*Lr34+*
2	T	N	TTC	T	206	156	362	*Lr34-*
3	A	N	TTC	T	11	37	48	*Lr34-*
4	T	A	TTC	T	31	40	71	*Lr34-*
5	T	N	N	T	0	2	2	*Lr34-*
Missing data	-	-	-	-	11	36	47	
Total					363	337	700	

3.5 Discussion

The *Lr34* gene has been an important source of resistance to leaf rust in wheat breeding programs due to its durability, its enhancement of other resistance genes, and the ability to confer resistance to some other major pathogens of wheat (i.e., yellow and stem rusts and powdery mildew). Developing diagnostic markers to determine the presence or absence of *Lr34* in wheat germplasm is an important objective for wheat breeders and geneticists worldwide because phenotypic characterization of this adult plant resistance gene can be challenging due to environmental conditions or genetic background. A good marker is defined as robust, user-friendly, co-dominant and closely linked. Ideally, it would be derived from the gene itself and be based on completely linked polymorphism(s). In some instances, genetic differences between resistant and susceptible alleles are small, rendering this task challenging (Krattinger et al. 2009). Here, we reported on the development of several new markers at the *Lr34* locus, provided recombination-based evidence for the identity of the ABC transporter as *Lr34*, identified new ABC transporter haplotypes and described a new mutation in this gene. We also described the development of marker caIND11, a co-dominant, robust, user-friendly marker that promises to be highly useful in breeding programs and for germplasm characterization. Alternative markers suitable for different technology platforms are also described.

3.5.1 Fine mapping of the *Lr34* locus

Cloning of *Lr34* has been a major goal of wheat geneticists, breeders and pathologists alike. Sequence analysis of the *Lr34* locus resulted in the identification of a gene-rich

region comprising six genes: HC, ABC transporter, CYP-1, CYP-2, LRK-1, and LRK-2 (Krattinger et al. 2009), of which the ABC transporter was shown to be the only possible candidate responsible for *Lr34* function. Postulation of the *Lr34* gene was based on the identification of a number of mutant lines, which had lost *Lr34* resistance and had mutations in the ABC transporter gene but not in any of the five adjacent genes of the csLVA1/SWSNP3-csLVE17 interval. Here, we developed seven novel markers (cam1, cam2, cam8, cam11, cam16, cam23 and ISBP1), of which the last five are interstitial to the XcsLVA1/SWSNP3-XcsLVE17 interval (Fig. 3.2). In addition, three markers (caSNP4, caIND11, and caSNP12) were developed to characterize the three previously identified ABC transporter polymorphisms. These markers were mapped on 1,742 recombinant lines from five populations thereby generating a fine-map of the locus showing the co-segregation of the new markers. A recombination event in line 98C18-SSD-K16 was identified in the 4.9 Kb interval between caSNP4, a marker developed in intron 4 of the ABC transporter gene and cam8, a microsatellite marker located just upstream of the ABC transporter (Fig. 3.4). This recombination, located between CYP-1 and ABC, ruled out the possibility that CYP-1, CYP-2, LRK-1 and LRK-2 were needed for *Lr34* function and thereby provided recombination based evidence to further support the *Lr34* functional identity of the ABC transporter. The hexose carrier of Renan, an *Lr34*- line, has a 6-bp deletion when compared to that of Chinese Spring (*Lr34*+). This polymorphism can be detected by marker SWDEL1 (Krattinger et al. 2009). Parental lines of the five populations used herein all had the HC of Chinese Spring and were monomorphic for SWDEL1 (data not shown). Therefore, no additional polymorphic markers for the HC-ABC transporter interval were identified between the parents of our

five segregating populations and recombination based evidence to rule out the hexose carrier could not be provided using these populations.

3.5.2 Marker development

Genomic DNA sequences can be mined for different types of markers. Microsatellites are ubiquitous in plant genomes and are found in coding and non-coding regions (Moxon and Wills 1999; Morgante et al. 2002). Putative microsatellites can be readily identified using software such as MISA (Thiel et al. 2003) and SSRIT (Temnykh et al. 2001). Although only a fraction of putative microsatellites are successfully developed into polymorphic markers, their sheer number makes them highly suitable for fine mapping. Indeed, we successfully developed four new polymorphic microsatellite markers (cam8, cam11, cam16, and cam23) in a short region of only 75 Kb (Fig. 3.2) of the *Lr34* locus of wheat located on the D genome, a genome known for its low polymorphism level (Banks et al. 2009; Chao et al. 2009).

Repetitive elements represent an estimated 90% of the wheat genome, of which transposable elements (TE) are the most important component (Li et al. 2004). The insertion points of these elements constitute unique signatures that can be capitalized upon for the development of markers referred to as Insertion Site Based Polymorphisms. While the majority of the ISBP markers are dominant, i.e., characterizing the presence or absence of a TE border, caISBP1 was designed to be co-dominant by using a second forward primer unique to the interstitial region between the *copia* and the CACTA elements in Renan. This robust and easy to use PCR marker is located only 13 Kb upstream of the ABC transporter. Easily resolved on an agarose gel due to the large

allele size difference, this co-dominant marker is well suited for laboratories that do not possess acrylamide or polymer based instruments.

Previous studies reported on several molecular markers closely linked to the *Lr34* locus including gwm1220, csLV34, csLVMS1, swm10, SWSNP3, csLVA1, and csLVE17 (Spielmeyer et al. 2005; Bossolini et al. 2006; Lagudah et al. 2006; Spielmeyer et al. 2008; Krattinger et al. 2009). These markers were used to map *Lr34* to its most restricted interval bordered by markers csLVA1/SWSNP3 and csLVE17 (Krattinger et al. 2009). Additional markers were developed from gene sequences. SWDEL3 was developed from the sequence of one of the LRK genes, SWDEL2, and csLVD2 originated from the ABC transporter and SWDEL1 from the hexose carrier. These last four markers all co-segregated with *Lr34* and did not enhance the fine mapping of the gene (Krattinger et al. 2009). The microsatellite and ISBP markers described herein provide alternate markers that can be readily used in germplasm characterization. Combined with segregating populations, they rendered it possible to further define the *Lr34* interval (Fig. 3.4).

3.5.3 ABC transporter specific markers

The ABC transporter gene proposed as the *Lr34* gene is predicted to have 1,401 amino acids in 24 exons spanning 11,805 bp (Krattinger et al. 2009). Sequencing of the gene from a few genotypes revealed three polymorphisms, namely an A/T SNP in intron 4, a 3 bp TTC indel in exon 11, and a C/T SNP in exon 12. Like microsatellites, SNPs are widely distributed and provide a rich source of markers (Phillips 2007). To evaluate the polymorphism at these three positions, we developed two co-dominant SNP markers for

SNP4 and SNP12 respectively and a co-dominant marker for the 3-bp indel in exon 11 (caIND11). This last marker generated fragments of 394 bp in *Lr34*+ lines and either 397 or 398 bp in *Lr34*- lines (Fig. 3.1C). Lagudah et al. (2009) reported markers cssfr1 to cssfr5 based on the indel polymorphism in exon 11 and cssfr6 marker based on the SNP in exon 12. They did not report on markers for the SNP in intron 4. The markers reported herein are therefore complementary or additional to these recently reported markers. Sequencing of two 7D specific amplicons spanning SNP4 and IND11/SNP12 confirmed the robustness of the SNP markers and revealed the presence of an A/- indel in exon 10 of lines that generated the 398 bp fragment with the caIND11 marker. The A indel in exon 10 would cause a frameshift and lines with the additional nucleotide may not produce a functional ABC transporter. Similar findings were recently reported for a G/T mutation in exon 22 of cultivar Jagger (Lagudah et al. 2009). This latter mutation was predicted to result in an early stop codon and be non-functional. The A indel in exon 10 is a widespread mutation and was found in 71 of the 700 accessions of the two collections (see below) while Jagger's exon 22 SNP mutation is rare. We tested the reported marker on both AM and WC collections and none of the 700 lines displayed this mutation (data not shown).

The *Lr34* phenotype may result from non-functional transcripts through various mechanisms such as frameshift mutation (e.g., indel in exon 10) and early stop codon mutation (e.g., SNP in exon 22). Alternate splicing, a well documented mechanism in mammalian ABC transporter genes (Stojic et al. 2007; Piehler et al. 2008) may also be involved in *Lr34* function in plants.

3.5.4 Germplasm characterization

Krattinger et al. (2009) hypothesized that *Lr34* had a single origin because they found only two haplotypes for the three mutations identified in the ABC transporter in 27 wheat lines of various origins. *Lr34+* lines had the A/Del/C haplotype for caSNP4, caIND11, caSNP12, and *Lr34-* lines displayed the T/TTC/T haplotype for the same mutation positions. Indeed, in the 700 accessions surveyed, these two haplotypes were the most common but they were not the only haplotypes. Recently, Lagudah et al. (2009) reported on a third haplotype (A/TTC/T) discovered in three winter and two spelt wheat accessions. In our survey of 700 accessions, we found 48 lines with this haplotype (Table 3.3). It was found in wheat accessions originating from all areas of the world. In agreement with Lagudah et al. (2009), we also did not observe the reciprocal allele of haplotype 3 supporting the hypothesis that it originated from mutation rather than recombination.

The discovery of a new mutation in exon 10 defined a new haplotype (haplotype 4, Table 3.3). This haplotype was only found in *Lr34-* lines (T/A/TTC/T). The reciprocal of this haplotype was also not observed, suggesting that it similarly arose through mutation (Lagudah et al. 2009) and that this mutation occurred in a T/TTC/T genotype, therefore after the formation of the original haplotype. This haplotype was surprisingly broadly distributed and represented more than 10% of the 700 lines tested. As with haplotype 3, its distribution was ubiquitous among the WC and AM collections. Marker caIND11 is diagnostic for both indels in exon 10 (A/-) and exon 11 (TTC/-) because the two mutations will result in different size fragments (Fig. 3.1C).

A rare fifth haplotype (T/N/N/T) was found in two of the 700 lines tested, i.e., Odess Kaja 13 and Koktunkulskaja 332. We believe these lines to be *Lr34*-. The Plant Gene Resource Centre (PGRC) reported them as susceptible to rust and that is in agreement with our own field observations where rust severity for these 2 lines, evaluated in a total of five environments over three years, ranged from 10-80% (data not shown). These lines lack the TTC indel in exon 11 typically associated with the *Lr34*+ phenotype. The phenylalanine residue encoded by the TTC indel may therefore not play a role in the *Lr34*- phenotype. On the other hand, we cannot rule out that these lines may possess other silencing or altering mutations such as the rare mutation in cultivar Jagger (Lagudah et al. 2009). Complete sequencing of their ABC transporter may provide such information. Other mutations in this ABC transporter will likely be discovered as more in-depth information on this gene from large germplasm collections is gathered and becomes available. Preliminary field assessment on the two collections indicated rust severity up to 70% on some of the lines with the A/N/N/C haplotype predicted to be associated with the *Lr34*+ phenotype. This brings the question whether such lines have a functional *Lr34* gene and if not, what are the additional causes for the non-functionality of *Lr34*. Additional phenotyping in different environments and more in-depth characterization of the ABC transporter sequence and gene expression will be necessary to confirm association.

3.6 Conclusion

We described the development of seven new markers located at the *Lr34* locus in addition to three markers specifically targeting the ABC transporter gene previously

identified as *Lr34*. Physical and genetic maps of the locus confirm the close proximity of these markers to *Lr34*. They provide multiple alternatives for marker assisted selection. Marker caIND11 is likely the most informative while caISBP1 is the most user-friendly. We were able to map *Lr34* distal to four of the six genes previously identified to the interval thereby ruling out CYP-1, CYP-2, LRK-1, and LRK-2 as having a role in *Lr34* function. We described a new mutation in exon 10 that would likely be non-functional and that constituted a new haplotype. The characterization of a large germplasm collection identified a fourth and fifth haplotype of the ABC transporter.

HAPLOTYPE DIVERSITY AND EVOLUTIONARY HISTORY OF THE *LR34* LOCUS OF A WORLD WHEAT GERMPLASM COLLECTION

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The author, Abdulsalam Dakouri, designed, performed the experiments, analysed and interpreted the data and drafted the manuscript. The major supervisor, Dr. Sylvie Cloutier, directed the design and the experiments, participated in data analysis and manuscript review.

The manuscript was submitted to **BMC Evolutionary Biology** and it is under review

4.0 HAPLOTYPE DIVERSITY AND EVOLUTIONARY HISTORY OF THE *LR34* LOCUS OF A WORLD WHEAT GERMPLASM COLLECTION

4.1 Abstract

The resistance gene *Lr34* has been a key gene in the genetic management of wheat leaf rust worldwide. However, little is known about the geo-genetic diversity, the history and the origin of this unique gene. This study was conducted to provide a comprehensive analysis of the genetic diversity at the *Lr34* locus of a world wheat germplasm collection employing 10 molecular markers located within the coding sequence of *Lr34* or closely linked to it. A total of 52 alleles were detected for the 10 markers in the germplasm. Marker gwm1220 was the most polymorphic with 21 alleles, and had the highest polymorphic information content value of 0.91. On the basis of the *Lr34*-specific markers, the world collection was divided into five major haplotypes (H) of which H1 was consistently associated with the resistance phenotype *Lr34*+. Combined analysis of the 10 molecular markers resulted in dividing the major haplotypes into 118 different sub-haplotypes. Structure and clustering analyses grouped these sub-haplotypes into two main clusters and seven sub-clusters. Variance among main clusters represented the largest proportion of the total variation. Based on the maximum parsimony and other observations, H2, an *Lr34*- haplotype, was hypothesized to be the most ancient haplotype and H1 the most recent, as it likely arose after the advent of hexaploid wheat. Analysis of geographical distribution showed that H1 was more frequent in the Asian germplasm while H2 was predominant in European germplasm. *Lr34*, a gain of function mutation, is hypothesized to have originated in Asia.

4.2 Introduction

The wheat leaf rust *Lr34* resistance gene has been a major component of the genetic resistance strategy for managing this disease on wheat worldwide. *Lr34*, an adult plant resistance (APR) gene, confers similar levels of resistance to all leaf rust races, i.e. it is race non-specific (Spielmeyer et al. 2005). No instances of new virulent races toward this gene have been reported for more than 40 years, hence its durability (McCallum et al. 2007). The importance of *Lr34* also lies in its pleiotropic effect providing resistance not only to leaf rust but also to yellow rust (*Yr18*), powdery mildew (*Pm*38) (Spielmeyer et al. 2005), stem rust (Hiebert et al. 2010), and barley yellow dwarf virus (Singh 1993a).

Lr34, first located on chromosome 7DS of wheat (Dyck 1987), has recently been isolated and corresponds to an 11,805 bp gene comprising 24 exons and encoding an ABC transporter of 1,401 amino acids of the pleiotropic drug resistance class (Krattinger et al. 2009). Sequence comparison of the *Lr34* gene between the resistant (*Lr34*+) wheat cultivar Chinese Spring and the susceptible (*Lr34*-) cultivar Renan revealed three mutations: two single nucleotide polymorphisms (SNPs) in intron 4 and exon 12 and a 3bp indel in exon 11. These three mutations formed two haplotypes: A/N/C for *Lr34*+ and T/TTC/T for *Lr34*- (Krattinger et al. 2009). A third haplotype (i.e. A/TTC/T) was later identified and, in the absence of lines carrying the reciprocal allele, Lagudah et al. (2009) concluded that the A/T transversion in intron 4 originated by mutation rather than recombination. Dakouri et al. (2010) identified another mutation site within *Lr34*, i.e. an "A" indel in exon 10 that defined a fourth haplotype in spring wheat. This frameshift mutation would cause amino acid changes of the following 26 residues and is also

predicted to be non-functional. They also reported a fifth rare haplotype (T/N/N/T) found in only two of the 700 lines tested that might help to unravel the putative functionality of exon 11 and 12 polymorphisms. Two homoeologous *Lr34* genes were detected on chromosomes 4A and 7A of hexaploid wheat and two orthologous genes were found in the rice and sorghum genomes (Krattinger et al. 2011).

Phenotype-based analysis of *Lr34* in wheat is challenging because of the pleiotropy of other *Lr* genes and the inherent difficulties in assessment of the APR reaction. Diagnostic molecular markers facilitate postulation of *Lr34* presence. Several molecular markers completely or tightly linked to *Lr34* have been developed including gwm1220 and gwm295 (Spielmeyer et al. 2005), swm10 (Bossolini et al. 2006), csLV34 (Lagudah et al. 2006), csLVMS1 (Spielmeyer et al. 2008), csLVE17 and csLVA1/SWSNP3 (Krattinger et al. 2009), cssfr1-6 (Lagudah et al. 2009), and cam1, 2, 8, 11, 16, 23, caISBP1, caSNP4, caSNP12, caIND10, and caIND11 (Dakouri et al. 2010).

Bread wheat (*Triticum aestivum* L. 2*n*=6*x*=42, AABBDD) is one of the most important crops worldwide. Genetic diversity of wheat has been studied at all ploidy levels including the polyploid genome level (Zhang et al. 2011) all the way to the single locus or gene level (Singh et al. 2007; Kolmer et al. 2008; Wicker et al. 2009; McCallum et al. 2012a).

Years of breeding practices often reduce genetic diversity (Fu et al. 2003). Molecular characterization of germplasm can be used to quantify the extent of inbreeding and assist in selection of genetically diverse germplasm from the existing gene pool to broaden the genetic background of breeding materials (Van Beuningen and Busch 1997). Molecular markers such as simple sequence repeat (SSR) and single nucleotide

polymorphism (SNP) have been used to assess the genetic diversity of wheat germplasm because of their high level of polymorphism, reliability, and reproducibility. Haplotype diversity using such markers is also useful to efficiently discriminate wheat cultivars and genotypes. Here, we present a detailed description of the molecular marker profiles and haplotype diversity at the *Lr34* locus in a world collection of hexaploid wheat accessions. Insights into the evolutionary history of *Lr34* and geographical distribution of its haplotypes are also provided.

4.3 Materials and methods

4.3.1 Plant materials

Germplasm comprising 310 hexaploid wheat lines collected from 42 countries representing the six continents was used in this study and is referred to as the world collection (WC). The accessions are distributed as follows: 97 from Europe, 59 from Asia, 47 from South America, 36 from North America, 27 from Africa, 29 from Oceania, and 15 are of unknown origin. A detailed list including accession number, name, and country of origin is provided (Appendix III).

4.3.2 Molecular marker analysis

Ten molecular markers spanning the *Lr34* genomic region including five SSRs, two SNPs, two indels and one insertion site based polymorphism (ISPB) were analysed. SSR marker gwm1220 was located distal to the hexose carrier gene flanking one side of *Lr34* (Spielmeyer et al. 2005). Markers caSNP4, caIND10, caIND11 and caSNP12 were diagnostic of the four mutations within *Lr34 per se* while caISBP1 and cam11 flanked the

other side of *Lr34* and were positioned between *Lr34* and the first cytochrome P450 (*CYP-1*) gene (Dakouri et al. 2010; Krattinger et al. 2009). SSR markers csLVMS1 (Spielmeyer et al. 2008), swm10 (Bossolini et al. 2006) and csLV34 (Lagudah et al. 2006) were located distal to the second cytochrome P450 (*CYP-2*) gene. A schematic location of the markers with respect to *Lr34* is provided (Fig. 4.1).

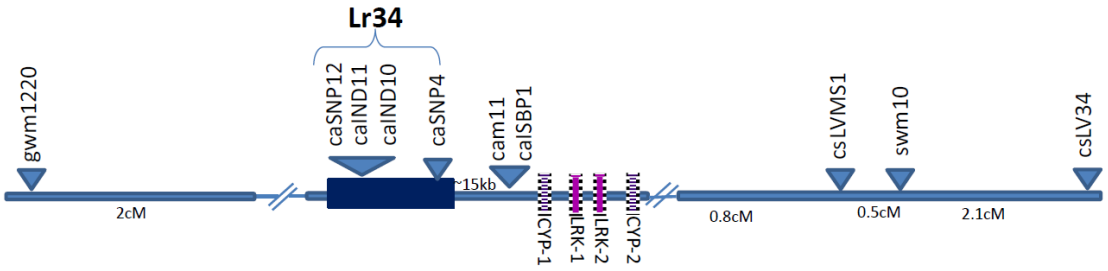

Fig. 4.1 A schematic diagram of the *Lr34* locus illustrating the locations of the ten molecular markers. Genetic distances are in cM and physical distance is in Kb.

Genomic DNA isolation and marker assessment were performed as previously described (Dakouri et al. 2010). Markers gwm1220, cam11, csLVMS1, swm10, and caIND11 were resolved on an ABI 3130x1 (Applied Biosystem, Foster City, CA). Amplicon sizes were determined using a modified version of Genographer (Benham et al. 1999) modified by Travis Banks and available at http://sourceforge.net/projects/geno-grapher) while markers caISBP1, csLV34, caSNP4 and caSNP12 were resolved on 1.5% agarose gels.

4.3.3 Genetic data analysis

Total allele number, observed and effective allele number per locus (Kimura and Crow 1964) were calculated using PopGene V.1.31 (Yeh et al. 2000). The effective allele

number refers to a hypothetical number of equally frequent alleles necessary to achieve the same gene diversity. Allele frequencies and polymorphic information content (PIC) values (Roussel et al. 2004) were computed using PowerMarker v.3.25 (Liu and Muse 2005).

A phylogenetic haplotype network was constructed using the maximum parsimony (MP) method implemented in NETWORK 4.6.0 (Fluxus Technology Ltd., Suffolk, England). The network was first constructed using a median joining (MJ) calculation (Bandelt et al. 1999). The default parameters were used where a weight of 10 was given to each character (i.e. each marker) and the transversion to transition weight ratio was set at 1:1. The Epsilon value specifying the weighted genetic distance was set at 10, equal to the highest genetic distance between data sets. The MJ network was post-processed using the MP calculation method (Polzin and Daneshmand 2003).

Structure analysis for the sub-haplotypes of the *Lr34* genomic region was performed using STRUCTURE v2.2 (Pritchard et al. 2000). The admixture model and correlated allele frequency with a burn-in period of 50,000 iterations and 100,000 replications of Markov Chain Monte Carlo (MCMC) were applied. Five runs were conducted to estimate the mean likelihood for a number of populations K ranging from 1 to 10. The average of the log-likelihood estimates LnP(D) for each K was computed. The *ad hoc* statistics ΔK was used to determine K (Evanno et al. 2005). STRUCTURE HARVESTER, a web-based program implementing the Evanno method, was used for visualizing STRUCTURE output (Earl and vonHoldt 2011). To study the genetic relationships of the *Lr34* sub-haplotypes, Nei's genetic distance (Nei 1973) and

unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal 1973) were performed with PowerMarker v.3.25.

The analysis of molecular variance (AMOVA) was conducted using Arlequin v.3.5 (Excoffier and Lischer 2010) to estimate the genetic variance among clusters and sub-clusters of sub-haplotypes. In this analysis, the distance matrix between samples was computed to estimate the genetic structure of the sub-haplotypes. Genetic variance components were estimated and the total variance was partitioned among major clusters, among sub-clusters within major clusters and within sub-clusters. Significance of variance components was tested using 1,000 permutations. The fixation index (*Fst*), an estimation of population differentiation and genetic distance based on genetic polymorphism data, was calculated. CONVERT V. 1.31 (Glaubitz 2004) was used to convert the molecular marker data files to PopGene, Arlequin (Escoffier and Lischer 2010), and STRUCTURE compatible data file formats.

Linkage disequilibrium (LD) between molecular markers was computed using GGT 2.0 software (Van Berloo 1999). LD was measured using squared correlation coefficient (r^2) between polymorphic sites of the whole WC and the *Lr34+* and *Lr34-* subsets separately (Hedrick 1987). The genetic distance values were obtained from the genetic map of the mapping population 98C18SSD (Dakouri et al. 2010).

4.4 Results

4.4.1 Molecular marker profiles at the *Lr34* locus

A total of 52 alleles were identified for all 10 marker loci among the 310 accessions (Table 4.1). The number of alleles and effective alleles, the PIC value and the major

allele frequency were calculated for all markers (Table 4.2). SSR marker gwm1220 was the most polymorphic marker with 21 alleles, 12 effective alleles and a PIC value of 0.91. Markers caSNP4, caIND10, caIND11 and caSNP12 mapped within *Lr34* and were all biallelic.

