Identification of Molecular Markers Associated with Rust Resistance in Bread Wheat (*Triticum aestivum* L. em. Thell)

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by

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To My
Father

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SURESH NAIK

DECLARATION

Certified that the work incorporated in the thesis "**Identification of Molecular Markers Associated with Rust Resistance in Bread Wheat (*Triticum aestivum* L. em Thell)"** submitted by Mr. Suresh V Naik was carried out by him under our supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "**Identification of Molecular Markers Associated with Rust Resistance in Bread Wheat (*Triticum aestivum* L. em Thell)"** submitted for Ph.D. degree of University of Pune has not been submitted by me for a degree of any other University.

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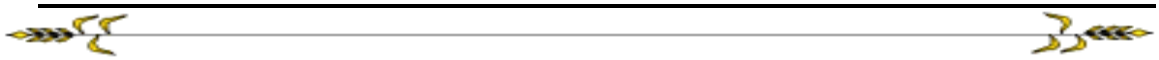
LIST OF ABBREVIATIONS

ABBREVIATIONS	WORD(S)
A	adenine
APR	adult plant resistance
AFLP	Amplified fragment length polymorphism
bp	base pairs
C	cytosine
CAPS	cleaved amplified polymorphic sequence
cv.	Cultivar
CIMMYT	Centro Internacionale de Mejoramiento de Maiz y Trigo
CTAB	Hexacetyltrimethyl ammonium bromide
dATP	deoxy ribose adenosine tri-phosphate
DNA	deoxy ribose nucleic acid
DWR	Directorate of Wheat Research
EDTA	Ethylenediamine tetrachloroacetic acid-sodium salt
EST	expressed sequence tag
G	guanine
H	heterozygous
ha	hectare
IAA	isoamyl alcohol
ISSR	inter-simple sequence repeat
IPTG	Isopropylthio-*b*-D-galactoside
ITMI	International Triticeae Mapping Initiative
kb	kilobases
LB	Luria-Bertani medium
Lr	Leaf rust
LRR	lucine rich repeats
M	mole
MAS	marker-assisted selection
mM	millimoles
$MgCl_2$	magnesium chloride
min	minutes
ml	millilitres
m.t	million tones
N	normal (solution)
NBS	nucleotide binding sites
NaCl	sodium chloride
NaOH	sodium hydroxide
NEB	neutral electrophoretic buffer
ng	nanograms
PCR	polymerase chain reaction
PVP	polyvinyl pyrrolidone
QTL	quantitative trait loci

R	resistant
RAPD	random amplified polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RIL	recombinant inbred line
RGAs	Resistance Gene Analogues
S	susceptible
s	seconds
SCAR	Sequence Characterized Amplified Region
SDS	sodium dodecyl sulphate
Sr	stem rust
SSC	saline sodium citrate
SSR	simple sequence repeat
STMS	sequence-tagged microsatellite site
STS	sequence-tagged site
T	thymine
TBE	Tris-borate EDTA
T_m	melting temperature
USA	United States of America
V	Volts
var	variety
W	Watts
X-gal	5-Bromo-4-chloro-3-indolyl-*b*-D-galactoside
Yr	yellow rust
µg	microgram
µl	microlitre



ABSTRACT

GENETIC IMPROVEMENT OF WHEAT INVOLVING PYRAMIDING OF RUST RESISTANCE GENES

Wheat surpassed rice in the 1970s to become the most consumed food grain in the world. This is evident from the 1996-97 worldwide production estimates of 579 million metric tons for wheat as compared to 573, 558, and 132 million metric tons worldwide for corn, rice, and soybeans, respectively. However, there is little doubt that the worldwide wheat production will have to be increased in the near future to help feed the world's growing population. It is also true that increases in wheat production will need to stem from genetic improvements because new arable croplands will not be available on a very large scale.

Leaf rust, stem rust and stripe rust represent the major diseases in wheat and their management continues to be a major challenge to the wheat breeders in India. One promising approach to overcome this problem is to develop germplasm carrying combination of several effective genes. The selection of genotypes carrying two or more genes using traditional host-parasite interaction is very time consuming and often not possible due to lack of isolates with specific virulence and difficulty of identifying one seedling resistance gene in the

presence of another gene. Identification of molecular markers for resistance genes can facilitate gene pyramiding into one cultivar in less time and make it more cost effective (Tanksley *et al.,* 1989).

There are now more then 50 various loci tagged using molecular markers, majority being those of disease resistance. The next important stage in the marker development will be the implementation of markers into breeding programs. A revision of current breeding methods and close interaction between the molecular laboratories and breeders will be crucial to this next stage. However, there are few, if any, published reports of disease gene pyramiding or deployment using DNA markers, though breeding, even with markers, can take years before the actual release of a new cultivar.

My thesis work represents a part of the continuing effort to identify molecular markers linked to the rust resistance genes important for Indian rust breeding programme. I have selected a leaf rust resistance gene *Lr28* of alien origin (from *Aegilops speltoides*) and a stem rust that is of wheat origin (from cv Webster).

IDENTIFICATION OF MOLECULAR MARKER LINKED TO *Lr28*

Riley and co-workers transferred a leaf rust resistance gene, *Lr28*, from *Ae. speltoides* into hexaploid wheat. The chromosome location of *Lr*28 was determined by monosomic analysis, and telocentric mapping, and this gene was mapped 39 centimorgens from centromere on the long arm of wheat chromosome 4A (McIntosh *et al*, 1982).

For present study, seven resistant NILs in different Indian varietal backgrounds, along with the donor source stock (CS 2A/2M#4/2) from which *Lr28* was transferred in the Indian varieties, were used to identify RAPD markers linked to the resistance gene *Lr28*. To confirm the linkage of the marker with the rust resistance gene, F_3 families developed as described below were tested.

NIL HW-2035 was crossed to the recurrent parent NI-5439, F_2 plants were generated from individual F_1 seeds and about 50 F_2s were advanced to the F_3 generation and were classified for resistance/susceptibility using a mixture of leaf rust pathotypes applied as a suspension at seedling stage.

DNA was extracted from all the above-mentioned plant material and subjected to PCR screening was done using 80 RAPD primers. The parental analysis was carried on the enriched DNA for the low copy sequences (Eastwood *et al*,. 1994; William *et al*., 1997). A band of 378 bp was observed to be polymorphic between resistant and susceptible lines.

The 378bp polymorphic band corresponding to the resistant phenotype (of the *Lr28* donor) was eluted from agarose gel, purified, cloned and transformed into the *E. œoli* strain. Plasmid was isolated from the transformed bacterium and was sequenced by Sanger's dideoxy chain termination method (*Sanger et al.*, 1977). This sequenced information was used to design specific primers that could serve as STS primers specific to the translocated fragment- a forward primer of 20bp *Lr*28-01, 5'CCCGGCATAAGTCTATGGTT3' having the original 10bp of RAPD primer along with the next 10 internal bases and a reverse primer of 20bp, *Lr*28-02, 5'CAATGAATGAGATACGTGAA3' having entirely internal sequence next to the RAPD primer, were designed.

The STS marker so obtained was further confirmed by bulk segregation analysis of F_3 families and was found to be consistently present in the NILs, resistant F_3 bulk and resistant F_3 lines while absent in recurrent parents, susceptible F_3 bulk and susceptible F_3 lines.

EFFORTS TO IDENTFY MARKERS LINKED TO THE *Sr30*

The origin of this gene is common wheat cv. Webster that was later transferred into other cultivated bread wheat varieties. It is genetically independent of *Pm2* (on 5DS) and *Lr*1 and located on 5DL (Knott & McIntosh, 1978).

In an effort to produce a better susceptible parent, a multiple cross program was taken up, resulting in LMPG-6 which was used as recurrent parent in the cross with Webster. During the backcrossing, plants or families were occasionally observed that were more resistant than normal. When these lines were selected and tested, there appeared small differences among the lines, although all lines gave resistant reaction with the nine races. Accordingly, they were numbered as

LMPG-1, LMPG-2 and LMPG-3. The difference in the reaction level was attributed to the possible presence of modifiers or a second gene linked to the *Sr*30 resistance (Knott & McIntosh, 1978).

The plant material to work on *Sr30* was identified from the "Wheat Rusts: an atlas of resistance genes"(McIntosh *et al.,*1995). The three NILs (LMPG-1, LMPG-2, LMPG-3), which are a product of 13 backcrosses, the recurrent parent LMPG-6, and the two populations, Population 1 with 21 lines and Population 2 with 28 lines were obtained from Dr. D. Knott, University of Saskatoon, Canada. The segregating population that would help the mapping of the putative marker was advanced to the F_3 and was scored for disease reaction. Since this population was small, we developed a second population by crossing LMPG-6 X LMPG-2 and generated 124 F_3 lines. NILs along with the donor parent Webster were used for the initial marker screening.

DNA was extracted from all the above-mentioned plant material and PCR screening was carried out using 800 RAPD and 100 ISSR primers. Several restriction enzymes were used to prepare the survey blots for the *Sr30* parent and NILs and hybridized with the RFLPs located on the 5DL. However, no polymorphism was observed in the entire comprehensive screening program.

Wheat sequence tagged microsatellites (STMS), located on the 5DL(Roder *et al.,* 1998b), were utilized to screen the NILs and cv. Webster for the *Sr30* work. Though, one microsatellite was identified segregating in the population, the polymorphism did not co-segregate with the gene, based on the field infection scores. When confirmed using 5DL deletion lines, the marker was located between deletion 2 and deletion 9, which is closer to the centromere than the gene, which is found to be located towards the tip of 5DL (Knott, 1990).

In another effort to identify a polymorphic band, AFLP analysis was carried on the parental lines of *Sr30* using 64 combinations of primer/enzyme and the polymorphic combinations were tested on the population. The segregating combinations were mapped along with mapped loci using mapmaker but no polymorphism could be located on 5DL.

FUTURE PERSPECTIVES

The main goal in identification of markers is their usefulness in the screening of the breeding material by determining the presence of a specific gene in a complex genetic background of other resistance genes apart from using tightly linked markers for chromosome landing, and gene cloning.

A marker assisted selection scheme could allow plant breeders to efficiently select for a resistance gene without waiting for its phenotypic expression of the gene in plants. Indirect selection using DNA markers would also be helpful in elucidating rarely-occurring recombination between resistance genes, thus facilitating the combination of these closely-linked resistance genes into cultivars. Although no virulence for *Lr28* has been found in India as of yet; glass-house cultures have produced virulent mutants in Australia (R.A. McIntosh, personal communication) indicating that development of virulent strains in India cannot be ruled out.

To prevent rapid breakdown of seedling resistance genes once they are integrated into new wheat varieties, it is suggested that such genes should be used in combination with other leaf rust resistant genes (Roelfs *et al*., 1992) preferably with adult plant resistance genes. However, there are no reports of attempts in India to pyramid rust resistance genes in wheat using molecular markers. In order to combine several genes in the same line, markers for all of these genes need to be identified. My future work will, therefore, concentrate on taking the *Sr30* work further and identifying markers for the additional leaf rust resistant genes that are effective in India.

CHAPTER 1

REVIEW OF LITERATURE

1.1 WHEAT: THE MOST IMPORTANT CEREAL CROP OF THE WORLD

1.1.1 World and Indian Scenario: - Bread wheat (*Triticum aestivum* L. emend Thell.) is a disomic allohexaploid (2n = 6X = 42, AABBDD) crop with seven pairs of chromosomes derived from three different related grass ancestors. Allopolyploids arise from processes of interspecific hybridization followed by spontaneous chromosome doubling and contain the entire genome of two or more species in the homozygous condition. Hexaploid wheat originated as a result of two separate amphiploidization events (Figure 1.1).

Wheat surpassed rice in the 1970s to become the most consumed and most important food grain in the world. It is the staple food of nearly 35 per cent of the world population, and it is predicted that demand for wheat will grow faster than for any other major crop (RAJARAM, 2000). The global estimate of wheat production is 584 million tons, which is higher than that for corn, rice, and soybeans (MARATHÉE & GOMEZ-MACPHERSON, 2001). Wheat production in India, which was just 6.4 million tons in 1950, reached 71 million tons in 1999 (RAO *et al.,* 2001). The major wheat producing area in the country are covered by states of Punjab, Haryana, Uttar Pradesh, Madhya Pradesh, Rajasthan, Bihar, West Bengal, Maharashtra and Karnataka. The Indo-Gangetic plains account for nearly 70% of the wheat area and about 90% of total wheat production in the country (Figure 1.2).

1.1.2 Challenges Ahead: - The forecasted global demand for wheat in the year 2020 varies between 840 and 1,050 million tons. To reach this target, global production will need to increase by 1.6 to 2.6 per cent annually from the present production level of 560 million tons. Increases in realized grain yield have provided about 90 per cent of the growth in world cereal production since 1950 and by the first decade of this century most of the increase needed in world food production must come from higher absolute yields. For wheat, the global average grain yield must increase from the current 2.5 t ha^{-1} to 3.8 t ha^{-1}. In 1995, only 18 countries worldwide had average wheat grain yields of more than 3.8 t ha^{-1}, the majority being located in northern Europe (RAJARAM, 2000).

Even though wheat production in India has been on the rise, with the burgeoning population, this cannot be sustained without further increase in productivity, particularly since the area under wheat is not likely to increase. The population of India has become about 1 billion by the end of last century, and with present growth rate of 1.8% it is estimated to reach 1.3 billion by 2020 AD. Assuming a 20 per cent more per capita requirement of wheat, India has to produce over 109 m.t. by 2020 AD. This poses a challenge of producing extra 40 m.t., which requires a growth rate of 1.8%/year as against the current 1%. Effectively, the current national average has to increase from 2700 Kg/ha to 4200 Kg/ha.

1.1.3 Impediments For Higher Yields: Two main factors, which destabilize wheat yields, are biotic and abiotic stresses, which the crop is subjected to (Table 1.1). Many environmental factors which induce abiotic stress in wheat are drought, causing poor seedling emergence/establishment and stress during life cycle; water logging; high and low (freezing) temperatures; wind (lodging); or mineral stress (deficiency or toxicity). In extreme cases, abiotic stress can cause very high yield loss in wheat. For example in Romania,

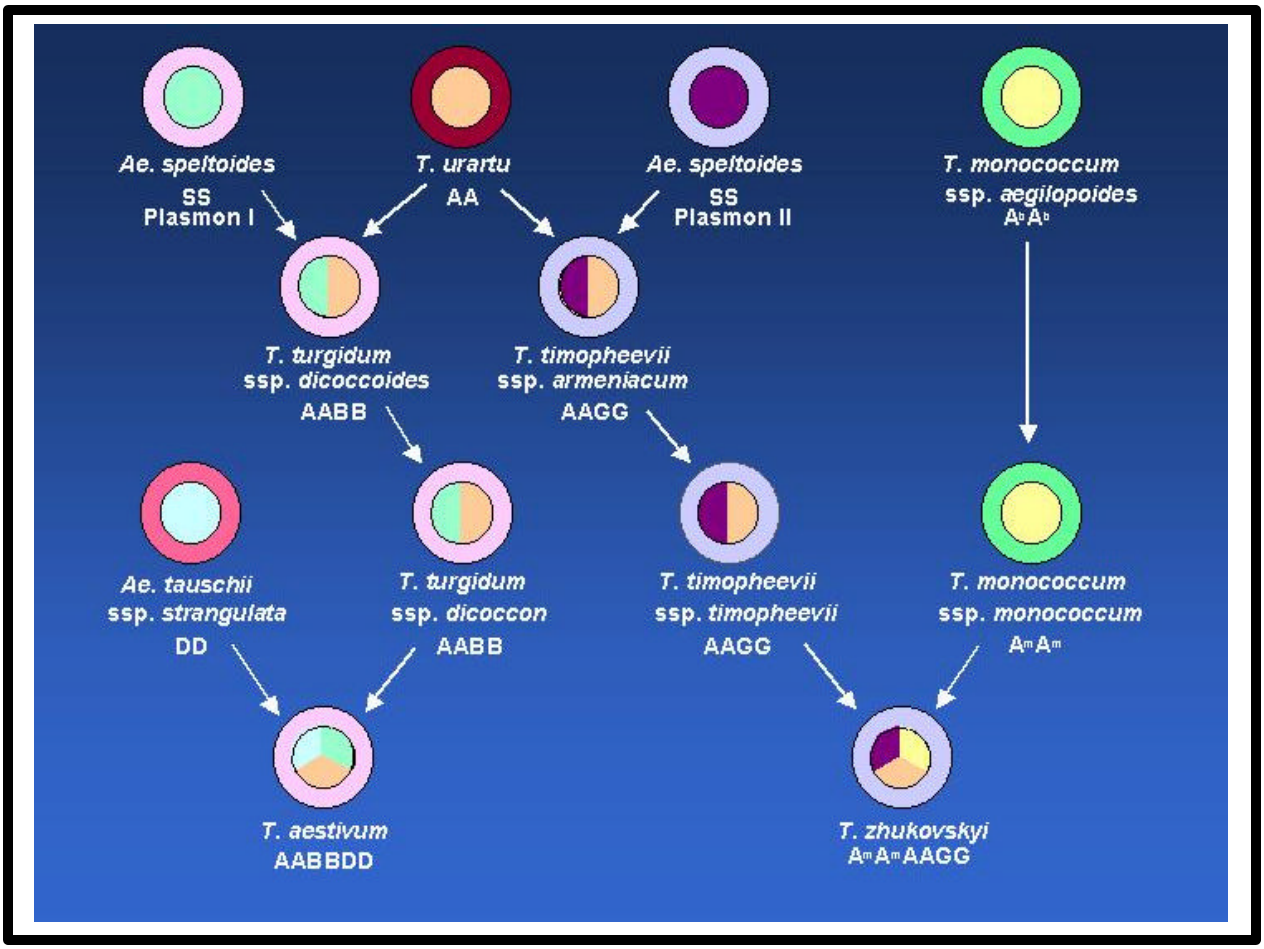


Figure 1.1: The two evolutionary lineages of wheat. In the first lineage, the diploid *Aegilops speltoides* (SS) with plasmon type I hybridized with the diploid *Triticum urartu* (AA) to form the tetraploid *T. turgidum* ssp. *dicoccoides* (AABB). *T. turgidum* ssp. *dicoccon* (AABB), a domesticated form of ssp. *dicoccoides*, hybridized with the diploid *Ae. tauschii* ssp. *strangulata* (DD) about 8,000 years ago to give rise to the hexaploid bread wheat *T. aestivum* (AABBDD). In the second lineage, the diploid *Ae. speltoides* (SS) with plasmon type II hybridized with *T. urartu* (AA) to give rise to the tetraploid *T. timopheevii* ssp. *armeniacum* (AAGG). *T. timopheevii* ssp. *timopheevii* is the domesticated form of ssp. *armeniacum*. The diploid *T. monococcum* ssp. *monococcum* (A^mA^m), which is the domesticated form of *T. monococcum* ssp. *aegilopoides* (A^bA^b), hybridized with *T. timopheevii* ssp. *timopheevii* (AAGG) to give rise to the hexaploid *T. zhukovskyi* (A^mA^mAAGG) (John Raupp, KSU, personal communication).

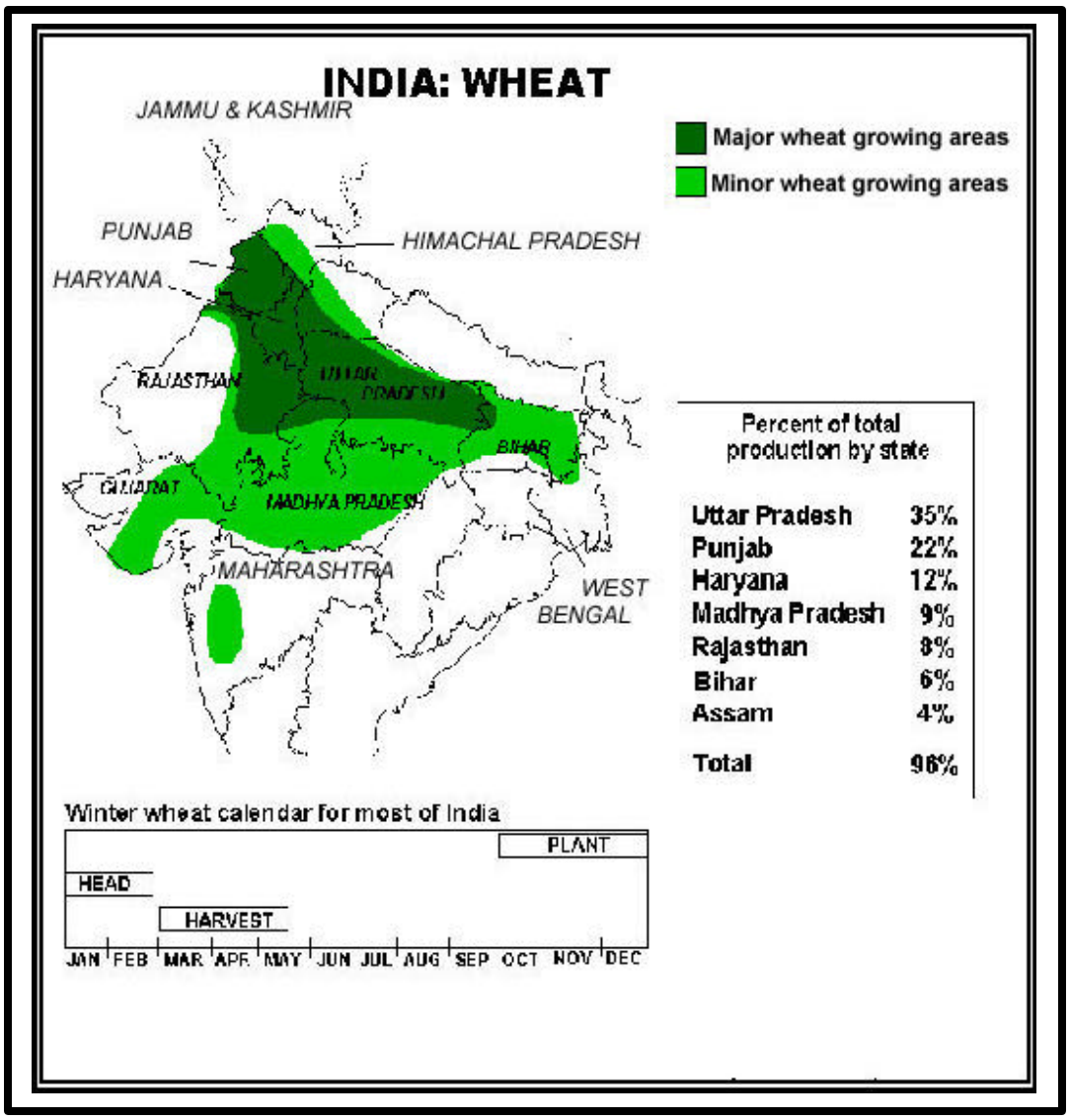


Figure 1.2: Wheat growing areas in India and individual contribution to total production.

that yield losses due to abiotic stress may amount to 3 million tons compared to 0.84 million tons for losses due to biotic stress (SAULESCU *et al.*, 1990).

