The identification of RAPD markers for the *Thinopyrum* derived *Lr19* translocation of wheat.

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November 1998

Thesis presented in partial fulfilment of the requirements for the degree Master of Natural Sciences at the University of Stellenbosch.

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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: 17/11/1998

ABSTRACT

Ongoing attempts to introduce novel traits in wheat necessitate the development of detailed genetic maps to facilitate these processes. *Thinopyrum ponticum* derived leaf rust resistance (*Lr19*) has been successfully incorporated as a terminal translocation onto chromosome 7DL of wheat (Sharma & Knott, 1966). Following the induction of allosyndetic pairing between the translocated segment and homoeologous chromosome arms, a shortened translocation designated *Lr19*-149, was recovered (Marais 1992c). A number of near isogenic lines (NILs) have been developed using the two forms of *Lr19* (Prins *et al.* 1997), as well as a set of 29 irradiation induced deletions of the original *Lr19* translocation (Marais, 1992a). These proved very useful in the process of mapping the chromosome region.

A poorly developed genetic map hamper attempts to study and modify the introgressed chromatin block. Polymorphic variation between a NIL and its recurrent parent can be detected on the molecular level making it possible to generate easily usable markers for mapping purposes. Since the Random Amplified Polymorphic DNA (RAPD) technique (Williams *et al.* 1990) requires very little knowledge of sequence information prior to its employment, and since its suitability has already been well demonstrated in a variety of species (Welsh and McClelland, 1990), it is a logical choice in the search for polymorphic markers.

In this study four *Lr19* RAPD markers have been identified, and mapped successfully and a further three repulsion phase markers for the corresponding wheat chromosome arm have been identified. The marker, *Xus-OPK15*$_{800}$*-7el*$_1$ is located the closest to the *Lr19* gene. This marker was used to confirm two putative *Lr19*-149 recombinants as *Lr19* derivatives and suggested that a third recombinant has apparently lost the marker and must be the most useful (shortest) recombinant.

OPSOMMING

Pogings om nuwe gene na koring oor te dra maak dit noodsaaklik om goed gedefiniëerde genetiese kaarte te ontwikkel om die proses te bespoedig. Die *Thinopyrum ponticum* verhaalde blaarroes weerstandsgeen, *Lr19*, is as 'n terminale translokasie op chromosoomarm 7DL van koring gevoeg (Sharma & Knott 1966). Nadat allosindetiese paring tussen die getranslokeerde segment en die homoeoloë koring chromosoomarms geïnduseer is, is 'n nuwe, verkorte translokasie herwin, naamlik *Lr19*-149 (Marais, 1992c). Naby isogeniese lyne (NILs) van die twee *Lr19* translokasies is in verskeie korings ontwikkel (Prins *et al*, 1997) asook 'n stel van 29 bestraling-geïnduseerde terminale delesies van die oorspronklike *Lr19* translokasie (Marais, 1992a). Laasgenoemde lyne kon nuttig gebruik word vir die kartering van die chromosoomarea.

'n Swak ontwikkelde genetiese kaart belemmer pogings om die spesie-verhaalde chromatienblok te bestudeer of om dit te verkort vir kommersiële gebruik. Polimorfiese variasie tussen 'n NIL en sy betrokke herhalende ouerlyn kan geïdentifiseer word op molekulêre vlak en sodoende is dit moontlik om bruikbare merkers te ontwikkel vir karterings-doeleindes. Aangesien die "Lukraak Geamplifiseerde Polimorfiese DNA" (RAPD) tegniek (Williams *et al*, 1990) baie min vooraf kennis van DNA volgordes vereis, en die bruikbaarheid van die tegniek goed gedemonstreer is in 'n verskeidenheid spesies (Welsh en McClelland 1990), is dit 'n logiese keuse vir die opsporing van nuwe, polimorfiese merkers.

In hierdie studie is vier *Lr19* RAPD merkers geïdentifiseer, en suksesvol gekarteer terwyl 'n verdere drie repulsiefase merkers met die verplaasde koring chromosoomarm geassosieer kon word. Die merker, *Xus-OPK15*$_{800}$-*7el*$_1$ is die naaste aan die *Lr19* geen. Hierdie merker is gebruik om twee vermoedelik verkorte *Lr19*-149 rekombinante te bevestig as van *Lr19* oorsprong. 'n Derde rekombinant is ook getoets en die data dui daarop dat dit waarskynlik die merker verloor het en dus die mees bruikbare (kortste) rekominant moet wees.

For my mother and father

ACKNOWLEDGEMENTS

I would like to thank the following people and institutions:

- Professor G.F. Marais for his support during the full term of this study and for supplying the seed stocks.
- Mrs A.S. Marais for providing some of the plant material.
- The Foundation for Research Development and the University of Stellenbosch for their generous funding.
- All the staff and students at the Department of Genetics for their contributions.
- My dear parents and brothers for their love and assistance during my studies.

ABBREVIATIONS USED

α	Alpha
µg	Microgram
µl	Microlitre
µM	Micromolar
°C	Degrees centigrade
A	Adenine
AMP	Ampicillin
BC	Backcross
Bps	Base pairs
C	Cytosine
cM	CentiMorgan
dA	Deoxyadenine
dATP	Deoxyadenosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidiumbromide
g	Gram
G	Guanine
H_3PO_3	Boric acid
HGP	Human genome project
IGH	Immunoglobulin heavy chain cluster
IPTG	Isopropylthiogalactoside
kb	Kilobase
LB	"Luria Bertani"
Lr	Leaf rust resistance locus
M	Molar
mA	Milliampere
Mb	Megabases
Mg	Milligram

$MgCl_2$	Magnesium chloride
ml	Millilitre
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
PB	Phosphate buffer
PCR	Polymerase chain reaction
pmol	Picomole
RAPD	Random amplified polymorphic DNA
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleicacid
Sd	Segregation distortion
SDS	Sodium dodecyl sulphate
STMS	Sequence tagged microsatellite site
STS	Sequence tagged site
Sr	Stem rust resistance locus
T	Thymine
TBE buffer	Tris borate EDTA buffer
Th.	*Thinopyrum*
Tm	Melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
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

1.1 INTRODUCTION

1.1.1 OVERVIEW

Wheat (*Triticum aestivum* L. em. Thell.) is cultivated across a wide range of climates and environments. It is the most adaptable of the grass species to extreme habitats and stress conditions. During cultivation, temperatures may vary from as little as 3°C to 4°C in higher latitudes and up to 30 to 32°C in equatorial regions, with an optimum of 25°C (Briggle, 1980). More land mass is being used for the cultivation of wheat than any other commercial crop in the world, and it serves as the most important food source for mankind, ranking higher than maize, rice and potatoes.

Wheat products are an excellent source of dietary nutrients such as carbohydrates, amino acids, minerals, starch, vitamins and fatty acids. It is, however, low in the essential amino acid, lysine. The consumption of grain in the early 1970's in third world countries stabilised at around 175 kg per capita (Lupton, 1987). This was attributed to changing preferences in the consumption of traditional foods and taste preferences for specific grain species, such as rice, maize and sorghum. Little or no wheat is produced in these countries. With the global population increasing, and more drastic population increases being expected in third world countries, food resource development encouraged by the developed countries will have to remain central to avoid famine. Presently, the supplies and imports to third world countries are mainly in the form of raw wheat quotas (CIMMYT, 1983). A great deal of effort is currently being employed to stimulate the production of wheat, particularly through the education and training of personnel and skilled labourers (Briggle and Curtis, 1987).

1.1.1.1 WHEAT IN SOUTH AFRICA

In South Africa the land area devoted to the production of wheat has increased steadily and averaged approximately 1 197 000 ha in the early sixties. It was estimated to be more than 1 800 000 ha in the 1980s (CIMMYT 1983, 1985). Wheat yield per hectare is generally low as a result of the dry climate. In areas of better rainfall, poor soils often result in low yields (Briggle and Curtis, 1987).

Globally, annual losses due to fungal, bacterial and viral diseases are substantial. Stem rust and yellow rust rank among the most destructive fungal diseases of wheat (CIMMYT 1978), whereas leaf rust and septoria generally result in less damage if not controlled. Stem rust and root diseases such as *Gaeumannomyces*, *Fusarium*, *Rhizoctonia* and *Periconia* spp. under favourable conditions can decrease yield by as much as 50%. Wheat aphids are probably the most harmful insect pests of wheat and may cause extensive losses.

Seeding generally occurs between April and August except for the Northern Transvaal, where the months February to March are best for planting. Here, harvesting is done in July. In the rest of the country wheat is harvested in November to January. South Africa is largely self sufficient with regard to wheat production.

Plant breeding dates back to when plants were first domesticated by mankind and plants selected on the basis of a variety of traits were used for planting, thus influencing the phenotypical and genotypical evolution of the species. With the introduction of molecular techniques (Williams *et al*, 1990; Welsh and McClelland, 1990; Caetano-Anollés *et al*, 1991; Hantula *et al*, 1996; Zietkiewicz *et al*, 1994) to facilitate classical and modern breeding strategies, a realisation of the vast contribution this could make to help solve the food crisis unfolded. Molecular technology can greatly speed up the process of trait oriented breeding and supplies incredibly powerful ways to reach this objective (Johnson, 1982).

1.1.1.2 WHEAT IN THE FUTURE

Increased wheat yield can be obtained by limiting the factors that influence wheat production negatively before and after harvests. The world population is estimated to reach between 8 and 10 billion people in the early 21st century (Briggle and Curtis 1987). Food production has to be doubled in the next 30-40 years to accommodate this growth. This can be achieved effectively only by means of:

i) The use of better agronomical practices.
ii) The expansion of the area devoted to grain production.
iii) The development of better wheat plants, i.e. with increased genetic yield potential, better disease resistance and increased tolerance to environmental stress.

1.1.1 CLASSIFICATION OF THE TRITICEAE

The annual grass genera *Triticum* and *Aegilops* were included along with five other genera in the tribe *Triticeae* by Linnaeus in 1753 with *Triticum* containing the cultivated wheat species and *Aegilops* encompassing the wild wheat relatives (Kimber and Feldman 1987). This was a purely artificial classification. Bowden (1959) reviewed it and included both *Aegilops* and *Triticum* in the genus *Triticum*. This classification was adopted essentially unchanged by Morris and Sears (1967). Further re-classification of the *Triticeae* was done by Löve (1982) in an attempt to have the taxonomical classification reflect phylogenic relationships within the tribe. This placed species that are genomically similar in the same genus. Problems with this approach included: i) What constitutes similar genomic constitutions? ii) How to choose names for the new and frequently monotypic genera. Presently, this classification has the widest acceptance, mainly since its basis is that of evolutionary relationships.

More than 100 of the perennial grass species used to be included in the very complex, heterogenous, and artificial genus *Agropyron* P. Beau vois (Pienaar, 1990). According to Dewey (1984), all species with genomes other than P should not be

included in *Agropyron*. Many former *Agropyron* species as well as those from other perennial genera were therefore reclassified according to the genomes they possess. A new genus, *Thinopyrum*, was proposed for the species having the J-genomes. *Thinopyrum* was derived from the Greek words meaning 'beach' and 'wheat', as this grass type is well adapted to the harsh conditions posed by coastal beaches. The species of the genus occur primarily on the shores of the Nordic, Mediterranean, and the Baltic seas where it grows in the exposed areas between the sand dunes and high water marks. The section *Thinopyrum* of the genus includes the *Thinopyrum junceum* complex, i.e. *Th. bessarabicum* (Savul and Rayss), *Th. distichum* , *Th. junceiforme*, *Th. junceum* and *Th. runemarkii*, of which *Thinopyrum distichum* is indigenous to the south western, southern and south eastern coastline of the Cape Province. As is the case with the most *Triticeae*, this grass species reproduces primarily by self pollination and has a brittle rachis (Pienaar *et al*, 1988).

The *Thinopyrum* section *Lophopyrum* consists of the *Thinopyrum elongatum* complex i.e. *Th. caespitosum*, *Th. curvifolium*, *Th. elongatum*, *Th. ponticum* en *Th. scirpeum* (Pienaar *et al*, 1988). These species do not grow exclusively in beach and coastal environments, but are also found in more inland habitats and also in the Middle East and European Russia. Another distinguishing factor is that these species are cross pollinating as well as self pollinating.

Trichophorae, the third section, is comprised of the *Thinopyrum intermedium* complex. The most important species include *Th. gentryi*, *Th. intermedium* and *Th. podperae*. This section, however, is cross pollinating and is adapted to conditions prevalent in the inland areas of Europe, the Middle East, and Central Asia (Pienaar *et al*, 1988).

1.1.2 WIDE HYBRIDISATION

The identification of genetic markers and the development of detailed genetic maps are essential for the application of trait-orientated breeding and the transfer of novel genes of importance to crops and species of interest. With advances in wide hybridisation techniques, it has become possible to transfer a wide array of genes

from alien plant species and genera to cultivated crops in order to engineer stronger plants. Embryo rescue, cytological manipulations and the application of molecular techniques are central to such transfers (Pienaar *et al*, 1977). Numerous useful genes occur in the *Thinopyrum* spp. These include genes for the perennial growth habit, resistance to fungal and viral diseases, salt tolerance, drought tolerance, etc. (Pienaar 1990). This approach for transferring foreign genes into wheat breeding lines has been the most successful (Devos and Gale, 1992). Several molecular genetic techniques have, however, also been developed for the transfer of resistance genes, or other genes to plants. The biggest advantage of these techniques is that it now becomes possible to transfer material across species boundaries. This has not been possible with wide hybridisation based approaches, since sexual compatibility remained central to its success. For this reason resistance and other favourable traits had to be identified in plants within the same genus, and this limited the amount of useful foreign genetic material available considerably. Vast wild hybridisation projects have been initiated internationally involving *Triticum aestivum* and an array of compatible grass species ranging from diploid to octaploid genera (Schachermayer *et al*, 1994; Vierling and Nguyen 1992; King *et al*, 1993). These efforts have been the only truly productive methods for transferring alien chromatin to wheat, since the complexity of the wheat genome, together with the gene dosage effect from the multiple genomes hamper molecular techniques in achieving this.

1.1.3 CHROMOSOME ENGINEERING

Common wheat is a hexaploid with genomes AABBDD and therefore its genetic material occurs in triplicate. This is of great advantage in chromosome engineering attempts, since it allows the wheat plant to tolerate the loss of chromosomes, as well as the addition thereof. While multiple genomes may mask chromosome losses, it may also mask the expression of alien genes introgressed in wheat, a problem which is often encountered in gene transfer attempts (Yu *et al*, 1991). The development of aneuploid lines used in chromosome engineering takes advantage of this ability of wheat to compensate for chromosomal loss (Gale and Miller, 1987). Sears (1954) has derived complete sets of aneuploid lines for wheat including monosomics, nulli-tetrasomics, telosomics etc, which facilitate attempts to achieve homoeologous

chromatin transfer through chromosome engineering. The various aneuploids may also be used very successfully to allocate genes to chromosomes and chromosome arms.

1.1.4 ALTERNATIVE METHODS OF GENE TRANSFER

Probably the most successful molecular system for transferring DNA into plant genomes, is the *Agrobacterium* system (Watson *et al*, 1992). This procedure utilises the Ti plasmid of *Agrobacterium* as a vector to deliver alien DNA to a host chromosome. Cereal cells have been infected successfully with the transfection system, but until recently the recovery of plants from the resulting callus has been problematic (Joshi and Joshi, 1991). Presently, transgenic plants can be recovered in wheat making use of the *Agrobacterium* system (Barcelo *et al*, 1998). Another method for alien DNA transfer is "shotgun transformation" or particle ballistics. In this application DNA coated onto the surfaces of heavy metal particles is shot into living cells. Protoplast fusion, micro-injection and electroporation, are other methods for achieving transformation (Watson *et al*, 1992). These methods are hampered by transient expression and the formation of chimeric tissues, which makes it difficult to recover stably transformed plants.

According to Gustafson (1984) the development of hybrids of compatible species is the only truly proven way to achieve successful genetic transformation in *Triticum*. This remained true until recently since limited success has been achieved with the use of molecular and transfection strategies (Joshi and Joshi, 1991). A survey by Barcelo *et al*, (1998) showed that *Agrobacterium* transformation and particle bombardment are being applied with growing success for the transformation of wheat. However, "chromosome engineering" is still the only realistic approach to the transfer of multigenic traits from wild species.

1.1.5.1 THE T4 TRANSLOCATION

The first hybrids of wheat with *Thinopyrum* was produced by Tsitsins, Vakar and Veruschkine in Russia in 1930 (Briggle 1980). Subsequent successes were obtained by Armstrong *et al.* (1937) in Canada, and Sando and Smith in the USA. Realising the potential benefits to wheat cultivars, a variety of *Thinopyrum* species have been included in hybridisation strategies with *Triticum*, with a great deal of success (Schachermayr *et al.* 1994; 1995). In 1957 a wheat/*Th. ponticum* translocation was induced by irradiation, and stem rust resistance found in backcross derivatives containing 42 chromosomes (Elliot, 1957). In a similar fashion Knott (1961) succeeded in transferring stem rust resistance gene *Sr2b* from *Thinopyrum ponticum* to chromosome 6D of wheat, also by irradiation. A wheat line 'Agatha' was produced by Sharma and Knott (1966) through irradiation induced translocation from *Thinopyrum ponticum*. This translocation is also termed the T4 translocation and carries the resistance genes *Lr19* and *Sr25*. The T4 translocation showed preferential transmission in heterozygotes caused by a segregation distortion gene, *Sd1*, mapped by Zhang and Dvorák (1990).

1.1.5.2 THE TRANSFER OF CHROMATIN FROM *THINOPYRUM DISTICHUM* TO *TRITICUM*

The primary hybrid between *Thinopyrum distichum* (Figure 1.1) and wheat is of no commercial use due to the detrimental effects of the complete *Thinopyrum* genomes. By backcrossing the F_1 amphiploid to the wheat parent, partial amphiploids with reduced numbers of alien chromosomes can be derived (Pienaar, 1990). Further backcrossing to wheat in association with selection for the trait of interest, may eventually produce wheat lines with monosomic and disomic additions of *Thinopyrum*. The latter may then be used to produce translocations to wheat chromosomes.

Successful hybridisation of common wheat cultivars 'Chinese Spring' and 'Inia 66' with *Th. distichum* (Pienaar *et al.* 1977) followed by colchicine treatment resulted in the production of allodecaploids. The resulting amphiploids, Inia 66/*Th. distichum* and Chinese Spring/*Th. distichum*, were backcrossed to the respective wheat parents. This was done to restore the *Triticum* genetic background, to reduce the number of detrimental *Th distichum* genes as well attempt to exploit any useful genes transferred. During these backcrosses the BC_2F_3 progeny produced two leaf rust resistant semi-dwarf plants. One of these plants was retained and used for further breeding (Pienaar *et al.* 1985) and later designated 'Indis'. Marais and Marais (1990) concluded that the resistance was due to a gene on chromosome arm 7DL. At this time it was believed that 'Indis' carried a translocated chromosome segment homoeologous to the T4 translocation derived from *Th. ponticum* by Sharma and Knott (1966).

