The genetic stability of a recombinant form of the *Lr19* translocation of wheat

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Date:...23./.11./99

OPSOMMING

Die *Lr19* geen is wêreldwyd 'n uitstekende bron van blaarroes-weerstand. Dit kom voor op 'n translokasie op chromosoom 7BL van *Triticum aestivum* en is verhaal van *Thinopyrum ponticum*. Bekende gene op die translokasie is: sentromeer - *Sd1* (segregasie distorsie) - *Xpsr 165* - *Xpsr 105* - *Xpsr 129* - *XcsIH81-1* - *Lr19* - *WSP-D1* (water oplosbare proteïen) - *Sr25/Y* (stamroes-weerstand/ geel endosperm). Nadat die meiotiese paring in 'n *Lr19* heterosigoot ontwrig is, is 'n rekombinant, *Lr19* (149), geselekteer (Marais 1992c). Tydens rekombinasie is *Lr19* (149) verplaas na chromosoom-arm 7BL in 'n dubbel-oorkruisings-gebeurtenis. In die proses is *Thinopyrum* chromatien verruil vir koring-chromatien aan beide kante van *Lr19*. *Lr19* (149) het *Y1*, *Sr25* en *Sd1* verloor. Gamete met *Lr19* (149) toon 'n sterk neiging om te self-elimineer in translokasie heterosigote.

Die doel van hierdie studie was om te bepaal of: (1) self-eliminasie van gamete in beide geslagte van heterosigote plaasvind, (2) die *Lr19* (149) translokasie, in die teenwoordigheid van die *Ph₁* (parings inhibeerder) geen, kan rekombineer met homoeoloë streke op chromosoom-arm 7BL, en (3) die translokasie se self-eliminerings-neiging gepaard gaan met 'n verhoogde voorkoms van mutasies.

Sterk self-eliminasie van *Lr19* in heterosigote is waargeneem in F_2 en F_3 populasies. Die self-eliminasie effek was sterker ten opsigte van oordrag deur die stuifmeel.

Geen rekombinasie is tussen *Lr19* en twee proksimaal geleë loki, *Xus-OPK9₁₃₅₀* en *XcsIH81-1* waargeneem nie, en ook nie tussen *Lr19* en twee distaal geleë loki, *Wsp-D1c* en *X12c*, nie. Hieruit kan afgelei word dat die translokasie gedurende meiose oorgeërf word as 'n enkel, groot koppelingsblok. Die rede hiervoor kan wees dat die homologie-vlak oor die hele chromosoom op molekulêre vlak getoets word voordat sinapse en oorkruisings plaasvind gedurende meiose. Daar word geglo dat die *Ph₁* geen sodanige homologie-herkenning reguleer. Geen mutasies is by die vier merker loki gevind nie. Dus, indien *Lr19* (149) in teling gebruik word, sal dit noodgedwonge as 'n koppelingsblok oorerf en seleksie vir verbeterde *Lr19* (149) homosigote sal oneffektief wees. 'n Voordeel is dat dit nie met 'n verhoogde mutasie vermoë gepaard sal gaan nie.

'n Poging wat aangewend is om die csIH81-1 peiler om te skakel na 'n STS ("sequence-tagged-site") merker was nie suksesvol nie omdat geen bruikbare polimorfisme verkry is nie. Selfs nadat verskillende restriksie-ensieme gebruik is om die amplifikasie-produk te sny, is daar steeds geen nuttige polimorfismes waargeneem nie.

SUMMARY

The *Lr19* gene is an excellent source of leaf rust resistance worldwide. It occurs on a translocated segment on chromosome 7DL in *Triticum aestivum* and was derived from *Thinopyrum ponticum*. Known genes on the translocation are: *centromere - Sd1 (segregation distortion) - Xpsr165 - Xpsr 105 - Xpsr129 - XcsIH81-1 - Lr19 - WSP-D1 (water soluble protein) - Sr25/Y (stem rust resistance/ yellow endosperm)*. Following the disruption of meiotic pairing behaviour in a *Lr19* heterozygote, a recombinant, *Lr19* (149), was selected (Marais, 1992c). In the recombination event *Lr19* (149) was relocated to chromosome arm 7BL with wheat chromatin on both sides of the translocation. *Lr19* (149) has lost *Y1, Sr25* and *Sd1*. In translocation heterozygotes, gametes with *Lr19* (149) have a strong tendency to self eliminate.

The purpose of this study was (1) to determine if self-elimination occurs in heterozygotes of both sexes, (2) if the *Lr19* (149) translocation can recombine with homoeologous regions on 7BL in the presence of Ph_1 (pairing inhibitor) gene, (3) to determine whether the self-elimination tendency of the translocation is accompanied by an increased incidence of mutations.

Strong self-elimination of *Lr19* was detected in F_2 and F_3 populations. The self-elimination, which is influenced by the genetic background, was found to be more pronounced when the segment was transmitted via pollen.

No recombination was detected between *Lr19* and two proximally located loci: $Xus-OPK9_{1350}$ and *XcsIH81-1*, and also not between two distally located loci: *Wsp-D1c* and *X12c*. This suggests that the translocation is transmitted as a single, large linkage block during meiosis. The reason for this is probably the Ph_1 gene which regulates homology recognition along the entire length of the chromosome. No mutations were found at the four marker loci. Thus, if *Lr19* (149) is used in breeding, its transmission will be impaired on the segregating generations and the selection of superior *Lr19* (149) homozygotes will be complicated. Fortunately, this will not be accompanied by an increased tendency for mutation.

An attempt to convert the csIH81-1 probe into a STS (sequence-tagged-site) marker was not succesful as no useful polymorphisms could be obtained, even after using different enzymes to cut the amplification product.

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LIST OF ABBREVIATIONS

α	Alpha
AFLP	Amplified fragment length polymorphism
avr	Avirulence
BC	Backcross
Bp	Basepair
Bps	Basepairs
°C	Degrees centigrade
CDP	Chemiluminescent alkaline phosphatase substrate
cM	CentiMorgan
cm	centimeter
dATP	Deoxyadenosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidiumbromide
g	Gram
HR	Hypersensitive response
kb	Kilobase
LB	"Luria Bertani"
Lr	*Leaf rust resistance locus*
MAS	Marker-assisted selection
Mb	Megabases
Mg	Milligram
min	minutes
ml	Milliliter
MT	Megaton
NaCl	Sodium chloride
NaOH	Sodium hydroxide

PCR	Polymerase chain reaction
pmol	picomole
RAPD	Random amplified polymorphic DNA
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SCAR	Sequence characterized amplified region
Sd	*Segregation distortion*
SDS	Sodium dodecyl sulphate
STS	Sequence tagged site
TAE buffer	Tris acetic-acid EDTA buffer
TBE buffer	Tris boric-acid EDTA buffer
TE	Tris EDTA
Th.	*Thinopyrum*
Tm	Melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
μg	Microgram
μl	Micrometer
μM	Micromolar
UV	Ultra violet
VNTR	Variable number of tandem repeats
X-Gal	5-Bromo-4-chloro-3-indolyn-β-D-galactoside
Y	*Yellow endosperm locus*

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CHAPTER 1

INTRODUCTION

Wheat belongs to the plant family Gramineae. It is the second most important cereal crop in the world (Pienaar, 1990). Barley, rice, maize, oat, sorghum and sugarcane are other crops of high commercial value which belong to the same family. Wheat is grown in a wide range of climates over an area of 228 945 thousand hectares and an estimated 535 842 MT are produced annually in the world.

The chromosomes of bread or common wheat, *Triticum aestivum* ($2n = 6x = 42$), can be grouped into seven homoeologous sets. Each set consists of three homologous pairs, one each of the A, B and D genomes. The genome size is 16×10^6 kilobase pairs (kb). More than 75% of the wheat genome consists of repeated DNA sequences and approximately 20% of unique sequences (Ranjekar *et al.* 1976).

Homoeologous chromosomes in wheat have similar gene contents and can replace and compensate for each other in nullisomic-tetrasomic combinations (Sears, 1952, 1966). In spite of the close relationship among the genomes of bread wheat, its chromosomes pair only homogenetically at metaphase I. The suppression of pairing between wheat homoeologous chromosomes is primarily due to the activity of the *Ph1* locus on chromosome 5B (Okamoto, 1957; Riley & Chapman, 1958; Riley, 1960; Sears, 1977).

The wheat genome is genetically poorly mapped in comparison to most of the other important crop species (Hart, 1994) due to polyploidy, a large genome and autogamy. Furthermore, the presence of genes in triplicate may prevent the expression of the morphological and physiological effects of recessive alleles - they are masked by the effects of dominant alleles at paralogous loci.

The cereal rusts rank among the most harmful pathogens of wheat. Wheat leaf rust, sometimes called brown rust, is caused by *Puccinia recondita* Rob. ex Desm *tritici* (Knott, 1989). It may cause serious economic losses and is the most widely distributed of the wheat rusts. Other types of rusts occurring in wheat are stem rust (*Puccinia graminis* Pers *tritici*) and yellow rust (*Puccinia striiformis* West *tritici*) (Quisenberry & Reitz, 1967).

Leaf rust primarily attacks the leaf blades and to a lesser extent leaf sheaths and glumes. Heavy infestations result in premature defoliation of the plants which leads to the shriveling of the kernels (Knott, 1989). Although total crop loss does not occur, yield reductions of up to 40% have been reported. Leaf rust tends to cause less severe damage than stem rust, but in some areas it occurs more frequently and it can cause greater losses overall. According to Quisenberry & Reitz (1967), leaf rust reduces the quality, protein and carbon contents of wheat and can also increase the rate of transpiration, thus increasing the water requirements of the plant.

The symptoms of leaf rust are small, round, orange-red pustules, usually about 0.2 cm in diameter (Fig.1.1). The pustules are largely found on the upper leaf surface (Knott 1989). They are readily distinguishable from stem rust pustules on leaves by their smaller size, round shape and orange-red color. In a severe epidemic, almost the entire surface of the leaf blade can be covered with pustules. The leaves senesce rapidly and dry out, depriving the plant of much of its photosynthetic area.

Leaf rust is primary controlled by either genetic resistance or the use of chemicals, and to a lesser extent by cultural methods (Knott 1989).
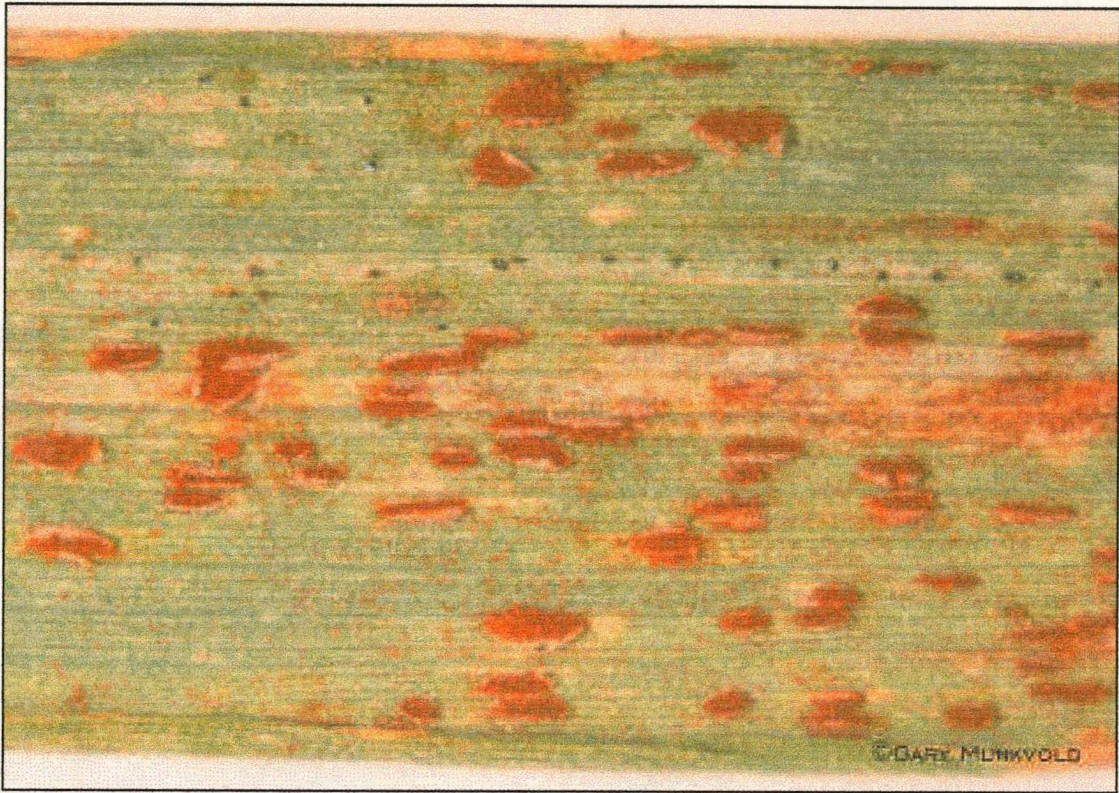
Figure 1.1 Leaf rust.
(http://www/ent.iastate.edu./imagegal/plantpath/wheat/leafrust)

1.1 PLANT DISEASE RESISTANCE

Breeding for disease resistance is of increasing importance world-wide. Plant disease resistance reduces yield losses caused by pests and diseases and limits the use of pesticides (Franck-Oberaspach & Keller, 1997).

Plant breeders have used disease-resistance genes (R-genes) to control plant diseases since the turn of the century. In contrast to animals, which have a circulatory system and antibodies, plants have evolved a unique system whereby each plant cell is capable of defending itself (Staskawicz *et al*., 1995). This is accomplished by a combination of constitutive and induced defenses. Resistance to a pathogen is manifested in a variety of ways. It is often correlated with a hypersensitive response (HR), which results in localized induced cell death in the host plant at the site of infection (Keen *et al*, 1993 as sited in Staskawicz *et al*., 1995). The HR is thought to be responsible for the limitation of pathogen growth.

The work of Flor (1971) provided the theoretical basis for the gene-for-gene hypothesis of plant pathogen interactions. Recent successes in the isolation and chracterisation of many avirulence (*avr*) and resistance genes have provided molecular support for this hypothesis (De Wit, 1997). An *avr* gene gives the pathogen an avirulent phenotype on a host plant that carries the corresponding R gene (Fig. 1.2). In gene-for-gene interactions, the induction of the plant defense response that leads to HR is initiated by the plant's recognition of specific signal molecules (elicitors) produced by the pathogen. These elicitors are encoded directly by avirulence genes, and R genes are thought to encode receptors for these elicitors. The recognition event activates a cascade of host genes that leads to HR and inhibition of the pathogen growth (Staskawicz *et al*, 1995).
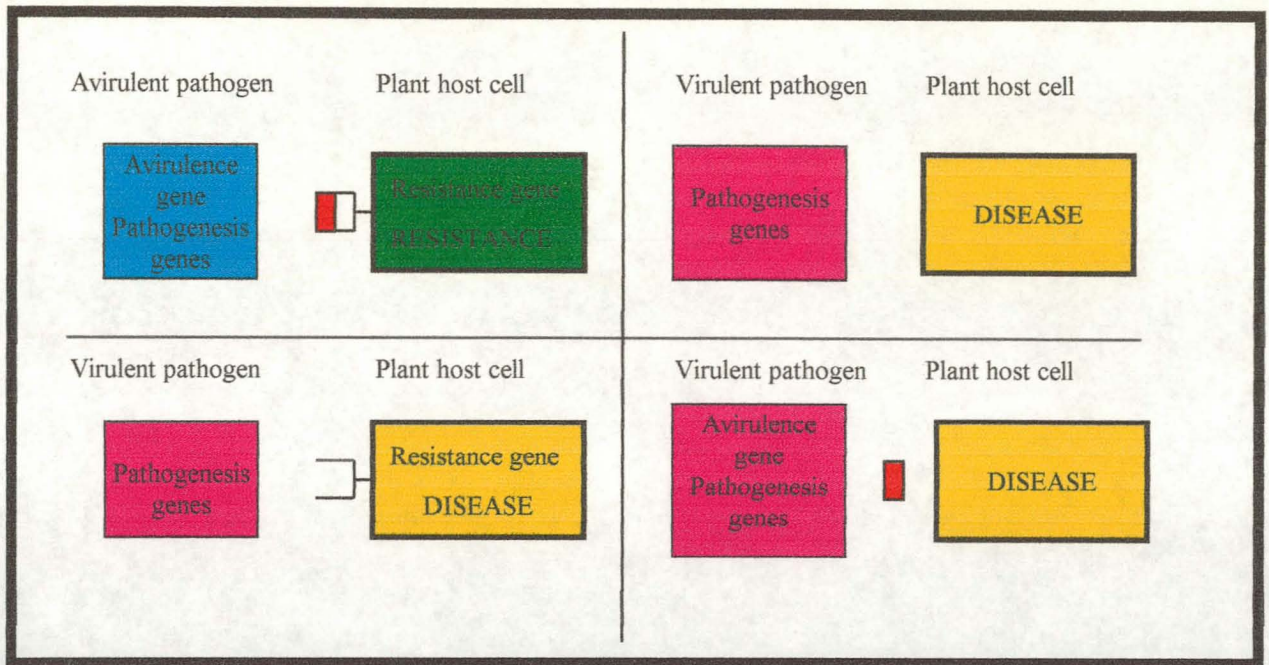
Figure 1.2 Gene-for-gene interactions specify plant disease resistance (from Staskawics *et al.*, 1995). Resistance is only expressed when a plant that contains a specific R gene recognizes a pathogen that has the corresponding avirulence gene (upper left panel). All other combinations lead to lack of recognition by the host, and the result is disease.

Key:　　　　　　　⅃⎺　　= Resistance gene product (receptor)

　　　　　　　　　▮　　　= Avirulence gene (*avr*) product (elicitor)

1.1.1 Transfer of resistance genes.

Small, fragmented and isolated populations of many plant and animal species, due to the activities of man, may lead to genetic erosion (Van Treuren *et al*, 1991). It can also reduce the fitness of individuals in a population and promote the extinction of the population. The deleterious effects of genetic erosion in populations, however, can be reversed by gene introduction, renewing the genetic variation.

Bread wheat can be successfully crossed with its wild relatives and other related species (Sharma & Gill, 1983; Baum *et al*., 1992; Jiang *et al*., 1994; Sharma, 1995). These species represent a large reservoir of useful traits that can be exploited for wheat improvement. The method for transferring genes from a related species to wheat depends on the evolutionary distance between cultivated wheat and the species involved (Friebe *et al*., 1996).

Donor species can belong to one of three gene pools. The first (primary) gene pool shares homologous genomes with wheat e.g. the donors of the A and D genomes of bread wheat. Gene transfer from these species can be achieved by direct hybridization, homologous recombination, backcrossing and selection, examples are *Sr22* (stem rust resistance) from *T. monococcum* (Kerber & Dyck, 1990) and *Gb5* (greenbug resistance) from *Ae. speltoides* (Lay *et al*., 1971; Wells *et al*., 1973, 1982).

The secondary gene pool of common wheat includes species that have at least one homologous genome in common with *T. aestivum* (Friebe *et al*., 1996). Gene transfer from these species by homologous recombination is possible if the target gene is also located on a homologous chromosome. *Sr40* (stem rust resistance) was transferred from *T. timopheevii* spp. *araraticum* (AAGG) (Dyck, 1992) which is a specie from the secondary gene pool.

Species belonging to the third gene pool are more distantly related. Their chromosomes are not homologous to those of wheat and gene transfer is very difficult because of the fact that the *Ph1* gene ensures that only homologous chromosomes pair and recombine. Radiation treatment can be used to induce chromosome breaks and translocations. Using this method, *Lr19* (leaf rust resistance gene) was transferred from *Thinopyrum ponticum* to wheat (Sharma & Knott, 1966). By disrupting normal meiotic chromosome pairing through hybridization

with a high pairing line of *Ae. speltoides,* a stripe rust resistance gene from *Ae. comosa* was transferred to wheat by induced homoeologous recombination (Riley *et al.* 1968).

