IDENTIFICATION OF MOLECULAR MARKERS ASSOCIATED WITH THE LEAF RUST RESISTANCE GENES Lr41 AND Lr42 IN WHEAT

By

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NOMENCLATURE

AFLP	Amplified fragment length polymorphisms
APR	Adult plant resistance
avr	Avirulence
BAC	Bacterial artificial chromosome
bp	Base pair
BSA	Bulked segregant analysis
CIMMYT	International Maize and Wheat Improvement Center
cM	Centi-Morgan
CTAB	Cethyl-trimethyl ammonium borate
DNA	Deoxy ribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylene diamide tetra acetate
EST	Expressed sequence tag
HR	Hyper-sensitive reaction
Kb	Kilo base
LGS	Large genome species
LOD	Linkage dis-equllibrium
Lr	Leaf rust
MAS	Marker assisted selection
MR	Moderately resistant
MS	Moderately susceptible
NBS-LRR	Nuclotide binding site-Leucine rich repeats
NIL	Near isogenic line
PCR	Polymerase chain reaction
QTL	Quantitative trait locus

R	Resistant
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RGA	Resistance gene analogs
S	Susceptible
Sr	Stem rust
SSR	Simple sequence repeats
STS	Sequence tagged sites
TBE	Tris-Boric acid-EDTA
TEMED	Tetramethyl, ethylene-diamine
TRIS	Tris (hydroxymethyl) aminomethane hydrochloride
WGRC	Wheat germplasm resource center

CHAPTER I

A LITERATURE REVIEW

Introduction

Economic impact

Wheat is a globally predominant food crop both in terms of acreage and production. In the year 1999, wheat ranked third among U.S. field crops in both acreage and gross farm revenue (http://www.ers.usda.gov/briefing/wheat/), with 596 million tons of production in the year 2002/03 (Wheat Outlook, 2002). The forecasted global demand for wheat in the year 2020 varies between 840 (Rosegrant et al. 1995) and 1,050 million tons (Kronstad, 1998). To reach this target, global production will need to increase 1.6 to 2.6 percent annually from the current average production level of 560 million tons (Rajaram, 2000). Leaf rust is one of the most important diseases in all major wheat-growing regions worldwide, causing significant reduction in yield potential. The pathogen responsible for wheat leaf rust is an obligate, biotrophic fungus called *Puccinia triticina* (http://www.crl.umn.edu/tritname.html). Yield losses due to leaf rust in individual fields can range from trace to over 40% (Knott, 1989). In the USA, losses in yield from leaf rust in winter wheat were estimated at 4.8% in 1992 and 1993 (Long et al. 1998). It is also estimated that an average of 150 million dollars is incurred every year in losses due to this disease to farmers in the southern Great Plains alone (McGraw, 2001). Wheat leaf rust damaged about 95 million bushels of wheat in Texas and Oklahoma in 1985 and over 40 million bushels in Kansas and Nebraska in 1993 (Mark, 1999). Such losses are considered to be significant to US economy since wheat is a major export commodity.

Nature of wheat leaf rust resistance-breeding challenges

Breeding wheat for leaf rust resistance is a challenging task, because resistance built into a wheat variety can be totally overcome by a shift in pathogenic races in the rust fungal population. There are more than 60 known genes for stem rust resistance and more than 45 genes for leaf rust resistance identified from wheat and its relatives (APPENDIX A). Each of these leaf rust resistance genes provides resistance against one or several rust pathogen races, but none of them is capable to combat all races. Genetic studies with rust disease system has shown that every resistance gene in the host plant matches with a corresponding avirulence gene (avr) in the fungal pathogen, thus showing resistance reaction on an infected plant. Any change to the avirulence gene in a pathogen may allow the fungus to overcome that resistance. A pathogenic race in a rust fungus is determined by the combination of avirulence genes present in the fungus and the resistance genes in the host. Therefore, a combination of avirulence genes in a pathogen determines which resistant varieties the rust race can attack and which varieties are resistant to it. Because new rust races can and do arise, there is always a need to discover new resistance genes and accumulate them into new varieties. The successful control of rust epidemic using genetic resistance has two dimensions: monitoring the dynamic change of pathogen population to identify new virulent races in rust populations and discovering the corresponding resistance genes from plant sources and incorporating them into elite breeding lines to defeat the new races. It is desirable, but difficult, to accumulate multiple resistance genes in a plant through conventional breeding process, because evaluation of large breeding populations for each of existing rust races is time consuming besides being a technical challenge.

Strategies

Two possible approaches are being proposed to tackle this problem. One is to identify durable resistance genes that can provide long-lasting resistance but partial protection from rust damage. APR (adult plant resistance) is a type of resistance that is gaining considerable attention among wheat researchers due to its longer lasting effects. APR is a quantitative trait and the development of such a trait involves continuous selections to accumulate several resistance genes with partial resistance in plants. Since this type of resistance may not be overcome by a single pathogen race, is considered to be durable. However, there could be a threat of severe rust damage in the fields if considerable amount of primary inoculum is deposited in the early plant growth stages given the fact that this type of resistance is effective only in the later stages of plant growth with partial protection. One promising strategy under study in CIMMYT in the case of cereal rusts is to breed for general resistance (slow rusting / APR) based on historically proven stable genes. This type of race-nonspecific resistance can be further diversified by accumulating several minor genes and then combining them with different specific genes to provide a certain degree of additional genetic diversity. This approach also is applied to other diseases such as septoria leaf blotch, helminthosporium spot blotch and fusarium head scab (Rajaram, 2000).

An alternative approach is to pyramid several different race-specific resistance genes into one cultivar to prevent the attack from the newly formed races or from an existing race to become a predominant race by reducing selection pressure on a specific race. This approach seems to offer sustainable protection to the crop for over a period of

time (Raupp et al. 2001), besides protecting the crop in the early growth stages. However, this has to be a constant up-gradation process of the newly identified genes.

In either approach mentioned above, stacking several genes in one plant is essential for variety development. To dissect and genetically manipulate individual genes is an essential step for gene pyramiding. This process is both time consuming and technically difficult for race specific resistance genes, since breeding populations need to be tested for resistance to each target race of the pathogen and may be impossible for APR genes when only conventional breeding approach is used. Therefore, a molecular approach is a necessity to manipulate these genes. Simple and user-friendly molecular markers serve as landmarks for the presence of different resistance genes, which may speed up the breeding, process and facilitate the pyramiding of these genes.

Pathogen and its life cycle

There are two predominant sources of inocula for the leaf rust epidemics. The primary source is the spore masses traveling through wind from distant places, often from different states and even countries, and getting deposited on conducive wheat plants. The secondary source is the spore masses that rapidly spread from plant to plant within a field, and eventually to other nearby fields. A considerable amount of build up of inoculum under a favorable climate would lead to severe epidemics (Lipps, 1998, http://www.ag.ohio-state.edu/~ohioline/)

Leaf rust causes very small (about 1/32 inch long by 1/64 inch wide) orange pustules (uredia) that erupt through the leaf surface. In some cases, pustules are surrounded by a narrow yellow or white halo. The pustules contain masses of powdery

orange spores called urediospores. Spores may spill out of pustules and form a grainy orange dust on the leaf surface around the pustule to initiate secondary infection. As leaves age, pustules begin to produce dark black spores instead of orange spores called teliospores which is an inactive stage. These black pustules look like tar spots and are most easily seen on the lower leaf surface and leaf sheaths. Although leaf rust may initiate tiny orange spots on culms and heads, it does not form large, open pustules on these organs. Leaf rust pustules occur randomly across a leaf. Leaf rust typically occurs uniformly across a field. In over wintering locations, it is most severe on the bottom leaves and when it blows in from distant fields, it will be most severe on upper leaves.

Survival and transmission

It has been observed in the USA that the pathogen does not have an alternate host for its survival during off-season. The leaf rust population is composed of distinct races that do not cross with each other, because they do not have an alternative host for the completion of their sexual stage (Bowden, 2000, http://www.oznet.ksu.edu/path-ext/factSheets). This slows the development of new races because mutation is the only means of genetic change. In the summer, it survives on volunteer wheat and the spores blow to newly planted wheat in fall. Early planted wheat sometimes sustains heavy rust infection and may turn yellow in the fall. Leaf rust can survive the winter as latent infections if green leaves survive the winter. In the early spring, pustules erupt and fresh spores blow to new leaves. It is observed that the severity of the disease depends often on the amount of inoculum carried over from the northern parts of Mexico into southern US and up into the northern states. However, delay in this transmission often reduces the

final severity of the disease. The rust fungus moves back to volunteer wheat around the harvest time in the southern states of US and survives in dormancy until the next season.

Leaf rust infection in wheat

Leaf rust severity increases exponentially over time, causing rust epidemics during favorable weather. Daytime temperatures from 15^0C to 24^0C favor rust development in the spring. The infection process requires moisture, which can be provided by rain or dew. Heavy rain is unfavorable for rust because it tends to wash the spores off the leaves. Infection can occur in few hours during favorable weather if the inoculum is available. Dispersal of spores to upper leaves and between fields is favored by dry and windy conditions. Leaf rust reduces yields and test weights because infected leaves die prematurely. The earlier the leaves are lost, the more severe the yield loss. Losses may vary depending on the variety's ability to fill from the stem, glumes, and awns, and rust severity on flag leaf at various stages of growth (APPENDIX B).

Susceptible wheat does not have the ability to retard the fungal growth inside leaf tissues. The fungus grows extensively and produces relatively large pustules on a wheat leaf. Such a pustule may produce about 1,000 spores daily and each of them is capable of reinfecting new wheat tissues to start secondary infection or a new cycle if these spores move great distances along with the wind currents. Therefore, this disease can increase rapidly and epidemics may occur when susceptible varieties are grown and climatic conditions are conducive for rust development.

Mechanism of leaf rust resistance in wheat

Race-specific resistance

Genetically inherited resistance is an efficient means of controlling leaf rust (McCallum, Variety Survey 2001-2002). Resistance levels may vary among the classes of wheat and varieties within a class. According to the degree of resistance, varieties can be classified as resistant (R), moderately susceptible (MS) and susceptible (S) varieties. Susceptible varieties can suffer substantial yield loss up to 30 percent when inocula are readily available and environmental conditions are favorable for rust infection, as indicated in a NDSU study (McMullen et al. 1998). Varieties with MS reaction should be used with caution as damage may occur in favorable environments. Resistant and susceptible wheat reacts differently to the fungal infection. In a resistant variety, the dominant 'R' gene interacts with the dominant 'Avr' gene in the pathogen, resulting in hypersensitive reaction (HR). HR reaction to rust attack is manifested by the death of the infected cell to deprive nutrients for the growth of the fungus in the cells, thus killing it during initial infection stage. This type of resistance limits infection and retards fungal growth and spore formation. Resistant varieties may develop yellowish-white "flecks" at the site of spore penetration. This type of resistance response is characteristic of race specific resistance and is 'specific' because of gene-gene interaction.