Figure 1.1 *Thinopyrum distichum* plants found on the shores of south-western Cape.

Both the 'Indis' and T4 translocations occur on chromosome arm 7DL and carry genes for stem rust resistance (*Sr25*), yellow flour pigmentation (*Y*) and segregation distortion (*Sd1*) (Marais 1992b; Marais 1992c; Sharma and Knott, 1966; Kibirige-Sebunya and Knott, 1983). Marais (1992c) proposed the suffix "d" to distinguish the 'alleles' in 'Indis' from those in the T4 translocation. However, it was later shown that the 'Indis' translocation was not a new translocation, but rather the existing T4 translocation (Prins *et al*, 1996).

Since the *Lr19* translocation in 'Indis' behaved as a simple, large linkage block and did not recombine with homoeologous wheat chromatin during meiosis, deleterious genes could not be separated from *Lr19d* through normal crossing over during meiosis (Marais, 1990). The presence of the gene for yellow endosperm pigmentation meant that the resistance gene could not be used commercially. An attempt was therefore made by Marais (1992c) to induce homoeologous pairing between the *Lr19d* translocation and homoeologous areas of the wheat genome. Diploid pairing in wheat is regulated by genes present throughout the genome. Some of the genes (for example Ph_1 on chromosome 5B and Ph_2 on chromosome 3D) are known to suppress pairing

of the homoeologues while others may promote the pairing of homoeologues (Gale and Miller, 1987).

Marais (1992c) made use of the 'Chinese Spring' monosomic 5B stock (the *Ph1* gene occurs on chromosome arm 5BL) and the Chinese Spring *Ph1b* deletion mutant to produce plants that were *Lr19d* heterozygotes and at the same time lacked the *Ph1b* suppressor of homoeologous pairing. Among the testcross progeny a single recombinant that had lost the *Sd1*, *Y* and *Sr25* loci, yet had retained *Lr19*, was recovered. This recombinant was subsequently designated *Lr19* –149 and was found to have resulted from a double crossover. In the crossover event it got relocated to chromosome arm 7BL.

In the absence of meiotic pairing between the *Lr19* translocation and the 7DL arm of wheat, conventional recombination mapping was not possible. Marais (1992a) therefore made use of irradiation to produce a set of deletion lines for physical mapping of the translocation. A total of 29 homozygous *Lr19* deletion mutant stocks were derived. These had different terminal deletions of the translocation and proved very useful for producing a physical map of the translocation. Marais (1992a) and Prins *et al*, (1996) used the deletion lines to deduce the relative positions of a number of loci on the translocation as:

Centromere- *Sd1 – Xpsr165 - Xpsr 105 - Xpsr 129 - XcslH 81 - Xwg 380 - Xmwg 2062 - Lr19 - Wsp-D1 - Sr25 -Y.*

The complete *Lr19* translocation showed preferential transmission in heterozygotes (Marais, 1990). The degree of segregation distortion associated with its presence appeared to be conditioned by polygenes (responder) genes in the genetic background of a *Lr19* heterozygote. Zhang and Dvorák (1990) attributed this property to the presence of the *Sd1* gene on the translocation. Following the derivation of the *Lr19*–149 translocation and its physical mapping it became clear that it must have lost *Sd1*. However, *Lr19*–149 shows very consistent self-elimination depending on the genetic background of a *Lr19*–149 heterozygote. This suggested that the shorter translocation must have retained at least one further segregation distortion gene, *Sd2* (Prins and Marais 1998a).

In an attempt to further reduce the amount of *Thinopyrum* chromatin associated with *Lr19* –149, Marais and Marais (1998) once again produced *Lr19*–149 heterozygotes lacking the *Ph1b* homoeologous pairing control locus. Derivatives from these plants are being tested for recombinants. Three putative recombinants have been recovered thus far and designated *Lr19*-149-462, *Lr19*-149-252 and *Lr19*-149-299.

1.1.5.3 TRANSFER 10

Sears (1973, 1977) induced homoeologous pairing between wheat chromosome 7D and chromosome $7el_1$ of *Th. ponticum*, and obtained 12 recombinant lines (referred to as 'Transfer' 1 to 12). It is believed that these contain translocations of the *Thinopyrum ponticum* chromosome $7el_1$ (=7Ag) and chromosome 7D of wheat. In 1987 Eizenga showed that the resistance in 'Transfer 12' was associated with chromosome 7A, and Sears (1977) suggested that the yellow pigment gene (*Y*) is located close to *Lr19*. Sears (1977) estimated the relative sizes of the *Th. ponticum* segment in the recombinant chromosomes as 6>4>7 and 10>8 and 9>1,2,3 and 5. To achieve this, he employed two genetic markers in his analysis, the seed protein locus, *Pro* on chromosome 7DS, and the leaf rust resistance locus, *Lr19*, in $7el_1L$ (Sears 1973, 1977). Zhang and Dvorák (1990) also characterised the 'Transfer' lines and used the interspersed nucleotide sequence, pLeUCD2, (*Thinopyrum* specific) for this purpose. They constructed a map using this probe and also estimated the relative sizes of the alien segments retained in the recombinant chromosomes. They concluded, as did Sears, that 'Transfer 6' had the largest *Th. ponticum* segment and 'Transfer 4' the second largest. The remaining 'Transfer' lines were, however, characterised differently. Zhang and Dvorák (1990) estimated the sizes of the *Th. ponticum* segments as 6>4>1>3>2 and 5>8>7 and 9>10, and also mapped the segregation distortion gene (*Sd1*) proximal to *Lr19*. Prins and Marais (1998b) concluded that 'Transfer 10' had retained the smallest amount of *Thinopyrum* chromatin proximally of *Lr19*, also when compared to the *Lr19*-149 recombinant.

1.1.6 VIRULENCE TO *LR19*

The leaf rust resistance gene, *Lr19*, was transferred to wheat from *Agropyron elongatum* (Host) P. Beauv (=*Thinopyrum ponticum* in newer classification systems – Pienaar, 1990) (Sharma and Knott, 1966) and for a long time proved to be effective against all isolates of *Puccinia recondita* Roberge ex Desmaz f. sp. *tritici* tested in Mexico, Canada, the United States as well as thirty two other countries around the world. However, virulence to the *Lr19* gene has since been reported in some countries (Huerta-Espino and Singh, 1994). Two cultivars, i.e. 'Sunnan' from Sweden and 'Oasis 86' from Mexico, carrying the *Lr19* gene, were released in Mexico in 1986 and were grown mainly in the north-western states (Sonora and Sinaloa). Leaf rust pustules were identified on seedlings of Oasis 86 inoculated with stripe rust collected in Ceaya (central Mexico). These tests were performed under greenhouse conditions and it was found that the stripe rust inoculum was contaminated with leaf rust. 'Thatcher' near-isogenic lines, a *Lr19* mutant in 'Thatcher' *Lr19* (RL6040) lacking in the yellow flour pigment, and 'Oasis 86', were used in this experiment. It is believed that this pathotype evolved from a previously known pathotype, CBJ/QL, and it was designated, CBQ/QQ (Singh, 1991a; 1991b). Reports of virulence to *Lr19* has since also come from Eastern Europe (McIntosh, 1998, personal communication). However, *Lr19* remains a potentially useful gene that can be used in countries where its resistance has not broken down as well as in gene pyramiding attempts.

1.1.7 THE LEAF RUST PATHOGEN

The leaf rust disease is characterised by a small, round, brownish-red, noncoalescent pustules (uredia) found mainly on the leaves of infected wheat plants. This is caused by the leaf rust fugus. The name given to it in 1899 was *Puccinia triticina* Erikss., but it was later noted that it had already been characterised as *P. rubigo-vera* in 1882 by Winter, and is currently known as *P. recondita* Rob. ex Desm. This name was used primarily for the leaf rust pathogen in rye, but applies to wheat since it is essentially the same pathogen found in wheat. The *forma specialis* name *tritici* was added to the name *P. rubigo-vera* as means to indicate the specialisation of the fungus on wheat

and is used in a similar way with the new nomenclature *P. recondita*. The species *P. recondita tritici* is subdivided into numerous physiologic races depending on the varieties of common wheat it infects (Loegering *et al*, 1967; McIntosh *et al*, 1995).

Effects of infection by this pathogen includes the shrivelling of grain under severe conditions and a reduction in kernel size (Loegering *et al*, 1967). A reduction in the number of kernels per head leads to considerable losses of grain. A decrease in the protein content and an increase in carbohydrate content of the grain are also common during heavy infections. Infected plants require more water to survive due to an increased transpiration rate which leads to hastened maturity and weakened plants.

1.1.8 GENETIC MARKERS

Genetic markers used for tagging vary greatly in their nature and utility. These include heritable biochemical, cytological and morphological features associated with specific genotypes. 'Molecular markers', a subclass of biochemical markers, exceed cytological and morphological markers in abundance and are not limited by tissue specificity, expression levels or arduous biochemical detection techniques (Demeke *et al*, 1992).

Molecular markers are a potentially inexhaustible source of markers for the generation for detailed genetic maps and the tagging of genes whose effects are hard to distinguish. With the advances made in DNA technology, polymorphisms based on differences in protein or DNA has become increasingly important in fields of research including plant breeding, genetics, ecology, phylogeny and taxonomy (Weising *et al*, 1995). Protein based markers, such as the conventional allozyme markers, have the disadvantage that they detect gene product differences rather than differences in the DNA template itself. Allozyme marker methodology cannot provide markers for the largest portion of the DNA found in complex organisms, namely non-coding DNA sequences. DNA based markers are unbiased to the target template and thus can generate a potentially large number of polymorphisms associated with a trait of interest (Clegg, 1989). Molecular markers should have specific characteristics in order to be useful in molecular laboratories. Weising *et al* (1995) concluded that presently no marker system fulfil all the properties sought in an ideal marker. The characteristics most sought after in markers are:

- They should be highly polymorphic.
- They should have co-dominant inheritance (which allows distinction of homo- and heterozygotes in diploid organisms).
- They should be abundant in the genome.
- They should be evenly distributed throughout the genome.
- They should have a selectively neutral behaviour (i.e. no pleiotropic effects) in all plant stages.
- They should be easily accessible (i.e. by purchasing or fast procedures).

- They should involve easy and rapid assays (e.g. by procedures amenable to automation).
- They should be highly reproducible.
- They should allow for easy exchange of data between laboratories.

1.1.8.1 PCR TECHNIQUES

The development of techniques to amplify DNA *in vitro*, revolutionised all search mechanisms available to the molecular scientist. RFLP has remained the premier technique for identifying polymorphisms in DNA sequences (Ahn *et al.* 1992). Its complexity and laborious nature, as well as the level of expertise required, often limited its application and use in extensive crop improvement programmes. The polymerase chain reaction (Mullis and Faloona, 1987; Saiki *et al.* 1985; 1988; Ochman, 1988) led to the development of many related amplification strategies for the detection of polymorphic DNA sequences. These techniques have had an impact on numerous disciplines, including forensics, medicine, population genetics and molecular biology (Weising *et al.* 1995).

The PCR technique is an *in vitro* method whereby genomic DNA sequences of a target genome is enzymatically amplified as directed by highly specific oligonucleotide primers, typically in the order of 20 base pairs. These primers are synthetically produced and the sequences chosen from specific regions within the target DNA. Amplification of the area between two primers leads to the generation of multiple copies (i.e. amplification) of the targeted template sequence. Investigation of the amplified fragment makes it possible to detect variation in the region between the annealing primers and thus differences in banding patterns, often visualised by gel electrophoresis and essentially creating a genetic polymorphism (Skolnick and Wallace, 1988). PCR based protocols are less sophisticated screening methods than RFLP technology and allows safe, efficient and fast screening of large populations. PCR techniques also require less DNA and highly pure template DNA is not a preliminary requirement for the reaction to be successful. The procedure is largely automated and is less labour intensive. This often allows a larger number of samples

to be tested at a fraction of the costs involved with conventional RFLP analysis. Radioactive materials are also not required.

One of the most limiting aspects of the PCR technique is that prior DNA sequence information is required. Several adaptations have, however, been made to circumvent this requirement i.e. AP-PCR ("arbitrary primed PCR") (Welsh and McClelland 1990), RAPD-PCR (Williams *et al*, 1990) and DAF (Ceatano-Anollés *et al*, 1991). Further variations include cases where semi-random primers were used in conjunction with specific primer sequences in plants (Weising and Langridge, 1991). Another limitation of PCR is the inability of the polymerase enzyme to synthesise very long sequences. Cheng *et al*, (1994) and Cohen (1994) introduced "long-PCR" as an alternative strategy to address this limitation. This can greatly increase the size of the amplification product and increases the likelihood to detect a polymorphism (Ye *et al*, 1996).

Several PCR related marker techniques followed the development of a reliable and thermostable PCR reaction and are widely used in molecular biology i.e. microsatellites (Hughs and Queller, 1993; Queller *et al*, 1993), restriction enzyme digestion of amplified products (Tragoonrung *et al*, 1992, Konreczny and Ausubel, 1993) and AFLP ('Amplified Fragment Length Polymorphisms") (Bassam *et al*, 1992). The digestion of amplified products has allowed another means of detecting base pair polymorphisms in amplified fragments. This procedure is also known as CAPS ("Cleaved Amplified Polymorphic Sequences"). The PCR technique has also proven invaluable in gene cloning strategies, i.e. inverse PCR (Triglia *et al*, 1988) and anchored PCR (Frohman *et al*, 1988; Loh *et al*, 1998).

1.1.8.2 RANDOM PRIMER SEQUENCE BASED PCR

The ability to amplify sequences from multiple amplicons in a target genome addressed one of the major disadvantages when employing the polymerase chain reaction. Caetano-Anollés (1994) termed this group of techniques "multiple arbitrary amplicon profiling (MAAP)". MAAP distinguishes itself from PCR by using limited base pair primers i.e. 5 bp (DAF) to 10 bp as is the case in the RAPD reaction. This

greatly reduces the primer specificity during the *in vitro* reaction, and results in fingerprints characteristic to certain primer and DNA template combinations. This has aided forensic sciences greatly and now allows this technique to be used with anonymous genomes and DNA of questionable origins. The following methodologies are MAAP techniques:

- Random amplified polymorphic DNA (RAPD : Williams *et al,* 1990)
- Arbitrarily primed PCR (AP-PCR : Welsh and McClelland 1990).
- DNA amplification fingerprinting (DAF : Caetano-Anollés *et al,* 1991).
- Random amplified microsatellites (RAMS : Hantula *et al,* 1996).
- Random amplified microsatellite polymorphisms (RAMP : Zietkiewicz *et al,* 1994).

MAAP techniques are universal in their application and each varies with regard to the number of DNA amplification products (and thus loci amplified) and characteristic fingerprints generated. RAPDs will be discussed as it is the technique utilised in this study and found to be the most versatile, informative and cost effective for the purpose.

1.1.8.3 RAPD ANALYSIS

The RAPD technique has contributed to the construction of genetic maps in areas where RFLP and conventional biochemical analyses failed to produce ample polymorphisms. Low levels of polymorphic variation are generally found in wheat using the conventional techniques and the problem was partially solved by RAPD analysis. RAPD assays have proven exceptionally useful in the taxonomic and comparative disciplines. Genetic variation can be measured on a regular basis exclusively with the aid of RAPDs. It has acclaimed status as a reliable technique for universal DNA fingerprinting and has also been used in specific gene targeting strategies (Gu *et al*, 1995). This methodology uses arbitrary nucleotide primers of 9 to 10 base pairs in length and amplifies random DNA segments from genomic template DNA. Thermal cycles and thermostable DNA polymerase enzyme are used similar to conventional PCR (Innis *et al*, 1990). The use of oligonucleotide primers removes the need for knowledge of specific primer sequences and provides a potentially unlimited number of markers. Another determining factor is that primer template annealing temperatures are considerably lower than that used in PCR reactions, namely 36 °C. This produces a spectrum of amplified products in the range of 0.5 to 2.5 kb. These products can also be used as probes in Southern hybridisation, and most other blotting strategies, i.e. *in situ* hybridisation. Depending on the primer and template combinations, the spectrum of fragments created varies uniquely, producing a specific repeatable fingerprint. After separating the products on a high percentage agarose gel, the product bands are visualised, either by means of ethidium bromide, or silver staining (polyacrylamide gel electrophoresis). The fingerprints created are used to detect polymorphisms (Williams *et al*, 1990; Welsh and McClelland, 1990).

Issues of importance in RAPD fingerprints include i) band intensity, ii) background artifices due to DNA degradation in the gel matrix, and iii) miss-amplification due to the lack of a proof reading mechanism in *in vitro* reactions. The presence of lighter bands in the fingerprints is speculated to be attributable to multiple copies of the fragment present during the initial stages of the reaction (Caetano-Anollés *et al*, 1991). A more satisfactory explanation was put forward by Thorman *et al*, (1994),

stating that these differences are not due to multiple template copies in the template DNA, but rather determined by the degree of homology between the primer and the template DNA. Sequencing of RAPD products (Vengupal *et al.* 1993) subsequently proved the latter theory. A study by Fukuoka *et al.* in 1992 suggested that it is possible that products may be amplified even if only one primer site is available on one end of the amplified DNA fragment. Excessive cycling during the reaction is also known to produce DNA smears and aberrations in the generated DNA fingerprint. It is known that the amplification pressure and the low stringency primer annealing nature of the RAPD reaction encourages amplified products to double as primers after the available primer concentrations have been depleted in the RAPD reaction (Bell and DeMarini, 1991). This leads to the annealing of products in such a manner during excessive cycles as to mimic primer behaviour and leads to the production of excessively long RAPD products of a variety of lengths.

Polymorphisms in RAPD reactions are induced by:

i) Single of multiple nucleotide changes preventing primer annealment to the template DNA and preventing amplification of the specified amplicon.
ii) Deletion of a template binding site.
iii) Insertions resulting in amplification sites exceeding the maximum distance that the enzyme is capable of amplifying.
iv) Deletions or insertions in the amplified fragment effectively changing the product size.