Another method for the transfer of resistance genes is the production of transgenic plants through the stable introduction of foreign DNA into the plant genome, followed by regeneration to produce intact plants which express the introduced gene(s) (Walden & Wingender, 1995). Methods used for transformation can be divided into two groups: indirect and direct. *Agrobacterium-tumefaciens*-mediated gene transfer is an indirect method because the gene of interest first has to be transferred to the bacterium. The bacterium cells then infect plant cells *in vitro* and transfer the gene into the nucleus of the plant cell. When the foreign DNA is delivered directly into the plant cell it is classified as a direct method. This is done by one of the following methods, (i) by converting donor and recipient plant cells into protoplasts, and then stimulating fusion by electroporation or chemical treatment, (ii) by particle bombardment or (iii), by microinjection. Dicotyledonous crops are normally transformed by using *Agrobacterium tumefaciens*, whereas monocotyledonous crops are usually transformed using direct methods.

Several transgenic crop varieties with agronomically useful levels of resistance to insects and viral pathogens have been generated through gene transfer. Transgenic wheat plants were first obtained by biolistic bombardment of long-term embryogenic callus cultures (Vasil *et al.* 1992). Further improvements in transformation were achieved by the bombardment of cultured immature embryos (Vasil *et al.*, 1993; Weeks *et al.* 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994). In cereals, as in other higher plants, one to several copies of transgenes are integrated at random and often at multiple sites in the host genome. For the practical use of transgenic plants in agriculture, it is not only important that the transgenes are stably integrated and expressed, but also that they are transmitted to progeny in a Mendelian fashion. The marker gene *bar* has been successfully transferred to wheat (Vasil *et al.*, 1992, 1993). This transgene was expressed stably and inherited as a single dominant locus in the wheat genome following Mendelian inheritance (Srivastavba *et al.*, 1996).

Wheat transformation has only recently been achieved, and only a few reports describing the long-term stability of transgenes in wheat are available (Cannell *et al.*, 1997 & Lörz *et al.*, 1998). An example of a transgene that is expressed successfully in a cereal is the sheath blight resistance gene of rice (Lin *et al.*, 1995).

1.2 MARKER ASSISTED SELECTION (MAS)

The primary objective of plant breeding is to develop cultivars that are high and stable yielding and can withstand biotic and abiotic stresses. Until recently progress in breeding has relied on phenotypic assays. However, a plant's phenotype is not only determined by its genetic composition but also by the environment in which it is grown. In many cases effects of the environment mask those of the genotype, so that the phenotype provides an imperfect measure of a plant's potential (Tanksley *et al.* 1989). Many such problems can be overcome by the use of molecular markers to improve selection efficiency.

Selection progress with MAS depends on the quality of the marker used. The most important properties of good markers are:

1) Easily scoreable alleles that allow for the identification of homo-and heterozygotes

2) Early expression in the development of the organism, and

3) Low or no interaction among markers, allowing the use of many markers at the same time in a segregating population (Arùs & Moreno-Gonzàlez, 1993).

Additional advantages can be expected from the use of markers in breeding for disease resistance. Misclassifications resulting from the inoculation method are avoided and breeding for resistance can be done in areas where field inoculation with the pathogen of interest is not possible.

MAS can be very efficient when there is tight linkage between the marker and the target gene (< 5 recombination units or cM). This will ensure that only a minor fraction of the selected individuals will be recombinants. Alternatively, when two flanking markers are used, it is only required that the interval between them is approximately 20cM, since selection for both markers at the same time results in the recovery of the target gene with a probability of at least 99% (Tanksley 1983 as cited in Arùs & Moreno-Gonzàlez, 1993).

1.3 MOLECULAR MARKERS

Molecular markers can identify polymorphisms present in a genome and can then become valuable tools in genetic mapping strategies. Ideally, molecular markers should have the following properties: (i) be highly polymorphic, (ii) show co-dominant inheritance (which allows for discrimination between the homo- and heterozygotic states in diploid organisms) (iii) occur frequently in the genome, (iv) be evenly distributed throughout the genome, (v) show selectively neutral behaviour, (vi) have easy access (e.g., by purchasing or fast procedures), (vii) have an easy and fast assay, (viii) ensure high reproducibility of results and (ix) easy reproducibility of data among laboratories (Weising *et al.*, 1995). Each marker has its advantages and disadvantages and it differs in variability, cost and technical simplicity.

1.3.1 Isozymes

Isozymes have been used to fingerprint individuals, confirm the purity of pure lines or hybrid seed and to do marker assisted selection. Isozymes were used as genetic markers during the last 20 years for plant germplasm management (Tanksley & Orton, 1983). Their application in MAS have been limited by scarcity of isozyme-gene linked pairs. The probability of finding an isozyme linked to an important gene is low because the number of isozymes are low. In wheat, isozyme loci *Ep-D1* and *Ep-A1* can be used for tagging strawbreaker foot rot resistance (genes *Pch1* and *Pch2*) (McMillin *et al.*, 1986). Other examples of the application of isozymes in MAS are the use of *Aps-1* in tomato to detect the nematode resistance gene *Mi* (Rick & Fobes, 1974), *Adh-1* in pea to detect enation mosaic virus resistance gene *En* (Weeden & Provvidenti, 1988) and *Dip-2* in barley to label the rust resistance gene, *Rph-11* (Feuerstein et al., 1990).

1.3.2 Restriction fragment length polymorphisms (RFLPs)

The use of RFLPs as genetic markers was first proposed in the context of human genetics (Botstein *et al.,* 1980). RFLPs have also been used to construct genetic maps in a number of major crop plants (Helentjaris, 1987). Unfortunately this technology is not compatible with the needs of breeders involved in resistance breeding who work with large populations and limited budgets.

The RFLP analysis entails digestion of DNA with restriction enzymes and electrophoresis of the digested DNA in an agarose gel. The DNA fragments are separated according to size and then transferred to a membrane by the Southern blotting procedure. A labeled probe is then hybridized to the DNA on the membrane and the resulting RFLP pattern can be visualized by autoradiography (Beckman & Soller, 1983).

According to Beckman & Soller (1983), RFLPs are detected when restriction fragments from a specific chromosomal locus vary in size between individuals. This variation can be caused by insertions or deletions that increase or decrease the distance between the two restriction sites. Single base changes can modify the sequence which results in the creation or elimination of a restriction site. RFLPs are co-dominant markers that follow a simple Mendelian inheritance.

RFLPs are well suited for the construction of linkage maps and synteny studies, because of their locus specificity. RFLP analyses are limited by the relatively large amount of high molecular weight DNA required for restriction enzyme digestion and the process of Southern blotting and hybridization makes it a time-consuming and laborious procedure.

1.3.3 Sequence-tagged-site (STS) and cleaved amplified polymorphic sequence (CAPS) markers

The concept of a sequence-tagged-site (STS) was first developed by Olsen *et al.* (1989) and can be described as a short region of DNA whose exact sequence is unique in the genome. A functional STS marker will amplify only a single target region in the genome. STSs can be developed from any clone with a unique DNA sequence by simply developing primers and using them to amplify the unique sequence of genomic DNA from the target organism by means of the polymerase chain reaction (PCR). Polymorphisms between lines can be distinguished by a difference in size of the amplified fragments, known as ALPs (amplicon length polymorphisms). If the amplified fragments do not vary in size, they can also be digested with a number of restriction enzymes to identify RFLPs among lines. This assay is named cleaved amplified polymorphic sequence (CAPS) or PCR-based RFLPs and it captures some of the advantages of the RFLP assay, while avoiding the disadvantages of Southern blot

analysis. The only shortcoming of this technique is the limit in the size of the fragment that can be amplified by PCR (Rafalski & Tingey, 1993).

Because the ends of the PCR products are defined by the primer sequence, ALPs must represent additions/deletions in the DNA between the primer sequences (Ghareyazie *et al.*, 1995). It is necessary to make sure that the locus being assayed by the primers is identical to that assayed by the RFLP probe.

In their classification of rice germplasm, Ghareyazie *et al.* (1995) used 15 pairs of primers of which only six showed ALPs. The other 9 amplification products were digested with 9 different restriction enzymes and seven showed polymorphism. No polymorphism was detected with two of the primers. In general it was found that the degree of polymorphisms was lower with PCR-based RFLP than with Southern-based RFLP. They suggested the reason for this could be that Southern hybridization detects polymorphisms many kilobases away from the site of hybridization of the probe, whereas PCR detects polymorphisms only within the region spanned by the PCR primers. Larson *et al.* (1996) also reported a clone which, when converted into a STS marker showed no polymorphism among barley varieties. Sequence analysis revealed a single base-pair polymorphism that would not be detected using restriction endonucleases. Blake *et al.* (1996) found in their wheat-barley introgression studies the conversion of RFLP markers to PCR-based markers to be a tedious process.

1.3.4 Random amplified polymorphic DNA (RAPDs)

Random amplified polymorphic DNA (RAPDs) is based on the polymerase chain reaction (PCR) and has been developed in two different laboratories (Welsh & McClelland 1990; Williams *et al.* 1990). Instead of using a pair of carefully designed and long oligonucleotide primers to amplify a specific target sequence, a single short oligonucleotide primer is used to amplify random sequences from a template DNA (Waugh & Powell, 1992). Genomic DNA serves as a template and when the single primer anneals to two complementary sequences on opposite strands, amplification of that area will take place, provided that the binding sites are close enough for successful PCR. Polymorphisms result from changes in either the sequence of the primer binding site, which prevents stable association with the primer, or from changes which alter the size or prevent the successful amplification of a target DNA. Because the

RAPD assay does not require knowledge of the target DNA sequence and does not require a Southern blot, it eliminates the technical limitation of RFLP analysis or PCR assays.

RAPD primers are usually 8 to 10 bases long. A very low annealing temperature is used in the PCR amplification reaction. Amplified fragments are detected on agarose gels after ethidium bromide staining (Rafalski & Tingey, 1993). Similar experimental procedures are followed with DNA amplification fingerprinting (DAFs) and arbitrarily-primed PCR (AP-PCR), with the differences being in the detection methods and primer length. According to Rafalski and Tingey (1993), AP-PCR products are usually analyzed on acrylamide gels with radioactive detection and primers are usually more than 10 bases long, while DAF products are analyzed on silver stained acrylamide gels and primers are 2 - 5 bp shorter.

RAPDs are commonly inherited as dominant markers and can therefore not distinguish between heterozygous and homozygous plants (Tingey & del Tufo, 1993). For most presence/absence banding polymorphisms, absence of a band can be attributed to the loss or alteration of one or both of the opposing pair of primer-binding sites needed to produce the PCR product. An insertion between the primer-binding sites may place them at a distance too great to allow amplification. In either case, the absence of a band effectively indicates a recessive allele. Co-dominant RAPD markers are rare. The dominant inheritance of RAPDs can be a disadvantage in mapping or MAS, especially when the markers are linked in repulsion. Therefore, when mapping with dominant markers, it is necessary to work with markers that are linked in coupling (Tingey & del Tufo, 1993).

Once a RAPD marker is found (linked to a specific trait), it is more practicable to convert it into a sequence characterized amplified region (SCAR) (Paran & Michelmore, 1993). The reason for this is because it is easier to score and more reproducible. These markers are produced by isolating the polymorphic fragment from the gel, sequencing the ends of the fragment and developing specific primers for PCR amplification.

1.3.5 Amplified fragment length polymorphisms (AFLPs)

Amplified fragment length polymorphisms (AFLPs) are based on selective PCR amplification of restriction fragments that have been created by total digestion of genomic DNA (Vos *et al.*, 1995). The AFLP technique has overcome many of the problems of the RFLP and RAPD analyse.

There are three major steps in the AFLP procedure:

      1) Restriction enzyme digestion of DNA and ligation of adapters.

      2) Selective amplification of sets of restriction fragments.

      3) Gel analysis of the amplified fragments.

The selectivity of the amplification step can be increased by incorporating one to three nucleotides at the 3'-end of the primers. These extra nucleotides at the 3'-end of the primers will extend beyond the restriction site into the fragment itself and this will cause only a subset of fragments to be amplified. Only restriction fragments that possess the same nucleotides adjacent to the restriction site, will provide the primer with a complementary binding site, which will reduce the number of fragments detected. The AFLP technique can amplify a large number of restriction fragments at the same time and the number of fragments increases, with the genome size (Vos *et al.*, 1995).

The only factor that will limed the number of fragments that can be detected, is the resolution power of the detection system. Polyacrylamide gels are used for detection, since they have a higher resolving power than agarose gels. Incomplete digestion of template DNA may be a problem with the AFLP technique, since it may produce apparent polymorphisms (Vos *et al.*, 1995).

According to Vos *et al.* (1995), AFLP-polymorphisms are inherited in a dominant Mendelian fashion and the bands are expected to segregate independantly of each other. Heterozygous and homozygous individuals cannot be distinguished. Differences in DNA sequences are observed by the presence or absence of bands. The differences are caused by absence or presence of a restriction site or changes in primer extension sites due to basepair changes. The bands can, however, be co-dominant when DNA fragments of different lengths are amplified from the same locus. This is because of changes in internal sequence length of

amplified fragments due to natural insert/deletion mutations or microsatellite repeat variations. These differences are characteristic and heritable.

Fingerprints can be generated and used to establish the identities of specific DNA samples or to asses the relatedness between individual samples (Maughan *et al.*, 1996). AFLP fingerprints are also useful as a source of DNA markers for genetic linkage maps. They can complement RFLP markers already mapped. The markers allow breeders to select desirable traits more effectively or tag genes at the seedling stage which saves time. AFLP patterns can be used for authentication and legal protection of patent strains or AFLP-based detection of DNA polymorphism in genome evolution studies. Conserved bands can be used in combination with co-dominant markers to map quantitative trait loci (QTLs).

1.3.6 Variable number of tandem repeats (VNTRs)

The biological function of many repetitive DNA sequences remain a mystery. However, due to the highly polymorphic nature of some of these units, molecular geneticists have, over the past twelve years, exploited them as markers. Their input to genetic mapping and fingerprinting has been invaluable. Minisatellites, which are about 15-75 bp long, were first discovered by Jeffreys and co-workers in 1985. Minisatellites are spread unevenly through the genome and therefore their usefulness is limited. Tandem repeats of shorter length (1-6 bp), such as (GT)n and (CAC)n, were also found to be abundant in human genomes and were termed microsatellites (also known as simple sequence repeats (SSRs)) (Tautz 1989; Weber & May 1989). Microsatellites show a codominant Mendelian inheritance and have been characterized in a variety of plant species, including tomato (Vosman *et al.* 1992), maize (Senior & Heun, 1993), *Arabidopsis thaliana* (Bell & Ecker, 1994) and wheat (Devos *et al.*, 1995).

The microsatellite sequence itself cannot be used as a primer because it can anneal and prime for DNA polymerase in a number of different registers, and the resultant sequence would be insignificant. Instead, PCR primers complementary to single copy DNA flanking the repeated element are used for the amplification of VNTR markers in mapping studies. The differences in PCR product lengths can easily be detected by electrophoresis.

1.3.7 Summary of different DNA markers

A summary of DNA markers with their features are presented in Table 1. The choice of marker depends on the purpose for which it is to be used.

Table 1: Properties of systems for generating genetic markers (Rafalski & Tingey, 1993).

	RFLP	RAPD	VNTR	CAPS	AFLP
Principle	Endonuclease restriction; Southern blotting; Hybridization	PCR amplification with random primers	PCR of simple sequence repeats	Endonuclease restriction of PCR products	Endonuclease restriction; Selective PCR
Type of polymorphism	Single base changes; Insertions; Deletions	Single base changes; Insertions; Deletions	Changes in number of repeats	Single base changes; Insertions; Deletions	Single base changes; Insertions; Deletions
Genomic abundance	High	Very High	Medium	High	Very High
Level of polymorphism	Medium	Medium	High	Medium	Medium/High
Dominance	Co-dominant	Dominant	Co-dominant	Co-dominant	Dominant
Amount of DNA required	2-10 µg	10-25 ng	50-100 ng	50-100 ng	250 ng
Sequence information required?	No	No	Yes	Yes	No
Radioactive detection required?	Yes/No	No	Yes/No	No	Yes/No
Development costs	Medium	Low	High	Medium/High	Medium/High
Start-up costs	Medium/High	Low	High	High	High

1.4 MEIOTIC CHROMOSOME PAIRING, RECOMBINATION AND SEGREGATION.

Chromosome pairing is an important event during meiosis because the chromosomes that are in close physical contact may cross-over, i.e., reciprocally exchange parts of their chromatids. Crossing over creates new complete chromosomes from pieces of the original parental chromosomes and helps to ensure the diversity and genetic variability of offspring.

According to Bascom-Slack *et al.* (1997), Jansens (1909) described chiasmata as cytologically observable "crosses" between the arms of chromosome pairs during late prophase I. The idea that chiasmata are formed at the sites where genetic exchange took place (chiasmatype theory) resulted from these observations (Bascom-Slack *et al.*, 1997). A debate that continued for over half a century and experiments supporting this theory followed. Tease and Jones (1987) provided the most convincing evidence that chiasmata result from crossovers. By using techniques to differentially label sister chromatids, they observed that chiasmata originated at sites where chromatids were broken and rejoined to non sister chromatids. The majority of visible exchanges (crossovers) coincided with the positions at which chiasmata were formed in the bivalents. Correlation between chiasmata and meiotic exchange was thus demonstrated. For the purpose of this thesis, the term crossover will be used to refer to the breaking and rejoining of DNA strands that results in a reciprocal exchange between a pair of homologous chromosomes (between two of the four chromatids present after DNA replication). The term chiasmata will refer to the cytological evidence of a meiotic crossover or exchange.

1.4.1 Meiosis

Meiosis is a special type of cell division that produces haploid gametes from diploid parental cells through two rounds of chromosome segregation. At the meiosis I division, homologous chromosomes move to opposite poles, while sister chromatids remain associated. Sister chromatids segregate during meiosis II (anaphase II).

Meiosis begins with a condensing of the chromosomes (Fig. 1.3) (Bascom-Slack *et al.*, 1997). In some organisms they can be seen to become attached to the nuclear envelope with their

telomeres. They then move towards the inner nuclear membrane (Loidl, 1990). Prior to leptotene homologous chromosomes come in contact at their centromeric sites. In early leptotene, all the telomeres aggregate and project a bundle of loops into the nuclear lumen. This is called the "bouquet" formation. In other organisms, mostly plants, a similar clustering of chromosomes - without the involvement of the membrane, the "synizetic knot" is formed. The replicated homologous chromosomes (each chromosome has two identical sister chromatids) begin to condense, coiling along a cytologically visible proteinaceous structure called the axial element. In zygotene the axial elements of the homologues are connected (synapsed) and arranged in parallel, at a distance of about 100 nm, by transverse filaments. At about the same time, a third longitudinal element, running halfway between the chromosomal axes (at this stage called lateral elements) is formed. This whole structure is called the synaptonemal complex (Moens 1973). In pachytene, the synaptonemal complex is complete. The first meiotic crossovers occur in late pachytene. In diplotene-diakinesis, the synaptonemal complex has been removed, revealing the individual homologues linked by chiasmata, and sister chromatids become apparent. Prometaphase begins as the bivalents begin to move to the metaphase plate. When all of the bivalents have achieved a bipolar attachment to the spindle metaphase begins. Anaphase is marked by the separation of the bivalents, followed by disjunction of univalents to opposite poles of the cell.

In a recent paper by Miller *et al.* (1998) it is suggested that the centomere structure may play a part in the initiation of chromosome pairing in wheat. However, Lukaszewski (Marais 1999, personal communication) found that pseudo-isochromosomes, which differs at the telomeres, are not able to autosinapse, which would suggest that telomeres rather than the centromeres are required for pairing initiation.

DNA replication

Axial elements appear

Double-strand breaks observed
in yeast (Initiation of recombination)

Tripartite synaptonemal complex
first appears

Full length synaptonemal complex

Mature recombinants detected in yeast

Synaptonemal complex breaks down,
achiasmate chromosomes fall apart

Sister chromatids are apparent but are
lightly associated

Movement to metaphase plate begins
(Congression)
Correct spindle fiber attachment is
tested

Homologue disjunction

Cohesion along sister chromatid arms
is relaxed

Sister chromatids remain attached
at centromeres
Segregation of sister chromatids

Leptotene
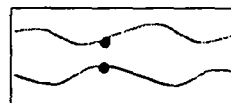
Zygotene

PROPHASE

Pachytene

Diplotene/Diakinesis
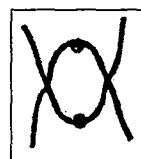
Prometaphase

METAPHASE
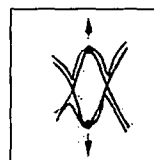
ANAPHASE
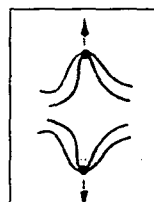
MEIOSIS II

Figure. 1.3 Generalized time line of meiosis (from Bascom-Slack *et al.*, 1997).