Durable resistance by APR

Adult plant resistance (APR) to rust is responsible for durable resistance or slow rusting in wheat. Plants with APR do not show complete resistance to the fungal infection as in the case of race-specific resistance. Moderately resistant varieties develop small reddish-orange pustules surrounded by a yellow-white halo a characteristic of APR. APR

protects wheat from severe fungal damage by increasing latent periods and reducing spore production during a disease cycle thus contributing to low levels of secondary infection and eventually reduced disease severity and therefore, yield loss from fungal infection. However, the yield losses are considered to be significantly lower than that in susceptible cultivars. Wilcoxson (1986) showed that slow rusting varied with environment as well as races of the pathogen prevalent. This type of resistance is usually controlled by several genes and a single gene may contribute to a small portion of resistance and hence, combining of other resistance genes becomes necessary.

Lr genes and their usage

So far, about 45 leaf rust resistance genes have been catalogued (McIntosh et al. 1995) and many of them (such as Lr1, Lr2, Lr3, Lr16, Lr17 and Lr10) were used either singly or in combination of widely grown varieties. But, individual resistance genes were not effective in controlling leaf rust, because some races of the pathogen have evolved to overcome these genes soon after their release (McCallum, Variety Survey 2001-2002). A change in the pathogen population could affect the longevity of popular varieties carrying a race-specific gene. AC Barrie, a popular Canadian variety carrying the resistance genes Lr16 and Lr13 (Kolmer, 2001) was highly resistant when it was released in 1994 (McCaig et al. 1996), but virulence has developed for both of these resistance genes shortly after their release. At the same time, combinations of Lr9 and Lr24 (Long et al. 1994; McVey and Long, 1993; Roelfs et al. 1992) were reported to provide relatively long lasting resistance.

For race non-specific genes such as Lr34, the case is different. It conditions an intermediate level of resistance in wheat, protects wheat through slowing the development of the rust, and thus results in a reduced final disease severity. In a report from CIMMYT, four partial resistance genes, including Lr34, provide a slow rusting resistance and have restrained the outbreak of leaf rust epidemics in many developing countries during the past 15 years wherever the cultivars carry these genes (Rajaram, 2000). Another study confirms that Lr34 has been used extensively throughout the world, and has never been overcome by virulent races of the pathogen so far (Kolmer, 1996). But again, the major race non-specific genes were complemented with either minor genes or race-specific genes in different cultivars for their success over a period of time in a particular geographical location. Cultivars containing Lr34 gene were always complemented with several other genes, (Bezostaya-Lr34, 3a, 10; Chinese Spring-Lr34, 12, 31; Chris-Lr34, 10, 13 and Ciano-Lr34, 1, 13, 14a) (http://www.crl.umn.edu/res_gene/res_gene.html)

In either case, race-specific and race-nonspecific genes will not be able to provide the required level of protection against leaf rust when used singularly. So, strengthening the genetic base to ensure additional stability to wheat against leaf rust infection by either combining the available resistance genes into a cultivar or alternatively, complimenting the durable resistance genes with race-specific genes are the possible solutions for preventing rust epidemics.

D-genome-contribution

Wheat has limited source of resistance to leaf rust attack (APPENDIX A). Fortunately, a treasure of resistance genes has been identified from its wild relatives, *Triticum tauschii* and *T. monococcum* that are resistant to various pests and diseases (Gill et al. 1986, Gill and Raupp, 1987) and have been suggested to be potential sources of useful alleles for bread wheat improvement (Raupp et al. 1983). Besides the fact that broad-based plant germplasm resources are imperative for a sound and successful breeding program, they serve as additional sources for crop improvement. To generate additional genetic diversity in hexaploid wheat, Mujeeb-Kazi et al. (1996) produced synthetic wheats through hybridization of durum wheat (*Triticum turgidum* L.), the donor of the B and A genomes with *T. tauschii* Coss., the donor of the D-genome (Kihara. 1944). The genus *Aegilops* L. has contributed two other bread wheat genomes (Hegde et al. 2002), and *Triticum tauschii* (Coss.) Schmal. (2n = 2x = 14, DD) (syn. *A. squarrosa* L.; *Aegilops tauschii*) is well known as the D-genome donor of bread wheat (*T. aestivum*, 2n = 6x = 42, AABBDD) (See APPENDIX C).

Several leaf rust resistance genes from the D-genome have been transferred into wheat and used in breeding programs. It has been postulated that recombination between the D-genomes' of *T. aestivum* and *T. tauschii* occurred at a level similar to that in an intraspecific cross (Fritz et al. 1995), making such a transfer easy and meaningful.

To date six leaf rust resistance genes have been located on to the D-genome, Lr21 (1DS), Lr22a (2DS), Lr32 (3D), Lr41 (1DS), Lr42 (1DS) (Cox et al. 1994) and Lr43 (7DS) (Hussien et al. 1997), and transferred into bread wheat from different *T. tauschii* accessions (Huang et al. 2001). Also, resistance to leaf rust was observed to be common

among goat grass accessions held in the Wheat Genetics Resource Center (WGRC) collection (Gill et al. 1986; Cox et al. 1993).

Because of conserved synteny, the current research extends to the construction of a high-density map of the *T. tauschii* genome, which is thought to be useful for breeding and genetics within the tribe Triticeae that besides bread wheat also includes barley and rye (Boyko et al. 2002). The diploid status of *T. tauschii* made possible the construction of a large DNA insert library [bacterial artificial chromosome (BAC)] of the D genome (Moullet et al. 1999). This BAC library, combined with genetic linkage maps of *T. tauschii*, is being used to investigate the relationship between genetic and physical distances for regions of interest including those contributing to leaf rust resistance. A detailed molecular analysis of D-genome is a significant effort to probe the importance of this part of the genome (Gill et al. 1991; Lagudah et al. 1991b). Recently, about 249 new loci have been incorporated into a high-density cytological and genetic map of *T. tauschii* for a total of 732 loci and is thought to be one of the most extensive maps produced to date for the Triticeae species. Of the mapped loci, 160 are estimated to be defense-related genes (Boyko et al. 2002) These efforts facilitate the simple and easy identification of markers and genes related to traits of importance because of the fact that, directly working with a diploid genome is much simpler than working with a hexaploid genome and recombination frequency within a given genetic interval may also increase in the diploid compared to the hexaploid genome, enhancing the resolution of tightly linked markers (Dubcovsky et al. 1995).

Accessions contributing to the non-allelic genes Lr41, Lr42 originated in North central Iran which provided the largest number of leaf rust resistant accessions (Cox et al.

1993) and is thought to be the most genetically diverse region of *T. tauschii's* range based on molecular marker data (Lubbers et al. 1991). Lr41 and Lr42 genes originated from the *T tauschii* accessions; TA2450 and TA2460 were transferred into bread wheat (Cox et al. 1994). In the current study we have identified molecular markers that are very closely linked to these two independent genes. Considering their diverse genetic background, we believe that these genes would provide additional genetic base for the already existing leaf rust resistance genes in the breeding programs and the identified molecular markers would serve as useful selection tools to facilitate their deployment.

Tagging leaf rust resistance genes with molecular markers

Implications and research

Common wheat (*Triticum aestivum* L. em Thell, 2n = 42, AABBDD) has a large genome with ~ 16 million kb nucleotides per haploid cell. The genome is ~ 35 times of rice and ~ 110 times larger than that of arabidopsis (Bennett and Smith, 1976). The gene-containing fraction of the wheat genome should, therefore be <2.7%. Since only a small fraction of the wheat genome is expected to represent genes, identification and marking of the gene-containing regions is invaluable for their characterization.

While research indicates that multigenic resistance may be more durable (Roelfs, 1988), a single gene of interest in a complex background of other resistance genes may be difficult to detect through traditional phenotypic analysis. Hence, specific genetic markers for each of these genes can be a valuable tool to allow gene pyramiding for marker-assisted selection (MAS) in breeding programs. Moreover, several resistance

genes can be tracked simultaneously by testing for the presence of multiple molecular markers, and marker-assisted selection could be performed at an early developmental stage (seed / seedling) instead of evaluating rust in the later stages of plant growth.

In wheat, comparisons between near-isogenic lines (NILs) and their recurrent parents have been used to identify molecular markers linked to pathogen resistance genes (Schachermayr et al. 1994, Autrique et al. 1995, Procunier et al. 1995). Identification of molecular markers for the Lr35/Sr39 resistance genes facilitated the transfer of these genes to elite wheat lines (Gold et al. 1999). There is considerable interest in finding markers that could ultimately be used to incorporate longer-lasting resistance to major wheat diseases, such as leaf rust, Karnal bunt, and fusarium head scab (McGraw, 2001). Several resistance genes effective against rust pathogens have been located on the group-1 chromosomes of wheat (McIntosh et al. 1998). Several RGA markers were mapped to the same chromosome locations (Spielmeyer et al. 1998). The integration of Lr24 gene into breeding lines has been facilitated by an STS marker linked to this gene (Schachermayr et al.1995). Wheat leaf rust resistance gene Lr10 was studied extensively by map-based cloning strategies and characterized (Feuillet et al. 2000). Lr19 provides effective resistance against all leaf rust pathotypes in South Africa (Prins et al. 1996, 1997) and was recently tagged by AFLP and STS markers (Prins et al. 2001). These markers would eventually facilitate pyramiding of these genes in breeding programs.

Identification of molecular markers has not been easy in wheat considering the limited level of polymorphism (Chao et al. 1989; Kam-Morgan et al. 1989; Liu et al. 1990; Cadalen et al. 1997). To-date, more than 45 leaf rust-resistance genes have been identified (McIntosh et al. 1995) but very few genes have been tagged and thus very few

markers are available for MAS. So far, nineteen Lr genes have been tagged by molecular/cytological markers (Langridge and Chalmers, 1998; Seyfarth et al. 1999; Seah et al. 2000; Spielmeyer et al. 2000). However, tightly linked PCR-based markers have been developed only for Lr1 (Feuillet et al. 1995), Lr10 (Feuillet et al. 1997), Lr25 and Lr29 (Procunier et al. 1995), Lr28 (Naik et al. 1998), Lr35 (Seyfarth et al. 1999) and Lr37 (Seah et al. 2000). PCR-based markers, allow for large-scale genotypic selection of individuals in breeding populations. These markers can be used to pyramid different leaf rust-resistance genes and as starting points for positional cloning of the genes (Martin et al. 1993; Song et al. 1995).

Detailed RFLP (restriction fragment length polymorphism) linkage maps (Chao et al. 1989; Devos and Gale, 1993; Xie et al. 1993; Nelson et al. 1995A, Nelson et al. 1995B, Nelson et al. 1995c; Van Deynze et al. 1995; Marino et al. 1996, Ahn and Tanksley, 1993) and physical maps (Gill et al. 1993; Kota et al. 1993; Hohmann et al. 1994; Ogihara et al. 1994 Delaney et al. 1995a, Delaney et al. 1995b; Mickelson-Young et al. 1995; Gill et al. 1996) have been published for all seven homeologous groups in wheat. However, the laborious procedure and low polymorphism among wheat cultivars, in this hybridization based marker identification tool besides the fact that only a few loci are detected per assay, limit its application for high-throughput mapping and marker–assisted selection.

RAPD (random amplified polymorhic DNA), a PCR-based method has been used in the construction of linkage maps and in the identification of specific loci of interest (Williams et al. 1990, Sonder et al. 1996, Millan et al. 1996 and Ratnaparkhe, 1995). However, the sensitivity of RAPD markers to subtle changes in reaction conditions is the

major limitation in the large-scale application of RAPD in genome research (Bai et al. 1999).