RAPDs are inherited in a Mendelian fashion and are therefore, traceable in progeny. RAPDs are dominant markers and are unable to distinguish among homozygotes and heterozygotes. This shortcoming of the RAPD technique has not made RAPDs a less used marker system, but rather its simplicity and fast methodology has made it popular in studies to detect polymorphisms among NILs, RILs and other homozygous or bulked populations (Martin *et al.* 1991). Evidence has, however, been supplied to question the Mendelian mode of inheritance of RAPD markers (Echt *et al.* 1992). A disadvantage of RAPD polymorphisms is that they are less informative for the determination of recombination frequencies than the conventional RFLP marker

system. It is therefore necessary to standardise RAPD parameters and to proceed only when they are producing optimal and repeatable results.

1.1.9.1 REPEATABILITY OF THE RAPD REACTION.

After an intensive study of the repeatability of RAPDs in rye (*Secale cereale* L.), Iqbal and Rayburn (1994) concluded that despite the high level of heterozygosity in the crop, RAPDs can indeed be reliably and effectively used to rapidly type segregating populations. A similar study (Hu and Quiros, 1991) with broccoli and cauliflower illustrated that RAPD profiles remain constant within a specific cultivar, but varies among different cultivars of the same species. In Iqbal and Rayburn's study (1994), the repeatability on their results was constantly emphasised. The reproducibility of the RAPD reaction is determined to a large extent by the concentration of the components in the reaction mix, such as the target DNA, magnesium chloride, primers, buffer, dNTP concentrations, as well as the thermal cycles employed. The RAPD reaction is also known to be influenced by competition for predominant template sequences (Halldén *et al*, 1996) and genotyping errors from this source may be as high as 14%. This does, however, correlate with the stringency of the polymerase chain reaction (Williams *et al*, 1990; Welsh and McClelland, 1990). Variation between laboratories and laboratory equipment has been known to pose problems for the reproduction of RAPD results. In studies comparing RAPDs and RFLPs in wheat, it was found that the levels of polymorphism among the two marker systems are very similar (He *et al*, 1992).

1.1.9.1.1 THE TARGET GENOME

Common (bread) wheat belongs to the tribe Triticeae Dumort of the *Gramineae* or grass family. The tribe includes 15 genera among which are the major agricultural crop species as well as a wide variety of wild grass species. Allohexaploid wheats such as *Triticum aestivum*, have 42 chromosomes (2n=6x=42) which are organised into 3 homoeologous genomes, namely A, B and D. Bennet (1972) estimated that the average wheat somatic cell contains as much as 18.1 picograms of genomic DNA. This is equal to approximately 16 x 10^6 kb with an average of 760 Mb per chromosome. Wheat chromosomes differ minimally in their relative sizes which makes it difficult to distinguish chromosomes during banding and cytological studies.

It is estimated that more than 75% of the wheat genome consists of high copy numbers or repetitive sequences (Smith and Flavell, 1975) which hampers genetic mapping of the genomes. Polyploidy, the large genome sizes and the generally low levels of genetic polymorphism has resulted in wheat being one of the most poorly mapped crop species (Hart, 1994). The number of RAPD fragments generated during an amplification reaction has proved to be unrelated to the complexity of the genome studied. This is due to the fact that competition for the available primer binding sites in the genome plays a bigger role than the total number of binding sites available therein (Rafalski *et al*, 1991). According to Sambrook *et al*, (1989) it is possible to estimate and predict the number of primer binding sites by means of a mathematical formula : $N = (1/4)^L C$. In the formula N = the number of primer sites, with the oligonucleotide primer length *L* as determined on a single DNA strand of the given genome with complexity *C*. Both the primer and template concentration influence the RAPD reaction quantitatively, and are critical in determining the number of fragments produced. It is not merely a question a increasing the concentrations of these variables and thus increasing the number of fragments generated, since fragments produced from a low template concentration often disappear when the same reaction is being completed with higher template and primer concentrations. This is in contrast with conventional PCR reactions where a quantitative effect on the amount of PCR product synthesised, can be induced by increasing the template and primer concentrations (Muralidharan *et al*, 1993)

Modern wheat mapping strategies are making good use of aneuploid mapping populations and NIL populations for effective gene tagging and mapping (Sears and Sears, 1978). Aneuploid stocks i.e. monosomic-, telosomic-, substitution-, addition and nullisomic lines, are available for chromosomes and chromosome arms (Sears, 1954; 1966; Sears and Sears, 1978) for rapid gene localisation. Of similar importance is comparative mapping strategies which rely on well mapped populations in related crops such as rice and maize to identify illusive and quantitative traits that could previously not be mapped (Briggle, 1980). The development of deletion aneuploids in wheat making use of the mutagenic effect of a *Triticum sharonense* addition chromosome has also been extremely useful for the mapping of a variety of genes (Gill *et al*, 1996a; 1996b; Werner *et al*, 1992).

1.1.10 INFLUENCES OF REAGENTS

1.1.10.1 THE PRIMER

Since no prior knowledge of the target genome is necessary for the development of primers, these can be obtained commercially as pre prepared kits (OPERON Technologies Inc.). Different primer base sequences lead to the generation of different DNA fingerprints. Changes made to the 5' side of the primer do not have a significant effect on the amplified profile, and therefore the first 8 nucleotides in a 10-mer primer form a domain which is mainly responsible for the profile generated. Changes made to the 3' side is the determining factor in the amplification reaction (Caetano-Anollés *et al,* 1992). In a study by Fukuoka *et al,* (1992) it was found that a high G + C content in the RAPD primer leads to a larger number of amplified products. Preference for G and C bases over A and T in the base pair makeup of the primer is expected to achieve this. Primers with a 60 to 80% G + C content are expected to produce more bands after gel analysis (Williams *et al,* 1990). This is explainable because the G + C base pair combinations employ three hydrogen bonds for binding with the template, thus generating a higher affinity than the A + T combination for the template DNA (Fukuoka *et al,* 1992).

It is also possible to use two RAPD primers simultaneously. The result, however, is not the sum of the products generated when the primers are used individually, but rather a new RAPD profile. This is to be expected as interaction and the sharing of intermediate products now occur. It has, however, been noted that only a small amount of products are being shared in such a fashion. This is explainable by the fact that each primer uniquely amplifies an area of the genome and the levels of interactions of such amplification events are largely dictated by the properties of the DNA template (Kaemmer *et al,* 1992; Caetano-Anollés *et al,* 1992). As is the case with the other reaction components in the RAPD reaction, primer concentration also has a crucial role in the amplification reaction. Higher primer concentrations lead to the amplification of smaller fragments as well where the opposite is usually true in the case where lower quantities are used (Ellsworth *et al,* 1993). This merely emphasises

the need to fully optimise the RAPD reaction for the generation of duplicable reaction results.

1.1.10.2 MAGNESIUM CHLORIDE CONCENTRATION

After extraction of DNA from somatic cells by means of most mini prep protocols, a small amount of magnesium chloride ions remains in the DNA solution. This is usually due the incomplete removal and precipitation of various metallic salts naturally present in the original tissue. High purity of the DNA template is therefore important and often plays a crucial role in the subsequent amplification reaction. $MgCl_2$ concentration in the reaction also has a large influence in the reaction mixture (Muralidharan *et al*, 1993) and is often the easiest way to quickly alter the quality and quantity of band fragments produced. It is speculated that $MgCl_2$ enhances the template primer binding reaction and increases the specificity thereof. The large effect of $MgCl_2$ on the RAPD reaction often causes reproducibility problems and it is for this reason that its concentration is crucial and should be kept to a minimum.

1.1.10.3 THERMAL CYCLES

The temperature at which the amplification reaction will be done is largely dependent on the type of polymerase enzyme being used in the reaction, since most enzymes have a specific half life and all the enzyme activity will be lost after a certain number of cycles. The composition of the genomic DNA involved and the length of the primers also determine the optimum temperatures (Welsh and McClelland, 1990). This is possibly due to the slower renaturing and denaturing of more complex DNA types. Higher annealing temperatures result in more specific template to primer binding. The first standard program was produced by Williams *et al*, (1990) : 45 cycles at 94°C for 1 min, 36 °C for 1 min (annealment) and 2 min at 72°C for elongation of the annealed primer.

1.1.11 APPLICATIONS OF RAPDS IN WHEAT

RFLP analysis has been applied with great success (Rogowski *et al.* 1991) in the tagging of inherited traits in wheat or chromosome segments derived through introgression. It also simplifies the construction of well developed genetic maps (Koebner *et al.* 1986). The method, however, is laborious and requires specialised equipment and hazardous chemicals (e.g. radioactive materials). PCR approaches only require primers designed to amplify specific amplicons as in the case of the well-known gamma-gliadin gene in wheat (D'Oridio *et al.* 1990) and the alpha amylase sequences (Weining and Lanridge, 1991) for the detection of useful polymorphisms. The RAPD method utilises arbitrary primers (Welsh and McClelland, 1990; Williams *et al.* 1990) and is currently being employed by an increasing number of established researchers. This method avoids the tedious costly strategies used by traditional RFLP technology.

1.1.12 ADVANTAGES AND DISADVANTAGES OF RAPD ANALYSIS.

In summary, the greatest advantages of the RAPD reaction methodology are its simplicity and the ability to rapidly screen large numbers of DNA samples. RAPDs are not dependent on expensive equipment and can be performed in any laboratory. Radioactive materials, such as those used with conventional RFLP techniques, are avoided and the screening process can be performed in a fraction of the time necessary to a complete a RFLP analysis. RAPDs conform to a totally random fashion of generating amplified fragments, leading to its widespread use for detecting polymorphisms in areas where genomes may be devoid of biochemical markers or restriction sites. There is almost no limit to the number of markers that can be generated by using RAPDs, since an almost inexhaustible number of primer sequence combinations can be generated for use in the detection screening. One of the problems associated with the use of a RAPD marker is its dominant nature and inability to distinguish between homo- and heterozygous genotypes. With the use of NIL screening populations, it may be possible to identify repulsion phase markers, which can be used in a complementary fashion to overcome this shortcoming, but this has not found widespread use. The conversion of RAPD markers into co-dominant

STS or SCAR (Blake *et al.* 1996) markers remains a powerful alternative to the use of RAPDs, and can enhance its utility in MAS considerably. Reproducibility is unarguably a primary shortcoming of the RAPD technique.

1.1.13.1 SEQUENCE CHARACTERISED AMPLIFIED REGIONS (SCARS)

The dominant nature of RAPD markers coupled with low reproducibility has led to the development of SCAR (Sequence Characterised Amplified Regions) markers. These are often no more than extended RAPD primers for use in a PCR reaction. The most favourable attribute hereof, is that this allows amplification of a specific locus in a given genome, and unlike the RAPD reaction, may be performed under much less optimised amplification conditions (Michelmore *et al.* 1994). They are derived by isolation of a RAPD fragment and its cloning into a vector for sequencing. RAPD fragments are usually of such length that it may not be possible to sequence the entire fragment. It is, for the construction of SCAR markers, only necessary to sequence the ends on the cloned fragment. This allows for the development of two longer PCR primers (now termed SCAR primers), to specifically amplify the region identified by the RAPD marker. These markers are free of the problems associated with the RAPD technique and are often co-dominant. Co-dominant markers make it possible to distinguish between homozygous and heterozygous genotypes. Allelic differences in the amplicon arise when chromosomal changes have occurred within the region flanked by the SCAR primers, base pair additions or deletions. The development of this technique has made the use of RAPDs a much more appealing strategy for the development of molecular markers closely associated with traits of interest.

1.1.13.2 ALLELE SPECIFIC ASSOCIATED PRIMERS (ASAP)

Gu *et al.* (1995) introduced a marker technique similar to SCARs, namely ASAP (allele specific associated primers). These markers associate exclusively with a specific allele and are generated by using very stringent amplification conditions. The main advantage with ASAPs is that it eliminates the need to perform gel electrophoresis analysis of the PCR reaction after its completion. Samples are treated

directly with ethiduim bromide (or similar DNA staining substance) and visualised under UV light. A positive sample would thus produce amplified DNA and a negative sample no amplification product. The stringent nature of the procedure does not allow contamination to be amplified readily, but negative control reactions are advisable since they may lead to false positive results. This technique allows the rapid screening of extremely large numbers of samples at very low costs.

1.1.13.3 SEQUENCE-TAGGED-SITE (STS) MARKERS

Sequence-tagged site markers are the most useful marker type for gene mapping. It involves a set of specific oligonucleotide primers used in a PCR reaction. The principle is similar to SCARs, except that the targeted area occurs nowhere else in the genome or genomic library. Converted RAPD markers are preferably labelled SCAR markers because of the difficulty in identifying the origin of the marker. STSs were originally conceived as DNA landmarks used in the Human Genome Project (Green *et al*, 1990). The reliability and specificity of this marker type has led to substantial STS maps being created for several organisms. STS markers have further use in that it is possible to use the PCR amplified fragments as probes by radioactively labelling them. This has the advantage that fresh single copy probe stocks can be generated whenever needed, and because of the longer length of the fragment, it saves in costs associated with the synthesis of long molecular probes. DNA clones constructed from RFLPs (after Southern blot analysis) (Saiki *et al*, 1985) have been used extensively in mapping studies (Litt and Luty, 1989; Tautz, 1989; Weber *et al*, 1989). STS-PCR markers were successfully introduced in crop species by Larson (1996). RFLP markers were converted into STS-PCR markers that were co-dominant and allelic. Cloned fragments from the PCR reactions were also sequenced from the flanking ends and used for designing primer sets to reamplify the fragments *in vitro*. This was used successfully to distinguish between certain wild type and cultivated rice species. The resultant amplified fragments were of similar size compared to the ones cloned and its polymorphic nature provided proof of this technique's success. STS primer sets have also been applied in comparative mapping strategies (Talbert *et al*, 1996) and have proven particularly useful in evolutionary studies of the origins of several grass species. Further advances made will likely include the use of STS primer sets

developed for genes already mapped in simpler genome species i.e. the rice genome, which has a well developed genetic map, to find the corresponding gene in less well mapped crop species (Blake *et al*, 1996). The generally easy conversion of classical RFLP markers to their less laborious STS counterparts in both the barley and wheat genomes will increase the availability of STS-based maps in the future.

1.1.13.4 CLEAVED AMPLIFIED POLYMORPHIC SEQUENCE (CAPS)

Sometimes the PCR products of STS primer sets are not polymorphic. The use of restriction enzymes to differentially digest these amplified products and thus produce polymorphic sequences has proven valuable in situations like these. This polymorphism type was termed a Cleaved Amplified Polymorphic Sequence (CAPS) and combines the advantages of RFLP based analysis with that of PCR based techniques. A large number of restriction enzymes are currently available, which facilitates the detection of RE based polymorphisms (Rafalski *et al*, 1993).

1.2 ENRICHMENT STRATEGIES

Hydroxyapatite chromatography is used primarily as a way to prepare radiolabelled cDNA probes, to prepare subtracted cDNA libraries, to isolate DNA from low melting temperature agarose (Wilkie and Cortini, 1976) and to remove contaminants from DNA. Calcium residues and hydroxyapatite binds the phosphate groups of the nucleotide backbone of DNA molecules (Sambrook *et al*, 1989). By means of phosphate buffer elution, it is possible to remove these bonded molecules selectively. Single-stranded nucleic acids bind less strongly than double-stranded DNA and can thus be removed at lower phosphate concentrations at elevated temperature, i.e. 60°C. This makes it possible to separate single and double stranded DNA, as well as DNA:RNA hybrids from single stranded molecules.

One of the major drawbacks of this technique is the low concentrations of the eluted DNA, as well as it being suspended in phosphate buffers of high ionic strength. Ethanol precipitation of the nucleic acids will result in simultaneous precipitation of

the phosphate ions in the solution, making recovery of the DNA almost impossible. In the past, these ions were removed by means of extended dialysis and led to the loss of the major part of the DNA sample against the wall of the dialysis tube. Sambrook *et al.* (1989) produced a simple way of circumventing this, i.e. by precipitation of the nucleic acids by 2-butanol and then desalting the samples by chromatography through Sephadex G-50 columns.

For optimum cleanness of single- and double stranded DNA, hydroxyapatite columns should be maintained at 60°C. Acceptable results have, however, been achieved at room temperatures in cases where small columns were utilised, i.e. columns prepared in pasteur pipettes and plugged with siliconized glass wool. Hydroxyapatite chromatography is more successful in cases where the nucleic acids are of a length short enough to successfully bind and still pass through the column during the phosphate buffer elution. Large DNA strands, like those found in isolates of genomic DNA, are known to be lost due to the sheer size and difficulty in passing through such columns. Sonication of genomic DNA has proven effective in eliminating this problem.

As a strategy for enrichment of DNA for low copy sequences, re-annealment kinetics can be used to selectively remove repetitive nucleic acid sequences from genomic DNA at elevated temperatures. Re-annealing at 60°C over a 24 hour period, would have a C_0t value (log value of moles of nucleotides per litre x the re-annealing time in seconds) of more than 100 (Smith *et al.* 1975). Separating the resulting single- and double stranded DNA will thus enable analysis of a DNA fraction suspected to carry more coding sequences, and making the identification of genes in this enriched DNA more likely. The use of the RAPD methodology in conjunction with enriched DNA has proven successful for amplifying DNA sequences within the coding regions of the genome. The large amount of repetitive sequences contained in the wheat genome makes it a suitable candidate for the use of such enrichment strategies.

1.3 MAPPING OF MOLECULAR MARKERS

The generation of comprehensive genetic maps lies central to marker-assisted selection (MAS) in breeding. The optimum genetic map should contain markers evenly spaced across the entire genome and include as many markers as possible (Young, 1994). Map and sequence data promises a wealth of new knowledge about biological processes and the engineering thereof for the benefit of mankind. The development of molecular marker technology has made an almost infinite recourse of markers available for the creation of such detailed genetic maps. QTL mapping and chromosome characterisation is also dependent on the successful mapping of genomes (Young, 1994). Evolutionary studies in comparative mapping has endless benefits from understanding various genomes and the investigation of protein evolution between species to understanding disease and even mental disorders in humans. Understanding disease genes will lead to the unravelling of their pathologies and ultimately suggest new rationales for treatment and correction. In the past, mapping was limited in extent, but this has escalated to a global realisation of the importance thereof and collaboration between laboratories has since set off an unparalleled growth in this area of research. For this reason, most crop species' genomes (Schwarzacher, 1994) are now used in extensive mapping activities.

1.3.1 NEAR-ISOGENIC LINES (NILs) AND TRUE BREEDING POPULATIONS

Special mapping populations have been used extensively for finding markers closely linked to specific traits in plants (McMullen and Louie, 1989; Ahn *et al.* 1992; Yu *et al.* 1991; Hauge *et al.* 1991; Schüller *et al.* 1992; Hartl *et al.* 1993; Williams *et al.* 1994). Near-isogenic lines were used by Young *et al.* (1988) as one of the first breeding population strategies to map genes of interest to certain chromosomes. These NIL populations are created by continuous backcrosses. Backcrossing and eventually selfing, lead to the fixation of the gene of interest after five to six generations and result in low levels of heterozygosity. This inbreeding, or positive assortative mating will always increase homozygosity in the population where it is in

induced. Unlike trait-orientated selection, which affects only those genes on which the selection is based (and the genes in linkage disequilibruim along with them) this affects all genes.

