



**CYTOGENETIC AND MOLECULAR GENETIC MARKERS FOR CHROMOSOME 6R
OF RYE LINKED TO CCN RESISTANCE**

by

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DECLARATION

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CONFERENCE PROCEEDINGS

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ABBREVIATIONS

Common abbreviations are listed in Current Protocols on CD-ROM (1993). Additional abbreviations are as follows:

μCi	:	microcurie
μg	:	microgram
μl	:	microlitre
μM	:	micromolar
1R ^{Imp}	:	chromosome 1R from rye cv. Imperial
1R ^{King}	:	chromosome 1R from rye cv. King
2R ^{Imp}	:	chromosome 2R from rye cv. Imperial
2R ^{King}	:	chromosome 2R from rye cv. King
6R ^{Imp}	:	chromosome 6R from rye cv. Imperial
6R ^{King}	:	chromosome 6R from rye cv. King
6R ^{Mon}	:	chromosome 6R from <i>Secale cereale</i> spp. <i>montanum</i>
6R ^{T701}	:	chromosome 6R from triticale T701-4-6
AP-PCR	:	arbitrarily primed polymerase chain reaction
ASP	:	amplified sequence polymorphism
BC ₁ -F ₂	:	backcross ₁ F ₂
CCN	:	cereal cyst nematode
DGGE	:	denaturing gradient gel electrophoresis
GISH	:	genomic <i>in situ</i> hybridisation
GOT	:	glutamate oxaloacetate transaminase
ISH	:	<i>in situ</i> hybridisation
LAF	:	long arm fusion
LOD	:	log likelihood
MAS	:	marker assisted selection

Mbp	:	mega base pairs
mCi	:	millicurie
MI	:	mitotic index
ml	:	millilitre
mM	:	millimolar
ng	:	nanogram
NIL	:	near isogenic line
nmole	:	nanomole
NOR	:	nucleolar organising region
pg	:	picogram
pmole	:	picomole
QTL	:	quantitative trait loci
RAPD	:	randomly amplified polymorphic DNA
RT	:	room temperature
SA	:	specific activity
SAF	:	short arm fusion
SAP	:	specific sequence polymorphism
SSCP	:	single strand conformation polymorphism
TC-F ₁	:	test-cross F ₁
TC-F ₂	:	test-cross F ₂
TGGE	:	temperature gradient gel electrophoresis
V	:	volts
V/cm	:	volts per centimetre
YAC	:	yeast artificial chromosome

SUMMARY

This thesis reports on the generation of molecular tools for the analysis of chromosome 6R of rye and the application of these tools in a structural analysis of 6RL. Results presented include physical and genetic maps of chromosome 6RL incorporating RFLP and PCR markers and *CreR*, the locus conferring resistance to cereal cyst nematode (CCN).

An important tool for both the physical and genetic mapping has been a clone containing an almost complete member of the R173 family of dispersed, repetitive rye-specific DNA sequences. This clone was used to identify rye chromosomes in wheat-rye hybrids via non-isotopic *in situ* hybridisation (ISH). Hybridisation of this clone to metaphase chromosomes of triticale cv. Carman and various Chinese Spring-Imperial disomic chromosome addition lines indicates that the R173 family is evenly dispersed on all seven rye chromosomes with the exception of some telomeres and centromeric regions. The ability to detect small introgressions of rye chromatin in wheat is demonstrated.

Molecular markers suitable for the development of physical and genetic linkage maps were generated. RFLP markers mapping to the long arm of chromosome 6R of rye were identified. Complementary and genomic DNA probes previously mapped to wheat and barley homoeologous group 6 chromosomes were obtained and screened across the Chinese Spring-Imperial 6R disomic addition line and Chinese Spring-T701-4-6 6R(-6D) disomic substitution line. A high proportion of clones were found to map to both chromosome 6R^{Imp} and chromosome 6R^{T701}. Importantly, high levels of polymorphism were detected between chromosome 6R^{Imp} and chromosome 6R^{T701} for both cDNA and gDNA clones. In spite of the success in developing RFLP markers for the long arm of chromosome 6R, the ever-present difficulties associated with RFLP technology led to the development of PCR-based molecular markers.

Three approaches to the generation of polymerase chain reaction (PCR)-based markers were utilised. An attempt was made to generate a PCR-based assay to replace RFLP analysis using a clone mapped to chromosome 6RL. Partial sequencing of this clone allowed the generation of primers for use in PCR studies on wheat, rye and barley genomic DNA. The second approach utilised primers derived from the flanking regions of cloned members of rye-specific dispersed, repetitive DNA sequences. PCR amplification products generated were mapped to all seven rye chromosomes. This study has provided some information regarding the local genomic organisation of the families of DNA sequences utilised. Finally, the 442 bp insert present in the clone pAW173 was sequenced. Primers were generated from this sequence in an attempt to develop markers from the internal regions of R173 elements. Southern analysis of amplification products generated in wheat and rye has provided information regarding the evolution of sequences related to the R173 family of repetitive DNA sequences.

A physical map of the long arm of chromosome 6R was generated. RFLP and PCR markers were subchromosomally localised to 6RL using the chromosome 6RL^{T701} terminal deletion lines described by Dundas *et al.* (1992). Comparative mapping indicates that an internal inversion is present in rye 6RL relative to the long arm of wheat group 6 chromosomes. Results suggest that segments of 3RL and 7RL known to be present at the distal end of 6RL constitute approximately 30% of the physical length of the arm. Significantly, the use of homoeologous group 6 RFLP probes indicates that at least 90% of the ancestral 6RL arm remains on the present day chromosome arm. The implications of these findings on the prospects of inducing homoeologous recombination between rye chromosome 6R and wheat group 6 chromosomes are discussed.

The generation of a mapping population for chromosome 6R of rye is described. Individuals demonstrating recombination around the *CreR* locus were identified through the use of flanking markers. Twelve individuals were selected and progeny tested to ascertain

their response to challenge with CCN. Unambiguous genotypic assignment for the *CreR* locus was possible for eight individuals.

A genetic linkage map comprising eight RFLP loci and five PCR loci has been constructed for the long arm of chromosome 6R. The *CreR* locus was mapped via the inclusion of progeny testing results and the assignment of genotypes to individuals upon visual inspection of the data set. This map spans approximately 60 cM and the order of loci established conforms to the order established via physical mapping. The RFLP locus *XksuF37* was mapped 3.5 cM proximal to *CreR* and represents a potentially useful marker for future introgression studies. Ten loci were found to form two clusters within a small internal region of the linkage map. Recombination was observed between these groups but not within groups. These observations indicate reduced levels of recombination between chromosome 6R^{T701} and chromosome 6R^{Imp}. These results are discussed with respect to the limitations of the TC-F₁ mapping population and the likely effects of structural differences between chromosomes 6R^{T701} and 6R^{Imp}.

PROJECT AIMS

The limited resistance of wheat cultivars to *H. avenae* is of importance both economically and culturally. Resistance to *H. avenae* has been reported in a small number of rye cultivars (Brown and Meagher, 1970; Meagher, 1972; Meagher and Brown, 1974; Brouwer and Castleman, 1981; Fisher, 1982a). Specifically, a gene conferring strong resistance to *H. avenae*, *CreR*, has been mapped to the long arm of chromosome 6R present in the triticales T701-4-6 (Asidue *et al.*, 1990; Dundas *et al.*, 1992). Initial attempts to introgress this gene into wheat via homoeologous recombination have been unsuccessful (Dundas *et al.*, 1992). It is presumed that the failure to isolate recombinants was due to differences in gene order on chromosome 6R relative to the wheat homoeologues. Consequently, the primary aim of the work presented in this thesis was to account for the problems concerning the lack of pairing between chromosome 6R of rye and chromosome 6D of wheat.

The secondary aim of the work presented was to develop a series of molecular tools which could be used in future attempts to introgress *CreR* from rye to wheat. The tools developed were:

- 1) A molecular-cytogenetic marker for the rye genome capable of allowing the rapid identification and characterisation of rye chromatin in alien backgrounds;
- 2) RFLP and PCR-based molecular markers for the long arm of chromosome 6R. A series of chromosome 6R long arm deletion lines was used to physically assign molecular markers to the long arm of chromosome 6R^{T701}.
- 3) A genetic linkage map of the long arm of chromosome 6R of rye incorporating RFLP markers, PCR-based markers and the locus, *CreR*.

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CHAPTER 1

REVIEW OF THE LITERATURE

1.1 CCN: HISTORY AND DISTRIBUTION

Members of the genera *Heterodera* belong to the "cyst forming" group of nematodes, are endoparasitic and in contrast to other nematode species, such as the root-knot nematodes (*Meloidogyne spp.*), tend to be very host specific (Schumann, 1991). The species *Heterodera avenae* Wollenweber, often referred to as cereal cyst nematode (CCN), is a major cause of disease in cereal crops throughout the world. This organism has been recorded in 31 countries and is believed to have originated in Europe (Meagher, 1977). While the distribution of cyst-nematodes is principally restricted to regions with temperate climates, a number of tropical and sub-tropical species have been described (Stone, 1977). *Heterodera avenae* Woll. is thought to have been introduced into Australia in the late 1800's (Meagher, 1977) and has since rapidly disseminated throughout the wheat growing regions of this country via the movement of cysts by wind (Meagher, 1974).

CCN has been recorded in all of the major wheat growing regions of Australia including Western Australia, South Australia, Victoria and New South Wales (Brown, 1982). It is, however, in the Southern wheat belt of Victoria and South Australia where this organism is of greatest concern where it infests over two million hectares of land and results in an estimated loss of between \$40 and \$80 million dollars annually to the wheat industry (Meagher, 1968; Rovira *et al.*, 1981). Nationally, this figure may be as high as \$173M (Stirling *et al.*, 1992).

Early work on this organism revealed that unlike the situation in Europe, this species of nematode caused greater damage to wheat than oats or barley (Davidson, 1930; Millikan, 1938a) leading these workers to regard this species of nematode as a "wheat race" due to its consistency and severity of attack on wheat (Millikan, 1938a). Variability both between and

within cereal species with respect to their response to attack by the nematode was also observed (Millikan, 1938*b*). This was the first demonstration of varying degrees of resistance and/or tolerance of cereals to this organism in Australia and extended the findings of Nilsson-Ehle (1920) who had earlier demonstrated differences in the levels of resistance between barley cultivars. Importantly, these authors recognised the existence of resistance and the use of resistant cultivars in the control of this organism.

1.2 PATHOTYPES

Andersen (1959) first demonstrated the existence of variation in the pathogenicity of nematode populations by comparing the reactions of different resistant barley varieties to nematode populations from various regions in Denmark. Cotten (1963) subsequently demonstrated the same phenomenon within British nematode populations.

Based on the International Test Collection of cereals at least ten different pathotypes have been identified. Of the ten recognised pathotypes, two have been observed in Sweden (Wählstedt, 1967), two in Denmark (Andersen, 1959; Jakobsen, 1981), three in Britain (Saynor, 1975), four in Germany (Lücke, 1976), four in The Netherlands (Kort *et al.*, 1964), one in Norway (Støen, 1971), two in Czechoslovakia (Sabová *et al.*, 1990) at least one in Spain (Romero, 1982) and four in France (Rivoal, 1977). Three pathotypes have been identified in India (Mathur *et al.*, 1974; Andersen and Andersen, 1982).

A single unique pathotype identified as Ha13 under the classification of Andersen and Andersen (1982) has been identified in Australia (Brown, 1969; Brown, 1974; Meagher, 1974; Brown, 1982) despite early suggestions that two pathotypes may exist (O'Brien and Fisher, 1974; Ellis and Brown, 1976).

1.3 CONTROL

The available methods of control are crop rotation (Millikan, 1938*b*), nematicides (Brown *et al.*, 1970; Brown, 1972; Gurner *et al.*, 1980), cultural practice (Andersson, 1982), host resistance (O'Brien and Fisher, 1974) and host tolerance (Fisher *et al.*, 1981). However, limitations in the current levels of understanding of nematode population dynamics, particularly multiplication rates and the relation between nematode densities and damage (Andersson, 1982; Fisher and Hancock, 1991), seasonal variation, particularly soil temperature and moisture (Meagher, 1970; Banyer and Fisher, 1971; Georg *et al.*, 1989) and environmental concerns arising from the use of nematicides (Brown, 1972; Schumann, 1991) emphasises the importance of the application of resistant and/or tolerant cultivars in the control of this organism.

1.3.1 Resistance

Resistance is defined as the ability of a plant to prevent or suppress nematode reproduction (Cotten and Hayes, 1969; Cook and York, 1982). As a consequence, cysts fail to develop on resistant cereal varieties. Consequently, resistant varieties are likely to yield better than susceptible cereals and carry-over of cysts in the field is reduced leading to better yielding crops, resistant or otherwise in subsequent years.

Nilsson-Ehle (1920) observed variation in cyst development on barley cultivars and demonstrated that resistance in the three cultivars Primus, Svanhals and Chevalier was dominant and inherited in a Mendelian fashion. Millikan (1938*a*) observed a number of "moderately resistant", "susceptible" and "very susceptible" barley varieties in field tests. The varieties Primus and Chevalier were found to be susceptible to the Australian pathotype, demonstrating (retrospectively) for the first time, variation in the effectiveness of resistance genes to different nematode populations.

Resistance to the Australian pathotype in wheat has been reported in Spring Wheat (AUS 10894), Loros (AUS 90248), Portugal 120 and Portugal 131 (O'Brien and Fisher, 1974); Loros (AUS 11577) and Psathias (AUS 881) (Brown, 1974; Cook and McLeod, 1980); Festiguay, Dural and Duramba (McLeod, 1976); seven lines of Mediterranean origin from the Australian Winter Cereals Collection (F. Green, personal communication); Algeria 33 and a number of *T. tauschii* accessions (E. Lagudah, personal communication). The wheat cultivar "Kaytil" was bred for cereal cyst nematode resistance from spring wheat AUS 10894 and released in Victoria in 1982 (Brown and Young, 1982)

Strong resistance to the Australian pathotype has been observed in barley cultivars Morocco, Marocain 079 and Sabarlis (Brown and Meagher, 1970; Ellis and Brown, 1976) and Athenais, Morocco, Nile, Orge-Martin 839 and CI 8147 (O'Brien and Fisher, 1977) while moderate resistance has been reported for a number of additional cultivars (Brown and Meagher, 1970). The barley cultivar "Galleon" released in 1981 represents the first commercial barley cultivar bred for cereal cyst nematode resistance in Australia (Sparrow and Dubé, 1981).

Resistance in cereal rye (*Secale cereale*) to the Australian pathotype has been described for a small number of cultivars. High levels of resistance have been observed in rye cv. South Australian (Brown and Meagher, 1970; Meagher and Brown, 1974). Brouwer and Castleman (1981) reported moderate resistance and tolerance in the triticale cv. Towan and Fisher (1982a) has reported a high level of resistance in the triticale line T701-4-6 with subsequent work (Asiedu, 1986) confirming the localisation of the resistance gene(s) to rye chromosome 6R.

1.3.2 Use of resistant cultivars

It has been suggested that reliance upon a single source of resistance may promote the appearance of new pathotypes of the nematode (Cook, 1974; Andersson, 1982). Therefore, a number of approaches for the use of resistant cultivars have been suggested. Andersen

(1980) suggests alternating between resistant and susceptible cereals, using resistant cereals only when required or cropping with mixtures of resistant and susceptible varieties. In contrast, Andersson (1982) argues that resistant cultivars carrying different sources of resistance should be used and should include resistant barley, oats and wheat. While the appearance of new pathotypes remains a distinct possibility, the Australian experience would suggest that this is an unlikely scenario (Meagher and Brown, 1974). In addition, it would be expected that the dissemination of any new pathotype would be a slow process leading to comparatively long lasting resistance (Cook, 1974; Cook and York, 1982).

1.4 INHERITANCE OF RESISTANCE

It is clear that inheritance of resistance to *H. avenae* in the cereals is under the control of a number of distinct genetic systems and includes both dominant and recessive, monogenic and polygenic modes of inheritance.

1.4.1 Wheat

Resistance to *H. avenae* in wheat is limited to a small number of cultivars. The two most utilised sources of resistance being the cultivar Loros (AUS 90248) and Spring Wheat (AUS 10894). Sloomaker *et al.* (1974) established that the mode of inheritance in Loros was monogenic dominant and localised the resistance gene to chromosome 2B via monosomic analysis. Resistance in Spring Wheat (AUS 10894) has been shown to be monosomic dominant and allelic to or identical with the resistance gene in Loros (O'Brien *et al.*, 1980; Nielsen, 1982). The presence of different modifier genes in these cultivars could not be ruled out and were suggested as being partly responsible for slight variations in response to the nematode observed within F₂ populations. Rivoal *et al.* (1986) identified resistance to pathotype Ha21 in the genome of *Aegilops ventricosa* and localised the gene(s) to chromosome 6M^V using disomic additions of the *A. ventricosa* chromosomes to the susceptible wheat cultivar Moisson. While this study demonstrated the existence of a

dominant gene, the existence of modifier genes within the *A. ventricosa* genome could not be excluded.

1.4.2 Barley

Sources of resistance in barley are varied, can be defined upon the basis of reaction to *H. avenae* pathotypes and fall into five broad groups: *RhaEmir*, resistant to pathotype B; *Rha1*, resistant to A, B and E; *Rha2*, resistant to A and C; *Rha3* resistant to A, B, C, E and G and a fifth class partially resistant to pathotypes A and C.

The genetic control of *RhaEmir* is unknown (Cook and York, 1982). *Rha1* is better understood. *Rha1* is monogenic dominant and probably located on the short arm of chromosome 2 (Andersen and Andersen, 1968, 1970; Cotten and Hayes, 1969). Resistance to pathotypes A and C (*Rha2*) was first identified in the barley cultivar L.P. 191 (Andersen, 1959; Andersen and Andersen, 1973). *Rha2* is not allelic to *Rha1* (Andersen and Andersen, 1968) and is monogenic dominant (Cotten and Hayes, 1969; Nielsen, 1982). *Rha2* has been mapped to chromosome 2 of barley and linkage with a number of loci including six-row, liguleless and a translocation breakpoint has been established (Cotten and Hayes, 1969; Andersen and Andersen, 1973).

Rha3 is characterised by the cultivar Morocco which is resistant to the ten recognised pathotypes (Andersen and Andersen, 1982) but susceptible to a small number of additional (virulent) populations (Andersen and Andersen, 1982; Cook and York, 1982). The nature of inheritance is unclear (Andersen and Andersen, 1970; O'Brien *et al.*, 1979).

Pathotype studies have differentiated *Rha2* from the resistance found in cultivars Morocco, Marocain, Bajo Aragon-1-1 and Martin 403-2 indicating either clear differences between the resistance genes identified or the presence of additional resistance or modifier genes. It is possible that resistance defined as *Rha3* is due to a combination of different genes and that the

range of resistances observed between those characterised by *Rha2* and *Rha3* are dependent upon the presence of additional resistance or modifying loci.

1.4.3 Rye

There have been very few reports on the inheritance of resistance found in rye. The resistance in rye cv. South Australian is unknown. However, Asiedu (1986) determined that resistance to the Australian pathotype Ha13, identified in the triticale T701-4-6, was monogenic dominant and located on chromosome 6R. Subsequent work localised this resistance gene to the long arm of 6R (Asiedu *et al.*, 1990; Dundas *et al.*, 1992)

1.5 TOLERANCE

Differences in tolerance of cereals to CCN has been known for many years (Franklin, 1951) and the overriding opinion is that oats are more tolerant than wheat and wheat more tolerant than barley (Gair, 1965; Kerry and Jenkinson, 1976; Vallotton, 1980). Traditionally tolerance is viewed as the ability of the plant to withstand attack by the nematode as defined by some measure of growth, usually yield. Variable levels of tolerance both between and within cereal species has been recognised (Mathison, 1966; Fisher *et al.*, 1981) and it is clear that genetic control of tolerance is distinct to that of resistance (Dropkin, 1955; Jones, 1956; Fox and Spasoff, 1976).

Analysis of tolerance has centred on the impact of nematode invasion on host plant root systems. Tolerance in some wheat cultivars appears to be positively correlated with a reduction in root growth and a slower utilisation of endosperm reserves (Stanton and Fisher, 1988). In oats, tolerance has been found to be due (in part) to a lesser impact of nematode invasion on seminal root extension and to earlier development of nodal roots (Volkmar, 1989) while in some barley cultivars, increased levels of tolerance is associated with the number of root tips and seminal root axes in a "dilution" effect (Rawsthorne and Hague, 1985). Fisher

(1982b) has demonstrated that an association exists between the relative tolerance of cereals and the number of seminal roots (mean of 3 for oats, 5 for wheat and 7 for barley) implicating the plant response to initial nematode invasion as the basis for tolerance.

1.5.1 Resistance and tolerance

Since most mechanisms of resistance operate *after* nematode invasion, the ability to combine resistance and tolerance offers the greatest potential for effectively controlling nematode levels in soils while achieving optimal crop yields. This has wide support and most workers advocate such a strategy (Andersson, 1982; Fisher, 1982b).

1.6 CYTOGENETICS OF *TRITICUM*, *HORDEUM* AND *SECALE*

1.6.1 Chromosome number and polyploidy

The genomes of wheat and other species within the Triticeae are assumed to have evolved from a common ancestral genome. Consequently, all are characterised by a haploid base number, $x=7$. Species within the genus *Triticum* form a three-level ploidy series; diploids have the genome constitution $2n=2x=14$, tetraploids have the genome constitution $2n=4x=28$ and hexaploids have the genome constitution $2n=6x=42$. Cultivated wheat (*Triticum aestivum* L. em Thell.) is an allohexaploid with the genome configuration $2n=6x=42$.

The genera *Secale* and *Hordeum* contain only diploid species having a genome configuration $2n=2x=14$. Within the genus *Secale* are cultivated and wild species which are annuals and perennials and include cultivated rye *S. cereale* L., *S. montanum* Guss., *S. vavilovii* Grossh., *S. africanum* Staph. and *S. silvestre* Host. *S. kuprijanovii* Grossh., *S. dalmaticum* Vis. and *S. anatolicum* Boiss. are considered sub-species of *S. montanum* (Zeller and Cermeño, 1993). *Hordeum* contains *H. spontaneum* species, cultivated barley *H. vulgare* and wild barley, *H. bulbosum*.

1.6.2 Genomic classification

Chromosome pairing studies are perceived as a reliable method of analysis of allopolyploid genomes (Kimber, 1984) and have formed the basis for genomic classification within the Triticeae. High numbers of univalents (little or no pairing) in species hybrids is regarded to infer a distant evolutionary relationship between genomes while high numbers of bivalents or multivalents (high levels of pairing) infers a relatively close evolutionary relationship.

Three genomes designated A, B and D have been recognised in hexaploid wheat (for review, see Lilienfeld, 1951). The A, B and D genomes are considered to have originated from distinct, wild diploid species. The A genome of diploid wheat (*T. monococcum*) is common to *T. turgidum* and *T. aestivum*, the B genome to *T. turgidum* and *T. aestivum* and the third genome, D is present in *Aegilops* spp. and *T. aestivum*. Consequently, *T. aestivum* has the genomic constitution AABBDD.

The genomes of cereal rye (*S. cereale* L. [2n=2x=14]) and barley (*H. vulgare* L. [2n=2x=14]) are designated R and H respectively. Thus, diploid rye has the genomic constitution RR and barley HH.

1.6.3 Homoeology

Substitution-compensation tests, analysis of homoeologous pairing at metaphase I and conservation of syntenic groups through comparative gene mapping allow the determination of homoeology.

Substitution-compensation tests in which specific chromosomes compensated for the phenotypic disturbances associated with nullisomy allowed the 21 chromosomes of wheat to be placed into seven "homoeologous" groups of three (Sears, 1952, 1954). The interrelationship between homoeologous groups and genomes is such that each chromosome

within the A, B and D genomes belongs to a specific homoeologous group. Homoeologous groups are defined numerically one through to seven resulting in a two-way genome classification (ie., 1A-7A, 1B-7B and 1D-7D).

The homoeologous grouping defined with regard to the A, B and D genomes of hexaploid wheat has also been extended to many other genera within the Triticeae including *Aegilops*, *Agropyron*, *Haynaldia*, *Hordeum* and *Secale* (for reviews, see Gupta, 1993; Sakamoto, 1993). This work has utilised the various series of alien-wheat chromosome addition and substitution lines available and has relied principally on substitution-compensation analyses along with assessment of meiotic pairing with wheat chromosomes. Chromosomes of *Secale cereale* and *Hordeum vulgare* are designated 1R-7R and 1H-7H respectively. In addition to the homoeology seen amongst members of the Triticeae, conservation of gene order or synteny has now been well documented throughout the Graminaea (Ahn and Tanksley, 1993; Ahn *et al.*, 1993; Devos *et al.*, 1994; Van Deynze *et al.*, 1995).

1.6.4 Chromosome nomenclature

The karyotype of somatic metaphase chromosomes as defined by their number, size and morphology has been established for many members of the Triticeae and until quite recently has been the principal tool for genome analysis within the tribe. The karyotypes of most cultivated species including *Triticum*, *Secale* and *Hordeum* are characterised by metacentric or submetacentric chromosomes (Gupta, 1993) with few morphological differences thereby complicating the positive identification of individual chromosomes. In addition, it is often difficult to correctly identify the long and short arms of metacentric chromosomes due to similarities in both arm length and morphology (Singh and Tsuchiya, 1982). Other problems include the normal variation of chromosome morphology in cultivars and populations. However, chromosomes 1A, 1B, 6B and 5D of common wheat, chromosome 1R of rye and 1H of barley are all characterised by nucleolus organising regions (NORs) and are readily identifiable on the basis of secondary structure (satellited regions).

1.6.5 Chromosome banding

The application of banding methods within the Triticeae has, in the main, been limited to C-banding, N-banding and Ag-banding. C-banding is a general method for the detection of constitutive heterochromatin (for definition, see Greilhuber, 1977) which in cereals is comprised of arrays of highly repeated, late-replicating DNA (Appels *et al.* 1978; John and Miklos, 1979; Bedbrook *et al.* 1980a; Jones and Flavell, 1982a, b). N-banding detects a more specialised class of heterochromatin within plant (and animal) chromosomes containing polypyrimidine-polypurine tract DNA sequences (Gerlach, 1977; Appels *et al.* 1978; Gerlach *et al.* 1978; Dennis *et al.* 1980; Singh and Tsuchiya, 1982; Chen and Gill, 1983). In contrast, Ag-banding selectively stains active nucleolar organising regions and has limited use as a cytological tool outside the identification of such regions.

Consequently, banding of cereal chromosomes has allowed the classification of heterochromatin on the basis of its ability to be stained through C-banding methods (C^+ , C^-) or N-banding methods (N^+ , N^-) and two types of heterochromatin can be differentiated; C^+N^- or C^+N^+ (Schlegel *et al.*, 1986; Gill and Sears, 1988; Lange, 1988). Comparison of C- and N-banding patterns between wheat, barley and rye chromosomes reveals quite divergent patterns of heterochromatin distribution between these species. In wheat and barley, C- and N-staining heterochromatin is predominantly intercalary while in rye, it is located at the telomeres with few intercalary sites.

Since the first reports of C-banding and N-banding of wheat, rye and barley chromosomes (Sarma and Natarajan, 1973; Merker, 1973; Gill and Kimber, 1974a, b; Natarajan and Sarma, 1974; Zurabishvili *et al.*, 1974; Linde-Laursen, 1975; Gerlach, 1977; Jewell, 1979), chromosome banding techniques have become a pivotal tool for cytological studies within the Triticeae (for review, see Gill and Sears, 1988). Of major benefit is the ability to identify chromosomes on the basis of a banding karyotype thereby eliminating the problems associated with differential contraction (Endo and Gill, 1984) and distortion of chromosomes

through preparation. However, present chromosome banding methodologies suffer a number of problems. For example, the C-banding pattern revealed by one method may differ from that of another for the same karyotype (Martin and Hesemann, 1988).

In contrast to the complex banding patterns observed for human and mammalian chromosomes, banding methods have, in general, revealed only relatively simple banding patterns in cereals. It is specifically the lack of interstitial banding of chromosomes of the A and D genomes of wheat and the R genome of rye which limit chromosome banding studies in cereals (Lukaszewski and Gustafson, 1983; Fukui *et al.*, 1988; Lange, 1988). Furthermore, C-banding identifies chromosomes on the basis of the presence or absence of late-replicating heterochromatin, irrespective of its nucleic acid composition (Rayburn and Gill, 1986). Thus, all C-bands appear the same, chromosomes lacking heterochromatin cannot be identified and euchromatic regions remain cytologically uncharacterised.

Improved resolution of banding methods has allowed accurate and reliable identification of all 21 wheat, 7 rye and 7 barley chromosomes on the basis of C-banding and, through N-banding, the identification of 16 chromosomes of the wheat complement as well as all 7 barley chromosomes (Linde-Laursen, 1975; Darvey and Gustafson, 1975; Sybenga, 1983; Endo, 1986; Schlegel and Gill, 1987; Gill, 1987; Lange, 1988). However, identification of wheat chromosomes, particularly those of the A and D genomes may often require additional cytological techniques such as chromosome pairing analysis, the use of aneuploid stocks and *in situ* hybridisation (Fukui *et al.*, 1988; Jiang *et al.*, 1993). Rye chromosomes may, through natural variation or terminal deletions possess little or no terminal heterochromatin (Darvey and Gustafson, 1975; Pilch, 1981; Appels *et al.*, 1982; Jouve *et al.*, 1989). Since it is the prominent telomeric bands which enable accurate identification of rye chromosomes in wheat-rye hybrids such as triticale, absence of these bands generally renders banding methods ineffectual.

G-banding in wheat, barley, rye and maize has been reported (Zhu *et al.*, 1986; Fukui *et al.*, 1989; Kakeda *et al.*, 1990), although the best examples have utilised prophase chromosomes. Banding of metaphase chromosomes remains poor, lending credence to the possibility that G-bands in plants may be unresolvable due to increased levels of contraction of plant chromosomes at metaphase (Greilhuber, 1977). While this theory is contested by the findings of Anderson *et al.* (1982) and Bennett *et al.* (1983), the number of G-bands in mammalian chromosomes is reduced significantly as a result of band fusion as chromosomes progress from prophase to metaphase (Yunis *et al.*, 1978, 1980; Sawyer *et al.*, 1987).

An additional, important factor limiting the use of chromosome banding analyses within the Triticeae is the variability between cultivars and populations for both C- and N-banding patterns (Weimark, 1975; Lelley *et al.*, 1978; Giraldez *et al.*, 1979; Endo and Gill, 1983; Pilch and Hesemann, 1986; Jouve *et al.*, 1989). While polymorphisms for C-bands provide useful cytological markers (particularly in chromosome pairing studies and gene mapping analyses) and may be significant enough to allow cultivar identification (Endo and Gill, 1983), they confound attempts to establish standard karyotypes. As a result of nomenclature proposals discussed at the 7th International Wheat Genetics Symposium (IWGS), Cambridge, England, Gill *et al.* (1991) have presented a standard karyotype of wheat based on *Triticum aestivum* L. Chinese Spring which includes reliable or landmark N-banding, C-banding and modified C-banding. The nomenclature system devised accounts for both chromosomal aberrations, band polymorphisms, present genetic nomenclature of wheat and remains speculative with respect to future methodologies such as G-banding and *in situ* hybridisation.

Similarly, the Chinese Spring-Imperial wheat-rye addition series (Driscoll and Sears, 1971), has been accepted as the standard set of rye chromosomes from which to establish a standard rye karyotype (Sybenga, 1983). From this, numerous workers have presented general karyogrammes according to the nomenclature system adopted at the 1st International Workshop on Rye Chromosome Nomenclature and Homoeology Relationships (Sybenga, 1983; Schlegel *et al.*, 1986; Schlegel and Gill., 1987). However, Mukai *et al.* (1992) using

designations similar to those recommended for wheat (Gill *et al.*, 1991), have re-analysed the Chinese Spring-Imperial wheat-rye addition series and presented the most detailed C-banded karyotype for rye to date.

1.6.6 *In Situ* Hybridisation

The technique of *in situ* hybridisation (ISH) involves the annealing of labelled nucleic acid probes to DNA or RNA targets preserved in cytological preparations followed by their detection; in the case of radio-labelled probes, via autoradiography. This represents the most direct approach for mapping DNA sequences at a subchromosomal level. The localisation of single-copy DNA sequences to human metaphase chromosomes was first realised by Harper and Saunders (1981) and Gerhard *et al.* (1981) using radio-labelled probes (^3H and ^{125}I , respectively). Subsequently, many low- and single-copy genes have been mapped to human chromosomes (Harper and Saunders, 1981; Gerhard *et al.*, 1981; Jhanwar *et al.*, 1983; Zabel *et al.*, 1983). However, isotopic ISH suffers a number of inherent problems. Spatial resolution is limited since visualisation of hybridisation is a function of both β -particle emission and resulting formation of silver grains *above* the target. Also, statistical analysis of many metaphases is usually required and detection of hybridisation often requires exposure times of many weeks. Additional concerns are chemical lability due to radioactive decay and personnel safety.

Non-isotopic ISH methods have been developed (for review see, Hopman *et al.*, 1988; Wilchek and Bayer, 1988; Leary and Ruth, 1989). Biotin has been the most extensively utilised hapten since it is readily incorporated enzymatically into nucleic acids through the use of biotinylated analogues of dTTP, UTP and dUTP with little effect on denaturation and reassociation properties (Langer *et al.*, 1981). Immunological detection commonly exploits the high affinity of biotin for the proteins avidin and streptavidin (Green, 1975). Both proteins are commonly used directly conjugated to an enzyme (ie., horseradish peroxidase, HRP) or fluorochrome label (ie., fluorescein isothiocyanate, FITC) (for review, see Hopman

et al., 1988; Wilchek and Bayer, 1988; Leary and Ruth, 1989; McNeil *et al.*, 1991). Indirect detection is also possible using anti-biotin antibodies together with secondary antibodies enzymatically or fluorescently labelled. Enzyme labels are colourimetrically detected by the addition of appropriate chromogenic substrates after hybridisation is complete, resulting in the site-specific formation of an insoluble precipitate (Langer-Safer *et al.*, 1982). Fluorescent labels require only fluorescent microscopy to visualise hybridisation (for review, see Lichter *et al.*, 1991; McNeil *et al.*, 1991).

Advantages of non-isotopic ISH are numerous and include greater stability of labelled nucleic acids, reduced levels of background hybridisation and the ability to manipulate detection sensitivity through the application of successive cytochemical layers. For example, avidin-FITC and biotinylated anti-avidin antibodies (Pinkel *et al.*, 1986). Consequently, using non-isotopic ISH, it is possible to detect unique DNA sequences as small as 1-6 kb on human metaphase chromosomes (Garson *et al.*, 1987; Albertson *et al.*, 1988; Lawrence *et al.*, 1988; Lichter *et al.*, 1988; Viegas-Pequignot *et al.*, 1989). Moreover, with the recent development of additional haptens such as digoxigenin and improved fluorescent labels, it is possible to simultaneously hybridise different DNA sequences to metaphase and interphase cells resolving gene order and linkage relationships (Lawrence *et al.*, 1990; McNeil *et al.*, 1991). Importantly both resolution and speed have been greatly enhanced and most non-isotopic methods can be completed within a day.

Within the Triticeae, as with all other plant genera, the application of ISH has largely been confined to the localisation of repetitive DNA sequences and re-iterated gene families (Appels *et al.*, 1978; Hutchinson *et al.*, 1980; Gerlach and Peacock, 1980; Dennis *et al.*, 1980; Bedbrook *et al.*, 1980a; Jones and Flavell, 1982a, b; Rayburn and Gill, 1985a, b, 1986; Lapitan *et al.*, 1986; Mukai *et al.*, 1990, 1991). However, the repeated sequence component of rye has been the most extensively studied. In addition to the six distinct families of tandemly repeated sequences which constitute both telomeric and interstitial heterochromatic regions (Bedbrook *et al.*, 1980a; Appels *et al.*, 1981; Jones and Flavell, 1982a, b), a number

of dispersed, repetitive sequences have been identified and characterised also (Appels *et al.*, 1986a; McIntyre *et al.*, 1988; Guidet *et al.*, 1991).

Sequences demonstrating differential hybridisation patterns in wheat and rye chromosomes or those demonstrating rye specificity have been used to assay for the presence of rye chromosomes in various wheat-rye hybrids (Bedbrook *et al.*, 1980a; Hutchinson *et al.*, 1980; Jones and Flavell, 1982a, b; Appels *et al.*, 1986a; Guidet *et al.*, 1991), identify wheat-rye translocations (May and Appels, 1980; Appels and Moran, 1984), quantitatively assess rye telomeric heterochromatin in *Secale* species, rye cultivars and wheat-rye hybrids (May and Appels, 1980; Appels *et al.*, 1982; Jones and Flavell, 1982b; Lapitan *et al.*, 1988) and to investigate the nature of chromosome pairing in *Aegilops-Secale* hybrids (Hutchinson *et al.*, 1980).

The localisation of repetitive sequences to wheat chromosomes using non-isotopic ISH was first demonstrated by Rayburn and Gill (1985a). Subsequently, non-isotopic ISH using repetitive DNA sequences has become an extremely useful cytological tool within the Triticeae. Chromosome-specific banding patterns produced by certain repetitive sequences allow identification of individual chromosomes and aid chromosome karyotyping (Rayburn and Gill, 1985a, b, 1986; Friebe *et al.*, 1991a; Mukai *et al.*, 1992). In addition, chromosome substitutions, additions and rearrangements have been identified (Lapitan *et al.*, 1986, 1988; Friebe *et al.*, 1991b; Mukai and Gill, 1991) as well as entire genomes within natural polyploids and interspecies hybrids such as triticale (Rayburn and Gill, 1986; Mouras *et al.*, 1991, this thesis). Since genome-specific, dispersed repetitive sequences allow cytological detection of euchromatin, it is possible to determine with precision, the breakpoints of translocated chromosomes (Lapitan *et al.*, 1986; McIntyre *et al.*, 1990) as well as the amount and physical location of introgressed chromatin (Gill and Sears, 1988). However, these studies are limited by the availability of suitable cloned, repetitive DNA sequences.

Genomic *in situ* hybridisation (GISH) utilises biotinylated total genomic DNA from the donor or parental line in combination with unlabelled total genomic *competitor* DNA from the recipient or second parental line. This method has wide application since it obviates the need for cloned, species or genome-specific, dispersed repetitive DNA sequences. GISH has been used to identify chromosomes from parental genomes in barley x rye species hybrids (Schwarzacher *et al.*, 1989; Anamthawat-Jónsson *et al.*, 1990), wheat x rye hybrids (Le *et al.*, 1989; Zhong *et al.*, 1991; Mukai *et al.*, 1992), wheat x barley hybrids (Mukai and Gill, 1991) and to identify chromosome segments in a number of wheat-rye translocation lines (Le *et al.*, 1989; Heslop-Harrison *et al.*, 1990; Zhong *et al.*, 1991; Mukai *et al.*, 1993) and wheat-*Agropyron* translocation lines (Friebe *et al.*, 1993; Jiang *et al.*, 1993). Importantly, this method allows precise determination of translocation breakpoints and is sufficiently sensitive to allow detection of small, intercalary introgressions of alien chromatin (Mukai *et al.*, 1993).

An essential pre-requisite for efficient *in situ* hybridisation is adequate access of labelled probe to denatured chromosomes. Most significantly, it has been the ability to prepare high quality metaphase spreads free of cell wall debris and cytoplasm through the application of protoplasting methods which has enabled the detection of low- and single-copy sequences to plant chromosomes (Ambros *et al.*, 1986; Mouras *et al.*, 1987; Huang *et al.*, 1988). In cereals, the localisation of low-copy sequences has been possible only through the development of protoplasting methods. However, detection efficiency remains low due to the poor quality and reduced mitotic index (MI) of such preparations.

Improved techniques have led to greater sensitivity for both isotopic and non-isotopic methods in plants. Consequently, numerous low- and unique-sequences have been localised to plant metaphase chromosomes (Ambros *et al.*, 1986; Mouras *et al.*, 1987; Shen *et al.*, 1987; Huang *et al.*, 1988; Huang *et al.*, 1989; Simpson *et al.*, 1988; Mouras *et al.*, 1989; Clark *et al.*, 1989; Gustafson *et al.*, 1990). For example, Clark *et al.* (1989) identified B-hordein loci on barley chromosome 5 using a 1 kb biotinylated cRNA probe while Gustafson

et al. (1990), using a biotinylated 900 bp cDNA clone mapped a number of endosperm-storage protein loci to rye metaphase chromosomes.

Efficiency of signal detection of low- and unique copy sequences in plants varies dramatically ranging from 60%-80% (Mouras *et al.*, 1987) to 0.025% (Clark *et al.*, 1989). Reasons for the extremes in variation can be attributed to the nature of target sought; as the size of both the probe and target decrease, so does the ability to detect hybridisation. A number of factors are critical to the sensitivity of ISH and include high specific activity of (isotopically) labelled probes (Mouras *et al.*, 1989), efficient denaturation of both probe DNA and chromosome DNA (Mouras *et al.*, 1989; Clark *et al.*, 1989) and the ability of labelled probe DNA to form "networks", effectively increasing the localised concentration of either isotope or biotin (Huang *et al.*, 1988). Consequently, choice of probes and labelling methods are important. However, perhaps the most important issue affecting ISH sensitivity in plants is the difficulty in obtaining high quality metaphases with a high MI. In species where this is possible detection efficiencies are comparable to human studies (Mouras *et al.*, 1987; Huang *et al.*, 1988).

1.7 CONSERVATION OF SYNTENY WITHIN THE TRITICEAE

1.7.1 *Triticum*

Sears (1954) alluded to the presence of duplicated genes on each homoeologue (homoeoloci) assuming that effective substitution was dependent upon the ability to restore normal gene dosage from four to six. This hypothesis has since been confirmed through the localisation of structural genes either through the analysis of isozymes (for review see Hart, 1987) or by classical and molecular genetic techniques (see McIntosh, 1988).

Both intra- and inter-chromosomal rearrangements within the wheat genome have been identified as a result of their effects on both pairing and ability to compensate (Riley and Chapman, 1966; Gill and Chen, 1987; Naranjo *et al.*, 1987). However, relatively few

chromosomal rearrangements have been identified within the genome of *T. aestivum* cv. Chinese Spring (Sears, 1954; Chapman *et al.*, 1976; Dvorák *et al.*, 1984). Consequently, *T. aestivum* cv. Chinese Spring is accepted as the standard cultivar for cytogenetic research.

Homoeology among the A, B and D genomes of wheat has been further elucidated through the localisation of structural genes encoding isozymes utilising the nullisomic-tetrasomic lines and ditelosomic lines of Chinese Spring (Hart, 1975, 1982, 1983; Hart and Tuleen, 1983; Hart, 1987 for review). Presently, at least 21 enzymes, encoded by 19 triplicate and 2 duplicate sets of paralogous genes have been localised to homoeologues of the A, B and D genomes with at least one set in each homoeologous group (Hart, 1979, 1982, 1983; Zeller and Cermeño, 1993). This data indicates a high degree of homoeology between the A, B and D genomes of hexaploid wheat with few exceptions.

The localisation of genes via molecular genetic techniques has established high levels of synteny between the three genomes of hexaploid wheat. Due to the greater number of loci analysed, a more detailed picture of the extent of the conservation of synteny between the A, B and D genomes has emerged. The generation of genetic linkage maps for wheat group 7 chromosomes (Chao *et al.*, 1989) and wheat group 3 chromosomes (Devos *et al.*, 1992) for example, reveals a high degree of co-linearity for loci across the A, B and D genomes for these chromosomes. Significantly, the study of Chao *et al.* (1989) identified the presence of a number of isozyme and RFLP loci mapping to 7AS, 7DS and 4AL. Both Anderson *et al.* (1992) and C. J. Liu *et al.* (1992) have since extended this work and have demonstrated, on the basis of chromosomal localisation of complementary DNA (cDNA) probes and genomic DNA (gDNA) probes, the occurrence of a series of reciprocal chromosomal translocations involving 4AL, 5AL and 7BS.

1.7.2 *Hordeum and Secale*

The chromosomes of common barley (*Hordeum vulgare* L.) fall into each of the seven homoeologous groups and appear largely unaltered to those of the wheat genome (Bothmer and Jacobsen, 1985). Chromosomes 2 and 7 of barley have been shown to be homoeologous with groups 7 and 5 of wheat through the comparison of phenotypes of wheat-barley addition lines with wheat tetrasomics (Islam and Shepherd, 1981). The localisation of homoeoloci for alcohol dehydrogenase (*Adh-1*), glutamic oxaloacetic transaminase (*Got-2*), aminopeptidase (*Amp-1*) and endopeptidase (*Ep-1*) indicate homoeology of barley chromosomes 4, 6 and 1 with the Chinese Spring chromosomes of homoeologous groups 4, 6 and 7 (Hart *et al.*, 1980). Analysis of additional isozyme loci indicates that there have been no structural changes within the barley genome relative to those of wheat with complete concordance regarding their chromosomal location (Hart and Tuleen, 1983, Hart, 1987). More recently, comparative RFLP mapping has highlighted the high levels of synteny shared between barley, wheat, rye and *T. tauschii* chromosomes (Devos and Gale, 1993; Devos *et al.*, 1993a; Namuth *et al.*, 1994; Hohmann *et al.*, 1995; Sherman *et al.*, 1995). Consequently, barley chromosomes 1, 2, 3, 4, 5, 6 and 7 are homoeologous to wheat analogues and are designated 7H, 2H, 3H, 4H, 1H, 6H and 5H, respectively.

The ability of individual rye chromosomes to substitute for their respective homoeologues is variable. Substitution-compensation analyses have demonstrated homoeology of rye chromosomes 1R, 2R, 3R, 5R and 6R to wheat groups 1, 2, 3, 5, and 6 (Riley, 1965; Sears, 1968; Gupta, 1971; Miller, 1984) while chromosomes 4R and 7R demonstrate partial reciprocal homoeology to wheat groups 4 and 7 (Koller and Zeller, 1976; Naranjo *et al.*, 1987). Subsequent work involving the analysis of homoeologous pairing between wheat and rye chromosomes and localisation of homoeoloci via isozyme and molecular genetic studies has further refined the relationship of individual rye chromosomes with those of wheat. While generally in support of the homoeologous assignment of rye chromosomes as described, there are many instances where homoeology of rye chromosomes to more than one

group has been identified (Miller, 1984; Zeller and Cermeño, 1993). In a study based on the pattern of allosyndetic pairing in *ph1b* mutant wheat-rye hybrids, Naranjo and Fernández-Rueda (1991) were able to conclude that only chromosome arms 1RS, 1RL, 2RL, 3RS and 5RL demonstrate normal homoeology to wheat.

Numerous studies have been carried out using RFLP technology comparing the degree of conservation of syntenic groups within the Triticeae, with particular emphasis on the genomes of wheat, rye and barley (Devos *et al.*, 1992; C. J. Liu *et al.*, 1992; Wang *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993a, b; Namuth *et al.*, 1994; Hohmann *et al.*, 1995). However, since the gene conferring resistance to *H. avenae* has been localised to the long arm of chromosome 6R of rye, homoeology within group 6 chromosomes of the Triticeae is discussed in more detail.

1.7.2.1 Chromosome 6R

Substitution-compensation studies involving rye 6R indicate high levels of homoeology between this chromosome and wheat group 6 chromosomes (Riley, 1965; Jenkins, 1966). Chromosome 6R from *S. montanum* has also been substituted for chromosomes 6A, 6B and 6D of Chinese Spring (cited in Miller, 1984). However, the ability of rye chromosome 6R to compensate for wheat group 7 chromosomes has also been demonstrated (cited in Koller and Zeller, 1976).

Short arm

The presence of homoeoloci *co* and *Amp-1* on both 6RS and 6WS indicates homoeology between these chromosome arms (Tang and Hart, 1975; Miller, 1984; McIntosh, 1988). However, pairing between 6RS and 6WS is seldom observed (Naranjo and Fernández-Rueda, 1991). The localisation of homoeoloci (Sharp *et al.*, 1989) and chromosome pairing studies (Naranjo and Fernández-Rueda, 1991) have identified a translocation between 6RS and 4RL.

This rearrangement appears to be non-reciprocal (Sharp *et al.*, 1989; Devos *et al.*, 1993b) with the segment of 6RS being distally located on the long arm of 4R (Naranjo and Fernández-Rueda, 1991; Devos *et al.*, 1993b). Furthermore, 7RS, a potential recipient of 6RS material through rearrangement with 4RL, displays no apparent homoeology with the short arm of group 6 chromosomes.

The structural genes encoding *Gli-2* have been localised to 2RS, 6AS, 6BS and 6DS (McIntosh, 1988; Devos *et al.*, 1993b) and to 6R^{mon} (Shewry *et al.*, 1985) suggesting a translocation involving chromosomes 2R and 6R in the evolution of rye relative to wheat. Devos *et al.* (1993b) have sought to explain these observations via a series of reciprocal translocations involving initially 6RS and 2RS, followed by a second rearrangement between 6RS/2RS and 7RL. These rearrangements are thought to have resulted in the transfer of the segment of 2RS to 7RL via 6RS with the reciprocal transfer of a segment of 7RL to 6RS. Finally, the 7RL segment present on the short arm of 6R has, through a pericentric inversion been transferred to the long arm of 6R. This model agrees well with the localisation of additional homoeoloci such as *Got-1* (Hart, 1975; Wehling, 1991) and explains the lack of pairing found for this chromosome arm with wheat group 6S chromosome arms (Naranjo and Fernández-Rueda, 1991).

Concerning synteny of loci on the short arm of chromosome 6R, all that may be presently concluded is that all loci mapping to 6RS have homoeoloci on wheat chromosomes 6AS, 6BS and 6DS (McIntosh, 1988; Sharp *et al.*, 1989; Devos *et al.*, 1993b). In addition to the 6RS/2RS translocation identified in rye, the localisation of the RFLP marker *Xpsr899* to 2BS, 6AS and 6DS indicates a translocation involving 2BS and 6BS (Devos *et al.*, 1992). However, *Xpsr899* maps to 4RL and not 2RS indicating that these rearrangements are the result of independent events since speciation.

Long arm

The long arm of rye chromosome 6R displays considerable homoeology with the long arms of wheat group 6 chromosomes. In addition to low levels of pairing observed between 6RL and the long arms of wheat group 6 chromosomes (Naranjo and Fernández-Rueda, 1991), homoeoloci *Got-2*, *α-Amy-1*, *Adh-3*, *Aco-1* and *Xpsr152* have been localised to the long arms of both wheat and rye group 6 chromosomes (Hart, 1975; Tang and Hart, 1975; Hsam *et al.*, 1982; Hart and Tuleen, 1983; Miller, 1984; McIntosh, 1988; Sharp *et al.*, 1989; Devos *et al.*, 1993b).

Based upon cytological and substitution-compensation data, Koller and Zeller (1976) proposed that the long arm of 6R had been involved in a translocation with the long arm of chromosome 7R. More recently, Benito *et al.* (1991) have provided biochemical evidence for a translocation involving 6RL and 7RL through the localisation of the structural gene encoding endopeptidase (*Ep-1*) to 6RL. Hart and Langston (1977) and Koebner *et al.* (1988) previously localised *Ep-1* homoeoloci to wheat chromosomes 7BL and 7DL. More recently, Chao *et al.* (1989) have demonstrated linkage between *Ep-1* and the wheat group 7L RFLP marker PSR121 while Devos *et al.* (1993b) have constructed a linkage map of the distal segment of 6RL comprising *Ep-1* and 4 7WL RFLP markers. Also indicative of this rearrangement is the low frequency of pairing observed between 6RL and 7WL (Naranjo and Fernández-Rueda, 1991). The inability to detect 6RL loci on 7RL is consistent with a non-reciprocal translocation providing evidence for a pericentric inversion.

The relatively high levels of pairing observed between 3RL and the long arm of wheat group 6 chromosomes (Naranjo *et al.*, 1987; Naranjo and Fernández-Rueda, 1991) and low levels of pairing observed between 6RL and the long arms of wheat group 3 chromosomes (Naranjo and Fernández-Rueda, 1991) indicate a reciprocal translocation involving 6RL and 3RL. Genes controlling esterase and red grain colour, both previously mapped to homoeologous group 3 chromosomes, have been localised to 6R (Miller, 1984). Moreover, linkage

mapping the long arm of 6RL using wheat group 6L, 3L and 7L loci indicate that the segment of 3RL translocated to 6RL is interstitial with respect to 6RL and 7RL (Devos *et al.*, 1993b). On this basis, the rearrangement involving 6RL and 3RL is believed to have preceded the translocation and subsequent pericentric inversion involving 7RL (Devos *et al.*, 1993b). No group 6L homoeoloci have been localised to the long arm of 3RL. Consequently, the breakpoint within 3RL can only be inferred by the inability to detect group 3 long arm loci distal of *XCxp1* (Devos *et al.*, 1993a, b).

1.8 MOLECULAR GENETICS OF *TRITICUM*, *HORDEUM* AND *SECALE*

1.8.1 Genome Size and Composition

The nuclear DNA content of hexaploid wheat (A, B and D genomes) is estimated at 18.1 pg per haploid nucleus, corresponding to 1.6×10^{10} bp (Bennett and Smith, 1976; May and Appels, 1987). The DNA content of each genome present in hexaploid wheat is not even, with ratios of 1.21 : 1.36 : 1.00 for the A, B and D genomes respectively (Furuta *et al.*, 1988), which are in good agreement with length measurements of somatic chromosomes. Typical of many plant species, a highly significant correlation ($r=0.9$) exists between DNA content and somatic chromosome length of wheat chromosomes allowing estimation of the DNA content of individual chromosomes (Gustafson and Bennett, 1976). Nuclear DNA content in 2x, 4x and 6x wheats does not conform to a simple 1:2:3 ratio (Furuta and Nishikawa, 1993) and the nuclear DNA content of the diploid progenitors of wheat has been shown to differ to that of their respective homoeologous genomes within hexaploid wheat (May and Appels, 1987). This has been interpreted to infer the amplification or deletion of DNA within the diploid progenitors during coevolution (Gupta *et al.*, 1993).

Barley has a 1C DNA value of 5.5 pg, corresponding to 5.3×10^9 bp per haploid genome while the 1C DNA content of rye has been determined to be 9.5 pg (Bennett and Smith, 1976). However, for rye, the figure of 33.14 pg per 4C nuclei is commonly used (Bennett *et al.*, 1977). The length of the haploid rye genome in base-pairs is in the order of 7.8×10^9 bp

(Bedbrook *et al.*, 1980a). The rye genome therefore has some 35% more DNA than the largest genome (ie., B) present in tetraploid and hexaploid wheat. Moreover, the smallest rye chromosome, 1R contains approximately 12% more DNA than the largest wheat chromosome, 5B (Gustafson and Bennett, 1976). A positive correlation between DNA content and somatic chromosome length of rye chromosomes has been demonstrated using ultraspectrophotometry (Heneen and Caspersson, 1973; Lukaszewski *et al.*, 1982). However, clear differences in the distribution of chromatin along rye chromosomes exist (Heneen and Caspersson, 1973). Principally, high amounts of chromatin are observed at centromeric and telomeric regions with reduced levels at sites of primary and secondary constrictions. The pattern of distribution is chromosome specific and corresponds to the heteropycnotic differentiation of late prophase chromosomes.

Constitutive heterochromatin (C-heterochromatin) represents a significant proportion of cereal genomes and has been demonstrated to be influential in shaping genome size and influencing chromosome pairing and chiasma formation (for review, see Rees, 1990; Furuta and Nishikawa, 1993). A positive correlation between the variation in telomeric heterochromatin and nuclear DNA content has been demonstrated within the genus *Secale* and it is assumed that increases in DNA content has occurred throughout the evolution of this genus (Bennett *et al.*, 1977). Variation within the telomeric heterochromatin of *S. cereale* cultivars and inbred lines is well documented (Weimark, 1975; Lelley *et al.*, 1978; Giraldez *et al.*, 1979; Pilch and Hesemann, 1986) and in wheat, polymorphic C- and N-banding patterns among cultivars have been observed (Seal, 1982; Endo and Gill, 1984; Friebe and Heun, 1988). Mechanisms such as saltatory amplification and unequal crossing-over involving both homologous and non-homologous chromosomes are likely to be involved (Weimark, 1975; Bennett *et al.*, 1977). While the frequency of such events is low (Gustafson *et al.*, 1983), once introduced, variant chromosomes are apparently stable and inherited according to Mendelian genetics (Giraldez *et al.*, 1979; Gustafson *et al.*, 1983; Pilch and Hesemann, 1986).

The ribosomal multigene families belong to the large fraction of the cereal genome defined as repetitive DNA and have been studied extensively in the Triticeae (for review, see Appels and Honeycut, 1986). The 18S.26S rRNA multigene families constitute the Nor locus present on homoeologous chromosome groups 1, 5 and (or) 6 (for reference list, see Appels *et al.*, 1989). More recently, ISH to wheat deletion lines has confirmed rDNA loci on chromosomes 1AS, 1BS, 5DS, 6BS and identified a new locus on 7DL (Mukai *et al.*, 1991). The 5S rRNA genes are arranged in tandem arrays and have been mapped in hexaploid wheat via ISH to homoeologous groups 1AS, 1BS, 1DS, 5AS, 5BS and 5DS (Mukai *et al.*, 1990). In 4x and 6x wheats, the repeat unit length is small (420 bp and 500 bp) and length variation has been attributed to the non-transcribed spacer region (Gerlach and Dyer, 1980; Scoles *et al.*, 1988). There are 10,000 copies within the genome of Chinese Spring and 5,000 in rye cv Imperial (Appels *et al.*, 1980; Gerlach and Dyer, 1980). In rye, 5S rRNA loci have been mapped to 1R and 5R and are arranged in tandem arrays of 460 bp and 480 bp repeating units respectively (Appels *et al.*, 1980; Reddy and Appels, 1989).

1.8.2 Repetitive DNA sequences

Repetitive DNA sequences have been identified as a major component of higher plant genomes (for review, see Flavell *et al.*, 1974). In the winter cereals rye, wheat, barley and oats, this fraction constitutes at least 75% of the genome (Flavell *et al.*, 1974; Flavell and Smith, 1976). Many different classes of repetitive DNA sequences have been recognised including a rapidly renaturing fraction accounting for 4%-10% of cereal genomes and consisting of highly repetitious and palindromic sequences distributed in clusters throughout the genome (Smith and Flavell, 1977; Appels *et al.*, 1978). However, most repetitive sequences appear to be moderately repeated with 10^2 to 10^5 copies per diploid genome (Bouchard, 1982).

Flavell *et al.* (1977) classified the repetitive DNA sequences of cereals into groups based on their abundance in oat, barley, wheat and rye genomes. While the homologies found

between repetitive DNA sequences of each species corresponds well to their phylogenetic relationship, variability between species regarding the proportion of the genome represented by each group of repetitive sequences demonstrates that further amplification (and divergence) has occurred since speciation. A species-specific fraction of repetitive DNA sequences has been identified in all species, the proportion of which increases with evolutionary distance (Flavell *et al.*, 1977). While considerable divergence (6%-10%) exists within most families of repetitive DNA, sequences within each group are more closely related within species than between species (Flavell *et al.*, 1977; Rimpau *et al.*, 1978).

Approximately 50% of the wheat and 60% of the rye genome are devoid of non-repeated DNA sequences, consisting of short, repeated sequences (50-2000 bp) interspersed with related or non-related repetitive DNA sequences (ie., tandemly arranged) at intervals of <4 kbp. Consequently, non-diverged repeat sequences are interspersed with longer regions of diverged repeat sequences (Rimpau *et al.*, 1978, 1980). Single or low copy sequences are also dispersed throughout the cereal genome with approximately 80% residing close (<4.0 kbp) to repetitive DNA sequences (Rimpau *et al.*, 1978). Non-repetitive DNA falls into two size classes with average lengths of 700 bp and 2.5 kbp however, while most non-repetitive DNA is short and interspersed, a small fraction (2%-10%) exists as long, uninterrupted blocks >10 kbp (Rimpau *et al.*, 1978, 1980). In addition, repetitive DNA sequences specific to cereal genomes (16% for wheat and 24% for rye) are also interspersed throughout the genome (Rimpau *et al.*, 1978).

1.8.2.1 Tandem repeats

Characteristic of eukaryotic genomes, the repetitive DNA sequences observed in cereal genomes falls into two broad groups; tandem arrays and dispersed repeats (for review, see Smyth, 1991). Appels *et al.* (1978) first demonstrated the presence of highly re-iterated, tandemly repeated sequences within the telomeric heterochromatin of rye chromosomes. In contrast, highly re-iterated, tandemly repeated sequences in wheat have been localised

predominantly to interstitial regions of chromosomes of the B genome (Gerlach and Peacock, 1980; Hutchinson and Lonsdale, 1982). Of the tandemly arranged sequences, the best studied are those from the genus *Secale* and include the 120 bp, 610 bp, 630 bp and 480 bp families (Bedbrook *et al.*, 1980a), the 350 bp family (Appels *et al.*, 1981) which incorporates the 480 bp family (Bedbrook *et al.*, 1980a; Appels *et al.*, 1981; Appels, 1983) and the 2.2 kbp family (Bedbrook *et al.*, 1980b). Analysis of these families has confirmed the general organisation of the rye genome as described by Rimpau *et al.* (1978). These families are complex sequences consisting of simple sub-repeats interspersed with unrelated, non-repeating sequences.

