CHARACTERIZATION OF YELLOW RUST (*PUCCINIA STRIIFORMIS)* RESISTANCE IN A F_6 DURUM WHEAT POPULATION

by

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To my family,

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ABSTRACT

CHARACTERIZATION OF YELLOW RUST (*PUCCINIA STRIIFORMIS)* RESISTANCE IN AN F_6 DURUM WHEAT POPULATION

Nearly half of the agricultural land in Turkey is devoted to wheat. Turkey is one of the leading durum wheat producers in the Middle East and one of the most important biotic stress factors restricting the production is yellow rust. An F_6 recombinant inbred line durum wheat population from a Kunduru-1149 X Cham-1 cross was previously characterized for field resistance by Göçmen *et al.* (2003). The 150 lines of the population showed differing reactions to yellow rust in the field. The work done in this M.S. thesis was aimed at characterizing the yellow rust seedling resistance of this population and developing markers linked to this resistance. The 150 lines were tested for seedling resistance using a yellow rust isolate virulent on yellow rust resistance genes *Yr2, Yr6, Yr7* and *Yr9* representing the virulence profile of the yellow rust isolate used by Göçmen *et al.* (2003). The 150 lines were categorized based on their seedling resistances, 4 categories were formed, the majority of the lines being resistant. Based on the ratios observed in the seedling test, 53 of the 150 lines were chosen to constitute a sub-population. This sub-population was used to screen for SSR and NBS-profiling markers. Three linkage groups were formed and alleles of the SSR and NBS-profiling markers were compared to seedling and adult phenotypes (Göçmen *et al.*, 2003) using the Kruskal-Wallis single marker regression analysis program in MapQTL version 5 for Windows. Two QTLs were found on chromosome 1BL where the NBS-profiling band NBS3 290 was linked to three SSR markers. Three of the NBS-profiling bands were excised and cloned into a pGEM-T Easy vector and sequenced. The obtained sequences were analyzed using the BLASTX program. These sequences show homology to known NBS-LRR resistance genes in plants. Due to this sequence homology and results of the linkage analysis, we predict the presence if two QTLs on chromosome arm 1BL.

ÖZET

F_6 DURUM BUĞDAYI POPÜLASYONUNDA SARI PAS (*PUCCINIA STRIIFORMIS*) DİRENÇ KARAKTERİZASYONU

Türkiye'deki tarım alanlarının yaklaşık yarısı buğday ekimi için kullanılmaktadır. Türkiye Yakın ve Orta Doğu bölgelerinin en önemli durum buğdayı üreticilerindendir ve bu önemli üretimi etkileyen en önemli hastalıklardan birinin sarı pas olduğu kabul edilmektedir. Kunduru-1149 X Cham-1 melezlenmesi ile geliştirilmiş, 150 hatlık F_6 durum buğday popülasyonunun yetişkin bitki sarı pas dayanıklılığı Göçmen *ve ark*. (2003) tarafından araştırılıp değişen dayanıklılıklara sahip oldukları bulunmuştur. Bu master tez çalışmasının amacı 150 hatta fide döneminde sarı pas dayanıklılığını araştırmak ve bu dayanıklılığa bağlı DNA markörler geliştirmektir. Bu araştırmada, Göçmen *ve ark.* (2003) tarafından kullanılan sarı pas izolatında bulunan *Yr2, Yr6, Yr7* ve *Yr9* dayanıklılık genlerine karşı virulens taşıyan bir sarı pas izolatı seçilip, 150 hatta fide döneminde test edilmiştir. 150 hat fide döneminde sarı pasa coğunlukla dayanıklı olup, dayanıklılıklarına göre 4 kategoriye ayrılmıştır. Fide dönemindeki dayanıklılık oranına dayanarak 150 hattın 53'ü bir alt populasyon için seçilmiş ve SSR ve NBS-profilleme teknikleri kullanılarak markör calışmalarında kullanılmıştır. Üç bağlantı grubu belirlenmiş ve MapQTL programının Kruskall-Wallis ve Interval Mapping fonksyonları kullanılarak fide ve yetiskin dönemlerinde görülen dayanıklılık karakterize edilmiştir. Bu araştirmalar sonucunda 1BL kromozomunda NBS3 290 NBS-profilleme bantı ve 3 SSR bantını kapsayan 2 QTL bulunmuştur. 1BL QTL'i icinde bulunan NBS3 290 bantı ve bu bölge dışında bulunan 2 diğer NBS-profilleme bantı pGEM-T Easy vektörü kullanılarak klonlanmış ve DNA dizin analizine yollanmıştır. Elde edilen sekanslar BLASTX programı kullanılarak bilinen sekanslarla karşılastırılmıştır. Sonuç olarak NBS-profilleme bantlarının tümünün bilinen NBS-LRR tipi dayanıklılık genlerine homoloji gösterdikleri görülmüştür. DNA dizin homolojisi ve linkage analizi sonucunda 1BL bölgesinde iki adet QTL bulunduğu sonucuna varılmıştır.

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LIST OF SYMBOLS / ABBREVIATIONS

°C	Degrees centigrade
µl	Microlitre
ml	Millilitre
AFLP	Amplified-fragment-length-polymorphism
APAF-1	Apoptotic peptidase activating factor
APR	Adult Plant Resistance
ATP	Adenosine 5'-triphosphate
Avr	Avirulence
bp	Base pair
BSA	Bovine Serum Albumin
CC	Coiled coil
cDNA	Complementary deoxyribonucleic acid
cM	Centimorgan
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosidetriphosphate
EST	Expressed sequence tag
FAS	Foreign Agricultural Service
GDP	Gross Domestic Product
HR	Hypersensitive Response
Hz	Hertz
ICARDA	International Center for Agricultural Research in the Dry Areas
kb	Kilo base
Lr	Leaf rust
LRD	Leucine rich domain
LRR	Leucine rich repeat
MAP	Mitogen activated kinase
MAS	Marker-assisted selection
MOCA	Monographies on Candidate Countries

MS	Moderately susceptible
NB-ARC	Nucleotide binding in APAF-1, R-gene products, and
NBS	Nucleotide binding site
NO	Nitrogen Oxide
ORF	Open reading frame
PCR	Polymerase chain reaction
PK	Protein Kinase
P-loop	Phosphate-binding loop
QTL	Quantitative trait locus
R	Resistance/Resistant
RGA	Resistance gene analog
RIL	Recombinant Inbred Line
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
S	Susceptible
SAR	Systemic Acquired Resistance
SSR	Single-sequence-repeat
STS	Sequence-tagged-site
TBE	Tris Borate EDTA
TIR	Toll-Interleukin- 1
UV	Ultraviolet
Yr	Yellow rust

1. INTRODUCTION

The world's population is increasing at a far more rapid rate than previously seen. By 2050 the world's population is predicted to double to nearly 12 billion people. The big question we face is whether we will be able to supply the world with enough food given this population increase. It has been estimated that the world will need to produce more than twice as much food in the next 50 years as was produced since the beginning of agriculture, 10 000 years ago (Hoisington *et al.*, 2002). Since the area of agriculturally usable land is limited, this exponential increase in food production will require advancements in breeding that lead to enormous increases in yield.

1.1. Wheat: The Host

Wheat is the most widely grown crop in the world. The majority of the world's population uses wheat-based products to obtain their daily caloric intake. The estimated wheat production for 2005/2006 is 609 million tons, while the forecasted consumption is 615 million tons (International Grains Council, Grain Market Report 2005). Historically wheat and coarse grain production has steadily increased through breeding for higher yields and through modernization of agriculture. Table 1.1 summarizes the world historical figures on wheat area harvested, yield, production and consumption.

Table 1.1. World historical wheat figures (Foreign Agricultural Service (http://www.fas.usda.gov)

Year	Area	Yield	Prod.	Cons.	Year	Area	Yield	Prod.	Cons.
77/78	227.2	1.66	377.8	396.0	**92/93**	222.8	2.52	561.6	547.6
78/79	228.9	1.92	438.9	413.3	**93/94**	222.1	2.51	558.0	552.4
79/80	227.8	1.83	417.5	431.9	**94/95**	214.4	2.44	523.2	542.4
80/81	236.9	1.84	435.9	444.1	**95/96**	218.8	2.46	537.9	544.8
81/82	238.9	1.86	445.0	445.1	**96/97**	230.2	2.53	582.6	573.4
82/83	238.4	1.98	472.7	455.6	**97/98**	228.4	2.67	610.0	577.3

83/84	229.9	2.11	484.3	468.9	**98/99**	225.1	2.62	590.0	579.0
84/85	231.7	2.20	508.9	486.2	**99/00**	215.4	2.72	585.8	585.0
85/86	229.8	2.15	494.8	484.5	**00/01**	217.6	2.67	581.5	583.9
86/87	227.9	2.30	524.1	511.3	**01/02**	214.6	2.71	581.1	585.2
87/88	219.7	2.27	497.9	530.3	**02/03**	214.6	2.65	567.7	604.0
88/89	217.4	2.28	495.0	519.6	**03/04**	209.9	2.64	554.7	588.6
89/90	225.8	2.36	533.2	531.0	**04/05**	217.0	2.88	625.7	608.6
90/91	231.4	2.54	588.0	553.7	**05/06**	216.6	2.82	610.6	620.2
91/92	222.5	2.44	542.9	551.5					

Year: calendar year; Area: Area harvested (millions of hectares); Yield: tons per hectare; Prod: Production (millions of metric tons); Cons.: Consumption (millions of metric tons)

1.1.1. Wheat in Turkey

Agriculture is of special importance to Turkey due to the increasing population and the great contribution agriculture makes to the national economy. In 2000, Turkey had 41.5 million hectares of land used for agriculture. This represents 53.5 % of the total land area. Agriculture accounts for 11.2 % of the national GDP (Gross Domestic Product), employing nearly 14 % of the population (MOCA (Monographies on Candidate Countries (http://agrifish.jrc.it/marsstat/crop%5Fyield%5Fforecasting/MOCA/INDEX.HTM).

Cereals, especially wheat, are the most important crops in Turkey (Figure 1.1). In 1998 Turkey was 7th in the world for wheat production and is the leading wheat producer in the Middle East. Along with Syria it is the only country in the region that does not import wheat, but instead is a net exporter (MOCA http://agrifish.jrc.it/marsstat/crop%5Fyield%5Fforecasting/MOCA/INDEX.HTM; FAS http://www.fas.usda.gov). The wheat growing areas of the Middle East are as seen in Figure 1.2.

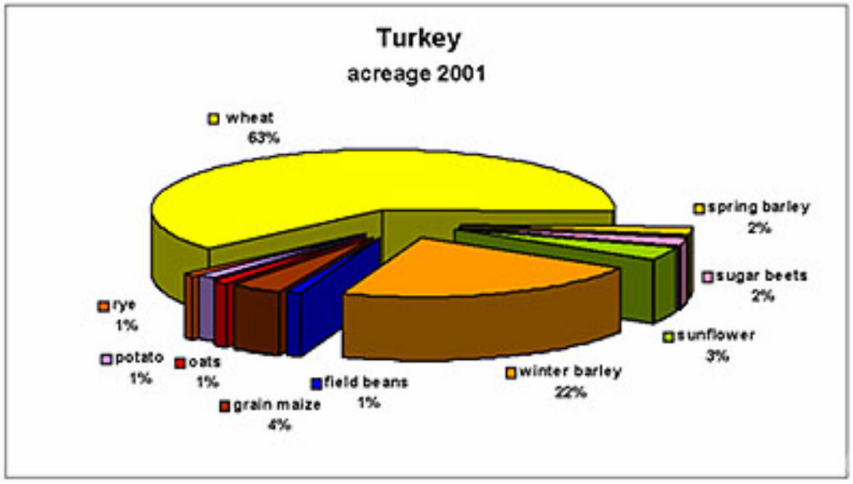


Figure 1.1. 2001 Crop production in Turkey (MOCA, http://agrifish.jrc.it/marsstat/crop%5Fyield%5Fforecasting/MOCA/INDEX.HTM).

In many regions of Turkey agricultural land is dedicated to wheat production. The most important region is Central Anatolia (including the Çukurova area), which accounts for nearly 48 % of total Turkish wheat production. The Aegean, Marmara and Mediterranean regions account for 41 % of wheat production, having high yields due to favorable environmental conditions (MOCA, http://agrifish.jrc.it/marsstat/crop%5Fyield%5Fforecasting/MOCA/INDEX.HTM).

Turkey took part in an agricultural modernization initiative, in conjunction with subsidized industrial development from the early 1960's to the mid 1970's, implementing the National Wheat Improvement program in 1969 (Bradsley and Thomas, 2005; MOCA). The most dramatic improvements took place in the first ten years of the program, due to new varieties and improved agronomic practices. Since then, wheat production in Turkey has nearly tripled, while land used for wheat production has only slightly increased in comparison (FAS, http://www.fas.usda.gov). Yield has more than doubled in the past 40 years, which explains the huge increase in production without a parallel increase in land use. Modern agriculture, it seems, has benefited Turkey enormously. The trends in wheat production for the past eight years can be seen in Table 1.2.

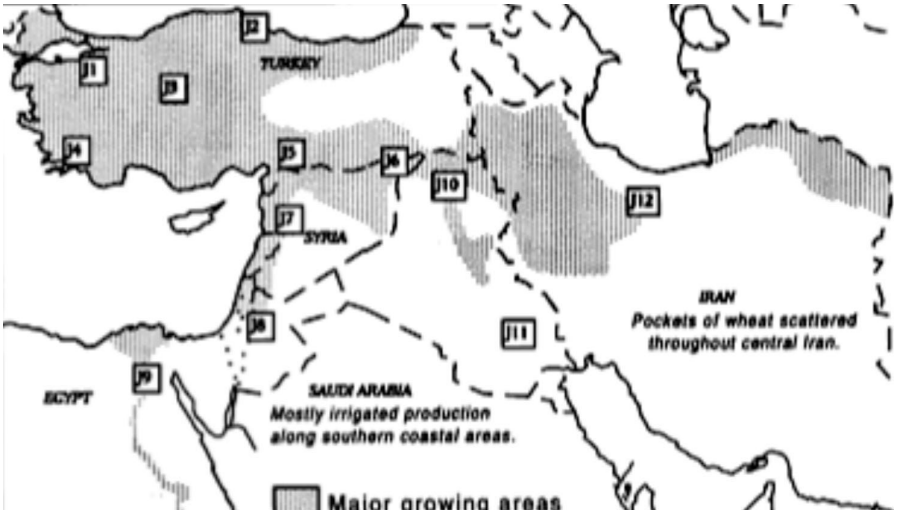


Figure 1.2. Wheat growing areas and winter wheat calendar for the Middle East (FAS, http://www.fas.usda.gov).

Table 1.2. Turkish wheat figures for past eight years (FAS, http://www.fas.usda.gov).

Year	Area	Yield	Prod.	Cons.	Imports	Exports
1997/98	8.5	1.9	16.0	16.8	1.8	1.3
1998/99	8.6	2.1	18.0	16.9	1.9	2.6
1999/00	8.7	1.9	16.5	16.8	1.5	2.2
2000/01	8.7	2.1	18.0	16.7	0.5	1.6
2001/02	8.5	1.8	15.5	16.5	1.0	0.8
2002/03	8.6	2.0	16.8	17.0	1.2	0.8
2003/04	8.6	2.0	16.8	17.0	1.1	0.8
2004/05	8.6	2.2	18.5	17.0	0.5	2.0
2005/06	8.6	2.1	18.0	17.0	0.5	2.0

Year: calendar year; Area: Area harvested (millions of hectares); Yield: tons per hectare; Prod: Production (millions of metric tons); Cons.: Consumption (millions of metric tons); Imports: (millions of metric tons); Exports: (millions of metric tons).

Modernization of agriculture has taken place predominantly in the geographically core regions of Turkey. In the periphery limited resources and techniques prevail and in these areas local landraces have remained important (Bradsley and Thomas, 2005). Most farmers prefer to continue using their traditional local landraces, which require little fertilizer and pesticide inputs compared to modern wheat varieties, which are also bred in

Turkey. From the standpoint of biodiversity, Turkey is a very important source of agro-biodiversity, as it retains more genetic diversity compared to other nations (Bradsley and Thomas, 2005).

1.1.2. Durum Wheat

The origins of durum wheat trace back to the Fertile Crescent of southwestern Asia. Cultivated tetraploid 'durum' wheat was derived from a wild 'emmer' tetraploid, a product of the hybridization of two diploid species, followed by a spontaneous doubling of the chromosomes. Cultivated durum wheat originated from *T. dicoccoides*, a wild emmer wheat, following the selection of its free-threshing character sometime around 300 B.C. (Göçmen, 2001)

Durum wheat (*Triticum turgidum* l. var *durum*) is an allo-tetraploid made up of the A and B genomes (AABB, 2n = 4X = 28). It is the main source of semolina for the production of pasta, couscous, bulgur and other Mediterranean local end products. It serves a major role in providing vitamin A in this region (Elouafi and Nachit, 2004). Pasta is the most common durum-derived product consumed in Europe and North America, while bulgur is one of the oldest cereal foods in Middle Eastern countries (e.g., Turkey, Syria, Jordan, and Lebanon) and couscous is made from durum wheat flour mostly in Morocco, Tunisia, Turkey, Algeria, and Libya (Göçmen, 2001).

Durum wheat occupies about 8 % of the total wheat-producing area in the world, and it is cultivated primarily in semiarid regions such as the Middle East and North Africa, the North American Great Plains and the Mediterranean Countries. Major production is located in the Mediterranean region covering 9 million ha. Even though the entire growing area devoted to durum wheat may appear small, the heavy concentration into localized growing regions makes it a very important crop. Over 40 % of the world durum wheat-growing area is located in the Middle East and North Africa (Göçmen, 2001).

Durum wheat is predominantly spring or semi-winter (facultative) in growth habit. Durum wheat is often grown in harsh environments with low levels of management and exposure to many pathogens and insects. Adverse climatic conditions limit yield and

undermine the quality, thereby pushing up market prices. World durum production generally fluctuates between 25 - 35 million tons per year (Göçmen, 2001).

1.1.3. Durum Wheat in Turkey

About half of the agricultural land in Turkey is devoted to wheat production and recent estimates indicate that approximately 85-90 % of the wheat area is devoted to bread wheat and 10-15 % to durum wheat. Turkey has an important place in the world as a durum wheat producer, being the leading durum wheat producer in the Near and Middle East (Göçmen, 2001; MOCA, http://agrifish.jrc.it/marsstat/crop%5Fyield%5Fforecasting/MOCA/INDEX.HTM).

Durum wheat is traditionally an important crop in Turkey. In the early 1930s, durum wheat covered 40–80 % of the total wheat growing area. After the introduction of high yielding bread wheat varieties, this pattern changed rapidly in favor of bread wheat. The effort afforded to bread wheat improvement was not seen for durum wheat until the late 1980s. The price policies applied by governments were also in favor of bread wheat until 1993. As a consequence durum wheat production has pushed into marginal areas. However, the consumption of durum wheat products is increasing and to meet this increased consumption Turkey has had to import durum wheat in recent years (Göçmen, 2001).

Durum wheat is grown primarily in 3 regions of Turkey; (1) The Central Plateau and Transitional Zones, (2) The Southeast, (3) The Coastal Regions including the Aegean, Marmara (except Thrace) and Mediterranean Regions. These three regions form approximately 50, 30 and 12 % of the total durum production area, respectively. Durum wheat has also been grown in other regions on a small scale, occupying the remaining 8 % (Göçmen, 2001).

Current production of durum wheat in Turkey depends on quite a limited number of varieties. Varieties Kunduru-1149, Çakmak-79, Kızıltan-91 and Ç-1252 are mainly grown in the highlands. The main disadvantage of these varieties is susceptibility to cold and disease (especially to rusts) and low grain quality (Göçmen, 2001).

The main constraints limiting the production of durum wheat in Turkey are: price policy, biotic and abiotic stresses. The prices paid for durum wheat in Turkey are not enough to compete with bread wheat, leading to farmers abandoning the crop. Cold and drought are major abiotic limiting factors, cold seasons harm durum wheat more than bread wheat and result in a poorly developed crop in spring. Diseases affecting durum wheat are rusts, loose smut, common bunt, septoria, barley yellow dwarf virus, some root diseases and powdery mildew. The importance of these diseases changes from region to region and from year to year (Göçmen, 2001; MOCA study).

1.1.4. Genomic Organization of Wheat

Bread wheat has a very large genome consisting of about 1.6x 10^{10} base pairs. Bread wheat is hexaploid, meaning it has three genomes (AABBDD), while durum wheat is tetraploid, having two genomes (AABB). Most of the wheat genome (95-99%) is non-transcribed, consisting mainly of transposons and duplicated pseudogenes. The remaining 1-5 % consists of genes, which are present in clusters (Dilbirliği and Gill, 2003). The genomic organization of wheat therefore consists of small gene rich regions interspersed with large gene poor regions made up of mostly repetitive DNA. It is estimated that there are between 75 000 to 150 000 genes in the hexaploid wheat genome, therefore only 1.2-2.4 % of the wheat genome is expected to contain functional genes (Erayman *et al*., 2004).

1.1.5. Diseases of Wheat

The survival of any organism essentially depends on two things: nourishment and health. Therefore, it is not surprising that apart from drought, diseases pose the biggest threat to agriculture. Disease resistance may ultimately determine the success of a crop species in agriculture.

