EVALUATING LINES IN A WHEAT BREEDING PROGRAM FOR RACE NONSPECIFIC RESISTANCE TO LEAF RUST, *PUCCINIA TRITICINA*

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CHAPTER I

INTRODUCTION

Breeding for resistance against leaf rust, *Puccinia triticina* (*P. recondita* Rob. Ex Desm), has Been an area of concern for wheat breeders since the advent of modern wheat breeding in the early part of the 20th century. A significant part of the increase in world wheat production since the Green Revolution has been from increased disease resistance. Therefore, continued yield gains from breeding in wheat depend in part on maintaining high levels of leaf rust resistance.

For most of its history, breeding for leaf rust resistance has been focused on simply inherited race specific leaf rust genes which confer immunity to prevailing rust strains. The problem with this approach is that race specific leaf rust resistance is not durable, as it breaks down once the virulence of rust pathotypes in rust populations changes. As a result, there has been a growing emphasis on using race nonspecific genes to breed for leaf rust resistance. Race nonspecific genes confer durable resistance but since they are genes with smaller effects more of them must be incorporated into lines to achieve adequate levels of resistance. Also, the presence of race specific genes can mask the effects of race nonspecific genes. These challenges make it more difficult to breed for leaf rust resistance using race nonspecific genes.

Therefore, for this research, a new screening method for the selection of race nonspecific genes was used. This method takes advantage of the differential seedling susceptible and adult plant resistant phenotype found in wheat plants that derive their resistance from race nonspecific genes.

Greenhouse seedling susceptibility and field adult plant readings were combined to determine which lines had the phenotype indicative of race nonspecific resistance. These results were then combined with genotypic data using molecular markers for one of the two race nonspecific genes currently available, *Lr34*. Markers for the second race nonspecific gene, *Lr46*, though effective, lack accuracy, and were therefore not used for this research.

The objectives of this study were to develop a novel screening method using adult plant field susceptibility readings and greenhouse seedling susceptibility readings to determine which advanced lines in an Oklahoma State University wheat breeding program had leaf rust resistance likely derived only from race nonspecific genes, and which lines had some combination of race nonspecific genes and race specific genes. Also, the number of genes likely present in the advanced lines with only race nonspecific resistance was postulated based on the level of resistance observed, and the presence or absence of the *Lr34* gene as determined using molecular markers.

CHAPTER II

LITERATURE REVIEW

Importance of Leaf Rust

Leaf rust, *Puccinia triticina* (*P. recondita* Rob. Ex Desm), is one of the oldest known pathogens of wheat. It is a heteroecious pathogen, requiring a telial/uredinial host (wheat or one of several grass species including wild relatives of wheat) and a pycnial/aecial host to complete its life cycle (Bolton et al. 2008). Since the native range of the pycnial/aecial host, *Thalictrum speciossimum*, and the wild relatives of wheat overlap in the Fertile Crescent - what is now part of Iraq, Syria, southern Turkey, Jordan and Israel - it is thought that wheat leaf rust originated there when wheat was domesticated (Bolton et al. 2008, Huerta-Espino et al. 2011). Ancient records, including the Bible, mention leaf rust epidemics in the Fertile Crescent. The ancient Romans were also aware of the importance of rust. According to Stakman and Christensen (1960), the Roman author Varro in his *Rerum Rusticarum* published around 0 B.C.E, recommended against planting wheat in lands that receive lots of fog. In modern times, the importance of leaf rust as a pathogen was not recognized until early in the 20th century (Mains and Jackson 1926, Meggs 1947). This may have been due to the fact that rain-fed wheat yields up to that time were much lower than they are today. Average yields in the U.S., Russia, and Argentina at the beginning of the 20th century were less than one ton/ha and yields in England barely exceeded two ton/ha (Biffen 1905) which must have made the effect of the pathogen on yield loss difficult to be observed.

Of the three rusts, stem rust, *Puccinia graminis*, stripe rust, *P. striiformis* and leaf rust (or black rust, yellow rust and brown rust, respectively), stem rust is the most destructive. However, leaf rust is often the most economically important rust due to the fact that it is both the most common rust in many wheat regions and usually causes damage every year in the regions where it is present (Kolmer and Long 2007). Stem and stripe rust, though common, are not prevalent every year and therefore, tend to cause less damage (Meggs 1947, Simmonds 1988, Williams and Littlefield 2007, Huerta-Espino et al. 2011). In Oklahoma, the three rust diseases together have been shown in an eleven year study comparing fungicide protected plots versus unprotected plots to result in annual grain yield decreases of between 14 and 16% per year, with the majority of these yield losses from leaf rust (Williams and Littlefield 2007). However, in some years damage from leaf rust can be much more severe. For example, in 1938 Chester (1939) estimated yield losses due to leaf rust of 25-30% in Oklahoma's hard red winter wheat crop.

Yield losses from leaf rust primarily result in fewer kernels per head , shrunken kernels, and lower kernel weights (Williams and Littlefield 2007, Bolton et al. 2008), largely due to premature senescence of infected leaves and reduced tiller numbers (Xu et al. 2005, Williams and Littlefield 2007). Timing and length of rust exposure affects severity of losses significantly. Infections early in the life cycle are the most damaging (Sayre et al. 1998, Kolmer and Long 2007, Williams and Littlefield 2007, Huerta-Espino et al. 2011,) and can sometimes result in yield losses greater than 50% (Huerta-Espino et al. 2011). For this reason, the severity of rust damage can be reduced by avoiding planting too early in the growing season (Sayre et al. 1998) and planting early-maturing varieties (McVey and Delong 1993, Sayre et al. 1998, Williams and Littlefield 2007). However, the only effective way to eliminate crop losses from leaf rust (other than the use of fungicides – rarely a cost effective option) are through planting leaf rust resistant wheat varieties.

Change in the Virulence in Leaf Rust Populations

Understanding the processes by which the virulence of pathogens change is essential to understand how to control them in the long-term (Burdon 1993). Therefore, how the virulence of rust populations change is very important to wheat breeders (Marshall 1992, Singh et al. 2011). Leaf rust occurs almost everywhere wheat is grown (Kolmer and Long 2007) and virulence in leaf rust populations is constantly undergoing change (Singh et al. 2011). As a result, the virulence of leaf rust populations throughout the world is extremely diverse (Singh et al. 2011). The factors that affect changes in the virulence of leaf rust populations are recombination, migration, mutation, and selection (Burdon 1993, McIntosh 1988, Singh and Rajaram 2002, Singh et al. 2011).

In nature, recombination from sexual reproduction is generally the most significant factor in population change (Darwin 1859). However, in North America, recombination is not a significant driver of change in leaf rust populations. This is due to the fact that reproduction of leaf rust is almost exclusively through asexual uredinospores, primarily because the aecial host of leaf rust, *Thalictrum speciossimum*, is not native(Kolmer and Long 2007). Also, the *Thalictrum* spp. that do grow in North America are not effective hosts as they are relatively resistant to basidiophore infection (Bolton et al. 2008, Jackson and Mains 1921) and because the aeciciospores that are produced from infections on them are rarely able to infect wheat (Saari et al. 1968).

Nevertheless, a large amount of diversity in leaf rust virulence exists in North American leaf rust populations. The United States Department of Agriculture – Agricultural Research Service Cereal Disease Laboratory reported the presence of 92 different races in the United States in 2011

(Kolmer 2011). Also, up to 70 virulences of leaf rust are identified each year in the U.S. (Kolmer and Long 2007). Therefore, the other forces of population change must be at work in North American leaf rust populations.

Migration is a significant source of diversity in leaf rust populations in North America. This is because wind-blown leaf rust spores can migrate over huge distances, which can greatly increase the diversity of pathotypes present in leaf rust populations (Kolmer 2005). Leaf rust pathotypes have arrived in Australia from Africa (McIntosh 1988). In North America, the wind blows the spores from Texas into the northern Great Plains and Canada (Williams and Littlefield 2007). Leaf rust pathotypes migrate from the South and remain in United States growing regions by overwintering in uredinial stage (Kolmer 2005). Throughout its growing range, from the Atlantic Seaboard to the Gulf Coast to the northern Great Plains, leaf rust overwinters as uredinia (Marshall 1989, Kolmer and Long 2007, Williams and Littlefield 2007,). Leaf rust pathotypes can also survive in volunteer wheat in summer on field margins or in ditches, which can serve both as additional reservoirs for reinfection and as sources for increased population diversity (Kolmer and Long 2007).

