Molecular Studies of Homologous Chromosome Pairing in *Triticum aestivum*

by

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ERRATA

Page 11 - last paragraph, line 7
contrast to the Holliday "model"

Page 15 - line 5
replace "shown to be" with "shown to have"

Page 23 - line 28
"initiation"

Page 25 - last paragraph, line 1
"polycomplexes"

Page 26 - 3rd paragraph, line 2
"extranuclear"

Page 31 - 3rd paragraph, line 4
replace "long arm of the chromosome" with "long arm of chromosome 5B"

Page 35 - 2nd paragraph, line 11
"non-homologous"

Page 35 - 2nd paragraph, line 14
replace "Furthermore in plants tri-isosomic 5BL plants" with "Furthermore in tri-isosomic 5BL plants"

Page 35 - 2nd paragraph, line 16
"intrachromosomal"

Page 36 - 2nd paragraph, line 1
"non-random"

Page 43 - line 2
"diethylpyrocarbonate"

Page 43 - 2nd paragraph, line 1
"precooled"

Page 46 - 4th paragraph, line 3
"solution"

Page 47 - 2nd paragraph, line 2
"manafacturer's"

Page 48 - line 2
and "was" incubated

Page 58 - line 5
"paraffin"

Page 59 - line 10
"identified"

Page 69 - paragraph 3, line 1
replace "has been utilised for cloning and allows" with "was utilised for cloning and allowed"

Page 75 - paragraph 2, line 14
"zygotene-pachytene"

Figure 3.5 - line 2
the phage "were" transferred

Figure 3.5 - line 6
clones which "did" not

Figure 3.9 - line 4
5 minutes "then" exposed

Page 84 - line 5
"advanced"

Page 88 - line 5
"both ends of"

Page 88 - paragraph 2, line 14
"pollen had been"

Page 116 - line 1
"Appendix 2"

Figure 5.17 - line 4
"polymorphisms observed"

Page 129 - line 3
"chromosomes"

Page 129 - 2nd paragraph, line 7
"production of antibodies is required"

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Stephen W. Thomas

Acknowledgments

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Dedication

This thesis is dedicated to my Grandfather F. J. Wauchope (1904-1989), I hope that this thesis would have made him proud.

Abbreviations

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

µCi	:	microcurie
µg	:	microgram
g	:	gram
µl	:	microlitre
µM	:	micromolar
bp	:	base pair
kb	:	kilobase
ml	:	millilitre
nm	:	nanometre
mM	:	millimolar
M	:	molar
ng	:	nanogram
Δ	:	null mutation
SC	:	synaptonemal complex
pfu	:	plaque forming unit
DSB	:	double strand break
SPB	:	spindle pole body
DEPC	:	di-ethyl-pyro-carbonate
AWWM	:	Australia, Waite, Wheat, Meiosis
UTR	:	untranslated region
Da	:	dalton
EMS	:	Ethyl methane sulphonate

Abstract

Allohexaploid bread wheat (*Triticum aestivum*) is composed of three closely related, homoeologous genomes, A, B and D. Despite the high degree of homology between the genomes, only bivalents form during chromosome pairing at meiosis. The diploid character of hexaploid wheat arises from the action of pairing genes (*Ph*) located predominantly on chromosomes 3 and 5. Although cytogenetic studies of the pairing genes in hexaploid wheat have been performed for a number of years, the understanding of the molecular action of these genes remains poorly understood. Here, two approaches to the molecular characterisation of the homologous pairing process in allohexaploid wheat have been described.

The isolation and characterisation of late replicating DNA in lily has indicated that this DNA species is required for correct chromosome pairing in this organism. Attempts here to isolate late replicating DNA from hexaploid wheat have proven to be unsuccessful. It is suggested that, if late replicating DNA is present in wheat, it is of a different structure than that observed in lily.

In addition to studying late replicating DNA, several meiotic genes were isolated from an early meiosis cDNA library screened with a probe prepared by subtractive hybridisation. One of these clones, AWWM5, was further characterised. The genes of this family are located within the *ph2a* deletion on the short arm of chromosome 3D and on the short arm of chromosome 3A. The *ph2a* deletion has been characterised using a barley consensus map and is demonstrated to be a sub-terminal deletion. The AWWM5 gene is expressed predominantly at premeiotic interphase and early meiosis in both the meiocytes and the tapetum. The protein encoded by AWWM5 has putative DNA binding and membrane binding characteristics. A speculative model on the action of the pairing genes in allohexaploid wheat, and the putative function of the AWWM5 gene is discussed.

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Chapter 1

Review of Literature

1.1 Introduction

In most organisms, chromosomes are only visible during cell division when the chromatin becomes sufficiently condensed to be viewed by light microscopy. Two different types of cell division have been identified in eukaryotes, mitosis and meiosis. Mitosis is characterised by a single cell division following DNA replication, resulting in the formation of two identical daughter cells. In contrast, during meiosis a single round of DNA replication is followed by two successive cell divisions to produce four cells containing the haploid compliment of chromosomes. In many lower eukaryotes, such as species of protozoa, algae and fungi, whose reproduction is asexual, mitosis is the only kind of cell division which occurs. In the higher eukaryotes, sexual reproduction is performed to provide successive generations with new combinations of genes. This is achieved through the processes of meiosis, which reduces the chromosome compliment by half, and fertilisation to restore the diploid compliment of chromosomes. Whilst many of the processes of mitosis have become increasingly clear, the mechanisms of meiosis remain debatable despite nearly a century of investigation (Rassmussen and Holm, 1980; John and Lewis, 1983). In particular, the events of early meiosis including homologous chromosome pairing, synaptonemal complex formation and recombination have been the focus of intensive research but remain poorly understood. This thesis attempts to identify DNA structures and genes involved in the process of homologous chromosome pairing in allohexaploid bread wheat (*Triticum aestivum* L.).

In order to explore the mechanism of homologous chromosome pairing, it is first necessary to understand the basic events of meiosis and how they are related to the chromosome pairing process. Consequently, the following review is aimed at outlining the deficiencies in the classical model of meiosis, describing the organisms which have been utilised for meiotic research and an overview of the current status of research into the mechanisms and genes involved in homologous chromosome pairing, synaptonemal complex formation and recombination.

1.2 The Classical View of Meiosis and Mitosis

Mitosis and meiosis have both been studied extensively by light microscopy and, while there are many parallels between the two types of division, fundamental differences of the processes are observed.

During mitosis, chromosomes replicate at the middle of the S-phase to form sister chromatids which are joined at the centromere. This is evident at prophase when the chromosomes are sufficiently condensed to be viewed by light microscopy. The nuclear membrane surrounding the chromosomes degrades towards the end of prophase, allowing the chromosomes to move within the cell. As the cell enters metaphase, the centromere of each replicated chromosome attaches to the spindle apparatus. The chromosomes are manoeuvred by the spindle to align on the equatorial plate of the cell. At anaphase, contraction of the spindle fibres separates the sister chromatids to opposite poles of the cell. The nuclear membrane reforms around both groups of chromosomes at telophase and a new cell wall forms between them, giving rise to two identical daughter cells.

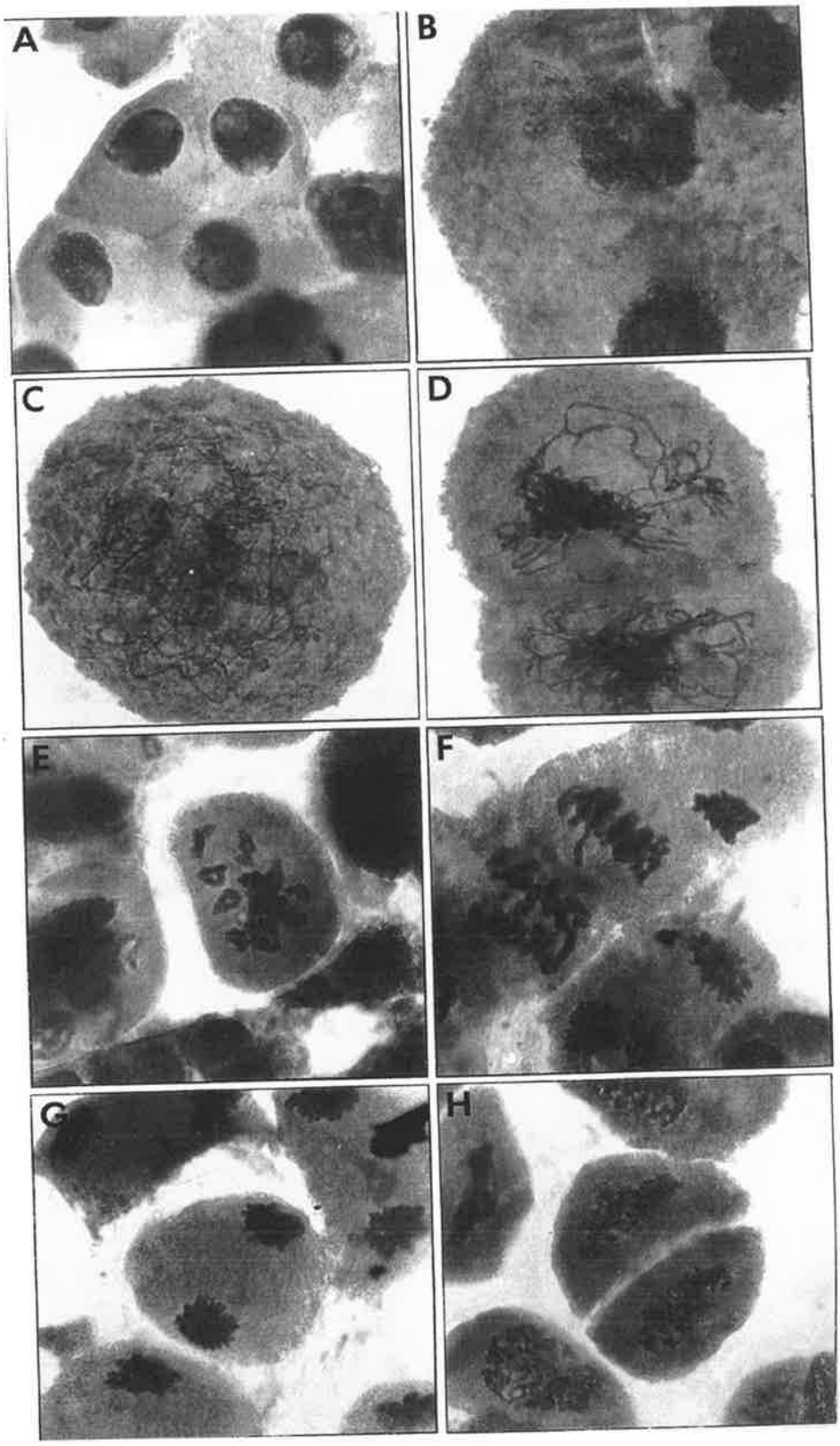
In contrast to mitosis, DNA replication during meiosis is followed by two nuclear divisions to produce cells containing the haploid number of chromosomes. Following DNA replication during meiosis, cells enter prophase I which has been divided into 5 stages based on the appearance of the chromosomes (John and Lewis, 1966) (Figure 1.1). At leptotene the chromosomes first become visible by light microscopy as long, thin threads. Chromosomes continue to condense during zygotene, and homologues begin to pair at one or more points along their length through an active and highly specific pairing mechanism resulting in the formation of the haploid number of bivalents. Often the telomeres of chromosomes become attached at a single point on the nuclear membrane at zygotene resulting in the characteristic "bouquet" appearance. At pachytene, homologues are completely paired, with synapsis being either facilitated by or resulting in the formation of a proteinaceous structure between the homologous chromosomes. This structure is termed the synaptonemal complex. Within the context of the synaptonemal complex recombination between homologues occurs. Maximum condensation of the chromosomes occurs at diplotene and

Figure 1.1

Pollen mother cells of *Triticum aestivum* stained with aceto-carmine and viewed by light microscopy. The distinct stages of the first meiotic division are apparent (from Letarte, 1996).

A Premeiotic interphase
B Leptotene
C Zygotene
D Pachytene
E Diplotene-Diakinesis
F MetaphaseI-AnaphaseI
G TelophaseI
H Dyads-MeiosisII.

Figure 1.1



diakinesis and crossovers are visible as chiasmata which begin to terminalise. The chromosome compliment is represented as the haploid number of bivalents. The following stages of metaphase I, anaphase I and telophase I closely resemble their mitotic counterparts although it is the bivalents which migrate to the equatorial plate and then segregate to opposite poles of the cell.

Meiosis II fundamentally represents a mitotic division of the two cells from meiosis I, resulting in the formation of four haploid cells which may undergo further mitoses and differentiation to become the gametes.

The above describes the classical views of the processes of meiosis and mitosis. However, recent research has demonstrated that the temporal order of the events of meiosis and the mechanisms underlying them, have been oversimplified and need to be studied further (Giroux, 1988; Rockmill and Röeder, 1990; Hawley and Arbel, 1993; Moens, 1994; Klein, 1994; Loidl, 1994; Orr-Weaver, 1995; Röeder, 1995; Yamamoto, 1996). The majority of research to date has focussed on understanding the events which occur during prophase I, including chromosome pairing, synaptonemal complex formation and recombination. Recent evidence supports a view that chromosome pairing, synaptonemal complex formation and recombination are all linked and may share common functions (see Hawley and Arbel, 1993 for discussion). Whilst this thesis is concerned predominantly with the study of chromosome pairing in *T. aestivum*, to fully understand the processes involved, it is first necessary to understand how other functions of prophase I occur and how they are related to chromosome pairing.

1.3 Model Organisms for the Study of Meiosis

1.3.1 The lower eukaryotes-yeasts and other fungi

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizzosaccharomyces pombe* have been the most extensively studied organisms in relation to meiosis. The yeasts provide an excellent system for studying meiosis for several reasons. Haploid cells of opposite mating types fuse to form a zygote which directly enters meiosis. It is possible, however, to grow zygotes which, when starved of carbon and nitrogen, initiate meiosis in a synchronous fashion (Röeder, 1995;

Yamamoto, 1996). This provides a virtually unlimited source of well defined material which can be used for temporal analysis of the cell as it undergoes meiosis. Yeasts are easily mutated via a variety of agents and are also easily transformed allowing rapid identification of mutated genes by complimentation (Burns *et al.*, 1994; Röeder, 1995). These genes can be readily identified through homology searches with the yeast genome mapping project data. The ease of transformation of the yeasts also allows the integration of synthetic constructs for the study of chromosome pairing as well as intragenic and intergenic recombination. Finally, the ability to isolate all four products of a single meiosis by tetrad dissection allows the detailed study of the events and mechanism of recombination (Baker *et al.*, 1976, Röeder, 1995).

Although advances in cytology have made it possible to view the yeast chromosomes, they are very small making detailed cytological examination of the chromosomes difficult though not impossible. In addition, the practicalities of translating the knowledge of meiosis in yeast to other, more advanced organisms, has not been tested, although some genes, first isolated in yeast, do seem to be present and functioning in higher eukaryotes (Kobayashi *et al.*, 1993). However, it is likely that higher eukaryotes would require additional functions for meiosis. Therefore, yeasts provide a good system for the study of the fundamental aspects of meiosis but may not yield adequate information to explain some characteristics of meiosis in the higher eukaryotes.

Fungal species such as *Neurospora crassa* (Raju, 1992), and *Coprinus cinereus* (Pukkila *et al.*, 1994) have also been utilised for the study of meiosis for many of the same reasons as yeast. *Coprinus cinereus* is particularly amenable to the study of meiosis being more suited to cytological examination.

1.3.2 Plants

Traditionally, meiotic studies of plants have utilised *Lilium* as a model (Ito *et al.*, 1967a). *Lilium* species possess very large anthers which undergo synchronous development and so provide a ready source of well defined material (Erickson, 1948). Lily microsporocytes can also be cultured *in vitro* (Ito *et al.*, 1967a) to provide a defined source of material. The use of lily for the biochemical study of meiosis is well

documented (Hotta *et al.*, 1984; Stern and Hotta, 1987). However, due to the large size of the lily genome and the lack of a suitable transformation system, genetic analysis is difficult (Kobayashi *et al.*, 1993).

The importance of cereal crops in world agriculture has ensured that research on the meiotic procedures of these species has been performed and there are numerous reports of the duration of meiosis (Bennett, 1971; Bennett and Smith, 1972; Bennett *et al.*, 1973) and meiotic disturbances (Baker *et al.*, 1976).

Whilst the asynchronous nature of meiosis in cereal species and the small size of most cereal anthers, makes collection of material and cytological examination difficult, research on these species is progressing. Several meiotic genes and mutations have been identified in cereal species including the *ameiotic* (Golubovskaya *et al.*, 1993) and *mac* (Sheridan *et al.*, 1993) genes of maize which control entry and commitment to meiosis. The *ds* mutation in *Triticum monococcum* (Smith, 1939) eliminates the reductional division of meiosis, similar to the *spo13* null mutation of *S. cerevisiae*, resulting in the formation of two diploid microspores. However, the most numerous class of mutants in higher plants are the asynaptic and desynaptic mutants which cause an elevated frequency of univalent formation at metaphase I. Mutations of these types have been most recently investigated in *Secale cereale* (Fedotova *et al.*, 1994).

A number of cereal species also display active regulation of chromosome pairing. This process is thought to require the interaction of several genes and is well defined in higher plants, in particular the Triticeae. *T. aestivum* is the most commonly studied cereal which exhibits regulation of chromosome pairing. Consisting of three related but distinct genomes, *T. aestivum* displays a high order of organisation at meiosis, with only homologous chromosomes from the same genome pairing at prophase I. *T. aestivum* is therefore an ideal organism for the study of the genetic control of chromosome pairing.

1.3.3 Animals

Drosophila melanogaster has been utilised for the study of a wide range of both genetic and biochemical anomalies. Investigations into meiosis in *Drosophila* have

been significant for two main reasons; the ability to visualise meiotic divisions in both male and female flies (Orr-Weaver, 1995) and the large collection of mutations which affect different components of the meiotic cycle (Hawley, 1993). *Drosophila* meiosis has been extensively studied at the cytological level predominantly in male flies where the chromosomes are accessible to both light and electron microscopic examination (Goldstein, 1981). Female chromosomes are smaller but advances in electron microscopy have permitted analysis of female meiosis. Consequently, the events of homologue pairing, synaptonemal complex formation and chiasma formation have been cytologically defined in *Drosophila* females (Carpenter, 1975). Male *Drosophila* provide important material for the study of early meiosis, as they fail to undergo synaptonemal complex formation and recombination (Ault and Rieder, 1994) but otherwise exhibit completely normal segregation of chromosomes and other events of meiosis. This has allowed the investigation of the relative importance of the other early events of meiosis, such as homologue pairing, synaptonemal complex formation and recombination, and their effect on latter components of the meiotic cycle. Mutations in *Drosophila* have also been important in the examination of several features of meiosis, including regulation of the meiotic cell cycle, sister chromatid attachment, and the segregation of non - exchange chromosomes (Baker *et al.*, 1976; Orr-Weaver, 1995).

While *Drosophila* has provided an excellent source of genetically well defined material, the small size of the chromosomes and the unsynchronous nature of meiosis in this organism have contributed to difficulties in studying meiosis on a cytological and molecular level.

Meiosis has also been investigated in other animals such as Australian plague locust (Peacock, 1970), newt (Wimber and Prensky, 1963), and rodents (Mukherjee and Cohen, 1968; Kofman-Alfaro and Chandley, 1970).

Given that meiosis has been studied in a vast range of organisms, it is surprising that we still understand very little about the processes which are involved in this highly complex process. It is accepted that future investigation of meiosis will continue in the lower eukaryotes such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as it has done in the past. However, it is important that research also focus on higher

eukaryotes such as plants and animals. In particular, research in the plant cereal species will provide an excellent source of material for the study of genetic regulation of chromosome pairing.

1.4 The Synaptonemal Complex

One of the unique features of meiosis in most eukaryotes is the formation of a proteinaceous scaffold called the synaptonemal complex (SC), which forms between homologous chromosomes in most organisms during prophase I. Because the synaptonemal complex is first observed at early meiosis (prophase I), it has been implicated in a number of processes including chromosome synapsis and promotion of recombination (reviewed by Petes *et al.*, 1991; Egel, 1995), conversion of crossovers into functional chiasmata (Maguire, 1978), mediation of crossover interference (Egel, 1978) and resolution of chromosome interlocks prior to segregation (Kleckner *et al.*, 1991). The exact function of the SC remains unknown and research into its constituents has been difficult.

The synaptonemal complex is morphologically highly conserved across a wide range of species and consists of two lateral elements flanking a central region which contains a central element and transverse filaments (Figure 1.2) (Moses, 1968; von Wettstein *et al.*, 1984; Westergaard and von Wettstein, 1972).

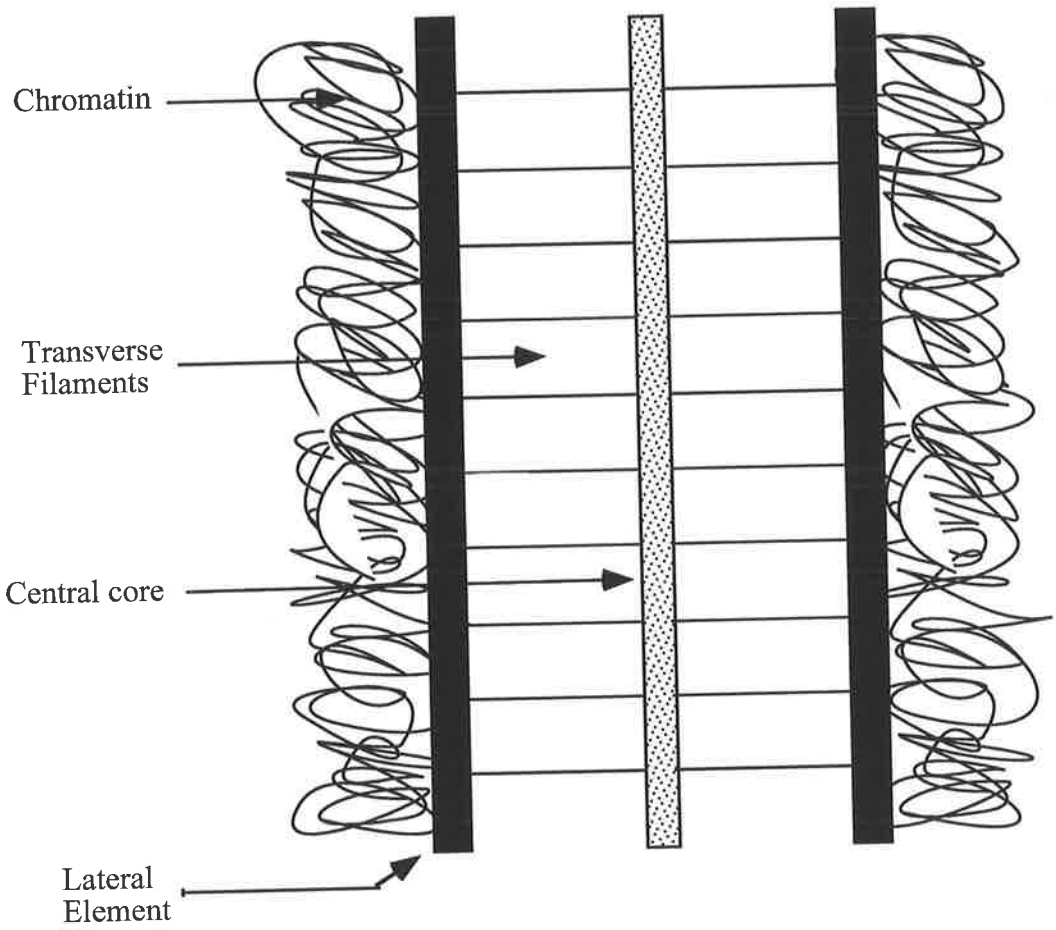
The earliest observable evidence of the SC occurs at leptotene when the axial elements, known as lateral elements within the context of the SC, become visible on paired but unsynapsed chromosomes. Atomic force and electron microscopy of synaptonemal complexes from rat and mouse reveal that the axial elements appear to be double and triple stranded structures (Putman *et al.*, 1993). This has led to the proposal that the axial elements form on a framework composed of individual sister chromatids (Putman *et al.*, 1993; Rufas *et al.*, 1992).

Recently, several proteins which appear to be specific constituents of the axial elements have been identified. These include HOP1 and RED1 of *S. cerevisiae* (Hollingsworth and Byers, 1989; Rockmill and Röeder, 1988), RAD3-1 of *Coprinus cinereus* (Pukkila *et al.*, 1992) and COR 1 from hamster (Dobson *et al.*, 1994).

Figure 1.2

Diagrammatic illustration of the synaptonemal complex. Lateral elements are aligned in parallel with loops of chromatin extending from them. A percentage of chromatin is also present in the central region but is not shown here. The transverse filaments and central core are also shown.

Figure 1.2


Chromatin
Transverse Filaments
Central core
Lateral Element

Additionally, proteins of the axial elements have been identified in lily (Anderson *et al.*, 1994) as well as rat and mouse (Heyting *et al.*, 1988; Moens *et al.*, 1992). Null mutations of *hop1* (*hop1*Δ) are defective in chromosome pairing and display a ten fold decrease in levels of meiotic crossing over and intragenic recombination leading to high levels of chromosome non-disjunction at meiosis I. Electron microscopy reveals that these mutants fail to form synaptonemal complexes (Hollingsworth and Byers, 1989). Deletion mutations of *red1* (*red1*Δ) differ from *hop1*Δ mutations in that, whilst they undergo high levels of chromosome non-disjunction at meiosis I, they display wild type levels of recombination (Rockmill and Röeder, 1988). Therefore, *RED1* might function in the attachment of the chromosomes to the spindle in preparation for disjunction or may also be a constituent of the spindle or spindle pole body such that defects in this gene product prevent attachment of the chromosomes to the spindle. Alternatively, *RED1* may be required for chiasma terminalisation such that bivalents in *red1*Δ mutants fail to undergo terminalisation and remain intact throughout anaphase I (Rockmill and Röeder, 1988). Both the *hop1*Δ and *red1* Δ mutations are alleviated by the *spo13*Δ mutation (Rockmill and Röeder, 1988; Hollingsworth and Byers, 1989) which causes diploid cells to undergo a single round of chromosome segregation to produce two spored asci containing diploid spores (Klapholz and Esposito, 1980). Recombination is not required in *spo13*Δ strains (Malone and Esposito, 1981) as the first meiotic division does not occur. This observation suggests that both *HOP1* and *RED1* are required for proper recombination indicating a dependence of recombination on formation of the synaptonemal complex (or at least the axial elements). Recently it has been demonstrated that over-expression of *RED1* can alleviate the phenotype of a temperature sensitive *hop1* mutation (*hop1-628*) (Hollingsworth and Johnson, 1993) suggesting that *RED1* acts to stabilise the HOP1 protein in the synaptonemal complex. Interestingly, the *hop1-628* mutant displays high levels of recombination and is defective specifically for chromosome segregation similar to *red1*. It is possible that both the *HOP1* and *RED1* genes act together during chromosome segregation with *HOP1* also playing a role in chromosome synapsis.

Synapsis of homologous chromosomes occurs at zygotene when homologues come into close apposition, the lateral elements of homologous chromosomes being separated by approximately 100nm. Synapsis initiates at a series of points along the

chromosomes, perhaps at sites of potential recombination, and proceeds at a rapid rate as compared to the rate at which new sites of initiation are established (Dobson *et al.*, 1994). Synapsis initiation can occur after full length axial elements are formed (von Wettstein *et al.*, 1984) or before the completion of axial element formation, as in *S. cerevisiae* (Dresser and Giroux, 1988; Alani *et al.*, 1990). During synapsis, the gap between the lateral elements is known as the central region and has been shown to be composed of a central element and transverse filaments (Solari and Moses, 1973). The central element runs as a protein core longitudinally between homologous chromosomes equidistant from each lateral element. Transverse filaments lie perpendicular to, and between the central and lateral elements. Proteins identified as specific to the central region include SYN1 from hamster (Dobson *et al.*, 1994) as well as two un-named proteins from rat (Heyting *et al.*, 1988; Smith and Benevente, 1992). ZIP1 from *S. cerevisiae* (Sym *et al.*, 1993) has been proposed as a component of the transverse filaments of the SC in this organism (Sym and Röeder, 1995). The role of ZIP1 in the central region is supported by electron microscopy of *zip1*Δ mutations. Extensive axial element formation is apparent in *zip1*Δ cells but no SC is formed and the distance separating paired chromosomes is significantly greater than in the wild type (Sym *et al.*, 1993). *zip1*Δ mutants display almost wild type levels of recombination even though they lack a major structural component of the SC (Sym *et al.*, 1993). Hence, fully formed SC is not a prerequisite for the initiation of recombination.

In addition to the proteins which are specific to the SC, several other ubiquitous proteins have been shown to be present in the structure. These include the heat shock protein HSP70-2 (Allen *et al*, 1996) which may play a chaperone role in the formation of the SC in mouse and hamster spermatocytes but is absent in oocytes. It is also demonstrated that topoisomerase II reacts with the SC (Moens and Earnshaw, 1989). Interestingly, topoisomerase II has been implicated in the recombination process (Klein *et al.*, 1992). Further analysis of the composition and function of the SC is currently being focussed on the use of monoclonal antibodies to identify SC components (Heyting *et al.*, 1988) and the isolation of the corresponding genes from expression libraries (Chen *et al.*, 1992). Preliminary results from these studies

indicates that the SC is composed of both rearranged nuclear structures and newly synthesised components.

1.5 Genetic Recombination at Meiosis

1.5.1 The recombination process

Genetic recombination is the general term used to describe the exchange of DNA sequences between chromatids. Recombination has been most extensively studied in the lower fungi as these allow the recovery and analysis of all of the recombination events of an individual meiosis. Examination of heterozygous markers in the meiotic products of fungi by tetrad dissection has allowed three different types of genetic exchange to be identified.

Reciprocal exchange, or crossing over is the most common form of recombination and results in the equal exchange of information between chromatids such that all markers exhibit 4:4 segregation. However, aberrant forms of segregation do occur and can produce 6:2, 5:3 or aberrant 4:4 segregation of markers. The 6:2 segregation of markers arises from the non reciprocal exchange of information between DNA duplexes. Other aberrant segregation ratios arise by post-meiotic segregation which occurs when the two strands of a DNA duplex carry different genetic information. The duplex in question is referred to as heteroduplex DNA and can be asymmetric if found on only one chromatid, or symmetric if it covers the same region on two chromatids. Cells carrying heteroduplex DNA divide to produce two genetically different daughter cells. If only one chromatid contains heteroduplex DNA, the marker segregation is a 5:3 ratio. If two chromatids carry heteroduplex DNA within the same region, two pairs of non-identical daughter cells are produced. This is not immediately apparent from the segregation ratio which appears to be a normal 4:4 segregation of heterozygous markers. However, tetrad analysis reveals that daughter cells are genetically different and hence the segregation is referred to as an aberrant 4:4 segregation.

Studies of the differing types of recombination have led to the formulation of a number of models to describe the events of recombination and explain how crossing over and aberrant segregation occur and are related (reviewed by Szostak *et al.*,

1983). A brief description of the Holliday and Meselson-Radding models is presented here and a more in depth analysis of the models is presented by Szostak *et al.* (1983)

1.5.1.1 The Holliday model

The Holliday model (Holliday, 1964, 1968) was the most widely accepted explanation of the recombination process. Holliday envisaged that after replication and general pairing of chromosomes, the DNA molecules of opposing homologous chromatids were nicked at defined sites to yield single strand gaps. Strand separation could then proceed along the length of homologous chromatids, stopping at any point. The single strands thus generated were capable of annealing to opposite complimentary strands to form symmetric heteroduplex DNA and a Holliday junction. Should a heterozygous marker fall within the region of symmetric heteroduplex, a mismatch of base pairs occurs. Repair of the mismatch in the same direction on both DNA duplexes could result in a 6:2 or 2:6 segregation ratio. Repair of only a single duplex would result in an aberrant 5:3 ratio, and correction of neither duplex would cause an aberrant 4:4 segregation ratio. It was postulated that resolution of the Holliday junction at the point of strand exchange was proceeded by the precise breakage and reunion of complimentary strands such that deletions and duplications were avoided. If breakage occurred in the exchanging strands, no crossover formed. If breakage occurred in the non-exchange strands, a crossover was formed and reciprocal recombination could occur.

In the last two decades however, the Holliday model has come under increasing scrutiny and several investigations have cast doubt on the accuracy of this model. Crosses in *Ascobolus* were used to investigate 5:3 segregation of wild type coloured and mutant colourless spores in asci where the flanking markers displayed a non-recombinant phenotype (Stadler and Towe, 1971). It was demonstrated that nearly all of the aberrant segregations were of the type where mismatch correction occurred predominantly on a single chromatid. This is in contrast to the Holliday which predicts the formation of heteroduplex on two chromatids proceeded by the repair of the resulting mismatch at an equal frequency on either chromatid. To explain this phenomenon, Stadler and Towe (1971) propose that heteroduplex DNA frequently occurs on only one chromatid (asymmetric heteroduplex).

1.5.1.2 The Meselson-Radding model

The Meselson-Radding model (Meselson and Radding, 1975) accounts for the data from *Ascobolus* by assuming that regions of both asymmetric and symmetric heteroduplex arise from a single initiation point. Recombination is initiated by a single strand nick on one of the two chromatid duplexes and this becomes the site for strand displacement by a DNA polymerase. The displaced strand invades the homologous duplex, displacing a D-loop and forming a region of asymmetric heteroduplex DNA. The D-loop is degraded and the invading single strand is ligated in place. Extension of the region of asymmetric heteroduplex occurs through the ongoing displacement of single stranded DNA from the donor duplex and enzymatic degradation of single stranded DNA on the recipient duplex. Following the formation of asymmetric heteroduplex, branch migration can bring the 5' and 3' ends into parallel positions so they can be ligated. Alternatively, the 5' and 3' ends can be aligned by isomerisation of the structure. The resulting Holliday junction can move by branch migration generating either asymmetric heteroduplex DNA or, if the structure has undergone isomerisation, symmetric heteroduplex DNA. Mutations within the asymmetric heteroduplex DNA would result in mainly 5:3 segregation and very few aberrant 4:4 segregation's. Mutations further from the site of initiation and in a region of symmetric heteroduplex would display higher proportions of aberrant 4:4 segregation in relation to 5:3 segregation.

1.5.1.3 The double strand break repair model

An alternative to the Meselson-Radding model has been proposed by Szostak *et al.* (1983) on the basis of results of experiments involving recombination between chromosomes and plasmids in *Escherichia coli* (Orr-Weaver *et al.*, 1981). The double strand break (DSB) repair model proposes that recombination is initiated by the formation of a DSB on the recipient chromatid by a double strand endonuclease. One of the resulting 3' ends then invades the donor duplex to displace a D-loop. The D-loop is enlarged by repair synthesis until it can anneal with the complimentary 3' end of the recipient duplex and repair synthesis from the opposite 3' end then completes the gap repair resulting in the formation of two regions of asymmetric heteroduplex DNA. The repair process also results in the formation of two Holliday junctions which

can move in either direction by branch migration producing regions of symmetric heteroduplex DNA. Resolution of the crossovers by cutting of either the inner or outer strands gives two possible non-crossover and two possible crossover configurations.

6:2 segregation of markers can occur by two possible courses. Markers within the region of a double strand gap are repaired by double strand transfer of information with no involvement of heteroduplex DNA. Alternatively, mismatches within a region of asymmetric heteroduplex will be corrected by mismatch repair giving either 6:2 or 2:6 segregation depending on the direction of correction. Heteroduplex DNA in which a mismatch is not repaired would result in the aberrant 5:3 and 4:4 ratios of segregation.

Whilst both the Meselson - Radding model and the Double Strand Break Repair model adequately explain the observed recombination in many studied organisms, several lines of evidence suggest that the DSB model of recombination is likely to be the most accurate.

The view that recombination is initiated by DSBs is supported firstly by the finding that a number of genes required for the repair of DSBs in vegetative cells, are also required for meiosis in *Saccharomyces cerevisiae*. These include the members of the *RAD50* epistasis group *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55* and *RAD57* (Resnick, 1987).

A second line of evidence for the involvement of DSBs in meiotic recombination comes from the examination of recombination hotspots. In *Saccharomyces cerevisiae*, and presumably in all other organisms, certain sites display higher rates of recombination than the remainder of the genome. These sites are termed recombination hotspots and are thought to correspond to regions of DNA that are more accessible to the enzymes and proteins required for recombination. Recent studies have defined many recombination hotspots in *S. cerevisiae* including the *ARG4* (Sun *et al.*, 1989) hotspot for initiation of gene conversion and the *HIS4-LEU2* hotspot created by insertion of a 2.8 kb segment from *LEU2,* containing the hotspot, downstream of *HIS4* (Cao *et al.*, 1990). Physical studies of these recombination

hotspots have demonstrated the presence of double strand breaks at these sites. The studied DSBs are most abundant after DNA replication and before the commitment to recombination. Deletion of the initiation site of *ARG4* or removal of *LEU2* from the *LEU2* - *HIS4* hotspot abolishes the formation of DSBs (Sun *et al.*, 1989; Cao *et al.*, 1990). Sun *et al.* (1989) have also demonstrated that the DSBs formed at the *ARG4* locus have single stranded 3' tails as would be required for duplex invasion in the double strand break repair model. Recent studies also suggest that DSBs are present at other loci which correspond to recombination hotspots and are a general feature of recombination at all genomic loci (Game *et al.*, 1989; Zenvirth *et al.*, 1992; Fan *et al.*, 1995; Wu and Lichten, 1995).

The double strand break repair model of recombination proposes the formation of double Holliday junctions as a key intermediate in the recombination process (Szostak *et al.*, 1983). Bell and Byers (1983) first isolated branched molecules from DNA undergoing meiotic recombination by 2D gel electrophoresis. The eye-like structure of these DNA molecules makes it likely that they represent double Holliday junctions. More recent studies utilising a procedure developed by Brewer and Fangman (1987) incorporating 2D electrophoresis and Southern blotting have resulted in the identification of joint molecules of DNA formed during meiosis and containing information from both parental chromosomes (Collins and Newlon, 1994; Schwacha and Kleckner, 1994). Temporal analysis of joint molecules from *S. cerevisiae* shows that they arise shortly after DSB formation and remain present until crossing over occurs (Byers and Hollingsworth, 1994). The implications of this result are that joint molecules may represent an intermediate between DSB's and mature recombination molecules. Joint molecules do not form in *spo11* or *rad50* null mutations or the *rad50*S mutation which are deficient for meiotic recombination (Collins and Newlon, 1994; Schwacha and Kleckner, 1994).

Analysis of the structure of joint molecules also supports a role in recombination. Joint molecules occur frequently between homologues but rarely between sister chromatids, as expected for a recombination intermediate (Schwacha and Kleckner, 1994). The joint molecules can also be separated and analysed as single strands by denaturing gel electrophoresis. Analysis of the DNA obtained in such a way has shown that joint molecules are comprised of four intact full length strands which are

non recombinant for markers flanking the DSB from which the joint molecule was proposed to have arisen. Such an observation implies that joint molecules can not consist of a single or odd number of Holliday junctions but instead are most likely the result of a double Holliday junction as envisaged by the DSB model of recombination (Schwacha and Kleckner, 1994). Indeed, joint molecules were recently shown to be double Holliday junctions which could be resolved into both parental and recombinant duplexes when treated *in vitro* with Holliday junction-resolving endonucleases RuvC or T4 endo VII (Schwacha and Kleckner, 1995).

The evidence gained to date from studies of *S. cerevisiae*, therefore, suggests that recombination occurs via a repair pathway which incorporates the formation of DSBs, their conversion to joint molecules and finally the formation and resolution of mature recombination molecules.

1.5.2 Genes required for recombination

Like other areas of meiosis, the majority of our knowledge of the genetic control of recombination comes from the study of various mutants, especially those of *Saccharomyces cerevisiae*. If the DSB model of recombination is accepted, the initiation of recombination requires the formation of a double strand gap with recessed 5' ends and the invasion of intact duplex by the single stranded 3' tail. Both of these events presumably require the action of suitable enzymes and proteins.

The *E. coli* RecA protein was the first DNA strand exchange protein discovered (Roberts *et al.*, 1978) and is the most extensively studied enzyme involved in genetic recombination (Roca and Cox, 1991; Kowalczykowski, 1991). In the presence of ATP, dATP or ATPγS, the RecA protein will polymerise onto ssDNA to form a right hand helical nucleoprotein filament (Ogawa *et al.*, 1979) and within the complex the DNA is unwound and extended increasing the distance between base pairs. The nucleoprotein filament is capable of invading dsDNA, recognising sequence homology and assimilating the homologous duplex DNA to form a three stranded nucleoprotein filament (Stasiak *et al.*, 1984). Following the establishment of homology, the region of heteroduplex can be enlarged in an ATP dependent manner by the switching of

base pairs between the duplex and invading ssDNA and the displacement of one of the strands of the duplex in a 5' to 3' direction (Howard-Flanders *et al.*, 1984).

Given the parallels between the predicted mechanics of the DSB recombination model and the actual function of the RecA protein, it is not surprising that recent studies have aimed at isolating RecA homologues from eukaryotic cells and determining a role in eukaryotic recombination. Original attempts to isolate RecA homologues from eukaryotes were based on the identification of proteins capable of catalysing strand transfer *in vitro* between single stranded viral DNA and homologous DNA. Using this approach, several strand exchange proteins were isolated including SEP1 from *S. cerevisiae* (Kolodner *et al.*, 1987), RRP1 from *Drosophila melanogaster* (Lowenhaupt *et al.*, 1989) and HPP1 from human cells (Fishel *et al.*, 1988). However, studies have since demonstrated that these proteins have exonuclease activity (Moore and Fishel, 1990; Johnson and Kolodner, 1991; Sander *et al.*, 1991) which could result in the single stranded nicking of the double stranded DNA substrate in the strand transfer reaction. This would allow the production of single strand regions in the target dsDNA enabling complimentary base pairing of the single strands mimicking strand exchange. To overcome this difficulty, Ogawa *et al.* (1993a) screened previously isolated radiation sensitive mutants of *S. cerevisiae* for recombination deficient mutants with pleiotropic phenotypes similar to RecA. Two mutants, *rad51* and *rad52* were isolated that were sensitive to ionising radiation and defective in both spontaneous and X-ray induced mitotic recombination. The corresponding genes of these mutants have been cloned and deletion alleles constructed (Adzuma *et al.*, 1984; Shinohara *et al.*, 1992). The RAD51 and RAD52 proteins have been purified (Shinohara *et al.*, 1992) and both proteins display RecA like activities. RAD51 displays ATP dependent DNA binding, ssDNA-dependent ATPase activity and nucleoprotein filament formation whilst strand exchange activity is a feature of RAD52 (Ogawa *et al.*, 1993a).

Whilst both the *RAD51* and *RAD52* genes are transcribed throughout the life cycle of yeast, evidence for a role in meiosis has been obtained. *rad51*Δ mutant cells display an accumulation of meiosis specific DSBs the 5' ends of which are more extensively processed than in wild type cells. *rad52*Δ mutants have a similar phenotype of over processed 5' ends, and in both mutants the level of meiotic recombinant molecules

formed is only 20% of wild type levels (Shinohara *et al.*, 1992). The simplest explanation for the observed similarities of the mutation of Rad51 and Rad52 is that the two proteins interact to function in recombination. Indeed, physical binding of Rad51 and Rad52 proteins has been demonstrated (Ogawa *et al.*, 1993b) suggesting that, in a complex Rad51 forms a nucleoprotein filament on the 3' tails of DSB's and Rad52 catalyses the invasion of the homologous duplex by the single stranded DNA.

A second RecA homologue of *S. cerevisiae* is *DMC1*, a meiosis specific gene isolated from an enriched prophase cDNA library. Evidence for view that *DMC1* functions in the recognition of homologous DNA and strand exchange comes from observations of *dmc1*Δ mutants that display similar phenotypes as *rad51*Δ with a decrease in the overall formation of recombination molecules (approximately 10% of wild type) and an accumulation of DSBs with greatly resected 5' ends (Bishop *et al.*, 1992). *DMC1* is also required for the formation of hybrid joint molecules *in vitro* (Story *et al.*, 1993). As a result of the failure to convert DSBs into stable recombination intermediates, *dmc1* mutants do not undergo either of the two meiotic divisions and display ineffective sporulation. Structurally, *DMC1* is very closely related to *Rad51* and shows primary sequence homology with the RecA protein (Bishop *et al.*, 1992; Shinohara *et al.*, 1992). However, the homology is not so great that differing functions of the two genes can be ignored. Indeed, *DMC1* is not only required for recombination but is also likely to be required for the formation of the synaptonemal complex as *dmc1*Δ mutants form only short stretches of tripartite SC whilst axial element formation appears normal. This does not imply that DMC1 and RAD51 do not interact, recent research suggests that the two proteins form multiple nuclear complexes at prophase I (Bishop, 1994) but have distinct roles in recombination. DMC1 may confer meiosis specific properties to the recombination repair of DSB's such as the promotion of crossover interference or the enhancement of interhomologue recombination over sister chromatid exchange (Schwacha and Kleckner, 1994). The infrequent co-localisation of ZIP1 and DMC1 proteins suggests that SC assembly (or at least the assembly of the central region) and the early steps of recombination are reliant upon each other but may be temporally separated. This is further supported by the observation that the number of DMC1 complexes detected reaches a maximum before SC assembly. It is possible that formation of the central region of the SC is associated with the disassociation of the DMC1 protein supported

by the observation of DMC1 persistence in *zip1*Δ cells (Bishop, 1994). In this case, *dmc1*Δ mutants would fail to produce normal SC's because the mutation would block a function required for the action of ZIP1.

Other genes required for recombination have been isolated in several organisms and can be classified into two distinct groups. One group comprises genes which only affect meiotic recombination and include *HOP1* (Hollingsworth and Byers, 1989), *MRE4* (Leem and Ogawa, 1992), *MER1* (Engebrecht and Röeder, 1989), *MER2* (Engebrecht *et al.*, 1990), *MEK1* (Rockmill and Röeder, 1991), *MSH4* (Ross-Macdonald and Röeder, 1994), *MSH5* (Hollingsworth *et al.*, 1995), *RED1* (Rockmill and Röeder, 1988) and *SPO11* (Klapholz *et al.*, 1985). The second group of genes display an additional mutant phenotype of defective mitotic recombination as well as a loss of meiotic recombination function. The majority of these genes belong to the *RAD50* epistasis group (*RAD50-RAD57*) but also includes *MRE11* (Ajimura *et al.*, 1993), *MEI9* (Sekelsky *et al.*, 1995) and *XRS2* (Ivanov *et al.*, 1992). The proposed functions of some of the above genes are outlined in Table 1.1.

1.6 Chromosome Pairing

It is a widely held belief that chromosome pairing is a two step process whereby homologous chromosomes become aligned at a distance before intimate synapsis occurs (vonWettstein *et al.*, 1984; Scherthan *et al.*, 1992). Several observations support presynaptic alignment of homologous chromosomes. In triploid *Allium sphaerocephalon*, only two of the three homologues are synapsed at any one region. However, the unsynapsed homologue remains aligned with the synapsed pair indicating that the processes of synapsis and alignment are distinct (Loidl and Jones, 1986). Regular anaphase I division of homologues has also been described in asynaptic organisms which undergo presynaptic homologue alignment (reviewed in vonWettstein *et al.*, 1984). This again indicates a synapsis independent relationship between homologues. Furthermore, Loidl *et al.* (1994a) demonstrate that homologue association occurs in *rad50*, *rad50*S and *spo11* mutants of *S. cerevisiae* which are defective in chromosome synapsis and recombination.

Table 1.1 Outline of some genes required for meiotic recombination

Gene	Organism	Mutant Defect	Proposed Function	Reference
MRE4	*S. cerevisiae*	Decreased recombination Inviable spores	DSB formation DSB maintenance	Leem and Ogawa, 1992
MER1	*S. cerevisiae*	Accumulation of axial elements No reciprocal crossovers Inviable spores	Constiuent of SC central region	Engebrecht and Roeder, 1989 Engebrecht *et al.*, 1990
MER2	*S. cerevisiae*	No DSB formation Decreased homologue alignment	Homologue alignment	Rockmill *et al.*, 1995
MEK1	*S. cerevisiae*	Decreased recombination	Unknown	Rockmill and Roeder, 1991
MSH4	*S. cerevisiae*	Decreased recombination	Homologous to *MutS* required for mismatch repair in *E. coli*	Ross-Macdonald and Roeder, 1994 Modrich, 1991
MSH5	*S. cerevisiae*	Decreased recombination	Homologous to *MutS* required for mismatch repair in *E. coli*	Hollingsworth *et al.*, 1995 Modrich, 1991
Rad50	*S. cerevisiae*	No DSB formation No tripartite SC	Homology search for initiation of DSBs and SC formation	Alani *et al.*, 1990
Mei19	*D. melanogaster*	No meiotic recombination	Cuts nucleoprotein filament to release single strand	Sekelsky *et al.*, 1995
Xrs2	*S. cerevisiae*	No DSB formation	DNA repair gene	Ivanov *et al.*, 1992

Assuming that chromosome pairing involves presynaptic alignment of homologues followed by intimate synapsis, two fundamental questions need to be answered if the process of homologous chromosome pairing is to be elucidated; How and when do homologous chromosomes become associated and aligned? and How is homology between chromosomes identified?

Addressing the first of these questions, the hypotheses on how and when homologous chromosomes become associated can be divided into four general categories as described by Loidl (1990):

- premeiotic association of chromosomes
- chance contacts between randomly distributed chromosomes
- long range interactions between chromosomes
- interaction of chromosomes with internal nuclear structures

1.6.1 Premeiotic or somatic association of homologous chromosomes

Premeiotic or somatic alignment of homologues is viewed favourably by a number of authors as a mechanism for homologous chromosome association and alignment. If homologous chromosomes were aligned prior to entry into meiosis, only a mechanism for testing that the homology is correct and conversion of the alignment into stable synapsed pairs would be required to ensure homologous chromosome pairing during meiosis. The requirement of a homology search at the beginning of meiosis would, in effect, be circumvented.

Support of premeiotic pairing comes from many organisms. The somatic and premeiotic association of homologous chromosomes has been described as early as 1916, when Metz (1916) reported on the somatic pairing of homologous chromosomes in *Drosophila*. It has now been established that most Diptera display premeiotic and somatic association of homologous chromosomes (eg., vonWettstein *et al.*, 1984; Hiraoka *et al.*, 1993). There are reports of premeiotic association of chromosomes in other organisms. Maguire (1983) reports on homologue pairing at premeiotic interphase in maize. Pairing in maize varies from intimate, extensive pairing to rough alignment on homologues and requires the involvement of internal nuclear structures. Similarly, the observed interaction, in wheat meiocytes, of

internuclear fibrillar material with both the chromosomes and the nuclear membrane at premeiotic interphase has led to speculation that this material is responsible for the maintenance of homologous chromosome associations formed at premeiotic interphase (Bennett *et al.*, 1974; Bennet and Smith, 1979).

Feldman and Avivi (1973) have identified that in *Triticum aestivum* root tip cells, all chromosomes are distributed non-randomly with homologues being more closely associated than homoeologues or unrelated chromosomes. The disruptive effect of premeiotic applications of colchicine on chromosome pairing at meiosis in wheat (Avivi and Feldman, 1973) also indicates that a mechanism for homologue alignment is active at premeiotic interphase in these species. That premeiotic colchicine applications do not effect structures required during meiosis is demonstrated by the observation that colchicine applied at premeiotic interphase in wheat does not effect synapsis or chiasma formation in an isochromosome where the arms are held together by virtue of a common centromere (Discal and Darvey, 1970, Feldman and Avivi, 1988).

The advent of fluorescent *in situ* hybridisation (FISH) has allowed the study of premeiotic pairing in a number of organisms in which the chromosome structure is diffuse and is not amenable to light microscopy. Using FISH, two groups of researchers have recently demonstrated premeiotic alignment of homologous chromosomes in *S. cerevisiae*. Weiner and Kleckner (1994) used probes to relatively small, and well separated targets to identify premeiotic association of homologous chromosomes which occurred on average every 65 kb in the yeast genome. In contrast, Loidl *et al.* (1994a) studied only the two smallest *S. cerevisiae* chromosomes and employed a large number of probes to cover almost an entire half of each chromosome.

Whilst there is growing support for a role of premeiotic pairing in providing for homologous pairing at meiosis, several lines of evidence suggest this may not be the case. One objection comes from researchers who have failed to detect somatic pairing (Del Fosse and Church, 1981; Heslop-Harrison *et al.*, 1988). An explanation may be that the association of homologous chromosomes only occurs in the germ line cells and even then only at the mitosis directly preceding meiosis. Indeed, Maguire (1983)

failed to detect homologous associations in root tip cells of maize but observed associations during premeiotic mitosis.

John (1990) opposes the premeiotic pairing concept based on a number of observations. Firstly, "in organisms where there is prophase condensation of chromosomes after G2 and before the onset of leptotene, it is possible to confirm that homologues consistently lie separate. This is true for the plants *Lilium* (Walters, 1970, 1976) and *Hordeum* (Bennett, 1984)." The argument here can be negated by the observations of Weiner and Kleckner (1994) who clearly demonstrate that the association of homologues before meiosis involves only limited regions of the chromosomes and does not necessarily reflect complete alignment. In addition, the premeiotic chromosome associations are likely to be temporarily disrupted during DNA replication and reform independently of the synaptonemal complex (Weiner and Kleckner, 1994). Re-formation of homologous associations is also demonstrated to be meiosis dependent in view of the almost complete disruption of meiotic pairing in yeast strains deficient for *spo11* which display normal premeiotic associations. Therefore, it is possible, that homologous associations are only re-established at leptotene after prophase condensation, but that the re-establishment of pairing is facilitated by the premeiotic associations drawing homologous chromosomes into domains which "topologically favour" their interaction (Weiner and Kleckner, 1994). John (1990) also argues that in several zygotic fungi, karyogamy, and therefore meiosis, are delayed. Hence, the diploid state is only restored immediately prior to the entry into meiosis and does not allow any time for the premeiotic association of chromosomes. This argument is accepted although zygotic fungi are the only organisms where homologous chromosome associations are absolutely known to occur after DNA replication. The possibility exists that many non-zygotic organisms may employ premeiotic chromosome associations as a precursor to intimate pairing during meiosis. Holm and Wang (1988) refute the evidence for premeiotic associations in wheat on the basis that axial elements were not aligned as the chromosomes became visible to light microscopy at leptotene. Again, it can be argued that homologous chromosome association at premeiosis do not necessarily reflect complete alignment and only act to place the chromosomes in favourable domains which then facilitates chromosome pairing at a later stage, presumably proceeding the beginning of leptotene.

Whilst several organisms would appear to display somatic and premeiotic association of chromosomes, the evidence for the involvement of premeiotic interactions in homologous chromosome alignment remains predominantly circumstantial and further studies will be required for any critical decision on the process. In addition, the mechanisms to achieve premeiotic interactions, should they occur directly before meiosis, are still not identified in these models.