There are many pests that plague wheat, they range from insects to bacteria, fungi and viruses. On average, these pests cause 20-37 % yield loss worldwide, translating to ~70 billion dollars a year (Dilbirliği *et al*., 2004). Global losses caused by pathogens are estimated to be 12 % of the potential crop production, despite the continued release of new resistant cultivars and pesticides. Fungi are becoming more and more resistant to

fungicides and larger numbers of resistance genes. In addition to reducing crop yield, fungal diseases often lower crop quality by producing toxins that effect human and animal health (Rommens and Kishore, 2000)

Some pathogens live off of the living plant. The term obligate biotroph characterizes a specific lifestyle in which the host as a whole suffers only minor damage over a longer period of time, as the pathogen is dependent on the living host plant to complete it's life cycle (Voegele and Mendgen, 2003). Some of the best-studied pathogenic fungal biotrophs belong to the group of obligate parasites known as the rust fungi. These belong to the division Basidiomycota, order Uredinales (Heath, 1997). Wheat rusts belong to the genus *Puccinia*, family Pucciniaceae and they are highly specialized plant pathogens, with narrow host ranges (Singh *et al*., 2002).

The rusts are considered to be the major constraint to wheat production in Turkey. There are three rust species of cereals, stem or black rust (*Puccinia graminis*), leaf or brown rust (*Puccinia triticina*) and stripe or yellow rust (*Puccinia striiformis*). Each species is further divided into *formae speciales*. *Formae species* are used to refer to the division of rusts based on the host genus that is attacks (Line, 2002). For example, in the case of yellow rust, *P. striiformis* f. sp. *hordei* attacks barley, while *P. striiformis* f. sp. *tritici* attacks wheat.

Of the three rust species occurring in Turkey, yellow rust is the most important and can cause yield losses in all parts of the country (Çetin *et al.*, 1999).

1.2. Yellow Rust

Puccinia striiformis is a pathogen of grasses and cereal crops: wheat, barley, Triticale and rye. It is thought to have originated in Transcaucasia (Georgia, Azebaijan and Armenia), where grasses were the primary host. From there the pathogen probably moved into Europe and along the mountain ranges to China and Eastern Asia (Line, 2002).

Because yellow rust has a low optimum infection temperature, it is limited as a major disease in many areas of the world. Yellow rust prefers to grow at lower temperatures than leaf or stem rust. Minimum, optimum and maximum temperatures for growth are 1,11 and 23 °C, respectively. Because of this, it is principally a disease of wheat grown in cooler climates (2° to 15° C), at higher elevations, northern latitudes, maritime regions and in cooler years (Singh *et al*., 2002; Boyd, 2005; Roelfs *et al.* 1992).

Yellow rust can cause severe losses on susceptible varieties. Historically, yellow rust caused and presently causes major losses in: East Africa, Far East and West Asia and Western Europe. Specific regions where yellow rust occurs are northern Europe, the Middle East, the east African highlands, China and the Indian sub-continent, the west coast of the USA, the Andean region of South America, Australia and New Zealand (Singh *et al*., 2002; Boyd, 2005). The world-wide distribution of yellow rust is shown in Figure 1.3.

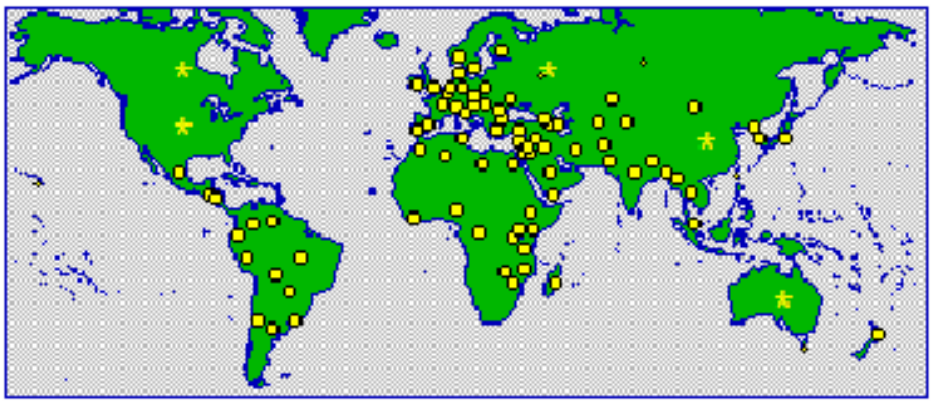


Figure 1.3. Distribution map of *P. striiformis* (CABI Crop Protection Compendium, 2000)

Yellow rust can be wind-transported over long-distances while maintaining a viable state; up to 2000 km in the case of spread from Australia to New Zealand. Yellow rust can sequentially migrate, such as virulence 8156, which originated in Turkey but was later traced to the Sub-continent of India and Pakistan (Roelfs *et al*., 1992).

Yellow rust is the only rust of wheat that consistently spreads beyond the initial infection point (Roelfs *et al*., 1992). Very low amounts of inoculum (one infected leaf per

acre) can start an epidemic (Line, 2002). Because of the disease's early onset, infected plants become stunted and weakened. Crop losses are caused by shriveled grain and damaged tillers and can be up to 50 –100 %.

Fifty-nine races of yellow rust have been detected in the western United States, pre-2000 (Line, 2002). Information on the races present in Turkey is rather limited. Races 2E16, 70E16, 6E16, 46E13, 46E15, 14E16, 70E0, 6E0, 86E16 2EO, 2E16, 6E16 and 6E150 were recorded between the years 1970 and 1991 (Göçmen, 2001).

1.2.1 Life Cycle of Yellow Rust

Yellow rust can only parasitize living host tissue, i.e. it is an obligate, biotrophic fungus. Unlike leaf and stem rust no sexual cycle has been identified for yellow rust and no alternate host is known. Yellow rust is most likely a hemiform rust, in that the life cycle only consists of the uredinial and telial stages (Singh *et al*., 2002). Figure 1.4 summarizes the life cycle of yellow rust.

1.2.2. Yellow Rust Infection Process

Asexual urediospores are the only known source of inoculum of yellow rust (Singh *et al*., 2002). The yellow rust infection process starts with a urediospore landing on the leaf surface and germinating. In other rust fungi the germ tube responds to topographical features of the leaf surface, enabling it to grow towards a stomata. In yellow rust there is no evidence for a topographical stimulus, so the signal that directs the germ tube growth towards the stomata is unknown.

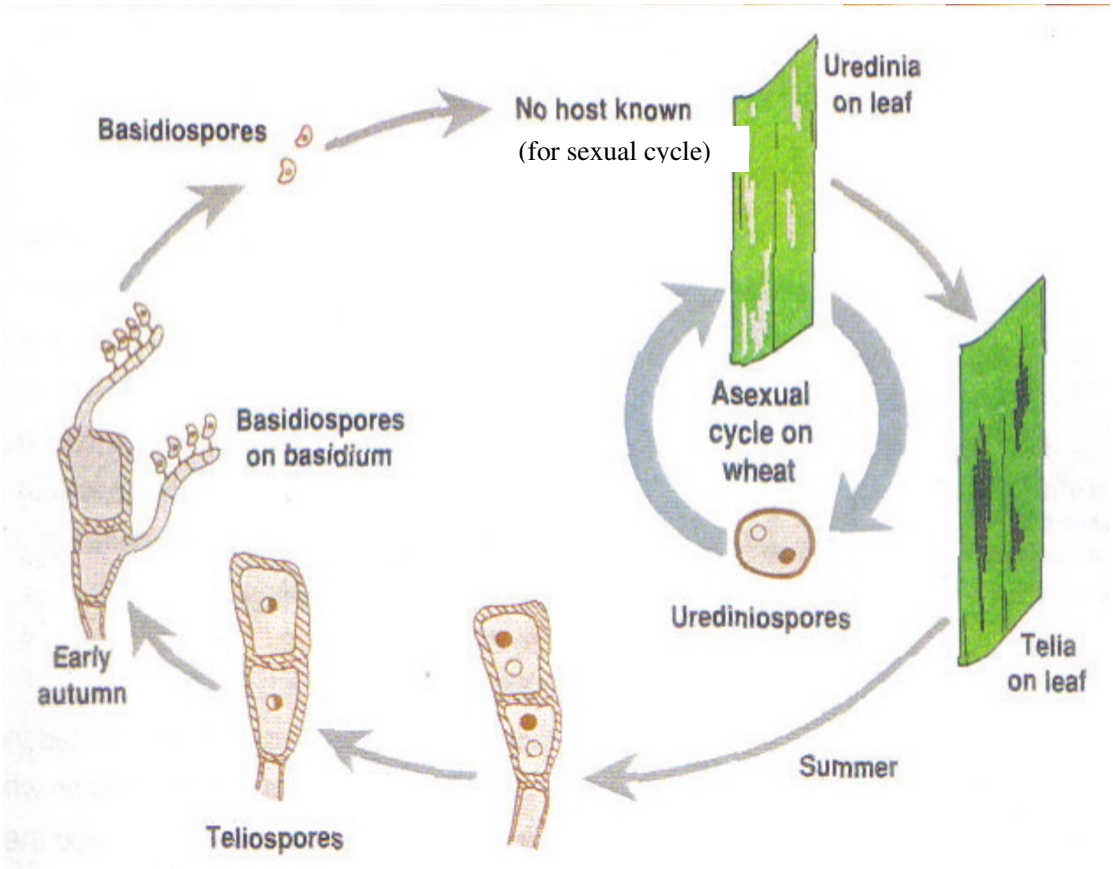


Figure 1.4. The life cycle of yellow rust (Roelfs *et al*., 1992)

The yellow rust germ tube enters the plant through the stomata. In other rusts a swelling, called an appressorium is formed over the stomatal opening, an infection peg then penetrating the stomatal opening, growing between the guard cells. However, for yellow rust a structure resembling an appressorium is rarely, if ever seen (Garrood, 2001). Eight to 12 hrs after infection a substomatal vesicle (SSV) is formed at the end of the infection peg, within the stomatal cavity. Sixteen hrs after infection this vesicle gives rise to three infection hypha that grow out from the SSV, into the mesophyll cell layer.

A haustorial mother cell (HMC) is formed by the differentiation of a septum across the tip of the infection hypha when it contacts a mesophyll cell. The HMC extends a penetration peg that crosses the plant cell wall (Heath, 1997; Mares and Cousen, 1977). A haustoria forms at the end of the penetration peg, pushing back the plasma membrane of

the living plant cell. Haustoria are thought to perform two main tasks: the regulation of the host-parasite interaction and the uptake of nutrients (Mendgen *et al.,* 2000).

Horizontal ramification of hyphae between neighboring cells allows the fungus to grow parallel to the leaf blade. Additional haustoria are formed. Hyphae begin to grow upwards, through the leaf epidermis and form uredia on the leaf surface approximately 11-14 days after infection. It is from these uredia that urediospores are released. The first macroscopic signs of infection appears six to seven days after inoculation in the form of small, yellowing circles of tissue on the leaf surface. Pustules (uredia) form 11 to 14 days after inoculation from these yellowing legions of the leaf (Mares and Cousen, 1977).

1.2.3. Yellow Rust in Turkey

Because yellow rust favors cooler and moist conditions, the wheat production areas of central and eastern parts of the Anatolian Plateau are more vulnerable to yellow rust. However, in certain years, when climatic conditions are suitable, the disease occurs in other parts of the country, such as coastal regions and the South East Anatolia.

Records indicate that a significant yellow rust epidemic occurs once or twice a decade, sometimes more often. The first record of yellow rust in Turkey was made by M. Rasim in 1886. Most of these were experienced in Central and Eastern Anatolia infections that occurred in 1936, 1940, 1950 and 1963 were of nationwide importance, while epidemics in 1975, 1976, 1977, 1984 and 1991 were of local and regional importance. Yield losses of up to 35 % occurred in Central and Eastern Anatolia in 1963 and 20-80 % losses were recorded between 1969 and 1980 (Göçmen, 2001).

There have been a number of fungicide trials in Turkey against yellow rust. Good control was achieved by combining fungicides, however there is no fungicide use against yellow rust in Turkey because the chemicals are too expensive (Bradsley and Thomas, 2005; Göçmen, 2001).

Crop failures in Turkey are mainly due to a single cultivar being grown over wide areas. When the resistance of these varieties fails, devastating losses ensue. Bradsley and

Thomas (2005) observed that numerous, widely used wheat varieties in Turkey rely on a single yellow rust resistance gene, rendering the country susceptible to wide-spread crop losses caused by mutation to virulence in the fungus. For example, the cultivar Gerek79 covers two million hectares in Turkey, it is the number one cultivar based on acreage, but has been very susceptible to yellow rust since 1990.

In 1995 the most recent, pronounced epidemic occurred in Çukurova, one of the major spring wheat growing regions of Turkey, when the resistance to *Yr9* was broken (Çetin *et al*., 1999). A loss of nearly 0.5 million tones occurred, solely due to yellow rust (Düşünceli *et al*., 1996). The virulence to *Yr9* has existed in all subsequent seasons. Virulence to *Yr6* and *Yr7* were recorded in 1998 and virulence to *Yr17* and *YrSk* was recorded in 2001. Resistances *Yr18, Yr24, Yr26, Yr1, Yr5, Yr15* and *YrSp* have remained effective in Turkey. Overall in 2001, 37 % of tested durum and bread wheat lines were resistant, 18 % were moderately susceptible and 44 % were highly susceptible (Çetin *et al*., 2001).

1.3. Disease Resistance

Plants have evolved co-linearly with the pathogens that attack them. During the course of evolution plants have defended themselves from each event of attack by the pathogen by mounting appropriate defense responses. The genes responsible for defending the plant against pathogens are termed resistance (R) genes. The pathogenesis genes that allow the pathogen to invade the plant are termed pathogenicity factors. Some of these pathogenicity factors have been adopted by the plant as pathogen recognition factors, known as effectors or virulence genes. If an effector enables the plant to recognize a normally pathogenic strain of a pathogen and mount a resistance response through a host R-protein, this virulence gene is now termed an avirulence gene (Avr).

It seems counter-intuitive that a pathogen would produce elicitors that enable the plant to recognize the pathogen. The primary function of the suites of Avr gene products produced by pathogens is to assist the pathogen in both colonizing and gaining nutrition from the host plant (Ellis *et al*., 2000b). Avr proteins originated as virulence (pathogenicity) factors that played a part in the infection process of the pathogen and

evolved to facilitate the pathogen's life cycle (Martin *et al.*, 2003). When attacking a host plant that lacks the R-gene product required to recognize the Avr gene product, the Avr gene product is now successful in it's pathogenicity role, resulting in disease. However, when attacking a host that does contain the corresponding R-gene product, the Avr gene product facilitates host recognition of the attacking pathogen, resulting in a resistant interaction.

R-genes appear to be very diverse, and we are just beginning to elucidate the various roles for these genes. So far 48 R-genes have been cloned that act against insects, nematodes, bacteria, fungi and viruses from 12 plant species (Dilbirliği and Gill, 2003, Table 1.3). Most cloned R-genes show structural conservation and can be grouped into 8 distinct classes. This classification is created based on the conserved domains and molecular characteristics of the R-genes (Hammond-Kosack and Parker, 2003).

Table 1.3. Examples of cloned R-genes in plants (modified from Dilbirliği and Gill, 2003)

Plant species	**R-gene**	**Structure**	**Plant species**	**R-gene**	**Structure**
Wheat	*CRE3*	NB/LRR	Rice	*XA21*	NB/LRR
	VRGA1	NB/LRR		*PIB*	NB/LRR
	LR10	NB/LRR		*RPR1*	NB/LRR
	LR21	NB/LRR	Maize	*RP1-D*	NB/LRR
	Pm3	NB/LRR	Arabidopsis	*RPS2*	NB/LRR
Barley	*MLA6*	NB/LRR		*RPP13*	NB/LRR
	RPG1	PK/PK		*RPM1*	NB/LRR
Tomato	*PRF*	NB/LRR	Flax	*L6*	NB/LRR
	PTO	PK		*M*	NB/LRR
	CF9	LRR		*P2*	NB/LRR
Potato	*RX*	NB/LRR	Sugarbeet	$HS1^{pro-1}$	LRR
	RGC1	NB/LRR	Tobacco	*N*	NB/LRR

	R1	NB/LRR	NB	=	Nucleotide-Binding
Lettuce	*DM3*	NB/LRR	LRR	=	Leucine Rich Repeat
Pepper	*BS2*	NB/LRR	PK	=	Protein Kinase

Evolution of R-genes is driven by selection for allelic variation caused by mutation and re-assortment, following recombination between alleles (intra allelic recombination). Recombination can also take place between different genes within a R-gene family complex. Selection favors genes that can recognize a gene product present in the pathogen population, which then, by definition becomes an avirulence gene (Hulbert *et al.,* 2001).

Plants respond to pathogens by activating local and some times systematic, broad-spectrum innate immune responses. Local responses can include a rapid, local programmed cell death called the hypersentitive response (HR), while systematic enhanced resistance to secondary infections is called systematic acquired resistance (SAR) (Maleck and Lawton, 1998). Despite the vast array of potential phytopathogens, resistance is the rule and susceptibility is the exception (Staskawicz *et al.,* 1995) due to fact that most plant species are resistant pathogens of different plant species (non-host resistance).

1.3.1. NBS-LRR Type Resistance Genes

The majority of isolated R-genes encode proteins with a nucleotide-binding site (NBS) region and a series of leucine-rich repeat residues (LRR). These NBS-LRR genes are a superfamily of R-genes present in both monocots and dicots. This gene family can be divided into two subgroups based on the content of the amino terminus: those that have homology to the Drosophila Toll protein and interleukin-1 receptor, a TIR domain and those that have a coiled-coil, CC domain, instead (Ayliffe and Lagudah, 2004).

NBS-LRR R-genes have been found that provide plant recognition of fungal, bacterial, viral and nematode pathogens in cereals. The NBS domain functions in signal transduction, while the LRR domain contributes to specificity of pathogen recognition. So

far, there has been no function other than disease resistance ascribed to this NBS-LRR gene family (Hulbert *et al*., 2001). NBS and LRR domains are present in other proteins in the plant, but their order and arrangement are unique in R-genes (Dilbirliği and Gill, 2003). Mapping studies have shown that NBS-LRR genes tend to cluster and are often linked to known disease resistance genes (Ayliffe and Lagudah, 2004).

The LRR region is made up of repeating leucine rich segments. Mutational and chimeric studies have shown that R-gene specificity can be attributed to this region. The generation of R-gene polymorphism involves gene duplication, DNA-sequence divergence caused by point mutation and by deletion and duplication of intragenic DNA repeats encoding blocks of leucine-rich elements (Ellis *et al*., 2000a).

The NBS domain is found in many proteins, including RAS proteins and ATPases. Mutational analysis indicates a critical role for the NBS domain in that it may affect R-protein function through nucleotide binding or hydrolysis (Martin *et al*., 2003). The NBS domain is composed of 8 characterized motifs, these motifs and their consensus sequences are given in Table 1.4. Three motifs: the P-loop (kinase 1a), kinase 2 and kinase 3a (Yun, 1999) are the best characterized. In addition to kinase 1a, 2 and 3a, the NBS region contains five other short motifs designated NB-ARC motifs. These motifs are of undefined function and align with APAF-1 and CED-4 cell-death regulatory proteins in animals. This suggests the possible involvement of R-proteins in cell-death pathways (Martin *et al*., 2003).

Table 1.4. Motifs present within NBS domain of NBS-LRR R-genes (Dilbirliği and Gill, 2003).

Motif	**Consensus sequence**	**Motif**	**Consensus sequence**
Kinase-1a	GGLGKTTL	RNBS-D	CFLYCSIFP
Kinase-2	LIVLDDVW	WIAEGF	WVAEGF
Kinase-3a	GSKIIVTTR	MHD	MHD
GLPL	GLPLAI	LRVLDL	LRVLDL

The N terminus of NBS-LRR genes can be composed of either a TIR domain or a CC domain. The TIR domain is only present in dicot R-genes. The absence of the TIR sequence in cereal genomes suggests that the TIR domain has been 'lost' from cereals (Pan *et al*., 2000). Despite extensive synteny in cereals, map locations of NBS-LRR analogues are present in non-syntenic positions in genomes of barley, rice and foxtail millet (Leister *et al.,* 1998).

1.3.2. Molecular Models Explaining Disease Resistance

There are two basic hypotheses that describe the interaction between host R-genes and pathogen avirulence genes: The gene-for-gene hypothesis and the guard hypothesis (summarized in Figure 1.5).

Flor (1949) first defined the gene-for-gene hypothesis from his studies on flax and flax-rust. He observed that resistance was not only defined by the expression of a R-gene in flax, but instead was due to the interaction between the R-gene in flax and the Avr gene in the rust. This theory states that for a plant to be resistant to a pathogen, for each R-gene in the host there must be a specific Avr gene in the pathogen. The gene-for-gene hypothesis was based on the assumption that there is a direct interaction between the R-protein and the Avr protein, however experimental evidence of this interaction is limited. There is some evidence of direct interaction, an example being the binding of the rice R-gene *Pi-ta* to the *Magnaporthe grisea* avirulence gene *Avr-Pita* (Martin *et al*., 2003). Physical interaction between R protein and avirulence effector has also been demonstrated for *Pto* with *AvrPto* and *RPS2* with *AvrRpt2* and *AvrB* (Martin *et al*., 2003).

The second concept is termed 'the guard hypothesis'. This hypothesis was formulated because of the lack of interaction seen between R and Avr proteins. This hypothesis suggests either indirect recognition of Avr products by R-proteins or that R proteins recognize Avr product/host complexes (Ayliffe and Lagudah, 2004). R-gene products may provide a surveillance mechanism by either recognizing host proteins interacting with Avr gene products or by recognizing host proteins which have been modified by the Avr product. In both cases the R-gene products trigger resistance (Ellis *et al*., 2000b). This model predicts that R-proteins activate resistance when they interact with

a ‘guardee’ host protein that has been modified by the pathogen in an attempt to attack the plant. This model suggests that R-genes actively monitor key physiological processes rather then idly waiting for signals from an invader (McDowell and Woffenden, 2003).