Microsatellites have emerged as an important source of ubiquitous genetic markers for many eukaryotic genomes (Wang et al. 1994). In plants, it has been demonstrated that microsatellites are highly informative, locus-specific markers in many species (Condit and Hubbell, 1991; Akkaya et al. 1992; Lagercrantz et al. 1993; Senior and Heun, 1993; Wu and Tanksley, 1993; Bell and Ecker, 1994; Saghai-Maroof et al. 1994; Rongwen et al. 1995; Liu et al. 1996; Morchen et al. 1996; Provan et al. 1996; Szewc-McFadden et al. 1996; Taramino and Tingey, 1996; Smulders et al. 1997). Microsatellites show a high level of polymorphism in hexaploid bread wheat (Plaschke et al. 1995; Roder et al. 1995; Ma et al. 1996; Bryan et al. 1997). The main constraint behind using these markers is the underlying technical difficulty, time and expenses involved in the design of SSR primers.

AFLP (amplified fragment length polymorphism) is a powerful tool for identification of markers associated with genes of interest. However, there has been only one instance till to date where they have been used for tagging Lr genes (Prins et al. 2001; Lr19), despite being ubiquitous in other studies (Alonso-Blanco, 1998; Bai et al. 1999; Castiglioni et al. 1999; Shan et al. 1999 and Vuylsteke, 1999). AFLP assays can detect a larger number of genetic loci per reaction than RFLP and SSR analysis (Becker et al. 1995; Mackill et al. 1996;Schondelmaier et al. 1996). A marker identification system with the capacity to assay large numbers of loci with minimal effort is especially important in genome mapping.

Future directions

More recently techniques such as chromosome walking and sequencing in hexaploid wheat (Stein et al. 2000) led to the identification of two resistance gene analogs (RGA1 and RGA2), which co-segregate with Lr10 (Wicker et al. 2001). There is also effort to isolate the resistance gene Lr1 located at the distal end of chromosome 5DL of wheat (Feuillet et al. 1995) using map-based gene cloning. The majority of resistance genes isolated belong to the class encoding nucleotide binding sites-leucine rich repeat proteins (NBS-LRR) (Martin, 1999). Short peptide sequences adjacent to the NBS are well conserved among gene members of this class and have been used to identify resistance gene analogs (RGAs) from many plant genomes (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Collins et al. 1998; Seah et al. 1998). There are reports of detailed mapping in *T. tauschii* gene family members belonging to two NBS-LRR classes and linkage of these markers to leaf rust resistance genes Lr21 and Lr40 located on chromosome 1DS and a stripe rust resistance gene from *T. vavilovi* located on chromosome 1BS (Spielmeyer et al. 2000).

Considering the large, hexaploid and repetitive genome of wheat, identification and isolation of agronomically important genes by map-based cloning is not an earthly task. A recent approach to this problem is to use a simpler model of the genome to identify candidate genes, comparative mapping. Given a model species for which the entire genome is sequenced, the information could be used to map and identify genes of a large genome species (LGS) such as wheat. One approach is to sequence previously mapped probes or generate new ESTs (expressed sequence tags) for the LGS, map a fraction of them in the LGS, and cross-reference both mapped and un-mapped sequences

by sequence matching using BLAST searches against the model species (Sorrels et al. 1999).

Consensus maps can be developed for related species with similar genomes. Such maps are based on conservation of linkage groups composed of homologous and homoeologous chromosomes. These maps merge information about closely related species and are useful for cross-referencing genetic information from more distantly related species. A consensus map has been developed for Triticeae species, based on a common set of markers mapped onto the respective linkage groups of *T. aestivum*, *T. tauschii*, and Hordeum species (Nelson et al. 1995ab; Van Deynze et al. 1995ac). Consensus maps have been constructed for several Gramineae species. Devos et al. (1993) demonstrated conservation of the wheat and rye genomes. The genomic regions of two wheat leaf rust disease resistance loci; Lr1 and Lr10 have been mapped using the putative model genomes of rice and barley (Gallego et al. 1998).

Even though there is an abundance of genetic potential available in the form of Lr genes, not all of them could be incorporated successfully into breeding programs due to lack of good selection tools. There is also an urgent need for development of workable germplasm for molecular marker identification. Since many genes associated with the leaf rust resistance have been identified on the D-genome of wheat, and working with a smaller sized genome may be relatively simpler. Therefore, the focus should be to identify molecular markers closely linked with these genes. There are a variety of marker identification tools, besides the available BAC library of the D-genome could be used for genetic characterization of the genes for which tightly linked markers are available. Also, the availability of complete genome sequence data of *T.tauschii* and ESTs of bread wheat

would be particularly beneficial in all the efforts aimed at identification of genes of interest such as, identifying closely linked markers, map-based cloning and comparative mapping

The current study is aimed to identify closely linked molecular markers to two different leaf rust resistance genes, Lr41 and Lr42 that would facilitate the pyramiding of these two useful leaf rust resistance genes. Multiple markers, segregating in a single plant will indicate that the respective resistance genes are also present. Lr42 is believed to be a partially dominant gene conferring durable resistance (Martin et al. 2000, unpublished results) and Lr41 is a single dominant race-specific gene (Cox et al. 1994). There were several efforts in the past to identify markers linked to different Lr genes and most of them by using RFLP technology (Helguera et al. 1999; Lr47, Messmer et al. 2000, Lr1 and Lr10; Feuillet et al. 1995, 2000) and RGA markers (Lr21 and Lr40; Spielmeyer et al. 2000). But still, there is an ever-increasing need to develop fast, reliable and high-throughput screening methods in order to identify the genes of inerest from a mixed population. In this study we have used AFLP technology and further optimized the protocol for high-throughput screening of mapping populations. The new protocol eliminates utilization of radio labeled primers, that simplifies the screening procedure, reduces the cost and at the same time ensuring safety to the people involved. Several tightly linked markers identified for the two Lr genes in this study would facilitate their usage in breeding programs.

CHAPTER II

AFLP MARKERS ASSOCIATED WITH THE LEAF RUST RESISTANCE GENES Lr41 AND Lr42 IN WHEAT

Introduction

Leaf rust caused by *Puccinia triticina* (Eriks. and E. Henn.), has been reported to cause yield losses up to 40% in susceptible wheat cultivars (Knott, 1989) and has been identified to be one of the most destructive disease of wheat worldwide (Kolmer, 1996). Severe yield losses from rust infection mainly result from premature defoliation, which affects photosynthetic and grain filling ability. Utilization of genetic resistance is the most environment-friendly strategy for the disease control (Huang et al. 2001). However, most of leaf rust resistance genes widely used in wheat breeding programs are race specific, and therefore tend to be effective only for a limited duration. The evolution of new pathological races is constant (Bayles and Stigwood, 1994, 1995, 1996) and therefore, the need for exploration of new resistance genes is always imminent. Pyramiding of major genes into a single cultivar is an effective solution to this problem (Boyko et al. 1999). Several wild ancestors of the modern bread wheat, such as *Triticum tauschii. Coss (Syns. Aegilops squarrosa* L. and *Aegilops tauschii* (Coss) Schmal. 2n=2x=14, DD) and *Triticum monococcum*, have been identified to carry resistance genes to several races of the leaf rust pathogen (Gill et al. 1986). To date, six leaf rust resistance genes, Lr21 (1DS), Lr22a (2DS), Lr32 (3D), Lr41 (1DS), Lr42 (1DS) (Cox et al. 1994) and Lr43 (7DS) (Hussien et al. 1997), were transferred into bread wheat from different *T. tauschii* accessions (Huang et al. 2001). The transfer of major genes of importance had been possible because of the contribution of D-genome to common wheat by *T.tauschii* (Gill and Raupp, 1987). Lr41 and Lr42 are two major leaf rust resistance genes identified on the short arm of chromosome 1D of *T. tauschii* (Cox et al. 1994; Rowland and Kerber, 1974) and were introgressed into the wheat germplasm from different *T. tauschii*

accessions.

The mechanism for durable resistance to leaf rust is poorly understood (Raupp et al. 2001), but durability appears to be enhanced when genes are combined. For example, the combination of Lr16 and Lr13 (Long et al. 1993; Samborski and Dyck, 1982) or Lr9 and Lr24 (Long et al. 1994; McVey and Long, 1993; Roelfs et al. 1992) were reported to provide relatively long-lasting resistance. Given the necessity of controlling multiple races of the rust pathogen, deploying different resistance genes against leaf rust can be achieved by stacking or pyramiding various resistance genes into individual adopted wheat cultivars. Since gene pyramiding with conventional breeding approaches is both laborious and time consuming, an efficient strategy to address this problem is to identify molecular markers tightly linked to the major resistance genes for marker-assisted selection (MAS). These markers could be used to predict the presence of target genes (Harms, 1992; Michelmore, 1995a).

The development of the polymerase chain reaction (PCR) (Saiki et al. 1988) has expanded the repertoire and efficiency of available methods for DNA marker identification tools (Vos et al. 1995). AFLP is a PCR based marker identification system, and combines both the advantages of RFLP (using restriction-site variation) and PCR (exponential amplification of DNA templates). In addition, AFLP is a multiplex marker identification tool (Powell et al. 1996; Pejic et al. 1998) and provides high levels of polymorphism per primer pair base. It provides equal or greatly enhanced performance in terms of reproducability, resolution and time efficiency. Probably the single greatest advantage of the AFLP technology is its sensitivity to polymorphism detection at the total-genome level, making it an ideal system for the construction of high-density linkage

maps. Normally, 50–100 AFLP loci can be analyzed with each primer combination. By increasing the number of primer and/or enzyme combinations, large numbers of AFLP loci can be screened throughout the entire genome, thus enabling AFLP markers tightly linked to the target gene to be identified. Thomas et al. (1995) screened about 42,000 AFLP loci and obtained two AFLP markers flanking the *Cf* gene in tomato only 15.5 kb apart. AFLP technique has been extensively used in many plant species to assess genetic diversity (Barrett and Kidwell, 1998; Barrett et al. 1998; Burkhamer et al. 1998; Ellis et al. 1997; Schut et al. 1997; Ajmone Marsan et al. 1998; Hill et al. 1996; Hongtrakul et al. 1997; Lu et al. 1996; VanTorai et al. 1997; Roa et al. 1997; Gaiotto et al. 1997), to construct high-resolution maps (Becker et al. 1995; Van Eck et al. 1995; Schondelmaier et al. 1996; Keim et al. 1997; Qi and Lindhout 1997; Maheswaran et al. 1997; Wang et al. 1997, Ballvora et al. 1995; Meksem et al. 1995; Thomas et al. 1995; Rouppe van der Voort et al. 1997,), to tag quantitative trait loci and other genes (Pakniyat et al. 1997; Powell et al. 1997; Roa et al. 1997; Meksem et al. 1995; Thomas et al. 1995; Cnops et al. 1996; Büschges et al. 1997; Schwarz et al. 1999). In addition, they could be used for integrating the genetic and physical maps of the complex genomes (Klein et al. 2000; Zobrist et al. 2000).