4.4.2 Haplotype analysis and evolutionary history of *Lr34*

The four markers located within *Lr34 per se*, i.e. caSNP4, caIND10, caIND11, and caSNP12 defined the recently described five *Lr34* haplotypes of wheat, namely H1 and H2 (Krattinger et al. 2009), H3 (Lagudah et al. 2009) and H4, and H5 (Dakouri et al. 2010) (Table 4.3). Haplotype H1 (A/N/N/C) represents the known *Lr34+* and comprises 22% of the accessions. H2 (T/N/TTC/T), H3 (A/N/TTC/T) and H4 (T/A/TTC/T) were all *Lr34-* and represented 14%, 14%, and 50% of the accessions, respectively. H5 (T/N/N/T), a rare haplotype, was found in only two accessions (0.6%) that have been postulated to be susceptible to wheat leaf rust (*Lr34*-?) (Dakouri et al. 2010). Additional field data on the APR response of these two accessions is required to confirm this hypothesis.

To evaluate the most probable evolutionary steps leading to the development of the *Lr34+* haplotype, a phylogenetic relationship network was obtained for the five known haplotypes using the maximum parsimony criterion (Figure 4.2). The network displayed the shortest paths and a median vector (mv1) required to connect the existing haplotypes within the network with maximum parsimony.

Table 4.1 Allele sizes and frequencies for 10 *Lr34* locus specific markers assessed in a world collection of 310 wheat accessions.

Allele No	gwm1220		caSNP12		caIND11		caIND10		caSNP4		cam11		caISBP1		csLVMS1		swm10		csLV34	
	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)
1	N	1.0	C	21.9	N	22.6	N	86.1	A	35.5	N	18.4	391	79.2	N	0.3	N	0.3	175	22.4
2	130	1.0	T	78.1	TTC	77.4	A	13.9	T	64.5	297	0.3	509	20.8	226	46.6	206	0.3	255	77.6
3	140	0.3									298	12.2			228	50.5	208	28.4		
4	143	2.6									299	22.9			230	2.6	210	45.5		
5	145	1.0									300	26.3					212	13.2		
6	146	2.3									301	14.2					214	12.3		
7	148	16.0									302	3.5								
8	150	1.0									303	1.9								
9	152	3.5									304	0.3								
10	154	2.9																		
11	156	1.0																		
12	157	5.8																		
13	158	1.0																		
14	159	9.0																		

Allele No	gwm1220		caSNP12		caIND11		caIND10		caSNP4		cam11		caISBP1		csLVMS1		swm10		csLV34	
	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)	allele	Freq (%)
15	161	31.6																		
16	163	3.9																		
17	165	8.7																		
18	167	4.8																		
19	169	1.3																		
20	170	0.3																		
21	172	1.0																		
Total	21		2		2		2		2		9		2		4		6		2	

Table 4.2 Extent of the genetic diversity at the *Lr34* locus as described by 10 molecular markers assayed on 310 accessions of a wheat germplasm collection.

Marker	no. alleles	no. effective alleles	PIC value	major allele frequency
gwm1220	21	12.0	0.91	0.19
caSNP4	2	1.8	0.34	0.67
caIND10	2	1.3	0.20	0.87
caIND11	2	1.4	0.25	0.82
caSNP12	2	1.4	0.23	0.84
cam11	9	5.4	0.77	0.28
caISBP1	2	1.3	0.23	0.85
csLVMS1	4	2.2	0.45	0.49
swm10	6	3.2	0.64	0.47
csLV34	2	1.4	0.25	0.82
Mean	5.2	3.1	0.43	0.63

Table 4.3 Genotypes of the five major *Lr34* haplotypes of the world collection as defined by the four genic markers identified to date in spring wheat germplasm.

Haplotype	caSNP4	caIND10*	caIND11*	caSNP12	total	*Lr34*
1	A	N	N	C	68	*Lr34*+
2	T	N	TTC	T	155	*Lr34*-
3	A	N	TTC	T	42	*Lr34*-
4	T	A	TTC	T	43	*Lr34*-
5	T	N	N	T	2	*Lr34*-?
total					310	

* N=null/deletion of the indel

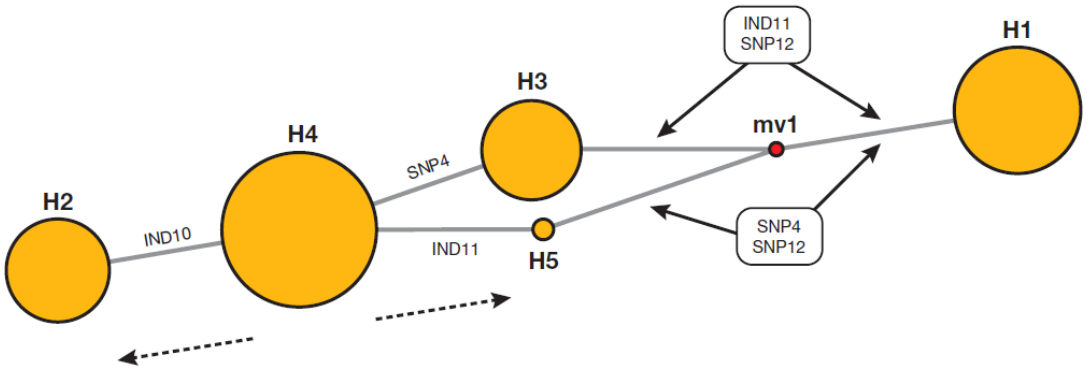

Fig. 4.2 Maximum parsimony tree of the five *Lr34* major haplotypes based on the four polymorphic sites assayed within the ABC transporter gene. Mutations are indicated on the lines joining the haplotypes. Circle sizes reflect the number of accessions representing each haplotype. The median vector (mv1) required to connect existing haplotypes within the network with maximum parsimony is indicated. The arrows indicate the evolutionary directions.

4.4.3 Genetic diversity of the WC at the *Lr34* locus

The analysis of six additional markers flanking *Lr34* divided the five major haplotypes described above into 118 sub-haplotypes (Table 4.4). H4 had the largest number of sub-haplotypes with 62. Each sub-haplotype was represented by one to 40 accessions.

Population structure analysis was performed to infer the number of groups included in the WC. The analysis indicated two major groups as also confirmed by the Evanno criterion (Evanno et al. 2005) method (Figure 4.3). The first group included haplotype H1 (*Lr34+*) while the second group contained the other four haplotypes.

Using Nei's genetic distance and UPGMA clustering method implemented in PowerMarker v3.0, a dendrogram describing the genetic relationships between the *Lr34* sub-haplotypes was constructed. The dendrogram also suggests the presence of two major clusters (Figure 4.4). Cluster I exclusively contains sub-haplotypes of H1 (*Lr34+*) and cluster II encompassed sub-haplotypes belonging to H2, H3, H4 (*Lr34-*), and H5

Table 4.4 *Lr34* sub-haplotypes based on 10 molecular markers

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
H1	H1_1	148	C	N	N	A	N	509	226	208	175	1	0.3
	H1_2	159	C	N	N	A	N	509	226	208	175	12	3.9
	H1_3	161	C	N	N	A	N	509	226	208	175	22	7.1
	H1_4	161	C	N	N	A	N	509	226	208	255	1	0.3
	H1_5	145	C	N	N	A	300	391	228	210	255	1	0.3
	H1_6	169	C	N	N	A	N	509	226	210	175	1	0.3
	H1_7	159	C	N	N	A	299	509	226	208	175	2	0.6
	H1_8	159	C	N	N	A	N	509	226	208	255	1	0.3
	H1_9	161	C	N	N	A	298	509	226	208	175	15	4.8
	H1_10	161	C	N	N	A	303	509	228	208	175	1	0.3
	H1_11	161	C	N	N	A	303	509	228	N	175	1	0.3
	H1_12	161	C	N	N	A	303	509	228	214	175	1	0.3
	H1_13	161	C	N	N	A	299	509/391	226/228	210	175/255	1	0.3
	H1_14	163	C	N	N	A	303	509	226	208	175	1	0.3

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
	H1_15	165	C	N	N	A	300	391	226	208	175	3	1.0
	H1_16	165	C	N	N	A	300	391	226	206	175	1	0.3
	H1_17	167	C	N	N	A	300	391	226	208	175	1	0.3
	H1_18	N	C	N	N	A	N	509	226	208	175	1	0.3
	H1_19	N	C	N	N	A	301	509	228	214	255	1	0.3
Sub-total												68	
H2	H2_1	148	T	TTC	N	T	300	391	228	208	175	1	0.3
	H2_2	148	T	TTC	N	T	299	391	228	210	175	2	0.6
	H2_3	148	T	TTC	N	T	298	391	228	210	255	40	12.9
	H2_4	159	T	TTC	N	T	299	391	226	210	255	3	1.0
	H2_5	159	T	TTC	N	T	299-300	391	230	210	255	2	0.6
	H2_6	161	T	TTC	N	T	299	391	228	210	255	8	2.6
	H2_7	165	T	TTC	N	T	300-301	391	226	212	255	6	1.9
	H2_8	143	T	TTC	N	T	299-300	391	226	210	255	2	0.6
	H2_9	143	T	TTC	N	T	299	391	228	210	255	1	0.3

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
	H2_10	143	T	TTC	N	T	299	391	228	212	255	4	1.3
	H2_11	143	T	TTC	N	T	N	391	226	210	255	1	0.3
	H2_12	145	T	TTC	N	T	299	391	228	210	255	1	0.3
	H2_13	148	T	TTC	N	T	300	391	226	208	175	1	0.3
	H2_14	148	T	TTC	N	T	300	391	226	210	255	1	0.3
	H2_15	148	T	TTC	N	T	N	391	228	210	255	1	0.3
	H2_16	148	T	TTC	N	T	N	509	228	210	255	1	0.3
	H2_17	150	T	TTC	N	T	300	391	226	210	255	1	0.3
	H2_18	150	T	TTC	N	T	300	391	228	210	255	1	0.3
	H2_19	150	T	TTC	N	T	300	391	228	214	255	1	0.3
	H2_20	152	T	TTC	N	T	298	391	226	210	255	2	0.6
	H2_21	152	T	TTC	N	T	300	391	228	210	255	4	1.3
	H2_22	152	T	TTC	N	T	N	391	228	210	255	1	0.3
	H2_23	154	T	TTC	N	T	297	391	226	210	255	5	1.6
	H2_24	154	T	TTC	N	T	302	391	226	212	255	1	0.3

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
	H2_25	154	T	TTC	N	T	300	391	228	208	255	1	0.3
	H2_26	154	T	TTC	N	T	300	391	230	210	255	1	0.3
	H2_27	154	T	TTC	N	T	302	391	228	210	255	1	0.3
	H2_28	156	T	TTC	N	T	299	391	226	212	255	1	0.3
	H2_29	156	T	TTC	N	T	300	391	228	210	255	1	0.3
	H2_30	157	T	TTC	N	T	299	391	226	210	255	3	1.0
	H2_31	157	T	TTC	N	T	302	391	226	212	255	2	0.6
	H2_32	157	T	TTC	N	T	299-300	391	228	208	255	2	0.6
	H2_33	157	T	TTC	N	T	299	391	228	210	255	2	0.6
	H2_34	157	T	TTC	N	T	N	391	226	210	255	1	0.3
	H2_35	158	T	TTC	N	T	298	391	226	210	255	1	0.3
	H2_36	158	T	TTC	N	T	N	509	226	208	175	1	0.3
	H2_37	159	T	TTC	N	T	299	391	228	212	255	2	0.6
	H2_38	159	T	TTC	N	T	299	391	230	212	255	1	0.3
	H2_39	159	T	TTC	N	T	N	391	228	210	255	1	0.3

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
	H2_40	159	T	TTC	N	T	N	391	228	208	255	1	0.3
	H2_41	159	T	TTC	N	T	N	391	226	210	255	1	0.3
	H2_42	161	T	TTC	N	T	301	391	226	208	255	1	0.3
	H2_43	161	T	TTC	N	T	300	391	226	210	255	7	2.3
	H2_44	161	T	TTC	N	T	300	391	226	212	255	1	0.3
	H2_45	161	T	TTC	N	T	299-301	391	228	214	255	3	1.0
	H2_46	161	T	TTC	N	T	N	509	226	208	175	1	0.3
	H2_47	161	T	TTC	N	T	N	391	226	210	255	1	0.3
	H2_48	161	T	TTC	N	T	N	391	228	210	255	3	1.0
	H2_49	163	T	TTC	N	T	298	391	226	210	255	3	1.0
	H2_50	163	T	TTC	N	T	299-300	391	228	210	255	2	0.6
	H2_51	165	T	TTC	N	T	299	391	226	210	255	1	0.3
	H2_52	165	T	TTC	N	T	300	391	228	214	255	1	0.3
	H2_53	165	T	TTC	N	T	300	391	230	212	255	1	0.3
	H2_54	167	T	TTC	N	T	300-302	391	226	208	255	5	1.6

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
	H2_55	167	T	TTC	N	T	300	391	228	210	255	1	0.3
	H2_56	167	T	TTC	N	T	300	391	230	208	175	1	0.3
	H2_57	167	T	TTC	N	T	300	391	230	212	255	2	0.6
	H2_58	167	T	TTC	N	T	N	391	226	208	255	3	1.0
	H2_59	167	T	TTC	N	T	N	391	228	210	255	1	0.3
	H2_60	169	T	TTC	N	T	301	391	228	210	255	1	0.3
	H2_61	169	T	TTC	N	T	299	391	228	212	255	1	0.3
	H2_62	172	T	TTC	N	T	298	391	228	210	255	1	0.3
Sub-total												155	
H3	H3_1	146	T	TTC	N	A	298	391	228	210	255	4	1.3
	H3_2	157	T	TTC	N	A	301	391	226	208	255	6	1.9
	H3_3	130	T	TTC	N	A	301	391	226	212	255	3	1.0
	H3_4	140	T	TTC	N	A	298	391	226	210	255	1	0.3
	H3_5	145	T	TTC	N	A	298	391	228	210	255	1	0.3
	H3_6	146	T	TTC	N	A	298	391	226	210	255	2	0.6

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
	H3_7	146	T	TTC	N	A	298	391	226	208	255	1	0.3
	H3_8	148	T	TTC	N	A	299-302	391	226	210	255	2	0.6
	H3_9	152	T	TTC	N	A	298	391	226	210	255	4	1.3
	H3_10	156	T	TTC	N	A	298	391	228	208	255	1	0.3
	H3_11	157	T	TTC	N	A	301	391	226	210	255	2	0.6
	H3_12	161	T	TTC	N	A	299	391	226	210	255	1	0.3
	H3_13	161	T	TTC	N	A	299	391	228	212	255	1	0.3
	H3_14	161	T	TTC	N	A	301	391	N	214	255	1	0.3
	H3_15	163	T	TTC	N	A	301	391	226	210	255	1	0.3
	H3_16	163	T	TTC	N	A	300-301	391	228	210	255	2	0.6
	H3_17	165	T	TTC	N	A	301	391	226	208	255	1	0.3
	H3_18	165	T	TTC	N	A	301	391	226	212	255	1	0.3
	H3_19	165	T	TTC	N	A	300-301	391	228	214	255	6	1.9
	H3_20	170	T	TTC	N	A	300	391	226	210	255	1	0.3
Sub-total												42	

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
H4	H4_1	161	T	TTC	A	T	299-301	391	228	214	255	6	1.9
	H4_2	169	T	TTC	A	T	300	391	228	212	255	1	0.3
	H4_3	158	T	TTC	A	T	300	391	228	212	255	1	0.3
	H4_4	159	T	TTC	A	T	300	391	228	212	255	1	0.3
	H4_5	161	T	TTC	A	T	300	391	228	210	255	1	0.3
	H4_6	161	T	TTC	A	T	299-302	391	228	212	255	6	1.9
	H4_7	161	T	TTC	A	T	299-302	391	228	214	255	15	4.8
	H4_8	163	T	TTC	A	T	299	391	228	212	255	1	0.3
	H4_9	163	T	TTC	A	T	301	391	228	214	255	1	0.3
	H4_10	165	T	TTC	A	T	299-300	391	228	210	255	4	1.3
	H4_11	165	T	TTC	A	T	299-300	391	228	212	255	1	0.3
	H4_12	165	T	TTC	A	T	299-300	391	228	214	255	1	0.3
	H4_13	167	T	TTC	A	T	303	391	228	214	255	1	0.3
	H4_14	172	T	TTC	A	T	300-301	391	228	212	255	2	0.6
	H4_15	N	T	TTC	A	T	300	391	228	212	255	1	0.3

Haplotype	sub-haplotype	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34	no. of lines	overall frequency (%)
Sub-total												43	
H5	H5_1	159	T	N	N	T	300	391	228	210	255	1	0.3
	H5_2	163	T	N	N	T	304	391	228	210	255	1	0.3
Sub-total												2	
Total												310	

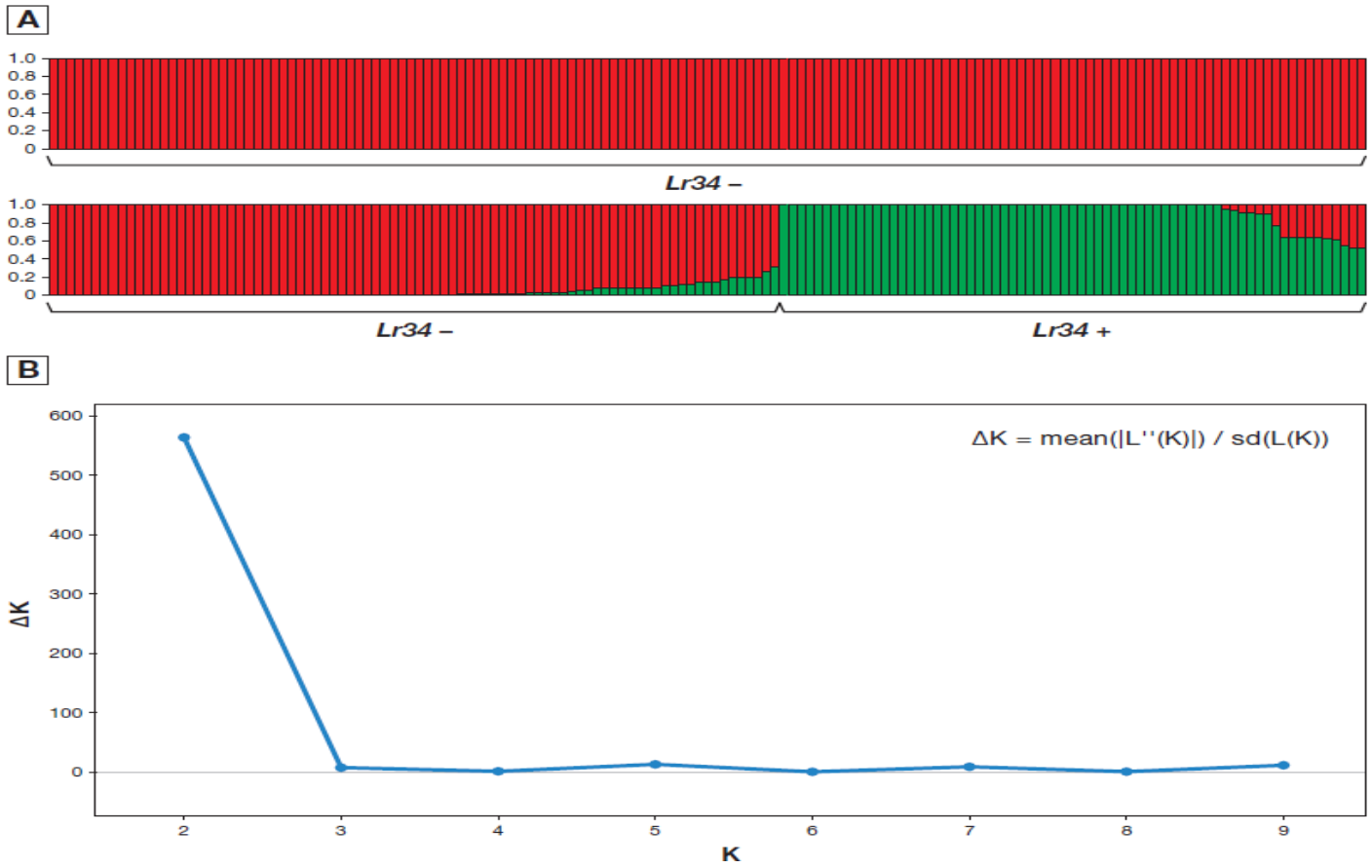

Fig. 4.3 Population structure analysis of the 310 accessions based on the 10 markers assessed at the *Lr34* locus. A: Model-based Bayesian clustering performed using STRUCTURE for $K = 2$ groups. Each of the 310 accessions is represented by a column broken into red and green segments with length proportional to each of the K inferred ancestral groups (*Lr34*+ and *Lr34*- lines) shown underneath. B: Estimation of the number of sub-populations based on the Evanno criterion. *Ad-hoc statistic* ΔK (Evanno et al. 2005) for K values of 1 to 10.

Fig. 4.4 A Nei's genetic distance-based UPGMA dendrogram of the 118 sub-haplotypes of the *Lr34* locus representing 310 accessions of a world collection of wheat. The genetic distance was calculated from the 10 molecular markers at the *Lr34* genomic region. Zoom in section shows *Lr34*+ sub-haplotype cluster. The sub-haplotype names are written on each sub-haplotype branch.

(*Lr34*-?). This clustering was mainly defined by the intergenic markers. The genetic distance ranged from 0.001 (closely related) to 0.4 (distantly related).

Cluster I contained 19 sub-haplotypes all representing H1 accessions and could be sub-divided into two sub-clusters CIA and CIB (Figure 4.4, zoomed in section). Pairwise differences between these sub-haplotypes ranged from one (i.e. closely related) to six different alleles (i.e. distantly related) (Table 4.4). Cluster II contained 99 sub-haplotypes representing 242 wheat accessions and was further subdivided into five sub-clusters labelled A to E. CIIE comprised the largest number of sub-haplotypes with 26 while CIIC contained only 8 (Figure 4.4).

4.4.4 Analysis of molecular variance (AMOVA)

The two major clusters and seven sub-clusters defined above were utilized to conduct an AMOVA. Significant genetic differences were found among sub-clusters within major clusters, within sub-clusters ($p<0.001$) and between major clusters ($p<0.05$) (Table 4.5). Major clusters accounted for the largest proportion (50.0%) of the total variance followed by within sub-cluster variance (26.5%), and among sub-cluster variance (23.5%). The fixation index (*Fst*) value was 0.74 suggesting that *Lr34* sub-haplotypes belong to two highly differentiated groups: *Lr34*+ and *Lr34*-.

Sub-clusters pairwise *Fst* values ranged from 0.36 between sub-clusters CIID and CIIE of the *Lr34*- sub-haplotypes to 0.82 between the sub-clusters CIA of *Lr34*+ and CIIA of *Lr34*- (Table 4.6).

Table 4.5 Analysis of molecular variance (AMOVA) of the 310 accessions of the world wheat collection based on the *Lr34* markers.

Source of variation	d.f.	Sum of Squares	Variance Components	Variation (%)	p-value
Between major clusters	1	565.82	2.05	50.0	<0.05
Among sub-clusters within major clusters	5	312.57	0.96	23.5	<0.001
Within sub-clusters	303	665.40	1.09	26.5	<0.001
Total	309	1543.792	4.10		
Fst = 0.74					

Table 4.6 Sub-clusters pairwise *Fst* differences.

Sub-clusters	CIA	CIB	CIIA	CIIB	CIIC	CIID	CIIE
CIA	0.00						
CIA	0.61	0.00					
CIIA	0.82	0.69	0.00				
CIIB	0.73	0.53	0.57	0.00			
CIIC	0.73	0.67	0.61	0.47	0.00		
CIID	0.80	0.67	0.52	0.42	0.44	0.00	
CIIE	0.74	0.59	0.46	0.40	0.42	0.36	0.00

4.4.5 Linkage disequilibrium at the *Lr34* locus

LD significance was estimated for the entire WC. Significant r^2 values for all markers with the exception of gwm1220 were observed (Appendix IV). To evaluate whether LD significance differed between markers of the *Lr34*+ and *Lr34*- subsets, the same analysis was conducted separately on both sub-haplotypes. The threshold for significant LD was $r^2>0.12$ for the *Lr34*+ group and $r^2>0.05$ for the *Lr34*- group. Significant LD between markers in the *Lr34*+ sub-haplotypes was detected with the strongest LD ($r^2=1$) detected between the *Lr34* markers caSNP12, caIND11, caIND10, and caSNP4 and LD decreasing with increasing the genetic distance (Appendix V). In contrast, much lower LD levels were observed between markers in the *Lr34*- sub-haplotypes (Appendix VI).