1.6.2 Specific interactions of homologous chromosomes at prophase

If homologous chromosomes are not associated prior to meiosis, the association and alignment process must occur at early prophase. The alignment of homologous chromosomes would then either represent the first stage of synaptonemal complex formation such that the synaptonemal complex is responsible for bringing homologues into alignment, or an independent mechanism preceding synaptonemal complex formation must act to allow homologue alignment (Loidl, 1990).

Studies of chromosome alignment and synaptonemal complex formation on a variety of organisms indicates that presynaptic alignment of chromosomes is not dependent on the formation of the synaptonemal complex. In the fungus *Sordaria macrospora*, homologous chromosome alignment has been demonstrated during leptotene, before the formation of the synaptonemal complex (Zickler, 1977). More strikingly, *Schizzosaccharomyces pombe* appears to maintain normal levels of chromosome pairing and recombination in the complete absence of the synaptonemal complex (Kohli and Bahler, 1994) indicating that the SC is not required for either chromosome alignment, pairing or recombination in this organism. The observations of homologous chromosome association in triploid *A. sphaerocephalon*, as described above (Loidl and Jones, 1986), where alignment occurs in the absence of synapsis also indicates that the synaptonemal complex does not participate in presynaptic alignment of homologues.

If the synaptonemal complex does not induce presynaptic alignment, specific interactions which may facilitate homologous alignment at meiosis include the long range interaction of homologous chromosomes, chance contacts between homologous

chromosomes in random motion, or the interaction between chromosomes and specific extra-chromosomal structures such as nuclear proteins and the nuclear membrane.

1.6.3 Long range interaction of chromosomes

The interaction of chromosomes across the nucleus can conceivably be mediated by either long strands of DNA (Smithies and Powers, 1986) or by DNA-protein (Comings and Okada, 1970) interactions. Presumably, the DNA strand or protein filament, attached to one homologue, searches the nucleus for a homologous chromosome and binds specifically to it. Following the preliminary establishment of homology, the interacting chromosomes are brought together either by condensation of the chromosome or contraction of the protein. Smithies and Powers (1986) propose a model for homologous chromosome association based on the observed high frequencies of gene conversion in human foetal globin genes. The details of the model include the formation of single stranded "feelers" which are extruded at multiple sites along DNA molecules probably at sites of double strand break occurrence. The feelers are able to invade any duplex encountered and search for homology by Watson-Crick interactions. Scanning is halted when homology is encountered. Should nearby invasions also encounter homologous segments, a series of stable heteroduplexes form and can act to zip the homologous chromosomes together. If nearby interactions fail to result in homology, as is the case if the original interaction was with a homoeologous chromosome, full pairing would not occur and the relative instability of a low number of interactions would result in the separation of the homoeologues. It is proposed that the high frequency of gene conversion observed would then be a consequence of stable heteroduplexes forming on homoeologous chromosomes giving rise to small gene conversions. This model is consistent with the observation that gene conversion occurs between closely linked genes within a single chromatid, between sister chromatids and between homologous chromosomes (Smithies and Powers, 1986). The role of intact DNA interactions in chromosome pairing is appealing as the condensation which would be required for chromosome movement is an ongoing process of prophase. In addition, the iniation of DSB formation required for the production of single stranded DNA has been demonstrated to occur in the absence of a homologous chromosome, indicating that DSB formation may occur in the absence

of homologue alignment (Gilbertson and Stahl, 1994). If this is the case, DSB formation to produce single stranded feelers may be a method of initiating alignment of homologues. However, it is difficult to consolidate the apparent deficiencies of the model, in particular whether DNA strands actively search for a homologue or if homologous interactions occur at random. Presuming that there must be several distinct sites of homology recognition on each chromosome to ensure accurate alignment of homologues, the effectiveness of attaining homologous interactions between all, or even a majority of pairing sites, must be questioned.

1.6.4 Chance contacts between homologous chromosomes in random motion

Models which propose random contacts as the method of initiating primary homologous associations have little supporting evidence. As described by Maguire (1974), the degree of chromosome movement at meiotic prophase is unlikely to be sufficient to effect the random association of homologous chromosomes. Considering a complex organism such as bread wheat containing 42 chromosomes, it is difficult to envisage that homologous chromosome interactions are initiated by relatively limited chromosome movement at prophase. Furthermore, random movement of chromosomes is unlikely to promote homologous chromosomes associations over homoeologous contacts in allopolyploid species. However, this does not imply that chromosome movement does not occur, indeed some movement of chromosomes could be initiated by internuclear microtubules (Chikashige *et al.*, 1994), although it is more likely that microtubule initiated movement is of a directional rather than random nature.

1.6.5 Interaction of chromosomes with intranuclear structures

The final proposal for the initiation of presynaptic alignment involves a method of restricting the placement of chromosomes to increase the possibility of homologous interactions. This could be achieved if the chromosomes were anchored to either a nuclear structure or to the nuclear membrane. Several authors report the occurrence of nuclear structures in meiotic cells (Bennett and Smith, 1979; Bahler *et al.*, 1993; Sym and Röeder, 1995). In *S. pombe*, it is known that karyogamy is preceded by the fusion of spindle pole bodies (SPBs) (Hirata and Tanaka, 1982)and that the centromeres of chromosomes are clustered near the SPBs in vegetative cells

(Takahashi *et al.*, 1992). Bahler and co workers (1993) propose that centromeres and SPBs are connected throughout the life cycle of *S. pombe* and that the fusion of SPBs before karyogamy serves to bring the centromeres of the two chromosome sets together as a single cluster. Within the cluster, homologous centromeres recognise each other and initiate alignment. Interestingly, Bahler *et al.* (1993) also suggest that the telomeres of *S. pombe* cluster in a different region of the meiotic nucleus resulting in the polarisation of centromere - telomere arrangement as has been described in *Drosophila* (Hiraoka *et al.*, 1990). Following the original recognition events at the centromeres, the formation of the observed axial like elements is initiated at several sites along the chromosomes and facilitates the alignment of homologues from centromere to telomere. The observed elements thus may facilitate alignment either by providing structural support for interacting chromosomes or alternatively, the organisation of chromatin on the elements, may result in the presentation of specialised sequences to the homologous partner which would allow the registration of homology and alignment of the partners.

Whilst Bahler *et al.* (1993) present a reasonable hypothesis for chromosome alignment in *S. pombe*, contradictory evidence comes from Chikashige and co workers (1994) who have recently demonstrated that the telomeres of chromosomes in *S. pombe* initiate movement and are clustered at the SPB. Whilst this evidence refutes the suggestion that centromeres are the sites of initial homology recognition, there is no evidence to suggest that initial homology recognition can not occur at the telomeres. In fact, the clustering of telomeres is reminiscent of the bouquet arrangement of meiotic chromosomes observed in many species (Loidl, 1990). Furthermore, the clustering of telomeres at the SPB and subsequent movement of the SPB may result in the linear alignment of chromosomes and thereby facilitate chromosome pairing and resolution of interlocking chromosomes (Chikashige *et al.*, 1994).

Unusual structures, referred to as poly complexes have also been described in *S. cerevisiae* (Sym and Röeder, 1995) and other organisms (Goldstein, 1987). These structures appear to be composed of SC material but do not seem to associate with the chromosomes (Goldstein, 1987) and are therefore unlikely to have an effect on

chromosome pairing. It is more likely that the polycomplexes represent aggregates of SC core material which has not been incorporated into the SC.

In a wide range of species, telomeres have been shown to be attached to the nuclear membrane during meiotic prophase I. This may promote alignment of homologues in a number of ways. Moens (1968) suggests that the attachment of telomeres to the nuclear membrane serves to restrict chromosome movement to a plane and therefore increases the chance encounters of homologous chromosomes. For this to be true, the attachment would need to be specific such that both homologues reside in the same plane. An extension of this model is that chromosomes only attach to specific sites of the membrane. Homologous chromosomes would attach at the same site which would facilitate their association (see review by Loidl, 1990). There is however, little evidence to support the existence of specific binding sites on the nuclear membrane. Indeed, it is unlikely that telomeres play a direct role in the establishment of homologous associations as chromosomes with terminal deletions, which can be assumed to lack telomeres, appear to undergo regular alignment and synapsis (Maguire, 1984). However, the possibility that sites for alignment reside in the subterminal regions of chromosomes could be a possible explanation for the effective alignment of chromosomes lacking telomeres.

Alternatively, the attachment of telomeres to the nuclear membrane may reflect their interaction with extra nuclear microtubules. Sheldon *et al* (1988) report that microtubules seem to interact with chromosomes through the nuclear membrane and may be responsible for chromosome movement during prophase I. It can be envisaged that homologous chromosomes are either moved into alignment, or into domains which facilitate alignment, by their interaction with microtubules. This is supported by the documented effects of colcemid inhibitors which prevent the polymerisation of tubulin. Several authors describe the effects of colchicine applied during or following premeiotic interphase. In *T. aestivum*, colchicine applied to pollen mother cells during premeiotic interphase induces a loss of homologous pairing and resultant univalency at metaphase I, although colchicine applications after premeiotic interphase appear to have no effect on the association of chromosomes (Driscoll *et al.*, 1967; Dover and Riley, 1973). However, Thomas and Kaltsikes (1977) propose that in *T. aestivum* the bouquet stage between leptotene and zygotene is also colchicine sensitive. In *Lilium*,

microsporocytes exposed to colchicine as late as zygotene show reduced chiasma formation and the presence of univalents (Shepard *et al.*, 1974). Disruption of meiotic pairing and chiasma formation is also reported in *Allium* sp. treated with colchicine (Loidl, 1988). Hence, although the actual time of effectiveness differs between species from premeiotic interphase to mid zygotene, the general effect of applied colchicine appears to be a disturbance of homologous chromosome alignment. It is therefore believed that microtubules function to align homologues, and that colchicine disrupts microtubule assembly and thereby causes defects in chromosome alignment (Loidl, 1990).

Of the proposed methods of homologous chromosome alignment outlined above, the directional movement of chromosomes mediated by extranuclear microtubules would appear to provide the best explanation of presynaptic chromosome alignment. The interaction of chromosomes with microtubules explains the disruption of chromosome distribution by colchicine, and could also result in the clustering of chromosomes at the nuclear membrane as the microtubules contract. The proposed alignment of chromosomes mediated by extranuclear microtubules could occur before meiosis, in those organisms which display premeiotic and somatic association of homologues, or the mechanism may act at prophase I in those organisms where homologous chromosome association is known to occur after the onset of meiosis.

1.6.6 The mechanism of homology recognition

Whilst the models proposed for presynaptic association of homologues differ widely, all of them require a mechanism for the determination of homology between interacting chromosomes. Two types of homology recognition have been identified in a variety of organisms. One is the existence of specific pairing sites which may be required for primary homology recognition during alignment, the other is the precise determination of homology at the DNA level. Depending on which model of chromosome association is accepted, and the organism being studied, one or both of these methods of homology recognition may be employed.

1.6.7 The presence of specific pairing sites

Currently there is growing support for the existence of pairing sites on homologues. This arises from observations in *S. cerevisiae* where premeiotic alignment of chromosomes appears to occur at multiple sites along the chromosome (Weiner and Kleckner, 1994). Observations of chromosome pairing in *Caenorhabditis elegans* have also demonstrated that homologue alignment requires the presence of a specific homologue recognition sequence located near the end of each chromosome as well as other, interstitial recognition sites (McKim *et al.*, 1993).

Late replicating DNA or zygDNA has been proposed to function in the recognition of homology at different sites along homologous chromosomes via the formation of DNA duplexes (Stern and Hotta, 1987). Late replicating DNA has only been definitively observed in *Lilium* (Hotta *et al.*, 1966), although some circumstantial evidence would appear to favour its presence in mouse also (Mukherjee and Cohen, 1968). Whilst the bulk of the genome undergoes DNA replication at S-phase, a small but significant portion of DNA (zygDNA) replicates at zygotene, at least in *Lilium* (Hotta and Stern, 1971). ZygDNA constitutes approximately 0.3% of the genome (Hotta *et al.*, 1966) and is characterised by a high buoyant density (Hotta and Stern, 1971). The length of zygDNA sequences has been estimated at between 3 and 5 kb (Hotta *et al.*, 1984) and they are generally distributed over all of the chromosomes (Ito and Hotta, 1973). These observations, as well the observed disruption of chromosome pairing by inhibition of zygDNA synthesis (Ito *et al.*, 1967b), have contributed to a model where zygDNA replication functions to align homologues. It is postulated that the recognition of homology arises from an assessment of complementarity between single stranded DNA of interacting chromosomes arising from the nicking of zygDNA sequences on each chromosome required for the initiation of their replication (Stern and Hotta, 1987). The evidence to support this conclusion is largely circumstantial and a defined function of zygDNA in chromosome pairing has not been established. However, it has been demonstrated that zygDNA itself, is not sufficient for chromosome pairing as mitotic revertants do not display pairing of chromosomes even when reverting after zygDNA replication (Hotta and Stern, 1971). The role of zygDNA in chromosome alignment and pairing therefore remains contentious, and further advances in this field will inevitably require the isolation of late replicating DNA from other organisms.

1.6.8 The intimate assessment of homology at the DNA level

If it is assumed that the double strand break model of recombination is correct, the mechanism of homology recognition by complimentary base pairing can be explained on the basis of single strand invasion of a homologous duplex as described earlier. If the invading strand encounters a complimentary template, strand invasion continues resulting in the displacement of a D-loop and formation of a crossover giving stable association of homologues. The production of single stranded DNA from DSBs may be regarded as the single stranded "feelers" which search for homology in long range interactions as proposed by Smithies and Powers (1986). Alternatively, DSBs may only occur at regions where homologous contacts have already formed (see Hawley and Arbel, 1993).

A second method for the determination of homology at the DNA level comes from Comings and Riggs (1971). They suggest that genes exist which code for pairing proteins which are capable of binding to unique base sequences. Pairing proteins binding to DNA would undergo allosteric changes in surface configuration that would allow them to bind only to a protein interacting with a homologous segment of DNA. A series of unique DNA sequences along the chromosome, and their interaction with specific pairing proteins would then facilitate homologous chromosome alignment in the nucleus. It is claimed that this method of homology recognition would allow interactions which are as specific and stable as Watson-Crick pairing of complimentary DNA strands. The above model, implies the presence of recognition sites which occur as single copies on haploid chromosome sets. The occurrence of such sequences has not been demonstrated although it is likely that such sequences exist but have not been isolated. The additional advantage of this model is that the allosteric proteins, after binding to the DNA, can be further modified by effector ligands such that the specificity of binding can be controlled.

1.6.9 A model for homologous chromosome alignment and synapsis

Although a large number of hypotheses have been suggested to explain presynaptic alignment of chromosomes and their intimate association following homology recognition, the most complete model of chromosome pairing favoured here is

proposed by Kleckner and Weiner (1993) studying chromosome pairing in *S. cerevisiae*. It is presented that unstable homologous interactions occur at multiple sites in the genome. The occurrence of multiple interstitial interactions has been identified in *S. cerevisiae* both before the onset of meiosis and for several hours during meiosis (Weiner and Kleckner, 1994). It is proposed that the observed premeiotic associations involve the side by side (paranemic), unstable alignment of homologous segments and that the multiple interactions serve to draw homologues chromosomes into "topologically favourable domains". The associations are disrupted during DNA replication but homologous chromosomes remain in domains which favour re-establishment of alignment during meiosis. The resumption of multiple interactions of homologues during meiosis is thought to mirror the probable sites of DSB formation as the number of interactions is similar to the number of meiotic recombination events. However, whether DSBs facilitate the re-formation of interactions or are a consequence of them is not known. Following the alignment of homologues, a more accurate assessment of homology is made as recombination continues. Invasion of one duplex by a single strand from the opposing duplex would allow direct comparison of DNA homology via Watson-Crick interactions and thereby allow the absolute assessment of homology between the duplexes. If the duplexes are truly homologous, synapsis is initiated and crossing over takes place resulting in the stable plectonemic (intertwining) interaction of homologous chromosomes. This model is supported by the observation of premeiotic pairing in *S. cerevisiae* mutants defective for recombination (eg. *rad50*) which display lower efficiencies in pairing because the interactions between homologues are paranemic and therefore less stable than plectonemic interactions resulting in a higher probability of separation of homologues before the onset of recombination.

1.7 Chromosome Pairing in *Triticum aestivum*

1.7.1 The genetic control of chromosome pairing

Triticum aestivum is perhaps the most utilised organism of researchers studying the control of chromosome pairing. *T. aestivum* is allohexaploid, consisting of three genomes A, B and D derived from 14 chromosome diploids. The A genome is believed to be derived from *T. monococcum* (Dvorak, 1976), the origin of the B genome donor is unclear but is likely to be *T. speltoides* or a related species (Riley *et*

al., 1958), and the D genome is assumed to be derived from *T. tauschii* (Dvorak, 1976). By the early 1950's, it was demonstrated that the corresponding chromosomes of the three different genomes were very closely related. Deficiency (nullisomy) of one chromosome could be compensated for by the increased dosage (tetrasomy) of either of the two related chromosomes (Sears, 1952). In addition, hybrids between the proposed ancestral diploids displayed high levels of pairing (Sears, 1941) suggesting that the homoeologous chromosomes of the diploid ancestors are closely related.

Despite the close relationship of the three composite genomes, *T. aestivum* behaves like a typical allopolyploid and forms only bivalents at meiosis. Homoeologous chromosome pairing is excluded, and even in haploids very little homoeologous association is observed (Riley, 1960).

The failure of homoeologous chromosomes to pair in *T. aestivum*, and in hybrids between this species and related species was demonstrated to be due to the suppression of homoeologous pairing by a gene or genes located on the long arm of the chromosome (Okamoto, 1957; Sears and Okamoto, 1958; Riley and Chapman, 1958). The gene on 5BL has been designated the *Ph1* (Pairing homoeologous) gene. Removal of *Ph1* in haploid *T. aestivum* results in a significant increase in pairing of homoeologous chromosomes probably through segmental homology (Riley, 1960) and is observed as the presence of one or more multivalents. Frequently multivalent formation in nullisomic 5B haploids is as trivalents which suggests that multivalency occurs through pairing of homoeologues (Riley, 1960). In addition, in hybrids between *T. aestivum* and *Secale cereale* deficient for each of the 21 wheat chromosomes, only plants nullisomic for 5B showed a serious difference in pairing (Riley, 1960).

It was believed for many years that the full control of chromosome pairing in *T. aestivum* was attained through the action of *Ph1*. Subsequently, several additional suppressors of homoeologous pairing as well as promoters have been identified. A minor suppressor has been identified on the short arm of chromosome 3D (Mello-Sampayo, 1968, 1971; Upadhya and Swaminathan, 1967), termed *Ph2*, as well as a suppressor of smaller magnitude on chromosome 3A (Driscoll, 1972). Mello-Sampayo and Canas (1973) have demonstrated that in crosses of *T. aestivum* with

Aegilops sharonensis and *Secale cereale*, higher pairing of chromosomes is observed if 5B, 3D or 3A is missing. Interestingly, removal of both 5B and 3D resulted in no more chromosome pairing than if only 5B were absent. It is possible that removal of 5B allows the maximum possible pairing. Plants nullisomic for 3DS displayed higher pairing values than those with 3D completely absent indicating the existence of a pairing promoter on 3DL as suggested by Mello-Sampayo and Lorente (1968) and Driscoll (1972). Using a similar procedure, a suppressor of homoeologous pairing was also identified on 3AS (Mello-Sampayo and Canas, 1973). The most interesting results of this study, however, was the demonstration that removal of both 3DS and 3AS from *T. aestivum* in the crosses with *S. cereale* resulted in a level of pairing almost as high as the presumed maximum in nullisomic 5B plants. The effect of the removal of 3DS and 3AS is beyond the sum of the individual effects of these two suppressors (Mello-Sampayo and Canas, 1973). Furthermore, in plants nullisomic for 5B, the 3AS and 3DS chromosome arms will pair and it is likely that the suppressors on these arms occupy syntenic positions. From these observations, Mello - Sampayo and Canas (1973) propose a mechanism of action of the suppressors whereby the absence of one suppressor is partially compensated for by increased activity of the other. A further minor suppressor of homoeologous chromosome pairing has been located on 4D (Driscoll, 1973).

In addition to the suppressors of homoeologous chromosome pairing, several promoters of this process have also been identified. Apart from the promoters on 3AL and 3DL, promoters are also present on the short arm of 5B (Feldman and Mello-Sampayo, 1967: Riley *et al.*, 1966), 5DL (Feldman, 1966, 1968; Riley *et al.*, 1966; Mello-Sampayo, 1972) and 5AL (Feldman, 1966, 1968; Riley *et al.*, 1966). As 5DL, 5AL and 5BL all show homology (Riley and Chapman, 1964), it is likely that all of the regulators on the long arms of homoeologous group five chromosomes arose from a single locus with the 5BL suppressor arising from mutation of an original promoter (Feldman, 1966).

The regulation of chromosome pairing in *T. aestivum* is achieved by the balance of genes acting to promote and suppress homoeologous pairing. It is proposed that *Ph1* performs the regulatory function possibly by activating inactive portions of the other suppressors (Mello-Sampayo and Canas, 1973). This is supported by the observation

of a dosage effect on the expression of the suppressors. Increasing the dose of 5BL to six copies causes maximum expression of suppressors and therefore complete asynapsis as observed by Feldman (1966). Alternatively, removal of 5BL does not allow expression of any suppressors and hence a high degree of homoeologous pairing is achieved as shown by Riley (1960).

1.7.2 The mechanism of *Ph* action

Considering that chromosome pairing requires the alignment of homologous chromosomes followed by their synapsis following an intimate assessment of homology, it is apparent that the *Ph* genes may act to facilitate homologous alignment of chromosomes over homoeologous alignments or they could have an effect on the stringency of homology required for synapsis. There is also the possibility that the *Ph* genes act both on alignment and synapsis to ensure homologous chromosome pairing.

Several models for the action of *Ph* genes have been proposed. The earliest model depicted an effect on the ratio of histones to DNA. Studying *Loxa flavicolis,* Ansley (1958) noticed that the ratio of histone to DNA was lower in meiotic cells destined to undergo synapsis as compared to those due to undergo asynapsis. However, cytophotometric evaluations of the ratio of histones to DNA in *T. aestivum* with and without 5B demonstrates no significant differences in the histone:DNA ratio (Riley, 1968).

Riley (1960) observed that the frequency and distribution of chiasmata in euploid and nullisomic 5B wheat were similar and that, because the chromosomes in euploid wheat appear to be fully paired throughout their lengths, there were few regions in which pairing was unsatisfied and a genetic alteration could have an effect. Based on these observations he proposed that the *Ph1* gene acts to reduce the long range forces which were thought to be required for the attraction of chromosomes at prophase. As the force of attraction between homoeologues could be assumed to be weaker than between homologues, two doses of *Ph1* would be sufficient to prevent homoeologous attraction but still allow homologous chromosomes to pair. Questions have been raised by Sears (1976) as to how the model explains some of the basic observations of chromosome pairing. How does it explain the prohibition of pairing

between homoeologues which by chance lie in close proximity whilst their homologues are distantly placed in the nucleus? As chromosome pairing is assumed to initiate at the ends, how do chromosomes approaching one another from opposite sides of the nucleus avoid interlocking, as no interlocking bivalents are observed? The answer to the second question may lie in the proposals of a mechanism to correct interlockings at zygotene (Rasmussen, 1986; Rasmussen and Holm, 1980).

A second model for the control of chromosome pairing was proposed by Riley (1968). Riley reasoned that the attraction phase may be variable in time being terminated by the initiation of synapsis as an independent event. The action of *Ph1* would then be to shorten the time during which attraction takes place. Consequently, the removal of *Ph1* would result in a long attraction phase and allow the association of homoeologous as well as homologous chromosomes. In contrast, high doses of *Ph1* would shorten the attraction phase to such an extent that even homologues fail to associate. Riley also suggested that this model explains the action of other suppressors and promoters, with suppressors shortening the period of attraction and promoters extending it. This model was rejected when the determination of the duration of meiosis in wheat with and without *Ph1* showed that it did not have a significant effect (Bennett *et al.*, 1974).

Feldman (1966) demonstrated that in wheat plants tri-isosomic for 5BL, homologous chromosome pairing was reduced but in addition, homoeologous pairing was induced and a high frequency of interlocking bivalents was observed. Riley's theory of long range attraction of chromosomes (Riley, 1960) can not account for the observed increase in homoeologous pairing and so a theory of spatial separation of the wheat genomes was proposed (Feldman, 1966). Briefly, the model proposes that the three genomes of wheat are not randomly distributed, even in somatic cells, but occupy separate domains in the nucleus such that homologous chromosomes are more closely associated than either homoeologues or unrelated chromosomes of differing genomes. Meiotic pairing, which follows as a second step then brings the associated chromosomes into a more intimate contact. Because of their close proximity, homologues pair at meiosis more readily than homoeologues which are more distantly separated. Following this model, the observations of chromosome pairing in wheat plants carrying different doses of 5BL can be explained in the following manner: in

plants nullisomic for 5BL, separation of the genomes breaks down, the chromosomes become more randomly distributed in the nucleus such that homoeologues as well as homologues lie in close proximity and homoelogous as well as homologous pairing occurs. With two doses of 5BL, as in euploid wheat, the effect of the *Ph1* gene is not strong enough to prevent homologous association but is sufficient to keep the homoeologues apart resulting in the exclusive formation of bivalents at meiosis. Six doses of *Ph1* is assumed to suppress premeiotic associations completely and again gives rise to the random distribution of chromosomes within the nucleus and wide separation of homologous chromosomes. The forces required for meiotic pairing of closely associated chromosomes are not capable of bringing widely separated homologues together and as a result many chromosomes fail to undergo pairing. The observed increase in homoeologous chromosome pairing is presumed to occur between homoeologues which by chance lie in close proximity to each other. Finally, Feldman (1966) suggests that the occurrence of interlocking bivalents, in spite of a reduction in the total number of bivalents, clearly demonstrates that a proportion of the pairing occurs between somewhat separated chromosomes before the onset of meiosis.

In support of this model, Feldman and Avivi (1973) demonstrate the close association of homologous chromosomes in root tip cells. This not only suggests the alignment of chromosomes prior to meiosis but also in somatic cells. Furthermore, it was observed that unrelated chromosomes of the same genome were more closely associated than related chromosomes from other genomes leading to the proposal that the three genomes did not intermix but tended to occupy different domains in the nucleus. Other researchers have also demonstrated premeiotic or somatic association of homologous chromosomes in a range of organisms including yeast (Loidl *et al.*, 1994a; Weiner and Kleckner, 1994), *Drosophila* (Hiraoka *et al.*, 1993) and maize (Maguire, 1983). In wheat, the behaviour of two telocentric chromosomes, either homologous or non homologous, in a disomic *Ph* plant were studied. In root tip cells, the non-homologous telocentric chromosomes were found to be distributed randomly with respect to each other whilst the two homologous telocentric chromosomes were closely associated (Feldman, 1966). Furthermore, in plants tri isosomic 5BL plants the *Ph1* gene suppresses the interchromosomal pairing of the three isochromosomes to the same extent as normal chromosomes, but fails to reduce intra chromosomal

pairing (Feldman and Avivi, 1988). Under these circumstances extra doses of *Ph1*, whilst capable of retarding interchromosomal pairing, has no effect on intra-chromosomal pairing of two arms held by the same centromere because it can not modify the alignment of the arms. Hence the action of *Ph1* is proposed to be on the alignment of chromosomes at premeiosis (Feldman and Avivi, 1988).

Several authors dispute the validity of non random chromosome placement in non-meiotic cells. The wheat line Compair, in which the 2D chromosome is recombined with the homoeologous chromosome 2M of *Aegilops comosa* was produced by Riley (1968). In crosses of Compair with Chinese Spring euploid wheat, the recombinant chromosome 2M/2D has both homologous and homoeologous pairing partners available. Riley observed that only completely homologous segments synapsed (based on the formation of chiasmata) and took this as evidence that premeiotic association does not occur. It would be expected that the homoeologous segments should synapse since their relative positions would be determined by the homologous segments to which they are linked. It should be noted that Feldman (1966) proposed a model whereby premeiotic associations were stabilised by a more intimate relationship of chromosomes at meiosis. It is possible that the *Ph* gene acts not only to regulate premeiotic associations but also affects the stringency of homology required for intimate synapsis as a prerequisite for chiasma formation.

Other researchers have been unable to confirm that homologous chromosomes are more closely associated then homoeologues in root tip cells of wheat (Darvey and Driscoll, 1971; Darvey *et al.*, 1973). However, as indicated by Sears (1976), it is of little relevance whether chromosomes associate in somatic cells. More important to the spatial theory of chromosome pairing is the degree of chromosome association in the final mitosis of premeiotic cells. The diffuse nature of chromosomes at premeiotic interphase as well as at the early stages of prophase at the beginning of meiosis, have so far made it extremely difficult to determine the extent of chromosomal associations at premeiotic interphase in wheat. With the advent of fluorescent *in situ* hybridisation, some observations on premeiotic alignment of chromosomes have been made, and preliminary results from *S. cerevisiae* would suggest that chromosomes do in fact align prior to meiosis (Loidl *et al.*, 1994; Weiner and Kleckner, 1994).

1.7.3 The biochemical mode of *Ph* action

The mode of action of the *Ph* genes is difficult due difficulties encountered with microscopical studies of chromosome at premeiotic interphase and leptotene. Nevertheless, it has been possible for a general consensus on the biochemical action of *Ph* to be made. Treatment of wheat meiocytes with antimicrotubulin drugs such as colchicine and vinblastine, has been demonstrated to cause partial asynapsis of homologues and interlocking of bivalents as well as a slight but significant increase in the frequency of homoeologous associations (Driscoll *et al.*, 1967; Dover and Riley, 1973). The periods of development which are susceptible to colchicine exposure differ between species. In wheat, the stages of premeiotic interphase and leptotene are susceptible (Avivi *et al.*, 1970; Avivi and Feldman, 1973; Dover and Riley, 1973; Driscoll *et al.*, 1967; Thomas and Kaltsikes, 1977). Cells treated before the final premeiotic mitosis resulted in the formation of 84 chromosome meiocytes which display regular pairing of chromosomes into bivalents. However, colchicine applied during premeiotic interphase appears to inhibit association of homologues but does not inhibit synapsis or chiasma formation (Driscoll *et al.*, 1967). Dover and Riley (1973) confirm these observations, adding that homoeologous pairing appears to be more susceptible to the action of colchicine than pairing between homologues. The results of these experiments support the view that premeiotic association of homologous chromosomes is required for the accurate pairing at prophase, and that the premeiotic associations are disturbed by the application of colchicine. The fact that colchicine does not effect synapsis at zygotene, suggests that once the homologous chromosomes have associated to some degree, microtubules are no longer required (Sears, 1976).

The effect of colchicine on homologous pairing was observed to parallel the effects of the *Ph1* gene in wheat. A series of experiments involving the interaction of different doses of *Ph1* with varying concentrations of colchicine demonstrate that increasing the dose of *Ph1* in wheat plants from 0 to 2 to 4 doses, progressively increases the resistance of the microtubules in these plants to the effects of colchicine (Avivi *et al.*, 1970; Avivi and Feldman, 1973; Ceolini *et al.*, 1984). *Ph1* must effect more than one site of the microtubules to enhance their resistance to colchicine, as plants with extra doses of *Ph1* also display resistance to vinblastine, an antimicrotubule drug which binds at different sites to colchicine (Borisy and Taylor, 1967; Weisenberg *et al.*,

1968; Wilson, 1970). Similarly, *Ph1* can not be influencing the uptake of colchicine into the plant (Ceolini *et al.*, 1984).

Ph1 therefore, interacts with the microtubules to make the pairing process less susceptible to the action of antitubulin drugs. Two early suggestions were that *Ph1* either effected the rate of tubulin polymerisation or altered the primary structure or allosteric conformation of microtubule subunits to render them more resistant to colchicine action (Avivi *et al.*, 1970). The discovery that O-isopropyl N-phenylcarbamate, which effects spindle organisation rather than microtubule formation, does not effect plants with different doses of *Ph1* in a differential manner (Gualandi *et al.*, 1984) indicates that *Ph1* acts on the dynamic process of microtubule assembly and disassembly. Currently, it is proposed that *Ph1* complexes with microtubules to stabilise them and reduce the binding of antimicrotubulin drugs (Gualandi *et al.*, 1984; Feldman, 1993). The actual method of how this is achieved remains unknown, although the effect of *Ph1* on the spindle to modify sensitivity to a number of different antimicrotubulin drugs which bind to different sites might indicate that *Ph1* causes some kind of significant change in the conformation of the microtubules.

A further complicating factor in elucidating the biochemical action of the *Ph* genes comes from the observation that the *Ph2* gene, whilst a suppressor like *Ph1*, interacts with colchicine in a manner almost opposite to that of *Ph1* (Ceolini and Feldman, 1987). It is difficult to believe that both genes act on the spindle with *Ph1* having a stabilising effects whilst *Ph2* has a destabilising effect and yet both cause changes in the microtublue structure which prevent homoeologous pairing. It is possible that the *Ph* genes, instead of acting directly with the microtubules, exert their influence on microtubule associated proteins. This aspect is currently being investigated (Feldman 1993).

1.7.4 *Ph* mutants

Cytological observation of wheat plants carrying various doses of both pairing suppressors and promoters, has contributed greatly to the understanding of the genetic control of homologous chromosome pairing. However, cytological

observations have been unable to provide sufficient information to allow an accurate assessment of both the mechanism and biochemical mode of action of the genes regulating chromosome pairing in wheat. In order to test the many models of chromosome pairing proposed, it has become necessary to isolate genes, their corresponding proteins, and DNA species which may influence the pairing process. Only when the actual *Ph* genes are isolated will we be able to fully understand the genetic regulation of chromosome pairing in wheat.

The identification of genes involved in the regulation of chromosome pairing in wheat is aided by the production of a number of mutations in the *Ph1* and *Ph2* genes. Sears (1977) induced a small interstitial deletion in 5BL by X-ray irradiation. Testing of this mutation has demonstrated that it includes the *Ph1* gene, the phenotype of the mutant being the same as nullisomic 5B and ditelosomic 5BS plants. The mutation has consequently been termed *ph1b*. A second X-ray deletion mutation produced by Sears (1977) has been shown to be a large terminal deletion of part of the 3DS arm. This deletion encompasses the *Ph2* gene and is termed ph2a. A point mutation of *Ph2*, *ph2b*, induced by EMS treatment (Wall *et al.*, 1971) is also available. Both *ph2a* and *ph2b* induce the same amount of homoeologous pairing in wheat hybrids but do not effect chromosome pairing in wheat itself (Sears, 1977).

The deletion mutations produced are of particular value as they allow the rapid screening of isolated genes to determine if they are located in the region of the *Ph* genes. In addition to this, the fast growing area of cereal transformation will soon allow the analysis of genes in the region of the *Ph* loci to determine if they are capable of compensating for the corresponding mutation. By employing molecular techniques, and incorporating the analysis of the *ph* mutants, it may be possible to begin to answer the question of how homologous chromosome pairing is achieved

1.8 Research aims

This aim of this research was to isolate genes expressed at premeiotic interphase and early meiotic prophase I of meiosis in bread wheat and to determine possible relations between the isolated genes and the processes of homologous chromosome pairing, recombination and synaptonemal complex formation. An assessment of the presence

of late replicating DNA in bread wheat was also undertaken to determine if this DNA structure has a function in the process of homology recognition during chromosome pairing.

Chapter 2

Materials and Methods

2.1 Plants and Growth Conditions

Triticum aestivum cultivar Chinese Spring was grown for the collection of meiotic material. Plants were grown in an inverted day/night growth room at 20°C. The growth room was programmed for 14 hours of light (2.00pm-4.00am) and 10 hours darkness (4.00am-2.00pm).

Plants for DNA extraction were grown in a glasshouse at 20°C under natural light conditions.

Barley addition lines, nullisomic-tetrasomic lines and ditelosomic lines were supplied by Dr. Ken Shepherd, Department of Plant Science. The barley double haploid population Clipper x Sahara was supplied by Dr. Rafiq Islam, Department of Plant Science.

2.2 Collection and Staging of Meiocytes

Whole spikes were collected from wheat plants grown in the inverted day/night growth room. Spikes were collected at 4.00pm each day to ensure consistency of the material. A single spikelet from the middle of the spike was fixed in freshly prepared, cold acetic acid:ethanol (1:3, v/v) overnight at 4°C. Two spikelets from above, and two spikelets from below the excised spikelet were placed in an eppendorf tube, snap-frozen in liquid nitrogen and stored at -80°C. The anthers from the primary and secondary florets of the fixed spikelet were excised and crushed with a needle in a drop of aceto-carmine stain (Appendix 1) on a microscope slide. The status of the meiocytes was made on the basis of the appearance of the chromosomes when viewed under a Zeiss light microscope.

2.3 Isolation of Nucleic Acids from Plant Material

2.3.1 Extraction of DNA from plant tissue

The procedures for the isolation of DNA from cereals are as described by Langridge *et al.* (1995b), and are briefly outlined below.

2.3.1.1 Miniprep isolation of DNA for small scale applications

A 10cm long piece of leaf material was placed in a 1.5ml eppendorf tube, frozen in liquid nitrogen and ground to a fine powder with a knitting needle. DNA extraction buffer (600µl) (Appendix 1) was added and the leaf material homogenised. The solution was extracted with phenol:chloroform:iso-amyl alcohol (25:24:1) and precipitated by the addition of 3M sodium acetate (pH4.8) and ice cold ethanol as described below. The pellet was resuspended in R40 (50µl) overnight at 4°C and stored at -20°C.

2.3.1.2 Midiprep extraction of DNA

For procedures requiring a greater amount of DNA, extraction was performed by the midiprep procedure as developed by Langridge *et al.* (1995b).

Two grams of leaf tissue was frozen in liquid nitrogen then ground to a fine powder with a mortar and pestle. The powder was allowed to begin to thaw, DNA extraction buffer (4ml) (Appendix 1) was added, the mixture was homogenised for 5 minutes then transferred to a 10ml centrifuge tube. The mixture was extracted with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) by mixing on a rotary mixer for 15 minutes, and the phases separated by centrifugation at 10,000 rpm in a Beckman 121 centrifuge. The supernatant was transferred to a silica matrix tube (Becton Dickison) and extracted with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) for 10 minutes. The tubes were centrifuged at 10,000 rpm for 10 minutes and the supernatant poured into a clean 10ml tube. DNA was precipitated by the addition of 3M sodium acetate (pH4.8) and cold ethanol. The pellet was air-dried then resuspended in R40 (350µl) (Appendix 1) overnight at 4°C and stored at -20°C.

2.3.2 RNA extraction from plant material

For RNA extractions, all glassware and grinding materials were baked at 160°C for 8 hours. Solutions were made using di-ethyl-pyro-carbonate (DEPC) treated water to remove RNase activity. Treated water was produced by the addition of DEPC to 0.1%, incubation at 37°C for 12 hours then autoclaving twice to remove the DEPC. Plasticware was treated by soaking in 0.4N KOH for 10 minutes then rinsing well with DEPC treated water.

Plant tissue (no more than 3 grams) was placed in a pre cooled mortar, frozen in liquid nitrogen, then ground to a fine powder. REB buffer (4ml) (Appendix 1) was added and the material was homogenised. The slurry was transferred to a 15ml corex tube and the mortar rinsed with an additional 2ml of REB buffer. Plant debris was pelleted by centrifuging at 5,000rpm for 15 minutes at 4°C in a Beckman 121 centrifuge using a precooled JA20 rotor. The supernatant (5ml) was transferred to a clean 15ml corex tube and baked CsCl (5g) was added and dissolved on ice. 3ml of CsCl cushion solution (9.65g baked CsCl in 10ml TE buffer pH7.0) was pipetted into the bottom of a treated 10ml ultracentrifuge tube (Nalgene). The supernatant/CsCl mix was carefully layed on top of the cushion and the tube sealed. Tubes were centrifuged at 38,000 rpm for 18 hours at 4°C in a 65Ti Beckman rotor. Following centrifugation, the supernatant was carefully poured off and the inside of the tube cleaned with cotton buds. The RNA pellet was resuspended on ice in REB (400µl) then transferred to an eppendorf tube. The RNA solution was extracted with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1), then with an equal volume of chloroform. RNA was precipitated overnight at -20°C by adding 3M sodium acetate (pH4.8) and ice cold ethanol. RNA was resuspended in nanopure water (30µl) and stored at -80°C.

2.4 Purification and Precipitation of Nucleic Acids

2.4.1 Phenol:chloroform extraction of nucleic acids

Organic contaminants were removed from nucleic acid preparations by extraction with phenol:chloroform. An equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) (Appendix1) was added to the nucleic acid solution and mixed for 5 minutes by inversion of the tubes by hand or placing the tubes on an orbital mixer. The organic and aqueous phases were separated by centrifugation at 12, 000 rpm for 5 minutes in an Eppendorf benchtop centrifuge. The aqueous phase was transferred to a clean tube and the phenol:chloroform extraction repeated. The aqueous phase was extracted with an equal volume of chloroform,

the phases were separated by centrifugation and the nucleic acid solution transferred to a clean tube to be precipitated.

2.4.2 Precipitation of nucleic acids

Nucleic acids were precipitated by the addition of either 0.1 volumes of 3M sodium acetate (pH 4.8) or 1 volume of 7.5M ammonium acetate (pH 4.8) and 2.5 volumes of cold ethanol. The DNA or RNA was allowed to precipitate at -20°C overnight or for 1 hour at -80°C, then pelleted by centrifugation for 15 minutes at 12,000rpm in an eppendorf benchtop centrifuge. The pelleted nucleic acid was washed twice with cold 70% ethanol (1ml), dried under vacuum, then resuspended in nanopure water or R40 (Appendix 1).

2.5 *E. coli* recombinant techniques

2.5.1 Ligation of DNA into plasmids

Ligation of restriction digested DNA into the appropriate site of pBluescript KS$^-$ (Integrated Sciences) was performed as described in Maniatis *et al.* (1982). De-phosphorylation of the vector was also carried out according to Maniatis *et al.* (1982).

2.5.2 Transformation of *E. coli*

2.5.2.1 Preparation of competent cells for transformation

A single colony of *E. coli* DH5α was inoculated into 5ml SOB (Appendix 1) and grown overnight at 37°C with rapid shaking. The starter culture (500µl) was used to inoculate 25ml SOB supplemented with 20mM $MgSO_4$ and the cells grown to OD_{600} 0.6 at 37°C with shaking. The culture was incubated on ice for 10 minutes and the cells pelleted by centrifugation at 2,500 rpm for 12 minutes at 4°C (JA20 rotor in Beckman 121 centrifuge). The supernatant was removed and the cells resuspended in TFB (8.5ml) (Appendix 1) and incubated on ice for 10 minutes. Cells were pelleted as above and resuspended in TFB (2ml). DMSO (70µl) was added and the cells incubated on ice. After 5 minutes, 1M dithiothreitol (157µl) was added and mixed, and after a further 10 minutes DMSO (75µl) was added and mixed.

2.5.2.2 Transformation of *E. coli*

Competent cells (200µl) were gently dispensed into a glass test tube and kept on ice. Ligation mix (5µl) was added and gently mixed. The cells were incubated on ice for 30 minutes, heat shocked in a 42°C water bath for 2 minutes, then returned to ice for 5 minutes. SOC (800µl) (Appendix 1) was added and the cells incubated at 37°C for 45 minutes. Transformed cells (200µl) were spread on LB plates (Appendix 1) containing 50µg/ml ampicillin, 10mM isopropyl beta-thiogalactopyranoside (IPTG) and 0.4% bromo-(5)-4-chloro-3-indolyl-B-D-galactopyranoside (X-gal). Plates were incubated overnight at 37°C and recombinant colonies identified by their white colour.

2.5.3 Isolation of plasmids from *E. coli*

2.5.3.1 Small scale isolation of plasmids

The protocol for small scale isolation of plasmid DNA was essentially the same as the alkaline lysis method as described (Birnboim and Doly, 1979; Maniatis *et al.*, 1982) although some modifications were incorporated.

Briefly, a single colony of *E. coli* strain DH5α containing the plasmid of interest was inoculated into Terrific Broth (4ml) (Appendix 1) containing ampicillin (50µg/ml). The culture was grown overnight at 37°C with rapid shaking. Cells were pelleted in a 2ml eppendorf tube by centrifugation of 2 ml of the culture, removing the supernatant, then pelleting the remaining culture in the same tube. Cells were resuspended in plasmid 1 solution (200µl) (Appendix 1) and incubated on ice for 10 minutes. Plasmid 2 solution (300µl) (Appendix 1) was added, mixed gently by inversion than incubated on ice for 5 minutes. Potassium acetate (3M, pH 4.8) (200µl) was added, mixed, and the solution incubated on ice for 15 minutes. The tubes were centrifuged at 12,000 rpm for 10 minutes and the supernatant transferred to a clean tube. DNA was precipitated by the addition of 2.5 volumes of ice cold ethanol followed by incubation at -20°C overnight. The DNA was pelleted by centrifugation at 12,000 rpm for 15 minutes, dried briefly under vacuum then resuspended in nanopure water (500µl). RNase A was added to a final concentration of 100µg/ml and the solution incubated for 2 hours at 37°C, extracted with an equal volume of phenol:chloroform:iso-amyl alcohol (24:24:1) and then with an equal volume of chloroform. The supernatant was transferred to a fresh tube and the DNA precipitated with 7.5M

ammonium acetate (pH 4.8) and ethanol. The DNA was dissolved in nanopure water (40µl) and was of sufficient purity for all molecular techniques including sequencing.

2.5.3.2 Large scale isolation of plasmids

The method adopted for the large scale isolation of intact plasmids is described by Skingle *et al.* (1990).

2.6 Bacteriophage recombinant techniques

2.6.1 Preparation of *E. coli* host strains for infection with phage

For infection with λgt10, the *E. coli* host strain C600*Hfl*⁻ was used, whilst for λDashII the host strain was XL1-Blue. Glycerol stocks of the strains were plated onto LB agar, with the addition of tetracycline (15µg/ml) for C600*Hfl*⁻ and grown in an inverted position overnight at 37°C. Single colonies were selected, inoculated in LB (5ml) (Appendix 1) and grown overnight at 37°C with shaking. Overnight culture (100µl) was used to inoculate 20ml LB (Appendix 1) supplemented with 0.4% maltose and 10mM $MgSO_4$. The cultures were grown to OD_{600}0.5 then used for infection.

2.6.2 Preparation of DNA from lambda phage

Lambda DNA was prepared by a modification of the Amersham protocol outlined in the λgt10 cloning manual. The method utilises DEAE-cellulose to absorb bacterial DNA, RNA and protein from the lysate to produce lambda DNA of high purity.

Plaques grown on LB agar were cored out using a sterile pasteur pipette and placed in 100µl SM buffer (Appendix 1). Phage were allowed to diffuse out of the agar core overnight at 4°C. Phage solutuion (50µl) was added to 500µl of the appropriate, prepared *E. coli* strain in a large glass test tube. The phage was allowed to adsorb to the bacterial cells at 37°C for 1 hour then a further 5ml of LB (Appendix 1), supplemented with 5mM $CaCl_2$, was added. The culture was incubated, with shaking, at 37°C for approximately 5 to 7 hours or until the culture lysed as visualised by a clearing of the media. The lysate was transferred to a 10ml plastic centrifuge tube and the cellular debris pelleted by centrifugation at 3,000 rpm for 10 minutes. The supernatant was transferred to a clean 10ml centrifuge tube, DNAse and RNAse A added to final concentrations of 1µg/ml, then incubated at 37°C

for 1 hour. Phage precipitation buffer (5ml) (Appendix 1) was added and the solution incubated on ice at 4°C overnight. Phage particles were pelleted by centrifugation at 9,000 rpm for 20 minutes in a JA21 rotor at 4°C. The supernatant was removed, and the tubes allowed to drain in an inverted position for 15 minutes. The pelleted phage was resuspended in LB (700µl) and transferred to a 2ml eppendorf tube. DE52 solution (700µl) (Whatman DEAE cellulose in LB) was added and mixed by gentle inversion of the tube. DEAE cellulose was pelleted by centrifuging at 12,000 rpm for 5 minutes in a benchtop centrifuge. The supernatant was transferred to a clean tube, 13µl Proteinase K (0.1mg/ml) and 10%SDS (32µl) were added and the solution incubated at room temperature for 10 minutes. Potassium acetate, (3M, pH4.8) (130µl) was added to the solution, mixed by inversion, and the solution incubated at 88°C for 20 minutes then on ice for a further 10 minutes. The denatured protein was pelleted by centrifugation at 12,000rpm for 15 minutes in a benchtop centrifuge and the supernatant transferred to a clean 1.5ml eppendorf tube. The supernatant was extracted twice with an equal volume of phenol:chloroform:iso-amyl alcohol (24:24:1) and once with an equal volume of chloroform. DNA was precipitated by the addition ethanol and resuspended in nanopure water (30µl).

2.7 Preparation of a cDNA Library

2.7.1 Isolation of polyA+ RNA from total RNA samples

PolyA$^+$ RNA was purified from total RNA using the Promega PolyA Tract mRNA Isolation System. PolyA$^+$ RNA was isolated according to the manufacturers recommendations.

2.7.2 Preparation of the cDNA library

2.7.2.1 Production of double stranded cDNA and ligation into λgt10

Double stranded cDNA with linked *Eco*R1/*Not*1 adaptors was prepared from purified polyA$^+$ RNA (3µg) using the Pharmacia Timesaver cDNA Synthesis Kit. The manufacturers protocol was followed precisely with Oligo dT primers being utilised for the synthesis. Linkered cDNA was ligated into λgt10 EcoR1 arms (Promega) following the Timesaver (Pharmacia) protocol. The vector and insert for each ligation, were co-precipitated by addition of sodium acetate (pH4.8) and ethanol and incubation at -70°C for 1 hour. The DNA was resuspended in 8µl of 1X Ligation Buffer (Appendix 1) and 1mM

ATP (1µl) was added. The ligation was started by the addition of T4 DNA ligase (2 units) and incubated overnight at 16°C.

2.7.2.2 Packaging and transfection of the phage library

The cDNA/λgt10 ligation mix was packaged using the Promega packaging mix and following the recommendations of the manufacturer. Aliquots (100µl) of a serial dilution of the packaged phage were added to prepared *E. coli* C600*Hfl*$^-$ cells and the phage allowed to adsorb to the cells at 37°C for 20 minutes. Warm, molten, supplemented top agarose (4ml) (Appendix 1) was added and mixed briefly, then overlayed onto a 9cm LB agar plate (Appendix 1). Plates were incubated in an inverted position at 37°C until plaques appeared (8-12 hours). After determining the highest yielding ligation mixture, the remaining phage were plated onto large (12cm) LB agar plates as described above using 12ml of Top agarose. Following screening, the plates were washed with SM Buffer (12ml) (Appendix 1) with gentle agitation overnight at 4°C. The buffer was removed with a pipette and the plates washed with an additional 4ml of SM Buffer (Appendix 1). The washes were pooled (approximately 200ml) and chloroform (5ml) added. The was transferred to 30ml Corex tubes and centrifuged for 10 minutes at 2,500 rpm at 4°C in a JA20 rotor to remove the cellular debris. DMSO (7% v/v) was added and the library stored in 10ml aliquots at -80°C.

2.8 Southern Transfer and DNA Hybridisation

2.8.1 DNA gel electrophoresis and transfer to membranes

DNA fragments were separated by gel electrophoresis on TAE/agarose gels in TAE buffer (Appendix 1) using a horizontal slab system. Separated DNA was stained for 20 minutes in ethidium bromide solution (1µg/ml) to allow visualisation and photography under short wave UV light.

DNA fragments separated by TAE/agarose gel electrophoresis were transferred to Hybond N^+ membranes (Amersham) using the capillary blotting method (Southern, 1975).

2.8.2 Transfer of phage plaques to membranes

Round, 13cm diameter Hybond N^+ membranes were purchased from Amersham. Phage were transferred to membranes by placing the membranes on the plate for thirty seconds.

Membranes were immediately placed, phage side up, on 3MM Whatman filter paper soaked in denaturing solution (Appendix 1) for 5 minutes. Membranes were transferred to 3MM Whatman filter paper soaked with neutralising solution (Appendix 1) for five minutes then washed in 2xSSC (200ml) for 5 minutes. Membranes were blotted dry and the DNA fixed by UV cross-linking for 5 minutes.

2.8.3 Prehybridisation of membranes

Membranes were placed in a Hybaid hybridisation bottle and prehybridised in 10ml of hybridisation solution (Appendix 1) at 65°C. Prehybridisation proceeded overnight with constant rotation in a Hybaid hybridisation oven.

2.8.4 Hybridisation of membranes

2.8.4.1 Preparation of radio-labelled probe

Probes for hybridisation were prepared by either random primed or M13 primed radiolabelling of double stranded DNA with α-^{32}P-dCTP. DNA template (50-100ng) was mixed with 3µl of the appropriate primer (0.1µg/µl), boiled in a water bath for 5 minutes then chilled on ice for 5 minutes. 2X Oligo-labelling Buffer (12.5µl) (Appendix 1) was added to the tube with 3µl α-^{32}P-dCTP (10µCi/µl). The reaction was started by the addition of the Klenow fragment of *E. coli* DNA polymerase I (1 unit) and continued at 37°C for 45-60 minutes. Following incubation, unincorporated α-^{32}P-dCTP was removed by size exclusion chromatography. A sterilised pasteur pipette was filled with Sephadex G-100 in TE buffer and washed with TE buffer (2ml). The labelling reaction was loaded onto the top of the column, eluted with TE buffer and the radioactivity of the eluted buffer monitored constantly with a hand-held Geiger-Mueller counter. Two peaks of radioactivity are observed, the first represents α-^{32}P-dCTP incorporated into the probe and was collected and stored. The second peak represents unincorporated α-^{32}P-dCTP and was discarded.

2.8.4.2 Hybridisation conditions

Salmon sperm carrier DNA (200µl) (5mg/ml) was added to purified probes and the probe denatured by boiling for 5 minutes then chilling on ice for 5 minutes. The hybridisation solution used for prehybridisation of the membranes was replaced with fresh hybridisation

solution (Appendix 1) containing the denatured probe. Hybridisation continued at 65°C overnight in a Hybaid oven with constant rotation.

2.8.5 Washing hybridisation membranes

Following hybridisation, membranes were first washed in the Hybaid bottles at 65°C with 100ml of wash buffer 1 (Appendix 1) for 20 minutes. Membranes were removed from the bottles and placed in a plastic box. Further washings were carried out for 20 minutes per wash in the box at 65°C with constant agitation. Membranes were washed through a series of wash buffers (Buffers 1-4) of decreasing SSC concentration (Appendix 1) until the signal to background noise ratio was deemed to be adequate. After the final wash, membranes were blotted dry on paper towel, sealed in plastic and exposed to X-ray film at -80°C between intensifying screens.

2.9 Northern Transfer and DNA/RNA Hybridisation

2.9.1 Electrophoresis of RNA

2.9.1.1 Gel preparation

RNA was separated by denaturing gel electrophoresis. RNAse free agarose (0.6g) was dissolved in DEPC treated water (45ml) in a 100ml conical flask by boiling in a microwave oven. The solution was cooled to 55°C, 5ml of 10X MOPS Buffer (Appendix 1) and 37% formaldehyde (1.5ml) was added and the solution mixed by gently turning the flask to avoid air bubbles. Gels were poured in treated trays and allowed to set in a fume hood. Gels were pre-run in 1X MOPS Buffer at 40mA for 30 minutes.

