MOLECULAR ANALYSIS OF STRUCTURE OF CHROMOSOME 6R OF TRITICALE T701-4-6

by

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ERRATA

Summary, paragraph 1, line 9 read as "might have prevented" instead of "might have prevent".

Gill et al 1993 a

Graminaea=Poaceae, consider Poaceae in parenthesis wherever the Gramineae is mentioned.

Line 9, change 'prevent' to 'prevented', 'was' to 'has'

Page 1, para 3, line 9 'facilitate' to 'facilitates'

Page 3, para 3, line 2, 'have' to 'has'

Page 5, para 4, line 1, 'require' to 'requires'

Page 6, para 4, line 1, delete 'is'

Page 8, para 2, line 7, change 'a' to 'the'

Page 10, para 3, lines 3 and 4 'crossibility' to crossability'

Page 10, para 3, line 5 'Scale to 'Secale'

Page 10, para 4, line 3, '1981' to '1891'

Page 10, para 4, line 5, delete 'both'

Page 13, line 2 "molecular" instead of "moelcular".

Page 14, para 5, line 3, 'coversion to 'conversion'

Page 16, parag 2, line 4, read as "comparative mapping studies" instead of "comaparative mapping studies"

Page 17, para 5, line 1, 'cruicial' to crucial'

Page 17, para 5, line 4, 'assissted' to 'assisted'

Page 20, para 3, line 3, 'defficient' to 'deficient'

Page 22, para 2, line 1, 'boat' to 'boot'

Page 24, para 2, line 2, 'freeze' to 'frozen'

Page 25, 2.8 title, 'endonleose' to 'endonuclease'

Page 25, para 1, line 4, 'voulme' to 'volume'

Page 25, para 3, line 4, 'hybridistions' to ' hybridisations'

Page 27, para 1, line 6, 'initialy' to 'initially'

Page 28, para 3, line 3, 'scalepel' to 'scalpel'

Page 28, section 2.12.2, line 3 remove (?)

Page 29, section 2.13.1, line 9 read as "containing a 3ml $CsCl_2$ cushion"

instead of "cotaining 3 ml CsCl2 cushion".

Page 29, "9.65 g" not "9.65 gm". ·

Page 30, para 2, line 4 and 7, 'temprature' to 'temperature'

Page 30, para 2, line 8, 'supernatnt' to 'supernatant'

Page 30, para 2, line 15, 'vaccum' to 'vacuum'

Page 31, "HCl" not "Hcl". · P64 "Wehling 1991" instead of "Wehling et al".

Page 31, $MgSO_4$ not MgSo4

Page 31, para 4, line 1 & 4, 'innoculating' to 'inoculating'

Page 31, para 4, line 5, 'was reached to' to 'had reached'

Page 32, para 2, line 1, 'screenig' to screening'

Page 32, para 2, line 3, 'crefully' to 'carefully'

Page 32, para 2, line 8, 'neutrilising' to 'neutralising'

Page 32, para 3, line 3, 'hybridistion' to 'hybridisation'

Page 32, para 4, line 2, 'get hardened' to 'set'

Table 3.2, footnote 1,2 , 'Templets' to 'Templates'

Table 3.2, footnote, 1,2, 'temprature to 'temperature

Page 36, Heading no 2, 'probes' to 'probe'

Page 37, para 4, line 4, 'comigrated' to 'comigrate'

Page 37, para 5, line 6, delete 'were'

Page 38, para 3, line 8, delete 'were

Page 39, para 1, line 2, replace 'at' with 'to a'

Page 39, para 1, line 3, 'indicated' to 'indicating'

Page 39, para 2, line 3, 'elsewere' to 'elsewhere'

Page 40, para 2, line 1, delete 'been'

Page 40, para 2, line 6, 'repititive' to 'repetitive'

Page 40, para 2, line 11, 'localised' to 'localise'

Page 40, para 3, line 1, 'temprature' to 'temperature'

Page 43, para 3, line 1, 'have' to 'had'

Page 44, para 1, line, 'suggesttions' to 'suggestions'

Page 52, para4, line 3 the Schomburgk is correct.

Page 53, para 1, line 3, 'wether' to 'whether'

Page 54, para 2, line 1, delete 'were'

Page 54, para 3, line 8, 'existance' to 'existence'

Page 54, para 4, line 2, syntaneous' to 'syntenous'

Page 60, para 3, line 4, 'It' to 'They'

Page 60, "cDNA" not "cNDA"

Page 61, para 1, line10, ‘polymorhism’ to ‘polymorphism’

Page 61, para 2, line 1, delete ‘that’

Page 62, para 2, line 4, delete ‘exist’

Page 62, para 2, line 8 and 10, ‘wether’ to ‘whether’

Page 62, para 3, line 4, delete ‘a’

Page 62, para 3, line 6, insert ‘is’ before ‘still’

Page 62, para 2, line 9, correct is " a further work is needed to confirm that cDNA clones isolated in this study represent a novel multigene family”.

Page 64, para 1, line 9, ‘transfered’ to ‘transferred’

Page 64, Sorrells et al. 1991.

Page 67, line 6 read "Eastwood" instead of "East wood".

Page 81, Kurata et al 1994 a read as "genetic map of" instead of "genetic map f".

Page 87, Rimpau reference, ‘1981’ to ‘1891’

In the memory of my Ammi

Feroza Mekhmoor

1942-1996

Kabir, Sadic

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Conference Proceedings

Following work from the experiments described in this thesis was presented in conferences

1)"Mapping resistance to cereal cyst nematodes in rye".
Sadia **Kabir**, Chris Taylor, Ken Shepherd, and Peter Langridge. 5th
Australasian Gene Mapping workshop, University of New England, Armidale.
July 6-8, 1994. Genetic Society of Australia, Abstracts.

2)"Introgression of rye genes into wheat: is that easy ?".
Sadia **Kabir**, Ian Dundas, Ken Shepherd and Peter Langridge.
1st Inaugral International Conference on Genetics, NARC, Islamabad
November 24-26, 1996. Pakistan Genetical Society, Islamabad -Pakistan

Summary

Cereal cyst nematode (CCN), *Heterodera avenae* Woll., is a most devastating pathogen of cereals causing serious crop losses. Chemical control of the disease is costly both to the environment and to the farmer. Resistant cultivars are considered the most economical component of a nematode management system. Wheat is susceptible to CCN attack whereas the hybrid triticale T701-4-6 possesses a useful resistance gene on the long arm of rye chromosome 6R. Earlier attempts using a whole chromosome $6R^{T701}$ as a starting material have failed to introgress the CCN resistance gene into wheat. Although pairing was induced in the absence of the *Ph1* locus, the structural differences between the rye chromosomes and its wheat homoeologues might have prevent the recombination. Thus it was become necessary to understand the complexities in the structure of rye 6R which caused major differences between rye and wheat chromosomes.

The recent advent in recombinant DNA technology provides an array of useful techniques to understand the complex genomes structure and evolutionary chromosomal rearrangements in related species. Employing conventional hybridisation methods and molecular tools, this thesis reports the evidence for the diverse structure of 6R relative to wheat homoeologous chromosomes thus minimising its exploitation in a wheat breeding program.

Using RFLP and PCR techniques, DNA based markers were developed and mapped to rye 6R. A PCR based marker C1 derived from R173 (a dispersed repeat from the rye genome) was useful in determining the sub microscopic deletion in the structure of Imperial rye 6R, proved the efficiency of PCR technique as a fast screening assay for breeders seeds stock. Five new RFLP markers CDO534, CDO1158, PSR113, Tam-6 and Tam-24 were assigned to the short arm of rye 6R. Since only few markers have been mapped to 6RS, the new rye markers mapped in this study will be useful in mapping genes on the short arm of rye 6R. However the map position of new rye markers is not known as a suitable mapping population was not available. The order of the marker loci was inferred by comparing their locations on wheat and other cereals maps.

The comparative genome analysis using RFLP markers provided an opportunity to understand the level of homoeology between homoeologous group 6 wheat and rye chromosomes. Analysis revealed that the short arm of rye 6R contains a putative inversion in proximal region and a deletion of the distal region might have been translocated to 4RL via a 2L interchange during speciation. The presence of PSR148 (groups 2 and 7) on the intact 6RL confirmed a non-group 6 translocation at distal regions of 6RL. However, the assignment of a large number of group 6 probes on 6RL using deletion lines, indicated that a fragment of 6RL present in these deletion lines (in variable length) may maintain a complete synteny with wheat homoeologues and assumed to be useful in introgressing CCN resistance gene into wheat.

An important aspect of this study was the use of five variable 6RL length mutant lines to introgress the 6RL segment into wheat via homoeologous recombination. The Sear's *ph1b* mutant (1977) was used in test crosses to induce recombination between 6RL and wheat homoeologous chromosomes in genetic stocks having monosomes of translocated 6RL and 6D in a homozygous *ph1b* background. The successful screening of Sears *ph1b* mutant plants and test-cross progenies with the RFLP probe PSR128 (mapped in the deletion of region containing the *Ph1* locus) confirmed the homozygous status of *ph1b* in the plants. Isozymes markers, α-amylase and GOT were used to establish dosage of 6R and 6D and select plants monosomic for 6R and 6D. The isozyme markers in conjunction with RFLP markers, provided an opportunity to identify the recombination pattern along the homoeologous chromosomes.

A total of five hundred TC-F2 progeny from the five different 6RL mutant lines and deficient for the *Ph1* locus, was screened using two isozyme markers and ten to twelve RFLP probes depending on the length of deleted fragment of 6RL. Initial screening used the isozyme markers α-amylase and GOT, and no dissociation was observed. Since these two markers are very close to each other and proximal to CCN resistance gene, RFLP markers which are distributed along the length of chromosome flanking the CCN resistance gene were used in secondary screening. However, no dissociation in RFLP markers was observed on 6RL and 6D chromosomes, although a total of eleven putative recombinants were isolated. These recombinants showed deletion of RFLP markers on chromosomes other than 6R and 6D. A putative cross-over point was identified between loci *Xpsr915* and *Xpsr149*. In most cases (7 of 11), the chromosomes

showing dissociation of marker loci were detected in the presence of two doses of 6R and 6D. In few cases a single dose of 6D was present (e.g. G7-17 and I5-24) or absent (e.g. I5-10); and a single dose of 6R was present (e.g. H1-10). The abnormal plants were isolated in four different families of 1411-54 line which possesses 6RL fragment containing *Got-R2* and *Cre-R* loci. The recombination status of these plants is not yet known and it was assumed that these plants involved the recombination between homoeologous wheat chromosomes. This study has also shown that RFLPs are superior and more reliable for the identification of recombinants than isozyme markers because of their increased ability to detect polymorphism. Although the pairing conditions were maximised using Sears *ph1b* mutant and 6RL deletion stock, the failure to isolate any wheat-rye recombinant chromosomes reconfirms the complex structure of 6R preventing its involvement in homoeologous recombination.

A molecular approach was taken to directly analyse the structure of 6R by constructing a cDNA library from ten days old roots of the wheat-$6R^{T701}$(-6D) substitution line. Differential screening of this library generated two hundred clones differentially expressed in wheat-$6R^{T701}$(-6D) substitution line. One hundred and five clones fall into four families, whereas relationships of the remaining 95 clones are still unknown. Southern hybridisation of representative clones from each family with two wheat varieties (Schomburgk and Chinese Spring), two rye varieties (Imperial rye and South Australian rye) and triticale T701-4-6 and its derived 6R lines identified homoeoloci on rye and wheat chromosomes. The results suggest that clones identified in this study represent genes expressed differentially in wheat-$6R^{T701}$(-6D) substitution line by repression of expression of homoeoalleles in Schomburgk.

In summary, this study shows that chromosome $6R^{T701}$ possesses a complex structure relative to its wheat homoeologues. This complexity suggests that introgression of the CCN resistance gene from $6R^{T701}$ into wheat may not be practicable.

ABBREVIATIONS

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

α-amylase:	alpha amylase isozyme
µg:	microgram
µl:	microlitre
µM:	micromolar
4R:	chromosome 4R from rye cultivar Imperial
6-pgd:	6-phosphogluconate dehydrogenase
$6R^{Imp}$:	chromosome 6R from rye cultivar Imperial
$6R^{T701}$:	chromosome 6R from triticale
AFLP:	amplified fragment length polymorphism
bp:	base pairs
CCN:	cereal cyst nematode
cDNA:	complementry DNA
cM:	centimorgan
DEPC:	diethylpyrocarbonate
gDNA:	genomic DNA
GOT:	glutamate oxaloacetate transaminase
IEF:	isoelectric focussing
ISH:	in situ hybridisation
ml:	millilitre
mM:	millimolar
min:	minutes
Mbp:	mega basepairs
MOPS:	morpholinopropane sulfonic acid
ng:	nanogram
PFGE:	pulse field gel electrophoresis
RAPD:	random amplified polymorphic DNA
RFLP:	restriction fragment length polymorphism
RT:	room temperature
RT-PCR:	reverse transcription based-polymerase chain reaction
TC-F1:	test-cross-F1
TC-F2:	test-cross-F2
YAC:	yeast artificial chromosome

CONTENTS

CHAPTER 1

1.1 General Introduction

Cereals (rice, wheat, barley, rye, oats, maize, and millets) constitute more than 50% of staple diet consumed by man. Wheat, *Triticum aestivum*, ranks next to the rice in importance as a cereal crop and is the principal food for about one third of the world's total population. It is grown world wide and exceeds all other cereals in total area and production. All cereal grasses belong to the Graminaea family that contains over 10,000 species, grouped into several tribes. Amongst these tribes, Triticeae has a significance in wheat breeding. Members of the tribe Triticeae (including wheat) are presumably derived from a single common ancestor and their basic chromosome number is seven. Therefore, wheat can be sexually hybridised with many of its cultivated and wild relatives.

The conventional breeding methods to improve the quality, yield and disease resistance of wheat have led to the reduction in natural genetic variability of this crop. Wheat breeders have turned to the wild relatives of wheat (including *Aegilops*, *Agropyron*, *Secale*, *Hordeum*) as rich sources of many useful genes conferring resistance to biotic (pests and diseases) and abiotic (cold, drought and salt) stresses (reviewed by Fedak, 1989). Such hybrids provide a bridge by which the gene pool of wheat can be increased. However, most of the wheat-alien derivatives carrying alien chromosomes as additions, substitutions or translocations have had a limited use in practical breeding.

Large segments of chromosome contain desirable genes linked with undesirable genes which usually have a detrimental effect on agronomic performance. A better alternative is to produce lines with relatively smaller alien fragment containing the desirable gene. This can be achieved by the induction of homoeologous pairing and recombination between wheat and alien chromosomes. However, although the genomes of wheat and alien species are related, pairing and recombination between homoeologous chromosomes rarely occur. The pairing control genes, particularly *Ph1*, located on the long arm of chromosome 5B, suppresses pairing between homoeologous chromosomes (Okamoto, 1957; Riley and Chapman, 1958; Sears and Okamoto, 1958). The removal of the *Ph1* locus facilitate the pairing and recombination between wheat and alien chromosomes, but the introgression of genes from certain species such as rye is difficult.

Cytological studies in *ph1b* background have proved that rye and wheat chromosomes seldom pair (reviewed by Naranjo, 1992). The late replicative nature of rye telomeric heterochromatin compounded with genetic interactions and structural differences between the rye and wheat

genomes are the basis of these problems (reviewed by Appels, 1982). The major structural differences between the homoeologous chromosomes are a consequence of the rapid turnover of non-coding repeated sequences during evolution (Flavell, *et al.*, 1980). Further, chromosomal rearrangements during speciation have disturbed the homoeology between the two species at the gross level (Devos, *et al.*, 1993).

Cereal rye is a potential donor of many useful genes and has been used extensively to generate addition, substitution and translocation lines. Using wheat-rye translocation lines as starting material, rare recombinants involving rye chromosome 1R and wheat 1D have been isolated (Koebner and Shepherd, 1985, 1986 a, b)). Rye chromosome 1R maintains complete collinearity with homoeologous 1 chromosomes of wheat. Another useful rye chromosome is triticale chromosome 6R that possesses a resistance gene for cereal cyst nematode (CCN) on its long arm (Asiedu, *et al.*, 1990). Cereal cyst nematode is a serious pathogen of wheat and causes considerable yield losses in many parts of the world. The most economical method of control is the selection of resistant varieties and their exploitation in breeding program. However, attempts to introgress the CCN resistance gene into wheat, using a wheat-$6R^{T701}$(-6D) substitution line have been unsuccessful. Although homoeolgous pairing was induced in the absence of the *Ph 1* locus, screening of large number of F3 and F4 progenies did not yield any useful recombinants involving rye $6RL^{T701}$ and wheat homoeologous chromosomes. Instead, successive deletions in the length of $6RL^{T701}$ were isolated as variable fragments of 6R (Dundas *et al.*, 1992).

The deletion length lines seem to lack most of the distal region of $6RL^{T701}$, known to contain 3L and 7L translocation mainly responsible for disturbed homoeology between rye and wheat chromosomes (Devos *et al.*, 1993). Progenies of the $6RL^{T701}$ length deletions contain $6RL^{T701}$ as a telosome or in translocation probably with 6DS. This invaluable cytogenetic stock have provided the basis for useful research to understand the structure of chromosome 6R. Utilising the approach of deletion mapping, several isozyme (Dundas and Shepherd, 1993) and DNA-based markers (RFLPs and PCR) (Taylor, 1996) have been mapped on the long arm of chromosome $6R^{T701}$, subsequently mapping CCN resistance gene *Cre-R* on the intercalary region.

The discovery that RFLP markers are useful in mapping genes and sequences across related crop species, shows that homoeologous relationships between wheat and rye chromosomes can be explored by comparative genome analysis (Devos *et al.*, 1995 b). The molecular structure of $6R^{T701}$, based on the comparative studies, can be useful in devising suitable strategies for its utilisation in wheat breeding. Further, the DNA-based markers mapped on $6R^{T701}$ can be useful tools for marker assisted selection in a gene introgression program. Another approach which can provide a direct insight into the structure of $6R^{T701}$ is to isolate sequences which map specifically to this chromosome.

1.2 Aims and Objectives

The present study was undertaken to investigate the molecular structure of $6R^{T701}$ to devise the best strategy to introgress the $6R^{T701}$ CCN resistance gene in wheat. The main aims and objectives of this research were:

1)Development of molecular markers (PCR and RFLP) for the short arm of $6R^{T701}$ to complete the molecular map of $6R^{T701}$.

2)RFLP-based comparative analysis of triticeae chromosomes 6 to understand the homoeologous relationships between rye, triticale and wheat.

3)Utilisation of $6RL^{T701}$ deletion mutant stocks in crosses with Sear's *ph1b* mutant to induce pairing between homoeolgous group 6 chromosomes with the practical aim of introgressing CCN resistance into wheat.

4)Isozymes and RFLP marker assisted selection and isolation of potential recombinants between $6RL^{T701}$ and 6D to understand the recombination pattern along homoeologous group 6 chromosomes.

5)Direct analysis of chromosome $6R^{T701}$ by mapping cDNA clones isolated from the $6R^{T701}$ library employing differential screening.

A considerable literature reviewed during the course of this project is detailed in next section. In this concise report, the author have tried to review the research published in some key areas related to this study.

1.3 Literature review

1.3.1 Cereal cyst nematode (CCN)-A problem and its control

Nematodes cause considerable annual losses to many crops including cereals. Although many species of plant parasitic nematodes have been recorded associated with cereals, the most important is *Heterodera avenae* Wollenweber, generally known as cereal cyst nematode (CCN). This pest has been detected in 31 countries distributed widely in tropical, subtropical, temperate and temperate semiarid regions of the world (Meagher 1977). The origin of the species was Europe, probably Germany, from where it may have been distributed to other parts of the world in soil particles adhering to seed or through other plant material (Brown, 1987). Within Australia, its spread has been attributed mainly to dust-storms (Meagher, 1972).

In Australia, CCN is considered a major pest of wheat in South Australia, Victoria, Western Australia and New South Wales (Brown, 1982). Over two million hectares of wheatland are infested with *H. avenae* (Brown, 1984), with annual losses in wheat estimated at AUS$ 72 million (Brown, 1981). The computed figures suggest that for every 10 eggs/g soil, there is a

loss of 188kg/ha in wheat and 75 kg/ha in barley (Duggan, 1961; Dixon 1969). The symptoms of infection are characterised by uneven patches of stunted plants with yellowish leaves randomly distributed throughout the field (Wallace, 1965). Root systems appear shallow and multibranched with slight swellings at the site of cyst attachment. Reduce tillering, premature ear formation, and poorly-filled heads are some other characteristics of infected plants.

The best approach to the control of this pest are crop rotation and use of resistant and tolerant cultivars. The use of nematicides is not only costly, it is environmentally undesirable and affect many of the soil micro-flora and fauna. It consequently upsets the general soil ecology by indirectly changing the nitrogen status of soil and causing a build up of ammonia to a level toxic to plants. Crop rotation is preferable because of the rapid decline in nematode number under fallow and non-host crops, such as clover, legumes, cumin and fenugreek (Brown, 1984). However use of resistant cultivars is considered the most economical method of nematode control. Their use results in significant increases in yield and a corresponding reduction in nematode population densities (Boerma and Hussey, 1992).

Use of resistant cultivars

Resistance can be described as an inefficiency of the host to allow the growth and reproduction of the nematode (Parlevliet, 1981), whereas tolerance can be defined as the ability of the host plant to grow and yield well despite of nematode infection (Schafer, 1971). Therefore to achieve maximum yeild, use of cultivars with both nematode resistance and tolerance is recommended (Fisher, 1982, a;Trudgill, 1991).

However resistance in the Australian cultivars only reduces the nematode to population levels that still cause a 15-30% reduction in yield. As a result breeders and growers have shown reluctance to use these crops. Consequently, the use of resistant cultivars has to be viewed with some caution, and pathotyping and preliminary tests are necessary for breeding programmes before resistant varieties are recommended to farmers (Fisher, 1982, a). So far, there is only one unique pathotype that is Ha13 has been identified in Australia (Brown, 1982). However it has been suggested that reliance upon a single source of resistance may promote the appearance of new pathotypes of the nematode (Andersson, 1982).

The evolution of new pathotypes can be explained in light of Flor's "gene-for-gene" model (reviewed by Jones, 1996). According to this, for a plant to exhibit resistance to a pathogen, a resistance (*R*) gene must be present in the plant and a corresponding avirulence (*avr*) gene must be present in the pathogen. An absence of either can cause disease. Since, nematode resistance in plants has often been mediated by single genes (Sidhu and Webster, 1981), it has been proposed that resistant cultivars carrying different sources of resistance should be used to avoid the emergence of new pathotypes (Andersson, 1982).

CCN resistance in cereals

Resistance to CCN has been described in various members of the Graminaea family, therefore a large pool of resistance genes is available for cereal breeders to use in their programs to develop CCN resistant cultivars. To date the only resistance gene which has been reported in hexaploid wheat is the locus, *Cre*, located on 2BL in the lines Loros (O'Brien and Fisher, 1974) and AUS10894 (O'Brien *et al.*, 1980). This gene is not regarded as fully effective against the unique Australian pathotype of CCN. An alternative approach is to look for a suitable gene in relatives of wheat.

Other sources of resistance include *Triticum tauschii* (Eastwood *et al.*, 1991) and *Aegilops* sp. (Dosba *et al.*, 1978), barley (Choe, 1996, also see refrences cited therein), rye and triticale (Asiedu *et al.*, 1990). The resistance identified in barley cultivars appears to be represented by the same locus as in wheat. Gene mapping studies suggested that *Cre* (in wheat) and *Ha* (in barley) may be homologous, which could have serious implications for breeders working with these genes (Williams, 1994; Kretschmer *et al.*, 1997).

Two CCN resistance genes have been mapped in *Triticum tauschii*: *Ccn-D2* mapped to the proximal region of 2DL, is a weak gene and probably a homoeolocus of the wheat *Cre*, and *Ccn-D1*, mapped to the long arm of 6D, which could be a different gene from wheat. Rye cultivar, South Australian has also shown strong resistance to the nematode while Weethalle rye and Towan triticale have moderate resistance (reviewed by Asiedu, 1986) . Fisher (1982, b) identified resistance to CCN in the triticale line T701-4-6. A subsequent work (Asiedu *et al.*, 1990) demonstrated that resistance in T701-4-6 is stronger than that in the AUS 10894 and the gene for CCN resistance, *Cre-R*, was localised to the long arm of rye chromosome 6R in T701-4-6. This rye gene could be a possible homologue of *Ccn-D1* in *T. tauschii* and an alternative source of resistance for susceptible cultivars like wheat.

The exploitation of rye CCN resistance gene, *Cre R*, in cereal breeding programmes require a complete understanding of molecular organisation and homoeologous relationships of cereals genomes particularly wheat and rye, and the knowledge of methods and tools used to transfer and trace the alien genes into wheat. Nevertheless these studies could not be possible without the aid of useful molecular and cytogenetic tools.

1.3.2 Molecular organisation of cereals genomes

Genome size

Genome size is one of the key feature which has restricted the molecular analysis of wheat and rye and also the study of the basis of their evolution. The genomes of hexaploid wheat (*Triticum aestivum*; 17000 Mbp) and rye (*Secale cereale*, 7000 Mbp) are relatively larger in contrast to rice (*Oryza sativa*, 960 Mbp) (reviewed by Heslop-Harrison, 1991). A substantial part of this interspecific variation in cereals DNA content is due to the repetitive DNA in the genome.

Repetitive sequences

DNA reassociation kinetics or renaturation analyses have revealed the presence of abundant repetitive sequences that account for the large size of cereal genomes. Approximately 20-30% of the total DNA is in single or low copy sequence classes (Flavell *et al.*, 1980) with the remainder composed of repetitive sequences (Flavell *et al.*, 1974). Different classes of repeated sequences have been observed, from very highly repetitious and palindromic sequences which can account for 5 to 10% (Appels and McIntyre, 1985) to moderately repeated elements which account for 60 to 70% (reviewed by McIntyre *et al.*, 1988) of the cereal genome. The remainder of the genome is organised with short lengths of single copy DNA (200-4000 bp long) interspersed with short lengths of repetitive sequence DNA (50-2000 bp long) with a smaller fraction, 2 to 10%, of the genome consisting of long stretches (>10 kb long) of single copy DNA (Appels and McIntyre, 1985).

The physical organisation of moderately repetitive sequences further classify them into two broad groups: tandem arrays and dispersed repeats. Long tandem arrays are localised at heterochromatic knobs/centromeres whereas dispersed repeats are dispersed throughout the genome. There is some speculation that dispersed repeats may have originated from the tandem repeats due to chromosomal breakage followed by inversions, translocations or substitution of the telomeric regions. However, recent results suggest that dispersed repeats may have evolved by 'infection' of an ancestoral plant with an RNA sequence which was reverse transcribed into DNA copies and became the dispersed repeat into genome (reviewed by Flavell *et al.*, 1987). Thus dispersed repeats have similarities to retrotransposon-like elements. Most of these dispersed sequences are not conserved across different genomes varying greatly in size. However, the actual element comprising the bulk of sequences may be related.

Retrotransposon make a major contribution to the bulk of repeated DNA which is remain conserved in cereal genomes (reviewed by Grandbastein, 1992). A specific retroelement, *BIS-*

1, was found to be the major component of many cereal genomes, and can be used to study their genome organisation (Moore *et al.*, 1991; Abbo *et al.*, 1995). Sparvoli *et al.* (1989) also reported the presence of sequences homologous to maize *Mu* transposable elements in wheat which were presumed to be the old components of the genome that had lost their transposition activity. Diploid wheat contained about 4 to 6 elements, while tetraploid and hexaploid species had 2 to 3 times more elements. A high copy number of *Mu* elements was also found in rye and triticale.

Another classification of families of repeated sequences in oats, barley, rye and wheat is based on their interspecies and intraspecies DNA/DNA hybridisation analyses (Flavell *et al.*, 1977). According to this, repeated sequences of related families are more closely related within a species than between species, because of their involvement in many rounds of amplification or quantitative change via unequal crossing over during species divergence in cereal evolution. The more closely related species, for example wheat and rye, share a greater proportion of the repeated sequence component in common than do more distantly related species, for example wheat and oats, (Appels and McIntyre; 1985). Dobrzanska and Szurmak (1992) described a 371 bp long fragment from wheat which shares the same sequence features as autonomously replicating sequence (ARS) element, and shows the same hybridisation and distribution pattern for both wheat and rye.

A substantial proportion of each genome consists of repetitive sequences in a category called "species specific", this includes 16%, 24%, 28%, and 55% of the genomes of wheat, rye, barley and oats, respectively (McIntyre *et al.*, 1988). The distribution and amount of "species specific" or shared families of repeated sequences make them a useful phylogenetic or taxonomic tool (Jones and Flavell, 1982; Dvorak and Appels, 1982; Arnold *et al.*, 1985). In addition, these sequences could serve as valuable probes for tracing alien chromatin introduced into wheat (reviewed by Anamthawat-Jonsson and Heslop-Harrison, 1992). One such example is rye specific dispersed repeat R173 family (Guidet *et al.*, 1991) have been used successfully in tracing rye introgressions into wheat (Shepherd *et al.*, 1994). R173 family is dispersed on all seven rye chromosomes, found to be absent from telomeres and their copy number is low on centromere, heterochromatins (Guidet *et al.*, 1991; Taylor, 1996).

Distribution of genes on cereal chromosome

A high proportion of cytosine residues in wheat (82%) is methylated and present in pericentromeric regions of chromosomes. This information came from analysis of wheat-rye recombinant chromosomes using *Not I* (methyl sensitive enzymes) which throw light on distribution of genes on cereal chromosomes (Moore *et al.* 1993). The *Not* I enzyme produced a high proportion of larger segments in the pericentromeric region and shorter segments in the sub terminal or distal region. The higher density of unmethylated sites present in distal regions

indicate that these regions are highly rich in genes. As compared to this abundance of highly methylated CpG nucleotides in peri-centromeric/proximal regions suggests the presence of highly repetitive sequences. Since the major proportion of species specific repetitive sequences is present in proximal regions, it reduce the chiasmata formation between the chromosomes arise from the common ancestor. Hence proximal regions exhibit a low rate of recombination as compared to distal regions. This has a direct implication on gene mapping and consequently in cereal breeding programs where recombination is induced between homoeologous chromosomes to transfer useful genes.

1.3.3 Homoeologous genomes of wheat and rye

Both wheat and rye belong to the tribe Triticeae, and are assumed to have evolved from the common ancestor with seven pairs of chromosomes. Bread wheat (*Triticum aestivum*) is an allohexaploid species with 2n=6x=42 organised into seven homoeologous groups of chromosomes in three genomes A, B and D, each arise from different diploid species. Recent investigations (Dvorak *et al.*, 1993) suggested that A genome is derived from *T. urartu* rather than *T. monococcum* which has long been considered as a source of A genome in wheat. Progenitor of B genome is yet not known with certainity, whereas *T. tauschii* is a donor of D genome in wheat. Cereal rye, *Secale cereale*, is a diploid species with 2n=2x=14. Genomes of wheat are designated as AABBDD and rye as RR .

Homoeology of wheat and rye chromosomes

Homoeology is a measure of genetic similarity between chromosomes of wheat and its relatives and is defined as the degree to which a given chromosome can overcome nullisomy for another chromosome. The homoeologous relationships between chromosomes of related species can be identified using three main approaches: substitution compensation tests, analysis of homoeologous pairing at metaphase I of meiosis and genetic maps (Naranjo, 1992).

On the basis of the ability of rye chromosomes to compensate when substituted for chromosomes of a single homoeologous wheat group, chromosomes 1R, 2R, 3R, 5R and 6R were assigned to homoeologous groups 1, 2, 3, 5, and 6 respectively, while 4R and 7R showed a partial homoeology to group 4 and 7 (Sybenga, 1983; Zeller and Hsam, 1983; Miller, 1984).

Analysis of homoeologous pairing can provide valuable information about the evolutionary relationship among Triticeae species though it is problematic. However, C-banding provides a tool for identification of rye chromosomes and wheat chromosomes. Due to translocation during the course of evolution, some chromosomes of rye show homoeology with more than one chromosome group in the Triticeae (Table 1.1, reviewed by Naranjo, 1992).

Table 1.1 Homoeology of rye chromosomes with wheat chromosomes based on pairing analyses (reviewed by Naranjo, 1992).

S. No	Rye chromosome	Wheat chromosomes	
		Homoeologous group	Non-homoeologous group
1	1R	1	No
2	2R	2	6
3	3R	3	6
4	4R	4	7
5	5R	5	4
6	6R	6	2, 3, and 7
7	7R	7	4

Analyses of homoeologous pairing have also been used to identify the arm homoeology between wheat and rye chromosomes. The arms have been identified by means of C-banding (Friebe and Larter, 1988). Only arms 1RS, 1RL, 2RL, 3RS, and 5RS showed normal homoeologous relationships to wheat, while the remaining arms of rye appeared to be involved in chromosome rearrangements that occurred during evolution of genus *Secale*. However a pericentric inversion in rye chromosome 4R, a reciprocal translocation between 3RL and 6RL and a multiple translocation involving 4RL, 5RL, 6RS, and 7RS are present in rye relative to wheat (Naranjo *et al.*, 1987; Naranjo and Fernandez-Rueda, 1991; Naranjo, 1992).

Other evidences of translocations in rye genome came from the extensive studies of mapping of the genes governing biochemical traits within these species for example isozymes and seed storage proteins. Jouve and Diaz (1990) demonstrated that Esterase 6 isozymes are specifically active in leaves controlled by *Est-6* gene set. Three homoeoallelic loci were mapped to homoeologous group 3 chromosomes in wheat. However, in rye, Est-R6 was located on chromosome 6R which indicate for the possible interchange between rye and wheat. Endopeptidase isozymes in wheat are controlled by genes (*Ep-1*) located on group 7 chromosomes. There are conflicts about the location of (*Ep-1*) genes in rye. Several workers mapped this gene on chromosome 7R (Hart and Tuleen, 1983; Koebner *et al.*, 1988), however Benito *et al.*, (1991) claimed the location of *Ep-1* on 6R, indicating a translocation of 7R on 6R.

Besides the biochemical loci, genetic maps based on molecular markers 'RFLP' also serve as powerful tools for determining homoeologous relationship between wheat and rye chromosomes. An RFLP-based map of *Secale cereale* has also provided evidence for multiple evolutionary translocations on chromosome rearrangements in the rye genome relative to wheat (Devos *et al.*, 1993). According to them 2RS, 3RL, 4RL, 5RL, 6RS, 6RL, 7RS and 7RL have

all been involved in at least one translocation. This approach of comparative mapping is becoming increasingly important in cereals genomes comparison, and discussed in detail in section 1.3.5.

1.3.4 Rye genome in wheat breeding

Characters like resistance to a variety of wheat diseases, drought tolerance, winter hardiness and tolerance to low pH make rye a favourable species as a donor for new genetic material for the agronomic improvement of commercial wheat (reviewed by Merker, 1984).

In wheat, two recessive genes *kr1* and *kr2*, present on 5B and 5A respectively, regulate the crossability not only with rye but also with *Hordeum bulbosum* (Sitch *et al.*, 1985 and literature cited therein). In contrast, dominant genes in rye control crossibility with wheat (Tanner and Falk, 1981; Schlegel and Boerner, 1991). A high correlation between the crossibility of specific wheat cultivars with *Scale cereale* has been reported (Falk and Kasha, 1981; Tanner and Falk, 1981).

Many efforts have been made to combine the best characters of rye and wheat in one species. Discovery of colchicine led to the production of synthetic and first fertile amphiploid "Triticale" (X *Triticosecale* Wittmack), (Rimpau, 1981). Since then, triticale developed from a more or less experimental plant to a present day commercial cereal crop (Bushuk and Larter, 1982; Gupta and Priyadarshan, 1982; Gustafson, 1983; Kaltsikes *et al.*, 1984). The genetic pool of both its constituent genomes can be exploited to broaden the genetic basis of this artificially synthesised crop.

Wheat-rye addition, substitution, and translocation lines

The traditional way of gene transfer from rye into wheat involves back crossing of triticale to wheat in order to isolate addition lines. This has been done over the past thirty years (Gustafson and Dera, 1989). The wheat-rye addition lines show meiotic instabilities and poor fertility (Riley, 1960 a). Substitution lines may arise spontaneously (reviewed by Khush, 1973) but their poor fertility also indicates that compensation in alien substitutions of rye chromosomes for those of wheat is not as good as in substitution of *Aegilops* sp. and *Agropyron* sp. (Bielig and Driscoll, 1970; 1971). The other problems associated with introduction of rye chromosome include the deleterious effects on endosperm development and grain filling (Kaltiskes and Roupakias, 1975) are because of late replicative nature of rye telomeric heterochromatin. This is evident from the observations that rye chromosomes which have lost their telomeric heterochromatin pair more readily than those which retains it (Singh and Robbelen, 1976; Bennet, 1977).

In wheat-rye additions and wheat-rye substitution lines, both rye and wheat chromosomes affect each other's homologous pairing. The decrease in homologous pairing can be due to factors like genes controlling meiotic pairing, constitutive heterochromatin, and cryptic wheat rye interactions (Orllena *et al.*, 1984). Schemes to produce wheat-rye addition and/or substitution lines can involve the univalent condition of rye chromosome arms leads to the formation of isochromosomes and translocations. Sears, 1973, specifically tested the possibility of obtaining spontaneous translocations through the union of telocentric chromosomes formed from univalent chromosomes. He recovered a 6BL-5RL translocation from wheat carrying 6B and 5R as univalents.