4.4.6 Geographical distribution of *Lr34* haplotypes

Asia, South America, and North America had the highest numbers of H1 (*Lr34*+) accessions with 24, 17 and 11, respectively (Figure 4.3, Appendix XII). Despite the fact that the number of accessions differed, these same three regions contained also the

highest proportions of H1 at 40.7%, 36.2% and 30.6%, respectively. The highest proportions of H4 were found in Africa (37.0%) and Asia (27.1%). Proportions of germplasm with H3 ranged from 6.3 (South America) to 20.7% (Oceania) (Figure 4.5, Table 4.7). A low frequency of H2 was observed in Asian germplasm (15.3%) but the opposite was true in European germplasm (68.0%) while frequencies for the other regions were close to the overall average of 50%.

4.5 Discussion

Better utilization of available genetic resistance resources of wheat to breed for improved rust resistance requires an in depth molecular characterization of its genetic diversity. *Lr34* is probably the single most important resistance gene in wheat because of its durability and pleiotropy.

Here, we presented a detailed molecular analysis of the *Lr34* locus in a world collection of 310 wheat accessions providing insights on the genetic variability in hexaploid wheat germplasm at this locus. Geographical distributions of *Lr34* haplotypes and historical mutation events leading to the development of *Lr34* have also been described.

4.5.1 Molecular marker profile analysis

Ten molecular markers spanning the *Lr34* genomic region were used to characterize a world collection. In total, 52 alleles were detected including six biallelic markers and four markers with four to 21 alleles. The average allele number per locus for SSR markers was 8.4 which is higher than in previous studies on genetic diversity of

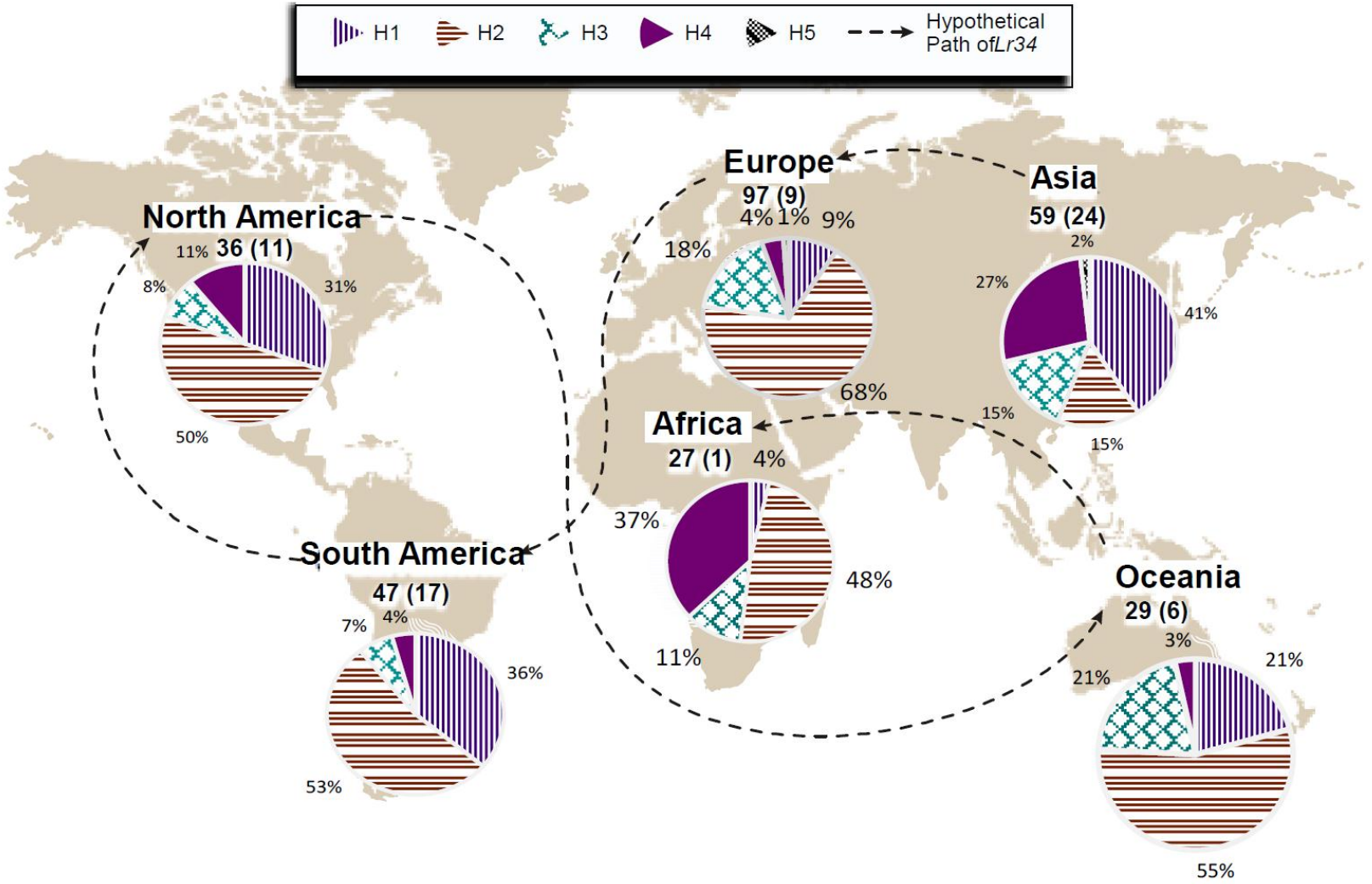

Figure 4.5 Geographical distribution of *Lr34* haplotypes. Numbers underneath each continent name represent the total number of accessions and numbers between brackets refer to the number of accessions with *Lr34*+. Pie charts show the frequencies of haplotypes within each continent. Dashed arrows refer to hypothetical paths of *Lr34* movement across the continents.

Table 4.7 Geographical distribution of *Lr34* haplotypes.

Haplotype	*Lr34*	Asia	Europe	North America	South America	Africa	Oceania	Unknown	Total
H1	*Lr34*+	24	9	11	17	1	6	0	68
H2	*Lr34*-	9	66	18	25	13	16	8	155
H3	*Lr34*-	9	17	3	3	3	6	1	42
H4	*Lr34*-	16	4	4	2	10	1	6	43
H5	*Lr34*-?	1	1	0	0	0	0	0	2
Total		59	97	36	47	27	29	15	310

hexaploid wheat, ranging from 3.6 to 6.2 (Christiansen et al. 2002). Marker gwm1220 was the most polymorphic with 21 alleles followed by cam11 with nine alleles, higher than recently reported for a germplasm collection of Canadian and historical accessions indicating the greater genetic variability of the WC (McCallum et al. 2012a). In the Canadian germplasm collection, all H1haplotype accessions except *Lr34* near isogenic line RL6058 and its donor PI58548, displayed the alleles cam11-N, caISBP1-509, csLVMS1-226, swm10-208 and csLV34-175 for the markers flanking *Lr34* (McCallum et al. 2012a) while only 36 of the 68 H1 haplotype accessions of the WC displayed this particular sub-haplotype make up. Considering only these five markers, the other 32 lines formed 11 distinct sub-haplotypes not observed in the Canadian germplasm collection further emphasizing the broader genetic variability of the WC. The four markers located within the *Lr34* coding sequence were biallelic.

The alleles caIND11-N and caSNP12-C are both diagnostic of *Lr34*+ with the exception of Odess Kaja 13 and Koktunkulskaja 332 which belong to H5. These lines had the caIND11-N allele associated with *Lr34*+ and the caSNP12-T associated with *Lr34*-. Additional phenotypic characterization of these lines in the field is required to precisely determine whether their adult plant resistance (APR) phenotype correspond to *Lr34*.

4.5.2 Haplotype analysis and evolutionary history of *Lr34*

Based on caSNP4, caIND10, caIND11 and caSNP12 marker profiles of the *Lr34* gene, five major haplotypes were identified. H1 represents *Lr34*+ lines while H2, H3 and H4 are *Lr34*- lines, and H5 remains unconfirmed. The first four major haplotypes were also

found in the Canadian germplasm collection (McCallum et al. 2012a) but H5 was not. The *Lr34+* haplotype was detected in three independent wheat breeding lineages thus suggesting that *Lr34* had a single origin (Krattinger et al. 2009).

Lr34 is located on the short arm of chromosome 7D genome (Dyck 1987), the most recent genome to be integrated into hexaploid wheat. Sequence comparison of *Lr34* containing BAC clones from wheat cultivars Chinese Spring, Glenlea and Renan as well as *Aegilops tauschii*, the donor of the D genome, revealed higher structural similarity between *Ae. tauschii* and Renan (H2, *Lr34–*) followed by Chinese Spring (H1, *Lr34+*) and Glenlea (H1, *Lr34+*). Indeed, the *Lr34* locus of Chinese Spring and Glenlea was estimated to have diverged approximately 700 years ago while divergence of these two lines with Renan was estimated at 6,339 years ago (Wicker et al. 2009). We tested all four *Lr34* genic markers on 112 *Ae. tauschii* accessions and discovered that they all had the haplotype H2 (caSNP4-T, caIND10-N, caIND11-TTC, and caSNP12-T) (data not shown), identical to hexaploid wheat Renan (Krattinger et al. 2009). We propose that (1) H2 is the ancestral haplotype in hexaploid wheat, (2) H1, H3, H4 and H5 arose after the formation of hexaploid wheat, (3) H1 (*Lr34+)* is the result of gain of function mutations, and (4) this latter haplotype originated in Asia. Although, we cannot rule out the existence of *Lr34+* in the *Ae. tauschii* gene pool, the fact that all 112 accessions tested had the same H2 haplotype strongly supports the hexaploid wheat origin of *Lr34+* (H1) as well as H3, H4 and H5. The maximum parsimony network built on the genic mutations in the hexaploid gene pool also corroborates H2 as the most ancient haplotype (Figure 4.2). Single but independent mutations of H2 gave rise to H3, H4 and H5. Cumulative mutations of, most likely an H3 haplotype, resulted in H1, the only *Lr34+*

haplotype, which therefore corresponds to a cumulative gain of function mutations that occurred only after the polyploidization of bread wheat (Kolmer et al. 2008). No accessions were identified to be intermediate between H3 and H1, hence the medium vector (mv1) node, and thus precluding the exact ordering of the last two mutations. The two ABC transporter DNA sequences that define H2 and H1 differ by only two nucleotide substitutions and one 3-bp indel. Based on the Kimura-2 model of nucleotide substitution and a substitution rate of 1.3×10^{-8} (Ma and Bennetzen 2004), a time of divergence of 6,516 years ago is in line with the advent time of origin of *Lr34+* described herein.

4.5.3 Genetic diversity of the WC germplasm at the *Lr34* genomic region

In this study, the genetic diversity of *Lr34* was assessed by looking at a broader *Lr34* locus region spanning at least six genes (including *Lr34*) and two pseudogenes. This was accomplished by the characterization of a broad germplasm collection for 10 molecular markers. Doing so, the five major *Lr34* haplotypes were further divided into 118 different sub-haplotypes. This extensive subdivision was mainly the result of the allelic diversity of gwm1220, csLVMS1 and swm10. The same 10 molecular markers analysed on the AM collection of mostly Canadian and historical wheat germplasm resulted in the identification of only 30 sub-haplotypes among the 375 accessions evaluated (McCallum et al. 2012a). Differences between the two collections are indicative of the breadth of the genetic diversity of the WC. Cam11-N, caISBP1-509, csLVMS1-226, swm10-208, and csLV34-175 were the most frequent alleles in H1 sub-haplotypes (*Lr34+*). These alleles were rare in the *Lr34-* sub-haplotypes indicating few historical recombination events

between *Lr34* and csLV34. Consequently, these five flanking markers are largely, although not perfectly, diagnostic of *Lr34* (Bossolini et al. 2006; Singh et al. 2007; Kolmer et al. 2008; Lagudah et al. 2009; Dakouri et al. 2010; McCallum et al. 2012a).

H4 lines did not have any of the alleles that seem to be characteristic of *Lr34+* at all the above-mentioned marker loci indicating that the 'A' indel in exon 10 resulted from a point mutation of an H2 accession (Figure 4.1). *Lr34-* haplotypes such as H2, H3 and H4 are still widely present in recent varieties and germplasm. This is particularly true in Europe (Figure 4.5) in part because leaf rust diseases were not economically important and selection for rust resistance was not a major focus of breeding programs (Singh et al. 2004b).

Structure and clustering analyses indicated the presence of two major clusters and seven sub-clusters among the sub-haplotypes defined by the six markers surrounding the *Lr34* gene. A total of 63.6% of sub-haplotypes contained a single line while the remaining had two to 40 lines. Geographic diversity within each sub-haplotype varied where some contained lines from the same geographical region while others comprised lines from diverse areas representing all continents. We suggest that some sub-haplotypes are well adapted to specific environments while others are more universal. Further analysis including additional traits may be required to verify this hypothesis.

AMOVA estimates population differentiation using molecular data or phylogenetic trees based on molecular data (Excoffier et al. 1992). AMOVA of the *Lr34* locus haplotypes showed substantial subdivision among the wheat germplasm with 50% of the total variation being detected among major clusters representing *Lr34+* (H1) and *Lr34-* (H2, H3 and H4) indicating a high level of divergence and limited gene flow

between *Lr34+* and *Lr34-* clusters. The high proportion of genetic variation among major clusters is likely due to selection for leaf rust resistance during the course of wheat breeding. The significant variation among sub-clusters within clusters and within sub-clusters is possibly because of selection for adaptation to local agronomic environments during wheat breeding processes (Roussel et al. 2005).

Several factors influence LD including population structure, recombination, mutation, population size, genetic drift, population mating pattern, admixture, and selection (Flint-Garcia et al. 2003). Taking into account the structure of the WC, we performed LD analysis on the *Lr34+* and *Lr34-* clusters separately. The high levels of LD in the *Lr34+* cluster as compared to the low levels in the *Lr34-* suggest that selection for *Lr34* resistance gene is recent. The strong LD values in the *Lr34+* cluster indicates that some historical recombinations have been fixed in homozygous states, while the presence of very low LD values in the same cluster reflects broad genetic diversity that is maintained in this gene pool (Hao et al. 2011).

4.5.4 Geographical distribution of *Lr34* haplotypes

The worldwide distribution of *Lr34* haplotypes was investigated using a world collection of wheat germplasm collected from 42 countries. Three breeding lineages of *Lr34* have been hypothesized: Far East germplasm (Asia), North and South American spring wheat, and European winter wheat cultivars (Krattinger et al. 2009). Here, we found that H1 (*Lr34+*) was the prevalent haplotype in Asia and South America while H2 (*Lr34-*) dominated the European germplasm. We propose that *Lr34* originated in Asia, specifically the Far East (China and/or Japan). This hypothesis is supported by H1 sub-

haplotype diversity in this region with 12/19 sub-haplotypes present, higher than any other regions. Analysis of 80 European winter wheat cultivars with csLV34 revealed that with a single exception all had the csLV34a allele associated with *Lr34*- suggesting a low level presence of *Lr34* in Western European winter wheat breeding germplasm presumably because leaf rust was not economically significant (Kolmer et al. 2008). Prolonged exposure to cold (8°C) of *Lr34*+ spring wheat appears to be detrimental to the growth of these lines (personal observation) which may partially explain its low frequency in winter wheat. From Asia, the *Lr34*+ material is hypothesized to have been transferred through human migration and germplasm exchange to South and North America and to Europe. The Brazilian cultivar Frontana, derived from crosses made in Italy by Nazareno and Strampelli in the early 1900s between the Italian cultivar Mentana (*Lr34*+) and the Brazilian cultivar Frontier (*Lr34*-), was the original source of *Lr34* in the majority of CIMMYT and North American hard red spring wheat (HRSW) cultivars (Kolmer et al. 2008). From South America, parental lines with *Lr34* were taken to Oceania (Australia and New Zealand) and were incorporated in breeding program (Kolmer et al. 2008). The recent origin of wheat in Kenya traced back mainly to the introduction of Australian cultivars at the beginning of the 20th century (Dixon 1960). Therefore, *Lr34*+ germplasm was probably transferred from Oceania to Africa through parental lines. Detailed pedigree analysis of the germplasm may be required to verify this hypothesis.

4.6 Conclusion

Lr34 remains a highly effective and durable leaf rust resistance gene. This work provides a detailed description of molecular markers and haplotype diversity at the *Lr34* locus in a broad wheat germplasm. *Lr34* specific markers and closely linked markers have proven useful in investigating the genetic diversity of this gene in wheat. Molecular markers located within the coding sequence of *Lr34* divided the germplasm into five major haplotypes where H1 was consistently associated with the *Lr34+* phenotype while at least three of the other four haplotypes (H2, H3, and H4) were *Lr34-*. We hypothesized that haplotype H2 is the ancestral haplotype that gave rise first to H3 and subsequently to H1 (*Lr34+*) following two successive mutations. The exclusive H2 haplotype found in *Ae. tauschii* provides further support for this hypothesis. H1 (*Lr34+*) is therefore the result of gain of function mutations hypothesized to have originated in Asia.

MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF SEEDLING AND PARTIAL LEAF RUST RESISTANCE IN A WORLD WHEAT COLLECTION

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The author, Abdulsalam Dakouri, designed, performed the experiments, analysed and interpreted the data and drafted the manuscript. The major supervisor, Dr. Sylvie Cloutier, directed the design and the experiments, participated in data analysis and manuscript review. Dr. Brent D. McCallum helped in field experiments, data collection, data interpretation and manuscript review. Mrs. Natasa Radovanovic contributed to field phenotyping, data collection and manuscript review.

5.0 MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF SEEDLING AND PARTIAL LEAF RUST RESISTANCE IN A WORLD WHEAT COLLECTION

5.1 Abstract

Wheat leaf rust caused by *Puccinia triticina* Eriks, is the most common rust disease of wheat. Genetic resistance has been acknowledged as the most effective approach to manage this disease. Knowledge of the number and identity of resistance genes is a prerequisite for effective use of germplasm in breeding. The aim of this study was to characterize seedling and adult plant resistance of a world wheat collection of 275 accessions from 42 countries using gene specific markers and gene postulation and to evaluate them for field resistance. In total, 14 seedling genes were determined or postulated to be present in the collection. *Lr1*, *Lr10*, *Lr3* and *Lr20* were the most prevalent genes while *Lr9*, *Lr14b*, *Lr3ka* and/or *Lr30* and *Lr26* were rare. Although possessing *Lr1* and/or *Lr10*, 60 accessions showed high infection types (HITs) to *P. triticina* race BBBD. In addition to known genes, 106 accessions contained unidentified or potential novel genes that could not be postulated in our study. Beside seedling tests, rust severity rating was collected from three locations over three years to evaluate field resistance. The rust rating in the field ranged from nearly immune, or highly resistant, with severity of 1% and resistant (R) host response, to highly susceptible, with severity of 84% and susceptible (S) host response. The adult plant resistance gene *Lr34* was found in 52 accessions of which, 40 had a maximum rust severity (MRS) of 35% similar to accession RL6058, a Thatcher-*Lr34* near isogenic line. Many accessions showed immune

to high levels of resistance under field conditions. Our data shows that the high levels of resistance were achieved as a result of synergy between adult plant resistance (APR) genes or between APR and seedling genes or with three or more seedling genes. Immune or highly resistant accessions are potential sources of resistance for wheat breeding programs to improve leaf rust resistance.

5.2 Introduction

Leaf rust, caused by the fungal pathogen *Puccinia triticina* Eriks., has been the most prevalent disease in wheat producing areas (Kolmer, 2005). This fungus is adapted to a wide range of environments and it can co-exist with wheat wherever it is grown (Wamishe and Milus, 2004). It can cause significant yield loss reaching 15% or more depending on the crop developmental stage at infection and the susceptibility of the cultivars (Samborski, 1985). Genetic resistance has been the most effective method to control this disease because it constitutes an environmentally friendly and cost effective long term strategy to minimize yield losses (Pink, 2002).

Leaf rust resistance genes named *Lr1* to *Lr68* have been characterized in bread wheat, durum wheat and diploid wheat species. These genes are located on 18 of the 21 chromosomes of hexaploid wheat (McIntosh *et al.* 2007). Most *Lr* genes confer race-specific resistance and follow the gene for gene theory leading to a hypersensitive response (HR) or programmed cell death (Flor 1942). Through co-evolution of host and pathogen, most of these genes have been overcome by new pathogen races. Between 1938 and 1964, Australia released many varieties containing single *Lr* genes which consequently resulted in an increase in the frequency of corresponding virulent isolates of

P. triticina (Park et al. 2001). Gene pyramiding is therefore a viable strategy to provide more durable resistance by slowing down the rate with which single resistance genes are overcome. *Lr* seedling genes may be postulated upon inoculation with leaf rust isolates with known avirulence and virulence patterns on wheat differential sets, i.e., cultivars with known *Lr* genes. Initially established by Loegering et al. (1971) and Browder (1973), gene postulation has been a method of choice for several decades. This method was used to survey leaf rust resistance genes in several collections including a world collection of winter wheats (McVey, 1992), American hard red spring wheats (Statler, 1984; Oelke and Kolmer, 2004), American hard red winter wheats (McVey and Long, 1993b), American soft red spring wheats (Kolmer, 2003; Wamishe and Milus, 2004), Mexican wheat cultivars (Singh and Rajaram, 1991; Singh, 1993), cultivars from Ethiopia and Germany (Mebrate et al. 2008), Chinese cultivars (Singh et al. 1999; Li et al. 2010), British cultivars (Singh et al. 2001), Argentinean wheat cultivars (Vanzetti et al. 2011) and Eastern, Western and Northern European germplasm (Bartos and Valkoun, 1988; Winzeler et al. 2000; Park et al. 2001; Herrera Foessel, 2001).

A few *Lr* genes conferring resistance at the adult plant stage have also been characterized. These genes include *Lr12* (McIntosh and Baker, 1966), *Lr13* (Dyck et al. 1966), *Lr22a,b* (Dyck, 1979), *Lr34* (Dyck, 1987), *Lr35* (Kerber and Dyck, 1990), *Lr37* (Bariana and McIntosh, 1993), *Lr*46 (Singh et al. 1998), *Lr67* (Herrera-Foessel et al. 2011; Hiebert et al. 2010) and more recently *Lr68* (Herrera Foessel et al. 2012). Genes *Lr34, Lr46* and *Lr67* provide partial resistance or slow rusting to leaf rust and are considered more durable than seedling resistance genes (Caldwell, 1968). The mode of action of these genes is characterized by a longer latent period, a lower infection

frequency, smaller uredinia size, a shorter period of sporulation and a lower spore density (Caldwell, 1968). Among these genes, *Lr34* has not only been durable, but it has also been demonstrated to act synergistically with other leaf rust resistance genes (German and Kolmer, 1992).

Efficient utilization of genetic resistance relies on an accurate and thorough knowledge of the leaf rust resistance genes and gene combinations and of their effectiveness in different environments. Such knowledge would undoubtedly increase our understanding of the durability of the genes and assist in pyramiding resistance genes in adapted germplasm. The goals of the current study were to characterize the seedling and adult plant *Lr* resistance genes present in a world collection (WC) and to evaluate their efficiency under field conditions in order to identify potential sources of novel resistance and superior gene combin ations for use in breeding programs.

5.3 Materials and methods

5.3.1 Seedling gene analysis

Two hundred and seventy five wheat lines collected from 42 countries were surveyed. These lines include wheat cultivars, breeding lines and landraces of hexaploid wheat. Additionally, 30 Thatcher near isogenic lines (NILs) of wheat with known *Lr* genes constituted the differential set (Table 5.1).

Ten races of the leaf rust pathogen *Puccinia triticina* were tested on the wheat germplasm and on the differential set. These races were BBBD, MBDS, MGBJ, TJBJ, TDBG, MBRJ, PBDQ, THMJ, TNRJ and TCRJ. The virulence/avirulence patterns of these races to differentials of seedling *Lr* genes can be found in Table 5.1.