In biotic stress, the main problem facing wheat breeders to develop varieties adapted to a particular environment is to minimize potential losses due to pests and diseases. Pests cause major losses in crop yields in all areas of wheat production, worldwide. In parts of the world including the African continent losses due to pests can be higher than those due to other diseases (WORLAND & SNAPE, 2001). The main pests attacking wheat include insects, mites, molluscs, nematodes, birds and mammals. Additionally insects are vectors for viral diseases that can be transmitted to wheat.

Among diseases, WIESE (1987) identified over 40 fungal, 32 viral, and 81 bacterial diseases that attack wheat plants at various growth stages. It is difficult to obtain accurate estimates of crop losses to the different diseases. The British Agrochemical Association in 1993 suggested that under farm conditions where crop rotations, good husbandry and applications of pesticides are practiced, losses due to diseases could be around 13%, whilst under conditions where crop protection measures are not taken, losses could be as high as 50% (WORLAND & SNAPE, 2001). It is the goal of plant breeders to introduce genetic resistance into their varieties to minimize the use of chemical protection measures, and to minimize losses due to diseases.

1.2 WHEAT RUSTS AS IMPORTANT YIELD REDUCERS: - STEM RUST, LEAF RUST & STRIPE RUST

In wheat, rusts have been diseases of great economic importance since the losses caused by these diseases have been widespread. The economical importance of the disease in India can be understood by a rather old quote from the book by SIR ALBERT HOWARD (1909):

"In India the ravages of rust vary greatly from year to year, and it is obvious that anything like an accurate estimate of the annual loss is impossible. Further, the damage done in the great wheat-growing tracts of the North-west is generally slight, while in Bombay, the Central Provinces, and in parts of the United Provinces and Bengal the crop may be reduced 50 per cent or even more. . . . Rust is by no means a modern occurrence in

Table 1.1 : Biotic and abiotic stresses of wheat in India

Zone	Biotic Stresses	Abiotic Stresses
Northern Hill Zone	Stripe rust; Leaf rust; Powdery Mildew; Hill Bunt; Loose smut	Salinity/alkalinity
North Western Plain Zone	Stripe rust; Leaf rust; Karnal Bunt; *Fusarium* head Scab (in durum); Loose smut; Flag smut; Powdery mildew; *Phalaris Minor*, Aphids; Brown wheat mite; Army worm; cutworm; stem borer	Salinity/alkalinity
North Eastern Plain Zone	Stripe rust; Leaf rust; Loose smut; Powdery mildew; *Phalaris Minor*, Ear Cockel disease	Salinity/alkalinity
Central Zone	Leaf rust; Stem rust; *Sclerotium* foot rot; Foliar blight; Termite; Stem borer; Cutworm	Moisture stress; high temperatures during crop period
Peninsular Zone	Leaf rust; Stem rust; *Sclerotium* foot rot; Foliar blight	Moisture stress; high temperatures during crop period
Southern Hill Zone	Leaf rust; Stem rust; Powdery mildew; *Septoria*. Glume blotch	

Source RAO *et al.,* 2001

the wheat fields of India. Sleeman, in 1839, speaking of rust in the Central Provinces, wrote: "I have seen rich sheets of uninterrupted wheat cultivation for twenty miles by ten in the valley of the Narbudda so entirely destroyed by this disease that the people would not go to the cost of gathering one field in four," and further "I believe that the total amount of the wheat gathered in the harvest of 1827 in the district of Jubbulpore was not equal to the total quantity of seed that had been sown."

Stem rust, one of the greatest diseases of all time and also known as black rust, has plagued mankind for thousands of years and is caused by *Puccinia graminis* Pers. f. sp. *tritici* Eriks. E. Henn. It is most important where dews are frequent during and after heading and temperatures are warm, 18-30º C. In India, black rust occurs in almost all the wheat growing areas. In northern India, this rust does not appear before March and by this time the crop reaches

the ‘dough’ stage and hence the damage is not severe. On the other hand, in the peninsular and southern part, it may appear as early as fourth week of November and hence losses to the crop are generally great in this region. Severe disease can cause straw breakage resulting in a loss of spikes with combine harvesting (SAARI & PRESCOTT, 1985) and premature ripening and shrinking of the grains (Figure 1.3) incurring the losses as much as 90% of grain yield.

Wheat leaf rust, an important disease of cooler climates, commonly known as brown rust, is caused by *Puccinia recondita* Robberge ex Desmaz. f.sp. *tritici*. It is most important where dews are frequent during the jointing through flowering stages and temperatures are mild of the order of 15-25º C. It spreads by wind blown uredospores from plant to plant and from field to field until the crop matures (BROWDER, 1971).

Epidemics of brown rust occurred in the north western parts of the country in 1971-72 and 1972-73 causing a loss of 0.8 to 1.0 million tons and 1.5 million tons, respectively (GOVINDU, 1977). Leaf rust causes losses by reducing the number and size of kernels per head, lowering test weights, and by lowering the protein content of the grain. (WATKINS, 1995). Wheat stripe rust, or yellow rust is caused by *Puccinia striiformis* West. Stripe rust resembles leaf rust except that the pustules develop along the leaf veins as long streaks. The pustules of stripe rust, which contain yellow to orange-yellow uredospores, usually form narrow stripes on the leaves in mild attacks, but in more severe attacks pustules also can be found on leaf sheaths, necks, and glumes. The green colour of the leaves fades in long streaks on which rows of small uredosori appear. Stripe rust can attack wheat, barley, triticale, and many other related grasses since the disease is found in temperate areas where cereals are grown. Severe infections can cause yield losses, mainly by reducing the number of kernels per spike, test weight, and kernel quality (SAARI & PRESCOTT, 1985; WATKINS, 1995).

1.3 STRATEGIES FOR RUST RESISTANCE BREEDING:

1.3.1 Problems Associated with Resistance Breeding: Breeding for resistance to rust in wheat has been relatively easy and successful and it

A **B**

Figure 1.3: Comparative figure of A: normal seeds; and B: seeds from rust infected plant (Source www.crl.umn.edu/gifs).

represents a small part of the genotype package that is delivered by the breeder. Breeding for rust resistance involves: the identification of potential sources of resistance, assessment of their effectiveness over sites, seasons, and pathotypes and incorporation of resistance into cultivars. The resistances deployed in agriculture have depended on single identifiable genes and combination of them. The problems come with the genetic plasticity of the pathogen where virulent pathotypes increase in frequency and render the resistant cultivars vulnerable to disease and cause crop losses.

1.3.2 Pyramiding Wheat Rust Resistance Genes: During last 20 to 30 years, much progress has been made in breeding for resistance to the rust disease and several varieties have been developed in India with one or combination of rust resistance genes (Table 1.2). However, genetic erosion caused by modern cultivation procedures has narrowed the genetic base of many crops, including wheat. Many wild relatives and related species represent a large reservoir of useful traits and can be successfully crossed with bread wheat leading to its improvement (JIANG *et al.,* 1994). So far, over 100 rust resistance genes have been identified in wheat and related species and some of them have been introgressed into wheat (MCINTOSH *et al.,* 1995). However, due to narrow genetic base and continuously evolving pathogen races, resistant varieties become susceptible to the disease when grown in vast areas (ROELFS *et al.,* 1992).

Pyramiding of genes is a resistance breeding procedure where more than one gene is brought together to enhance the resistance life of an otherwise better performing variety against the pathogenic races. Thus to prevent the rapid breakdown of seedling resistant genes once they are integrated into new wheat varieties, it is suggested that such genes should be used in combination with other rust resistant genes (ROELFS *et al.,* 1992, JIANG *et al.,* 1994) preferably with an adult plant resistance gene. However, the selection of genotypes carrying two or more genes using traditional host-parasite interaction is very time consuming and often not possible due to lack of isolates with specific virulence and difficulty of identifying one resistance gene in the presence of another gene.

Table 1.2: Genes for rust resistance in some Indian bread wheat varieties.

S. No	Variety	*Lr*	*Sr*	*Yr*
1	C 306	*Lr34+*		*Yr18+*
2	NI 5439	*Lr34+*	*Sr11+*	*Yr2+ Yr18+*
3	UP 262	*Lr23+Lr34+*	*Sr11+*	*Yr2+ Yr18+*
4	WH 147	*Lr13+Lr34+*	*Sr7a+Sr11+*	*Yr18+*
5	K 7410	*Lr13+Lr1+*	*Sr11+*	*Yr2+(Ks)+*
6	HD 2189	*Lr13+Lr34+*		*Yr2+ Yr18+*
7	Lok 1	*Lr13+*	*Sr2+Sr9b+Sr11+*	*Yr18+*
8	HUW 234	*Lr14a+*	*Sr9b+ Sr11+*	*Yr2+(Ks)+*
9	HUW 206	*Lr26+Lr23+*	*Sr31+*	*Yr9+*
10	HD 2285	*Lr23+*	*Sr9b+ Sr11+*	*Yr2+*
11	HD 2329	*Lr13+Lr10+*	*Sr8+Sr9b+ Sr11+*	*Yr2+ Yr18+*
12	VL 616	*Lr3+*	*Sr7a+ Sr11+*	*Yr2+*
13	K 8027	*Lr13+*	*Sr2+ Sr11+*	*Yr2+(Ks)+*
14	HS 240	*Lr26+Lr34+Lr1+*	*Sr31+*	*Yr9+Yr18+*
15	HDR 77	*Lr23+Lr10+*	*Sr9b+ Sr11+*	*Yr2+(Ks)+*
16	K 8804	*Lr26+Lr23+*	*Sr2+ Sr11+*	*Yr9+*
17	MACS 2496	*Lr26+Lr23+Lr1+*	*Sr2+ Sr11+*	*Yr9+*
18	HP 1633	***Lr9+***	*Sr2+Sr7a+ Sr11+*	*Yr2+*
19	WH 542	*Lr26+Lr23+Lr34+*	*Sr31+*	*Yr9+Yr18+*
20	GW 190	*Lr26+Lr23+Lr1+*	*Sr2+Sr31+*	*Yr9+*
21	UP 2338	*Lr26+Lr34+*	*Sr31+*	*Yr9+Yr18+*
22	DL 803-3	*Lr26+Lr23+*	*Sr31+*	*Yr9+*

Source RAO *et al.,* 2001

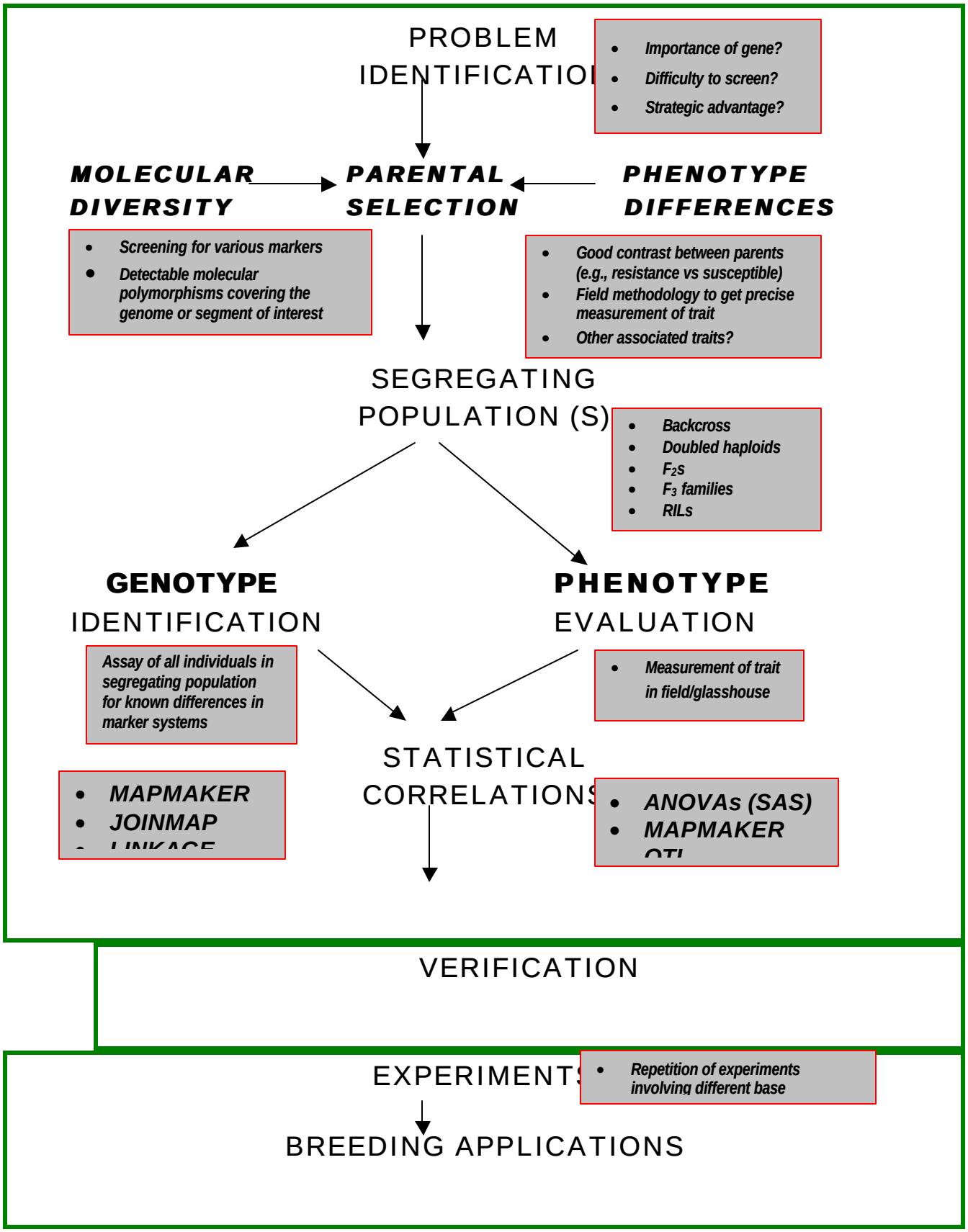
1.3.3 DNA Markers as New Tools for Resistance Breeding: In recent years, DNA based markers have shown great promise in expediting plant breeding procedures. One such application is to determine the presence of the gene in a complex genetic background of other resistance genes. The power to select desirable individuals in a breeding program based on genotypic configuration can be especially helpful if a target trait is complex and time consuming to score. Marker-based breeding may revolutionize the process of cultivar development (Figure 1.4) by eliminating or reducing the need for field trials and making it possible to select individuals or lines with crossovers very near to a gene of

interest, potentially removing “linkage drag” that frequently comes from the donor parent (ZEVEN *et al.,* 1983).

Identification of molecular markers for resistance genes can efficiently facilitate pyramiding major genes into a valuable background in less time and make it more cost effective. In such special cases of disease resistance breeding, marker assisted selection (MAS) takes on special roles, whereby pyramiding several major resistance genes into a valuable genetic background is simplified (TANKSLEY *et al*., 1989). Rather than screening sequentially for the inheritance of single resistance (or simultaneously through progeny screens), individuals that have retained all of the genes of interest can be selected based solely on DNA marker genotype. Similarly, gene deployment can be accelerated through the use of MAS which aims at achieving durable disease protection, in which farmers can grow cultivars with complementary sets of resistance genes with different race-specificities.

For plant breeding purpose, selection for these resistance genes could profitably be employed by selecting them indirectly with adjacent molecular markers. This would speed up the breeding programs (MAZUR and TINGEY, 1995), as one type of molecular analysis could replace several disease tests. Also, indirect selection with molecular marker is very helpful in elucidating rarely-occurring recombination between resistance genes, and in facilitating the combination of these closely-linked resistance genes into cultivars. In many crop species, genes that confer resistance to disease or insect often exhibit dominance and epistasis. As a result, it is generally considered difficult or impossible to accumulate, or pyramid resistance genes with both large and small effects in a single population or cultivars. Selection at major loci generates large nonadditive genetic variation and genetic disequilibria. Phenotypic selection at minor loci is generally ineffective when major genes are also present. Pleiotropic penalties on resistance genes, while major alleles are selected, cause minor resistance alleles to be eliminated from the population (TANKSLEY *et al*., 1989). The cost of producing and releasing a new variety being high, it is not surprising that breeders are now turning to molecular genetic studies to help them assemble optimum combinations of genes that will allow them to produce the ideal varietal phenotype for the farmer of the 21st century. Identification of molecular markers for resistance genes can, therefore, facilitate gene pyramiding into one cultivar in less time

Figure 1.4: OUTLINE FOR THE MARKER ASSISTED BREEDING



- ***Marker Assisted Selection (MAS)***
  -recurrent genome selection
  -single segment introgretion
  -different QTL selection
  -reduction/avoidance of linkage drag

and make it more cost effective. However, there are few, if any, published reports of disease resistance gene pyramiding or deployment using DNA markers, since breeding, even with markers, can take years before the actual release of a new cultivar. Another important point is that in order to combine several genes in the same line, markers for all of these genes need to be identified.

1.4 DNA MARKERS LINKED TO RESISTANCE GENES IN WHEAT: SOME EXAMPLES

During last decade different technologies such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR) or Microsatellites and others have been developed and applied to range of crop species, including wheat. In last few years newer approaches such as Single Nucleotide Polymorphism (SNP), Expressed Sequence Tag (EST), DNA chips and MALDI-TOF mass spectrometry (BONJEAN & LACAZE, 2001) are expected to be widely used for various applications.

Wheat, being a complex genome, the development and application of molecular markers in this species has been lagging as compared to that in other cereals. The haploid DNA content of hexaploid wheat is approximately 1.7 X 10^{10} bp which is about 100 times larger than the *Arabidopsis* genome, 40 times that of rice and about 6 times that of maize. The average wheat chromosme is around 810 Mb, 25 times that of the average rice chromosome (AMURUGANATHAN & EARLE, 1991; BONJEAN & LACAZE, 2001). This huge size arises from the origin of the wheat genome (TALBERT *et al.*, 1998), of its triple structure (ABD genomes) and the inclusion of extensive duplications such that more than 85% of the whole genome is composed of repetitive, highly methylated sequences (Ranjekar *et al.,* 1978; MOORE *et al.*, 1993). The breeding history, including the use of wide crosses to introgress disease resistance genes that has brought extensive

translocations (like 1B/1R) has hampered a clear understanding of this whole complex genome. Non-homoeologous translocations involving 4,5 and 7 group chromosomes in the ancestors of wheat further complicate the wheat genome (LIU *et al.*, 1992).

Though the progress of research in the area of Marker Assisted Selection (MAS) is more in maize, barley or rice, it is no longer recalcitrant to molecular analysis. With utilization of newer and improved DNA markers more than 50 different loci are now tagged, and majority of these loci represent disease those of resistance genes (Table 1.3) (LANDGRIDGE and CHALMERS, 1998, NAIK *et al.,* 2001). Leaf rust resistance gene, *Lr1* (FEUILLET *et al.,* 1995) is an example where RFLP marker was converted into allele specific STS marker. Here, probe PSR 567 was completely linked with the gene in one population with 156 F_2 plants and the marker was assigned to the single copy locus on chromosome 5DL. Twenty-seven breeding lines containing *Lr1* resistance gene in different backgrounds showed the same band as *Lr1*/6*Thatcher when hybridized with pTAG621. There was no recombination between the gene and the marker in the second F_2 population with 165 plants. As stated above, though, the RFLP was converted into STS marker; the marker allele was also found to be present in 50% of the lines not containing *Lr1*. The reason postulated by the authors is the possible origin of the resistance gene as the result of a point mutation from the locus carrying the marker allele (FEUILLET *et al.,* 1995).

Another example is of adult plant resistance gene, *Lr35,* where RFLP marker has been identified to be linked with the locus (SAYFARTH *et al.,* 1999) The polymorphic band BCD260/0.9 was sequenced to construct the STS primer which was used to amplify the DNA from NIL, susceptible parent and the population. The product of STS amplification was again digested with tetra-cutter restriction enzyme *Dde*I and the fragments were separated on the gel to obtain a 450 bp dominant marker, called CAPS (cleaved amplified polymorphic sequence) specific to the resistance. HELGUERA *et al.,* (2000) identified a RFLP marker *Xabc465* linked to the leaf rust resistance gene *Lr*47 and converted it to STS marker, which was completely linked to the gene.

WIILLIAM and co-workers (1997) identified RAPDs for the *Lr34* gene and converted them into RFLP to remove the repeatability problems. The two

Table 1.3 : Reports of resistance gene tagging in wheat.