Translocation can occur fairly commonly as shown by Shepherd (1973) who found 3 out of 13 putative substitution lines of chromosome 1R for 1D of wheat involve translocation between 1DL and 1RS. Several Europeans cultivars also possess a spontaneous 1B/1R translocation (Mettin *et al.*, 1973; Zellar, 1973). Using in situ hybridisation, May and Appels (1980) identified a reciprocal translocation involving 2BS/2RL and 2BL/2RS in hexaploid wheat with the loss of telomeric heterochromatin. The probe use also showed that rye heterochromatin has little effect on the pairing of translocated wheat arm to its wheat homologue during meiosis. They also characterised the chromosome resulting from a 1B-1R translocation event.

By means of wheat-rye translocations, genes for resistance to pathogens such as powdery mildew (Mettin *et al.*, 1973; Zeller, 1973), stem and leaf rust resistance (Sears, 1956; Driscoll and Jensen, 1964; Stewart, 1968; Friebe, 1993) insects including wheat curl mite (Martin *et al.*, 1976) and green bug (Sebesta and Wood, 1978) and Hessian fly (Mukai *et al.*, 1993), and Cereal cyst nematode (I. Dundas, personal communication) have been transferred into wheat.

Gene introgression using induced homoeologous recombination

The detrimental effects associated with large linkage blocks containing deleterious gene reduce the commercial importance of addition, substitution and translocation lines. The larger then necessary amount of alien genetic material can be minimised by inducing allosyndetic recombination between wheat and rye chromosomes. However, pairing is the prerequisite for achieving recombination (Sears, 1983).

The discovery of genetic control of pairing in wheat (Okamoto, 1957; Sears and Okamoto, 1958; Riley and Chapman, 1958) made it possible to develop improved and effective method for transferring genes from alien chromosomes. Several pairing controlling genes present on different chromosomes have been identified in wheat (reviewed by Feldman, 1993). The pairing promoter genes are present on short arm of chromosome 5 and long arm of chromosome 3 whereas the pairing suppressor genes have been reported on short arms of group 3 chromosomes and long arm of chromosome 5B. Control of meiotic pairing in wheat is

a complex process based on interaction between pairing suppressor and promoter genes (reviewed by Feldman, 1993). However, the *Ph1* gene located on 5BL (Riley and Chapman, 1958; Sears and Okamoto, 1958) near the middle of arm (Jampetese and Dvorak, 1986), about 1 centimorgan (cM) away from the centromere (Sears, 1985) seems to be the single gene with the largest influence on pairing. It is the major factor responsible for the cytological diploid like behaviour of polyploid wheat (Riley, 1960 b), suppress the pairing of homoeologous chromosomes despite their close cytogenetic relationship and promote the homologous pairing. The recent investigations on mode of action of *Ph1* gene suggest that *Ph1* effects the pre meiotic alignment of both homologous and homoeologous chromosomes and thereby controls the regulation and pattern of pairing (reviewed by Feldman, 1993). Due to its major role in meiotic pairing, *Ph1* gene is the one that has been used more extensively in gene transfer program involving allosyndetic recombination.

The utilization of the *Ph1* gene for gene introgression is usually conducted in different ways. One approach is to utilise the nullisomic condition of chromosome 5B. This is often made by using nullisomic-tetrasomic line, however a complex aneuploidy result from the use of this procedure. The second approach is to use *Ph1* mutants, where the gene have been made non-functional. Such mutants have been described by Sears (1985). The *Ph1* gene mutants in both hexaploid and (*ph1b*) and tetraploid (*ph1c*) wheats are available and can be used to replace the normal *Ph1* gene in order to activate homoeologous pairing and recombination. The utility of these mutants in alien gene tranfer has been demonstrated in many species (Sears, 1967; Riley *et al.*, 1968; Naranjo and Lacadena, 1980; Knott, 1981; Lu *et al.*, 1992; Ceoloni and Donini, 1992). The Sears *ph1b* mutant (Sears, 1977) has been used successfully in introgressing rye chromosome 1R into wheat (Koebner, *et al.*, 1986; Rogowsky *et al.*, 1993; Shepherd *et al.*, 1994), however no success has been achieved using chromosome 6R (Dundas *et al.*, 1992).

Another approach is to make crosses with alien species carrying the promoter genes that are epistatic to wheat meiotic pairing control system. In such cases homoeologous pairing occur even in the presence of *Ph1* gene. Situations have been reported in hybrids of wheat with *Aegilops* (Riley, 1966, Farooq *et al.*, 1990), *Agropyron* (Dvorak, 1987; Charpentier *et al.*, 1988), and rye (Lelley, 1976; Naranjo *et al.*, 1979; Jouve, *et al.*, 1980).

However the abovementioned approaches have difficulties in scoring of plants lacking the effect of *Ph1* locus. This demand quite a lot of cytogenetic work as meiosis has to be studied in order to keep track of *ph1b* mutants. Use of molecular markers have overcome this problem. Since the *ph1b* mutation represent an interstitial deletion on chromosome 5BL containing *Ph1* gene (Gill and Gill, 1991), several useful RFLP probes HVKSU75, TKSUS1 (Gill *et al.*, 1993), HVKSU8 (Gill and Gill, 1996), PSR1201 (Xie *et al.*, 1993) mapped in this region can provide an efficient scoring of plants with *ph1b* mutation. A PCR marker based on RFLP probe HVKSU8 have also developed provide an alternate mean of scoring (Gill and Gill, 1996).

Molecular (RFLP and PCR) markers are gaining importance because of their multidimensional applications. An over view on different moelcular markers and their use in gene mapping (particularly comparative mapping) and marker assisted selection is included in forthcoming section.

1.3.5 Genetic markers and their applications

Genetic markers have become an indispensible tool in plant genetic research and practical crop breeding programs. Both biochemical and molecular markers offer a diversified role. However, their major use to enhance selection in gene intrgression program is dependent on their relative location (order and map distance) on linkage map. Gale *et al.*, 1989, listed all possible aspects of chromosome manipulation in intervarietal and wide crosses where genetic maps and markers are required. Examples of biochemical markers are isozymes and protein markers. While, the advancement of DNA-based technologies provides a plentiful array of molecular markers includes RFLPs and Polymerase Chain Reaction (PCR) based markers such as RAPD, SCAR, SSR, SPLAT, and AFLP.

Isozyme markers

Isozyme studies in the Triticeae tribe have provided valuable insights into the phylogeny of genera and species (Asins and Carbonell 1986; Mc Intyre, 1988). Extensive use of isozyme markers in genetic analyses have been reported in wheat (*Triticum aestivum* L.) (Ainsworthy and Gale, 1987). Over a hundred isozymes and storage protein loci have been located on wheat chromosomes by various aneuploid analyses (reviewed by Hart and Gale, 1987).

Genetic maps based on seed protein polymorphisms and isozyme loci have also been reported in rye (Singh and Shepherd, 1984, 1988 a and b; Lawrence and Appels, 1986; Benito *et al.*, 1990; Vaquero *et al.*, 1990). Data on translocation and morphological markers have been reviewed by DeVeries and Sybenga, 1984, and Figuiras *et al.*, 1985. However, facts that isozyme markers demonstrate a low level of polymorphism and their use requires separate and different electrophoresis and staining techniques (Forster *et al.*, 1987), the need for a single technique of higher resolution highly exists. The use of DNA-based markers represents a new approach capable of overcoming this limitation.

RFLP markers

RFLPs have been used extensively to detect DNA polymorphism in plant species. The RFLP technique depends on the use of probes to identify single or low copy gene sequences in genomic DNA. In this method, polymorphism is relied on change in restriction endonuclease sites attributed to insertions or deletions in the sites or chromosoml rerrangements involving the

restriction sites. Polymorphism can be revealed by hybridising radioactive or non-radioactively labelled sequences of cloned genomic DNA (gDNA) or complementry DNA (cDNA) with known or unknown functions, using method of Southern (1975).

The efficiency of mapping largely depends on the level of DNA variation and the probe source (Liu and Musial, 1995). Predominantly cross-pollinating species such as maize (Helentjaris *et al.*, 1985) and pearl millet (Liu *et al.*, 1994), possess more abundant RFLPs than self-pollinating species such as wheat (Devos et al., 1992) and barley (Graner *et al.*, 1991), which contain fewer RFLPs. On the other hand, genomic clones detect a higher level of polymorphism in some species like wheat (Devos *et al.*, 1992), while cDNA clones are superior in other species like lentils (Havey and Muehlbauer, 1989).

RFLP markers have been used extensively in constructing detailed linkage maps of several crop species including maize (Coe *et al.*, 1993 ; Burr *et al.*, 1993), rice (Mc Couch, 1988; Kurata *et al.*, 1994, a; Causse *et al.*, 1994), barley (Graner *et al.*, 1991; Heun *et al.*, 1991; Klienhofs *et al.*, 1993), rye (Devos *et al.*, 1993; Philipp *et al.*, 1994, Loarce, 1996), wheat (Devos and Gale, 1993; Gale, *et al.*, 1995; Nelson, *et al.*, 1995; Cadalen, 1997) and *T. tauschii* (Gill *et al.*, 1991; Lagudah *et al.*, 1991). RFLPs have also been used in the analysis of phylogenetic relationships (Monte *et al.*, 1993).

RFLPs have several advantages as these are abundant, codominant, and phenotypically neutral. In addition to this, their ease of detection irrespective of the plant's developmental stage make them superior over isozymes. However, RFLP markers are time consuming and expensive to assay which make them unsuitable for large scale screening. Technical difficulties with their use in species with large and complex genomes like wheat and barley also indicate a need for an alternative method (Weining and Langridge, 1991).

PCR-based markers

Polymerase chain reactions (PCR) (Saiki *et al.*, 1988) offer a less technically demanding and more rapid methodology for cereal genome mapping (Williams *et al.*, 1990). The direction of mapping programs therefore tends to be focussed on the coversion of an RFLP- to a PCR-based assay (Olson *et al.*, 1989; Williams, 1994). However, DNA sequence analysis is required for designing primers and the polymorphism is usually low (Williams, 1994; Taylor, 1996).

The PCR technique is based on the use of oligonucleotides that anneal to the target sequences to prime the polymerase chain reactions. Polymorphisms are detected as differences in length of DNA sequences between two annealing primers or the absence of one or both primer annealing sites. Several marker have been developed based on polymerase chain reactions but these markers have had limited use in genetic mapping.

Random Amplified Polymorphic DNAs (RAPDs)

This method was introduced by Williams *et al.*, 1990, involves the random annealing of single primer usually 10mers to reveal dominant markers. Polymorphisms are monitored by the presence or absence of an amplified DNA fragment. As compared to RFLP assay, the protocol requires less DNA and no radioactivity. In contrast with isozymes, RAPDs provide accurate determination in pair-by-pair comparisons of relationships between accessions that are too close to be accurately resolved by isozymes (Heun *et al.*, 1994). However, RAPDs have problems of limited repeatibility and scoring error confounded with amplification of repetitive DNA sequences. Several reseachers are trying to address these problems through variations of the technique (Skroch and Nienhuis, 1995). However, for complex genomes, RAPDs have been shown to be unreliable and are not readily transferable between crosses (Langridge, 1994).

Sequence characterized amplified regions (SCARs)

Paran and Michelmore (1992) introduced Sequence Characterised Amplified Regions (SCARs) by sequencing the two ends of a RAPD fragment and synthesizing two longer (24 mer) primers homologous to each end. These primers could be used in more traditional PCR cycles to amplify RAPD fragments specifically and reliably.

Microsatellites or Simple sequence repeat (SSR)

Eukaryotic genomes are ubiquitously interspersed with simple sequence repeats (SSR) such as (GT)n or (CT)n also called microsatellites (Tautz and Renz, 1984). Their presence within stretches of unique DNA exhibits an extensive length variation easily detectable, using specific primers in the flanking regions of such loci and subsequent amplification via the PCR (Weber and May, 1989; Litt and Liuty, 1989). The high level of polymorphism combined with a high interspersion rate makes them an abundant source of genetic markers. Their codominant nature and potential for automated analysis make them superior over other PCR-based markers. In plants, microsatellite markers have been reported in soybean (Akkaya *et al.*, 1992; Morgante and Olivieri, 1993), *Brassica* (Lagercrantz *et al.*, 1993) and *Arabidopsis* (Bell and Ecker, 1994). Among cereals, rice was extensively investigated (Wu and Tanksley, 1993; Zhao and Kochert, 1993) but microsatellites were also reported in wheat (Plaschke, 1995; Devos, *et al.*, 1995 a; Roder, *et al.*, 1995), barley (Saghai Maroof *et al.*, 1994; Becker and Heun, 1995) and maize (Senior and Heun, 1993).

Amplified Length Polymorphism (AFLPs)

Vos *et al.* (1995), combinined the strength of different types of markers and inventented new type of markers generally known as AFLPs. The method to generate AFLPs involves the digestion of DNA with one or two restriction enzymes followed by ligation of suitable adapters

with fragments. These ligated fragments are then selectively amplified using different primers combinations. Polymorhic bands are separated as AFLPs on polyacrylamide gels. These markers allow efficient DNA finger printing and have been useful in genetic and taxonomic studies of cereals (Becker *et al.*, 1995).

A brief review on genetic markers outlined above indicate that RFLPs are by far the most appreciated markers because of their reproducibility and ability to show useful level of polymorphism within species and their ability to be transferred to different crosses and species. Thus RFLPs have been used most extensively in gene mapping, comaparative mapping studies and gene introgression programs

Mapping in cereals

A dense linkage map (physical or molecular) provide efficient and accurate cytogenetic manipulation for gene transfer and is very useful in planning the proper crosses to combine several genes of agronomic importance in one cultivar.

Genetic maps have been constructed in various cereals species using morphological, biochemical and molecular markers (reviewed in previous section). Physical maps can be constructed either by using molecularly based techniques such as the construction of cosmids, yeast artificial chromosome (YAC) libraries and long range restriction mapping (Cheung *et al.*, 1991), or by cytogenetically based techniques like *in situ* hybridisation (Leitch *et al.*, 1991), deletion mapping (Gill *et al.* 1993; Taylor, 1996; This study) and translocation mapping (Kim *et al.*, 1993). The existance of many intentionally constructed wheat alien addition, substitution and translocation lines (Shepherd and Islam, 1988) greatly aided in constructing the cytologically based physical map of wheat (Werner *et al.*, 1992) as well as genetic maps of various Triticeae members .

However, investigations relating genetical and cytogenetical distances of genetic markers for many species have shown a non-correspondance between the two types of measurements (Dvorak and Chen, 1984; Gill *et al.*, 1993; Taylor, 1996). A compression of proximal and expansion of distal regions on genetic maps is also well documented (Gustafson *et al.*, 1990; Gale *et al.*, 1992; Lukaszewski, 1992). This non correspondence between the physical and genetic maps of chromosomes is because of the chiasmata interference in proximal regions (Lukaszewski, 1995) and clustering of recombination events in the distal regions of cereal chromosomes. The proximal regions of cereal chromosomes contain repetitive sequences which account for major structural differences in related species.

Comparative mapping and coservation of synteny in cereal species

In contrast to repetitive sequences, cereal genes have been highly conserved in related species during evolution. About 44-100% of cDNA fragments cloned from one species cross-hybridise at moderate stringency to homologous squences in the related species (Hulbert, *et al.*, 1990; Kurata, *et al.*, 1994, b; Van Deynze, *et al.*, 1995). The sequences identified in two related species by a single cDNA probe represent the orthologous genes which are derived from a single sequence in an ancestoral species (Hulbert, *et al.*, 1990). The relative positions of orthologous sequences can be determined by mapping these sequences as RFLP markers using common sets of probes. This approach is called comparative mapping.

Comparative genetic mapping using common RFLP markers has shown a significant conservation in genomes of related, but sexually incompatible plant species such as tomato and pepper (Tanksley *et al.*, 1988) and tomato and potato (Gebhardt *et al.*, 1991). Comparative mapping is also becoming increasingly important in cereal breeding in determining the gene synteny on homoeologous chromosomes of related species. It allows an understanding of the relationships between genomes within polyploids, between species within tribes, and between species within families, and throw light on evolution of cereal chromosomes.

Rice, maize and wheat have been isolated by more than 60 million years of evolution. Because of its small genome size, rice can be an ideal tool to analyse genetic relationships within the Graminae (Poaceae). The genetic maps of rice chromosomes can be dissected into small linkage segments like lego blocks. By using these segments, it is now possible to reconstitute the genetic maps of wheat, barley and maize (reviewed by Moore, 1995). Comparative studies between rice, maize and wheat have indicated that extensive conservation of gene order exists, despite large differences in DNA content and different basic chromosome number (Ahn and Tanksley, 1993; Ahn *et al.*, 1993; Kurata *et al.*, 1994, b; Van Deynze, *et al.*, 1995).

The genetic map of hexaploid wheat is itself a comparative map and depicts the collinearity of markers within its three constituent genomes A, B and D (Gale *et al.*, 1992; Devos and Gale, 1992). However, certain evolutionary rearrangements are identified in homoeologous groups 4, 5 and 7 (Nelson, *et al.*, 1995). Within the Triticeae, comparison between the molecular maps of wheat, rye, barley (Devos, and Gale, 1993, b; Devos *et al.*, 1993;) has revealed that apart from major translocations, gene order is highly conserved.

Since that gene order is highly conserved among grass species, it is likely to prove cruicial in the application of map based cloning to isolate important genes whose introgression is difficult using routine breeding strategies. The detailed information on genome and gene organisation provided by molecular markers enhance their role in marker assissted selection and particularly in the analysis of alien chromosome segments. The markers provide good understanding of

problems associated with recombination and alien chromosome introgression (reviewed by Langridge, 1994; this study, Chapter 4).

Marker assisted analysis of alien chromosome introgression

Both isozyme loci and seed storage proteins have been reliably used in selecting recombinants in gene introgression program (Koebner and Shepherd, 1986; Marais, 1992; Islam and Shepherd, 1992). However, these are less polymorphic and limited in number donot allow selection along the entire length of alien chromosome. Molecular markers, RFLPs and PCR offer a powerful role in characterising wheat-alien recombinant involving any region of the homoeologous chromosomes.

Hospital, *et al.* (1992) proposed an optimal strategy for a hastened recovery of recombinant chromosomes using molecular markers in gene introgression program. They stressed on the use of markers mapped farther from gene of interest. According to them, a density of 2-3 markers per 100cM is sufficient to allow identification of recombinants after two generations of back-crossings. Similarly, use of distal markers is highly desirable in early generations of selection and proximal markers can be used in later generations as recombination events accumulate over time.

Particularly, RFLP markers have been extensively used in determining the alien genes from variety of related species including rye (Rogowsky *et al.*, 1993; Shepherd, *et al.*, 1994; Lelley *et al.*, 1995), *Agropyron intermedium* (Banks, *et al.*, 1995; Khan, 1996). Besides molecular, cytogenetic markers also provide efficient means of detection of alien chromatin introgressed in wheat genome. C-banding and *in situ* hybridisation detect the cytogenetical loci on metaphase spread of cereal chromosomes.

C-banding and insitu hybridisation (ISH) are sensitive tools for chromosome and genome identifications in plants. C-banding have been widely used to identify rye chromatin in wheat-rye amphiploids, chromosome addition and substitution and translocation lines (Gustafson, 1983; Lukaszewski and Gustafson, 1983; Lapitan *et al.*, 1984; Merker, 1984; Mukai *et al.*, 1992, Friebe *et al.*, 1993). Since the first report of Rayburn and Gill (1985) using biotin labeling and ISH to map a highly repeated DNA sequences of rye on wheat chromosomes, *in situ* hybridisation with biotinylated total genomic probes have been used to detect the presence of alien chromatin in wheat genome. (Lapitan *et al.*, 1986; Mukai and Miki, 1989; Anamthawat-Jonsson *et al.*, 1990; Mukai and Gill, 1991; Zhong *et al.*, 1991; Friebe *et al.*, 1992; Mukai *et al.*, 1992, Khan, 1996).

Chapter 2

Materials and methods

2.1 Seeds-Sources and History

All of the seeds stocks used in this project were recieved from Dr Ian Dundas, Plant Sciences Department, Waite Agriculture Research Institute, except Imperial rye, South Australian rye and Sears' *ph1bph1b* mutant which were kindly provided by Dr. K. W. Shepherd Plant Sciences Department, Waite Agriculture Research Institute. The details of seeds stocks are as following:

T701-4-6

This is a hexaploid triticale line originally bred by CYMMIT at Mexico. It has the parentage:KoalaXTCL MAYA II- ARM "S". The only rye named in its pedigree was "Merced". T701-04-6 is highly resistant to cereal cyst nematode, *Heterodera avenae*. (Fisher *et al.*, 1989) Resistance gene is localised on chromosome 6R (Asiedu *et al.*, 1990) and mapped specifically to interstitial region of its long arm (Dundas *et al.*, 1992;Taylor, 1996).

Disomic $6R^{T701}$(-6D) substitution-wheat line

This is a T701-4-6 derivative CCN resistant line developed by Asiedu *et al.*, (1990) after one back cross generation and one generation of selfing with Chinese Spring as a recurrent parent and T701-4-6 as a donar parent. This is a disomic line have a nullisomy for 6D substituted with a pair of triticale 6R ($6R^{T701}$).

$6RL^{T701}$ deletion length mutant lines

Dundas *et al.*, 1992 isolated several $6RL^{T701}$ deletion length mutant lines after screening critical F3 and F4 seeds derived from crosses between wheat-disomic $6R^{T701}$(-6D) substitution line and Sears' *ph1b ph1b* mutant (Aseidu *et al.*, 1990). Mutant lines were characterised using α-amylase, GOT and 6pgd isozymes. Hence CCN resistance gene was mapped on $6RL^{T701}$ using these deletion mutants and isozyme markers. Variant forms of $6RL^{T701}$ present as a telocentric or translocation were isolated from the selfed progeny of these mutant lines (Dundas *et al.*, 1993). Five different lines used in the present study are listed below (for detailed illustrations see Chapter 4 Figure 4.1):

1)2373-21XS-2XS-1XS-5XS-2

This is a translocation of whole chromosom arm $6RL^{T701}$ on 6DS of wheat, posses CCN resistance gene, *α-amy-R2, got-R2* and *6pgd-R2* loci.

2)657-3 X S-115

This is a $6RL^{T701}$ telocentric line carrying a deletion of long arm with missing of its distal region along half of the distal C-band. This line carries CCN resistance gene, *α-amy-R2*, and *got-R2* but defficient for *6pgd-R2* locus.

3)791-6-64-51XS-26

This is another $6RL^{T701}$ translocation carrying a deletion of long arm with two C- bands missing at terminal. Hence this line lacks little more of 6RL content as compared to 657-telodeletion line. It possess CCN resistance gene along with *α-amy-R2* and *got-R2* but defficient of *6pgd-R2* locus.

4)1411-54XS-1-1-146-3XS-2

This is a $6RL^{T701}$ translocation carrying a deletion of long arm with three C-bands missing at the terminal. It also carries CCN resistance gene along with *α-amy-R2* and *got-R2* but lacks *6pgd-R2* locus.

5)1411-95XS-16-4-57XS-4

This is a CCN susceptible $6RL^{T701}$ translocation line carrying the longest deletion of $6RL^{T701}$ fragment which confers the loss of CCN resistance character as well as *got-R2*.

Wheat-$6RS^{T701}$ditelosomic addition and wheat-$6RS^{T701}$ tritelosomic adition lines

These are also $6R^{T701}$ mutant lines isolated during screening of selfed progenies of 6RL deletion mutant lines (Dundas *et al.*, 1993). These mutant lines possess short arm of $6R^{T701}$ as a telosome in two and three doses, respectively in a mixed wheat background. Hence these can be ideal tool to localise genes to short arm of 6R.

Chinese Spring wheat-$6R^{Imp}$ disomic addition line and other rye chromosome addition lines:

These are wheat-rye addition lines originally produced by Driscoll and Sears (1971). The Chinese Spring wheat line possesses an additional pair of 6R of Imperial rye and the remaining lines possess an addition of pair of each rye chromosomes 1R, 2R, 3R, 4R and 7R.

Chinese Spring wheat and Schomburgk wheat:

These are CCN susceptible control wheat varities. Chinese Spring is a euploid hexaploid spring wheat. Schomburgk wheat is a backcross derivative from the hexaploid wheat cultivar Aroona selected by Dr A J Rathjen, Waite Agriculture Research Institute (Mackay, 1987).

Chinese Spring Sears' *ph1bph1b*:

This is a mutant line (Sears, 1977) of wheat cultivar Chinese Spring which is deficient for the *Ph1* locus on 5BL (Sears, 1981).

Imperial Rye and South Australian Rye:

South Ausralian rye is a rye cultivar which is known to have a high resistance to *Heterodera avenae* (Brown, 1974). Imperial rye which is also a rye cultivar has a susceptiblity to cereal cyst nematode attack.

2.2 Crossing

All crossess were made between plants growing in Containment glasshouse at approximately 22°C under natural and sometimes in artificial light.

2.3 Cytological analysis of chromosomes

2.3.1 Mitosis

Seeds were soaked on moist filter paper in sterile petri dishes at 25°C for one day then shifted at 4°C for overnight to break the dormancy. Seeds were brought back at 25°C and germinated until the seminal roots were approximately 1-2 cm long.At least two roots were cut off and prefixed in ice cold water at 0°C on ice in cold room (circa 4°C). After 26 hours these roots were fixed in a 3:1 mixture of ethanol (99.8%) and glacial acetic acid for one hour and if not proceed for Feulgen staining, was stored at -20°C for several months.

Feulgen Staining

For Feulgen staining, 1 gm of basic fuchsin was gradually dissolved in 200ml boiling distilled water on hot plate with continous stirring. Solution was cooled to 50°C and filtered. 30 ml 1NHCl were added to filterate followed by 3g potassium metabisulphate. Solution was stored in an air tight dark container at 4°C.

Fixed root tips were washed once in distilled water to remove the fixative. These were hydrolysed with 1NHCl (60°C) for 8 mins. After hydrolysis these were rinsed once with distilled water to remove excess water and stained with Feulgen stain.

2.3.2 Meiosis

An immature spike was removed when the distance between flag leaf and boat leaf was 2 inches. Starting from the middle an anther was removed from secondary floret and placed in a drop of one percent acetocarmine on a glass slide. Pollen mother cells were gently teased from the anther with the help of pair of fine needles. Cover slip was applied and silde was warmed gently over the flame. Then pressed hard between layers of filter paper to spread the cells. Cells were observed under 40X and 100X (oil immersion). Coverslip edges were sealed with nail enamel to prevent drying and slides were stored at 4°C.

2.4 Isozyme analysis

2.4.1 α-amylase analysis by Isoelectric focusing

Isozymes of α-amylase were separated by isoelectric focusing of endosperm samples of 5-day-old seedlings using the method of Gale *et al.,* (1983). Chemicals and solutions used in this experiment are described in (Appendix 1).

Gel Preparation

The horizontal slab gel was made by mixing solutions and polymerising them between two glass plates of different thickness. Prior pouring the gel solution, the plates were cleaned with pyroneg and ethanol. Repel silane (LKB#1850-252) was applied to large glass plate to avoid the stickness of gel while removing process and silane to thin galss plate. Plates were allowed to dry and leave in a warm place until ready. Chemicals were added (as given in Appendix 1) and gel solution was poured. Let it stand for 10 mins and then checked for polymerisation. After 20 mins the upper glass plate was carefully removed.

Preparation of Samples

Endosperm halves were excised carefuly from the germinating seedlings when the coleoptiles were about 2cm long. At sometimes these halves were also stored at -20°C until required. Samples were extracted by squashing in 50 ml $CaCl_2$ (1mM) with the help of sterile knitting needle in eppendorf tubes. Later heated in water bath at 70°C for 10 min. After that samples were centrifuged at 12,000 rpm for 2 min.

Gel Run

Thermatic Circulator was cooled to 4°C. Electrode wicks were soaked thoroughly with electrode buffers then carefully blotted on the piece of paper towel to avoid excess of buffer solution. These were applied on terminals of gel. Gel plate was then placed on cooler plate according to right orientation. Voltage was set at 2000 volts, Current to 50milli Ampere and Power to 10Watts. Gel was prefocussed initially for 30min and then samples were loaded using 10X3mm wicks (Whattman #3MM) 5-6µl per wick. Gel was allowed to run and it was then discontinued after 30min to remove the wicks. Run was started again and finally stopped after 1.5hrs. Gel was removed and then proceed for staining to observe the results.

Gel Staining

For staining gel was first overlayed smoothly with 40-50°C warm starch solution for 1-2 min. Carefully washed under running tap water and then IKI (staining solution) was poured over the gel in tray. As the bands started appearing, fixed with 10% acetic acid and bands were immediately scored. The gels were covered with plastic film and stored at 4°C in refrigerator.

2.4.2 Glutamate Oxaloaceate Transaminase

The analysis for isozyme glutamateoxaloacetate transaminase (GOT) was carried out by using the procedure adapted from Hart (1975) and modified by Asiedu (1986). The chemicals and solutions used in the procedure are given in the Appendix 2. The recipie used for the preparation of gel is also given in the same appendix.

For an early screening of GOT, 5-days old leaves were taken as samples. These were extracted by grinding in Carlson's buffer+Dithiothreitol (DTT) (approximately 60 ml per 20 mm leaf) in an eppendorf tube. Samples were then centrifuged at 12,000 rpm for 2min. Supernatants from the samples were applied to wells in the stacking gel. Gel apparatus was transfered to fridge at 4°C. The power pack was connected as cathodal eletrode at top and anodal electrode at bottom. The gel was run for about 1.5 hours or until green front moves off the gel at the bottom. For visualising the results, the gel was stained by dissolving α-ketoglutaric acid (*Sigma # K-1875*) and L-aspartic acid (*BDH # 37021*) in a solution of Phosphate buffer (PO4) (1M pH 7.0). Fast blue and Pyridoxal-5-phosphate was added just before staining. The proportions of chemicals used in making staining solution is given in Appendix 2. The gel was stained in dark. After 5 mins the bands were scored for the presence or absence and the number of dose of chromosome 6R. The gel was fixed with 10% acetic acid, sealed in plastic bag and keep at 4°C.

2.5 Bacterial strains, Plasmids and Clones

Bacterial strains containing plasmids and cloned inserts were grown in Luria-Bertaini (LB) media (Sambrook *et al.*, 1989) in the presence of antibiotics (required). Ampicillin was included at 50 μg/ml.

2.6 Isolation and purifiction of DNA

2.6.1 Cereal genomic DNA isolation

DNA was extracted preferably from fresh, young and healthy leaves of growing plants at the stage of tillering. However, plants leaves were collected also, sealed in plastic bags, freeze in liquid nitrogen and stored at -20°C or -80°C until use. DNA was extracted on large (maxi), medium (midi) and small (mini) scale depending on the requirement of sample. Methods of maxiprep, midiprep and miniprep of isolation are described in detail by Langridge, *et al.*, 1992.

2.6.2 Plasmid DNA isolation

Plasmid DNA was isolated on small scale from 2ml overnight grown culture by the alkaline extraction method as described by Birnboim and Doly (1979). At some stages, this method was scaled-up to maximise the yield of plasmid DNA.

2.7 Gel electrophoresis

To visualise the quality of DNA, gel electrophoresis was carried out through 0.8-3.0% (w/v) agarose horizontal slab gels. Samples were mixed with 1/10 volume of 6X ficoll loading buffer (100 mM Tris-HCl, 200 mM EDTA, 15% Ficoll type 4000, 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-acetate (TAE; 40 mM Tris-acetate, 1mM Na2 EDTA [pH 8.0]) or Tris-borate (TBE; 90mM Tris-borate, 1mM Na2 EDTA [pH 8.0]) for PCR analysis. DNA was visualised by staining the gels subsequent to electrophoresis in a solution of eithidium bromide (0.5 μg/ml). Gels were photographed either using a Polaroid landpack camera and Polaroid 665 positive film. Different markers were used as molecular weight standards viz:

Lambda DNA restricted with *HindIII*
pTZ18U restricted with *DraI* and *RsaI*
pUC19 restricted with *MspI*

2.8 Restriction endonulease digestion

Plant genomic DNA and the plasmid DNA were digested with restriction endonucleases (restriction enzymes) as recommended by the manufacturer. Typically 5-10 µg of genomic DNA was digested with 20 units of restriction enzyme in a 10 ml of reaction volume at 37°C in water bath for 2-6 hours. The reaction voulme was scaled-up when a larger amount of DNA was required to be digested.

2.9 Southern Hybridisation

Approximately 5-10 µg of DNA was digested to completion as explained above and separated by electrophoresis in 1.0% agarose gels in TAE buffer for 16 hrs at 25 mA (2.0 V/cm). The gels were then stained with ethidium bromide (0.5 µg/ml) for 10 mins on a rocking platform, the DNA visualised on a short wave ultraviolet transilluminator and photographed. Gels were then proceed for denaturation in a 200 mls of denaturing solution (1.5 M NaCl, 0.5 M NaOH) by shaking at a rocking platform for 20 mins. Denaturing solution was poured off and gels were rinsed in 10x SSC for 2 mins to washoff the excess of denaturing solution. DNA was transfered to nylon membrane (Hybond N+ Amersham) using 10x SSC. Assembly for Southern transfer was followed as described by Langridge et al., 1992. DNA transfer was carried out for 4 hrs to overnight. Blots were disassembled and membrane were rinsed for 2 mins in 5x SSC and air dried by lying DNA-side up on Whatman 3 MM paper. DNA was fixed to the membranes by placing them on the pad of Whatman 3 MM paper densly saoked with 0.4 M NaOH for 20 mins at RT. Membranes were neutralised in 50 mls of neutralising solution (1.5 M Na Cl, 0.5 M Tris-HCl, 0.001 M Na2 EDTA, pH 7.2) for 5 mins and further rinse in 2x SSC for 5 mins. Blot dried among the stack of Whatman 3 MM paper, air dried, sealed in plastic bag and stored in dark in refrigerator until use.

Hybridisation of 32P-labelled probes to N+ membranes was carried out as follows: membranes were soaked in 5x SSC for 1 min and laid flat on a lid of lunch box, rolled-up into a cylinder of about 2cm diameter and placed inside the glass hybridisation bottle (HY-OUBLA, Hybaid). An alternative approach was taken for multiple membrane hybridistions. Membranes were incuabted at 65C in shaking water bath for 30 mins with 100 mls of pre-pre-hybridisation solution(5x SSC, 2% [w/v] ficoll 400, 2% [w/v] polyvinyl-pyrrolidone 360 (PVP), 2% [w/v] BSA, 1% SDS, 5 mg/ml salmn sperm DNA) After that removed and rolled each one over the next into a cylinder of about 2 cm diameter. Excess liquid was drained off using the absorbent paper and then proceed for pre-hybridisation. Ten millilitres of pre-hybridisation solution (3 M Na Cl, 100 mM PIPES, 25 mM Na2EDTA [pH 6.8], 2% [w/v] ficoll 400, 2% [w/v] polyvinyl-pyrrolidone 360 (PVP), 2% [w/v] BSA, 1% SDS, 5 mg/ml denatured salmon sperm DNA) prewarmed to 65°C was added to each hybridisation bottle. These bottles were placed in

rotating oven (Model HB-OV-1, Hybaid) at 65°C in reverse orientation so the membranes will not be unrolled. After 20 mins the orientation of bottles were reversed and membranes were allowed to unwind. Pre-hybridisation was resumed for 2-6 hrs at 65°C. Denatured 32P-labelled probe was then added and bottles were returned to oven to incubate overnight.

Hybridisation solution was then poured off and membranes were washed with 40 ml of 2x SSC, 0.1% SDS for 20 min in oven. Membranes were then removed and washed successively with 200 ml of the following buffers in a shaking water bath (100 rpm) at 65°C;

2nd wash 1x SSC, 0.1% SDS for 20 min
3rd wash 0.5x SSC, 0.1% SDS for 15-20 min
4th wash 0.2x SSC, 0.1% SDS for 10-15 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.

After autoradiography, labelled probe DNA was stripped from membrane by pouring boiling stripping solution over the membranes and leave it for 30 mins on rocking platform at room temprture. Membranes were then checked for signal, if any left, this step was repeated and membranes were then sealed in plastic and stored at 4°C.

2.10 Oligolabelling of nucleic acids

2.10.1 Oligo labelling of DNA probes

The random priming method described by Feinberg and Vogelstein (1983) was used to label DNA probes with [α-32 P] dCTP (3000 Ci/mmol, Amersham). Each reaction was carried out in a 25 µl volume containing approximately 50-100 ng of denatured DNA templates, 12.5 µl of oligo labelling mix (0.3 µg Primers, 60 µM dATP, dTTP, dGTP, 150 mM Tri-HCl [pH 7.6], 150 mM NaCl, 30 mM MgCl, 300 µg/ml BSA), 9-mers random primer mix (0.1µg/ml) [synthesised on an Applied Biosystems 391 DNA synthesiser] and 1 U of Klenow enzyme (Boehringer Mannheim or Pharmacia). Reactions were incubated at 37°C for 1 hr. 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 geiger counter and identifying the peak relating to the labelled fraction compared with that relating to unincorporated nucleotides.