Mutation and selection are also important factors affecting the diversity of virulence in North American leaf rust populations. Mutation can lead to rapid changes from avirulence to virulence, when mutation rates are high enough (Vanderplank 1981b). Also, a kind of unintentional artificial selection caused by planting large acreages of resistant varieties with selected leaf rust (*Lr*) genes (Samborski 1967 called this phenomenon "cultivar-directed" selection) is a major driver of virulence changes in leaf rust populations. Wheat rust surveys conducted in Texas provide evidence of cultivar-directed selection in the southern Great Plains. Long et al. (1985) found that virulence to *Lr1* and *Lr16* increased in Texas after planting Probrand 812**,** a variety

that most likely had *Lr1*, and *Lr16* resistance genes. Later, Marshall (1989) found that virulence to *Lr1* and *Lr16* significantly decreased after acreages of Probrand 812 were significantly reduced from 1986 to 1987. Similarly, Kolmer (2005) found changes in the proportion of leaf rust pathotypes present in different regions of the American Great Plains corresponding to the leaf rust resistance genes deployed in varieties planted in those regions.

Breeding for Rust Resistance with Race Specific Genes

Most of the work on breeding for leaf rust resistance has been focused on simply inherited oligogenic genes that confer resistance due to a hypersensitive response (Caldwell 1968, Das et al. 1992) that is often, but not always, characterized by necrotic flecking (Heath 1976, Rubiales and Niks 1995, Bolton et al. 2008). Hypersensitive genes work by giving the plant the ability to recognize the invading rust and trigger rapid cell death of infected cells, thereby preventing infection from establishing in the plant (Heath 1976, Bolton et al. 2008). Since *Lr* genes that confer this ability to the plant are effective only for a specific leaf rust virulence, the term "race specific" resistance has been used for this type of resistance. Most of the *Lr* genes identified up to the present are race specific genes (McIntosh et al. 1995, Navabi et al. 2003).

The problem, however, is that race specific resistance has long been known to be short-lived (Das et al. 1992, Singh and Rajaram 2002, Xu et al. 2005) largely because ever-changing leaf rust populations are able to rapidly develop virulence that plants with the hypersensitive race specific genes are unable to overcome (Schafer et al. 1963, Caldwell 1968, Simons 1972, Knott 1988, Singh and Rajaram 2002, Oelke and Kolmer 2005, Kolmer and Long 2007, Vanderplank 1981b). Changes in virulence of leaf rust populations is so rapid that it is common for race specific resistance to last only five years or less (Singh and Rajaram 2002, Singh et al. 2011). Despite this, wheat breeders have continued to use these genes in breeding because, being oligogenic

genes, they are simply inherited through Mendelian genetics. Selection for resistance, therefore, is relatively straight forward (Biffen 1905, Meggs 1947, Simmonds 1988, Vanderplank 1963, Singh and Rajaram 2002).

The use of race specific genes has been aided somewhat by incorporating more than one of them into the same variety, a strategy that has been thought to give varieties a broader range of race specific resistance (Vanderplank 1978). An early proponent of this strategy was Flor (1956), who devised a method of backcrossing to incorporate known genes for rust resistance into lines of interest in flax. In wheat, a good example of the use of this approach is the Agripro variety "Ivan" which has been shown to possess good leaf rust resistance because it has race specific genes (*Lr16* and *Lr24*) for which virulence was rare in the northern Great Plains (Kolmer and Oelke 2006) . The result of using only race specific genes in this way to control leaf rust is that many of the varieties planted in the Great Plains today have only race specific resistance (Bockus et al. 2009). Having only race specific resistance**,** however**,** is a problem because when virulence emerges for which no resistances have yet been found, boom and bust cycles emerge (Vanderplank 1963). In years in which leaf rust is not well controlled by the *Lr* genes in deployed varieties, yields can decrease dramatically.

Another difficulty with using only race specific genes in varieties is the fact that there are very likely only a finite number of race specific genes available for deployment and there may be a time when breeders run out of new ones (McIntosh 1988, Simmonds 1988). In North America, leaf rust virulence has already emerged that can overcome race specific leaf rust genes *Lr1, Lr2a, Lr3, Lr3ka, Lr9, Lr10, Lr11, Lr13, Lr14a, Lr17, Lr17a, Lr23, Lr24, Lr26, Lr41, Lr50* (Singh and Rajaram 1992, Kolmer 2005, Oelke and Kolmer 2005, Kolmer and Long 2007, Zhang et al. 2008). Also, by serving as reservoirs for mutation and selection, large acreages of deployed resistance genes increases the speed at which race specific genes can lose their effectiveness

(Vanderplank 1963, Marshall 1992, Marshall 1988, Marshall 1989, Kolmer 2005). Finally, deploying varieties with multiple race specific genes to try to achieve broader resistance can lead to the creation of leaf rust pathotypes with virulence to a broad range of race specific genes. For these reasons, Schafer et al. (1963) concluded that for leaf rust, incorporating many race specific genes into varieties would not work in the long term because races with virulence to all the race specific genes would eventually emerge.

Nevertheless, wheat breeders have achieved some success with rust virulence monitoring coupled with rapid deployment of appropriate resistance genes to prevent rust epidemics. This approach has been successfully used to control stem rust in the cereal growing region of northeastern Australia (McIntosh 1988, McIntosh 1992). In Australia, breeders use the diversity of race specific genes as "insurance" against lack of durability of these genes (McIntosh 1988).

However, the practice of incorporating many rust specific genes in varieties as a hedge against the breakdown of effectiveness of individual genes is not a viable approach everywhere. A salient example of this is the Ug99 stem rust epidemic that started in Africa and that has now spread throughout the continent as well as to the Middle East, and is now threatening wheat production in Europe and Central Asia (Singh et al. 2011). Ug99 emerged when a catastrophic loss of effectiveness of several race specific stem rust genes occurred simultaneously (Singh et al. 2011). Intensive monitoring of the virulence of rust populations coupled with rapid deployment of varieties with the appropriate race-specific genes might have prevented this catastrophe. But this method is possible neither in Africa nor in many other areas of the developing world because monitoring and rapid deployment of seed are difficult. Indeed, McIntosh (1988) notes that this strategy has only been effective in Australia due to extensive support of the agricultural industry there. No such well-developed agricultural industry exists in many developing nations. All of

these limitations are leading breeders to look into using other types of genes for rust resistance: race nonspecific resistance genes.

Breeding with Race Nonspecific Genes

Breeding with race nonspecific genes is more complicated since it involves multiple genes with smaller effects that confer resistance against all rust virulence pathotypes. Consequently, mechanisms for how these genes operate –polygenetically, additively, through partial dominance–and the number of genes required for effective levels of resistance took some time to work out (Schafer et al. 1963, Parlevliet and Zadoks 1977, Kuhn et al. 1978, Browder and Eversmeyer 1980 ,Kuhn et al. 1980, Lee and Shaner 1985b, Knott 1988, Simmonds 1988, Das et al. 1992, McIntosh 1992 ,Singh and Rajaram 1992, Dyck 1997, Shaner et al. 1997, Knott and Padidam 1998, Singh et al. 2000, Hsam and Zeller 2002, Singh and Rajaram 2002, Al-Maaroof et al. 2005). Therefore, adoption and use of race nonspecific genes came about gradually. Singh and Rajaram (2002) gave a review of research developments that led to the increased use of race nonspecific genes in breeding for rust resistance in wheat. These developments included the discovery of a genetic basis for resistance in wheat (Biffen 1905); Vanderplank's (1963) theoretical basis for resistance; and the discovery of physiological specialization of leaf rust pathogens by Mains and Jackson (1926), and stem rust by Stakman and Piemeisel (1917). The same developments also led to an understanding of gene-for-gene interaction between rust virulence and corresponding genes in wheat plant hosts (Flor 1956). Flor's gene-for-gene model – "for every gene for resistance in the host there is a gene for virulence in the pathogen" – was thought by Vanderplank (1978) to hold true for all the rusts, stem rust, leaf rust and stripe rust. Browder (1985) reviews some of the work showing that this is indeed the case for stem rust:wheat, leaf rust:wheat and stripe rust:wheat, as well as some other pathogen:host systems. All of this work led to a greater interest in the use of race nonspecific resistance in crop

improvement (Caldwell 1968, Singh and Rajaram 2002). Borlaug (1972) developed the uses of race nonspecific resistance for stem rust. Caldwell (1968) and Johnson (1988) were the first to apply the concept to leaf rust and stripe rust, respectively. Over time, the use of race nonspecific genes to breed for leaf and stripe rust resistance has gained more acceptance. In CIMMYT's bread wheat breeding program**,** the use of race nonspecific resistance has been the predominant breeding method for leaf rust resistance for the last thirty years (Singh and Rajaram 2002).