Pairs of NILs are constructed such that the backcross-derived NIL will be genetically similar to its recurrent parent counterpart except for the chromosome segment introgressed therein. Since the degree of heterozygosity is so small in these NIL pairs, it can be expected that any polymorphism identified by a random marker strategy, is most probably linked to the targeted introgressed segment. The development of homozygous lines derived from doubled haploid genotypes and recombinant inbred lines (RILs) (Burr *et al*, 1988) are alternative strategies for achieving this.

Due to RAPDs being dominant markers, it makes for a good choice of marker identification system to use in conjunction with NIL populations. The leaf rust resistance genes, *Lr9* and *Lr24* in wheat, were tagged using both RFLP and RAPD markers and NIL populations (Schachermayr *et al*, 1994; 1995).

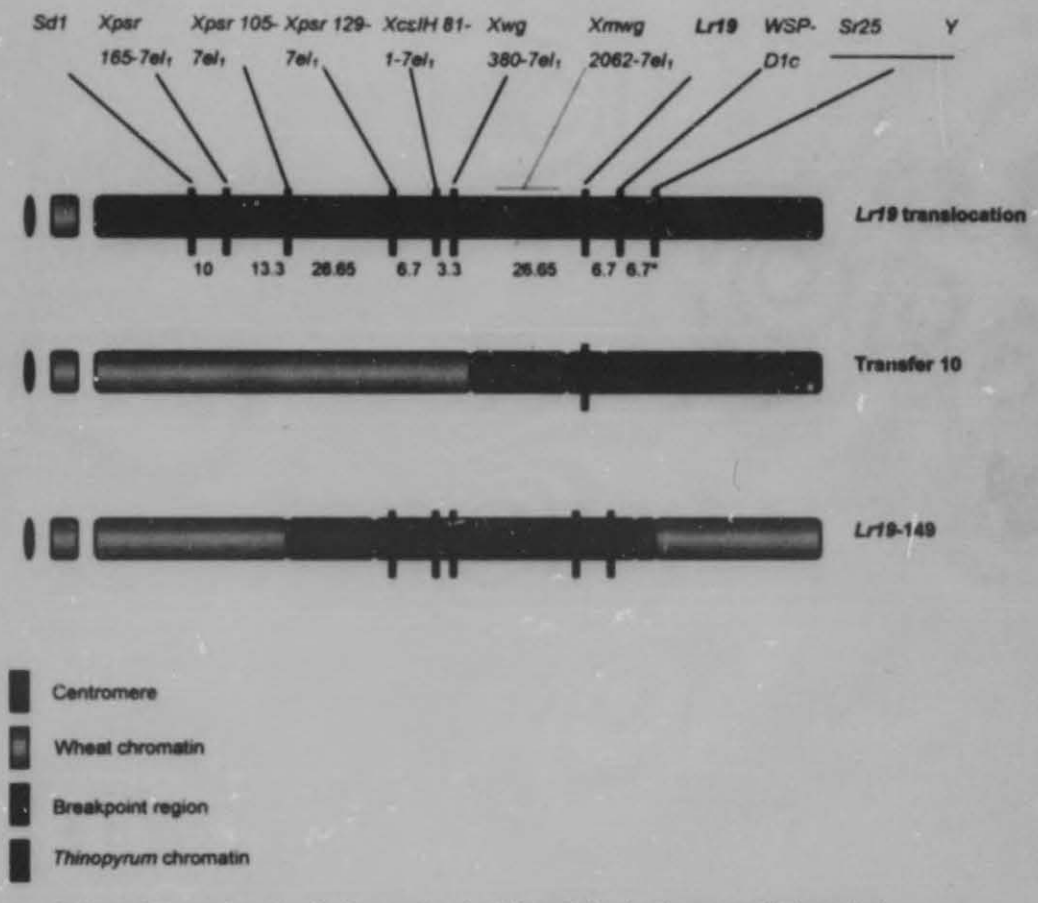
1.3.2 GENETIC MAP THE OF *THINOPYRUM* DERIVED *LR19* TRANSLOCATION

Marais (1992a) and Prins *et al*, (1996; 1997) physically mapped a number of loci on the *Lr19* translocation making use of a set of 29 deletion mutants (Figure 1.2).

1.3.3 DELETION MAPPING

The reasons for the construction of deletion mapping populations are similar to that of constructing other mapping populations, namely that they greatly simplify the localisation of certain traits to specific chromosome areas. This has had a major advantage for the construction of genetic maps in wheat.

Figure 1.2 A physical map of the original *Lr19* (or T4) translocation and two modified forms (Prins *et al*, 1996, 1997).



* *Arbitrary distances based on the frequencies at which irradiation breaks occurred between loci.*

Deletions are induced in such populations by means of irradiation or gametocidal gene effects, but have also been known to occur spontaneously (Payne *et al.* 1984; Snape *et al.* 1985; Kota and Dvorák, 1986; Marais, 1992a). Irradiation was used to induce deletions in the wheat line, 'Indis', containing the *Lr19* translocation (Marais 1992a). Irradiated 'Indis' plants were pollinated with the variety 'Inia 66'. The testcross F_1 and F_1-derived F_2 seeds were screened for the presence of *Lr19*, *Sr25*, *Y* and *WSP-D1c*. Radiation induced chromosome breaks appear to be totally random and in most cases the deletions resulting from irradiation are of a terminal type (Friebe *et al.* 1993). When some or all of the marker genes could not be recovered in a testcross family, a terminal deletion of the *Lr19* translocated segment was suspected. Deletion mutant homozygotes were then recovered by selecting for the absence of *Ep-D_1* (endopeptidase) products. The *Ep-D_1* locus occurs within the translocated region (Marais 1992a) and the *Lr19* translocation does not possess/express this gene.

1.3.3.1 The segregation distortion locus, *Sd1*

The *Lr19* translocation causes its preferential transmission in heterozygotes (Zhang and Dvorák, 1990; Marais, 1992c). This is the result of the selective abortion of gametes lacking *Lr19*. Zhang and Dvorák (1990) as well as Prins *et al.* (1996) mapped a gene contributing to the effect, *Sd1*, proximal to the *Lr19* locus.

1.3.3.2 Water soluble protein locus, *Wsp-D1c*

This is a protein that can be extracted in water from the endosperm. Three homoeoloci encoding the protein occur on the group 7L chromosome arms of wheat. The protein has an unknown function (Liu *et al.* 1989), and its isoforms can be separated by iso electric focusing.

1.3.3.3 Resistance Genes

The genes *Sr25* (stem rust resistance) and *Lr19* (leaf rust resistance) also occur on the T4 translocation and their presence can be detected using appropriate pathogen isolates.

1.3.3.4 RFLP markers

Six RFLP markers have been mapped to the *Lr19* translocated segment (Prins *et al*, 1996). Their order was determined as: *Xpsr165 – Xpsr104 – Xpsr129 – XcSIH81-1 – Xwg380 – Xmwg2062*.

1.3.3.5 Yellow Endosperm

This locus encodes for carotenoid pigments that result in yellow coloured endosperm. Since yellow coloured flour is undesirable in the wheat industry, the complete translocation is not used commercially in most industrialised countries.

1.4 AIM OF THE STUDY

The shortened *Lr19*-149 translocation exhibits strong self-elimination due to the loss of *Sd1* and the retention of the *Sd2* gene. This hampers attempts to recover homozygotes in a practical breeding program. It is also known that gametocidal genes often induce mutagenic effects, and this is undesirable when used in a breeding program. Consequently, efforts are being made to minimise the alien chromatin associated with *Lr19* by means of the induction of homoeologous recombination with wheat chromosomes (Prins *et al.* 1998a; 1998b). For this purpose, it is necessary to have appropriate markers which can be used to identify and characterise recombinants.

The set of 29 deletion mutants (Marais 1992a) for the *Lr19* translocation is a useful tool for the physical mapping of the genes on this chromosome arm. Due to the limited number of markers available for this large translocation it has not been possible to arrange the mutants correctly according to the sizes of their deletions. The use of new markers will facilitate in the classification of the deletion mutants.

The aim if this study was to identify more markers for the *Lr19* translocation and especially for the shortened *Lr19*-149 translocation and to utilise the set of 29 deletion mutants to map these new marker loci physically with regard to existing markers.

The target gene, *Lr19*, is transcriptionaliy active and is expected to reside within an area of coding DNA. Attempts were therefore made to enrich DNA for low copy sequences, which is likely to originate from coding areas in the wheat genome. It was expected that this may increase the likelihood of identifying markers closely linked to the *Lr19* gene.

CHAPTER 2

2.1 MATERIALS AND METHODS

2.1.1 PLANT MATERIAL

Near isogenic lines of both the original *Lr19* translocation (Marais, 1990), as well as the reduced *Lr19*-149 translocation, in the genetic backgrounds of the varieties 'Inia 66', 'Chinese Spring', 'SST66' and the breeding line, 'W84-17' (Marais, 1990; Prins *et al.* 1996), were used in the search for polymorphisms associated with the *Lr19* translocation (Table 2.1). A set of 29 deletion mutants (Marais, 1992a) of the original *Lr19* translocation was then used to physically map polymorphic RAPD loci found.

Table 2.1 Near isogenic lines having either the *Lr19* translocation or the shortened *Lr19*-149 translocation in the genetic background of 'Inia 66', 'Chinese Spring', 'SST66' or the breeding line 'W84-17'.

	*Lr19d/6** Inia 66	*Lr19d/6** C Spring	*Lr19d/6** SST66	*Lr19d/6** W84-17
	*Lr19#149/5** Inia 66	*Lr19#149/6** C Spring	-	*Lr19#149/7** W84-17

NILs have been used extensively to screen for markers where an introgressed alien chromatin segment is involved (Iqbal and Rayburn, 1995). The NILs differ from their parental lines only with regard to the translocated segment (Marais, 1992a) and are

genetically similar otherwise. Heterozygosity and background genetic variation between members of a NIL pair are expected to be negligible after 4-6 consecutive backcrosses and with the added control of having the translocated segment in several breeding lines, the chances of detecting false polymorphisms are even less.

2.1.1.1 DNA ISOLATION

A number of protocols are available for DNA extraction in plants and vary depending on the tissue type and age. The near isogenic lines and deletion mutants were grown in a greenhouse to provide leaf tissue for DNA extraction. Extractions were performed on the NILs by employing a modified version of the Doyle and Doyle liquid nitrogen protocol (1990). Adaptations were made to include the use of a lyophiliser to allow leaf tissue to be stored for extended periods of time. Tissue samples were ready for harvesting approximately 3 weeks after germination. Following liquid nitrogen freezing of the leaf tissue, the samples were placed in a lyophiliser for 5 days. Approximately 500 mg of the freeze-dried tissue were ground and supplied adequate DNA quantities of high quality. Ten ml preheated (60°C) CTAB (5% w/v cetyltrimethylammonuim bromide), 1.4M NaCl, 0.2% v/v 2-mercaptoethanol, 20mM EDTA, 100mM Tris-HCl pH 8.0) were added to the ground tissue. Following incubation for 30 min with occasional mixing at 60°C, it was extracted once with 1 volume of chloroform-isoamyl alcohol (24:1 v:v) and mixed thoroughly but gently. This was centrifuged at 5000g for 10 min at room temperature. The aqueous phase was transferred to a glass centrifuge tube by means of a wide-bore pipette and 2/3 volume of ice cold isopropanol (2-propanol) was added and mixed gently. A DNA spindle became visible which could be hooked by means of a sterile needle and transferred to 10 to 20 ml of wash buffer (76% v/v ethanol, 10mM ammonium acetate). This was the preferred method of transfer, yet if this was not possible, the solution was centrifuged at 5000g for 10 min and the supernatant removed by aspiration, leaving the pellet as dry as possible. The wash buffer was added thereafter. Washing was done for at least 20 min. This was then centrifuged at 12000g for 10 min and the buffer removed by decantation. The pellet was vacuumed (or air-dried) and resuspended in 500 µl deionised water. DNA degradation was

examined by gel electrophoresis, and quantified by means of a fluorometer (Hoefer) and spectrophotometer (Ultraspec III).

A simpler and considerably faster technique for DNA extraction from leaf tissue was used to extract DNA from leaves of the deletion mutants. The carborandum method for DNA extraction allows for the extraction of DNA from leaf tissue in a similar fashion to that of the Doyle and Doyle extraction protocol. It is a mini-preparation protocol for rapidly isolating smaller amounts of DNA of lower quality, yet adequate for RAPD amplification purposes. Seedlings were grown in a greenhouse and leaf tissue was harvested. A microfuge tube's cap was used to press 2 leaf disks from the harvested leaf and to place it in the tube. A pinch of carborandum was added and the material was ground with a grinding stick (Whitehead Scientific) to a fine mesh. Four hundred microlitres of preheated (60°C) 2% CTAB buffer (2% w/v cetyltrimethylammonuim bromide) as well as 1% β-mercapto-ethanol were added and the mixture incubated at 60°C for 30 to 60 min with occasional mixing. This was followed by three chloroform-isoamyl (24:1) extractions. The DNA was then precipitated with 0.8 volume of isopropanol (room temperature) and left overnight. Additional precipitation was done by allowing a 10 min cooling at -80°C. Following centrifugation at 12 000 g for 30 min the isopropanol was removed by decantation. The DNA was dissolved in 20 - 50 µl of deionised water depending on the amount of DNA harvested and inspection of the relevant pellet size. DNA was quantified as previously described.

2.1.2 DNA ENRICHMENT

2.1.2.1 SONICATION

As discussed in the introduction (1.2), large molecular weight DNA is not suited for hydroxyapatite chromatography. An appropriate method for breaking up genomic DNA is the sonication thereof. Numerous techniques to do this have been published (Sambrook *et al*, 1989). The present protocol was adopted from a protocol for the sonication of salmon sperm DNA and human placental DNA (Ramachandra, 1990).

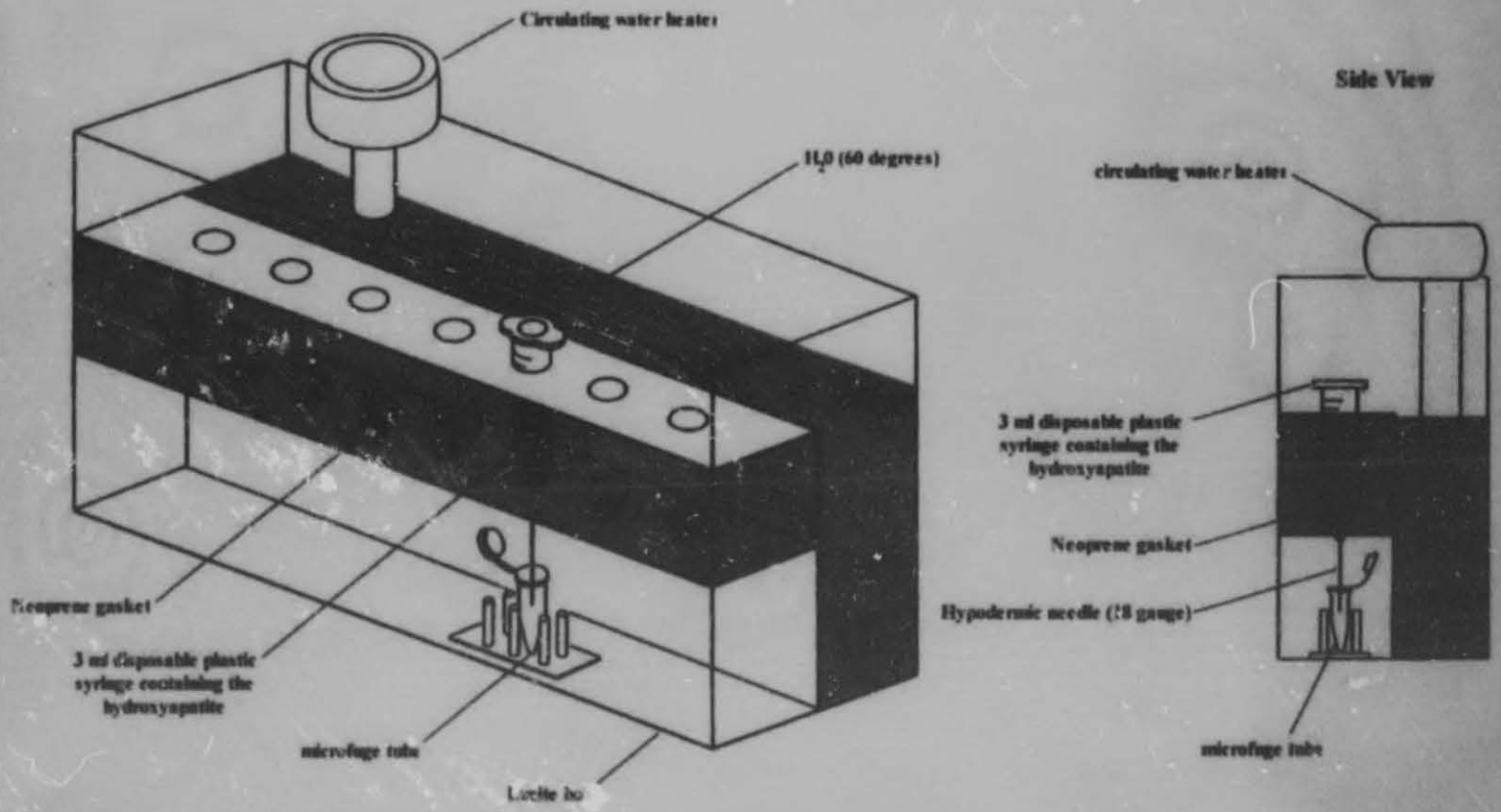
A sonicator (Heat Systems Inc. Sonicator - model for cell distribution W-220F) with micro-tip placed in a sound controlled chamber was used. Specifications differ depending on the machine. Approximately 2ml of a 500 mg/ml DNA solution isolated from the wheat leaf tissue were transferred to a strong glass tube for sonication. The settings selected on the sonicator were: Output = 6, Duty cycle = 60% and Hold = "continuous". The micro-tip was sterilised with ethanol and "Kimwipes". After putting on earmuffs, the micro-tip was immersed halfway into the DNA solution. Sonication was performed on ice for 5 min in 1 min pulses, cooling the solution after every pulse (a minute on ice). The micro-tip was cleaned after each sample. The DNA size was verified by electrophoresis on a 0.8% L.E. agarose gel with a standardised BRL 1 kb ladder. The sonication methodology is effective for unbiasedly breaking up DNA and produced almost identical fragment sizes. The bulk of the fragmented DNA should be around 600 bp, however, re-sonication was sometimes necessary to obtain the right sizes.