1.4.2 Possible pathways of chromosome pairing

Several hypotheses on chromosome pairing, which differ by the mechanism of primary contact and the time of operating, have been suggested (Loidl, 1990). A diagram of suggested pathways of chromosome pairing is shown in Fig.1.4. Three situations are supposed as possible first steps in chromosome pairing at meiotic prophase. The first possibility is that the chromosome positions at the onset of meiosis are not random (A). This would require a mechanism to "guide" the already existing relationship into synapsis. It could be straightforward (path 1) if homologues are already grouped, and a more elaborated process if the ordered chromosome position were of a different kind (path 2). Different structures have been proposed to guide or affect the union of homologues. Synapsis could be achieved directly (path 3) or via presynaptic alignment (paths 4 and 5). A bouquet could also be formed of this movement (path 6), or be a relic of the Rabl orientation (path 7). During the Rabl orientation the telomeres tend to associate, more or less in pairs, become attached to the nuclear envelope. The bouquet could facilitate homologous recognition at intercalary sites as well and thereby promote the formation of presynaptic alignment (path 8) or directly result in the formation in SC initiation (path 9).

The second (B) possibility for the initiating step is that randomly positioned homologous chromosomes "cross-talk" and then move towards each other. As cited in Loidl (1990), Fussell (1987) suggested that premeiotic chromosomes do not have a random position, but this possibility is included anyway to give the reader a complete picture. Alternatively, interactions could exist between chromosomes and specific sites in the nuclear membrane (path 10) with various possible pathways towards synapsis.

The third scenario (C) is the occurrence of mechanisms that ensure a sufficiently high probability of accidental homologous contacts. These mechanisms are thought of as undirected "stirring" movements (paths 12, 13 and 14). A further condition for accidental homologous contacts could be a general clustering whereby chromosomes form a synizetic knot or bouquet cluster at the nuclear membrane (paths 15 and 16). The Rabl orientation could again contribute to the formation of the bouquet (path 17). All this could then be followed by SC formation (path 18) or otherwise create presynaptic alignment first (path 19).

A
Somatic (premeiotic)
chromosome disposition

B
Specific interactions
at prophase

C
Random contacts
at prophase
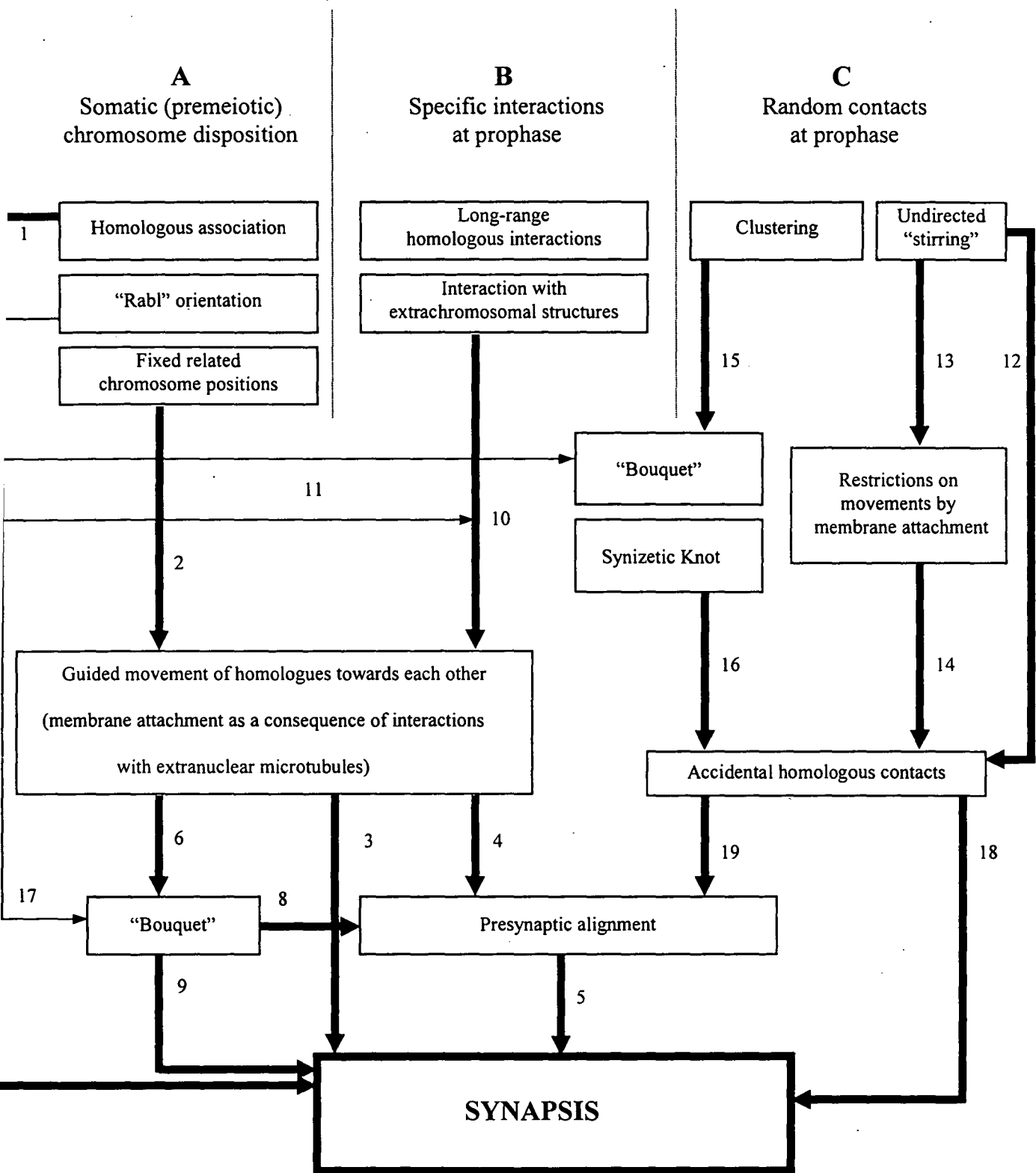
Figure 1.4. Possible pathways of chromosome pairing (from Loidl, 1990).

1.4.3 Recombination and chiasma formation

A number of models have been proposed to describe the events in the formation of a crossover (Bascom-Slack *et al.*, 1997). Most models for describing recombination suggest that the process is iniated by precise double strand breaks (DSBs) (Fig. 1.5). A free 3' end invades a nonsister chromatid, displacing one of the strands. DNA synthesis occurs using the 3' ends as primers. The ends are healed, forming two Holliday junctions. A crossover is seen when the strands are resolved.
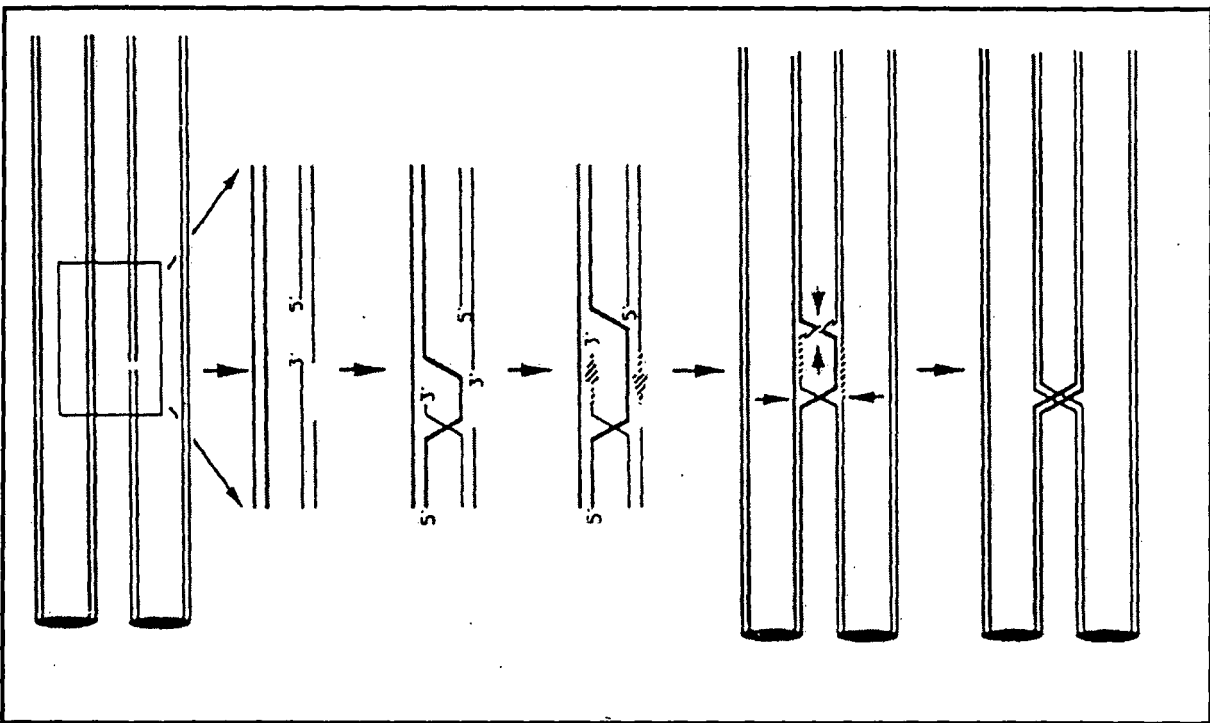
Figure 1.5. Model of meiotic recombination (from Bascom-Slack *et al.*, 1997).

The *MER2* gene was identified as a meiotic gene in *Saccharomyces cerevisiae* (Malone *et al.* 1991& Engebrecht & Roeder 1990). Rockmill *et al.* (1995), concluded that *MER2* is absolutely essential for the initiation of meiotic recombination. *MER2* mutants do not have synaptonemal complex formation or recombination. It may be possible that *MER2* acts indirectly, by regulating the activity of several gene products.

1.4.4 Chiasma function

The relationship between crossing over and chromosome segregation appear to be consistent (Carpenter, 1994). The resulting chiasma are used to orient homologous chromosomes to opposite poles at metaphase I. The stable configuration of a bivalent on the metaphase plate has one kinetochore being pulled toward one pole and the other kinetochore pulled to the other pole (Bascom-Slack *et al.*, 1997). These pulls are resisted by unit-internal bonding. At anaphase this bond is released and the onset is usually delayed until all chromosome units within the cell have attained stability (bipolar orientation). In mitosis and meiosis II, this bonding is between sister chromatids and occurs close to the sister kinetochores themselves. In meioisis I, this bonding is provided by the chiasmata closest to the homologous kinetochores.

When chiasma formation fails, each of the resulting pairs of sister chromatids is called a univalent. Organisms that use chiasma to direct meiosis I segregation also have strategies to ensure that each bivalent has at least one chiasma (Carpenter, 1994). When achiasmate bivalents occur, which are rare, the univalents move to the poles independently. According to Carpenter (1994), half of the time the univalents go to the same pole, resulting in nondisjuction and therefore aneuploid gametes. In most organisms, mutations that result in a reduction in meioitic exchange are correlated with reductions in viability of the gametes produced (Bascom-Slack *et al.*, 1997). Studies in *Drosophila* done by Merriam and Frost (1964), showed that bivalents with multiple exchanges were 5-fold more likely to missegrate than homologues with single exchanges, possibly due to chromosome entangling. Homologous chromosomes without exchanges were 16-fold more likely to nondisjoin than single-exchange bivalents.

1.4.4.1 How is homology recognized?

Chromosomal homology is based on similarity at the DNA level (Loidl, 1990). DNA sequences for the mutual recognition of homologous chromosomes may be in individual sites distributed along chromosomes and function autonomously. It is not known if recognition works by direct contact of DNA, whereby corresponding DNA sequences are matched. These

DNA sequences could also interact via proteins. The sites of homologous recognition may correspond with the association sites at which presynaptically aligned chromosomes come together.

According to Bascom-Slack *et al.* (1997), Fussell (1987) reported that premeiotic chromosomes do not have a random orientation within the nucleus. Centromeres and telomeres are maintained in specific locations which result in alignment of the homologous chromosome arms. Such arrangements of chromosomes may allow easier comparisons of sequences in homological searches.

A question that is often asked is 'how perfect must the sequence homology be to define a homologous chromosome?' Do small allelic differences, measured in single base pair differences matter, or are they ignored? Outbreeding species have a higher degree of polymorphism than inbreeders and therefore must have a more flexible checking system. In conclusion, there must be a preprogrammed checking level built into the organism, but how this is defined or regulated is as yet unknown (Clark and Wall, 1996).

1.4.4.2 Establishment of proper chromosome attachment to the meiotic spindle

Chromosomes have a stable spindle attachment when fibers from one pole attach to the kinetochore of one homologue and fibers from the other pole attach to the kinetochore of the other homologue. Experiments in *Drosophila* oocytes have shown that recombination is important for the assembly of the meiotic spindle around the condensed chromosomes (Bascom *et al.*, 1997). The experiments showed that mutants lacking the chromokinesin, nod (chromatin-associated motor protein that pushes the chromosomes towards the center of the spindle), could assemble bipolar spindles as could recombination mutants. Double mutants never developed spindles, thus, nod and crossovers are both needed to keep chromosomes associated. Crossovers prevent chromosomes from physically moving apart, whereas nod pushes them together. These two factors probably play a role in meiotic spindle formation. They hold the chromatin mass together while the other elements of the meiotic machinery shape the microtubules that assemble around the chromatin to form a spindle.

1.4.4.3 <u>Chiasma binder</u>

Maguire (1974), argued that a chiasma alone is insufficient to link homologous chromosomes - there must be some sort of binding mechanism to hold the chiasma in place. There are three possible chiasma binding mechanisms: firstly, sister-chromatid cohesion; secondly, a chiasma binder may act at the site of the crossover to fix the chiasma in place, or finally, binding action can be provided by base pairing between DNA strands when the crossover remains unresolved.

1.4.5 Cohesion of sister chromatids

Metaphase chromosome alignment not only depends on "splitting" forces exerted by microtubles on kinetochores but also on an opposing "cohesive" force (Michaelis *et al.*, 1997). These forces hold sister chromatids together. It is suspected that eventual splitting does not happen from any major changes in the forces applied by microtubles but rather from a sudden loss of cohesion between sisters (Miyazaki & Orr-Weaver, 1994). Prior to metaphase I/anaphase I the sister chromatids are attached along their length, and their kinetochores are constrained so that they attach to the same pole. At metaphase I/anaphase I the cohesion at the arms is lost as the chiasma resolves and homologues segregate. At metaphase II/anaphase II the cohesion at the kinetochore is lost.

According to Miyazaki & Orr-weaver (1994) two models can explain sister chromatid cohesion. The first one implies that sister chromatids are linked through DNA structure or chromatin topology. The second model suggests a direct role for chromatid-linking proteins.

Many proteins which are candidates for cohesion functions, have been isolated (Michaelis *et al.*, 1997) which makes the second model more acceptable. The first model can, however, not be totally ignored considering the two-step loss of cohesion in meiosis I. This raises the possibility that different mechanisms of cohesion may exist early and late in meiosis.

Four proteins necessary for sister chromatid cohesion were discovered by Michaelis *et al.*, 1997. In *scc1*, *scc2*, *smc1* and *smc3* mutants, sister chromatids separate soon after the

formation of bipolar spindles. *Smc1*, *smc3* and *scc1* proteins are associated with chromosomes and are essential for the cohesive force that opposes microtubule-induced chromosome splitting. *Scc1* dissociates from the chromosomes at the beginning of anaphase. This explains the loss of cohesion between sisters and is thought to be the trigger for their separation.

1.5 The *Ph* system

The *Ph* system involves one major gene, *Ph1*, on 5BL which has the most pronounced effect in suppression of pairing between wheat homoeologous chromosomes (Okamoto, 1957; Riley & Chapman, 1958; Sears & Okamoto, 1958; Riley, 1960; Sears, 1977; Giorgi & Cuozzo, 1980). Thus, only bivalents are formed at meiosis and common wheat behaves like a typical genomic allopolyploid. The removal of this arm or mutation of *Ph1* to *ph1* not only permits the wheat homoeologues to pair with each other, but also to pair with introduced alien homoeologues. *Ph2*, a gene with intermediate effect, located on 3DS, and a number of genes with minor effects are also part of the *Ph* system. These genes, found on a number of chromosomes (e.g. 5D, 5BS, 5AL, 3D) can either suppress or promote homoeologous pairing (Sears, 1976; Feldman & Sears, 1981). It is interesting to note that chromosomes of *T. speltoides* (S genome) suppress the *Ph1* gene of common wheat. They have no effect on homologous chromosome pairing, but they increase the amount of homoeologous pairing in hybrids with wheat (Friebe *et al.*, 1995).

The mechanism by which *Ph1* suppresses heterogenetic chromosome pairing has been the subject of much speculation. Riley suggested in 1960 that *Ph1* strengthens the forces which bring chromosomes together in prophase. His second hypothesis was based on the Faberge (1942) theory. Chromosome pairing occur in two phases, first the potential partner chromosomes pair and then precise matching of DNA base sequences occurs. Riley (1960) suggested that *Ph1* may shorten the duration of meiotic prophase, allowing homologues to pair but not providing enough time for homoeologues to pair. The latter hypothesis was abandoned when it was found that meiosis was of similar duration in *Ph1* and *ph1* plants (Bennett *et al.*, 1974 as cited in Luo *et al.*, 1996).

Feldman *et al.* (1966) observed that when the dose of *Ph1* is increased it also suppresses pairing between homologous chromosomes. He concluded that *Ph1* controls heterogenetic chromosome pairing by suppressing premeiotic (somatic) association of homoeologous chromosomes. Measurements of distances between telocentrics in root tip metaphase plates supported this hypothesis since homologous telocentrics were found to be closer to each other than homoeologous telocentrics (Feldman *et al.*, 1966). Feldman (1968,1993) also found that telocentrics of opposite arms were associated in *Ph* plants. He also investigated the influence of antimicrotubule drugs like colchicine on *Ph1*. Such treatments induced partial asynapsis of homologues, pairing of homoeologues, and interlocking of bivalents. The effect of extra doses of *Ph1* on pairing has been duplicated by premeiotic treatments with colchine. He concluded that the resemblance between the effect of the genetic and the chemical treatments suggests that the microtuble system is the subcellular target of *Ph1*.

Increasing the dosage of *Ph1*, from zero to two to four, progressively increased the resistance of the microtuble system to colchicine. However, O-isopropyl N-phenylcarbanate which affects spindle organization, did not cause differential effects in plants carrying different doses of *Ph1* (Gualandi *et al.*, 1984). It was therefore concluded that *Ph1* acts on the dynamics of microtubule assembly and disassembly.

Holboth (1981), as cited in Luo *et al.* (1996), and Gillies (1987) questioned the concept of premeiotic alignment of wheat chromosomes. They suggested that *Ph1* may regulate the rate of pairing or the duration of crossing over. Holm & Wang (1988) suggested that *Ph1* contributes to both synapsis and crossing over by regulating heteroduplex formation.

Luo *et al.* (1996) cited experiments done by Dubcovsky and Dvorak (1995) to investigate the role of the centromeres and telomeres in the recognition of homoeology, meiosis I pairing and recombination. *Ph1* plants of two types were used, i.e. (i) plants having chromosome pairs in which terminal homologous segments were present in otherwise homoeologous chromosomes with a homoeologous centromere or (ii) terminal homoeologous segments were present in otherwise homologous chromosomes with homologous centromeres. In such chromosomes, recombination was absent in the homoeologous segments in the *Ph1* state.

Luo *et al.* (1996) concluded that the activity of *Ph1* prevents recombination of homoeologous segments in chromosome pairs composed of homologous and homoeologous segments in the same way as it does prevent completely homoeologous chromosomes from pairing. Recombination is absent from the homoeologous region(s) irrespective of whether the telomere is homologous and the centromere homoeologous, or the centromere homologous and the telomere homoeologous, or both the telomere and the centromere homologous. The absence of recombination from a homoeologous segment is also independent of its length (the lengths of homoeologous segments ranged from 2.6 to 111.7 cM). These observations do not agree with the hypothesis that *Ph1* regulates homoeologous meiotic pairing by premeiotic alignment of chromosomes. Recombination was absent in the interstitial homoeologous segments when the centromeres and both telomeres were homologous. Therefore it is very unlikely that an interaction of mitotic spindle with the centromere is the target of the *Ph1* gene (as is assumed by the premeiotic association hypothesis). No alignment of chromosomes was seen at the onset of meiotic prophase, which also argues against this hypothesis.