Considering the serious losses incurred to wheat due to the leaf rust pathogen, there is an urgent need to combine various resistance genes into cultivars to effectively combat the threat. To improve the breeding efficiency for leaf rust resistance, an effective screening method to evaluate breeding populations for Lr genes need to be developed. Molecular markers tightly linked to rust genes may provide an efficient tag for these genes and could be used as an indirect selection tool during gene pyramiding. Objectives

of this study are to: 1) develop a high throughput protocol for molecular marker identification by using automated DNA analyzer and 2) identify molecular markers associated with the Lr41 and Lr42 rust resistant loci by using NILs.

Materials and Methods

Plant materials and disease evaluation

TA2460 and TA2450 are two *T.tauschii* accessions, harboring Lr41 and Lr42 leaf rust resistance genes (Cox et al. 1994). To develop near-isogenic lines contrasting in Lr41 and L42 alleles, both the tauschii accessions were backcrossed three times to the wheat cultivar Century. The lines, KS90WGRC10 and KS91WGRC11 (PI 56668) were selected to carry the Lr41 and Lr42 alleles in Century background (Cox et al. 1994). KS93U62 and KS93U50, selections from KS91WGRC10 and KS91WGRC11, were further crossed to OK92G205, an awnletted near-isoclines of Century derived from Century*5/McNair1003 without the alleles, Lr41 and Lr42 (Carver et al. 1993). McNair1003 was a soft red winter wheat carrying the awnletted gene. The F2 population from the crosses of KS93U62/OK92G205 and KS93U50/OK92G205 each segregated at both the Lr and awn production loci (Table 2). The seedlings of more than 200 F2 plants were inoculated with *P. triticina* in the controlled greenhouse environment (Martin et al. 2002). The $F_{2:3}$ families were further tested for rust segregation through natural infection under field conditions at Stillwater, OK. Four classes of homozygous genotypes in the population were identified: awned resistant and susceptible, and awnletted resistant and susceptible (Martin et al. 2002). Another awned series segregating only at the leaf rust

resistance locus in Lr41 and Lr42 lines was also developed by crossing KS93U62 and KS93U50 independently to OK92G206, an awned near-isoline of OK92G205 (Carver et al. 1993). The two series of $F_{2:3}$ and $F_{2:4}$ wheat lines were tested for rust resistance through natural infection at the field of Stillwater, OK in 1998 and Lahoma, OK in 1999 (Martin et al. 2002). The inocula were bulk collections of urediospores from Kingfisher, Apache and Lahoma in Oklahoma. Leaf rust reaction was determined using modified Cobb scale (Peterson et al. 1948). The bulk spores of *Puccinia triticina* were also used for inoculation on a set of single gene differentials as well as appropriate checks to determine the virulence/avirulence formula of the bulk collections The result indicated the presence of the race with avirulence gene corresponding to Lr41 and Lr42 resistance genes in the inocula (Martin et al. 2002).

DNA extraction and preparation for AFLP

Genomic DNA of 51 Lr41 and 45 Lr42 $F_{2:5}$ series was extracted with the automatic FastPrep DNA Isolation System (Q.BIOgene, Carlsbad CA) by using a modified CTAB extraction protocol (Maroof et al. 1984). For AFLP analysis, 500 ng DNA was double digested with *Pst*I and *Mse*I restriction enzymes. *Pst*I is a rare cutter restriction enzyme with a 6-base recognition site and *Mse*I is a frequent cutter with a 4-base recognition site. This step was follows the ligation of corresponding adapters to both ends of the digested fragments. The sequences for *Pst*I adapterare 5'-CTCGTAGACTGCGTACATGCA and 5'-CATCTGACGCATGT, and the sequences for *Mse*I adapter are 5'-GACGATGAGTCCTGAG and 5'-TACTCAGGACTCAT. The primers with or without a selective nucleotide at the 3'-end were used for pre-

amplification. However, the same banding patterns were produced for selective amplification with both preamplified DNA templates. Therefore, primers without a selective nucleotide were used for the remainder of the experiment. The pre-amplified fragments were further selectively amplified using the various primer combinations (Table 1)

AFLP marker analysis

All the PCR reactions were carried out in the MJ PTC-100 thermocycler. For preamplification, a 40 µl of PCR mixture consisting of 1 x PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTP mix, 75 ng each of unlabeled *Pst*I (5'-GACTGCGTACATGCAG) and *Mse*I (5'-GATGAGTCCTGAGTAA) primers, 0.75 u of *Taq* Polymerase and 10 µl of ten-fold diluted and ligated DNA. PCR was run for 25 cycles at 94 °C for 30 s, at 56 °C for 60s and at 72 °C for 60s.

Tenfold diluted preamplified PCR product was then used as a template for further selective amplification. A 10 µl of PCR mixture contained 2 µl of diluted preamplified DNA, 1 X PCR buffer, 2.5 mM of $MgCl_2$, 0.2 mM of dNTP mix, 10 ng of unlabeled *Mse*I primer, 0.35 pmol of IR florescent-labeled *Pst*I primer and 0.20 unit of *Taq* Polymerase. The reactions were run at 94 °C for 2 min followed by 13 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 C for 60s with a touchdown temperature of –0.7 °C/cycle as annealing temperature in each following cycle, followed by another 23 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60s.

To detect AFLP products, *Pst*I primers were end-labeled with IR700 or IR800 fluorescent dye compatible with the LI-COR DNA analyzer. Both *Pst*I and *Mse*I selective

primers are listed in the Tab 1. Following amplification, PCR products were mixed with 5 µl of formamide loading dye (98% formamide, 100 m*M* EDTA, pH 8.0, and bromophenol blue, and xylene cyanol). The resulting mixture was denatured for 5 min at 94°C, and then quickly cooled on ice. To a 20 ml, 6.5% gel solution (4.75% acrylamide, 0.25% methylene bisacryl- amide, and 7.5 *M* urea in 1X TBE buffer), 75 µl of 20% ammonium persulfate and 15 µl TEMED were added right before the gel were cast with LI-COR 25 cm glass plates. The gel is cast at least one hour before running or overnight preferably. Gel was pre-run in 1L of 1X TBE buffer (50 mM TRIS, 50 m*M* boric acid, 1 m*M* EDTA) for 10 min before the samples were loaded. A sample of 0.8 µl was loaded into each lane of the gel. The electrophoresis condition was set at 1500 V, 40 W at 50 °C. The gel image was collected simultaneously as the electrophoresis progressed by a scanner inside the analyzer and visualized on a computer screen. The AFLP images were visualized through the AFLPSCAN software from LI-COR Inc. (Lincoln, NE). The identified markers were named according to the standard AFLP marker nomenclature (Zabeau, 2000) with the format '*XxyzAN1N2N3*, where *'X'* is the usual symbol for a DNA marker (e.g., Lr41 and Lr42); '*xyz*' is the laboratory designator (osu); A is a single upper-case letter denoting the rare-cutter enzyme used, e.g., P for *PstI*, etc (If the same enzyme was used in more than one mark, labeled as P1, P2 etc). N1 and N2 are two-digit numbers identifying standard one, two or three base-pair extensions and N3 is a three-digit number corresponding to the molecular weight of the fragment.

Bulked segregant analysis

In the current study, bulked segregant analysis coupled with AFLP was used to identify putative markers linked to Lr41 and Lr42 genes. For the initial screening, DNA from 12 resistant lines and 12 susceptible lines was pooled to form two representative bulks for each of the two populations. A total of 17 labeled *Pst*I primers in combination with 36 *Mse*I primers (Table 2) were used for the screening of the bulks, KS93U62 (Lr41 donor), KS93U50 (Lr42 donor) and two Century isolines contrasting in awn production. Primer pairs that amplify polymorphic bands between bulks and parents were selected for further screening of 51 lines for Lr41 and 45 lines segregating for Lr42 alleles.

AFLP mapping of Lr41 and Lr42 genes

Two sets of NILs each segregating for leaf rust resistance at either Lr41 or Lr42 locus was subjected to AFLP analysis. The polymorphic marker loci were scored as 1 for the presence and 0 for the absence of the band. The disease and marker data from the populations were used for genetic linkage analysis using Mapmaker 3.0 software (Lander et al. 1987. Macintosh version 2.0) with the LOD value set at 3.0. Genetic distance was expressed as a Kosambi function (Kosambi, 1944) in cM

Results

AFLP analysis of wheat using LI-COR DNA analyzer

Because the parents of the mapping populations shared the same Century background and contrasted only in the region containing Lr gene, polymorphism levels between the parents of both populations was expected to be very low, therefore, the

multiplex AFLP marker identification tool was the only choice for this experiment. Among 612 combinations of *PstI* and *MseI* primers screened, about 95% of them amplified AFLP products, and most of the gels amplified from 50 to 100 loci with an average of about 70 loci per gel when florescent-labeled AFLP fragments were analyzed with LI-COR DNA analyzer. To improve throughput of population screening, this was the first time that LI-COR DNA analyzer was used for AFLP analysis in wheat. Due to the high sensitivity of IR fluorescent system and large genome size of wheat, too many bands in each reaction result in a low-resolution gel image with many overlapped bands in initial experiment. Therefore, existing AFLP protocol has been optimized to accommodate fluorescent screening using the LI-COR DNA analyzer. Gel running conditions were optimized and DNA concentration for selective amplication was increased. In addition, many primers with four selective nucleotides were used when primers with two or three selective nucleotides amplified too many bands. As a result, most primers studied provided scorable DNA bands and the banding pattern was comparable to radioisotope-labeled AFLP or silver stained gels (Figures 1-10). The result demonstrated that fluorescent labeling provided certain advantages over radioisotope-labeled AFLP such as cheaper and faster for data collection and environmentally friendly and safer for laboratory operation. In radioisotope labeled AFLP primers, visualization of AFLP takes 2-3 days and costs 40 ¢ per reaction, while it only takes 3 hours to get data and costs about 20 ¢ per reaction in fluorescent-labeled AFLP. When AFLP is analyzed in LI-COR DNA analyzer, two sets of primers labeled with dyes at different wavelengths could be analyzed in a single reaction and run simultaneously in a single gel, which not

only increases throughput of analysis and also reduces cost. In addition, it provides more accurate measurement of molecular sizes for each fragment.

Mapping of Lr41 gene

A total of 612 primer combinations of *PstI* and *MseI* were used to screen the bulked resistant and susceptible NILs carrying the Lr41 gene and the respective parents, and eight primer combinations showed polymorphism. These primers were used further to screen 51 $F_{2:5}$ NILs including both homozygous resistant and susceptible classes. Four AFLP markers tightly linked to the Lr41 gene were identified (Figures 1-4) and they were all mapped on one linkage group that covered 4 cM distance (Figure 11). Two markers (Lr41osuPAG200 and Lr41osuPCATG140) completely co-segregated with the Lr41 gene. Three AFLP markers for Lr41 gene were dominant (Figures 1, 3, 4). Molecular weights for these markers ranged from 200 bp to 230 bp. However, the primer combination P-CATG/M-CGCT amplified co-dominant bands at about 60 bp (Figure 2) in that, it has amplified two fragments with different size in resistant and susceptible genotypes, respectively.

Mapping of Lr42 gene

After an initial screening of 612 pairs of AFLP primers (Table 1), 15 of them amplified at least one polymorphic band between two parents and the bulks. Further analysis of the $F_{2:5}$ mapping population with these primers indicated that six of them amplified a polymorphic band associated with rust resistance in the population. These bands were about 200 bp in molecular size (Figures 5, 6, 7, 8, 9 and 10). When these

marker data and disease ratings were analyzed together using the MapMaker program, Lr42 locus and all the six markers were mapped on a single linkage group that covered 51.3 cM in genetic distance (Figure 12).

