A Study of Meiosis in Allohexaploid Wheat:

The Molecular Aspects

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ABBREVIATIONS

aa	Amino acid
bZip	Basic-leucine zipper protein
cDNA	Complementary DNA
cpm	Counts per minute
DNA	Deoxyribonucleic acid
DT	Ditelosomic
DTT	Dithiothreitol
g	Gram
HLH	Helix-loop-helix protein
HTH	Helix-turn-helix protein
IPTG	Isopropyl β-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
ml	Millilitre
MOPS	Morpholino propanesulfonic acid
ng	Nanogram
NT	Nullisomic-tetrasomic
ORF	Open reading frame
PCR	Polymerase chain reaction
PMC	Pollen mother cell
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
TEMED	Tetramethylethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane

X-Gal	Bromo-(5)-4-chloro-3-indolyl-β-D-galactopyranoside
µg	Microgram
µl	Microlitre

To Fang Yun, my dearest wife,

for extraordinary sacrifice. endurance and love.

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Chapter Two Materials and Methods

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STATEMENT OF ORIGINALITY AND CONSENT TO PHOTOCOPY OR LOAN

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(Liang-Hui Ji)

SUMMARY

Meiosis occupies only a very short period of the life cycle of eukaryotes but it is a very important developmental process that ensures correct passage and maintenance of genetic information of parents to their offspring. However, very little is known about the molecular aspects of this cell division, especially in plants.

The main aim of this study is to investigate possible approaches for the study of meiosis in wheat (*Triticum aestivum*), a plant with very small anthers but of great economic importance. The experiments were designed to minimise the amount of material required for studies at the molecular level by avoiding the direct use of biochemical methods. Rather, information on meiosis from other organisms was applied to meiosis in wheat.

The study has focused on two aspects of meiosis in wheat: chromosome pairing and genetic recombination.

A modification to the classical method of synthesis of cDNA has been made. The use of the moloney murine leukemia virus (M-MLV) reverse transcriptase improved the efficiency of synthesis of cDNA from an unpurified RNA sample and simplified the procedures for construction of cDNA library from limited materials. With this method, a cDNA library was constructed from florets at early meiotic prophase I.

Using a maize probe of unknown function, pZm9, which was

isolated with a *Lilium* meiosis-specific cDNA clones (Appels *et al.*, 1984; Appels, unpublished result), two cDNAs were isolated. The cDNAs, designated pAWJL1 and pAWJL3, were assigned to chromosome arms of wheat (*Triticum aestivum*), rye (*Secale cereale*) or barley (*Hordeum vulgare*). pAWJL3 belongs to a small family of genes with over 20 members that mapped to the short arm group 3 and group 5 chromosomes (except 3BS). The chomosomal assignments of the genes related to pAWJL3, coincide with two *Ph* genes of wheat, *Ph2* and *Ph3*, but the linkage is unknown. The genes were shown to be expressed uniquely after leptotene and predominantly at zygotene and pachytene. pAWJL3 has been sequenced and the deduced protein revealed two separate domains, one with three leucine-rich 24 aa repeats and the other with four leucine heptad repeats that resemble the basic leucine-zipper (bZip) proteins. Both are potentially involved in protein-protein interactions.

The SPO11 gene was also used as a probe. This gene is involved in meiotic recombination and the formation of synaptonemal complex (SC) in budding yeast (*Saccharomyces cerevisiae*) (Atcheson *et al.*, 1987; Dresser and Giroux, 1988). The gene was shown to cross-hybridise to wheat (*Triticum aestivum* cv. Chinese Srping), rye (*Secale cereale* cv Imperial Rye) and barley (*Hordeum vulgare* cv Betzes) at reduced stringency of hybridisation. A genomic library of wheat was constructed and the sequences that cross-hybridised, isolated. The lambda clones were very unstable in *E.coli* but a *recD* mutant greatly improved the efficiency of cloning. A 4.1 kb fragment (pAWJL4.1) that hybridised to the *SPO11* gene has been analysed. Sequence analysis has identified 21 bases with perfect homology to the yeast *SPO11* gene and the amino acid sequence

around the region revealed 69.2% homology including conservative substitutions over a 26 aa stretch.

In addition, within pAWJL4.1, a C+G-rich medium repetitive sequence (about 4000 copies in wheat) with a size of about 1.5 kb has been identified. The sequence, about 700 nt away from the low copy number region that hybridised to the *SPO11* gene, was termed Transcript A and was shown to be expressed preferentially (but not uniquely) after leptotene. However, the sequence is not able to code for a long ORF due to the interruption of two in-frame stop codons.

From the same genomic library, clones that hybridised to pAWJL1 and pAWJL3 were isolated. Interestingly, the clones were also highly unstable in *E.coli* but the *recD* mutant rescued the clones. Furthermore, the repetitive sequence in pAWJL4.1, Transcript A, was found to hybridise to these genomic clones in regions located closely to the those that hybridised to pAWJL1 and pAWJL3. In at least one case, a sequence homologous to the Transcript A was found to be present in two copies within a 10 kb interval around low copy sequences. Indirect evidence suggests that Transcript A, or its related sequences, are partially responsible for the instability of the corresponding lambda clones.

Two types of meiosis-specific DNA replication have been observed in *Lilium*. One occurs at zygotne (zygDNA) and the other at pachytene (PDNA). These events were proposed to be associated with chromosome pairing and genetic recombination (Stern and Hotta, 1984). However, the replication has been demonstrated only in *Lilium* and partly in mouse and its applicability to other

eukaryotes remains questionable (Loidl, 1991). Preliminary evidence indicates that similar DNA replication occurs in wheat. A DNA band of about 7-8 kb with high bouyant density was identified in material containing prophase I meiocytes in wheat. The DNA was assumed to be the equivalent of the *Lilium* zygDNA. In addition, with pAWJL4.1 as a probe, which contains a DNA subfragment of a genomic clone isolated with the yeast *SPO11* gene, extra DNA fragments were revealed on a Southern blot of DNA extracted from anthers between leptotene and diplotene. This result may be caused by meiotic DNA replication and repair.

A theoretical model of the molecular basis of origin of promoters and suppressors of homoeologous chromosome pairing of wheat is discussed.

Chapter One

Literature Review

1.1. Chromosome Pairing and Meiotic Recombination

1.1.1. Chromosome Pairing

1.1.1.1. Introduction to Meiosis and Chromosome Pairing

Meiosis is programmed to occur only once in a life cycle after countless cell divisions. It normally occupies a very short period of the life cycle; only a few days in most plants. Synapsis, genetic recombination and chromosome reduction are major features of meiosis. To date, although considerable visual data has been recorded and a lot of the events known to be under genetic control (Golubovskaya, 1989), the underlying mechanisms are poorly understood. This is in sharp contrast to mitosis, where the molecular basis for the control of DNA replication, chromosome condensation, chromosome movement and many other aspects have become increasingly clear.

Chromosome pairing is generally regarded as the close association of homologous chromosomes at the prophase I of meiosis. However, it has also been used in other contexts, such as the coarse alignment of homologues (somatic association or

somatic displacement) in premeiotic mitosis or in root tip cells (Avivi and Feldman, 1980; Giroux, 1988). Giroux (1988) suggested that the term synapsis should be used to describe synaptonemal complex (SC) formation. Accordingly, the following can best be regarded as a review of chromosome synapsis.

Synapsis is mediated by a special meiosis-specific structure called the synaptonemal complex (SC) which is initiated at late leptotene and degraded from early diplotene. The structure of the SC seems to be universal among the extremely diverse eukaryotic world: one proteineous central element (CE) is sandwiched between two parallel lateral elements (LE). The SC is normally initiated at multiple sites on a chromosome especially in organisms with large genomes. However, it shows a preference for initiation near the telomeres (Gillies, 1984; Wettstein *et al.*, 1984). The mechanism of chromosome recognition, matching and subsequent crossover remains the focal point of interests.

1.1.1.2. Models for the Homology Search

One of the most intense debates on the topic of synapsis relates to the time sequence of homologue recognition, formation of the SC and genetic exchange. It is presently not known whether these three events occur sequentially or simultaneously. An acceptable model would be that formation of the SC is preceded by a homology signaling and receiving process. Sybenga (1966) and Hotta *et al.* (1984) proposed that single-stranded DNA (ssDNA), generated during early meiosis, acts as the basis for the homology signal. Once complement homology is found and strands re-

associated, the ssDNA can act as a nucleation site for the SC proteins. It can be inferred from the model that extension of the SC is not possible without homology. However, this is not the case. The SC formation in haploids, for example, can proceed to near completion (Jong *et al.*, 1991; Loidl *et al.*, 1991; Wang, 1988). Furthermore, in polypoids, multivalents are common and a subsequent correction stage is needed to yield the required stringency of pairing (Gillies, 1984; Holm, 1988). It seems that the formation of the SCs, as judged by the appearance under electron microscope, requires little or no homology and homology testing occurs after the SC formation.

Other models proposed that homology testing occurs before the SC formation. Gene conversion was believed to act as the basis of homology search, which implies that homology is searched and tested after formation of SC (Smithy and Powers, 1986; Carpenter, 1987). Recent studies in yeast (Alani *et al.*, 1990; Engebrecht *et al.*, 1990) have supported this proposal. However, it remains unclear why bivalents are predominantly formed under normal conditions.

1.1.1.3. The Molecular Basis of Homology Signalling during Chromosome Pairing

In normal diploids, pairing between nonhomologous chromosomes is rare. Homologues find their counterparts precisely in a short time, often within hours. This is a remarkable process since the nucleus is often crowded with chromosomes that are usually interlocked during pairing and there is a wealth of repeated

sequences that many chromosomes share. Homology is probably reflected at the DNA level; that is, the determining factor in the choice of synapsis partner is DNA.

Several results suggest that there exist certain DNA sequences that are vital for homology recognition. A good illustration of this is the observation that the re-introduction of ribosomal RNA gene (rDNA) into the *Drosophila* X chromosome restores pairing that was lost due to a deletion in the rDNA locus (McKee and Karpen, 1990).

Particular groups of sequences (zygDNA, refer to Section 1.2.5 for description) may have a more general role in homology recognition (Hotta and Stern, 1971; Stern and Hotta, 1984). It was shown that inhibition of zygDNA synthesis lead to a failure of initiation of the SC. Moreover, continuation of zygDNA synthesis is necessary for elongation of the SC development (Roth and Ito, 1967). ZygDNA is indeed a tempting candidate for the sequence that provides the homology signal not only because of its coincidence with the time of synapsis, but also because of its structural organisation. Roth and Ito (1967) have shown that these sequences are dispersed throughout the genome and, most importantly, they are generally closely linked (more than 50% are within 1 kb) to another group of DNA sequences called PDNA that undergo repair synthesis during pachytene (Stern and Hotta, 1984). In addition, a protein with single-stranded endonuclease activity, termed the L-protein, was found to bind to the ends of zygDNA before zygotene to inhibit the replication of this DNA. Therefore, single-stranded DNA generated in the zygDNAs was thought to serve as the signal for

chromosome pairing via complementary base pairing between homologues (Hotta *et al.*, 1984). Unfortunately, zygDNA synthesis has been demonstrated only in *Lilium* and mouse and its general occurrence remains to be investigated. Furthermore, the model failed to explain why initial SC formation seems to be unspecific (Loidl, 1990).

Stern (1986) proposed a new function for zygDNA. Here the importance of these sequences is not to directly participate in DNA association but to serve as the binding sites for proteins that are involved in the formation of the SC. Nevertheless, the relationship between replication and DNA-protein interaction remains unexplained.

The new proposal for the function of the zygDNA avoids a conflict between the low copy nature of the zygDNA and the non-specificity of initial SC formation. In Section 1.1.1.2., it was suggested that the initial SC formation requires little homology. From this it can be inferred that if DNA sequences directly participate in homology search prior to SC formation, they must be repetitive sequences and of considerable length in order to re-associate efficiently. However, the *Lilium* zygDNAs are low or single copy sequences (Hotta *et al.*, 1984). In addition, yeast (*Saccharomyces. cerevisiae*) contains very little repetitive sequence and yet nonhomologous SCs form just as efficiently as in haploids of higher eukaryotes (Loidl *et al.*, 1991).

Comings and Riggs (1971) proposed a protein that becomes competent in pairing (protein oligomerisation) due to

conformational change upon binding to specific DNA sequence. This cannot be excluded. However, a protein whose conformational change is induced by DNA binding, is rare. Certainly the picture would be clearer if the major SC proteins could be analysed at molecular level.

From *S. cerevisiae*, Hollingsworth and Byers (1989) recently isolated a gene called *HOP1* that is essential for SC formation and meiotic recombination. DNA sequence analysis revealed that the gene has a zinc finger motif and it was postulated that it binds to chromatin. *In situ* hybridisation demonstrated that the *HOP1* proteins are distributed over the whole SC without any preference to the central element or the inner side of the lateral element. Therefore, this protein is unlikely to play a direct role in the initial homologue interaction. It may be a structural protein whose function is to create chromatin loops or it may be involved in the regulation of transcription or DNA replication (Hollingsworth and Byers, 1989).

Moens *et al.* (1987) injected isolated rat SCs into mice and two antibodies specific to the SC proteins have been identified. One antibody (III15B8) has detected proteins localized on the central element of the SC. The molecular information on this protein is still lacking.

1.1.1.3. Synapsis Correction

Electron microscopic studies have revealed two types of synapsis correction. One results in an overall increase in

specificity while the other acts in reverse. The first type is most frequently observed in polyploids, e.g., *Triticum aestivum* (Wang and Holm, 1988), *Lolium* (Jenkins , 1985b) and *Bombyx mori* (Rasmussen, 1977). At zygotene, the SCs can repeatedly switch between complete or partial homologues but at pachytene, they are corrected into strict bivalents or univalents. The univalents are often self-synapsed as foldbacks (Rasmussen, 1977).

The second type of synapsis correction was first reported by Moses and coworkers (1982) using mice containing heterozygous inversions, deletions or duplications. It was found that loops formed in zygotene, gradually diminish or become synapsed as straight heterozygous bivalents at late pachytene. Similar corrections have been reported in other animals such as chicken (Kaelbling and Fechheimer, 1985) and human (Guichaoua *et al.*, 1985) but has not been confirmed in plants (Anderson *et al*, 1988). Even in animals, synapsis in these regions is variable, showing either no correction (Chandley, 1982) or delayed heterologous synapsis at late pachytene (Ashley *et al.*, 1981; Saadallah and Hulten, 1986).

While the correction that leads to an increased specificity of synapsis seems easily understandable so that Holm and Wang (1988) believed that it was a natural and ongoing process, the reverse type of correction, that leads to decreased specificity of synapsis, is mysterious. An immediate question is, what controls these variabilities and what significance (if any) these corrections have? Moses and Poorman (1984) suggested that correction may be related to crossing-over. If this is the case, lack of correction may

be a result of lack of crossing-over in the vicinity of branch points or in the loops.

1.1.2. Genetic Recombination

It has long been observed that genetic recombination is greatly promoted during meiosis with an increase from several hundred to over one hundred thousand fold compared to vegetative cell division (Giroux, 1988; Junker *et al.*, 1987). This seems to contradict the mission of meiosis where genetic conservation is the theme. Such an activity has evolved to become indispensable to a eukaryote's survival. It is essential for proper disjunction and segregation of chromosomes. Abnormality in recombination will ultimately result in infertility. Meiotic recombination is known to be non-random (Maguire, 1988) and is often associated with gene conversion (intrachromatid exchange or non-reciprocal exchange). It is generally accepted that there exist some discrete sites of initiation for meiotic recombination (Stern and Hotta, 1984; Rouyer *et al.*, 1990). Much of the debate about recombination centres around the molecular basis of recombination and its relationship with the SC.

1.1.2.1. Initiation of Recombination

It is generally accepted that recombination occurs during pachytene, after the assembly of the SC. A number of enzymes that are potentially involved in strand exchange increase in amount dramatically after mid-zygotene and reach a peak at pachytene. These are the meiosis-specific RecA-like proteins of *Lilium* and

yeast (Hotta *et al.*, 1985b), RNA dependent meiosis-specific endonuclease of *Lilium* (Stern and Hotta, 1978); DNA reassociating protein (R-protein) and DNA unwinding protein (U-protein) of *Lilium* and mouse (Hotta *et al.*, 1977; Stern and Hotta, 1978; Hotta *et al.*, 1979). Furthermore, single-stranded DNA breaks in *Lilium* chromatin have been found predominantly during pachytene (Hotta and Stern, 1971) and prolongation of pachytene induces a rise in the frequency of recombination (Byer and Goetsch, 1982). However, since non-reciprocal exchange (gene conversion) and reciprocal exchange (crossing-over) are traditionally both referred to as recombination, the time of recombination may be as early as late leptotene if gene conversion is regarded as a mechanism for homology search.

Another controversial aspect regarding recombination concerns the nature of the initial DNA break. Current understanding of genetic recombination seems to suggest that the whole process involves generation of nicks or gaps, D-loop formation by invasion of a single strand tail from the nick, repair synthesis and resolution of the Holliday junction by cutting of the four-stranded structure (Szostak *et al.*, 1983; Thaler and Stahl, 1988). Recently, it was observed that transient double-stranded breaks are generated during the time of genetic recombination (Sun *et al.*, 1989; Cao *et al.*, 1990). This result supports the double-stranded break initiation model (Szostak *et al.*, 1983; Thaler and Stahl, 1988) and is in sharp contrast to corresponding research in *Lilium*, in which single-stranded nicks are observed in a family of medium repetitive sequence called PsnDNA that is transcribed into RNA (PsnRNA) during pachytene and activate a single-stranded

endonuclease (Hotta and Stern, 1981). Noticeably, such nicks are not generated at random. Symmetrically distributed nicks are located about 300 bp apart on complementary strands (Hotta and Stern, 1984).

1.1.2.2. Meiotic Recombination in Relation to the SC

Meiotic mutants have contributed much to the information of the relationship between recombination and the SC. Various mutants are available in a variety of organisms including maize (Golubovaskya, 1989), tomato (Golubovaskya, 1979), wheat (Sears, 1977; 1982; La-Cour and Wells, 1970) and *Drosophila* (King, 1970; Carpenter, 1982) but the best genetic material today comes from the artificially manipulated mutants of yeast that enable dissection and analysis of individual genes. While failure to form SC abolishes crossing-over, the presence of the SC does not guarantee recombination. The yeast *mer1* mutant strain with multiple copies of *MER2* gene, for example, produces morphologically normal SCs but crossing-over is nearly abolished (Engebrecht *et al.*, 1990). A similar result was observed in the *Drosophila mei9* mutant (Carpenter, 1982). Furthermore, in haploids, nonhomologous SCs are abundant but recombination is rare (Loidl *et al.*, 1991; Jong *et al.*, 1991). Apparently, the formation of the SC alone is insufficient for recombination and many believed that it only provides a structural framework that accommodates the recombinational machinery as well as bringing the recombination targets together in close vicinity (Giroux, 1988; Loidl, 1990).

Following the discovery of the SC (Moses, 1956, 1958), Carpenter (1975) described another meiosis-specific micro-structure within or alongside the SC which she called it recombination nodule (RN). Since then the RN has been assumed a role in mediating recombination. In other words, such a structure is a recombinational enzyme complex (Carpenter, 1975; Rasmussen and Holm, 1978). It has been demonstrated, by eletron microscopic autoradiography, that DNA repair is active in the RN (Carpenter, 1981). This is consistent with most models of recombination. The number and location of RNs from early to mid pachytene also correlates well with chiasmata (Carpenter, 1975; Byers and Goestsch, 1975) whereas the earlier zygotene nodule, parallels gene conversion (Carpenter, 1979). Moreover, in the *Drosophila* recombination defective mutants *mei41* and *mei218*, decreased crossing-over was accompanied by a reduction in the number RNs (Carpenter, 1979). However, many aspects remain under debate. For example, the RNs persist from zygotene to late diplotene (Carpenter, 1979). This is inconsistent with the general belief that recombination occurs at pachytene (See previous section).

Two types of RNs with different shapes and time of occurrence have been observed and it was proposed that the early RNs become precursors of the late RNs, which are destined to form crossing-over points (Carpenter, 1979). This is at variance with most models of recombination in that the choice between reciprocal and non-reciprocal exchange is not predetermined before initiation of recombination. Instead, it is a matter of random events (Messelson and Radding, 1975). In addition, Carpenter's (1979) proposal would imply that the DNA breaks, that is the site

for recombination, is chosen 'long' before nicking and it is not the nicks that initiate recombination. Rather, it is the modification of the early RN that initiates nicking and subsequent crossing-over. On the other hand, this proposal is in line with the finding that crossing-over is non-random (Maguire, 1988). All these questions await an answer for the mechanism of the initiation and termination of crossing-over or conversion.

1.1.2.3. Genetic Control of Meiotic Crossing-over and Gene Conversion

Gene conversion is associated with crossing-over in up to 50% of the cases (Hurst *et al.*, 1972; Maguire, 1988). However, crossing-over interferes with gene conversion whereas gene conversion does not (Mortimer and Fogel, 1974; Holliday,1977). The current models of genetic recombination believe that these two events are alternative resolutions of a four-stranded DNA hybrid (Holliday Intermediate) (Szostak *et al.*, 1983; Thaler and Stahl, 1988). But why do the two events differ greatly in distribution? Maguire (1988) suggested that the first successful crossing-over transmits a signal for the SC to release the RNs that have not yet established crossing-over intermediates and to prevent installation of additional RNs. However, the conversion-only nodules will not be affected as long as they are sufficiently distant from crossing-over sites. This model is intriguing but it suffers from the lack of an understandable basis for signal transmission. Giroux (1988) suggested that a crossing-over occurs preferentially near a conversion event or, alternatively, there exist

two classes of gene conversions, one precedes and the other is coincident with crossing-over.

Studies in yeast and *Drosophila* have identified a number of genes that have been found to exert a differential effect on gene conversion and crossing-over. Among them is the *MER2* gene of *S. cerevisiae*. In high copy number, this gene restores the SC formation and the frequency of gene conversion in the *mer1* mutant, but crossing-over remains defective (Engebrecht *et al.*, 1990). The *Drosophila Mei9* and *Mei218* genes act similarly. They both have drastically reduced crossing-over frequency but gene conversion is not affected (Carpenter, 1982). The yeast *RAD52* gene may belong to the same group of genes. However, the differential effects of this gene on the two types of genetic exchange, have only been observed during mitosis (Jackson and Fink, 1981) and its effect on meiotic recombination remains to be investigated.

Genetic recombination is not only subject to the action of regulatory enzymes but also to the action of undefined *cis*-acting DNA sequences. Molecular studies have revealed a numbers of *cis*-acting sequences capable of either promoting or inhibiting meiotic recombination. These sequences are normally found in the promoter regions of genes, such as *CEN3, ARG4, LEU2* genes of *S. cerevisiae* (Lambie and Roeder, 1988; Cao *et al.* 1990, Sun *et al.*, 1989); *ADE6* gene of *Schizosaccharomyces pombe* (Gutz, 1971) but the *COG* region of *Neurospora crassa* is downstream of HIS3 gene (Catcheside, 1974). The *ARG4* locus and the *LEU2-HIS4* interval of yeast were known to be recombination hot spots (Newlon *et al.*, 1986; Nicolas *et al.*, 1989) and double-stranded breaks have been observed at the *ARG4* and *LEU2* genes

when cells are at the zygotene-pachytene interval (Sun *et al.*, 1989; Cao *et al.*, 1990).

The sequence important for the high frequency of gene conversion in the *ARG4* locus has now been localised. Interestingly, the major contribution to the enhanced level of recombination comes from a 8 bp poly(dA·dT) tract in the promoter region (Schutes and Szostak, 1991). Similar tracts can be found in the sequence of other recombination hotspots, for example, *HIS4, LEU2* genes and a new fragment found to be responsible for the enhanced level crossing-over between *LEU2* and *CEN3*. However, attempts to detect similar breaks at these loci as well as the *ADE6* locus of fission yeast (*Schizosaccharomyces pombe*) have failed (Schuchert *et al.*, 1991; Symington *et al.*, 1991). In addition, the hotspot in the *ADE6* locus was found to be caused by a heptanucleotide ATGACGC in the gene's translation initiation site (Schuchert *et al.*, 1991). While it seems reasonable to assume that these sequences serve as the binding sites of some recombination catalysing protein, it is puzzling that deletion of the 142 bp sequence in the promoter of *ARG4* did not completely abolish recombination at the locus (Nicolas *et al.*, 1989) and the *cis*-acting sequence in the *ADE6* locus has no resemblance to that of *ARG4*. A likely explanation for these results is that these short DNA sequences induce a conformation change in the neighbouring chromatin rather than serve as the cutting sites of recombinational enzymes. The change may make the chromatin more susceptible to attack by endonuclease.

In *Lilium,* the generation of nicks in chromatin at pachytene was found to be regulated by a class of small nuclear RNA called

PsnRNA (Hotta *et al.*, 1979; Hotta and Stern, 1981). The PsnRNA makes the corresponding chromatin susceptible to attack by the endonuclease. It is possible that the *cis*-acting sequences found in yeast and *Neurospora* could serve as a similar function Unfortunately, similar endonuclease and PsnRNAs have not been isolated from yeast but it will be interesting to see if similar short nuclear RNAs are transcribed from the recombination hotspots.

Alternatively, the structural RNAs themselves of *ARG4* and *LEU2* and possibly other hotspots, may directly perform the function of PsnRNAs, thereby making the coding sequences accessible by the enzyme. Indirect support for this is seen in the observation that all recombination promoting sequences found so far are located in the promoter regions genes that are active during meiosis. Conceivably, the *cis*-acting sequences, e.g., the poly(dA·dT) tract of *ARG4* and the ATGACGC motif of *ADE6*, may promote recombination by enhancing transcription so that endonuclease is competent (sufficiently active) to attack the corresponding chromatin. It has been shown that mitotic recombination stimulated by *HOT1*, a short sequence in the promoter region of ribosomal DNA of budding yeast (Keil and Roeder, 1984), was operated by enhancing transcription (Stewart and Roeder, 1989; Lin and Keil, 1991). Similar results were observed in the mating-type switch and *GAL10* locus of budding yeast (Klar *et al.*, 1984; Thomas and Rothstein, 1989) and the mammalian immunoglobulin genes (Blackwell *et al.*, 1986; Schlissel and Baltimore, 1989). On the other hand, the deletion in the promoter of *HIS4* gene has no significant effect on gene

conversion on this locus (Stapleton and Petes, 1991). Therefore, the mechanistic basis for the stimulation of recombination by these short *cis*-acting sequences remains a mystery.

1.1.3. The Future of Meiosis Research

The study of meiosis is falling behind many aspects of biology, particularly other forms of cell division in which the controlling mechanisms are partially understood at the molecular level and rapid progress is being made. The study of chromosome synapsis and recombination has been hampered by the difficulty in observing the SCs easily in the highly versatile organism *S. cerevisiae*. However, the development of surface spreading techniques (Dresser and Giroux, 1988) now allow rapid progress.

The current controversy on chromosome synapsis and genetic recombination may be resolved if more diverse organisms are studied. The pioneering research in *Lilium*, for example, has provided interesting information. However, most of the meiosis-specific elements (see Table1 in Section 1.1.5) have been demonstrated only in *Lilium* with a few found in mouse and yeast. This causes concern about the general occurrence of these elements (Loidl, 1990).

It should be noted that some diversity of genetic mechanism between different organisms should be expected, as is the case for somatic association in *Diptera* (Liodl , 1991). Even within one species, meiosis in female and male can differ enormously, for example, differences between male and female in the recombina-

tion level in silkworm (*Bombyx mori*) (Sturtevant, 1915) and the sub-telomeric regions of human chromosomes (Rouyer *et al.*, 1990). In cereals, differences in transmission rate of telosomic chromosomes between pollen and ovary may reflect sex differences in pairing or recombination (Sears and Sears, 1978). Data from interspecific hybrids and polyploids should therefore be interpretated with caution since pairing is irregular and hence a fundamental change in biochemical status may have resulted.

To date the mechanisms of chromosome pairing and recombination remain unknown. Indeed the short duration of meiosis and limitations of materials makes the study unattractive, especially in higher eukaryotes. Biochemical investigations analogous to those undertaken in *Lilium* may reveal clues to the mechanism of initiation of recombination and synapsis if performed in fungi such as yeast. The differences between haploid and normal diploid may be especially important for showing the relationship between synapsis and recombination.

1.2. Chromosome Pairing in Wheat

1.2.1. Introduction

The genome of bread wheat (*Triticum aestivum*) is built up of three separate genomes termed A, B and D. Each genome derived from a different ancestor but they are all interrelated and the three related chromosome set are called homoeologues (Sears, 1952). Although there is extensive sequence homology between the homoeologues, chromosome pairing is restricted to true

homologues. In bread wheat a mechanism exists to allow pairing despite the low level of sequence divergence of homologues but to prevent it where the higher divergence of homoeologues chromosomes occurs (Okamoto, 1957). The diploid-like behaviour of hexaploid wheat has been studied genetically since the late 1960's. The control of pairing resides with a complex group of genes that promote or suppress the pairing of homoeologues. These genes are largely located on homoeogroups 3 and 5 (Sears, 1976). The strongest effect resides with a suppressor located on the long arm of chromosome 5B (5BL), called *Ph1* (Riley and Chapmam, 1958; Sears and Okamoto, 1958). Another, somewhat weaker suppressor (*Ph2*) has been identified on the short arm of 3D (3DS) (Mello-Sampayo, 1971), while the short arm of 5B (5BS) holds an enhancer (Feldman and Mello-Sampayo, 1967; Riley and Chapman, 1967). This gene has not been named. For convenience, it be referred to as $Ph3_p$ (P stands for promoter) hereafter because it regulates the same pairing process as the suppressors but produces the opposite effect. These three genes are the best studied and exert the strongest effects. The removal of chromosome 5B (as in 5B nullisomic plants) or the deletion of the region containing *Ph1*, will induce homoeologous pairing (Riley and Chapman, 1958). This has practical applications in wheat breeding where the transfer and introgression of alien chromosomal segments into wheat is desired (Riley *et al.*, 1968; Sears, 1973; Koebner and Shepherd, 1986).

1.2.2. The Cytological Effects of *Ph* Genes

The process of synaptonemal complex formation in euploid wheat compares well to other eukaryotic systems with respect to the timing and the sites of initiation. This applies to the frequency of chromosome interlocking and partner exchange during the initial stages of the chromosome alignment and correction (Holm and Wang, 1988). However, in wheat, abnormalities will occur if the balance of suppressor and promoter genes is altered. The hexaploid nature of wheat is ideally suited to the manipulation of the number of individual chromosomes because of the compensation by the homoeologues. For example, the copy number of 5BL can be manipulated to give plants possessing zero to six copies (Feldman, 1966). Holm and Wang (1988) and Holm (1988 a, b) undertook a comprehensive study of the effects of *Ph1* gene dosage on chromosome synapsis in wheat. Both an increase or a decrease in copies of 5BL resulted in changes in the degree of synapsis and multiple chromosome association. Promoters of pairing had the reverse effect but the results showed that there is no simple proportional correlation between dosage of suppressor or promoter. Two copies of the *Ph1* gene gave the highest level of pairing or synapsis and the lowest level of multiple associations. Electron microscopic (EM) studies have also revealed that altering the balance of promoter and suppressor can cause synapsis arrest either in wheat itself or in interspecies hybrids (Gillies, 1987; Holm and Wang, 1988).

1.2.3. The Mechanism of Action of *Ph* Genes

Several models have been proposed for the mechanism of action of the *Ph* genes. The earliest model suggested an effect on

the ratio of DNA to histones since cells undergoing synapsis tend to have lower ratios (Ansley, 1958). Support for the model was lost as a result of experiments by Feldman and colleagues who proposed that *Ph* genes act by regulating chromosome proximity (Avivi and Feldman, 1973a; 1973b; Feldman and Avivi, 1973). Based on an analysis of the effect of *Ph* gene dosage and colchicine treatment on somatic chromosome association (Avivi *et al.*b, 1970), it was proposed that pairing suppressors destabilise the spindle and microtubule system whereas pairing enhancers increase stability. In the absence of the *Ph1* gene, chromosomes appeared to lie closer together, while with six copies of the *Ph1* genes, chromosomes lie nearly at random during early meiotic prophase stages. Indeed it explained some curious features of specificity of pairing and the interlocking of chromosomes. The difference in the inter-chromosomal distance is, however, so small that other researchers such as Darvey and Driscoll (1972), could not observe it. In addition, Feldman (1966) found that autododecaploid wheat, which was produced by colchicine treatment of hexaploid wheat, formed only bivalents. Because four chromosomes are identical in cells of such plants, the somatic association hypothesis should predict the formation of quadrivalents. Moreover, recent cytogenetic studies, particularly at the electron microscopic level (Holm, 1986; 1988a), have also cast doubt upon the validity of the chromosome proximity model.

Hobolth (1981) believed that the *Ph* genes control the time of crossing-over. He observed that multivalent SCs regularly form at zygotene but become corrected into strict bivalents at pachytene in euploid wheat. However, the number of multivalents increased in

tri-isosomic 5BL plants (with six copies of 5BL). This lead him to suggest that the *Ph* genes affect the time at which crossing-over occurs, that is, with two copies of *Ph1* crossing-over is delayed until after multivalents are corrected while with six copies of *Ph1* crossing-over is delayed until diplotene when the SCs begin to degrade and condition for recombination has become sub-optimium. The presence of multivalents in *ph1* genotype is therefore contributed to earlier initiation of recombination than euploid, making multivalent correction impossible or inefficient because of the tightly bound crossover. This was in part supported by Gillies (1987) with the observation that hybrids of *ph1 T. aestivum* and *T. tauchii* tended to synapse slower than the *Ph1* hybrids. Holm and Wang (1988), on the other hand, disagreed with Hobolth's timing model based on two lines of evidence. Firstly, in monosomic 5B and occasionally euploid wheat, multiple associations persisted through the crossing-over interval but no chiasmata formed between homoeologues. Secondly, reduction of chiasmata corresponded to pairing arrest in tri-isosomic 5BL.

Holm and Wang (1988) could only restate the observations of the nature of the *Ph* gene effect, rather than provide a biochemical mode of action. They assign two functions to *Ph1*: one is to raise the stringency of synapsis and the other is to suppress crossing-over between partially homologous chromosome segments. This still leaves the problem of how the *Ph* genes can distinguish between homologous and homoeologous chromosomes.

1.2.4. The Biochemical and Physiological Aspects of *Ph* Genes

Colchicine and vinblastine both interfere with polymerization of tubulin, the subunit of microtubules. Both chemicals show similar effect but bind to different sites (Creasely and Chau, 1968; Olmsted *et al.*, 1970). Their effect can be easily observed at metaphase. The resultant chromosomes are more condensed than their normal counterparts and the sister chromatids are separated from each other except at the centromere. When root tip cells of wheat were treated with vinblastine or colchicine, it was observed that the cells became more tolerant with increasing dosages of 5BL or decreasing dosages of 5BS. This lead to the conclusion that *Ph* genes affect the stability of microtubules (Avivi and Feldman, 1973a; 1973b; Avivi *et al.*, 1970b). Recently, this experiment was repeated in *ph1* and *ph2* mutants and the results were similar (Ceoloni *et al.*, 1984; Ceoloni and Feldman, 1987), re-enforcing the earlier conclusion. In addition, it was suggested that the *Ph* gene product may be some component of the microtubules or able to modify the microtubule components, such as the microtubule associated proteins (MAPs) or tubulin, thereby altering the tubulin-microtubule equilibrium towards polymerisation (Ceoloni *et al.*, 1984; Gualandi *et al.*, 1984; Ceoloni and Feldman, 1987).

GTP and ATP were found also to bind to tubulin and stabilise the colchicine binding capacity of tubulin (Stevens, 1967; Shelenski and Taylor, 1968). A synergistic effect of colchicine was observable when applied to root-tip cells of wheat possessing zero to two doses of 5BL, that is zero to two copies of *Ph1*. Interestingly, with four doses of 5BL, this effect was no longer noticeable (Avivi *et al.*, 1970a). The author suggested that GTP and

ATP may affect the equilibrium between microtubule subunits and microtubules.

Colchicine can also interfere with the behaviour of meiotic chromosomes. It causes reduction in synapsis as well as chiasma frequency (Shephard *et al.*, 1974; Bennett *et al.*, 1979) when applied at a particular stage. The appropriate stage varies between species (Bennett *et al.*, 1979). Early (but not late) premeiotic interphase was the most sensitive for wheat (Dover and Riley, 1973) and *Secale cereale* was similar but sensitivity extended to as late as leptotene (Bowman and Rajhathy, 1977). In *Triticale* sensitivity extends to early zygotene (Thomas and Kaltsikes, 1977) and in *Lilium* late zygotene (Bennett *et al.*, 1979).

Heat shock treatment of premeiotic or early meiotic tissues induces a similar effects to colchicine in a number of organisms including wheat (Bayliss and Riley, 1972a; 1972b), *Locusta* (*Locusta migratiria*) (Buss and Henderson, 1971) and *Allium ursinum* (Loidl, 1989). Surprisingly, 5BL (and presumably *Ph1*) was able to phenocopy the effect of colchicine or heat shock (Feldman, 1966; Yacobi *et al.*, 1982).

The tubulin-microtubule equilibrium model can not be confirmed at present but the mechanism may be much more complex. Firstly, colchicine has much more radical effects than merely binding to microtubules. It has already been demonstrated that it inhibits transport of nucleosides through cytoplasmic membranes (Mizel and Wilson, 1972). Another potentially important protein for recombination, the R-protein of *Lilium*, was found to

have reduced in concentration after colchicine treatment (Hotta and Shephard, 1973). Secondly, GTP and ATP are versatile regulators of gene expression and of metabolic pathways and it is difficult to decide whether the effects observed are the indirect or direct consequence of these nucleotides (Albert *et al.*, 1989). Thirdly, isopropyl-N-phenyl-carbamate (IPC), a chemical which disturbs microtubule orientation and patterning (Helper and Jackson, 1969; Coss and Pickett-Heaps, 1974; Coss *et al.*, 1975), was unaffected by *Ph* genotypes when applied at low concentration (Gualandi *et al.*, 1984). Finally and most importantly, the *Ph2* gene is functionally similar gene *Ph1* but it showed decreased rather than increased tolerance to colchicine, just as $Ph3_p$ do (Ceoloni and Feldman, 1987).

Nonetheless, the co-relationship of *Ph* genes to chemicals known to affect microtubules has been repeatedly demonstrated in somatic tissues. The chromosome morphology is very different when cell are treated with anti-tubulin drugs and the effects can be easily scored (Avivi and Feldman, 1973b; Gualandi *et al.*, 1984). Therefore, it is unlikely that the co-relationship resulted from experimental artifacts. This puzzle, it seems, is beyond reach of the traditional approaches for the study of the *Ph* genes.

1.2.5. A Need for Molecular Study of Meiosis

Many of the problems associated with the elucidation of the control of chromosome pairing in wheat may be clarified by a molecular rather than a cytogenetic approach to the control of meiosis. It is difficult, however, to isolate genes involved in the

process of meiosis without first understanding the biochemistry of the process. In this case, the causal factors of meiosis are likely to be of two broad types. Clearly, there will be a range of proteins involved. We would predict the bulk of these to be the same as those operating during mitosis, for example the enzymes of DNA repair. Some, however, will be specific to meiosis. It is also clear that many of the features of meiosis will require specific chromosomal structures. These will again include a range of proteins that will be involved in the structure of the meiotic chromatin but there are also likely to be specific DNA sequences involved in meiotic events.

Two approaches have been taken to develop an understanding of molecular events involved in meiosis. In yeast it has been possible to isolate genes using mutant complementation. Mutants in meiosis have been identified amongst sporulation deficient (*spo*) or radiation sensitive (*rad*) cells. This type of analysis has been possible due to the small size of the yeast genome, the ease of mutant isolation and the efficiency of yeast transformation. None applies to wheat.

The second approach to the construction of a molecular picture of meiosis, is from a more biochemical attack. The anthers of lilies are large and can be prepared from specific stages of meiosis. In addition, Ito and Stern (1967) have developed a system for the *in vitro* culturing of micromeiocytes. Extensive work by Hotta and Stern has led to the identification of a range of proteins, DNA and RNA sequences that appear to be specific for meiosis. These can be used as probes to study the wheat counterparts. The

elements that have specificity for prophase I of meiosis are summarised in Table 1.

