Tagging and mapping of prominent structural genes on chromosome arm 7DL of common wheat

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DECLARATION

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

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ABSTRACT

Chromosome arm 7DL of common wheat carries genes for agronomically important traits such as leaf rust, stem rust, Russian wheat aphid and eye spot resistance. Some of these genes occur on introgressed foreign chromatin, which restricts their utility in breeding. The 7DL genetic maps are poorly resolved, which seriously hampers attempts to manipulate the genes and introgressed regions in breeding. This dissertation represents an attempt to improve our knowledge of the relative map positions of three resistance genes that have significant potential for use in local breeding programmes.

The leaf rust resistance gene, *Lr19*, is located on a *Thinopyrum ponticum*-derived translocation which occupies a large part of the terminal end of 7DL. The translocation also carries genes for less favourable traits such as yellow flour colour. Attempts have been made to reduce the size of the translocation through allosyndetic pairing induction; the primary aims being to remove deleterious genes and to minimise the amount of foreign chromatin associated with *Lr19* so it can be recombined with other useful 7DL genes. Twenty-nine 'Indis'-derived *Lr19* deletion mutants were previously produced by gamma irradiation and a physical map was constructed. In this study, the set of mutant lines were further analysed using 144 *Sse*8387I/*Mse*I and 32 *Eco*RI/*Mse*I amplified fragment length polymorphism (AFLP) primer combinations. The previous physical map, which was based on five restriction fragment length polymorphism (RFLP) markers and five structural gene loci, was extended and now includes 95 novel AFLP markers (86 *Sse*8387I/*Mse*I and 9*Eco*RI/*Mse*I markers), of which seven map close to *Lr19*. Most of the deletions could be ordered according to size and the improved map has already been used to characterise shortened recombinant forms of the *Lr19* translocation. An unsuccessful attempt was made to convert one of the seven markers closest to *Lr19* into a sequence-specific marker. However, an AFLP marker located distally from *Lr19* was successfully converted into a sequence-specific marker in collaboration with other researchers.

An attempt was also made to map and tag the Russian wheat aphid (RWA) resistance gene, *Dn5*. A doubled haploid mapping population consisting of 94 lines was created and typed for *Dn5*, four microsatellite loci and the endopeptidase locus, *Ep-D1*. The *Dn5* locus mapped 25.4 cM and 28.6 cM distally from *Xgwm111* and *Xgwm437*, respectively, but was not linked to

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Xgwm428, *Xgwm37* or *Ep-D1*. Tagging of *Dn5* was attempted by screening twelve homozygous resistant and seven homozygous susceptible F_2 lines from a cross between 'Chinese Spring' and 'PI 294994' with 70 *Sse*8387I/*Mse*I AFLP primer combinations. Only two potentially useful polymorphisms (one in coupling and one in repulsion phase) were identified. Conversion of the coupling phase marker to a sequence-specific marker was not successful.

The eyespot resistance gene, *Pch1*, was derived from *Triticum ventricosum* and is present in the wheat VPM-1. Close association between *Pch1* and the endopeptidase *Ep-D1b* allele has been reported previously. *Pch1*/*Ep-D1* was tagged by screening ten wheat genotypes (each homozygous for the confirmed presence or absence of *Pch1* and/or *Ep-D1b*) with 36 *Sse*8387I/*Mse*I AFLP primer combinations. Three AFLP markers were closely associated with *Pch1*/*Ep-D1*, one of which was targeted for conversion into a sequence-specific marker. The sequence-specific marker contained a microsatellite core motif and was found to be useful for tagging *Pch1*/*Ep-D1*. A genetic distance of 2 cM was calculated between the novel microsatellite marker and *Ep-D1*. The microsatellite marker was also polymorphic for the *Lr19* translocation and it was possible to map it between the *Wsp-D1* and *Sr25* loci.

In this dissertation, mapping and/or tagging of three important resistance genes were achieved. Due to the fact that all markers used in these studies were not polymorphic between all of the targeted regions, it was not possible to fully integrate the data obtained for the three regions.

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OPSOMMING

Chromosoom arm 7DL van broodkoring dra gene vir agronomies-belangrike kenmerke soos blaarroes, stamroes, Russiese koringluis en oogvlek weerstand. Sommige van hierdie gene kom voor in blokke spesie-verhaalde chromatien wat hul bruikbaarheid in teling beperk. Die genetiese kaarte van 7DL is swak ontwikkel en dit maak dit baie moeilik om hierdie gene en spesie-verhaalde streke tydens teling te manipuleer. Hierdie proefskrif verteenwoordig 'n poging om kennis van die relatiewe kaart liggings van drie weerstandsgene, met betekenisvolle potensiaal in plaaslike teelprogramme, te verbreed.

Die blaarroes weerstandsgeen, *Lr19*, kom voor op 'n *Thinopyrum ponticum*-verhaalde translokasie wat 'n groot terminale gedeelte van 7DL beslaan. Die translokasie dra ook gene vir minder gewensde kenmerke soos geel meelkleur. Pogings is aangewend om die translokasie deur homoeoloë parings-induksie te verkort. Die doel was om nadelige gene te verplaas en die hoeveelheid vreemde chromatien geassosieer met *Lr19* te minimiseer sodat dit met ander nuttige gene op 7DL gerekombineer kan word. Nege-en-twintig 'Indis'-verhaalde *Lr19* delesie mutante is vroeër met gamma bestraling geproduseer en gebruik om 'n fisiese kaart op te stel. Teenswoordig is die stel mutante verder ontleed met behulp van 144 *Sse*8387I/*Mse*I en 32 *Eco*RI/*Mse*I amplifikasie-fragment-lengte-polimorfisme (AFLP) inleier kombinasies. Die bestaande fisiese kaart, wat gebaseer was op vyf restriksie-fragment-lengte-polimorfisme (RFLP) merkers en vyf strukturele geen loki, is uitgebrei en sluit nou 95 unieke AFLP merkers (86 *Sse*8387I/*Mse*I en 9*Eco*RI/*Mse*I merkers) in, waarvan sewe naby aan *Lr19* karteer. Die meeste van die delesies kon op grond van hulle grootte gegroepeer word en die verbeterde fisiese kaart is alreeds gebruik om verkorte rekombinante vorms van die *Lr19* translokasie te karakteriseer. 'n Onsuksesvolle poging is aangewend om een van die sewe merkers naaste aan *Lr19* om te skakel na 'n volgorde-spesifieke merker. 'n AFLP merker wat distaal van *Lr19* karteer is egter wel suksesvol in samewerking met ander navorsers omgeskakel na 'n volgorde-spesifieke merker.

'n Poging is ook aangewend om die Russiese koringluis (RKL) weerstandsgeen, *Dn5*, te karteer en merkers gekoppel aan die geen te identifiseer. 'n Verdubbelde-haploïede karteringspopulasie van 94 lyne is geskep en getipeer vir *Dn5*, vier mikrosatelliet loki en die endopeptidase lokus, *Ep-D1*. Die *Dn5* lokus karteer 25.4 cM en 28.6 cM distaal van *Xgwm111* en *Xgwm437*,

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respektiewelik, maar was nie gekoppel met *Xgwm428*, *Xgwm37* of *Ep-D1* nie. Twaalf homosigoties weerstandbiedende en sewe homosigoties vatbare F_2 lyne uit die kruising: 'Chinese Spring' / 'PI 294994' is met 70 *Sse*8387I/*Mse*I AFLP inleier kombinasies getoets in 'n poging om merkers vir *Dn5* te identifiseer. Slegs twee moontlik bruikbare polimorfismes (een in koppelings- en een in repulsie fase), is geïdentifiseer. Omskakeling van die koppelingsfase merker na 'n volgorde-spesifieke merker was onsuksesvol.

Die oogvlek weerstandsgeen, *Pch1*, is uit *Triticum ventricosum* oorgedra en kom voor in die koringlyn, VPM-1. Noue koppeling van *Pch1* en die endopeptidase alleel, *Ep-D1b*, is vantevore gerapporteer. Merkers is vir *Pch1*/*Ep-D1* gevind deur tien koring genotipes (elkeen homosigoties vir die bevestigde teenwoordigheid of afwesigheid van *Pch1* en/of *Ep-D1b*) te toets met 36 *Sse*8387I/*Mse*I AFLP inleier kombinasies. Drie AFLP merkers is gevind wat nou koppel met *Pch1*/*Ep-D1*, waarvan een gekies is vir omskakeling na 'n volgorde-spesifieke merker. Die volgorde-spesifieke merker het 'n mikrosatelliet kernmotief bevat en was nuttig as merker vir *Pch1*/*Ep-D1*. 'n Genetiese afstand van 2 cM is tussen die unieke mikrosatelliet merker en *Ep-D1* bereken. Die mikrosatelliet merker was ook polimorfies vir die *Lr19* translokasie en dit is tussen die *Wsp-D1* en *Sr25* loki gekarteer.

Kartering en/of identifikasie van merkers vir drie belangrike weerstandsgene was suksesvol in hierdie studie. Omdat al die merkers wat gebruik is, nie polimorf was tussen al die streke van belang nie, was dit nie moontlik om die data vir elk van die drie streke ten volle te integreer nie.

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LIST OF COMMON ABBREVIATIONS

A	adenine
Ae.	*Aegilops*
AFLP	amplified fragment length polymorphism
AP-PCR	arbitrary primed PCR
ASAP	allele specific associated primer / arbitrary signatures from amplification profiles
BAC	bacterial artificial chromosome
BMV	brome-mosaic virus
BSA	bulked segregant analysis
C	cytosine
CAPS	cleaved amplified polymorphic sequence
cDNA	copy DNA
CEPH	Centre d'Etude du Polymorphisme Humaine
CS	'Chinese Spring'
dATP	deoxyadenosine 5'-triphosphate
DAF	DNA amplification fingerprinting
ddH_2O	deionized distilled water
DH	doubled haploid
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ds	double-stranded
e.g.	*exempli gratia* (Latin: for example)
et al.	*et alii* (Latin: and others)
etc.	*et cetera* (Latin: and so forth)
FISH	fluorescent *in situ* hybridisation
F_n	n^{th} generation
gDNA	genomic DNA
G	guanine
GISH	genomic *in situ* hybridisation
ISH	*in situ* hybridisation
ISSR	inter-simple sequence repeat
ITMI	International Triticeae Mapping Initiative
L	long arm of chromosome

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LB	Luria-Bertani
LRR	leucine rich repeat
MAAP	multiple arbitrary amplicon profiling
mRNA	messenger RNA
NBS	nucleotide binding site
NIL	near isogenic line
nt	nucleotide
P.	*Pseudocercosporella*
p.	page
pp.	pages
PCR	polymerase chain reaction
QTL	quantitative trait locus
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RGA	resistance gene analog
RIL	recombinant inbred line
RNA	ribonucleic acid
RWA	Russian wheat aphid
S	short arm of a chromosome
SAMPL	selective amplification of microsatellite polymorphic DNA
SCAR	sequence characterised amplified region
ss	single-stranded
ssp.	sub-species
SSCP	single-strand conformational polymorphism
SSD	single seed descent
SSR	simple sequence repeat
STS	sequence tagged site
syn.	synonym
T	thymine
T.	*Triticum*
tecMAAP	template endonuclease cleavage MAAP
tRNA	transfer RNA
UV	ultra violet
var.	variant
viz	*vidalicet* (namely)
YAC	yeast artificial chromosome

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LIST OF ABBREVIATIONS USED FOR MEASUREMENT

bp	base pair
°C	degrees Centigrade
Ci	Curie
cm	centimetres
cM	centiMorgan
g	grams / gravity
hr	hour
kb	kilobase pairs
M	molar (moles per litre)
mA	milliAmpére
Mbp	megabase pairs
MBq	megaBecquerel
mCi	milliCurie
mg	milligrams
min	minute
ml	millilitres
mM	millimolar
mm	millimetres
mmol	millimol
ng	nanogram
nmol	nanomol
OD_{xxx}	optical density at wave length xxx
rpm	revolutions per minute
s	second(s)
TBq	teraBecquerel
v	volume
w	weight
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre
V	Volt
w/v	weight per volume
w/w	weight per weight

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LIST OF ABBREVIATED LOCUS AND GENE NAMES

α -Amy	α-amylase isozyme
avr	avirulence gene
Bdv	barley yellow dwarf virus resistance gene
Cf	resistance to *Cladosporium fulvum*
cn-N	chlorina mutant, chromosome N
Cre	cereal cyst nematode resistance gene
Dn	Russian wheat aphid resistance gene
Ep	endopeptidase
Gli	storage gene
Gm	gall midge resistance gene
H	Hessian fly resistance gene
Hm	resistance to *Cochliobolus carbonum*
Hs	sugar beet cyst nematode resistance gene
I_2-*C*	resistance to *Fusarium oxysporum* f.sp. *lycopersicon*
Kr	crossability gene
L	resistance to *Melampsora lini*
Lr	leaf rust resistance gene
Ltn	leaf tip necrosis gene
M	resistance to *Melampsora lini*
Meu	potato aphid resistance gene
Mi	root knot nematode resistance gene
Mla	powdery mildew resistance gene
N	tobacco mosaic virus resistance gene
Pch	eyespot resistance gene
ph	pairing homologous gene
Pm	powdery mildew resistance gene
Pto	resistance to *Pseudomonas syringae* pv. *tomato*
Pvr	potyvirus resistance gene
R	resistance gene
RPM	resistance to *Pseudomonas syringae*
RPS	resistance to *Pseudomonas syringae*
Sd	segregation distortion

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Sr	stem rust resistance gene
Wsp	water soluble protein gene
Xa	resistance to *Xanthomonas oryzae*
Y	yellow endosperm
Yr	yellow rust resistance gene

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CHAPTER ONE

LITERATURE REVIEW

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1. INTRODUCTION

Wheat is an annual grass that can be grown from sea level to altitudes of more than 3,000 metres. It prefers well drained, clay-loam soils and is adapted to temperate, arid or semi-arid environments. As a food source, it provides about 20% of the world's food calories, is staple food for nearly 40% of the world's population and supplies almost 55% of the carbohydrates consumed world-wide (Wiese, 1977 p. 1; Gupta *et al.*, 1999).

1.1 CLASSIFICATION OF BREAD WHEAT

Wheat belongs to the genus *Triticum* L. of the grass family *Gramineae* or *Poaceae* (Figure 1). The grass family comprises of more than 10 000 species, of which hexaploid bread (common) wheat (*Triticum aestivum* L. em Thell) is highly valued for bread making while tetraploid durum wheat (*Triticum durum*) is more suitable for the production of pasta products. Other cultivated cereals include spelt (*Triticum spelta*), emmer (*Triticum dicoccum*), einkorn (*Triticum monococcum*), barley (*Hordeum vulgare*), triticale (*Triticosecale*), oat (*Avena sativa*) and rye (*Secale cereale*). All of these are taxonomically grouped in the tribe *Triticeae*. The subtribe *Triticinae* is made up of the genera *Aegilops*, *Agropyron*, *Eremopyron*, *Haynaldia*, *Triticum* and *Secale* (Morrison, 1995). Cultivated wheat can be divided into three ploidy groups containing 7, 14 and 21 chromosome pairs, called di-, tetra- and hexaploids, respectively. Common and club wheats have 21 chromosome pairs (consisting of three genomes - AABBDD). Durum wheat has 14 chromosome pairs, consisting of two genomes (AABB) (Wiese, 1977 p. 1). Diploid wheat comprises a single genomic group (AA) which is common to all the polyploid wheats.

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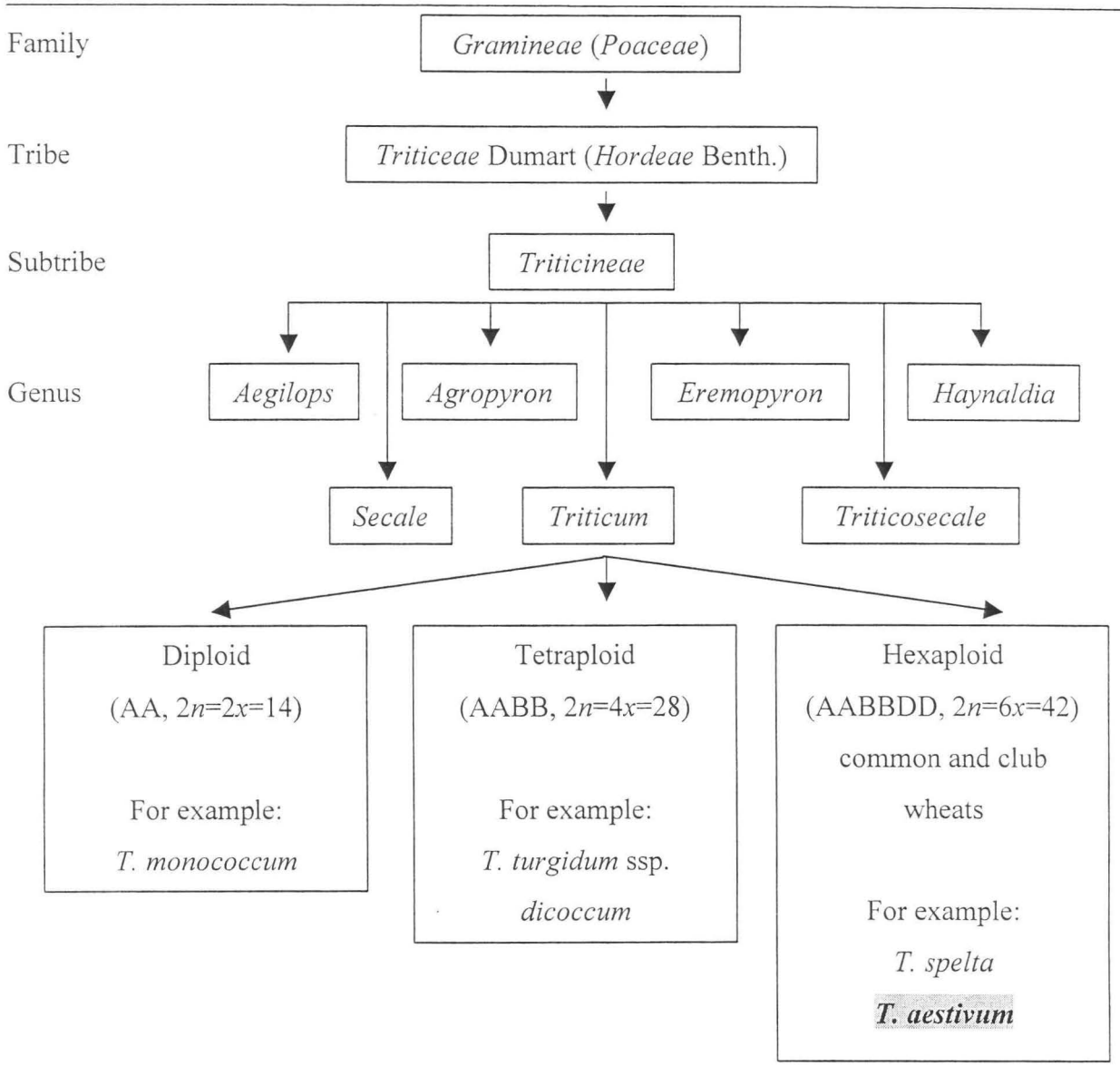

Figure 1. Partial schematic representation of the classification of common wheat (*Triticum aestivum*). Adapted from Miller (1987) and Morrison (1995)

1.2 The Genome of Bread Wheat

Bread wheat is a self-pollinating hexaploid species. The genome (2n = 6x = 42, AABBDD) contains approximately 3.2 x 10^{10} base pairs of DNA (Bennett and Smith, 1976; May and Appels, 1987) and the total length of the haploid chromosome complement is 235.4 μm (Gill *et al.*, 1991). Each 4C euploid bread wheat nucleus (somatic nucleus after DNA replication but before cell division) contains approximately 69.3 picograms of DNA (Bennett and Smith, 1976). Three average wheat chromosomes are equal in size to the haploid maize genome and the haploid rice genome is equal to one half of an average wheat chromosome (Gill and Gill, 1994).

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The ratio of the DNA contents of the three diploid genomes in hexaploid wheat is 1.0 : 1.2 : 0.8 (Furata *et al.*, 1988). Up to 90% of the wheat genome contains repetitive DNA and the three genomes share approximately 30% sequence homology (Flavell *et al.*, 1977; Moore *et al.*, 1993; Donini *et al.*, 1997). The wheat genome contains an abundance of $(AT)_n$ repeats (Wang *et al.*, 1994). Cytosine residues in the nuclear DNA of wheat are highly methylated (30% compared to 1-7% in animals - Shapiro, 1976) with 82% of CpG dinucleotides being methylated (Gruenbaum *et al.*, 1981). The number of genes present in the hexaploid wheat genome has been estimated at 150,000 (Kamalay and Goldberg, 1980), although a more recent estimate of the number of genes in cereals is 20,000 to 30,000 genes (Moore, 1995) with a density of approximately one gene in every 5 - 50 kb (Keller and Feuillet, 2000). Distribution of recombination is highly variable on the wheat chromosome: approximately 60% of recombination events occur on only 18% of the arm length with no recombination within the closest 20% of the arm surrounding the centromere (group 2 - Delaney *et al.*, 1995; group 5 - Gill *et al.*, 1996).

Cultivated diploid (*T. monococcum*), tetraploid (*T. turgidum* ssp. *durum*) and hexaploid (*T. aestivum*) wheat species have one (AA), two (AABB) and three (AABBDD) pairs of seven chromosomes each. The A genome is present in both tetra- and hexaploid wheat species, the B genome is shared by tetra- and hexaploid wheat and the D genome is only present in hexaploid wheats (McFadden and Sears, 1946). The B genome contains more heterochromatin and repeated sequences when compared to the A and D genomes (reviewed by Flavell *et al.* 1987; May and Appels, 1987). The donor of the D genome of common wheat is the Asian goatgrass *Triticum tauschii* (syn. *Aegilops squarrosa* (Coss.) Schmal. or *Aegilops tauschii* L., genome 2n = 2x = 14, DD) (Kihara, 1944; McFadden and Sears, 1946). It has been shown in several cases that the D genome suppresses leaf rust resistance conferred by genes on the other two genomes (Kihara, 1944; Kerber, 1983; Boyko *et al.*, 1999).

Homoeologous recombination among chromosomes of the three genomes is largely suppressed due to the presence of the *Ph1* gene on chromosome 5BL (Okamato, 1957; Riley and Chapman, 1958). This gene causes the 42 chromosomes of hexaploid wheat to pair preferentially as 21 bivalents (7 per genome) and in its absence the occurrence of reciprocal translocations in 'Chinese Spring' is enhanced (Naranjo *et al.*, 1987). Clustering of markers around the centromere has been observed for a number of marker systems, for example RFLPs (restriction fragment length polymorphisms - Chao *et al.*, 1989; Devos *et al.*, 1992; Hart, 1994) and AFLPs

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(amplified fragment length polymorphisms - Moore *et al.*, 1993; Hart, 1994). The gene synteny across the three genomes is normally conserved, except for ancestral translocations involving chromosomes 4, 5 and 7 (Anderson *et al.*, 1992). A large portion of the *Gramineae* genomes is composed of ancestral transposable elements such as inactive retrotransposons (Moore *et al.*, 1991a and 1991b; Röder *et al.*, 1998). Wheat cells also contain chloroplasts and mitochondria: wheat chloroplast genomes are approximately 135 kb in size and the mitochondrial genome approximately 430 kb. The genomes of both organelle have been characterised (reviewed by May and Appels, 1987).

A low level of molecular polymorphism is usually found within cultivated wheat (Chao *et al.*, 1989; Kam-Morgan *et al.*, 1989; He *et al.*, 1992) and isolines carrying alien introgressions are generally recognised as better germplasm for gene tagging due to the large size normally associated with the introgressed chromatin and the increased levels of polymorphism present (William *et al.*, 1997). However, the amount of alien genetic material linked to introgressed alien genes and its genetic divergence from wheat chromatin poses difficulties in mapping and recombination studies (Riley and Chapman, 1958; Davoyan and Ternovskaya, 1996). Genetic divergence refers to the phenomenon that the more remote common wheat is from its wild relative, the more likely the possibility that the relative will have genes that are not present in common wheat, and the more disruptive the co-transfer of these genes to common wheat becomes (Davoyan and Ternovskaya, 1996). A low success rate has been achieved when trying to tag genes of common wheat origin. This is mainly caused by the large genome size in bread wheat, low levels of molecular polymorphisms and the large number of repetitive sequences present (Smith and Flavell, 1975; Gale *et al.*, 1990; Devos and Gale, 1992; William *et al.*, 1997). A technique that can be used to overcome the limitations of low polymorphism in the mapping of wheat is to produce synthetic hexaploids derived from hybridisation of *T. turgidum* and *T. tauschii*. The latter species are evolutionarily closely related to common wheat and hybridisation of common wheat with a synthetic hexaploid results in informative mapping populations.

Reproducibility, especially when using RAPDs (random amplified polymorphic DNAs), is often also a problem when attempting to tag genes or traits (Devos and Gale, 1992). A number of molecular maps for wheat has been described in literature, for example microsatellite maps (Plaschke *et al.*, 1995; Röder *et al.*, 1995; Bryan *et al.*, 1997; Röder *et al.*, 1998; Stephenson *et*

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al., 1998), RFLP maps (Hohmann *et al.*, 1994; Gale *et al.*, 1995; Nelson *et al.*, 1995) and cytogenetically based maps (Werner *et al.*, 1992). An example of an RFLP map is shown in Figure 2. Röder *et al.* (1998) mapped 115 microsatellites to the B genome of wheat, 93 to the A genome and 71 to the D genome. The lowest number of microsatellite markers was identified on chromosomes 1A, 4A, 6A, 1D, 4D, 6D and 7D. Huang *et al.* (2000) used 256 AFLP primer combinations (*Eco*RI and *Mse*I) to screen 'Chinese Spring' nulli-tetrasomic stocks for chromosome specific AFLP markers. Of the 256 possible combinations, 15 randomly chosen *Eco*RI + ANN and *Mse*I + CNN primer combinations detected 928 (39.9%) out of 2328 AFLP markers that could be assigned to specific chromosomes with 131 (14.1% or 5.6% of total) out of the 928 markers mapping to specific chromosome arms (see Table 1 for summary). All wheat chromosomes were identified with any of the 15 random combinations, showing that the markers covered the whole genome.

Table 1. Marker distribution observed with 15 randomly chosen *Eco*RI + ANN and *Mse*I + CNN AFLP primer combinations. (Adapted from Huang *et al.* (2000))

Number (out of 928)	Percentage (out of 100 %*)	Homoeologous group	Genome
356	38.4 %		A
294	31.7 %		B
278	30.0 %		D
113	12.2 %	1	
130	14.0 %	2	
143	15.4 %	3	
139	15.0 %	4	
133	14.3 %	5	
117	12.6 %	6	
153	16.5 %	7	

* Figures do not add up to 100% due to rounding error.

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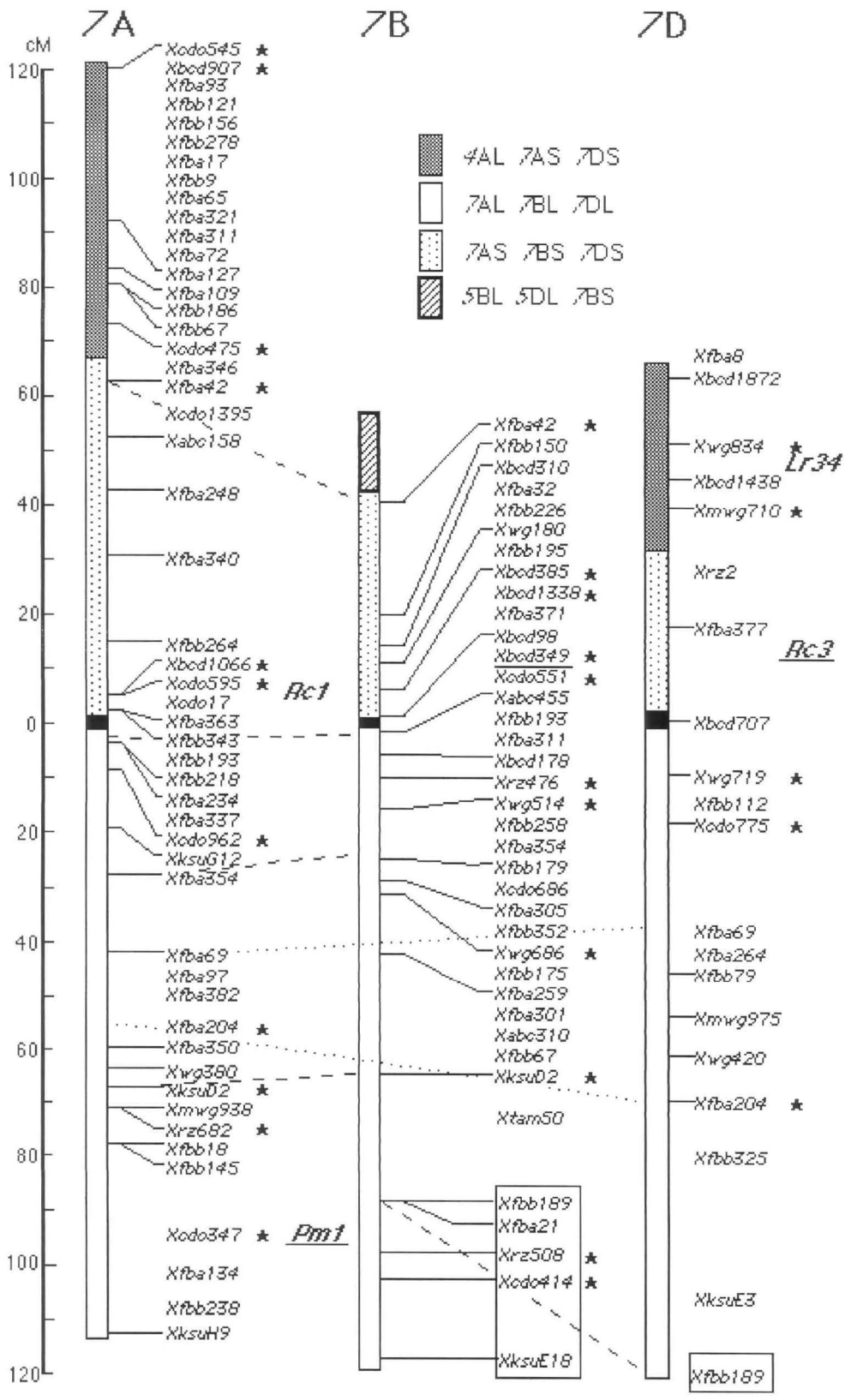

(internet address: http://wheat.pw.usda.gov)

Figure 2. RFLP map of the group 7 chromosomes from the cross 'Synthetic' × 'Opata'.

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RFLP studies by Anderson *et al.* (1992) revealed that arm homoeologies were as previously deduced, except for the group 4 chromosomes where 4AL=4BS=4DS and 4AS=4BL=4DL. This can be ascribed to a pericentric inversion in a part of chromosome 4A during the evolution of wheat.

Recently, a wheat genetics resources database called KOMUGI (from the Japanese for 'wheat') has been established that covers most wheat stocks in Japan (Yamazaki *et al.*, 1998) and can be contacted at the world-wide web address http://www.shigen.nig.ac.jp/wheat/wheat.html. Additional and more comprehensive grain databases are also available from:

1) 'International Grass Genome Mapping Initiative' (http://www-iggi.bio.purdue.edu/) and
2) the GrainGenes database (http://wheat.pw.usda.gov/ggpages/)

1.3 Chromosome Arm 7DL

Marker maps for chromosome 7DL have been published by Chao *et al.* (1989) and Boyko *et al.* (1999) and D-genome RFLP maps by Chao *et al.* (1989), Kam-Morgan *et al.* (1989), Gill *et al.* (1991), Hart *et al.* (1993), Hohmann *et al.* (1994) and Chen and Gustafson (1995). Large discrepancies exist between the physical and genetic maps of chromosomes 7B and 7D (Werner *et al.*, 1992). Loci that map genetically close to the centromere are physically localised to more distal chromosome regions, with distortion observed for chromosome 7D probably due to its lack of a centromeric heterochromatin block. A consensus map of the genes of the group 7 chromosomes of common wheat is shown in Figure 3.

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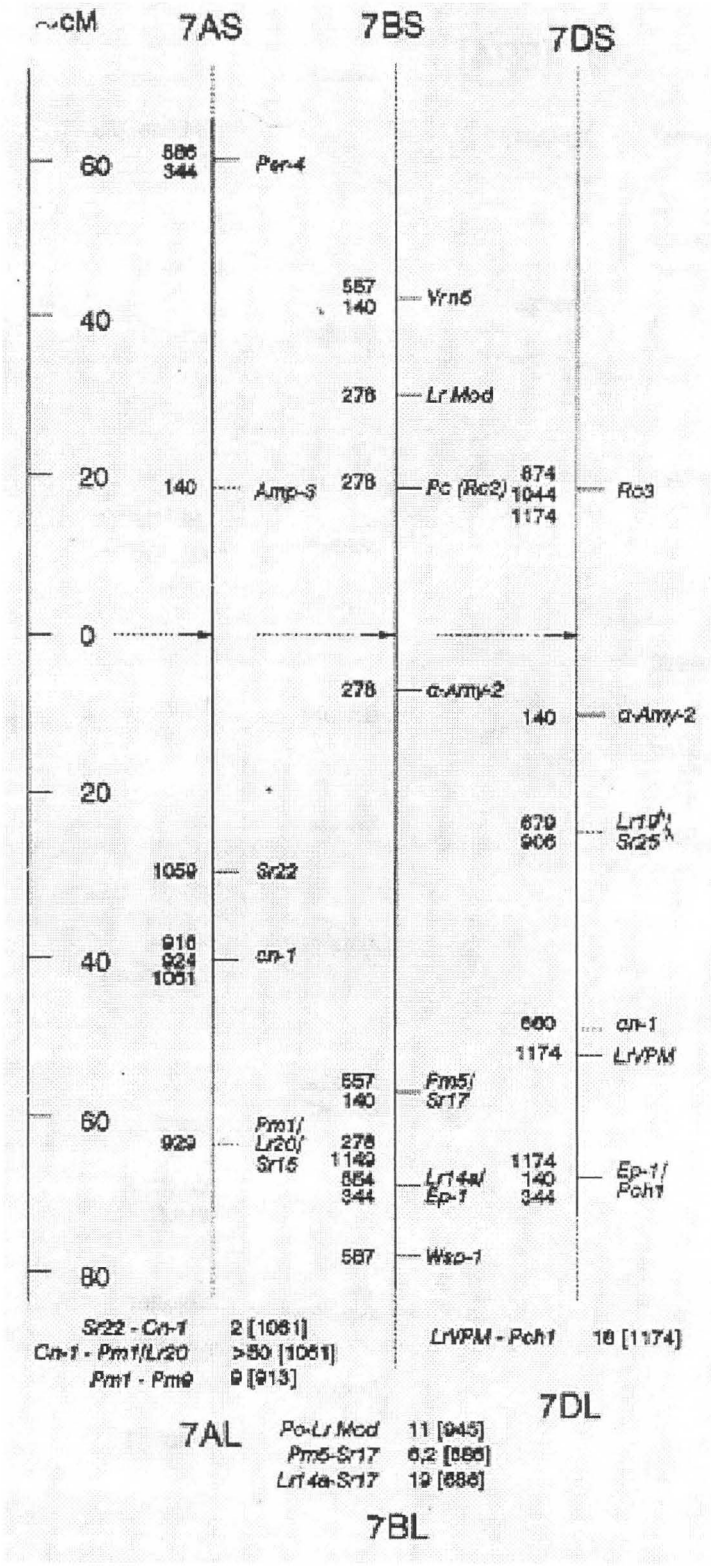

(internet address: http://wheat.pw.usda.gov/ggpages/linemaps/wheat/wheat7.html)

Figure 3. Consensus map of the genes of the group 7 chromosomes of common wheat (*Triticum aestivum*).

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Several structural genes have been mapped to or localised on chromosome arm 7DL and some of these will be discussed briefly:

1. Chlorina mutants have reduced chlorophyll levels, especially chlorophyll *b*, and seedlings are pale yellow-green, darkening with maturity. The mutant genes were localised to the long arms of the group 7 chromosomes (*cn-A1*, *cn-B1* and *cn-D1*) (Sears and Sears, 1968; McIntosh, 1988). The chlorina mutant gene is not a null allele, as group 7 nullisomics have normal green leaves. In three doses, extreme chlorina phenotypes with golden leaves are formed, but the mutant allele is partially dominant to the normal one as heterozygotes have light green leaves except in cases of aneuploidy (Pettigrew *et al.*, 1969).
2. A major gene associated with preharvest sprouting tolerance has been assigned to 7DL by Roy *et al.* (1999) using a STS developed from RFLP probe *Xmst101*.
3. An isozyme locus, *Ep-D1* (McIntosh, 1988) and associated Russian wheat aphid resistance gene, *Dn5*, (Du Toit *et al.*, 1995) maps to 7DL and will be discussed in more detail in later sections. Russian wheat aphid resistance gene, *Dn2*, has also been assigned to 7DL (Ma *et al.*, 1998).
4. The α-amylase structural gene series *α-Amy-2* (Nishikawa and Nobuhara, 1971) which encodes sixteen *α-AMY-2* isozymes (Gale et al., 1983).
5. Gene(s) that suppress stem rust resistance (Kerber and Green, 1980; Dyck, 1987).
6. Effective resistance against eyespot has been introduced from *Aegilops ventricosa* into bread wheat (Maia, 1967) and the resistance is mainly controlled by a single gene located distally on chromosome 7D (Jahier *et al.*, 1978; Worland *et al.*, 1988). Also transferred were genes for α-amylase production (Gale *et al.*, 1984), red coleoptile colour (Worland and Law, 1986) and leaf rust resistance (*LrVPM* - Worland *et al.*, 1988). The eyespot resistance gene *Pch1* will be discussed in more detail in a later section.
7. An important leaf rust resistance gene, *Lr34*, and the associated yellow rust resistance gene *Yr18* (Dyck, 1987; Singh, 1992a; McIntosh, 1992) were initially mapped to 7DL. A gene for leaf tip necrosis in adult plants, *Ltn*, is associated with *Lr34*. *Ltn* is, however, not a reliable marker for the presence of *Lr34* due to the fact that other biotic and abiotic stresses can cause the same symptoms (Dyck, 1991; Singh 1992b). A barley yellow dwarf

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virus tolerance gene, *Bdv1*, is also associated with *Lr34* (Singh, 1993). More recent mapping results reassigned *Lr34* to 7DS (Dyck *et al.*, 1994).

8. The leaf rust resistance gene, *Lr19*, and an associated stem rust resistance gene, *Sr25*, have been transferred by irradiation from *Agropyron elongatum* to bread wheat (Sharma and Knott, 1966). The *Lr19* gene will be discussed in more detail in a later section.

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2. WHEAT DISEASE RESISTANCE

The primary factors causing yield loss are weather, insects, viruses, fungi, nematodes, bacteria and weeds. Wheat disease was defined by Wiese (1977) as the abnormal condition(s) resulting from injuries and stresses that interfere with the normal functioning and development of the wheat plant at any stage(s) of growth. Of these factors, rust-causing fungi and insects are economically more important.

Initially, chemical control of a large number of pathogens was possible, but more isolates resistant to the various control measures are being isolated (Knott, 1989, p. 13). The public disapproval of the use of chemical control, cost considerations, as well as the acquired resistance by pathogens, are forcing plant breeders to turn to genetic control of diseases. A major breeding objective is therefore the identification of new sources of resistance for use in breeding programs in order to maintain genetic resistance to various pathogens.

2.1 LEAF RUST RESISTANCE

Fungi belong to a very diverse group of organisms and are adapted to a large variety of environmental conditions. They normally infect the plant through openings, wounds or by direct penetration, causing rots, blights, deformations, lesions, spots, rusts, smuts or mildews (Wiese, 1977 p. 10).

The rust diseases are fungal infections of wheat and include stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Erikss. & E. Henn), yellow rust (*Puccinia striiformis* Westend.) and leaf rust (*Puccinia recondita* Roberg ex Desmasz f. sp. *tritici* Erikss. & E. Henn) (Wiese, 1977 p. 35). They are characterised by the eruption of sori or pustules (Figure 4a) of the fungus through the plant epidermis, causing discoloration of the leaf or stem (Figure 4b). The amount of damage depends on the age of the plant at the onset of the disease and, normally, rusts decrease the value of the crop's forage.

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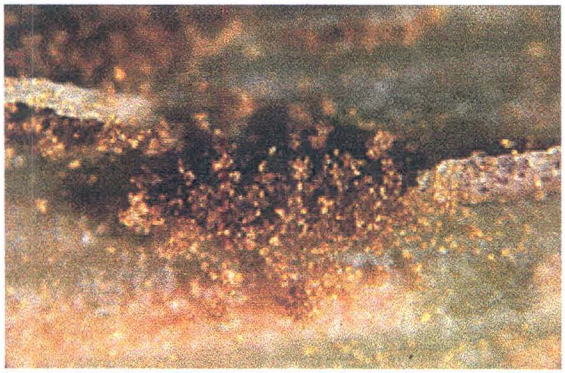
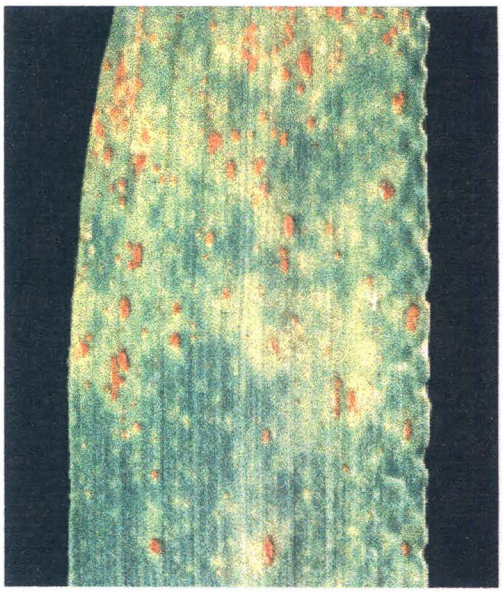
a)



b)



Source (both figures):
http://www.ppws.vt.edu/stromberg/smallgrain/biology/wlrust.html

Figure 4. a) Close-up of a sporulating rust pustule and b) a wheat leaf infected with leaf rust.

2.1.1 Pathogen and Life Cycle

Leaf rust (brown rust, dwarf rust or orange rust) is probably the most important wheat disease on a worldwide basis (Samborski, 1985). The pathogen is adapted to a wide range of climates and is found in diverse wheat growing areas of the world (Kolmer, 1996). It is most prevalent in spring wheat regions where the wheat matures late.

Spores of the fungus germinate overnight on the wheat leaf surface under high humidity and with an optimal temperature range between 10 and 22 °C (Kramer and Eversmeyer, 1992). The fungal germ tube is directed to grow perpendicularly to the venation of the leaf (thigmotropic reaction) to the nearest stoma in response to the topography and chemical features of the leaf surface (Hoch and Staples, 1987; Hoch *et al.*, 1987; Allen *et al.*, 1991). At the stoma the apex of the germ tube forms an appressorium. A substomatal vesicle develops after penetration of the stoma, from which the intercellular space is invaded by the primary hypha. It produces haustorial mother cells that develop haustoria in the host cells. The haustorial mother cells give rise to secondary hyphae that grow into the intercellular space and produce new haustorial mother cells and haustoria. The number of haustoria produced within each colony increases exponentially in a susceptible host. Under optimal conditions the fungus undergoes reproduction within a few days and the typical symptoms become visible as sporulating pustules on the leaf surface 7 to 8 days after infection. (Ortelli *et al.*, 1996)

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2.1.2 Genetic control of the Pathogen

To date, numerous genes conferring leaf rust resistance have been identified and designated *Lr1* to *Lr47*, while an additional 22 genes were given temporary designations (Cereal Research Laboratory: http://www.crl.umn.edu/res_gene/wlr.html). Some leaf rust resistance genes, for example *Lr34* in combination with *Lr12*, *Lr13* or other leaf rust resistance genes, could confer durable resistance to leaf rust in wheat (Roelfs, 1988a; German and Kolmer, 1992). It has been suggested that the use of gene combinations, irrespective of whether major or minor genes are used, is the best genetic control of leaf rust (Dyck and Samborski, 1974; Samborski and Dyck, 1982). In wheat cultivars that have a combination of rust resistance genes, the genes normally act independently and exhibit the infection type of the gene that conditions the lower infection type when used on its own (Dyck and Kerber, 1985; Roelfs, 1988b). Gene interaction has generically been defined as the combination of two or more genes resulting in higher resistance than that conferred by the individual genes (Schafer *et al.*, 1963; Dyck, 1977; Samborski and Dyck, 1982) and this effect may be additive (Dyck and Kerber, 1985). Samborski and Dyck (1982) reported interactions between seedling and adult plant wheat leaf rust resistance genes.

Leaf rust resistance genes have been located in different chromosomes of the three genomes of wheat and other genes are known to be located in homoeologous chromosomes at similar distances from the centromere (McIntosh, 1988), suggesting that allelic variants may be present at different homoeologous loci. It is also possible, in a hexaploid background like wheat, that three doses of these genes might be present (Roelfs, 1988b). Most of the resistance genes have been identified in cultivated common wheat, although some have been derived from wheat relatives. However, genes transferred from wild relatives often introgress along with less desirable traits from the donor genome, normally decreasing its usability.

2.1.3 Leaf Rust Resistance Gene, *Lr19*

The *Lr19* (=T4) translocation was originally transferred to common wheat from *Thinopyrum ponticum* (=*Agropyron elongatum*) by Sharma and Knott (1966). *Lr19* is worldwide a highly effective resistance gene (Winzeler *et al.*, 1995), but leaf rust isolates virulent on *Lr19* have

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been reported (Huerta-Espino and Singh, 1994; Sibikeev *et al.*, 1996). Lobachev (1992) reported that *Lr19* might influence the grain yield by increasing the number of kernels per spike.

Marais *et al.* (1988) reported that an apparently spontaneous translocation between a *Thinopyrum distichum* and a wheat chromosome probably resulted in the leaf rust resistance of wheat selection 'Indis'. However, the 'Indis' translocation showed many similarities with the *Thinopyrum ponticum* derived *Lr19* translocation, for example similar leaf and stem rust resistances, segregation distortion, yellow endosperm, null conditions for the endopeptidase locus *Ep-D1* and the *α-Amy-D2* locus. A water-soluble (WSP) protein allele, *Wsp-D1a*, has also been demonstrated in both the 'Indis' and T4 translocations (Marais, 1992). Using RFLP markers, Prins *et al.* (1996) could demonstrate that the translocation in 'Indis' is in reality the T4 translocation originally produced by Sharma and Knott (1966), thus *Lr19* = T4 = 'Indis'. The *Lr19* translocated segment is homoeologous to the distal part of 7DL (Dvorák and Knott, 1977; Kim *et al.*, 1993).