Clarification of Terms

Research in the use of race nonspecific or general resistance has taken place over many years with numerous researchers looking at the same problem from different perspectives in different pathogen:host systems (Schafer et al. 1963, Caldwell 1968, Parlevliet and Zadoks 1977, Parlevliet 1981, Browder 1985, Knott 1988, Simmonds 1988, Das et al. 1992, Singh and Rajaram 1992, Dyck 1997, Shaner et al. 1997, Singh et al. 2000, Hsam and Zeller 2002, Singh and Rajaram 2002). As a result, there are several terms used for race nonspecific resistance, as well as race specific resistance which have caused confusion in the terminology used for rust resistance (Knott 1982). Even the designation of physiologic rust races as distinct taxa is a concept open to debate, due to the fluid nature of gene flow within rust populations (Vanderplank 1981a). It is therefore necessary to discuss the terms that have been used in order to explain what is meant by the terms race specific and race nonspecific resistance. Vanderplank's (1963) terms "horizontal resistance" and "vertical resistance", are convenient starting points for this discussion because his development of the theoretical basis for resistance plays an important role in the current understanding of race specific and race nonspecific resistance used by crop breeders today (Singh and Rajaram 2002).

Horizontal resistance (Vanderplank 1963) is resistance whereby the "pathogen varies independently of the differences in the varieties of the host". In other words, resistance of the

host is effective against all pathogen strains or pathotypes, i.e. this resistance is race nonspecific (Vanderplank 1978). Horizontal resistance is synonymous with "slow-rusting", general or generalized resistance, late-rusting, partial resistance, adult plant resistance, polygenic resistance, quantitative and usually but not always "minor-gene" resistance (Stakman and Christensen 1960, Caldwell 1968, Luke et al. 1972, Parlevliet and Zadoks 1977, Parlevliet 1981, Bjarko and Line 1988, Knott 1988, Rajaram et al. 1988, Simmonds 1988, Das et al. 1993, Rubiales and Niks 1995, Hsam and Zeller 2002, Singh and Rajaram 2002).

Vertical resistance as defined by Vanderplank (1963, 1978) is "variation in pathogen that is associated qualitatively with variation in the host." Or put another way, there is an interaction between pathogen strains and host lines (Vanerplank 1978) i.e. the resistance of the host is race specific (Vanderplank 1963). Vertical resistance is synonymous with race specific resistance, simply inherited resistance**,** single-gene resistance, oligogenic resistance, and "hypersensitive" resistance (Vanderplank 1963, Caldwell 1968, Borlaug 1972, Heath 1976, Kuhn et al. 1978, Vanerplank 1978, Knott 1988, Simmonds 1988, Hsam and Zeller 2002). Resistance of this type is usually, but not always "major-gene" resistance (Singh and Rajaram 2002). When Vanderplank (1978) introduced the concepts of vertical and horizontal resistnce, he estimated that there were at least twenty vertical or race specific genes for stem rust and leaf rust. Currently a much larger number of race specific genes for both rusts have been identified (McIntosh 1988, McIntosh et al. 1995).

To clarify terminology, Caldwell (1968) advocated the use of the terms **"**general resistance**"** and **"**specific resistance**"** respectively to replace Vanderplank's horizontal and vertical resistance as well as other terms being used at the time to describe resistance responses such as "slow-rusting" resistance. The use of the terms general and specific resistance clears up some of the confusion around studies on resistance types, as they describe the type of resistance in question in a more

intuitively appealing way than Vanderplank's terms "vertical" and "horizontal" resistance and they have the advantage in that similar terms had been used for many years to describe the different resistance responses in wheat (see for example Stakman and Christensen (1960)). More recently, the terms race nonspecific and race specific have been introduced to replace Caldwell's "general" and "specific" resistance terms.

Finally the term "durable" resistance was introduced by Johnson (1988) and others to describe resistance that does not break down in response to changes in the virulence of rust populations over time. Durable resistance is "effective resistance that persists for generations in a favorable environment for the pathogen" (Johnson 1988). The genetic basis for durable rust resistance was slow to be recognized, due in part to the fact that the existence of durable rust resistance only became apparent in the 1990's (Singh and Trethowan 2007). But it is now understood that durable resistance is synonymous with horizontal resistance (Vanderplank 1981b), race nonspecific resistance (Simmonds 1988, McIntosh 1992, Singh and Trethowan 2007) as well as the equivalent terms adult plant resistance (Parlevliet and Zadoks 1977), and slow-rusting resistance (Bjarko and Line 1988, Das et al. 1993). An example of durable resistance is found in the variety "Knox" which was derived from a Chinese Spring land race (Kuhn et al. 1978). This variety ceased to be grown when other higher yielding varieties replaced it, but it has retained its resistance, and lines derived from it retained durable leaf rust resistance as well (Kuhn et al. 1978). Durable adult plant resistance to stem rust was first found in lines derived from "Hope" and "H44" and is described by Caldwell (1968), Borlaug (1972), and Singh et al. (2011).

A corollary of Johnson's definition of durable resistance is that race specific resistance, a type of resistance in which there exists, or will exist, one or more races of rust possessing virulence for which host plants have no resistance, is not durable resistance (Vanderplank 1981b). According to Vanderplank (1981b), "vertical" or race specific resistance can never be stable when mutation

rates in the pathogen are great enough, because mutation will make the differential interaction between host plants with race specific resistance and the pathogens unstable. Race specific resistance is therefore useful only when no virulence in the rust population is present to defeat it. As soon as virulence that can overcome it emerges, either through mutation, recombination, migration or selection, race specific virulence becomes "broken down" or "defeated". However, this does not mean that race specific genes are useless. Kolmer and Oelke (2006) noted that the "defeated" race specific resistance found in the variety "Ivan" was effective because virulence to the race specific genes it possesses were not present in the northern Great Plains where the variety was grown. This variety is an example of how race specific genes can be deployed through varieties to effectively control leaf rust if the virulence present in the leaf rust population is monitored. In fact, monitoring of the virulence of leaf rust populations coupled with deployment of the appropriate race specific leaf rust genes has been successfully used in Australia for many years to protect wheat from stem rust (McIntosh 1988, McIntosh 1992). Therefore, the terms "broken down" and "defeated" should not be applied to race specific genes. The terms specific resistance or race specific resistance are far more useful to describe this type of resistance.

The confusion of terminology of the various terms used to describe race specific and race nonspecific resistance have led to misunderstandings on the nature of resistance in particular lines or varieties over the years, in part because they are often found together in the same plant. Indeed, Vanderplank (1963), when first promulgating his ideas that became the theoretical basis for the inheritance of resistance (Vanderplank 1978) noted that many wheat varieties have genes for both "vertical" (race specific) resistance and "horizontal" (race nonspecific) resistance. The first time this phenomenon was observed was when the first systematic studies of leaf rust was conducted by Mains and Jackson (1926). Mains and Jackson (1926) discovered the difference between what is now understood as "vertical" or race specific and "horizontal" or race nonspecific resistance when they demonstrated that seedling/greenhouse resistance is not always

the same as field/adult plant resistance. The variety Mains and Jackson (1926) used to illustrate this point was the variety "Kanred", which had high resistance in the greenhouse/seedling stage but low resistance at the field/adult plant stage. According to Vanderplank (1963) this is because the variety Kanred had a "high degree" of "vertical" or race specific resistance, since it exhibited good resistance in the greenhouse when inoculated with several leaf rust strains of known virulence, but very little "horizontal" or race nonspecific resistance because it had very low field/adult plant resistance when exposed to an entire population of rust spores with very diverse virulence. Another good example of this type of confusion in the terminology of leaf rust resistance is the leaf rust resistance in the variety "Knox". Ohm and Shaner (1976) called leaf rust resistance in Knox "mature-plant, polygenic, hypersensitive" resistance and said that although resistance in Knox had broken down it retained some level of resistance. It is probably the case that Knox has a combination of race specific and race nonspecific genes. The resistance of the race specific genes has broken down as virulence has emerged in rust populations that could defeat it, but the race nonspecific resistance has retained its effectiveness, giving the variety the low level, but durable resistance that Ohm and Shaner (1976) observed. Many other examples of this type exist in the various host:pathogen systems. So many instances of plants containing both horizontal or race nonspecific and vertical or race specific resistances have been documented in the literature that , according to Vanderplank (1981b), the coexistence of both types of resistances within plants is well-established.