Table 1. A summary of meiosis-specific elements in *Lilium*

Meiosis-specific Element	Characteristic	Function proposed	Reference
ZygDNA	Replication delayed to zygotene, ss gaps or nicks remain at both ends after pachytene, 3-8 kb, unique or low copy sequence, 0.3% genome (0.9% transcribed).	Synapsis	1,2,3,7
PDNA	Repair replication at pachytene, 0.8-3 kb moderate repeat, Flanked by PsnDNA.	Recombination	2,3,7
PsnDNA	150-300 bp moderate repeat, Transcribed into RNA (PsnRNA).	Recombination (Nicking site)	3,4,7
PsnRNA	Complementary to PsnDNA, Level affected by homologous pairing.	R-protein co-factor	3,4,7
EMPR	Abundant transcripts at zygotene Diverse repeat Related to soybean small heat-shock protein	Synapsis?	5,10
L-protein	Site-specific topology-dependent DNA binding protein, Generates ss-nicks in the presence of ATP, Inactive before leptotene and after pachytene.	Suppresses zygDNA replication	1
Psnprotein	Binds to PsnDNA replacing histone, Causes PDNA accessible to endonuclease attack, Level affected by homologous pairing.	Recombination	3,6
R-protein	Level affected by homologous pairing, Catalyses ss-DNA re-association, Binding to DNA regulated by phosphorylation.	Recombination	3,6,9
U-protein	DNA unwinding enzyme.	Recombination	3,6
RecA-like protein	Involves in DNA duplex and DNA strand exchange, Level affected by homologous pairing.	Recombination Synapsis?	8

References: 1, Hotta *et al.*, 1984; 2, Hotta and Stern, 1971; 3, Stern and Hotta, 1984; 4, Hotta and Stern, 1981; 5, Appels *et al.*, 1982; 6, Stern and Hotta, 1978; 7, Hotta and Stern, 1984; 8, Hotta *et al.*, 1985b; Hotta *et al.*, 1979; 10, Bouchard, 1990.

The findings in lilies are particularly attractive when we consider the situation in wheat, since both are monocotyledonous plants and show several common features with respect to meiotic chromosome behaviour; for example, the presence of *Ph*-like genes (Aung and Evans, 1987) and the effects of colchicine on pairing and chiasmata frequency (Hotta *et al.*; 1979). Indeed, there has been a slow accumulation of evidence to suggest that the sequences identified in *Lilium* have their equivalents in several other eukaryotes(Appels *et al.*, 1982; Friedman *et al.,* 1982; Hotta *et al.*, 1985c). From Table 1 there are two types of meiotic elements that are of particular relevance to a consideration of chromosome pairing in wheat. These are the zygotene DNA (zygDNA) and the Expressed Meiotic Prophase Repeats (EMPRs), both of which appear to be involved in synapsis. The remaining elements all appear to be associated with recombination. This conclusion was reached after studies involving an achiasmatic *Lilium* variety, Black Beauty. The achiasmatic lily showed reduced levels of the zygotene RNAs (zygRNA), which are transcripts of zygDNA, and the pachytene small nuclear RNA (PsnRNA). There were also lower concentrations of the various meiosis-specific proteins. However, no clear evidence was found for an effect on the zygDNA or the EMPR RNA levels.

The broad features of the zygDNA and EMPRs can be described as follows:

1. Zygotene DNA Most DNA replication has been completed well before cells enter meiosis. However, a small proportion of

DNA synthesis occurs during meiosis in *Lilium* (see references in Table1 for details). In *Lilium* this proportion is only 1.3% of the total DNA. The DNA synthesis at zygotene comprises about 0.3% of the total DNA and this late replicating DNA has been termed zygDNA. Although the zygDNA appears to be associated with other sequences involved in crossing-over, it has been linked to chromosome synapsis for two reasons. Firstly, the timing of zygDNA replication ties in well with synapsis and secondly, inhibition of zygDNA replication with chemicals, such as deoxyadenosine and hydroxyurea, leads to a disruption of chromosome pairing (Roth and Ito, 1967; Stern and Hotta, 1969).

2. Expressed Meiotic Prophase Repeats (EMPRs) Meiosis is an active developmental stage for gene expression. Not only are there major structural changes occurring in the chromosomes but there is also the synthesis of various meiosis-specific proteins. Surprisingly, the bulk of early meiotic gene transcription in *Lilium* is devoted to the production of a class of mRNAs of which neither protein nor function has been ascribed. These are the zygRNAs and EMPR RNAs. They comprise more than 40% of the total mRNA population at prophase of meiosis and are synthesised at no other time or location in the plant (Hotta *et al.*, 1985c). As their name implies, the EMPRs are transcribed from a family of repeat DNA sequences. Although most EMPR genes cross-hybridise, they show extensive sequence diversity. Nevertheless, EMPR cDNA clones from *Lilium* hybridise to DNA from a range of plants including maize and wheat (Appels *et al.*, 1982).

1.2.5. Discussion

The elucidation of the mechanisms underlying the control of chromosome pairing in wheat have, to date, relied almost exclusively on genetic and cytogenetic approaches. These results have allowed the formation of several models to explain the observed action of control but they have failed to reveal the mechanism. Any attempt to characterise the molecular basis for the control of chromosome pairing in wheat, will require either biochemical or molecular genetic data; ideally both. The small size of the wheat anthers and ovaries at meiosis virtually eliminates a direct biochemical approach to this problem. The size limitation also applies to molecular genetic methods but to a lesser extent. These are three potential approaches to a molecular study.

1. Differential Screening One could make use of this technique to identify sequences specific for meiosis in normal wheat but missing in the aneuploid or meiosis mutant plants, e.g., nulli5B-tetra5D or the Sears *ph1* mutant. This procedure is technically difficult given the small amounts of material available and the possible low level of expression of the desired sequences. There is also no guarantee that homologous sequences are not present on the homoeologous chromosomes. Therefore, this approach is not very appealing.

2. Probing with yeast clones Over 30 genes involved in the control of meiosis and genetic recombination have been isolated from yeast using complementation of mutant cells (Kao *et al.*, 1989). Although the evolutionary distance between yeast and cereals is great, some meiosis controlling genes might be

conserved enough to allow detection by cross-hybridisation, especially using antibodies.

3. Probing with *Lilium* clones Sequences from *Lilium* thought to be associated with the control of chromosome pairing appear to have analogous counterparts in wheat: the zygDNA and the EMPR RNAs. The identification and cloning of these sequences from wheat could offer an entry point to the genes controlling chromosome pairing. Identification of a gene can lead to isolation of a group of regulatory genes and effector genes.

Table 2. Pairing Mutants of Cereals

Species	Phenotype	Mutation	Location	Reference
T. aestivum	homoeologous pairing	deletion? (*ph1b*)	*Ph1* (5BL)	Sears, 1977
T. aestivum	slightly increased univalents	deletion	$Ph3_p$ (5BS)	Kota & Dvorák, 1986
T.aestivum	intermediate pairing	deletion (*ph2a*)	*Ph2* (3DS)	Sears, 1982
T. aestivum	intermediate pairing	point mutation (*ph2b*)	*Ph2* (3DS)	Wall *et al.*, 1971,
T. durum	homoeologous pairing	deletion (*ph1c*)	*Ph1* (5BL)	Dvorák and Chen, 1984
T. durum	homoeologous pairing	5B-5D translocation	*Ph1* (5BL)	Mello-Sampayo, 1972
T.durum	asynapsis	deletion?	unknown	Martini&Bozzini, 1966; La-Cour and Wells, 1970
A. sativa	desynapsis	point mutation	*Syn1* (IV)	Rines & Johnson, 1988
A. sativa	asynapsis	point mutation	*Syn5*(XII)?	As above

No matter what procedure is used, correct identification of a gene depends on the availability of specific mutants and an efficient method to transform cereals. To date a limited number of such mutants have been isolated. These are summarized in Table 2. However, information about the nature of the mutations is largely lacking. Deletion mutants are of particular value since they allow fast mapping of genes. Progress will, to a great extent, depend upon the co-ordination of mutagenesis and cytogenetics research. On the other hand, a method for the transformation of cereals may be available in the near future, this will provide a powerful aid to the study of meiosis in cereals.

The genes controlling chromosome pairing are required to function during only a very short period of the organism's life cycle. In wheat these genes are probably transcribed for only a few hours and only in a small number of highly specialised cells. Nevertheless, the correct and efficient control of pairing is essential for sexual reproduction. It is likely that these genes are amongst the most finely regulated of all eukaryotic genes. No genes controlling chromosome pairing have yet been isolated from higher eukaryotes.

Chapter Two

Materials and Methods

2.1. Materials

2.1.1. Plant Genetic Stocks

All the wheat, rye, barley, rye addition lines, barley addition lines and Chinese Spring (CS) aneuploids were kindly supplied by Dr K.W. Shepherd, Department of Plant Science, Waite Agricultural Research Institute. All plants were grown in a glasshouse in 25 cm pots without artificial light. The day and night temperatures were 20-25°C and 16°C respectively.

2.1.2. Sources of Enzymes

2.1.2.1. Restriction Enzymes

Most restriction enzymes were purchased from Boehringer Mannheim, Promega and Pharmacia.

2.1.2.2. Other Enzymes

Alkaline phosphatase:	Boehringer Mannheim
E.coli DNA polymerase I:	Pharmacia
Human placental RNase inhibitor:	Promega, Pharmacia
Klenow DNA polymerase:	Amersham, Pharmacia

Moloney murine leukemia virus (M-MLV) reverse transcriptase:	BRL
RNase A	Boehringer Mannheim
RNase H	Pharmacia
T4 DNA ligase	Boehringer Mannheim, Promega
Taq DNA polymerase	Promega

2.1.3. Blotting Membranes

Hybond-N+:	Amersham
Hybond-N:	Amersham
Zetaprobe:	Bio-Rad

2.1.4. Plasmids used as Probes

pZm9:

A maize cDNA clone containing a gene homologous to the *Lilium* meiosis-specific gene (Appels, unpublished result)

pMR1:

9kb maize ribosomal operon in pBR328 (Toloczyki and Feix, 1986).

pGB436:

the *Acc* I-*Spe* I fragment containing the *SPO11* gene of yeast (*S. cerevesiae*) in pBluescript KS (Giroux, personal Comm.).

2.2. General Methods

2.2.1. Ethanol Precipitation of DNA and RNA

One tenth volume of 3M sodium acetate pH 4.8 or 5.2 (for RNA) was mixed with the DNA or RNA solution and 2.5 volumes of pure ethanol was added and mixed. For very dilute DNA samples the mixture was incubated at -20°C for 30 minutes to overnight. To recover the nucleic acids, the sample was centrifuged at 10,000 to 15,000g for 10 minutes. The supernatant was removed and the pellet washed twice with 70% ethanol. Short spinning is required between each washes. The pellet was dried under vacuum for 2-5 minutes and resuspended in appropriate solvent (usually 1X TE or water).

2.2.2. Iso-propanol Precipitation of Nucleic Acids

This method is essentially the same as the ethanol precipitation except that the 2.5 volumes of ethanol was replaced by 1 volume of iso-propanol.

2.2.3. Preparation of Plasmid Vector for Cloning

Plasmid (usually 10 µg) was digested with the appropriate restriction enzyme to completion. This was extracted once with phenol/chloroform and precipitated with ethanol. The DNA was resuspended in 44 µl water and dephosphorylated with 0.1 to 0.2 units calf intestinal alkaline phosphotase (CIP) in a 50 µl reaction volume at 37°C (for blunt ends or recessed 5' ends, the incubation

was at 56°C) for 30 minutes. The sample was extracted with phenol/chloroform and electrophoresed on a 0.8% agarose gel. The DNA band was exercised with a surgical blade and purified with Geneclean (Bio 101). Finally, the sample was quantified by the ethidium bromide fluorescence method (see section 2.2.11.1) and adjusted to 20 ng/µl.

2.2.4. Ligation of Insert to Cloning Vector

The routine ligation reaction was performed in 20 µl volume with 20 ng of vector, 60 ng of insert and 1 unit of T4 DNA ligase at 12°C overnight. For cloning of inserts larger than 3 kb, the ratio of vector to insert and concentration of vector were altered as described (Zimmerman and Pheiffer,1983; Revie *et al.*, 1988).

2.2.5. Transformation of *E. coli*

A simplified method (Chung and Miller, 1988) was usually used. An overnight culture (0.5 ml) was added to 50 ml LB medium. This was incubated at 37°C with vigorous shaking for approximately 2.5 hour. When the OD_{600nm} had reached 0.3 to 0.5, the culture was chilled on iced water and transferred to a clean sterile centrifuge tube. The cells were pelleted at 1100g for 10 minutes and resuspended in 1/10 volume of TSB buffer (Mg^{2+} ion and DMSO were added freshly). Up to 10 ng circularised DNA was mixed with 100 µl cell suspension and incubated on ice for 30 minutes. TSB (900 µl) without DMSO but supplemented with 50 mM glucose was added and the cells were incubated in a 37°C shaker for 45 minutes. The cells were plated onto 90 mm LB-agar Petri dishes

with the appropriate antibiotic, IPTG and X-gal. The plates were incubated overnight at 37°C.

2.2.6. Preparation of *E.coli* Plating Cells for Infection with Bateriophage Lambda

A single bacterial colony was inoculated into 2 ml LB and grown overnight in a 37°C shaker. An aliquot (0.5 ml) was inoculated into 50 ml LB supplemented with 0.4% maltose and 10 mM $MgSO_4$ and incubated with vigorous shaking at 37°C for 2-3 hours. When the OD_{600nm} reached 0.5, the culture was chilled on ice and then centrifuged at 1100g, 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in 15 ml ice cold 10 mM $MgSO_4$. The cells could be stored at 4°C for up to one week but, for the cDNA library, fresh cells (strain NM514) were used.

2.2.7. Mini-prep of Plasmid DNA

A single colony was inoculated into 1.5 ml TYP medium and allowed to grow overnight in a 37°C shaker. The alkaline lysis method for mini scale plasmid isolation was as described (Birnboim and Doly, 1979; Maniatis *et al.*, 1982).

2.2.8. Large Scale Isolation of Plasmid

The DNA isolation method was the same as described (Maniatis *et al.*, 1982). Again TYP was usually used since, with this medium, culture volume can be reduced by 5 to 10 fold without sacrificing yield.

2.2.9. Small Scale Isolation of Bacteriophage Lambda DNA

This method is described by Amersham in a λgt10 cDNA cloning manual and uses DEAE-cellulose to absorb chromosomal DNA, RNA and protein from the *E. coli* lysate. The resultant lambda DNA is easily digestable and free of RNA and chromosomal DNA.

The *E.coli* host (0.5 ml) (NM514, NW2 or ER1647) was infected with a single plaque (or half of the overnight elution in SM) for 15 minute at room temperature and added to 5 ml LB supplemented with 5 mM $CaCl_2$. The mix was incubated at 37°C shaker until complete lysis of the cells had occurred. After 10 minutes centrifugation at 10,000g, the supernatant was transferred to a fresh tube and an equal volume of phage precipitating buffer was added. After one hour incubation on ice, the phage particles were pelleted by centrifugation at 8000g for 20 minutes. The pellet was gently resuspended in 750 µl LB broth and an equal volume of DEAE-cellulose (Whatman DE52) LB suspension was added. The phage remained in solution after centrifugation and the supernatant was transferred to a fresh tube. The DEAE-cellulose extraction step was repeated once and the phage DNA were released by the addition of 13 µl 0.1 mg/ml proteinase K and 32 µl SDS. After 5 minutes digestion at room temperature, 130 µl 3M potassium acetate was added. This solution was treated at 88°C for 20 minutes. SDS and protein were precipitated after chilling on ice and separated from the DNA by centrifugation. Finally, the DNA was precipitated by iso-propanol and washed with 70% ethanol before resuspending in 20 µl TE.

2.2.10. Purification of DNA Fragments from Agarose Gels

2.2.10.1. Freeze-thaw Method (Gaastra and Jϕrgensen, 1984)

The DNA fragment separated by agarose gel eletrophoresis was excised with a surgical blade and placed in an Eppendorf tube which had been punched with a needle at the bottom and fitted with a small plug of glass fibre. The tube was frozen in liquid nitrogen and then mounted on top of a capless Eppendorf tube and spun for 10 minutes at room temperature. The liquid that passed into the lower tube was extracted with phenol/chloroform. The DNA was precipitated with ethanol and then resuspended in an appropriate volume of 1X TE.

2.2.10.2. Geneclean Method

For DNA fragment larger than 2 kb, a commercial Geneclean kit (Bio 101) was used. The DNA was purified according to the manufacturer's instruction. Briefly, the agarose block containing the DNA was dissolved with 6 M NaI at 50°C. DNA was then bound to 5 µl glass milk and the DNA-glass complexes were pelleted by centrifugation and washed with the solution (NEW wash) provided. DNA was eluted in 1X TE at 50°C.

2.2.11. Quantification of DNA

2.2.11.1. Sub-microgram Amounts

This method is based on that described by Maniatis *et al.*, (1982). The DNA sample was diluted 10 and 100 folds in 1X TE and a 2 µl aliquot was mixed with equal volume of 1 µg/ml ethidium bromide that had been spotted on a sheet of polyethylene. Standard amounts of DNA (1ng, 2ng, 4 ng, 6 ng and 10 ng of pUC19) were included for comparison. The sheet was photographed under UV light and the brightness of the samples was compared to standards to estimate the concentration. This method is generally used to quantify DNA substrates for ligation and radiolabelling.

2.2.11.2. Quantification of Nucleic Acid by Spectroscopy

The DNA or RNA sample was diluted N fold in water to 1 ml. The absorbance was determined by scanning the sample against a water blank between 200-300 nm wavelength and the concentration was calculated by the following formula.

Double strand DNA: OD_{260nm} x 50 x N

Single strand DNA: OD_{260nm} x 33 x N

Single strand RNA: OD_{260nm} x 40 x N

2.2.12. Microscopic Examination of Anthers

The flower spike was removed from the stem sheath and fixed in ethanol:acetic acid (3:1) from 30 minutes to several days

at 4°C. Pollen mother cells were squeezed out of the anther wall in 45% acetic acid under a dissecting microscope, squashed in aceto-orcein and examined under a microscope.

2.3. Detection of Nucleic Acid on Membranes

2.3.1. Southern Blot Analysis

2.3.1.1. Plant DNA Extraction

2.3.1.1.1. Mini-scale Extraction

One fresh, young leaf (10-15 cm long) was folded into a 2 ml Eppendorf tube. This was frozen in liquid nitrogen and the leaf was crushed into powder with a small rod. DNA extraction buffer (600 µl) was added and the leaf powder was homogenised. An equal volume of Tris-HCl saturated phenol was added to the tube and mixed gently for 30 minutes at 4°C. Following centrifugation, the supernatant was transferred to a fresh tube and re-extracted with phenol/chloroform/iso-amyl alcohol. The DNA was then precipitated with ethanol, re-suspended in 1X TE buffer and then re-precipitated with ethanol. Finally, the sample was dried briefly under vacuum and dissolved in 50 µl R40 buffer.

2.3.1.1.2. Large Scale DNA Extraction

Wheat leaves (5-10 g) were cut into about 1 cm long pieces, frozen in liquid nitrogen in a mortar and ground to a fine powder. This was transferred to a 200 ml centrifuge tube and immediately,

100 ml DNA extraction buffer and 100 ml Tris-HCl saturated phenol were added. The two phases were gently mixed at 4°C on a rotatory mixer for 30 minutes. The lower phase was removed following centrifugation for 5 minutes at 5000 rpm, 4°C. The extraction was repeated with 100 ml of phenol/chloroform/iso-amyl alcohol. The two phases were separated by centrifugation and the upper phase was carefully poured off and filtered through a nylon mesh into a conical flask. The DNA was precipitated with ethanol followed by 2 washes with 70% ethanol. The DNA precipitate was dried briefly with tissue paper, transferred to a 10 ml polycarbonate ultracentrifuge tube and resuspended in 7 ml of TE. To separate DNA from RNA, the sample was then run on an equilibrium CsCl gradient. CsCl (7.5 g) was added to the DNA suspension which was inverted repeatedly to dissolve the salt. Finally, 0.5 ml 10 mg/ml ethidium bromide was added. After thorough mixing the sample was centrifuged to equilibrium at 40,000 rpm for 40 hours. The DNA band was transferred to a 20 ml tube and extracted with water saturated butanol until no pink colour was visible. The DNA sample was dialysi ed against 3 changes of 1X TE for about 16 hours. The concentration of DNA was determined by spectroscopy.

2.3.1.2. Restriction Digestion of DNA and Fractionation on Agarose Gel

DNA (5-10 µg) was digested with 20 units of restriction enzyme in 20 µl 1X restriction buffer supplied by the manufacturer for 4 hours. The enzymes commonly used are *Bam* HI, *Bgl* II, *Dra* I, *Eco* RI, *Eco* RV, *Hind* III, *Kpn* I, and *Xba* I. DNA sample loading buffer

(2 µl) was added before loading onto a 0.8% to 1% agarose gel. For Southern hybridisation, the gel was run overnight at 25 mAmp.

2.3.1.3. Transfer of DNA to Nylon Membrane

After electrophoresis the gel was stained in 1 µg/ml ethidium bromide for 15 minutes and photographed over UV light. The gel was soaked for 30 minutes in denaturing solution followed by 30 minutes in neutralising solution. The DNA was transferred in 20X SSC for 6 hours and fixed to the membrane by soaking in 0.4 M NaOH with DNA side up for 30 minutes. The membrane was rinsed twice with 4X SSC and blotted dry with Whatman 3MM paper.

2.3.1.4. Prehybridisation and Hybridisation

Prehybridisation and hybridisation, unless specified, was performed in a plastic box at 42°C overnight in 20-30 ml 3X SSC, 40% formamide, 1% SDS, 0.5% Blotto (low fat milk powder, Johnson *et al.*, 1984), 0.25 mg/ml salmon sperm DNA. Gentle mixing was achieved with a rocker-mixer. The advantages of this setup are easy of handling of membrane and low background (normally little background is visible over two weeks exposure).

Before hybridisation, the prehybridisation mix was drained and the filter blotted dry with Whatman 3MM paper. Fresh mix was prepared and 20-30 ng radiolabeled probe was boiled and immediately chilled in iced water before being added to the mix. The mix was poured into the plastic box and the membrane

carefully layed in. Hybridisation was normally carried out overnight.

After hybridisation, the filter was rinsed twice with 2X SSC, 0.1% SDS, then washed three times in 0.5X SSC, 0.1% SDS at 60°C for 30 minutes, blotted dry, wrapped in plastic wrap and exposed at -70°C for 3-10 days between intensifying screens.

2.3.2. Northern Blot Analysis

2.3.2.1. Plant RNA Extraction

2.3.2.1.1. Large Scale RNA Extraction

The method has been described (Jury, 1985). Briefly, 0.5-5 g of leavies or florets were ground to a fine power under liquid nitrogen in a mortar. RNA extraction buffer (6 ml) was added and mixed thoroughly. The icy slurry was transferred to a pre-cooled 15 ml Corex tube and centrifuged at 5000 rpm, 4°C for 10 minutes. The supernatant was transferred to a KOH treated (0.5 M KOH, then thoroughly rinsed with sterilised water) ultracentrifuge tube containing 5 g CsCl. The tube was repeatedly inverted to dissolve the CsCl and then 3 ml CsCl cushion (0.1 M Tris-HCl, pH8.0, 0.965g/ml CsCl) was carefully layered at the bottom of the centrifuge tube. The RNA passed through the CsCl cushion and was pelleted by centrifugation for 16 hours at 30,000 rpm, 4°C in a Ti 65 rotor. DNA and protein were left in the supernatant. The sticky top layer of the supernatant was first removed with sterile cotton buds and then the tube quickly inverted to pour out the remaining

supernatant. The tube wall was wiped dry while the tube was kept inverted. The pellet was re-suspended in 500 µl 0.1X RNA extraction buffer and then extracted with phenol/chloroform. The RNA was precipitated with 50 µl 3 M NaAcetate pH5.2 and 500 µl iso-propanol. To further remove contaminants such as Sarkosyl and CsCl from RNA, the sample was re-dissolved in 300 µl TE and re-precipitated with ethanol. The pellet was finally resuspended in the appropriate volume of TE buffer. The RNA concentration was determined by spectroscopy.

2.3.2.1.2. Small Scale RNA Extraction

This method is best suited for the extraction of RNA from a few anthers or from small amounts of other tissues. It is based on the ability of LiCl to differentially precipitate RNA (Barlow *et al.*, 1963).

Wheat anthers (usually 12) was collected under a dissecting microscope and immediately placed in a 1.5 ml Eppendorf tube and frozen in liquid nitrogen. The sample was stored in a -80°C freezer until processing.

The material was crushed with an oven-sterilised metal crusher and immediately 50 µl RNA extraction buffer with 1-5 µg yeast tRNA as carrier RNA was added. The sample was homogenized further with the crusher and then extracted with 50 µl phenol/chloroform/iso-armyl alcohol by vortexing vigorously for 1-2 minutes. When all samples were processed, they were centrifuged in 4°C for 5 minutes and the supernatant transferred to

fresh tubes. The extraction was repeated and 50 µl of 6 M LiCl was added to the supernatant and kept on ice for one hour. RNA was pelleted by centrifugation. After two washes with 80% ethanol, the pellet was dried briefly under vacuum and dissolved in 80 µl diethyl pyrocarbonate (DEPC) treated double distilled water and re-precipitated with ethanol. Residual DNA was eliminated by treatment with RNase-free DNase. (for Northern blot analysis this step was not necessary). The samples were treated at 75°C for 5 minutes then chilled on ice and 10 µl 10X DNase buffer, 0.5 µl RNasin (20 units) and 4 µl (40 units) RNase free DNase I were added and allowed to digest at 37°C for one hour. The samples were boiled for 2 minutes and again chilled before adding the same amount of enzymes as above. It was found that this step is essential for total elimination of chromosomal DNA for the RT-PCR assay.

2.3.2.2. Purification of Poly(A)+ mRNA with Oligo(dT)-cellulose

The binding and elution condition used were based on those described by Slater (1984). The whole process was done in an 2 ml Eppendorf tube for fast washing and elution. This allowed multiple samples to be processed simultaneously.

About 0.2 g of oligo(dT)-cellulose was suspended in 0.3 M NaOH in a 2 ml Eppendorf tube for 15 minutes. The NaOH was washed off with 1X binding buffer until the pH was about 7.6. Total RNA, up to a volume of 900 µl in 1X TE was heated to 70°C for 5 minutes then chilled on ice and mixed with equal volume of 2X binding buffer before being added to the cellulose. The 2 ml

Eppendorf tube was placed in a rotatory mixer at low speed for 5 minutes and the unbound RNA was removed after centrifugation for 1 minute. The mRNA-bound oligo(dT)-cellulose pellet was washed five times with 1.5 ml 1X binding buffer, each with 5 minutes mixing and 1 minute centrifugation. Finally, mRNA was eluted with aliquots of 300 µl elution buffer, each with 5 minutes mixing and 5 minutes centrifugation. The poly(A)$^+$-RNA was recovered by iso-propanol precipitation and resuspended in 1X TE buffer.

2.3.2.3. RNA Electrophoresis

Agarose gels (1-1.5%) containing 2.2 M formaldehyde were prepared according to Gerard and Miller (1986). Total RNA (20 µg) was lyophilised and then dissolved in 4.5 µl Buffer A and 9.5 µl formamide:formaldehyde (250:89), heated at 70°C for 5 minutes and chilled on ice. To each sample, 2 µl sample buffer was added and then loaded onto a gel which had been pre-run in 1X MOPS/EDTA buffer at 60 Volts for 30 minutes. The gel was run at 60 volts for 2-3 hour then stained in 2 µg/ml ethidium bromide for 20 minutes followed by destaining in water for 30 minutes.

2.3.2.4. Blotting RNA to Nylon Support

The gel was soaked in 20X SSC for 20 minutes and then transferred in the same solution overnight. The RNA was fixed to membrane by irradiation under a 15 Watt UV light (254 nm, 24 cm above the filter) for 7 minutes.

2.3.2.5. Northern Hybridisation

Northern prehybridisation and hybridisation was essentially as described for Southern hybridisation except that the following hybridisation solution was used: 1X SSPE, 1% SDS, 0.5% Blotto, 250 µg/ml salmon sperm carrier DNA, 50% formamide. The final washing of the filter was in 0.2X SSC, 0.1% SDS at 65°C.

2.3.3. Screening Bacteriophage Lambda Library

2.3.3.1. Transfer of Phage DNA to Membrane

The plate was dried in a laminar flow hood for 5 minutes and then Hybond N circle membrane was carefully laid over the plate. Four marks on the outer edge of membrane were made using a 18 gauge syringe needle. The transfer was allowed to continue for 1-2 minutes. The membrane was lifted off the plate and laid for 10 minutes on a sheet of Whatman 3MM paper saturated with denaturing solution (DNA side up) and then an neutralising solution for 5 minutes. The membrane was rinsed in 2X SSC for a few seconds, blotted dry on Whatman 3MM and dried at 42°C for 1-2 hours before being fixed under a 15 Watt, 254 nm UV light (24 cm above) for 7 minutes. The membrane was wetted with 4X SSC before proceeding to pre-hybridisation.

2.3.3.2. Hyridisation of Plaque Lift

Hybridisation was performed essentially as for the Southern blot but the stringency was sometimes altered by changing the

concentration of formamide and salt. Up to 10 membranes could be put in a single 19x26 cm box containing 120-150 ml hybridisation mix. (Some were done in Petri dishes.)

2.3.4. Preparation of DNA Probe for Hybridisation

2.3.4.1. Labelling Probe by Random Priming

Multiprime kits were purchased from Amersham. Labelling mix (7.5 µl) and 3 µl α-^{32}P-dCTP (10 µCi/µl, 3000 Ci/mMole) were mixed with 13.5 µl boiled template DNA (approximately 20 ng). Klenow enzyme (1µl, 1-2 units) was added and incubated in a 37°C water bath for 90 minutes. The reaction was terminated by the addition of EDTA to 10 mM.

2.3.4.2. Labelling of DNA Probes by Nick Translation

The method was as described (Rigby *et al.*, 1977). The reaction (for 0.5 µg DNA) was performed in 1X nick translation buffer, 5 µl ^{32}P-dCTP (3000 Ci/mMole, 10 µCi/µl), 25 ng DNase I, 2.5 units *E.coli* DNA polymerase I in 25 µl at 16°C for 1 hour.

2.3.4.3. Separation of Unincorporated Radionucleotide from Probe

A mini Sephadex G100 column was packed into a pasteur pipette and equilibrated with 1X TE supplemented with 0.1% SDS, before adding the multiprime reaction mix to the column. Fractions (0.5 ml) were collected and the first peaks of radioac tivity were

pooled. These were boiled for 10 minutes to denature the DNA and added to the hybridisation mix.

2.4. DNA Sequencing

2.4.1. Small Scale Isolation of Single-stranded Phagemid DNA.

pTZ18U recombinant plasmids were first re-transformed into JM103 and plated onto M9 agar plates. A single colony was picked, inoculated into 1 ml of M9 medium (with ampicillin) and allowed to grow at 30°C with vigorous shaking overnight. An aliquot (100) µl was removed to a scintillation vial containing 3 ml TYP medium with 50 µg/ml ampicillin and about $2x10^{11}$ pfu of helper phage M13 K07. After 2 hours shaking at 30°C, kanamycin was added to 70 µg/ml and the culture was incubated with vigorous shaking overnight. The culture was transferred to two 2 ml Eppendorf tubes and centrifuged for 10 minutes. The supernatant was transferred to a fresh tube and spun again. The supernatant (1.2 ml) was removed to a 1.5 ml Eppendorf tube containing 10 µg DNase I and 10 µg RNase A. After 30 minutes at 37°C, 300 µl Phage Precipitating Buffer was added. The phages were pelleted by centrifugation at 10,000g for 10 minutes and then incubated for 15 minutes at room temperature. The tubes were drained and centrifuged again so that residual supernatant could be removed. The pellets were dissolved in 350 µl TE and the two tubes combined. The phage DNA was released by extracting the solution once with Tris-HCl saturated phenol and once with chloroform. Finally, the DNA was precipitated with iso-propanol and resuspended in 50 µl TE.

2.4.2. DNA Sequencing with Taq DNA Polymerase

A Taq DNA sequencing kit was purchased from Pharmacia and both single-stranded or double-stranded DNAs were sequenced with the "thermal cycler method" as described in the manufacture's manual with certain modifications of the program (see below for the two different programs). The first was for normal templates and the second for difficult templates which produced low signal and/or artificial terminations.

Program 1. 70°C, 10 min. - 30°C, 10 min. (annealing) - hold 2 min. at 30°C - 70°C, 4 min. - 37°C, 4.5 min. - 70°C, 5 min. (for sequencing two templates).

Program 2. 70°C, 5 min. - 60°C, 5 min. (annealing) - hold 2 min. at 60°C - 70°C, 4 min. - 42°C, 4.5 min. - 75°C, 5 min. (for sequencing two templates).

Briefly, about 1 µg single-stranded or 1.8 µg denatured double-stranded templates were annealed with 80 ng universal primer at 30°C for 10 minutes (for difficult templates, 5 minutes at 60°C) in a total volume of 16 µl. ^{32}P-dATP (10 µCi/µl, 3000Ci/mMole) or ^{35}S-dATP (10 µCi/µl, 1000Ci/mMole) (1 µl) and 1 µl Taq DNA polymerase (about 4.5 units) were added and 4 µl fractions were transferred to four tubes labelled A, C, G, and T. The tubes were capped and placed on a Hybaid IHB 2024 thermal cycler at 70°C for 4 minutes (to denature any secondary structures). When the temperature had dropped to about 45°C, all tubes were uncapped and, when at 38°C (43°C for difficult templates), a stop-watch was started and 2 µl labelling mix was mixed into each tube

by pipetting up and down. As soon as the stopwatch reached 2.5 minutes, 3 µl A, C, G, or T termination mix was added to the corresponding tubes. The termination reaction was allowed to proceed for 5 minutes and then terminated by the addition of 3 µl stop mix.

2.4.3. Generation of Progressive Deletion Clones for Sequencing

A double-stranded nested deletion kit was purchased from Pharmacia and all procedures followed those recommended by the manufacturer. The λ exonuclease III deletions were performed at 30°C in 75 mM NaCl and fractions were collected at 2 minute intervals (expecting 200 bp deletion for each interval). The re-circularised plasmids were transformed in *E.coli* strain DH5α. Three clones from each deletion were screened and desirable clones were purified on a Sephadex G200 mini-column after RNase A treatment and phenol extraction or, re-transformed into JM103 to isolate single-stranded phagemid DNA.

2.4.4. Glass Plate Surface Treatment for Polyacylamide-urea Gel

The surface of glass plates for sequencing gel were treated so that one plate tightly hold the gel and the other repelled the gel. The treatment simplified the pouring of a gel and the separation of glass plates from the gel.

Stick: 10 ml ethanol, 300 µl acetic acid and 50 µl methacrylic acid 3-trimethoxy-silypropyl ester were mixed and poured onto a 60 x 30 cm glass plate. This was spreaded evenly

with tissue paper and allowed to dry for 30 minutes. The surface was then thoroughly cleaned with ethanol.

Repel: 10 ml carbon tetrachloride was mixed with 500 µl dimethyl-dichlorosilane and the same procedure was followed for the stick solution.

2.4.5. Preparation of 8% Sequencing Gel

The treated glass plates were assembled using 0.5 mm spacers. Gel mix (75 ml) was prepared by mixing 30 ml 20% acylamide-urea solution and 37.5 ml 46% urea solution. This was then filtered through Whatman 541 filter paper and degassed for 10 minutes. After mixing with 50 µl TEMED and 375 µl 10% ammonium persulphate, the solution was slowly poured between the glass plates and two combs were inserted. The comb area was clamped and the gel was allowed to polymerise for 30-60 minutes. The gel could be stored at 4°C overnight or used immediately.

2.4.6. Electrophoresis

The combs were carefully removed and the wells flushed with water before the gel and thermal plate were attached to an electrophoresis tank. The wells were flushed with gel running buffer (1X TBE) and a saturated urea solution was layed over the wells. The gel was pre-run for about 30 minutes at 2500 Volts (or until the gel temperature had reached about 55°C). The denatured DNA samples were loaded after the wells had been flushed with buffer. The gel was run at 2500 Volts for about 8 hours and fixed in

10% acetic acid for 15 minutes. The gel was allowed to dry in a fume hood overnight and exposed to a X-ray film for 0.5-7 days.

2.5. RT-PCR Reaction for Detection of Gene Expression

The method was based on that described by Kaswasaki and Wang (1989), with certain modifications.

2.5.1. Synthesis of single-stranded cDNA

Half the amount of total RNA isolated from 12 anthers was heated to 70°C for 5 minutes and mixed with 20 units RNasin, 0.2 μg of 3' primer (20-mer) or 1 μg oligo$(dT)_{8-12}$ and 100 or 200 units of reverse transcriptase (BRL) in 15 μl 1X M-MLV reverse transcriptase buffer with 5 mM dNTPs. The reaction was performed for 10 minutes at 37°C and then 30 minutes at 42°C followed by phenol/chloroform extraction, ethanol precipitation and resuspension in 4-10 μl 1X TE buffer.

2.5.2. PCR Reaction

The product from the reverse transcription reaction was boiled for 5 minutes. One microlitre was added to 20 or 30 μl PCR mix containing 20 mM Tris-HCl (pH8.4), 50 mM KCl, 330 μM dNTPs, 1.67 mM $MgCl_2$, 0.15-0.3 μg upstream and downstream primers and 0.5 units of Taq DNA polymerase. The reactions were performed in a Hybaid IHB 2024 thermal cycler programmed as follow:

1. pAWJL3 Primers 4 min. denaturation at 95°C followed by 40-45 cycles with 1 min. denaturation at 94°C, 2 min. at 55°C for annealing and 2 min. at 74°C for chain extension. The chain extension reaction in the final cycle was allowed to proceed for 10 min.

2. pAWJL4.1 (Transcript A) and Ubiquitin Gene Primers 4 min. denaturation at 95°C followed by 30 cycles with 1 min. denaturation at 94°C, 2 min. at 60°C for annealing and 2 min. at 74°C for chain extension. The chain extension reaction in the final cycle was allowed to proceed for 10 min.

The ubiquitin gene primers UB1 (CATGCAGATCTTCGTGAAGA) and UB2 (CCTCCAAGCCTGAGCACCAG) are based on the sequence of barley (Gausing and Barkardottir, 1986).

2.6. Isolation ZygDNA from Wheat

The method was based on the assumption the density of wheat zygDNA would be greater than the bulk DNA.

About 500 mg of florets ranging from late leptotene to pachytene were ground to fine powder in a mortar and transferred to a 20 ml Corex tube to which 8 ml DNA extraction buffer and 8 ml phenol/chloroform/iso-amyl alcohol (24:24:1) were added. The mixture was vortexed vigorously for about two minutes and then mixed for 15 minuets at 4°C. The supernatant (5 ml) was transferred to a 10 ml ultracentrifuge tube containing 5 g of CsCl. The tube was inverted repeatedly to dissolve the salt and a CsCl

cushion with a density of 1.712 g/ml was underlayed to the bottom the tube. The sample was centrifuged in a Beckman Ti 65 rotor at 4°C, 30,000 rpm for 16 hours. ZygDNA, RNA and any other DNA that are denser than 1.8 g/ml would pass through the 1.712 g/ml CsCl cushion and be pelleted at the bottom of the centrifuge tube while the bulk of wheat DNA remained in the supernatant.

After centrifugation the supernatant was poured off and the tube wiped clean with cotton buds. The pellet was dissolved in 300 µl TE buffer, extracted with phenol/chloroform and precipitated with ethanol. The sample was finally resuspended in 40 µl TE buffer overnight at 4°C.

2.7. Construction of cDNA Library from the Florets of Wheat

2.7.1. First Strand cDNA Synthesis

The reaction was done essentially as described (D'Alessio *et al.*, 1987). The condition used was 5 µg/µl total RNA, 50 ng/µl oligo(dT)$_{12-18}$, 5 mM dATP, dCTP, dGTP and dTTP, 10 µCi α-^{32}P-dCTP, 40 units human placetal RNase inhibitor and 200 units moloney murine leukemia virus reverse transcriptase in 20 µl of 1X M-MLV reverse transcriptase buffer (A 5X buffer was supplied by the manufacturer). The RNA was denatured at 70°C for 5 minutes and chilled on ice prior to being added to the mixture. The reaction was performed at 37°C for 45 minutes. When the reaction was completed, a 1 µl sample was removed to a new Eppendorf tube containing 9 µl 10X TE buffer. After mixing, 1 µl was spotted onto a DE50 filter (T_0) and another 4 µl onto a second filter (T_1) for

later use. The remainder was loaded onto a 5% polyacrylaminde-urea gel (Maniatis *et al.*, 1982) in order to visualize the cDNA.

2.7.2. Second Strand cDNA Synthesis

This reaction is as described (D'Alessio *et al.*, 1987). After the first strand synthesis had been confirmed, the following components were added to the 19 µl first strand mixture: 8 µl 10X second strand buffer, 1 µl 10 mM dNTPs, 20 µCi α-^{32}P-dCTP, 0.8 units RNase H, 20 units *E.coli* DNA polymerase I. Double distilled water was added to bring the volume to 80 µl. After mixing, 1 µl was immediately removed to mix with 9 µl 10X TE. The enzymes was then inactivated by heat and spotted onto a DE50 filter ($T_{0'}$). The remainder was incubated at 16°C for 120 minutes. When the reaction had been completed, the product was treated at 70°C for 10 minutes to inactivate the enzymes. A 2 µl aliquot was transfered to a new tube and mixed with 8 µl 10X TE buffer. Half was spotted onto a DE50 filter ($T_{1'}$) and the second half was used for size determination on a denaturing 5% acrylamide gel.

2.7.3. Monitoring the Yield of cDNA Synthesis

The filter T_0 and $T_{0'}$ were set aside for counting without washing whereas all the remaining three filters were washed 5 times with 5% TCA and once with 80% ethanol. After drying, The Cerenkov radiation of the filters was measured in a Beckman LS 3801 scintillation counter.