Luo *et al.* (1996) furthermore concluded that homology is checked very carefully along the entire length of the chromosome because crossing over ceased at the borders of homoeologous and homologous regions. This argument corresponds with the conclusion of Holm and Wang (1988) (as cited in Luo *et al.*, 1996) that *Ph1* regulates homology recognition at the level of individual DNA heteroduplexes.

Miller *et al*, 1998 proposed that *Ph1* operate pre-meiotically to guarantee the association and alignment of homologues. Thus, it restricts synapsis and recombination to homologues rather than between homoeologues. This implies that, before homoeologous chromosomes can pair, *Ph1* must be removed. This do not guarantee synapsis and recombination. This is reflected in the relatively small number of wheat-alien introgressions that have been achieved in the absence of *Ph1*. It is also reported that studies done by Snape *et al.* (unpublished), as cited in Miller *et al.* (1998), have shown that the *Ph1* locus is complex. Preliminary analysis indicates that there are at least three genes at this locus that have an effect on chromosome organization and homoeologous pairing. Unfortunately, the exact mechanism of *Ph1* action remains unknown.

1.6 ORIGIN OF THE *LR 19* TRANSLOCATION OF COMMON WHEAT

1.6.1 The T4 translocation

In 1966 Sharma and Knott transferred the leaf rust resistance gene, *Lr19*, to wheat from a *Thinopyrum ponticum* chromosome (7el₁). They used thermal and soft X-ray treatments to produce translocations between 7el₁ and 'Thatcher' chromosomes. Four translocation lines that showed leaf rust resistance, were recovered. Translocation 4 appeared to be the most useful as it was transmitted normally through the gametes. Monosomic analyses revealed that the resistance gene in translocation 4 was located on chromosome 7D. This line was renamed 'Agatha'. *Thinopyrum* chromosome 7el₁ was shown to be homoeologous to the group 7 chromosomes of wheat (Quinn & Driscoll, 1967; Nanda in Knott, 1970; Dvorák, 1975). It was shown by Dvorák and Knott in 1977 that 7DL is homoeologous to the 7el₁ long arm of *Thinopyrum ponticum* which carries the *Lr19* gene.

The leaf rust resistance gene (*Lr19*) in 'Agatha' is associated with a gene (Y) coding for yellow endosperm pigmentation (Knott, 1968) and a stem rust resistance gene (*Sr25*) (McIntosh *et al*. 1976). The yellow pigmented flour renders *Lr19* useless for wheat breeding in many countries. McIntosh and co-workers (1976) showed that *Lr19* and *Sr25* do not recombine with the *Cn-D1* locus on 7DL. They suggested that the translocation includes this locus as well.

Knott (1980) found that the order of the genes on the translocated segment is *Lr19-Sr25-Y*. These results were confirmed by Bournival *et al* (1994). According to Autrique *et al*. (1995) a number of RFLP markers co-segregate with the *Lr19* resistance.

Sharma and Knott (1966) found that significantly more than 75% (80.9%) of the progeny were resistant when plants heterozygous for the T4 translocation were selfed. Sears (1972a, 1972b, 1973, 1977) observed that some of the recombinant versions of the T4 translocation had a significantly higher than 50% transmission through pollen. Zhang and Dvorák (1990) mapped a segregation distortion gene (*Sd1*) proximal to the *Lr19* gene.

1.6.2 The 'Indis' translocation

Pienaar *et al.* (1977) successfully crossed wheat cultivar 'Inia 66' with *Thinopyrum distichum*. The 'Inia 66'/*Th. distichum* amphiploid which was susceptible to leaf rust, was backcrossed to 'Inia 66'. Two plants that showed resistance to stem rust and leaf rust were selected from the B_2F_3 population, one of which gave rise to the true breeding germplasm line 'Indis' (Pienaar *et al.*, 1985).

It was concluded that a spontaneous translocation had occurred between a *Th. distichum* chromosome segment and chromosome 7D of 'Inia 66' (Marais *et al.*, 1988). The 'Indis' translocation was thought to be homo(eo)logous to the T4 translocation and was found also to carry a stem rust resistance gene, *Sr25,* and a gene coding for yellow endosperm pigmentation (Y) (Marais, 1992c). Further similarities with the T4 translocation were the presence of the *Wsp-D1* gene, that codes for a water-soluble protein of unknown function and a segregation distortion gene (*Sd1*). Both translocations produce null alleles for the *Ep-D1* and α-*Amy-D2* loci and show homoeology to 7DL of common wheat (Dvorák and Knott 1977; Marais and Marais 1990; Marais 1992a). However, it was subsequently shown (Prins *et al*, 1996) that the pedigree of 'Indis' is suspect and that the 'Indis' translocation is in reality the T4 translocation.

Marais (1992a) isolated 29 deletion lines after gamma-irradiation of 'Indis'. Each line was homozygous for a different deletion of the translocation. This material enabled Marais (1992c) to determine the linear order of the genes on the translocation as: *centromere - Lr19 - Wsp-D1 - yellow pigment*. Prins *et al.* (1996) extended the physical map of the region and determined the relative position of various other marker loci as; *centromere - Sd1 - Xpsr 165 - Xpsr 105 - Xpsr 129 - Lr19 - WspD1 - Sr25/Y*. This data agree with the published chromosome 7D maps of wheat (Chao *et al.*, 1989; Werner *et al.*, 1992; Hart *et al.*, 1993; Hohmann *et al.*, 1994; Chen and Gustafson, 1995).

1.6.3 Segregation distortion

If the alleles of a locus are normally transmitted through the gametes in equal numbers it is called Mendelian segregation. Deviations from normal can result from competition between pollen grains (Hornaza & Herrero 1992), self-incompatibility mechanisms (Savolainen *et al.* 1992) or meiotic drive. There are also examples where gametes that do not carry a specific allele are inviable. This allele can either be a lethal allele or a gametocidal gene. If a gamete carries a lethal allele it will die. Gametocidal (*Gc*) genes eliminate non-carrier gametes to cause their own preferential transmission (Endo, 1990).

Pollen competition can take place at two levels. It can be either through direct competition among haploid gametophytes or through interaction between the haploid and diploid genes (Hornaza and Herrero 1992). Direct competition can either be a physical competition based on the rate of pollen growth or a chemical competition based on pollen inhibition. According to Hornaza and Herrero (1992) a number of reports have demonstrated a genetic relationship between pollen tube growth (determined by the gametophytic genome) and several sporophytic traits. This clearly shows that pollen competition, as influenced by pollen tube growth rate, may result in segregation distortion of these and linked genes.

Meiotic drive describes a mechanism where events at meiosis are not random. It usually occurs in females, where asymmetry during oogenesis results in the furher development of only one of the four products of meiosis (Lyttle, 1991). In many organisms, one of the two outermost of the four meiotic products becomes the functional egg. These cells tend to contain those chromosomes which have an advantage in movement on the spindle. This results in the production of an excess of particular genotypes (megaspores).

Whereas meiotic drive predetermines the genotype of the gametes produced, a gametocidal (*Gc*) gene selects the type of gamete that partakes in fertilisation (Lyttle, 1991). By eliminating a portion (or in some cases all) non carrier gametes, the *Gc* gene is transmitted to more progeny than is expected with Mendelian segregation. Even though the precise mechanism of gamete elimination in the Gramineae is unclear, chromosome fragmentation during the early stages of meiosis is a possibility. A *Gc/gc* (heterozygote) is marked by a reduction in fertility and chromosome mutation is a possibility (Miller *et al.*, 1982). A *Gc/Gc* homozygote on the other hand is usually (not necessarily) normal in appearance and fertility

(Marais & Pretorius, 1996).

Interspecific hybridization provides a means to introgress novel traits into cultivated crops. The aim is to transfer only the gene of interest and to minimize the co-introduction of linked deleterious or unnecessary chromatin. Gene transfer can be complicated by the presence of gametocidal genes, especially when the genes are linked or when exclusive transmission of the *Gc* gene precludes the survival of all *Gc* noncarrier gametes. The deleterious nature of the *Gc* gene (i.e. semi-sterility and chromosome mutation) necessitates its removal prior to the utilisation of other transferred genes. *Gc* genes may also cause seed shrivelling which is another reason why it should be separated from introduced genes (Tsujimoto & Tsunewaki, 1985). Marais and Pretorius (1996) utilized two methods in an attempt to inactivate a *Gc* gene through mutagenesis. One method involved the soaking of mature seed (heterozygous for the *Gc* gene) in N-nitroso-N-methyl-urea. The other method involved the pollination of irradiated *Gc/Gc* homozygous plants with *gc/gc* pollen. Progeny with improved fertility was expected to have impaired *Gc* genes.

Sometimes gametocidal genes could be useful in breeding (Endo, 1990). Agronomically desirable genes that are closely linked to a *Gc* gene will be transmitted preferentially. Selection for such genes will be unnecessary and in this way breeders will be able to focus their attention on other traits.

Some of the gametocidal genes was found to induce chromosome mutations in common wheat (Endo, 1990). Chromosomal structural changes, including deletions, translocations, dicentric chromosomes and ring chromosomes occur in hybrid zygotes. These abnormalities were more abundant in the progeny of a cross where the gametocidal gene was passed on through pollen. Chromosome mutations such as aneuploidy, telocentric-, ring- and dicentric chromosomes were reported in somatic metaphase chromosomes obtained from root tip cells of F_6 *GcGc* (91M56) plants (Middleton, 1998). Only 42% of 40 F_7 families contained plants with no chromosome mutations.

Mutations induced by *Gc* genes can be very useful in chromosome mapping. Disappearance of a trait otherwise present in the hybrid indicates the loss of the chromosomal segment on which a dominant gene responsible for the trait is located. When a cytological study is to determine the location of the deletion it is possible to assign the gene to the specific region of

a chromosome. The association of the deletion with the absence of the trait can be confirmed in the progeny of the structural heterozygotes. Using this approach, Endo and Mukai (1988) mapped a gene suppressing the speltoid character of wheat spikes to the distal 46% region of the long arm of chromosome 5A. The region in which the locus occurs was further narrowed down to the distal arm 13% section of the 5AL with deletions induced by other gametocidal chromosomes (Tsujimoto & Noda, 1990).

1.6.4 Removal of the yellow endosperm pigmentation gene on the T4 translocation

The *Lr19* translocation does not recombine with homoeologous areas of the wheat genome but is inherited as a single large linkage block (Knott, 1980; Marais & Marais 1990). Dvorak (1975) tried to break the linkage between *Lr19* and the yellow pigment gene in the T4 translocation but was unsuccessful. Pairing between the translocation and a *Thinopyrum ponticum* group 7 chromosome, which did not carry the *Lr19* and yellow pigment genes, was observed but no *Lr19* recombinants that lacked the pigment gene could be recovered. In another attempt 'Agatha' was treated with ethyl methanesulphonate to mutate the gene which codes for the yellow pigment (Knott, 1980). He identified two such mutants ('Agatha'-28 and 'Agatha' 235) which unfortunately were associated with a detrimental effect on yield (Knott, 1986, 1989).

Marais (1992c, 1992d) used the *ph1b* and *ph2b* (pairing-inhibitor genes) mutants to induce allosyndetic pairing and crossovers between the *Lr19* segment and homoeologous areas of the wheat genome. He obtained eight putative recombinants of the translocation in 'Indis', four of which were associated with white endosperm. Only one of the 4 white endosperm selections, recombinant 88M22-149, was subsequently found to be a true *Lr19* derivative. It has retained the *Lr19* resistance but has lost *Sr25* and appeared to have an altered segregation distortion effect (Marais, 1992 ; Prins *et al.*, 1997).

1.6.5 Recombinant *Lr19* (149)

A comparison of the *Thinopyrum*-derived genes on the translocation in 'Indis' and the *Lr19* (149) recombinant are given in Fig.1. 6. Recombinant *Lr19* (149) appears to be the product of a double crossover. It has retained a reduced *Thinopyrum* segment with the complete *Lr19* resistance. During recombination the *Xpsr129-7B* locus has been replaced with the *Thinopyrum*-derived *Xpsr129-7el$_1$* locus (Prins *et al.*, 1997). Thus, the segment has been relocated to chromosome 7B. It is not known exactly where the break occurred between *Xpsr105* and *Xpsr129*. The recombination event did not replace the α-*Amy-B2* locus on chromosome 7B, which would suggest that this locus does not occur within the recombined region. Both the *Wsp-B1* and *Wsp-D1* loci are expressed in *Lr19* (149) which would suggest that an unequal crossover occurred which created a duplicated region (Marais, 1992).

Lr19 (149) has lost *Sd1*, but it seems that the recombinant has acquired a strong tendency to self-eliminate in heterozygotes (Prins *et al.*, 1997). In backcrosses to two local wheats the F$_2$ segregation of *Lr19* (149) was found to be 0,01 RR: 0,53 Rr: 0,46 rr. If, however, the *Lr19* (149) segment is in the homozygous condition it has a perfectly normal phenotype and fertility. It is not clear whether the self-elimination stems from a disrupted complex of *Sd* genes or whether it is due to chromosomal modifications (structural and/or genetical) that occurred during recombination.
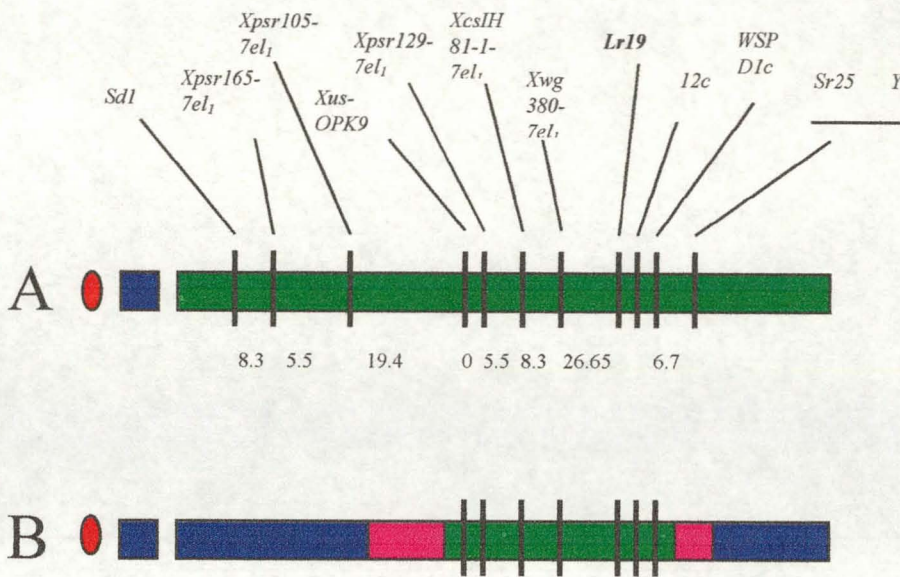
A: *Lr19* translocation - (chromosome arm 7DL)
B: *Lr19* (149) translocation - (chromosome arm 7BL)

■ Centromere

■ Wheat chromatin

■ Breakpoint region

■ *Thinopyrum* chromatin

* Estimates of relative physical distances between loci (Prins *et al.*, 1996 & Barkhuizen *et al.*, 1997). Gene to centromere and gene to telomere distances are unknown and are not reflected by the diagram

Figure 1.6 Comparison of *Lr19* and *Lr19* (149) translocations

1.7 AIM OF STUDY

An attempt was made to study the stability of the *Lr19* (149) recombinant in heterozygotes. The segment has been reduced significantly in size, has lost the yellow pigment locus, and now contains wheat chromatin on both ends. This may impact on its pairing with homoeologous wheat chromatin in heterozygotes and may promote crossing over in this region. Furthermore, the *Sd1* gene has been lost which completely altered the segregation effect of the translocation. As gametocidal genes are sometimes associated with high mutation rates, the altered condition of the *Sd* complex may result in increased mutations. In order to determine the stability of the translocation and its associated marker genes, the study aimed to answer the following with respect to *Lr19* (149) heterozygotes:

i) Does self-elimination of gametes occur in both sexes?

ii) Is a certain level of homoeologous recombination possible in the presence of the *Ph*-genes?

iii) Is the translocation accompanied by an increase in the incidence of mutations?

CHAPTER 2

MATERIALS AND METHODS

2.1 Gametic transmission of *Lr19* (149)

Segregation for leaf rust resistance was used to study self-elimination of the *Lr19* (149) translocation in different genetic backgrounds. For this purpose use was made of F_1 heterozygotes for *Lr19* (149) derived from backcrosses to Inia 66, W84-17, Chinese Spring and SST 66. The male and female transmission of the resistance was studied by further backcrossing the BF_1 reciprocally to the respective recurrent genotypes (Table 2.1).

Table 2.1 Pedigrees of the plant material used.

Cross number	Pedigree
91M71	*Lr19* (149)/ 5* Inia 66
93M97	*Lr19* (149)/ 7* W84-17
93M3	*Lr19* (149)/ 6* Chinese Spring
93M101	*Lr19* (149)/ 7* SST 66

The BF_1 and BF_2 progeny were tested for resistance to the leaf rust pathotype UVPrt 8 (=3SA 132) which is virulent on each of the recurrent parents.

2.2 Segregation of *Lr19* (149) in B₆F₂ population: 93M97

A segregating **B₆F₂** population (93M97) was screened for translocation heterozygotes that could be used to study the stability of marker loci on the translocation (Table 2.2). Alleles at the marker loci may change as a result of: (a) crossing over during meiosis, and (b), mutation since gametocidal genes often induce high mutation rates. The **B₆F₂** was used as source of heterozygotes as there was not enough F_1 seeds available and it is known that the high level of self-elimination of *Lr19* (149) in the **F₁** 93M97 results in a **F₂** in which the resistant plants are predominantly heterozygotes. **F₃** progeny of each resistant **F₂** plant was tested for seedling resistance to determine which were resistant homozygotes and which were resistant heterozygotes..

Table 2.2 Loci tested on the heterozygotes of B₆F₂ population: 93M97 to study the stability of marker loci on translocation *Lr19* (149).

Marker	Locus	Allele
XcsIH81-1	*XcsIH81-1*	*XcsIH81-1-7el₁*
*X12c		
XusOPK9₁₃₅₀	*XusOPK9₁₃₅₀*	*XusOPK9₁₃₅₀₋7el₁*
WSP-D1c	*Wsp-D1*	*Wsp-D1c*

* This locus is not named yet. The lab name (X12c) is used in the thesis.

2.2.1. Plant material used in marker studies

About 300 seeds of the F_2: 93M97 (*Lr19* (149)/ 7* W84-17) were labelled and each carefully halved to provide enough endosperm for the determination of WSP (water-soluble-protein) polymorphisms yet leaving the embryo intact and viable. To detect *Lr19* leaf rust resistance, the embryos were planted and the seedlings infected with UVPrt 8. Resistant plants were then moved to a greenhouse. Leaves of the one-month-old seedlings were harvested for DNA extractions and the plants were left to mature. Cross pollination was prevented by covering spikes with glassine bags prior to anthesis. Approximately 70-80 F_3 seeds were harvested from each F_2 plant. Leaf rust resistance tests (UVPrt 8) was done on the F_3. DNA samples from 80 heterozygotes resistant F_2 plants were tested for the presence of the XcsIH81-1-7el$_1$, XusOPK9$_{1350}$-7el$_1$ and 12c alleles. An outline of the procedure is provided in Fig. 2.1.

2.2.2 Seedling screening for leaf rust resistance

Seven days after planting the plants (two-leaf stage) were inoculated with leaf rust pathotype UVPrt 8. Fresh spores were suspended in approximately 300-400 ml dH$_2$O to which a drop of Triton was added. A fine nozzle was used to spray the suspended spores onto the seedlings.   The inoculated plants were covered with a plastic bag to create a suitable environment (high humidity, 22-25°C) for the pathogen to grow. After twenty four hours the plastic bags were removed and the plants placed at 22-25°C continuous temperature (day/night cycle = 12/12h) in a growth chamber. Ten days after inoculation, resistant and susceptible plants could be distinguished.