Resistance Gene Tagged	Technique	Reference
Lr1	PCR, RFLP	Feuillet *et al.,* 1995
Lr3	RFLP	Parker *et al.,* 1998
Lr9	RAPD, PCR	Schachermayer *et al.,* 1994
Lr10	RFLP/STS	Schachermayer *et al.,* 1997
Lr19	RFLP	Autrique *et al*., 1995
Lr20	RFLP	Parker *et al.,* 1998
Lr23	RFLP	Nelson *et al.,* 1997
Lr24/Sr24	RAPD, PCR	Schachermayer *et al.,* 1995
Lr25	RAPD/DGGE	Procunier *et al.,* 1995
Lr27	RFLP	Nelson *et al.,* 1997
Lr28	RAPD/PCR	Naik *et al.,* 1998 (Thesis work)
Lr29	RAPD/DGGE	Procunier *et al.,* 1995
Lr32	RFLP	Autrique *et al.,* 1995
Lr34	RFLP	Nelson *et al.,* 1997
Lr35	ISSR/PCR	Gold *et al.,* 1999; Sayfarth *et al.,* 1999
Lr37	RGA clone	Seah *et al.,* 2001
Lr47	RFLP/STS	Helguera *et al.,* 2000
Yr15	RAPDRFLP	Sun *et al.,* 1997
Yr17	RGA clone	Seah *et al.,* 2001
YrH52	Microsatellite	Peng *et al.,* 1999
Sr5	RFLP	Parker *et al.,* 1998
Sr9e	RFLP	Parker *et al.,* 1998
Sr22	RFLP	Paull *et al.,* 1994
Sr36	RFLP	Parker *et al.,* 1998
Sr38	RGA clone	Seah *et al.,* 2001
Sr39	ISSR	**Gold *et al.,* 1999**
Pm1	RAPD/RFLP	Ma *et al.,*, 1994,Hartl *et al.,* 1995, Hu *et al*., 1997,
Pm2	RFLP	Ma *et al*., 1994,Hartl *et al.,* 1995, Mohler & Jahoor, 1996
Pm3	RFLP	Hartl *et al.,* 1993, Ma *et al*., 1994
Pm4	RAPD	Li *et al.,* 1995
Pm18	RFLP	Hartl *et al.,* 1995
Pm21	RAPD	Qi *et al.,* 1996
T10	RFLP/SCAR	Procunier *et al.,* 1997
Bt-10	RAPD	Demeke *et al*., 1996
Pch-2	RFLP	Delapena *et al*., 1996
H3,H5,H6,H9,H10,H11,H12,H13,H14,H16,H17	RAPD/DGGE	Dweikat *et al.,* 1994, 1997
H21	RAPD	Seo *et al*., 1997
Cre1	RFLP/STS	Williams *et al*., 1994
Cre3/Ccn-D1	RAPD/PCR	Eastwood *et al.,* 1994
Wms1	RAPD/STS	Talbert *et al*., 1996

RAPD markers linked with the QTLs associated with *Lr34* gene putatively conferring leaf rust resistance. DNA bulk segregation analysis was conducted on the population of 77 recombinant inbred lines (RILs) using 400 RAPDs. The polymorphic bands were mapped and loci separated by 2cM were found with 1:1 observed segregation in each of the marker and the leaf tip necrosis loci (*Ltn*) which is considered either as very tightly linked trait or pleiotropic with *Lr34* (SINGH, 1992).

A similar approach was used for tagging the *Lr24* gene, where the RAPD marker OPJ-09_{550} was converted into RFLP that mapped into translocated fragment from *Agropyron elongatum* on the 3DL in wheat (SCHACHERMAYR *et al.,* 1995). Two tightly linked RAPD markers were identified for two alien genes introgressed into wheat *viz. Lr25* (from rye) and *Lr29* (from *A. elongatum*) (PROCUNIER *et al.,* 1995). Similarly another RAPD marker was identified for *Yr*15, a stripe rust resistance gene from *T.* dicoccoides (SUN *et al.,* 1997).

MICHELMORE and co-workers (1991) and MARTIN *et al.* (1991) introduced the modified technique of sequence characterized amplified region (SCAR), wherein the RAPD marker termini are sequenced and longer primers are designed (22-24 nucleotide bases long) for specific amplification of a particular locus. These are similar to STS (OLSON *et al.,* 1989) markers in construction and application. This approach is used in my thesis research to identify marker linked to the translocated segment on 4 AL of bread wheat carrying the leaf rust resistance gene *Lr28.*

A relatively new stripe rust resistance gene *YrH52* from emmer wheat, *T. dicoccoides* was transferred into durum wheat, *T. durum* (THE *et al.,* 1993). Wheat microsatellite markers were screened to identify tightly linked marker for *YrH52* which was mapped on chromosome 1B of wheat (PENG *et al.,* 1999). GOLD *et al.* (1999) also identified completely linked ISSR markers for *Lr*35 and *Sr*39 genes.

1.5 RESISTANCE GENE ANALOGUES (RGAs) IN WHEAT

In recent years, several genes conferring resistance to a wide range of pathogens have been isolated from diverse plant species, and have been classified based on the predicted protein products. Conserved amino acid motifs have been identified based on the sequence comparisons within the classes (BAKER *et al.,* 1997). Nucleotide binding sites (NBS) and leucine rich repeat regions (LRR) are most common motifs and are predominantly present in the protein products taking part in signal transduction and protein-protein interactions. Classical genetic and molecular data show that genes determining disease resistance in plants are frequently clustered in the genome (MICHELMORE & MEYERS, 1998). LAGUDAH and co-workers (1997) reported presence of NBS-LRR type of motif at the *cre3* locus conferring cyst nemetode resistance in wheat. With the availability of around 100 rust resistance genes in wheat (MCINTOSH *et al.,* 1998), the comparison of conserved motifs of these genes can provide information to design the primers to amplify the RGAs using PCR. Similar approach has been reported in potato to isolate RGAs (LEISTER *et al.,* 1996) and to identify the RGA superfamily in wheat using the genomic and cDNA libraries of *Triticum tauschii*, the D genome donor of wheat (SPIELMEYER *et al.,* 1998). The RGA loci so identified mapped across all seven homoeologous chromosome groups of wheat. A resistance gene analog family was cloned from wheat and barley (SEAH *et al.,* 1998). Similarly, major conserved regions, NBS and LRR, from the dicots were used to isolate related gene regions from rice and barley using PCR (LEISTER *et al.,* 1998) As it is evident that the resistance genes appear as clusters of homologues (MARTIN *et al.,* 1993), co-segregating polymorphic fragments may reflect the presence of such clusters. The peptide sequence comparison of dicot and monocot resistance genes revealed shared motifs and did not show any monocot specific motifs. This information can be used to identify closely linked markers to the putative resistance genes and eventually clone them.

Although, monocots and dicots diverged from each other 120-200 million years ago (LEISTER *et al.,* 1998) there is considerable homology found between the R genes. All the R genes isolated till date are from dicots, except *Xa-21,* a bacterial

leaf blight resistance gene in rice (LEISTER *et al.,* 1998). The information from the dicot species can be used in the monocots to isolate and clone resistance (R) genes. Based on the peptide sequence information, one homologue has been found in bread wheat where the kinase/LRR-like homologue genetically co-segregated with the rust resistance locus of *Lr10* (FEUILLET *et al.,* 1997). A receptor-like kinase (*wlrk*) gene family in wheat was characterized at molecular level (FEUILLET *et al.,* 1998). In this work the wheat probe encoding serine/threonine protein kinase was screened on the NILs carrying different rust resistance genes. The probe hybridized with a unique band on NIL carrying *Lr10* and the fragment mapped to the genetic locus of *Lr10*, encoding receptor like protein kinase, which was named as LRK10 (FEUILLET *et al.,* 1997) and was tested as RFLP for the *Lr10* resistance gene. The most specific fragment was converted into PCR based STS marker which showed presence in all the lines carrying *Lr10* only and was absent in those carrying other resistance genes (SCHACHERMAYR *et al.,* 1997) making it a *Lr10* specific marker mapping on the same locus.

SPIELMEYER *et al.,* (2000) created a detailed RFLP map of the distal end of the short arm of chromosome 1D of *Aegilops tauschii* where wheat and two of unrelated RGAs mapped close to known leaf rust resistance genes, *Lr*21 and *Lr*40 located distal to seed storage protein genes on chromosome 1DS. Also, one RGA clone, which was shown to be a part of candidate gene for stripe rust resistance (*Yr*10) located within the homoeologous region on 1BS, identified at least three gene family members on chromosome 1DS of *Ae. tauschii.* One of the gene members co-segregated with the leaf rust resistance genes, *Lr*21 and *Lr*40, in *Ae. tauschii* and wheat segregating families. Hence, RGA clone with NBS-LRR sequence derived from a candidate gene for stripe rust resistance located on chromosome 1BS detected candidate genes for leaf rust resistance located in the corresponding regions on 1DS of wheat. SEAH *et al.,* (2001) were successful in assaying *Yr*17, *Lr*37 and *Sr*38 using a cloned NBS-LRR class of disease resistance gene sequence super family, Vrga1D.

1.6 GENESIS OF THE THESIS

The wheat rusts, historically, have been diseases of great importance and the losses caused by these diseases worldwide, over the centuries, have been substantial. They are widely distributed and are most frequently occurring diseases in all the wheat growing areas of India. They can take a heavy toll of wheat production particularly in epidemic years, where the susceptible varieties can incur 5-30% loss in yield. Various cultural and chemical methods have been advocated to contain the occurrence and severity of rust, the most economical and cost-effective method being the genetic control, i.e., incorporation of resistance genes in agronomically superior varieties.

Though breeding for resistance to rust in wheat has been relatively easy and successful, narrow genetic base and continuously evolving pathogen races are the two main impediments in breaking the disease resistance in wheat. One promising approach to overcome problem of resistance breakage is to develop germplasm carrying combination of several effective genes especially those from wild relatives and related species that can be successfully crossed with cultivated genotypes (JIANG *et al.,* 1994). Availability of differential varietie for diseases or insects or molecular markers linked to major and minor resistance loci may allow such loci to be pyramided sufficiently along with minor genes in a cost effective manner.

Several rust resistance genes have been tagged in wheat using various molecular markers (Table 1.3). My thesis work represents a part of the continuing effort to identify molecular markers linked to those rust resistance genes which are important for Indian rust resistance breeding programme. I have selected a gene for leaf rust resistance *Lr28* of alien origin (from *Aegilops speltoides*) and a gene for stem rust resistance, *Sr*30, which is of wheat origin (from cv. Webster) and no information was available about the molecular markers associated with these genes.

***Ae. speltoides* Derived Leaf Rust Resistance Gene, *Lr28*:** Riley and co-workers used a high pairing line of *Ae. speltoides* for inducing homoeologous recombination to transfer *Yr*B, a gene for stripe rust resistance, from *Ae. comosa* to wheat translocation line designated Compair (RILEY *et al.,* 1968a, b). Besides the Compair-translocation, several other wheat-*Ae.comosa* translocations were produced (MILLER *et al*., 1988) and two of them, 2D/2M#3/8 and 2A/2M#4/2 were shown to have a leaf rust resistance gene, *Lr*28, derived from *Ae. speltoides*. The chromosome location of *Lr*28 was determined by monosomic analysis, and by telocentric mapping this gene was mapped 39 centimorgens from centromere on the long arm of wheat chromosome 4A (MCINTOSH *et al*., 1982).

Chromosome 4A of *T. turgidum* and *T. aestivum* is involved in a cyclical translocation with chromosomes 5A and 7B (MICKELSON-YOUNG *et al.,* 1995) while the distal region of 4AL arm is derived from 7BS. Since the *Lr*28 transfer is produced by homeologous recombination, the transferred segment having *Lr*28 is most likely derived from the short arm of the *Ae. speltoides* chromosome 7S#2, resulting in the translocation chromosome T4AS.4AL-7S#2S. This gene is not associated with deleterious characters and is widely effective in South Asia and Europe (FRIEBE *et al.,* 1996). In India, it has been found to be effective against a wide spectrum of prevalent leaf rust pathogen races and hence, its presence, in combination with other resistance genes, is desirable in new cultivars to be released.

***Sr*30, a Stem Rust Resistance Gene From cv. Webster: -** The origin of this gene is common wheat cv Webster (CI 3780), introduced to the US from Russia by USDA, which was tested by STAKMAN *et al.,* (1925) with 19 races of stem rust and was found to have some resistance to all of them. Webster gives fairly high percentage rust reading, but because the pustules are small and rarely coalesced, damage is not severe. Infection response ranges from 1^+ to 3 and some lines produce lower response than others (KNOTT, 1990).

The resistance of Webster to North American and Australian races of stem rust is apparently due to a single gene located on the long arm of chromosome 5D

(KNOTT & MCINTOSH, 1978). It is genetically independent of *Pm2* (on 5DS) and *Lr*1 and the gene order appears to be *Pm2*-centromere-*Lr1-Sr30*. LUIG (1983) reported that Sr30 was genetically effective in North America and Europe, although race 11 RHR was virulent. In Australia (LUIG, 1983) and South Africa, several virulent pathotypes became prevalent (MCINTOSH, *et al.*, 1995). HUERTA-ESPINO (1992) found virulence in a number of countries with moderate to high levels among samples from Spain, Ethiopia, Turkey, Pakistan and a number of South American countries.

Work at the University of Sydney Plant Breeding Institute has demonstrated the presence of *Sr*30 in several Mexican wheats and Australian derivatives. Because of absence of virulence, Webster was considered to be almost universally resistant (HART, 1931). However, when the resistance was developed in the Australian cultivar Festiguay, virulent pathotypes increased on the cultivar but declined after it was withdrawn from cultivation. Recently, a distant virulent pathotype was isolated in eastern Australia (PARK and WELLINGS, 1992). Although this pathotype could overcome the resistance of some current wheats with *Sr*30, remained at very low levels. The pathotype was identified in Klein Cometa and its presence was suspected in certain CIMMYT-produced wheats such as Inia 66, Pavon, and Cheel (MCINTOSH, *et al.*, 1995).

In an effort to produce a better susceptible parent, a multiple cross program was taken up at University of Saskatoon by Dr. DR Knott, leading to LMPG-6. Marquis. which carried several stem rust resistance genes and was day-length sensitive, was used as susceptible recurrent parent in the production of NILs by backcrossing. Since Marquis was resistant to Race 48A, Prelude (susceptible to Race 48A) was crossed to Marquis and F_1 was backcrossed 7 times to Marquis while selecting for susceptibility to Race 48A. The resultant line, resistant to relatively avirulent Race 111, was crossed to Little Club (susceptible to Race 111) and three times backcrossed to Pld/8*Mq, selecting for susceptibility to Race 111. To eliminate day-length sensitivity, a susceptible line was crossed to Gabo (day-length insensitive) and resultant F_6 were selected for day-length insensitivity. The resultant line, susceptible to prevalent races and day-length

insensitive, was named LMPG-6 and was used as a recurrent parent in the cross with Webster to produce NIL. Webster is noted for its moderate resistance to many races. During the backcrossing, plants or families were occasionally observed that appeared to be more resistant than normal. When these lines were selected and tested, there appeared to be small differences among the lines, although all lines gave resistant reaction with the nine races, which were accordingly numbered as LMPG-1, LMPG-2 and LMPG-3. The difference in the reaction level was attributed to the possible presence of modifiers or a second gene linked to the *Sr*30 resistance (KNOTT, 1990).

Studies by HART (1931) showed that Webster carried all: protoplasmic (physiological), morphological, and functional resistance, and that it was likely to be almost universally resistant. Thus, *Sr30* gene lets the fungus grow on it, thereby, avoiding the possibility of pathogen mutating to a virulent strain against it, which would give a longer life to the variety that would carry *Sr30.*

With above mentioned background and information on *Lr28* and *Sr30*, I planned my thesis research to identify molecular markers linked to them using several approaches viz., RAPD-PCR, RFLP, AFLP, SSR-PCR, and Microsatellites.

CHAPTER 2

MATERIALS & METHODS

2.1 PLANT MATERIAL & DISEASE SCORING

2.1.1 **Lr*28* from Aegilops speltoides**: Eight resistant NILs in different Indian wheat varietal backgrounds (Table 2.1), along with the donor source stock (CS 2A/2M#4/2) from which Lr28 was transferred in the Indian varieties, were obtained from Dr. MK Menon, Head, IARI Regional Station, Wellington, The Nilgiris, TN, India. Among these, only one NIL, HW-2035 was crossed to the recurrent parent NI-5439, F_2 plants were generated from individual F_1 seeds and 50 F_2s were advanced to the F_3 generation. The progeny rows were classified for resistance/susceptibility (R/S) (Table 3.1) using a mixture of leaf rust pathotypes applied as a suspension at seedling (3-4 leaf) stage. Disease scoring was carried out ten days after inoculation (ROELFS et al., 1992) at Wellington (TN) and at ARI, Pune.

Table 2.1: List of **N**ear **I**sogenic **L**ines (NILs) and corresponding recurrent parents for leaf rust resistant gene *Lr28.*

NILs	Recurrent parents	No. of Backcross
HW-2031	Sonalika	8
HW-2032	Lok-1	7
HW-2033	WH-147	7
HW-2034	C-306	9
HW-2035	NI-5439	7
HW-2036	J-24	7
HW-2037	HD-2329	7
HW-2038	HD-2285	7

Plant material received from Dr. MK Menon, Head, IARI Regional Station, Wellington, The Nilgiris, TN, India

2.1.2 *Sr30* from Webster: The plant material including 1. Webster 2. Festiguay 3. Lerma Rojo-64 4. Sunstar containing *Sr*30 (MCINTOSH *et al.,* 1995) was obtained from DWR Flowerdale, Shimla. The three NILs (LMPG-1, LMPG-2, and LMPG-3), which are a product of 13 backcrosses (as detailed in chapter 1, section 1.6), the recurrent parent LMPG-6, and the two populations thereof derived from LMPG-6 X Webster cross, Population 1 with 28 lines and Population 2 with 21 lines were obtained from Dr. D. R. Knott, University of Saskatoon, Canada. Since this population was small, I developed another population by crossing LMPG-6 X LMPG-2 and generated 124 F_2s. NILs, parents and the F_3 population were scored for disease reaction using several pathotypes of *P. recondita* in the glasshouse. Infection types were recorded 10 days after inoculation using a scale similar to that of STAKMAN *et al.* (1962) (Table 3.2). The selection of pathotype for disease scoring was done using various stem rust pathotypes and testing them on the Webster, NILs, LMPG-6, and Festiguay. The pathotypes used for initial screening were: 11(79G31), 122 (7G11), 40 (104G13) and 117-4 (166G3). Only pt. 122 (7G11) could produce a score of 1-2+ on

resistant genotypes and of 3-3+ on susceptible genotype (Table 2.2) this race was used to score the populations at DWR, Flowerdale Station, Shimla.

Table 2.2: Reaction of stem rust pathotypes used in the study on the various parental genotypes.

Genotypes	*Virulent*		*Avirulent*	
	11	**40**	**117-4**	**122**
LMPG-1	3^+	$2,2^+$	2	2
LMPG-2	$3,3^+$	2	2	1,2
LMPG-3	3^+	2	2	2^+
LMPG-6	3^+	$3,3^+$	3^+	$3,3^+$
Festiguay	2^+	2^+	2^-	2
Webster	3^+	2	2	2
Pusa-4	3^+	3^+	3^+	$3,3^+$

The International Triticeae Mapping Initiative (ITMI) population is also known to segregate for the *Sr30* gene from var. Opata-85 (Dr. Harbans Bariana, PBI, Cobbitty, Australia, personal communication). This population was derived by single seed decent (F_8) from a cross of W-7984, an amphihexaploid wheat synthesized from *Triticum tauschii* (DD) and *T. durum* (AABB) variety Altar 84, with the Mexican variety Opata-85 from CIMMYT.

The mapping population consisting of RILs (NELSON *et al.,* 1995) and was obtained from Dr. BS Gill, KSU. Sixty RILs from this population along with the parents, Opata-85 and synthetic hexaploid (W-7984) were used for the present study. Further 7 lines of Chinese Spring (CS) with terminal chromosomal deletions in the long arms of group *5*D chromosome were also obtained from Dr. BS Gill, KSU. These were, 1: 0.29 5DL-7; 2: 0.35 5DL-10; 3: 0.48 5DL-12; 4: 0.60 5DL-1; 5: 0.69 5DL-2; 6: 0.72 5DL-8; 7: 0.74 5DL-9 and were either homozygous or heterozygous for the deletion chromosome.

2.2 DNA ISOLATION:

Leaf tissue for DNA isolation was collected from either 7-10 day glasshouse grown seedlings or 3-4 week old plants from field. DNA was isolated by CTAB method as described by ROGERS & BENDICH (1988) with little modifications. Approximately 5 g of leaf tissue was frozen in liquid nitrogen, ground with a mortar and pestle, and transferred to 30 ml polypropylene tubes. 1% of β-Mercaptoethenol was added to the extraction buffer (100 mM Tris.Cl-8 pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2%CTAB). Extraction buffer (10-15 ml), preheated to 60^0C was added to frozen tissue, mixed to form slurry and incubated at 60^0C for 30 to 45 min. Equal volumes of 24:1 solution of chloroform:isoamyl alcohol was added, mixed and centrifuged at 8,000 x g for 10 min. The upper phase was removed, and the DNA was precipitated with equal volume of precipitation buffer (1% CTAB, 50 mM Tris.Cl pH 8.0, 10 mM EDTA pH 8.0). The spool of DNA was removed, air dried and dissolved in high salt TE buffer [1mM NaCl, 10mM Tris-HCl (pH 8.0) and 1mM EDTA (pH 8.0)]. The DNA was re-precipitated with 2 volumes of cold 95% (v/v) ethanol, dried, washed with 70% ethanol, and dissolved in TE buffer [10mM Tris-HCl (pH 8.0) and 1mM EDTA (pH 8.0)]. This DNA solution was given an RNase treatment (with DNase free RNase A) at 37^0C for 2 h and extracted with 24:1 Chloroform:Isoamylalcohol. The DNA was reprecipitated and quantified on a 0.9% agarose gel in 1X TAE [40Mm Tris-acetate, 1mM EDTA (pH 8.0)] buffer.