2.10.2 Oligo labelling of RNA probes

A method based on initial reaction of RT-PCR reaction (Li *et al.*, 1997) was employed to label total RNA as a probe via its conversion into cDNA by using Super Script II RNAase H- Reverse Transcriptase (RTase) (Gibco-BRL). Reactions were carried out in a 20 µl volume, containing approximately 5-10 µg of total RNA, 100 ng oligo dT, 10 mM of each of dATP, dTTP, dGTP and 0.1 mM of dCTP, 4 µl 5x buffer (50mM Tris HCl pH3.8, 75 mM KCl, and 3 mM $MgCl_2$), 3 µl 32P dCTP, and 2 µl RTase. Initialy samples were incubated without RTase at 45°C in water bath for 2 min. Two µl of RTase was then added and incubation resumed at 45°C for 1 hr. Labelled probe was collected by monitoring column with geiger counter as described above in the section 2.10.1.

2.11 Polymerase Chain Reaction (PCR)

2.11.1 RFLP Probe preparation

Probes for RFLP analysis were prepared by amplification (of the cloned inserts) using the polymerase chain reaction with the M13 forward and reverse primers. The following sequences were used for the primers:

M13 Forward:	GTA AAA CGA CGG CCA G
M13 Reverse:	CAG GAA ACA GCT ATG AC

One µl of 1:20 or 1:100 dilution of miniprep plasmid DNA was included in a reaction volume of 50 µl which included: 0.15 µg/ml of M13 forward and 0.15 µg/ml of M13 reverse primer, 25mM of Mg Cl2, 200 mM of each of the nucleotides dATP, dTTP, dCTP and dGTP, 1/10 volume of 10x reaction buffer (Promega), 1/10 volume of Glycerol and 0.5 units of Taq polymerase (Promega) and nanopure water , overlaid with approximately 30 µl paraffin oil and placed in a programmable Thermal Cycler (MJ Research Inc., Watertown, Mass.). DNAs were denatured initialy at 94°C for 2 min, followed by 35 cycles consisting of 1 min denaturation at 94°C, 2 mins annealing with primer at 55°C and 2 mins extension at 72°C were performed prior to a final extension step of 5 min at 72°C and cooling to 25°C. Analysis was performed on 1% TAE agrose gels as described in Section 2.7.

Similar protocol with some modifications was followed for analysis of cDNA library and check the insert size. Differential plaques were cored out with sterile pasteur pippette and transferred to 100µl SM buffer. Phages were allowed to diffuse for one hour at 25°C. Afterwards 30µl of the soluble plaque was denatured for 5 mins and spin at full speed. Of this 5µl of supernatant

was taken in total volume of 50µl and proceed for the polymerase chain reaction, as described above. For PCR of inserts from λgt10, the following primers were used:

λgt10 Forward:	GCA AGT TCA GCC TGG TTA AG
λgt10 Reverse:	TGA GTA TTT CTT CCA GGG T

2.11.2 PCR amplifiction of plant genomic DNA

PCR reactions were carried out to amplify plant genomic DNA so the polymorphism could be revealed. The method used was similar as described by Williams *et al.*, 1990. The total volume of reaction was 25 µl and reaction conditions were similar as mentioned above. Since a variety of primers including random primers, specific primers and Intron Splice Junction (ISJ) primers were used, minor modifications were adopted to the protocol. In a standard reaction, annealing temperature was varied by using a range of 45°C-58°C. Variations in the number of reaction cycles was sought also. Where necessary, template DNA was predigested with restriction endonuclease according to manufacturer's recommendations prior to use in reaction. Specific details are given in Chapter 3, Table 3.1. PCR products were analysed on 3% TBE gels as described in section 2.7.

2.12 Recovery of DNA

2.12.1 Using Glass-Milk

DNA probes as amplified inserts from PCR reactions were recovered following gel electrophoresis and visualisation of gel on a long wave ultraviolet transilluminator (340 nm). The required band of insert .was excised using a sterile scalepel. The agarose block was transferred to 1.5 ml tube, weighed and DNA recovered according to standard protocol supplied with the Geneclean kit (Bio 101) [Bresatec, Australia]. The quantity of DNA isolated was established usually after gel electrophoresis.

2.12.2 Using Spin Clean TM

cDNA was concentrated and recovered by using Spin-Clean kit (Wizard probe, Spin clean^{TM}) according to standard protocol supplied with the kit. To one volume of cDNA, 4 volumes of binding buffer (?) was added and loaded on spin clean columns suspended in sterile 2 ml eppendorf tube. Column was centrifuged at full speed for 2 mins. Flow throw was discarded. Column was washed three times by adding 500µl of isopropanol, centrifuging at full speed for 2 mins and discarding flow throw each time. After third wash, 20µl of nano pure H_20 was

added to saturate the matrix of column and DNA was eluted by centrifugation at full speed. This step was repeated. Final volume of cDNA collected was 40µl.

2.13 Construction and analysis of cDNA library

2.13.1 Total RNA extraction

Total RNA was extracted from roots of 12 days old germinating seedlings. About 2cms of roots was cut, weighed and 3gms of root material was proceeded for RNA extraction. Roots were quickly ground to fine powder in mortar frozen with liquid N2. Powder was homogenised to slurry by adding 4mls of RNA extraction buffer (REB=100mM Tris-Hcl, pH 8.4, 4% sarkosyl, 10mM EDTA) buffer. The slurry was transferred to sterile 15ml corex centrifuge tube. Sample was then centrifuged in a JA20 rotor at 4°C for 10 mins at 5000 rpm. Exactly 3mls of supernatant was transferred to 15ml corex centrifuge tube containing 3.5gm of $CsCl_2$ previously baked at 160C. Tubes were vortex for 10sec to mix solution and $CsCl_2$ and transferred to RNA centrifuge tubes (ultra centrifuge tubes) cotaining 3ml CsCl2 cushion. The $CsCl_2$ cushion was prepared by adding Tris EDTA buffer (TE= 10mM Tris-HCl, pH 7.5 + 1 mM EDTA) to 9.65gm baked $CsCl_2$ to a final volume of 10ml. The ultracentrifuge tubes containing RNA solution were centrifuged at 40,000 rpm for 18 hrs at 4°C. After the run, sticky layer was removed carefully with the help of sterile cotton bud and supernatant was poured off carefully without disturbing the pellete. The tube walls were also wiped to remove any extra debris. Pellet was resuspended in 400µl REB and transferred to a fresh sterile 1.5ml eppendorf tube. Proteins were separated by adding 400µl Phenol/chloroform solution and centrifugation at 12,000 rpm for 5 mins. Upper phase was transferred carefully to a fresh tube and 40µl of 3M sodium acetate (NaAc pH 4.8) and 99% ethanol were added. Samples were left at -20°C for overnight. Then centrifuged at 12,000 rpm for 15 mins. Supernatant was discarded and pellet was washed with 1ml of 70% ethanol. Another 300µl TE and 30µl 3M NaAc were added. Sample was mixed gently and then 99% ethanol was added to finally precipitate the RNA. Sample was centrifuged for 5 mins and pellet was washed twice with 1ml of 70% ethanol. Sample was briefly dried under vaccum and resuspended in 30µl TE buffer.

2.13.2 RNA gel electrophoresis

A gel system based on 2 M formaldehyde and 50% formamide was used to visualise RNA. 0.6 gm agarose (SEAKEM GTG) was weighed and 45mls of DEPC treated water was added. Agarose was dissolve by boiling in microwave. Cooled down to 55C and 5ml of 10XMOPS EDTA buffer(0.5 M MOPS, 0.01 M EDTA, pH 7.0), 1.5ml of 37% Formaldehyde were added. Gel was poured and left at room temprature for 1 hr. After that gel was prerun for 30mins to get rid of any contaminants at 60 volts. Meanwhile RNA samples were prepared by

aliquoting 5µg of RNA and 2µl of 10XMOPS EDTA buffer and 13.5µl of a solution of formamide/formaldehyde/H20 (3.5:0:3.5) was added. RNA was gently dissolved by heating at 65-70°C for 10 mins and then cooled on ice and 1 µl gel loading buffer (322 µl buffer A+ 5mg xylene cyanol+ 5 mg bromocresol green+ 78µl 37% formaldehyde and 500µl formamide+400 mg sucrose) was added. RNA sample was loaded on the gel, initially ran for 30 mins at 60 volts then 100 volts for 1-2 hrs. Gel was then rinsed with DEPC treated water for about 10 mins and stain in 0.1 mg/ml eithidium bromide for 10 mins. Destained for 30 mins in water and then visualised on long wave 360 nm UV transilluminator and photographed.

2.13.3 mRNA synthesis

Total RNA was converted to mRNA by using mRNA synthesis kit (Promega), according to manufacturer's advice. Volume of total RNA extracted was raised to 500µl by adding DEPC treated nanopure water. Solution was heated at 65°C for 10 mins and then 3 µl dT (biotinylated) oligo and 13 µl 20XSSC were added. Sample was left to cool down at room temprature. Meanwhile SA-PMPs were washed thrice with 400µl 0.5XSSC. SA-PMPs were suspended in 100µl 0.5XSSC and entire content of RNA solution was added. Solution was mixed gently and left at room temprature for 10 mins for adsorption. SA-PMPs were collected through magnetic power. Supernatnt was collected and stored at -20°C until the surity of result. SA-PMPs were washed four times with 0.1XSSC (300µl) after final wash. Particles were suspended in 100µl DEPC treated nanopure water. SA-PMPs were collected again through magnetic power and mRNA was separated in aqueous phase and transferred to fresh RNAse free tube. This step was repeated for final yield of 250µl reaction mixture. To this, 25µl 3M NaAc and 250µl isopropanol was added and incubated at -20C for precipitation of mRNA for overnight. Sample was centrifuged at 12,000 rpm for 15 mins at 4°C. Supernatant was discarded and pellet was washed twice with 75% ethanol. Dried in vaccum and dissolved in 10 µl nanopure water and then stored at -80°C.

2.13.4 cDNA synthesis

cDNA was synthesised from poly A+mRNA using cDNA synthesis kit (Time saver, Pharmacia) according to manufacturer's protocol. mRNA sample was first thawed on ice and heated at 65°C for 10 mins and then chilled on ice. mRNA content was added to first strand reaction mix (reverse transcriptase) along with 1 µl of DTT (Dithiothreitol) and 1 µl of pdN6 primer. Reaction contents were mixed by pipetting up and down and incubated at 37°C for 1 hour. The entire content was then trnsferred to second strand reaction mix (ribonuclease H and DNA polymerase I), gently mixed and incubated initially at 12°C for 30 mins and then shifted to 22°C for one hour. Spun columns were prepared with due care according to manufacturer's advice. Columns with their tips immersed in 1.5 ml eppendorf tube were suspended in 15 ml

sterile corex tube and centrifuged at 1500 rpm for 2 min. Sample was applied in the center of column and proceed for centrifugation with a fresh eppendorf tube. The purified cDNA was collected and proceed for adaptor addition.

2.13.5 Addition of adaptor *Eco* RI/*Not* I

In a clean sterile eppendorf tube, 3 µl diluted adaptor solution, 30 µl PEG (20% poly ethylene glycol 6000, 2M NaCl) buffer, 1 µl ATP solution and 1 µl T4 DNA ligase were added to cDNA content. Sample was mixed gently, briefly spinned and incubated at 16°C for 1 hour. Samples were then heated at 65°C to denature DNA ligase and chilled on ice. To this reaction mixture, 1.5 µl ATP solution and 1 µl T4 PNK (polynucleotide kinase) was added, gently mixed and incubated at 37°C for 30 mins. Sample was heated at 65°C for 10 mins and chilled on ice. 140 µl phenol:chloroform:isoamylalcohol (25:24:1) was added and vortex and then centrifuged for 1 min. The supernatant was removed and applied to spun column and the adaptor ligated cDNA was recovered by spinning the columns as described in section above. Effluent was collected in sterile eppendorf tube and 100 µl ligation buffer (66mM Tris-Hcl, pH 7.6, 0.1 mM spermidine, 6.6 mM $MgCl_2$ 10 mM DTT and 150 mM Na Cl) was added and sample was stored at -20°C.

2.13.6 Cloning into λgt10 vector

For the construction of library, cDNA from wheat-$6R^{T701}$(-6D) disomic substitution was cloned into λ gt10 (*Eco* RI arms). In total 10 µl reaction, 1 µl of each of λ gt10 *Eco* RI arms, 10x ligation buffer and T4 DNA ligase was added to 7 µl of double stranded cDNA. The sample was incubated at 15°C for 2 hrs. The packaging extract (Promega) was thawed on ice quickly and entire ligation reaction was added into 50 µl packaging extract. Sample was incubated at room temprature for 3 hrs. Phage buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl and 10 mM $MgSO_4$) was added to make a final volume of 500 µl and overlaid with 25 µl chloroform. The reaction was genly mixed by inversion and chloroform was allowed to settle. A dilution series was prepared from 1:10 to 1:1,000 for each packaged phage preparation using SM buffer (Phage buffer+0.1% gelatin).

A bacterial strain LE392 was grown by innoculating a single colony into 50mls LB medium (Luria-Bertaini) (1% bactotryptone, 0.5% bacto-yeast extract, 0.5% NaCl) (pH 7.5) supplemented with 1% 20% maltose and 1%1M $MgSo_4$. Bacterial cultures were shaken overnight at 37C and next morning fresh cultures were started by innoculating 1 ml of overnight grown culture into 50 mls supplemented LB medium. When the bacterial density was reached to 0.6 at O. $D._{600}$, bacterial cells were stored at 4°C. To 100 µl of each diluted phage, 100 µl of prepared bacterial cells was added and phage was left for 30 mins at 37°C to get adsorbed. 3 mls of molten (45°C) TB top agarose (0.5% yeast extract, 1% bacto-tryptone, 0.5% NaCl,

0.7% agarose (pH=7.0) was added, gently vortexed and poured immediately on LB (medium) plates. Top agarose was allowed to harden and incubated overnight inverted at 37°C. Number of plaques were scored and titre of the phage was calculated. On the basis of inferences drawn from calculations, the library was immediately plated on ten 15cmØ plates at a density of 20, 000 pfu/plate.

2.13.7 Differential screening

For differential screenig, two sets of membrane were prepared by hybridising phage DNA with hybond N+ membranes (circle) . The plate was dried in a laminar flow hood for 5 mins and then membrane was crefully laid over the plate. Four marks on the outer edge of membranes were made using a 18 gauge sterile needle. The transfer was allowed to continue for 10-20 seconds for first membrane for 30 seconds to 1 min for second membrane, sequentially. Membranes were then lifted off and transfered on filter papers (3MM) soaked with denaturing solution and left for denaturation of plaques DNA for 2 mins. Then transferred onto filter papers (3MM) soaked with neutrilising solution and left for another 2 mins. Membranes were then rinsed with 2XSSC for 2-3 mins on shaker, dried between sheets of filter papers and fixed with ultraviolet radiations for 2 mins.

The membranes were used in hybridisation studies with labelled cDNA probes (prepared as described in section 2.10.2) of Schomburgk and wheat-$6R^{T701}$ (-6D) disomic substitution line according to Southern hybridistion method described in Section 2.9. The solutions used in the plaque DNA transfer were also the same as used in Southern transfer (Section 2.9). Membranes were then autoradiographed for five days. Results were obtained and compared. The experiment was repeated and membranes were used in alternate manner after stripping of probes. The plaques that gave differential signals that is hybridised only to wheat-$6R^{T701}$ (-6D) disomic substitution line were selected as putative 6R clones. Further classification of these plaques were made by cross-hybridisation studies.

2.13.8 Cross-hybridisation

Freshly grown (4 hours) competent cells were mixed with Top agarose and plated on LB medium plates. When the agarose get hardened, the putative clones (2 µl) were spotted on the plate and incubated at 37°C for 6 to 12 hours. Plaque lifts were prepared as described in section above. The membranes were used in hybridisation studies with labelled probes (Section 2.10.1) of selected insert ($6R^{T701}$-putative clones) according to the method described in Section 2.9.

Chapter 3

A comparison of rye chromosome 6 (T701) and wheat group 6 chromosomes based on molecular markers

3.1 Introduction

DNA based molecular markers reveal abundant polymorphisms thus they are very useful in mapping genes of interest and much research has been directed at the production of dense genetic linkage maps in many crop species including members of the Triticeae (reviewed in Section 1.2.4 of this thesis). The applications of DNA markers and maps include marker-assisted selection in plant breeding programs. In recent years the utility of RFLP maps has been recognised in comparative genome analyses of cereals (reviewed by Moore, 1995, Devos *et al.*, 1995). A comparison of genomic maps based on homoeologous DNA probes that identify related genes and sequences across groups of distantly related crop species, provides an understanding of homoeologous and evolutionary relationships and may shed light on ancestoral chromosomal rearrangements. Comparative mapping experiments have shown that *Secale cereale* genome has undergone multiple chromosomal rearrangements during its evolution from the basic Triticeae genome (Devos *et al.*, 1993). Such a finding has a direct implication in wheat breeding programs where one seeks to introgress rye genes through induced homoeologous pairing.

This project was aimed to introgress a cereal cyst nematode (CCN) resistance gene from the triticale chromosome 6R into the wheat genome. Homoeology between two genomes is a prerequisite to achieve such a goal. Earlier investigations based on chromosomal substitution compensation tests proposed that 6R efficiently compensate the loss of any group 6 chromosome in wheat (reviewed by Miller, 1984). However, analysis of homoeologous pairing between wheat and rye chromosomes indicated that rye 6R shows a large degree of homoeology with wheat group 6 chromosomes but also exhibits affinity with group 2, 3, and 7 chromosomes (Naranjo *et al.*, 1987). Considering chromosome arms, a substantial amount of pairing was observed between 4RL/6WS, 3RL/6WL, and 6RL/7WL whereas 6RS/6WS and 6RL/6WL paired infrequently (Naranjo and Fernandez-Rueda, 1991). Evidences of localisation of biochemical markers (Ainsworth *et al.*, 1985; Jouve and Diaz, 1990; Benito *et al.*, 1991) and molecular markers (Liu *et al.*, 1992; Devos *et al.*, 1993; Martinez *et al.*, 1994) eventually confirmed the rearranged structure of 6R.

It is now well established that the present conformation of 6R consists of a reciprocal translocation of 3L in interstitial and a non-reciprocal translocation of 7L in distal regions of the

Table 3.1: Primers screened against triticale T701-4-6, wheat and rye lines.

S. No	Primers	Size	Primer Sequences
1	A1	19 mer	5' GCA CGC CGG CGG GTG GTA C 3'
2	B1	20 mer	5' GAG CCC AGA ACG ACG CCC GG 3'
3	C1	18 mer	5' GAC CGT CAT TGC TCT CTT 3'
4	C2	18 mer	5' GGC CCA ATG TTC TTC TCT 3'
5	C5	18 mer	5' GCC TCA GAT TCA ATC ACC 3'
6	E3	15 mer	5' GAA TTC CAG GTA AGT 3'
7	E4	15 mer	5'CGA ATT CCA CCT GCA 3'
8	L1	24mer	5' CTT ATG AGT ATT TCT TCC AGG GTA 3'
9	R1	18 mer	5' TCG TGG CTG ACT TAC CTG 3'
10	R2	18 mer	5' TGC T_G[1]T_G[1]T_G[1] T_G[1]T GCA GGT 3'

Table 3.2: Summary of the results of PCR reactions carried out against triticale T701-4-6, Imperial rye, South Australian rye, Chinese Spring, Schomburgk, wheat-$6R^{T701}$(-6D) disomic substitution, Chinese Spring-$6R^{Imp}$ disomic addition, wheat-$6RS^{T701}$ disomic and trisomic addition lines and 6RL length mutants. Primers, their combinations and conditions in PCR reactions are also specified.

S. No	Primers	Annealing Temprature	Number of PCR Cycles	Amplification Products observed			
				wheat	rye	$6R^{T701}$	$6R^{Imp}$
1	C1 and C2	50	35	N	Y	N	N
2	C2 and C5	55	36	N	N	N	N
3	E3 and E4[1]	58[1]	34[1]	N	N	N	N
4	A1 and R1[2]	56[2]	36[2]	Y	N	N	N
5	B1 and R1[2]	56[2]	34[2]	N	N	N	N
6	R2 and L1[2]	56[2]	34[2]	N	N	N	N
7	C1	48	36	Y	Y	Y	Y
8	C2	48	35	N	N	N	N
9	C5	50	35	N	N	N	N
10	B1	55	36	N	N	N	N
11	R1[2]	58[2]	34[2]	N	N	N	N

[1]Templets DNA was digested with *Eco* RI prior to be used in reaction. Annealing temprature was 40°C for first 6 cycles.
[2]Templets DNA was digested with *Eco* RV prior to be used in reaction. Annealing temprature was 40°C for first 6 cycles.

long arm whereas a terminal portion of short arm has been translocated on 4RL. In explaining the differences in the genomes of *Secale cereale* and *S. montanum* related to interchanges involving 2R, 6R and 7R (Van Heemert and Sybenga, 1972), Devos *et al.*, 1993 postulated two hypotheses. The first hypothesis assumed that during speciation of *S. cereale* from *S. montanum*, *Got*-1 gene localised on 6S (Hart, 1975) may have undergone duplication. One copy was translocated to 7RL followed by a pericentric inversion in chromosome 6R transferring it to the distal part of long arm and the other copy translocated first to 2RS and subsequently to 4RL as evidenced by the location of *Got-1* genes in 4R and 7R in rye (Wehling, 1991). The second model proposed that initial interchanges occured between 2S/7L and 6S/4L followed by a 2S/6S rearrangement. In the Triticeae, known locations of CCN resistance genes {on chromosomes: 2D in *Triticum. tauschii* (*Ccn-D2*) (Eastwood *et al.*, 1994); 2H in barley (*Ha*) (Choe, 1996; Kretschmer *et al.*, 1997), and 2BL in wheat (*Cre*) (Slootmaker *et al.*, 1974; Williams *et al.*, 1994); 6RL in rye (*Cre R*) (Asiedu *et al.*, 1990) and 6D in *T. tauschii* (*Ccn-D1*) (Eastwood *et al.*, 1994)} may provide support for this hypothesis. Detailed molecular analyses of these chromosomes can throw some light on homoeology of CCN resistance genes and ancestoral rearrangements in Triticeae.

Given the evolutionary rearrangements in Imperial rye chromosome 6, the present study was undertaken to analyse the structure of chromosome $6R^{T701}$ so that the chromosome could be manipulated to introgress the CCN resistance gene into wheat. A linkage map of long arm of 6R based on PCR and RFLP markers has been produced in this lab (Taylor, 1996). There was a need to generate mapping data for the short arm of 6R so that the complete structure of $6R^{T701}$ could be elucidated. This chapter describes the development of molecular markers for the short arm of 6R, in particular. Development of a PCR-based marker is described in Section 3.2.1. Section 3.2.2 describes the development of RFLP markers for 6RS in conjunction with the utility of these and other RFLP based markers of 6R to understand the comparative organisation of the homoeologous T701 rye chromosome 6 ($6R^{T701}$) and wheat group 6 chromosomes. This chapter also highlights the problems in using breeders stock and focuses particularly on the use of molecular markers, PCR-based and RFLPs, to test for chromosomal anomalies not revealed by cytological tools.

3.2 Results

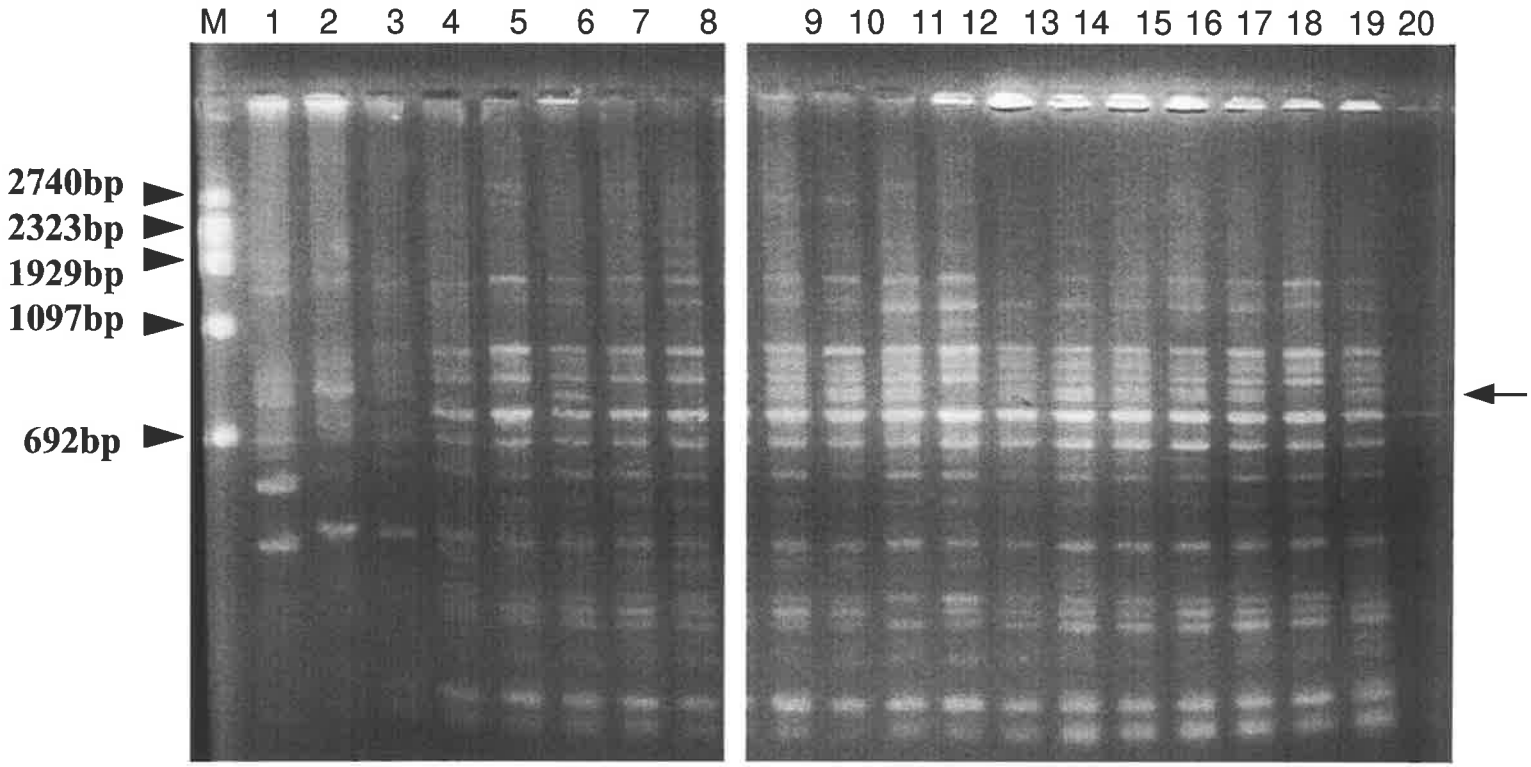
3.2.1. Development of PCR based markers for short arm of $6R^{T701}$

Ten primers in combinations and five primers used singly were assayed in PCR-reactions with DNA from triticale T701-4-6, South Australian rye, Imperial rye, wheat-$6R^{T701}$(-6D) disomic substitution line, Chinese Spring $6R^{Imp}$ disomic addition, Chinese Spring, Schomburgk, $6RL^{T701}$ length deletions mutants and wheat-$6RS^{T701}$ disomic and trisomic addition lines. The sequences of these primers are given in Table 3.1and Table 3.2 summarises the results.

Figure 3.1 Single Primer PCR

Amplification products generated using the primer C1. Amplification products were separated on 3% agarose gel. The size standard (M) was PTZ8U DraI+RsaI. The size of bands generated in standard is shown in base pairs on the left. The 6R specific amplified products generated using C1 is indicated by an arrow.(◄—). Lanes 1-19 contained the following DNA samples:

- Lane 1. South Australian rye
- Lane 2. Imperial rye
- Lane 3. Triticale T701
- Lane 4. Chinese Spring (CS) wheat
- Lane 5. Schomburgk wheat
- Lane 6. CS-$6R^{Imp}$ disomic addition
- Lane 7. CS-$6R^{Imp}$ disomic addition P1
- Lane 8. CS-$6R^{Imp}$ disomic addition P2
- Lane 9. Wheat-$6R^{T701}$(-6D) disomic substitution
- Lane 10. Wheat-$6RS^{T701}$ disomic addition
- Lane 11. Wheat-$6RS^{T701}$ trisomic addition
- Lane 12. Wheat-$6RL^{T701}$ telosome addition
- Lanes 13-20. a sub population of TC-F1-progenies
- Lane 20. H2O


M 1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16 17 18 19 20
2740bp
2323bp
1929bp
1097bp
692bp

Intron splice junction primers E3 and E4, R1 and R2 were used in combinations with random primers (B1) and specific primers (A1 and L1) as these primers have generated bands in barley specific to 6H and 3H (Weining and Langridge, 1992). With E3 and E4, DNA was digested with *Eco* RI prior to use in the PCR reaction, while with B1 and R1, DNA was pre digested with *Eco* RV. These primers generated very complex banding pattern which were not reproducible. None of the primers revealed useful polymorphism even after altering the annealing tempratures and number of cycles.

Use of primers (C1 and C2) derived from the border sequences of a repeated DNA sequence belonging to rye specific R173 family (Rogowski *et al.*, 1991, 1992) have yielded polymorphisms mapped to 6R (Taylor, 1996). In this study, these primers were tested either individually or in combinations with the C5 {primer (Taylor, 1996) derived from AWBMA15 (Murphy *et al.*, 1995)} in variable PCR conditions. Only primer "C1" yielded useful results. When used singly at an annealing temperature of 48°C, this primer detected a polymorphism between wheat-6R^{T701}(-6D) disomic substitution and Chinese Spring-6R^{Imp} disomic addition lines (Figure 3.1). This primer generated two bands in wheat-6R^{T701}(-6D) disomic substitution and a single band in Chinese Spring-6R^{Imp} disomic addition lines. The polymorphism was localised to 6RS using wheat-6RS^{T701} disomic and trisomic addition lines. Absence of this band in 6RL^{T701} translocation line indicates that it may be a 6RS band. The precise location of this marker is not known, as the mapping population based on test cross progenies (Taylor, 1996), either showed the inheritance pattern of parent wheat-6R^{T701}(-6D) disomic substitution or the polymorhic band was completely absent. The second situation was also observed in two individuals of Chinese Spring-6R^{Imp} disomic addition lines which suggest that perhaps the parent used in the test cross was lacking the short arm of 6R or had a minor deletion.

3.2.2 RFLP based-homoeology between 6W, 6R^{Imp} and 6R^{T701}

3.2.2.1 Polymorphism detected by RFLP probes

Since it is well known that rye 6R shares partial homoeology with groups 6, 2, 3, 4 and 7, 59 probes from group 6, 2, 3, 4 and 7 were selected to elucidate the homoeologous and non-homoeologous regions of rye 6R^{T701}. These clones have been assigned to wheat (Sorrells *et al.*, 1992), barley (Langridge *et al.*, 1995), and rye (Devos *et al.*, 1993) chromosome/ chromosome arms. All 59 clones were analysed using eight restriction enzymes on DNA of Schomburgk, Chinese Spring, triticale T701, rye cultivar Imperial, wheat-6R^{T701}(-6D) disomic substitution and Chinese Spring-6R^{Imp} disomic addition lines to identify probes revealing RFLPs. Table 3.3 lists all these probes, their location on chromosome/chromosomal arm, and summarises results of intervarietal polymorphisms identified and probes mapped to 6R^{T701} in this study. All these

Table 3.3: List of clones screened against triticale T701-4-6, Imperial rye, Chinese Spring, Schomburgk, wheat-6R^{T701}(-6D) disomic substitution, Chinese Spring-6R^{Imp} disomic addition lines. Locations of these clones on chromosome/chromosomal arm are given. Intervarietal polymorphisms detected in this study are symbolised as ♣ (wheat-wheat); ♦ (wheat-rye); ♥ (triticale-Imperial rye); ♠ (6R^{T701}-6R^{Imp}). Clones mapped to 6R are highlighted in shade. Lightly shaded areas represent gDNA clones.

S. No	Clones	Location	Intervarietal Polymorphism
1	ABG 466	6S	♣♦♥
2	BCD 0021	6S	♣♦♥
3	BCD 0342	6S	♣♦♥
4	CDO 1158	6S	♦♥♠
5	CDO 0534	6S, 3LS, 7S, 1S	♣♦♥♠
6	PSR 0627	6S	♣♦♥♠
7	PSR 113	6S	♣♦♥
8	PSR 167	6S	♣♦♥
9	PSR 312	6S	♣♦♥
10	PSR 964	6S	♣
11	Tam 6	6S	♣♦
12	Tam 10	6S	♣♦♥
13	Tam 24	6S	♣♦
14	Tam 31	6S	-
15	BCD 1	6L	♣♦♥
16	BCD 758	6L	♣♦♥♠
17	BCD 1426	6L	♣♦♥♠
18	CDO 1380	6L	♣♦♥
19	Amy-1	6L	♣♦
20	α-amylase	6L	-
21	AWBMA 15	6L	♣♦♥♠
22	AWBMA 16	6L	♣♦♥♠
23	PSR 142	6L	♣♦♥♠
24	PSR 148	6L	♣♦♥♠
25	PSR 149	6L	♣♦♥♠
26	PSR 154	6L	♣♦♥♠
27	PSR 371	6L	♣♦♥
28	PSR 915	6L	♣♦♥♠
29	WG 933	6L	♣♦♥♠

Contd.....

S. No	Clones	Location	Intervarietal Polymorphism
30	xksu F037	6L	♣♦♥
31	ABC 152	6	-
32	ABG 474	6	♦
33	BCD 269	6	♣♦♥♠
34	BMA 31	6	-
35	CDO 676	6	♣♦♥♠
36	PSR 106	6	♣♦♥♠
37	Tam 26	6	-
38	Tam 57	6	♣♦♥
39	WG 282	6	♣♦♥
40	ksu D001	6	♣♦♥
41	ksu D006	6	♣
42	ksu F043	6	♣
43	ksu H011	6	♣♦♥
44	BCD 221	2S, 6B	♣♦♥
45	BCD 433	2S	-
46	BCD 438	2S	♦♥
47	PSR 109	2S	♦♥
48	WG 222	2BS/6H	-
49	CDO 0537	2BL/S	♣♦♥
50	WG 996	2BL/S	♦♥
51	BCD 339	2BL, 3BL, 6	♣♦♥
52	AWBMA 28	2	-
53	AWBMA 33	2	♣♦♥
54	BCD 127	3HS	♣♦♥
55	TAG 512	4A/6A	♣
56	TAG 752	4A/6B	♣♦
57	AWBMA 29	4	♣♦♥
58	AWBMA 30	4	♣♦♥
59	AWBMA 34	7	♣♦

probes showed cross hybridisation with rye DNA hence they detected homoeoloci on rye chromosomes in relation to wheat, barley and oat chromosomes.

Relative efficiencies of restriction enzymes used

Of 472 probe/enzyme combinations used, 84 (17.79%) detected polymorphism. Table 3.4 summarises the efficiency of each restriction enzyme in generating RFLPs for the probes used in this study. Of all enzymes used, *Sac* I (30.508%) was the most effective and *Eco* RI (3.389%) was the least effective in revealing RFLPs using all the 59 probes. Over all *Bam* HI (23.728%) and *Dra* I (22.033%), *Bgl* II (20.338%) and *Eco* RV (20.338%), and *Hin* dIII (10.169%) and *Xba* I (11.864%) produced polymorphisms at almost equal rates.

Table 3.5 summarises the relative efficiencies of these enzymes in revealing different types of polymorphism among the varieties. Though restriction enzyme *Eco R*V was found to be very consistent in revealing polymorhism between wheat-wheat, wheat-rye and rye-rye its performance was found to be lower (8.4%) in mapping loci to 6R as compared to *Dra* I, *Bam* HI, *Bgl* II and *Sac* I (10.16%).

Efficiency of different probes types used

Of 59 probes, two known function clones (α-amylase and Amy-1) were used however their source and origin is not known. The remaining 57 cDNA and gDNA probes derived from wheat (ksu, PSR, Tam, TAG, WG), barley (ABC, ABG, AWBMA, BCD) and oats (CDO) libraries were used. Excluding two known function clones, Table 3.6 summarises the number of remaining 57 clones (obtained from various resoures) used in this study and the frequencies of probes types which produced RFLPs. Of 57 probes used, 50 (87.65%) revealed useful RFLPs. Of 33 cDNA types, 29 (87.8%) demonstrated RFLPs whereas, of 24 gDNA type, 21 (87.5%) showed polymorphism. cDNA probes were found to be more efficient than gDNA in generating different intervarietal polymorphism with the exception of wheat-wheat polymorphism. Table 3.7 summarises the relative proportions of intervarietal polymorphism revealed by cDNA and gDNA probes. cDNA probes detected 78.7% (wheat-wheat), 84.8% (wheat-rye), 84.8% (Imp.-triticale) and 39.3% ($6R^{Imp}$-$6R^{T701}$) polymorphism. In comparison, gDNA probes revealed 79% (wheat-wheat), 62.5% (wheat-rye) and 54.1% (Imp.-triticale), and 12.1% ($6R^{Imp}$-$6R^{T701}$) polymorphisms.