In *S. cereale*, the four families described by Bedbrook *et al.* (1980a) constitute some 8%-12% of the rye genome and are present in high copy number (7.4×10^4 - 1.5×10^6 /haploid genome). The 630 bp and 610 bp families are found exclusively in constitutive telomeric heterochromatin while both the 350-480 bp and 120 bp families are found at interstitial heterochromatic sites also. Chromosomal location of these sequences corresponds to the location of C-banding heterochromatin with the exception of centromeric and NOR associated heterochromatin (Jones and Flavell, 1982a). In wheat, 120 bp sequences have been localised to both telomeric and interstitial sites on chromosomes of the B and D genomes corresponding to light C-bands (Bedbrook *et al.*, 1980a; Rayburn and Gill, 1985a, b). This sequence is absent from *H. vulgare*, *H. agriocrithon* and *H. spontaneum* (Gupta *et al.*, 1989) but has been localised to the telomeres of *H. bulbosum* (Xu *et al.*, 1990). The 120 bp family also forms the simple sub-repeating unit of the 2.2 kbp family found in rye in which 120 bp sequences are interspersed with unrelated, repetitive sequences (Bedbrook *et al.*, 1980b). The 2.2 kbp family is not highly re-iterated in wheat and has presumably arisen from the rearrangement of 120 bp sequences with other repetitive sequences through recombination prior to re-amplification in *Secale* since speciation. High levels of heterogeneity within this family suggest either independent amplification events or insertion and/or deletion events or both (Bedbrook *et al.*, 1980b).

Further analysis within the genus *Secale* has identified both inter- and intravarietal polymorphisms in the distribution and copy number of the 120 bp, 350-480 bp, 610 bp and 630 bp families (Jones and Flavell, 1982b). The variation in telomeric heterochromatin found to exist between *S. cereale* and *S. silvestre* is accounted for by the absence and/or reduced quantities of 610 bp, 630 bp and 350-480 bp families in *S. silvestre* (Bedbrook *et al.*, 1980a). The 350-480 bp family is best characterised and a consensus sequence of 29 units has been established confirming both a high level of sequence heterogeneity within the family and clustering of sequence variants within the genome (Appels and Moran, 1984; Appels *et al.*, 1986a). Linkage with non-heterochromatic sequences has also been observed suggesting that the distribution of this family of sequences is not confined to telomeric heterochromatin (Appels *et al.*, 1986a). Both amplification and deletion of rye telomeric heterochromatin has been assayed using the 350-480 bp family (Koebner *et al.*, 1986; Lapitan *et al.*, 1988) and the possible order of families at telomeres and interstitial chromosomal regions has been suggested; the 350-480 bp family being the most distal (Jones and Flavell, 1982a; Koebner *et al.*, 1986; Appels *et al.*, 1989). This family of sequences has been localised to the telomeres of numerous species other than rye (Xin and Appels, 1988; Appels *et al.*, 1989) and extensive sequence divergence observed between species suggests an ancient origin.

The observations of Flavell *et al.* (1977), Bedbrook *et al.* (1980b) and Appels *et al.* (1989) indicate that amplification (or contraction) of tandemly repeated sequences appears to be a common phenomenon associated with cereal genome evolution. Both amplification and contraction of telomeric heterochromatin has been observed in rye (Gustafson *et al.*, 1983; Koebner *et al.*, 1986; Lapitan *et al.*, 1988). Unequal crossing-over, either post-replicatively between sister chromatids or during meiosis between homologous chromosomes (Smith, 1976) and/or saltatory amplification (Britten and Kohne, 1968; Fry and Salser, 1977) is believed to be responsible for the generation and maintenance of tandemly repeated sequences and can account for the variation within families and apparent clustering of sequence variants within cereal genomes (Flavell *et al.*, 1977; Appels *et al.*, 1986a). While mechanisms are unknown, rolling circle replication, "slippage" and "directed" recombination

involving short, highly recombinogenic sequences may be involved (Benslimane *et al.*, 1986; Hardman, 1986). In rye, Jones and Flavell (1982*b*) speculate that tandemly repeated sequences originated at the telomeres and through evolution, chromosome breakage and fusion, translocation and inversion has resulted in the transfer of a number of these sequences to interstitial sites.

1.8.2.2 Dispersed repetitive sequences

The structural analysis of a number of repeated sequences isolated from plant genomes has revealed strong similarities to both prokaryote transposons and retrotransposons (for review, see Smyth, 1991). Numerous retrotransposons have been isolated and characterised from hexaploid wheat. The WIS-2 family of elements (Moore *et al.*, 1991*a*) was first identified through the cloning of an 8 kbp element, WIS-2-1A interrupting the *Glu-1* locus of chromosome 1A of the hexaploid wheat variety Chinese Spring (Harberd *et al.*, 1987). Short (5-6 bp) duplications have been identified flanking WIS-2 elements and short terminal repeats including the conserved 5' TG-CA 3' motif identified for all retroviral proviruses and retrotransposons studied (Warmington *et al.*, 1985; Panganiban, 1985; Adams *et al.*, 1987). Deduced protein sequences have identified an integrase domain with sequence similarity to *Drosophila copia*-like elements and a reverse transcriptase domain has been identified (Moore *et al.*, 1991*a*). The WIS-2 family is comprised of approximately 400 elements in wheat which demonstrate significant structural heterogeneity. Related elements have been identified in rye, barley, oats and *Aegilops* species (Moore *et al.*, 1991*a*).

Other elements characterised include WIRE-1 (Flavell, 1986), WIS-1 (Martienssen and Baulcombe, 1989) and BIS 1 (Moore *et al.*, 1991*b*). These families of elements possess long LTR's and putative integrase domains while WIRE-1 elements possess terminal inverted repeats including the 5' TG-CA 3' motif. WIRE-1 is present in about 10,000 copies/diploid wheat genome while BIS 1 is much more highly re-iterated at 1×10^5 copies/diploid barley genome. BIS 1 type elements have been identified in wheat and rye. In wheat, a family of

elements defined by the clone *pTag546* (Y. G. Liu *et al.*, 1992) are similar to CIN-4 elements of *Z. mays* (Schwarz-Sommer *et al.*, 1987) in lacking LTR's. These elements are analogous to the long interspersed sequences (LINE's) found in mammalian genomes. However, unlike LINE sequences, they are not highly re-iterated. Sequence analysis of the clone pHv7161 isolated from barley reveals a repeating unit of 999 bp characterised by the inclusion of short sub-repeats, two inverted repeats flanked by short, direct repeats, CAAT and TATAA boxes and a number of transcription initiation signals for RNA polymerase II and III (Vershinin *et al.*, 1990). Consequently, as with mammalian genomes, it is likely that reverse transcription has played an important role in the evolution of cereal genomes. However, similarities and differences regarding the types of elements present, their level of re-iteration and distribution appear to exist between higher plant and mammalian genomes.

A number of dispersed, highly re-iterated repetitive sequences have been isolated from cereal genomes which lack clear structural features distinguishing them as transposable elements. One such family is the R173 family of rye (Guidet *et al.*, 1991). The R173 family is moderately repeated with approximately 15,000 copies per diploid rye genome but is absent from wheat and barley (<20 copies/diploid genome). This family of repeats is dispersed on all 7 rye chromosomes with the exception of some telomeres and lower copy number at the centromeres and NOR's (Guidet *et al.*, 1991; Mouras *et al.*, 1991; this study). Elements within the R173 family have a repeat unit length of 3.0-6.0 kbp (Rogowsky *et al.*, 1991a, 1992a) and structural features such as potential target site duplications and terminal direct repeats have been identified. While these structures are indicative of transposable elements, they fail to fulfil the size and position criteria associated with such elements (Rogowsky *et al.*, 1991a, 1992a). Specifically, the 5' TG-CA 3' motif conserved at the terminal ends of retrotransposons and retroviral proviruses has not been identified.

Less well characterised dispersed, repetitive sequences include the rye-specific 5.3-H3 family which was initially isolated as part of a more complex repeating unit (Appels and Moran, 1984). This family appears heterogeneous and is dispersed throughout the rye genome on all

7 chromosomes. In contrast to other repetitive sequences characterised, it is generally not found in telomeric heterochromatin and is not tandemly arranged (Appels *et al.*, 1986a). RIS 1 (Moore *et al.*, 1993) and Dialect-1 (Sonina *et al.*, 1989) elements are also highly re-iterated and dispersed throughout the rye and barley genomes, respectively. These elements may represent a novel class of sequences or prove similar to retrotransposons or R173-like elements. Suggestive of recent evolution, is the apparent genome specificity of this latter group of sequences.

Despite the vast amount of information gathered in recent years regarding the structure and spatial organisation of cereal genomes and the families of repetitive sequences which constitute the greater proportion of the genome, there is little information at the molecular level regarding the environment directly associated with these families of sequences. In the case of rye specific repetitive sequences, it is expected that 84% of the flanking sequences are interspersed with repetitive sequences common to both wheat and rye with only the remaining 16% consisting of single or low copy DNA (Rimpau *et al.*, 1978). Recent limited studies indicate that this picture is essentially correct with few low copy sequences being observed flanking members of the R173 family (Rogowsky *et al.*, 1992a) and the 5.3-H3 family (Appels *et al.*, 1986a).

1.9 GENOME MAPPING

1.9.1 Genetic and physical maps

While the concept of linkage and the methods used to determine linkage are well established (see Ott, 1991), renewed interest in linkage mapping complex genomes has been generated through advances in recombinant DNA technology. Linkage analysis allows the mapping and ordering of genetic loci (markers) in a linear fashion on a chromosome by observing segregation resulting from recombination at meiosis. Genetic distances are expressed in centimorgan (cM) units which correspond to an observation of 1% recombination in gametes within a sample and are assumed to contain, on average 1000 kbp (Stranzinger, 1990). For

loci separated by small distances, the recombination frequency is virtually identical to map distance and is additive. As map distances increase, this relationship becomes more complex, largely through the occurrence of multiple cross-over events. Recombination frequency may be equated to cM distances through the use of a mapping function (see Ott, 1991). However, recombination frequencies per Mbp of DNA vary considerably being influenced by factors including sex, age and chromosomal region (Rees, 1990; Kidd, 1991).

In contrast, physical maps of complex genomes relate information regarding both the physical position (cytogenetic) of genetic loci as well as the physical distance separating such loci. Molecular distances between loci are ideally measured in nucleotide bases and consequently, are additive between loci along a chromosome. Physical maps rely on techniques which establish overlaps between clones or DNA segments which allow reconstitution of the original genomic order. Consequently, cytogenetic and molecular methods including chromosome banding, *in situ* hybridisation (ISH), pulsed field gel electrophoresis (PFGE), contiguous mapping of yeast artificial chromosomes (YACs) and cosmid clones are used.

1.9.2 Genetic markers

Factors important for the effective and wide application of the use of genetic markers in breeding and mapping programmes include high levels of polymorphism, the co-dominant expression of marker phenotype and the absence of pleiotrophic effects on traits under examination. Morphological and biochemical markers have been used successfully in both animal and plant studies. However, their use in breeding programmes and in constructing genetic linkage maps is limited by low levels of polymorphism (Beckmann and Soller, 1983), and relative technical difficulty in their analysis. Moreover, many biochemical markers suffer from being developmentally regulated or demonstrate tissue specific expression.

1.9.2.1 RFLP markers

The ability to identify DNA polymorphisms between homologous chromosomes through various molecular biological methods has revolutionised gene mapping and construction of genetic linkage maps as well as allowing the integration of genetic and physical maps. Botstein *et al.* (1980) first proposed that restriction fragment length polymorphisms (RFLPs) detected using cloned DNA sequences and Southern hybridisation (Southern, 1975) could be used in human linkage studies since they defined discrete genetic loci, inherited in simple Mendelian fashion. Since then, RFLP-based linkage maps have been generated for a wide variety of organisms (see O'Brien, 1990) and have proven immeasurably successful in human studies where RFLP markers have been obtained for numerous genetic disorders (for review, see Watkins, 1988).

RFLPs detect changes in homologous DNA sequences affecting the length of fragments obtained upon digestion with type II restriction endonucleases. Differences may result from base changes affecting restriction sites (deletion or addition), insertions or deletions internal to sites or chromosomal rearrangements encompassing restriction sites. Polymorphism is assayed by the method of Southern (1975) using cloned genomic DNA (gDNA) or complementary DNA (cDNA) sequences of known or anonymous function, labelled isotopically or non-isotopically. RFLPs demonstrate a number of qualities amenable to their use as genetic markers; they are generally silent in their effect on the phenotype allowing potential epistatic interference to be ignored (Helentjaris, 1987) and the number of detectable loci is large with at least 1×10^5 for the human genome (Beckmann and Soller, 1983). Importantly, RFLP markers demonstrate a codominant mode of inheritance (Botstein *et al.*, 1980; Beckmann and Soller, 1983).

RFLP analysis has proven particularly amenable to plants (for review, see Tanksley *et al.*, 1989; Chang and Meyerowitz, 1991). Currently, RFLP linkage maps have been constructed for many plants including arabis, maize, tomato, potato, pepper, soybean, rice, cabbage,

and lettuce (for reference list, see Chang and Meyerowitz, 1991). Tanksley *et al.* (1992) have extended the linkage maps for the potato and tomato genomes with a map comprising in excess of 1000 markers with an average map distance of 1.2 cM between loci. RFLP mapping within the Triticeae has lagged behind other crop plants for a number of reasons (see later). However, RFLP linkage maps of varying saturation are emerging for hexaploid wheat (Liu and Tsunewaki, 1991; Hart *et al.*, 1993), barley (Shin *et al.*, 1990; Graner *et al.*, 1991; Heun *et al.*, 1991; Kleinhofs *et al.*, 1993; Langridge *et al.*, 1995), rye (Devos *et al.*, 1993b) and wild relatives of wheat such as *T. tauschii* (K. S. Gill *et al.*, 1991a, b; Lagudah *et al.*, 1991). Importantly, RFLP markers tightly linked to a number of disease resistance loci have been identified in various crop plants including tomato (Messeguer *et al.*, 1991), maize (Bentolila *et al.*, 1991), soybean (Weismann *et al.*, 1992) and potato (Gebhardt *et al.*, 1993).

The time required for analysis and technical complexity, restrict to some degree the large scale use of RFLP markers. Furthermore, the prospect of complete automation is not high. However, more importantly, the low levels of allelism and uneven distribution often encountered with RFLP markers limit their use in the development of high resolution genetic linkage maps (Kidd, 1991).

1.9.2.2 PCR-based markers

The polymerase chain reaction (PCR) first described by Sakai *et al.* (1985) has transformed most areas of molecular biology research. The development of molecular markers and their use in genetic linkage mapping studies is no exception (for review, see Nelson, 1991). Of its many attributes, PCR is exquisitely sensitive requiring only minute amounts of DNA. Moreover, DNA purity is less critical; degraded samples may be successfully assayed. Genotyping of individuals is much more rapid and, for small sample sizes PCR-based markers are more cost efficient than RFLPs (Ragot and Hoisington, 1993). Importantly, PCR has allowed the development of novel DNA-based markers.

Skolnick and Wallace (1988) initially described three classes of amplified sequence polymorphisms (ASPs) which could potentially be detected using PCR; restriction site polymorphisms, DNA base changes not associated with restriction sites and sequence length polymorphisms. PCR-RFLP analysis enables base changes affecting restriction enzyme sites within amplified DNA sequences to be assayed, replacing classical RFLP analysis. This approach has been successful in mammalian species (Medrano and Aguilar-Cordova, 1990), wheat (D'Ovidio *et al.*, 1990), barley (Shin *et al.*, 1990) and rice (Williams *et al.*, 1991). Single nucleotide polymorphisms can also be assayed through their affect on the physical behaviour of DNA during electrophoresis in acrylamide gels. The use of denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and single strand conformation polymorphism analysis (SSCP) in detecting polymorphisms in PCR-amplified products has recently been reviewed (Lessa and Applebaum, 1993). Allele-specific amplification allows determination of single base polymorphisms when sequence information is available to design appropriate oligonucleotide primers (for review, see Sommer *et al.*, 1992). However, while these methodologies have been extensively utilised for mutation analyses (for review, see Gibbs, 1991), they have limited application to gene mapping studies due to relative technical complexity.

Short tandem repeats (STRs) or microsatellites represent a subclass of repeated DNA sequences found within eukaryote genomes. STRs are composed of iterations of very short motifs (1-5 bp), exhibit site-specific variation in repeat number (Litt and Luty, 1989; Webber and May, 1989) and are readily assayed using PCR. Within the mammalian genome, the (CA)_n(GT)_n motif [designated (CA)_n] is the most abundant with one repeat every 50 kbp (Webber and May, 1989). Genome distribution is widespread and random (Webber and May, 1989) and in absolute terms, within the human genome, microsatellites longer than 20 bp are present every 6 kbp (Beckmann and Webber, 1992). Microsatellite markers demonstrate codominant Mendelian inheritance (Webber and May, 1989; Tautz, 1989; Litt and Luty, 1989) and have become the preferred type of marker for the construction of linkage

maps due to increased levels of heterozygosity, simplicity of analysis and the ability to provide markers for genomic regions where other markers are rare (Webber, 1990).

Recent publications (Weising *et al.*, 1989; Condit and Hubbell, 1991; Akkaya *et al.*, 1992; Zhao and Kochert, 1992; Lagercrantz *et al.*, 1993; Morgante and Olivieri, 1993; Wu and Tanksley, 1993; Wang *et al.*, 1994) have demonstrated the widespread occurrence of microsatellite repeats in many plant species. High levels of allelism have been observed along with codominant Mendelian inheritance. Based on EMBL and GenBank database searches for di- and trinucleotide repeat motifs, Morgante and Olivieri (1993) estimated the occurrence of microsatellite repeats in plant genomes to be in the order of one every 50 kbp with (AT)_n repeat motifs the most abundant. These results have since been supported and extended by others (Lagercrantz *et al.*, 1993; Wang *et al.*, 1994). Database searches by Lagercrantz *et al.* (1993) suggest that in plants, microsatellites longer than 20 bp exist every 29 kbp. Importantly, the proportion of repeats longer than 32 bp for (AA)_n, (GA)_n and (AT)_n motifs identified in plant genomes was not significantly different from estimates in human studies (Beckmann and Webber, 1992).

Wang *et al.* (1994) estimated that microsatellites were distributed about every 23 kbp throughout plant genomes with (AT)_n repeats most common and distributed every 62 kbp. Collectively, tri- and tetranucleotide repeats were found to be as abundant as (AT)_n repeats. Microsatellite repeats were found to be more abundant in dicotyledons than in monocotyledons with one microsatellite every 21 kbp compared with 65 kbp. Seven microsatellite repeats were identified within sequence data from *T. aestivum* and estimated to be present about every 20 kbp within the wheat genome. Importantly, of the trinucleotide microsatellite repeats identified containing G-C nucleotides, 57% were found to be distributed within coding regions. Since the informativeness of microsatellite markers increases with repeat length (Webber, 1990), the confirmation of repeats with lengths greater than 20 bp indicates that microsatellites will provide informative markers for plant genome studies.

The arbitrarily primed-polymerase chain reaction (AP-PCR) developed by Welsh and McClelland (1990) and randomly amplified polymorphic DNA (RAPD) analysis developed simultaneously by Williams *et al.* (1990) both utilise arbitrarily chosen oligonucleotide primers coupled with PCR to identify a novel class of DNA polymorphisms. The former approach relies upon two cycles of low stringency amplification followed by PCR at high stringency. In contrast, RAPDs are generated through the use of short oligonucleotides, typically 8-10 nucleotides in length under conditions employing a single annealing temperature. The detection of polymorphism in both cases is dependent on the degree of mis-match between primer and template DNA, with as little as one base-pair difference preventing amplification at a given locus (Williams *et al.*, 1990).

Both forms of analysis are claimed to result in a reproducible pattern of amplified genomic fragments for a given primer which can be used as a fingerprint for varietal identification (Welsh and McLelland, 1990; Williams *et al.*, 1990), parentage determination (Welsh *et al.*, 1991), pedigree analysis (Stiles *et al.*, 1993; Tinker *et al.*, 1993) or the generation of genetic linkage maps (Williams *et al.*, 1990). RAPD analysis of crop plants has demonstrated that it is relatively easy to generate polymorphic markers between closely related individuals (Heun and Helentjaris, 1993; Stiles *et al.*, 1993; Tinker *et al.*, 1993). RAPD markers have been used to construct primary linkage maps (Torres *et al.*, 1993) or to increase the level of saturation of existing RFLP-based maps (Williams *et al.*, 1990). The ability to detect polymorphisms in middle- and highly repetitive DNA (Williams *et al.*, 1990; Devos and Gale, 1992; Paran and Michelmore, 1993) allows linkage maps to be extended towards telomeres and gaps to be filled since these regions are generally not accessible by other methods.

One of the most powerful aspects of RAPD analysis is the ability to generate markers for specific genomic regions rapidly. Michelmore *et al.* (1991) developed a DNA bulked segregant analysis strategy in lettuce utilising individuals from a segregating F₂ population.

Three RAPD markers linked to the *DM5/8* gene conferring resistance to downy mildew were identified, the closest being mapped to within 4.2 cM. In attempting to identify molecular markers more closely linked to genes of interest, many workers have employed the use of near-isogenic lines (NILs). This approach has proven successful in tomato (Martin *et al.*, 1991), lettuce (Paran *et al.*, 1991), bean (Miklas *et al.*, 1993), oat (Penner *et al.*, 1993a) and barley (Barua *et al.*, 1993) with RAPD markers demonstrating linkage to a number of disease resistance loci being identified. However, the generation of RAPD markers using NILs is dependent upon the degree of sequence divergence for the introgressed region (Martin *et al.*, 1991). Often, many primers must be screened in order to identify useful polymorphisms. Other, more universal problems concern the dominant nature of RAPD markers.

Except for the study of Tinker *et al.* (1993) which identified a single co-dominant RAPD marker in barley, RAPDs are scored as dominant markers (presence or absence). Consequently, the heterozygous genotype cannot be identified, yielding less information than other co-dominant (ie., RFLP) markers. This reduces the mapping efficiency of RAPD markers, particularly within F₂ populations since RAPD markers in repulsion cannot be mapped (Torres *et al.*, 1993). A number of studies have identified RAPD markers deviating from Mendelian segregation ratios (Carlson *et al.*, 1991; Echt *et al.*, 1992; Reiter *et al.*, 1992; Heun and Helentjaris, 1993). Work by Heun and Helentjaris (1993) indicates that this phenomenon is the result of the combined probabilities associated with the competitive nature of annealing and subsequent extension of product under specific conditions. In this way, the genetic background of the individual has been implicated in the variation observed.

1.10 THE APPLICATION OF MOLECULAR MARKERS

There are essentially two broad areas of application of molecular markers; map-based or positional cloning and marker assisted selection (MAS). Map-based or positional cloning is an emerging technique which allows the isolation of genes for which only genetic information is available. Marker assisted selection covers a wide range of techniques including the use of

markers linked to agronomic traits, quarantine breeding, pyramiding of resistance genes, accelerated backcrossing, parent building, multi-trait breeding and the analysis of quantitative trait loci (QTL). These latter techniques are now well established and are reviewed elsewhere (Langridge *et al.*, 1996). However, the three techniques at the centre of recent developments are positional cloning, QTL analysis and comparative mapping. Consequently, it is aspects of these three techniques which will be discussed in detail here.

1.10.1 Positional cloning

The integration of genetic and physical maps allows the assignment of linkage groups to chromosomes and an assessment of the genome coverage of linkage maps. Moreover, comparisons between physical and linkage data allow an assessment of recombination rates across and between genomes. Most significantly, the ability to initiate map-based cloning of genes known only by their phenotype (ie., reverse genetics) by chromosome walking is only possible through the integration of both maps (Rommens *et al.*, 1989; Martin *et al.*, 1992).

RFLP and PCR-based markers provide a link between genetic and physical maps; resolution of meiotic linkage analysis is equivalent to 500 kbp-1 Mbp (Stranzinger, 1990; Evans, 1991), allowing molecular markers used in linkage studies to be employed in the development of physical maps. The most direct approach is to physically localise RFLP markers using ISH. However, PFGE has been used extensively in human studies, allowing the generation of long range physical maps of regions of interest and the cloning of loci responsible for genetic disorders (for review, see Evans, 1991). While PFGE has had limited application in plants, due in part to difficulties in the preparation of DNA and the paucity of suitable markers, a number of studies have demonstrated its utility. In a preliminary analysis, Jung *et al.* (1990) used PFGE to map RFLP probes to a chromosome fragment derived from *Beta procumbens* carrying a gene for nematode resistance while Ganai *et al.* (1989) have identified suppression of recombination within the tomato genome. Within the region of the genome surrounding the *Tm-2a* locus, 1 cM was found to correspond to more than 4 Mbp which is more than six

times greater than expected. In contrast, Segal *et al.* (1992) identified a greater than 10-fold reduction in the expected correlation between physical and genetic distance around the *I2* locus on chromosome 11 of tomato.

YAC clones have become the preferred tool for the assembly of physical maps since large segments of genomic DNA may be cloned and propagated allowing continued analysis (Burke *et al.*, 1987; Nelson, 1991). Individual clones can be physically anchored to chromosomal regions using ISH, an approach extensively utilised in mammalian studies (Burke, 1991; Cohen *et al.*, 1993). While the ability to characterise and identify overlapping clones via PCR has greatly simplified the process of chromosome walking and gene localisation (for review, see Nelson, 1991), the identification of sequence tagged sites (STSs; Olson *et al.*, 1989) and microsatellite repeats within cosmid and YAC clones also allows their inclusion in genetic linkage maps (Cohen *et al.*, 1993; Ellegren *et al.*, 1994). Currently, the human physical map based on contiguous YAC clones covers almost 90% of the genome (Cohen *et al.*, 1993). However, in plants, the development of YAC clones has been limited. The small genome size (140 Mbp) of *Arabidopsis thaliana* has allowed the generation of a number of YAC libraries with good genome coverage making physical mapping and map-based cloning of genes in this plant species possible (Guzman and Ecker, 1988; Ward and Jen, 1990; Grill and Sommerville, 1991). In tomato, Martin *et al.* (1992) have constructed a YAC library which provides a three-fold genome coverage. The average insert size of clones is about 200 kbp, which is significantly smaller than that of human YAC clones. Using RFLP markers closely linked to the *Tm-2a* and *Pto* loci, Martin *et al.* (1992) have identified 5 YAC clones allowing the initiation of chromosome walks to these genes.

1.10.2 Marker assisted selection

The polygenic nature of most traits of economic importance in higher organisms (Weller, 1986) makes them refractory to standard genetic manipulation. Moreover, traditional methods of selection are often expensive, time consuming and ineffectual since selection is

based on phenotype (Beckmann and Soller, 1983; Soller, 1990). The generation of high density genetic linkage maps based on polymorphic molecular markers allows the detection and mapping of loci affecting the expression of qualitative (ie., disease resistance) and quantitative (ie., yield) traits (quantitative trait loci or QTL). Importantly, closely linked markers may be exploited through marker assisted selection (MAS) of desirable alleles in breeding programmes (Burr *et al.*, 1983; Tanksley *et al.*, 1989). In plant species, RFLP markers have been shown to facilitate the transfer of numerous qualitatively expressed genes, such as those conferring pest and disease resistance from wild species into cultivated backgrounds (Young and Tanksley, 1989).

The identification of QTL is a more difficult undertaking. Modification of standard linkage analysis (ie., the application of maximum likelihood methods) and the development of powerful biometrical methods such as iterative mapping (Lander and Botstein, 1989), has enabled the application of molecular markers for mapping QTLs in populations derived from crosses between inbred lines (ie., F₂ or backcross). In this way, QTL affecting fruit weight, concentration of soluble solids and fruit pH have been mapped in tomato using RFLP markers separated by approximately 20 cM and covering about 95% of the genome (Paterson *et al.*, 1988). Recently, a QTL affecting shoot differentiation rate in *Hordeum vulgare* has been mapped (Komatsuda *et al.*, 1993). In soybean, QTL for reproductive, morphological and seed traits were mapped using an interval mapping approach (Mansur *et al.*, 1993).

Recently, improvements to iterative mapping (Lander and Botstein, 1989) have been proposed. The ability to incorporate random effects due to environment or multiple, linked QTLs has been shown to improve the power of QTL identification (Jansen, 1992; Knott and Haley, 1992; Rodolphe and Lefort, 1993). Increasing marker heterozygosity and reducing interval size lead to improvements in power (Knott and Haley, 1992) while the ability to use information from all markers within a linkage group improves the sensitivity of the test statistic and reduces bias in the estimated position and effect of a QTL (Knott and Haley, 1992; Zeng, 1993; Haley *et al.*, 1994). Beckmann and Soller (1988) proposed a method

allowing QTL mapping in outbred populations based on tracing marker alleles through three generations (ie., parents, F₁ and F₂) of a cross. However, this process is limited by the availability of informative markers since only markers polymorphic in both parents can be utilised. These limitations were realised in a recent study in potato (Van Eck *et al.*, 1994). Haley *et al.* (1994) using least squares analysis rather than maximum likelihood methods have developed an interval mapping-based approach which allows all markers within a linkage group to be utilised and incorporates fixed effects and effects of background genotype. Principal benefits are reduced demands on computation complexity and time. Darvasi and Soller (1992) demonstrated that selective genotyping provided increases in the power to map QTL. These authors showed that almost 100% of the information used in linkage analysis was obtained from the top and bottom 25% tails of the distribution. Since selective genotyping results in increased disequilibrium between marker-QTL genotypes and phenotypes (Mackinnon and Georges, 1992), greater efficiencies in marker utilisation are also gained.

In wheat, it has been estimated that for a linkage map comprising of markers placed at 10 cM intervals, 150 triplicated loci would be required (Gale and Sharp, 1988). Presently, most RFLP maps are of low or moderate density consisting of markers (50-300) spaced at intervals >5 cM. In contrast, the current linkage map of tomato consists of >1000 markers at intervals of about 1.2 cM (Tanksley *et al.*, 1992). This and other studies (Ganal *et al.*, 1989; Segal *et al.*, 1992) have revealed regions of suppressed and increased recombination resulting in dramatic differences in genetic and physical distances within the tomato genome. Success in chromosome walking and cloning strategies is consequently improved with the availability of high density linkage maps since such maps increase the probability of uniform genome coverage and the identification of markers physically more proximal to the locus of interest. Importantly, the power to detect QTL of small to moderate affect is also increased (Paterson *et al.*, 1988; Tanksley *et al.*, 1992).

While MAS is potentially a very powerful tool, a number of factors affecting the efficiency of such an approach have been identified. In order for a molecular marker to be useful, it must be tightly linked to a locus of interest in linkage *disequilibrium* since markers identified in linkage equilibrium are not informative outside of the pedigree structure which enabled their identification (Taylor *et al.*, 1995). This is particularly true for QTL analyses of outbreeding populations for which inbred lines are unavailable. The degree of polymorphism observed for molecular markers also impacts on the efficiency of MAS by restricting genotypes which may be utilised in constructing crosses and genome coverage of linkage maps (Paterson *et al.*, 1988; Lander and Botstein, 1989). Within the Triticeae, low levels of polymorphism are often encountered necessitating the use of wide crosses in the construction of mapping populations. Since it has been observed that genetic distance between loci may not be conserved across genomes (Chao *et al.*, 1989; Devos *et al.*, 1992; Wang *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993a), the utility of such maps in breeding programmes may be limited.

The application of selected markers in screening potential genotypes for the presence of QTL and their suitability in establishing mapping populations may allow a more pertinent selection of parental types (Paterson *et al.*, 1988). Other problems have been discussed by Rocha *et al.* (1995) and include trapping effects which concern the presence or absence of QTL alleles across breeds, populations or experimental samples and the subsequent ability to manipulate such loci. Epistasis (genetic architecture effects) and genotype by environment interactions are also of concern (Paterson *et al.*, 1988; Rocha *et al.*, 1995). In a theoretical analysis of the use of molecular markers in aiding selection in introgression breeding programmes, Hospital *et al.* (1992) demonstrated that gains of about two generations may be achieved in the recovery of the recipient genome in a backcrossing strategy. Not surprisingly, mapped markers were found to be more efficient. Importantly, when selection intensity was moderate, selection applied to specific loci or chromosomal regions was more efficiently controlled through the use of more distally located markers. However, early selection on such loci adversely affected the selection efficiency of non-carrier chromosomes.

Consequently, under conditions of moderate selection intensity, these authors advocate an approach allowing early selection with markers more distally located from the locus of interest or selection at a later stage using more closely linked markers.

1.10.3 Comparative genome mapping

The comparison of genetic maps between species is shedding light on patterns of evolution from common ancestral genomes. Genome homologies and, more specifically, conservation of syntenic groups within higher vertebrates has been established through cytogenetic methods including chromosome banding and gene mapping of conserved Type I loci via ISH as well as linkage analysis of both Type I and Type II loci (for review, see Stranzinger, 1990; O'Brien, 1991). As a result, genome evolution within this group has been shown to be conservative (Kidd, 1991). The ability to use microsatellite markers across species due to conservation of flanking primer binding sites has vastly improved the ability to construct interspecies genome maps (J. Taylor, personal communication). This approach allows chromosomal regions to be targeted and gaps to be filled, improving the resolution and application of such maps.

Sears (1954) first identified genome and chromosomal homologies within the Triticeae. More recently, comparative linkage maps based on biochemical and RFLP loci has revealed extensive conservation of syntenic groups within the tribe (Nelson *et al.*, 1995a, b; Van Deynze *et al.*, 1995; Marino *et al.*, 1996). Importantly, conservation of synteny has been extended to the genomes of maize and rice (Ahn and Tanksley, 1993; Ahn *et al.*, 1993; Devos *et al.*, 1994; Sherman *et al.*, 1995). A more comprehensive understanding of the extent of syntenic homologies will not only provide a more detailed understanding of genome evolution within this group but should also open the way for positional cloning studies of agronomically important genes possibly through the manipulation of smaller genomes (ie., rice). The potential to utilise microsatellite markers across species within this group would allow the generation of region-specific markers and construction of high density comparative

maps containing numerous anchored loci. Consequently, comparative gene mapping within the Triticeae is becoming an important feature of cereal mapping studies and should serve to improve map-based strategies for characterising and mapping quantitative trait loci (QTL).

1.11 GENETIC MAPPING THE TRITICEAE

Wheat genetics has lagged behind that of other crop plants such as maize, tomato and barley due to the problems posed by polyploidy; the triplication of many gene loci greatly complicates the isolation and analysis of morphological mutants and masks effects caused by homoeoalleles. However, the production of a complete set of aneuploid lines in common wheat (Sears, 1954) and the various interspecies addition and substitution lines (see Shepherd and Islam, 1988) have provided an unparalleled resource for gene mapping studies within this group. More than 100 morphological, isozyme and disease resistance loci have been assigned to individual wheat chromosome arms (Hart *et al.*, 1993) as well as the chromosomes of numerous wild and cultivated relatives of wheat. The complete set of nullisomic-tetrasomic chromosome lines has allowed the rapid assignment of loci to carrier chromosomes while telosomes have allowed the mapping of loci relative to centromeres (Singh and Shepherd, 1988*a, b*; Nelson *et al.*, 1995*b*).

Isozymes have been particularly useful in providing markers for the introgression of alien chromatin into wheat (see Gale and Sharp, 1988; Islam and Shepherd, 1993). However, while numerous loci have been genetically mapped in wheat (Hart, 1987; Miller, 1987), low levels of intervarietal polymorphism and the numerous methods required to assay individual loci, limit their application as genetic markers in wheat breeding. In recent years, the systematic mapping of genes within Triticeae species has progressed through the development and utilisation of RFLP markers. While limited levels of polymorphism detected in wheat have restricted the development and application of RFLP markers (Chao *et al.*, 1989; Kam-Morgan *et al.*, 1989; Liu *et al.*, 1990), the ability to rapidly characterise RFLP probes and assign them to chromosomal arms via aneuploid and addition line stocks has aided the

progress of linkage mapping within the tribe (Anderson *et al.*, 1992). This approach to marker assignment is now well established, allowing a more judicious choice of markers in addition to providing rapid chromosomal localisation of linkage groups.

1.11.1 Linkage mapping

Earlier, low-resolution RFLP linkage maps generated in wheat revealed a high degree of collinearity across the A, B and D genomes of wheat (Chao *et al.*, 1989; Liu and Tsunewaki, 1991; Devos *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993a). Significantly, Kam-Morgan *et al.* (1989) demonstrated the benefits of linkage mapping *T. tauschii*, the D-genome progenitor of wheat. Consequently, the development of linkage maps for wheat has also proceeded through the use of diploid relatives of hexaploid wheat. K. S. Gill *et al.* (1991a, b) and Lagudah *et al.* (1991) have adopted this approach using F₂ mapping populations derived from divergent *T. tauschii* accessions to develop moderately dense linkage maps of the D genome. Significantly, many loci mapped in *T. tauschii* have now been localised in wheat aiding comparative mapping efforts (Nelson *et al.*, 1995a, b, c; Van Deynze *et al.*, 1995; Marino *et al.*, 1996).

Currently, moderately-dense linkage maps are available for wheat group 1 (Van Deynze *et al.*, 1995), group 2 (Nelson *et al.*, 1995a), group 3 (Nelson *et al.*, 1995b), groups 4, 5 and 7 (Nelson *et al.*, 1995c) and group 6 chromosomes (Marino *et al.*, 1996). These studies have confirmed that extremely high levels of synteny exist among wheat A, B and D genome homoeologues with few exceptions. Significantly, the application of loci common to linkage maps of other species such as *T. tauschii*, barley and rye has allowed the derivation of consensus maps for a number of the homoeologous chromosome groups. These maps indicate considerable conservation of synteny across the tribe. This is particularly true for homoeologous group 1 chromosomes. Van Deynze *et al.* (1995) observed only 12 inconsistencies in locus order among seven wheat, four barley and two rye maps. These observations are consistent with the conclusions of Naranjo and Fernández-Rueda (1991)

regarding chromosome 1R of rye. Further, these consensus maps have led to an increased understanding of the homoeologous relationships found within the genomes of oats and rice. Such maps represent an important tool in the selection of markers for specific chromosomal regions and the future manipulation of agronomically important genes.

The most detailed RFLP-based linkage map of the rye genome to date consists of 156 loci spanning about 1000 cM generated using an F₂ population consisting of 120 individuals (or their F₃ derivatives) derived from two inbred rye lines (Devos *et al.*, 1993b). This map has provided the most detailed description of rye chromosomes relative to their wheat homoeologues and indicates that chromosome arms 2RS, 3RL, 4RL, 5RL, 6RS, 6RL, 7RS and 7RL have all been involved in at least one translocation since speciation. A number of relatively detailed RFLP-based genetic linkage maps have been developed for barley (Shin *et al.*, 1990; Heun *et al.*, 1991; Kleinhofs *et al.*, 1993; Langridge *et al.*, 1995). The map of Kleinhofs *et al.* (1993) incorporates 295 loci including 152 cDNA and 114 gDNA RFLP, 14 RAPD, five isozyme, two biochemical, one disease resistance and seven specific amplicon polymorphism (SAP) markers. Total map length is 1,250 cM with an average of 4.2 cM between markers. Centromeres of all barley chromosomes except chromosome 5 have been localised to within a few markers and telomeric regions of 1S, 2S, 3S and 7L have been mapped also. More recently, the genome coverage of the barley map has been extended via the generation of a consensus RFLP-based linkage map utilising common loci between 7 barley maps (Langridge *et al.*, 1995). The consensus map contains a total of 587 RFLP loci, of which 87 are of known function. Significantly, almost perfect co-linearity was observed between the consensus map and the individual maps from which it was derived.

In addition to clarifying inter-genomic relationships, RFLP-based linkage mapping within the Triticeae has revealed a number of additional phenomenon. Anderson *et al.* (1992) observed non-uniform distribution of mapped loci from randomly chosen clones across homoeologous chromosome groups, with homoeologous group 6 chromosomes the least populated. Similarly, Liu and Tsunewaki (1991) observed a paucity of markers mapping to wheat D

genome chromosomes. In barley, Heun *et al.* (1991) observed that 24.5% of loci mapped to chromosome 1H compared to 5.8% to chromosome 6H. Anderson *et al.* (1992) has suggested a general lack of coding sequences and/or the presence of a greater proportion of repeated DNA sequences relative to other chromosomes for the under-represented regions. However, other workers have attributed similar observations to factors including bias introduced during the construction of mapping populations (ie., lack of polymorphism between parental lines at specific chromosomal regions) or the under-representation of specific chromosomal regions in clone libraries (Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). The generation of high density linkage maps in barley (Kleinhofs *et al.*, 1993; Langridge *et al.*, 1995) and to lesser extent *T. tauschii* (K. S. Gill *et al.*, 1991a, b) displaying even marker distribution and few gaps would support this view.

1.11.2 Levels and nature of polymorphism

Data regarding relative levels of polymorphism between cDNA and gDNA clones as well as individual restriction enzymes is conflicting. Wang *et al.* (1992) have observed greater levels of polymorphism with cDNA clones. However in general, the tendency of cDNA clones to target more conserved genomic regions leads to reduced levels of polymorphism. In accordance, a number of workers have reported substantially higher levels of polymorphism using gDNA clones (Hart, 1990; K. S. Gill *et al.*, 1991a; Devos *et al.*, 1992). While there is apparently no difference regarding the distribution of loci mapped using either cDNA or gDNA clones (Heun *et al.*, 1991; Kleinhofs *et al.*, 1993), cDNA clones appear more useful in comparisons across genomes due to the existence of homoeoalleles and stronger hybridisation resulting presumably from greater levels of conservation (Liu and Tsunewaki, 1991; Wang *et al.*, 1992). In addition, problems associated with the use of gDNA probes include the high number of repeat sequences present within libraries, non-uniform hybridisation across genomes, non-syntenous hybridisation patterns and complex hybridisation patterns (Gale, 1990; Harcourt and Gale, 1991; Devos *et al.*, 1992).

Variability in the efficacy of individual restriction enzymes in detecting RFLPs has been observed (Chao *et al.*, 1989; Heun *et al.*, 1991; Wang *et al.*, 1992). However, this phenomenon has not been observed in all studies (Liu and Tsunewaki, 1991) and no clear pattern has emerged. The choice of restriction enzyme used to generate genomic libraries has been found to influence relative levels of polymorphism observed (Liu and Tsunewaki, 1991). These workers reported that clones derived from a *Pst*I generated library were 20% more efficient than those generated from either an *Eco*RI or *Hind*III generated library. However, while the choice of restriction enzyme influences the level of polymorphism detected, the use of diverse genotypes for the development of mapping populations is of most significance (Chao *et al.*, 1989; K. S. Gill *et al.*, 1991a; Hart, 1990).

In addition to identifying gross chromosomal rearrangements, RFLP mapping has revealed more subtle differences in chromosomal architecture. Liu and Tsunewaki (1991) observed a significant proportion of hemizygous loci. Such loci follow a 3:1 segregation pattern and are scored as dominant markers by their presence or absence and, while useful in mapping studies (Wu *et al.*, 1992), presumably reflect partial or complete deletion of DNA sequences at specific chromosomal regions. Dominant RFLP markers have also been observed in barley (Shin *et al.*, 1990).

Graner *et al.* (1990) suggest that most RFLPs found between diverse barley varieties are due to insertion/deletion events, while Shin *et al.* (1990) and Heun *et al.* (1991) have both commented on the significance of insertion/deletion events in accounting for RFLPs observed in barley. In *T. tauschii*, both insertion/deletion events and point mutations have been found to be equally important (K. S. Gill *et al.*, 1991a) while in wheat, insertions and deletions account for much of the polymorphism observed (Chao *et al.*, 1989; Liu *et al.*, 1990).

The presence of numerous intra- and interchromosomal duplications have also been identified through RFLP mapping via the observation of multiple hybridising fragments in wheat (Liu and Tsunewaki, 1991; Anderson *et al.*, 1992), *T. tauschii* (K. S. Gill *et al.*, 1991a) and rye (C.

J. Liu *et al.*, 1992). K. S. Gill *et al.* (1991a) observed intrachromosomal duplications to be more frequent than interchromosomal duplications and separated by distances ranging from 1 cM to 100 cM. Other problems include the observation of non-Mendelian segregation of RFLP markers (Kam-Morgan *et al.*, 1989; Shin *et al.*, 1990; Graner *et al.*, 1991; Heun *et al.*, 1991; Liu and Tsunewaki, 1991; Kleinhofs *et al.*, 1993). This phenomenon has been attributed to sampling error (Shin *et al.*, 1990) or potentially the methods used to derive mapping populations (ie., anther culture) (Heun *et al.*, 1991).

1.11.3 Genetic and physical mapping

The emerging RFLP linkage maps of cereal genomes demonstrate clearly that departures from synteny within the tribe are due principally to inter- and intrachromosomal rearrangements. Significantly, these skeletal genetic maps are characterised by a clustering of loci around the centromeres indicating that recombination is concentrated in the distal regions of cereal chromosomes. Importantly, the clustering of markers results in inflated intervals between distal loci, reducing overall map resolution and utility. This is particularly true of rye (Devos *et al.*, 1993b). Physical mapping studies within the Triticeae have demonstrated a non-uniform distribution of recombination along chromosomes. While numerous studies have utilised chromosome deletion and translocation lines to establish the physical location of genetic loci (Payne *et al.*, 1984; Endo and Mukai, 1988; Sybenga *et al.*, 1990), the ability to use polymorphic C-bands as genetic markers in linkage analyses provides a more direct means of integrating physical and genetic maps since both genetic and physical positions of loci can be deduced simultaneously. Such studies have been carried out in barley (Linde-Laursen, 1982), wheat (Curtis and Lukaszewski, 1991) and rye (Singh *et al.*, 1990; Alonso-Blanco *et al.*, 1993; Orellana *et al.*, 1993).

In all studies to date, comparison has revealed disparity between genetic and physical distances between loci. Due to the agronomic importance of chromosome 1R of rye (see Baum and Appels, 1991), much attention has been paid to the development of genetic and

physical maps of this chromosome. Genetic linkage maps of chromosome 1R including the consensus map constructed using data from numerous sources (Baum and Appels, 1991) and those of other workers (Lawrence and Appels, 1986; Benito *et al.*, 1990; Wang *et al.*, 1991) reveal clustering of loci around the centromere and a lack of concordance with physical distance. The genetic map distance between the centromere and *NOR-RI* located on the short arm of chromosome 1R is 2.7-10.2 cM (Lawrence and Appels, 1986; Wang *et al.*, 1991). Physically the secondary constriction is two-thirds of the arms length away from the centromere (Sybenga, 1983). Comparison between physical mapping studies of Gustafson *et al.* (1990), Sybenga *et al.* (1990) and Rogowsky *et al.* (1993) with genetic linkage data for this chromosome arm reveals similar disparity. Most significantly, genetic distances proximal of the NOR are reduced while those distal are expanded.

Genetic mapping of C-bands allows an assessment of the distribution of recombination along chromosomes. Curtis and Lukaszewski (1991) utilised 11 C-bands and two seed storage protein loci to assess the distribution of recombination along chromosome 1B from four *T. dicoccoides* accessions. Higher rates of recombination were observed in distal chromosomal regions. In the short arm, 75.7% of all crossovers occurred in the distal 30% of the chromosome arm while in the long arm, 88% of all crossovers occurred in the distal 51.4% of the chromosome arm. Comparison between physical and genetic maps for this chromosome indicate that loci located physically within the proximal 70% of this chromosome arm are clustered around the centromere. In contrast, the genetic distance between loci within the satellite are expanded. These observations have been extended across chromosomes 2B, 3B, 5B, 6B and 7B where recombination in general was concentrated in the distal 20% of chromosome arms (Lukaszewski and Curtis, 1993). Similar results have been reported for wheat group 6 chromosomes (Gill *et al.*, 1993), barley (Linde-Laursen, 1982) and rye (Lukaszewski, 1992). Consequently, genetic linkage maps of cereal chromosomes are essentially derived from recombination events occurring in the distal 20 to 30% of the arms (Lukaszewski and Curtis, 1993).

The physical distribution of recombination is a continuous variable with a positive correlation existing between recombination and distance from the centromere (Curtis and Lukaszewski, 1991; Lukaszewski and Curtis, 1993). Shorter chromosome arms demonstrate a greater proportion of distal recombination and, consequently greater disparity between genetic and physical distances. This has been observed for chromosome 6B in the study of Gill *et al.* (1993). Possible explanations for such observations include telomeric pairing initiation and strong positive chiasma interference Lukaszewski and Curtis (1993). In physically longer chromosome arms, sufficient distance exists for interference to weaken allowing the formation of interstitial chiasmata thereby increasing the relative proportion of proximal recombination. Significantly, both the frequency and distribution of recombination along cereal chromosomes are affected by genetic background and degree of homology between chromosomes (Curtis and Lukaszewski, 1991; Lukaszewski, 1992). Such observations may explain reduced recombination frequencies and often widely divergent linkage estimates between loci.

The studies of Curtis and Lukaszewski (1991) and Lukaszewski (1992) have also revealed sex differences in linkage estimates. Male meiosis leads to greater estimates of genetic distance between loci than those obtained via female meiosis. These workers have suggested that selection against aneuploid gametes which may be produced at elevated levels due to reduced pairing, particularly on the male side, would lead to over-estimates in recombination frequencies. Consequently, the approach taken in the construction of mapping populations will undoubtedly account for some of the variability observed in the literature.

The relationship between genetic distance and physical (molecular) distance in the Triticeae has been estimated (Lukaszewski and Curtis, 1991; Gill *et al.*, 1993). One centimorgan may vary from 440-1,530 kbp in distal regions to about 172,000-234,000 kbp in proximal regions. Importantly, the lack of correspondence between physical and genetic distance has serious repercussions for genetic studies within the Triticeae including chromosome walking to, and positional cloning of, agronomically important genes. Significantly, the results of Gill *et al.*

(1993) also reveal that the distal regions of wheat chromosomes are enriched in undermethylated single/few copy transcribed sequences. Similar patterns in the distribution of undermethylated DNA sequences has been demonstrated for rye chromosomes via PFGE studies (Moore *et al.*, 1993). However, in wheat such loci tend to be non-homoeologous and wheat-specific. Loci which have homoeoloci detectable in barley and rye tend to map more proximal to the centromeric region (Devos *et al.*, 1993a). Consequently, genes mapping proximal to the centromere may prove refractory to manipulation.

CHAPTER 2

MATERIALS AND METHODS

2.1 SEED STOCKS

All seed stocks, unless otherwise stated, were maintained by Dr. K. W. Shepherd and were supplied by him as required.

T701-4-6

T701-4-6 is a hexaploid triticales originally bred and obtained from CYMMIT. It has the parentage: KOALA X TCL MAYA II - ARM 'S'; 'MERCED' is the only rye named in its pedigree (Asiedu, 1986). Seed stock of this line is maintained by Dr. I. S. Dundas, Department of Plant Science, Waite Agricultural Research Institute and was provided by him when required. This triticales has been shown to be highly resistant to the Australian pathotype of the cereal cyst nematode, *Heterodera avenae* Wollenweber. Asidue *et al.* (1990) demonstrated that the gene for resistance is contained in the rye complement of the genome, specifically chromosome 6 (6R). The resistance gene has since been mapped to the long arm of chromosome 6R (Dundas *et al.*, 1992).

Carman

Carman triticales (*x Triticosecale* Wittmack) is a high yielding spring hexaploid triticales line selected from *Triticosecale* Wittmack cv. 'Beagle' originally bred by Dr. F. J. Zillinsky at the International Maize and Wheat Improvement Centre (CIMMYT), Mexico (Gustafson *et al.*, 1982). Seed was kindly provided by Dr. J. P. Gustafson, University of Columbia, Missouri, USA.

Amphiploid (-6R)

This line was a variant recovered in the laboratory of Dr. K. W. Shepherd from material originally derived by Dr. E. R. Sears, Department of Agronomy, University of Columbia, Missouri, USA from a cross between *Triticum aestivum* cv. Chinese Spring and *Secale cereale* cv. Imperial. The line described is nullisomic for rye chromosome 6.

Imperial Rye

Secale cereale cv. Imperial is a rye line established by Dr. E. R. Sears. It is highly self-compatible but has never been inbred by bagging for 7 generations or more (J. P. Gustafson, personal communication).

Petkus-R5

Secale cereale cv. Petkus-R5 is a sister line to the European rye variety 'Petkus' which has been used extensively in European breeding programmes (J. P. Gustafson, personal communication).

South Australian Rye

Secale cereale cv South Australian has been shown to have a high resistance to *H. avenae* (Brown and Meagher, 1970).

***Secale cereale* cv. 'Vila Pouca'**

The rye cultivar 'Vila Pouca' is an Portuguese land race population. Seed was kindly supplied by Professor Henrique Guedes-Pinto, Theoretical and Applied Genetics Division, Universidade de Tras-os-Montes e Alto Douro, 5000 Vila Real, Portugal.

Blanco Rye

Secale cereale cv. Blanco is a morphologically uniform rye population homozygous for aluminium tolerance obtained from B. Metzger (J. P. Gustafson, personal communication). Seed was kindly provided by Dr. J. P. Gustafson, University of Columbia, Missouri, USA.

Chinese Spring Wheat

Triticum aestivum cv. Chinese Spring is a euploid hexaploid spring wheat.

Schomburgk Wheat

Schomburgk wheat is a backcross derivative from the hexaploid wheat cultivar Aroona selected by Dr. A. J. Rathgen, Waite Agricultural Research Institute (Mackay, 1987). Seed of Schomburgk selection #784 was obtained from J. Lewis (Waite Institute). Selection #784 is a late generation selection and differs from the commercial cultivar Schomburgk in its 1D glutenin bands. Seed was provided by Dr. I. S. Dundas.

Egret

Egret is an early maturing semidwarf hexaploid spring wheat released by the New South Wales Department of Agriculture (Fisher and Martin, 1974). It is high yielding and resistant to many stem rust strains. However, Egret is highly susceptible to CCN (Fisher, 1982*b*). Seed was obtained from Ms. F. Green.

Chinese Spring-Imperial Rye Addition Lines

The complete set of disomic additions of each rye homologue (1R-7R) from *Secale cereale* cv. Imperial to *Triticum aestivum* cv. Chinese Spring (Driscoll and Sears, 1971) was obtained for both cytogenetic and molecular analyses.

186/15.1

The line 186/15.1 was isolated from tissue culture as a putative deletion of chromosome 6R (P. Langridge, personal communication). The Chinese Spring-Imperial 6R disomic addition line was used for culture. Immature embryos were used to generate embryogenic callus and plants were recovered from the callus. The recovered plants were selfed and the progeny screened with the clone pAW161 to identify lines where the whole chromosome had been lost or where there had been deletion of both telomers. Seed was provided by Dr. P. Langridge.

Chinese Spring-T701-4-6 6R(-6D) Substitution Line

This line was derived from a BC₁F₂ derivative from crosses between the hexaploid triticales T701-4-6 and *Triticum aestivum* cv. Chinese Spring (Asidue *et al.*, 1990). It contains a disomic substitution of the 6R chromosome from the triticales T701-4-6 with 6D from wheat and is resistant to CCN. Seed stocks of this line are maintained and were provided by Dr. I. S. Dundas.

Chinese Spring-T701-4-6 6R Deletion and Translocation Lines

The deletion mutants of rye chromosome 6R from triticales line T701-4-6 were supplied by Dr. I. S. Dundas. These lines were found after screening for the disassociation of 6RL isozyme markers in several thousand critical wheat seedlings representing progeny of plants monosomic for the rye chromosome and homozygous for Sears' *ph1b* mutant (Sears, 1977). The initial cross was described by Asidue *et al.* (1990). This screening procedure was conducted in an attempt to transfer the resistance gene against the cereal cyst nematode (*Heterodera avenae* Woll.) from chromosome 6R to wheat chromosome 6D by homoeologous recombination (Dundas *et al.*, 1990, 1992). The isozyme markers studied were α -amylase (α -Amy-R1), glutamate oxaloacetate transaminase (*Got-R2*) and 6-phosphogluconate dehydrogenase (*6-pgd-r1b*). Cytological examination of C-banded root-tip squash preparations of these isozyme disassociation lines confirmed the loss of terminal segments of the long arm of the 6R chromosome.

The translocation lines involving fusion of the 6RL and 6RS chromosome arms with 6DS and 6DL chromosome arms, respectively, were found after routine screening of C-banded mitotic chromosome preparations of progeny of critical seedling #2373. This seedling was derived from the same cross as that for the 6RL deletion lines. Sib-seedlings containing each of the translocation chromosomes were crossed with wheat line Schomburgk selection #784.

The isozyme expression patterns and CCN resistance reactions of the four deletion mutants are as follows (I. S. Dundas, personal communication):

Deletion Line	Isozyme Marker/CCN Resistance Reaction			
	<i>α-Amy-R1</i>	<i>Got-R2</i>	<i>6-pgd-r1b</i>	<i>CreR</i>
del6RL22	+	+	-	+
del6RL21	+	+	-	+
del6RL1807	+	+	-	+
del6RL1801	+	-	-	-

Alg/809 and L18913

Alg/809 and L18913 are both accessions of *Triticum tauschii* which have been shown to harbour a high level of resistance to the Australian pathotype of *H. avenae* (R. Appels, personal communication). Seed for both Alg/809 and L18913 was kindly provided by Dr. R. Appels.

Chinese Spring-Betzes Addition Lines

The complete set of disomic additions of each barley homologue (1H-7H) from *Hordeum vulgare* cv. Betzes to *Triticum aestivum* cv. Chinese Spring (Islam *et al.*, 1981) was obtained for molecular analyses.

Barley Cultivars (*Hordeum vulgare*)

The barley cultivars 'Galleon', 'Clipper', 'Betzes' and 'Franklin' were obtained for molecular analyses from Dr. R. Lance.

Wheat-rye chromosome 1R-1D short and long arm recombinant lines

Seed stocks of the 1R-1D short arm recombinant lines I-93, 82-180 and DRA-1 and the 1R-1D long arm recombinant lines R1 and RL2 were provided by Dr. K. W. Shepherd. The

isolation and characterisation of the short arm recombinant lines and long arm recombinant lines has been described previously (Koeber and Shepherd, 1985, 1896a, b; Shepherd *et al.*, 1990; Rogowsky *et al.*, 1993).

2.2 CROSSING

All plant crosses were carried out using plants grown in a glasshouse at approximately 25°C day and 15°C night temperatures with a 16 hour day-length (maintained during winter using artificial lighting).

2.3 CYTOGENETIC ANALYSIS

2.3.1 Mitosis

Seeds were surface sterilised for 15 minutes in a 2% solution of sodium hyperchlorite containing one drop of Tween 20. The seeds were then rinsed thoroughly in water and placed on moist filter paper (which had been sterilised by boiling water) at 25°C overnight, transferred to 4°C for 24 hours and then returned to 25°C in the dark until the seminal roots were approximately 1.5 cm long. Roots were cut off (approximately 1 cm) and pre-fixed in ice cold water for 24 hours prior to fixation in a 3:1 mixture of absolute ethanol and glacial acetic acid for at least 1 hour but not longer than 4 days.

Preparations of metaphase cell spreads for cytological examination or *in situ* hybridisation (ISH) were carried out by either of the following methods:

- i) Root tips were placed in a drop of 45% acetic acid on an alcohol-cleaned or when used for isotopic *in situ* hybridisation, on a pre-coated (Gerhard *et al.*, 1981) microscope slide and allowed to soften for 1-2 minutes. Meristematic cells were teased from the tip of the root using watchmakers forceps under a dissecting microscope. A coverslip (22mm x 22mm) was then carefully placed over the cell suspension and the slide warmed gently over a flame. Cells

were squashed by placing the slide between three layers of Whatman 1MM (Maidstone, England) filter paper and using the thumb, applying gentle, even pressure. The coverslip was removed by freezing the slide either on a block of dry ice or by immersing in liquid nitrogen and popping the coverslip off with a scalpel blade. The slide was then dried by gently warming over a flame. This last process also served to fix the cells to the slide. Slides were then stored overnight in a desiccator if they were not to be used immediately.

ii) Root tips were transferred to water briefly before being placed in 0.2 N HCl at room temperature (RT) for 1 hour hydrolysis. Following hydrolysis, root tips were processed as described above. Slides were stored overnight in a desiccator or if they were not going to be used immediately, were placed at -80°C for up to 6 months. Slides prepared as described in section i) were not stored at -80°C .