Similarly, the terms "major" and "minor" genes have created confusion. The term "major genes" has been used to describe genes that give high levels of resistance (Rubiales and Niks 1995, Singh and Rajaram 2002). Race specific resistance is almost always conferred by dominant genes (Caldwell 1968) and race specific genes are almost always considered "major" genes because they give a hypersensitive or other response that either makes lines with these genes immune or gives them a very strong resistance to specific strains of leaf rust. The term "minor

genes" has been used to describe race nonspecific genes because they are often recessive genes, and because they do not confer much rust resistance themselves, but can confer adequate levels of resistance when they are present in combination (Singh and Rajaram 2002). Confusion is created because sometimes race nonspecific genes are called genes with "major effects". For example, *Lr34* a gene which confers a particularly high level of resistance for a race nonspecific gene (Singh et al. 1998) has been called a "major gene" by Rubiales and Niks (1995). Due to the misleading nature of the terms "major" and "minor" resistance, these terms should not be used. The the terms general or specific resistance proposed by Caldwell (1968) or the terms race nonspecific and race specific resistance should be used instead and will be used hereafter.

Characteristics of Race Nonspecific Resistance

The use of race nonspecific genes in breeding programs has been complicated because both the biology and the mode of the inheritance of race nonspecific resistance is less well understood than that of race specific resistance. The nature of race specific resistance is relatively straightforward by virtue of the fact that it is simply-inherited, oligogenic and manifests due to hypersensitive response of the host plant to the leaf rust pathogen (Heath 1976, Rubiales and Niks 1995, Bolton et al.).

Race nonspecific resistance, on the other hand, possesses none of these characteristics. The inheritance of race nonspecific resistance is more complicated as it requires multiple genes to achieve adequate levels of resistance (Singh et al. 2000, Navabi et al. 2003). Also, unlike race specific genes, race nonspecific genes confer resistance additively, operating on a gene-for-gene basis (Schafer et al. 1963, Browder and Eversmeyer 1980, Knott 1982, Browder 1985, and Shaner 1985b, Bjarko and Line 1988, Das et al. 1992, Singh and Rajaram 1992, Dyck 1997, Shaner et al. 1997, Singh et al. 2000, Singh and Rajaram 2002, Al-Maaroof et al. 2005), with

each recessive gene conferring a small cumulative effect (Knott 1982, Singh et al. 2000, Navabi et al. 2003). Finally, partial dominance also plays a role in the inheritance of race nonspecific resistance (Kuhn et al. 1980, Singh et al. 1998).

Furthermore, the biology of race nonspecific resistance is also less well understood than that of race specific resistance, because research into the biology of race nonspecific resistance has been limited to a single race nonspecific gene, *Lr34*. This research indicates that race nonspecific resistance, like race specific resistance, can manifest both before haustorium and after haustorium formation (Rubiales and Niks 1995, Jacobs 1989). However, unlike race specific resistance, race nonspecific resistance does not operate by rapid cell death. Instead Jacobs (1989) and Rubiales and Niks (1995) found that the race nonspecific gene *Lr34* works by slowing down intracellular hyphal development prior to haustorium formation, thereby reducing the number of haustorium formed in cells. The reduced haustorium formation during infection is the cause of the slow rusting response in plants with *Lr34* (Rubiales and Niks 1995). Jacobs (1989) speculated that *Lr34* resistance may be from some inhibiting host substance, which has cumulative effects that are slow to develop. This is a possible reason why the race nonspecific resistance of *Lr34* is greatest when wheat plants with the *Lr34* gene are mature, i.e. it provides an explanation why the race nonspecific resistance conferred by *Lr34* and possibly other race nonspecific genes confer adult-plant resistance or late-rusting resistance. Jacobs (1989) also found that slower passage through cell wall or "pre-haustorial exclusion" of the invading sporelings is not the cause of race nonspecific resistance and concluded that slowed haustorial development and abortion of infecting structures as well as a longer latent period is the cause of race nonspecific resistance (Jacobs 1989). Rubiales and Niks (1995) noted pale flecks are associated with resistance from *Lr34*, but noted that these are not the same as the necrotic flecks observed in race specific resistance and should not be confused with a hypersensitive response.

Despite the more complicated nature of inheritance of race nonspecific resistance and the different mode of action of race nonspecific genes, the additive effects of race nonspecific genes have been shown to be heritable (Navabi et al. 2003, Xu et al. 2005) and this has made it possible for breeders to select for race nonspecific resistance (Lee and Shaner 1985b, Das et al. 1993, Navabi et al. 2003). Many breeders have used this approach, breaking down the slow-rusting phenotype into components that can be more easily measured, and then by determining the heritability of these to assess their usefulness in breeding (Caldwell 1968, Luke et al. 1972, Ohm and Shaner 1976, Shaner et al. 1978, Shaner et al. 1980,Kuhn et al. 1980, Shaner et al. 1980, Parlevliet 1981, Knott 1988, Rajaram et al. 1988).

Of the components of slow-rusting studied, latent period and uredinium size have been found the most useful in breeding for the slow-rusting phenotype. (Kuhn et al. 1980, Shaner et al. 1997, Das et al. 1993). However, use of the components of slow-rusting in selection, can be labor intensive (Xu et al. 2005). Therefore, Navabi et al. (2003) and others have suggested using marker assisted selection instead of selecting based on heritable phenotypic characteristics, and molecular markers for the components of the slow-rusting phenotype have therefore been sought (William et al. 1997, Xu et al. 2005).

Marker Assisted Selection

The use of marker assisted selection has potential to aid in incorporation of race nonspecific leaf rust genes into lines (William et al. 2003, William et al. 2007, Lagudah et al. 2009, Cao et al. 2010). Unfortunately, few molecular markers for race nonspecific genes exist (William et al. 2003, Cao et al. 2010), and the cost of their use in incorporating race nonspecific leaf rust resistance genes into lines is, at present, prohibitive (William et al. 2007). At least a dozen as yet uncharacterized race nonspecific genes are thought to exist (Singh et al. 1998, Singh and Rajaram

2002), but markers for only two race nonspecific leaf rust genes, *Lr34* and *Lr46*, are currently available, and the markers for *Lr46* lack accuracy (Singh et al. 1998, Xu et al. 2005). William et al. (2007) suggested that increasing availability of automated high-throughput markers as more molecular markers for race nonspecific leaf rust genes become available over time will reduce the cost and difficulty associated with using marker assisted selection. Therefore, there is great need for more research like that of Cao et al. (2010), and Lagudah et al. (2009) to develop more molecular markers for race nonspecific genes.

The lack of markers for more race nonspecific genes is unfortunate because the use of molecular markers has the advantage that it allows the breeder to make selections based on observing the genotype directly, rather than forcing the breeder to rely on the use of heritable phenotypic characteristics which can be difficult to measure and are not always predictive of the slow-rusting phenotype of race nonspecific resistance (Kolmer and Liu 2001). Therefore, progress in breeding with race nonspecific genes could be significantly improved if more molecular markers for race nonspecific genes were available. Breeders would then be able to select directly for race nonspecific genes without being encumbered by the more complicated nature of the inheritance of race nonspecific genes, or deal with problems associated with the presence of race specific genes which mask the effects of race nonspecific genes lines (Vanderplank 1978, Knott 1982, Simmonds 1988, Oelke and Kolmer 2005, Kolmer and Oelke 2006, Kolmer and Long 2007), or with other problems associated with breeding with race nonspecific genes.

Pyramiding Race Nonspecific Genes

Fortunately, breeders have not had to wait for the development of molecular markers to breed using race nonspecific genes, and have managed to overcome the problems associated with breeding with them. Incorporating several different genes or "pyramiding" genes into lines

(Hsam and Zeller 2002) has proven effective in producing lines with durable race nonspecific leaf rust resistance (Kuhn et al. 1978, McIntosh 1992, Navabi et al. 2003, Singh et al. 2011). Researchers at CIMMYT have been pyramiding race nonspecific genes into lines for over thirty years, leading to the creation of many lines with high levels of race nonspecific resistance (Rajaram et al. 1996, Singh and Trethowan 2007, Krattinger et al. 2009). The availability of these race nonspecific CIMMYT lines has led to worldwide improvements in leaf rust resistance (Rajaram et al. 1988) and dramatic yield gains worldwide (Sayre et al. 1998).