In the presence of *Ph1*, the *Lr19* translocation does not recombine with homoeologous regions of the wheat genome and is therefore inherited as a single large linkage block (Knott, 1980; Marais and Marais, 1990). Since the translocated region does not pair and recombine with homoeologous 7DL chromatin during meiosis it cannot be mapped using crossover frequencies and physical mapping had to be attempted. Marais (1992) irradiated spikes of the translocation line 'Indis' with gamma rays and pollinated these spikes with 'Inia 66' pollen. F_2 and F_3 generations were screened for translocation mutants, using leaf rust resistance, stem rust resistance, yellow endosperm pigmentation genes and *Wsp-D1* loci, as markers. Twenty-nine deletion mutants were identified and characterised with these markers. Making use of the *Ep-D1* null allele, homozygous stocks were obtained for each mutation. These were subsequently used for physical mapping purposes and it was found that most of the mutations resulted from terminal deletions, while two intercalary deletions may also have occurred (Marais, 1992; Prins *et al.*, 1996). Shortened recombinants which retained the *Lr19* gene, but have exchanged some of the proximal and distal *Thinopyrum* chromatin for wheat chromatin, have been developed and further shortening of these recombinants is in progress (Marais *et al.*, 2001).

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2.2 *EYESPOT (STRAW BREAKER FOOT ROT)*

Eyespot (also called strawbreaker foot rot) of common wheat (*Triticum aestivum*) is caused by the necrotrophic fungus, *Pseudocercosporella herpotrichoides* (Fron.) Deighton. It is a major disease of wheat and barley grown in cool, moist climates, resulting in yield losses of up to 50% (Scott and Hollins, 1974; Fitt *et al.*, 1990). The hyphomycete causative of eyespot disease was originally called *Cercosporella herpotrichoides* (Fron.) (Fron, 1912), but was later re-examined, placed in a newly erected genus and called *P. herpotrichoides* (Fron.) Deighton (Deighton, 1973). A teleomorph for *P. herpotrichoides* (Fron.) Deighton, namely *Tapesia yallundae* (Wallwork & Spooner), has also been reported and characterised (Wallwork, 1987; Wallwork and Spooner, 1988; Nicholson *et al.*, 1997).

2.2.1 Pathogen and Life Cycle

A wide host range among small grain cereals and grasses has been reported (Murray, 1992). Morphologically, *P. herpotrichoides* has curved and straight conidia (Nirenberg, 1981). Four main types can be distinguished, usually on the basis of cultural characteristics and isozyme polymorphisms: i) the wheat type (W-type or N-type) which is more virulent to wheat than to barley and less virulent to rye; ii) the rye type (R-type or L-type) which is approximately equally virulent to wheat, barley and rye; iii) the S-type which is virulent to both wheat and *Aegilops squarrosa* L.; and iv) the C-type which is pathogenic on wheat, *Ae. squarrosa*, *Elymus repens* (formerly *Agropyron repens*) and couch grass (*Elymus repens* (L.) Gould) (Cunningham, 1981; Hollins *et al.*, 1985; Nicholson *et al.*, 1997). The W-type has been renamed to *Tapesia yallundae* and the R-type *Tapesia acuformis* (Campbell *et al.*, 1996; Dyer *et al.*, 1996; Nicholson *et al.*, 1997). It is possible to differentiate between the two types using molecular markers and so far, only the W-type has been identified in South Africa (Campbell *et al.*, 1996),

Some of the major problems with the identification of eyespot are that early detection of infection is difficult as 4-12 weeks go by between infection and manifestation of the disease (Macer, 1966; Rowe and Powelson, 1973a) and seasonal rainfall variations have a large influence on the appearance of the disease symptoms (Gorter, 1941). In winter wheat, four stages of infection can be identified: sporulation, spore dispersal, infection and development of lesions. A wide temperature range of 1 - 20 °C (with an optimum of approximately 5 °C) can support sporulation of naturally infected wheat stem bases and water is essential for sporulation,

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especially when wet and dry conditions alternate (Glynne, 1953). Spore dispersal is dependent on water droplets and due to the greater number of spores produced (Rowe and Powelson, 1973b) on infected debris ('primary inoculum') in comparison to leaf sheath lesions ('secondary inoculum'), spores are normally dispersed from the primary inoculum to other plants in the crop (Fatemi and Fitt, 1983). Infection of the plant is initiated after the production and dispersal of conidia, but only if sufficient primary inoculum as well as suitable weather conditions are present (Rowe and Powelson, 1973a). After infection, several weeks will pass before the disease manifests in large enough lesions to affect crop yield (Scott and Hollins, 1974). The development of dark-coloured, elliptical or eye-shaped lesions also takes place in four stages: establishment of a leaf sheath lesion, penetration of the leaf sheath, establishment of stem lesions and finally development of stem lesions (Fitt and White, 1988). After the host has died, the pathogen survives saprophytically on the debris where it produces conidia and the cycle starts again with the dispersal of spores.

2.2.2 Control of the Pathogen

Eyespot is only partially controllable with fungicides such as benzimidazole or prochloraz and cultural practices and therefore genetic sources of resistance are extremely valuable (Koebner and Martin, 1990). Cultural practices recommended for the control of eyespot include later sowing dates, lower plant densities and, to a lesser extent, nitrogen rate and fertiliser form (Colbach and Saur, 1998). Resistance to eyespot is known to occur in genera related to *Triticum*, for example *Aegilops* and *Secale* (Macer, 1966). Eyespot resistance may take one of two forms: 1) direct resistance to pathogen growth in the stem base or 2) indirect tolerance to eyespot through resistance to lodging (Scott and Hollins, 1974 and 1985). In resistant cultivars like 'VPM-1' and 'Capelle Desprez', the hypodermis is wider, while more cell layers and larger lignified cell wall appositions (lignitubers) are formed in greater profusion at penetration sites (Murray and Bruehl, 1983).

In contrast to other fungal pathogens of wheat, only three resistance genes for eyespot have been reported previously, namely *Pch1* to *Pch3*. The first resistance gene, *Pch1*, will be discussed in more detail in the next session. The second resistance gene, *Pch2*, is the source of resistance in Cappelle Desprez (Vincent *et al.*, 1952) and an association between this gene and the endopeptidase allele *Ep-A1b* on chromosome arm 7AL of common wheat has been demonstrated (Koebner and Martin, 1990). *Pch2* is also linked to the RFLP marker *Xpsr121* (Law *et al.*, 1975; De la Peña *et al.*, 1996). The third resistance gene, *Pch3*, which is not effective against

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the R-type pathogen, is located on the long arm of chromosome 4V of *Dasypyrum villosum* (2n=2x=VV) (Murray *et al.*, 1994). Unfortunately, the majority of *D. villosum* accessions are resistant to eyespot and it could therefore be difficult to map or tag *Pch3* (Yildirim *et al.*, 2000). Additional resistance has also been found in *T. tauschii* (2n=2x=DD) and *T. monococcum* (2n=2x=AA) (Sprague, 1936).

Good cultivation practice also reduces the incidences of eyespot epidemics. Rotation of wheat with medics (*Medicago* spp.), lupins (*Lupinus albus* L.) and oats, instead of wheat monoculture, is very important (Bester, 1990). In the wheat growing regions of South Africa, grass weeds must be controlled in legume crops when rotated with wheat, as many of these weed grasses and small grains are susceptible to eyespot (Bester, 1990).

2.2.3 Eyespot Resistance Gene, *Pch1*

The most potent eyespot resistance gene, *Pch1* (Worland *et al.*, 1988), is present in VPM-1, a 42 chromosome derivative of a bridging cross between *Aegilops ventricosa* and the tetraploid species *T. persicum*; and *T. aestivum* cv 'Marne' (Maia, 1967). *Ae. ventricosa* has also been used as the source of eyespot resistance in the winter wheat cultivar 'Rendezvous' (Hollins *et al.*, 1988). *Pch1* is located on chromosome 7DL (Jahier *et al.*, 1978; Doussinault *et al.*, 1983a; Hollins *et al.*, 1988) and close linkage between this gene and the endopeptidase allele *Ep-D1b* has been observed (Koebner *et al.*, 1988; Worland *et al.*, 1988). It has been proposed that the resistance could be a product of this endopeptidase locus (Worland *et al.*, 1988), but Mena *et al.* (1992) used isozyme markers and DNA probes and confirmed that *Pch1* is indeed on chromosome 7D and that, although linkage between *Pch1* and *Ep-D1b* exists, the two loci can be separated from each other. The resistance of the wheat variety 'Rendezvous' was obtained from *Ae. ventricosa*-derived VPM1 and this highly resistant variety carries both the *Ep-D1b* and *Ep-A1b* alleles (Hollins *et al.*, 1988; Koebner and Martin, 1990). Due to its more effective suppression of disease development, *Pch1* has been used more widely than *Pch2* (Doussinault, 1973; Allan *et al.*, 1989 and 1990). However, even in the presence of both *Pch1* and *Pch2*, severe eyespot infections can still result in substantial yield loss (Hollins *et al.*, 1988). Delibes *et al.* (1988) reported that the *Ae. ventricosa*-derived eyespot resistance gene in the wheat line H-93-70 is not allelic to the resistance gene locus on chromosome 7D of the VPM1-derived wheat cultivar 'Roazon' described by Jahier *et al.* (1978). H-93-70 was one of the wheat lines with 42

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chromosomes obtained by selfing the progeny of an intermediate self-sterile hybrid between *T. turgidum* and *Ae. ventricosa* rescued with hexaploid wheat pollen (Delibes and García-Olmedo, 1973; Delibes *et al.*, 1977a and b; Doussinault *et al.*, 1983b). However, in a follow-up study by Mena *et al.* (1992), lack of segregation of the resistance gene in progeny from reciprocal crosses between H-93-70 and 'VPM1' suggested allelism of their respective resistance factors.

2.3 *Russian Wheat Aphid (RWA) Resistance*

The Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), is worldwide a serious pest of wheat and barley (*Hordeum vulgare* L.). The RWA, indigenous to Southern Russia (originally identified in the Caucasus in 1900) and countries bordering the Mediterranean, was first reported as a pest of wheat in South Africa during 1978. By September 1979 it had spread from the Eastern Free State to most of the Western Orange Free State and also Lesotho, with isolated cases reported in the Transvaal and Natal. Other countries subsequently infected include Mexico, United States of America, Chile, Argentina, the Middle East, Pakistan, Afghanistan, China, Ethiopia and Mozambique (Walters *et al.*, 1980; Robinson 1992).

2.3.1 Biology and Life Cycle

The Russian wheat aphid is a small (less than 2 mm long) pale green aphid that has an elongated spindle-shaped body (Figure 5). The presence of an extremely short antenna above the cauda ("tail"), as well as the absence of prominent siphunculi, makes it easy to distinguish this aphid from other aphids infesting South African wheat. Preferred hosts of the pest are wheat, barley and triticale (Walters *et al.*, 1980; Dürr, 1983; Kindler and Springer, 1991; Nkongolo *et al.*, 1996). It has been implicated as a transmitter of plant viruses, for example brome-mosaic virus (BMV) (Von Wechmar and Rybicki, 1991) but is a poor vector for barley yellow dwarf virus.

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Source: Department of Entomology, University of Nebraska-Lincoln (http://entomology.unl.edu/images/smgrains/aphids/russwht.htm)

Figure 5. The Russian wheat aphid on a leaf.

The Russian wheat aphid occurs in two forms in South Africa, namely winged (alate) and wingless (apterous) females. Males are not found locally and reproduction takes place parthenogenetically (without fertilisation). The viviparous winged females develop under certain conditions, for example when the host plant is under stress or the habitat is no longer favourable, and this form allows the insect to move to more suitable conditions. The winged aphid can only fly short distances by itself and therefore makes use of prevailing winds or convection currents to travel longer distances. Immediately on reaching suitable conditions, the aphid starts to feed and gives birth to small nymphs that grow quickly and develop into wingless females. Under warm spring conditions these new wingless females can give birth to up to four small nymphs per day after just two weeks, with typically a minimum of 70 nymphs per female lifetime. The increasing population is fed by the emerging young leaves of the plant while dense colonies may occur in the rolled-up leaves. As soon as the ears of the plant appear, the population density of the insect starts to decline and winged forms develop that migrate in search of new host plants. (Walters *et al.*, 1980; Robinson, 1992)

2.3.2 Symptoms of Infection

Seedlings are very susceptible to RWA, especially when grown under drought stress or nitrogen deficiency. Damage to the plant occurs from direct feeding of the insect on the phloem of the plant (results in a reduced photosynthetic capacity by destruction of the chloroplasts) and/or from the effect of a phytotoxin injected into the plant during feeding (Fouche *et al.*, 1984; Zemetra *et al.*, 1993, Rafi *et al.*, 1996). The exact nature of the toxin is not known but, if similar

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to other aphid toxins, would consist of a mixture of cellulases, lipases, pectinases and proteolytic enzymes (Dreyer and Campbell, 1987; Robinson, 1992). More recent investigations revealed an increased β-1,3-glucanase activity in lines containing RWA resistance gene *Dn1* after infestation (Van der Westhuizen *et al.*, 1998). Infestations of susceptible plants occur mainly on the newest growth, in the axils of the leaves or within curled-up leaves. These infestations are normally accompanied by white, yellow and purple to reddish-purple longitudinal streaks on the wheat leaves (Figure 6) and can even be caused by a single aphid. In cases of heavy infestation the plants often exhibit a flattened appearance, the young tillers almost lying parallel to the ground.

(Source: http://entoplp.okstate.edu/ddd/insects/russianwheataphid.htm)

Figure 6. Russian wheat aphids feeding on a leaf (note the longitudinal streaks in the leaf).

In later growth stages, the flag leaf often gets infested and the ears then become bent and turn white, indicative of poor yield. Under favourable conditions, the infestation can vary from 20% to 80% infested plants (Walters *et al.*, 1980). RWA infestation can also interfere with cold hardening and lead to a predisposition to winterkill (Thomas and Butts, 1990). Typical winter wheat losses in South Africa range between 35 to 60% and approximately 40% of the wheat crop is treated annually for RWA infestation (Du Toit and Walters, 1984; Robinson, 1992). Wheat seedlings can be visually scored for resistance on a one to six scale (Table 2) three weeks after infestation (Du Toit, 1987).

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Table 2. Scoring system for RWA infestation of wheat plants (Du Toit, 1987).

Score	Symptoms	Classification
1	small isolated chlorotic spots on the leaves	highly resistant
2	larger chlorotic spots on the leaves	resistant
3	chlorotic spots tend to become streaky	moderately resistant
4	mild streaks visible and leaves tend to roll lengthways	moderately susceptible
5	prominent white/yellow streaks present and leaves tightly rolled	susceptible
6	severe white/yellow streaks present and leaves tightly rolled and starting to die from the tips	highly susceptible

2.3.3 Control of the Insect

Several means of controlling the insect are available, usually with varied efficiency. These include cultural practices (for example choice of planting date and the control of alternative hosts), biological control (for example ladybirds and wasp parasites), chemical control and the breeding of resistant cultivars.

In the Orange Free State, it is recommended that cereals should not be planted before May to restrict the build-up of aphid populations and that only winter and intermediate types should be planted. Well-fertilised wheat planted in high densities under favourable soil moisture conditions also appears to be more resistant to infestation. Rescue grass (*Bromus wildenowii*) is a weed found generally in and around wheat fields and is fairly readily attacked by the insect and can therefore play an important part in the survival of the RWA during periods when cereals are not cultivated. *Agrotricum*, a winter pasture grass, is also attacked by the insect, while rye and oats appear to be less favourable hosts. Temperature also plays an important role in the survival of the insect: the low winter temperatures during June to August restrict the increase of the population, while the high temperatures and rainfall of the Highveld during January and February lead to high mortality and a reduction in the numbers of the population (Walters *et al.*, 1980). Laboratory research has indicated that increased levels of nitrogen fertiliser reduce grain yield loss to RWA (Riedell, 1990). However, field trials showed that there was a lack of significant interaction between nitrogen fertiliser treatment and RWA infestation and that grain yield was affected similarly by the infestation for all fertiliser treatments studied (Riedell and

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Kieckhefer, 1993). These results were confirmed by Archer *et al.* (1995), who found that water stress plays a more important role in RWA increase than the amount of nitrogen fertiliser available to the crop and that supplemental irrigation during periods with low rain fall might reduce the RWA increase rate.

Due to the seclusion of the RWA in rolled leaves, the application of contact aphicides or biological control agents (for example parasitoid wasps and coccinellid species) is not very effective (Walters *et al.*, 1980; Zemetra *et al.*, 1993). Systemic insecticides, for example disulfoton and dimethoate, have proven to be successful, but the price tag makes these insecticides extremely uneconomical (Robinson, 1992). RWA is a suitable host for most fungi (for example species of *Erynia*, *Conidiobolus* and *Verticillium*) known to attack other aphid species and this control approach might have some potential (Feng and Johnson, 1991). However, the use of moderately to highly resistant cultivars together with predators like wasp parasites appear to have better potential for controlling population sizes (Tolmay, 1996).

The financial implications, as well as the negative effect on the environment, of applying systemic aphicides also offers no easy solution. Insecticides often have to be used in combinations to be effective or are only effective during certain periods of the growth season, for example soil treatments might only be effective up to a fairly advanced growth stage. The only and most effective solution that is also financially healthy and environmentally safe in controlling RWA is the use of resistant cultivars (Walters *et al.*, 1980, Du Toit, 1987, Zhang *et al.*, 1998). Due to a scarcity of effective resistance genes, additional sources of resistance are needed to establish a wider range of resistance genes for crop improvement. Alternative sources of resistance include related species, for example *T. monococcum*, *T. dicoccum* and *T. tauschii*, and unimproved germplasm of central Asia. Resistance to RWA has also been reported in barley (*Hordeum vulgare*) (Webster *et al.*, 1987; Assad *et al.*, 1999). Total immunity to RWA is rare, the main mechanism is often tolerance rather than total resistance. Following the release of resistant varieties, the aphid will be placed under selective pressure and biotypic variation might occur, resulting in the need for cultivars that do not contain monogenic resistance (Bush *et al.*, 1989). An additional problem is that RWA is often found in the presence of other aphids that cause additional damage through direct feeding or acting as vectors for plant viruses, therefore RWA resistance should be combined with resistance against other aphids as well as plant viruses (Du Toit and Van Niekerk, 1985; Souza *et al.*, 1991; Robinson, 1992). A

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potential problem of using resistant lines is that biotype differences exist for RWA collected from different global locations and therefore a line should first be typed using the local biotype before including it in a breeding program (Dreyer and Campbell, 1987; Puterka *et al.*, 1992).

Currently there are seven designated RWA resistance genes, denoted *Dn1* through to *Dn7*. Two dominant genes, *Dn1* and *Dn2*, were identified in wheat lines 'PI 137739' (a hard white spring wheat from Iran) and 'PI 262660' (a hard white winter wheat from Bulgaria), respectively (Du Toit, 1989). A number of genes for resistance to RWA appear to be located in the D genome of common wheat. In 'PI 137739', resistance is conferred by *Dn1* on chromosome 7D and also by a lesser gene on chromosome 7B (Schroeder-Teeter *et al.*, 1994). A single recessive gene, *dn3*, has also been identified in *Triticum tauschii* (accession SQ24) (Nkongolo *et al.*, 1991a). Nkongolo *et al.* (1991b) reported the presence of a single dominant resistance gene, designated *Dn4*, in the wheat line 'PI 372129'. Ma *et al.* (1998) mapped *Dn2* to chromosome 7D and *Dn4* to chromosome 1DS using RFLP markers. Myburg *et al.* (1998) identified four RAPD markers linked to *Dn2*. *Dn5*, identified in 'PI 294994' (a hard red winter wheat accession from Bulgaria), was mapped to chromosome 7DL (Du Toit, 1990, Marais and Du Toit, 1993; Du Toit *et al.*, 1995). Another single dominant resistance gene, *Dn6*, has been reported in 'PI 243781' by Saidi and Quick (1994 and 1996). A dominant resistance gene, designated *Dn7*, on *Secale cereale* chromosome 1RS was transferred to 1RS of the 1BL.1RS translocation in 'Gamtoos' by homologous recombination by Marais *et al.* (1994). A single dominant gene, located on chromosome 4R of *Secale montanum*, has been reported and used for introgression by Quick *et al.* (1993). The existence of RWA resistance gene(s) on chromosome 4R has also been reported by Nkongolo *et al.* (1996). The triticale accession 'PI 386156' also contains Russian wheat aphid resistance and a co-dominant RAPD marker associated with chromosome 4R was identified (Fritz *et al.*, 1999). A spring wheat accession collected from Iran, 'PI 140207', was reported to be an excellent source of resistance to RWA (Porter *et al.*, 1993), but allelism tests showed that the resistance gene in 'PI 140207' is the same as *Dn1* (Porter *et al.*, 1998).

A number of resistant cultivars and accessions are available. In Colorado, the wheat cultivar 'Halt', containing a resistance gene from 'PI 372129' has proved to be very successful (Quick *et al.*, 1996). In December 1996, South Africa had seven RWA resistant cultivars available, namely 'Tugela-DN', 'Betta-DN', 'SST 333', 'Gariep', 'Limpopo', 'SST 936' and 'PAN 3235' (Tolmay, 1996).

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2.3.4 RWA Resistance Gene, *Dn5*

RWA resistance gene, *Dn5*, is derived from the hard red winter wheat accession 'PI 294994'. 'PI 294994' appears to be a heterogeneous germplasm containing more than one gene for resistance. Different laboratories working with genotypes derived from the 'PI 294994' collection came to different conclusions regarding the genetic base of its resistance.

Elsidaig and Zwer (1993), illustrated that a dominant allele at one locus and a recessive allele at a second locus were present in their 'PI 294994' source, using results obtained from random F_2-derived F_3 families from crosses between 'PI 294994' and two RWA-susceptible cultivars. This model was also strongly supported by Dong and Quick (1995) using F_2 segregation data from six crosses between 'PI 294994' and several other RWA-resistant materials. Elsidaig and Zwer (1993) also suggested that the dominant allele for resistance in 'PI 294994' might be the same as the gene (*Dn4*) characterised in 'PI 372129' by Nkongolo *et al.* (1991b).

A second study favoured allelism between the genes in 'PI 294994' and *Dn1*, *Dn2, Dn4* and *Dn6* or the presence of two dominant genes (Saidi and Quick, 1994). Saidi and Quick (1996) obtained no susceptible F_2 plants in crosses of 'PI 137739' (*Dn1*), 'PI 262660' (*Dn2*), 'PI 372129' (*Dn4*) and 'PI 243781' (*Dn6*) with 'PI 294994' and therefore concluded that the genes in 'PI 294994' might be allelic to *Dn1*, *Dn2, Dn4* and *Dn6*. Furthermore, F_2 data from the cross 'PI 294994' × 'Carson' suggested the presence of two dominant genes in 'PI 294994'.

A third study indicated the presence of a single dominant gene on chromosome arm 7DL. Based on F_2 and backcross data from a cross between 'SA463' ('PI 294994') and 'Chinese Spring', as well as monosomic analysis of 'PI 294994', Marais and Du Toit (1993) concluded that a single dominant gene on chromosome 7D was responsible for RWA resistance. Comparisons with previously published information led these researchers to conclude that the resistance gene in 'PI 294994' is not allelic to resistance genes *Dn1, Dn2, Dn3* or *Dn4* and they then designated it *Dn5*. Based on the low frequency of susceptible plants in the F_2 progeny of the cross 'PI 294994' × 'PI 137739', it was furthermore suggested that *Dn1* and *Dn5* were linked on chromosome 7D. Du Toit *et al.* (1995) did a telosomic analysis and found that recombined phenotypes occurred frequently among progeny derived from 7DL, which suggested that the location of *Dn5* is on chromosome arm 7DL.

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These conflicting results were attributed to i) heterogeneity of the original 'PI 294994' wheat accession for the resistance genes, ii) differences due to the parents used in the crosses with 'PI 294994' and iii) differences in the RWA biotype used by the researchers. To determine whether genetic variation was the cause for the conflicting reports, Zhang *et al.* (1998) crossed 22 RWA-resistant plants from the 'PI 294994' accession with the susceptible cultivar 'Carson'. F_2 and backcross resistance test data showed a varied number of resistance genes in the original individual 'PI 294994' plants: (i) five plants favoured two dominant independent genes, (ii) four plants favoured a single dominant gene, (iii) two plants favoured one dominant and one recessive gene, (iv) nine plants fitted either a one dominant gene or one dominant and one recessive gene model and (v) in the remaining two plants the number of resistance genes could not be determined but fitted a linked dominant gene model. Furthermore, the data which fitted the one dominant and one recessive gene model also fitted the linked dominant gene model. It was then concluded that variation in the model for resistance was based on the use of different RWA-resistant selections within 'PI 294994' and it was therefore proposed that the original 'PI 294994' wheat accession be regrouped into four sub-accessions.

A number of markers associated with *Dn5* has been reported. An endopeptidase allele, *Ep-D1e*, is linked to *Dn5* with a recombination percentage of 32 ± 4.97 (Marais *et al.*, 1998). Ma *et al.* (1998) found an allelic relationship between *Dn2* and a gene in 'PI 294994' and speculated that a linkage relationship between RFLP marker *Xksua1* and the gene in 'PI 294994' might exist. Venter *et al.* (1998) identified two repulsion phase and one coupling phase RAPD marker associated with *Dn5*.

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3. MAPPING OF TRAITS OR GENES

The mapping of traits in a genome requires a comprehensive genetic map with markers covering all chromosomes evenly. This is a necessity when trying to map complex traits or quantitative trait loci (QTLs), for the characterisation of chromosomes and for marker-assisted breeding (Young, 1994). The choice of a mapping population is also important and is influenced by the ultimate goal of the mapping project, for example identification of markers linked to genes, creation of a framework map, *etc*. Additional factors critical to a mapping population include: which parents are chosen for the cross, the population size, what cross type is used and which generation will be used for DNA and phenotype analysis (Young, 1994).

3.1 MAPPING POPULATIONS

A wide variety of mapping populations is available, and they differ in various aspects, for example ease of establishment, suitability, stability, time and cost, *etc*. A few of the more commonly used populations and techniques will be highlighted in the following sections.

3.1.1 F_2 populations

F_2 populations (Figure 7) are derived by selfing F_1 hybrids and this has been the mapping population of choice for the construction of a number of plant genome RFLP maps (Kochert, 1994). One of the main advantages of this population type is the relatively ease and short time required to produce a F_2 population. For a F_2 population, only bagging is required whereas crossing is needed to create a backcross population. A F_2 population provides almost twice as much information for co-dominant markers as a backcross due to the fact that markers are segregating in both the male and female gametic populations generating the F_2 (Tanksley *et al*., 1988a). However, this is also a complication as the genotype will segregate again in the next generation, making it difficult to exchange the mapping population indefinitely between groups (Kochert, 1994). As a temporary solution, F_3 progeny from each F_2 line can be pooled to reconstruct the genotype and phenotype of the original F_2 line.

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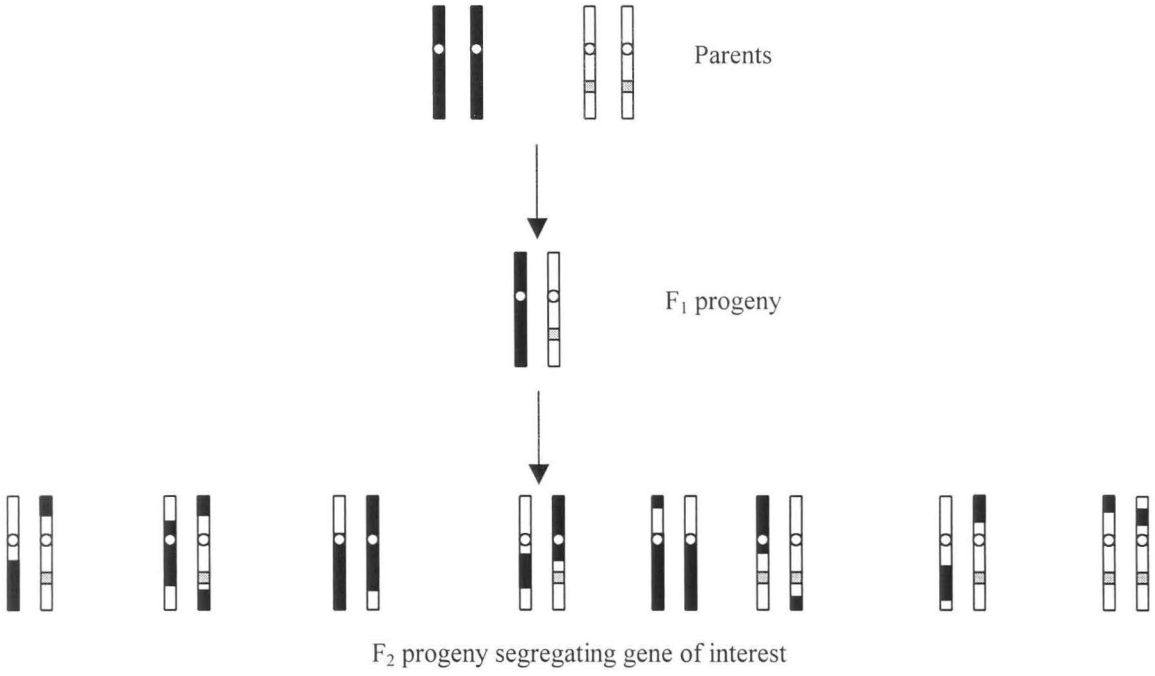

Figure 7. The production of a F_2 population.

3.1.2 Near Isogenic Lines (NILs)

Pairs of near isogenic lines (NILs - Figure 8) are established by repeated backcrossing of a gene or trait of interest from a donor parent to a recurrent parent followed by self-pollination of the final backcross F_1 and are therefore time consuming and expensive to develop (Young *et al.*, 1988; Hu *et al.*, 1997). However, even after five backcrosses, only half the loci polymorphic between NILs are expected to map to a selected region (Muehlbauer *et al.*, 1988) and the donor segment in the NILs comprises 22.8% of the NIL target chromosome (Stam and Zeven, 1981). A pair of NILs consists of a line(s) without the target gene and its counterpart with the target gene, the only difference between them being the introgressed chromosome segment from the donor where the target gene is located. Linkage drag, where large regions linked, or sometimes even unlinked, to the area of interest still remains in the NILs after several backcrosses, can complicate screening for polymorphisms and will result in false positives (Brinkman and Frey, 1977; Young and Tanksley, 1989). Regions of the donor genome can also inadvertently be co-introgressed into the NIL, resulting in the identification of markers not linked to the trait being studied (Young and Tanksley, 1989) or agronomic properties not caused by the introgressed gene being ascribed to the gene (Stam and Zeven, 1981). This problem can be limited by applying bulked segregant analysis (BSA) to the NILs (Michelmore *et al.*, 1991).

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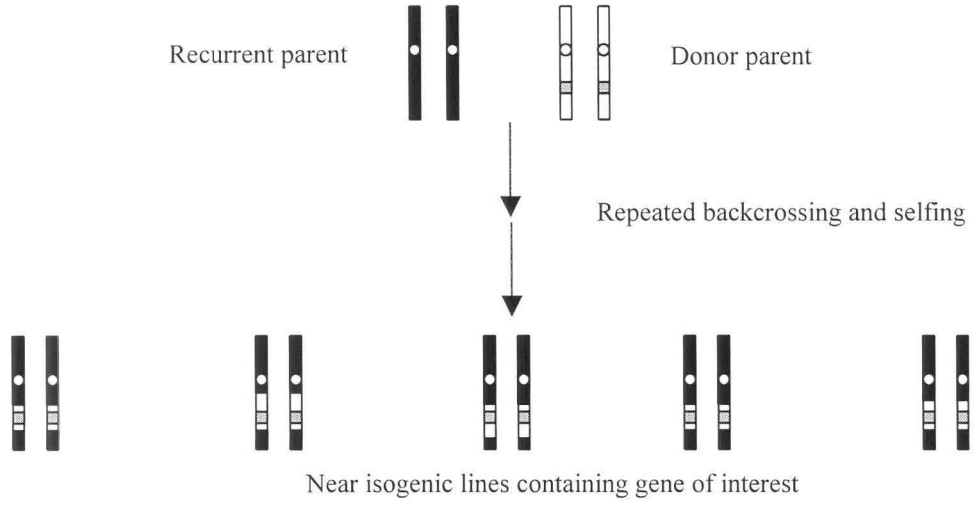

Figure 8. The production of near isogenic lines (NILs).

Near isogenic lines (NILs) are valuable for tagging genes using molecular marker technology as they normally contain a small amount of donor chromatin around the gene of interest (Young *et al.*, 1988). NILs are stable and seed can easily be exchanged between groups. However, due to the difficulty of creating such lines, a limited number of backcross-derived NILs are available for marker screening in wheat (Hu *et al.*, 1997)

3.1.3 Recombinant Inbred Lines (RILs)

Recombinant inbred lines (RILs - Figure 9) are derived through single seed descent of selfed or sib-mated progeny of individual F_2 or backcross plants for many generations, resulting in the fixation of a recombination event in a near-homozygous background (Burr *et al.*, 1988; Burr and Burr, 1991). A recombinant inbred population is therefore a permanent population in which segregation is fixed and this population can therefore easily be shared between groups. If the original parents of the F_2 plants were homozygous, only two alleles will segregate in the RIL population for a given locus. This simplifies mapping in RILs: each RIL is typed for a parental allele at every mapped locus and two loci are linked when alleles from the same parent occur more frequently together than would be expected by random distribution. RILs have two major advantages over conventional F_2 or backcross populations: 1) RILs can be propagated indefinitely without further segregation after homozygosity has been attained and 2) The large number of meiotic events before homozygosity is achieved results in a greater probability that

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linked genes will undergo recombination (Burr and Burr, 1991). The larger number of meioses results in more precise estimates of map distances, but unfortunately also makes it rather time-consuming to produce a population of RILs.

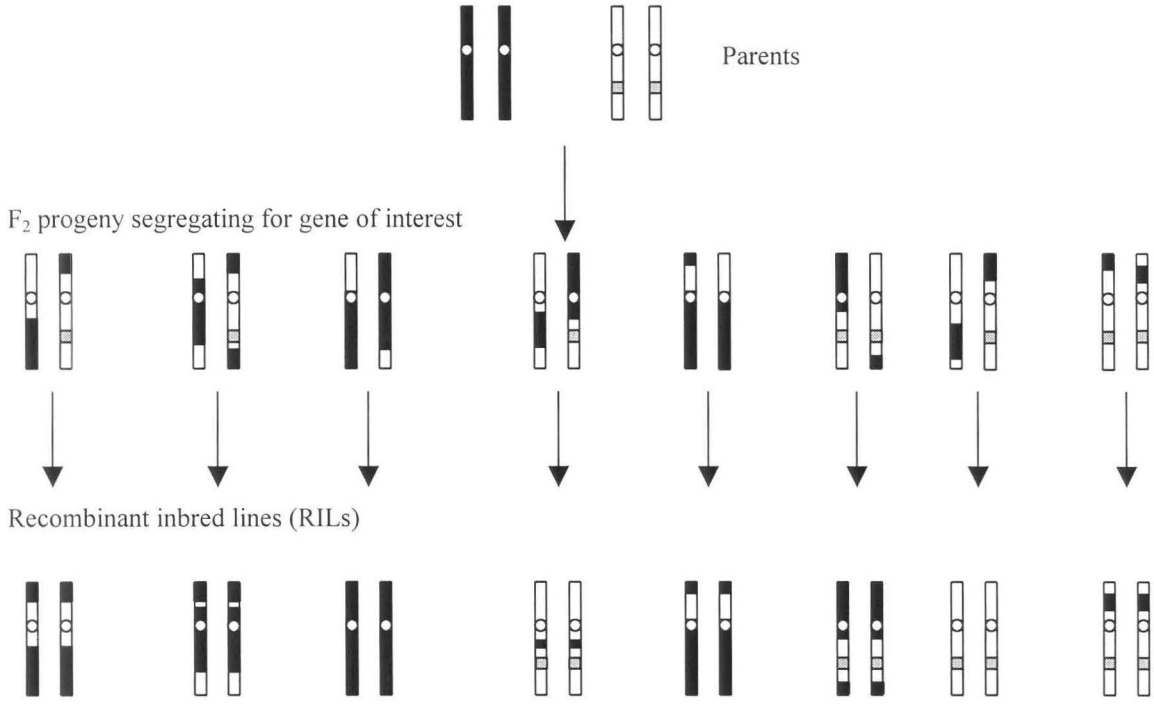

Figure 9. The production of recombinant inbred lines (RILs).

Single seed descent (SSD) is a procedure that can be used to accelerate the production of nearly homozygous lines. Seed is grown in growth rooms at high plant densities to produce plants with only a few grains in a very short generation time. Only one or two seeds are harvested from each plant and the process is repeated as many times as necessary. SSD has a need for extensively controlled environment facilities, is not extremely labour intensive and is more time and cost efficient than some of the other techniques (Bingham and Lupton, 1987).

3.1.4 Deletion Mutant Lines

Deletion mutants (Figure 10) can occur spontaneously (Payne *et al.*, 1984; Snape *et al.*, 1985; Kota and Dvorák, 1986) or can be induced by gametocidal genes (Endo, 1985, 1988a,b and 1990), gamma (Pienaar and Van Niekerk, 1973) or fast neutron (Okubara *et al.*, 1994) irradiation.

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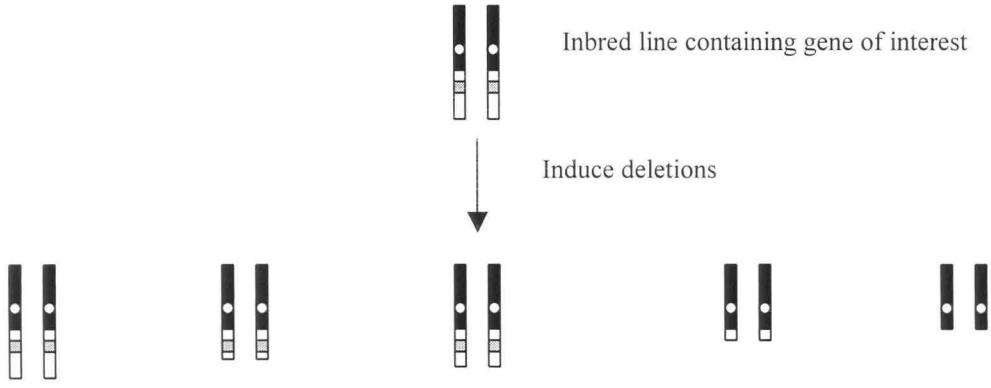

Figure 10. The production of deletion mutant lines (example using an alien introgression containing the gene of interest).

Deletion mapping is a simple and rapid method to construct cytogenetically based physical maps of marker loci (Werner *et al.*, 1992). Mapping can be done in either hemizygous or homozygous chromosome conditions and most genetic markers can be used without the necessity of polymorphisms, allowing for the sub-arm localisation of genes or markers. The genome of wheat is buffered by its polyploidy and can therefore tolerate structurally and numerically modified chromosomes over generations (Werner *et al.*, 1992). A large number of wheat deletion stocks was produced after the discovery of a gametocidal chromosome breakage system in wheat (Endo, 1990; Endo and Gill, 1996). Examples of genes characterised by deletion mapping includes leaf rust resistance *Lr19* (discussed in a previous section) and the storage gene locus *Gli-B1* (Payne *et al.*, 1984).

3.1.5 Doubled Haploid population

A doubled haploid (DH) plant is obtained by doubling the chromosome number of a haploid plant with colchicine, instantly creating a homozygous plant (Nei, 1963; Pienaar *et al.*, 1997). DHs are normally developed from a F_1-derived population, but this material has only had a single parental meiosis event and might therefore reduce the variability and frequency of new gene combinations. However, if little recombination is required, F_1 material is sufficient. F_2 and F_3 populations, or much bigger DH populations, are required if a larger frequency of recombination is necessary (Wenzel *et al.*, 1987). DHs give rise to pure breeding lines that can advance breeding programmes aimed at homozygosity by four to six generations (Pienaar *et al.*,

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1997). Additive and recessive traits can more easily be characterised using DH plants (Wenzel *et al.*, 1987). The recovery of a specific recombinant from x independently segregating loci is calculated to be 2^x times more efficient using DHs than using conventional breeding methods (Jensen, 1977).

The primary methods used to produce haploid wheat plants are anther culture (De Buyser *et al.*, 1987), ovary culture (Comeau *et al.*, 1988) and chromosome elimination following wide crosses (Barclay, 1975; Matzk and Mahn, 1994). Examples of the latter are crosses between wheat and wild barley (*Hordeum bulbosum* - Inagaki and Tahir, 1990), wheat and maize (Laurie and Bennett, 1988a; Inagaki and Tahir, 1990; Pienaar *et al.*, 1997) or wheat and pearl millet (Inagaki and Hash, 1998). The cross between wheat and maize is usually more reliable and stable than anther culture (Kisana *et al.*, 1993). Anther culture is also limited by factors such as few responding genotypes and a low haploid recovery rate (Kisana *et al.*, 1993). Only 0.3% of cultured anthers are useful for practical breeding purposes while 4.8% of pollinated florets produce plantlets in wheat × maize crosses (Kisana *et al.*, 1993). Crosses between hexaploid wheat and *Secale cereale* (Falk and Kasha, 1981 and 1983) or *Hordeum bulbosum* (Falk and Kasha, 1981 and 1983; Sitch *et al.*, 1985) are often hampered by a drastically reduced fertilisation frequency due to the presence of the wheat crossability genes *Kr1* and *Kr2* on chromosomes 5B and 5A, respectively (Riley and Chapman, 1967; Falk and Kasha, 1983). The system used to develop DH lines determines which sex's genotype is fixed, for example anther culture will fix the male gametes while interspecific crosses will fix the female gametes (Wang *et al.*, 1995).

The preferred system for inducing haploids in wheat is the wheat × maize system (Figure 11 - Snape, 1998). In a cross between wheat and maize, the maize chromosomes are eliminated from the developing embryo during the initial rounds of cell division (Laurie and Bennett, 1986). Zygotes from this cross contain one complete haploid chromosome set from each parent, but the maize chromosomes have poorly defined centromeres, appear to have little affinity for the spindle microtubules and are therefore lost after the first few cell divisions resulting in the production of haploid wheat (Laurie and Bennett, 1988b). Because the endosperm of these caryopses is either absent or highly abnormal, the embryos have to be rescued, otherwise they will degenerate if left to develop on the wheat plant (Laurie and Bennett, 1987 and 1988a). Chromosomes are doubled either after the plantlets have been transferred to soil (Inagaki, 1985) or *in vitro* (Barnabás *et al.*, 1991). DH lines produced from wheat × maize crosses were found

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to be equivalent to their wheat parent in agronomic performance, with a slight degree of gametoclonal variation in some of the DH lines (Kisana *et al.*, 1993). It appears as if there might be an interaction between the wheat and maize genotypes used in wheat × maize crosses for the production of haploid plants, with some wheat genotypes responding better than others (Bitsch *et al.*, 1998). Verma *et al.* (1999) found that, under field conditions, differences in efficiency exist in maize genotypes while the influence of wheat genotypes was mild. In their study, the maize pollinator that produced the largest number of haploid plants was five times more efficient than the average of the 15 pollinators tested.

DH technology enables the rapid generation of populations for applications such as linkage mapping (Moharramipour *et al.*, 1997). In a DH population, dominant markers such as AFLPs are as informative as codominant ones such as RFLPs (Voorrips *et al.*, 1997). Increased selection pressure on deleterious alleles in a DH compared to a F_2 population, as well as selection pressure due to culture and plant regeneration processes, might cause an increased frequency of distorted segregation ratios, as found in *Brassica oleracea* by Voorrips *et al.* (1997).

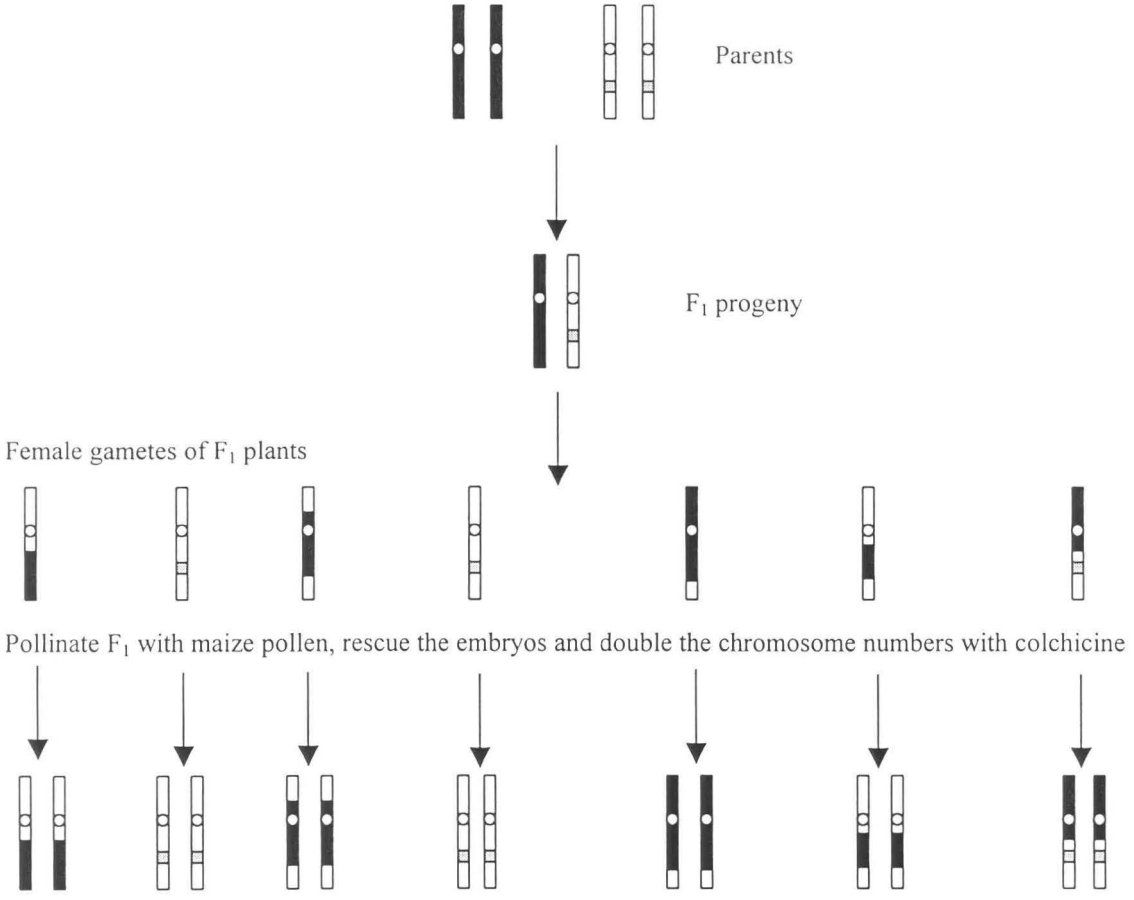

Figure 11. The production of a doubled haploid population using wheat × maize hybridisation.