Among the six markers, markers Lr42osuP1CGA110, Lr42osuP2CGA200 and Lr42osuPGTG200 were linked to Lr42 in repulsion phase, while markers Lr42osuPCATG200, Lr42osuP2CGA200 and Lr42osuP3CGA210 were linked to Lr42 in coupling phase. The marker Lr42osuPGTG200 completely co-segregated with resistance. Two markers (Lr42osuP1CGA110 and Lr42osuPCATG200) were very close to Lr42 gene and apart at 2.2 and 6.6 cM to the Lr42 gene respectively. The three remaining were further apart from the Lr42 locus.

Discussion

Major leaf rust resistance genes have been introgressed into wheat through breeding approaches to strengthen the genetic assembly of the currently available germplasm against this pathogen. To beat the pathogen outbursts, gene-pyramiding process seems to be an effective approach. Only a few resistance genes have been pyramided into the commercial cultivars (Lr 1, 2a, 9, 12, 13, 19, 22a, 24, 25, 28, 29, 34, 35, and 37; Mesterhazy et al. 2000). A constant shift in the pathogen population as a result of mutations leads to the formation of new races or existing races becoming dominant, hence the focus rests inadvertently on the search for new and durable leaf rust resistance genes and their application in elite cultivars. Molecular markers tightly linked to a specific gene may facilitate gene pyramiding and improve selection efficiency by shortening the time of selection in breeding programs (Mohan et al. 1997).

Previous studies indicate that Lr41 is a completely dominant gene based on the segregation analysis and that the resistance it imparts was evident in the seedling stage. Wheat plants hosting this gene showed no visible symptoms of disease when inoculated in the green house with bulk spores of *Puccinia triticina* and also under conditions of natural infection in the field (Martin et al. 2002). The same study indicated that the difference in rust severity between the NILs was larger for Lr41 than that for Lr42 gene. The presence of Lr41 gene contributed to an increase in grain yield by 63%, test weight by 5% and kernel weight by 14%. It was concluded that, resistance to leaf rust was critical to maintain maximum yield, test weight and kernel size. Similar studies suggest that Lr42 gene could be conditioning resistance in adult plants (Martin et al. 2002; Cox et al. 1994). In such a case, the gene would provide durable resistance when incorporated into wheat cultivars.

Although, it has been identified that both Lr41 and 42 genes are confined to the short arm of chromosome 1D (APPENDIX 1) (Cox et al. 1994) and thus belong to a single linkage group. None of the markers identified in the current study were common to both of them and we hypothesize that either they could be separated from each other by considerable distance that makes them segregate independently or they could be present on separate chromosomes, so the markers did not represent a common locus. In order to further confirm that these genes are located on the short arm of chromosome 1D, the AFLP markers identified in the current study may need to be converted into locus-specific PCR based markers to localize their positions using wheat nullitetrosomic and ditelosomic genetic stocks.

Working with the genome of wheat is considered quite a challenge due to its large size and complexity. In this study, several of such factors were considered prior to the initiation of the task and AFLP protocol was optimized at several steps for high-throughput screening. The expected number of genetic polymorphisms was low, since each working population is a set of near-isogenic lines among which only genetic loci closely linked the two Lr genes segregated. The use of selective nucleotides 'A' and 'C' in pre-applification step did not increase resolution any further. Additional studies were carried out without the selective nucleotides at the 3' end of the preamplification primers. For selective amplification, 2-4 selective nucleotides were used, but primers with 4 selective nucleotides amplified less, but clear bands than that from two selective nucleotides. Bulked segregant analysis (BSA) is a fast approach to identify markers linked to target traits (Li et al. 1998). This technique is very powerful and has been used extensively in different plant species in combination with various marker identification techniques since its first discovery (Michelmore, 1991). Combination of AFLP technique with BSA to detect the polymorphic loci was especially useful in this study considering its robustness with an abundance of primers for screening without the need of any prior sequence information.

The use of fluorescent-labeled primer screening for Lr loci was experimented in this study that served to screen a large number of loci in a very short span of time.

The automated DNA analyzer from LI-COR uses IR fluorescent labeling of PCR primers to generate labeled amplicons, which simplifies the procedure for data generation with higher accuracy. Data can be collected in 3 hr after PCR. In addition, two PCR reactions with different IR dyes can be run simultaneously or alternatively, two different

sets of primers could be used in a reaction. Both the methods yield similar results and no contamination whatsoever was observed in our experiments. Conventional visualization by autoradiography or silver staining has low throughput, because only one sample per gel lane can be analyzed. Also accurate allele typing is often not feasible due to poor resolution and migration-variability from lane to lane, as well as from one run to another (Schwarz, 2000). In LI-COR DNA analyzer, the fluorescence dyes with distinguishable wavelength emissions allows one to electrophorese two different samples simultaneously in a single lane, and a computer connected to the machine monitors all gel-running conditions. An acceleration of genotyping was also demonstrated for SSRs in *Brassica* spp by combining several fluorescent-labeled primers in a single PCR reaction when an automated DNA analyzer was used (Mitchell et al. 1997). When AFLP products are labeled with a radioisotope and visualized on an autoradiograph to detect the polymorphisms, handling radioactive waste becomes a major safety concern for lab operations. In addition, it takes at least 2-3 d to collect data and manual inspection of radioisotopic data is time-consuming and with low accuracy. The automated DNA analyzer from LI-COR uses IR fluorescent labeling of PCR products, which simplifies the procedure for data generation with higher accuracy. Data collection starts at about 45 min. after loading the samples. The quality of gel images is comparable to radioisotope-labeled AFLP and also the silver stained gels. In this study, we use NIL population in which extremely low polymorphism levels were expected. AFLP coupled with a LI-COR DNA analyzer serves as a high throughput marker identification system and enables to identify molecular markers to loci of agronomic importance by simplifying the screening process. In this study, ten AFLP markers linked to two Lr genes were successfully

identified. We thus further conclude that this optimized AFLP technique is a very efficient tool to identify molecular markers even in those species with low polymorphisms such as wheat.

Due to the proximity of the markers identified to the genes, we assume they could be used as efficient tools for the selection of the respective genes in breeding population. The markers that co-segregated with the respective resistance genes in coupling phase would serve to select for resistance specifically and those that segregated with susceptibility in repulsion phase will help establish the absence of resistance imparted by these genes. Also there is potential for further conversion of the AFLP markers to high-throughput PCR-based markers for MAS and map based cloning of the genes.

Table 1. List of Parents and population used for marker analysis of Lr41 and Lr42 genes

Population	# of lines	Lr gene (a)	Awn type (b)
OK92G205 (Parent)	1	Lr41-/Lr42-	A-
OK92G206 (Parent)	1	Lr41-/Lr42-	A+
KS93U62 (Parent)	1	Lr41+	A+
KS93U62/OK92G206 $F_{2:5}$	11	Lr41+	A+
KS93U62/OK92G206 $F_{2:5}$	8	Lr41-	A+
KS93U62/OK92G205 $F_{2:5}$	16	Lr41+	A+/A-
KS93U62/OK92G205 $F_{2:5}$	16	Lr41-	A+/A-
KS93U50 (Parent)	1	Lr42+	A+
KS93U50/OK92G206	6	Lr42+	A+
KS93U50/OK92G206	7	Lr42-	A+
KS93U50/OK92G205	19	Lr42+	A+/A-
KS93U50/OK92G205	13	Lr42-	A+/A-

(a) Lr41 and Lr42 +/- indicate resistance or susceptibility conferred due to the presence or absence of the respective genes based on penotypic data.

(b) A+ / A- indicate the presence or absence of awn in the progeny based on phenotypic data.

Table 2. A list of AFLP primers used for parent screening by bulked segregant analysis

*Pst*I primer	*Mse*I primer	*Mse*I primer	*Mse*I primer
P-ACT	M-ACGC	M-GCAG	M-GGCT
P-ACTG	M-AGC	M-GTG	M-GCAT
P-AGT	M-CAA	M-ATGC	
P-CAT	M-CAC	M-ACAG	
P-CATG	M-CACG	M-ACGT	
P-CTC	M-CAG	M-ACTG	
P-TGC	M-CAGT	M-AGAC	
P-AGC	M-CAT	M-AGCT	
P-CGA	M-CGAC	M-AGGC	
P-AG	M-CTA	M-AGTG	
P-ACA	M-CTC	M-CACG	
P-AGG	M-CTG	M-CGCT	
P-CAG	M-CTGA	M-CGAT	
P-CGT	M-CTT	M-CGTA	
P-CTCG	M-GAC	M-CTCG	
P-GCTG	M-GCG	M-TGCG	
P-GTG	M-TGC	M-TCGA	

Note: P- and M- are abbreviations for pre-amplification primers at the *Pst*I site and *Mse*I site. Their complete sequence is GACTGCGTACATGCAG and GATGAGTCCTGAGTAA, respectively

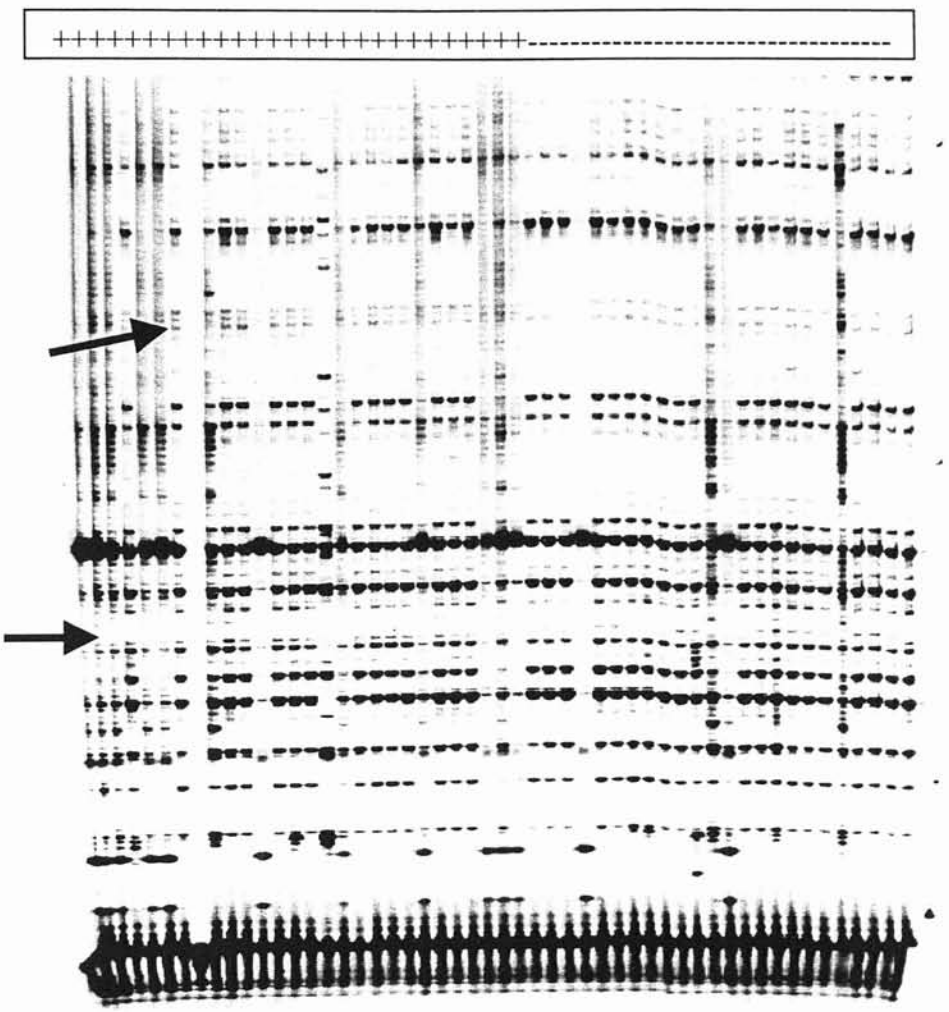

Figure 1. An AFLP gel showing markers, (Lr41osuPCATG60) and (Lr41osuPCATG140) closely linked to Lr41 gene and amplified by the primer combination CATG/CGCT. Sign "+" indicate the lines that carry the Lr gene and "–" indicate the lines that do not carry the Lr gene.