2.2.1 Enrichment of The Genomic DNA for Low Copy Sequences: Genomic DNAs for *Lr28* work were enriched for low copy sequences essentially by following the procedure described by EASTWOOD *et al.* (1994). The genomic DNA was first sonicated for about 10s to get the size range of 0.6 to 6.0 kb. The sonicated DNA was then resuspended in phosphate buffer, denatured at 100^0C and reannealed at 60^0C for about 20h to a C_ot value of >100. The DNA samples were then loaded onto DNA grade hydroxyapatite (Bio-Rad, USA) columns maintained at 60^0C and the single stranded DNA was eluted with 0.15 M phosphate buffer at 60^0C. The low copy DNA in phosphate buffer was dialyzed against TE buffer (pH 8.0) overnight at 4^0C and then used for DNA amplifications.

2.3 RAPD ANALYSIS:

Decamer random primers (Operon Technologies, USA) were used to screen NILs and parental DNAs for *Lr28* and *Sr30*. RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction) was performed in a 25 µl reaction mixture containing 30-50 ng of template DNA, 1.0 unit of *Taq* DNA polymerase (Bangalore Genei, India), 5 pmoles of primer, 100µM each of dNTPs, and 1X reaction buffer containing 1.5 mM $MgCl_2$. The reaction mixture was overlaid with 30 µl of mineral oil (Sigma, USA) and run on a DNA Thermal Cycler-480 (Perkin Elmer, USA) essentially by following the conditions described by Eastwood *et al.* (1994). The PCR cycle included initial denaturation of 94^0C for 5 min followed by five cycles of denaturation at 92^0C for 30 s, annealing at 35^0C for 2 min, and extension at 72^0C for 1.5 min.; 35 cycles of denaturation at 92^0C for 5 s, annealing at 40^0C for 20 s, and extension at 72^0C for 1.5 min; one cycle of denaturation at 92^0C for 10 s, annealing at 40^0C for 20 s, and final extension at 72^0C for 5 min. The amplified products were separated on a 2% agarose gel, viewed on a UV transilluminator after staining with ethidium bromide and photographed using 35 mm SLR camera.

2.4 CLONING AND SEQUENCING OF THE RAPD PRODUCT:

The polymorphic band corresponding to the resistant phenotype (of the *Lr28* donor) was eluted from the agarose gel slice by the freeze-thaw method and part of the eluted DNA was re-amplified in a 100µl reaction. The amplified reaction mix was extracted once with each of phenol, phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). The DNA was finally precipitated with two volumes of ethanol.

A portion of the reamplified DNA was ligated to the PCR product-cloning vector (Figure 2.1) from the commercially available pMOSBlue T-vector cloning kit (Amersham Inc., U.K) as per the manufactures' instructions. The ligation reaction was carried out at 16^0C for 16 h in 20µl volume with 2 units of T4 DNA ligase, 50 ng of pMOSBlue T-vector in 660 mM Tris HCl (pH 7.6), 66mM $MgCl_2$, 100mM DTT and 660mM ATP. The ligated mixture was transformed into the *E.*

coli strain, XL-1 Blue (Stratagene, Switzerland) and the transformed cells were plated on X gal-IPTG-LB (Luria Bertani) agar medium [0.5% NaCl and 0.5% Bacto-yeast extract (pH 7.2) 1.5% Bacto-agar]. Resulting white colonies on X gal-IPTG-LB Agar plates were screened for the presence of an insert by restricting the recombinant plasmid using appropriate enzymes as described by SAMBROOK *et al.* (1989). A colony positive for the insert was chosen, grown in a large culture and the plasmid DNA was isolated by alkaline lysis method.

For sequencing, the plasmid DNA was first purified using polyethylene glycol as described by SAMBROOK *et al.,* (1989). It was then sequenced by Sanger's dideoxy chain termination method (SANGER *et al.,* 1977) using Sequenase®

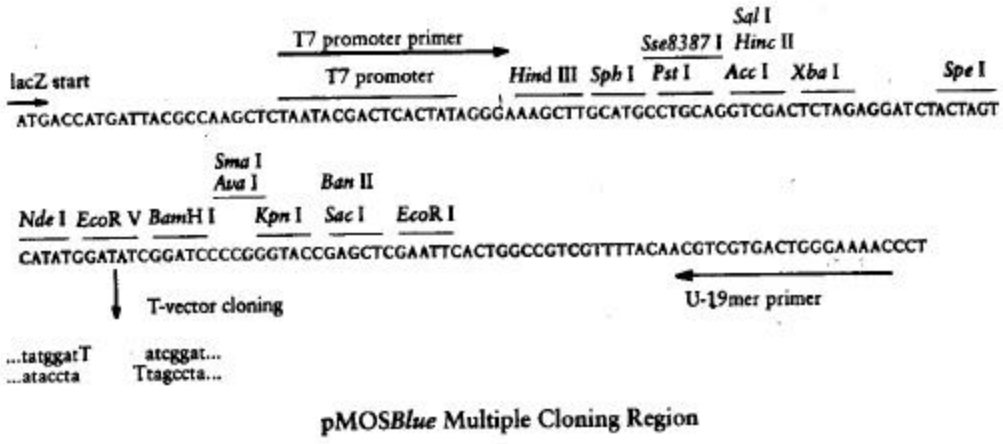


Figure 2.1: pMOS*Blue* vector cloning region (adopted from manufacturers catalogue).

version 2.0 sequencing kit (USB). 5µg of purified plasmid DNA was denatured at 37^0C for 30 min after adding 40µl of denaturing solution (0.2M NaOH and 0.2mM EDTA). One-tenth volume of 3M sodium acetate (pH 5.2) and two and half volumes of chilled absolute ethanol were added and the mixture was kept at -70^0C for 30 min. DNA was precipitated by centrifugation at 10,000 rpm for 10 min at 4^0C and the pellet was washed with 70% ethanol and dissoled in 7µl sterile distilled water.

The second step was primer annealing in which 1µl (0.5 pmoles) of primers (pUC forward, reverse or specific) and 2µl of 5X reaction buffer were added, incubated at 65^0C for 2 min and then allowed to cool gradually to 37^0C over 15-30 min. To this annealed template-primer mix which was then chilled on ice, 1µl of 0.1 M DTT, 2µl labeling mix, 1µCi α^{35}S dATP and 2µl of diluted T7 DNA polymerase (3.7 units) were added and incubated at 21^0C for 2-5 min. Then 3.5µl of the mix was added to four tubes containing 2.5 µl of respective dideoxynucleotide and incubated at 37^0C for 5 min for chain termination. Finally, 4µl of stop solution was added to terminate the reaction. The reaction mixtures were heated at 75-80^0C for 2 min to denature and were then loaded on 6% denaturing polyacrylamide gels containing 7M urea and 0.5X TBE and electrophoresed at 2000V. Successive loadings were performed so that the entire sequence could be read. The gels were dried on a vacuum gel dryer at 80^0C and exposed to X-ray films for 24-48h. The sequence from both the strands was read manually.

2.5 SCAR DESIGN AND ANALYSIS:

The sequence information of the cloned product was used to design the SCAR (Sequence Characterized Amplified Region) primers. A forward primer of 20 bp *Lr28*-01, 5'CCCGGCATAAGTCTATGGTT3' had the original 10 bp of RAPD primer along with the next 10 internal bases and a reverse primer of 20 bp, *Lr28*-02, 5'CAATGAATGAGATACGTGAA3' had an entirely internal sequence next to the RAPD primer. The two STS primers were custom synthesized from Organic Chemical Synthesis Division, NCL. PCR reactions were performed in a 25 µl reaction mixture containing 30-50 ng of non-enriched template DNA, 1.0 unit of Taq DNA polymerase, 40 nM of each primer; 100 µM each of dNTP, and 1X reaction buffer containing 1.5 mM $MgCl_2$. The reaction mixture was overlaid with 30 µl of mineral oil and run on a thermocycler where it was incubated at 94^0C for 6 min for initial denaturation, followed by 35 cycles of 94^0C for 1 min, 50^0C for 1 min, and 72^0C for 2 min. The amplification reaction was concluded by a final extension of 72^0C for 5 min. The amplification products were separated on a 2% agarose gel in TAE buffer, viewed on a UV

transilluminator after ethidium bromide staining and photographed using 35 mm SLR camera.

2.6 ISSR ANALYSIS:

One hundred Inter Simple Sequence Repeat (ISSR) primers (University of British Columbia, Canada) were screened for polymorphism with *Sr30* parents. PCR reactions were performed in a 25 µl reaction mixture containing 30-50 ng of non-enriched template DNA, 1.0 unit of Taq DNA polymerase, 40 nM of each primer; 100 µM each of dNTP, and 1X reaction buffer containing 1.5 mM $MgCl_2$. The reaction mixture was overlaid with 30 µl of mineral oil and run on a thermocycler where it was incubated at 94^0C for 6 min for initial denaturation, followed by 35 cycles of 94^0C for 1 min, annealing for 1 min (annealing temperature varied with the primer used; based on GC/AT ratio), and 72^0C for 2 min. The amplification reaction was concluded by a final extension of 72^0C for 5 min. The amplification products were separated on a 2% agarose gel, viewed on a UV transilluminator after ethidium bromide staining and photographed using 35 mm SLR camera.

2.7 SOUTHERN AND RFLP ANALYSIS:

For the *Lr28* work, the gel of amplification products obtained with a specific polymorphic random primer was Southern blotted (Sambrook *et al.,* 1989) and hybridized with a doned polymorphic band as a probe. Hybridization conditions used were essentially similar to those previously described by KAM-MORGAN *et al.,* (1989). Probes were labeled by the random hexamer method with [α^{32}P]dCTP (FEINBERG and VOGELSTEIN, 1983), purified through spun columns containing Sephadex G50, denatured by boiling for 2 min, added to the membranes, and allowed to hybridize for 18 to 22 h. Membranes were washed at 65^0C for 20 min each in 2X SSC and 1X SSC followed by 1 h in 0.5X SSC (1X SSC: 0.15 M NaCl plus 0.015 M sodium citrate). All washing solutions also contained 0.1% (w/v) SDS. Membranes were placed in plastic sheets and exposed to X-ray film for 3 to 7 days at -70^0C with intensifying screens.

For Restriction Fragment Length Polymorphism (RFLP) analysis on *Sr30* material, 20 µg of DNA was digested with 40 units of various restriction endonucleases one at a time, in the presence of the appropriate buffer in a final volume of 35 µl. After incubation of 16 h at 37^0C, the reactions were stopped by adding 8 µl of gel loading buffer [7.6 M glycerol, 0.5X neutral electrophoresis buffer (NEB) (1X NEB: 0.1 M Tris, 1 mM EDTA, 12.5 mM sodium acetate $3H_2O$, pH 8.1), 0.02 mM EDTA, 0.2% (w/v) SDS, and 6 g l^{-1} bromophenol blue]. The resulting mix was loaded on a 0.9% agarose gel made using 1X NEB and run for 16 h at 22 V in a horizontal gel apparatus. Gels were stained with ethidium bromide, rinsed in distilled water, and photographed. DNA was transferred from gels to Hybond N+ membranes (AMERSHAM INT., USA) according to manufacturer's instructions, using alkaline transfer, except that a large sponge soaked in 0.4 M NaOH served as the base of the blot. The prehybridization and hybridization conditions were as described in KAM-MORGAN *et al.,* (1989). All of the 134 RFLP markers mapped on 5DL were used to screen the NILs and Webster (Figure 2.2) (XIE *et al.,* 1993; OGIHARA *et al.,* 1994; DEVOS *et al.,* 1995; NELSON *et al.,* 1995; FARIS *et al.,* 1996; GILL *et al.,* 1996; KOJIMA and OGIHARA 1998; LI *et al.,* 1999; BOYKO *et al.,* 1999; J. FARIS, KSU, Personal communication).

2.8 MICROSATELLITE ANALYSIS:

The chromosome *5DL* microsatellite markers were selected based on the map positions (Figure 2.3) determined by RÖDER *et al.* (1998 b). PCR reactions were performed (RÖDER *et al*., 1998 a) in a 25 µl reaction volume. The reaction mixture contained 250 nM of each primer (forward and reverse), 0.2 nM of each dNTP, 1.5 mM $MgCl_2$, 1 unit *Taq* DNA polymerase, and 50-100 ng of template DNA. After 3 min at 94^0C, 45 cycles were performed with 1 min at $94^{0,}$C, 1 min at $50^{0,}$C, $55^{0,}$C, or $60^{0,}$C (depending upon the individual microsatellite), 2 min at $72^{0,}$C, and final extension step of 10 min at $72^{0,}$C. Amplified products were run on a 2.3% metaphor agarose gel made with 1X TBE [22.5 mM Tris-borate,

0.5mM EDTA (pH 8.0)] buffer at 57 V for 4 h. Gels were stained with ethidium bromide, visualized under UV light and photographed.

2.9 AFLP ANALYSIS:

The AFLP™ (Amplified Fragment Length Polymorphism) Analysis System-I and the AFLP Starter Primer Kit (LIFE TECHNOLOGIES INC., USA) were used according to the manufacturer's instructions. Briefly, genomic DNA was digested with the restriction enzymes *Eco*RI and *Mse*I simultaneously, followed by ligation of *Eco*RI and *Mse*I adapters to serve as primer binding sites on the restriction fragments. A preamplification reaction was then performed using primers each having one selective nucleotide as anchors. The preamplification products were diluted 1:50 and used to perform a selective amplification reaction consisting of an *Eco*RI and an *Mse*I primer with three selective nucleotides. The *Eco*RI primer was labeled by phosphorylating the 5' end with [γ-^{33}P] ATP and T_4 kinase. The kit contained eight *Eco*RI primers and eight *Mse*I primers for a total of 64 possible primer

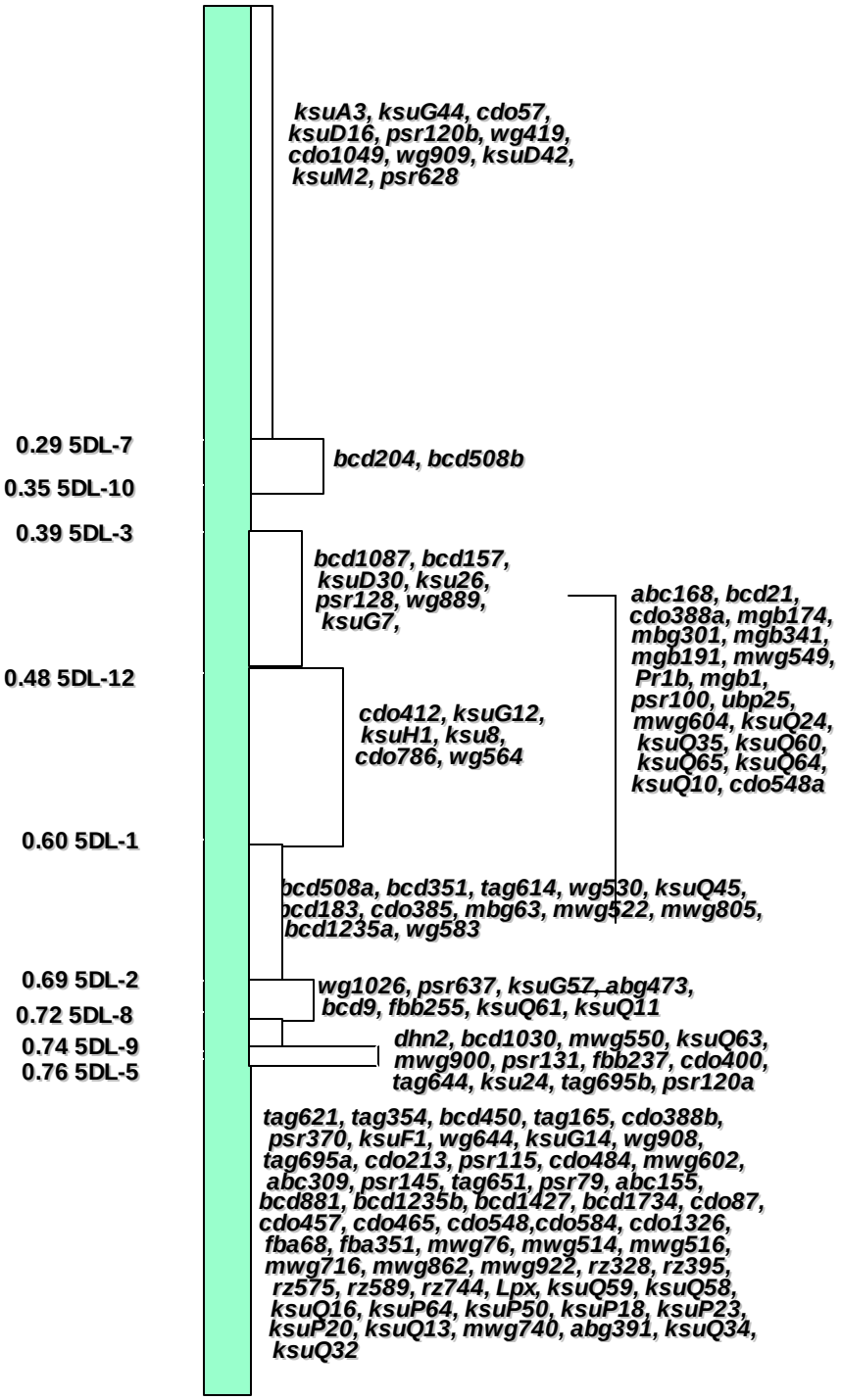

ksuA3, ksuG44, cdo57, ksuD16, psr120b, wg419, cdo1049, wg909, ksuD42, ksuM2, psr628
0.29 5DL-7
bcd204, bcd508b
0.35 5DL-10
0.39 5DL-3
bcd1087, bcd157, ksuD30, ksu26, psr128, wg889, ksuG7,
abc168, bcd21, cdo388a, mgb174, mbg301, mgb341, mgb191, mwg549, Pr1b, mgb1, psr100, ubp25, mwg604, ksuQ24, ksuQ35, ksuQ60, ksuQ65, ksuQ64, ksuQ10, cdo548a
0.48 5DL-12
cdo412, ksuG12, ksuH1, ksu8, cdo786, wg564
0.60 5DL-1
bcd508a, bcd351, tag614, wg530, ksuQ45, bcd183, cdo385, mbg63, mwg522, mwg805, bcd1235a, wg583
0.69 5DL-2
wg1026, psr637, ksuG57, abg473, bcd9, fbb255, ksuQ61, ksuQ11
0.72 5DL-8
0.74 5DL-9
dhn2, bcd1030, mwg550, ksuQ63, mwg900, psr131, fbb237, cdo400, tag644, ksu24, tag695b, psr120a
0.76 5DL-5
tag621, tag354, bcd450, tag165, cdo388b, psr370, ksuF1, wg644, ksuG14, wg908, tag695a, cdo213, psr115, cdo484, mwg602, abc309, psr145, tag651, psr79, abc155, bcd881, bcd1235b, bcd1427, bcd1734, cdo87, cdo457, cdo465, cdo548,cdo584, cdo1326, fba68, fba351, mwg76, mwg514, mwg516, mwg716, mwg862, mwg922, rz328, rz395, rz575, rz589, rz744, Lpx, ksuQ59, ksuQ58, ksuQ16, ksuP64, ksuP50, ksuP18, ksuP23, ksuP20, ksuQ13, mwg740, abg391, ksuQ34, ksuQ32

Figure 2.2: consolidated 5DL map, showing various deletion intervals and markers mapped.