2.6.4. Cloning of cDNA into λgt10

After the second strand synthesis, the sample was treated with 5 μg RNase A for 30 minutes at 37°C followed by extraction with phenol/chloroform. The cDNA was precipitated with 0.7 volume of iso-propanol, which removed ribonucleotide monomers and oligomers digested by the RNase A.

The cloning procedures was followed precisely as described by the supplier. The cDNA was first methylated with *Eco* RI methylase then ligated to *Eco* RI linker. Cohesive *Eco* RI ends were regenerated after *Eco* RI digestion and the free linkers were separated from the cDNA by a Sephadex mini column. The first radioactive peak was collected and the cDNA recovered by iso-propanol precipitation. The cDNA was resuspended in 6 μl TE buffer. λgt10 arms (0.5 μg) was ligated overnight at 15°C to linkered cDNA (40 or 80 ng) in a final volume of 5 μl. The ligation mix was precipitated with ethanol and packaged as described by the supplier. The packaging mixes were diluted to 500 μl with SM buffer and 10 μl chloroform was added to prevent contaminating bacteria from growing. A 2 μl aliquot was diluted serially from 10^{-2} to 10^{-5} fold and 100 μl of which were plated on L87 (wildtype) or NM514 (*hfl* strain that discriminates recombinant against parental phage) in Petri dishes (ø 90 mm). After overnight incubation in 37°C, pfu (plaque forming unit) was counted for each plate (as only very small number of turbid plaques were apparent, no attempt was made to differentiate them from clear ones). The

library was then plated on NM514 in Petri dishes (ø 90 mm) at a density of 10,000 pfu per plate.

2.8. Construction of an Enriched Genomic Library of Wheat

2.8.1. Preparation of λEMBL4 Arms for Cloning

λEMBL4 DNA (10 μg) was purchased from Promega and double digested with *Eco* RI and *Sal* I. After extraction with phenol/-chloroform/iso-amyl alcohol, the DNA was precipitated with 0.7 volume of iso-propanol twice to removed the resultant *Eco* RI-*Sal* I small fragments from the polycloning sites. The DNA was resuspended in 10 μl 1X TE buffer. The concentration of lambda arms was thus about 0.3 μg/μl.

2.8.2. Preparation of *Eco* RI Fragments of Wheat Enriched for 15 kb Region on Sucrose Gradient

Total DNA of Chinese Spring (250 μg) was restricted with *Eco* RI to completion in a volume of 500 μl and extracted once with phenol/chloroform. A 10 ml 10%-34% sucrose gradient was prepared with a gradient mixer. The restricted DNA was carefully loaded on top of the gradient and centrifuged in a SW40.1 rotor at 20°C, 26,000 rpm for 24 hours. After centrifugation, a 10 gauge syringe needle was punched in the bottom of the centrifuge tube and approximately 500 μl aliquots were collected. A small aliquot of each fraction (10 μl) was assayed by agarose gel electrophoresis and the DNA was recovered from the fractions of interest by ethanol precipitation. The DNA was dissolved in 10 μl

of 1X TE buffer and the concentration was estimated to be 0.75 μg/μl by the ethidium bromide fluorescence method.

2.8.3. Ligation of Inserts to λEMBL4 Arms

λEMBL arms (0.3 μg) and insert DNA (0.25 or 0.5 μg) was ligated overnight in 10 μl with 1 unit T4 DNA ligase at 16°C. The ligated DNA was precipitated with ethanol and resuspended in 3 μl of 1X TE buffer before being packaged with 25 μl packaging extract (Promega) at 22°C for 2 hours. SM buffer (475 μ) and 10 μl chloroform were added. A small aliquot was diluted 100 fold and 10 μl was taken to infect 100 μl plating *E.coli* host (NW2, KW251 or ER1647) and plated in a Petri dish (ø 90 mm). The pfu of each plate was counted after overnight incubation at 37°C.

After the titration, the library was immediately plated on Petri dishes (ø 145 mm) at a density of about 40,000 pfu/plate.

Chapter Three

A General Evaluation of Potential Approaches to the Study of Meiosis in Wheat

3.1. Introduction

Hexaploid wheat completes a meiotic division cycle in about 24 hour at 20°C. Nearly half of meiotic time (10.4 hours) is spent in leptotene, about 3.4 hours in zygotene and 2.2 hour in pachytene. The second meiotic division is so rapid that synchrony is usually lost by tetrad stage. Diploid cereals such as barley and rye require about twice this time (Bennett and Smith, 1973). However, animals and some other plants are slower, taking up to 12 days (Alberts *et al.*, 1989).

The development of meiosis within a spike and within a spikelet is uneven. In euploid wheat, meiosis starts from the upper-middle part of a spike, spreading to each end. However, within a spikelet the bottom floret is the fastest in meiotic development. Nonetheless, up to five of the middle spikelets from the primary shoot or two to three from the secondary or tertiary tillers, develop at a similar rate (personal observation). This is taken as the basis for the determination stage of anthers within a spike.

The biochemical and molecular aspects of meiosis in cereals, including *T. aestivum* has been little studied. However, as has been discussed in Chapter One, a number of meiosis-specific genes from

Saccharomyces cerevisiae and a class of abundant messenger RNA, the Expressed Meiosis Prophase Repeats (EMPRs), have been isolated from *Lilium*. Some information on EMPR genes is available: they are expressed predominantly at zygotene and the putative proteins are found to contain a domain that is homologous to the small heat shock proteins of soybean (Bouchard, 1990). Nevertheless, the function of these genes remains unknown although one may logically associate them with a major task of zygotene, i.e., chromosome pairing. The genes from yeast are better understood. The *SPO11* gene, for example, has been characterised genetically and at molecular level and is known to be required for SC formation and meiotic recombination (Klapholz and Esposito, 1982; Klapholz *et al.*, 1985; Wagstaff *et al.*, 1985; Dresser *et al.*, 1986; Atcheson *et al.*, 1987). The genetic distance between yeast and wheat, however, might be too great to allow direct nucleic acids cross-hybridisation. Nonetheless, since some of the meiosis regulating proteins may be among the best conserved and a wide range of meiosis-specific probes have been isolated from yeast, these are potentially valuable for the study of wheat counterparts.

Another aspect of relevance to meiosis concerns the replication of zgyDNA and PDNA (refer to Chapter One for details). This is a potentially interesting subject and can be investigated with available techniques.

In this chapter, preliminary investigations on the three aspects described above are presented.

3.2. A Preliminary Investigation of EMPR Genes in Cereals

3.2.1. Southern Hybridisation of pZm9 to Wheat, Rye and Barley

A cDNA has been isolated from maize using one of the *Lilium* EMPR clones as a probe (Appels, personal commun.). The maize clone, pZm9, was used to probe wheat, rye and barley DNAs digested with various restriction enzymes. The results are shown in Fig. 3.1.A. Under normal hybridisation conditions, a smear was produced without any distinct bands. A similar result was obtained when the original EMPR cDNA clones from *Lilium* were used as probes (Appels *et al.*, 1982).

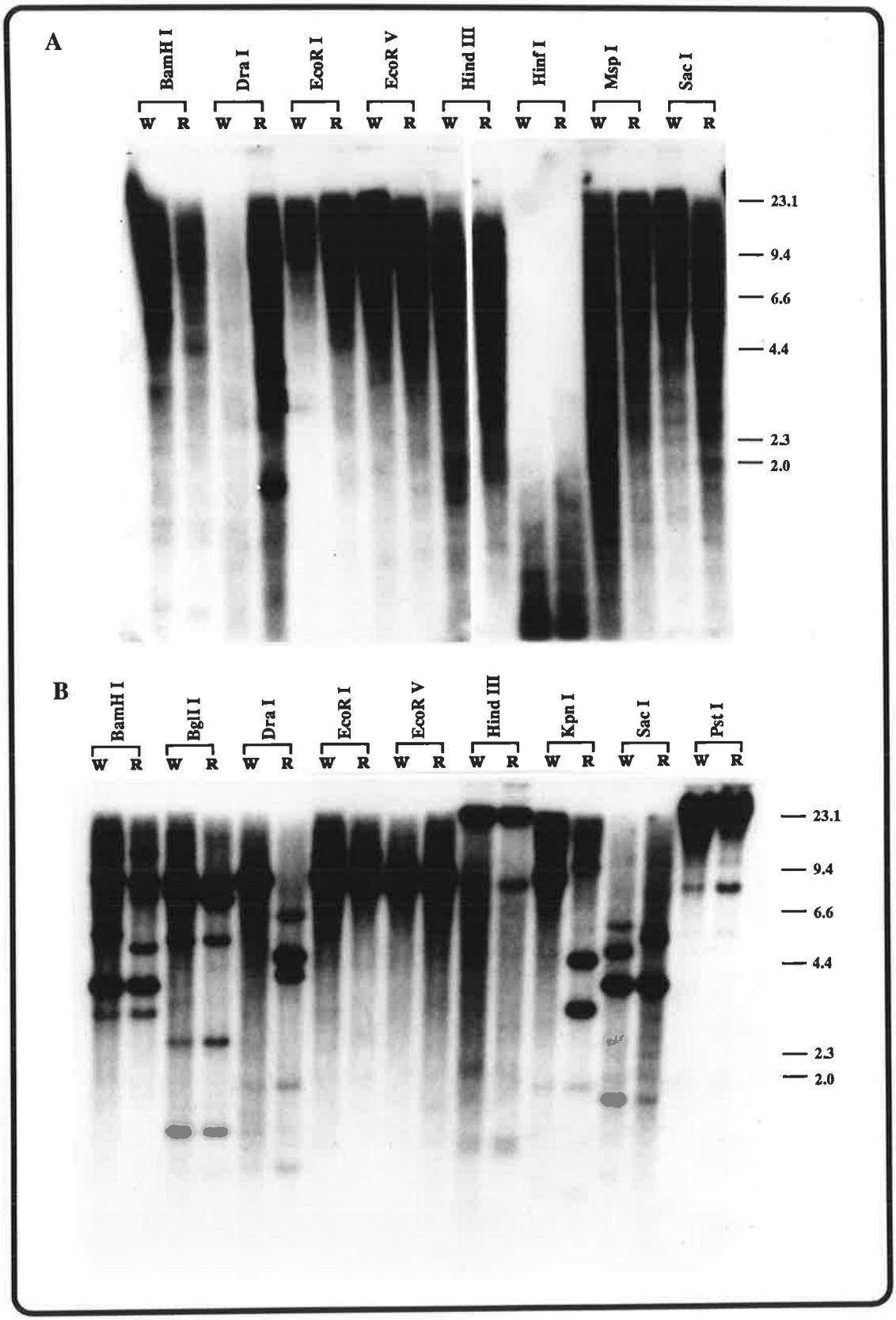
However, if the stringency of hybridisation is reduced, discrete bands are revealed. The autoradiograph shown in Fig. 3.1.B was obtained when the T_m of hybridisation was lowered by 9°C and when the post-hybridisation washing was at lower stringency (1X SSC, 0.1% SDS, at 50°C). Despite the weak smear of background hybridisation, restriction fragment length polymorphisms (RFLPs) are evident between wheat and rye. Shorter exposure time was used for the low stringency hybridisation and washing compared to that done in normal conditions.

Fig. 3.1. Hybridisation of pZm9 to Wheat and Rye

Total DNA of wheat (W) and rye (R) (5 µg) was digested to completion with various restriction enzymes, fractionated on 1% agarose gels, transfered to Zetaprobe membranes and probed with pZm9 radiolabeled by nick translation. The membranes were exposed for 2 and 7 days at -70°C. DNA size marker (λ *Hin*d III) is shown on the left (sizes are given in kbp).

A. Hybridisation was performed overnight in 3X SSPE (0.54 M Na^+), 50% formamide, 1% SDS, 0.5% Blotto, 500 µg/ml salmon sperm DNA at 42°C. The final washing was performed in 0.2X SSC, 0.1% SDS at 60°C.

B. Hybridisation was performed overnight in 4X SSPE (0.76 M Na^+), 40% formamide, 1% SDS, 0.5% Blotto, 500 µg/ml salmon sperm DNA at 42°C. The final washing was performed in 1X SSC, 0.1% SDS at 50°C.


A
BamH I
Dra I
EcoR I
EcoR V
Hind III
Hinf I
Msp I
Sac I
W R W R W R W R W R W R W R W R
23.1
9.4
6.6
4.4
2.3
2.0
B
BamH I
Bgl I
Dra I
EcoR I
EcoR V
Hind III
Kpn I
Sac I
Pst I
W R W R W R W R W R W R W R W R W R
23.1
9.4
6.6
4.4
2.3
2.0

3.2.2. Expression of EMPR Related Genes in Anthers and Somatic Tissues

It is unlikely that all the signal detected by the Southern hybridisation reflects genes that are active during meiosis. Some may be pseudogenes while others are probably genuine small heat shock protein genes. Evaluation of pZm9 as a probe to study wheat meiosis was extended by the analysis of expression of EMPR homologous genes in meiocytes of wheat.

The expression of genes related to pZm9 in wheat meiocytes were studied using RNA isolated from wheat spikelets with anthers at different stages of meiosis. Spikelets containing interphase to leptotene (Early) and zygotene to late pachytene (Late) meiocytes were used in similar amounts to extract RNA which was fractionated on a 1.5% formaldehyde-agarose gel. Fig. 3.2 shows the result obtained when the gel was blotted and probed with pZm9. It indicates that the genes are expressed at low level in the spikelet RNA but not in leaves. The level of expression at zygotene and pachytene was higher than interphase and leptotene. Since whole spikelets were used, it is not possible to relate the signal specifically to meiocytes. Other floral tissues may also be expressing these genes.

pZm9 has clearly been shown to be homologous to some genes expressing in wheat meiocytes. But it is of interest to see if it, as with the *Lilium* counterparts, is also homologous to small heat shock genes in wheat. Seedlings of normal Chinese Spring and plants lacking the *Ph1* gene were heated to 42°C for two hours and the leaves are immediately collected and frozen in liquid nitrogen. Small scale RNA preparations and Northern analysis were conducted and the result is

presented in Fig. 3.3. No difference was seen between the different wheat genotypes, although transcripts of about 1000 bases were induced by the heat shock treatment.

Fig. 3.2. Detection of Homologous Gene Expressed in Anthers of Wheat

A. Small scale RNA prepared by LiCl precipitation method was fractionated on a 1% formadehyde-agarose gel. The four different tissues used are: Early1, spikelets of NT 5B-5D of which most anthers were before leptotene; Early2, spikelets of Chinese Spring of which most anthers were before leptotene; Late, florets of Chinese Spring of which most anthers were between leptotene to metaphase I. Leaves, fresh young leaves of Chinese Spring.

B. The above gel transferred to Zetaprobe membrane and hybridised against pZm9 labeled by nick translation. The membrane was exposed for 10 days at -70°C between intensifying screens.

The positions of the 26S and 18S rRNA are indicated.

A
Early1
Early2
Late
Leaf
B
Early1
Early2
Late
Leaf
26S
18S

Fig. 3.3. Detection of Homologous Gene Expressed in Leaves of Wheat Treated with Heat Shock

1.5-month-old plants of Chinese Spring (CS), NT 5B-5A, NT 5B-5D and Sears *ph* mutant (*ph*)were treated at 42°C for two hours and the leaves were immediately collected and frozen in liquid nitrogen.

A. RNA samples extracted by the small scale LiCl precipitation method fractionated on a 1.2% formaldehyde-agarose gel. Asterisks indicate the heat-shocked samples.

B. An autoradiograph of the above gel probed with pZm9 (10 days exposure).

The positions of the 26S and 18S rRNAs are indicated.

A
CS
CS
CS*
NT 5B-5A
NT 5B-5A*
NT 5B-5D
NT 5B-5D*
ph
ph*
B
CS
CS
CS*
NT 5B-5A
NT 5B-5A*
NT 5B-5D
NT 5B-5D*
ph
ph*
18S
26S

3.3. Investigation of Sequences in Wheat Homologous to the *SPO11* Gene of Yeast

A genomic clone of the *S. cerevisiae SPO11* gene, pGB436, containing the entire coding sequence and a small AT-rich flanking region was obtained from Dr C. N. Giroux (Wayne State University, USA). The genomic organisation of the clone is illustrated in Fig. 3.4.

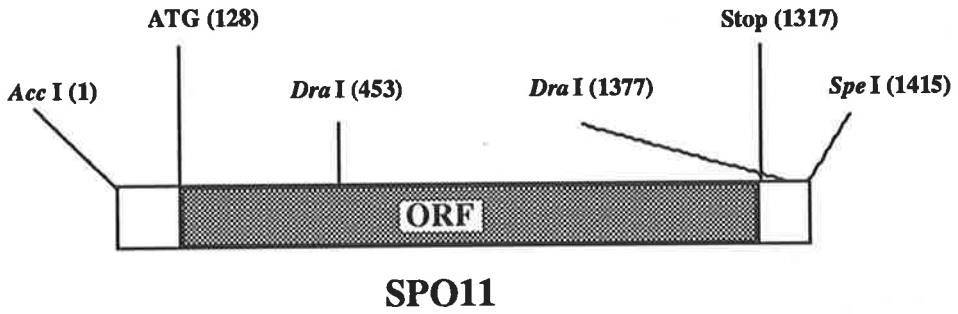


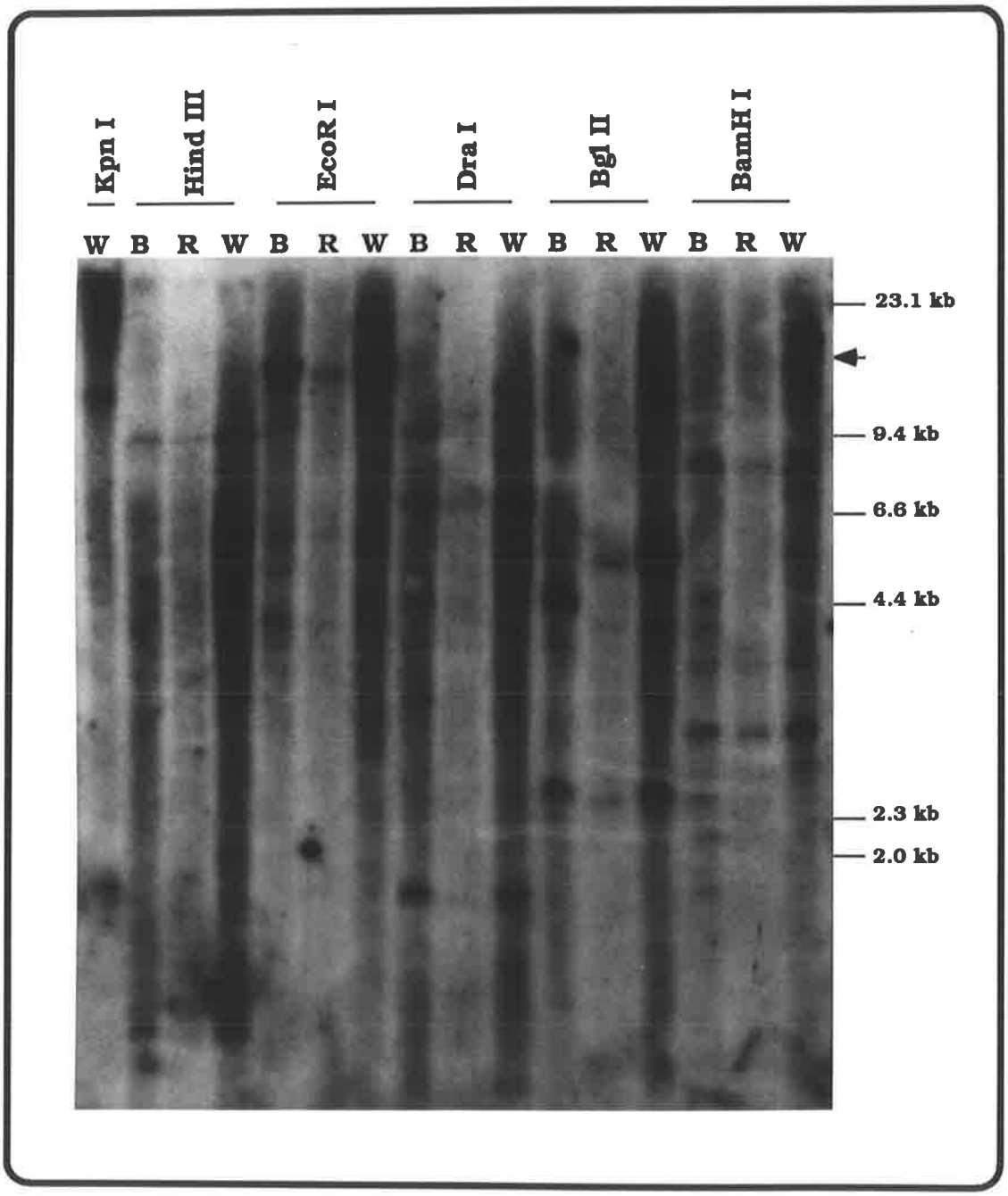
Fig. 3.4. The structure of pGB436 The restriction map of the gene is based on Atcheson *et al.*, 1987.

Cross-hybridisation of the yeast *SPO11* gene to cereal DNAs was tested using a gradient of stringency of hybridisation. This was done by altering the concentration of formamide in the hybridisation mix. The concentration of formamide varied from 25% to 40% at a 5% interval while the rest of the components were kept constant. Hybridisation has been seen when the concentration of formamide had been reduced to 25-30% (Fig. 3.5). In most restriction enzyme digests, one or two bands were revealed. In *Eco* RI digest of either wheat, rye and barley, the bands were about 15 kb. RFLPs between wheat, rye and barley were rare. Only one was seen with six restricton enzymes (between wheat and barley in *Bgl* II digest).

Fig. 3.5. Cross-hybridisation of *S. cerevisiae SPO11* Gene (pGB436) to Wheat, Rye and Barley

Total DNA of wheat (cv. Chinese Spring) (W), rye (cv. Imperial Rye) (R) and barley (cv. Betzes) (B) (5 µg) was digested with *Bam* HI, *Bgl* II, *Dra* I, *Eco* RI, *Hin*d III and *Kpn* I, separated on a 1% agarose gel, transferred to Hybond-N+ membrane and probed with pGB436. The hybridisation was performed in 6X SSPE (1.14 M Na^+), 25% formamide, 1% SDS, 0.5% Blotto, 500 µg/ml salmon sperm DNA at 42°C. The membrane was washed three times in 2X SSC, 0.1% SDS at 42°C and exposed for 14 days at -70°C between intensifying screens. Arrow indicates the 15 kb band in the *Eco* RI digest that was used for cloning (Chapter Five).

Positions of DNA size markers is given on the right.


Kpn I
Hind III
EcoR I
Dra I
Bgl II
BamH I
W B R W B R W B R W B R W B R W
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

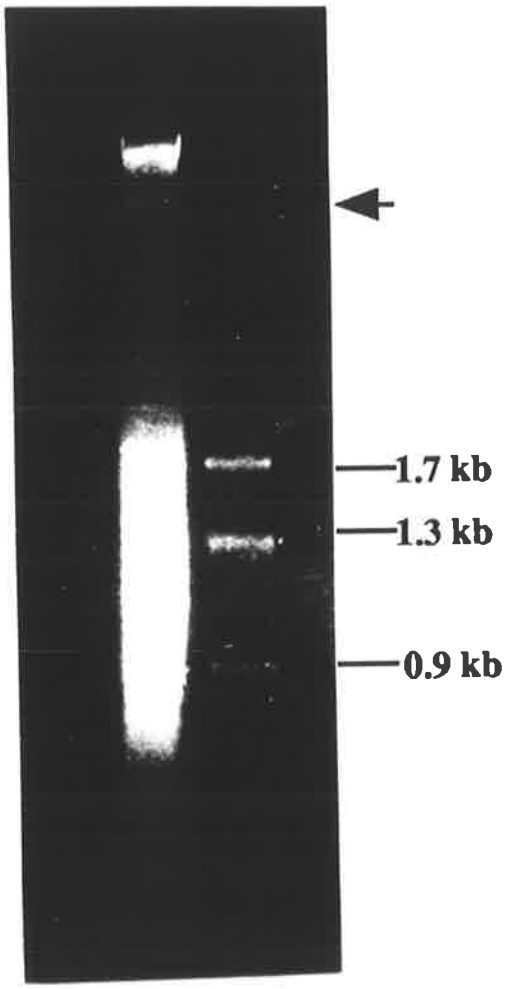
3.4. An investigation into the Occurrence of Delayed Chromatin Replication During Meiosis in Wheat

In *Lilium*, the zygDNA has a higher bouyant density and can be separated from the bulk of chromatin on a CsCl gradient. *T. aestivum* has an average DNA density of 1.702 g/ml (Wells and Ingle, 1978), identical to that of *Lilium longiflorum* (Hotta and Stern, 1971). The method used to isolate the wheat zygDNA was based on the assumption that the zygDNA of *T. aestivum* has a similar density to *L. longiflorum*, that is, 1.712 g/ml (Hotta and Stern, 1971).

With DNA extracted from florets containing meiocytes at early meiotic prophase I, a very faint diffuse band of about 7-8 kb is visible just below a major band using the method described in Chapter Two (Fig. 3.6). The small band could not be isolated when leaves or florets containing post-prophase I meiocytes were used. It was, therefore, postulated that the band is the equivalent of the zygDNA found in *Lilium*. However, extensive further experimental data would be needed to established this.

Fig. 3.6. Isolation of zygDNA from Wheat.

Total DNA and RNA were extracted with vigorous vortexing from florets of wheat (c.a. 500 mg) ranging from leptotene to pachytene. ZygDNA, RNA and any other C+G-rich DNA were pelleted through a CsCl density cushion of 1.712 g/ml by centrifugation. The pellet was dissolved in TE buffer, extracted with phenol/-chloroform and precipitated with ethanol. The sample was resuspended in 40 µl TE buffer overnight at 4°C and resolved on a 1.5% agarose gel. The separate DNA band (c.a. 7-8 kb) is indicated by arrow. The sizes of marker DNAs are given on the right.


1.7 kb
1.3 kb
0.9 kb

3.5. Discussion

3.5.1. The pZm9 Homologous Sequences in Wheat

Data obtained with the pZm9 resembles those obtained with the genuine EMPR clones of *Lilium* in three aspects: the clone represents a member of a large family; it is expressed exclusively during meiotic prophase I and it shares homology to the small heat shock genes of soybean (Bouchard, 1990). The clear bands on Southern blots obtained by hybridisation at reduced stringency, are likely to represent genes that are more distantly related to the probe and the visualisation of the bands above the smeared hybridisation background suggests that the observed bands will have a high copy number and are likely to be a sub-family of the EMPRs. The probe may be useful for the isolation of these genes. However, the analysis of the isolated clones is likely to be difficult due to the complexity of the gene family.

3.5.2. The *SPO11* Homologous Sequences in Wheat

It has been clearly shown by Southern hybridisation that cereal genomes contain DNA sequences that are homologous to the *SPO11* gene of budding yeast (*S. cerevisiae*). It was noticed that the probe used contained about 100 bp AT-rich non-coding sequence at both termini of the gene causing concern that the hybridisation signal might result from these sequences. However, when the 900 bp *Dra* I subfragment that contains little sequence outside the protein coding region was used (refer to Fig. 3.4), an identical result was obtained (data not shown).

Furthermore, the autoradiograph shown in Fig. 3.5 indicates that the homologous sequences in wheat, rye and barley are likely to be present in low copy number as the signal was very weak and in digestion with many restriction enzymes, only one or two bands were seen. In addition, all the bands revealed were indistinguishable in size between wheat, rye and barley (except *Bgl* II digest). This is similar to the result obtained with mammalian DNAs (Giroux, unpublished results). These results suggested that the signal detected with pGB436 may represent a true *SPO11* homologous sequence and the probe may be useful in the isolation of cereal counterparts to *SPO11* gene using the established conditions of hybridisation.

3.5.3. The Delayed Replicating Chromatin in Wheat Meiocytes

The demonstration of a special class of DNA extractable from tissues containing meiocytes extended the findings in *Lilium*. The faint DNA band at 7-8 kb may well be the wheat analogue of the *Lilium* zygDNA although the possibility that it represents PDNA, another class of DNA undergoing intense repair type replication in *Lilium* (Hotta and Stern, 1971; 1984), or some other type of sequences can not be excluded in this experiment. However, this is unlikely because the *Lilium* PDNA is only 0.8-3 kb long. On the other hand, the size of the wheat DNA is close to the zygDNA of *Lilium*, i.e., 3-8 kb (depending on the conditions for DNA extraction) (Hotta *et al.*, 1984; Hotta and Stern, 1984; Stern and Hotta, 1984). The faint band is not ribosomal DNA as it did not hybridise to the maize rDNA probe, pMr1 (data not shown). The major band above the putative zygDNA is likely to represent other C+G-rich DNA.

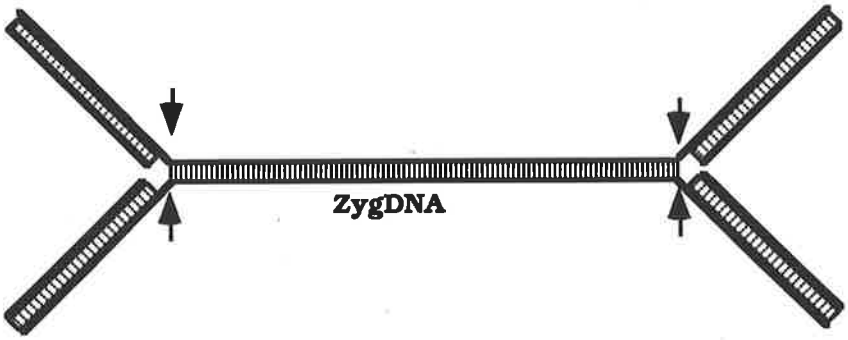


Fig.3.7. The structure of chromatin around zygDNA Arrows indicate the fragile single-stranded gaps at the junctions between replicated and non-replicated chromatin.

The isolation of a DNA band of putative zygDNA at the heavy end of the density gradient and its considerable size uniformity suggest that the wheat zygDNA, like its *Lilium* counterpart, is C+G-rich and is flanked by nicks or breaks. However, the nature of the breaks and the mechanism of breakage is unclear. The breaks may be a property of the DNA itself, alternatively, they could be generated artificially. In *Lilium*, the replication of zygDNA is inhibited before zygotene. The situation in wheat is possibly similar. At the junction between replicated and non-replicated chromatin, there must be a region that is single-stranded. This region will be very fragile and is vulnerable to breakage by shearing during the process of DNA extraction (see Fig. 3.7).

The demonstration of the presence of zygDNA in wheat male meiocytes matched the situation in *Lilium* and mouse. This reinforced Hotta and Stern's suggestion that this class of DNA sequences may be widespread in eukaryotes and may play an important role during meiosis (Hotta *et al.*, 1985 b). Interestingly, high C+G content seems to be a general property of the zygDNAs as it could be isolated by the

same principle as the *Lilium* and mouse sequences. This leads to a question: Is the high C+G content a functional requirement of the sequences? Hotta and Stern (1984) proposed that zygDNA may represent the DNA sequences that directly participate in the chromosome pairing process. If this is the case, the richness of C+G nucleotides may be able to form a special structure that facilitates chromosome recognition and pairing.

The function of the zygDNA remains a mystery but the ability to isolate this class of DNA has permitted an approach to the analysis of these sequences by cloning and *in vitro* manipulation.

3.6. Conclusion

The series of experiments described in this chapter were designed to allow an assessment of possible entry points to isolate sequences involved in the early stages of meiosis in wheat. Three techniques were employed; the use of lily derived clones for the EMPR sequences, the use of a meiosis gene isolated from yeast and the physical fractionation of meiotic DNA to isolate zygDNA sequences. While all three approaches appear to have been successful, only the first two have been pursued further. The remaining chapters described the analysis of wheat EMPR and *SPO11* related sequences.

Chapter Four

Isolation and Characterisation of Meiosis-specific Genes in *T. aestivum*

4.1. Introduction

Two groups of genes in wheat are homologous to pZm9. One group is expressed in meiocytes while the other in heat shocked tissue. The results seem to have provided indirect evidence for the existence of meiosis-specific genes related to the small heat shock genes in wheat. The EMPR genes in *Lilium* remain poorly characterised especially in the region outside the heat shock related domain and the functions of these genes is unknown (Bouchard, 1990). It is possible that the analysis of the variable regions will reveal important information on the function of a particular gene.

Bouchard (1990) has shown that EMPRs are expressed predominantly during zygotene, a stage when the cell machinery is busy organising homologous chromosome pairing. This suggests a possibility that these genes may play a role in chromosome pairing. Wheat is a plant in which chromosome pairing genes have been intensively studied. Also, chromosome assignment of a cloned gene is straight forward since a wide range of alien chromosome addition lines and aneuploids of wheat are available.

The aim to obtain meiosis-specific cDNA clones can be achieved by differential screening, or direct screening of a cDNA library with a cloned meiosis-specific probe, such as pZm9. The latter approach was chosen because the former will require large amount of mRNA as a probe. This is apparently impractical in wheat.

4.2. The Development of an Efficient and Simple Method for cDNA Cloning from Small Quantity of Tissue

4.2.1. Principle of the Method

The standard methods for cDNA cloning used AMV reverse transcriptase. This requires the purification of poly(A)$^+$ RNA since rRNA and tRNA will interfere with cDNA synthesis (Gerard, 1987). However, the wheat anther is so small at meiosis that it is extremely difficult to isolate sufficient poly(A)$^+$ RNA for purification through an oligo(dT)-cellulose column. A further difficulty arises from the lack of synchrony in anther development both between spikelets and within a spikelet. For cDNA library construction, uniformity in materials can reduce the number of clones needed to be screened and, hence, is an advantage. On the other hand, over-emphasis in uniformity would add further problems to RNA preparation. A compromise must be made between the specifity and the size of the library. Since the targets are expected to be relatively abundant, effort was biased towards reducing labour and risk.

The limitation of material for cDNA library construction prompted modifications to the traditional method of cDNA synthesis. Although PCR-aided cloning, including cDNA library construction, is now widely-used for microcloning, PCR techniques were not fully developed at the time this project was in progress. Cloned moloney murine leukemia virus (M-MLV) reverse transcriptase was found to be much less sensitive to rRNAs and tRNAs than AMV reverse transcriptase (Gerad, 1987). In addition, a one-tube double-stranded cDNA synthesis method had been described for this enzyme (D'Alessio *et al.*, 1987). This indicated that cDNA synthesis may be possible without purification of poly(A)$^+$ mRNA. Total RNA was isolated from selected florets at or near meiosis. cDNA was synthesized directly from unpurified mRNA using oligo(dT)$_{8\text{-}12}$ as primers and cloned into the highly efficient λgt10 cloning system from Amersham.

4.2.2. Construction and Analysis of the cDNA Library

Starting from about 100 mg florets, 165 µg total RNA was isolated. The efficiency of the cDNA synthesis was monitored through the incorporation of ^{32}P-dCTP. It was estimated that 133 ng cDNA was synthesised by the M-MLV reverse transcriptase (about 8.1% of the poly(A)$^+$ RNA converted to cDNA) and 256 ng double-stranded cDNA was produced (96.2% of the cDNA converted into double-stranded DNA). The double-stranded cDNA was methylated with *Eco* RI methylase and then cloned into λgt10 after ligation with *Eco* RI linkers and *Eco* RI digestion.

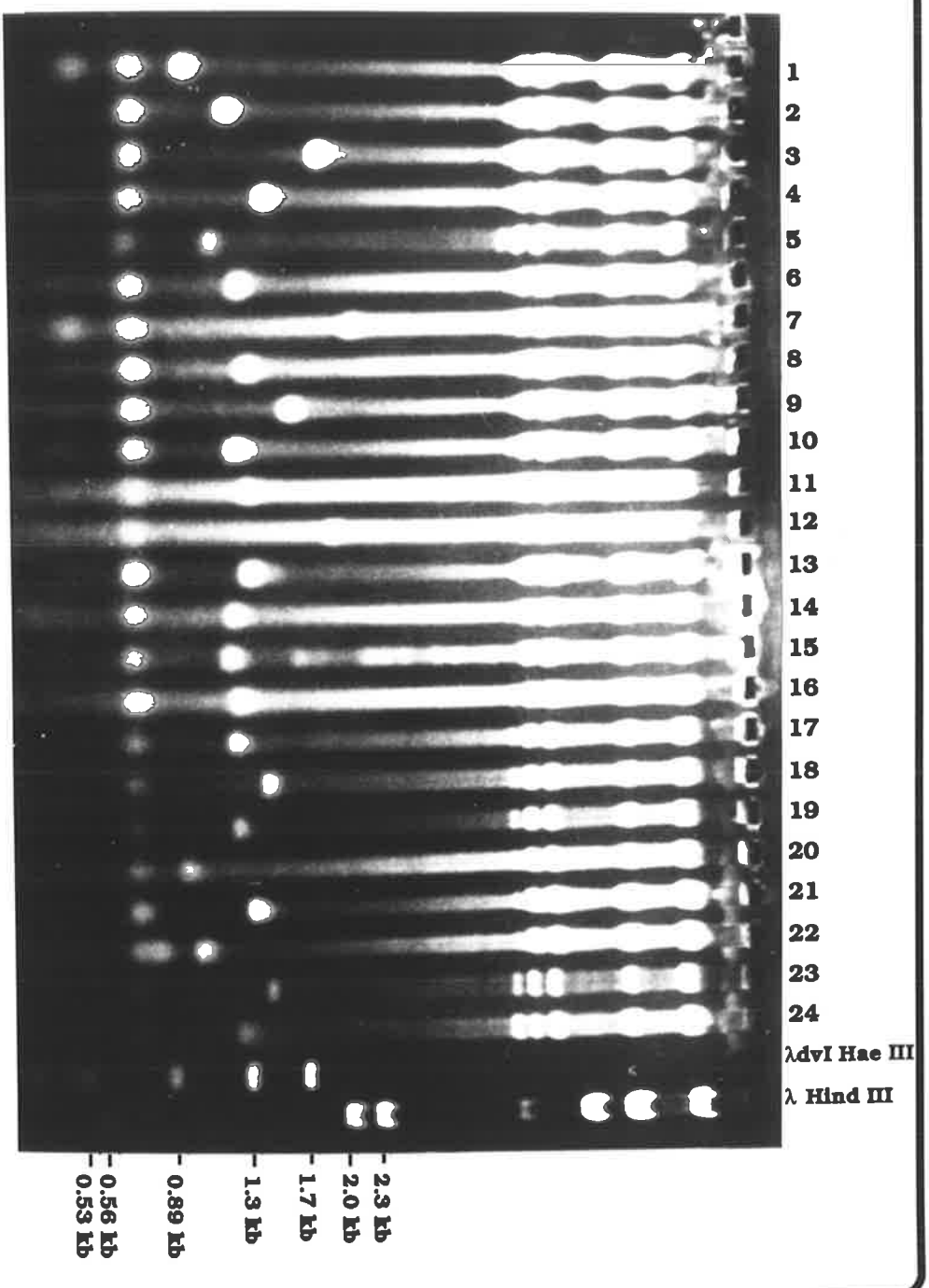
Table 4.1. Cloning of cDNA into λgt10

Insert (ng)	*E.coli* Strain and Dilution			L87/NM514 Ratio	Yield (NM514)
	L87 (pfu) 10^{-3}	NM514 (pfu) 10^{-3}	L87 (puf) 10^{-5}		
0		53	88	167	2.63×10^7
50 (Control)	1218	523		2.23	1.57×10^5
40	223	141		1.58	4.23×10^4
80	378	338		1.12	1.01×10^5

As can be seen from Table 4.1, both the ligation and packaging reactions (1.41×10^8 in the control lambda DNA) were successful. The pfu ratio between wildtype strain (L87) and high frequency lysogeny strain (NM514) in the null insert control was 167, i.e., only one in 167 phages would form a plaque if it did not contain an insert, indicating that the plating cells (NM514) were competent in the selection against parental phage. In addition, the L87/NM514 ratios of pfu in the cDNA ligations were close to that of the ligations with control insert. All these parameters are indications of successful library construction. This was confirmed by direct analysis of the clones. Among 24 randomly selected plaques, 83% were found to contain inserts in the size range between 50 and 1300 base pairs (Fig. 4.1).