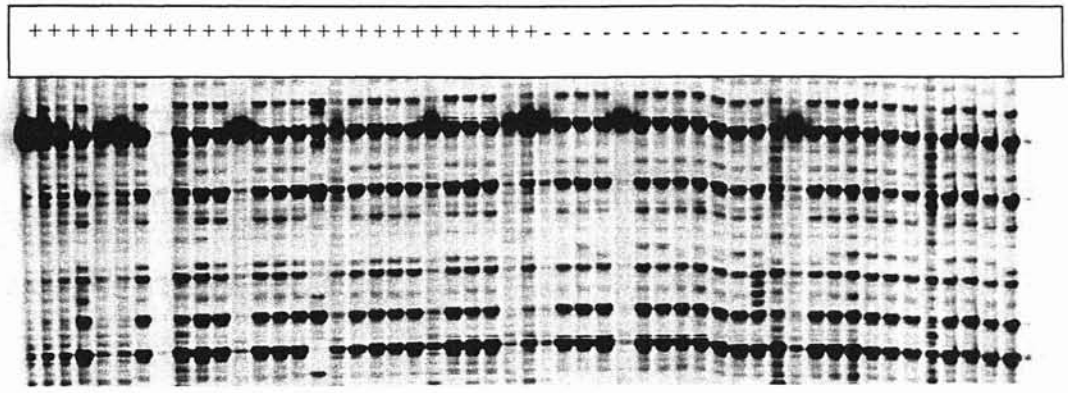

Figure 2. An AFLP gel showing a marker (Lr41osuPCATG60) closely linked to Lr41 gene and amplified by the primer combination CATG/CGCT.

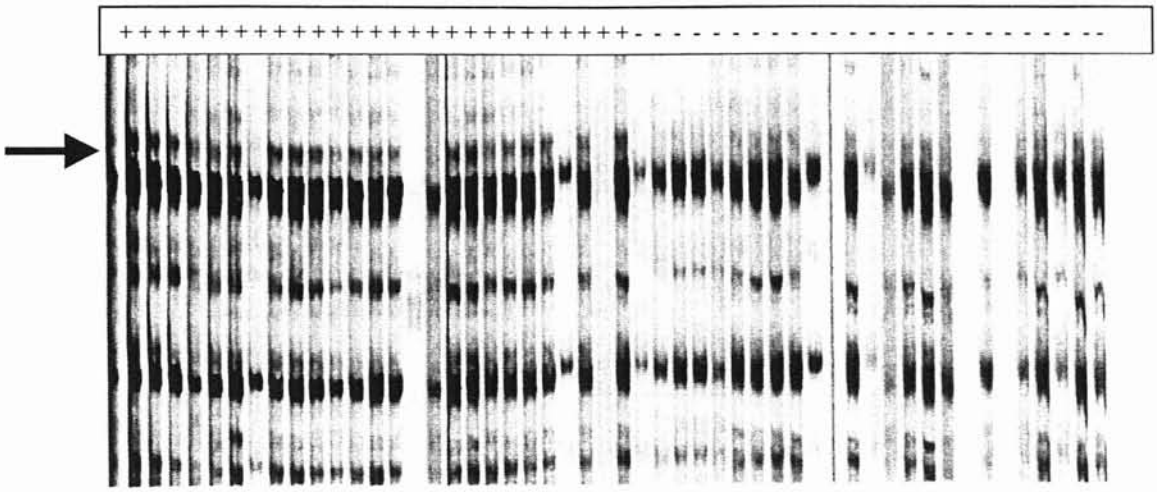

Figure 3. An AFLP gel showing a marker (Lr41osuPAG200) linked to Lr41 gene and amplified by the primer combination AG/TCGA.

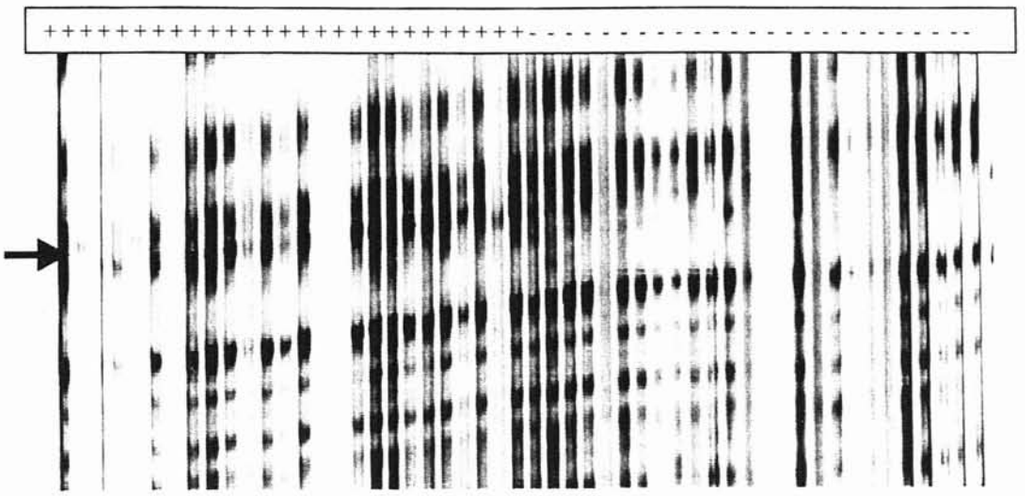

Figure 4. An AFLP gel showing a marker (Lr41osuPCATG230) linked to Lr41 gene and amplified by the primer combination CATG/CGAT.

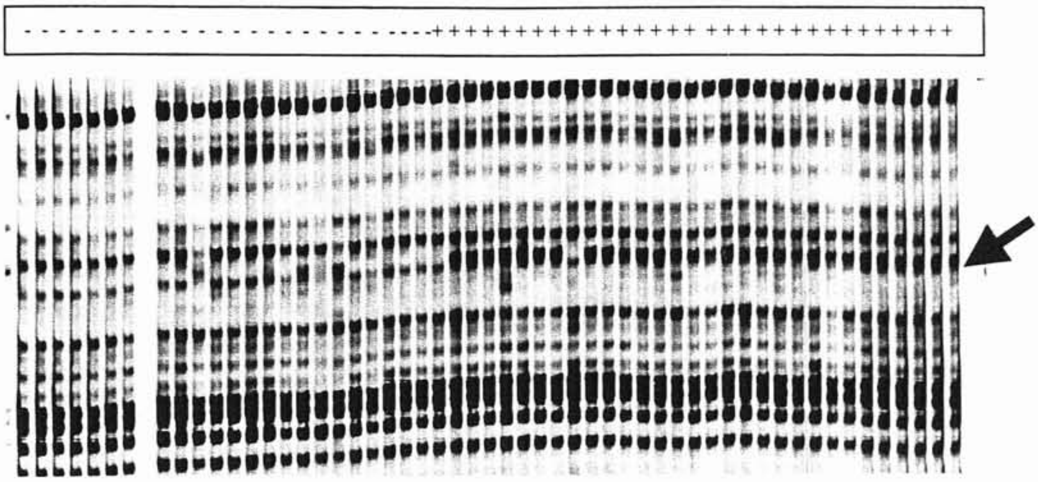

Figure 5. An AFLP gel showing a marker (Lr42osuPGTG200) closely linked to Lr42 gene and amplified by the primer combination GTG/CAG.

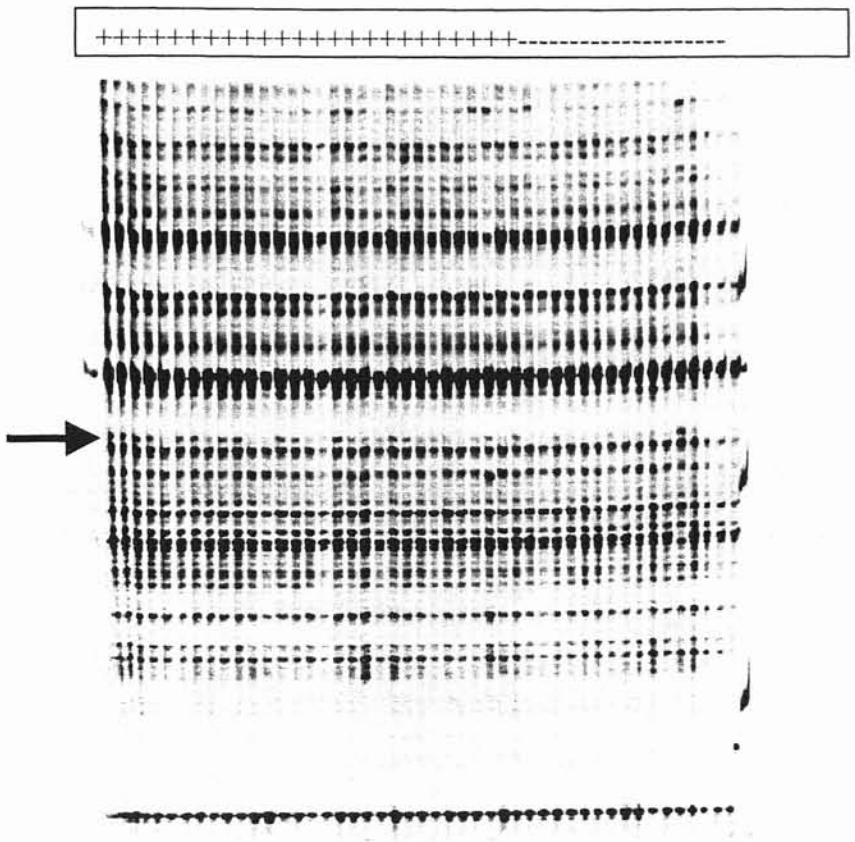

Figure 6. An AFLP gel showing a marker (Lr42osuP1CGA110) closely linked to Lr42 gene and amplified by the primer combination CGA/AGCT.