2.9.1.2 Sample preparation

RNA (3.5µl) was mixed with 10X MOPS (2µl) (Appendix 1), deionised formamide (10µl) and 37% formaldehyde (3.5µl). The solution was heated at 65°C for 10 minutes then cooled on ice for 5 minutes and 1µl of RNA gel loading buffer (Appendix 1) was added. Samples were loaded onto a pre-run gel and electrophoresed for 30 minutes at 40mA then at 60mA. Gels were stained with ethidium bromide and the RNA viewed under ultraviolet light.

2.9.2 Northern Transfer and hybridisation

2.9.2.1 Transfer of RNA to membranes

Following staining and photography, gels were soaked in 1X HETS Buffer (Bresatec) for 15 minutes. Hybond N^+ membranes (Amersham) were cut to the size of the gel then soaked in warm water for 5 minutes. Transfer of RNA was accomplished by capillary blotting with 1X HETS Buffer. Following the transfer, membranes were washed for 5 minutes in 5X SSC then blotted dry on 3MM Whatman paper. RNA was fixed to the membranes by UV crosslinking for 5 minutes.

2.9.2.2 Prehybridisation conditions

Membranes were placed in Hybaid bottles containing 20ml of Northern prehybridisation solution (Appendix 1). Membranes were prehybridised for 48 hours at 42°C in a Hybaid oven with constant rotation.

2.9.2.3 Hybridisation conditions

Following prehybridisation, the solution was replaced with hybridisation solution (10ml) containing the prepared, denatured DNA probe. Membranes were hybridised at 42°C for 48 hours with constant rotation. Washing of the membranes was as described for Southern hybridisations.

2.10 Isolation of Late Replicating DNA from Wheat Meiocytes

2.10.1 DNA preparations

DNA was extracted from a range of material using the method described by Ji (1992). The material was frozen in liquid nitrogen then ground to a fine powder using a precooled mortar and pestle. The powder was homogenised with 8ml of DNA extraction buffer (Appendix 1) and the slurry transferred to a 30ml Corex tube. Phenol:chloroform:iso-amyl alcohol (25:24:1) (8ml) was added and mixed for 5 minutes by gentle inversion of the tube. The phases were separated by centrifugation at 10,000rpm for 15 minutes at 4°C and the aqueous phase removed to a clean tube. The phenol extraction was repeated then the aqueous phase extracted with an equal volume of chloroform. DNA in the supernatant was either used directly for experiments, or precipitated with sodium acetate and ethanol and resuspended in nanopure water.

2.10.2 Isopycnic centrifugation

Florets (400mg each) containing anthers with pollen mother cells at pre-meiotic interphase, leptotene, zygotene-pachytene, diplotene-telophase II and mature pollen were collected and DNA was extracted as described above. DNA in the supernatant (6ml) was lightly sheared by drawing it up a pipette several times. The supernatant was then transferred to an ultracentrifuge tube containing caesium chloride (6g) and swirled gently to dissolve the solid. A caesium chloride cushion (3ml) in TE buffer (density 1.71 g/ml) was carefully layered into the bottom of each tube and the samples were centifuged in a Beckman Ti 65 rotor at 30,000 rpm for 16 hours at 4°C. Following centrifugation, the supernatant was removed and the inside of each tube cleaned with cotton buds. The pellet of high density material at the bottom of the tube was resuspended in TE buffer (1ml) and transferred to a 2ml eppendorf tube. RNase A was added to a final concentration of 40µg/ml and the samples incubated at 37°C for 2 hours. Samples were subsequently extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and once with an equal volume of chloroform. The DNA was precipitated with sodium acetate and ethanol then resuspended in TE buffer (20µl). Each sample (15µl) was mixed with 6X DNA gel loading buffer (3µl) (Appendix 1) and electrophoresed on a 1.2% agarose TAE gel run at 60mA. The DNA was visualised by staining in ethidium bromide for 30 minutes then viewing the gel under UV light.

2.10.3 Digestion of DNA with Mung Bean Nuclease I

DNA was isolated from 400mg of zygotene florets and precipitated with sodium acetate and ethanol as described previously. The resulting DNA pellet was resuspended in 40µl of nanopure water and the concentration was adjusted to 0.5µg/µl as determined by UV spectrophotometry (Maniatis *et al.*, 1982). DNA was digested with either 5units/µg, 10units/µg or 15units/µg of Mung Bean Nuclease I before separation by gel electrophoresis. DNA (10µg) was mixed with 5X buffer (150mM Na acetate, pH5.0, 250mM NaCl, 5mM $ZnCl_2$, 25%(v/v) glycerol), the appropriate volume of Mung Bean Nuclease (50U/µl) and nanopure water to 50µl. The DNA was digested at 37°C for 20 minutes and the reaction neutralised by the addition of 5µl of 1.0M Tris-HCl, pH 8.0. The DNA was extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol and once with an equal volume

of chloroform. DNA was precipitated overnight by the addition of sodium acetate and ethanol. The resulting DNA was resuspended in TE buffer (15µl). Each DNA sample (7.5µl) was mixed with 6X DNA gel loading buffer (1.5µl) (Appendix 1) and separated by gel electrophoresis on a 1.0% agarose TAE gel run at 60mA, then stained with ethidium bromide for 20 minutes and visualised under UV light.

2.10.4 Fractionation of DNA on a continuous caesium chloride gradient

DNA was extracted from 300mg of zygotene florets and precipitated by the addition of sodium acetate and ethanol. The DNA was resuspended in TE buffer (100µl) and gently sheared by vortexing for 10 seconds. Sheared DNA was applied to the top of a continuous caesium chloride (CsCl) gradient (8ml) (density ranging from 1.60g/ml to 1.75g/ml) in an ultracentifuge tube. Tubes were centrifuged in a Beckman SW41 swing-out rotor at 30,000 rpm for 16 hours at 20°C. The CsCl gradients were fractionated into 200µl aliquots from the bottom of the tube as described by Maniatis *et al.* (1982). The volume of each aliquot was adjusted to 2ml with TE buffer and the DNA precipitated with ethanol to remove excess CsCl. DNA was resuspended in nanopure water (50µl) and 15µl separated by electrophoresis on a 1% agarose/TAE gel run at 15mA.

2.10.5 End labelling of DNA with α-^{32}P-dCTP

DNA was isolated from 100mg zygotene florets and 200mg leaf as described above. The DNA was dissolved in TE buffer, pH 7.6 (Appendix 1) and the concentration of each DNA preparation determined by UV spectrophotometry (Maniatis *et al.*, 1982) then adjusted to 0.25µg/µl. Both DNA preparations (20µl) were lightly sheared by pipetting up and down several times. $MgCl_2$ was added to a final concentration of 5mM followed by the addition of 1µl of a solution of dATP, dGTP and dTTP (each dNTP at a concentration of 1mM) and 3µl of α-^{32}P-dCTP (30µCi). Klenow fragment of DNA polymerase I (2 units) was added and the reaction incubated at 37°C for 30 minutes. 6X DNA gel loading buffer (4µl) (Appendix 1) was added to each reaction and the DNA separated by gel electrophoresis on a 1.0% agarose/TAE gel run at 40mA. The gel was allowed to run until unincorporated dNTP's had migrated into the running buffer. The DNA was then transferred from the gel to a Hybond N^+ membrane. The membrane was washed in 5X SSC for 5 minutes, blotted

dry on a piece of 3MM Whatman paper and exposed to X-ray film at -80°C between intensifying screens for 1 hour.

2.10.6 Preparation of a lambda library of high density DNA from wheat

DNA was isolated from wheat florets at zygotene as described above and the concentration adjusted to 0.5 µg/µl. The extracted DNA was made blunt ended following the procedure of Maniatis *et al.* (1982). Blunt end DNA (5µg) was ligated to *EcoR1/Not1* linkers from a "Time Saver cDNA Kit" (Pharmacia) according to the manufacturers protocol. Following ligation, excess linkers were removed by spun column chromatography (Pharmacia Time Saver Kit). The resulting DNA was cloned into λgt10 *EcoR1* arms (Promega) following the manafacturers guidelines and the phage packaged and titrated as described above using the C600*Hfl*⁻ host strain. The titre of the library was calculated to be approximately $2.8x10^5$ plaque forming units (pfu)/ml. $1x10^5$ pfu were plated onto large (15cm) LB plates at a density of approximately 5,000 pfu per plate.

The phage were transferred to Hybond N^+ membranes, hybridised with pMR1 (Tolocyzki and Feix, 1986) and washed to 0.5X SSC, 0.1%SDS before being exposed to X-ray film for 4 days at -80°C. Clones which did not hybridise to pMR1 were cored from the plates, placed in 100µl SM buffer (Appendix 1) and incubated at 4°C overnight. Large LB plates were prepared for secondary screening by overlaying them with 14ml of warm, molten Top agarose (Appendix 1) containing competent C600*Hfl*⁻cells (500µl). The plates were incubated at 37°C until a bacterial lawn covered the entire plate surface. SM buffer (1µl) containing a selected phage was spotted onto a grid on the plate and the plates incubated at 37°C until lysis of the bacteria occurred. Phage were again transferred to Hybond N^+ membranes and hybridised with pMR1. The membranes were washed to 1X SSC, 0.1%SDS and exposed to X-ray film at -80°C for 5 days. DNA was extracted from the phage which did not hybridise with pMR1 and 10µl was digested with *Not*1 at 37°C for 3 hours. The resulting DNA fragments were separated by gel electrophoresis on a 1% agarose/TAE gel run at 40mA.

2.11 Preparation of an early meiosis subtractive probe from wheat meiocytes

2.11.1 Production of mRNA populations with subtractive oligolinkers

Total RNA was isolated from 300 wheat anthers containing pollen mother cells at either leptotene or zygotene and 600 anthers containing immature pollen. The RNA was resuspended in TE buffer (60µl) and stored at -80°C. Total RNA (2µl) was separated on a 1.2% agarose denaturing gel to determine if any degradation occurred during extraction, and the concentration of the RNA was determined by UV spectrophotometry (Maniatis *et al.*, 1982). Poly (A^+) RNA was isolated from each of the total RNA populations and resuspended in nanopure water to a final concentration of 1µg/µl.

cDNA was synthesised from 2.5µg of zygotene/ leptotene poly (A+) RNA and 2.5µg of poly(A+) RNA from immature pollen using the Pharmacia Time Saver cDNA Synthesis Kit. The cDNA was purified by spun column chromatography on a Sepharose CL4B column (Pharmacia) following the manufacturers recommendations and the column effluent stored at -20°C.

Two subtractive oligonucleotides, a 21mer and a 25mer (Diguiud and Dinauer, 1989) were synthesised by Dr N. Shirley (Department of Plant Science).

- 21mer - dCTCTTGCTTGAATTCGGACTA
- 25mer - dTAGTCCGAATTCAAGCAAGAGCACA

The concentration of each oligonucleotide was determined by UV spectrophotometry (Maniatis *et al.*, 1982) and adjusted to 1µg/µl. The oligonucleotides were phosphorylated (Maniatis *et al.*, 1982) using T4 polynucleotide kinase, then hybridised in kinase buffer at 45°C for 10 minutes. This yielded a duplex subtractive oligo-vector which was blunt on one end, had a 3' overhang at the opposite end and contained an internal *Eco*R1 recognition site (Diguiud and Dinauer, 1989). Following phosphorylation, the reaction volume was adjusted to 200µl with TE buffer (pH7.5), the reaction was extracted twice with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and the aqueous phase transferred to a clean tube after each extraction. The organic phase was re-extracted with 100µl of TE buffer (pH 7.5) and the aqueous phases pooled. The pooled aqueous phases (approximately 400µl) was extracted once with an equal volume of chloroform and the DNA precipitated by the addition of an equal volume of 7.5M ammonium acetate (pH 4.8) and 2.5 volumes of ice

cold ethanol. The linkers were precipitated overnight at -80°C, centrifuged at 12,000 rpm in a bench top centrifuge and the pellet washed twice with cold 70% ethanol (1ml). The pellet was dried under vacuum and resuspended in nanopure water (10µl) to give a final concentration of 1µg/µl.

Subtractive linkers were ligated to both cDNA populations by adding to the column effluent; 5µl subtractive oligovector (5µg), 30µl PEG buffer (Appendix 1), 1µl ATP (10mM) and 1 unit of T4 DNA ligase. The reaction was incubated at 16°C for 2 hours then purified by spun coulmn chromatography on a Sepharose CL4B column (Pharmacia) to remove the excess linkers. The cDNA was precipitated overnight by the addition of sodium acetate and ethanol and resuspended in nanopure water (20µl).

2.11.2 Polymerase chain reaction amplification of cDNA populations

In a total volume of 50µl, the following reagents were mixed; 2µl cDNA with oligolinkers, 1µl of the 21mer oligonucleotide (1µg), 8µl dNTP's (1.25mM), 3µl $MgCl_2$ (25mM), 5µl 10 X reaction buffer (Applied Biosystems), 2 units of Taq polymerase (Applied Biosystems) and nanopure water to 50µl. The cDNA was amplified in a MJ Research thermal cycler using the following parameters; denaturation-30 seconds, 94°C; annealing-30 seconds, 50°C; elongation-2 minutes 72°C. The cDNA was amplified through either 20 cycles or 32 cycles and the final elongation period was extended to 5 minutes.

2.11.3 Amplification of immature pollen cDNA and photobiotin labelling

Three reactions, using 2µl of immature pollen cDNA each, were amplified through 32 cycles of PCR as described above. After amplification, the reactions were pooled in a 1.5ml eppendorf tube and extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and once with an equal volume of chloroform. The DNA was precipitated by the addition of sodium acetate and ethanol and resuspended in nanopure water (60µl). The concentration was determined to be 0.5µg/µl by UV spectrophotometry (Maniatis *et al.*, 1982). The amplified DNA was digested with a 10 fold excess of *Eco*R1 in a 100µl volume for 3 hours at 37°C, extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and once with an equal volume of chloroform, then precipitated

overnight by the addition of ammonium acetate and ethanol. The resulting DNA was resuspended in 30µl of nanopure water (1µg/µl).

Amplified immature pollen cDNA (30µg) was labelled with biotin employing photo-activated biotin purchased from Bresatec. The cDNA was labelled by passing it through three cycles of photobiotin labelling as outlined in the manufacturers protocol. According to the manufacturer, the resulting cDNA should have a biotin molecule incorporated every 50 bases. An aliquot of the final reaction (2µl) was removed for determining the efficiency of labelling. The bulk cDNA was precipitated with sodium acetate and ethanol for 30 minutes at -80°C then resuspended in 6µl of PAB hybridisation buffer (Appendix 1).

2.11.4 Detection of biotin incorporation into cDNA

cDNA (2µl) was separated on a 1.5% agarose/TAE gel together with 0.2µg of positive biotin control (Bresatec). The DNA was transferred to a Hybond N^+ nylon membrane as described above and incorporated biotin detected using a modification of the streptavidin-alkaline phosphatase detection kit (Gibco BRL). The membrane was dried at 80°C under vacuum for 30 minutes, then rehydrated in Detection Buffer 1 (Appendix 1) for 1 minute. The filter was blocked by incubating in Detection Buffer 2 (Appendix 1) for 1 hour at 65°C then blotted dry between two sheets of 3MM Whatmann paper. Incorporated biotin was detected by incubating the filter in stretavidin-alkaline phosphatase solution (10ml) (1µg/ml in Detection Buffer 1) for 10 minutes at room temperature with gentle agitation. The membrane was washed in 20 ml of Detection Buffer 1 (Appendix 1) for 5 minutes at room temperature, then rinsed briefly in Detection Buffer 3 (Appendix 1). Detection Buffer 4 (7.5ml) (Appendix 1) was added to the filter in a petri dish (9cm). The dish was wrapped in foil and incubated in the dark at room temperature for 30 minutes. The filter was washed for 1 minute in Stop Buffer (Appendix 1) to inhibit development after detection.

2.11.5 Subtractive hybridisation

The hybridisation and removal of hybrids procedures are essentially as described by Sive and St John (1988) and employ the addition of streptavidin and phenol extraction to remove biotin labelled sequences.

cDNA in the leptotene/zygotene population was precipitated overnight with sodium acetate and ethanol. The resulting cDNA was resuspended in PAB hybridisation buffer (2.5µl) (Appendix 1) to give a final concentration of 1µg/µl. A 1µg aliquot of leptotene/zygotene cDNA was mixed with 3µl (10µg) of biotinylated immature pollen cDNA in a 0.5ml eppendorf tube and the reaction overlayed with parrafin oil. The reaction was heated to 94°C for 2 minutes to denature the cDNA species, and hybridisation was performed at 65°C for 48 hours.

Following hybridisation, the reaction was transferred to 100µl of PAB buffer without SDS (PAB-SDS; Appendix 1). Streptavidin (5µg) (Gibco BRL) was added and the mixture incubated at room temperature for 1 minute. The reaction was extracted with an equal volume of TE-saturated phenol:chloroform (1:1) and the aqueous phase, containing unhybridised cDNA, was transferred to a clean tube. The organic phase was washed with PAB-SDS buffer (25µl), and the aqueous phase pooled with the original aqueous solution. The pooled aqueous phases were extracted twice more with streptavidin and phenol:chloroform as described above. Following the final extraction, the pooled aqueous phases were extracted with an equal volume of chloroform and precipitated overnight at -20°C by the addition of sodium acetate and ethanol. The DNA was resuspended in PAB buffer (1µl) and the subtractive hybridisation repeated twice more. Following the final round of subtractive hybridisation, the cDNA was resuspended in PAB-SDS buffer (2µl). The cDNA was amplified through 15 cycles of PCR as described above using the 21mer as a primer. Following the final amplification, the subtracted cDNA was extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and once with an equal volume of chloroform. The DNA was precipitated overnight at -20°C with sodium acetate and ethanol and the DNA resuspended in PAB-SDS buffer (20µl).

2.12 Characterisation of WM27

2.12.1 Subcloning the meiosis specific region of pWM27

A restriction map of pWM27 based on the sequence data available (Chapter 4) was produced using the DNA Strider programme for the Apple MacIntosh computer. The restriction sites *Eco*RV and *Mlu*1 were identifed which allow the separation of the putative meiosis specific region from the L11 region.

Plasmid DNA was isolated from a bacterial culture (4ml) containing pWM27. The DNA was resuspended in nanopure water (20µl) and an aliquot (10µl) digested with *Mlu*1 (20 units) in a 20µl volume at 37°C for 3 hours. A sample (2µl) was separated on a 1.5% TAE/agarose gel to assess the efficiency of the digestion. The remaining DNA was extracted with an equal volume of phenol:chloroform:iso-amyl alcohol then an equal volume of chloroform and precipitated by the addition of sodium acetate and ethanol and incubation at -80°C for 2 hours. The DNA was resuspended in nanopure water (16µl) and digested with *Eco*RV (20 units) for 3 hours at 37°C. The DNA fragments were separated by gel electrophoresis on a 1.5% TAE/agarose gel. The released band representing the meiosis specific region of pWM27 was identifed and excised from the gel under long wave UV light. The DNA was recovered from the gel slice by the gene clean procedure and stored at 4°C.

The isolated DNA fragment was rendered blunt ended by treatment with the Klenow fragment of DNA polymerase I as described by Maniatis *et al.* (1982), and cloned into the *Sma*1 site of pBluescript (KS^-) as described above. The plasmid was transformed into *E. coli* strain DH5α as described and 24 white colonies randomly selected for plasmid isolation. DNA (10µl) isolated from the selected colonies was digested with *Hind*III and *Bam*H1 and the resulting fragments farctionated on a 1.5%TAE/agarose gel run at 40mA. The DNA was transferred to a Hybond N^+ membrane and hybridised with the *Eco*R1 isolated insert from pWM27 containing both the meiosis specific and ribosomal regions of the original clone. Plasmids identified as being recombinant, and hybridising with the pWM27 *Eco*RI insert, were identified and re-named pWM27ms (meiosis specific).

2.12.2 Northern hybridisation with WM27ms

The insert of pWM27ms was isolated by digestion with *Hind*III and *Bam*H1, gel electrophoresis of the fragments and recovered using the gene clean procedure. The insert was labelled with α-^{32}PdCTP using random primers, and was used to probe a Northern membrane with representative RNA from numerous tissues as described earlier.

2.12.3 Preliminary mapping of the pWM27ms clone

Preliminary mapping was performed using wheat/barley addition lines, wheat nullisomic/tetrasomic and ditelosomic lines and also the *ph2a* and *ph2b* mutant wheat lines. The plants were grown as described and DNA extracted using the midiprep procedure. The addition lines are composed of a Chinese spring background with an additional chromosome from the barley cultivar Betzes. To identify a polymorphism between Chinese spring and Betzes, DNA (5µg) from the two cultivars was digested at 37°C for 3 hours with 20 units of *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV or *Hind*III in a 20µl volume. The DNA was fractionated on a 0.8% TAE/agarose gel run overnight at 10mA, and transferred to a Hybond N^+ membrane. The membrane was hybridised overnight with labelled pWM27ms insert, washed to 0.2X SSC, 0.1% SDS and exposed to X-ray film for 5 days at -80°C.

DNA from each addition line (5µg) was subsequently digested with *Bam*HI (20 units) at 37°C for 3 hours in a 20µl volume. The DNA fragments were separated by electrophoresis at 10mA on a 0.8% TAE/agarose gel and transferred to a Hybond N^+ membrane. The membrane was hybridised with labelled insert from pWM27ms and washed to 0.2X SSC, 0.1% SDS before being exposed to X-ray film at -80°C for 4 days.

The addition line Southern analysis revealed that the clone is located on chromosome 3. Analysis with the following lines was then performed:

- nullisomic 3A - tetrasomic 3B
- nullisomic 3A - tetrasomic 3D
- nullisomic 3B - tetrasomic 3A
- nullisomic 3D - tetrasomic 3A
- nullisomic 3D - tetrasomic 3B
- ditelosomic 3AS
- ditelosomic 3AL
- ditelosomic 3BL
- ditelosomic 3DS
- ditelosomic 3DL
- *ph2a* mutant
- *ph2b* mutant

DNA was extracted using the midiprep procedure outlined above and resuspended in nanopure water. An aliquot of each DNA sample (5µg) was digested with *Dra*I (20 units) in a 20µl volume at 37°C for 3 hours then fractionated on a 0.8% TAE/agarose gel. The DNA was transferred to a Hybond N^+ membrane and hybridised with labelled insert from pWM27ms. The membrane was washed to 0.2X SSC, 0.1% SDS then exposed to X-ray film at -80°C for 4 days.

2.12.4 Re-screen of the cDNA library

Aliquots of the cDNA library constructed above, were removed from -80°C and the titre determined as described using *E. coli* strain C600*Hfl*⁻ as the host. The library ($3.1x10^5$ pfu) was plated at 5000 pfu per plate and transferred to Hybond N^+ membranes. The membranes were hybridised overnight with randomly labelled insert from pWM27ms and washed to 0.5X SSC, 0.1%SDS then exposed to X-ray film for 4 days at -80°C. Positive phage were isolated and purified by secondary screening at low density as described.

2.12.5 Production of a wheat genomic library and isolation of genomic and full length cDNA clones

2.12.5.1 Partial digestion with *Eco*R1

T. aestivum cv Chinese Spring DNA (50µg) was digested with 4 different concentrations of *Eco*R1 (1unit/µg, 0.3units/µg, 0.1units/µg and 0.03units/µg) at 37°C for 30 minutes in a final volume of 100µl. The reactions were stopped by the addition of 0.5M NaEDTA (2µl) and the degree of digestion of each DNA sample was determined by fractionating an aliquot (10µl) on a 0.8% agarose/TAE gel run at 30mA. The reaction producing a majority of restriction fragments in the size range of between 9 and 25kb was extracted once with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) and once with an equal volume of chloroform. The DNA was precipitated using ammonium acetate and ethanol then resuspended in nanopure water to a final concentration of 0.5µg/µl.

2.12.5.2 Fractionation on a sucrose gradient

*Eco*RI digested genomic DNA was fractionated on a 10%-40% sucrose gradient following the procedure of Maniatis *et al.* (1982).

2.12.5.3 Ligation to vector and packaging

DNA, partially digested with *Eco*R1, was ligated into λDASH II *Eco*R1 arms (Stratagene) following the protocol of the manufacturer. The resulting phage particles were packaged using an *in vitro* packaging module purchased from Amersham and following the manufacturers instructions.

2.12.5.4 Plating and titre of phage

Packaged phage were diluted 1:100, 1:1,000, 1;10,000 with SM buffer (Appendix 1). Aliquots (100µl) of each dilution were added to prepared XL1-Blue cells and incubated at 37°C for 45 minutes to allow the phage to adsorb to the cells. Warm, molten top agarose (12ml) (Appendix 1) was added, the solution gently mixed then overlayed onto a large LB (Appendix 1) plate. Plates were incubated inverted at 37°C until phage lysis was observed. The titre of the library was calculated to be $8x10^6$ pfu/ml. $4x10^6$ pfu were plated onto large petri dishes at a density of 10^5 pfu per plate.

2.12.5.5 Screening of the genomic library

The genomic library produced above, was transferred to Hybond N^+ membranes and hybridised overnight with randomly labelled insert from pWM27ms. The membranes were washed to 0.2X SSC, 0.1% SDS and exposed to X-ray film for 4 days at -80°C. An area of agarose $1cm^2$ surrounding positively identified clones was transferred to an eppendorf tube containing SM Buffer (1ml) (Appendix 1). The phage were allowed to diffuse from the agarose at 4°C overnight. The phage from each clone identified by the first screen were plated onto large LB plates at a density of 500 pfu/plate, transferred to Hybond N^+ membranes and re-hybridised with the insert from pWM27ms. Positive clones identified in the second screen were cored from the plate with a pasteur pippete, placed into SM Buffer (100µl) in an eppendorf tube, and stored at 4°C.

2.12.6 Subcloning the genomic insert

DNA was extracted from each of the phage positively identified in the screen of the genomic library. The DNA (10µl) was digested with *Eco*R1 (20units) in a 20µl reaction volume at 37°C for three hours and the fragments separated by electrophoresis on a 1.2% TAE/agarose gel run overnight at 20mA. The DNA was transferred to a Hybond N^+ membrane and hybridised overnight with labelled insert from pWM27ms. The membrane

was washed to 0.2X SSC, 0.1%SDS then exposed to X-ray film overnight at -80°C. The hybridising fragments were identified and isolated by restriction digest of a second sample of phage DNA (10µl) with *Eco*R1 (20 units) and excision of the bands under long wave UV light. DNA was extracted from the gel slices using the geneclean procedure and was ligated into the the *Eco*R1 site of pBluescript (KS$^-$) as described above.

2.12.7 Construction of a nested deletion library

A nested deletion library of the genomic clone was constructed using the Erase-a-base system (Promega). Circular plasmid DNA was extracted from p27/16a using alkaline lysis and HPLC as described (Skingle *et al.*, 1990) and the resulting DNA resuspended in nanopure water (2µg/µl). Samples of the plasmid DNA (6µg each) were digested with 20 units of *Bam*H1, *Bst*X1, *Eco*R1, *Not*1, *Pst*1, *Sac*1 and *Xba*1 in a 20 µl reaction volume at 37°C for 3 hours. Aliquots (1µg) of the digests were separated by electrophoresis on a 0.8% TAE/agarose gel. The remaining *Xba*1 digested DNA (5µg) was extracted once with phenol:chloroform:iso-amyl alcohol and once with an equal volume of chloroform, then precipitated with sodium acetate and ethanol at -80°C for 1 hour. The DNA was resuspended in 1X Klenow Buffer (60µl) (Appendix 1) and a mixture of all four α-phosphorothioate DNTPs added to a final concentration of 40µM each. 1M Dithiothreitol (0.7µl) was added, and the reaction started by the addition of Klenow enzyme (7 units). The reaction was incubated at 37°C for 15 minutes then extracted with phenol:chloroform:iso-amyl alcohol and chlorofrom and precipitated by adding sodium acetate and ethanol and incubating at -20°C overnight. The DNA was resuspended in nanopure water (10µl) and digestsed with *Bam*H1 (20 units) at 37°C for 3 hours in a 20µl reaction volume. The DNA was extracted with an equal volume of phenol:chloroform:iso-amyl alcohol and an equal volume of chloroform then precipitated overnight at -20°C by the addition of sodium acetate and ethanol.

The DNA pellet was resuspended in ExoIII Buffer (60µl) (Appendix 1), and the nested deletion performed as described in the manufacturers protocol, utilising 400 units of ExoIII and performing the digest at 30°C. The rate of deletion should be approximately 200bp/minute. Aliquots (4µl) of DNA removed from each time point were separated on a 0.8% TAE/agarose gel to determine the extent of digestion of the clone. Following analysis of the digestion, the deleted plasmids were circularised, transformed into *E. coli* strain

DH5α and plated onto selective media as described. Two white colonies were randomly selected from each plate and inoculated into Terrific broth (Appendix 1) supplemented with ampicillin (50μg/ml). The bacteria were grown overnight at 37°C with vigorous shaking, and plasmid DNA isolated as described. The DNA was resuspended in nanopure water (0.25μg/μl) and sequencing reactions performed using the M13 forward primer as outlined below.

2.12.8 Reverse transcription PCR amplification

Using the sequence data derived from the genomic clone, two primers were designed for PCR amplification;

27upper - 5' CGTCCCGTGGCGTGCTT 3'

27lower - 5' TCCCCTCGTTGGCTTTCTTGA 3'

Total RNA was isolated from florets containing pollen mother cells at premeiotic interphase (30 florets), leptotene (30 florets), zygotene (30 florets), metaphase I (30 florets), tetrads (30florest), mature pollen (30 florets), root tips and leaf. First strand cDNA was synthesised from 5μg of total RNA using Superscript II RNase H^- Reverse Transcriptase (GIBCO BRL) and following the manufacturers recomendations. Following the production of the first strand cDNA, complimentary RNA was removed by the addition of *E. coli* RNase H (2 units) and incubation of the reaction at 37°C for 20 minutes. The first strand cDNA (2μl) was used as a template for PCR amplification using the genomic primers. In addition, templates of DNA from Chinese Spring (30ng), *ph2a* mutant (30ng), *ph2b* mutant (30ng) and the genomic clone (40ng) were included in the amplification. The PCR reaction mix was as follows; 10μl 10X PCR Buffer (Appendix 1), 3μl $MgCl_2$ (25mM), 2μl DNTPs (10mM), 1μl each primer (10μM), DNA template (2μl), *Taq* polymerase (2 units) and nanopure water to 100μl total volume. The reactions were heated to 94°C for 3 minutes to denature the DNA then subjected to 32 cycles of 30 seconds at 94°C denaturing, 1 minute at 62°C primer annealing and 1 minute at 72°C chain extension in a MJ Research thermal cycler equipped with a hot bonnet. The final chain extension was prolonged to 5 minutes before the reactions were cooled to 4°C. Subsequent PCR amplifications were performed using the same parameters except that the primer annealing temperature was lowered to 60°C and 58°C respectively.

2.12.9 Screening of a cDNA library with the genomic clone

A second cDNA library constructed from RNA extracted from anthers containing pollen mother cells at leptotene was supplied by Jocelyne Letarte. 50, 000 pfu of the library were plated onto large LB plates, as described earlier, at a density of 5, 000 pfu per plate. The plaques were transferred to Hybond N^+ membranes in triplicate with the first transfer being allowed to proceed for 30 seconds and the subsequent transfers being extended to 1 minute and 2 minutes respectively. The three replicate membranes were hybridised with three different regions of the genomic probe. Following the hybridisation, the membranes were washed to 0.2X SSC, 0.1% SDS and exposed to X-ray film for 4 days at -80°C. 4 clones which hybridise to all three of the probes were identified and purified by subsequent screening at lower density. DNA was extracted from the phage and a sample (10µl) digested with *Not*1 (20 units) for 3 hours at 37°C. The fragments were separated on a 1.0% TAE/agarose gel and DNA from the clone representing the longest cDNA was extracted from the gel using the gene clean procedure. The DNA was ligated into the *Not*1 site of pBluescript (KS^-) as described and the clone named AWWM5 (Australia, Waite, Wheat Meiosis). The insert was used in hybridisations to the nullisomic-tetrasomic and ditelosomic lines as well as in Northern hybridisation as described earlier to verify the validity of the isolated cDNA.

2.12.10 Sequencing of the cDNA clone

Plasmid DNA (10µg) from pAWWM5 was digested with *Hind*III (20 units) as well as double digests with 20 units of *Sal*I and *Not*I. The fragments were separated on a 1.2% TAE/agarose gel, extracted from the gel using the geneclean procedure and subcloned into pBluescript (KS). Recombinant plasmids were extracted as described and sequenced using the M13 forward and reverse sequencing primers.

2.12.11 Spatial expression of AWWM5

In situ hybridisation of RNA transcripts was kindly performed by Mrs. R. Guo.

2.12.11.1 Fixation and sectioning of tissue

Wheat florets with anthers containing pollen mother cells at either leptotene or zygotene were collected and incubated in fixation buffer (Appendix 1) at 4°C for 16 hours on an orbital rotor. The samples were washed for 15 minutes in 50mM PIPES (pH 7.0) to remove

excess aldehydes. The samples were washed three times then dehydrated through a series of ethanol washes as outlined below:

- 20 minutes in 20% ethanol, 25mM PIPES
- 20 minutes in 30% ethanol, 10mM PIPES
- 20 minutes in 50% ethanol
- 20minutes in 70% ethanol

Samples were placed in plastic cassettes and subjected to automated processing involving incubations as follows:

- 1 hour in 70% ethanol
- 1 hour in 89% ethanol
- 1.5 hours in 95% ethanol
- 4 hours in 99% ethanol
- 1 hour in histoclear:ethanol (50:50, v/v)
- 4 hours in histoclear
- 4 hours in liquid paraffin (DIFCO)

The florets were transferred to metal moulds containing melted (57°C) embedding wax (Polywax, DIFCO) and cooled rapidly. The florets were cut using a rotary microtome (Leitz 1512) to produce sections of 6-10 μm thickness. Sections were floated in a waterbath (45°C) onto microscope slides coated with 2% aminopropylethoxysilane and dried overnight at room temperature.

2.12.11.2 Preparation of labelled RNA transcripts

Digoxigenin labelled RNA transcripts were produced by *in vitro* transcription of the AWWM5 insert in pBluescript (KS^-). pAWWM5 was linearised by digesting with either *Bam*HI or *Sac*II. Linearised plasmid was separated by electrophoresis on a 1.0% TAE/agarose and purified by the geneclean procedure.

RNA transcripts were produced in a 20μl reaction volume containing 1X transcription buffer (Promega), linearised plasmid (1.0μg), 10mM DTT, RNasin (20U), 1mM each of ATP, CTP and GTP, 0.65mM UTP, 0.35mM digoxigenin-11-UTP (Boehringer) and either T3 or T7 RNA polymerase (50U) to produce a sense or antisense transcript. The reactions were incubated at 37°C for 2 hours then stopped by the addition 0.2M Na_2EDTA (2μl). RNA was precipitated by the addition of 4M LiCl (2.5μl) and cold ethanol (75μl) and

incubation at -80°C for 1 hour. RNA was recoverd by centrifugation at 12,000 rpm for 15 minutes at 4°C, then washed with 70% ethanol (1ml) and dried under vacuum. RNA was resuspended in RNase-free nanopure water (100µl).

The quality and approximate concentration of the synthesised RNA was assessed by fractionation on a 1.5% agarose denaturing gel. The length of the RNA probes was then reduced to approximately 150bp by partial alkaline hydolysis as described (Cox *et al.*, 1984).

2.12.11.3 Prehybridisation treatments

Wax was removed from tissue sections by submersion in xylene (5 minutes), washing in pure ethanol and 70% ethanol (5 minutes each) then air drying. The sections were incubated in proteinase K solution (Appendix 1) at 37°C for 15 minutes then washed three times with 2X SSC (5 minutes each) and rinsed with nanopure water. Excess water was removed by gently wiping the slide with blotting paper and the sections acetylated in 0.25% acetic anhydride, 0.1M triethanolamine (pH8.0) for 10 minutes at room temperature. The sections were rinsed 5 times in 2X SSC, rinsed in nanopure water then rapidly dehydrated through an ethanol series of 25%, 40%, 55%, 70% and 100% ethanol. Following dehydration, the sections were prehybridised by heating in *in situ* hybridisation solution (Appendix 1) to 80°C for 5 minutes then applying hybridisation solution (40µl) to the section, covering with a cover slip and incubating at 45°C for 2 hours.

2.12.11.4 Hybridisation and washing

Following prehybridisation, the slides were dipped quickly into 2X SSC to remove the coverslips and residual hybridisation solution removed from the edges of the section with tissue. RNA probes were added to fresh hybridisation solution (8-10ng/µl), heated to 80 °C for 5 minutes and applied to the section. The section was covered with a cover slip and incubated at 45°C in a humid chamber for 16 hours. The cover slips were removed and the sections washed as following

- 5 minutes in 2X SSC at room temperature
- 15 minutes in RNase A solution (Appendix 1) at 37 °C
- 45 minutes in 2X SSC at 42 °C
- 45 minutes in 1X SSC at 42 °C

- 30 minutes in 0.5X SSC at room temperature

2.12.11.5 Immunological detection

Detection of hybridisation was performed using anti-digoxigenin alkaline phosphatase (anti-DIG-ap) purchased from Boehringer Mannheim. Slides were immersed in Detection Buffer 1 (Appendix 1) for 5 minutes at room temperature then incubated at 37°C in Detection Buffer 2 (Appendix 1) for 60 minutes. Anti-DIG-ap (1:500 in Detection Buffer 2) (100µl) was applied to each section and incubated at room temperature for 2 hours. Unbound antibody conjugate was removed by washing twice in Detection Buffer 1 containing Tween 20 (0.3% v/v) for 15 minutes and rinsing in Detection Buffer 3 (Appendix 1) for 2 minutes. Excess washing solution was removed with a tissue, 100µl of Detection Buffer 4 (Appendix 1) was placed on each section and the slides incubated in the dark until colour development occurred. Colour development was stopped by rinsing the slides in several volumes of TE buffer and nanopure water. Sections were dehydrated through an ethanol series (10%, 20%, 30%, 50%, 70%, 90% and pure) then mounted with coverslips in DPX mountant (BDH, England) and photographed under bright field conditions using a Zeiss Axioscope microscope.

2.12.12 Mapping of the *ph2a* deletion

Three different double haploid barley populations were available for mapping purposes. The Galleon X Haruna Nijo and Chebec X Harrington populations were produced by Dr. S. Logue, Department of Plant Science and the Clipper X Sahara population was produced by Dr. R. Islam, Department of Plant Science. DNA (10µg) from all of the parents was digested with five different restriction enzymes, *Eco*RI, *Dra*I, *Hind*III, *Bam*HI or *Eco*RV (20 units of each) at 37°C for 3 hours in 1X SDB buffer (Appendix 1). The DNA was separated on a 0.8% TAE/agarose gel and transferred to a Hybond N^+ membrane. The membrane was hybridised overnight with AWWM5 then washed to 0.1X SSC, 0.1% SDS and exposed to X-ray film for 5 days at -80°C.

Mapping of AWWM5 was performed using the Clipper X Sahara population. DNA (10µg) from each individual was supplied by Dr R. Islam and was digested with *Eco*RV (20 units) in 1X SDB buffer (Appendix 1) at 37°C for 3 hours. The DNA fragments were separated by electrophoresis on 0.8% TAE/agarose gels and transferred to Hybond N^+ membranes. The

membranes were hybridised with AWWM5, washed to 0.1X SSC, 0.1% SDS and exposed to X-ray film for 5 days at -80°C. The polymorphisms were scored and the location of AWWM5 determined using the Mapmaker programme designed for the Apple MacIntosh computer and incorporating the data available through the barley mapping project (Langridge *et al.*, 1995a).

Probes surrounding AWWM5 were identified from the chromosome map. Three replicates of DNA (10µg) from Chinese Spring and the *ph2a* and *ph2b* mutants was digested with *Bam*HI, *Dra*I, *Eco*RI and *Eco*RV (20 units each) in 1X SDB buffer (Appendix 1) at 37°C for 3 hours. The DNA was separated on 0.8% TAE/agarose gels and transferred to Hybond N^+ membranes. The membranes were hybridised with probes ABG460, BCD828 and CDO395 washed to 0.2X SSC, 0.1%SDS and exposed to X-ray film at -80°C for five days. The membranes were stripped and hybridised with the probes PTAG683, PSR598 and ABG309. The washing and exposure conditions were the same as described above. All of the probes were obtained from the Waite Institute mapping collection.

2.13 General Methods

2.13.1 Isolation of DNA from agarose gels

The geneclean (BIO 101) protocol was adopted for the recovery of DNA from gel slices. Gel slices were excised under longwave UV light and dissolved at 60°C in 6M NaI (700µl). Glass milk (5µl) was added and the solution incubated on ice for 1 hour. The glass milk was pelleted by centrifugation at 12,000 rpm, the supernatant was removed by aspiration and the pellet washed thoroughly three times with new wash solution (1ml). Following the final wash, the pellet was dried briefly under vacuum and the DNA eluted by resuspension in TE buffer (20µl) and incubation at 60°C for 5 minutes. DNA was stored at -20°C.

2.13.2 PCR amplification of λgt10 clones

The unique *Eco*RI site within the *imm434* region of λgt10 has been utilised for cloning and allows direct selection of recombinant bacteriophage. Primers have also been designed which are specific for and complimentary to the *imm434* region on either side of the cloning site as outlined below:

gt10F - 5' AGCAAGTTCAGCCTGGTTAAGT 3'

gt10R - 5' CTTATGAGTATTTCTTCCAGGGTA 3'

Single phage plaques were cored from an LB plate using a sterile pasteur pippette and transferred to a 1.5ml eppendorf tube containing SM buffer (100µl) (Appendix 1). The tubes were incubated overnight at 4°C to allow the phage to diffuse into the surrounding medium. An aliquot (10µl) of phage solution was transferred to a 0.5ml Eppendorf PCR tube, boiled for 10 minutes, then chilled on ice to denature the phage protein coat and DNA. A PCR reaction mix (40µl) containing 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.25mM $MgCl_2$, 250mM of each dNTP, 0.1µg gt10R primer, 0.1µg gt10F primer and *Taq* DNA polymerase (0.5 units), was added to the denatured phage and the reactions performed in a MJ-Research thermal cycler programmed as follows: denaturation of DNA at 94°C for 4 minutes followed by 36 cycles of 1 minute denaturation at 94°C, 2 minutes primer annealing at 55°C and 2 minutes DNA chain extension at 72°C. The final chain extension was allowed to proceed for 5 minutes then the tubes were cooled to 4°C.

2.13.5 Sequencing reactions

Recombinant pBluescript plasmids were isolated as described above and the concentration of the DNA adjusted to approximately 0.25ng/µl with nanopure water. The DNA thus prepared was used for subsequent sequencing reactions based on a modified dideoxy method (Sanger *et al.*, 1977) incorporating fluorescent dye linked primers, buffer and *Taq* polymerase of the Prism sequencing kit from Applied Biosystems. Reaction products were combined and precipitated following the manafucturers specifications. The sequencing products were separated on an Applied Biosystems automated sequencer by Dr. N. Shirley. Sequence data was analysed using SeqEd computer software (Applied Biosystems) designed for the Apple MacIntosh computer.

Chapter 3

Investigation of delayed chromatin replication in wheat

3.1 Introduction

Although replication of the bulk of the nuclear genome occurs before meiotic prophase, just as replication occurs before prophase of mitosis, additional periods of DNA synthesis during meiosis have been detected by autoradiography during leptotene, zygotene and pachytene in newt (Wimber and Prensky, 1963), human (Lima de Faria *et al.*, 1968) and mouse (Mukherjee and Cohen, 1968). The nature of late replicating DNA and its role during meiosis remains largely unknown. However, studies of late replicating DNA (zygDNA) in the monocotyledonous plant *Lilium* has resulted in the proposal that zygDNA acts as the sites of homology recognition between chromosomes during homologue alignment and synapsis (Chapter 1). In this chapter, attempts were made to isolate late replicating DNA from wheat to allow an assessment of a putative role in homology recognition and a possible interaction with the genes controlling homologous chromosome pairing in this species.

Two stages of DNA synthesis after premeiotic S phase have been identified in *Lilium*, occurring at zygotene and pachytene (Hotta *et al.*, 1966). The two types of synthesis are readily distinguishable not only on a temporal basis but also because they exhibit distinct patterns of synthesis (Hotta and Stern, 1971).

DNA synthesised at zygotene has been termed zygDNA and represents between 0.1% and 0.2% of the entire genome (Hotta *et al.*, 1984). Its synthesis is of a semi-conservative nature and represents a delayed replication of sequences not replicated at pre-meiotic S phase (Hotta and Stern, 1971). In contrast, DNA synthesised at pachytene (pDNA) appears to be of the replication repair type as the synthesis does not result in a change in the net amount of DNA (Hotta and Stern, 1971). pDNA is of a moderately repetitive nature, being 170 bases long and reiterated 2000 times per genome (Hotta *et al.*, 1984). pDNA is putatively assigned a role in recombination and chiasmata formation

It is postulated that zygDNA sequences are duplexes and are joined to the adjacent nuclear DNA by single stranded DNA links (Hotta and Stern, 1976) (Figure 3.1). The comparatively high G/C content of zygDNA gives it a buoyant density of 1.712 g/cc as compared to bulk nuclear DNA density of 1.702g/cc (Hotta and Stern, 1971). These characteristics have allowed the isolation of zygDNA by gentle mechanical shearing followed by isopycnic centrifugation with the zygDNA identifiable as a high density satellite band separate from the remainder of the genome. The resulting zygDNA has an average length of 5 kilobases (Hotta *et al.*, 1984) and Cot analysis of the extracted zygDNA demonstrates that is composed of unique and low copy sequences (Hotta and Stern, 1975).

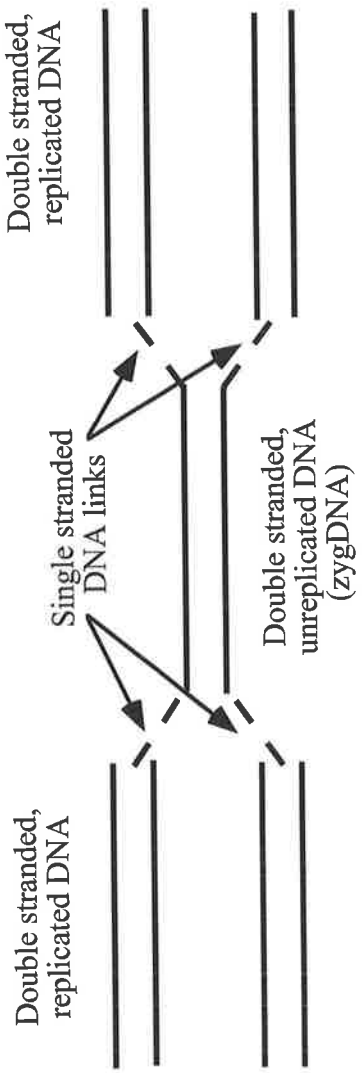
ZygDNA has been shown to be chromosomal and is thought to be distributed over the entire length of the chromosome (Ito and Hotta, 1973), an assumption which is backed by the observed general fragmentation of chromosomes upon partial inhibition of its synthesis (Ito *et al.*, 1967b). Inhibition of zygDNA synthesis in lily prevents the initiation and continuation of chromosome pairing (Ito *et al.*, 1967b; Roth and Ito, 1967). It is known that zygDNA is a component of the synaptonemal complex in lily (Kurata and Ito, 1978) and functions in both its formation and maintenance. The inhibition of zygDNA synthesis prevents the proper formation of the synaptonemal complex as well as possibly preventing the maturation of the synaptonemal complex as identified by the prevention of homologue disjunction at diplotene (Roth and Ito, 1967).

DNA synthesis in wheat anthers has also been investigated by autoradiography methods. Wheat anthers containing pollen mother cells in various meiotic stages ranging from leptotene to telophase II have been cultured in the presence of tritiated thymidine. The results of such investigations show the incorporation of label in pollen mother cells at all stages of meiosis studied (Riley and Bennett, 1971). Riley and Bennett (1971) took the presence of label to indicate that DNA synthesis occurs at all stages of meiosis in wheat. It is impossible to prove this assumption as the detected label could result from the incorporation of thymidine during DNA repair, such as that proposed for pDNA, arising during meiotic recombination. A recent report (Ji, 1992) however, suggests that a DNA species of approximately 5-8kb length is extractable from zygotene florets of wheat by isopycnic centrifugation and that the DNA isolated displays many of the attributes of zygDNA from lily. To further investigate the possible presence of late replicating DNA in

Figure 3.1

Theoretical structure of late replicating DNA from lily (Hotta and Stern, 1976). The unreplicated DNA is joined to the adjacent genomic DNA by single strand DNA links which can be broken either by mechanical shearing or by nuclease treatment.

Figure 3.1


Double stranded, replicated DNA
Single stranded DNA links
Double stranded, unreplicated DNA (zygDNA)
Double stranded, replicated DNA

wheat, attempts have been made to extract zygDNA via several methods. This chapter outlines the methods applied for the extraction of zygDNA and their relative success.

3.2 Materials and Methods

Four general methods were employed to attempt the isolation of zygDNA from wheat. They include isopycnic centrifugation, digestion with single stranded DNA nuclease, end labelling and cloning into bacteriophage as described in Chapter 2.

3.2.1 Isopycnic centrifugation

Ji (1992) reported the isolation of a high buoyant density DNA species from wheat meiocytes during early prophase. The method of isolation is outlined in Chapter 2 and includes the mechanical shearing of extracted total DNA followed by the separation of high buoyant density DNA by centrifugation through a CsCl cushion. To confirm the result of Ji (1992), DNA was extracted from 400mg of wheat florets containing anthers with pollen mother cells at either pre-meiotic interphase, leptotene, zygotene - pachytene, diplotene - telophase II or mature pollen, and was subjected to the isopycnic centrifugation procedure. DNA resulting from this procedure was separated by gel electrophoresis, transferred to a Hybond N^+ nylon membrane (Amersham) and hybridised with pMR1 which contains a 9kb maize ribosomal operon in pBR328 (Tolocyzki and Feix, 1986). In addition, DNA from 700mg of wheat florets at zygotene was also applied in the above isopycnic centrifugation procedure.

Another method, alternative to the separation of high buoyant density DNA molecules by passage through a CsCl cushion of constant density, is the fractionation on a continuous CsCl gradient of variable density. This method was utilised with DNA extracted from 300mg of florets containing meiocytes at zygotene. The DNA was sheared mechanically and separated on a continuous CsCl gradient, ranging in density from 1.60g/ml to 1.75g/ml, which was then fractionated into 200µl aliquots. The DNA in each aliquot was analysed by gel electrophoresis.

3.2.2 Digestion of DNA with Mung Bean Nuclease I

An alternative to the use of mechanical force for the breakage of the single strand links thought to link late replicating DNA with the remainder of the genome, is digestion of the DNA with a single stranded DNA nuclease such as Mung Bean Nuclease I. Accordingly, 10μg of DNA isolated from florets containing zygotene meiocytes was digested with either 5U/μg, 10U/μg or 15U/μg of Mung Bean Nuclease I. The DNA was digested at 37°C for 20 minutes then separated by gel electrophoresis.

3.2.3 Labelling of sheared DNA

Considering that the model of the structure of late replicating DNA contains regions of single stranded DNA, it is possible that these regions could be labelled by the incorporation of α-^{32}P-dCTP. To address this possibility, DNA isolated from florets containing meiocytes at zygotene and from leaf were sheared lightly to produce segments with overhanging single stranded regions. The single stranded regions of both DNA populations were labelled by the incorporation of α-^{32}P-dCTP using the Klenow fragment of DNA polymerase I. The DNA was separated by gel electrophoresis, transferred to a Hybond N^+ membrane, and the pattern of label incorporation determined by autoradiography.

3.2.4 Lambda cloning of zygDNA

An attempt was made to clone the DNA isolated by the isopycnic procedure outlined above. The DNA resulting from the isopycnic centrifugation was rendered blunt ended and ligated to *Eco*R1/*Not*1 linkers from a Time Saver cDNA Synthesis Kit (Pharmacia). The DNA was purified to remove excess linkers and cloned into the *Eco*R1 site of λgt10 (Promega). The resulting phage were packaged and plated onto LB plates then probed with pMR1 (Tolocyzki and Feix, 1986) to determine which phage contained inserts with homology to ribosomal DNA. DNA was extracted from phage which did not hybridise with pMR1 and was digested with *Not*1 then subjected to gel electrophoresis to determine the size of the phage insert.

3.3 Results and Discussion

Autoradiographic studies with a range of species have demonstrated DNA synthesis during meiosis, however the results arising from many of these studies is inconclusive. DNA

synthesis during zygotene was observed by Mukherjee and Cohen (1968) in male mice when ^{3}H thymidine was injected directly into the testes. Further experiments by Kofman-Alfaro and Chandley (1970) did not substantiate these findings, although the later experiments involved intraperitoneal injection of ^{3}H thymidine. In addition, research with ^{3}H thymidine incorporation during meiosis into the DNA of many species may be misleading due to a lack of control experiments to demonstrate that the label is not bound by proteins or incorporated into newly synthesised DNA during recombinational repair. The only unchallenged evidence for the existence of late replicating DNA comes from *Lilium*.

Late replicating DNA, or zygDNA was first isolated from Lily by isopycnic centrifugation (Hotta and Stern, 1971). This method of isolation was applied by Ji (1992) for the isolation of zygDNA from wheat. As the buoyant density of the bulk genomic DNA in lily is the same as that in wheat, Ji (1992) assumed that the zygDNA should also be of similar buoyant density. On this basis, DNA was isolated from wheat florets containing anthers at zygotene by shearing the single stranded links then centrifuging the DNA through a CsCl cushion with a density of 1.712g/ml. Centrifuging allows only high density DNA and RNA to pass through the cushion. When a sample of DNA from the bottom of the tube was separated by gel electrophoresis, a distinct band was observed at about 8kb. Whilst the size of this band is larger than that of zygDNA from lily (Hotta *et al.*, 1984), it may represent wheat zygDNA. Attempts here to confirm the findings of Ji (1992) have been unsuccessful. Utilising the above procedure, a DNA species as reported by Ji (1992) was not evident in preparations from 400mg of florets containing meiocytes at pre-meiotic interphase, leptotene, zygotene, pachytene, diplotene - telophase II or mature pollen (Figure 3.2). A band which is apparent at approximately 20Kb is present in all of the preparations. The size of this band, and the fact that it is represented in late meiotic tissues and non-meiotic tissues suggests that it does not represent late replicating DNA. Late replicating DNA should not be extractable by shearing after prophase 1 as at this time it is believed that the single stranded links between the zygDNA and the genome are repaired into double stranded molecules following the replication of the zygDNA (Hotta and Stern, 1976). Similarly, the links between zygDNA and the surrounding genome in non-meiotic tissues are double stranded as, in these tissues, the zygDNA replicates with the bulk nuclear genome. The transition of the links to double stranded DNA makes them resistant to breakage by mechanical shearing or digestion with single stranded DNA nucleases. Ribosomal DNA, like the proposed zygDNA, contains a high percentage of guanine and cytosine residues and

therefore also has a high buoyant density. Therefore, ribosomal DNA would also pass through the CsCl cushion during the isopycnic procedure and may be present in the pellet of material extracted for this study. Indeed, a band evident at approximately 20Kb in the preparation of zygDNA described by Ji (1992) may be representative of rDNA. Hybridisation of the DNA extracted by the isopycnic procedure with the probe pMR1, which contains a 9Kb insert from the maize ribosomal operon (Tolocyski and Feix, 1986), indicates that the DNA band is indeed ribosomal DNA (Figure 3.3).