F₂: 93M97 (*Lr19-149/*7W84-17*)

Embryos

Endosperm

Screening for presence of *Lr19* (UVPrt8-resistant)

Iso electric focusing of WSPs (only on resistant heterozygotes)

Susceptible -rr-

Resistant - RR or Rr -

Plant in greenhouse

Select random 80 Rr individuals

Plants selfed at maturity

DNA extraction

Gather F₃ seed of each F₂ plant

RFLP marker csIH81-1 detection

RAPDs

PCR (STS marker)

Plant F₃ seed and screen for the *Lr19* locus

Restriction digestion

Conversion to a PCR-based marker

OPK9

12c

Discard resistant homozygotes

Southern blot

Sequencing of probe

rehybridization

Primer design

Hybridization

Evaluation of STS marker

Autorad

Figure 2.1 Summary of procedures followed.

2.3 Marker loci

2.3.1 Water soluble proteins (WSPs)

WSPs were separated by iso-electric focusing. The protocol used was based on the method described in Liu *et al.* (1989) and adapted by Marais (1992). The endosperms were crushed and incubated overnight in 70 µl of distilled water at room temperature. The following day it was centrifuged at 15 000 rpm for 25 minutes. Flat-bed isoelectric focusing was carried out on 0.25mm thick, 17 cm wide polyacrylamide gels containing 2% of the total ampholytes (Pharmalyte pH 5-8; Pharmalyte pH 8-10,5 and Servalyte pH 9-11 in the ratio 3:1:1). Hepes and ethylene diamine were used for anolyte and catholyte, respectively. The gels were pre-run for 30 min at 13 Watts. After the pre-run 30 µl of the sample was loaded onto the surface of the gel. Electrophoresis was carried out at 4°C for three hours (same setting).

After electrophoresis the gels were fixed in 20% TCA for 10 minutes. The gels were separated from the glassplate with running water. Staining was done for two min in Serva Violet dissolved in 50ml of solvent (2.5 methanol: 6.5 water: 1 glacial acetic acid). Gels were destained in solvent (2.5 methanol: 6.5 water: 1 glacial acetic acid) until the background was clear.

Two controls, Indis and Chinese Spring, were included on every gel.

2.3.2 *XcsIH81-1*

2.3.2.1 DNA extraction for RFLP analysis

The protocol described in the CIMMYT Applied Molecular Genetics Laboratory Manual, which is based on the method of Saghai-Maroof *et al.* 1994, was used for DNA extraction. The procedure is as follows:

Leaves were harvested from one month old seedlings planted in a greenhouse. The leaves were quick-frozen with liquid nitrogen and the frozen leaf samples transferred to a lyophilizer for 4 days. The dried leaf samples were then ground to a fine powder with the use of liquid nitrogen. Three hundred to four hundred mg lyophilized tissue was used for each extraction. 9.0ml of preheated (65°C) CTAB extraction buffer (1% CTAB, 100mM Tris - pH7.5, 700mM NaCl, 50mM EDTA - pH8.0, 140 nM BME) was added to the lyophilized tissue. Incubation was carried out for 90min., with continuous gentle rocking in a 65°C waterbath. The tubes were removed and cooled down for 5min. Chloroform/octanol (24:1) was added and gently rocked for 10 minutes. It was spinned in a table-top centrifuge for 10min at 1500xg at room temperature. The aqueous layer was poured into new 15ml tubes. Chloroform/octanol (24:1) was added and gently rocked for 10min. Then it was spinned in a table-top centrifuge for 10min at 1500xg at room temperature. The top aqueous layer was pipetted into new 15ml tubes containing 40µl of 10mg/ml Rnase A. It was gently mixed and incubated for 30min at room temperature. Isopropanol (2-propanol) (6ml) was added. Precipitated DNA was removed with a glass hook. DNA was dissolved overnight in a 1ml TE-buffer at room temperature. The DNA was precipitated by adding 50µl of 5M NaCl. 2.5ml EtOH was added and mixed by gentle inversion. The DNA was removed with a glass hook. The hook with DNA was placed in 3.5ml WASH 1 (76% EtOH, 2.5M NaOAc) for 20 minutes. The DNA on hook was briefly rinsed in 1ml of WASH 2 (76% EtOH, 10mM NH₄OAc). DNA was transferred to a tube containing 0.5 - 1.0ml TE. It was gently rocked overnight at room temperature to dissolve the DNA.

Genomic DNA concentrations were estimated using a Pharmacia Genequant Spectrophotometer. The degree of DNA degradation was determined by gel electrophoresis (0.8% agarose gel). Electrophoresis was carried out in 1 x TBE running buffer.

2.3.2.2 Attempt to convert the XcsIH81-1 marker into a cleaved amplified polymorphic sequence (CAPS) marker.

2.3.2.2a <u>Probe csIH81-1</u>

The probe csIH81-1 detects a RFLP locus linked to the *Lr19* gene and was obtained from the CSIRO division of plant Industry (Australia). csIH81-1 is an anonymous gDNA clone from a wheat genomic library which maps to the long arm of the group 7 chromosomes (Lagudah *et al.* 1991). The probe was cloned into the *Pst1* site of the poly linker of the vector pBluescript SK. The recombinant vector was transformed to *Escherichia coli*.

To facilitate its detection an attempt was made to convert it to a CAPS marker.

2.3.2.2b <u>Isolation of the probe</u>

A 50 ml LB medium bottle was inoculated from freeze cultures of the RFLP probe. Ampicillin was added to the medium as a selective agent at a concentration of 50 µg/ml. Cultures were grown overnight at 37°C on a shaking platform at 200rpm.

Plasmid isolations were done using the Nucleobond® AX PC-kit 100 for the purification of plasmids and cosmids (Machery & Nagel, 1994). The pellet was dissolved in 60 µl dH$_2$O.

Concentration of the vector was estimated by loading it together with lambda DNA of known concentrations, as control, onto a 0.6% agarose gel. After electrophoresis the gel was stained in 1 x TBE buffer containing ethidium bromide (EtBr) at a concentration of 0.5µg/ml. The concentration was estimated under UV light by comparing the band sizes and intensity to those of the lambda control.

2.3.2.2c Sequencing of the probe

Two different protocols were used for the sequencing of probe csIH81-1.

The Sequenase Version 2.0 DNA sequencing kit (Amersham) based on Sanger's dideoxynucleotide method was used for sequencing. The bacteriophage M13 forward and reverse primers were used as sequencing primers. α-^{32}S dATP (Amersham) was used for labeling and the reaction products were loaded onto a 6,6% denaturing polyacrylamide gel and electrophoresed at $\pm$ 33mA. The gel was dried at 80°C for 2 hours and autoradiography was carried out using Cronex medical X-ray film with an exposure time of 24-96 hours.

The Perkin Elmer ABI PRISM^{TM} 377 automatic DNA sequencer was also used for sequencing of probe csIH81-1. M13 universal and reverse primers (5pmol/µl) were used as sequencing primers. A graphical output from the computer enabled the reading of the sequence.

2.3.2.2d PCR reactions with unique primers of probe csIH81-1

Unique primers were designed from the sequence information by using the Primer Designer-version 1.01 software program (Copyright 1990, Scientific & Educational Software Serial number: 50132). The forward primer 5'- GCC TTG ATT GCT GCT TGT AG-3' and the reverse primer 5'-TTG TCA GGT CAG AGC ATA CT-3' of CSIH81-1 were synthesized by GIBCO BRL Custom Primers.

The following PCR parameters were optimized for the primer combination: DNA template, MgCl$_2$, PCR program, dNTPs and *Taq* polymerase. The optimized PCR reactions were performed in a reaction mixture of 25 µl. It contained the following: 15pmol of each primer, 20ng genomic DNA, 200µM of each dNTP, 2.5mM MgCl$_2$ and 2.5 µl of Bioline 10x buffer, and 0.5U *Taq* DNA polymerase (Bioline). The mixture was briefly centrifuged and a drop of mineral oil was added after which the mixture was placed in a thermal cycler (Hybaid Omnigene). After an initial denaturation step at 94°C for 5 min, 30 cycles, each consisting of 30 seconds at 92°C, 30 seconds at 56°C and 1 minute at 72°C, was performed, followed by a final elongation step of 5 minutes at 72°C. The amplified products were separated on a 0.7 %

agarose gel. The gels were run in a 1 x TBE buffer (containing EtBr) at 70V for 2 hours. The amplified bands were viewed under UV light and photographed. The 1 Kb DNA ladder was used as size marker with fragments of 12.216kb, 11.198kb, 10.180kb, 9.162kb, 8.144kb, 7.126kb, 6.108kb, 5.090kb, 4.072kb, 3.054kb, 2.036kb, 1.636kb, 1.1018kb, 0.506kb, 0.517kb and smaller fragments (range 0.396kb - 0.075kb).

2.3.2.2e Attempts to find cleaved amplified polymorphic sequences (CAPS) for the *XcsIH81-1* locus

The primers amplified fragments of the same size of the three homoeologous loci in wheat. In an attempt to find a *Thinopyrum* specific fragment the amplified products were digested with different enzymes (*Hae III, Hinf I, Taq I, Sac I, Sma I, Eco RI, Dra I, Xho I, Hind III, Bam HI, Eco RV, Cfo, Alu, Sty 1* and *Msp 1*). One microliter enzyme and 2μl of the appropriate buffer were added to the PCR product. The mixture was incubated in a waterbath at the appropriate temperature (the temperatures differs for the different ensymes used) for two hours. Digested PCR products were mixed with loading buffer and separated on a 6% mini acrylamide gel. The gels were run in a 1.5 x TBE buffer. The gels were stained after electrophoresis in EtBr for approximately 20 minutes. The bands were viewed under UV light and a photograph was taken. The 1 Kb DNA ladder was used as size marker for the fragments.

The CAPS were tested on the following near isogenic lines (NILs):

W806: 93M108-R (*Lr19/ 6* W84-17*)

W807: 93M108-S (*W84-17* background without *Lr19*)

W808: 94M38-R (*Lr19/ 6* SST66*)

W809: 94M38-S (*SST 66* background without *Lr19*)

W810: 93M6-R (*Lr19/ 6* Chinese Spring*)

W811: 93M6-S (*Chinese Spring* background without *Lr19*)

W823: 91M71-R (*Recombinant 149/ 5* Inia 66*)

W824: 91M71-S (*Inia 66* background without *Lr19*(149))

W825: 93M97-R (*Recombinant 149/ 7* W84-17*)

W826: 93M97-S (*W84-17* background without *Lr19*(149))

W828: 93M3-R (*Recombinant 149/ 6* Chinese Spring*)

W829: 93M3-S (*Chinese Spring* background without *Lr19*(149))

DNA extracts of the near isogenic lines were made using the Superquick DNA extraction method (Machery & Nagel). Two leaf discs were collected and transferred to a tube. Carborandum was added and the leaves ground to a pulp. Superquick extraction buffer (200mM Tris-Cl, 250mM NaCl, 25mM EDTA and 0.5% SDS) was added and mixed with the ground material. It was then incubated for 20 min in a 60°C waterbath. Chloroform/isoamylalcohol (24:1) was added and mixed by inversion. The tubes were centrifuged at 10000 rpm for 10 min. The aqueous supernatant was transferred to a clean microfuge tube and 150 µl of 250mM NaCl and 1 ml EtOH were added. It was mixed by inversion and put at -20°C for 30 min and centrifuged at 8000 rpm for 10 min. The ethanol was removed, 70% EtOH (500 µl) was added and centrifuged at 8000 rpm for 10 min. The tubes were inverted on tissue paper to allow the pellet to air dry. The pellet was resuspended in 50 µl dH$_2$0. The concentration of the DNA was determined on a 0.8% agarose gel together with lambda DNA of known concentrations.

2.3.2.3 Restriction fragment length polymorphism (RFLP) analyses of *XcsIH81-1*

Two procedures were used for the RFLP detection. The first was radioactive labeling and the second method used the DIG (digoxigenin) system. The following radioactive procedure was used to detect the presence of *XcsIH81-1* :

A. Radioactive protocol

2.3.2.3a Restriction digestion and Southern blot

For RFLP analyses 10µg plant DNA was digested with *HindIII* (Promega) overnight at 37°C. The digested DNA fragments were separated on a 0.8% agarose gel at 35V overnight. Lambda DNA digested with *HindIII* was used as size marker with fragments of 23.13kb, 9.416kb, 6.682kb, 4.361kb, 2.322kb, 2.072kb and 0.564kb. The alkaline transfer method from the Amersham protocol, with slight modifications was used for Southern blotting.

Following overnight electrophoresis the agarose gel was put in 0.2N HCl for 20 min (depurination step), the 0.2N HCl was poured off and the gel rinsed with distilled water. The gel was then covered with the denaturation solution (1.5M NaCl; 0.5M NaOH) for 15 min on a shaker. This step was repeated. The gel was covered with transfer buffer (0.4M NaOH) for 30 min on a shaker. The capillary blot was set up as follows: a dish was filled with transfer buffer. A platform was covered with a wick made from Whatman 3MM filter paper, saturated with transfer buffer. The gel was placed bottom up on the wick. A sheet of Hybond-N+ membrane was cut to the exact size of the gel and placed on top of it. The gel and membrane were surrounded with cling film to prevent direct absorbtion of the blotting buffer by the paper towels. A stack of absorbent paper towels and a 0.5-1kg weight was placed on top. The transfer was allowed to proceed overnight (usually 20-24 hours). The next day the paper towels and 3MM paper were removed. The membrane was briefly washed in 2 x SSC to remove adhering agarose. It was now possible to proceed directly with the prehybridization step or to cover the membrane in a plastic bag for storage at 4°C.

2.3.2.3b Hybridization

The plasmid containing the CSIH81-1 probe was extracted as in 2.1.3.3.2b. The probe was amplified with the use of M13 universal and reverse primers. The product was run on a 0.8% agarose gel. The ±1200 bp fragments were recovered from the agarose gel with the aid of Nucleo Spin Extract 2 in 1 (Machery and Nagel). The CSIH81-1 probe was radiolabeled with α-^{32}P-dATP using a random primed DNA labeling kit (Boehringer-Mannheim) and the unincorporated nucleotides were removed using a Sephadex G50 column.

New membranes were prehybridized overnight at 65°C and reused membranes were prehybridized at 65°C for 6h. The prehybridizations and hybridizations were performed in a Techne Hybridizer Oven. Prehybridization was carried out in 6 x SSC (1 x SSC: 0.15 M NaCl plus 0.015 M sodium citrate), 50 mM Tris-HCL (pH 8), 10 mM EDTA (pH 8), 0.2% SDS, 5 x Denhardt's solution (1 x Denhardt's solution: 0.02% polyvinylpyrrolidone, 0.02% (w/v) BSA, plus 0.02% Ficoll), and 170 µg/mL salmon sperm DNA, while the hybridization solution contained less salmon sperm DNA (20 µg/mL), 0.2% dextran sulfate, and the labelled denatured DNA probe. After an overnight hybridization at 65°C, the membranes were washed for 10 min and 20 min at 65°C with 2 x SSC plus 0.1% SDS. The membranes

were exposed to Cronex X-ray film for 5-10 days, using intensifying screens. Probes were stripped off the membranes by washing them for 30 min in a 0.2 M NaOH - 0.1% SDS solution at 37°C.

B. DIG-system protocol

The Southern blot was done in the same way as for the radioactive detection method. However, the labelling of the probe, the hybridization and detection differed.

The probe was labelled by PCR using the PCR DIG Probe synthesis kit (Boehringer Mannheim). The prehybridization was done in fresh DIG easy hyb for 3 to 4 hours at 42°C. The rollers were used. Hybridization was done in fresh DIG easy hyb containing the DIG labelled probe. The hybridization was carried out overnight at 42°C. Post-hybridization washes were as follows: 2 x SSC/0.1% SDS for 5 minutes at room temperature and 4 times with 0.5 x SSC/0.1% SDS at 68°C for 10 minutes.

Detection was done by using the DIG wash and Block buffer set (Boehringer and Mannheim). Probe-target hybrids were detected by an enzyme-linked immunoassay. The membrane was blocked to prevent non-specific interaction of the antibody with the filter. Antibody specific for digoxigenin recognized the labeled hybrid. Alkaline phosphatase conjugated to the antibody allowed detection of the complex. CDP-star is a chemiluminescent substrate for alkaline phosphatase. The light emitted from the blot was detected with X-ray film.

The blot was first transferred to Buffer 1 (1 x Maleic acid buffer) and shaked at room temperature for 2 minutes. Thereafter it was transferred to Buffer 2 (1 x blocking solution) for 30 minutes at room temperature. In the meantime the DIG alkaline phosphatase reagent was prepared. The antibody was spinned for 5 minutes at full speed. This conjugate was added to fresh buffer 2 and shaked for another 30 minutes at room temperature. After this the membrane was washed in washing buffer (2 x 15 minutes). The washing buffer was replaced with buffer 3 (1 x detection buffer), and shaked for 3 minutes. The CDP-star was prepared by diluting it 1:100 with buffer 3 and applied on the membrane for 5 minutes. The blot was exposed to x-ray film.

2.3.3 *X12c*

To detect the presence of the X12c allele a PCR reaction was carried out (unpublished data Prins & Groenewald 1999). The forward secquence was: 5'-CAT CCT TGG GGA CCT C - 3' and the reverse sequence: 5'-CCA GCT CGC ATA CAT CCA - 3'. The optimized PCR reactions were performed in a reaction mixture of 25μl that contained the following: 50-100ng DNA, 12.5pmol of each primer, 5mM dNTPs, 50mM MgCl$_2$ and 2.5ul of Bioline 10x buffer, and 0.5U of Taq polymerase (Bioline). The mixture was placed in a thermal cycler (Hybaid OmniGene). After an initial step at 94°C for 1 minute, 30 cycles, each consisting of 30 seconds 94°C, 30 seconds at 63°C and 30 seconds at 72°C, were performed, followed by a final elongation step of 5 minutes at 72°C. The length of the amplified product was 130bp. The products were separated on a 1.5 % LE agarose gel and visualized with ethidium bromide. The gels were run at 100 V for 2 hours.

2.3.4 *Xus-OPK 9$_{1350}$*

OPK 9 primer (from the Operon primer kit) with the sequence 5' CCCTACCGAC 3' was used to screen 80 *Lr19* heterozygotes for the *Xus-OPK9$_{1350}$-7el$_1$* allele (Barkhuizen, 1998). RAPD amplification was performed in a total reaction volume of 25μl. Optimal amplification was obtained by using 25 ng of template DNA and 10 pmol primer. The following chemicals were included in the amplification reaction: 200 μM of each dNTP, 1.5 mM MgCl$_2$, 2.5 μl of Bioline 10x buffer and 0.5 Units of *Taq* DNA polymerase (Bioline). Control reactions (in which all the reaction components except DNA template were present) were included on each gel. Any contamination could thus be detected. A thermal cycler (Hybaid OmniGene) was used for performing the amplification reactions. Cycles of 1 min at 94°C, 60 sec at 94°C, 20 sec at 35°C and 60 sec at 72 °C for 45 cycles were included in the program, ending in a 10 min cycle at 72°C to complete any incomplete fragments synthesised during the reaction.

The RAPD products were mixed with 2 μl of loading buffer, of which 15 μl were loaded on a 1.5 % LE agarose gel. Running buffer consisted of 0.5 x TAE (containing EtBr). Separation was achieved at 100V for 4 hours. The 1 Kb+ DNA ladder was used as size marker.

Visualisation of the RAPD products were done by using an ultraviolet transilluminator and the profiles were photographed.

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CHAPTER 3
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RESULTS AND DISCUSSION

3.1 Gametic transmission of *Lr19* (149)

Self-elimination of *Lr19* (149) is clearly demonstrated by the segregation ratios observed in progeny of the testcrosses (Table 3.1). Self-elimination occured in both sexes. Chi-square tests showed that the observed segregation ratios differed significantly from the expected 1:1 ratio.