Fig. 4.1. Cloning of Wheat Floret cDNA from Unpurified Total RNA

cDNA synthesised from unpuriifed total RNA of wheat florets was cloned after the addition of *Eco* RI linker and ligation into λgt10. Clones established on *E.coli* strain NM514 were chosen randomly and small scale DNA was prepared by the DEAE-cellulose method, digested with *Bgl* II and *Hin*d III and electrophoresed on a 1% agarose gel. The size markers are indicated on the right.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
λdvI Hae III
λ Hind III
0.53 kb
0.56 kb
0.89 kb
1.3 kb
1.7 kb
2.0 kb
2.3 kb

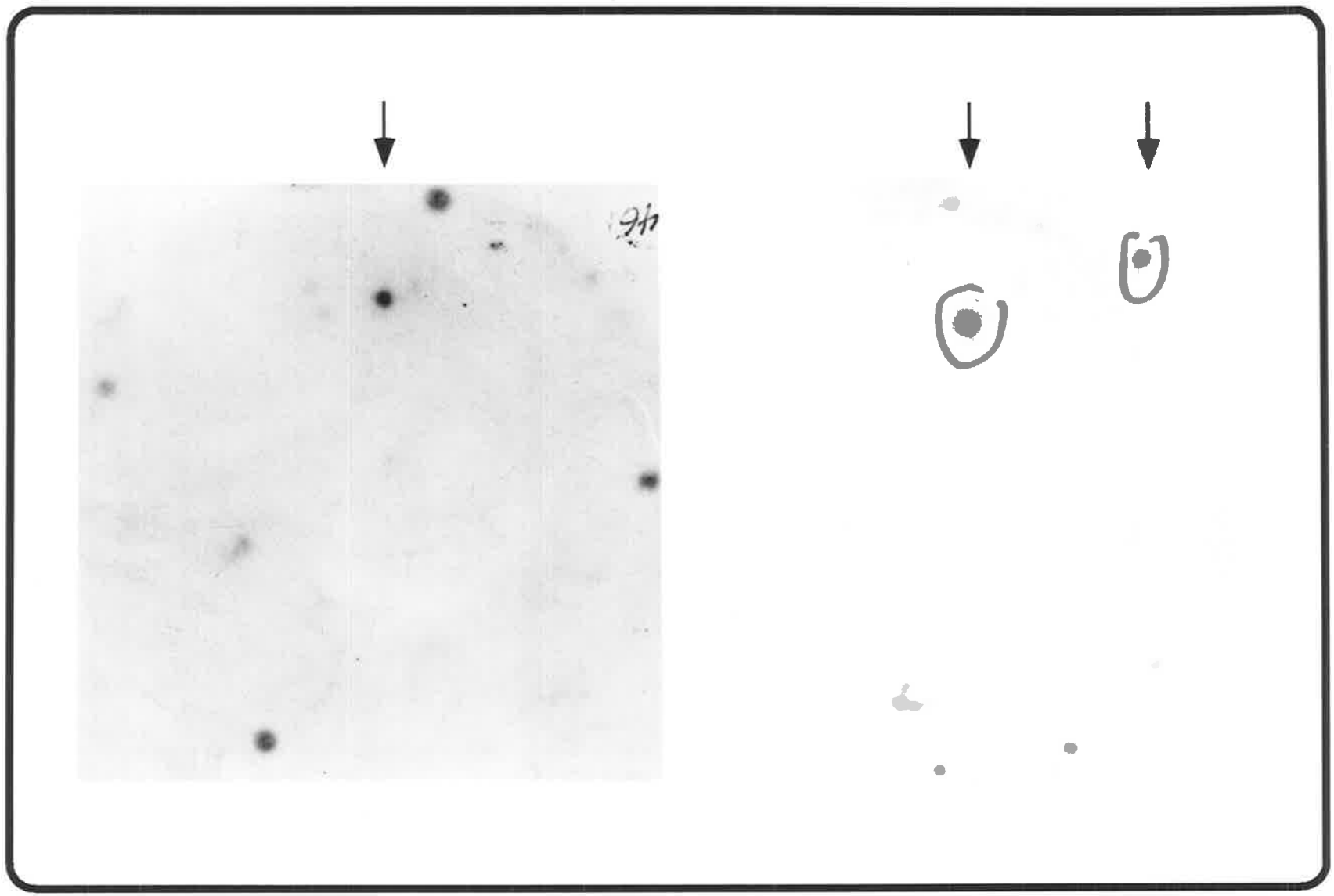
The library was immediately plated onto 14 Petri dishes (ø 90 mm) at a density of about 10,000 pfu per plate. Half of the plates were eluted in SM buffer and stored over chloroform at 4°C as an amplified library stock and the remainder was used directly for screening.

4.3. Identification of cDNA Clones Homologous to pZm9

The library was screened with pZm9 and three positive plaques were identified (Fig. 4.2). These were designated λAWJL1, λAWJL2 and λAWJL3. The clones were purified by two rounds of plating and screening and confirmed to contain pZm9 homologous sequences by Southern blot analysis of the lambda DNA. Both λAWJL1 and λAWJL2 failed to release cDNA inserts after *Eco* RI digestion but double digestion with *Eco* RI and *Hind* III exercised inserts of the same size from each clone. Since these phage were derived from the same amplified stock, it was assumed that they stemmed from a common parental clone. λAWJL2, therefore, has not been analysed further. λAWJL1 and λAWJL3 were re-cloned into pTZ18R for further analysis. The two plasmids were called pAWJL1 and pAWJL3, respectively.

Fig. 4.2. Identification of Three cDNA Clones Homologous to pZm9

Plaques of the wheat floret cDNA library were transferred to Zetaprobe membrane, fixed by UV irradiation and hybridised against pZm9 in a mix containing 4X SSPE, 40% formamide, 1% SDS, 0.5% Blotto, 500 μg/ml salmon sperm DNA and 1 ng/ml radiolabeled probe (c.a. $7X10^8$ cpm/μg) at 40°C. The final wash was done in 2X SSC, 0.1% SDS at 50°C. The membranes were exposed overnight at -70°C between intensifying screenings. Arrows indicate the three positive clones.



No cross-hybridisation was detected between pAWJL1 and pAWJL3 even at reduced stringency of hybridisation. When the cDNA inserts were isolated and used as probes for Southern blot analysis against wheat genomic DNA, discrete bands were seen for both clones with negligible smearing in the background (Fig. 4.3 and Fig. 4.4). The pattern of banding was very similar between pZm9 and pAWJL1. However, unlike pZm9, pAWJL1 revealed bands of similar intensity. pAWJL3 produced a more complex pattern. Nonetheless, the bands revealed by hybridisation to pAWJL1, are amongst those seen when pAWJL3 was used as the probe. It can be seen from Fig. 4.3 and Fig. 4.4 that RFLPs are evident between wheat and rye or wheat and barley. This allows the identification of the chromosomal locations on which the cDNAs reside.

Fig. 4.3. Hybridisation of pAWJL1 to Wheat and Rye

Total DNA of wheat (cv. Chinese Spring) and rye (cv. Imperial Rye) (5 μg) was digested with nine restriction enzymes, separated on a 1% agarose gel, transferred to Zetaprobe membrane and probed with pAWJL1 labelled by random priming. The membrane was exposed for one week at -70°C between intensifying screens. W and R denote wheat and rye, respetively.

The positions of a DNA size marker (λ *Hin*d III) are given on the left.

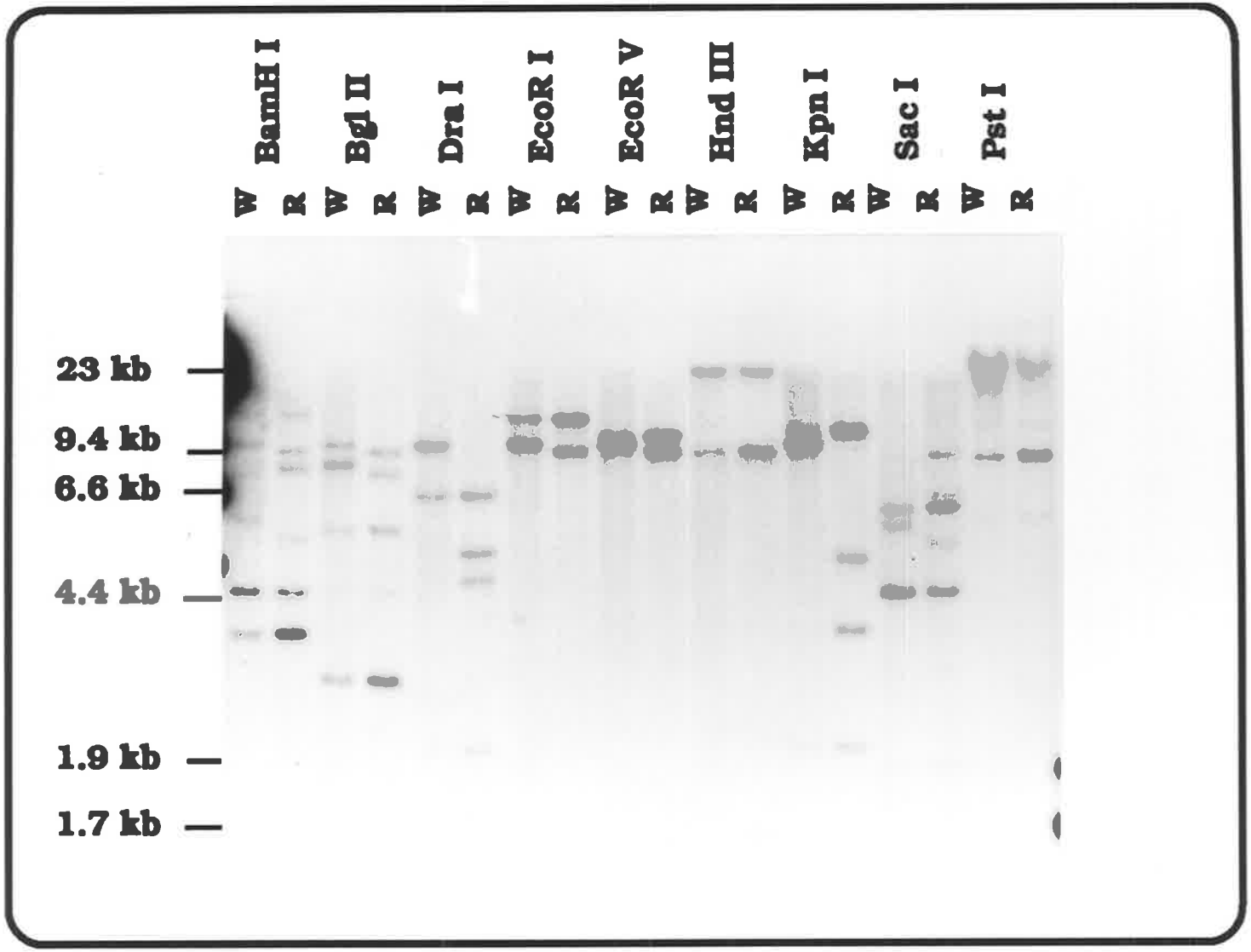
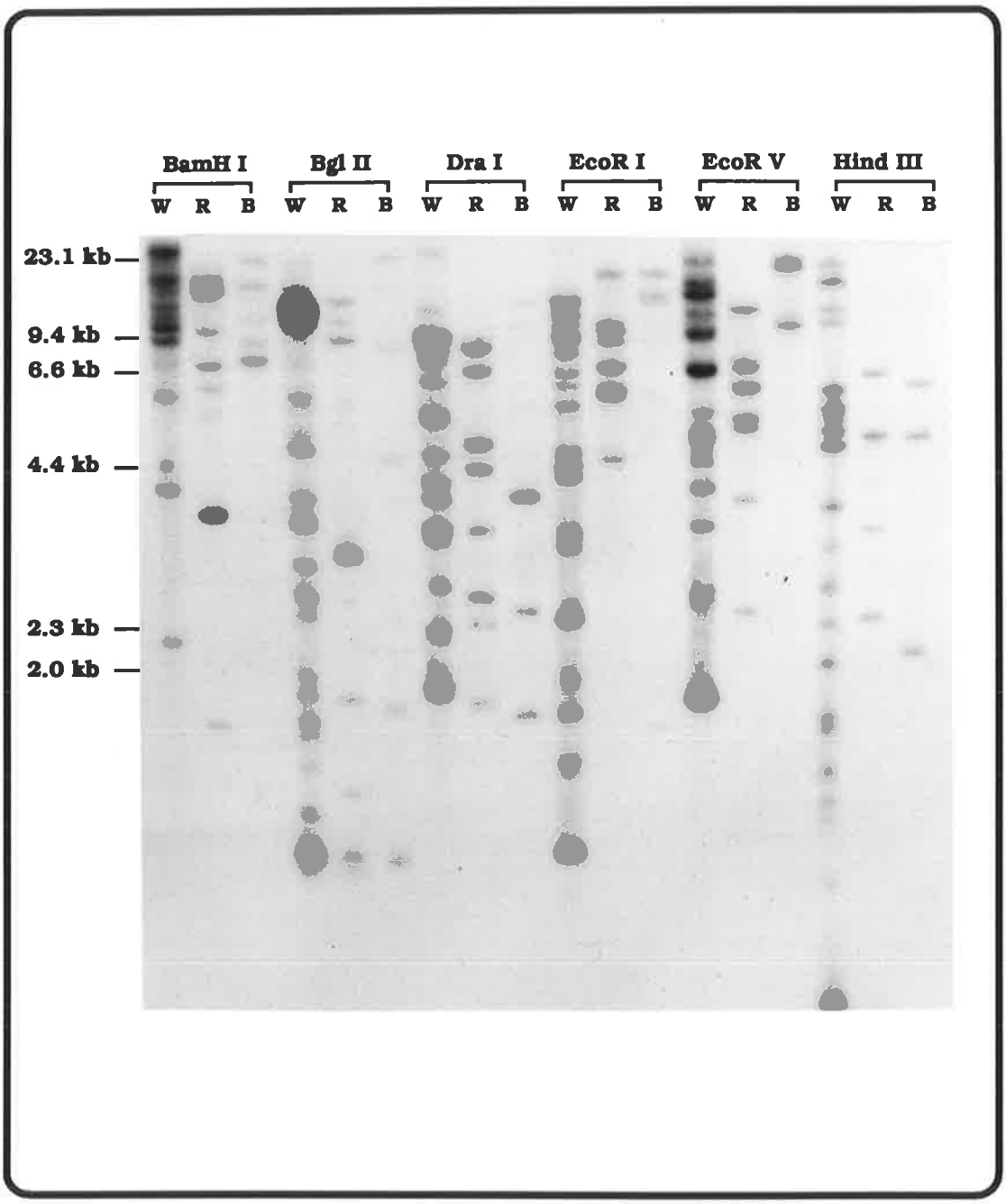

BamH I
Bgl II
Dra I
EcoR I
EcoR V
Hnd III
Kpn I
Sac I
Pst I
W R W R W R W R W R W R W R W R W R
23 kb
9.4 kb
6.6 kb
4.4 kb
1.9 kb
1.7 kb

Fig. 4.4. Hybridisation of pAWJL3 to Wheat, Rye and Barley.

Total DNA of wheat (W), rye (R) and barley (B) was restricted with *Bam* HI, *Bgl* II, *Dra* I, *Eco* RI, *Eco* RV and *Hin*d III, transferred to Hybond-N+ membrane and probed with pAWJL3 I radiolabelled by random priming. The membrane was exposed for one week at -70°C between two intensifying screens.

The positions of DNA size markers is given on the left.


BamH I
Bgl II
Dra I
EcoR I
EcoR V
Hind III
W R B W R B W R B W R B W R B W R B
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

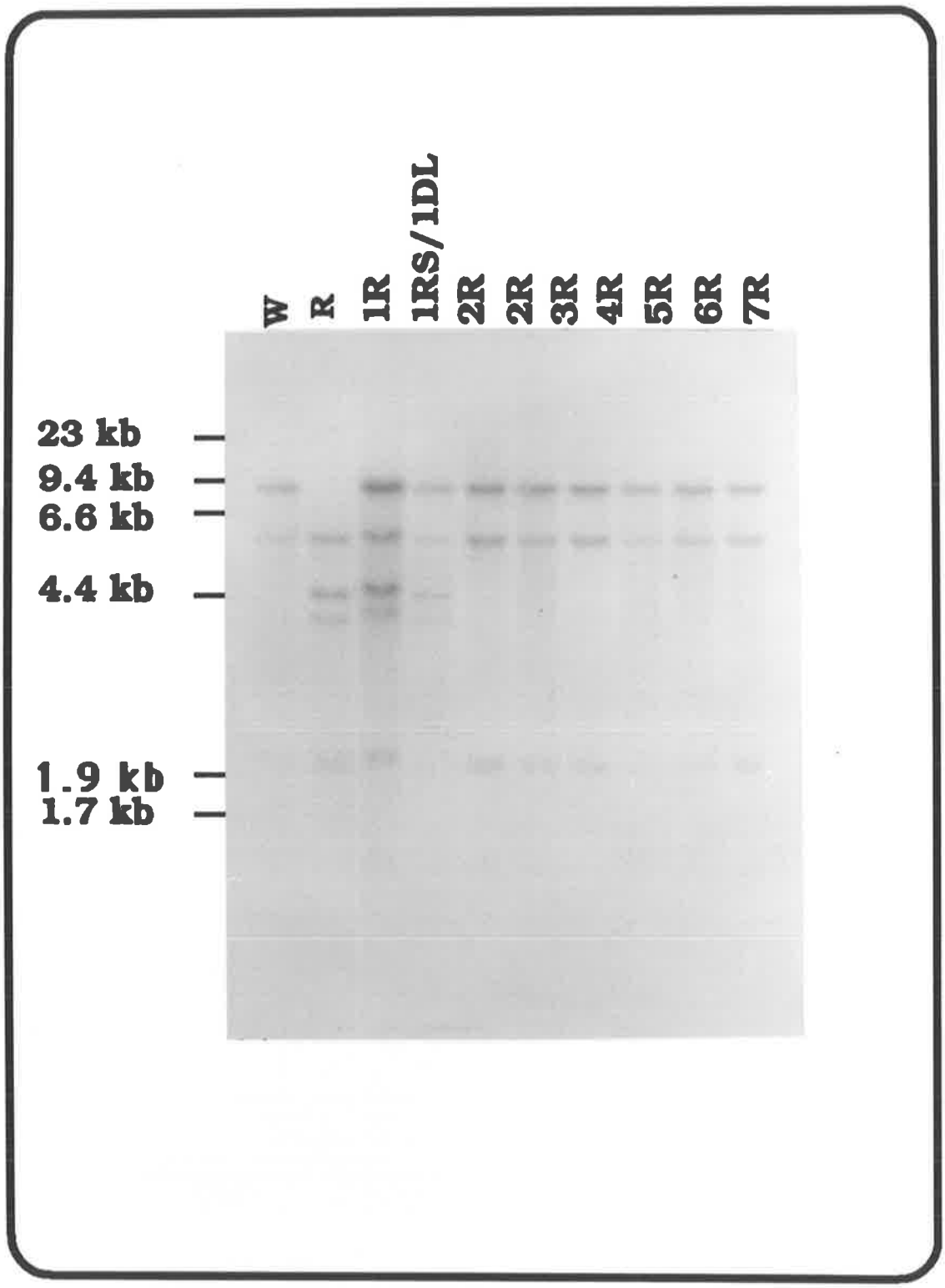
4.4. Chromosomal Assignment of the Genes Corresponding to the cDNA Clones

4.4.1. Mapping of pAWJL1

It has been shown that pAWJL1 revealed distinct RFLPs between wheat and rye in the *Dra* I and Kpn I digests. When the rye addition lines were digested with these two enzymes and probed with pAWJL1, the rye bands appear in both wheat-rye amphiploid and 1R addition lines, indicating that one of the loci is on chromosome 1R. The locus was further mapped to 1RS using 1RS-wheat translocation lines (Fig. 4.5). Unfortunately, it is not possible to assign the bands to particular wheat chromosomes because the same banding pattern was seen from all the six nullisomic-tetrasomic lines of group 1 chromosomes (data not shown).

Fig. 4.5. Chromosomal Mapping of pAWJL1.

Total DNA of wheat (cv. Chinese Spring), rye (cv. Imperial Rye), rye addition lines in wheat and the 1RS/1DL translocation line (5 μg) was digested with *Dra* I, separated on 1% agarose gel, transferred to Zetaprobe membrane and probed with pAWJL1 labelled by random priming. The membrane was exposed for one week at -70°C between intensifying screens. W, R and 1R to 7R represent wheat, rye and rye addition lines 1R to 7R, respectively .


W
R
1R
1RS/1DL
2R
2R
3R
4R
5R
6R
7R
23 kb
9.4 kb
6.6 kb
4.4 kb
1.9 kb
1.7 kb

4.4.2. Mapping of pAWJL3

Extensive RFLPs for pAWJL3 were seen between wheat, rye and barley digests probed with pAWJL3. By using rye and barley addition lines in wheat, several alleles or loci can be assigned to chromosomes 3R and 5R or 3H and 5H. An autoradiograph of *Dra* I digests of the barley addition lines and *Eco* RV digests the rye addition lines is shown in Fig. 4.6.

Since the wheat, rye and barley chromosomes are homoeologous (Islam *et al.*, 1981; Miller, 1984), genes located on the addition lines should be located on group 3 and group 5 chromosomes of wheat. This was confirmed using nullisomic-tetrasomic lines for group 3 and group 5 chromosomes. Each group 5 nullisomic-tetrasomic line and nulli 3D-tetra3A missed 3 or 4 bands when the DNA was digested with *Hind* III and probed with pAWJL3 (Fig. 4.7.A). No band could be assigned to 3A in the *Hind* III digest, however, in the *Bgl* II digest, two bands corresponded to 3A (Fig. 4.7.B). Interestingly, no band could be assigned to 3B with the six restriction enzymes tested.

The mapping was extended to chromosome 3 and 5 ditelosomic lines. Again all DNAs were restricted with *Hind* III and probed with the pAWJL3 insert. The result is shown in Fig. 4.8. It can be seen that the bands of each chromosome are missing in the corresponding long arm ditelosomic lines, but present in

the short arm ditelosomic lines. This indicates that both loci are located on the short arms of chromosomes 3 or 5 in wheat. (5BL ditelo was not available in this experiment, however, the normal homoeologous relationship is probably applicable. This needs to be confirmed with further experiment.)

Fig. 4.6. Mapping of pAWJL3 to Rye and Barley Chromosomes

A. Total DNA (7.5 μg) was digested with *Dra* I, separated on a 0.8% agarose gel, transferred to Hybond-N+ membrane and probed with pAWJL3 radiolabelled by random priming. The membrane was exposed at -70°C for one week between two intensifying screens.The symbols shown in the figure are: W, wheat (cv. Chinese Spring); B, barley (cv. Betzes); 1H to 7H, barley chromosome (1H to 7H) addition lines in wheat.

B. Total DNA (7.5 μg) was digested with *Eco* RV and handled as described above. The symbols W+R, 1R to 7R, denote amphiploid of wheat and rye chromosome (1R to 7R) addition lines in wheat.

The chromosomal locations of the barley and rye DNA bands are indicated arrows.

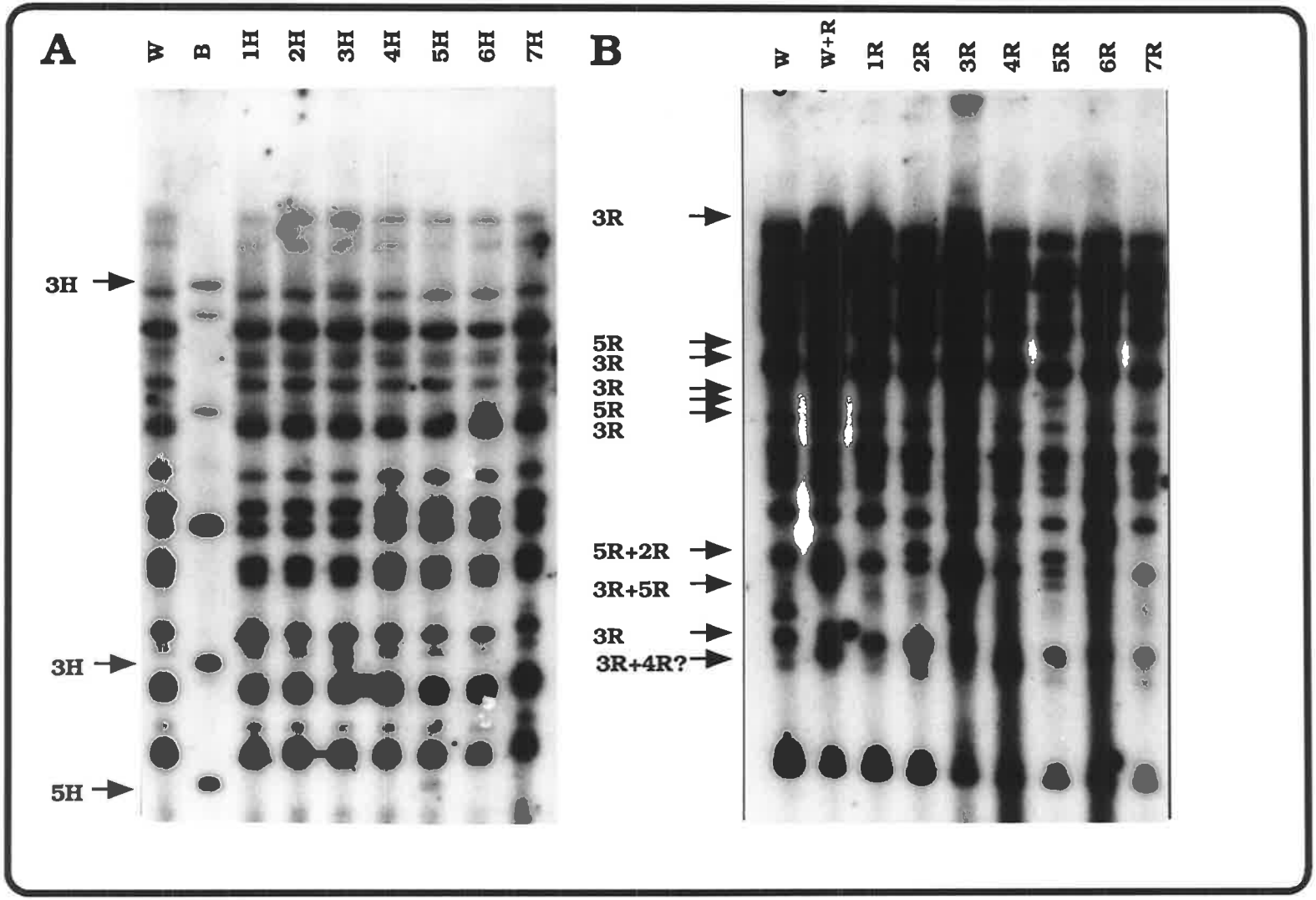

A
W
B
1H
2H
3H
4H
5H
6H
7H
3H
3H
5H
B
W
W+R
1R
2R
3R
4R
5R
6R
7R
3R
5R
3R
3R
5R
3R
5R+2R
3R+5R
3R
3R+4R?

Fig. 4.7. Chromosomal Mapping of pAWJL3 in Wheat

Total DNA (7.5 μg) digested with *Hin*d III (A) or *Bgl* II (B), resolved on 0.9% ararose gels, transferred to Hybond-N+ membrane and probed with pAWJL3 radiolabeled by random priming. The membranes were exposed at -70°C for one week between two intensifying screens. The chromosomal locations of the bands are indicated by arrows. All lines prefixed with NT are nullisomic-tetra-somics and DT are ditelosomics. CS is Chinese Spring (euploid wheat).

A. *Hin*d III digest.

B. *Bgl* II digest.

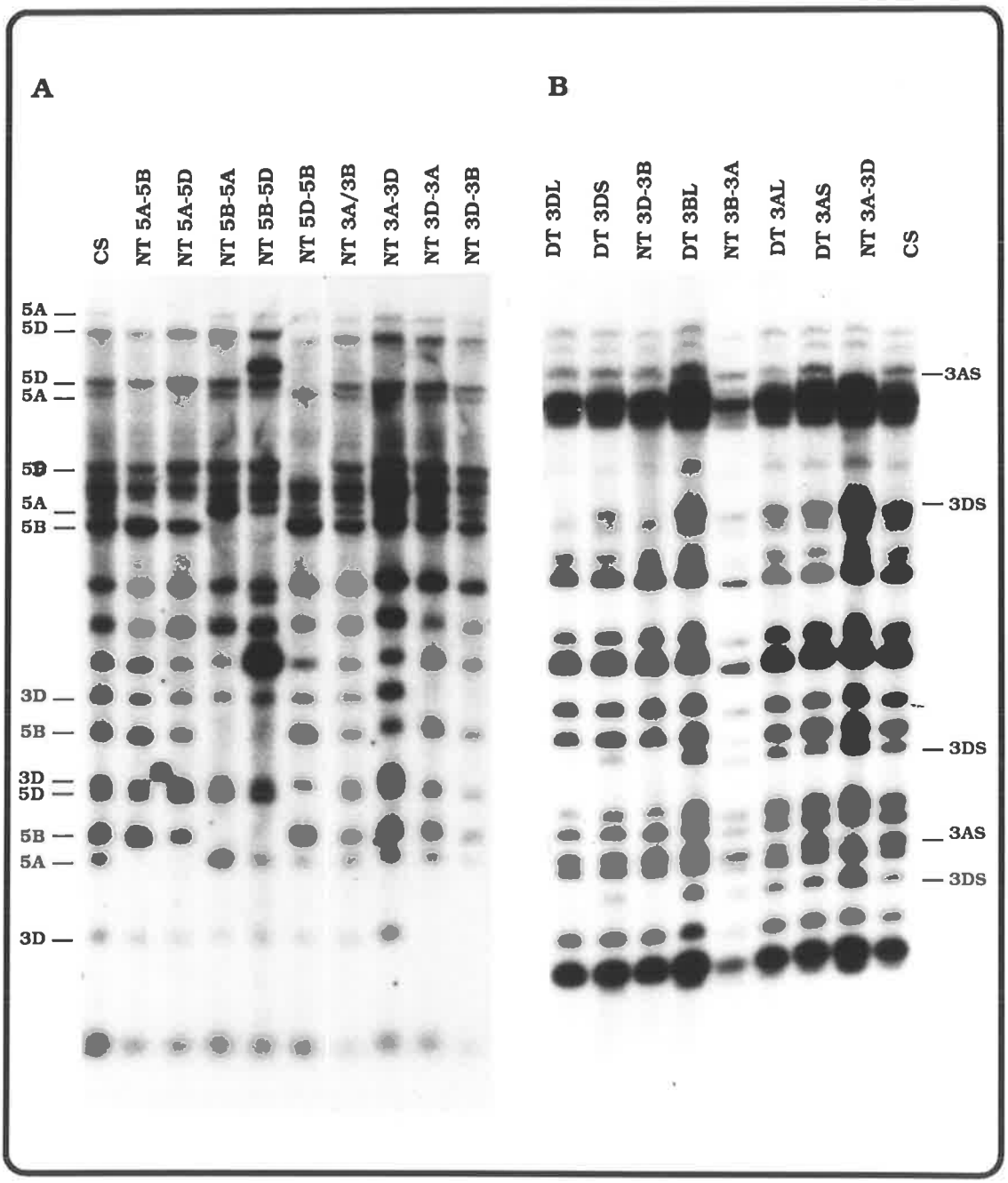
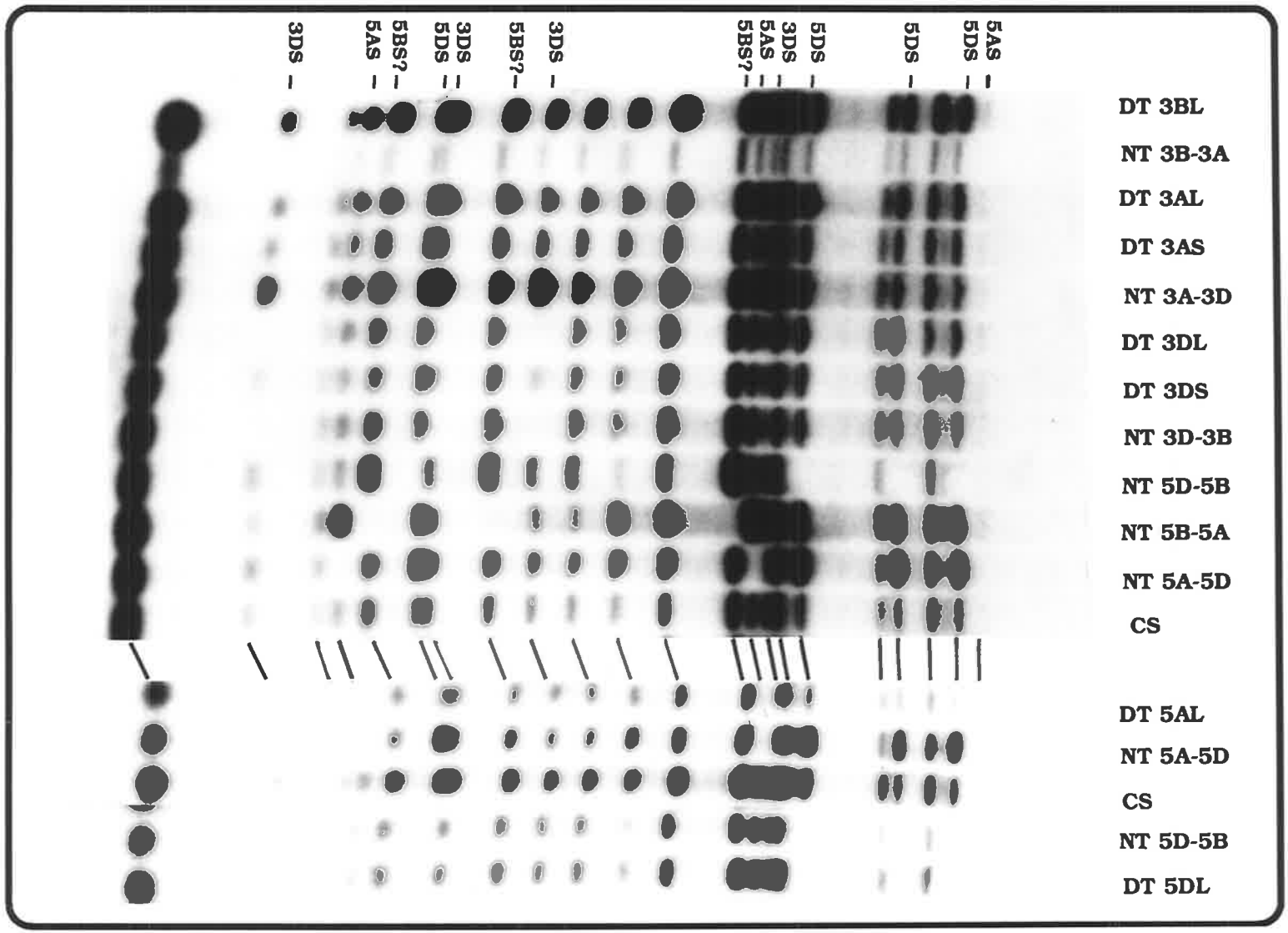

A
CS
NT 5A-5B
NT 5A-5D
NT 5B-5A
NT 5B-5D
NT 5D-5B
NT 3A/3B
NT 3A-3D
NT 3D-3A
NT 3D-3B
5A
5D
5D
5A
5D
5A
5B
3D
5B
3D
5D
5B
5A
3D
B
DT 3DL
DT 3DS
NT 3D-3B
DT 3BL
NT 3B-3A
DT 3AL
DT 3AS
NT 3A-3D
CS
3AS
3DS
3DS
3AS
3DS

Fig. 4.8. Chromosomal Arm Mapping of pAWJL3 in Wheat

Total DNA (7.5 μg) was digested with *Hind* III, resolved on 0.9% agarose gels, transferred to Hybond-N+ membranes and probed with pAWJL3 radiolabeled by random priming. The membranes were exposed at -70°C for one week between intensifying screens. The arm locations of the bands are indicated on the left of the autoradiographs. DT and NT denote ditelosomic and nullisomic-tetra-somic lines, respectively.


3DS
5AS
5BS?
5DS
3DS
5DS
3DS
5BS?
3DS
5DS
3DS
5AS
5BS?
5DS
5DS
5AS
DT 3BL
NT 3B-3A
DT 3AL
DT 3AS
NT 3A-3D
DT 3DL
DT 3DS
NT 3D-3B
NT 5D-5B
NT 5B-5A
NT 5A-5D
CS
DT 5AL
NT 5A-5D
CS
NT 5D-5B
DT 5DL

4.5. Nucleotide Sequence and the Deduced Amino Acid Sequence of pAWJL3

pAWJL3 was chosen for detailed analysis since it is located on the same chromosomes as some of the *Ph* genes. In a restriction analysis of the cDNA, it was found to contain two *Hin*d III sites which cut the insert into three fragments of about 150, 450 and 250 bp. The cDNA was cut with *Hin*d III and the three fragments subcloned into pTZ18U in two different orientations. The sequencing strategy is illustrated in Fig. 4.9.A. Sequencing was performed with Taq DNA polymerase as described in Materials and Methods using double-stranded DNA and α-^{32}P-dATP as label. Most sequence information on both strands was obtained by the above strategy. However, the uncertain region in the middle of 450 bp Hind III fragment, was confirmed with clones generated by nested deletion. The full nucleotide sequence of the cDNA clone pAWJL3 is presented in Fig 4.9.B.

A.

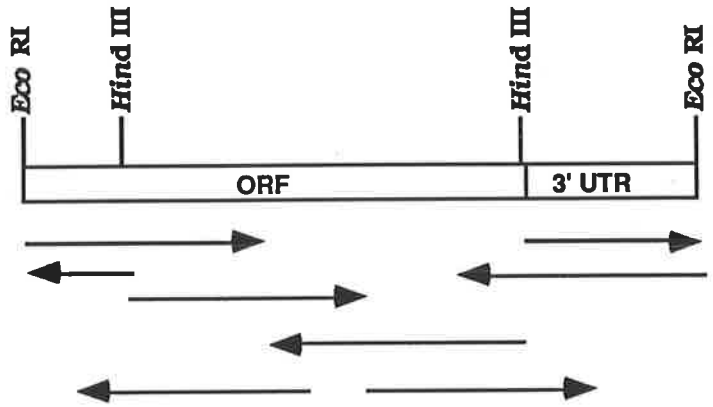


Fig.4.9.A. Sequencing strategy for pAWJL3 Arrows indicate the direction and extent of the sequencing reactions.

B.

```
CCA TCA GAT ATC ACT TCC CTT GAT GCA GTA ATA AGT TTG AAT TTA TCA TCA 51
 Pro Ser Asp Ile Thr Ser Leu Asp Ala Val Ile Ser Leu Asn Leu Ser Ser 17

AAC CAT CTG AGC GGA ATG ATT CCA AAT GAT ATT GGT GCC TTG CAT TCA TTG 102
 Asn His Leu Ser Gly Met Ile Pro Asn Asp Ile Gly Ala Leu His Ser Leu 34

GAA TCT CTT GAC CTC TCC ATG AAC AAG CTT ACT AGT GAA ATC CCA TCG AGT 153
 Glu Ser Leu Asp Leu Ser Met Asn Lys Leu Thr Ser Glu Ile Pro Ser Ser 51

TTA TCA AGT TTG ACA TCC CTG AGT TAC TTG AAC TTG TCC TAC AAC AAT TTA 204
 Leu Ser Ser Leu Thr Ser Leu Ser Tyr Leu Asn Leu Ser Tyr Asn Asn Leu 68

TCT GGA AGG ATA CCC TCA AGC CGC CAA CTT GGC ACC CTC AAT GTA GAC AAC 255
 Ser Gly Arg Ile Pro Ser Ser Arg Gln Leu Gly Thr Leu Asn Val Asp Asn 85

CCA GCA CTT ATG TAC ATT GGC AAC AGT GGA CTT TGT GGG CCT CCT CTC CAG 306
 Pro Ala Leu Met Tyr Ile Gly Asn Ser Gly Leu Cys Gly Pro Pro Leu Gln 102

AAG AAT TGT TCA GGA AAT GAT ACG GGC ACT AGT CAT GTT GGA AGC AAC AAC 357
 Lys Asn Cys Ser Gly Asn Asp Thr Gly Thr Ser His Val Gly Ser Asn Asn 119

CAT GAG GAA TTT GAA CTG ATG ACC TTC AAA TTT GGT CTT GTC CTG GGA CTT 408
 His Glu Glu Phe Glu Leu Met Thr Phe Lys Phe Gly Leu Val Leu Gly Leu 136

GTG GCG GGG CTT TGG AGT GTG TCC TGT GCA CTT TTG TTC AAG AAG GCA TGG 459
 Val Ala Gly Leu Trp Ser Val Ser Cys Ala Leu Leu Phe Lys Lys Ala Trp 153

AGG ACA GCT TAT TTT CAA CTC TTT GAT GAG ATG TAT GAT GCA GAA TCT ATG 510
 Arg Thr Ala Tyr Phe Gln Leu Phe Asp Glu Met Tyr Asp Ala Glu Ser Met 170

TAT ACG TGG TTG TGA AAT GGG CAA GCT TGG TAA GGA AAA CAG ATG AAA ATG 561
 Tyr Thr Trp Leu TER                                                 174

AAC AAG TTG CCC TGA AGG AGC CAA TGT CTG TGC CAC GAC CAA TAG AAT GGG 612

GGC TCT ATA CAC AAC GCG TTG TCT AGC TAG CGT GCC TTG CCT AAT TTT TTA 663

TAT TCT GAA TAA AGA TGG AGA TGT TGA TTA GTC CTT CTT ATA CAT GTA AAA 714

CTT CAG ATG TTA TAA ATC AGA ACT TCA ATG ACC AGT TAT TTT GGT GAA TAA 765

GGA ATT 771
```

Fig. 4.9.B. Nucleotide and deduced amino acid sequences of pAWJL3. A putative polyadenylation site (AATAAA) is underlined and a motif that resembles the upstream regulating sequence of frog maternal mRNA polyadenylation (TTTTTAT) is dotted-underlined.