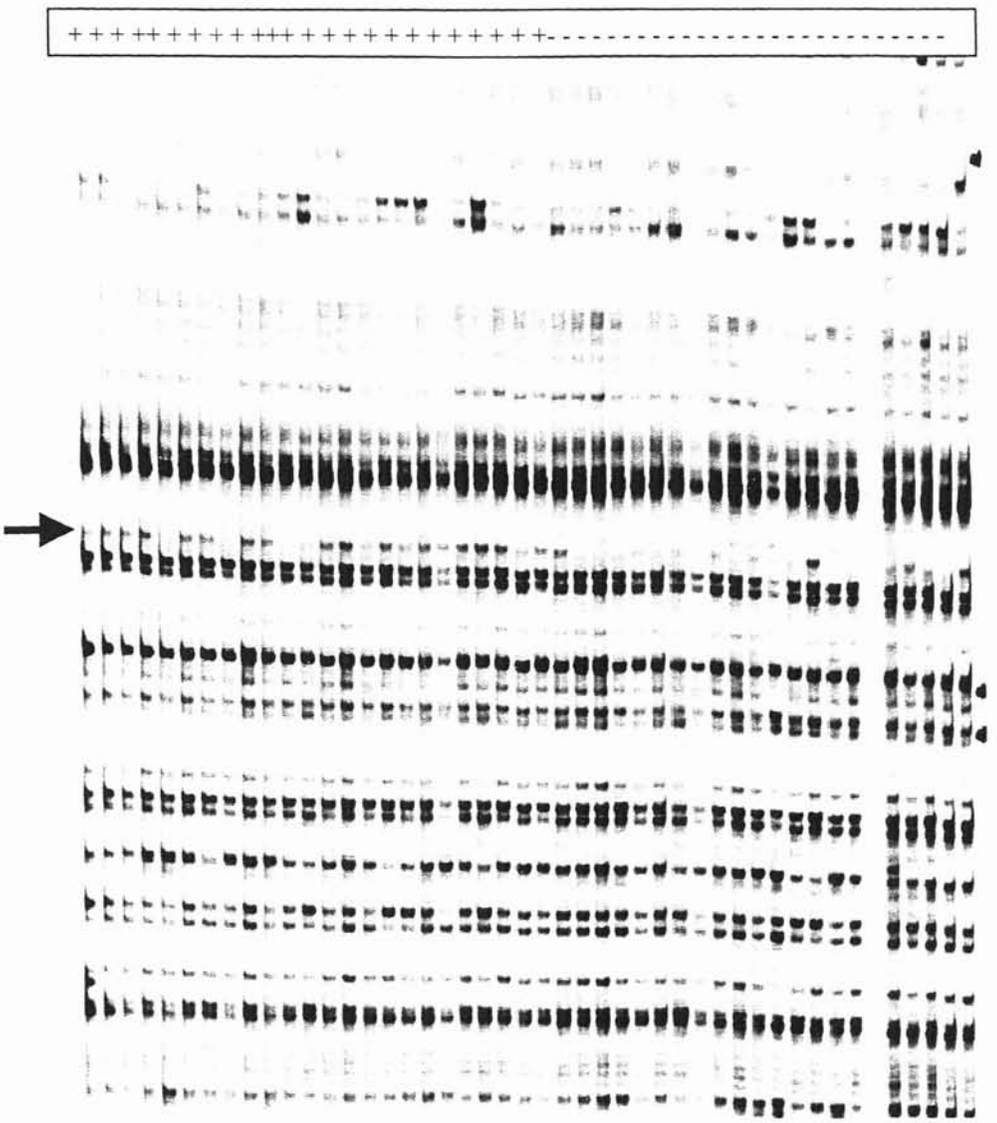

Figure 7. An AFLP gel showing a marker (Lr42osuP3CGA210) linked to Lr42 gene and amplified by the primer combination CGA/AGGC.

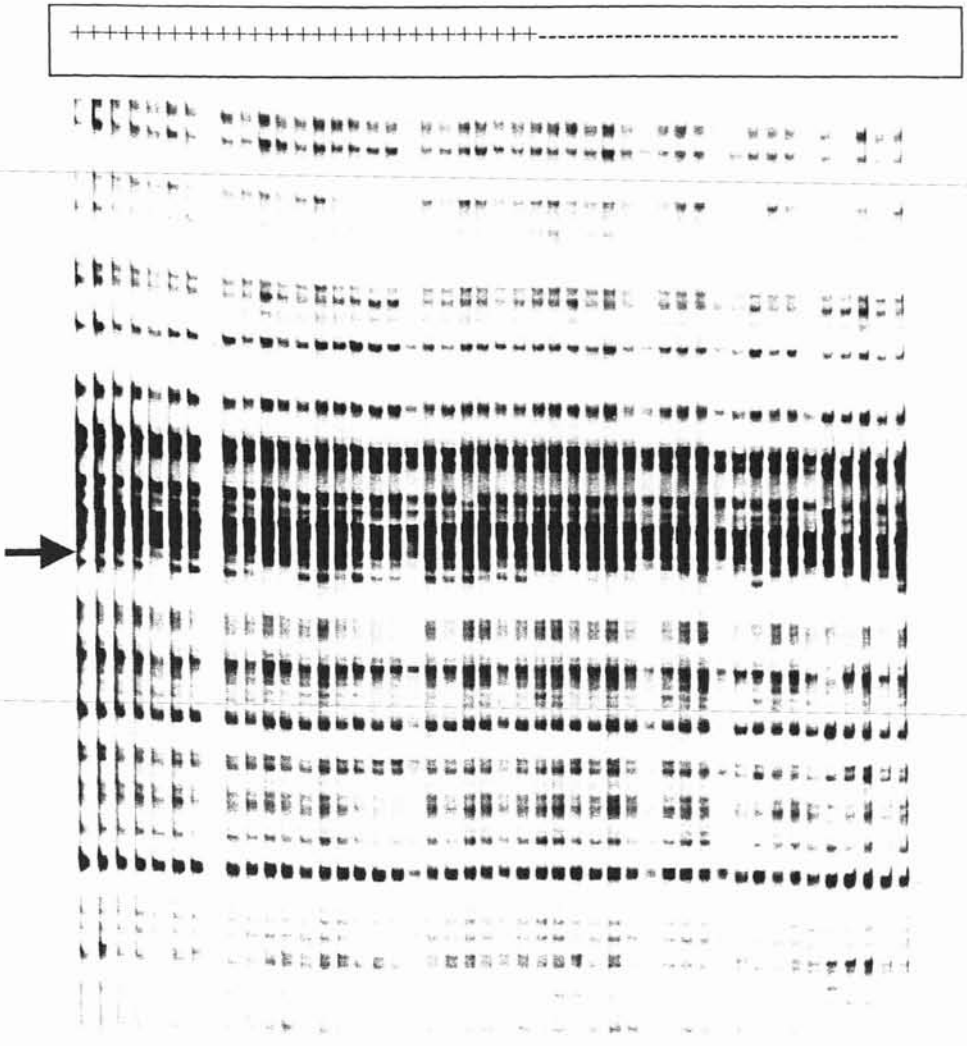

Figure 8. An AFLP gel showing a marker (Lr42osuPCATG200) closely linked to Lr42 gene and amplified by the primer combination CATG/GCAT.

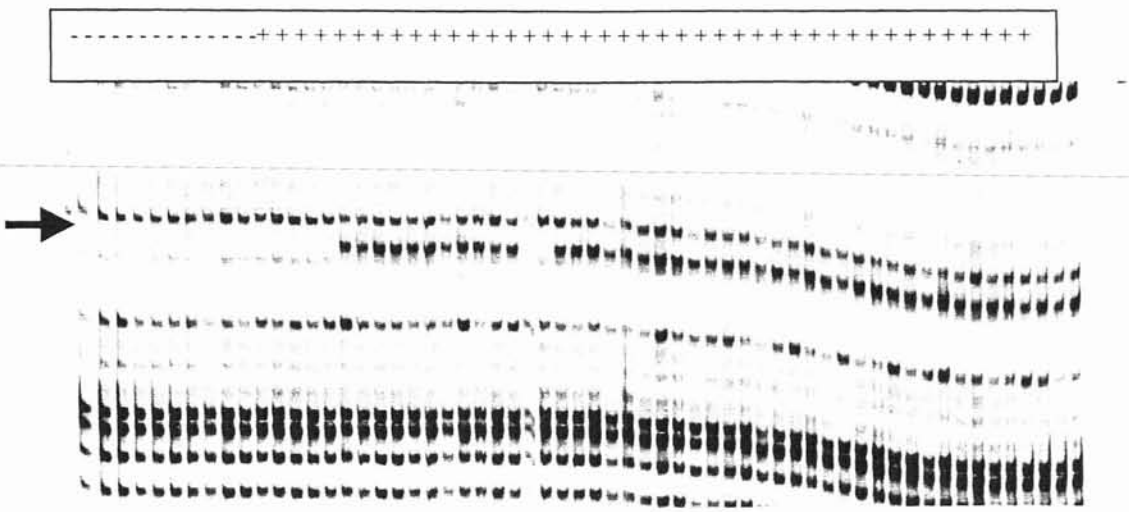

Figure 9. An AFLP gel showing a marker (Lr42osuP2CGA200) closely linked to Lr42 gene and amplified by the primer combination CGA/CAGT.

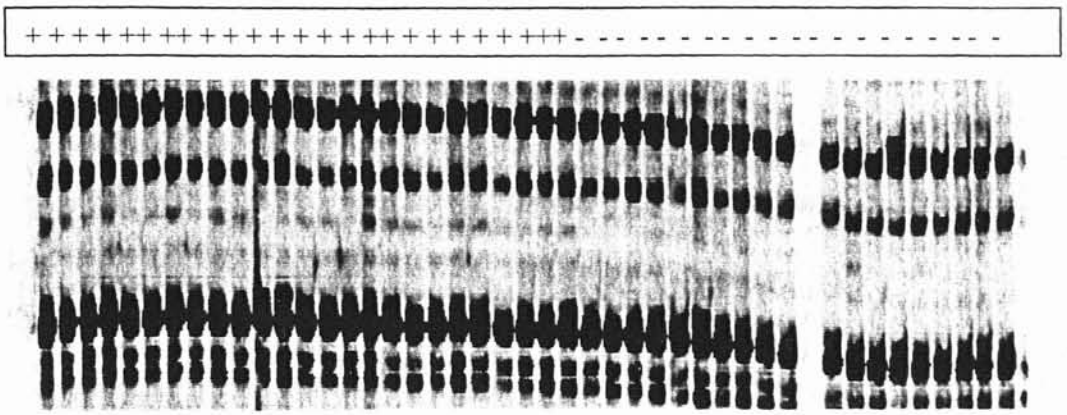

Figure10. An AFLP gel showing a marker (Lr42osuPGTG200) linked to Lr42 gene and amplified by the primer combination GTG/CAC.