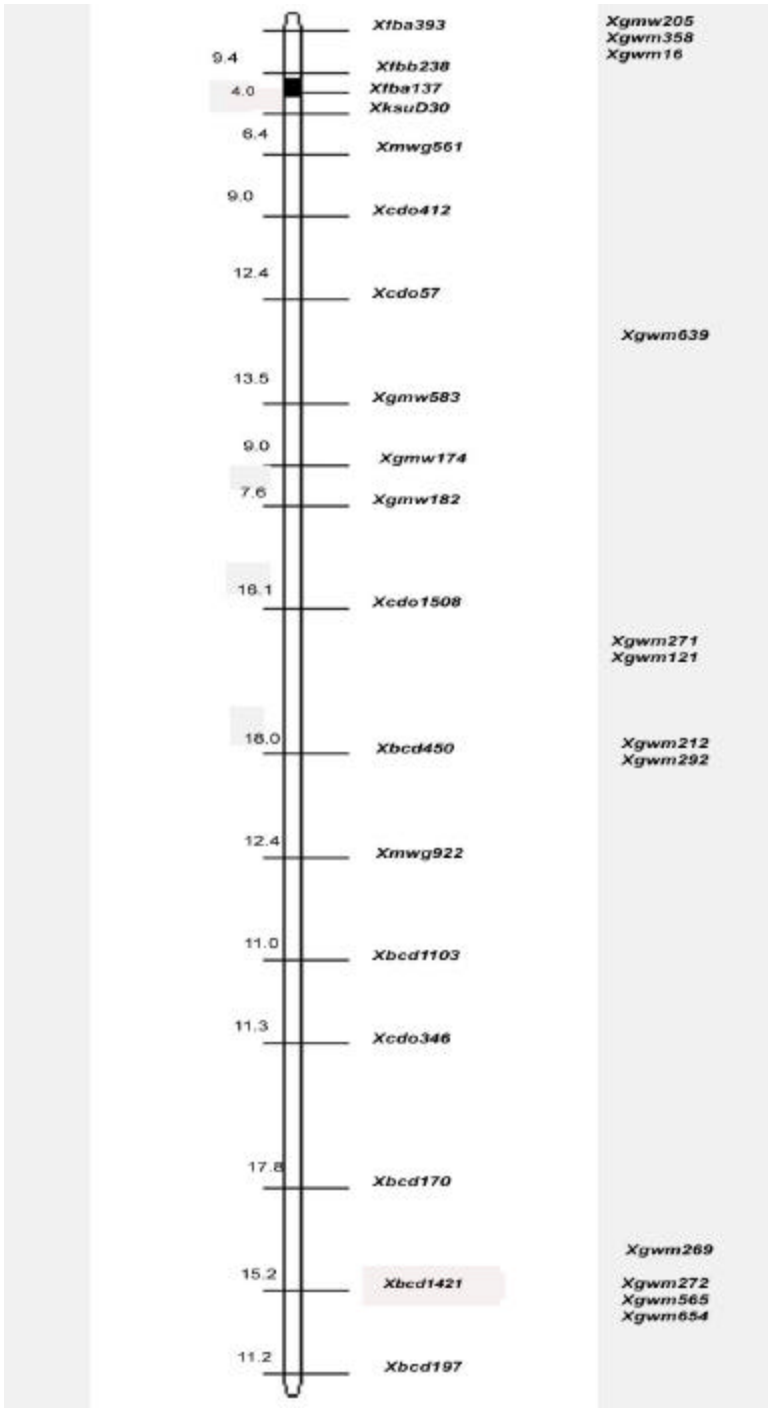


Figure 2.3: Microsatellite linkage map of 5D of wheat. Unmapped microsatellites are placed in the most probable place. (Adopted from RODER *et al.* 1998b)

combinations. Selective amplification products were run on a 5% polyacrylamide gel at 80 W for 3.5 hr, dried on filter paper for 2 hr, and exposed to X-ray film for 3 to 7 days. Only one NIL, LMPG-2 and recurrent parent LMPG-6 were used for this analysis. Reactions yielding fragments, which were present in the NIL and absent in recurrent parent were repeated two times for verification and only repeatable reactions were performed on the population DNA.

2.10 MAPPING AND CALCULATIONS:

Linkage analysis of microsatellite marker with the *Sr30* gene was carried out using the computer program MAPMAKER (LANDER *et al.,* 1987) V2.0 to calculate linkage distances using the Kosambi mapping function (KOSAMBI,1944) and an LOD of 3.00.

CHAPTER 3

RESULTS

3.1 IDENTIFICATION OF MOLECULAR MARKERS FOR *Lr28*

3.1.1 RAPD Analysis With *Lr28* Parents: A total of 80 decamer primers of series A, F, J and V, with 20 primers each (from Operon technologies, USA), were screened to identify polymorphism between eight resistant NILs and the respective recurrent parents (Table 2.1) along with the donor source stock (CS

2A/2M#4/2). Primers that gave clear and distinguishable patterns were considered for further analysis. In addition, polymorphism was confirmed by at least six repetitions to ensure the reproducibility of RAPD results.

Out of total 80 primers, 17 did not amplify any product, while others amplified about 5-7 fragments each with size ranging from 200 bp to 2500 bp. Most of the primers produced monomorphic RAPD profiles. Some primers produced polymorphic bands with few NILs. Only one primer, OPJ-01 showed polymorphism corresponding to all the resistant genotypes including donor in comparison to the recurrent

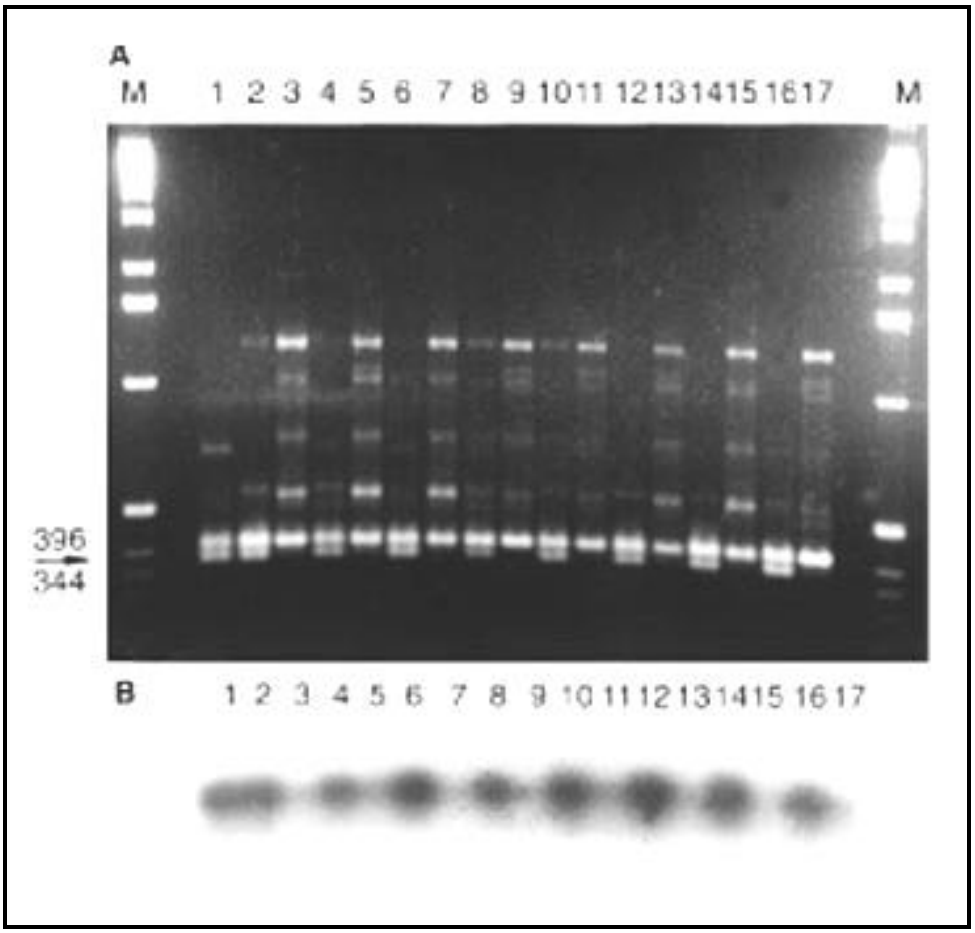


Figure 3.1: A) RAPD profiles obtained by OPJ-01. A polymorphic band, present in all NILs but absent in respective recurrent parent genotypes, is indicated by the arrow. Products were separated on a 2% agarose gel. 1: *Lr28* source stock (CS 2A/2M#4/2), 2: HW-2031, 3: Sonalika, 4: HW-2032, 5: Lok-1, 6: HW-2033, 7: WH-147, 8: HW-2034, 9: C-306, 10: HW-2035, 11: Nl 5439, 12: HW-2036, 13: J-24, 14: HW-2037, 15: HD-2329, 16*:* HW-2038, 17: HD-2285. Lane M DNA molecular-weight marker X (Boehringer Mannheim)
B) Autoradigram of the Southern blot prepared from the above gel and hybridized using a plasmid with the OPJ-01 378 insert as a probe. Only the 378-bp specific band in all the resistant genotypes shows intense hybridization.

parents (Figure 3.1A). As shown in Figure 3.1A, very few prominent bands were obtained in the amplification products of OPJ-01 with enriched DNA. Band sizes ranged between 380 bp to 1300 bp and showed differences in intensity between NILs and recurrent parents. Compared to recurrent parents, high molecular weight bands were less intense in NILs. One band of approximately 380 bp was present only in the source stock and in all the NILs but absent in the recurrent parents. This band was selected as a putative marker and hence was characterized in detail as described below.

3.1.2 Cloning and Characterization of Polymorphic RAPD Band: To convert OPJ-01$_{\sim 380}$ band into SCAR (Sequence Characterized Polymorphic Region) marker, it was necessary to clone the polymorphic fragment from the RAPD profile. This band was eluted from the agarose gel and purified for the purpose of cloning into pMOSBlue T-vector (AMERSHAM Inc., USA). This vector system exploits the template-independent activity of thermostable polymerase that preferentially adds a single adenosine nucleotide to the 3' end of the double stranded DNA.

Clone was characterized by restricting it by mapped restriction enzymes on the vector. *Hind*III is present 50 bp upstream of T-cloning site and *Bam*HI is present 5 bp down stream (Figure 2.1). Thus the cloned fragment after a double digestion with *Hind*III and *Bam*HI produced a fragment larger by 55 bp than the insert and polymorphic RAPD band, which was about 380 bp long (Figure 3.2). The mobility of restricted insert was less compared to purified polymorphic band because a portion of plasmid was also included in the insert when restricted out. Also a double digest using *Hind*III/*Eco*RI having only one site each on the vector (Figure 2.1) is expected to produce a single band larger than *Hind*III/*Bam*HI since site for *Eco*RI lies further down than for *Bam*HI. But it produced two smaller fragments of about 260 bp and 190 bp (Figure 3.2). The lower 190 bp band corresponds to the band produced by *Eco*RI digestion of clone (Figure 3.2, lane 5),

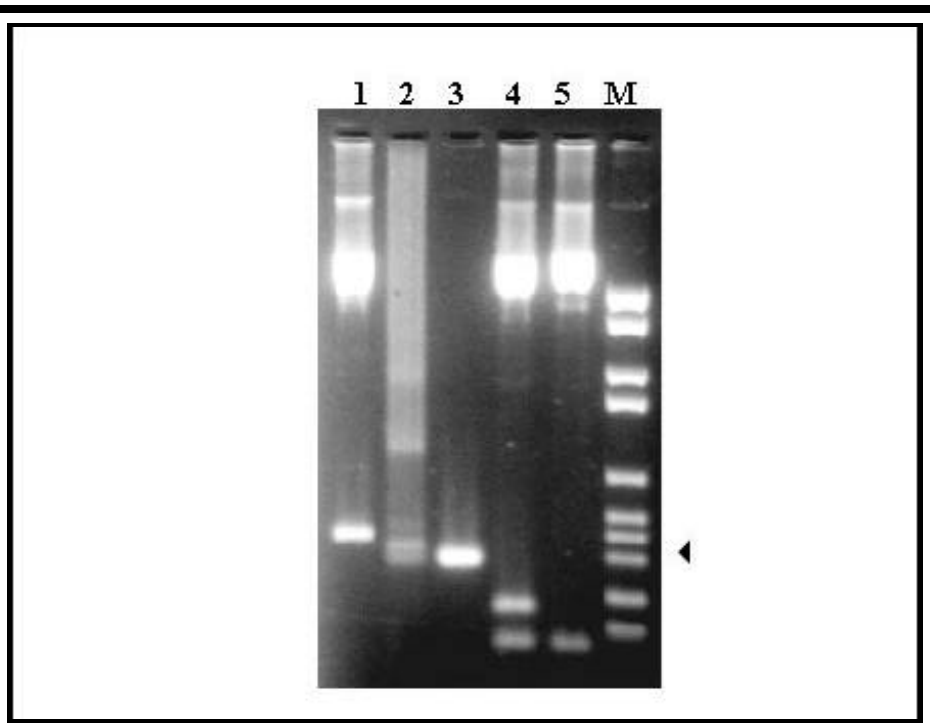


Figure 3.2: Agarose gel showing size comparison of cloned insert. Lane 1: Clone digested with *Bam*HI/*Hind*III; 2: OPJ-01 RAPD profile of resistance donor; 3: re-amplified insert; 4: clone digested with *Hind*III/*Eco*RI; and 5: clone digested with *Eco*RI. Lane M: molecular-weight marker X (Boehringer Mannheim)

confirming an *Eco*RI site into the insert at the position of approximately 210 bp (Figure 3.2).

Further to verify the identity of cloned product, the plasmid with OPJ-01 insert was labeled with [α^{32}P]dATP to be used as a probe and hybridized to the Southern blot prepared from the gel of the RAPD profiles obtained with OPJ-01 (Figure 3.1B). As seen in figure, only resistant genotypes specifically showed intense hybridization at a position corresponding to the band of approximately 380 bp, thus confirming the identity of the cloned product and indicating the absence of sequence homology with other bands in the RAPD profiles obtained by primer OPJ-01.

3.1.3 Conversion of Polymorphic Band Into SCAR: The selected clone was sequenced by Sanger's dideoxy chain termination method (SANGER *et al.,* 1977). A sequence of 378 bp (Figure 3.3) was read from the sequencing gel, with both ends having original RAPD primer, OPJ-01. Based on the sequence information, four 20 bp primers were designed:

A) Forward primer with OPJ-01 primer sequence along with ten inner bases

B) Reverse primer with OPJ-01 primer sequence along with ten inner bases

C) Forward primer without OPJ-01 primer sequence, all 20 inner bases next to the RAPD primer

D) Reverse primer without OPJ-01 primer sequence, all 20 inner bases next to the RAPD primer

After testing all possible combinations such as A/C, B/C, A/D, and B/D, considering the PCR conditions and repeatability of the reactions, two primers, one forward primer consisting of 20 bp *Lr28*-01, 5'CCCGGCATAAGTCTATGGTT3' having the original 10 bp of RAPD primer along with the next 10 internal bases and a reverse primer of 20 bp, *Lr28*-02, 5'CAATGAATGAGATACGTGAA3' having an entirely internal sequence next to the RAPD primer were selected for further analysis.

3.1.4 Linkage Analysis of the SCAR Primers Based Amplified Product and the *Lr*28 Resistance: When the SCAR primers were tested against non-enriched DNAs of all the NILs and the donor source, only one band of 378 bp length was amplified in the resistant lines and it was absent in the recurrent

5´CCC GGCA TAA	GTCTATGGTT	TGCATAATTC	AGTGGCCCA T	CTGCTTGCTA	50
TTTCAATGCA	TACTCTCTTG	ATTCATTTGT	GTTCCGTTGT	ACCCTGCTGA	100
TTCTGTCTAT	GACATGTGCT	AGGTTTTTCA	TTCGACTGGA	TCTCCTCACC	150
TTGTCATCTG	ATTGCTCTTC	GTTTGCTGCT	CTGCTTACCA	AGGTTGGTGT	200
TTGCTCTTTA	GAATTCTCTA	TTACTCCTGG	TCTTTCGTAT	TGGGTGCCTA	250
TTTCAATTTC	ACTTTATGAA	CTATTTCAAT	TATTACCTAT	ATATCGCCTG	300
TCTCACAACG	AGCTTACCTT	CTTTAGCTAC	TTCACTGAAC	ACATGTATTT	350
		AT GC			

parents (Figure 3.4). The intensity differences of the bands between lanes were probably due to slight difference in the amounts of non-enriched template DNA.

To investigate segregation of the identified marker and *Lr28*, 50 F_3 progeny lines of the cross HW 2035/NI 5439 were scored for the disease infection and classified for resistance/susceptibility (Table 3.1) using a mixture of leaf rust isolates avirulent to *Lr*28 resistance. As shown in the table 3.1, the resistance segregated confirming to 3:1ratio.

DNAs extracted from F_3 progeny lines were used to confirm the linkage of the SCAR marker. The parents, resistant and susceptible bulk DNAs (containing five lines each), and individual progeny line DNA was used to analyze the presence of SCAR marker. Figure 3.5 shows representative photograph of five resistant (lanes 3-7) and five susceptible lines (lanes 8-12) along with the parents (lanes 13-14), and resistant and susceptible DNA bulks (lanes 1-2). As seen in the figure, the marker was present in resistant lines, the resistant bulk and HW 2035, but absent in the recurrent parent (NI 5439), susceptible lines and susceptible bulk.

Table 3.1: Infection scores for HW 2035/NI 5439 F_3 population segregating for *Lr*28 using mixture of pathogen races carried out on fields at IARI Regional Station, Wellington, The Nilgiris, and ARI Pune, based on ROELFS *et al.,* (1992). R=Resistant, S=Susceptible.

Line No.	Score	Line No.	Score	Line No.	Score	Line No.	Score	Line No.	Score
1	R	11	R	21	R	31	R	41	R
2	R	12	R	22	S	32	R	42	R
3	S	13	R	23	R	33	S	43	R
4	R	14	S	24	R	34	S	44	R
5	S	15	R	25	R	35	R	45	S
6	S	16	S	26	S	36	R	46	R
7	R	17	R	27	R	37	S	47	R
8	S	18	R	28	R	38	R	48	S
9	R	19	S	29	R	39	R	49	S
10	R	20	R	30	R	40	R	50	R

3.2 IDENTIFICATION OF MOLECULAR MARKERS FOR *Sr30*

3.2.1 Disease Scoring of Parental lines and Populations: After the initial screening with the pathotypes as described in materials and methods, pt. 122 (7G11) was selected to screen 1: Population obtained from Dr. Knott (population 1-28 lines, population 2-21 lines), 2: ITMI population (60 RILs), and 3: LMPG-6 X LMPG-2 (124 lines). The scores for all these populations are presented in the Table 3.2.

3.2.2 Parental analysis for Polymorphism:

3.2.2.1 RAPD and ISSR analysis: All the three NILs, LMPG-1, LMPG-2, LMPG-3, along with the susceptible recurrent parent LMPG-6 and donor Webster were used for PCR screening using RAPD and ISSR primers. A total of 800 RAPD primers (from Operon Technologies, USA) and 100 ISSR primers (from UBC, Canada) were screened. All the primers failed to detect any polymorphic band pattern.

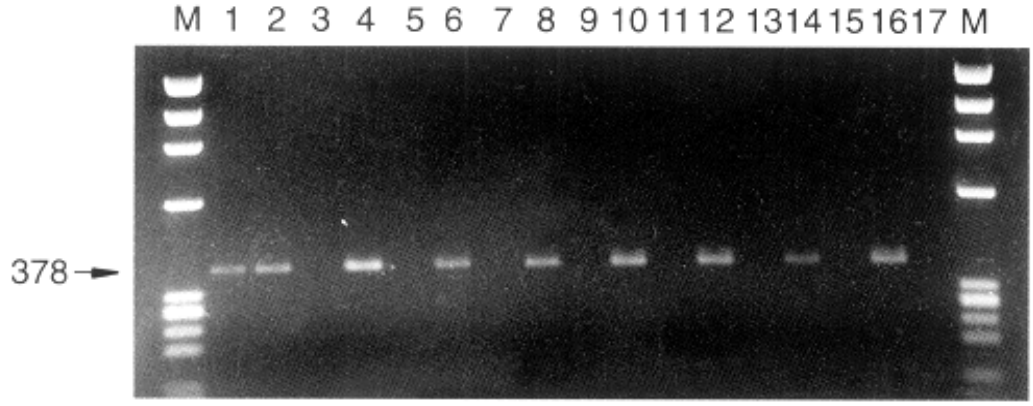


Figure 3.4: Amplification products obtained by SCAR primers *Lr2*8-01 and *Lr2*8-02 showing a polymorphic band only in resistant lines possessing *Lr2*8. Products were separated on a 2% agarose gel. The sequence of wheat genotypes is similar to that mentioned in Figure 3.1. Lane M is molecular-weight marker-ϕX-174 RF DNA/*Hae* lll degest.

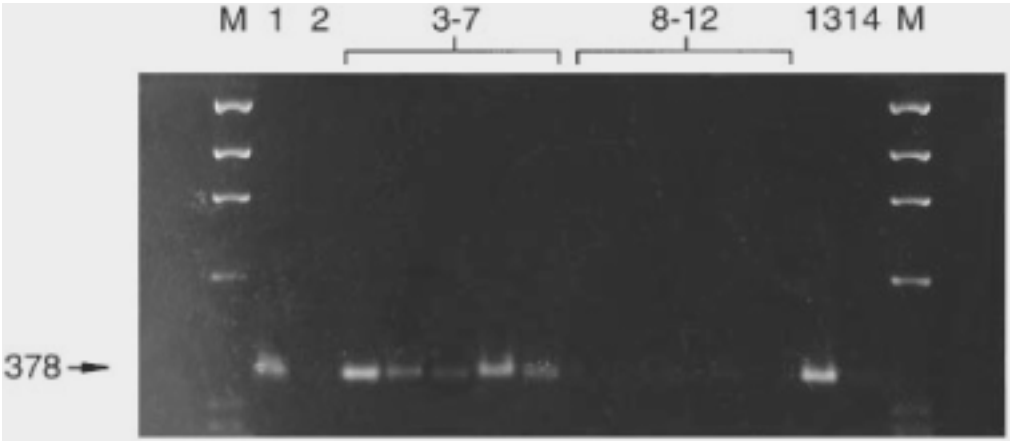


Figure 3.5: Segregation of amplification products in F_3 lines. Amplifications were performed using primers *Lr2*8-01 and *Lr2*8-02. Products were separated on a 2% agarose gel. Resistant and susceptible lines were clearly distinguishable. *1* Resistant bulk, *2* Susceptible bulk, *3-7* Individual resistant lines, *8-12* Individual susceptible lines, *13* HW-2035, *14* NI-5439, Lane M is molecular-weight marker- ϕX-174 RF DNA/*Hae* III digest.