The nucleotide sequence was searched for potential ORFs (Open Reading Frames) with McMolly-Translate program (Schöneberg and Priedemuth, 1989) and a 152 aa (amino acid) ORF was found. The sequence flanking the first ATG, i.e., GCGGAATGA, however, is very different from the consensus sequence of eukaryotic translation initiation site CC(A/G)CCATGG (Kozak, 1984) and probably does not function as an initiation codon. The 5' sequence can be translated in-frame to add an extra 22 aa. It seems likely that the real initiation methionine is located further upstream and has not been included in this clone. The ORF was translated using universal codons and the deduced protein is shown in Fig. 4.9.B.

4.6. Sequence Analysis of pAWJL3

4.6.1. Sequence Comparison of pAWJL3 to Existing Sequences

The deduced protein sequence of pAWJL3 was compared with known sequences by the Pearson method (Pearson and Lipman, 1988) using the FASTA computer program. No homologous protein was found after searching the latest Swiss-Prot data base (version 17) and Genepep data base (version 64.3). However, the N-terminal 70-90 aa revealed considerable homology to a number of proteins with identities varying from 20% to 40.6%. The results of the search are summarised in Table 4.2. The best match (40.7% identity over 69 aa) is with the adenylate cyclase of fission yeast (*Schizosaccharomyces pombe*). The identical matches are mostly between leucine and asparagine residues.

Comparison of the N-terminus of pAWJL3 protein to the proteins listed in Table 4.2 also shows a high percentage of residues that could represent conservative substitutions (about 40-50%).

Table 4.2. A summary of pAWJL3 protein homology search

Protein	Identity (%)	Similarity (%)	Function+Reference
Adenylate cyclase (*Sch. pombe*)	40.6	75.4	cAMP synthesis, regulation of cell metabolism (Yamawaki-Kataoka *et al.*, 1989)
Adenylate cyclase (*S.cerevisiae*)	30.4	78.2	As above (Kataoka *et al.*, 1985)
Toll (*Drosophila*)	26.2	77.0	Dorsal-ventral development (Hashimota *et al.*, 1988)
Chaoptin (*Drosophila*)	28.6	79.2	Cell-specific photoreceptor, cell morphogenisis (Reinke *et al.*, 1988)
LRG (Human)	29.0	64.0	Unknown function (Takahashi *et al.*, 1985)
GPIbα (Human)	29.9	79.2	Cellular ahension (Lopez *et al.*, 1987)
Carboxypeptidase (Human)	31.4	71.4	Protein hydrolysis at basic amino acid residues (Tan *et al.*, 1990)
RNase inhibitor (Pig)	26.3	65.8	RNase inhibition (Hofsteenge *et al.*, 1988).

Surprisingly, neither the *Lilium* EMPRs nor small heat shock genes appeared in the lists of best match to either protein or nucleotide sequence. When sequences of the *Lilium* EMPR genes and the small heat shock genes of soybean were extracted from the data base and compared to pAWJL3 by PC/Gene "Alignment" program (IntelliGenetics, 1989), no significant homology was found. Nonetheless, the Southern blot result clearly indicated that the clone cross-hybridised to pZm9 at about 20°C above the T_m of the pAWJL3 (data not shown). The low degree of homology of the wheat to the small heat shock genes or *Lilium* EMPR genes may be due to the probe, pZm9, having only intermediate homology to the *Lilium* EMPRs as well as to the pAWJL3.

4.6.2. Primary Structure of the Deduced pAWJL3 Protein

The pAWJL3 protein sequence was analysed with PC/Gene computer program package (IntelliGenetics, 1989), and revealed a number of interesting features. Firstly, the overall protein is very acidic, with an isoelectric point of 4.72, and leucine-rich (17.2%). Secondly, the N-terminus is serine-rich (23.8% in the first 80 aa). And thirdly, the C-terminus is highly hydrophobic. This became apparent when the average hydrophobicity of every 5 residues were plotted (Fig. 4.10): there is a high concentration of alanine, isoleucine, phenylalanine, methionine, proline, valine and trytophane from aa 125 to aa 162, immediately following a hydrophilic stretch of about 10 aa.

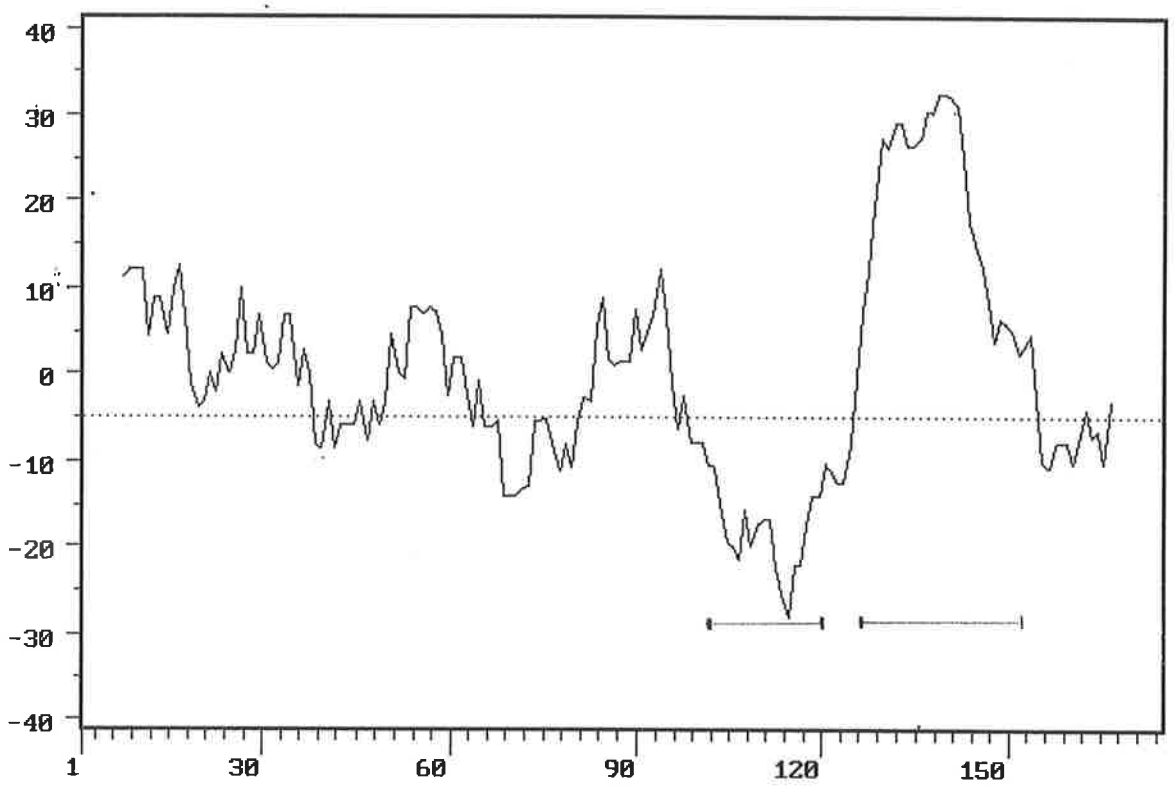
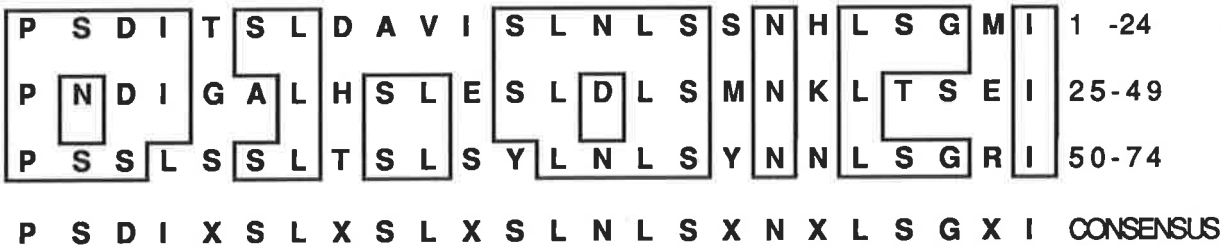


Fig. 4.10. The hydrophobicity plot of the deduced pAWJL3 protein The average hydrophobicity of every 5 aa was calculated and plotted using the PC/Gene computer program. The highly hydrophobic and hydrophilic regions are indicated.

The most interesting feature of the protein sequence is the presence of three 24 aa repeats at the N-terminus (Fig. 4.11.A). The repeats resemble the consensus repeat sequences of a number of proteins involved in a wide range of cellular functions. These include the yeast sds22+ (Ohkura and Yanagida, 1991), yeast

adenylate cyclase (Kataoka *et al.*, 1985; Yamawaki-Kataoka *et al.*, 1989), *Drosophila* chaoptin (Reinke *et al.*, 1988), *Drosophila* Toll protein (Hashimota *et al.*, 1988), human platelet glycoprotein receptor a (GPIbα) (Lipez *et al.*, 1987) and human platelet leucine rich glycoprotein (LRG) (Takahashi *et al.*,1985). The alignment of the sequences is illustrated in Fig. 4.11 (B).

A.



B.

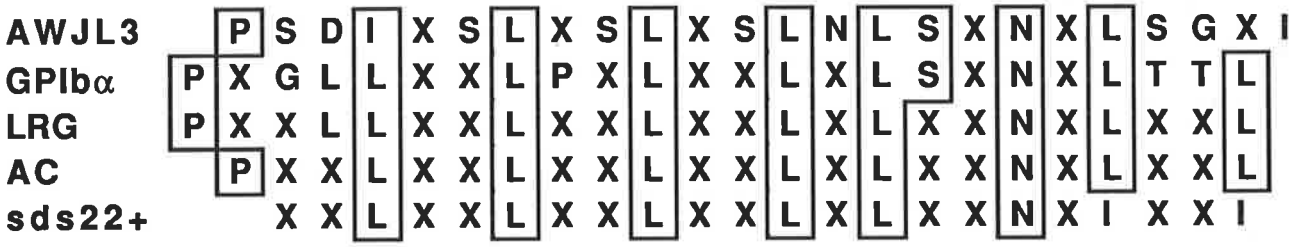


Fig. 4.11. Amino acid sequence organisation at the N-terminus of the putative pAWJL3 protein A. Alignment of the 24 aa repeats. B. Alignment of pAWJL3 24 aa repeat consensus with other leucine-rich repeats. Identical residues are boxed. GPIbα, human platelet glycoprotein receptor α (Lipez *et al.*, 1987); LRG, leucine-rich glycoprotein (Takahashi *et al.*, 1985); AC, *S. cerevisiae* adenylate cyclase (Kataoka *et al.*, 1985); sds22+, *Sch. pombe* suppressor of *dis2*+ (protein phosphatase) gene (Ohkula and Yanagida, 1991).

In addition, the C-terminus hydrophobic stretch contains four leucine heptad repeats (Fig. 4.12.A). This is a structure very similar to leucine-zipper proteins (Landschulz *et al.*, 1988). The alignment of the leucine repeats with those of plant leucine-zippers is presented in Fig. 4.12.B.

A.

1	2	3	4	5	6	7	Position
N	H	E	E	F	E	L	120-126
M	T	F	K	F	G	L	127-133
V							134
L	A	L	V	A	G	L	135-141
W	S	L	S	C	A	L	142-148

Fig. 4.12 A. Alignment of the leucine heptad repeats of pAWJL3 protein. Periodic leucine and conserved residues are boxed.

B.

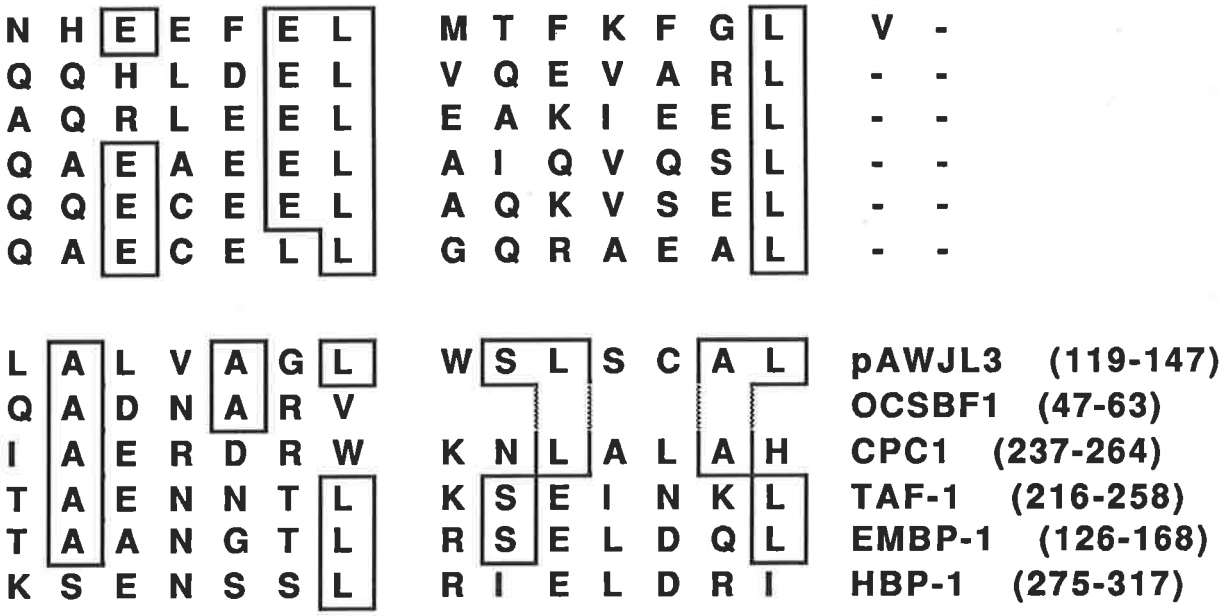


Fig. 4.12 B. pAWJL3 leucine heptad repeats compared with leucine-zipper repeats. OCSBF-1, maize Ocs sequence binding factor (Singh *et al.*, 1990); CPC1, *N. crassa* cross-pathway control gene of amino acids biosynthesis; TAF-1, Tobacco *trans*-acting factor (Oeda *et al.*, 1991); EmBP-1, wheat Em1a binding protein (Guiltinan *et al.*, 1990); HBP-1, wheat histone $H3^a$ gene binding protein (Tabata *et al.*, 1989).

4.6.3. Secondary Structure and Post-translational Modifications of the Deduced pAWJL3 Protein

Using sequence analysis program, an attempt was made to predict the secondary structure of the pAWJL3 protein. Garnier's method (Garnier *et al.*, 1978) suggests that the N-terminal region is extended conformation, which is of dubious significance. However, the region spanning the heptad leucine repeats at the C-terminus would form a continuous helix (Fig. 4.13). This agrees with models of leucine-zipper proteins.

```
                        (P)                                                 Cho
                         |                                                   |
1   Pro-Ser-Asp-Ile-Thr-Ser-Leu-Asp-Ala-Val-Ile-Ser-Leu-Asn-Leu-
                                                                           Myr
                                                                            |
16  Ser-Ser-Asn-His-Leu-Ser-Gly-Met-Ile-Pro-Asn-Asp-Ile-Gly-Ala-

31  Leu-His-Ser-Leu-Glu-Ser-Leu-Asp-Leu-Ser-Met-Asn-Lys-Leu-Thr-

46  Arg-Glu-Ile-Pro-Ser-Ser-Leu-Ser-Ser-Leu-Thr-Ser-Leu-Ser-Tyr-
        Cho                   Cho      (P)  Myr              (P)
         |                     |        |    |                |
61  Leu-Asn-Leu-Ser-Tyr-Asn-Asn-Leu-Ser-Gly-Arg-Ile-Pro-Ser-Ser-

76  Arg-Gln-Leu-Gly-Thr-Leu-Asn-Val-Asp-Asn-Pro-Ala-Leu-Met-Tyr-
                                                                           Cho
                                                                            |
91  Ile-Gly-Asn-Ser-Gly-Leu-Cys-Gly-Pro-Pro-Leu-Gln-Lys-Asn-Cys-
    (P)
     |
106 Ser-Gly-Asn-Asp-Thr-Pro-Thr-Ser-His-Val-Gly-Ser-Asn-Asn-His-
                               (P)
                                |
121 Glu-Glu-Phe-Glu-Leu-Met-Thr-Phe-Lys-Phe-Gly-Leu-Val-Leu-Ala-

136 Leu-Val-Ala-Gly-Leu-Trp-Ser-Leu-Ser-Cys-Ala-Leu-Leu-Phe-Lys-

151 Lys-Ala-Trp-Arg-Thr-Ala-Tyr-Phe-Gln-Leu-Phe-Asp-Asp-Met-Tyr-

166 Asp-Ala-Glu-Ser-Met-Tyr-Thr-Trp-Leu
```

Fig. 4.13. Predicted secondary structure and potential sites for post-translational modifications in the deduced pAWJL3 protein. Conformation prediction was done by the Garnier method (Garnier *et al.*, 1978) and potential post-translational modification sites are shown above the amino acid sequence. All predictions were done with PC/GENE program. Cho, possible glycosylation sites; Myr, potential sites for myristylation; (P), potential phosphorylation sites. The areas double underlined are predicted to form a helical conformation, underlined, extended conformation, dotted underlined, turn conformation and plain text, coil conformation.

Leucine-zipper sequences are normally linked to a short basic motif, in which the basic motif serves for DNA binding whereas the leucine repeats serves for homologous or heterologous protein dimerisation or oligomerisation (Landschulz *et al.*, 1988). Besides the helical conformation and leucine repeats, the amphipathic arrangement of hydrophobic and hydrophilic residues is another feature of leucine-zippers and this allows helix mediated protein interactions such as those seen in the helix-turn-helix (HTH) motif. The disposition of the hydrophobic residues on one face of a helix provides a contiguous array of stabilising force whereas the configuration of oppositely charged amino acids pairs allows the formation of a salt bridge. (Lassar *et al.*, 1989; Williams and Tjian, 1991).

The position of residues around a helix can be predicted by the PC/GENE HelWheel program. When the residues of peptide 120-148 were placed on a helix wheel, it became apparent that a strong hydrophobic face would form between position 7 and 4 (Fig. 4.14). Position 7 of the helix would consist of all hydrophobic residues and at position 4, three are hydrophobic residues and the remaining one is a uncharged polar amino acid. Most noticeably, residues at position 7 all the have the potential to be the teeth of a leucine-zipper (Landschulz *et al.*, 1988).

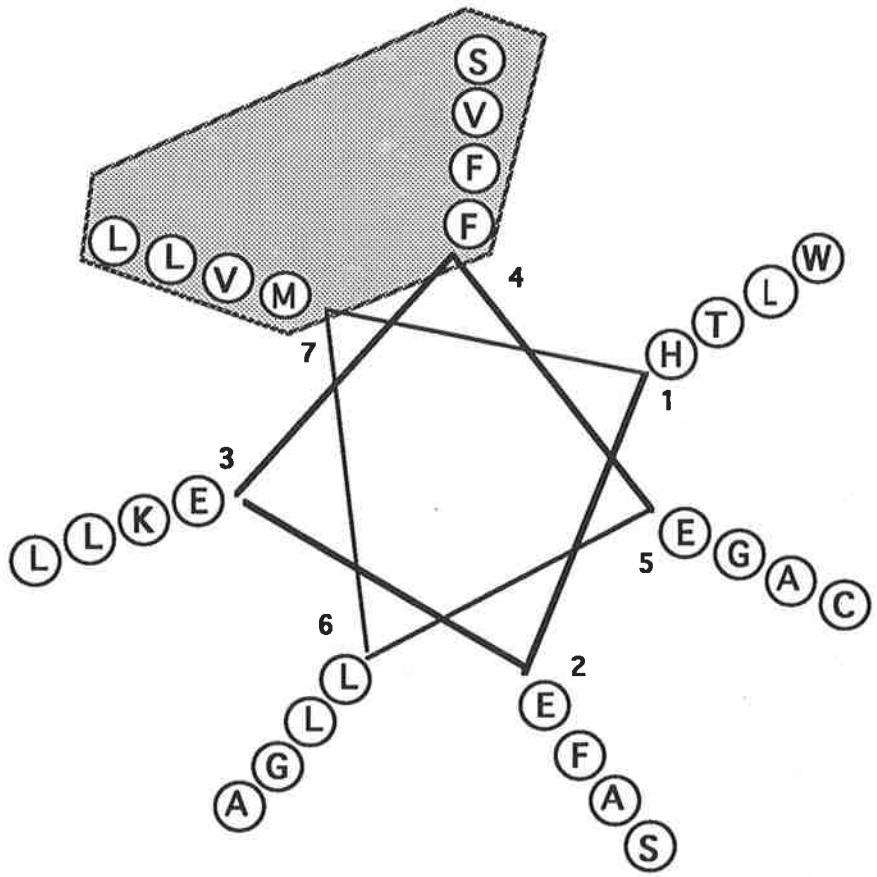


Fig. 4.14. Arrangement of aa 120-149 of pAWJL3 on an idealised helix. The wheel represents a view looking down from the N-terminus. The hydrophobic face of the helix is indicated by the shaded area.

4.7. Tissue-specific Expression of pAWJL3 Gene

The pattern of expression of the pAWJL3 gene was studied using RNA from different stages of meiosis by Northern hybridisation. The result was ambiguous due to difficulties in obtaining sufficient RNA and the possible low level of transcription of the genes (Data not shown). In addition, the tissues used were not only meiocytes. Other floral and anther organs may also be expressing these genes. However, it is possible that the expression seen reflects transcription in the meiotic cells.

The PCR has become a powerful tool in molecular biology and has been used as an alternative to Northern blot analysis (Kawasaki and Wang, 1989). This technique provides a solution to the problem of Northern analysis for the small amount of RNA obtainable from anthers of wheat. Barley was chosen for the investigation since the duration of meiosis in this plant is twice that of hexaploid wheat (Bennett and Smith, 1973). The results are shown in Fig. 4.15.A. No expression of the pAWJL3 gene was detectable before leptotene. However, expression was seen at zygotene and pachytene, after which the level starts to decline. The profile of expression at different stages of meiosis is quite similar to that the *Lilium* EMPR genes (Bouchard, 1990). The RT-PCR method is less accurate for quantification than Northern (Becker-André and Hahlbrock, 1989). Therefore, it is not possible to identify the peak of expression from this experiment. Transfer of the PCR gel to membrane and hybridised with pAWJL3 confirmed that the RNA that had been amplified was indeed homologous to pAWJL3 (Fig. 4.15.B).

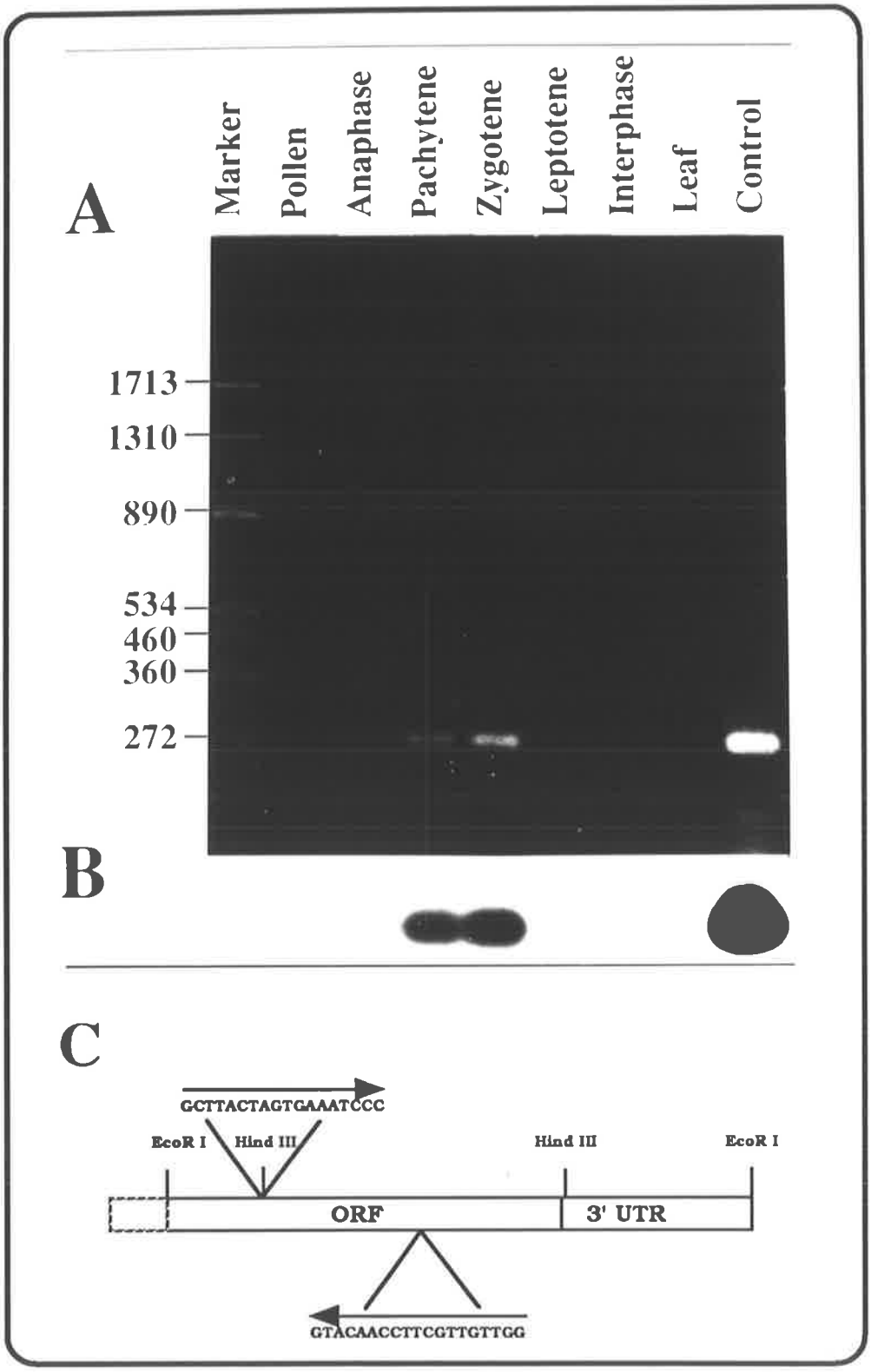
Fig. 4.15. The Expression of pAWJL3 at Different Stages of Meiosis

Total RNA was extracted from 12 barley (*Hordeum vulgare* cv. Betzes) anthers. Half of the RNA was converted into single-stranded cDNA with M-MLV reverse transcriptase and Primer JL3-1. One-tenth of the product was amplified by 45 cycles of PCR.

A. Half of the PCR product fractionated on a 2.0% agarose gel. The stage of the anthers is indicated above the lanes. Leaf and pAWJL3 DNA served as control. The sizes of marker DNAs are given on the left.

B. The gel above transfered to membrane and probed with pAWJL3.

C. An illustration of the locations of the two primers used. The box in broken lines at the left indicates unknown sequence. ORF and 3'UTR stands for open reading frame and 3' untranslated region, respectively.


A
Marker
Pollen
Anaphase
Pachytene
Zygotene
Leptotene
Interphase
Leaf
Control
1713
1310
890
534
460
360
272
B
C
GCTTACTAGTGAAATCCC
EcoR I
Hind III
Hind III
EcoR I
ORF
3' UTR
GTACAACCTTCGTTGTTGG

4.8. Discussion

4.8.1. The Implications of the Modified Method for cDNA Library Construction

The main modification of the traditonal cDNA construction method in this experiment is the omission of a poly(A)+ RNA purification step. Since the moloney murine leukemia reverse transcriptase is less sensitive to inhibition by rRNA and tRNA (Gerard, 1987), the conversion of mRNA to cDNA (about 8.1%) was quite low when compared to the "normal" methods (10-40%) (Gerard, 1985). The amount of cDNA generated from rRNA or tRNA has not been tested, but a method similar to that used in this experiment showed that the percentage of clones with rRNA gene is negligible (about 2-3%) (Lu and Werner, 1988).

In this experiment, the cloning efficiency was 1.1×10^5 per 50 ng cDNA or 20 µg total RNA, i.e., about 5.5×10^5 clones per µg mRNA. In addition, 83% of the clones contained inserts. The presence of rRNA and tRNA did not seem significantly affect the efficiency of cloning, although the ligase has been reported to use RNA as a substrate (Engler and Richardson, 1982).

The cDNA library contains a large proportion of small inserts. This problem may be solved by altering the ratio of RNA to reverse transcriptase (200 units enzyme per 100 µg RNA was used). Nonetheless, the method has been used for two other cDNA libraries in this laboratory: one from *Phalaris* pollen and the

other for myccorrhyza infected barley roots. Both have produced large libraries. Most importantly, the inserts are on average large, possibly because of the reduced RNA/reverse transcriptase ratio.

The number of positive clones (2-3 out of $1.52x10^5$) identified with pZm9 was low. This represents only about 1 in 70,000. However, it must be remembered that whole florets were used to prepare RNA for cloning. The low frequency of positive clones may reflect the low uniformity and small proportion of pollen mother cells to the tissue used.

4.8.2. The Possible Implications of the Locations of pAWJL1 and pAWJL3

pAWJL1 has been mapped to 1RS. To date no *Ph* genes have been found on group 1 chromosomes in wheat or rye although a minor effect of chromosome 1BS on pairing was reported (Thomas and Kaltsikes, 1977). The gene is probably present in single or low copy as it, unlike pZm9, revealed only a very weak signal on the Southern blots. Two possibilities may account for the failure to assign bands to wheat chromosomes using nullisomic-tetrasomic lines of group 1 chromosomes. It could be that all the bands seen are shared by more than one homoeologue of group 1 chromosomes. In this case, the genes on different chromosomes would be highly conserved since all six restriction enzymes failed to reveal a band belonging to a particular group 1

chromosomes. Alternatively, the genes related to pAWJL1 are not located on group 1 chromosomes in wheat.

The mapping result for pAWJL3 is more interesting than for pAWJL1 because the locations of the genes coincide with a number of the *Ph* genes. The second major suppressor of homoeologous chromosome pairing is located on 3DS and the first major promoter is on 5BS (Sears, 1976). Three bands have been mapped to 3DS and 5BS in the *Hin*d III digest. Strangely, no bands have been able to map to 3B although seven restriction enzymes (*Bam* HI, *Bgl* II, *Dra* I, *Eco* RI, *Eco* RV, *Hin*d III and *Kpn* I) have been used. However, cytogenetical data has also failed to observe a *Ph* gene on chromosome 3B (Cuarado *et al.*, 1991). One of the possible reasons for the lack of an allele on 3B is non-homologous translocation. This remains to be investigated.

4.8.3. The Structure of the Gene Encoding pAWJL3

The sequence of pAWJL3 lacks a poly(dA) tail. An AATAAA polyadenylation signal was found 94 bp upstream of the 3' end of the cDNA, but it may not be the functional signal for this particular mRNA since such signals usually lies just 15-23 bases upstream of the poly(A) tail (Messing, 1987). An additional polyadenylation signal probably exists further downstream and this assumption is consistent with the observation in *Lilium* that the EMPR transcripts are heterogeneous in length (Bouchard, 1990). This may result from multiple processing of the precursor mRNA. The lack of poly(dA) tail in the cDNA is uncommon but not

without precedent, e.g., TGFβ1 cDNA (Kanzaki *et al.*, 1990). The cause of the loss is unknown. It could be a consequence of incomplete second strand cDNA synthesis, degradation of mRNA from the RNA-cDNA hybrid by the RNase H activity of the reverse transcriptase (Kotewicz *et al.*, 1988) or deletion by the methylation restriction system of the *E.coli* host. The motif TTTTTAT was found in a number of mRNAs expressed in frog oocytes (Wickens, 1990) and has been shown to be essential for poly(A) addition in the cytoplasm of frog oocytes (Fox *et al.*, 1989; McGrew *et al.*, 1989). This sequence is also present in pAWJL3, six bases upstream of AATAAA. It is possible that the TTTTTAT motif in wheat meiocytes would play a function similar to in frog oocyte.

4.8.4. The Possible Functions of the pAWJL3 Gene

The genes encoding pAWJL3 have been assigned to the short arms of 5A, 5B and 5D as well as 3A and 3D. It appears that the genes are transcribed predominantly during the zygotene-pachytene interval. This suggests these may be *Ph* genes. However, *Ph* genes on the short arms of these two homoeologous groups are of a totally different character. Those of group 5 are promoters while those on group 3 are suppressors (Sears, 1976). Can this group of genes fulfil two opposite functions for chromosome pairing?

It has been proposed in section 4.6.2 and 4.6.3 that the putative pAWJL3 protein contains a leucine-zipper-like motif.

However, this stretch of peptides is unusual in that it is not linked to a short basic motif in its N-terminus. This makes it highly unlikely that it is a DNA binding protein. However, it is quite likely to be involved in protein interactions. The protein contains not only a leucine-zipper-like structure but also three 24 aa repeats. Both have the potential to interact with other proteins.

4.8.4.1. Is the Leucine-zipper-like Structure Functional?

It has been demonstrated that the region spanning the leucine-zipper-like motif is a continuous helical conformation and the hydrophobic residues are arranged predominantly on one face of the helix. The structure is imperfect only due to an extra valine residue in the middle of the four repeats. Would this extra residue abolish its dimerisation or oligomerisation function? Mutational analysis of the GCN4 leucine-zipper, a *S. cerevisiae* general control gene for amino acids biosynthesis, demonstrated that a minimum of 3 perfect leucine repeats are required in order to be functional (Hu *et al.*, 1990). Recent reports, however, point out that this is not always true. The leucine-zippers of the maize OCSBF-1 [Ocs sequence binding factor. Ocs is a 16 bases palindrome sequence found in the promoter of a number of genes active in plants (Bouchez *et al.*, 1989)] and *N. crassa* CPC1 (cross-pathway control gene for amino acids biosynthesis), for example, contain only two complete repeats and the third one is substituted by a conservative residue valine in OCSBF-1 and in CPC1, by a structurally very different residue, trytophan (Paluh

et al., 1988; Singh *et al.*, 1990). The ZEBRA protein of Epstein-Barr virus is an even more extreme case. It contains no apparent periodic leucine repeats and few charged residues adjacent to the hydrophobic face, but it is indeed a functional protein dimerisation motif via coiled-coil helix interaction (Flemington and Speck, 1990).

The disadvantage of an amino acid insertion in the pAWJL3 protein may be compensated by the richness of leucine. Each repeat contains on average 1.75 leucines. This figure is higher than the average for 51 repeats listed in two papers (Schmidt *et al.*, 1990; Oeda *et al.*, 1991), in which there are only an average of 1.26 leucines per repeat.

The structure can be looked at from another angle. It can be seen from Fig. 4.14 that residues at position 4 are also quite hydrophobic. This would enlarge the surface of protein contact, thereby resulting in a more stable dimer or oligomer. In addition, the leucine-zipper-like structure can be arranged in a different way from Fig. 4.11 by shifting one residue forward. This would make the leucine repeats appear as: HEEFELM TFKFGLV LALVAGL WSLSCAL. In such a situation, the leucine positions in the first two repeats are replaced by the analogous substitutes methionine and valine containing bulky side-chain that can potentially be the teeth of a zipper (Landschulz *et al.*, 1988)

4.8.4.2. The Biological Significance of the 24 AA Repeats

Leucine-rich periodic repeats have been found in an increasing number of proteins. The repeats vary from 22 to 28 aa and are highly divergent apart from the positions of leucine, asparagine and, in most cases, proline (Gay *et al.*, 1991). The functions of these repeats is poorly-understood. However, two lines of evidence suggest that they are involved in protein-protein interactions. In adenylate cyclase, for example, the repeats are essential for interaction with *ras2* protein and important for the activation of adenylate cyclase (Young *et al.*, 1989; Suzuki *et al.*, 1990). Recently, a synthetic 23 aa repeat unit from the *Drosophila* Toll protein was shown to form a gel via a β-sheet structure (Gay *et al.*, 1991). If the 24 aa repeats are involved in an interaction between proteins, identification of the interacting partner may provide important information on the function of pAWJL3.

4.8.4.3. Possible Biological Function of the pAWJL3 Gene

It should be noted that the complete N-terminus of the gene is lacking and the number of repeats remains to be determined. The function will remain a mystery until at least the full sequence is known and the protein studied biochemically. The proposition that the clone represents a *Ph* gene remains to be confirmed. Furthermore, the sequence information is confusing due to the presence of two potential protein-protein interaction domains.

An explanation for the function of pAWJL3 gene will first require an understanding of the nature of its role, i.e., structural or regulatory. It has been demonstrated that the gene is first expressed after the end of leptotene. Cytologically, formation of the SC and chromosome synapsis are the main tasks at this stage. Can the pAWJL3 protein be a structural component of the SC? This would be unlikely. Components of the SC are likely to be crucial for fertility. However, all the chromosome arms to which pAWJL3 maps are not of vital importance for fertility. In fact, the ditelosomics of the long arms of chromosomes 3 and 5 are amongst the most fertile of the ditelosomic plants (Sears and Sears, 1978). One may argue, however, that the SC structural components of homoeologous chromosomes are sufficiently conserved for interchange. Unfortunately, the long arm ditelosomics of chromosomes 3 and 5 are not available in diploid species and the importance of the short arms of chromosomes 3 and 5 for fertility is unknown. In addition, as has been discussed in Chapter one, the biochemistry of the SC components is poorly understood.

A further argument against a structural role for the pAWJL3 protein lies in the sequence data. The protein of pAWJL3 can be divided into two major domains, the leucine-zipper-like repeats and the 24 aa repeats. The former is unlikely to have a structural role since these kinds of repeats are normally short and, so far, no protein has been found to contain such motif and be a structural protein. The latter type of repeats are generally present in greater numbers but to date only one, the bovine bone

proteoglycan, was a structural protein of bone (Krusius and Ruoslahti, 1986). In the bone proteoglycan, the repeat unit was proline-less, cysteine-rich at the ends and contained 10 repeat units. These features, that would make a very stable structure, are not present in the pAWJL3 protein.

In conclusion, the function of the pAWJL3 gene is unclear. For the indirect reasons and inferences described above, it is possible that it plays a role in regulating other genes during meiosis. pAWJL3 is only one member of a family of which several others remain to be analysed. It will be important to determine the true size of the mRNA and protein. It is possible that some members of this family, but not necessarily the pAWJL3 itself, represent a *Ph* gene such as *Ph2* or *Ph3p*.

Chapter Five

Isolation and Preliminary Characterisation of Wheat Genomic Sequences Homologous to the *SPO11* Gene of Yeast, pAWJL1 and pAWJL3

5.1. Introduction

It has been demonstrated that a single or low copy sequence of wheat, rye and barley cross-hybridises to the *SPO11* gene of yeast (*S. cerevisiae*) under reduced stringency (Chapter Three). In addition, RFLPs between these three cereal species are rare, suggesting a high degree of conservation for this sequence. These results were assumed to be the consequence of evolutionary pressure on the *SPO11* gene.

The demonstration of cross-hybridisation between the yeast *SPO11* gene and cereal total DNA did not guarantee detection of a homologous gene between the two organisms, since more than 85% of the wheat genome comprises of repetitive sequences (Flavell *et al.*, 1974). The result would become clearer if a direct hybridisation to the cereal mRNA could be achieved. However, in *S. cerevisiae*, the *SPO11* mRNA is expressed only weakly (about 5 molecules per cell at the peak of expression) (Atcheson *et al.*, 1987). If the *SPO11* homologues in wheat are expressed at a similar level, detection of the mRNA by Northern hybridisation will be highly unlikely due to difficulties in

obtaining a large amount of tissues at a particular stage of meiosis. Similarly, it will be highly unlikely to isolate cDNA clones from the floret cDNA library that was made previously.

It has been demonstrated that the yeast *SPO11* gene hybridises to an *Eco RI* band of about 15 kb in wheat, rye and barley genomic DNAs (Chapter Three). The two cDNAs isolated with pZm9, pAWJL1 and pAWJL3, also detected bands at or near 15 kb in wheat (Chapter Four). The size of the bands are ideal for cloning into EMBL lambda vectors. The size of interest can be enriched and selected on a sucrose gradient.

In this chapter, the cloning and preliminary analysis of the wheat genomic sequences that cross-hybridise to the yeast *SPO11* gene, and to pAWJL1 and pAWJL3 will be presented.

5.2. Isolation of Genomic Sequences Homologous to pGB436, pAWJL1 and pAWJL3

5.2.1. Construction of an *Eco* RI Genomic Library

Wheat (cv. Chinese Spring) DNA was digested to completion with *Eco* RI and fractionated on a sucrose gradient. Aliquots from the 15 kb region were precipitated with ethanol and resuspended in TE buffer. The DNA was ligated to λEMBL4 arms double digested with *Eco* RI and *Sal* I, packaged *in vitro* and plated on appropriate *E.coli* host. Two libraries were constructed. The first one was plated on a *RecD+* strain, NW2, which yielded 1.2×10^6 pfu from 0.5 μg insert DNA, whereas the second was plated on a *recD-* strain,

ER1647, which yielded $3.7x10^5$ pfu from the same amount of insert.

5.2.2. The Instability of the Target Sequences in *E.coli* Host

About $8x10^5$ pfu of the NW2 library were initially screened with the clone pGB436 (Giroux *et al.*, 1986), which contains the entire *SPO11* gene of *S. cerevisiae*. It was found that the f1 replication origin of the cloning vector cross-hybridised to λEMBL4 at reduced stringency. The probe, therefore, was subcloned into pBluescribe (Stratagene) to reduce background hybridisation.