Of 27 group 6 probes tested on $6RS^{T701}$ and $6RL^{T701}$ addition lines, 13 were tested on both lines. The remaining 14 probes were different (as have been previously assigned to short and long arms) therefore tested on the correseponding arm addition lines. More cDNA clones were mapped to 6RS (27.2%) and 6RL (86.6%) as compared to gDNA clones. A proportion of 25%

Table 3.4: Summary of level (%) of RFLPs detected by various restriction enzymes tested against DNAs of triticale T701-4-6, Imperial rye, South Australian rye, Chinese Spring, Schomburgk, wheat-6R^{T701}(-6D) disomic substitution, Chinese Spring-6R^{Imp} disomic addition probed with 59 clones. Frequencies of most efficient restriction enzyme i.e. *Sac* I and least efficient restriction enzyme i.e. *Eco* RI are highlighted in bolds.

S. No	Restriction enzyme	Number of Probes tested	Number of Probes detecting RFLP	Frequency of Probes detecting RFLP (%)
1	*Bam*HI	59	14	23.728
2	*Bgl* II	59	12	20.338
3	*Dra* I	59	13	22.033
4	***Eco* RI**	**59**	**2**	**3.389**
5	*Eco* RV	59	12	20.338
6	*Hind* III	59	6	10.169
7	***Sac* I**	**59**	**18**	**30.508**
8	*Xba* I	59	7	11.864
	Total	**472**	**84**	**142.367**
	Average			**17.79%**

Table 3.5: Relative efficiency of restriction enzymes in revealing intervarietal polymorphisms: wheat-wheat, wheat-rye, Imperial rye-triticale and $6R^{Imp}$-$6R^{T701}$.

S. No	Polymorphism types	Restriction enzyme							
		Bam HI	*Bgl* II	*Dra* I	*Eco* RI	*Eco* RV	*Hin* dIII	*Sac* I	*Xba* I
1	wheat-wheat	23.72	16.94	22.03	3.3	20.03	5	29.82	3.3
2	wheat-rye	23.72	20.03	23.72	3.3	20.03	10.16	30.50	10.16
3	Imp-triticale	16.94	20.03	22.03	3.3	20.03	6.7	23.72	5
4	$6R^{Imp}$-$6R^{T701}$	10.16	10.16	10.16	3.3	8.4	6.7	10.16	1.6
	Average	18.63	17.26	19.48	3.3	17.12	8.43	23.55	5.01

Table 3.6: Summary of the results of using cDNA and gDNA types of clones obtained from various sources (reference). Number of probes tested and frequency (%) of clones detected RFLPs are given. Total frequencies (%) of cDNA and genomic DNA type clones detected RFLPs is highlighted in bolds.

S. No	Clones	Reference	Number tested	Number of clones detecting RFLP	Frequency of clones detecting RFLP (%)
1	ABC (Barley cDNA)	Kleinhofs *et al.*, 1993	1	0	0
2	AWBMA (Barley cDNA)	Murphy *et al.*, 1995	8	6	75
3	BCD (Barley cDNA)	Heun *et al.*, 1991	11	10	90.9
4	CDO (Oat cDNA)	Heun *et al.*, 1991	5	5	100
5	PSR 1-200 (Wheat cDNA)	Sharp *et al.*, 1988	8	8	100
Sub total			**33**	**29**	**87.8**
6	ABG (Barley gDNA)	Kleinhofs *et al.*, 1993	2	2	100
7	KSU (*T. tauschii* genomic)	Gill *et al.*, 1991	5	5	100
8	PSR>200 (Wheat gDNA)	Devos *et al.*, 1993	5	5	100
9	TAG (Wheat gDNA)	Liu and Tsunewaki, 1991	2	2	100
10	WG (Wheat gDNA)	Heun *et al.*, 1991	4	3	75
11	TAM (Wheat gDNA)	Devey and Hart, 1993	6	4	66.66
Sub total			**24**	**21**	**87.5**
Total			**57**	**50**	**87.65**

Table 3.7: Summary of frequencies (%) of cDNA and gDNA clones in revealing different intervarietal polymorhisms: wheat-wheat, wheat-rye, Imperial rye-triticale, $6R^{Imp}$-$6R^{T701}$.

S. No	Clone type	Number of clones tested	Proportions of Intervarietal polymorphism scored.			
			wheat-wheat	wheat-rye	Imp.-triticale	$6R^{Imp}$-$6R^{T701}$
1	cDNA	33	26 (78.7%)	28 (84.8%)	28 (84.8%)	13 (39.3%)
2	gDNA	24	19 (79%)	15 (62.5%)	13 (54.1%)	4 (12.1%)
	Total	57	45 (79%)	43 (75%)	41 (72%)	17 (29.8%)

Table 3.8: Proportions of group 6 cDNA and gDNA clones mapped to 6RL and 6RS.

S. No	Clones type	6RL		6RS	
		Number of clones tested	Number of clones mapped	Number of clones tested	Number of clones mapped
1	cDNA	15	13 (86.6%)	11	3 (27.2%)
2	gDNA	12	6 (50%)	16	4 (25%)
	Total	27*	19 (70.3%)	27*	7 (25.9%)

*Of 27 clones tested, 13 were same and 14 were different for 6RS and 6RL.

and 50% gDNA clones was mapped on 6RS and 6RL The results are summarised in Table 3.8. The proportion of clones mapped to 6RS is smaller (25.9%) as compared to the proportion of clones (70.3%) mapped to 6RL.

3.2.2.2 Mapping of RFLP markers to 6R

RFLP markers for 6RST701

Of 14 6S probes, seven were localised to the short arm of $6R^{T701}$, using the wheat-$6RS^{T701}$ trisomic addition lines. Of the seven, four probes showed RFLPs between wheat-disomic $6R^{T701}$(-6D) substitution and Chinese Spring-$6R^{Imp}$ disomic addition. Two of them PSR627 and PSR312 selected from the $6R^{Imp}$ published map by Devos, *et al.* (1993) were also mapped to the short arm of $6R^{T701}$.

The other two probes 'CDO0534' and 'CDO1158' were localised for the first time to 6RS using 2 or 3 restriction enzymes. *Sac* I digested DNA of wheat-$6RS^{T701}$ trisomic addition hybridised with CDO534 showed a strong band of size "12 Kb" comigrated with wheat-disomic $6R^{T701}$(-6D) substitution, triticale and Imperial rye band. Whereas its polymorphic band in Chinese Spring-$6R^{Imp}$ disomic addition, comigrated with a band of South Australian rye (Figure 3.2). There could be a few basepair difference in the size of bands of $6R^{T701}$ and $6R^{Imp}$. The other probe CDO1158 mapped to 6RS with a weak band in wheat-$6RS^{T701}$ trisomic addition comigrated with strong bands in Imperial rye, triticale and wheat-$6R^{T701}$(-6D) substitution and detected a null allelle in $6R^{Imp}$.

Three other new markers, 'Tam-6', 'Tam-24' and 'PSR113' had not been previously localised to rye 6RS before the commencement of this study. These markers did not show any polymorphism between $6R^{Imp}$ and $6R^{T701}$ with any of the eight enzymes tested. A rye specific 6RS band was found to comigrated in triticale, Imperial, South Australian, Chinese Spring-disomic $6R^{Imp}$ addition and wheat-disomic $6R^{T701}$(-6D) substitution. Like some gDNA clones, 'Tam-6' (Figure 3.3) and Tam-24' showed a high background hybridisation smear as with other Tam clones (Taylor, 1996). This could be attributed to the presence of repetitive elements in these probes.

Five other 6S clones (ABG466, BCD21, BCD342, PSR167) revealed RFLPs only between triticale T701 and Imperial rye that could not be mapped to 6R. However, BCD21was found to be located on chromosome 4R when hybridised with Chinese Spring-disomic $4R^{Imp}$ addition (Figure 3.4). Probe Tam-10 detected bands in triticale T701 and wheat-$6R^{T701}$(-6D) disomic substitution lines and $6RS^{T701}$ trisomic addition lines which comigrated with the Schomburgk band. Two probes PSR964 and Tam-31 were failed to detect any polymorphism between wheat

Figure 3.2 Chromosomal localisation of CDO534

The size standard (M) was λ *Hin* d III . The size of bands generated in standard is shown in base pairs on the left. The polymorphic bands are indicated by arrows: 6R (◄—) and 6D (←—). Lanes 1-13 contained the following DNA samples digested with *Sac*I

- Lane 1. South Australian rye
- Lane 2. Imperial rye
- Lane 3. Triticale T701
- Lane 4. Chinese Spring (CS) wheat
- Lane 5. Schomburgk wheat
- Lane 6. Wheat-6R^{T701}(-6D) disomic substitution
- Lane 7. CS-6R^{Imp} disomic addition
- Lane 8. Wheat-6R^{T701} addition
- Lane 9. Wheat-6RL telosome addition
- Lane 10. Wheat-6RS^{T701} trisomic addition
- Lane 11. Wheat-6RL^{T701} translocation mutant
- Lane 12. Wheat-6RL^{T701} del. 1 translocation mutant
- Lane 13. Wheat-6RL^{T701} del. 2 translocation mutant

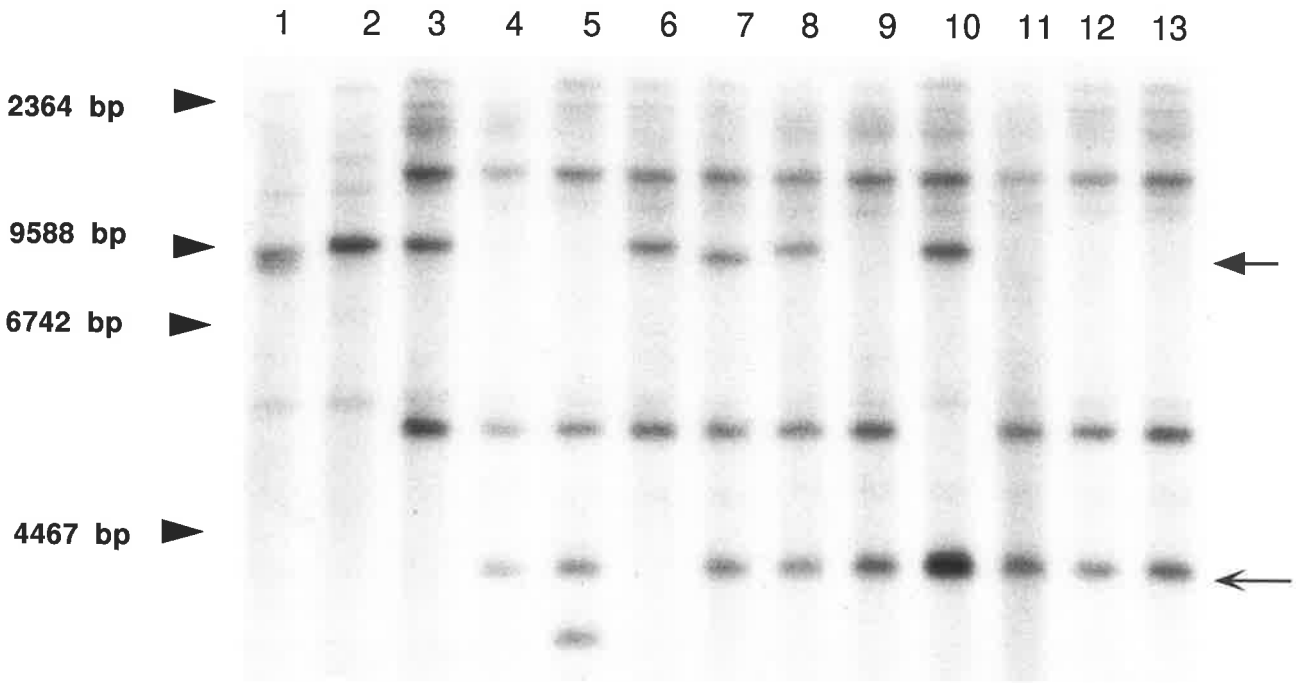
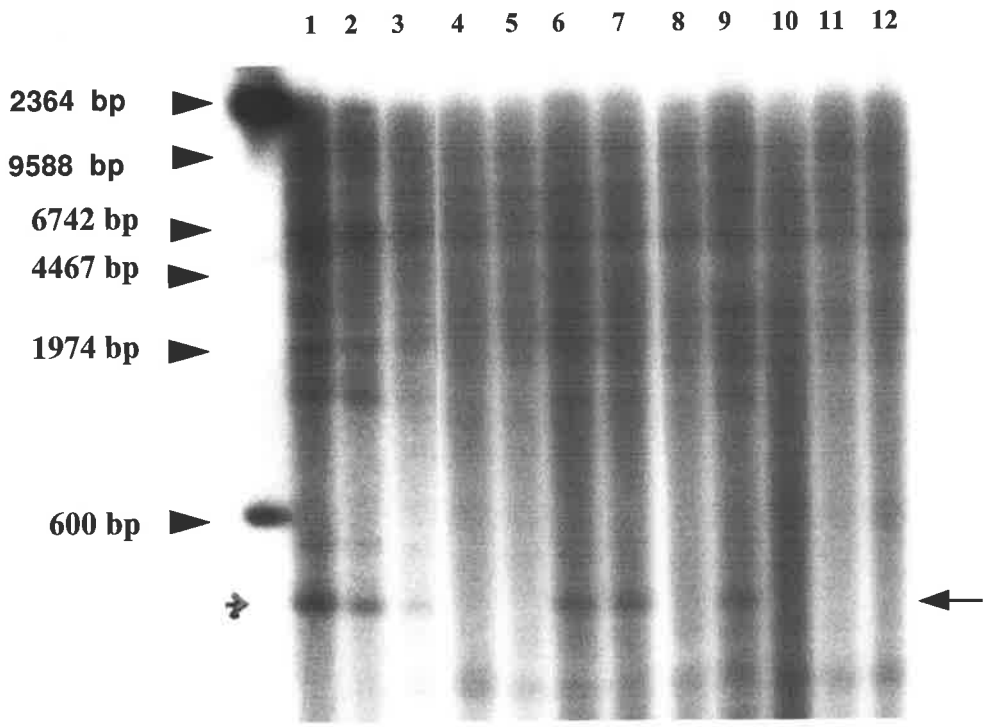

1
2
3
4
5
6
7
8
9
10
11
12
13
2364 bp
9588 bp
6742 bp
4467 bp

Figure 3.3 Chromosomal localisation of Tam-6

The size standard (M) was λ *Hin* d III. The size of bands generated in standard is shown in base pairs on the left. The polymorphic band is indicated by 6R arrow (◄—). Lanes 1-13 contained the following DNA samples digested with *Sac*I

- Lane 1. South Australian rye
- Lane 2. Imperial rye
- Lane 3. Triticale T701
- Lane 4. Chinese Spring (CS) wheat
- Lane 5. Schomburgk wheat
- Lane 6. Wheat-$6R^{T701}$(-6D) disomic substitution
- Lane 7. CS-$6R^{Imp}$ disomic addition
- Lane 8. Wheat-$6RL^{T701}$ telosomic addition
- Lane 9. Wheat-$6RS^{T701}$ trisomic addition
- Lane 10. Wheat-$6RL^{T701}$ translocation mutant
- Lane 11. Wheat-$6RL^{T701}$ del. 1 translocation mutant
- Lane 12. Wheat-$6RL^{T701}$ del.2 translocation mutant


1 2 3 4 5 6 7 8 9 10 11 12
2364 bp
9588 bp
6742 bp
4467 bp
1974 bp
600 bp

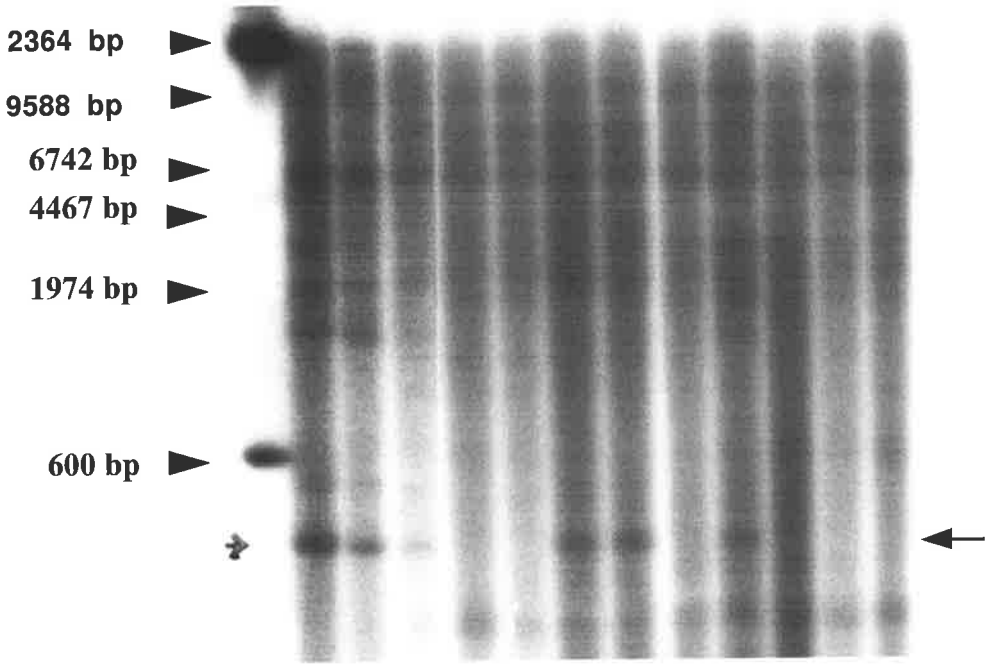

2364 bp
9588 bp
6742 bp
4467 bp
1974 bp
600 bp

Figure 3.4 Chromosomal assignment of BCD21 using CS-Imperial rye addition lines. The size standard (M) was λ *Hin* d III. The size of bands generated in standard is shown in base pairs on the left. The polymorphic band is indicated by an arrow (◄—). Lanes 1-8 contained the following DNA samples digested with *Bgl*II.

- Lane 1. Chinese Spring (CS)
- Lane 2. Triticale T701-4-6
- Lane 3. CS-1R Imperial rye
- Lane 4. CS-2R Imperial rye
- Lane 5. CS-3R Imperial rye
- Lane 6. CS-4R Imperial rye
- Lane 7. CS-6R Imperial rye
- Lane 8. CS-7R Imperial rye

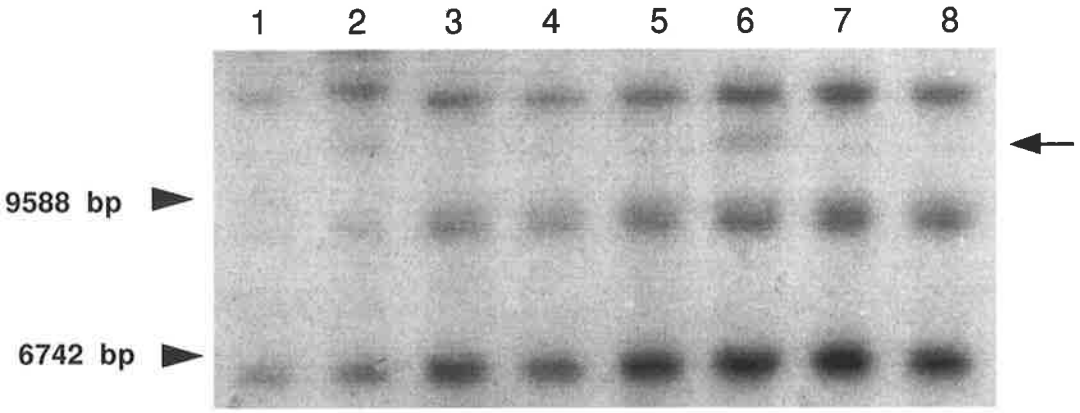
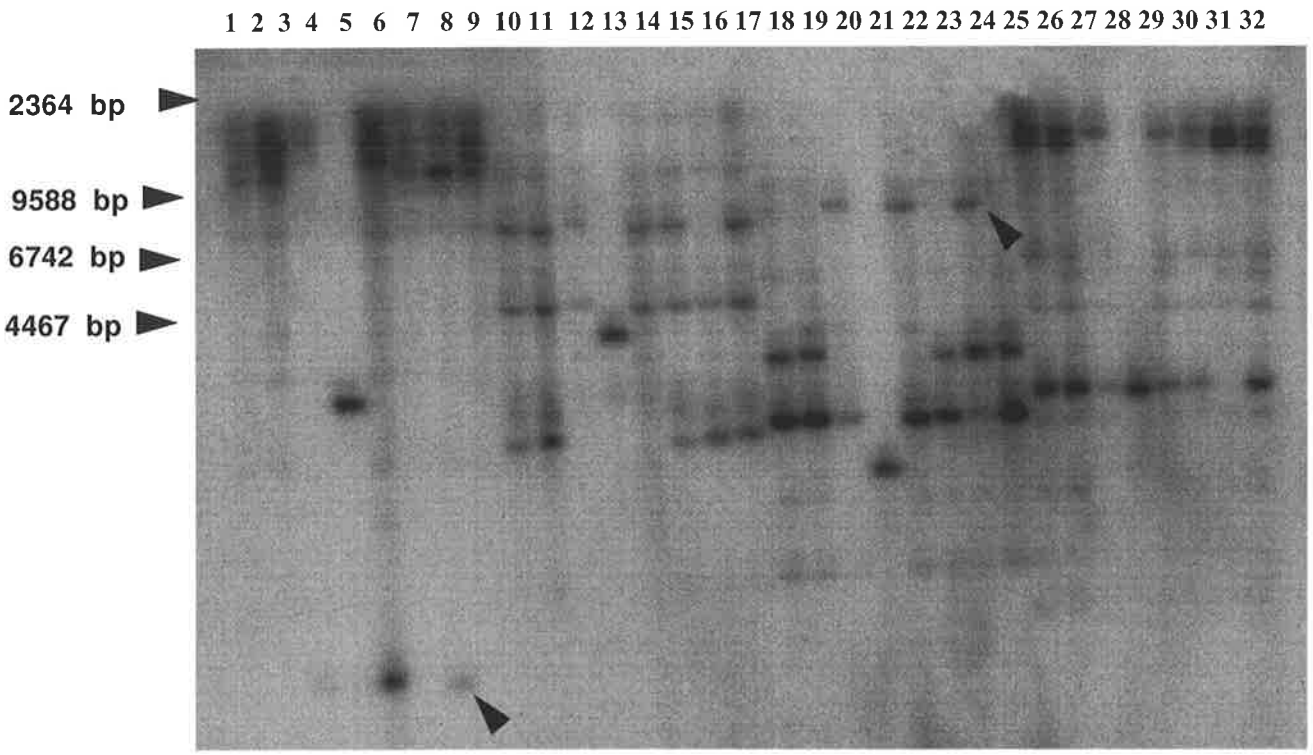

1
2
3
4
5
6
7
8
9588 bp
6742 bp

Figure 3.5 Detection of anomaly in CS-6R^{Imp} disomic addition line.

Hybridisation with (a)6S marker CDO534 and (b)6R marker BMA15. The size standard (M) λ *Hin* d III. The size of bands generated in standard is shown in base pairs on the left. The 6R polymorphic band is indicated by an arrow (◄). Following DNA samples digested with *Bam* HI (1-8), *Bgl* II (9-16), *Dra* I (17-24) and *Eco* RI (25-32).

- 1. Chinese Spring (Lanes 1,9,17 and 25)
- 2. Schomburgk (Lanes 2,10,18 and 26)
- 3. Triticale T701-4-6 (Lanes 3, 11,19 and 27)
- 4. Imperial rye (Lanes 4, 12, 20 and 28)
- 5. Wheat-6R^{T701}(-6D) disomic substitution (Lanes 5, 13, 21 and 29)
- 6. CS-6R^{Imp} disomic addition (Lanes 6, 14, 22 and 30)
- 7. Wheat-6RS^{T701} trisomic addition (Lanes 7, 15, 23 and 31)
- 8. Wheat-6RL^{T701} telosome addition (Lanes 8, 16, 24 and 32)


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
2364 bp
9588 bp
6742 bp
4467 bp

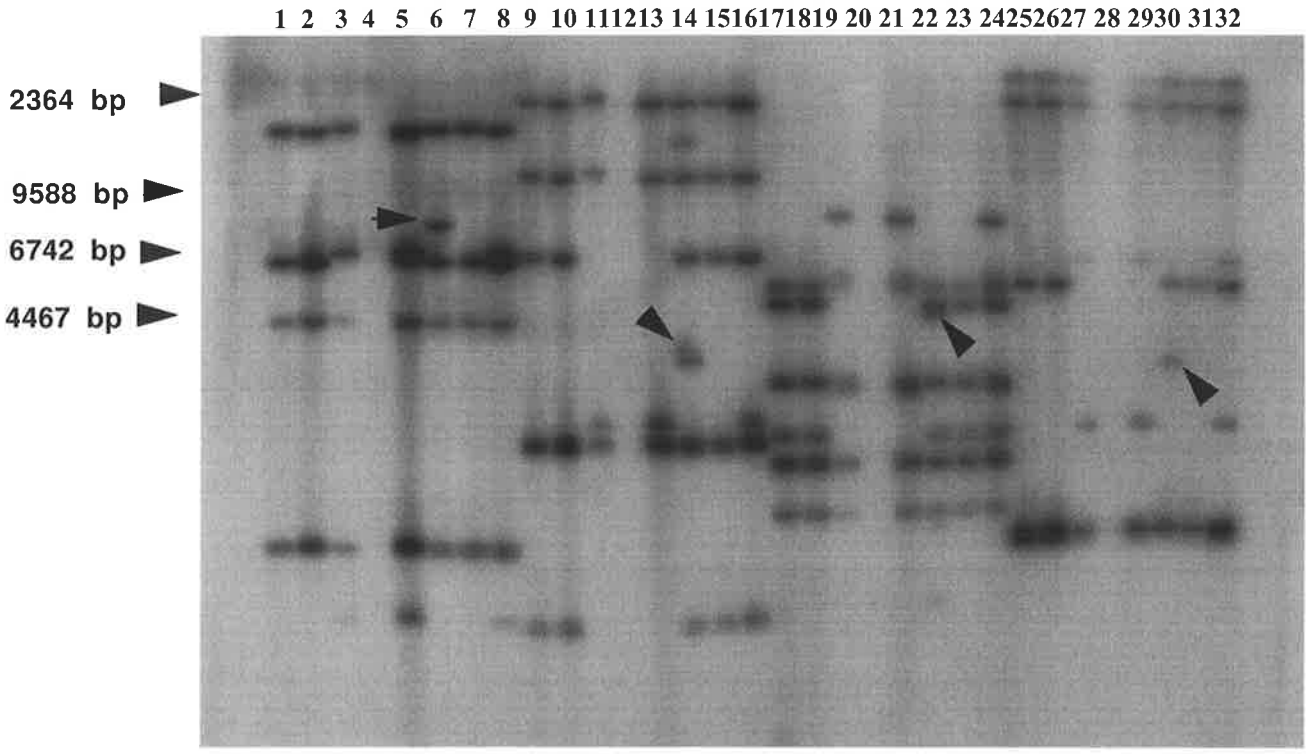
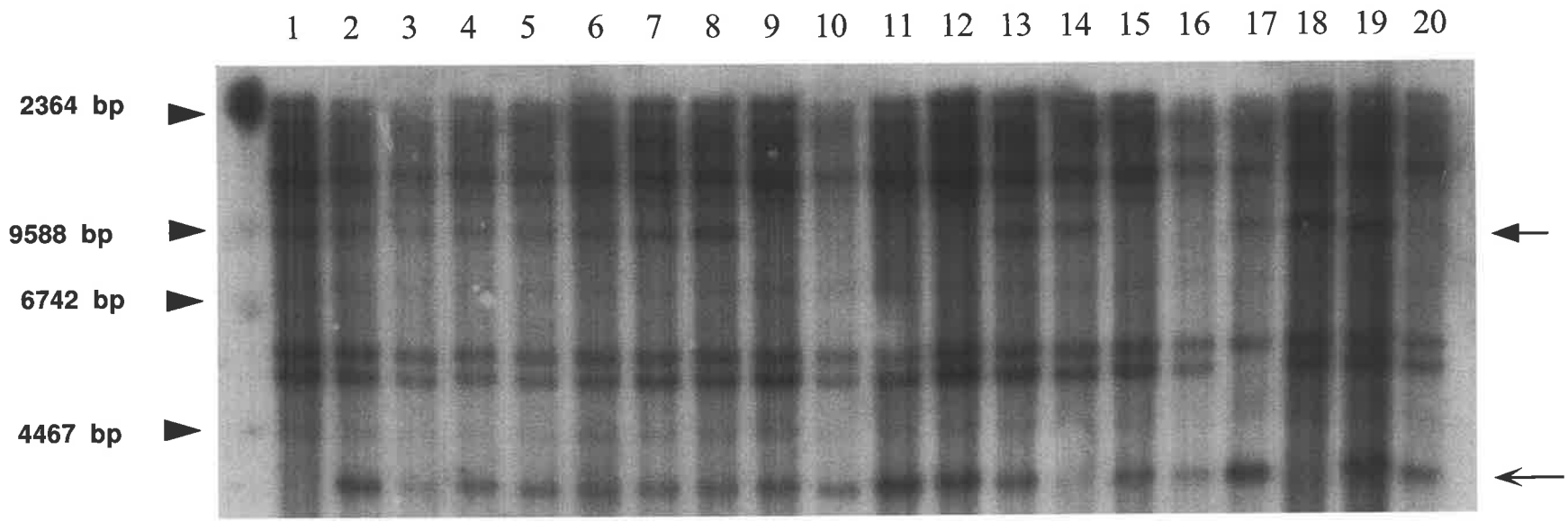

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
2364 bp
9588 bp
6742 bp
4467 bp

Figure 3.6 Segregation analysis of *Xcdo534* in TC-F1 progenies

The size standard (M) was λ *Hin* d III. The size of bands generated in standard is shown in base pairs on the left. The 6R polymorphic band is indicated by an arrow (◄—). Lane 1-20 contained the following DNA samples digested with *Sac*I

- Lanes 1-16. a sub population of TC-F1 progenies
- Lane 17. Wheat-6RS^{T701} trisomic addition
- Lane 18. Wheat-6R^{T701}(-6D) disomic substitution
- Lane 19. CS-6R^{Imp} disomic addition
- Lane 20. Chinese Spring (CS) wheat


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
2364 bp
9588 bp
6742 bp
4467 bp

and rye with all the restriction enzymes used in the present study. Tam-31 showed high copy number with the high background hybridisation.

Certain anomalous findings were obtained in this study with 6S probes which precluded further mapping of 6RS markers with precision. In an initial screening, when 6S probes were tested against the DNA of a Chinese Spring-disomic $6R^{Imp}$ addition line plant used as one of the parent in the crosses made to produce mapping population (Taylor, 1996), the 6RS specific bands were found to be missing. However all 6RL probes showed rye specific 6RL bands in the same parental DNA. Sequential use of the same membrane with 6RS marker 'CDO534' followed by a 6RL marker 'BM15'(Figure 3.5) provided a clear indication that a portion of the short arm of Imperial rye 6R might have been deleted in the particular parent used to produce mapping population. Use of 6RS specific markers including PSR627 (Devos *et al.*, 1993) and CDO534 (this study), detected $6RS^{Imp}$ specific bands polymorphic with $6RS^{T701}$ band, in another plant of Chinese Spring-$6R^{Imp}$ disomic addition (seeds were kindly provided by Dr I Dundas, Waite Institute). This plant was then used as a control in further mapping studies. Hybridisation with CDO534 demonstrated a dominant pattern of inheritance in mapping population. Individuals of the TC1-progenies (Taylor, 1996), either showed a 6RS specific band corresponding to $6RS^{T701}$ or the band was missing (Figure 3.6). PCR results with C1 primer (Section 3.2.1) also support this finding.

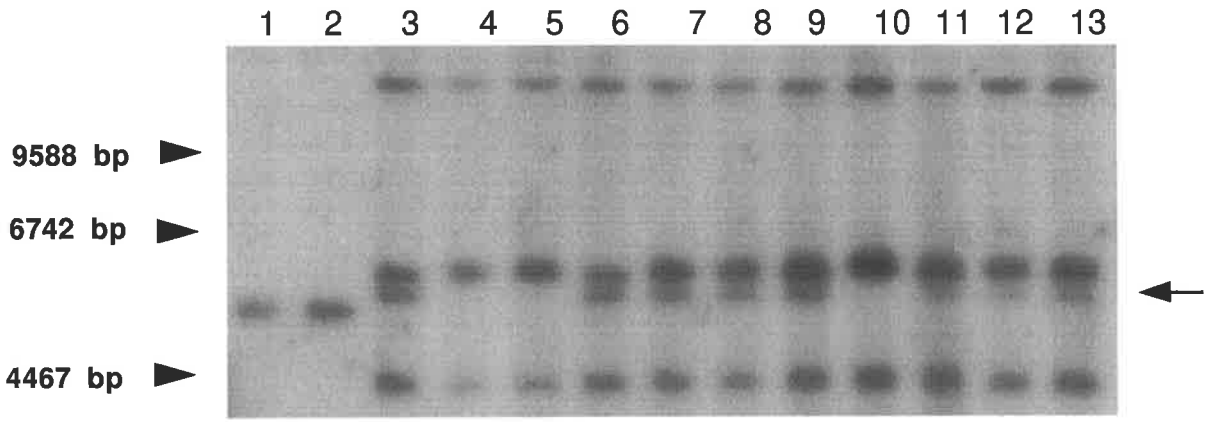
RFLP markers for $6RL^{T701}$

A total of sixteen probes was selected from the 6L linkage maps; 15 were localised to 6RL using different 6RL length deletion mutants. 6RL probes (PSR149, PSR154, and PSR148) selected from the Devos *et al.* (1993) published map were found to be localised on $6RL^{T701}$, indicated the structure of $6R^{T701}$ and $6R^{Imp}$ are similar. Furthermore, locations of previously mapped RFLP markers {BCD1, BCD758, WG933, BCD1426, BM15, CDO676, ksuD001, ksuF043 (Taylor, 1996)} and {PSR142, PSR915 and PSR371 (Dr PJ Sharp, personal communication)}were also confirmed on the long arm of $6R^{T701}$. Known functional gene clone Amy-1 also detected loci mapped to $6RL^{T701}$ using *Xba* I. 6R specific bands were co-migrated in triticale and Imperial rye. An additional RFLP marker, ABG474 (Figure 3.7), identified in this study was mapped to the proximal region of 6RL using 6RL translocation deletion mutants. Hybridisation of this clone with *Sac* I digest DNAs revealed a rye specific band in triticale co-migrated with bands in its derived lines and Chinese Spring-$6R^{Imp}$ disomic addition line. This rye specific triticale band was polymorphic with one present in Imperial rye and South Australian rye.

Figure 3.7 Chromosomal localisation of ABG474

The size standard (M) was λ *Hin* d III . The size of bands generated in standard is shown in base pairs on the left. The 6R polymorphic bands is indicated by an arrow (◄—). Lanes 1-13 contained the following DNA samples digested with *Sac*I

- Lane 1 South Australian rye
- Lane 2. Imperial rye
- Lane 3. Triticale T701
- Lane 4. Chinese Spring (CS) wheat
- Lane 5. Schomburgk wheat
- Lane 6. Wheat-6R^{T701}(-6D) disomic substitution
- Lane 7. CS-6R^{Imp} disomic addition
- Lane 8. Wheat-6R^{T701} addition
- Lane 9. Wheat-6RL telosome addition
- Lane 10. Wheat-6RS^{T701} trisomic addition
- Lane 11. Wheat-6RL^{T701} translocation mutant
- Lane 12. Wheat-6RL^{T701} del. 1 translocation mutant
- Lane 13. Wheat-6RL^{T701} del. 2 translocation mutant


1 2 3 4 5 6 7 8 9 10 11 12 13
9588 bp
6742 bp
4467 bp

Conservation of synteny between 6W and $6R^{T701}$

Mapping of 26 of 43 (60.4%) group 6 probes on $6R^{T701}$ indicate that this chromosome shares homoeology with group 6 chromosomes at large extent. However only a small proportion of 6S clones could be mapped to $6RS^{T701}$ indicated that synteny pertains largely for long arms as compared to short arms of 6W and $6R^{T701}$. As far as the short arm of $6R^{T701}$ concerned, homoeology seems to be incomplete particularly in distal regions. As 6S RFLP markers, ABG466, BCD21, BCD342 and PSR167 located on distal regions of 6S could not be localised to 6S though mapped to Imperial rye and triticale genomes.

Other group 6 clones whose arm locations were only known recently, Tam-57, ksuH11(Devey *et al.*, 1994) and WG282 also detected loci on Imperial rye and triticale genomes not linked to 6R, indicating for their presence elsewere. Group 2 markers CDO537, WG996, BCD433 and BCD438 (K. J Williams, personal communication) were selected for this study to elucidate homoeolgy of CCN resistance genes in rye and wheat, and to detect any evolutionary chromosomal interchanges between 6R and group 2 chromosomes. For the same reason, some other clones which identify non-homoeologous loci (2,3,4 and 7) (Table 3.3) as well as detect loci on group 6 chromosomes were also included in this study. None of these clones from groups 2, 3, 4 or 7 revealed any RFLP that could be localised to 6R, although showed RFLPs between Imperial rye and triticale T701. However, RFLP marker "CDO0534", localised to 6RS in this study, also identified loci on chromosome arms 1S, 3LS, and 7S on wheat map (Anderson *et al.*, 1992). Similarly 6RL probes "AWBMA15" (Figure 3.8) and "PSR148" also identify loci on other wheat chromosomes. BMA 15 whose position was confirmed in this study identifies loci on groups 3 (Collins, *et al.*, 1996) and 7 in the barley linkage maps (A. Karakousis, personal communication). Another marker BCD127 mapped closely to BM15 on 3H (Collins, *et al.*, 1996) however did not show any linkage with rye 6R rather showed polymorphism in triticale and Imperial rye. Clone PSR148 identify loci on 2A and 7B in wheat (Gale *et al.*, 1995)

In this study, most of the rye specific 6R bands in Chinese Spring-$6R^{Imp}$ disomic addition line did not show comigration with $6R^{Imp}$ bands in Imperial rye, indicate that the structure of $6R^{Imp}$ is different in these two cultivars.