2.3.2 Meiosis

An immature inflorescence was removed and placed in a small amount of water in a plastic petri dish. Anthers at the appropriate stage (Metaphase I) were identified by making temporary aceto-carmine preparations of one of the three anthers present in a single floret; one anther of a primary or secondary floret was placed in a drop of 1% aceto-carmine on a glass microscope slide. The pollen mother cells were teased from the anther, a coverslip (22mm x 22mm) was placed on the cell suspension and the coverslip was tapped gently to spread the cells. Cells were squashed by placing the slide between three layers of Whatman 1MM filter paper and gently applying pressure with the thumb. Slides were examined under the microscope to determine the stage of cell division. If cells were not in Metaphase I, anthers from florets above and below the previous one were checked. Once an anther was identified to be at the correct stage, the remaining two anthers were placed in a 3:1 mixture of absolute ethanol and glacial acetic acid for at least 30 min. Anthers were stored in this manner at 4°C for up to 2 weeks.

2.3.3 C-Banding

The following method was used for C-banding meiotic chromosome preparations; slides were placed in a saturated solution of barium hydroxide (6 g/100 ml) at 60°C and incubated for 4-6 minutes. Slides were washed thoroughly of the barium hydroxide solution with tap water and transferred to a 2X solution of standard saline citrate (SSC; 0.15 M NaCl, 0.015 M trisodium citrate) at 60°C and incubated for at least 20 minutes. Slides were agitated gently at this stage to prevent the formation of bubbles on the surface of the slide. Slides were then washed gently with water and stained with geimsa. Geimsa staining was according to the method of Dr. I. S. Dundas (personal communication); slides were air dried and geimsa stain (Gurrs Improved R66), diluted 1:3 with water, was pipetted directly onto the cellular material. After 60 seconds, the geimsa stain was rinsed off the slide. Slides were air dried and coverslips were mounted using immersion oil and examined under phase contrast on a Zeiss Axiophot Microscope using a Plan-Neofluar 100x/1,30 oil objective.

2.4 SCREENING FOR RESISTANCE TO *HETERODERA AVENAE* WOLL.

The assay procedure reported by Fisher (1982a) was used with minor modifications. Seeds were surface sterilised and germinated as described in 2.3.1. When the first three seminal roots were approximately 1 cm long, the seedlings were planted in Palmer soil (sandy red soil) in polyvinyl chloride tubes (27 mm diameter x 125 mm long). Each seedling was inoculated with 100 larvae per 1 ml of water (prepared by Heather Fraser) at the time of planting and four times thereafter with alternating intervals of three and four days. Once inoculation was complete, the tubes were arranged in trays on a base of potting mix.

Plants were grown at 15°C constant temperature with a 16 h day and 8 h night regime maintained with fluorescent light (12 x 500 Watt quartz halogen). Plants were grown for 9 weeks after the last inoculation before harvesting.

At harvest, the tubes were placed in a beaker of water (ca. 600 ml) and allowed to soak for 10 min. The plant was removed from the tube and the water decanted over a set of two sieves (22 mesh; 710 μ m and 60 mesh; 250 μ m). Soil and cysts were dislodged from the roots with a strong spray of water over the sieves. Cysts were collected in the lower sieve and were counted under 10X magnification. Selected resistant plants were re-potted and placed in the glasshouse for further analysis.

2.5 ACRYLAMIDE GEL ELECTROPHORESIS

2.5.1 Glutamate oxaloacetate transaminase (E. C. 2.6.1.1.)

The presence or absence and number of doses of chromosome 6R was established in seedlings by screening for the isozyme glutamate oxaloacetate transaminase (GOT) using the procedure adapted from Hart (1975) and described in detail by Asiedu (1986).

2.5.2 Seed storage proteins

Reduced proteins were extracted from seed endosperm as described by Lawrence and Shepherd (1980). For extraction of unreduced proteins, β -mercaptoethanol (1.5% v/v) was excluded and samples were heated at 65°C for 2 h. Electrophoresis was carried out as described by Lawrence and Shepherd (1980) with the following modifications; the percentage of acrylamide was increased to 10% w/v and the percentage of bisacrylamide increased to 0.8% w/v. Electrophoresis was carried out at 80 mA (200V), 4°C for approximately 2 h followed by a further 20 min at 10 mA. Gels were then stained and destained as described by Lawrence and Shepherd (1980).

2.6 BACTERIAL STRAINS, PLASMIDS AND CLONES

Bacterial strains were grown in Luria-Bertaini (LB) media (Sambrook *et al.*, 1989) and when required, the antibiotics ampicillin and tetracycline were included at 50 µg/ml and 12.5 µg/ml, respectively. Plasmids were propagated in the *E. coli* strain DH5α.

2.7 ISOLATION AND PURIFICATION OF DNA

2.7.1 Large scale cereal genomic DNA preparation

DNA was extracted from fresh leaves (1.0-10.0 g) from plants grown as described by Guidet *et al.* (1991).

2.7.2 Small scale cereal genomic DNA preparation

DNA was extracted using the mini-prep method described in detail by Rogowsky *et al.* (1991*b*). The yield of DNA was approximately 25 µg.

2.7.3 Large scale plasmid isolation

Five millilitre cultures were incubated overnight at 37°C. From this culture, 0.5 ml was inoculated into 30 ml of LB media which was incubated shaking at 37°C until the OD₆₅₀ reached 0.6. Four and a half millilitres of this culture was used to inoculate 500 ml of media. This was incubated shaking at 37°C until the OD₆₅₀ reached 0.6-0.8. At this point, 7.5 ml of 20% glucose and 7.5 ml chloramphenicol (10 mg/ml in 50% ethanol) were added and the cultures allowed to incubate shaking at 37°C overnight for plasmid amplification. The cells were then chilled on ice and pelleted by centrifugation at 10,000 rpm for 10 min at 4°C (Sorval type SS-34 rotor). Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly (1979) and further purified by ultra-centrifugation to equilibrium in cesium chloride-

ethidium bromide solution (refractive index = 1.390-1.396) using a Beckman type 65 rotor at 40,000 rpm for 43 h at 20°C.

2.7.4 Small scale plasmid isolation

Plasmid DNA was isolated from 1.0-1.5 ml of an overnight culture by the alkaline extraction method of Birnboim and Doly (1979).

2.7.5 Recovery of DNA from agarose gels

A) Glass-Milk

Following gel electrophoresis of restricted DNA, the required band was visualised after ethidium bromide staining on a long wave ultraviolet transilluminator (340 nm) and excised using a scalpel. The agarose block was transferred to a 1.5 ml tube, weighed and DNA recovered according to the standard protocol supplied with the GeneClean kit (Bio 101) [Bresatec, Australia]. The quantity of DNA isolated was established usually after gel electrophoresis.

B) "Freeze-squeeze"

The required band was isolated as described above, however, DNA was recovered as described by Koenen (1989). DNA was resuspended in TE buffer and its concentration and quality checked on a 1% agarose gel.

2.8 RESTRICTION ENDONUCLEASE DIGESTS

2.8.1 Cereal genomic DNA

Plant genomic DNA was digested to completion with restriction endonucleases as recommended by the manufacturer. Typically, 5-10 μg of genomic DNA was digested with 10-20 U of restriction endonuclease in a 15 μl reaction volume over-night at 37°C.

2.8.2 Other DNA

Plasmid DNA was digested to completion with restriction endonucleases as recommended by the manufacturer.

2.9 GEL ELECTROPHORESIS

Gel electrophoresis was carried out through 0.8-3.0% (w/v) agarose horizontal slab gels. Samples were mixed with 1/10 volume of 10X ficoll loading buffer (100 mM Tris-HCl, 200 mM EDTA, 30% [w/v] ficoll 4000, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol FF, pH 8.0) prior to loading the gel. The running buffer was either Tris-borate (TBE; 90 mM Tris-borate, 1 mM Na₂EDTA [pH 8.0]) for PCR analysis or Tris-acetate (TAE; 40 mM Tris-acetate, 1 mM Na₂EDTA [pH 8.0]). DNA was visualised by staining the gels subsequent to electrophoresis in a solution of ethidium bromide (0.5 $\mu\text{g}/\text{ml}$). If a permanent record was required, gels were photographed using a Polaroid Landpack camera and Polaroid 665 positive film. Lambda DNA restricted with *Hind*III or $\lambda\text{dv}1$ DNA (Streeck and Hobom, 1975) restricted with *Hae*III were used as molecular weight standards.

2.10 SOUTHERN HYBRIDISATION

Approximately 3.0-5.0 μg of total plant DNA was digested to completion as described above and separated by electrophoresis in 1.0% agarose gels in TAE buffer for approximately 16 h at

25 mA (2.0 V/cm). The gels were then stained with ethidium bromide (0.5 µg/ml) for 20 min on a rocking platform, the DNA visualised on a short wave ultraviolet transilluminator and photographed. Gels were then denatured for 30 min in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and neutralised for 30 min in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl, 0.001M Na₂EDTA, pH 7.2). DNA was transferred to nylon membrane (Hybond N⁺, Amersham) using 20X SSC and was carried out for at least 7 hours before disassembly. DNA was fixed to the membranes by air drying for 15 min and then lying DNA-side up on two sheets of Whatman 3MM paper moistened with 0.4 N NaOH for 20 min at RT. Membranes were then rinsed in 5X SSC for 1 min, air dried and stored between 2 sheets of Whatman 3MM paper at 4°C.

Hybridisation of ³²P-labelled probes to nylon membranes was carried out as follows; nylon membranes were wetted in 5X SSC, sandwiched between nylon mesh (HY-OV-RM, Hybaid, Middlesex, UK), rolled up and placed inside a glass hybridisation bottle (HY-OUBLA, Hybaid). Ten millilitres of pre-hybridisation fluid (0.6 M NaCl, 20 mM PIPES, 5 mM Na₂EDTA [pH 6.8], 0.2% [w/v] ficoll 400, 0.2% [w/v] polyvinyl pyrrolidone (PVP), 0.2% [w/v] BSA, 1% SDS, 0.5 mg/ml denatured salmon sperm DNA) pre-warmed to 65°C was added to each hybridisation bottle and incubated in a rotating oven (Model HB-OV-1, Hybaid) at 65°C for 3-6 h. Following pre-hybridisation, the fluid was exchanged for 5 ml of hybridisation fluid (0.6 M NaCl, 20 mM PIPES, 5 mM Na₂EDTA [pH 6.8], 0.2% [w/v] ficoll, 0.2% [w/v] polyvinylpyrrolidone (PVP), 0.2% [w/v] BSA, 1% SDS, 7.5% [w/v] dextran sulphate, 0.1 mg/ml denatured salmon sperm DNA, denatured ³²P-labelled probe) pre-heated to 65°C. Membranes were incubated in a rotating oven at 65°C for 12-18 h.

Following hybridisation, membranes were washed with 100 ml of 2X SSC, 0.1% SDS for 30 min in the hybridisation bottle and then successively with 100 ml per membrane of the following buffers in a shaking water bath (100 rpm) at 65°C; 1X SSC, 0.1% SDS for 30 min and 0.5X SSC, 0.1% SDS for 15-30 min depending upon the degree of hybridisation present on individual membranes. Membranes were then sealed in plastic and placed in an X-Ray

cassette with X-Ray film (Fuji New Nif RX, Fuji, Japan) and exposed at -80°C for the appropriate length of time.

Labelled probe DNA was stripped from the membranes following autoradiography by pouring boiling stripping solution (0.1% SDS, 2 mM Na₂EDTA) over the membranes and incubating at 65°C in a shaking water bath (100 rpm) for 30 min. This step was repeated and the membranes sealed in plastic and stored at 4°C.

2.11 SUBCLONING

2.11.1 Isolation of pAW173 in forward and reverse orientation

The clone pAW173 containing a 442 bp fragment from Imperial Rye (Guidet *et al.*, 1991) was subcloned in order to obtain clones containing this fragment in both forward and reverse orientation to facilitate sequencing. The 442 bp *Bam*HI insert was isolated from pAW173 by *Bam*HI digestion and purification as described in section 2.7.5 (A). One µg of vector pTZ19R (USB) was linearised by restriction endonuclease digestion with 5 U of *Bam*HI in a volume of 50 µl at 37°C for 1.5 h. Linearized plasmid DNA was then extracted with phenol:chloroform (1:1) once and precipitated by the addition of 2.5 volumes of ethanol. DNA was pelleted by centrifugation (13,000 rpm, 15 min), washed with 70% ethanol, air dried and resuspended in 50 µl TE (10 mM Tris-HCl pH 8.0, 0.1 mM Na₂EDTA). Dephosphorylation of linearised vector DNA was carried out by the addition of 0.3 U calf intestinal alkaline phosphatase (CIP, Boehringer Mannheim) and subsequent incubation at 37°C for 30 min. This step was repeated prior to the enzyme being inactivated by incubation at 80°C for 10 min. DNA was then extracted with phenol:chloroform (1:1) once, precipitated by the addition of 2.5 volumes of ethanol, pelleted by centrifugation (13,000 rpm, 15 min), washed with 70% ethanol, air dried and resuspended in 30 µl TE buffer. The ligation reaction was carried out as follows; 50 ng of vector DNA and 25 ng of purified insert DNA (vector:insert 2:1) was incubated with 1.0 U of T4 ligase in a 12 µl volume in a buffer containing 66 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 15 mM dithiothreitol (DTT), 1 mM

spermidine, 0.2 mg/ml BSA (DNase free, Boehringer Mannheim) and 1 mM ATP for 30 min at RT followed by 1 h at 4°C.

2.11.2 Isolation of pAW173 *Bst*EII subclones

Sequencing data obtained from λ clones containing members of the R173 rye specific repetitive DNA family (Rogowsky *et al.*, 1992a) and from the clone pAW173 indicated that the fragment contained in pAW173 possessed an internal *Bst*EII site. Therefore, to facilitate the use of the Applied Biosystems automated sequencing system (ABI model 373A), subclones of pAW173 in the vector pUC19 (Gibco BRL) were created utilising the internal *Bst*EII site.

A) Digestion with *Eco*RI/*Bst*EII and *Hind*III/*Bst*EII

Six micrograms of plasmid DNA was digested with either *Eco*RI and *Bst*EII or *Hind*III and *Bst*EII in order to create the two possible segments of this fragment. Both reactions were carried out in a 50 μ l volume in a buffer containing 10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 100 mM NaCl and 1 mM β -mercaptoethanol with 30 U of each restriction endonuclease and were incubated at 37°C for 1 h followed by 60°C for 2 h. Products were checked on a 3% TBE gel (5 V/cm) revealing the expected fragments of 235 bp and 265 bp for the *Eco*RI/*Bst*EII and *Hind*III/*Bst*EII digestions, respectively. The DNA was extracted with phenol:chloroform (1:1), precipitated by the addition of 2.5 volumes of ethanol, pelleted by centrifugation (13,000 rpm, 15 min), washed with 70% ethanol, air dried and resuspended in 34 μ l TE buffer.

B) Ligation

Non-compatible, recessed 3' termini resulting from digestion were filled in by incubation (of the DNA) in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 50 mM NaCl, 1 mM dithioerythritol [DTE], 250 μ M dATP, dCTP, dGTP, dTTP and 2 U Klenow (Boehringer

Mannheim) at 30°C for 30 min followed by heat inactivation of the enzyme at 60°C for 10 min. DNA was recovered by ethanol precipitation and centrifugation (13,000 rpm, 30 min) and was resuspended in 20 µl of TE buffer. Ligation of blunt-ended linearised vector and deleted insert (0.9 µg) was carried out as described in section 2.11.1 with minor modifications; reactions were carried out in a 20 µl volume and were incubated at RT for 2 h.

2.12 COMPETENT CELLS

Competent DH5α cells were prepared as described by Sambrook *et al.* (1989) with the following modification; cells were cultured in LB media not SOB. Cells were incubated shaking at 37°C for approximately 2 h until the OD₆₀₀=0.45-0.55. The cells were then placed on ice for 10-15 min and were harvested by centrifugation at 2,500 rpm for 12 min at 4°C (Sorval SS-24 rotor). The cells were then resuspended in 8 ml of TFB buffer (10 mM MES [pH 6.3], 45 mM MnCl₂, 100 mM RbCl, 10 mM CaCl₂, 3 mM hexamine CoCl₂) and placed on ice for 10-15 min. Cells were then pelleted as described above and resuspended in 2 ml TFB. Seventy microlitres redistilled DMSO was then added, the cells gently mixed and incubated on ice for 5 min. One hundred and fifty seven micolitres of 1M DTT was added, the cells mixed gently and incubated on ice for 10 min. A further 75 µl of redistilled DMSO was added and the cells incubated on ice for 5 min. At this stage cells were ready for use in transformation of plasmid DNA.

2.13 TRANSFORMATION

Plasmid DNA was transformed into competent DH5α cells using one of the following methods;

- i) 1.0 µl of DNA was mixed with 50 µl of competent cells and incubated on ice for 20 min. The cells were then subjected to a heat shock at 42°C for 2 min followed by the addition of

100 μ l of SOC (Sambrook *et al.*, 1989). Cells were then incubated at 37°C for 20-60 min prior to plating out on LB plates containing the appropriate antibiotic.

ii) As described above except 1.0-10.0 μ l of DNA was mixed with 210 μ l of competent cells, 800 μ l of SOC was added to the cell suspension after the heat shock and cells were incubated at 37°C for 50-60 min prior to plating out.

2.14 LABELLING OF NUCLEIC ACIDS

2.14.1 Tritium

DNA probes were labelled with [³H]TTP (1 mCi/ml, Amersham) using the random priming method of Feinberg and Vogelstein (1983). Labelling reactions were carried out in a 30 μ l volume. Forty microcuries of [³H]TTP (300 nmole) were lyophilised in an eppendorf tube and resuspended in 15 μ l 2X labelling buffer (100 mM Tris-HCl [pH 7.5], 10 mM NaCl, 10 mM MgCl, 0.2 mM DTE, 80 μ M dATP, dCTP, dGTP and 12.0 μ g/ml random hexamers [Bresatec, Australia]). Approximately 100 ng of DNA template was denatured by boiling for 5 min, rapidly chilled on ice and then added to this mixture. The volume was adjusted to 28 μ l with sterile water and 2 U of Klenow (1 U/ μ l, Boehringer Mannheim) was added. This mixture was incubated at 37°C for 2 hours.

Labelled DNA was purified by G-100 Sephadex chromatography; 28 μ l of the reaction mixture (2 μ l was reserved for determination of incorporation efficiency) was eluted through a G-100 column using approximately 1 ml of TE buffer. Fractions were collected as follows; fraction one containing the first 500 μ l (25 drops), the following 6 fractions each containing approximately 100 μ l (5 drops) and finally, the last fraction containing approximately 500 μ l (25 drops). Fractions containing the labelled DNA were identified via scintillation counting and pooled for use in *in situ* hybridisation experiments.

2.14.2 Biotin

DNA probes were labelled with either Biotin-11-dUTP (Enzo Biochem) or Biotin-14-dUTP (Gibco BRL) via nick translation (Rigby *et al.*, 1977). Reactions were carried out in a 50 μ l volume according to the method described by Rayburn and Gill (1985a) with the following modifications; biotinylated dUTP (30 μ M) was used to label 1.5 μ g of probe DNA for only 2 h at 15°C rather than 2.5 h at 18°C. The amount of DNaseI used in each reaction was increased to 3000 pg. [³H]dTTP was not included in the reaction to ascertain the efficiency of the nick translation reaction, rather the success of each labelling reaction was determined via a dot blot assay (Section 2.15.1).

Labelled DNA was purified on a Sepharose (CL-6B, Pharmacia) spin column. Briefly, an 0.5 ml eppendorf tube pierced at the base with an 18 gauge syringe was "plugged" with a small quantity of acid washed sand. Approximately 750 μ l of Sepharose slurry (Sepharose CL-6B, washed 6 times with 1 volume of TE buffer and finally resuspended in 1 volume of TE buffer) was pipetted into the plugged tube with a wide bore pipette. This tube was placed inside a 1.5 ml eppendorf tube and centrifuged (Hettich Universal 2S Centrifuge) at 1,500 rpm for 10 min. The contents of the nick translation reaction (50 μ l) were then loaded onto the Sepharose column and the tube centrifuged again at 1500 rpm for 10 min. Labelled DNA was collected in a clean 1.5 ml eppendorf tube.

2.14.3 Phosphorous

DNA probes were labelled with [α -³²P]dCTP (3000 Ci/mmol, Amersham) using the random priming method of Feinberg and Vogelstein (1983). Each reaction was carried out in a 30 μ l volume containing approximately 50-100 ng of denatured DNA template, 15 μ l of a 2X buffer (100 mM Tris-HCl [pH 7.6], 100 mM NaCl, 20 mM MgCl, 200 μ g/ml BSA, 60 μ M dATP, dTTP, dGTP and 16.6 μ g/ml random primers (Bresatec, Australia) or random 9-mers [synthesised on an Applied Biosystems 391 DNA Synthesiser]) and 2 U of Klenow enzyme

(Boehringer Mannheim or Pharmacia). Reactions were incubated at 37°C for 1 h. Unincorporated nucleotides were removed by chromatography on a Sephadex G-100 column prepared in pasteur pipettes. The fraction containing labelled probe DNA was collected by monitoring the column with a gieger counter and identifying the peak relating to the labelled fraction compared with that relating to the unincorporated nucleotides.

2.15 EFFICIENCY OF LABELLING

2.15.1 Biotin: Dot blot

Efficiency of the biotin labelling reaction was determined via the dot blot assay using the BluGENE[®] Nonradioactive Nucleic Acid Detection System (Gibco BRL) according to the manufacturers instructions. Briefly, a dilution series of labelled DNA was prepared in the dilution buffer supplied and 1500 pg, 15 pg and 3 pg of DNA spotted onto a nylon membrane in a 5 µl volume (Hybond N⁺, Amersham). Detection of biotinylated DNA was carried out according to the manufacturers instructions and after 1 h incubation, the membrane observed. If the smallest quantity of DNA (3 pg) could be detected, the probe was considered to be adequately labelled and was retained; probes were otherwise discarded.

2.15.2 Tritium: Scintillation counting

The efficiency of labelling was monitored via scintillation counting. Two microlitre samples of the labelling reaction were taken prior to Sephadex G-100 chromatography, diluted (1:50) in water, 5 µl spotted onto Whatman 1MM filter paper (1 cm x 1 cm) and air dried. Total radioactivity was determined by placing air dried filters in a 1.5 ml eppendorf tube containing 1 ml of a toluene-based scintillation fluid and counting in a Hewlett Packard scintillation counter. In order to determine the proportion of incorporated nucleotides, a second set of filters were prepared as described above and then washed 3 x in ice cold 5% trichloroacetic acid (TCA), once in 100% ethanol and air dried. Counts were determined as described above. The specific activity of labelled DNA was calculated using the following equation;

Assuming that each of the four oligonucleotides are incorporated with equal efficiency;

$$\text{Total [DNA] in ng (A)} = \frac{\mu\text{Ci input} \times 13.2 \times \% \text{ incorporation}}{\text{specific activity } ^3\text{H}} + [\text{Template DNA}]$$

Since: $2.22 \times 10^4 \text{ dpm} \approx \mu\text{Ci}$, then;

Total radioactivity incorporated (B) = $\mu\text{Ci input} \times 2.22 \times 10^4 \times \% \text{ incorporation}$

Therefore, specific activity (SA) of labelled DNA is;

$$\text{SA} = \text{B/A} \times 10^3 \text{ dpm}/\mu\text{g}$$

Typical determinations for SA were $3.0 \times 10^7 \text{ dpm}/\mu\text{g DNA}$ - $1.0 \times 10^8 \text{ dpm}/\mu\text{g DNA}$

2.16 SIZE DETERMINATION OF BIOTINYLATED PROBES

Approximately 30 ng of biotinylated DNA was denatured by boiling for 5 min and quenching immediately on ice for 5 min. This step was repeated once prior to the addition of 1/10 volume of 10X ficoll loading buffer. Samples were electrophoresed through 1.5% agarose gels in TAE buffer for 1h at 100 mA (2.5 V/cm). Non-biotinylated λdv1 DNA (Streck and Hobom, 1975) restricted with *Hae*III and non-denatured biotinylated DNA were run as a size standards. DNA was visualised by staining in ethidium bromide and photographed as described in Section 2.9 prior to Southern transfer. DNA was transferred to nylon membrane (Hybond N⁺, Amersham) in 20X SSC for at least 8 h, omitting both the denaturing and neutralising steps. The membrane was then rinsed in 5X SSC for 1 min and the DNA fixed to the filter by baking at 80°C for 2 h. Detection of biotinylated DNA was as described in section 2.15.1. All probes tested in this way were found to range in size from 1700 bp-200 bp with a median of 530 bp-350 bp.

2.17 SILONISATION OF COVERSLIPS FOR *IN SITU* HYBRIDISATION

Glass cover-slips (20 mm x 20 mm) were rinsed in distilled water, air dried and siliconised using Sigmacote (Sigma) according to the manufacturers instructions.

2.18 *IN SITU* HYBRIDISATION

2.18.1 Tritiated probes

A) Pre-treatment of chromosome slides

RNA present in the cellular material on slides was removed by digestion with RNaseA (Sigma). Slides were incubated in a solution of RNaseA (100 µg/ml) in 2X SSC at 37°C for 60 min. Slides were then rinsed once in 2X SSC at RT and subsequently dehydrated in 70% ethanol at RT for 1 minute followed by 96% ethanol at RT for 1 min (Mouras, 1987).

B) Chromosome denaturation

DNA on the slides was denatured in a solution of 70% deionised formamide in 2X SSC, pH 7.0 at 70°C for 2.5 min as described by Rayburn and Gill (1985a). No more than 5 slides were denatured at any one time as this caused too great a drop in temperature of the denaturing solution. Typically, the solution would drop to 66°C-67°C and over the 2.5 min incubation return to 70°C. Slides were then transferred to a solution of 2X SSC, pH 7.0 at 0°C for 2 min followed by ethanol dehydration in 70% and 90% ethanol at 0°C for 2 min. Slides were then air dried.

C) Hybridisation

The tritiated probe was denatured by boiling for 5 minutes followed by rapid cooling on ice for 5 min. DNA was then added to the appropriate volume of chilled hybridisation mixture (50% [v/v] formamide, 2X SSCP [0.24 M NaCl, 0.03 M sodium citrate, 0.04 M NaH₂PO₄, pH 6.0],

10% [w/v] dextran sulphate, 1.25-5.0 µg salmon sperm DNA (denatured), 0.01% [w/v] ficoll 400 and 0.01% [w/v] PVP) to give a final concentration of 0.1 µg/ml-0.4 µg/ml depending upon the experiment. Twenty five microlitres of denatured hybridisation mixture (2.5-10 ng probe DNA) was then aliquoted onto the slide and covered carefully avoiding air bubbles with a siliconised coverslip which was then sealed using rubber gum (Earth Brand). Slides were incubated at 42°C for 12-18 hours in a 2X SSC saturated environment.

D) Post-hybridisation washing

Following hybridisation, coverslips were carefully removed with watchmakers forceps and the slides washed. Conditions for post-hybridisation washes and slide dehydration were as described by Huang *et al.* (1988).

E) Autoradiography and staining

For autoradiography, slides were individually dipped for 3 s into photo-emulsion (Kodak, NTB2) diluted with distilled water (1:1) at 42°C. Slides were removed slowly (5 s) from the emulsion, the emulsion wiped from the back of the slide and the slide placed onto an ice-cold tray for 20 min. Slides were transferred to a light-tight box for additional air drying at RT for 90 min prior to being placed in racks inside black light-tight boxes containing silica gel. These boxes were wrapped inside black plastic bags and exposed at 4°C for the required period of time.

Slides were developed in Kodak D-19 developer diluted with H₂O (1:1) at 15°C for 2-5 min, rinsed in 2% acetic acid for 30 s and fixed in Kodak Unifixer (undiluted) at 15°C for 4 min. After washing in H₂O for 5-10 min, slides were stained for 30-45 min with 5% Giemsa stain in 0.15 M phosphate buffer and air dried prior to viewing under phase contrast on a Zeiss Axiophot microscope. Photographs were taken using Fuji 64T film and a Zeiss Plan Neofluar 100 x 1,30 oil objective.

2.18.2 Biotinylated probes

A) Pre-treatment of chromosome slides

Slides were pre-treated as described in section 2.18.1 (A).

B) Chromosomal denaturation

Denaturation of chromosomes in preparation for non-isotopic *in situ* hybridisation was as described in section 2.18.1 (B) except that slides were dehydrated through an ethanol series at -20°C as described by Rayburn and Gill (1985a).

C) Hybridisation

In contrast to the protocol used to prepare the hybridisation solution for isotopic *in situ* hybridisation, biotinylated probes were prepared in the following manner; non-denatured biotinylated DNA was added to the appropriate volume of hybridisation mixture (50% [v/v] deionised formamide, 10% [w/v] dextran sulphate, 2X SSC [pH 7.0], 100X salmon sperm DNA) to give a final concentration of 2.85 µg/ml (100 ng/slide). This mixture was then denatured at 95°C for 10 min followed by rapid chilling in ice water for 5 min. Thirty-five microlitres (100 ng labelled probe DNA) of this mixture was pipetted onto the chromosome preparation and a clean coverslip placed over the preparation. Slides were placed in a humid chamber (saturated with 2X SSC) and incubated at 80°C for 10 min in order to denature the preparations and the probe; slides were then transferred to 42°C for hybridisation for 6-16 h.

D) Post-hybridisation washing

Following hybridisation, coverslips were floated off in 2X SSC at RT. Slides were then subjected to either of the following washing regimes;

- i) Slides were washed in 2X SSC at RT for 5 min, 2X SSC at 42°C for 10 min, 2X SSC at RT for 5 min, phosphate buffered saline (PBS)/0.1 % (v/v) Triton X-100 at RT for 4 min and finally in 1X PBS at RT for 5 min or;
- ii) Slides were washed each for 5 min in 2X SSC at RT, 2X SSC at 45°C, 50% formamide (v/v) in 2X SSC at 45°C, 2X SSC at RT, PBS/0.1 % (v/v) Triton X-100 at RT and finally PBS at RT.

The latter regime is the more stringent of the two and was used in the latter part of this study.

E) Detection and visualisation of hybridised probes

Detection of hybridised probes was done enzymatically using a streptavidin-horseradish peroxidase conjugate (Gibco, BRL) as the reporter molecule. In order to amplify the signal, primary and secondary antibodies were employed. One hundred microlitres of a solution of goat anti-biotin IgG (Sigma) diluted in PBS/0.1% (w/v) BSA (30 µg/ml) was applied and the slides incubated at 37°C for 30 min. Unbound antibodies were removed by washing slides in PBS/0.1% (v/v) Triton-X 100 at RT for 5 min followed by PBS at RT for 5 min. One hundred microlitres of biotin-conjugated rabbit anti-goat IgG (Sigma) diluted 1:400 in PBS/1% (w/v) BSA was then applied to the slides and slides incubated at 37°C for 30 min. Unbound antibodies were removed by washing as described above. Streptavidin-horseradish peroxidase was diluted 1:400 in 4X SSC and 100 µl applied to each slide and incubated at 37°C for 30 min. Unbound streptavidin-horseradish complex was removed by washing slides in 2X SSC at RT for 5 min followed by a 5 min wash in PBS/0.1% (v/v) Triton-X 100 at RT and then in PBS at RT followed by a final rinse in PBS for 2 min at RT immediately prior to cytochemical detection.

Detection of hybridisation was carried out by pipetting 500 µl of a 0.05% solution of 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.03% hydrogen peroxide in dd H₂O

which had been filtered through a 0.2 μm filter (Millipore) directly onto the slides and incubated at RT in the dark for 30 min. The slides were rinsed in PBS at RT for 1 min and stained immediately in a 5% giemsa solution for 1 min. Slides were air dried, mounted with DPX (Sigma) and viewed under phase contrast on a Zeiss Axiophot microscope. Photographs were taken with Fuji 64T film using a Zeiss Plan Neofluar 100 x 1,30 oil objective.

2.19 POLYMERASE CHAIN REACTION (PCR)

2.19.1 Oligonucleotide synthesis

All oligonucleotides used in this study (including M13 forward and reverse sequencing primers) were synthesised on an Applied Biosystems 391 DNA Synthesiser. Oligonucleotides were purified and deprotected according to the manufacturers instructions. Purified oligonucleotides were resuspended in 1 ml of sterile nanopure water and their concentration determined by absorbance spectrophotometry at λ 260 nm.

2.19.2 RFLP Probe preparation

Where the probe DNA was cloned into vectors containing the M13 forward and reverse primer sites, probes for RFLP analysis were prepared by amplification (of the cloned inserts) using the polymerase chain reaction with the M13 forward and reverse primers. Five microlitres of a 1:100 dilution of miniprep plasmid DNA was included in a reaction volume of 100 μl which included 1 pmol M13 forward sequencing primer, 1 pmol M13 reverse sequencing primer, 200 μM each of dATP, dTTP, dCTP and dGTP, 1/10 volume of 10X reaction buffer (Promega, Madison, Wis.) and 2.5 units *Taq* polymerase (Promega), overlaid with approximately 50 μl paraffin oil and placed in a Programmable Thermal Controller (MJ Research Inc., Watertown, Mass.). After an initial denaturation step of 5 min at 94°C, 32 cycles consisting of 1 min denaturation at 94°C, 2 min annealing at 50°C and 2 min extension at 72°C were performed prior to a final extension step of 5 min at 72°C and cooling to ambient temperature. Analysis

was performed on 1% TAE agarose gels as described by Rogowsky *et al.* (1992b) and amplified fragments purified as described in 2.7.5 (A).

2.19.3 PCR amplification of plant genomic DNA

A large number of primers were tested during the course of this study. PCR reactions were carried out as described in 2.19.2. However, a number of minor modifications to this protocol were adopted such as altered annealing temperature for various primers and variations in the number of reactions cycles. Specific details including primer sequence, annealing temperature and number of cycles are listed in Chapter 5, Tables 1 and 2. PCR products were analysed on 3% TBE gels as described by Rogowsky *et al.* (1992b).

2.19.4 Hot-start PCR

Hot-start PCR was carried out as described by D'Aquila *et al.* (1991) with minor modifications. Reaction mixes contained 1 pmol each of forward and reverse primers and were pre-heated at 70°C for 5 min prior to the addition of Taq polymerase and genomic DNA. Reactions were carried out in a total volume of 25 µl. After the addition of Taq polymerase and DNA, the reaction mixes were overlaid with approximately 50 µl of paraffin oil and subjected to a denaturation step of 5 min at 94°C. PCR consisted of 36 cycles of 1 min at 94°C, 2 min annealing at 55°C and 2 min extension at 72°C. A final extension step of 5 min at 72°C was performed before cooling to ambient temperature.

2.20 SEQUENCING

2.20.1 pAW173 in forward and reverse orientation

Individual subclones containing the 442 bp fragment in pTZR19 in both the forward and reverse orientation were sequenced using the T7 Sequencing kit (Pharmacia) according to the manufacturers instructions.

2.20.2 pAW 173 *Bst*EII subclones

Individual subclones containing either the *Eco*RI/*Bst*EII or the *Hind*III/*Bst*EII fragments were sequenced (double-stranded) using dye labelled primers following the standard cycle sequencing protocol (ABI). Sequencing reactions were analysed using the 373A automated sequencing system (ABI).

2.20.3 AW15

The 1.8 kb insert contained in the recombinant clone AW15 was partially sequenced (using dye labelled primers) following the standard cycle sequencing protocol (ABI). Sequencing reactions were analysed using the 373A automated sequencing system (ABI). From this partial sequence, oligonucleotide primers were designed.

2.21 LINKAGE ANALYSIS AND MAP CONSTRUCTION

Data were collected within the TC-F₁ mapping population for 5 PCR and 10 RFLP markers. Marker phenotypes were scored as "a" for 6R^{T701} alleles, "h" for 6R^{Imp} alleles and "-" for missing or uncertain data. Due to the nature of the crosses used in the generation of the TC-F₁ population, segregation of all markers is restricted to 1:1 (female). Linkage analysis and map construction was carried out using the computer programme JoinMap (Stam, 1993). Initial map construction utilised segregation data for all 280 individuals and all 15 molecular markers (ie., including loci *AawC2*, *Xtam36B* and *Xbcd1426* which demonstrated distorted segregation). Allocation of loci into linkage groups was based on a LOD threshold of 3.0. Map construction was initially carried out using a maplod threshold of 0.05. Data was then reanalysed using a maplod threshold of 1.0 in order to ensure the exclusion of information derived from weak linkages and to identify "floating" loci. The marker input data file derived from all 280 TC-F₁ individuals utilised in initial analysis by JoinMap map is shown in Appendix 2. When departures in the gross order of markers as deduced from physical mapping studies

were observed, the data was analysed for possible scoring errors. JoinMap placed all loci except *Xtam36B* into a single linkage group. This locus demonstrated segregation distortion and was removed from further analysis.

Initial map construction indicated two major inconsistencies in the data. Loci *AawS5/FG3a* and *AawS5/FG3b* and loci *Xbcd758* and *AawC2* were inverted with respect to other loci as deduced from physical mapping data. In addition, locus *Xpsr154* consistently mapped immediately proximal of *Xaw15*, which conflicts with physical mapping data (Appendix 3A). Removal of locus *Xpsr154* from the analysis resulted in loci *AawS5/FG3a* and *AawS5/FG3b* being flipped with loci *Xbcd758* and *AawC2* (Appendix 3B). The order shown in Appendix 3B is consistent with physical mapping data and indicates likely errors associated with the scoring of phenotypes for marker locus *Xpsr154*. This marker was therefore removed from further analysis. Increasing the maplod threshold from 0.05 to 1.0 resulted in locus *AawC2* being placed distal of *XksuF37* along with a rearrangement of locus order for loci mapping distal of *Xbcd758* and proximal of *Xaw15* (Appendix 3C). While the latter changes do not conflict with physical mapping data, the position of locus *AawC2* does. Using JoinMap "fixed sequences" option, it was possible to override JoinMap and force *AawC2* to map distal of *Xbcd758* (Appendix 3D). Consequently, *AawC2* was included in final map construction. The recombination frequencies were converted to map units (cM) with the Kosambi mapping function. The computer programme DrawMap (Van Ooijen, 1994) was used for graphic representation of the map. The locus order shown in Appendix 3D was used to assign phenotypes for *CreR*.

In order to map the *CreR* locus, phenotypes determined through the bioassay were included for eight lines only. Phenotypes for individuals TC-F₁-99, TC-F₁-116 and TC-F₁-158 were omitted. *CreR* phenotypes were then assigned to individuals within the data set possessing parental phenotypes for markers mapping both proximal and distal of the locus *XksuF37* (ie., loci *Xcdo1380*, *AawS5/FG4-1*, *AawS5/FG4-2*, *Xbcd1426*, *Xwg933*, *Xbcd340*, *Xaw15*, *Xbcd1*, *AawS5/FG3b* and *AawS5/FG3a*). Individuals which could not be assigned to a particular

parental type due to insufficient marker information were scored as unknown. Linkage mapping of *CreR* was carried out as described above. However, of the 280 individuals screened, 135 possessed phenotype information for PCR markers alone. Consequently, these individuals could not be assigned a phenotype for *CreR* and were therefore not informative for this locus or any of the RFLP loci. Furthermore, 3 of the 135 individuals were putative disomics and 6 individuals were putative nullisomics. Therefore, subsequent linkage analysis utilised a subpopulation of plants consisting of individuals with PCR *and* RFLP data with two exceptions. Individuals TC-F₁-209 and TC-F₁-284 were retained as they had been successfully phenotyped for *CreR* by inclusion in the CCN bioassay.

2.22 STATISTICAL ANALYSIS

Statistical analysis of cyst counts observed on TC-F₂ seedlings derived from TC-F₁ plants 99, 116 and 158 were carried out using the Students *t*-test as described in expression 9.2 of Sokal and Rohlf (1969).

CHAPTER 3

MOLECULAR CYTOGENETIC MARKERS FOR THE RYE GENOME

3.1 INTRODUCTION

The ability to routinely and reliably identify introgressed alien chromatin in breeding lines is a primary concern of plant breeders. Identification of plant chromosomes has in the past relied upon the analysis of chromosome size, arm length ratios, pairing and more recently, chromosome banding. The later technique is based principally on the methods of C-banding and N-banding which, as previously discussed, are inadequate, particularly in identifying small chromosome segments. *Secale cereale* represents a particularly rich gene pool which wheat breeders may access in improving the agronomical characteristics of wheat. While it has been possible to identify small, intercalary introgressions of rye chromatin into wheat using chromosome pairing and C-banding analysis (Friebe *et al.*, 1990; 1991b), it is, in general, extremely difficult and always laborious due principally to the lack of interstitial C-and N-bands present on rye chromosomes.

In situ hybridisation has proven indispensable in establishing the chromosomal localisation of repetitive DNA sequences within cereal genomes. While sequences such as the telomeric repeats initially isolated and characterised by Bedbrook *et al.* (1980a) have proven useful in assessing the presence of rye chromosomes and, to a lesser extent, the characterisation of chromosomal rearrangements in intergeneric hybrids such as Triticale (Appels *et al.*, 1982), these sequences have limited application due to their localisation to heterochromatic regions. A number of moderately repeated, dispersed, repetitive sequences have been isolated and characterised from rye (Appels and Moran, 1984; Appels *et al.*, 1986a; McIntyre *et al.*, 1990; Guidet *et al.*, 1991; Moore *et al.*, 1993). The clone pSc119 has proven useful in characterising rye chromatin present in wheat lines (Lapitan *et al.*, 1986, 1988). Hybridisation to wheat chromosomes occurs at specific, interstitial sites involving sequences homologous to

the 120 bp family from rye (Bedbrook *et al.*, 1980a) while hybridisation to rye chromosomes is characterised by a dispersed pattern along the entire length of all rye chromosomes (Lapitan *et al.*, 1986). The hybridisation pattern observed on rye chromosomes results from a 645 bp fragment recovered from pSc119 subcloned in pSc119.1 (McIntyre *et al.*, 1990). Consequently, the subclone pSc119.1 is a more valuable cytogenetic tool. Other sequences isolated from the rye genome and characterised via ISH include the 5.3-H3 family represented by the clone pSc5.3H3 (Appels *et al.*, 1986a) and the RIS 1 family (Moore *et al.*, 1993). Both sequences are moderately repeated and are dispersed on all rye chromosomes.

Recently, genomic *in situ* hybridisation has been used to characterise interspecies hybrids including those between wheat and rye (Heslop-Harrison *et al.*, 1990; Zhong *et al.*, 1991). This technique has been used widely since it obviates the need for suitable cloned sequences. However, since probe DNA is not cloned, further analysis is impossible. Cloned BIS 1 and RIS 1 sequences have for example, allowed the identification of a higher order of genome organisation in which specific cereal chromosome regions containing interspersed repetitive sequences have themselves been amplified (Moore *et al.*, 1991b, 1993). Consequently, dispersed, species-specific repetitive DNA sequences are still sought as they have the potential to provide information pertaining to the structure and evolution of cereal chromosomes in addition to providing useful cytogenetic markers.

This chapter describes the application of *in situ* hybridisation to establish the chromosomal distribution of the R173 family of rye-specific repeats within the genome of *S. cereale*. An assessment of the potential for this family of repeat sequences to be used as a molecular cytogenetic marker for the identification and characterisation of small intercalary introgressions into wheat was carried out. To this end, a series of both short and long arm 1D-1R recombinant lines, previously described (Koebner and Shepherd, 1985, 1986a, b; Rogowsky *et al.*, 1991b) have been characterised. Both isotopic and non-isotopic IHS methods are compared along with methods of probe preparation.

3.2 RESULTS

3.2.1 Chromosomal distribution of the R173 family of sequences

3.2.1.1 Isotopic ISH

Initial experiments utilised the 450 bp *Bam*HI fragment contained in the clone pAW173 (Guidet *et al.*, 1991). Figure 1A shows a complete metaphase spread of rye cv. Imperial hybridised with 8 ng (9.5×10^4 dpm) of [³H]TTP-labelled probe. Slides were exposed for 14 days at 4°C in light-tight boxes containing silica gel and developed as described in Chapter 2, Section 2.18.1 (E). Specifically, slides were immersed in developer for 2 min. While a certain degree of background hybridisation is apparent, it is possible to conclude that the sequence contained in the plasmid pAW173 is dispersed on all rye chromosomes. Signal intensity is low at both telomeric and centromeric regions on all rye chromosomes. Prominent clusters associated with sequences arranged in large blocks of tandem repeats are not obvious. These observations are demonstrated far more conclusively in Figure 1B. Slides of metaphase cells prepared from the Chinese Spring-Imperial 2R disomic addition line (Driscoll and Sears, 1971) were hybridised and treated as described for Figure 1A. The R173 family is apparently absent from the centromeric region of chromosome 2R and the short arm telomere. There is apparent signal at the long arm telomere. The distribution of silver grains along both chromosome arms indicates that the R173 family of sequences are dispersed.

3.2.1.2 Non-isotopic ISH

Figure 2A shows a complete metaphase cell from the rye cv. Blanco. The clone pScR4-T1 (Appels *et al.*, 1986b) was used as a probe for *Nor-R1* enabling conditions appropriate for non-isotopic ISH to rye metaphase chromosomes, to be determined. Hybridisation was detected on chromosome 1R at the NOR's on the short arm of both homologues (marked with arrows). This result is consistent with previous reports (Leitch *et al.*, 1991; Zhong *et al.*, 1991) and indicates that the protocol in use is effective. Importantly, chromosomal denaturation is sufficient and chromosome morphology is good.

Figure 1A-B. *In situ* hybridisation of [3H]TTP-labelled pAW173 probe to metaphase chromosomes of;

A) *Secale cereale* cv. Imperial. Hybridisation signal indicates a dispersed pattern of distribution for the probe pAW173 on all 7 rye chromosomes.

B) Chinese Spring-Imperial 2R disomic addition line. Both rye chromosome 2R homologues are shown (filled arrows). Hybridisation signal is detected on both rye chromosomes and is more intense than in Figure 1A. A dispersed pattern of distribution is observed for the probe pAW173 on chromosome 2R of rye. Signal intensity appears most intense in the distal half of both the short and long arms.

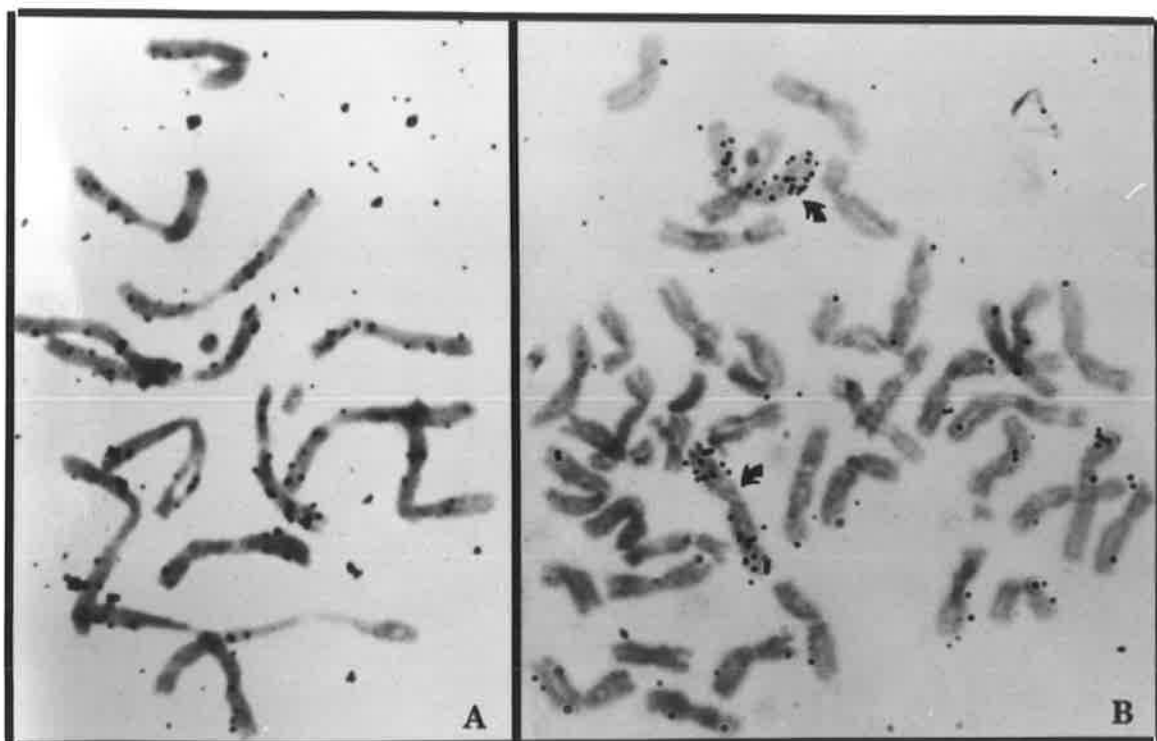
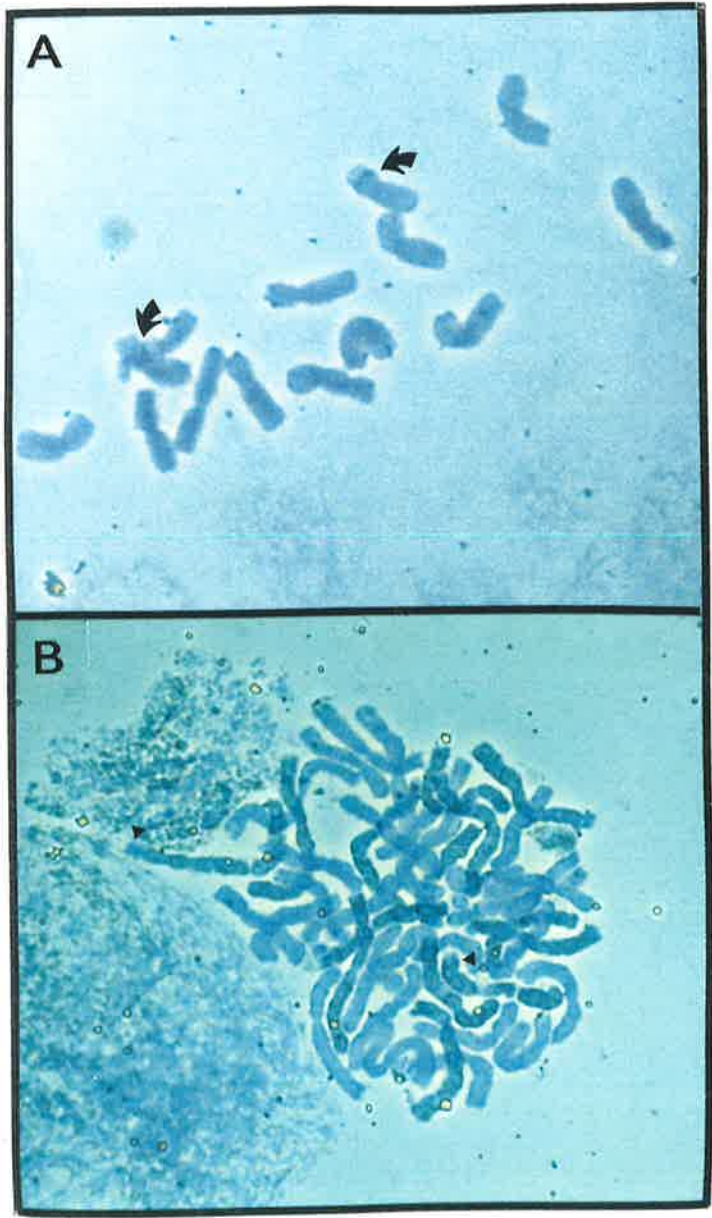


Figure 2A. *In situ* hybridisation of biotinylated pScR4-T1 probe to metaphase chromosomes of *Secale cereale* cv. Blanco. Hybridisation is observed at the NOR of rye chromosome 1R (arrows) as a dark blue/green band against the light blue colour of unlabelled rye chromatin. Both homologues are shown.

Figure 2B. *In situ* hybridisation of biotinylated pAW173.1 probe to metaphase chromosomes of Triticale cv. Carman. Hybridisation is observed along the length of all 14 rye chromosomes with the exception of some telomeres (filled arrows). Unlabelled wheat chromosomes appear light blue compared to the darker green/brown colour of labelled rye chromosomes.



Initial non-isotopic ISH experiments using the clone pAW173 involved labelling the entire clone with biotin 11-dUTP via nick translation. Direct detection through the application of streptavidin conjugated HRP failed to enable visualisation of hybridisation (data not shown) as did cytochemical amplification (data not shown). Consequently, a second clone including almost a complete R173 element was used (Rogowsky *et al.*, 1992a). This clone contained approximately 2.9 kbp of R173 sequence and was labelled with biotin 11-dUTP via nick translation in its entirety. Figure 2B shows a complete metaphase cell from the triticale cv. Carman hybridised with 100 ng of biotinylated pAW173.1. Unlabelled wheat chromosomes appear light blue due to staining with Giemsa. Labelled rye chromosomes appear a much darker green/brown colour, resulting from deposition of insoluble precipitate at the site of hybridisation. Careful analysis identifies a granular appearance to the rye chromosomes and specific sites of hybridisation can be determined. In a number of instances it is possible to observe telomeres which are unlabelled, identified by their light blue appearance (filled arrows). Also, some centromeric regions are less intensely stained, indicating lower copy number of R173 sequences. Resolution of hybridisation is much higher and background greatly reduced compared to earlier results (ie., Figures 1A and 1B). The distribution of R173 elements is even. In agreement with isotopic results, signal intensity is reduced at some centromeric and telomeric sites.

3.2.2 The R173 family of elements as a molecular-cytogenetic tool

3.2.2.1 Wheat-rye 1D-1R recombinant chromosomes

Figure 3A demonstrates hybridisation of the probe pAW173.1 nick translated with biotin 14-dATP to an incomplete metaphase cell from the Chinese Spring-Imperial 1R disomic addition line (Driscoll and Sears, 1971). Both rye 1R homologues are present and are identified by the dark blue/brown precipitate formed. A number of significant points are observed. Hybridisation is observed at the short arm telomere but is absent from the long arm telomere (open arrows). Hybridisation proceeds from the short arm telomere, through the NOR, centromere and long arm including the secondary constriction. The intensity of hybridisation

Figure 3A-F. *In situ* hybridisation of biotinylated pAW173.1 probe to metaphase chromosomes of;

A) Chinese Spring-Imperial 1R disomic addition line. Both rye chromosome 1R homologues are shown (filled arrows). Hybridisation is even and extends from the short arm telomere through the centromere and long arm but is absent from the long arm telomere of each homologue (open arrows).

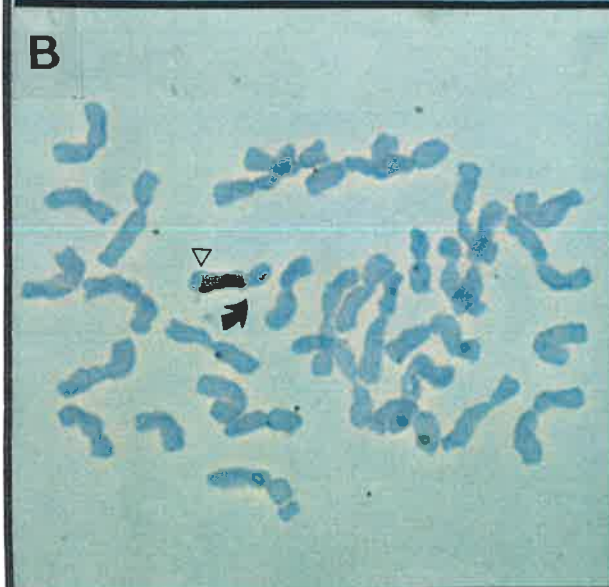
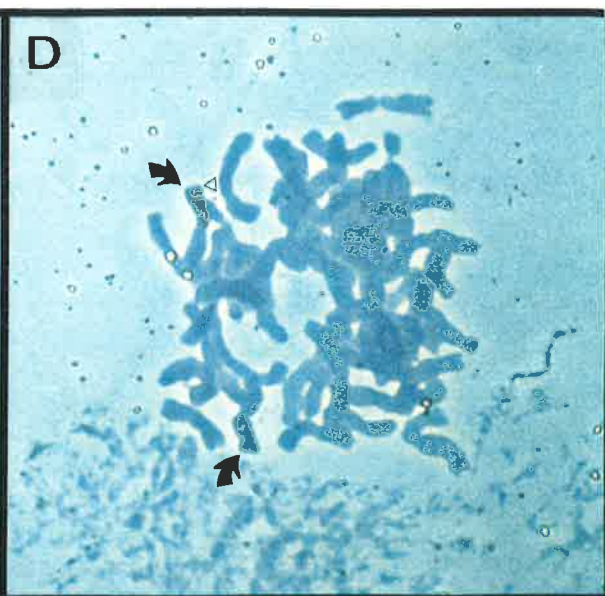
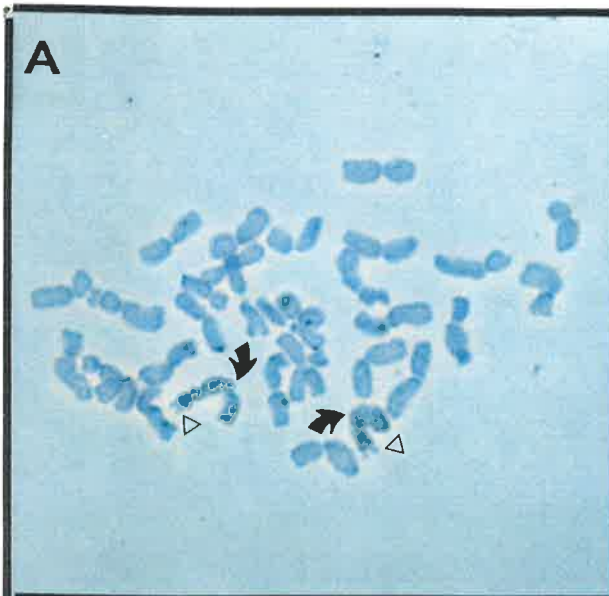
B) Chinese Spring-Imperial 1DL-1RL recombinant line RL2. A single recombinant chromosome is shown (filled arrow). Hybridisation is absent from the centromere (filled arrow) but is observed immediately distal of the centromere. The long arm telomere is unlabelled (open arrow).

C) Chinese Spring-Imperial 1DL-1RL recombinant line R1. Both recombinant homologues are shown (filled arrows). Hybridisation is confined to a small interstitial segment in the distal 20% of the chromosome arm. Both long arm telomeres remain unlabelled (open arrows).

D) Chinese Spring-Imperial 1DS-1RS recombinant line 82-180. Both recombinant homologues are shown (filled arrows). Hybridisation extends from the centromere through the NOR to the satellite region of the short arm of both recombinant chromosomes. The distal telomeric region of both recombinant chromosomes remain unstained (open arrows) indicating the presence of wheat chromatin.

E) Chinese Spring-Imperial 1DS-1RS recombinant line I-93. A single recombinant chromosome is shown (filled arrow). Hybridisation is detected by the dark brown precipitate at the distal telomeric region (open arrow).

F) Chinese Spring-Imperial 1DS-1RS derived recombinant line DRA-1. A single recombinant chromosome is shown (filled arrow). Hybridisation is detected as a very thin light brown band distal of the NOR in the satellited region of the short arm (open arrow).



is decreased at both the NOR and centromere. At sites both immediately proximal and distal of the NOR, signal intensity is increased, possibly indicating higher copy number of R173 sequences at these sites.

In Figure 3B, pAW173.1 has been hybridised to the 1DL-1RL recombinant line RL2 (Koebner and Shepherd, 1985). A single recombinant chromosome is observed in an incomplete metaphase cell. The hybridisation pattern observed is the same in all aspects to that observed in Figure 3A with one exception. Hybridisation at the centromere is apparently completely absent (filled arrow) while signal immediately distal of the centromere is observed. Analysis of other recombinant chromosomes from this line validates this observation (data not shown).

A complete metaphase cell of the 1DL-1RL recombinant line R1 (Koebner and Shepherd, 1985) is shown in Figure 3C. Hybridisation with pAW173.1 is observed as a thin band of dark brown precipitate and identifies both recombinant homologues. The hybridisation signal is confined to a small interstitial segment approximately 4/5 of the way along the long arm towards the telomere. Consequently, the proximal 80% of this chromosome arm is wheat while the remaining distal 20% is rye and probably includes rye telomeric sequences.

Hybridisation of pAW173.1 to the short arm recombinant 82-180 (Koebner and Shepherd, 1986*a, b*) is shown in Figure 3D. Hybridisation is observed as a dark brown/green precipitate, evenly distributed along the short arm of this chromosome. Three points are important to note from this photograph; 1) hybridisation is observed at the centromere; 2) hybridisation is less intense at the NOR, and clearly while hybridisation is intense on either side of the NOR, the light blue appearance of this region indicates low levels of hybridisation and; 3) the distal region of the short arm is unstained, indicating the presence of wheat chromatin since from Figure 3A it may be concluded that pAW173.1 hybridises to the entire length of the short arm of chromosome 1R.