Most R-genes, whether through direct or indirect interaction with effector molecules are believed to function by regulating a signal transduction pathway leading to the activation of defense responses. The components of this pathway are still being elucidated, however recent findings indicate roles for: ubiquitination, MAP (Mitogen Activated Kinase) kinase cascades, glycosylation, ROS (Reactive Oxygen Species) production and NO (Nitrogen Oxide) production (Martin *et al*., 2003).

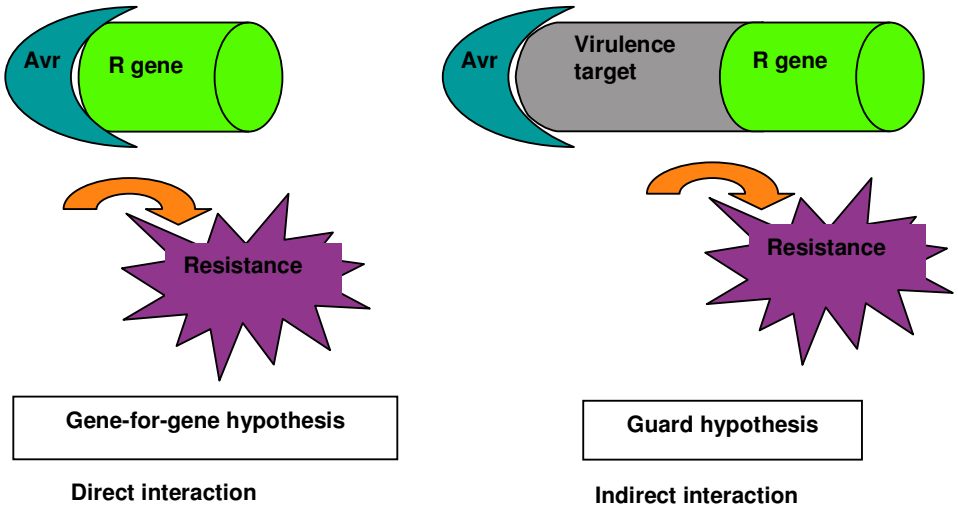


Figure 1.5. The two main hypotheses describing the interaction between host resistance genes and pathogen avirulence genes. The gene-for-gene and guard hypotheses differ in the type of interactions leading to resistance.

1.3.3. Race-Specific Resistance

The ability of an Avr gene to mutate to a virulent form, no longer recognizable by the corresponding R-gene, implies a type of resistance termed race-specific resistance. A cultivar carrying certain R-genes will be resistant against pathogen isolates carrying the

corresponding Avr gene, i.e. the cultivar carries race-specific resistance to that particular race of the pathogen. Race-specific resistances are mostly expressed from the seedling growth stage onward and are thus also known as seedling resistance. This type of resistance is usually defined by major genes that show up in a population against a specific race of pathogen.

When resistance fails in the short term, a phenomenon termed 'the boom and bust cycle' is seen (Figure 1.6). This cycle comes about in the following manner: a single R-gene is widely distributed over a large agricultural area, this is called the "boom". The pathogen evolves to adapt to the presence of this major resistance and a new population of pathogen that can overcome this resistance if formed, this is the "bust". In these cases, the 'breakdown' of the resistance was due to the pressure exerted by the major resistance, driving the pathogen to select for mutants, recombinants or immigrants that were better adapted (McDonald and Linde, 2002).

One of the hallmarks of gene-for-gene race-specific resistance is the commonly short-lived agronomic usefulness of these R-genes, as virulent pathogens can evolve by either loss or alteration of avirulence gene products (Ayliffe and Lagudah, 2004).

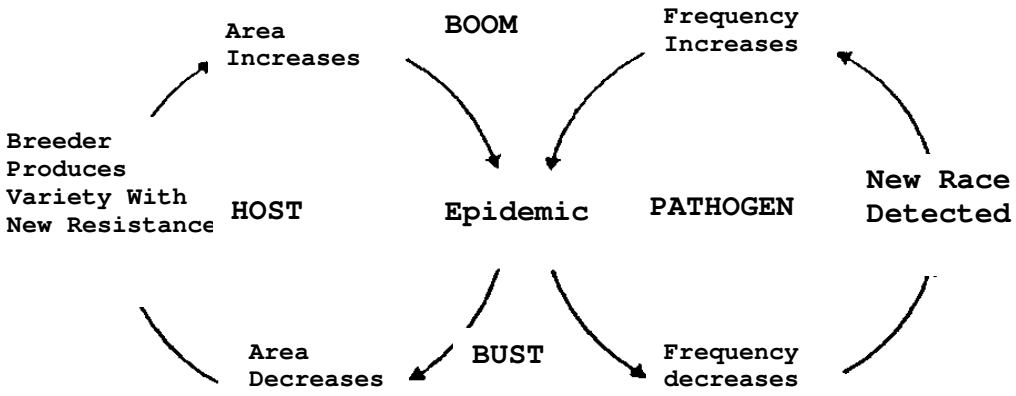


Figure 1.6. The boom-and-bust cycle seen following the deployment of major, race-specific R-genes in new crop varieties (Hertz, UK).

1.3.4. Non Race Specific Resistance

Complete disease resistance is generally manifested through the action of many genes, each with a minor, additive effect. Some non race specific resistances do however confer major effects that fit Mendelian distributions, e.g. *mlo* gene.

Quantitative resistance

Quantitative resistance refers to resistance which exhibits a continuous phenotype through low penetrance, partial resistance or a large environmental effect. Resistance to pathogens can be due to the action of preformed chemical or physical barriers, induced hydrolytic enzymes or other chemicals such as phytoalexins. The resistance conveyed in this manner is often termed "quantitative". Quantitative resistance, while often conveying a partial phenotype tends to be effective against all strains of a pathogen population (McDonald and Linde, 2002). Quantitative resistance is often characterized by a reduced rate of epidemic development (Singh and Rajaram, 2002). With rust diseases this slow disease progression is referred to as slow-rusting.

Slow-rusting resistance leads to a retarded rate of disease progression resulting in an intermediate to low resistance levels against all races of the pathogen. The components that characterize slow rusting are: longer latent period, low receptivity or infection frequency, reduced uredial size and reduced duration and quantity of spore production (Singh and Rajaram, 2002). Complete quantitative resistance can be obtained by accumulating minor, partial resistance genes into one genetic background (Bartos *et al*., 2002).

Adult Plant Resistance

Cultivars showing quantitative resistance will probably show susceptibility at seedling growth stages, becoming more resistant as the plant matures. This phenotype is referred to as adult plant resistance (APR; Broers and Lopez-Atilano, 1996). While there is no optimal growth stage for resistance expression, race-specific resistance tends to be expressed throughout the whole life of the plant, while partial, quantitative resistance tends to be expressed at some time after GS 12-13 (two leaf seedlings), i.e. APRs. APR genes

can however show a major resistant phenotype (Borner *et al*., 2000) and be race-specific, but generally are partial in phenotype and often have remained effective for long periods.

Durable Resistance

A resistance is termed "durable" if the cultivar that carries the resistance has remained resistant despite being grown widely and for a number of years (Johnson, 1981, 1988). The terms non-specific, generalized, field and horizontal resistance all describe features generally considered to relate to durability (Law *et al*., 1978). Durable resistance can either be complete or partial. However, the term durable does not imply that the cultivar will remain resistant indefinitely. It could mean that the mutation needed to convert the Avr gene to a virulent form (required to overcome a race-specific R-gene) has a significant cost in terms of fitness or pathogenicity and may be detrimental to the pathogen's infection ability (Boyd, 2005).

Cultivars may be durable because they carry many different minor genes that have an additive effect, creating a long-lasting resistance, which is hard for the pathogen to overcome. For example, durable resistance can be created by combining slow-rusting resistance genes, which results in reduced epidemics. Durable resistance may also be due to genes that alter the host's physical properties, thus rendering the pathogen unable to penetrate and infect the plant, which may be related to another type of non race specific resistance, termed non-host resistance. On average, race-specific resistance lasts 3 to 4 years against yellow rust, while durable resistance can last decades.

Non-Host Resistance

Non-host resistance is defined as the ability of most genotypes of a plant species to resist infection by most isolates of a potential pathogen (Heath, 1997). In non-host resistance, the lack of pathogenicity is thought to be due to the absence of compatibility between pathogen and plant, the pathogen lacking the required pathogenicity factors to overcome the plant's basic defenses, and/or the plant does not provide the environment required by the pathogen for successful infection (Rodrigues *et al*.,2004).

Non-host resistance is thought to be genetically very complex and believed to be based on additive effects of minor genes (Singh and Rajaram, 2002). Non-host resistance genes may be more durable if they recognize general or genera-specific elicitors, or e.g. if the resistance genes have an effect that is independent of pathogen recognition, having an effect on plant physiology that prevents pathogen colonization (Hulbert *et al*., 2001).

1.3.5. Resistance to Yellow Rust

In 1905, Biffen was the first to confirm Mendelian inheritance of disease resistance by showing that the resistance to yellow rust in a segregating population of wheat was recessive and monogenic. Seemingly an important study of disease resistance at the time, henceforth less work has been carried out on resistance to yellow rust than the other rust diseases of wheat.

Most of the identified yellow rust resistance genes have proven to be race-specific (Boyd, 2005). Wheat yellow rust race-specific resistance genes characterized thus far are listed in Table 1.5.

Yellow rust resistance expression can be sensitive to environmental conditions such as temperature, light and humidity. An example of the temperature sensitive nature of yellow rust resistance is High Temperature Adult Plant Resistance (HTAP). This is a durable resistance and 90 % of the cultivars grown in the western U.S. have HTAP (Line, 2002). As plants mature they become more resistant when grown at high temperatures, however remain susceptible at lower temperatures (Milus and Line, 1986). A number of adult plant expressed, and often partial yellow rust R-genes have also been characterized (Table 1.6).

Mechanisms of yellow rust developmental arrest

Theoretically, resistance to rusts can act at any stage of fungal development. The low infection rate of yellow rust on quantitatively resistant wheat cultivars can be attributed in part to a mechanism that prevents the recognition of stomata by germ tubes and the subsequent formation of infection sites, either because of the failure to find the stomata or failure to form infection structures. It is possible that disintegration of sub-stomatal

vesicles and delayed development of infection hyphea may also play a role in prevention of disease establishment (Broers and Lopez-Atilano, 1996)

Mares and Cousen (1977) noted that yellow rust resistant interactions could involve cessation of fungal growth at some infection sites prior to, or just following the production of the first haustoria, reduced rates of rust hyphal growth, hypersensitive host cell chlorosis and/or necrosis and reduced sporulation.

Table 1.5. Yellow rust race-specific resistance genes and their chromosomal locations (modified from Catalogue of Gene Symbols for Wheat-Mac gene 2004 and Boyd 2005).

Yellow Rust Resistance Gene	**Chromosomal Location**	**Yellow Rust Resistance Gene**	**Chromosomal Location**
Yr1	2AL	*Yr37*	2DL
Yr2	7B	*YrA*	-
Yr3	5B	*YrCle*	4B
Yr4	3B	*YrCv*	2BS
Yr5	2BL	*YrDa1*	1A
Yr6	7BS	*YrDa2*	5D
Yr7	2BL	*YrD*	6A
Yr8	2A/2M and 2D/2M	*YrDru*	5B/6B
Yr9	1BL	*YrDru2*	6A
Yr10	1BS	*YrHVII*	4A
Yr15	1BS	*YrH46*	6A
Yr17	2AS-6M	*YrH52*	1BS
Yr19	5B	*YrMin*	4A
Yr20	6D	*YrMor*	4B
Yr21	1B	*YrND*	4A
Yr22	4D	*YrS*	3B
Yr23	6D	*YrSp*	2BS
Yr24	1BS	*YrSte*	2B

Yr25	1D	*YrSte2*	3B
Yr26	1BS	*YrTye*	6D
Yr27	2BS	*YrTr1*	6D
Yr28	4DS	*YrTr2*	3A
Yr31	2BS	*YrYam*	4B
Yr32	2AL	*YrYam.*	4B
Yr33	7DL	*YrV23*	2B
Yr35	6BS		

Table 1.6. Yellow rust adult plant resistance genes and chromosomal locations (modified from Catalogue of Gene Symbols for Wheat-Mac gene 2004; Boyd 2005 and Cereals Disease Lab)

Yellow Rust Resistance Gene	**Chromosomal Location**	**Yellow Rust Resistance Gene**	**Chromosomal Location**
Yr11	Unknown	*Yr30*	3BS
Yr12	Unknown	*Yr34*	5AL
Yr13	Unknown	*Yr36*	6BS
Yr14	Unknown	*Yrns-B1*	3BS
Yr16	2D	*YrKat*	-
Yr18	7DS	*YrCk*	2DS
Yr29	1BL		

1.3.6. Breeding for Disease Resistance

Plant breeding has been very successful in improving both quantitative and qualitative traits that affect agronomic performance and market requirements (Martin, 1998). The long-term success of breeding for disease resistance is influenced by the following factors: the infection process and rate of mutation of the pathogen and diversity of virulence in the pathogen population; availability, diversity and type of genetic resistance; the effectiveness of the methodology used to screen the population for

resistance genes and the selection of environment in which the resistance will be assessed (due to the environmental variability of resistance expression) (Singh and Rajaram, 2002).

R-gene mediated resistance has several advantages, eliciting defense responses that stop pathogen progress with minimal damage to the plant and gives a complete, resistant phenotype, requiring no input of pesticides from the farmer (McDowell and Woffenden, 2003). The limited durability of single R-gene however, makes it necessary to continue to discover and introgress new R-genes. Strategies developed to overcome the lack of durability seen with the application of single R-genes includes pyramiding of R-genes within a single cultivar. However, evolution of rust pathogens through recombination, migration and mutation is frequent and would eventually create virulence gene pyramids that match the R-gene pyramids (Singh and Rajaram, 2002).

Another approach is to form R-gene polycultures (varietal mixing) that are heterogeneous for pathogen recognition, but homogeneous for commercial traits (Jones, 2001). However, these methods have not been used extensively in breeding programs due to the amount of time needed (15-20 years) to breed assortments of R-genes into elite cultivars and the chance that R-genes may be linked to undesirable traits (McDowell and Woffenden, 2003; Rommens and Kishore, 2000).

Many breeders now avoid the use of seedling R-genes, but aim instead to breed cultivars with good field resistance (Boyd, 2005) due mainly to quantitative resistance genes. McDonald and Linde (2002) proposed a model whereby they demonstrated how a pathogen would overcome quantitative resistance. They saw that unlike the boom-and-bust breakdown of major gene resistance, quantitative resistance would gradually ‘erode’. This model is due to the effect of the environment on quantitative characteristics and characterized by a gradual increase in pathogenicity of the pathogen to a host with quantitative resistance through an increase in avirulence alleles in the population (McDonald and Linde, 2002).

Traditional breeding strategies favor deploying cultivars with a single R-gene at a time, in the form of a monoculture, thereby placing an enormous selection pressure on the pathogen and for mutation of Avr genes. If the pathogen can afford to alter or discard the

single Avr gene corresponding to the deployed R-gene with little or no fitness penalty then the R-gene is rapidly rendered ineffective (McDowell and Woffenden, 2003). Therefore, in using major genes for formation of resistant cultivars, it is favorable to select resistances that have a high fitness cost for the pathogen. Although there have been many technological advancements that have helped facilitate breeding, resistant varieties have never completely surplanted the use of fungicides.

Environmental effects on expression of quantitative resistance make selection and pyramiding of R-genes and quantitative trait loci (QTLs) for rust resistance difficult, but pyramiding can be greatly aided by the use of physical markers closely linked to the gene/QTL. These markers can be readily identified using a rapid, high-through-put screening procedure as part of the breeding program, an approach referred to as Marker Assisted Selection (MAS).

1.4. Molecular Marker Techniques

1.4.1. Molecular Markers

Differences in DNA sequence among individual plant genotypes are often associated with specific genes and can function as molecular markers for these genes (Hoisington *et al*., 2002). Molecular markers have been successfully used to identify and locate both genes of major and minor (QTLs) effect. Markers can be used to dissect polygenic traits onto their Mendelian components or QTLs, thus increasing the understanding of the inheritance and gene action.

Two DNA-based, molecular marker systems were used in this study, Simple Sequence Repeats (SSRs) or Microsatellites and NBS-profiling.

1.4.2. SSRs

SSRs consist of repeats of small units, consisting of di-,tri- or tetra-nucleotides and show high levels of polymorphism between genotypes. SSR markers detect changes in the length of these repeats using specific primers. The high level of polymorphism, combined

with a high interspersion rate, makes SSRs a good source of genetic markers. They are also highly informative and chromosome-specific in many species (Elouafi and Nachit, 2004).

Plants carry SSRs at relatively lower frequencies then mammals. Even so, they are ubiquitous, dispersed throughout the genome and have the potential to provide extremely polymorphic, co-dominant marker systems in plants (Bryan *et al.,* 1997). They are also advantageous in that they only require small amounts of DNA for analysis. These markers are useful for QTL mapping, MAS, qualitative gene tagging and are very reliable and repeatable (Hoisington *et al*., 2002).

1.4.3. NBS-profiling

R-genes appear to be conserved among monocots and dicots (van der Linden *et al*., 2004). The identification of conserved domains in NBS-LRR type R-genes has enabled markers systems to be developed that allow the specific tagging of these genes (van der Linden *et al*., 2004). The NBS-profiling procedure looks for polymorphisms between restriction enzyme cut sites associated with NBS conserved motifs within the genome. It is a PCR based approach that efficiently targets R-genes and R-gene analogs (RGAs), while at the same time identifying polymorphism between genotypes associated with the NBS sites within the genome (van der Linden *et al*., 2004).

Genomic DNA is digested with a restriction enzyme and adapters ligated to each end of the cut DNA. An NBS-specific primer is used to amplify towards an adapter-specific primer (Figure 1.7) which generates a reproducible, polymorphic multilocus marker profile, separated on a sequencing gel, that is highly enriched for R-genes and RGAs (van der Linden *et al*., 2004).

NBS-profiling can be used to produce markers tightly linked to R-genes and R-gene clusters for genomic mapping and positional cloning and to mine for new alleles and new sources of disease resistance (van der Linden *et al*., 2004)

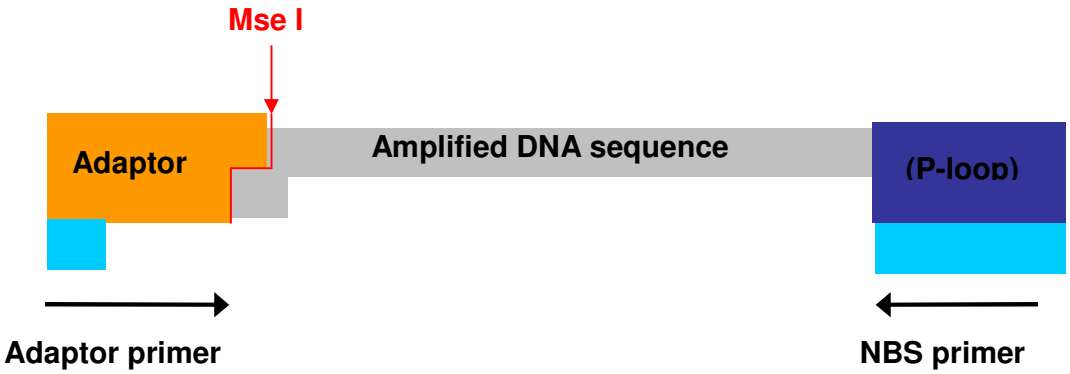


Figure 1.7. NBS-profiling technique targets DNA sequences between conserved NBS motifs and restriction enzyme cut sites (*Mse* I in this case).

1.4.4. Marker-Assisted Selection

Conventional breeding uses phenotype to assess whether a cultivar contains a desired resistance gene. However, because of the complex genetics of useful resistances such as quantitative, partial, APR these phenotypes can be variable and misleading. Molecular markers have proven to be very useful tools in plant breeding by serving as "flags" to mark the presence/absence of specific resistance genes/QTLs.

There are certain criteria that a molecular marker must meet in order to be eligible for MAS. The marker must be suitable for high-throughput screening at a low cost, be closely linked to the gene/QTL controlling the trait of interest and be polymorphic for a large number of potential parent lines that are likely to be used in the breeding program (Boyd, 2005). Peng *et al.*, (2000) found that a single SSR marker was sufficient for effective MAS where genotypes were homozygous for the resistance gene and the map distance was <5.0 cM. SSRs have been used to transfer the *T. turgidum* derived yellow rust resistance, *Yr26* into Turkish wheat genotypes (Yıldırım *et al.,* 2004).

Immediate breakdown of resistance and the failure of some cultivars in the field have in some instances been due to the disease screening protocol being inadequate to identify and select resistant wheat lines (Singh *et al*., 2002). MAS offers many possibilities for the intensification of breeding, providing an alternative means for the selection of important genes in breeding programs in the absence of pathogens (Bariana *et al*., 2001), particularly

in cases where field tests take too long and are expensive. MAS programs have been estimated to reduce the time-to-market by 50-70 % (Rommens and Kishore, 2000). MAS offers an opportunity to select for important traits such as resistance or yield that are major and/or quantitative (due to low penetrance of the pathogen, minor phenotypic effects or environmental variability) enabling these traits to be pyramided into modern cultivars.