Since few molecular markers are available to directly determine the genotype of lines, a major challenge of pyramiding race nonspecific genes into lines to achieve resistance is that race specific genes must be eliminated either from the parental lines or from the progeny. The elimination of race specific genes is necessary because their presence masks the effects of race nonspecific genes in lines (Vanderplank 1978, Knott 1982, Simmonds 1988, Oelke and Kolmer 2005, Kolmer and Oelke 2006, Kolmer and Long 2007). Therefore, if race specific genes are not removed from parental lines or the progeny it will not be possible for the breeder using only phenotypic selection methods to screen for the presence of race nonspecific genes because there will be no way for the breeder to determine whether the resistance was derived from pyramided race nonspecific genes or from one or more race specific genes that may be present (Vanderplank 1978, Knott 1982, Simmonds 1988, Oelke and Kolmer 2005, Kolmer and Oelke 2006, Kolmer and Long 2007). This is not to imply that race specific genes should not be used to develop rust resistance in varieties, however. Once desired levels of resistance from pyramided race nonspecific genes is reached, the breeder can then cross these lines to adapted lines or varieties with useful race specific genes. Indeed, a major advantage of having molecular markers for race nonspecific genes, should more of them become available, would be that a breeder would not have to eliminate race specific genes from lines at all to assess for resistance from race nonspecific genes, since the genotypes could be read directly.

Breeders using phenotypic selection methods to screen for race nonspecific genes can remove race specific genes by testing lines against leaf rust pathotypes (Simmonds 1988), or by testing with isogenic lines such as the ones first created by P.L. Dyck (Dyck et al. 1966, Huerta-Espino et al. 2011), or by other methods (Flor, H.H. 1956, Dyck, P.L. 1977, Singh R.P. and Rajaram S. 1991, Dyck et al. 1994,). Another approach is to "work on a broad genetic base, under enhanced recombination" to produce useful parental lines (Simmonds 1988). This is the approach used at CIMMYT, where race nonspecific resitance is maintained lines in segregating populations, keeping only those segregates that show low levels (10-30%) of rust severity. This method eliminates lines with race specific genes (McIntosh 1992), generating lines with high levels of race nonspecific resistance (Singh and Trethowan 2007). At CIMMYT selection is done under "artificially induced rust epidemics", generating parents that were crossed to adapted lines (Navabi et al. 2003). Pyramiding of race nonspecific genes is achieved via 3-way and 4-way crosses to incorporate many race nonspecific genes into lines (Singh and Trethowan 2007). Adapted varieties are chosen as the recurrent parent, allowing the additively inherited race nonspecific genes to be transferred into adapted varieties, without losing any yield potential or grain quality (Navabi et al. 2003, Singh and Trethowan 2007).

The Use of Area Under Disease Progress Curve to Pyramid Genes

The pyramiding of race nonspecific genes into lines has been much aided by the development of the area under disease progress curve (AUDPC) method used at CIMMYT to observe the progression of the "slow-rusting" characteristic of race nonspecific resistance over time in the field (Knott 1988, Rajaram et al. 1988). The use of AUDPC became possible after Caldwell et al. (1970) observed that the slow-rusting effect of race nonspecific resistance is often characterized by a pattern of infection in which pustules occur mainly at the bottom ten to twenty-five percent of leaf blades at the onset of the infection, and then gradually increase to cover more of the leaf as

the disease progresses. Caldwell et al. (1970) referred to the slow-rusting response as "Mentana" type infection pattern after the variety on which it was first observed. The Mentana infection pattern affects plants with one or more race nonspecific genes. The rust infection pattern starts on the basal portion of the leaves but over time, and if rust severity is great enough, can cover the entire leaves, even in lines with more than one race nonspecific gene conferring reasonable levels of race nonspecific rust resistance. Rust coverage is especially high after kernel ripening/leaf senescence but rust infection at this stage usually does not reduce yields. AUDPC is used to monitor disease progression and make selections based on the slow rusting phenotype. The use of AUDPC to select lines with pyramided race nonspecific genes has been a routine activity at CIMMYT since the 1980's (Knott and Padidam 1988, Rajaram et al. 1988).

AUDPC is an effective selection tool because it is highly heritable (Das et al. 1992, Das et al. 1993, Navabi et al. 2003), and unlike latent period and other components of the slow-rusting phenotype that must be measured in the seedling stage in juvenile plants, AUDPC allows field, adult-plant determinations of the race nonspecific phenotype to be made. This is convenient because the use of AUDPC means that seedling readings are not necessary in breeding programs in which race specific genes are removed from breeding populations.

Over the course of their work, CIMMYT researchers found that high variation in AUDPC readings in segregating F_1 populations indicates that many race nonspecific resistance genes were present in the populations (Singh and Rajaram 1992). They also were able to roughly correlate the level of resistance observed in field readings with the number of race nonspecific genes likely to be present in advanced lines (Knott 1988, Singh and Rajaram 1992, Singh and Trethowan 2007). Singh and Trethowan (2007) and Singh and Rajaram (1992) found that under heavy leaf rust disease pressure two to three race nonspecific genes gave some level of resistance but that four to five genes were needed to achieve higher levels of resistance (see Table 1). Singh and

Trethowan (2007) and Navabi et al. (2003), found that if the race nonspecific *Lr34* was also present, it considerably increased the resistance of lines so that fewer additional race nonspecific genes were needed to achieve higher levels of resistance. Navabi et al. (2003) found that as few as two to four race nonspecific *Lr* genes are enough to generate reasonable levels of resistance. This is comparable to the work of Knott and Padidam (1998) who found that three to four race nonspecific genes were needed to confer adequate levels of resistance to stem rust.

Researchers at CIMMYT also found that pyramiding as few as 4 to 5 race nonspecific genes into lines could result in near-immunity to leaf rust (Singh et al. 2000, Singh and Trethowan 2007, Singh et al. 2011). Near-immunity derived from race nonspecific genes is advantageous to immunity conferred from race specific genes because unlike race specific genes, race nonspecific genes confer durable resistance (Singh and Trethowan 2007). Another advantage of pyramiding multiple race nonspecific genes into lines is that durability may increase with the addition of each race nonspecific gene (Singh et al. 2000).

TABLE 1. Changes in degree of susceptibility to leaf rust as more race nonspecific genes are incorporated into lines, with and without *Lr34*.

Approximate degree of susceptibility	Number of race nonspecific genes	Presence of *Lr34*
10%	4 to 5	No
30%	2 to 3	No
100%	0	No
1-5%	3 to 4	Yes
10-15%	2 to 3	Yes
40%	1	Yes

Source: Knott (1988), Singh and Rajaram (1992), Sing et al. (2000), Navabi et al. (2003), Singh and Trethowan (2007), Singh et al. (2011).

Combining Seedling and Adult Plant Resistance Readings

Combining adult plant field readings and juvenile plant greenhouse seedling readings has been shown to be an efficient procedure for selection of race nonspecific resistance (Das et al. 1993). This method takes advantage of the differential adult plant or field phenotype versus the seedling plant or greenhouse phenotype that was first observed by Mains and Jackson (1926) in their foundational work on the pathology of leaf rust. Combining adult plant field readings and juvenile plant greenhouse readings and the use of AUDPC to screen for race nonspecific resistance are comparable methods since both methods screen for race nonspecific genes using the "slow rusting" phenotype of race nonspecific resistance (Das et al. 1993, Singh et al. 2000). Combining adult plant and seedling resistance readings has already been used by Knott (1982) to produce lines with race nonspecific resistance to stem rust, and by Oelke and Kolmer (2005), Kolmer and Oelke (2006), Zhang et al. (2008) and others to produce lines with race nonspecific resistance to leaf rust.

For selection using adult plant field readings combined with juvenile plant greenhouse readings to be successful, the use of heritable traits of leaf rust resistance in the field and in the greenhouse are necessary (Das et al. 1993). Final rust status is a heritable trait useful for selecting for adult plant race nonspecific resistance in the field (Das et al. 1993). Heritable components of the "slow rusting" phenotype used in the greenhouse to assess juvenile plant greenhouse readings include latent period and uredinium size (Lee and Shaner 1988, Das et al. 1993). Since seedling rust resistance readings including the one developed by Mains and Jackson (1926) use heritable components of the slow rusting phenotype, namely uredinium size, to designate infection types, these methods are useful greenhouse tools for making selections based on seedling resistance.