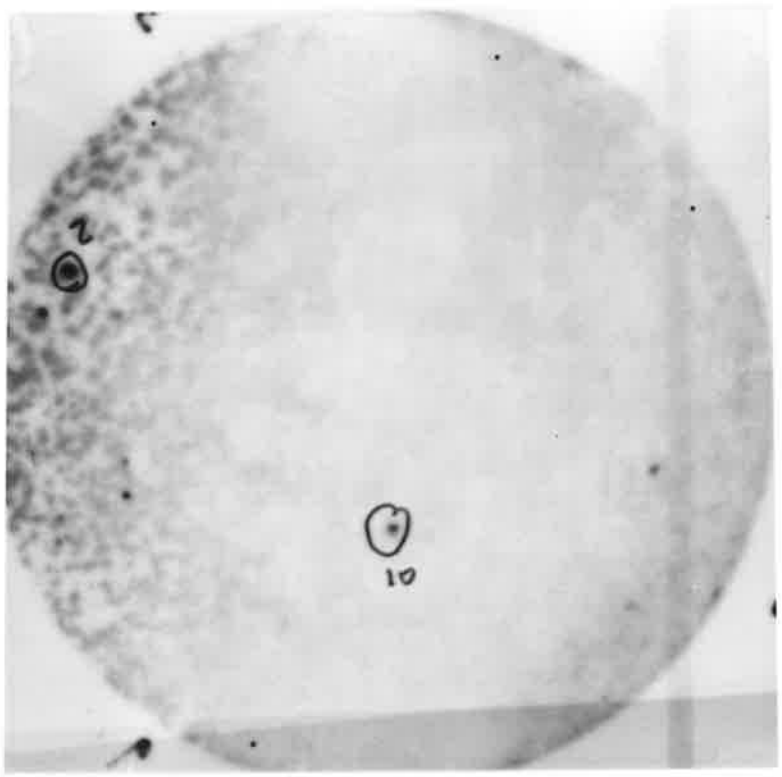
Potentially positive clones (16) were selected and re-screened. Unfortunately, none was truly positive. Although more than 30 were expected for a single gene on an assumption that the enrichment is 33 fold (7.5 μg out of 250 μg). A similar result was obtained when the library was screened with pAWJL1. This suggested that the target sequences were unstable in the *E.coli* host. The plating host, NW2, which is tolerant of methylation at cytosine and adenosine residues in the input DNA due to the deletions at the *McrA, McrB* and *Mcrr* loci, has been particularily useful for construction of clone libraries of eukaryotic genomes. However, it is *RecA+* and *RecD+*, which would make the insert vulnerable to recombination and deletion if it contained duplicated sequences (Woodcock *et al.*, 1989). This lead to the speculation that the fragments of interest may contain duplicated sequences. The strain ER1647 is another recommended host for the construction of genomic libraries. It contains not only all the

advantages of NW2 has but is also *recD*$^-$. This mutation will stabilise clones containing inverted repeats (Woodcock *et al.*, 1989). Therefore, a new library was made in this strain.

The screening method with pGB436 was as before. Most potential plaques in this library were confirmed to be truly positive. Typically, one strong and 2-4 weak hybridising signals were seen on a plate containing about 40,000 pfu (Fig. 5.1). Twenty-four were selected and re-screened. Sixteen were found to be positive and were purified to homogeneity by another two rounds of screening.

Fig. 5.1. Screening of the Genomic Library with pGB436

Plaques of the genomic library established on *E.coli* strain ER1647 were transferred onto Hybond-N+ membrane and hybridised against pGB436. The hybridisation was performed overnight in 6X SSPE (1.14 M Na^+), 25% formamide, 1% SDS, 500 μg/ml salmon sperm DNA, 0.5% Blotto and 0.7 ng/ml radiolabeled probe (c.a. $1X10^9$ cpm/μg). After being washed three time in 2X SSC, 0.1% SDS at 42°C, the membranes were exposed three days between intensifying screens at -70°C. The representative strong and weak hybridisation signals are circled.


2
10

Small scale lambda DNA preparations from each positive clone were made. Digestion of these DNA and fractionation by agarose gel electrophoresis resulted in the photograph shown in Fig. 5.2.A. Most clones contained more than one insert instead of the single 15 kb insert expected. In addition, the additive size of all the inserts from each clone varied. However, when the gel was blotted to Hybond-N+ and probed with pGB436, most clones showed a strong hybridising band (Fig. 5.2.B). Furthermore, most clones that hybridised to the entire *SPO11* insert, also hybridised to the *Dra* I fragment (coding sequence only) although some less strongly (see Fig. 5.2.C). These clones were, therefore, regarded as truly positive and designated λAWJL4 to λAWJL19. For convenience, these clones will be termed WSPs (Wheat SPO11).

Fig. 5.2. Hybridisation of pGB436 to the WSPs

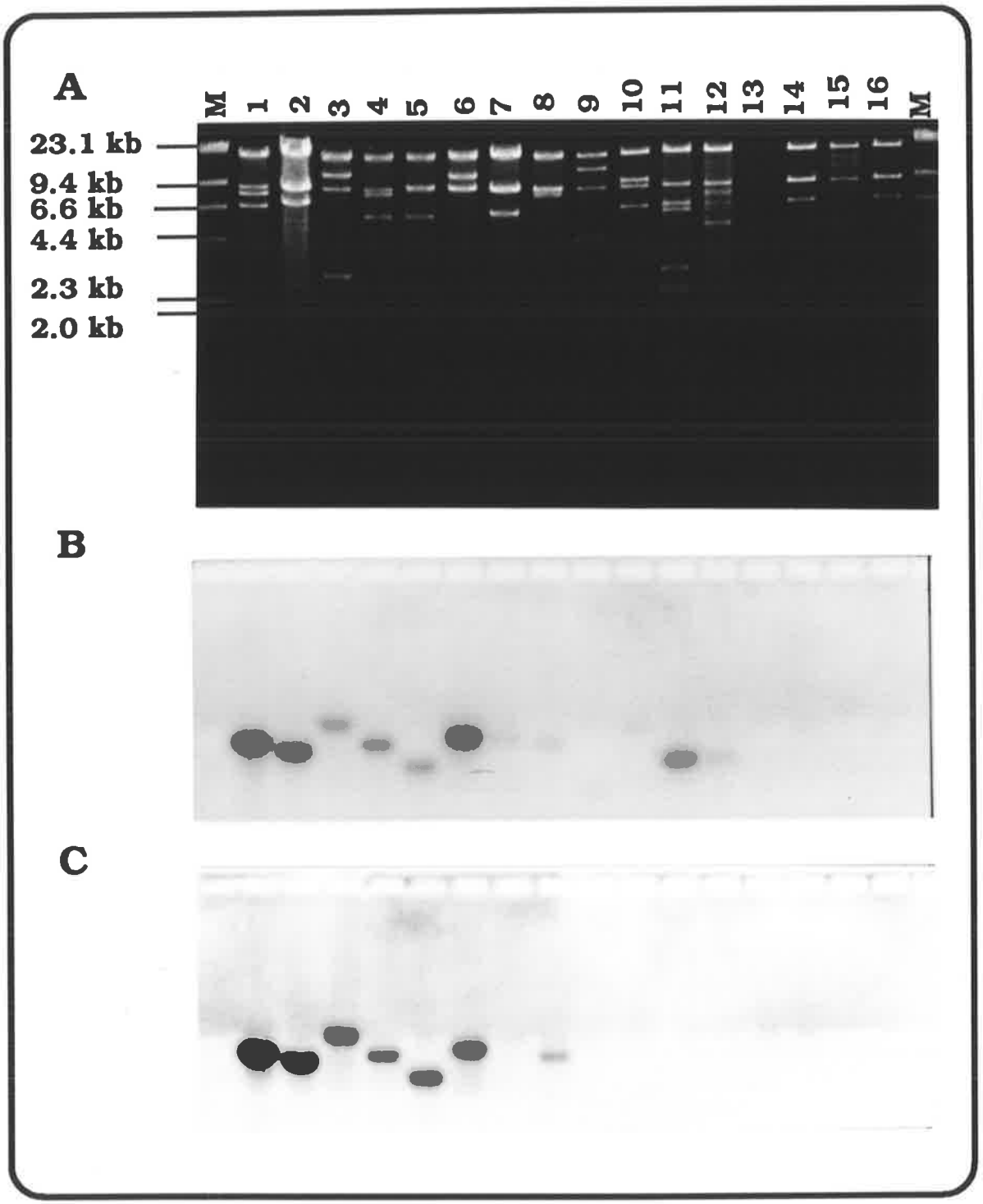
A. Positive clones identified by plaque hybridisation were propagated in strain ER1647 (5 ml liquid culture) and lambda DNA was isolated by the DEAE-cellulose method. One third of DNA was digested with *Eco* RI and fractionated on a 1% agarose gel.

B. The above gel was transferred to Hybond-N+ membrane and probed with pGB436.

C. The same membrane probed with the middle *Dra* I subfragment of pGB436.

The autoradiographes were exposed overnight at -70°C between intensifying screens. Size marker lanes are labeled M and the sizes are given on the left of Panel A.

The clones (except number 13) are designated λAWJL4 to λAWJL16, respectively.


A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 M
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
B
C

The second library (in ER1647) was also screened with the wheat cDNA clones pAWJL1 and pAWJL3. Typically, one potentialy positive plaque was detected per plate (40,000 pfu) with pAWJL1 and three or four positive clones with pAWJL3. The plaques (12 for pAWJL1 and 24 for pAWJL3) were re-screened, resulting the isolation of 7 pAWJL1 positive clones and 13 pAWJL3 positive clones. Based on the banding patterns of the lambda clone DNA digested with *Eco* RI, *Bam* HI or *Sal* I, the clones were classified into 3 groups for those isolated with pAWJL1 and 7 groups for those isolated with pAWJL3. One clone from each group was chosen and designated λAWJL1-1 to λAWJL1-3 and λAWJL3-1 to λAWJL3-7 (Fig. 5.3).

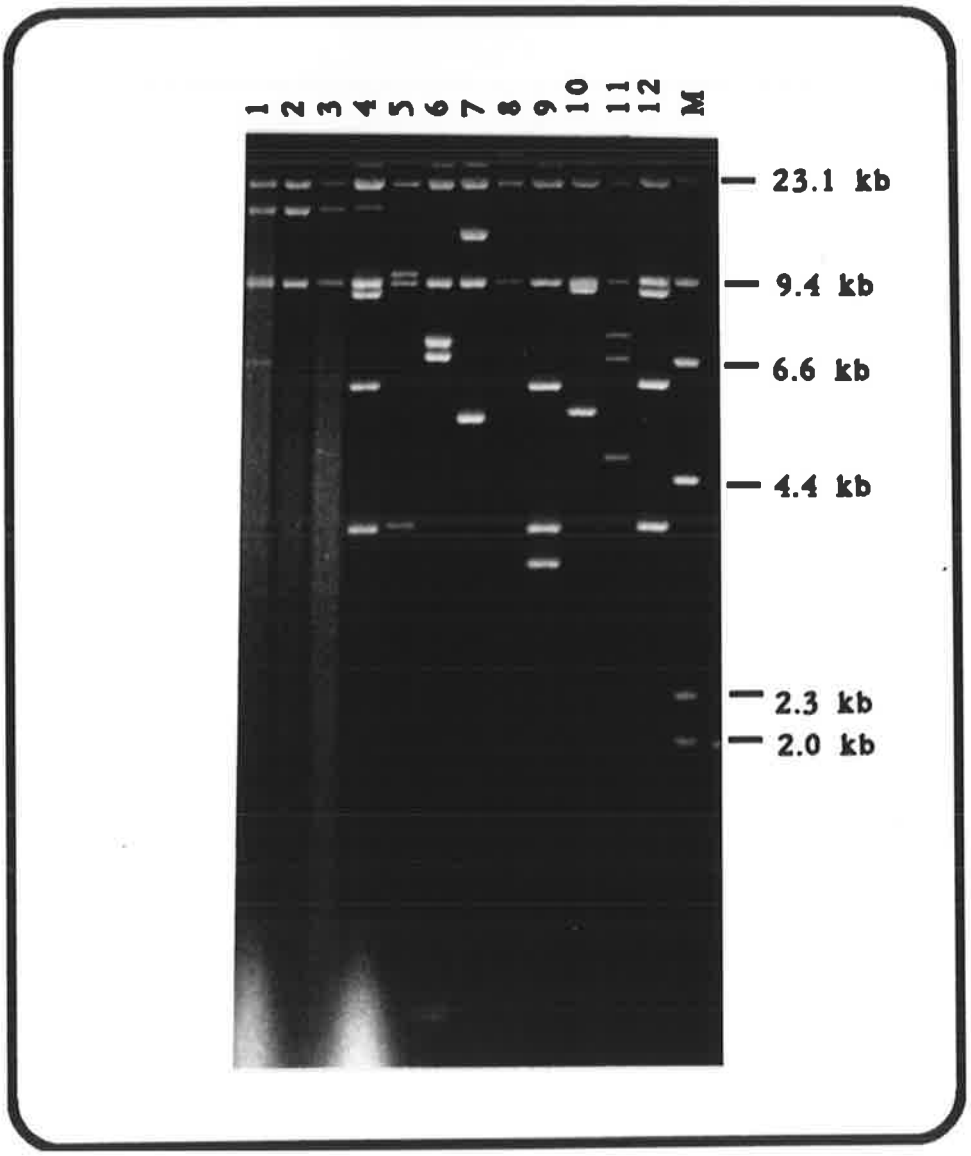
Fig. 5.3. The Genomic Clones Isolated with pAWJL1 and pAWJL3

One third of the small scale DNA preparation was digested with *Eco* RI and resolved on a 0.7% argarose gel.

Clones numbered 1-5 represent the three groups isolated with pAWJL1. They are designated λAWJL1-1, λAWJL1-2, λAWJL1-3 (isolate 1), λAWJL1-3 (isolate 2) and λAWJL1-3 (isolate 3).

Clones numbered 6-12 represent the seven groups isolated with pAWJL3. They are designated λAWJL3-1 to λAWJL3-7, respectively.

The DNA size marker is labeled M and the sizes of the DNA fragments are given on the right.


1
2
3
4
5
6
7
8
9
10
11
12
M
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

It can be seen from Fig. 5.3 that, like the WSPs, most clones did not release a single band upon digestion with *Eco* RI but 2-3 bands smaller than 15 kb and the additive sizes of all the bands of a clone varied. Southern blots of wheat genomic DNA probed with both pAWJL1 and pAWJL3 revealed bands at or near 15 kb region (see Fig. 4.3 and Fig. 4.4). Most clones were expected to contain only one band at or near 15 kb in size since the inserts for the construction of the library had been enriched in this size region. If more than one insert were ligated into the same lambda, it would contain a 30 kb insert which would make the clone inviable (Frischauf *et al.*, 1983). It is likely that the target sequences for all three probes, pGB436, pAWJL1 and pAWJL3, were highly unstable and the *recD* mutation in the host bacterium only improved the stability and recovery rate of the clones. Nearly all of the clones recovered appeared to have undergone rearrangement, leading to the variability of insert sizes and multiple inserts. However, three of the pAWJL1 positive clones, λAWJL1-1, λAWJL1-2 and λAWJL1-3, have recovered as a single 15 kb insert, although, instability was still apparent since different isolates of the same clone (re-screened three times with pAWJL1) contained inserts of different sizes (Fig. 5.3 lane 4 and 5).

5.3. Restriction Mapping of the Lambda Clones

In order to define more precisely the regions in the lambda clones that cross-hybridise to pGB436 and pAWJL1, λAWJL4, λAWJL5, λAWJL1-1 and λAWJL1-2 were selected for restriction

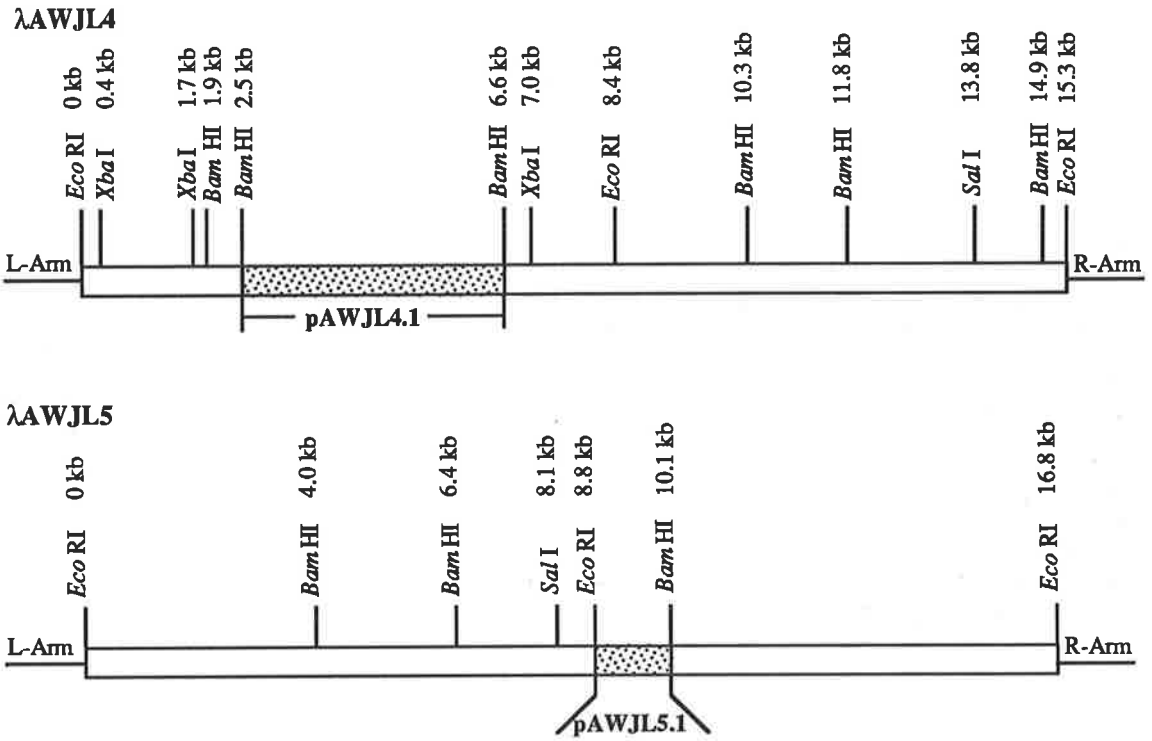
mapping. The first two clones were selected because they hybridised to pGB436 more strongly than the remaining WSPs while the latter two clones were selected because they contained only a single 15 kb insert. They may have undergone little or no structural rearrangement. Restriction mapping of the genomic clones of pAWJL3 was not attempted because they showed extreme rearrangement.

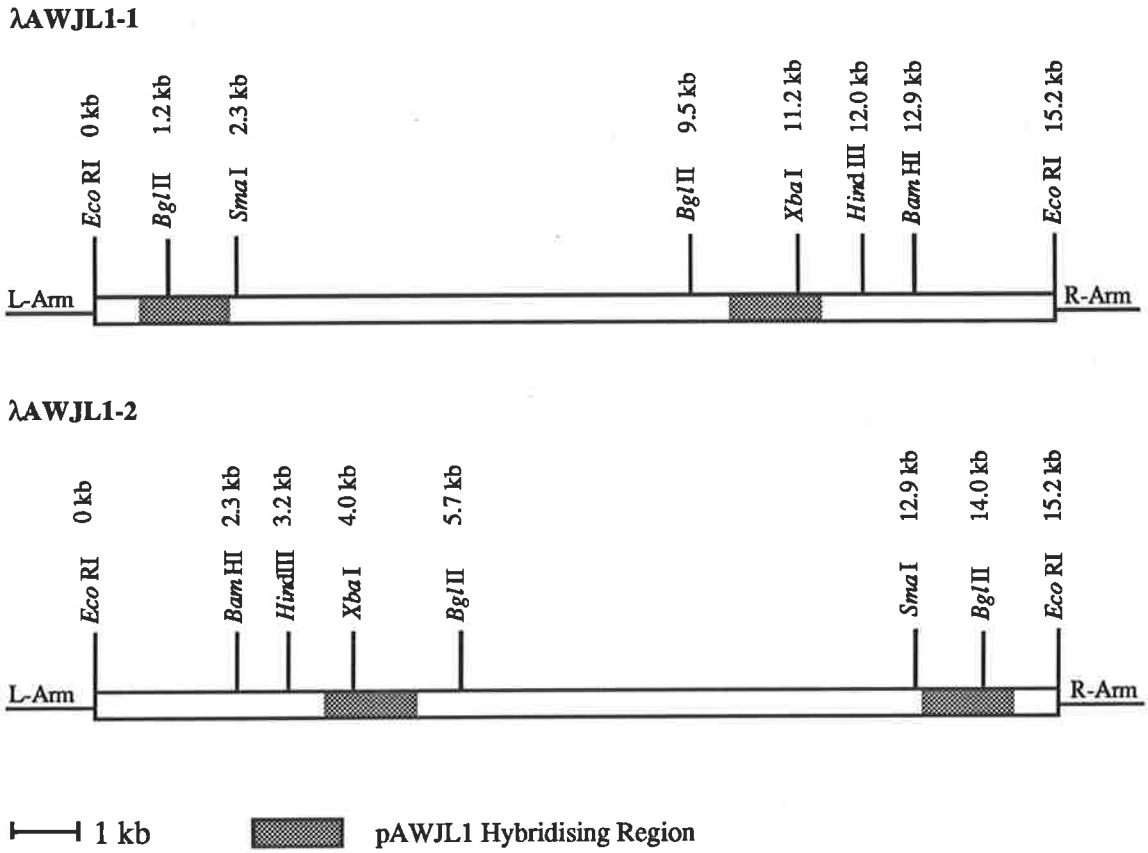
The phage DNA of the various wheat clones was digested with *Eco* RI, *Sal* I, *Bam* HI and *Xba* I or a combination of two enzymes and separated on a 0.8% agarose gel. The gel was blotted onto Hybond-N+ and probed with pGB432 (entire insert) or pAWJL1. The restriction maps and the regions that hybridised to the probes are illustrated in Fig. 5.4. The *SPO11* homologous regions in λAWJL4 and λAWJL5 were localised to within 4.1 kb and 1.3 kb respectively. The two clones do not appear to share a common restriction digestion pattern. The 4.1 kb *Bam* HI fragment of λAWJL4 and the 1.3 kb *Eco* RI/*Bam* HI fragment of λAWJL5 to which pGB436 hybridised were subcloned into pTZ18U and designated pAWJL4.1 and pAWJL5.1.

λAWJL1-1 and λAWJL1-2 appeared to contain identical inserts but inserted in opposite orientations. Both have two regions that hybridised to pAWJL1. The precise end-points of cross-hybridisation of the lambda clones to pAWJL1 in the *Xba* I/*Hin*d III, *Xba* I/*Bgl* II, *Bgl* II/*Sma* I and *Bgl* II/*Eco* RI intervals remain undetermined. The regions in λAWJL1-1 and λAWJL1-2 that hybridised to pAWJL1 were also found cross-

hybridised to pAWJL3, although the cDNA clones pAWJL1 and pAWJL3 did not cross-hybridised (Chapter Four).

Fig. 5.4. Restriction maps of λAWJL4, λAWJL5, λAWJL1-1 and λAWJL1-2. The locations of regions homologous to the yeast *SPO11* gene and pAWJL1 are hatched.


λAWJL4
0 kb
0.4 kb
1.7 kb
1.9 kb
2.5 kb
6.6 kb
7.0 kb
8.4 kb
10.3 kb
11.8 kb
13.8 kb
14.9 kb
15.3 kb
Eco RI
Xba I
Xba I
Bam HI
Bam HI
Bam HI
Xba I
Eco RI
Bam HI
Bam HI
Sal I
Bam HI
Eco RI
L-Arm
R-Arm
pAWJL4.1
λAWJL5
0 kb
4.0 kb
6.4 kb
8.1 kb
8.8 kb
10.1 kb
16.8 kb
Eco RI
Bam HI
Bam HI
Sal I
Eco RI
Bam HI
Eco RI
L-Arm
R-Arm
pAWJL5.1
1 kb
pGB436 Hybridising Region


λAWJL1-1
0 kb
1.2 kb
2.3 kb
9.5 kb
11.2 kb
12.0 kb
12.9 kb
15.2 kb
Eco RI
Bgl II
Sma I
Bgl II
Xba I
Hind III
Bam HI
Eco RI
L-Arm
R-Arm
λAWJL1-2
0 kb
2.3 kb
3.2 kb
4.0 kb
5.7 kb
12.9 kb
14.0 kb
15.2 kb
Eco RI
Bam HI
Hind III
Xba I
Bgl II
Sma I
Bgl II
Eco RI
L-Arm
R-Arm
1 kb
pAWJL1 Hybridising Region

5.4. Characterisation of pAWJL4.1

5.4.1. Cross-hybridisation of pAWJL4.1 to Other WSPs

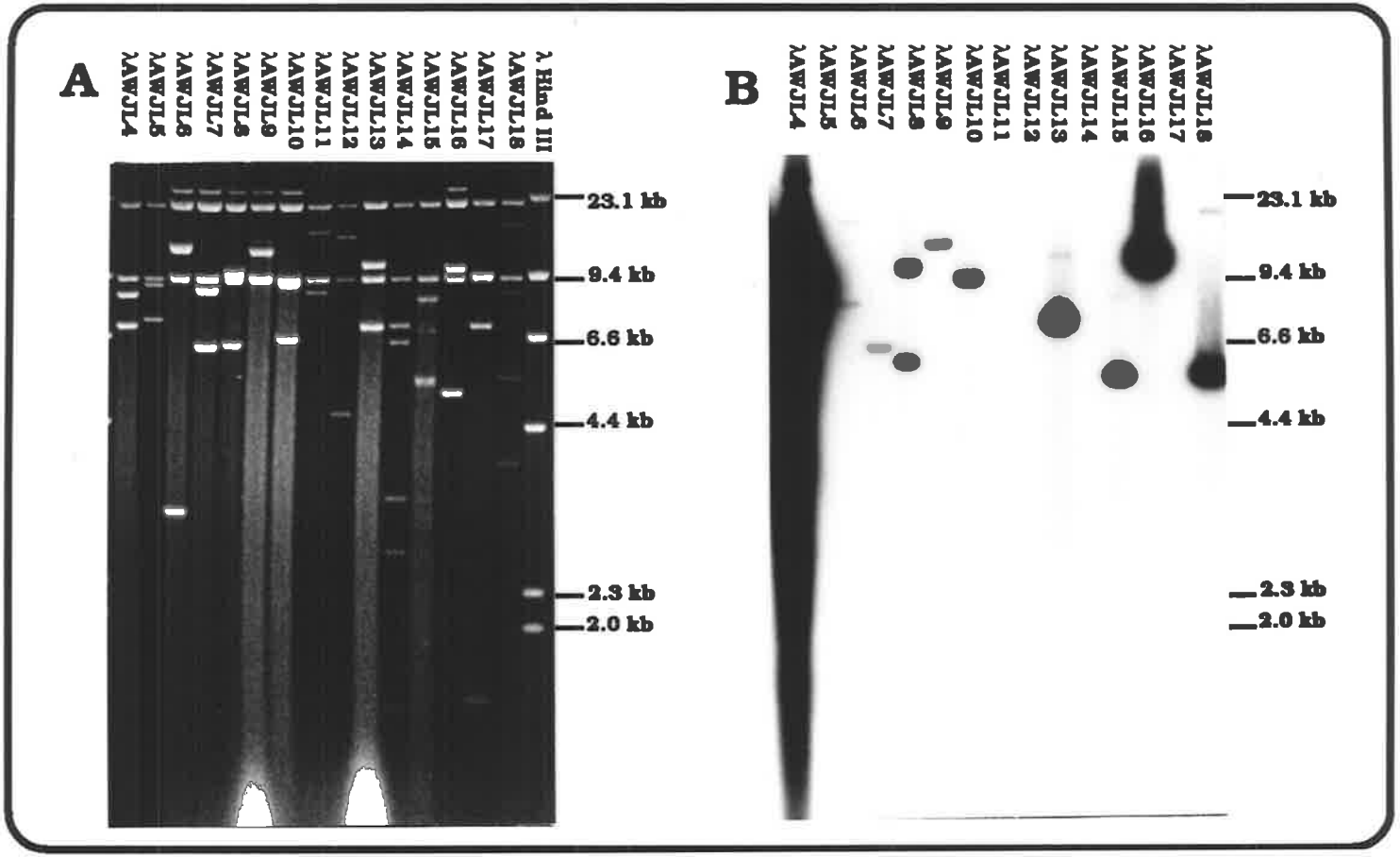
When insert of pAWJL4.1 was isolated and used to probe the other WSP lambda clones, variable degrees of hybridisation were observed (Fig. 5.5). λAWJL16, λAWJL18 and λAWJL13 hybridised strongly to the probe whereas λAWJL5, λAWJL6, λAWJL7 and λAWJL9 hybridised only weakly while hybridisation to λAWJL11, λAWJL12, λAWJL14 and λAWJL17 could only be seen after extended exposure. Some clones, including λAWJL4 itself, showed two hybridising bands in the *Eco* RI digest when probed with pAWJL4.1, e.g., λAWJL6, λAWJL13 and λAWJL18. However, some of these hybridising bands (Fig. 5.5.B) are not visible in the ethidium bromide stained gel (Fig. 5.5.A), e.g., c.a. 7.0 kb band in λAWJL6. This provides further indication of the instability of the WSP lambda clones. It is noticeable, from Fig. 5.5, the clones that show the strongest hybridisation to the yeast *SPO11* gene, e.g., λAWJL5 and λAWJL6, are not necessarily those that are most closely related to pAWJL4.1, although λAWJL4 was also one of the clones that showed strong hybridisation to pGB436 (c.f. Fig. 5.2).

Fig. 5.5. Hybridisation of pAWJL4.1 to Other WSPs

A. Mini-prep DNA of WSPs digested with *Eco* RI, resolved on a 0.8% agarose gel.

B. The above gel blotted onto Hybond-N+ membrane and probed with random-primed pAWJL4.1. The membrane was exposed for 6 hours at room temperature.

The position of DNA size marker, λ *Hin*d III, is given on the right of each panel.


A
λAWJL4
λAWJL5
λAWJL6
λAWJL7
λAWJL8
λAWJL9
λAWJL10
λAWJL11
λAWJL12
λAWJL13
λAWJL14
λAWJL15
λAWJL16
λAWJL17
λAWJL18
λ HindIII
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
B
λAWJL4
λAWJL5
λAWJL6
λAWJL7
λAWJL8
λAWJL9
λAWJL10
λAWJL11
λAWJL12
λAWJL13
λAWJL14
λAWJL15
λAWJL16
λAWJL17
λAWJL18
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

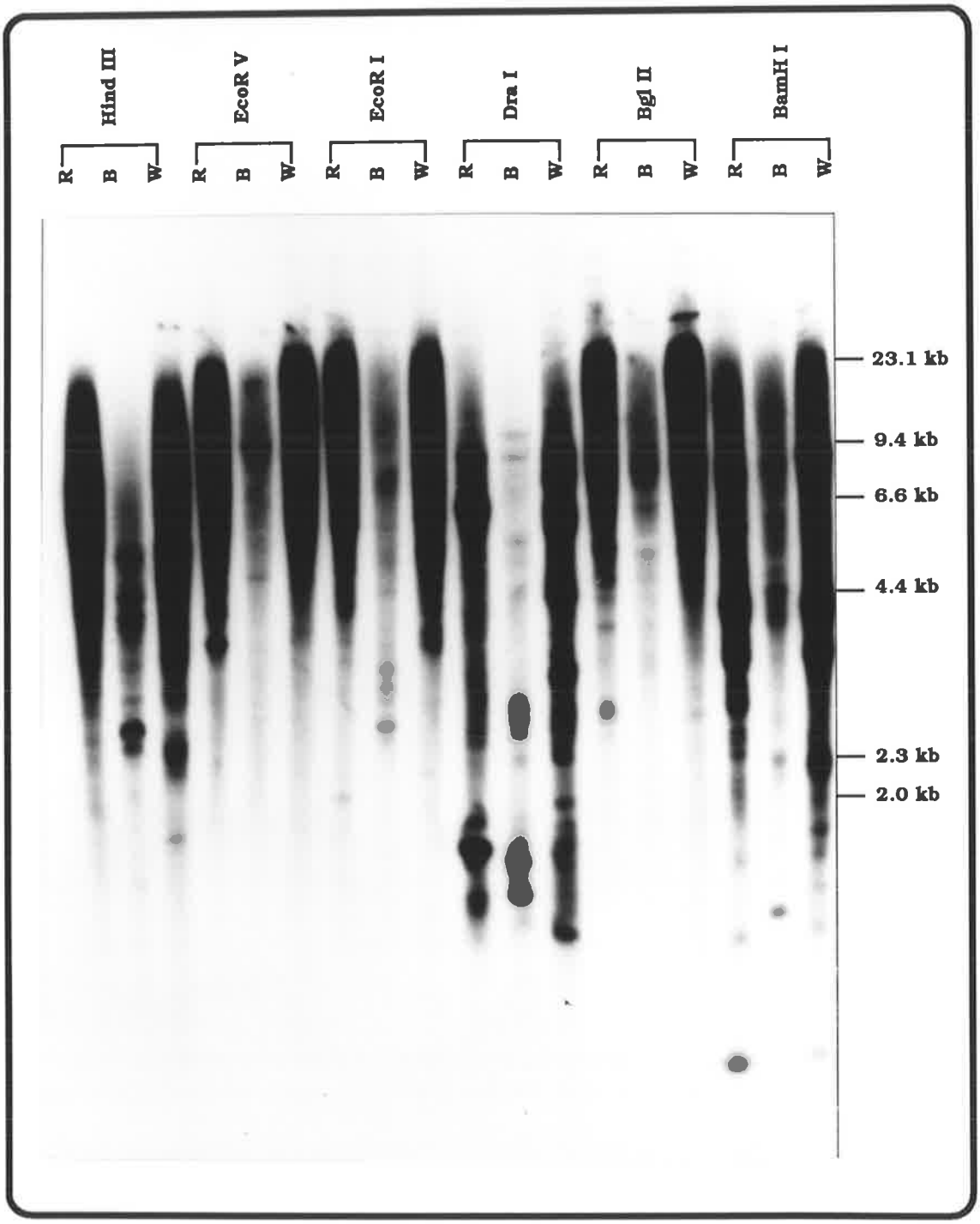
5.4.2. Hybridisation of pAWJL4.1 to Wheat, Rye and Barley

A complex pattern of hybridisation was revealed when Southern blots of cereal DNA was probed with pAWJL4.1. In most restriction digests, a strong smear was produced (Fig. 5.6). The DNA detected by the probe represented a class of moderate repetitive sequences. Only 2-4 hours exposure of the membrane was required to produce an intense signal on the autoradiographs. Some RFLPs could be seen between wheat, rye and barley, however, these could not be mapped to particular chromosomes.

Fig. 5.6. Hybridisation of pAWJL4.1 to Wheat, Rye and Barley

Total DNA (5 µg) wheat (cv. Chinese Spring), rye (cv. Imperial Rye) and barley (cv. Betzes) was digested with *Bam* HI, *Bgl* II, *Dra* I, *Eco* RI, *Eco* RV and *Hin*d III, resolved on a 1% agarose gel, transferred onto Hybond-N+ membrane and probed with pAWJL4.1 radiolabeled by random priming. The final wash was done in 0.2 X SSC, 0.1% SDS at 6o°C. The membrane was exposed at room temperature for four hours.

W, R and B represent wheat, rye and barley, respectively. The positions of DNA size marker are given on the right.


Hind III
EcoR V
EcoR I
Dra I
Bgl II
BamH I
R
B
W
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb

5.4.3. Localisation of the Repetitive Sequence and *SPO11* Hybridising Region within pAWJL4.1

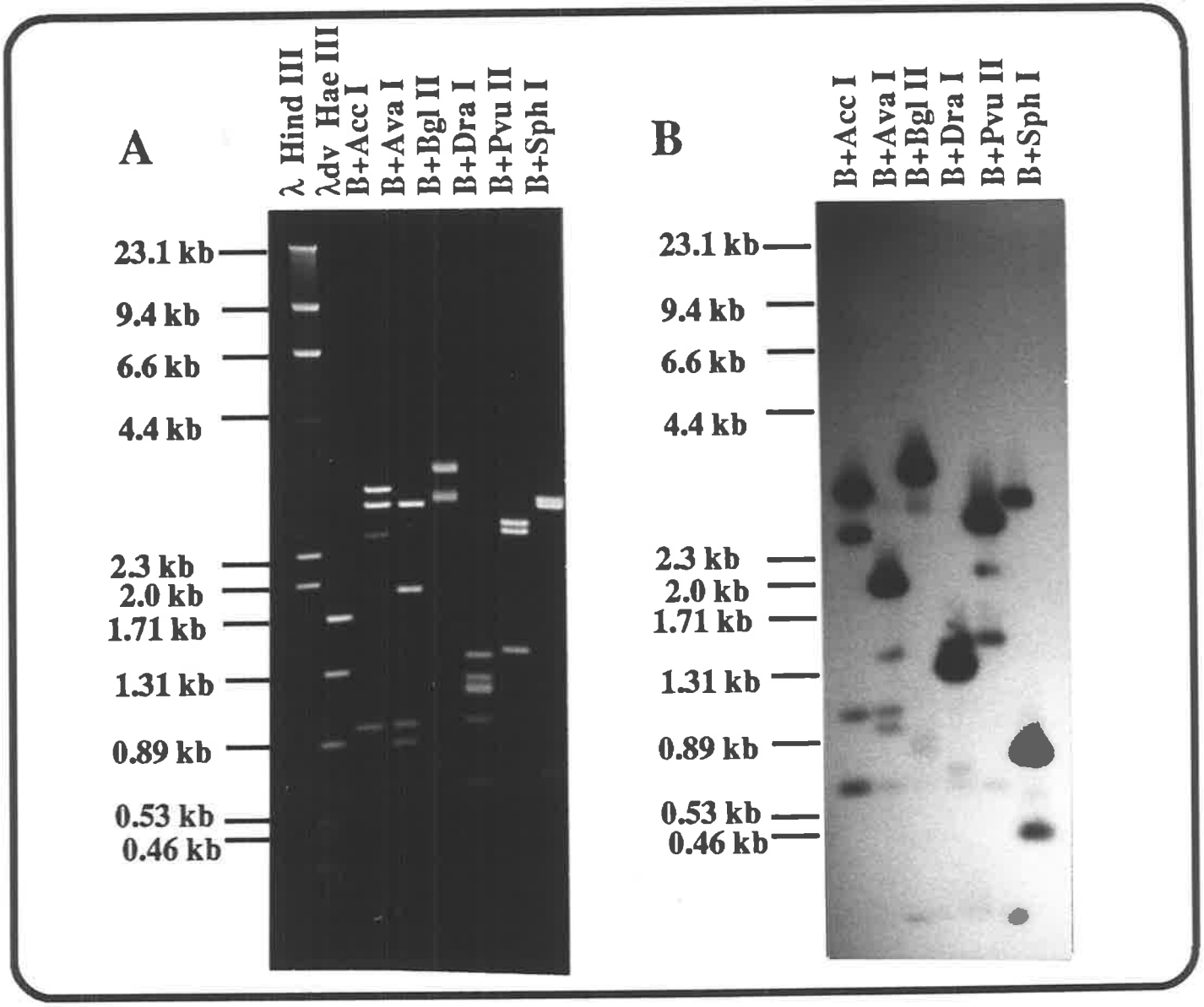
In order to determine whether the high-copy nature of the pAWJL4.1 homologous regions is a characteristic of the whole 4.1 kb insert or only part of it, the plasmid was double-digested with *Bam* HI in conjunction with *Acc* I, *Ava* I, *Bgl* II, *Dra* I, *Pvu* II, *Sph* I and *Pst* I. The DNA was fractionated on an 1% argarose gel, transferred to Hybond-N+ and probed with nick-translated wheat total DNA. As can be seen Fig. 5.7, the signal intensity varied greatly with different sub-fragments of pAWJL4.1. The most intensely hybridising region was the 1.4 kb *Dra* I fragment, to which the signal was about 100 times stronger than for the remainder of the plasmid. With some additional restriction mapping data, the high-copy region was localised to the central region of pAWJL4.1 (Fig. 5.8). The membrane shown in Fig. 5.7 was stripped and re-probed with pGB436 and the region homologous to the *SPO11* gene of yeast was localised to be the *Bgl* II/*Bam* HI fragment, at the right hand side of pAWJL4.1 (Fig. 5.8).

Fig. 5.7. Identification of High Copy Number Sequence within pAWJL4.1

A. pAWJL4.1 digested with *Bam* HI in conjunction with *Acc* I, *Ava* I, *Bgl* II, *Dra* I, *Pvu* II or *Sph* I, resolved on a 1% agarose gel.

B. The above gel transferred to Hybond-N+ and hybridised against wheat (cv. Chinese Spring) total DNA radiolabeled by nick translation.

The positions of DNA size marker are given on the left of each panel.