2. PURPOSE

A F_6 durum wheat population, derived from the cross Kunduru-1149 X Cham-1 was provided by Prof. Dr. Zeki Kaya (Department of Biological Sciences, Middle East Technical University) and Dr. Belgin Göçmen (Present addres: Department of Biology, Muğla University, Muğla) to allow the molecular analysis of yellow rust resistance in this population. The field resistance in this population had been previously characterized by Göçmen *et al*. (2003), but the molecular basis of the observed resistance and the seedling resistance to yellow rust had not been characterized. NBS-profiling was seen as a potentially powerful tool for molecular resistance characterization of resistance, and was subsequently used to analyze yellow rust resistance in this durum wheat population.

The objective of this study was to characterize seedling yellow rust resistance in the F_6 population and along with the previously characterized adult plant resistance in this population identifying one or multiple QTLs to explain the observed resistances using SSR markers to identify chromosomal location and NBS-profiling to target NBS-type resistance.

3. MATERIALS AND METHODS

3.1 The Wheat Population

The durum wheat population used in this study was an F_6 RIL population developed by ICARDA (Göçmen *et al*., 2003). The population was developed from a cross between the yellow rust resistant durum wheat Cham-1 and the yellow rust susceptible wheat Kunduru-1149. The attributes of the parents, development of the lines and a description of the field resistance to yellow rust segregating in this population can be found in Göçmen *et al*., (2003). This population and the parents were kindly provided by Prof. Dr. Zeki Kaya and Dr. Belgin Göçmen.

3.2. Characterization of Seedling Yellow Rust Resistance

3.2.1. Sowing of Plant Material

Eight seed of each of the 150 lines were sown onto filter paper (Sartorius; 90mm) disks, pre-wet with tap water, in Petri dishes (Sterilin, 50 mm). The seed were allowed to germinate in the dark at 25°C. Three days after sowing, about five germinated seed of each line were transferred into John Innes No2 compost in 7x7 cm pots. The plants were grown in a spore-free greenhouse, with a 16/8 hour photoperiod cycle supplemented with sodium lighting at temperatures of 18° C during the day and 15°C during the night (Boyd and Minchin, 2001). The same sowing procedure was applied to sets of the International and European wheat differentials and a set of near isogenic lines (NILs), which were used to verify the virulence profile of the *Puccinia striiformis* f. sp. *tritici* isolate tested (Table 3.1).

Table 3.1. List of differentials and NILs used in this study and R-genes they contain. + signs next to R-genes indicates there are unidentified resistances in those lines.

Source	Differential	R-genes	Source	Differential	R-genes
International	Chinese 166	*Yr1*	European	Heines Peko	*Yr2, Yr6*
	Lee	*Yr7*		Nord Desprez	*Yr3*

	Heines Kolben	*Yr2, Yr6*		Compair	*Yr8*
	Vilmorin 23	*Yr3*		Carstens V	*YrCV*
	Moro	*Yr10*		Spalding Prolific	*YrSp*
	Clement	*Yr9*		Heines VII	*Yr6+*
	Strubes Dickkopf	*YrSd*	NILs	Avocet S* 3/*Yr 17*	*Yr17*
	Suwon92/ Omar	*YrSu*		Avocet S* 6/*Yr 8*	*Yr8*
	T. spelta	*Yr5*		Avocet S* 6/*Yr 15*	*Yr15*
	Kavkas	*Yr9*		Avocet S* 6/*Yr 5*	*Yr5*
	VPMI	*Yr17*		Avocet S* 6/*Yr 10*	*Yr10*
	Kalyasona	*Yr2*		Avocet S* 6/*Yr 7*	*Yr7*
European	Hybrid 46	*Yr4*		Avocet S* 6/*Yr 9*	*Yr9*
	Reichersberg 42	*Yr7+*		Avocet S* 6/*Yr1*	*Yr1*

3.2.2. Yellow Rust Inoculation

When the seedlings were 14 days old they were inoculated with the *P. striiformis* isolate WYR 85-22 ($6E0A^+$) containing virulences for *Yr2, Yr6, Yr7, Yr9* and *YrA*. The isolate was obtained from the John Innes Center isolate collection. Urediospores of the isolate had been stored over liquid nitrogen, so to break dormancy the urediospores were heated to 42°C for 5 minutes. The urediospores were then mixed with an equal volume of talcum powder. The plants were sprayed with a fine mist of ddH_2O containing a few drops of Tween 20 (Sigma) as a wetting agent. After this, the urediospore and talcum powder mixture was air blown (using a puffer device) onto the plant material. The inoculated plants were placed in an incubator room at 10°C and 95 % humidity for 24 hours in

darkness, conditions which optimize urediospore germination. After the 24-hour period the plants were returned to the spore-free greenhouse (Boyd and Minchin, 2001).

3.2.3. Yellow Rust Disease Scoring

Fourteen days after inoculation the plants were scored for yellow rust infection. The University of Sydney, Plant Breeding Institute, Cobbity (PBI) infection type scale was used (McIntosh *et al*., 1995, Rodrigues *et al*., 2004; Table 3.2). Scores were entered into an excel spreadsheet.

Table 3.2. Scoring scale used in this study (McIntosh *et. al*., 1995, Rodrigues *et al*., 2004).

Infection Type	Host Response	Symptoms
0	Immune	No visible symptoms (no uredia/necrosis/chlorosis)
;	Very resistant	Necrotic flecks
;n	Resistant	Necrotic areas greater than 1mm in diameter
0^{n}	Resistant	Necrotic areas greater than 2 mm in diameter
0^{nn}	Resistant	Spreading necrotic regions greater than 4 mm in diameter
1	Resistant	Necrotic and chlorotic areas with restricted sporulation
2	Moderately resistant	Moderate sporulation with necrosis and chlorosis
3	Moderately susceptible	Sporulation with chlorosis
4	Susceptible	Abundant sporulation without chlorosis
n	-	Necrotic tissue
c	-	Chlorotic tissue

3.2.4. Photography of Infected Leaves

Leaves were cut off of individuals that showed representative yellow rust infection phenotypes. Detached leaves were mounted using double sided tape onto rectangular metal frames, approximately 5 cm wide. After mounting the leaf segments the metal frames were placed in a plastic box lined with H_2O moistened filter papers to maintain humidity in the box. The box was placed at 15°C overnight to allow the yellow rust pustules to resporulate, giving a good infection phenotype for photography. Photographs were taken by the John Innes Centre photography department.

3.2.5. Formation of the Wheat Subpopulation

The 150 lines were categorized based on their reaction to *P.striiformis* isolate WYR85-22. This produced four phenotypic categories. A subpopulation of 53 lines was randomly selected which represented the four categories and maintained the same ratio of lines within each category. Lines that did not give a clear phenotype or were still segregating for yellow rust resistance were excluded, otherwise the 53 lines were selected at random.

3.3. Marker Analysis of Yellow Rust Resistance

3.3.1. DNA Isolation

DNA was isolated from fresh leaf tissue of 14 day-old, uninfected seedlings using a modified protocol that combined the protocols for DNeasy 96 Plant Kit and the DNeasy Plant Mini kit (both Qiagen). All reagents used were supplied with the Qiagen kits. The DNeasy 96 Plant kit protocol for isolation of DNA from fresh plant leaves using the Mixer Mill MM 300 was used to homogenize the leaf tissue using the following protocol: ~50 mg of fresh leaf tissue from the 55 lines (53 lines plus 2 parents) were placed into microtube racks with tungsten carbide beads. 400 µl of Buffer AP1 (preheated to 65 °C) and 100 µg RNase A were added to each sample. Samples were disrupted by shaking at 30 Hz. for 1 min using the Mixer Mill 300, then centrifuged (SIGMA 4-15 C) briefly at 3000 rpm to collect plant material off the caps of the microtube racks. Buffer AP2 (130 µl) was added

to each sample and centrifuged briefly at 3000 rpm. Samples were incubated for 10 min at –20 °C then centrifuged for 5 min at 6000 rpm. The protocol for the DNeasy 96 Plant kit was followed up until this step, but from this step onward the DNeasy Plant Mini kit was used.

The supernatant lysate (450ul) from samples were applied to QIAshredder spin columns from the DNeasy Plant Mini Kit and centrifuged (ALC 4214) for 2 min at maximum speed (14 000 rpm). The supernatant of the flow-through was mixed with 1.5 volumes of Buffer AP3/E and mixed by pippetting. This mixture was applied onto a DNeasy Mini column and centrifuged for 1 min at 14 000 rpm. The column was washed twice with Buffer AW (500 µl) by centrifugation for 1 min initially, then 2 min at 14 000 rpm. DNA was eluted into a 1.5 ml eppendorf tube (Starlab) by application of Buffer AE (100 µl, preheated to 65 °C) onto the washed DNeasy column.

The concentration of the isolated DNA was visually estimated on a 1% agarose gel. The gel was prepared by melting 1 g agarose in 100 ml 1X TAE buffer (40 mM Tris, 1 mM EDTA, 20 mM acetic acid) using a microwave oven. Ethidium bromide (1 mg/ml final concentration) was added to the agarose mix just before it was poured. Two µl of each DNA sample was mixed 1/6 (v/v) with 6X loading dye (250 mg bromophenol blue, 550 mg xylene cyanol in 33 ml 150 M Tris (pH 7.6), 60 ml glycerol and 7 ml H_2O) and then loaded into the agarose gel. The gel was run at 100 V until the bromophenol blue and xylene cyanol bands were separated sufficiently (approximately 30 min- 1hour). An Invitrogen 1 kb ladder (1 µg/µl) was used as a size marker. Bands were visualized by phosphoimaging (Molecular Dynamics, Typhoon 8600). Due to the high variability of total DNA concentration from the samples, an average concentration of 80 ng/µl was assumed for all samples for ease of experimentation.

3.3.2. NBS-Profiling

The NBS-profiling technique (van der Linden *et al.*, 2004) was modified to create the GEDIFLUX protocol (Simon Orford, personal communication). This modified NBS-profiling protocol was followed in this study. All PCR reactions contained Roche Taq polymerase, Roche 10X buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3),

Promega dNTPs (original stock 100mM each, prepared 2 mM dNTP mix as stock) and amplifications were performed on a PTC-200 Thermocycler (MJ research, Waltham, Mass., USA) unless otherwise indicated. All primers and adaptor strands were synthesized by SIGMA.

Genomic DNA was digested with the restriction enzyme *Mse*I, and in the same reaction an adapter was ligated to the cut site. Prior to the start of the digestion/ ligation reaction the adapters were prepared as follows: 1.25 nmols of upper and 1.25 nmols of lower strand were mixed and sterile ddH_2O was added to give a final concentration of 0.125 µM of upper and lower adapters. This mixture was placed in a water bath at 95°C and incubated for 3 min. The mixture was then allowed to gradually cool to room temperature in the water bath to ensure full annealing of upper to lower strands (Figure 3.1).

Upper strand 5’- ACTCGATTCTCAACCCGAAAGTATAGATCCCA -3’
Lower strand NH_2-3’-TTCATATCTAGGGTAT -5’-P

Figure 3.1. NBS-profiling MseI adaptor

The digestion/ligation reaction was carried out in a 30µl reaction volume containing; ~320 ng of template genomic DNA, 1X RL buffer (10 mM Tris.HAC pH 7.5, 10 mM MgAc, 50 mM KAc, 5 mM DTT and 50 ng/µl BSA), 15 pmoles adaptor, 1 mM ATP (Invitrogen), 5 U MseI (New England Biolabs), 0.5 U T4 DNA ligase (Invitrogen). The reaction was incubated at 37°C for 3 hours, followed by inactivation of the ligase and restriction enzyme by incubation at 65°C for 15 min. The digestion/ligation reaction was diluted 2X and stored at 4°C, or -20°C for long-term storage.

Amplification of the NBS-specific fragments involves a two-step procedure. The first PCR reaction was carried out in a 25 µl reaction volume: 1X PCR reaction buffer, 0.2 mM dNTPs, 20 nmoles domain specific primers (NBS2, NBS3 or NBS5), 20 nmoles adapter (pigtail) primer and 0.40 U HotStar Taq (Qiagen) were added to 5 µl of diluted

digestion/ligation reaction. PCR conditions were 95°C for 15 min, followed by 30 cycles of 95°C for 30 sec, X°C for 1min 40sec and 72°C for 2min, and a final elongation step of 72°C for 20 min. X°C represents the annealing temperature, which differed for each domain specific primer (Table 3.3).

Table 3.3. Primers used, their sequences and annealing temperatures. Primer sequences were based on alignments of conserved motifs of R-genes (van der Linden *et al*., 2004).

Primer Type	**Sequence**	**Annealing Temp**
Domain specific primer NBS2	5'-GTWGTYTTICCYRAICCISSCAT-3'	60°C
Domain specific primer NBS3	5'-GTWGTYTTICCYRAICCISSCATICC-3'	60°C
Domain specific primer NBS5	5'-YYTKRTHGTMITKGATGAYGTTTGG-3'	55°C
Adaptor (pigtail) primer	5'-GTTTACTCGATTCTCAACCCGAAAG-3'	-

Fourteen µl of the 1st round PCR product were run on a 1% agarose gel to observe uniformity of the PCR product, which should give a smear ranging from about 0-500 bps. The agarose gel was run exactly as described before except that 2 µl of 6 X loading dye was added to 14 µl of 1st round PCR product. Fifty-four µl of sterile ddH_2O were added to the remaining PCR product to give a ~10 X dilution.

The second round PCR reaction was carried out in a 20 µl volume: 1 X PCR reaction buffer, 0.2 mM dNTPs, 2 nmoles pigtail primer, 5 nmoles domain specific primer (NBS2, NBS3 or NBS5) and 0.4 U Taq polymerase were added to 5 µl 10X diluted 1st round PCR product. PCR conditions were 30 cycles at 95°C for 30 sec, 55°C for 1min 40 sec and 72°C for 30 sec, and a final extension step of 72°C for 20 min. The 2nd round PCR product was

run on an acrylamide gel as described in 3.3.4 and visualized by silver staining (Section 3.3.5).

3.3.3. SSR analysis

The parents of the RIL population, Kunduru-1149 and Cham-1, were screened with 75 different SSR markers to identify polymorphic markers. The SSR markers were selected to represent each chromosome arm of the A and B genomes. SSR markers were selected from wheat consensus maps (Roder *et al*., 1998, Nachit *et al*., 2001 and www.graingenes.org) and with extensive help from Claire Lewis (personal communication). GWM, WMC and BARC SSR markers were used from the John Innes Centre collection. From the initial screening, 33 polymorphic markers were selected, representing at least one marker per chromosome arm.

Table 3.4. List of SSR markers used, with chromosomal locations and annealing temperatures. Location= chromosomal location, Temp= annealing temperature.

Marker	Location	Temp	Marker	Location	Temp	Marker	Location	Temp
BARC148	1AS	52 °C	**GWM148**	2BS	60 °C	**BARC151**	5AL	55 °C
WMC312	1AL	61 °C	**GMW526**	2BL	55 °C	**GWM443**	5BS	55 °C
BARC61	1BS	52 °C	**GWM369**	3AS	60 °C	**GWM499**	5BL	60 °C
GWM153	1BL	60 °C	**GWM391**	3AL	55 °C	**GWM459**	6AS	55 °C
GWM268	1BL	55 °C	**GWM376**	3BS	60 °C	**GWM169**	6AL	60 °C
GWM140	1BL	55 °C	**GWM181**	3BL	50 °C	**GWM132**	6BS	60 °C
WMC156	1BL	51 °C	**BARC106**	4AS	50 °C	**WMC182**	6BL	51 °C
BARC188	1BL	58 °C	**WMC262**	4AL	61 °C	**WMC83**	7AS	61 °C
BARC181	1BL	58 °C	**GWM368**	4BS	60 °C	**WMC525**	7AL	61 °C
WMC177	2AS	61 °C	**WMC47**	4BL	61 °C	**GWM297**	7BS	55 °C
WMC181	2AL	61 °C	**BARC180**	5AS	52°C	**GWM333**	7BL	55 °C

For all SSR markers the following protocol was applied. PCR amplifications were carried out in 15 µl reaction volumes using ~240 ng of genomic DNA, 1X PCR reaction buffer (with 15 mM $MgCl_2$, Roche), 0.13 mM dNTPs, 0.2 µM forward primer, 0.2 µM reverse primer, 0.35 Units Taq polymerase (Roche). PCR reactions conditions were 94°C

for 3 min, followed by 35 cycles of 94°C for 1 min, X°C for 1 min and 72°C for 2min. A final elongation step of 72°C for 4 min was included. The X°C represents the annealing temperature of each SSR primer combination (Table 3.4). SSR PCR reactions were run on acrylamide gels as described in 3.3.4 and visualized by silver staining (Section 3.3.5).

3.3.4. Polyacrylamide Gel Analysis

Polyacrylamide gel analysis was carried out on 4.75 % acrylamide gels. Gel mix containing 480 g/L urea (Duchefa Biochimie), 1X TBE (diluted from 10X TBE, Severn Biotech) and 5 % 19:1 acrylamide/bisacrylamide solution (40 % stock solution, Severn Biotech) was prepared. The gel mix was filtered through a 0.22 μm membrane (Millipore) and stored at 4°C. To induce polymerization, 0.05 % (w/v) Ammonium Persulfate (SIGMA) and 0.05 % (v/v) TEMED (Pharmacia) were added.

The gel mix was poured between assembled sequencing gel plates (Whatman) cleaned with ethanol and treated with Repelcote (BDH) and Bind silane (Pharmacia). The poured gel was allowed to polymerize for 1 hour and 30 minutes at room temperature.

PCR samples were mixed with equal amounts of formamide loading dye, composed of 98 % (v/v) formamide (SIGMA), 0.01 M EDTA (BDH), 1 % (w/v) bromophenol blue (BDH) and 1 % xylene cyanol (SIGMA). Following dye addition, samples were denatured at 95°C for at least 3 minutes then placed on ice. The denatured samples (3-4ul) were loaded per lane onto the polyacrylamide gel. The sequencing gels were run in 1 X TBE buffer for 1 hour 30 min to 2 hours at 80 Watts.

3.3.5. Silver Staining of Polyacrylamide Gels

Polyacrylamide gels were silver stained to visualize DNA bands. All solutions applied to the gel were done so on a shaker. The gel was fixed in 10 % acetic acid (Fuka) for 30 mins and then washed quickly, twice with ddH_2O (Elga filtration system) and a third time for 10 to 20 min. One last rinse was performed before adding the silver nitrate staining solution composed of 0.0057 N silver nitrate (Aldrich) and 0.15% (v/v)

formaldehyde (FISHER). The gel was left to stain for 30 mins. The gel was developed with ice-cold developer solution, composed of 0.73 M sodium carbonate, 0.0151 N sodium thiosulfate (Aldrich) and 0.15 % (v/v) formaldehyde (FISHER) chilled to 4°C. Once the bands became visible, the reaction was terminated by adding 10 % acetic acid. The gel was dried overnight. A permanent record of the band pattern was taken by exposing the gel to X-Ray film (Kodak, Duplicating film) for 8-12 sec in direct light. The X-Ray films were developed in a developer machine (Fuji, RG II).

3.3.6. Scoring of SSR and NBS-Profiling Bands

For SSR markers, polymorphism was detected based on a shift in the size of the bands amplified by the SSR primers in each parent. SSR markers are generally co-dominant, so homozygous individuals have either of the parent alleles and heterozygous individuals have both parental alleles. The allele contributed by the susceptible parent, Kunduru-1149 was designated as the ‘a’ allele, while the allele contributed by the resistant parent, Cham-1 was designated as the ‘b’ allele. The subpopulation was scored for which allele each line carried using this designation.

NBS-profiling bands are dominant, so the lines of the subpopulation will either show a band representing an allele from one of the parents or no band at all. NBS-profiling gels were scored based in the presence/absence of bands in the lines of the subpopulation. Bands present the resistant parent, but absent from the susceptible parent were designated ‘c’, with lines without the band being designated ‘a’. Bands present in the susceptible parent, but absent from the resistant parent were designated ‘d’ with lines without the band being designated ‘b’.

3.4 Linkage Analysis of Scored Bands

Scores from SSR and NBS-profiling marker analyses were entered into JoinMap version 5.0 for Windows (Kyazma). Linkage groups were determined using a minimum LOD score of 3.0 and a maximum recombination frequency (REC) of 0.45. The recombination values were converted into genetic distances using the Kosambi mapping function. Three linkage groups were obtained and the rest of the markers remained unlinked.

Linkage groups formed in JoinMap, along with phenotypic data and marker scores were entered into the QTL mapping program MapQTL version 5.0 for Windows (Kyazma B.V., the Nederlands). This program was used to locate QTLs for resistance to yellow rust in the population. Kruskall-Wallis and interval mapping functions were used. The Kruskall-Wallis test is a non-parametric test in which no assumptions are made about the probability distribution of the quantitative trait (Rodrigues *et al*., 2004). For QTL mapping an imposed significance value of 5 % gave an upper LOD significance threshold of 1.7 through a permutation analysis (1000 permutations, MapQTL).

3.5. Cloning and Sequencing of NBS-profiling Bands

3.5.1. Band Excision from Polyacrylamide Gels

Three bands of interest were identified from NBS-profiling. These bands were cut from the polyacrylamide gels using razor blades. Each gel piece was placed in 100 µl 1X TE overnight at 4°C. Each gel slice was heated at 50°C for 10 min. The solution was then centrifuged at 16 000 rpm for 5 min. The supernatant was used directly as a source of DNA for PCR (Smith *et al*., 2002). The corresponding region of the gel, from lines not carrying the NBS band, were also cut from the gel as a background, negative control. This negative control was taken to ensure that the clones and sequences obtained were not due to background bands, undetected by silver staining.