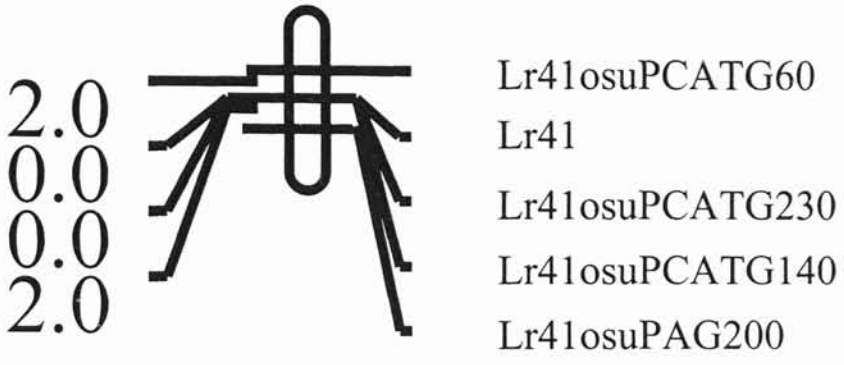

Figure 11. Genetic linkage map of AFLP markers associated with Lr41 gene on the chromosome 1DS. The numbers to the left indicate genetic distance between the markers and the names on the right indicate the markers identified and the locus of Lr41 gene on the genetic linkage map.

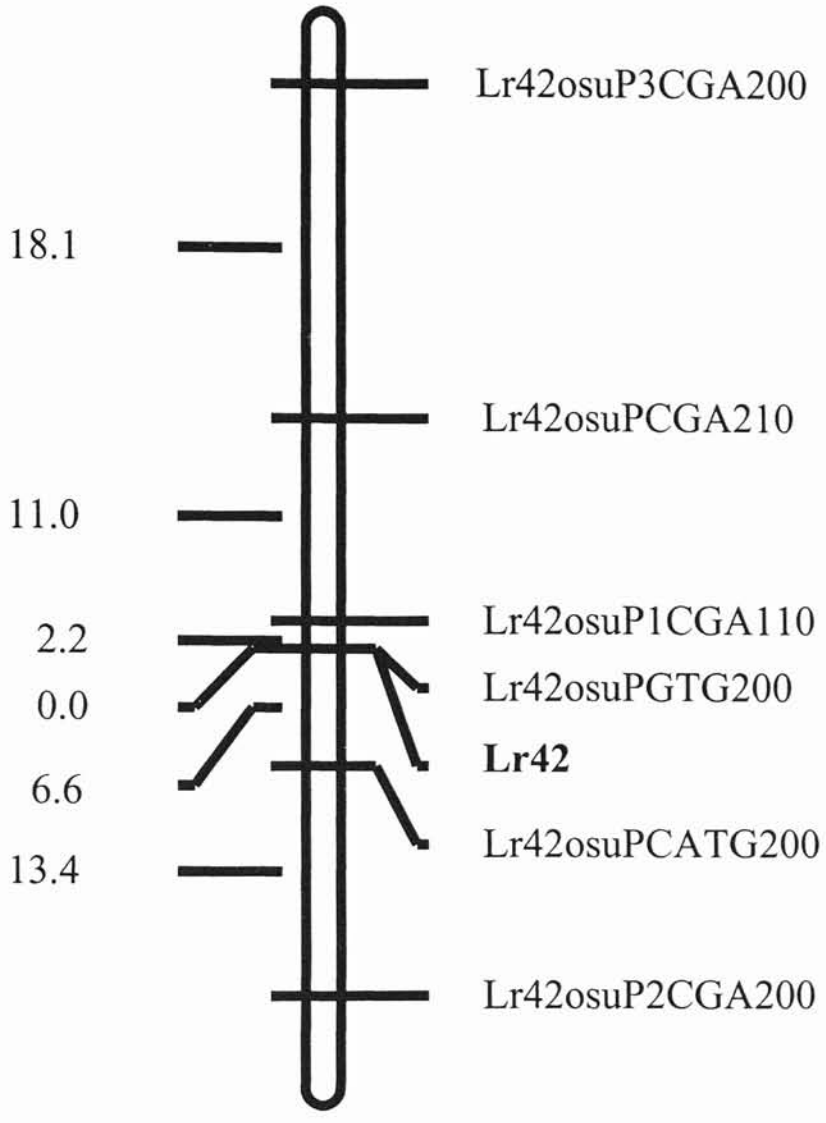

Figure 12. Genetic linkage map of AFLP markers associated with Lr42 gene on the chromosome 1DS. The numbers to the left indicate genetic distance between the markers and the names on the right indicate the markers identified and the locus of Lr42 gene on the genetic linkage map.

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APPENDIX A
Wheat Leaf Rust Resistance Genes, Genome Location, Source, Infection Type and References

Gene	Chromosome	Original source	Resistance	Gene references
1	5DL	Malakof	I	Ausemus, ER et al. Amer Soc of Agron 38:1083-1099
2a	2DS	Webster	I,MR	Dyck, PL and Samborski, DJ Can J Genet Cytol 16:323-332
2b	2DS	Carina	R,MR	"
2c	2DS	Brevit	MR-R	"
3a	6BL	Democrat	R,MR	Browder, LE Crop Sci. 20:775-779
3bg	6BL	Bage	MR-MS	"
3ka	6BL	Klein Aniversario	MR-MS	"
9	6BL	Triticum umbellulatum	I	Soliman, AS et al. Crop Sci 3:254-256
10	lAS	Lee	R-MS	Choudhuri, HC Ind J Genet 18:90-115
11	2A	Hussar	MR	Soliman, AS et al. Crop Sci 3:254-256

12	4BS	Exchange	R	Dyck, PL et al. Can J Genet Cytol 8:665-671
13	2BS	Frontana	R	"
14a	7BL	Hope	MS	Dyck, PL and Samborski, DJ Can J Genet Cytol 12:689-694
14b	7BL	Bowie	MS	"
15	2DS	Kenya 1-12 E-19-J	R	Luig, NH and McIntosh, RA Can J Genet Cytol 10:99-105
16	2BS	Exchange	MS-MR	Dyck, PL and Samborski, DJ Pcro 3rd Int Wheat Genet Symp, pp 245-250
17	2AS	Klein Lucero	MR-MS	"
18	5BL	T. timopheevi	MS	"
19	7DL	Agropyron elongatum	R	Sharma, D and Knott, DR Can J Genet Cytol 8:137-143
20	7AL	Thew	R	Browder, LE Crop Sci 12:705-706
21	1DL	T.tauschii	I	Rowland, GG Can J Genet Cytol 16:137-144
22a	2DS	Thatcher	MR	"
22b	2DS	T. tauschii	R	Dyck, PL Can J Pl Sci 59:499-501
23	2BS	Gabo	MR,MS	McIntosh, RA Aust J Biol Sci 28:201-211

24	3DL	A. elongatum	R	Browder, LE Crop Sci 13:203-206
25	4AB	Rosen rye	I	Driscoll, CJ and Anderson, LM Can J Genet Cytol 9:375-380
26	lBL	Imperial rye	MR	Singh, RP et al. Theor Appl Gent 80:609-616
27	3BS	Gatcher	MR	Singh, RP and McIntosh, RA Can J Genet Cytol 26:736-742
28	4AL	T. speltoides	I	McIntosh, RA Z Pflanzenzuchtung 89:295-306
29	7DS	A. elongatum	R	Sears, ER Proc4th Intl Wheat Genet Symp pp.191-199
30	4BL	Terenzio	R	Dyck, PL and Kerber, ER Can J Genet Cytol 23:405-409
31	4BS	Gatcher	MR	Singh, RP and McIntosh, RA Can J Genet Cytol 26:736-742
32	3D	T. tauschii	MR	Kerber, ER Crop Sci 27:204-206
33	1BL	Pl58458	MR	Dyck, PL Genome 29:463-466
34	7D	Terenizo	MR-MS	Dyck, PL Genome 29:467-469

35	2B	T. speltoides	?	McIntosh, RA Z Pflanzenzuchtung 92:1-14
36	6BS	T. speltoides	?	Dvorak, J and Knott. DR Genome 33:892-897
37	2AS	T. ventricosa	I	Bariana, HS and McIntosh, RA Euphytica 76:53-61
38	2AL	A. intermedium	?	Friebe Zeller
39*	2DS	T. tauschii	?	Raupp, WJ
40*	1D	T. tauschii	?	Raupp, WJ
41	1D	T. tauschii	?	Cox, TS et al. Crop Sci 34:339-343
42	1D			"
43	7D	T. tauschii		"
44	1BL	T. aestivum spelta 7831	MR	Dyck, PL and Sykes, EE Can J Plt Sci 74:231-233
45	2AS	rye		McIntosh, RA
46	1BL	Pavon 76		Singh, R.P. and Huerta-Espino, J. Phytopathology 88:S82
47	7AS	T. speltoides		Dubcovsky, J. et al. Crop Sci (in press 1998)

APPENDIX B

Estimate of percent yield loss for different leaf rust severities

Estimate of Percent Yield Loss For Different Leaf Rust Severities

Growth Stage	**Rust Severity on Flag Leaf**				
	10%	25%	40%	65%	100%
Flowering	10	15	20	30	35
Milk	2	5	8	14	20
Soft Dough	1	3	4	7	10
Hard Dough	1	1	2	3	5

APPENDIX C

Introgression of D-Genome into wheat

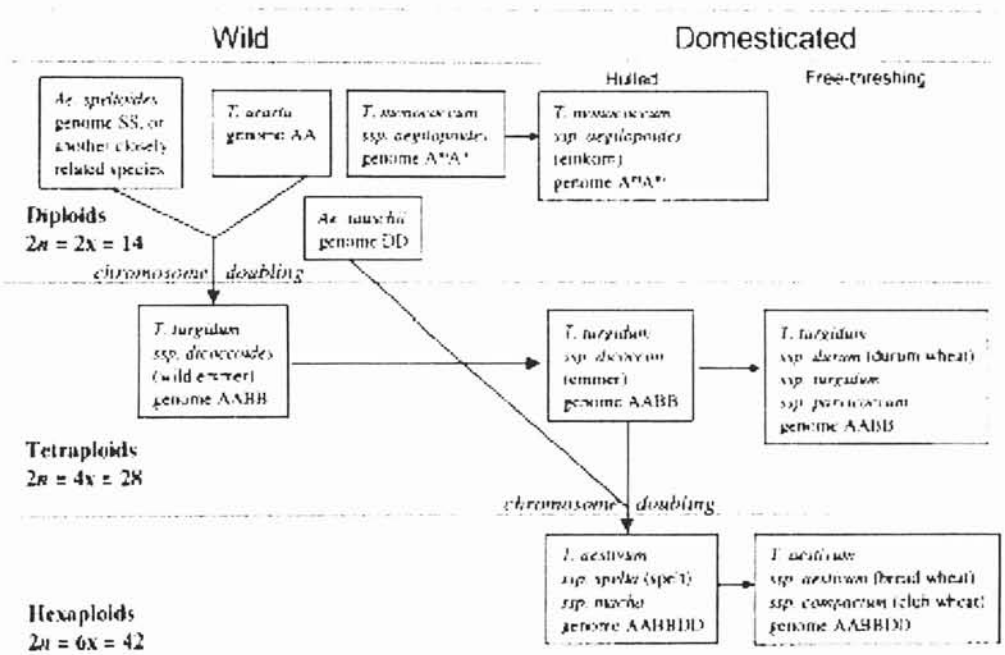

Figure 1. Evolution of Hexaploid Bread Wheat *T. aestivum* and Other Wheat Cultivars.

The S genome of *Ae. speltoides* or of another closely related Aegilops species is the closest known relative of the B genome. Thus, B was either contributed by an unknown and now extinct genome, or it diverged from the S genome progenitor after polyploidization. Adapted from Feldman (2001).

VITA

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