Figure 3E shows an incomplete metaphase spread of the recombinant line I-93 (Koebner and Shepherd, 1986a, b). Only one recombinant chromosome is observed. Hybridisation with pAW173.1 reveals a thin band of dark brown precipitate at the telomere of the short arm of this chromosome.

Finally, the recombinant line DRA-1 which contains a small, intercalary segment of rye chromatin (Shepherd *et al.*, 1990) was analysed and an example is shown in Figure 3F. An incomplete metaphase cell from this line is demonstrated and a single recombinant chromosome is identified (filled in arrow). The open arrow indicates a very thin region of hybridisation, identified as a light brown precipitate confirming both the size and position of the rye introgression.

3.2.2.2 Chromosome 6R

Chromosome 6R from the triticale T701-4-6 present as a disomic substitution in Chinese Spring wheat (Asidue, 1986) was characterised via hybridisation with the clone pAW173.1. Figure 4A shows a complete metaphase cell in which both rye $6R^{T701}$ chromosomes are heavily labelled. The long arm of chromosome $6R^{T701}$ is labelled including the telomere and the secondary constriction. Hybridisation is apparent at the centromere although the signal intensity is somewhat reduced. Hybridisation extends through the short arm proximal to the satellited region. The hybridisation signal observed immediately proximal of the satellited region is of greater intensity. Hybridisation is absent from the satellite on the short arm of chromosome $6R^{T701}$ (open arrows).

Figures 4B and 4C show hybridisation of pAW173.1 to a wheat line carrying a potential translocation chromosome involving chromosome $6R^{T701}$ and a wheat group 6 chromosome, likely to be 6DS (I. Dundas, personal communication). Incomplete metaphase cells are shown in both cases. Hybridisation is observed on the long arm of a single chromosome only in Figure 4B (filled arrows). In Figure 4C, the chromosome in question has broken at the

Figure 4A-C. *In situ* hybridisation of biotinylated pAW173.1 probe to metaphase chromosomes of;

A) Chinese Spring-T701-4-6 6R(-6D) disomic substitution line. Both rye chromosome 6R homologues are shown (filled arrows). Hybridisation extends from the long arm telomere through the secondary constriction. Signal intensity is reduced at the centromere. Hybridisation extends from the centromere through the short arm to the satellited region. Signal intensity is increased immediately proximal of the satellited region. Hybridisation is absent from the satellited region and short arm telomere (open arrow).

B-C) Chinese Spring-T701-4-6 6WS-6RL translocation line. A single recombinant chromosome is shown in both plates (filled arrow). The hybridisation pattern observed on the long arm of the recombinant chromosome is identical to that observed for the long arm of chromosome 6R^{T701}. Hybridisation is absent from the short arm of the recombinant chromosome (open arrow).

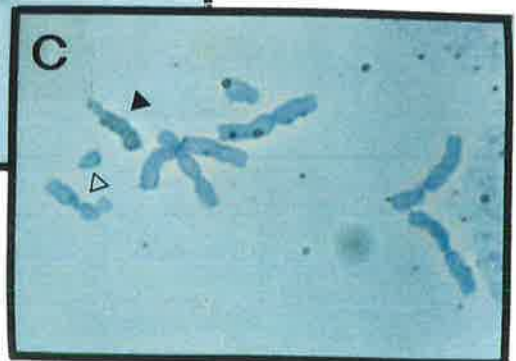
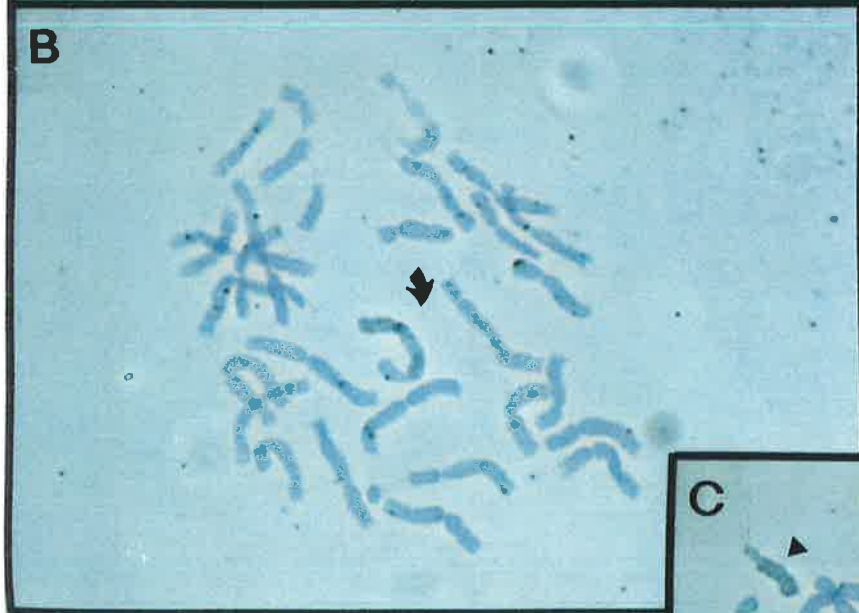
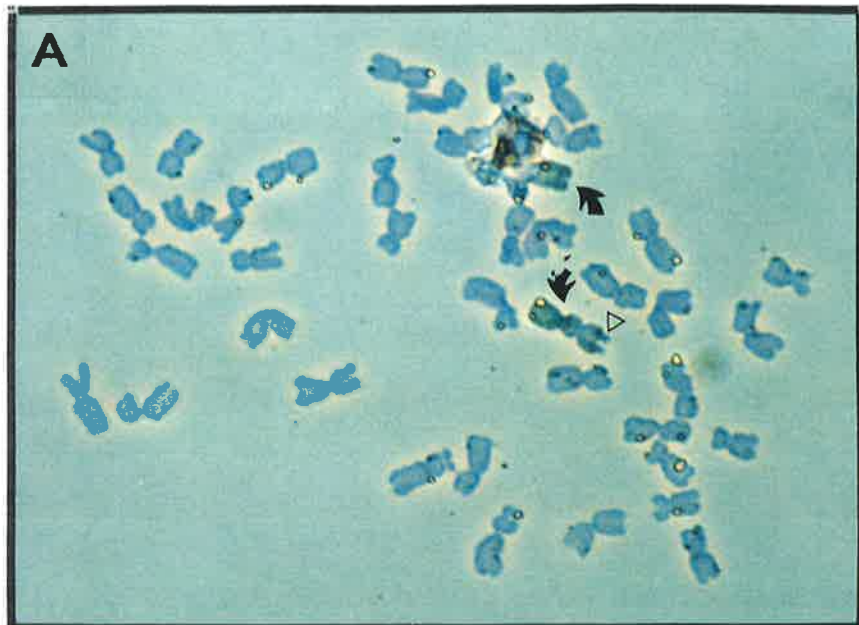
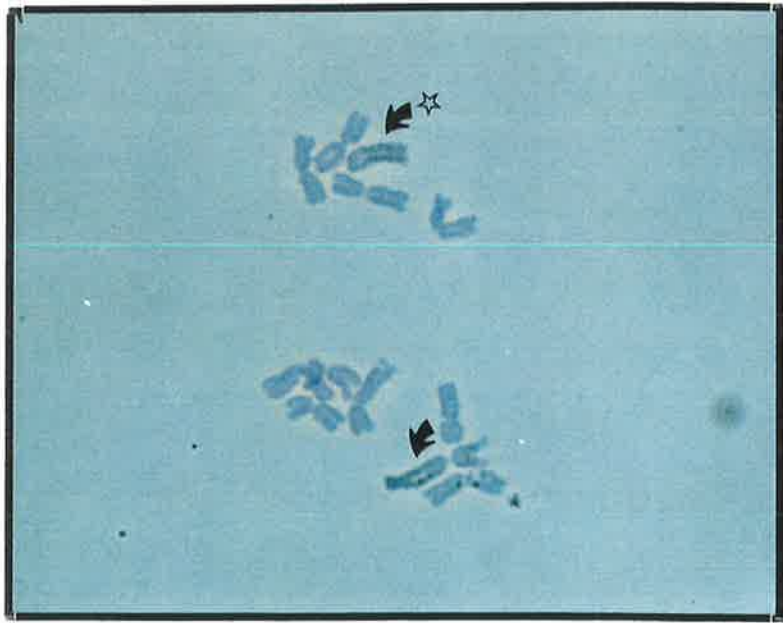


Figure 5. *In situ* hybridisation of biotinylated pAW173.1 probe to an incomplete metaphase spread of an F₁ hybrid produced by crossing Chinese Spring-Imperial 6R disomic addition line with Chinese Spring-T701-4-6 6R(-6D) disomic substitution line. Hybridisation signal is detected on two chromosomes (filled arrows). Chromosome 6R originating from the Chinese Spring-Imperial 6R disomic addition line is identified as a result of its differing morphology (ie., lack of obvious satellited region on the short arm; open star). Hybridisation extends from telomere to telomere. The morphology of the second rye chromosome 6R identifies it as being derived from the Chinese Spring-T701-4-6 6R(-6D) disomic substitution line while the hybridisation pattern is also characteristic of chromosome 6R^{T701} as the short arm telomere remains unlabelled.



centromere, presumable as a result of slide preparation. Hybridisation is not as intense as seen in Figure 4A. However, the hybridisation pattern and chromosome morphology is consistent with this chromosome arm originating from 6R^{T701}. In both plates, the short arm is unlabelled indicating that it is of wheat origin (open arrows).

Finally, ISH with biotinylated pAW173.1 was used to confirm the status of a number of potential F₁ hybrids produced by crossing Chinese Spring-Imperial 6R disomic addition (Driscoll and Sears, 1971) as female to Chinese Spring-T701-4 6R(-6D) disomic substitution (Asidue, 1986). Figure 5 shows an incomplete metaphase cell from one such line. Two chromosomes demonstrate hybridisation with this probe (filled arrows). Clearly, chromosome 6R^{T701} is identified by its distinctive karyotype which includes a prominent satellited region on the short arm. Typically, hybridisation is observed from the long arm telomere through to the short arm, proximal to the satellite. In the upper portion of the plate a second chromosome demonstrating hybridisation is observed (open star). Hybridisation is continuous from telomere to telomere and is characteristic of hybridisation observed to chromosome 6R of rye cv. Imperial (data not shown).

3.3 DISCUSSION

While the results presented in this chapter are apparently straightforward, they hold a number of important implications with respect to our current understanding of cereal chromosome structure. As a consequence, it has been deemed necessary to discuss at length, a number of these implications.

3.3.1 Isotopic ISH

A number of factors were identified as being essential to the success of isotopic hybridisation of repetitive DNA sequences to cereal chromosomes. Initial experiments led to high levels of background. Consequently, the hybridisation temperature was increased from 37°C to 42°C, the amount of labelled probe added to each slide was reduced to no more than 10 ng and the stringency of post-hybridisation washes was increased, following the protocol of Huang *et al.* (1988). The specific activity of probes has been identified as important in successful localisation of DNA sequences (Mouras *et al.*, 1989). Therefore, probes of high specific activity were ensured through random priming (Feinberg and Vogelstein, 1983) of isolated insert DNA with [³H]TTP along with the determination of specific activity upon probe purification. Consequently, subsequent experiments in which the 450 bp *Bam*HI fragment from the clone pAW173 was labelled to a high specific activity and hybridised to rye cv Imperial and the Chinese Spring-Imperial 2R disomic addition line demonstrated that this family of sequences was dispersed throughout all seven rye chromosomes. The absence of large clusters of silver grains also provided evidence that this family of sequences is not tandemly arranged. Results from the experiments using the Chinese Spring-Imperial 2R disomic addition line also indicate that this family of sequences is absent from some telomeric heterochromatic regions and is either absent or present in low copy number at the centromeres of rye chromosomes.

Low levels of signal were detected on rye chromosomes after hybridisation to pAW173. Increased exposure times may have increased the level of signal. Also, other parameters known to affect the efficacy of hybridisation detection sensitivity have been previously identified and include the labelling of probe DNA complete with vector sequences to allow the formation of probe "networks" (Zabel *et al.*, 1983; Huang *et al.*, 1988). These modifications were not attempted in this study since it was likely that the results observed were due to a number of factors other than those attributed to the methodology *per se*. The data presented here and elsewhere (Guidet *et al.*, 1991) demonstrate that R173 elements are not tandemly

arranged. Consequently, in this case, deposition of silver grains results from the hybridisation of the 450 bp *Bam*HI fragment to individual R173 elements and consequently is comparable to the localisation of a unique sequence of 450 bp. In addition, the sequencing of a number of R173 elements (Rogowsky *et al.*, 1992a) has revealed that the 450 bp *Bam*HI fragment is absent from approximately 25% of the members of this family. Consequently, this sequence does not allow the full complement of the R173 family of sequences to be assayed.

Characteristic of isotopic ISH was the low resolution of hybridisation observed in all experiments. Also, the technical demands of isotopic ISH severely limited the application of this method as a means of rapidly identifying rye chromatin present in a wheat background. However, the results from this study indicate the potential application of cloned R173 elements for the molecular-cytogenetic characterisation of rye chromatin and non-isotopic ISH methods were developed.

3.3.2 Non-isotopic ISH

The failure to detect hybridisation with nick translated pAW173 is indicative of the lower levels of sensitivity observed with non-isotopic relative to isotopic ISH methods in plants (Ambros *et al.*, 1986; Clarke *et al.*, 1989; Mouras *et al.*, 1989; Gustafson *et al.*, 1990). This result is also compatible with the present understanding of the distribution and sequence organisation of the R173 family of repetitive sequences in the rye genome (Guidet *et al.*, 1991; Rogowsky *et al.*, 1992a). However, subsequent experiments using the clone pAW173.1 containing a much longer segment from a member of the R173 family, revealed high levels of hybridisation to all seven rye chromosomes. Factors found important to the success of non-isotopic ISH with pAW173.1 include high incorporation rates of biotinylated analogues along with a high concentration (50-100 ng/25 μ l) of labelled probe per slide. However, care in probe quantification is necessary since excessive background resulted above 100 ng/25 μ l. It was also important to maintain the hybridisation temperature at 42°C. Below 42°C, background labelling of wheat chromosomes was observed, presumably as a result of cross-

hybridisation with sequences demonstrating partial homology to the R173 family of sequences present within the wheat genome. Other factors important to the success of non-isotopic ISH have been documented elsewhere (Hopman *et al.*, 1988; Lichter *et al.*, 1991).

3.3.2.1 Distribution of R173 elements within the rye genome

While a detailed study of individual rye chromosomes present as additions to wheat (ie., Chinese Spring-Imperial addition lines) was not carried out in the present study, a number of conclusions can be drawn from the data obtained. As judged from ISH data, R173 elements are present at the telomeres of many rye chromosomes including the long arm of chromosome 6R^{T701}, both the long and short arm of 6R^{Imp} and the short arm of chromosome 1R^{Imp}. Isotopic ISH indicates that R173 elements are also distributed within the telomeric region on chromosome 2RL^{Imp}. The corollary is that R173 elements are absent from many rye telomeres in particular, chromosomes 1RL^{Imp}, 2RS^{Imp} and 6RS^{T701}. Significantly, this pattern of distribution appears to be correlated with the presence or absence of large blocks of constitutive heterochromatin defined by C-banding. C-banding of the chromosomes of the rye cultivar Imperial reveals prominent C-bands at the telomeres of chromosomes 1RL, 2RS and 6RS while only faint or reduced C-bands are observed at the telomeres of chromosomes 1RS, 2RL and 6RL (Mukai *et al.*, 1992). The C-banding pattern of 6R^{T701} has been described by Asidue (1986) and is consistent with that described for 6R^{Imp} by Mukai *et al.* (1992). Consequently, it may be concluded that R173 elements are restricted from regions comprised of large amounts of constitutive heterochromatin.

Regarding the sequence composition of rye telomeric heterochromatin, Jones and Flavell (1980a), using the rye cultivar King II have established that at least one of the four highly repeated, tandemly arranged sub-telomeric families initially described by Bedbrook *et al.* (1980a) is present within these regions. Jones and Flavell (1980b) extended this observation to other species within the genus *Secale*. Specifically, only the 480 bp family of repeats are observed at the long arm telomere of chromosome 6R^{King}, while both the 480 bp and 120 bp

families are observed at the short arm telomere of chromosome 2R^{King}. In contrast, the 120 bp, 480 bp, 610 bp and 630 bp families of repeats have been demonstrated to reside at the short arm telomeres of chromosomes 1R^{King}, 2R^{King} and 6R^{King} (Jones and Flavell, 1980a). These observations suggest that the class of tandemly repeated sequences present at the telomeres of rye chromosomes, notably the 610 bp and 630 bp families, may play some role in restricting the interspersion of R173 elements within these regions. However, Rogowsky *et al.* (1991a) have demonstrated linkage between individual R173 elements and the telomere-specific 610 bp family contained in λ clones cloned from the short arm of chromosome 1R^{Imp}. The 120 bp, 480 bp, 610 bp and 630 bp families make up only 44-65% of the DNA in telomeric heterochromatin (Bedbrook *et al.*, 1980a). Consequently, it is possible that as yet uncharacterised sequences may be influencing the distribution of R173 elements.

Within mammalian genomes, the distribution of LINE or L1 elements has been studied in detail (for review, see Hardman, 1986). L1 elements are a major family of long, interspersed repeat elements found in primate genomes. They vary in size from 6-7 kbp and are reiterated (1-4) $\times 10^4$ times within the human genome accounting for 2-3% of the total complement (Singer, 1982). While L1 elements lack LTR's, they demonstrate some of the salient features of elements which replicate via reverse transcription including the duplication of target sites and potential internal reading frames which may encode transcription functions (Hardman, 1986). However, other evidence points to at least some L1 elements being transcribed adventitiously as part of other RNA molecules (Hardman, 1986). Within the mouse genome, homologues to L1 elements have been shown to have been amplified as part of a larger repeating unit indicating that such sequences are affected by mechanisms operating on other classes of repeated DNA (Nasir *et al.*, 1991). L1 sequences appear to preferentially integrate into G-positive (dark) bands (Korenberg and Rykowski, 1988) which are A+T rich, late replicating regions devoid of genes (Bickmore and Sumner, 1989; Nasir *et al.*, 1991). In contrast, short interspersed elements (SINE) such as *Alu1* are highly repeated throughout the mammalian genome (1×10^6) and appear to preferentially integrate into G-negative (light) bands which are relatively G+C rich, harbour most of the genes and are early replicating (Bickmore and

Sumner, 1989; Korenberg and Rykowski, 1989; Nasir *et al.*, 1991). The mammalian genome, therefore, appears compartmentalised and consequently, G-banding appears to reflect an underlying chromosome structure (Bickmore and Sumner, 1989).

Like L1 elements, R173 elements are also long, interspersed repeated elements which, on the whole, lack LTR's and are flanked by short duplications (Rogowsky *et al.*, 1992a). Both families of repeats are structurally heterogeneous. However, R173 elements do not appear to possess ORF's of any significance and their mode of replication is presently unknown (Rogowsky *et al.*, 1992a). In addition to an apparent absence from some telomeres, lower levels of hybridisation is observed at the centromeres and NOR's of rye chromosomes. More intense hybridisation signal localised to regions immediately proximal and distal to the NOR on the short arm of chromosome 1R^{Imp} and proximal to the satellited region on the short arm of chromosome 6R^{T701} are obvious and suggest the presence of localised areas within the rye genome containing a higher density of R173 elements. However, with the exception of these regions, the overall distribution of R173 sequences is strikingly even.

The chromosomal distribution of a number of dispersed, rye-specific repetitive DNA sequences has been described in some detail. Appels *et al.* (1986a) for example have demonstrated via isotopic ISH that the 5.3 family is dispersed across all rye chromosomes. However, one subclone, designated 5.3-H2 was observed to be absent from the short arm telomeres of chromosomes 1R and 7R from the rye cv Snoopy. The 5.3-H3 sequence was present in the telomeric heterochromatin but in much lower copy number. Both sequences were absent from the NOR present on chromosome 1R. However, it should be said that the resolution of this study was limited through the use of isotopic ISH. Moore *et al.* (1993) reported the cloning of a dispersed, repetitive DNA sequence from the rye genome, RIS 1. Using fluorescent ISH, these authors demonstrated that RIS 1 is dispersed throughout all rye chromosomes but observed either reduced or no signal at centromeres, telomeres and NOR's. The same pattern of distribution has been observed for BIS 1 on barley chromosomes (Moore *et al.*, 1991b). Both BIS 1 and RIS 1 elements have been implicated in a higher level of genome organisation

involving the amplification of specific chromosomal regions containing such interspersed repeats. However, BIS 1 is structurally similar to retrotransposons possessing LTR's and a putative integrase domain (Moore *et al.*, 1991*b*) and is likely to move (or have moved) via reverse transcription. Consequently, interspersion of BIS 1 may simply be superimposed over a pre-existing higher order of structure through a preference for integration sites. It may thus be argued that any evenly dispersed, repetitive sequence will reveal the same form of structural organisation as observed with BIS 1. Indeed, Moore *et al.* (1991*b*) have observed similar results in wheat with a second barley sequence unrelated to BIS 1. With the exception of R173 and some intensely staining C-banding telomeric regions, it would seem that none of the dispersed, repetitive sequences characterised to date are strictly compartmentalised to any notable structural feature of rye chromosomes such as C⁺ or C⁻ chromatin. This implies that sequences analogous to L1 and *Alu1* elements have not yet been identified or do not exist within plant genomes. This observation may therefore indicate a fundamental structural difference between the chromosomes of mammals and those of cereals and possibly higher plants in general. The inability to demonstrate clear G-banding, R-banding and Q-banding in plant chromosomes would seem to support this conclusion.

3.3.2.2 R173 family of elements as a molecular-cytogenetic tool

Rayburn and Gill (1985*a, b*) initially described non-isotopic methods in plants and demonstrated the ability to detect chromosomal translocations involving rye and wheat chromosomes using a dispersed rye-specific repeat found in the clone pSc119. Since this initial study, the use of non-isotopic ISH for the identification and characterisation of introgressed chromatin, rye in particular has become widespread. The application of R173 elements in analysing introgressions of rye chromatin into wheat has been demonstrated using a series of wheat-rye 1D-1R recombinant lines. The hybridisation patterns identified for 1D-1R long arm recombinants are consistent with data obtained using isozyme, RFLP and PCR markers for these chromosome arms (Koebner and Shepherd, 1985; Rogowsky *et al.*, 1993). However, ISH with R173 has provided information regarding the structure of the recombinant

chromosomes which would otherwise be extremely difficult to obtain through molecular methods such as RFLP analysis. Hybridisation of pAW173.1 to the recombinant line RL2 is consistent with that observed for the long arm of chromosome 1R^{Imp} except for an apparent lack of hybridisation at the centromere. This observation has been confirmed in other individual plants from this line indicating that the translocation involved is not centromeric. Since RL2 recombinant chromosomes carry *Glu-R1*, this locus must lie distal to the breakpoint. ISH to the recombinant line R1 has identified that a small distal region of chromosome 1RL has been translocated to chromosome 1DL. Rye chromatin represents approximately 20% of this chromosomal arm. Consequently, both the position and relative amount of rye chromatin introgressed in both long arm recombinant lines has been determined.

Koebner and Shepherd (1986a, b) first described the isolation and characterisation of a series of 1DS-1RS recombinant lines derived through the induction of allosyndesis. Of the recombinant classes identified, the lines 82-180 and I-93 were perhaps the most significant. Recombinant lines were characterised using a number of isozyme and molecular markers. It was established that recombinant line 82-180 contained almost the entire 1RS arm with the exception of a small distal region which had been replaced by wheat chromatin; the wheat loci *Gli-D1* and *Glu-D3* mapped to this chromosome while, rye telomeric sequences were absent. In contrast, I-93 contained most of the 1DS arm but had a small segment of 1RS translocated distally which included rye telomeric sequences. Importantly, both recombinant chromosomes possessed a number of common loci including *Gpi-R1*, *Sec-1*, 5S DNA and *SrR* indicating that these two recombinant classes contained overlapping rye chromatin. Rogowsky *et al.* (1991b) has confirmed and extended the observations of Koebner and Shepherd (1986b) through a more detailed molecular analysis of the recombinant lines 82-180 and I-93 and the recombinant line DRA-1 derived from them. In an attempt to quantify the amount of rye chromatin present in each recombinant line, the copy number of R173 elements was calculated (Rogowsky *et al.*, 1991b). From this analysis, the recombinant lines I-93 and DRA-1 appear to contain similar amounts of rye chromatin while 82-180 could not be distinguished from the entire short arm of chromosome 1R. The amount of rye chromatin present within I-93 and DRA-1, as estimated

by the number of R173 elements present, represents approximately 50% of the entire short arm; a result which apparently contradicts the data obtained through the localisation of both isozyme and RFLP loci (Koebner and Shepherd, 1986b; Rogowsky *et al.*, 1993).

ISH using pAW173.1 allowed the characterisation of the short arm recombinant lines. It can be concluded that the recombinant line 82-180 does indeed contain almost the entire short arm of chromosome 1R. The small, distal region which remains unlabelled after ISH is identified as wheat chromatin since this region of 1RS is always labelled after hybridisation with pAW173.1. Importantly, hybridisation was detected at the centromere, indicating that this recombinant chromosome contains a rye centromere. This conclusion has recently been confirmed through the localisation via ISH of a rye centromeric repeat to the centromere of this chromosome (P. Langridge, personal communication). Hybridisation to the recombinant chromosome contained in I-93 identified a small amount of rye chromatin distally translocated to which rye telomeric sequences have been localised (Koebner and Shepherd, 1986b). However, the most significant observation was that the derived recombinant DRA-1 contained a very small intercalary introgression of rye chromatin, apparently significantly smaller than that found in I-93. This result directly challenges the data of Rogowsky *et al.* (1991b) since the intensity of the signal observed in DRA-1 would indicate significantly lower numbers of R173 elements present within this line as compared to I-93. This result may indicate either that the data presented by Rogowsky *et al.* (1991b) are subject to large error or that the distribution of R173 elements is not as even as suggested. However, considering the latter proposition, results presented in this chapter indicate that the distribution of R173 elements throughout the short arm of chromosome 1R is even.

In an earlier study, Moore *et al.* (1993) characterised the short arm recombinant lines using GISH. These workers came to similar conclusions regarding the relative size and location of rye chromatin present. However, upon comparison with this study, differences in the amount of rye chromatin present in lines I-93 and DRA-1 are apparent. Specifically, using GISH there appears to be more rye chromatin present in both lines. Since GISH utilises biotinylated total

genomic DNA, the hybridisation signal detected will be the result of many repetitive DNA sequences present within the rye genome including tandemly arranged sequences such as the 120 bp, 480 bp, 610 bp and 630 bp families and dispersed families such as RIS 1, 5.3 and R173. As a consequence, a larger fraction of the introgressed rye chromatin may be targeted resulting in an increase in signal intensity. Since assaying I-93 with pAW173.1 leads to the characterisation of a smaller segment of rye chromatin, and it has been established that the distribution of R173 elements is even throughout 1RS, it must be assumed that the increased size determined via GISH is due solely to an increase in signal intensity. This may be compounded through the use of fluorescent reporter molecules. As a consequence, the amount of rye chromatin present in these lines is likely to be less than that established by Moore *et al.* (1993) but more than indicated in this study. The results of this study conclude that the breakpoint of 1RS in the recombinant line 82-180 is centromeric. Moore *et al.* (1993) were unable to reach this conclusion due to the low levels of hybridisation observed at centromeric regions using GISH.

ISH using pAW173.1 also allowed the classification of wheat-rye hybrid lines containing chromosome 6R of rye in various forms. Both chromosome 6R^{T701} and chromosome 6R^{Imp} were characterised. Clear morphological differences were identified between these chromosomes including a prominent secondary restriction found on the long arm of 6R^{T701} but not 6R^{Imp} and the presence of a prominent satellited region on the distal region of 6RS^{T701}. This structure was not observed on 6RS^{Imp}. The ability to identify both chromosomes in putative F₁ hybrids is significant and demonstrates the application of this technology to breeding programmes. The morphological structure of 6R^{Imp} raises some concerns over its structural integrity since the results of Mukai *et al.* (1992) indicate that chromosome 6R^{Imp} used in their analysis possessed the same morphological structures as chromosome 6R^{T701} in this study. It has been demonstrated that rye chromosomes present as additions in wheat background may undergo structural rearrangements including terminal deletions (Gustafson *et al.*, 1983; Koebner *et al.*, 1986). In addition, some variability with respect to the morphological structure of individual rye chromosomes present within the Chinese Spring-

Imperial wheat-rye addition series (Driscoll and Sears, 1971) has been documented previously (Sybenga, 1983). The reason for the apparent structural differences observed with regard to chromosome 6R^{Imp} (this study) are presently unknown.

The use of R173 elements to obtain information on the amount and structural arrangement of rye chromatin introgressed into wheat has been clearly demonstrated through both isotopic and non-isotopic ISH. Non-isotopic methods were found to be superior to isotopic methods in a number of areas. Increased speed and resolution are obvious and are essential if this methodology is to be used in screening programmes. Furthermore, while the sensitivity of non-isotopic ISH in this study was below that of isotopic methods, the ability to detect a very small segment of rye chromatin introgressed in the recombinant line DRA-1 demonstrates that this is unlikely to limit the use of this technique. While GISH studies are also capable of similar results (Moore *et al.*, 1993), the use of total genomic DNA over cloned sequences limits the application of this method, at least in the pursuit of a greater understanding of cereal chromosome structure. Hybridisation to the long arm recombinant line RL2 revealed a further advantage of the use of cloned sequences such as R173. Since R173 does not hybridise to the long arm telomere of 1R, the amount of euchromatin present in this line was more accurately and easily estimated. Furthermore, the results using R173 also indicate that a cloning strategy utilising R173 as a screening tool would allow the isolation of low and single copy sequences from this region of chromosome 1RL. GISH would not have provided this information.



CHAPTER 4

MOLECULAR MARKERS FOR THE LONG ARM OF CHROMOSOME 6R OF RYE: RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) MARKERS

4.1 INTRODUCTION

Fisher (1982a) initially described a line of triticale (x *Triticosecale* Wittmack; T701-4-6) showing high levels of resistance to CCN. The production of individual rye chromosome addition lines through backcrossing T701-4-6 triticale to wheat revealed the resistance character to be controlled by a single dominant gene located on rye chromosome 6R, probably the long arm (Asidue *et al.*, 1990). Subsequent work utilising a series of long arm deletion lines of the rye chromosome 6R^{T701} has confirmed the location of this gene, *CreR* to the long arm of chromosome 6R of rye (Dundas *et al.*, 1992; 1993).

The knowledge of chromosomal location of genes conferring traits of interest enables a more directed approach to the generation of molecular markers. Many cDNA and gDNA clones have been characterised with respect to their chromosomal arm location in hexaploid wheat using the aneuploid and nullisomic-tetrasomic lines developed by Sears (1954). Moreover, wheat-alien chromosome addition and substitution lines allow an assessment of chromosomal location of RFLP probes within homoeologous genomes. This approach to the generation of molecular markers and gene mapping within the Triticeae has been applied with great success in recent years (Sharp and Soltes-Rak, 1988; Sharp *et al.*, 1989; Heun *et al.*, 1991; Anderson *et al.*, 1992). The generation of RFLP-based linkage maps for individual homoeologous chromosome groups within hexaploid wheat and related species such as *T. tauschii*, barley and rye (Chao *et al.*, 1989; K. S. Gill *et al.*, 1991a; Lagudah *et al.*, 1991; Liu and Tsunewaki, 1991; Wang *et al.*, 1991; Devos *et al.*, 1992; Wang *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993a, b; Plaschke *et al.*, 1993) has provided a greater understanding of the nature and

extent of homoeology within the tribe. As a consequence, such maps are valuable for selecting molecular markers potentially useful in mapping studies.

While non-homologous exchanges between rye chromosomes have occurred throughout the evolution of the genus *Secale*, the long arm of chromosome 6R has remained relatively intact with respect to both wheat and barley homoeologous group 6 chromosomes. Numerous homoeoloci have been localised to the long arms of both wheat and rye group 6 chromosomes and low levels of pairing has also been observed. However, a number of studies indicate that exchanges have occurred between 6RL and other rye chromosome arms. Koller and Zeller (1976) suggested a translocation involving 6RL and 7RL. Low levels of pairing between 6RL and 7RL (Naranjo and Fernández-Rueda, 1991) and the localisation of biochemical and RFLP markers to 6RL and 7WL (Benito *et al.*, 1991; Devos *et al.*, 1993b) confirm this rearrangement. However, studies by Devos *et al.* (1993b) indicate that this rearrangement is non-reciprocal and distal. An interstitial, reciprocal rearrangement between 6RL and 3RL has also been demonstrated through chromosome pairing studies and mapping of biochemical and RFLP markers (Miller, 1984; Naranjo and Fernández-Rueda, 1991; Devos *et al.*, 1993b).

Rather than initiating the lengthy and difficult process of generating RFLP markers within this laboratory, the emerging maps and information regarding the nature and extent of homoeology observed between chromosome 6R of rye and group 6 chromosomes of wheat and 6H of barley were utilised to select RFLP markers. This chapter describes the mapping of both genomic and complementary DNA probes previously localised to the long arms of wheat and barley group 6 chromosome arms to chromosome 6RL of rye using the Chinese Spring-Imperial 6R disomic addition line (Driscoll and Sears, 1971) and the Chinese Spring-T701-4-6 6R disomic substitution line described by Asidue (1986) in initial screening experiments.

4.2 RESULTS

4.2.1 Screening chromosome group 6 clones

Table 1 lists all cDNA and gDNA clones previously assigned to chromosome 6 of wheat and/or barley used in this study. A total of 43 clones were analysed, 26 of which had been mapped to the long arm of homoeologous group 6 chromosomes. The chromosomal arm location of the remaining 17 clones was unknown prior to the commencement of this study.

Preliminary screening utilised the rye cultivar Imperial, the Chinese Spring-Imperial 6R disomic addition line and the Chinese Spring-T701 6R disomic substitution line carrying the *CreR* resistance gene. Since the wheat background of the substitution line contains both a small amount of Schomburgk material (Asidue, 1986), this hexaploid wheat cultivar was included. Figures 1A and 1B show two autoradiograms obtained after hybridising screening membranes with the cDNA clone TAM36. All restriction enzymes used, except *HindIII*, revealed RFLPs in rye cv. Imperial with respect to those identified in hexaploid wheat cultivars Chinese Spring and Schomburgk. Importantly, all enzymes used except *HindIII* identified polymorphic bands in both the 6R^{Imp} and 6R^{T701} addition and substitution lines. The restriction enzymes *BamHI*, *BglIII* and *DraI* revealed the same bands for both the 6R^{Imp} and 6R^{T701} addition and substitution lines as identified in rye cv. Imperial. However, *EcoRI*, *KpnI* and *EcoRV* identified RFLPs between the 6R^{Imp} line and rye cv. Imperial. The restriction enzymes *EcoRI*, *KpnI*, *BglIII*, *EcoRV* and *DraI* also identified additional RFLPs between 6R^{T701} and rye cv. Imperial and the 6R^{Imp} addition line. With respect to *HindIII*, the relative intensity of the 5.0 kbp band identified in the 6R^{T701} substitution line suggests that this line possesses the same allele as rye cv. Imperial in addition to a 12.0 kbp band. In the 6R^{Imp} addition line, the 11.0 kbp wheat fragment appears more intense suggesting co-migration of the rye band with that observed in hexaploid wheat.

Across all probe/enzyme combinations employed, cDNA clones demonstrated stronger hybridisation in rye and resulted in less complex banding patterns than those observed with

Table 1. A list of cDNA and gDNA clones, their chromosomal arm location in either hexaploid wheat or barley, the restriction enzyme used and source/reference from which clones were obtained. Clones mapped to chromosome 6R of rye are indicated by an asterix.

Clone	cDNA or gDNA	Restriction Enzyme	Chromosomal Location	Additional Chromosomal Locations	Source and Reference ¹
AW1	C	Unknown	6H		1
AW9	C	Unknown	6H		1
AW13	C	Unknown	6H		1
AW15*	C	Unknown	6H		1
AW19	C	Unknown	6H		1
AW23*	C	Unknown	6H		1
BCD1*	C	<i>EcoRV</i>	6AL, BL, DL		2
BCD102*	C	<i>DraI</i>	6AL, BL, DL	3BS, 3DS	2
BCD221	C	<i>EcoRV</i>	6H	2HS	2
BCD269*	C	<i>XbaI</i>	6H		2
BCD276*	C	<i>DraI</i>	6AL, BL, DL		2
BCD339	C	<i>EcoRV</i>	6H	2HL, 3HL	2
BCD340*	C	<i>EcoRI</i>	6AL, BL, DL		2
BCD758*	C	<i>EcoRV</i>	6AL, BL, DL		2
BCD1426*	C	<i>EcoRV</i>	6AL, BL, DL	3BL	2
CDO419*	C	<i>EcoRI</i>	6H	3HL	2
CDO497*	C	<i>EcoRV</i>	6AL, BL, DL		2
CDO676*	C	<i>DraI</i>	6DL	7AS, 7BS, 7D	2
CDO772*	C	<i>EcoRV</i>	6AL, BL, DL		2
CDO1091*	C	<i>EcoRV</i>	6AL, BL, DL		2
CDO1380*	C	<i>EcoRV</i>	6AL, BL, DL		2
CDO1400*	C	<i>DraI</i>	6D	4AS, 5D, 7AS, 7D	2
WG222	G	Unknown	6H	2HS	2
WG282	G	<i>EcoRV</i>	6H		2
WG286*	G	<i>EcoRI</i>	6AL, BL, DL		2
WG341	G	<i>EcoRI</i>	6BL	5AS, 5DS, 7BL	2
WG405	G	<i>DraI</i>	6DL	5BL, 2H, 3HL	2
WG522*	G	<i>EcoRI</i>	6AL, DL	7AS, 7BS, 7D	2
WG933*	G	<i>EcoRI</i>	6AL, BL, DL		2
KSUF37*	G	Unknown	6DL		3
KSUD1*	G	Unknown	6DL		3
TAM3	G	<i>HindIII</i>	6A, B, D		4
TAM9	G	<i>BamHI</i>	6AL, BL		4
TAM17*	G	<i>BamHI</i>	6AL, BL, DL		4
TAM21*	C	<i>HindIII</i>	6AL, BL, DL		4
TAM25*	G	<i>HindIII</i>	6AL, BL, DL		4
TAM26	G	<i>HindIII</i>	6A, B, D		4
TAM28	G	<i>SacI</i>	6BL, DL		4
TAM30*	C	<i>HindIII</i>	6AL, BL, DL		4
TAM36*	C	<i>HindIII</i>	6AL, BL, DL		4
TAM57	G	<i>BamHI</i>	6A, B, D		4
CSIH90	G	Unknown	6D		5
PSR154*	C	<i>HindIII</i>	6AL, BL, DL	6HL, 6RL	6

¹1) P. Murphy, University of Adelaide, Australia (Murphy *et al.*, 1995); 2) M. E. Sorrells, Cornell University, USA (Heun *et al.*, 1991); 3) B. S. Gill, Kansas State University, USA (K. S. Gill *et al.*, 1991a); 4) G. E. Hart, Texas A&M University, USA; 5) R. Appels, CSIRO, Australia (Lagudah *et al.*, 1991); 6) P. Sharp, University of Sydney, Australia (Sharp *et al.*, 1988).

Figure 1A. Hybridisation of probe TAM36 to screening membrane. Five micrograms of genomic DNA was digested with the restriction enzymes indicated. Membranes were exposed at -80°C for 7 days. The position of lambda *Hind*III markers are shown on the right. DNA samples digested with individual restriction enzymes include;

Lane 1: Chinese Spring wheat

Lane 2: Schomburgk wheat

Lane 3: Imperial rye

Lane 4: Chinese Spring-Imperial 6R disomic addition line

Lane 5: Chinese-Spring-T701-4-6 6R(-6D) disomic substitution line

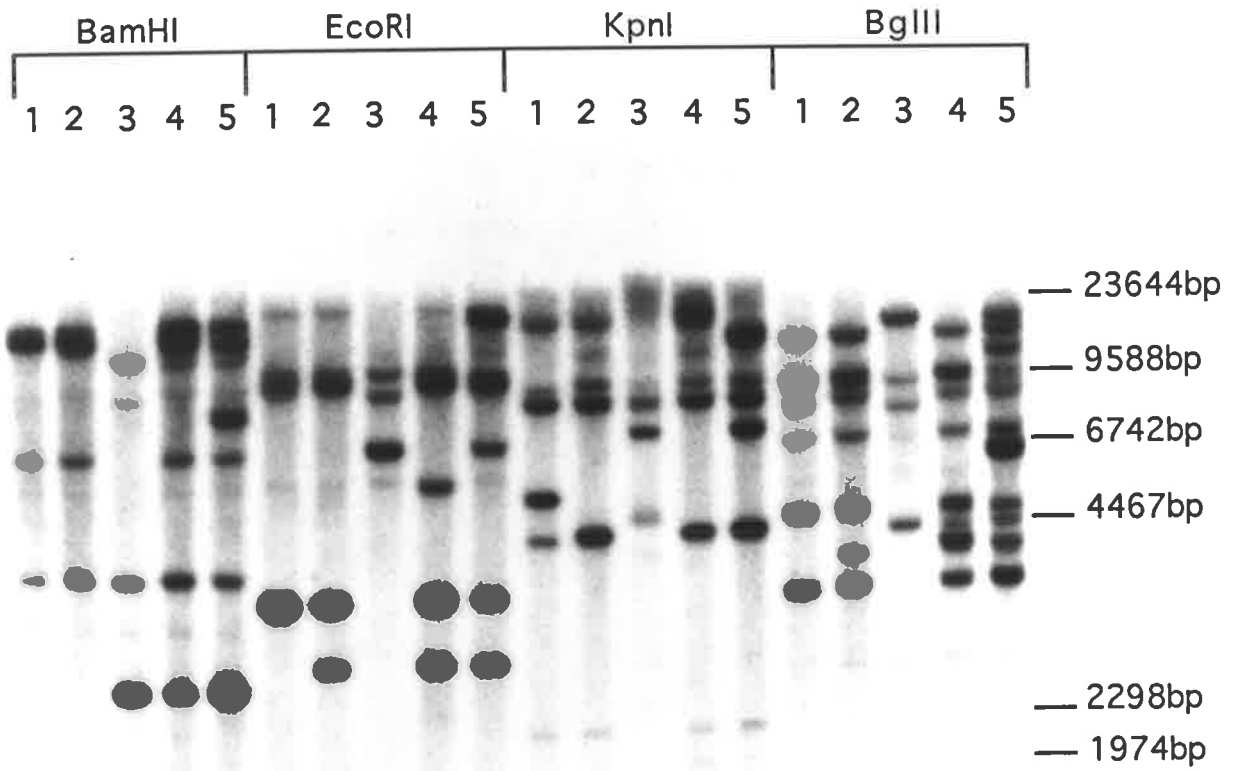


Figure 1B. Hybridisation of probe TAM36 to screening membrane. Five micrograms of genomic DNA was digested with the restriction enzymes indicated. Membranes were exposed at -80°C for 7 days. The position of lambda *Hind*III markers are shown on the right. DNA samples digested with individual restriction enzymes include;

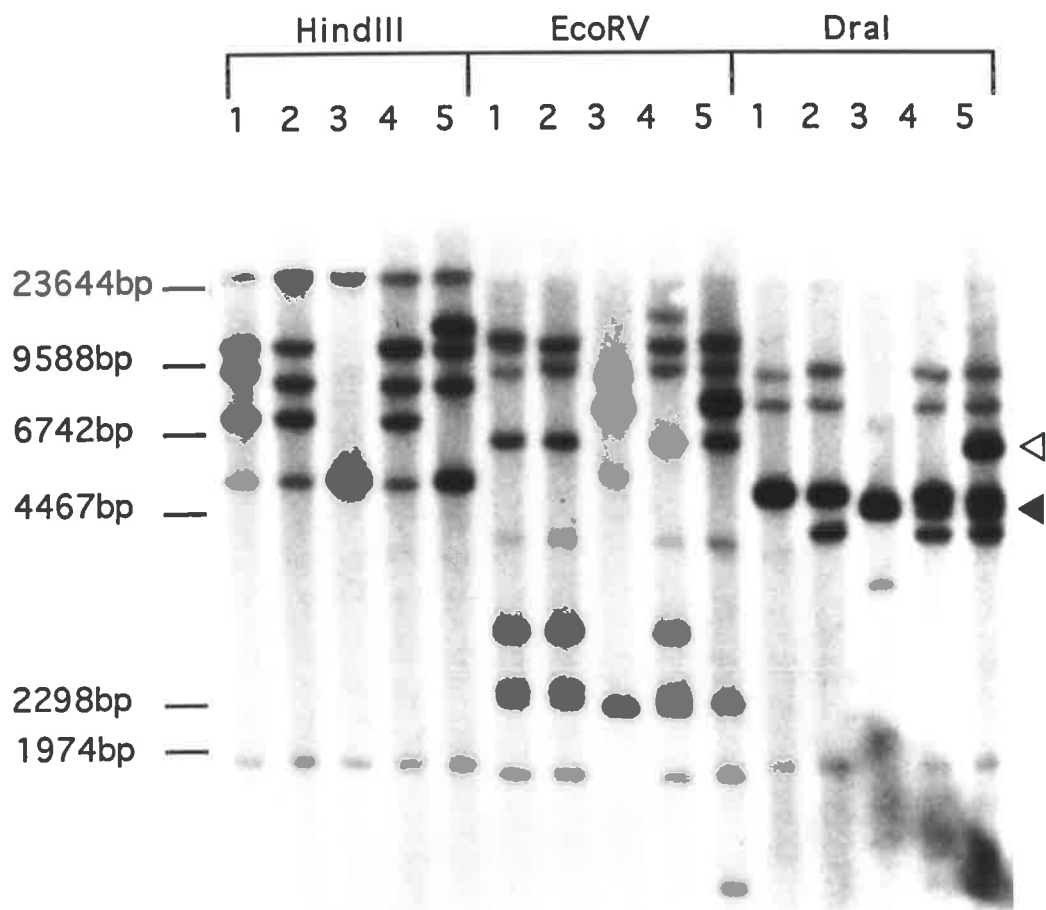
Lane 1: Chinese Spring wheat

Lane 2: Schomburgk wheat

Lane 3: Imperial rye

Lane 4: Chinese Spring-Imperial 6R disomic addition line

Lane 5: Chinese-Spring-T701-4-6 6R(-6D) disomic substitution line



gDNA clones. However, 5 cDNA clones (AW9, AW13, BCD221, BCD339 and CDO419) revealed more than six bands for most enzymes used. Of these clones, only CDO419 could be mapped to chromosome 6R of rye. The clone CDO1400 was mapped to chromosome 6R via a single, weakly hybridising fragment observed in 6R^{Imp} after digestion with *EcoRI*. Similarly, clones AW23 and CDO676 were mapped to chromosome 6R^{T701} with five and four restriction enzymes, respectively. No hybridising fragments were observed in chromosome 6R^{Imp} for either of these clones. AW9 and AW13 could not be mapped to chromosome 6R and BCD339 failed to hybridise to rye under the conditions employed. Many of the genomic DNA clones that mapped in rye cv. Imperial demonstrated simple banding patterns with good levels of hybridisation (ie., WG933, WG522). However, of the gDNA clones mapped to chromosome 6R, most revealed bands demonstrating reduced levels of hybridisation. Moreover, the genomic clones TAM3, TAM9, TAM17, TAM28 and WG405 hybridised poorly to rye and/or were characterised by high levels of background. The clone WG341 failed to hybridise to rye under the conditions employed.

Table 2 summarises the number of homoeologous group 6 cDNA and gDNA clones demonstrating RFLPs between rye cv. Imperial and wheat as well as the number of cDNA and gDNA clones which could be mapped to chromosome 6R^{Imp} and chromosome 6R^{T701} for all restriction enzymes utilised in this study. In total, 41 of 43 (95.3%) of clones revealed RFLPs between rye and wheat. The proportion of cDNA and gDNA clones demonstrating polymorphism was similar (96.2% and 94.1%, respectively). Of the 43 clones used, 27 (62.8%) could be mapped to chromosome 6R of rye (Table 1). Twenty of the 26 (76.9%) cDNA clones were mapped to chromosome 6R of rye while only seven of the 17 (41.2%) gDNA clones could be mapped to this chromosome.

4.2.2 Intervarietal polymorphism: Wheat and Rye

No significant difference was observed in the efficacy of any of the restriction enzymes used in this study in identifying RFLPs in rye with respect to wheat. Table 3 summarises the percent

Table 2. Summary of polymorphism scored for cDNA and gDNA clones used in this study. Numbers in parentheses are percentages calculated as a proportion of all cDNA and/or gDNA clones used.

	Number clones used in study	Number of clones revealing polymorphism between rye cv. Imperial and wheat	Number of clones mapping to either chromosome 6R ^{T701} or chromosome 6R ^{Imp}
cDNA	26	25 (96.2)	20 (76.9)
gDNA	17	16 (94.1)	7 (41.2)
Total	43	41 (95.3)	27 (62.8)

Table 3. Relative efficiency of individual restriction enzymes to detect polymorphism between wheat and rye cv. Imperial for all cDNA and gDNA clones used in this study. Figures indicate the number of clones identifying polymorphism expressed as a percentage of all cDNA and/or gDNA clones used.

	<i>Bam</i> HI	<i>Eco</i> RI	<i>Kpn</i> I*	<i>Bg</i> III	<i>Hind</i> III	<i>Eco</i> RV	<i>Dra</i> I	Total
cDNA	92.3	88.5	90.0	88.5	84.6	92.3	96.2	96.2
gDNA	94.1	88.2	91.7	82.4	82.4	82.4	88.2	94.1
Total	93.0	88.4	90.6	86.0	83.7	88.4	93.0	95.3

*11 clones were not screened with *Kpn*I.

cDNA and gDNA clones demonstrating polymorphism between Imperial rye and wheat. Extremely high levels of polymorphism were observed and of the probe/enzyme combinations used, 83.7% of clones used identified polymorphism between wheat and rye after digestion with *HindIII* while 93.0% of clones used identified polymorphism between wheat and rye after digestion with both *BamHI* and *DraI*. The proportion of cDNA clones demonstrating polymorphism was slightly higher than gDNA clones for all enzymes except *BamHI* and *KpnI*. Since the number of probes screened with *KpnI* was reduced, it is likely that this figure is biased. Polymorphism was detected for all clones hybridising to rye cv. Imperial and for all clones except CSIH90, polymorphism was detected with at least 2 restriction enzymes.

4.2.3 Localisation of homoeologous group 6 clones to chromosome 6R of rye

Table 4 summarises the number of cDNA and gDNA clones mapped to chromosome 6R using the 6R^{Imp} addition line and 6R^{T701} substitution line for all restriction enzymes employed. In all instances, a greater proportion of cDNA clones were mapped to chromosome 6R than gDNA clones. The least efficient enzyme used was *KpnI* for both cDNA and gDNA clones. However, all six cDNA clones and three of the five gDNA clones not screened with *KpnI* were mapped to chromosome 6R. Consequently, figures derived for this enzyme are likely to be biased. *DraI* was the most efficient enzyme for mapping cDNA clones to 6R^{T701} (69.2%) while *DraI* and *HindIII* were equally efficient in mapping cDNA clones to 6R^{Imp} (57.7%). *DraI*, *BglII* and *HindIII* were equally efficient in mapping gDNA clones to 6R^{Imp} and 6R^{T701} (29.4% and 35.3%, respectively). In overall terms, *DraI* was the most efficient enzyme used; 24 of 27 (88.9%) clones mapped to chromosome 6R could be mapped with this enzyme. No single restriction enzyme revealed RFLPs for all clones mapping to chromosome 6R of rye.

The arm location of five clones previously mapped only to homoeologous group 6 chromosomes was established. The clones AW15, AW23, BDC269, CDO419 and CDO1400 were all localised to the long arm of rye chromosome 6R.

Table 4. Relative efficiency of individual restriction enzymes used to map cDNA and gDNA clones to chromosome 6R^{Imp} and 6R^{T701}. Figures indicate the number of clones mapped as a percentage of all cDNA and/or gDNA clones used. The overall number of clones mapped to either chromosome 6R^{Imp} and/or 6R^{T701} for each enzyme is also shown (lower section). This figure is expressed as a percentage of the total number of clones mapped to chromosome 6R.

	<i>Bam</i> HI		<i>Eco</i> RI		<i>Kpn</i> I*		<i>Bgl</i> II		<i>Hind</i> III		<i>Eco</i> RV		<i>Dra</i> I	
	6R ^{Imp}	6R ^{T701}	6R ^{Imp}	6R ^{T701}	6R ^{Imp}	6R ^{T701}	6R ^{Imp}	6R ^{T701}	6R ^{Imp}	6R ^{T701}	6R ^{Imp}	6R ^{T701}	6R ^{Imp}	6R ^{T701}
cDNA	53.8	61.5	53.8	61.5	25.0	50.0	53.8	57.7	57.7	57.7	50.0	57.7	57.7	69.2
gDNA	29.4	29.4	23.5	17.6	8.3	8.3	29.4	35.3	29.4	35.3	29.4	29.4	29.4	35.3
Total	44.2	48.8	41.9	44.2	18.8	34.4	44.2	48.8	46.5	48.8	41.9	46.5	46.5	55.8
Percent clones mapped to 6R ^{Imp} and/or 6R ^{T701}	85.2		77.8		64.7		77.8		81.5		77.8		88.9	

* Of the 27 clones mapped to either chromosome 6R^{Imp} or chromosome 6R^{T701}, 10 were not screened with *Kpn*I

The dominant expression of marker phenotype was observed for a number of enzyme/probe combinations between 6R^{Imp} and 6R^{T701}. Table 5 summarises the number of dominant polymorphisms scored as the presence of an RFLP in one line (ie., 6R^{Imp}) relative to the absence of an RFLP in the other line (ie., 6R^{T701}) for each restriction enzyme used. The majority of dominant polymorphisms were identified in the 6R^{T701} substitution line (30.3%) compared to 7.9% identified in the 6R^{Imp} addition line. In broad terms, dominant polymorphisms accounted for 38.2% of all polymorphisms observed between the 6R^{Imp} addition line and the 6R^{T701} addition line for the 27 clones mapped to chromosome 6R of rye. Significantly, three clones (AW23, CDO676 and CDO1400) demonstrated dominant expression of marker phenotype for all enzymes tested. All other clones revealed the expected co-dominant expression of marker phenotype for at least one restriction enzyme.

4.2.4 Intervarietal polymorphism: 6R^{Imp} versus 6R^{T701}

Levels of polymorphism were assessed between chromosome 6R^{Imp} and chromosome 6R^{T701} for the 27 clones mapped to chromosome 6R of rye. Seventeen of 20 cDNA clones (85.0%) and 5 of 7 gDNA clones (71.4%) demonstrated polymorphism between the rye chromosome 6R present in these lines (Table 6). Variability in the efficacy of each restriction enzyme was observed with no one enzyme superior for both cDNA and gDNA clones. The proportion of clones demonstrating polymorphism ranged from 33.3% (*Bgl*III and *Hind*III) to 59.3% (*Bam*HI) and in total, 22 of 27 (81.5%) group 6 clones localised to rye chromosome 6R, demonstrated polymorphism between the 6R^{Imp} addition line and the 6R^{T701} substitution line.

The number of hybridising bands observed in 6R^{Imp} and 6R^{T701} for clones mapped to chromosome 6R was determined for all probe/enzyme combinations (Table 7). Overall, a total of 139 bands were observed in 6R^{Imp} while 174 bands were observed in 6R^{T701}. Of these, 90 were monomorphic between 6R^{Imp} and 6R^{T701}. A greater number of bands was observed in 6R^{T701} for both cDNA and gDNA clones. With respect to cDNA clones, 105 of 247 (42.5%) bands observed were polymorphic and for gDNA clones, 28 of 66 (42.4%) bands

Table 5. Summary of dominant probe phenotypes scored in 6R^{Imp} and 6R^{T701} relative to each other for each probe/restriction enzyme combination. The total number of dominant polymorphisms observed in each line is expressed as a proportion of all dominant polymorphisms scored between 6R^{Imp} and 6R^{T701} or as a proportion of all polymorphisms scored between 6R^{Imp} and 6R^{T701} (figures in parentheses).

	<i>Bam</i> HI	<i>Eco</i> RI	<i>Kpn</i> I	<i>Bgl</i> II	<i>Hind</i> III	<i>Eco</i> RV	<i>Dra</i> I	Total
6R ^{Imp}	2	2	0	0	1	1	0	20.7 (7.9)
6R ^{T701}	4	3	5	2	2	3	4	79.3 (30.3)
Total	6	5	5	2	3	4	4	100.0 (38.2)

Table 6. Summary of polymorphism scored between 6R^{Imp} and 6R^{T701} using cDNA and gDNA clones mapped to chromosome 6R of rye. Figures indicate the number of clones identifying polymorphism expressed as a percentage of all cDNA and/or gDNA clones mapped to chromosome 6R.

	<i>Bam</i> HI	<i>Eco</i> RI	<i>Kpn</i> I*	<i>Bg</i> III	<i>Hind</i> III	<i>Eco</i> RV	<i>Dra</i> I	Total
cDNA	65.0	50.0	57.1	25.0	30.0	45.0	50.0	85.0
gDNA	42.9	28.6	0.0	57.1	42.9	14.3	42.9	71.4
Total	59.3	44.4	44.4	33.3	33.3	37.0	48.1	81.5

*Of the 20 cDNA clones and 7 gDNA clones mapped to chromosome 6R, 6 cDNA and 3 gDNA clones were not screened with *Kpn*I

Table 7. Summary of the number of RFLPs observed between 6R^{Imp} and 6R^{T701} using cDNA and gDNA clones mapped to chromosome 6R for all probe/enzyme combinations. The number of bands monomorphic between 6R^{Imp} and 6R^{T701} is also shown.

	RFLPs Scored		RFLPs Monomorphic
	6R ^{Imp}	6R ^{T701}	
cDNA	108	139	71
gDNA	31	35	19
Total	139	174	90

observed were polymorphic. Bands identified in 6R^{Imp} and 6R^{T701} using cDNA clones BCD276, CDO1380 and TAM30 and gDNA clones KSUD1 and TAM17 were monomorphic for all restriction enzymes tested.

4.3 DISCUSSION

In total, 41 of the 43 homoeologous group 6 clones revealed RFLPs between wheat and rye cv. Imperial. All seven restriction enzymes used revealed polymorphism with similar, high levels of efficacy (83.7% to 93.0% of clones used). With the exception of AW1 and CSIH90, polymorphism was detected for each clone with at least 5 restriction enzymes. As a consequence, it may be concluded that deletion and/or insertion events account for the majority of polymorphism observed between wheat and rye. Deletion and/or insertion events have been attributed to much of the polymorphism observed in barley (Graner *et al.*, 1990; Shin *et al.*, 1990; Heun *et al.*, 1991) and also wheat (Sharp *et al.*, 1988; Chao *et al.*, 1989; Liu *et al.*, 1990). Two clones, one cDNA and one gDNA failed to hybridise to rye under the conditions employed. Hybridisation was carried out at 65°C and final post-hybridisation washes were carried out in 0.5X SSC, 0.1% SDS at 65°C for 15-30 min. Consequently, as hybridisation stringency was high, it cannot be concluded that these sequences are absent from the rye genome since sequence divergence between wheat and rye could account for these results.

Only 62.8% of the homoeologous group 6 clones could be localised to chromosome 6R of rye. Not surprisingly, the proportion of cDNA clones localised to chromosome 6R of rye was significantly higher than that observed for gDNA clones (76.9% and 41.2%, respectively). Other workers have attributed similar observations between cDNA clones and gDNA clones to the reduced level of sequence conservation and non-homoeologous behaviour of gDNA clones when used as probes in species other than from which the clones were isolated (Gale, 1990; Harcourt and Gale, 1991; Wang *et al.*, 1992). In contrast, this study and others (Liu and Tsunewaki, 1991; Wang *et al.*, 1992) indicates that cDNA clones are of greater use when

studying homoeologous genomes due to greater levels of conservation and the presence of homoeoalleles. Consistent with this was the overall superior hybridisation signal observed with cDNA clones; cDNA clones demonstrated stronger hybridisation signals, less complex banding patterns and less background than gDNA clones.