The failure to extract zygDNA from wheat meiocytes by utilising the isopycnic centrifugation procedure may be due to a number of factors. It is possible that zygDNA can be isolated by this procedure but, due to a lack of starting material, only in very small amounts which could not be visualised by gel electrophoresis. This possibility was addressed in two ways. Firstly, the amount of starting material for the isopycnic centrifugation procedure was increased so that DNA was extracted by isopycnic centrifugation from 700mg of florets containing meiocytes at zygotene (almost double the amount of material as used by Ji (1992). This experiment also failed to reveal the expected presence of a DNA species between 5 and 10Kb (Figure 3.4) although the higher concentration of rDNA at the top of the gel reflects the increase in the amount of starting material.

The second approach is based on the hypothesis that, although the yield of zygDNA from wheat may be so low as to be undetectable by gel electrophoresis, it may be sufficient for cloning purposes. Attempts to clone zygDNA extracted by the isopycnic centrifugation procedure were also unsuccessful. A λgt10 library was constructed with DNA extracted from florets containing meiocytes at zygotene and purified by isopycnic centrifugation. Of 5,000 pfu probed with a maize ribosomal DNA operon (pMR1), 40 clones did not hybridise to ribosomal DNA (Figure 3.5). Extraction of DNA from the 40 phage and digestion with *Not*1 reveals that all of the selected clones are non-recombinant (Figure 3.6). This suggests that all of the viable clones obtained from DNA after processing by the isopycnic procedure contain ribosomal DNA inserts. However, the possibility that any zygDNA clones obtained were unstable and hence lost their insert can not be discounted. Indeed Ji (1992) demonstrates that many meiotic clones from wheat are very unstable in a Rec^+ host such as C600*Hfl*$^+$ and may be lost through recombination or deletion. Despite the possibility that zygDNA clones may be unstable in the host used for the cloning method, it is suspected that

Figure 3.2

DNA was extracted from 400mg of florets containing pollen mother cells at premeiotic interphase, leptotene, zygotene - pachytene, diplotene - telophase II and mature pollen using the isopycnic centrifugation procedure described. The DNA was electrophoresed on a 1.2% agarose/TAE gel, stained with ethidium bromide and visualised under UV light. The lanes are as marked and λ *Hind*III is included as a size marker.

Figure 3.3

The DNA extracted from wheat florets (Figure 3.2) was transferred to a Hybond N^+ membrane and hybridised with the ribosomal DNA probe pMR1. The hybridisation reveals that the DNA extracted is homologous with rDNA.

Figure 3.2

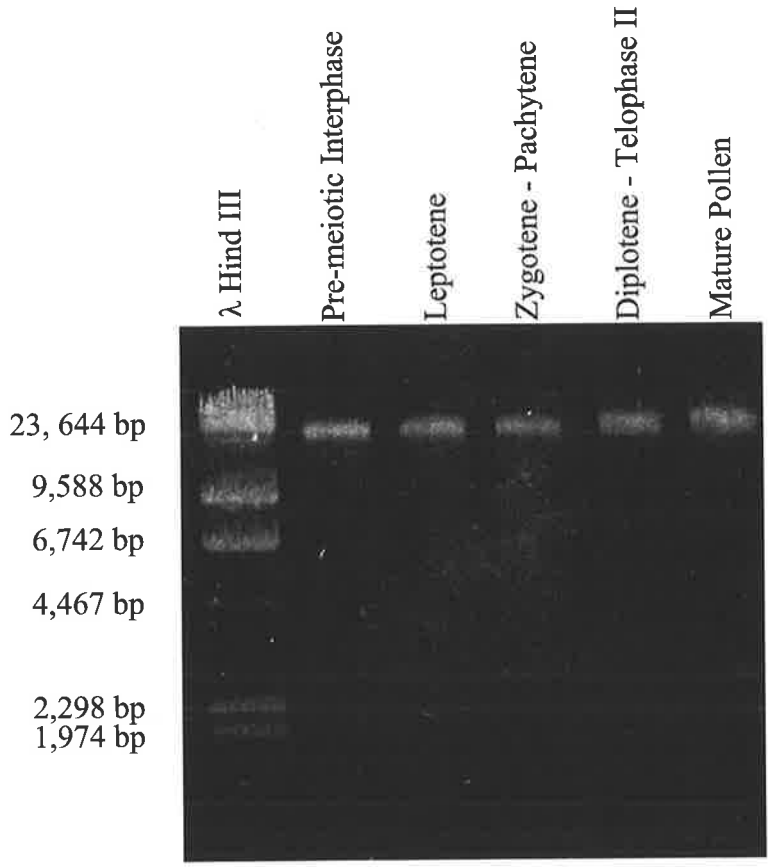


Figure 3.3

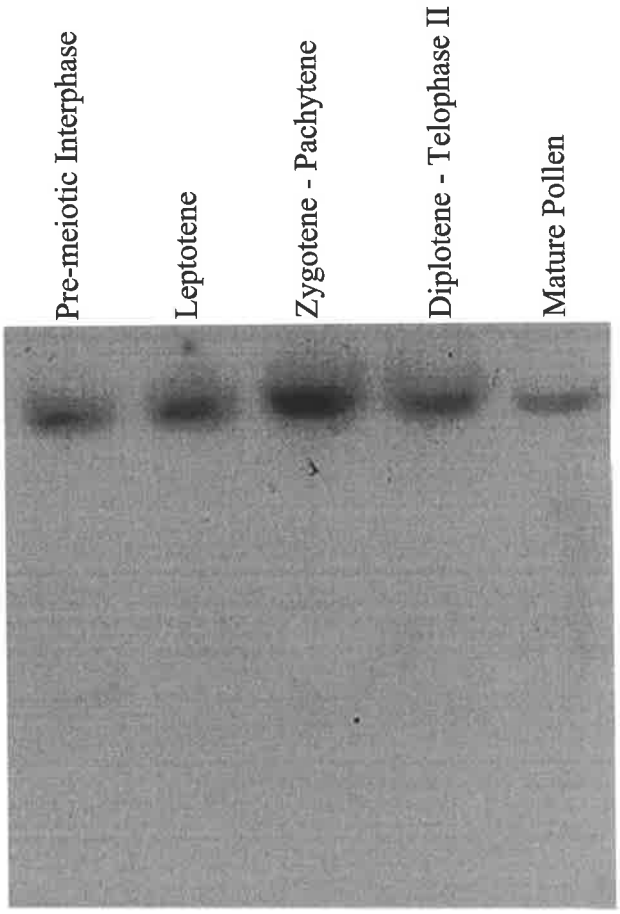


Figure 3.4

DNA was extracted from 700mg of florets containing pollen mother cells at zygotene using the isopycnic procedure described. The DNA was separated by gel electrophoresis on a 1.2% agarose/TAE gel, stained with ethidium bromide and visualised under UV light.
Lane 1: DNA isolated from 400mg of florets
Lane 2: DNA isolated from 700mg of florets

Figure 3.4

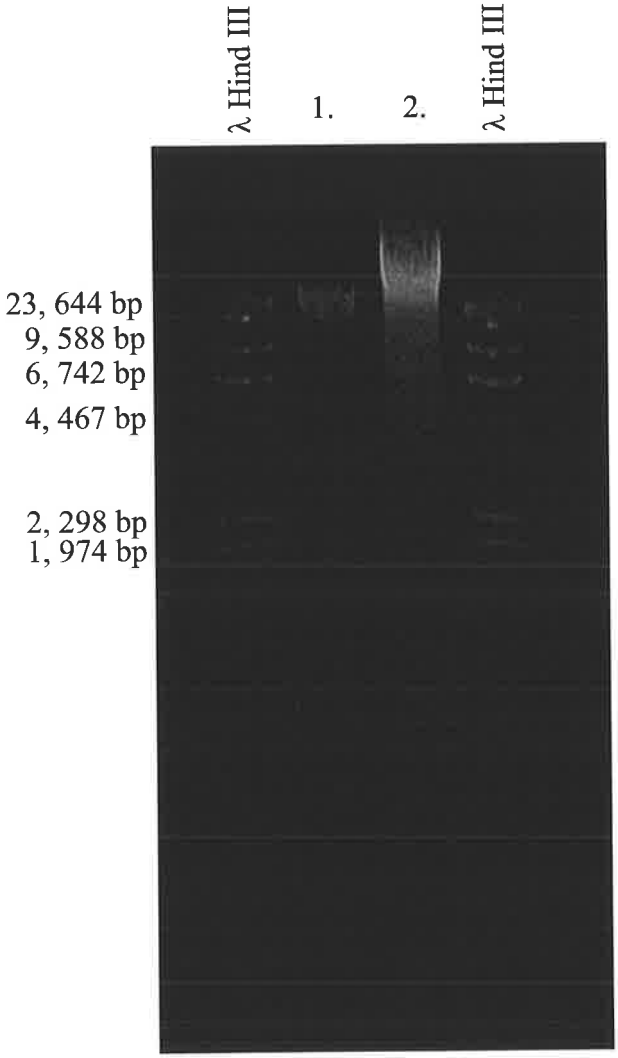

λ Hind III
1.
2.
λ Hind III
23, 644 bp
9, 588 bp
6, 742 bp
4, 467 bp
2, 298 bp
1, 974 bp

Figure 3.5

A bacteriophage library of high bouyant density DNA was constructed in lambda gt10. The phage transferred to Hybond N^+ membranes and were initially screened with the ribosomal DNA probe *pMR1*. Selected clones from the intial screen were plated onto LB plates in a grid fashion. The phage were transferred to Hybond N^+ membranes and hybridised with *pMR1* overnight at 65°C. The membranes were washed to 1X SSC, 0.1%SDS and exposed to X-ray film at -80°C for 5 days. Clones which do not hybridise with ribosomal DNA are highlighted by the arrows.

Figure 3.5

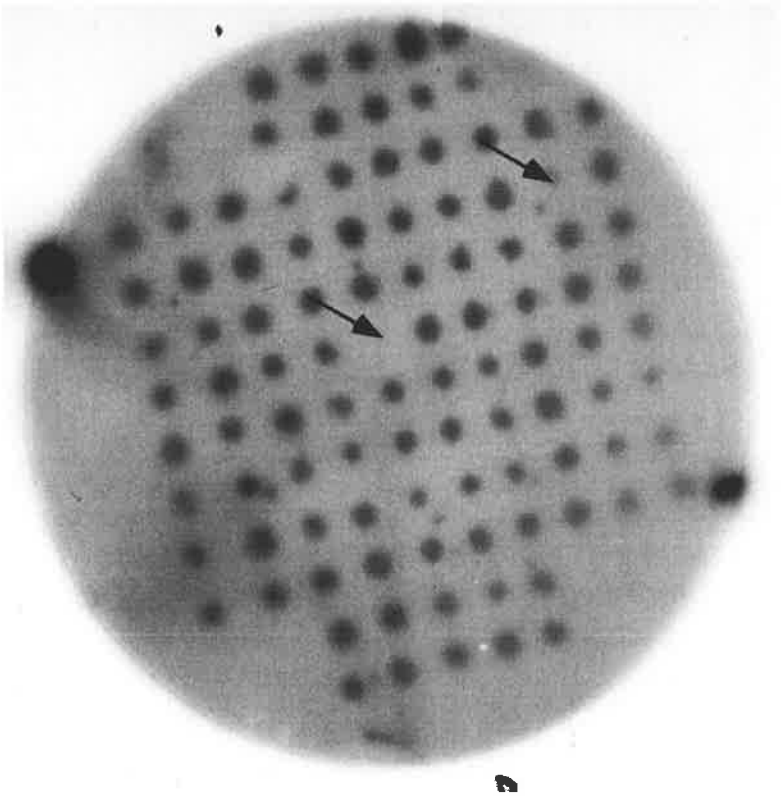
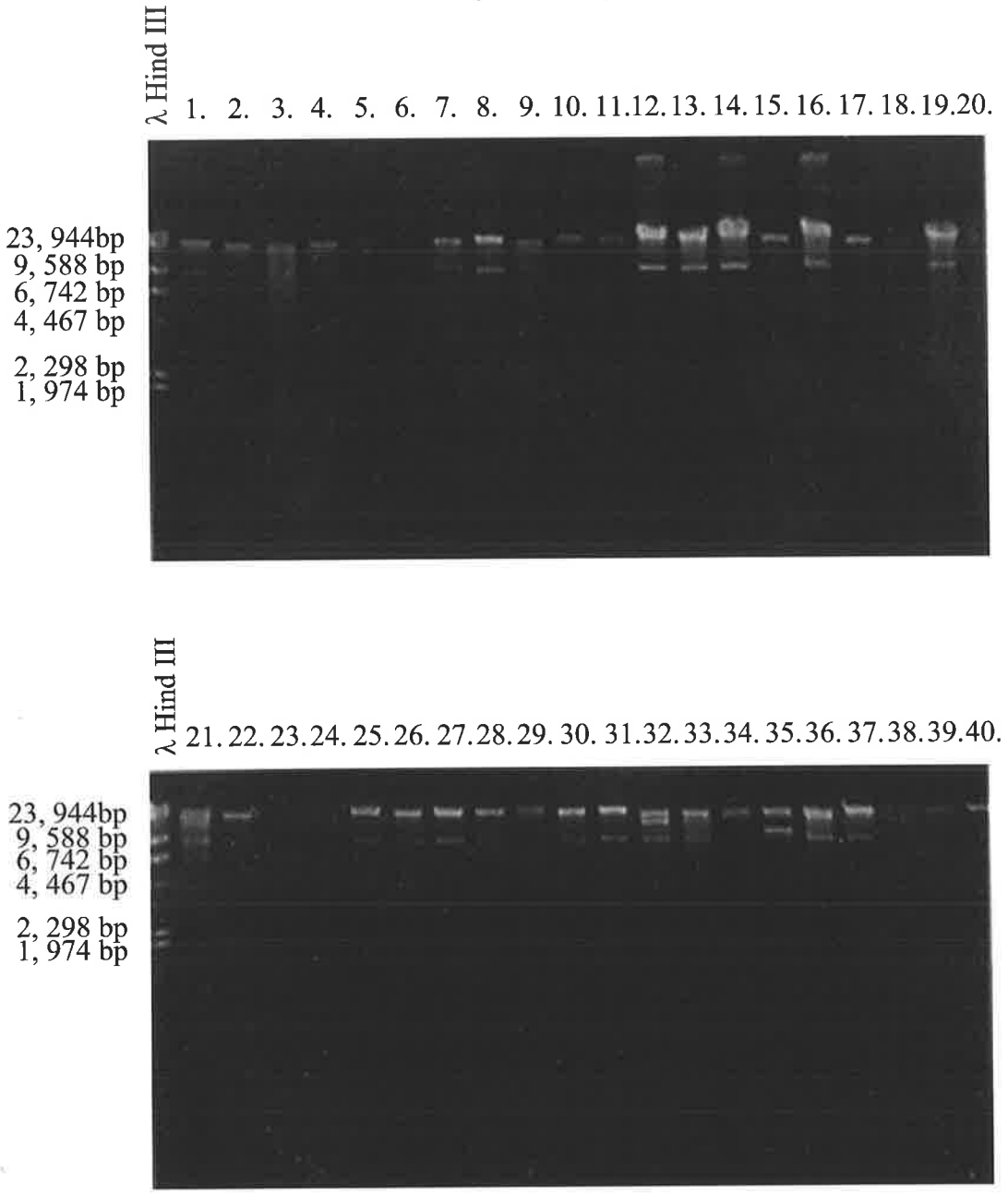


Figure 3.6

DNA was extracted from phage which did not hybridise with a ribosomal probe *pMR1*. The DNA was digested with *Not*1 for 3 hours at 37°C and the fragments separated by electrophoresis on a 1% agarose/TAE gel. DNA was stained with ethidium bromide and visualised under UV light. λ *Hind*III is included as a size marker. No recombinant phage are evident.

Figure 3.6


λ Hind III
1. 2. 3. 4. 5. 6. 7. 8. 9. 10. 11. 12. 13. 14. 15. 16. 17. 18. 19. 20.
23, 944bp
9, 588 bp
6, 742 bp
4, 467 bp
2, 298 bp
1, 974 bp
λ Hind III
21. 22. 23. 24. 25. 26. 27. 28. 29. 30. 31. 32. 33. 34. 35. 36. 37. 38. 39. 40.
23, 944bp
9, 588 bp
6, 742 bp
4, 467 bp
2, 298 bp
1, 974 bp

the isopycnic centrifugation method for the isolation of zygDNA from wheat was unsuitable. However, it is possible that zygDNA may be extractable for only a short period during zygotene. Zygotene in lily extends for between 2 and 5 days (Ito and Stern, 1967) and zygDNA replication may only occur during part of this period. Therefore the temporal window in which zygDNA is extractable may be much shorter than the overall duration of zygotene. In wheat, the duration of zygotene is only 3.4 hours (Bennett *et al.*, 1971). Hence the period of time in which zygDNA is extractable may be very short. It is plausible that the material collected for this experiment, whilst covering a wide range of meiotic stages, was not at a stage where zygDNA could be extracted by an isopycnic procedure.

Alternative explanations for the apparent failure to release zygDNA from wheat meiocytes using the isopycnic procedure are based on possible errors in the assumed structure of late replicating DNA in wheat.

Whilst the buoyant density of bulk nuclear DNA in both lily and wheat is very similar, the assumption that zygDNA in wheat would mimic zygDNA in lily by having a higher buoyant density then the surrounding genome (Ji, 1992) may not be accurate. Should the buoyant density of zygDNA in wheat be lower than the density of the CsCl cushion used for the centrifugation, it would remain in the supernatant with the bulk nuclear DNA and not be detectable in the DNA sample pelleted during centrifugation. This possibility was addressed by separating lightly sheared DNA, extracted from zygotene material, by centrifugation through a continuous CsCl gradient. Fractionation of the CsCl gradient (Figures 3.7a and 3.7b) demonstrated that zygDNA was not detectable in the higher buoyant density fractions of the gradient. A high concentration of DNA in the lower density portion of the gradient probably represents the bulk genomic DNA but may contain late replicating DNA which would be masked by bulk nuclear DNA of a similar density. The presence of rDNA in the higher density fractions indicates that the density range of the gradient was appropriate to separate zygDNA if it is of higher density than the bulk of the wheat genomic DNA. The results suggest that zygDNA, if present in wheat, is of a similar density to the surrounding genome and hence, during fractionation of a continuous CsCl gradient, its presence is masked by the bulk genomic DNA.

A second possible error in the assumption of the structure of late replicating DNA in wheat focuses on the links between the late replicating DNA and the surrounding genome. In lily,

Figure 3.7a

Aliquots of DNA from the fractionation of continuous CsCl density gradient were precipitated overnight and resuspended in 50µl of nanopure water. 15µl of each sample which contained DNA was subjected to gel electrophoresis on a 1% agarose/TAE gel. The order of the lanes is such that lane 1 represents DNA from the top of the gradient whilst lane 13 contains DNA in an aliquot from the bottom of the gradient. The lanes in between represent sequential 200µl aliquots of the gradient. DNA was stained with ethidium bromide and visualised under UV light. λ *HindIII* is included as a size marker.

Figure 3.7b

DNA from aliquots represented by lanes 1 to 9 in figure 3.7a was diluted 1:25 with nanopure water. 15µl of the diluted DNA was separated by electrophoresis on a 1% agarose/TAE gel. DNA was stained with ethidium bromide and visualised under UV light. λ *HindIII* is included as a size marker.

Figure 3.7a

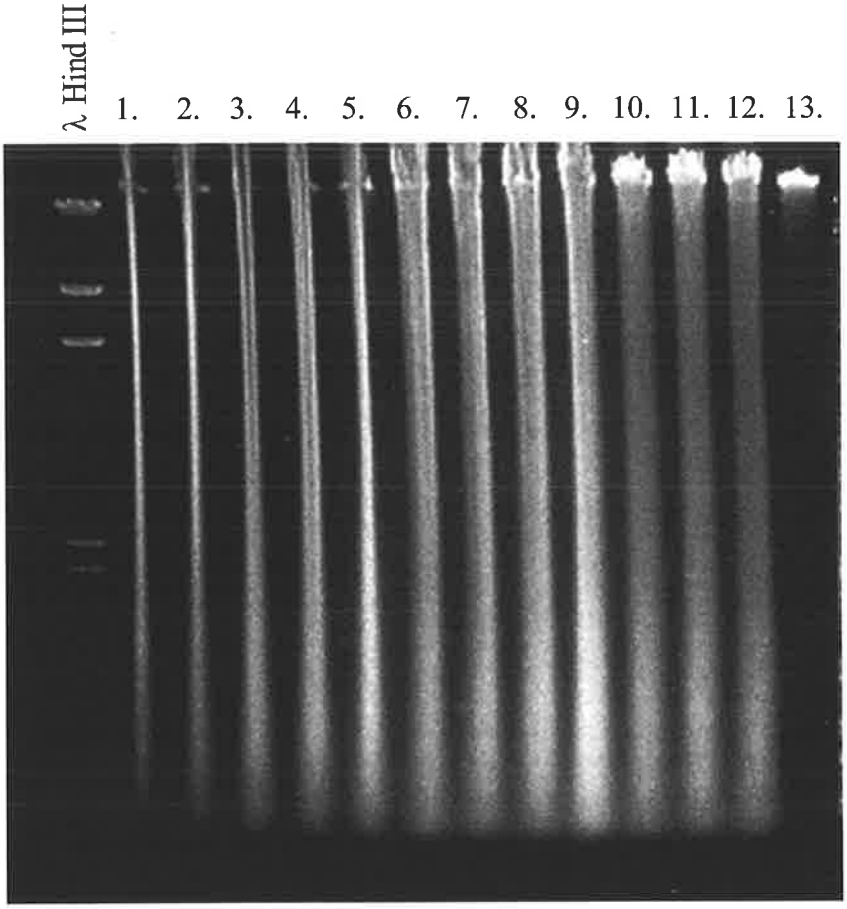
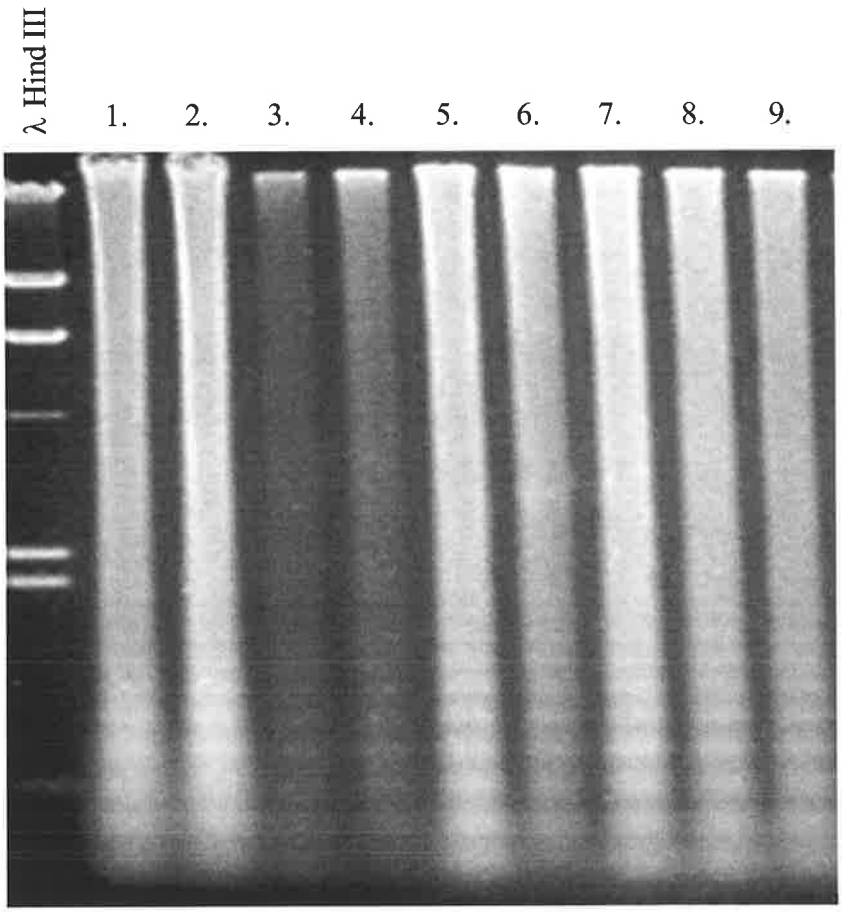


Figure 3.7b



zygDNA is linked to the surrounding DNA by single strand links, as evidenced by the ability to release it by mechanical shearing (Hotta and Stern, 1976) or treatment with S1 nuclease (Hotta *et al.*, 1984). DNA preparations made by lysis of protoplasts, which minimises shearing of the DNA, do not release zygDNA presumably because the DNA remains linked to the remainder of the genome. Furthermore, "curing" of the single stranded links by incorporating nucleotides also caused a loss of satellite behaviour of zygDNA during centrifugation (Hotta and Stern, 1976). If single stranded links are not a characteristic of late replicating DNA in wheat, shearing of the DNA would be ineffective in releasing the fragments before centrifugation. Two approaches were used to test for the presence of single strand links in late replicating DNA of wheat; digestion with Mung Bean Nuclease I, and incorporation of labelled nucleotides into the DNA.

Mung Bean Nuclease I enzymatically degrades extended stretches of single stranded DNA in preference to double stranded DNA (Kedzierski *et al.*, 1973). If late replicating DNA in wheat contains single stranded DNA links to the surrounding genomic DNA, the links could be degraded by treatment with mung bean nuclease I, leaving the surrounding DNA intact and separable by gel electrophoresis. DNA prepared with minimal shearing, was digested with a range of concentrations of Mung Bean Nuclease I. Subsequent separation of the DNA by gel electrophoresis did not reveal any bands which may be representative of zygDNA (Figure 3.8). The high molecular weight, indistinct bands in all of the preparations, but especially evident in the DNA digested with the lowest concentration of nuclease, are indicative of DNA which has undergone very little degradation. This demonstrates that not only has the DNA been extracted with very little shearing, but that there are very few sites in the genomic DNA which are susceptible to digestion with Mung Bean Nuclease I. The results of this experiment support the proposal that, in wheat, the links joining late replicating DNA to the surrounding DNA are not susceptible to digestion with Mung Bean Nuclease I and therefore are not single stranded DNA. This observation is supported by experiments aimed at incorporating labelled dCTP into any single stranded gaps in DNA isolated from zygotene florets.

Should late replicating DNA in wheat be joined to the remainder of the genome by single stranded links, the gaps could be filled using the Klenow fragment of DNA polymerase I and labelled with α-^{32}P-dCTP. Assuming that approximately half of the overhangs generated by shearing of single stranded DNA would have 5' overhangs, the ends of late

Figure 3.8

DNA was isolated from florets containing pollen mother cells at zygotene with a minimum of shearing. 10μg of the DNA was digested with 5U/μg of mung bean nuclease I (Lane 1) 10U/μg of mung bean nuclease I (Lane 2) or 15U/μg of mung bean nuclease I (Lane 3) for 20 minutes at 37°C. The DNA fragments were separated by gel electrophoresis on a 1% agarose/TAE gel, stained with ethidium bromide and visualised under UV light. λ*Hind III* is included as a size marker.

Figure 3.9

DNA, isolated from 100mg of zygotene florets and 200mg of leaf material, was sheared by vortexing then end-labelled with α-^{32}P-dCTP. The DNA was fractionated on a 1.0% agarose/TAE gel then transferred to a Hybond N^+ nylon membrane. The membrane was washed in 5X SSC for 5 minutes than exposed to X-ray film for 1hour at -80°C.

Figure 3.8

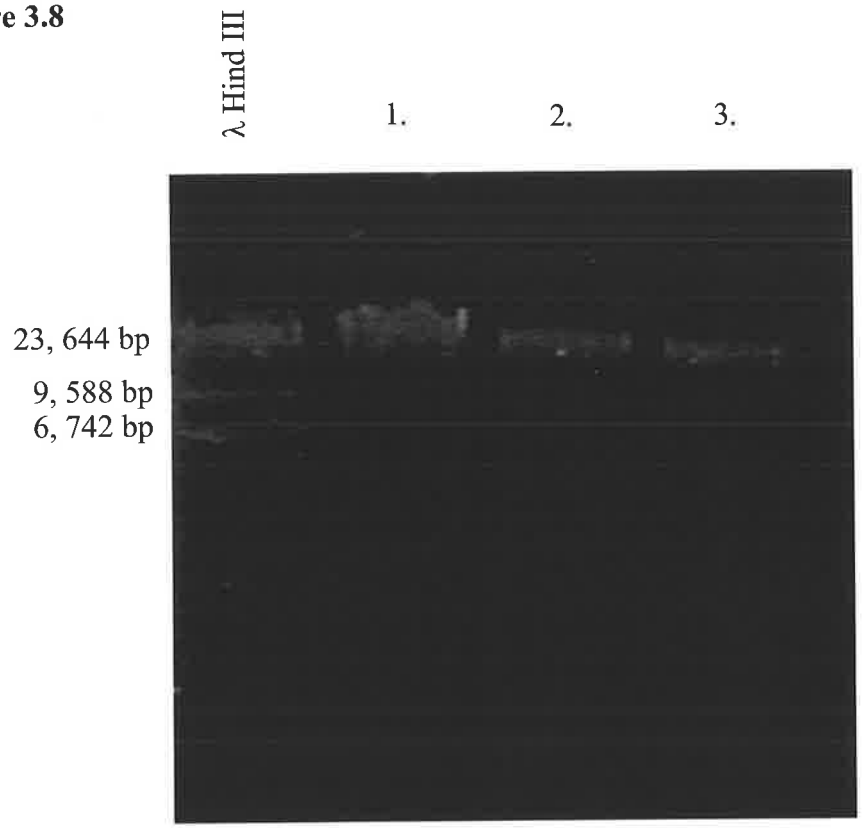
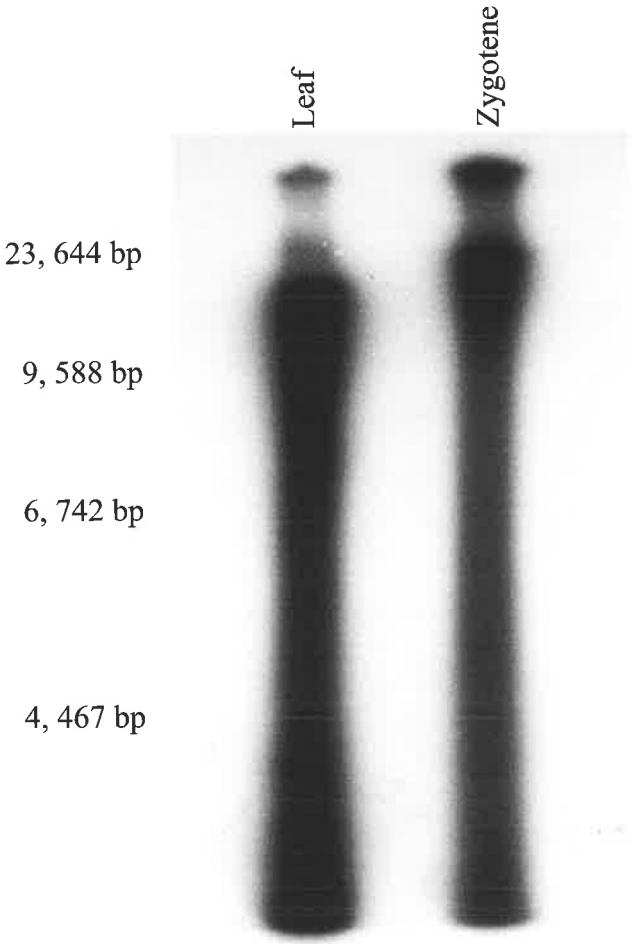


Figure 3.9



replicating DNA molecules could be tagged radioactively. The shearing of DNA extracted from both meiocytes at zygotene and from leaf would inevitably result in DNA molecules of highly diverse size containing guanine residues in the 5' overhangs in both DNA populations. It was postulated that the shearing of DNA from leaf would be random and hence, end labelling of the DNA could be detected as a smear of signal coming from the incorporation of label into randomly sheared bulk nuclear DNA. If zygDNA in wheat was flanked by single stranded links, then the shearing of DNA in this population would not be completely random as the single strands would prove to be more susceptible to mechanical disruption. It was postulated that the zygDNA molecules should then be visible as a band, representing a discrete population of DNA bordered by single stranded DNA, in a background smear of randomly sheared genomic DNA. Incorporation of α-^{32}P-dCTP into sheared DNA from leaf and zygotene florets showed no major differences between the two DNA sources (Figure 3.9). This result may indicate that zygDNA in wheat is not flanked by single stranded DNA linkers, as a discrete band is not present in the labelled zygotene DNA preparation. However, the smear of labelled DNA in the zygotene preparation may mask the presence of faint bands which might represent zygDNA.

3.4 Conclusion

On the basis of the above results, one would assume that late replicating DNA in wheat, if it exists, shares little similarity with the zygDNA of lily. However, the observations are based on a single assumption, that late replicating DNA in wheat is of a uniform size similar to zygDNA of lily. If it is considered that late replicating DNA in wheat is of random length, then it is unlikely that it can be visualised either by gel electrophoresis or radiolabelling. It can be argued that construction of the λgt10 library would allow the cloning of random length zygDNA. However, the cloning technique employed was reliant on the assumption that zygDNA is of high density and is extractable by the method outlined by Ji (1992). This assumption, to date, appears to be inaccurate. The fact that no clones of late replicating DNA were observed in the library suggests that either the structural characteristics of late replicating DNA in wheat are divergent from those of zygDNA in lily, or that all nuclear DNA replication in wheat meiocytes occurs before the onset of prophase I.

An alternative method for examining the possible existence of late replicating DNA in wheat, would be a strictly biochemical approach. Hotta *et al.* (1984) have demonstrated

that in lily, the replication of zygDNA is delayed by the binding of a specific protein, the L protein. This protein may allow the extraction of late replicating DNA from wheat via protein-DNA interactions. However, the validity of the experimental procedure employed for the isolation of L protein has recently been challenged as the zygDNA clones isolated from a L protein-DNA interaction in lily, do not display late replicating characteristics (Jaywardene *et al.*, 1994). Direct isolation of late replicating DNA from wheat by cross hybridisation with zygDNA from lily is also untenable until the replication characteristics of the lily zygDNA clones can be more fully analysed.

Whilst there is little supporting evidence of late replicating DNA outside of lily, many modern researchers still view zygDNA as an important DNA species which may effect a range of meiotic processes. It is suggested that zygDNA may be required for homologue recognition during chromosome pairing, acting to allow the precise checking of homology by gene conversion (Engebrecht *et al.*, 1990). Hollingsworth *et al.* (1990) describe the isolation of the HOP1 protein of *S. cerevisiae* which is required for chromosome pairing and synaptonemal complex formation. Further characterisation demonstrates that HOP1 and the L protein of lily are the same size, and it is possible that HOP1 and the L protein may have similar roles during meiosis and that *S. cerevisiae* may therefore contain zygDNA sequences. The isolation of zygDNA sequences from a model organism such as *S. cerevisiae* would be of great benefit to the understanding of chromosome pairing and synaptonemal complex formation. However, before attempts are made to isolate late replicating DNA from other species, it is important that the significance of the L protein of lily be established. If this is accomplished, homologues of the L protein can be extracted from other organisms, and can in turn be used to isolate late replicating DNA sequences via a protein/DNA interaction system.

Chapter 4

Isolation of meiotic genes from Triticum aestivum

4.1 Introduction

Studies with lower eukaryotes have led to the isolation of genes required for many meiotic processes including chromosome pairing, synaptonemal complex formation and the progressive steps of recombination. In contrast, few genes required for these processes have been isolated from higher eukaryotes.

The cereal, *Triticum aestivum* provides a novel system for the study of chromosome pairing in higher eukaryotes as it displays active genetic control of homologous chromosome pairing. *T. aestivum* is an allohexaploid species consisting of three closely related, homoeologous diploid genomes. Despite the high degree of homology between the genomes, chromosome pairing in *T. aestivum* is restricted to completely homologous chromosomes and the species acts as a diploid during meiosis. The diploidising effect in *T. aestivum* arises from the interaction of a number of genes which both suppress and promote homoeologous pairing (see Chapter 1).

There are several molecular genetic methods which can be employed for the isolation of genes which contribute to the control of chromosome pairing in *T. aestivum*.

4.1.1 Isolation of known homologues

As meiosis is a highly conserved process, genes isolated from, and characterised in lower eukaryotes can be used to screen for homologues in other species. Several yeast genes have a corresponding gene in higher plants. These include *Lim15* of *Lilium longiflorum* which is a homologue of *Rad51* (Kobayashi *et al.*, 1993) and homologues of *Spo11* in *T. aestivum* (Ji, 1992). It is likely that other genes are also common to both lower and higher eukaryotes. Whilst this method provides a rapid approach to the isolation of genes which may contribute to chromosome pairing in higher eukaryotes, it is unlikely to reveal novel features of chromosome pairing in *T. aestivum*. Given that none of the lower eukaryotes currently being studied appears to display genetic control of chromosome pairing, it is

unlikely that a homology screen will result in the isolation of genes controlling homologous pairing in wheat.

4.1.2 Mutation and complementation studies

The majority of meiotic genes isolated from lower eukaryotes have been isolated by a mutation/complementation approach. Strains displaying novel meiotic phenotypes such as defects in chromosome pairing, SC formation or recombination, are produced typically by mutation with chemical agents or by irradiation with X-rays. Transformation of such mutants with DNA from wild type strains and identifying transformants with restored wild type activity allows the isolation of the gene associated with the mutation. This method not only allows the isolation of meiosis specific genes but characterisation of the gene function is assessed during the screening process.

The use of a mutation/complementation approach to the isolation of meiotic genes in *T. aestivum* is difficult. Although transformation of *T. aestivum* is possible, the efficiency of transformation remains low. As the genome of *T. aestivum* is very large, not only would large libraries need to be constructed, but an enormous number of transformations would be required. At the current transformation efficiency of *T. aestivum* this is inviable so that, whilst mutations of the homologous pairing genes are available in *T. aestivum* (see Chapter 1), isolation of the mutated gene by complementation is impractical. An alternative would be to complement fungal meiotic mutants with DNA from *T. aestivum* but, as with homology screening, this is unlikely to result in the isolation of genes novel to *T. aestivum*.

4.1.3 Transposon tagging

In transposon mediated mutagenesis, mutations are induced by the insertion of a transposable genetic element in to the gene of interest (Walbot, 1992). The movement of the transposon is reliant on defined border sequences which can be used to identify the location of the mutation and the mutagenised gene can be isolated by either oligonucleotide screening of a genomic library or by PCR amplification using primers specific to the border regions.

Whilst this method of generating tagged mutations has been extensively employed in some plants such as maize, it has not been widely applied for two major reasons. Transposon

tagging in maize has been successful mainly because active transposons are present in this species and can be utilised for mutagenesis. Most plant species, including *T. aestivum*, lack active forms of transposons, and the degree of technical implementation required to introduce an active transposon makes this approach difficult. In addition, by their very nature, transposons move throughout the genome causing gene mutations. It is often difficult to identify which transposable element, of the many that may be present, has inserted into the gene of interest.

4.1.4 Differential screening

Complementary DNAs (cDNAs) are double stranded DNA molecules produced by reverse transcription of messenger RNA (mRNA) and can be ligated to either phage or plasmid vectors to form libraries. Clones within the library can either be randomly analysed to detect tissue specific clones or the library can be screened with appropriate probes to detect clones of interest. Usually, the amount of mRNA in a given tissue reflects the expression of the gene from which it arose although some genes are regulated at a translational level. The relative abundance of gene expression can therefore be approximated from the amount of mRNA present in a given tissue. Differential screening involves the screening of complementary DNA (cDNA) libraries from materials of interest with reverse transcribed mRNA from other tissues in order to identify genes which are specific to, or more abundant in, one material as compared to the other. In order to identify genes expressed in a particular tissue, an mRNA population is extracted, converted into double stranded cDNA and cloned. The resulting clones are analysed by hybridisation with reverse translated mRNA from the original population as well as populations from differing sources. The relative intensity of the signal each clone emits after hybridisation can be used to gauge the relative abundance of the clone in each mRNA population. Using this approach, genes which are differentially expressed during meiosis have been isolated in higher plants (Appels *et al.*, 1982; Bouchard, 1990; Roberts *et al.*, 1991). The major difficulty associated with this approach, is that it requires extensive quantities of well defined starting material, a requirement which is not easily met in *T. aestivum* due to the structure of the wheat floral organs.

The floral organs of wheat are associated in a spike, the typical ear of wheat. The wheat spike itself is composed of spikelets arranged in an alternate manner along the length of the

spike. Within each spikelet, there are three florets termed the primary, secondary and tertiary florets, arranged with the tertiary floret lying between the primary and secondary. Meiosis in *T. aestivum* is asynchronous, with the spikelets in the middle of a spike being more advanced than those below and above. Meiotic development within a spikelet is also asynchronous. The primary floret of a spikelet is more advance than the secondary floret which in turn is more mature than the tertiary floret. This, in addition to the fact that some of the individual stages of meiosis in wheat can have a duration of less than one hour (Bennett *et al.*, 1971), makes it very difficult to collect well defined material. The staging of many anthers is required to collect a sample of well defined material, and this process is both labour intensive and time consuming.

Several new methods for differential screening have been developed that require only very small amounts of mRNA. One such procedure, which is reliant on the amount of mRNA reflecting gene expression for the isolation of specific cDNA clones, is the technique of differential display (Liang and Pardee, 1992). Essentially, the differential display method utilises an anchored oligo-dT primer for reverse transcription of mRNA. The oligo-dT primer is composed of 11 or 12 T residues plus two additional nucleotides at the 3'end which provide specificity to the primer and allow it to anneal only to a subset of the mRNA population. The primer is then used in conjunction with an arbitrarily defined decamer for PCR amplification of the reverse transcribed cDNAs. Amplified cDNA fragments representing the 3' termini of mRNAs are then separated by size on a polyacrylamide gel. Using this procedure, differences in the amplification of cDNAs from two different mRNA populations can be identified which represent genes with differing expression in the two populations. Differential display has been heavily utilised since its inception because of its simplicity, sensitivity and low RNA requirement. However, the technique does produce a high incidence of false positives, and characterisation of genes identified is labour intensive especially when large scale screening, as would be required in wheat, is used.

Subtractive hybridisation (Kavathas *et al.*, 1984) is a powerful alternative to the process of differential screening as it requires relatively little starting material. Subtractive cloning has been applied to the isolation of genes expressed during meiosis (Kobayashi *et al.*, 1994; Crossley *et al.*, 1995). The technique resembles an *in vitro* form of differential screening as it allows the isolation of genes based on the relative abundance of the mRNA of the gene in a given population. During subtractive hybridisation, cDNAs from a mRNA population to

be studied are hybridised *in vitro* with biotin labelled cDNAs from different mRNA populations. Sequences common to both populations hybridise with each other and can be removed by binding with streptavidin thus enriching the studied population for unique sequences. As subtraction reactions are carried out in small volumes and unlimited supplies of subtracting cDNA can be produced by the polymerase chain reaction, the subtractive hybridisation procedure requires little starting material. In addition, the availability of extensive quantities of subtracting cDNA allow the subtractive hybridisation procedure to be repeated many times on the same cDNA population being analysed so that genes with very low expression can be detected.

In this chapter, a subtractive hybridisation procedure has been utilised for the isolation of cDNA clones from *T. aestivum* specific for early meiotic prophase I. The procedure described is a modified version of the subtractive hybridisation method outlined for the isolation of genes expressed during scrapie infection in hamster (Diguid *et al.*, 1988; Diguid and Dinauer, 1989).

4.2 Methods

4.2.1 Production of a subtractive probe

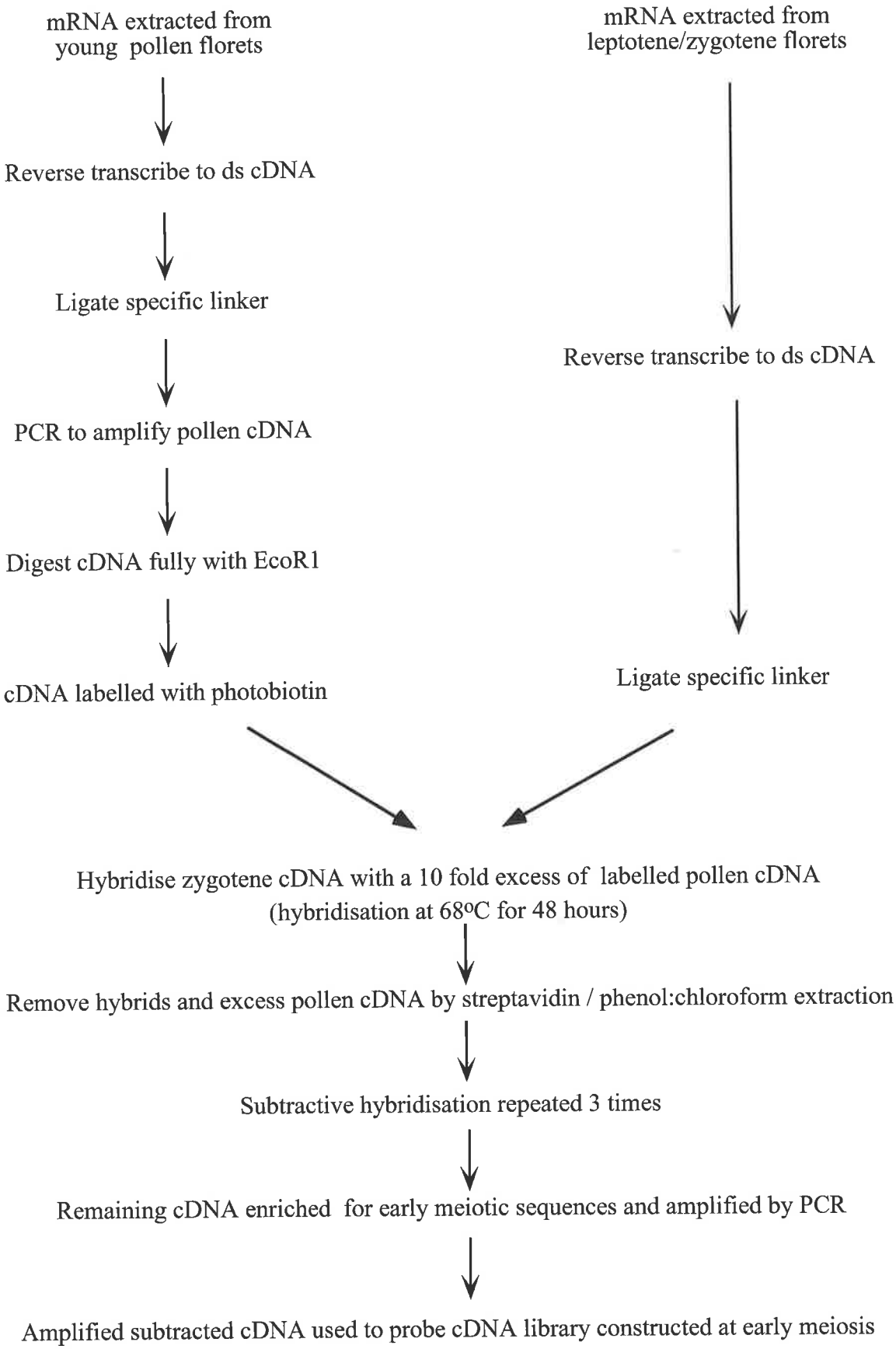
The outline of the procedure employed for the production of an early meiosis subtractive probe is given in Figure 4.1 and is described in detail in Chapter 2.

Briefly, total RNA was extracted from 300 wheat anthers containing meiocytes at leptotene or zygotene and 600 anthers containing immature pollen. Poly (A^+)RNA was isolated from the total RNA populations, converted to double stranded cDNA using a Time Saver cDNA Synthesis Kit (Pharmacia), and ligated to prepared subtractive oligo linkers (Diguid and Dinauer, 1989). The efficiency of the ligation reaction was checked by PCR amplification of both of the cDNA populations using the 21mer as a primer. Following assessment of the ligation reaction, the immature pollen cDNA population was amplified through 32 cycles of PCR and digested with *EcoR1*. The resulting immature pollen cDNA population was biotinylated using photoactivated biotin (Bresatec). The biotin labelling reaction was assessed by transferring a sample of the DNA to a Hybond N^+ membrane and detecting the biotin with streptavidin-alkaline phosphatase.

Figure 4.1

Flow diagram of the subtractive hybridisation procedure applied for the isolation of early meiosis specific clones. The technique is a modified version of the procedure applied by Diguid and Dinauer (1989) for the isolation of clones expressed during scrapie infection in mice.

Figure 4.1


mRNA extracted from young pollen florets
Reverse transcribe to ds cDNA
Ligate specific linker
PCR to amplify pollen cDNA
Digest cDNA fully with EcoR1
cDNA labelled with photobiotin
mRNA extracted from leptotene/zygotene florets
Reverse transcribe to ds cDNA
Ligate specific linker
Hybridise zygotene cDNA with a 10 fold excess of labelled pollen cDNA
(hybridisation at 68°C for 48 hours)
Remove hybrids and excess pollen cDNA by streptavidin / phenol:chloroform extraction
Subtractive hybridisation repeated 3 times
Remaining cDNA enriched for early meiotic sequences and amplified by PCR
Amplified subtracted cDNA used to probe cDNA library constructed at early meiosis

Leptotene/zygotene cDNA was hybridised with a 10 fold excess of biotinylated immature pollen cDNA at 65°C for 48 hours. Hybrid molecules forming between the two cDNA populations were removed by streptavidin/organic solvent extraction as described by Sive and St John (1988). The leptotene/zygotene cDNA was submitted to three rounds of subtractive hybridisation with immature pollen cDNA and then amplified by PCR using the 21mer oligonucleotide as a primer.

4.2.2 Production of a leptotene/zygotene cDNA library

Poly(A^+) RNA (3μg) from anthers containing meiocytes at leptotene and zygotene was used for the production of a cDNA library employing the Time Saver cDNA Synthesis Kit (Pharmacia). *EcoR1/Not1* linkers ligated onto the ends of the cDNA allowed ligation into the *EcoR1* site of bacteriophage vector λgt10 (Huynh *et al.*, 1984). The ligation, packaging of phage and titre of the library was performed as described with *E. coli* strain C600*Hfl*$^-$ being employed as a host for transfection of the library. The cDNA library was plated onto 15cm LB plates at a density of 5,000 pfu per plate for screening.

4.2.3 Screening of a cDNA library with the subtractive probe

$3.1x10^5$ pfu of the leptotene/zygotene cDNA library were transferred to Hybond N^+ membranes and hybridised overnight at 65°C with the subtractive probe. Positive clones were purified by a secondary screening of diluted phage then amplified by PCR. Each clone was subjected to a third screen by hybridisation of the amplified insert with the subtractive probe at high stringency. Positive clones were identified from the third round screen and a subgroup of the clones selected for further analysis. Inserts were ligated into the *Not*1 cloning site of pBluescript (KS^-) or pTZ18U and further analysed by Northern hybridisation and sequencing.

4.2.4 Northern hybridisation using the selected clones

Total RNA was extracted from wheat anthers containing meiocytes at pre-meiotic interphase (300 anthers), leptotene (300 anthers), zygotene (310 anthers), pachytene - metaphase I (330 anthers), anaphase I - telophase I (280 anthers), meiosis II (290 anthers), tetrads (300 anthers), immature pollen (300 anthers), mature pollen (240 anthers), 3 day old root tips (30mg) and leaf (60mg). The RNA was separated by denaturing gel

electrophoresis and transferred to Hybond N^+ membranes. Inserts of the clones to be characterised were isolated from the plasmid vector, by digestion with *Not*I, and used as probes for hybridisation with the membranes.

4.2.5 Sequencing of selected clones

Sequencing reactions were performed utilising Applied Biosystems materials and incorporating the M13 forward and reverse priming sites of pBluescript (KS^-) or pTZ18U.

4.3 Results and Discussion

4.3.1 Production of the early meiosis probe

The production of a subtractive probe requires the performance of many molecular biological techniques including nucleic acid extraction, ligation, PCR amplification and biotin labelling. In order to produce a subtractive probe, it is essential that each of the constituent techniques be employed successfully. It is therefore necessary to regularly determine the effective accomplishment of each step of the subtractive hybridisation procedure.

The preliminary step in production of a subtractive probe is the isolation of total RNA from the two or more tissues to be incorporated in the hybridisation. For this experiment total RNA was extracted from anthers containing meiocytes at leptotene and zygotene and anthers containing immature pollen. Twice the amount of starting material was used for the isolation of RNA from immature pollen as compared to the early meiosis material. Despite this, the early meiosis material yielded a total of 282µg of RNA whilst 270µg of RNA was extracted from the immature pollen. This reflects higher gene expression during early meiosis than at the latter stages of meiosis, probably due to the large number of proteins which are required for recombination, chromosome pairing and synaptonemal complex formation. Separation of a sample of RNA from both populations on a denaturing gel (Figure 4.2) reveals that the RNA is of high quality as no degradation of the ribosomal RNA is apparent.

Following the extraction of high quality total RNA from early meiosis and immature pollen florets, poly (A^+) RNA was isolated from both populations and converted into double

stranded cDNA with subtractive oligolinkers ligated to both ends. As the oligolinker is composed of a 21mer and a 25mer, the 21mer can be used as a primer for PCR amplification of the cDNA. The smear of products resulting from PCR amplification of both cDNA populations (Figure 4.3) demonstrates that the cDNA synthesis from both RNA populations has been successful and that the cDNA is ligated at both ends to the subtractive oligolinkers. It can also be concluded that the majority of cDNA molecules in the early meiosis population are between 500bp and 1.2Kb in length indicating that numerous cDNAs in the population probably represent full length sequences. Having demonstrated the effective amplification of both cDNA populations, the immature pollen population was amplified to approximately 30μg of DNA. The advantage of the subtractive hybridisation procedure is that very little starting material is needed as both cDNA populations can be amplified by PCR to yield an unlimited supply of cDNA. Whilst this is theoretically possible, practically it has been observed that the process of PCR amplification tends to select for shorter length cDNAs so that the population will eventually become biased and unrepresentative after continued amplification. To minimise this problem, three separate amplifications of the immature pollen were undertaken in preference to the re-amplification of a single reaction. It is proposed that by using this procedure, most of the cDNAs present in the immature pollen population will be represented in the amplified mixture.