3.3 Discussion

Both RFLP and PCR techniques have been widely used to construct detailed linkage maps in various plant species. Apart from determining the organisation of genes and markers along the chromosomes, these molecular maps particularly, RFLP-based, provide an understanding of the evolutionary relationships between species. This study used a similar approach to elucidate the structure of triticale 6R chromosome and its homoeologous relationship with wheat group 6

chromosomes. Since a linkage map of 6RL have been already constructed (Taylor, 1996), this study reports development and assignment of PCR and RFLP markers for the short arm of $6R^{T701}$. A brief account of the efficiency of enzymes and type of clones used in RFLP analysis is also included. The results presented in Sections 3.2.1 and 3.2.2 are discussed below and indicate that the structrure of $6R^{T701}$ is far more complex then originally thought.

3.3.1 C1 a PCR-based marker for 6RS derived from the border region of rye specific R173 element

PCR-based markers particularly, 'RAPDs', have been emerged as an extra source of markers to fill in gaps which remain in RFLP linkage maps (Loarce *et al.*, 1996). The use of RAPDs in the construction of rye maps have been demonstrated previously (Philipp *et l.*, 1992; Loarce *et al.*, 1996, Taylor, 1996). In the present study, a C1-primer based PCR marker was used to reveal polymorphisms. The chances of obtaining useful polymorphism with C1 were very high as it is derived from the flanking region of R173, a rye specific, high copy, repititive sequence (Rogowsky *et al.*, 1992). Other workers have also attributed the large proportion of RAPDs to repetitive sequences (Williams *et al.*, 1990; Devos and Gale, 1992). Taylor (1996) have demonstrated the development of PCR markers using primers derived from flanking regions of dispersed repetitive sequences including R173. He identified a complex banding pattern with C1 primer at a 50°C annealing temperature. However he could not localised these bands to any rye chromosome.

This study showed that shifting of annealing temprature to 48°C revealed a stable banding pattern with C1 primer and polymorphic bands were localised to 6RS. However, this PCR marker showed a dominant polymorphism when checked against test-cross progenies and the parental Chinese Spring $6R^{Imp}$ addition line. Taylor (1996) also reported PCR markers for 6RL showed dominance, being amplified in $6R^{T701}$ and not in $6R^{Imp}$. However when tested on another individual of Chinese Spring $6R^{Imp}$ disomic addition, C1 generated a 6RS-specific Imperial rye band. This finding indicated a submicroscopic deletion in the short arm of 6R present in the Chinese Spring $6R^{Imp}$ disomic addition line used as a parent in the crosses to produce mapping population. Such deletions are spontaneous and could have arisen while selecting the addition and substitution lines.

In an intial screening of RFLP probes mapped to 6RS, several clones could not be mapped in this particular plant of Chinese Spring $6R^{Imp}$ addition line and detected loci segregating with null alleles. When the mapping population (Taylor, 1996) was screened with these probes, progenies also showed the dominant pattern of inheritance for probes CDO 534, PSR 627 and PSR 312 which detect rye specific-6RS markers. Anomalous findings using RFLP probes have also been reported previously for 6B (Chen *et al.*, 1994). Deletion/insertion or other smaller

rearrangements are frequent in the rye genomes and are responsible for many RFLPs in rye (Philipp *et al.*, 1994).

3.3.2 Efficiencies of restriction enzymes and probes in revealing RFLPs within Triticeae

Polymorphism in wheat and rye was studied using Triticeae chromosomes 6 in RFLP analysis. About 17.79% of probe-enzyme combinations revealed polymorphisms. Of eight enzymes used, 3 to 4 identified the useful polymorphisms with each probe (demonstrating RFLPs). In all the enzymes used *Sac* I (GAGCTC) was found to be the most effective (30.50%) in detecting intervarietal polymorphism between wheat and rye. However *Dra* I (TTTAAA) and *Bam* HI (GGATCC) and *Bgl* II (AGATCT) and *Eco* RV (GATATC) also demonstrated significant polymorphism between wheat and rye. In plant species wheat (Chao, et al., 1989), maize and tomato (Helentjaris *et al.*, 1986), rice (Mc Couch *et al.*, 1988) and lettuce (Landry *et al.*, 1987), A-T rich sequences are known to generate more polymorphism than G-C rich sequences.

About 90% of the probes revealed useful polymorphism. cDNA probes demonstrated more intervarietal polymorphism than gDNA probes. Similarly the proportion of cDNA clones mapped to 6R was higher than gDNA clones. This is in agreement with the findings of other workers (Taylor, 1996; Liu and Tsunewaki, 1991; Wang *et al.*, 1992). However, the proportion of gDNA localised to 6RS was higher than cDNA clones. Although characterstics such as non-homoeologous behaviour, poor hybridisation signals and complex banding pattern with higher background discourage the use of gDNA probes, this study found them a greater source for mapping loci to 6RS on which only few markers have been placed previously.

3.3.3 Comparative analysis of $6R^{T701}$ and group 6 homoeologous chromosomes using RFLP markers

Use of a common set of RFLP probes on the genomes of related species demonstrate their homoelogous relationships and evolutionary links with one another. Information from complete RFLP linkage maps of rye (Philipp *et al.*, 1994; Devos *et al.*, 1993; Loarce *et al.*, 1996) and wheat (Van Deynze *et al.*, 1995) provides comparison of the four genomes. At the commencement of this project little information was available regarding the order of various loci on group 6 chromosomes of Triticeae. But in recent years, several reports on wheat group 6 chromosomes (Gill *et al.*,1993; Chen *et al.*, 1994; Jia *et al.*, 1996, Marino *et al.*, 1996) and 6R (Wanous and Gustafson, 1995, Delaney *et al.*, 1995) have provided an opportunity to examine homoeology between rye and wheat group 6 chromosomes. RFLP markers assigned to $6R^{T701}$ in this study identify homoeoloci on wheat linkage maps thus very useful in elucidating the comparative organisation of $6R^{T701}$ with its homoeologous group 6 chromosomes. A consensus map of group 6 chromosomes canbe drawn to establish presumptions about the structure of

$6RS^{T701}$. The comparative analysis of 6R and wheat group 6 chromosomes based on RFLP markers is discussed below.

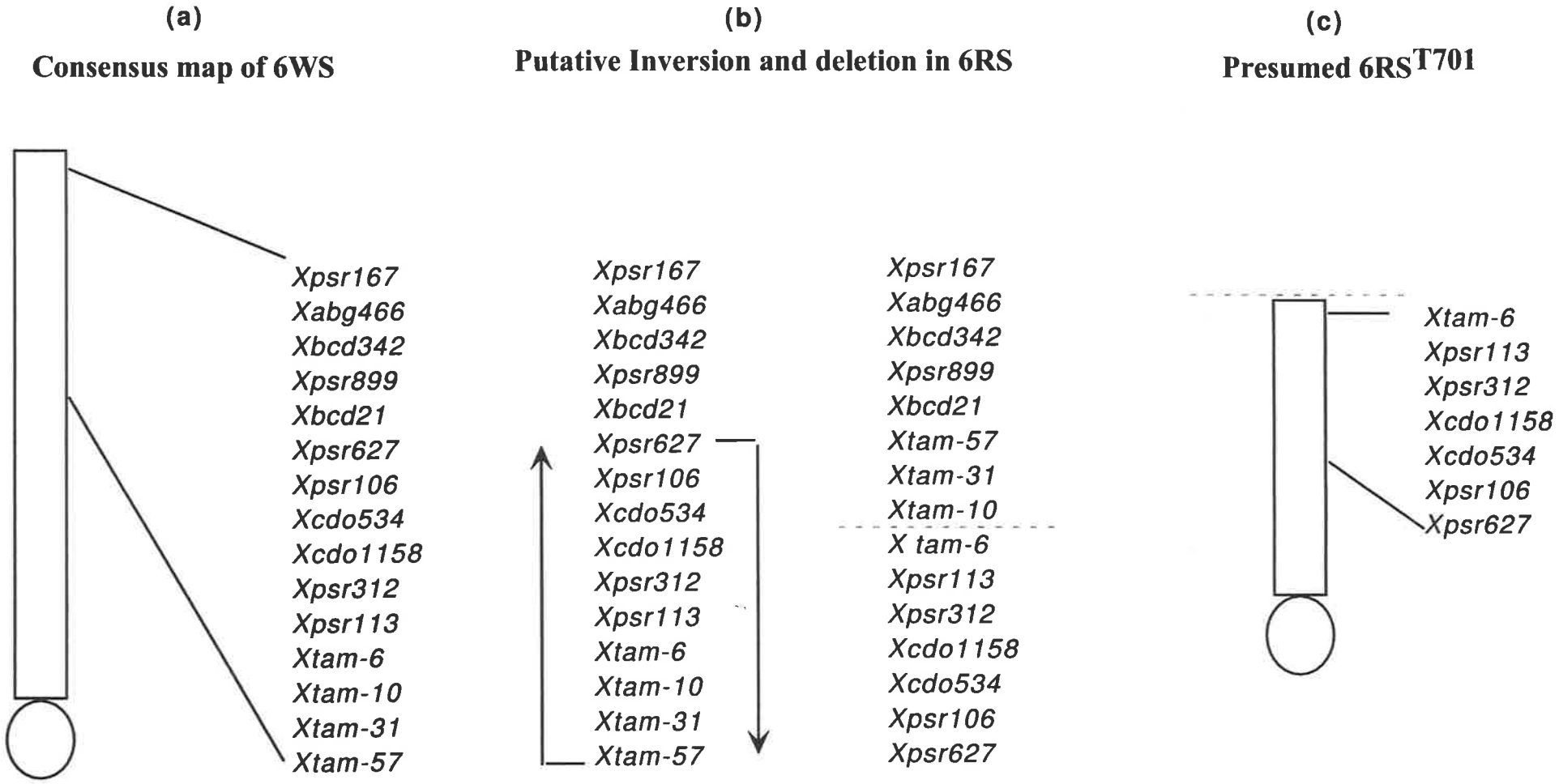
Comparison of rye $6RS^{T701}$ with homoeologous 6WS

Including *Xpsr627* and *Xpsr312* (Devos *et al.*, 1993), new RFLP markers *Xcdo534*, *Xcdo1158*, *Xpsr113*, *Xtam-6* and *Xtam-24* were assigned to the short arm of $6R^{T701}$ in this study. The linear order of these markers on $6RS^{T701}$ is not known since these markers could not be mapped with the available mapping population. RFLP probe, CDO534, identified duplicate loci on 1S and 3S/L in addition to 6S (Sorrells *et al.*, 1992) in wheat. The utility of this probe as an 'anchor' probe was shown recently and proposed for comparative mapping to evaluate homoeology and conservation of sequences among the distantly related grass species, rice, maize and oats (Van Deynze *et al.*, 1995). However this probe was not reported on rye 6S, previously. This study reports the identification of *Xcdo534* and *Xcdo1158* as rye 6RS markers. These marker loci are closely linked on the 6S on consensus wheat map (Van Deynze *et al.*, 1995). The region on wheat 6S carrying *Xcdo534* and *Xcdo1158* shows homoeology to chromosome B of oats (VanDeynze *et al.*, 1995 a, b). Both probes (CDO534 and CDO1158) were isolated from an oat cDNA library (Heun *et al.*, 1991). The homoeologous locations of *Xcdo534* in other grasses are chromosomes 2 and 11 in rice and chromosome 4 and 5 in maize. There are no published reports of the location of *Xcdo1158* on rice and maize chromosomes.

The other RFLP markers for 6RS identified in this study are *Xpsr113*, *Xtam-6* and *Xtam-24*. Location of *Xtam-24* is still unknown on wheat map though it is derived from a wheat genomic library. However the location of *Xtam-6* in relation to other *Xtam* markers, *Xtam-10*, *Xtam-31* and *Xtam-57* has been identified on a wheat map (Chen *et al.*, 1994). The linear order of these markers from centromere is *Xtam-57*, *Xtam-31*, *Xtam-10* and *Xtam-6* on 6BS. However, this order has been reversed on 6AS suggesting an inversion relative to 6BS (Chen *et al.*, 1994). There are inconsistencies in the location of *Xtam-31* on genetic and physical maps. Chen *et al.*, 1994 and Marino *et al.*, 1995 mapped *Xtam-31* on proximal regions of 6BS and 6DS, respectively. While Gill *et al.*, 1993, mapped *Xtam-31* to a distal region of 6AS and 6DS and on intercalary region of 6B. This inconsistency arises due to low level of recombination in the pericentric region (Lukaszewski, 1995). Another marker *XksuF43* is located distal to *Xtam-31* on 6S in *T.tauschii* (Gill *et al.*, 1993). Locations of *Xpsr312* and *Xpsr627* (Devos, *et al.*, 1993) are distal to *Xtam-10* and *Xtam-31* on the consensus map of group 6 chromosomes of Triticeae (Marino *et al.*, 1996). The locus *Xcdo534* is located between *Xpsr627* and *Xtam-31* on 6DS (Marino *et al.*, 1996). Location of *Xpsr113* is proximal to *Xpsr312* and *Xpsr627* on 6WS (Jia *et al.*, 1996).

In this study, a large number of 6S probes {group a (ABG466, BCD21, BCD342, and PSR167) and group b (Tam-10, Tam-31, Tam57 and ksuF043)} could not be localised to

Figure 3.8. (a) Consensus map of 6WS. (b) Model of putative inversion and deletion identified in proximal region of 6RS is illustrated and (c) presumed structure of $6RS^{T701}$ is also given.



Marino *et al.*, 1996
Jia *et al.*, 1996
VanDeynze *et al.*, 1995
Cheun *et al.*, 1994
Gill *et al.*, 1993

$6RS^{T701}$ although polymorphisms between Imperial rye and triticale were revealed. This suggest that loci identified by these probes are on chromosomes other than 6R. The group (a) loci are mapped to the distal region of 6S of wheat (Marino *et al.,* 1996).

A consensus physical map of 6S in Triticeae was drawn based on known locations of all 6S markers. Assumptions can be inferred for the linear order of markers on $6RS^{T701}$ by comparing the location of unmapped markers in relation to the markers mapped to 6R in this study. The presumed map of 6R is shown in Figure 3.8. It indicates an inversion on 6RS in the proximal region and a deletion of the distal region which might have translocated to 4RL. However not all unmapped 6S probes were tested on 4RL except PSR167, BCD21 and BCD342. The putative break points for inversion may lie between *Xbcd21* and *Xpsr627* and the centromere. It can be speculated that inversion followed by a deletion event was responsible for failure to map most of the 6S probes. The presumed inversed order of *Xtam 57*, *Xtam 10* and *Xtam 6* on $6RS^{T701}$ is identical to the order of these loci on 6A in contrast to the order on 6B present in *T. turgidum* (Chen *et al.,* 1994). Inversions have been reported previously in the proximal regions of 6DL of wheat (Gill *et al.,* 1993) and $6RL^{T701}$ of wheat-$6R^{T701}$ disomic substitution (Taylor, 1996). However the conclusions made here regarding structure of $6RS^{T701}$ need confirmation. A suitable mapping population involving $6R^{T701}$ or $6RS^{T701}$ deletion line (I. Dundas, personal communication) may be useful in clarifying the structure of $6RS^{T701}$.

Comparison of $6RL^{T701}$ with 6WL

When this study was initiated a considerable number of RFLP markers from group 6 have been already mapped to 6RL (Taylor, 1996). However comparative RFLP studies of wheat-rye chromosomes (Devos *et al.*, 1993) have shown that rye 6RL possesses a reciprocal translocation of 3L in its interstitial region and a non-reciprocal translocation of 7L in its distal region. In this study, four RFLP probes, PSR149, PSR154, PSR106 and PSR148 selected from the rye map (Devos *et al.*, 1993) also detected loci on 6RL. Since PSR154 and 149 are group 6 probes, their assignment to 1411-54 line, a $6RL^{T701}$ deletion.6WS translocation, indicated that 70% of 6RL shares homoeology to 6WL.

Another probe PSR106 though detected duplicate loci on chromosome 6R (Devos *et al.*, 1993), identified a single locus on 6RL indicated a slight inconsistency with Devos *et al.*, 1993 map. Using the 657XS deletion line, PSR106 was assigned to the region distal to the breakpoint of 1411-54 line. Duplicated loci were also observed for PSR142 and PSR915 on 6RL mapped to the region proximal to deletion break point of the 1411-54 line. While using the 2373 line, a $6RL^{T701}$ intact.6WS translocation line, PSR148 was mapped to very distal region of 6RL which contain the 10% of $6RL^{T701}$. Since PSR148 identifies loci on 2A and 7B in wheat (Gale, *et al.*, 1995), its location on 6R confirms the presence of non-group 6 translocation on the distal region of 6R.

The presence of marker AWBMA 15 (Murphy, 1994) also confirmed on 6R which was previously shown to be located proximal to the CCN resistance gene (Taylor, 1996) on 6RL. This marker was mapped to 3HS in barley (Collins *et al.*, 1996), and groups 6, 3 and 7 in wheat (Angelo Karakousis, personal communication). Considering the location of BM15 in Triticeae, two possible suggesttions can be made: first, this marker detects multiple loci for GOT isozymes on non-homoeologous chromosomes; second, marker AWBMA15 is present on the region which shares some homoeology to wheat groups 3L and 7L, and located at or near the junction of 3L and 7L in the non-group 6 region of chromosome 6R. Recently another anchor probe BCD147 (3BL) (mapped distally on rye 6RL (Loarce *et al.,* 1996) at the same position where BCD276 and BCD1 are mapped. Taylor (1996) mapped BCD1 and BCD276 on the 57-69% of 6RL These markers are distal to BM15 (Taylor, 1996). This result provide further evidence of 3L translocation on 6RL and confirms the non-homoeology of rye 6R with wheat group 6 chromosomes.

Since rye 6R was involved in multiple chromosomal rearrangements, RFLP probes assigned to group 2, 3, 4 and 7 were selected to reveal non-homoeologous regions in 6R. However, these probes detected polymorphism only between triticale and Imperial rye suggesting their locations are synteneous with the wheat genome. Group 2 probes used in this study to elucidate the synteny in the region containing CCN resistance gene on wheat and rye chromosomes could not be mapped with the exception of PSR148. These results further suggest that location of CCN resistance genes in rye is at critical region.

This study has shown the value of molecular markers in understanding cereal genome structure and gene organisation. Numerous rearrangements studied on rye chromosome 6R indicates a disturbed gene order on rye $6R^{T701}$ relative to wheat chromosomes. The lack of collinearity of group 6 maps of wheat and rye studied here provides an understanding of the homoeology between wheat and rye chromosomes. This has direct application to wheat breeding where alien gene transfer programs utilise homoeologous recombination to exploit genetic variability of distant relatives such as rye. RFLP markers allow an efficient detection of rye translocations and recombinations in wheat background. The next chapter describes their use in conjunction with isozyme markers to trace the introgression of 6R.

Chapter 4

Induction of homoeologous recombination between chromosomes rye 6R and wheat 6D using 6RL^{T701} deletion mutants and Sears' *ph1bph1b* mutant.

4.1 Introduction

Introgression of a desired alien gene into wheat genome can be achieved by homoeologous recombination induced by manipulating, the *Ph1* locus (homoeologous pairing barrier) on the long arm of chromosome 5B (Okamato, 1957; Riley and Chapman, 1958; Okamoto and Sears, 1958). Earlier investigations have proved that Sears'*ph1b* mutant (deficient of *Ph1b* locus) is more superior to nulli5B-tetra5D for inducing the transfer of alien genes as it makes chromosome 5B available for possible involvement in transfers (Wu, *et al.*, 1989). Using Sears' *ph1b* mutant several useful genes have been introgressed into wheat from different related species of Triticeae (Islam and Shepherd, 1991, 1992; Dundas and Shepherd, 1994; Khan, 1996; Naranjo and Maestra, 1995; Koebner and Shepherd, 1985, 1986; Shepherd *et al.*, 1994). However, the success of introgression depends on factors such as the extent of homoeology between wheat and the alien chromosomes and the pattern of recombination along the chromosomes. For example, in cereal rye, homoeology is marred by gross chromosomal rearrangements that leads to a low level of pairing between wheat and rye chromosomes thus making transfer of certain genes very difficult. The rye CCN resistance gene is located on the interstitial region of 6RL which has disrupted homoeology attributed to non-group 6 translocations. Furthermore, manipulation of an interestitially located gene requires a double cross-over and there is a particularly low level of multiple cross-over events between homoeologous chromosomes (Lukaszewski, 1995).

The uneven distribution of recombination along the homoeologous chromosomes also has significance in homoeologous transfer. Distal regions are more capable of recombination than the proximal regions (Curtis and Lukaszewski, 1991; Lukaszewski, 1992) indicating positive chiasmata interference in the proximal regions (Lukaszewski 1995). However chiasmata form more frequently in the proximal regions of wheat chromosomes deficient for their distal segments suggesting that the proximal region is capable of recombination (Curtis *et al.*, 1991).

Dundas *et al.*, (1992) described the isolation of variant forms of deletions of the long arm of chromosome 6R^{T701} in F3 and F4 progenies from the crosses between Sears *ph1bph1b* mutant and wheat-6R^{T701} (-6D) disomic substitution lines. The deletion breakpoints are concentrated in the distal region of 6RL^{T701} thus the chromosomes retain 6RS^{T701} and the proximal portion of 6RL^{T701} in variable lengths. Progeny from these deletion lines were shown to contain 6RL^{T701}

with variable lengths as telocentric chromosomes (Dundas and Shepherd, 1993) as well as in the form of translocations with the short arm of wheat chromosomes probably 6D; $6RS^{T701}$ telocentric arms were also recovered (Dr I. Dundas, personal communication).

The 6RL deletion mutants were useful in determining the physical location of CCN resistance gene (*Cre -R*). While characterising these deletion mutants, numerous isozyme markers (*α-Amy-R1, Got-R2* and *6-Pgd-r1b*) (Dundas and Shepherd, 1993) and several molecular markers (RFLPs and PCR based) (Taylor, 1996; this study chapter 3) were assigned to deleted fragments subsequently mapping CCN resistance gene *Cre* R to an intercalary region of $6RL^{T701}$. The $6RL^{T701}$ deletion lines can be ideal tools for the introgression of rye chromatin into wheat as the terminal deletions of non-syntenous regions may have left a complete synteny in the remaining segment with the wheat homoeologues. This chapter describes experiments designed to use different $6RL^{T701}$ deletion length mutant lines as female parents to introgress the $6RL^{T701}$ chromatin, thereby introducing CCN resistance gene into wheat by induced homoeologous recombination in a *ph1bph1b* background.

Characterisation of $6RL^{T701}$ deletion mutants have already demonstrated the array of useful genetic markers present on $6RL^{T701}$. These genetic markers are linked, co-dominantly expressive, and spread widely over the target chromosomes, therefore they can be useful to isolate and characterise recombinants. Any dissociation in the linkage of markers can provide an indication of putative cross-over points between the chromosomes facilitating the selection of recombinants. This chapter demonstrates the use of isozyme markers in conjunction with RFLP markers (Taylor, 1996; this study Chapter 3) to isolate the putative recombinants obtained as a result of homoeologous pairing. Two isozyme markers α-amylase and GOT were used in an initial screening to detect the presence of 6RL. Further analysis was achieved using RFLP markers.

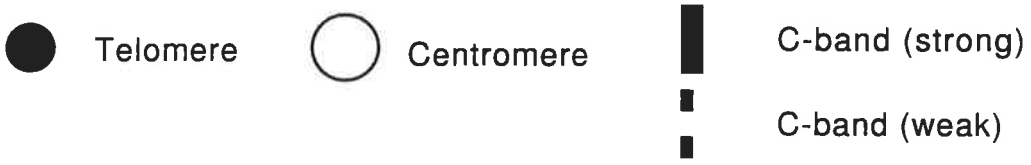
4.2 Results

4.2.1 The generation of a TC-F2 population: A strategy to induce recombination between wheat and rye chromosomes

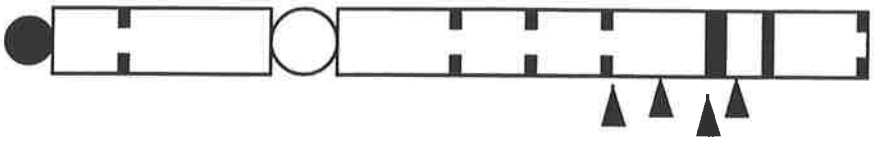
Wheat-rye translocation lines can be good starting material to induce recombination between wheat and rye chromosomes. Based on the method of Koebner and Shepherd (1985; 1986 b), different $6RL^{T701}$ translocation lines (with variable 6RL lengths) and a telodeletion line were used in back-crosses with Sears' *ph1bph1b* mutant to induce homoeologous recombination between $6RL^{T701}$ and 6D. These lines are illustrated in Figure 4.1 and their detailed description is given in Chapter 2, Section 2.1. Two lines, 1411-95 carrying the shortest deletion of $6RL^{T701}$ and the 2373 line carrying the intact translocation of 6RL were used as controls to compare the

Figure 4.1. (a)An illustration of rye chromosome $6R^{T701}$ showing C-bands designations and four breakpoints resulting into deletion mutants. (b)-(f) Different 6WS.6RL translocation mutants arise due to centric fusion of 6WS and variable fragments of 6RL.The data was kindly provided by Dr Ian Dundas, Waite Institutte, South Australia.

Key: *6pgd-r1b*= 6phospho gluconate dehydrogenase
Got-R2=glutamate oxaloacetate transaminase
α-*amy-R1*=α-amylase
Cre-R=CCN resistance gene



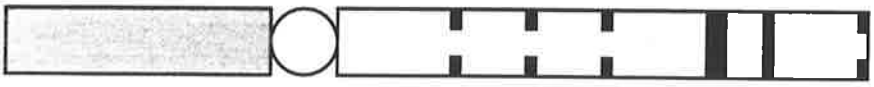
(a)



+6pgd-r1b
+Got-R2
+Cre R
+α-amy-R1

(b)

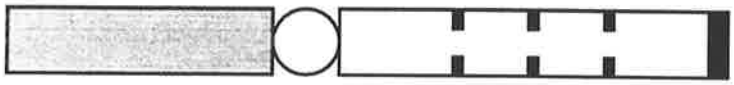
2373-21XS-2XS-1XS-5XS-2



+6pgd-r1b
+Got-R2
+Cre R
+α-amy-R1

(c)

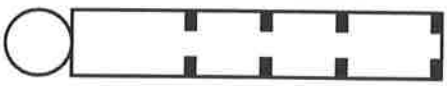
657XS-3-115



-6pgd-r1b
+Got-R2
+Cre R
+α-amy-R1

(d)

791-6-64-51XS-26



-6pgd-r1b
+Got-R2
+Cre R
+α-amy-R1

(e)

1411-54XS-1-1-146-3XS-2

-6pgd-r1b
+Got-R2
+Cre R
+α-amy-R1

(f)

1411-95XS-16-4-57XS-4

-6pgd-r1b
-Got-R2
+Cre R
+α-amy-R1

Table 4.1: Summary of seed set and germination frequencies (%) of plants in F1, TC-F1 and TC-F2 populations of different the 6RL deletion mutant lines.

S.No	Lines	F1		TC-F1		TC-F2	
		Seed Set	Germination (%)	Seed Set	Germination (%)	Seed Set	Germination (%)
1	2373	158	95	93	100	54	96
2	791	72	80	61	65	46	89
3	657	176	100	208	95	109	98
4	1411-54	239	100	564	90	256	98
5	1411-95	61	90	34	88	59	93
Total		706	93	960	87.6	524	94

efficiency of lines in producing recombinants. Figure 4.2 illustrates the method used to generate the TC-F2 with *ph1bph1b* .

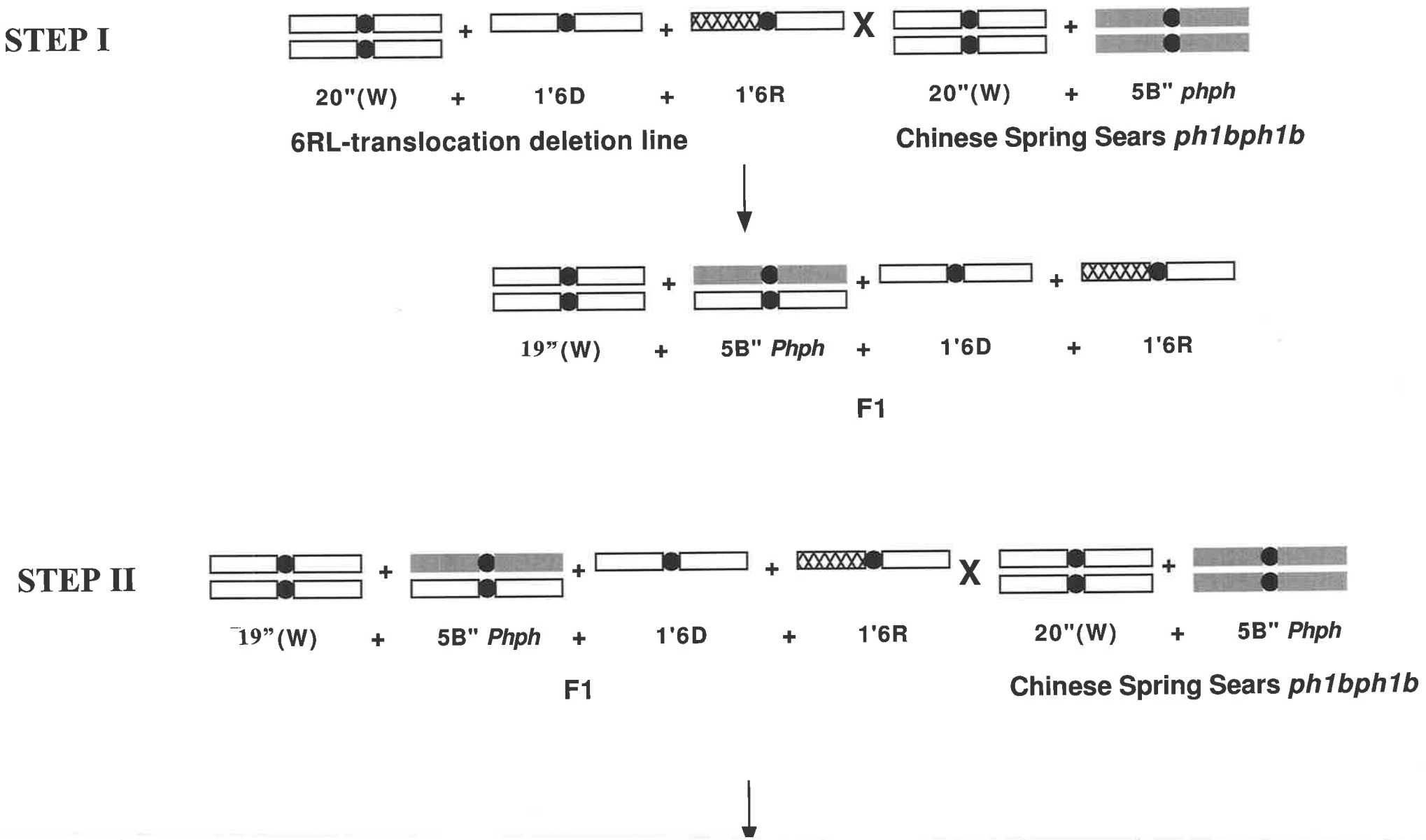
The method consists of three distinct steps. In the first step, variant forms of the 6RL length mutants as females were crossed with Chinese Spring Sears' *ph1bph1b* to produce heterozygous *Ph1bph1b* F1s. In the F1 population, double monosomics of $6R^{T701}$ and 6D were selected and taken to the second step. In this step, selected F1s were test-crossed with Sears' *ph1bph1b* mutant lines to generate a large frequency of TC-F1 with homozygous *ph1b*. Double monosomics of 6R and 6D, with homozygous *ph1b*, were selected and allowed to self in the third and final step to generate the TC-F2 population. Table 4.1 summarises the total number of seeds set and frequency (%) of seeds germinated for mutant lines in each generation. Overall the frequency of seed set was very low for all the lines. However, it is difficult to establish a correlation between the length of $6RL^{T701}$ fragment and the frequency of seed set in these lines. Germination percentage of all the lines except 2373XS line was found to be reduced as a result of the test-cross. Although selfing of TC-F1 enhanced the germination frequency in TC-F2 of all the lines (except 2373XS), poor seed set was observed.

In the first step fifty seeds from each of the five mutant lines were initially screened for the double monosomic condition of 6R and 6D. Thirty seedlings for each line (except 791XS and 1411-95) were selected and used in initial crosses with twenty five Sears *ph1b* mutant (grown at different stages for the fresh supply of pollen). For 791XS only six and 1411-95 only four plants were pursued in initial crosses. In the second step, for each line, thirty seedlings, pooled from at least three crosses, were screened to isolate double monosomics of $6R^{T701}$ and 6D. Ten plants were selected and then used in test-crosses with Sears *ph1b* plants. Except 1411-95, only one desirable seedling carrying 6RL was used in test-crosses. Thirty TC-F1 seedlings from each line were screened and the double monosomics were taken for further studies. After confirming the *ph1b* status, at least five plants homozygous for *ph1b* were identified, bagged and allowed to set self seeds. Except for 791XS and 1411-95, only one desirable seedling carrying 6RL and homozygous for *ph1b* was used in selfing. Total 524 seeds were obtained from selfing TC-F1. Of the 524 TC-F2s, 507 were germinated and tested with isozyme markers. Seven seeds could not be transplanted and 500 plants represent the TC-F2 population analysed for phenotypes resulting from a recombinational event.

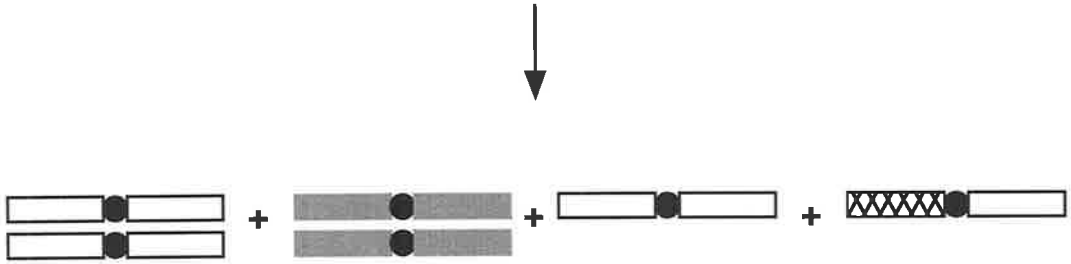
4.2.2 Selection of plants homozygous for *ph1b* using RFLP marker PSR 128

The conventional method (Koebner and Shepherd, 1985; Asiedu *et al.*, 1990; Dundas and Shepherd, 1994) of identification of *ph1b* homozygotes is not only laborious but also delays the identification process. In this study, a fast screening assay based on an RFLP marker was used to select *ph1b* homozygotes at earlier stages.

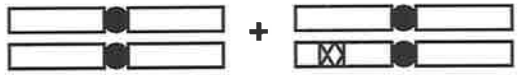
Figure 4.2 An schematic representation of the method used to generate homoeologous recombinants between wheat 6 and 6R^{T701} using Sears' *ph1bph1b* mutant and 6RL^{T701}(deletion mutant lines). Double monosomics for 6R and 6D were selected in each generation and used in the crosses.



STEP III

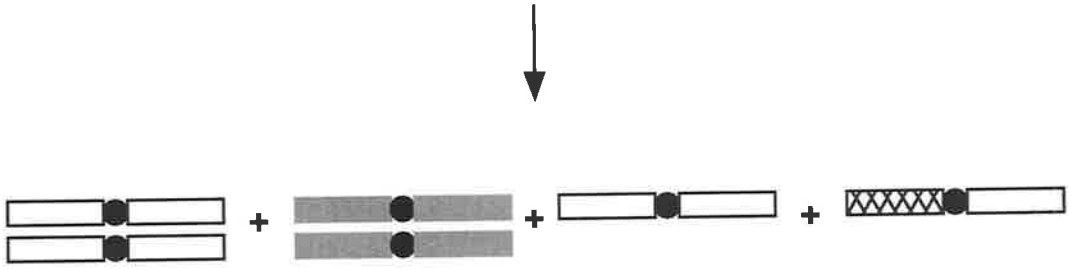


BC1-F1

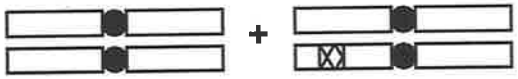


BC1-F2

STEP III



BC1-F1



BC1-F2

Table 4.2: Summary of frequencies (%) of homozygous *ph1bph1b* plants in TC-F1 and TC-F2 progenies of different 6RL deletion mutants. Data is based on the observations obtained from hybridisation of clone PSR128 with plants DNA samples digested with *Eco* RV.