2.1.2.2 HYDROXYAPATITE CHROMATOGRAPHY

Sonicated DNA was recovered by ethanol precipitation and centrifugation. The DNA was re-suspended in 0.12M phosphate buffer. Phosphate buffer (PB) stock (2M) was prepared from equal amounts of 2M NaH_2PO_4 and 2M Na_2HPO_4 (pH 6.8) A buffer series of increasing concentrations of sodium phosphate (0.12, 0.15 and 4.0 M) is necessary for the elution of DNA from the hydroxyapatite column. Usually single-stranded DNA elutes in 0.14 - 0.16 M PB (pH 6.8) and double-stranded DNA at concentrations exceeding 0.36 M. Hydroxyapatite chromatography columns were prepared (Fig. 2.1) with disposable plastic syringes. The syringe was blocked with Whatman GF/C filters after removing the plunger. A needle was attached to the Neoprene gasket ensuring that the filter was plugged securely. As with most other reactions, optimisation of the chromatography process was also necessary since batches of hydroxyapatite differ slightly in characteristics from one to the other.

The DNA sample (suspended in PB), was heat-denatured by boiling for 10 min and then re-annealed at 60°C for 24 h. DNA grade hydroxyapatite powder (Bio-Rad DNA grade) was suspended in 0.12 M PB (1g in 5 ml 0.12 M PB). DNA-grade Bio-Gel HTP gel is a dry powder with small particle size to allow for increased DNA binding capacity, it is autoclaveable and recommended for small-scale chromatography in very short columns. Its flow rate is less than 5 cm /hr and the hydrated volume is 2-3 ml/g. The suspension was well mixed and placed in boiling water for equilibration for 30 min. The buffer was removed and replaced with fresh buffer. The suspension and the column were maintained at 60°C. The column was loaded with the prepared slurry and the neoprene stopper removed to allow the PB to flow through. The column was washed with 3 volumes of 0.12 M PB until the slurry had settled. The column was plugged again before the sample was applied to allow ample time for the DNA binding process. Single-stranded DNA (signifying the complex or slow annealing DNA) could be eluted by 4 column volumes of 0.15 M PB and were kept for RAPD analysis. The double-stranded DNA could be eluted with 3 column volumes of 0.4 M PB. Fractions were tested by ethidium bromide spot fluorescence (Sambrook *et al.*, 1989).

Figure 2.1 Apparatus used during the hydroxyapatite chromatography with Bio-gel. (Sambrook *et al*, 1989)



The 0.15 M PB eluates containing the single-copy or complex DNA were pooled. The DNA was concentrated by adding one volume of 2-butanol, vortexing, and centrifuging for 20 sec at 12 000 g. The top (organic) phase was discarded and the procedure repeated until an end volume of approximately 200 µl was obtained. Sephadex G-50 spin columns (Boehringer Mannheim) were used to remove the PB salt residues and the DNA was recovered through precipitation with 2 volumes 100% ethanol at 0°C and dissolved in deionised water.

2.1.3 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

2.1.3.1 PRIMERS

Synthesised oligo-nucleotides with randomly generated sequences are available from Operon Technologies ™ (Alamede, California) and are distributed in primer kits containing 20 primers each. The primers used and their sequence data are listed in Table 2.2. A total of 80 primers were tested on the NIL lines described.

Table 2.2 Table detailing the sequence data of the Operon primer kits used, totalling 80 primers.

Primer Code	Sequence 5' to 3'	Primer Code	Sequence 5' to 3'
OPA 1	CAGGCCCTTC	OPK 1	CATTCGAGCC
OPA 2	TGCCGAGCTG	OPK 2	GTCTCCGCAA
OPA 3	AGTCAGCCAC	OPK 3	CCAGCTTAGG
OPA 4	AATCGGGCTG	OPK 4	CCGCCCAAAC
OPA 5	AGGGGTCTTG	OPK 5	TCTGTCGAGG
OPA 6	GGTCCCTGAC	OPK 6	CACCTTTCCC
OPA 7	GAAACGGGTG	OPK 7	AGCGAGCAAG
OPA 8	GTGACGTAGG	OPK 8	GAACACTGGG
OPA 9	GGGTAACGCC	OPK 9	CCCTACCGAC
OPA 10	GTGATCGCAG	OPK 10	GTGCAACGTG
OPA 11	CAATCGCCGT	OPK 11	AATGCCCCAG
OPA 12	TCGGCGATAG	OPK 12	TGGCCCTCAC
OPA 13	CAGCACCCAC	OPK 13	GGTTGTACCC
OPA 14	TCTGTGCTGG	OPK 14	CCCGCTACAC
OPA 15	TTCCGAACCC	OPK 15	CTCCTGCCAA
OPA 16	AGCCAGCGAA	OPK 16	GAGCGTCGAA
OPA 17	GACCGCTTGT	OPK 17	CCCAGCTGTG
OPA 18	AGGTGACCGT	OPK 18	CCTAGTCGAG
OPA 19	CAAACGTCGG	OPK 19	CACAGGCGGA
OPA 20	GTTGCGATCC	OPK 20	GTGTCGCGAG

Figure 2.3 (continued)

Primer Code	Sequence 5' to 3'	Primer Code	Sequence 5' to 3'
OPL 1	GGCATGACCT	OPY 1	GTGGCATCTC
OPL 2	TGGGCGTCAA	OPY 2	CATCGCCGCA
OPL 3	CCAGCAGCTT	OPY 3	ACAGCCTGCT
OPL 4	GACTGCACAC	OPY 4	GGCTGCAATG
OPL 5	ACGCAGGCAC	OPY 5	GGCTGCGACA
OPL 6	GAGGGAAGAG	OPY 6	AAGGCTCACC
OPL 7	AGGCGGGAAC	OPY 7	AGAGCCGTCA
OPL 8	AGCAGGTGGA	OPY 8	AGGCAGAGCA
OPL 9	TGCGAGAGTC	OPY 9	AGCAGCGCAC
OPL 10	TGGGAGATGG	OPY 10	CAAACGTGGG
OPL 11	ACGATGAGCC	OPY 11	AGACGATGGG
OPL 12	GGGCGGTACT	OPY 12	AAGCCTGCGA
OPL 13	ACCGCCTGCT	OPY 13	GGGTCTCGGT
OPL 14	GTGACAGGCT	OPY 14	GGTCGATCTG
OPL 15	AAGAGAGGGG	OPY 15	AGTCGCCCTT
OPL 16	AGGTTGCAGG	OPY 16	GGGCCAATGT
OPL 17	AGCCTGAGCC	OPY 17	GACGTGGTGA
OPL 18	ACCACCCACC	OPY 18	GTGGAGTCAG
OPL 19	GAGTGGTGAC	OPY 19	TGAGGGTCCC
OPL 20	TGGTGGACCA	OPY 20	AGCCGTGGAA

2.1.3.2 AMPLIFICATION

Optimisation of the RAPD protocol and verification of its reproducibility, were done using a standard amplification Taq protocol. Five random primers were selected to do preliminary reactions. Several protocols have been described for the successful utilisation of the RAPD technique in wheat (Wei and Wang, 1995; Iqbal and Rayburn, 1995; Transue *et al,* 1994; Tinker *et al,* 1993; King *et al,* 1993; Dweikat *et al,* 1993, Vierling and Nguyen, 1992; Devos and Gale, 1992; Rafalski and Tingey, 1991; Weining and Langridge, 1991). After optimisation, it was noted that the DNA isolation method often influences the subsequent amplification during the RAPD reaction. This is mainly due to phenol residues or even traces of chloroform. To assess the reproducibility of results, reactions were done in duplicate on different occasions. Control reactions were included on each gel in which all the reaction components except DNA template were present. Any contamination could thus be

detected and the reaction repeated if necessary. It is important to be careful to avoid contamination. For this reason aerosol resistant tips and a laminar flow cabinet were used.

RAPD amplification reactions were performed in autoclaved thin-walled PCR tubes, using a total volume of 24µl. Optimally amplified fragments were obtained by using 20 ng of template DNA and 10 pmol primer. In cases where few amplified products were obtained, conditions were changed to compensate. Increases in the primer concentration (20 pmol) were in most cases adequate to achieve this. Buffer (from Advanced Biotechnologies, London) to a total of 10% of the reaction volume (2,5 µl) was used. The buffer contained 200mM $(NH_4)_2SO_4$, 750 mM Tris-HCl pH 9.0 (25°C) 0.1% Tween and 1.5 mM $MgCl_2$. In addition, 100mM of each deoxy-nucleotidephosphate (dNTP) and 0.5 Units of AB Taq DNA polymerase (Advanced Biotechnologies, London) were used in the reaction mix. A Techne PHC-3 thermal cycler was used for performing the amplification reactions. Cycles of one 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.

Profiling was done on completion of the cycles by using 24 µl of the RAPD amplification reaction and mixing it with 6.5 µl "blue" loading Buffer type IV (0.25% bromophenol blue and 40% (w/v) sucrose on water - Sambrook *et al*, 1989), and loading the mixture on an electrophoresis 1.4% LE agarose gel. Running buffer consisted of 0.5 x TBE (1 x TBE : 0.089M Tris-borate, 0.089 Boric Acid, 0.002M EDTA) and Ethidium bromide (10 µl/dm^3) and the gels were run at 80 Volts for 5 to 6 hours. Visualisation of the RAPD products were done by using an ultraviolet transilluminator (UVP Inc.) and results photographed by means of thermal printing.

2.1.3.3 PRESENTATION OF RESULTS

Thermal prints (thermal photographs) of agarose gels were used to document gels. Polymorphisms were identified and scored either by the presence or absence of a specific amplification product. Polymorphic amplification products exhibiting

differences in intensity, but which were equal in size, were not regarded as polymorphic. Markers were named in accordance with its primer origin and the base pair size of the amplified fragment.

2.1.3.4 SCREENING FOR RAPD MARKERS LINKED TO *LR19*

A total of 80 primers were used in the RAPD reactions for screening the NIL DNAs. Reactions were completed in duplicate, and on suspicion of containing a polymorphism, repeated again, thus verifying reproducibility in triplicate. Only reproducible fragments were considered for mapping.

2.1.4 RAPDs ON ENRICHED DNA

After completing the DNA sonication (2.1.2.1) and enrichment processes (2.1.2.2) the DNA were aliquotted. Quantification was done by means of fluorometer (Hoefer) and verified with Lambda DNA standard gel electrophoresis. It was, however, necessary to pool some of the DNA stocks because of the low amounts of DNA eluted via the enrichment process. This proved to be a major problem with this procedure.

The RAPD technique was applied, as described (2.1.3), but further optimisation was necessary to achieve results comparable to that of the full DNA RAPDs. To increase the amplified fragment quantities, the reagent concentrations were slightly modified. Twenty nanograms of DNA were also used, but the primer concentrations were increased from 10 pmol to 20 pmol primer. All the other RAPD reaction conditions remained as previously. Amplified RAPD products were run on a 1.4% agarose gel containing EtBr at 80 volts for 5 to 6 hours.

2.2. ATTEMPT TO CONVERT THE DOMINANT *Xus-OPK15$_{800}$-7EL$_1$* MARKER INTO A CODOMINANT SCAR MARKER

Cloning and the partial sequencing of a polymorphic RAPD fragment are the first steps towards its conversion into a co-dominant SCAR marker (Paran & Michelmore 1993). The RAPD marker *Xus-OPK15$_{800}$-7el$_1$* was found to be the closest to *Lr19* of the isolated polymorphic fragments and was therefore used in this attempt.

2.2.1.1 FRAGMENT ISOLATION AND PURIFICATION

The polymorphic fragment was amplified by means of the standard RAPD procedure, but performed in a larger volume (75 µl) and run on a 3.0% LE agarose gel. The use of a larger slot size was necessary to accommodate the larger sample volume. Lambda ladder standards were included together with the original NIL template DNA to provide additional controls. The target DNA fragments were excised from the agarose gel using a sterile scalpel blade and processed with a DNA fragment isolation kit (Amercham). This is recommended, since it also removes any PCR products, i.e. primers left after the completion of the reaction. The gel slice was treated with an agarose dissolving agent, incubated at 50°C and centrifuged slowly through a DNA-binding matrix. The DNA was recovered after cleaning the mini column with ethanol, re-dissolving the DNA in water and centrifuging the DNA-binding column again. Gel electrophoresis quantification of the subsequent isolation was performed in order to be able to calculate the volume of DNA necessary for setting up the ligation reaction and cloning.

2.2.1.2 CLONING OF THE POLYMORPHIC FRAGMENT

The use of PCR based cloning systems greatly simplify the arduous conventional cloning procedures, i.e. super-competent cells and a suitable plasmid vector. pGEM - T Easy Vector Systems (Promega) are designed for cloning of PCR produced fragments. Vectors are prepared from pGEM-5Zf(+) vectors (Promega), cut with *Eco*R V. This procedure adds 3' terminal thymidine to both ends of the vector. The efficiency of the ligation of the PCR product is thus greatly improved by the resulting 3'T overhangs and the prevention of recircularization of the vector. A number of Taq DNA polymerase enzymes (exhibiting 5' - 3' exonuclease activity i.e. AB Taq Polymerase as used here) add these overhanging ends to the PCR amplification products they produce. This results in the quick and effective cloning of PCR fragments.

2.2.1.2.1 Ligation

A molar ratio of 1:1 of insert DNA to vector DNA is necessary for successful ligation, even though ratios of 1:8 and 8:1 have also been found to be effective. This was done by comparing the isolated polymorphic DNA from the RAPD gel to lambda DNA mass standards. The following equation was used for determining the quantity of insert DNA required for ligation :

$$\frac{\text{ng of vector x size (kb) of the insert}}{\text{size (kb) of the vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

The ligation reaction was set up for overnight ligation in accordance with the cloning kit protocol. Additional reactions included 1:3 and 3:1 vector : insert ratios as well as the standard positive and background controls.

2.2.1.2.2 Transformation

The pGEM easy vector II system uses the β-galactosidase coding system found in *E. coli* JM109 super competent cells to screen for recombinant cells. Transformations were completed and a transformation efficiency control was included in the form of linearised pUC18 vector. LB Ampicillin plates (100 µg/ml) were used for transformation (10g bacto-tryptone; 5g bacto-yeast extract; 10g NaCl; 15g Agar per 1 litre water pH=7.0). Forty microlitres of X-Gal (5-Bromo-4-chloro-3-indoyl-β-D-galactoside - 50mg/ml) per 9cm plate and 40 µl 0.024g/ml IPTG (isopropyl-thiogalactoside) were plated out for screening. After heat-shock transformation the cells were plated out and incubated at 37°C for 16 hours.

2.2.1.2.3 Plasmid Extraction

Plasmid cloning was done by utilising a standardised plasmid mini-preparation protocol (Promega). Ten millilitres of LB medium (1g bacto-tryptone; 0.5g bacto-yeast extract; 0.5g NaCl to 1 litre of water) containing 50 µg/ml Ampicillin were inoculated with recombinant colonies picked from the transformation (2.3.1.2.2). These were incubated at 37°C overnight and 1.5 ml of the culture were centrifuged at 12 000g for 1 min. The remaining culture was stored at 4°C. The medium was removed by careful decantation and the bacterial pellet resuspended by vortexing in 100 µl of ice-cold mini-preparation lysis buffer. It was then incubated at room temperature for 5 min and 200 µl of a 0.2N NaOH; 1% SDS solution were added. This was followed by incubation for 5 min after gentle mixing. Hundred and fifty microlitres of ice-cold Potassium-acetate solution (29.4g Potassium-acetate and 11.5ml Glacial Acetic Acid to total of 100 ml pH 4.8) were added and the mix placed on ice for 5 min. Following centrifugation for 5 min at 12 000 g the supernatant was transferred and RNAse A added to a final concentration of 20 µg/ml followed by incubation at 37°C for 20 min. One volume of TE-saturated phenol/chloroform was added, the mixture vortexed for 1 min and centrifuged for 2 min at 12 000g. The upper aqueous phase was transferred to a fresh tube and 1 volume of chloroform:isoamyl alcohol (24:1) added. The mixture was vortexed for 1 min and

centrifuged and transferred as in the previous step. Two and a half volumes of ethanol were added, mixed and allowed to precipitate at -80°C for 5 min. The supernatant was removed and the pellet washed with 70 % ethanol. The pellet was dried under a vacuum and dissolved in 10 - 20 µl of deionised water.

As an alternative for analysing the plasmid DNA, the bacterial clones may be subjected to 1 minute of boiling in a water bath and 1 µl used for a PCR run with M13 forward and reverse sequence primers. Considering the risk of amplifying non-targeted bacterial DNA, it was decided to rather complete the entire plasmid mini-preparation, and then to confirm that the plasmid contained the correct insert making use of the RAPD primer sequences.

2.3.1 SCREENING OF FRAGMENT

Theoretically the RAPD primer sites should be included in the cloned fragment, but considering the nature of the fragment isolation and cloning procedures, it is quite possible that the primer ends may be damaged or at least shortened. Amplification of the cloned insert was achieved by using the M13 forward and reverse sequences at the MCS (Multiple Cloning Site) in the vector at 2959-2975 and 176-192 bp, respectively (the total plasmid contains 3018bp). The expected amplified product should therefore have a fragment size of approximately 800bps plus the sequences flanking the insert up to the M13 sequences. The PCR reaction was performed with a Techne thermal cycler, and involved one cycle of 1 min at 91 °C and 40 cycles at 91°C, 60°C and 72°C and a final elongation cycle at 72°C for 10 minutes. The PCR products were mixed with the standard "blue juice" loading buffer (Sambrook *et al*, 1989) and were run on a 1.4 % agarose electrophoresis gel at 80 volts, for 5 hours with 0.5 x TBE and EtBr (10 µl/L). The gel was documented by means of UV transilluminance and a thermal print was obtained.

2.3.2 SEQUENCING

One positive clone of the appropriate fragment size was selected and its culture used to prepare a freeze culture for later use. The freeze culture contained the LB medium and sterile glycerol (end concentration 25% of the volume) and was stored at -80°C. The remainder of the culture was used to re-inoculate 50 ml of LB medium followed by ultra pure Nucleobond plasmid extraction for sequencing. However, the amplified fragment was found to be of the wrong size and its sequencing was not attempted.