Of the clones mapped to chromosome 6R of rye, a greater proportion of cDNA clones detected polymorphism between 6R^{Imp} and 6R^{T701} than gDNA clones (85.0% and 71.4% respectively). Overall, 81.5% of all clones mapped to chromosome 6R identified polymorphism between 6R^{Imp} and 6R^{T701}. These figures are comparable to those reported by Hart (1990) between *T. turgidum* var. *durum* cv. Langdon and *T. turgidum* var. *dicoccoides* for homoeologous group 3 and 6 chromosomes and by K. S. Gill *et al.* (1991a) between two divergent accessions of *T. tauschii*. However, both studies utilised gDNA clones. This study identified extremely high levels of polymorphism for cDNA clones which is contrary to expectations (Gale, 1990). Such high levels of polymorphism are therefore likely to reflect the diverse nature of the material under investigation rather than any intrinsic feature of the clones or restriction enzymes used *per se*. Indeed, in terms of the actual number of RFLPs detected in 6R^{Imp} and 6R^{T701}, there was found to be no difference in the proportion of polymorphic bands identified with either cDNA or gDNA clones (42.5% and 42.4% respectively)

K. S. Gill *et al.*, (1991a) reported *DraI* as being the most efficient in revealing polymorphism between two divergent *T. tauschii* accessions while Chao *et al.*, (1989) identified *DraI* as being one of the most efficient in detecting polymorphism for wheat group 7 chromosomes along with other restriction enzymes having 4 of the 6 bases of their respective recognition sequences as A or T. The percentage of clones localised to chromosome 6R of rye with individual restriction enzymes was high; 77.8% for *EcoRI* and *BglII* up to 88.9% for *DraI*. *DraI* was the most efficient restriction enzyme used in localising cDNA clones to either 6R^{Imp} or 6R^{T701} and equally efficient, along with *HindIII* and *BglII*, in localising gDNA clones. However, *BamHI* (GGATCC) was observed to be more efficient than *DraI* in detecting

polymorphism between 6R^{Imp} and 6R^{T701}. Consequently, this study revealed no clear association between restriction enzymes and levels of polymorphism.

More RFLP bands were scored in 6R^{T701} than 6R^{Imp}. This can be attributed largely to the higher incidence of dominant probe phenotypes observed in 6R^{T701}. This phenomenon also led to a greater number of clones being localised to chromosome 6R^{T701} for any given restriction enzyme. Hemizygous loci have been observed in *T. tauschii* (K. S. Gill *et al.*, 1991a) and hexaploid wheat (Liu and Tsunewaki, 1991). In *T. tauschii*, the proportion of such loci was 16%. Of the 27 clones mapped to chromosome 6R, three (11.1%) demonstrated dominant phenotypes for all restriction enzymes revealing RFLPs. The cDNA clones AW23 and CDO676 were localised to chromosome 6R^{T701} only with four and three restriction enzymes, respectively. The cDNA clone CDO1400 was mapped to chromosome 6R^{Imp} with only a single restriction enzyme. While the true nature of these loci is unknown, it is tempting to speculate that they may indicate small rearrangements in one chromosome relative to the other. Such rearrangements could take the form of small deletions which is consistent with the observation regarding the importance of deletion/insertion events for the generation of polymorphism in rye.

Additional dominant probe phenotypes scored in chromosome 6R^{T701} relative to chromosome 6R^{Imp} are most likely the result of rye bands (ie., in 6R^{Imp}) co-migrating with bands observed in wheat since the expected co-dominant expression of probe phenotypes was observed with at least one restriction enzyme. This is the case for the probe/enzyme combination TAM36/*Hind*III (Figure 1B) which shows that the rye fragment from 6R^{Imp} co-migrates with the 11.0 kbp wheat fragment through an increase in hybridisation intensity. Such increases in band intensity were observed for other probe/enzyme combinations (data not shown). As a consequence, much of the difference observed between 6R^{Imp} and 6R^{T701} is likely to be due to the co-migration of rye bands with wheat bands rather than the presence of hemizygous loci.

With the exception of the small structural differences identified with the clones AW23, CDO676 and CDO1400, no major structural rearrangement differentiates these two chromosomes as determined by this study. Moreover, each clone which was mapped to chromosome 6R was done so with at least three restriction enzymes. Consequently, it is unlikely that the 16 homoeologous group 6 clones which failed to map to rye chromosome 6R did so due to insufficient screening. Of the 16 clones not mapped, six were cDNA and ten were gDNA. The most likely explanation for this observation is that these clones reside on those segments of chromosome 6RL present on chromosome arms 3RL and 2RS. Since the arm location of 12 clones is unknown (Table 1), these clones may map to either chromosome 3RL or chromosome 2RS. However, the remaining four clones (WG341, WG405, TAM9 and TAM28) have been localised to the long arm of wheat group 6 chromosomes indicating that they are likely to map to the segment of 6RL present on the long arm of chromosome 3RL. It is also possible that some or all of the gDNA clones may map to chromosomes other than 6R as a result of non-syntenous hybridisation patterns (Gale, 1990; Harcourt and Gale, 1991; Devos *et al.*, 1992).

The results presented in this chapter would indicate that selection of clones based upon their chromosomal arm position in wheat and/or barley is an efficient means of identifying clones of potential use in alien genomes such as rye. A more complete understanding of the homoeologous relationships shared between such genomes (as has been revealed in the few years since this study was instigated) will serve to increase the power of this approach. Not surprisingly, far more homoeologous group 6 cDNA clones were mapped to chromosome 6R than gDNA clones. This observation along with the high levels of polymorphism observed with cDNA clones and the superior hybridisation results obtained with such clones argues strongly for their use over gDNA clones in mapping studies in alien species.

CHAPTER 5

MOLECULAR MARKERS FOR THE LONG ARM OF CHROMOSOME 6R OF RYE: PCR-BASED MARKERS

5.1 INTRODUCTION

The polymerase chain reaction has allowed the development of novel DNA-based markers. Typically, primers used for the development of molecular markers fall into two categories; specific and random. Specific primers rely on the availability of sequence data for their design and, as a consequence, target the sequence of interest. In contrast, random primers are not typically generated from sequence data. Rather, the generation of markers using such primers is the result of fortuitous (random) primer annealing and extension. An example would be the so-called RAPD markers.

RAPD markers have been utilised extensively in plant genetic mapping studies due principally to the efficiency and simplicity of the approach. Although RAPD markers have proven extremely useful in providing molecular markers for specific genomic regions (Williams *et al.*, 1990; Michelmore *et al.*, 1991; Martin *et al.*, 1991; Paran *et al.*, 1991), a number of problems are inherent with their use. RAPD markers are almost exclusively inherited in a dominant manner and deviation from Mendelian segregation, a consequence of the competitive nature of annealing has often been observed (Carlson *et al.*, 1991; Echt *et al.*, 1992; Reiter *et al.*, 1992; Heun and Helentjaris, 1993).

The development of markers using specific primers has proven successful in crop plants. Importantly, a number of workers have demonstrated the feasibility of developing PCR-based markers from RFLP clones. In barley, Shin *et al.* (1990) have utilised primers derived from a gDNA clone to allow a deletion/insertion event to be assayed via PCR. Similarly, in rice, Williams *et al.* (1991) have utilised primers derived from the 5' and 3' ends of mapped gDNA

clones to identify both length and sequence variation in amplification products between rice varieties. However, the major limitation with this approach to the generation of markers is the requirement of accurate sequence data.

Rogowsky *et al.* (1992b) have reported a novel type of PCR marker based upon the R173 family of interspersed, repetitive elements found within the rye genome. The combination of a primer derived from terminal sequences *within* R173 elements (internal primer) with a second primer derived from sequences flanking R173 elements (external primer) has been shown to allow the amplification of unique fragments which may be mapped to individual rye chromosomes. Due to the high proportion of repetitive DNA within the rye genome, external primers are most often derived from such sequences. Since R173 elements are dispersed, apparently uniformly throughout the rye genome, it is likely that individual elements will be inserted at random, throughout families of repetitive DNA sequences thereby creating a range of possible amplification products for any given combination of internal and external primers. Indeed, using a number of primer combinations derived from R173 elements cloned from the short arm of rye chromosome 1R, Rogowsky *et al.* (1992b) identified an additional 18 markers for rye chromosomes other than 1R.

Structural analysis of R173 elements has revealed significant levels of sequence heterogeneity within this family (Rogowsky *et al.*, 1992a). Of interest is the observation that the sequence contained within the plasmid pAW173 defining the R173 family (Guidet *et al.*, 1991) may be absent from approximately 25% of the elements within this family. This finding suggests that internal rearrangement of some form has occurred during the evolution of this family of sequences.

Consequently, three approaches have been taken in the development of PCR-based molecular markers for the long arm of chromosome 6R of rye. Since PCR markers allow for more rapid assaying and have less stringent requirements (ie., DNA quality) than RFLP methods, an attempt was made to generate PCR-based markers from an RFLP clone previously mapped to

chromosome 6R of rye. The second approach centred on the use of flanking primers derived from the junction of R173 elements contained in lambda clones. Markers generated using this approach have been shown to be specific, very reliable and inherited in a Mendelian fashion (Rogowsky *et al.*, 1992*b*). Additional primers derived from a second dispersed, repetitive DNA sequence contained in the clone pSc119.1 (McIntyre *et al.*, 1990) were also included in the present study. Finally, oligonucleotide primers derived from an internal region of R173 were used to exploit potential structural rearrangements within individual R173 elements for additional PCR-based markers.

5.2 RESULTS

5.2.1 Screening

PCR conditions were determined empirically using DNA from Chinese Spring and Schomburgk wheats and rye cv. Imperial. When banding patterns observed in rye cv. Imperial were too complex or characterised by excessive levels of background smearing, annealing temperatures were increased. Conversely, when banding patterns observed in rye cv. Imperial were non-existent or faint and not reproducible, annealing temperatures were reduced. Table 1 lists the origin and sequence information for individual primers used in this study while Table 2 summarises the primer combinations and conditions employed.

After establishing optimal PCR conditions, primer combinations were screened across additional DNA samples which typically included the complete set of Chinese Spring-Imperial disomic addition lines (Driscoll and Sears, 1971), rye cv. Imperial, rye cv. Petkus-R5, rye cv. South Australian, rye cv. Vila Pouca, Triticale T701-4-6, Chinese Spring-Triticale T701-4-6 6R(-6D) disomic substitution line and Chinese Spring-T701-4-6 6RL ditelosomic addition line. The inclusion of the Chinese Spring-T701-4-6 6RL ditelosomic addition line allowed unambiguous assignment of PCR-generated bands to the long arm of chromosome 6R. In reactions employing two primers, each primer was used independently on wheat and rye DNA to establish the origin of resulting amplification products.

Table 1. Origin and sequence of oligonucleotide primers used in this study.

Primer	Length	5'-3' Sequence	Origin
PawC1	18	GACCGTCATTGCTCTCTT	Clone pAW173, nucleotides 332-349
PawC2	18	GGCCCAATGTTCTTCTCT	Clone pAW173, nucleotides 35-52
PawC3	18	ACCTCGACTGTCATGAGA	Clone AW15, nucleotides 35-52
PawC4	18	AGAGCTGTCTCTCCATCT	Clone AW15, nucleotides 333-350
PawC5	18	GCCTCAGATTCAATCACC	Clone AW15, nucleotides 20-37
PawFG3	20	ATGCTGGAGCCCGAGGCTCA	Clone pSC119.1, nucleotides 30-50
PawFG4	20	CTGTTTCGCTTCTGTTGCTC	Clone pSC119.1, nucleotides 690-710
PawS5	18	AACGAGGGGTTCGAGGCC	Border of R173 in clones R173-2 and R173-3
PawS6	18	GAGTGTCAAACCCAACGA	Flanking R173 in clone R173-3
PawS11	18	GAATCTTGAAAAATGTA	Flanking R173 in R173-2
PawS13	18	GATCATATTTGGACTAAC	Border R173 in R173-1
PawS14	18	AATCCAATACATAGAGGA	Border of LTR reading towards R173 in R173-1
PawS15	18	CCGGGTCCGCACTGGATC	Border of R173 in R173-3

Table 2. Summary of the results obtained with the primer combinations and conditions employed in this study.

Primer Combination	Annealing Temperature	Number of PCR Cycles	Amplification Products Observed						Size of Amplification Products Mapped to Chromosome 6R	
			Wheat	Rye	Barley	6R ^{Imp}	6R ^{T701}	Other	6R ^{Imp}	6R ^{T701}
PawC1	50	35	Y	Y	NT	N	N	N		
PawC2	50	35	Y ¹	Y	NT	N	Y	2R		710 bp
PawC1/PawC2	55	32	Y ¹	Y	NT	N	Y	N		380 bp
PawC3/PawC4	55	36	N	N	Y	N	N	1H, 7H		
PawC4/PawC5	55	36	N ²	N ²	Y	N	N	1H, 7H		
PawS5/PawS6	55	40	Y	Y	NT	Y	Y	1R, 2R, 4R, 5R, 7R	125 bp	125 bp
PawS5/PawS11	50	40	Y	Y	NT	N	N	N		
PawS5/PawS14	55	32	Y	Y	NT	Y	Y	N	630 bp	630 bp
PawS5/PawS15	55	32	Y	Y	NT	N	N	1R, 2R		
PawS5/PawFG3	55	32	Y	Y	NT	N	Y	4R		170 bp & 280 bp
PawS5/PawFG4	55	32	Y	Y	NT	Y	Y	1R, 2R, 3R, 4R, 7R	400 bp	450 bp
PawS6/PawS14	55	32	Y	Y	NT	N	N	N		
PawS6/PawS15	55	32	Y	Y	NT	N	N	N		
PawS6/PawFG3	50	40	Y	Y	NT	N	N	1R, 4R, 5R, 7R		
PawS6/PawFG4	50	40	Y ³	N	NT	N	N	N		
PawS11/PawFG3	55	32	Y	Y	NT	N	N	N		
PawS11/PawFG4	55	32	Y	Y	NT	N	N	N		
PawS13/PawS14	50	32	N	Y	NT	N	N	N		
PawS14/PawS15	55	32	Y	Y	NT	Y	Y	5R ⁴	310 bp	310 bp
PawS14/PawFG3	55	32	Y	Y	NT	N	N	N		
PawS14/PawFG4	55	32	Y	Y	NT	N	N	N		
PawS15/PawFG3	55	32	Y	Y	NT	N	N	7R		
PawS15/PawFG4	55	32	Y	Y	NT	N	N	N		

N=Amplification products not observed/mapped

Y=Amplification products observed/mapped

NT=Not tested

¹ Few, faint bands observed

² Faint, non-reproducible bands observed

³ Single 140 bp band observed

⁴ Band mapped to 5R present in all rye varieties tested

5.2.2 PCR-RFLP analysis: AW15

The cDNA clone AW15 contains an insert of approximately 1.8 kbp isolated from a cDNA library prepared from mRNA extracted from the roots of barley cv. Galleon. AW15 has been localised to chromosome 6H of barley (P. Murphy, personal communication) and chromosome 6R of rye (Chapter 4). The 1.8 kbp insert contained in the clone AW15 was sequenced in both directions using the Universal sequencing primer and the T7 sequencing primer as described in Chapter 2.0, Section 2.20.3. Since sequence obtained using the Universal sequencing primer was more reliable, oligonucleotide primers were designed from the region of the clone sequenced with this primer (Figure 1).

5.2.2.1 Oligonucleotide primer design

Primers PawC3 and PawC4, were expected to amplify a 315 bp fragment (Figure 1). Initial experiments using a standard thermocycling protocol and an annealing temperature of 55°C revealed faint, non-specific bands when genomic DNA was used as template. However, the expected 315 bp product was amplified when plasmid AW15 was used as template. Modifications to reaction conditions including annealing temperatures, thermostable polymerase concentration, primer concentration or DNA template concentration failed to resolve the problem. A third primer, PawC5 was designed and, in combination with PawC4 was expected to amplify a 330 bp fragment (Figure 1). Initial experiments revealed similar results to those described for primers PawC3 and PawC4.

5.2.2.2 Hot-start PCR

The presence of a small, intensely staining band of approximately 40 bp in reactions using either PawC3/PawC4 or PawC4/PawC5 primer combinations indicated that under standard PCR conditions, at least one primer was able to form stable dimers or hairpin-loop structures capable of disrupting primer mediated extension. Consequently, Hot-start PCR was carried

Figure 1. Sequence of AW15. Single-stranded sequence was generated using the Universal sequencing primer as described in Chapter 2, Section 2.20.3. The nucleotide position and sequence of primers PawC3, PawC4 and PawC5 is shown (bold, italics; bold, lower case; bold, underlined, respectively). The *EcoRI* cloning site is also shown (bold).

GAATTCGCGG CCGGTCACCG CCTCAGATTC AATCACCTCG ACTGTCATGA 50
GAACAAGGAG CATGCCCTGC GAATTAAAGA TGCTGAAACG AGTGGCCTGG 100
AGAGTTCATC AGGAAAGGAT GCCATGATTA GGGGAGATTT CAGTAATGAA 150
GCTGGCAATG ATGAGAGAAC ATCTGGAGAA GGGGTAGTC CTATGGCACA 200
AGAGCTTGTA GAACCTGCAC CTGATAATGT CCACATTGAC GCAGTTTTCA 250
TTACAGAAGT TAGTGGCTCC ATTTCCAGT CTGATTTCCA CTCCTCGCTG 300
ATCACATCTG AAAGGATCAT GCCTGGCTTG GAAGATGGAG AGACAGCTCT 350
tctacctc tctgtcgaga
CCGCAGACTT TCATCCAGAG ATGTTATGTC AAGTGAAAGC TCAGGATGTT 400
TCTCAATCCA GCTTAACATC TTTGTTGCCT GCTACTTCAA GTTGCATCAT 450
CCATTATTTG GAGATCAATT CCAGATGCAC TACTAGGCAG AGAAGTACTT 500
CCACCATATT CAGTGTGTCA CATGGCAGAT TGGTGGAGGA CAGTGCATGA 550
GGCATCATTT CTCAATTTGG CCAAGTTGGG TGGATGAGAC GATTAGGGCC 600
TTTGGTTTTT AAACGGGGTT GGGTTTGGTA CTTGTGCTGG TTTAAGGTTT 650
CTTGACTTTG GGGGTTGGG CTTACATAT 680

out as described in Chapter 2, Section 2.19.4. An example of a Hot-start PCR using both PawC3/PawC4 and PawC4/PawC5 primer combinations is shown in Figure 2. The expected amplification product using primers PawC3/PawC4 (315 bp) can be seen in the positive control lane (Figure 2A, lane 12) and for all barley DNA samples used (Figure 2A, lanes 6-10). Interestingly, the 40 bp band was still apparent and importantly, can be observed in the minus DNA control (Figure 2A, lane 11). Similar results were obtained with PawC4/PawC5 primer combination (Figure 2B). Length polymorphisms were not observed for any of the barley varieties tested under the electrophoresis conditions employed. Significantly, both primer pairs failed to amplify the expected products from wheat and rye DNA.

5.2.2.3 Mapping PawC3/PawC4 and PawC4/PawC5 amplification products

Figure 3 shows the results of Hot-start PCR carried out using primer combinations PawC3/PawC4 and PawC4/PawC5 on DNA extracted from the Chinese Spring-Betzes addition lines (Islam *et al.*, 1981). Both primer combinations revealed the expected amplification product from the Chinese Spring-Betzes 6H addition line (lanes 7 and 17). However, the expected bands (ie., 315 bp and 330 bp) were also observed in the 1H addition line (lanes 2 and 12), the 4H addition line (lanes 5 and 15) and the 7H addition line (lanes 8 and 18).

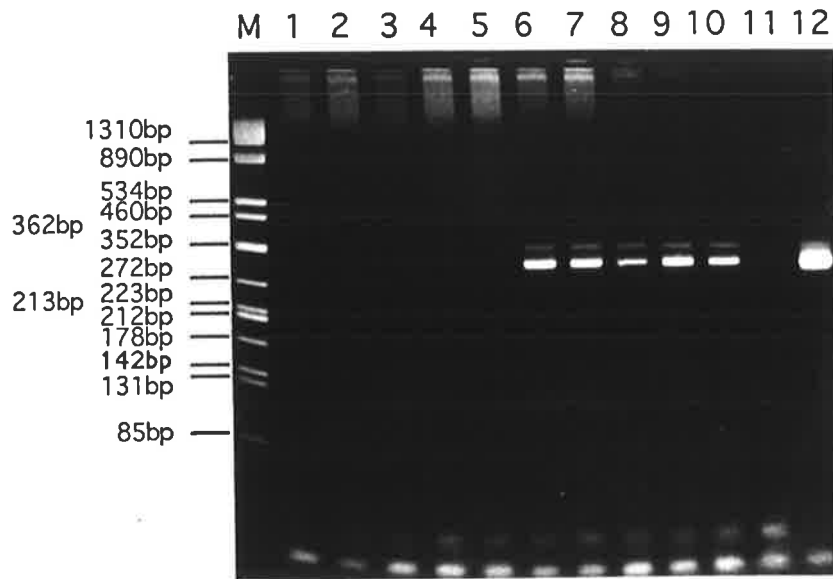
5.2.3 R173 flanking primers

Oligonucleotide primers previously synthesised from the junction of individual R173 elements contained within lambda clones were obtained from Dr. P. Rogowsky. Primers, PawFG3 and PawFG4 derived from the rye specific, dispersed repetitive element contained within the clone pSC119.1 (McIntyre *et al.*, 1990) were obtained from Dr. F. L. Y. Guidet. Both PawFG3 and PawFG4 were used in combination with R173 internal and external primers. Primer combinations tested are shown in Table 2.

Figure 2. Hot Start PCR. A) Primer combination PawC3/PawC4. B) Primer combination PawC4/PawC5. Amplification products were separated on 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard are shown in base-pairs on the left. Lanes 1-12 in A) and B) contained the following DNA samples:

Lane 1: Schomburgk wheat
Lane 2: Chinese Spring wheat
Lane 3: rye cv. Imperial
Lane 4: 6R^{Imp}
Lane 5: 6R^{T701}
Lane 6: barley cv. Betzes
Lane 7: barley cv. Galleon
Lane 8: barley cv. Franklin
Lane 9: barley cv. Clipper
Lane 10: barley cv. Galleon
Lane 11: H₂O
Lane 12: AW15

A



B

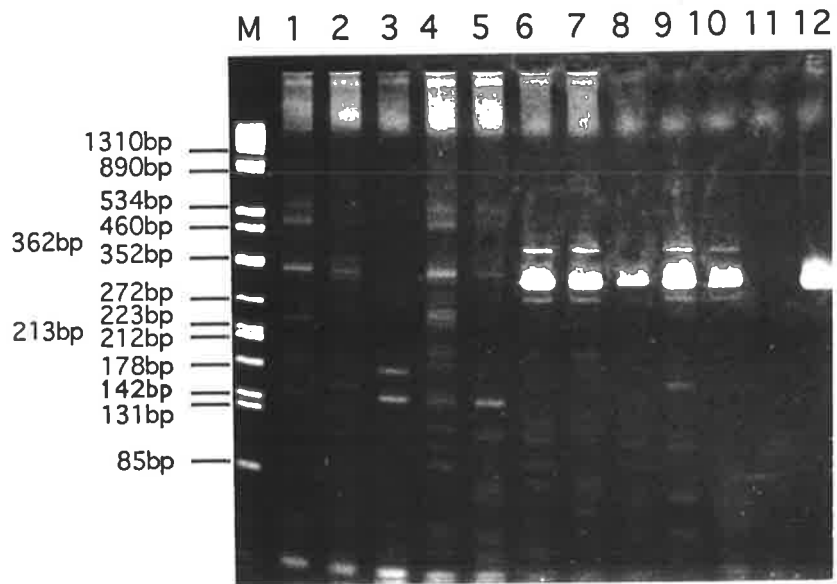
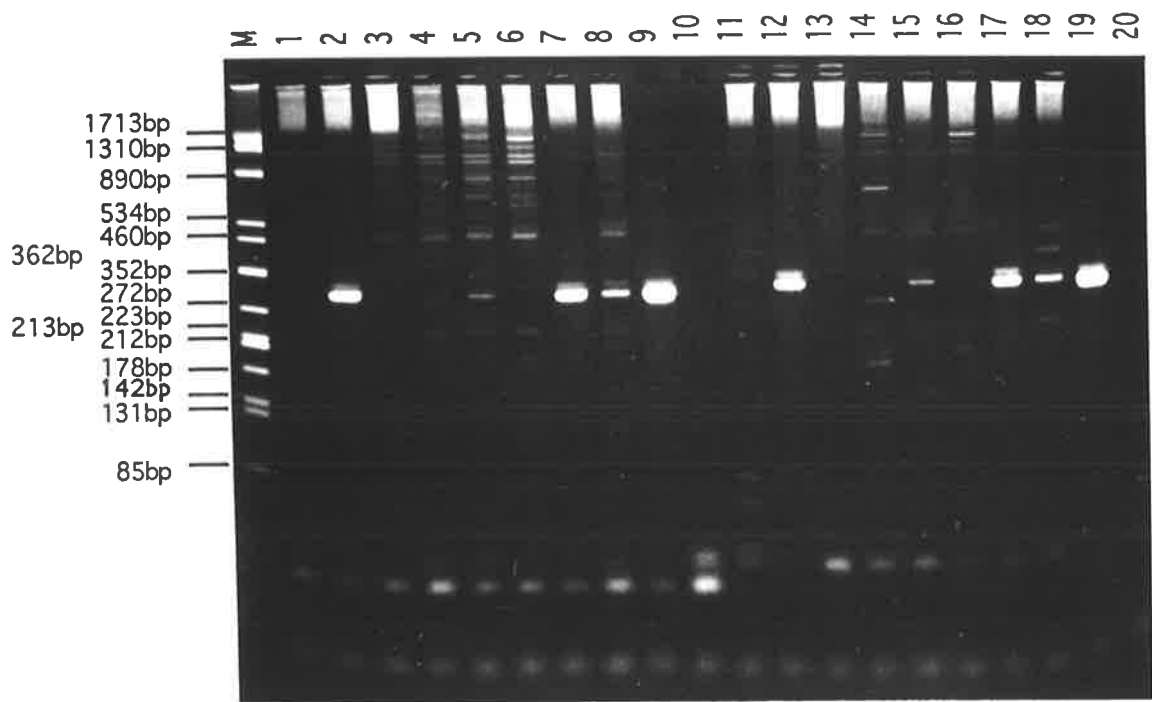


Figure 3. Chromosomal localisation of Hot-start PCR amplification products. Amplification products were separated on 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hoborn, 1975). The size of bands generated in the standard is shown in base-pairs on the left. Lanes 1-10 show products generated using the primer combination PawC3/PawC4 while lanes 11-20 show products generated using the primer combination PawC4/PawC5. Lanes 1-20 contained the following DNA samples:

Lanes 1,11: Chinese Spring wheat
Lanes 2,12: Chinese Spring-Betzes 1H addition line
Lanes 3,13: Chinese Spring-Betzes 2H addition line
Lanes 4,14: Chinese Spring-Betzes 3H addition line
Lanes 5,15: Chinese Spring-Betzes 4H addition line
Lanes 6,16: Chinese Spring-Betzes 5H addition line
Lanes 7,17: Chinese Spring-Betzes 6H addition line
Lanes 8,18: Chinese Spring-Betzes 7H addition line
Lanes 9,19: AW15
Lanes 10,20: H₂O



5.2.3.1 Primer combination PawS5 and PawFG3

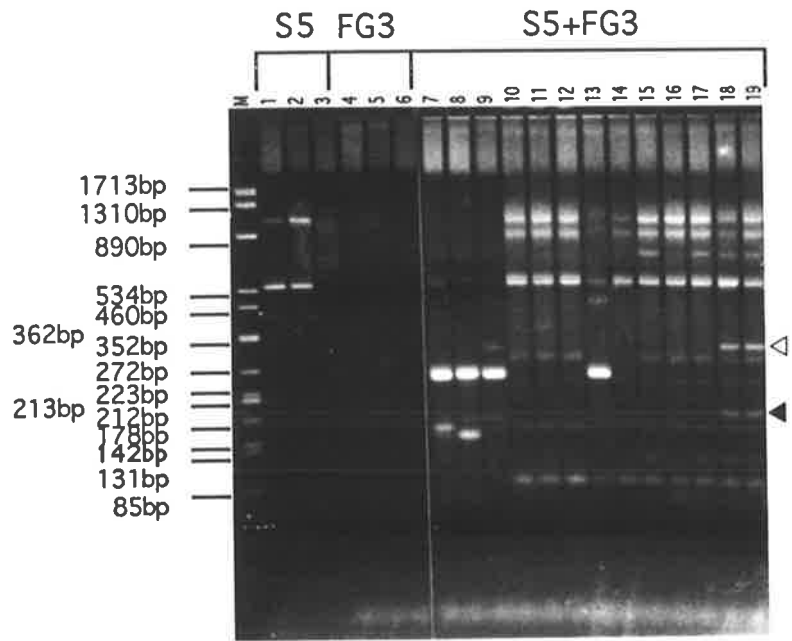
Figure 4 shows results obtained using the primer combination PawS5/PawFG3. Under the reaction conditions employed, the primer PawS5 when used independently always resulted in the generation of two strong bands of 560 bp and 1120 bp in both Chinese Spring and Schomburgk wheats. However, experimental variation often led to increased intensity of amplification products and the observation of additional bands for both primers when used independently. In combination, PawS5 and PawFG3 produced a profile which included bands identified when each primer was used independently as well as a number of bands in both wheat and rye which were clearly the result of amplification with both primers. Specifically, two bands of 170 bp and 280 bp were observed in both the 6R^{T701} addition line and the 6RL^{T701} ditelosomic addition line (lanes 18 and 19). While neither band was observed in the 6R^{Imp} addition line, both were observed in rye cv. Imperial (lane 9). A band of about 240 bp was amplified from all three rye cultivars tested. This band was mapped to rye chromosome 4R using the Chinese Spring-Imperial disomic addition lines (lane 13). Two additional bands were identified, one in the rye cv. Vila Pouca and the other in rye cv. Petkus-R5. Neither band was observed in rye cv. Imperial nor could they be mapped to individual rye chromosomes.

5.2.3.2 Other primer combinations

The results observed with the primer combination PawS5 and PawFG3 are indicative of those observed using other primer combinations. Typically, amplification products were observed to be a mixture of bands produced by both primers *individually* as well as in *combination*. However, the banding patterns produced using some primer combinations, notably PawS11/PawFG3 and PawS11/PawFG4 were almost exclusively the result of primers PawFG3 and PawFG4, respectively. All approaches to the combination of primers (ie., the use of R173 internal primers with their respective external primers; the combination of R173 internal primers with external primers derived from different R173 elements and the combination of

Figure 4. Chromosomal localisation of PCR amplification products generated using the primer combination PawS5/PawFG3. Amplification products were separated on 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard are shown in base-pairs on the left. Lanes 1-3 show the products generated when PawS5 was used independently while products generated when PawFG3 was used independently are shown in lanes 4-6. Lanes 7-19 show the products generated when PawS5 and PawFG3 were used in combination. The open and filled arrow on the right indicate the 280 bp and 170 bp amplification products mapped to the long arm of chromosome 6R^{T701}, respectively. Lanes 1-19 contained the following DNA samples:

- Lanes 1,4: Chinese Spring wheat
- Lanes 2,5: Schomburgk wheat
- Lanes 3,6: rye cv. Imperial
- Lane 7: rye cv. Vila Pouca
- Lane 8: rye cv. Petkus-R5
- Lane 9: rye cv. Imperial
- Lane 10: Chinese Spring-Imperial 1R disomic addition line
- Lane 11: Chinese Spring-Imperial 2R disomic addition line
- Lane 12: Chinese Spring-Imperial 3R disomic addition line
- Lane 13: Chinese Spring-Imperial 4R disomic addition line
- Lane 14: Chinese Spring-Imperial 5R disomic addition line
- Lane 15: Chinese Spring-Imperial 6R disomic addition line
- Lane 16: Chinese Spring-Imperial 7R disomic addition line
- Lane 17: Chinese Spring-Imperial 6R disomic addition line
- Lane 18: Chinese Spring-T701-4-6 6R(-6D) disomic substitution line
- Lane 19: Chinese Spring-T701-4-6 6RL ditelosomic addition line



internal and external primers derived from R173 elements with primers PawFG3 and PawFG4) were successful in generating specific amplification products which could be mapped to individual rye chromosomes. Most primer combinations generated amplification products which were polymorphic between the rye cultivars tested. However, a number of the products generated could not be mapped to individual rye chromosomes using the Chinese Spring-Imperial rye disomic addition lines.

Of the 26 amplification products mapped to individual rye chromosomes, 19 (65.5%) were generated using PawS5 in combination with either PawS6, PawS14, PawS15, PawFG3 or PawFG4 (Table 2). The remaining seven mapped products were generated using the primer combinations PawS6/PawFG3, PawS14/PawS15 and PawS15/PawFG3. Primer combinations involving PawFG3 and PawFG4 generated 8 (30.8%) and 7 (26.6%) of all markers observed. Moreover, the primer combinations PawS5/PawFG3 and PawS5/PawFG4 accounted for 10 (38.5%) of all amplification products mapped. In total, 7 new PCR-based markers were generated for chromosome 6R of rye. However, of the 5 primer combinations resulting in amplification products mapped to chromosome 6R of rye, only PawS5/PawFG3 and PawS5/PawFG4 yielded products which were polymorphic between 6R^{Imp} and 6R^{T701}.

5.2.4 pAW173 as a source of markers for chromosome 6R

5.2.4.1 Nucleotide sequence of pAW173

Sequencing of the rye-specific, repetitive DNA sequence contained in the plasmid pAW173 (Guidet *et al.*, 1991) was carried out to enable the generation of oligonucleotide primers for PCR analysis. Clones containing the *Bam*HI insert in both orientation were obtained as well as *Bst*EII subclones (Chapter 2, Section 2.11) and sequenced as described (Chapter 2, Section 2.20). Figure 5 shows the complete double-stranded sequence of the 442 bp insert contained in the plasmid pAW173. The nucleotide position and sequence of the primers PawC1 and PawC2 are highlighted (bold and underlined) as is the *Bst*EII site used to create the subclones (bold and italicized). Orientation is relative to the sequence data of Rogowsky *et al.* (1992a).

Figure 5. Sequence of pAW173. Double-stranded sequence of the 442 bp rye-specific insert contained in the clone pAW173 was generated as described in Chapter 2, Section 2.20. The nucleotide position and sequence of primers PawC1 and PawC2 is shown (bold; bold, lower case, respectively) as is the *Bst*EII site used to create the subclones used for sequencing (bold, italicized). Orientation is relative to the sequence data of Rogowsky *et al.* (1992a).

GATTCAATAT	TGCTTTGTCTG	CTCTGCATGA	TTAT GACCGT	CATTGCTCTC	50
CTAAGTTATA	ACGAAACAGC	GAGACGTACT	AATACTGGCA	GTAACGAGAG	
TTAGTTGGTC	GCTCCCAGTC	TTTTGCTAAC	CTCCACCTGC	ACTAAGCAAA	100
AATCAACCAG	CGAGGGTCAG	AAAACGATTG	GAGGTGGACG	TGATTCGTTT	
CTAACCATGC	AATACATAGC	TCATGGGAAA	GTAGCCTAAA	AAATATTGTG	150
GATTGGTACG	TTATGTATCG	AGTACCCTTT	CATCGGATTT	TTTATAACAC	
GTAAGAATA	TGTTGCTTAT	GSTATCTTATT	TCTTATAAAGT	TGCTTGCTGA	200
CATTTCTTAT	ACAACGAATA	CATAGAATAA	AGAATATTCA	ACGAACGACT	
GCGGTAACCA	TGTTTCTGGG	GACGCCATCA	ACTGTCACAC	TTTTGTTGAA	250
CGCCATTGGT	ACAAAGACCC	CTGCGGTAGT	TGACAGTGTG	AAAACAACCTT	
TATCATGTGA	GTTGCTATGC	ATGTTTCGTCT	TGTCTGAAGT	AAGGGTGATT	300
ATAGTACACT	CAACGATACG	TACAAGCAGA	ACAGACTTCA	TTCCCCTAA	
TACAGTGGGT	TGAATTAATA	TGCATATTGT	TAGAGAAGAA	CATTGGGCCG	350
ATGTCACCCA	ACTTAATTAT	ACGTATAACA	Atctcttctt	gtaaccggc	
CCAACCAAAG	CCATGCATCA	TGGTGGAAGT	TTTAGCTTGG	ACCTTAATCC	400
GGTTGGTTTC	GGTACGTAGT	ACCACCTTCA	AAATCGAACC	TGGAATTAGG	
TCAATCTCTC	ATGAGAATAT	GCTTATGCAT	TAAAGAGGAG	CT	442
AGTTAGAGAG	TACTCTTATA	CGAATACGTA	ATTTCTCCTC	GA	

5.2.4.2 PCR using primers PawC1 and PawC2

Primers PawC1 and PawC2 were expected to amplify a 316 bp fragment (Figure 5). Figure 6A shows the results of PCR reactions using the primer combination PawC1/PawC2 on DNA extracted from test-cross (TC)-F₁ plants. The derivation of the TC-F₁ population is detailed in Chapter 7. However, each individual shown in Figure 6A was confirmed to have a single chromosome 6R homologue present in a wheat background. A band of the expected size was observed in all samples (open arrow) although the intensity of this band is clearly stronger when rye DNA is present. Similarly, two bands of approximately 420 bp and 480 bp (bands 1 and 2, respectively) were observed in all samples but were of greatly increased intensity when rye DNA was present. A number of strongly fluorescing bands of approximately 725 bp, 910 bp and 1100 bp were also observed in Schomburgk wheat (lane 2). A strong background smear was observed after PCR on samples containing rye DNA. Within this smear, three diffuse bands of approximately 590 bp, 1000 bp and 1200 bp (bands 3, 4 and 5, respectively) were observed. A faint band of approximately 380 bp was observed in some samples containing chromosome 6R of rye (arrow; lanes 4, 5, 10, 13). This product was not present in rye cv. Imperial or any of the Chinese Spring-Imperial disomic addition lines but was amplified from DNA isolated from Triticale T701-4-6 and the 6R^{T701} disomic substitution line (data not shown).

In order to ascertain the nature of the amplification products observed, gels were transferred to nylon membranes and probed using the *Bam*HI insert from the plasmid pAW173. Figure 6B is a Southern hybridisation of PCR products shown in the gel in Figure 6A. The most intense hybridisation signal results from the 316 bp amplification product in both wheat and rye DNA (open arrow). Strong hybridisation with the 420 bp and 480 bp bands in both wheat and rye indicates significant homology to the probe pAW173. A clear hybridisation signal can also be observed at 590 bp, 1000 bp and 1200 bp indicating a degree of homology between these amplification products and the probe, pAW173. However, most significantly, the strongly fluorescing bands observed in Schomburgk wheat failed to hybridise with the probe even after

Figure 6. Primer combination PawC1/PawC2.

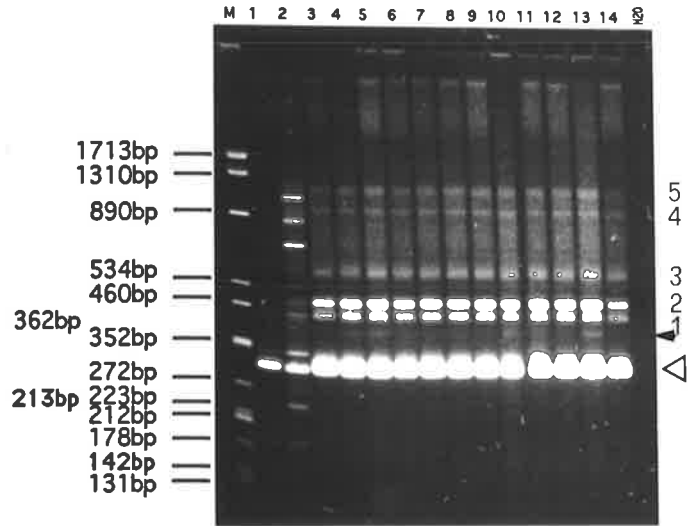
A) PCR using the primer combination PawC1/PawC2 on DNA extracted from Chinese Spring and Schomburgk wheats and 12 individuals from the Test-Cross F₁ population. All 12 TC-F₁ individuals were confirmed to be carrying at least one chromosome 6R homologue. Amplification products were separated on 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard is shown in base-pairs on the left.

B) Southern hybridisation of the gel shown in Figure 6A probed with the *Bam*HI fragment contained in the clone pAW173. Southern transfer and hybridisation were carried out as described in Chapter 2, section 2.10.

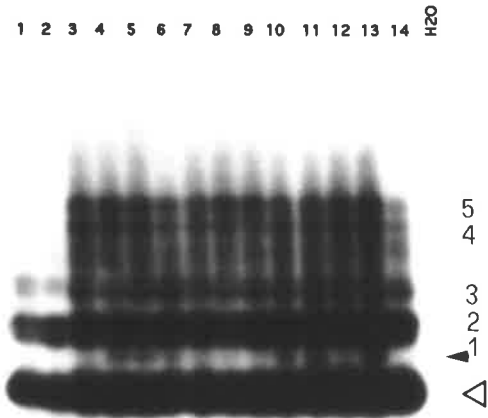
Lanes 1-14 contained the following DNA samples:

- Lane 1: Chinese Spring wheat
- Lane 2: Schomburgk wheat
- Lane 3: Test-Cross F₁-1
- Lane 4: Test-Cross F₁-2
- Lane 5: Test-Cross F₁-3
- Lane 6: Test-Cross F₁-4
- Lane 7: Test-Cross F₁-5
- Lane 8: Test-Cross F₁-6
- Lane 9: Test-Cross F₁-7
- Lane 10: Test-Cross F₁-8
- Lane 11: Test-Cross F₁-9
- Lane 12: Test-Cross F₁-10
- Lane 13: Test-Cross F₁-11
- Lane 14: Test-Cross F₁-12

A



B



prolonged exposure. The faint 380 bp band, mapped to chromosome 6R^{T701} also showed specific hybridisation indicating that this amplification product is derived from sequences related to the R173 family of elements (arrow).

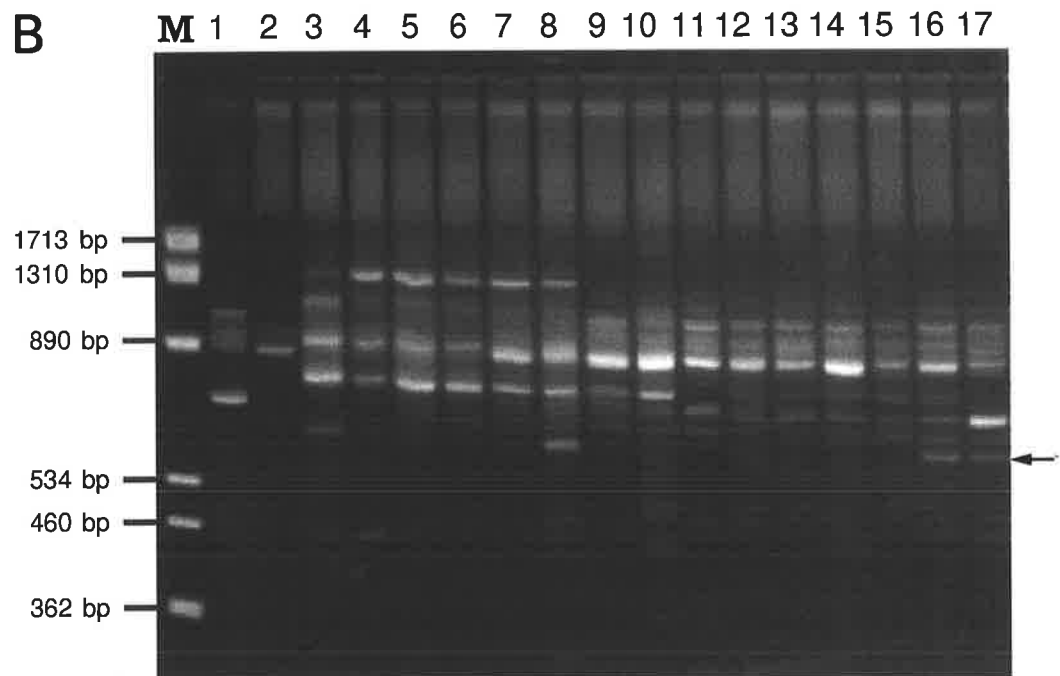
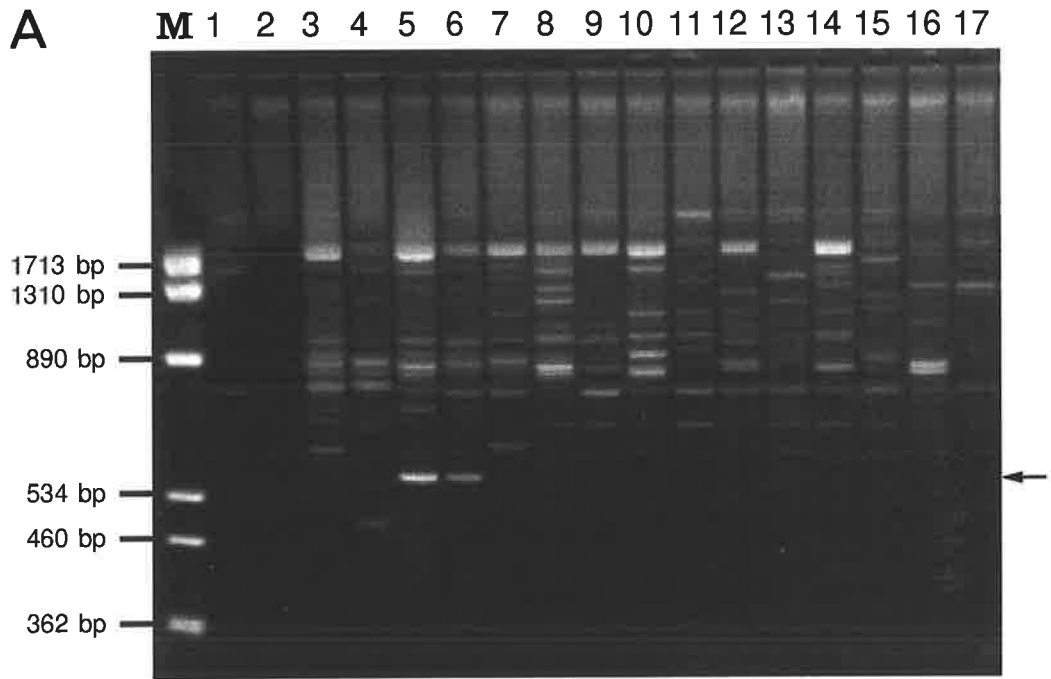
5.2.4.3 Single-primer PCR: Primers PawC1 and PawC2

Primers PawC1 and PawC2 were used independently in an attempt to identify additional polymorphisms and to confirm the origin of bands amplified when these primers were used in combination. PCR using an annealing temperature of 55°C were unsuccessful (data not shown). Reducing the annealing temperature to 50°C resulted in reproducible, complex banding patterns for both PawC1 and PawC2 from both wheat and rye although the intensity and complexity of products was reduced in wheat relative to rye (Figures 7A and 7B). PCR amplification using PawC1 is shown in Figure 7A. Amplification resulted in few faint bands in Chinese Spring and Schomburgk wheats (lanes 1 and 2). In contrast, many polymorphic products were amplified from rye DNA using this primer (lanes 3, 4, 5 and 6). However, none could be conclusively mapped to individual rye chromosomes including the strong amplification product of about 580 bp observed in rye cultivars South Australian and Imperial (lanes 6 and 7).

PCR amplification with PawC2 resulted in a number of wheat-specific and rye-specific products (Figure 7B). While most of the bands amplified from samples containing rye DNA appeared common, a unique band of about 710 bp was identified and mapped to the long arm of chromosome 6R^{T701} (lane 17). This band was also amplified from the rye cv. Vila Pouca (lane 3). A band of about 1410 bp was observed in all rye cultivars as well as Triticale T701-4-6 (lanes 4, 5, 6, 7 and 8) but could not be mapped to a specific rye chromosome.

Figure 7. PCR using primers PawC1 and PawC2 independently. A) Amplification products generated using the primer PawC1. B) Amplification products generated using the primer PawC2. Amplification products were separated on 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard is shown in base-pairs on the left. The 580 bp product generated using PawC1 and the 710 bp product generated using PawC2 are indicated by an arrow in A) and B) respectively. Lanes 1-21 in A) and B) contained the following DNA samples:

- Lane 1: Schomburgk wheat
- Lane 2: Chinese Spring wheat
- Lane 3: rye cv. Vila Pouca
- Lane 4: rye cv. Petkus-R5
- Lane 5: rye cv. South Australian
- Lane 6: rye cv. Imperial
- Lane 7: Chinese Spring-Imperial amphiploid
- Lane 8: Triticale T701-4-6
- Lane 9: Chinese Spring-Imperial 1R disomic addition
- Lane 10: Chinese Spring-Imperial 2R disomic addition
- Lane 11: Chinese Spring-Imperial 3R disomic addition
- Lane 12: Chinese Spring-Imperial 4R disomic addition
- Lane 13: Chinese Spring-Imperial 5R disomic addition
- Lane 14: 6R^{Imp}
- Lane 15: Chinese Spring-Imperial 7R disomic addition
- Lane 16: 6R^{T701}
- Lane 17: 6RL^{T701}



5.3 DISCUSSION

5.3.1 PCR-RFLP Analysis

The clone AW15 has been localised to chromosome 6H of barley and chromosome 6R of rye including chromosome 6R^{Imp} and chromosome 6R^{T701}. Moreover, polymorphism detected by this clone in rye is likely the result of insertion/deletion events. However, no attempt was made to characterise the polymorphism detected in RFLP studies. Rather, it was anticipated that polymorphism would be detected in PCR studies via fortuitous positioning of oligonucleotide primers or subsequent digestion of amplification products with frequently cutting restriction enzymes.

Since AW15 is a cDNA clone, the number of nucleotides separating forward and reverse primers were minimised in order to reduce the risk of intervening intronic sequences in genomic cereal DNA. Initial experiments with primer combinations PawC3/PawC4 and PawC4/PawC5 failed to generate the expected 315 bp and 330 bp products from genomic DNA of barley, wheat or rye. While initial results suggested the presence of intronic sequences or other structural rearrangements, the presence of a number of small, strongly fluorescing bands in all samples including the water control suggested possible problems with primer design. Therefore, Hot-start PCR was used since this methodology typically reduces the ability of primers to form stem-loop or primer-dimer structures through the pre-incubation of reaction substrates including primers, nucleotides and reaction buffer at relatively high temperatures (ie., 70°C) for short periods prior to thermocycling (D'Aquila *et al.*, 1991; Chou *et al.*, 1992).

Under Hot-start PCR conditions, both primer combinations resulted in the amplification of expected fragments from barley genomic DNA. Since computer analysis of the primers used in this study indicates that PawC4 is capable of forming stable primer-dimers and hairpin-loop structures, the success of Hot-start PCR is presumably due to the reduced ability of this primer to form such structures under the conditions employed. Both the 315 bp and 330 bp

fragments were mapped to barley chromosome 6H indicating the specificity of PCR for both primer combinations. However, amplification products of similar size were also mapped to barley chromosomes 1H, 4H and 7H. While cytogenetic analysis of the Chinese Spring-Betzes addition lines has confirmed the presence of 6HL in the 1H addition line (Islam and Shepherd, 1990), there is no such evidence for the presence of chromosome 6H in either the 4H or 7H addition line. Consequently, evidence for the presence of related sequences or duplicated loci on barley chromosomes 4H and 7H is presented.

The inability to amplify the expected products from wheat and rye using primer combinations PawC3/PawC4 and PawC4/PawC5 was surprising. While the reason for this observation is unknown, it is possible that a breakdown in sequence homology exists within the primer binding sites in wheat and rye relative to barley. Alternatively, the deletion of one or both primer binding sites or the presence of a large insertion within the sequence flanked by primers PawC4/PawC5 in wheat and rye relative to barley would cause PCR to fail under the conditions employed. Recent data (including this thesis) indicates that much of the polymorphism observed in wheat, rye and barley results from insertion and deletion events.

Due to the difficulties experienced in developing a PCR-based marker for rye chromosome 6R from the RFLP clone AW15, this strategy was abandoned. However, a number of issues have been raised by this study. Clearly, efficient conversion of Southern to PCR analysis requires that the RFLP in question be characterised. Shin *et al.* (1990) have demonstrated the feasibility of this approach. Also, the choice of probe, cDNA or gDNA, appears significant. In contrast to the study of Williams *et al.* (1991), primers were designed from sequence generated from one end of a cDNA clone. As a result, the ability to identify length polymorphisms in amplified products directly is likely to be greatly diminished. Moreover, although only one clone was included, the results from this study suggest that the ability to use primers derived from cDNA sequences across genomes may be diminished.

5.3.2 R173 Flanking primers

Six primers derived from regions flanking R173 elements and two primers derived from the sequence contained in the clone pSc119.1 were used in 18 different permutations. Of the numerous PCR amplification products generated, 26 could be mapped to individual rye chromosomes. Therefore, on average, each primer revealed 3.25 new markers for the rye genome while each primer combination revealed 1.44 new markers for the rye genome. Seven (26.9%) markers were generated for chromosome 6R of rye. However, amplification products were mapped to all seven rye chromosomes highlighting the generality of this approach. These results compare favourably to other PCR-based approaches including RAPD analysis (Williams *et al.*, 1990) and the semi-random approach of Weining and Langridge (1991).

The initial study of Rogowsky *et al.* (1992b) has been extended via the inclusion of primers derived from the dispersed, repetitive sequence contained within the clone pSc119.1. The results presented confirm that primers derived from R173 elements are an efficient and essentially limitless source of molecular markers for the rye genome. This clearly extends to primers derived from other dispersed, repetitive DNA sequences such as pSc119.1. However, while reliability of PCR reactions involving R173-derived primers was high, presumably due to the relatively long primer length (18 bp) and stringent reaction conditions, it remains unclear whether amplification products identified reflect amplification events at the border of R173 elements or of unrelated sequences cross-hybridising to the primers.

The fact that some primers produced essentially identical banding patterns when used individually or in combination is indicative of cross-hybridisation. However, primer combinations involving PawFG3 and PawFG4 account for some 57.7% of all markers scored. Since these two primers were derived from a dispersed, repetitive family of DNA sequences also found within the rye genome, it seems reasonable to conclude that sequences defined by the primers PawFG3 and PawFG4 occur in the vicinity of R173 elements in slightly different

configurations. This rationale also applies to the results observed with other external primers derived from multicopy sequences such as PawS6. Therefore, it is likely that many of the PCR products observed are the result of specific amplification events at the border of individual R173 elements. PawFG3 was positioned at the 5' end of the sequence contained in the clone pSc119.1 while PawFG4 was positioned at the 3' end of this sequence. Both primers are oriented away from each other with primer extension resulting in the targeting of flanking sequences as judged from the sequence data of McIntyre *et al.* (1990). Consequently, the generation of specific amplification products with both PawS5/PawFG3 and PawS5/PawFG4 primer combinations indicates that R173 elements and sequences related to that contained within the clone pSc119.1 occur in both orientations relative to each other within the rye genome.

When used independently, PawFG3 and PawFG4 resulted in amplification products from both wheat and rye which were found to be both reproducible and polymorphic. Using *in situ* hybridisation, McIntyre *et al.* (1990) reported slight cross-hybridisation of the probe pSc119.1 to the centromeric regions of some wheat chromosomes while in rye, this family of repeats is dispersed throughout the genome on all chromosomes with the exception of some telomeres and the nucleolar organising region. Presumably, the primers PawFG3 and PawFG4 are targeting homologous sequences within the wheat genome. While speculative, the ability to generate amplification products with these two primers independently suggests that the sequence present in the clone pSc119.1 may comprise a repeating unit which is present in wheat and rye genomes in both orientations in a head to tail manner. Length polymorphisms of PCR amplification products identified between wheat and rye apparently indicate differences in the structural arrangement of such sequences within the genomes of these two cereals.

Sequencing of individual R173 elements has revealed significant structural and sequence heterogeneity within the R173 family of repeats (Rogowsky *et al.*, 1992a). Specifically, one element (R173-1) is apparently structurally distinct from two other elements (R173-2 and R173-3) in being flanked by two almost perfect direct repeats. The primer PawS14 was

designed from the border of the direct repeat identified in R173-1 reading towards R173 while primers PawS5 and PawS15 were both designed from the border of elements R173-2 and R173-3 and R173-3, respectively, reading away from R173. As such, the primer combinations PawS5/PawS14 and PawS14/PawS15 have the ability to identify R173 elements possessing direct repeats. Numerous amplification products were generated with both primer combinations. However, most significantly, both PawS5/PawS14 and PawS14/PawS15 resulted in the generation of individual amplification products which could be mapped to chromosome 5R and chromosome 6R, respectively. These results would indicate that at least two other R173 elements structurally analogous to either R173-2 or R173-3 possess direct repeats homologous to those found in the clone R173-1.

5.3.3 pAW173 as a source of markers for chromosome 6R

Primers were generated from the sequence contained in pAW173 in an attempt to exploit structural variation observed within R173 elements. Specifically, it was hoped that length polymorphisms within the region flanked by primers PawC1 and PawC2 could be detected and mapped to individual rye chromosomes. The primer combination PawC1/PawC2 was expected to amplify a 316 bp fragment from rye. This band was observed in all samples containing rye DNA. However, numerous other products were amplified from rye DNA. With the exception of a 380 bp product which was mapped to chromosome 6R^{T701}, none could be mapped to a specific rye chromosome. Therefore, while it has been possible to utilise structural variation within R173 elements to generate markers for the rye genome, it would appear that the ability to identify unique variants within the region targetted is poor.

Observations with respect to the nature of amplification products generated using PawC1 and PawC2 have implications regarding the structure and evolution of the R173 family of sequences which warrant further discussion. PCR amplification of samples containing rye DNA with primers PawC1 and PawC2 resulted in the generation of three major bands. In addition to the 316 bp band expected, bands of 420 bp and 480 bp were also observed.

Hybridisation of pAW173 to both the 420 bp and 480 bp bands indicates that they are likely to be the result of amplification from R173 structural variants. Moreover, the relative intensity of both bands in ethidium bromide-stained gels indicates that the copy number of such elements is high. Consequently, while it is known that the R173 family is structurally heterogeneous, these results suggest that a number of major classes exist within the group.

The 316 bp, 420 bp and the 480 bp bands were amplified wheat also. All three bands in both cereals appear to be derived from R173-related sequences as judged by hybridisation to pAW173. Therefore, rearrangements such as insertions/deletions occurring within the sequence defined by the clone pAW173 and flanked by the primers PawC1 and PawC2 must have occurred prior to amplification of the R173 family in rye. Guidet *et al.* (1991) estimated there to be less than 20 copies of R173 in hexaploid wheat. The amplification of the 316 bp, 420 bp and 480 bp bands in wheat suggests that some of these elements are structurally analogous to those found in rye. In contrast, three slightly diffuse bands of about 590 bp, 1000 bp and 1200 bp were identified in rye only. Hybridisation to pAW173 indicates that these fragments are R173-related. Again, these results are suggestive of insertions within the sequence defined by the clone pAW173 and flanked by the primers PawC1 and PawC2. However, these events have occurred *since* speciation. Such observations argue that the evolution of the R173 family of sequences has been via a cassette-type mechanism in which repetitive DNA sequences common to both wheat and rye have been further amplified in concert with additional DNA sequences since speciation.

5.3.4 Single-primer PCR amplification

PCR amplification using primers PawC1 and PawC2 individually at an annealing temperature of 55°C resulted in few faint bands suggesting that neither sequence is present in an inverted orientation within R173 elements. These results also indicate that the products generated when using PawC1 and PawC2 in combination are the result of specific priming within R173 elements. Reducing the annealing temperature by 5°C resulted in complex banding patterns

for both primers in rye. The number of bands amplified in wheat was limited to three or four. Numerous false priming sites (in inverted orientation) have been identified within the three R173 elements sequenced for primers PawC1 and PawC2. Consequently, false priming within individual R173 elements would seem the likely origin of many if not all of the amplification products observed including the product generated with the primer PawC2 mapped to the long arm of chromosome 6R^{T701}. Differences observed in band complexity between wheat and rye presumably reflects the higher copy number of R173 elements in rye relative to wheat.

5.3.5 PCR Markers: General comments

Observations in the present study suggest that a number of the PCR amplification products identified in different rye cultivars are derived from the same locus. For example, amplification products mapped to chromosomes 6R^{Imp} and 6R^{T701} using the primer combinations PawS5/PawS6, PawS5/PawS14 and PawS14/PawS15 were the same molecular weight. Also, the 180 bp fragment and 270 bp fragment generated by the primer combination PawS5/PawFG3 were observed in rye cv. Imperial and 6R^{T701}. Importantly, neither product was not observed in 6R^{Imp}. Consequently, the primer combination PawS5/PawFG3 generated a marker demonstrating a dominant polymorphism phenotype. Significantly, four of the eight chromosome 6R markers generated demonstrated a dominant polymorphism phenotype, being amplified in 6R^{T701} and not 6R^{Imp}. These observations may be further indication of the diverse nature of the chromosomes under analysis or, alternatively, due to the presence of small deletions or insertions in chromosome 6R^{T701} relative to chromosome 6R^{Imp}.