A
λ Hind III
λdv Hae III
B+Acc I
B+Ava I
B+Bgl II
B+Dra I
B+Pvu II
B+Sph I
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
1.71 kb
1.31 kb
0.89 kb
0.53 kb
0.46 kb
B
B+Acc I
B+Ava I
B+Bgl II
B+Dra I
B+Pvu II
B+Sph I
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
1.71 kb
1.31 kb
0.89 kb
0.53 kb
0.46 kb

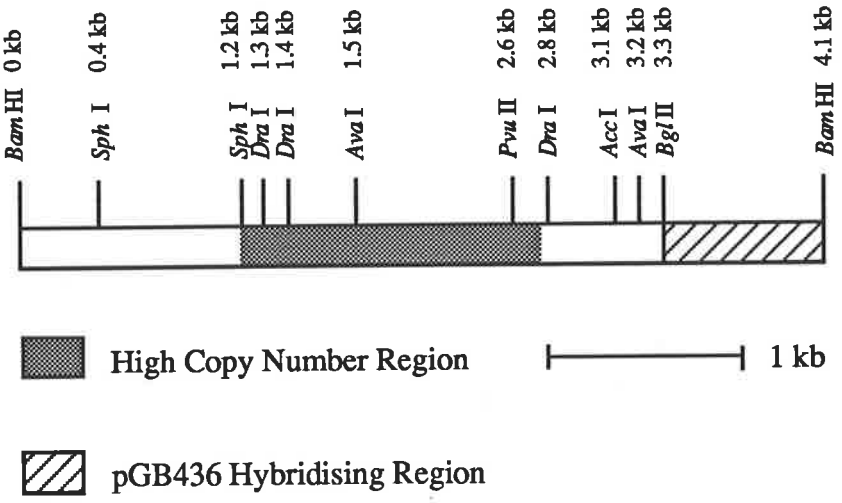


Fig. 5.8. The restriction map of pAWJL4.1. The regions with high copy number in wheat and related to the yeast *SPO11* gene are indicated.

5.4.4. The Copy Number of the 1.4 kb *Dra* I Sub-fragment of pAWJL4.1 in Wheat and Rye

Total DNA of wheat (0.36 µg per dot) and rye (0.19 µg per dot) and the purified 1.4 kb *Dra* I fragment of pAWJL4.1 (140 pg) were dot-blotted onto Hybond-N+ using a dot-blotting apparatus. The membrane was hybridised against the 1.4 kb *Dra* I fragment of pAWJL4.1 and the radioactivity retained after extensive washing (0.5 x SSC, 0.1% SDS, 60°C) was measured with a Beckman LS 3801 scintillation counter. The Cerenkov radiation of wheat (cv. Chinese Spring), rye (cv. Imperial rye), 1.4 kb *Dra* I fragment and null DNA control (average of 5 assays) were 1929, 676, 4854 and 72 cpm, respectively. Since the genome of wheat and rye contain approximately 36 and 19 pg of nuclear DNA (Bennett, 1972), the

copy number of the 1.4 kb *Dra* I fragment was estimated to be about 4000 copies in wheat and 1200 copies in rye.

5.4.5. Nucleotide Sequence of pAWJL4.1

Successive deletions at about 300 bp intervals were generated with a nested deletion kit (Pharmacia) from pAWJL4.1 and a clone with an inversely orientated insert, pAWJL4.2. Single-stranded phagemid DNA was made from the selected clones by infection with helper phage of M13, K07.

The sequencing reactions used ^{35}S-dATP as label and the sequencing strategy is illustrated in Fig. 5.9.A. The sequence at both ends was available on only one strand due to incomplete nested deletions. However, since the sequencing was done with Taq DNA polymerase and the sequence at these regions had shown little ambiguity, the whole sequence of pAWJL4.1 is sufficiently reliable for general analysis.

A.

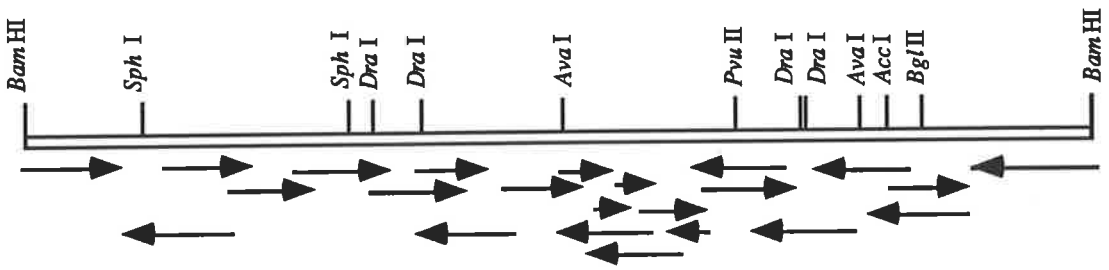


Fig. 5.9.A. Sequencing strategy for pAWJL4.1. Arrows indicate the direction and extent of a sequencing reaction.

B.

```
GATCCACTCC CGTCTCCCTA TGAGAAGGCC AGCCATAGCA CTCACACTTA TCTTGCGAGT 60
CTAGGTGAGG GCAGAGGGAT ACTCTTCCGG TCGGTATCGT GAGTGTGAAT AGAACGCTCA

TTTAGAGTAT CCACTTTCAC TTTTCTATGA ACTGTTATAG GCAACCCAGA AGTCCATTAC 120
AAATCTCATA GGTGAAAGTG AAAAGATACT TGACAATATC CGTTGGGTCT TCAGGTAATG

CGCGGACACG GCTATTTGAA TAGATGATGT TAACCCTGCA GGGGTGTACT TCTTCACACA 180
GCGCCTGTGC CGATAAACTT ATCTACTACA ATTGGGACGT CCCCACATGA AGAAGTGTGT

CGCTCTCACC ACTTACCGTC GTTTACACGA CATGTACTCG GCAACCTTCA AGCGGAAGCC 240
GCGAGAGTGG TGAATGGCAG CAAATGTGCT GTACATGAGC CGTTGGAAGT TCGCCTTCGG

CAACGAGGGT GTCGGCCACG GCCTACCTAA ACACTCAAGT CTCTAGTCCA GGTTTATCGC 300
GTTGCTCCCA CAGCCGGTGC CGGATGGATT TGTGAGTTCA GAGATCAGGT CCAAATAGCG

CTATCCAGGT TCCATCCGCA GGAGTCCGGC CGAGGTTTCC ACATACGGCC CCGGACGATG 360
GATAGGTCCA AGGTAGGCGT CCTCAGGCCG GCTCCAAAGG TGTATGCCGG GGCCTGCTAC

TGTACAGGGT TCCGAGACAC CAAACGGGCG CCCAGCATGC CCGGCCATGG TGTATCTACC 420
ACATGTCCCA AGGCTCTGTG GTTTGCCCGC GGGTCGTACG GGCCGGTACC ACATAGATGG

GCATCATAGC CCACCCCTAG GGTTAGCGTA CGCACGGTCG CCAACACATA TCCTATAAAC 480
CGTAGTATCG GGTGGGGATC CCAATCGCAT GCGTGCCAGC GGTTGTGTAT AGGATATTTG

ACCAGAAACT AGTTGCAACT CCTGGACAGA GGACAAGGGT GATCAAGAAG CCGAGAGGGT 540
TGGTCTTTGA TCAACGTTGA GGACCTGTCT CCTGTTCCCA CTAGTTCTTC GGCTCTCCCA

CCATTGGTTT CGGGCCCAAT GTGTGGTAGT AACTATTTCA AGGATCACAA ACACAGAACT 600
GGTAACCAAA GCCCGGGTTA CACACCATCA TTGATAAAGT TCCTAGTGTT TGTGTCTTGA

CGGTTCCTAA GGACGGTTTC AATGAGACAA CCCACCATGT ACTCCTACAT GGCCTCTCAC 660
GCCAAGGATT CCTGCCAAAG TTACTCTGTT GGGTGGTACA TGAGGATGTA CCGGAGAGTG

CGCTACCTTT ACCAAATCGT GTTCACACAC TTAGCTCACA CACAGTAGGA CATGTTCATC 720
GCGATGGAAA TGGTTTAGCA CAAGTGTGTG AATCGAGTGT GTGTCATCCT GTACAAGTAG

ACTGTTCCAA TTCATCCCCG ATGAATCAGA CCTGACTCAA CTCTAAGCAG TAGCAGGCAT 780
TGACAAGGTT AAGTAGGGGC TACTTAGTCT GGACTGAGTT GAGATTCGTC ATCGTCCGTA

GACAAATAAG CATGAATGAG TAGGCACATC AGGGCTCAAA CAACTCCTAC TCATCTAGTG 840
CTGTTTATTC GTACTTACTC ATCCGTGTAG TCCCGAGTTT GTTGAGGATG AGTAGATCAC

GGTTTCATCT ATTTACTGTG GCAATGACAG GTCATGTAGA GGAAAGGGGT TCAACTACCG 900
CCAAAGTAGA TAAATGACAC CGTTACTGTC CAGTACATCT CCTTTCCCCA AGTTGATGGC

CGACATGTAA CAGTTGAATC GTTGTTGCCA ATGCAGTAAA AGAGAGCAGG AGTGAGAGAG 960
GCTGTACATT GTCAACTTAG CAACAACGGT TACGTCATTT TCTCTCGTCC TCACTCTCTC

TGGGATTGTA TCAGAATGAA CAAGGGTGTT TTGCTTGCCT GGCACTTCTG AAGATATTAT 1020
ACCCTAACAT AGTCTTACTT GTTCCCACAA AACGAACGGA CCGTGAAGAC TTCTATAATA

AGCTCTTCAT CGGTGTCATC GAACCCATCG TCGAAACCAC GTCTATCGAG AGGGGACAAA 1080
TCGAGAAGTA GCCACAGTAG CTTGGGTAGC AGCTTTGGTG CAGATAGCTC TCCCCTGTTT

TACCGGCTAC AGGAGAAAGA AACACAATCA ATGCAATGCA CAATATGAGG CATGCTCATG 1140
ATGGCCGATG TCCTCTTTCT TTGTGTTAGT TACGTTACGT GTTATACTCC GTACGAGTAC

ACATGTCAAT ATGCTATGGT TTGAGCTAAT GCAACTAGCA ACATGTTAAA TGGGGTTGGT 1200
```

TGTACAGTTA TACGATACCA AACTCGATTA CGTTGATCGT TGTACAATTT ACCCCAACCA

TTGAACCCTA GGTTCAAATT CAAACTCCAT ATGTGAGAGT TTAAATTCCA TTTATATGAA 1260
AACTTGGGAT CCAAGTTTAA GTTTGAGGTA TACACTCTCA AATTTAAGGT AAATATACTT

TTGGCCTAAT CAGCAGCCAT AAGTTGTTCT AACATGCATG AAAATGCCAT AGACAATTCC 1320
AACCGGATTA GTCGTCGGTA TTCAACAAGA TTGTACGTAC TTTTACGGTA TCTGTTAAGG

TTGAATTTTT CTGATAATTT TTCATATATA ATTTATTTCA TTTGGAGTTA CGTTGAATTT 1380
AACTTAAAAA GACTATTAAA AAGTATATAT TAAATAAAGT AAACCTCAAT GCAACTTAAA

CTATGATTTT TAGAAGTTTA AATTGTTTCC TGGAATTTCC TGTATTATTT TAAATCCAGA 1440
GATACTAAAA ATCTTCAAAT TTAACAAAGG ACCTTAAAGG ACATAATAAA ATTTAGGTCT

AATTCCTTTA CTGCGTCAGA CTACGTCATT ATGATGTTAG CAGGTCAACA GGGCTGGTTA 1500
TTAAGGAAAT GACGCAGTCT GATGCAGTAA TACTACAATC GTCCAGTTGT CCCGACCAAT

GAGTCAAACC TGACACATGG GACCCACCGG TCAGTGTCAT TAGAATAACT AATTTTTTGT 1560
CTCAGTTTGG ACTGTGTACC CTGGGTGGCC AGTCACAGTA ATCTTATTGA TTAAAAAACA

TAGTCCTAAT TCTAGGGTAA TTAGTAGGGC GGGCCCACAT GTCAGTGGCT CATCTAATTA 1620
ATCAGGATTA AGATCCCATT AATCATCCCG CCCGGGTGTA CAGTCACCGA GTAGATTAAT

GCTAAGTTAA TTAACACTAA CTAAACCTAA CACTAATTAG ACAGGCGCCT GGGCCCACAC 1680
CGATTCAATT AATTGTGATT GATTTGGATT GTGATTAATC TGTCCGCGGA CCCGGGTGTG

GTCAGTGGAG GTCAAACCCC TGGTCAACCA GGGCTAAACC ACCAGCGTTT AGCCGCCGGC 1740
CAGTCACCTC CAGTTTGGGG ACCAGTTGGT CCCGATTTGG TGGTCGCAAA TCGGCGGCCG

GATGACCAAA CACGGCGGAG GCCGTGGGAA TGGGCTCTGG GGCCCCGTTC GTAGCGTGGT 1800
CTACTGGTTT GTGCCGCCTC CGGCACCCTT ACCCGAGACC CCGGGGCAAG CATCGCACCA

TGGGCTCGTT CGAGAGCCCA GACCAGCACG CATCGGATGG TGGCACCAGC CGGGCCTGGG 1860
ACCCGAGCAA GCTCTCGGGT CTGGTCGTGC GTAGCCTACC ACCGTGGTCG GCCCGGACCC

GCGGCCGGAG CGACGGCGGG GAGCTCGGCC ACGGCGGCCG GAGCTCGGGC GTGCTCGGGT 1920
CGCCGGCCTC GCTGCCGCCC CTCGAGCCGG TGCCGCCGGC CTCGAGCCCG CACGAGCCCA

TCAGCGCTGG GATGCACAAC AGGGGCGTGG AGAGGGGCTA TGGCCTCCTG GGAGTGTGGC 1980
AGTCGCGACC CTACGTGTTG TCCCCGCACC TCTCCCCGAT ACCGGAGGAC CCTCACACCG

AAACACGGTG ACGCGCTCGG TTTCGAGCCC GCGTGGCTGT GGCCACGACG ACGGCATCGC 2040
TTTGTGCCAC TGCGCGAGCC AAAGCTCGGG CGCACCGACA CCGGTGCTGC TGCCGTAGCG

CGGCGGAGCA GAGTGCTCGG TCTCGGTGGA GATGGCGGCT AGGGGGCGCA ACCAAGGCGT 2100
GCCGCCTCGT CTCACGAGCC AGAGCCACCT CTACCGCCGA TCCCCCGCGT TGGTTCCGCA

AGAGGAGGGG GGTTAGGGGC AGGGGCTCAC GGTGGATGCA AAGGTGGCCT CGGCGTGCTC 2160
TCTCCTCCCC CCAATCCCCG TCCCCGAGTG CCACCTACGT TTCCACCGGA GCCGCACGAG

GGGGAAGCAC CGGAGCGAGC GGGGCGGCGA GAGGCATCTC CGATGGACAA AGGTTGAAGA 2220
CCCCTTCGTG GCCTCGCTCG CCCCGCCGCT CTCCGTAGAG GCTACCTGTT TCCAACTTCT

AGACCTCGAA GGCGACGCTG TAGGGCTTCC GGCGGGGCAT GGTTCGGTGA GGTGGTCAAG 2280
TCTGGAGCTT CCGCTGCGAC ATCCCGAAGG CCGCCCCGTA CCAAGCCACT CCACCAGTTC

GACGACGTCG CGGAACTCGT GGGCACAACA GAGGGGCGAG GGGGTAGCTG TGGCCGTGGT 2340
CTGCTGCAGC GCCTTGAGCA CCCGTGTTGT CTCCCCGCTC CCCCATCGAC ACCGGCACCA

GGTTCTCATC GGCGGCGGCG GACGCGTTCG GTGGTGCGAG GGAGAGAGAC AGGGGAGGGG 2400
CCAAGAGTAG CCGCCGCCGC CTGCGCAAGC CACCACGCTC CCTCTCTCTG TCCCCTCCCC

ATGAGCACGG GGGGGGGGGG GGGGGGAAGG AGTGAGAGGG CCTGGTGGTG CGTGGCCGTC 2460

Primer WSP1

TACTCGTGCC CCCCCCCCCC CCCCCCTTCC TCACTCTCCC GGACCACCAC GCACCGGCAG

TCCGTCGCGC TGGAAGAGAG CCTTAGGGGC GCGAGGCAGG CAGGGAGGAG GTGGCGGCCT 2520
AGGCAGCGCG ACCTTCTCTC GGAATCCCCG CGCTCCGTCC GTCCCTCCTC CACCGCCGGA

CAGCACGCGC CGTGCCTGCC TTCGCCTCTG CCTACTGGCA GAGGTTGAAG ACGACTGGCA 2580
GTCGTGCGCG GCACGGACGG AAGCGGAGAC GGATGACCGT CTCCAACTTC TGCTGACCGT

CTGGCCAGCT GGGCTAGGCT GGCTGGAGGA GCTGGGCCGC TACAGTAACA GGCCAGGTGG 2640
GACCGGTCGA CCCGATCCGA CCGACCTCCT CGACCCGGCG ATGTCATTGT CCGGTCCACC

Primer WSP2

GCTTTGCCAG GTAGGTTCCT CTCTTTCTAA TTCTGTTTCT GTTTTCTATT TATGTTATTT 2700
CGAAACGGTC CATCCAAGGA GAGAAAGATT AAGACAAAGA CAAAAGATAA ATACAATAAA

TGTTTTGATT TAGTTTATAA CCCAAACCAT TTTAATAAAT CCTGAAAATA ATTGTGGGCA 2760
ACAAAACTAA ATCAAATATT GGGTTTGGTA AAATTATTTA GGACTTTTAT TAACACCCGT

TTAAATGAAA TTATTGCAGA TGCCCTTAAC TATTTTCAGA ATTATTGGAG CATTTAAAAA 2820
AATTTACTTT AATAACGTCT ACGGGAATTG ATAAAAGTCT TAATAACCTC GTAAATTTTT

TATTAATAGT ATTTAAAGGC CCCAATTCAA ATACAATATG GGTTATTCAA AAATCCAGAA 2880
ATAATTATCA TAAATTTCCG GGGTTAAGTT TATGTTATAC CCAATAAGTT TTTAGGTCTT

TGGCCTAAAA GTACGTGCAT CATTTTTGGC AGAGGTTTCA CCTTTATCAA AATAATGAAC 2940
ACCGGATTTT CATGCACGTA GTAAAAACCG TCTCCAAAGT GGAAATAGTT TTATTACTTG

TTTTCTAAAG GGCATTATGG GTTCATTGAA AATATTTTTA TTTTGATCCT AATTACATTT 3000
AAAAGATTTC CCGTAATACC CAAGTAACTT TTATAAAAAT AAAACTAGGA TTAATGTAAA

CATTTAGTGC TAGGGCTTGT CATCCCCATT TCAAATTTTC TGAGTAAAGT AGACATGATG 3060
GTAAATCACG ATCCCGAACA GTAGGGGTAA AGTTTAAAAG ACTCATTTCA TCTGTACTAC

CAACACCATA TGTTAGCACT AGGCATTACC AGAACTTGGG ATGTGACAAC TCACCCCCAC 3120
GTTGTGGTAT ACAATCGTGA TCCGTAATGG TCTTGAACCC TACACTGTTG AGTGGGGGTG

TAAAACAAGA ATCTCGTCCC GAGATTCAGG AAGTGGAGTA AGAAGACAGA GGGGTCACAA 3180
ATTTTGTTCT TAGAGCAGGG CTCTAAGTCC TTCACCTCAT TCTTCTGTCT CCCCAGTGTT

AACTATCACA GTCTTATCGA TTGAACTGTC CTTCTCGAAG ATACCGGTAT ATTGCATCAC 3240
TTGATAGTGT CAGAATAGCT AACTTGACAG GAAGAGCTTC TATGGCCATA TAACGTAGTG

ATTGCTCTTG ACGTCTTTGT CTTCGAGATC TTCATCCAAC ACGATGAAGA GGGAAGGAAA 3300
TAACGAGAAC TGCAGAAACA GAAGCTCTAG AAGTAGGTTG TGCTACTTCT CCCTTCCTTT

CTCTACAGAA AACGATCTTC TCAAAGATCG AGCAACTTAA GATCAACTCA TGGAGTGAGA 3360
GAGATGTCTT TTGCTAGAAG AGTTTCTAGC TCGTTGAATT CTAGTTGAGT ACCTCACTCT

TATTGGAACA TCTCTTGAGC TGAGACACTA AACACACATC GAGAGAGATG GAAGGTAATA 3420
ATAACCTTGT AGAGAACTCG ACTCTGTGAT TTGTGTGTAG CTCTCTCTAC CTTCCATTAT

GATGACAAGG GTTCAAATTG GTAGGCAACA ATTCCACGTT GGAGTAGGAT GGTGCATGGT 3480
CTACTGTTCC CAAGTTTAAC CATCCGTTGT TAAGGTGCAA CCTCATCCTA CCACGTACCA

Identical to *SPO11*

TTCAAAGTAG CGAGGAGATA ATTGCCATGA TTCCATAATG GGGCACCTTA GGGAAAAATG 3540
AAGTTTCATC GCTCCTCTAT TAACGGTACT AAGGTATTAC CCCGTGGAAT CCCTTTTTAC

ACTCGTAGAG CATTTCCCTT AAGTGGCAAA AAGAATTACT TTTGATACAA AGATCATTGA 3600
TGAGCATCTC GTAAAGGGAA TTCACCGTTT TTCTTAATGA AAACTATGTT TCTAGTAACT

```
AACTCTCTAT ACCAGCCTAG GTAATGACNG GTATAACACA ATCGTACGTT GGAAGGGGTT 3660
TTGAGAGATA TGGTCGGATC CATTACTGNC CATATTGTGT TAGCATGCAA CCTTCCCCAA

TGAAAGAATG GCATACTCCG ACTAGGTGGA TGATGTGGAC TATGTTGTTG AAGACAAGAA 3720
ACTTTCTTAC CGTATGAGGC TGATCCACCT ACTACACCTG ATACAACAAC TTCTGTTCTT
                                                  Primer WSP3

TGGGATTGAT TTTTGCCTTA TCATCGGGAA TGGATGAGAC CCATGGTAAG TTCACTTTTG 3780
ACCCTAACTA AAAACGGAAT AGTAGCCCTT ACCTACTCTG GGTACCATTC AAGTGAAAAC

AAGATGATCC TGAACAACAA CTACTGAGGT GTAAACTGGG AACAAAATGC AAATTGTTGG 3840
TTCTACTAGG ACTTGTTGTT GATGACTCCA CATTTGACCC TTGTTTTACG TTTAACAACC

GAATGATTCT AGTAATGGGG AGAAGGTTCA CCAGCAAAGG TGATTCCACC AATTGGTATG 3900
CTTACTAAGA TCATTACCCC TCTTCCAAGT GGTCGTTTCC ACTAAGGTGG TTAACCATAC

GCTATCGAAG AAGTGAGAGA ACGGAGGCAC ATTCCAGTTA ACGACAACAT CAGAACCTAG 3960
CGATAGCTTC TTCACTCTCT TGCCTCCGTG TAAGGTCAAT TGCTGTTGTA GTCTTGGATC

TGCCTGAGCG TGCTCTCAAG AACTTGAGCA TTTCCATACG CATCACGGTC TTACCAACAT 4020
ACGGACTCGC ACGAGAGTTC TTGAACTCGT AAAGGTATGC GTAGTGCCAG AATGGTTGTA

CCGTGCCGAG GATC
GGCACGGCTC CTAG
```

Fig. 5.9.B. Nucleotide sequence of pAWJL4.1. Potential signature sequences regulating gene expression are double underlined (TATA box, CAAT box and polyadenylation signal). The sequences dotted underlined are aditional CAAT boxes are and an ATTTA motif that implies instability and rapid turn-over (Shaw and Kamen, 1986). The putative translation initiation and termination codons are shown in **Bold**. The sequence of the three primers used for PCR (section 5.4.8) and the 21 bases sequence identical to the yeast *SPO11* gene are indicated beneath the sequences.

5.4.6. Sequence analysis of pAWJL4.1

When the sequence of both strands of pAWJL4.1 was scanned for potential ORFs using the PC/Gene computer program, the longest ORF with a potentially functional translation initiation codon lies between base 1742 and 2113 (Fig. 5.9, upper row). This ORF will be

referred to as ORFA. Upstream of ORFA, a putative TATA box and CAAT box were found (double-underlined). The distance between the two boxes is consistent with most eukaryotic promoters (Messing, 1986). Interestingly, further upstream of the TATA box, there were an additional 11 potential CAAT boxes (underlined), with four are arranged in a cluster (bases 1105-1124). Downstream of ORFA, there is a potential polyadenylation signal AATAAA (base 2734, double-underlined). The distance between the stop codon of the ORFA and the AATAAA motif is unusually long (626 base) but the sequence in-between is interupted by only two in-frame stop codons at base 2402 and 2711 and has a similar C+G content to ORFA. The region from TATATAAT (base 1353) to AATAAA (base 2734) will be called Transcript A and the translated amino acid sequence from the putative translation initiation signal to the third in-frame stop codon (base 2711) within the Transcript A will be called ORFB. Transcript A lies within the high copy number region of pAWJL4.1 and the sequence contains a 18 bases long poly(dG) tract (bases 2409-2426). The potential activity and function of this transcript will be discussed later.

The nucleotide sequences of pAWJL4.1 and the yeast *SPO11* gene were compared using the MacMolly-Compare program. The best match was a 21 bases perfect homology located in the reverse complement of the sequence shown in Fig. 5.9.B. The alignment of the sequences around the region is shown in Fig. 5.10.A.

A.

```
W (3478)  CAT GCA CCA TCC TAC TCC AAC GTG GAA TTG TTG CCT ACC AAT TTG AAC
              |       | | ||| ||| ||| ||| ||| ||| ||  |   |    |      |||
Y (391)   AGA GAT ATC TTC TAC TCC AAC GTG GAA TTG TTT CAA AGA CAA GCA AAC

W (3430)  CCT TGT CAT CTA TTA CCT TCC ATC TCT CTC GAT GTG TGT TTA GTG TCT
                  ||       |          ||       |   ||  |       |
Y (439)   GTA GTC CAG TGG CTG GAC GTT ATA CGC TTT AAT TTC AAG CTC TCT CCA
```

B.

```
Wheat   H A P S Y S N V E L L P T N L N P C H L L P S I S L D V C L V S
        : :     | | | | | | :   : : : |     :   |     | : : :     |
Yeast   R D I F Y S N V E L F Q R Q A N V V Q W L D V I R F N F K L S P
```

Fig. 5.10. Sequence alignment of yeast *SPO11* gene to a putative partial exon of the wheat homologue. Identities are indicated by | and conservative substitutions are indicated by :. W and Y represent wheat and yeast. A. Alignment of nucleotide sequences (Atcheson *et al.*, 1987). A potential end (GT) of the exon is underlined (Brown, 1986). B. Alignment of amino acid sequences.

When the sequence surrounding the 21 nt homologous sequence was translated and compared to the yeast *SPO11* gene, the two sequences displayed 38.5% identity or 69.2% homology including conservative substitutions over a 26 aa overlap (Fig. 5.10 .B). The 5' of the wheat sequence was interrupted by an in-frame stop codon (TGA) but no potential 5' splicing site (AG:GT) could be found.

5.4.7. The Primary Structure of the Putative ORFA and ORFB

The amino acid sequence of the ORFA protein would contain 124 aa (13.3 kDa). The motif Gly-Asn-Gly-Leu-Trp-Gly-Pro-Val at the N-terminus (underlined) is similar to the nucleotide binding site of protein kinases, (Leu, Iso or Val)-Gly-X-Gly-X-X-(Gly, Ala or ser)-X-Val (Hanks *et al.*, 1988), and is identical to the consensus sequence of general nucleotide binding site, Gly-X-Gly-X-X-Gly (Wierenga and Hol, 1983). The ORFA is rich in glycine (18.5%) and arginine (16.1%). No zinc finger, helix-loop-helix or leucine-zipper motifs are evident. The biological activity of putative nucleotide binding sites cannot be determined from sequence data alone.

```
Met Thr Lys His Gly Gly Gly Arg Gly Asn Gly Leu Trp Gly Pro Val Arg 17
ATG ACC AAA CAC GGC GGA GGC CGT GGG AAT GGG CTC TGG GGC CCC GTT CGT

Ser Val Val Gly Leu Val Arg Glu Pro Arg Pro Ala Arg Ile Gly Trp Trp 34
AGC GTG GTT GGG CTC GTT CGA GAG CCC AGA CCA GCA CGC ATC GGA TGG TGG

His Gln Pro Gly Leu Gly Arg Pro Glu Arg Arg Arg Gly Ala Arg Pro Arg 51
CAC CAG CCG GGC CTG GGG CGG CCG GAG CGA CGG CGG GGA GCT CGG CCA CGG

Arg Pro Glu Leu Gly Arg Ala Arg Val Gln Arg Trp Asp Ala Gln Gln Gly 68
CGG CCG GAG CTC GGG CGT GCT CGG GTT CAG CGC TGG GAT GCA CAA CAG GGG

Arg Gly Glu Gly Leu Trp Pro Pro Gly Ser Val Ala Asn Thr Val Thr Arg 85
CGT GGA GAG GGG CTA TGG CCT CCT GGG AGT GTG GCA AAC ACG GTG ACG CGC

Ser Val Ser Ser Pro Arg Gly Cys Gly His Asp Asp Gly Ile Ala Gly Gly 102
TCG GTT TCG AGC CCG CGT GGC TGT GGC CAC GAC GAC GGC ATC GCC GGC GGA

Ala Glu Cys Ser Val Ser Val Glu Met Ala Ala Arg Gly Arg Asn Gln Gly 119
GCA GAG TGC TCG GTC TCG GTG GAG ATG GCG GCT AGG GGG CGC AAC CAA GGC

Val Glu Glu Gly Gly TER 125
GTA GAG GAG GGG GGT TAG
```

Fig. 5.11. The amino acid sequence of ORFA. The potential nucleotide binding site is underlined.

When the nucleotide sequence of both strands was scanned against the EMBL and Genebank nucleotide data bases using the FASTA computer program (Pearson and Lipman, 1988), no significant match was found. The best matches are those that contain C+G-rich sequences, such as the mammalian immunoglobulin gene and the *Alu*-type repeats. Similarly, no analogous protein was found for ORFA.

Interestingly, two entries showed a high rate of similarity to the hypothetical ORFB. One is the human immune system gene, *Bat2* (Banerji *et al.*, 1990) and the other is a hypothetical ORF of *Micrococcus luteus* overlapping the UV repair gene, *UvrA* (Shiota and Nakayama, 1989). Although the identical amino acids are only about 20%, the homology is over 70%, if conserved substitutions are included (Fig. 5.11).

Micrococcus luteus ORF1 (23.8% identity in 206 aa overlap)

```
              20         30         40         50         60
ORFB     LWGPVRSVVGLVREPRPARIGWWHQPGLGRPERRRGARPRRPELGRARVQRWDAQ-----
                                        | ||:  ||||::|||| :|: ::
ORF1     GETPVSCPCPPLCWPRAQELLHHRLLRRRGPRRRPRLRPRRRALGRAGPHRRPGRPRAQP
         40         50         60         70         80         90

          70         80         90        100        110        120
ORFB     QGRGEGLWPPGSVANTVTRSVSSPRGCGHDDGIAGGAECSVSVEMAARGRNQGVEEGGGQ
         :|||  | |:|: ::: ::   ::   |  :::  |::   :|::  | : :| :: :|:
ORF1     EGRGCQL-PAGRHGRVHGSVRLGQVLPGLRHDLRRGPAALRRVALLLRPHVPGPGGQAGR
         100        110        120        130        140        150

         130        140        150        160        170         180
ORFB     GLTVDAKVASACSGKHRSERGGERHLRWTKVEEDLEGDAVGLPAGHGSV--RWSRTTSRN
         ||    : : : : :::: :  :: |: :: : ||  :| :| : :|::    :|:::::
ORF1     GL---HRGPVPGRVHRPEVHQPQPALHSGDHHRDLRLHAPALGTCRGAALPAVRRAGEPA
           160        170        180        190        200        210

           190        200        210        220        230        240
ORFB     SWAQQRGEGVAVA-VVVLIGGGGRVRWCEGERQGRGARGGGGGEGVRGPG-GAWPSPSRW
         : |::|| : ::| :  | |: :| :: :|  : ||:  |   :|:| :| |::|:
ORF1     DPAADRGPARGAARAHPLPGARARGPRPQG--RVRGPVQGPVHAGLRRRGRGDRPALGPA
           220        230        240         250        260        270
```

```
Human Bat2 (19.0% identity in 210 aa overlap)

          20        30        40        50        60        70
ORFB      RSVVGLVREPRPARIGWWHQPGLGRPERRRGARPRRPELGRARVQRWDAQQGRGEGLWPP
                                        |:|||||:  |||: |   ::  | :  :
Bat2      EPLKEKLIPGPLSPVARGGSNGGSNVGMEDGERPRRRRHGRAQQQDKPPRFRRLKQEREN
              1200      1210      1220      1230      1240      1250

          80        90        100       110       120       130
ORFB      GSVANTVTRSVSSP-RGCGHDDGIAGGAECSVSVEMAARGRNQGVEEGGGQGLT-VDAKV
          :: ::: ::|:: | :: |:::::::: :  :::   ||:::: : :::: :: :  :|:
Bat2      AARGSEGKPSLTLPASAPGPEEALTTVTVAPAPPRAAAKSPDLSNQNSDQANEEWETASE
              1260      1270      1280      1290      1300      1310

            140       150       160       170       180       190
ORFB      ASACSGKHRSERGGERHLRWT-KVEEDLEGDAVGLPAGHGSVRWSRTTSRNSWAQQRGEG
          :|: ::::|::::: :::  | |: :: :|:: |  :| :::::  : |::   :|: :
Bat2      SSDFTSERRGDKEAPPPVLLTPKAVGTPGGGGGGAVPGISAMSRGDLSQRAKDLSKRSFS
              1320      1330      1340      1350      1360      1370

                200       210       220       230       240
ORFB      VAVAVV----VLIGGGGRVRWCEGERQGRGARGGGGGEGVRGPGGAWPSPSRWKRALGAR
           : : :       | ||::  ::::: | |:  || ::  ||   :||||::::|: ::|
Bat2      SQRPGMERQNRRPGPGGKAGSSGSSSGGGGGGPGGRTGPGRGDKRSWPSPKNRSRPPEER
              1380      1390      1400      1410      1420      1430
```

Fig. 5.12. Alignment of the hypothetical ORFB to search sequences. Identical residues are indicated by | and conservative substitutions are indicated by :.

5.4.8. Detection of Gene Expression from pAWJL4.1

In order to determine if any part of the pAWJL4.1 is transcribed and whether transcription occurs preferentially in meiocytes, crude total RNA was extracted from leaves, spikelets at early meiotic stages (mostly at prophase I) and spikelets at late meiotic stages (mostly after prophase I). mRNA of the samples were isolated by oligo(dT) chromatography. Both poly(A)$^+$ and poly(A)$^-$ RNA were fractionated on a formaldehyde-agarose gel and transferred to Hybond-N+. When the blot was probed with pAWJL4.1, two faint bands of about 1000 and 1500 bases and a weak diffuse band of about 200-300 bases were observed in the poly(A)$^-$ RNA fraction from spikelets at the early meiotic stages but not from leaves or spikelets at late

meiotic stages. Similarly, a weak smear was visible throughout the lane with poly(A)+ RNA isolated from early-stage spikelets but not from leaves or late-stage spikelets. Interestingly, the 200-300 bases signal seen in the poly(A)- RNA was not present in the poly(A)+ fraction (Fig. 5.13).

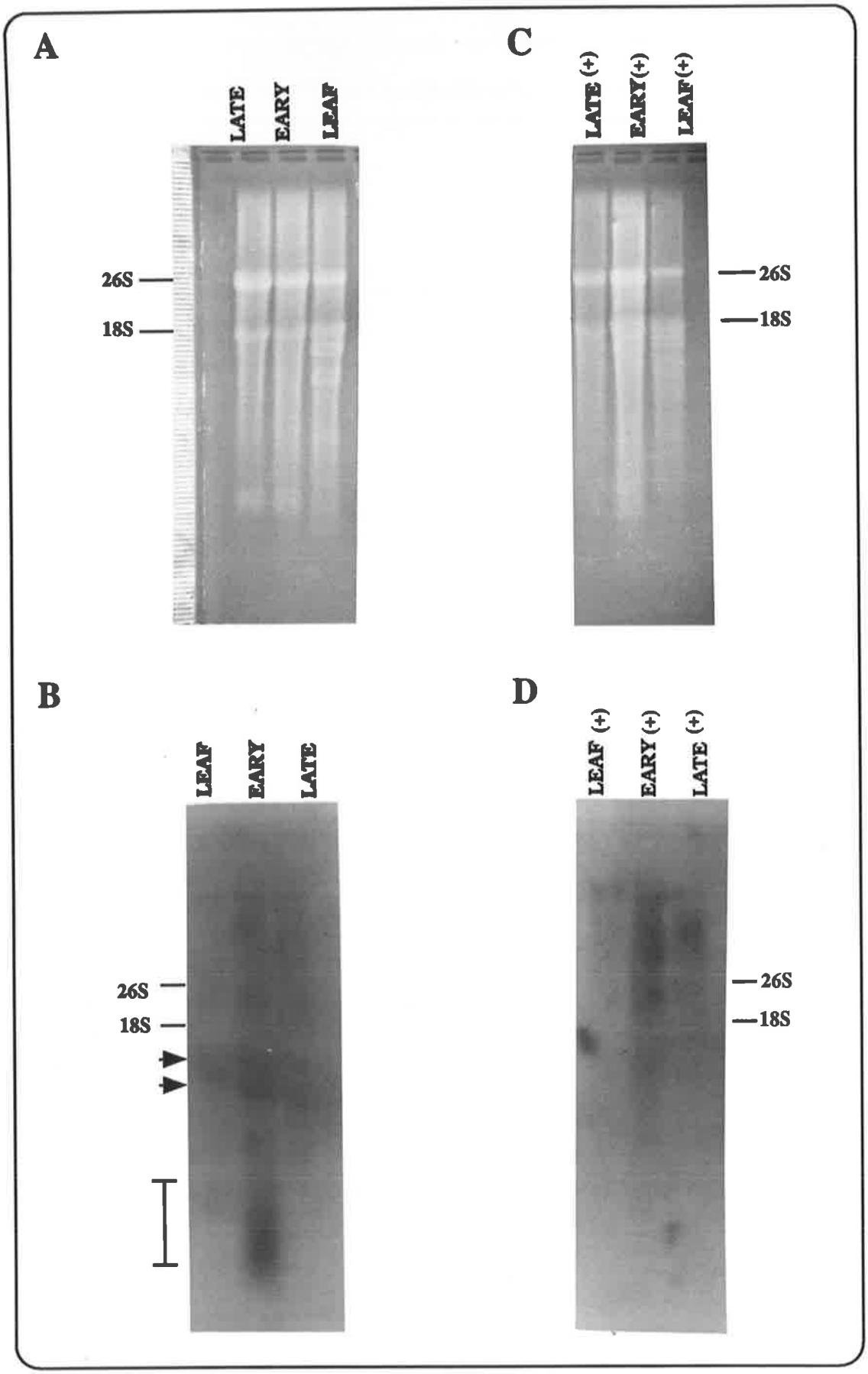
Fig. 5.13. Detection of Gene Expression within pAWJL4.1 by Northern Hybridisation

Total RNA (c.a. 20 μg) or oligo(dT)-cellulose purified mRNA [i.e., about 15 μg poly(A)+ RNA] extracted from different tissues were fractionated on a 1.5% formadehyde-agarose gel, transferred onto Hybond-N+ membrane and probed with pAWJL4.1. The membrane was exposed for 11 days at -70°C between intensifying screens.

EARLY and LATE represent spikelets with most anthers at prophase I and post-prophase I.

A. Ethidium bromide stained gel of the total RNA.
B. Autoradiograph of the gel shown in A probed with pAWJL4.1. The weak. hybridisation signals are indicated by arrows and the bar low down the gel.
C. Ethidium bromide stained gel of the poly(A)+ RNA.
D. Autoradiograph of the gel shown in C.

The positions of the 26S and 18S rRNAs are indicated.


A
LATE
EARY
LEAF
26S
18S
C
LATE (+)
EARY(+)
LEAF(+)
26S
18S
B
LEAF
EARY
LATE
26S
18S
D
LEAF (+)
EARY (+)
LATE (+)
26S
18S

A more precise analysis of the time of expression of the genes detected on the Northern blot was undertaken using RNA from specific meiotic stages (Fig. 5.15) isolated from 12 anthers. The total RNA was converted into single strand cDNA using oligo(dT_{8-12}) as primer and moloney murine leukemia virus reverse transcriptase. The region to be amplified lies in the 3' region of the putative Transcript A.