Table 3.1 Segregation for leaf rust resistance (*Lr19*) in the progeny of test crosses

PARENTS (Female/Male)	Genotype	Observed Resistant R	Observed Suscept. S	Observed R:S ratio	Expected R:S values	Chi-square	P-value (1:1)
W84-17/F$_1$: 93M97	rr x Rr	4	23	15:85	13.5:13.5	13.37	0.00
F$_1$: 93M97/W84-17	Rr x rr	14	37	27:73	25.5:25.5	10.37	0.00
Inia/F$_1$: 91M71	rr x Rr	1	10	9:91	5.5:5.5	7.36	0.00
F$_1$: 91M71/Inia	Rr x rr	0	1	-	0.5:0.5	1.00	0.32
SST 66/F$_1$: 93M101	rr x Rr	4	34	11:89	19:19	23.68	0.00
F$_1$: 93M101/SST 66	Rr x rr	6	11	35:65	8.5:8.5	1.47	0.23
C. Spring/ F$_1$: 93M3	rr x Rr	4	30	12:88	17:17	19.88	0.00
F$_1$: 93M3/ C.Spring	Rr x rr	11	16	41:59	13.5:13.5	0.93	0.34
Total:male transmission	rr x Rr	13	97	12:88	55:55	64.14	0.00
Total:female transmission	Rr x rr	31	65	32:68	48:48	12.04	0.00

Heterogeneity in the male and female transmission of the *Lr19* (149) segment was tested according to Snedecor and Cochran (1989). Only the 'Chinese Spring' and 'W84-17' data sets were analyzed because the other two data sets were too small. 'Chinese Spring' showed heterogeneity between reciprocal crosses (P = 0.009), but in 'W84-17' no proof was found for differences (P = 0.208). The total transmission values shows a very strong suicidal tendency of the translocated chromosome in the genetic background of the male parent where 88% of

the progeny were susceptible. Marais (1992c) crossed 'Inia 66' as the female parent with a plant having telosome 7DL and a *Lr19* (149) translocated chromosome. This resulted in 94% susceptible progeny.

A test for heterogeneity (Little & Hills, 1978) fitted on the full dataset shows that self-elimination of *Lr19* (149) is similiar for the four genotypes (Table 3.2). These findings confirm the results of Prins *et al.* (1997).

It was found by Prins & Marais (1999) that the degree of segregation distortion is determined by the interaction of the *Sd* genes with polygenes (response genes) on various wheat chromosomes. Responder alleles derived from 'Inia 66' or 'Indis' (chromosomes 2A, 2B, 3B, 5B, 5D and 6D) appeared mostly to be partially dominant to overdominant over the Chinese Spring derived alleles. A specific allele did not necessarily have the same effect (suppression or enhancement) in different genetic backgrounds. Also, responder genes may not fully compensate for the absence of a homologue in a hemizygote which may then produce effects quite different from those of the homo- and heterozygotes. Thus, it is possible to find a genetic background in which *Lr19* (149) inherits normally or even preferentially.

Table 3.2 Heterogeneity analysis of the reciprocal test cross results.

Cross	Observed Resistant	Observed Suscept-ible	Expected Resistant	Expected Suscept-ible	Deviation Chi-square	P-value	Heterogenity Chi-square	P-value
rr/Rr	13	97	55	55	64.145	0.000	0.155	0.984
Rr/rr	31	65	48	48	12.042	0.001	1.727	0.631

The expected F_2 segregation of *Lr19* (149) (R_:rr) can be predicted from the observed transmission rates in the reciprocal crosses by developing a "Punnett square" (Table 3.3). The average male and female transmission values were used for this purpose. The expected segregation pattern thus dereived was compared to the observed F_2 segregation of the data in section 3.1.2.

Table 3.3 Numbers of expected resistant and susceptible plants in the F_2 of a *Lr19* (149) heterozygote.

		Pollen	
		R (0.12)	r (0.88)
Egg	R (0.32)	0.04	0.28
cells	r (0.68)	0.08	0.60

Of the expected 40% resistant F_2 plants, 36% will be heterozygotic. Alternatively, 90% of the resistant F_2 progeny can be expected to be heterozygotes. The observed F_2 segregation of 93M101 was 0.05RR:0.53Rr:0.42rr (section 3.2.1).

3.2 Segregation of *Lr19* (149) in B$_6$F$_2$ population: 93M97

The stability of the *Lr19* (149) was studied by testing for the presence of *Thinopyrum* specific alleles at four loci on the translocation, i.e. *Wsp-D1, X12c, XcsIH81-1* and *Xus-OPK9$_{1350}$*. The loci, *Xus-OPK9$_{1350}$* and *XcsIH81-1* are located proximally to *Lr19* while *Wsp-D1* and *X12c* are located distally from *Lr19* (Fig.3.1). Eighty *Lr19* heterozygotes from the cross: 93M97 (*Lr19*-149/*7 W84-17) were characterized for the presence/absence of the four marker loci. The results are summarized in addendum 1. Each marker is discussed separately in sections 3.3.1-3.3.4.
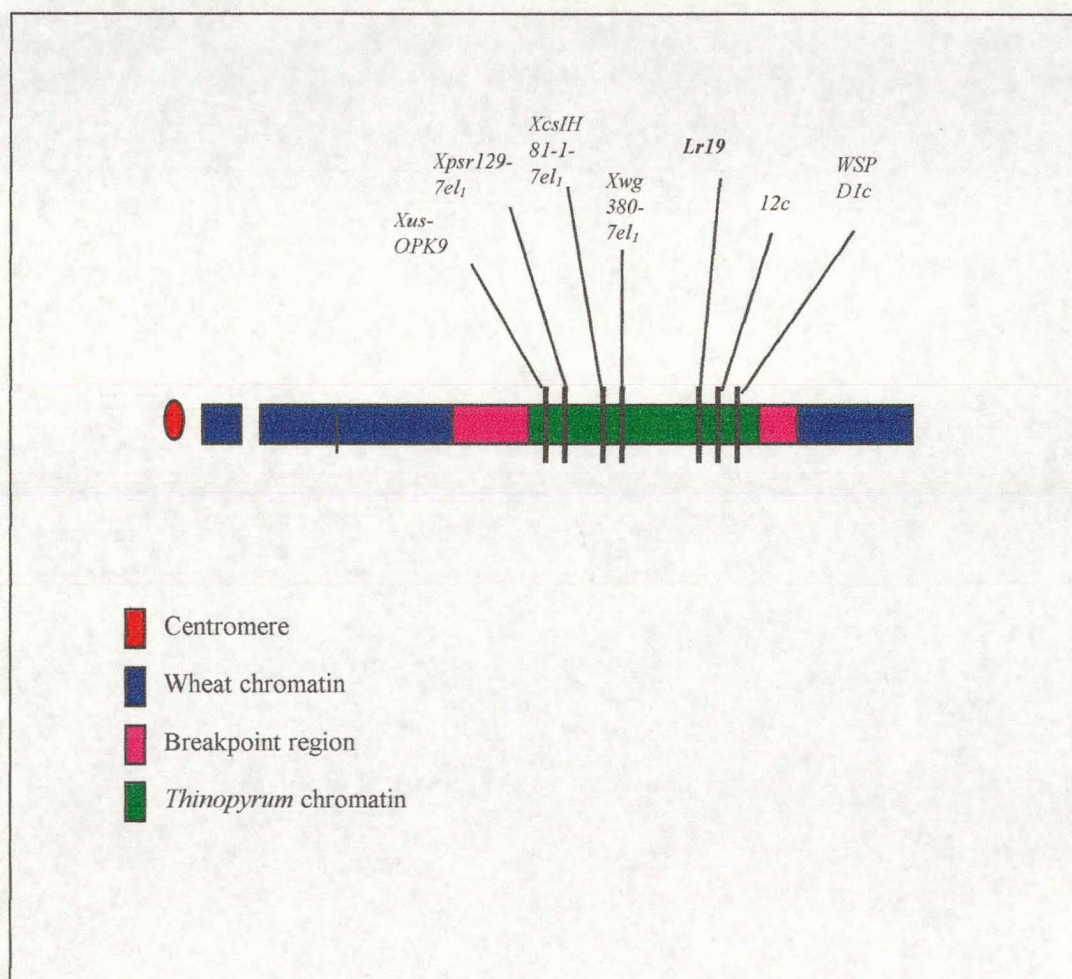
Figure. 3.1 Shortened *Lr19* (149) translocated segment on chromosome arm 7BL

A total of 233 B_6F_2 93M97 plants were tested for resistance. One hundred and thirty five were found to be resistant. Resistant plants were grown and allowed to self-pollinate. At least 50 F_3 progeny from each resistant F_2 plant were tested for leaf rust resistance. The genotype (RR/Rr) of each F_2 plant could then be established by observing the segregation of *Lr19* in the F_3 family. A total of 123 plants were found to be heterozygous for *Lr19* (Figure 3.2).
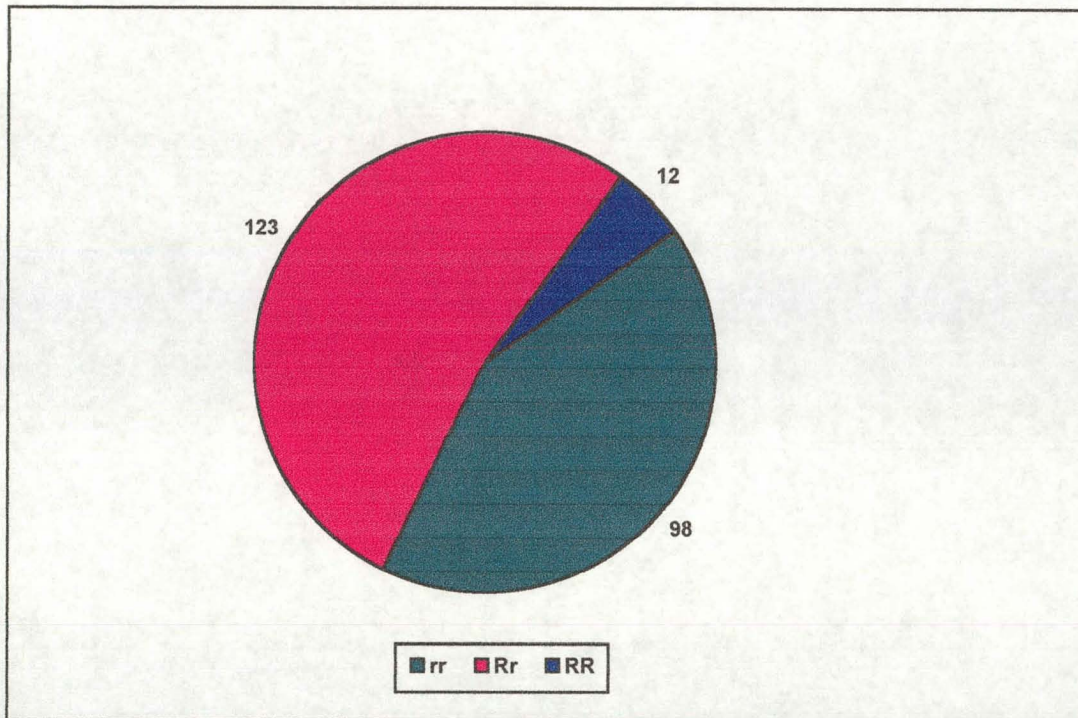
Figure 3.2 Pie chart showing the segregation of *Lr19* (149) leaf rust resistance in the F_2: 93M97 population.

The segregation ratio of 0.58R-:0.42rr (2.3:1.7) differs from the expected 0.75R-:0.25rr (3:1) ratio (P=0.000) of a F_2 population. With Mendelian segregation it is expected that one out of every four plants will be homozygous resistant, therefore 25 out of 100. Out of 233 plants one would expect 58 to be RR which differs from the actual value of 12 RR plants. This confirms the work done by Prins *et al.* 1997, where 2 out of 78 resistant F_2 plants were homozygous and emphazises strong self-elimination of the *Lr19* translocation in the F_2 population.
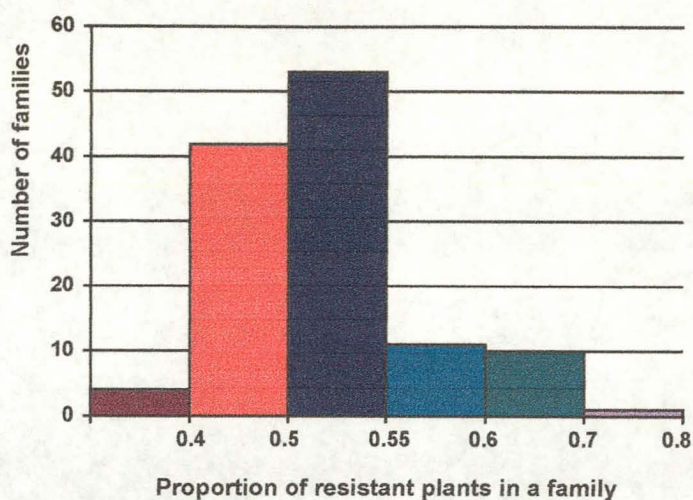
Figure 3.3 Segregation of *Lr19* (149) in F_2 derived F_3 families from the backcross population 93M97 (*Lr19- 149*/7*W84-17)

An analysis of segregation in F_3 families derived from heterozygous F_2 plants (Addendum 2), confirmed self elimination in the material as described for the F_2 segregation. The segregation ratios observed in the F_3 families derived from heterozygous F_2 plants are summarized in Figure 3.3. More than 100 of the 123 (>73%) families contained proportions of 0.4 to 0.6 resistant plants. The average of all the F_2 families was 51 resistant: 49 susceptible (3387:3294). The highest proportion of resistant plants was 0.76, which occurred in a single family. Thus the overall segregation ratio deviated strongly from the expected 0.75R-:0.25rr ratio. The observed segregation ratio was close to a 1:1 ratio and was also found in previous studies (Marais 1992a; Marais, 1993; Prins *et al*. 1997).

However, a test for heterogeneity did not suggest differences in the extent of segregation distortion among F_3 families (P=0.072).

3.3 Marker loci

3.3.1 Water soluble proteins (WSPs)

The presence of WSP-D1c protein was investigated in the endosperm halves of all the resistant F_2. It appeared that WSP-D1c was present when *Lr19* was present and absent if *Lr19* was absent. Only the two controls which was used on all the gels, are shown in Figure 3.4. The arrow shows the polymorphism which was scored. The polymorphic band (present in 'Indis') was present in all 233 *Lr19* carrying plants, which confirms complete linkage in coupling between *Lr19* and *Wsp-D1c*.
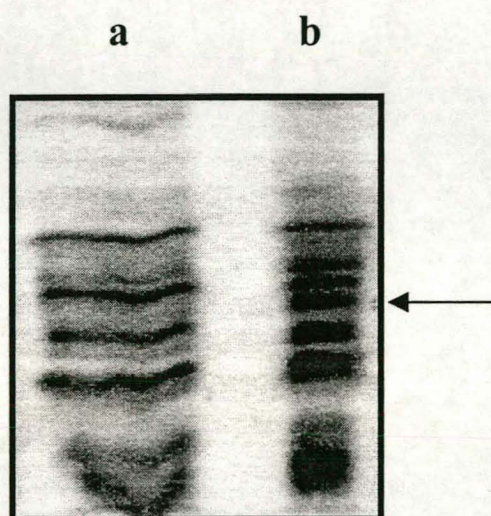
Figure. 3.4 WSP-1 profiles of **a**, Chinese Spring and **b**, Indis.

3.3.2 *XcsIH81-1*

3.3.2.1 DNA extractions

Excellent results were obtained from lypholized plant material, using the CTAB method published by Saghai-Maroof *et al.* (1984). Up to 0.5 - 0.6 µg/µl DNA was extracted from 2 g plant material. Agarose gels confirmed that the DNA was intact.

3.3.2.2 Sequence-tagged-site (STS) and cleaved amplified polymorphic sequence (CAPS) analysis

Because of the laborious nature of RFLP analysis an attempt was made to convert the *XcsIH81-1* marker into a PCR-based marker. The conversion of mapped RFLP markers, such as csIH81-1, to their sequence-tagged-site (STS) counterparts has proven an effective method of obtaining easy-to-use, reliable markers (Rafalski & Tingey, 1993).

3.3.2.2a Sequencing of probe csIH81-1

To develop STS primers from a probe, partial or complete sequence information of the probe is necessary. Following its amplification, large amounts of plasmid DNA were obtained with a Nucleobond kit. From both the results of the Perkin Elmer ABI PRISM^{TM} automatic Sequencer and manual sequencing with the Sequenase Version 2.0 DNA sequencing kit, about 500 bp from each side of probe csIH81-1 (± 1400 bp) could be sequenced. A section of the double-strand DNA sequence of probe csIH81-1 is given in Figure 3.5. The forward and reverse primers which were selected with the Primer Design software program are double underlined. The approximate length of the expected amplicon was calculated at ± 1100bp.

A

5' GGTGAT ATATCG AGCATT CGGACA TATTAT TTCCAA TGCTCC AGT

3' CCACTA TATAGC TCGTAA GCCTGA ATAATA AAGGTT ACGAGG TCA

GTCmsC AGTTAT AACAGA AGCCCA TCAGGT TAATTA CTTCTT GAT

  CAGmxG TCAATA TTGTCT TCGGGT AGTCCA ATTAAT GAAGAA CTA

  TGAAGC ATGCMC AACCCT <u>TTGTCAG GTCAG AGCATA</u> CTAATT GCC

  ACTTCG TACGMG TTGGGA AACAGT CCAGTC TCGTAT GATTAA CGG

  CAATTG CTTCAC TAATTG TTTTTT CAGATT TGCTCG TGATGG TTG

  GTTAAC TAAGTG ATTAAC AAAAAA GTCTAA ACGAGC ACTACC AAC

  ATATCT TAGCCA AATTGA GYGTGG ATAATC AGGATA .... 3'

  TATAGA ATCGGT TTAACT CYCACC TATTAG TCCTAT .... 5'

B

5'...........GAAGAC TTCCTT ATTTTC TCTTGA GTAACG TCTTAC CAT

3'... ......CTTCTG AAGGAA TAAAAG AGAACT CATTGC AGAATG GTA

ATAAGA ACGTCC AGG<u>ATG TTCGTC GTTAGT TCC</u>GAG AACTAC

TATTCT TGCAGG TCCTAC AAGCAG CAATCA AGGCTC TTGATG

TCTTTT CCCGAC AAGCGG TTCTCC GACAAG CTACAG CTGTTT GTA

AGAAAA GGGCTG TTCGCC AAGAGG CTGTTC GATGTC GACAAA CAT

CCATAC TTTTGA TATAGG ATAAAG ACCCTC AAGAAC ACGATA AGA

GGTATG AAAACT ATATCC TATTTC TGGGAG TTCTTG TGCTAT TCT

CACCGT TGTACT TGGTGG ACTACA TTATAA TCCCGT ....3'

GTGGCA ACATGA ACCACC TGATGT AATATT AGGGCA.....................5'

Figure. 3.5 A partial sequence of probe csIH81-1 with the derived forward (A) and reverse (B) primers underlined.

3.3.2.2b PCR reactions with unique primers for csIH81-1

Only one major band (± 1100bp) was amplified using primers for probe csIH81-1.

Purified genomic DNA of Indis, Inia 66, Chinese Spring (CS), CS Nulli 7A, CS Nulli 7B, CS Nulli 7D, near isogenic lines (W806-828) and F_2 plants of cross 93M97 were used as template DNA for PCR amplification with the derived primers of csIH81-1. Fig. 3.6 shows the amplified fragments of the different lines with an equal size of about 1100 bp. From the results obtained with the nullisomics it can be concluded that amplicons of similar size are probably produced at the three homoeoloci.
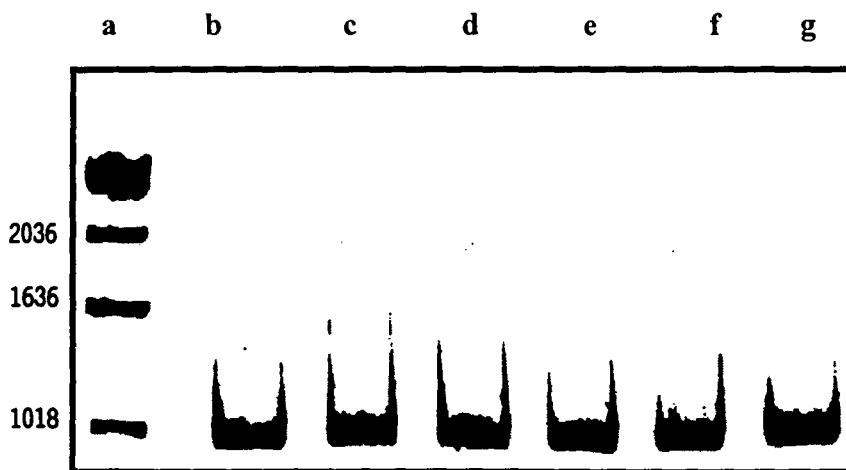
Figure. 3.6 STS csIH81-1 PCR product from different genomic DNAs: **a**, 1 kb ladder size marker; **b**, Indis; **c**, Inia 66; **d**, Chinese Spring; **e**, CS Nulli 7A; **f**, CS Nulli 7B and **g**, CS Nulli 7D.