The amplified immature pollen cDNA was labelled with biotin using photo-activatable biotin. Although biotin could be incorporated into the DNA by synthesis of labelled strands using the Klenow fragment of DNA polymerase I or by direct incorporation of a biotin labelled nucleotide during the PCR amplification, the use of photo-activated biotin labelling allows the rapid incorporation of biotin into the DNA without the need for denaturation and production of newly synthesised DNA strands. The photoactivated procedure also allows for incorporation of a biotin molecule onto more than one type of nucleotide and provides some control over the degree of labelling. A biotin molecule was introduced to the immature pollen cDNA at approximately every 50bp. Higher rates of labelling were not considered because of the possibility that high levels of incorporated biotin in the immature pollen cDNA may adversely effect the hybridisation with the early meiosis cDNA. A sample of the labelled cDNA electrophoresed on a 1.2%TAE/agarose gel, transferred to a nylon membrane and detected with streptavidin-alkaline phosphatase demonstrates that the immature pollen has been effectively labelled with biotin (Figure 4.4).

Figure 4.2

Denaturing gel electrophoresis of RNA - Total RNA was extracted from anthers containing pollen mother cells at early meiosis and immature pollen. An aliquot of the RNA (2µl) was separated by denaturing gel electrophoresis to assess its quality. The RNA appears to be of high quality with little degradation of the 25S and 18S ribosomal RNA bands marked.

Figure 4.3

PCR amplification - Samples (2µl) of the immature pollen and early meiotic cDNA populations were amplified by PCR after ligation of the subtractive oligo linkers. 5ml of the resulting DNA was separated by gel electrophoresis on a 1.2% TAE/agarose gel run at 60mA. Smears of amplified products are apparent in both populations consistent with the amplification of a mixed size population. The amplification indicates that both the cDNA synthesis and the ligation of oligo linkers to the cDNA has been successful.

Figure 4.2

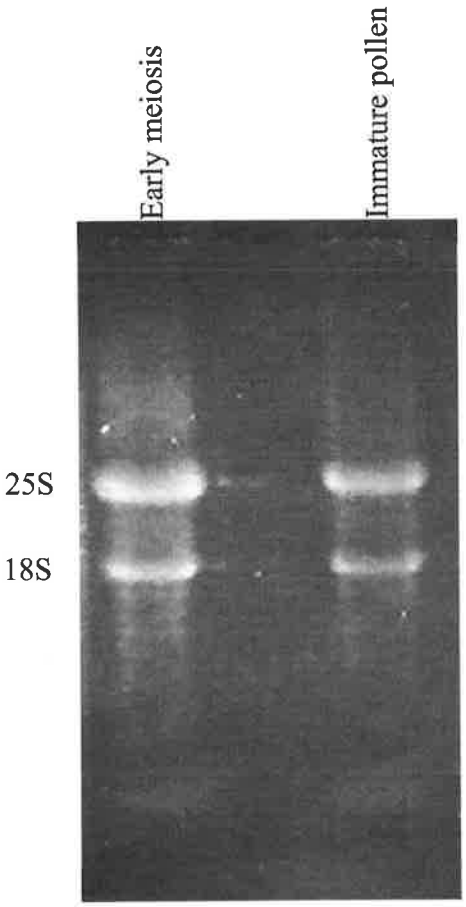
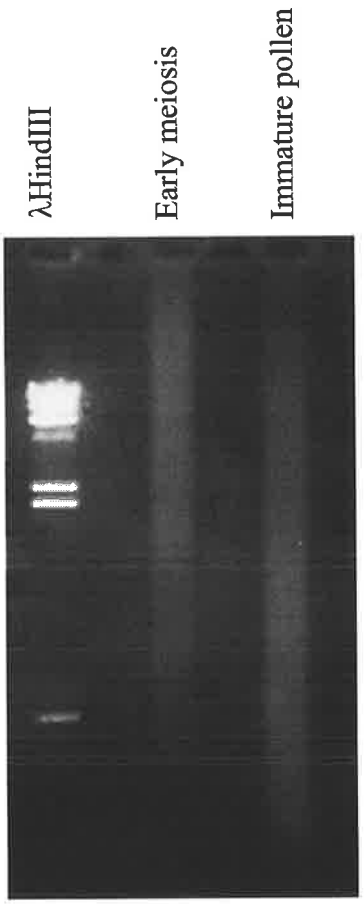


Figure 4.3



The subtractive hybridisation was carried out at 65°C and in 500mM NaCl. It is noted that the degree of hybridisation during the subtractive procedure can be altered by varying the hybridisation temperature or salt concentration. It is also evident that more than one tissue could be used for the subtractive hybridisation so that early meiosis cDNA might be hybridised with both immature pollen cDNA and cDNA from another source. By controlling the parameters of the hybridisation and also by including cDNA from several sources, it is theoretically possible to obtain a probe which is highly specific for a restricted period of meiosis. To this extent, subtractive hybridisation with root tip cDNA after the hybridisation with immature pollen cDNA was considered. Root tips contain cells which are actively undergoing mitosis, so that subtraction with root tip cDNA would provide a probe which is specific for meiosis. This consideration was eventually dismissed as many genes which are thought to be essential for the early events of meiosis are also required for mitosis. Hence, subtraction with root tip cDNA may remove some of the cDNAs which are the target of this research.

Following the subtractive hybridisation, a sample of the remaining early meiosis cDNA population and a sample of *Eco*R1 digested immature pollen cDNA was amplified using the 21mer as a primer. Gel electrophoresis of both samples of the amplified DNA (Figure 4.5) indicates that the average length of the cDNA in the population is shorter than the original, being between 300 and 500 base pairs. The immature pollen cDNA fails to amplify as expected because digestion with *Eco*R1 cuts in the middle of the oligo linker and therefore removes the priming site for the 21mer. The remaining subtracted cDNA was amplified so that any immature pollen cDNA not removed by the streptavidin treatment would be under-represented in the population due to the removal of the 21mer priming site.

It is possible that the remaining cDNA in the early meiosis population could be directly cloned and analysed. However, due to the relatively short length of most of the subtracted cDNAs present, it was believed that using the subtracted cDNA as a probe to screen a cDNA library would be more effective in isolating longer length clones. Whilst this method overcomes the problems associated with isolating longer length clones, the small size of the subtracted cDNA may give rise to a certain degree of cross hybridisation with non-meiotic clones which share homology.

Figure 4.4

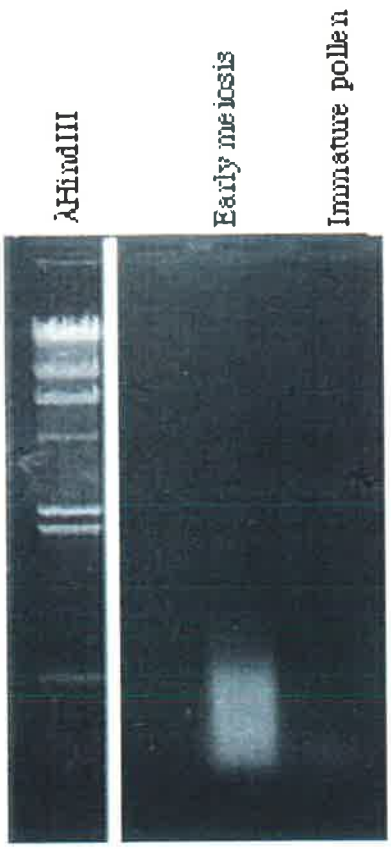
Biotin detection - Immature pollen cDNA, labelled with photo-activated biotin, and labelled control DNA (Bresatec) were separated by gel electrophoresis and transferred to a Hybond N^+ membrane. Incorporated biotin was detected using a modification of the streptavidin - alkaline phosphatase detection procedure (GIBCO-BRL). Efficient labelling of the control and the immature pollen cDNA is demonstrated.

Figure 4.5

PCR amplification - Subtracted cDNA and *Eco* R1 digested immature pollen cDNA were amplified by PCR using the 21mer as a primer. 5µl of each reaction was separated by gel electrophoresis on a 1.0% TAE/agarose gel. The subtracted cDNA is amplified and has an average length less than 500bp. The immature pollen cDNA does not amplify because the *Eco* R1 digestion removes the priming site.

Figure 4.4

Figure 4.5



4.3.2 Production of an early meiosis cDNA library

The immunity insertion vector λgt10 was chosen for the construction of the early meiosis cDNA library. λgt10 contains a single *Eco*R1 site within the phage repressor gene *imm434* (Huynh *et al.*, 1984) such that inserts into the *Eco*R1 site generate phage which form plaques with a clear centre. Phage without a disruption of the *imm434* gene form turbid plaques hence recombinant phage can be identified on the basis of their morphology. By utilising C600*Hfl*⁻, which carries the high frequency lysogeny mutation *hflA150* (Hoyt *et al.*, 1982), as the *E. coli* host strain for λgt10, non-recombinant phage can be eliminated because the *hflA150* mutation represses growth of phage with an intact *imm434* gene so efficiently that a plaque is not formed (Winnacker, 1987). The titre of each of three different ligation reactions, incorporating different ratios of insert and vector, are displayed in Table 4.1. Reaction number one gives approximately 6.1×10^5 pfu/ml with the resulting library being approximately 3.1×10^5pfu.

Table 4.1 Titration of the early meiosis cDNA library prepared in λgt10 and plated using *E. coli* C600*Hfl*⁻ as the host.

Insert:Vector Dilution	3:1	2:1	1:1	Negative control
1:10	Confluent	Confluent	Confluent	5
1:100	Confluent	~ 1,000	280	0
1:1,000	~ 590	9	35	0
1:10,000	61	0	0	0

4.3.3 Screening of the cDNA library

Following screening of the early meiosis cDNA library (3.1×10^5 pfu) with the subtractive probe, 94 positive clones were identified, numbered 1 to 94 and purified by a second, low density screen with the subtractive probe. Fourteen clones, 10, 20, 26, 29, 41, 42, 43, 45, 53, 54, 57, 65, 71 and 80 did not hybridise with the subtracted probe in the second screening. There are two possible explanations for this. The positive clones may not have been present on the plate after dilution of the original phage solution. In this case the phage would still be present in the original solution and could be isolated by repeating the

secondary screening procedure. A second possibility is that the positive inserts have been lost from the vector. The recombination systems of both the vector and the host can cause sequence rearrangements or loss of the insert during the initial and subsequent platings (Watson and Jackson, 1985). As C600*Hfl*$^-$ is *recA*$^+$ and λgt10 is *red*$^+$ it is possible that the influence of the two recombination systems causes instability of some sequences. Indeed, Ji (1992) reports on the instability of some meiotic clones from wheat when present in vectors and hosts capable of recombination.

The 80 positive clones isolated in the second screen were further analysed by PCR amplification to determine their length. The clones were also subjected to a final screen of the amplified inserts by hybridisation with the subtracted probe at high stringency. The results of the third screen are outline in Table 4.2.

It must be noted that the size of the inserts given in Table 4.2 are actual sizes and not the size of the PCR product as determined by gel electrophoresis. Because the λgt10 primers used for the amplification anneal to the phage approximately 50 bp outside of the *EcoR1* site, the PCR products are approximately 100bp longer than the length of the insert.

The results of the hybridisation shown in Table 4.2 indicate that most of the clones amplified hybridise with the subtracted probe. However, some of the PCR amplified inserts fail to hybridise with the subtractive probe when the hybridisation is carried out at high stringency. This may arise through loss of the insert as described above but more probably reflects a lower degree of homology between these clones and the subtractive probe.

The PCR amplification of boiled whole phage allows a rapid determination of the size of the isolated clones. However, because the amplification does not utilise purified phage DNA as a template, the contaminating proteins may cause an appreciable degree of inhibition of the PCR amplification. This appears to be true for several of the clones (11, 17, 23, 27, 37, 82, 86, 88 and 91) which show either limited amplification during PCR or result in a smear of products, but which are still highly homologous to the subtracted probe as determined by hybridisation. Digestion with *Not*1 of DNA extracted from some of these phage and subsequent separation of the DNA fragments be gel electrophoresis (Figure 4.6) shows that most carry inserts of a large size. This confirms that the absence of a discreet band following PCR amplification was due to poor amplification and not because the phage were

Table 4.2 Outline of the clones selected from the secondary screening of an early meiosis, wheat cDNA library. The inserts were amplified by PCR then hybridised with the subtractive probe at high stringency.

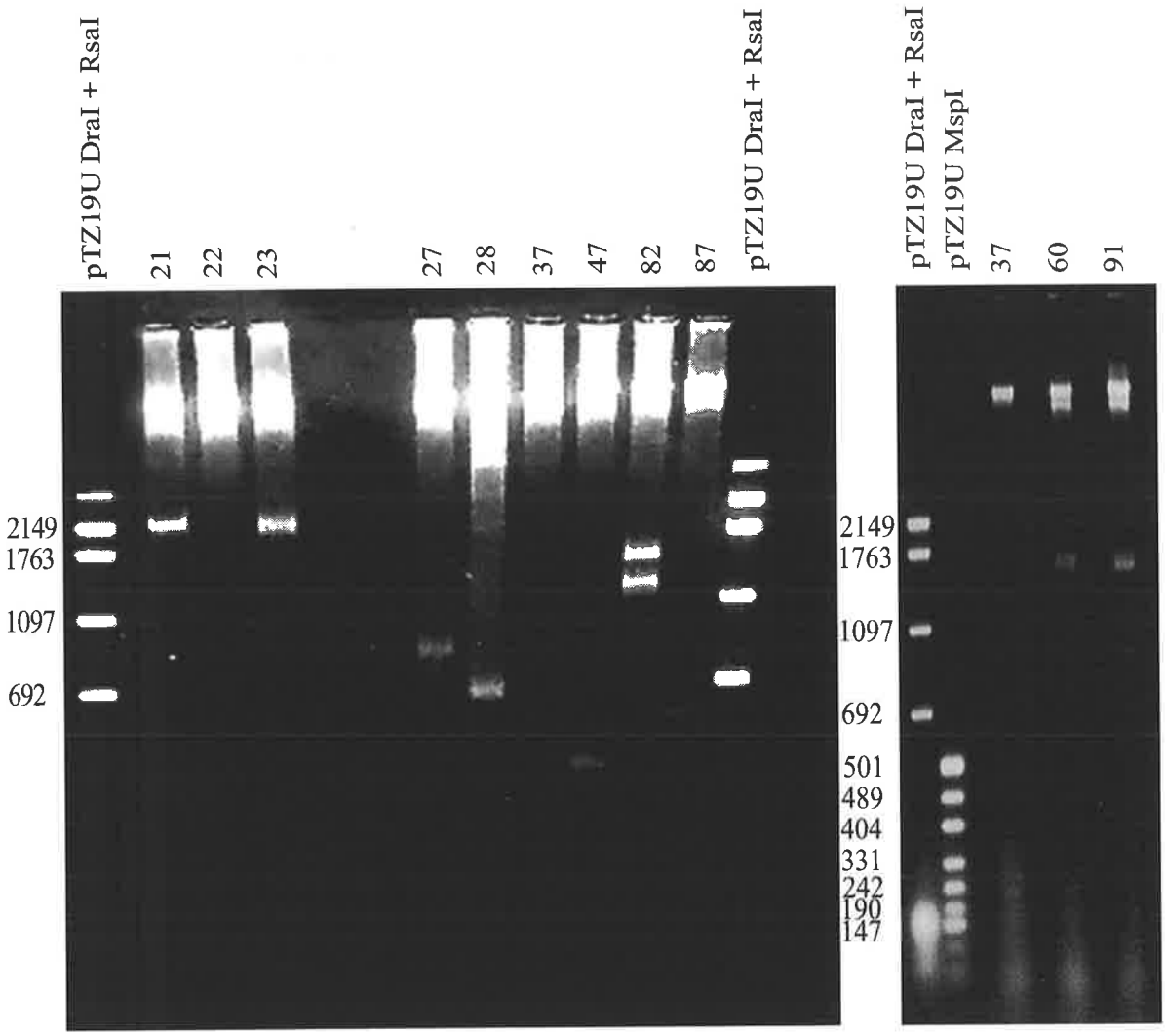
Clone	Amplification	Approximate size (bp)	Hybridisation with Subtractive Probe
1	Yes	200	No
2	Yes	250	Yes
3	Yes	200	No
4	Yes	150	No
5	Yes	400	Yes
6	No	-	-
7	Yes	200	No
8	Yes	250	No
9	Yes	250	No
11	Smear		Yes
12	No	-	-
13	Yes	270	Yes
14	Yes	100	No
15	No	-	-
16	Yes	200	Yes
17	Smear		Yes
18	Yes	100	No
19	Yes	450	Yes
21	Smear		No
22	No	-	-
23	Smear		Yes
24	No	-	-
25	Yes	500	Yes
26	No	-	-
27	Smear		Yes
28	No	-	-
29	Yes	330	Yes
31	Yes	800	Yes
32	Yes	450	Yes
33	Yes	270	Yes
34	Yes	280	Yes
35	Yes	100	No
37	Smear		Yes
38	No	-	-
40	Yes	280	Yes
41	Yes	250	Yes
42	Yes	330	Yes
43	Yes	280	Yes
44	Yes	100	No
45	Yes	280	Yes
46	Yes	250	Yes
47	Yes	500	Yes
48	Yes	200	No
49	Yes	270	Yes
50	Yes	550	Yes
54	Yes	480	Yes
56	Yes	150	Yes
57	Yes	100	No
58	Yes	280	No
59	No	-	-
60	Yes	750	Yes
61	Yes	250	No

62	Yes	280	Yes
65	Yes	400	Yes
66	Yes	200	Yes
68	No	-	-
69	Yes	150	No
70	Yes	200	Yes
71	Yes	100	No
72	Yes	200	No
73	Yes	280	Yes
74	Yes	250	Yes
76	Yes	280	Yes
77	Yes	250	No
78	Yes	300	Yes
79	Yes	300	Yes
80	Yes	200	No
82	Smear		Yes
84	Yes	550	Yes
85	Yes	500	Yes
86	Smear		Yes
87	Yes	600	Yes
88	Smear		Yes
89	No	-	-
91	Smear		Yes
92	Smear		No
93	Yes	200	No
94	Yes	100	Yes

Figure 4.6

Restriction digestion - Phage DNA was isolated from a sample of clones which failed to amplify during PCR but displayed hybridisation with the subtracted probe. The DNA was digested with Not 1 and the fragment separated by gel electrophoresis on a 1.2% TAE/agarose gel. All of the clones except 22 and 37 contain inserts ranging in size from approximately 500bp to 2.1Kb. 22 does not hybridise with the subtractive probe and was included in the digests as a negative control representing a non-recombinant clone.

Figure 4.6


pTZ19U DraI + RsaI
21
22
23
27
28
37
47
82
87
pTZ19U DraI + RsaI
2149
1763
1097
692
pTZ19U DraI + RsaI
pTZ19U MspI
37
60
91
2149
1763
1097
692
501
489
404
331
242
190
147

non-recombinant. It is also observed that two bands arise when WM82 is cut with *Not*1. It is possible that WM82 contains an internal *Not*1 site but, as *Not*1 has an eight base pair recognition sequence, it is more probable that two clones with *Eco*R1/*Not*1 linkers ligated to their ends have ligated to each other to produce an insert with an internal *EcoR1/ Not1* linker.

4.3.4 Northern and sequence analysis

A subset of the longer positive clones was selected for further analysis by Northern hybridisation and sequence analysis. These clones included numbers 5, 19, 23, 25, 27, 47, 54, 60, 82A, 82B, 87 and 91 and were given the prefix WM (wheat meiosis). Table 4.3 outlines the results of the Northern hybridisation and Table 4.4 details the results of the sequencing analysis. The actual Northern films and sequencing data are presented in Appendices 2 and 3, respectively. Sequence homologies were determined using both the BlastX (Altschul *et al.*, 1990; Gish and States, 1993) and BlastN (Altschul *et al.*, 1990) programmes to search for homology in the protein and nucleotide databases. Alignments were performed using the MSA programme at Washington University.

All inserts cloned into pTZ18U failed to produce reliable sequencing data from the M13 forward priming site. Sequencing of pTZ18U in the region of the M13 forward priming site has revealed that this vector contains a mutation responsible for the lack of sequencing data in the forward direction.

4.3.5 WM19 and WM47 - Clones of unknown function and expression

WM19 and WM47 display no signal when used as probes in Northern analysis (Table 4.3) even when the autoradiographs were exposed for 14 days at -80°C. This would suggest that the genes represented by WM19 and WM47 have very low or no expression in the tissues examined. This may arise in several ways. The genes may have inherent low expression such that the Northern hybridisation technique employed is not sufficiently sensitive to detect their expression. Should this be true, more sensitive techniques such as reverse transcription PCR (RT-PCR) may be employed, although this procedure makes it difficult to achieve a quantitative demonstration of the gene expression. Observation of low expression of meiotic genes is not unique, Ji (1992) demonstrates that the expression of a wheat homologue of *Spo11* can only be detected by RT-PCR. Alternatively, the expression of the genes

Table 4.3 Outline of the pattern of expression of selected clones at a number of meiotic stages and in other tissues as determined by Northern hybridisation. PMI = pre-meiotic interphase; LEP = leptotene; ZYG = zygotene; PI - MI = pachytene - metaphase I; AI - TI = anaphase I - telophase I; MII = meiosis II; T = tetrads; IP = immature pollen; MP = mature pollen; RT = root tips; L = leaf. The level of expression is displayed on an arbritary scale with +++ being high expression, + being low expression and - representing no detectable expression. Two bands were detectable after Northern hybridisation with pWM27. The corresponding autoradiographs are displayed in Appendix 2.

Clone	PMI	LEP	ZYG	PI-MI	AI-TI	MII	T	IP	MP	RT	L
WM5	++	++	++	++	+	+	+	+	+	+	-
WM19	-	-	-	-	-	-	-	-	-	-	-
WM23	+++	+++	+++	++	+	+	+	+	+	+++	-
WM25	+++	+++	+++	+	+	+	+	+	+	+++	-
WM27											
band1	++	++	++	+	+	+	+	+	+	-	-
band2	+	+	+	+	+	+	+	+	+	+	+
WM47	-	-	-	-	-	-	-	-	-	-	-
WM54	+++	+++	+++	+++	++	++	++	++	++	++	-
WM60	+++	++	++	++	++	++	++	++	++	+++	-
WM82A	++	++	++	++	+	+	+	+	+	+	-
WM82B	-	-	-	-	-	-	-	-	-	-	+
WM87	+	+	+	+	+	+	+	+	+	++	-
WM91	+++	+++	+++	++	++	N/A	+++	++	N/A	+++	-

Table 4.4 Homologies of selected clones as determined by sequencing data from the M13 reverse and forward primers.

Clone	Vector	M13 Primer	Sequence Length	Homology (BlastX/BlastN)
WM5	pTZ18U	Reverse	245bp	BlastX - Wheat Histone H3 100% identity over 19 amino acids BlastN - Wheat Histone H3 72.9% identity over 188 nucleotides
WM5	pTZ18U	Forward	-	-
WM19	pTZ18U	Reverse	270bp	BlastX - None BlastN - None
WM19	pTZ18U	Forward	-	-
WM23	pTZ18U	Reverse	306bp	BlastX - Rice ADP Ribosylation Factor 100% identity over 60 amino acids BlastN - Rice ADP Ribosylation Factor 69.1% identity over 366 nucleotides
WM23	pTZ18U	Forward	-	-
WM25	pBluescript	Reverse	413bp	BlastX - α Tubulin-mouse testes specific 75% identity over 41 amino acids BlastN - Barley α Tubulin 93% identity over 246bp
WM25	pBluescript	Forward	253bp	BlastX - Maize α Tubulin 97% identity over 35 amino acids BlastN - Barley α Tubulin 78.7% identity over 280 nucleotides
WM27	pBluescript	Reverse	310bp	BlastX - None BlastN - None
WM27	pBluescript	Forward	345bp	BlastX - Alfalfa ribosomal protein L11

				89% indentity over 38 amino acids BlastN - Alfalfa ribosomal gene L11 77% identity over 115 nucleotides
WM47	pTZ18U	Reverse	298bp	BlastX - None BlastN - None
WM47	pTZ18U	Forward	-	-
WM54	pBluescript	Reverse	410bp	BlastX - Wheat Histone H3 73% identity over 56 amino acids BlastN - Wheat Histone H3 70.1% identity over 180 nucleotides
WM54	pBluescript	Forward	314bp	BlastX - Wheat Histone H3 92% identity over 56 amino acids BlastN - Wheat Histone H3 99% identity over 105 nucleotides
WM60	pTZ18U	Reverse	300bp	BlastX - None BlastN - None
WM60	pTZ18U	Forward	-	-
WM82A	pBluescript	Reverse	360bp	BlastX - None BlastN - None
WM82A	pBluescript	Forward	375bp	BlastX - None BlastN - None
WM82B	pTZ18U	Reverse	270bp	BlastX - Wheat Histone H3 97% identity over 40 amino acids BlastN - Wheat Histone H3 89% identity over 236 nucleotides
WM82B	pTZ18U	Forward	-	-
WM87	pBluescript	Reverse	421bp	BlastX - No homology BlastN - Barley *cam* gene for calmodulin 90.3% identity over 165 nucleotides

WM87	pBluescript	Forward	348bp	BlastX - *A. thaliana* Ribosomal Protein L18 76% identity over 52 amino acids BlastN - *A. thaliana* Ribosomal Protein L18 70.4% identity over 153 nucleotides
WM91	pBluescript	Reverse	330bp	BlastX - Wheat Histone H2B 99% identity over 90 amino acids BlastN - Wheat Histone H2B 91.7% identity over 333 nucleotides
WM91	pBluescript	Forward	370bp	BlastX - Wheat Histone H2B variant 2 67% identity in 40 amino acids BlastN - Wheat Histone H2B variant 2 61.6% identity over 114 nucleotides

represented by WM19 and WM47 may be confined to a very limited time frame. Considering the relatively short duration of male meiosis in wheat (Bennett *et al.*, 1971), it is possible that numerous genes required for highly specific meiotic processes may be expressed for periods shorter than one hour. Should the expression of a gene be highly temporally restricted, it is plausible that it would be difficult to detect using standard procedures. A third explanation for the lack of detection of expression might be that the genes are not expressed in any of the tissues examined. This would suggest that the clones represent artefacts of the cloning procedure and is unlikely. It is proposed that WM19 and WM47 represent a genes whose expression is severely restricted either in a quantitative or temporal manner.

Comparison of sequence data for WM19 and WM47 with the international gene databases did not reveal any homology identified for either clone at the nucleotide or amino acid level. WM19 sequence data (Appendix 3) does not include a poly(A^+) tail and may therefore represent the 5' end of the clone. WM47 sequence data shows the presence of a polyA+ tail and represents the 3' region of the gene. It is difficult to determine from the available sequence data if this clone contains an open reading frame or is representative of the 3' untranslated region (3'UTR). The results indicate that both WM19 and WM47 represent genes which have not otherwise been isolated either from wheat or other organisms. This is not unexpected if it is considered that wheat is composed of a large, polyploid genome, and is likely to display novel mechanisms for the control of meiosis in such as those needed for homologous chromosome pairing. The genes encoding the proteins required for these mechanisms may be represented by WM19 and WM47.

4.3.6 WM60 and WM82A - Clones with no known function

The expression pattern of WM60 does not reflect the type of gene which is likely to actively control early meiotic functions such as recombination, synaptonemal complex formation and chromosome pairing. WM60 displays a moderate to high degree of expression throughout meiosis and mitosis as deduced from Northern analysis. While several recombination genes are expressed during both mitosis and early meiosis (Ogawa *et al.*, 1993a), the high degree of expression of WM60 throughout meiosis does not indicate that it is required for recombination but rather reflects a gene which appears to be required during cell division but has no specific meiotic function.

The 300bp of sequence of WM60 shows no homology with known genes when compared to the databases at both the nucleotide and amino acid level. The sequence available includes the polyadenylated 3' end of the clone and reflects the 3'UTR of the gene. It is not possible to determine if part of the open reading frame of the gene represented by WM60 is present in the limited amount of sequence data available as the reading frame can not be accurately identified over such a small area. In addition, it is possible that WM60 represents a known gene but has a divergent 3'UTR such that comparison with the sequence databases fails to reveal any homology.

Clone 82A displays no homology to known genes in either the 360bp of the reverse primed sequence or 375bp of the forward primed sequence. However, the expression pattern of this clone makes it of interest. The differential expression between early and late meiosis and the low expression in root tips suggests that the clone represents a gene which functions during both mitosis and meiosis but which is required to a greater extent at early meiosis. A possible role for a gene displaying such an expression pattern, is a function in recombination. Recombination can, and does, occur during both mitosis and meiosis but is much more active during meiosis. Providing that the assumption that both meiotic and mitotic forms of recombination utilise at least some of the same genes, the expression pattern of WM82A can be explained as representing a gene required for mitotic and meiotic recombination. Several other recombination genes required for both meiosis and mitosis have been documented, the most notable being the nucleotide excision repair type genes of the *Rad50* epistasis group (Ogawa *et al.*, 1993a), but also including genes such as *MRE11* (Ajimura *et al.*, 1993) and *MEI19* (Sekelsky *et al.*, 1995). Alternatively, WM82A may function in chromosome condensation which is evident during both meiosis and mitosis.

4.3.7 WM23 - A putative ADP ribosylation factor

Clone WM23 displays differential expression during meiosis having higher expression at the early stages of prophase I than in subsequent stages of meiosis. However, the highest level of expression of the gene represented by this clone is in root tip material suggesting that the gene functions in both meiotic and mitotic processes. WM23 also displays some expression in leaf suggesting that a low level of expression of the gene is required in all tissues, implying a "housekeeping" function.

At the amino acid level WM23 displays high homology with an ADP-ribosylation factor (ARF) from rice (Higo *et al.*, 1994) having 100% identity with 60 amino acids at the C-terminal end of the rice protein (Figure 4.7a) which includes the conserved NKXD motif specific for guaninyl binding (Kaziro *et al.*, 1991). Homology searches at the nucleotide level also demonstrate high homology with the 3'UTR of the rice ADP-ribosylation factor (Figure 4.7b).

Small GTP binding proteins have been identified in a wide range of eukaryotic organisms and are thought to play a key role in vesicle mediated protein transport within animal and yeast cells and protein secretion (Balch *et al.*, 1992). GTP binding proteins can be divided into two classes based on their structural features. The *rab* family of homologues include *rab*, *ypt1* and *sec4* which are common to animals and yeast. The second family are the *ras* and ARF types.

More than 40 GTP binding proteins have been isolated from plants mainly by degenerate PCR amplification or by low stringency hybridisation (Ma, 1994). The GTP binding proteins isolated to date from plants have predominantly been members of the *rab* family (Redhead and Palme, 1996). Only four *ARF* genes have been identified in the plant kingdom. *ARF1* of *Arabidopsis thaliana* was the first *ARF* isolated from a plant (Regad *et al.*, 1993), and more recently a second *ARF* (*ARF3*) has also been isolated from *A. thaliana* (Lebas and Axelos, 1994). Both *ARF1* and *ARF3* were isolated by random sequencing of clones in a cDNA library, but are quite divergent with *ARF3* displaying higher homology to the *ARF*-like genes of *Drosophila* and *Pisum sativum* (Lebas and Axelos, 1994) than to mammalian *ARF* homologues. *ZmARF* was isolated from a maize cDNA library in a screen to identify genes which complement a mutation in the *FabD* gene of *E. coli* required for fatty acid biosynthesis (Verwoert *et al.*, 1995). The other *ARF* identified in plants is the rice *ARF* (Higo *et al.*, 1994) cloned from a cDNA library whilst attempting to isolate homologues of phospholipase A2. A comparison of all of the *ARF*s from plants with homologues from animals (Figure 4.7a) demonstrates that the plant *ARF*s differ from their mammalian counter parts at the C-terminal end of the protein although all of the *ARF*s display the conserved guaninyl binding motif (NKXD).

Figure 4.7a

Comparison of the deduced protein of WM23 with ADP-ribosylation factors from rice (Higo *et al.*, 1994), *Arabidopsis* (Regad *et al.*, 1993) maize (Verwoert *et al.*, 1995), bovine (Adamik *et al.*, 1988) and human (Bobak *et al.*, 1989). The conserved NKQD guaninyl binding motif is double underlined. Conserved amino acids across the proteins are represented by dots and missing amino acids are represented by dashes.

Figure 4.7b

Comparison of the 3' untranslated regions of WM23 and the rice ADP-ribosylation factor (Higo *et al.*, 1994). Conserved nucleotides are shown as dots whilst missing nucleotides are represented by dashes

Figure 4.7a

```
WM23        LVFANKQDLPNAMNAAEITDKLGLQSLRQRHWYIQSTCATSGKGLY
ARF-Rice    ...............................................
ARF1-Ara    ........................H..................E...
ARF3-Ara    .I........G.LDD.AV.EA.E.HKIKS.Q.A.FK...VK.E..F
ARF-Maize   ........................N................T.E...
ARF2-Bov    ...V............................N....A......D...
ARF1-Hum    ..............................H.N....A......D...

WM23        EGLDWLANNIANKA-
Rice        ..............-
ARF1-ARA    ......S....S..-
ARF3-Ara    ......S.TLKSGSG
ARF-Maize   ......SS...T..-
ARF2-Bov    ........QLK.QK-
ARF1-Hum    ........QLR.QK-
```

Figure 4.7b

```
                    715                                        750
WM23                ACATTCT CGGCAGGGC TTCTATGGAT CTGGGATAAG
Rice                .GCA... T...C.... ..AA...A.A GGAA..C.T.

      751                                                      800
WM23   CTGGATAT-- TCGGACTAAT ACTGCCTTT- -ACGTATATA AACTATATAG
Rice   GC......CC CT.AGTG..C G........T T......CGA ..........

      801                                                      850
WM23   TCTTGACAGA TA---ATGTG GGTGTTTTGG AAAGATAAAG CTCT------
Rice   ..A....... ..ATG..... .AT....... G......... ....TTAGGT

      851                                                      900
WM23   -----CTGTC AAAGAGCTAG CTATCAGATT GTTTT-CGTG GG-TGTT---
Rice   TCCAC.ACCA ..G....... .......... .....T.... ..G....AAA

      901                                                      950
WM23   AAATTGTCCG TGGCGCTGTT TGTTTGCCCA TC--ATGGCT TTACTATGTC
Rice   ....C..G.. ..TTT.A... ..G..TTTA. ..GA.A.... .ACGGTGCCT

      951                                                     1000
WM23   CGCTC-TGTA -CTCTGTACT CGCCACTCCG ACAGAC--TG ATACATTGTA
Rice   GA..TT.... CC........ .....-------CAGA---TG ------TG..

      1001                                                    1050
WM23   GTATTGACTT GTCTTGA-CA T..................... ..........
Rice   .......... ......GG.. .AGCGTATTG TTACGTAATC CTGTTCTGTG

      1051                          1079
WM23   .......... .......... .........
Rice   CAGCCACCCC CAATATCCTT ACCAGTTTT
```

The exact function of *ARF*s has yet to be defined, but recent studies have suggested that they may be involved in a number of processes including regulation of vesicular protein trafficking in the exocytic and endocytic pathways (Serafini *et al.*, 1991; Balch *et al.*, 1992; Kahn *et al.*, 1993), nuclear envelope assembly (Boman *et al.*, 1992), regulation of phospholipase D (Brown *et al.*, 1993), regulation of cellular ADP-ribosyltransferases (Regad *et al.*, 1993) and other basic processes such as cell growth and proliferation (Hall, 1990). Stearns *et al.*(1990) has demonstrated that the *ARF*s of mammalian cells are localised to the golgi apparatus, leading to the speculation that *ARF*s are required for vesicular transport of proteins between the endoplasmic reticulum and golgi apparatus (Balch *et al.*, 1992), and in the modulation of vesicle budding and uncoating within the golgi apparatus (Serafini *et al.*, 1991). In plants, the golgi apparatus is responsible for the formation of the cell wall components hemicelluloses (Moore *et al.*, 1991) and pectins (Zhang and Staehelin, 1992) as well as modifying and transporting glycoproteins. It is suggested that *ARF*s in plants are also involved in the regulation of the synthesis of these cell wall components (Higo *et al.*, 1994). The expression of the *ARF* in wheat is linked to phases requiring the formation of cell walls, that is at premeiotic interphase and in root tips, but is weakly expressed at the end of meiosis I and in immature pollen where cell wall formation also takes place. It therefore seems unlikely that this *ARF* is involved in the regulation of cell wall construction.

An interesting hypothesis on the action of plant *ARF*s involves regulation of DNA synthesis and cell proliferation (Verwoert *et al.*, 1995). It is known that in mammalian systems, *ARF*s can influence the phospholipid content of membranes by activating phospholipase D and yielding phosphatidic acid and choline (Kahn *et al.*, 1993). Phophatidic acid has been demonstrated to induce DNA synthesis and cell proliferation (Yu *et al.*, 1988) so it is possible that *ARF*s act to regulate DNA synthesis. This theory complements the expression pattern of the isolated wheat *ARF* with expression being high in tissues where active cell division and DNA synthesis are occurring. Low expression of the *ARF* is apparent in other tissues where active DNA synthesis does not occur including the later stages of meiosis where, although there is active cell division, DNA synthesis does not occur. The lack of expression in mature pollen may be surprising considering that mitotic divisions of the meiotic products does occur after meiosis in the formation of gametes. However, given that the pollen collected was classed as mature only if the pollen pore was apparent, it is likely that this material had already completed the mitotic events following meiosis.

The *ARF* isolated here represents the fifth *ARF* identified in a plant and the first isolated from an organism with a genome as complex as *T. aestivum*. It is unlikely that the *ARF* gene plays a role in chromosome pairing. However, until the exact function of the *ARF* family is deduced, it is not possible to completely exclude this possibility.

4.3.8 WM5, WM54, WM82B, and WM91 - Putative histone variants

The fundamental function of histones in eukaryotic cells is the packaging of DNA into nucleosomes. Five major classes of histones have been identified and include the core histones H2A, H2B, H3 and H4 and the linker histone H1. H3 and H4 histones form the central tetrameric block of the nucleosome and have a highly conserved primary structure (Brandt *et al.*, 1988). H2A and H2B are added to the tetramer as H2A/H2B dimers. The H2A and H2B histones have conserved C-terminal hydrophobic regions which may interact to form the histone core (Iwai *et al.*, 1970). In contrast, the N-terminal regions of both the H2A and H2B histones are highly variable. The linker histone H1 interacts with intranucleosomal DNA and can be highly variable (Joanin *et al.*, 1992). All of the histones are encoded by multigene families (Nakayama and Iwabuchi, 1993).

The accumulation of data from a number of experiments on animal and yeast histone genes originally resulted in the conclusion that all histone mRNAs are not polyadenylated but had a T-hyphenated palindromic sequence in the 3'UTR required for the regulation of 3' processing of pre-mRNAs (Gigot, 1988). It was also accepted that the expression of the histone genes was temporally restricted to the S-phase of the cell cycle, being dependent on DNA replication and was coordinately regulated at the transcriptional and post-transcriptional levels (reviewed in Osley, 1991).

Recently, it has been demonstrated that the H3 and H4 histones in maize (Chaubet *et al.*, 1988), *Arabidopsis* (Chaboute *et al.*, 1988), barley (Chojecki, 1986) and alfalfa (Wu *et al.*, 1989) are polyadenylated and do not contain the T-palindromic sequence conserved in animal histones (Gigot, 1988). The coupling of histone gene expression to DNA synthesis is also not as tight as was first assumed. Sittman *et al.* (1983) demonstrate that in the mouse lymphoma cell line S49, there are cells in G-phase with relatively large amounts of histone mRNA and high rates of histone synthesis.

In addition to the cell cycle dependent histones, variants exist whose expression is cell cycle independent or tissue specific (Schümperli, 1986). The histone variants are characterised as often being single copy genes, having mRNAs with polyadenylated 3' ends and having a variable amino acid composition (Schümperli, 1986). Histone variants have been described in *Tetrahymena* (Thatcher *et al.*, 1994), plants (Chaubet *et al.*, 1991), invertebrates (Fretzin *et al.*, 1991) and vertebrates (Wellman *et al.*, 1987). The effect of the alterations in the amino acid composition of the histone variants is not known, but it is possible that the change in expression pattern of these histones may be more important than the changes in the protein structure (Akhmanova *et al.*, 1995). Alternatively, the changes in protein structure of the histone variants may function to change the packaging of the DNA into nucleosomes such that different areas become accessible or inaccessible to a variety of proteins. This may result in the regulation of gene expression through a mechanism which prevents access of transcriptional enzymes to the DNA (Old and Woodland, 1984). Histone variants appear to be more abundant on DNA which is transcriptionally active (Gabrielli *et al.*, 1981) and may represent the histone form required in areas of high nucleosome turn over.

In yeast and animal cells (Osley, 1991), sequences in both the 5' and 3' flanking regions of the major and variant histone genes have been identified which are required for the regulation of gene expression. In contrast, very little is known about the regulation of the histone genes in higher plants.

Study of the accumulation of histone gene mRNA transcripts during germination of wheat seedlings has demonstrated an increase in mRNA levels concomitant with DNA synthesis. This has prompted the suggestion that at least some regulation of the plant histone genes occurs at the transcription level (Nakayama and Iwabuchi, 1993). Analysis of the primary structure of several H3 genes from plants have indicated the presence of a number of conserved sequences in the 5' and 3' regions which may act as *cis* regulators of gene expression and post-transcriptional modification of pre-mRNA's (Chaubet *et al.*, 1986; Tabata and Iwabuchi, 1986; Gigot *et al.*, 1987; Wu *et al.*, 1988). Within the 3'UTR, the consensus sequence $TTT(N)_{13\text{-}16}GAT(T/C)$ is highly conserved in most plant histones, although there is some variation in the number of nucleotides between the two conserved regions. Deletion of this motif has been demonstrated to result in a decrease in the

maturation of histone H3 pre-mRNA (Nakayama and Iwabuchi, 1993). The consensus sequence ATG(G)AAATG is reported as a putative polyadenylation signal for plant histone genes (Chaubet *et al.*, 1988). Whilst this sequence is less highly conserved in the H1 (Yang *et al.*, 1991), H2A (Huh *et al.*, 1995) and H2B (Yang *et al.*, 1991), removal of the consensus sequence from the H3 gene results in the incorrect formation of mRNA transcripts (Nakayama and Iwabuchi, 1993). These results, contrasted to the lack of polyadenylation of animal histone mRNA, suggests the presence of post-transcriptional regulation of histone genes which is specific to plants.

Three histone H3 genes and one histone H2B gene were isolated from wheat using the subtractive hybridisation procedure. WM91 shows very high homology to wheat histone H2B (Figure 4.8a). The C-terminal region is highly conserved, with variability arising from amino acid changes in the N-terminal end of the protein. This is not unexpected as variability in the N-terminus of most eukaryotic H2B proteins has been reported with the exception of vertebrates (Wells and McBride, 1989). Multiple variants of H2B genes have particularly well documented in the Graminae (Langenbuch *et al.*, 1983; Spiker, 1982) and alignment of WM91 with some of the wheat H2B variants is shown in Figure 4.8a. It is observed that there are seven proline residues evenly distributed in the first 40 amino acids of all plant histone H2B proteins. These proline residues are part of the pentamer motif P(A/K)X(E/K)K which is repeated five times in all animal, plant and yeast histone H2B proteins (Joanin *et al.*, 1992). The proline amino acids in the motif may interact to induce an extended helical structure making the surrounding charged residues accessible to interaction with the DNA and allowing tighter packaging of the chromatin (Joanin *et al.*, 1992). The predicted amino acid sequence of WM91 displays a substituted proline residue at position 22 as compared to the other wheat H2B sequences. The affect of this proline is to induce another turn in the protein structure which is likely to effect the interaction of the protein with DNA although the nature of the effect is not known.

Analysis of the 3'UTR of WM91 (Figure 4.8b) reveals two variations of the conserved $TTT(N)_{13\text{-}16}GAT(TC)$ motif which is thought to be involved in 3' processing of wheat H3 pre-mRNAs (Nakayama *et al.*, 1989). The variable middle portion of this motif is shorter in WM91 but is still likely to regulate 3' processing of this gene. The (A/T)(G/A)AAAT(A/G) consensus sequence proposed to be required for polyadenylation of plant histone transcripts (Nakayama *et al.*, 1989; Ohtsubo and Iwabuchi, 1994) is not present in the 3'UTR of

Figure 4.8a

Comparison of the deduced protein sequence of WM91 with wheat H2B variants: H2B2 (Brandt *et al.*, 1988), H2B1 (Yang *et al..*, 1991), H2B and H2B153 (Yang *et al..*, 1995). Conserved amino acids are represented as dots and missing amino acids are shown as dashes. The C-terminal of all of the H2B histones is conserved. The five P(A/K)X(E/K)K motifs are double underlined, and the inserted proline residue in WM91 is in bold.

Figure 4.8b

Comparison of the 3'untranslated regions of WM91 and wheat histone H2B (Yang *et al.*, 1995). Dots represent conserved nucleotides while dashes represent missing nucleotides. Variations of the TTT$(N)_{13-16}$GAT(T/C) motif in WM91 are underlined.

Figure 4.8a

```
WM91      --PKAEKKPAA-KKPAEEEPAPEKAAEKTPAAKKPKAEKRLPAGKTASKEA
H2B2      MA.........-....A.NKV-.........G.....................
H2B1      MA.........-.........-...-.....G.............S.A..G
H2B       MA.........-.........-AE--K....G.............S.A..G
H2B-153   MA..------..E..------V-..-------..V.......KV.---T-...G

WM91      GDEAKTRGRKKGSKAKKSVETYKIYIFKVLKQVHPDIGISSKAMSIMNSFI
H2B2      .G.G.............G.....................................
H2B1      .---DKK.K..A---.......................................
H2B       .-------K..-----......................................
H2B-153   .EKK---.K..-..--..M....................................

WM91      NDIFEKLAGEAAKLARYNKKPTITSREIQTSVRLVLPGELAKHAVSEGTKA
H2B2      ....................................................
H2B1      ....................................................
H2B       ....................................................
H2B-153   ....................................................

WM91      VTKFTSS
H2B2      .......
H2B1      .......
H2B       .......
H2B-153   .......
```

Figure 4.8b

```
WM91      AGTGCATCTGCATTGCTTGTATCTATCTAGTAGTAGCGACTCTAGCTGGTT
H2B       ..G...C.AAGGCC.TCACC.AG.TCACCTCCTCGTAG.G.GCTC...C.C

WM91      CTGTTGCT.TTAGTTAGTGCTTTTATTGCTGGATGGGGAATTGATTGATGG
H2B       ....GT..CG.......CTG.CCGGG..TGTTTGT..CCG.GT.A.CG..T

WM91      ATGACTGCCAATGCTTTGTTGCCTCTTTGGGTGTAACA------------
H2B       .A..A.CG..CCGTGA....T.TGGA.CTC.A.A..TAGTAACGGTGCCTG

WM91      ACTGAATTTGTGCTTCTTTTGGCAAACTGATATTTCAAAAGTACGGATTC
H2B       C..C...G.TC.G.A..ACAT.TTCGTCTTA.....TTCTCGT.TTG.GA

WM91      TTTCAAAAAAAAAAAAAAAAAAA
H2B       G.CTTGATTCAAAAAAAAAAAAAA
```

WM91. However, this motif is less highly conserved among the plant histone genes as discussed earlier.

Northern analysis of WM91 expression suggests that this gene is closely linked to DNA synthesis, similar to the major histone groups. However, as the whole clone was utilised for hybridisation in the Northern analysis, there would be an appreciable degree of cross hybridisation with other H2B transcripts in the coding region for the highly conserved C-terminal end of the protein. This makes it difficult to determine the actual expression pattern of the clone. In addition, the high expression of WM91 during the latter stages of meiosis might arise through increased stability of the mRNA such that the sequence is detected over a longer period of time. Therefore, whilst WM91 represents a novel variant of histone H2B from wheat, it is difficult to accurately determine its expression. The exact expression of WM91 could be determined by subcloning the 3'UTR of the clone, which is divergent from other histone H2Bs , and using this region as a probe in Northern analysis.

Three H3 histone variants were also identified, WM5, WM54 and WM82B. The expression of WM82B is limited to leaf tissue which identifies this clone as a highly specific H3 variant. As many of the histone H3 genes have divergent 3'UTRs which determine their expression (Chaubet *et al.*, 1991), analysis of the 3' end of WM82B may reveal a high degree of variation from the cell-cycle dependent H3 form. Further sequencing of WM82B will be necessary to determine the sequence of the 3'UTR of this gene as the existing sequence does not contain the poly(A^+) tail. Comparison of the deduced amino acid sequence of WM82B with the international protein databases shows 100% homology with the C-terminal region of wheat H3 (Figure 4.9a) (Tabata *et al.*, 1984).

Surprisingly, WM82B is not expressed in meiotic tissues as determined by Northern analysis. This is unexpected for two reasons: Firstly, it is believed that the high degree of conservation between this clone and the cell cycle dependent H3 indicates significant homology between WM82B and other H3 genes. Cross hybridisation between WM82B and other H3 genes would be expected to result in false signals in other tissues during Northern analysis as is suspected for WM91. However, it is possible that the 231 nucleotides representing the conserved 77 amino acids did not allow for significant cross hybridisation at the stringency of hybridisation employed. This is in contrast to the highly conserved 504bp in clone WM91 which are available for cross hybridisation. The second surprising

Figure 4.9a

Comparison of the deduced protein sequences of WM82B, WM54 and WM5 with the known sequence of the C-terminal region of H3 from wheat (Tabata *et al.*, 1984). Conserved amino acids are represented as dots, while missing amino acids are shown as dashes.

Figure 4.9b

Comparison of the 3' untranslated regions of WM54, WM5 and H3 from wheat (Tabata *et al.*, 1984). Conserved nucleotides are shown as dots and missing nucleotides as dashes. The putative $TTT(N)_{21}GATC$ motif in wheat H3 is dotted underlined. The identified (A/T)(G/A)AAAT(A/G) motif in WM5 is underlined. The proposed enhancers of meiotic expression identified in WM5 and WM54 are double underlined.

Figure 4.9a

```
     70                                                                  116
H3    KYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVSALQEAAEAYLVGLF
WM82B ------.....................................................
WM54  ---------------------------------.........PG.........P.
WM5   ---------------------------------------------------------

     117                                  147
H3    EDTNLCAIHAKRVTIMPKDIQLARRIRGERA
WM82B ................................
WM54  ................................
WM5   ------------.....................
```

Figure 4.9b

```
WM54 GCTGCTGCAT CTGCAATCCA TGCGTCGTCT GTTAGATCGT TGTTAGGAAG
WM5  ---------- ------G.TC ..TGCGTCG. CG.CTG.TAG .CG.C.TCTT
H3   .........C .........G ..TTAG.CTG AG.TC....G ..GA.AAT..

WM54 TGTGTGTGCT CGTAGTGTTT CAGAATGTGC CTGTGT---- ----TGTTCT
WM5  A.CCA.ATG. GT.CAGCGG. G.A.T..... G...T.GTAG TGTC.....G
H3   ..GTGT.T.A GAAT...CG...GTGT.AGAT..CA...TAGA TCCC.....C

WM54 TGTCATGGTG GG----TCTG TTATGCCTAA TCTGATGGTA CCTTCTTGTT
WM5  ..C....... ..---A.G.A G.G.T...G. .......TC. ...C....C.
H3   .C........ ..TGGA.G.. .......... .......... ..........

WM54 GCCAAAAAAA AAA.AA.... .......... .......... ..........
WM5  .T.ATCAGTA ACTGAAATGA AAATGTTTGT TATCTGTTCT AAAAAAAAAA
H3   ...
```

aspect of the leaf specific expression of WM82B is the fact that the clone was isolated from an early meiosis cDNA library using a probe enriched for early meiotic sequences. This can be explained by the fact that WM82B is derived from a clone containing two inserts. The high expression of WM82A in early meiotic tissues allowed the identification of the original clone in the subtractive hybridisation and WM82B was isolated only because it was ligated to WM82A. Therefore WM82B, whilst displaying some interesting attributes as a histone variant, is of little value in the search for genes operating to control homologous chromosome pairing.

The deduced expression of WM5 and WM54 is probably more accurate than the expression patterns determined for WM91 and WM82B. This is because the clones are much shorter than the other histones isolated and cover more of the divergent 3'UTR compared to the more highly conserved coding regions.

WM5 and WM54 display differential gene expression between meiotic and other tissues. These genes are highly expressed at premeiotic interphase and early meiosis with the level of expression dropping sharply as meiosis progresses. There is no expression in leaf material and the lower levels of expression in root tips compared to early meiosis indicate that the clones represent genes which have a specific meiotic function. The clones may even represent meiosis specific genes, with the expression in root tip arising from a limited amount of cross hybridisation with cell cycle dependent H3 genes. However, this is speculative and would need to be further analysed by Northern analysis of the 3'UTRs of the two clones.

Several meiosis specific histones have been identified in both plants and animals. Meistrich *et al.* (1985) report on variants of H2B and a H3 in rat which are testis specific and named TH2B and TH3. TH2B is first detected in pre-leptotene primary spermatocytes with rapidly increasing expression at early prophase and decreasing as meiosis progresses. This pattern of expression has led to speculative roles of TH2B in chromosome pairing or recombination. TH3 is present in spermatogonia and has been linked with programming of cells to the germ line. Sheridan and Stern (1967) describe the identification of a meiotic histone from *Lilium longiflorum*. The histone was identified as a variant of histone H1 and was proposed to be synthesised only during the interval directly preceding meiosis. The variant did not cause any qualitative change associated with chromosome condensation and

therefore its role during meiosis was not linked to changes in its composition. Recently, other meiotic histone variants have been identified in *Lilium*, including the gamete specific variants gH2B and gH3 (Ueda and Tanaka, 1995) and meiotin 1 (Riggs and Hasenkampf, 1991; Hasenkampf *et al.*, 1992; Riggs, 1994). Meiotin 1 is a chromatin associated protein showing a degree of homology to histone H1 but varying from H1 on the basis of size (meiotin 1 is almost 5,000 Da larger than H1), immunoblotting (meiotin 1 antiserum does not recognise H1 and H1 antiserum does not recognise meiotin 1) and form (meiotin 1 found in nucleosomes is stoichiometrically different to H1). Meiotin 1 has been proposed to play a role in limiting the degree of chromosome condensation during meiotic prophase (Hasenkampf *et al.*, 1992) which is viewed as being important for the synapsis of homologous chromosomes and the process of recombination (Riggs, 1994). The composition and possible function of the gamete specific gH2B and gH3 histone variants is not known (Ueda and Tanaka, 1995) although they might also regulate the degree of chromosome condensation during early meiosis.