Table 3.2: Infection scores for various populations segregating for *Sr*30 using pt. 122 (7G11) carried out in glasshouse at DWR, Flowerdale station, Shimla, using a scale similar to that of STAKMAN *et al.,* (1962). P1: population 1, P2: population2, HR: Homozygous Resistant, HS: Homozygous susceptible, SEG: Segregating line

Line number	**LMPG-6 X Webster populations (F_3 lines scored)**	**ITMI Population (RILs)**	**LMPG-6 X LMPG-2 (F_3 lines scored)**
1	SEG (first 28 lines-P1)	HR	HR
2	SEG	HR	HR
3	HR	HR	SEG
4	SEG	HR	SEG
5	SEG	HS	SEG
6	SEG	HR	HS
7	SEG	HS	SEG
8	HR	HS	SEG
9	SEG	HR	SEG
10	SEG	HR	SEG
11	SEG	HR	SEG
12	HR	HS	SEG
13	HS	HR	SEG
14	HR	HR	SEG
15	HR	HR	SEG
16	HR	HR	HS
17	SEG	HS	SEG
18	HR	HR	SEG
19	HR	HS	SEG
20	SEG	HR	SEG
21	SEG	HR	SEG
22	HR	HR	SEG
23	HR	HS	SEG
24	SEG	HR	SEG
25	SEG	HR	SEG
26	SEG	HS	SEG
27	SEG	HS	SEG
28	SEG	HS	SEG
29	HR (onwards P2, 21 lines)	HR	HR
30	SEG	HR	SEG
31	HR	HS	HR
32	SEG	HR	SEG
33	SEG	HR	SEG

34	SEG	HR	HR
35	SEG	HS	HR
36	SEG	HR	HR
37	HR	HR	SEG
38	SEG	HS	HR
39	SEG	HS	SEG
40	SEG	HS	SEG
41	SEG	HR	HS
42	HS	HR	HS
43	HR	HS	SEG
44	SEG	HS	SEG
45	SEG	HR	SEG
46	HR	HR	SEG
47	HR	HS	SEG
48	HR	HS	SEG
49	HR	HR	SEG
50		HS	SEG
51		HR	HR
52		HR	SEG
53		HR	SEG
54		HR	SEG
55		HR	SEG
56		HR	SEG
57		HS	SEG
58		HR	SEG
59		HS	HS
60		HS	SEG
61			SEG
62			SEG
63			SEG
64			SEG
65			SEG
66			SEG
67			SEG
68			SEG
69			SEG
70			SEG
71			HS
72			SEG
73			SEG
74			HR
75			SEG
76			HR
77			SEG
78			SEG

79			SEG
80			SEG
81			HR
82			SEG
83			SEG
84			SEG
85			SEG
86			HS
87			SEG
88			HS
89			SEG
90			SEG
91			SEG
92			SEG
93			SEG
94			SEG
95			SEG
96			SEG
97			SEG
98			SEG
99			SEG
100			SEG
101			SEG
102			SEG
103			SEG
104			SEG
105			SEG
106			HS
107			SEG
108			SEG
109			SEG
110			HS
111			SEG
112			SEG
113			SEG
114			SEG
115			SEG
116			SEG
117			HR
118			SEG
119			SEG
120			SEG
121			SEG
123			SEG
124			HR

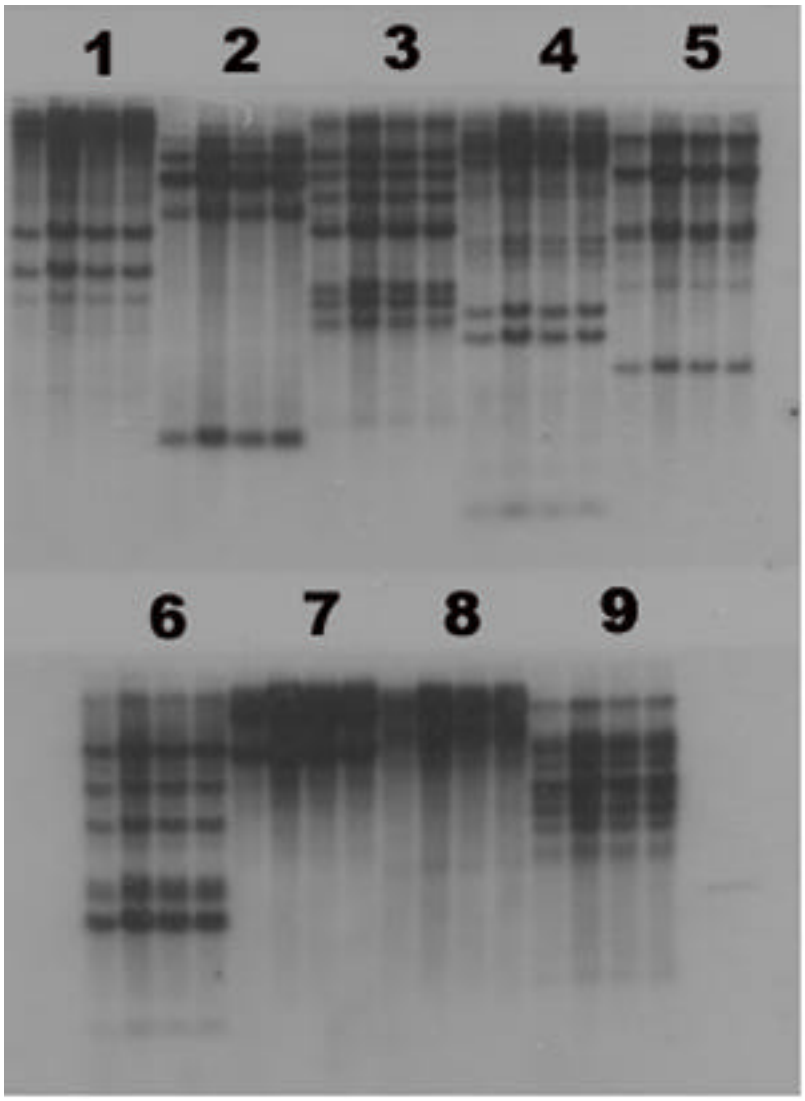


Figure 3.6: RFLP profile with nine restriction enzymes and a probe WG 1026 located on 5DL. LMPG-1, LMPG-2, LMPG-3, LMPG-6 represent every four lanes under all the restriction enzymes; 1: *Eco*RI; 2: *Eco*RV; 3: *Xba*I; 4: *Dra*I; 5: *Bam*HI; 6: *Hind*III; 7: *Apa*I; 8: *Kpn*I; and 9: *Sca*I

3.2.2.2 RFLP and AFLP analysis: Three NILs and recurrent parent were used for the RFLP analysis. Southern blots were prepared for these DNAs using *Eco*RI, *Eco*RV, *Xba*I, *Dra*I, *Bam*HI, *Hind*III, *Apa*I, *Kpn*I, and *Sca*I restriction enzymes. Since the *Sr*30 is located on chromosome 5DL(Figure 2.2), the 134 RFLP markers mapped on this chromosome arm were used to screen for the polymorphism. Bands with varied intensity were observed on the autoradiograms. However, none of the combinations produced a polymorphic profile with the NILs/recurrent parent. A representative autoradiogram is shown in Figure 3.6.

Since no polymorphism was obtained with RFLPs, AFLP analysis was performed considering the detection power of this system. Analysis was carried on the parental lines of *Sr30* using 64 combinations of primers/enzyme including *Eco*RI and *Mse*I linkers. Since about 80 bands per primer pair were produced, approximately 5,120 fragments were visually observed. Very low molecular weight bands were very faint, while very high molecular weight bands were not well separated; hence these were eliminated from the analysis. Six primer combinations produced 8 polymorphic bands. The primer combinations included Eacc/Mcag, Eacg/Mcag, Eacg/Ecaa, and Eact/Mcac producing one band each, while Eact/Mctg, and Eaca/Mcaa producing two bands each. Polymorphic combinations were tested on the Webster X LMPG-6 population (Figure 3.7). As seen in the figure, the bands showed segregation in the population (Table 3.3, data sheet I).

3.2.2.3 Microsatellite analysis: Wheat microsatellites located on the 5DL (Figure 2.3) (RODER *et al.,* 1998b) were used to identify the polymorphism in the parental combination. Out of the 12 microsatellites analyzed, only one, GWM-174 was found to be polymorphic in between the parents. As shown in Figure 3.8, a band of 230 bp was present in the NILs, LMPG-2 (lane no. 2)

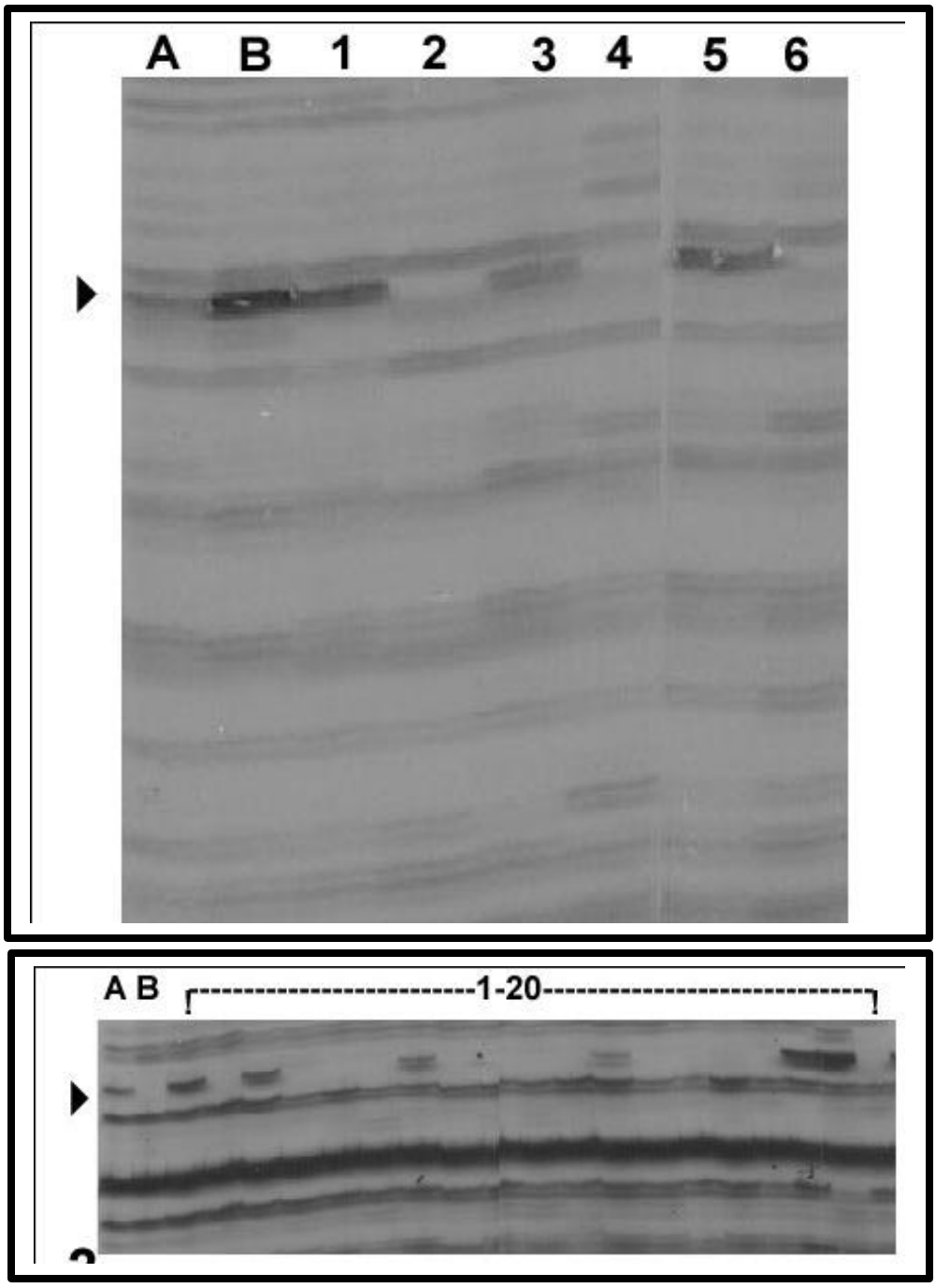


Figure 3.7: AFLP profiles of representative polymorphic primer combinations for *Sr*30 population (LMPG-6 X Webster) A: LMPG-2; B: LMPG-6. **1:** Profile of primers E-ACC/M-CAG on population lines serial no. 1-6 (lanes 1-6). **2:** Profile of primers E-ACG/M-CAG on population lines serial no. 1-20 (lanes 1-20). Arrows represent the segregating band.

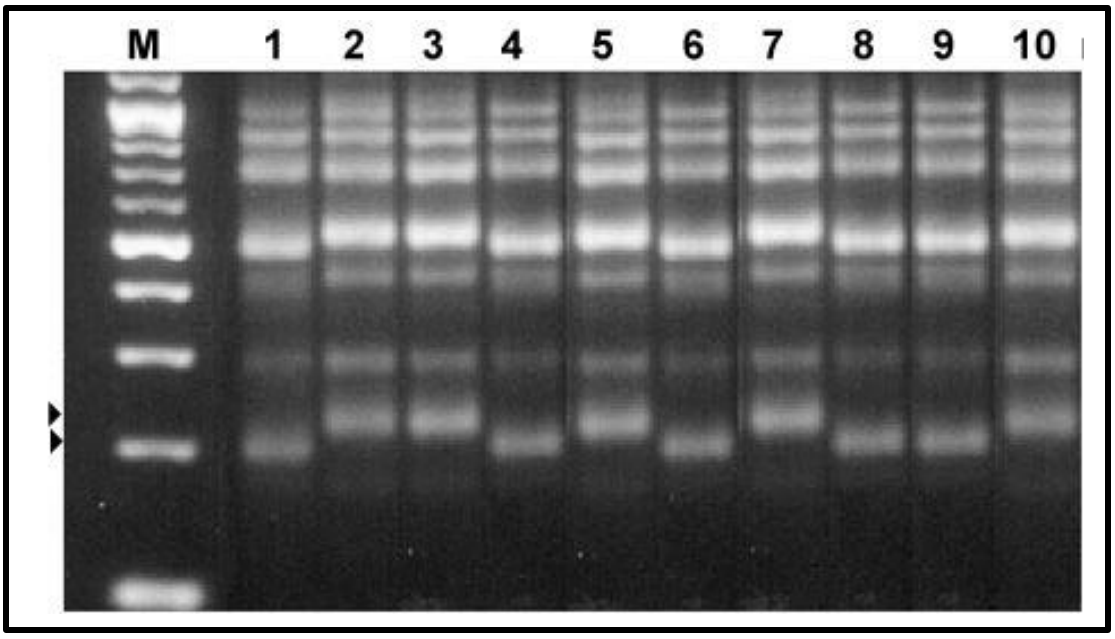


Figure 3.8: Microsatellite profile for the *Sr*30 parental screening with GWM-174: 1: LMPG-1; 2: LMPG-2; 3: LMPG-3; 4: LMPG-6; 5: Opata-85; 6: W-7984; 7: Webster; 8: Festiguay; 9: Sunstar; 10: Lerma Rojo-64. M: Molecular weight marker, 100 bp Ladder, Gibco BRL (Life Technologies, USA) Arrows represent the polymorphic co-dominant bands.

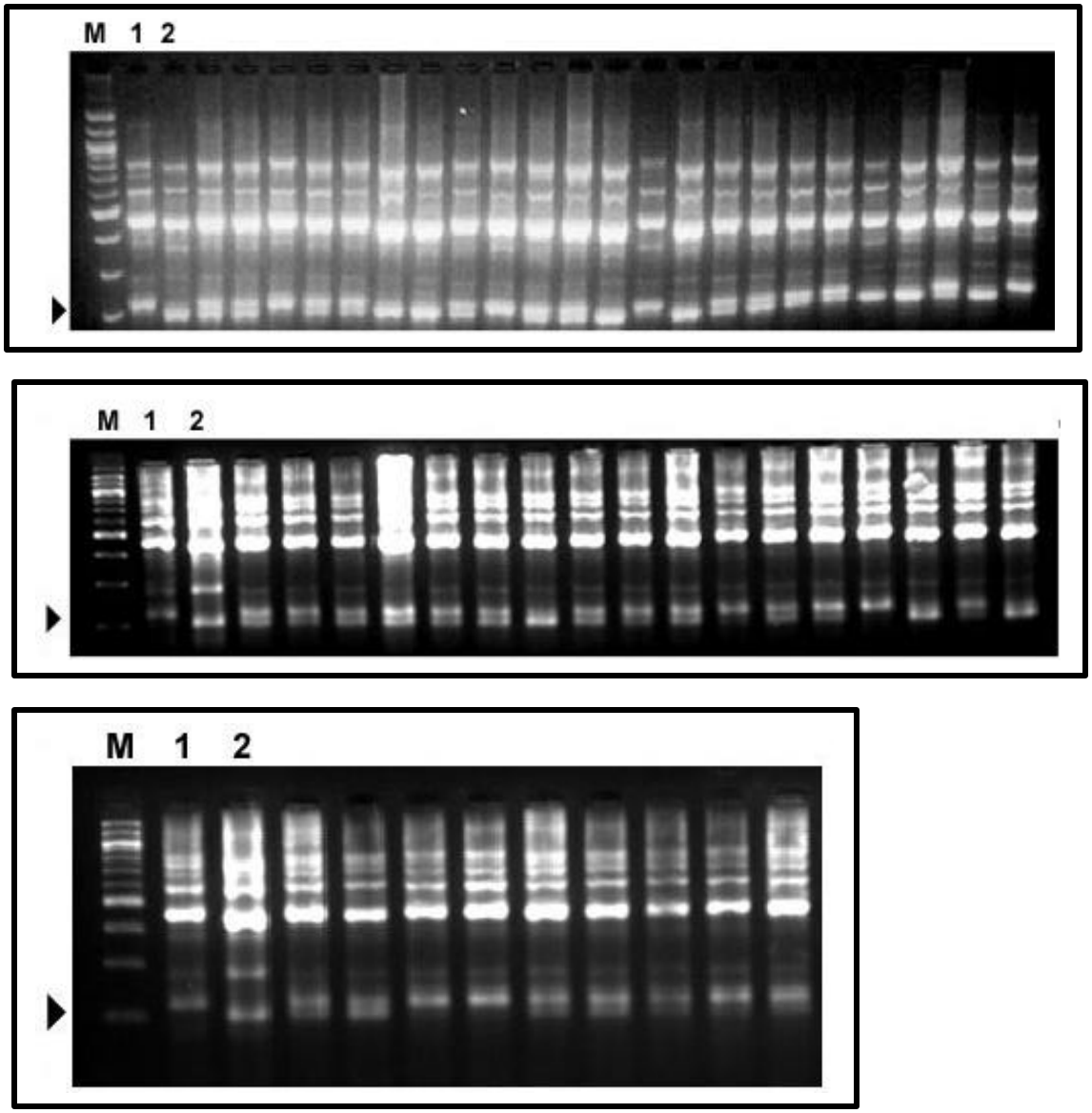


Figure 3.9: Assessment of microsatellite marker GMW-174 on LMPG-6 X Webster population of 49 F_3 lines segregating for *Sr*30. 1: Webster; 2: LMPG-6; M: Molecular weight marker, 100 bp Ladder, Gibco BRL (Life Technologies, USA) Arrows represent the segregating co-dominant band.

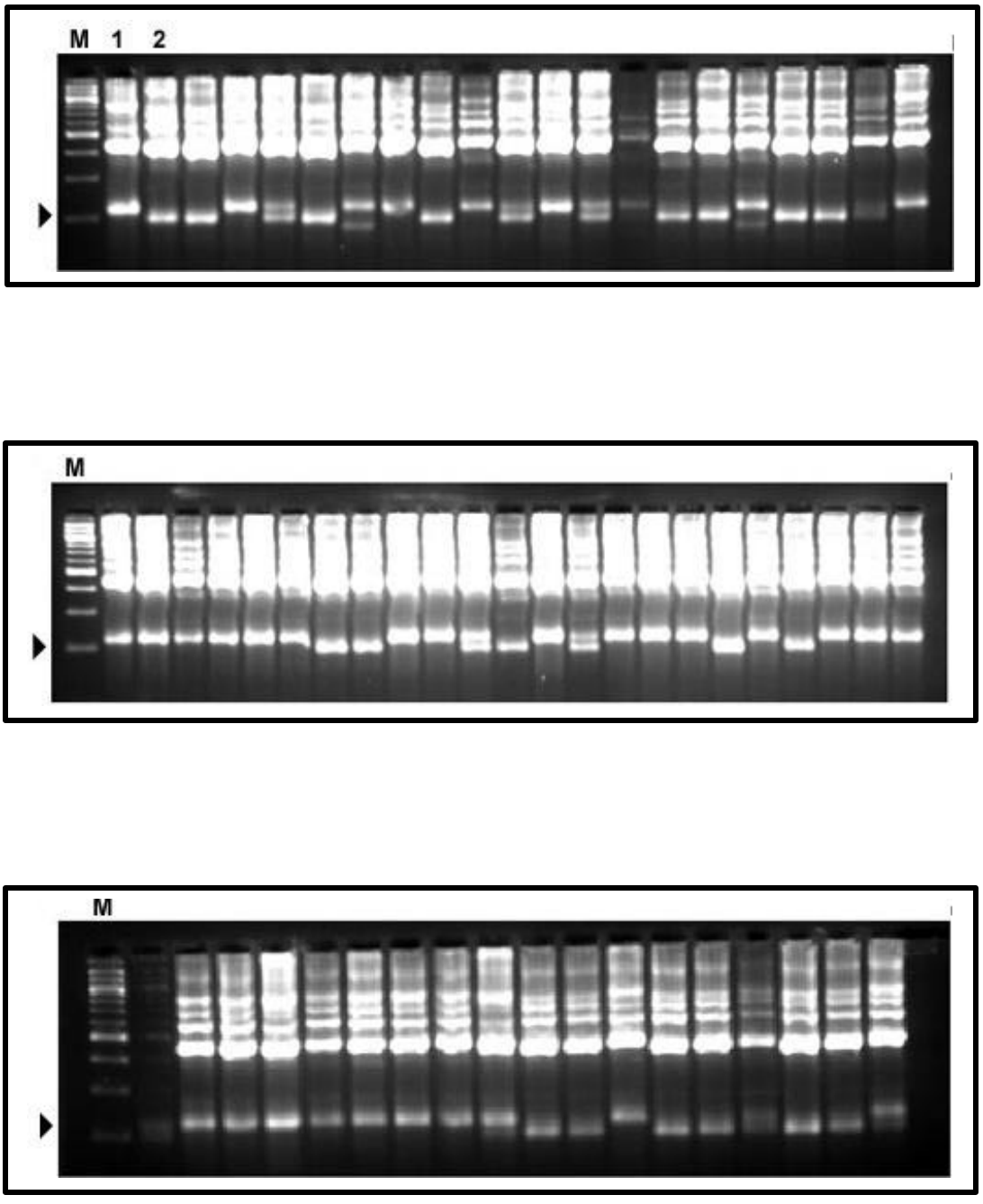


Figure 3.10: Assessment of microsatellite marker GMW-174 on ITMI population of 60 RILs segregating for *Sr*30. 1: Opata-85; 2: Synthetic Hexaploid; M: Molecular weight marker, 100 bp Ladder, Gibco BRL (Life Technologies, USA) Arrows represent the segregating co-dominant band.