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3.1.6 Bulk Segregant Analysis (BSA)

In bulked segregant analysis, seeds, plant material or DNA of individuals that share a common trait or gene of interest is bulked together in two contrasting pools (*e.g.* resistant and susceptible) which are tested for association with a second characteristic or molecular marker to determine possible linkage (Michelmore *et al.*, 1991; Eastwood *et al.*, 1994). BSA has been called "local mapping" by Reiter *et al.* (1992). One major advantage of using BSA is the ability to focus on regions of interest or poorly mapped regions when mapping with arbitrary selected markers become less efficient (Michelmore *et al.*, 1991). This procedure is also sufficiently robust to cope with a low level of phenotypic misclassification when enough individuals are bulked together (Poulsen *et al.*, 1995). BSA can also be used for 'genetic walking' along a chromosome, where new loci identified in one round of BSA is used to generate the bulks for the next round (Michelmore *et al.*, 1991). In large genomes, like that of wheat, the tagging of genes by BSA can be enhanced by enriching the DNA for low-copy sequences (Eastwood *et al.*, 1994). However, it has been found that not all polymorphisms detected between bulks are reliable and repeated amplification might be required for confirmation (Gresshoff and Caetano-Anollés, 1997). BSA has been used to identify a large number molecular markers linked to genes or traits of interest. Examples of such genes or traits include leaf rust resistance in barley (Poulsen *et al.*, 1995) and a gall midge resistance gene (*Gm2*) in rice (Nair *et al.*, 1995).

3.2 Physical Mapping

Physical maps show the actual position of a marker on a chromosome. It is important to establish physical maps because genetic linkage distances do not always correspond to the actual physical distance between loci, since those mapped genetically close to centromeres are often found to be more distal on chromosomes after physical mapping (Werner *et al.*, 1992). The unit of recombination, used for measuring genetic distance, might represent anything between 10 to 100,000 kb of DNA in a specific region of the genome (Werner *et al.*, 1992). Lukaszewski and Curtis (1993) calculated that 1 cM may vary in wheat from about 1.530 Mbp in distal chromosome regions to approximately 234 Mbp in centromeric regions. This can be ascribed to non-random distribution of recombination along the length of chromosomes (Meagher *et al.*, 1988). In wheat, genetic maps of the short chromosome arms are mainly made up from the most distal regions of the arms and 70 - 75% of short arms are at best poorly represented in genetic maps (Lukaszewski and Curtis, 1993). For physically long chromosome arms, the figures are

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slightly more positive with the proximal 35 - 40% of the arm not appearing to contribute to the genetic map of the arm while the interstitial 35 - 40% of their length makes a minor but clearly identifiable contribution. The distal 20 - 30% of the long arm makes out the largest proportion of the genetic maps for these chromosome arms (Lukaszewski and Curtis, 1993). It is therefore extremely important to combine genetic mapping with physical mapping. Physical mapping is recommended for resolving the order of loci in proximal regions of wheat chromosomes, while genetic analysis is extremely useful for distally located genes due to the high recombination frequency towards the chromosome ends (Werner *et al.*, 1992).

Physical maps can be constructed by various methods which can be categorised as either cytogenetically based or molecularly based (Delaney *et al.*, 1995). Cytogenetically based methods include chromosome banding, the use of modified chromosome numbers, deletion mutants and *in situ* hybridisation. Molecular based methods include the cloning of DNA fragments into contigs, cosmids, yeast artificial chromosome (YAC; Schlessinger, 1990) libraries and bacterial artificial chromosome (BAC) libraries (Shizuya *et al.*, 1992; Xoconostle-Cázares *et al.*, 1993), restriction mapping using rare cutting restriction endonucleases (Cheung *et al.*, 1991), specialised DNA separation procedures such as pulse-field gel electrophoresis (Cantor *et al.*, 1988) and ultimately DNA sequence analysis. A few of these physical mapping procedures will be highlighted in the following sections.

3.2.1 Chromosome Banding

Chromosome recognition makes it possible to investigate phenomena such as intervarietal variation and evolutionary patterns within a genus (Gill and Kimber, 1974a,b).

Wheat metaphase chromosomes are approximately 5.6 μm long (Sears, 1954) and are therefore very suitable for cytological analysis. Two staining techniques, namely C- (C = constitutive heterochromatin - Figure 12) and N-banding, are mainly used in wheat, revealing alternating dark (heterochromatin) and light (euchromatin) stained regions. It is possible to identify each wheat chromosome by its banding pattern, with the standard karyotype based on 'Chinese Spring' (Gill, 1987). *In situ* hybridisation banding techniques (see section 3.2.3) that employ repetitive

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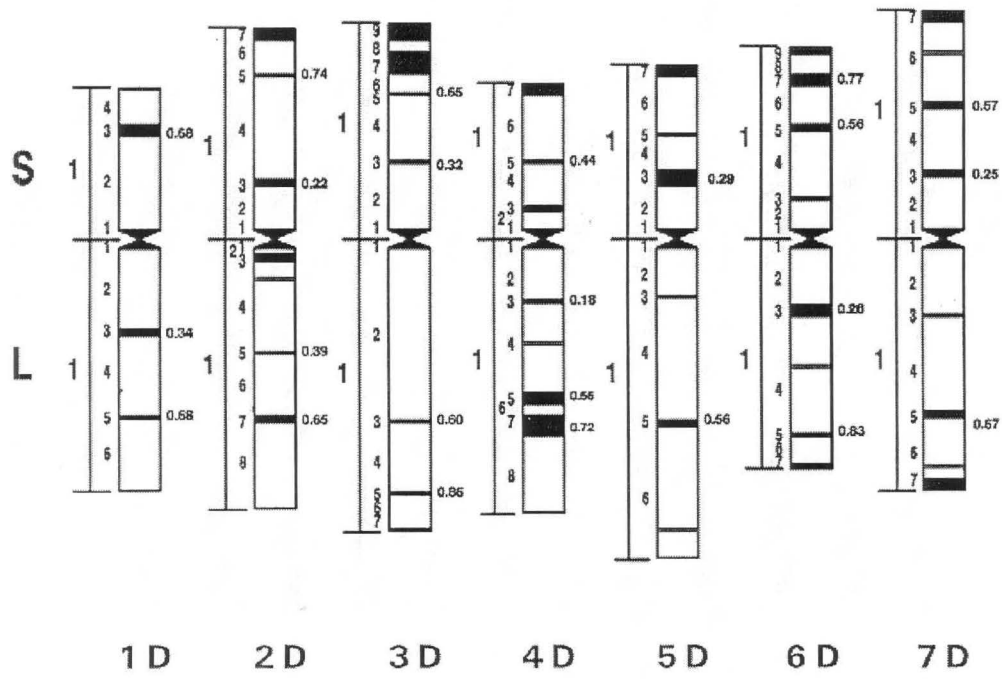
sequences concentrated in tight clusters, *e.g.* FokI in field bean (Fuchs *et al.*, 1994), are gaining popularity over the more traditional banding methods (Busch *et al.*, 1996; Kamstra *et al.*, 1997).

The staining of chromosomes allows the ability to identify specific chromosomes, for example in wheat-rye addition lines (Gill and Kimber, 1974a and 1974b). The use of chromosome banding techniques allowed for the identification of a N-band marker in common wheat for a leaf rust resistance gene derived from a related species (Yamamori, 1994).



(Source: http://wheat.pw.usda.gov)

Figure 12. C-banding of the chromosomes of the D genome of common wheat.

3.2.2 Aneuploidy

Wheat is an excellent crop for constructing a chromosomal arm map as there is an abundance of aneuploid stocks available for it (Sears, 1954; Kimber and Sears, 1970). The genome is buffered by its polyploidy and can therefore tolerate structurally and numerically modified chromosomes (Werner *et al.*, 1992). Sears (1939, 1954 and 1966) obtained 42 nullisomic-tetrasomic compensating combinations of 'Chinese Spring' using monosomics (one chromosome

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of a homologous pair present), nullisomics (homologous chromosome pair absent) and tetrasomics (additional homologous chromosome pair present). This was followed by a series of 42 ditelosomics of which a small number are non-viable or sterile and are maintained as heteromorphic pairs (Sears and Sears, 1978). Ditelosomics are homologous chromosome pairs from which either the short or long arms are missing. Monosomic analysis is frequently used to assign genes to chromosomes whereas ditelosomics can be used to determine the chromosome arm location (Sears and Sears, 1978).

The major advantage of an aneuploid stock is the enhanced tagging of qualitatively inherited traits by initial assignment to a chromosome arm, followed by fine-mapping of the locus using chromosome arm specific markers (Anderson *et al.*, 1992). A disadvantage of mapping to chromosome arms (for example with ditelosomic stocks) is that the linear arrangement and genetic linkage between the marker or gene being mapped and the markers on the specific arm map are not known during the screening process and further molecular mapping will be required after the chromosome arm has been identified.

3.2.3 *In situ* Hybridisation

Isolated DNA sequences can be used as hybridisation probes on chromosomes mounted on glass slides (*in situ*), allowing for a detailed molecular cytogenetic analysis of the wheat genome. The probes can be labeled with radioisotopes (Gall and Pardue, 1969; Appels *et al.*, 1980; Hutchinson and Lonsdale, 1982) or biotin (Rayburn and Gill, 1985). Variations of the *in situ* hybridisation (ISH) protocol include fluorescence ISH (FISH - Reader *et al.*, 1994) and genomic ISH (GISH - Durnam *et al.*, 1985; Pinkel *et al.*, 1986) and these allow for the identification and mapping of repetitive DNA sequences, specific DNA probes and alien chromatin on wheat chromosomes (reviewed by Gill, 1995). FISH uses region-specific probes, for example centromeric or chromosome specific probes to detect specific sequences on a chromosome. GISH, on the other hand, uses labeled total DNA from one parent or species as probe on the unlabeled chromosomes of the target parent or species, allowing for the identification of 'alien' DNA present in the unlabeled chromosomes.

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3.2.4 Sequencing

DNA sequences are the highest level of physical mapping. DNA is characterised at nucleotide level, allowing for future manipulation of DNA, such as in plant transformation. DNA sequencing can be based on either the Maxam-Gilbert chemical degradation method (Maxam and Gilbert, 1977) or the Sanger enzymatic sequencing method (Sanger and Coulson, 1975; Sanger *et al.*, 1977a). Initially, sequencing reactions were visualised by autoradiography or silver staining after polyacrylamide gel electrophoresis but now semi-automated machines using fluorescent dyes are available for running and analysing sequencing data (Wilson *et al.*, 1990).

At the moment there are a number of genome sequencing projects operational worldwide, examples include the Human Genome Project and projects aimed at obtaining the DNA sequences of various yeast, plants, viruses, *Drosophila* and nematodes. The ultimate goal of these projects is to obtain the full sequence for all the DNA contained in each organism. The complete nucleotide sequences of some genomes have already been determined and published, for example simian virus genomes (*e.g.* Fiers *et al.*, 1978), bacteriophage genomes (*e.g.* Sanger *et al.*, 1977b), the fruit fly *Drosophila melanogaster* (http://www.fruitfly.org/) and bacterial plasmids (*e.g.* Sutcliffe, 1979), as well as the sequence of human chromosomes *e.g.* 21 (Clancy and Patterson, 1999). A draft sequence comprising 97% of the human genome has also been announced in June 2000 and updated information can be found on the internet at the address http://www.ncbi.nlm.nih.gov/genome/guide/human/.

With the complete sequence of *Arabidopsis thaliana* reported recently, the first plant sequence is now available (Walbot, 2000). The sequence was found to contain a lot of genetic redundancy, with at least 70% of the genome being duplicated. A project aimed at sequencing and deciphering the chromosomal location and biological function of all genes in the wheat genome is also currently underway. The web site of the wheat genome project can be found at:

http://wheat.pw.usda.gov/NSF/project.html

The complete sequence of the chloroplast genome of 'Chinese Spring' has also been determined recently and is available electronically at the following web address:

http://ftp2.ddbj.nig.ac.jp:8000/getstart-e.html (Accession Number AB042240).

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3.3 GENETIC MAPPING

Selection or genetic analysis based solely on phenotypic traits can be influenced by the environment, multigenic and quantitative inheritance or partial and incomplete dominance, is time-consuming and can lead to misclassification of agronomically important genes (Procunier *et al.*, 1997; Tingey and Del Tufo, 1993). A large number of these factors can be eliminated or reduced if genotype can be identified directly with a DNA-based diagnostic assay, for example using molecular markers linked to a trait of interest. Molecular markers are extremely useful in a breeding program when linked to traits that are difficult or time-consuming to select for using conventional breeding methods and can also be used as a starting point for the physical mapping of genes (Tanksley *et al.*, 1989; Martin *et al.*, 1993; William *et al.*, 1997). These markers are normally developmentally stable, can be detected in all tissues and are unaffected by environmental conditions (Lu *et al.*, 1998). Markers linked to specific resistance genes can be used to determine which resistance genes are present in breeding lines with more than one resistance gene or when pyramiding two or more resistance genes (Feuillet *et al.*, 1995; Hu *et al.*, 1997). Unfortunately, the genetic basis of a particular disease resistance in economically important breeding lines is often not known and this complicates breeding for different resistance gene combinations (Feuillet *et al.*, 1997).

Genetic mapping was revolutionised with the discovery of the polymerase chain reaction (PCR) (Saiki *et al.*, 1985; Mullis and Faloona, 1987; Saiki *et al.*, 1988, *etc.*). The PCR technique makes it possible to amplify, in one reaction, large numbers of DNA sequences distributed randomly throughout a genome (Paran and Michelmore, 1993; Cho *et al.*, 1996). Several PCR-based fingerprinting procedures make use of a single short primer of arbitrary sequence, for example AP-PCR (arbitrarily primed PCR - Welsh and McClelland, 1990), RAPDs (random-amplified polymorphic DNA - Williams *et al.*, 1990) and DAFs (DNA amplification fingerprinting - Caetano-Anollés *et al.*, 1991a), and these were collectively called multiple arbitrary amplicon profiling (MAAP) by Caetano-Anollés (1994). Evenly spaced molecular markers are also useful for the analysis of quantitative traits by partitioning complex traits into quantitative trait loci (QTLs) (William *et al.*, 1997).

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Genetic markers used for marker-assisted selection or QTL analysis must meet certain criteria (Shin *et al.*, 1990; Maheswaran *et al.*, 1997; Mueller and Wolfenbarger, 1999):

1. Should be cheap and time efficient
2. Multiple independent markers should be generated
3. Adequate resolution of genetic differences should be provided (must have clear phenotypes)
4. The system must be reliable and replicable
5. Extremely small and even partly degraded tissue and DNA samples should be sufficient
6. Little operator experience should be required
7. No prior information about the organism's genome should be required
8. If prior information is available, the markers must map to specific loci
9. High levels of polymorphisms should be generated
10. It may not influence or modify the character(s) being evaluated

Very few existing techniques meet all of these criteria. A number of these techniques will be discussed in the following pages and some are summarised in Table 3.

Table 3. Summary of genetic marker systems as reviewed by Ridout and Donini (1999) and Mueller and Wolfenbarger (1999).

	Genetic marker system				
Criterion	Isozyme	RFLP	RAPD	AFLP	SSR
Quantity of information	Low	Low	High	High	High
Replicability	High	High	Variable	High	High
Resolution of differences	Moderate	High	Moderate	High	High
Ease of use and development	Easy	Difficult	Easy	Moderate	Difficult
Development time	Short	Long	Short	Short	Long
Amount of DNA required (μg)	none	10.0	0.02	0.5-1.0	0.05-0.10
PCR-based	No	No	Yes	Yes	Yes

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3.3.1 Isozymes

Isozymes can be differentiated by their relative migration during gel electrophoresis because of amino acid charge differences (Mueller and Wolfenbarger, 1999). The particular enzyme is visualised on the gel by supplying the appropriate substrate and cofactors and involving the product in a colour-producing reaction, after which the coloured product is deposited in the gel to form a visible band at the location of the enzyme (Staub *et al.*, 1996). Several isozymes have been reported in wheat, examples include alcohol dehydrogenase and acid phosphatase (Hart, 1973; Tang and Hart, 1975), as well as lipoxygenase (Hart and Langston, 1977), esterase (Ainsworth *et al.*, 1984), aminopeptidase and α-amylase (Ainsworth *et al.*, 1985; Chao *et al.*, 1989). The various isozymes and proteins differ in extraction, electrophoretic and staining techniques. Once the assay has been established, it is rapid, economical and potentially highly informative as it can provide information as co-dominant markers. A disadvantage is that the number of isozyme and storage protein markers is limited and only a low number of protein-based marker loci has been mapped (Milne and McIntosh, 1990). Another disadvantage is that the expression of these markers is often tissue and developmental specific and influenced by the environment.

Endopeptidase markers were used in this study and it will therefore be discussed in more detail. Hart and Langston (1977) first described four endopeptidase bands for the zymogram of 'Chinese Spring' and each band was assigned in order of increasing mobility to chromosomes 7DL, 7AL, 7BL and 7BL respectively. Three structural endopeptidase alleles on chromosome 7AL and two on 7BL of the wheat 'PI 357307' were reported by McMillin and Tuleen (1977). Three endopeptidase bands were observed on a zymogram of 'Chinese Spring' with the intermediate band a product of two isozymes (Tang and Hart, 1975). Two alleles (*Ep-D1a* and *Ep-B1a*) were identified by Koebner *et al.* (1988) in embryo halves of mature seed from 'Chinese Spring', while a third band (occurring in some plants) corresponded to the position of the 7AL band described by Hart and Langston (1977). Four more *Ep-B1* alleles and two more *Ep-A1* alleles, one of which was a null allele, were identified in other hexaploid wheats. The *Aegilops ventricosa*-derived translocation in 'Rendezvous' and a null allele (*Ep-D1c*) proposed to exist in 'Synthetic' contribute to variation at the *Ep-D1* locus. An additional endopeptidase allele, *Ep-D1e*, has also been identified (Marais *et al.*, 1998).

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3.3.2 Restriction Fragment Length Polymorphisms (RFLPs)

Restriction fragment length polymorphism (RFLP) has been shown to be a powerful marker technique for establishing linkage relationships to important qualitative and quantitative genes (Botstein *et al.*, 1980; Beckman and Soller, 1983; Paterson *et al.*, 1991). Polymorphisms are usually attributed to differences in the length of DNA fragments after digestion with restriction endonucleases and gel electrophoresis. Differences can normally be attributed to the presence or absence of restriction sites in the sequence (Figure 13) or variation in fragment length due to insertions or deletions (Botstein *et al.*, 1980; Beckman and Soller, 1983). In cereals, RFLPs appear to be caused by insertions and deletions rather than mutations of restriction sites (Gale and Sharp, 1988). The polymorphisms are revealed after Southern blotting (Southern, 1975) to a membrane by hybridisation of a specific labeled fragment (normally 500 to 3,000 base pairs in length) to the digested DNA fixed on a membrane. Depending on the detection method used, the filter can be placed against a photographic film and the film is developed after autoradiography (Staub *et al.*, 1996; Mueller and Wolfenbarger, 1999).

RFLP markers are often co-dominant, show Mendelian segregation and recognise low-copy sequences (Chao *et al.*, 1989; William *et al.*, 1997). These markers are locus specific, but unfortunately the procedure involved is very laborious and expensive and only a few loci are detected per assay (Bai *et al.*, 1999a). Large amounts of high quality DNA are needed and the use of radioactivity is often involved in the procedure (Singh *et al.*, 1999). However, nonisotopic chemiluminescent detection systems of digoxigenin-labeled DNA fragments have also been developed (Höltke *et al.*, 1992). A move towards the conversion of RFLP markers to PCR-based allele-specific markers are currently underway (Helguera *et al.*, 2000) and these markers are less expensive, easier, faster and requires much less DNA (refer to section 3.3.7). The detection limit of RFLPs is determined by the DNA binding capacity of the membranes used for Southern blotting (Southern, 1975) and the labeling efficiency of the DNA probes (Han *et al.*, 1999). In a study by Ma and Lapitan (1998), an average RFLP polymorphism level of 61% has been suggested for a population of 48 wheat individuals, requiring 14 RFLP probes to screen the parents to give 8 RFLP probes useful in the population. Gill *et al.* (1991) detected 75% polymorphisms between two *Triticum tauschii* accessions digested with four different restriction endonucleases. Eighteen cDNA clones were used by Chao *et al.* (1989) to detect

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variation at the group 7 loci between pairs of six common wheat varieties examined with 13 restriction enzymes and polymorphism was found in only 8.7% of the comparisons. A wheat chromosomal arm map that comprises of more than 800 restriction fragments has been reported by Anderson *et al.* (1992). Cereal genes tend to occur in clusters and it often happens that RFLPs fail to detect these clusters (Gill *et al.*, 1996). However, closely linked RFLP markers have been reported for, amongst others, leaf rust resistance gene *Lr9* (Schachermayr *et al.*, 1994) and powdery mildew resistance gene *Pm3* (Hartl *et al.*, 1993).

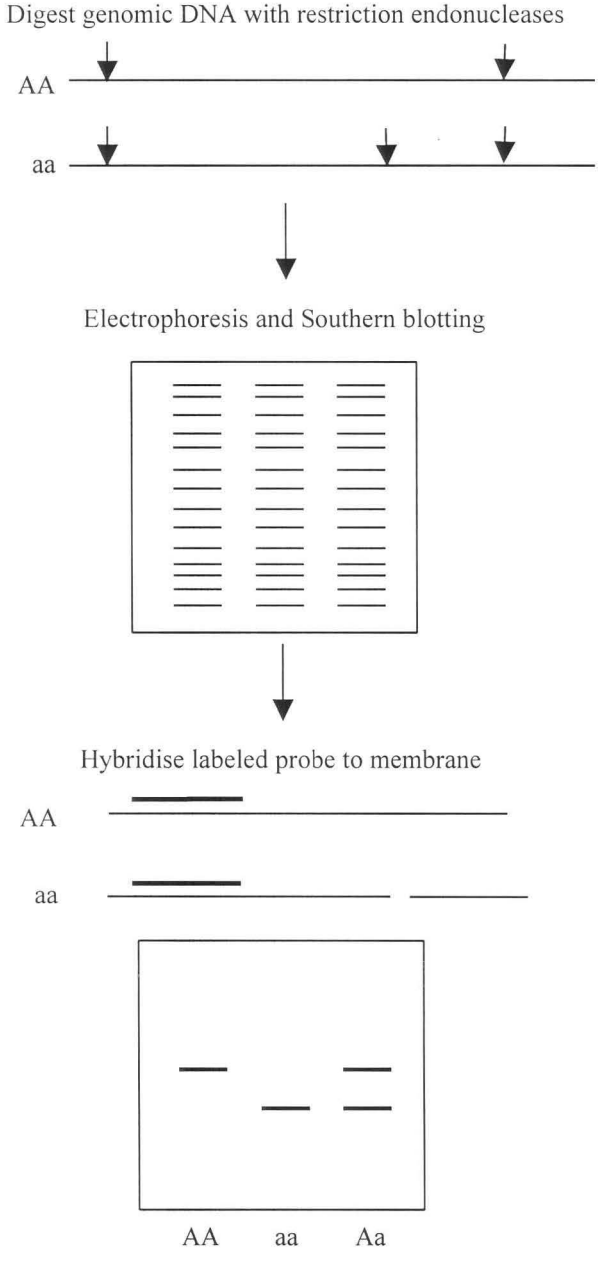

Figure 13. Restriction fragment length polymorphism (RFLP).

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3.3.3 Arbitrarily primed PCR (AP-PCR)

Arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990; Williams *et al.*, 1990) uses single arbitrarily chosen primers and two cycles of low stringency PCR followed by many cycles of high stringency PCR to produce simple and reproducible fingerprints of complex genomes (Figure 14). An AP-PCR fingerprint is generated by PCR amplification of a small amount of DNA using relaxed stringency with a single randomly selected PCR primer (Welsh *et al.*, 1991). A different fingerprint pattern is revealed because a somewhat different set of sites in the genome matches the primer best or because of deletions and insertions. AP-PCR is easy, fast and polymorphisms can easily be identified with almost every primer used, without prior knowledge of the genome. Polymorphic fragments can easily be isolated directly from the gel, reamplified and used as probes. Welsh and McClelland (1991) used consensus tRNA gene primers to amplify regions between tRNA genes, generating fingerprints that are generally invariant between strains of the same species and are often conserved between related species. Two AP-PCR techniques that are used more commonly, namely RAPDs and DAFs, will be discussed in the next two sections.

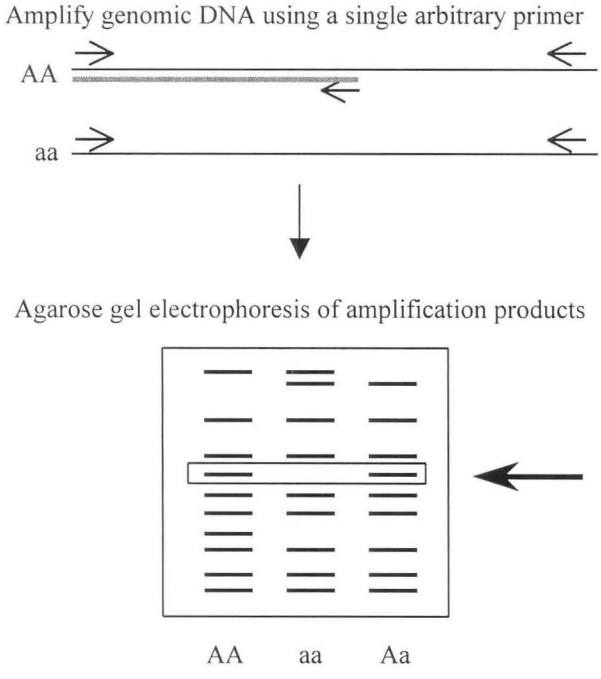

Figure 14. Dominant mode of inheritance of AP-PCR, including RAPDs and DAFs markers.

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3.3.4 AP-PCR : Random-Amplified Polymorphic DNA (RAPD)

Random-amplified polymorphic DNA (RAPD - Williams *et al.*, 1990; Rafalski *et al.*, 1991) is a PCR-based technique that involves the use of a single arbitrary primer in an amplification mixture under relatively low stringency conditions (Figure 14). A number of discrete DNA fragments is amplified when areas in the genome, in an inverted orientation, contain sequences complementary to the primer sequence and it is possible for the DNA polymerase to synthesise an amplification product (Jones *et al.*, 1997). Primers bind competitively to the template DNA and will therefore bind more often to repetitive DNA sequences than low-copy ones (William *et al.*, 1997). The low stringency of the PCR increases the chances for non-specific priming and artifactual polymorphisms of up to 60% have been reported (Pérez *et al.*, 1998). Many primers are commercially available in the form of kits. Amplification products can be size-fractionated on either agarose or polyacrylamide gels (Welsh and McClelland 1990; Williams *et al.*, 1990). William *et al.* (1997) and Nair *et al.* (1995) recommended that RAPD polymorphisms should rather be converted to more dependable RFLP or STS assays in problematic crops like wheat.

RAPDs can be used for a variety of applications, including the following examples:

1. Estimate the outcrossing rate in a breeding population (Gaiotto *et al.*, 1997)
2. Fingerprinting of cultivars (Sosinski and Douches, 1996)
3. Identification of markers linked to genes (Nair *et al.*, 1995; Poulsen *et al.*, 1995; Hu *et al.*, 1997)
4. Calculating the genetic distance between cultivars (Tinker *et al.*, 1993; Myburg *et al.*, 1997)

RAPDs have several advantages above other techniques such as RFLPs and isozymes: 1) very small amounts of DNA are required, 2) the technique is nonisotopic, 3) it is relatively cheaper, 4) it is a relatively simpler and quicker laboratory procedure and 5) a larger number of polymorphisms can be revealed (Sosinski and Douches, 1996). One of the main drawbacks of RAPDs is the need for consistent reaction conditions (especially template DNA and $MgCl_2$ concentrations) in order to get reproducible results (Nair *et al.*, 1995; Jones *et al.*, 1997; Pérez *et*

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al., 1998). Minor bands that are not repeatable are sometimes observed after agarose gel electrophoresis and has been ascribed to the formation of heteroduplexes between multiple amplified fragments or non-specific amplification (He et al., 1992). RAPDs are mainly of dominant nature as the fragments are normally scored as present or absent but can differ in length when present (Williams *et al.*, 1990; Nair *et al.*, 1995; Mueller and Wolfenbarger, 1999).

RAPD polymorphism frequencies of 0.3 per primer in *Arabidopsis thaliana*, 0.5 per primer in soybean, 1 per primer in corn and 2.5 per primer in *Neurospora crassa* have been reported (Tingey and Del Tufo, 1993). Due to the complexity of the wheat genome, it has been predicted by Devos and Gale (1992) that RAPDs will be of limited use for linkage mapping in wheat but may be useful for the characterisation of introgressed chromosome fragments. Schachermayr *et al.* (1994) used 395 RAPD primers to identify markers linked to leaf rust resistance gene *Lr9* and only three of the primers differentiated between the resistant NILs and 'Arina'. However, RAPD markers linked to the powdery mildew resistance gene *Pm1* (Hu *et al.*, 1997), leaf rust resistance gene *Lr9* (Schachermayr *et al.*, 1994), a number of Russian wheat aphid resistance genes (Myburgh *et al.*, 1998; Venter and Botha, 2000) and Hessian fly resistance gene *H9* (Dweikat *et al.*, 1994) have been reported in wheat. Conversion of wheat RAPD markers to more effective SCAR markers has also been reported, but with a rather low success frequency (Schachermayr *et al.*, 1994; Myburgh *et al.*, 1998; Venter and Botha, 2000).

3.3.5 AP-PCR : DNA Amplification Fingerprinting (DAF)

DNA amplification fingerprinting (DAF) is a PCR-based procedure that uses a single arbitrary GC-rich primer of 5 to 8 nucleotides in length (Figure 14) to generate relatively complex profiles of 40 to 60 bands per polyacrylamide gel lane after silver staining (Bassam *et al.*, 1991; Caetano-Anollés *et al.*, 1991a and 1991b; Weaver *et al.*, 1995). Major advantages of DAFs include: silver stained gels can be archived for future reference (Prabhu *et al.*, 1997), the technique is nonisotopic, does not require the use of membranes and no knowledge of the target genome is required (Caetano-Anollés *et al.*, 1991a). Nonisotopic gels have the additional advantage that fragments can be isolated directly from the gel and used as probes for Southern blotting (Southern, 1975; Weaver *et al.*, 1994; Men and Gresshoff, 1998). DAFs are very reproducible,

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less than 2% variation in mobility and less than 3% variation in product yield was found for flowering dogwood (Caetano-Anollés *et al.*, 1999).

To improve the detection of polymorphic DNA and allow for fingerprinting using very small DNA fragments, several variations of the DAF procedure have been used. Among these are restriction endonuclease digestion of template DNA (tecMAAP - Caetano-Anollés *et al.*, 1993) or the use of mini-hairpin sequences at the 5' end of primers (Caetano-Anollés and Gresshoff, 1994). Another variation called ASAP (arbitrary signatures from amplification profiles) utilises a dual-step 'fingerprint of fingerprint' amplification procedure where 5'-anchored short simple repeat (SSR) primers are used for reamplification of DAF amplicons (Caetano-Anollés and Gresshoff, 1996).

DAFs can be used for a variety of applications, including the following examples:

1. Estimation of the genetic relationships between species (Caetano-Anollés *et al.*, 1995; Prabhu *et al.*, 1997)
2. Fingerprinting cultivars (Weaver *et al.*, 1995)
3. Analysis of genetic diversity (He *et al.*, 1995)
4. Identification of markers linked to a specific locus (Men *et al.*, 1999)

In wheat, Sen *et al.* (1997) obtained 20-35 scorable bands (smaller than 2 kb) with nine out of ten linear primers tested on bread wheat. They concluded that primers with low GC content amplified better DAF products with more distinct bands and less background smear. This was, however, not in direct correlation with the degree of polymorphism revealed by the primers. Four of the ten mini-hairpin primers tested by Sen *et al.* (1997) gave excellent DNA profiles while the remaining six primers gave only a few faint bands and a lot of background smear. This suggested that mini-hairpin primers will be of limited use for detecting polymorphisms in bread wheat. However, the authors' final conclusion was that DAF technology may become a useful tool for the detection of polymorphisms and the development of molecular markers for gene tagging and marker aided selection in wheat breeding.

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3.3.6 Amplified Fragment Length Polymorphisms (AFLPs)

AFLP markers are a very recent innovation in genetic marker technology and can be used with greater success in large-scale mapping (Vos *et al.* 1995; Keim *et al.*, 1997). The AFLP system is based on the selective amplification of fragments of gDNA cut with rare (typically *Sse8387*I, *Pst*I or *Eco*RI) and frequent (typically *Mse*I) cutter restriction endonucleases. This is accomplished by ligating DNA adaptors to the restriction fragment ends, followed by amplification with primers homologous to the adapter sequences. The addition of arbitrary nucleotides to the 3' end of the primer increases selectivity of amplification and decreases the complexity of the resulting amplification fingerprint (Figure 15). Typically, the addition of three selective nucleotides to each primer will result in 50 to 100 loci being amplified (Vos *et al.* 1995; Law *et al.*, 1998). A single AFLP primer combination can detect up to eight times more polymorphism than a polymorphic RFLP probe (Ma and Lapitan, 1998). However, this is dependent on the specific primer combination utilised as well as the complexity of the genome and the polymorphism frequency of the species being studied (Keim *et al.*, 1997). The use of up to four selective nucleotides has been reported for wheat (Bai *et al.*, 1999b), Scots pine (Lerceteau and Szmidt, 1999) and sugarbeet (Hansen *et al.*, 1999) and up to eight selective nucleotides for the Inca lily (Han *et al.*, 1999). The complexity of an AFLP fingerprint can also be decreased by using three restriction endonucleases instead of two (Lindstedt *et al.*, 2000; Van der Wurff *et al.*, 2000)

The amplification fingerprints are size-fractionated on a denaturing polyacrylamide sequencing gel, allowing size discrimination as small as one base pair. AFLP patterns can be visualised by autoradiography when using a radioisotope or by silver staining. Both techniques were shown to give identical patterns in rice, although silver staining produced a larger number of scorable bands (Cho *et al.*, 1996). A fluorescence-based semi-automated AFLP approach in barley and wheat (Huang *et al.*, 2000; Schwarz *et al.*, 2000) and evergreen azaleas (De Riek *et al.*, 1999) has also been reported recently. In lower organisms, for example *Bacillus cereus*, AFLP fingerprints have been analysed on a 1.5% agarose gel after staining with ethidium bromide (Ripabelli *et al.*, 2000). AFLP fingerprinting has been shown to be robust, extremely reproducible and highly polymorphic in *Eucalyptus* (Gaiotto *et al.*, 1997, Singh *et al.*, 1999). Approximately 7% of the primer combinations used in wheat amplified at least one polymorphic band between DNA bulks for scab resistance (Bai *et al.*, 1999b).

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Normally, AFLP markers behave in a dominant fashion. In soybean, 87% of AFLP bands were found to be dominant, with some markers being co-dominant (Keim *et al.*, 1997). A disadvantage of AFLPs is that it is difficult to identify homologous markers (alleles) and they are therefore not optimal for use in heterozygosity studies (Mueller and Wolfenbarger, 1999). However, several cases have been reported where co-dominant AFLP markers were identified at frequencies of 4 - 15% (Waugh *et al.*, 1997; Lu *et al.*, 1998; Boivin *et al.*, 1999, Bai *et al.*, 1999a, Caranta et al., 1999). Another disadvantage is the co-migration of non-allelic fragments of the same size especially in complex organisms, however it has been found that this occurs at very low levels in potato (Rouppe van der Voort *et al.*, 1997a; Qu *et al.*, 1998).

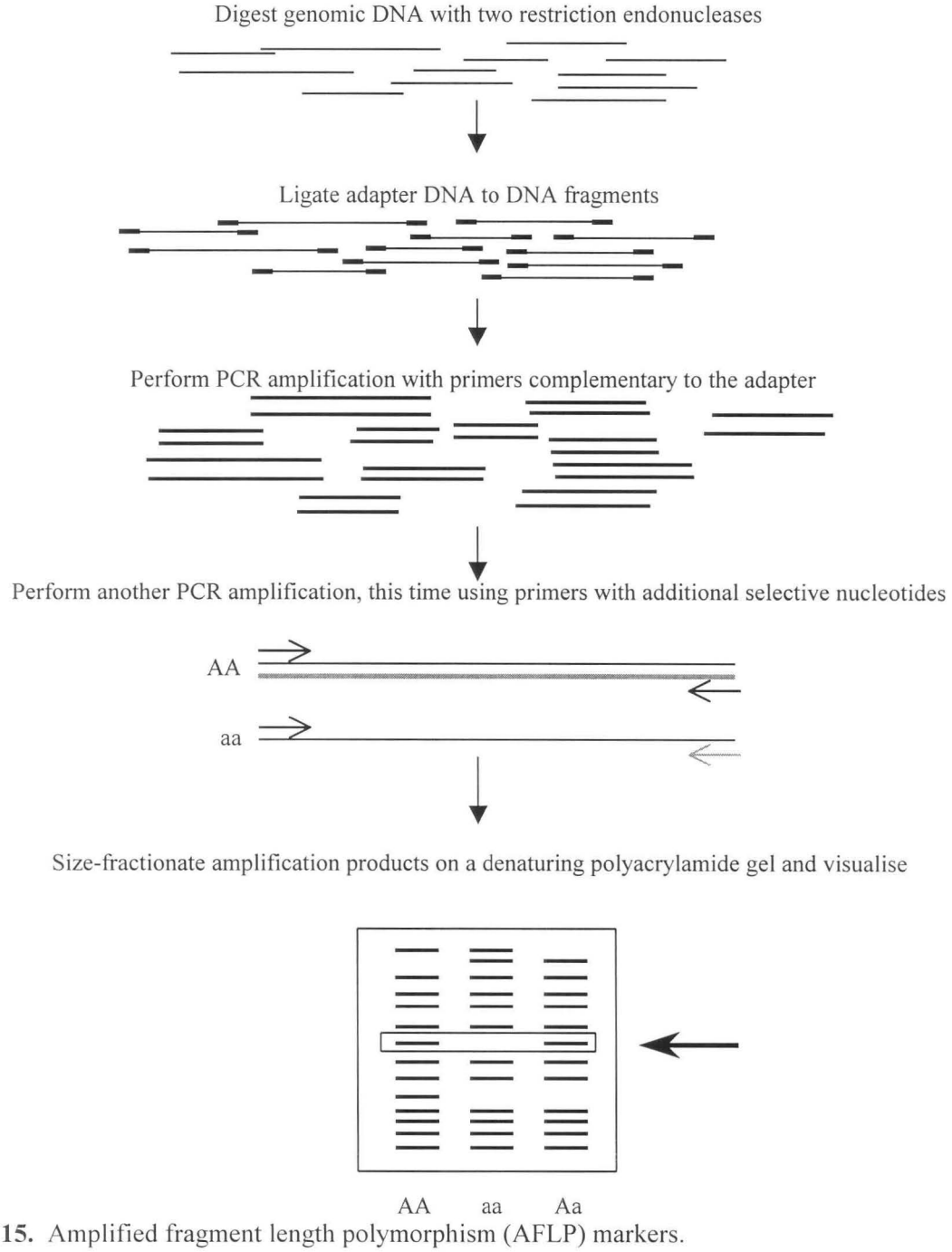

Figure 15. Amplified fragment length polymorphism (AFLP) markers.

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AFLPs can be used for a variety of applications. Among these are:

1. Create high-density genetic maps (Keim *et al.*, 1997; Cho *et al.*, 1998)
2. Estimation of the outcrossing rate in a breeding population (Gaiotto *et al.*, 1997)
3. Assessment of genetic differences (Jackson *et al.*, 1999; Mueller and Wolfenbarger, 1999)
4. Mapping of quantitative trait loci (Powell *et al.*, 1997; Voorrips *et al.*, 1997; Yin *et al.*, 1999)
5. Identification of markers linked to important genes (Rouppe van der Voort *et al.*, 1997b; Lu *et al.*, 1998)
6. Determination of the genomic contribution of parents to a population (VanToai *et al.*, 1997)
7. Characterisation of somaclonal variation (Vendrame *et al.*, 1999)
8. Fingerprinting and genotyping of important pathogens and other organisms (Duim *et al.*, 1999; Hansen *et al.*, 1999)

Currently, all evidence suggests that AFLPs are as reproducible as RFLPs (Jones *et al.*, 1997; Law *et al.*, 1998). Schondelmaier *et al.* (1996) tested two different *Taq* DNA polymerase enzymes (Pharmacia and Perkin-Elmer) and found that low molecular weight patterns were identical for both enzymes, but amplification products above 400 bp were more reliable using the Perkin-Elmer enzyme. It has been shown that variation in AFLP fingerprints due to differences in the wheat plant organs used as template exists. It was illustrated that the complexity of the fingerprint, when using a methylation-sensitive enzyme, increases from leaf DNA to seed DNA to seedling root DNA (Donini *et al.*, 1997). It has been estimated that 5-methyl cytosine is the most common methylated base in plants and represents up to 30% of all C in a plant genome (Gruenbaum *et al.*, 1981). In a study on maize by Castiglioni *et al.* (1999), it was found that *Eco*RI/*Mse*I-generated AFLP markers tended to localise preferentially at the centromeric regions while *Pst*I/*Mse*I-generated markers were randomly distributed across the chromosomes with the exception of one chromosome where they were associated with telomeric sequences. Barrett and Kidwell (1998) found that the methylation-sensitive combination *Pst*I/*Mse*I detected significantly lower levels of diversity in wheat than the methylation-insensitive combination *Eco*RI/*Mse*I and ascribed it to the possibility that hypomethylated

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regions might contain less DNA variation than hypermethylated regions. This is contrary to Powell *et al.* (1997), who found that *Pst*I/*Mse*I-generated markers were more efficient in identifying polymorphisms than *Eco*RI/*Mse*I in barley. Clustering of AFLP markers in the centromeric region has, for example, been reported for wheat (Moore *et al.*, 1993; Hart, 1994), barley (Castiglioni *et al.*, 1998), rice (Nandi *et al.*, 1997), soybean (Keim *et al.*, 1997), ryegrass (Bert *et al.*, 1999), and tomato (Haanstra *et al.*, 1999), but not in tef (Bai *et al.*, 1999a), a DH rice population (Maheswaran *et al.*, 1997), or pine (Remington *et al.*, 1999). The use of a methylation-sensitive restriction enzyme like *Sse*I has been recommended by Law *et al.* (1998) to prevent bias of fragments to the repetitive sequences. Castiglioni *et al.* (1999) postulated that *Pst*I/*Mse*I-generated markers might be associated with the hypomethylated non-centromeric regions of the chromosomes. Clustering of the markers around the centromere has also been attributed to the centromeric suppression of recombination (Tanksley *et al.*, 1992), heavy methylation in these regions (Keim *et al.*, 1997) and/or the presence of repeated sequences in the peri-centromeric areas (Alonso-Blanco *et al.*, 1998; Qi *et al.*, 1998). Implications therefore are that, depending on the type of chromatin a gene is localised in, the use of certain restriction enzyme combinations might be favoured, for example *Pst*I/*Mse*I markers were used to locate the *Cf-9* gene in tomato euchromatin (Thomas *et al.*, 1995) while *Eco*RI/*Mse*I markers were used to locate the *Mi* gene in tomato heterochromatin (Kaloshian *et al.*, 1998) Similar results (regions void of AFLP markers on one map will have AFLP markers on the other map) was observed by Virk *et al.* (1998) after comparison of their *Eco*RI/*Mse*I AFLP marker map for rice with the map obtained with *Pst*I/*Mse*I AFLP markers by Maheswaran *et al.* (1997) and by Young *et al.* (1999) for soybean. Young *et al.* (1999) found that 34% of *Eco*RI/*Mse*I markers displayed dense clustering, while only 10.9% of the *Pst*I/*Mse*I markers mapped to these clusters with no apparent clustering of the remaining *Pst*I/*Mse*I markers. A more even genomic distribution of *Pst*I/*Mse*I markers when compared to *Eco*RI/*Mse*I markers was also found in maize by Vuylsteke *et al.* (1999). AFLP markers tend to sample the same regions of the genome as RAPDs and RFLPs (Becker *et al.*, 1995; Powell *et al.*, 1996). However, this was not true for a DH rice population (Maheswaran *et al.*, 1997) and a sorghum population (Boivin *et al.*, 1999) where little intermingling of AFLP and RFLP loci were found

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AFLPs have a number of advantages over other marker systems (reviewed by Mueller and Wolfenbarger, 1999; Maheswaran *et al.*, 1997):

1. It is possible to generate AFLP fingerprints for any organism containing DNA without any knowledge of its genomic composition
2. AFLPs show near perfect repeatability and overall errors (including mispriming and scoring errors) are generally less that 2%
3. Minimal amounts of DNA are required and it is possible to use partially degraded DNA
4. The generation of AFLP markers are quick and potentially large numbers of polymorphisms are generated per primer combination
5. AFLP markers segregate in a Mendelian fashion
6. The large number of combinations that can be tested with AFLP primers increases the chance that some markers will be located in variable regions

Conversion of AFLP markers to sequence-specific markers has been reported in literature, but only a few of the corresponding converted markers retained their specificity (Meksem *et al.*, 1995; Cho *et al.*, 1996; Qu *et al.*, 1998). An investigation by Shan *et al.* (1999) into the conversion of AFLP markers in wheat revealed that sequence-specific primers were successful for only 6 of 26 chromosome-specific AFLP fragments and led them to conclude that AFLP polymorphisms are often not efficiently converted to sequence-specific markers in wheat. However, an AFLP marker linked to the Y_2 locus in carrot has been converted successfully to a co-dominant PCR-based marker (Bradeen and Simon, 1998), while Caranta *et al.* (1999) converted a co-dominant AFLP marker linked to potyvirus resistance in pepper into a co-dominant cleaved amplified polymorphic sequence (CAPS) marker. Qu *et al.* (1998) developed a PCR-based method from an AFLP marker for scoring the *ph1b* deletion in common wheat. Raemon-Büttner and Jung (2000) developed AFLP-derived STS markers linked to sex in asparagus (*Asparagus officinalis* L.) and found that it was important to first determine the copy number and distribution in the genome before attempting conversion.

A variation of the AFLP method, used to increase the number of co-dominant markers, is called 'microsatellite AFLP' or SAMPL (selective amplification of microsatellite polymorphic DNA - Paglia and Morgante, 1998; Witsenboer *et al.*, 1997). This technique basically combines AFLP primers with anchored compound short simple repeat primers. Another interesting AFLP-based

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technique is AFLP-based mRNA fingerprinting (Money *et al.*, 1996; Bachem *et al.*, 1996 and 1998) and involves the use of double-stranded cDNA instead of gDNA as template for a standard AFLP protocol. This modification can be used to display mRNAs and isolate sequences mapped to deleted chromosome segments.

3.3.7 Sequence Tagged Sites (STSs)

A sequence tagged site (STS) is a DNA region of 200-500 base pairs with a known sequence that is found nowhere else in the genome and that can be amplified from genomic DNA (Figure 16) using specific oligonucleotide primers (Olson *et al.*, 1989; Shin *et al.*, 1990). The amplification product of a STS should correspond to the size of the target region and the same product size must be amplified from any DNA sample. Due to the random distribution of repetitive DNA sequences throughout the genome, the primer sequence of the amplification product of a STS may not contain any repetitive DNA sequences. STSs can be physically mapped by PCR (Green and Olson, 1990) or by using it as a single-copy hybridisation probe (Primrose, 1995). In the first application lies one of the main advantages of STS technology: all information necessary for an STS assay can be retrieved from a database entry, no biological material such as probes is required (Olson *et al.*, 1989). STSs can therefore act as a common language for physical mapping projects between laboratories, especially if existing probes can be converted into STSs.