Five seeds from each accession were planted in 30 x 25 cm fiber flats with 25 accessions in each flat. A mixture of soil, sand and peat moss was used as a growing medium. The fungicide Captan was applied at seeding to prevent fungal infection at germination. The seeded flats were placed in a greenhouse (20-25°C and 16 hours light) and watered as required. Twelve days after planting, i.e., at the two leaf stage, the seedlings were inoculated with a single race. The inoculation was conducted by spraying the spore solution onto the seedlings using micro-inoculators (University of Minnesota, Minneapolis, USA). The inoculated seedlings were left at ambient environment for 1 hr prior to being placed in a humidity chamber for 24 hr under conditions of 100% humidity and 20°C. After incubation, the flats were returned to the greenhouse and watered daily. Fourteen days after inoculation (DAI), the infection types (ITs) were scored for each race using the 0 to 4 modified Stakman Scale (Roelfs and Singh, 1992). ITs of 3 or higher refer to a high infection type (HIT) while ITs of 2 or less were low infection type (LIT). The seedling gene postulation was conducted by comparing the IT patterns of the WC accessions to those of the differential lines.

5.3.2 Molecular marker analysis

DNA extraction of the WC was performed as described by Dakouri et al. (2010). Four molecular markers specific to *Lr1* (Cloutier et al. 2007), *Lr10* (Schachermayr et al. 1997), *Lr21* (Huang and Gill, 2001) and *Lr34* (Dakouri et al. 2010) were assessed to confirm the presence or absence of these genes in the WC. Primer names, sequences and PCR conditions for each marker are listed in Appendix VII.

5.3.3 Field experiments

The world collection was planted in a randomized complete block design (RCBD) with two replications at each of the three locations, namely Winnipeg (WPG), Glenlea (GLN) and Portage La Prairie (POR), Manitoba, Canada in 2009, 2010 and 2011. Ten seeds of each accession were seeded per hill in WPG and GLN and single 50-cm rows in POR with 25 cm between hills or rows. The wheat cultivars Mckenzie, Thatcher and Thatcher-*Lr34* NIL RL6058 were used as checks. Spreader rows of the highly leaf rust susceptible cultivar Morocco were planted between groups of five rows or hills. Leaf rust epidemics were established by inoculating the entire experiments with a mixture of leaf rust races. In 2009, a mixture of the following races was used: MBDS, MBPS, MBTS, MDNS, MDPS, MFDS, MFNS, MFPS, MLDS, PDBB, TDBG, TDBJ, TFBJ, TFBS, TGBJ, TJBG and TLDS. In 2010 experiments, the following races were used: MBPS, MDNS, MDPS, MFDS, MFNS, MFPS, MJDS, MLDS, MNDS, PBDG, PBDQ, TBBJ, TBJS, TDBG, TDBJ, TFBG, TMGJ, TNBQ and TNPS. In 2011, the following races were used: MBPS, MCNS, MDDS, MDNS, MDPS, MDTS, MFDS, MFNS, MFPS, MLDS, PBDQ, PBJQ, TBBG, TBBJ, TDBG, TDBJ, TDGJ, TNBG and TNBJ. Rust severity ratings and reaction types using a modified Cobb scale (Peterson et al. 1948) were collected at maximum rust severity (MRS) which was determined to be when the susceptible check Thatcher showed maximum severity. The host reaction type was evaluated as follows: R , no uredinia present; MR, small uredinia with necrosis and light sporulation; MR-MS, small to medium size uredinia with moderate sporulation; MS, medium size uredinia with moderate to intensive sporulation; S, large uredinia with abundant sporulation.

5.3.4 Data analysis

The SAS software was used to perform an analysis of variance (ANOVA) using the PROC MIXED model. Each site × year was considered one environment. All factors and their interactions were random effects.

5.4 Results

5.4.1 Seedling and adult plant resistance genes

The presence of seedling genes *Lr1*, *Lr10* and *Lr21* in the WC were determined using gene specific markers. Marker analysis revealed that *Lr1* and *Lr10* were present in 74 and 69 accessions, respectively including 24 accessions that had both genes while *Lr21* was not present in the collection (Table 5.2). Gene postulation was applied to determine the other seedling resistance genes in the WC. The ITs of 30 differential lines to the ten races of *P. triticina* are shown in Table 5.1. A total of 14 genes were postulated: *Lr1*, *Lr2a*, *Lr2c*, *Lr3*, *Lr3ka*, *Lr9*, *Lr10*, *Lr14a*, *Lr14b*, *Lr15*, *Lr20*, *Lr26*, *Lr28* and *Lr30* (Table 5.2). *Lr20* was postulated in 86 accessions, *Lr3* in 61, *Lr28* in 19, *Lr15* in 13, *Lr14a* in 12, *Lr2c* in 4 and *Lr30* and/or *Lr3ka* in two accessions while *Lr2a*, *Lr9*, *Lr14b* and *Lr26* were each postulated in a single accession (Table 5.2). *Lr2a*, *Lr2b*, *Lr11*, *Lr16*, *Lr17*, *Lr24*, *Lr*25, *Lr3bg* and *LrB* were not postulated in any accessions because none of the accessions showed IT patterns similar to their corresponding differential lines. *Lr18*, *Lr19*, *Lr21*, *Lr29* and *Lr32* had LITs to the ten races and also may not be present in the WC. Marker

Table 5.1 Seedling infection types of 30 differential lines with known genes tested with 10 races of *Puccinia triticina*

Differential sets	*Lr* gene	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ
RL 6003	*Lr1*	0	3	3	3	3	33+	33+	3	3	3
RL 6016	*Lr2a*	0	0	0	3	33+	0	2	3	3	3
RL 6019	*Lr2b*	0	0	0	3	3	1	2	3	3	3
RL 6047	*Lr2c*	1	1	1	3++	3	0	3	3	3	3
RL 6002	*Lr3*	1-	3-	3	3	3	33+	3	3	3	3
RL 6010	*Lr9*	0	0	0	0	0	;	0	0	3	0
RL 6005	*Lr16*	1+	2+	3-	3	2	22+	;1	3	2	2
RL 6064	*Lr24*	0	0	0	3++	33-	0	0	;	3	;
RL 6078	*Lr26*	1-	2+	1	2	23	;	2	3	;	3
RL 6007	*Lr3Ka*	1	2	2	2	2	33+	2	3	3	3
RL 6053	*Lr11*	2	2+	2	2+	2	33+	2	2	3	3
RL 6008	*Lr17*	1	3	1	1+	21	2	3	;1	0	;1
RL 6049	*Lr30*	2	2	2	2+	2	33+	2	3	3	3

Differential sets	*Lr* gene	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ
RL 6051	*LrB*	2	3	2+	2+	23	23	3	2	2	2
RL 6042	*Lr3bg*	;	3	3	2	2	2	3	x	X	X
RL 6004	*Lr10*	1-	3	3-	3	33-	33+	3	3	3	3
RL 6013	*Lr14a*	3	3	3	3	X	33+	2	3	3	3
RL6006	*Lr14b*	3	3	3	3S	X	33+	;1	3	0	3
RL 6052	*Lr15*	3-	3	3	3	33+	33+	3	3	2	3
RL 6009	*Lr18*	2	2+	1	2	21	22+	22+	22+	2	23
RL 6040	*Lr19*	0	0	0	0	0	0	0	0	0	0
RL 6092	*Lr20*	3	3	3	3	2	33+	3	3	3	3
RL 6043	*Lr21*	2	2+	1	1	2	;	2	2	0	2
RL 6012	*Lr23*	3-	3	2	3	33+	33+	2	3	0	3
RL 6094	*Lr25*	0	0	0	1	;	;	3	0	0	2
RL 6079	*Lr28*	0	0	3	3	33+	33+	33+	3	3	3
RL 6080	*Lr29*	1	1	1	1	13-	2	x	;2	;	2

Differential sets	*Lr* gene	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ
RL 5497	*Lr32*	2	2	1	1+	2	3	x	x	0	2
Thatcher	Thatcher	3	3	3	3++S	33+	33+	3	3	3	3
-	*LrW*	2	2	2+	1	33-	33+	;	;	0	;1

Infection types: 0 = no flecks or uredinia, 0; = faint hypersensitive flecks, ; = hypersensitive flecks, 1 = small uredinia with necrosis, 2 = small to medium uredinia with necrosis, 3 = moderate to large size uredinia with/without chlorosis, 4 = very large uredinia without chlorosis,"+" = indicates slightly larger uredinia, "-" = indicates slightly smaller uredinia, ITs with two symbols denote a range in IT: 22+= indicates a mixture of 2 sizes of uredinia with chlorosis and slightly larger uredinia with chlorosis.

Table 5.2 Seedling gene postulation based on infection types of 10 races of *P. triticina* and molecular data, and evaluation of maximum rust severity and host response of a world collection of 275 wheat accessions

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
Group1																			
Neepawa	*none*	3	3	3	3	33+	33+	33+	3	33+	3				61	4	MRMS	S	S
Kanred	*none*	3	3	3	3	3	3	33+	3	33+	3				4	1	R	MS	R
Chinese Spring	*none*	3	3	3	3	3+	3	33+	3	33+	3			*Lr34*	2	1	MS	?	R
CN33802	*none*	3	3	3	3	4	33+	33+	3	3	3				78	3	?	?	MS
CN11739	*none*	3	3	3	3	33+	33+	33+	3	3	3				70	4	S	MSS	MSS
G3893	*none*	3	3	3	3	33+	33+	3	3	3	3				30	5	MR	MSS	S
CWI42988	*none*	3	3	3	3	33+	3	33+	3	3	3				41	5	MRMS	MS	R
Surkh	*none*	3	3	3	3	33+	33+	3	3	3	3				78	2	S	S	S
Li Yang Wong Shu Bai	*none*	3	3	3	3	33+	3	33+	3	3	3			*Lr34*	4	1	MS	R	MS
Ajelea	*none*	3	3	3	3	33+	33+	33+	3	3	3				2	1	?	R	R
Nyu Bay	*none*	3	3	3	3	33+	33+	33+	3	3	3			*Lr34*	14	3	MS	MS	MS

Accession name	Seedling genes	BB BD	MB DS	MG BJ	TJ BJ	TD BG	MB RJ	PB DQ	TH MJ	TN RJ	TC RJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
CN29856	*none*	3	3	3	3	43+	33+	33+	3	3	3				63	3	MS	S	MS
CN29854	*none*	3	3	3	3	33+	33+	33+	3	3	3				53	4	S	S	MSS
CN9934	*none*	3	3	3	3	33+	33+	3	3	3	3				21	4	R	R	MS
CN9936	*none*	3	3	3	3	33+	3	33+	3	3	3				40	8	R	MS	MS
CN12251	*none*	3	3	3	3	33+	33+	3	3	3	3				13	6	MRMS	R	MR
Dvina	*none*	3	3	3	3	33+	33+	3	3	3	3				70	6	S	S	S
Golubka	*none*	3	3	3	3	33+	33+	33+	3	3	3				36	6	?	MS	MS
Gorkorskaja 15	*none*	3	3	3	3	3	33+	33+	3	3	3				25	3	MRMS	MS	MS
Narinskajal	*none*	3	3	3	3	33+	33+	33+	3	3	3				66	5	S	S	S
Odess kaja 13	*none*	3	3	3	3	3	33+	33+	3	3	3				48	7	S	S	S
Pamjat Urala	*none*	3	3	3	3	33+	33+	33+	3	3	3				65	4	MSS	S	S
Severodvinskaja 1	*none*	3	3	3	3	33+	33+	33+	3	3	3				70	6	S	S	S
Udarnica	*none*	3	3	3	3	33+	33+	33+	3	3	3				65	7	S	S	S

Accession name	Seedling genes	BB BD	MB DS	MG BJ	TJ BJ	TD BG	MB RJ	PB DQ	TH MJ	TN RJ	TC RJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
CN11461	*none*	3	3	3	3	3	33+	33+	3	3	3				41	4	S	S	MSS
Comeback	*none*	3	3	3	3	33+	33+	33+	3	3	3				56	5	MS	S	S
Fylby	*none*	3	3	3	3	33-	33+	33+	3	3	3				57	4	S	S	S
CN40750	*none*	3	3	3	3	33-	33+	3	3	3	3				50	3	MRMS	MS	S
Lada	*none*	3	3	3	3	33-	33+	3	3	3	3				9	3	MR	R	MS
Falerio	*none*	3	3	3	3	33+	33+	3	3	3	3			*Lr34*	21	6	MSS	R	R
Albimonte	*none*	3	3	3	3	33+	33+	33+	3	3	3			*Lr34*	7	2	MSS	R	MS
CN11642	*none*	3	3	3	3	33+	33+	3	3	3	3				39	6	S	S	MS
CN12361	*none*	3	3	3	3	33+	33+	33+	3	33+	3				52	5	MS	?	?
CN11720	*none*	3	3	3	3	33+	33+	3	3	3	3				38	7	MS	MS	MS
CN10661	*none*	3	3	3	3	33+	33+	3	3	3	3			*Lr34*	35	5	?	MS	MS
Bunyip	*none*	3	3	3	3	33+	33+	3	3	3	3				57	5	S	S	MS
Bungulla	*none*	3	3	3	3	33+	33+	3	3	3	3				46	6	MS	S	MS

Accession name	**Seedling genes**	**BB BD**	**MB DS**	**MG BJ**	**TJ BJ**	**TD BG**	**MB RJ**	**PB DQ**	**TH MJ**	**TN RJ**	**TC RJ**	**Marker data**			**MRS**	**SE**	**HR**		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
Vijay	*none*	3	3	3	3	33+	3	33+	3	?	3				24	4	MS	S	R
Dorziyeh karak	*none*	33+	3	33-	?	33+	3	3	3	3	3				6	1	?	R	?
Krasnaja Svenzda	*none*	3	3	33-	33+	33+	33+	33+	3	3	3				54	5	MS	S	S
Group2																			
CN99032	*N*	3	3	2	3	33+	3	33+	3	3	3				1	0	R	R	R
Preludio	*N*	3	3	3	1	33+	33+	3	3	33+	3				3	1	MR	R	R
Kenya Farmer	*N*	3	2	3	3	3	33+	3	3	3	3				28	4	MR	R	MS
Santa Elena	*N*	33+	3	3	3	21	3	3	3	3	3				53	5	MS	R	MS
CN9940	*N*	3	2	33-	3	33+	3	33+	3	3	3				10	2	MRMS	MS	R
Beloglina	*N*	3	3	3	3	33-	3	;2	3	3	3				34	4	S	S	?
Strubes Fortschritt	*N*	3	3	33-	3	33+	33+	;1	3	3	3				42	5	MS	MS	MS
Atacatzo 1	*N*	3	3	3	33+	33+	33+	;1	3	3	3				76	3	S	S	S
CN10750	*N*	3	3	2	2	33+	33+	3	3	3	3				44	6	MSS	MSS	S

Accession name	Seedling genes	BB BD	MB DS	MG BJ	TJ BJ	TD BG	MB RJ	PB DQ	TH MJ	TN RJ	TC RJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
Klein Credito	*N*	3	2	1	2	33+	3	33+	3	3	3				17	3	MR	MS	MS
CN12624	*N*	3	3	2	2	33+	2	3	;12	3	3				7	2	MR	R	R
Group 3																			
Subgroup 1																			
Red River 68	*Lr1*	0	3	3	1	33+	3	33+	3	33+	3	*Lr1*			13	3	MR	MRMS	MS
Shu Chou Wheat #3	*Lr1*	;	3	3	3	33+	33+	3	33+	3	3	*Lr1*		*Lr34*	21	3	MS	MSS	MSS
Shinchunaga	*Lr1*	2	3	3	3	33+	33+	3	3	3	3	*Lr1*			37	5	MSS	MSS	MS
Kostoff's Triple hybrid	*Lr1*	1	3	3	3	3	33+	33+	3	3	3	*Lr1*			29	4	MS	S	MS
Iskra	*Lr1*	2	3	3	3	33+	33+	33+	3	3	3	*Lr1*			56	6	R	MR	?
Fruher Tiroler Bin	*Lr1*	2	3	3	3	3	33+	33+	3	3	3	*Lr1*			51	5	MS	MSS	S
Ventura	*Lr1*	1	3	3	3	33+	33+	3	3	3	3	*Lr1*			44	6	MS	S	S
Endeavor	*Lr1*	0	3	3	3	3	33+	3	3	3	3	*Lr1*			29	6	MS	MS	S
cailloux	*Lr1*	3	3	3	3	33+	33+	33+	3	33+	3	*Lr1*			55	3	?	S	S

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
CN12213	*Lr1*	3	3	3	3	33+	33+	33+	3	3	3	*Lr1*			65	4	MSS	MSS	S
Shahpassand	*Lr1*	3	3	3	3	33+	33+	33+	3	3	3	*Lr1*			84	2	S	S	?
Moskovka	*Lr1*	3	3	3	3	33+	33+	33+	3	3	3	*Lr1*		*Lr34*	53	5	MS	MS	S
Buffum	*Lr1*	3	3	3	3	33-	33+	3	3	3	3	*Lr1*			48	4	?	S	S
Maja	*Lr1*	3	3	3	3	33+	33+	3	3	3	3	*Lr1*			48	4	MS	S	S
Kinsman	*Lr1*	3	3	3	3	3+	33+	3	3	3	3	*Lr1*			8	3	?	S	S
Heines Peko	*Lr1*	3	3	3	3	33+	33+	3	3	3	3	*Lr1*			32	6	S	MSS	MSS
CN12425	*Lr1*	3	3	3	3	3	33+	3	3	3	3	*Lr1*			16	4	MR	R	R
Ho chun No. 12	*Lr10*	2	3	3	3	33+	33+	33+	3	3	3		*Lr10*		48	5	MS	S	MS
Etoile De Choisy	*Lr10*	2	3	3	3	33+	33+	33+	3	3	3		*Lr10*		58	6	S	MS	S
Magueija	*Lr10*	2	3	3	3	33+	33+	3	3	3	3		*Lr10*	*Lr34*	3	2	R	?	R
Bonza	*Lr10*	0	3	3	3	33+	33+	3	3	3	3		*Lr10*	*Lr34*	18	4	MRMS	R	5jw
Chara	*Lr10*	2	3	3	3	33+	33+	3	3	3	3		*Lr10*	*Lr34*	30	6	?	S	?

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
Federation	*Lr10*	1	3	3	3	33+	33+	3	3	3	3		*Lr10*		60	6	MS	S	MSS
CN33892	*Lr10*	2	3	3	3	33+	?	33+	3	3	3		*Lr10*		25	5	S	S	R
CN33893	*Lr10*	3	3	3	3	33+	33+	33+	3	3	3		*Lr10*		7	3	R	R	R
Kenphad 25	*Lr10*	3	3	3	3	33+	3	33+	3	3	3		*Lr10*		46	4	MRMS	MSS	MSS
Sonalika	*Lr10*	33+	3	3	3	3	33+	33+	3	3	3		*Lr10*		47	6	MS	S	R
Irkutskaja 49	*Lr10*	3	3	3	3	33+	33+	33+	3	3	3		*Lr10*		67	6	S	S	S
Krasnokutka 3	*Lr10*	3	3	3	3	33+	33+	3	3	3	3		*Lr10*		72	4	MSS	S	S
CN9794	*Lr10*	3	3	3	3	33+	3	33+	3	3	3		*Lr10*		25	6	?	?	R
Hard Federation	*Lr10*	3	3	3	3	33+	33+	3	3	3	3		*Lr10*		50	5	S	S	MS
Hofed	*Lr10*	3	3	3	3	33+	3	3	3	3	3		*Lr10*		44	4	MRMS	S	S
Sinvalocho	*Lr3*	0	3	3	3	33+	33+	3	3	33+	3			*Lr34*	18	3	S	MS	R
CN2758	*Lr3*	1	3	3	3	33+	33+	33+	3	33+	33+				7	2	MS	MS	MS
CN11647	*Lr3*	2	3	3	3	33+	33+	33+	3	3	3				43	6	S	S	S

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
CN33803	*Lr3*	2	3	3	3	33+	33+	33+	3	3	3				17	5	MS	R	R
Su Mai No 3	*Lr3*	1	3	3	3	33+	33+	3	3	3	3			*Lr34*	47	7	S	S	S
Azar	*Lr3*	2	3	3	3	33+	33+	3	3	3	3				73	3	S	S	S
Norin 75	*Lr3*	2	3	3	3	33+	3	4	3	3	3				71	4	?	?	S
Nobeoka Bozu	*Lr3*	2	3	3	3	33+	33+	33+	3	3	3			*Lr34*	14	2	MS	MS	S
Ikuzai #1	*Lr3*	2	3	3	3	33+	33+	33+	3	3	3			*Lr34*	42	7	?	S	?
CN42522	*Lr3*	2	3	3	3	33+	33+	3	3	3	3				70	3	MS	R	MS
CN29871	*Lr3*	2	3	3	3	33+	33+	33++	3	3	3				61	5	MS	R	MS
CN29858	*Lr3*	2	3	3	3	33+	33+	33++	3	3	3				69	2	S	S	S
CN10241	*Lr3*	2	3	3	3	33+	33+	3	3	3	3				15	4	?	R	?
Ferrugineum87	*Lr3*	2	3	3	3	33+	33+	33+	3	3	3				18	3	MS	MS	MS
karagandinskaja	*Lr3*	2	3	3	3	33+	33+	33+	3	3	3				44	6	MS	MS	MSS
Vega	*Lr3*	2	3	3	3	33+	33+	33+	3	3	3				46	5	?	S	MSS

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
71GN No 115	*Lr3*	2	3	3	3	3	33+	33+	3	3	3				51	7	MR	MS	MS
Sandra	*Lr3*	1	3	3	3	33+	33+	3	3	3	3				51	4	MS	S	S
CN32504	*Lr3*	2	3	3	3	33+	33+	3	3	3	3			*Lr34*	31	6	S	RMR	R
Argentina	*Lr3*	1	3	3	3	33+	33+	3	3	3	3			*Lr34*	17	5	MS	R	R
Klein otto Wulff	*Lr3*	0	3	3	3	33+	33+	3	3	3	3				17	4	MS	MSS	MS
CN11307	*Lr3*	;	3	3	3	33+	33+	3	3	3	3				52	6	S	S	MS
Gabo	*Lr3*	1	3	3	3	33+	33+	3	3	3	3				59	3	MS	S	S
Hilgendorf	*Lr3*	1	3	3	3	33+	3	3	3	3	3			*Lr34*	1	0	MS	R	R
Sunbird	*Lr9*	0	;	;	0	23	0	X	;	3	;			*Lr34*	35	6	MS	R	MS
Onohoiskaja 4	*Lr20*	3	3	3	3	22+	33+	33+	3	3	3				45	4	MS	MS	S
Ladoga	*Lr20*	3	3	3	3	X	33+	33+	3	3	3				55	5	S	S	S
Janetzkis Probat	*Lr20*	3	3	3	3	22+	33+	3	3	3	3				41	5	MSS	S	S
Da Maia	*Lr20*	3	3	3	3	22+	33+	3	3	3	3				41	4	MS	MSS	MS

Accession name	Seedling genes	BB BD	MB DS	MG BJ	TJ BJ	TD BG	MB RJ	PB DQ	TH MJ	TN RJ	TC RJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
Red Marvel	*Lr20*	3	3	3	3	22+	33+	33+	3	3	3				12	4	MS	R	?
Little Joss	*Lr20*	3	3	3	3	22+	33+	3	3	3	3				46	6	MS	MS	MSS
Holdfast	*Lr20*	3	3	3	3	22+	33+	3	3	3	3				1	0	MS	R	MS
CN6030	*Lr20*	3	3	3	3	22+	33+	3	3	3	3			*Lr34*	41	7	MR	S	MS
John Brown	*Lr20*	33+	3	3	3	2	33+	3	3	3	3			*Lr34*	43	5	MRMS	S	S
Cross 7	*Lr20*	3	3	3	3	;	33+	3	3	3	3				39	7	MS	MS	S
Oroua	*Lr20*	3	3	3	3	21	33+	3	3	3	3				14	2	MR	MS	MS
Giza 141	*Lr20*	3	3	3	?	22+	33+	33+	3	3	3				18	5	?	MSS	R
Fronteira	*Lr20*	3	3	3	33+	22+	33+	3	3	3	3				25	3	MR	S	S
Janetzkis Fruher S	*Lr28*	2	2	3	3	33+	33+	33+	3	3	3				45	4	S	S	S
Prospur	*Lr28*	0	2	3	3	33-	33+	33+	3	3	3			*Lr34*	36	4	MR	MSS	MS