CHAPTER 6

CHROMOSOME 6R LONG ARM DELETION LINES: THEIR USE IN MARKER ASSIGNMENT

6.1 INTRODUCTION

In recent years, intrachromosomal localisation of genes has progressed through the use of chromosomal deletion lines. For example, the gene(s) responsible for speltoid suppression (Q) along with β -*Amy-A2* have been mapped to the distal 13% of the long arm of chromosome 5A using partial chromosome deletions of the long arm of chromosome 5AL in the wheat cv. Chinese Spring (Endo and Mukai, 1988; Tsujimoto and Noda, 1990). Similarly, using a series of chromosome 5B deletion lines, K. S. Gill *et al.* (1991c) have reported the physical localisation of *Ph1* and three RFLP markers to the same interstitial chromosomal region on 5BL. In a more detailed analysis, Kota *et al.* (1991) reported the generation of 226 independent deletion lines for all 21 chromosomes of hexaploid wheat using a chromosome of *Triticum cylindricum* as described by Endo (1988). Cytogenetic characterisation of deletion lines (ie., the expression of breakpoint positions as a fraction length from the centromere) combined with screening of RFLP markers has allowed the systematic assignment of DNA probes to chromosome regions throughout the wheat genome and, consequently, the construction of cytologically-based physical maps. This approach has been particularly well demonstrated for homoeologous group 6 chromosomes of wheat (Gill *et al.*, 1993).

The physical assignment of markers requires only that they be scored by their presence or absence. Consequently, monomorphic markers or markers demonstrating limited heterozygosity may be localised. Assignment to specific subchromosomal regions is carried out by defining the breakpoint of the largest deletion in which the marker is observed and the breakpoint of the next smallest deletion in which it is absent. Significantly, the physical localisation of markers mapped genetically allows the correlation of genetic and physical

distances. Such information is essential to the success of chromosome walking and positional cloning of agronomically important genes. Recent data suggests that there is little recombination in regions proximal to the centromere (Lukaszewski and Curtis, 1993). Such a phenomenon will undoubtedly affect the accuracy of genetic linkage maps of these regions. Providing that deletion lines of sufficient resolution are available, it is likely that markers may be ordered with greater accuracy in regions of reduced or suppressed recombination through the development of high resolution physical maps. Chromosomal deletion lines can also be used to target specific chromosomal regions (and hence genes of interest) in the development of useful molecular markers (K. S. Gill *et al.*, 1991c).

CreR, the gene conferring resistance to the cereal cyst nematode (*Heterodera avenae* Woll.) has been localised to the long arm of chromosome 6R derived from the triticale T701-4-6 (Asidue *et al.*, 1990). Significantly, Dundas *et al.* (1992) reported the isolation of four chromosome 6RL^{T701} deletion mutants. In each case, terminal segments of the long arm of chromosome 6R^{T701} had been deleted with breakpoints at unique positions as deduced by the dissociation pattern of isozyme markers and C-banding. These lines have allowed the physical mapping of three isozyme marker genes and *CreR*. By associating cytological features of the deletion lines with isozyme results, the order of the marker genes was found to be α -*Amy-R1* (proximal), *Got-R2* and *6-pgd-r1b* (distal) (Dundas *et al.*, 1992). *CreR* was localised to an interstitial segment adjacent to *Got-R2* (Dundas *et al.*, 1992).

The deletion lines described by Dundas *et al.* (1992) and the telocentric deletion lines derived from them (Dundas *et al.*, 1993) represent an extremely useful tool in the identification of molecular markers for the *CreR* locus. Consequently, this chapter describes the use of the chromosome 6RL deletion lines in the physical localisation of RFLP and PCR markers to discrete regions of this chromosome arm. The construction of a cytologically-based physical map of the long arm of chromosome 6R using molecular markers developed in chapters 4 and 5 is presented.

6.2 RESULTS

6.2.1 Physical assignment of group 6 RFLP markers to 6RL

For deletion analysis, 21 of the 27 DNA probes previously mapped to chromosome 6R were used (Table 1). The clones CDO419, CDO1400, WG286, WG522 and TAM21 were omitted due either to poor hybridisation signal or dominant phenotype. The clone CDO1091 was mistakenly omitted. Table 1 lists the probe/enzyme combinations used. Where possible, the probe/enzyme combinations giving the simplest and clearest signal were chosen. Twelve probes were localised with two restriction enzymes.

Subarm mapping of the clone TAM36 is demonstrated in Figure 1. A single band of about 4.5 kbp previously scored in rye cv. Imperial and which mapped to both chromosome 6R^{Imp} and 6R^{T701} was observed in the three additional rye cultivars screened (lanes 3, 4 and 5). However, a band of about 6.0 kbp, localised to chromosome 6R^{T701} was only observed in the Triticale from which this substitution line was derived (lane 8). Both the 4.5 kbp and 6.0 kbp bands were present in the chromosome 6RL^{T701} monotelosomic addition line (lane 11) confirming their localisation to the long arm chromosome 6R. The 4.5 kbp band is present in all deletion lines indicating that the locus maps proximal to the breakpoint in del6RL1801. The 6.0 kbp band is missing from all of the deletion lines used indicating that the locus detected maps distal of the breakpoint in del6RL22. Therefore, these bands are non-allelic due to their localisation to different subchromosomal regions.

Subarm mapping of the clone WG933 is shown in Figure 2. A band of about 7.9 kbp, previously mapped to chromosome 6R^{T701} was also identified in rye cvs. Vila Pouca and Petkus-R5 (lanes 3 and 4) while a band of about 12.6 kbp, mapped previously to 6R^{Imp} was observed in rye cultivars Imperial and South Australian (lanes 5 and 6). The 7.9 kbp band mapping to chromosome 6R^{T701} is also present in the chromosome 6RL^{T701} monotelosomic addition and chromosome 6RL^{T701} long arm fusion lines (lanes 14 and 15), confirming the localisation of this clone to the long arm of chromosome 6R^{T701}. Accordingly, this band is

Table 1. Subchromosomal localisation of group 6 clones to the long arm of chromosome 6R^{T701}. Genotypes were scored by the presence (+) or absence (-) of bands for each deletion line. The molecular weight (MW) of bands identified in 6R^{Imp} and 6R^{T701} is listed. The restriction enzyme(s) used for genomic DNA digestion is included under "Restriction Enzyme".

Clone	Locus ¹	Restriction Enzyme	MW of band(s) mapped to 6R ^{Imp} (kbp)	MW of band(s) mapped to 6R ^{T701} (kbp)	Subchromosomal Localisation			
					Deletion Line ²			
					1	2	3	4
AW15	<i>Xaw15</i>	<i>Hind</i> III	3.8	4.3+4.0	+	+	+	-
		<i>Bam</i> HI	5.3	1.5	+	+	+	-
AW23	<i>Xaw23</i>	<i>Eco</i> RV	nm	6.9	+	+	-	-
BCD1	<i>Xbcd1</i>	<i>Bam</i> HI	20.4	20.4	+	+	+	-
BCD102	<i>Xbcd102</i>	<i>Dra</i> I	6.2+5.4	6.2+5.4	+	+	+	-
		<i>Hind</i> III	17.4+3.0	17.4+3.0	+	+	+	-
BCD269	<i>Xbcd269</i>	<i>Hind</i> III	13.8+2.4	13.8+2.4	+	+	+	+
		<i>Eco</i> RV	26.6	23.7	+	+	+	+
BCD276	<i>Xbcd276</i>	<i>Eco</i> RV	24.5	24.5	+	+	+	-
BCD340	<i>Xbcd340</i>	<i>Bgl</i> II	3.9	3.9	+	+	+	-
		<i>Bam</i> HI	17.0	23.6	+	+	+	-
BCD758	<i>Xbcd758</i>	<i>Dra</i> I	18.6	10.5	+	+	+	+
		<i>Eco</i> RV	6.3	5.8	+	+	+	+
BCD1426	<i>Xbcd1426</i>	<i>Bgl</i> II	15.9	8.1	+	+	+	-
		<i>Hind</i> III	11.5	10.0	+	+	+	-
CDO497	<i>Xcdo497</i>	<i>Eco</i> RV	3.6	3.6	+	+	+	-
		<i>Hind</i> III	13.2	13.2	+	+	+	-
CDO676	<i>Xcdo676</i>	<i>Dra</i> I	nm	4.2	+	+	+	+
CDO772	<i>Xcdo772</i>	<i>Dra</i> I	6.0	6.0	+	+	+	+
CDO1380	<i>Xcdo1380</i>	<i>Dra</i> I	2.8+2.1	2.8+2.1	+	+	+	-
WG933	<i>Xwg933</i>	<i>Bgl</i> II	12.6	7.9	+	+	+	-
		<i>Eco</i> RV	23.6	19.1	+	+	+	-

KSUF37	<i>XksuF37A</i>	<i>HindIII</i>	10.0	13.5	+	+	-	-
	<i>XksuF37B</i>	<i>DraI</i>	nm	3.7	-	-	-	-
KSUD1	<i>XksuD1</i>	<i>HindIII</i>	5.0	5.0	+	+	+	-
		<i>EcoRV</i>	7.1	7.1	+	+	+	-
TAM17	<i>Xtam17</i>	<i>BamHI</i>	2.5	2.5	+	+	+	+
TAM25	<i>Xtam25</i>	<i>HindIII</i>	4.3	4.3	+	+	+	-
TAM30	<i>Xtam30</i>	<i>HindIII</i>	3.1+1.7	3.1+1.7	+	+	+	-
		<i>EcoRV</i>	3.8	3.8	+	+	+	-
TAM36	<i>Xtam36A</i>	<i>DraI</i>	4.5	4.5	+	+	+	+
	<i>Xtam36B</i>	<i>DraI</i> ³	nm	6.0	-	-	-	-
	<i>Xtam36A</i>	<i>HindIII</i>	10.7	5.3	+	+	+	+
	<i>Xtam36B</i>	<i>HindIII</i> ⁴	nm	13.5	-	-	-	-
PSR154	<i>Xpsr154</i>	<i>HindIII</i>	4.0+1.6	1.4	+	+	+	+

¹RFLP bands mapping to the same subchromosomal region using more than one probe/enzyme combination are considered to represent the same locus

²Deletion line 1 = del6RL22, deletion line 2 = del6RL21, deletion line 3 = del6RL1807, deletion line 4 = del6RL1801

³The probe/enzyme combination TAM36/*DraI* identified an additional band of 6.0 kbp in 6R^{T701}. This band is considered non-allelic to the 4.5 kbp band identified since it was mapped to a different subchromosomal region

⁴The probe/enzyme combination TAM36/*HindIII* also identified two loci in 6R^{T701} confirming the results obtained with the probe/enzyme combination TAM36/*DraI*

nmRFLP loci could not be mapped to chromosome 6R^{Imp}

Figure 1. Subchromosomal localisation of the clone TAM36 to the long arm of chromosome 6R^{T701} of rye. The size standard was λ *Hind*III. The size of bands generated in the standard are given on the left in base-pairs. The filled in arrow shown on the right indicates the 4.5 kbp mapped to the long arm of chromosomes 6R^{Imp} and 6R^{T701}. The open arrow shown on the right indicates the 6.0 kbp band mapped to the long arm of chromosome 6R^{T701}. The filter contains genomic DNA digested with *Dra*I from the following lines:

- Lane 1: Schomburgk wheat
- Lane 2: Chinese Spring wheat
- Lane 3: rye cv. Petkus-R5
- Lane 4: rye cv. South Australian
- Lane 5: rye cv. Vila Pouca
- Lane 6: rye cv. Imperial
- Lane 7: Chinese Spring-Imperial (-6R) amphiploid
- Lane 8: Triticale T701-4-6
- Lane 9: 6R^{Imp}
- Lane 10: 6R^{T701}
- Lane 11: 6RL^{T701} monotelosomic addition
- Lane 12: del6RL22
- Lane 13: del6RL21
- Lane 14: del6RL1807
- Lane 15: del6RL1801

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

23644bp —

9588bp —

6742bp —

4467bp —

2298bp —

1974bp —

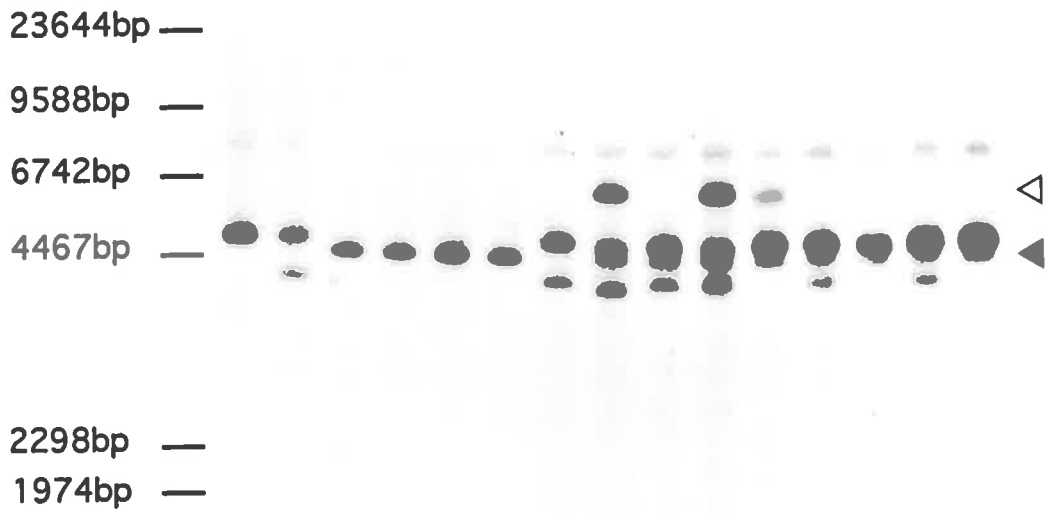
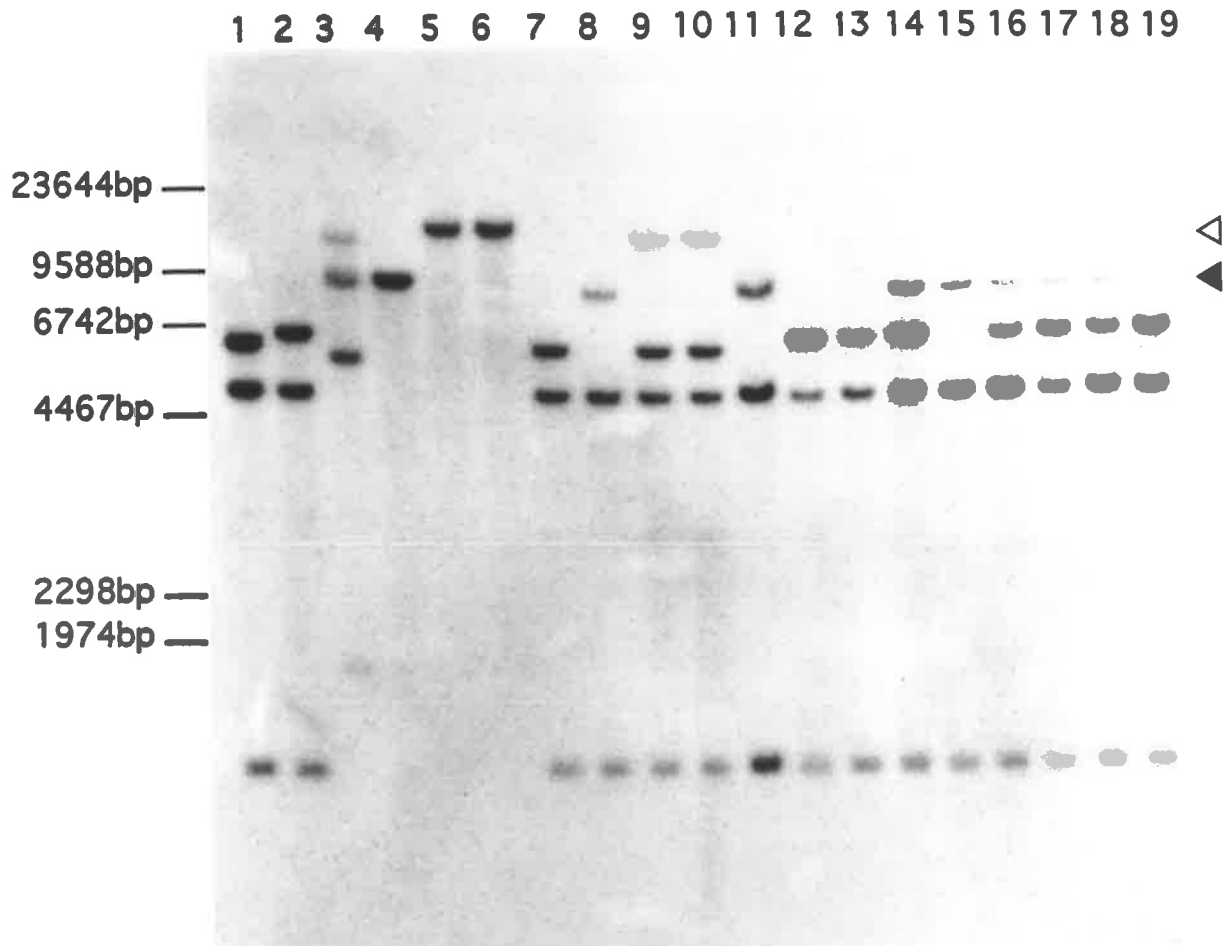


Figure 2. Subchromosomal localisation of the clone WG933 to the long arm of chromosome 6R^{T701} of rye. The size standard was λ *Hind*III. The size of bands generated in the standard are given on the left in base-pairs. The open arrow shown on the right indicates the 12.6 kbp band mapped to chromosome 6R^{Imp} while the filled arrow indicates the 7.9 kbp mapped to the long arm of chromosome 6R^{T701}. The filter contains genomic DNA digested with *Bgl*II from the following lines:

- Lane 1: Chinese Spring wheat
- Lane 2: Schomburgk wheat
- Lane 3: rye cv. Vila Pouca
- Lane 4: rye cv. Petkus-R5
- Lane 5: rye cv. South Australian
- Lane 6: rye cv. Imperial
- Lane 7: Chinese Spring-Imperial (-6R) amphiploid
- Lane 8: Triticale T701-4-6
- Lane 9: 6R^{Imp}
- Lane 10: 186/15.1
- Lane 11: 6R^{T701}
- Lane 12: 6RS^{T701} disomic addition
- Lane 13: 6RS^{T701} disomic short arm fusion
- Lane 14: 6RL^{T701} monotelosomic addition
- Lane 15: 6RL^{T701} monosomic long arm fusion
- Lane 16: del6RL22
- Lane 17: del6RL21
- Lane 18: del6RL1807
- Lane 19: del6RL1801



absent in the chromosome 6RS^{T701} ditelosomic addition and the chromosome 6R^{T701} short arm fusion lines (lanes 12 and 13). The 7.9 kbp band was observed in deletion lines del6RL22, del6RL21 and del6RL1807 (lanes 16, 17 and 18) but is missing in del6RL1801 (lane 19). Therefore, the locus identified with the clone WG933 maps distal to the breakpoint in deletion del6RL1801 and proximal of the breakpoint in deletion del6RL1807. The 12.6 kbp band mapped to 6R^{Imp} was also observed in the putative deletion line, 186/15.1 indicating that if a deletion event has occurred in this line, then the breakpoint lies distal of the locus identified by the clone WG933.

Subchromosomal localisation of the remaining group 6 clones mapped to chromosome 6R was carried out as described for clones TAM36 and WG933. All clones could be localised intrachromosomally to the long arm of 6R^{T701} (Table 1). Two clones detected non-allelic loci. Using the restriction enzyme *DraI*, the clone TAM36 detected two bands, one of 4.5 kbp and the second, 6.0 kbp which mapped to different subchromosomal regions on 6RL^{T701}. This result was confirmed using the restriction enzyme *HindIII* (Table 1). The clone KSUF37 also identified non-allelic loci (Table 1). In initial screening experiments, this clone identified either a single, strongly hybridising band or one strong and one weakly hybridising band in rye cv. Imperial depending on the restriction enzyme used. For all probe/enzyme combinations (excluding *KpnI*), RFLPs showing reduced hybridisation intensity could be localised to both chromosome 6R^{Imp} and chromosome 6R^{T701}. However, strongly hybridising bands could be localised to chromosome 6R^{T701} only. Digestion with *HindIII* revealed weakly hybridising bands of 10.0 kbp and 13.5 kbp in 6R^{Imp} and 6R^{T701} respectively. The 13.5 kbp band identified in chromosome 6R^{T701} was localised to the long arm to the subchromosomal region defined proximally by the breakpoint in del6RL21 and distally by the breakpoint in del6RL1807 (Table 1). In contrast, digestion with *DraI* revealed a single, intensely hybridising band of 3.7 kbp which mapped to chromosome 6R^{T701} only and was found to be absent from all four chromosome 6RL deletion lines (Table 1). This band could not be mapped to the 6RL^{T701} monotelosomic addition line, suggesting that the locus identified maps to the short arm of chromosome 6R^{T701}.

6.2.2 Physical assignment of PCR markers to 6RL

For deletion analysis, seven of the eight PCR markers developed for chromosome 6R^{T701} were used (Table 2). The 380 bp amplification product mapped to chromosome 6R^{T701} using the primer combination PawC1/PawC2 was omitted from this study due to its dominant phenotype and the requirement of Southern hybridisation for screening. PCR on genomic DNA samples including the four chromosome 6RL^{T701} deletion lines was carried out using the conditions described previously.

The primer combination PawS5/PawFG4 generated a 400 bp band which was mapped to chromosome 6R^{Imp} and a 450 bp band which was mapped to chromosome 6R^{T701}. Figure 3 shows the subarm mapping of the 450 bp amplification product generated with this primer combination. The 400 bp band mapping to chromosome 6R^{Imp} can be observed (lane 19) while the 450 bp band can be observed in T701-4-6 (lane 13), 6R^{T701} (lane 21), 6RL^{T701} monotelosomic addition (lane 24) and 6RL^{T701} long arm fusion (lane 25) confirming its localisation to the long arm of chromosome 6R. Accordingly, the 450 bp band was not observed in either 6RS^{T701} ditelosomic addition or 6RS^{T701} short arm fusion lines (lanes 22 and 23). The 450 bp band was observed in deletion lines del6RL22, del6RL21 and del6RL1807 but not del6RL1801 (lanes 26, 27, 28 and 29). These results indicate that the locus identified maps to the chromosomal region defined proximally by the breakpoint in del6RL1801 and distally by the breakpoint in del6RL1807.

Subarm mapping of the 125 bp amplification product generated using the primer combination PawS5/PawS6 is shown in Figure 4. Experimental variation in reaction efficiency is evident since the amplification profile of the primer PawS5 is not as complex as observed for this primer in Figure 3. The 125 bp band mapped to both 6R^{Imp} and 6R^{T701} was observed in rye cvs. Vila Pouca, South Australian and Petkus-R5 (lanes 10, 11 and 12). While amplification using genomic DNA from rye cv. Imperial failed in this experiment, this band is amplified using rye cv. Imperial genomic DNA as template (data not shown). The 125 bp band was observed

Table 2. Subchromosomal localisation of PCR markers to the long arm of chromosome 6R^{T701}. Genotypes are scored by the presence (+) or absence (-) of bands for each deletion line. The molecular weight (MW) of amplification products mapped in 6R^{Imp} and 6R^{T701} is listed.

Primer Combination	Locus ¹	MW of 6R ^{T701} Amplification Product(s) (bp)	Subchromosomal Localisation			
			Deletion Line ²			
			1	2	3	4
PawS5/PawS14	<i>AawS5/S14</i>	630	+	+	+	+
PawC2	<i>AawC2</i>	710	+	+	+	+
PawS5/PawFG4	<i>AawS5/FG4-1</i>	450	+	+	+	-
PawS5/PawS6	<i>AawS5/S6</i>	125	+	+	+	-
PawS5/PawFG3	<i>AawS5/FG3a</i>	170	-	-	-	-
	<i>AawS5/FG3b</i>	280	-	-	-	-
PawS14/PawS15	<i>AawS14/S15</i>	310	+	+	U	U

¹Nomenclature of loci detected via PCR follows Rogowsky *et al.* (1992b) except individual primer names are retained due to the uncertain nature of loci targeted.

²Deletion line 1=del6RL22, deletion line 2=del6RL21, deletion line 3=del6RL1807, deletion line 4=del6RL1801.

^{mm}Amplification products not mapped to chromosome 6R^{Imp}.

UUnknown.

Figure 3. Subchromosomal localisation of the 450bp amplification product generated by the primer combination PawS5/PawFG4 to the long arm of chromosome 6R^{T701}. Amplification products were separated in 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard are shown in base-pairs on the left. Lanes 1-4 show the products generated when PawS5 was used independently while products generated when PawFG4 was used independently are shown in lanes 5-8. Lanes 9-30 show the products generated when PawS5 and PawFG4 were used in combination. The arrows on the right indicate the amplification products mapped to chromosome 6R^{T701} and chromosome 6R^{Imp}. Lanes 1-30 contained the following DNA samples:

- Lanes 1, 5 and 9: Schomburgk wheat
- Lanes 2, 6 and 10: Chinese Spring wheat
- Lanes 3, 7 and 19: 6R^{Imp}
- Lanes 4, 8 and 21: 6R^{T701}
- Lane 11: rye cv. Imperial
- Lane 12: Chinese Spring-Imperial (-6R) amphiploid
- Lane 13: Triticale T701-4-6
- Lane 14: Chinese Spring-Imperial 1R disomic addition
- Lane 15: Chinese Spring-Imperial 2R disomic addition
- Lane 16: Chinese Spring-Imperial 3R disomic addition
- Lane 17: Chinese Spring-Imperial 4R disomic addition
- Lane 18: Chinese Spring-Imperial 5R disomic addition
- Lane 20: Chinese Spring-Imperial 7R disomic addition
- Lane 22: 6RS^{T701} disomic addition
- Lane 23: 6RS^{T701} disomic short arm fusion
- Lane 24: 6RL^{T701} monosomic addition
- Lane 25: 6RL^{T701} monosomic long arm fusion
- Lane 26: del6RL22
- Lane 27: del6RL21
- Lane 28: del6RL1807
- Lane 29: del6RL1801
- Lane 30: ddH₂O

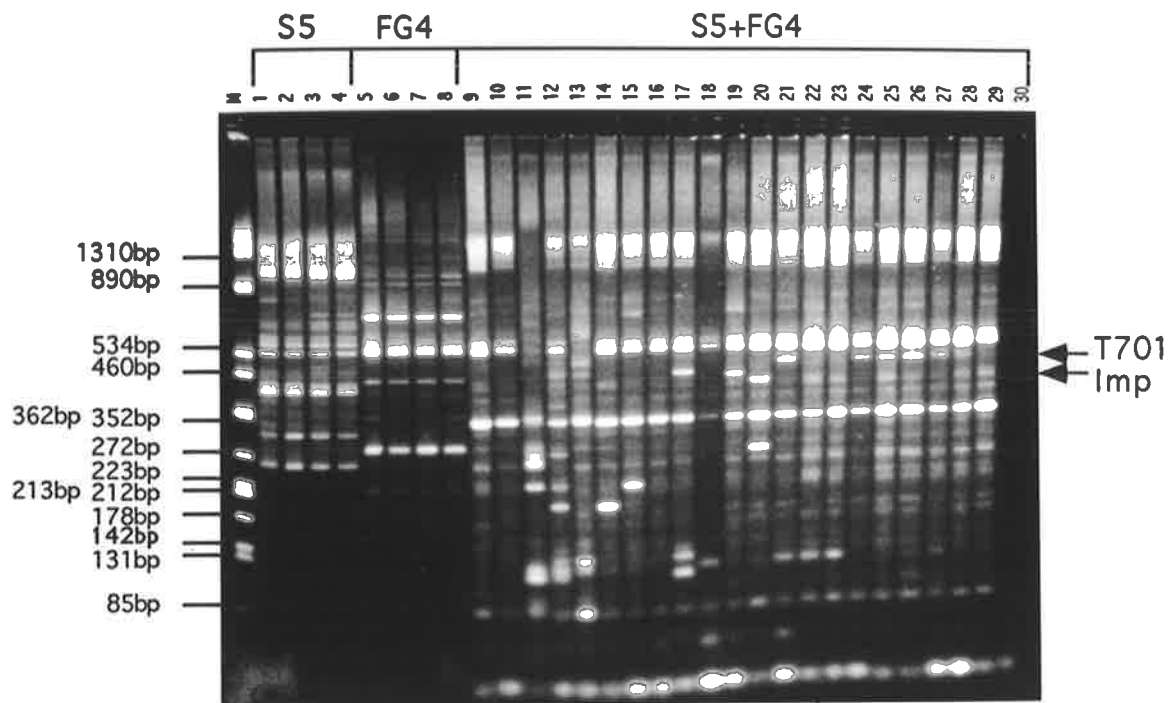
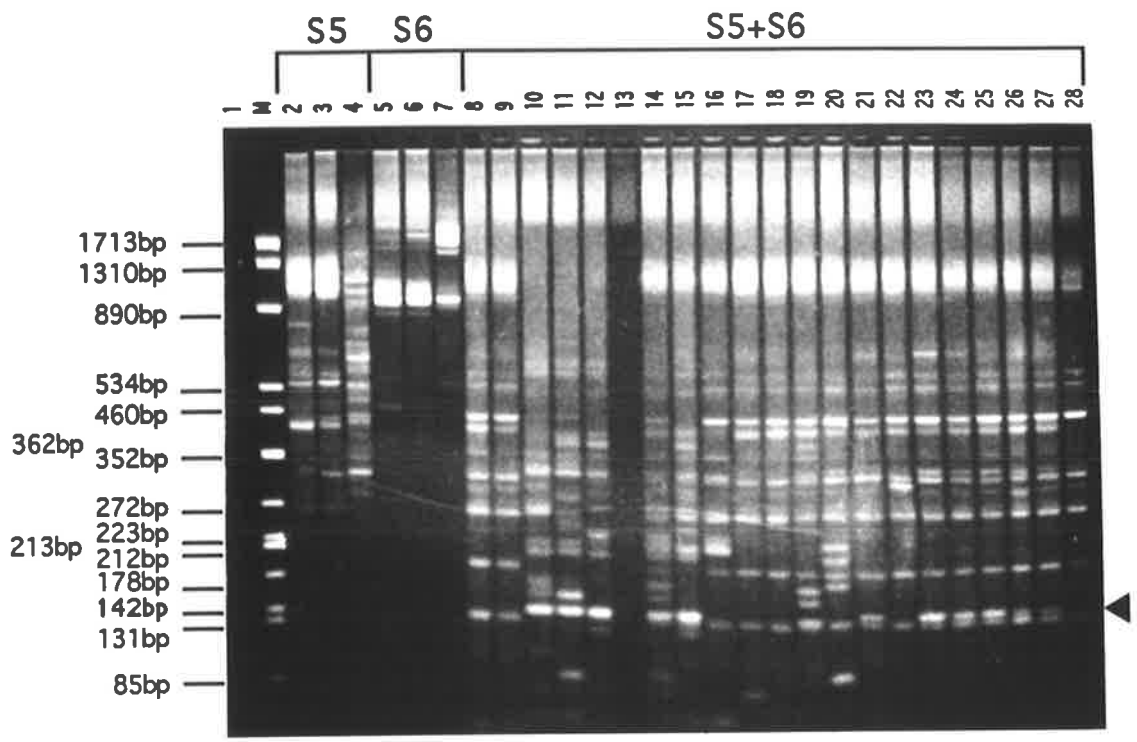


Figure 4. Subchromosomal localisation of the 125bp amplification product generated by the primer combination PawS5/PawS6 to chromosome 6R^{T701}. Amplification products were separated in 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard are given in base-pairs on the left. Lanes 2-4 show the products generated when PawS5 was used independently while lanes 5-7 show the products generated when PawS6 was used independently. Lanes 1, 8-28 show the products generated when PawS5 and PawS6 were used in combination. The arrow on the right indicates the 125 bp amplification product mapped to chromosome 6R^{Imp} and 6R^{T701}. Lanes 1-28 contained the following DNA samples:

- Lane 1: ddH₂O
- Lanes 2, 5 and 8: Schomburgk wheat
- Lanes 3, 6 and 9: Chinese Spring wheat
- Lanes 4, 7 and 13: rye cv. Imperial
- Lanes 10: rye cv. Vila Pouca
- Lane 11: rye cv. Petkus-R5
- Lane 12: rye cv. South Australian
- Lane 14: Chinese Spring-Imperial (-6R) amphiploid
- Lane 15: Triticale T701-4-6
- Lane 16: Chinese Spring-Imperial 1R disomic addition
- Lane 17: Chinese Spring-Imperial 2R disomic addition
- Lane 18: Chinese Spring-Imperial 3R disomic addition
- Lane 19: Chinese Spring-Imperial 4R disomic addition
- Lane 20: Chinese Spring-Imperial 5R disomic addition
- Lane 21: 6R^{Imp}
- Lane 22: Chinese Spring-Imperial 7R disomic addition
- Lane 23: 6R^{T701} (1)
- Lane 24: 6R^{T701} (2)
- Lane 25: del6RL22
- Lane 26: del6RL21
- Lane 27: del6RL1807
- Lane 28: del6RL1801



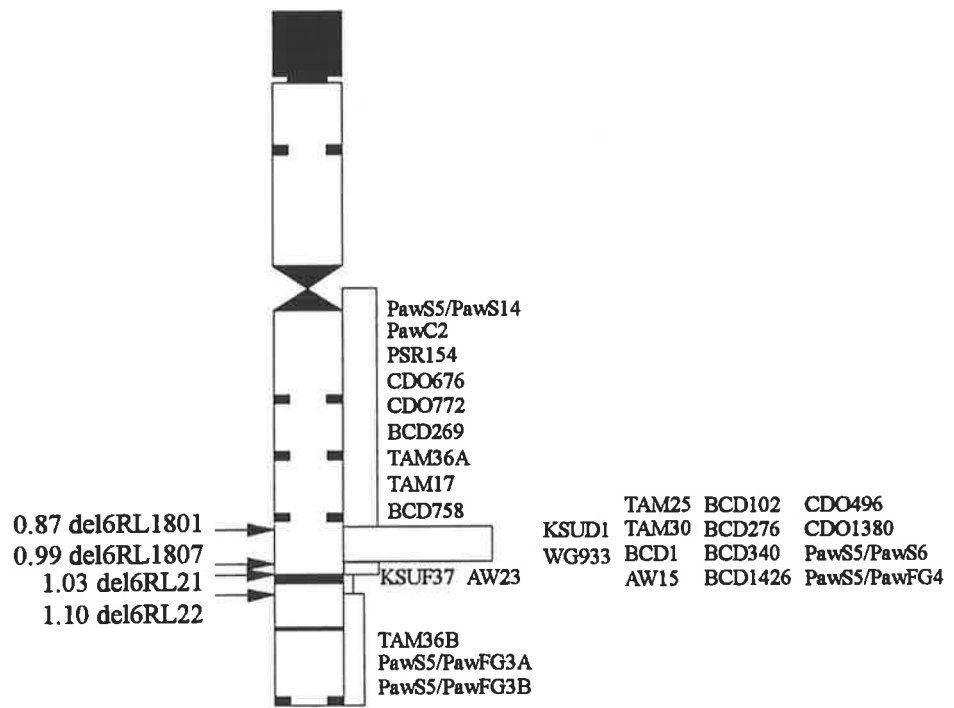
in 6R^{T701} (lanes 23 and 24) and was present in deletions del6RL22, del6RL21 and del6RL1807 but was absent in deletion del6RL1801 (lanes 25, 26, 27 and 28). Consequently, the locus identified maps to the same subchromosomal region as the amplification product generated using the primer pair PawS5/PawFG4.

With the exception of the amplification product generated using the primer combination PawS14/PawS15, all PCR products could be subchromosomally localised to the long arm of chromosome 6R^{T701} (Table 2). PCR using the primer combination PawS14/PawS15 resulted in a 310 bp amplification product which was mapped to both 6R^{Imp} and 6R^{T701}. This band was observed in the 6RL^{T701} monotelosomic addition line confirming its localisation to the long arm of this chromosome. Since amplification failed in samples using genomic DNA from del6RL1807 and del6RL1801 as template, subchromosomal localisation was limited to concluding only that this band maps proximal to the breakpoint in del6RL21. As this marker was deemed to be of little value in genetic linkage analysis (ie., monomorphic between chromosome 6R^{Imp} and chromosome 6R^{T701}), attempts to further localise this amplification product were not carried out. Both the 170 bp and 240 bp bands generated using the primer combination PawS5/PawFG3 mapped to the most distal region of the long arm of chromosome 6R^{T701} defined by the breakpoint in deletion line del6RL22. The 630 bp amplification product generated with the primer combination PawS5/PawS14 was amplified from genomic DNA from all four deletion lines.

6.2.3 Construction of a cytologically-based physical map of chromosome 6RL

Using cytogenetic data kindly provided by Dr. I. Dundas (Appendix 1), a cytologically-based physical map of the long arm of chromosome 6R^{T701} was constructed utilising all molecular markers subchromosomally localised to this chromosome arm (Tables 1 and 2). The position of C-bands and breakpoints of each of the deletion lines relative to the length of the short arm are incorporated (Figure 5). From this information, the amount of chromatin deleted in each of the 6RL deletion lines was estimated (see Appendix 1).

Figure 5. Physical map of chromosome 6R^{T701}. The C-bands (A-G) are drawn to scale and according to their intensities. The breakpoints of the four chromosome 6RL^{T701} deletions, along with their respective arm ratios are marked by arrows on the left. The positions of clones and chromosome regions defined by the deletion breakpoints are shown on the right.



Of the 22 RFLP loci mapped to the long arm of chromosome 6R, 7 (31.8%) were localised to the proximal 59% of the long arm and 15 (68.2%) mapped to the distal 41% of the long arm. Significantly, 12 (54.5%) of the RFLP loci identified were localised to an interstitial region comprising only about 8% of the length of the long arm (Figure 5). Only 3 (13.6%) loci were mapped to the distal third of this chromosome arm.

The PCR loci mapped in the present study did not demonstrate the same extreme level of bias in their chromosomal distribution, possibly due to the smaller number of markers screened (Figure 5). Two loci (*AawC2* and *AawS5/S14*) were localised to the proximal 59% of the long arm. Two loci (*AawS5/S6* and *AawS5/FG4*) were localised to an intercalary region comprising about 8% of the long arm while the remaining loci (*AawS5/FG3a* and *AawS5/FG3b*) were localised to the distal 25% of the long arm. Incorporating the PCR markers, a total of nine RFLP and PCR loci (32.1%) mapped physically to the proximal 59% of the long arm of 6R^{T701} while nineteen (67.9%) mapped to the distal 41% of the chromosome arm. However, as observed with RFLP loci, the inclusion of PCR markers did not alter the disproportionate number of loci found to map to the intercalary 8% of the chromosome arm defined by the breakpoints in del6RL1801 and del6RL1807. Fourteen of 28 loci (50.0%) analysed were localised to this chromosomal region.

6.3 DISCUSSION

6.3.1 Distribution of RFLP and PCR-based markers on chromosome 6RL

The four deletion lines utilised in this study divide the long arm of chromosome 6R^{T701} into five subarm regions. Twenty-one probes detected 23 loci on chromosome 6R^{T701}. One locus detected by the probe KSUF37 could not be mapped to the long arm and presumably lies on the short arm of chromosome 6R^{T701}. Consequently, 22 loci mark four of the five chromosome regions defined for the long arm since no loci were localised to the chromosomal region defined proximally by the breakpoint in del6RL21 and distally by the breakpoint in del6RL22. This region has been estimated to contain about 3% of the long arm (Appendix 1)

and includes a prominent C-band. Since this region is largely heterochromatic and the majority of clones used were cDNA, these results are not surprising. However, only 3 (13.6%) of 22 RFLP loci studied mapped in the distal third of the long arm of this chromosome. These results are in complete contrast to the study of Gill *et al.* (1993). Of the 29 RFLP loci studied, 20 (68.9%) were physically assigned to the distal 35% of the long arm of group-6 wheat chromosomes.

While it is possible that the uneven distribution of markers observed along rye chromosome 6RL is the result of random effects, a number of chromosomal rearrangements involving chromosome 6RL and other rye chromosomes have been identified and may account for the present observations. Koller and Zeller (1976) first suggested a translocation involving 6RL and 7RL. More recent studies including chromosome pairing and the localisation of the *Ep-1* locus and numerous RFLP loci confirm this rearrangement and indicate that it is distal and non-reciprocal (Naranjo and Fernández-Rueda, 1991; Benito *et al.*, 1991; Devos *et al.*, 1993b). Five homoeologous group 7 loci have been mapped genetically to the distal region of 6RL and span at least 30 cM (Devos *et al.*, 1993b). A reciprocal translocation involving 6RL and 3RL has also been demonstrated (Miller, 1984; Naranjo *et al.*, 1987; Naranjo and Fernández-Rueda, 1991; Devos *et al.*, 1993b). The segment of 3RL present on 6RL is interstitial with respect to 6RL and 7RL chromatin. RFLP mapping with homoeologous group 3 clones indicates that genetically, this segment spans at least 40 cM (Devos *et al.*, 1993b). Combined, the non-homoeologous segments identified at the distal end of 6RL account for at least 70 cM.

Gill *et al.* (1993) concluded that the distal 11% of the long arm of group 6 wheat chromosomes accounted for 90 cM. By extrapolation, this would imply that segments of 3RL and 7RL present in 6RL would account for at least 9% of this chromosome arm. Therefore, it is likely that the inability to localise homoeologous group 6 clones to the distal region of 6RL in the present study is due to the presence of a large segment of non-homoeologous chromatin derived from chromosomes 3RL and 7RL. The most distal locus was identified by the clone TAM36. This locus was identified only on 6R^{T701} and may indicate the presence of a small

duplication in 6RL^{T701} or possible cross-hybridisation to non-syntenous sequences. The lack of RFLP loci common to this study and that of Devos *et al.* (1993b) makes it difficult to estimate the amount of 3RL and 7RL chromatin present. However, the ability to localise homoeologous group 6 RFLP clones AW23 and KSUF37 to del6RL21 would indicate that physically, no more than 30% of the arm is derived from segments of 3RL and 7RL.

Physical and genetic mapping of C-bands in wheat and rye chromosomes has revealed that recombination occurs predominantly in the distal 20 to 30% of cereal chromosomes (Lukaszewski and Curtis, 1993). As a consequence, the relationship between physical and genetic distances is non-linear with recombination demonstrating a positive correlation with distance from the centromere (Curtis and Lukaszewski, 1991; Lukaszewski and Curtis, 1993). In the study of Devos *et al.* (1993b), five homoeologous group 6 clones were mapped genetically to chromosome 6RL spanning about 50 cM. Physically, the distal region of 6RL is comprised of segments from 3RL and 7RL which may account for as much as 30% of this chromosome arm. Therefore, using the genetic linkage data of Devos *et al.* (1993b), the proximal 70% of 6RL would account for only about 40% of the total genetic length of this chromosome arm indicating that the long arm of chromosome 6R also demonstrates reduced recombination in proximal regions.

Clones KSUF37, KSUD1 and TAM30 were physically localised to the distal 10% of group 6 wheat chromosomes in the study of Gill *et al.* (1993). Both KSUF37 and KSUD1 have been mapped genetically in *T. tauschii* with KSUF37 being the more distal of the two (K. S. Gill *et al.*, 1991a). However, the order of TAM30 relative to KSUD1 and KSUF37 is unknown. In the present study, TAM30 was physically localised to the same subchromosomal region as KSUD1. Both clones mapped proximal to KSUF37. Since recent genetic mapping studies have demonstrated high levels of synteny between wheat, rye and barley chromosomes (Devos *et al.*, 1992; Wang *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993a), the order of loci determined in rye indicates that TAM30 should map proximal of KSUF37 in wheat homoeologous group 6 chromosomes. The order of TAM30 and KSUD1 remains unknown.

Most significantly, the loci identified by the clones KSU37, KSUD1 and TAM30 are likely to serve as markers for the distal region of the long arm of homoeologous group 6 cereal chromosomes in general. Therefore, as much as 90% of ancestral 6RL may remain on the present-day long arm of chromosome 6R. The observation that over 50% of homoeologous group 6 clones used in this study were localised to the same subchromosomal region as TAM30 supports such a hypothesis since Gill *et al.* (1993) have demonstrated the clustering of loci in the distal 20% of group 6 wheat chromosomes. Assuming that much of the ancestral arm remains, these clones would actually reside on the distal 20 to 30% of the *ancestral* chromosome arm. Their present "intercalary" localisation is a consequence of subsequent rearrangements with segments of rye chromosome arms 3RL and 7RL. Devos *et al.* (1993b) presented a model for the evolution of the *S. cereale* genome in which the rearrangement between 3RL and 6RL occurred prior to that involving 7RL, resulting in a reciprocal translocation between 3RL and 6RL. Significantly, it was suggested that this rearrangement resulted in only a very small terminal region of 6RL being lost, thereby predicting that ancestral 6RL had remained essentially intact. Therefore, the results presented in this chapter are in agreement with the model described by Devos *et al.* (1993b).

The value of PCR-based markers was demonstrated in this study. Although chromosomal rearrangements involving non-homoeologous exchanges can only be identified via the application of mapped RFLP markers, such exchanges dramatically affect the efficiency of such an approach. In this study for example, no homoeologous group 6 clones except for TAM36 were mapped to the distal third of chromosome 6RL. However, two useful PCR markers were developed for this region. Random PCR markers therefore bypass potential problems caused by non-syntenous chromosomal rearrangements.

6.3.2 Evidence of an intercalary inversion in 6RL

Of the clones physically localised to 6RL in the present study, WG933, PSR154 and KSUF37 have also been mapped genetically and physically in *T. tauschii* and wheat, respectively (Gill *et al.*, 1993). Results from these studies have been adapted and are shown in Figure 6A and 6B. In wheat, the order of loci is: WG933-PSR154-KSUF37. Physically, WG933 has been localised to a small subchromosomal region within the proximal 45% of the chromosome arm (Gill *et al.*, 1993). This clone maps genetically about 40 cM from the centromere (Figure 6A). Both PSR154 and KSUF37 have been localised to the distal 10% of the long arm of wheat group 6 chromosomes (Gill *et al.*, 1993). The clone PSR154 maps genetically about 108 cM distal of WG933 while KSUF37 maps about 43 cM distal of PSR154 (Figure 6A). Consequently, both the genetic and physical maps of the long arm of wheat group 6 chromosomes are in agreement. In rye, PSR154 maps genetically about 25 cM from the centromere (Devos *et al.*, 1993b; Figure 6D). This clone was localised to the proximal 59% of the long arm in this study (Figure 6B). Consequently, PSR154 maps more proximal in rye than in wheat. In rye, the clone WG933 was localised to the same subchromosomal region as KSUD1 and TAM30 (Figure 6C). This region maps 59-67% of the distance from the centromere along the *present-day* long arm of chromosome 6R. Consequently, WG933 maps more distal in rye than in wheat. Therefore, results from physical mapping studies indicate that the order of loci in rye chromosome 6RL is: PSR154-WG933-KSUF37. These results indicate an inversion within the long arm of chromosome 6R relative to wheat.

The order of clones (ie., WG933-PSR154-KSUF37) is conserved in wheat 6AL and *T. tauschii* (K. S. Gill *et al.*, 1991a) and probably wheat 6BL. The clones PSR154 and KSUD17 have been genetically mapped in chromosome 6D of *T. tauschii* and chromosome 6H of barley (K. S. Gill *et al.*, 1991a; Kleinhofs *et al.*, 1993). In both cases, the order of these two clones is conserved with PSR154 mapping distal of KSUD17. Consequently, the order established in wheat is the likely ancestral order. Assuming this, putative breakpoints in the ancestral arm of chromosome 6RL can be inferred. The proximal breakpoint would lie between the

Figure 6. Comparison between genetic and physical maps of the long arm of wheat group 6 chromosomes and the long arm of rye chromosome 6R. For simplicity, only clones common to this study and the studies of Gill *et al.* (1993) and Devos *et al.* (1993b) are shown.

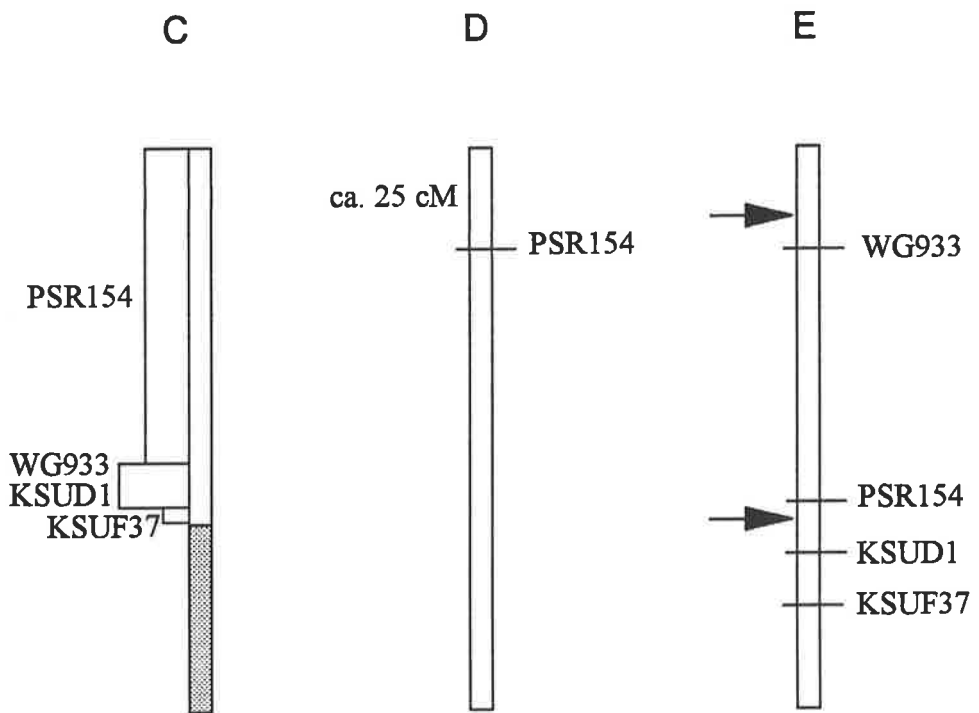
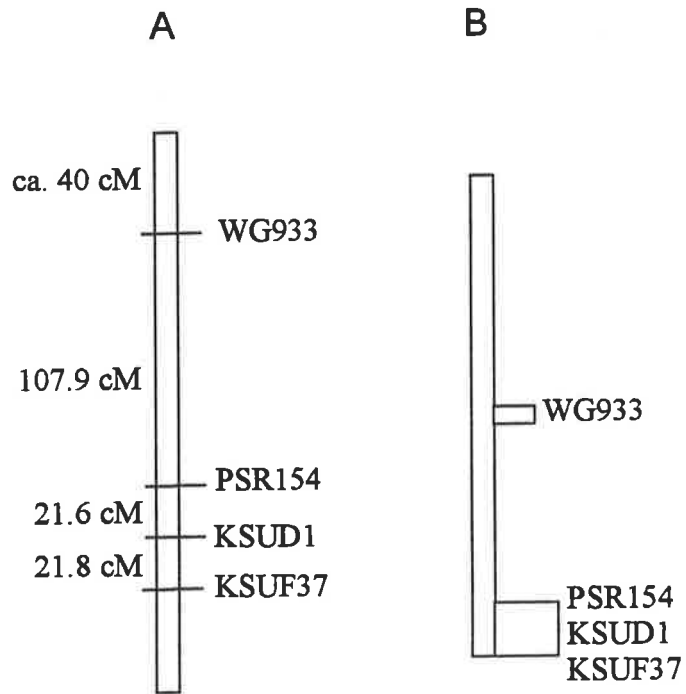
A) Genetic linkage map of the long arm of wheat group 6 chromosomes adapted from Gill *et al.* (1993). The genetic distance between the centromere and the locus detected by the clone WG933 is estimated since the centromere was not localised in the study of Gill *et al.* (1993).

B) Consensus physical map of the long arm of wheat group 6 chromosomes adapted from Gill *et al.* (1993).

C) Physical map of the long arm of rye chromosome 6R adapted from Figure 5 (this study). The stipled region distal of the clone KSUF37 indicates the possible extent of 3RL and 7RL chromatin present on the long arm of rye chromosome 6R.

D) The clone PSR154 has been genetically mapped to rye chromosome 6R (Devos *et al.*, 1993b). However, the position of the centromere is unknown and, consequently, the genetic distance shown is an estimate only.

E) The position of putative proximal and distal breakpoints in the long arm of ancestral rye chromosome 6R are shown (arrows). The proximal breakpoint must lie between the centromere and the locus detected by the clone WG933 while the distal breakpoint must lie between the loci identified by clones PSR154 and KSUD1.



centromere and WG933 while the distal breakpoint must lie between PSR154 and KSUD1 (and TAM30) since in the *present-day* chromosome 6RL arm, KSUD1 maps proximal to KSUF37 which is consistent with the order established in wheat. Gill *et al.* (1993) inferred that an inversion had occurred in the long arm of chromosome 6D relative to chromosomes 6A and 6B. This conclusion was based on the physical localisation of the clone KSUD17. In chromosome 6D this clone maps to the proximal 10% of the chromosome arm. However, in chromosomes 6A and 6B this clone maps in the region FL 0.415 and FL 0.549. While the clone KSUD17 was not used in the present analysis of chromosome 6RL, it is possible to conclude that the putative rearrangements identified in wheat 6DL and rye 6RL are not the result of a single event. Since the clone WG933 was mapped to the same subchromosomal region in both 6DL and 6AL (Gill *et al.*, 1993), this locus appears not to have been involved in the putative rearrangement identified in the long arm of chromosome 6D.

6.3.3 Implications of the structural rearrangements identified in 6RL

Information pertaining to structural rearrangements of cereal chromosomes is useful to the development of molecular markers and the construction of a high density molecular genetic linkage maps. Clearly, the molecular genetic map of chromosome 6RL will be enriched via the application of homoeologous group 3 and group 7 clones as well as additional homoeologous group 6 clones. However, most significantly, the structural integrity of alien chromosomes relative to those of wheat has considerable bearing on the available mechanisms and approaches which may be used by breeders for the introgression of agronomically important characters. Such information is particularly relevant to the application of homoeologous recombination.

The principal of homoeologous recombination relies on the increase in pairing observed between homoeologous chromosomes in the absence of *Ph1*, the major pairing suppressor in hexaploid wheat (Riley and Chapman, 1958; Sears and Okamoto, 1958). Increased levels of pairing can be induced between homoeologous chromosomes either through nullisomy for

chromosome 5B or the presence of the *ph1b* mutation (Sears, 1977). The potential for this method in wheat breeding has been demonstrated using *Aegilops* spp. (Riley *et al.*, 1968; Dvorák, 1977), *Agropyron* spp. (Sears, 1973, 1981; Kibirige-Sebunya and Knott, 1983) and more recently, for rye (Koebner and Shepherd, 1985, 1986a; Naranjo *et al.*, 1989). Koebner and Shepherd (1985) induced allosyndesis between the long arm of chromosomes 1RL and 1DL using the *ph1b* mutant (Sears, 1977) while Koebner and Shepherd (1986a) utilised both the *ph1b* mutant and nullisomy for chromosome 5B. In both studies, long arm and short arm wheat-rye translocation lines were used since it was believed that homoeologous pairing between wheat and rye arms would be increased through the induction of normal synapsis of homologous wheat chromosome arms. In a detailed analysis, Naranjo *et al.* (1989) observed chiasmatic associations between 1RL and the long arms of wheat group 1 chromosomes from all three genomes. However, 1RL-1AL and 1RL-1DL associations were rare, a result possibly vindicating the approach of Koebner and Shepherd (1985). Recombinant chromosomes were isolated in all three studies.

The success observed in studies involving wheat group 1 chromosomes and chromosome 1R of rye has not been reproduced in studies involving other rye chromosomes. Specifically, attempts to induce homoeologous pairing and recombination between the long arm of chromosome 6D of wheat the long arm of chromosome 6R of rye have failed (Dundas *et al.*, 1992, 1993). While rye chromosomes have been shown to induce homoeologous pairing with wheat chromosomes (Riley *et al.*, 1973; Jouve *et al.*, 1980; Naranjo and Palla, 1982), pairing between wheat and rye chromosomes in the presence of *Ph1* is low (Naranjo and Lacadena, 1980; Jouve *et al.*, 1980; Naranjo and Palla, 1982). Naranjo (1982) analysed homoeologous pairing between wheat and rye chromosomes in ABRR and (0-7)A(0-7)BRR plants. Chromosomes of homoeologous group 1 were found to pair preferentially in (0-7)A(0-7)BRR plants. Overall, pairing between wheat and rye chromosomes from homoeologous groups 2-7 was low with a maximum of 0.78%. In contrast, pairing between wheat group 1 chromosomes and chromosome 1R of rye was significantly higher reaching a maximum of 16.25%. Greater affinity between wheat and rye chromosomes of homoeologous group 1 or

more efficient pairing initiation due to common nucleolar organising regions were cited as possible reasons for these observations (Naranjo, 1982).

Naranjo and Ferández-Rueda (1991) have further elucidated the arm homologies of rye chromosomes relative to wheat via an analysis of chromosome pairing between rye and wheat chromosome arms at metaphase I in *ph1b* mutant wheat x rye hybrids. By also incorporating the available data on the chromosomal location of homoeoloci in wheat and rye, these authors were able to conclude that only arms 1RS, 1RL, 2RL, 3RS and 5RS demonstrated normal homoeologous relationships to wheat. These conclusions are supported by recent comparative mapping studies (Devos *et al.*, 1992; Wang *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993a, b). Both the short and long arms of chromosome 1R, the long arm of chromosome 2R and the short arm of chromosome 3R of rye have remained largely unaltered with respect to wheat chromosomes. As a consequence, the method of homoeologous recombination appears best suited to the introgression of genetic material from these chromosome arms due to higher levels of pairing observed between these chromosome arms and their wheat homoeologues.

Naranjo and Ferández-Rueda (1991) observed pairing between 6RL and wheat chromosomes 6WL, 7WL and 3WL. However, in all cases, the level of pairing was low. Significantly, the level of pairing observed between 6RL and 7WL was higher (0.9%) than that observed between 6RL and 3WL (0.2%) and 6RL with 6WL (0.2%). The non-homoeologous segments from 3RL and 7RL present at the distal end of 6RL are likely to affect levels of pairing with wheat group 6 chromosomes through pairing competition with the long arms of homoeologous groups 3W and 7W. Also, since pairing initiation sites appear to be distributed in the distal halves of wheat chromosomes and that efficient chromosome pairing may require the alignment of telomeres (Curtis *et al.*, 1991), higher levels of pairing between 6RL and 7WL should be anticipated. Furthermore, it is likely that the low levels of interstitial pairing observed between the long arm of chromosome 6R with the long arm of wheat group 6 chromosomes is also affected by the inversion identified in the long arm of chromosome 6R of rye in this study.

Consequently, the failure to induce homoeologous pairing between 6RL and 6DL is probably attributable to the significant structural rearrangement of this chromosome arm relative to wheat.

While it may be possible to improve the level of pairing between the long arm of chromosome 6R and wheat group 6 chromosomes via the use of deletion lines in which the distal 3RL and 7RL segments are deleted, pairing is still likely to be very low due to the influence of the interstitial inversion. Also, while it appears that 6RL is essentially intact, a small amount of distal 6RL chromatin has been lost. Curtis *et al.* (1991) have shown that in wheat, small distal chromosomal deletions are capable of adversely affecting homologous pairing frequency in structural heterozygotes, possibly through the interference of pairing initiation. Consequently, it would appear that the method of homoeologous recombination will have limited application in the introgression of useful agronomic characters such as the gene for cereal cyst nematode resistance (*CreR*) from chromosome 6R of rye to wheat group 6 chromosomes.

CHAPTER 7

THE DEVELOPMENT OF A GENETIC LINKAGE MAP OF THE LONG ARM OF CHROMOSOME 6R OF RYE

7.1 INTRODUCTION

Requisite to the generation of linkage maps is the availability of segregating populations. To date, the majority of genetic linkage maps developed for species within the Triticeae have utilised F₂ populations (or bulked F₃ lines) established through the crossing of two inbred or highly divergent lines. For example, linkage maps have been developed for *T. tauschii* (K. S. Gill *et al.*, 1991*a, b*; Lagudah *et al.*, 1991; Namuth *et al.*, 1994), *T. aestivum* (Chao *et al.*, 1989; Lui and Tsunewaki, 1991; Williams *et al.*, 1994), and barley (Shin *et al.*, 1990) utilising F₂ populations. In particular, RFLP-based linkage maps developed for the rye genome have utilised an F₂ population derived from crossing two Polish inbred lines, Ds2 and RxL 10 (Wang *et al.*, 1991; Devos *et al.*, 1992; C. J. Liu *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993*a, b*; Plaschke *et al.*, 1993). With respect to the long arm of rye chromosome 6R, the genetic linkage map of Devos *et al.* (1993*b*) is the most detailed comprising 15 loci spanning approximately 133 cM.