3.5.2. Band Reamplification

The excised bands were reamplified using the original primer combinations by which the NBS-band was identified. The reamplification PCR reaction was carried out in a 40 µl volume. 1 X Mg-free PCR reaction buffer (Promega), 0.2 mM dNTPs, 2 nmoles pigtail primer, 5 nmoles domain specific primer, 1.56 mM $MgCl_2$ (Promega) and 0.4 U Taq polymerase (Promega) were added to 5 µl of excised band supernatant. PCR conditions were 30 cycles of 95°C for 30 sec, 55°C for 1min 40 sec and 72°C for 30 sec, and a final extension step of 72°C for 20 min, carried out on Techne Techgene PCR machine. The

reamplified PCR products (5µl) were checked on a 1% agarose gel. The reamplified PCR products gave bands the same size as the bands originally excised, confirming the success of band excision. Bands giving the correct band size were PCR purified using a Qiagen PCR purification kit.

3.5.3. Cloning of Bands

PCR products were cloned into pGEM-T Easy vector (Promega, Mannheim, Germany, Figure 3.2.) with a vector: insert ratio of 6:1. The protocol supplied with the pGEM-T Easy vector was followed. In brief, 50 ng of vector, one unit of T4 DNA ligase and 1X buffer (30 mM Tris-HCL, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 5 % polyethylene glycol) were added to 2 µl of reamplification PCR product in a final volume of 10 µl. The ligations were incubated overnight at 4^0C. Competent cells provided with the kit were thawed on ice for 5 minutes. Two µl of ligation mix was added to 50 µl of competent cells and incubated on ice for 20 minutes. The cells were heat shocked at 42^0C for 45 seconds and incubated on ice for an additional 2 minutes. Subsequently, 0.9 ml of SOC medium (2 g bacto tryptone (DIFCO), 0.5 g bacto yeast extract (DIFCO), 1 ml 1 M NaCl (Merck), 0.25 ml 1 M KCl,(SIGMA) 1 ml 1 M $MgCl_2$ (SIGMA), 1 ml 2 M glucose (SIGMA) in 1 L ddH_2O) was added and the cells were grown at 37^0C for 1.5 hours, with shaking at 150 rpm.

The transformed cells were then plated onto LB agar plates (5 g yeast extract (DIFCO), 10 g peptone (DIFCO), 10 g NaCl (Merck), 15 g bacto-agar (DIFCO) in one L ddH_2O) containing 100 µg/ml ampicillin (SIGMA) that were supplemented with 40ul of 20 ng/ml X-Gal and 5 µl of 200 ng/ml IPTG (Promega) immediately prior to plating. A 100 µl aliquot of transformed cells were plated. The remaining bacterial culture was concentrated by centrifugation at 5000 rpm for 2 minutes, the supernatant was poured off and the cells were resuspended in the residual media prior to plating. The plates were incubated overnight at 37^0C.

Cells transformed with native pGEM-T Easy plasmid generate blue colonies, whereas recombinant plasmids form white colonies due to the interruption of the expression of β-Galactosidase by the DNA insert. Sixty white colonies for each excised band and 15 white

colonies from background, negative control samples were selected. These colonies were then single colony propagated onto plates. Liquid LB (5 g yeast extract (DIFCO), 10 g peptone (DIFCO), 10 g NaCl (Merck) in one L of ddH_2O) cultures of the single colonies were seeded for plasmid isolation.

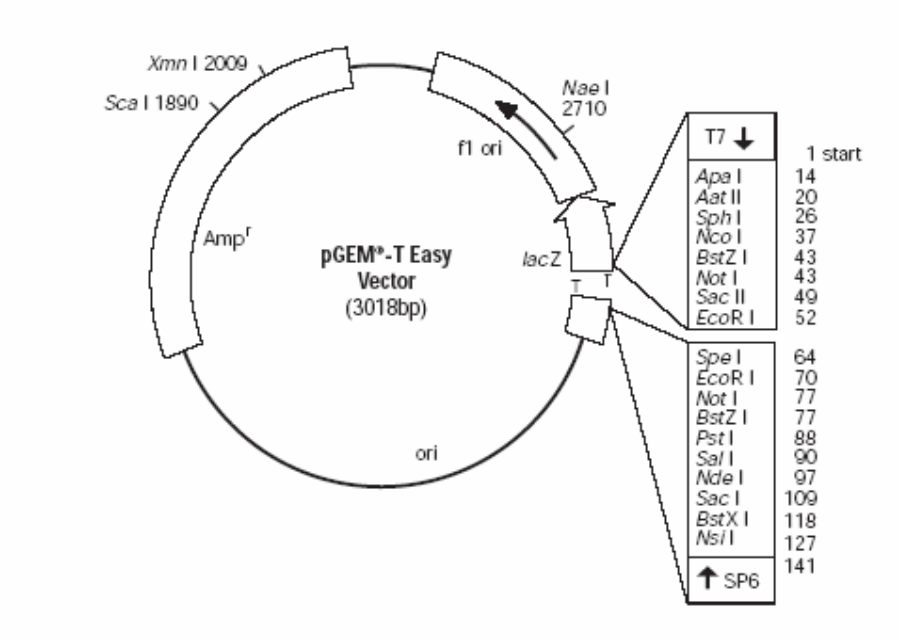


Figure 3.2. pGEM-T Easy Vector Map (Promega)

3.5.4. Plasmid Isolation of Selected colonies

Plasmids were isolated from 1 ml of the single colony liquid cultures using Wizard *Plus* SV Minipreps DNA purification system (Promega). The protocol was followed as described in the manual using solutions provided in the kit. Bacterial cells were harvested from the 1 ml culture in 1.5 ml eppendorf tubes (Axygen Scientific), by centrifugation for 5 min at 14 000 rpm. Cell Resuspension Solution (250 µl) was added to each sample and cells were completely resuspended. Cell Lysis Solution (250 µl) was added and samples were mixed by inversion and incubated 1-5 min until lysate clears. Alkaline Phophatase

(10 µl) was added, samples were mixed by inversion and incubated for 5 min. Neutralization Solution (350 µl) was added and inverted 4 times to mix. The cleared lysate was then centrifuged at 14 000 rpm for 15 min.

The supernatant (~850 µl) was applied onto Spin Columns by decanting, then columns were centrifuged at 14 000 rpm for 1 min. Column wash solution was applied twice (750 µl, then 250 µl) centrifuging for 1 min the first and 2 min the second time. Plasmid DNA was eluted into 1.5 ml eppendorf tubes (Axygen Scientific) by addition of 100 µl Nuclease-free Water to the spin column. Plasmid DNA was stored at –20 °C.

3.5.5. PCR and Agarose Gel Verification of Plasmid Insert

To check the contents of the vector from each colony isolate, a T7- Sp6 PCR amplification was used. T7 (5'-ATTTAGGTGACACTATAG-3') and Sp6 (5'-TAATACGACTCACTATAGGG-3') primers were obtained from SIGMA. 1 X PCR reaction buffer, 0.2 mM dNTP's, 5.3 pmoles each of T7 and Sp6 primers and 0.655 U of Taq polymerase were added to 36.5 ng of plasmid isolate. The amplification conditions were 95 °C for 4 min followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec with a final elongation step of 72 °C for 20 min. The PCR product (10µl) was run on a 1 % agarose gel as described previously. Each of the 60 colony isolates per band and 15 negative control colony isolates were checked for size and empty vectors were omitted from the rest of the analysis.

3.6. SSCP Gel Analysis of Isolated Plasmids

Single Strand Confirmation Polymorphism analysis was carried out on plasmid insert DNA to identify a predominant clone type (Smith *et al.,* 2002). SSCP analysis is a PCR based technique that can distinguish most conformational changes in single stranded DNA folding structure caused by subtle sequence differences (Hayashi, 1991). This method was chosen because even though most of the plasmid inserts of the 60 colony isolates were of the expected size, they may be completely different in sequence. SSCP can help classify the inserts into clone types and determine the predominant clone type that most probably represents the excised band.

PCR products from the T7-Sp6 primer amplification were loaded onto SSCP gels. SSCP gel cassettes were prepared exactly as described for polyacrylamide gels, except that the gel mix used consisted of 25 % (v/v) MDE gel solution (Combrex), 9 % (v/v) glycerol (Fluka), 1 X TTE buffer (National Diagnostics) 0.054 % TEMED and 0.06 % APS. This gel mix was allowed to polymerize for an hour.

An equal amount of formamide dye was added to PCR samples, which were denatured as described for polyacrylamide gel analysis. Samples were loaded onto the gel and run in 1 X TTE buffer for 16 hours at 5 Watts. The gels were stained by silver staining as described previously (Section 3.3.5). Plasmid inserts were classified based on the distance traveled in the gel and the banding pattern.

3.7. DNA Sequencing of Inserts

The DNA inserts of 12 isolated plasmids from each excised and cloned polymorphic band were sequenced. BigDye (Applied Biosystems) sequencing reaction system was used. Four µl of BigDye mix, 1 X BigDye buffer and 5 pmoles of T7 primer were added to 292 ng of plasmid DNA. PCR amplification conditions were 96 ℃ for 5 min followed by 25 cycles of 96 ℃ for 10 sec, 50 ℃ for 5 sec and 60℃ for 4 min. Products of this PCR amplification were sent to the Genome Center (Norwich Research Park) for sequencing.

ABI files obtained of each plasmid insert sequenced were analyzed and a contig of each cloned polymorphic band constructed using Gap 4 (version 4.10) (Bonfield *et al*., 1995) program. Most inserts of the same clone were almost identical in sequence. Contigs were assembled in Gap 4 v 4.10 and sequences that were excluded from the major contig were excluded from sequence analysis. Consensus sequences were formed from the contigs. These consensus sequences were TBLASTX (NCBI) searched for homology to other DNA and amino acid sequences held within the NBCI databases. Amino acid sequences were obtained using Gap 4 v 4.10 translation tool and were selected based on the presence of the p-loop consensus sequence detected by the NBS-profiling primers.

4. RESULTS

4.1. Characterization of Seedling Yellow Rust Resistance

The Kunduru-1149 X Cham-1 RIL population was characterized for seedling yellow rust resistance using the yellow rust isolate WYR 85-22 (virulent for *Yr2, Yr6, Yr7, Yr9*). This isolate was chosen because it's virulence profile represented the isolate mixture used by Göçmen *et al.,* (2003) in their field tests. The yellow rust resistance phenotype was scored using the modified Cobb scale described in Materials and Methods, section 3.2.3. The Cobb scale values were converted to numerical values based on the conversion scale shown in Table 4.1, as only numerical values can be used in QTL analysis. The numerical equivalents represent the severity of infection relative to the overall severity of infection. An average was taken of the five seedling scores obtained for each line. This average constitutes the seedling resistance to yellow rust seen in this population and is represented in Table 4.2.

Table 4.1. Numerical conversion of Cobb scale for seedling yellow rust resistance. IT= Infection type

Seedling yellow rust resistance score	Numerical conversion
Flecking IT	1
IT 0	2
IT 1	4
IT 2	5
IT 3 and 4	7

Seedling scores for the 150 lines were compared to field scores previously observed by Göçmen *et al*., (2003). The averaged coefficient of infection (CoI) of three field resistance scores (Göçmen *et al*., 2003) were compared to the averaged seedling scores (Table 4.2.), plotted as a regression (Figure 4.1.). Many of the lines deviated from the predicted linear correlation, indicating that these lines were susceptible at the seedling stage, but were resistant to yellow rust in the field. This suggests that a portion of the yellow rust resistance in this population is likely to be APR (Adult Plant Resistance).

Table 4.2. Seedling and field resistance phenotypes of the 150 lines and parents to yellow rust. Avrg= Average seedling score of 5 seedlings, CoI= Coefficient of infection from Göçmen *et al.,* (2003). 151= Kunduru-1149, 152= Cham-1. Lines with asterisks were segregating for seedling yellow rust resistance phenotype, lines marked 'a' were selected for marker analysis.

Line	Avrg	CoI	Line	Avrg	CoI	Line	Avrg	CoI	Line	Avrg	CoI
1*	4.40	10.13	**40**	7.00	17.50	**79[a]**	1.80	3.47	**118**	2.20	2.27
2*	1.80	4.47	**41**	1.00	1.33	**80[a]**	1.20	0.13	**119**	4.00	10.25
3*	3.60	3.90	**42***	2.00	19.25	**81**	1.20	1.47	**120[a]**	5.30	16.00
4*	2.20	1.47	**43[a]**	1.00	0.13	**82**	5.20	1.80	**121***	3.60	21.00
5	1.00	0.13	**44**	1.00	0.13	**83**	1.00	7.13	**122***	3.40	15.33
6	1.00	0.00	**45[a]**	1.00	0.27	**84**	6.33	13.13	**123[a]**	1.00	0.00
7[a]	1.00	0.00	**46**	4.00	21.15	**85**	1.00	10.15	**124**	4.20	19.42
8[a]	1.00	0.00	**47[a]**	1.00	0.13	**86[a]**	1.00	0.20	**125***	3.20	25.50
9[a]	1.00	0.00	**48**	1.60	5.90	**87***	5.50	3.25	**126***	5.20	50.00
10	1.00	0.00	**49[a]**	1.40	0.00	**88[a]**	1.60	6.00	**127**	6.60	34.00
11[a]	2.60	6.73	**50**	1.00	0.00	**89[a]**	2.00	8.53	**128***	5.25	17.33
12	1.00	4.27	**51*[a]**	3.25	17.58	**90***	3.80	4.33	**129**	4.50	7.33
13[a]	1.80	21.00	**52**	1.00	0.27	**91**	1.25	18.48	**130***	2.50	0.00
14*	4.20	9.75	**53[a]**	6.00	33.00	**92***	2.00	3.23	**131[a]**	6.50	64.67
15	1.00	0.00	**54**	1.20	0.13	**93**	4.40	5.60	**132**	1.00	0.00
16	1.60	0.00	**55**	1.00	0.00	**94[a]**	2.00	4.33	**133**	5.25	14.43
17*	4.00	22.30	**56**	6.50	21.00	**95**	1.00	1.13	**134**	4.40	22.50
18[a]	1.00	0.00	**57**	1.20	1.50	**96[a]**	1.00	0.13	**135[a]**	7.00	18.75
19[a]	7.00	37.50	**58***	5.30	30.00	**97[a]**	7.00	16.68	**136**	6.00	21.33
20*	5.00	3.40	**59**	1.00	0.00	**98**	1.00	6.67	**137**	4.40	6.65
21*	3.40	24.33	**60**	1.00	0.27	**99[a]**	1.00	0.13	**138**	1.25	3.77
22	7.00	7.33	**61[a]**	7.00	11.40	**100[a]**	1.00	0.13	**139**	4.00	26.17
23[a]	6.20	11.83	**62[a]**	1.00	0.00	**101**	1.00	0.00	**140[a]**	7.00	15.33
24	3.00	8.98	**63***	3.20	7.00	**102**	1.00	5.20	**141[a]**	2.00	0.00
25	1.00	0.30	**64**	5.80	8.85	**103*[a]**	1.80	12.47	**142**	1.20	0.40
26[a]	1.00	0.13	**65***	2.75	4.90	**104**	2.33	7.37	**143**	1.00	0.67
27	5.75	19.00	**66***	1.88	16.13	**105**	2.75	2.65	**144***	3.60	18.00
28[a]	1.00	0.00	**67**	6.20	27.83	**106**	1.00	4.58	**145***	6.16	13.08
29[a]	1.60	0.00	**68[a]**	1.00	0.00	**107**	5.80	4.47	**146**	1.00	0.53
30	7.00	2.73	**69***	4.00	8.13	**108[a]**	1.00	0.00	**147***	4.00	18.83
31[a]	1.50	3.20	**70*[a]**	3.40	7.33	**109[a]**	2.00	0.73	**148[a]**	1.60	0.00
32	2.00	16.08	**71[a]**	6.30	10.00	**110[a]**	1.00	0.13	**149**	2.00	0.87
33	1.00	0.40	**72[a]**	6.50	22.50	**111[a]**	5.60	11.05	**150**	1.00	0.40
34[a]	6.60	10.33	**73**	1.00	0.00	**112[a]**	2.25	11.25			
35[a]	2.60	19.10	**74**	1.20	1.60	**113**	1.00	4.00	**151[a]**	5.50	60.00
36	1.20	0.27	**75[a]**	6.60	21.92	**114***	1.60	0.40	**152[a]**	1.00	0.00
37*	2.33	58.00	**76**	5.00	27.75	**115[a]**	1.00	0.00			
38	1.00	0.13	**77**	4.00	14.40	**116[a]**	1.00	1.00			
39*	5.00	2.27	**78**	1.33	0.13	**117**	3.60	11.47			

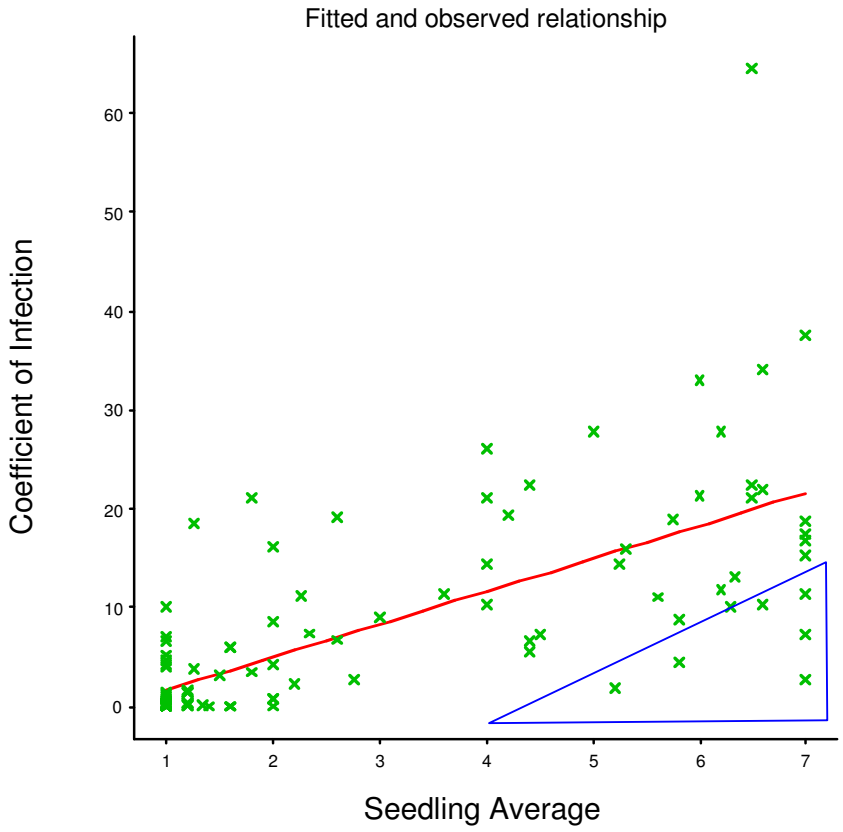


Fig 4.1. Adult CoI (Göçmen *et. al.*, 2003) vs. seedling resistance nominal (Table 4.2). The area outlined by the blue triangle represents the approximate region encompassing lines that show APR. The red line represents an approximate linear correlation with r-value=0.705.

Values for seedling resistance phenotype nominals range from 1 to 7. Low values indicate greater resistance to yellow rust. Values for field Coefficient of Infection (CoI) range from 0 to ~65, and were taken from Göçmen *et al.*, (2003). The lower values indicate more resistant lines.

Thirty-two of the 150 lines were segregating for seedling yellow rust resistance (Table 4.2). Twenty-five of the 150 lines were more susceptible, i.e. had a higher average seedling coefficient, than the susceptible parent Kunduru-1149. This was not observed by Göçmen *et al.* (2003) in the field, which suggests that Kunduru-1149 may contain seedling resistance to yellow rust detected by WYR 85-22. Also, allelic discrepancies observed during SSR analysis (Section 4.2.3.) suggests that the parental line Kunduru-1149 used in

the cross to produce the 150 RIL may not be the same seed stock given as the Kunduru-1149 parental line for this study.

The 150 lines were classified into 4 categories based on their yellow rust seedling resistance. Category IV contained 8 lines that were susceptible to isolate WYR85/22, having Infection Type (IT) 4. Category I contained 59 resistant lines with flecking IT. Category II contained 22 resistant lines with IT0, while Category III contained 61 lines showing an intermediate resistance phenotype with IT 1/2. Phenotypic examples of the yellow rust resistance of each category are illustrated in Figure 4.2.