Accession name	**Seedling genes**	**BBBD**	**MBDS**	**MGBJ**	**TJBJ**	**TDBG**	**MBRJ**	**PBDQ**	**THMJ**	**TNRJ**	**TCRJ**	**Marker data** *Lr1*	*Lr10*	*Lr34*	**MRS**	**SE**	**HR** WPG	GLN	POR
Subgroup 2																			
Glenavon	*Lr1,Lr10*	0	3	3	3	33+	33+	3	3	33+	3	*Lr1*	*Lr10*	*Lr34*	28	5	RMR	?	MS
Koktunkuskaja 332	*Lr1,Lr10*	0	3	3	3	33+	33+	33+	3	3	3	*Lr1*	*Lr10*		47	4	MSS	S	MS
Cargimarec	*Lr1,Lr10*	0	3	3	3	33+	33+	3	3	3	3	*Lr1*	*Lr10*		22	5	?	S	S
Hohenhein	*Lr1,Lr10*	;	3	3	3	3	33+	3	3	3	3	*Lr1*	*Lr10*		50	6	S	S	MSS
Jakutjanka	*Lr1,Lr10*	3	3	3	3	3	33+	33+	3	3	3	*Lr1*	*Lr10*		65	4	80	S	S
Kamalinka	*Lr1,Lr10*	3	3	3	3	33+	33+	33+	3	3	3	*Lr1*	*Lr10*		49	5	MS	?	MS
Krasnojarskaja 1103	*Lr1,Lr10*	3	3	3	3	33+	33+	3	3	3	3	*Lr1*	*Lr10*		69	3	S	S	S
Penkop	*Lr1,Lr10*	3	3	3	3	33+	33+	3	3	3	3	*Lr1*	*Lr10*		64	4	MSS	S	MSS
White Federation	*Lr1,Lr10*	3	3	3	3	?	33+	3	3	3	3	*Lr1*	*Lr10*		59	6	?	S	R
Carina	*Lr1,Lr2c*	0	0	0	3	33+	0	33+	3	3	3	*Lr1*			65	4	S	MSS	S
Hsin ShuKuan#1	*Lr1,Lr14a*	3	3	3	3	X	33+	x	3	33+	3	*Lr1*			50	5	MSS	S	MS
New Pusa	*Lr1,Lr20*	2	3	3	3	;	33+	3	3	3	3	*Lr1*			25	4	MR	MS	MS
Retacon INTA	*Lr1,Lr20*	0	3	3	2	33+	33+	33+	3	3	3	*Lr1*			22	5	MS	S	MS

Accession name	**Seedling genes**	**BB BD**	**MB DS**	**MG BJ**	**TJ BJ**	**TD BG**	**MB RJ**	**PB DQ**	**TH MJ**	**TN RJ**	**TC RJ**	**Marker data** *Lr1*	*Lr10*	*Lr34*	**MR S**	**S E**	**HR** WPG	GLN	POR
CN12666	*Lr1,Lr20*	1	3	3	3	0	33+	33+	3	3	3	*Lr1*		*Lr34*	46	4	?	S	MS
CN6025	*Lr1,Lr20*	0	3	3	3	22+	33+	3	3	3	3	*Lr1*			58	5	S	S	S
Sabre	*Lr1,Lr20*	1	3	3	3	2	3	3	3	3	3	*Lr1*		*Lr34*	34	7	S	?	MS
Pobeda	*Lr1,Lr20*	3	3	3	3	2	33+	33+	3	3	3	*Lr1*			66	4	MSS	S	S
CN12442	*Lr1,Lr20*	3	3	3	3	22+	33+	3	3	3	3	*Lr1*			47	6	MS	S	S
Kenya Governor	*Lr1,Lr20*	3	3	3	3	22+	3	3	3	3	3	*Lr1*		*Lr34*	30	4	MS	S	MSS
NING 8331	*Lr1,Lr26*	1	2	1	;	X	;	;	3	;	33-	*Lr1*			3	1	?	?	?
Wildcat	*Lr1,N*	0	3	3	3	33+	33+	X	3	33+	3	*Lr1*			66	5	MR MS	MSS	S
Xiang Mai No 1	*Lr1,N*	1	3	3	2	33+	3	3	3	3	3	*Lr1*			9	3	MR	MS	R
Touse	*Lr1,N*	2	3	3	0	33+	33+	33+	3	3	3	*Lr1*			69	3	S	S	MS
Buck Pucara	*Lr1,N*	0	3	3	3	33+	33+	;1	3	3	3	*Lr1*			19	4	MR	R	MS
Kukri	*Lr1,N*	0	3	3	2	33-	33+	3	3	3	3	*Lr1*			26	5	MR MS	MS	MSS
Tobari F66	*Lr1,N*	0	3	3	2	33-	33+	;	3	3	3	*Lr1*			26	5	MR MS	MS	R
CDC Teal	*Lr1,N*	2	3	2	1	33+	X	0	3	21	3	*Lr1*		*Lr34*	16	4	MR	MS	MS

Accession name	Seedling genes	BB BD	MB DS	MG BJ	TJ BJ	TD BG	MB RJ	PB DQ	TH MJ	TN RJ	TC RJ	Marker data *Lr1*	*Lr10*	*Lr34*	MR S	S E	HR WPG	GLN	POR
Klein Universal	*Lr1,N*	3	3	0	2	33-	3	3	3	3	3	*Lr1*			10	4	MS	R	MSS
Fengcheung No. 1	*Lr1,N*	3	3	2	3	33+	3	33+	3	3	3	*Lr1*			78	2	S	S	S
Jufy 2	*Lr1,N*	3	3	3	2	33+	33+	3	3	3	3	*Lr1*			65	4	SMS	S	S
Bersee	*Lr10, Lr14a*	3	3	3	3	33+	33+	3	3	3	3		*Lr10*		15	3	MR	RMR	R
Pirana	*Lr10,Lr20*	2	3	3	3	;	33+	3	3	3	3		*Lr10*	*Lr34*	15	3	MS	S	MS
Dominator	*Lr10,Lr20*	1	3	3	3	22+	33+	33+	3	3	3		*Lr10*		70	4	SMS	S	S
CN6024	*Lr10,Lr20*	1	3	3	3	;12	33+	3	3	3	3		*Lr10*		41	4	S	S	S
CN12388	*Lr10,Lr20*	2	3	3	3	22+	33+	3	3	3	3		*Lr10*	*Lr34*	27	3	MS	MS	MS
Daeraad	*Lr10,Lr20*	1	3	3	3	0	3	3	3	3	33-		*Lr10*	*Lr34*	36	5	MR MS	S	S
CD87	*Lr10,Lr20*	;	3	3	3	2	33+	3	3	3	3		*Lr10*		36	4	MS	S	S
CN12035	*Lr10,Lr20*	1	3	3	3	0	33+	3	3	3	3		*Lr10*		47	7	MR	R	R
Trintani	*Lr10,Lr20*	3	3	3	3	22+	33+	33+	3	3	3		*Lr10*		37	6	MR MS	S	S
Olaeta Calandria	*Lr10,Lr20*	3	3	3	3	X	33+	33+	3	33+	3		*Lr10*		69	4	MS	S	MS
CN1849	*Lr10,Lr28*	2	1	3	3	33+	33+	3	3	3	3		*Lr10*		47	4	MS	MSS	MS

Accession name	**Seedling genes**	**BBBD**	**MBDS**	**MGBJ**	**TJBJ**	**TDBG**	**MBRJ**	**PBDQ**	**THMJ**	**TNRJ**	**TCRJ**	**Marker data** *Lr1*	*Lr10*	*Lr34*	**MRS**	**SE**	**HR** WPG	GLN	POR
CN12261	*Lr10,N*	1	3	1	3	33+	22+	33+	3	33+	33-		*Lr10*		20	3	MR MS	R	MS
Hybride Du Jubile	*Lr10,N*	3	3	3	3	33-	22+	3	3	3	3		*Lr10*		56	3	S	S	MSS
Phoebus	*Lr10,N*	3	3	1	3	33-	33+	3	3	3+	33-+		*Lr10*		9	4	MR	R	R
Werna	*Lr10,N*	3	2	3	3	33+	2	33+	3	3	3		*Lr10*		31	3	MR	R	S
Combate	*Lr10,N*	3	3	3	3	33+	3	x	3	3	33+		*Lr10*		22	3	MR	S	S
Arawa	*Lr3,Lr14a*	2	3	3	3	21	33+	;1	3	3	3				13	2	MR MS	R	R
Ladoga	*Lr3,Lr15*	0	3	3	3	33+	33+	33+	3	;12	3				71	5	MS	MS	S
KVL 2263	*Lr3,Lr20*	2	3	3	3	;	33+	3	3	3	3			*Lr34*	8	1	R	R	MR MS
Chengdu Guangtou	*Lr3,Lr20*	1	3	3	3	;	3	3	3	3	3				6	2	MR MS	R	S
Nan Tong DA Huang PI	*Lr3,Lr20*	0	3	3	3	;1	33+	3	3	3	3				13	3	MR	MS	?
Minskaja	*Lr3,Lr20*	;	3	3	3	;	33+	33+	3	3	3				50	4	S	S	MSS
Setter	*Lr3,Lr20*	2	3	3	3	0	33+	3	3	3	3				18	4	S	R	MS
Musket	*Lr3,Lr20*	2	3	3	3	22+	33+	3	3	3	3				24	5	MS	MSS	MS
Tonic	*Lr3,Lr20*	2	3	3	3	0	33+	3	3	3	3				34	5	MR MS	MS	MS

Accession name	**Seedling genes**	**BBBD**	**MBDS**	**MGBJ**	**TJBJ**	**TDBG**	**MBRJ**	**PBDQ**	**THMJ**	**TNRJ**	**TCRJ**	**Marker data** *Lr1*	*Lr10*	*Lr34*	**MRS**	**SE**	**HR** WPG	GLN	POR
Hope	*Lr3,Lr20*	0	3	3	3	;12	33+	3	3	3	3			*Lr34*	52	4	MRMS	MSS	MSS
Klein Toledo	*Lr3,Lr20*	2	3	3	3	1	33+	3	3	3	3				4	1	R	R	R
Calidad	*Lr3,Lr20*	0	3	3	3	X	33+	33+	3	3	3				30	6	MS	MS	MS
Surpresa	*Lr3,Lr20*	1	3	3	3	22+	33+	3-	3	3	3				25	4	MR	S	MSS
Toropi	*Lr3,Lr20*	2	3	3	3	22+	33+	3	3	3	3				8	2	?	MS	MS
Menkemen	*Lr3,Lr20*	0	3	3	3	22+	3	3	3	3	3			*Lr34*	22	4	?	MSS	R
Maribal 50	*Lr3,Lr20*	;	3	3	3	22+	33+	3	3	3	3				38	5	MS	S	S
Sunstar	*Lr3, N*	2	3	3	3	3	33+	3	3	3	0				37	6	MS	MS	MS
Prinqual	*Lr3, N*	;	3	0	3	33+	33+	3	3	3	3			*Lr34*	25	3	MR	MS	MS
CN12268	*Lr3, N*	2	3	3	3	33+	33+	;	3	3	3				31	4	R	S	MS
Rayhany	*Lr3, N*	`1	3	2	3	33+	?	33+	3	3	3				76	6	S	S	S
Froubou	*Lr3, N*	1	3	3	1	33+	33+	3	3	3	3				2	1	R	R	R
Gogatsukomugai	*Lr3, N*	2	3	2	3	33+	33+	33+	3	3	3				39	6	MS	S	MSS

Accession name	**Seedling genes**	**BB BD**	**MB DS**	**MG BJ**	**TJ BJ**	**TD BG**	**MB RJ**	**PB DQ**	**TH MJ**	**TN RJ**	**TC RJ**	**Marker data** *Lr1*	*Lr10*	*Lr34*	**MRS**	**SE**		**HR**	
																	WPG	GLN	POR
HY 320	*Lr3,N*	2	3	3	2	33+	3	3-	3	33+	0				39	5	R	MRMS	?
Funo	*Lr3,N*	2	3	3	3	33+	22+	3	3	33+	2				38	4	MS	MS	MS
Squarhead's Master	*Lr3,N*	2	3	2	3	33+	33+	x	3	3	3				60	5	S	?	S
Bola Picota	*Lr3,N*	1	3	3	3	33+	2	;	3	3	3				31	5	MS	R	MS
El Gaucho	*Lr2c,N*	2	1	0	;	3	1	3	3	3	3			*Lr34*	2	1	MRMS	R	R
Tropeano	*Lr14a,N*	3	2	3	2	22+	33+	x	3	3	3			*Lr34*	5	2	R	MS	R
NING 8026	*Lr15, N*	2	3	12	2	33+	;	;12	3	;1	3				52	4	MRMS	MS	MS
CN11698	*Lr3,Lr15, N*	3	3	2	2	3	1	3	;	;	;				10	2	MR	R	R
Warden	*Lr20,Lr28*	2	1	3	3	0	33+	33+	3	33+	3				45	5	MS	S	MS
DART	*Lr20,Lr28*	3	2	3	3	21	33+	3	3	3	3				54	6	MS	S	S
BAART	*Lr20,Lr28*	1	2	3	3	22+	33+	3	33+	3	3				51	5	S	S	MS
Girija	*Lr20, N*	3	3	3	2	0	3-	3	3	3	3			*Lr34*	11	3	MS	R	R
Kozlodui	*Lr20, N*	3	2	3	3	2;	33+	3	3	3	3				61	7	S	S	S
Acadimia 48	*Lr20, N*	3	3	3	3	22+	22+	3	3	3	3				37	5	MS	MSS	MS

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data *Lr1*	*Lr10*	*Lr34*	MRS	SE	HR WPG	GLN	POR
Mentana	*Lr20, N*	3	2	3	3	22+	33+	3	3	3	3			*Lr34*	36	5	MS	S	S
Equator	*Lr20, N*	3	2	3	3	22+	3	3	3	3	3				30	7	S	S	S
Kenya 58	*Lr20, N*	3	3	3	2	;12	33+	3	3	3	3			*Lr34*	17	3	MS	MS	MS
Pacific Bluestem	*Lr20, N*	33+	2	3	3	22+	3	3	3	3	3				35	5	MS	R	MS
CN30213	*Lr20, N*	3	2	2	3	;	3	3	3	3	3				31	4	MRMS	MSS	MS
Benvenuto 3085	*Lr20,N*	3	2	3	2	X	33+	33+	3	3	3			*Lr34*	4	1	MS	R	R
Colonias	*Lr20,N*	1	3	2	2	21	33+	3	3	33+	3				16	3	MRMS	S	R
Camadi	*Lr20,N*	3	1	;	2	22+	2	3	3-	3	3				4	1	R	MR	R
Encruzilhada	*Lr28,N*	2	0	2	2	33+	33+	33+	3	3	3				6	2	MR	R	R
Arcola	*Lr28, N*	2	1	2	3	33+	3	3	33+	3	3				3	1	R	?	MS
Lom	*Lr28, N*	2	1	1	3	3	33+	3	3	3+	3				17	5	R	R	R
Dorziyeh Safra	*Lr28,N*	1	1	0	2	33+	3	3	3	3	3				5	1	R	MS	?
Pampa INTA	*Lr28,N*	2	2	2	3	2	33+	3	3	3	3			*Lr34*	3	1	MR	R	MS
AC Minto	*Lr28,N*	1	2	1	2	33+	33+	;3	3	3	3				8	2	R	RMR	R

Accession name	**Seedling genes**	**BB BD**	**MB DS**	**MG BJ**	**TJ BJ**	**TD BG**	**MB RJ**	**PB DQ**	**TH MJ**	**TN RJ**	**TC RJ**	**Marker data** *Lr1*	*Lr10*	*Lr34*	**MRS**	**S E**	**HR** WPG	GLN	POR
Subgroup 3																			
Jumbuck	*Lr3, Lr20, N*	1	3	3	2	;	33+	3	3	3	3			*Lr34*	48	5	MRMS	S	MS
Bluesky	*Lr1,Lr10,N*	0	3	2	3	33+	33+	33+	3	3	3	*Lr1*	*Lr10*		19	3	MS	MS	MR
RL4452	*Lr1,Lr10,N*	0	3	2	3	33+	3	3	3	33+	3	*Lr1*	*Lr10*		28	5	MRMS	MS	S
Roblin	*Lr1,Lr10,N*	0	3	3	2	33+	33+	3	3	3	3	*Lr1*	*Lr10*	*Lr34*	30	5	MR	SMS	MS
Corrine	*Lr1,Lr10,N*	0	3	2	3	33+	3	3	3	3	3	*Lr1*	*Lr10*		16	2	R	SMS	MS
Laura	*Lr1,Lr10,N*	0	3	2	3	33+	33+	3	3	3	3	*Lr1*	*Lr10*	*Lr34*	5	2	MR	R	R
BW 90	*Lr1,Lr10,N*	2	3	3	12	33+	3	3	3	3	3	*Lr1*	*Lr10*		13	2	MRMS	MS	MS
Otane	*Lr1,Lr10, Lr20*	;	3	3	3	2	3	33+	3	3	3	*Lr1*	*Lr10*		31	5	MRMS	S	MS
Biggar	*Lr3, 15, N*	2	3	3	2	33+	3	3	3	22+	3				28	3	?	R	S
CN11204	*Lr1,Lr20,N*	2	3	3	3	;	22+	33+	3	3	3	*Lr1*		*Lr34*	8	2	MS	MS	MS
Katja A1	*Lr1,Lr20, Lr28*	;	2	3	3	32	33+	33+	3	3	3	*Lr1*			24	6	S	S	MS
Parana	*Lr10,Lr20, N*	;	3	3	2	22+	3	3	3	3	3		*Lr10*		19	4	MS	R	R
CN6171	*Lr3,Lr20,N*	1	3	3	2	22+	33+	3	3	3	3				53	5	S	MSS	MS

Accession name	**Seedling genes**	**BBBD**	**MBDS**	**MGBJ**	**TJBJ**	**TDBG**	**MBRJ**	**PBDQ**	**THMJ**	**TNRJ**	**TCRJ**	**Marker data** *Lr1*	*Lr10*	*Lr34*	**MRS**	**SE**	**HR** WPG	GLN	POR
Marquillo	*Lr10,Lr20, N*	2	3	3	3	22+	22+	3	3	3	3		*Lr10*		24	3	MR	MRMS	MS
AC Vista	*Lr3,Lr14a, N*	2	3	3	2	X	33+	;1	3	3	3				29	5	MR	MSS	S
HY358	*Lr3,Lr14a, N*	;	3	3	2	X	33+	x	3	33+	3				21	2	R	MS	MS
Fu Mai-No 3	*Lr1,Lr15,N*	2	3	3	3	33+	2	;1	3	23	3	*Lr1*			37	4	MS	MS	R
Bajio	*Lr1,Lr14a, N*	0	3	3	12	0	33+	X	3	3	3	*Lr1*		*Lr34*	18	3	MR	MS	MS
Victoria INTA	*Lr1,Lr14a, N*	1	3	2	3	;	33+	;	3	3-	3	*Lr1*			16	3	MS	MS	MS
Tezanos Printos Precoz	*Lr3, Lr20,N*	0	3	2	2	21	33+	3	3	3	3			*Lr34*	6	1	MR	MRMS	R
Fortaleza	*Lr3, Lr20,N*	2	3	2	1	0	33+	3	3	33+	3			*Lr34*	3	1	R	R	R
Helvia	*Lr10,Lr14a ,N*	2	3	3	3	22+	22+	;1	3	3	3		*Lr10*		56	5	S	MR	?
Duiker	*Lr1,Lr14a, Lr28*	2	2	3	3	22+	33+	x	3	3	3	*Lr1*			71	3	MR	MRMS	MS
Columbus	*Lr3, Lr15,N*	1	3	3	3	33+	2	;12	3	21	2				49	5	MRMS	MS	S
Yang Mai No.1	*Lr3,Lr15, N*	2	3	3	3	33+	33+	;1	3	12	3				46	5	MS	MSS	S
Buck Nandu	*Lr10,Lr28, N*	0	1	3	3	33+	1	;	3	33+	2		*Lr10*		23	5	MSS	MR	MS
Universal II	*Lr1,Lr28,N*	2	0	2	2	33-	33+	;2	3	3	3	*Lr1*			14	4	S	R	MS

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data			MRS	SE	HR		
												Lr1	*Lr10*	*Lr34*			WPG	GLN	POR
Trintecinco	*Lr10,Lr20, N*	1	3	2	2	0	22+	3	3	33+	3		*Lr10*		11	3	MR	MRMS	MS
Cinquentenario	*Lr3,Lr14a, N*	2	3	3	2	22+	22+	;1	3	33+	3				7	2	MR	MS	R
Burnside	*Lr10,Lr3ka /Lr30*	1	1	;	;	0	33+	0	3	33+	3		*Lr10*		4	1	MR	R	R
Tambillo 1	*Lr20, Lr28, N*	1	2	2	?	21	2	x	3	33+	;12				5	1	?	?	R
Oslo	*Lr3, Lr20, N*	1	3	1	2	X	2;	33+	3	;	2				24	4	MR	MS	?
CN12358	*Lr3, Lr15, N*	;	3	1	2	3	1	3	;	0	;1				6	2	MR	R	R
Americano 44D	*Lr1, Lr20, N*	3	3	2	2	12;	2	33+	3	3	3	*Lr1*			8	3	MRMS	R	R
Alfy 2	*Lr1, Lr20, N*	3	2	3	3	21	33+	33+	3	3	3	*Lr1*			48	5	S	S	MSS
Wheaton	*Lr1,Lr15, N*	3	3	1	3	33+	1	;1	3	;	23	*Lr1*			30	5	R	S	?
Major	*Lr1, Lr20,N*	3	2	3	3	2	33+	3	3	3	3	*Lr1*			42	6	MRMS	R	R
Indur compactum	*Lr10, Lr20, N*	3	2	1	3	0	1	3	3	3	3-		*Lr10*		57	7	S	S	MSS
Rio Negro	*Lr10, Lr14a, N*	3	3	3	2	X	33+	;	3	3	3		*Lr10*	*Lr34*	3	1	R	R	R
Warigo	*Lr10, Lr20, N*	3	3	3	3	22+	3	3	3	3	3		*Lr10*		44	5	MS	SMS	MS
Cobbs 1066	*Lr10, Lr20, N*	3	3	3	3	22+	33+	3	3	3	3		*Lr10*		23	4	MS	MS	MS

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data *Lr1*	*Lr10*	*Lr34*	MRS	SE	HR WPG	GLN	POR
Kenya	*Lr1,Lr10,N*	3	3	3	2	3	33+	3	3	3	3	*Lr1*	*Lr10*		46	5	MSS	S	S
Bencubbin	*Lr1,Lr10, Lr20*	3	3	3	3	X	33+	3	3	3	3	*Lr1*	*Lr10*		45	6	S	S	MS
Klein Trou	*Lr1,Lr10, Lr20*	3	3	3	33+	;	33+	33+	3	3	3	*Lr1*	*Lr10*		34	6	MS	?	MS
Subgroup 4																			
Frontana	*Lr10,Lr20, Lr28,N*	1	2	3	2	22+	33+	3	3	3	3		*Lr10*	*Lr34*	3	1	R	R	R
CN37599	*Lr10,Lr15, Lr20,N*	1	3	2	3	;	2	3	3	;12	33-		*Lr10*		31	3	MS	S	MS
Bage	*Lr1,Lr10, Lr20,N*	2	3	2	2	0	;1	33+	3	3-	3	*Lr1*	*Lr10*	*Lr34*	22	5	MS	S	R
Katepwa	*Lr1,Lr10, Lr3ka/Lr30*	1	1	2	2	22+	33+	x	3	3	3	*Lr1*	*Lr10*		36	5	MR	MS	MS
CN12229	*Lr10,Lr2c, Lr15, N*	1	2	12	3	33-	22+	3	3	;12	21				18	4	MRMS	R	MS
Klein Sendero	*Lr15, Lr20, Lr28, N*	1	2	;	;	21;	1	33+	3	0	;				14	4	MR	R	MS
CN40895	*Lr10,Lr15, Lr20,N*	3	2	;	1	22+	1	3	;12	;12	;12		*Lr10*		8	3	?	MR	R
Koalisie	*Lr1,Lr10, Lr20, N*	3	2	3	3	22+	33+	33+	3	3	3	*Lr1*	*Lr10*		21	5	?	MSS	S
Dromedaris	*Lr1,Lr10, Lr20, N*	3	3	2	3	0	33+	3	3	3	3	*Lr1*	*Lr10*		53	4	?	?	MS
Stoa	*Lr10,Lr2c, Lr15, N*	0	;	;	3	33+	0	3	;	;1	0		*Lr10*		46	4	MRMS	MS	S

Accession name	Seedling genes	BBBD	MBDS	MGBJ	TJBJ	TDBG	MBRJ	PBDQ	THMJ	TNRJ	TCRJ	Marker data *Lr1*	*Lr10*	*Lr34*	MRS	SE	HR WPG	GLN	POR
Nordic	*Lr1,Lr10, Lr2a, Lr15, N*	0	2	2	3	33-	2	2	3	;	2	*Lr1*	*Lr10*		16	3	MRMS	MS	R
NING 7840	*Lr1,Lr14b, Lr15,Lr20, N*	3	2	;	12	22+	;	;	33+	;	3	*Lr1*		*Lr34*	3	1	MR	R	R

Infection types: 0 = no flecks or uredinia, 0; = faint hypersensitive flecks, ; = hypersensitive flecks, 1 = small uredinia with necrosis, , 2 = small to medium uredinia with necrosis, 3 = moderate to large size uredinia with/without chlorosis, 4 = very large uredinia without chlorosis, "+" = indicates slightly larger uredinia, "-" = indicates slightly smaller uredinia, ITs with two symbols denote a range in IT: 22+= indicates a mixture of 2 size uredinia with chlorosis and slightly larger uredinia with chlorosis, 'X'= mixture of all infection type, '?'= missing data, (Note: combinations of infection types are shown when there are more than one postule sizes). Host response (HR): 0 = no flecks or uredinia, R = small uredinia with necrosis, MR = moderate size uredinia with necrosis, MRMS = small to medium size uredinia with moderate sporulation, MS = moderate size uredinia with chlorosis, S = large uredinia. MRS: Maximun rust severity. SE: standard error. WPG=Winnipeg, GLN=Glenlea, POR= Portage la prairie

analysis of *Lr1* and *Lr10* were as expected based on the gene postulation for the majority of the accessions. However, 51 accessions possessing *Lr1* and/or *Lr10* showed unexpected HITs to race BBBD (Table 5.2).