The disadvantages and/or low repeatability of some marker systems like RAPDs can be overcome by converting such a marker into a more reliable and easier scorable STS marker (Staub *et al.*, 1996; Hu *et al.*, 1997). The marker can be converted into a cleaved amplified polymorphic sequence (CAPS), a sequence characterised amplified region (SCAR) or a marker with allele-specific associated primers (ASAP). One disadvantage of STSs is the need to sequence the fragment of interest before it can be converted to a PCR-based assay (Hu *et al.*, 1997).

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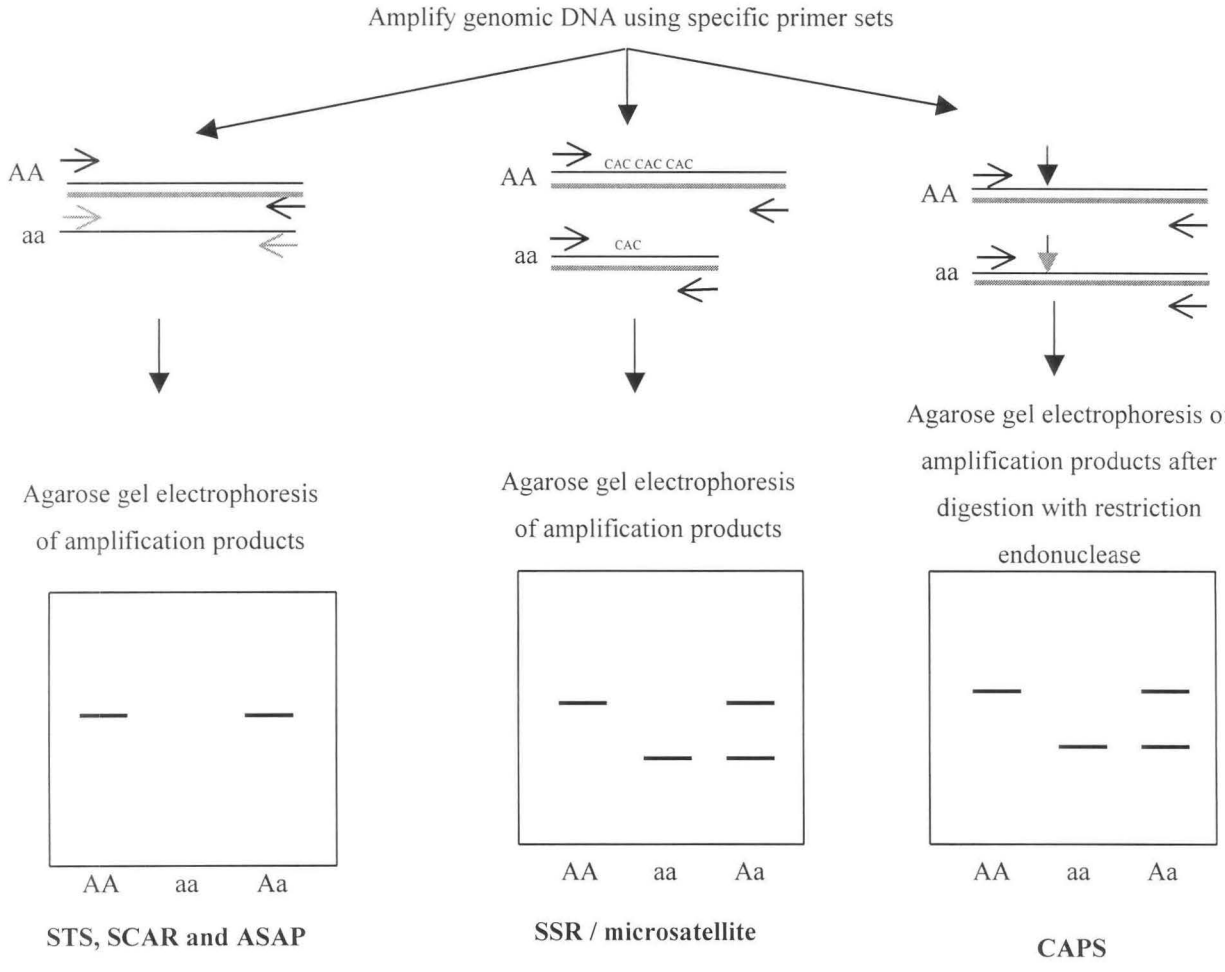

Figure 16. Sequence tagged site (STS) markers and STS-based markers (for example CAPS, SCAR, ASAP and SSR).

A CAPS marker system (Williams *et al.*, 1991; Konieczyn and Ausubel, 1993; Jarvis *et al.*, 1994) involves the amplification of a specific PCR product, followed by restriction endonuclease digestion and agarose gel electrophoresis (Figure 16). This technique is especially useful when the initial PCR amplification does not reveal any polymorphisms, for example converted RFLPs (Helguera *et al.*, 2000). An advantage of restriction endonuclease digestion of PCR products is that the amplification products do not contain methylated DNA and therefore does not influence methylation-sensitive enzymes (Williams *et al.*, 1991). The possibility of the marker being co-dominant is also high.

A SCAR (Paran and Michelmore, 1993; Wechter and Dean, 1998) is a genomic DNA fragment that is specifically amplified at a single genetically defined locus by a pair of long oligonucleotide primers (*e.g.* 24 nucleotides in length). These markers can amplify alleles from both parents and can therefore turn a dominant locus into a co-dominant SCAR locus (Figure

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16). A SCAR is distinguished from a STS in that it is primarily defined by linkage association although they can be used as physical landmarks and its amplification product may contain repetitive DNA sequences (Paran and Michelmore, 1993). SCARs that were converted from RAPDs allowed for the high resolution mapping of the *Dm* region in lettuce (Paran and Michelmore, 1993).

ASAP (Gu *et al.*, 1995; Yu *et al.*, 1995) is a PCR-based method that uses primers at stringent annealing temperatures to amplify only a single DNA fragment, corresponding to individuals possessing the appropriate allele (Figure 16). An error rate of 1.5% has been determined for the ASAP procedure (Gu *et al.*, 1995). ASAP markers are normally dominant markers and can be observed directly in micro-titre plates or PCR tubes after staining with ethidium bromide. A single fragment is amplified by specific primers in individuals possessing the relevant allele and detection can take place without the need for gel electrophoresis. Mayer *et al.* (1997) successfully converted a RAPD marker into an ASAP marker, but a second converted RAPD marker did not reveal any differences between resistant and susceptible lines even after a limited survey with restriction enzymes and was therefore classified as locus rather than allele specific.

3.3.8 Microsatellites or Simple Sequence Repeats (SSRs)

These markers have a known position in the genome. They have a co-dominant mode of inheritance (Litt and Luty, 1989; Weber and May, 1989 and Wu *et al.*, 1994). Microsatellites normally consist of a core motif with a few nucleotides (normally 1-6 base pairs) that are repeated multiple times in tandem (up to a total length of less than 60 base pairs, normally 10-60 repeat units). They are usually flanked by unique sequences from which PCR primers can be designed (Figure 16) (Litt and Luty, 1989; Weber and May, 1989). SSRs are very useful where RFLP polymorphisms are low (Akkaya *et al.*, 1992) or in cases such as the detection of tumour specimens in normal tissue where the sample DNA is tainted with background DNA (Gruis *et al.* 1993). The alleles are a result of the variation in the number of tandem repeat units and it is possible to amplify the repeated region as well as the flanking unique regions between the single-copy primers by means of the polymerase chain reaction (Litt and Luty, 1989; Weber and May, 1989). Sometimes non-inheritance of parental alleles is observed in some offspring and this phenomenon is called 'null' (Callen *et al.*, 1993) or 'novel' alleles. 'Null' alleles have been ascribed to deletions or mutations in the flanking DNA that contains the primer binding site,

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resulting in heterozygotes being mistyped as homozygotes (Callen *et al.*, 1993). 'Novel' alleles are alleles carried by the offspring individual(s) that are not present in either of the parents and can be caused by incorrect identification of the parent(s), outcrossing, molecular slippage or mutation. Electrophoresis on a high-resolution gel allows the discrimination of alleles with a size difference of up to one base pair (Weber and May, 1989). Microsatellites are not restricted to telomeric or centromeric regions, are multi-allelic and very small amounts of template DNA, which can even be partly degraded, is needed (Gruis *et al.* 1993).

Although there is a lot of speculation on the stability of microsatellites, it was reported by Fuentes *et al.* (1991) that the alleles at the microsatellite loci are very stable in the human population studied and that the evolutionary mutation rate is apparently lower than the recombination rate (Morral *et al.*, 1991). However, certain hypervariable loci do occur in mammals and it was observed that mutation events are biased towards alleles with a larger number of repeat units, causing an expansion rather than a reduction in the repeat length (Weber and Wong, 1993; Primmer *et al.*, 1996). Extreme hypervariability has not been observed for plants, probably due to shorter repeat lengths (Lagercrantz *et al.*, 1993). A major disadvantage of microsatellite markers is that the identification and development of these markers are expensive and time-consuming.

It is estimated that there are a total of 5×10^3 to 3×10^5 microsatellites per plant genome, probably five times less abundant than in mammals (Condit and Hubbell, 1991; Lagercrantz *et al.*, 1993). In maize, SSRs carry two-fold more information than AFLPs and RAPDs, and 40% more information than RFLPs when using the number of alleles per locus as criterion (Pejic *et al.*, 1998). Approximately 22-36% of microsatellite markers developed in wheat are polymorphic (Röder *et al.*, 1995; Ma *et al.*, 1996; Bryan *et al.*, 1997). In wheat, the absence of homoeologous SSRs loci in most cases makes SSRs likely to be inappropriate for comparative analyses or introgression studies involving wild species related to wheat (Stephenson *et al.*, 1998). However, the locus specificity and high levels of polymorphism should make it useful in practical wheat breeding (Stephenson *et al.*, 1998). Microsatellites have been used to map dwarfing genes (Korzun *et al.*, 1997 and 1998) and an anonymous gene for preharvest sprouting tolerance (Roy *et al.*, 1999) in bread wheat; and also to detect genetic diversity in closely related bread wheat cultivars and lines (Plaschke *et al.*, 1995).

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An interesting variation of SSRs, called inter-simple sequence repeat (ISSR) markers or anchored microsatellites, uses simple sequence repeats anchored at either 5' or 3' ends by short arbitrary SSR sequences as PCR primers and generate mostly dominant markers (Wu *et al.*, 1994; Zietkiewicz *et al.*, 1994; Nagaoka and Ogihara, 1997). Amplification products originate from the regions between two closely spaced, oppositely oriented simple sequence repeats and can be size-fractionated on a polyacrylamide gel. In general, the most common repeat motif is $(AA)_n$, followed by $(AT)_n$ and $(CT)_n$ (Lagercrantz *et al.*, 1993). Ma *et al.* (1996) reported a relative abundance of $(AC)_n$ and $(AG)_n$ in wheat and estimated 1.3×10^5 number of $(AC)_n$ and $(AG)_n$ sites combined. It was found by Nagaoka and Ogihara (1997) that $(AG)_n$, $(CT)_n$ and $(GT)_n$ sequences proved most useful and more reliable compared to RAPDs in wheat. Other interesting variations using microsatellites and their applications in wheat breeding were recently comprehensively reviewed by Gupta and Varshney (2000).

3.3.9 Single-strand conformation polymorphism (SSCP)

The power of single-strand conformation polymorphism (SSCP) is in its ability to detect mutations in amplified DNA fragments as a mobility shift of radio-labeled single-stranded DNA after size-fractionation on nondenaturing polyacrylamide gels (Orita *et al.*, 1989). Therefore, SSCP is based on the relation between the mobility of a ssDNA molecule and its folded conformation, which is a reflection of its nucleotide sequence, during electrophoresis (Spinardi *et al.*, 1991). However, SSCP does not detect all sequence changes with a single electrophoresis condition (Liu *et al.*, 1999). Nonradioactive SSCP protocols have been developed, involving the use of heat denatured dsDNA separated in a nondenaturing polyacrylamide gel followed by ethidium bromide staining (Hennessy and Pullinger, 1997). Nonradioactive gels have the advantage that fragments can readily be cut from the gel and enriched for sequencing by further PCR (Calvert *et al.*, 1995). Another improvement to SSCP analysis was the addition of denaturants, for example urea and formamide, to increase the sharpness of bands where fuzzy bands were present (Yip *et al.*, 1999). In a study by Iacopetta and Hamelin (1998), it was found that PCR-SSCP reduced gel and PCR manipulation by 10-fold and that size variation of as little as two base pairs was readily detected with mini-sized, silver-stained SSCP gels run for 2.5 hours. They concluded that PCR-SSCP is a specific, sensitive and rapid method for determining the Replication Error Phenotype status of human tumours.

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3.4 COMPARATIVE MAPPING

Comparative mapping can be defined as the comparison of maps within and across species and it provides the basis for understanding the evolution of genomes (Keller and Feuillet, 2000). Colinearity of markers is often conserved between species of the same family, although inter-chromosomal translocations and inversions might be observed (Tanksley *et al.*, 1988a; Weeden *et al.*, 1992). It is essential that a common set of molecular markers must be used to map the genomes of the different species for comparative mapping. A comparison of RFLP maps of wheat, barley and rye (all members of the family *Gramineae*) revealed extensive synteny along the chromosomes with a tendency to break up towards the distal part of the chromosomes (Devos *et al.*, 1993). A comparative 'grass-genome' map will greatly simplify the identification and tagging of important genes and facilitate the cloning of these genes in smaller and simpler genomes (such as rice) rather than large genomes (such as wheat) by using the homologous sequence (Moore, 1995; Moore *et al.*, 1995). A consensus grass map that aligns the genomes of seven different grass species was prepared by Moore *et al.* (1995) and extended by Gale and Devos (1998). The extended grass map uses only 25 rice linkage blocks to describe the genomes of oat, barley, wheat, maize, sorghum, sugarcane, foxtail millet and rice (Devos and Gale, 1997). Originally, the hope was that comparative mapping would make the isolation of important traits in large genomes less labour-intensive if the large genome could be compared with a simpler genome. This is unfortunately not as simple as would be hoped for, for a surprising level of rearrangements and deletions at microlevel has been observed between the model genome of rice and other genomes, and also between the genomes of closely related species such as sorghum and maize (Keller and Feuillet, 2000). Although significant portions of the lentil and pea genomes are co-linear, numerous translocations and inversions were also observed for these genomes (Weeden *et al.*, 1992). Also, little conservation exists between linkage groups of tomato and pepper (two species of the same family) even though they have the same chromosome numbers and gene repertoire (Tanksley *et al.*, 1988b).

An application of comparative mapping is the use of resistance gene analogs (RGAs). Interaction between a host plant and a pathogen isolate is often explained by a postulated "gene for gene" interaction where the plant gene ("*R*") that confers resistance to a particular isolate appears to have a matching pathogen gene that confers cultivar-specific avirulence, called an avirulence gene ("avr") (Flor, 1971). Up to 1992, cloning and characterisation of resistance

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genes were unheard of, but in the following years a number of resistance genes has been cloned (reviewed by Staskawicz *et al.*, 1995). RGAs are based on the conservation of nucleotides observed within disease resistance genes cloned from widely diverse taxa. Often overall sequence homology between the genes is low, but the nucleotides in conserved domains can be used to isolate sequences with similar motifs from species from which no disease resistance genes have been cloned (Kanazin *et al.*, 1996; Chen *et al.*, 1998). These sequences can be classified in seven classes (see Table 4 for the five more important classes) and mostly involve nucleotide binding sites (NBS), protein-protein interaction sites (leucine-rich repeat regions - LRR) and / or intracellular signalling sites (kinase domains) (Pflieger *et al.*, 1999). Resistance genes from these classes possess conserved amino acid motifs consisting of a nucleotide binding site (NBS) domain and a hydrophobic domain (HD) with the consensus amino acid sequence Gly-Leu-Pro-Leu (GLPL) downstream of the NBS (Mago *et al.*, 1999).

Table 4. Descriptions and examples of the five more important RGA classes (Adapted from Baker *et al.*, 1997).

Class	Description	Examples	References
I	Genes that encode cytoplasmic receptor-like proteins that contain a leucine rich repeat (LRR) domain and a nucleotide-binding site (NBS).	*RPS2* and *RPM1* (resistance to *Pseudomonas syringae* in *Arabidopsis*)	Mindrinos *et al.*, 1994; Bent *et al.*, 1994; Grant *et al.*, 1995
		L6 and *M* (resistance to *Melampsora lini* in flax)	Lawrence *et al.*, 1995; Anderson *et al.*, 1997
		I_2-*C* (resistance to *Fusarium oxysporum* f.sp. *lycopersicon* in tomato)	Ori *et al.*, 1997
		Cre3 (cereal cyst nematode resistance in wheat)	Lagudah *et al.*, 1997
		N (tobacco mosaic virus resistance in tobacco)	Whitham *et al.*, 1994
II	The *Pto* gene encodes a serine-threonine kinase (STK) domain product that does not possess any LRR or NBS, but is dependent on *Prf*, which is an NBS-LRR-containing protein, for its function.	tomato *Pto* gene (resistance to *Pseudomonas syringae* pv. *tomato*)	Martin *et al.*, 1993
III	Encodes putative transmembrane receptors with large extracytoplasmic protein containing LRRs.	Tomato *Cf* genes (resistance to *Cladosporium fulvum*)	Jones *et al.*, 1994; Dixon *et al.*, 1996
		$HS1^{pro-1}$ (sugar beet cyst nematode resistance gene)	Cai *et al.*, 1997; Hammond-Kossack and Jones, 1997

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IV	Encodes a putative transmembrane receptor with an extracellular LRR domain and an intracellular serine-threonine kinase (STK) domain.	rice *Xa21* gene (resistance to *Xanthomonas oryzae*)	Song *et al.*, 1995
		wheat *Lr10* gene (resistance to *Puccinia recondita*)	Feuillet *et al.*, 1997
V	Encodes a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductase.	*Hm1* (resistance to *Cochliobolus carbonum* in maize)	Johal and Briggs, 1992

Degenerate oligonucleotides can be designed from the conserved domains and used to amplify similar regions from a number of diverse species, for example potato, tomato, pepper, barley, *Arabidopsis*, *Antirrhinum*, *Rosa*, yeast and human (Leister *et al.*, 1996; Chen *et al.*, 1998). For a given primer pair, mean polymorphisms of 31% in rice, 38% in barley and 21% in wheat have been reported (Chen *et al.*, 1998). Cloned specific resistance genes can be used as cosegregating or 'perfect' markers in selection or as intergeneric sources of transgenes in transformation breeding (Lagudah *et al.*, 1997; Seah *et al.*, 1998). Spielmeyer *et al.* (1998) showed that a superfamily of NBS-LRR RGAs is located on all homoeologous groups of wheat, but they also recommended that all other classes of RGAs should also be screened in order to identify RGAs linked to a specific gene. Mapping of RGAs revealed that their map positions correlated to several disease resistance loci, although clustering has been observed for some plant species while others mapped to several chromosomes (Mago *et al.*, 1999). However, a lack or decrease in conservation of the RGAs and resistance genes among the grass genomes has been reported (Leister *et al.*, 1998) and this might be suggestive of possible difficulties for using comparative analysis for genes that evolve rapidly. An example where a lack of colinearity was found is between rice and wheat at the wheat leaf rust resistance gene locus *Lr1* on chromosome 5DL (Gallego *et al.*, 1998).

The advantage of RGAs over arbitrary DNA markers is that RGAs target potentially useful genes (Chen *et al.*, 1998). RGAs were used by Kanazin *et al.* (1996) to identify sequences in soybean related to conserved sequences from coding regions of disease resistance genes *N*, *RPS2* and *L6*. However, RGA sequences might identify pseudogenes that map close to R-genes and constitute landing markers (Mago *et al.*, 1999). Kanazin *et al.* (1996) concluded that genetic mapping of RGAs might be more useful for the identification of markers close to resistance genes for subsequent map-based cloning than for the direct identification of resistance genes. Shariflou and Sharp (1999) used an imperfect $(AT)_n$ repeat motif near the 3' end of wheat waxy

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cDNA sequence that partly aligned with an $(AT)_4$ in the barley waxy gene to develop a polymorphic microsatellite that successfully amplified genomic DNA from barley, rye, triticale and potentially from other distantly related species. Chen *et al.* (1998) used denaturing polyacrylamide gel electrophoresis and silver staining to detect products amplified using primers based on conserved regions of resistance genes. Reproducible DNA fingerprinting patterns with high levels of polymorphism were revealed for rice, barley and wheat by this approach. As early as 1996, Leister and co-workers speculated that homologues of resistance genes might, for example, confer resistance to a fungal pathogen in one species but to nematodes in another. This was also confirmed by Seah *et al.* (1998), who hypothesised that RGAs might be useful for cloning candidate genes for nematode or aphid resistance in wheat if an RGA for the one is known, but not for the other. This hypothesis is supported in tomato, where tight linkage was found between root knot nematode (*Mi*) and potato aphid (*Meu*) resistance loci (Kaloshian *et al.*, 1995). These two loci were later shown to be the same gene (Rossi *et al.*, 1998). If the molecular and functional basis of a number of resistance and susceptibility alleles of genes isolated from diverse plant species are known, it might help to identify structural features important for pathogen specificity and might ultimately result in resistance genes tailored according to needs in a variety of crops (Leister *et al.*, 1996).

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4. SIGNIFICANCE AND AIMS OF THIS STUDY

Chromosome arm 7DL carries very important disease and pest resistance loci, among which the *Lr19* translocation (*Thinopyrum ponticum* - leaf rust resistance and yield advantage), the *VPM1* segment (*Triticum ventricosum* - *Pch1*, eye spot resistance), Russian wheat aphid resistance genes (*Dn1* and *Dn5*) and the *TCI4* translocation (*Thinopyrum intermedium*-derived - barley yellow dwarf virus resistance). The alien translocations do not pair, or has reduced recombination, with homoeologous wheat chromatin during meiosis and can therefore not be mapped by conventional means. Also, the translocations appear to overlap with one another and with useful "native" genes on 7DL, which means that they cannot be used simultaneously in the development of pure breeding lines. It is therefore necessary to obtain detailed maps of the 7DL arm and translocations in order to modify them for better utility in breeding. In addition, the need for polymorphic markers, especially in a crop known for low levels of polymorphism, as well as improved genetic maps of wheat are of utmost importance for marker assisted selection and gene pyramiding in a breeding program.

Working toward these goals, the structural loci for leaf rust resistance (*Lr19*), Russian wheat aphid resistance (*Dn5*) and endopeptidase protein (*Ep-D1* - which is closely linked to the eyespot resistance gene, *Pch1*) were targeted. The following were attempted with regard to each gene:

a) The *Lr19* translocation

1. To improve the resolution of the deletion map of the *Lr19* translocation produced by Marais (1992) and Prins *et al.* (1996) by identifying and mapping further molecular markers.
2. Use the improved deletion map to: i) characterise recombinant forms of the *Lr19* translocation produced by Marais and Marais (1990), and ii), to identify and use appropriate AFLP fragments present on the shortest recombinants for conversion into STS markers. Such markers can be used in attempts to further shorten the translocation or for the purpose of marker assisted selection.

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b) The *Dn5* locus

1. Establish a DH mapping population that will be polymorphic for important structural loci on 7DL making use of the F_1:'Chinese Spring' × 'SA463' ('PI 294994').
2. Attempt to identify AFLP markers linked to *Dn5* and convert them into PCR-based markers.
3. Use the DH population in an attempt to map the *Dn5*, *Ep-D1*, as well as several other RFLP, SSR and PCR-based markers.

c) The *Pch1* gene

1. Convert an AFLP marker linked to the *Ep-D1* locus (an thus to eyespot resistance gene, *Pch1*) into a sequence-specific marker and test the converted marker in segregating material.
2. Determine the linkage distance between the new marker and *Ep-D1*.

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Chapters Two to Four

The experimental parts of this study are written in the form of research articles with the intent to submit it to a journal for publication, where applicable. For the purpose of this dissertation, the experimental procedures and research results are discussed in more detail than necessary for publication.

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CHAPTER TWO

AFLP ANALYSIS EXTENDS A PHYSICAL MAP OF THE *LR19* TRANSLOCATION

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Abstract

Twenty-nine deletion mutant lines (produced by gamma irradiation) were used to extend a physical map of the *Thinopyrum*-derived *Lr19* translocated chromosome segment. One hundred and forty-four *Sse*8387I/*Mse*I and 32 *Eco*RI/*Mse*I primer combinations were used to obtain 95 useful AFLP markers. The physical map confirmed that terminal deletions had mostly occurred, however, it appears that several intercalary deletions and a number of primer or restriction site mutations were also induced. The markers allowed for the discrimination of the deletion mutant lines into 19 clusters, with seven AFLP markers mapping close to leaf rust resistance gene *Lr19*. An unsuccessful attempt was made to convert one of the latter markers into a sequence-specific marker. The extended physical map should be useful for further characterisation of recombinant forms of the *Lr19* translocation and for physical mapping of other molecular markers.

Key words wheat, deletion mapping, leaf rust resistance

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INTRODUCTION

Leaf rust is caused by *Puccinia* Roberge ex Desmasz f. sp. *tritici* Erikss. & E. Henn and is probably the most important wheat disease on a world-wide basis (Samborski, 1985). Numerous leaf rust resistance genes have been catalogued, of which *Lr19* is regarded world-wide as highly effective (Winzeler *et al.*, 1995), although virulence has been reported by Huerta-Espino and Singh (1994) and Sibikeev *et al.* (1996). The *Lr19* translocation (initially known as the T4 translocation, later named 'Agatha') was transferred from *Thinopyrum ponticum* (=*Agropyron elongatum* Host.) to wheat by Sharma and Knott (1966).

The *Lr19* translocation is homoeologous to 7DL (Dvorák and Knott, 1977) and is associated with genes for stem rust resistance (*Sr25*), segregation distortion (*Sd1*), yellow endosperm (*Y*) and water-soluble protein (*Wsp-D1*); but shows null conditions for the endopeptidase (*Ep-D1*) and α-amylase (α-*Amy-D2*) loci (Marais, 1992; McMillin *et al.*, 1993; Winzeler *et al.*, 1995). In the presence of *Ph1*, the translocation does not recombine with homoeologous regions of the wheat genome and is therefore inherited as a single large linkage block (Knott, 1980; Marais and Marais, 1990).

Since the translocated region does not pair and recombine with homoeologous 7DL chromatin during meiosis it cannot be mapped using crossover frequencies (Marais, 1992), and physical mapping had to be attempted. Marais (1992) irradiated spikes of the translocation line 'Indis' with gamma rays and pollinated these spikes with 'Inia 66' pollen. Test cross progenies were screened for translocation mutants, using the leaf rust resistance and yellow endosperm pigmentation genes as markers. The *EP-D1* null condition was used to recover mutant translocation homozygotes which were then also characterised for the presence/absence of the *Sr25*, *Sd1* and *Wsp-D1* loci. Twenty-nine deletion mutants were identified and used to construct a physical map. Prins *et al.* (1996) expanded the physical map with the use of RFLP markers. Thus, it was possible to assign the mutant lines to seven deletion size groups containing (from the largest deletions to the smallest deletions) 3, 2, 7, 11, 2, 2 and 2 lines, respectively. All of the mutations apparently involved terminal deletions, while two of them may also have intercalary deletions (Marais, 1992; Prins *et al.*, 1996).

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A larger number of polymorphic markers specific for the translocated chromosome arm will be required to arrange all the deletions according to size. Amplified fragment length polymorphism (AFLP - Vos *et al.* 1995; Keim *et al.*, 1997) technology is a powerful screening tool that has two important advantages: it requires no prior knowledge of the genome and typically amplifies 50 to 100 loci in a single round of screening. Polyacrylamide gel electrophoresis of amplification products allows high-resolution size fractionation of fragments. Different applications of AFLP markers have been reviewed extensively by Shan *et al.* (1999) and include assessment of genetic diversity, construction of high-density genetic maps, quantitative trait analysis and identification of additional DNA markers near a locus of interest.

The aim of the present study was to identify AFLP markers occurring on the *Lr19* translocation in order to improve the resolution of the physical map of Prins *et al.* (1996). Such a map will make it possible to characterise the substantially shortened forms of the *Lr19* translocation already produced through allosyndetic pairing induction (Marais *et al.*, 2001). It may also yield new *Thinopyrum* specific markers that can be used in marker assisted selection or future attempts to shorten the translocation.

MATERIAL AND METHODS

In this study, twenty-nine homozygous deletion lines (Marais, 1992; Prins *et al.*, 1996), as well as the wheat accession 'Indis', which has the complete *Lr19* translocation, (Marais *et al.*, 1988) and the cultivar 'Inia 66' were used to identify molecular markers associated with the translocation. Genomic DNA was extracted from each of the lines and then subjected to AFLP analyses. Initially, a core group consisting of 'Indis', 'Inia 66', four mutants carrying the smallest deletions (AA:87M23-225, Z:89M2-426, AB:89M1-18 and AC:87M23-266) and two mutants with the largest deletions (C:87M23-145 and E:87M23-198) were used for preliminary polymorphism screening. Potentially useful polymorphisms were then tested on the full set of twenty-nine lines. One of the polymorphic fragments, closely associated with the resistance gene, was excised from the gel, the fragment was purified, cloned and sequenced. Specific primers were designed from the sequence, PCR conditions were optimised and the primer combination tested on the mutants to confirm association of the marker with the *Lr19* locus. An AFLP marker located distally to *Lr19* was succesfully converted to a sequence specific PCR marker in collaboration with other researchers (Prins *et al.*, 2001).

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DNA EXTRACTION

Total genomic DNA was extracted from approximately 4-week-old seedlings grown in a greenhouse, using the procedure of Doyle and Doyle (1990) with slight modifications. Approximately 0.5 g lyophilised or 1.5 g fresh leaf tissue was ground in 15 ml of preheated CTAB extraction buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% beta-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 8.0), followed by incubation in a shaking waterbath at 60 °C for approximately 1 hour. This mixture was then extracted once with an equal volume of chloroform:isoamylalcohol (24:1), centrifuged for 10 minutes at 7000 rpm (room temperature), followed by the precipitation of the nucleic acids in the upper aqueous phase with 2/3 volumes isopropanol at -20 °C for up to 1 hour. The precipitate was spooled and left to dissolve in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8). The dissolved DNA was treated with RNase A for 30 minutes at 37 °C, diluted with TE buffer, 7.5 M NH_4OAc (pH 7.7) was added and followed by 100% ethanol precipitation. After the sample was centrifuged for 10 minutes at 10000 rpm (10-25 °C), the supernatant was removed and the pellet was air-dried briefly. The pellet was dissolved in TE buffer (pH 8.0) overnight and then subjected to a phenol:chloroform:isoamylalcohol (25:24:1) treatment, a chloroform:isoamylalcohol (24:1) treatment and finally precipitated at -20 °C for 60 minutes in the presence of 0.5 volumes 7.5M NH_4OAc (pH 7.7) and double the total volume cold 100% ethanol. After centrifugation for 30 minutes at 12000 rpm (4 °C optional), the genomic DNA was washed twice with washing buffer (76% ethanol; 10mM NH_4OAc (pH 7.7)) and centrifuged for 30 minutes at 12000 rpm (4 °C optional) (Sambrook *et al.*, 1982). The resulting pellet was air-dried and dissolved in ddH_2O. DNA concentrations were determined on a Hoefer DyNA Quant 200 fluorometer according to the manufacturer's instructions.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

The technique involves three main steps: 1) the restriction of genomic DNA and the ligation of oligonucleotide adapters; 2) amplification of the restriction fragments using selective primer pairs and 3) the detection of the amplified products (Vos *et al.*, 1995). The protocol described by Donini *et al.* (1997), as modified from Vos *et al.* (1995), was used. Primer sequences were kindly supplied by the John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom.

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500 ng of total genomic DNA was digested with 5 units *Sse*8387I (AEC Amersham) and 5 units *Mse*I (New England Biolabs) restriction enzymes in a total reaction volume of 40.0 µl containing 0.1 µg/µl BSA (New England Biolabs), 1× One-Phor-All buffer (Pharmacia) and AFLP-grade water. The reaction was incubated for two to three hours at 37 °C, after which 10 µl of adapter ligation solution was added. The adapter ligation solution consisted of 5 pmoles *Sse*8387I adaptor (5'- CTC GTA GAC TGC GTA CAT GCA -3' / 3'- CAT CTG ACG CAT GT -5'), 50 pmoles *Mse*I adaptor (5'- GAC GAT GAG TCC TGA G -3' / 3'- TA CTC AGG ACT CAT -5'), 1 mM ATP (Pharmacia), 1 unit T4 DNA ligase (Pharmacia), 0.1 µg/µl BSA, 1× One-Phor-All buffer (Pharmacia) and AFLP-grade water. The restriction-ligation reaction was then incubated overnight at 37 °C. A 1:10 dilution using 1× $TE_{0.1}$ buffer (10 mM Tris-HCl, 0.1 mM Na_2EDTA, pH 8.0) was performed on 45 µl of the restriction-ligation reaction and the remaining 5 µl of each reaction was tested on a 1.5% agarose gel (1× TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM Na_2EDTA, pH 8.3), 3 hours at 75 V) to confirm complete digestion of the DNA.

Pre-selective amplification of the diluted reactions was performed in thin-walled 0.2 ml PCR tubes. The total volume of each amplification reaction consisted of 13 µl diluted restriction-ligation template, 75 ng S00 primer (5'- AGA CTG CGT ACA TGC AGG -3'), 75 ng M00 primer (5'- GAT GAG TCC TGA GTA A -3'), 50 µM of each dNTP, AFLP-grade water, as well as 1 unit of *Taq* polymerase (GIBCO), 1× GIBCO PCR buffer and 1.5 mM $MgCl_2$. The PCR profile was run on a Hybaid PCR Express and consisted of elongation at 72 °C for 5 minutes, 30 cycles of 94 °C for 30 seconds, 56 °C for 60 seconds, 72 °C for 60 seconds, followed by final extension for 5 minutes at 72 °C and a soak temperature of 4 °C. A 1:10 dilution using 1× $TE_{0.1}$ buffer (pH 8.0) was done on 45 µl of each of the amplification reactions and the remaining 5 µl of each reaction was tested on a 1.5% agarose gel (1 × TBE buffer, 3 hours at 75 V) to confirm successful amplification.

For each selective reaction, 2.5 ng of the *Sse*8387I selective primer was end-labeled with 0.5 µCi {γ-^{33}P}ATP (74 TBq/mmol, 2000 Ci/mmol, 370 MBq/ml, 10 mCi/ml; NEN Life Science Products, Inc.) in the presence of 0.05 units of T_4 polynucleotide kinase (Pharmacia), 1× One-Phor-All buffer (Pharmacia) and AFLP-grade water. The reaction was incubated for 2 hrs at 37

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°C, after which the enzyme was heat-inactivated by incubation for 10 min at 65 °C. The labeled primer was stored at +4 °C until required. 30-330 bp AFLP ladder (GIBCO BRL) was labeled according to the manufacturer's instructions.

Selective amplification ('hot reaction') was performed in 0.2 ml PCR tubes in a 10 µl total reaction volume. This consisted of 2.5 µl diluted template DNA (diluted pre-selective amplification product), 15 ng unlabeled *Mse*I primer (5'- GAT GAG TCC TGA GTA ANN N -3', where N denotes the specific selective nucleotides), 2.5 ng labeled *Sse*8387I primer (5'- GAC TGC GTA CAT GCA GGN NN -3', where N denotes the specific selective nucleotides), 50 µM of each dNTP, 0.25 units *Taq* DNA polymerase (Bioline), 1× PCR buffer, 1.5 mM $MgCl_2$ and AFLP-grade water. The PCR profile was run on a Hybaid PCR Express and consisted of 1 cycle of 94 °C for 30 seconds, 65 °C for 30 seconds, 72 °C for 60 seconds, followed by 12 cycles in which the annealing temperature in each cycle was lowered by 0.7 °C. This was followed by 23 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 60 seconds and a final soak temperature of 4 °C. The amplification reactions were stored at +4 °C until size-separation on a denaturing polyacrylamide gel.

For the screening of mutant lines with the restriction endonuclease combination *Eco*RI and *Mse*I, the AFLP Analysis System I (GIBCO BRL) was used according to the manufacturer's instructions.

Samples were size-fractionated on a 0.4 mm thick 6% w/v acrylamide:*bis*-acrylamide (19:1 w/w) denaturing sequencing gel containing 6 M urea in 1× TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM Na_2EDTA, pH 8.3) using a Model S2001 sequencing gel electrophoresis apparatus (Life Technologies) according to the manufacturer's instructions. Prior to loading the samples, an equal volume of AFLP loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol FF) was added to each sample after which the samples were denatured at 90 °C for 3 to 5 minutes and then immediately quenched on ice. Five to 6.5 µl of the denatured samples and 2 µl of the labeled 30-330 bp AFLP ladder (GIBCO) were loaded on the gel. Denaturing polyacrylamide gel electrophoresis was then performed at a constant power of 80 W for approximately 2 hours (when the xylene

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cyanol front was approximately 7 cm from the bottom of the glass plate). The gel was transferred to Whatmann 3MM Chr chromatography paper and dried on a Savant Gel Dryer for 2 hours at 80 °C. Amplified products were visualised after exposure to an X-ray film (Kodak BioMax MR) for up to five days.

Fragments were scored manually for the absence and presence of an amplification product. Fragments present as a 'doublet' were scored as a single marker. Potentially useful polymorphisms were fragments that were amplified from 'Indis' and from one or more of the mutants with smaller deletions, but not from 'Inia 66' or any of the mutants with the largest deletions. Potentially useful polymorphisms were tested on all 29 deletion mutants and the data were integrated with an existing physical map in order to determine the location of the polymorphism. Two AFLP markers associated with the *Lr19* gene were selected to be converted into a simpler PCR-based marker, one of which was converted in collaboration with other researchers (Prins *et al.*, 2001).

Conversion of AFLP Markers to STS Markers

A doublet AFLP marker 27b:S-AC/M-AT_{191} (Figure 2a) was identified to be converted to a STS ('sequence tagged site') marker. AFLP reactions containing the fragment of interest, as well as reactions without the fragment, were loaded and electrophoresed on a polyacrylamide gel. The orientation of the X-ray film was clearly marked on the dried gel in such a way that the autoradiograph could easily be aligned on the dried gel after development. The fragment of interest was cut out from the dried gel using the aligned autoradiograph as guide. The dried gel was again exposed to a new X-ray film to confirm that the correct fragment was excised. The DNA in the dry gel piece was isolated using the protocol of Shan *et al.* (1999). Fragments were isolated from the higher molecular weight fragment of A:'Indis', Z:89M2-426 and AC:87M23-266, as well as the lower molecular weight fragment of A:'Indis' and AB:89M1-18 (Figure 2a). The excised gel piece was rehydrated in 100 µl ddH_2O for 10 minutes at room temperature, after which the water was replaced with 100 µl elution buffer (0.5 M ammonium acetate, 5 mM EDTA (pH 8.0)), the gel piece was crushed with a pipet tip and the mixture boiled for 3 minutes. Following centrifugation, the DNA was ethanol precipitated, air dried and resuspended in 10-20

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µl ddH_2O. Five microlitres of the dissolved DNA was used in a PCR amplification identical to the original selective AFLP reaction (this time with 50 µl total reaction volume), but without labeling the *Sse*8387I primer first. The amplification product was run on a 1% agarose gel (0.5× TAE buffer (20 mM Tris-acetate, 0.5 mM Na_2EDTA, pH 8.0)) for approximately 3 hours at 75 V, stained with ethidium bromide and excised from the gel with a sterile blade, purified using the Wizard PCR Purification Kit (Promega) and cloned using the pGEM-T Easy vector system (Promega) according to the manufacturer's instructions.

Following blue-white screening, a transformed colony was selected for each of the isolated fragments. Plasmids were isolated from the selected clones using the Wizard Mini-Prep Kit (Promega) according to the manufacturer's instructions. These clones were sequenced with T7 and SP6 primers on an ABI 377 DNA Analyzer using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, CA, USA). Only sequences containing the selective primer binding sites of the original *Sse*8387I and *Mse*I primers were considered for primer design. Primers of sixteen to twenty-five nucleotides in length were designed with Primer Express (Applied Biosystems) using the DNA PCR document system.

The designed primers were tested in a total amplification reaction volume of 25 µl consisting of 50 ng gDNA, 12.5 pmol of each primer, 1.25 mM of each dNTP, AFLP-grade water, as well as 0.625 units of *Taq* polymerase (Bioline), 1× PCR buffer and 2.5 mM (2.0 mM for the ACH primer set) $MgCl_2$. The PCR profile was run on an Eppendorf Gradient Cycler and consisted of denaturation at 94 °C for 4 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, followed by final extension for 5 minutes at 72 °C and a soak temperature of 4 °C. The annealing temperature was increased to 60 °C for the ACH primer set. The amplification reaction was size-fractionated on a 3 % w/v agarose gel (1× TBE buffer, 4 hours at 70 V) to determine if there is a size difference between the marker present and marker absent samples. In cases where the marker was not polymorphic, nine microlitres of the amplification reactions were digested for three hours with 1 unit of restriction endonuclease in the presence of 1× reaction buffer at 37 °C. Test digestions were done with three different restriction endonucleases: *HaeIII* (Roche), *MseI* (New England Biolabs) and *PstI* (Roche). Following digestion, the samples were loaded onto a 1.4 % agarose gel using 1× TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM Na_2EDTA, pH 8.3) as running buffer.

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Electrophoresis was allowed to proceed for 3 - 5 hours at 70 V, after which the gel was stained in ethidium bromide and photographed under UV light.

The second AFLP marker identified for conversion, *viz.* 12c:S-AG/M-AC_{174}, was converted in collaboration with other researchers (Prins *et al.*, 2001). The AFLP marker was identified at the John Innes Centre, United Kingdom, by R. Prins and subsequently excised from the gel and cloned, the clones were screened and promising clones were sequenced by RP and co-workers at John Innes Centre. Primer sets were designed from the sequence of the most representative clone (called 'Group A' by Prins *et al.*, 2001), but failed to amplify any polymorphisms between the parental genotypes. It was at that stage that this PhD (Agric) candidate got involved in the project. I designed primer sets for the remaining groups and optimised the PCR conditions for each primer set. I tested the parental genotypes with the primer set from each group using the optimised conditions and found that the 'Group B' primer set amplified a fragment of the correct size from the *Lr19* carrying line 'Indis' but not from the non-carrying line 'Inia 66'. I then tested the 'Group B' primer set on all 29 deletion mutants as well as a panel of *Lr19* carrier and non-carrier lines and thereby confirmed the specificity of the primer set to *Thinopyrum* chromatin.

RESULTS

Two *Sse*8387I +1, two *Sse*8387I +2, four *Sse*8387I +3, twelve *Mse*I +2 and six *Mse*I +3 AFLP primers in a total of 144 primer combinations were used to screen a population consisting of 'Indis', 'Inia 66', four deletion mutants carrying the smallest deletions (AA:87M23-225, Z:89M2-426, AB:89M1-18 and AC:87M23-266) and two mutants with the largest deletions (C:87M23-145 and E:87M23-198). Four *Eco*RI +3 and eight *Mse*I +3 AFLP primers in a total of 32 primer combinations were used to screen a population consisting of 'Indis', 'Inia 66' and seventeen mutants from groups that could not be distinguished using *Sse*8387I as rare cutter. Potentially useful polymorphisms were tested on all twenty-nine mutant lines.

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Sse*8387I*/Mse*I primer combinations*

All *Sse*8387I selective primers amplified useful polymorphisms when used in conjunction with most of the *Mse*I selective primers that were tested (Table 1). The *Sse*8387I selective primers S04+T and S13+AG amplified the largest number of useful polymorphisms in combination with thirteen of the eighteen *Mse*I selective primers, while S12+AC and S27+AGA only amplified useful polymorphisms in combination with six and five *Mse*I primers, respectively. The lowest number of polymorphisms was detected with S27+AGA (7 polymorphisms). The number of polymorphisms per useful combination was highest for S02+C and S27+AGA (1.50 and 1.40, respectively) and lowest for S13+AG (1.08), S28+AGC and S30+AGT (both 1.14). Likewise, all the *Mse*I selective primers yielded useful polymorphisms when used in combination with *Sse*8387I selective primers. M15+CA, M21+GG and M88+TGC were useful with six of the eight possible combinations with *Sse*8387I selective primers, while M64+GAC was only useful in combination with one *Sse*8387I selective primer. The highest number of polymorphisms was found with M21+GG (nine useful polymorphisms) and the lowest number with M16+CC and M22+GT (two each) and M64+GAC (only one useful polymorphism). The highest number of polymorphisms per useful primer combination was with M19+GA (1.60) and the lowest number of 1.00 was found for combinations with *Mse*I selective primers M12+AC, M16+CC, M18+CT, M22+GT, M53+CCG, M64+GAC and M88+TGC.

Fifty-one of the 68 (75%) useful primer combinations yielded a single useful polymorphism, 16 (23.5%) yielded two useful polymorphisms and only one (1.5%) combination resulted in three useful polymorphisms. If all 144 combinations are considered (Table 1), the useful polymorphisms per combination when testing *Sse*8387I selective primers against all *Mse*I selective primers ranged from 0.39 to 0.94 while a range of 0.13 to 1.13 was observed when testing *Mse*I selective primers against all *Sse*8387I selective primers. The overall average was 0.60 useful polymorphisms per combination.

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Table 1. Summary of results obtained for the 144 *Sse* 8387I / *Mse* I primer combinations tested.