CHAPTER 3

3.1 RESULTS

3.1.1 DNA ISOLATION

The near isogenic lines and deletion mutants were grown in a greenhouse. Leaf tissue was harvested from the seedlings and used to prepare lyophilised leaf tissue stocks which were stored at -80°C. The DNA extraction procedure produced intact DNA of high quality. Minimal shearing of the DNA as well as limited enzymatic activity in the isolates are preferable, although this is not crucial for the generation of reliable RAPD profiles. The use of RNase A during the extraction process also improved the procedure since RNA is often associated with the presence of proteins that could be destructive during excessive thawing as is done with the DNA used during RAPDs. An average yield of 500µg/µl was obtained and verified by both gel electrophoresis and fluorometer quantification.

3.1.2 ENRICHMENT STRATEGY

In an attempt to maximise the likelihood of finding suitable genetic markers close to the *Lr19* translocation (located within the coding regions of the chromosome), hydroxyapatite columns were used to enrich the DNA for unique sequences. Following sonication, denaturing and re-annealing of the DNA, the unique sequences were retained whereas the repetitive DNA was mostly eluted. This significantly reduced the amount of DNA available for amplification. The use of the enrichment process therefore required larger amounts of DNA and often necessitated additional DNA isolation.

3.1.3.1 Sonication of DNA

Sonication was done using a sonic probe and the process worked very well. It was, however, necessary to repeat the sonication steps two to three times to ensure that the DNA was not sonicated beyond the desired fragment size. A problem with this procedure is that it has to be completed in an open tube, greatly increasing the risk of contamination, whether by the open-air environment, or from other samples also sonicated by means of the sonication probe. Some probes, like the one used in this study, were not autoclavable and conventional ethanol cleansing had to be used instead, making the risk factor even higher. An alternative to the use of a Micro-probe sonicator would be to use a sonic bath as used for dispersing cell volumes coagulated during growth phases. However, most sonic baths are not powerful enough to shorten and break genomic DNA. The sonication probe used in the experiment has proven effective in generating evenly sized fragments of the desired length in the wheat template DNA. The fragment sizes were checked on a 0.8% L.E. agarose gel stained with EtBr by comparing them to a 1kb DNA ladder.

3.1.3.2 Hydroxyapatite chromatography

The method described for hydroxyapatite chromatography was executed under stringent conditions and the DNA elutes were concentrated and used in the subsequent RAPD analyses. The chromatography columns were prepared and set up as described in Figure 2.1. Some temperature fluctuations were noted, but the procedure worked well in general.

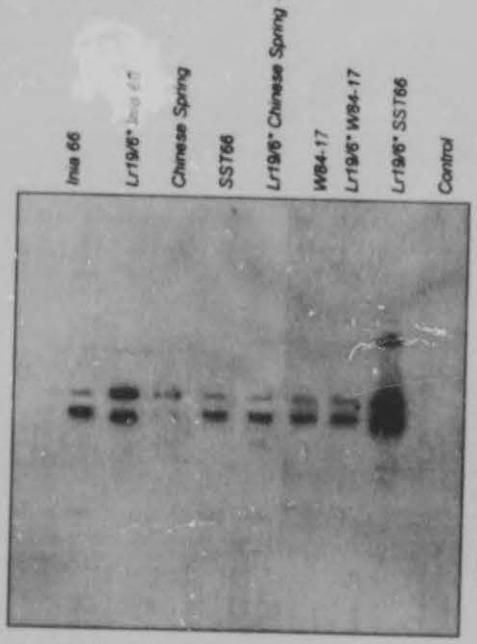
3.1.4.1 COMPARISON OF RAPD PROFILES OBTAINED WITH THE USE OF NORMAL AND ENRICHED DNA.

RAPD profiles were obtained before and after enrichment of the genomic DNA. For this purpose random primers were selected. Figure 3.1 shows the differences between the profiles obtained from the primers OPK11 and OPK12. It is evident from the results that a large amount of DNA was removed by the process, as is reflected by the

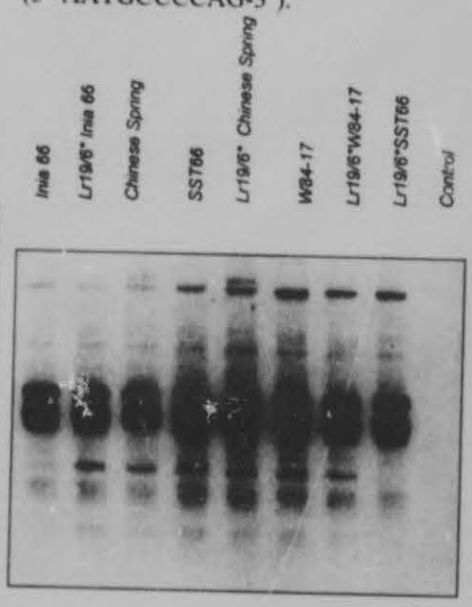
considerably smaller number of primer loci being amplified. This was seen with most of the primers tested. Also, certain primers produced similar amplification products prior to and after enrichment. RAPDs on enriched DNA did not produce any fragments not visible with the full DNA complement, but rather caused a reduction in the number of fragments and their resolutions. It was also found that some primers tested did not produce any amplification products, implying the absence of any homology between the primer and the enriched DNA. In some instances the intensity of a band improved with enrichment of the DNA. All in all, ambiguous results were produced, since bands disappeared in some samples but remained in others completed in duplicate, even if they were only very faint bands. Clearly, the enrichment procedure could not be reproduced satisfactorily. This may be attributable to inadequate temperature control in the water bath and the sample column during the enrichment process. The complete genomic DNA is denatured (by boiling) and results in all of the DNA becoming single stranded. Lowering the temperature will allow the high copy DNA (repetitive or "simple sequences") to reanneal over the 24 hours allowed. A too low sample temperature leads to a considerably larger fraction of DNA renaturing and being removed as high copy or repetitive DNA. The problems associated with RAPD amplification of enriched DNA, i.e. (i) the large DNA quantities necessary, (ii) the fact that very few novel bands appeared as compared to the use of untreated DNA, (iii) the very poor resolution and reproducibility of the majority of amplification products, and (iv) the amount of effort required by this technique, has led to its discontinuation.

Figure 3.1 Examples of RAPD profiles generated using (a) enriched DNA (b) full genomic DNA (next page).

(a) Enriched DNA using primer OPK11 (5'-AATGCCCCAG-3').

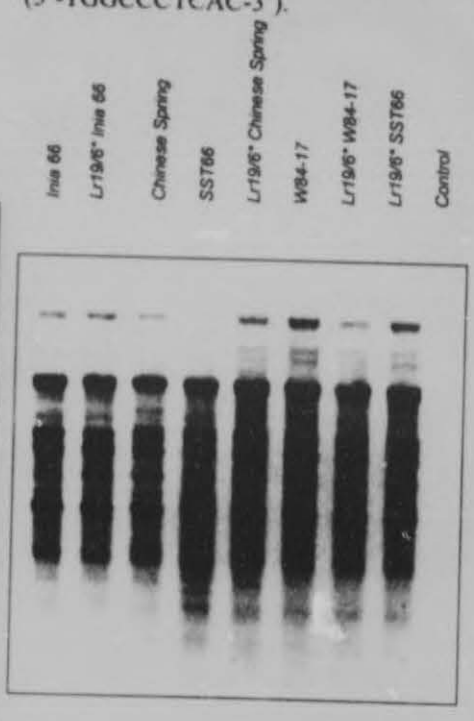


(b) Genomic DNA using primer OPK11 (5'-AATGCCCCAG-3').



(a) Enriched DNA using primer OPK12 (5'-TGGCCCTCAC-3').

(b) Genomic DNA using primer OPK12 (5'-TGGCCCTCAC-3').



3.1.4.2 RAPD ANALYSIS USING TOTAL GENOMIC DNA

Agarose gel electrophoresis allows for clear distinction among RAPD amplification products from approximately 150 to 2000 bp. Only RAPD fragments that were clearly visible and repeatable were considered. A total of 80 primers were used in amplification reactions resulting in more than 1000 distinguishable RAPD fragments. The fragments generated were tested for their association with the *Lr19* translocation in the different near isogenic lines. Even though results varied for some primers, and the RAPD conditions had to be re-optimised, the conditions as described under the section Materials and Methods could be used for the majority of primers.

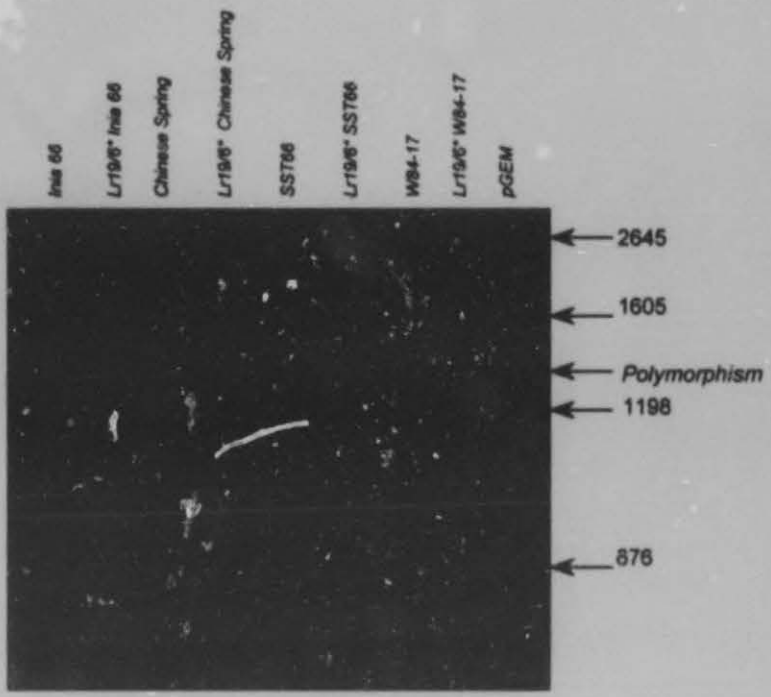
3.2 POLYMORPHIC RAPD MARKERS

Both the detection of repulsion phase markers (markers generated from the displaced wheat chromatin) and non-repulsion markers are possible when working on a NIL population. Four RAPD markers were identified to be polymorphic for the *Lr19* translocation segment. These markers produced bands of comparable intensities with all the NIL lines used and also proved to be repeatable. A further three repulsion phase markers were also identified for the *Lr19* translocation.

3.2.1 RAPD marker *Xus-OPK9*$_{1350}$*-7el*$_1$

The first polymorphism was identified with the RAPD primer OPK9 (5'-CCCTACCGAC-3') and produced a large amplification fragment of 1350 bp (Figure 3.2). This fragment has an ideal size for its conversion into a more usable SCAR marker. Larger fragments are in general more useful than shorter ones, since they allow the retrieval of more sequence data. If the amplified sequence comes from within a gene, it may allow for the identification of possible intron and exon sequences. This may be particularly useful for designing allele specific markers. Larger fragments also increase the likelihood of developing co-dominant markers.

Figure 3.2 RAPD profile indicating the size and authenticity of the polymorphism identified with the RAPD marker OPK9. The last lane contains the DNA size standard pGEM.

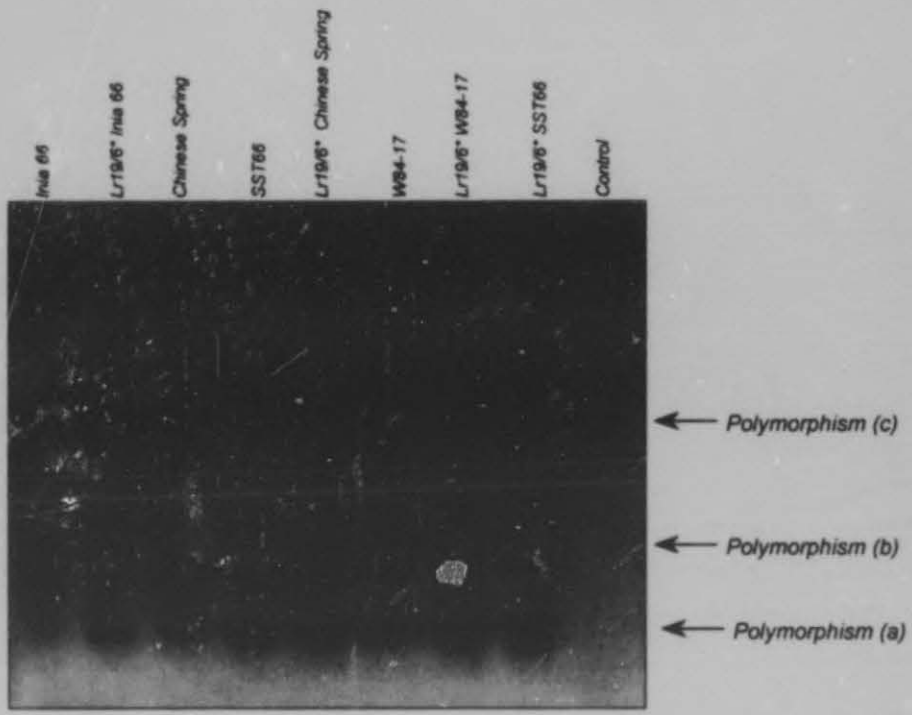


3.2.2 RAPD marker *Xus-OPK10$_{350}$-7el$_1$*

The second marker identified, OPK10$_{350}$ (5'-GTGCAACGTG-3') was a small fragment with a size of approximately 350 bp very clearly detected in all the NIL lines used and marked as (a) in the thermal print (Figure 3.3). Initial results indicated a further 2 possible markers (marked b and c in Figure 3.3) also generated by this primer. The likelihood of three markers detected for the *Lr19* segment by only one primer is small, but not unlikely since the translocation represents a rather large chromosomal region.

However, the polymorphic fragments (b) and (c) shown in the thermal print (Figure 3.3) were not repeatable in subsequent reactions performed under the same conditions. This was confirmed when an attempt was made to map the polymorphisms using a deletion mapping population. Upon re-optimisation of the amplification reaction, it may be possible to stably reproduce these polymorphisms. During the course of the study more than one brand of polymerase enzyme was used due to availability and cost considerations. Use of a different amplification enzyme, may be another possible way to consistently reproduce these fragments.

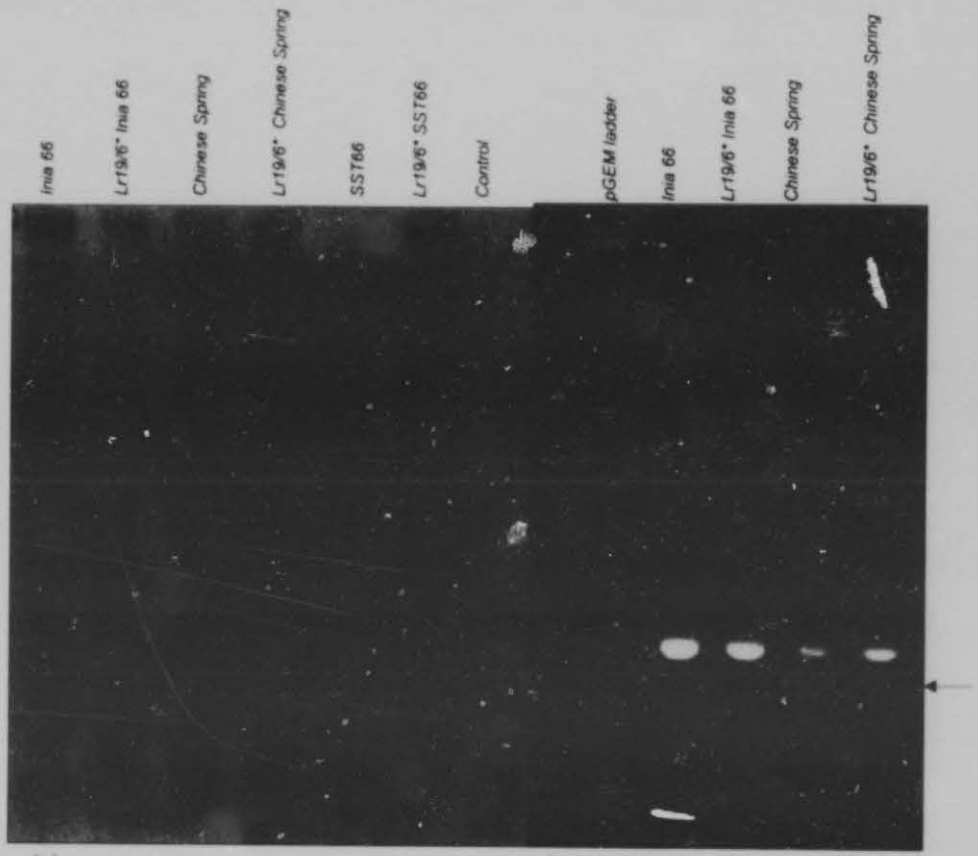
Figure 3.3 RAPD profiles of the NIL lines with the primer OPK10 showing three polymorphisms. The polymorphisms indicated (b) and (c) were not repeatable. The last lane contains the DNA negative control reaction.



3.2.3 RAPD marker *Xus-OPK15$_{800}$-7el$_1$*

Polymorphic locus *Xus-OPK15$_{800}$-7el$_1$* was identified with the primer sequence 5'-CTCCTGCCAA-3' and is shown in Figure 3.4a. The fragment was, however, closely associated with a second, slightly larger fragment. Additional higher percentage agarose gels were run (Figure 3.4b) to separate the two fragments and to clearly distinguish between them (small arrows). This polymorphism was also reproducible and later proved to be the closest to the *Lr19* gene.

Figure 3.4 The RAPD profiles generated by the OPK15 primer. The last lane contains the DNA negative control reaction. The arrows indicate the polymorphism. Fragments were separated on (a) 1.4% agarose gels and (b) 3% agarose gel to better resolve the polymorphic band.