Analysis of the deduced protein structures of WM54 and WM5 (Figure 4.9a) identifies three compositional differences in the C-terminal of the WM54 protein compared to H3. Two of the substitutions involve the addition of proline residues which would cause major conformational changes in the structure of the protein. The affect of these changes can not be readily identified at the molecular level and would require biochemical analysis. The coding region of WM5 covers only a short region of H3 and is completely homologous to it. Analysis of the 3'UTR of WM5 and WM54 (Figure 4.9b) reveals that the degree of divergence between the two variants and H3 is much lower than that observed between WM91 and H2B. This is in contrast to the H3 variants of *Drosophila* which display a very high degree of divergence in the 3'UTR sequences (Akhmanova *et al.*, 1995). The consensus sequence (A/T)(G/A)AAAT(A/G) can be identified in WM5 but not in WM54. Interestingly, the highly conserved $TTTN_{(13\text{-}16)}GATC$ motif which is required for pre-mRNA maturation in pea H3 genes (Nakayama and Iwabuchi, 1993) is not present in either WM54 or WM5 but is present in wheat H3 although there are 21 intervening bases between the TTT and GATC sequences (Figure 4.9b). A new consensus sequence has been identified in WM5 and WM54 which is absent from the cell-cycle dependent H3 gene. The CGTCGTCTGTTAG motif is present in both of the histone variants and may function to increase expression of these genes during meiosis in contrast to other tissues.

The isolation of four histone variants using the early meiosis subtractive hybridisation procedure supports the hypothesis that major histones in plants are replaced by variants during meiosis in a similar fashion to the replacement of histones with protamines in animals. The role of the histone variants is unclear although, as already mentioned, they may function in limiting the degree of chromosome condensation during meiosis and possibly regulate gene transcription through changes in the accessibility of the DNA to transcriptional enzymes.

4.3.9 WM25 An α-tubulin homologue

Sequence data for the full length of clone WM25 spanning 453bp was obtained. Comparison of the nucleotide data, and the deduced amino acid sequence with the databases reveals that WM25 is highly homologous with α-tubulin. At the nucleotide level, WM25 most closely resembles α-tubulin from barley (Close and Choi, 1996) displaying 94% homology in two separate regions of 136bp and 110bp. Interestingly, at the amino acid level the highest degree of homology is with the testis specific α-tubulin from mouse (Villasante *et al.*, 1986). The distinction between the homologies as determined at the nucleotide and amino acid level may have arisen from different codon usage in wheat compared to barley. However, comparing the deduced protein sequence of barley α-tubulin (Figure 4.10) with WM25 reveals that the two genes are identical except for an additional 10 amino acids at the C-terminal of WM25. Therefore, it is likely that the failure to detect homology between WM25 and barley α-tubulin arises because the α-tubulin sequence of barley was submitted as a nucleotide sequence and hence was not present in the protein databases scanned during a BlastX search. This highlights the need to perform homology searches at both the DNA and protein levels to produce an accurate result.

The isolation of a tubulin homologue is not unexpected as tubulins are a primary component of the spindle apparatus required for correct segregation of chromosomes during meiosis and mitosis. Analysis of the expression pattern of WM25 reveals that it is expressed at high levels at early meiosis until metaphase I when the spindle is assembled and in root tips which are actively undergoing mitosis. The lack of expression during meiosis II tends to suggest that all of the α-tubulin transcripts are produced at the beginning of meiosis and are either very stable allowing the synthesis of spindle proteins at meiosis II, or are translated immediately and the spindle proteins recycled for the second meiotic division.

Figure 4.10

Comparison of the deduced protein sequence of WM25 with α-tubulin from barley (Close and Choi, 1996).

Figure 4.10

```
392                                                                446
PGVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGM   H.vulgare
----------------------------VFSRIDHKFDLMYAKRAFVHWYVGEGM   WM25

447                              475
EEGEFSEAREDLAALEKDYEEVGAEFDEStop                          H.vulgare
EEGEFSEAREDLAALEKDYEEVGAEFDDGEDGDEGDEYStop                WM25
```

4.3.10 WM87 - Ribosomal protein or calmodulin homologue?

Northern analysis of WM87 reveals that the clone is highly expressed in root tips and at premeiotic interphase but shows a low, and generally stable, level of expression during meiosis. The full length nucleotide sequence of this clone has been determined (Appendix 4.). The clone is 674bp long and interestingly possesses poly(A^+) regions at both ends. Original comparison of sequence from the whole clone against the nucleotide and protein databases gave contradictory results with protein searches, displaying high homology at the amino acid level with the cytoplasmic ribosomal protein L18 from *Arabidopsis thaliana* (Figure 4.11a) (Baima *et al.*, 1995). In contrast, nucleotide based searches (Figure 4.11b) returned results showing high homology between WM87 and the calmodulin gene of barley in a reading frame of opposite direction to that showing homology with L18 (Ling and Zielinski, 1989). The observation that WM87 displayed high homology to the ribosomal L18 protein when translation was performed in a frame reading in the opposite direction to that showing homology to the calmodulin gene, suggests that the clone consists of two genes which have ligated together during the cloning process. This hypothesis is supported by the identification of two poly(A^+) sequences indicative of two genes, although the lack of two signals after Northern analysis contradicts the assumption of two genes in the same clone. However, it is possible that the single band identified by Northern analysis represents hybridisation to the calmodulin gene, which hybridises to mRNA approximately 850bp long in barley (Ling and Zielinski, 1995), and also the L18 ribosomal gene which displays an mRNA transcript of 900bp in *A. thaliana* detectable in all tissues (Baima *et al.*, 1989). An underlying signal displaying equal intensity in all tissues could represent the expression of the L18 ribosomal gene as the regulation of ribosomal protein production is performed at the translation level, and the mRNA transcripts of the different ribosomal proteins tend to be equally expressed in all tissues. Masking the L18 ribosomal signal would be the signal arising from hybridisation to the calmodulin gene and displaying differential expression in the tissues examined. The isolation of a calmodulin homologue is not unexpected as Ca^{2+}, which is bound and transported by calmodulin, has been implicated in regulating expression of a variety of protein kinases (Poovaiah and Reddy, 1987) which are likely to be involved in signal transduction and other processes during meiosis. Indeed, the ATR and ATM protein kinases of humans and mouse are expressed in the testes during meiosis I and

Figure 4.11a

Comparison of the protein sequence, deduced from the reverse sequence data, of WM87 with the ribosomal protein L18 from *Arabidopsis thaliana*. (Baima *et al.*, 1995). Conserved amino acids are shown as dots.

Figure 4.11b

Comparison of the nucleotide sequence from the forward sequencing primer of WM87 with the 3' untranslated region of the calmodulin gene from barley (Ling and Zielinski, 1989). Conserved nucleotides are shown as dots while missing nucleotides are represented by dashes.

Figure 4.11a

```
          147                                                        187
WM87R      GPKNAREAVRHFGKAPGVPHSHTKPYVRSKGRKFEKARGRRNSRGFKV
ARATH L18 ....S....K...P........S.....A..........K.K......
```

Figure 4.11b

```
WM87F   CTGCTATGCTCTCCAGTGTGTTTCCAGACCTCTTGTGTTTTATGTGAACTT
BARCAM  ..................................................

WM87F   GTGTCCACCCTGGTGTATCCTGATTGCTGTTGGGCCCGTTTGTAGTATATT
BARCAM  ......T.............................C...........

WM87F   TTTTCATCAACTTCTCGTTCACTCTTCTA----GTATCAATGGAAAACCGG
BARCAM  ..C.................G........TCTA..................

WM87F   TGTTTCGTAAAA----------------------------------------
BARCAM  ..........TTCTTTGACTCTTTATTAGATGTTATCCTGTGCTTCGGGTA

WM87F   ----------------------------------------
BARCAM  CGTGTTTTGCGTGTGAATGGCAGATAAAGATACATTTGAT
```

interact directly with synapsed and synapsing chromosomes recognising and responding to DNA strand interruptions that occur during meiotic recombination (Keegan *et al.*, 1996).

The isolation of WM87 highlights one of the differences between classical differential screening and subtractive hybridisation. In a normal differential screen, a clone such as WM87 which contains two inserts would be removed from the screening procedure because the ribosomal protein region, which is constitutively expressed, would hybridise to probes produced by reverse transcription of RNA from other tissues. Subtractive hybridisation enriches for genes expressed during early meiosis and thus may include calmodulin homologues which are expressed during meiosis. By using the subtracted cDNA to probe a cDNA library, WM87 was isolated due to hybridisation with a calmodulin homologue in the subtracted cDNA pool.

4.3.11 WM27 - Another clone containing two inserts

The difference between classical differential screening and subtractive hybridisation is again highlighted by the isolation of WM27. Northern analysis using WM27 as a probe reveals two differently sized signals after hybridisation (Appendix 3). One of the signals is of equal intensity in all of the tissues examined, whilst the other varies in intensity in anthers containing pollen mother cells at different stages of development and is not apparent in either root tips or leaf. This indicates that WM27 contains two inserts, one encoding a gene which is constitutively expressed, and the other encoding a gene which is differentially expressed only during meiosis and pollen maturation. Sequence analysis of WM27 (Figure 4.12) reveals that this clone displays very high homology to the ribosomal protein L11 of alfalfa (Asemota *et al.*, 1994) when sequencing data obtained from the forward M13 primer is submitted for the homology search. However, when sequence data obtained from the M13 reverse primer is submitted for homology searching in the databases, no homology with known genes is detected. This indicates that WM27 consists of two cDNA clones which are ligated together. One of these encodes the L11 ribosomal protein and the other represents a gene of unknown function whose expression is induced during early meiosis. The expression pattern of WM27 warrants further characterisation of the clone. This has been performed and is outlined in Chapter 5.

Figure 4.12a

Comparison of the 5' end of the WM27 clone with the cytoplasmic L5 ribosomal protein from alfalfa (Asemota *et al.*, 1994) at the amino acid level. Conserved amino acids are shown as dots.

Figure 4.12b

Comparison of the 5' end of the WM27 clone with the cytoplasmic L5 ribosomal protein from alfalfa (Asemota *et al.*, 1994) at the nucleotide level. Conserved nucleotides are shown as dots.

Figure 4.12a

```
WM27          RRRRCKARVGIHQRVTKEDAMKWFQVKYEGVILNKSHA
M. sativa      ...........QH....D.................Q.
             142                                  179
```

Figure 4.12b

```
            1                                                  50
WM27        CGCCGGCGCCGATGCAAGGCCCGTGTTGGGATTCACCAAAGGGTGACCAA
M. sativa   ..T..T..TA.G........A..A.....A..C..A..TC.T..C..A..
            436                                                485

            51                                                 100
WM27        GGAAGACGCCATGAAGTGGTTCCAGGTCAAGTATGAGGGTGTCATCCTGA
M. sativa   ...C..T.................A..T..A.....A.....G.....A.
            486                                                535

            101          115
WM27        ACAAGTCCCACGCTA
M. sativa   ..........G..A.
            536          550
```

4.4 General Discussion

Although differential screening has proven to be valuable for the isolation of meiotic clones from wheat (Letarte, 1996), it will only detect relatively abundant transcripts estimated to represent 0.1% of the mRNA population (Sargent, 1987). The result is that clones representing rare mRNAs, which are often those of greatest interest, are lost during the screening procedure. Subtractive hybridisation screening permits the isolation of cDNA clones which are present in as little as 0.01% of the mRNA population (Sargent, 1987). This, together with the ability to produce subtractive hybridisation populations from a small amount of starting material, makes subtractive hybridisation an attractive option to classical differential screening when attempting to isolate meiotically induced genes. Indeed, subtractive hybridisation has been applied for the isolation of meiotically induced genes from lily (Kobayashi *et al.*, 1994) with a great deal of success. The procedure adopted by Kobayashi *et al.* (1994) is similar to that outlined here although the subtracting population contained biotinylated mRNA rather than cDNA. The approach used here was based on subtraction of cDNA with biotin labelled cDNA as this has been demonstrated to reduce the chance of thermally induced degradation of the nucleic acids in the subtracting population of nucleic acids (Sargent and Dawid, 1983). In contrast to the subtractive hybridisation procedure outlined by Diguid *et al.* (1988), hybrid molecules forming during the subtractive hybridisation were removed by the addition of streptavidin and organic solvent extraction rather than chromatography on an avidin column which might have caused dilution of the probe and the resultant loss of sequences of interest.

The subtractive procedure applied here incorporates a PCR amplification of the subtracting cDNA population so that minimal amounts of starting material can be employed. Whilst this approach assists screening where only small amounts of material are available, there are some inherent problems. The most obvious problem is that some cDNAs in the subtracting population are preferentially amplified thus skewing the population away from normal. There is little that can be done to overcome this, but the degree of disproportionate representation of cDNAs can be minimised by reducing the number of amplification cycles applied. It is for this reason that the immature pollen cDNA was amplified in three separate reactions rather than one reaction being amplified three times. A second effect of the PCR amplification is that some under-represented genes in the subtracting cDNA population will be amplified to a stage where they become common. Thus genes of interest which are not meiosis specific, but are differentially expressed during meiosis, may be lost from the

subtracted population because of the abundance of the amplified transcript in the subtracting population. For this reason, young anthers were not selected as the source of subtracting DNA, as for lily (Kobayashi *et al.*, 1994), as it was thought that subtraction with the amplified cDNA would result in the removal of too many of the genes which display differential expression at the beginning of meiosis. Immature pollen was selected for the isolation of the subtracting cDNA population as it represents the first stage following meiotic division but maintains a high level of gene expression, allowing for the efficient subtraction of transcripts from the early meiosis population. If a highly specific meiotic probe was required, a second subtractive hybridisation incorporating cDNA from root tips could be applied to the subtracted cDNA. Cells in root tips are actively undergoing mitosis, so that subtraction with cDNA from this material would remove many of the genes which are required for both mitosis and meiosis. However, given the recent reports of somatic pairing of homologous chromosomes (see Chapter 1), root tips cDNA was not incorporated in the initial screen as the chromosome pairing genes may be expressed in such a tissue which is undergoing a high rate of mitosis.

Another consequence of the subtractive procedure used pertains to the fact that whole anthers were collected for the isolation of mRNA and its subsequent conversion to cDNA. Consequently, cDNAs specific to the tapetum but showing differential expression during meiosis could also be isolated. This is accepted as being unavoidable, as the technology for the isolation of meiocytes from wheat with no contaminating tapetal cells is not currently available to allow the purification of even the small amounts of starting material required for subtractive hybridisation. In addition, should pure meiocytes be prepared from wheat anthers, tapetum specific cDNAs may still be isolated as has been demonstrated in subtractive hybridisations involving only lily meiocytes (Crossley *et al.*, 1995). This is not necessarily a problem, as proteins expressed in the tapetum may subsequently be transported to the meiocytes where they play role in the control of meiosis. Indeed, the identification of genes meiotically expressed in the tapetum, from a meiocyte subtractive library in lily, provides strong evidence for the transport of proteins and mRNA from the tapetum to the meiocytes during meiosis (Crossley *et al.*, 1995).

The results of the subtractive hybridisation reveal that a high percentage of clones are differentially expressed at early meiosis and also in root tips. This highlights the large number of genes which are required for both cell divisions indicating a close relationship

between meiosis and mitosis. Interestingly, three clones, WM27, WM82 and WM87, containing two inserts were isolated from the subtractive hybridisation. WM27 and WM87 do not contain an internal *Eco*R1/*Not*1 linker indicating that the two inserts were ligated together before the addition of the linkers. WM82, in contrast does contain an internal *Eco*R1/*Not*1 linker and represents two inserts which ligated during cloning. The high percentage of clones which contain two inserts is another anomaly of the subtractive hybridisation procedure. During differential screening, these clones would normally have been removed during the screening procedure as they contain inserts which would hybridise to cDNA obtained from non-meiotic tissues. A clone such as WM27 which contains a meiosis specific insert and an insert which represents a ubiquitously expressed gene would therefore be removed from the screening process and a potentially interesting clone would have been lost.

Subtractive hybridisation overcomes the major limiting factor in isolating meiosis specific genes from wheat; a lack of accurately staged starting material. Several clones isolated using an early meiosis subtracted probe have been discussed in this chapter. Of the clones isolated, WM19, WM27 and WM47 would appear to be the most likely to represent genes which might regulate homologous chromosome pairing in wheat and warrant further analysis.

CHAPTER 5

Characterisation and further analysis of WM27

5.1 Introduction

Three clones representing genes with putative meiotic functions in wheat were isolated from an early meiosis cDNA library using a subtracted probe, WM19, WM27 and WM47. WM27 differs from the other clones in that it is thought to be composed of two cDNAs ligated together. One of the cDNAs displays high homology to the L11 ribosomal protein of alfalfa (Asemota *et al.*, 1994), whilst the other cDNA has no known homology with characterised genes (Chapter 4). In this chapter, a detailed analysis of WM27 is presented. The putative meiosis specific cDNA has been identified and genomic and full length homologous cDNA clones isolated. The temporal and spatial expression of the full length cDNA clone has been determined by Northern analysis and *in situ* RNA hybridisation. The clone has been mapped to the *ph2a* deletion region on the short arm of chromosome 3D as well as the short arm of chromosome 3A, and a possible correlation between the gene and the *Ph* genes of wheat is discussed. The clone has also been mapped in barley using the double haploid mapping population Clipper X Sahara.

5.2 Materials and Methods

5.2.1 Isolation of the meiosis specific region of WM27

A restriction map of WM27 was produced and *Mlu*I was identified as cutting once within the insert adjacent to the proposed ribosomal cDNA. *Eco*RV was also demonstrated to cut in the poly-linker region of the pBluescript vector but not within the insert. These enzymes were used to isolate the proposed meiosis specific cDNA which was subsequently subcloned into the *Sma*I site of pBluescript (KS^-).

To determine that the cDNA isolated was meiosis specific, it was used as a probe in a Northern hybridisation to representative RNA from numerous tissues. Following positive identification of the cDNA as being meiosis specific, the clone was named WM27ms (meiosis specific).

5.2.2 Chromosomal location of WM27ms

Preliminary mapping was performed using wheat cv. Chinese Spring/barley cv. Betzes addition lines, wheat nullisomic/tetrasomic lines and ditelosomic lines supplied by Dr. K. Shepherd, Department of Plant Science. The *Ph* mutants *ph2a* and *ph2b* were supplied by Dr. M. Feldman and were also included in the screening procedure.

5.2.2.1 Mapping in wheat/barley addition lines

Polymorphisms between Chinese Spring and Betzes were identified by digesting DNA from the two cultivars with *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV or *Hind*III and hybridising with WM27ms. Following identification of a polymorphism between *Bam*HI digested DNA from Chinese Spring and Betzes, DNA from the barley addition lines was digested with *Bam*HI and hybridised with WM27ms.

5.2.2.2 Mapping with nullisomic, ditelosomic and mutant wheat lines

To determine which of the chromosome 3 homoeologues of wheat carried copies of WM27ms, and to localise the gene to separate chromosome arms, analysis of the following lines was performed:

- nullisomic 3A - tetrasomic 3B
- nullisomic 3A - tetrasomic 3D
- nullisomic 3B - tetrasomic 3A
- nullisomic 3D - tetrasomic 3A
- nullisomic 3D - tetrasomic 3B
- ditelosomic 3AS
- ditelosomic 3AL
- ditelosomic 3BL
- ditelosomic 3DS
- ditelosomic 3DL
- *ph2a* mutant
- *ph2b* mutant

The *ph2a* and *ph2b* mutants were included in the analysis to explore a possible correlation of WM27ms with the *Ph2* gene. DNA from each of the lines was digested with *Dra*I and hybridised with WM27ms.

5.2.3 Rescreen of the cDNA library

The original cDNA library (Chapter 4), was screened with the insert of WM27ms. 10^5 pfu (5000 pfu/plate) were transferred to Hybond N^+ membranes and hybridised with the WM27ms probe. The membranes were washed to 0.5X SSC, 0.1% SDS then exposed to X-ray film for 2 days at -80°C. Phage which hybridised with WM27ms were purified by a second screen at lower density.

DNA was extracted from the isolated phage as described and digested with *Not*I. The DNA was fractionated on a 1.0% TAE/agarose gel and the size of the inserts determined by comparison with the size marker pTZ18U *Dra*I/*Rsa*I.

5.2.4 Isolation and analysis of a homologous genomic clone

5.2.4.1 Production of a wheat genomic library

DNA from Chinese Spring was partially digested with *Eco*R1 to produce a majority of fragments in the size range from 9kb to 25kb. The DNA was fractionated on a continuous 10%-40% (w/v) sucrose gradient and DNA of appropriate size was ligated into λDASHII *Eco*RI arms (Stratagene). The resulting phage were packaged using an *in vitro* packaging module (Amersham) and the titre of the library determined to be $8x10^6$ pfu/ml. $4x10^6$ pfu were plated onto large LB plates at a density of 10^5 pfu/plate.

5.2.4.2 Screening of the genomic library

Plaques were transferred to Hybond N^+ membranes and hybridised with WM27ms. Hybridising clones were purified by a low density screen as described. DNA was extracted from the phage containing positive inserts and was digested with *Eco*RI. The resultant DNA fragments were separated by gel electrophoresis, transferred to Hybond N^+ and hybridised with WM27ms. The hybridising bands were identified and a second digest of the phage DNA with *Eco*RI was performed. The DNA fragments were separated by gel

electrophoresis, and the bands identified as homologous to WM27ms extracted from the gel by the geneclean procedure and subcloned into the *Eco*RI site of pBluescript (KS$^-$).

To determine if the genomic clone isolated was indeed a homologue of WM27ms, the *Eco*RI insert was used to probe the nullisomic/tetrasomic mapping membrane produced earlier to determine if it gave the same pattern of hybridisation as WM27ms. The membrane was washed to 0.2X SSC, 0.1% SDS then exposed to X-ray film at -80°C for 4 days. After positive identification, the genomic clone was named WM27g (genomic).

5.2.4.3 Sequencing of the genomic clone

The Erase-a-base system (Promega) was used to produce a nested deletion library of the genomic clone. Circular plasmid DNA was isolated from WM27g using a HPLC procedure (Skingle *et al.*, 1990). Samples of the DNA were digested with a range of restriction enzymes including *Bam*HI, *Bst*XI, *Eco*RI, *Not*I, *Pst*I, *Sac*I or *Xba*I. *Bam*HI and *Xba*I, which cut the clone only once, were used in a double digest of the genomic plasmid. The *Xba*I overhanging end was filled with α-phosphorothioate nucleotides and the clone deleted from the *Bam*HI site using the Erase-a-base kit (Promega) to produce a set of sequentially deleted plasmids. The plasmids were transformed into *E. coli* strain DH5α and plated onto selective media. Three randomly selected, recombinant clones, corresponding to each time point of the deletion, were selected and plasmid DNA was isolated. The protected M13 forward primer was utilised to obtain sequence information for each clone selected from the nested deletion library. The sequence data was edited and overlapping alignments made using the SeqEd programme for the Apple MacIntosh computer.

5.2.4.5 RT PCR amplification

Utilising the sequence data available for the WM27 genomic clone, two specific primers were designed to be used for RT-PCR amplification of first strand cDNA. Primer design was performed using the OLIGO v4.0 programme (National Biosciences, Inc., Plymouth, MN) for the Apple MacIntosh computer.

27 upper - 5' CTTCTGCTCCCCGCTATTTAT 3'

27 lower - 5' TCCCCTCGTTGGCTTTCTTGA 3'

Total RNA was extracted from anthers containing pollen mother cells at premeiotic interphase (90 anthers), leptotene (90 anthers), zygotene (90 anthers), metaphase I (90

anthers), tetrads (90 anthers) or mature pollen (90 anthers), as well as root tips and leaf. First strand cDNA was synthesised using Superscript II RNase H$^-$ reverse transcriptase (GIBCO-BRL) and the complimentary RNA removed by RNase treatment. PCR reactions were performed using the designed primers and the cDNA as template. DNA from pWM27g, Chinese Spring, the *ph2a* mutant and the *ph2b* mutant was also included in the amplification. Three different primer annealing temperatures were used; 62°C, 60 °C and 58 °C. The parameters for the amplification are described in Chapter 2. A sample (10µl) of each reaction was separated by gel electrophoresis on a 1.2% TAE/agarose gel following amplification.

5.2.5 Screening of a cDNA library with WM27g

A cDNA library constructed from mRNA extracted from wheat pollen mother cells at leptotene was screened with three different regions of the WM27g clone. $5x10^4$ pfu of a cDNA library produced by J. Letarte (1996) were plated onto large LB plates at a density of 5,000 pfu/plate. The phage were transferred to three replicate membranes and hybridised with three regions of the genomic clone covering the 5' end, the middle and the 3' end. The membranes were washed to 0.2X SSC, 0.1% SDS and exposed to X-ray film for 4 days at -80°C. The X-ray films were aligned and clones which hybridised with all three probes identified. The positive phage were purified by a second, low density screen using the 5' end of the genomic clone as a probe.

DNA was extracted from the isolated phage, digested with *Not*I and fractionated on a 1.0% TAE/agarose gel. The largest insert was isolated from the gel using the geneclean procedure (BIO101) and subcloned into the *Not*I site of pBluescript (KS$^-$). The expression of the full length cDNA was verified by Northern analysis as described and the clone named AWWM5 (Adelaide Waite Wheat Meiosis) in accordance with the other four meiotic clones isolated in the laboratory. Sequencing of the clone was achieved by digesting the plasmid with *Hind*III and a double digest with *Sal*I and *Not*I, and sequencing of the fragments after subcloning into pBluescript (KS$^-$).

5.2.6 *In situ* RNA hybridisation of AWWM5

Whole florets containing anthers with pollen mother cells at either leptotene or zygotene were harvested and placed in fixation buffer. The samples were dehydrated through an

ethanol series then embedded in paraffin wax. 6-10μm thick cross sections were cut with a Leitz 1512 rotary microtome and the sections floated onto treated microscope slides. Wax was removed from the sections by submersion in xylene and rinsing in 70% ethanol. Sections were treated with proteinase K then acetylated before being dehydrated through an ethanol series as described. Sections were prehybridised at 45°C for 2 hours.

In vitro transcripts of AWWM5 were prepared by digesting pAWWM5 with either *Bam*HI or *Sac*II and purifying the linearised plasmid by the geneclean procedure. RNA transcripts representing the sense and anti-sense strands were produced, incorporating digoxigenin-11-UTP. Sections were hybridised with 8-10 ng/ml of labelled RNA in hybridisation buffer at 45°C for 16 hours then washed to 0.5X SSC for 30 minutes at room temperature. Labelled RNA was detected using anti-digoxigenin alkaline phosphatase as described.

5.2.7 Mapping the *ph2a* deletion

The Waite Institute has three barley double haploid populations available for mapping purposes; Galleon X Haruna Nijo, Chebec X Harrington and Clipper X Sahara. DNA from each of the parents was digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV or *Hind*III, fractionated on a 0.8% TAE/agarose gel and transferred to a Hybond N^+ membrane. The DNA was hybridised with AWWM5 to determine which combination of parents and restriction enzymes gave an identifiable polymorphism. Following identification of an acceptable polymorphism in *EcoRV* digested DNA from Clipper and Sahara3771, DNA from 90 double haploid individuals in the Clipper X Sahara3771 population was digested with *Eco*RV and hybridised with AWWM5. Scoring of the polymorphism allowed the inclusion of AWWM5 in the consensus linkage map (Langridge *et al.*, 1995a) produced using the Mapmaker computer programme.

Probes surrounding AWWM5 on the barley consensus map were identified and utilised in an analysis of the position and size of the *ph2a* deletion. Three replicates of DNA from Chinese Spring, the *ph2a* mutant and the *ph2b* mutant were digested with *Bam*HI, *Dra*I, *Eco*RI or *Eco*RV. The DNA was fractionated by electrophoresis on 0.8% gels and transferred to Hybond N^+ membranes. The membranes were hybridised with the probes PTAG653, BCD828 and CDO395, then stripped and hybridised with ABG460, PSR395 and ABR334. The polymorphisms resulting from each hybridisation allowed a determination of the

probe's presence or absence in the *ph2a* deletion and thus allowed the analysis of the size of the deletion in *ph2a*.

5.3 Results and Discussion

5.3.1 Isolation of the meiosis specific cDNA of WM27

WM27 was isolated from a wheat early meiosis cDNA library using a subtractive hybridisation technique (Chapter 4). Northern analysis of WM27 reveals that it hybridises to two different mRNAs. Preliminary sequence analysis shows that the 5' end of the clone has extensive homology with the L11 ribosomal protein (Asemota *et al.*, 1994), however there is little homology detected in the 3' end of the clone when compared to sequences in the international databases. Further analysis of the preliminary sequence data (Appendix 3), revealed the presence of an internal poly(A^+) stretch. This, together with the Northern results and sequence comparisons, indicate that the clone is composed of two cDNA inserts, one representing the L11 ribosomal gene and the other representing a meiosis specific gene of unknown function.

From a restriction map of WM27 produced in DNA Strider (not shown), digestion of pWM27 with *Mlu*I and *Eco*RV should produce a fragment of approximately 470 bases representing the meiosis specific cDNA. Digestion of pWM27 with *Mlu*I and *Eco*RV did release the desired fragment (Figure 5.1) which was subcloned into the *Sma*I site of pBluescript (KS^-). Digestion of plasmid DNA from selected recombinant colonies with *Bam*HI and *Hind*III released the cloned insert and hybridisation with WM27 revealed a high degree of homology (data not shown). Therefore, it was concluded that the putative meiosis-specific 470bp insert isolated, originated from the WM27 clone.

5.3.2 Northern analysis of the putative meiosis specific cDNA

Whilst it is known that the *Mlu*I/ *Eco*RV fragment containing the putative meiosis specific cDNA originated from the WM27 clone, it does not indicate that the cDNA represents the meiosis specific region of WM27. To address this, the *Mlu*I/ *Eco*RV insert was randomly labelled and hybridised with RNA from numerous tissues, including anthers containing pollen mother cells at various stages of meiosis (as indicated), root tips and leaf (Figure 5.2). The insert hybridises to a single mRNA species of approximately 1700bp, with the expression pattern and size of mRNA corresponding to the meiosis specific band displayed

Figure 5.1

Restriction digestion of plasmid DNA from pWM27 with *Mlu*I and *Eco*RV. The fragments were separated on a 1.2% agarose/TAE gel and stained with ethidium bromide for visualisation. The 470bp band is thought to represent the meiosis specific region of pWM27.

Figure 5.2

Northern Analysis of the putative meiosis-specific cDNA
Total RNA (10μg) from tissues (as indicated) was separated by denaturing gel electrophoresis, transferred to a HybondN$^+$ membrane and hybridised with the putative meiosis specific region of pWM27. The meiosis specific nature of the probe is evident. The positions of the 18S and 25S ribosomal RNA bands are marked. The probe hybridises to a mRNA species approximately 1700bp in length. The lower film shows consistent loading of the total RNA between lanes as judged by hybridisation with ribosomal DNA.

Figure 5.1

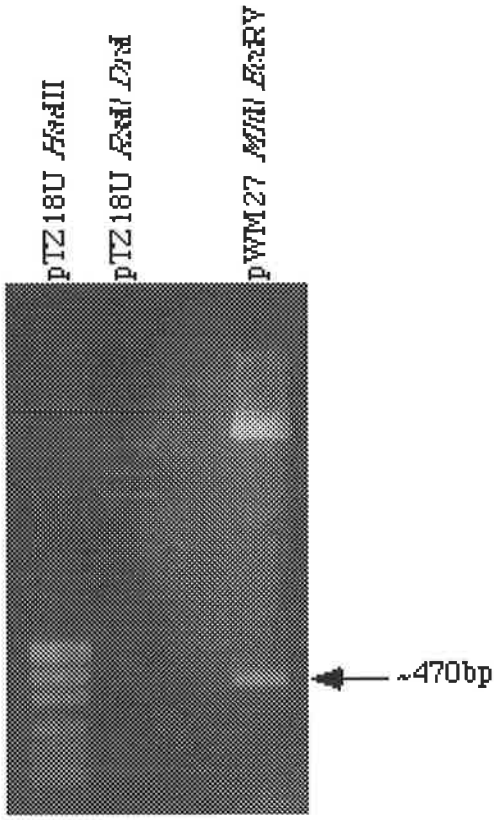
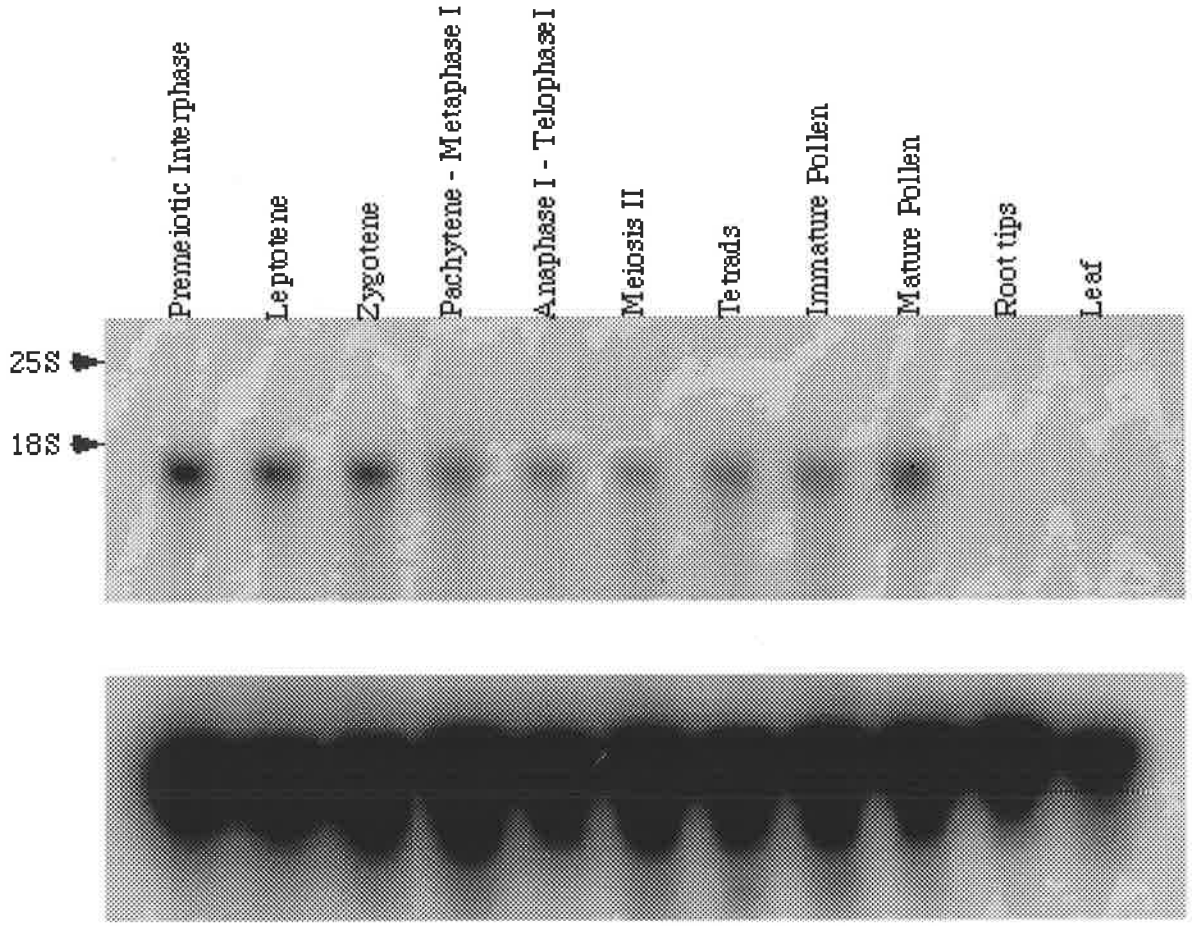


Figure 5.2



in the Northern analysis of WM27 (Appendix 3). It was concluded that the *Mlu*I/ *Eco*RV DNA fragment isolated represented the meiosis specific region of WM27 and the clone containing the cDNA was subsequently named WM27ms (meiosis specific).

5.3.3 Chromosomal location of WM27ms

The diploid nature of meiosis in wheat is achieved through the action of several pairing genes located predominantly on chromosomes 3 and 5 (Chapter 1). To determine if WM27ms shows any correlation with the pairing genes, the chromosomal location of the clone was analysed using barley addition lines as well as wheat nullisomic/tetrasomic and ditelosomic lines. The high degree of homology between the three related genomes of wheat allows the production of such lines because the absence of one chromosome can be compensated for by the increased dosage of either of its two homoeologues (Sears, 1972). The incorporation of a related chromosome from an ancestral diploid such as barley (Sears, 1941; Kimber and Riley, 1963) is also tolerated because of the hexaploid nature of *T. aestivum*.

Mapping of WM27ms to a chromosome was achieved using barley addition lines produced from Chinese Spring wheat and incorporating chromosomes from the barley cultivar Betzes (supplied by Dr. K. Shepherd). The use of the barley addition lines in the mapping procedure requires the identification of a polymorphism between Chinese Spring and Betzes. DNA from Chinese Spring and Betzes, digested with different restriction enzymes, (as indicated) and hybridised with WM27ms (Figure 5.3), reveals several polymorphisms between the two parental plants with all of the restriction enzymes used. The presence of between 3 and 6 bands in all of the Chinese Spring DNA digests suggests that WM27ms represents a single or low copy gene. The digests with DNA from Betzes barley reveal only a single major band which hybridises with WM27ms. Hybridisation of *Bam*HI digested DNA from Betzes with WM27ms gives a single band which is easily recognised whilst minor bands in the other digests are located at positions corresponding to bands in Chinese Spring wheat. Mapping with these enzymes is likely to prove difficult for some of the bands. Subsequently, DNA from the barley addition lines was digested with *Bam*HI and probed with WM27ms (Figure 5.4). It is evident that a copy of WM27ms maps to chromosome three in barley. This is of interest as it has been demonstrated that chromosome 3 of barley is homoeologous with the group three chromosomes of wheat (Sears, 1941; Nelson *et al.*,

Figure 5.3

DNA from *T. aestivum* cv. Chinese Spring (CS) and *Hordeum vulgare* cv. Betzes (B) was digested with restriction endonucleases as indicated. The DNA fragments were separated by electrophoresis on a 0.8% agarose/TAE gel, transferred to a HybondN$^+$ membrane and hybridised with the insert from pWM27ms. Polymorphisms between Chinese Spring and Betzes are evident in all of the restriction digests.

Figure 5.4

DNA from *T. aestivum* cv. Chinese Spring (CS),*Hordeum vulgare* cv. Betzes (B) and the indicated barley addition lines was digested with *Bam*HI. The DNA fragments were separated by electrophoresis on a 0.8% agarose/TAE gel, transferred to a HybondN$^+$ membrane and hybridised with the insert from pWM27ms. It is evident that a copy of WM27ms is located on chromosome 3 of barley.

Figure 5.3

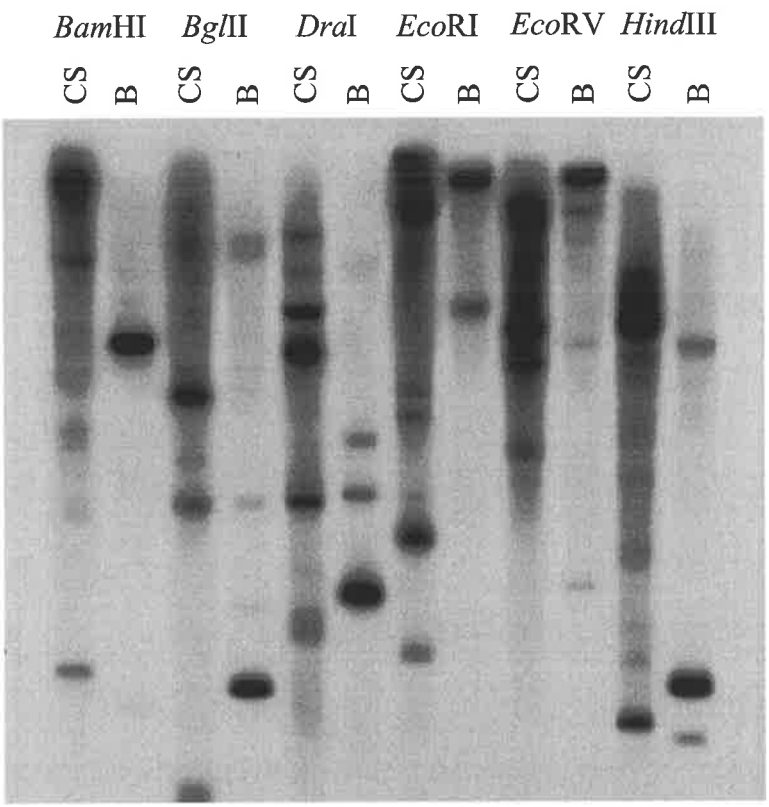
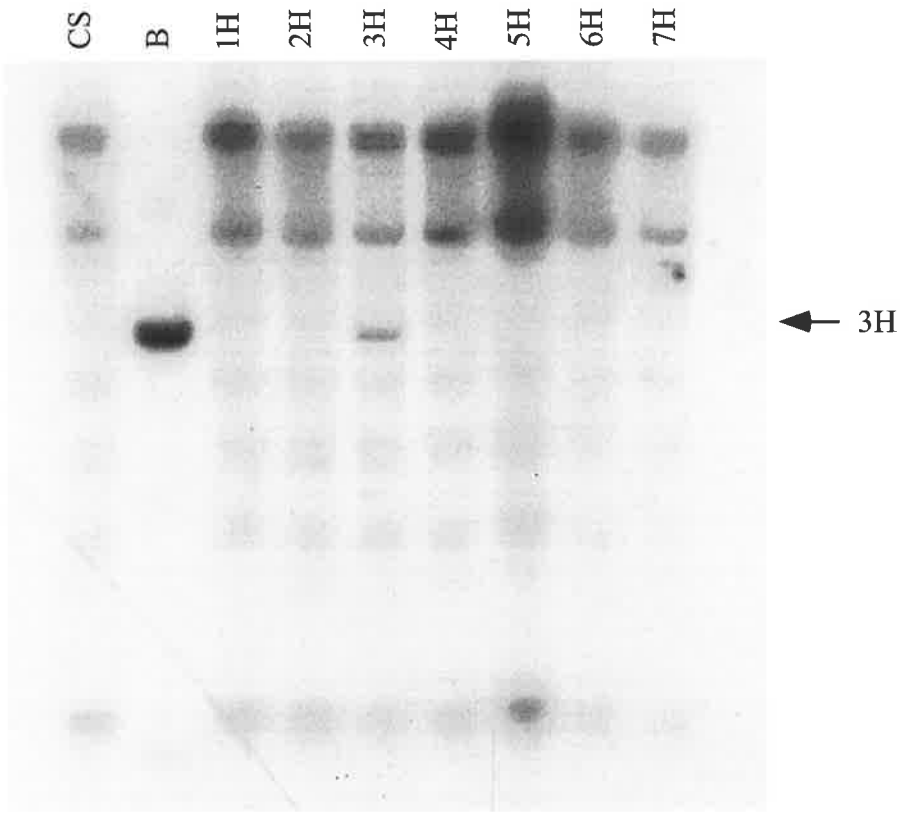


Figure 5.4



1995) on which the *Ph2* suppressor of homoeologous pairing is located (Upadhya and Swaminathan, 1967; Mello-Sampayo and Lorente, 1968; Mello-Sampayo, 1971). However, it should be noted that the low degree of polymorphism in Betzes barley generally, and the low degree of polymorphism between barley and wheat, may not allow the detection of copies of WM27ms on other chromosomes. This could be addressed in the future by either digesting the barley addition lines with different enzymes in an attempt to identify other polymorphisms, or by the use of rye addition lines which may provide a higher degree of polymorphism.

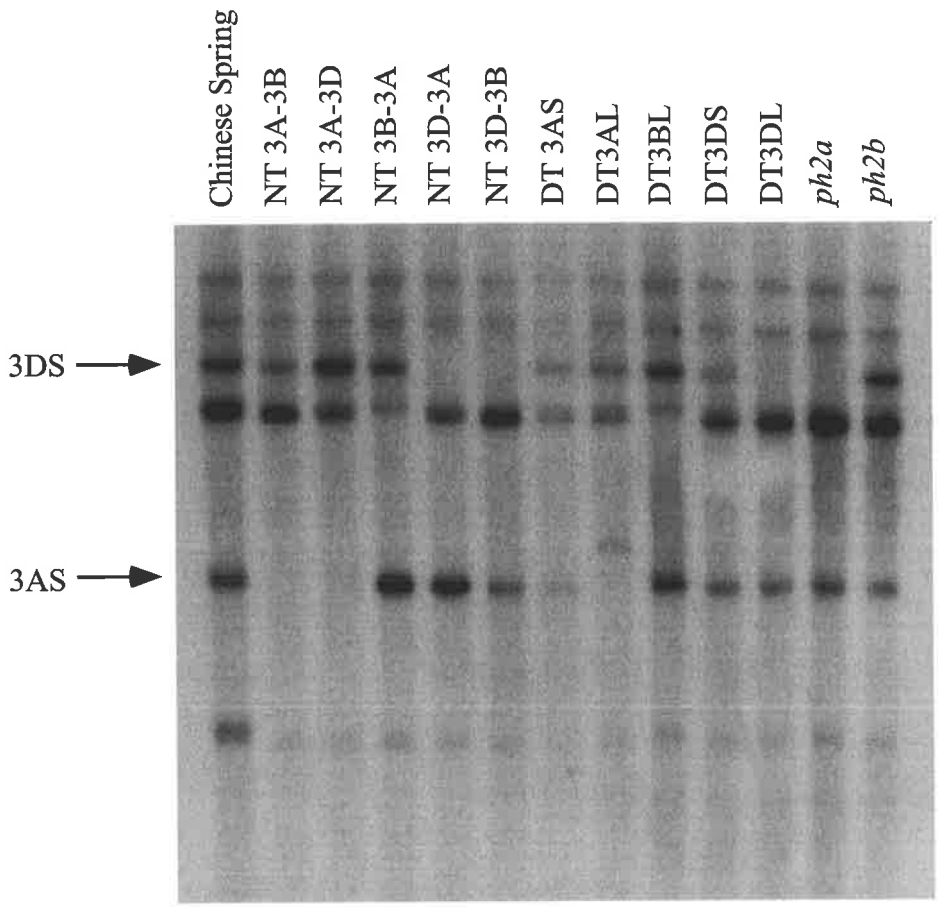
To determine which of the homoeologous group three chromosomes contain copies of WM27ms, and to localise the gene to a chromosome arm, the clone was used to screen a variety of nullisomic/tetrasomic and ditelosomic lines. Two mutants of the *Ph2* suppressor on the short arm of chromosome 3D, *ph2a* (Sears, 1982) and *ph2b* (Wall *et al.*, 1971) are available and these mutants were also included in the screening. It has been demonstrated (Figure 5.3) that multiple, well defined bands are visible when *Dra*I digested DNA from Chinese Spring is probed with WM27ms. The nature of the hybridisation pattern in *Dra*I digested wheat DNA provides the best possibility of identifying the chromosomal location of WM27ms homologues. Subsequently, DNA from the various wheat lines was digested with *Dra*I and probed with WM27ms (Figure 5.5). A copy of WM27ms is located on the short arm of chromosome 3D in the region of the *ph2a* mutation but a polymorphism with the *ph2b* mutation is not evident. This is not unexpected as the *ph2a* mutation is thought to be a large terminal deletion produced by X-ray irradiation (Sears, 1982), whilst *ph2b* is presumed to be a point mutation or very small deletion produced through treatment with EMS (Wall *et al.*, 1971). A second copy of WM27ms is present on the short arm of chromosome 3A. Interestingly, another suppressor of homoeologous chromosome pairing in wheat has been localised to 3AS (Mello-Sampayo and Canas, 1973). This suppressor is thought to occupy a position on 3AS syntenic to the *Ph2* gene on 3DS. Furthermore, in crosses with *S. cereale*, the removal of both the *Ph2* gene and the suppressor on 3AS results in levels of chromosome pairing almost as high as that attained when the *Ph1* gene is absent (Mello-Sampayo and Canas, 1973) suggesting that the suppressors on 3DS and 3AS interact.

Other bands which are apparent in Figure 5.5 do not display any polymorphism between the various group three mapping lines. This can be explained in two ways: If the gene

Figure 5.5

DNA from *T. aestivum* cv. Chinese Spring, a selection of nullisomic-tetrasomic and ditelosomic lines and the *ph2a* and *ph2b* mutants was digested with *Dra*I. The fragments were separated by electrophoresis on a 0.8% agarose/TAE gel, transferred to a HybondN$^+$ membrane and hybridised with the insert from pWM27ms. Two genes homologous to WM27ms are located on the short arms of chromosome 3D and 3A as indicated by the arrows.

Figure 5.5


Chinese Spring
NT 3A-3B
NT 3A-3D
NT 3B-3A
NT 3D-3A
NT 3D-3B
DT 3AS
DT3AL
DT3BL
DT3DS
DT3DL
ph2a
ph2b
3DS
3AS

represented by WM27ms is highly conserved on all three group three homoeologues, digesting with *Dra*I may produce hybridising bands of the same size in all of the lines tested. This is most likely and may be reflected in the differing intensities of hybridisation of some bands in both the nullisomic/tetrasomic and ditelosomic lines. Alternatively, there may have been duplication of the gene in wheat such that it is present on chromosomes other than group three homoeologues in wheat. These hypotheses could be tested in the future by digestion of the various mapping lines with different enzymes to identify a polymorphism in the bands which are currently present in all of the nullisomic/tetrasomic and ditelosomic lines. As the aim of the mapping experiments was to determine if the WM27ms gene was located in the region of known *Ph* genes, the additional mapping of non-polymorphic bands was considered unnecessary at this stage.

5.3.4 Screening of the original cDNA library to identify a full length homologue of WM27ms

The general methods which can be applied for the isolation of full length clone homologous to WM27ms include include screening of a cDNA library with the partial clone, screening of a genomic library and random amplification of cDNA ends (RACE) techniques.

The screening of a cDNA library with the partial cDNA clone can be a rapid method for the isolation of a full length homologue, provided that the cDNA library has already been constructed. Screening of the original cDNA library would require only that the phage be plated and hybridised with WM27ms in order to identify homologous clones. The disadvantage of this approach, is that often the clones isolated are not full length and in order to isolate a full length homologue, a new cDNA library needs to be produced. If a new cDNA library is required for screening, the procedure can be very time consuming considering the difficulty in collecting well defined meiotic material, and the time and care needed to extract RNA, purify mRNA, synthesise cDNA and construct the library. In addition, the production of a new cDNA library does not guarantee the isolation of a full length cDNA clone, as the size of the clones present is reliant on the quality of the RNA used as starting material, and the efficiency of the reverse transcription reaction. However, as a cDNA library had been constructed for the subtractive hybridisation procedure (Chapter 4), screening of the library with WM27ms might result in the rapid isolation of a full length homologous cDNA clone.

Three phage hybridising to WM27ms were identified in a screen of 10^5 pfu from the original cDNA library, and the size of their respective inserts determined by extraction of the phage DNA, digestion with *Not*I and electrophoresis of the DNA fragments (Figure 5.6). This reveals that the three clones identified, contain inserts which are approximately 600bp, 400bp and 700bp in length. Considering that WM27ms hybridises to an mRNA of approximately 1700bp (Figure 5.2), it is evident that none of the clones isolated contained a full length cDNA. Considering the time required, and likely difficulties in the production and screening of another early meiosis cDNA library, the possibility of utilising genomic screening and RACE approaches to isolating a full length homologue were explored.

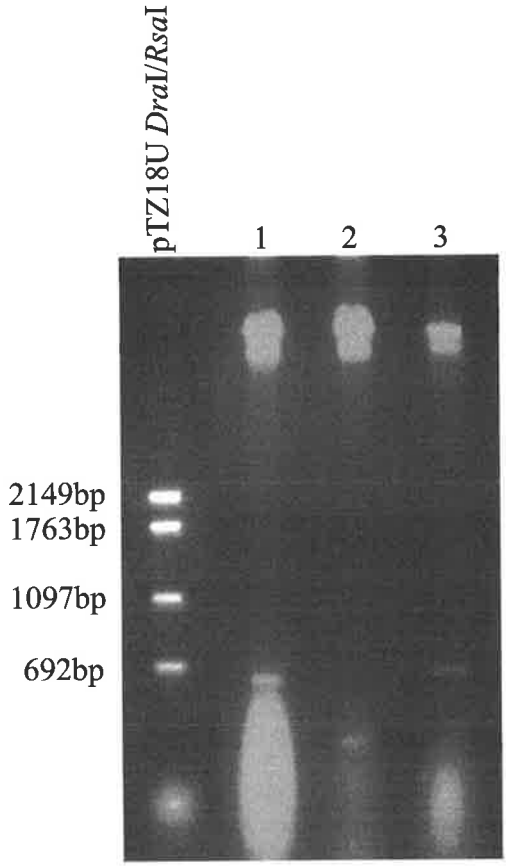
5.3.5 Isolation and analysis of a genomic clone homologous to WM27ms

The production and screening of a genomic DNA library overcomes the problem of producing a cDNA library containing a full length cDNA clone homologous to WM27ms. In contrast to cDNA library construction, genomic libraries are relatively easily produced and a large genomic library will theoretically contain representatives of all genes in the studied genome. Furthermore, because a genomic library is constructed from genomic DNA rather than reverse transcribed cDNA, all of the genes represented are full length, although the gene may bridge more than one clone. Clones containing the gene of interest can be isolated by hybridisation with the partial cDNA clone and can be sequenced quickly using deletion techniques. An added advantage of the isolation of a genomic clone, is that it allows the identification of promoter elements not present in the cDNA population. However, although the production of a library from genomic DNA ensures that the gene isolated is full length, the use of genomic DNA for library construction does have complications. Introns, which are present in genomic DNA separating open reading frames, can make it difficult to determine the exact protein encoded by the gene as the reading frame can shift in the region of the introns and the actual beginning and ends of the introns are often unclear. The production of cDNA from mRNA, where the introns have been spliced out, circumvents this problem. Therefore, to determine the exact sites of introns in the genomic DNA sequence, and the reading frame of the encoded protein before and after the intron, the cDNA overlapping the site of a potential intron needs to be analysed. This can be achieved by producing PCR primers flanking the putative introns for use in reverse transcription PCR (RT-PCR) of single stranded cDNA. Comparison of the sequence of the

Figure 5.6

Three positive phage showing cross-hybridisation to the insert of pWM27ms were identified in a screen of an early meiosis, wheat anther cDNA library. The phage DNA was digested with *Not*I and the fragments separated by electrophoresis on a 1.2% agarose/TAE gel. Staining of the DNA with ethidium bromide and viewing under UV light reveals that the phage contain inserts which are 600bp, 400bp and 700bp in length, respectively.

Figure 5.6



amplified cDNA and the genomic sequence allows the accurate determination of the position of an intron as well as the reading frame of the gene before and after the intron.

The third method for the isolation of a full length clone homologous to WM27ms utilises first strand cDNA similar to RT-PCR. Rapid amplification of cDNA ends (RACE) techniques theoretically provide a rapid method for the cloning of full length cDNAs as large amounts of first strand cDNA can be rapidly screened for full length homologues without the need to construct a cDNA library. The RACE procedure was first described by Frohman *et al.* (1988) and Belyavsky *et al.* (1989) and employed terminal deoxyribonucleotide transferase (TdT) to add a homopolymeric tail of dA's or dG's to the 3' end of the first strand cDNA (equivalent to the 5' end of the mRNA). The cDNA can then be amplified using a gene specific primer and a universal primer homologous to the generated homopolymeric tail. The disadvantage of this procedure are that it is difficult to control the production of the homopolymeric tail on the cDNA which can reduce the effectiveness of amplification. In addition, the universal primer used for amplification may anneal within some genes giving rise to unspecific amplification. Procedures have been described to overcome these problems but the RACE techniques remain difficult to prepare and somewhat unreliable.