Combining greenhouse seedling resistance readings with adult plant field resistance readings has the potential to be a useful way to screen for race nonspecific resistance in breeding programs where advanced materials with pyramided race nonspecific genes are crossed with locally adapted cultivars that have race specific genes. This is particularly important when advanced spring wheat lines with race nonspecific resistance are crossed with adapted winter wheat lines because a second cross or "top cross" to a winter parent is often needed to recover the winter type.

CHAPTER III

MATERIALS AND METHODS

Leaf Rust Breeding Program at Oklahoma State University

Combining greenhouse and field resistance readings to screen for race nonspecific resistance was used in a new breeding program that was established at Oklahoma State University, whereby locally adapted winter wheat lines were crossed to advanced CIMMYT spring wheat lines with high levels of pyramided race nonspecific resistance. In addition to other objectives, the goal of the breeding program was to transfer the pyramided race nonspecific resistance from CIMMYT spring wheats into adapted winter wheats in the Southern Great Plains. The development of the breeding program required a new selection methodology since race specific genes are present in the adapted winter wheat lines being used as the parent in the crosses. Therefore, selections were made based on relatively high levels of susceptibility in the early generations and for each subsequent generation selections were made for less and less susceptibility, or higher and higher levels of resistance. Just as in the CIMMYT breeding program, in every generation plants that showed 0% susceptibility or 100% resistance were not kept, since these segregates would have oligogeneticly-inherited race specific resistance (Vanderplank 1978, Knott 1982, Simmonds 1988, Oelke and Kolmer 2005, Kolmer and Oelke 2006, Kolmer and Long 2007). Also, only the plants that exhibited the "Mentana-type" infection pattern indicative of the slow-rusting phenotype of race nonspecific resistance described by Caldwell et al. (1970) were kept. The

objective of the selection procedure was to select plants showing 40 to 50% susceptibility (or 40 to 50MS in the modified Cobb scale, Peterson et al. 1948) or less in the F_2 generation, plants showing less than 20 to 30% susceptibity (20 to 30MS) in the F_3, and plants showing less than 20% susceptibility (20MS) in the F_4 generation . Finally, plants showing 20MR or less were selected in the F_5 generation.

Final Rust Reaction Measured Using Modified Cobb Scale

In the breeding program at Oklahoma State University, lines are evaluated at F_2, F_4, F_5, F_7, and F_8 generations for rust resistance in a severe rust environment in south Texas, and other characteristics such as agronomic type, and winter hardiness were evaluated in Oklahoma in all generations except F_2. Final rust status was determined on advanced lines at approximately Feekes stage 10.5 (Large 1954) by readings taken in April in Bexar, County Texas, using the modified Cobb scale as described in Peterson et al. (1948). This approach is justified since Das et al. (1993) showed that final rust status at pollination is just as effective as AUDPC when selecting for race nonspecific resistance. The modified Cobb scale has two components,a percent leaf coverage from trace to 100%, and a qualitative component rated from resistant (R) to susceptible (S). Moderately resistant (MR) and moderately susceptible (MS) ratings are given for intermediate levels of resistance and susceptibility (Peterson et al. 1948).

Greenhouse Seedling Readings

Spores collected from the research farm at Oklahoma State University in 2010 were used to evaluate the seedling resistance of all of the lines. These spores were stored in liquid nitrogen from the time of collection the previous spring. Prior to use, the spores were heat shocked at 42^{o} C for 15 minutes. A variety very susceptible to leaf rust, TAM 110, was planted very densely in 8 inch pots. These plants were inoculated to greatly increase the amount of spores available to

inoculate the lines under evaluation. The plants, innoculated to increase the amount of spores for innoculation of lines, were allowed to grow in the greenhouse for 14 days. Then the plants were inoculated using the paint brush method after the plants had been wetted thoroughly with a solution of 1 ml tween in 1 L water purified by reverse osmosis. After inoculation, the plants were placed in a mist chamber and misted for 24 hours using water purified by reverse osmosis.

Advanced lines were planted in 60 cm by 90 cm flats one to two days after the TAM 110 plants were inoculated. Final rust status data from 2011 and 2012 were used to identify the most promising advanced lines for seedling susceptibility evaluations. A total of 435 advanced lines and thirteen checks from the south Texas field trials were evaluated in the greenhouse. The field checks were the varieties Greer, Cedar, Jackpot, Santa Fe, TAM 401, Overley, Endurance, TAM 111, Duster, Fannin, Billings, Armor, and Everest. The lines and the field checks were planted in flats in two replicates each. Three very susceptible varieties, TAM 110, Jagger, and Jagalene, were planted in every flat with the lines and field checks as susceptible checks for the seedling susceptibility readings. When the lines and checks reached the 2-4 leaf stage they were wetted with a 1 ml tween in 1 L water purified by reverse osmosis and inoculated by plant to plant contact with the inoculum increase plants. After inoculation, the flats were placed in a mist chamber and misted for 24 hours using water purified by reverse osmosis.

Infection type data were taken at the seedling stage seven to ten days following inoculation using the methods described by Mains and Jackson (1926). Infection type was recorded on a 0 to 4 scale. A description of the infection types used following Mains and Jackson (1926) is shown in Table 2. Mains and Jackson defined readings of 0-2 as highly to moderately resistant, and readings 3-4 as moderately to highly susceptible. Jacobs (1989), Kolmer and Liu (2001), Das et al. (1993), and Das et al. (1992) have shown that adult plant resistant / slow rusting race nonspecific resistance phenotype manifests in seedlings as high infection types. Plants with race

nonspecific resistance show resistance when mature, rather than at the seedling stages. Therefore, only plants with infection type readings of 3 or higher were considered when greenhouse and field resistance readings were combined to determine phenotypes consistent with race nonspecific resistance.

Table 2. Description of infection types used

Infection Type	Resistance	Phenotype
0	Highly Resistant	no uredinia formed, small flecks of chlorotic or necrotic areas only
1	Very Resistant	few, small uredinia formed in small necrotic spots, many necrotic spots formed do not produce uredinia
2	Moderately Resistant	uredinia fairly abundant, of moderate size , always in necrotic or very chlorotic spots, almost all necrotic spots have uredinia
3	Moderately Susceptible	uredinia fairly abundant of moderate size, no necrosis produced but sometimes slight chlorosis surrounding uredinia
4	Very Susceptible	uredinia abundant and large, no necrosis surrounding uredinia, infection areas (of several uredinia formations) sometimes form “islands” surrounded by chlorotic rings

Source: Mains and Jackson (1926).

Combining Adult Plant and Seedling Readings to Determine Phenotype

After adult plant field resistance readings and greenhouse seedling resistance readings were recorded for all 435 advanced lines and 13 field checks in this study, they were combined to determine the phenotype of each line. Phenotypes were considered consistent with race specific readings when replicates had average greenhouse Mains and Jackson resistance readings of 2 or less and modified Cobb scale readings of 20S/30MS or more resistant. Lines were considered to have phenotypes consistent with race nonspecific resistance when they had average greenhouse Mains and Jackson resistance readings greater than 2 and modified Cobb scale readings of 20S/30MS or more resistant. Plants were considered to have susceptible phenotypes when they showed high levels of susceptibility in both the greenhouse and the field (average Mains and Jackson resistance readings greater than 2 and modified Cobb scale readings more susceptible than 30S/40MS). Finally, a fourth phenotype, consistent with race specific resistance based on race specific genes for which virulence existed in the population of rust spores or "defeated race specific resistance" was determined. Lines were placed in this group if they showed resistance in the seedling stages but susceptibility in the field (average Mains and Jackson resistance readings of 2 or less, and modified Cobb scale readings more susceptible than 20S/30MS).

Marker Assisted Selection

Molecular sequencing work done by Krattinger et al. (2009) and molecular mapping work done by Cao et al. (2010) on the *Lr34* gene has allowed the development of molecular markers and characterization of haplotypes for this gene. Krattinger et al. (2009) and Lagudah et al. (2009) found susceptible and resistant haplotypes resulting from three polymorphisms, a 3 bp deletion in exon 11, and single nucleotide polymorphisms in exon 12 and intron 4. Lagudah et al. (2009) and Cao et al. (2010) found an additional G/T single nucleotide polymorphism in exon 22 that produces a stop codon in the variety Jagger. Markers developed by Lagudah et al. (2009)

based on the 3 bp indel polymorphism in exon 11, as well as markers developed by Cao et al. (2010) to identify the Jagger type allele based on the SNP in exon 22 were used in this research.