The fragment's lengths obtained following amplification on the plasmid and genomic DNA correlated with the anticipated fragment length as calculated from the sequence information. Since the STS products for homoeoloci and genotypes were of the same size, the possibility of sequence differences among amplicons had to be investigated. If amplicons differed for restriction enzyme recognition sites, this could serve to differentiate between them.

3.3.2.2c <u>Cleaved amplified polymorphic sequences (CAPS)</u>

PCR products were then digested with each of 15 restriction enzymes in an attempt to find DNA variation not detectable as STSs. Variation of this nature may include nucleotide substitutions creating or destroying a recognition site for one of the restriction endonucleases or additions/deletions too small to be detected in the undigested PCR products. In Figs. 3.7 and 3.9 the results obtained with two restriction enzymes, which digested the amplified PCR product, are represented. The other 13 enzymes (*Hinf I, Taq I, Sac I, Sma I, Eco RI, Dra I, Xho I, Hind III, Bam HI, Cfo, Alu, Sty I, Msp I*) did not digest the PCR product at all. With the enzyme *Hae III*, three different DNA fragments can be detected in Indis, while only two different fragments were detected in Inia, Chinese Spring, CS Nulli 7A, CS Nulli 7B and CS Nulli 7D (Fig. 3.7). Two different fragments (only one visible on gel) can be detected with the *EcoRV* restriction enzyme in all the different entries (Fig. 3.9).

Use of the enzyme *HaeIII*, resulted in three DNA fragments (± 1000bp, ± 600bp and ± 400kb) for Indis and two different fragments (± 600bp and ± 400kb) for Inia 66, Chinese Spring, CS Nulli 7A, CS Nulli 7B and CS Nulli 7D. Thus, it appeared that the amplicon from the *Thinopyrum* locus may lack the *HaeIII* restriction site present in the wheat homoeo-alleles. A group of *Lr19* near isogenic lines (NILs) were also studied to determine whether the undigested fragment in Indis is in fact associated with the *Lr19* translocation (Fig.3.8). The observed banding patterns did appear to correlate with the presence/absence of *Lr19*, however, the procedure did not always allow for unambiguous identification of the *XcsIH81-1* locus as in the absence of *Lr19* bands of lower intensity rather than no bands were produced (Fig. 3.8). It was thought of been undigested material, but after more units of enzyme were used and the incubation time was changed from two hours to three hours the band was still present. This marker could therefore not be reliably used to test for the presence of *Lr19*.
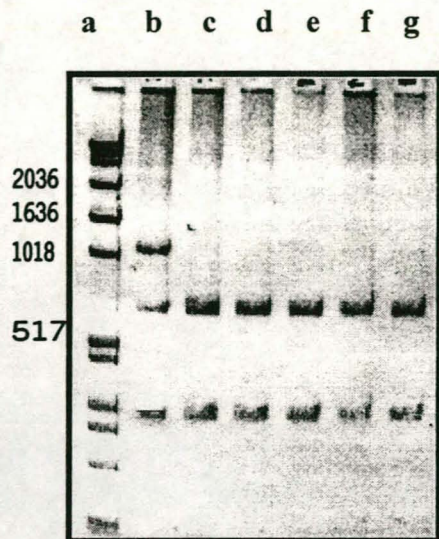
Figure.3.7 *Hae III* restriction digested DNA fragments of amplified PCR products with the STS csIH81-1 primers: **a**, 1kb ladder size marker; **b**, Indis; **c**, Inia 66; **d**, Chinese Spring; **e**, CS Nulli 7A; **f**, CS Nulli 7B and **g**, CS Nulli 7D.
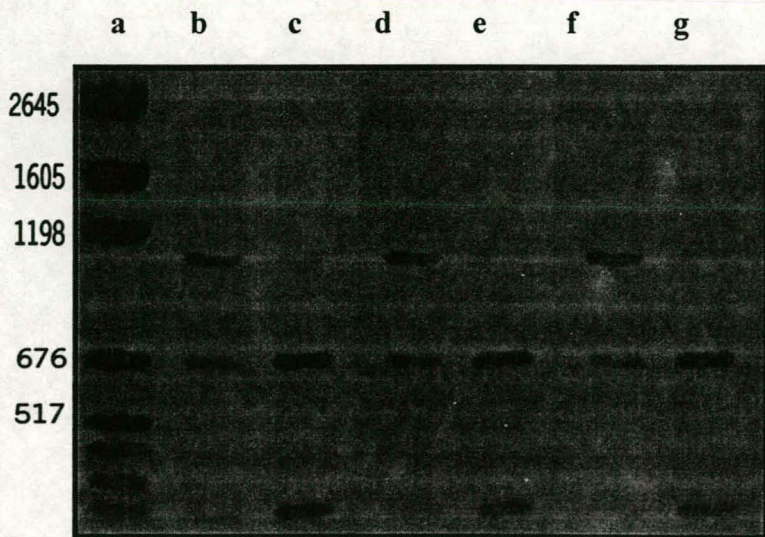
Figure.3.8 *HaeIII* restriction digested DNA fragments of the near isogenic lines: **a**, pGEM size marker; **b**, W806 (W84-17-*Lr19*); **c**, W807 (W84-17); **d**, W808 (SST66-*Lr19*); **e**, W809 (SST66); **f**, W825 (W84-17-*Lr19*(149)); **g**, W826 (W84-17)
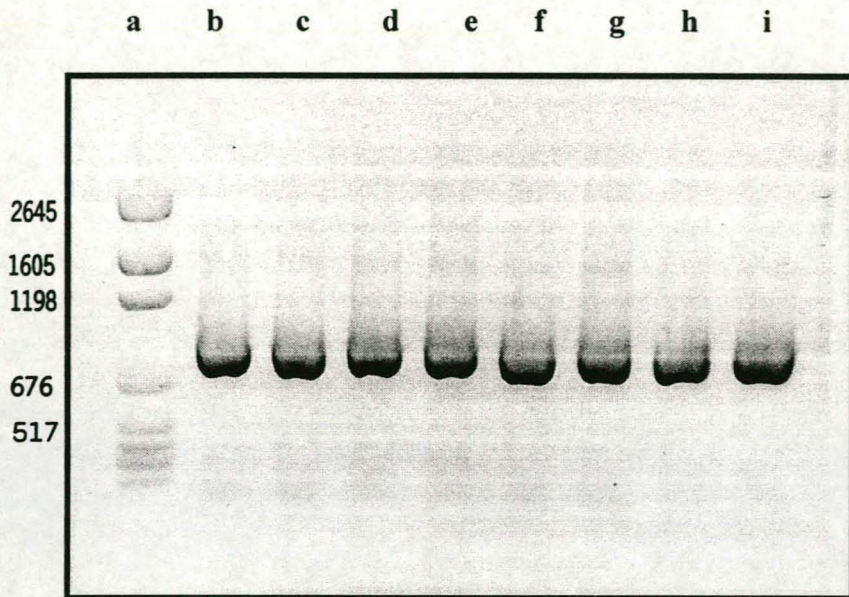
Figure. 3.9. *EcoRV* restriction digested DNA fragments of amplified PCR products with the STS csIH81-1 primers: **a**, pGEM size marker; **b**, Indis; **c**, Inia 66; **d**, Chinese Spring; **e**, CS Nulli 7A; **f**, CS Nulli 7B; **g**, CS Nulli 7D; **h**, W806 (W84-17-*Lr19*) and **i**, W807 (W84-17).

With the restriction enzyme *EcoRV*, only one distinct fragment was detected at approximately 800bp with all the different entries (Figure 3.9). The other fragment is not visible on the gel and is approximately 200bp in length. No polymorphism could be detected between resistant and susceptible plants.

If the csIH81-1 RFLP marker could be converted to a PCR-based marker it would have been very useful in this study and in MAS breeding. Yet, restriction digestion of the amplification products revealed no useful polymorphisms. The results obtained would suggest a high degree of conservation of sequence homology at the three homoeoloci. Due to time and money considerations no other restriction enzymes were tested.

3.3.2.3 Restriction fragment length polymorphism (RFLP) analysis of XcsIH81-1

Failing to convert the RFLP marker (CSIH81-1) to a STS marker, it was decided to use csIH81-1 as a RFLP marker for detection of the *Thinopyrum* locus.

Prins (1997) used aneuploids to identify the fragment associated with each group 7 chromosome. The *Lr19* translocation produces a unique fragment with size of approximately 20 kb (Figure 3.10).

The 7D and 7B wheat fragments, respectively, disappear in *Lr19* and *Lr19(149)* homozygotes (Prins, 1997). *Lr19* and *Lr19 (149)* heterozygotes (Rr) exhibit the unique *Lr19* fragment as well as all of the wheat group 7 fragments. Recessive individuals (rr) show only the wheat group 7 bands. RFLP analyses were done on 80 Rr F_2 individuals, to determine whether the *Thinopyrum* derived fragment was present or not.
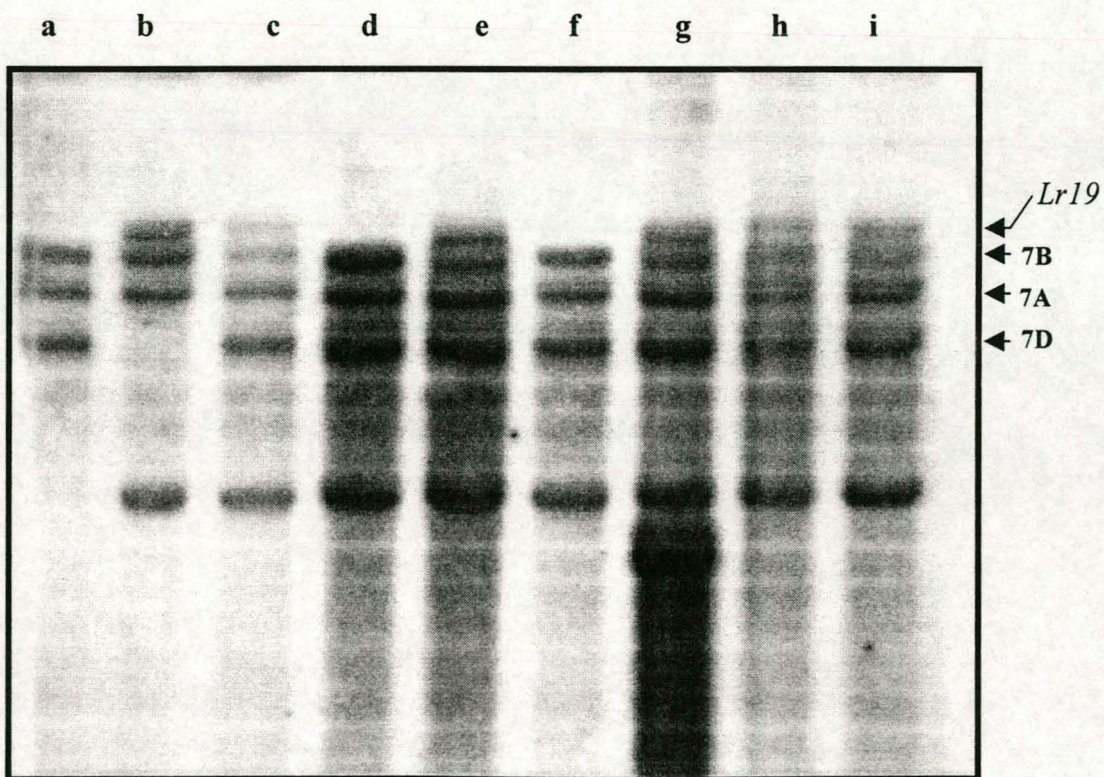
Figure. 3.10 Autoradiogram showing hybridisation of probe csIH81-1 on total genomic DNA of: **a**, Chinese Spring; **b**, Indis; **c-i**, different F_2 individuals. Plants **d** and **f** are recessive homozygotes (rr) for the *Lr19*(149) locus. The other plants are heterozygotes (Rr).

Initially, hybridization of probe csIH81-1 was detected using radio-activity (^{32}P). However, the Dig-system was used to visualize polymorphisms. This afforded an opportunity to compare the advantages and disadvantages of the two methods which can be summarized as follows: (Table 3.6).

Table 3.6 Comparison of the Dig system with radioactive labeling in RFLP analysis.

DIG detection	Radioactive detection
1. Safe	1. Hazardous, mutagenic
2. Labeled probes can be stored for at least one year	2. The labeled probe cannot be stored because ^{32}P looses its activity in fourteen days
3. No column used	3. The labeled probe has to be separated from the unincorporated bases through a sephadex column
4. Hybridization solutions can be reused several times	4. Hybridization solutions cannot be reused due to radioactivity
5. Quick result	5. May take up to 2 weeks before a good result can be seen. This also complicates problem solving.
6. Several steps in detection where things can go wrong	6. Detection is simpler. After the stringent washes the membrane is directly transfered to the X-ray film and cassette

3.3.3 *X12c*

To score the *X12c* allele as absent or present, polymerase chain reactions were carried out with unique primers for a STS marker, developed from an AFLP marker (Unpublished data; Prins (ARC Small Grain Institute) and Groenewald (Department of Genetics, University of Stellenbosch), 1999). The PCR reactions amplified a fragment of 130 bp in resistant (*Lr19* +) plants (Figure 3.11). Eighty resistant *Lr19* heterozygotes each scored positive for this fragment.

a b c d e f g h i j k l m n o p q r s t u v
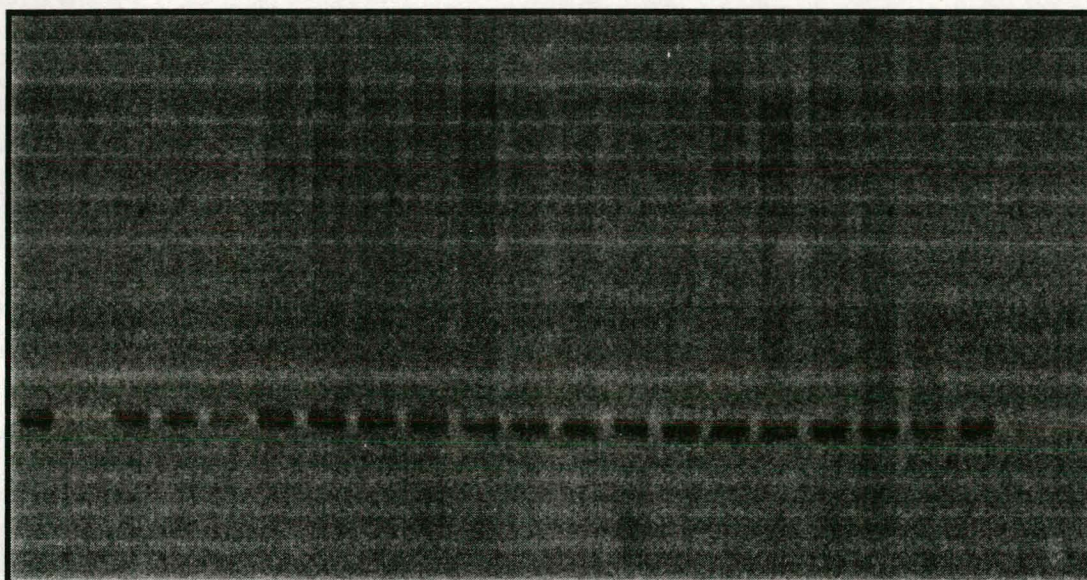
Figure. 3.11 *X12c* PCR product from different genomic DNAs: **a & t:** Indis, **b & u:** Inia 66, **c-s:** F$_2$ samples and **v:** negative control.

3.3.4 *Xus-OPK 9$_{1350}$*

RAPD analyses were used to score for the presence of allele *Xus-OPK9$_{1350}$-7el$_1$* in the 80 heterozygotes. A fragment of 1350 bp is amplified in resistant plants but is absent in susceptible plants as shown in Figure 3.12 (Barkhuizen, 1998).

a   b   c   d   e   f   g   h   i   j
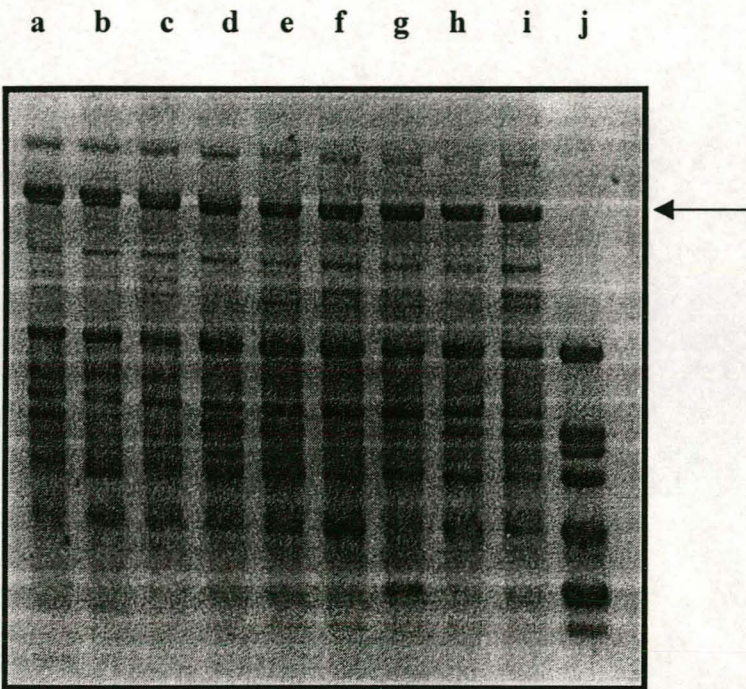
Figure. 3.12 RAPD profile showing the OPK9$_{1350}$ polymorphism in *Lr19* (149) carriers. **a-i:** resistant F$_2$ plants and **j:** Inia 66 (susceptible).

3.4 Stability of *Lr19* (149)

The data obtained for the four marker loci are summarized in Table 3.4. Each of the 80 F_2 heterozygotes received the normal *Thinopyrum* allele at each of the four loci. A further 42 heterozygous F_2 progeny were tested only for the presence of *Wsp-D1c*. In all, no mutations occurred at the 366 loci studied which would suggest that unlike the high incidence of mutations in the progeny of heterozygotes for *Gc*-genes (Tsujimoto & Noda, 1990; Middleton, 1998), *Lr19* (149) heterozygotes are fairly stable. It would seem that the altered *Sd* complex of *Lr19* (149) (which has lost *Sd1*) did not visibly increase the mutation rate at the four loci.

Table 3.4 Summary of data obtained (Addendum 1) following the evaluation of 233 F_2 progeny of the cross: 93M97

Number of F_2 progeny tested	*Xus OPK9-7el₁*	*XcsIH 81-1-7el₁*	*Lr19*	*X12c*	*Wsp-D1c*	Genotype
42	?	?	+	?	+	Rr
80	**+**	**+**	**+**	**+**	**+**	**Rr**

The lack of recombination among the marker loci suggests that the translocation is transmitted as a single, large linkage block during meiosis. The original *Lr19* translocation has a terminal location. If chromosome pairing is initiated from the telomeres (section 1.4.1) synapsis may be discouraged by the presence of *Thinopyrum* derived and wheat 7DL telomeres in a heterozygote. This together with the presence of *Ph1* could rule out the possibility of recombination between the *Lr19* translocation and homoeologous wheat chromatin. However, *Lr19*(149) is a considerably shorter version of *Lr19*; which had chromatin both proximally and distally of *Lr19* replaced with wheat chromatin. Thus, in *Lr19* (149) heteroygotes the two normal 7BL telomeres may be conducive to synapsis. From the present results it appears, however, that the shortened fragment is as resistant to homoeologous recombination as the original translocation was.