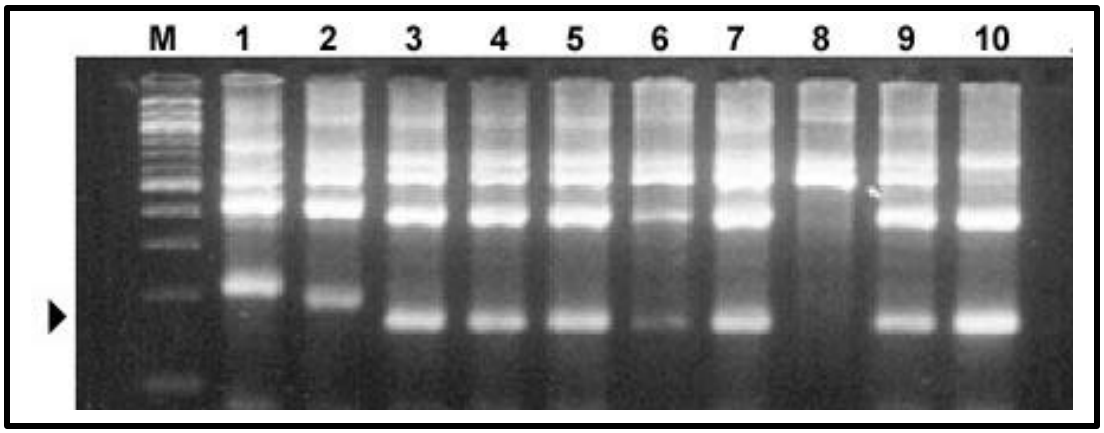


Figure 3.11: Deletion mapping of microsatellite marker GWM-174 using CS deletion lines of 5DL. 1: Webster; 2: LMPG-6; 3: Chinese Spring; 4: 0.29 5DL-7; 5: 0.35 5DL-10; 6: 0.48 5DL-12; 7: 0.60 5DL-1; 8: 0.69 5DL-2; 9: 0.72 5DL-8; 10: 0.74 5DL-9. M: Molecular weight marker, 100 bp Ladder, Gibco BRL (Life Technologies, USA). Arrow represent the co-dominant band.

and LMPG-3 (lane no. 3) but its allele of 200 bp was present in LMPG-1 (lane no. 1) and susceptible recurrent parent LMPG-6 (lane no. 4). This primer was tested against the donor of *Sr*30 Webster and other genotypes carrying *Sr*30. The band of 230 bp was associated with resistant genotypes, Webster (lane no. 7), Opata-85 (lane no. 5), and Lerma Rojo-64 (lane no. 10) while a band of 200 bp was present with the susceptible genotype W-7984 (lane no. 6) and with resistant genotypes, Festiguay (lane no. 8) and Sunstar (lane no. 9). Moreover, this marker was found to be segregating in the populations: LMPG-6 X Webster (Figure 3.9) and W-7984 x Opata-85 (Figure 3.10). However, the polymorphism did not co-segregate with the gene based on the field infection scores. When confirmed using 5DL deletion lines, the marker was located between deletion 2 and deletion 9 of 5DL, which was closer to the centromere (RODER *et al.,* 1998b) (Figure 3.11) than the gene, which was found to be located towards the 5DL tip (KNOTT, 1990).

Table 3.3:

DATA SHEET I Microsatellite (GMW-174) and AFLP genotype data in the population of F_2 intercross population derived from the cross LMPG-6 X Webster segregating for stem rust resistance gene *Sr*30. F_3 family lines 1-49. 1 = homozygous Webster allele; 0 = homozygous LMPG-6 allele; 2 = heterozygote; 3 = missing data; 4 = either 0 or 2; 5 = either 1 or 2.

GWM-174	22122002122010222200201222222022212210202211222 22
Eacc/Mcag	55005050550555500555505555555050505005500000000 05
Eacg/Mcag	55005050550555500555505555555050505000500000500 00
Eact/Mctg-1	05505000055555500005555505550555505055553555555 05
Eact/Mctg-2	14144444144444411114444441441114411411443444114 44
Eact/Mcac	50505055550555555555350505505555350505005500555 55
Eaca/Mcaa-1	05050505000550555000005055005050555005005500555 50
Eaca/Mcaa-2	14141414144411411141414144441444444414144414411 14
Eacg/Ecaa	41444411441143414114441114444111444444441144444 11

DATA SHEET II: Microsatellite (GMW-174) genotype data in the population of recombinant inbred (RI) lines derived from the cross W-7984 x Opata-85. RI lines 1-60. 1 = homozygous Opata-85 allele; 0 = homozygous W-7984 allele; 2 = heterozygote; 3 = missing data.

3.2.3 MAPMAKER ANALYSIS: All the segregating polymorphic combinations from sequence tagged microsatellites (STMS) (GWM-174) and AFLP markers were used for co-segregation analysis with the resistance phenotype as well as

among themselves (Table 3.3, data Sheet, I, II). The segregating AFLPs were mapped along with mapped locus GWM 174 which is known to be located on the chromosome 5DL (RODER *et al.,* 1998b), using MAPMAKER (LANDER *et al.,* 1987) V2.0, However, they did not show co-segregation with each other as well as with the GWM-173 and the resistance phenotype indicating that none of the AFLP markers was located on chromosome 5DL and associated with resistance gene *Sr*30.

CHAPTER 4

DISCUSSION

4.1 RUST: MAJOR DISEASE IN WHEAT LEADING TO YIELD LOSSES

The intensive agricultural management practices associated with high yielding varieties have also accentuated many disease problems due to favorable micro-climatic conditions. In such situation, in addition to the agroclimatic superiority of wheat varieties, effective disease management also gains importance for stable and sustainable production. PAL (1978) and SWAMINATHAN (1978) rightly emphasized the need for reducing losses caused by disease and pests and reducing the vulnerability of the wheat crop to major diseases in order to achieve stability in production.

Of the various diseases attacking wheat crop, the most important ones are the three rusts, *viz*. leaf rust or brown rust, caused by *Puccinia recondita* Rob. ex Desm. f. sp. *tritici*, stem rust or black rust, caused by *Puccinia graminis* Pers. f. sp. *tritici* Ericks. & E Henn, and stripe rust or Yellow rust, caused by *Puccinia striiformis* West. These can take a heavy toll of wheat production particularly in

epidemic years. In India, stem and leaf rusts are prevalent throughout the country. Stem rust is very destructive in Peninsular and Central India when it occurs, but not so in the main northern wheat belt, where it comes late and in low intensity. Leaf rust is the most important, widely distributed and most frequently occurring disease of wheat in Indian sub-continent. However, its importance has been overlooked in the past not only in India (JOSHI *et al.*, 1986) but also in other countries (CHESTER, 1946).

4.2 GENE PYRAMIDING: - A SOLUTION TO INCREASE FIELD LIFE OF THE GENOTYPE:

The severe leaf rust development on the predominant wheat varieties of Punjab during the year 1995-96 was said to be one of the primary reasons for reduction in productivity of wheat in the state as well as in the country. One of the reasons for this is the excessive dependence on highly effective seedling resistance genes in isolation from that of the adult plant resistance. The effectiveness of major seedling resistance genes is limited due to ever-evolving pathotypes. In recent past, breakdown of the gene *Lr*26 for leaf rust resistance present in many Indian varieties (Table 1.2) to new virulence necessitates a different approach for increasing the life span of Indian wheat varieties. To prevent rapid breakdown of seedling resistance genes once they are integrated into new wheat varieties, it is suggested that such genes should be used in combination with other leaf rust resistance genes (ROELFS *et al.,* 1992) preferably with adult plant resistance genes so that the variety does not become completely susceptible in the event of evolution of a new pathotype. By combining, or pyramiding a seedling resistance gene with another seedling and/or adult plant resistance (APR) gene, the field life of a variety will be extended, and in the event of origin of new pathotype against one seedling resistance, other seedling resistance or APR will hold the variety for some more time till complete break down of its resistance occurs in 3-4 years. This period can be used for withdrawing the genotype and replacing it by another resistant variety. Several genes are considered to be important for Indian rust resistance breeding programs and some of these include leaf rust resistance genes, *Lr15, Lr22A,*

Lr23, Lr28, Lr34 and *Lr46* and stem rust resistance genes *Sr5, Sr6, Sr30* to name a few.

Based on the suggestions from the Directorate of Wheat Research, Karnal, India, I selected two rust resistance genes namely *Lr*28, the *Ae. speltoides* derived alien leaf rust resistance gene, and *Sr*30, a cv. Webster derived stem rust resistance gene for my thesis research.

4.3 LOCUS SPECIFIC SCAR FOR *Lr28*:- FIRST REPORT FROM INDIA

4.3.1 Convenience of RAPD System Over Other Marker Systems: Detection of RFLPs previously used as predominant markers, by DNA blot hybridization is laborious and incompatible with the high throughput required for many breeding applications (BECKMANN 1988). Furthermore, radioactive isotopes (e.g. ^{32}P) used in Southern analysis are potentially harmful as well as expensive and, in any case, may not be available in many laboratories in developing countries. Other polymorphism assays based on PCR require sequence information of the target DNA to design primers for PCR amplification (SKOLNICK & WALLACE 1988). RAPD analysis is a PCR technique that requires neither sequence information nor isotopes. Usually, arbitrary decamer oligonucleotides are used in the PCR procedure at a low annealing temperature (e.g. 36^0C), which enables a number of DNA fragments to be amplified from several regions in the genome (WILLIAMS *et al.,* 1990, WELSH & MCCLELLAND, 1990). RAPD is fast, non-radioactive, relatively inexpensive, and needs much less genomic DNA than RFLP analysis. Thus it is convenient for a wide range of research applications and is hailed as a potential marker system (WILLIAMS *et al.,* 1993).

In my research, I have attempted 80 RAPD primers on the 8 NIL/recurrent parent combinations (Table 2.1) where the NILs were the products of separate backcross program in different genetic backgrounds. If polymorphism is obtained in all the 8 combinations of NILs/recurrent parents it would identify a marker probably linked to *Lr*28 resistance and would reduce further screening of larger population.

4.3.2 Enrichment Of Wheat DNA For Low Copy Sequences: Though, good amount of polymorphism was obtained when enriched DNA was used for parental analysis, it was difficulty to get consistent results while standardizing the PCR conditions for the wheat. This may be attributed to the fact that in wheat most of the genomic DNA is composed of highly repetitive DNA sequences (SMITH & FLAVELL, 1975) and when the RAPD assay is used in wheat, it often amplifies repetitive sequences. Another possible reason is that repetitive sequences lead to the formation of secondary structures in the template DNA rendering many sites inaccessible to the primer. Removal of highly repetitive and abundant DNA from wheat genomic DNA, or enrichment for low copy DNA fraction, could reduces the competition of highly repetitive sequences for RAPD primer sites facilitating detection of potential differences near the target region of the genome (EASTWOOD *et al.,* 1994; WILLIAM *et al.,* 1997). In my work, I have enriched the wheat DNA for low copy sequences and this probably has led to the high reproducibility of RAPD data in my analysis leading to the distinct polymorphic bands.

4.3.3 Locus Specific SCAR Marker: A Preferred System for MAS: RAPD produces numerous bands on the gel which makes it difficult to analyze when bands are not separated well and population size is larger. Compared to random primers, SCAR primers are longer, making them more specific to the site of interest. Identification of a desired phenotype also becomes much easier due to the production of a specific band. In addition, it provides a higher degree of repeatability compared to RAPDs. Therefore, I converted the RAPD marker to SCAR marker, which identified a locus of interest on the chromosome representing a reliable and efficient assay. While designing the SCAR marker, care was taken to ensure that the GC content of the primer was at least 50 %. Moreover, I did not use enriched DNA while analyzing SCAR marker, making it easier in application. SCAR/STS markers have been used successfully to detect polymorphisms in wheat for traits such as resistance to rusts (SCHACHERMAYR *et al.,* 1994, 1995, 1997; FEUILLET *et al.,* 1995; DEDRYVER *et al.,* 1996), wheat streak mosaic virus (TALBERT *et al.,* 1996), loose smut (PROCUNIER *et al.,* 1997) and

powdery mildew (HU *et al.,* 1997). The marker fragments amplified by these primers ranged from *28*2 bp (SCHACHERMAYR *et al.,* 1997) to 1.1kb (SCHACHERMAYR *et al.,* 1994).

4.4 ATTEMPT TO IDENTIFY MOLECULAR MARKERS FOR *Sr*30*:*

4.4.1 The Stem Rust Resistance Gene, *Sr30* from Webster: This gene was found to be a very effective resistance gene against wide range of races due to its uniqueness (HART 1931). CLAUDE *et al.,* (1986) and SHANG *et al.,* (1988) found *Sr*30 gene to be ubiquitous and fairly wide spread as it was detected in collections from Egypt, Crete, Turkey, Poland, India, Sardinia, Greece, and Syria. KNOTT & MCINTOSH (1978) while studying the F_1 between Chinese Spring monosomics and Webster found that the seedlings gave infection types ranging from 2^+ to 4^- with race 56 of *P. graminis*. They concluded that heterozygous plants were variable in reaction and the gene showed little dominance and was nearly recessive in nature.

A susceptible and day-length insensitive line LMPG-6, developed through various crosses involving Marquis, Prelude, Little Club, and Gabo, was crossed with Webster to develop NIL. However, during the backcrossing, lines or families were occasionally observed that appeared to have more resistance than normal. These lines were selected, tested and numbered as LMPG-1, LMPG-2 and LMPG-3. LMPG-1 was found to be more resistant to the race 56 compared to LMPG-2, and LMPG-3. LMPG-2 was more resistant to Race C15 than LMPG-3. These differences in resistances were attributed to modifiers, possibly linked to *Sr30*, or another gene of resistance (KNOTT, 1990), which was missed out during the selection for susceptible lines while developing LMPG-6. The NILs produced through 13 backcrosses would have minimal linkage drag and would lead to identification of molecular marker tightly linked to the gene of interest.

Therefore, initially these three NILs, LMPG-1, LMPG-2, and LMPG-3 with susceptible recurrent parent LMPG-6 (as described in KNOTT, 1990) and 49 lines of a 13-backcross F_2 material were obtained from Dr. Knott, University of Saskatoon which were advanced to F_3. Since this population was small, I

developed another population by crossing LMPG-6 X LMPG-2 that generated 124 F_2s. I also used Webster, the source, and three cultivars: Festiguay, Sunstar and Lerma Rojo-64 for the study. The International Triticeae Mapping Initiative (ITMI) population that also segregates for the *Sr30* gene from var. Opata-85 consisted of RILs (NELSON *et al.,* 1995) and was obtained from Dr. BS Gill, KSU. Sixty RILs from this population along with the parents, Opata-85 and synthetic hexaploid (W-7984) were used for the present study.

4.4.2 Exhaustive Use of Marker Systems to Reveal Polymorphism: Due to a large number of backcrosses given to the NILs, little linkage drag was expected around the locus of *Sr*30 gene and need of large number of different types of markers was deemed necessary to detect polymorphism. Initially I used a total of 800 decamer random RAPD primers and 100 ISSR primers to screen the parental material of NILs and recurrent parent. However, no polymorphism was obtained from this study.

Since several RFLP loci are already mapped on the wheat 5D chromosome (XIE *et al.,* 1993; OGIHARA *et al.,* 1994; DEVOS *et al.,* 1995; NELSON *et al.,* 1995; FARIS *et al.,* 1996; GILL *et al.,* 1996; KOJIMA and OGIHARA 1998; LI *et al.,* 1999), I selected 134 RFLPs mapped on this chromosome (a consolidated map is shown in figure 2.2 along with deletion intervals) for hybridization to the blots prepared using *Eco*RI, *Eco*RV, *Xba*I, *Dra*I, *Bam*HI, *Hind*III, *Apa*I, *Kpn*I, and *Sca*I restriction enzymes on all the NILs and recurrent parent. Although, good hybridization was observed, none of the enzyme-probe combination produced a polymorphic pattern. Nonetheless, KNOTT & MCINTOSH (1978) located the *Sr*30 beyond *Lr*1 on the tip of 5DL and the density of mapped markers towards the tip was poor (GILL *et al.,* 1996). This could be the reason why no polymorphism was observed by the RFLPs mapped on 5DL.

In my next effort, 64 combinations of primers from the AFLP were attempted to screen the parental material. Only six polymorphic primer/enzyme combinations were obtained which were tested on the LMPG-6 X Webster population (Figure

3.9) (Table 3.3, Data Sheet I), and no polymorphism was found linked to the resistance.

After trying RAPD, ISSR, RFLP and AFLP, I used already mapped wheat microsatellite markers located on the 5DL. Twelve microsatellite markers are reported to have mapped on 5DL (Figure 2.3) out of which only three are integrated on the map framework with a LOD >2.5 and remaining 9 are placed in the most probable intervals (RODER *et. al..,* 1998b). Out of 12 primer pairs tested on the NILs, only one pair GWM-174 produced polymorphic pattern of a band of 230 bp with two of the NILs, LMPG-2 and LMPG-3 and donor Webster, compared to susceptible recurrent parent LMPG-6 with a band of 200 bp as described in results. When tested on the cultivars, only cv. Lerma Rojo-64 produced band corresponding to Webster, whereas Festiguay and Sunstar produced band corresponding to LMPG-6. Parent pair for the ITMI population also produced the same polymorphic pattern (Figure 3.10; Table 3.3, data Sheet II).

4.4.3 Segregation of GWM-174 Derived Polymorphic Band With *Sr30*: The polymorphic primer pair, GWM-174 was tried on all the populations segregating for the *Sr30* using the computer program MAPMAKER (LANDER *et al.,* 1987) V2.0 to calculate linkage distances with Kosambi mapping function (KOSAMBI, 1944) and an LOD of 3.00. Although the marker segregated in 3:1 ratio (Figure 3.3; Table 3.3, data sheets I & II), the polymorphism could not be associated with the resistance segregating in the populations scored for the phenotype using the pt. 122 (7G11).

Interestingly, the microsatellite marker GWM-174, which has been mapped by RODER *et al.* (1998b) was shown to be located on 5DL closer to centromere, while the *Sr*30 gene was found to be located towards the tip of 5DL after *Lr*1 (KNOTT & MCINTOSH, 1978). Therefore, in an attempt to physically locate the polymorphic microsatellite marker GWM-174, I used 7 deletion lines of 5D long arm from Chinese Spring deletion line set. When confirmed using 5DL deletion lines, the marker was located between deletion 2 and deletion 9 of 5DL (Figure

3.11). Although deletion 5DL-8 showed the band, it being a heterozygous deletion, I had to assume the location of the marker between deletion 5DL-2 and 5DL-9.

I also tried to map the microsatellite markers GWM-182 and GWM-583 flanking GWM-174 at a distance of 7.6 cM and 9 cM (RODER *et al.,* 1998b, Figure 2.3). In case of GWM-583, based on the microsatellite map (Figure 2.3), its position should be towards centromere. However, the physical location of GWM-583 could not be ascertained due to the presence of band in all the deletion lines of 5DL used in my analysis indicating its location on 5DL probably beyond deletion 12 towards the centromere (Figure 4.1). Although, microsatellite GWM-182 could be mapped into the same deletion interval (Figure 4.1) as GWM-174 (Figure 3.11), it did not show any polymorphism with the resistant and susceptible *Sr*30 genotypes in my analysis. Similarly, 16 RFLP markers located on the same deletion interval (Figure 2.2), when used for the parental analysis, did not show polymorphism although the microsatellite GMW-174 located in the same region showed polymorphism. The possible explanation could be the fact that the deletion interval 5DL-2 and 5DL-8 comprises recombination hotspot (Justin Faris, KSU, personal communication) and the recombination hotspots on a chromosome are known to have high gene density, and

have preferentially participated in recombination (GILL *et al.,* 1993, 1996; HOHMANN *et al.,* 1994; DELANEY *et al.,* 1995 a, b; MICKELSON-YOUNG *et al.,* 1995).

4.4.4 *Sr*30, albeit important, a Complex Trait to Establish Molecular Markers: My intense efforts to identify DNA markers associated with *Sr30*, though unsuccessful, have thrown light on several aspects of the trait: --

It has been reported that although the resistance of Webster is governed by a single gene, it does not produce a hypersensitive reaction producing clear classes of resistant and susceptible lines and the heterozygous plants are variable in reaction ((KNOTT & MCINTOSH, 1978). I observed a similar discrepancy while scoring the population for the phenotype where the field scores did not show a segregation of 3:1 ratio. This could be the reason why I

could not show the co-segregation of microsatellite marker with the phenotype data of the *Sr*30 gene. The result was the same for both Webster derived LMPG populations and W-7984 X Opata-85 derived ITMI population.