First, all lines were analyzed using the exon 11 markers cssfr1 and cssfr2 described by Lagudah et al. (2009), following the protocols described by those authors. The markers cssfr1 and cssfr2 are dominant markers; therefore any failed PCR reaction can be wrongly read as the absence of the allele (Lagudah et al. 2009). For this reason, Lagudah et al. (2009) multiplexed these markers with a codominant sequence tagged site marker, *cs*LV34. However, Cao et al. (2010) found that *cs*LV34 does not work well with Jagger derivatives. Since several of the lines in this study were made from crosses with Jagger derivatives, the *cs*LV34 marker was not used. Instead, any ambiguous results obtained with the cssfr1 and cssfr2 markers were analyzed again using cssfr1/cssfr2 and a novel second set of primers developed by Cao et al. (2010). Finally, a modified cssfr7 (Lagudah et al. 2009) exon 22 CAPS marker using the restriction enzyme PstI developed by Cao et al. (2010) was used to test all lines that tested positive for exon 11 markers to separate the lines that had the susceptible Jagger type alleles from those with the resistant allele.

CHAPTER IV

RESULTS AND DISCUSSION

Combining Adult Plant and Seedling Readings to Determine Phenotype

The greenhouse seedling resistance readings and adult plant field resistance readings were used to classify the phenotypes of the 435 advanced lines in this study into four phenotype classes. These data are presented in Table 3. Of the 435 lines, 154 showed a phenotype consistent with race specific resistance. The majority of lines, 237, displayed phenotypes consistent with race nonspecific resistance. Of the remaining lines, 44 displayed a susceptible phenotype and 13 lines showed resistance in the seedling stage in the greenhouse but susceptibility in the field, a phenotype typical of resistance based on one or more "defeated" race specific genes.

The 13 field checks were also classified based on the seedling and field resistance readings obtained. The varieties Cedar, Duster, Fannin, Billings, Armour and Everest all displayed high field resistance. Of these, Duster, Fannin, Billings, Armour, and Everest showed susceptibility in the seedling stage and therefore displayed a phenotype consistent with race nonspecific resistance. It is therefore likely that the resistance in these varieties is, at least to some degree, based on race nonspecific genes.

The other variety with high field resistance, Cedar, showed high resistance in the greenhouse and therefore displayed a phenotype consistent with race specific resistance. If race nonspecific genes are present in Cedar, then they were masked by the action of race specific genes in the greenhouse resistance studies.

Three checks, TAM 111, Santa Fe, and TAM 401 demonstrated moderate to intermediate levels of field resistance and greenhouse susceptibility, a phenotype that would also be associated with race nonspecific resistance. It is possible that these three varieties possess race specific genes that are no longer effective against the prevalent strains of rust that exist in south Texas. However, due to the relatively high degree of resistance they showed in the field, there is some evidence which indicates that they have at least some resistance based on some combination of race nonspecific genes that is still functioning.

Finally, the varieties Jackpot, Endurance, and Overley showed susceptibility in the field and the greenhouse and were therefore classified as susceptible. The resistance of these varieties is almost certainly based on race specific genes which are no longer effective against the virulence present in the current population of rust spores in south Texas.

Table 3. Phenotypic Classes

Phenotype Class	Lines	Checks
Race Specific	154	Cedar
Race Nonspecific	237	Duster, Fannin, Billings, Armour, Everest
Defeated Race Specific	13	TAM111, Santa Fe, TAM 401
Susceptible	44	Endurance, Overley

The number of check varieties with phenotypes consistent with race nonspecific resistance was somewhat surprising, but previous research has indicated that Duster and the Oklahoma variety 2174 have *Lr34* (Cao et al. 2010). Both varieties were derived from crosses made by the private breeding company Pioneer, which made extensive use of CIMMYT germplasm. Some of the varieties with phenotypes consistent with race nonspecific resistance may be crosses to Pioneer germplasm or have CIMMYT germplasm in their pedigrees, or may have received race nonspecific genes from unknown sources.

Molecular Analysis with Lr34 Markers

Of the 435 lines in this study, a subset of 192 lines and 6 of the 13 field checks from south Texas were analyzed with *Lr34* exon 11 markers. These 192 lines were chosen for marker analysis because they were made from crosses with CIMMYT lines known to have high levels of leaf rust resistance derived from pyramided race nonspecific genes. A total of 94 of the 192 lines analyzed tested positive for *Lr34* exon 11 markers.

The 94 lines that tested positive for the exon 11 markers were analyzed for the presence of the exon 22 Jagger-type allele using the cssfr7_F3/cssfr7_r, *Lr34E22*, PstI fragment CAPS marker developed by Cao et al. (2011). Of these, 81 lines tested positive for the *Lr34*(+) allele. Of the others, only 11 had markers consistent with the Jagger type allele. Out of the 192 lines tested, 81 had the *Lr34*(+) allele.

Analysis of Phenotypic Classes Using *Lr34* Markers

The subset of 192 of the 435 lines in the study that was selected for analysis with *Lr34* markers was chosen before any of the lines were classified by phenotype. As a result, *Lr34* markers

were run on lines that were later found to belong to each of the phenotypic classes. Of the 192 lines analyzed, 94 lines displayed the phenotype consistent with race nonspecific resistance, 71 displayed the phenotype consistent with race specific resistance, 22 displayed a susceptible phenotype and 5 displayed a phenotype consistent with defeated race specific resistance. These data are presented in Table 4.

Lines testing positive for the presence of *Lr34* were found in each phenotypic class. A slightly higher proportion (46%) of lines with the phenotype consistent with race nonspecific resistance tested positive for *Lr34* than for any other phenotype class. A relatively large proportion (38%) of lines displayed the race specific phenotype, yet still carried the markers for *Lr34*(+). One or more effective race specific genes in addition to the *Lr34* gene must therefore be present in these lines.

Finally, several lines with the susceptible phenotype carried *Lr34*. Lines were classified as susceptible if their field readings exceeded 30S/40MS. The high levels of field susceptibility in these lines indicate that these lines most likely had *Lr34* as their only source of resistance. This is also likely true of the one line showing high levels of seedling resistance but with high susceptibility in the field, a phenotype consistent with "defeated" race specific resistance, but having the*Lr34* gene. This one line apparently possessed *Lr34* plus one or more "defeated" race specific genes that conferred resistance in the greenhouse but not in the field.

Table 4. *Lr34* marker results grouped by phenotype class.

Phenotype Class	Lines Tested	Presence of *Lr34* markers	Percentage with *Lr34*
Race Specific	71	27	38%
Race Nonspecific	94	43	46%
Defeated Race Specific	5	1	20%
Susceptible	22	10	45%

Estimating Number of Genes Present in Lines

Several researchers have been able to roughly correlate the number of race nonspecific genes likely present in advanced lines using final rust status when it is known that the resistance of these lines is due to the presence of race nonspecific genes (Knott 1988, Knot and Padidam 1998, Singh and Rajaram 1992, Navabi et al. 2003, Singh and Trethowan 2007). Levels of susceptibility these authors associated with the number of race nonspecific genes likely present is given in Table 1. The advanced lines in this study were analyzed using similar methods with final rust status readings taken in 2010 and 2011. The results of these analyses are given in Tables 5 and 6.

Table 5. Number of race nonspecific genes postulated in lines analyzed with *Lr34* markers

Level of Susceptibility	Number of Race Nonspecific Genes	*Lr34*(+)	Lines	Percentage of Lines
Trace to 20MS	4 to 5	No	42	40%
20MS to 30MS	2 to 3	No	8	8%
30MS or higher	0 to 1	No	0	0%
Trace to 5MS	3 to 4	Yes	3	3%
5MS to 20MS	2 to 3	Yes	33	32%
20MS to 30MS	1 to 2	Yes	7	7%
30MS to 50S	1	Yes	11	11%

The subset of 192 lines tested with *Lr34* markers could be analyzed for the number of genes likely present with a greater degree of precision since it was known whether or not *Lr34* was also present. The number of race nonspecific genes for the remaining 237 lines could also be postulated, but the higher resistance / lower susceptibility levels associated with the presence of *Lr34* could not be determined. Therefore, the more conservative estimate of the number of race nonspecific genes present was used to estimate the number of race nonspecific genes that may be present. That is, the data for the remaining 237 lines was analyzed as if one of the postulated race nonspecific genes was *Lr34*, giving a lower estimate of the number of race nonspecific genes present. The result of this analysis is given in Table 6.