Based on this overall gene postulation, the collection was divided into three major groups. Group 1 contained 40 accessions that had HITs to all ten races and thus may not have any of the seedling genes of the differential set. Group 2 contained 11 accessions with ITs that did not match any of the differential lines with known genes thereby representing unidentified known or potentially novel (N) *Lr* genes. Group 3 contained the remaining 224 lines which may possess one to five seedling genes.

Based on the number of genes, group 3 was further subdivided into four subgroups. Subgroup1 contained 72 accessions postulated to have a single *Lr* gene. Within this subgroup, 17 and 15 accessions possessed *Lr1*and *Lr10,* respectively. Twenty four accessions were negative for *Lr1* and *Lr10* but showed LIT to BBBD and were consequently postulated to have *Lr3*. Thirteen accessions had a LIT to race TDBG and hence were postulated to have *Lr20* alone. Accessions Janetzkis Fruher S and Prospur were postulated to only have *Lr28* and Sunbird was postulated to only have *Lr9* (Table 5.2). Subgroup 2 contained 96 accessions that possessed combinations of two seedling genes including *Lr1*, *Lr2c*, *Lr3*, *Lr10*, *Lr14a*, *Lr15*, *Lr20*, *Lr26* and *Lr28* as well as unidentified or novel (*N*) genes. Fifty accessions were postulated to have two of these known genes and 46 accessions had one known gene and one unidentified or novel gene. Subgroup 3 included 44 accessions with three genes, of which, six had three known genes while 38 had combinations of known and unidentified or novel genes. Subgroup 4

encompassed 12 accessions with more than three genes including 10 accessions with four genes and two with five (Table 5.2).

The adult plant resistance gene *Lr34* was assessed in the WC using the three gene specific markers caSNP4, caIND11 and caIND12 (Dakouri et al. 2010) and 52 accessions possessed *Lr34* while the remaining 223 did not (Table 5.2).

5.4.2 Field resistance

All accessions analysed for seedling and adult plant resistance genes were also evaluated for field resistance at three locations over three years. The ANOVA showed significant differences ($P<0.05$) between accessions and between environments (Table 5.3). Also, significant differences were observed between accessions with *Lr34* and those without *Lr34* as well as between accessions within the *Lr34+* and *Lr34-* groups. The averaged maximum rust severity (MRS) and host response (HR) ranged from ~ 1R to 84S. Overall ~17% of the accessions were considered highly resistant to leaf rust with MRS <10%, ~15% were resistant with MRS ranging from 10-20%, ~28% were moderately resistant with MRS between 20 and 40%, 16% moderately susceptible with MRS of 40-50%, 20% susceptible with MRS of 50-70% and 5% highly susceptible with MRS >70%. Host response was not entirely correlated with MRS where some accessions, for example Albimonte, had very low MRS but large pustules. In general, accessions possessing three or more genes did not have high severity ratings as compared to those having zero, one and two seedling genes (Figure 5.1). Similarly, accessions containing *Lr34* were more resistant to leaf rust than those lacking it regardless of their gene complement (Figure 5.2). Of the 52 accessions with *Lr34*, 41 had an average maximum rust severity (MRS)

Table 5.3 Analysis of variance of rust severity rating of 275 accessions of a world collection of wheat tested in nine environments

Source of Variation	DF	SS	MS	F Value	Pr>F	Variance components	%
Environments	8	231276	28909.0	20.6	<.0001	57.5	7.7
Accessions	274	1526625	5571.6	11.7	<.0001	324.3	43.4
With *Lr34*	51	168505	3304.0	8.6	<.0001		
Without *Lr34*	222	1235863	5566.9	11.3	<.0001		
With vs. without *Lr34*	1	122257	122257	553	<.0001		
Block(Environment)	9	10188	1132.0	5.1	<.0001	3.9	0.5
Environments by Accessions	2102	1019685	485.1	2.2	<.0001	140.5	18.8
Residual	2105	465480	221.1	.	.	221.1	29.6

S.E. Mean = 0.41

ranging from 0 to 35% which was similar to RL6058, a Thatcher near isogenic line with *Lr34.* The other *Lr34+* accessions showed MRS ranging from 36 to a maximum of 53%.

Group 1 accessions, i.e., accessions without identified seedling genes, displayed MRS ranging from 2 to 78%. Seven group 1 accessions were classified as nearly immune or highly resistant with an average MRS ranging from 2 to <10% and a R to MS reaction type (Table 5.2, Figure 5.1). Six group 1 accessions possessed *Lr34* and exhibited MRS of 2-35%. Group 2 with unidentified or putative novel (N) genes showed rust reading ranging from 1R to 76S. Accessions CN99032, Preludio and CN12624 were rated nearly immune or highly resistant with rust reading 1R, 3RMR and 7RMR, respectively (Table 5.2).

Within group 3, accessions possessing the gene combinations *Lr1+Lr26* and *Lr28+N* were particularly highly resistant to leaf rust with MRS <10%. Accessions containing *Lr2c+N*, *Lr14a+N*, *Lr20+N*, *Lr28+N*, *Lr1+Lr20+N*, *Lr1+Lr14a+N*, *Lr10+Lr14a*, *Lr1+Lr20+Lr28+N* and *Lr1+Lr14a+Lr15+Lr20+N* in combination with *Lr34* were highly resistant when compared to other gene combinations.

5.5 Discussion

Puccinia triticina, the leaf rust causing pathogen, is present in most of the wheat growing areas. Genetic resistance remains the most effective, economical and environmentally friendly strategy for controlling the negative impact of this disease. A survey of the seedling and adult plant resistance genes in a world collection of wheat accessions using molecular markers and gene postulation accompanied by an evaluation of field resistance at three locations during three years is reported.

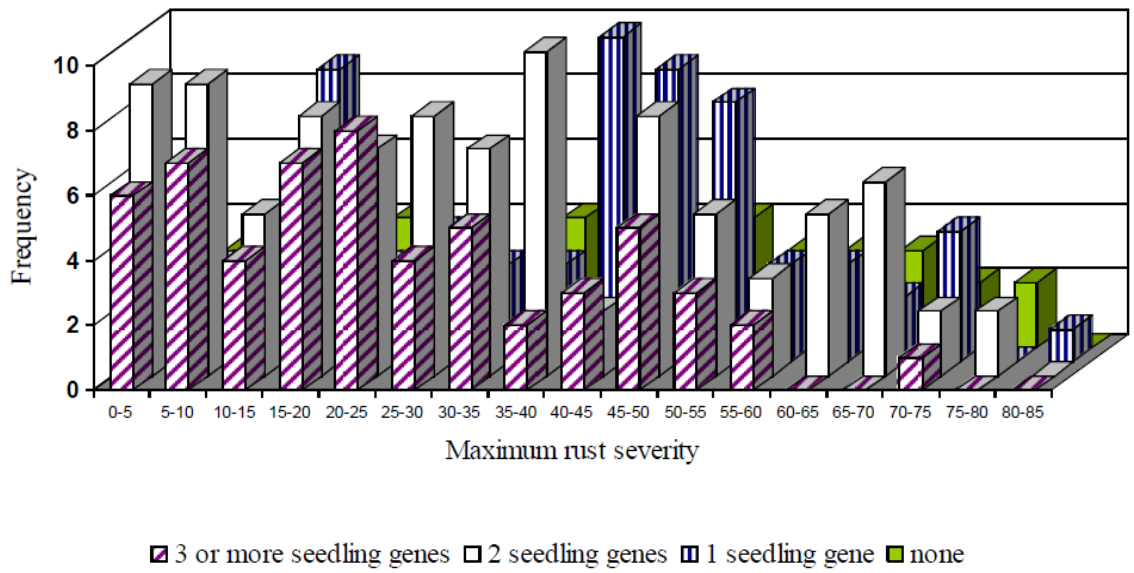

Fig. 5.1 Frequency distribution of accessions plotted against maximum rust severity rating and the number of seedling genes they possess.

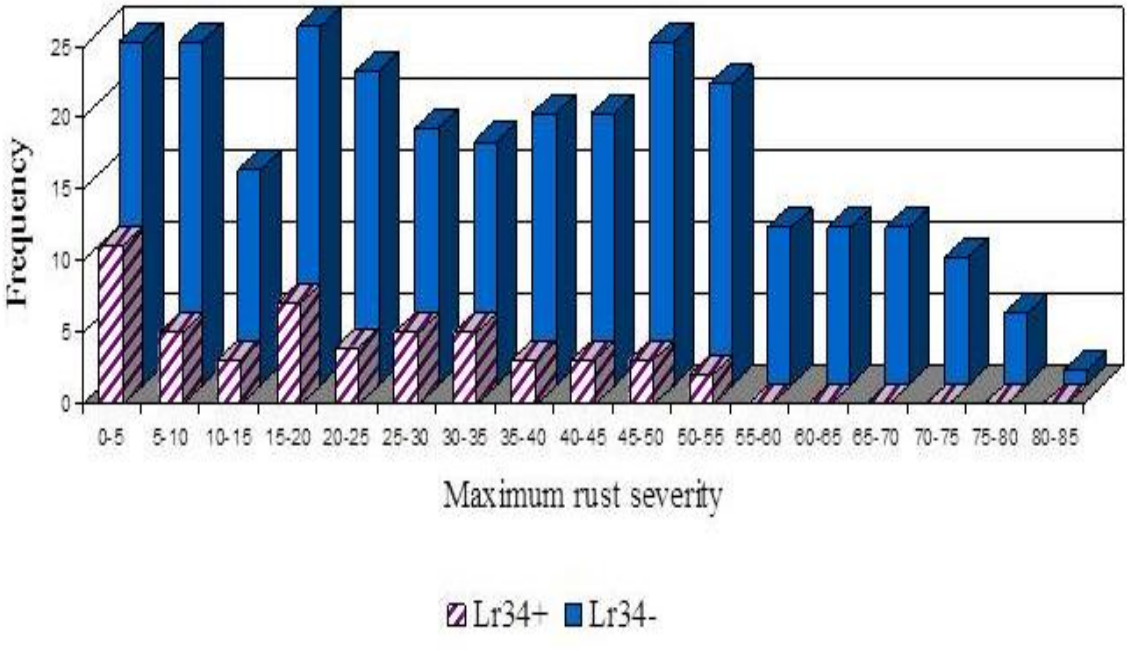

Fig. 5.2 Frequency distribution of accessions plotted against maximum rust severity rating in the presence or absence of the *Lr34*.

5.5.1 Seedling and adult plant resistance genes

Gene postulation has been applied widely as a quick method to hypothesize the seedling gene composition of germplasm. This method follows the gene-for-gene specificity theory that was first described by Flor (1942). Comparison of infection type profiles of unknown genotypes to various rust races with those of Thatcher near isogenic lines, each with a single known gene, forms the basis of gene postulation. However, this method does not enable differentiation between genes that have similar infection type profiles, e.g., *Lr1, Lr3* and *Lr10*. This problem can be overcome using gene specific markers or additional races that can distinguish these genes but such markers are limited because truly diagnostic markers exist only for *Lr* genes that have been cloned which are *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003), *Lr21* (Huang et al. 2003) and *Lr34* (Krattinger et al. 2009). Considering the minimum number of genes required to explain phenotypes (McVey and Long, 1993; Singh, 1993b), combining gene postulation with molecular marker analyses allowed us to identify 14 known seedling genes in the WC, either as single genes or in combinations. They were *Lr1, Lr2a, Lr2c, Lr3, Lr9, Lr10, Lr14a, Lr14b, Lr15, Lr20, Lr26, Lr28* and *Lr3ka* and/or *Lr30*.

Lr1, 3, 10 and *20* were the most common genes in the WC of which the first three were also the most common in American soft red winter wheats (Roelfs et al. 2000; Wamishe and Milus, 2004). *Lr20* was the most frequent gene in Ethiopian wheat germplasm (Mebrate et al. 2008) but was found at low frequency in British wheat (Singh et al. 2001) and was rare in the American soft red winter wheat and Australian germplasm (Wamishe and Milus, 2004; Singh et al. 2007). A high *Lr1* frequency was

previously reported in Chinese cultivars, American hard red spring wheats, Australian wheat cultivars, Mexican bread wheats and Indian and Pakistani wheat cultivars (Singh et al. 1999; Oelke and Kolmer 2004; Singh et al. 2007; Singh and Rajaram, 1991; Singh and Gupta, 1991). *Lr10* was commonly postulated in international winter wheat nurseries, Mexican bread wheat and Indian and Pakistani wheat cultivars (McVey, 1992; Singh and Rajaram, 1991; Singh and Gupta, 1991). Conversely, *Lr2a* and *Lr9* were rare in the WC with one accession each but were common in American soft red winter wheats (Roelfs et al. 2000; Wamishe and Milus, 2004) and in cultivars from the UK (Singh et al. 2001). Located on the 1BL.1RS translocation, *Lr26* was reported at a high frequency in Chinese wheat germplasm (Singh et al. 1999) and British cultivars (Singh et al. 2001) but was present in a single accession (NING 8331) of the WC. The WC may simply have a low frequency of the rye translocation, a feature we did not evaluate. Fifty-two accessions though possessing *Lr1* and/or *Lr10* had HITs to BBBD. Previous studies on *Lr26* and *Pm8* reported similar results which were explained by the presence of inhibitor genes (Li et al. 2010; Hanusova et al. 1996). A similar mechanism may be at play for *Lr1* and *Lr10* but inhibitor genes have not been reported to date for these two genes.

Accessions El Gaucho, Bage, AC Minto, Americano 44D and Biggar postulated to have *Lr2c+N*, *Lr1+10+20+N*, *Lr28+N*, *Lr1+10+N* and *Lr3+15+N*, respectively, were previously reported to possess *Lr11*, *Lr3bg*, *Lr11+13+21+22a*, *Lr13+34* and *Lr14a+13* in the same order (Long and Kolmer. 1989; Roelfs1988; Kolmer1994). The *N* gene(s) in these accessions could be *Lr13* or *Lr22a* which are known gene(s) that were not in the differential set or they could be novel gene(s).

Lr19, *Lr21*, *Lr29* and *Lr32* displaying LITs to all ten races have not been deployed in wheat varieties (Mebrate et al. 2008; Wamishe and Milus, 2004; McIntosh et al. 1995), and so their absence in the WC is not surprising. The same can be said for *Lr36* and *Lr38*. *Lr3ka* and/or *Lr30* were postulated in accessions Katepwa and Burnside but discrimination between the two genes was precluded by the lack of differentiating race(s).

The ten races and the 30 differential lines used in this study permitted gene postulation of 14 seedling genes but these races and differential lines were not sufficient to postulate and determine the identity of all known resistance genes indicative of the limitations of the gene postulation method (Hysing et al. 2006; Mebrate et al. 2008). A total of 96 accessions possessed combinations of known and unidentified (*N*) seedling genes and 11 accessions were postulated to only have one or more *N* genes. The unidentified but known genes could be *Lr13*, *Lr27+Lr31* (Browder, 1980; Pretorius et al. 1984; Dyck, 1991) or undescribed alleles of known genes (Wamishe and Milus, 2004). Allelism tests are appropriate alternatives but they are lengthy to perform (McCallum et al. 2010). Developing molecular markers tightly linked and, ideally, perfectly linked to genes and alleles of interest is the method of choice to determine the identity of known genes (Tanksley et al. 1989).

Variability in the LIT scores (i.e., 0, ;, 1; 2, 22+) is a common indication of heterozygosity for avirulence among *P. triticina* isolates (Samborski and Dyck, 1968; Wamishe and Milus, 2004). Temperature sensitivity is another possible cause of LIT score variability such as previously reported for *Lr11* and *Lr18* (Long and Kolmer, 1989; McIntosh et al. 1995). Temperatures as low as 17°C were used to postulate *Lr11* when tested with homozygous avirulent races (Wamishe and Milus, 2004). In our study all

races were tested within a temperature range of 18-22°C which might explain the lack of detection of *Lr11*.

Adult plant resistance is conferred by genes that are effective at the adult plant stage. They are mostly race non-specific and provide similar levels of resistance to all leaf rust races (Ohm and Shaner, 1976; Das et al. 1993). Using gene specific markers (Dakouri et al. 2010), the APR gene *Lr34* was identified in 52 of the 275 accessions of the WC. This gene has been widely utilized in breeding for leaf rust resistance and it is commonly found in the world wheat germplasm (Kolmer et al. 2008) and at a higher frequency in Asian and South American germplasm (Singh et al. 1999).

5.5.2 Field resistance

To better understand the effect of seedling and APR genes on leaf rust under field conditions, the WC was evaluated in the field at three locations over three years. Field data showed that 60% of the accessions were highly (1-10%) to moderately (11-40%) resistant to leaf rust while 40% were moderately (41-55%) to highly (56-85%) susceptible.

Overall, the rust severity decreased as the number of seedling genes increased indicating the importance of gene pyramiding in reducing the damage caused by leaf rust (Slikova et al. 2004). Gene pyramiding, a process of combining more than one resistance gene in a single genotype has been a promising strategy for developing long lasting resistance to plant disease (Singh et al. 2000). This strategy has been exploited for resistance to bacterial blight and bacterial blast in rice (Huang et al. 1997; Hittalmani et al, 2000), powdery mildew and leaf rust in wheat (Liu et al. 2000; Slikova et al. 2004)

and stripe rust in barley (Castro et al. 2003). In our study, accessions of the WC contained one to five genes and, in total, 43 gene combinations were observed including 19 two-gene, 19 three-gene, 6 four-gene and 2 five-gene combinations. Accessions possessing three genes or more clearly displayed an overall lower rust severity rating compared to those with zero, one or two genes (Figure 5.1).

Subgroup1 composed of accessions with one seedling gene including *Lr1, 3, 9, 10*, *20* and *28* that have already been overcome by the pathogen (Kolmer, 1996). Within this subgroup, correlations between the gene content and low MRS were not consistent which indicates that low levels of MRS in some accessions can not solely be accounted for by the presence of these genes alone but possibly by the presence of one or more APR genes (Kolmer, 1996). *Lr20* did not contribute significantly to leaf rust resistance (McIntosh et al. 1995; Wamishe and Milus, 2004). The same conclusion may apply to *Lr1*, *3*, *10* and *28*.

Subgroup 2 encompassed accessions with two genes each. Two-gene combinations also provided variable levels of MRS ranging from 2 to 78%. Six accessions possessing *Lr28+N* consistently showed low levels of MRS ranging from 3 to 17% indicating the potential of this combination in leaf rust resistance. Accessions El Gaucho and NING 8331with *Lr2c+N* and *Lr1+Lr26* respectively, were highly resistant with MRS of 2 and 3% and may also represent good combinations for improved leaf rust resistance.

Despite the fact that most combinations within subgroup 3 provided high to moderate levels of resistance, the gene combination *Lr1+Lr10+N* seems to provide better levels of resistance. The resistance could be solely accounted by *N* because *Lr1* and *Lr10*

have been overcome (Kolmer 1996). The only exception was observed in the case of accession Kenya which had a moderately susceptible phenotype.

Subgroup 4 with four and five seedling genes was overall more resistant than the other subgroups. The best levels of resistance were observed for combinations with *N* genes, stressing once more the need to further investigate them.

Lr34 has been the most important leaf rust resistance gene identified to date because of its race non specificity, its durability, its synergistic and pleiotropic effects and its positive effect on yield (Samborski, 1985; Dyck, 1987; Dyck 1991; Singh 1992a,b; McIntosh, 1992; Singh 1992b; Dyck et al. 1994; Singh and Huerta-Espino,1997; Spielmeyer et al. 2005; McCallum et al. 2007). The majority of *Lr34+* accessions (79%) had high to moderate levels of resistance with MRS ranging from trace to 35% similar to RL6058 (i.e., Thatcher +*Lr34*). The remaining eleven *Lr34+* accessions (~21%) showed averaged MRS ranging from 36 to 52%. The specific reason(s) for the relatively higher rust severity in these *Lr34+* accessions are unknown. DNA methylation was reported to play a role in the expression of APR genes in rice (Sha et al. 2005) where a correlation was observed between hypermethylation and repression of gene expression, a mechanism that might explain the higher severity rate in some of the *Lr34* lines. Gene expression, sequencing and methylation pattern analyses would be required to investigate these hypotheses.

The interactions between *Lr34* and other leaf rust resistance genes both seedling and APR genes were described in previous reports and also herein. Here, we highlighted gene combinations that provide the excellent levels of resistance. The relatively high levels of resistance in *Lr34+* accessions with no seedling genes might be explained by the

presence of additional APR genes (Li et al. 2010) such as *Lr12*, *Lr13*, *Lr22a*, *Lr46*, *Lr*67 and *Lr*68, or novel APR genes. Synergy between *Lr34* and other APR genes was previously reported (Sawhney, 1992; Kloppers and Pretorius, 1997). Cultivars possessing combinations of *Lr34+Lr12* (e.g., Chinese Spring) and *Lr34+Lr13* (e.g., Roblin) were highly resistant with 5RMR and 10MRMS rust reading, respectively (Dyck, 1991, 1992). Accession El Gaucho which has the gene combination *Lr34+Lr2c+N* was highly resistant with a severity of 2% and an RMR reaction type indicative of the potential synergy between these genes. Seven accessions had the combination of *Lr3+Lr34* with rust rating ranging from 1RMS to 48MSS. Because *Lr3* is defeated, additional APR genes are hypothesized in accessions with the lowest MRS. *Lr9*, previously reported as an important leaf rust resistance gene in soft red winter wheat (Kolmer, 2003) was only postulated in Sunbird and, in combination with *Lr34*, provided only moderate level of field resistance to leaf rust.

The high levels of resistance in some accessions without *Lr34* may be caused by various gene combinations not fully characterized herein. Beside *Lr34*, several APR genes have been reported including *Lr12*, *Lr13*, *Lr22a*, *Lr22b*, *Lr35*, *Lr37*, *Lr46*, *Lr67* and *Lr68*. *Lr13* which originated from South America germplasm, was commonly found in wheat germplasm worldwide (Singh et al. 2001; Singh et al. 1998; McIntosh et al. 1995). Despite being defeated, *Lr13* in combination with other APR genes may provide an acceptable level of field resistance (Kolmer, 1992). *Lr12* is also frequent in wheat cultivars from Australia, China, North America and South America (Kolmer 2003; McIntosh et al. 1995; Park and McIntosh 1994; Wamishe and Milus 2004). The WC may contain some or all known APR genes as well as novel genes, alone or in combinations.

The accession Kanred for example, had no seedling genes but displayed a low severity of 5% and host response (HR) of R in two locations and MS in one indicating the presence of unidentified genes. The variability in host response (i.e., R in two locations and MS in one) could be due to environmental effects. The same can be said for accessions Lada and Ajelea which did not possess seedling genes but showed MRS of 10-11%. In the case of Ajelea, the host response was R in all locations indicating the environmental insensitivity of its putative APR genes.