S.No	Lines	% of *ph1bph1b* plants	
		Generations	
		TC-F1	TC-F2
1	2373XS	54	100
2	791	42	100
3	657XS	52	100
4	1411-54	55	100
5	14111-95	48	100

The DNA probe PSR128 (Sharp *et al.*, 1989), which had been mapped to chromosome arm 5BL in the deleted region carrying *Ph* allele (Dr RMD Koebner, personal communication) was used to confirm the *ph1b* homozygous status of the Sears' *ph1b* used as parents, TC-F1s and TC-F2s. Genomic DNA of euploid Chinese Spring *Ph1* parent, Chinese Spring Sears *ph1b* mutant line and progeny TC-F1 and TC-F2 were digested with *Eco* RV and membranes were prepared. When hybridised to *Eco* RV digested euploid Chinese Spring (*Ph1*) DNA, PSR 128 showed three bands, while in the case of Sears' *ph1b* mutant, the lowest molecular band of size 2.3 kb was missing (Figure 4.3). A total of 500 TC-F2 plants were tested for PSR 128 and all lacked the band associated with *Ph1* locus, confirming their homozygous *ph1b* status (Figure 4.3). The frequency (%) of *ph1bph1b* plants in the TC-F1 and TC-F2 populations of each of the 6RL mutant lines summarised in Table 4.2 indicate that plants segregate well for *Ph* locus and observed frquencies were as expected.

4.2.3. Selection of plants monosomic for $6R^{T701}$ and 6D using isozyme markers

A prerequisite for pairing between $6R^{T701}$ and 6D is their monosomic status in a wheat background. However, the low transmission frequency of chromosome 6R (Lukaszewski, *et al.*, 1982; de Vries, 1984) and meiotic instability in double monosomic plants due to asynapsis or desynapsis can result in progeny nullisomic for 6R or 6D or both chromosomes. Consequently, it was necessary to screen the population routinely to select plants monosomic for $6RL^{T701}$ and 6D before using them as parents in the selection program.

The isozyme markers α-amylase was used to select plants with 6RL. Glutamate Oxaloacetate Transaminase (Got) was used to select the double monosomics of $6RL^{T701}$ and 6D by monitoring the segregation pattern of *GotR2* and *GotD2* loci associated with segregation of $6R^{T701}$ and 6D. Table 4.3 lists the all expected genotypes of F1 and TC-F1 (a), and TC-F2 (b), arise from fusion of various types of gametes.

4.2.3.1 α-amylase analysis to screen for the presence of 6RL

Alpha–amylase (α–1,4-glucan-4-glucanhydrolase EC. 3.2.1.1) is involved in the breakdown of starch granules in the endosperm of germinating cereal grain. On the basis of their pI (isoelectric point), α–amylases can be classified into two groups; namely, α–AMY1 (high pI) and α–AMY2 (low pI) isozymes controlled by: *α–Amy1* family on group 6 and *α–Amy2* genes on group 7 chromosomes (Nishikawa and Nobuhara, 1971; Lazarus *et al.*, 1985). A third group of *α–Amy3* is present as a single copy on group 5 chromosomes (Baulcombe *et al.*, 1987). In wheat, the estimated numbers of α–amylase genes at:*α–Amy1* loci is five to six on 6B and 6D, two on 6A; three to four at the *α–Amy2* loci on 7A, 7B and 7D (Martienssen, 1986), and one at the *α–Amy3* loci on each of the group 5 homoeologues (Baulcombe *et al.*,

Figure 4.3 Screening of sub population of TC-F2 for *ph1bph1b* phenotype.

Hybridisation with PSR128. The size standard (M) was λ *Hin* d III. The size of bands generated in standard is shown in base pairs on the left. The 5BL band associated with *Ph* locus is indicated by arrow (⟶). Lane 1-20 contained the following DNA samples digested with *Eco* RV:

- Lane 1. Schomburgk
- Lane 2. euploid Chinese Spring
- Lane 3. mutant Chinese Spring Sear's *ph1bph1b*
- Lane 4. Wheat-6R^{T701}(-6D) disomic substitution line
- Lane 5. 6RL^{T701}(del).6WS translocation-1411-54-H1
- Lanes 6-20. a subpopulation of TC-F2 progenies

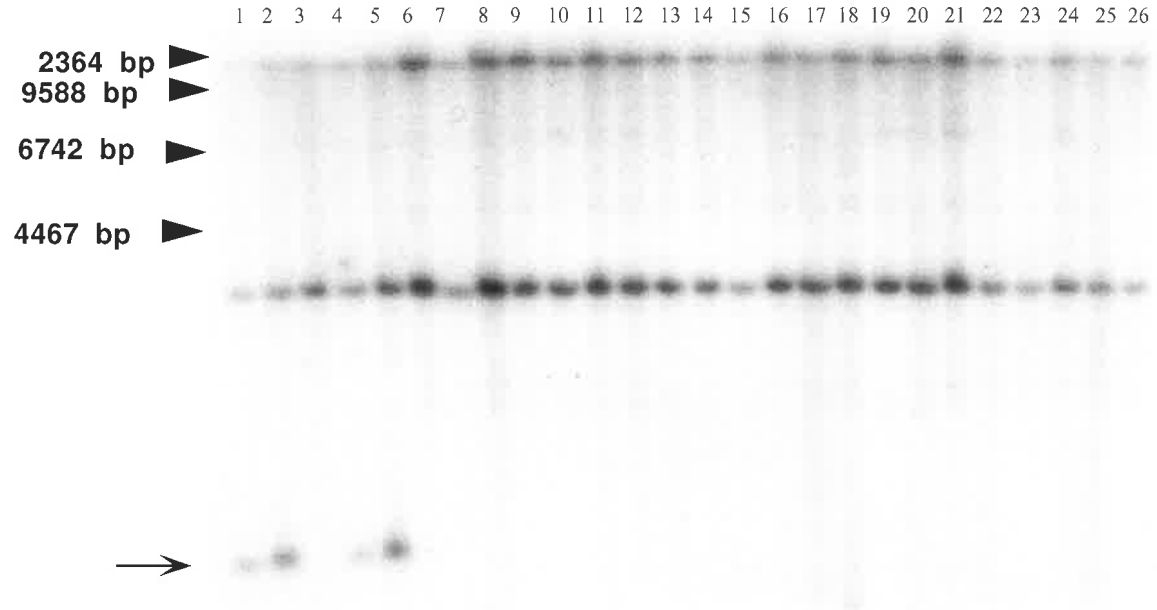

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
2364 bp
9588 bp
6742 bp
4467 bp

Table 4.3 List of all the possible genotypes arise from the fusion of various gametes resulting from asynapsis or desynapsis.

(a) Expected genotypes in F1 and TC-F1 populations shown in shaded areas. Expected proportion of all the genotypes is 25%.

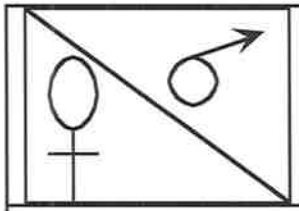
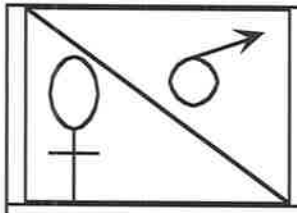
	6D'+20W' Got-D2
6R'+20W' Got-R2	6R'+6D'+20W" Got-R2 Got D2
6D'+20W' Got-D2	6D"+20W" Got-D2 Got-D2
6R'+6D'+20W' Got-R2 Got-D2	6R'+6D"+20W" Got-D2 Got-D2 Got-R2
20W'	6D'+20W" Got-D2

Table 4.3 cntd...

(b) Expected genotypes in TC-F2 population. Intensity of shading represents the variable proportion of these genotypes in population.

	6R'+20W' Got-R2	6D'+20W' Got-D2	6R'+6D'+20W' Got-R2 Got-D2	20W'
6R'+20W' Got-R2	6R"+20W" Got-R2 Got-R2	6R'+6D'+20W" Got-R2 Got-D2	6R"+6D'+20W" Got-R2 Got-R2 Got-D2	6R'+20W" Got-R2
6D'+20W' Got-D2	6R'+6D'+20W" Got-R2 Got-D2	6D"+20W" Got-D2 Got-D2	6R'+6D"+20W" Got-D2 Got-D2 Got-R2	6D'+20W" Got-D2
6R'+6D'+20W' Got-R2 Got-D2	6R"+6D'+20W" Got-R2 Got-R2 Got-D2	6R'+6D"+20W" Got-D2 Got-D2 Got-R2	6R"+6D"+20W" Got-R2 Got-R2 Got-D2 Got-D2	6R'+6D'+20W" Got-R2 Got-D2
20W'	6R'+20W" Got-R2	6D'+20W" Got-D2	6R'+6D'+20W" Got-R2 Got-D2	20W"

1987). In rye, there are three *α–Amy1* genes on 6R, two or three *α–Amy2* genes on 7R and three *α–Amy3* genes on 5R (Masojc, 1987; Masojc and Gale, 1991).

In an initial study, 50 seeds from the line 1411-95XS-16-4-57XS-4 were screened for the presence of 6RL using the α–amylase isozyme marker. This particular line posseses the shortest fragment of 6RL (see Section 2.1), containing the *α–Amy1* locus and lacking the *Got-R2* locus. Figure 4.4 shows the α–amylase zymogram phenotypes of a subpopulation of plants from the line 1411-95XS-16-4-57XS-4. The presence of 6RL can be identified by the appearance of two bands of pI 6.15 and 6.2. Bands associated with 6D are absent in the wheat-$6R^{T701}$ (-6D) disomic substitution line. This enzyme was then used routinely for screening 1411-95 lines for the presence of 6RL.

4.2.3.2 Glutamate oxaloacetate transaminase (GOT) analysis to screen for the presence of 6RL

Hart (1975) localised three triplicate sets of glutamic-oxaloacetic transaminase (GOT) structural genes on the 3L, 6S and 6L chromosome arm groups of wheat. Other genes were located on chromosomes of homoeologous group 7. In Chinese Spring, the structural genes encoding GOT-2 have been localised to the long arm of group 6, designated as *Got-A2, Got-B2* and *Got-D2*. In other members of the Triticeae, rye chromosome 6R and barley chromosome 6H possess *Got-R2* and *Got-H2*, respectively. These groups of GOT genes code subunits are designated as α^2, β^2 , δ^2 (wheat), ρ^2 (rye) and θ^2 (barley).

In Chinese Spring wheat, the GOT-2 isozymes are dimers composed of all possible combinations of subunits coded by the triplicate genes, revealed as three forms on polyacrylamide gels, GOT-2a, GOT-2b and GOT-2c (Hart, 1975). In rye, a single form, GOT-2e, is observed as the most cathodal band which has a slower mobility than all three forms obtained in Chinese Spring wheat (Tang and Hart, 1975). An additional form, GOT-2d, is identified in the Chinese Spring- $6R^{Imp}$ disomic addition line (Tang and Hart, 1975) resulting from the combinations of dimers α^2 and β^2 with δ^2 in an intermediate mobility band. This information provides criteria for selecting plants possessing 6RL.

Initially, 50 seeds from each of the four lines 2373-21XS-3X-1XS-3, 791-6-64-51XS-26, 657XS-3-115-354 and 1411-54XS-1-146-3XS-2 were screened using GOT analysis. In the intermediate mobility zone, the wheat-$6R^{T701}$(-6D) disomic substitution line displays a pattern of three closely spaced bands, the slowest of which characterizes GOT-2 encoded by 6RL. The bottom band, with relatively fast mobility in the GOT-3 zone, is associated with 6D present in wheat but absent in the wheat-$6R^{T701}$(-6D) disomic substitution line. These were used as controls (Figure 4.5). The summary of expected and observed frequency (%) of four different

Figure 4.4 Zymogram of α–amylase of sub-population of 6RL(del).6WS (1411-95XS-16-4-57XS-4). Bands are indicated by 6R (◄—) and 6D (←—). Plants containing 6R and 6D chromosomes are represented by schematic diagram on top of the picture.Black chromosomes represent 6D and white chromosomes represent 6R. Lanes 1-12 contain the following isozyme samples:

- Lanes.1and 14. Chinese Spring
- Lanes.2 and 13. Wheat-$6R^{T701}$(-6D) disomic substitution line
- Lanes.3-12. a sub-population of 6RL(del).6WS (1411-95XS-16-4-57XS-4)

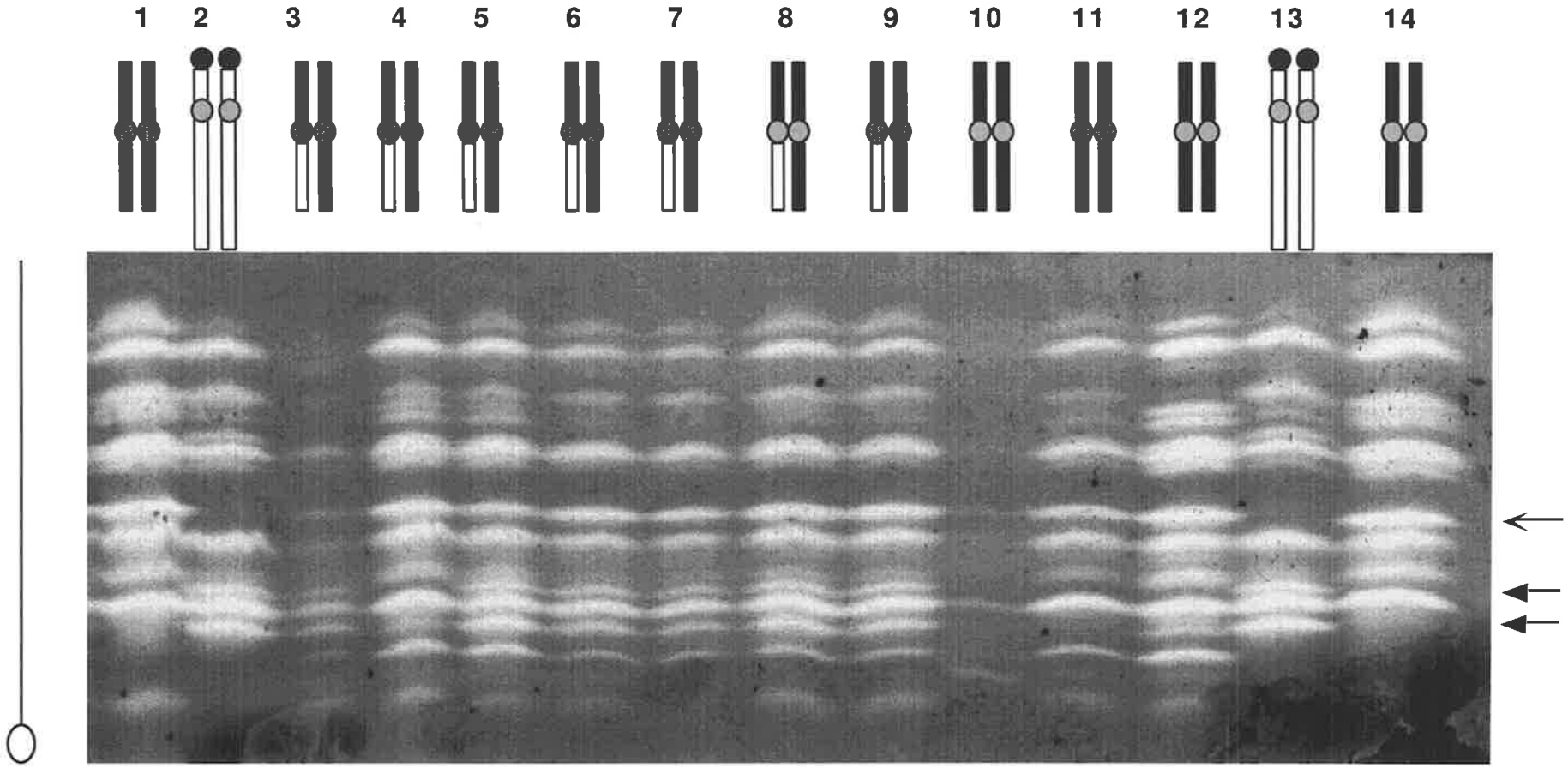

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Table 4.4: The expected and observed frequencies (%) of plants with different genotypes in F1 and TC-F1 progeny of 6RL deletion mutants. Doses of 6R and 6D were established on the basis of GOT analysis.

S. No	Line	Genotypes	Expected frequencies (%)	Observed frequencies	
				% of F1 population	% of TC-F1 population
1	2373	Got R2/Got D2	25	21	10
		Got-R2 /GotD2GotD2	25	20	40
		Got r2/Got D2GotD2	25	50	40
		Got-r2/Got-D2	25	5	10
2	791	Got R2/Got D2	25	10	3
		Got-R2 /GotD2GotD2	25	30	30
		Got r2/Got D2GotD2	25	50	66
		Got-r2/Got-D2	25	10	0
3	657	Got R2/Got D2	25	20	16
		Got-R2 /GotD2GotD2	25	40	46
		Got r2/Got D2GotD2	25	33	33
		Got-r2/Got-D2	25	7	3
4	141-54	Got R2/Got D2	25	50	15
		Got-R2 /GotD2GotD2	25	40	33
		Got r2/Got D2GotD2	25	6	40
		Got-r2/Got-D2	25	4	12

genotypes obtained in F1 and TC-F1 populations of each of the four 6RL lines is give in Table 4.4

Table 4.5: The expected and observed frequencies (%) of plants with different genotypes in TC1-F2 progeny of different 6RL deletion mutant lines. Doses of 6R and 6D were established on the basis of GOT analysis.

S. No	Genotypes	Expected frequencies (%)	% of observed genotypes in TC-F2			
			Lines			
			2373XS	791	657XS	1411-54
1	Got R2/Got D2	25	32	30	30	28
2	Got R2/Got D2 Got D2	12.5	18	15	24	9
3	Got R2 Got R2/Got D2	12.5	8	5	11	10
4	Got R2/Got d2	12.5	8	15	3	8
5	Got r2/ Got D2	12.5	22	20	21	25
6	Got R2/GotR2/Got d2	6.25	6	5	4	5
7	Got r2/Got D2 Got D2	6.25	2	5	0	7
8	Got R2Got R2/Got D2Got D2	6.25	4	5	6	5
9	Got r2/Got d2	6.25	0	0	1	3

4.2.4 Screening of TC-F2s using isozymes and RFLP markers to characterize putative recombinants

A total of 500 TC-F2s representing 3 to 5 different families of each of the five 6RL mutant lines were screened with RFLP marker PSR128 to confirm the *ph1b* homozygous status. All were found to be of *ph1bph1b* genotype. Meiotic configurations at metaphase I, anaphase I and telophase I in anthers squashes stained with acetocarmine indicated multivalent and rod bivalents (Figure 4.6). However, no attempt was made to check the pairing of $6RL^{T701}$ with wheat group 6 chromosomes, as it was not possible to confidently identify the 6RL due to the faint C-bands in the fragment of $6RL^{T701}$ remaining on these chromosomes.

Of 500 TC-F1 seedlings: 50 were screened from line 2373-21XS representing three families, 40 seedlings from line 791-6-64-51XS-26 representing one family, 105 from 657XS-3-155 line representing five families, and 250 TC-F2 seedlings from the line 1411-54-XS-1-146-3XS-2 representing five families. Although 55 TC-F2s seedlings were screened from the line 1411-95 using α–amylase analysis, only 2 possessed the 6RL fragment.

All the available TC-F2s seeds were initially screened for the presence of $6RL^{T701}$ and 6D using α–amylase and GOT isozymes. The results of Got analysis are summarised in Table 4.5 with expected and observed frequencies of genotypes arise as a result of vaious gametic fusions. No dissociation between *α–Amy* and *Got* loci was observed on $6R^{T701}$ and 6D chromosomes for four mutant lines. These plants were further characterised using RFLP markers. Ten RFLP markers (ABG474, BCD758, WG933, BCD1426, PSR142, PSR915, PSR149, PSR154, BM15, BCD1) were tested in various combinations comprising sets of 4-6 markers on all the lines. Further, two additional markers, (ksuF037and PSR148) were tested on TC-F2 of line 2373 and only ksuF037 was tested on 657 and 791 lines as it is the most distal marker found on these lines. Table 4.6 lists the RFLP markers tested on TC-F2, and the enzyme used to detect co-dominant loci for $6R^{T701}$and 6D. No dissociation was observed in the linkage of these RFLP markers along $6R^{T701}$ or 6D chromosomes. However, deletions were observed for certain loci, *Xbcd758* (proximal), *Xwg933*, *Xbcd1426*, *Xpsr142* and *Xpsr915* (interstitial) in wheat group 6 chromosomes other than 6D (Figure 4.7 a, b, c). The distal loci were retained in these lines (Figure 4.8 a, b). The new lines were obtained in TC-F2 of four different families (H1, H10, G7, I5) of 1411-54 lines indicating either a deletion of the region carrying these loci or a putative recombination in the proximal region of these wheat chromosomes. Dissociation in the linear order indicated that a putative cross-over point may lie between *Xpsr915* and *Xpsr149* . Interestingly, the majority of these lines have *Got-R2Got-R2 /GotD2 GotD2* genotype indicating that these plants possess a double dose of $6R^{T701}$ and 6D, therefore there is a possibility that any recombination involving $6R^{T701}$ or 6D is masked by the presence of intact 6R and 6D chromosomes. Four plants were different: containing a single dose of 6D (G7-17

Figure 4.5 Zymogram of GOT of sub-population of 6RL(del).6WS (1411-54XS-1-1-146-3XS-2 57XS-4). Bands are indicated by arrows 6R (◄—) and 6D (←). *Got-R2* is designated as (+) and *Got-D2* is designated as (0). Plants containing 6R and 6D chromosomes are represented by schematic diagram on top of the picture.Black chromosomes represent 6D and white chromosomes represent 6R. Double monosomics are indicated by (+0) genotype. Lanes 1-11 contain the following isozyme samples:

- Lanes.1and 11. Wheat-6R^{T701}(-6D) disomic substitution line
- Lanes.2-9. a sub-population of 6RL(del).6WS (1411-54X)
- Lanes.10. Chinese Spring

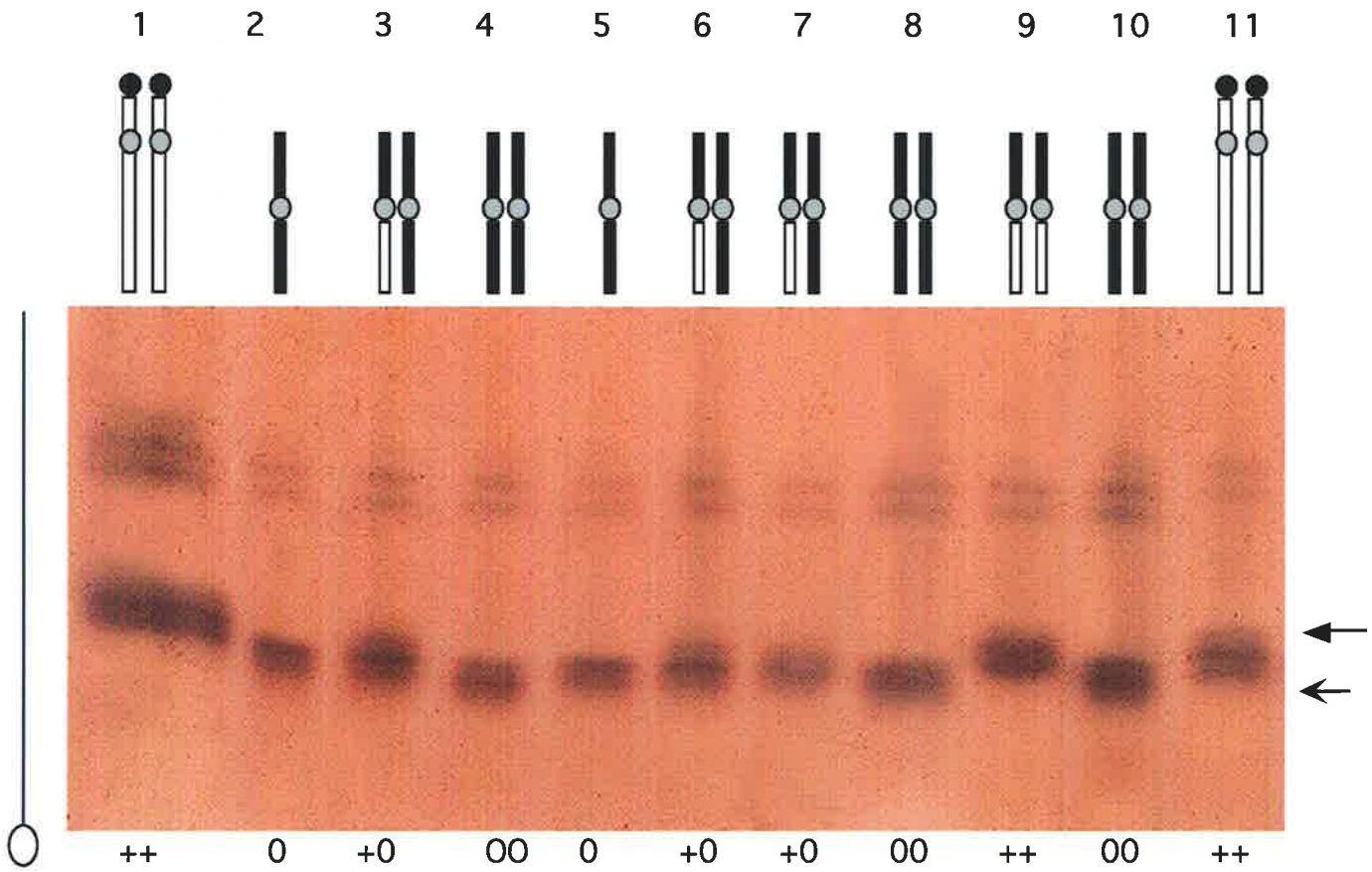
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Table 4.6: List of 6L probes used to screen the TC-F2 population of each 6RL deletion line. Restriction enzymes used to reveal RFLPs with each probe are listed.

S. No	Clones	Locus	Restriction enzyme
1	BCD758	*Xbcd758*	*Dra I*
2	WG933	*Xwg933*	*Sac I/Hind III*
3	PSR142	*Xpsr142*	*BamH I*
4	PSR915	*Xpsr915*	*Dra I/Sac I*
5	BCD1426	*Xbcd1426*	*Dra I/ Hind III*
6	PSR149	*Xpsr149*	*Sac I*
7	BCD1	*Xbcd1*	*Dra I/ Hind III*
8	PSR154	*Xpsr154*	*Sac I*
9	ksuF037	*XksuF037*	*Hind III*
10	PSR148	*Xpsr148*	*BamH I*

Fig 4.6 Meiosis in TC-F1-H10

Configuration of chromosomes at Metaphase I in PMCs of TC-F1-H10. Note the occurance of substantial number of rod bivalents and multivalents. Diagonostic rod bivalents and mutivalents are arrowed (◄).

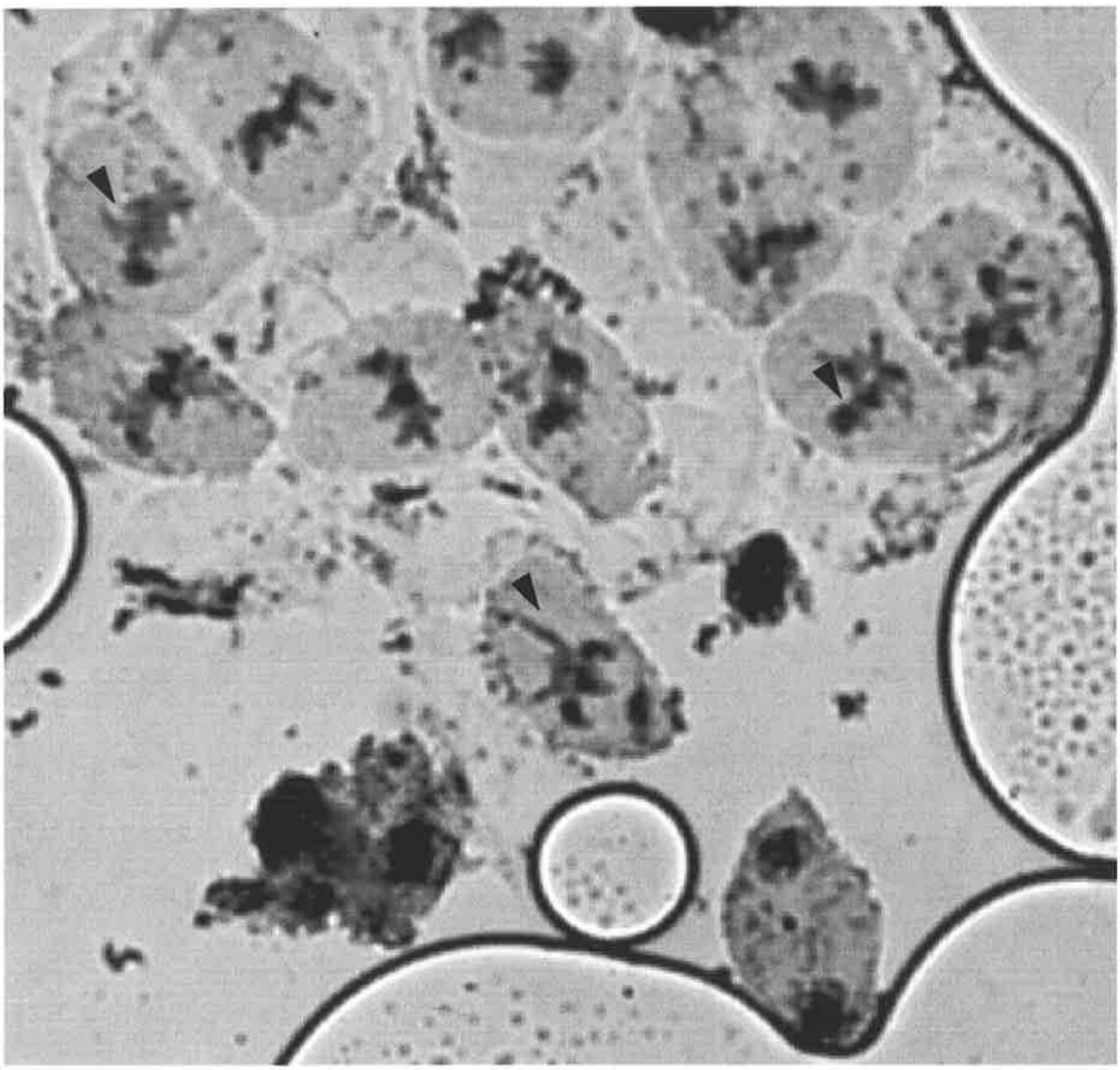


Fig 4.7 Screening of sub population of TC-F2 with RFLP markers:(a) BCD758, (b)BCD1426 and (c) PSR915.

The specific chromosomal bands are indicated by arrows 6R (⟶) and 6D (⟶). Putative deletion in the chromosome other than 6R and 6D is indicated by arrow (◄) in individual H10-70 (*) (lane-21). Lane 1-21 contained the following DNA samples digested with *Dra* I:

- Lane 1. Chinese Spring
- Lane 2. Wheat-6R^{T701}(-6D) disomic substitution line
- Lane 3. 6RL^{T701}.6WS translocation (2373XS)
- Lane 4. 6RL^{T701}(del-1).6WS translocation (657XS)
- Lane 5. 6RL^{T701}(del-3).6WS translocation-(1411-54)
- Lane 6. 6RL^{T701}(del-4).6WS translocation-(1411-95)
- Lane 7. 6RL^{T701}(del-3).6WS translocation-1411-54---TC-F1-H10
- Lanes 8-21. a subpopulation of TC-F2 progenies
- Lane 21. TC-F1-H10-70 with putative deletion in proximal region of 6WL (AL or BL)

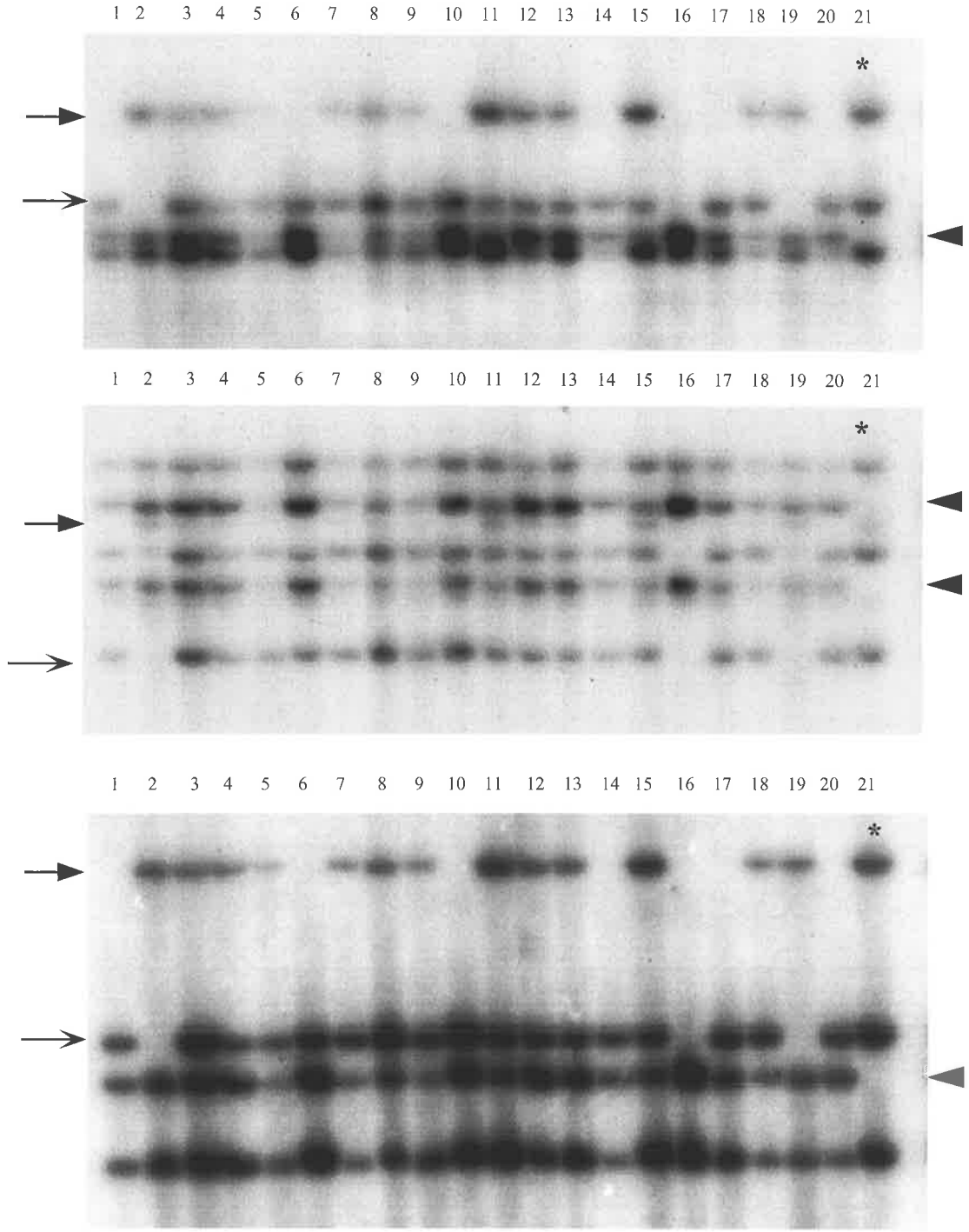

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
*

Fig 4.8 Screening of sub population of TC-F2 with RFLP markers:

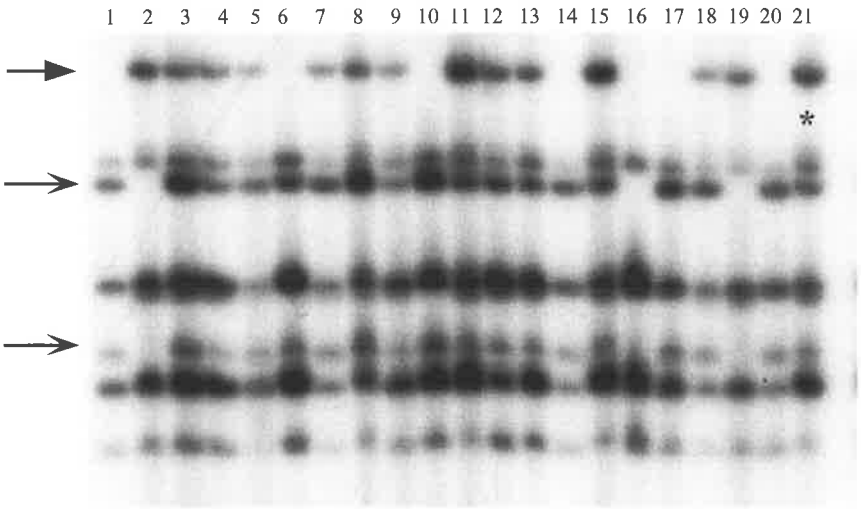
(a)BM15 hybridised with *Dra* I (b)PSR154 hybridised with *Sac* I.

The specific chromosomal bands are indicated by arrows 6R (⟶) and 6D (⟶).

Individual TC-F1-H10-70 with putative proximal deletion is indicated by an aestrik (*).