In barley, doubled haploid lines (DHL) derived via anther culture or, alternatively, embryo rescue techniques have provided an additional approach to the development of mapping populations. Populations based on DHL have been used extensively in recent years for the development of linkage maps of moderate density of the barley genome (Heun *et al.*, 1991; Kleinhofs *et al.*, 1993). Benefits of this approach include the simplification of analysis, the provision of an immortal reference population for future studies and the ability to analyse both female and male recombination (Kleinhofs *et al.*, 1993; Wang *et al.*, 1995). The use of single chromosome recombinant populations in the development of genetic linkage maps has also been reported (Chao *et al.*, 1989; Joppa, 1991). Similar to DHL, recombinant substitution

lines (RSL) are valuable as they provide an immortal reference population allowing further studies such as the identification of QTL.

Benefits of genetic mapping studies reside in the ability to genetically localise genes conferring traits of agronomic importance and particularly, the identification of molecular markers linked to these genes. Resistance to *H. avenae* pathotype Ha13 has been described for a small number of rye cultivars. High levels of resistance have been observed in rye cv. South Australian (Brown and Meagher, 1970). Brouwer and Castleman (1981) reported moderate resistance and tolerance in the triticale cv. Towan and Fisher (1982a) has reported a high level of resistance in the triticale T701-4-6. The gene conferring resistance to CCN identified in the triticale T701-4-6 (ie., *CreR*), demonstrates monogenic dominant inheritance and has been localised to the long arm of chromosome 6R^{T701} (Asidue, 1986; Asidue *et al.*, 1990). More recently, Dundas *et al.* (1992) physically mapped *CreR* to an intercalary region on the long arm of chromosome 6R^{T701} using chromosome 6RL deletion lines. Presently, screening for resistance to CCN relies on a bioassay which is both time consuming and unreliable (Fisher, 1982a). The development of a molecular marker linked to *CreR* could allow for more rapid screening of resistance in rye cultivars. However, the application of linked markers to enable introgression of *CreR* from rye into wheat in breeding programmes is of most significance.

Consequently, the Chinese Spring-T701-4-6 6R(-6D) disomic substitution line represents a valuable resource for studies aimed at genetically mapping *CreR* and identifying linked molecular markers. An approach to the generation of a Test-Cross F₁ (TC-F₁) mapping population using this line is described. The resulting TC-F₁ population was screened using RFLP and PCR markers described in Chapters 4, 5 and 6 allowing the generation of a genetic linkage map for the long arm of chromosome 6R. Finally, CCN bioassays carried out on a subpopulation of plants allowed *CreR* to be mapped genetically as well as identify a number of RFLP markers showing linkage to *CreR*.

7.2 RESULTS

7.2.1 The generation of a TC-F₁ population: Rationale

Joppa (1991) has described the generation of homozygous recombinant substitution lines (RSL) using Langdon-*T. dicoccoides* disomic substitution lines (LDN(DIC)), Langdon durum (LDN) and Langdon D-genome disomic substitution lines. This method is significant since it allows genetic linkage maps to be constructed for individual chromosomes in a relatively homogeneous genetic background. While the method of Joppa (1991) results in the generation of BC-F₂ lines, it is theoretically possible to use lines isolated earlier in the process (ie., BC-F₁) for the development of a genetic linkage map since loci should segregate 1:1 in a population of BC-F₁ plants.

The generation of a series of chromosome 6R RSL using the Chinese Spring-T701-4-6 disomic 6R(-6D) substitution requires a second wheat-rye disomic 6R(-6D) substitution line. Crossing Chinese Spring-T701-4-6 disomic 6R(-6D) with a second wheat-rye chromosome 6R(-6D) substitution line would allow the production of an F₁ in which both 6R chromosomes are able to pair and recombine at meiosis. Since rye chromosomes rarely pair with wheat homoeologues in the presence of *Ph1* (Naranjo and Lacadena, 1980; Jouve *et al.*, 1980; Naranjo and Palla, 1982), the generation of BC-F₁ lines is simplified; the F₁ can be backcrossed to Chinese Spring wheat producing TC-F₁ plants monosomic for chromosome 6D and (recombined) 6R. Test-Cross F₂ lines (ie., RSL for chromosome 6R) could then be generated by selfing TC-F₁ individuals.

Two additional wheat-rye chromosome 6R substitution lines are listed in the Fourth compendium of wheat-alien chromosome lines (Shepherd and Islam, 1988). However, the resistance status of these lines to CCN is unknown. Moreover, the level of polymorphism between chromosome 6R^{T701} and chromosome 6R present in these lines for RFLP and PCR loci is unknown and was not assessed in the present study. Consequently, neither line was suitable for establishing a chromosome 6R mapping population. In contrast, the Chinese

Spring-Imperial 6R disomic addition line was utilised in all preliminary screening experiments involving RFLP and PCR markers allowing detailed assessment of polymorphism between 6R^{Imp} and 6R^{T701} chromosomes. Most importantly, chromosome 6R^{Imp} does not confer resistance to *H. avenae* (J. Fisher, personal communication). Consequently, it was decided to devise a method to allow the generation of a mapping population using the Chinese Spring-T701-4-6 6R(-6D) disomic substitution line and the Chinese Spring-Imperial 6R disomic addition line.

Figure 1 details the crosses utilised in the development of a TC-F₁ population. Pollen from the Chinese Spring-T701-4-6 6R(-6D) disomic substitution was used to fertilise Chinese Spring-Imperial 6R disomic addition (Figure 1A). A total of eighteen seeds were set of which, six were selected from an individual spike. Successful hybridisation was confirmed via PCR and ISH. Figure 2 shows PCR carried out on DNA samples extracted from the six putative F₁ plants using the primer combination PawS5/PawFG4. PCR using this primer combination results in the generation of a 400 bp band mapping to 6R^{Imp} and a 450 bp band mapping to 6R^{T701} (Chapter 6, Table 2). Both bands are present in seedlings F₁-1, F₁-2, F₁-4, F₁-5 and F₁-6 (lanes 3, 4, 6, 7 and 8) confirming successful hybridisation of Chinese Spring-Imperial 6R disomic addition with Chinese Spring-T701-4-6 6R(-6D) disomic substitution. However, seedling F₁-3 possesses only the 450 bp 6R^{T701} band (lane 5). Chinese Spring-Imperial 6R disomic addition was crossed as a female. Consequently, plant F₁-3 is likely to have resulted from the functioning of a gamete, deficient for rye chromosome 6R^{Imp} arising through desynapsis. Successful fertilisation by Chinese Spring-T701-4-6 6R(-6D) disomic substitution has yielded plant F₁-3 monosomic for chromosome 6R^{T701}. Further confirmation of the hybrid status of seedlings F₁-5 and F₁-6 was obtained via ISH using the rye-specific repetitive DNA probe, pAW173.1 (Chapter 3, Figure 5). Consequently, individuals F₁-5 and F₁-6 were selected for further crossing.

Due to the structural differences observed between chromosome 6R^{Imp} and chromosome 6R^{T701}, it was considered important to establish that these chromosomes paired at meiosis.

Figure 1. Illustration of procedures utilising the Chinese Spring-T701-4-6 6R(-6D) disomic substitution line for the production of a TC-F₁ mapping population.

A. Generation of F₁ hybrid plants. P₁ (C. S.-Imp 6R addition) was crossed as a female to P₂ (C. S.-T701-4-6 6R[-6D] substitution). Of the 18 seed set, six were germinated and seedlings screened via PCR to verify hybridisation. Successful hybridisation was observed for five of the six seedlings. The hybrid status of two F₁ plants (F₁-5 and F₁-6) was also determined via ISH. Genomic configuration of F₁ plants is indicated (framed). Chromosomes 6R^{T701} and 6R^{Imp} are depicted schematically and morphological differences observed between 6R^{T701} and 6R^{Imp} are shown. Recombination between chromosome 6R^{T701} and chromosome 6R^{Imp} is depicted (X).

B. F₁ plants F₁-5 and F₁-6 were backcrossed to P₃ (Chinese Spring wheat) in order to generate a TC-F₁ population. Collectively, four hundred and thirty nine seeds were set. Six genomic configurations are possible for TC-F₁ individuals (framed). Plants possessing genomic configurations 1 and 2 will be segregating for a 6R monosome and therefore be suitable for constructing a genetic linkage map of the long arm of chromosome 6R. Plants possessing genomic configurations 3-6 are of no value since they will be either disomic or nullisomic for chromosome 6R.

A) P_1 (C. S.-Imp 6R Addition) X P_2 (C. S.-T701-4-6 6R Substitution)

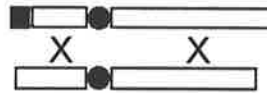


F_1

(18 seeds set; 6 tested)

$20W''+6R^{T701}+6R^{Imp}+6D'$

♀ Gametes



6R T701-4-6

6R Imperial

B) F_1 Plant (F_1 -5) x P_3 (C. S.)



TC- F_1
(314 seeds set)

F_1 Plant (F_1 -6) x P_3 (C. S.)



TC- F_1
(125 seeds set)



1. $20W''+6R'+6D'$
2. $21W''+6R'$
3. $20W''+6R''+6D'$
4. $21W''+6R''$
5. $20W''+6D'$
6. $21W''$



Select TC- F_1 6R monosomic plants

Figure 2. Screening putative F_1 hybrid plants derived from a cross between Chinese Spring-Imperial 6R disomic addition and Chinese Spring-T701-4-6 6R(-6D) with the primer combination PawS5/PawFG4. Amplification products were separated in 3% agarose gels. The size standard (M) was λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard are given on the left in base-pairs. Lanes 1-14 show the products generated using both primers. Lanes 15 and 16 show the products generated when only primer PawFG4 was used in reactions and lanes 17 and 18 show the products generated when only primer PawS5 was used in reactions. The arrows on the right indicate the amplification products mapped to chromosome 6R^{T701} (filled) and chromosome 6R^{Imp} (open). Lanes 1-18 contained the following DNA samples:

Lanes 1, 2, 10, 15, and 17: Chinese Spring wheat
Lane 9: Schomburgk wheat
Lane 3: F_1 -1
Lane 4: F_1 -2
Lane 5: F_1 -3
Lane 6: F_1 -4
Lane 7: F_1 -5
Lane 8: F_1 -6
Lane 11: 6R^{T701}
Lane 12: 6R^{Imp}
Lane 13: 6R^{T701} + 6R^{Imp} (1:1)
Lane 14: ddH₂O
Lanes 16 and 18: 6R^{Imp}

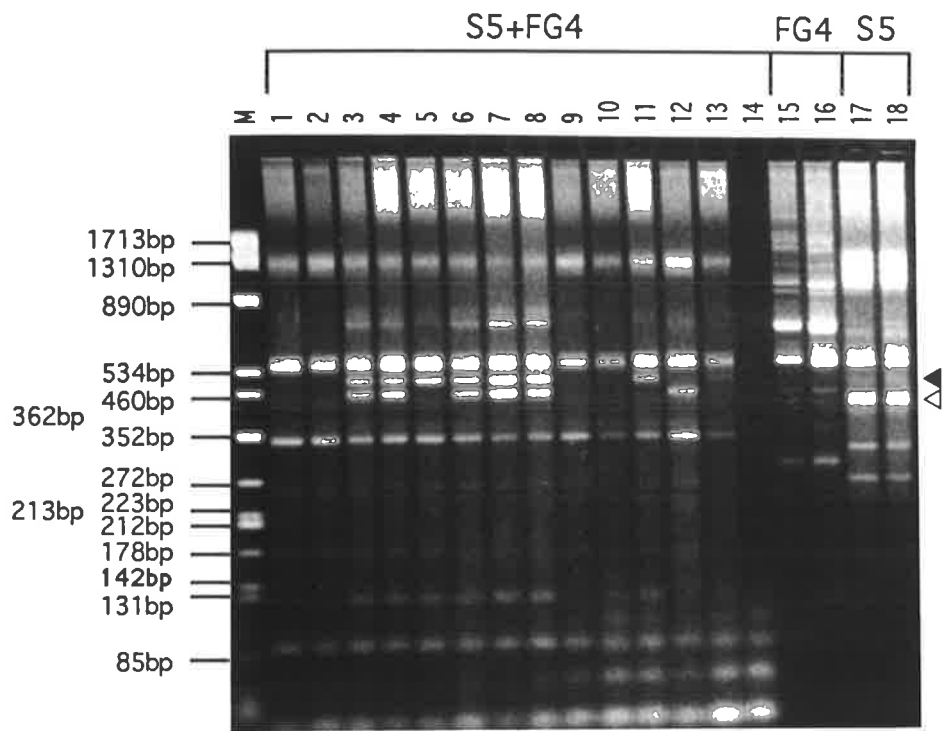


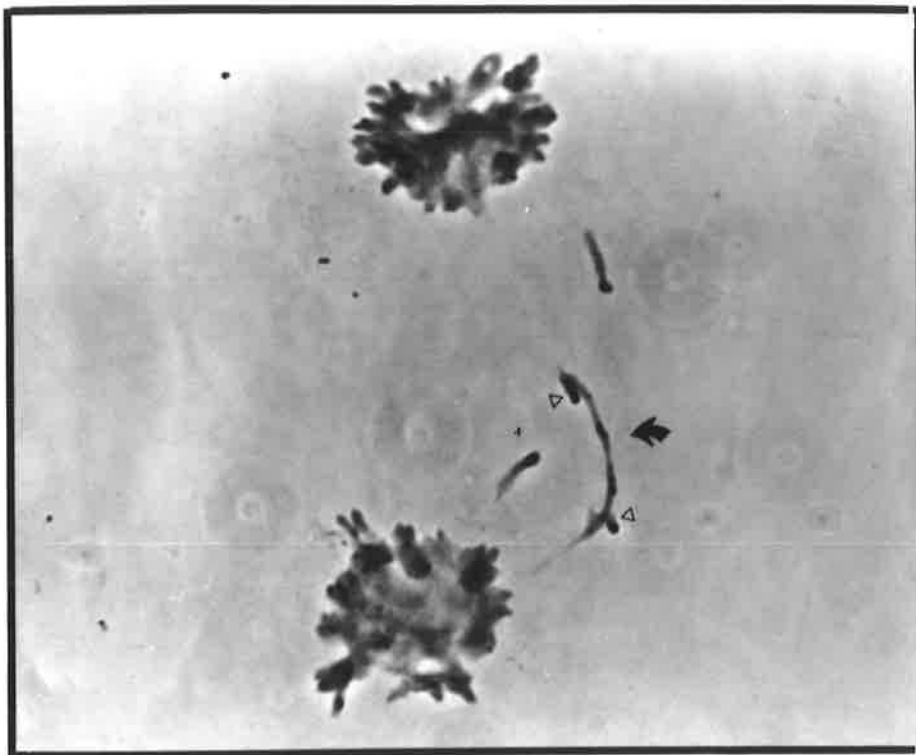
Figure 3 shows C-banded telophase cell from plant F₁-5. Chromosome 6R is identifiable due to the heavily stained short arm telomeres (open arrows). Shown is an open bivalent involving 6R^{T701} and 6R^{Imp}. The chiasmatic association is located at the distal end of the 6RL chromosomal arm (filled arrow). Most pairing associations between 6R^{T701} and 6R^{Imp} chromosomes appeared as open bivalents with the long arms being paired. The 6R^{T701} and 6R^{Imp} chromosomes were also observed as univalents at MI in some cells, indicating the occurrence of either asynapsis or desynapsis.

Figure 1B details the second step in the generation of a chromosome 6R mapping population. F₁ plants will have the genomic constitution 20Wⁿ+6R^{T701}+6R^{Imp}+6D^l. Consequently, to ensure the most efficient transmission of chromosome 6R to TC-F₁ plants, individuals F₁-5 and F₁-6 were crossed as a female to Chinese Spring wheat. A total of four hundred and thirty nine seeds were set. All possible genomic configurations for TC-F₁ plants are listed in Figure 1B. While it would be possible to self individual plants carrying chromosome 6R as described by Joppa (1991), the unbalanced genomic constitution of TC-F₁ plants and the low transmission rates of monosomic alien chromosomes in pollen are likely to have a significant, adverse affect on the efficacy of recovering plants disomic for chromosome 6R. The characterisation of RFLP and PCR markers with respect to the alleles identified on chromosome 6R^{Imp}, chromosome 6R^{T701} and wheat group 6 chromosomes greatly simplifies the screening of these markers in a TC-F₁ population. For these reasons, the generation of a TC-F₂ population was not carried out.

7.2.2 Selection of TC-F₁ plants monosomic for chromosome 6R of rye

Due to the unbalanced chromosome number of the F₁ plants used in the generation of the TC-F₁ population, backcrossing to Chinese Spring will result in TC-F₁ plants possessing six distinct genomic configurations (Figure 1B). Most significantly, asynapsis and/or desynapsis will result in TC-F₁ plants possessing both chromosome 6R^{Imp} and chromosome 6R^{T701}. Such plants have no value in the present analysis. Asynapsis or desynapsis will also lead to the

Figure 3. C-banded telophase cell from plant F₁-5. The rod bivalent (filled arrow) is identified as chromosome 6R due to the heavily stained short arm telomeres (open arrows). Pairing between chromosome 6R^{T701} and chromosome 6R^{imp} is localised to the distal region of the long arm.



generation of plants nullisomic for chromosome 6R. Consequently, it was necessary to screen the TC-F₁ population in order to select only plants monosomic for chromosome 6R.

The structural genes encoding GOT-2 have been localised to the long arm of wheat chromosomes 6A, 6B and 6D (Hart, 1975), rye chromosome 6R (Tang and Hart, 1975) and barley chromosome 6H (Hart *et al.*, 1980). Designated *Got-A2*, *Got-B2*, *Got-D2*, *Got-R2* and *Got-H2* respectively, these genes code subunits designated α^2 , β^2 , δ^2 , ρ^2 and θ^2 respectively. In Chinese Spring wheat, the active GOT-2 isozymes are dimers composed of all possible combinations of subunits coded by the triplicate structural genes. Electrophoresis in acrylamide gels reveals three forms, designated GOT-2a, GOT-2b and GOT-2c (Hart, 1975). In rye cv. Imperial, a single form, GOT-2e composed of dimers $\rho^2\rho^2$ is observed (Tang and Hart, 1975). GOT-2e has a slower mobility to all three forms identified in Chinese Spring wheat and is the most cathodal band observed in the Chinese Spring-Imperial disomic 6R addition line. However, an additional form, GOT-2d is resolved in the Chinese Spring-Imperial disomic 6R addition line (Tang and Hart, 1975) and results from the association of dimers α^2 and β^2 with ρ^2 .

The ratio of the five forms, GOT-2a, GOT-2b, GOT-2c, GOT-2d and GOT-2e is 1/16, 4/16, 6/16, 4/16 and 1/16 respectively and is reflected by the intensity of staining of bands in acrylamide gels (Tang and Hart, 1975). Significantly, a linear relationship exists between the quantity of each of the possible types of subunit contained in the active enzyme and the dosage of the chromosomes and chromosome arms carrying the structural genes for the subunits (Hart, 1975; Tang and Hart, 1975). GOT-2a is comprised only of subunits $\delta^2\delta^2$. Similarly, GOT-2e is comprised only of subunits $\rho^2\rho^2$. Both forms are present in the ratios 1/16 when chromosomes 6D and 6R are disomic and are observed as faint bands in acrylamide gels. However, the ratios of these forms will be reduced in plants monosomic for either or both chromosomes (Table 1). Consequently, it should be possible to select TC-F₁ plants monosomic for chromosome 6R based upon the intensity of the isozyme form GOT-2e and the presence of isozyme form GOT-2d. Similarly, it should be possible to classify TC-F₁ plants

Table 1. The expected distribution of the isozymes which produce the GOT-2 bands 1, 2, 3, 4 and 5 in TC-F₁ individuals when, A) chromosome 6R is present as a monosome; B) chromosome 6D is present as a monosome and C) both chromosome 6R and 6D are present as monosomes. Band nomenclature and isozyme subunit composition follows Tang and Hart (1975).

A. Monosomic for chromosome 6R

Isozymes	Band	Subunit composition
GOT-2a	1	2/25 $\delta^2\delta^2$
GOT-2b	2	8/25 $\alpha^2\delta^2, \beta^2\delta^2$
GOT-2c	3	10/25 $\alpha^2\alpha^2, \beta^2\beta^2, \alpha^2\beta^2, \delta^2\rho^2$
GOT-2d	4	4/25 $\alpha^2\rho^2, \beta^2\rho^2$
GOT-2e	5	1/25 $\rho^2\rho^2$

B. Monosomic for chromosome 6D

Isozymes	Band	Subunit composition
GOT-2a	1	1/25 $\delta^2\delta^2$
GOT-2b	2	4/25 $\alpha^2\delta^2, \beta^2\delta^2$
GOT-2c	3	10/25 $\alpha^2\alpha^2, \beta^2\beta^2, \alpha^2\beta^2, \delta^2\rho^2$
GOT-2d	4	8/25 $\alpha^2\rho^2, \beta^2\rho^2$
GOT-2e	5	2/25 $\rho^2\rho^2$

C. Monosomic for chromosome 6R and chromosome 6D

Isozymes	Band	Subunit composition
GOT-2a	1	1/19 $\delta^2\delta^2$
GOT-2b	2	4/19 $\alpha^2\delta^2, \beta^2\delta^2$
GOT-2c	3	9/19 $\alpha^2\alpha^2, \beta^2\beta^2, \alpha^2\beta^2, \delta^2\rho^2$
GOT-2d	4	4/19 $\alpha^2\rho^2, \beta^2\rho^2$
GOT-2e	5	1/19 $\rho^2\rho^2$

for chromosome 6D based upon the relative staining intensities of isozyme forms GOT-2a and GOT-2b.

Of the 439 seed set, 423 (96.4%) germinated. Figure 4 shows GOT-2 zymogram phenotypes of a subpopulation of plants from the TC-F₁ population. Band nomenclature follows that of Tang and Hart (1975). Bands 1, 2 and 3 are observed in Chinese Spring wheat (lane 1). Bands 2, 3, 4 and 5 are observed in the triticale T701-4-6 (lane 2). Band 1 appears absent from the triticale T701-4-6 indicating possible monosomy for chromosome 6D. As expected, bands 4 and 5 are present and bands 1 and 2 are absent from the Chinese Spring-T701-4-6 6R(-6D) substitution line (lane 18). All possible genomic configurations are observed in this subpopulation of 15 TC-F₁ plants.

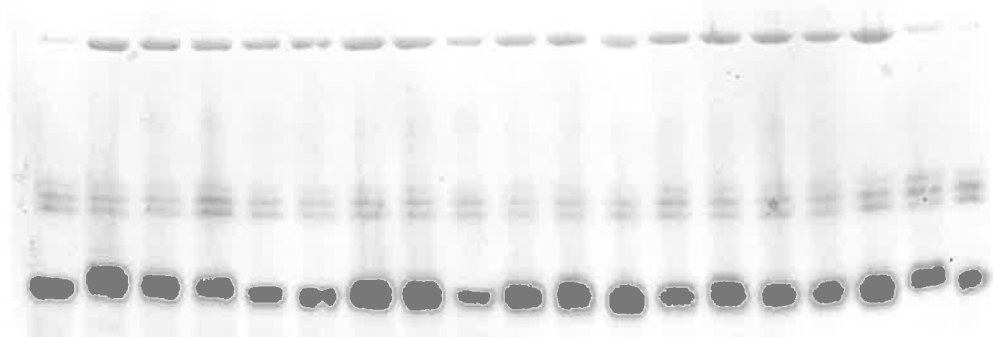
Table 2 shows the results obtained from screening the entire TC-F₁ population for GOT-2. Individual plants were classified based on their genomic configuration as deduced from GOT-2 zymogram phenotypes. About sixty-eight percent (288/423) of the TC-F₁ population proved to be monosomic for chromosome 6R, indicating that bivalents are frequently maintained in the F₁ until the first meiotic division. However, a significant proportion of plants were found to be nullisomic (21.75%) or disomic (10.16%) for chromosome 6R, indicating relatively high levels of asynapsis and/or desynapsis.

Chromosome 6D is present as a monosome in the F₁ (Figure 1A). Consequently, backcrossing to Chinese Spring will yield TC-F₁ individuals either monosomic or disomic for this chromosome. Female transmission of wheat monosomes is about 25% (Sears, 1952). Therefore, the proportion of TC-F₁ plants *disomic* for chromosome 6D should be about 25%. Within the TC-F₁ population, 36.64% of the individuals were observed to be disomic for chromosome 6D as determined by GOT-2 zymogram phenotypes (Table 2). This figure is higher than expected and may indicate preferential selection for chromosome 6D in female gametes.

Figure 4. Glutamate oxaloacetate transaminase zymogram phenotypes observed for a subpopulation of TC-F₁ plants. Band nomenclature follows Tang and Hart (1975) and is shown on the right. Band 4 (filled arrow, right) results from the association of subunits coded by the structural genes *Got-2A* and *Got-2B* with subunits coded by *Got-2R*. Band 1 (arrow head, right) results from the association only of subunits encoded by the structural gene *Got-2D* while band 5 (open arrow, right) results from the association only of subunits coded by the structural gene *Got-2R*. Intense staining for bands 1 and 5 indicate disomy for chromosomes 6D and 6R, respectively. Nomenclature for genomic configuration of TC-F₁ plants is described in Figure 1B. The genomic configuration scored for each TC-F₁ plant is shown (bottom). The following samples are included:

Lane 1: Chinese Spring
Lane 2: T701-4-6
Lane 3: TC-F₁-338
Lane 4: TC-F₁-339
Lane 5: TC-F₁-340
Lane 6: TC-F₁-341
Lane 7: TC-F₁-342
Lane 8: TC-F₁-343
Lane 9: TC-F₁-344
Lane 10: TC-F₁-345
Lane 11: TC-F₁-346
Lane 12: TC-F₁-347
Lane 13: TC-F₁-348
Lane 14: TC-F₁-349
Lane 15: TC-F₁-350
Lane 16: TC-F₁-351
Lane 17: TC-F₁-352
Lane 18: 6R^{T701}
Lane 19: Chinese Spring

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19



1 1 5 6 2 2 5 2 4 2 1 3 1 1 2

5
4
3
2
1

Table 2. GOT-2 screening. Genomic classification of TC-F₁ individuals. Nomenclature for genomic configuration is described in Figure 1B. Plants were scored for the presence or absence of GOT-2a, GOT-2d and GOT-2e.

Genomic Configuration	Number of Plants	Percent of TC-F ₁ Population
1. 20W''+6R'+6D'	179	42.32
2. 21W''+6R'	109	25.77
1+2	288	68.09
3. 20W''+6R''+6D'	22	5.20
4. 21W''+6R''	21	4.96
3+4	43	10.16
5. 20W''+6D'	67	15.84
6. 21W''	25	5.91
5+6	92	21.75
1+3+5	268	63.36
2+4+6	155	36.64
Total	423¹	100.00

¹ Sixteen out of 439 seeds failed to germinate.

7.2.3 Screening TC-F₁ population plants with group 6 RFLP and PCR markers

On the basis of GOT analysis, 288 seedlings were classified as being monosomic for chromosome 6R. These seedlings were transplanted and genomic DNA was extracted one week later. Four plants died prior to DNA extraction and DNA samples from two plants were lost during extraction. DNA was not re-extracted from these plants. Consequently, DNA samples were available for 282 *Got-R2*-positive individuals.

A total of 16 cDNA and five gDNA clones were localised subchromosomally to chromosome 6R^{T701} (Table 1, Chapter 6). However, of these, three gDNA clones and four cDNA clones were non-polymorphic between chromosome 6R^{T701} and chromosome 6R^{Imp}. A further two cDNA clones were hemizygous revealing RFLPs in chromosome 6R^{T701} only. In addition to the hemizygous and non-polymorphic clones, clones CDO497 and BCD269 were omitted from the linkage analysis due to likely difficulties in scoring (ie., differences in the size of RFLP bands observed between chromosome 6R^{T701} and chromosome 6R^{Imp} were small). Consequently, eight cDNA and two gDNA clones were selected for linkage mapping studies in the TC-F₁ population (Table 3). Of the seven PCR-based markers localised subchromosomally to chromosome 6R^{T701} (Chapter 6, Table 2), four were suitable for linkage mapping studies (Table 4). The primer combination PawS5/PawFG4 revealed two loci, *AawS5/FG4-1* and *AawS5/FG4-2* (Table 4) mapping to chromosome 6R^{T701} and 6R^{Imp}, respectively. Consequently, the TC-F₁ population was screened with a total of ten RFLP markers and five PCR-based markers.

It should be noted that the restriction enzyme *DraI* was used to map the clone TAM36. This probe/enzyme combination revealed two loci, *Xtam36A* and *Xtam36B* as determined via physical mapping studies. However, *DraI* did not reveal polymorphism between chromosome 6R^{T701} and chromosome 6R^{Imp} for *Xtam36A*. Consequently, this locus was not mapped genetically. In hindsight, the enzyme *HindIII* should have been used as this would have allowed both *Xtam36A* and *Xtam36B* to have been mapped.

Table 3. Homoeologous group 6 cDNA and gDNA RFLP clones used to screen the TC-F₁ population. The restriction enzymes used to digest DNA are listed for each clone.

Clone	Locus	Restriction Enzyme
AW15	<i>Xaw15</i>	<i>EcoRI</i>
BCD1	<i>Xbcd1</i>	<i>DraI</i>
BCD340	<i>Xbcd340</i>	<i>BamHI</i>
BCD758	<i>Xbcd758</i>	<i>DraI</i>
BCD1426	<i>Xbcd1426</i>	<i>BgIII</i>
CDO1380	<i>Xcdo1380</i>	<i>EcoRI</i>
WG933	<i>Xwg933</i>	<i>BgIII</i>
KSUF37	<i>XksuF37</i>	<i>BgIII</i>
TAM36	<i>Xtam36B</i>	<i>DraI</i>
PSR154	<i>Xpsr154</i>	<i>HindIII</i>

Table 4. PCR primer combinations used to screen the TC-F₁ population.

Primer Combination	Locus/Loci
PawS5/PawFG4	<i>AawS5/FG4-1</i> <i>AawS5/FG4-2</i>
PawS5/PawFG3	<i>AawS5/FG3a</i> <i>AawS5/FG3b</i>
PawC2	<i>AawC2</i>

Figure 5 shows the results obtained screening a sub-population of TC-F₁ plants with the PCR primer combination PawS5/PawFG4. Lanes 1 and 2 are 6R^{Imp} and 6R^{T701}, respectively. Of the plants shown, ten have the 450 bp band (ie., 6R^{T701} genotype) while 16 have the 400 bp band (ie., 6R^{Imp} genotype). Lane 7 shows a sample in which both the 400 bp and 450 bp bands have been amplified while lane 30 shows a sample in which neither the 6R^{Imp} nor 6R^{T701} bands have been amplified even though the PCR reaction appears to have worked efficiently, as judged by the other bands present in this lane. Of the 282 individuals within the TC-F₁ population, five were found to possess both bands while six possessed neither. These results suggest one of two possibilities. If the bands detected in 6R^{T701} and 6R^{Imp} by the primer combination PawS5/PawFG4 are allelic, then plants possessing both bands will be disomic for chromosome 6R while plants possessing neither bands will be nullisomic for chromosome 6R. Alternatively, if the bands detected by this primer pair are non-allelic, then the alternate phenotypes (ie., both bands or neither band) would indicate that recombination has occurred around these loci. The products generated with the primer pair PawS5/PawFG4 were scored as independent, dominant markers.

Figure 6 shows the results obtained screening a sub-population of TC-F₁ plants with the primer combination PawS5/PawFG3. Lanes 1 and 2 are 6R^{T701} and 6R^{Imp}, respectively. Lane 3 is a water control. Of the plants shown, 16 demonstrate the 6R^{Imp} phenotype (lanes 4, 5, 11, 12, 13, 14, 15, 16, 20, 21, 23, 25, 27, 28, 31 and 33), 11 demonstrate the 6R^{T701} phenotype (lanes 6, 7, 8, 9, 10, 17, 19, 24, 26, 30 and 32) while a single plant (lane 18) possess only the 280 bp band. The PCR failed for two samples (lanes 22 and 29). Of the 282 individuals within the TC-F₁ population, seven were observed to possess only the 280 bp band while eight possessed only the 170 bp band. These results indicate that the bands amplified with the primer combination PawS5/PawFG3 are not allelic.

Initial screening experiments with RFLP probes utilised membranes prepared using all 282 individuals. Four clones BCD758, BCD1, PSR154 and TAM36 were screened using these membranes. However, the DNA from 133 individuals was found to be degraded and not of

Figure 5. Screening TC-F₁ individuals with the primer combination PawS5/PawFG4. TC-F₁ plants included are listed (lanes 3-30). Amplification products were separated in 3% agarose gels. The size standard (M) is λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard are shown on the left in base-pairs. The arrows on the right indicate the 400 bp amplification product derived from chromosome 6R^{Imp} and the 450 bp amplification product derived from chromosome 6R^{T701}. Lanes 1-30 contained the following DNA samples:

Lane 1: 6R^{Imp}
Lane 2: 6R^{T701}
Lane 3: TC-F₁-121
Lane 4: TC-F₁-122
Lane 5: TC-F₁-123
Lane 6: TC-F₁-125
Lane 7: TC-F₁-126
Lane 8: TC-F₁-127
Lane 9: TC-F₁-128
Lane 10: TC-F₁-129
Lane 11: TC-F₁-130
Lane 12: TC-F₁-131
Lane 13: TC-F₁-132
Lane 14: TC-F₁-133
Lane 15: TC-F₁-134
Lane 16: TC-F₁-135
Lane 17: TC-F₁-136
Lane 18: TC-F₁-137
Lane 19: TC-F₁-138
Lane 20: TC-F₁-139
Lane 21: TC-F₁-140
Lane 22: TC-F₁-141
Lane 23: TC-F₁-142
Lane 24: TC-F₁-143
Lane 25: TC-F₁-145
Lane 26: TC-F₁-146
Lane 27: TC-F₁-148
Lane 28: TC-F₁-147
Lane 29: TC-F₁-149
Lane 30: TC-F₁-64

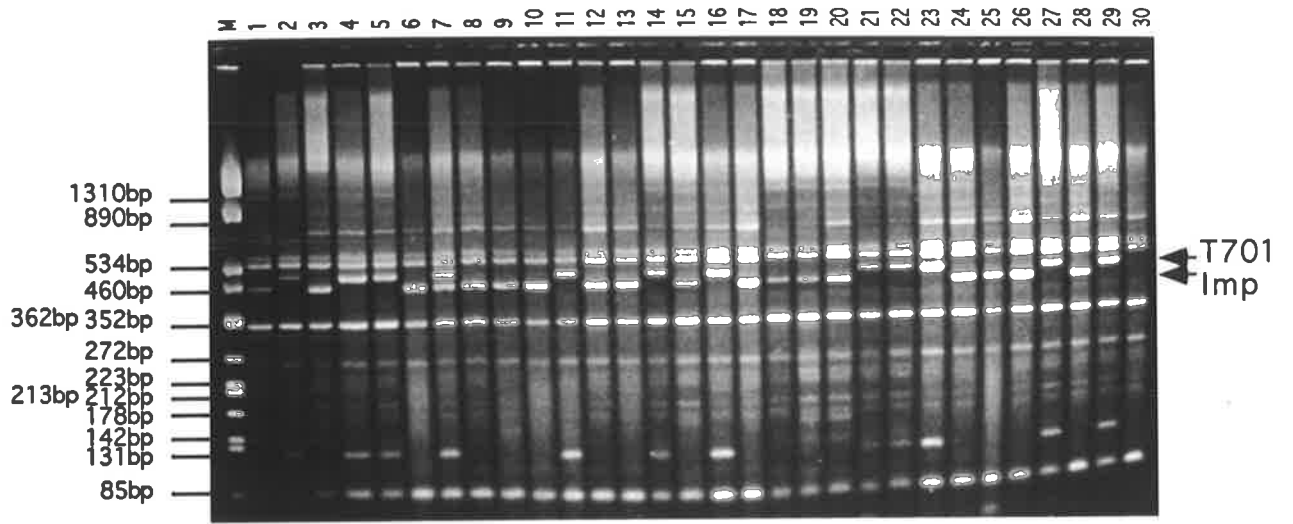
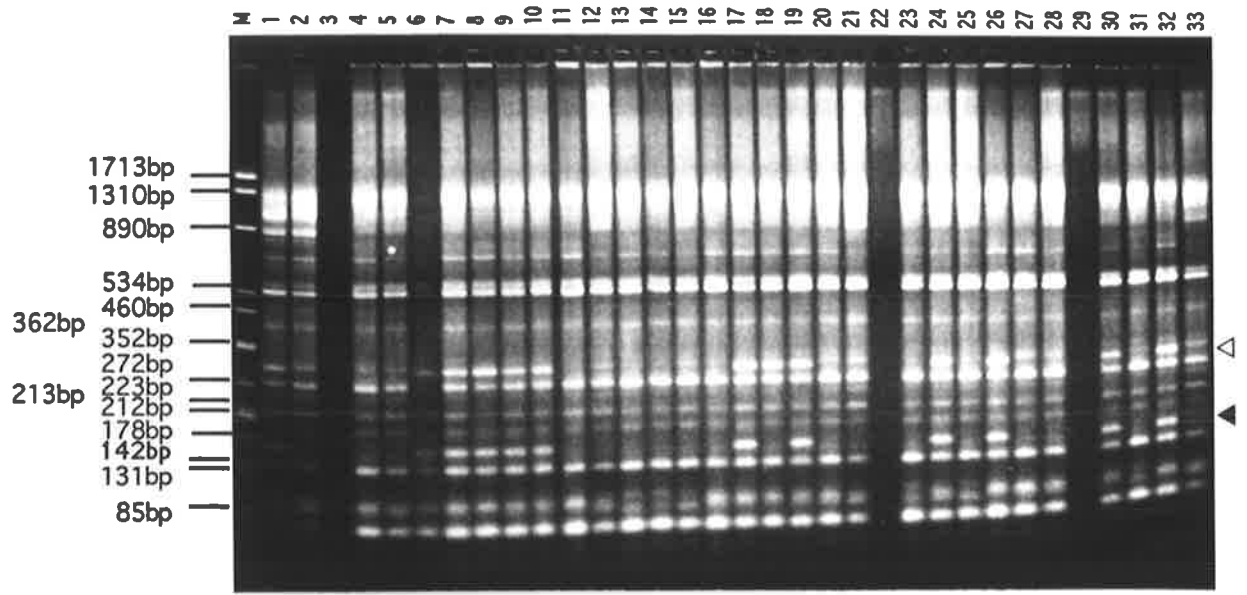


Figure 6. Screening TC-F₁ individuals with the primer combination PawS5/PawFG3. TC-F₁ plants included are listed (lanes 3-30). Amplification products were separated in 3% agarose gels. The size standard (M) is λ dv1 (Streeck and Hobom, 1975). The size of bands generated in the standard are shown on the left in base-pairs. Both the 280 bp band (open arrow, right) and the 170 bp band (filled arrow, right) are derived from 6R^{T701}. Lanes 1-33 contained the following DNA samples:

Lane 1: 6R^{T701}
Lane 2: 6R^{Imp}
Lane 3: ddH₂O
Lane 4: TC-F₁-273
Lane 5: TC-F₁-273
Lane 6: TC-F₁-274
Lane 7: TC-F₁-275
Lane 8: TC-F₁-276
Lane 9: TC-F₁-276
Lane 10: TC-F₁-277
Lane 11: TC-F₁-278
Lane 12: TC-F₁-279
Lane 13: TC-F₁-280
Lane 14: TC-F₁-281
Lane 15: TC-F₁-282
Lane 16: TC-F₁-283
Lane 17: TC-F₁-284
Lane 18: TC-F₁-285
Lane 19: TC-F₁-286
Lane 20: TC-F₁-287
Lane 21: TC-F₁-288
Lane 22: TC-F₁-289
Lane 23: TC-F₁-290
Lane 24: TC-F₁-291
Lane 25: TC-F₁-292
Lane 26: TC-F₁-293
Lane 27: TC-F₁-294
Lane 28: TC-F₁-295
Lane 29: TC-F₁-296
Lane 30: TC-F₁-297
Lane 31: TC-F₁-298
Lane 32: TC-F₁-301
Lane 33: TC-F₁-302

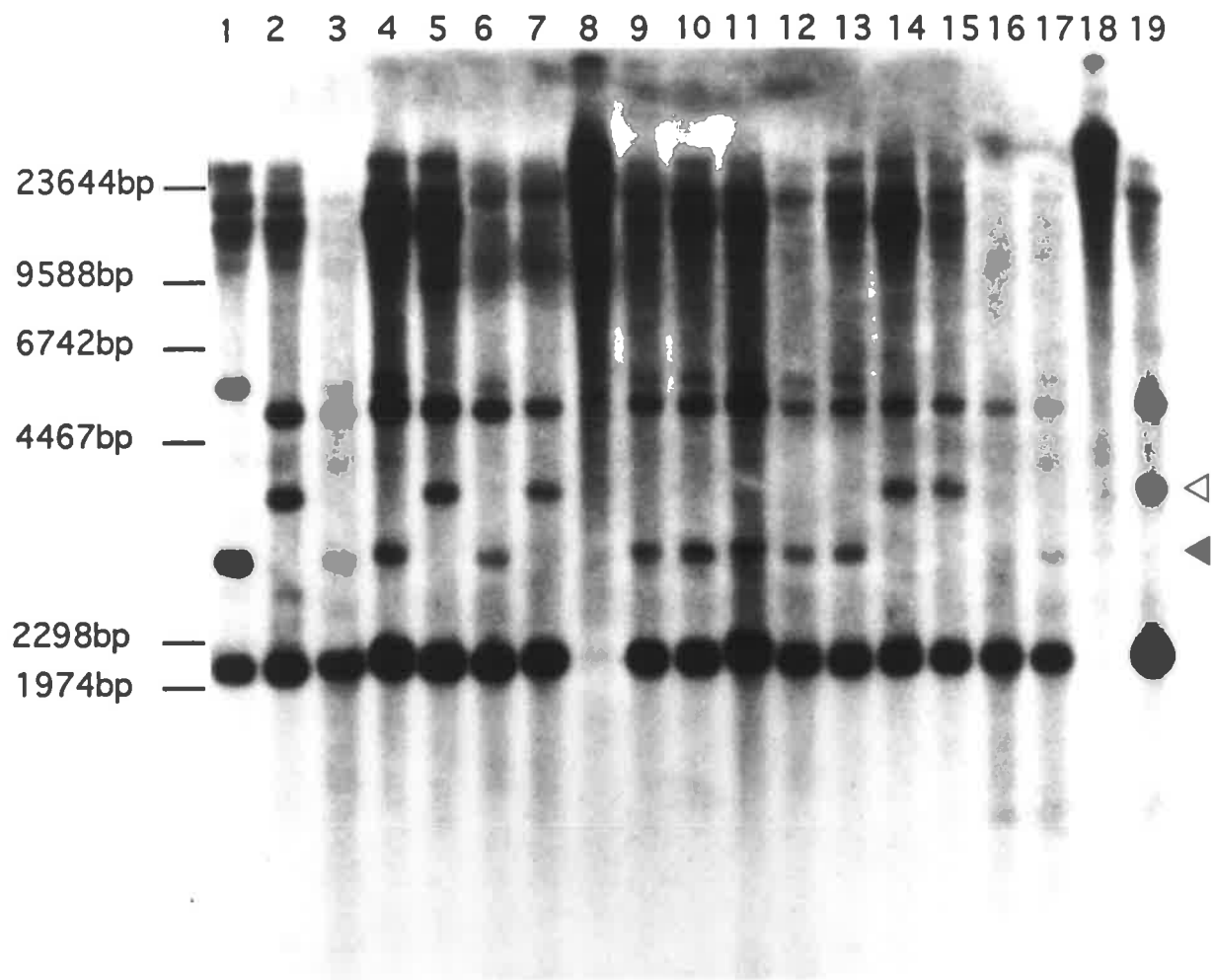


high enough quality for RFLP studies. Consequently, RFLP data could not be obtained for these individuals. Subsequent membranes were prepared using only DNA from the 149 individuals for which DNA quality was sufficiently high to enable analysis. The remaining clones AW15, KSUF37, WG933, CDO1380, BCD1426 and BCD340 were screened using these latter membranes. Figure 7 shows results from screening experiments using the clone AW15. Lanes 1 and 2 are $6R^{T701}$ and $6R^{Imp}$, respectively. Of the seventeen TC-F₁ individuals included, nine possess the $6R^{T701}$ allele (lanes 3, 4, 6, 9, 10, 11, 12, 13 and 17) while five possess the $6R^{Imp}$ allele (lanes 5, 7, 14, 15 and 19). Two samples were undigested (lanes 8 and 18). Of the 149 individuals screened with the clone AW15, one plant (# 91) possessed both the $6R^{T701}$ allele and the $6R^{Imp}$ allele suggesting disomy for chromosome 6R.

Of the RFLP probes used, difficulties were encountered with the clone PSR154. While the band(s) mapped in rye cv. Imperial were of equivalent intensity to those mapped in wheat, bands mapping to either chromosome $6R^{T701}$ or chromosome $6R^{Imp}$ were of reduced intensity. In addition, a faint band was observed in both wheat cultivars Chinese Spring and Schomburgk which was of similar size to the band mapping to chromosome $6R^{Imp}$. As a result, phenotypes were determined conservatively for 105 individuals. The clone KSUF37 also presented problems due to the weakness of intensity of signal. Consequently, phenotypes were recorded conservatively for only 45 individuals. Of interest, phenotypes for all ten RFLP clones screened were obtained for plant TC-F₁-91. In every instance, this individual was found to possess both the $6R^{T701}$ allele and the $6R^{Imp}$ allele confirming that this plant was disomic for chromosome 6R. A second plant, TC-F₁-94 possessing both the 400 bp and 450 bp amplification products derived from the primer pair PawS5/PawFG4 was also found to possess both $6R^{T701}$ and $6R^{Imp}$ alleles for clones BCD758 and BCD1 indicating that this plant was also disomic for chromosome 6R. Therefore, data derived from individuals TC-F₁-91 and TC-F₁-94 was omitted from linkage analysis.

Figure 7. Screening TC-F₁ individuals with the clone AW15. Genomic DNA was digested with the restriction enzyme *EcoRI*. TC-F₁ plants included are listed (lanes 3-19). The size standard was λ *HindIII*. The open arrow on the right indicates the 3160 bp band mapped to chromosome 6R^{Imp} while the filled arrow indicates the 2690 bp band mapped to chromosome 6R^{T701}. Lanes 1-19 contained the following DNA samples:

Lane 1: 6R^{T701}
Lane 2: 6R^{Imp}
Lane 3: TC-F₁-1
Lane 4: TC-F₁-11
Lane 5: TC-F₁-21
Lane 6: TC-F₁-23
Lane 7: TC-F₁-24
Lane 8: TC-F₁-27
Lane 9: TC-F₁-28
Lane 10: TC-F₁-29
Lane 11: TC-F₁-30
Lane 12: TC-F₁-31
Lane 13: TC-F₁-32
Lane 14: TC-F₁-35
Lane 15: TC-F₁-42
Lane 16: TC-F₁-45
Lane 17: TC-F₁-51
Lane 18: TC-F₁-78
Lane 19: TC-F₁-80



7.2.4 CCN bioassay

7.2.4.1 Selection of TC-F₁ population plants

Since it was impossible to undertake large-scale screening for *CreR* within the TC-F₁ population, a strategy was devised in order to genetically map this locus via the selection and screening of individuals demonstrating recombination around the *CreR* locus. The gross order of markers has been established from physical mapping studies described in Chapter 6. Consequently, the order: [*Xbcd758*, *Xpsr154*][*AawS5/FG4-1*, *AawS5/FG4-2*, *Xbcd340*, *Xcdo1380*, *Xbcd933*, *Xbcd1426*, *Xaw15*, *Xbcd1*][*XksuF37*][*AawS5/FG3b*, *AawS5/FG3a*, *Xtam36B*] was assumed. It must be stressed that the genetic order of loci mapped physically to the same subchromosomal region (ie., loci within parentheses) with the exception of loci *AawS5/FG3b* and *AawS5/FG3a* as shown is putative since linkage analysis of the data set was not carried out at this stage. Loci *AawS5/FG3b*, *AawS5/FG3a* could be ordered relative to each other through the visual inspection of the data set.

CreR has been physically localised to the same subchromosomal region as *Got-R2* (Dundas *et al.*, 1992). RFLP loci *Xbcd340*, *Xcdo1380*, *Xbcd933*, *Xbcd1426*, *Xaw15* and *Xbcd1* and PCR loci *AawS5/FG4-1* and *AawS5/FG4-2* have also been localised to this subchromosomal region. Recombination events occurring between loci flanking this region (ie., *Xbcd758* and *AawS5/FG3b*) will also be most likely to be informative for the *CreR* locus. Marker phenotypes for all 280 plants within the TC-F₁ population were examined and, based upon the order described above, plants demonstrating recombination distal of *Xbcd758* and plants demonstrating recombination proximal of *AawS5/FG3b* were identified. Where possible, individuals possessing information for both PCR and RFLP markers were selected. However, a number of individuals were selected on the basis of disassociation of PCR loci *AawS5/FG4-1* and *AawS5/FG4-2* with loci *AawS5/FG3a* and *AawS5/FG3b* only. The individuals selected and their marker phenotypes are shown in Table 5. A total of 12 TC-F₁ plants were selected, of which six possessed phenotype information for both RFLP and PCR loci while the remaining six plants were selected on phenotype information derived from PCR loci only.

Table 5. Phenotypes of PCR and RFLP markers scored for a sub-population of TC-F₁ plants. Nomenclature used for coding phenotypes scored is described in Chapter 2, Section 2.21. Plants were selected based upon the identification of putative cross-over events. Locus order, the number of putative cross-over events and their position is based upon physical mapping data and visual inspection of marker phenotype data.

Plant	Loci ¹														Number of putative crossover events
	<i>Xbcd</i> 758	<i>Xpsr</i> 154	<i>AawS5/</i> <i>FG4-1</i>	<i>AawS5/</i> <i>FG4-2</i>	<i>Xbcd</i> 340	<i>Xcdo</i> 1380	<i>Xwg</i> 933	<i>Xbcd</i> 1426	<i>Xaw</i> 15	<i>Xbcd1</i>	<i>XksuF</i> 037	<i>AawS5/</i> <i>FG3b</i>	<i>AawS5/</i> <i>FG3a</i>	<i>Xtam36</i>	
TC-F ₁ -99	-	a	a	a	-	-	-	-	-	-	-	h	h	h	1
TC-F ₁ -116	a X	h	h	h	h	-	h	h	h	-	-	h	h	h	1
TC-F ₁ -124	-	-	a	a	-	-	-	-	-	a X	-	h	h	-	1
TC-F ₁ -158	-	-	h	h	-	-	-	-	-	-	-	a	a	-	1
TC-F ₁ -186	-	-	h	h	-	-	-	-	-	-	-	a	a	-	1
TC-F ₁ -209	-	-	a	a	-	-	-	-	-	-	-	h	h	-	1
TC-F ₁ -210	a	a X	h	h X	a	a	a	a	a	a	a X	h	h	h	3
TC-F ₁ -254	-	a X	h X	a	-	-	-	-	-	a	-	a	a X	h	3
TC-F ₁ -260	-	-	a	a	-	-	-	-	-	-	-	h	h	-	1
TC-F ₁ -274	h	h	h	h	-	-	-	-	-	-	-	a	a	-	1
TC-F ₁ -284	-	-	h	h	-	-	-	-	-	a	-	a	a	-	1
TC-F ₁ -295	a	-	a	a	a	a	a	a X	h	h	h	h	h	-	1

¹The order of loci is from centromere to telomere, left to right. Loci mapping to the same subchromosomal region are shown grouped within the table. The order of loci within each grouping is putative, since linkage analysis was not undertaken at this stage.

X = Indicates the position of putative cross-over events.

The phenotypes of a number of informative individuals indicates the likely order of three loci relative to others within the same subchromosomal region. For example, individual TC-F₁-295 indicates that loci *Xaw15* and *Xbcd1* should map distal of all other loci within this subchromosomal region (Table 5). Marker phenotypes of a number of individuals indicates that the locus *Xtam36B* should map distal of both *AawS5/FG3b* and *AawS5/FG3a* while data from individual TC-F₁-116 would indicate that *Xpsr154* should map distal of *Xbcd758* (Table 5). Based on the assumed locus order, the marker phenotypes of ten individuals could be completely accounted for by a single cross-over event. While the position of the event cannot be accurately inferred for all ten individuals, a single cross-over event can account for the marker phenotypes observed irrespective of the *actual* order of loci *within* each subchromosomal region. However, the marker phenotype information obtained for individuals TC-F₁-210 and TC-F₁-254 cannot be easily accounted for. For both individuals, a minimum of three cross-over events is required to account for the marker phenotypes based on the assumed locus order. Significantly, it is impossible to reduce the number of cross-over events by re-ordering loci within subchromosomal regions. If the marker phenotypes for loci *AawS5/FG4-1* and *AawS5/FG4-2* are correct for both individuals, these loci cannot be allelic.

The 12 selected plants (Table 5) were grown on in the glasshouse, heads were bagged thereby generating TC-F₂ seed. Seed was germinated and TC-F₂ seedlings screened for *Got-R2*. Table 6 shows the results from *Got-R2* screening of TC-F₂ progeny. A germination rate of 100% was observed for all seed generated. However, in families where the number of seedlings exceeded 20, a sub-set of (≤ 20) seedlings were selected for screening. No chromosome 6R-positive TC-F₂ individuals were isolated from individual TC-F₁-260. Transmission of chromosome 6R therefore ranged from 0.00% (TC-F₁-260) to 50.00% (TC-F₁-158). In general, higher transmission rates were observed for plants possessing the genomic configuration 20W''+6R'+6D' (Table 6).

Table 6. GOT-2 screening of progeny plants from selected TC-F₁ individuals. For each plant, the number of seedlings tested is shown along with the number testing positive for *Got-R2*. The percentage of progeny seedlings testing positive for *Got-R2* is shown in parentheses. The genomic configuration determined in initial screening of the TC-F₁ population for GOT-2 is shown for each TC-F₁ individual.

Plant	# Seedlings Tested	# Seedlings <i>Got-R2</i> Positive	Genomic Configuration
BC-F ₁ -99	17	4 (23.53%)	20W''+6R'+6D'
BC-F ₁ -116	20	4 (20.00%)	20W''+6R'+6D'
BC-F ₁ -124	15	4 (26.67%)	20W''+6R'+6D'
BC-F ₁ -158	20	10 (50.00%)	20W''+6R'+6D'
BC-F ₁ -186	19	2 (10.53%)	21W''+6R'
BC-F ₁ -209	20	6 (30.00%)	20W''+6R'+6D'
BC-F ₁ -210	12	1 (8.33%)	21W''+6R'
BC-F ₁ -254	17	4 (23.53%)	20W''+6R'+6D'
BC-F ₁ -260	4	0 (0.00%)	21W''+6R'
BC-F ₁ -274	20	5 (25.00%)	21W''+6R'
BC-F ₁ -284	9	1 (11.11%)	20W''+6R'+6D'
BC-F ₁ -295	19	5 (26.32%)	20W''+6R'+6D'

7.2.4.2 Screening of selected TC-F₂ population plants

Based on the GOT-2 results, sib-plants with and without chromosome 6R were selected. All *Got-R2* positive progeny seedlings identified were selected along with five to six *Got-R2* negative sib-lines. These plants were assayed for CCN resistance as described in Chapter 2, Section 2.4. A completely randomised design was used. Also included in this assay were wheat cultivars Chinese Spring, Egret and Schomburgk (susceptible), two resistant *T. tauschii* accessions Alg/809-6 and L18913, rye cultivars Imperial and Vila Pouca (unknown), triticale T701-4-6 (resistant), Chinese Spring-T701 6R(-6D) disomic substitution line (resistant) and Chinese Spring-Imperial 6R disomic addition line (susceptible). The chromosome 6RL deletion lines were also included in the bioassay. Deletion lines del6RL22, del6RL21 and del6RL1807 were screened for the presence or absence of the isozymes of *Got-R2* to confirm the presence of 6RL chromatin in seedlings to be assayed (ie., *Got-R2*-negative sib-lines were included in the bioassay for del6RL1807 only). Since the locus encoding *Got-R2* has been localised to the chromosome segment bordered by the breakpoints in deletion lines del6RL1801 and del6RL1807 (Dundas *et al.*, 1992), PCR markers generated using the primer combinations AawC2 and AawS5/AawS14 were used to screen for sib-plants with and without this deletion chromosome since both markers had been mapped proximal to the breakpoint in deletion line del6RL1801. Only seedlings testing positive for both markers were included in the bioassay.

The results of CCN bioassays conducted on the cultivars listed above are shown in Table 7. The number of seedlings tested for each line/cultivar is listed as is the mean number of female nematodes and the standard deviation for each group. Although the number of individuals tested was small, control plants with the whole 6R chromosome had few or no female nematodes per plant while susceptible control plants of cultivars Schomburgk and Egret had high numbers of females present. The wheat cultivar Chinese Spring had fewer than expected females. However, the absence of or low incidence of females (viz. 1 or 2) on a healthy plant known to have a segment of 6R rye chromatin was considered as evidence for the presence of

Table 7. Number of white female nematodes on the roots of TC-F₂ individuals with and without chromosome 6R derived from selfed TC-F₁ plants selected based upon putative recombination events. Number of white female nematodes on the roots of deletion mutants of rye chromosome 6R^{T701}, control susceptible and resistant lines and rye cultivars Vila Pouca and Imperial are also shown.

Line	Number of <i>H. avenae</i> females per plant (Mean \pm SD ¹)	Number of seedlings tested	Range of <i>H. avenae</i> females
<i>Control lines:</i>			
Schomburgk	20.00 \pm 6.81	6	13-28
Egret	8.67 \pm 3.14	6	4-13
Chinese Spring	4.80 \pm 2.59 ²	5	1-8
Alg/809-6	2.33 \pm 3.38	6	0-8
L18913	0.17 \pm 0.41	6	0-1
Imperial Rye	0.83 \pm 1.33	6	0-3
Portuguese Rye	1.83 \pm 3.25	6	0-8
T701-4-6	0.00 \pm 0.00	2	0
Chinese Spring - Imp. 6R	3.33 \pm 2.58	6	1-7
Chinese Spring - T701 6R	1.00 \pm 0.00	1	1
<i>Sib-lines \pm 6R chromatin:</i>			
del6RL22			
+ deletion chromosome	0.80 \pm 0.84	5	0-2
- deletion chromosome	NT	0	
del6RL21			
+ deletion chromosome	0.00 \pm 0.00	6	0
- deletion chromosome	NT	0	
del6RL1807			
+ deletion chromosome	5.17 \pm 3.25	6	2-10
- deletion chromosome	7.67 \pm 2.88	6	4-12
del6RL1801			
+ deletion chromosome	8.83 \pm 6.55	6	1-20
- deletion chromosome	NT	0	

Table 7. cont.

Line	Number of <i>H. avenae</i> females per plant (Mean \pm SD)	Number of seedlings tested	Range of <i>H. avenae</i> females
<i>Sib-lines \pm 6R chromatin:</i>			
BC-F ₂ -99			
+ 6R	0.25 \pm 0.50	4	0-1
- 6R	1.50 \pm 0.84*	6	1-3
BC-F ₂ -116			
+ 6R	1.25 \pm 2.50	4	0-5
- 6R	3.00 \pm 2.97	6	0-7
BC-F ₂ -124			
+ 6R	0.50 \pm 0.57	4	0-1
- 6R	9.83 \pm 4.71	6	4-15
BC-F ₂ -158			
+ 6R	0.10 \pm 0.32	10	0-1
- 6R	1.38 \pm 2.13*	8	0-6
BC-F ₂ -186			
+ 6R	0.00 \pm 0.00	2	0
- 6R	4.60 \pm 1.95	5	2-6
BC-F ₂ -209			
+ 6R	3.00 \pm 2.83	5	1-7
- 6R	6.33 \pm 3.56	6	1-11
BC-F ₂ -210			
+ 6R	0.00 \pm 0.00	1	0
- 6R	3.60 \pm 3.21	5	0-8
BC-F ₂ -254			
+ 6R	0.00 \pm 0.00	4	0
- 6R	3.33 \pm 2.88	6	0-8
BC-F ₂ -274			
+ 6R	0.00 \pm 0.00	5	0
- 6R	7.33 \pm 6.31	6	1-16
BC-F ₂ -284			
+ 6R	1.00 \pm 0.00	1	1
- 6R	7.17 \pm 6.31	6	3-10
BC-F ₂ -295			
+ 6R	6.00 \pm 4.53	5	3-14
- 6R	9.17 \pm 6.19	6	3-17

¹SD=Standard deviation.

²Mean number of white female nematodes observed on Chinese Spring was compared with the mean number of white female nematodes observed on chromosome 6R-deficient lines TC-F₂-99, TC-F₂-116 and TC-F₂-158 using Students t-test.