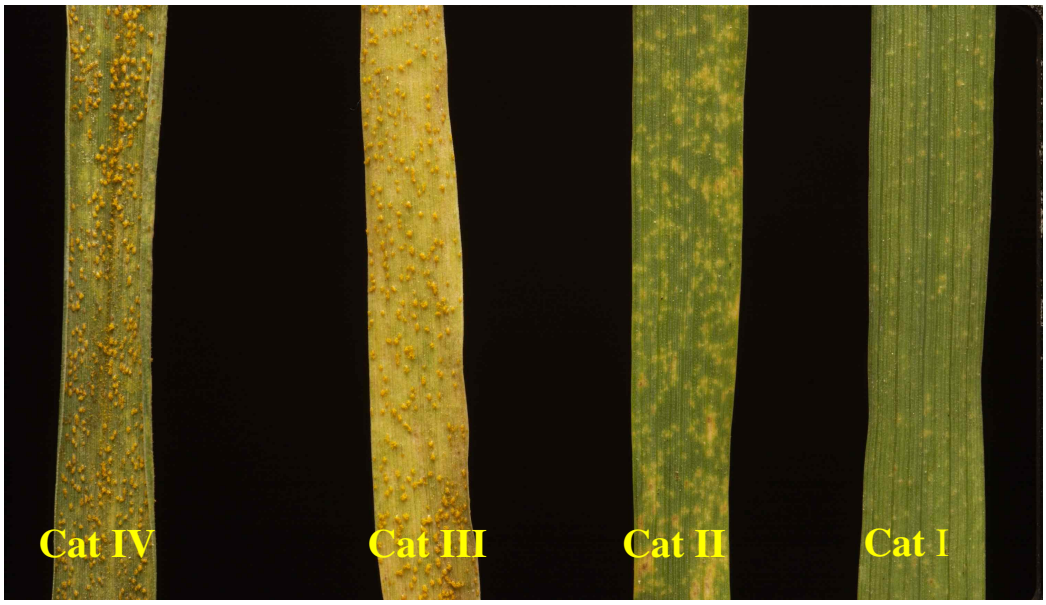


Figure 4.2 Seedling yellow rust resistance phenotypes. Scores given to the four individuals are as follows: Cat IV=IT 4; Cat III=IT 2; Cat II=IT 0 and Cat I=IT fleck (;)

The 150 lines formed a ratio of categories I:II:III:IV of 59:22:61:8. Since Cat III, Cat II and Cat I all represent a resistant phenotype, we combined the numbers from these categories and relate this to the number of susceptible individuals. This gives a ratio of Resistant : Susceptible of 142:8, or 17.75:1 (Resistant : Susceptible). The predicted X^2 values for possible numbers of dominant seedling resistance genes in this population are given in Table 4.3. As seen in Table 4.3, the X^2 value closest to 0 (zero) is for a R : S ratio of 15 : 1 that corresponds to 4 dominant seedling resistance genes. This X^2 value corresponds to a 50-75 % chance that the observed number of resistant/susceptible lines

would be equal to the expected number of resistant/susceptible lines. In conclusion, the ratio obtained would fit the Mendelian distribution for 4 dominant genes (15:1).

Table 4.3. X^2 values for predicted number of seedling resistance genes in this population. R : S ratio represents the ratio of expected yellow rust resistant : susceptible plants in this population. The number of dominant genes corresponds to the number of dominant seedling resistance genes that would produce this ratio.

R : S Ratio	**Number of dominant genes**	**X^2 Value**
1 : 1	1	119.7066
3 : 1	2	30.9416
7 : 1	3	7.0438
15 : 1	4	0.2151
31 : 1	5	2.41633

A sub population of 53 individuals was formed. Lines that were still segregating for yellow rust resistance or did not show a clear phenotype were omitted before making a random selection of lines from each category. As Category IV comprised the susceptible lines, without resistance genes and would have been very small, containing only 2 lines in a sub-population of 53 lines, it was decided to select 6 of the 8 lines from Category IV. The final number of lines chosen from each category was: six lines from Cat IV, 12 lines from Cat III, 12 lines from Cat II and 23 lines from Cat I. All 8 susceptible lines were not used due to lack of germination.

4.2. Marker Analysis of Yellow Rust Resistance

4.2.1. Genomic DNA Isolation

Genomic DNA from each of the 53 lines and the parents is shown in Figure 4.3. The DNA was of good quality and estimated to be approximately 80 ng/µl.

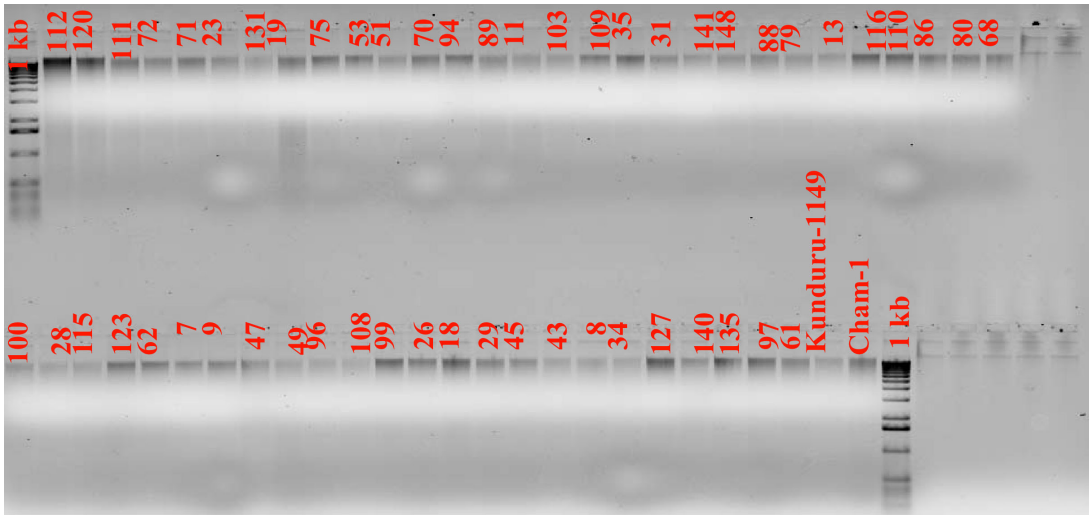


Figure 4.3 Genomic DNA from the 53 lines and the parents, Kunduru-1149 and Cham-1. One µl of 1µg/µl 1kb ladder was loaded to obtain an approximate estimate of the DNA concentrations.

4.2.2. NBS-Profiling

Genomic DNA of the 53 lines and the parents were screened using *Mse*I and three different NBS primers (NBS 2, 3 and 5, van der Linden *et al*., 2004). Bands present in the resistant parent, Cham-1, but absent from the susceptible parent, Kunduru-1149 were designated ‘c’ when present and ‘a’ when absent from the population lines. Bands present in the susceptible parent, but absent from the resistance parent were designated ‘d’ when present and ‘b’ when absent from the population lines. The band profile produced by NBS-profiling is shown in Figure 4.4.

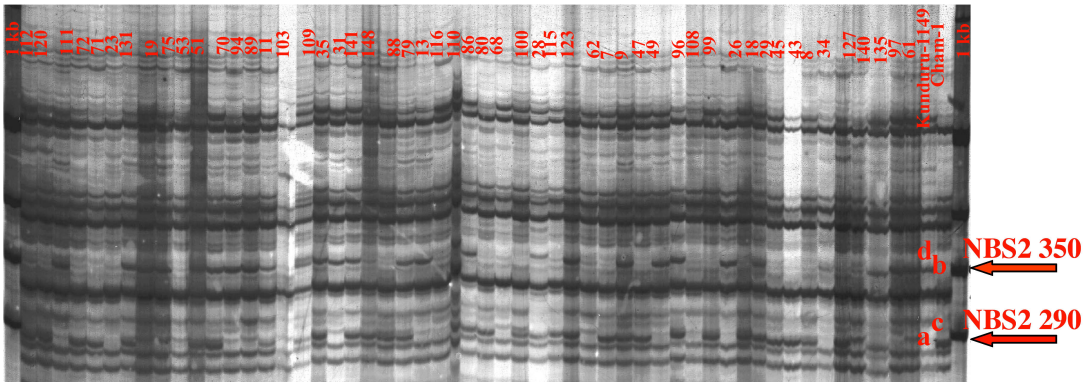


Figure 4.4. NBS-profile of the 53 lines and parents. Two of the polymorphic bands observed in this NBS-profile are marked NBS2 290 and NBS2 350.

In total, 14 polymorphic bands were identified as segregating within the sub-population of 53 lines (Table 4.4). Five bands originated from the NBS2 primer-*Mse*I combination, 6 from NBS3 and 3 from NBS5. Designations of the bands are based on the NBS-specific primer used to obtain the band and the approximate size of the observed band. Two polymorphic NBS bands were seen to segregate between the 53 lines, but neither band was amplified in either parent (Table 4.4). This initially indicated that one or both the parental lines supplied with the population may not be the parental lines used in the cross to produce the population, so the polymorphic band would have been derived from the original parent(s) used in the cross, but would not be present in the parental line(s) supplied with the population.

Table 4.4. Polymorphic bands identified by NBS-profiling.

Band designation-Size in bp	**Origin**	**Band designation-Size in bp**	**Origin**
NBS2-290	Cham-1	NBS3-350	Kunduru-1149
NBS2-325	Cham-1	NBS3-500	Kunduru-1149
NBS2-350	Kunduru-1149	NBS3-800	Kunduru-1149
NBS2-750	Kunduru-1149	NBS3-1300	Neither
NBS2-1010	Kunduru-1149	NBS5-290	Cham-1
NBS3-270	Neither	NBS5-370	Kunduru-1149
NBS3-290	Cham-1	NBS5-375	Cham-1

Cham-1 = Yellow rust resistant, Kunduru-1149 = Yellow rust susceptible

The profiles obtained using the 3 NBS primers were assessed for the degree of polymorphism that they showed, i.e. the number of bands obtained in the profile compared to how many of those bands were polymorphic. Profiles obtained using the primer NBS5 were 2 % (3/168) polymorphic, while NBS3 was 6 % (7/126) polymorphic and NBS2 was 3 % (5/168) polymorphic.

4.2.3. SSR Marker Analysis of the Subpopulation

The 53 lines were analyzed for SSR marker polymorphisms (Figure 4.5). Seventy-five SSR markers were screened for polymorphisms between parents, 39 being polymorphic. Thirty-three of these polymorphic markers were used for SSR analysis of the subpopulation, giving at least one SSR marker per chromosome arm. Alleles donated by the resistant parent, Cham-1 were designated 'b', while alleles donated by the susceptible parent, Kunduru-1149 were designated 'a'. Subpopulation lines were scored based on which allele they carried.

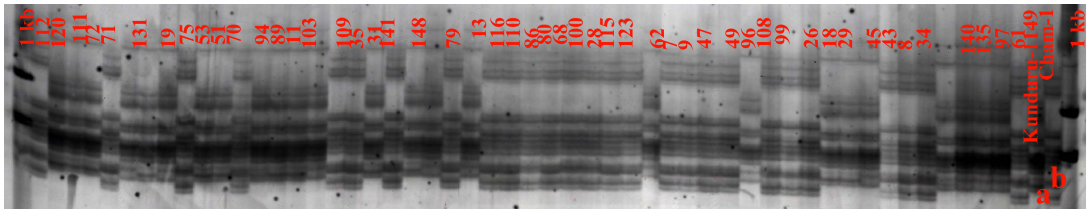


Figure 4.5 SSR profile of GWM 268 across 53 lines and parents. Cham-1= Resistant parent donating the 'b' allele, Kunduru-1149=Susceptible parent donating the 'a' allele.

Almost all SSR markers used showed simple band-shift polymorphisms. One marker, BARC81 was monomorphic in the population, even though that marker was polymorphic for the parents, again indicating that one or both of the parental lines supplied with the population were not the original parent(s) used to produce the cross. WMC262 showed 4 alleles segregating between the 53 lines. These two markers were omitted from further analysis.

The question of whether one or both of the parental lines supplied were different from the parental lines used to make the population was clarified when it was observed that for some SSR markers (GWM 153, GWM 526, GWM 368 and WMC 83) the lines showed an 'a' allele different from that observed in the susceptible parent Kunduru-1149. For these markers the b allele was contributed by Cham-1 and was present in half of the 53 lines, while the other allele, found in the other half of the population, showed a different shift pattern from that contributed by Kunduru-1149 (Figure 4.6). This shows that the Kunduru-1149 parental line supplied with the population is different from the original Kunduru-1149 line used in the cross from which the population was made.

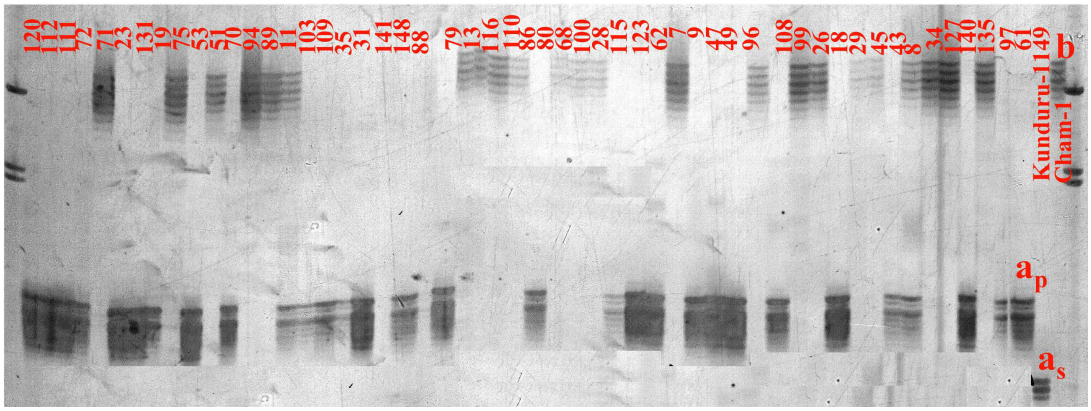


Figure 4.6 SSR marker WMC83 identified an allele in Kunduru-1149 different from that present in the population. Cham-1 donates the 'b' allele, the supplied Kunduru-1149 donates the allele 'a_s' which contrasts to 'a_p', the allele found in the population.

4.3. Linkage Analysis and Mapping of Yellow Rust Resistance Loci

Scores from SSR and NBS-profiling analyses were entered into JoinMap program to check for linkage between markers. Three mapping groups were found. Four NBS markers (NBS2 350, NBS2 750, NBS3 350 and NBS3 800) were linked. Three SSR markers (BARC188, GWM268 and GWM153), located on 1BL, were linked to the NBS-profiling marker NBS3 290. This indicated that NBS3 290 was probably located on 1BL. The last mapping group linked 2 SSR markers known to be located on the same chromosome. The

NBS-profiling marker NBS2 290 is in the exact same position as GWM 333 (7BL) and both are linked to GWM 297 (7 BS) in a mapping group.

These mapping groups were entered into the MapQTL program along with seedling and adult phenotype scores and genotype scores. Four lines were excluded from linkage analysis because they appeared to be segregating based on seedling yellow rust tests. Even though segregating lines were excluded from the subpopulation, upon further analysis it was revealed that even though these four lines were not showing obvious phenotypic segregation, their removal made a difference in the linkage analysis. As a result, the four lines were excluded from further analysis. As a result, a total of 48 lines were entered into MapQTL analysis program. Seedling and adult phenotype were analyzed separately and Kruskall-Wallis and Interval Mapping functions were used to locate QTLs for seedling and adult resistances.

Analysis of the seedling phenotypes, using Kruskall-Wallis function revealed significant QTLs on 1BL, encompassing the 3 linked SSR bands and NBS3 290 (Figure 4.7) while interval mapping of the seedling resistance data confirmed this as a QTL with a significant LOD value (above 1.7) was observed (Figure 4.8) Two other SSR markers, both located on 1BL (WMC 156 and BARC 181) but which did not form part of the linkage group, were linked to resistance with a high level of significance (Kruskall-Wallis).

Group	Position	Locus	K*	Signif.
1	0	NBS2350bp	5.558	**
1	2.217	NBS2750bp	2.968	*
1	5.819	NBS3350bp	3.65	*
1	10.315	NBS3800bp	5.327	**
2	0	barc188	5.634	**
2	2.617	gwm268	7.133	***
2	5.03	gwm153	8.385	****
2	19.714	NBS3290bp	6.428	**
3	0	gwm297	0.03	-
3	5.202	gwm333	0.289	-
3	5.202	NBS2290bp	0.289	-
U	1	gwm140	0.226	-
U	2	wmc156	13.275	******
U	3	barc181	11.452	*****
U	4	barc148	1.737	-
U	5	wmc312	2.763	*
U	6	barc61	4.625	**
U	7	wmc177	0.078	-
U	8	wmc181	4.891	**
U	9	gwm148	0.178	-
U	10	gwm526	1.24	-
U	11	gwm391	0.181	-
U	12	gwm376	0.424	-
U	13	gwm181	1.462	-
U	14	barc106	0.359	-
U	15	gwm368	3.215	*
U	16	wmc47	0.907	-
U	17	barc180	0.048	-
U	18	barc151	0.005	-
U	19	gwm443	0.306	-
U	20	gwm499	1.314	-
U	21	gwm459	0.037	-
U	22	gwm169	0.49	-
U	23	gwm132	0.069	-
U	24	wmc182	0.017	-
U	25	wmc83	1.919	-
U	26	wmc525	0.528	-
U	27	NBS2325bp	2.183	-
U	28	NBS21010bp	4.654	**
U	29	NBS3500bp	1.95	-

U= Unlinked Markers
P-values:

*	0.1
**	0.05
***	0.01
****	0.005
*****	0.001
******	0.0005

Figure 4.7. Seedling Kruskall-Wallis analysis.

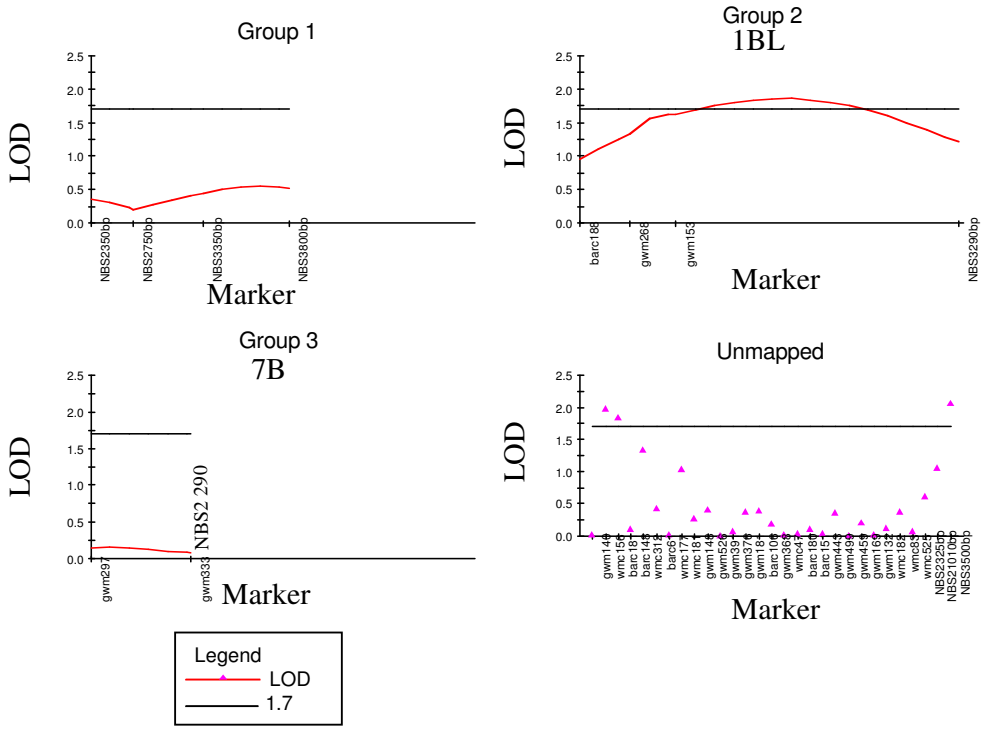


Figure 4.8. Seedling Interval Mapping analysis. The marker NBS2 290 cosegregates with GWM333 and is thus mapped to the same location.

Analysis of the APR using Kruskall-Wallis also revealed a significant QTL on 1BL, encompassing the 3 linked SSR markers and NBS3 290 (Figure 4.9), but interval mapping of the APR showed that the QTL did not have a significant LOD value (above 1.7) (Figure 4.10). The two SSR markers located on 1BL (WMC 156 and BARC 181), found in the seedling analysis to show a significant association were also observed to be significantly linked to resistance in the adult phenotype QTL analysis.

Group	Position	Locus	K*	Signif.
1	0	NBS2350bp	2.464	-
1	2.217	NBS2750bp	2.67	-
1	5.819	NBS3350bp	2.818	*
1	10.315	NBS3800bp	3.04	*
2	0	barc188	2.87	*
2	2.617	gwm268	5.937	**
2	5.03	gwm153	9.669	****
2	19.714	NBS3290bp	6.564	**
3	0	gwm297	0.025	-
3	5.202	gwm333	0.097	-
3	5.202	NBS2290bp	0.097	-
U	1	gwm140	0.173	-
U	2	wmc156	10.119	****
U	3	barc181	8.909	****
U	4	barc148	0.563	-
U	5	wmc312	2.683	-
U	6	barc61	0.373	-
U	7	wmc177	1.499	-
U	8	wmc181	1.046	-
U	9	gwm148	0.05	-
U	10	gwm526	0.843	-
U	11	gwm391	1.113	-
U	12	gwm376	0	-
U	13	gwm181	0.628	-
U	14	barc106	0.785	-
U	15	gwm368	0.47	-
U	16	wmc47	1.215	-
U	17	barc180	0.028	-
U	18	barc151	0	-
U	19	gwm443	0	-
U	20	gwm499	1.972	-
U	21	gwm459	0.084	-
U	22	gwm169	3.155	*
U	23	gwm132	1.623	-
U	24	wmc182	0.02	-
U	25	wmc83	0.254	-
U	26	wmc525	0.022	-
U	27	NBS2325bp	2.045	-
U	28	NBS21010bp	5.553	**
U	29	NBS3500bp	1.673	-

U= Unlinked Markers
P-values:

*	0.1
**	0.05
***	0.01
****	0.005
*****	0.001
******	0.0005

Figure 4.9. APR Kruskall-Wallis analysis.