The difference in the level of polymorphism which I observed using microsatellite GWM-174 within NILs LMPG-1, 2 and 3 could be attributed to the fact that these lines almost certainly carried different linkage blocks around *Sr*30 (Dr. D R Knott, personal communication). The latter is based on the observation that the three lines showed small differences in infection types with some specific races used by Dr. Knott. In this case LMPG-1 was found to be more resistant to the race 56 compared to LMPG-2, and LMPG-3, while LMPG-2 was more resistant to Race C15 than LMPG-3 (KNOTT, 1990). This may mean that during the backcrossing program selecting for the most resistant plants in different segregating families, they probably carried different modifiers along. If the markers are linked to these modifiers, they

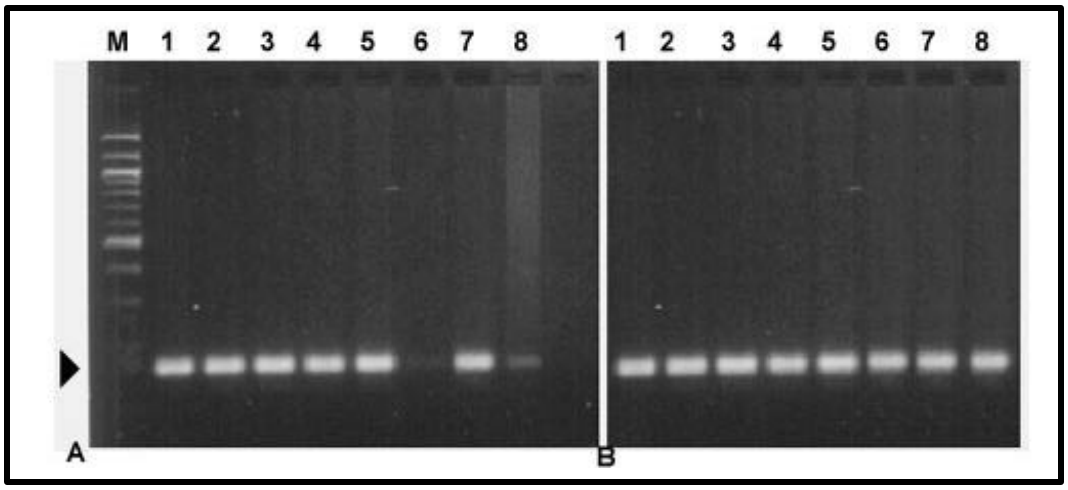


Figure 4.1: Deletion mapping of microsatellite markers; **A**: GWM-182 and **B**: GWM-583 using CS deletion lines of 5DL. ; 1: Chinese Spring; 2: 0.29 5DL-7; 3: 0.35 5DL-10; 4: 0.48 5DL-12; 5: 0.60 5DL-1; 6: 0.69 5DL-2; 7: 0.72 5DL-8; 8: 0.74 5DL-9. M: Molecular weight marker, 100 bp Ladder, Gibco BRL (Life Technologies, USA)

may not co-segregate with the phenotypic data of *Sr*30 gene in the populations. If the postulation that either this gene and/or the modifiers are located on a recombination hot spot, the region will take longer to get fixed since recombination events are frequent here than rest of 5D. This could be the reason of accumulation of different modifiers in different lines.

Observations on single plants within segregating lines indicated that the allele for resistance showed greater dominance in Festiguay crosses than in Webster crosses. This may be due to interactions in different genetic backgrounds (KNOTT & MCINTOSH, 1978) or a difference in the modifier (inhibitor) present in Webster and absent in Festiguay. This could also explain the polymorphism derived by microsatellite GWM-174 associated with two groups of parental lines, one consisting of Webster, Opata-85, Lerma Rojo-64 and two NILs (LMPG-1 and LMPG-2) and the second consisting of recurrent parent LMPG-1, Festiguay, Sunstar and more resistant NIL LMPG-1 to race 56 (KNOTT, 1990).

CLAUDE *et al.* (1986) and SHANG *et al.* (1988), while studying a F_2 population derived from the cross RL6071 (a susceptible Marquis selection) X V614, found it to be segregating for stem rust reaction according to a ratio of 9 resistant: 7 susceptible suggesting the presence of two complementary genes. When V614 was later crossed with NIL *Sr*30, no susceptible F_2 plant was observed which demonstrated the presence of *Sr*30 in V614. The explanations for this behaviour were: 1) that V614 carried *Sr30* gene that was partially dominant and the heterozygous plant inconsistently classified; 2) two complementary genes, of which one was *Sr30;* or 3) one dominant gene, *Sr30*, which was influenced by an inhibitor in RL6071. Marquis was one of parents used during the development of susceptible line LMPG-6 for transfer of various genes at University of Saskatchewan (KNOTT, 1990) and it is likely that the modifiers (inhibitors) were transferred from Marquis to the LMPG-6 line. Using Festiguay derived population, segregating 3:1 for *Sr*30, and identifying markers associated with *Sr*30 could prove this hypothesis.

4.5 SUMMARY:

This thesis documents my attempt to identify molecular markers for a leaf rust resistance gene *Lr*28 and a stem rust resistance gene *Sr*30.

Lr28: I could identify a RAPD marker linked to the *Aegilops speltoides* translocated fragment on chromosome 4AL in bread wheat carrying *Lr*28. However, I could not ascertain the recombination distance between the gene *Lr*28 and the marker since there was no segregation observed, as the translocation was inherited as a single unit and the RAPD/SCAR markers are dominant and cannot differentiate heterozygotes in the population. The RAPD was converted into a more specific SCAR marker that would enable an easy and faster screening of large population.

Sr30: In spite of an extensive screening of *Sr*30 parent material using more than 1100 diverse marker systems, only one microsatellite GWM-174 produced a polymorphic band and six AFLP primer combinations produced 8 polymorphic bands. Although, marker GWM-174 was mapped on 5DL (RODER *et al.,* 1998b) where *Sr*30 is also located (KNOTT, 1990), no linkage was ascertained between the two. Also, none of the polymorphisms obtained by AFLPs were mapped on 5DL and none were found linked to the *Sr*30 gene indicating the complexity of the locus containing *Sr*30.

CHAPTER 5

GENERAL DISCUSSION & FUTURE PERSPECTIVE

5.1 CHALLENGES BEFORE PLANT BREEDERS:

For all developing countries, wheat yields have grown at an average annual rate of over 2 per cent between 1961 and 1994. In Western Europe and North America, the annual rate of growth for wheat yield was 2.7 per cent from 1977 to 1985, which decreased to 1.5 per cent from 1986 to 1995. Recent data have indicated a decrease in the productivity gains being achieved by major wheat-producing countries. In western Europe, where the highest average wheat grain yield is obtained in the Netherlands (8.6 t ha^{-1}), yield increased from 5 to 6 t ha^{-1} in 5 years, but it took more than a decade to raise yields from 6 to 7 t ha^{-1}. Worldwide, annual wheat grain yield growth decreased from 3.0 per cent between 1977-1985, to 1.6 per cent from 1986-1995, excluding the USSR. Degradation of the land resource base, together with a slackening of research investment and infrastructure, have contributed to this decrease. Though, production constraints affected by physiological or genetic limits are hotly debated, future increases in food productivity will require substantial research and development investment to improve the profitability of wheat production systems through enhancing input efficiencies. Due to a continuing necessity for multidisciplinary team efforts in plant breeding, and the rapidly changing development of technologies, three overlapping avenues which can be considered for raising the yield frontier in wheat are: continued investments in "conventional breeding" methods; use of current and expanded genetic diversity; and investigation and implementation of biotechnology-assisted plant breeding (RAJARAM, 2000).

5.2 QUESTIONS TO BE ANSWERED:

On a worldwide basis, plant breeding has been one of the most successful technologies developed in modern agriculture. Its methods are opportunistic and adaptable to myriad production schemes, they require relatively inexpensive input, and their products have pervasive social benefits. Imminent global developments will demand continued and perhaps greater success because agriculture systems will be required to maintain or increase production with fewer resources. The human population is projected to grow from 5.7 to 10 billion by the year 2050, the supply of high quality land is diminishing, and concerns about environmental quality will encourage production practices that require less energy (e.g., chemical and fuel). To assess and develop strategies for continued progress, several questions should be addressed. How have the gains been achieved in past? Can the present rates of gain be maintained or improved? What are the sources and costs of future gains? Substitutive answers to these questions become progressively difficult to obtain (LEE, 1995) and the most difficult to answer is, can the present rates of gain be maintained or improved? SINCLAIR (1993) and others have argued that only marginal opportunities remain for genetic improvements in crop yield potential. Instead, greater benefits may be realized through genetic improvements in crop tolerance to biotic and abiotic stresses.

5.3 INVOLVEMENT OF DNA MARKERS IN CURRENT PLANT BREEDING METHODOLOGY:

Much, if not most, of the success of plant breeding has been accomplished by less use of basic knowledge of plant biology. But in an era of heightened expectations from crop genetics, the demand should increase for firm data and more complete understanding of genetic gains. Basic plant biology will be the source of much new information about genomics, genes, pathways, and interactions of direct relevance to crop improvement. In many instances, DNA markers will be the vital link between a nascent tributary and mainstream plant breeding (LEE, 1995). DNA markers offer considerable advantages for backcross

(BC) breeding such as (1) indirect selection of desirable gene(s) from donor parents, (2) selection for regions of recurrent parent genome unlinked to the introgressed region, and (3) reduction of linkage drag of unwanted donor parent genome near the introgressed region(s).

5.4 MARKER ASSISTED SELECTION (MAS):

Molecular marker assisted selection facilitates the creation of new varieties with more directed improvements. It allows breeders to handle larger populations and select lines with desired attributes and with appropriate agronomic performance. This application of biotechnology does not fundamentally change what wheat breeders have been doing for years, namely selecting for desirable traits in new varieties. Nevertheless, it increases the efficiency of that selection (Figure 5.1). Plant breeders need to handle an ever-increasing number of wheat classes designed for different end-use markets. Molecular markers offer an effective alternative means of selecting for traits where conventional selection techniques are difficult to perform, costly, or time consuming (MAZUR & TINGEY, 1995).

Among the two stresses to which crops are exposed, biotic stress is important since losses incurred due to diseases and pests are larger and wide spread in the worst cropping season. Resistance genes represent a small part of the genotype package that is of interest to the wheat breeder. Development of the genetic map using molecular marker systems has revolutionized the power of genetic analysis in wheat, enabling agronomic trait loci, whether major genes or QTLs, to be identified, located and tagged. They have been refined further in 1990's particularly by the application of molecular cytogenetics and molecular marker techniques (SNAPE, 1998). The power to select desirable individuals in a breeding program based on genotypic configuration is an extremely important application of DNA markers and QTL mapping (TANKSLEY *et al.*, 1989). DNA markers in genomic regions of interest enable breeders to select on the basis of genotype rather than phenotype, which can be especially helpful if a target trait is time consuming to score.

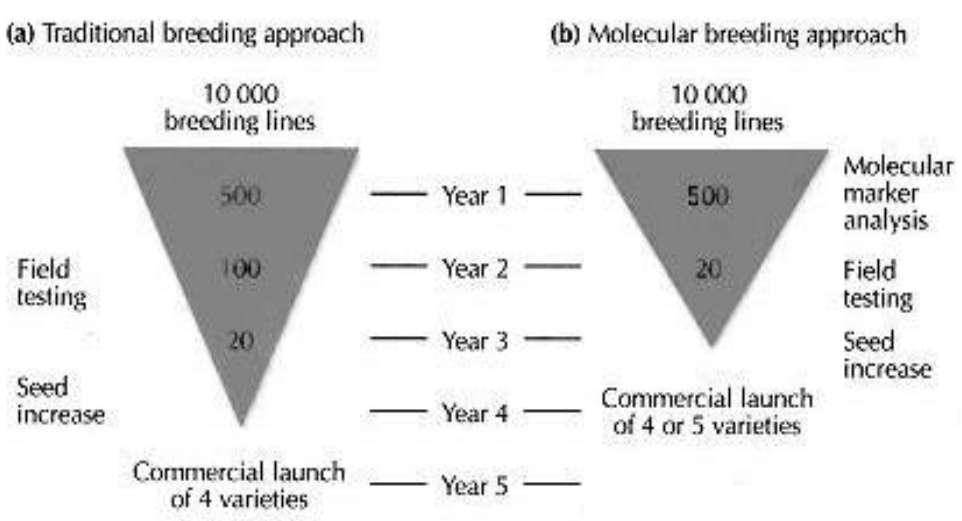


Figure 5.1: Comparison of traditional and molecular breeding approaches to variety development. Molecular marker technology can be applied to reduce the most expensive cycle of yield testing, and thereby reduce development time by one year (Adapted from MAZUR & TINGEY, 1995).

5.5 THE ERA OF MOLECULAR MARKERS:

Is this the dawn of the "biology" era of plant breeding? Previous innovations and technologies that had major impacts on the efficiency and productivity of plant breeding may be inherently different from today's biotechnologies and related basic information. Computers and NIR analyzers were easily inducted into existing plant breeding methods and they allowed plant breeders to look at more genotypes in more environments. The new biotechnologies are different and they will require plant breeders and their colleagues to look within the plant and understand its architecture before routine, beneficial, and predictable advancements.

How will we assess the efficacy of DNA markers as a tool for crop improvement? In some situations, the accounting process may be easy. For example, important benchmarks have been established for several crops, for traits such as; rate and cost of gain, genetic component of gain, and time and cost required for cultivar

development. Situation lacking such history and databases will be more difficult to assess.

The first phase of the encounter between plant breeding and DNA markers has been concluded. While the markers have unquestioned benefits for basic research, their utility for plant breeding remains to be established in several aspects. Plant breeders have been justifiably skeptical given the history of hype associated with previous biotechnologies and other derivatives from basic biology (SIMMONDS, 1991). The efficiency of plant breeding programs, often with several decades of organized activity, will be difficult to enhance in a direct manner, but there is sufficient evidence to maintain an optimistic forecast (LEE, 1995). Also, the comprehensive scope and countless challenges of breeding programs will require an equally broad and critical assessment of new approaches and resources of information.

5.6 NEW ADVANCES AND FUTURE PROSPECTS OF THE MOLECULAR TOOLS:

The transition to a new era of biological research is underway, and both the public and private sectors are moving to exploit the new tools and opportunities presented by genomics. In response to the promise of both fundamental advances and profitable applications, there has been an infusion of funding that is enabling large-scale experimentation and rapid progress. New technologies are also permitting experimentation on a scale that was previously unimaginable. The massive amounts of data beginning to be generated are providing new insights and challenges. The transition will affect most areas of biology, and disease resistance in plants is no exception. Genomic approaches are already beginning to have impact on fundamental and applied plant biology. Over the next ten years, there will be paradigm

Table 5.1 . Plant genomics, now and in five to ten years.

Activity	Now	Five to ten years
Sequencing	De novo generation	Rice, Arabidopsis and large portions of other crop species sequenced Resequencing of allelic variants
Genetic mapping	Based on segregation analysis	Hybridization to contiguous arrays of genomic clones
	Low density maps for many species High density maps for a few	Extensive inferences between species based on macro- and micro-synteny
Gene expression	Predominantly at mRNA level Sequencing of random cDNAs	Quantitative catalogs of all expressed genes will exist for many species and situations
	Microarray analysis beginning Proteomics in its infancy	Routine global analyses using DNA chips, protein arrays and/or other technologies
Gene discovery	Gene-by-gene basis	Candidate gene approaches by relating phenotypes to sequenced genomes
	High-throughput phenotyping of mutants beginning	Bulk discovery relating differentially expressed sequences to phenotypes
Comparisons between homologues	Emphasize similarities for functional inferences	Analyses of allelic differences to explain variation in function
Focus of research	Basic biology of model species	Transfer of paradigms from model species to crops and study of crop problems in model species

shifts in experimentation (Table 5.1) (MICHELMORE, 2000). A transitory change is occurring from hypothesis-driven research to a period of descriptive study involving the generation of large amounts of data. Subsequently, hypotheses will often be derived from *in silico* analyses of databases, and testing may initially involve computer simulations prior to actual experimentation. Testing of hypotheses will still require detailed phenotyping, but experimental studies will access a broad range of new tools capable of global analyses of RNAs, proteins, and metabolites rather than a gene-by-gene or protein-by-protein approach.

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Academic Qualifications:

DEGREE	UNIVERSITY	YEAR	SUBJECT	PER CENT/ GRADE	DIVISION
B.Sc.	Indira Gandhi Agricultural University, Raipur, India	1985-1989	Agriculture	71.17	First
M.Sc.*	Indira Gandhi Agricultural University, Raipur, India	1989-1993	Plant Breeding & Genetics	7.53/10	First

* Thesis research work done at ICRISAT

Research Experience: (See Annexure I, II, III)**:**

1. 1989-1990: Worked as a Post-graduate student on plant cell and tissue culture studies on ***Brassica***, ***lathyrus***, and **chickpea.**
2. 1991-1993: Worked as a Research Scholar on the Project *'Interspecific hybridization and characterization of hybrids in genus CICER*" for masters thesis work, at the **International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)**
3. June 1994 –1997 worked as Project Assistant at **Agharkar Research Institute (ARI)** and

   April 1997-2000: **Council for Scientific and Industrial Research (CSIR)** Senior Research Fellowship on:

   "*Identification and Localization of Molecular Markers Linked to the Stem & Leaf Rust Genes in Wheat***"**, a collaborative project with **Kansas State University (KSU) USA, University of Saskatoon (Saskachewan) Canada, and National Chemical Laboratories (NCL), Pune, India.**
4. September 1999-March 2000: Worked as Visiting Research Assistant at, **Department of Plant Pathology, Kansas State University, USA.**
5. **Currently holding PROJECT ASSISTANT II position at NCL.**

Awards/Scholarships:

1. 1999-2000: **Visiting Research Assistantship at Kansas State University, USA.**
2. 1997 onward: **Council for Scientific and Industrial Research (CSIR) Senior Research Fellowship**
3. 1994-1997: **ARI Project Assistantship**
4. 1995: International Maize & Wheat Research Institute (**CIMMYT**)/International Triticeae Mapping Initiative (**ITMI**)/Pioneer Hi-bred Intl. **Scholarship** for training course on "MOLECULAR MARKER APPLICATIONS TO PLANT BREEDING", at CIMMYT, Mexico.
5. 1991-1993: **ICRISAT Research scholarship**
6. 1991 - Madhya Pradesh (India) Council of Science and Technology (MAPCOST, Govt. of M.P) & Department of Science and Technology (DST, Govt. of India) **Young Scientist Award**.

Published work:

[A] BOOK CHAPTER:

1. **S Naik**, Tiwari R, Gill BS, Gupta VS, Rao VSP, Ranjekar PK, Keller B, Nagarajan S (2001) Utility of molecular markers in breeding rust resistance wheat material. In-"Role of Resistance in Intensive Agriculture" Nagarajan, S and D.P. Singh (eds), Kalyani Publishers, India, New Delhi. pp- 205-215.

[B] JOURNAL PAPERS:

1. **S Naik**, KS Gill, VS Prakasa Rao, VS Gupta, S Tamhankar, S Pujar, BS Gill and PK Ranjekar (1998) Identification of STS markers linked to the *Triticum speltoides*-derived leaf rust resistance gene *Lr28* in wheat.Theor. Appl. Genet. Vol.97(4):535-540.
2. S Pujar, SA Tamhankar, VS Prakasa Rao, VS Gupta, **S Naik** and PK Ranjekar (1999) Arbitrarily primed-PCR based on diversity assessment reflects hierarchical groupings in Indian tetraploid wheat genotypes. Theor. Appl. Genet. 99:868-876.

[C] CONFERENCE PAPER:

1. Vidya Gupta, PK Ranjekar, VSP Rao, BS Gill, **Suresh Naik**, Aditi Galande, SA Tamhankar, MD Lagu, KS Gill, R. Tiwari. Molecular marker assisted wheat improvement in India: A progress report. (1998) 9th International Wheat Genetics Symposium.(Proceedings)
2. **Naik S** (1991) *In vitro* studies on plant regeneration in *Lathyrus sativus* L." In: 1991 MAPCOST, DST Young scientist Congress, Raipur, India (Proceedings)

[D] POSTER PRESENTATIONS:

1. **Suresh Naik**, V S Rao, V S Gupta, S A Tamhankar, S Pujar & P K Ranjekar (1996) Identification of molecular markers linked to the leaf rust resistance gene *Lr28* of bread wheat (*Triticum aestivum* L.) 1996 International Public Workshop of International Triticeae Mapping Initiative (ITMI) August 30-31, 1996, Sydney, Australia.
2. **Naik S** , S K Katiyar and J) P Moss (1994) A new hybrid: *Cicer arietinum* X *C. Pinnatifidum* through embryo rescue. Proc. Second Asia-Pacific Conference on Agricultural Biotechnology, 6-10 March 1994, Madras, India (Proceedings)
3. Katiyar S K, **S Naik**, and H Sharma (1994) *In vitro* regeneration of genetically variable plants of *Lathyrus sativus* L.. Proc. Second Asia-Pacific Conference on Agricultural Biotechnology, 6-10 March 1994, Madras, India (Proceedings)
4. Katiyar S K and **S Naik**, (1992) *In vitro* Plant Regeneration in *Lathyrus sativus* L. Proc. 2nd International Food Legume Research Conference, 12-16 April 1992, Ramses Hilton, Cairo, Egypt (Proceedings)

[E] PATENT:

1. An Indian patent on “A Process for the preparation of a semisynthetic amplicon useful for the identification of the presence of rust resistance gene Lr28 in wheat”: VS Prakasa Rao, PK Ranjekar, **Suresh Naik**, VS Gupta. (Patent #380Del98, filed with CSIR, New Delhi)