		Sse 8387I selective primers								Number of useful polymorphisms	Number of combinations		Number of polymorphisms per combination	
		S02+C	S04+T	S12+AC	S13+AG	S27+AGA	S28+AGC	S29+AGG	S30+AGT		Useful	Total	Useful	Total
Mse I selective primers	M11 +AA	1	1					2		4	3	8	1.33	0.50
	M12 +AC		1		1	1		1		4	4	8	1.00	0.50
	M13 +AG		2		1		1		1	5	4	8	1.25	0.63
	M14 +AT	1	1	2	1					5	4	8	1.25	0.63
	M15 +CA		2	1	1	2		1	1	8	6	8	1.33	1.00
	M16 +CC				1			1		2	2	8	1.00	0.25
	M17 +CG		1	1		1	1		2	6	5	8	1.20	0.75
	M18 +CT	1		1			1			3	3	8	1.00	0.38
	M19 +GA	2	1	2	1			2		8	5	8	1.60	1.00
	M20 +GC		1		1			2	1	5	4	8	1.25	0.63
	M21 +GG	3		1	1	2	1		1	9	6	8	1.50	1.13
	M22 +GT				1				1	2	2	8	1.00	0.25
	M32 + AAC	1	2		2		1			6	4	8	1.50	0.75
	M53 + CCG		1		1			1		3	3	8	1.00	0.38
	M64 + GAC					1				1	1	8	1.00	0.13
	M74 + GGT		1				2			3	2	8	1.50	0.38
	M88 + TGC	1	1		1		1	1	1	6	6	8	1.00	0.75
	M90 + TGT	2	2		1			1		6	4	8	1.50	0.75
Number of useful polymorphisms		12	17	8	14	7	8	12	8	86				
Number of combinations	Useful	8	13	6	13	5	7	9	7		68			
	Total	18	18	18	18	18	18	18	18			144		
Polymorphisms per combination	Useful	1.50	1.31	1.33	1.08	1.40	1.14	1.33	1.14				1.26	
	Total	0.67	0.94	0.44	0.78	0.39	0.44	0.67	0.44					0.60

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Eco*RI*/Mse*I primer combinations*

Only *Eco*RI selective primers E+AAC and E+ACA amplified useful polymorphisms when used in combination with two and four of the eight *Mse*I selective primers, respectively, while E+AAG and E+ACC in combination with all *Mse*I selective primers did not amplify any useful polymorphisms (Table 2). The largest number of useful polymorphisms was detected using the *Eco*RI selective primer E+ACA (6 polymorphisms), while no useful polymorphisms were detected with E+AAG and E+ACC. The number of useful polymorphisms per combination was highest for E+AAC and E+ACC (1.50 with two and four primer combinations, respectively) and none for E+AAG and E+AAC. Likewise, for the *Mse*I selective primers, all primers but M+CAA, M+CTA, M+CTG and M+CTT yielded useful polymorphisms when used in combination with *Eco*RI selective primers. Four of the *Mse*I selective primers were useful in combination with *Eco*RI selective primer E+ACA and two in combination with *Eco*RI selective primer E+AAC. The highest number of polymorphisms was found with M+CTC (four useful polymorphisms) and none with M+CAA, M+CTA, M+CTG and M+CTT. The highest number of polymorphisms per useful primer combination was with M+CTC (2.00) and none was found for combinations with *Mse*I selective primers M+CAA, M+CTA, M+CTG and M+CTT. On average, three of the six (50%) useful primer combinations yielded a single useful polymorphism and another three (50%) yielded two useful polymorphisms.

Three of the six (50%) useful primer combinations yielded a single useful polymorphism, while the other half yielded two useful polymorphisms. If all 32 combinations are taken into account (Table 2), 0.00 to 0.75 useful polymorphisms per combination were found when testing *Eco*RI selective primers against all *Mse*I selective primers while a range of 0.00 to 1.00 were found when testing *Mse*I selective primers in combination with all *Eco*RI selective primers, with a total average of 0.28 useful polymorphisms per combination.

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Table 2. Summary of results obtained for the 32 *Eco* RI / *Mse* I primer combinations tested.

		Eco RI primers				Number of useful polymorphisms	Number of combinations		Number of polymorphisms per combination	
		E+AAC	E+AAG	E+ACA	E+ACC		Useful	Total	Useful	Total
Mse I primers	M+CAA					0	0	4	0.00	0.00
	M+CAC			1		1	1	4	1.00	0.25
	M+CAG	1		2		3	2	4	1.50	0.75
	M+CAT			1		1	1	4	1.00	0.25
	M+CTA					0	0	4	0.00	0.00
	M+CTC	2		2		4	2	4	2.00	1.00
	M+CTG					0	0	4	0.00	0.00
	M+CTT					0	0	4	0.00	0.00
Number of useful polymorphisms		3	0	6	0	9				
Number of combinations	Useful	2	0	4	0		6			
	Total	8	8	8	8			32		
Polymorphisms per combination	Useful	1.50	0.00	1.50	0.00				1.50	
	Total	0.38	0.00	0.75	0.00					0.28

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Comparison of Sse*8387I/*MseI *and* Eco*RI/*MseI *primer combinations*

A comparison of the results of the *Sse*8387I/*Mse*I and *Eco*RI/*Mse*I primer combinations reveals a difference in the number of useful polymorphisms per total number of combinations screened (0.60 and 0.28, respectively). Two *Eco*RI and four *Mse*I selective primers did not reveal any useful polymorphisms when used in combination with *Mse*I and *Eco*RI selective primers, respectively, while all *Sse*8387I and *Mse*I selective primer combinations yielded at least one useful polymorphism. However, this may be a peculiarity of the specific primer combinations tested and the specific chromosome segment that was studied.

Physical mapping of AFLP markers and characterisation of mutants

Two mutant lines, namely 87M23-175 and 89M2-416, were found to produce inconsistent results, which may be the result of heterogeneous seed stocks (data not shown). These two lines were not used in subsequent mapping. A total of 95 (86 *Sse*8387I-derived and 9 *Eco*RI-derived) useful translocation-specific polymorphisms were amplified by 74 primer combinations (68 *Sse*8387I-derived and 6 *Eco*RI-derived) and were integrated with the physical map constructed by Prins *et al.* (1996). The seven main clusters of the previous map were re-organised into 19 clusters and the revised map is shown as a line drawing in Figure 1 (a detailed map is given in Appendix 1). The distances among the groups of loci are given in arbitrary units which were deduced from the distribution of irradiation breakpoints in the deletion lines.

No polymorphic AFLP markers were associated with RFLP markers *Xpsr165* and *Xpsr105* (marker intervals X4 and X5, respectively), sixteen with RFLP marker *Xpsr129* (marker interval X10), twenty-three with RFLP markers *XcsIH81-1* and *XmWG380* (marker interval X12), seven grouped with *Lr19* (marker interval X15) and none with *Wsp-D1* (marker interval X17). Two AFLP markers, one mapping to interval X15, *viz* 27b:S-AC/M-AT_{191}, and the other to interval X12, *viz* 36b:S-AG/M-CA_{205}, are shown in Figure 2.

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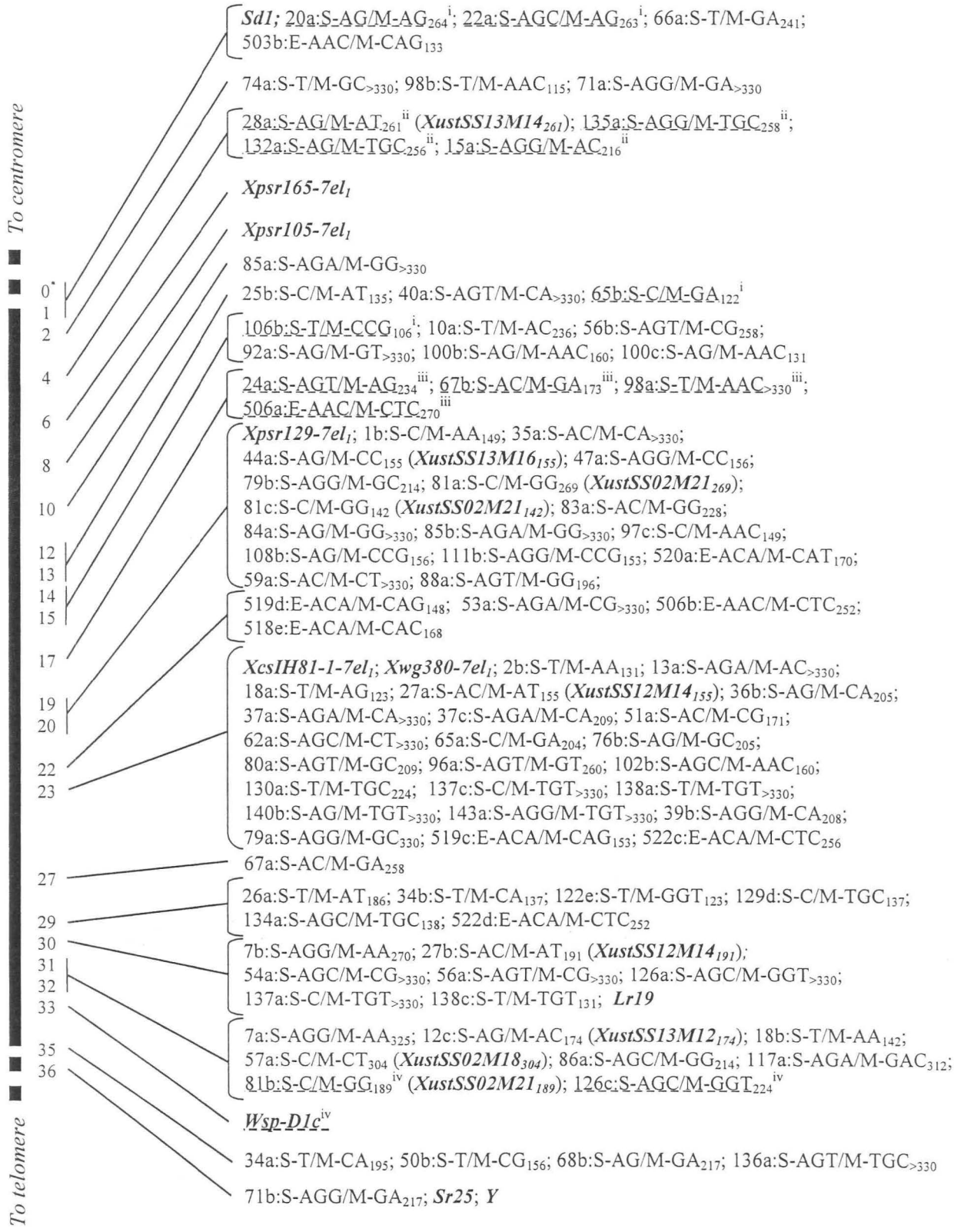

* Arbitrary distance units (refer to text)
i Intercalary deletions in mutant X:87M23-103
ii Intercalary deletion in mutant J:87M23-273
iii Intercalary deletion in mutant Q:87M23-3
iv Intercalary deletion in mutant AA:87M23-225
bold Markers used by Marais *et al.* (2001)

Figure 1. Extended physical map of the *Lr19* translocation (order of loci in intervals is unknown).

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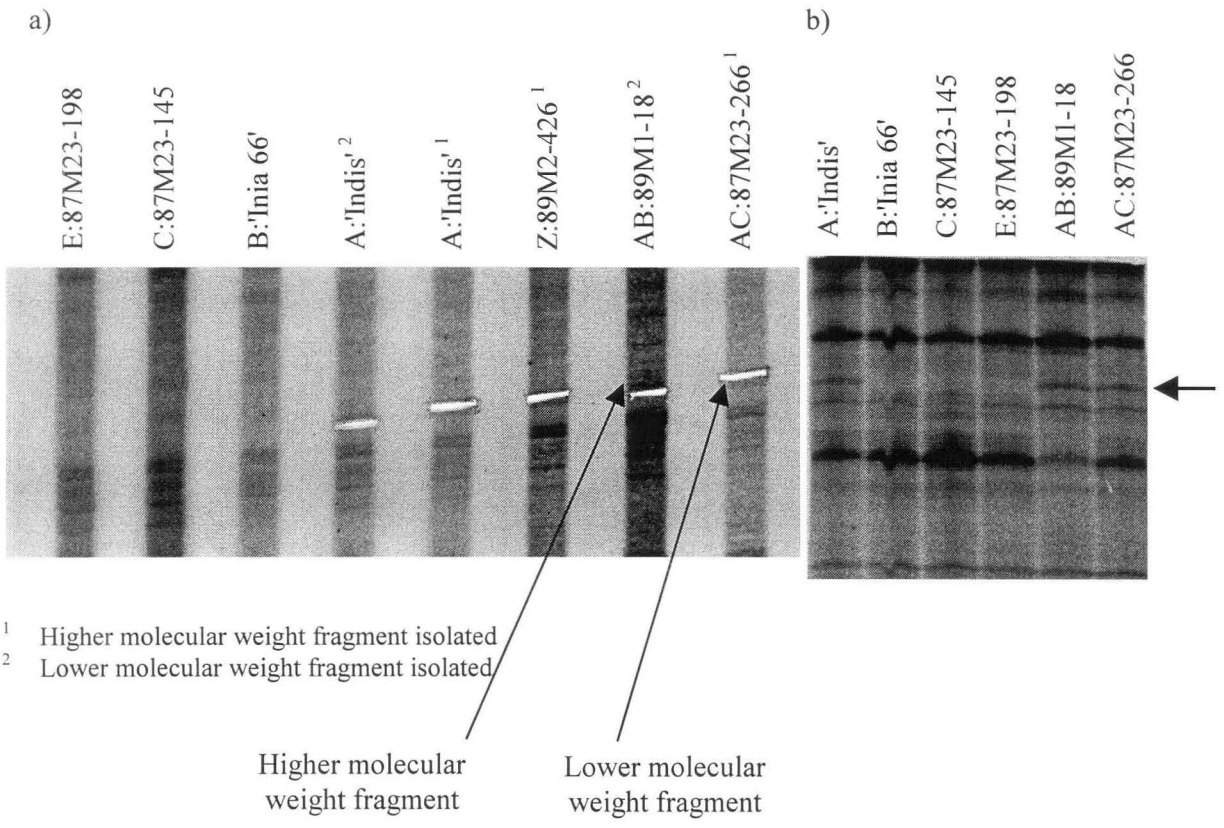

Figure 2. Sections from autoradiographs showing AFLP markers a) 27b:S-AC/M-AT_{191} and b) 36b:S-AG/M-CA_{205}.

The marker shown in Figure 2a, *viz.* 27b:S-AC/M-AT_{191}, was selected for marker conversion as described in the *'Conversion of AFLP Markers to SCAR Markers'* section of the Material and Methods. This marker appeared as a doublet on a polyacrylamide gel and therefore both higher and lower molecular weight fragments were isolated and cloned, namely the higher molecular weight fragment from A:'Indis', Z:89M2-426 and AC:87M23-266, and the lower molecular weight fragment of A:'Indis' and AB:89M1-18.

It was not possible to size the mutants in some clusters, for example deletion mutants J:87M23-273 and K:87M23-227 in cluster Y6 and Q:87M23-3, R:87M23-128, S:87M23-108 and T:87M23-219 in cluster Y12, while other clusters only contained one mutant, for example mutants W:89M2-40 to Z:89M2-426 in clusters Y14 to Y17. Prins *et al.* (1996) reported intercalary deletions in two mutants, namely 87M23-175 and 89M2-416. As these were the two lines that produced inconsistent results in this study and that were subsequently excluded, this was not confirmed. However, in several mutants, the possibility of intercalary deletions exists, although it was not always possible to determine this with absolute certainty (Appendix 1). For example, mutants J:87M23-273 (interval X3) and Q:87M23-3 (interval X9) may both have intercalary deletions spanning four AFLP markers, while mutants P:89M2-39 (interval X10) and

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X:87M23-103 (intervals X1 and X7/8) appear to have intercalary deletions involving two AFLP markers in each deletion. In mutant AA:89M2-225 (cluster Y16/17), two AFLP markers and the *Wsp-D1* locus were deleted. In other intervals single markers were deleted, for example AFLP marker 15a:S-AGG/M-AC_{216} for mutant F:87M23-1 in interval X3 and the AFLP marker 85a:S-AGA/M-$GG_{>330}$ for mutants L:87M23-314 and Q:87M23-3 in interval X6, probably due to mutations in the restriction site or at positions in the selective AFLP primers' binding site. However, it is also possible that some of these may be due to small intercalary deletions. Overall, both *Sse*8387I/*Mse*I and *Eco*RI/*Mse*I markers are evenly distributed throughout the map.

Conversion of AFLP markers to sequence-specific markers

One of the AFLP markers located distally to *Lr19* (12c:S-AG/M-AC_{174}) has been converted successfully into a dominant PCR-based marker (Prins *et al.*, 2001), and an attempt to convert another AFLP marker completely linked to *Lr19*, *viz.* 27b:S-AC/M-AT_{191}, is reported here. At the stage when conversion was attempted, the latter marker was the only AFLP marker identified to be completely linked to *Lr19*. Only three of the six clones that were sequenced had sequences suitable for primer design, the sizes of the remaining clones were not correct. The other three clones All three sequences were different and the sequence of the higher molecular weight fragment of 89M2-426 contained a compound $(AC)_n$-repeat motif (Figure 3) of approximately 50 base pairs (14 bp + 14 bp + 22 bp) in length. Sequence-specific primers of sixteen to twenty-five nucleotides in length were designed to amplify each of the three clones (Table 3).

Primer set ACH-F and ACH-R (spanning the repeat motifs) amplified more than one fragment after several rounds of optimisation from both of the parental lines and no polymorphisms could be detected between these lines (Figure 4). The remaining two marker sets were also not polymorphic (Figure 4) and their amplicons were subsequently subjected to restriction endonuclease analysis. Digestion with three restriction endonucleases (two of which have known cutting site(s) in the amplicon(s), *viz.* *Pst*I for primer set ADL1 and *Hae*III for both primer sets AEH and ADL1) did not result in any fragments that were polymorphic between the parental lines.

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Table 3. Results of amplification reactions with primers designed from the sequences of the three clones.

Deletion mutant	AFLP position	Primer identifier	Product size	G:C:A:T	Sequence of primer (5' - 3')	Polymorphic
89M2-426	Higher	ACH-F	131 bp	14:64:43:7	ACA TCC GCC ACC GAG G	No
		ACH-R			GGG TTG ATT TGG GTG TTG C	
87M23-266	Higher	AEH-F	148 bp	44:27:48:29	GAC GAA AAT ATA CGA AAC AGA GAA G	No
		AEH-R			ATA GGC CAG GCC CAG C	
89M1-18	Lower	ADL1-F	137 bp	27:29:51:30	GCC CAT GGT TAC AAC AGA	No
		ADL1-R			CAT TAT TGT GTT GTT TAG TTG TTT G	

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MseI primer *Forward primer*

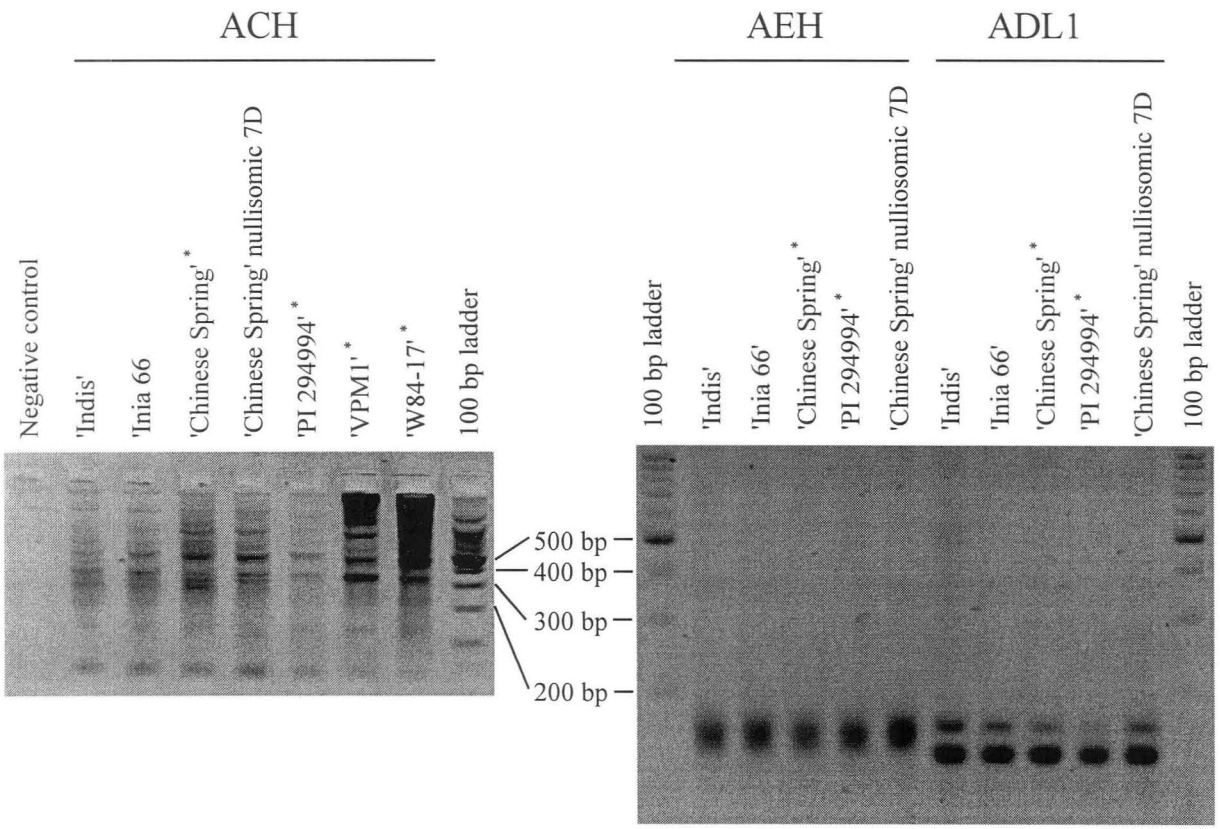
GATGAGTCCTGAGTAAATTACATCCGCCACCGAGGACCCCGYGTY(*ACACACACACACAC*)GCG

CGCGCGCGCACCCCCCCCC(*ACACACACACACAC*)TAAGACTYCATCTC(*ACACACACACACAC*

ACACACAC)**GCAACACCCAAATCAACCC**AAATGTATATGAACGAC**GTYCTGCATGTACGCAGTC**

Reverse primer *Sse8387I primer*

Figure 3. Sequence of cloned insert of the higher molecular weight amplicon from Z:89M2-426 with the ACH forward and reverse primers indicated.



- 'Chinese Spring', 'PI 294994', 'VPM1' and 'W84-17' were included in the test panel as these lines were parental genotypes for populations segregating for Russian wheat aphid resistance gene, *Dn5* (Chapter 3), and eyespot resistance gene, *Pch1* (Chapter 4), respectively. These resistance genes co-occur on chromosome arm 7DL, therefore markers mapping to this chromosome arm might be useful for tagging them as well.

Figure 4. Agarose gel with products amplified using the sequence-specific primers.

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DISCUSSION

Cultivated wheat is not very polymorphic and this hampers the mapping of specific wheat chromosomes. RFLP maps covering large regions of the wheat genome are available, but screening with RFLP probes is time-consuming and, because they are not always polymorphic, not very effective. AFLP marker technology offers a reproducible and accelerated screening method that facilitates sampling of a larger number of loci in a single round than would ever be possible using current RFLP technology.

In organisms with complex genomes, like wheat, the complexity and scorability of the fingerprint resulting from AFLP analysis are important. The addition of more selective nucleotides on the 3' end of the *Sse*8387I or *Mse*I primers used for selective PCR amplification will decrease the complexity of the fingerprint as the selectivity of the primers increases. In the present study, *Sse*8387I +1 *Sse*8387I +2 and *Sse*8387I +3 selective primers, as well as *Eco*RI +3 selective primers, in combination with *Mse*I +2 and *Mse*I +3 primers were used for the selective amplification of fragments. The largest number of polymorphisms was found using *Sse*8387I +1 and *Sse*8387I +2 primers. Combinations of *Sse*8387I and *Mse*I were better suited for analysis of the translocation than *Eco*RI and *Mse*I, as 68 (47.2%) of the 144 primer combinations resulted in useful polymorphisms versus 6 (18.75%) out of 32 for the latter. The low number of useful polymorphisms detected (0.60 and 0.28 per combination over all combinations tested) is to be expected if only a comparatively small region of the genome is targeted. Schwarz *et al.* (1999) used 256 *Eco*RI/*Mse*I (16 primers of each) primer combinations to screen barley NILs with resistance to powdery mildew and identified six polymorphic fragments associated with a large *Mla1*-carrying donor fragment in NIL P01 and four fragments for the small *Mla12*-carrying donor fragment in NIL P10. Only three of these fragments were found to be useful after subsequent testing in a segregating F_2 population. AFLPs were also used by Bai *et al.* (1999) to identify markers associated with a major QTL controlling scab resistance in wheat: eleven markers from twenty of 300 *Eco*RI/*Mse*I primer combinations (15 *Eco*RI and 20 *Mse*I selective primers) resulted in significant association. Qu *et al.* (1998) utilised AFLP markers to develop a PCR-based method for scoring the *ph1b* deletion in wheat: six useful polymorphisms were found with 128 *Pst*I/*Mse*I primer combinations (2 *Pst*I and 64 *Mse*I selective primers), of which some of the polymorphisms were subsequently converted to sequence-specific markers. In pepper crops (*Capsicum annuum* L.), eight useful AFLP markers were identified with five of

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145 *Eco*RI/*Mse*I primer combinations when tested for association with resistance to the potyvirus resistance gene *Pvr4* (Caranta *et al.*, 1999).

Using 176 primer combinations, the existing physical map of the *Lr19* translocation has been extended to include an additional 95 AFLP markers derived from 74 useful combinations and allowed the ordering of 19 of the 27 deletion mutants. Seven clusters, consisting of fourteen mutants, remain in which lines could still not be distinguished from one another. To eliminate the possibility that the methylation status of these regions might have influenced the cutting preference of the methylation-sensitive rare cutting enzyme *Sse8387I* used, the methylation-insensitive enzyme combination *Eco*RI/*Mse*I were also tested. Barrett and Kidwell (1998) found that the methylation-sensitive combination *Pst*I/*Mse*I detected significantly lower levels of diversity in wheat than the combination *Eco*RI/*Mse*I and ascribed it to the possibility that hypo-methylated regions might contain less DNA variation than hypermethylated regions. A combination of *Pst*I/*Mse*I was used to locate the *Cf-9* gene in tomato euchromatin (Thomas *et al.*, 1995) while *Eco*RI/*Mse*I was used to locate the *Mi* gene in tomato heterochromatin (Kaloshian *et al.*, 1998). Screening the set of mutants with *Eco*RI/*Mse*I primer combinations did not break up the clustering any further than did the *Sse*8387I/*Mse*I combinations. It is therefore unlikely that methylation is the main cause of clustering of the mutants. A more plausible explanation is that some of the mutation breakpoints may simply be close to one another, reducing the chance of co-occurrence of markers. Attempts to order the mutants that are still clustered may therefore require the screening of many more primer combinations.

The attempt to convert AFLP marker 27b:S-AC/M-AT_{191} into a sequence-specific marker was not successful. Few successful conversions of AFLP markers have been reported in the literature (Bradeen and Simon, 1998; Qu *et al.*, 1998; Shan *et al.*, 1999; Raemon-Büttner and Jung, 2000). Qu *et al.* (1998) sequenced twenty clones from six markers identified and found that all clones contained both the rare and frequent cutter enzymes' primer sequence. Four of the sequences were found to be too short for successful conversion, eight were eliminated because of sequence similarity to others determined and the remaining eight were used for primer synthesis. Two of the eight sequences yielded primer pairs that were useful for the tagging of the *ph1b* deletion. Prins *et al.* (2001) converted an 'Indis' derived *Lr19* translocation-specific AFLP marker (fragment 12c:S-AG/M-AC_{174} in Figure 1) into a useful absence/presence PCR-based marker after screening 40 clones of which only two carried the correct insert. The latter authors found the AFLP conversion process to be technically difficult and ascribed the difficulties to the presence of contaminating fragments and also the fact that the dominant clone was not the one carrying the 'correct' insert, which might be a reason for the low number of successful

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conversions of AFLPs to sequence-specific markers reported in literature. In our laboratory, other attempts to convert AFLP markers to sequence-specific markers had varied results: an AFLP marker associated with RWA resistance gene, *Dn5*, could not be successfully converted into a sequence-specific marker even though all the clones sequenced contained the same insert (Chapter 3). An attempt to convert an AFLP marker linked to eyespot resistance gene *Pch1* and the endopeptidase locus *Ep-D1* (Chapter 4) using the same approach yielded a microsatellite marker that was useful for tagging the two loci with which it was associated. A probable reason for the lack of success in converting AFLP fragment 27b:S-AC/M-AT_{191} might be the small number of clones that yielded sequences suitable for primer design. It is possible that sequencing of a larger number of clones might increase the chance of identifying the correct insert. However, the profile of the doublet AFLP marker targeted for conversion in this study is reminiscent of a microsatellite marker profile with a stutter band. It is therefore very probable that the ACH primer set amplifies the correct fragment, but that the source of the AFLP polymorphism is present in the AFLP restriction site rather than within the repeat motif. This is also a likely scenario if it is taken into account that the ACH primer set amplifies a number of distinct fragments with a wide range of sizes, most likely from more than one locus.

At the stage when the conversion of AFLP marker 27b:S-AC/M-AT_{191} was initiated, it was the only AFLP marker available that was closely associated with *Lr19*. Currently, there are six other AFLP markers co-occurring with *Lr19* and it might be worthwhile to attempt conversion of all or some of these markers. The downside is that there is no sure way to predict whether an AFLP marker will readily convert to a sequence-specific marker. If the polymorphism is caused in the restriction site of one of the enzymes used for AFLP analysis, designing primers within the fragment is only likely to yield a useful sequence-specific marker in cases where alien chromatin is involved and/or little homoeology to the corresponding wheat regions is present. In such cases, the converted marker will be scored as a dominant marker for its absence or presence. The problem of restriction site associated polymorphisms can sometimes be overcome by obtaining flanking sequence information from AFLP markers using ligation-mediated suppression PCR (Schupp *et al.*, 1999) and should be useful in species where a very small number of AFLP markers are found and need to be converted to an easier PCR-based assay. The flanking sequence information can then be used to design primers that will amplify a fragment which includes the restriction site and post-PCR restriction endonuclease digestion of the amplicon will then result in a scorable polymorphism. An alternative source of an AFLP polymorphism occurs when the polymorphism is caused by insertions or deletions and, if the sequence-specific primers span the insertion / deletion site, the resulting amplification product

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would differ in size between the genotypes used to identify the AFLP fingerprint and such converted markers could be scored as co-dominant markers.

Unfortunately, the microsatellite marker identified in this study does not appear to be associated with the *Lr19* translocation. However, the microsatellite nature of this marker may make it useful for tagging other traits, provided that its chromosomal location(s) can be determined. Ma *et al.* (1996) reported a relative abundance of $(AC)_n$ and $(AG)_n$ microsatellite motifs in wheat and estimated the total number of sites of both types to be 1.3×10^5. It is calculated that these two motifs occur in common wheat on average every 292 to 704 kb for $(AC)_n$ and 212 to 440 kb for $(AG)_n$ microsatellite motifs (Gupta and Varshney, 2000). A total number of 23,000 $(AC)_n$ and 36,000 $(AG)_n$ microsatellite motifs are predicted to occur in common wheat (Röder *et al.*, 1995). Major advantages of microsatellite markers include that they are not restricted to telomeric or centromeric regions, are multi-allelic and very small amounts of template DNA, which can even be partly degraded, is needed (Gruis *et al.*, 1993). Recent genetic and physical mapping of microsatellites in wheat indicates that the microsatellites are uniformly distributed in different regions of wheat chromosomes rather than clustered in specific regions (Röder *et al.*, 1998a,b). A recent review paper lists a total of 349 microsatellite loci mapped in common wheat (Gupta and Varshney, 2000) and examples where microsatellites were used to tag traits include genes for dwarfing (Korzun *et al.*, 1997,1998); preharvest sprouting tolerance (Roy *et al.*, 1999) and to detect genetic diversity in closely related bread wheat cultivars and lines (Plaschke *et al.*, 1995). It should be possible to use the wheat nullisomic-tetrasomic series to map the different fragments amplified by the microsatellite PCR primer set.

While *in situ* hybridisation provides an alternative way to characterise the deletion mutants and allosyndetic recombinants of the *Lr19* translocation, it lacks the resolution to resolve small differences in length. In contrast, AFLPs are valuable to identify such differences and simultaneously increase the number of molecular markers on a physical map. The improved physical map has already been used very effectively to find the shortest and most useful allosyndetic recombinants of *Lr19* (Marais *et al.*, 2001). The mutant set has also proved suitable for the physical mapping of molecular markers (such as RFLPs) that are difficult to map genetically on wheat chromosome arm 7DL. Markers suggested by the map to be located close to *Lr19* may prove very useful in attempts to further shorten the translocation and in marker aided selection where it will allow rapid identification of lines containing the locus.

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CHAPTER THREE

ESTABLISHMENT AND CHARACTERISATION OF A DOUBLED HAPLOID MAPPING POPULATION AND TAGGING OF RUSSIAN WHEAT APHID RESISTANCE GENE, *DN5*

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Abstract

A doubled haploid mapping population consisting of 94 lines was established from the F_1 progeny of a cross between 'Chinese Spring' and 'PI 294994'. This population was typed for Russian wheat aphid seedling resistance (*Dn5*), endopeptidase (*Ep-D1*) and four microsatellite (*Xgwm37*, *Xgwm111*, *Xgwm428*, *Xgwm437*) loci. Several RFLP probes as well as two PCR-RFLP markers were tested on the parental lines, but were not polymorphic. Twelve F_2 lines homozygous for resistance and seven homozygous for susceptibility to the Russian wheat aphid were also used in an attempt to identify AFLP markers associated with *Dn5*. Seventy *Sse*8387I/*Mse*I selective primer combinations were used to obtain one repulsion and one coupling phase AFLP marker. The latter marker could not be successfully converted into a sequence-specific marker. *Dn5* was found to be linked to *Xgwm111* and *Xgwm437* at distances of 25.4 and 28.6 cM, respectively, but segregated independently from *Xgwm428*, *Xgwm37* and *Ep-D1*.

Key words *Diuraphis noxia*, RFLP, SSR, AFLP, endopeptidase

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INTRODUCTION

The Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko), is world-wide a serious pest of wheat and barley (*Hordeum vulgare* L.). Under favourable conditions, the infestation can vary from 20% to 80% infested plants (Walters *et al.*, 1980). With typical winter wheat losses in South Africa ranging from 35 to 60%; approximately 40% of the wheat crop is sprayed annually to combat RWA infestation (Du Toit and Walters, 1984; Robinson, 1992). Several means of controlling the insect are employed, usually with varied efficiency. These include cultural practices (for example choice of planting date and the control of alternative hosts), biological control (for example ladybirds and wasp parasites), chemical control and the breeding of resistant cultivars. Due to the seclusion of the RWA in rolled leaves, neither the application of contact aphicides nor biological control agents (for example parasitoid wasps and coccinellid species) is very effective (Zemetra *et al.*, 1993). Systemic insecticides, for example disulfoton and dimethoate, have proven to be successful, but the price and the negative effect on the environment make the use of these insecticides inappropriate (Robinson, 1992). The only and most effective solution that is both financially viable and environmentally safe is the use of resistant cultivars (Walters *et al.*, 1980; Du Toit, 1987; Zhang *et al.*, 1998).

Currently there are seven designated RWA resistance genes, denoted *Dn1* through *Dn7*. *Dn5* is derived from the hard red winter wheat accession 'PI 294994'. 'PI 294994' appears to be a heterogeneous germplasm containing more than one gene for resistance (Zhang *et al.*, 1998). Based on F_2 and backcross data from a cross between 'SA463' ('PI 294994') and 'Chinese Spring', as well as monosomic analysis of 'PI 294994', Marais and Du Toit (1993) concluded that a single dominant gene on chromosome 7D, *viz. Dn5*, was responsible for RWA resistance in 'PI 294994'. Comparisons with previously published information led these researchers to conclude that resistance gene *Dn5* is not allelic to resistance genes *Dn1, Dn2, Dn3* or *Dn4*. Based on the low frequency of susceptible plants in the F_2 progeny of the cross 'PI 294994' × 'PI 137739', it was furthermore suggested that *Dn1* and *Dn5* were linked on chromosome 7D. Du Toit *et al.* (1995) did a telosomic analysis and concluded that *Dn5* occurs on chromosome arm 7DL.

Only a small number of markers associated with *Dn5* has been reported. An endopeptidase allele, *Ep-D1e*, is linked to *Dn5* with a recombination percentage of 32 ± 4.97 (Marais *et al.*, 1998). Ma *et al.* (1998) found an allelic relationship between *Dn2* and a RWA resistance gene in 'PI 294994' and speculated that the RFLP marker *Xksua1* and *Dn5* might be linked. Venter *et*

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al. (1998) identified two repulsion phase and one coupling phase RAPD markers associated with *Dn5*. The three RAPD markers were subsequently converted to sequence-specific markers (Venter and Botha, 2000).

Mapping of a trait or gene on a chromosome arm requires the identification of polymorphic markers that map to the region of interest. Several marker techniques can be considered with this aim in mind. Restriction fragment length polymorphism (RFLP - Botstein *et al.*, 1980; Beckman and Soller, 1983) analysis has been used extensively in wheat and numerous RFLP probes and maps are available, but unfortunately the level of polymorphism detected is low and screening of additional RFLP probes is laborious and time consuming. Microsatellite or SSR (short simple repeat) markers have a co-dominant mode of inheritance (Litt and Luty, 1989; Weber and May, 1989; Wu *et al.*, 1994) and normally consist of a core motif with a few nucleotides (normally 1-6 base pairs) that are repeated multiple times in tandem. They are usually flanked by unique sequences from which PCR primers can be designed (Litt and Luty, 1989; Weber and May, 1989). SSRs are very useful where RFLP variation is rare (Akkaya *et al.*, 1992); unfortunately SSRs are expensive to find and develop, but relatively easy to use. Microsatellites are not restricted to telomeric or centromeric regions, are multi-allelic and very small amounts of template DNA, which can even be partly degraded, is needed (Gruis *et al.*, 1993). Random-amplified polymorphic DNA (RAPD - Williams *et al.*, 1990; Rafalski *et al.*, 1991) markers do not require knowledge of the genome and it is easy to screen a larger number of loci in a single round, but the procedure is hampered by lack of reproducibility and might require the use of a large number of primers to be screened in order to cover an adequate number of loci. Amplified fragment length polymorphism (AFLP - Vos *et al.* 1995; Keim *et al.*, 1997) technology provides a powerful alternative to RAPDs: it also requires no prior knowledge of the genome, and typically amplifies 50 to 100 loci in a single screening attempt. High-resolution size fractionation of amplification fragments is accomplished by polyacrylamide gel electrophoresis. Applications of AFLP markers include assessment of genetic diversity, construction of high-density genetic maps, quantitative trait analysis and identification of additional DNA markers near a locus of interest and have been reviewed extensively by Shan *et al.* (1999).

Several populations can be considered with mapping of a trait or gene in mind. The most commonly used are F_2 populations (Kochert, 1994), which can be produced in a relatively short time. A F_2 population provides almost twice as much information for co-dominant markers as a

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backcross due to the fact that markers are segregating in both the male and female gametic populations generating the F_2 (Tanksley *et al.*, 1988). Near isogenic lines (NILs) and recombinant inbred lines (RILs) can also be used for mapping purposes. A pair of NILs consists of a line(s) without the target gene and its counterpart with the target gene, the only difference between them being the introgressed chromosome segment from the donor where the target gene is located. Linkage drag, where large regions linked, or sometimes even unlinked, to the area of interest still remains in the NILs after several rounds of backcrossing, can complicate screening for polymorphisms between NILs and may result in false positives (Brinkman and Frey, 1977; Young and Tanksley, 1989). RILs are derived through single seed descent of selfed or sib-mated progeny of individual F_2 or backcross plants for many generations, resulting in the fixation of a recombination event in a near-homozygous background (Burr *et al.*, 1988; Burr and Burr, 1991). A recombinant inbred population is therefore a permanent population in which segregation is fixed and this population can therefore easily be shared between groups.

A very popular mapping population is doubled haploids (DHs), which are pure breeding lines (Nei, 1963; Pienaar *et al.*, 1997). The primary methods used to produce haploid wheat plants are anther culture (De Buyser *et al.*, 1987), ovary culture (Comeau *et al.*, 1988) or chromosome elimination following wide crosses (Barclay, 1975; Matzk and Mahn, 1994), for example between wheat and maize (Laurie and Bennett, 1988; Inagaki and Tahir, 1990; Pienaar *et al.*, 1997). The use of wheat × maize wide crosses is usually a more reliable and stable procedure than anther culture (Kisana *et al.*, 1993). In a cross between wheat and maize, the maize chromosomes are eliminated from the developing embryo during the initial rounds of cell division (Laurie and Bennett, 1986). Chromosomes are doubled either after the plantlets have been transferred to soil (Inagaki, 1985) or *in vitro* (Barnabás *et al.*, 1991). DH lines produced from wheat × maize crosses were found to be equivalent to their wheat parent in agronomic performance, with a slight degree of gametoclonal variation in some of the DH lines (Kisana *et al.*, 1993).

The aim of the present study was to establish a doubled haploid mapping population segregating for the Russian wheat aphid resistance gene, *Dn5*, characterise it with available markers and then attempt to map the *Dn5* locus. The AFLP technique was also used in an attempt to identify novel AFLP markers associated with *Dn5*.

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MATERIALS AND METHODS

A doubled haploid mapping population was derived from F_1 progeny of the cross: 'PI 294994' / 'Chinese Spring'. 'Chinese Spring' is susceptible to the Russian wheat aphid (RWA) and is homozygous for the *Ep-D1a* allele, whereas 'PI 294994' is the source of *Dn5* and possesses the *Ep-D1e* allele (Marais *et al.*, 1988). Seeds of the F_1 progeny were planted in a green house; spikes were emasculated and pollinated with pollen from the sweet corn hybrid cultivar 'Seneca 60'. Embryos were rescued and grown to plantlets on growth medium. The haploid plantlets were transferred to soil, tiller-cloned and treated with colchicine to produce doubled haploid plants. Root tips were cut from the clones prior to colchicine treatment to confirm the plant's haploid status. An untreated haploid clone was kept as a back-up. The doubled-haploid plants were selfed and the seed harvested. Seedling RWA resistance reactions were subsequently determined for each of the doubled-haploid lines. Genomic DNA was extracted from the plant leaves and used for RFLP and microsatellite analysis. Iso-electric focusing of seed (embryo) endopeptidase was used to determine which of the *EpD1a* or *Ep-D1e* alleles occurred in a doubled haploid.

For AFLP analyses, the F_3 progeny of thirty-eight F_2: 'Chinese Spring' × 'PI 294994' lines were tested for segregation of the Russian wheat aphid resistance gene, *Dn5*. Genomic DNA, extracted from 19 homozygous F_2 plants, were then subjected to AFLP analyses. In the initial stage of the study, resistant and susceptible bulks were made up using respectively, genomic DNA from 12 homozygous resistant F_2 lines and 7 homozygous susceptible F_2 lines. The two bulks were utilised for preliminary polymorphism screening. The resistant and susceptible F_2 bulks were constructed from equal volumes of the respective diluted pre-selective amplification products. Genomic DNA of the wheat accession 'PI 294994' and the wheat cultivar 'Chinese Spring' were included as controls. Any potentially useful polymorphisms were then tested on the individual resistant and susceptible lines. A polymorphism, associated with *Dn5*, was excised from the gel; the fragment was purified, cloned and sequenced. Sequence-specific primers were designed from the sequence; PCR conditions were optimised and the primer combination tested on the lines to confirm association of the marker with the *Dn5* resistance locus.

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THE DOUBLED HAPLOID POPULATION

Doubled haploid lines were established according to a protocol adapted from Pienaar *et al.* (1997). The two outer florets of the primary wheat spikes of F_1: 'Chinese Spring' × 'PI 294994' plants were emasculated on emergence from the boot. The spike was covered with a glassine bag and labeled with the date of emasculation. Fresh 'Seneca 60' pollen was collected as soon as the central florets of the spike began to open, the upper half of the florets were removed with a small pair of scissors and the wheat pistils were pollinated with a fine paint brush using 'Seneca 60' pollen in excess. After pollination the spikes were covered with brown paper envelopes and labeled with the date of emasculation, as well as the date and time of pollination. After 24 to 30 hours, an insulin syringe was used to fill the floral cups with a hormone solution containing 50 mg/litre 2,4-dichlorophenoxyacetic acid and 100 mg/litre gibberellic acid (GA_3). The treated spikes were harvested after approximately 18 days and the green parthenocarpic caryopses (GPCs) were removed from the florets using surgical pincers.

A laminar flow cabinet was used for all tissue culture work to maintain sterile conditions. The GPCs were sterilised in 70% ethanol for 30 seconds followed by treatment with 1% sodium hypochlorite for 10 to 12 minutes. The GPCs were then washed two or three times with autoclaved dH_2O, each wash lasting three minutes. The GPCs were dissected and the embryos placed on modified Murashige and Skoog medium supplemented with 10% NH_4NO_3 (Pienaar *et al.*, 1997). The tissue culture jar was incubated in a dark shoe box at the bottom of an incubator running at 23 - 25 °C with a 14 hours light / 10 hours dark cycle for a week after which the jar was removed from the box and placed in the light on a higher rack for four to six weeks. When the plantlets had developed to the three-leaf stage, they were transplanted into small pots containing sterile sand, covered with a plastic bag to maintain humid conditions and kept in an incubator with a photoperiod of 16 hours day length at 23 - 25 °C. Acclimatisation was accomplished over a period of one week by gradually opening the plastic bag. Plants in the three or more tiller stage were lifted from the pot, cloned and their roots were washed and trimmed to 10 cm if necessary (at this stage root tips were cut for chromosome counting). One clone from each plant was not doubled and was kept in soil as a back-up. The clones were then treated in an aerated 0.05% (w/v) colchicine solution without DMSO for 24 hours at room temperature. At the end of the treatment, the plants were washed under running water and then placed in a refrigerator for two to three days (water was replaced every morning). The shoots of the plants were cut back to approximately 20 cm and the plants repotted and placed back in the 23 - 25 °C

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incubator. After one to two weeks after colchicine treatment, the plants were moved to a green house under natural light conditions, but kept under a 50% shade screen for the first week. All spikes emerging from a doubled haploid plant were covered with glassine bags to prevent cross-pollination.

Root Tip Chromosome Counts

Cytological preparations of mitotic root tip chromosomes were accomplished using an adaptation of the protocol described by Darlington and La Cour (1960). Clean scissors were used to cut two to three roots from each haploid plant before cloning and chromosome doubling, after which the roots were placed in cold dH_2O in numbered 18 mm × 50 mm glass vials on ice at 4 °C in a refrigerator for 29 hours. The cold dH_2O was drained after this period and replaced with fresh fixative solution (3 parts methanol : 1 part propionic acid) for approximately one week until staining.

Staining of the root tips were accomplished by replacing the fixative with dH_2O for 30 minutes. The roots were then transferred to numbered vials containing 1 N HCl at 60 °C for 7½ minutes, after which the roots were returned to the vials containing the dH_2O to stop the hydrolysis of the DNA. The roots were rinsed twice with new dH_2O for two to three minutes and then the dH_2O was replaced with Feulgen solution and the vials were placed in the refrigerator for either two hours or overnight. The Feulgen solution was removed and the roots rinsed twice with dH_2O. The dH_2O was drained and the roots rinsed with 7.5 mM sodium acetate buffer (pH 4.5) for three to five minutes. The sodium acetate buffer was replaced with fresh 2.5% w/v pecticlear solution (0.5 g pecticlear from Serevac and 20 ml of the 7.5 mM sodium acetate buffer, pH 4.5) for 30 minutes at 37 °C. After the required time period, the 2.5% pecticlear solution was replaced with dH_2O until the roots were mounted on slides.

The roots were mounted on slides cleaned with ethanol by cutting off a stained root tip in a small drop of Rosner 1% w/v aceto-carmine prepared with an equal volume of propionic acid. The root tip was gently tapped with a hard wood needle to release the cells from the root tip, after which a cover slip was placed on the slide. The slide and cover glass were placed in folded filter paper and then the top of the cover glass was gently pressed to remove excess aceto-carmine and spread the chromosomes. The chromosomes were then counted under the 40 times magnification lens of a light microscope using a pink filter in the filter holder.