5.6 Conclusion

Efficient exploitation of genetic resistance to leaf rust requires a detailed examination of the occurrence and distribution of both seedling and adult plant resistance genes. Using molecular markers combined with gene postulation, 14 seedling genes and one adult plant resistance gene were determined to be present in a world collection of wheat. Additional seedling and APR genes may be present but could not be postulated based on the differential lines, leaf rust races and diagnostic molecular markers. The world collection potentially contains several novel resistance genes, both seedling and APR. The majority of these accessions seemingly have multiple genes, with some of them providing excellent levels of resistance. However, the actual number of genes contained in each accession can only be determined by genetic analysis. This study confirmed *Lr34* as being the single most significant and durable gene in breeding programs for leaf rust resistance and highlight the importance of gene pyramiding to provide acceptable long-term resistance to wheat leaf rust with emphasis on the important role of APR genes.

6.0 GENERAL DISCUSSION AND CONCLUSION

An essential pillar of food security, wheat is an important crop for food industry worldwide. It is a staple food for 35% of the world inhabitants providing more nourishment than any other food crop. Leaf rust caused by *P. triticina* a major constraint to wheat production around the world, causing significant yield loss in excess of 15%. *P. triticina* is adapted to a range of environments and can co-exist with wheat wherever it is grown. Genetic resistance has been the method of choice to control leaf rust and has been pursued all over the world. To date, leaf rust resistance genes designated *Lr1* to *Lr68* have been identified and mapped to chromosomes. The majority of these genes are race-specific and a number of them have been overcome by new races of *P. triticina*. However, a few genes were found to be race non-specific and have remained effective over longer periods of time. In this regard, *Lr34* has been a key component in breeding for leaf rust resistance. Besides conferring resistance to leaf rust, this gene also enhances resistance to other wheat diseases such as stripe rust, powdery mildew, stem rust and barley yellow dwarf virus (BYDV). Incorporating *Lr34* in adapted wheat germplasm will contribute to reduce yield loss caused by leaf rust but its nature as an adult plant resistance (APR) gene poses a challenge to the breeding process. Developing molecular markers tightly linked to or located within *Lr34* has facilitated its introgression in adapted wheat germplasm.

Lr34 was first mapped to the short arm of wheat chromosome 7D. Several molecular markers closely linked to *Lr34* were subsequently developed including gwm1220, csLV34, csLVMS1, swm10, SWSNP3, csLVA1 and csLVE17. However,

none of these markers were perfectly diagnostic for *Lr34*. The *Lr34* locus bordered by markers SWSNP3 and csLVE17 contains a gene-rich island encompassing six genes including a hexose carrier (HC), an ABC transporter (ABC), two cytochrome P450s (CYP-1, CYP-2) and two lectin receptor kinases (LRK-1, LRK-2) of which, the ABC transporter was the only candidate for *Lr34* function.

Here, we reported on the development of molecular markers utilizing DNA sequence information from four wheat genotypes: Chinese Spring, Glenlea, Renan and *Aegilops tauschii,* the donor of the D genome. Ten novel molecular markers were developed including six microsatellites (cam1, cam2, cam8, cam11, cam16 and cam23), one insertion site based polymorphism (caISBP1), two single nucleotide polymorphisms (caSNP4, caSNP12) and one insertion/deletion (caIND11). Markers cam8, cam11, cam16, cam23 and caISBP1 were interstitial to the XcsLVA1/SWSNP3-XcsLVE17 interval covering only 75kb of the *Lr34* locus. Markers caSNP4, caIND11 and caSNP12 were located within the ABC transporter, targeting the polymorphisms of the ABC transporter. The three co-dominant markers caSNP4, caSNP12 and caISBP1 are all easily resolved on agarose gels making them appropriate for laboratories that do not have access to acrylamide or polymer-based devices. Overall, these eight markers represent the most recent, highly diagnostic molecular markers that can be applied for marker-assisted selection.

The newly developed markers were exploited to fine map *Lr34*. Even though several gene specific markers were previously established, their co-segregation with *Lr34* did not permit the fine mapping of the gene. The newly developed markers as well as four previously developed markers namely gwm1220, csLVMS1, swm10 and csLV34 were

mapped on 1,742 recombinant lines from five populations. One line, named 98C18SSD-K16, showed a recombination event between caSNP4 of the ABC transporter and cam8, an SSR marker located between the ABC transporter and CYP-1. This recombination eliminated the possibility that any of the four genes located upstream of the ABC transporter (i.e., CYP-1,2 and LRK-1,2) might play an essential role in *Lr34* function. Recombination-based evidence was thus provided to further support the ABC transporter as sole candidate for *Lr34* function.

Lr34+ lines were reported to differ from *Lr34*- lines by only three mutations: a SNP in intron 4, a SNP in exon 12 and a 3-bp indel in exon 11. The latter two mutations occurred in the first transmembrane domain linking the two nucleotide binding domains of the ABC transporter. The SNP in exon 12 resulted in an amino acid substitution of a histidine to a tyrosine while the 3-bp indel in exon 11 corresponded to the deletion of a phenylalanine residue. These three mutations gave rise to the first two haplotypes: A/N/C found in *Lr34*+ lines and T/TTC/T in *Lr34*- lines. A third haplotype was later detected that resulted from a point mutation (T/A) of the SNP in intron 4 in the T/TTC/T haplotypes. The three gene specific markers (i.e., caSNP4, caIND11 and caSNP12) targeting the three mutations were assessed for polymorphism on ten parental lines. The marker caIND11 produced three fragments of 394, 397 and 398bp. Sequencing of the 398bp fragment revealed the presence of an A/- indel in exon 10. The A/- mutation in exon 10 would cause a frameshift and likely be non-functional. We further characterized a large collection of 700 wheat accessions of diverse origin for the ABC–specific markers. The characterization confirmed the presence of the first three haplotypes, i.e., A/N/C, T/TTC/T and A/TTC/T. Additionally, two novel haplotypes were discovered: a

fourth haplotype (T/A/N/T) resulted from an A/-, and a fifth one (T/N/N/T) found only in two accessions. H2 was the most common haplotype while H5 was found in only two accessions. H1 was the only haplotype with *Lr34*+ function while H2, 3, and 4 were found to be *Lr34*-. The two H5 accessions had an intermediate severity rating and could not be categorically clasified. If they were *Lr34*-, then the caSNP12 (C/T) would constitute the only functional unit differentiating *Lr34*+ and *Lr34*- lines.

Genetic diversity studies are important for the understanding of the genetic structure of plant germplasm and to assist in widening the genetic basis of breeding material. Gene evolution studies can provide valuable information regarding the causality of the trait, i.e., how sequential mutations and/or recombinations can lead to the development of functional genes and vice-versa. A world wheat collection (WC) of 310 accessions of *Triticum aestivum* and a collection of 112 accessions of *Aegilops tauschii,* the donor of the wheat D genome, were characterized at the *Lr34* locus to study the genetic and geographical diversity of *Lr34* and to shed light on its evolutionary history. The four markers located within *Lr34* divided the WC into five major haplotypes namely H1, H2, H3, H4 and H5. A maximum parsimony network was constructed for the five haplotypes to evaluate the most probable evolutionary steps leading to the origin of the *Lr34*+ haplotype. The network showed that H2 is the ancestral haplotype. Of the five haplotypes, H2 was the only haplotype present in *Ae. tauschii* thus supporting it as the most ancient haplotype. The other four haplotypes likely arose after the formation of hexaploid wheat, and H1 (*Lr34*+) is the result of a gain of function mutation. To the best of our knowledge, this is the first detailed picture of *Lr34* evolution.

The geographical distribution of *Lr34* haplotypes was investigated exploiting the availability of a world collection of wheat accessions representing the six continents. Haplotype H1 (*Lr34*+) occured at a high frequency in Asia followed by South America while haplotype H2 was most frequent in Europe. There was more diversity at the *Lr34* locus in Asia than in the other regions. Asia was hypothesized as the place of origin of H1 (*Lr34*+) from which it was distributed to the rest of the world. This hypothesis is further supported by this region having the highest number of H1 sub-haplotypes.

To better understand the impact of *Lr34* on resistance to leaf rust, seedling and adult plant resistance in a world collection of wheat accessions were characterized. The field resistance of the world collection was evaluated at three locations over three years. Molecular markers specific to *Lr1*, *Lr10*, *Lr21* and *Lr34* were assessed on a world collection of 275 wheat accessions. The results show that while *Lr21* was totally absent, *Lr1*, *Lr10* and *Lr34* were commonly present in the germplasm. Gene postulation was further performed to identify the other seedling genes in the WC. In total, 14 seedling genes were postulated to be present in the germplasm including *Lr1*, *Lr2a*, *Lr2c*, *Lr3*, *Lr9*, *Lr10*, *Lr14a*, *Lr14b*, *Lr15*, *Lr20*, *Lr26*, *Lr28*, and *Lr3ka* and/or *Lr30*. Additionally, many accessions were found to have unidentified or novel genes. In agreement with previous studies, *Lr1*, *Lr3*, *Lr10* and *Lr20* were very common in the WC while *Lr2a* and *Lr9* were rare. *Lr18*, *Lr19*, *Lr21*, *Lr29* and *Lr32* were not postulated because they had low infection types to all leaf rust races which were not the case in any of the tested accessions. Seedling genes were present either singly or in combinations of two to five genes. In general, accessions possessing three or more seedling genes displayed better field resistance to leaf rust than those with zero, one or two seedling genes, highlighting

the importance of gene pyramiding. Accessions including gene combinations *Lr2c+N*, *Lr1+Lr26*, *Lr28+N* and *Lr1+Lr10+N* consistently showed lower levels of leaf rust severity indicating the potential of these gene combinations.

Lr34 is the most significant leaf rust resistance gene characterized to date due to its race non-specificity, its durability, its pleiotropic effect and its positive effect on yield. Accessions containing *Lr34* were more resistant than those without it emphasizing *Lr34* as an essential component of resistance to leaf rust under field conditions. *Lr34* has been known to interact with other seedling and adult plant leaf rust resistance genes to increase the level of resistance.. Synergistic interactions between *Lr34* and other genes were previously reported and this observation has been confirmed here between *Lr34* and Lr2c+N and between *Lr34* and other unidentified or novel seedling or adult plant resistance genes.

The world collection represents a potential source of novel seedling and adult plant resistance genes. Most accessions have more than one gene and some of them confer high levels of resistance. However, more extensive detailed genetic analyses will be required to determine the nature and the exact number of genes present in each accession. Our result supports *Lr34* as a very important, if not the most important, leaf rust resistance gene identified to date. Its importance of gene pyramiding to provide acceptable long lasting resistance to leaf rust is obvious, especially in view of synergestic effects seen where *Lr34* combined with certain other genes.

Lr34 has been cloned and its sequence is publicly available, however, the biochemical and molecular aspects of the *Lr34*-based defense mechanism remains elusive and so far hypothetical. *Lr34*-based resistance was hypothesized to be the result of

senescence-like mechanism or it could be directly involved in resistance by exporting metabolites essential for fungal development. Detailed investigations of the possible molecules involved, i.e., salicylic acid, or others are required.

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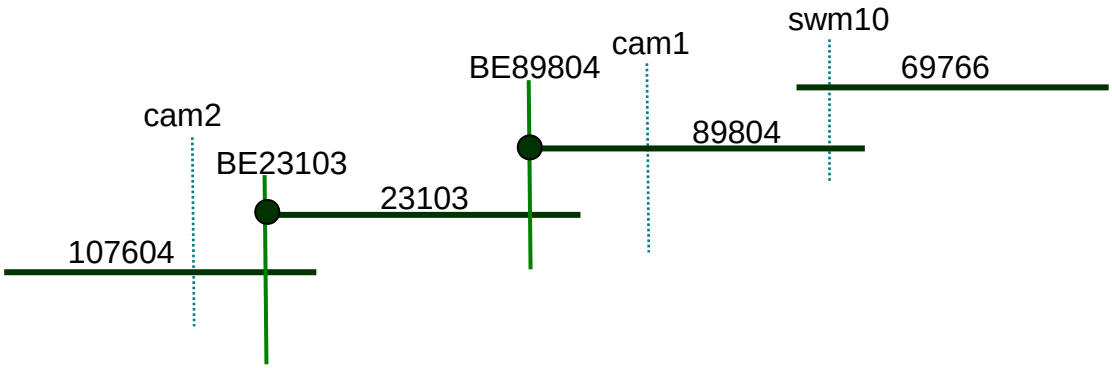
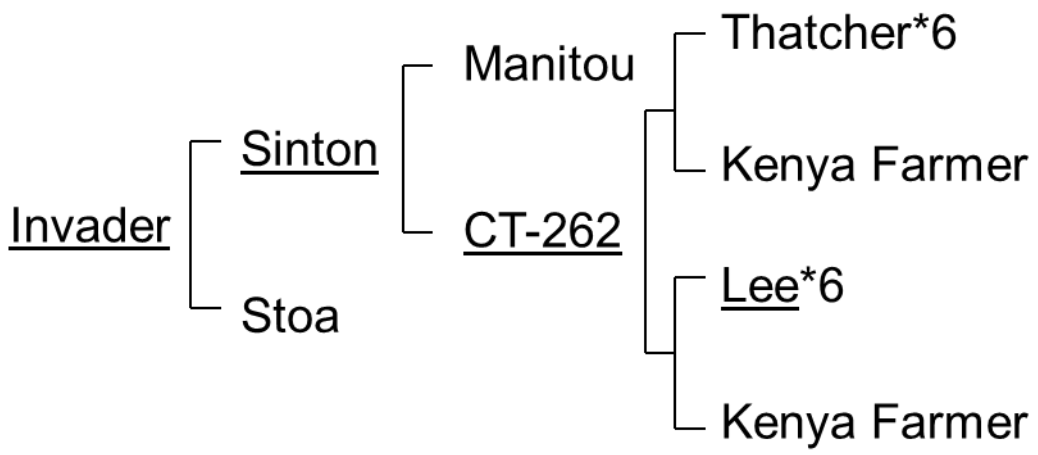
APPENDICES



Appendix I Physical map representing four overlapping BAC clones contiguous to the swm10 microsatellite marker. BAC clone names are labelled on each clone. BAC end (BE) markers used to extend the contigs and novel microsatellite markers cam1 and cam2 are positioned on the contig assembly.



Appendix II Pedigree of spring wheat cultivar 'Invader' which has the ABC transporter T/A/TTC/T haplotype corresponding to the additional A in exon 10. This haplotype can be traced into its pedigree. Underlined accessions all have the T/A/TTC/T haplotype.

Appendix III Description of germplasm including, accession number, name, country of origin and genotypic data for 10 *Lr34* locus markers.

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN43694	BW 90	Canada	148	C	N	N	A	N	509	226	208	175
CN 44437	Bluesky	Canada	159	C	N	N	A	N	509	226	208	175
-	Corrine	Canada	159	C	N	N	A	N	509	226	208	175
CN 113488	Burnside	Canada	159	C	N	N	A	N	509	226	208	175
CN 106396	Glenavon	Canada	159	C	N	N	A	N	509	226	208	175
CN10020	El Gaucho	Argentina	159	C	N	N	A	N	509	226	208	175
_	Endeavor	New Zealand	159	C	N	N	A	N	509	226	208	175
PI462141	Li Yang Wong Shu Bai	China	159	C	N	N	A	N	509	226	208	175
CItr14108	Chinese Spring	China	159	C	N	N	A	N	509	226	208	175
CItr1667	Beloglina	USA	159	C	N	N	A	N	509	226	208	175
CN11043	Magueija	Portugal	159	C	N	N	A	N	509	226	208	175
-	RL4452	Canada	159	C	N	N	A	N	509	226	208	175
CN32504	CN32505	Denmark	159	C	N	N	A	N	509	226	208	175
CN44167	Laura	Canada	161	C	N	N	A	N	509	226	208	175
PI531191	NING 8331	China	161	C	N	N	A	N	509	226	208	175
PI483064	Sunstar	Australia	161	C	N	N	A	N	509	226	208	175

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN9511	Albimonte	Switzerlan	161	C	N	N	A	N	509	226	208	175
BW386	Bajio	Mexico	161	C	N	N	A	N	509	226	208	175
CWI33350	Retacon INTA	Argentina	161	C	N	N	A	N	509	226	208	175
CN11098	Menkemen	Colombia	161	C	N	N	A	N	509	226	208	175
CN11323	Preludio	Brazil	161	C	N	N	A	N	509	226	208	175
CN12082	Tropeano	Brazil	161	C	N	N	A	N	509	226	208	175
CN10084	Fortaleza	Brazil	161	C	N	N	A	N	509	226	208	175
Aus30426	Otane	New Zealand	161	C	N	N	A	N	509	226	208	175
Aus91169	CD87	Australia	161	C	N	N	A	N	509	226	208	175
Aus30031	Chara	Australia	161	C	N	N	A	N	509	226	208	175
01C0204206	Sunbird	Australia	161	C	N	N	A	N	509	226	208	175
CN6030	CN6030	Ethiopia	161	C	N	N	A	N	509	226	208	175
-	CDC Teal	Canada	161	C	N	N	A	N	509	226	208	175
CN44171	Girija	India	161	C	N	N	A	N	509	226	208	175
PI481544	Xiang Mai No 1	China	161	C	N	N	A	N	509	226	208	175
CN11204	CN11204	Jordan	161	C	N	N	A	N	509	226	208	175
CN10098	Froubou	Germany	161	C	N	N	A	N	509	226	208	175

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
-	TAA36	Israel	161	C	N	N	A	N	509	226	208	175
CN11791	Rio Negro	Argentina	161	C	N	N	A	N	509	226	208	175
CN9544	Argentina	Argentina	161	C	N	N	A	N	509	226	208	255
CN10059	Faleria	Italy	145	C	N	N	A	300	391	228	210	255
PI462149	Nan Tong DA Huang PI	China	169	C	N	N	A	N	509	226	210	175
CN12268	CN12268	Peru	159	C	N	N	A	299	509	226	208	175
CN11057	Maria Escobar	Argentina	159	C	N	N	A	299	509	226	208	175
CN11969	Sinvalocho	Argentina	159	C	N	N	A	N	509	226	208	255
CItr12470	Frontana	Brazil	161	C	N	N	A	298	509	226	208	175
CN39713	Lovrin 32	Romania	161	C	N	N	A	298	509	226	208	175
CN9524	Andes	Mexico	161	C	N	N	A	298	509	226	208	175
CN9591	Bage	Brasil	161	C	N	N	A	298	509	226	208	175
PI462151	Shu Chou Wheat #3	China	161	C	N	N	A	298	509	226	208	175
PI447402	Cai Zi Huang	China	161	C	N	N	A	298	509	226	208	175
PI531188	NING 7840	China	161	C	N	N	A	298	509	226	208	175
CN9666	Bonza	Colombia	161	C	N	N	A	298	509	226	208	175
BW464	Calidad	Argentina	161	C	N	N	A	298	509	226	208	175

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
01C0105549	Maja	Czech Republic	161	C	N	N	A	298	509	226	208	175
BW779	Tezanos Printos Precoz	Argentina	161	C	N	N	A	298	509	226	208	175
CN9631	Benvenuto 3085	Argentina	161	C	N	N	A	298	509	226	208	175
PI481542	Su Mai No 3	China	161	C	N	N	A	298	509	226	208	175
PI197128	Shinchunaga	Japan	161	C	N	N	A	298	509	226	208	175
CN32076	Nobeoka Bozu	Japan	161	C	N	N	A	298	509	226	208	175
CN44024	Gogatsukomugai	Japan	161	C	N	N	A	303	509	228	208	175
CN32077	Nyu Bay	Japan	161	C	N	N	A	303	509	228	N	175
CN44025	Ikuzai #1	Japan	161	C	N	N	A	303	509	228	214	255
CItr12699	Norin10	Japan	161	C	N	N	A	299	509/391	226/228	210	175/255
PI447403	Wan Nian No.2	China	163	C	N	N	A	303	509	226	208	175
PI197129	Shirasaya No1	Japan	165	C	N	N	A	300	391	226	208	175
PI197130	Sanshukomugi	Japan	165	C	N	N	A	300	391	226	208	175
PI157584	Seu Seun 27	Japan	165	C	N	N	A	300	391	226	208	175
CN42881	Chengdu Guangtou	China	165	C	N	N	A	300	391	226	206	175
CN42882	Mazha Mai	China	167	C	N	N	A	300	391	226	208	175