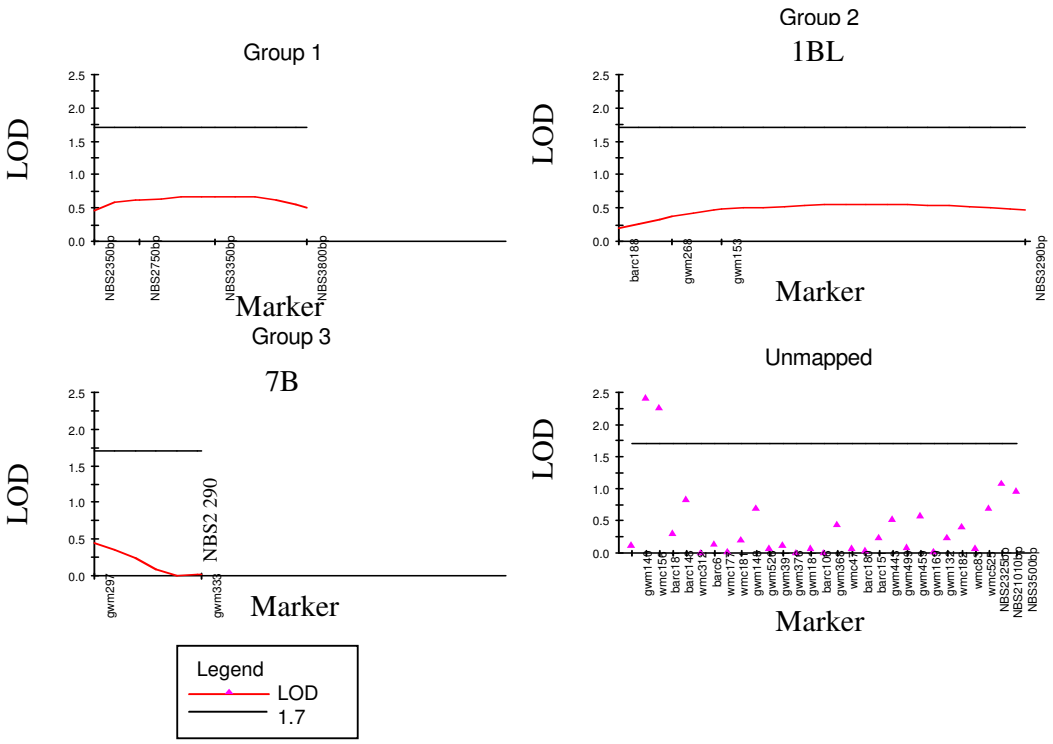


Figure 4.10. APR Interval Mapping analysis. The marker NBS2 290 cosegregates with GWM333 and is thus mapped to the same location.

Results from Kruskall-Wallis and Interval mapping analyses were assessed and positions of the significantly linked markers (WMC 158, BARC 181, GWM 268, BARC 188 and GWM 153) were compared to known locations based on a recent consensus map (www.shigen.ing.ac.jp). The approximate location of a putative QTL on 1BL was projected for seedling resistance (Figure 4.11) and adult plant resistance (Figure 4.12). There were not enough markers to fill in the gap between the markers known to all be on 1BL but were too far away to map together in JoinMap analysis performed, therefore a predicted positioning of the mapped markers is shown linked with a dashed line.

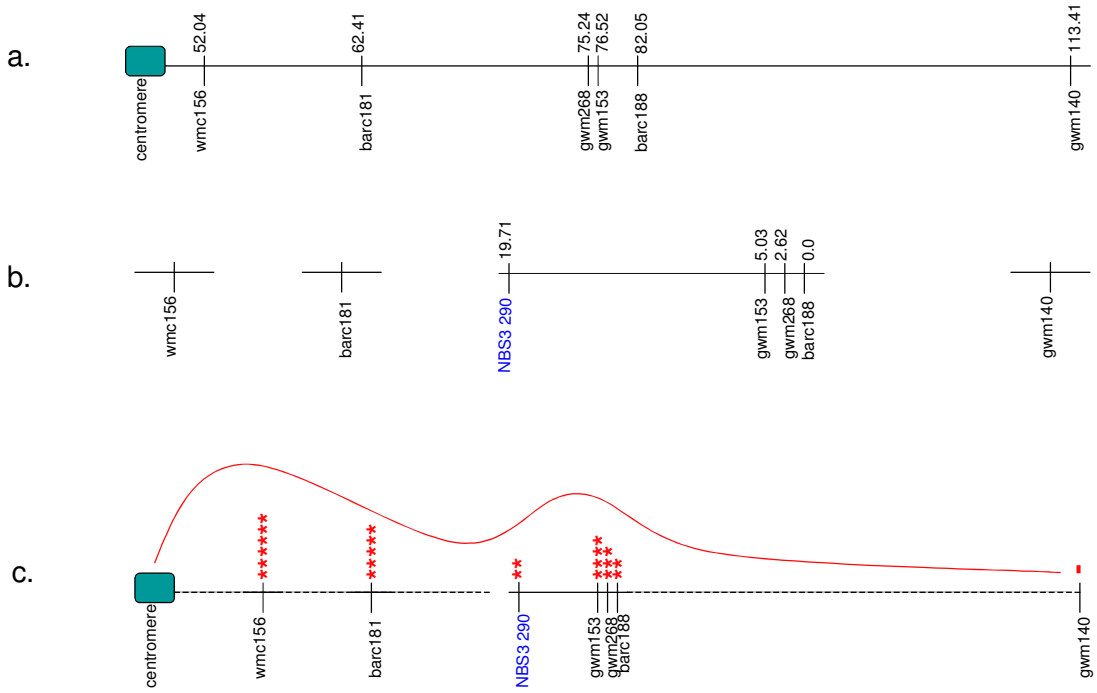


Figure 4.11. Predicted seedling resistance QTL location. a. The genomic map of chromosome 1BL as given in www.shigen.ing.ac.jp. b. Map produced in JoinMap of the markers used on the Kunduru-1149 X Cham-1 population. Linkage is seen between 3 of the markers already known to be on 1BL and one NBS-profiling marker. c. The predicted location of the seedling QTL characterized in this cross. The asterisk represent the degree of significance of the linkage to resistance as predicted by Kruskall-Wallis analysis.

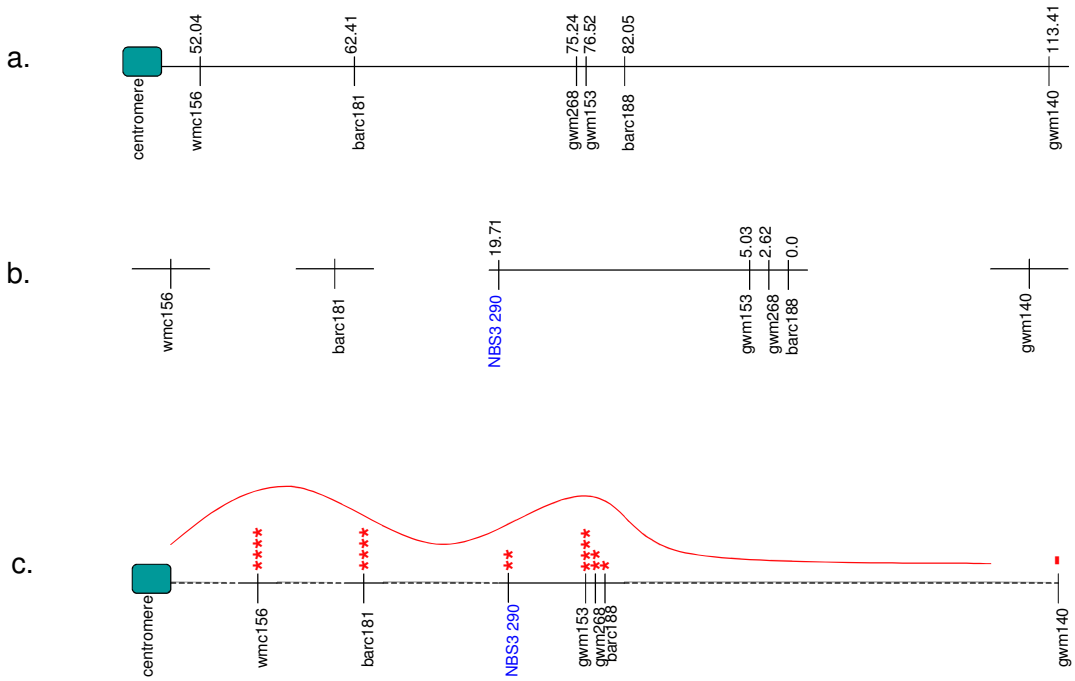


Figure 4.12. Predicted adult plant resistance QTL location. a. The genomic map of chromosome 1BL as given in www.shigen.ing.ac.jp. b. Map produced in JoinMap of the markers used on the Kunduru-1149 X Cham-1 population. Linkage is seen between 3 of the markers already known to be on 1BL and one NBS-profiling marker. c. The predicted location of the APR QTL characterized in this cross. The asterisk represent the degree of significance of the linkage to resistance as predicted by Kruskall-Wallis analysis.

As seen in Figures 4.11 and 4.12, there could be 2 QTLs on 1BL, both expressed at the seedling and adult plant growth stages.

4.4. Cloning of NBS-Profiling Bands

4.4.1. Isolation of NBS-Profiling DNA Bands

Four polymorphic bands, obtained through NBS-profiling were designated to be of interest based on the results of the linkage analysis. These four bands were: NBS2 290, NBS2 350, NBS3 290 and NBS3 500. The main consideration for choosing these bands was the ease with which they could be excised from polyacrylamide gels. NBS2 290 and

NBS3 500 were not linked to yellow rust resistance at seedling or adult stages, whereas NBS3 290 was linked to yellow rust resistance at both seedling and adult stages and NBS2 350 was linked only at the seedling stage. For each of these bands one line of the population that carried the band and one that lacked the band were selected. For all 4 bands these lines and both parents, Kunduru-1149 and Cham-1, were rerun on a polyacrylamide gel. For each of the four polymorphic bands, the band was excised from the parent that donated the band and the line that carried the band. As a negative, background control, the corresponding region of the gel was also cut from the parent and a line that did not carry the band. Table 4.4 is a summary of bands excised from NBS-profiling.

Table 4.5. Bands cloned from NBS-profiling. +Parent=Parent that donated the band, -Parent=Parent from which background area was cut, +Line= Line of the population from which the band was cut, -Line= Line of the population from which background area was cut.

Band	+ Parent	-Parent	+Line	-Line
NBS2 290	Cham-1	Kunduru-1149	99	110
NBS2 350	Kunduru-1149	Cham-1	89	68
NBS3 290	Cham-1	Kunduru-1149	18	68
NBS3 500	Kunduru-1149	Cham-1	141	123

The isolated DNA was reamplified before ligation into the cloning vector pGEM-T Easy to verify that a single band of the correct size was cut from the polyacrylamide gel. This was done for both the positive band and negative, background control (Figure 4.13).

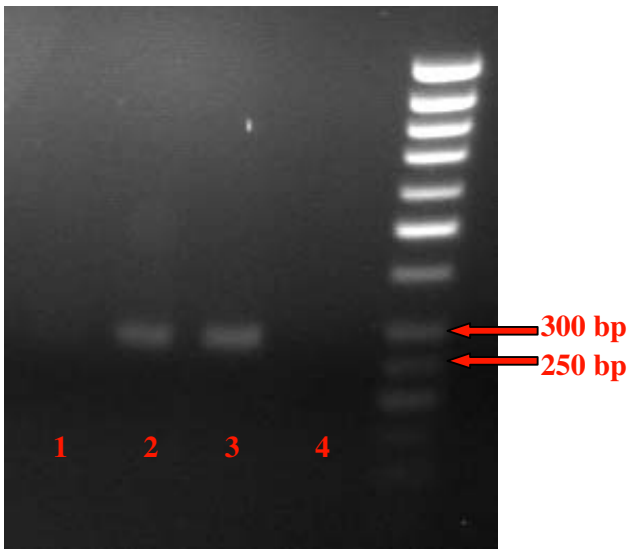


Figure 4.13 Reamplified NBS-profiling bands. The reamplified band is NBS2 290, which is expected to have a 290 bp PCR product. Lane 1= Line110 (negative control), Lane 2=Line 99 (positive band), Lane 3= Cham-1 (positive band) and Lane 4= Kunduru-1149 (negative control).

4.4.2. Cloning of NBS-Profiling Bands

As expected, many more white clones were observed for cloned bands than for the background controls. For NBS2 290 and NBS3 290 60 white colonies were selected from each cloned band and 15 white colonies from each background control. Only 50 white colonies were selected for NBS2 350, the background cloning not being analysed because this band was cloned at a later date and it had been previously observed through study of the NBS2 290 and NBS3 290 bands that the negative controls contained no background bands of real significance. The band NBS2 500 was not studied as it was discovered after cutting and reamplifying this band that is was not significantly linked to yellow rust resistance and was thus omitted from further studies.

4.4.3. Verification of Plasmid Inserts

Insert sizes were checked using the T7-SP6 promoters present within the pGEM-T Easy vector. An empty plasmid would produce a PCR amplified product of 177bps. The cloned NBS bands NBS2 290, NBS3 290 and NBS3 350 produced bands of the expected size (Table 4.6).

Table 4.6. Numbers of plasmids containing an insert of expected size for each cloned NBS band and negative, background controls containing inserts. (+) and (-) signs indicate bands and background controls respectively.

Band/Background control	**Number of plasmids containing an insert of the correct size/ number of screened clones**
NBS2 290 Cham-1 (+)	48/60
NBS2 290 Kunduru-1149 (-)	0/15
NBS2 290 Line 99 (+)	52/60
NBS2 290 Line 110 (-)	6/15
NBS3 290 Cham-1 (+)	48/60
NBS3 290 Kunduru-1149 (-)	10/15
NBS3 290 Line 18 (+)	56/60
NBS3 290 Line 68 (-)	15/15
NBS2 350 Kunduru-1149 (+)	13/50
NBS2 350 Line 89 (-)	47/50

For NBS2 290 and NBS3 290 a plasmid with an insert would produce an amplification product of 467 bps. For NBS2 350, a plasmid carrying and insert would produce a 527 bps product (Figure 4.14).

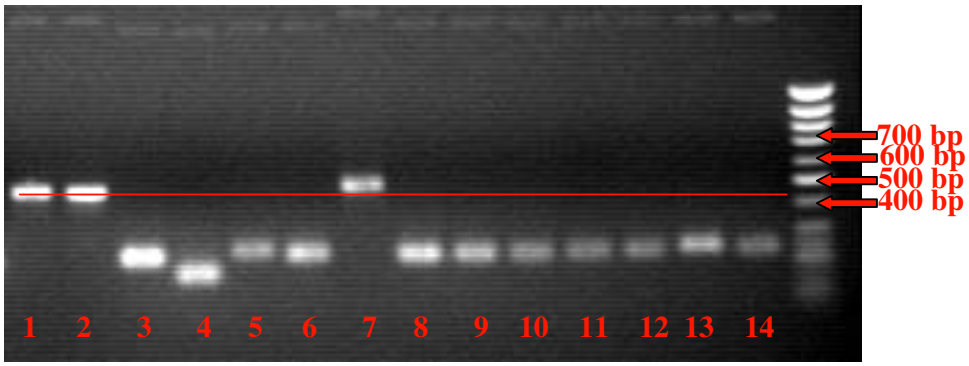


Figure 4.14. T7-SP6 amplification of NBS2 350 plasmid isolates. The expected insert size is 527 bps, while the empty plasmid would give a PCR product of 117 bps. Lanes 3-6 and 8-14 contain empty plasmids. The plasmid insert in lane 7 probably contains a background band as the insert is about 600bp. Plasmid inserts in lanes 1 and 2 contain the cloned 350 bp band (~467bp band).

4.4.4. SSCP Gel Analysis of Isolated Plasmids

The content of the plasmids were previously verified based on insert size but the exact nature of the insert remained to be determined. Due to the large number of isolated plasmids, sequencing of them all was not feasible. SSCP gel analysis is a useful tool to differentiate inserts based on single nucleotide differences (Smith *et al.*, 2002). T7-SP6 PCR products of all the plasmid inserts were loaded onto SSCP gels (Figure 4.15). Background controls were included in the SSCP analysis to ensure that the selected, predominant clone type was not from a background band. Background samples mostly displayed outlying banding patterns (Figure 4.15).

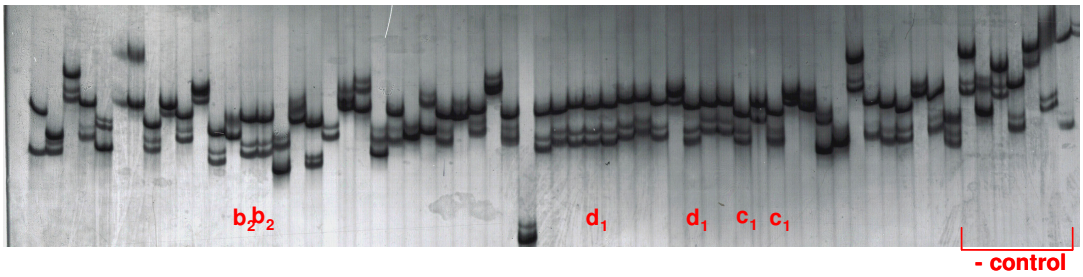


Figure 4.15 SSCP gel. Samples labeled '-control' are T7-SP6 PCR products of NBS3 290 line 68, a negative, background control. The rest of the gel corresponds to NBS3 290 line 18, positive band, for which insert 'd_1' was determined to be the predominant clone-type. Insert types 'c_1' and 'b_2' were close 2nd and 3rd predominant clone-types. Inserts from the 6 labeled lanes were sequenced.

The SSCP banding patterns, for all three cloned and analyzed NBS bands (NBS2 290, NBS3 290 and NBS2 350) were very complex and it was difficult to distinguish a single dominant clone-type (Figure 4.15). In order to distinguish a predominant clone-type from the SSCP gels a scoring system was developed whereby both distance between the allelic bands of each sample and the total distance traveled from the top of the gel were taken into account. This enabled clones to be classified into categories (Figure 4.16)

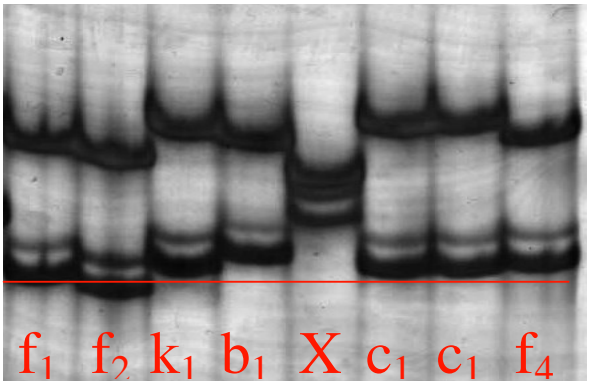


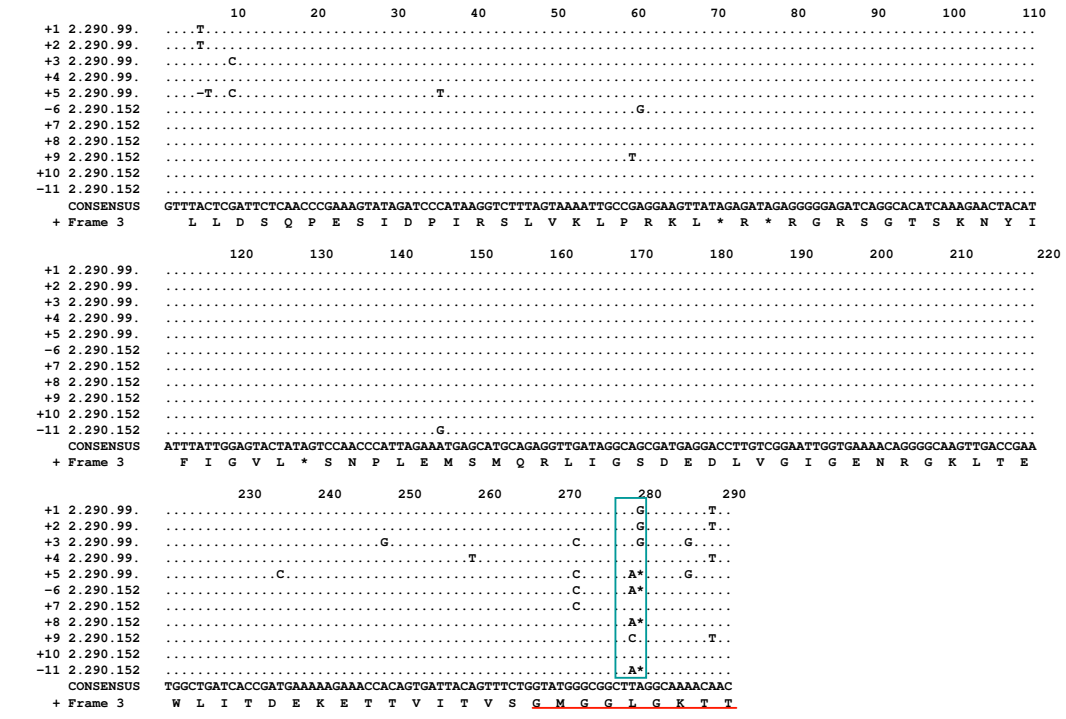
Figure 4.16. SSCP scoring method. Letters represent classes of clones based on banding pattern. The red line represents a measure of how far the bands have traveled in the gel, and the subscript number represents the sub-classification based on the distance traveled relative to the ruler. The lane labeled 'X' is an example of an outlier band with no

similarities to the others. In this example the f type banding pattern is observed 3 times, each traveling a different distance.