Taking into account the likely difficulties which would arise in attempting to prepare and employ RACE techniques for the amplification of a full length cDNA, it was decided that the isolation of a genomic clone would be the most suitable method for the isolation of the gene represented by WM27ms, even if a larger degree of analysis of the clone is required to identify the actual coding sequence. In addition, it was considered that the identification of the promoter elements of the gene in the genomic clone may provide additional information on the control of gene expression.

5.3.5.1 Construction and screening of the genomic library

Partial digestion of DNA from Chinese Spring wheat with *Eco*RI, and fractionation of the DNA fragments on a sucrose gradient, was used to separate fragments between 9kb and 25kb which were cloned in the λDASHII replacement vector (Stratagene). The resultant genomic library contained approximately $8x10^6$ pfu/ml. $4x10^6$ pfu were screened with labelled insert from pWM27ms and five hybridising clones (2, 14, 16, 21 and 29) were

Figure 5.7a

Five bacteriophage, which hybridise with the insert of pWM27ms, were isolated from a genomic wheat library and purified by low density screening. DNA was isolated from each of the identified phage and digested with *Eco*RI. The DNA fragments were separated on a 1.2% agarose/TAE gel and visualised under UV light after staining with ethidium bromide. It is apparent that each of the phage contain internal *Eco*RI sites.

Figure 5.7b

EcoRI digested DNA from the gel in Figure 5.7a was transferred to a HybondN$^+$ membrane and hybridised with randomly labelled insert from pWM27ms.

Figure 5.7a

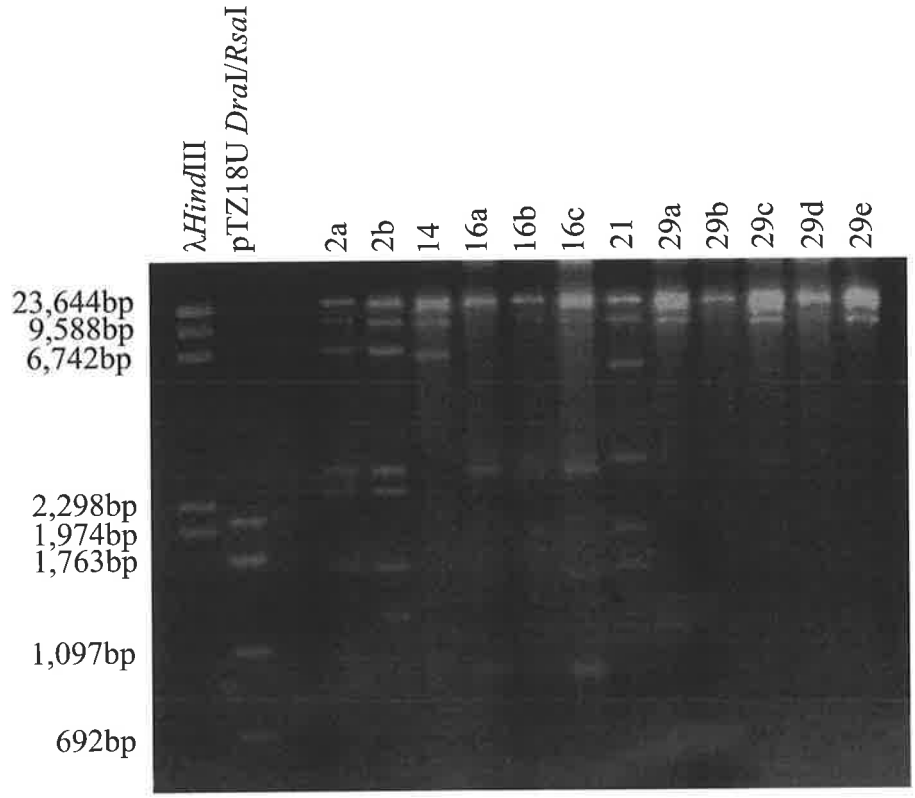
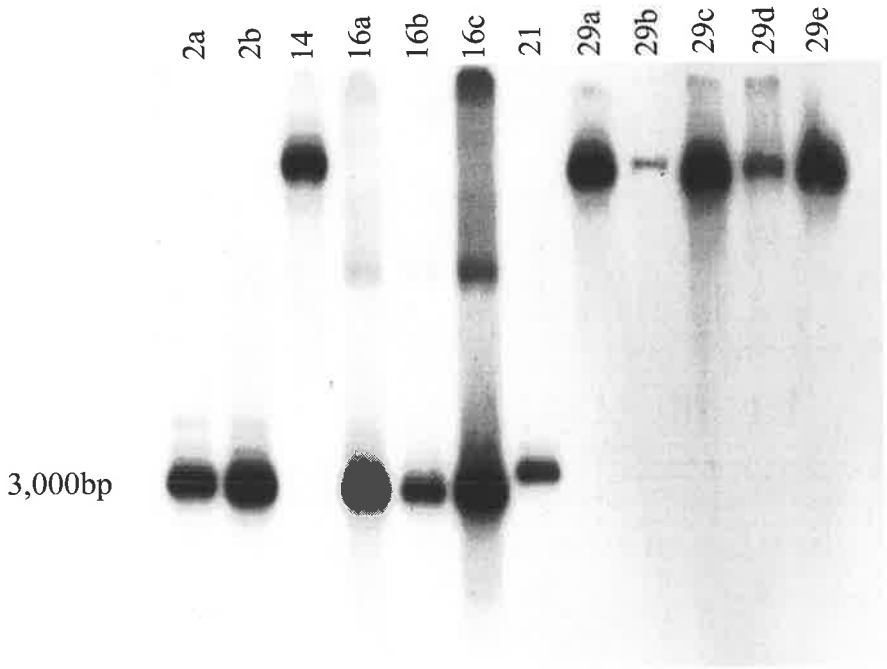


Figure 5.7b



identified. Extraction of DNA from each of the identified phage and digestion with *Eco*RI reveals that all of the clones except number 29 contain internal *Eco*RI sites (Figure 5.7a). Hybridisation of the digested phage DNA with WM27ms (Figure 5.7b) shows that in clones 2 and 16, the homologous genomic DNA is located on a fragment approximately 3kb long. A fragment slightly longer than 3kb in *Eco*RI digested DNA from clone 21 is also homologous to WM27ms. In clones 14 and 29, WM27ms hybridises with *Eco*RI fragments which are approximately 9kb in length. The 3kb homologous segment from clone 16a was subsequently cloned into the *Eco*RI site of pBluescript (KS^-). The insert was used as a probe to the ditelosomic mapping membrane to assess that the hybridisation pattern was the same as for WM27ms (data not shown), and was named WM27g.

5.3.5.2 Sequence analysis of WM27g

Sequence analysis of WM27g was performed using the Erase-a-base system (Promega) to produce a set of sequentially deleted plasmids. Digestion of circular plasmid DNA, purified by a HPLC procedure (Skingle *et al.*, 1990), with the enzymes described revealed that *Bam*HI and *Xba*I cut only adjacent to the genomic insert in the poly linker (data not shown). Protection of the *Xba*I site with α-phosphorothioate nucleotides and deletion of the insert from the *Bam*HI site produced a set of overlapping deletions used for sequencing of the genomic clone from the M13 forward primer. A diagrammatic outline of the resulting sequence alignments and overlaps is displayed in Figure 5.8. The genomic sequence, aligned with the known sequence of WM27ms is displayed in Figure 5.9.

Alignment of the established sequence of the genomic clone and the sequence determined for WM27ms demonstrates that WM27ms is homologous to a region in the middle of the genomic clone. 1758bp of genomic sequence preceding and including the homologous region has been identified. A small insertion of 12bp is located between 1715bp and 1727bp, and several mismatches between the genomic and cDNA sequence are apparent. This indicates that the genomic clone isolated is slightly different than the isolated cDNA. The position of the identified 12bp insertion close to the poly(A^+) tail of the cDNA indicates that it is likely to be located in the untranslated 3 prime end of the gene, and hence is unlikely to effect the composition of the translated protein. Because WM27ms is homologous to the central region of WM27g, it is unlikely that the genomic clone contains the promoter region of the gene, the analysis of which might provide information on the

Figure 5.8

Outline of the overlapping clones generated from a sequential deletion of the WM27g plasmid. Sequencing of the clones allowed the full sequence of the genomic clone homologous to WM27ms to be identified.

Figure 5.8

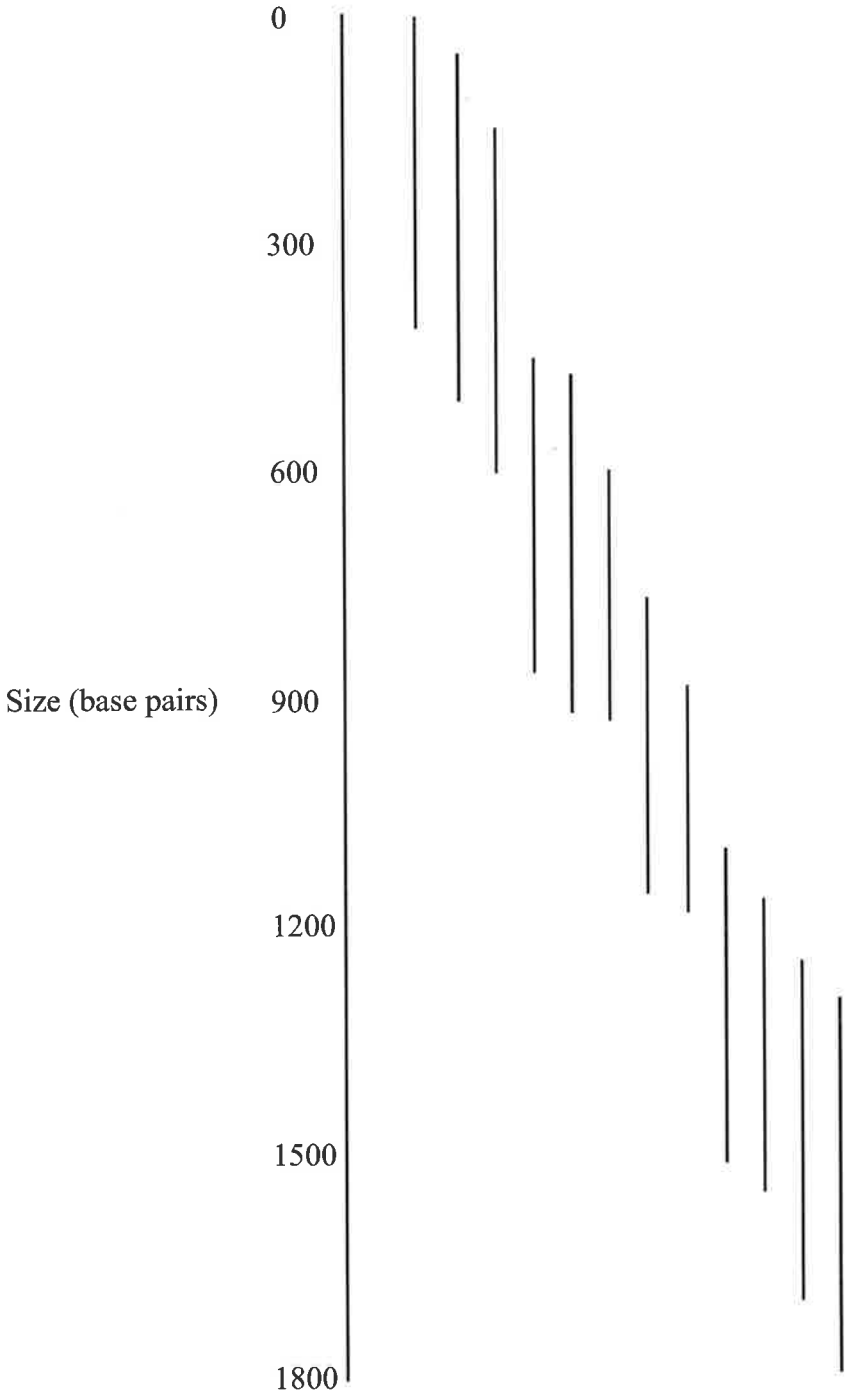


Figure 5.9

Sequence of the genomic clone homologous to WM27ms. The alignment with the known sequence of WM27ms is displayed and the apparent 12 base insertion is underlined. The putative start and stop codons of the cloned gene are dotted underlined. The position of the upper and lower primers designed for RT-PCR are double underlined.

Figure 5.9

```
WM27G     1 ATCATCCTGCAGCACACGAATTGAAGCCATGCACATCTCCGCCTCAAGAT
WM27G    51 CGATAGCTGGAACCCGAATCAAAAACTTCAAGAGGAAAATGAATTTATTG
WM27G   101 TCACTAGGTAGGTGATAAACACGCAAGGCCAGTCCACCATGAGTCCATGA
WM27G   151 CCTTTGCCCTGTCTACTCGGATCGGTTGAGCGTTTCATGGCGCGCAATGA
WM27G   201 ATGCCCTGGCCAACCACTCGGTGGTGTCATCTCTGCCTCTGCGACCTCCA
WM27G   251 TTTACCACGCTCTCTCACGCAGACCTCATCAATTTTCATGGCGCGTCGGT
WM27G   301 CTGGTAGCTTCTGCTCCCCGCTATTTATACCCCGCTCTCTTCGCCTGTGT
WM27G   351 CGTTCAGTTAACTCCACCTCTCCACTTCACTCCCACTGAAACAGCTCCCA
WM27G   401 CTGAAGCAGCGGCGGAAGAAGAAGAAGAAGCTAGAGGACATGGGGGCGCA
WM27G   451 GAGAGCTCTCCTCGCGAGCTTCCTGCTCGCCGCGTTGGCCACCCAGGCCT
WM27G   501 TCGTCTCCGTCTCGGCCAGGACCGGCCCGACGGACAAGGCAGGCCAAGGT
WM27G   551 GCGTACCTAGCTAGATCTTGTGCATGAAGCTTGTGGTGACTGGTGAGTAG
WM27G   601 TGAGCTGGGTTATAACAAATGCCGTCCCGTGGCGTGCTTGCAGATGACGT
WM27G   651 GAAGAAGCCGGACTGCGTGCCGTCGCTCGACCCGCACAATTTCCCCGGCC
WM27G   701 ACGGGGGCACCACCGTGCCGCTTCCCTCGCACGGAGGCTCCTCCGGCACG
WM27G   751 CCCCCCTACCACGGAGGCTCCGGCACGACGCCGTCGCACGGCGGCTCCGG
WM27G   801 CTCGACTCCGTCGCACAGCGGCTCTGGTTCGTTGCCTGACCCGTCGCACG
WM27G   851 GCTGGTACGGGACGCCGCCTTCGCACGGGGGCTCTGGTTCGTTGCCTGAC
WM27G   901 CCGTCGCACAGCGGACGTGGTTACGGGTCCACTCCGGATGCCCGTCACCA
WM27G   951 CGGCGAAGGCGCGTACGGGAGCTCCCCGACGCCTTCCACTGGCGGTCTAC
WM27G  1001 GGCAGCTCACCCACGCCGTCCCACGACGGAGGCGCCTACGGGAGCTCCCC
WM27G  1051 GACGCCGGCCCACGACGGAGGCTCCTACAGCGGAACTCCGGCTGCACCGT
WM27G  1101 CGCACAGCAGCCACGGGTCTGTGACACCGACGCCGCTCATCCCAGTCGAC
WM27G  1151 CCCAACAGCCTCGGGACATGCGAGTAAGCCATTTTACACCGGCACGTAAC
WM27G  1201 ATTTTCTTTCTTCTACGAATACCACGAGTCAAGTTATGACGTGTTGAGCC
WM27G  1251 CGTGACGCTGCATTTGTTTTTGCACGTAGCTACTGGAGGACGCACCCCAT
WM27G  1301 GCAGATCTGGTCGGCGCTGGGGAGCTGGCCGAGCTCAGTGAGCCACTTCT
WM27ms                                                CTTCT

WM27G  1351 TCGGCGCCGCCGGTGGCGCGGTCGCCGGCGGGCCGAGCATGAGCATCCAG
WM27ms      TCGGCGCCGCCGGTGGCGCGGTCGCCGGCGGGCCGAGTATGAGCATCCAG

WM27G  1401 GACGCGCTGGCGAACACGAGGACCGACGGCGCCGGTGCGCTGCTGCGCGA
WM27ms      GACGCGCTGGCGAACACGAGGACCGACGGCGCCGGCGCGCTGCTGCGCGA

WM27G  1451 GGGCACCGCGCGCCTGCTCAACTCCATGACCCGTCCGGGGGTTCGCCTACA
WM27ms      GGGCACCGCAGCCCTGCTCAACTCCATGACCCGCCCGGGGTTCGCCTACA

WM27G  1501 CCACCCAGCAGGTGAGGGACGCGTTCGCGGCGGCCGCGGCCGGCGGCGT-
WM27ms      CCACCCAGCAGGTGAGGGACGCATTCGCGGCGGCCGTGGCCGGCGGCTCT

WM27G  1551 AGACAGCGCCGCGGCGGCGCAGGCGGCCTCGTTCAAGAAAGCCAACGAGG
WM27ms      -GACAGTGCCGCGGCGGCGCAGGCGGCGGCGTTCAAGAAAGCCAACGAGG
```

Figure 5.9 cont.

```
WM27G 1601  GGAGGAAGGCGTAGATCGACCGGATCGCCATGGAGAGGGCTTTAGCTAGC
WM27ms      GGAGGAAGCCGTAGATCGACCGGATCGCCATCGAGAGGGCTTTAGCTAGC

WM27G 1651  TGGCTACCTATATTTGTGGCATGTCTGCATGCATGCGTTTAGGCCGGACG
WM27ms      TGGCTACCTATATCTGTGGCATGTCTGCATGCATGCGTTTAGGCCGGACG

WM27G 1701  GACGTGTGCGTTTTGATGTGCGTTTTGATTTGGTTGTTTCGTTTGAGTTA
WM27ms      GACGTGTGCGTTGC------------GATTTGGTTGTTTCGTTTGAGTTA

WM27G 1751  TGGTTT
WM27ms      TGGTTTGGTTGTGTACGTTGGTGTCTTCGTGGGCTAGCTTCGATCTATCG

WM27ms      TACTGTGTTGTAATGAATCTCGTTTAAGTTTTTTGCAGTTCCGTACCCTG

WM27ms      TCGCATTGAATGGAATATATTGAAGCGAACTATTCCTAGCTCAAAAAAAA
```

control of its expression. While this is unfortunate, the genomic clone is still of value as Open Reading Frame analysis can be used to identify introns in the structure of the gene and a putative deduced protein sequence encoded by the gene can be established.

Construction of an Open Reading Frame (ORF) map of the genomic sequence, using DNA Strider, identified three putative ORFs in the first phase. These are separated by two putative introns reflected in the groups of stop codons located in the regions at 550bp to 600bp and 1200bp to 1300bp (Figure 5.10). The putative start codon is located 189 bases into the sequence and the putative termination codon is located at 1650bp.

To determine if the putative introns are real, and to identify the reading frame of the translated gene on either side of the putative introns, RT-PCR of single strand cDNA was performed using two primers flanking both possible introns as indicated in Figure 5.9. The forward primer is situated at base 308 while the reverse primer is located at position 1584 in the region known to be homologous with WM27ms. Amplification of first strand cDNA between these two primers should produce a product 1276bp in length if the putative introns are not present, and a product shorter than this if the gene does contain introns. Amplification of first strand cDNA from various tissues, genomic DNA from Chinese Spring and the *ph2a* and *ph2b* mutants, as well as plasmid DNA from WM27g was performed at three different primer annealing temperatures. RT-PCR of the templates using the optimal primer annealing temperature of 62°C, as calculated in the OLIGO programme fails to produce consistent amplification of any of the templates (Figure 5.11). No amplification of the cDNA templates is evident, and the major bands at approximately 1300bp and 700bp present when *ph2b* DNA is used as template are not present in the amplification of DNA from Chinese Spring and WM27g as expected. This indicates that the effective amplification of the templates did not occur and the amplification of DNA from *ph2b* is non-specific. Decreasing the primer annealing temperature to 60 °C and 58 °C resulted in amplification of numerous, non-specific products (data not shown).

Further analysis of the genomic sequence has subsequently demonstrated that the guanine-cytosine rich regions of the gene form long and stable hairpin loops which would disrupt any amplification of the gene. In order to remove all of the loops, the DNA would need to be heated to 72 °C at which temperature the primers would fail to anneal and amplification could not be achieved. Following this discovery, it is evident that attempts to analyse the

Figure 5.10

Open Reading Frame map of WM27g. Reading in the first phase appears to give the most likely gene product. The stop codons which may indicate the localisation of introns are located at approximately bases 550-600 and 1200-1300. The putative start codon is located at base 189 and the putative stop codon is located at base 1650.

Figure 5.10

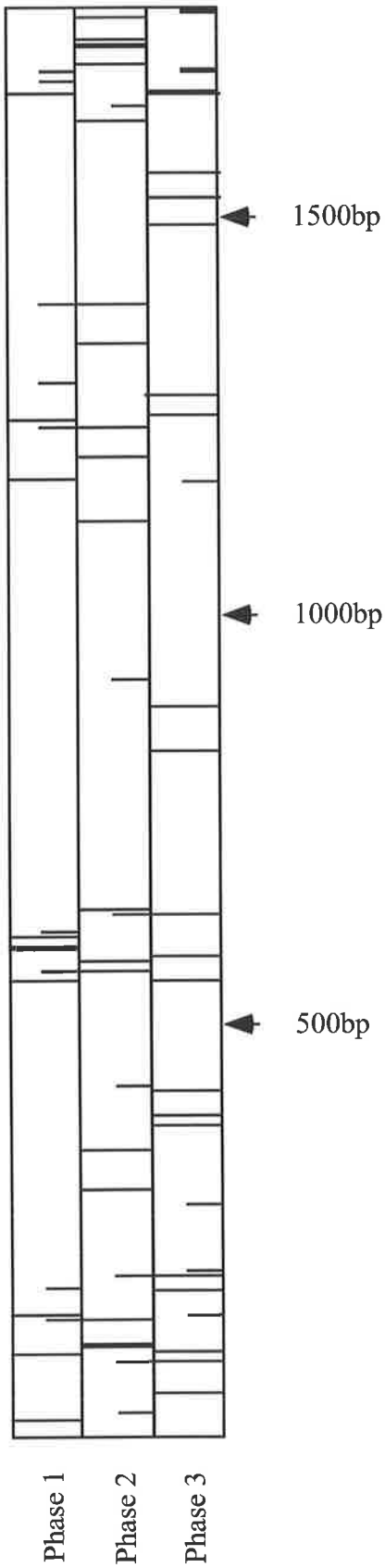
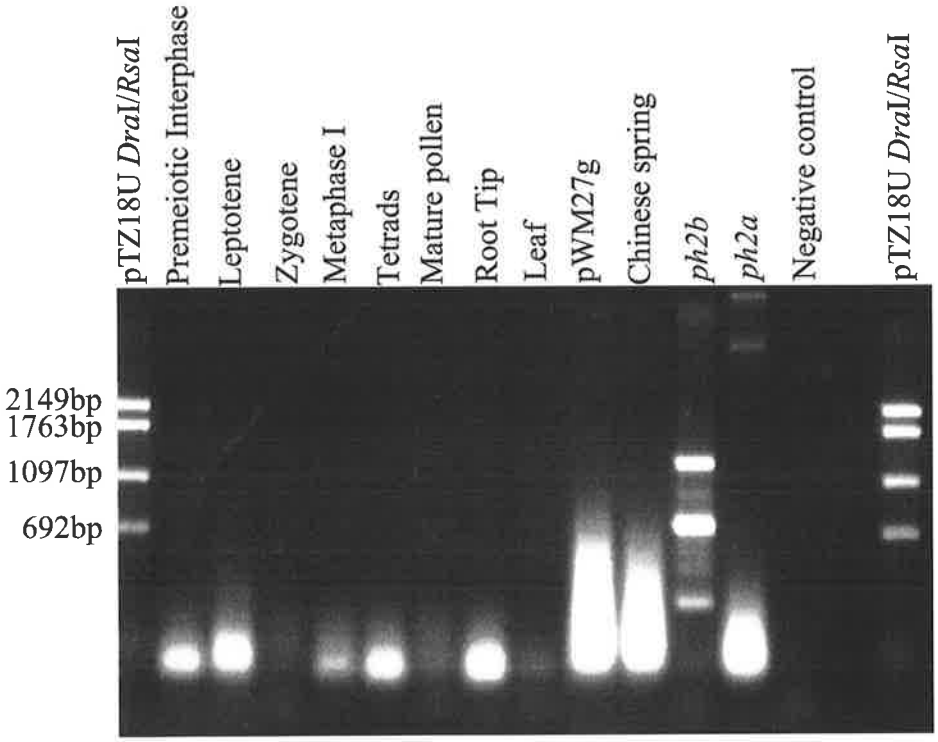


Figure 5.11

PCR amplification of reverse transcribed RNA from various tissues (as indicated), as well as DNA from WM27g, Chinese Spring, *ph2a* and *ph2b*. A sample (10µl) was separated by electrophoresis on a 1.2% TAE/ agarose gel and visualised under UV light following staining with ethidium bromide.

Figure 5.11


pTZ18U DraI/RsaI
Premeiotic Interphase
Leptotene
Zygotene
Metaphase I
Tetrads
Mature pollen
Root Tip
Leaf
pWM27g
Chinese spring
ph2b
ph2a
Negative control
pTZ18U DraI/RsaI
2149bp
1763bp
1097bp
692bp

position of putative introns via RT-PCR would not be successful. Considering that PCR amplification of first strand cDNA, as well as genomic and plasmid DNA was unsuccessful, the use of PCR based RACE techniques is also considered inappropriate for the isolation of a full length cDNA homologue.

5.3.6 Screening of a cDNA library with the genomic clone WM27g

The screening of a second cDNA library was originally viewed as an undesirable method for the isolation of a full length cDNA because the collection of new material and production of the library is labour intensive and time consuming. However, the low efficiency and inconsistency associated with PCR amplification necessitates the use of this approach for the isolation of a full length cDNA homologue of the gene. Fortunately a wheat early meiosis cDNA library produced by Letarte (1992), became available so that construction of a second cDNA library was unnecessary.

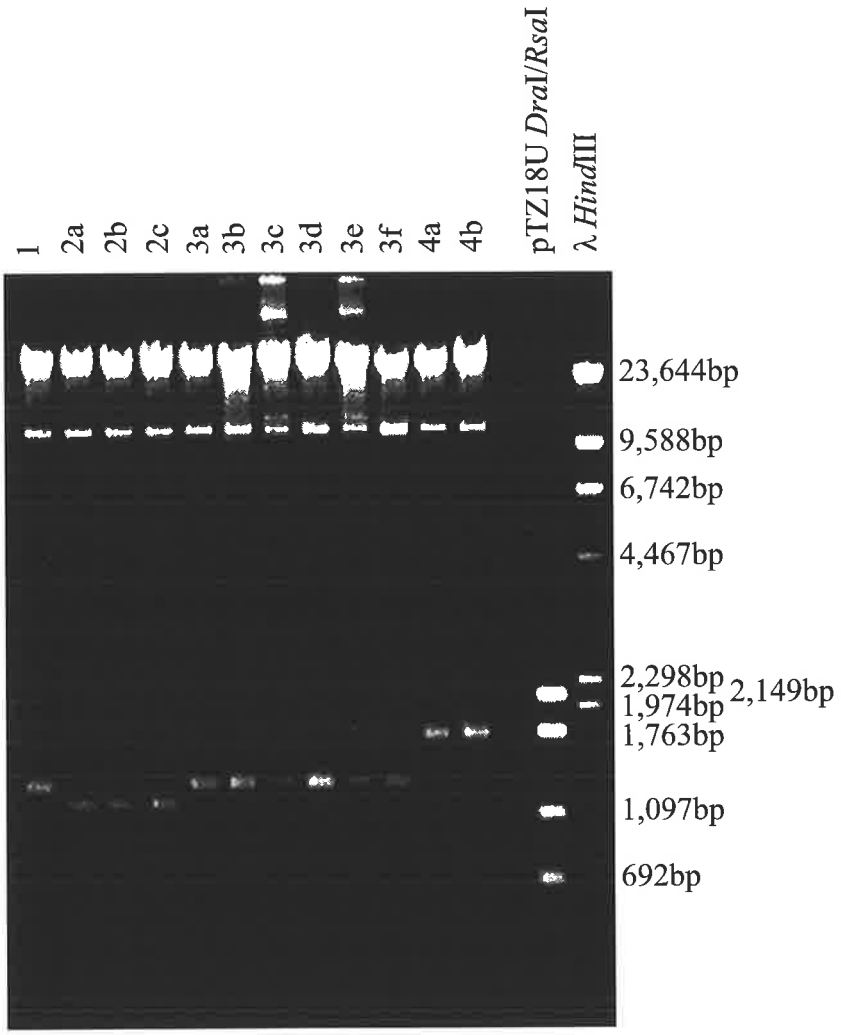
To limit the isolation of clones from the new cDNA library to those containing cDNAs of longer length, the genomic insert was digested with *Hind*III and *Sal*I to produce a number of regions spanning the clone so that each region could be used as a probe separately. Digestion of the insert with *Hind*III produces a fragment representing 570bp of the 5' end of the genomic clone, while *Sal*I digestion gives a fragment of 1100bp representing the 5' end and middle of the genomic clone. The 3' end of the gene was represented by the partial cDNA clone WM27ms. Hybridisation of $5x10^4$ pfu of the cDNA library with all three probes allowed the identification of four phage which hybridise to all of the probes. Electrophoresis of *Not*I digested DNA isolated from the purified phage revealed that the inserts ranged in size from approximately 1100bp to 1700bp (Figure 5.12). The largest insert corresponds to the size of the mRNA detected by Northern analysis (Figure 5.2) and this clone is most likely to contain a full length cDNA homologous to WM27ms.

To be assured that the cDNA isolated was homologous to WM27ms, it was used as a probe for hybridisation to the nullisomic/tetrasomic and ditelosomic Southern membrane as outlined above. The hybridisation demonstrates that the insert hybridises to the same bands in the Southern analysis (data not shown). The cDNA was subcloned into pBluescript (KS^-) and named AWWM5 (Adelaide Waite Wheat Meiosis) in accordance with other wheat meiosis genes isolated (Ji, 1992; Letarte, 1996).

Figure 5.12

Four phage were isolated in the screen of a cDNA library with the genomic clone. DNA was isolated from each of the phage and digested with *Not* I. The resulting fragments were separated by electrophoresis on a 1.0 % TAE/agarose gel and visualised under UV light following staining with ethidium bromide. The size of the inserts in the phage range from 1100bp to 1700bp.

Figure 5.12


1
2a
2b
2c
3a
3b
3c
3d
3e
3f
4a
4b
pTZ18U *DraI/RsaI*
λ *Hind*III
23,644bp
9,588bp
6,742bp
4,467bp
2,298bp
2,149bp
1,974bp
1,763bp
1,097bp
692bp

Digestion of pAWWM5 with *Hind*III and double digestions with *Sal*I/*Not*I were performed to dissect the clone into fragments which were subsequently sequenced. The full length sequence of AWWM5 and the deduced protein are presented in Figure 5.13. Comparison of the cDNA sequence with that of the genomic clone reveals that the gene is interrupted by two introns. The first intron is 96bp long situated between bases 552 and 648 and covers the first region of stop codons in the genomic clone identified as a putative intron in the ORF map (Figure 5.10). The second intron begins at base 1178, extends to base 1283 and covers the region of the second two stop codons identified in Figure 5.10.

Analysis of the sequence of AWWM5 reveals a high percentage of guanine and cytosine residues. Comparison of the AWWM5 sequence with the international gene databases demonstrates that AWWM5 shares no homology with any known genes either at the DNA (Altschul *et al.*, 1990) or protein level (Altschul *et al.*, 1990; Gish *et al.*, 1993). However, the high guanine and cytosine contents of some genes in *E. coli*, *S. cerevisiae* and humans has been related to the early replication of the DNA (Deschavanne and Filipski, 1995). It would be of interest to determine the time of replication of AWWM5 to allow the investigation of the possibility that the G/C content of AWWM5 causes early replication and allows active transcription of the gene during premeiotic interphase as would be required for a gene regulating proposed premeiotic chromosome associations.

AWWM5 encodes a peptide of 385 amino acids (Figure 5.13), the most interesting feature of which is the high percentage of positively charged arginine residues (14.8%) which are arranged in blocks of between 3 and 7 residues. Comparisons using MacPattern (Fuchs, 1994) reveals that no other characterised gene displays this structure of blocks of arginine residues.

The high percentage of arginine residues indicates that AWWM5 encodes a protein which is probably DNA or RNA binding (Burd and Dreyfuss, 1994). Binding of DNA or RNA by the AWWM5 protein is likely to be very stable, as the most stable amino acid - nucleotide interaction is between arginine and guanine (Lustig and Jernigan, 1995). The arrangement of the arginine residues in blocks is also likely to have a profound effect on the protein structure. As arginine is a positively charged amino acid, closely positioned arginine

Figure 5.13

Nucleotide and deduced protein sequence of AWWM5. The putative nuclear targeting motifs are underlined and the putative membrane binding motif is dotted underlined. Comparison of the nucleotide sequence with the genomic sequence (data not shown) indicates the presence of two introns situated between bases 552-648 and 1178-1283.

Figure 5.13

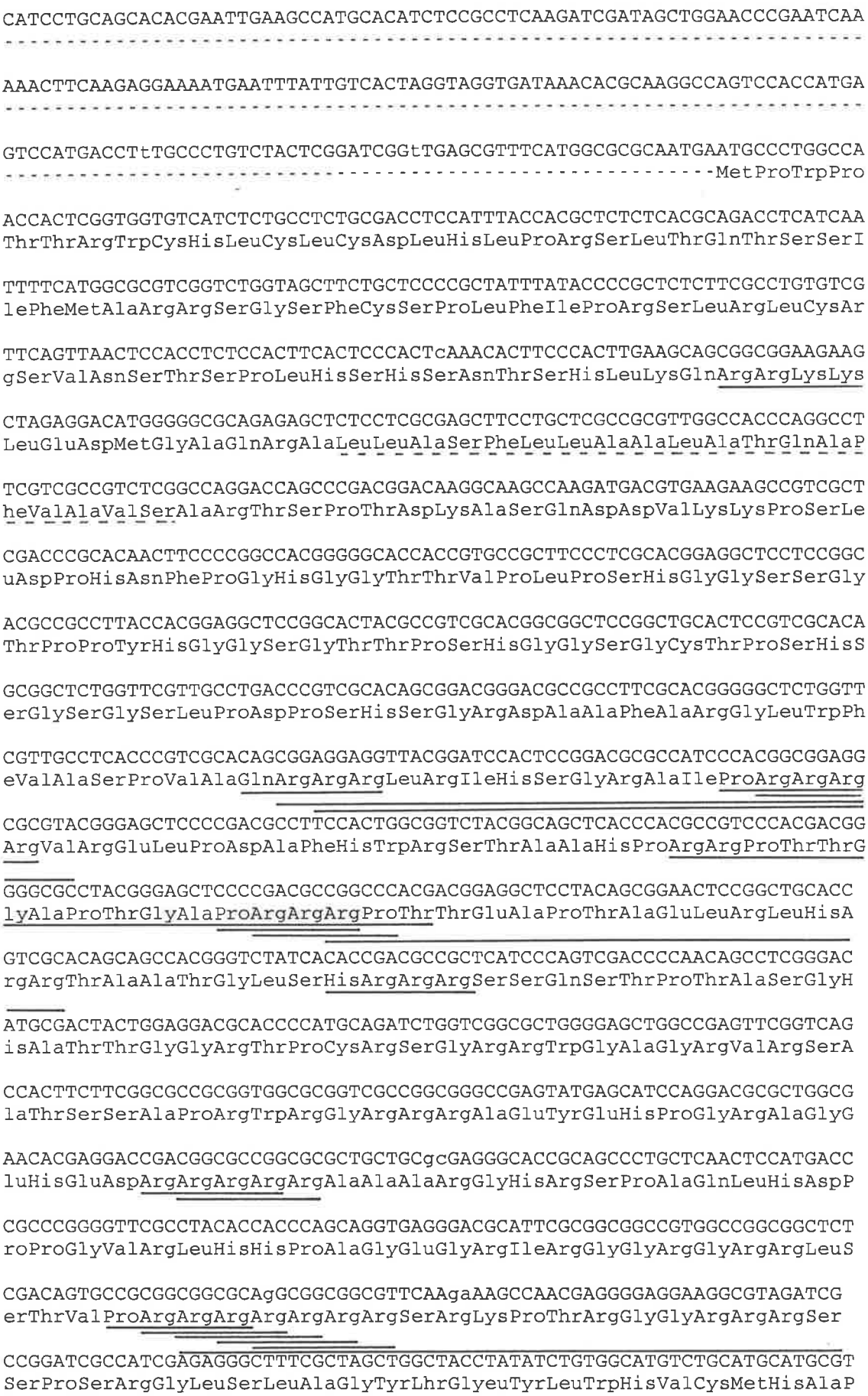

CATCCTGCAGCACACGAATTGAAGCCATGCACATCTCCGCCTCAAGATCGATAGCTGGAACCCGAATCAA
AAACTTCAAGAGGAAAATGAATTTATTGTCACTAGGTAGGTGATAAACACGCAAGGCCAGTCCACCATGA
GTCCATGACCTtTGCCCTGTCTACTCGGATCGGtTGAGCGTTTCATGGCGCGCAATGAATGCCCTGGCCA
MetProTrpPro
ACCACTCGGTGGTGTCATCTCTGCCTCTGCGACCTCCATTTACCACGCTCTCTCACGCAGACCTCATCAA
ThrThrArgTrpCysHisLeuCysLeuCysAspLeuHisLeuProArgSerLeuThrGlnThrSerSerI
TTTTCATGGCGCGTCGGTCTGGTAGCTTCTGCTCCCCGCTATTTATACCCCGCTCTCTTCGCCTGTGTCG
lePheMetAlaArgArgSerGlySerPheCysSerProLeuPheIleProArgSerLeuArgLeuCysAr
TTCAGTTAACTCCACCTCTCCACTTCACTCCCACTcAAACACTTCCCACTTGAAGCAGCGGCGGAAGAAG
gSerValAsnSerThrSerProLeuHisSerHisSerAsnThrSerHisLeuLysGlnArgArgLysLys
CTAGAGGACATGGGGGCGCAGAGAGCTCTCCTCGCGAGCTTCCTGCTCGCCGCGTTGGCCACCCAGGCCT
LeuGluAspMetGlyAlaGlnArgAlaLeuLeuAlaSerPheLeuLeuAlaAlaLeuAlaThrGlnAlaP
TCGTCGCCGTCTCGGCCAGGACCAGCCCGACGGACAAGGCAAGCCAAGATGACGTGAAGAAGCCGTCGCT
heValAlaValSerAlaArgThrSerProThrAspLysAlaSerGlnAspAspValLysLysProSerLe
CGACCCGCACAACTTCCCCGGCCACGGGGGCACCACCGTGCCGCTTCCCTCGCACGGAGGCTCCTCCGGC
uAspProHisAsnPheProGlyHisGlyGlyThrThrValProLeuProSerHisGlyGlySerSerGly
ACGCCGCCTTACCACGGAGGCTCCGGCACTACGCCGTCGCACGGCGGCTCCGGCTGCACTCCGTCGCACA
ThrProProTyrHisGlyGlySerGlyThrThrProSerHisGlyGlySerGlyCysThrProSerHisS
GCGGCTCTGGTTCGTTGCCTGACCCGTCGCACAGCGGACGGGACGCCGCCTTCGCACGGGGGCTCTGGTT
erGlySerGlySerLeuProAspProSerHisSerGlyArgAspAlaAlaPheAlaArgGlyLeuTrpPh
CGTTGCCTCACCCGTCGCACAGCGGAGGAGGTTACGGATCCACTCCGGACGCGCCATCCCACGGCGGAGG
eValAlaSerProValAlaGlnArgArgArgLeuArgIleHisSerGlyArgAlaIleProArgArgArg
CGCGTACGGGAGCTCCCCGACGCCTTCCACTGGCGGTCTACGGCAGCTCACCCACGCCGTCCCACGACGG
ArgValArgGluLeuProAspAlaPheHisTrpArgSerThrAlaAlaHisProArgArgProThrThrG
GGGCGCCTACGGGAGCTCCCCGACGCCGGCCCACGACGGAGGCTCCTACAGCGGAACTCCGGCTGCACC
lyAlaProThrGlyAlaProArgArgArgProThrThrGluAlaProThrAlaGluLeuArgLeuHisA
GTCGCACAGCAGCCACGGGTCTATCACACCGACGCCGCTCATCCCAGTCGACCCCAACAGCCTCGGGAC
rgArgThrAlaAlaThrGlyLeuSerHisArgArgArgSerSerGlnSerThrProThrAlaSerGlyH
ATGCGACTACTGGAGGACGCACCCCATGCAGATCTGGTCGGCGCTGGGGAGCTGGCCGAGTTCGGTCAG
isAlaThrThrGlyGlyArgThrProCysArgSerGlyArgArgTrpGlyAlaGlyArgValArgSerA
CCACTTCTTCGGCGCCGCGGTGGCGCGGTCGCCGGCGGGCCGAGTATGAGCATCCAGGACGCGCTGGCG
laThrSerSerAlaProArgTrpArgGlyArgArgArgAlaGluTyrGluHisProGlyArgAlaGlyG
AACACGAGGACCGACGGCGCCGGCGCGCTGCTGCgcGAGGGCACCGCAGCCCTGCTCAACTCCATGACC
luHisGluAspArgArgArgArgArgAlaAlaAlaArgGlyHisArgSerProAlaGlnLeuHisAspP
CGCCCGGGGTTCGCCTACACCACCCAGCAGGTGAGGGACGCATTCGCGGCGGCCGTGGCCGGCGGCTCT
roProGlyValArgLeuHisHisProAlaGlyGluGlyArgIleArgGlyGlyArgGlyArgArgLeuS
CGACAGTGCCGCGGCGGCGCAgGCGGCGGCGTTCAAgaAAGCCAACGAGGGGAGGAAGGCGTAGATCG
erThrValProArgArgArgArgArgArgArgSerArgLysProThrArgGlyGlyArgArgArgSer
CCGGATCGCCATCGAGAGGGCTTTCGCTAGCTGGCTACCTATATCTGTGGCATGTCTGCATGCATGCGT
SerProSerArgGlyLeuSerLeuAlaGlyTyrLhrGlyeuTyrLeuTrpHisValCysMetHisAlaP

Figure 5.13 cont.

```
TTAGGCCGGACGGACGTGTGCGTTGCGATTTGGTTGTTTCGTTTGAGTTATGGTTTGGTTGTGTACGTT
heArgProAspGlyArgValArgCysAspLeuValValSerPheGluLeuTrpPheGlyCysValArgT

GGTGTCTTCGTGGGCTAGCTTCGATCTATCGTACTGTGTTGTAATGAATCTCGTTTAAGTTTTTTGCAG
rpCysLeuArgGlyLeuAlaSerIleTyrArgThrValLeu<O>

GCAGTTCCGTACCCTGTCGCATTGAATGGAATATATTGAAGCGAACTATTCCTAGCT
```

residues will repel each other causing the protein to adopt a rod-like structure (M. Tate, pers. comm.).

The high percentage of arginine residues is also reflected in the hydrophobicity analysis of the putative protein (Figure 5.14) which shows that the C-terminal two thirds of the protein is very hydrophilic, indicating a cytoplasmic localisation. In contrast, the N-terminus of the protein is hydrophobic and may be membrane bound. Indeed, further analysis of the deduced protein using the PSort programme at Expasy (Nakai, 1991; Nakai and Kanehisa, 1992) indicates that the C-terminal region is cytoplasmic, and that the blocks of arginine residues (indicated in Figure 5.13) act as nuclear targeting signals whilst the N-terminus is identified as having a membrane binding capacity. It is suggested that the deduced AWWM5 protein is located in the nucleus, presumably being bound to the nuclear membrane, and may bind DNA through its C-terminus which possibly projects into the nuclear cytoplasm as a rod-like structure. The *in vitro* expression of the AWWM5 protein in *E. coli* and analysis of its DNA binding properties, as well as *in situ* localisation of the protein in wheat anthers through the production of antibodies, should allow a more accurate assessment of the function and distribution of the protein during meiosis.

5.3.7 *In situ* localisation of AWWM5 RNA transcripts

Whilst Northern analysis of AWWM5 demonstrates that the gene is transcribed during meiosis and preferentially during early prophase and premeiotic interphase, the use of whole anthers, containing both tapetal cells and pollen mother cells, for the extraction of RNA means that the actual location of the transcribed mRNA can not be determined from this analysis. *In situ* RNA hybridisation of digoxigenein labelled AWWM5 RNA sense (Figure 5.15a) and antisense (Figure 5.15b) transcripts, to sections of whole wheat florets reveals that AWWM5 is expressed in both the tapetal layer and the pollen mother cells.

The *in situ* localisation of AWWM5 to the tapetum and the meiocytes does not mean that the gene does not act during meiosis. Porter *et al.*, (1984) demonstrate that transcriptional activity in developing meiocytes is greatly reduced in comparison to the surrounding somatic cells and germ line cells not committed to meiosis. It is likely that the tapetum is recruited to produce proteins which may be required for a meiotic process. Indeed, Crossley *et al.* (1995) demonstrate that meiosis-specific transcripts isolated from *Lilium henryii*

Figure 5.14

Hydrophobicity plot of the deduced AWWM5 protein as determined by DNA Strider. The N-terminus is hydrophobic and may represent a membrane binding domain, whilst the C-terminus is hydrophilic and is possibly located in the nuclear cytoplasm (Nakai, 1991; Nakai and Kanehisa, 1992).

Figure 5.14

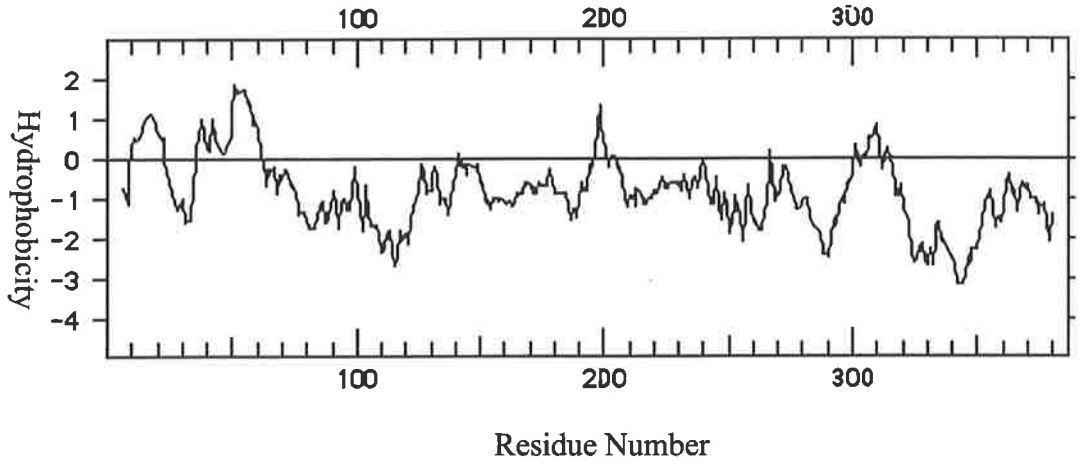

100
200
300
2
1
0
-1
-2
-3
-4
Hydrophobicity
100
200
300
Residue Number

Figure 5.15

In situ localisation of RNA transcripts of AWWM5 to cross sections of a wheat whole floret containing anthers with pollen mother cells at leptotene. Hybridisation's were made using sense (5.15a) and anti-sense (5.15b) transcripts. It is evident that AWWM5 is transcribed both in the tapetum and the pollen mother cells. Some transcription may also occur in the stigma.

Figure 5.15a

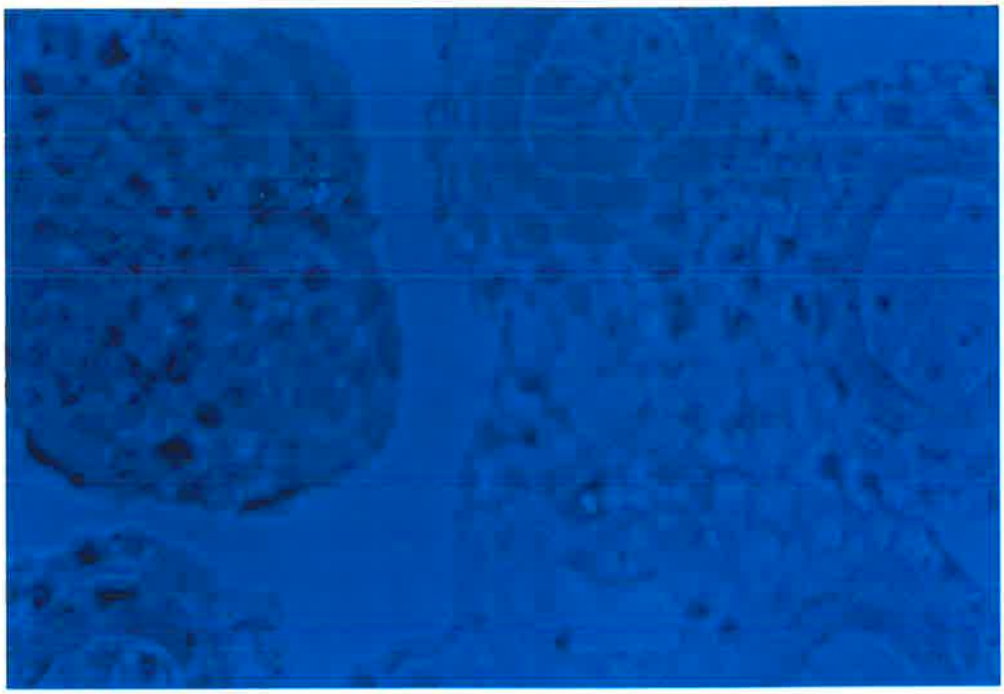
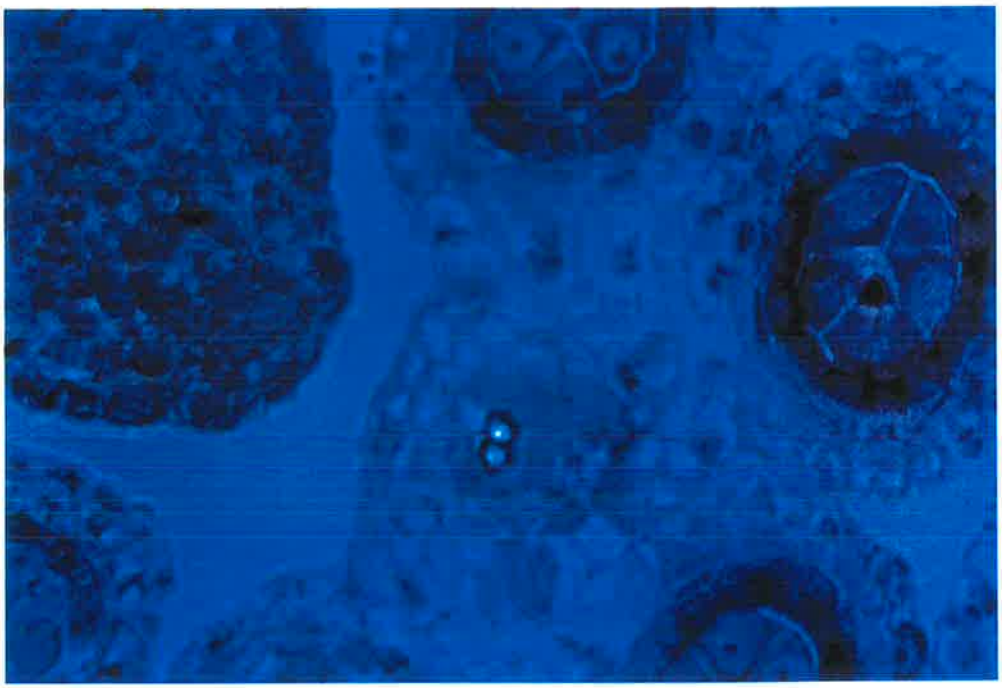


Figure 5.15b



meiocytes by subtractive hybridisation, are located in the tapetal layer and not in the meiocytes themselves. In addition, wheat meiosis-specific transcripts have also been located to the tapetal cells (Letarte, 1996). The presence of secretion signal motifs in the deduced proteins of these transcripts suggests that the protein is transferred to the meiocytes during meiosis (Letarte, 1996).

The observed transcriptional activity of AWWM5 within the meiocytes and the tapetum might indicate that the gene product is required at low levels during much of meiosis and that at premeiotic interphase and early meiotic prophase I, when high levels of the gene product are required, transcription in the tapetum is recruited to meet the increased demand. If the tapetum is recruited to produce additional amounts of the AWWM5 gene product which are transferred to the developing meiocyte, it could be assumed that the translated protein would contain a signal secretion motif. Analysis of the deduced AWWM5 protein fails to reveal the presence of a known secretion signal (Nakai, 1991). However, it has been demonstrated that a high degree of variation exist amongst secretion signal peptides, including those targeted to the same secretion pathway (Izard and Kendall, 1994), and it is possible that the AWWM5 protein possesses a signal sequence which is divergent form the known forms. Alternatively, the high degree of expression of the AWWM5 protein in the tapetum may be required during the mitotic division of cells in this tissue which has been demonstrated to occur during meiosis (Bennett *et al.*, 1971). However, the lack of expression in root tips could be assumed to indicate that the protein has no function during mitosis. It therefore seems likely that the AWWM5 protein is transferred, by an unknown pathway, from the tapetal cells to the meiocytes where it is targeted to the nucleus.

5.3.8 Consensus mapping of the *ph2a* deletion

The *ph2a* deletion mutant was originally isolated in an X-ray irradiated population during an attempt to isolate mutants of the *Ph1* locus (Sears, 1977). A mutant which displayed homoeologous pairing with *T. kotschyi* was identified but the effect of the deficiency was substantially lower than that expected for removal of *Ph1*. Subsequently, the deletion was shown to be located on the short arm of chromosome 3D and involved the *Ph2* locus (Sears, 1982). The mutation was named *ph2a* and was believed to involve a large, terminal deletion of the chromosome arm because no pairing between the mutant and a ditelosomic 3DS chromosome was observed (Sears, 1982). Copies of the AWWM5 gene have

previously been localised within the *ph2a* deletion on the short arm of chromosome 3D and the short arm of chromosome 3A.

To further characterise the *ph2a* deletion, the AWWM5 gene was mapped onto a barley consensus map (Langridge *et al.*, 1995a). The barley consensus map was utilised because a map of this quality is not currently available for wheat. In addition, as there is a high degree of conservation of gene order between wheat and barley chromosome 3 homoeologues (Sears, 1966; Hart *et al.*, 1980; Devos *et al.*, 1992; Nelson *et al.*, 1995), the barley consensus map was viewed as a practical method of mapping the extent and position of the *ph2a* deletion.