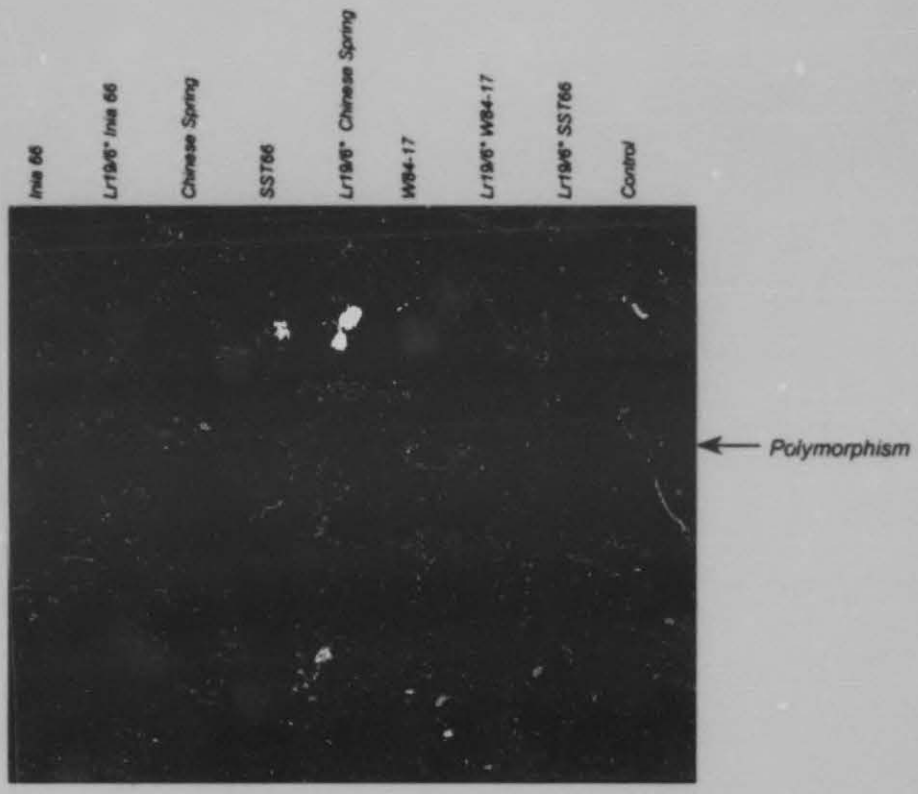
(a) (b)

3.2.4 RAPD marker *Xus-OPY14$_{950}$-7el$_1$*

The use of RAPD marker OPY14 (5'-GGTCGATCTG-3') produced a polymorphism labelled Xus-OPY14$_{950}$-7el$_1$ (Figure 3.5). This polymorphic fragment was very clearly distinguishable and also proved to be very repeatable. The RAPD profile

generated by OPY14 fingerprinted the NILs and as in all the other cases confirmed the homogenous genetic backgrounds in the different NIL pairs.

Figure 3.5 Polymorphism *Xus-OPY14*$_{950}$-*7el*$_1$ and the RAPD profile generated by the primer OPY14. The last lane contains the DNA negative control reaction.



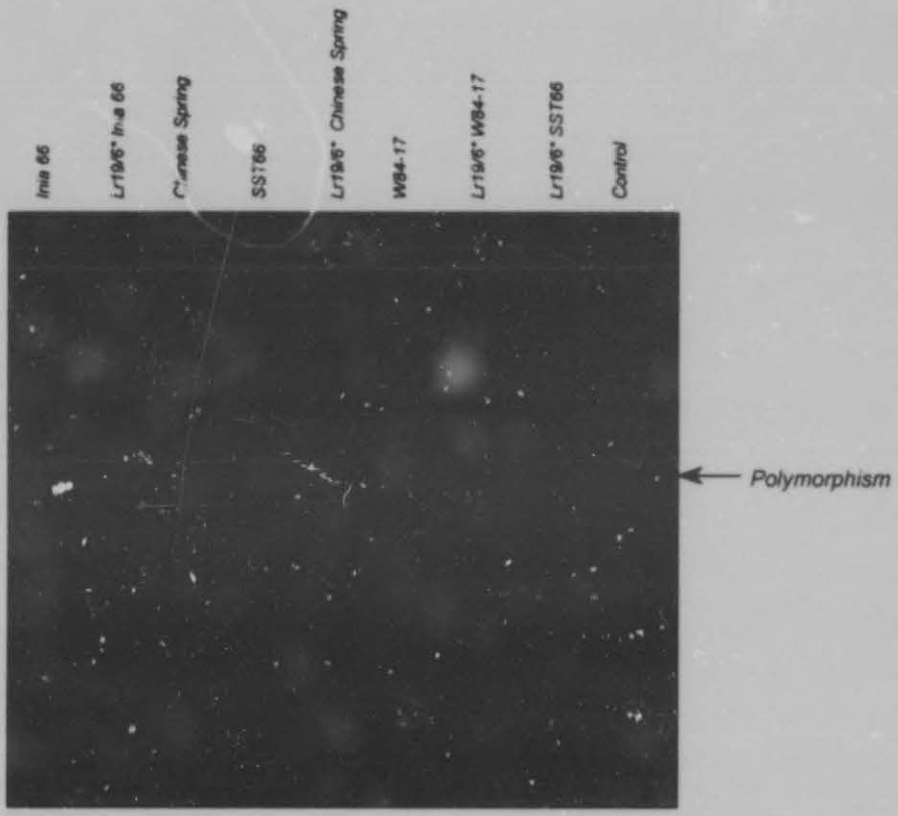
3.3 REPULSION PHASE RAPD MARKERS.

Fragments originating on the member of a NIL pair not containing the *Lr19* translocated segment are associated with the wheat chromatin replaced during the translocation event. This has approximately a 50% likelihood of occurring, since the method of fragment amplification is a totally random process, and the replaced wheat chromosome segment is approximately equal in size to the alien *Thinopyrum* derived segment. These markers are not informative for the purpose of this study since they cannot be mapped onto the translocation segment using the deletion stocks. This was explained in more detail in chapter 4. These markers can, however, be used to identify the replaced wheat segment on chromosome 7DL, and even if they cannot indicate the heterozygous condition of the *Lr19* translocation (this would be possible only if the markers were co-dominant in nature i.e. VNTR and RFLP markers), they can indicate the presence or complete absence of the replaced wheat segment. Repulsion phase markers can only serve to identify heterozygous breeding lines if used in conjunction with RAPD markers specific for the *Lr19* segment. It may also be possible to convert these markers into wheat and chromosome specific probes. The repulsion phase markers were also labelled with the primer identification code, the size of the fragment amplified, and a '-R' added to indicate the marker as repulsion type.

3.3.1 RAPD fragment OPL1-R₇₀₀

This RAPD marker was the first repulsion phase marker identified (Figure 3.6). Primer OPL1 (5'-GGCATGACCT-3') was used. The fragment size is approximately 700 bp.

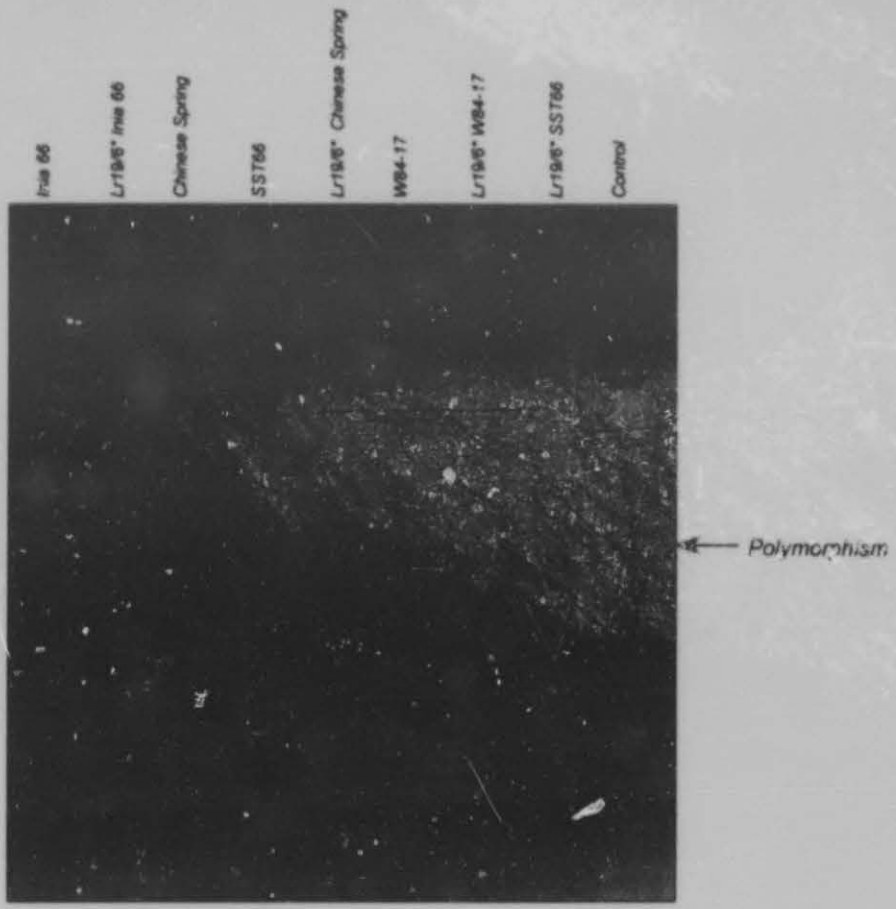
Figure 3.6 RAPD profile generated on the NIL lines by using primer OPL1. The last lane represents the DNA negative control.



3.3.2 RAPD fragment OPL3-R_{850}

This polymorphism is exceptionally clear and it is approximately 850 bp in size (Figure 3.7).

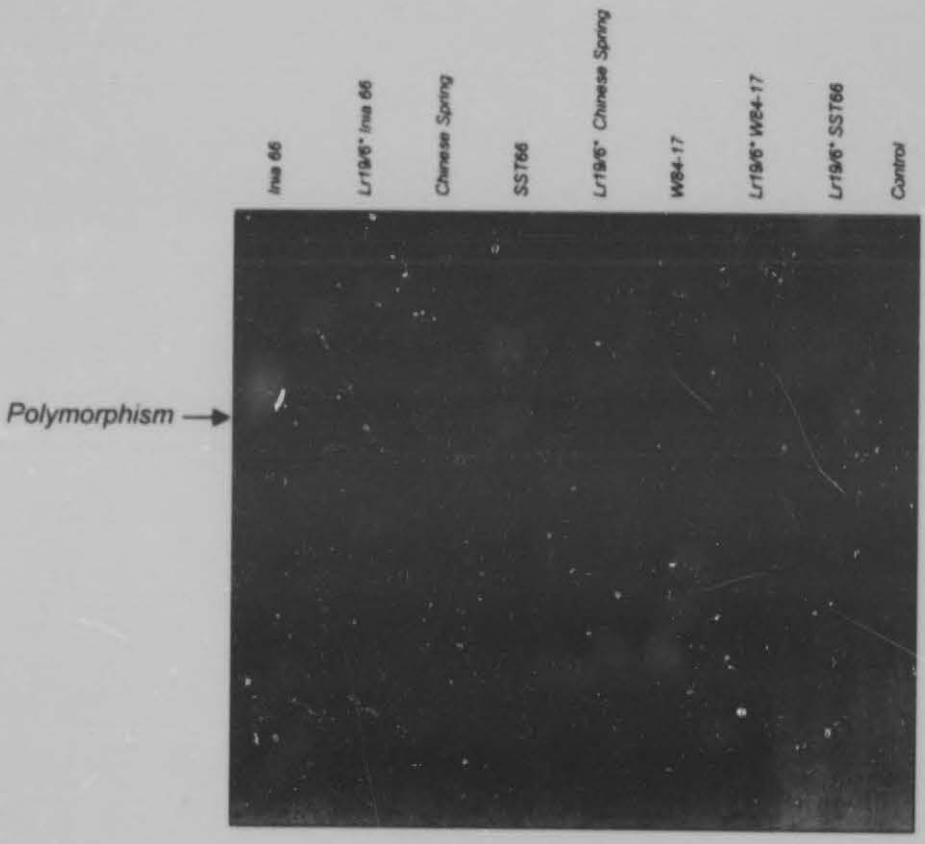
Figure 3.7 RAPD profiles produced with the NIL lines by using RAPD primer OPL3. The last lane shows the negative DNA control reaction.



3.3.3 RAPD fragment OPY17-R_{850}

This is another repulsion phase marker identified with primer OPY17. This fragment, like the previous ones is specific to wheat and is not amplified on the *Thinopyrum* translocation (Figure 3.8).

Figure 3.8 RAPD profiles of the NIL lines with OPY17. The final lane shows the DNA negative control reaction.



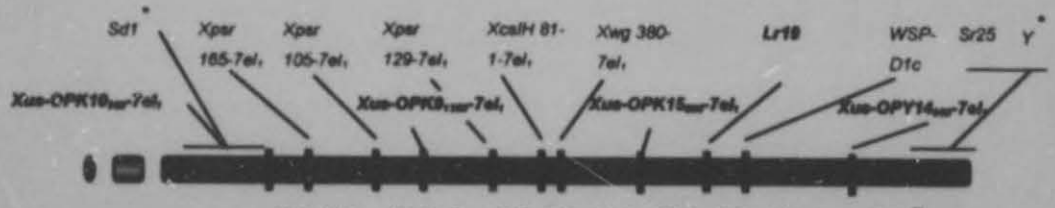
3.4 SUMMARY

A total of four reproducible, coupling phase RAPD markers for the *Lr19* translocation on chromosome 7DL were identified as well as three repulsion phase markers associated with the replaced wheat segment. Another four markers have also been identified (only two shown, Figure 3.3), but were found not to be reproducible and were excluded from further study. It may be possible, however, to adjust the amplification conditions and to consistently reproduce the latter polymorphisms.

3.5. MAPPING OF FOUR RAPD MARKERS.

The four RAPD markers were mapped onto the *Lr19* translocated segment with the use of the deletion mapping population. RAPDs were performed using the same conditions as those used in the original screening process. The approximate size order of the deletion mutants for the translocation (largest to smallest) were known (Prins *et al*, 1996). All 29 mutants were used to amplify fragments with the four RAPD primers and the locations of the polymorphic loci relative to the markers already mapped, were deduced. The map positions of the identified RAPD markers are shown in Figure 3.9. Results obtained with this study were integrated with the existing physical maps of the *Lr19* translocation (Prins *et al*, 1996; Prins *et al*, 1997).

Figure 3.9 Results of a deletion mapping experiment to add the four RAPD markers to a physical map produced by Prins *et al.* (1996).



1. 87M23-145	+	a	-	-	-	-	-	-	-	-	-	-	-	-	
2. 87M23-198	+	a	-	-	-	-	-	-	-	-	-	-	-	-	
3. 89M1-51	+	b	-	-	-	-	-	-	-	-	-	-	-	-	
4. 87M23-1	+	b	+	-	-	-	-	-	-	-	-	-	-	-	
5. 89M2-327	+	a	+	-	-	-	-	-	-	-	-	-	-	-	
6. 87M23-178	+	a	+	+	-	-	-	-	-	-	-	-	-	-	
7. 87M23-227	+	c	+	+	-	-	-	-	-	-	-	-	-	-	
8. 87M23-273	+	a	+	+	-	-	-	-	-	-	-	-	-	-	
9. 89M1-25	+	a	+	+	-	-	-	-	-	-	-	-	-	-	
10. 89M1-69	+	d	+	+	-	-	-	-	-	-	-	-	-	-	
11. 89M1-78	+	a	+	+	-	-	-	-	-	-	-	-	-	-	
12. 87M23-314	+	a	+	+	-	-	-	-	-	-	-	-	-	-	
13. 87M23-118	+	a	+	+	+	+	-	-	-	-	-	-	-	-	
14. 89M2-39	+	c	+	+	+	+	-	-	-	-	-	-	-	-	
15. 87M23-27	+	d	+	+	-	+	+	-	-	-	-	-	-	-	
16. 87M23-3	+	a	+	+	+	+	+	+	-	-	-	-	-	-	
17. 87M23-173	+	c	-e	+	+	+	+	+	-	-	-	-	-	-	
18. 87M23-108	+	d	+	+	+	+	+	+	+	-	-	-	-	-	
19. 87M23-115	+	a	+	+	+	+	+	+	+	-	-	-	-	-	
20. 87M23-128	+	d	+	+	+	+	+	+	+	-	-	-	-	-	
21. 89M2-416	+	b	+	-e	+	+	+	+	+	-	-	-	-	+f,g	
22. 87M23-219	+	d	+	+	+	+	+	+	+	-	-	+f	-	-	
23. 89M2-40	+	c	+	+	+	+	+	+	+	-	-	-	-	-	
24. 87M23-103	+	c	+	+	+	+	+	+	+	+	-	+f	-	-	
25. 89M2-245	+	d	+	+	+	+	+	+	+	+	-	-	-	-	
26. 87M23-225	+	d	+	+	+	+	+	+	+	+	+	-	-	-	
28. 89M2-426	+	d	+	+	+	+	+	+	+	+	+	-	+	-	
27. 89M1-18	+	b	+	+	+	+	+	+	+	+	+	+	-	+f,g	
29. 87M23-266	+	a	+	+	+	+	+	+	+	+	+	+	+	+	

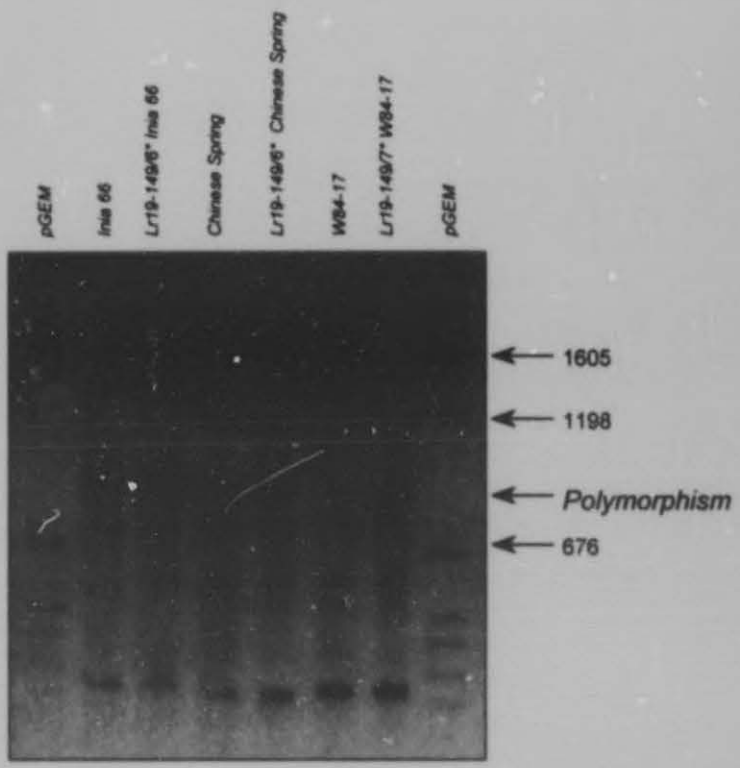
(a-d are based on segregation of the mutated translocation in different full sib families.)
a Preferential/normal transmission and self-elimination.
b Only self-elimination observed.
c Apparently normal segregation ratios - low seed set.
d Only preferential transmission observed.
e Intercalary deletion.
f Dubious classifications.
g Intermediate pherotypes suggesting the possibility of more than one locus.
* Order unknown.
** Arbitrary physical map units.