Recombination in a given organism can vary considerably due to many factors (both environmental and genetic). For example, it is known that the amount of recombination between marker loci can be increased or decreased by environmental factors such as temperature. Furthermore, it has been shown that moderate doses of irradiation (X-rays) or treatment with mutagenic chemical agents are able to increase the amount of recombination between marker loci. However, most frequently differences in the amount of recombination have a genetic basis (Baker *et al* 1976). In many organisms, recombination during male and female gametogenesis occurs at different rates (van Ooijen *et al.*, 1994 , Busso *et al.*, 1995). In humans and other mammalian species, the recombination rate during male gametogenesis is much lower than during female gametogenesis (Donnis-Keller et al., 1987). In *Drosophila,* as an extreme case, no recombination is observed in male gametes at all. Very little is known about different rates of recombination in male and female gametogenesis in plants, and it has only been with the advent of genetic maps based on isoenzymes (Gadish and Zamir, 1987) and restriction fragment length polymorphisms (RFLPs) that it has been possible to study this phenomenon in detail for many regions of a genome or even the entire genome. In tomato, data are now available that suggest a generally higher rate of recombination during female gametogenesis (de Vincente and Tanksley 1991; van Ooijen *et al.*, 1994). However, data from other plants such as potato and pearl millet suggest that this is not a general phenomenon (Busso *et al.*, 1995).

If differences in recombination exist between the sexes, these might be exploited in crossing-schemes to either reduce crossing-over (e.g., in the construction of chromosome substitution / addition lines) or to increase recombination (e.g. where undesirable linkages need to be broken or for the construction of high-resolution RFLP maps around genes targeted for cloning) (de Vincente and Tanksley, 1991). If recombination rates are higher in females, then existing backcross breeding using the recurrent parent as the male should minimize linkage drag. By the same reasoning, the recurrent parent could be used as the female in cases where it is desirable to minimize recombination.

In some cases, it is known that the amount of recombination is controlled by specific genes. In *Drosophila*, it has been possible to breed strains with differing amounts of recombination for specific marker loci (Baker *et al.*, 1976). The same has been found for certain plant

species where specific genes affect recombination. As described in section 1.5, in wheat the genes with the most drastic effects on recombination are the *Ph* genes, especially *Ph1*, that controls the pairing of homoeologous chromosomes and thus the occurrence of recombination between the different genomes. This gene also keeps wheat chromosome arm 7BL from pairing with the *Thinopyrum* derived *Lr19*(149) translocation during meiosis. The data in Table 3.4 emphasize this.

If premeiotic alignment of homologous was the strategy by which *Ph1* prevented heterogenetic chromosome pairing, interstitial homoeologous segments would be expected to behave as homologous, and recombine with a similar frequency as they do in the absence of *Ph1*. Alternatively, homoeologous segments could cause the rest of a chromosome pair to behave as homoeologous and recombine poorly or not at all, so that such chromosomes would act entirely as homoeologous. In the heterozygous F_1 plants both the telomeres and the centromere were homologous, only the translocation segment (which is interstitial) was homoeologous and no recombination occurred in that area. These facts strongly argue against a possibility that the only effect of *Ph1* is to regulate homoeologous meiotic pairing by premeiotic alignment of chromosomes as suggested by Feldman *et al.* (1966, 1973). It would be interesting to do a study on the same DNA, but on markers in the non-translocation area, to see if the homoeologous segment have any effect on the recombination of the otherwise homologous chromosomes. Dubcovsky (1995) reported that recombination was significanty reduced in the homologous segment of a chromosome with a distal homoeologous insertion, compared to that in completely homologous chromosomes.

However, if the conclusion made by Luo *et al.* (1996) that the *Ph* gene regulates a mechanism to check homology along the entire length of the chromosome is correct, the lack of recombination in this area can be contibuted to this mechanism. It can be assumed that the level of homology between chromosome 7BL (*T. aestivum*) and the translocation segment (derived from *Thinopyrum ponticum*) has been checked by a mechanism (probably involving *Ph1*) and was found inadequate. This could have prevented synapsis and crossovers in the relevant area (Fig. 3.13).

The length of the translocation (homoeologous area) can not be considered as a factor for the absence of recombination because Luo *et al.* (1996) used homoeologous segments as small as 2.6 cM and still recombination did not occur.

Thus, the activity of *Ph1* effectively prevents recombination of the homoeologous segment (translocation) in the chromosome pair 7B in the same way as it does in completely homoeologous chromosome pairs e.g. 7A and 7D. The homology-check system in wheat may also be more strict because of its inbreeding nature.

Lr19

In a reproductive cell of a Rr plant, during MEIOSIS I (leptotene/zygotene) :
One wheat chromosome 7BL and one translocated chromosome 7BL (*Lr19* (149)) are grouped

"Homology check" along entire length of the chromosomes (*Ph1* could be involved)

System finds low homology between chromosomes in the translocation area

Lr19

Recombination is prevented in the area with low homology

Figure 3.13 Possible regulation of chromosome pairing and recombination in a *Lr19* (149) translocation heterozygote.

Another example of a recombinationally inactive introgressed segment is the root-knot nematode resistance gene (*Mi*) on chromosome 6 of *Lycopersicon esculentum* (wild tomato). In crosses between *Lycopersicon esculentum* (wild tomato) lines which contain an introgressed *Mi* gene and lines that do not, a severely reduced level of recombination has been demonstrated in this region. The material could therefore not be used for fine mapping this gene to a resolution that is necessary for map-based cloning (Ganal & Tanksley, 1996).

Inadequate levels of homology in regions of a bivalent will contribute to the phenomenon of 'linkage drag', which results in larger than expected segments being retained during backcross breeding (Brinkman and Frey, 1977). Repeated backcrossing simultaneously accomplishes two essential goals. It allows segregation to remove donor parent chromosomes unlinked to the target gene and it allows recombination to remove donor parent segments which are linked to the target gene. Fifty percent of unlinked DNA is removed in each generation, so that by the eighth backcross generation, less than 0.2% of the unlinked donor genome is expected to persist. By contrast, the removal of linked segments occurs in a complex fashion that was described first by Hanson 1959, and further elaborated by Stam and Zeven 1981. Their work showed that it takes many generations to remove the linked donor segments. After 20 generations, for example, it is predicted that the target gene will be flanked by an introgressed segment extending approximately 5 cM in both directions (depending on homology). The products of introgression will therefore be a pair of NILs which are identical except for a region near the target gene. If crossing over in the target area is partially or completely suppressed as in the case with an introgressed alien segment (e.g. *Lr19* (149)), the situation is augmented.

CHAPTER 4

CONCLUSIONS

The following conclusions can be drawn from this study:

- The tendency for the translocation to self-eliminate appears to be stronger in pollen

- *Lr19-149* self-eliminate (segregate in a 1:1 ratio in a F_2 population) and the majority of the resistant plants are heterozygotic

- It appears that the shortened translocation does not recombine with the corresponding region on homoeologous group 7B chromosome arm in the presence of *Ph1* and is inherited as a single, large linkage block

- The translocation is stable and no evidence could be found of an increased mutation rate because of the altered *Sd* complex.

CHAPTER 5

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CHAPTER 6

ADDENDA

ADDENDUM 1. Data obtained following the evaluation of 233 F_2 progeny of the cross: 93M97. Marker alleles were determined on a random sample of 80 resistant heterozygotes and are depicted in **BOLD**.

[(+) *Thinopyrum* allele present   (-) *Thinopyrum* allele absent]

F₂: (progeny): #	Xus-OPK 9_{1350}-7el₁	XcsIH 81-1-7el₁	Lr19	12c	Wsp-D1c	Genotype
Lr19-149-						
100	**+**	**+**	**+**	**+**	**+**	**Rr**
101	**+**	**+**	**+**	**+**	**+**	**Rr**
102	**+**	**+**	**+**	**+**	**+**	**Rr**
103	**+**	**+**	**+**	**+**	**+**	**Rr**
104			-		-	rr
105			-		-	rr
106			-		-	rr
107			-		-	rr
108			-		-	rr
109	**+**	**+**	**+**	**+**	**+**	**Rr**
110			-		-	rr
111			-		-	rr
112	**+**	**+**	**+**	**+**	**+**	**Rr**
113			-		-	rr
114	**+**	**+**	**+**	**+**	**+**	**Rr**
115			-		-	rr
116	**+**	**+**	**+**	**+**	**+**	**Rr**
117	**+**	**+**	**+**	**+**	**+**	**Rr**
118			-		-	rr
119	**+**	**+**	**+**	**+**	**+**	**Rr**
120	**+**	**+**	**+**	**+**	**+**	**Rr**
121	Dead					
122			+		+	RR
123	**+**	**+**	**+**	**+**	**+**	**Rr**
124	**+**	**+**	**+**	**+**	**+**	**Rr**
125			-		-	rr
126			+		+	Rr
127			-		-	rr
128			-		-	rr
129	**+**	**+**	**+**	**+**	**+**	**Rr**
130			-		-	rr
131	**+**	**+**	**+**	**+**	**+**	**Rr**
132	Dead					
133	**+**	**+**	**+**	**+**	**+**	**Rr**
134			-		-	rr
135			+		+	Rr
136			+		+	Rr
137	**+**	**+**	**+**	**+**	**+**	**Rr**
138			-		-	rr
139			+		+	

F$_2$:# (progeny)	Xus-OPK 9$_{1350}$-7el$_1$	XcslH 81-1-7el$_1$	Lr19	12c	Wsp-D1c	Genotype
142	+	+	+	+	+	Rr
143	+	+	+	+	+	Rr
144			-		-	rr
145	+	+	+	+	+	Rr
146			+		+	Rr
147			+		+	Rr
148			+		+	Rr
149			-		-	rr
150			+		+	Rr
151	+	+	+	+	+	Rr
152	+	+	+	+	+	Rr
153	+	+	+	+	+	Rr
154	+	+	+	+	+	Rr
155	+	+	+	+	+	Rr
156	Dead					
157			+		+	Rr
158			-		-	rr
159	+	+	+	+	+	Rr
160			-		-	rr
161			-		-	rr
162			-		-	rr
163			-		-	rr
164			-		-	rr
165			-		-	rr
166			+		+	Rr
167			+		+	RR
168			-		-	rr
169			+		+	Rr
170			-		-	rr
171	Dead					
172	Dead					
173			-		-	rr
174			+		+	Rr
175			-		-	rr
176	Dead					
177			-		-	rr
178			-		-	rr
179	+	+	+	+	+	Rr
180	Dead					
181	Dead					
182			-		-	rr
183			-		-	rr
184			-		-	rr
185			-		-	rr
186			-		-	rr
187	+	+	+	+	+	Rr
188			-		-	rr
189	+	+	+	+	+	Rr
190			-		-	rr
191	+	+	+	+	+	Rr

F₂ (progeny): #	Xus-OPK 9₁₃₅₀-7el₁	XcslH 81-1-7el₁	Lr19	12c	Wsp-D1c	Genotype
193			+		+	Rr
194	+	+	+	+	+	**Rr**
195	+	+	+	+	+	**Rr**
196			-		-	rr
197	+	+	+	+	+	**Rr**
198			+		+	Rr
199			+		+	Rr
200	+	+	+	+	+	**Rr**
201			-		-	rr
202			-		-	rr
203			+		+	RR
204			+		+	Rr
205			-		-	rr
206			-		-	rr
207	+	+	+	+	+	**Rr**
208	+	+	+	+	+	**Rr**
209	+	+	+	+	+	**Rr**
210			+		+	RR
211	+	+	+	+	+	**Rr**
212	+	+	+	+	+	**Rr**
213			+		+	RR
214	Dead					
215	+	+	+	+	+	**Rr**
216			-		-	rr
217			-		-	rr
218	Dead					
219			+		+	RR
220	Dead					
221			+		+	RR
222	+	+	+	+	+	**Rr**
223			+		+	Rr
224			-		-	rr
225			-		-	rr
226			-		-	rr
227	Dead					
228	+	+	+	+	+	**Rr**
229	+	+	+	+	+	**Rr**
230			-		-	rr
231			+		+	RR
232	+	+	+	+	+	**Rr**
233			-		-	rr
234			+		+	Rr
235	Dead					
236			+		+	Rr
237			-		-	rr
238			+		+	Rr
239			+		+	Rr
240			-		-	rr
241			+		+	Rr
242			+		+	Rr
243			+		+	RR

F$_2$ (progeny): #	Xus-OPK 9$_{1350}$-7el$_1$	XcslH 81-1-7el$_1$	Lr19	12c	Wsp-D1c	Genotype
245			-		-	rr
246			-		-	rr
248	Dead					
249	+	+	+	+	+	**Rr**
250			-		-	rr
251	+	+	+·	+	+	**Rr**
252			-		-	rr
253			-		-	rr
254			-		-	rr
255			+		+	Rr
256			+		+	Rr
257			+		+	Rr
258	+	+	+	+	+	**Rr**
259	+	+	+	+	+	**Rr**
260			-		-	rr
261			+		+	Rr
262	Dead					
263	+	+	+	+	+	**Rr**
264			+		+	Rr
265			+		+	Rr
266	+	+	+	+	+	**Rr**
267			+		+	Rr
268			-		-	rr
269			-		-	rr
270			-		-	rr
271			+		+	Rr
272			+		+	Rr
273			-		-	rr
274			+		+	Rr
275			+		+	Rr
276			+		+	RR
277	+	+	+	+	+	**Rr**
278			-		-	rr
279			-		-	rr
280			-		-	rr
281			+		+	Rr
282			+		+	Rr
283		+	+		+	RR
284	+	+	+	+	+	**Rr**
285			+		+	Rr
286			+		+	RR
287	+	+	+	+	+	**Rr**
288			-		-	rr
289	+	+	+	+	+	**Rr**
290			-		-	rr
291		-	-		-	rr
292			-		-	rr
293			-		-	rr
294			-		-	rr
295		-	-		-	rr
296			-		-	rr

F$_2$ (progeny): #	Xus-OPK 9$_{1350}$-7el$_1$	XcslH 81-1-7el$_1$	Lr19	12c	Wsp D1c	Genotype
297	+	+	+	+	+	Rr
298			-		-	rr
299	+	+	+	+	+	Rr
300	+	+	+	+	+	Rr
302	+	+	+	+	+	Rr
303		-	-		-	rr
304			-		-	rr
305			+		+	Rr
306			-		-	rr
307	+	+	+	+	+	Rr
308	+	+	+	+	+	Rr
309	+	+	+	+	+	Rr
310	+	+	+	+	+	Rr
311	Dead					
312			-		-	rr
313		-	-		-	rr
314	+	+	+	+	+	Rr
315	+	+	+	+	+	Rr
316	+	+	+	+	+	Rr
317	+	+	+	+	+	Rr
318	+	+	+	+	+	Rr
319	+	+	+	+	+	Rr
320	+	+	+	+	+	Rr
321	+	+	+	+	+	Rr
322	+	+	+	+	+	Rr
323			-		-	rr
324			-		-	rr
325			+		+	Rr
326			-		-	rr
327			-		-	rr
328	+	+	+	+	+	Rr
329	+	+	+	+	+	Rr
330			-		-	rr
331			-		-	rr
332			-		-	rr
333	+	+	+	+	+	Rr
334			-		-	rr
335			-		-	rr
336	+	+	+	+	+	Rr
337			-		-	rr
338	Dead					
339	+	+	+	+	+	Rr
340			-		-	rr
341			+		+	Rr
342			-		-	rr
343			-		-	rr
344	+	+	+	+	+	Rr

F_2 (progeny): #	Xus-OPK 9_{1350}-7el$_1$	Xcs IH81-1 7el$_1$	Lr19	12c	Wsp-D1c	Genotype
346			+		+	Rr
347			-		-	rr
348			-		-	rr
349			+		+	Rr
350			-		-	rr

ADDENDUM 2. F_3 segregation data of 123 Rr F_2 individuals (Cross 93M97)

F_2: #	F_3 segregation data R	S	Total	P	P(3:1)
100	26	24	50	0.854	0.000173
101	23	27	50	0.507	0
102	25	25	50	0.922	0
103	25	25	50	0.922	0
109	32	28	60	0.683	0.000106
112	30	31	61	0.813	0
114	9	16	25	0.142	0
116	10	15	25	0.285	0
117	30	44	74	0.081	0
119	45	45	90	0.895	0
120	30	39	69	0.230	0
123	44	37	81	0.514	0
124	34	35	69	0.813	0
126	40	35	75	0.643	0
129	34	35	69	0.816	0
131	35	35	70	0.907	0
133	14	14	28	0.941	0.00225
135	20	20	40	0.930	0.000261
136	19	21	40	0.686	0
137	18	22	40	0.471	0
139	35	35	70	0.907	0
140	37	31	68	0.540	0
142	25	20	45	0.514	0.002593
143	19	14	33	0.429	0.0208
145	30	25	55	0.568	0.00046
146	25	25	50	0.922	0
147	20	19	39	0.942	0.000625
148	20	19	39	0.942	0.000625
150	25	15	40	0.135	0.067889
151	20	20	40	0.930	0.000261
152	21	19	40	0.819	0.001015
153	22	18	40	0.585	0.003487
154	25	21	46	0.620	0.001217
155	18	27	45	0.151	0
157	25	20	45	0.514	0.002593
159	31	19	50	0.110	0.033763
166	32	32	64	0.911	0
169	25	26	51	0.811	0
174	60	46	106	0.224	0
179	34	36	70	0.722	0
187	25	25	50	0.922	0
188	28	23	51	0.548	0.000918
189	25	26	51	0.811	0
191	26	24	50	0.854	0.000173
193	25	25	50	0.922	0
194	23	27	50	0.507	0
195	20	24	44	0.487	0
197	17	25	42	0.185	0

F₂:	F₃ segregation data		Total	P	P(3:1)
#	R	S			
198	38	46	84	0.317	0
199	52	60	112	0.366	0
200	48	51	99	0.660	0
204	21	23	44	0.694	0
207	25	21	46	0.620	0.001217
208	18	26	44	0.194	0
209	20	23	43	0.583	0
211	19	26	45	0.256	0
212	31	19	50	0.110	0.033763
215	26	26	52	0.920	0
222	26	25	51	0.968	0
223	25	26	51	0.811	0
228	28	21	49	0.367	0.003892
229	19	29	48	0.124	0
232	34	46	80	0.143	0
234	33	37	70	0.552	0
236	29	26	55	0.763	0.000136
238	25	25	50	0.922	0
239	24	36	60	0.097	0
241	28	23	51	0.548	0.000918
242	27	26	53	0.971	0
249	23	27	50	0.507	0
251	31	19	50	0.110	0.033763
255	24	26	50	0.703	0
256	33	17	50	0.030	0.141645
257	22	28	50	0.344	0
258	26	26	52	0.920	0
259	31	28	59	0.777	0
261	38	12	50	0.000	0.870283
263	20	29	49	0.167	0
264	12	8	20	0.405	0.121335
265	12	9	21	0.555	0.058782
266	6	14	20	0.064	0
267	27	23	50	0.640	0.000605
271	25	25	50	0.922	0
272	24	25	49	0.810	0
274	27	24	51	0.748	0.000275
275	28	22	50	0.453	0.001918
277	25	25	50	0.922	0
281	26	24	50	0.854	0.000173
282	29	21	50	0.302	0.005502
284	31	20	51	0.150	0.019052
285	30	20	50	0.188	0.014306
287	21	28	49	0.272	0
289	31	19	50	0.110	0.033763
297	25	26	51	0.811	0
299	35	27	62	0.365	0.000744
300	24	27	51	0.603	0
301	27	24	51	0.748	0.000275
302	34	28	62	0.514	0.000246
305	26	25	51	0.968	0

F_2:	F_3 segregation data		Total	P	P(3:1)
#	R	S			
307	26	35	61	0.207	0
308	32	28	60	0.683	0.000106
309	30	30	60	0.914	0
310	30	30	60	0.914	0
314	33	27	60	0.505	0.000347
315	22	39	61	0.022	0
316	30	30	60	0.914	0
317	32	28	60	0.683	0.000106
318	35	25	60	0.237	0.002869
319	30	29	59	0.981	0
320	29	31	60	0.714	0
321	34	26	60	0.355	0.00104
322	33	27	60	0.505	0.000347
325	30	27	57	0.770	0
328	34	28	62	0.514	0.000246
329	31	30	61	0.985	0
333	26	34	60	0.254	0
336	34	26	60	0.355	0.00104
339	26	34	60	0.254	0
341	40	22	62	0.030	0.056597
344	36	24	60	0.149	0.00729
345	32	33	65	0.813	0
346	22	37	59	0.039	0
349	26	38	64	0.107	0
	3387	3294			