Table 6. Number of race nonspecific genes postulated in lines not analyzed with *Lr34* markers

Level of Susceptibility	Number of Race Nonspecific Genes	Lines	Percentage
Trace to 5MS	3 to 4	17	12%
5MS to 20MS	2 to 3	100	71%
20MS to 30MS	1 to 2	20	14%
40MR to 50MS	1	4	3%

Advanced lines with race specific phenotypes were not analyzed using this method since a single race specific gene can mask the presence of all race nonspecific genes that may be present. It is therefore not possible to postulate the number of genes that might be present in lines that have effective resistance conferred by one or more race specific genes. However, since many of the lines with the race specific phenotype were analyzed with *Lr34*, it was possible to determine which of these lines with the race specific phenotype also had *Lr34*. Of the 71 lines with the race specific phenotype analyzed, 38%, or 27 of these lines possessed the *Lr34*(+) allele. The relatively large proportion of lines displaying the race specific phenotype possessing the *Lr34*(+) allele likely indicates that many of the race specific lines not tested with *Lr34* markers have *Lr34* and possibly other race nonspecific genes as well.

Taken together, these results indicate that the combination of adult plant field readings and seedling plant greenhouse readings is a very useful tool for evaluating the type of resistance present in lines segregating from crosses of parents with pyramided race nonspecific genes and parents that may have one or more race specific genes and other race nonspecific genes. This screening method identified 57 advanced lines (44 lines with the susceptible phenotype and 13

lines with the phenotype consistent with defeated race specific resistance) for immediate elimination from the breeding program. Also, although *Lr34* markers were not run on all the lines in the study, it was possible to determine that as many as 141 of the 435 lines tested carried resistance due to 2 to 3 race nonspecific genes. In an additional 20 lines, resistance was conferred by 3 to 4 race nonspecific genes. Finally, testing with *Lr34* markers revealed that 4 to 5 pyramided race nonspecific resistances conferred resistance to 42 lines.

The results also revealed an unexpectedly large number of advanced lines displaying a phenotype consistent with race specific resistance. Since the identification of resistance based solely on race nonspecific genes requires race specific to be eliminated from breeding population, the relatively large proportion of lines displaying the race specific phenotype and having *Lr34*(+) is an indicator that refinements in the selection methodology used in this breeding program may need to be made. It is possible that the relatively high proportion of lines displaying phenotype consistent with race specific resistance demonstrates that the selection methodology based on gradually increasing the level of resistance selected for in each generation and never saving lines with very low levels of susceptibility was not always rigorously adhered to. A total of 154 of 435 displayed phenotypes consistent with race specific resistance and only a slightly greater number or 237 of the 435 lines displayed a phenotype consistent with race nonspecific resistance. This indicates that a relatively large proportion of lines with race specific genes for resistance were not eliminated by the breeding methodology.

Although this breeding program was designed to use a phenotypic selection method to eliminate lines with race specific genes from breeding populations in order to identify lines which derived their resistance from race nonspecific genes, it is very difficult for a breeder to throw out visibly superior plants or segregating populations if they also have higher levels of resistance than is allowed by the methodology. Furthermore, the breeding methodology requires selecting for very

low levels of resistance but not zero resistance in the later generations since zero resistance is a phenotype associated with race specific resistance. Therefore, unless the selection protocol is very stringently adhered to, it is therefore almost inevitable that some selections of lines with race specific genes will be made. On the other hand, the breeder lacking access to molecular markers for race nonspecific genes and having to rely instead on a selection method based on heritable phenotypic characteristics , would want to take care not to eliminate lines that carried high levels of pyramided race nonspecific resistance. The resistance of such lines might easily be misinterpreted as race specific resistance derived from the race specific resistance known to exist in many of the adapted winter wheat parents. The breeder would therefore be more likely to accept the risk of allowing a line with race specific resistance to stay in the breeding program longer, rather than eliminating a line with rust resistance based on pyramided race nonspecific genes.

Futhermore, since greenhouse seedling resistance studies were only added to the breeding methodology in 2011, seedling resistance was not evaluated in earlier generations, and this meant that more lines with race specific resistance were likely kept than there would have been had screening based on greenhouse seedling readings been done throughout the course of the program. Again, not eliminating race specific genes from lines would not normally be a problem, but it would be in a breeding program such as this one, where the goal is to eliminate race specific genes so that race nonspecific genes could be pyramided in adapted winter wheat lines using the selection methods described. This result however, is not as problematic as it otherwise might be because all of the advanced lines in the breeding program are derived from one parent known to have resistance based only on multiple genes for race nonspecific resistance combined with a locally adapted advanced line or variety. Therefore, it is very likely that many of the advanced lines displaying a phenotype consistent with race specific resistance have resistance based both on one or more race specific genes pyramided with race nonspecific genes.

Any race specific genes they carry will likely be eliminated from their progeny kept in the breeding program in future years by continued selection using the same methodology combined with the addition of the greenhouse seedling resistance readings.

Also, now that 195 promising winter wheat advanced lines likely having resistance derived from race nonspecific genes have been identified, and because these are known to derive from parents having different combinations of race nonspecific genes, these can now be intercrossed in an attempt to achieve winter wheat lines with even higher levels of pyramided race nonspecific resistance. Many of the more promising lines classified as having a phenotype consistent with race specific resistance may also have a high number of pyramided race nonspecific genes, the action of which are masked by the presence of one or more race specific genes. Therefore, some of the lines classified as having race specific resistance will also likely be intercrossed to each other and to the lines identified as having high levels of race nonspecific resistance.

Finally, these results confirmed the results of Singh and Rajaram (1992) and others who found that *Lr34* by itself can confer low levels of resistance, and it is therefore necessary to pyramid it with other resistance genes. Lines found to have *Lr34* were found to have field resistance readings as low as 60S in this study, while others containing *Lr34* had much higher levels of resistance, indicating more genes than just *Lr34* were present in these lines.

Having the ability to analyze these advanced lines with *Lr34* added an additional tool useful in selection. As more and more molecular markers for leaf rust race nonspecific genes are developed, the ability of breeders to identify which genes and the number of genes that are present will improve, and this will help them to refine their breeding methods. Until then, breeders must continue to rely on selection methods employing heritable phenotypic

characteristics, such as the approach used by breeders at CIMMYT or methods utilizing combinations of greenhouse and field studies like the one used at Oklahoma State University.

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VITA

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Scope and Method of Study:

Breeding resistance to leaf rust (*Puccinia triticina*) into wheat varieties has long depended on the the incorporation of one or more race specific or "major" genes that confer complete resistance to specific rust pathotypes. This approach, though successful, is problematic because virulence quickly emerges in leaf rust populations to overcome resistance in extant varieties. Therefore, a new approach in breeding for leaf rust resistance is to pyramid many recessive race nonspecific or "minor" genes into wheat lines to produce spring wheat varieties with durable race nonspecific resistance was developed at International Center for Maize and Wheat Improvement (CIMMYT). CIMMYT has made these lines available to wheat breeders throughout the world. However, the use of these lines to breed for resistance in winter wheats lines is made difficult because race nonspecific genes are often recessive and have very little effect individually and are therefore, very difficult to select for using field selection methods such as the modified Cobb scale. To alleviate this problem and to allow selections for race nonspecific genes to be made, molecular markers for one race nonspecific leaf rust gene, *Lr34*, rust reactions of seedlings grown in the greenhouse, and adult plant field readings were combined to identify winter wheat lines with recessive race nonspecific genes. One hundred ninety-two advanced winter wheat lines made from crosses with the CIMMYT Spring wheat lines were screened for the presence of race nonspecific genes using this method. An additional two 243 lines were screened using greenhouse and field reactions alone.

Findings and Conclusions:

A total of 237 advanced winter wheat lines were found to have resistance derived from race nonspecific resistance, with 62 of these lines having resistance derived from three or more pyramided race nonspecific genes. These results indicate that combining greenhouse seedling rust reactions with field rust readings and molecular markers is an effective method to identify lines with leaf rust resistance derived from pyramided race nonspecific genes.