*Significant at P \leq 0.05.

NT=Not tested.

the resistance gene. Both *T. tauschii* accessions were observed to have low numbers of females although L18913 appeared to demonstrate a stronger degree of resistance than Alg/809-6. Significantly, both rye cultivars Imperial and Vila Pouca were observed to have low numbers of female nematodes per plant suggesting the presence of hitherto previously unknown resistance to CCN.

As expected, deletion lines del6RL22 and del6RL21 demonstrated high levels of resistance with mean numbers of females of 0.80 and 0.00 respectively (Table 7). However, in contrast to previous results (Dundas *et al.*, 1992), a mean number of 5.17 females was recorded for the deletion line del6RL1807 indicating susceptibility. The deletion line del6RL1801 was observed to be susceptible with a mean number of 8.83 females in agreement with previous results (Dundas *et al.*, 1992). This result is significant since it indicates that the *CreR* locus maps more distal than previously shown (Dundas *et al.*, 1992). Of the progeny plants derived from the selected TC-F₁ individuals, the CCN status can be determined for eight of the 11 families screened (Table 7). TC-F₂ progeny derived from TC-F₁ individuals 124, 186, 254 and 274 are clearly resistant while TC-F₂ progeny derived from TC-F₁ individuals 209 and 295 are clearly susceptible. A single plant possessing chromosome 6R was isolated for CCN screening for individuals TC-F₁-210 and TC-F₁-284. No females were found on plant TC-F₂-210 while a single female nematode was observed on plant TC-F₂-284. Chromosome 6R-deficient sib-plants derived from both families possessed mean numbers of females comparable to Chinese Spring (3.60 and 7.17 respectively). Consequently, individuals TC-F₁-210 and TC-F₁-284 could be scored as resistant.

The difference between the mean number of females observed on Chinese Spring wheat and chromosome 6R-deficient TC-F₂ progeny derived from TC-F₁-99 and TC-F₁-158 individuals was found to be significant at $P \leq 0.05$ for both families (Table 7). Consequently, the bioassay failed for this group of plants. In contrast, the difference between the mean number of female nematodes observed on Chinese Spring wheat and chromosome 6R-deficient TC-F₂ progeny derived from individual TC-F₁-116 was found to be not significant indicating that the bioassay

had been successful for this group of plants. Consequently, the difference between the mean number of females observed on TC-F₂-116 (+6R) individuals and TC-F₂-116 (-6R) individuals was tested and found to be not significant ($P \leq 0.05$). Therefore, individual TC-F₁-116 is likely to be susceptible. However, the marginal nature of these results (ie., the requirement for statistical analyses) led to their exclusion in genetic linkage analysis.

7.2.5 Linkage analysis

7.2.5.1 Segregation distortion

Distorted segregation of markers was determined using the chi-square test. Due to the nature of the crosses used in the generation of the TC-F₁ population, segregation of all markers should be restricted to 1:1 (female). Consequently, this hypothesis was tested at all loci using a single locus goodness-of-fit test ($df=1$; Table 8). The results were calculated using; 1) the complete data set (ie., 280 individuals) and; 2) an amended data set which included only individuals for which PCR *and* RFLP or *CreR* phenotype data is available (ie., 145 individuals). Three loci (*AawC2*, *Xtam36B* and *Xbcd1426*) demonstrated segregation distortion in both data sets (Table 8). Segregation of all other markers fitted the expected 1:1 ratio.

7.2.5.2 Construction of a genetic linkage map of chromosome 6R

A genetic map of the long arm of chromosome 6R was constructed for a sub-set of the TC-F₁ mapping population (see Chapter 2, Section 2.21) using the computer programme JoinMap (Stam, 1993). As with most commonly available computer programmes for genetic mapping, JoinMap was conceived for studies of inbreeding species in which genetic markers follow either an "F₂"(3:1 or 1:2:1) or "BC" (1:1)-type segregation. In the TC-F₁ population used, segregation of all markers is 1:1. Consequently, this data set can be coded within the JoinMap programme as a BC-type. Maps generated via the analysis of the amended data set (145 individuals) and the complete data set (280 individuals) are shown in Figure 8 and Appendix

Table 8. Phenotypic frequencies for PCR and RFLP markers mapped in the TC-F₁ population. Figures shown are for the complete data set of 280 individuals and the amended data set of 145 individuals. χ^2 values shown in parentheses are for the amended data set.

Locus	Expected	Observed				χ^2 -values
		Complete		Amended		
		6R ^{T701}	6R ^{Imp}	6R ^{T701}	6R ^{Imp}	
<i>AawS5/FG4-1</i>	1:1	148	131	76	69	1.036 (0.338)
<i>AawS5/FG4-2</i>	1:1	151	128	77	68	1.896 (0.559)
<i>AawC2</i>	1:1	78	146	53	77	20.643** (4.431*)
<i>AawS5/FG3a</i>	1:1	142	131	72	68	0.443 (0.114)
<i>AawS5/FG3b</i>	1:1	144	129	74	66	0.824 (0.457)
<i>Xbcd758</i>	1:1	60	42	60	41	3.176 (3.574)
<i>Xpsr154</i>	1:1	48	58	48	57	0.943 (0.771)
<i>Xaw15</i>	1:1	46	34	-	-	1.800
<i>XksuF37</i>	1:1	25	20	-	-	0.556
<i>Xwg933</i>	1:1	48	35	-	-	2.036
<i>Xcdo1380</i>	1:1	48	32	-	-	3.200
<i>Xtam36B</i>	1:1	12	77	9	76	47.472** (52.812**)
<i>Xbcd1426</i>	1:1	44	26	-	-	4.696*
<i>Xbcd1</i>	1:1	56	45	56	44	1.198 (1.440)
<i>Xbcd340</i>	1:1	44	30	-	-	2.649
<i>CreR</i>	1:1	54	40	-	-	2.085

**Significant at P≤0.01.

*Significant at P≤0.05.

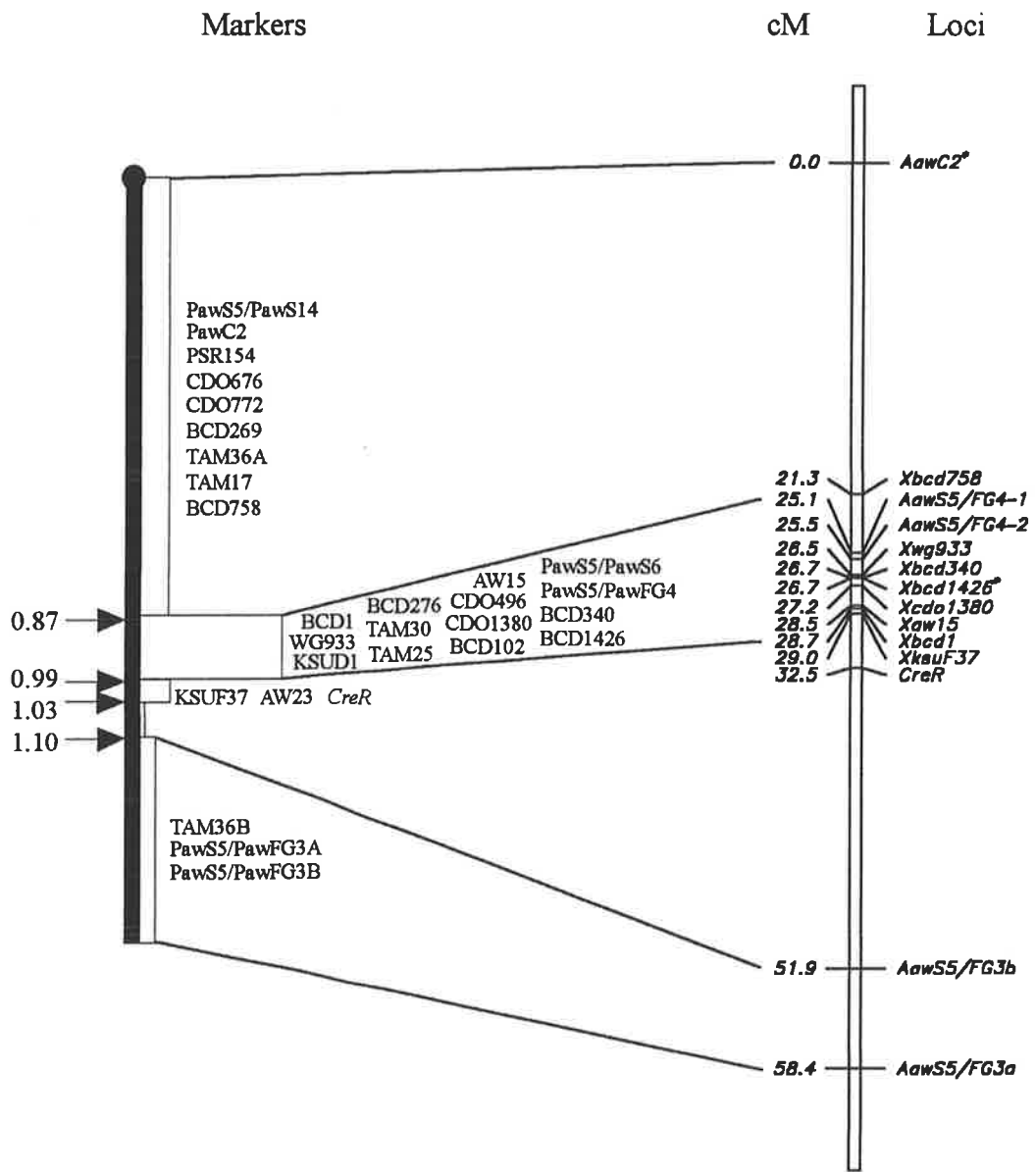
-Phenotypic frequencies unchanged in the amended data set.

3D, respectively. Both data sets yielded maps with similar locus orders. In both maps the relative position of loci *AawC2*, *Xbcd758*, *Xaw15*, *Xbcd1*, *XksuF37*, *AawS5/FG3b* and *AawS5/FG3a* is the same. However, differences are observed in the order of loci mapping distal of *AawS5/FG4-1* and proximal of *Xaw15*.

Figure 8 shows the most likely map derived from the amended data set of 145 individuals. Initial analysis included all 15 marker loci and the locus conferring resistance to *H. avenae*, *CreR*. Fifteen loci (including *CreR*) were placed by JoinMap into a single linkage group; the locus *Xtam36B* was a singleton, being placed into a second linkage group. This locus segregated in a dominant fashion and demonstrated segregation distortion in both the complete and amended data sets. As was observed in initial analysis of the complete data set (Appendix 3A), *Xpsr154* was localised adjacent to *Xaw15*. This result contradicts physical mapping data. Attempts to impose the expected order based on physical mapping results using the JoinMap option "fixed sequences" were unsuccessful in overruling JoinMap. Consequently, this marker was removed from further analysis. The final locus order as depicted in Figure 8 is in complete agreement with the order expected from physical mapping studies.

In contrast to the map derived from the complete data set (Appendix 3D), the order derived from the amended data indicates that *AawS5/FG4-2* maps 0.4 cM distal of *AawS5/FG4-1* (Figure 8). Significantly, two crossover events must have occurred, one between loci *Xbcd758-AawS5/FG4-1* and the other between loci *AawS5/FG4-2-Xwg933* in order to account for the phenotypes scored for loci *AawS5/FG4-1* and *AawS5/FG4-2* in TC-F₁-210. Since genotype information for markers mapping *genetically* proximal of *AawS5/FG4-1* is unavailable for TC-F₁-254 (ie., phenotype information for *Xpsr154* has been deleted), only a single crossover event between loci *AawS5/FG4-1* and *AawS5/FG4-2* can be deduced with certainty. Deleting the genotype information for loci *AawS5/FG4-1* and *AawS5/FG4-2* for individuals TC-F₁-210 and TC-F₁-254 resulted in *AawS5/FG4-1* and *AawS5/FG4-2* mapping to the same locus and the rearrangement of loci *Xcdo1380*, *Xbcd1426*, *Xwg933* and *Xbcd340* (Appendix 4A).

Figure 8. Genetic linkage map of the long arm of chromosome 6R and its comparison with the physical map of the long arm of chromosome 6R^{T701}. The breakpoints of the four chromosome 6RL^{T701} deletions, along with their respective arm ratios are indicated by arrows on the left of the physical map. The genetic linkage map includes five PCR markers, eight RFLP markers and the cereal cyst nematode resistance gene, *CreR*. An amended data set of 145 individuals from the TC-F₁ population was used. Linkage groups were established with a LOD \geq 3.0 and a maplod \geq 1.0. Loci demonstrating distortion segregation are indicated by an *asterisk* following the locus name (P \leq 0.05). The map position of locus *AawC2* was forced using the "fixed sequences" option as described in Chapter 2, Section 2.21.



The bioassay results obtained using the chromosome 6RL^{T701} deletion lines (Table 7) indicate that *CreR* maps to the same subchromosomal region as the clone KSUF37. Linkage analysis places the *CreR* locus 3.5 cM distal of the locus *XksuF37* (Figure 8). Consequently, both data are in agreement. *CreR* phenotypes for lines TC-F₁-210 and TC-F₁-284 were assigned based upon the reaction of a single TC-F₂ (+6R) plant (Table 7). As a result, the phenotypic assignment for these two lines cannot be regarded as conclusive. However, deleting the *CreR* phenotypes for either or both TC-F₁ lines had a minimal affect on the map position of this locus (data not shown). Specifically, deleting this information for line TC-F₁-210 resulted in *CreR* being placed 3.6 cM distal of *XksuF37*, an increase of 0.1 cM. Deleting the *CreR* phenotype information for line TC-F₁-254 resulted in *CreR* being placed 3.3 cM distal of *XksuF37*, a decrease of 0.2 cM. This was also the case when phenotype information was deleted for both TC-F₁ lines.

7.3 DISCUSSION

7.3.1 The development of a TC-F₁ population segregating for *CreR*

The methodology for generating homozygous recombinant substitution lines (RSL) as described by Joppa (1991) was utilised in this study to allow the development of a TC-F₁ population for chromosome 6R of rye using the Chinese Spring-T701-4-6 6R(-6D) substitution line and the Chinese Spring-Imperial 6R addition line. The Chinese Spring-T701-4-6 6R(-6D) substitution was crossed as a male to the Chinese Spring-Imperial 6R disomic addition producing F₁ plants with the genomic constitution 20W"+6R^{T701}+6R^{Imp}+6D'. PCR analysis with the primer combination PawS5/PawFG4 was used in conjunction with *in situ* hybridisation to metaphase cells using the rye-specific dispersed repetitive DNA sequence pAW173.1 to verify hybridisation between these two lines.

The unbalanced genomic composition of the F₁ plants used in the crosses led to the generation of TC-F₁ progeny with six possible genomic configurations; 1) 20W+6R'+6D'; 2) 21W"+6R'; 3) 20W"+6R"+6D'; 4) 21W"+6R"; 5) 20W"+6D' and; 6) 21W". Rather than undertake the

tedious task of selecting TC-F₁ progeny monosomic for chromosome 6R using cytogenetic methods, the TC-F₁ population was screened using GOT-2 since it was expected that differentiation between plants disomic for chromosome 6R and plants monosomic for chromosome 6R would be possible based on relative intensity of the GOT-2e isozyme band. While no attempt was made to quantify the intensity of any of the isozyme bands observed in acrylamide gels, it was found that bands GOT-2a and GOT-2e appeared to be absent for plants monosomic for chromosomes 6D and 6R, respectively when inspected visually.

Two hundred and eighty-eight plants (68.09%) were classified as being monosomic for chromosome 6R based upon their GOT-2 isozyme phenotype (ie., genomic configurations 1 and 2) and genomic DNA was prepared from 282 of these plants. Screening with RFLP and PCR markers identified two plants disomic for chromosome 6R while using PCR data alone, three additional putative disomic plants and six putative nullisomic plants were identified. Misclassification of TC-F₁ plants based upon GOT-2 isozyme phenotypes therefore ranged between 0.71% (2/282) and 3.90% (11/282). The relative age of the leaf tissue, amount of tissue extracted and amount of extract loaded in each lane are factors likely to be influencing the accuracy of this method. However, classification of the dosage of chromosome 6R based upon GOT-2 isozymes appears to be a relatively robust and efficient technique.

The dosage of chromosome 6D for TC-F₁ progeny was also ascertained via GOT-2 screening. Of the 423 plants screened, 268 (63.36%) were classified as being monosomic for chromosome 6D. In contrast, 155 (36.64%) were classified as being disomic for this chromosome. Female transmission of wheat monosomes is about 25% (Sears, 1952). Consequently, the figure of 36.64% derived from GOT-2 screening is higher than expected even if one assumes an error rate similar to that observed for chromosome 6R. Therefore, these observations may indicate differential selection for chromosome 6D and hence, selection against chromosome 6R in female gametes.

7.3.2 CCN bioassay

The relatively high number of female nematodes per plant on Schomburgk and the variation from 13-28 females per plant suggest that this was a reliable assay. O'Brien *et al.* (1980) reported 30-60 females per plant on the susceptible wheat variety Halberd. However, these authors employed an inoculation regime in which increasing number of juveniles were applied at 0, 7 and 21 days. In the current study, individual seedlings were inoculated with approximately 100 juveniles at the time of planting and then four times thereafter at alternating intervals of three and four days. Although only two individuals of triticale T-701-4-6 and a single Chinese Spring-T701-4-6 6R(-6D) disomic substitution individual were available for screening, both cultivars were clearly resistant with 0 and 1 females per plant, respectively.

The mean of 4.8 female nematodes observed on Chinese Spring wheat individuals was fewer than expected. Variability in the response of this wheat cultivar to *H. avenae* has been observed previously. Asiedu (1986) observed lower than expected mean number of cysts on Chinese Spring and suggested that slow early growth of this cultivar and consequent slower root production may have contributed in limiting support for growth of females or the number of available infection sites. Alternatively, the observations in this study may indicate reduced efficiency of the bioassay for other reasons such as variation in the genetic constitution of larvae (Asiedu, 1986).

Previous results indicate that the *CreR* locus maps to the same subchromosomal region as *Got-R2* (Dundas *et al.*, 1992). In the study of Dundas *et al.* (1992), deletion lines del6RL22, del6RL21, and del6RL1807 were found to be resistant while the deletion line del6RL1801 was susceptible. In contrast, CCN bioassays carried out on the chromosome 6R^{T701} long arm deletion lines in the current study indicate that *CreR* maps to the more distal subchromosomal segment defined proximally by the breakpoint in deletion line del6RL1807 and distally by the breakpoint in del6RL21. The bioassay results for all four deletion lines are unequivocal. For each deletion line, at least five chromosome 6R-proficient individuals were tested while for

del6RL1807, six chromosome 6R-deficient individuals were also tested. The mean number of females observed on del6RL1801 of 8.83 indicates susceptibility while mean numbers of 0.80 and 0.00 of females for lines del6RL22 and del6RL21, respectively, indicate the presence of the resistance gene, *CreR*. The mean number of females observed on chromosome 6R-proficient sib-lines derived from del6RL1807 was 5.17. This figure is not significantly different from the number observed on chromosome 6R-deficient individuals (7.67; $P \leq 0.05$). Importantly, the observation of a high mean number of females on chromosome 6R-deficient sib-lines derived from del6RL1807 is an indication of the reliability of this bioassay.

The inconsistencies observed between the CCN bioassays reported here and those reported by Dundas *et al.* (1992) are difficult to explain. However, Dundas *et al.* (1992) conducted CCN bioassays on newly-derived chromosome 6RL^{T701} deletion stocks whereas the deletion lines used herein had been subjected to backcrossing and selfing for several generations. Interestingly, similar results involving bioassays with advanced lines containing the deletion chromosome del6RL1807 as well as a telocentric deletion line and a translocation line derived from del6RL1807 have been observed previously (I. Dundas, personal communication). Recent experiments using selfed and F₁ progeny derived from the original isolate containing the deletion chromosome del6RL1807 indicate the presence of two different types of deletion chromosome within this line which differ, at least, by the *CreR* locus (I. Dundas, personal communication). Given the poor resolution of the C-banding pattern of rye chromosome 6R, it would be difficult to identify such deletions.

CCN bioassay results were unequivocal for eight of the 11 TC-F₁ lines selected. For two lines the bioassay was deemed to have failed as judged by the lower than expected mean number of females observed on chromosome 6R-deficient sib-lines. Although the mean number of females observed on chromosome 6R-deficient sib-lines derived from TC-F₁-116 was not significantly different from the Chinese Spring control, the inability to determine the *CreR* status of this line as a result of the indeterminate mean number of cysts observed on chromosome 6R-proficient sib-lines led to its exclusion from genetic linkage analysis.

A potentially new source of resistance to *H. avenae* has been identified. The mean number of females observed on rye cv. Vila Pouca was low (1.83) indicating putative resistance. Of the six plants tested, four were found to have no females present while two plants had three and eight cysts respectively indicating possible segregation of the locus or loci responsible. Rye cv. Vila Pouca is a land-race variety from Portugal and as such has not been extensively inbred (H. Geudes-Pinto, personal communication). Strong resistance to *H. avenae* was also identified in rye cv. Imperial. However, genetic control of this resistance must be different from that observed in the triticales T701-4-6 since it is not localised to chromosome 6R as judged by the results obtained using the Chinese Spring-Imperial 6R disomic addition line. This line was found to be susceptible, as expected although with a lower than expected mean of 3.33 females per plant. This is likely to be the result of the Chinese Spring background present in this line.

7.3.3 A genetic linkage map of the long arm of chromosome 6R of rye

The genetic linkage map of the long arm of chromosome 6R of rye presented includes eight RFLP loci, five PCR loci and the locus *CreR* and spans approximately 60 cM. With the exception of the locus *Xpsr154*, there are no markers in common with the chromosome 6RL linkage map presented and the maps generated by Devos *et al.* (1993b) and Philipp *et al.* (1994). Consequently, a direct comparison between these linkage maps is not possible.

Importantly, two of the four deletion breakpoints in the chromosome 6RL^{T701} deletion lines have been characterised with respect to flanking loci. The breakpoint in deletion line del6RL1801 is flanked proximally by *Xbcd758* and distally by *AawS5/FG4-1* while the breakpoint in deletion line del6RL1807 is flanked proximally by *Xbcd1* and distally by *XksuF37*. Genetically, loci *Xbcd758* and *AawS5/FG4-1* mapped 3.8 cM apart while loci *Xbcd1* and *XksuF37* mapped 0.3 cM apart. The remaining two breakpoints were not characterised due to the inability to identify clones mapping to the region of chromosome

6RL^{T701} defined proximally by the breakpoint in del6RL21 and distally by the breakpoint in del6RL22.

7.3.3.1 RFLP loci

The locus *Xtam36B* demonstrated extreme distortion segregation. Of the 89 individuals typed, 12 revealed the 6R^{T701} allele while 77 revealed the 6R^{Imp} (ie., null) allele. It was clear from the phenotypic data that chromosomal exchange involving the locus *Xtam36B* had occurred between chromosome 6R^{T701} and chromosome 6R^{Imp} since four TC-F₁ individuals were observed to have 6R^{Imp} phenotypes for all loci proximal of *Xtam36B* but the 6R^{T701} phenotype for *Xtam36B*. In contrast, the reciprocal exchange was difficult to ascertain since this locus was inherited in a dominant fashion with the 6R^{Imp} allele being null. The dominant PCR loci *AawS5/FG3b* and *AawS5/FG3a* also mapped physically to the distal third of the long arm of chromosome 6R and are in coupling with *Xtam36B*. While high levels of recombination were observed between these loci and more proximal RFLP loci (ie., *Xaw15-AawS5/FG3b*; 24.4% and *Xaw15-AawS5/FGa*; 27.3%), neither locus demonstrated distorted segregation. It is unclear then whether individuals possessing 6R^{T701} phenotypes for loci proximal of *Xtam36B* but the 6R^{Imp} phenotype for this locus are true recombinants or, represent 6R^{T701} chromosomes with terminal deletions. It is possible, for example, that the morphological differences observed between chromosome 6R^{T701} and chromosome 6R^{Imp} may lead to chromosomal aberrations during meiosis and hence, be the cause of the segregation distortion observed for *Xtam36B*. Alternatively, since *Xtam36B* may result from cross-hybridisation, the observed segregation distortion of this locus may be the result of poor hybridisation.

7.3.3.2 PCR loci

Rogowsky *et al.* (1992b) generated PCR markers for the short arm of rye chromosome 1R also using primers derived from the junction of R173 elements. The PCR products identified

were found to be polymorphic between rye cultivars Imperial, Petkus and King II. Linkage mapping revealed that at least the King II and Petkus products were not allelic, mapping approximately 18.2 cM apart. In this study, PCR loci *AawS5/FG3a* and *AawS5/FG3b* were amplified using the primer combination PawS5/PawFG3. Both loci mapped to chromosome 6R^{T701} and were separated by a genetic distance of 6.5 cM. PCR loci *AawS5/FG4-1* and *AawS5/FG4-2* were amplified using the primer combination PawS5/PawFG4 and were mapped to chromosome 6R^{T701} and chromosome 6R^{Imp}, respectively. However, these loci mapped less than 1 cM apart. These results also indicate that PCR markers derived from R173 elements are not allelic, in agreement with previous observations (Rogowsky *et al.*, 1992b).

In the present study individuals TC-F₁-210 and TC-F₁-254 were observed to be critical in determining the map positions derived for loci *AawS5/FG4-1* and *AawS5/FG4-2*. The reliability of the primer combination PawS5/PawFG4 was found to be extremely high. However, since reactions were not repeated for these individuals, it remains possible that the phenotypes scored for loci *AawS5/FG4-1* and *AawS5/FG4-2* are erroneous either as a result of reaction failure in the case of individual TC-F₁-254 or, due to sampling errors in the case of TC-F₁-210. In order to account for the phenotypic information observed for these two loci in individuals TC-F₁-210 and TC-F₁-254, recombination events between loci *Xbcd758* and *AawS5/FG4-1* and *AawS5/FG4-2* and *Xwg933* must have occurred in the case of individual TC-F₁-210. In individual TC-F₁-254, a recombination event must have occurred between loci *AawS5/FG4-1* and *AawS5/FG4-2* while a second recombination event is inferred to have occurred proximal of *AawS5/FG4-1* as a result of the phenotype scored for *Xpsr154*.

Under the assumption of no interference, the product of the recombination frequencies observed between two pairs of loci approximates the probability of a double recombination event around the loci in question. Consequently, using the direct recombination frequencies calculated between loci *Xbcd758* and *AawS5/FG4-1* and *AawS5/FG4-2* and *Xwg933* (ie., 0.05941%, 0.01205%, respectively), the probability of a double recombination event which would account for the chromosome type observed in TC-F₁-210 is 0.00072. While such a

figure argues against such an occurrence, it is my opinion that the phenotypic data scored for individuals TC-F₁-210 and TC-F₁-254 for loci *AawS5/FG4-1* and *AawS5/FG4-2* are correct. Therefore, this data was retained.

7.3.3.3 Map construction

During map construction, JoinMap calculates a goodness-of-fit value which allows an assessment of the integrity of individual markers. A large increase in the chi-square value calculated after the inclusion of any single marker indicates "internal friction" or non-conformity (Stam, 1993). Large increases were not observed in the present analysis using either the complete or amended data sets. However, initial analysis using the programme JoinMap with LOD ≥ 3.0 and maplod ≥ 0.05 resulted in an ordering of loci which conflicted with the order established via physical mapping studies. Specifically, loci *AawC2* and *Xbcd758* and loci *AawS5/FG3b* and *AawS5/FG3a* were inverted with respect to the remaining loci. Also, the map position derived for *Xpsr154* (ie., proximal to *Xaw15*) conflicted with the physical mapping data.

The inability to force the locus *Xpsr154* into a position consistent with physical mapping data using the JoinMap option "fixed sequences" indicates that the genotype data conflicts with the forced sequences used. The scoring of this marker was observed to be complicated by an RFLP band in wheat which co-migrated with the RFLP band observed in 6R^{Imp}. Although *Xpsr154* did not demonstrate distortion segregation, it is likely that a number of individuals were mis-typed for this marker. Such inconsistencies in the data could then lead to an incorrect ordering of this and other loci through false double recombinants. Indeed, deletion of *Xpsr154* from the data set resulted in the correct placement (relative to the order expected from physical mapping studies) of loci. Previous linkage mapping studies place *Xpsr154* approximately 30 cM from the centromere (Devos *et al.*, 1993b). Consequently, the results of Devos *et al.* (1993b) and the physical mapping data presented in this thesis suggest that the

genetic map position of *Xpsr154* derived in this study is incorrect. This marker was removed from further analysis.

7.3.3.4 Factors affecting locus order

Increasing the maplod value from 0.05 to 1.0 resulted in a reordering of some loci. Using the option "fixed sequences" it was possible to force *AawC2* back to the position originally derived using a maplod value ≥ 0.05 . Increasing the maplod to 1.0 results in the exclusion of weak linkage information (ie., recombination values between loci which are slightly less than 50%; P. Stam, personal communication). Since the LOD values calculated by JoinMap are used as weights in mapping calculations, the maplod value has the potential to influence both locus order and map distances between loci. Data sets which are internally consistent and possess sufficient "anchor" loci are relatively insensitive to the maplod value (P. Stam, personal communication). Therefore, the changes in the order of loci observed within the chromosome 6RL linkage map as a result of increasing maplod values suggest inconsistencies in the data.

Loci *AawC2* and *Xbcd1426* demonstrated segregation distortion. Furthermore, *AawC2* was scored as a dominant marker. Both segregation distortion and the mode of inheritance influence the derivation of LOD values via maximum likelihood methods and, as a consequence, locus order (Lorieux *et al.*, 1995; P. Stam, personal communication). Deleting *Xbcd1426* from the data set had no effect on the order of remaining loci. Consequently, it is likely that inconsistencies observed in locus order due to increasing maplod values are largely due to *AawC2*. Philipp *et al.* (1994) reported distortion segregation for chromosome 6R markers, although they did not specify which markers or the chromosome region involved. It is possible that segregation distortion observed for *AawC2* is due to linkage with lethal or sublethal genes present on chromosome 6RL. However, dominant RAPD markers have been observed to demonstrate non-conformity with Mendelian segregation (Carlson *et al.*, 1991; Echt *et al.*, 1992; Reiter *et al.*, 1992; Bucci and Menozzi, 1993). Work by Heun and Helentjaris (1993) indicates that this phenomenon is the result of the combined probabilities

associated with the competitive nature of annealing and subsequent extension of product under specific conditions. Other critical components of the PCR reaction such as the temperature profiles and annealing temperatures have also been implicated (Ellsworth *et al.*, 1993; Penner, 1993b). In the case of *AawC2*, the majority of individuals were observed to possess the null (ie., 6R^{Imp}) allele. Since this marker is the result of amplification using a single primer, it is comparable to dominant RAPD markers. Consequently, it is conceivable that PCR using the primer PawC2 will be influenced in a manner similar to RAPD markers. Segregation distortion observed for *AawC2* is probably the result of failed reactions.

7.3.3.5 Map distances and robustness

In calculating map distances, JoinMap uses a least squares procedure which utilises the LOD values calculated between individual pairs of loci as weights to derive a "joint best estimate" of recombination frequency (P. Stam, personal communication). Consequently, specific linkages will contribute more to the derivation of map distances between particular pairs of loci. In the present data set, a direct recombination frequency of 0.00% was observed between loci pairs *Xwg933-Xbcd340*, *Xwg933-Xbcd1426*, *Xbcd340-Xbcd1426*, *Xbcd1426-Xcdo1380*, *Xaw15-Xbcd1*, *Xaw15-XksuF37*, *Xaw15-CreR*, *Xbcd1-XksuF37*, *Xbcd1-CreR* and *XksuF37-CreR*. These loci form two clusters or blocks of loci comprising loci *Xwg933*, *Xbcd340*, *Xbcd1426* and *Xcdo1380* in the proximal group and *Xaw15*, *Xbcd1*, *XksuF37* and *CreR* in the distal group. The two groups of loci were separated by a number of recombination events and can clearly be ordered relative to each other. For example, the direct recombination frequency observed between loci *Xcdo1380-Xaw15* was 2.532%. However, recombination between loci *within* either group was not observed. Using the procedure outlined, JoinMap derives a "joint best estimate" recombination frequency greater than zero for all pairs of loci listed above. As a result, only loci *Xbcd340-Xbcd1426* were genetically mapped to the same position.

The physical and genetic map positions for loci *Xbcd758*, *Xaw15*, *Xbcd1*, *XksuF37*, *AawS5/FG3b*, *AawS5/FG3a* and *CreR* are completely consistent. Moreover, the relative

order of loci *Xbcd758*, *Xaw15*, *Xbcd1*, *XksuF37*, *AawS5/FG3b*, *AawS5/FG3a* and *CreR* remained unchanged irrespective of the maplod value used, indicating a high level of stability within the data set for these loci. Consequently, these loci, perhaps with the exception of *CreR* can be classified as "anchor" loci. However, it is possible that the relative order of loci within each group as presented in Figure 8 may be at odds with the true order of these loci. For example, in the case of *CreR*, it is possible that this locus may map proximal of *XksuF37*. Therefore, the map position of this locus is not conclusive since the map distances (and locus order) calculated by JoinMap between loci with a true recombination frequency of 0.00% are, to some extent arbitrary. Moreover, locus order and map distances are a function of the quality of the data (Lorieux *et al.*, 1995) and it is clear that inconsistencies exist for certain markers within the data set. Therefore, both the locus order and map distances for loci mapping within either of the two clusters of loci described should be viewed as preliminary.

7.3.4 Chromosome pairing and recombination

Pairing between chromosomes 6R^{T701} and 6R^{Imp} at meiosis was observed in F₁ plants selected for further crosses, although no attempt was made to establish the frequency or nature (chiasmate or nonchiasmate) of the meiotic associations between these two chromosomes. About 68% of TC-F₁ plants isolated were monosomic for chromosome 6R and hence, had apparently undergone normal assortment of that chromosome. This figure is likely to be an underestimate due to possible gametic selection against chromosome 6R in the F₁.

Pairing and formation of chiasmata in rye chromosomes is greatly reduced in wheat-rye derivatives (Benavente and Orellana, 1989). In addition, heterozygosity for interstitial and terminal C-heterochromatin has been shown to reduce the frequency of chiasmata, possibly due to differences in chiasmata localisation patterns (Naranjo and Lacadena, 1980; Orellana and Giraldez, 1981). Moreover, major structural differences between homologues, such as translocations, have also been shown to reduce levels of meiotic pairing (Sybenga, 1990). Significantly, reduced MI pairing of rye chromosomes in wheat-rye derivatives has been shown

to affect chiasma distribution and preferential pairing of rye chromosomes when in competition (Benevente and Orellana, 1992). Consequently, the structural differences between chromosomes 6R^{Imp} and 6R^{T701} observed in this study may act to further reduce chiasma frequency and possibly chiasma distribution.

In wheat and rye, recombination frequency has been shown to increase exponentially with the physical distance from the centromere. In addition, the distribution of recombination is dependant upon the physical length of the chromosome arm (Lukaszewski, 1992; Lukaszewski and Curtis, 1993). Since the orientation of loci within the linkage group is known, an assessment of recombination frequency along the long arm of chromosome 6R is possible. Excluding the locus *XawC2*, which demonstrates segregation distortion, the proximal two thirds of the chromosome arm has a recombinational length of only 7.4 cM. In contrast, the distal third of the arm has a recombinational length of 29.4 cM. While these figures may to some extent be artefactual due to the selection criteria used for identifying clones and the methodology employed by JoinMap in calculating map distances, they nevertheless indicate that the recombination rate is much greater in the distal third of the long arm of chromosome 6R.

CHAPTER 8

GENERAL DISCUSSION

The homoeologous genome relationships found within the Triticeae, particularly those of wheat, rye and barley are well documented (Miller, 1984; Bothmer and Jacobsen, 1985; Naranjo *et al.*, 1987; Naranjo and Fernández-Rueda, 1991). Significantly, the more recent application of RFLP markers within this tribe has expanded and refined our understanding of these relationships. Such studies have allowed the degree of synteny shared by specific homoeologous chromosomes to be established via the generation of genetic and in some cases, physical maps (Gill *et al.*, 1993; Namuth *et al.*, 1994; Hohmann *et al.*, 1995; Sherman *et al.*, 1995). Numerous loci have been chromosomally localised in rye (Melz *et al.*, 1992). However, the genetic linkage map of rye remains less developed than the maps of other diploid cereals such as barley (Shin *et al.*, 1990; Heun *et al.*, 1991; Kleinhofs *et al.*, 1993) or *T. tauschii* (K. S. Gill *et al.*, 1991*a, b*; Lagudah *et al.*, 1991). Chromosomes 1R, 2R, 3R and 5R have been studied in relative detail and genetic linkage maps utilising RFLP markers have been generated (Wang *et al.*, 1991; Devos *et al.*, 1992; Devos and Gale, 1993; Devos *et al.*, 1993*a, b*; Plaschke *et al.*, 1993; Philipp *et al.*, 1994). However, with the exception of the genetic linkage map of Devos *et al.* (1993*b*), information for chromosome 6R is limited.

Structural rearrangements involving rye chromosomes 6R, 3R and 7R has resulted in the translocation of segments of chromosomes 3RL and 7RL to the distal end of 6RL (Koller and Zeller, 1976; Miller, 1984; Schlegel *et al.*, 1986; Benito *et al.*, 1991; Naranjo and Fernández-Rueda, 1991; Devos *et al.*, 1993*b*). Consequently, there is a good understanding of the departures from homoeology observed between rye chromosome 6RL and the long arm of wheat group 6 chromosomes. In contrast, little is known regarding the amount of ancestral 6RL remaining or the degree of synteny shared between this and other homoeologous group 6 chromosomes. This study was primarily concerned with an observed lack of homoeologous pairing between rye chromosome 6R and wheat chromosome 6D. Since chromosomal

rearrangements are likely to have an adverse affect on homoeologous pairing, a series of molecular tools were developed to enable the structural analysis of chromosome 6RL.

A cytogenetic molecular marker capable of identifying very small introgressions of rye chromatin in wheat was developed (Chapter 3). Non-isotopic ISH using the clone pAW173.1 established that the R173 family of sequences is dispersed on all 7 rye chromosomes. However, this family of sequences was shown to be absent from the telomeres of some rye chromosomes, notably 1RL, 2RS and 6RS and in reduced copy number at centromeric regions.

Recently, genomic *in situ* hybridisation (GISH) techniques appear to have become the preferred method for detecting introgressions of rye chromatin into wheat (Moore *et al.*, 1993; Miller *et al.*, 1994; Islam-Faridi and Mujeeb-Kazi, 1995). The clone pAW173.1 was used to characterise a number of 1RS-1DS and 1RL-1DL recombinant chromosomes. This analysis clearly demonstrated the ability of this probe to identify rye euchromatin in a wheat background. These studies indicate that ISH studies using cloned sequences possess a number of distinct advantages over GISH studies. For example, the characterisation of hybridisation of pAW173.1 to rye chromosome 1R allowed the identification of the presence of a small intercalary segment of rye euchromatin in the 1RL-1DL recombinant chromosome, R1. This would not have been possible had GISH methods been used. However, in more general terms, cloned sequences allow more specific questions to be asked regarding cereal chromosome structure.

While R173 allowed a physical image of rye chromosomes to be developed, an indication of the genetic organisation was provided by RFLP and PCR markers. A series of homoeologous group 6 RFLP probes were selected and assessed. Probes were screened across genomic DNA from wheat cultivars Chinese Spring and Schomburgk, rye cv. Imperial, the Chinese Spring-Imperial 6R disomic addition line and the Chinese Spring-T701-4-6 6R (-6D) disomic substitution line (Chapter 4). Consequently, clones mapping to chromosome 6R (6R^{T701} and/or 6R^{Imp}) were readily identified as were clones revealing polymorphism between

chromosome 6R^{T701} and chromosome 6R^{Imp}. A high proportion of the homoeologous group 6 probes selected were localised to rye chromosome 6R indicating considerable homoeology with wheat and barley group 6 chromosomes.

The generation of PCR-based markers for cereal genomes has relied heavily on the use of RAPD technology (Kleinhofs *et al.*, 1993; Philipp *et al.*, 1994; Francis *et al.*, 1995; Hohmann *et al.*, 1995). In contrast, this study has utilised the approach of Rogowsky *et al.* (1992b) in the use of primers derived from the flanking regions of R173 elements (Chapter 5). The results presented confirm the findings of Rogowsky *et al.* (1992b). Significantly, these results indicate that primers derived from other families of rye-specific, dispersed repetitive sequences may also provide an extremely rich source of PCR markers. Other approaches to the development of PCR markers were utilised with varying degrees of success. Primers derived from regions within R173 elements led to the generation of markers for chromosome 6RL, although at greatly reduced efficiency. Surprisingly, primers derived from the barley cDNA clone AW15 failed to amplify in rye. It is unclear whether this was due to a breakdown in sequence homology or deletion/insertion events affecting primer binding sites. It would seem that characterisation of the polymorphism identified in Southern hybridisation is required prior to the design of primers.

A physical map of the long arm of chromosome 6R^{T701} was generated using the long arm deletion lines described by Dundas *et al.* (1992) (Chapter 6). This allowed the comparative mapping of rye and wheat group 6 chromosomes via a comparison with the consensus physical map of wheat homoeologous group 6 chromosomes of Gill *et al.* (1993). As a result, an interstitial inversion within rye 6RL relative to wheat was identified. Gill *et al.* (1993) reported an interstitial inversion in wheat 6DL relative to chromosomes 6AL and 6BL. However, evidence presented in this study indicates that the events identified in rye and wheat are distinct.

A number of homoeologous group 6 probes used in this study have been previously localised to the distal 10% of wheat group 6 chromosomes (Gill *et al.*, 1993). Physically, these probes were localised to a small interstitial region, residing in the proximal two-thirds of the long arm of chromosome 6RL (Chapter 6). This result is significant for two reasons. Firstly, it indicates that the segments of chromosomes 3RL and 7RL present at the distal end of 6RL comprise some 30% of the present-day long arm of chromosome 6R. However, most importantly this result indicates that as much as 90% of ancestral 6RL remains on the present-day 6RL arm. Devos *et al.* (1993b) speculated that a small segment of chromosome 6RL had been lost as a result of the rearrangements with chromosomes 3RL and 7RL. Results presented here indicate that if this is the case, then no more than 10% of 6RL has been lost.

In order to further elucidate the structure of chromosome 6RL, a genetic linkage map was constructed for this chromosome arm using a sub-population of molecular markers. Consequently, a Test-Cross F₁ population was generated using the Chinese Spring-T701-4-6 6R (-6D) disomic addition line and the Chinese Spring-Imperial 6R disomic addition line (Chapter 7). This population was screened with the isozyme marker GOT-2. Differences observed in the intensity of isozyme bands resulting from the products encoded by the structural genes *Got-R2* and *Got-D2* proved to be a reliable and accurate means of determining the status of chromosomes 6R and 6D within these plants. As a result, individual plants possessing a single rye chromosome 6R homologue were selected without cytological analysis. These plants were screened using a total of 10 RFLP and 5 PCR markers. Progeny testing of selected TC-F₁ individuals has allowed *CreR*, the locus conferring resistance to CCN to be incorporated into the genetic linkage map also.

The order of loci established genetically conforms to the physical order derived using the chromosome 6RL^{T701} deletion lines (Chapter 7). Ten loci were mapped to a region spanning 7.7 cM (Chapter 7). These loci formed two groups. Recombination was observed *between* these two groups, thereby allowing their ordering relative to the centromere. However, recombination between loci *within* either group was not observed. The genetic order of loci

shown is a function of the algorithms used by the computer programme, JoinMap (Stam, 1993) and should therefore be regarded as preliminary. While speculative, these observations may indicate the presence of a recombination "hot spot". However, rye chromosomes rarely pair in a wheat background in the presence of *Ph1*, the major pairing suppressor gene (Riley and Chapman, 1958; Sears and Okamoto, 1958). Therefore, reduced levels of recombination are to be expected in the F₁ individuals from which the TC-F₁ population was derived. Also, recombination in these individuals may have been further reduced as a result of the morphological differences observed between chromosome 6R^{Imp} and chromosome 6R^{T701}.

Clearly, the TC-F₁ mapping population derived in this study will not allow the generation of a high density linkage map of the long arm of rye chromosome 6R. Since a more accurate genetic linkage map will be required in any future attempt to isolate the CCN locus, a more suitable population such as the F₂ rye population described by Devos *et al.* (1993b) should be sourced and screened using the markers generated in this study. An analysis using such a population would also allow further characterisation of the interstitial inversion identified.

The results presented in this thesis provide a more detailed understanding of the molecular structure of the long arm of chromosome 6R than previously available. Notably, an interstitial inversion has been identified in the more proximal region of this chromosome arm. Naranjo and Fernández-Rueda (1991) observed extremely low levels of homoeologous pairing between chromosome 6RL and the long arm of wheat group 6 chromosomes. Significantly, these workers observed chromosome 6RL to pair most frequently with the long arm of wheat group 7 chromosomes. While the presence of segments of rye chromosomes 3RL and 7RL at the distal end of 6RL is likely to interfere with pairing between 6RL and wheat group 6 chromosomes, it is also likely that the internal inversion present in chromosome 6RL relative to the long arm of wheat group 6 chromosomes is contributing to the extremely low levels of pairing observed between these chromosomes. It may therefore be possible to improve the prospects for homoeologous recombination between rye 6RL and the long arm of wheat group 6 chromosomes through the use of deletion chromosomes devoid of chromosome 3RL and

chromosome 7RL segments. Furthermore, while the interstitial inversion identified in rye chromosome 6RL differs from that identified in chromosome 6DL, chromosome 6DL should be the target chromosome for introgression due to the likely increased synteny relative to chromosomes 6AL and 6BL.

Both the physical and genetic data concerning *CreR* are in agreement and indicate that this locus maps more distally than previously shown (Dundas *et al.*, 1992). Importantly, a number of molecular markers showing linkage to *CreR* have been identified in this study. Specifically, the locus *Xksuf37* was mapped 3.5 cM distal of *CreR*. Since it is doubtful that *CreR* will ever be introgressed into wheat via homoeologous recombination, future studies should be directed towards the physical localisation and cloning of this locus. For example, it should be possible, using the chromosome 6RL deletion lines to saturate the segment of 6RL carrying *CreR* with additional molecular markers. A high-resolution linkage map of this region could then be constructed using the markers identified. High-resolution linkage maps have been generated in a number of plant species around various disease resistance loci (Messeguer *et al.*, 1991; Gebhardt *et al.*, 1993), and are essential to the identification of tightly linked flanking markers. Importantly, the generation of such maps represent the first step in any attempt to clone the locus of interest. The study of Gill *et al.* (1993) indicates that for wheat group 6 chromosomes, 1 cM translates into approximately 0.44-172 Mb. Similar variability in the relationship of genetic and physical distances are anticipated for rye chromosome 6R. Consequently, it is important that the region of 6RL carrying the *CreR* locus be mapped both genetically and physically.

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Appendix 1

Ratios of positions of C-bands and deletion breakpoints on chromosome 6R relative to the length of the short arm of chromosome 6R. Markers A to G refer to features on chromosome 6R^{T701} indicated in Figure 5. Numbers in parentheses indicate the number of chromosomes analysed for each measurement. Data kindly provided by Dr. I. Dundas.

Chromosome	Physical Markers							6RL Breakpoint	Percentage 6RL deleted
	A	B	C	D	E	F	G		
Whole 6R	0.52)0.021 (15)	0.42)0.032 (14)	0.63)0.034 (15)	0.83)0.031 (15)	1.06)0.034 (15)	1.23)0.041 (15)	1.48)0.047 (15)	N/A	0.0%
del6RL22*	-	0.36)0.030 (13)	0.58)0.032 (20)	0.84)0.035 (20)	1.06)0.042 (20)			1.10	25.7%
del6RL21	0.53)0.017 (19)	0.42)0.024 (19)	0.61)0.027 (19)	0.83)0.031 (19)	1.03)0.034 (19)			1.03	30.4%
del6RL1807	0.54)0.013 (9)	0.41)0.036 (7)	0.60)0.027 (9)	0.83)0.028 (9)				0.99	33.1%
del6RL1801	0.46)0.018 (14)	0.43±0.000(2)	0.65)0.036 (14)	0.87)0.034 (14)				0.87	41.2%

N/A Not Applicable.

- Not measured.

* Ratios from raw data multiplied by a factor of 0.716 to adjust for an apparent interstitial deletion on the short arm of this chromosome type.

Appendix 2

1

population_label

280 15

AawS5/FG4-1 HxA

AAHAN	AHANH	AAAHN	AHANH	AAAAA	HHHHH	HAANA	HANAH	AAHAN
HHHAA	AAHAN	HHHAA	AHHAN	AHAAN	AHHAA	AAAAA	AAHHH	HAAHN
AAHHH	HHHHA	HAAHN	AAANA	AAHAN	HHANH	AHANH	HHAAA	HANHA
HANAA	AHAAN	AAHAA	HHHAA	AAHNA	HAANA	HANAH	AAHNA	AAHHH
AHAAA	AHAAN	HHAAN	A-AAH	AAAHN	HHHHH	AAAAA	HAAAA	AAAHN
AHANA	AAHHH	AAHAN	AHHHA	HANAH	HHAAA	AHANH	HHHHH	AHAAA
AAAAA	AHANA							

AawS5/FG4-2 HxA

AAHAN	AHANH	AAAHN	AHANH	AAAAA	HHHHH	HAANA	HANAH	AAHAN
HHHAA	AHHAN	HANAA	AHHAN	AHAAN	AHHAA	AAAAA	AAAAA	HAAHN
AAHHH	HHHHA	HAAHN	AAANA	AAHHH	HHANH	AHANH	HHAAA	HANHA
HANAA	AHAAN	AAHAA	HHHAA	AAHNA	HAANA	HAAAH	AAHNA	AAHHH
AHAAA	AHAAN	HHAAN	A-AAH	AAAHN	HHHHH	AAAAA	HAANA	AAAHN
AHANA	AAHHH	AAHAN	AHHHA	HANAH	HHAAA	AHANH	HHHAN	AHAAA
AAAAA	AHANA							

AawC2 HxA

AHHHH	HAANA	- - - H	AHA-H	AHANA	H-H-H	HHHHH	AAHHH	HHH-A
H - - HH	HHAAN	H-HAN	-HH-H	AHHHH	AHHH-	AHANH	HAAHN	AHANH
HHHHH	HHHAN	HANHA	HHAN-	H - - HH	HANAH	AAHAN	HHA - -	HHHHA
HHA-A	HHHHH	HHH-H	HAN-H	HAA - -	HHHH-	-AAA-	-AHHA	- - HAA
HAANA	-HA - -	-HAN-	A-AHH	AH-HH	HANHH	H-AAA	AA-A-	-AHH - -
- - HH	HANHA	-H - - H	A-AH-	HHH-A	HHAAN	AHAAA	AHHHH	HH-H - -
-AH	AAAHN							

AawS5/FG3a HxA

AAAAH	HHHHA	AAAHN	AAHAA	AAHAA	HHHAN	HAAAA	AAAAH	HHHAN
AHAAN	AAA-A	HHHAN	AHHNA	HHHAN	AHHAA	HAANA	HHHHH	HAAHN
HANHH	AHHAA	AAHAA	H-AHA	AAAAH	HANHA	AAANA	-HAAH	HANAH
HAAAH	AHHAA	AAHHH	HHHAN	AAHNA	HANHA	HAAHN	HANAH	AAHNA
AAAHN	HAAAH	H-AAH	H-AAH	AAAHN	AHANH	HAAAH	HAAAA	AHHHH
AHAAA	AAHHH	AAAAA	AAHAA	HAN-H	HHA-A	AHANH	AHHHH	HAAAA
HANHA	HANHA							

AawS5/FG3b HxA

AAHAN	HHHHA	AAAHN	AHHAA	AAHAA	HHHAN	HAAAA	AAAAH	HANAH
HHAAN	AAA-A	HHHAN	AHHAA	HHHAN	AHHAA	AAAAA	HANHH	HAAHN
HANHH	AHHAA	AAHAA	H-AHA	AAAAH	HANHH	AAANA	-HAAA	HANAH
HAAAH	AHHAA	AAHNA	HHHAN	AAHNA	HAANA	HAAHN	HANAH	AAHNA
AAAHN	HANAH	H-AAH	H-AAH	AAAHN	AHANH	HAAAH	HAAAA	AHANH
AHAAA	AAHHH	AAHAA	AHHAA	HAN-H	HHA-A	AHANH	AHHHH	HAAAA
HANHA	HANHA							

Xbcd758 HxA
A----- --A-- -----H AH--H AAAAA --H-- ---HA ----- -A---- -
----- A--AH ---A- -HHAH A---H A-A-- A--AA -A--- ----H
AAH-H HHH-A -AA-- A--H- A----- AH--H HHAA- -----
AA--H ----- H---A --H-- -A--- ---A- ----H A-AHH -
----- --AH H-A-- AHAH -A-HH --H-- --AAA -A--- -AA--
AA-H- -A-H- ---A- A----- --HA- HHA-- AH--- -----
--A- AH-H-

Xpsr154 HxA
A-H-- --A-- -----H AH--H ----- --H-- ---H- -----
----- --HAH ---A- -HH-H A-A-H A-H-- AA--A ----- -A-HH
AAH-H HHH-A -AAHH HAAH- A----- AH--H HHAA- ---H-
AA--A ----- H-H-A --H-- HA-H- --A-- --HA A-HHH -
----- A-AAH HHA-- -HAAH -----H -----
H-H- -A-H- ---A- AHHH- HAAH HHA-A AHA-- -----
-A- AH-H-

Xaw15 HxA
A----- --A-- -----H AH--- AAAAA --H-- ---H- ----- -A---- -
----- ---H ---A- -HH-H A---H A-H-- A---A -----
AA--H HHH-A -A--- ---HA A----- AH--H HHAA- -----
AA--A ----- H---A ----- ---A --H-- -
----- --AH H-A-- H-A-H -A--H --H-- --A-- -A--- -AA--
AH-H- -A-H- ---A- A----- --HA- H-A-A AH--- -----
--A- A--H-

XksuF37 HxA
A----- --A-- -----H AH--H AAA- -----
----- ---A- -H--H A---H A-----
--H HHH-A -A--- ----- A---H -HAA- AA-
-A ----- H---A ----- -A----- -A --HH-
----- H-A-- H-A-H -----
---H- ---A- A----- --HA- -----

Xwg933 HxA
A----- --A-- -----H AH--H AAAAA --H-- ---H- -A--- -A--- -
----- --AH ---A- -H--H A---H A----- A--AA ----- -H
AAH-H HHH-A -AA-- ---H- A----- AH--H HHAA- -----
AA--A ----- H---A ----- -A----- -A --HH- -
----- --H H-A-- A-A-H ---HH --H-- --A-- -A--- -AA-- -
-H- -A-H- ---A- A----- --HA- H-A-A AH--- -----
A- A--H-

Xcdo1380 HxA
A----- --A-- -----H AH--- AAAAA --H-- ---H- -A--- -A--- -
----- ---H ---A- -HH-H A---H A-H-- A---A -----
AA--H HHH-A -A--- ---HA A----- AH--H HHAA- -----
AA--A ----- H---A ----- ---A --H-- -
----- --AH H-A-- A-A-H -A--A --H-- --A-- -A--- -AA--
AH-H- -A-H- ---A- A----- --HA- H-A-A -H--- -----
--A- A--H-

Xbcd1426 HxA
 A----- --A-- -H A----- AAAAA --H-- --H- -----
 ----- --A- -A- -H-H A---H A-H-- A-AA ----- --H
 AAH-H HHH-A -AA-- --H- A----- --H HHAA- -----
 A--A ----- H--A ----- -A----- --A --H--
 ----- --AH H-A-- A-A-H ----- --A-- -A-- -A--
 --- -A-H- --A- A----- --HA- H-A-- A-----
 A- A--H-

Xbcd1 HxA
 ----- --AH- -----H AH--H AAAAA --H-- --HA --H- -----
 ----- --AH- --A- -H-AH A---H --H-- A-AA AA--- --H
 AAH-H HHH-- -AA-- -AAH- A----- --H HHAA- -----
 AA--A ----- H----- --H- --A-- --HA -AHH-
 -A-A -AAAH HHA-- HHA-H -A-HH -HH-- --AAA HAA-- -AA--
 AH-H- -AHH- --AH A----- -AHA- -----
 --A- AH-H-

Xbcd340 HxA
 A----- --AH- -----H A----- AAAAA --H-- --H- ----- -A---
 ----- --AH- --A- -H-H A---H A-H-- A-AA -----
 AAH-H HHH-A -A--- --H- A----- --H HHAA- -----
 AA--A ----- H--A ----- --A----- --A --H--
 ----- --A- H-A-- A-A-H --HH ----- --A-- -A-- -A--
 -H- -A-H- --A- A----- --HA- H-A-A -----
 A- A--H-

CreR HxA
 A----- --H- -----H AH--- AA-AA --H-- ----- -A---
 ----- --A- --A- -HH-- -----H A----- A-AA AA--- --H
 AH-H -HH-A -A--- --AHA A----- -A--- A--- HHAA- -----H
 AA--- ----- HH--- --H-- HA-H- --A-- ----- A-HHA-
 -A-- -AAAH H-A-- H-AAH -A-H- -H--- --AA- HAA-- -A--
 AH--- -AHH- --A- A----- HAHAA HHA-A AHA-- -----
 ----- --H-

Appendix 3

A. maplod = 0.05

<u>Locus</u>	<u>cM (cumulative)</u>
<i>AawS5/FG3a</i>	0.0
<i>AawS5/FG3b</i>	6.3
<i>AawS5/FG4-1</i>	34.1
<i>Xcdo1380</i>	35.4
<i>AawS5/FG4-2</i>	36.0
<i>Xbcd1426</i>	36.5
<i>Xbcd340</i>	36.6
<i>Xwg933</i>	36.8
<i>Xpsr154</i>	37.0
<i>Xaw15</i>	37.4
<i>Xbcd1</i>	37.8
<i>XksuF37</i>	38.0
<i>Xbcd758</i>	42.4
<i>AawC2</i>	64.9

B. maplod = 0.05 (- *Xpsr154*)

<u>Locus</u>	<u>cM (cumulative)</u>
<i>AawC2</i>	0.0
<i>Xbcd758</i>	21.8
<i>AawS5/FG4-1</i>	26.1
<i>Xcdo1380</i>	26.8
<i>Xbcd1426</i>	27.1
<i>Xwg933</i>	27.2
<i>Xbcd340</i>	27.4
<i>AawS5/FG4-2</i>	28.2
<i>Xaw15</i>	29.1
<i>Xbcd1</i>	29.4
<i>XksuF37</i>	29.7
<i>AawS5/FG3b</i>	57.2
<i>AawS5/FG3a</i>	63.6

Appendix 3 Cont.

C. maplod = 1.0 (- *Xpsr154*)

<u>Locus</u>	<u>cM (cumulative)</u>
<i>Xbcd758</i>	0.0
<i>AawS5/FG4-1</i>	4.1
<i>Xcdo1380</i>	5.5
<i>AawS5/FG4-2</i>	5.8
<i>Xwg933</i>	5.8
<i>Xbcd340</i>	6.4
<i>Xbcd1426</i>	6.5
<i>Xaw15</i>	7.8
<i>Xbcd1</i>	7.9
<i>XksuF37</i>	8.7
<i>AawC2</i>	33.5
<i>AawS5/FG3b</i>	35.4
<i>AawS5/FG3a</i>	41.8

D. maplod = 1.0 (- *Xpsr154*; JoinMap option "fixed sequences")

<u>Locus</u>	<u>cM (cumulative)</u>
<i>AawC2</i>	0.0
<i>Xbcd758</i>	21.5
<i>AawS5/FG4-1</i>	25.8
<i>Xcdo1380</i>	26.5
<i>Xbcd1426</i>	26.8
<i>Xwg933</i>	26.9
<i>Xbcd340</i>	27.1
<i>AawS5/FG4-2</i>	27.8
<i>Xaw15</i>	28.8
<i>Xbcd1</i>	29.1
<i>XksuF37</i>	29.4
<i>AawS5/FG3b</i>	56.8
<i>AawS5/FG3a</i>	63.2

Appendix 4

A. maplod = 1.0 (- *Xpsr154*; JoinMap option "fixed sequences")

<u>Locus</u>	<u>cM (cumulative)</u>
<i>AawC2</i>	0.0
<i>Xbcd758</i>	21.6
<i>Xcdo1380</i>	26.0
<i>AawS5/FG4-1</i>	26.4
<i>AawS5/FG4-2</i>	26.4
<i>Xbcd1426</i>	26.5
<i>Xwg933</i>	26.6
<i>Xbcd340</i>	26.7
<i>Xaw15</i>	28.3
<i>Xbcd1</i>	28.4
<i>XksuF37</i>	28.6
<i>CreR</i>	32.3
<i>AawS5/FG3b</i>	49.3
<i>AawS5/FG3a</i>	55.8