AWWM5 was used to probe digested DNA from the parents of the three barley double haploid populations available at the Waite Institute, Galleon X Haruna Nijo, Chebec X Harrington and Clipper X Sahara. The only polymorphism detected was between Clipper and Sahara DNA when digested with *Eco*RV. Subsequently, DNA from 90 individuals from the Clipper X Sahara double haploid population was digested with *Eco*RV and hybridised with AWWM5. Analysis of the results using the Mapmaker programme, confirmed the location of AWWM5 on the short arm of chromosome 3 approximately 2.9cM from the centromere (Figure 5.16).

Probes surrounding AWWM5 were identified from the consensus map (Figure 5.16) and were utilised to probe digested DNA from Chinese Spring, and the *ph2a* and *ph2b* mutation lines. The *ph2a* deletion has been determined to lie between the probes BCD828 (Figure 5.17a) and ABG460 (Figure 5.17b) and encompasses the probes ABG391 (Figure 5.17c) and PSR598 (Figure 5.17d) as well as AWWM5. Unfortunately, several of the MWG and ABG probes were not available. Hybridisations using these probes would allow a more accurate determination of the extent of the *ph2a* deletion. However, from the existing data it can be concluded that the *ph2a* deletion is relatively large but is not terminal as was believed.

Chromosomal *in situ* hybridisation using the meiosis specific AWWM1 clone, which is also located within the *ph2a* deletion (Ji, 1992), has revealed that AWWM1 is physically located very close to the telomere (Pederson, pers. comm.). In contrast, the position of the deletion as determined by linkage mapping, places it in the proximal half of the short arm of

Figure 5.16

Consensus linkage map of the short arm of barley chromosome three. The map was constructed using Mapmaker and incorporated markers from the laboratory mapping project (Langridge *et al.*, 1995a). Probes found to lie outside of the *ph2a* deletion are shown in bold whilst probes within the deletion are bold underlined.

Figure 5.16

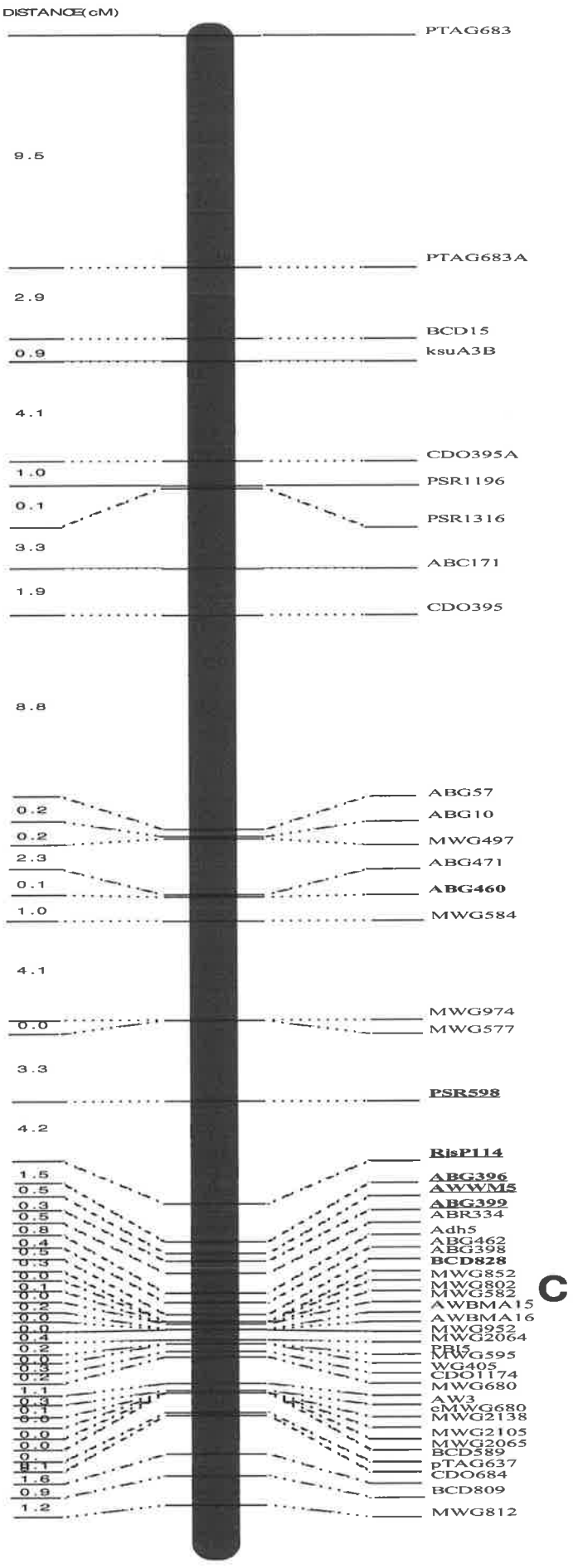

DISTANCE(cM)
PTAG683
9.5
PTAG683A
2.9
BCD15
0.9
ksuA3B
4.1
CDO395A
1.0
PSR1196
0.1
PSR1316
3.3
ABC171
1.9
CDO395
8.8
ABG57
0.2
ABG10
0.2
MWG497
2.3
ABG471
0.1
ABG460
1.0
MWG584
4.1
MWG974
0.0
MWG577
3.3
PSR598
4.2
RisP114
1.5
ABG396
0.5
AWWM5
0.3
ABG399
0.5
ABR334
0.8
Adh5
0.4
ABG462
0.5
ABG398
0.3
BCD828
0.0
MWG852
0.1
MWG802
0.0
MWG582
C
0.2
AWBMA15
0.0
AWBMA16
0.0
MWG952
0.4
MWG2064
0.2
PRI5
0.0
MWG595
0.3
WG405
0.2
CDO1174
1.1
MWG680
0.3
AW3
0.1
cMWG680
0.0
MWG2138
0.0
MWG2105
0.0
MWG2065
0.1
BCD589
1.6
pTAG637
0.9
CDO684
1.2
BCD809
MWG812

Figure 5.17

Mapping of the *ph2a* deletion - DNA from Chinese Spring, *ph2a* and *ph2b*, was digested with the enzymes indicated then hybridised with BCD828 (5.17a), ABG460 (5.17b), ABG399 (5.17c) and PSR598 (5.17d). No polymorphism's between the different lines are apparent in hybridisation's with BCD828 and ABG460. The polymorphism's observed after hybridisation with PSR598 and ABG399 are indicated by arrows.

Figure 5.17a

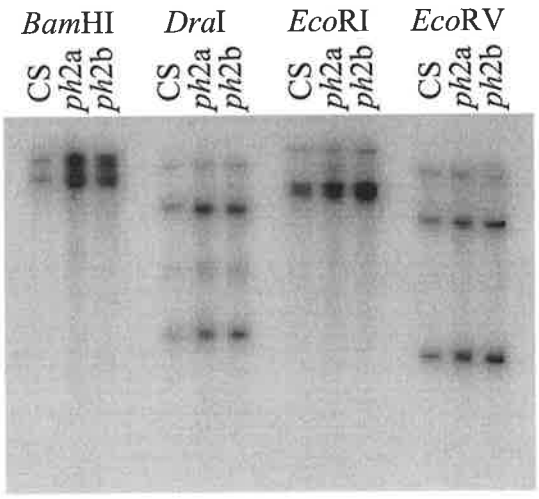


Figure 5.17b

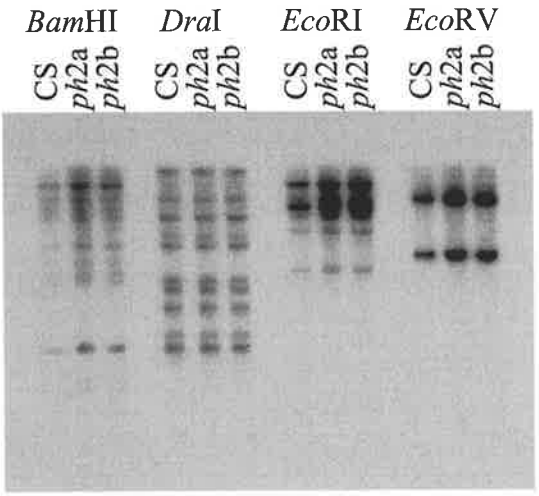


Figure 5.17c

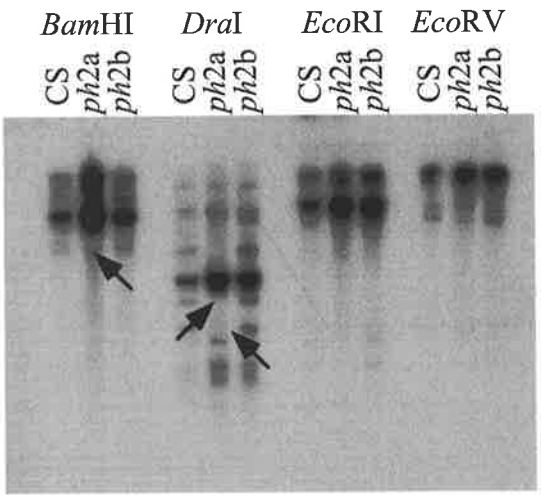
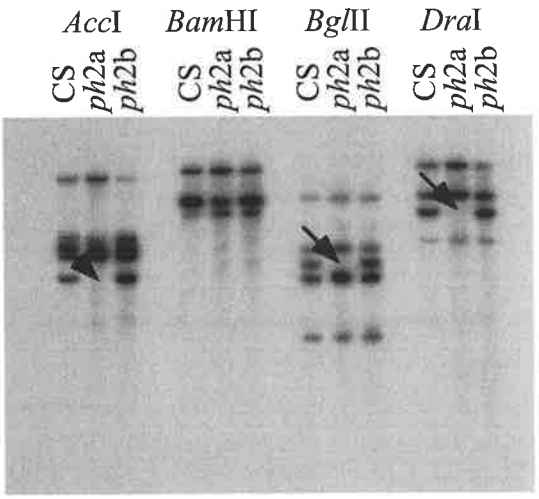


Figure 5.17d



chromosome 3D. This is by no means a rare phenomenon in wheat. Analysis of telocentric chromosome pairing in ditelosomic lines of Chinese Spring have demonstrated that almost all chiasmata are either terminal or subterminal, and that proximal recombination is extremely rare. Hence, there is often a distortion in the comparison of physical and genetic localities. Mapping of the *ph1b* mutation (Sears, 1984) has revealed a 1% recombination frequency with the centromere of chromosome 5B, yet physical mapping of the mutation shows it to be located midway along the long arm (Dvorak *et al.*, 1984).

5.4 Conclusion

The premeiotic association of homologous chromosomes as a mechanism to favour eventual intimate, meiosis-specific pairing has been proposed by several authors (Avivi and Feldman, 1980; Maguire, 1983; Hiraoka *et al.*, 1993; Kleckner and Weiner, 1993) but has been strongly opposed by others (Rasmussen and Holm, 1980; John, 1990) as discussed in Chapter 1. Recent research using fluorescent *in situ* hybridisation to *S. cerevisiae* chromosomes has identified multiple, premeiotic interactions in this organism (Loidl *et al.*, 1994a; Weiner and Kleckner, 1994) providing evidence for premeiotic chromosome associations.

Should the *AWWM5* gene isolated from *T. aestivum* function in homologous chromosome pairing, the premeiotic pairing proposal would be most attractive as analysis of *AWWM5* expression indicates that maximum expression of the gene occurs during premeiotic interphase and early meiotic prophase I. The presence of lower levels of the mRNA during latter stages of meiosis may then reflect continuing transcription of the gene or may be a consequence of high stability of the mRNA species. The absence of expression in root tips indicates that the gene is not required for mitotic functions. It is suggested that transcription of the gene at premeiotic interphase reflects a requirement of the gene product at this stage that is not associated with mitotic cell divisions known to occur in the tapetum at this time (Bennett *et al.*, 1971).

Premeiotic association of homologous chromosomes is an attractive hypothesis for the establishment of homologous synapsis at meiotic prophase. However, as noted by Giroux (1988), the hypothesis does little to address the mechanisms required for the establishment of association of homologous chromosomes; it simply assumes that the mechanism

functions before meiosis. The uncertainty surrounding the mechanism of chromosome association has resulted in an abundance of hypotheses to explain how homologous chromosome first come into contact. As outlined in Chapter 1, the homologue association models can be separated into three general categories: (1) long range specific attractions, (2) random associations of homologues in motion, and (3) interaction of homologues with specialised, extrachromosomal structures. It has long been recognised that intermolecular forces of attraction are extremely weak (Faberge, 1942) making mechanisms based on this assumption unlikely. Similarly, the evidence against passive, random movement of chromosomes resulting in homologue pairing is also strong (Maguire, 1974). The bulk of research into chromosome pairing has therefore concentrated on the interaction of chromosomes with various nuclear structures including the nuclear membrane (Avivi and Feldman, 1980) and other internal nuclear structures (Maguire, 1983).

The speculative targeting of the deduced AWWM5 protein to the nucleus, putative membrane binding characteristics of the N-terminus and the putative DNA binding capacity of the C-terminus, makes it attractive to assume an interactive role of the protein between the nuclear membrane and the chromosomes. It is possible that the initial contacts between homologous chromosomes are initiated through a specific interaction with the nuclear membrane mediated by the AWWM5 protein. However, the *in vitro* expression of the protein and the production of antibodies required so that the putative DNA binding properties and localisation of the protein can be more accurately assessed.

Chapter 6

General Discussion and Conclusion

The relative lack of information on the processes of early meiotic prophase and in particular chromosome paring, as well as the contradictory observations of these processes in different organisms, have resulted in numerous hypotheses to explain the mechanism of homologue pairing during meiosis. Whilst conjecture remains over the mechanisms acting to ensure homologous chromosome pairing during meiosis, it is apparent that pairing is a two step process involving the association of chromosomes followed by an intimate assessment of homology at the DNA level. The action of the *Ph* genes of wheat in ensuring homologous chromosome pairing could therefore occur at either the chromosome association stage and/or during the assessment of homology. If the Kleckner and Weiner (1993) model of chromosome pairing (Chapter1) is accepted, the putative action of the *Ph* genes in controlling initial homologue association would be likely to occur at premeiotic interphase and leptotene. The isolation of genes preferentially expressed during these periods of development may therefore provide important information on the genetic control of determination homologous chromosome pairing. Alternatively, if it is considered that the *Ph* genes act to increase the stringency of homology recognition, achieved through the single strand invasion of two interacting duplexes as proposed in the double strand break model of recombination (Chapter 1), the isolation of *RecA* homologues in wheat might allow the isolation of *Ph* proteins through their putative interaction.

The conflicting views on the timing of *Ph* gene action requires that molecular studies to identify these genes in wheat must encompass a broad range of meiocyte development from premeiotic interphase to the end of pachytene of prophase I. The rapid progression of meiosis in *T. aestivum*, and the small size of the anthers, makes the collection of suitable meiotic material covering such a large range of developmental stages difficult and time consuming. During this research, subtractive hybridisation has been employed for the isolation of genes expressed during premeiotic interphase and early prophase I. Subtractive hybridisation circumvents much of the laborious task of collecting and staging meiotic

material for use in traditional differential expression studies, as a relatively small amount of starting material is required in comparison to other techniques such as differential screening of cDNA libraries. Subtractive hybridisation also allows the isolation of genes with low expression and thus is viewed as a desirable technique for the isolation of *Ph* genes where the level of expression is unknown.

The use of subtractive hybridisation resulted in the isolation of a number of genes expressed preferentially during early meiosis in *T. aestivum*. The wide range of genes isolated reflects the complex nature of meiosis in bread wheat and further characterisation of all of the clones would provide new information on the proteins required for efficient meiotic division and the functions they play. In particular, further characterisation of the identified histone variants should provide excellent information on the process of chromosome condensation during chromosome pairing which may act to bring homologues into apposition following the initial establishment of recognition (Scherthan *et al.*, 1992). Analysis of the expression patterns of WM19 and WM47 would also enable a determination of the possibility that the genes represented by these clones are also required for a meiotic process.

None of the current chromosome pairing hypotheses can explain all of the observations on homologue pairing or the action of the *Ph* genes. However, the theories of premeiotic association of homologous chromosomes would appear to provide the best current explanation of chromosome pairing. Here, a speculative model based on several premeiotic association hypotheses (Feldman and Avivi, 1973, 1988; Maguire, 1988; Feldman, 1993; Kleckner and Weiner, 1993) has been suggested.

The model outlined here proposes that the processes leading to premeiotic association of homologous chromosomes, in domains which facilitate their interaction, are under genetic control and are active. It is suggested that the preliminary interaction of homologous chromosomes is mediated by a microtubule associated motor protein which actively moves chromosomes into domains where their interaction is "topologically favourable" during meiosis (Weiner and Kleckner, 1993). Several lines of evidence support the hypothesis that the association of homologous chromosomes is a result of the interaction of chromosomes with microtubules. Avivi and Feldman (1973) demonstrate the loss of non-random chromosome distribution in root tip cells of *T. aestivum* treated with colchicine. As colchicine specifically binds tubulins (Borisy *et al.*, 1967; Weisenberg *et al.*, 1968; Wilson,

1970), they conclude that microtubules are responsible for the movement and arrangement of chromosomes during premeiotic interphase and also in somatic cells. Similarly in rats, microtubule initiated chromosome movement has been observed during early meiosis (Sheldon *et al.*, 1988) and can be disrupted by the application of colchicine co-comittant with damage to the nuclear membrane (Salonen *et al.*, 1982). In fission yeast, telomere-led chromosome movement is a feature of premeiosis being caused by the migration of the spindle pole body on an array of microtubules (Chikashige *et al.*, 1994). The frequently observed attachment of telomeres to the nuclear membrane in many species could also occur as a consequence of their interaction with extranuclear microtubules (Loidl, 1990).

It is speculated that, following the establishment of a spatial relationship between chromosomes, a preliminary test of homology may be initiated at specific sites in the distal ends of chromosome arms under the influence of a membrane bound, DNA binding protein. If homology is detected, the chromosomes could be "locked" into their domains by disruption of the microtubules required for their movement. Further stabilisation of the homologue association might then be accomplished by the formation of weak multiple interstitial interactions. If homology was not detected at the specific pairing sites, the chromosomes could actively move apart under the influence of the motor protein, and be repositioned for further tests of homology. As the cell enters prophase, the association of homologous chromosomes could facilitate their interaction and a strict test of homology may be performed at the DNA/DNA level (Smithies and Powers, 1986) through the action of DNA strand invasion. DNA strand invasion is thought to be mediated by the formation of double strand breaks at the sites of multiple interactions established during premeiotic interphase (Weiner and Kleckner, 1994). DSB formation is co-commitant with the formation of axial elements of the synaptonemal complex along the homologues (Padmore *et al.*, 1991), and following positive identification of homology, the central core of the synaptonemal complex forms and is thought to "zip" the chromosomes together (Sym *et al.*, 1993) and provide an environment in which crossovers occur and recombination is completed.

It has been proposed that late replicating zygDNA may represent the sites where multiple interstitial interactions occur between homologues as zyg DNA has been reported to be present at multiple sites along the length of chromosomes in lily (Ito *et al.*, 1967a; Ito and Hotta, 1973) and the inhibition of zygDNA replication prevents the initiation and

continuation of chromosome pairing in this species (Ito *et al.*, 1967b). Alternatively, zygDNA may represent those areas in the lily genome which are most susceptible for DSB formation required for homology checking, recombination, and possibly the formation of tripartite synaptonemal complex. In agreement with this, zyg DNA has been demonstrated to be required for SC formation (Roth and Ito, 1976; Kurata and Ito, 1978).

Regardless of the possible functions of zygDNA in lily, it has been demonstrated here that late replicating DNA in wheat, if it exists, is of a different structure to the zygDNA of lily. The putative zygDNA of wheat either lacks the single stranded DNA links to the surrounding genome or has a buoyant density similar to bulk nuclear DNA. At present there is no conclusive evidence to suggest that late replicating DNA is present in wheat.

If homologous chromosomes are drawn into topologically favourable domains (Kleckner and Weiner, 1993) which facilitate their interaction at early meiosis, a mechanism must exist which not only serves to initiate chromosome movement but also acts to keep the chromosomes in their domains during DNA replication. Maguire (1983) observed that the association of chromosomes at premeiotic interphase in maize is reliant on their interaction with nuclear structures. Similarly, in the pollen mother cells of *T. aestivum*, bundles of fibrillar material are observed connecting masses of chromatin with each other and with the nuclear membrane from premeiotic interphase until zygotene (Bennett *et al.*, 1974). The intranuclear fibrillar material does not seem to be composed of microtubules (Bennett and Smith, 1979). However, the application of colchicine at premeiotic interphase affects fibrillar material assembly, indicating that it is either associated with microtubules or is reliant on a preceding function dependent upon microtubule assembly.

It is proposed that the deduced AWWM5 protein described here represents the intrafibrillar material of *T. aestivum*, possibly forming rod like structures which bind to the chromosomes through their putative DNA binding domain in the arginine rich C-terminus, and interacting with the nuclear membrane through the putative membrane binding N-terminus. Homologous chromosomes entering domains which favour their interaction at early meiosis could then be locked into position through their interaction with the nuclear membrane via the AWWM5 protein, thus maintaining the association through premeiotic DNA replication. The observed interaction of the telomeres with the nuclear membrane and the bouquet configuration of chromosomes present at zygotene in some organisms, could then be the

result of all telomeres being drawn to a restricted site on the nuclear membrane as proposed by Loidl (1990). Based on the hypotheses of premeiotic chromosome interactions (Kleckner and Weiner, 1993; Feldman, 1993) a hypothetical mechanism for the genetic control of homologue pairing is proposed below.

As described earlier (Chapter 1), the diploid like nature of wheat is controlled by the action of several pairing genes. Of these, the effects of the *Ph1* gene on 5BL, the *Ph2* gene on 3DS and the suppressor on 3AS are the most studied. Removal of *Ph1* has been demonstrated to result in the formation of multivalency, often as trivalents suggesting the interaction of homoeologues (Riley, 1960), a phenomenon which can be phenocopied by the treatment of meiocytes with anti-microtubulin drugs such as colchicine and vinblastine (Driscoll *et al.*, 1967; Dover and Riley, 1973). In addition, increasing the dosage of *Ph1* to six copies causes a decrease in homologous pairing whilst allowing infrequent pairing of homoeologues (Feldman, 1966). Removal of *Ph2* allows homoeologous pairing only with chromosomes of related species but does not appear to interfere with homoeologous pairing in wheat itself (Mello-Sampayo and Lorente, 1968). However, removal of both *Ph2* and the suppressor on 3AS results in a level of homoeologous pairing almost as high as that observed in plants deleted for *Ph1* (Mello-Sampayo and Canas, 1973). Interestingly, removal of *Ph2* makes plants less sensitive to the effects of colchicine than those with an active *Ph2* gene (Ceolini and Feldman, 1987). This is in direct contrast to the observations of *Ph1,* where removal of the *Ph1* gene results in a substantial increase in sensitivity to colchicine (Avivi and Feldman, 1973; Ceolini *et al.*, 1984). Therefore, it can be concluded that the *Ph* genes of wheat interact with microtubules but that, while both *Ph1* and *Ph2* are suppressors of homoeologous pairing, they have opposite effects on the sensitivity of the plant to the anti-microtubule agent colchicine.

It is proposed that *Ph1* is possibly a microtubule associated protein which draws homologous chromosomes into domains which facilitate their intimate association at early meiotic prophase. Movement of the chromosomes could therefore be active and directional to establish homologous over homoeologous interactions, and could be reliant both on the action of *Ph1* and on microtubule arrays. Following placement of the chromosomes by the *Ph1* action, the *Ph2* gene product, suggested to be the AWWM5 protein, could bind to the distal end of each chromosome arm, establishing contact with the nuclear membrane through the N-terminus. Testing of homology between associated chromosomes might

occur at two types of sites as proposed by McKim *et al.* (1993). Specific homology recognition regions (HRR) in the distal ends of chromosomes could be brought together by the interaction of chromosomes with the nuclear membrane mediated by the AWWM5 protein. The second level of homology testing could occur at multiple interstitial sites and is proposed to be less stringent than that proposed for *Ph2* and be under the control of the suppressor on 3AS. In the hypothetical associations, where the specific HRR's are homologous, the microtubules required for chromosome movement would be destabilised thus "locking" the homologous chromosomes in the domains which ensure their interaction at early meiotic prophase. Interstitial interactions would serve to stabilise the chromosome association. Non-homologous interactions between the HRR's would not cause microtubule destabilisation and the chromosomes would be free to move apart through the action of *Ph1* and would become available for other interactions.

Analysing the speculative chromosome paring model presented, the effects of different doses of the *Ph* genes and the effect of antimicrotubule drugs could be explained as follows. When all three suppressors are functioning, homologous chromosomes are drawn into domains which favour their interaction by the action of the putative microtubule associated *Ph1* protein. The homology recognition regions are brought together by the interaction of *Ph2* with the nuclear membrane and, if homology is recognised, the chromosomes are "locked" into position. The suppressor on 3AS could encode a protein which facilitates less stringent interactions at interstitial sites within the chromosomes which serve to further stabilise the interaction. The removal of *Ph1* may not allow the formation of the microtubule associated protein resulting in minimal movement of chromosomes. Homoeologous contacts, which might form through random contacts, would not have homologous HRR's but could not be resolved because of the lack of chromosome movement. Increasing the dose of *Ph1* to six copies could cause an excessive degree of chromosome movement that would not allow the homologous chromosomes to be locked into place thus resulting in a high level of univalency as well as occasional multivalency caused by the chance contacts of homoeologues. As colchicine causes a depolymerisation of the microtubules (Borisy and Taylor, 1967; Weisenberg *et al.*, 1968; Wilson, 1970), in cells treated with colchicine, the *Ph1* protein would lack the structural array of microtubules required to initiate effective chromosome movement. Chromosome movement in colchicine treated cells would decrease, random homoeologous contacts would not be resolved and homologous contacts would not be actively initiated. Increasing the dose of *Ph1* could render the meiotic

movement of chromosomes less susceptible to the action of colchicine either by the increased presence of the Ph1 protein allowing more efficient utilisation of the few microtubules which might form, or through a stabilising effect of Ph1 proteins on the microtubules conferring increased resistance to colchicine as proposed by Feldman (1993).

In plants lacking *Ph2*, homologous chromosomes would still hypothetically be actively manoeuvred to domains which facilitate their interaction by the *Ph1* protein. However, the specific homology recognition regions would not be brought into contact and hence a stringent assessment of homology would not occur and the chromosomes would not be locked into position. Despite this, the relative positions of homologous chromosomes would be maintained by *Ph1* gene action and the 3AS suppressor which is proposed to initiate interstitial associations. Therefore, homologues could still pair efficiently at early meiotic prophase. In substitution lines carrying an alien chromosome and lacking *Ph2*, the alien chromosome might possibly move to the same domain as the unpaired wheat homoeologue as all other homologues become associated under the influence of *Ph1*. The absence of *Ph2* would not allow for the stringent testing of homology but some association between the alien chromosome and its wheat homoeologue could occur and might be stabilised to some degree by the less stringent action of the 3AS suppressor. This could allow for a degree of pairing at meiotic prophase.

The observation that removal of *Ph2* seems to decrease the susceptibility of the cell to colchicine can be explained if chromosome associations, even homoeologous associations, in colchicine effected cells provide some form of stability. In this case, the lack of *Ph2* may confer some kind of stability to the few microtubules that are present, and thus allow a degree of *Ph1* mediated chromosome movement resulting in at least some homologous or homoeologous chromosome associations which stabilise the cell.

Whilst the actual function of the *AWWM5* gene remains unknown, there is some evidence to support the proposal that it represents the *Ph2* gene of wheat. The expression pattern of the gene is consistent with the homologue premeiotic association models described (Feldman and Avivi, 1973, 1988; Maguire, 1988; Scherthan *et al.*, 1992; Feldman, 1993; Kleckner and Weiner, 1993) suggesting that mechanisms act to influence the relative association of homologous and homoeologous chromosomes during premeiotic interphase. *AWWM5* genes have been located to the region of the *ph2a* deletion and also on 3AS. It has been

shown previously that the suppressor genes on 3DS (*Ph2*) and 3AS interact to limit homoeologous pairing and the genes probably occupy syntenic positions (Mello-Sampayo and Canas, 1973). Further mapping of the positions of copies of *AWWM5* genes should allow a more precise determination of the relationship between the copies on 3DS and 3AS, as well as the identification of positions of other genes in this family which might reflect an involvement in homologous chromosome pairing.

In situ localisation of *AWWM5* mRNA reveals that the gene is expressed in both the tapetal layer and the developing meiocytes. This is consistent with the expression of two other meiosis specific genes isolated from wheat, *AWWM1* (Ji, 1992) and *AWWM3* (Letarte, 1996) and does not necessarily indicate that the translated protein also resides in both the tapetum and the meiocytes. The production of antibodies to the AWWM5 protein would allow a determination of the location of the protein by *in situ* hybridisation.

The putative structure of the deduced AWWM5 protein is consistent with a possible function in attaching chromosomes to the nuclear membrane thereby facilitating their interaction. The deduced protein sequence contains a high percentage of arginine residues arranged in blocks in the C-terminal region indicating DNA binding properties. Expression of the protein in an *in vitro* system such as *E. coli* would allow a more detailed characterisation of the DNA binding capacity of the protein. The N-terminus of the deduced AWWM5 protein appears to have membrane binding capabilities. Again however, *in situ* localisation of the protein using antibodies would be needed to confirm this. *In situ* localisation of the protein in meiocytes of different developmental stages using electron microscopy would reveal if the protein was associated with the nuclear membrane and the chromosomes, and could also be used to demonstrate any association with the synaptonemal complex.

Whilst the preliminary characterisation of the AWWM5 clone has been completed, the actual function of the encoded protein remains speculative until *in situ* localisation of the protein and characterisation of the putative DNA binding capacity is performed. The increasing feasibility of wheat transformation should also be utilised in the future to produce plants expressing antisense constructs of AWWM5. Characterisation of meiosis in such plants would enable comparison with the effects of the *ph2* mutations to determine if there are any parallels.

The research described here, should provide the basis for a more accurate analysis of meiosis in *T. aestivum*. This might be by further characterisation of AWWM5 and some of the other clones isolated, or an extension of the deletion mapping in an attempt to isolate *Ph2* by map based cloning techniques.

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Appendix 1

Sources and composition of materials and solutions

Escherichia coli strains	Source
C600*Hfl*	Promega
DH5α	Clonetech, Palo Alto, C.A.
XL1-Blue	Stratagene

Enzyme	Source
Calf intestinal phosphatase	Promega
DNase I	Promega
Klenow DNA polymerase	Boehringer Mannheim
Mung Bean nuclease	Promega
Proteinase K	Sigma
Restriction endonucleases	Promega and Boehringer Mannheim
RNase A	Promega
Taq polymerase	New England Biolabs
T4 DNA ligase	Boehringer Mannheim
T4 Polynucleotide kinase	Boehringer Mannheim

Radio-Nucleotides

α-^{32}P-dCTP was supplied by Amersham

Membranes

Hybond N^+ membranes were purchased from Amersham

Buffers, solutions and growth media

Aceto-Carmine solution:	45ml Glacial acetic acid 55ml nanopure water 0.5g Carmine boiled for 5 minutes in a reflux condenser then cooled shake well and filter
Denaturing solution:	1.5M NaCl 0.5M NaOH
Denhardts III solution:	2% Bovine serum albumin 2% Ficoll 400 2% Polyvinylpyrolidone

	filter sterilised and stored at -20°C
Denhardts (50X):	10g/L Bovine Serum Albumin 10g/L Ficoll 400 10g/L Polyvinylpyrolidone filter sterilised and stored at -20°C
Detection Buffer 1:	0.1M Tris.HCl pH 7.5 0.15M NaCl
Detection Buffer 2:	3% Bovine Serum Albumin in detection buffer 1
Detection Buffer 3:	0.1M Tris.HCl pH 9.5 0.1M NaCl 50mM $MgCl_2$
Detection Buffer 4:	33µl stock NBT solution (75mg/ml) 7.5ml detection buffer 3 vortex gently and rest on ice add 25µl BCIP solution (50mg/ml) in subdued light and mix
DNA gel loading buffer (6X):	15% Ficoll 4000 0.25% Bromophenol blue 0.25% Xylene cyanol FF
DNA extraction buffer:	100mM Tris-HCl 10mM Na_2EDTA 100mM NaCl 1% Sarkosyl pH 8.5
Exo III buffer (10X):	660mM Tris.HCl (pH 8.0) 6.6mM $MgCl_2$
Fixation buffer:	4% paraformaldehyde 0.25% glutaraldehyde 50mM PIPES (pH 7.0)
HSB (5X):	3M NaCl 100mM PIPES 25mM Na_2EDTA pH 6.8
In situ Hybridisation buffer:	50% ultra pure deionised formamide 4X SSC 1X Denhardts III solution 200µg/ml tRNA 0.1M DTT 5% Dextran sulphate
LB complete medium:	10g/L Tryptone

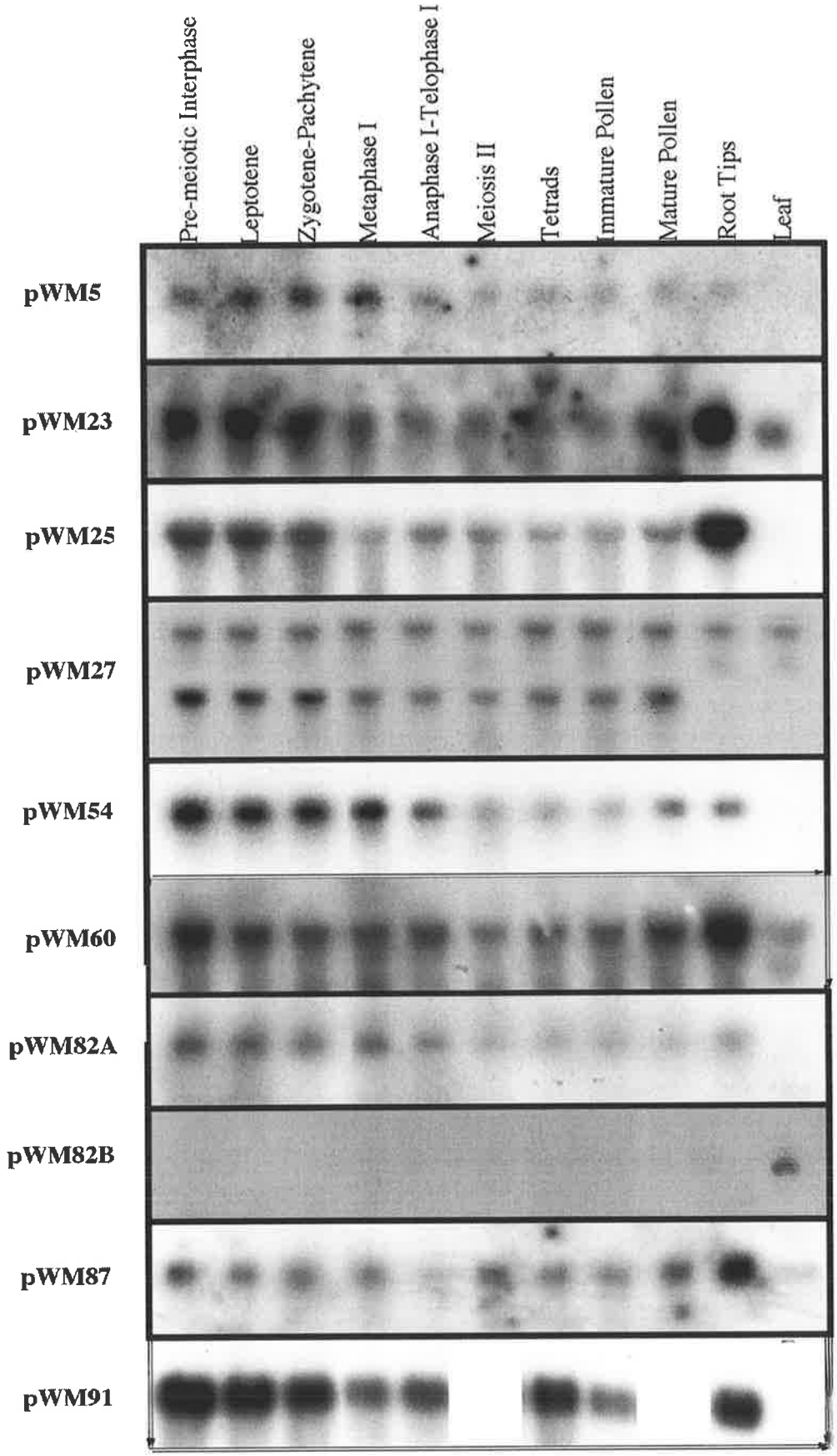
	5g/L Yeast extract 10g/L NaCl for LB agar add 15g/L agar pH to 6.8 then autoclave
Ligation buffer:	as supplied by Promega
MOPS buffer (10X):	0.5M MOPS 10mM Na_2EDTA pH 7.0 then autoclave
Neutralisation buffer:	1.0M Tris-HCl (pH 6.5) 2.0M NaCl pH 5.0
Northern prehybridisation solution:	5ml Deionised formamide 5ml 20X SSPE 2ml Denhardts solution (50X) 1ml 10% SDS 6ml nanopure water 200µl denatured salmon sperm DNA (10mg/ml) warm to 42 °C
Northern hybridisation solution:	5ml 20X SSPE 2ml Denhardts solution (50X) 1ml 10% SDS 9ml Deionised formamide 2ml Dextran sulphate (25%) 1ml denatured salmon sperm DNA (10mg/ml) warm to 42 °C
Oligolabelling buffer (2X):	60µM each of dATP, dGTP and dTTP 150mM Tris-HCl (pH 7.6) 150mM NaCl 30mM $MgCl_2$ 300µg/ml Bovine Serum Albumin
PAB Hybridisation buffer:	50mM Hepes (pH 7.6) 0.2% SDS 2mM Na_2EDTA (pH 8.0) 500mM NaCl
PAB Hybridisation buffer (-SDS):	as above with no SDS
PCR buffer:	as supplied by Applied Biosystems
PEG buffer:	as supplied by Promega
Phage precipitation buffer:	20% (w/v) PEG6000 2M NaCl

Phenol:	Phenol equilibrated with 1xTE 0.1% Hydroxy-quinoline added
Phenol:chloroform: iso-amyl alcohol:	25:24:1 mix of phenol:chloroform:iso-amyl alcohol
Plasmid I solution:	25mM Tris-HCl (pH 8.0) 10mM Na_2EDTA 50mM Glucose
Plasmid II solution:	0.2N NaOH 0.1% SDS
Proteinase K solution:	1µg/ml proteinase K in 0.1MTris.HCl (pH7.5), 50mM Na_2EDTA
REB:	100mM Tris-HCl (pH8.0) 10mM Na_2EDTA 1% Sarkosyl
RNA gel loading buffer:	322µl 3X MOPS buffer 5mg xylene cyanol 5mg bromocresol green 178µl 37% formaldehyde 500µl formamide 400mg sucrose
RNase A solution:	40mg/ml RNaseA in 0.5M NaCl, 10mM Tris.HCl (pH 8.0) 1mM Na_2EDTA
SDB buffer (10X):	1M Tris.HCl (pH 7.8) 5M Potassium acetate 1M Magnesium acetate 0.1M Spermidine 0.1M Dithiothreitol adjust pH to 7.8
Sephadex G100 solution:	10g Sepadex G100 in 300ml TE buffer incubate with gentle shaking at 65 °C for 2 hours store at room temperature
SM buffer:	20mM Tris-HCl (pH7.4) 100mM NaCl 10mM $MgSO_4$ 1% gelatin
SOB medium:	20g/L Tryptone 5g/L Yeast extract

	0.5g/L NaCl pH 7.0 and autoclave add 5ml 1M $MgCl_2$ and 1M $MgSO_4$ solution before use
SOC medium:	SOB with the addition of 20ml/L 1M glucose
Southern hybridisation solution:	1.8ml nanopure water 3ml 5X HSB 2ml Denhardts III solution 3ml Dextran sulphate (25%) 200µl denatured salmon sperm DNA (10µg/ml) warm to 65 °C
SSC (20x):	3M NaCl 0.3M Na_3citrate.$2H_2O$ pH 7.0
SSPE (20x):	3.6M NaCl 0.2M $NaH_2PO_4.H_2O$ 20mM Na_2EDTA (pH 8.0) pH 7.4
Stop Buffer:	20mM Tris.HCl pH 7.5 0.5mM Na_2EDTA
TAE buffer:	400mM Tris-HCl (pH 7.5) 10mM Na_2EDTA 50mM Na Acetate
TB Phosphate (10x):	0.17M KH_2PO_4 0.72M K_2HPO_4
TE buffer (pH 7.4, 7.5, 8.0):	10mM Tris-HCl (pH 7.4, 7.5, 8.0) 1mM Na_2EDTA (pH 8.0)
Terrific broth medium:	12g Tryptone 24g Yeast extract 4ml Glycerol 900ml nanopure water pH 7.0 then autoclave add 100ml 10x TB phosphate before use
TFB:	10mM MES (pH 6.3) 45mM $MnCl_2.4H_2O$ 100mM $RbCl_2$ 10mM $CaCl_2.2H_2O$ 3mM Hexylamine cobalt chloride filter sterilised and stored at -20°C
Top agarose:	10g/L Tryptone 5g/L Yeast extract

	5g/L NaCl 7g/L Agarose 10mM MgSO4 0.4% Maltose pH 7.0
Wash buffer 1:	2X SSC 0.1%SDS
Wash buffer 2:	1X SSC 0.1%SDS
Wash buffer 3:	0.5X SSC 0.1%SDS
Wash buffer 4:	0.2X SSC 0.1%SDS
Wash buffer 5:	0.1X SSC 0.1%SDS

Appendix 2


Pre-meiotic Interphase
Leptotene
Zygotene-Pachytene
Metaphase I
Anaphase I-Telophase I
Meiosis II
Tetrads
Immature Pollen
Mature Pollen
Root Tips
Leaf
pWM5
pWM23
pWM25
pWM27
pWM54
pWM60
pWM82A
pWM82B
pWM87
pWM91

Appendix 3

Sequence information for each of the isolated cDNA clones. For clones where the insert has been fully sequenced, the forward sequence data is underlined and the overlap between forward and reverse sequence data is double underlined.

WM5 Reverse

```
1                                                                   50
 GCGTCACCATCATGCCCAAGGACATCCAGCTCGCCCGCCGCATCCGTGGA
51                                                                  100
 GAGAGGGCCTAAGCTCTGTGCGTCGTCGTCTGTTAGTCGTCGTCTTAGCC
101                                                                 150
 AGATGTGTTCAGCGGTGAAATTGTGCGTGTTTGTAGTGTCTGTTCGTGCC
151                                                                 200
 ATGGTGGATGTAGTGTTCCTGATCTGATGTCACCTCCTTGCTGTCATCAG
201
 TAACTGAAATGAAAATGTTTGTTATCTGTTCTAAAAAAAAAAAA
```

WM19 Reverse

```
1                                                                   50
 GCCTGATGACGATGACGATCTGATCGAGTAGAGAGAGGATCATGAGTCTG
51                                                                  100
 TCTTATCAATCAATACTTTGAGAGCCAGAGACTATGCGAGTCGGTTTTAG
101                                                                 150
 GTTGANGGCTTGATATGGAGTAGTCAAGCCGGATGTTTTTCCTGTCTGCC
151                                                                 200
 TTGCCTTGTCTGGTGGGCTGCAAACTGTTTGTGTGACATTGATCTACATC
201                                                                 250
 ATGTTGCTTGCCATGGATTTGTTGTTCGCCACCTTGATGGTTCATCAGTA
251
 TTATTATGCCTGTTGGCTCAAAAAAAAA
```

WM23 Reverse

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1                                                                   50
 AACAACATTGCCAACAAGGCTTGAACATTCTCGGCAGGGGCTTCTATGGA
51                                                                  100
 TCTGGGATAAGCTGGATATTCGGACTAATACTGCCTTTACGTATTATAAA
101                                                                 150
 CTATATAGTCTTGACAGATAATGTGGGTGTTTTGGAAAGATAAAGCTCTC
151                                                                 200
 TGTCAAAGAGCTAGCTATCAGATTGTTTTCGTGGGTGTTAAATTGTCCGT
201                                                                 250
 GGCGCTGTTTGTTTGCCCATCATGGCTTTACTATGTCCGCTCTGTACTCT
251                                                                 300
 GTACTCGCCACTCCGACAGACTGTGATACATTGTAGTATTGACTTGTCTT
301
 GAGCATAAAAAAAAAAAAA
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WM25 Full Clone

1 50
GGTCTTCTCCCGCATCGACCACAAGTTTGACCTGATGTACGCCAAGCGTG
51 100
CCTTCGTCCACTGGTACGTGGGTGAGGGCATGGAGGAGGGAGAGTTCTCT
101 150
GAGGCCCGTGAGGACCTCGCTGCCCTGGAGAAGGACTATGAAGAAGTTGG
151 200
TGCTGAGTTCGACGAGGGTGAGGACGGTGATGAGGGCGATGAGTATTAAG
201 250
CCTGCCTCCTGGTGCTTTCCCAAGGCTTGCTACTGCTATCCTATGATCTG
251 300
CCCGAGTGGCTTTATCTGTTATCTGTCTGTTTGAACGTTTGCTTTGTGGT
301 350
GTTTGTTTTACAACCTGTTGTGTTGTAAGAACCTTGTATCTTTGAACCTG
351 400
CTTTGCACCTTGGTTAATATGCATGCTATCTGGTTATCTAAAAAAAAAAA
401
AAAAAAAAAAAA

WM27Full Clone

1 50
CGCCGGCGCCGATGCAAGGCCCGTGTTGGGATTCACCAAAGGGTGACCAA
51 100
GGAAGACGCCATGAAGTGGTTCCAGGTCAAGTATGAGGGTGTCATCCTGA
101 150
ACAAGTCCCACGCTAGCTAGTAACCTTTTGAGTGATTGAGTTGTCGTTAG
151 200
AGCTTAAGTCTGTCAAGCTTAATCTGGATCATGGAATCGTATGTTTATTG
201 250
GATACAAAGTTGCACTATCAAAAAAAAAAAAAAAAAACTTCTTCGGCGCC
251 300
GCGGTGGCGCGGTCGCCGGCGGGCCGAGTATGAGCATCCAGGACGCGTGG
301 350
CGAACACGAGGACCGACGGCGCCGGCGCGCTGCTGCGCGAGGGCACCGCA
351 400
GCCCTGCTCAACTCCATGACCCGCCCGGGGTTCGCCTACACCACCCAGCA
401 450
GGTGAGGGACGCATTCGCGGCGGCCGTGGCCGGCGGCTCTGACAGTGCCG
451 500
CGGCGGCGCAGGCGGCGGCGTTCAAGAAAGCCAACGAGGGAGGAAGCCGT
501 550
AGATCGACCGGATCGCCATCGAGAGGGCTTTAGCTAGCTGGCTACCTATA
551 600
TCTGTGGCATGTCTGCATGCATGCGTTTAGGCCGGACGGACGTGTGCGTT
601 650
GCGATTTGGTTGTTTCGTTTGAGTTATGGTTTGGTTGTGTACGTTGGTGT
651 700
CTTCGTGGGCTAGCTTCGATCTATCGTACTGTGTTGTAATGAATCTCGTT
701 750
TAAGTTTTTTGCAGTTCCGTACCCTGTCGCATTGAATGGAATATATTGAA
751
GCGAACTATTCCTAGCTCAAAAAAAAAAA

WM47 Reverse

1 50
TTCCTATCTCTCTCCTCCTCCCCTCCTCTTCTCTTCTTCCTCATGAAGAA
51 100
GAAGAGCAATAAGGATGTCATGTGTTTGTCTGGACTTGGATTAGATGGGT
101 150

CGGTCGATGGGGTGTTGCATTGTTTTGCTTTGACGCCTCGTGTCTTGTGA
151 200
TGGGCGTGCTTGTAGTATGAACTGCCAAGTGTTATGTAGTTCCTTTCCGG
201 250
TTGAATTGTTATTCCCTGTCGAGGACGAAACGGAGATTTGGTTGCCCTCA
251
TTTGGCCATTGAGCTCATGTCATTGTTTGTTTTAAAAAAAAAAAAAAA

WM54 Reverse

1 50
CTTCCAGTCCTCCGCCGTCTCCGCGCTGCAGGAGGCCGCCGAGGCCTACC
51 100
TCGTCGGCCTCTTCGAGGACACCAACCTCTGCGCCATCCACGCCAAGCGC
101 150
GTCACCATCATGCCCAAGGACATCCAGCTCGCCCGCCGCATCCGTGGCGA
151 200
GAGGGCCTAGGCTGCTGCATCTGCAATCCATGCGTCGTCTGTTAGATCGT
201 250
TGTTAGGAAGTGTGTGTGCTCGTAGTGTTTCAGAATGTGCCTGTGTTGTT
251 300
CTTGTCATGGTGGGTCTGTTATGCCTAATCTGATGGTACCTTCTTGTTGC
301
CAAAAAAAAAAAAA

WM60 Reverse

1 50
TTTTGAAAAACAAGCACAAGTGCCAAATGCTTATTACATCGGATAGCTCG
51 100
CCATAGCAGGACACAACGACAGGCCAGAAACTAAACACACACTCACGGAC
101 150
ACATCACCAACCGCACACGACTTTTATTCTAACGACACGGCAACAGNTCA
151 200
CAGGCCGATCTTTACCAACTCCGGGTCACGGTGGCGATCAACTCAGCTTC
201 250
AGCAGCATGACACGTTTACCAGGGAAGGATCTTCCAGCGCTGGTTGTCGC
251 300
CCTTGGGACCAGTTCCACAGCACGAGGGAGGTGCCGTCGAGTACGACGAC

WM82A Forward

1 50
TTTGGTATGAGCCGTCAGCAGAGTACAGATGACATATTTTCGATTTTTGA
51 100
TGAGCTGCGTCCGAGTGAAGAGCCTGCCATTAAGGAGATCGAGCAGTGCT
101 150
ACAAGAACAGCTCCACTGTGTGTGAATCAGAGATCCAAGAGGCCAGCCCA

151 200
ATGACAGATATATTCCTGGAGAAGCCAGCCAAGGCGGCGTACTCGAATAA
201 250
GAACCCTCACAATGAGAGCGAAGTGGAAGATGCCAGCAGCTGGGAGACAA
251 300
TAAGCCATGANGAGATGCAGGGTTCAAGTGGTTCACCCGATGGAAGCCAG
301 350
TCGTCGATCAACAAGATCTTTGACGGGAGCATCTCCTGGACGAGCAGAAT
351
GATTTCGAGTATGGGGAGATCGAGA

WM82A Reverse

1 50
ACCAAAGGTGCACTCCCCCGGTGCTAAGCAGAAATCTAGGTGAAACGGTG
51 100
CAACCTAGAGAAATAATCTCAGAAATTACTCGCCGCTTCAGTTTTTTTCC
101 150
TGTTGTTGTCTTAGGGTCACTTGTAAGTTGTAATTGATGTACTAGTAAAG
151 200
AACTGAAATAGATCAATCGAGTCGGCCTTTGTGGGCCGTGCTGCTTCTGT
201 250
ATCTTGCCGTGGCTCTGGGGATATGGTTGCATTTTGATAGGCTTCGGTGA
251 300
GATCCGGGCATGATGTAATATAGACATAAACAGGCAGCAATGTTCAAAAA
301 350
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
351
AAAAAAAAAA

WM82B Reverse

1 50
TCGAGCTGCTCATCCGCAAGCTCCCCTTCCAGCGCCTGGTGAGGGAGATC
51 100
GCCCAGGACTTCAAGACCGACCTCCGCTTCCAGTCCTCCGCCGTCTCCGC
101 150
GCTGCAGGAGGCCGCCGAGGCTACCTGGTGGGGCTCTTCGAGGACACCAA
151 200
CCTGTGCGCCATCCACGTCAAGCGCGTCACCATCATGCCCAAGGACATCC
201 250
AGCTCGCACGACGAATCCGTGGCGAGAGGGCCTAAGCTGCTCTGTGCGCG
251
TCGTCGTCTTAGCCAGATGT

WM87 Full Clone

1 50
AAAAATTTAGTTTTGTCAACTGTCGTTATTCTTAGAACGTTTTATAGTAA
51 100
GCTTGTGACCCCGCTATCTAAAAGGATGAGAGTCATGTTTCATGAAATGA
101 150
TTAATGATAGTTAACGAGTTTGTCTTGTTGTCGGCGAGGTGTGTTTGTCG
151 200
CTGATTGTTCTTTCCCGCTGAATTTGGAACTTCGGTGCCGACAAGGAGGA

201 250
TGGTGCTCGGAAGAGTTTGAAGGATGGGAACCTCGCGTGTATCCCGAACC
251 300
ACACCGACACGCCGTGTGGTCCGCGGAACGGCTTCACGGAGTGGCGAGTG
301 350
CCCGTAAGAACCCAGGGAGTGACGATACAAAGAGGTCACACAAAGGTCTG
351 400
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401 450
ACCCGGGCAAACATCAATAAAACTTCCTACCCTTGGAGCGCAAATAGGGC
451 500
ATGGTGTGGCTGTACGGCACACCAGGCACCTTGCCGAAGTAGCCTCACCG
501 550
CCTAACAGCGCAAACTTGGGTCCCCTCACTGCTATGTTCACCAGTGTGTT
551 600
TCCAGACCTCTTGTGTTATATGTGAACTTGTGTCCACCCTGGTGTATCCT
601 650
GATTGCTGTTGGGCCCCGTTTGTAGTATAATTTTTCATCAACTTCTGCTT
651 700
CACTCTTCTAGTATCAATGGAAAACCGATGTTTCGTAAAAAAAAAAAAAA
701
AAAAAAAAAAAAAAAAAAAAAAAAA

WM91 Full Clone

1 50
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51 100
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101 150
CGGAGAAGCGGCTGCCGGCGGGCAAGACGGCGTCCAAGGAGGCCGGCGAC
151 200
GAGGCGAAGACGAGGGGCCGAAAGAAGGGCAGCAAGGCGAAGAAGAGCGT
201 250
GGAGACGTACAAGATCTACATCTTCAAGGTGCTGAAGCAGGTGCACCCCG
251 300
ACATTGGCATCTCCTCCAAGGCCATGTCCATCATGAACTCCTTCATCAAC
301 350
GACATCTTCGAGAAGCTCGCCGGCGAGGCCGCCAAGCTGGCCCGGTACAA
351 400
CAAGAAGCCCACCATCACGTCCCGGGAGATCCAGACCTCCGTCCGCCTCG
401 450
TCCTCCCCCGGCGAGCTCGCCAAGCACGCCGTCTCCGAGGGCACCAAGGCC
451 500
GTCACCAAGTTCACCTCCTCCTAGAGTGCATCTGCATTGCTTGTATCTAT
501 550
CTAGTAGTAGCGACTCTAGCTGGTTCTGTTGCTTTAGTTAGTGCTTTTAT
551 600
TGCTGGATGGGGAATTGATTGATGGATGACTGCCAATGCTTTGTTGCCTC
601 650
TTTGGGTGTAACAACTGAATTTGTGCTTCTTTTGGCAAACTGATATTTCA
651
AAAGTACGGATTCGTTTCAAAAAAA