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Russian Wheat Aphid Seedling Resistance Screening

Five to ten seedlings of each of the doubled haploid lines and 20 seedlings of the F_2-derived F_3 lines were tested for RWA resistance by dr. F. du Toit at PANNAR (PO Box 17164, Bainsvlei, 9388, South Africa) using standard protocols (Du Toit, 1987). Seedlings at the one leaf stage were evenly infested with Russian wheat aphids cultured in a greenhouse on susceptible wheat seedlings under day/night temperature conditions of 22 °C / 15 °C. The seedlings for each line were scored visually 21 days after infestation as being resistant or susceptible (Du Toit, 1987). F_2 lines were scored as homozygous if there was no segregation of resistance/susceptibility in the F_3 progeny of that specific line.

DNA Extraction

Total genomic DNA was extracted from approximately 4-week-old seedlings as described in the relevant section of Chapter 2.

Isoenzyme Screening

Endopeptidase analyses were performed according to Marais and Marais (1990). Endosperm sections (one to three per sample) were cut from each mature kernel and incubated in 120 µl dH_2O overnight at 4 °C. Following maceration, the extracts were centrifuged at 10 000 rpm for 25 minutes at 2 - 5 °C, after which 30 µl of supernatant was loaded directly on the cathodal end of a 150 mm × 125 mm × 0.25 mm 8% w/v acrylamide:*bis*-acrylamide (37.5:1 w/w) gel with 2% w/v ampholyte (consisting of 2 parts Pharmalyte 4.2 - 4.9 and 1 part Pharmalyte 4 - 6.5) and 13% v/v glycerol. 1 M glycine was used as catholyte and 0.5 M glacial acetic acid as anolyte. Electrophoresis was performed at 4 °C on a Hoefer Isobox unit after prefocusing for 30 minutes at 13 W and running for 3 hours at the same setting. Staining of the gel was done for 30 minutes according to Tang and Hart (1975). The stained gel was washed from the glass plates with water, spread onto white paper sheets and air-dried.

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RFLP ANALYSIS

Ten RFLP markers that mapped to chromosome arm 7DL, *viz. Xpsr56*, *Xpsr72*, *Xpsr105*, *Xpsr121*, *Xpsr129*, *Xpsr165*, *Xpsr169*, *XcsIH60-1*, *XcsIH81-1* and *XmWG380*, were screened. Dr. E. Lagudah (CSIRO Division of Plant Industry, Canberra ACT, Australia) supplied the csIH60-1 and csIH81-1 probes and the Plant Science Laboratory, Norwich supplied the PSR probes. The mWG380 probe was obtained from Prof. M.E. Sorrells (Department of Plant Breeding and Biometry, 252 Emerson Hall, Ithaca, NY 14853-1902). The markers were tested on the two parental lines 'Chinese Spring' and 'PI 294994' and also 'Chinese Spring' nullisomic 7D to determine which fragment was 7D specific and whether they were polymorphic between the parental lines used.

Approximately 10 µg of gDNA was digested overnight to completion with 60 units *HindIII* restriction endonuclease (Roche) at 37 °C, followed by size fractionation at 40 V in a 0.8% agarose gel using 1× TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM Na_2EDTA, pH 8.3) as running buffer. Following gel electrophoresis, the gel was depurinated for 15 minutes at room temperature in 0.2 N HCl, submerged twice with gentle shaking in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 15 minutes each at room temperature. The gel was then rinsed with sterile dH_2O and submerged twice with gentle shaking in neutralisation solution (0.5 M Tris-HCl (pH 7.5), 3.0 M NaCl), again for 15 minutes each at room temperature. The DNA was transferred overnight onto a Hybond-N+; positively charged nylon membrane (Amersham Life Science) using 20× SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0) as transfer buffer. The DNA was UV fixed to the blot by cross-linking the DNA side of the blot for 3 minutes on an UV transilluminator. The blot was rinsed briefly in sterile 2× SSC, sealed between plastic sheet protectors and stored at +4 °C for future use.

The insert of the plasmid containing the probe was PCR labeled using a dNTP stock mix consisting of 1 mM each of dATP, dGTP, dCTP, 0.75 mM dTTP and 0.3 mM digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (alkali labile - Roche). PCR amplification was performed in 0.2 ml PCR tubes in a 50 µl total reaction volume consisting of 2 ng plasmid DNA, 15 pmol each of M13 forward and reverse primer, 20% v/v dNTP mix, 2.5 units *Taq* DNA polymerase

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(Bioline), 1× PCR buffer, 1.5 mM $MgCl_2$ and AFLP-grade water. The PCR profile was run on a Hybaid PCR Express and involved (a) 7 minutes at 94 °C; (b) 36 cycles each consisting of 45 seconds at 94 °C, 1 minute at 60 °C, 2 minutes at 72 °C; (c) 2 minutes at 72 °C and (d) a final soak temperature of +4 °C. Membranes were prehybridised with DIG Easy Hyb (Roche) for at least 3 hours at 42 °C and reused membranes for 1 - 3 hours at the same temperature in a Techne Hybridizer oven. The hybridisation solution contained DIG Easy Hyb and approximately 300 ng denatured labeled probe. Following overnight hybridisation at 42 °C, the membrane was washed twice in a solution consisting of 2× SSC and 0.1% SDS, each wash lasting 5 minutes at room temperature. This was followed by another two washes, this time in a solution consisting of 0.5× SSC and 0.1% SDS, each wash lasting 15 minutes at 68 °C. Prior to detection, the membrane was rinsed in 2× SSC to avoid the formation of salt precipitants. Detection of the hybridised probe was performed using CDP Star (Roche) and the DIG Wash and Block Buffer Set (Roche) according to the manufacturer's instructions. Hybridisation products were visualised after exposure to an X-ray film (Sterling Cronex 4) for one to two hours. The membrane was rinsed in dH_2O for one minute at room temperature, the probe was stripped from the membrane by washing the membrane twice for 15 minutes at 37 °C in a solution consisting of 0.2 N NaOH and 0.1% SDS and then the membrane was rinsed in 2× SSC for 5 minutes at room temperature. The stripped membrane was sealed between plastic sheet protectors and stored at +4 °C.

MICROSATELLITE ANALYSIS

Four microsatellite markers that map to chromosome 7DL (Röder *et al.*, 1998), *viz. Xgwm37*, *Xgwm111*, *Xgwm428* and *Xgwm437*, were selected for screening of the doubled haploid population. The markers were first tested on the two parental lines 'Chinese Spring' and 'PI 294994' to determine whether they were polymorphic. All of the individuals in the mapping population were then tested for the polymorphic markers.

8.25 pmol of the forward primer in each set was end-labeled with 0.25 µCi $\{\gamma\text{-}^{33}P\}$ATP (74 TBq/mmol, 2000 Ci/mmol, 370 MBq/ml, 10 mCi/ml; NEN Life Science Products, Inc.) in the presence of 1 unit of T_4 polynucleotide kinase (AEC Amersham), 1× reaction buffer (AEC

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Amersham) and AFLP-grade water. The reaction was incubated for 2 - 3 hours at 37 °C, after which the enzyme was heat-inactivated by incubation for 10 min at 65 °C. The labeled primer was then stored at +4 °C. 30-330 bp AFLP ladder (GIBCO BRL) was labeled according to the manufacturer's instructions.

PCR amplification of the microsatellite locus was performed in 0.2 ml PCR tubes in a 12.5 µl total reaction volume. This consisted of 25-50 ng template DNA, 6.25 pmol of each primer, 200 µM of each dNTP, 0.5 units *Taq* DNA polymerase (Bioline), 1× PCR buffer, 1 - 2.5 mM $MgCl_2$ and AFLP-grade water. Optimal $MgCl_2$ concentrations were 1 mM for both *Xgwm37* and *Xgwm111*, while 2.5 mM was used for *Xgwm428* and 1.5 mM for *Xgwm437*. The PCR profile for *Xgwm37* and *Xgwm428* was run on an Eppendorf Gradient Cycler and involved (a) 3 minutes at 94 °C; (b) 45 cycles each consisting of 1 minute at 94 °C, 1 minute at the optimal annealing temperature, 2 minutes at 72 °C; (c) 10 minutes at 72 °C and (d) a final soak temperature of +4 °C. The PCR profile for *Xgwm111* and *Xgwm437* was also run on an Eppendorf Gradient Cycler and consisted of 1 cycle of 94 °C for 30 seconds, 65 °C for 30 seconds, 72 °C for 60 seconds, followed by 12 cycles in which the annealing temperature in each cycle was lowered by 0.7 °C. This was followed by 23 cycles of 94 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 60 seconds and a final soak temperature of 4 °C. The amplification reactions were stored at +4 °C until size-separation on a denaturing polyacrylamide gel.

Samples were size-fractionated on a 0.4 mm thick 6% w/v acrylamide:*bis*-acrylamide (19:1 w/w) denaturing sequencing gel containing 6 M urea in 1× TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM Na_2EDTA, pH 8.3) using a Model S2001 sequencing gel electrophoresis apparatus (Life Technologies) according to the manufacturer's instructions. Prior to loading the samples, an equal volume of AFLP loading buffer (98% formamide, 10 mM EDTA pH 8.0, 0.05% w/v bromophenol blue, 0.05% w/v xylene cyanol FF) was added to each sample after which the samples were denatured at 90 °C for 3 to 5 minutes and then immediately quenched on ice. 5 µl of the denatured samples and 2 µl of the labeled 30-330 bp AFLP ladder (GIBCO) were loaded on the gel. Denaturing polyacrylamide gel electrophoresis was then performed at a constant power of 80 W for approximately 2 hours (when the xylene cyanol front was approximately 7 cm from the bottom of the glass plate). The gel was transferred to

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Whatmann 3MM Chr chromatography paper and dried on a Savant Gel Dryer for 2 hours at 80 °C. Amplified products were visualised after exposure to an X-ray film (Kodak BioMax MR) for up to five days. Markers were scored manually and ambiguous results were double-checked and / or repeated.

PCR-RFLP ANALYSIS

The primer sequences of the PCR-RFLP markers developed by Venter and Botha (2000) from RAPD markers linked with *Dn5* (Venter *et al.*, 1998) were kindly supplied by Eduard Venter, Genetics/Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, Gauteng, 0002, Republic of South Africa.

Fifteen picomoles of each primer were used for PCR amplification of the two parental lines in 0.2 ml PCR tubes. The reaction was performed in a total volume of 30 µl and consisted of 25-50 ng template DNA, 200 µM of each dNTP, 0.5 units *Taq* DNA polymerase (Bioline), 1× PCR buffer, 1.5 mM $MgCl_2$ and AFLP-grade water. The PCR profile was run on an Eppendorf Gradient Cycler and involved (a) 7 minutes at 94 °C; (b) 36 cycles each consisting of 45 seconds at 94 °C, 1 minute at 60 °C, 2 minutes at 72 °C; (c) 2 minutes at 72 °C and (d) a final soak temperature of +4 °C.

Eighteen microlitres of the amplification reactions were digested overnight with 2 units of restriction endonuclease in the presence of 1× reaction buffer at 37 °C (except for *TaqI* which was incubated at 65 °C). Test digestions were done with nine different restriction endonucleases: *BamHI* (Roche), *DraI* (Roche), *EcoRI* (Promega), *HaeIII* (Roche), *HindIII* (Roche), *MseI* (New England Biolabs), *PstI* (Roche), *PvuI* (Roche) and *TaqI* (Roche). 300 ng lambda DNA was also digested as control for the efficiency of the digestion. Following overnight digestion, the samples were loaded onto a 0.8% agarose gel using 1× TBE buffer (100 mM Tris-HCl, 100 mM boric acid, 2 mM Na_2EDTA, pH 8.3) as running buffer. Electrophoresis was allowed to proceed for 3 hours at 75 V, after which the gel was stained in ethidium bromide and photographed under UV light.

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Characterisation of the Mapping Population

Polymorphic markers were tested on all 94 doubled haploid lines and the data generated were used to test all possible marker loci combinations for linkage. Linkage analysis and LOD scores were calculated using the software package MAPMAKER /EXP version 3.0b with default parameters (Lander *et al.* 1987; Lincoln *et al.*, 1992). The Kosambi function was used to correct recombination frequencies (Kosambi, 1944) and the results were integrated with existing chromosome arm 7DL maps.

AFLP analysis and Marker Conversion

AFLP analysis (Table 1) was performed as described in the relevant section of Chapter 2 using the protocol of Donini *et al.* (1997), as modified from Vos *et al.* (1995). Marker conversion from AFLP reactions containing the fragment of interest (lines 'PI 294994', 2, 18 and 23), as well as reactions without the fragment (lines 'Chinese Spring', 6 and 11), was attempted as outlined in the relevant section of Chapter 2. The isolated fragments were purified and the fragment isolated from resistant line '18' was cloned using the pGEM-T Easy vector system (Promega) according to the manufacturer's instructions. Ten transformed colonies were tested for correct insert size, three of which were subsequently used for plasmid isolation, automated sequencing and primer design.

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Table 1. *Sse*8387I and *Mse*I adaptor-specific oligonucleotides used as selective primers in this study.

*Mse*I	*Sse*8387I primers							
primers	S02 +C	S04 +T	S12 +AC	S13 +AG	S27 +AGA	S28 +AGC	S29 +AGG	S30 +AGT
M11 +AA	1	2	3	4	5	6	7	8
M12 +AC	9	10	11	12	13	14	15	16
M13 +AG	17	18	19	20	21	22	23	24
M14 +AT	25	26	27	28	29	30	31	32
M15 +CA	33	34	35	36	37	38	39	40
M16 +CC	41	42	43	44	45	46	47	48
M17 +CG	49	50	51	52	53	54	55	56
M18 +CT	57	58	59	60	61	62	63	64
M19 +GA	65	66	67	68	69	70	71	72
M20 +GC	73	74	75	76	77	78	79	80
M21 +GG	81	82	83	84	85	86	87	88
M22 +GT	89	90	91	92	93	94	95	96
M32 + AAC	**97**[1]	98	99	100	101	102	103	104
M53 + CCG	105	106	107	108	109	110	111	112
M64 + GAC	113	114	115	116	117	118	119	120
M74 + GGT	121	122	123	124	125	126	127	128
M88 + TGC	129	130	131	132	133	134	135	136
M90 + TGT	137	**138**[1]	139	140	141	142	143	144

The primers are labeled in such a way that the first three characters represent the primer identifier, followed by the selective nucleotides. For example S02-C represents Sse adaptor specific primer number S02 and the selective nucleotide that followed the common adapter sequence was a 'C'. The number written in italics in the body of the table designates a specific primer combination. For example, 'combination 10' will use primers S04 and M12. Darker shaded blocks indicate combinations that were tested on the bulked groups and lighter shaded primer combinations that were tested on the 19 individual lines. The superscript number indicates the number of useful polymorphisms amplified by the specific primer combination.

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RESULTS

A doubled haploid population consisting of 94 lines was created and characterised for RWA resistance, endopeptidase profiles and four microsatellite markers. The ten RFLP markers were not polymorphic and the PCR-RFLP markers developed by Venter *et al.* (1998) were found not to be useful in this population. The raw data of the markers for each individual line is shown as Table 8. Twelve of the 38 F_2-derived F_3 lines were homozygous for RWA resistance and seven homozygous for susceptibility. Screening of these lines with 70 AFLP selective primer combinations yielded one coupling phase and one repulsion phase marker. An unsuccessful attempt was made to convert the coupling phase marker to a sequence-specific marker.

Doubled Haploid Population

A total of 487 spikes of F_1's from the cross 'Chinese Spring' × 'PI 294994' were emasculated and 15,249 florets were pollinated with maize pollen. 10,908 GPCs developed from which 1,045 embryos were excised and placed on growth medium. Only 204 of the embryos developed into haploid plantlets, of which 94 survived the transfer to soil and subsequent colchicine treatment to become adult plants. DH43 was almost completely sterile and produced only a few shrivelled seeds. All of the progeny were completely sterile and late-flowering.

Root Tip Chromosome Counts

The haploid status of all plantlets was confirmed with root tip chromosome counts. At least three to five metaphase chromosome spreads per root tip squash were counted, and in the case of dubious or difficult chromosome spreads, this number was increased or more root tips were checked. All the haploid lines had 21 chromosomes (Figure 1), except for line number 111, which had 22 chromosomes. This line was also used in subsequent characterisation procedures.

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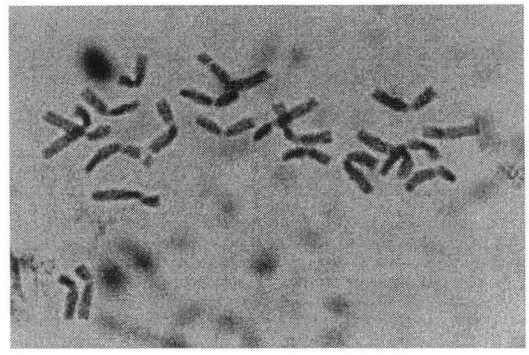

Figure 1. Metaphase chromosome spread of one of the haploid plantlets with 21 chromosomes.

RWA Seedling Resistance Screening

All 94 DH lines were tested for Russian wheat aphid seedling resistance and the summarised results are shown in Table 2. The results of each individual line can be found in Table 8. One of the lines, *viz.* DH43, produced only a few seeds and only one seed could be tested for resistance. Three lines, namely DH7 (8 susceptible to 1 resistant), DH67 (7 resistant to 2 susceptible) and DH110 (6 resistant to 2 susceptible), showed variation for resistance, which may have been the result of misclassification. The classification of the majority of seedlings in each of the three lines was taken to be the correct one. The seedlings of two lines, namely DH96 and DH102, developed poorly and their resistance could not be determined without some doubt. An overall segregation of 55 resistant to 39 susceptible DH lines (1.41:1) were found in the mapping population, which is in agreement with a Mendelian segregation of 1:1 ($\chi^2 = 2.7234$; $P = 0.0990$).

Table 2. Segregation for RWA resistance in 94 doubled haploid lines.

Number of resistant lines	Number of susceptible lines	Total number of lines	Ratio tested	Chi square	*P*
55	39	94	1:1	2.7234	0.0990
			3:1	13.6312	0.0002

Seedling reactions of 17 to 20 seeds from F_2-derived F_3 progeny of each of the 38 lines tested for RWA resistance revealed that twelve lines were homozygous resistant, seven lines were homozygous susceptible and resistance segregated in the remaining nineteen lines (Table 3).

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Table 3. Data obtained following the screening of 38 F_2-derived F_3 lines from the test cross: 'Chinese Spring' / 'PI 294994' for seedling reaction to the Russian wheat aphid.

Line number	Used for which bulk	Percentage resistant seedlings	Percentage Susceptible seedlings
1		77.78% (14/18)	22.33% (4/18)
2	Resistant	100.00% (20/20)	-
3		90.00% (18/20)	10.00% (2/20)
4		78.95% (15/19)	21.05% (4/19)
5	Susceptible	-	100.00% (19/19)
6	Resistant	100.00% (19/19)	-
7	Susceptible	-	100.00% (20/20)
8		85.00% (17/20)	15.00% (3/20)
9		90.00% (18/20)	10.00% (2/20)
10		83.33% (15/18)	16.67% (3/18)
11	Resistant	100.00% (20/20)	-
12		75.00% (15/20)	25.00% (5/20)
13	Susceptible	-	100.00% (20/20)
14		85.00% (17/20)	15.00% (3/20)
15		80.00% (16/20)	20.00% (4/20)
16		60.00% (12/20)	40.00% (8/20)
17	Resistant	100.00% (20/20)	-
18	Resistant	100.00% (19/19)	-
19	Resistant	100.00% (20/20)	-
20		82.35% (14/17)	17.65% (3/17)
21	Susceptible	-	100.00% (19/19)
22		44.44% (8/18)	55.56% (10/18)
23	Resistant	100.00% (20/20)	-
24		75.0% (15/20)	25.00% (5/20)
25	Susceptible	-	100.00% (20/20)
26		85.00% (17/20)	15.00% (3/20)
27	Resistant	100.00% (20/20)	-
28	Resistant	100.00% (17/17)	-
29		77.78% (14/18)	22.33% (4/18)
30		88.89% (16/18)	11.11% (2/18)
31	Resistant	100.00% (19/19)	-
32		73.68% (14/19)	26.32% (5/19)
33	Resistant	100.00% (20/20)	-
34	Susceptible	-	100.00% (18/18)
35		57.89% (11/19)	42.11% (8/19)
36		82.35% (14/17)	17.65% (3/17)
37	Susceptible	-	100.00% (19/19)
38	Resistant	100.00% (18/18)	-

Chi-square analysis of the seedling reactions (Table 4) was in agreement with the segregation ratio of a single dominant gene (1:2:1; $\chi^2 = 1.3158$; $P = 0.5179$).

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Table 4. Chi-square analysis of 38 F_2-derived F_3 RWA seedling reactions.

Number of homozygous resistant lines	Number of segregating lines	Number of homozygous susceptible lines	Total number of lines	Ratio tested	Chi square	*P*
12	19	7	38	1:2:1	1.3158	0.5179
				7:8:1	10.2932	0.0058

Isoenzyme Screening

Extracts of the full set of 94 DH lines, as well as the parental lines, were iso-focused and screened for the expression of endopeptidase alleles *Ep-D1a* ('Chinese Spring') and *Ep-D1e* ('PI 294994'). As the 'Chinese Spring' allele, *Ep-D1a*, could be scored unambiguously (Figure 2), the zymograms were scored for the absence/presence of this allele. The summarised results are shown in Table 5 and the results of each individual line can be found in Table 8. A segregation of 44 lines with the *Ep-D1e* ('PI 294994') allele to 50 with the *Ep-D1a* ('Chinese Spring') allele (0.88:1) were found in the mapping population, which is in agreement with a Mendelian segregation of 1:1 ($\chi^2 = 0.3830$; $P = 0.5360$).

Table 5. Segregation at the *Ep-D1* locus for 94 doubled haploid lines.

Number of *Ep-D1e* alleles	Number of *Ep-D1a* alleles	Total number of lines	Ratio tested	Chi square	*P*
44	50	94	1:1	0.3830	0.5360
			3:1	39.8440	<0.0001

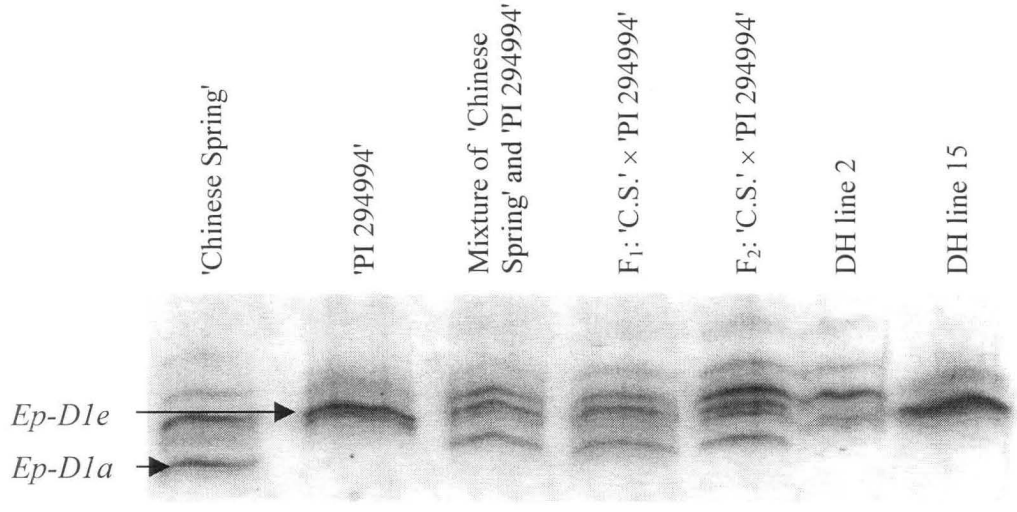

Figure 2. Zymogram of lines used in this study.

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RFLP Analysis

Five RFLP markers on chromosome arm 7DL, *viz. Xpsr105*, *Xpsr129*, *Xpsr165*, *XcsIH81-1* and *XmWG380*, were tested on the two parental lines 'Chinese Spring' and 'PI 294994' and also 'Chinese Spring' nullisomic 7D. None of the markers were polymorphic between the parental lines and could therefore not be used to characterise the mapping population. The results obtained with *Xpsr129* is shown in Figure 3.

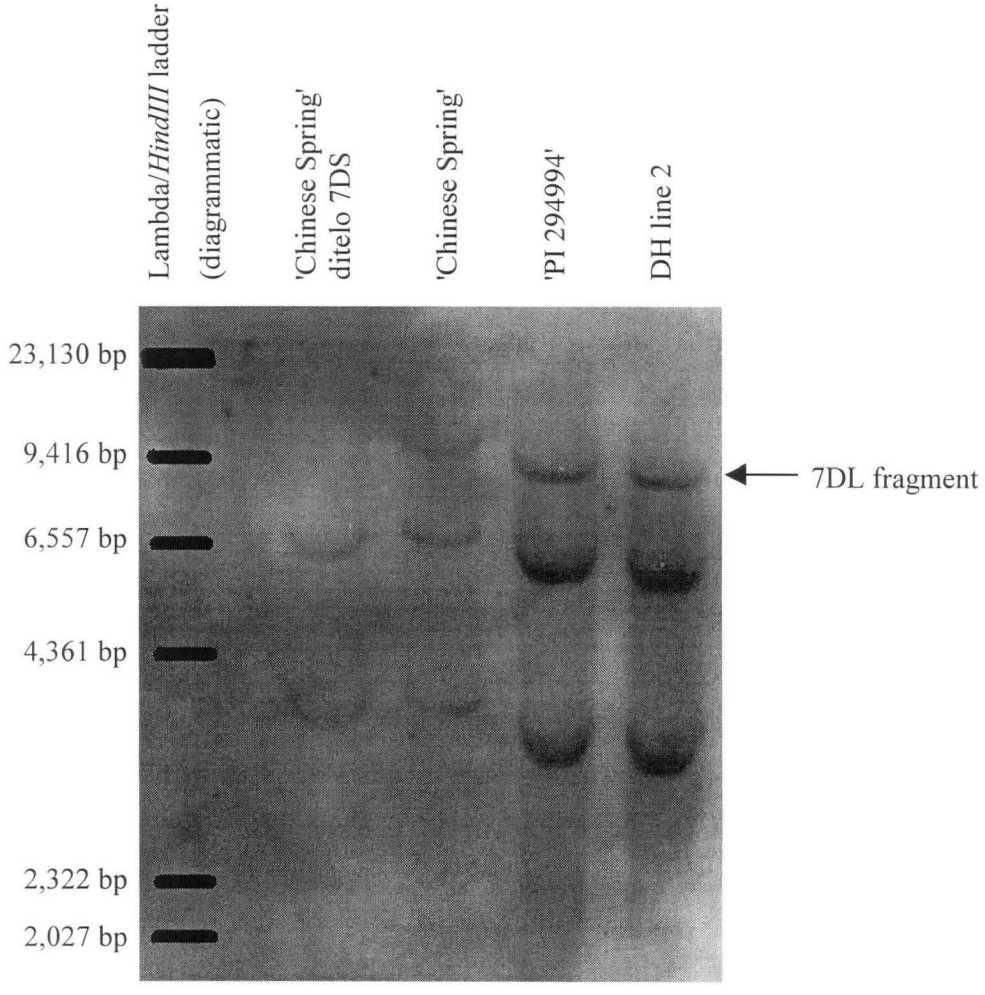

Figure 3. Partial Southern blot hybridised with probe *Xpsr129*.

Microsatellite Analysis

Four microsatellite markers, *viz. Xgwm37*, *Xgwm111*, *Xgwm428* and *Xgwm437* were tested on the two parental lines 'Chinese Spring' and 'PI 294994' and all four microsatellite markers were found to be polymorphic and were subsequently mapped in the doubled haploid population. Marker *Xgwm37* amplified a fragment of 186 base pairs in 'Chinese Spring' and a fragment of

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176 base pairs in 'PI 294994', while *Xgwm111* amplified a fragment of 207 base pairs in 'Chinese Spring' and a fragment of 213 base pairs in 'PI 294994'. For markers *Xgwm428* and *Xgwm437*, the alleles for 'Chinese Spring' and 'PI 294994' were 144 and 140 base pairs; and 108 and 100 base pairs, respectively. The observed segregation data for each marker is shown in Table 6. The microsatellite markers were all found to segregate as single genes (χ^2 = 0.0426 - 2.7234; *P* = 0.0989 - 0.8366). A partial autoradiograph of microsatellite marker *Xgwm111* is shown in Figure 4 and the results of each individual line can be found in Table 8.

Table 6. Segregation of four microsatellite loci in a population of 94 doubled haploid lines.

Number of 'PI 294994' alleles	Number of 'Chinese Spring' alleles	Total number of lines	Ratio tested	Chi square	*P*
Xgwm37					
44	50	94	1:1	0.3830	0.5360
			3:1	39.8440	<0.0001
Xgwm111					
40	54	94	1:1	2.0851	0.1478
			3:1	52.7801	<0.0001
Xgwm428					
48	46	94	1:1	0.0426	0.8366
			3:1	28.7234	<0.0001
Xgwm437					
39	55	94	1:1	2.7234	0.0989
			3:1	56.2979	<0.0001

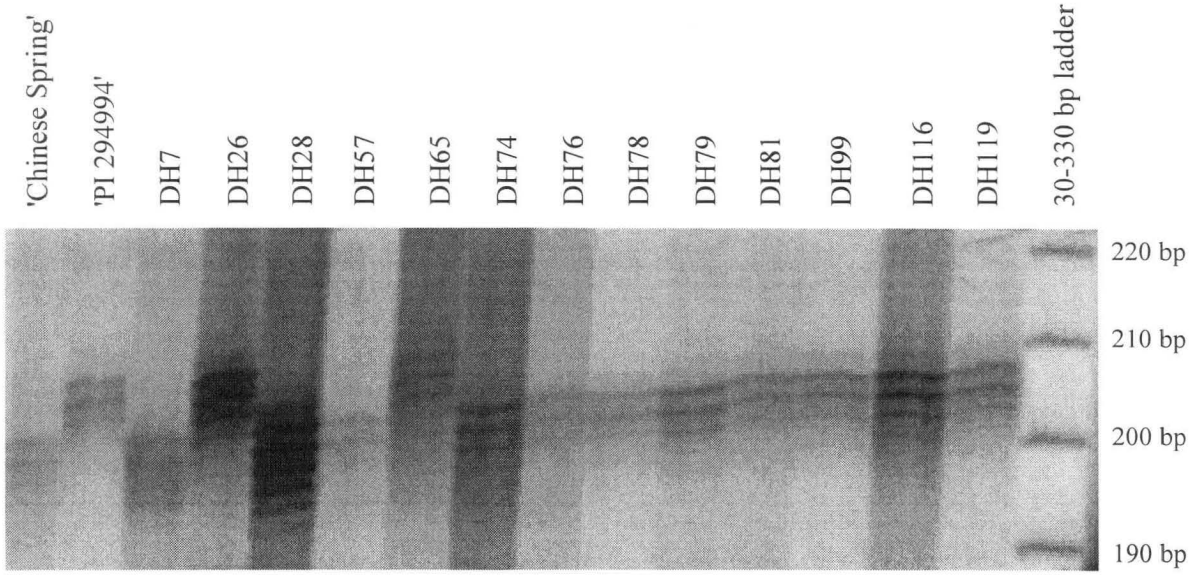

Figure 4. Partial autoradiograph of microsatellite marker *Xgwm111*.

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PCR-RFLP Analysis

The PCR-RFLP markers developed by Venter and Botha (2000) were tested and produced PCR products of the correct size after amplification. The amplification products were digested with *Eco*RI (Promega), as reported by the authors as well as eight other restriction endonucleases (Table 7). It was not possible to produce a polymorphic fragment between the parental lines with any of the restriction endonucleases tested. Lambda DNA was test-digested with the restriction endonucleases and always confirmed that the digestions were successful (Figure 5).

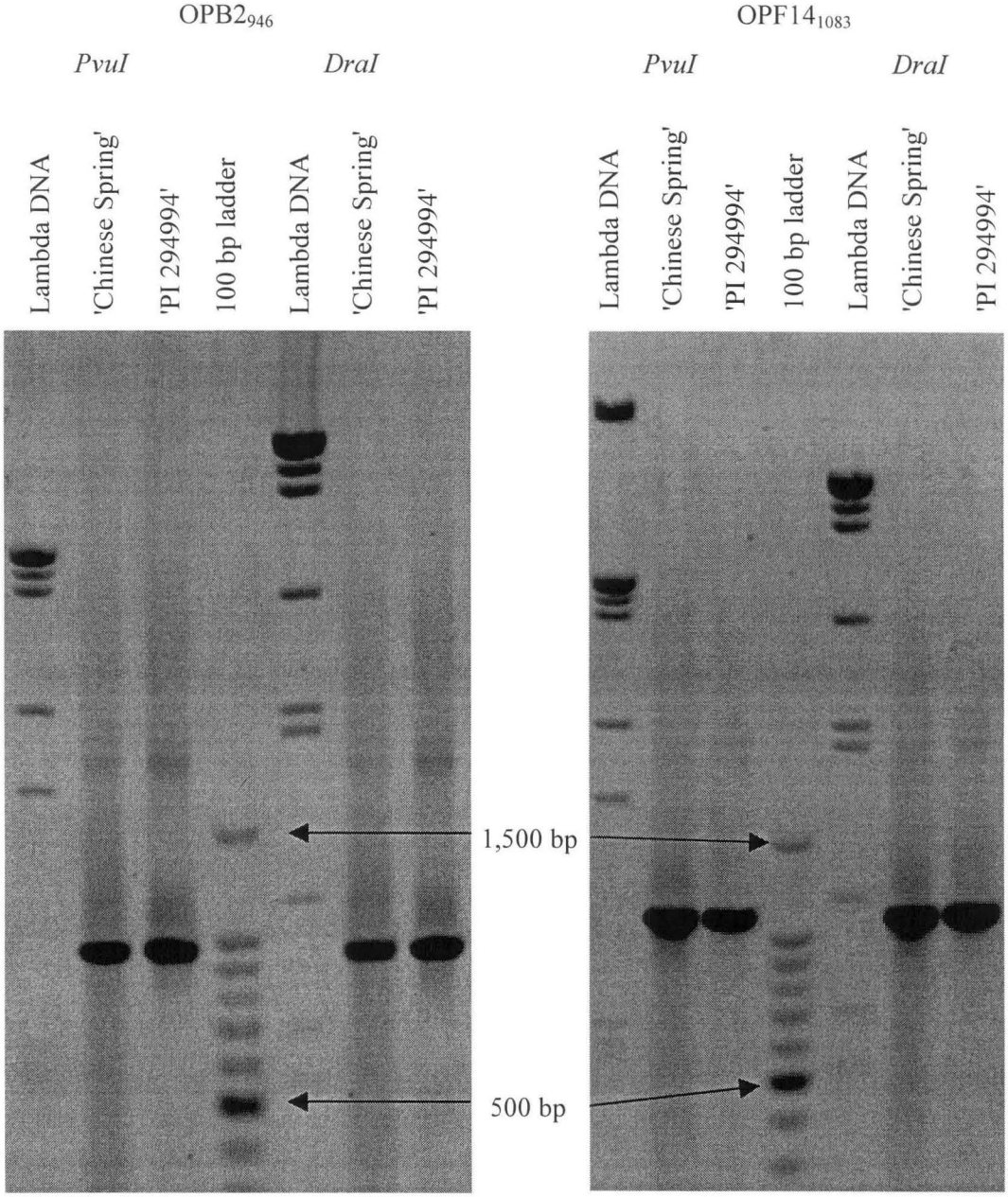

Figure 5. Agarose gel of amplification products of markers $OPB2_{946}$ and $OPF14_{1083}$ after restriction digestion with *PvuI* and *DraI*.

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Table 7. Restriction endonucleases used for PCR-RFLP.

Restriction endonuclease	Manufacturer	Recognition sequence	Number of sites in lambda DNA	Number of sites in OPB2	Number of sites in OPF14
*Bam*HI	Roche	G↓GATCC	5	-	-
*Dra*I	Roche	TTT↓AAA	13	-	-
*Eco*RI	Promega	G↓AATTC	5	-	2
*Hae*III	Roche	GG↓CC	149	3	2
*Hind*III	Roche	A↓AGCTT	6	-	-
*Mse*I	New England Biolabs	T↓TAA	195	1	1
*Pst*I	Roche	CTGCA↓G	28	-	2
*Pvu*I	Roche	CGAT↓CG	3	-	-
*Taq*I	Roche	T↓CGA	121	2	3

Characterisation of the Mapping Population

The entire mapping population was typed for the endopeptidase locus, RWA resistance gene *Dn5* and four microsatellite markers (Table 8). These markers were analysed with Mapmaker /EXP version 3.0b to determine the most likely locus order, pairwise linkage and LOD scores (Table 9). All distances were determined with LOD scores higher than 3.0, except for the distance between *Dn5* and *Xgwm428* where a LOD score was not calculated by the software because the recombination frequency between the two loci was higher than 40%. *Dn5* is linked to *Xgwm111* and *Xgwm437* at distances of 25.4 and 28.6 cM, respectively, but not at all to *Xgwm428*, *Xgwm37* or the endopeptidase locus. The typing data is shown as a diagram in Figure 6 and illustrates recombination events. Fourteen (14.9 %) of the doubled haploid lines have the same haplotype as the susceptible parent 'Chinese Spring', while eleven (11.7 %) have the same haplotype as the RWA resistance donor 'PI 294994'. Most of the remaining doubled haploid lines have evidence of a single recombination event (DH3, 39, 120, *etc.*), while double crossovers have occurred in some (DH9, 31, 67, *etc.*) and triple crossovers in other DH lines (DH93, 95, 85, *etc.*).

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Table 8. Raw data of the markers typed on the individual DH lines.

Line	Loci and alleles											
	Xgwm437		*Xgwm111*		RWA		*Xgwm428*		*Xgwm37*		*Ep-D1*	
	100	108	213	207	*Dn5*	*dn5*	140	144	176	186	e	a
'PI 294994	100		213		*Dn5*		140		176		e	
'C.S.'		108		207		*dn5*		144		186		a
DH2		108		207	*Dn5*		140			186	e	
DH3		108		207	*Dn5*			144		186	e	
DH4		108		207	*Dn5*		140		176		e	
DH6	100		213		*Dn5*			144		186		a
DH7[1]		108		207		*dn5*		144		186		a
DH9		108		207	*Dn5*			144		186		a
DH10	100		213		*Dn5*		140		176		e	
DH12		108		207		*dn5*		144		186		a
DH13	100		213		*Dn5*		140		176			a
DH14		108		207	*Dn5*		140		176			a
DH15		108		207		*dn5*	140		176		e	
DH16	100		213		*Dn5*			144		186		a
DH18	100		213		*Dn5*		140		176			a
DH21		108	213		*Dn5*		140		176		e	
DH22		108		207		*dn5*		144		186		a
DH23		108		207		*dn5*		144		186		a
DH26		108		207		*dn5*	140		176		e	
DH27	100		213		*Dn5*			144		186		a
DH28	100		213		*Dn5*			144		186		a
DH29		108		207		*dn5*		144		186		a
DH31		108		207	*Dn5*			144		186		a
DH32	100		213		*Dn5*			144		186		a
DH34		108		207		*dn5*		144	176		e	
DH35		108		207		*dn5*		144		186		a
DH37		108		207		*dn5*		144		186		a
DH39		108		207	*Dn5*			144	176		e	
DH40		108		207		*dn5*	140		176		e	
DH43*	100		213			*dn5*	140		176		e	
DH45		108		207		*dn5*		144		186		a
DH46	100		213		*Dn5*		140		176		e	
DH47		108		207		*dn5*		144	176		e	
DH48	100		213		*Dn5*		140		176		e	
DH49		108		207		*dn5*	140		176		e	
DH50		108	213		*Dn5*		140			186		a
DH51	100		213		*Dn5*			144		186		a
DH52		108		207	*Dn5*		140		176		e	
DH53		108		207	*Dn5*		140		176		e	
DH54		108		207	*Dn5*		140		176		e	
DH55		108		207	*Dn5*		140			186		a
DH56	100		213		*Dn5*		140			186		a
DH57		108		207		*dn5*	140		176		e	
DH58	100			207		*dn5*		144		186		a
DH59	100		213		*Dn5*			144		186		a
DH60	100		213		*Dn5*			144		186		a
DH62	100		213		*Dn5*		140		176		e	
DH63	100		213		*Dn5*		140		176		e	
DH64		108		207	*Dn5*		140		176		e	
DH65	100		213		*Dn5*		140		176		e	

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Table 8. *Continued.*

Line	Loci and alleles											
	Xgwm437		*Xgwm111*		RWA		*Xgwm428*		*Xgwm37*		*Ep-D1*	
	100	108	213	207	*Dn5*	*dn5*	140	144	176	186	e	a
DH66		108		207		dn5		144		186		a
DH67[1]		108		207	Dn5			144		186		a
DH68	100		213		Dn5		140		176		e	
DH70	100		213		Dn5		140			186		a
DH73		108		207		dn5	140		176		e	
DH74		108		207	Dn5		140		176			a
DH76		108		207		dn5	140		176		e	
DH77	100		213		Dn5			144		186		a
DH78		108		207		dn5	140			186		a
DH79		108		207		dn5	140			186		a
DH81		108		207	Dn5		140			186		a
DH82		108		207		dn5		144		186		a
DH84	100		213		Dn5			144	176		e	
DH85	100		213			dn5	140			186		a
DH86	100		213		Dn5			144		186		a
DH87	100		213		Dn5			144	176		e	
DH88	100		213		Dn5		140		176		e	
DH89		108		207		dn5		144		186		a
DH90		108		207		dn5	140		176		e	
DH91	100		213		Dn5		140		176		e	
DH92		108		207		dn5	140		176		e	
DH93		108		207		dn5	140			186	e	
DH94		108		207		dn5		144		186		a
DH95		108		207		dn5	140			186	e	
DH96[2]	100		213		Dn5			144		186	e	
DH97	100		213		Dn5			144	176		e	
DH99		108		207		dn5		144	176			a
DH100		108		207		dn5		144	176		e	
DH102[2]	100		213		Dn5		140			186		a
DH103	100		213		Dn5		140		176		e	
DH104	100		213		Dn5		140		176		e	
DH105		108		207		dn5		144		186		a
DH108		108		207		dn5	140		176		e	
DH109	100		213		Dn5		140			186		a
DH110[1]	100		213		Dn5			144		186		a
DH111		108		207	Dn5			144		186		a
DH112	100		213			dn5	140			186		a
DH113		108		207	Dn5			144		186		a
DH114	100		213		Dn5			144		186		a
DH116		108		207		dn5		144	176		e	
DH117		108		207		dn5		144	176		e	
DH119		108		207		dn5		144		186		a
DH120		108		207	Dn5			144	176		e	
DH121		108		207		dn5	140		176		e	
DH125	100		213		Dn5		140			186		a
DH129	100		213		Dn5			144		186		a

* only one seedling tested

[1] heterogeneity of resistance in sample (predominant classification taken to be correct)

[2] seedlings could not be scored with certainty

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Line	Loci					
	Xgwm437	*Xgwm111*	RWA	*Xgwm428*	*Xgwm37*	*Ep-D1*
'PI 294994'	100	213	*Dn5*	140	176	e
'C.S.'	108	207	*dn5*	144	186	a
DH58	100	207	*dn5*	144	186	a
DH7	108	207	*dn5*	144	186	a
DH12	108	207	*dn5*	144	186	a
DH22	108	207	*dn5*	144	186	a
DH23	108	207	*dn5*	144	186	a
DH29	108	207	*dn5*	144	186	a
DH35	108	207	*dn5*	144	186	a
DH37	108	207	*dn5*	144	186	a
DH45	108	207	*dn5*	144	186	a
DH66	108	207	*dn5*	144	186	a
DH82	108	207	*dn5*	144	186	a
DH89	108	207	*dn5*	144	186	a
DH94	108	207	*dn5*	144	186	a
DH105	108	207	*dn5*	144	186	a
DH119	108	207	*dn5*	144	186	a
DH9	108	207	*Dn5*	144	186	a
DH31	108	207	*Dn5*	144	186	a
DH67	108	207	*Dn5*	144	186	a
DH111	108	207	*Dn5*	144	186	a
DH113	108	207	*Dn5*	144	186	a
DH3	108	207	*Dn5*	144	186	e
DH39	108	207	*Dn5*	144	176	e
DH120	108	207	*Dn5*	144	176	e
DH34	108	207	*dn5*	144	176	e
DH47	108	207	*dn5*	144	176	e
DH100	108	207	*dn5*	144	176	e
DH116	108	207	*dn5*	144	176	e
DH117	108	207	*dn5*	144	176	e
DH99	108	207	*dn5*	144	176	a
DH78	108	207	*dn5*	140	186	a
DH79	108	207	*dn5*	140	186	a
DH93	108	207	*dn5*	140	186	e
DH95	108	207	*dn5*	140	186	e
DH2	108	207	*Dn5*	140	186	e
DH50	108	213	*Dn5*	140	186	a
DH55	108	207	*Dn5*	140	186	a
DH81	108	207	*Dn5*	140	186	a
DH14	108	207	*Dn5*	140	176	a
DH74	108	207	*Dn5*	140	176	a
DH15	108	207	*dn5*	140	176	e
DH26	108	207	*dn5*	140	176	e
DH40	108	207	*dn5*	140	176	e
DH49	108	207	*dn5*	140	176	e
DH57	108	207	*dn5*	140	176	e
DH73	108	207	*dn5*	140	176	e
DH76	108	207	*dn5*	140	176	e

Line	Loci					
	Xgwm437	*Xgwm111*	RWA	*Xgwm428*	*Xgwm37*	*Ep-D1*
DH90	108	207	*dn5*	140	176	e
DH92	108	207	*dn5*	140	176	e
DH108	108	207	*dn5*	140	176	e
DH121	108	207	*dn5*	140	176	e
DH4	108	207	*Dn5*	140	176	e
DH52	108	207	*Dn5*	140	176	e
DH53	108	207	*Dn5*	140	176	e
DH54	108	207	*Dn5*	140	176	e
DH64	108	207	*Dn5*	140	176	e
DH21	108	213	*Dn5*	140	176	e
DH43	100	213	*dn5*	140	176	e
DH10	100	213	*Dn5*	140	176	e
DH46	100	213	*Dn5*	140	176	e
DH48	100	213	*Dn5*	140	176	e
DH62	100	213	*Dn5*	140	176	e
DH63	100	213	*Dn5*	140	176	e
DH65	100	213	*Dn5*	140	176	e
DH68	100	213	*Dn5*	140	176	e
DH88	100	213	*Dn5*	140	176	e
DH91	100	213	*Dn5*	140	176	e
DH103	100	213	*Dn5*	140	176	e
DH104	100	213	*Dn5*	140	176	e
DH13	100	213	*Dn5*	140	176	a
DH18	100	213	*Dn5*	140	176	a
DH70	100	213	*Dn5*	140	186	a
DH109	100	213	*Dn5*	140	186	a
DH56	100	213	*Dn5*	140	186	a
DH102	100	213	*Dn5*	140	186	a
DH125	100	213	*Dn5*	140	186	a
DH6	100	213	*Dn5*	144	186	a
DH16	100	213	*Dn5*	144	186	a
DH27	100	213	*Dn5*	144	186	a
DH28	100	213	*Dn5*	144	186	a
DH32	100	213	*Dn5*	144	186	a
DH51	100	213	*Dn5*	144	186	a
DH59	100	213	*Dn5*	144	186	a
DH60	100	213	*Dn5*	144	186	a
DH77	100	213	*Dn5*	144	186	a
DH86	100	213	*Dn5*	144	186	a
DH110	100	213	*Dn5*	144	186	a
DH114	100	213	*Dn5*	144	186	a
DH129	100	213	*Dn5*	144	186	a
DH85	100	213	*dn5*	140	186	a
DH112	100	213	*dn5*	140	186	a
DH96	100	213	*Dn5*	144	186	e
DH84	100	213	*Dn5*	144	176	e
DH87	100	213	*Dn5*	144	176	e
DH97	100	213	*Dn5*	144	176	e

Figure 6. Graphic representation of typing data on the doubled haploid mapping population.