Lanes 1-21 contained the following DNA samples:

- Lane 1. Chinese Spring
- Lane 2. Wheat-6R^{T701}(-6D) disomic substitution line
- Lane 3. 6RL^{T701}.6WS translocation (2373XS)
- Lane 4. 6RL^{T701}(del-1).6WS translocation (657XS)
- Lane 5. 6RL^{T701}(del-3).6WS translocation-(1411-54)
- Lane 6. 6RL^{T701}(del-4).6WS translocation-(1411-95)
- Lane 7. 6RL^{T701}(del-3).6WS translocation-1411-54---TC-F1-H10
- Lanes 8-21. a subpopulation of TC-F2 progenies
- Lane 21. TC-F1-H10-70with putative deletion in proximal region of 6WL (AL or BL)



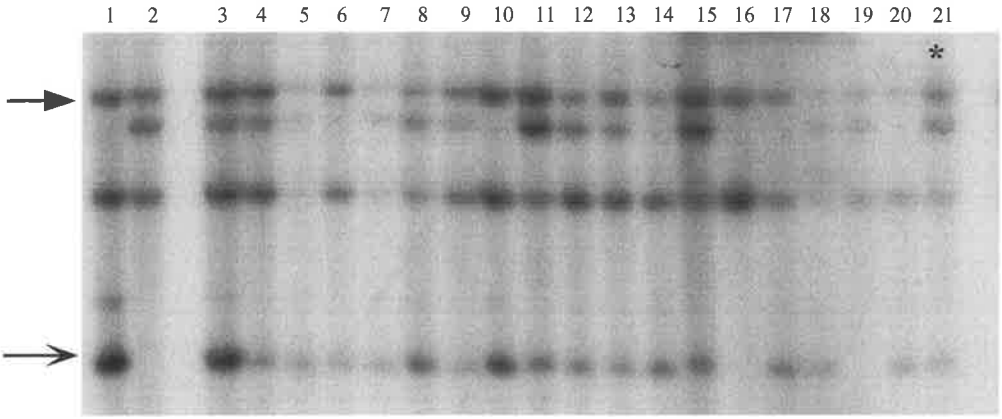


Table 4.7: Putative recombinants /deletion mutants of wheat group 6 chromosomes isolated in TC-F2 of line 1411-54. Deletions of certain RFLP markers are shown, indicating a breakpoint between *Xpsr915* and *Xpsr149*.

S.No	Plant	Loci								
		Xbcd758	*Xwg933*	*Xbcd1426*	*Xpsr142*	*Xpsr915*	*Xpsr149*	*Xpsr154*	*Xbma15*	*Got-R2/G0t-D2*
1	TC-F2-H1-10	–	–	–	–	–	+	+	+	+/++
2	TC-F2-H10-70	–	–	–	–	–	+	+	+	++/++
3	TC-F2-G7-9	–	–	–	–	–	+	+	+	++/++
4	TC-F2-G7-17	–	–	–	–	–	+	+	+	++/+
5	TC-F2-G7-21	–	–	–	–	–	+	+	+	++/++
6	TC-F2-G7-28	–	–	–	–	–	+	+	+	++/++
7	TC-F2-G7-35	–	–	–	–	–	+	+	+	++/++
8	TC-F2-G7-36	–	–	–	–	–	+	+	+	++/++
9	TC-F2-I5-10	–	–	–	–	–	+	+	+	++/--
10	TC-F2-I5-23	–	–	–	–	–	+	+	+	++/++
11	TC-F2-I5-24	–	–	–	–	–	+	+	+	++/+

and I5-24) or 6D absent (I5-10); and a single dose of $6R^{T701}$ was present (H1-10). The gentoypic characterisation of these lines is given in Table 4.7.

4.3 Discussion

The present study was undertaken to introgress 6RL into the wheat genome with the practical aim of introducing the cereal cyst nematode resistance gene. The method involved the use of Sears *ph1bph1b* mutant and $6RL^{T701}$(deletion).6WS translocation/telocentric lines to enhance the transfer of $6RL^{T701}$ segment into wheat genome. Isozyme and RFLP markers were used to assist the selection for $6R^{T701}$ and 6D and to isolate recombinants. This section discusses the comparative efficiency of isozyme and RFLP markers as selection tools and outlines the effects on the transfer of $6RL^{T701}$ by Sears *ph1bph1b* mutant and $6RL^{T701}$ translocation deletion mutants.

4.3.1 Comparative use of isozyme and RFLP markers as selection tools in isolation of recombinants

Genetic markers which are co-dominant expressive, easy to score and spread widely over the target chromosomes can be useful to isolate recombinants. These markers should be necessarily linked so the dissociation of markers indicating putative cross-over points between the chromosomes can facilitate the selection of recombinants. Isozyme markers are co-dominant and economical, but have limited use because of low polymorphism and limited number. As compared to this, RFLP markers are highly polymorphic and unlimited although not readily used for large scale screening. Furthermore, isozyme markers assays are tissue specific and stage dependent whereas RFLP analysis is not specific to time and tissue. Good quality DNA can be isolated from any tissue of plant at any stage.

In this study both isozyme and RFLP markers were used as selection tools to isolate putative recombinants between $6R^{T701}$ and 6D chromosomes. Two isozyme markers, α–amylase and GOT were used on young seedlings to detect the dissociation between two loci. Information from $6RL^{T701}$ deletion mutants suggests that these two loci flank the first proximal deletion break-point on $6RL^{T701}$ indicating a possible fragile site. However, no dissociation was observed between these two loci in the initial screening. Detection of any recombination event occuring proximal to α–amy or distal to Got-2 required further useful genetic markers that flank both sites of these two loci. This limitation of the isozyme markers was overcome by using large number of RFLP markers that extend over the entire length of 6R and 6D chromosomes.

A further drawback in working with isozyme markers was the reproducibility of results attributed to loss of enzyme activity from the samples, particularly α–amylase (Dr. I. Dundas, personal communication). However the isozyme marker GOT was more advantageous as it

allowed detection of chromosome dosage for 6R and 6D. In this study some putative homoeologous recombinants were observed and GOT provided an efficient detection of dosage of 6R and 6D in establishing wether or not intact chromosome (6R or 6D) could mask the involvement of 6R and 6D in recombination. In the case of RFLP markers, dosage detection could be established only when an intervarietal polymorphism exist between the chromosomes as intensity of signal was not a good criterion. For example, use of WG933 (data not shown) with different restriction enzymes was helpful in detecting the double dose of 6D because of the polymorphism between Chinese Spring and Schomburgk. Therefore, in this study isozymes coupled with RFLP markers were used to offset the limitation of eachother.

4.3.2 Strategy used for introgression of $6RL^{T701}$ segment into wheat

4.3.2.1 Effects of using Sears' *ph1b* mutant

In this study, Sears *ph1b* mutant (Sears, 1977) was used to induce pairing between 6R and 6W chromosomes. Sears *ph1bph1b* is a high pairing mutant (HPM) and not only restricts homologous pairing but also increases homoeolgous pairing between wheat genomes and wheat with related alien genomes. Chinese Spring wheat and Sears *ph1b* mutant are isogenic except for the *ph1b* mutation. This mutation has been specified as a deletion on the long arm of chromosome 5B. In recent years the number of RFLP and PCR markers mapped within this deletion region (Clark *et al.*, 1992; Gill and Gill, 1991, 1993) provided a fast and early detection system for *ph1b* homozygous plants as compared to conventional cytogenetics studies. In this study, the RFLP probe PSR128 (Sharp *et al.*, 1989) was reliably used to select the parents and progeny before their use in the recombination studies. The absence of the PSR128 band confirmed the homozygous state of *ph1b* in all TC-F1 and TC-F2 lines. The presence of multivalent and rod bivalent associations at metaphase I, anaphase I and telophase I observed in TC-F1 parents also suggested that optimal conditions for homoeologous chromosomes pairing and recombination had been obtained.

4.3.2.2 Test-cross with Sears' *ph1b* mutant

The test-cross strategy used has advantages over previously described methods (Koebner and Shepherd, 1985; Khan, 1996). Earlier studies used selfing to transfer desired gene of interest. In this study a modified approach was used. Instead of selfing, F1 progenies were test-crossed with Sears *ph1bph1b* and a recovery of 50% TC-F1 with *ph1bph1b* was achieved thus completely eliminating the *Ph1bPh1b* types. While selfing of F1 generates only 25% *ph1bph1b* homzygotes as a result of 1:2:1 segregation of the F1s at the *Ph1bPh1b* locus. However, a reduced number of tillers, reduced seed set and poor transmission rate of 6R was associated with test-crosses where as selfing generated large numbers of seeds (Khan, 1996). In this study

selfing of TC-F1 plants did not generate large number of viable seeds and the final population size of 500 was small. The poor seed set can be attributed to reduced fertility in homozygous *ph1b* plants associated with high level of non homoeologous pairing.(Sears, 1981).

4.3.2.3 Effects of using various 6RL^{T701}(deletion).6WS translocation lines

Previous attempts using Sears *ph1bph1b* and entire chromosome 6R^{T701} as a substitution were failed to introgress 6R into wheat genome, but produced useful 6R^{T701} deletion lines. Analysis of these lines indicated that deletion breakpoints are concentrated in the subterminal region (Dundas *et al.*, 1992) and may be due to incomplete crossing over at non-homoeologous distal regions (Devos *et al.* 1993). Since the 6R^{T701} deletions are deficient for most of the non group 6 region it was proposed that the chances of pairing between 6RL^{T701} and group 6 homoeologues were increased in the *ph1b* state. In this study four different 6RL^{T701}(deletion).6WS translocation and one 6RL^{T701}(del) telocentric lines were tested for their recombining ability with 6W.

Comparison of different 6RL mutants in producing recombinants

When this project was initiated little information was available on the 6RL^{T701} deletion lines. The array of isozyme markers mapped along the different lengths of 6RL^{T701} (Dundas and Shepherd, 1993) in these lines indicated for complete homoeology with the wheat 6DL. No efforts were made to confirm the presence of structural genes for esterases {(3A, 3B, 3D in wheat (Jouve and Diaz, 1990)} and endopeptidase {7BL and 7DL wheat (Koebner *et al*, 1988)} which has been mapped on rye 6RL (reviewed by Benito *et al.*, 1991). Instead, RFLP markers were used and presence of PSR148 on the intact 6RL translocation, (this study Chapter 3; Devos *et al.*, 1993) confirmed the existance of incomplete homoeology at the distal regions of chromosomal arms.

In comparison, assignment of large number of group 6 RFLP markers to 791, 657 and 1411-54 deletion lines, indicated that a large proportion of 6RL^{T701} is syntaneous with 6WL and the use of these deletion lines particularly 1411-54 could facilitate pairing between chromosomes. However, recently Taylor (1996) identified an inversion on 6RL^{T701} by mapping PSR154 to a telocentric line of 1411-95 carrying the largest deletion of 6RL. However, several proximal markers including WG933 could not be mapped to this line indicating that the order of PSR154 and WG933 may be reversed on 6RL and this could reduce the probability of recombination between 6R and 6W. The control line 2373 provided a useful opportunity to check the homoeologous recombination pattern along the length.

In this study a total of 500 TC-F2s from the different deletion mutants were screened with isozymes and RFLP markers but no dissociation of markers was observed on $6R^{T701}$ and 6D. Based on GOT analysis, a higher frequency for types possessing both $6R^{T701}$ and 6D was observed which indicates that these chromosomes were transmitted efficiently. Significant frequencies of other types possessing either $6R^{T701}$ or 6D were also observed. There could be two possibilities, these types are the product of either desynapsis or a potential recombination between wheat $6R^{T701}$ and 6D chromosomes. However, 1 to 3% of TC-F2 (in 1411-54 and 657XS families) showed 6R-/6D- type, which probably arose due to desynapsis. The expected frequency of such types was 6.25%. RFLP analysis with probes BCD758, WG933, BCD1426, PSR915, PSR142, PSR149, BCD001, BM15 and PSR154 did not show any recombination between 6R and 6D chromosomes. In addition, RFLP marker ksuF037 for TC-F2 of 657XS-3-115 and 791 lines and PSR148 for 2373-21XS lines were also used but no dissociation of markers was observed for the distal region of $6RL^{T701}$.

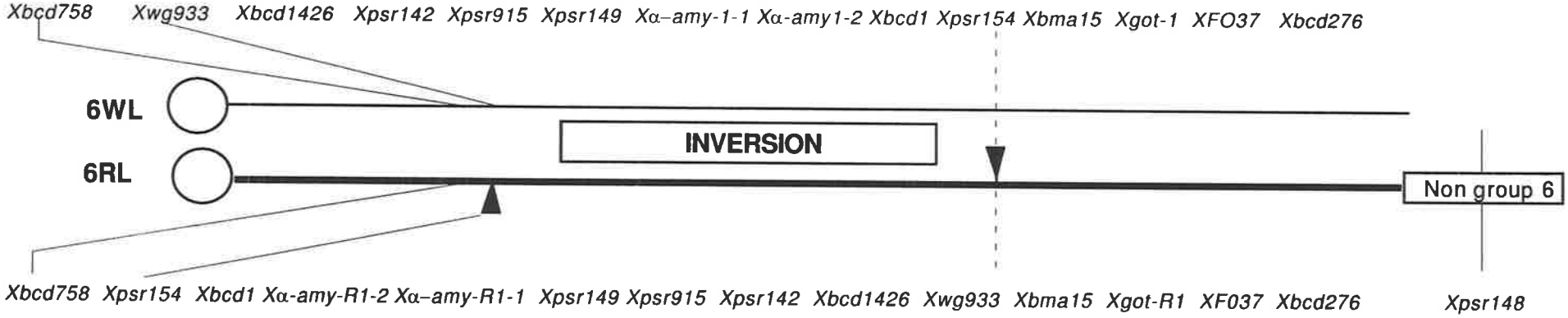
Although no dissociation between the markers was observed on $6R^{T701}$ and 6D, some lines for abberant wheat 6A or 6B chromosomes in the TC-F2 of 1411-54 were obtained with double doses of $6R^{T701}$ and 6D. RFLP data indicated a deletion of fragment comprising of loci *Xbcd758, Xwg933* , *Xbcd1426*, *Xpsr142* and *Xpsr915,* but retaining the more distal loci *Xpsr149* , *Xpsr154* and *Xbm15.* The recombination seems to occur between *Xpsr915* and *Xpsr149* loci. A cross-over involving *Xbcd1426* and *Xbm15* between $6R^{Imp}$ and $6R^{T701}$ was also observed in TC-F1-295 of mapping population (Taylor, 1996), which indicates that this is a possible hot site for recombination. TC-F1-295 was not tested for *Xpsr142* , *Xpsr915* , *Xpsr149* and *Xpsr154* .

Although 6R and 6D were present in a hemizygous state in TC-F1 facilitating pairing between them, the putative inversion on $6RL^{T701}$ involving a segment between PSR154 and WG933 might have reduced the chiasmata formation between 6WL and $6RL^{T701}$. If a cross-over occured, it may result in a dicentric chromosome and an acentric fragment as these are the typical consequences of crossing-over in an individual heterozygous for a paracentric inversion, reported in maize and other plants (reviewed by Burnham, 1962). The acentric fragment is usually lost at anaphase or either forms a micronucleus and included into spore, permits it to develop normally. Whereas the dicentric chromosome usually forms a bridge at anaphase which may break at any point during the division and the broken chromosome transmitted through pollens. However both acentric and broken chromosome do not persists through very many cell divisions. The classical work of Mc Clintock (reviewed by Burnham, 1962) on maize have provided evidences for the lost fates of dicentric chromosomes and acentric fragments. In maize, paracentric inversions are characterised by pollen abortions and usually the plants are partially sterile.

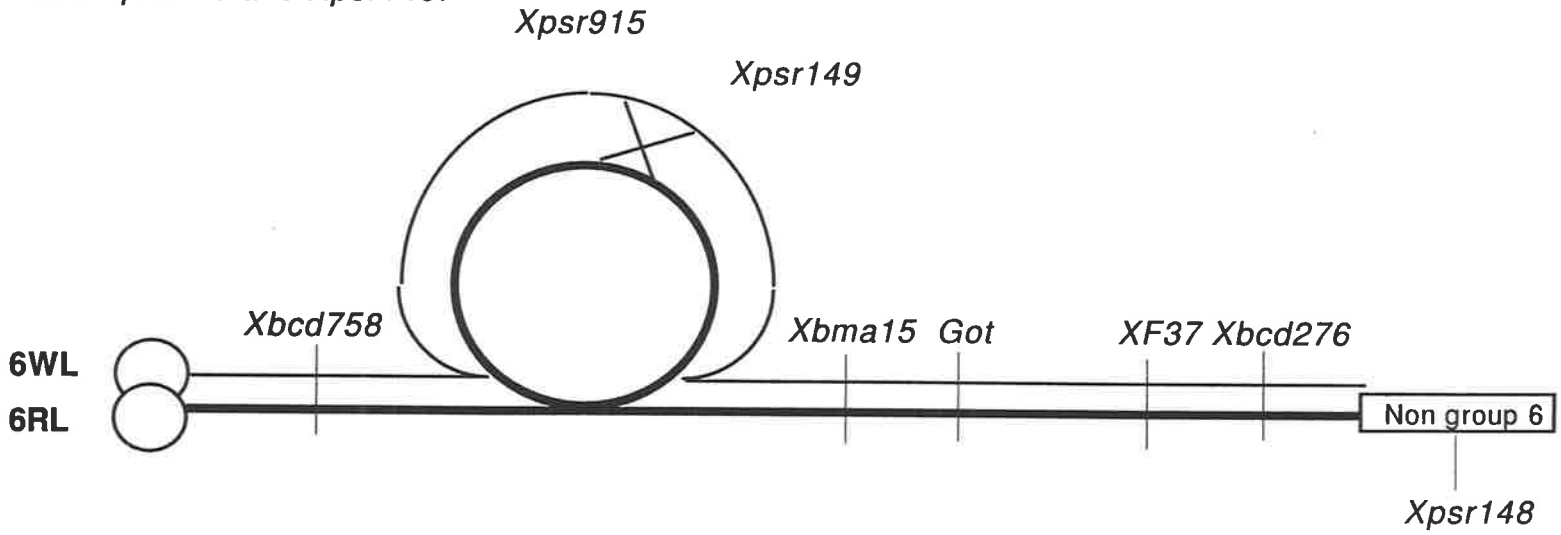
A model of putative recombination between 6WL and an inverted $6RL^{T701}$ with cross-over point between *Xpsr915* and *Xpsr149*, is illustrated in Figure 4.9. Since the order of wheat loci

Figure 4.9: A model of recombination in individual heterozygous for paracentric inversion on 6RL^{T701}.

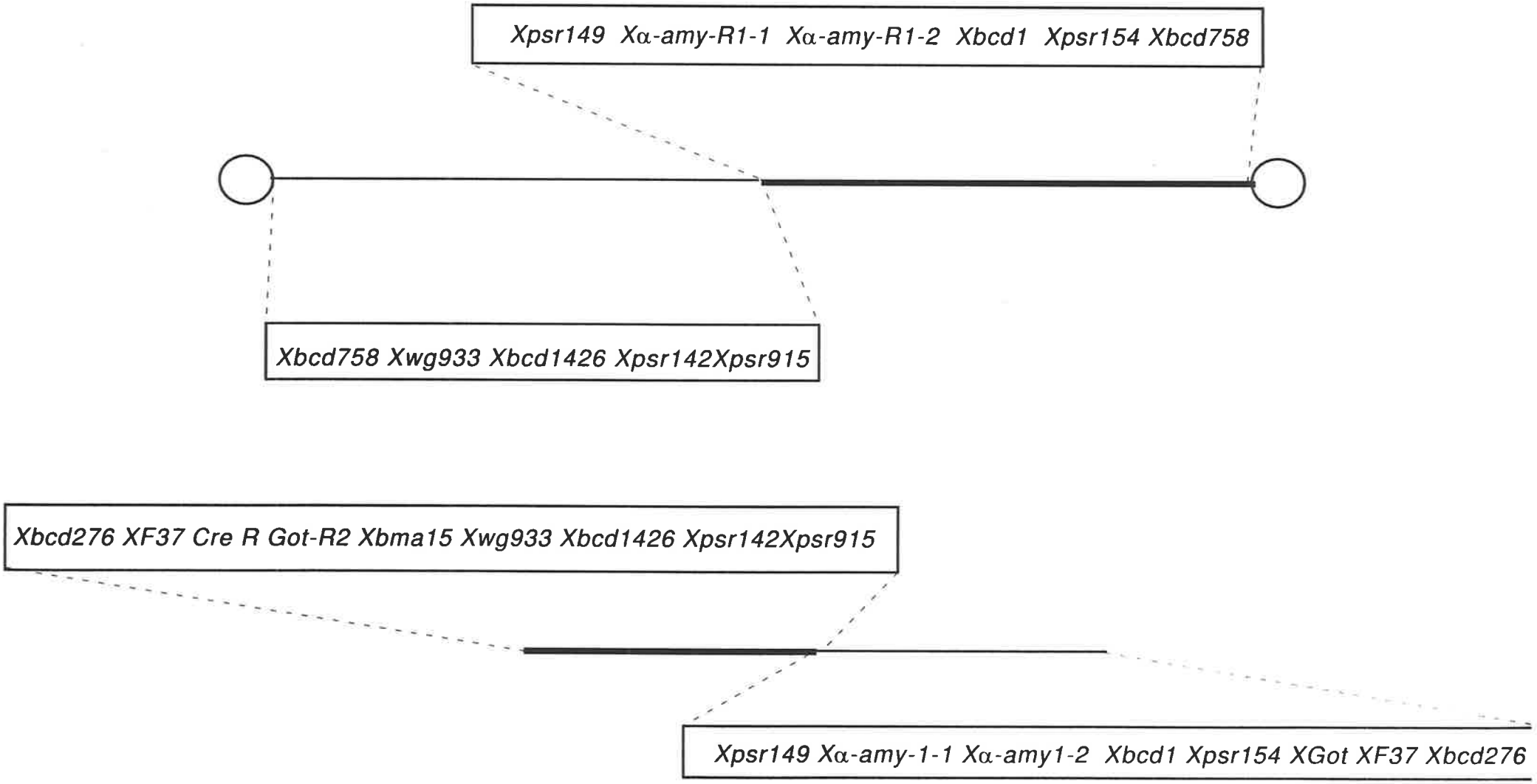
(a) A heterozygous individual with a paracentric inversion on 6RL. The inverted segment between the arrows indicating two break points on 6RL.



(b) Meiotic pachytene configuration in the inversion heterozygote showing a typical reverse loop pairing. The putative cross-over is shown between loci *Xpsr915* and *Xpsr149*.



(c) The chromatids resulting from a cross-over are shown. Upper: a dicentric fragment; Lower: an acentric fragment. Note that the order of loci on acentric fragment is identical to the one that is observed on abberant chromosomes isolated in this study.



observed on abberant 6W chromosomes isolated in this study is identical to the one supposed to be present on the recombined acentric fragment, it can be assumed that these recombinants may involve segment of $6RL^{T701}$ carrying CCN resistance gene. However, in the light of fate of acentric fragment, as discussed above, or given that inversion on 6RL reduced the chance of recombination between $6RL^{T701}$ and 6WL, it can be concluded that manipulation of CCN resistance gene through conventional breeding methods is quite impossible.

Further, it can be established that wheat aberrant lines isolated in this study are presumed recombinants between the wheat chromosomes. These results imply that hemizygosity of homoeologous chromatin is not sufficient to preferentially facilitate the homoeologous recombination (Anderson, 1985). It seems that 6D may have been recombined with 6A or 6B rather than with 6R. Since A and D genomes are more closely related (Jauhar, *et al.* 1991), therefore these recombinants may involved 6A and 6D chromosomes. Confirmation of the existence of new group 6 recombinants will require progeny tests after crossing with wheat nullitetra 6A, 6B and 6D tester lines, and observing co-segregation of markers in the F2 progeny. Furthermore, these results support the observation of partial suppression of homologous pairing and induction of homoeologous pairing in *ph1b* state (Anderson, 1985; reviewed by Feldman, 1993) between the homoeologous genomes in wheat.

The results described above strengthen the previous suggestions that non-homoeology of the long arm of chromosome 6R in relation with wheat group 6 chromosomes (Devos *et al.*, 1993; Taylor, 1996; Chapter 3, this study) is a main cause of interference in chiasmata formation. In desynaptic mutants of rye, *Secale cereale* L., chiasmata formation was prevented between non-homologous chromosomes by differences in nucleotide sequence although synaptonemal complexes (SC) were formed irrespective of homology (Fedotova *et al.*, 1994). Besides the structural differences (inversion and non-group 6 translocation), C-heterochromatin differences between 6R and wheat group 6 chromosomes could also account for reduced chiasmata frequency. A low level of pairing frequency and crossing-over, was observed between chromosomes 6R of Imperial rye and triticale T701 (Taylor, 1996). These two different rye chromosomes display different C-banding patterns. However, no attempts were made in this study to check pairing between $6R^{T701}$ and 6W as it was difficult to identify the meiotic configuration of translocated $6RL^{T701}$ due to low intensity of interstitial C-bands in deleted chromosomes.

The failure in obtaining 6R.6W recombinants, suggests that only a low level of homoeology exists between $6R^{T701}$ and wheat group 6 chromosomes due to chromosomal rearrangements. Comparative mapping to establish the relationships between $6R^{T701}$ and wheat group 6 (Chapter 3) supports this finding, but it is not clear exactly where in 6R synteny is lost. The following chapter addressed this problem by describing a construction of cDNA library together to the isolation of putative $6R^{T701}$ clones.

Chapter 5

Construction of cDNA library and isolation of 6R putative clones from a wheat-6R^{T701}(-6D) disomic substitution line

5.1 Introduction

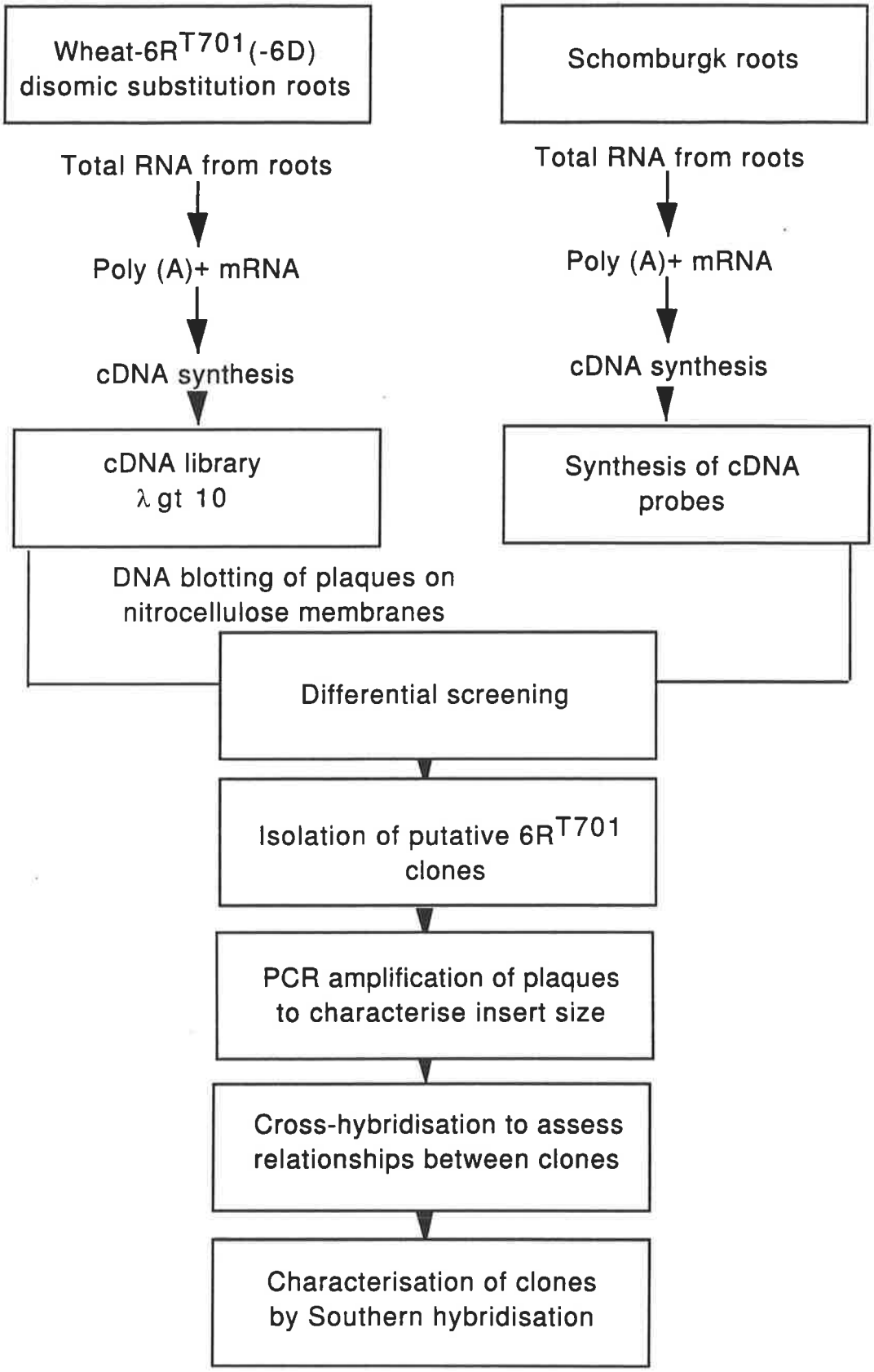
Numerous rearrangements in the structure of 6R^{T701}described here (Chapter 3) and by other researchers (Taylor, 1996, Devos *et al.*, 1993, Loarce, 1996), coupled with the failure to induce recombination between 6RL^{T701} and wheat group 6 chromosomes (Chapter 4), suggested that the 6R was insufficiently similar to its wheat homoeologs for recombination in the absence of *Ph1* locus.

In Chapter 3, RFLP loci used to elucidate the structure of 6R^{T701}, were detected with probes developed by the other researchers. Non-group 6 probes were used to understand the nature and extent of the translocation on 6RL^{T701} but the majority of these clones detected homoeologous loci elsewhere making this procedure tedious. Therefore an attempt was made to develop RFLP markers directly from chromosome 6R^{T701}. Such clones isolated could then be used in wheat and other cereals to elucidate their homoeologous relationships with rye 6R.

cDNA or gDNA libraries have provided the means for generating probes for RFLP studies. Since the large portion (70-80%) of cereal genomes is contributed by repetitive sequences (Flavell *et al.*, 1987), gDNA libraries usually contain many clones with repetitive sequences. Several methods have been designed to remove the repetitive sequences prior to cloning (Kohne *et al.*, 1977, Clarke *et al.*, 1992). By using substraction DNA hybridisation and representational DNA analysis (RDA), with Chinese Spring-6R Imperial rye addition line as a tester DNA, Delaney *et al.*, (1995) generated numerous and useful RFLP probes to construct the high density map of region containing a gene for resistance to Hessian fly (*H25*) on 6R. However, this method is not suitable for plants with a mixed genetic background.

Chromosome 6R^{T701} is present in the mixed wheat background in triticale, and in addition and substitution lines. These lines were originaly constructed in Chinese Spring wheat but several generations of crossings with Schomburgk wheat and selfing have introduced Schomburk as the dominant wheat genome (I. S. Dundas, personal communication). The construction of cDNA library and isolation of 6R clones by differential screening against Schomburgk DNA could circumvent problems with repetitive sequences and the mixed wheat background.

Figure 5.1. A scheme for generation of 6R clones by construction and differential screening of cDNA library.



The technique of differential hybridisation allows the isolation of those mRNAs (in the form of cDNA) that differ in abundance between the two mRNA populations, tester and control. It has therefore been widely used to identify and study developmentally regulated and tissue specific genes and has also been successfully employed in the isolation of plant genes which play an important role in plant-pathogen interactions (Niebel *et al.*, 1995; Herbers *et al.*, 1995). However, Many modifications of the differential screening methodology are reported (Hodge *et al.*, 1992; Tagu, *et al.*, 1993; Kochi *et al.*, 1995). A simple approach was taken in this study to initially isolate the clones which shows differential hybridisation signals between Schomburgk and wheat-$6R^{T701}$(-6D) substitution line. This chapter describes the construction and analysis of a cDNA library from wheat $6R^{T701}$ (-6D) disomic substitution line carrying $6R^{T701}$.

5.2 Results

The flow diagram (Figure 5.1) illustrates the scheme for generation of 6R clones by construction and analysis of cDNA library and differential screening.

5.2.1 Construction and analysis of cDNA library from a wheat-$6R^{T701}$(-6D) substitution line

A cDNA library in the λgt10 vector was prepared from poly (A^+)mRNA of wheat-$6R^{T701}$ (-6D) substitution. Starting with 3 g of root material, 103 μg total RNA from wheat-$6R^{T701}$(-6D) substitution and 96 μg total RNA for the Schomburgk were isolated from ten days old roots. RNAs quality was assessed by electrophoresis (Figure 5.2, a), converted to mRNA and used for cDNA synthesis (Chapter 2, Section 2.13). cDNA of wheat-$6R^{T701}$(-6D) substitution was then cloned into the λgt10 vector to construct a library. The initial titer of the library was 20, 000 pfu. Therefore, this library was amplified 10 fold and plated on five large plates of Ø15cm.

5.2.2 Isolation of differentially expressed putative 6R clones

Duplicate sets (A and B) of membranes were prepared from each plate. Each set of membranes corresponded to $4x10^5$ plaques each were differentially screened using 32P labelled cDNA probes. Set A was hybridised with cDNA probe derived from poly (A+) RNA of 12 days old Schomburgk roots (control) and Set B was hybridised with the cDNA derived from poly (A+) RNA from wheat-$6R^{T701}$(-6D) substitution roots (tester). The results were obtained by autoradiography. The membranes were then stripped to remove radioactivity and exposed overnight to check for signals carrying over. After establishing that no signals remained, the duplicate membranes were re-used in an alternate manner; Set A was hybridised with the tester probe and Set B with the control probe. The results were compared with the previous experiment to avoid ambiguity in the selection of differential clones.

Fig 5.2 Construction and analysis of cDNA library.

(a)Total RNA isolation. The samples are fractinonated on 1% formaldehyde-agarose gel. Lanes 1-5 contain following RNA samples:

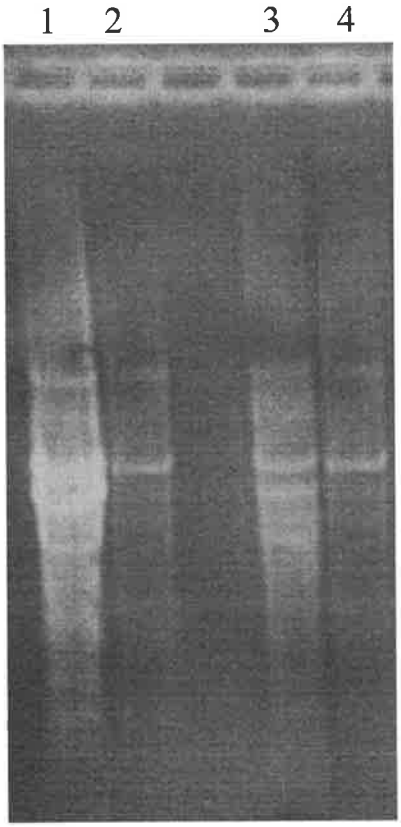
- Lane 1. Wheat-6R^{T701}(-6D) disomic substitution line (leaves)
- Lane 2. Wheat-6R^{T701}(-6D) disomic substitution line (roots)
- Lane 3. Chinese Spring (leaves)
- Lane 4. Chinese Spring (roots)

(b)PCR analysis of differential clones.