3.6 TESTING OF RECOMBINANT FORMS OF *LR19* AND *LR19*-149 TRANSLOCATION FOR THE PRESENCE OF THE *Xus-OKP15$_{800}$-7el$_1$* LOCUS

RAPD locus *Xus-OPK15$_{800}$-7el$_1$* (proximal) and *Xus-OPY14$_{950}$-7el$_1$* (distal) appear to be the closest to *Lr19*. The third locus *Xus-OPK9$_{1350}$-7el$_1$* mapped between the existing RFLP loci, *Xpsr 129-7el$_1$* and *Xpsr 105-7el$_1$*. *Xus-OPK10$_{350}$-7el$_1$* mapped closest to the centromere and was the only marker present in all the deletion mutants, except two.

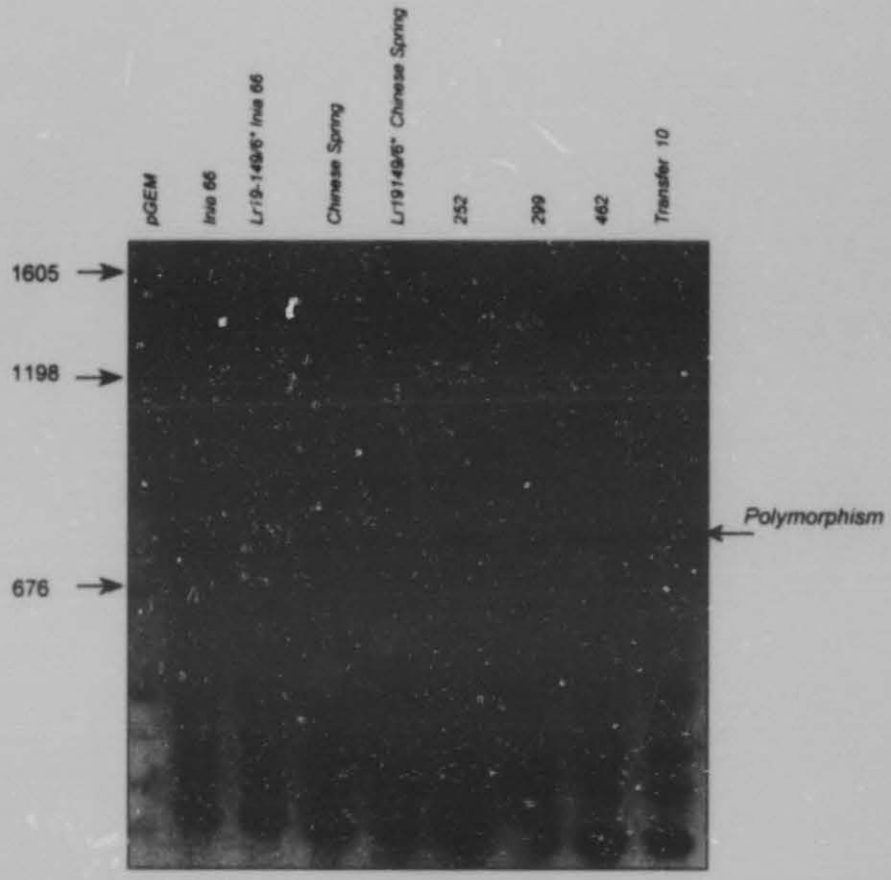
The four RAPD markers were also tested on the shortened *Lr19*-149 translocation (Figure 3.10). This was possible, making use of NIL lines similar to the NILs for the complete translocation used during the original RAPD screening process. The *Lr19*-149 translocation is available in the genetic backgrounds of Chinese Spring, Inia 66 and breeding line W84-17. This provides additional molecular evidence that the the shortened *Lr19*-(149) segment is in fact derived from the original longer *Lr19* translocation segment. The RAPD locus *Xus-OPK15$_{800}$-7el$_1$* was the only marker to be verified on this translocation.

Figure 3.10 The locus *Xus-OPK15$_{800}$-7el$_1$* verified on the shortened *Lr19*-149 translocation. The first and last lines indicates the DNA sizes as indicated by the pGEM DNA ladder standard.



Plant material of the recombinant 'Transfer 10' produced by Sears (1977) and the three putative *Lr19*-149 recombinants produced by Marais & Marais (1998) was used to test for the RAPD marker *Xus-OPK15$_{800}$-7el$_1$*. The marker tested positive (Figure 3.11) on Transfer 10 and two of the *Lr19*-149 recombinants (nos. 252 and 462). The *Lr19*-149 recombinant no 299, did not produce the OPK15$_{800}$ fragment. This would imply that the latter recombinant has lost the region on the translocation carrying this locus and must therefore be substantially shortened. All three of the recombinant *Lr19*-149 lines still carry the *Sd2* segregation distortion locus (Marais, 1998 – personal communication).

Figure 3.11 RAPD profiles generated by applying the OPK15 primer to three putative recombinants of the *Lr19*-149 translocation and the *Lr19* recombinant, 'Transfer 10' produced by Sears (1977).



CHAPTER 4

4.1 DISCUSSION

The RAPD technique has been shown to provide an effective means of identifying markers linked to a gene of interest (Williams *et al*, 1990; Welsh and McClelland 1990), this has been especially true with regard to alien translocations. The advantages of a RAPD approach include inexpensive materials and equipment while the use of dangerous chemicals and radioactive substances is not required. The screening process can be highly mechanised, as is the case with most PCR based procedures. The identification of markers linked closely to a trait of interest becomes a more realistic and achievable goal where an alien chromosome segment is involved. Such translocations may be relatively large and may resist recombination with homoeologous wheat chromatin. In this study, four new markers could be added to the genetic map of the *Lr19* translocation, while three new repulsion phase markers were found for the corresponding displaced *Triticum aestivum* chromatin.

The initial NIL screening populations used were based on the complete *Lr19* translocation. The genetic backgrounds of these NILs were Chinese Spring, Inia 66, SST66 and the breeding line W84-17. These NIL lines have been characterised and were used to map RFLP markers on the *Lr19* translocation (Prins and Marais, 1998b). The respective isogenic lines resulted from five or more backcrosses to the recurrent parents. This implies that less than 1% of the chromatin of the donor parent has remained. The RAPD profiles obtained confirmed that there was very little background variation between members of a pair of NILs. The reliability of the *Lr19* NILs plus the fact that four NIL pairs were available ensured that the polymorphisms detected were real.

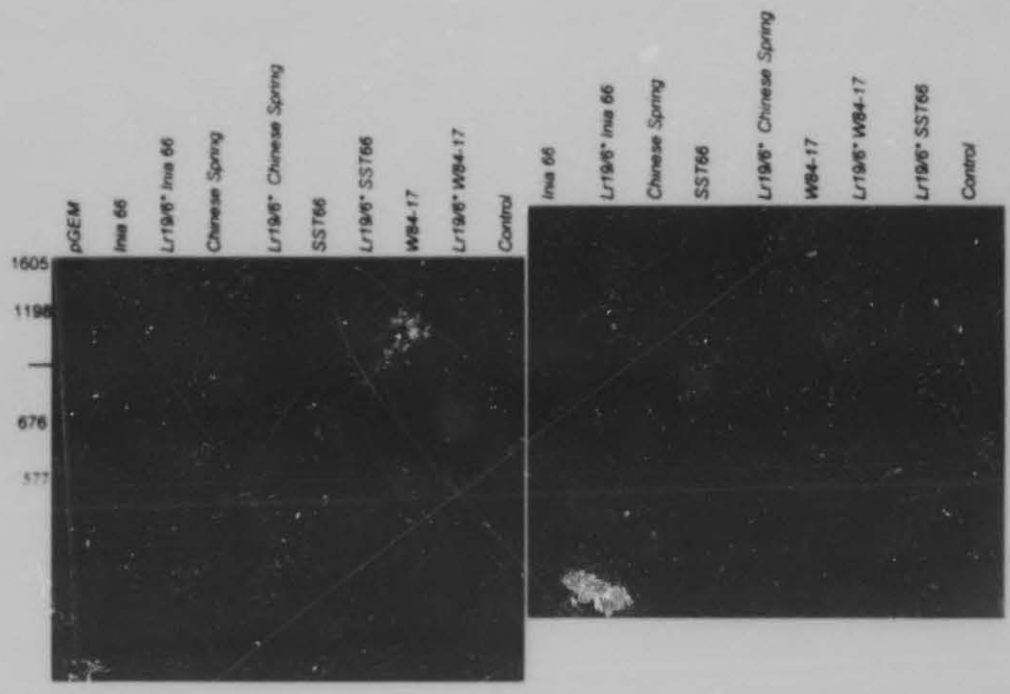
4.2 REPRODUCIBILITY OF THE RAPD REACTION

Experimental artefacts and miss-amplified fragments can cause variability in RAPD profiles. Contamination from external DNA sources can also present problems during RAPD amplification, which can be overcome by building in replication. When working with a specific NIL or DNA screening population, contamination if it occurs is more likely to be present in all the DNA samples in use and will seldom result in false polymorphisms. Tanksley *et al.* (1995) concluded that RAPDs can be considered one of the best high volume marker techniques available for screening large NIL pairs or bulked genotypes.

Following the initial optimisation of the RAPD procedure, and experimenting with different Taq amplification enzymes, it was noted that certain enzymes amplify certain fragment sizes preferentially, and this was very evident when comparing the RAPD profiles generated from different enzymes. The profiles did, however, not differ with regard to the majority of amplified products, merely to the size scale of the fragments amplified i.e. adding smaller or larger fragments. Taq enzymes isolated from different bacterial strains may differ in the preferred sizes of fragments being amplified (Cheng *et al.* 1994). It is also evident that differences in enzymes (Figure 4.1) may lead to the resulting RAPD reaction producing more or less bands more or less 'relaxed' profiles. Slight differences among thermal cyclers in controlling the amplification temperature can also lead to differences in the amplified RAPD profiles. Repeatability and the ability to repeat RAPD results between laboratories have proven to be a problem (Weising *et al.* 1995).

The mapping of the four *Thinopyrum ponticum* specific markers contributes to the ongoing global effort to construct detailed genetic maps of all the important agronomical crop species. The RAPD marker locus *Xus-OPK10*$_{350}$*-7el*$_1$ was mapped closest to the centromere and was the only marker visible in nearly all of the deletion mutant mapping population individuals. The relative length of *Thinopyrum* and wheat chromatin located proximally of the *Sd1* and *Xpsr 165-7el*$_1$ loci is unknown (Figure 3.9). The distance of the marker from the centromere can therefore not be determined.

Figure 4.1 A comparison of two different amplification enzymes using the same DNA and reaction conditions. The arrow indicates the RAPD marker fragment $OPY14_{950}$.



The second RAPD locus *Xus-OPK9*$_{1350}$-*7el*$_1$ is located between the RFLP markers *Xpsr 105-7el*$_1$ and *Xpsr 129-7el*$_1$. This chromosomal area is one of the largest inter-marker segments, spanning an area largely devoid of markers. Eight deletion lines have breakpoints in this region and up to now it was impossible to order them according to size. With the mapping of the *Xus-OKP9*$_{1350}$-*7el*$_1$ locus it now becomes possible to reorder and reclassify the mutants to some extent.

Marker *Xus-OPK15*$_{800}$-*7el*$_1$ was mapped between the existing RFLP markers *Xwg 380-7el*$_1$ and *Lr19*. *Xus-OPK15*$_{800}$-*7el*$_1$ is the closest proximal marker to *Lr19* and it may be worth while to convert it into a SCAR marker. However, it will be difficult to isolate the single fragment since the polymorphism is closely associated with other fragments of similar sizes (Figure 3.4). Problems encountered because of this have hampered the cloning of this polymorphic band. Attempts were made to better

separate the fragments on higher percentage gels to enable cloning. However, only small amounts of DNA could be recovered from these gels.

The three putative recombinant lines *Lr19*-149-299, *Lr19*-149-252 and *Lr19*-149-462 which have exchanged chromatin proximal to *Lr19* for wheat chromatin in an allosyndetic pairing experiment (Marais and Marais, 1998) were also tested with the *Xus-OPK15*$_{800}$-*7el*$_1$ marker (Figure 3.11). Positive polymorphisms were detected on lines *Lr19*-149-252 and *Lr19*-149-462. The third line *Lr19*-149-299 does not contain the marker implying that this is the shortest of the three recombinants. These plants have been tested with a virulent isolate to confirm the presence of resistance comparable to *Lr19*. The presence of the marker *Xus-OPK15*$_{800}$-*7el*$_1$ therefore serves as evidence of the authenticity of the two lines containing the marker. Figure 3.10 also shows that *Xus-OPK15*$_{800}$-*7el*$_1$ is amplified on the 'Transfer 10' translocation (Sears *et al*, 1977). This translocation differs from the T4 (*Lr19*) and *Lr19*-149 translocations in that it contains less *Thinopyrum* chromatin proximal to *Lr19*. This could therefore be used in crosses with the *Lr19*-149 translocation in an attempt to further reduce the amount of *Thinopyrum* chromatin through recombination. However, the present results would suggest that the recombinant, *Lr19*-149-299 contains even less *Thinopyrum* chromatin proximal of *Lr19* as compared to Transfer 10.

The final RAPD marker OPY14$_{950}$ mapped to the distal side of *Lr19* and appears to be the most distal marker. The size of the area distally to the two phenotypic markers, *Sr25* (stem rust resistance) and the yellow endosperm is largely unknown.

4.2.1 ENRICHMENT OF DNA FOR RAPD ANALYSIS

Enrichment strategies to increase the likelihood of detecting polymorphisms among different genotypes have been used to a limited degree in cases where high-copy DNA sequences can be easily removed. Chromatography combined with selective DNA binding methods is usually used for this purpose. This approach is based on the principle of complex DNA vs. non-complex DNA and their relation to coding and non-coding sequences found in the genome. In plant species with extensive genomes,

such as is the case in wheat, the repetitive sequences may account for up to 75% of the DNA and thus greatly outnumber the non-repetitive or 'coding DNA sequences' (Smith and Flavell, 1975). As genomes are studied and more insight is gained in the makeup of larger genomes, speculation increases regarding the evolutionary significance of repeated sequences. When working with large plant genomes, one would expect the loss of DNA after enrichment to be more than 2/3 because of the large number of repeated sequences found in wheat. This was found after the completion of RAPDs on the enriched DNA, since approximately this proportion of fragments, and thus RAPD loci detected by the specific primers, were effectively removed (Figure 3.1). Given that a RAPD reaction is largely driven by the availability of sequences complementing that of the primer, the removal thereof, will lead to a similar reduction in the number of fragments amplified. The initial purpose of the enrichment strategy was to amplify RAPD loci present in enriched DNA, as expected in coding regions that may be under-represented during the RAPD reaction due to the availability of multiple sequences of the same sort being preferentially amplified in the RAPD reaction. Evidence to support this could, however, not be found since no new fragments were observed using the enriched DNA as opposed to the DNA that was not enriched. DNA binding in the hydroxyapatite column is highly dependent on the temperature of the column since rapid renaturing of the denatured DNA will occur even with the slightest of temperature decreases. This will inevitably lead to a much larger fraction of the DNA being removed in the double stranded form and discarded.

The RAPD reaction methodology is strongly influenced by the template concentration, unlike its PCR counterpart where minimal template quantities are required for successful amplification at high annealing temperatures, leading to very specific reaction amplification, regardless of the amount of DNA used. Even if the samples are treated in exactly the same way during enrichment a temperature gradient may develop in the water bath leading to small differences in the amounts of DNA recovered. This can be expected to result in RAPD loci being present in some samples and absent from others, making the detection thereof inconsistent. They may then be classified as polymorphic, when the fragments are in fact not.

As explained in the second chapter, a major problem experienced in the enrichment of DNA on a hydroxyapatite column was the low amounts of DNA recovered and the need to concentrate it. For this reason, very large amounts of DNA are required initially, which have to be produced using the more laborious 'maxi-preparation' DNA isolation procedures. However, optimisation of the enriched protocol may improve DNA retrieval. The results obtained with the RAPDs performed on the enriched DNA suggested that a considerably decreased number of fragments is likely to result. The number of amplification fragments scored and analysed in the screening process remains central to the chances of a valid polymorphic fragment being identified and tested. The low number of amplified fragments obtained from the enriched DNA, and the large number of fragments needed for scoring, has made this technique less suitable for the study.

4.3 EXTENT OF THE STUDY

Schaechermayr *et al.* (1994) tested 395 RAPD primers in a similar study to find markers for *Lr9* (carried on a chromosomal translocation from *Triticum umbellulatum*). NILs were constructed with the *Lr9* gene in *Triticum aestivum* and *Triticum spelta* backgrounds. Only three of the primers produced polymorphic bands. In a later study Iqbal and Rayburn, (1995) also used RAPDs in an attempt to find markers on the 1RS introgressed rye chromosomal segment in wheat and identified polymorphisms in a similar way. The latter translocation was similar to the *Lr19* translocated segment used here in that an entire chromosome arm was translocated, and it required 120 arbitrary primers to identify two repeatable polymorphisms.

4.4 IMPACT ON BREEDING STRATEGIES

The complete *Lr19* translocation has no detrimental effects on the quality and agronomic performance of a wheat plant. However, in many western countries the yellow endosperm pigmentation associated with its use is commercially unacceptable. Also, the translocation has replaced a large portion of chromosome arm 7DL of wheat meaning that in breeding the *Lr19* resistance cannot be combined with other genes such as those for Russian wheat aphid resistance (*Dn2* and *Dn5*) or eyespot resistance *Pch1*. For this reason an attempt was made to reduce the amount of foreign chromatin. *Xus-OPK15$_{800}$-7el$_1$* has been useful in the process of deciding which of three putative shortened forms will be the most useful. Since the shortest form, *Lr19*-149-299, does no longer have the *Xus-OPK15$_{800}$-7el$_1$* locus, it cannot be used in marker assisted selection.

The original *Lr19* (T4) translocation is used as such in some countries. It can also be used to provide resistance in feed wheats. Any of the 4 RAPD markers identified here can be used to follow the translocation.

CHAPTER 5

5.1 CONCLUSION

The widespread application and usefulness of the RAPD technique, has led to large numbers of RAPD markers being mapped in many species. A total of four polymorphic RAPD markers have been identified on the *Lr19* translocated chromosome arm. In addition, three repulsion phase markers have also been associated with the displaced wheat chromosome arm 7DL. Three more markers were found not to be sufficiently repeatable, and may produce more consistent results under specifically optimised conditions.

The four coupling phase markers have been physically mapped relative to the other known loci on the *Lr19* translocation. The RAPD loci *Xus-OPK15*$_{800}$*-7el*$_1$ is the closet to the *Lr19* gene and could be used to identify the shortest of three putative recombinants of *Lr19*-149.

CHAPTER 6

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