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Table 9. Mapping results generated by (i) Mapmaker /EXP version 3.0b and (ii) calculated by hand.

Name of Locus	Mapmaker /EXP Distance*	LOD	Distances* calculated from recombination frequencies[1]
Xgwm437			
	3.2	45.05	3.2
Xgwm111			
	25.4	12.17	24.0
Dn5			
	72.0	<0.5	77.8
Xgwm428			
	31.2	8.45	31.1
Xgwm37			
	10.8	28.92	10.8
Ep-D1			

* Distance in Kosambi centiMorgans

[1] Recombination frequencies were determined by hand

The most likely map order of the loci (Figure 7) was determined by Mapmaker /EXP to be *Xgwm437* - 3.2 cM - *Xgwm111* - 25.4 - *Dn5* - 72.0 - *Xgwm428* - 31.2 - *Xgwm37* - 10.8 - *Ep-D1*. Except for markers *Xgwm437* and *Xgwm111* that are swapped around, the order of the microsatellite markers is as published by Röder *et al.* (1998). However, the genetic distance between *Xgwm437* and *Xgwm111* is calculated to be much less in this DH mapping population than the population used by Röder *et al.* (1998).

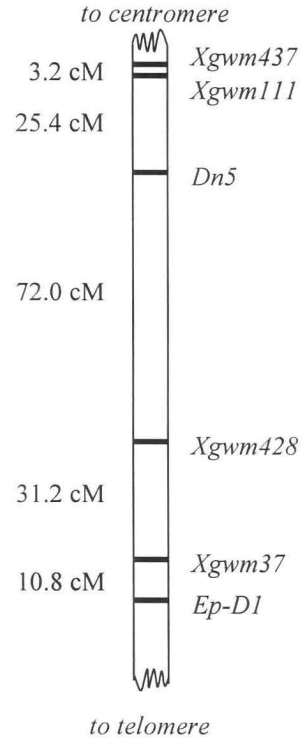

Figure 7. Mapping order and distances determined for the loci used in this study.

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AFLP analysis of the homozygous F_2 lines

Two *Sse*8387I +1, two *Sse*8387I +2, four *Sse*8387I +3, eight *Mse*I +2 and four *Mse*I +3 AFLP primers in a total of 70 primer combinations were used to screen a population consisting of 'PI 294994', 'Chinese Spring', a bulk consisting of 12 homozygous resistant lines (lines 2, 6, 11, 17, 18, 19, 23, 27, 28, 31, 33 and 38) and a bulk consisting of 7 susceptible lines (lines 5, 7, 13, 21, 25, 34 and 37) (Tables 1 and 3). Potentially useful polymorphisms were tested on all nineteen individual homozygous lines. Only two potentially useful polymorphisms (one in coupling and one in repulsion phase) were identified from the seventy primer combinations tested (Table 10). The repulsion phase marker was identified using primer combination 97 (S02+C / M32+AAC) and not only amplified fragments in all susceptibles, except line 37, and in resistant lines 2, 11 and 38 (three of the twelve resistant lines). The coupling phase marker was amplified using primer combination 138 (S04+T / M90+TGT) and was present in seven (lines 2, 18, 19, 23, 27, 28, 33 and 38) of the twelve resistant lines, and in susceptible lines 5, 13 and 34 (three of the seven susceptible lines). Even though the data were not suggestive of tight linkage with RWA resistance, an attempt was made to convert this marker into a sequence-specific marker (Figure 8 and 9). The three clones with the correct insert size of the ten checked were sequenced and all three contained the same insert. Both the *Mse*I and *Sse*8387I primer sequences were present. Primers were designed from the clone sequence to amplify the isolated fragment. The forward primer designed was 5' - AGT GAG AGC TAG CAG TCG AG - 3' and the reverse primer 5' - CTA GTG GAG GCT TAG GTA TG - 3'. After optimisation, a fragment of the correct size (approximately 80 bp) was amplified in both parental lines. As this marker was not polymorphic in the parental lines, it was additionally tested on the lines 'Chinese Spring' nullisomic 7D, 'Chinese Spring' ditelosomic 7DS, 'Indis', 'Inia 66', 'W84-17' and 'VPM1'. A fragment of the same size as the parental allele was amplified in all lines tested, even in 'Chinese Spring' nullisomic 7D (Figure 9).

Table 10. Results of AFLP analysis of the Russian wheat aphid resistance gene, *Dn5*.

Combination	Fragment size (bp)	Present in lines	Linkage phase
138	140 - 150	Resistant lines 2,18,19,23,27,28,33 and 38; also in susceptible lines 5,13 and 34	Coupling
97	220 - 230	All susceptible lines but 37; also in resistant lines 2,11 and 38	Repulsion

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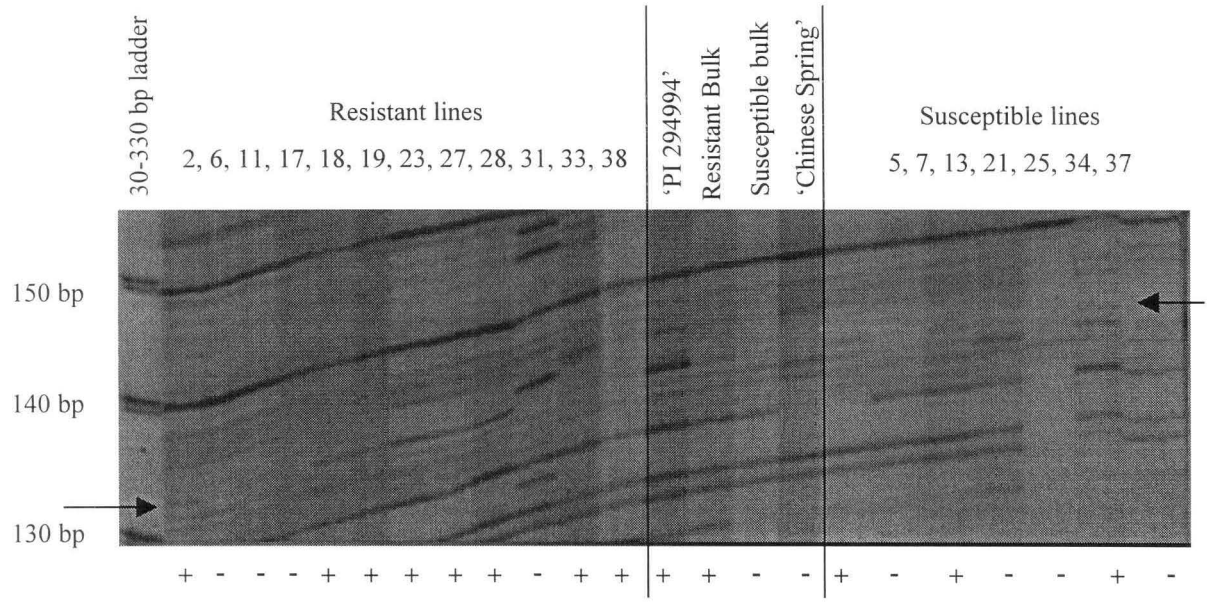

Figure 8. Partial AFLP fingerprint showing the segregation of the AFLP marker targeted for conversion to a sequence-specific marker.

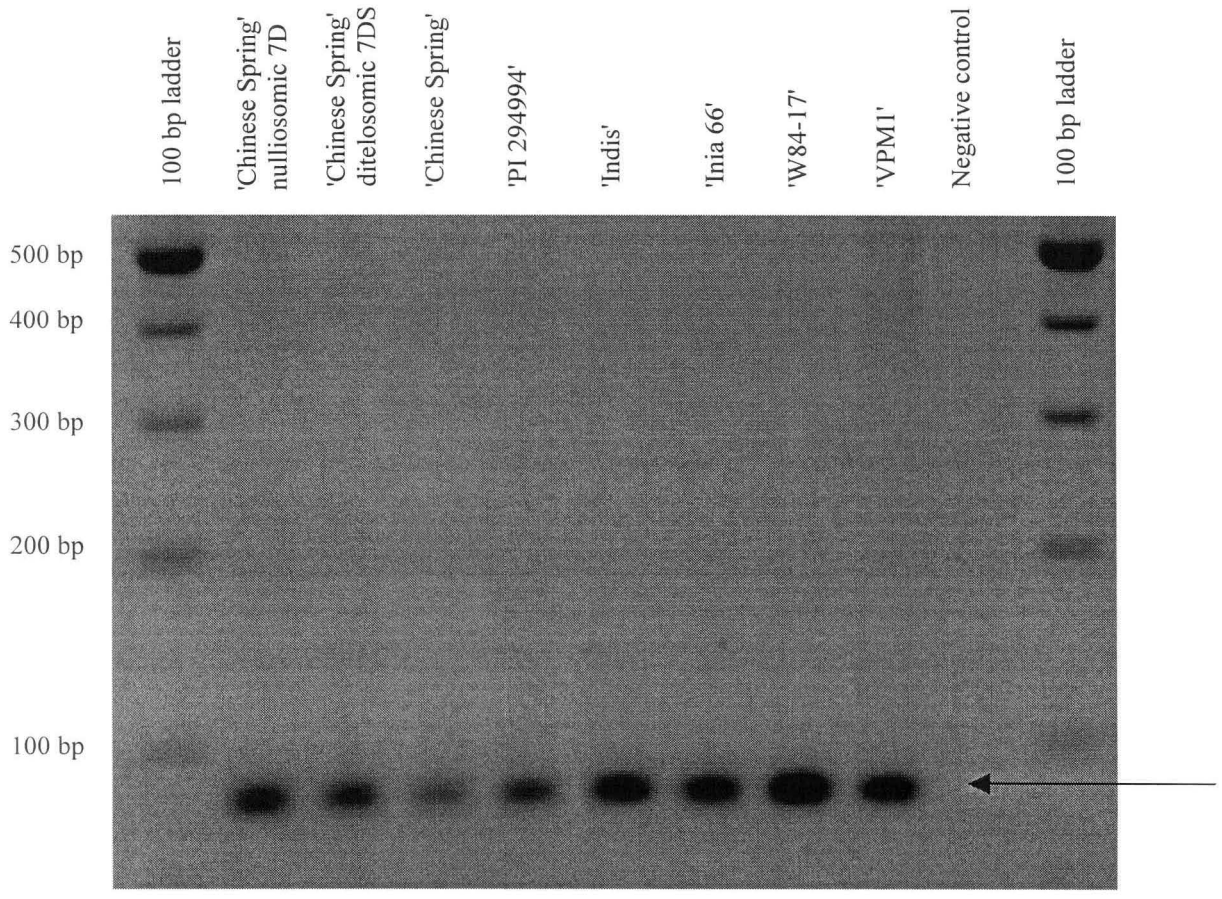

Figure 9. Amplification products of the converted AFLP marker on an agarose gel.

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DISCUSSION

The primary goals of this study were to establish a F_1-derived doubled haploid mapping population and to attempt the mapping of Russian wheat aphid resistance gene *Dn5*. A total of 94 doubled haploid plants were recovered and typed for traits and markers on chromosome arm 7DL of common wheat. This population was only typed for *Dn5*, the endopeptidase locus and four microsatellite markers, but should also be useful for mapping other important traits on any other chromosome as the parents of the cross differed considerably in phenotype and should segregate at numerous loci. Secondary to the main goals, a F_2-derived F_3 population was subjected to AFLP analysis in an attempt to identify novel molecular markers associated with *Dn5*.

Doubled haploid mapping population

Either an embryo, an endosperm or both can be expected in 20 - 30% of wheat florets pollinated with maize pollen (Laurie and Bennett, 1988) and approximately 60% (20.1 % of pollinated florets) of the embryos can develop into seedlings (Laurie and Reymondie, 1991). However, the percentage of florets producing embryos can be as low as 10.3%, of which 13.8% (1.4% of pollinated florets) can develop into plantlets (Kisana *et al.*, 1993). The statistics reported by Kisana *et al.* (1993) were based on crosses that included, amongst others, the RWA resistant parent 'PI 294994' used in this study and can therefore be useful for comparison with the statistics for the population developed here. In the present study, 1,045 embryos were excised from 15,249 florets (6.9% of pollinated florets), of which 9.0% survived to mature doubled haploid plants (0.62% of pollinated florets). The lower success rate achieved can be ascribed to the relative inexperience of this researcher with the procedure. One of the haploid lines, *viz.* DH111, had 22 chromosomes prior to doubling, which might have been caused by a centromeric break producing two telocentrics (Laurie and Reymondie, 1991).

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Mapping the DH population

The low levels of polymorphism found in cultivated wheat makes it difficult to find suitable markers to map a specific wheat chromosomal region (Kam-Morgan *et al.*, 1989). Chao *et al.* (1989) used 18 group 7 cDNA probes to screen six wheat varieties digested with thirteen restriction endonucleases and could only detect polymorphisms in 8.7% of the comparisons. It is therefore not unexpected that RFLP screening of the parental genotypes in this study using the most commonly used restriction endonucleases did not result in the detection of any polymorphisms. Even though the chances of identifying polymorphic probes are low and the screening process tedious, RFLP markers are often established framework markers that might be useful for comparative mapping. However, all four microsatellite markers tested were polymorphic and could therefore be used for typing the DH mapping population. The existing genetic maps of chromosome arm 7DL show poor resolution: currently only a small number of microsatellite markers map on this chromosome arm, but the increased chance of an SSR marker being polymorphic between any given lines make acquisition of these markers worthwhile. Another alternative towards extending the genetic map is to screen for AFLP markers, a technique which facilitates sampling of a larger number of loci in a single round than would ever be possible with current RFLP technology. An advantage of a doubled haploid population is that dominant marker types such as AFLPs can be as informative as the co-dominant type of most RFLPs (Voorrips *et al.*, 1997).

In a doubled haploid population, segregation ratios of 1:1, 3:1 or 15:1 are caused by segregation of one, two or three loci, respectively (Virk *et al.*, 1998). From the Chi square analyses of the RWA seedling data (Tables 2 and 4) and segregation of marker loci (Tables 5 and 6), it appears as if a Mendelian segregation ratio of 1:1 fits the observed segregation patterns. This confirms the segregation of a single dominant resistance gene, called *Dn5*, in 'PI 294994' that has previously been reported using the same parental material as that used in this study (Marais and Du Toit, 1993). However, heterogeneity in the original 'PI 294994' germplasm has been noted (Zhang *et al.*, 1998). Various authors using different variants of 'PI 294994' reported different mechanisms of resistance, for example a dominant allele at one locus and a recessive allele at a second locus (Elsidaig and Zwer, 1993; Dong and Quick, 1995); and allelism between the genes in 'PI 294994' and *Dn1*, *Dn2, Dn4* and *Dn6* or the presence of two dominant genes (Saidi and Quick, 1994 and 1996).

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RAPD markers that were converted to sequence specific markers by Venter and Botha (2000) were also tested on this mapping population. These authors used near-isogenic lines and F_2 mapping populations of crosses between a susceptible South African cultivar 'Palmiet' and the *Dn5* donor 'PI 294994' (SA463) to identify RAPD markers in repulsion and coupling phase with RWA resistance. Two RAPD markers, *viz.* $OPB2_{946}$ in repulsion phase and $OPF14_{1083}$ in coupling phase, were cloned, sequenced and sequence-specific primers were designed (Venter and Botha, 2000). The primer sets of Venter and Botha (2000) were tested on the parental lines used in this study, test digested using nine restriction endonucleases and checked on an agarose gel. No polymorphisms were found for either of the converted RAPD markers tested with any of the restriction endonucleases. Digesting the converted $OPF14_{1083}$ marker with *EcoRI* yielded a repulsion phase marker in the material used by Venter and Botha (2000) and it is therefore not surprising that it was not repeatable in our DH population; which used a different susceptible line. This is also true for the converted repulsion phase marker $OPB2_{946}$. The lack of polymorphism using a PCR-RFLP marker developed for another mapping population raises the concern that such converted RAPD markers might often only be useful in material with the same parents in the pedigree as those used in the original population in which the marker was identified.

The *Dn5* locus was mapped 72.0 cM proximally of *Xgwm428* and 25.4 cM distally from *Xgwm111*. Marais *et al.* (1998) reported a recombination percentage of 32 ± 4.97 between *Dn5* and *Ep-D1* whereas the two loci were not linked in this study. It must, however, be noted that the *Ep-D1b* allele typed by Marais *et al.* (1998) appeared to be transmitted preferentially, which could have affected the estimate. Also, the *Ep-D1b* allele is located on a *Triticum ventricosum*-derived chromosome segment and might therefore be surrounded by an area of recombinationally less active foreign chromatin that could have resulted in an underestimation of the recombination frequency (Marais *et al.*, 1998). It is therefore believed that the genetic distances reported in this study are more accurate estimates of the true map distances.

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AFLP analysis of the F_2 population and marker conversion

AFLP analysis is an approach that can be followed to identify markers associated with a gene of interest. The principles of the AFLP system allows that the addition of more selective nucleotides on the 3' end of the *Sse*8387I or *Mse*I primers used for selective PCR amplification will decrease the complexity of the fingerprint as the selectivity of the primers increase. In the present study, twelve *Mse*I+2 primers in 36 of a possible 96 combinations with *Sse*8387I+1, *Sse*8387I+2 and *Sse*8387I+3 selective primers were used for the selective amplification of fragments, as well as six *Mse*I+3 primers in 34 of a possible 48 combinations with *Sse*8387I+1, *Sse*8387I+2 and *Sse*8387I+3 selective primers. Two polymorphisms possibly linked to *Dn5* were identified using the latter 34 combinations, probably due to the decreased complexity of the fingerprint revealing more 'hidden' polymorphisms than with the *Mse*I+2 primer combinations.

Probable reasons for the small number of linked polymorphisms (0.029 useful polymorphisms per combination over all combinations tested) found in this study can be:

i) an insufficient number of selective primer combinations was screened - screening of more primer combinations might improve the recovery of useful markers, *e.g.* Bai *et al.* (1999) screened 300 *Eco*RI/*Mse*I primer combinations (15 *Eco*RI and 20 *Mse*I selective primers) and identified eleven markers associated with a major QTL controlling scab resistance in wheat from twenty primer combinations (0.037 polymorphisms per combination).

ii) hyper/hypo-methylation of the region containing *Dn5* might influence the effectiveness of the restriction endonuclease combination used in AFLP analysis - *e.g.* the methylation-sensitive combination of *Pst*I/*Mse*I was used to locate the *Cf-9* gene (resistance to *Claudosporium fulvum*) in euchromatin (Thomas *et al.*, 1995) while the methylation-insensitive combination *Eco*RI/*Mse*I located the *Mi* gene (resistance to the root-knot nematode) in tomato heterochromatin (Kaloshian *et al.*, 1998).

iii) *Dn5* is a "native" wheat gene which might make it more difficult to tag - genes located in large blocks of alien or introgressed chromatin (*e.g. Lr19* - Chapter 2; Marais *et al.*, 2001; Prins *et al.*, 2001) or genes that are spanned by a deletion (*e.g. ph1b* - Qu *et al.*, 1998), may be more amenable to AFLP analysis.

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The unsuccessful conversion of an AFLP marker into a sequence-specific marker in this study is disappointing and may be the result of the following: (i) The small size of the marker - Schwarz *et al.* (1999) eliminated two of three AFLP markers associated with powdery mildew resistance as candidates for conversion to sequence-specific because of their sizes (101 and 151 base pairs, respectively); (ii) A band of varied intensity was present in susceptible lines and the AFLP marker derived in this study amplified a product in 'Chinese Spring' nullisomic 7D. It is therefore possible that the amplified sequence is homoeoallelic for the three genomes of common wheat or not located on chromosome 7DL; (iii) The sequence from which the primers has been designed were identical for all three clones sequenced. Possibly, the inclusion of clones from other resistance associated fragments of the same AFLP marker in other resistant lines, or simply the sequencing of more clones with the correct insert size, might have improved the chances of obtaining a useful marker. However, because of the reasons listed above, the conversion of this fragment was not further pursued. Only a small number of successful conversions of AFLP markers into sequence-specific markers (discussed in Chapter 2) have been reported (Bradeen and Simon, 1998; Qu *et al.*, 1998; Shan *et al.*, 1999; Raemon-Büttner and Jung, 2000). The loose association between the AFLP marker and *Dn5*, as well as the small number of AFLP markers identified, did not rationalise an attempt to transfer this marker to the DH population.

Conclusions

Typing a DH population with four microsatellite markers and an isoenzyme marker made it possible to assign a putative map position for the Russian wheat aphid resistance gene, *Dn5*. It is believed that the DH population is diverse enough to allow more precise mapping of *Dn5*. Although a putative map position for *Dn5* has been determined in this study, marker assisted selection requires the tagging of a gene with a closely associated marker or a marker within the gene of interest. Therefore, more markers that occur between the microsatellite markers *Xgwm111* and *Xgwm428* should be found to fine-map *Dn5*. Unfortunately, none of the RFLP markers tested was useful for mapping *Dn5* and AFLP analysis failed to provide further markers suitable for tagging *Dn5*.

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CHAPTER FOUR

A NOVEL AFLP-DERIVED MICROSATELLITE SEQUENCE IS LINKED TO THE *PCH1* AND *EP-D1* LOCI IN COMMON WHEAT

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Abstract

AFLP markers were identified that are linked with the *Aegilops ventricosa* derived chromosome segment in VPM1 on which the eye spot resistance gene, *Pch1*, and the endopeptidase gene, *Ep-D1b*, occur. One of the markers thus identified was isolated from the gel, cloned and sequenced. Sequence analysis revealed a microsatellite repeat motif in the cloned fragment. Sequence-specific primers were designed to amplify a product containing the repeat motif and the microsatellite marker was tested for co-segregation with the *Ep-D1b* allele. Distinct amplification products were produced by the *Pch1* sources, normal wheat and wheat containing the *Lr19* translocation. A recombination frequency of 0.02 was observed between the novel microsatellite marker and the endopeptidase locus *Ep-D1*.

Key words Endopeptidase, eye spot resistance, genetic mapping

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INTRODUCTION

Eyespot (also called strawbreaker foot rot) of common wheat (*Triticum aestivum*) is caused by the necrotrophic fungus *Pseudocercosporella herpotrichoides* (Fron.) Deighton and is a major disease of wheat, resulting in yield losses of up to 50% (Fitt *et al.*, 1990). It has a wide host range among small grain cereals and grasses (Murray, 1992) and in wheat only three resistance genes (*Pch1* to *Pch3*) have been reported. *Pch1* derives from *Aegilops ventricosa* and has been transferred to hexaploid wheat in two independent studies (Mena *et al.*, 1992). Firstly, *Pch1* is present in VPM-1, derived from a cross between an amphiploid (*Ae. ventricosa* × *T. persicum*) and *T. aestivum* cv 'Marne' (Maia, 1967). *Pch1* has been mapped to chromosome arm 7DL (Jahier *et al.*, 1978; Doussinault *et al.*, 1983; Hollins *et al.*, 1988). Close linkage between *Pch1* and the endopeptidase allele, *Ep-D1b,* has been noted by Worland *et al.* (1988) with both genes residing on the introgressed segment. *Pch1* also occurs among a number of wheat lines (H-93-1 through H-93-70) obtained by repeated selfing of the progeny of a *T. turgidum*/*Ae. ventricosa*//hexaploid wheat hybrid (Mena *et al.*, 1992). Using isozyme and DNA markers, Mena *et al.* (1992) showed that all the resistant H-93 lines also carried the *Ep-D1b* marker that occurs on the long arm of chromosome $7D^V$. With one exception (H-93-51), the *Ep-D1b* marker was absent in the susceptible lines. H-93-51 was found to have 7D substituted by a $7M^V$ $7D^V$ 7D recombinant chromosome of which the $7D^V$ region included *Ep-D1b* but not the more distal *Pch1* locus. Thus, the tight linkage between *Pch1* and *Ep-D1b* had been broken during meiosis in the interspecific hybrids through recombination of chromosomes 7D and $7D^V$.

Due to its more effective resistance, *Pch1* has been used more widely than *Pch2*, a common wheat gene that occurs on chromosome 7A of Cappelle-Desprez (Doussinault, 1973; Allan *et al.*, 1989 and 1990). However, even in the presence of both *Pch1* and *Pch2*, severe eyespot infections can still result in substantial yield loss (Hollins *et al.*, 1988). *Pch3* occurs on chromosome 4V of *Dasypyrum villosum* and its disomic addition to 'Chinese Spring' (Murray *et al.*, 1994). Resistance has also been found in other *Triticum*, *Aegilops*, *Secale* and *Hordeum* species (Sprague, 1936; Macer, 1966).

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Marais (1992) recovered a set of twenty-nine homozygous deletion mutants of the *Lr19* translocation that proved to be extremely useful for physical mapping of the area. Using the material, Marais *et al.* (2001) showed the approximate positions of 18 loci in the translocated area and could also determine the relative sizes of five allosyndetic recombinants of *Lr19* induced through the use of the *ph1b* mutant.

Ep-D1b is a useful co-dominant marker for the presence of *Pch1* and its detection involves a simple and rapid assay, however, in segregating progeny it is often difficult to distinguish it from certain commonly occurring *Ep-A1* and *Ep-B1* alleles (Koebner *et al.*, 1988). An attempt was therefore made to find a more specific DNA marker for routine use in selection programmes. In this paper, the identification of AFLP markers linked to the *Ep-D1b* allele and the VPM1 gene and the conversion of one of these into a simpler and easier to use PCR-based screening system are reported.

MATERIALS AND METHODS

Ten wheat genotypes (Table 1), each homozygous for the confirmed presence or absence of *Pch1* and/or *Ep-D1b* were used for the identification of linked AFLP markers. One of the polymorphic fragments obtained was used to derive a converted marker which was then confirmed on a subset of the lines in Table 1 (numbers 2, 4, 6, 7 and 8). Linkage of the converted marker with the *Ae. ventricosa*-derived *Ep-D1b* allele was studied making use of a testcross population which was derived by pollinating 'Chinese Spring' nullisomic 7D (lacking *Ep-D1*) with the F_1 plants of the crosses: 'VPM1'/6*'W84-17'//*Lr19*/6*'Inia 66' and 94M116': 'VPM1'/3*'W84-17'//'Palmiet'. Both male populations were heterozygous *Pch1pch1*, *Ep-D1aEp-D1b*. Twenty days after pollination the testcross seeds were removed, the embryos excised and placed on embryo rescue medium (Bajaj, 1990) and the green caryopses frozen for endopeptidase determination. Plantlets obtained from the rescued embryos were transplanted to a greenhouse and used for DNA extraction and evaluation with the newly developed marker.

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Table 1. Homozygous genotypes and near-isogenic lines used to search for AFLP polymorphisms linked to *Aegilops ventricosa* derived chromatin (+ and - indicate presence or absence of the respective allele).

No.	Line number/ Pedigree	*Ae. ventricosa* allele	
		Pch1	*Ep-D1b*
1	94M42R-1 = 'VPM1'/6*'W84-17'	?	+
2	'VPM1'	+	+
3	92M166R-1 = 'VPM1'/3*'W84-17'	+	+
4	'W84-17' = 'Inia'/5/'El Gaucho'/'Son64'/4/Tg/3/'Son64'//'Tzpp'/'Nai60'	-	-
5	92M166R-2 = 'VPM1'/3*'W84-17'	+	+
6	94M42S = 'VPM1'/6*'W84-17'	?	-
7	94M42R-2 = 'VPM1'/6*'W84-17'	?	+
8	94M43R = 'VPM1'/5*'W84-17'//'SST66'/4*'W84-17'	?	+
9	'PI 511676' = 'Madsen'	+	?
10	'Chinese Spring' nullisomic 7D	-	-

DNA Extraction

Total genomic DNA was isolated from young seedlings as described in the relevant section of Chapter 2.

Amplified Fragment Length Polymorphisms

Screening of the genotypes for suitable AFLP markers (Table 2) was performed by Mrs A.S. Marais as described in the relevant section of Chapter 2 using the protocol of Donini *et al.* (1997), as modified from Vos *et al.* (1995). An AFLP marker was converted by a Honours student (Mr R. Bonthuys) under my supervision from AFLP reactions containing the polymorphic fragment (genotypes 2 and 7 in Table 1), as well as a reaction with the fragment absent (genoype 4 in Table 1), as outlined in the relevant section of Chapter 2. The isolated fragments were purified and the fragments isolated from each of the two resistant lines (genotypes 2 and 7) were cloned using the pGEM-T Easy vector system (Promega) according to the manufacturer's instructions. Eight transformed colonies from each transformation were tested for correct insert size, three of which were subsequently used for plasmid isolation, automated sequencing and primer design. PCR conditions for the primers were optimised and tested on the lines listed in Table 1 by a Honours student (Miss M. Fourie) under my supervision.

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Table 2. *Sse*8387I and *Mse*I adaptor-specific oligonucleotides used as selective primers in this study.

*Mse*I	*Sse*8387I primers					
Primers	S04 +T	S12 +AC	S27 +AGA	S28 +AGC	S29 +AGG	S30 +AGT
M32 + AAC	*98*	*99*	*101*[1]	*102*	*103*	*104*
M53 + CCG	*106*	*107*	*109*[1]	*110*[1]	*111*[2]	*112*
M64 + GAC	*114*	*115*	*117*	*118*[1]	*119*[1]	*120*
M74 + GGT	*122*	*123*	*125*	*126*[1]	*127*[2]	*128*
M88 + TGC	*130*	*131*	*133*	*134*[1]	*135*[2]	*136*
M90 + TGT	*138*	*139*	*141*	*142*[1]	*143*[1]	*144*[1]

The primers are labeled in such a way that the first three characters represent the primer identifier, followed by the selective nucleotides. For example S04+T represents Sse adaptor specific primer number S04 and the selective nucleotide that followed the common adapter sequence was a 'T'. The number written in italics in the body of the table designates a specific primer combination. For example, 'combination 98' will use primers S04 and M32. Darker shaded blocks indicate all combinations that were tested and lighter shaded blocks primer combinations that were tested and found to be polymorphic. The superscript number indicates the number of polymorphisms amplified by the specific primer combination.

Isoenzyme Screening

Extracts for endopeptidase analysis were made from frozen endosperm sections according to Marais and Marais (1990), as described in the relevant section of Chapter 3.

Screening with the Microsatellite Marker

The marker was amplified in a total reaction volume of 25 µl consisting of 20-50 ng gDNA, 12.5 pmol of each primer, 50 µM of each dNTP, 1 unit of *Taq* polymerase (Bioline), 1× PCR reaction buffer and 2.5 mM $MgCl_2$. The PCR profile consisted of denaturation at 94 °C for 3 minutes, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, followed by final extension for 5 minutes at 72 °C and a soak temperature of 4 °C. The amplification reaction was size-fractionated on a 2-3% w/v agarose gel (1× TBE buffer, approximately 5 hours at 100 V) and stained with ethidium bromide.

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RESULTS

Six *Sse*8387I +3 and six *Mse*I +3 AFLP primers (Table 2) in a total of 36 primer combinations were used to screen the population listed in Table 1 for markers associated with the *Ae. ventricosa* derived chromatin. Thirteen primer combinations yielded sixteen polymorphic markers of which three (two of which are shown in Figure 1) were closely associated with *Pch1* and *Ep-D1b*: a fragment smaller than 120 bp amplified with combination 135 (S29+AGG/M88+TGC) and two fragments of approximately 480 bp each amplified with combinations 142 (S28+AGC/M90+TGT) and 144 (S30+AGT/M90+TGT), respectively. The size and distinctness of the larger fragment produced by combination 144 made it more suitable for cloning and it was therefore selected for conversion into a sequence-specific marker. The combination 135 marker was too small to guarantee successful conversion.

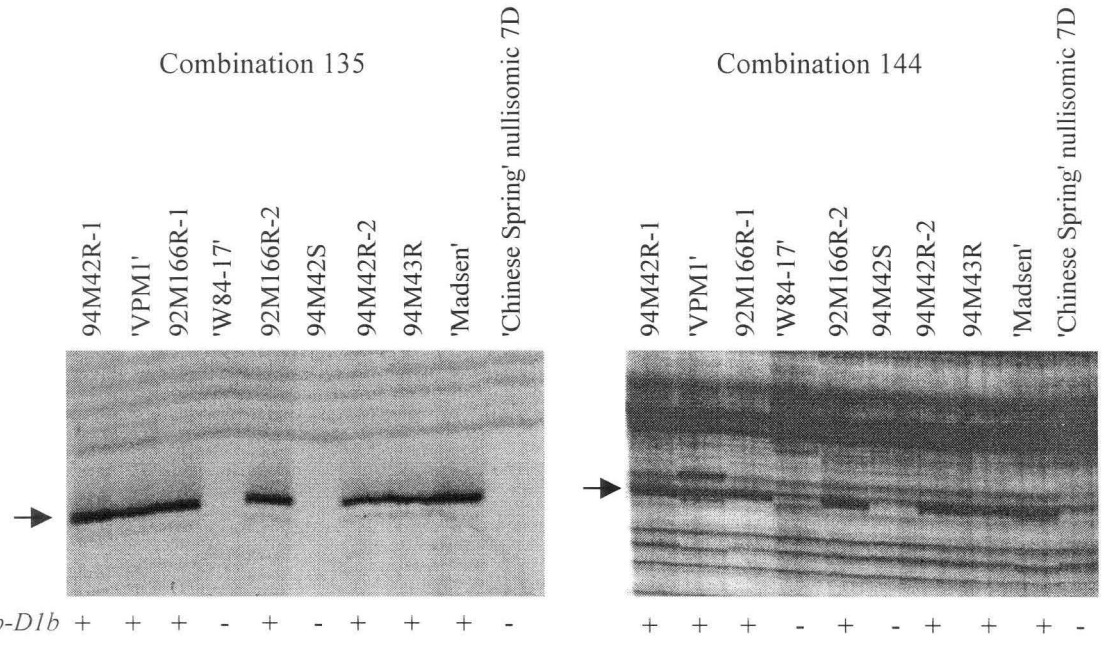

Figure 1. AFLP polymorphisms detected with selective primer combinations 135 and 144.

Blue-white screening was used to identify transformed colonies and the inserts of eight of the white colonies from both the fragment of genotype 2 and genotype 7 (16 colonies in total) were size-fractionated along with the original AFLP fingerprint. Plasmid extractions were done on two of the clones from genotype 7 and one clone from genotype 2. All three clones were sequenced and contained the same insert sequence and both the *Mse*I and *Sse*8387I primer sequences were present. The insert was found to contain a $(AG)_n$ core motif of approximately 70 bp in length.

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Primers were designed to amplify the microsatellite motif and flanking sequences. The new SSR (short simple repeat / microsatellite) locus was designated *XustSS30M90*$_{AG240}$. Its forward and reverse primer sequences are 5' - CAT CGT GTG GCC AAC TTG TT - 3' and 5' - TTC CTC GTG TCT AGT GTC TC - 3', respectively. After optimisation, a fragment of 240 bp co-segregated in resistant lines, while a fragment of 222 bp co-segregated in susceptible lines (Figure 2). An evaluation of 36 testcross progeny from the cross between 'Chinese Spring' nullisomic 7D and F_1:94M117 and 62 progeny from the cross between 'Chinese Spring' nullisomic 7D and 94M116 were tested for the presence of *Ep-D1a* and *XustSS30M90*$_{AG240}$. It was found that 47 plants received the *Ep-D1a* ('Chinese Spring') allele whereas 51 plants did not receive this allele (Figure 3) while half of the plants received the 'Chinese Spring' and the other half the VPM1 microsatellite allele (Table 3). A recombination frequency of 2% was calculated between *XustSS30M90*$_{AG240}$ and *Ep-D1*.

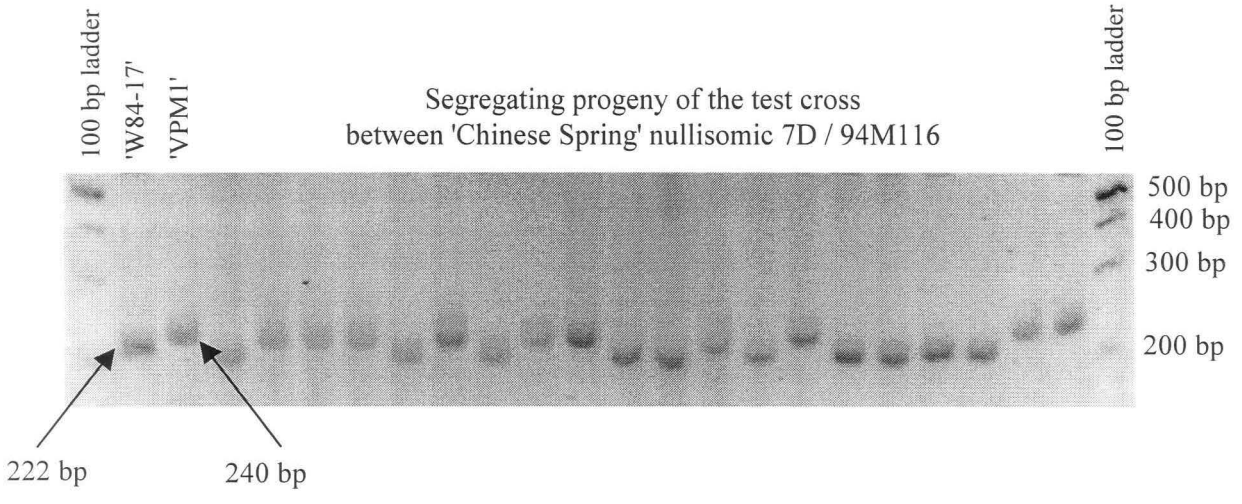

Figure 2. Amplification products of the converted AFLP marker on an agarose gel.

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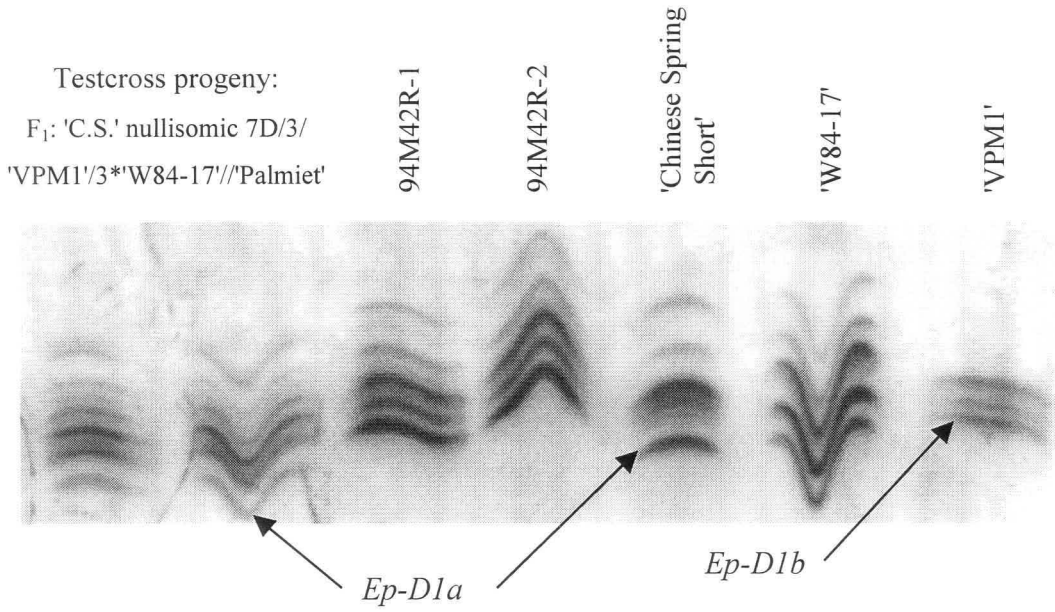

Figure 3. Endopeptidase zymogram of some of the genotypes used.

Table 3. Summarised results and chi-square analysis of the endopeptidase and microsatellite profiles.

Description	Number of lines
Allele combination	
Ep-D1b, VPM1-*XustSS30M90*$_{AG240}$	49
Ep-D1b, CS-*XustSS30M90*$_{AG240}$	2
Ep-D1a, VPM1-*XustSS30M90*$_{AG240}$	0
Ep-D1a, CS-*XustSS30M90*$_{AG240}$	47
Totals:	98
Ep-D1b (VPM1)[1]	51
Ep-D1a ('Chinese Spring')[1]	47
VPM1-*XustSS30M90*$_{AG240}$[2]	49
CS-*XustSS30M90*$_{AG240}$[2]	49

[1] Segregation of the endopeptidase alleles fits a 1:1 ratio ($\chi^2 = 0.1633$; $P = 0.6862$)
[2] Segregation of the microsatellite alleles fits a 1:1 ratio ($\chi^2 = 0.0000$; $P = 1.0000$)

The primer set was also tested on the common wheats 'Chinese Spring', 'Indis' (*Lr19* source), 'Inia 66' and 'PI 294994' (*Dn5* source) to determine whether the SSR is polymorphic in other genotypes as well. No product was amplified in 'Chinese Spring' nullisomic 7D. However, an allele of 224 bp was amplified in both 'Chinese Spring' and 'PI 294994' while a fragment of 214 bp was found for 'Indis' and one of 226 bp for 'Inia 66'. *XustSS30M90*$_{AG240}$ was subsequently tested on 29 'Indis'-derived *Lr19* deletion mutants and five recombinant forms. Based on the presence / absence of the

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214 bp fragment, amplified in the *Lr19* chromatin, the locus was found to map between the *Wsp-D1* and *Sr25* loci (Figure 4). However, unexpected fragments of higher molecular weight than the parental alleles were amplified in two of the mutants (87M23-145 and 89M1-51); whilst six others (89M2-327, 89M1-25, 87M23-118, 87M23-3, 87M23-27 and 89M2-245) amplified a fragment of lower molecular weight. The presence of these unexpected and non-typical *Lr19* bands are difficult to explain. They showed erratic occurrence that was not consistent with the well-established physical map data. The genetic background of the 87M23, 89M1 and 89M2 crosses was diverse and it is possible that these amplifications are associated with background, or represents a homoeoallele or an unrelated locus.

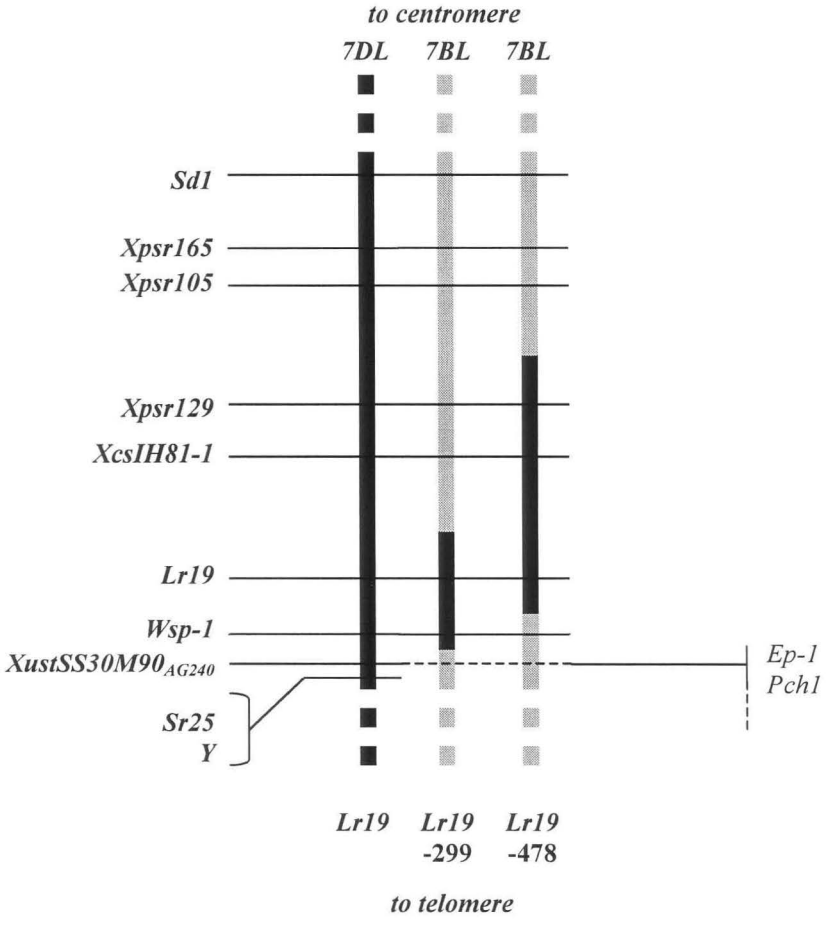

Figure 4. Line drawing map showing the map position of *XustSS30M90*$_{AG240}$.

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DISCUSSION

Cultivated wheat is characterised by little genetic polymorphism which hampers the application of marker aided selection for agronomically important loci (Hartl *et al.*, 1999). Comprehensive RFLP maps are available, but screening of RFLP probes is time-consuming and, due to the low levels of molecular polymorphism, is not very productive. AFLP analysis offers a reproducible and relatively fast screening method that assays numerous loci in a single round (Caranta *et al.*, 1999). Using 36 primer combinations, three AFLP markers closely linked to the *Pch1* and *Ep-D1* loci were identified, one of which was successfully converted to a sequence-specific marker. Interestingly, all of the polymorphisms detected in this study were identified using *Sse*8387I+3 and *Mse*I+3 primer combinations, probably due to the decreased complexity of the fingerprint revealing more 'hidden' polymorphisms than with primer combinations using less selective nucleotides. *Sse*8387I selective primers S28+AGC and S29+AGG produced polymorphisms with almost all *Mse*I+3 selective primers tested. The number of useful polymorphisms detected (0.083 polymorphisms per combination over all combinations tested) is comparatively high, possibly because of the lower number of combinations screened and the fact that *Pch1* is located in alien chromatin. Qu *et al.* (1998) utilised AFLP markers to develop a PCR-based method for scoring the *ph1b* deletion in wheat: they identified six useful polymorphisms (0.047 polymorphisms per combination) by screening 128 *Pst*I/*Mse*I primer combinations (2 *Pst*I and 64 *Mse*I selective primers).