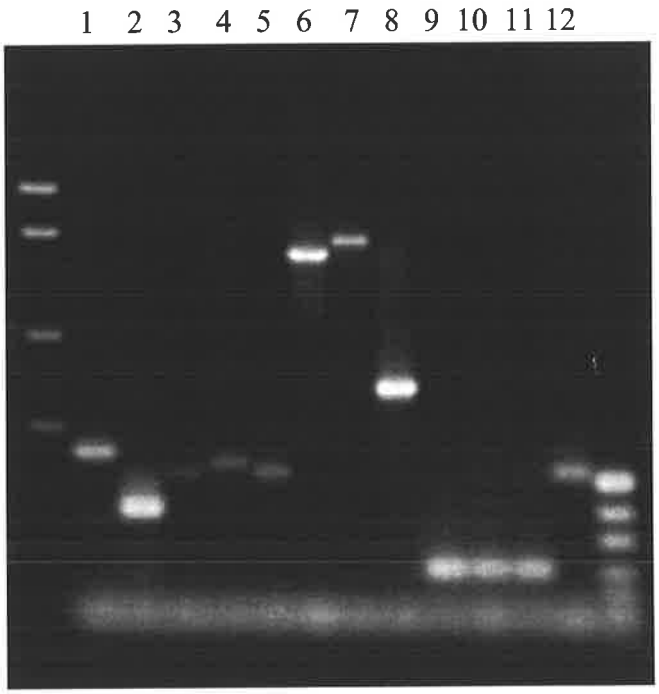
Differential clones were used in PCR reactions with λgt10 forward and reverse. Samples were separated on 1% agarose gel. The size markers PTZ18U *Dra* I+ *Rsa* I and PUC19*Msp* I are indiacted on left and right, respectively. Lanes contain following inserts:

- Lane 1. MK10
- Lane 2. MK19
- Lane 3. MK22
- Lane 4. MK24
- Lane 5. MK25
- Lane 6. MK26
- Lane 7. MK31
- Lane 8. MK33
- Lane 9. MK36
- Lane 10. MK38
- Lane 11. MK39
- Lane 12. MK40

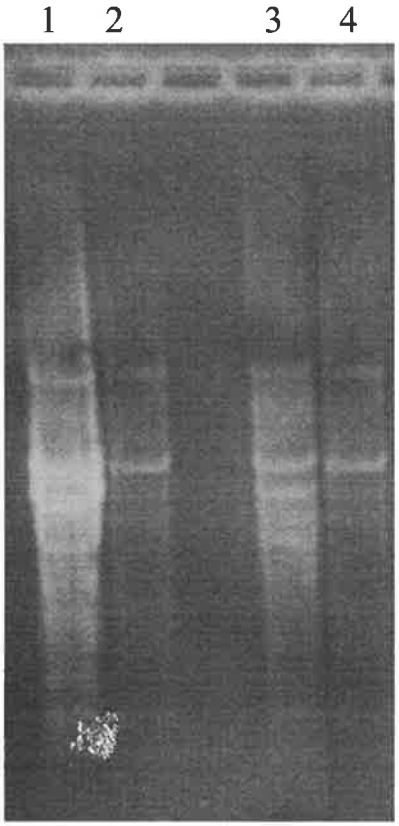
(a)


1 2 3 4

(b)


1 2 3 4 5 6 7 8 9 10 11 12
2149 bp
1763 bp
1097 bp
692 bp
501+489 bp
404 bp
331 bp
242 bp
190 bp
147+111bp

(a)


1 2 3 4

(b)

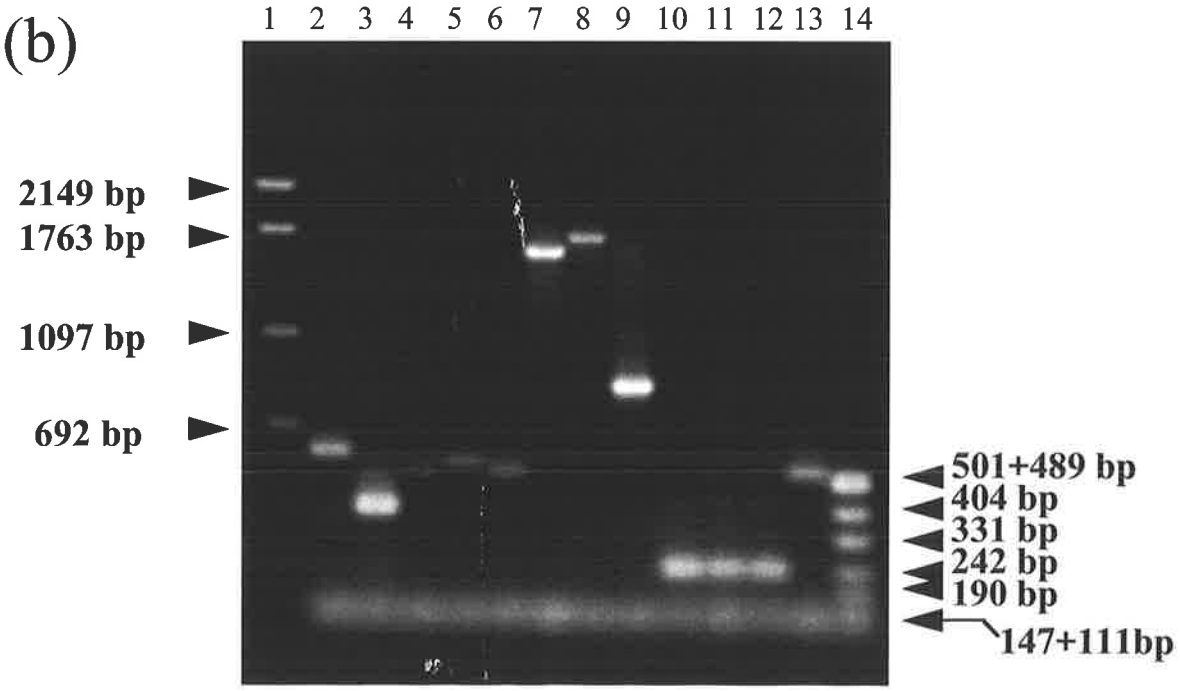

1 2 3 4 5 6 7 8 9 10 11 12 13 14
2149 bp
1763 bp
1097 bp
692 bp
501+489 bp
404 bp
331 bp
242 bp
190 bp
147+111bp

Table 5.1: Insert size in randomly selected 6R differential clones.

S.No	Clone	Size
1	MK6.1.2-1	142 bp
2	MK6.1.2-2	no insert
3	MK6.1.2-3	no insert
4	MK6.1.2-4	no insert
5	MK6.1.2-5	120 bp
6	MK6.1.2-6	110 bp
7	MK6.1.2-7	145 bp
8	MK6.1.2-8	145bp
9	MK6.1.2-9	no insert
10	MK6.1.2-10	650 bp
11	MK6.1.2-11	150 bp
12	MK6.1.2-12	147 bp
13	MK6.1.2-13	145 bp
14	MK6.1.2-14	no insert
15	MK6.1.2-15	147 bp
16	MK6.1.2-16	135 bp
17	MK6.1.2-17	no insert
18	MK6.1.2-18	no insert
19	MK6.1.2-19	490 bp
20	MK6.1.2-20	no insert
21	MK6.1.2-21	145 bp
22	MK6.1.2-22	500 bp
23	MK6.1.2-23	145 bp
24	MK6.1.2-24	500 bp
25	MK6.1.2-25	500 bp
26	MK6.1.2-26	1750 bp
27	MK6.1.2-27	147 bp
28	MK6.1.2-28	no insert
29	MK6.1.2-29	135 bp
30	MK6.1.2-30	120 bp
31	MK6.1.2-31	1760 bp
32	MK6.1.2-32	145 bp
33	MK6.1.2-33	800 bp
34	MK6.1.2-34	no insert
35	MK6.1.2-35	147 bp
36	MK6.1.2-36	190 bp
37	MK6.1.2-37	145 bp
38	MK6.1.2-38	190 bp
39	MK6.1.2-39	190 bp
40	MK6.1.2-40	500 bp

A total of 475 clones that showed strong differential signals between tester and control isolated, were submitted to secondary screening. During secondary screening, these 475 phages were spotted on master plates. Duplicate membranes were prepared and hybridised with control and tester probes. The subsequent re-screening finally verified two hundred plaques: 6 gave no signal and the remaining 194 gave very weak hybridisation signals with cDNA of Schomburgk. These plaques were isolated and considered as putative clones differentially expressed in wheat-$6R^{T701}$(-6D) substitution line.

5.2.3 PCR amplification of inserts

For direct analysis of these clones, and determination of size of inserts, the plaques were subjected to PCR amplification, using the standard λgt10 forward and reverse primers (Saiki *et al.*, 1988). Among 40 randomly selected putative clones, 30(75%) contained the inserts ranging in size from 110 bp and 1760 bp as shown in Table 5.1. Twelve clones had an insert size over 0.15kb (Figure 5.1 b), and these were used in further analyses.

5.2.4 Relationships between the clones by cross-hybridisation

As the cDNA clones represent mRNA sequences abundantly expressed in different proportions in the original prepration of mRNA, their expression levels can be determined by cross-hybridisation to assess the diversity of clones and size of the clonel families. Since the clones isolated from the library were derived from an amplified stock, it was neccassary to determine their relationships by cross-hybridisation. Cross-hybridisation between clones (Table 5.2) showed that these twelve clones represent only four distinct groups. Further relationships were established by hybridising four clones, MK40, MK26, MK33 and MK36 (representing each group) to all 200 clones. Several clones were found to be related but 95 clones showed very weak homology. In summary from 200 putative 6R clones, 105 clones fell into four groups (Table 5.3). These four groups and the remaining 95 clones (the relationships of 95 clones to each other was not investigated) were considered as putative 6R clones and stored at 4°C under chloroform.

5.2.5 Assignment of putative 6R specific clones

The chromosomal location of clones was determined by Southern hybridisation analysis. A representative clone from each family was hybridised with sets of membranes containing DNA of Schomburgk, Chinese Spring, South Australian rye, Imperial rye, Triticale T701-4, wheat-$6R^{T701}$(-6D) substitution, Chinese Spring-$6R^{Imp}$ addition, different 6RL deletion length mutants and 6RS trisomic addition lines digested with *Bam* HI, *Bgl* II, *Dra* I, *Eco* RV and *Sac* I. Use of

Table 5.2: Relationships of 12 putative 6R clones based on cross hybridisation. Strong and low hybridisation signals are designated as (++) and (-), respectively.

S.No	Clones	MK10	MK19	MK22	MK24	MK25	MK26	MK31	MK33	MK36	MK38	MK39	MK40
1	MK10	+ +	+ +	+ +	+ +	+ +	-	-	-	-	-	-	+ +
2	MK19	+ +	+ +	+ +	+ +	+ +	-	-	-	-	-	-	+ +
3	MK22	+ +	+ +	+ +	+ +	+ +	-	-	-	-	-	-	+ +
4	MK24	+ +	+ +	+ +	+ +	+ +	-	-	-	-	-	-	+ +
5	MK25	+ +	+ +	+ +	+ +	+ +	-	-	-	-	-	-	+ +
6	MK26	-	-	-	-	-	+ +	+ +	-	-	-	-	-
7	MK31	-	-	-	-	-	+ +	+ +	-	-	-	-	-
8	MK33	-	-	-	-	-	-	-	+ +	-	-	-	-
9	Mk36	-	-	-	-	-	-	-	-	+ +	+ +	+ +	-
10	MK38	-	-	-	-	-	-	-	-	+ +	+ +	+ +	-
11	MK39	-	-	-	-	-	-	-	-	+ +	+ +	+ +	-
12	MK40	+ +	+ +	+ +	+ +	+ +	-	-	-	-	-	-	+ +

Table 5.3: Classification of 105 putative clones in four groups. Total number of clones in each group is listed.

S.No	Groups	No. of clones
1	MK-L26	16
2	MK-L33	18
3	MK-L36	40
4	MK-L40	31

these clones with the five restriction enzymes did not reveal any RFLPs between Schomburgk and wheat-$6R^{T701}$(-6D) disomic substitution line. Probes showed strong bands comigrated in wheat and rye and $6R^{T701}$-derived lines, but use of clone MK33 (800 bp) revealed weak polymorphic bands of size 2100 bp and 600 bp present in only rye and 6R-derived lines (Figure 5.3). However, it is difficult to establish that the clone MK33 is 6R specific clone.

5.3 Discussion

Both cDNA and genomic DNA libraries have been used as a source of probes for RFLP mapping. Although more difficult to construct than genomic DNA libraries, cDNA libraries contain fewer repetitive sequences and actual genes making them more popular as a source of RFLP probes. Due to these potential advantages over gDNA libraries, a cDNA library was constructed from the wheat-$6R^{T701}$ (-6D) disomic substitution line and differential screening in an attempt to isolate cDNA clones which could map specifically to 6R.

Based on differential hybridisation signals between two wheat lines, initially 200 clones were isolated. However a proportion (25%) of these putative clones did not show any insert upon PCR amplification although showed hybridisation signals during differential screening procedure. It can be considered as artifacts of screening procedure or these clones may contain a very small insert that could not be detected upon PCR amplification. The cross-hybridisation studies, finally resulted into four groups and a further 95 putative 6R specific clones. The relationships of 95 clones are yet not known. As these clones detected very weak hybridisation signals with representatives of four groups, it could be possible that these clones either represent other gene families or the proportion of differential clones which contain a very small cDNA insert.

Based on earlier observation that a large proportion of cNDA probes detected polymorphic loci on 6R, it was thought that cDNA library developed in this study would be useful in generating probes that could be mapped to 6R. Although the differential clones were isolated after several rounds of screening against Schomburgk cDNA, RFLP analysis using representative clones from each group, did not reveal any useful polymorphism between wheat and rye that could be mapped to 6R. However, a clone MK 33 detected a very weak polymorphism between Schomburgk and wheat-$6R^{T701}$ (-6D) disomic substitution line using *Sac* I. Previously, mapping studies (Chapter 3) have shown *Sac* I as a useful enzyme in locating probes to chromosome 6R and revealing polymorphism between wheat and rye.

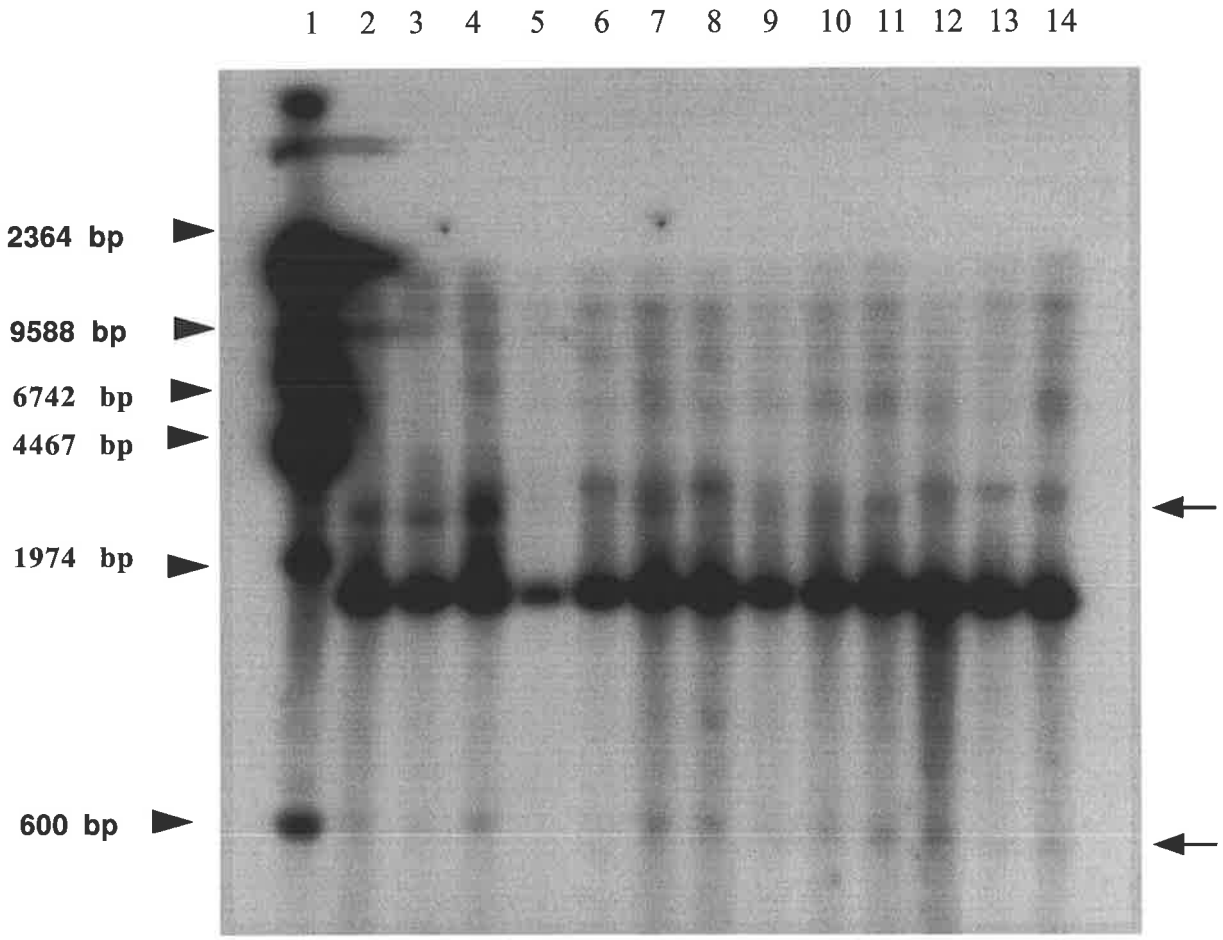
The differential clones detected complex loci on wheat and rye chromosomes corresponding to large number of monomorphic bands found close together and comigrating with each other. It can be inferred that these clones may represent clustered multigene family in Triticeae. Several genes are reported in wheat present in small families that are usually clustered together for

Fig 5.3 Characterisation of putative 6R clones

Hybridisation of MK33 with *Sac* I digested DNA samples.

The size standard (M) was λ *Hin* d III. The size of bands generated in standard is shown in base pairs on the left. The polymorphic bands are indicated by arrow (←). Lane 1-14 contained the following DNA samples

- Lane 1 South Australian rye
- Lane 2. Imperial rye
- Lane 3. Triticale T701
- Lane 4. Chinese Spring (CS) wheat
- Lane 5. Schomburgk wheat
- Lane 6. Wheat-$6R^{T701}$(-6D) disomic substitution
- Lane 7. CS-$6R^{Imp}$ disomic addition
- Lane 8. Wheat-$6R^{T701}$ addition
- Lane 9. Wheat-6RL telosome addition
- Lane 10. Wheat-$6RS^{T701}$ trisomic addition
- Lane 11. Wheat-$6RL^{T701}$ translocation mutant
- Lane 12. Wheat-$6RL^{T701}$ del. 1 translocation mutant
- Lane 13. Wheat-$6RL^{T701}$ del. 2 translocation mutant


1 2 3 4 5 6 7 8 9 10 11 12 13 14
2364 bp
9588 bp
6742 bp
4467 bp
1974 bp
600 bp

example those encoding storage proteins, α-amylases, other isozymes, histones and ribosomal RNA genes (reviewed by Flavell, *et al.*, 1987). Particularly, the complex loci of α-amylase have been duplicated and translocated during evolution, so that several closely related complex loci exist in the chromosome complement of wheat (Lazarus, *et al.*, 1985). The α-amylase gene family is also present in rye detect duplicated loci on chromosomes 6R and 7R. The RFLP analysis (this study Chapter 3, Taylor, 1996, Devos, *et al.*, 1993) have also revealed the presence of number of sequences (CDO534, BMA15, PSR915, PSR142, PSR106, Tam36, PSR148) on chromosome 6R that identify duplicated loci either intrachromosomally or on non-homoeologous chromosomes. Although clones isolated in this study failed to detect a clear polymorhism in wheat and rye specific to 6R, it is kown that RFLP analysis is not a good tool to study polymorphism in clustered multigene families. The advent of Pulse field gel electrophoresis (PFGE) can aid in the study and comparison of homoeologous regions that are poorly detected or invisible to RFLP analysis (Cheung *et al.*, 1991; Funke, *et al.*, 1993).

However, it is not known that why many genes are present in small families, it may be possible that different members of the locus are regulated differentially during development. This could be a reason of differential expression of these clones in wheat-$6R^{T701}$(-6D) disomic substitution line. It may also throw light on the control of gene expression in cereals as well as genetic interactions between rye and wheat. Therefore it can be further proposed that cDNA sequences isolated in this study represent multigene family that expressed differentially in a wheat-rye hybrid examplified by wheat-$6R^{T701}$(-6D) disomic substitution line. There could be two plausible explanations for the dormancy of Schomburgk alleles. First, it can be presumed that product of 6R allele automatically inactivated the product of Schomburgk allele. Second, the RNA polymerase of the 6R could have blocked the promoter or control site on the homoeologous Schomburgk gene. These hypotheses have been proposed previously for explaining allelic exclusion phenomenon in the synthesis of human immunoglobulin and ribosomal RNA in amphibian hybrids *Xenopus laevis X mulleri* (reviewed by Norman Maclean, 1976).

Studies on the structure and expression of multigenes coding rRNA in wheat have also contributed a valuable evidence to the differential gene expression. The 5' sequences upstream from a coding sequences are known as promoter sequences and play important role in gene regulation. Variation in such upstream sequences exist in wheat and is likely to be responsible for differential gene expression (dominance), and the tissue- and cell-specific expressions. It is known that different ribosomal RNA genes in wheat are active to different extents. This differential expression may be due to the differential affinity of the array of 135-bp repeats for the regulatory protein. Genes whose repeats have a sequence which binds the protein more efficiently or which have more DNA-binding sites would attract more proteins and be more active (reviewed by Flavell *et al.*, 1987)

Variation in number of rRNA genes at homologus loci and other loci causes variation in the expression of the rDNA loci indicates that many other genetic factors are involved. The rRNA genes though appear similar but differ greatly in carrying methylated cytosines which make the genes inactivated or dormant. The introduction of the NOR chromosome from *Aegilops umbellulata* into Chinese Spring wheat have also resulted in suppression of the wheat rRNA gene activity (nucleolous formation) and increased methylation of the wheat, but reduced methylation of the *Ae. umbellulata* genes (reviewed by Flavell *et al.*, 1987). Conversely, in wheat-rye hybrids, transcription of rye rRNA genes always repressed in the presence of the wheat NOR (Capesius and Appels, 1988).

However evidence of increased methylation of wheat genes by *Ae umbellulata* still hold promising and it can be speculated that may be a similar process is imposed by the chromosome $6R^{T701}$ in regulating the expression of Schomburgk genes. It could also be assumed that if there is no such interaction exist between wheat and rye, Schomburgk genes remain inactivated as 80-82% of cytosine residues in wheat are methylated. However, it requires the analysis of these clones in Northern hybridisation studies with Schomburgk and wheat-$6R^{T701}$(-6D) disomic substitution line RNAs extracted from different tissues. A further work is needed to confirm that wether the cDNA clones isolated in this study do represent a novel multigene family or not ?. It involves DNA sequencing of these clones to gain information concerning the possible function of these genes and secondly to investigate wether the clones represent different genes or pseudogenes.

Differential cDNA clones isolated in this study are useful to provide information about the interactions between wheat and rye genes which may throw light on the expression of multigenes families in related species of Triticeae. However, these clones do not exhibit a a suitable polymorphism between wheat and rye which could be localised specifically to 6R, therefore, these clones can not be used in mapping $6R^{T701}$. In conclusion, there still a need to generate 6R clones for fine mapping of chromosome $6R^{T701}$ so more elucidations can be made regarding the structure of $6R^{T701}$. That will be useful in map based cloning and isolation of CCN resistance gene.

Chapter 6

General Discussion and Future Prospects

Cereal cyst nematode (CCN) is amongst the economically most damaging pathogens of wheat and barley in South Australia, where it is found in all soil types. To date, there has been only one resistance gene reported in wheat, and this has not been completely effective. An alternative source of CCN resistance for use in wheat is the gene *Cre-R*, present in the triticale line T701-4-6. *Cre-R* is located on the long arm of chromosome $6R^{T701}$, that is, on the rye component of the triticale genome. Substitution and translocation lines with this $6R^{T701}$ have not been successfully exploited, because detrimental agronomic effects associated with the rye chromatin outweigh the advantages of the disease resistance. Future breeding of wheat with the rye resistance to CCN requires introgression of the gene as a recombinant segment. Removal of the homoeologous pairing barrier (*Ph* locus) can induce meiotic pairing between the homoeologous chromosomes of wheat and rye, allowing wheat-rye recombination to occur and alien introgression. However, attempts to transfer the CCN resistance gene into wheat using the $6R^{T701}$ (-6D) substitution line have been unsuccessful (Dundas, *et al.*, 1990). Earlier reports of reduced meiotic pairing between rye and wheat chromosomes in the absence of *Ph* locus (Riley and Kimber, 1966; Orellana, 1985; Naranjo and Fernandez-Rueda, 1991), have suggested that rye chromosome arms differ structurally from their wheat counter parts.

At the commencement of this project there was no concrete evidence for there being any structural differences between rye chromosome 6 and wheat group 6 chromosomes. However in recent years, a plethora of research (Naranjo, 1992; Devos *et al.*, 1993; Martinez *et al.*, 1994; Loarce *et al.*, 1996) suggested that rye chromosome 6R possesses non-group 6 translocations and a deletion. Further, Taylor (1996) reported a putative inversion on $6RL^{T701}$ in relation to wheat group 6 chromosomes. This project has increased our understanding of structure of chromosome $6R^{T701}$ and its homoeologous relationships between wheat group 6 chromosomes.

The absence of several wheat homoeologous 6S markers on the triticale chromosome arm $6RS^{T701}$, including BCD21 which was found to be located on the rye chromosome 4R (Chapter 3), confirms the deletion of the terminal segment of rye chromosome arm $6RS^{T701}$ and its translocation to 4RL. Results of comparative analyses of rye chromosome $6R^{T701}$ and wheat group 6 chromosomes using RFLP markers selected from group 6 and other chromosome maps (Chapter 3) have also provided evidence of multiple rearrangements in chromosome $6R^{T701}$ relative to its wheat homoeologs which in turn can throw light on the evolution of this chromosome. A putative inversion identified in this study indicated that $6R^{T701}$ may possess two independent paracentric inversions, one on the short arm and the other on long arm (Taylor, 1996). These studies led to a model for rearrangements in 6R. It appears that the

present structure of $6R^{T701}$ is due to two independent inverions on proximal/interstitial regions of the chromosomal arms, followed by deletion of terminal segments. Given the model of evolutionary rearrangements in the rye genome reported by Devos *et al.*, (1993), it can further be suggested that terminal regions of the chromosome $6R^{T701}$ might have undergone reciprocal translocations with 2S on the short arm and 3L on the long arm. The translocated fragment of 3L remained on 6RL whereas the 2S fragment underwent another reciprocal translocation with 7L, shifting 7L to 6S. This translocated fragment of 7RL later followed by a pericentric inversion transferred to the long arm of 6R. While the fragment of 6RL which was previously translocated onto 2S, was transfered to chromosome 4R due to an independent interchange between 2S and 4L.

The reliability of the above model for $6R^{T701}$ structure is based on the markers which identify duplicated loci on non homoeologous chromosomes. Two such markers, CDO534 (1, 3 and 7, Sorrells *et al.*, 1992; VanDeynze, *et al.*, 1995), and AWBMA15 (1, 3 and 7, Angelo Karakousis, personal communication) were found to be localised to $6RS^{T701}$ and $6RL^{T701}$, respectively. The physical assignment of AWBMA15 and Got on $6RL^{T701}$ in the 1411-54 line (Chapter 3) mapped these two loci interstitially on chromosome arm $6RL^{T701}$. Since CDO534 and AWBMA15 identify loci on the same group of non-homoeologous chromosomes viz., groups 3 and 7, their locations on $6R^{T701}$ open the question that whether these probes represent the DNA sequences which by any chance are the members of triplicate copies of the structural genes of isozymes, glutamic-oxaloacetic transaminase (GOT).

Earlier Devos *et al.*, (1993), have presumed that duplication of Got-1 genes was the event leading to numerous and multiple rearrangements to produce the present altered structure of 6R. In their review, they cited the locations of triplicate sets of GOT structural genes on 3L, 6S and 6L in wheat (Hart, 1975) and on 4RL and 7RL in rye (Wehling *et al.*, 1991). It is suggested here that the presence of duplicated loci increased the pairing affinities between these non-homoeologous chromosomes which might have resulted in breakage and translocations of non-group 6 fragments on 6R. PSR148 is a further example. This probe identify loci on 2AS and 7BL in wheat (Gale *et al.*, 1995) and was found to be present on $6RL^{T701}$ (Chapter 3) in agreement with Devos *et al.* (1993). This also suggests the translocation of group 7 on the distal region of the long arm of rye chromosome $6R^{T701}$. However further work is required to support the hypothesis that duplicated gene are involved in rearrangements. This could include the use of a common set of probes flanking Got gene on 6R in mapping group 3 chromosomes in wheat and 4R and 7R in rye. The important feature of this work is that, homoeology between wheat and rye chromosomes is highly disrupted thus prevented pairing between 6WS and 6RS, and 6WL and 6RL (Naranjo and Fernandez-Rueda, 1991).

Given the non-homoeology in distal regions of $6RL^{T701}$, a strategy was devised to introgress and monitor $6RL^{T701}$ in a wheat background (Chapter 4). The importance of using the

translocation and telocentric lines containing variant forms of terminally deleted 6RL^{T701} offered 6RL^{T701} fragment with varying level of homoeology. 1411-54XS was the critical line in this program as it is known to possess a comparatively shorter segment of 6RL carrying the *Cre-R* locus (Dundas and Shepherd, 1992). The most important of the other lines, 1411-95, was susceptible to CCN, it contained the shortest fragment of 6RL. A poor transmission of 6RL^{T701} was observed in progenies of 1411-95, indicating a preferential gametic selection against this fragment of 6RL.

Use of Sears' *ph1b* did induce pairing between the 6RL^{T701} segments and wheat chromosomes, as rod bivalents and multivalent associations between chromosomes were observed (Chapter 4). However, multivalent associations are not a good indicator of *ph1b* homozygosity. The use of RFLP marker, PSR128 mapped in a deletion of a fragment containing *Ph* locus, proved to be a reliable tool for scoring the *ph1b* homozygotes. A recently developed PCR-based screening assay for the *Ph1* gene (Gill and Gill, 1996) will provide an alternative for rapid scoring of *ph1b* homozygotes, but owing to its very recent introduction it could not be used in this study.

The application of RFLP markers coupled with isozyme markers was also used in this study (Chapter 4) to determine rye introgression as a consequence of recombination events. No dissociation was observed in the linkage of these RFLP or isozyme markers on 6RL^{T701}, which initially suggests recombination did not occur. However isolation of some 6W deletion mutants (Chapter 4) indicate that crossing-over occured between homoeologous wheat chromosomes, probably 6A and 6D. Interestingly, the majority of these recombinants (7/11) possessed two doses of 6RL^{T701} and 6DL based on GOT assays. Therefore, it is possible that any recombination involving 6RL^{T701} was masked by the presence of other intact 6RL^{T701}.

Confirmation of the new wheat recombinants isolated in this study can be made by progeny testing after crossing these plants with 6A, 6B and 6D nullisomic/tetrasomic lines. Nevertheless, these putative 6A or 6B recombinants indicate that distal regions are more capable of undergoing crossing-over if the level of homoeology is maintained. In the case of deleted rye chromosome arm 6RL, it seems that non homoeology was still present on a gross level, and this prevented crossing-over.

The non-homoeology of 6RL deleted fragments could be attributed to inversed order of RFLP loci on the proximal region of 6RL (Taylor, 1996) reducing the probability of cross-overs between homoeologous chromosomes. Furthermore, if the crossing-over did occur, there is an increased chance of loss for the acentric fragment containing *Cre-R* locus as suggested by a model in Chapter 4. The complex structure of 6R probably prevent the introduction of *Cre-R* into wheat using the breeding strategy described here.

The alternative strategies for the introduction of *Cre R* are to actually isolate the gene and introduce it into wheat via transformation or to attempt further fragmentation of $6RL^{T701}$. Both strategies involve detailed analysis of $6R^{T701}$, particularly in the region of *Cre R* and the production of fine linkage map. Therefore, it was presumed that markers should be generated directly from the chromosome $6R^{T701}$. These markers further be mapped on $6RL^{T701}$ can be useful in elucidating the complexities in structure of $6R^{T701}$.

Subtractive hybridisation and representational DNA analysis (RDA) (Delaney *et al.*, 1995) can be a useful approach in the isolation of gene as well as generating RFLP probes for mapping $6R^{T701}$. Progenies of deletion lines 1411-54 which showed susceptibility to CCN (I. S Dundas, personal communication) can be used as a deletion line in such experiments for the isolation of sequences containing CCN resistance gene. However, experiments used to generate markers close to Yd2 region in barley by using smaller fragment of 3H as tester DNA did not yield polymorphic clones (Collins, 1996). Therefore, a cDNA library was constructed from the wheat line containing whole $6R^{T701}$ and differential screening was employed to isolate putative $6R^{T701}$-specific cDNA clones (Chapter 5). In total four distinct groups of clones, together with a further 95 (yet to be classified) were isolated, which set the foundation for direct analysis of the altered structure of $6R^{T701}$. However when a representative differential clone from each of the group was used in RFLP analysis, these clones didnot reveal any polymorphism between wheat and rye chromosomes. A weak polymorphism between wheat and rye was identified by a probe MK33, which can be mapped to 6R.

The RFLP analysis with probes generated from $6R^{T701}$ (Chapter 5), detected several bands comigrating with each other indicated that these cDNA sequences may represent the clustered multigene family. The clustered genes usaully found close together in a complex locus is the characteristic of multigene family. These genes have duplicated/multiple loci on the same or homoeologous or nonhomoeologous chromosomes. A detailed RFLP analysis (Chapter 3) have also proved that chromosome $6R^{T701}$ contain DNA sequences which have duplicated copies on homoeologous and non-homoeologous chromosomes.

Several groups of workers have proposed that different members of the multigene family are expressed differentially during development. These reports also provide evidence for the differential expression of clones isolated in this study (Chapter 5). It can be concluded that there may be a competition between genes of two lines, and wheat-$6R^{T701}$(-6D) disomic substitution lines genes may repressed the Schomburgk genes. However Northern hybridisation studies are required to further analyse the expression pattern of these clones in different parts of plants of Schomburgk and Wheat-$6R^{T701}$(6D) substitution line. Furthermore PFGE followed by Southern hybridisation with the probes can be carried out to reveal the hidden polymorphism between wheat and rye remain undetected by RFLP analysis. DNA sequence analysis of these clones is also required to provide knowledge about the possible function of these genes.

cDNA sequences isolated here (Chapter 5) indicate the conservation of genes in wheat and rye but their location on 6R is not clearly known. Although existing information about $6R^{T701}$ provide a sound knowledge about its complex structure, a considerable effort will still be needed to develop a fine linkage map to isolate the CCN resistance gene and its use in transformation of wheat. It is likely that wheat breeding programs will concentrate on the use of resistance from more closely related sources, such as *Triticum. tauschii* (East wood *et al.*, 1991) and *Aegilops ventricosa* (Delibs, *et al.*, 1993).

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Appendix 1

Solutions and chemicals used in α-amylase separation are listed below

a)Chemicals for the preparation of polyacrylamide gel were added as given below:

Deionised water	2.4ml
Glycerol (BDH 10118)	0.80ml
Ampholine (LKB# 1818-1011)	0.18ml of pH 6-8
	0.09ml of pH 5-7
Acrylamide Stock:	3.5ml
Prepared as follows:	
Acrylamide (Bio-Rad# 161-0101)	4.85g
Bis-acrylamide (Bio-Rad# 1611-0201)	0.15g

Dissolved and made upto 50mls with deionised water.

Temed (Sigma#T8133)	13ml
Ammonium persulphate	15ml

b)For samples preparation, 1mM $CaCl_2$ was used.

c)For electrode buffers:

pH 3.5 to 9.5 (or pH 3.5 to 10.0; pH 5-7, 6-8)	
-ve 1M NaOH	(1g/25ml)
+ve 1M H3PO4	(1.7 ml upto 25ml)

d)For starch overlay:

2g Sigma starch (Sigma# S-4501) was dissolved in 50ml deionized water by boiling in microwave.

e)For the preparation of IKI solution:

Stock solution was prepared as:

I_2	6.5g
KI	9.5g

were added and dissolved in deionised water made upto 1 litre.

(Sargent and Walker, Starch 30 (1978), 160-163)

Working solution was prepared in ratio of 30ml IKI stock solution: 150ml H2O

GEL PREPARATION

Separating Gel:

Solution	7% for 2 sides
A	3.75ml
C	7.50ml
H2O	18.75ml

Mix thoroughly on stirrer/hot plate

TEMED (in fume hood) (Sigma # T-8133)	10µl
APS (mix while adding)	0.25ml

Stacking Gel:

Solution	2.5% for 2 sides
B	1.25ml
D	2.50ml
H2O	6.25ml

Mix thoroughly on stirrer/hot plate

TEMED (in fume hood)	12ml
APS (mix while adding)	40µl

Sample Extraction

Carlson's Buffer:

0.1M Tris	1.21g
0.01M KCl	0.0745g
0.005M EDTA	0.186g
Sucrose	2.736g

Add about 90ml deionized H2O and adjust to pH7.5 with conc. HCl.
Make volume up to 100ml.

Appendix 2

Stock solutions: Solutions were prepared and stored at refrigerator at 4°C.

A= 3.0 M Tris Hcl buffer, pH 8.9 at 23C (SEPARATING GEL)

Tris 36.6 g (Sigma # T-1378)
Make up to about 90ml with deionized water
Titrate with conc. HCl to pH= 8.9
Total volume should be 100ml

B= 0.4 M Tris HCl buffer, pH= 6.9 at 23°C (STACKING GEL)

Tris 5.0 g
Make up to about 90ml with deionized water
Titrate to pH= 6.9 with conc. HCl
Total volume should be 100ml

C= Acrylamide solution for SEPARATING GEL

Acrylamide	28.0g (Bio-Rad # 161-0101)
Bis-acrylamide	0.735g (Bio-Rad # 161-0201)
H2O	Up to 100ml
Total volume should be 100ml	

D= Acrylamide solution for STACKING GEL

Acrylamide	10.0g
Bis-acrylamide	2.5g
H2O	Up to 100ml
Total volume should be 100ml	

Ammonium persulphate (APS) 0.1g/1ml (must make fresh each day).

RESERVOIR BUFFER:= B. J. Davis (1964) Ann. N. Y. Acad. Sci. 121: 404-427
1X Electrode buffer= Tris/ Glycine: pH= 8.57 at 23°C

Glycine	= 4.32g (BDH-Analar # 10119)(Anax)
Tris	= 0.50g (Sigma # T-1378)

Adjust pH at 8.57 and make up to 1500 ml with deionized water.