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN11302	Pirana	Portugal	N	C	N	N	A	N	509	226	208	175
CN11932	San Marino	Italy	N	C	N	N	A	301	509	228	214	255
CN51812	Biggar	Canada	161	T	TTC	A	T	299-301	391	228	214	255
CN42929	HY 320	Canada	161	T	TTC	A	T	299-301	391	228	214	255
CN12624	CN12624	Colombia	161	T	TTC	A	T	299-301	391	228	214	255
CN11720	CN11720	Egypt	161	T	TTC	A	T	299-301	391	228	214	255
CN6025	CN6025	Ethiopia	161	T	TTC	A	T	299-301	391	228	214	255
CN6622	Oroua	New Zealand	161	T	TTC	A	T	299-301	391	228	214	255
CN10624	Janetzkis Probat	Germany	169	T	TTC	A	T	300	391	228	212	255
CN9940	CN9940	Unknown	158	T	TTC	A	T	300	391	228	212	255
CN9934	CN9934	Pakistan	159	T	TTC	A	T	300	391	228	212	255
CN9946	Daeraad	South Africa	161	T	TTC	A	T	300	391	228	210	255
CWI42988	CWI42988	Unknown	161	T	TTC	A	T	299-302	391	228	212	255
CN11739	CN11739	Unknown	161	T	TTC	A	T	299-302	391	228	212	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN29871	CN29871	Nepal	161	T	TTC	A	T	299-302	391	228	212	255
CN29854	CN29854	Nepal	161	T	TTC	A	T	299-302	391	228	212	255
CN33893	CN33893	Unknown	161	T	TTC	A	T	299-302	391	228	212	255
G3893	G3893	Unknown	161	T	TTC	A	T	299-302	391	228	212	255
CN44146	HY358	Canada	161	T	TTC	A	T	299-302	391	228	214	255
CN29858	CN29858	Nepal	161	T	TTC	A	T	299-302	391	228	214	255
CN30213	CN30213	Pakistan	161	T	TTC	A	T	299-302	391	228	214	255
CN10750	CN10750	Kenya	161	T	TTC	A	T	299-302	391	228	214	255
CN11191	New Pusa	India	161	T	TTC	A	T	299-302	391	228	214	255
CN10679	Kenphad 25	India	161	T	TTC	A	T	299-302	391	228	214	255
PI337371	Sonalika	India	161	T	TTC	A	T	299-302	391	228	214	255
CN9936	CN9936	Pakistan	161	T	TTC	A	T	299-302	391	228	214	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CWI3767	Tobari F66	Mexico	161	T	TTC	A	T	299-302	391	228	214	255
BW12005	Victoria INTA	Argentina	161	T	TTC	A	T	299-302	391	228	214	255
CN6024	CN6024	Ethiopia	161	T	TTC	A	T	299-302	391	228	214	255
01C0203832	Sabre	Australia	161	T	TTC	A	T	299-302	391	228	214	255
CN33898	Hsin ShuKuan1	China	161	T	TTC	A	T	299-302	391	228	214	255
CN10719	Kenya Farmer	Kenya	161	T	TTC	A	T	299-302	391	228	214	255
CN12213	Oslo	Canada	161	T	TTC	A	T	299-302	391	228	214	255
CN10905	Krasnaja Svezda	Kazakhstan	163	T	TTC	A	T	299	391	228	212	255
CN12425	CN12425	Egypt	163	T	TTC	A	T	301	391	228	214	255
CN9497	Ajelea	Iraq	165	T	TTC	A	T	299-300	391	228	210	255
CN10241	CN10241	Pakistan	165	T	TTC	A	T	299-300	391	228	210	255
CN12251	CN12251	Pakistan	165	T	TTC	A	T	299-300	391	228	210	255
CN10868	Klein Trou	South Africa	165	T	TTC	A	T	299-300	391	228	210	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN42522	CN42522	Nepal	165	T	TTC	A	T	299-300	391	228	212	255
CN12388	CN12388	Kenya	165	T	TTC	A	T	299-300	391	228	214	255
CN10150	Giza 141	Egypt	167	T	TTC	A	T	303	391	228	214	255
01C0200129	Maja	CSK	172	T	TTC	A	T	300-301	391	228	212	255
CN11150	Narinskajal	Russia	172	T	TTC	A	T	300-301	391	228	212	255
CN29856	CN29856	Nepal	N	T	TTC	A	T	300	391	228	212	255
CN11206	Norin 75	Japan	146	T	TTC	N	A	298	391	228	210	255
CN11309	Pobeda	Russia	146	T	TTC	N	A	298	391	228	210	255
CN44173	Prinqual	France	146	T	TTC	N	A	298	391	228	210	255
CWI35704	Wheaton	USA	146	T	TTC	N	A	298	391	228	210	255
CN9995	Duiker	South Africa	157	T	TTC	N	A	301	391	226	208	255
CN9984	Dromedaris	South Africa	157	T	TTC	N	A	301	391	226	208	255
CItr1697	BAART	Australia	157	T	TTC	N	A	301	391	226	208	255
CN10112	Gabo	Australia	157	T	TTC	N	A	301	391	226	208	255
CN9682	Bungulla	Australia	157	T	TTC	N	A	301	391	226	208	255
CN42403	Arcane	France	157	T	TTC	N	A	301	391	226	208	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN12036	Tabasi	Iran	130	T	TTC	N	A	301	391	226	212	255
CN11401	Rayhany	Iran	130	T	TTC	N	A	301	391	226	212	255
CN11962	Shahpassand	Iran	130	T	TTC	N	A	301	391	226	212	255
CWI33193	Buck Nandu	Argentina	140	T	TTC	N	A	298	391	226	210	255
CItr4795	Ladoga	Russia	145	T	TTC	N	A	298	391	228	210	255
CN10099	Fruher Tiroler Bin	Austria	146	T	TTC	N	A	298	391	226	210	255
CN10028	Equator	Kenya	146	T	TTC	N	A	298	391	226	210	255
PI243192	Hybrid 46	UK	146	T	TTC	N	A	298	391	226	208	255
CN40617	Katja A-1	UK	148	T	TTC	N	A	299-302	391	226	210	255
CItr13723	Druchamp	France	148	T	TTC	N	A	299-302	391	226	210	255
CN33802	CN33802	Unknown	152	T	TTC	N	A	298	391	226	210	255
CN9541	Arawa	New Zealand	152	T	TTC	N	A	298	391	226	210	255
CN10210	Holdfast	UK	152	T	TTC	N	A	298	391	226	210	255
CN11461	CN11461	Austria	152	T	TTC	N	A	298	391	226	210	255
CN9950	Da Maia	Portugal	156	T	TTC	N	A	298	391	228	208	255
CN12035	CN12035	New	157	T	TTC	N	A	301	391	226	210	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
		Zealand										
CN5904	CN5904	Iran	157	T	TTC	N	A	301	391	226	210	255
PI382144	Encruzilhada	Brazil	161	T	TTC	N	A	299	391	226	210	255
CN10209	Hohenhein	Germany	161	T	TTC	N	A	299	391	228	212	255
01C0102956	Gamin	France	161	T	TTC	N	A	301	391	N	214	255
CItr6731	Benefactor	UK	163	T	TTC	N	A	301	391	226	210	255
CN52356	Kutnowianka	Poland	163	T	TTC	N	A	300-301	391	228	210	255
01C0103940	Sabre	Australia	163	T	TTC	N	A	300-301	391	228	210	255
CWI3764	Klein Toledo	Argentina	165	T	TTC	N	A	301	391	226	208	255
CItr11666	Cheyenne selection	USA	165	T	TTC	N	A	301	391	226	212	255
CN10641	Kanred	USA	165	T	TTC	N	A	300-301	391	228	214	255
PI447404	Yang Mai No.1	China	165	T	TTC	N	A	300-301	391	228	214	255
CN12124	Vijay	India	165	T	TTC	N	A	300-301	391	228	214	255
PI213833	Funo	Italy	165	T	TTC	N	A	300-301	391	228	214	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
PI447405	Fu Mai No 3	China	165	T	TTC	N	A	300-301	391	228	214	255
PI531193	JG1	China	165	T	TTC	N	A	300-301	391	228	214	255
CN42949	Cargimarec	France	170	T	TTC	N	A	300	391	226	210	255
CN11642	CN11642	Poland	148	T	TTC	N	T	300	391	228	208	175
-	AC Minto	Canada	148	T	TTC	N	T	299	391	228	210	175
-	AC Vista	Canada	148	T	TTC	N	T	299	391	228	210	175
CN38927	Katepwa	Canada	148	T	TTC	N	T	298	391	228	210	255
CItr5125	Bunyip	Australia	148	T	TTC	N	T	298	391	228	210	255
CItr4733	Hard Federation	Australia	148	T	TTC	N	T	298	391	228	210	255
CN10529	Irkutskaja 49	Russia	148	T	TTC	N	T	298	391	228	210	255
01C0200103	Sandra	CSK	148	T	TTC	N	T	298	391	228	210	255
-	Columbus	Canada	148	T	TTC	N	T	298	391	228	210	255
CN51820	Wildcat	Canada	148	T	TTC	N	T	298	391	228	210	255
PI520297	Stoa	USA	148	T	TTC	N	T	298	391	228	210	255
CN11189	Neepawa	Canada	148	T	TTC	N	T	298	391	228	210	255
CN30572	Beijing No 6	China	148	T	TTC	N	T	298	391	228	210	255
CN10000	Dvina	Russia	148	T	TTC	N	T	298	391	228	210	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN52375	Betabanmuti	Hungary	148	T	TTC	N	T	298	391	228	210	255
CN9604	Baudi	Italy	148	T	TTC	N	T	298	391	228	210	255
CN12186	CN12186	Romania	148	T	TTC	N	T	298	391	228	210	255
CItr8178	Hope	USA	148	T	TTC	N	T	298	391	228	210	255
BWI1255	Buck Pucara	Argentina	148	T	TTC	N	T	298	391	228	210	255
CN12358	CN12358	Peru	148	T	TTC	N	T	298	391	228	210	255
CN11058	Maribal 50	Peru	148	T	TTC	N	T	298	391	228	210	255
CN10856	Klein Credito	Argentina	148	T	TTC	N	T	298	391	228	210	255
CN11243	Olaeta Calandria	Argentina	148	T	TTC	N	T	298	391	228	210	255
CN11137	Mokhtar Improved	Egypt	148	T	TTC	N	T	298	391	228	210	255
CN10207	Hofed	Australia	148	T	TTC	N	T	298	391	228	210	255
CItr4734	Federation	Australia	148	T	TTC	N	T	298	391	228	210	255
CN40750	CN40750	Bulgaria	148	T	TTC	N	T	298	391	228	210	255
CN11132	Minskaja	Bulgaria	148	T	TTC	N	T	298	391	228	210	255
CN11141	Moskovka	Russia	148	T	TTC	N	T	298	391	228	210	255
CN11698	CN11698	Peru	148	T	TTC	N	T	298	391	228	210	255
-	Nordic	USA	148	T	TTC	N	T	298	391	228	210	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN9711	cailloux	Australia	148	T	TTC	N	T	298	391	228	210	255
CN29735	FengcheungNo. 1	China	148	T	TTC	N	T	298	391	228	210	255
CN11961	Severodvinskaja1	Russia	148	T	TTC	N	T	298	391	228	210	255
CN12102	Udarnica	Russia	148	T	TTC	N	T	298	391	228	210	255
01C0101031	Bankuti 1201	Hungary	148	T	TTC	N	T	298	391	228	210	255
CN39039	Thatcher	Canada	148	T	TTC	N	T	298	391	228	210	255
CN11288	Phoebus	Belgium	148	T	TTC	N	T	298	391	228	210	255
CN9794	CN9794	Egypt	148	T	TTC	N	T	298	391	228	210	255
CN9834	Comeback	Australia	148	T	TTC	N	T	298	391	228	210	255
CN10622	Janetzkis FruherS	Germany	148	T	TTC	N	T	298	391	228	210	255
CN12168	Werna	France	148	T	TTC	N	T	298	391	228	210	255
CN11059	Marquillo	USA	148	T	TTC	N	T	298	391	228	210	255
CItr12471	Kenya 58	Kenya	159	T	TTC	N	T	299	391	226	210	255
CItr4981	White Federation	Australia	159	T	TTC	N	T	299	391	226	210	255
CN40618	Lada	Bulgaria	159	T	TTC	N	T	299	391	226	210	255
CN11917	Sabanero	Kenya	159	T	TTC	N	T	299-300	391	230	210	255
CItr4984	Major	Australia	159	T	TTC	N	T	299-300	391	230	210	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
PI320108	Santa Elena	Mexico	161	T	TTC	N	T	298	391	228	210	255
CN52368	Eszterhazai No.18	Hungary	161	T	TTC	N	T	298	391	228	210	255
PI193125	Little Joss	UK	161	T	TTC	N	T	298	391	228	210	255
CN31019	Parana	Argentina	161	T	TTC	N	T	298	391	228	210	255
CN9832	Combate	Brazil	161	T	TTC	N	T	298	391	228	210	255
CN12090	Trintani	Brazil	161	T	TTC	N	T	298	391	228	210	255
PI201195	Heines VII	Germany	161	T	TTC	N	T	298	391	228	210	255
CN9821	Colonias	Brazil	161	T	TTC	N	T	298	391	228	210	255
PI294982	Buffum	USA	165	T	TTC	N	T	300-301	391	226	212	255
CN43797	Ventura	France	165	T	TTC	N	T	300-301	391	226	212	255
CN371171	Kinsman	UK	165	T	TTC	N	T	300-301	391	226	212	255
01C0203281	Musket	UK	165	T	TTC	N	T	300-301	391	226	212	255
01C0203285	Tonic	UK	165	T	TTC	N	T	300-301	391	226	212	255
CN10193	Heines Peko	Germany	165	T	TTC	N	T	300-301	391	226	212	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CItr6316	Gold Drop	Australia	143	T	TTC	N	T	299-300	391	226	210	255
PI262231	Etoile De Choisy	France	143	T	TTC	N	T	299-300	391	226	210	255
PI278562	Victor	UK	143	T	TTC	N	T	299	391	228	210	255
CN37599	CN37599	Unknown	143	T	TTC	N	T	299	391	228	212	255
CN12229	CN12229	Unknown	143	T	TTC	N	T	299	391	228	212	255
CN12261	CN12261	Unknown	143	T	TTC	N	T	299	391	228	212	255
CN1849	CN1849	Unknown	143	T	TTC	N	T	299	391	228	212	255
-	Atacatzo 1	Ecuador	143	T	TTC	N	T	N	391	226	210	255
CN52397	Lovaszpatonai 160	Hungary	145	T	TTC	N	T	299	391	228	210	255
CN9514	Alfy 2	Belgium	148	T	TTC	N	T	300	391	226	208	175
CN52357	Malgorzatka Udycka	Poland	148	T	TTC	N	T	300	391	226	210	255
CN11307	CN11307	Ethiopia	148	T	TTC	N	T	N	391	228	210	255
CWI16281	Prospur	USA	148	T	TTC	N	T	N	509	228	210	255
PI294962	Poljana	Yugoslavia	150	T	TTC	N	T	300	391	226	210	255
01C0100319	Vega	CSK	150	T	TTC	N	T	300	391	228	210	255
CN10167	Golubka	Kazakhstan	150	T	TTC	N	T	300	391	228	214	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN10661	CN10661	Kenya	152	T	TTC	N	T	298	391	226	210	255
CN11281	Penkop	South Africa	152	T	TTC	N	T	298	391	226	210	255
CN9491	Acadimia 48	Romania	152	T	TTC	N	T	300	391	228	210	255
CN33803	CN33803	Unknown	152	T	TTC	N	T	300	391	228	210	255
CN10644	karagandinskaja	Kazakhstan	152	T	TTC	N	T	300	391	228	210	255
CN10068	Ferrugineum-87	Kyrgyzstan	152	T	TTC	N	T	300	391	228	210	255
CN11263	Pamjat Urala	Russia	152	T	TTC	N	T	N	391	228	210	255
CItr4996	DART	UK	154	T	TTC	N	T	297	391	226	210	255
CN2646	Camadi	Ethiopia	154	T	TTC	N	T	297	391	226	210	255
PI278572	Benefactress	UK	154	T	TTC	N	T	297	391	226	210	255
CN42519	Cinquentenario	Brazil	154	T	TTC	N	T	297	391	226	210	255
PI351654	Surpresa	Brazil	154	T	TTC	N	T	297	391	226	210	255
CItr4067	Pacific Bluestem	Australia	154	T	TTC	N	T	302	391	226	212	255
01C0100613	Bankuti	Hungary	154	T	TTC	N	T	300	391	228	208	255
CN33892	CN33892	Unknown	154	T	TTC	N	T	300	391	228	210	255
CN10118	Galego Barbado	Portugal	154	T	TTC	N	T	302	391	230	210	255
CN11647	CN11647	Unknown	156	T	TTC	N	T	299	391	226	212	255
CN5835	71GNNo115	Syria	156	T	TTC	N	T	300	391	228	210	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
PI262228	Poncheau	France	157	T	TTC	N	T	299	391	226	210	255
CN10169	Gorkovskaja15	Russia	157	T	TTC	N	T	299	391	226	210	255
CItr9351	Yeoman II	UK	157	T	TTC	N	T	299	391	226	210	255
CN10907	Krasnojarskaja 1103	Russia	157	T	TTC	N	T	302	391	226	212	255
CN10908	Krasnokutka3	Russia	157	T	TTC	N	T	302	391	226	212	255
CN10892	Koalisie	South Africa	157	T	TTC	N	T	299-300	391	228	208	255
CN12153	Warigo	Australia	157	T	TTC	N	T	299-300	391	228	208	255
CN10202	Hilgendorf	New Zealand	157	T	TTC	N	T	299	391	228	210	255
CN9577	Azar	Iran	157	T	TTC	N	T	299	391	228	210	255
CN9635	Bersee	France	157	T	TTC	N	T	N	391	226	210	255
CN10691	Kenya	Kenya	158	T	TTC	N	T	298	391	226	210	255
CN10722	Kenya Governor	Kenya	158	T	TTC	N	T	N	509	226	208	175
CN10234	Hybride Du Jubile	France	159	T	TTC	N	T	299	391	228	212	255
01C0100187	Samorinska	CSK	159	T	TTC	N	T	299	391	228	212	255
CN10631	Jufy 2	Belgium	159	T	TTC	N	T	299	391	230	212	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CN10921	Ladoga	Russia	159	T	TTC	N	T	N	391	226	210	255
CN11765	CN11765	Romania	159	T	TTC	N	T	N	391	228	208	255
CN10196	Helvia	Peru	159	T	TTC	N	T	N	391	228	210	255
CN10963	Libero	Italy	161	T	TTC	N	T	301	391	226	208	255
CItr3756	Carina	USA	161	T	TTC	N	T	300	391	226	210	255
PI167419	Nord Desprez	France	161	T	TTC	N	T	300	391	226	210	255
PI262223	Cappelle Desprez	France	161	T	TTC	N	T	300	391	226	210	255
PI531189	NING 8026	China	161	T	TTC	N	T	300	391	226	210	255
01C0100320	Torysa	Slovakia	161	T	TTC	N	T	300	391	226	210	255
PI125093	Vilmorin 27	France	161	T	TTC	N	T	300	391	226	210	255
PI201196	Minister	Australia	161	T	TTC	N	T	300	391	226	210	255
CItr8885	Cheyenne	USA	161	T	TTC	N	T	300	391	226	212	255
CItr4121	John Brown	Australia	161	T	TTC	N	T	299-301	391	228	214	255
CN44009	Trintecinco	Brazil	161	T	TTC	N	T	299-301	391	228	214	255
CN12666	CN12666	Congo	161	T	TTC	N	T	299-301	391	228	214	255
CItr14193	Red River 68	USA	161	T	TTC	N	T	N	509	226	208	175

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
Aus29472	Kukri	Australia	161	T	TTC	N	T	N	391	226	210	255
CItr12019	Fronteira	Brazil	161	T	TTC	N	T	N	391	228	210	255
CN44011	Toropi	Brazil	161	T	TTC	N	T	N	391	228	210	255
CN12149	Warden	UK	161	T	TTC	N	T	N	391	228	210	255
CN9975	Dominator	UK	163	T	TTC	N	T	298	391	226	210	255
CN11998	Squarhead's Master	UK	163	T	TTC	N	T	298	391	226	210	255
CN10104	Fylby	Belgium	163	T	TTC	N	T	298	391	226	210	255
CN11255	Onohoiskaja4	Russia	163	T	TTC	N	T	299-300	391	228	210	255
CN10620	Jakutjanka	Russia	163	T	TTC	N	T	299-300	391	228	210	255
CItr6017	Touse	USA	165	T	TTC	N	T	299	391	226	210	255
CN33902	Ho chun No12	China	165	T	TTC	N	T	300	391	228	214	255
CItr4608	Jumbuck	Australia	165	T	TTC	N	T	300	391	230	212	255
CN10060	Farrpo	Portugal	167	T	TTC	N	T	300-302	391	226	208	255
CItr12606	Klein Universal	Argentina	167	T	TTC	N	T	300-302	391	226	208	255
PI191937	Americano 44D	Uruguay	167	T	TTC	N	T	300-302	391	226	208	255

Accession	variety name	country of origin	gwm1220	caSNP12	caIND11	caIND10	caSNP4	cam11	caISBP1	csLVMS1	swm10	csLV34
CWI77253	Klein Sendero	Argentina	167	T	TTC	N	T	300-302	391	226	208	255
BW4689	Klein Atlas	Argentina	167	T	TTC	N	T	300-302	391	226	208	255
CNI42787	KVL 2263	Denmark	167	T	TTC	N	T	300	391	228	210	255
CItr7338	Red Marvel	UK	167	T	TTC	N	T	300	391	230	208	175
CItr6709	Japhet	France	167	T	TTC	N	T	300	391	230	212	255
CN10904	Kozlodui	Bulgaria	167	T	TTC	N	T	300	391	230	212	255
CWI14048	Universal II	Argentina	167	T	TTC	N	T	N	391	226	208	255
CN10862	Klein otto Wulff	Argentina	167	T	TTC	N	T	N	391	226	208	255
CWI14942	Klein Sin Rival	Argentina	167	T	TTC	N	T	N	391	226	208	255
CN9661	Bola Picota	Colombia	167	T	TTC	N	T	N	391	228	210	255
CN2758	CN2758	Unknown	169	T	TTC	N	T	301	391	228	210	255
CN12003	Strubes Fortschritt	Germany	169	T	TTC	N	T	299	391	228	212	255
CN9818	Cobbs 1066	Kenya	172	T	TTC	N	T	298	391	228	210	255
CN11239	Odess kaja 13	Ukraine	159	T	N	N	T	300	391	228	210	255
CN10898	Koktunkulskaja 332	Kazakhstan	163	T	N	N	T	304	391	228	210	255

Appendix IV LD and r^2 values for the ten markers at the *Lr34* locus for the entire WC

LD (R^2)	**gwm1220**	**caSNP12**	**caIND11**	**caIND10**	**caSNP4**	**cam11**	**caISBP1**	**cslvMS1**	**swm10**	**cslv34**
gwm1220										
caSNP12	0.01									
caIND11	0.01	0.89*								
caIND10	0.00	0.03	0.03							
caSNP4	0.01	0.39*	0.32*	0.07*						
cam11	0.00	0.03	0.02	0.01	0.01					
caISBP1	0.00	0.59*	0.51*	0.03	0.19*	0.12*				
cslvMS1	0.01	0.05*	0.03	0.13*	0.12*	0.01	0.04			
swm10	0.00	0.18*	0.14*	0.00	0.08*	0.00	0.21*	0.02		
cslv34	0.00	0.47*	0.41*	0.03	0.13*	0.03	0.42*	0.05*	0.27*	

r^2 threshold of significance =0.05

* Significant LD

Appendix V LD and r^2 values for the ten markers at the *Lr34* locus for the *Lr34+* sub-haplotypes.

LD (R^2)	**gwm1220**	**caSNP12**	**caIND11**	**caIND10**	**caSNP4**	**cam11**	**caISBP1**	**cslvMS1**	**swm10**	**cslv34**
gwm1220										
caSNP12	0									
caIND11	0	1*								
caIND10	0	1*	1							
caSNP4	0	1*	1	1						
cam11	0.02	0.02	0.02	0.02	0.02					
caISBP1	0.13*	0.13*	0.13*	0.13*	0.13*	0.15*				
cslvMS1	0.15*	0.15*	0.15*	0.15*	0.15*	0.12*	0.05			
swm10	0.26*	0.01	0.01	0.01	0.01	0.02	0.11	0.26*		
cslv34	0.15*	0.15*	0.15*	0.15*	0.15*	0.01	0.05	0.39*	0.14*	

r^2 threshold of significance = 0.12

* Significant LD

Appendix VI LD and r^2 values for the ten markers at the *Lr34* locus for the *Lr34*- sub-haplotypes.

LD (R^2)	**gwm1220**	**caSNP12**	**caIND11**	**caIND10**	**caSNP4**	**cam11**	**caISBP1**	**cslvMS1**	**swm10**	**cslv34**
gwm1220										
caSNP12	0.00									
caIND11	0.00	0.33*								
caIND10	0.00	0.00	0.00							
caSNP4	0.03	0.02	0.00	0.05*						
cam11	0.01	0.00	0.00	0.00	0.08*					
caISBP1	0.00	0.00	0.00	0.00	0.00	0.06*				
cslvMS1	0.00	0.00	0.00	0.12*	0.08*	0.02	0.01			
swm10	0.00	0.00	0.01	0.09*	0.02	0.01	0.02	0.00		
cslv34	0.00	0.00	0.00	0.01	0.01	0.01	0.18*	0.00	0.05*	

r^2 threshold of significance =0.05

* Significant LD

Appendix VII Primer names, sequences, PCR conditions, amplicon sizes and references for four *Lr* gene specific markers.

Gene	Primer Name	Sequence	PCR condition	Resolution method	Amplicon sizes	Reference
Lr1	Glenlea5_F233 Glenlea5_R712	AGTCTGCACAATCTTTTCCGG ATCTGTAGTTGGTCCACCAAGG	Initial denaturation: 94˚C, 4 min Denaturation: 94˚C, 15 sec Annealing: 65˚C, 30 sec Extention: 72˚C, 30 sec Cycles: 35 Final extension: 72˚C, 10 min End: 15˚C constant	1.5% agarose gel	500/N	Cloutier et al. 2007
Lr10	Lrk10D1 Lrk10D2	GAAGCCCTTCGTCTCATCTG TTGATTCATTGCAGATGAGATCACG	Initial denaturation: 94˚C, 3 min Denaturation: 94˚C, 45 sec Annealing: 57˚C, 45 sec Extention: 72˚C, 30 min Cycles: 35 Final extension: 72˚C, 10 min End: 15˚C constant	1.5% agarose gel	300/400/500	Schachermayr et al. 1997
Lr21	Lr21-227F Lr21-550R	TATTTGACATCAGTCATTGAGG CATCTTGAGATAGCAGTAAGG	Initial denaturation: 95˚C, 5 min Denaturation: 95˚C, 1 min Annealing: 54˚C, I min Extention: 72˚C, 1 min Cycles: 34 Final extension: 72˚C, 10 min End: 15˚C constant	1.5% agarose gel	400/N	Huang et al. 2001

Gene	Primer Name	Sequence	PCR condition	Resolution method	Amplicon sizes	Reference
			Initial denaturation: 93˚C, 2 min			
		F: GTCTCCCAATCTGCATGCTC	Denaturation: 94˚C, 1 min			
	caIND11	R: TACCTCCCAAAAGCCAGTTG	Annealing: 58˚C, 50 sec			
			Extention: 72˚C, 1 min	ABI3130*x*1	394/397	
			Cycles: 30			
			Final extension: 72˚C, 10 min			Dakouri et al. 2010
			End: 15˚C constant			
Lr34	caSNP4	F: GCGTTTCTGTCACCAGAAGT	Initial denaturation: 94˚C, 5 min		390/N	
		R: AATAAACTCGCGCCTCTTGA	Denaturation: 94˚C, 1 min			
	caSNP12	F: TCCCCAGTTTAACCATCCTG	Annealing: 65˚C, 30 sec	1.5% agarose gel	234/N	
		R: CATTCAGTCACCTCGCAGC	Extention: 72˚C, 1 min			
			Cycles: 35			
			Final extension: 72˚C, 10 min			
			End: 15˚C constant			