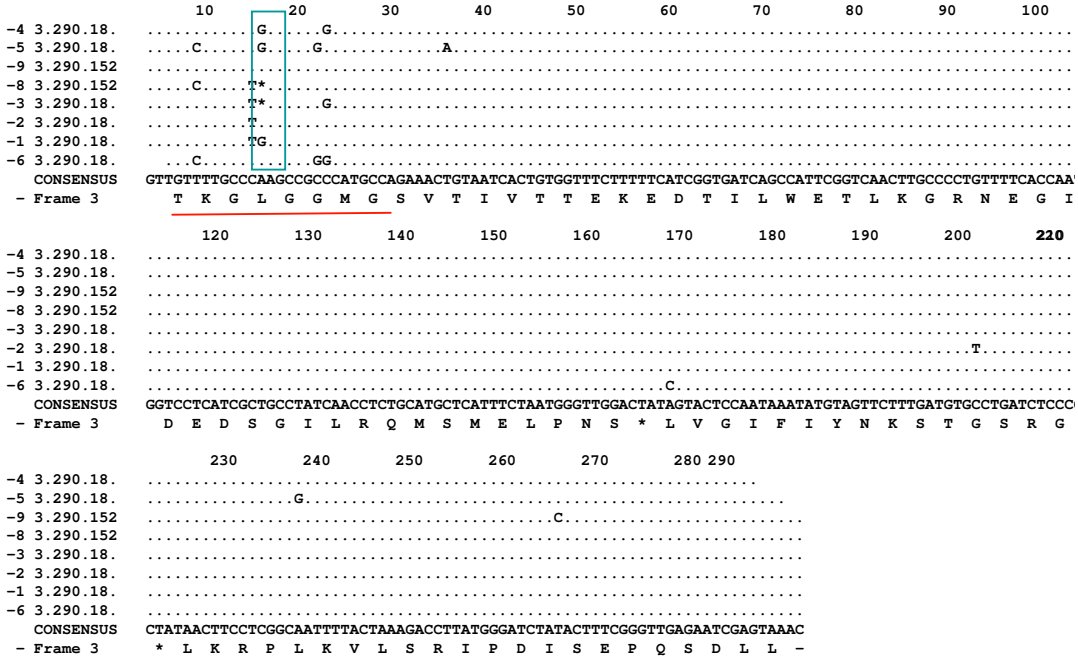
Using this SSCP scoring system, it was observed that most of the isolated plasmids showed unique banding patterns, thus it was difficult to determine one predominant clone type. In retrospect, it was realized that NBS-profiling primers are degenerate and will thus be highly variable in sequence and so using SSCP as a classification tool for clone types would not be useful. It was decided to take pairs of clone types that were identical in banding pattern and distance traveled, which resulted in 3 pairs taken for each cloned band (i.e. 6 clones were sequenced for each original cloned NBS band).

4.5. Sequencing and Analysis of Inserts

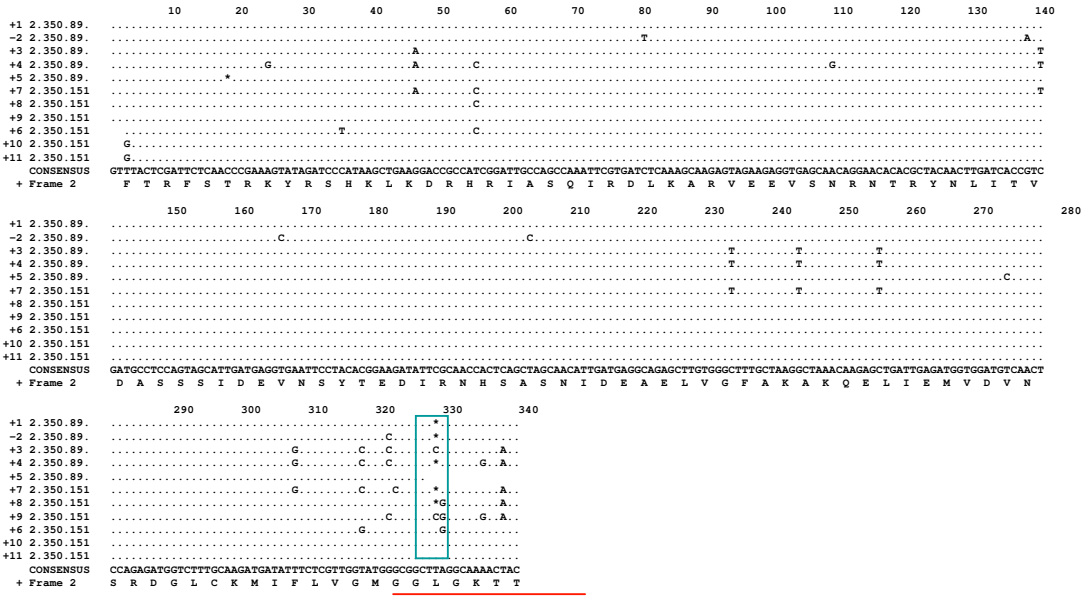
For each cloned NBS band, 6 plasmid inserts from the donating parent and 6 plasmid inserts from the line containing the band were sequenced. For each NBS band sequences were contiged and a consensus sequence and amino acid translations determined (Figure 4.17) using Gap 4 (version 4.10). Sequences from each sequenced band that did not fall into the same contig as the others (as determined by Gap 4.10) was omitted from remaining analysis. The amino acid reading frames were selected based on which of the 6 readouts contained the NBS-primer consensus sequence (~GGMGGLGKT) (Figure 4.17). Sequence analysis indicated that NBS2 290 and NBS3 290 appear to have the exact same DNA and amino acid sequence, although linkage analysis did not place them on the same linkage group. However, NBS2 290 and NBS3 290 PCR in two different directions off of two different DNA strands and therefore could not represent the same gene. These two bands may be the result of duplication after mutation of a NBS-LRR type resistance gene, forming the pseudogene that was partially cloned in this study.



a. NBS2 290 alignment contig and amino acid translation of the consensus sequence.



b. NBS3 290 alignment contig and amino acid translation of the consensus sequence.



c. NBS2 350 alignment contig and amino acid translation of the consensus sequence.

Figure 4.17 Consensus sequences and amino acid translations of NBS-profiling bands. The p-loop domains, which the NBS-specific primers NBS2 and 3 target, is underlined in red. Regions that showed high degrees of variability are boxed in blue. a. NBS2 290, b. NBS3 290 and c. NBS2 350.

The consensus sequences of all three NBS bands were BLASTX searched for homology with DNA and amino acid sequences held in the NCBI database. The top three BLASTX hits for each NBS band, along with hits to known resistance genes and important sequences are given in Table 4.7.

Table 4.7. BLASTX hits of the three cloned NBS bands. The three tables illustrate the 1st three most significant homologies and hits to important proteins.

A. BLASTX hits for NBS3 290 and NBS2 290 consensus sequences

Predicted gene	Accession number	E-value	Per cent homology	Species of origin
NBS- LRR type disease resistance protein	AAX85457	$1e^{-10}$	75 %	Zea mays
NBS-LRR disease resistance protein	CAD45034	$5e^{-10}$	87%	Hordeum vulgare
NBS-LRR type disease resistance protein	AAX94877	$2e^{-08}$	68 %	Oryza sativa
RPR1	BAA75812	$3e^{-07}$	84 %	Oryza sativa
Disease resistance gene homologue 9N and 1A	AAC99466 and AAC99464	$8e^{-04}$	62%	Brassica napus
LRR14	AAK20742	0.003	60 %	Triticum aestivum
RPM1 homologue	BAA87943	0.032	59 %	Arabidopsis thaliana
NBS/LRR disease resistance protein RPM1	AAD41050	0.041	58%	Arabidopsis lyrata
RPM1	CAA61131	0.041	58 %	Arabidopsis thaliana
Rust resistance protein M	AAB47618	0.12	60 %	Linum usitatissimum
Resistance protein RPP13	AAK62782	0.041	66%	Arabidopsis thaliana

B. BLASTX hits for NBS2 350 consensus sequence

Predicted gene	Accession number	E-value	Per cent homology	Species of origin
NBS-LRR type disease resistance protein	CAD45030	$5e^{-39}$	85 %	Hordeum vulgare
NBS-LRR type disease resistance protein	BAD69099	$2e^{-29}$	83 %	Oryza sativa
NBS-LRR type disease resistance protein	BAD69096	$2e^{-28}$	81%	Oryza sativa
Disease resistance protein RPM1 homologue	AAD27570	$4e^{-07}$	56%	Sorgum bicolor
Pib	BAA76282	$8e^{-06}$	57%	Oryza sativa
Stripe rust resistance Yr10	AAG42168	$2e^{-05}$	54%	Triticum aestivum

The majority of BLASTX sequence homology hits for the NBS bands were for NBS-LRR type resistance proteins. Of the few known resistance genes that the NBS2 350 band showed homology to, the stripe rust resistance gene Yr 10 was the most important due to the fact that it is a wheat yellow rust resistance gene. The BLASTX alignment of NBS2 350 and Yr 10 are shown in Figure 4.18. NBS2 290 and NBS3 290 showed the same BLASTX hits and of the few known resistance genes that they showed homology to, LRR14 was most important because it is a wheat protein. The BLASTX alignment of NBS2 290 and LRR14 are shown in Figure 4.19. As seen in both figures, the p-loop motif (GMGGLGKT) is an area of strict conservation, but more importantly there is homology outside of this motif between the cloned bands and known resistance genes.

```
gi|11990500|gb|AAG42168.1|  stripe rust resistance protein Yr10 [Triticum aestivum]
      Length=824

 Score = 50.1 bits (118),  Expect = 2e-05
 Identities = 33/102 (32%), Positives = 56/102 (54%), Gaps = 7/102 (6%)
 Frame = +2

Query  38   KLKDRHRIASQIRDLKARVEEVSNRNTRYNLITVDASSSIDEVNSYTEDIRNHSASNIDE  217
            K K RH+I   I+D+K+R++EVS+R  RY + +V    +     + T+ +R  +     E
Sbjct  112  KGKIRHKIGIDIKDIKSRIKEVSDRRERYKVDSVAPKPT----GTSTDTLRQLALFKKAE  167

Query  218  AELVGFAKAKQELIEMVDVNSR--DGLCKMIFLVGMGGLGKT  337
             EL+G  +   ++++M+           KM+ +VG GGLGKT
Sbjct  168  -ELIGTKEKSLDIVKMLTEGDEVFKKHLKMVSIVGFGGLGKT  208
```

Figure 4.18. NBS2 350 BLASTX hit to Yr 10. The sequence homology between the two sequences is indicated in between the sequences. Query = Yr 10 stripe rust resistance protein, Subject = NBS2 350 cloned band.

```
gi|13377505|gb|AAK20742.1|  LRR14 [Triticum aestivum]
      Length=926

 Score = 43.1 bits (100),  Expect = 0.003
 Identities = 23/48 (47%), Positives = 29/48 (60%), Gaps = 9/48 (18%)
 Frame = +3

Query  171  DEDLVGIGENRGKLTEWLI---------TDEKETTVITVSGMGGLGKT  287
            DEDLVGI EN+GKL +WL           ++  + V TV GM G+GKT
Sbjct  163  DEDLVGIEENKGKLVKWLTPGAGGDGDDLEQSSSKVTTVWGMPGVGKT  210
```

Figure 4.19. NBS2 290 BLASTX hit to LRR14. The sequence homology between the two sequences is indicated in between the sequences. Query = LRR14 wheat protein, Subject = NBS2 290 cloned band.

5. DISCUSSION

In poor, more marginal wheat growing areas of Turkey, where durum wheats are preferred because of their drought tolerance, the deployment of disease resistance genes is the only economically viable option. The utilization of yellow rust resistance genes seems to be the most reliable, economic and environmental friendly means of yellow rust disease control (Göçmen, 2001). This study has strived to characterize yellow rust resistance in a durum wheat population using a marker system that targets regions of the wheat genome which may be functionally related to disease resistance.

5.1. Characterization of Seedling Yellow Rust Resistance

The extensive of resistance to Yr seen in the Kunduru-1149 X Cham-1 cross (Göçmen *et al*. 2003) in the field was also observed at the seedling stage, indicating that much of the resistance segregating in this population is probably seedling expressed. The virulence profile of WYR 85/22 indicates that the seedling resistance in the Kunduru-1149 x Cham-1 population is not due to *Yr2, Yr6, Yr7* or *Yr9* but to unidentified seedling expressed resistances, probably due to multiple genes of major and minor effect leading to a genetically complex resistance. The X^2 value obtained with a R:S ratio of 17.75:1 was 0.2151 which indicates 50-75 % probability of the yellow rust resistance in this population being explained by four major genes.

The isolate used in this study, WYR 85/22, was only a representative of the isolate used by Göçmen *et al.* (2003). The isolate WYR 85/22 may have recognized yellow rust resistance genes in the population that are different from those recognized by the isolates used by Göçmen *et al.*,(2003). The field isolate mixture used by Göçmen *et al*. (2003) may have been virulent to the two additional seedling resistance genes found in this study. However, if that is not the case, then much of the field resistance in the population could be due to these two seedling resistance genes.

The yellow rust resistance phenotype was generally conserved between adult and seedling growth stages. However, the 8 seedling susceptible lines differed considerably for

field resistance including two lines (22 and 30) that were resistant in the field (Göçmen *et al*., 2003). This may indicate that this population contains APR in addition to the seedling resistance. However, due to the different yellow rust isolates used in the adult and seedling studies, no definitive conclusions can be made to the nature of resistance in this population, i.e. whether the adult plant resistance seen in the field (Göçmen *et al*., 2003) was also expressed at the seedling growth stage. Although, the location of the seedling and adult plant resistance to the same markers on 1BL would suggest that some of the field R genes were seedling expressed.

Seedling yellow rust resistance tests revealed that 25 lines of the population were more susceptible than the susceptible parent Kunduru-1149. In field tests, performed by Göçmen *et al.* (2003) only one line (Line 131) was more susceptible than Kunduru-1149 in the field. This suggests that Kunduru-1149 has seedling resistance to yellow rust, which was detected by WYR 85/22. Residual resistances in lines generally considered susceptible have been observed previously, for example several reports suggest that APR genes to powdery mildew in wheat have been contributed by the susceptible parent, attributed to effects of defeated seedling resistance genes. Ramburan *et al.* (2004) propose that resistance genes to yellow rust may be retained even when a line has been selected for susceptibility. A number of important Australian wheat cultivars, that were considered susceptible, were shown to have significant degrees of resistance (Bariana and McIntosh 1995). This led to the decision that only cultivars that developed a disease rating of 80 MS-S or higher at the post- heading stages should be regarded as susceptible. Kunduru-1149 had a score of 60 S in field trials and by this definition may not be fully susceptible to yellow rust and may have seedling resistance to yellow rust.

Another explanation, equally plausible, stems from an observation during SSR marker analysis. Four of the 39 SSR markers tested on the lines of the subpopulation carried a different allele in the population from that observed for Kunduru-1149. This suggests that the Kunduru-1149 seed batch given for this study may be different from the Kunduru-1149 seed batch used to produce the cross, which is possible as Kunduru-1149 has 8-10 different sub-types (Belgin Göçmen, personal communication). Other well-know varieties also have many sub-types, such as Chinese Spring (Lesley Boyd, personal communication) and Bobwhite. However, this possibility does not affect the results of this

study as markers linked to resistance were found and there were no discrepancies associated with the Cham-1 alleles.

Even though the population was an F7 RIL population at the time of the seedling yellow rust resistance tests, 32 of the 150 lines were segregating for the seedling yellow rust resistance phenotypes. These lines are not useful for genetic dissection of resistance as they are not yet homozygous and were thus omitted from the marker analysis. This reduced the number of lines usable for marker analysis and coupled with the limit on time and costs, marker analysis was carried out on a subpopulation of the 150 RIL population.

5.2 Linkage Analysis of Yellow Rust Resistance

Linkage analysis of SSR and NBS-profiling markers revealed two QTLs on the long arm of chromosome 1B. In durum wheat, as is the case for bread wheat, group 1 chromosomes are important in wheat breeding as they contain regions of major interest to breeders for biotic stress resistance and grain quality, including several genes for resistance against the three rusts and powdery mildew (Nachit *et al*., 2001).

There are numerous rust resistance genes on chromosome arm 1BL; The leaf rust resistance genes *Lr 24, Lr 26, Lr 33* and *Lr 44*, the stem rust resistance genes *Sr 14 ,Sr 24* and *Sr 31* and finally, the yellow rust resistance gene *Yr 29* (www.cdl.umn.edu). *Yr 29* was mapped to the distal region of chromosome 1BL (Bariana *et al*., 2001), but is an APR gene and the resistance in this population appears to be seedling resistance.

These factors indicate that the yellow rust QTL characterized on 1BL may be a novel resistance locus.

5.3 Sequence Analysis of Cloned NBS Bands

A study conducted by Meksem *et al.,* (2001) involving AFLP analysis on hexaploid wheat revealed that for the sequence analysis performed an average of 6 bands, with a range of 1-15 bands, composed each AFLP band. These fragments were of identical size but differed in sequence, with the same selective bases at the restriction enzyme cut sites.

Only one of these bands corresponded to the original polymorphism, so during cloning there were superimposing bands in addition to the actual polymorphic band inserted into the cloning vector. Random cloning of isolated inserts may be misleading due to this contamination of superimposing bands. It was predicted that this possibility might be overcome through the use of SSCP analysis. However it was observed that a large number of clone types were seen with SSCP analysis due to the degeneracy of NBS-profiling primers.

The degeneracy of the NBS-profiling primers would increase the number of same size bands that are different in sequence but observed as a single band during scoring and cloning. Another reason may be amplification of mistakes introduced into the inserts during PCR. Even though the clones looked so dissimilar on the SSCP gels, upon sequencing, it was observed that these classes all actually were of essentially the same sequence, with only single base-pair differences, mostly seen in the domain specific primer (NBS2, NBS3 and NBS5) regions.

In general, the sequence homology between NBS LRR type resistance genes seems to be limited to small conserved motifs and domains, while the interspersing regions are usually very diverse and lack conservation (Dilbirliği and Gill, 2003). The NBS-profiling primers NBS2 and NBS3 amplify away from the p-loop towards the CC domain and thus amplify an interspersing region, so little or no level of conservation is expected. The sequence conservation level of various domains of NBS LRR type resistance genes seems to be variable, the NBS domain is 23 % conserved, while the CC and LRR domains show 31 and 28 % conservation respectively (Dilbirliği and Gill 2003). Considering this overall low level of conservation, the cloned polymorphic NBS-profiling bands showed a large amount of homology to putative and confirmed resistance gene sequences in the NCBI database.

5.4 Validation of NBS-Profiling as a Marker System

A recent study comparing NBS-profiling and AFLP and SSR profiling in a durum wheat collection performed by Mantovani *et al*. (2004) has shown that NBS-profiling can be used with confidence to assess genetic diversity. But the usefulness of this method to

characterize resistances in wheat populations remains to be confirmed. In this study, 14 polymorphic bands, all generated using the p-loop consensus sequence were identified in a population segregating for resistance to yellow rust. These 14 polymorphisms were found using only 3 primer-restriction enzyme combinations, indicating a high rate of polymorphism for NBS-profiling as a marker technique.

NBS-LRR genes are the most abundant resistance gene family in plants. Apart from disease resistance, no other function has been ascribed to these genes, suggesting they may all be involved in plant defense (Ayliffe and Lagudah, 2004). To date about 150 NBS-LRR encoding genes representing ~0.5 % of all predicted open reading frames (ORFs) have been identified in Arabidopsis (Meyers *et al*., 2003). 600 NBS-LRR genes have been identified in the rice genome (Ayliffe and Lagudah, 2004). The wheat genome is about 35 times larger that rice (Oryza sativa L.), although both belong to the Poacaea family (Erayman, 2004). Despite this, because most of the extra DNA in wheat is non-coding, the number of expressed NBS-LRR type resistance genes in rice (~600) is probably the same as the number of expressed NBS-LRR type resistance genes in diploid wheat. This leads to a predicted number of NBS-LRR genes in wheat to be approximately 1 200. Dilbirliği *et al*., (2004) conducted a study to identify expressed resistance genes in wheat in which they identified 184 putative expressed resistance genes, 87 being of the NB-LRR gene type. This indicates a very low percentage of expressed NBS-LRR genes, which limits NBS-profiling as a functional gene marker system.

Of the three sequenced NBS bands, two (NBS2 290 and NBS3 290) encoded protein sequences with many stop codons, suggesting that neither represents an expressed NBS-LRR gene. This may be due to the fact that *MseI* was used to produce the polymorphic bands and the *Mse I* enzyme restriction sites are known to be rare in coding sequences (Mantovani *et al*., 2004). As an improvement of this study, replacement of *MseI* with another restriction enzyme would be considered based on these findings.

Functional R-genes appear to be nestled among multi-copy non-functional R-genes and pseudogenes that show structural similarity to expressed R-genes. During random cloning and amplified fragment (band) isolation the abundance of these sequences appears to out-compete functional R-gene sequences (Dilbirliği *et al.,* 2004). For example of the

~200 RGAs isolated and analyzed, only one was shown to be functional (Dilbirliği *et al.,* 2004) and for the *RPP5* locus in Arabidopsis only three full-length and functional genes have been identified out of 16 paralogues studied (Ellis *et al*., 2000a). However, RGAs can be useful in MAS as RGAs can be near functional genes and so can be used as tags for these genes in MAS. NBS-profiling offers an improvement on this line of thinking as a potential source of markers that are linked to resistance genes to be introgressed into varieties that are more reproducible that RGAs.

The fact that interspersing regions between conserved NBS-LRR motifs are not conserved (Dilbirliği *et al.,* 2004) makes the finding of this study very relevant. This is because, even though the cloned bands encompassed regions of a possible gene that are not predicted to be conserved, BLASTX homology searched revealed that the cloned bands were up to 85 % homologous to NBS-LRR type resistance genes and that this homology is not only restricted to the p-loop motif used as an NBS-specific primer.

In summary, a high level of seedling resistance was seen in the population which could be explained by 4 major genes. In quantifying resistance, two QTLs on chromosome 1BL (APR and seedling) were detected. 3 NBS-Profiling bands were cloned and sequenced. These sequences showed homology to known and predicted NBS-LRR type resistance genes in plants. This data confirms NBS-profiling as a useful marker technique to identify RGAs.

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