Few other successful conversions of AFLP markers have been reported (Qu *et al.*, 1998; Bradeen and Simon, 1998; Raemon-Büttner and Jung, 2000; Prins *et al.*, 2001). Only six sequence-specific primer sets derived from 26 AFLP markers specific to wheat 3BS and 4BS chromosome arms retained the specificity of the original AFLP markers (Shan *et al.*, 1999) and the authors ascribed it to the source of the AFLP polymorphism being lost when internal primers are designed. They concluded that, although the conversion of AFLP markers to sequence-specific marker is feasible, it is not a very efficient process in wheat. A procedure to obtain flanking sequence information from AFLP markers using ligation-mediated suppression PCR has been described by Schupp *et al.* (1999) and should be useful in cases where the original AFLP polymorphism was caused by a mutation(s) in the restriction site.

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The relative ease with which the marker described in this paper was converted might be ascribed to any of a number of factors, such as: 1) The relatively large size of the fragment may have precluded co-migration of *Mse*I/*Mse*I amplification products, 2) Comparison of clone sequences of fragments isolated from two different source lines might have increased the chance of identifying the correct sequence. 3) The genotypes of the lines used might have influenced the number of co-migratory amplification products. 4) The use of primers with +3 selective nucleotides might have decreased the complexity of the AFLP fingerprints and 5) The internal sequence was the basis for the polymorphism rather than the enzyme cutting site.

The microsatellite nature of *XustSS30M90*$_{AG240}$ should make it a useful marker, as it could be highly polymorphic with co-dominant expression. Microsatellites normally consist of a core motif with a few nucleotides (normally 1-6 base pairs) that are repeated multiple times in tandem (up to a total length of less than 60 base pairs, normally 10-60 repeat units). They are usually flanked by unique sequences from which PCR primers can be designed (Litt and Luty, 1989; Weber and May, 1989). Null alleles, *i.e.* where a loss of amplification product due to mutation in the primer binding site occurs, have been found in wheat microsatellites at a frequency of up to 25 % of the alleles at 13 % (Plaschke *et al.*, 1995) and 10 % (Prasad *et al.*, 2000) of microsatellite loci. In wheat, the microsatellite motifs $(AC)_n$ and $(AG)_n$ are found in relative abundance, with an estimated total number of 1.3×10^5 sites for both types (Ma *et al.*, 1996). Nagaoka and Ogihara (1997) concluded that $(AG)_n$, $(CT)_n$ and $(GT)_n$ sequences are most useful and more reliable than RAPDs. Microsatellites are not restricted to specific regions on a chromosome, are multi-allelic and require very small amounts of template DNA, which can even be partly degraded (Gruis *et al.*, 1993). SSRs are very useful where RFLP polymorphisms are low (Akkaya *et al.*, 1992), but unlike RFLP probes which allows for a relatively high degree of mismatch (up to 20 % - Anderson *et al.*, 1992), several studies have shown microsatellites to be locus specific without corresponding homoeoloci on other related genomes (Bryan *et al.*, 1997; Stephenson *et al.*, 1998; Röder *et al.*, 1998). Microsatellite markers were used to map genes for dwarfing (Korzun *et al.*, 1997 and 1998) and preharvest sprouting tolerance (Roy *et al.*, 1999) and to detect genetic diversity in closely related bread wheat cultivars and lines (Plaschke *et al.*, 1995).

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In the wheats tested, *XustSS30M90*$_{AG240}$ appeared to be chromosome 7DL specific and its usefulness in other genetic backgrounds and segregating populations must still be determined. In a study by Caranta *et al.* (1999), a co-dominant AFLP marker linked to the pepper potyvirus resistance gene, *Pvr4,* was converted into a useful co-dominant CAPS marker that identified the *Pvr4*-genomic region in a number of pepper breeding lines. The authors predicted that their CAPS marker could be used in many susceptible genetic backgrounds. Similar predictions were made by Hartl *et al.* (1999) for co-dominant AFLP markers associated with powdery mildew resistance genes *Pm1b* and *Pm1d* in common wheat. In wheat, the locus specificity and high levels of polymorphism of SSRs should make it useful in practical wheat breeding but the absence of homoeologous loci in most cases makes SSRs likely to be insufficient for comparative analyses or introgression studies involving wild species related to wheat (Stephenson *et al.*, 1998). However, it may not always be the case as present results indicate that *XustSS30M90*$_{AG240}$ also amplifies polymorphic fragments in homoeologous chromosome 7L regions of *Ae. ventricosa* and *Th. ponticum* and therefore has wider application than only tagging *Pch1*-related eyespot resistance. Unfortunately, *XustSS30M90*$_{AG240}$ amplified alleles of similar electrophoretic ability in 'Chinese Spring' and 'PI 294994' and it could therefore not be mapped in the doubled haploid population derived from a cross between these two lines. The utility of this marker in a much larger variety of cultivars should be examined.

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CONCLUSIONS

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Crosses are seldom made between genotypes of similar agronomic performance: one of the parents will have some trait, *e.g.* for pest resistance, that the other one lacks and a number of repeated crosses or backcrosses are required to develop a progeny line of suitable quality. Often the required trait is not available in adapted breeding lines and must then be introgressed from related species or genera. Because of linkage drag, the associated chromatin often contains traits of lesser quality or traits that decrease the value of introgressed chromatin. Both crosses with wheat accessions and crosses to related species will yield a trait(s) with surrounding chromatin that must be decreased in size until just the useful gene(s) remain(s) in the background of the required genotype. Molecular and other markers are important tools that can be used to characterise an introgressed region or to help in marker-assisted selection of seedlings in a breeding program. Unfortunately, one of the major problems of cultivated wheat is that the level of molecular polymorphism is very low. This causes problems when mapping attempts are made, as a large percentage of markers, especially RFLP markers, are often not polymorphic between lines used in a specific cross. It is therefore extremely important to have maps that are as comprehensive as possible and also to know which markers are polymorphic in a given population. In comparison to human genetics, which has standard mapping populations like the Centre d'Etude du Polymorphisme Humaine (CEPH) populations, wheat genetics has few commonly used mapping populations. One such mapping population is the International Triticeae Mapping Initiative (ITMI - http://www.scri.sari.ac.uk/ITMI/FirstAbt.htm) population (Van Deynze *et al.*, 1995). Markers tested on the parents of these wheat populations are often not polymorphic, making it impossible to map a new marker in relation to an existing map. Existing maps are often of little use when planning a mapping experiment using other parental lines, mainly because of non-polymorphic markers but also because different anchor probes/markers are used by different research groups to map novel markers onto a newly established mapping population. Such novel markers mapped in a new mapping population can therefore not readily be superimposed onto established mapping populations. At present, the genetic and physical maps of common wheat are poorly developed. Numerous attempts will still have to be made to identify additional polymorphic markers that can be used for mapping and marker assisted selection.

With the above taken into account, the main purpose of this study was to expand the current knowledge of the genetic map of chromosome arm 7DL of common wheat. This chromosome

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arm was targeted as part of an ongoing attempt to improve the utility of agronomically important genes on this chromosome, *e.g. Dn2*, *Dn5*, *Lr19* and *Pch1*. Both *Lr19* and *Pch1* are located on translocations and are associated with large regions of linked introgressed chromatin that do not recombine at all or show very limited recombination with homoeologous wheat regions. This makes the simultaneous incorporation of these resistance genes in a homozygous genotype difficult, as the introgressed and native regions overlap. Attempts are being made to reduce the amount of unnecessary species-derived chromatin associated with *Lr19* through allosyndetic pairing induction. However, the maps of the wheat group 7L are poorly developed, which make it very difficult to i) select appropriate markers for the recovery of recombinants, ii) identify the most useful recombinant forms and iii) determine when the modified translocation will be short enough.

It was therefore decided to further develop an existing physical map of the *Lr19* translocation which could improve our ability to manipulate and shorten the segment. Secondly, an attempt was made to find and genetically map new and existing markers relative to the *Pch1* and *Dn5* loci. It was hoped that pivotal map positions could be established that would eventually make it possible to relate the positions of the individual genes and the borders of the foreign chromosome regions. The attempt was made from three angles: i) a set consisting of 29 'Indis' derived deletion mutants was used to identify polymorphic AFLP markers that mapped to the translocated *Thinopyrum* chromatin; ii) a doubled haploid population segregating for Russian wheat aphid resistance gene, *Dn5*, and other loci was created and characterised; AFLP analysis of F_2 progeny was performed in an attempt to find and map further polymorphic loci linked to *Dn5*; and iii) an attempt was made to find polymorphic markers mapping close to the eyespot resistance gene, *Pch1*, and the endopeptidase locus, *Ep-D1*. An AFLP marker associated with the targeted loci was converted into a sequence-specific marker; the latter may prove to be a more versatile marker for marker assisted selection than the presently used *Ep-D1* marker.

AFLP analysis of deletion mutants of the 'Indis' derived *Lr19* translocation resulted in the identification of a large number of AFLP polymorphisms associated with the introgressed chromatin. An existing physical map of the region could be improved considerably. Although not all of the mutants could be distinguished from one another, the revised map provides important information for future mapping attempts. With the extended physical map now

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available for the *Lr19* translocation, the size of most shortened recombinant forms can readily be deduced and the markers identified in this study will also be very useful for further characterisation of these recombinant forms. Some of the disadvantages of the AFLP technique are its requirement for good quality DNA and the relatively long time that passes between leaf material collected and the visualisation of the marker. These disadvantages prompted an attempt to convert some of the more useful markers into sequence-specific PCR-based markers that can easily be detected from crude DNA extracts in a short period of time. Conversion of two of the markers were attempted: the first attempt is described in Chapter 2 and the second was reported in a multi-authored paper (Prins *et al.*, 2001).

A doubled haploid mapping population was also developed in this study. The population has been characterised for the structural loci *Dn5* and *Ep-D1*, as well as four microsatellite markers. Two of the microsatellite markers, *viz. Xgwm437* and *Xgwm111*, were found to be loosely associated with the *Dn5* locus. The RFLP markers and converted RAPD markers did not reveal any polymorphisms between the parental genotypes used to create the doubled haploid and F_2 populations. A segregating F_2 population was subjected to AFLP analysis, but only two putative markers were identified. An attempt to convert the coupling phase marker associated with *Dn5* into a sequence-specific marker failed, most likely due to the small size of the original AFLP amplicon. Previously reported linkage between *Ep-D1* and *Dn5* could not be confirmed using the doubled haploid population. As a result, the mapping data could not be related to that obtained in another mapping experiment involving *Ep-D1* and *Pch1*.

AFLP markers associated with eyespot resistance gene *Pch1* and the endopeptidase locus *Ep-D1* were identified. One of the markers thus identified was selected, cloned and sequenced. The sequence contained a microsatellite repeat motif and flanking primers were designed and tested on the material. The microsatellite marker ($XustSS30M90_{AG240}$) was able to distinguish between the parental genotypes used and a map distance of 2 cM was calculated between it and the endopeptidase locus in an extended mapping population. The marker was also tested on the cultivars 'Indis', 'Inia 66', 'Chinese Spring' and 'PI 294994'. It was not polymorphic between 'Chinese Spring' and 'PI 294994', but did amplify different alleles in 'Indis' and 'Inia 66' which made it possible to map the marker between the *Wsp-D1* and *Sr25* loci. Because of the close

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linkage between *Ep-D1* and *Pch1*, the map order of these two loci has not been determined with certainty and no unambiguous reports were found in the literature (Hart *et al.*, 1992; Gale *et al.*, 1995; Boyko *et al.*, 1999). The exact size of the VPM1 translocation has not been reported in literature.

The AFLP technique amplifies a large number of loci per combination and primers with different selective nucleotides can be used to change the fingerprint and resulting amplified loci. For a total genome screen or diversity testing, these are significant advantages but, for the identification of a specific chromosome region, it is important to know that the number of polymorphisms per primer combination could vary among the regions targeted. In this study, three loci in different genetic backgrounds were studied: the *Lr19* translocation, which spans approximately one quarter of the chromosome arm, was analysed using both *Sse*8387I and *Eco*RI restriction endonucleases in combination with *Mse*I and yielded 0.60 and 0.28 useful polymorphisms per combination, respectively. Using the combination *Sse*8387I/*Mse*I, the numbers for *Dn5*, a wheat gene located in wheat chromatin, and *Pch1*, located on the VPM1 translocation, were 0.029 and 0.083, respectively. If an attempt is made to map a specific locus using AFLP markers, it should be kept in mind that these proportions translate to a range of one useful polymorphism per almost two combinations (1.67 combinations) to one in 35 combinations (34.48 combinations) tested. Even though this implies that for some traits, screening could involve testing a very large number of primer combinations, the lack of a better screening technique than AFLPs should make it a commonly used screening system for some years to come.

Comparative mapping between the three regions targeted in this study is currently not possible, mainly due to the fact that none of the framework markers occurred, or were polymorphic, in all three regions (Figure 1). The microsatellite marker (*XustSS30M90*$_{AG240}$) associated with *Pch1* was polymorphic for *Lr19* and could therefore be used to anchor the *Lr19* and VPM1 maps on the distal end. The marker was not polymorphic in the *Dn5* population, although the *Ep-D1* locus (linked to the *XustSS30M90*$_{AG240}$ locus) was and it could therefore be used as a

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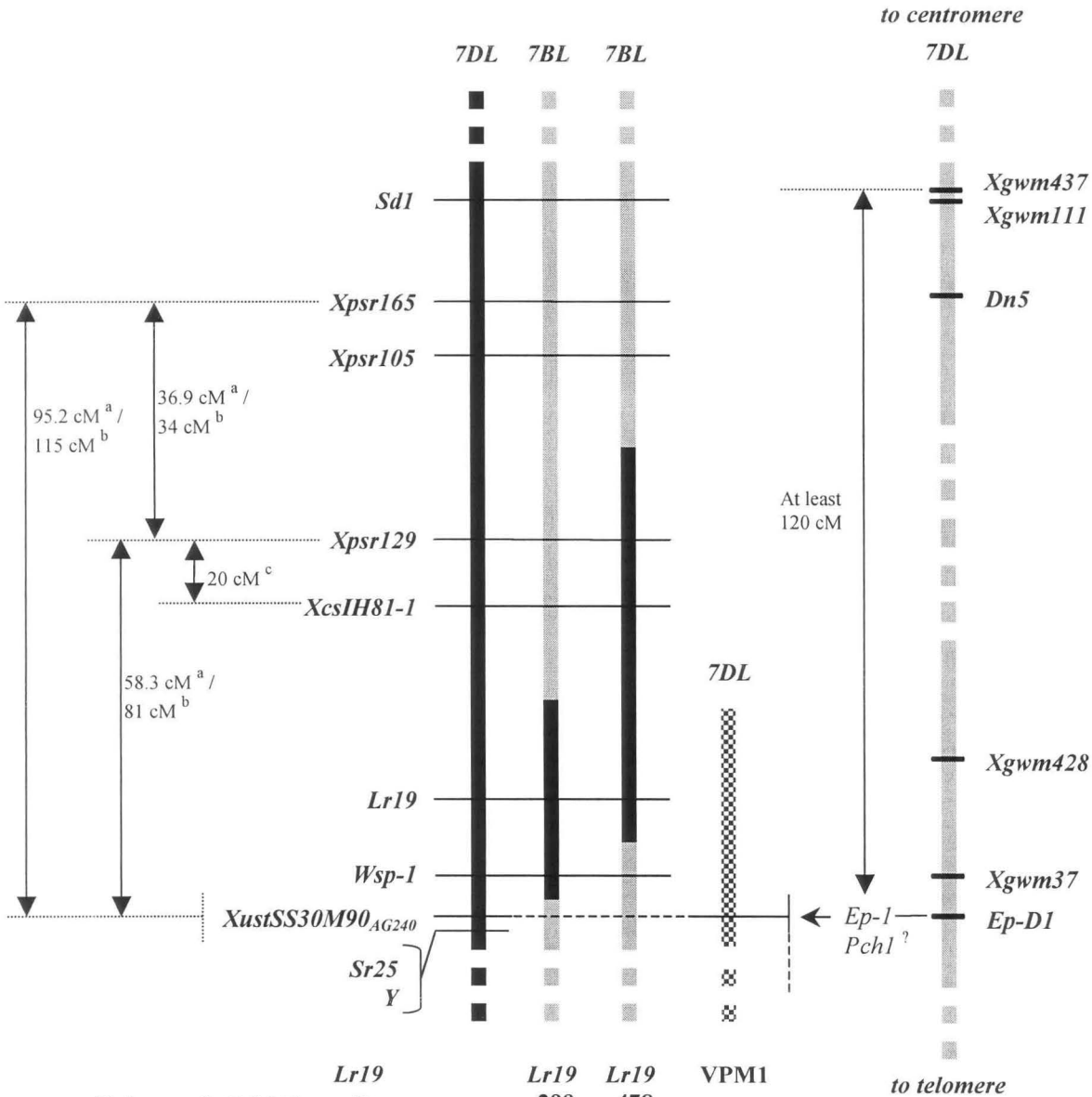

a Gale *et al.* (1995)
b Chao *et al.* (1989)
c Lagudah *et al.* (1991)

} Distances are shown as reported for chromosome 7D

? Relative order of *Pch1* and *Ep-D1* is unknown

Lr19 translocation and recombinants
Wheat chromatin
VPM1 translocation (size is unknown)

(Map distances not drawn to scale)

Figure 1. Comparison of the three line drawing maps of this dissertation.

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distal anchor for *Dn5*. Unfortunately, the different regions could not be anchored with any of the proximal markers used in this study. The map distance on 7D between *Xpsr129* and *Ep-D1* was estimated at 58.3 cM (Gale *et al.*, 1995) and 81 cM (Chao *et al.*, 1989), respectively. The *XcsIH81-1* locus is located 20 cM distally from *Xpsr129* (Lagudah *et al.*, 1991). If it is assumed that the homoeoloci on the *Lr19* translocation is spaced similarly, it can be concluded that a shortened translocation derived from homologous recombination between lines 299 and 478 may reduce the *Lr19* translocation to well below 50 cM in size. Recombinant line 299 has retained the *Wsp-D1c* (*Lr19* translocation-specific) allele, but recovered the wheat *Ep-B1* allele and it is therefore very likely that the endopeptidase locus is located distally from the *Wsp-1* locus. The mapping order of these two loci has been reported only in a consensus map of Hart *et al.* (1993) and shows an inverse order to the one shown here. The genetic distance between *Ep-D1* and *Xpsr165* was calculated to be 95 cM by Gale *et al.* (1995) and 115 cM by Chao *et al.*, 1989), while the total map distance between *Ep-D1* and *Xgwm437* was calculated in the *Dn5* population to be 142.6 cM (Chapter 3). *Dn5* and *Xgwm428* was found not to be linked and the calculated distance between these two loci may therefore be unreliable. However, assuming the closest distance between the two loci to be 50 cM (instead of the calculated 72 cM), the total distance between *Ep-D1* and *Xgwm437* may be at least 120 cM. This implies that *Xgwm437* may be located closer to the centromere than *Xpsr165*. The four microsatellite markers used to map the *Dn5* locus were also tested on the twenty-nine *Lr19* deletion mutants. Amplification occurred in the parental genotypes, but not in the mutant set, implying that the chromatin containing *Dn5* must be located distally from *Sr25/Y*. This contradicts the conclusion drawn above when comparing the genetic distances reported in literature to the distance between *Ep-D1* and *Xgwm437* calculated in this study. The reason for this discrepancy may be two-fold: firstly, the nature of the mapping populations used for the calculation of the genetic distances differs. In our *Dn5* population, distances were calculated for a population derived from a cross of two common wheats, whereas the population of Gale *et al.* (1995) had as its one parent the allohexaploid hybrid between *T. dicoccoides* and *T. tauschii*, named 'Synthetic' (McFadden and Sears, 1946). Chao *et al.* (1989) used 366 lines developed from six different mapping populations, but is is not clear whether their estimates were also based on mapping populations that involved species-derived parents. It is possible that recombination between 7DL of wheat and 7DL of *T. tauschii* is reduced, resulting in an underestimation of the true genetic distances.

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Xgwm437 and *Dn5* may therefore be located closer to *Lr19* than the data in Figure 1 would suggest. Secondly, the order of the loci in the *Dn5* map may be inverse to that reported in literature (Röder *et al.*, 1998), with *Xgwm37* rather than *Xgwm437* being closest to the centromere.

The material and data generated in this study may be used as follows in future work:

i) The AFLP map generated on the *Lr19* deletion mutants provides markers that can be used to further reduce the translocation and characterise recombinant forms.

ii) There are still deletion mutants that could not be distinguished from one another and additional *Sse*8387I/*Mse*I primer combinations can be tested to determine their relative sizes.

iii) The segregating doubled haploid population developed in this study can be typed for other key loci or traits as well and a skeleton molecular marker map covering all chromosomes can be constructed.

iv) Due to the fact that few useful AFLP polymorphisms associated with Russian wheat aphid resistance were identified in the F_2 population, it could be better to rather screen the doubled haploid mapping population with AFLP combinations. The advantage of this is that any polymorphisms identified anywhere in the genome can then immediately be mapped relative to the other molecular markers without having to convert the new AFLP marker first. Other directed marker attempts such as resistance gene analogs could also be used.

v) The eyespot and endopeptidase associated microsatellite marker reported in this dissertation should be tested against a wider background of genotypes to assess its potential for marker assisted selection.

vi) The four microsatellite markers used in the *Dn5* population should be tested on the VPM1 translocation in order to attempt comparative mapping between *Dn5* and *Pch1*.

vii) An attempt should be made to identify polymorphic marker loci that can be used to align the *Dn5* microsatellite and *Lr19* RFLP maps.

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APPENDIX ONE

EXTENDED PHYSICAL MAP OF THE *LR19* TRANSLOCATION

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Appendix 1. Extended physical map of the 'Indis' derived *Lr19* translocation.

Cluster	Line code	Line name	*Sd1*	20a:S-AG/M-AG$_{264}$	22a:S-AGC/M-AG$_{263}$	66a:S-T/M-GA$_{241}$	503b:E-AAC/M-CAG$_{133}$	74a:S-T/M-GC$_{>330}$	98b:S-T/M-AAC$_{115}$	71a:S-AGG/M-GA$_{>330}$	28a:S-AG/M-AT$_{261}$	135a:S-AGG/M-TGC$_{258}$	132a:S-AG/M-TGC$_{256}$	15a:S-AGG/M-AC$_{216}$
	A	Indis		+	+	+	+	+	+	+	+	+	+	+
	B	Inia 66		-	-	-	-	-	-	-	-	-	-	-
Y1	C	87M23-145	a	+	+	+	+	-	-	-	-	-	-	-
Y2	D	89M1-51	b	+	+	+	+	+	+	+	-	-	-	-
Y3	E	87M23-198	a	+	+	+	+	+	+	-	+	+	+	+
Y4	F	87M23-1	b	+	+	+	+	+	+	-	+	+	+	-
	G	89M2-327	a	+	+	+	+	+	+	+	+	+	+	+
Y5	H	87M23-178	a	+	+	+	+	+	+	+	+	+	+	+
	I	89M1-78	a	+	+	+	+	+	+	+	+	+	+	+
Y6	J	87M23-273	a	+	+	+	+	+	+	+	-	-	-	-
	K	87M23-227	c	+	+	+	+	+	+	+	+	+	+	+
Y7	L	87M23-314	a	+	+	+	+	+	+	+	+	+	+	+
Y8	M	89M1-25	a	+	+	+	+	+	+	+	+	+	+	+
Y9	N	89M1-69	d	+	+	+	+	+	+	+	+	+	+	+
Y10	O	87M23-118	a	+	+	+	+	+	+	+	+	+	+	+
Y11	P	89M2-39	c	+	+	+	+	+	+	+	+	+	+	+
Y12	Q	87M23-3	a	+	+	+	+	+	+	+	+	+	+	+
	R	87M23-128	d	+	+	+	+	+	+	+	+	+	+	+
	S	87M23-108	d	+	+	+	+	+	+	+	+	+	+	+
	T	87M23-219	d	+	+	+	+	+	+	-	+	+	+	+
Y13	U	87M23-27	d	+	+	+	+	+	+	+	+	+	+	+
	V	87M23-115	a	+	+	+	+	+	+	+	+	+	+	+
Y14	W	89M2-40	c	+	+	+	+	+	+	+	+	+	+	+
Y15	X	87M23-103	c	-	-	+	+	+	+	+	+	+	+	+
Y16	Y	89M2-245	d	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	89M2-426	d	+	+	+	+	+	+	-	+	+	+	+
Y18	AA	87M23-225	d	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	89M1-18	b	+	+	+	+	+	+	+	+	+	+	+
	AC	87M23-266	a	+	+	+	+	+	+	+	+	+	+	+
	***Sse* 8387I / *Eco* RI :**			**3 / 1**				**3 / 0**			**4 / 0**			
	AFLP markers :			**4**				**3**			**4**			
	Structural loci :			**0**				**0**			**0**			
	Total number:			**4**				**3**			**4**			
	Marker interval :			**X1**				**X2**			**X3**			

Appendix 1. (continued)

Cluster	Line code	*Xpsr165-7el$_1$*	*Xpsr105-7el$_1$*	85a:S-AGA/M-GG$_{>330}$	25b:S-C/M-AT$_{135}$	40a:S-AGT/M-CA$_{>330}$	65b:S-C/M-GA$_{122}$	106b:S-T/M-CCG$_{106}$	10a:S-T/M-AC$_{236}$	56b:S-AGT/M-CG$_{258}$	92a:S-AG/M-GT$_{>330}$	100b:S-AG/M-AAC$_{160}$	100c:S-AG/M-AAC$_{131}$
	A	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	+	-	-	-	-	-	-	-	-	-	-	-
	G	+	-	-	-	-	-	-	-	-	-	-	-
Y5	H	+	+	-	-	-	-	-	-	-	-	-	-
	I	+	+	-	-	-	-	-	-	-	-	-	-
Y6	J	+	+	+	-	-	-	-	-	-	-	-	-
	K	+	+	+	-	-	-	-	-	-	-	-	-
Y7	L	+	+	-	+	+	+	-	-	-	-	-	-
Y8	M	+	+	+	+	+	+	+	+	+	+	+	+
Y9	N	+	+	+	+	+	+	+	+	+	+	+	+
Y10	O	+	+	+	+	+	+	+	+	+	+	+	+
Y11	P	+	+	+	+	+	+	+	+	+	+	+	+
Y12	Q	+	+	-	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	-	-	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+
		0	0	1 / 0	3 / 0			6 / 0					
		0	0	1	3			6					
		1	1	0	0			0					
		1	1	1	3			6					
		X4	X5	X6	X7			X8					

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Appendix 1. (continued)

Cluster	Line code	24a:S-AGT/M-AG$_{234}$	67b:S-AC/M-GA$_{173}$	98a:S-T/M-AAC$_{>330}$	506a:E-AAC/M-CTC$_{270}$	*Xpsr129-7el$_1$*	1b:S-C/M-AA$_{149}$	35a:S-AC/M-CA$_{>330}$	44a:S-AG/M-CC$_{155}$	47a:S-AGG/M-CC$_{156}$	79b:S-AGG/M-GC$_{214}$	81a:S-C/M-GG$_{269}$	81c:S-C/M-GG$_{142}$	83a:S-AC/M-GG$_{228}$	84a:S-AG/M-GG$_{>330}$	85b:S-AGA/M-GG$_{>330}$
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Y10	O	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y11	P	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y12	Q	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
		3 / 1				15 / 1										
		4				16										
		0				1										
		4				17										
		X9				X10										

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Appendix 1. (continued)

Cluster	Line code	97c:S-C/M-AAC$_{149}$	108b:S-AG/M-CCG$_{156}$	111b:S-AGG/M-CCG$_{153}$	520a:E-ACA/M-CAT$_{170}$	59a:S-AC/M-CT$_{>330}$	88a:S-AGT/M-GG$_{196}$	519d:E-ACA/M-CAG$_{148}$	53a:S-AGA/M-CG$_{>330}$	506b:E-AAC/M-CTC$_{252}$	518e:E-ACA/M-CAC$_{168}$	*XcsIH81-1-7el$_1$*	*Xwg380-7el$_1$*	2b:S-T/M-AA$_{131}$	13a:S-AGA/M-AC$_{>330}$	18a:S-T/M-AG$_{123}$
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Y11	P	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-
Y12	Q	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
									1 / 3							
									4							
									0							
									4							
		X10 (continued)							X11				X12			

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Appendix 1. (continued)

Sub-group	Line code	27a:S-AC/M-AT$_{155}$	36b:S-AG/M-CA$_{205}$	37a:S-AGA/M-CA$_{>330}$	37c:S-AGA/M-CA$_{209}$	51a:S-AC/M-CG$_{171}$	62a:S-AGC/M-CT$_{>330}$	65a:S-C/M-GA$_{204}$	76b:S-AG/M-GC$_{205}$	80a:S-AGT/M-GC$_{209}$	96a:S-AGT/M-GT$_{260}$	102b:S-AGC/M-AAC$_{160}$	130a:S-T/M-TGC$_{224}$	137c:S-C/M-TGT$_{>330}$	138a:S-T/M-TGT$_{>330}$	140b:S-AG/M-TGT$_{>330}$
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y11	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y12	Q	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
									21 / 2							
									23							
									2							
									25							
									X12							

(continued)

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Appendix 1. (continued)

Cluster	Line code	143a:S-AGG/M-TGT$_{>330}$	39b:S-AGG/M-CA$_{208}$	79a:S-AGG/M-GC$_{330}$	519c:E-ACA/M-CAG$_{153}$	522c:E-ACA/M-CTC$_{256}$	67a:S-AC/M-GA$_{258}$	26a:S-T/M-AT$_{186}$	34b:S-T/M-CA$_{137}$	122e:S-T/M-GGT$_{123}$	129d:S-C/M-TGC$_{137}$	134a:S-AGC/M-TGC$_{138}$	522d:E-ACA/M-CTC$_{252}$
	A	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	-	-	-	-	-	-	-	-	-	-	-	-
Y11	P	-	-	-	-	-	-	-	-	-	-	-	-
Y12	Q	+	+	+	+	+	-	-	-	-	-	-	-
	R	+	+	+	+	+	-	-	-	-	-	-	-
	S	+	+	+	+	+	-	-	-	-	-	-	-
	T	+	+	+	+	+	-	-	-	-	-	-	-
Y13	U	+	+	+	+	+	+	-	-	-	-	-	-
	V	+	+	+	+	+	+	-	-	-	-	-	-
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+
							1 / 0			5 / 1			
							1			6			
							0			0			
							1			6			
				X12 (continued)			X13			X14			

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Appendix 1. (continued)

Cluster	Line code	7b:S-AGG/M-AA_{270}	27b:S-AC/M-AT_{191}	54a:S-AGC/M-$CG_{>330}$	56a:S-AGT/M-$CG_{>330}$	126a:S-AGC/M-$GGT_{>330}$	137a:S-C/M-$TGT_{>330}$	138c:S-T/M-TGT_{131}	*Lr19*	7a:S-AGG/M-AA_{325}	12c:S-AG/M-AC_{174}	18b:S-T/M-AA_{142}	57a:S-C/M-CT_{304}	86a:S-AGC/M-GG_{214}	117a:S-AGA/M-GAC_{312}	81b:S-C/M-GG_{189}	126c:S-AGC/M-GGT_{224}
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y11	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y12	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y13	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y14	W	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
Y15	X	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
					7 / 0									8 / 0			
					7									8			
					1									0			
					8									8			
					X15									X16			

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Appendix 1. (continued)

Wsp-D1c	34a:S-T/M-CA_{195}	50b:S-T/M-CG_{156}	68b:S-AG/M-GA_{217}	136a:S-AGT/M-$TGC_{>330}$	71b:S-AGG/M-GA_{217}	*Sr25*	*Y*	Line name	Line code	Cluster
+	+	+	+	+	+	+	+	Indis	A	
-	-	-	-	-	-	-	-	Inia 66	B	
-	-	-	-	-	-	-	-	87M23-145	C	Y1
-	-	-	-	-	-	-	-	89M1-51	D	Y2
-	-	-	-	-	-	-	-	87M23-198	E	Y3
-	-	-	-	-	-	-	-	87M23-1	F	Y4
-	-	-	-	-	-	-	-	89M2-327	G	
-	-	-	-	-	-	-	-	87M23-178	H	Y5
-	-	-	-	-	-	-	-	89M1-78	I	
-	-	-	-	-	-	-	-	87M23-273	J	Y6
-	-	-	-	-	-	-	-	87M23-227	K	
-	-	-	-	-	-	-	+	87M23-314	L	Y7
-	-	-	-	-	-	-	-	89M1-25	M	Y8
-	-	-	-	-	-	-	-	89M1-69	N	Y9
-	-	-	-	-	-	-	-	87M23-118	O	Y10
-	-	-	-	-	-	-	-	89M2-39	P	Y11
-	-	-	-	-	-	-	-	87M23-3	Q	Y12
-	-	-	-	-	-	-	-	87M23-128	R	
-	-	-	-	-	-	-	-	87M23-108	S	
-	-	-	-	-	-	+	-	87M23-219	T	
-	-	-	-	-	-	-	-	87M23-27	U	Y13
-	-	-	-	-	-	-	-	87M23-115	V	
-	-	-	-	-	-	-	-	89M2-40	W	Y14
-	-	-	-	-	-	+	-	87M23-103	X	Y15
-	-	-	-	-	-	-	-	89M2-245	Y	Y16
+	-	-	-	-	-	-	-	89M2-426	Z	Y17
-	+	+	+	+	-	-	-	87M23-225	AA	Y18
+	+	+	+	+	+	+	+	89M1-18	AB	Y19
+	+	+	+	+	+	+	+	87M23-266	AC	
0	**4 / 0**				**1 / 0**			**Totals :**		
0	**4**				**1**			**95**	**AFLP markers**	
1	**0**				**2**			**9**	**Structural loci**	
1	**4**				**3**			**104**	**Total number**	
X17	**X18**				**X19**					

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Appendix 1. Legend

The order of loci within an interval is unknown.

Sd1	a	**Preferential / normal transmission and self-elimination**
	b	**Only self-elimination observed**
	c	**Apparently normal segregation ratios, but low seed set**
	d	**Only preferential transmission observed**
	a-d	**Notation as used by Prins *et al*. (1996)**
+		***Sse* 8387I / *Mse* I AFLP polymorphism**
+		***Eco* RI / *Mse* I AFLP polymorphism**
+		**RFLP marker**
+		**Structural loci**
-		**Possible intercallary deletion**
-		**Possible deletion or mutation**
+		**Fragment targeted for conversion**

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Appendix 1. Extended physical map of the 'Indis' derived *Lr19* translocation.

Cluster	Line code	Line name	*Sdl*	20a:S-AG/M-AG$_{264}$	22a:S-AGC/M-AG$_{263}$	66a:S-T/M-GA$_{241}$	503b:E-AAC/M-CAG$_{133}$	74a:S-T/M-GC$_{>330}$	98b:S-T/M-AAC$_{115}$	71a:S-AGG/M-GA$_{>330}$	28a:S-AG/M-AT$_{261}$	135a:S-AGG/M-TGC$_{258}$	132a:S-AG/M-TGC$_{256}$	15a:S-AGG/M-AC$_{216}$
	A	Indis		+	+	+	+	+	+	+	+	+	+	+
	B	Inia 66		-	-	-	-	-	-	-	-	-	-	-
Y1	C	87M23-145	a	+	+	+	+	-	-	-	-	-	-	-
Y2	D	89M1-51	b	+	+	+	+	+	+	+	-	-	-	-
Y3	E	87M23-198	a	+	+	+	+	+	+	-	+	+	+	+
Y4	F	87M23-1	b	+	+	+	+	+	+	-	+	+	+	-
	G	89M2-327	a	+	+	+	+	+	+	+	+	+	+	+
Y5	H	87M23-178	a	+	+	+	+	+	+	+	+	+	+	+
	I	89M1-78	a	+	+	+	+	+	+	+	+	+	+	+
Y6	J	87M23-273	a	+	+	+	+	+	+	+	-	-	-	-
	K	87M23-227	c	+	+	+	+	+	+	+	+	+	+	+
Y7	L	87M23-314	a	+	+	+	+	+	+	+	+	+	+	+
Y8	M	89M1-25	a	+	+	+	+	+	+	+	+	+	+	+
Y9	N	89M1-69	d	+	+	+	+	+	+	+	+	+	+	+
Y10	O	87M23-118	a	+	+	+	+	+	+	+	+	+	+	+
Y11	P	89M2-39	c	+	+	+	+	+	+	+	+	+	+	+
Y12	Q	87M23-3	a	+	+	+	+	+	+	+	+	+	+	+
	R	87M23-128	d	+	+	+	+	+	+	+	+	+	+	+
	S	87M23-108	d	+	+	+	+	+	+	+	+	+	+	+
	T	87M23-219	d	+	+	+	+	+	+	-	+	+	+	+
Y13	U	87M23-27	d	+	+	+	+	+	+	+	+	+	+	+
	V	87M23-115	a	+	+	+	+	+	+	+	+	+	+	+
Y14	W	89M2-40	c	+	+	+	+	+	+	+	+	+	+	+
Y15	X	87M23-103	c	-	-	+	+	+	+	+	+	+	+	+
Y16	Y	89M2-245	d	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	89M2-426	d	+	+	+	+	+	+	-	+	+	+	+
Y18	AA	87M23-225	d	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	89M1-18	b	+	+	+	+	+	+	+	+	+	+	+
	AC	87M23-266	a	+	+	+	+	+	+	+	+	+	+	+
	***Sse* 8387I / *Eco* RI :**			**3 / 1**				**3 / 0**			**4 / 0**			
	AFLP markers :			**4**				**3**			**4**			
	Structural loci :			**0**				**0**			**0**			
	Total number:			**4**				**3**			**4**			
	Marker interval :			**X1**				**X2**			**X3**			

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Appendix 1. (continued)

Cluster	Line code	*Xpsr165-7el*$_1$	*Xpsr105-7el*$_1$	85a:S-AGA/M-GG$_{>330}$	25b:S-C/M-AT$_{135}$	40a:S-AGT/M-CA$_{>330}$	65b:S-C/M-GA$_{122}$	106b:S-T/M-CCG$_{106}$	10a:S-T/M-AC$_{236}$	56b:S-AGT/M-CG$_{258}$	92a:S-AG/M-GT$_{>330}$	100b:S-AG/M-AAC$_{160}$	100c:S-AG/M-AAC$_{131}$
	A	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	+	-	-	-	-	-	-	-	-	-	-	-
	G	+	-	-	-	-	-	-	-	-	-	-	-
Y5	H	+	+	-	-	-	-	-	-	-	-	-	-
	I	+	+	-	-	-	-	-	-	-	-	-	-
Y6	J	+	+	+	-	-	-	-	-	-	-	-	-
	K	+	+	+	-	-	-	-	-	-	-	-	-
Y7	L	+	+	-	+	+	+	-	-	-	-	-	-
Y8	M	+	+	+	+	+	+	+	+	+	+	+	+
Y9	N	+	+	+	+	+	+	+	+	+	+	+	+
Y10	O	+	+	+	+	+	+	+	+	+	+	+	+
Y11	P	+	+	+	+	+	+	+	+	+	+	+	+
Y12	Q	+	+	-	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	-	-	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+
		0	0	1 / 0	3 / 0			6 / 0					
		0	0	1	3			6					
		1	1	0	0			0					
		1	1	1	3			6					
		X4	X5	X6	X7			X8					

Appendix 1. (continued)

Cluster	Line code	24a:S-AGT/M-AG$_{234}$	67b:S-AC/M-GA$_{173}$	98a:S-T/M-AAC$_{>330}$	506a:E-AAC/M-CTC$_{270}$	*Xpsr129-7el$_1$*	1b:S-C/M-AA$_{149}$	35a:S-AC/M-CA$_{>330}$	44a:S-AG/M-CC$_{155}$	47a:S-AGG/M-CC$_{156}$	79b:S-AGG/M-GC$_{214}$	81a:S-C/M-GG$_{269}$	81c:S-C/M-GG$_{142}$	83a:S-AC/M-GG$_{228}$	84a:S-AG/M-GG$_{>330}$	85b:S-AGA/M-GG$_{>330}$
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Y10	O	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y11	P	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y12	Q	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			3 / 1										15 / 1			
			4										16			
			0										1			
			4										17			
			X9										X10			

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Appendix 1. (continued)

Cluster	Line code	97c:S-C/M-AAC$_{149}$	108b:S-AG/M-CCG$_{156}$	111b:S-AGG/M-CCG$_{153}$	520a:E-ACA/M-CAT$_{170}$	59a:S-AC/M-CT$_{>330}$	88a:S-AGT/M-GG$_{196}$	519d:E-ACA/M-CAG$_{148}$	53a:S-AGA/M-CG$_{>330}$	506b:E-AAC/M-CTC$_{252}$	518e:E-ACA/M-CAC$_{168}$	*XcsIH81-1-7el$_1$*	*Xwg380-7el$_1$*	2b:S-T/M-AA$_{131}$	13a:S-AGA/M-AC$_{>330}$	18a:S-T/M-AG$_{123}$
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Y11	P	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-
Y12	Q	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
								1 / 3								
								4								
								0								
								4								
		X10 (continued)						X11				X12				

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Appendix 1. (continued)

Sub-group	Line code	27a:S-AC/M-AT$_{155}$	36b:S-AG/M-CA$_{205}$	37a:S-AGA/M-CA$_{>330}$	37c:S-AGA/M-CA$_{209}$	51a:S-AC/M-CG$_{171}$	62a:S-AGC/M-CT$_{>330}$	65a:S-C/M-GA$_{204}$	76b:S-AG/M-GC$_{205}$	80a:S-AGT/M-GC$_{209}$	96a:S-AGT/M-GT$_{260}$	102b:S-AGC/M-AAC$_{160}$	130a:S-T/M-TGC$_{224}$	137c:S-C/M-TGT$_{>330}$	138a:S-T/M-TGT$_{>330}$	140b:S-AG/M-TGT$_{>330}$
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y11	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y12	Q	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y13	U	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
									21 / 2							
									23							
									2							
									25							
									X12							

(continued)

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Appendix 1. (continued)

Cluster	Line code	143a:S-AGG/M-TGT$_{>330}$	39b:S-AGG/M-CA$_{208}$	79a:S-AGG/M-GC$_{330}$	519c:E-ACA/M-CAG$_{153}$	522c:E-ACA/M-CTC$_{256}$	67a:S-AC/M-GA$_{258}$	26a:S-T/M-AT$_{186}$	34b:S-T/M-CA$_{137}$	122e:S-T/M-GGT$_{123}$	129d:S-C/M-TGC$_{137}$	134a:S-AGC/M-TGC$_{138}$	522d:E-ACA/M-CTC$_{252}$
	A	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	-	-	-	-	-	-	-	-	-	-	-	-
Y11	P	-	-	-	-	-	-	-	-	-	-	-	-
Y12	Q	+	+	+	+	+	-	-	-	-	-	-	-
	R	+	+	+	+	+	-	-	-	-	-	-	-
	S	+	+	+	+	+	-	-	-	-	-	-	-
	T	+	+	+	+	+	-	-	-	-	-	-	-
Y13	U	+	+	+	+	+	+	-	-	-	-	-	-
	V	+	+	+	+	+	+	-	-	-	-	-	-
Y14	W	+	+	+	+	+	+	+	+	+	+	+	+
Y15	X	+	+	+	+	+	+	+	+	+	+	+	+
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+
							1 / 0			5 / 1			
							1			6			
							0			0			
							1			6			
				X12 (continued)			X13			X14			

Appendix 1. (continued)

Cluster	Line code	7b:S-AGG/M-AA_{270}	27b:S-AC/M-AT_{191}	54a:S-AGC/M-$CG_{>330}$	56a:S-AGT/M-$CG_{>330}$	126a:S-AGC/M-$GGT_{>330}$	137a:S-C/M-$TGT_{>330}$	138c:S-T/M-TGT_{131}	*Lr19*	7a:S-AGG/M-AA_{325}	12c:S-AG/M-AC_{174}	18b:S-T/M-AA_{142}	57a:S-C/M-CT_{304}	86a:S-AGC/M-GG_{214}	117a:S-AGA/M-GAC_{312}	81b:S-C/M-GG_{189}	126c:S-AGC/M-GGT_{224}
	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y1	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y2	D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y3	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y4	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y5	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y6	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y7	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y8	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y9	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y10	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y11	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y12	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y13	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y14	W	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-
Y15	X	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
Y16	Y	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y17	Z	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y18	AA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Y19	AB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
					7 / 0									8 / 0			
					7									8			
					1									0			
					8									8			
					X15									X16			

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Appendix 1. (continued)

Wsp-D1c	34a:S-T/M-CA_{195}	50b:S-T/M-CG_{156}	68b:S-AG/M-GA_{217}	136a:S-AGT/M-$TGC_{>330}$	71b:S-AGG/M-GA_{217}	*Sr25*	*Y*	Line name	Line code	Cluster
+	+	+	+	+	+	+	+	Indis	A	
-	-	-	-	-	-	-	-	Inia 66	B	
-	-	-	-	-	-	-	-	87M23-145	C	Y1
-	-	-	-	-	-	-	-	89M1-51	D	Y2
-	-	-	-	-	-	-	-	87M23-198	E	Y3
-	-	-	-	-	-	-	-	87M23-1	F	Y4
-	-	-	-	-	-	-	-	89M2-327	G	
-	-	-	-	-	-	-	-	87M23-178	H	Y5
-	-	-	-	-	-	-	-	89M1-78	I	
-	-	-	-	-	-	-	-	87M23-273	J	Y6
-	-	-	-	-	-	-	-	87M23-227	K	
-	-	-	-	-	-	-	+	87M23-314	L	Y7
-	-	-	-	-	-	-	-	89M1-25	M	Y8
-	-	-	-	-	-	-	-	89M1-69	N	Y9
-	-	-	-	-	-	-	-	87M23-118	O	Y10
-	-	-	-	-	-	-	-	89M2-39	P	Y11
-	-	-	-	-	-	-	-	87M23-3	Q	Y12
-	-	-	-	-	-	-	-	87M23-128	R	
-	-	-	-	-	-	-	-	87M23-108	S	
-	-	-	-	-	-	+	-	87M23-219	T	
-	-	-	-	-	-	-	-	87M23-27	U	Y13
-	-	-	-	-	-	-	-	87M23-115	V	
-	-	-	-	-	-	-	-	89M2-40	W	Y14
-	-	-	-	-	-	+	-	87M23-103	X	Y15
-	-	-	-	-	-	-	-	89M2-245	Y	Y16
+	-	-	-	-	-	-	-	89M2-426	Z	Y17
-	+	+	+	+	-	-	-	87M23-225	AA	Y18
+	+	+	+	+	+	+	+	89M1-18	AB	Y19
+	+	+	+	+	+	+	+	87M23-266	AC	
0	4 / 0				1 / 0			**Totals :**		
0	4				1			95	AFLP markers	
1	0				2			9	Structural loci	
1	4				3			104	Total number	
X17	X18				X19					

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Appendix 1. Legend

The order of loci within an interval is unknown.

Sd1	a	**Preferential / normal transmission and self-elimination**
	b	**Only self-elimination observed**
	c	**Apparently normal segregation ratios, but low seed set**
	d	**Only preferential transmission observed**
	a-d	**Notation as used by Prins *et al*. (1996)**

+	***Sse* 8387I / *Mse* I AFLP polymorphism**
+	***Eco* RI / *Mse* I AFLP polymorphism**
+	**RFLP marker**
+	**Structural loci**
-	**Possible intercallary deletion**
-	**Possible deletion or mutation**
+	**Fragment targeted for conversion**