Since the method for RNA quantification by PCR did not directly visualise the RNA in the sample, a control experiment used to monitor the efficiency of the reactions and the recovery rate of cDNA in individual samples. The ubiquitin gene of plants is one of the genes required for many cellular activities and is expressed in all tissues with little variation in abundance (Berke *et al.*, 1988; Callis and Vierstra, 1989). It is probable that the gene will be maintained at quite a constant level during meiosis. Therefore, two primers were designed to amplify the coding region of the wheat ubiquitin gene according the sequence of the barley gene (Gausing and Barkardottir, 1986). The ubiquitin gene sequence amplified from different tissues were used as a control for the efficiency of RNA extraction, cDNA synthesis and DNA amplification of the Transcript A. As can be seen in Fig. 5.14, the signal for the ubiquitin gene was the strongest in root tips but quite uniform in the anthers of various meiotic stages. This contrasts the signal profile produced using primers WSP1 and WSP2. With these primers, the signal was the weakest in root tips but started to increase upon entry into meiosis (leptotene) and stayed at high level even after completion of meiosis (pollen). This indicated that Transcript A or a related sequence was transcribed *in vivo* and the expression is related to meiosis.

Fig. 5.14. RT-PCR Detection of Expression of the pAWJL4.1 gene at Different Stages of Meiosis

Total RNA was isolated from 12 anthers of wheat (Chinese Spring) and half of the sample was converted into single-stranded cDNA with M-MLV reverse transcriptase and oligo$(dT)_{8\text{-}12}$ as primer. The cDNA (one third) was amplified by 30 cycles of PCR with primers WSP1 and WSP2. For ubiquitin gene, one tenth of the cDNA was amplified by 30 cycles of PCR with primers UB1 and UB2. All conditions for PCR were the same as the primers WSP1 and WSP2 except that the annealing was performed at 65°C and Taq DNA polymerase added only after the whole mix had been heated to 65°C before amplification was started.

A. The PCR product resolved on a 1.8% agarose gel. Samples 1 to 9 represent root tip, 1; interphase (A), 2; leptotene (B), 3; zygotene (C), 4; early prophase II (F and G), 5; tetrad (H), 6; pollen (I), 7; no template control, 8; and positive control, 9 (pAWJL4.1 for the Transcrit A and total Chinese Spring DNA for ubiquitin gene) (the letters in bracket represent the stages recorded in Fig. 5.15). M denotes size marker (λdv I *Hae* III). The results obtained for Transcript A are shown on the left of M and the results with ubiquitin primers on the right.

B. An illustration of the locations of primers WSP1 and WSP2.

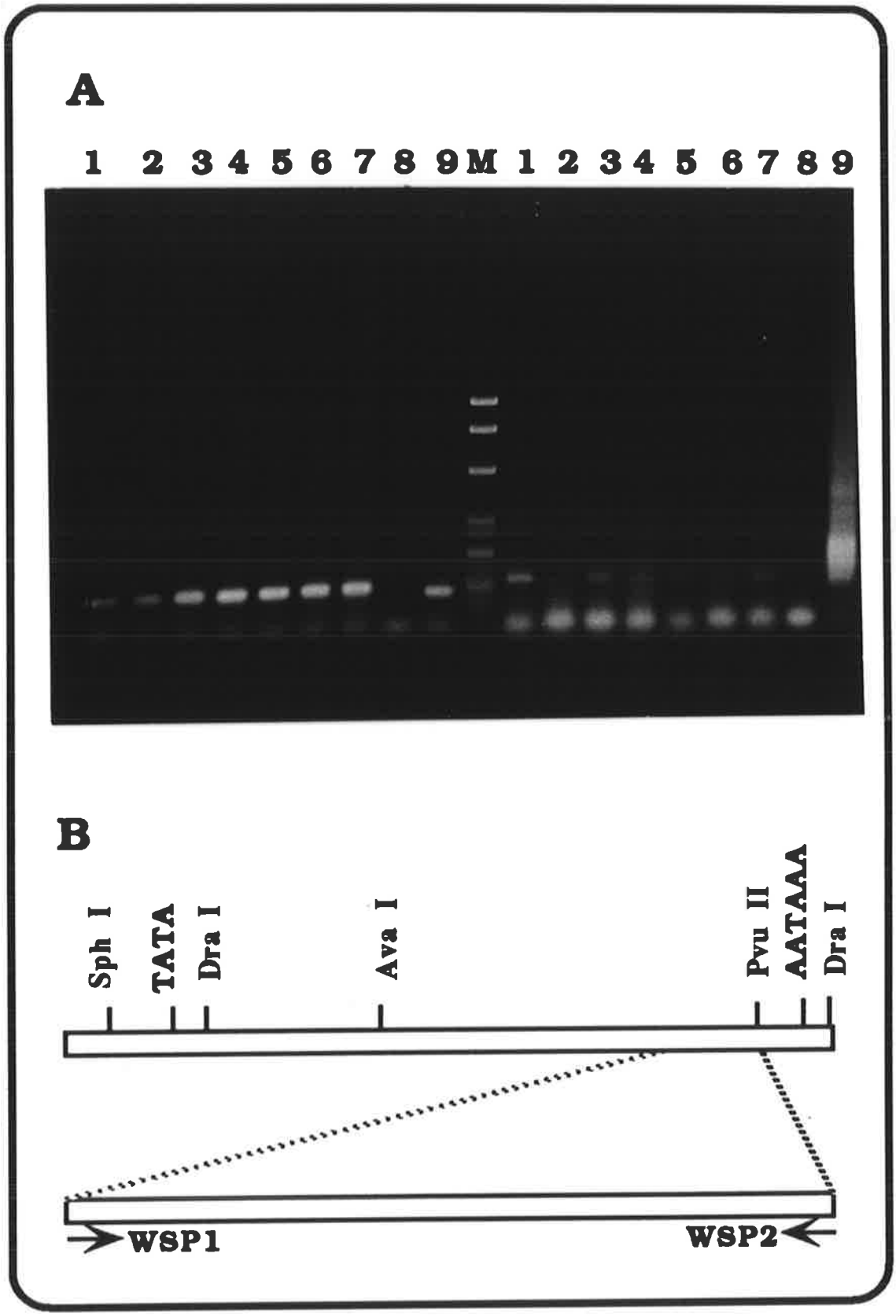

A
1 2 3 4 5 6 7 8 9 M 1 2 3 4 5 6 7 8 9
B
Sph I
TATA
Dra I
Ava I
Pvu II
AATAAA
Dra I
WSP1
WSP2

Fig. 5.15. A Record of the Meiotic Stages of the Anthers Used to Obtained the RNA and DNA for the Experiments Shown in Figurs 5.14. and 5.18

One of the florets was removed from each spike and fixed in 3:1 ethanol:acetic acid for 30 min.. One anther was squashed in aceto-orcein and photographed.

A. Interphase.

B. Leptotene.

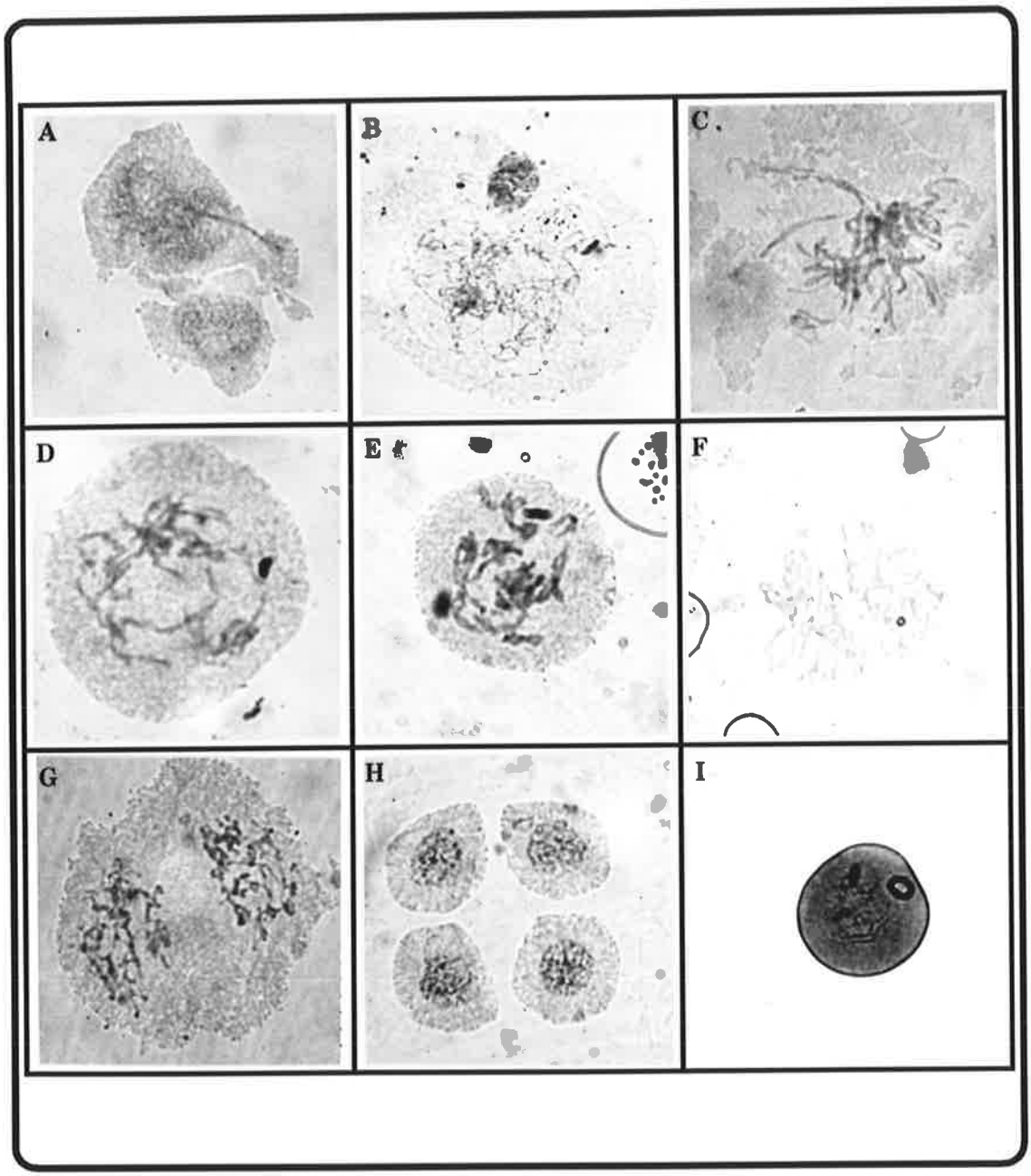
C. Zygotene.

D and E. Pachytene-diplotene.

F and G. Early prophase II.

H. Tetrad.

I. Pollen.


A
B
C
D
E
F
G
H
I

5.5. Identification of a Common DNA Sequence in the WSPs and the Genomic Clones of pAWJL1 and pAWJL3

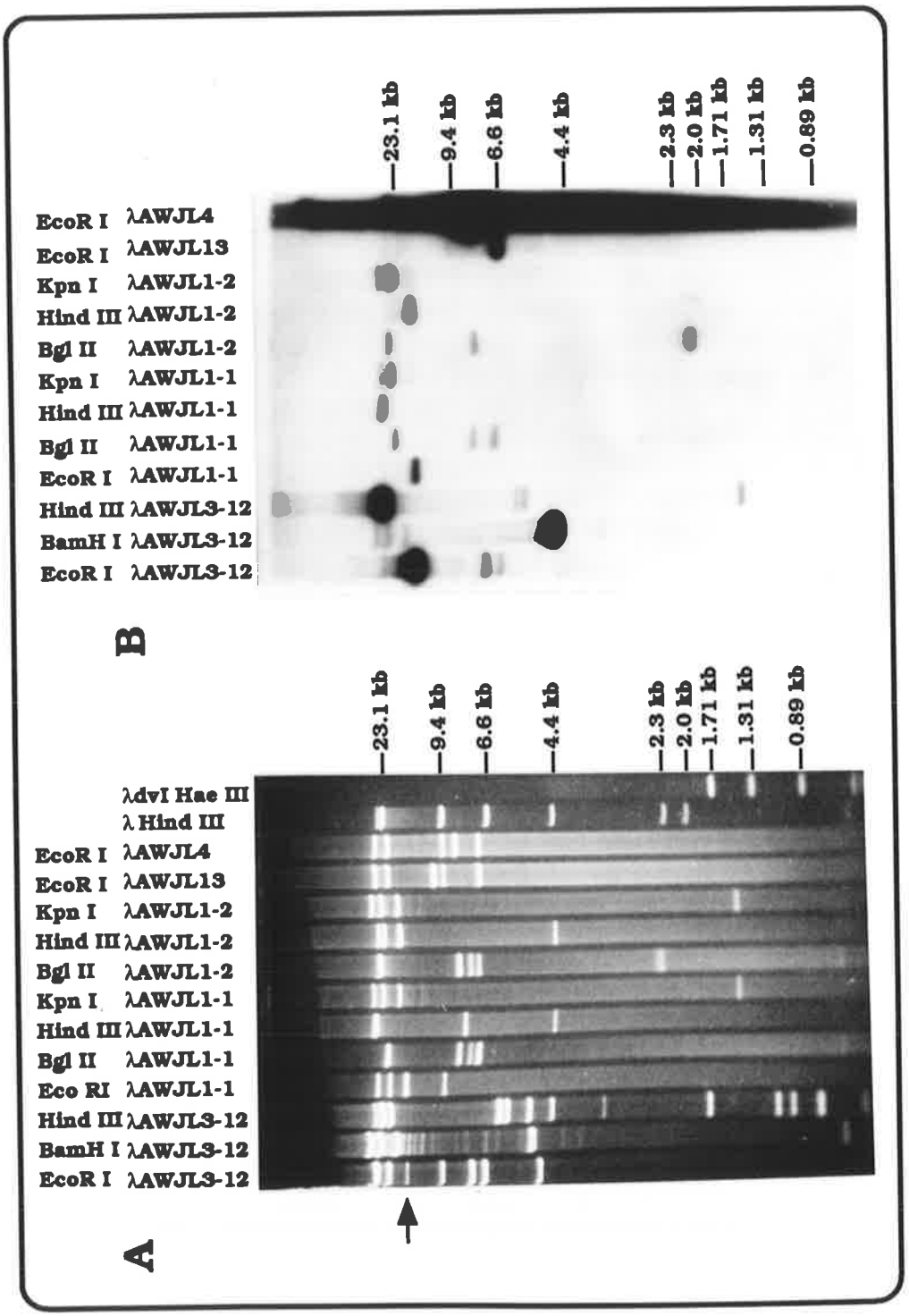
pAWJL4.1 has been shown to reveal a complex banding pattern on the Southern blot (Section 5.4.2). Interestingly, a sub-set of the bands were found to align with those revealed by hybridisayion to pAWJL1 and pAWJL3. It was postulated that the DNA hybridising to pAWJL4.1 was closely linked to sequences that hybridised to pAWJL1 and pAWJL3. This was confirmed by direct hybridisation of pAWJL4.1 to the genomic clones of pAWJL1 and pAWJL3. All the lambda clones isolated with the two cDNA clones cross-hybridised to the 1.4 kb *Dra* I sub-fragment of pAWJL4.1. Fig. 5.16 shows a representative pattern of cross-hybridisation. Most clones have two separate regions that hybridise to pAWJL4.1. However, the low copy number regions of pAWJL4.1, e.g. the 800 bp *Bgl* II/*Bam* HI fragment, did not cross-hybridise to the lambda clones (Data not shown).

Fig. 5.16. Cross-hybridisation of the Genomic Clones of pAWJL1 and pAWJL3 to the 1.4 kb *Dra* I Fragment of pAWJL4.1

A. λAWJL1-1, λAWJL1-2, λAWJL3-12, λAWJL4 and λAWJL13 digested with a range of restriction endonucleases and fractionated on a 0.8% agarose gel.

B. The above gel probed with the 1.4 kb *Dra* I fragment of pAWJL4.1 with an overnight exposure.

The positions of DNA size markers are given on the right of each panel.


23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
1.71 kb
1.31 kb
0.89 kb
EcoR I λAWJL4
EcoR I λAWJL13
Kpn I λAWJL1-2
Hind III λAWJL1-2
Bgl II λAWJL1-2
Kpn I λAWJL1-1
Hind III λAWJL1-1
Bgl II λAWJL1-1
EcoR I λAWJL1-1
Hind III λAWJL3-12
BamH I λAWJL3-12
EcoR I λAWJL3-12
B
23.1 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
1.71 kb
1.31 kb
0.89 kb
λdvI Hae III
λ Hind III
EcoR I λAWJL4
EcoR I λAWJL13
Kpn I λAWJL1-2
Hind III λAWJL1-2
Bgl II λAWJL1-2
Kpn I λAWJL1-1
Hind III λAWJL1-1
Bgl II λAWJL1-1
Eco RI λAWJL1-1
Hind III λAWJL3-12
BamH I λAWJL3-12
EcoR I λAWJL3-12
A

Using the restriction map of λAWJL1-1, the region of cross-hybridisation could be localised by digestion of the clones with a range of restriction endonuclease and probing with pAWJL4.1. It was found that the regions that hybridised to pAWJL4.1 were less than 1.5 kb away from the region that hybridised to pAWJL1 (Fig. 5.17).

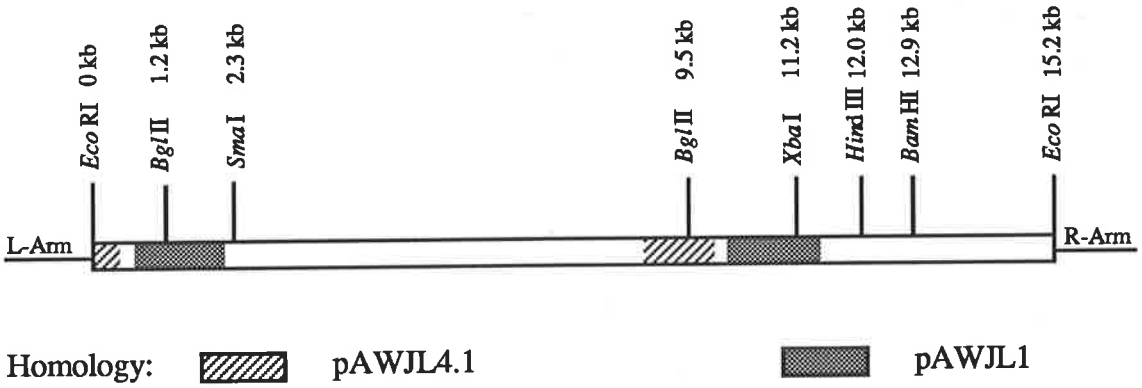


Fig. 5.17. The locations of homology to pAWJL4.1 and pAWJL1

5.6. Identification of Genomic DNA Fragments Unique to Meiotic Prophase I

Wheat genomic DNA was extracted from root tips, heat-shocked root tips and anthers at various stages from interphase to immature pollen, digested with *Eco* RI and probed with pAWJL4.1. Two extra bands were observed in samples from leptotene to diplotene compared to DNA prepared from other tissues (Fig. 5.18.A). DNA prepared from PMCs at leptotene showed an extra band of 3.7 kb. The band is quite diffuse and is also present in the DNAs from interphase to zygotene PMCs but the intensity is much reduced. Interestingly, the band disappeared after zygotene. However, a band at about 2.0 kb increased in intensity at pachytene-diplotene. Although the amount of DNA loaded on each lane is not constant, the increase of signal intensity of the 2.0 kb bands is not due to difference in DNA concentration (c.f. singal intensity of other bands at leptotene and "pachytene II" in Fig. 5.18.A and Fig. 5.18.B). The tissue-specific bands were also observed when the 1.4 kb *Dra* I fragment of pAWJL4.1 was used as a probe. When the membrane was stripped and probed with pAWJL3, no extra bands were seen (Fig. 5.18.B).

Fig. 5.18. Identification of Genomic DNA Fragments Unique to Early Meiotic Prophase I

The DNA was prepared from the same tissues as used for the extraction of total RNA for the RT-PCR assay shown in Fig. 5.14 (except "PACHYTENE I").

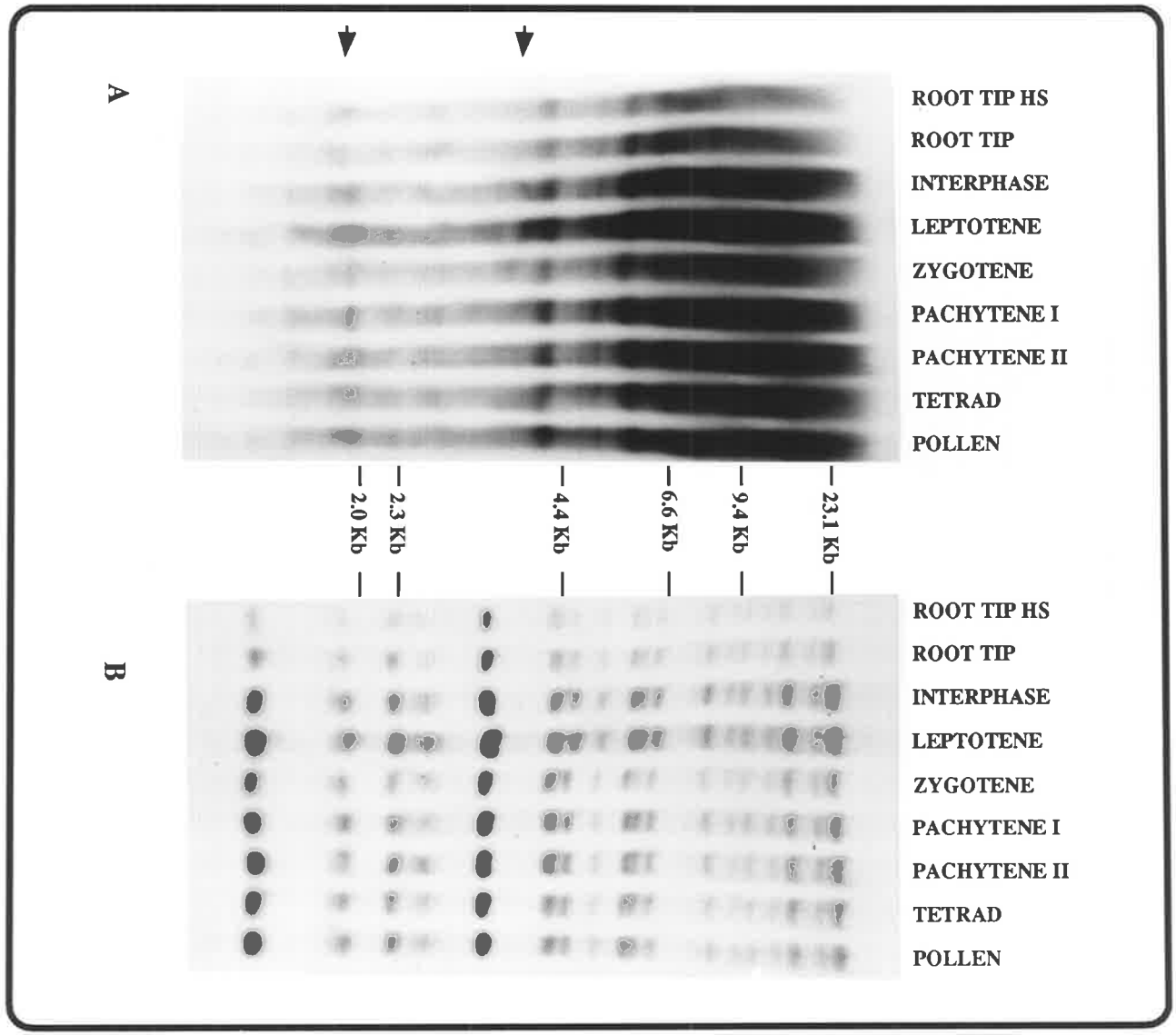
Anther DNA was recovered by ethanol precipitation from the supernatant after LiCl precipitation of RNA. The DNA was restricted with *Eco* RI, resolved on a 1% agarose gel and transferred to Hybond-N+ membrane and probed with pAWJL4.1. The membrane was then stripped and probed with pAWJL3.

A. Hybridisation against pAWJL4.1 with overnight exposure.

B. Hybridisation against pAWJL3 with one week exposure.

ROOT TIP HS, root tips that had been treated at 42°C for two hours. PACHYTENE1, late pachytene but also contains PMCs at diplotene (D and E in Fig. 5.15). "PACHYTENE II", prophase II as the cells were quite asynchronous (F and G in Fig. 5.15). The other meiotic stages of the anthers are shown in Fig. 5.15.

The position of the DNA size markers are indicated.


A
ROOT TIP HS
ROOT TIP
INTERPHASE
LEPTOTENE
ZYGOTENE
PACHYTENE I
PACHYTENE II
TETRAD
POLLEN
2.0 Kb
2.3 Kb
4.4 Kb
6.6 Kb
9.4 Kb
23.1 Kb
B
ROOT TIP HS
ROOT TIP
INTERPHASE
LEPTOTENE
ZYGOTENE
PACHYTENE I
PACHYTENE II
TETRAD
POLLEN

5.7. The Occurence of DNA Sequences Rearranged or Amplified *In Vivo*

It has been shown that the WSPs and the genomic clones of pAWJL1 and pAWJL3 are very unstable in *E.coli*. The instability could also be observed *in vivo*. Southern hybridisations with pAWJL1 and pAWJL3 frequently revealed bands with altered size or intensity using DNA extracted from the same batch of seed but from different plants or different tissues. Some examples are shown in Fig. 5.19. Sometimes, the intensity of bands can be increased by hundreds of fold (see Fig. 5.19.A). This would suggest that these genes are located near or at recombinational hotspots.

5.8. Discussion

5.8.1. The Wheat Gene Homologous to *SPO11*

Using the yeast *SPO11* gene as a probe, a candidate for a wheat *SPO11* homologue has been isolated. Although only a 21 nt perfectly conserved region was found when the wheat and yeast sequences were compared, more extensive homology exists at the amino acid level. Unfortunately, the 5' sequence lacks any recognizable intron splicing site and this casts some doubt on the true identity of the sequence cloned.

It is, however, worth noting that the lambda clones are unstable and it is possible that the recombination occurred just in front of the 21 bases conserved sequence. There is evidence to support this proposition. From the sequence of pAWJL4.1, primer WSP1 and WSP3 (see Fig. 5.9.B) should generate a 1.27 kb fragment containing the 21 nt conserved sequence from wheat genomic DNA. However, the largest fragment amplified, is only about 800 bp and there are other smaller bands (data not shown). Therefore, the sequence between the two primers does not seem to represent the *in vivo* organisation of λAWJL4.

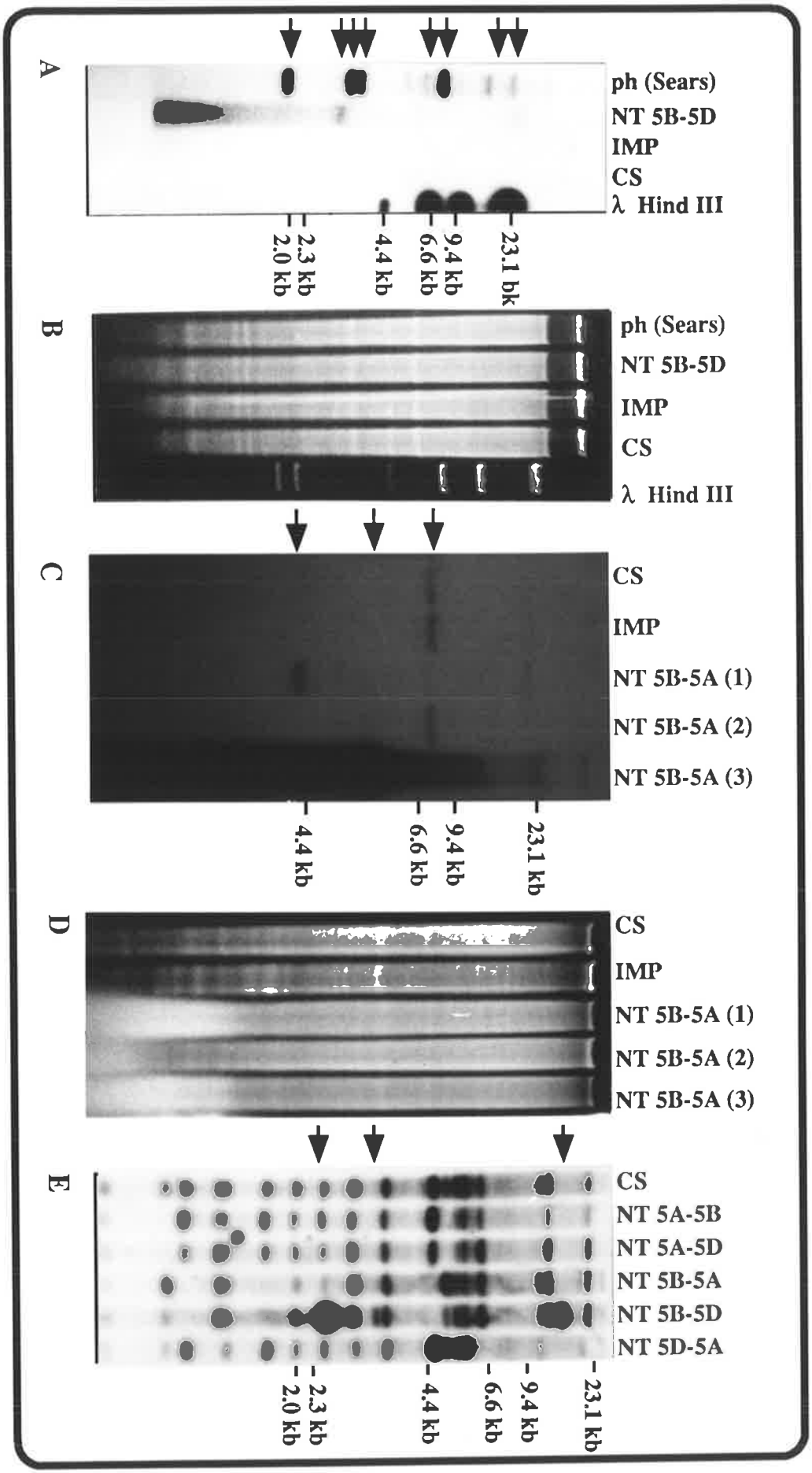
Several aspects of the genomic clone support the proposition that this contains a wheat homologue of the yeast *SPO11* gene. Firstly, the sequence around the 21 bases conserved motif is a low copy sequence although the sequence 700 bp away is reiterated many thousands of times in the genome. Secondly, the perfect conservation of a 21 bases nucleotide sequence between the two organisms (random

Fig. 5.19. Detection of Amplified or Rearranged DNA in Wheat Associated with pAWJL1 and pAWJL3

A and B. DNA (c.a. 5 μg) of the Sears *ph1* mutant, NT 5B-5D, Imperial Rye (IMP) and Chinese Spring (CS) digested with *Bgl* II (B), transferred to Zetaprobe membrane, probed with pAWJL1 and exposed overnight (A). Arrows indicate amplified DNA in *ph* mutant and NT 5B-5D.

C and D. The DNA (5 μg) was digested with *Hin*d III, resolved on a 0.9% agarose gel (D), transferred to Zetaprobe membrane and probed with pAWJL1 with three days exposure at -70°C between two intensifying screen (C). The middle arrow indicates amplified DNA sequence in NT 5B-5A and the others indicate rearrangements.

E. The DNA (5 μg) digested with *Hin*d III, resolved on a 0.9% agarose gel, transferred to Hybond-N+ membrane and probed with pAWJL3. Arrows indicate amplified and rearranged DNA.


A
ph (Sears)
NT 5B-5D
IMP
CS
λ Hind III
2.0 kb
2.3 kb
4.4 kb
6.6 kb
9.4 kb
23.1 bk
B
ph (Sears)
NT 5B-5D
IMP
CS
λ Hind III
C
CS
IMP
NT 5B-5A (1)
NT 5B-5A (2)
NT 5B-5A (3)
4.4 kb
6.6 kb
9.4 kb
23.1 kb
D
CS
IMP
NT 5B-5A (1)
NT 5B-5A (2)
NT 5B-5A (3)
E
CS
NT 5A-5B
NT 5A-5D
NT 5B-5A
NT 5B-5D
NT 5D-5A
2.0 kb
2.3 kb
4.4 kb
6.6 kb
9.4 kb
23.1 kb

probability of $1:4.4x10^{12}$) is likely to be significant. Thirdly, although nucleotide sequence homology beyond the 21 nt region is low, the translated amino acid sequence displays 69.2% similarity in that region.

5.8.2. The Cause of Instability of the Lambda Clones

Although *SPO11*, pAWJL1 and pAWJL3 are three different genes, their genomic structures share one feature in common, i.e., they are all highly unstable in *E.coli* and the *recD* mutation improves the recovery rate of the lambda clones. The most likely explanation for this is the presence of inverted repeats in the fragments containing these sequences. It has been shown that λAWJL1-1 contains two C+G-rich repetitive sequences that are closely linked to sequences related to pAWJL1 and pAWJL3. The instability could result from the inverted arrangement of this C+G-rich sequence, which is at least 1.5 kb long. An inverted repeat of such length would make a lambda clone very vulnerable to rearrangement in *E. coli* (Arlene and Wertman, 1987).

The cross-hybridisation of pAWJL4.1 to λAWJL15 provided some evidence that the C+G-rich repeat sequence is the main cause of clone instability. When the lambda clone was digested with *Eco* RI, three bands (c.a. 7.2, 6.6 and 4.6 kb) were released. However, a weak band of about 15 kb is also visible (indicated by arrow in Fig. 14.A). It is possible that the small bands are the rearranged products of the large band. Interestingly, on the autoradiograph of the gel probed with pAWJL4.1, the 15 kb band is the strongest (refer to Fig. 5.14.B) (same result was obtained when the 1.4 kb *Dra* I fragment of the

plasmid was used as a probe). Therefore, recombination of the lambda clone resulted in the loss of most of the C+G rich repetitive sequence. The organisation of the Transcript A sequence in the WSPs is likely to be similar. Take λAWJL6 for example, the c.a. 7.0 kb band (Fig. 5.5.B) was lost after extended propagation of the clone.

5.8.3. The Transcript A Sequence

The nucleotide sequence spanning Transcript A is highly C+G-rich. (40.33% G and 64.83% G+C from nt 1480 to nt 2640). Strangely, this is also the most repetitive part of pAWJL4.1. The putative transcript has the potential to code for a polypeptide of only 124 aa (13.3 kDa), leaving a long 3' untranslated tail with several possible stop codons. If this particular clone represents a pseudogene, the second and third stop codons could result from mutations of a functional gene. In this case, the RNA could encode for a protein of 324 aa (34.6 kDa) that is rich in glycine (19.8%).

The Transcript A RNA is not specific to meiotic cells but was shown to increase in concentration upon entry into meiosis. This appears to suggest that this RNA or its encoded protein plays a role on meiosis or pollen development. The similarity of the hypothetical ORFB (translated from the putative functional translational initiation codon to the third stop codon ignoring the interrupting stop codons) to the *Bat 2* gene and the *Micrococcos luteus* ORF1 might suggest that the gene is involved in the cellular defense system, such as DNA repair, since DNA damage may increase due to the intense chromosome synapsis and genetic recombinational activities. This is consistent with the presence of a nucleotide binding motif in the

ORF. It is also possible that Transcript A plays a role in genetic recombination as part of a PDNA element. Several aspects of the sequence resemble those of the *Lilium* PDNA in that it belongs to a family of moderate repeat sequence with high C+G content, closely associated with low copy sequences that may be transcribed at meiotic prophase I (e.g. pAWJL1 and pAWJL3) (refer to Chapter One). In addition, pAWJL4.1 hybridised to a family of poly(A)$^-$ small RNAs from spikelets at meiotic prophase I. These RNAs are similar to the PsnRNA of *Lilium* in their size and lack of a poly(A) tail (Hotta and Stern, 1984).

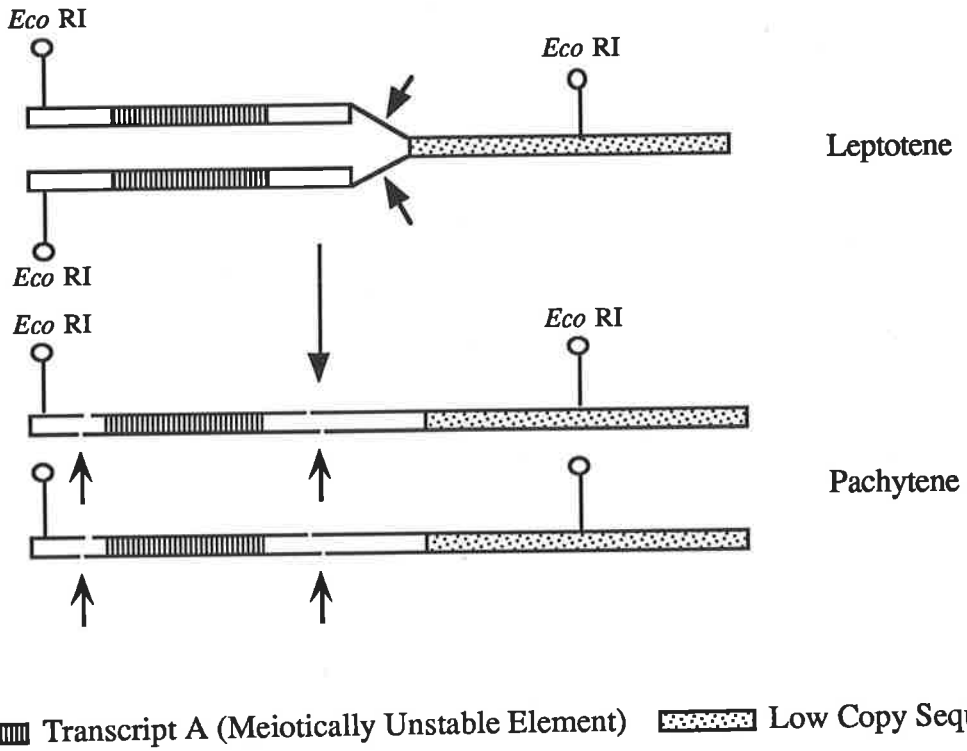


Fig. 5.20. A model of the chromatin structure housing the meiotically unstable elements and low copy sequence during meiosis. Large arrows indicate fragile single-stranded regions and the small arrows indicate nicks at pachytene.

The proposal that Transcript A is part of a meiotically unstable element may provide an explanation for the presence of extra bands observed with hybridisation of pAWJL4.1 to DNA extracted from early meiotic prophase I. If we assume that Transcript A is located just in front of a highly recombin-genic DNA then, at leptotene, the sequence is likely to have completed replication. The chromatin housing this sequence would have a Y-shaped structure with two fragile unreplicated regions (Fig. 5.20). The 3.7 kb band is likely to have resulted from breakage of the single-stranded region and a nearby *Eco* RI cutting site, since the band was quite diffuse. With the replication of the unstable elements, the chromatin becomes double-stranded and resistant to breakage. Therefore, the 3.7 kb band started to disappear. However, at pachytene the region was nicked, either at one or both ends, releasing the 2.0 kb band. The sharpness of this band may be due to the high sequence conservation of the meiotically unstable elements. This has been demonstrated in *Lilium* (Friedman *et al.*, 1982). After diplotene, the nicks were sealed and DNA banding pattern returned to normal.

The above proposal can also explain the frequent occurrence of DNA rearrangement in the DNA encoding pAWJL1 and pAWJL3. It has been shown that the DNA encoding pAWJL1 (possibly pAWJL3 too) is closely linked to a Transcript A sequence. If Transcript A is part of a PDNA element, which was thought to be site for meiotic recombination (Hotta and Stern, 1984), high frequency of DNA rearrangement at or near the sequences coding for pAWJL1 and pAWJL3 will be inevitable.

pAWJL3 was shown to be unable to detect meiosis-specific DNA bands similar to those revealed with pAWJL4.1. However, since

PMCs comprises only a very small proportion of a anther's mass, it is unlikely one would detect DNA breakage with a low copy probe. On the other hand, pAWJL4.1 belongs to a family with about 4000 members in the wheat genome. This should greatly increase the sensitivity of detection of the meiosis-specific genomic DNA fagments by Southern blot.

It could be argued that the extra bands observed from leptotene to diplotene resulted from DNA demethylation. Indeed, demethylation has often been found to be associated with active genes (Doerfler *et al.*, 1990; Boyes and Bird, 1992). Since Transcript A is active during meiosis, this may occur and the new bands may result from the loss of 5-methyl-cytosine in the *Eco* RI recognition site (GAATTC). DNA demethylation is generally thought to enhance gene transcription (Boyes and Bird, 1992). This implies that the level of Transcript A RNA would decline after diplotene since all the sequences became re-methylated (resistant to digestion by *Eco* RI). This was not observed from the PCR data. It can be seen from Fig. 5.14 that the level of Transcript A RNA was not significantly changed after diplotene. It is unlikely that the RNA transcribed before diplotene could remain undegraded till post-meiotic stages since these RNA is likely to be highly unstable. This is due to the presence of an ATTTA motif (base 2708) just before AATAAA motif. The ATTTA motif was found to cause instability and rapid turn-over of the RNA molecules (Shaw and Kamen, 1986).

In conclusion, convincing evidence for the function of this family of repetitive sequences remains to be found. It will be important to show unambiguously the presence of pachytene DNA

break and zygotene DNA replication in or near these sequences. For this, a very gentle procedure for extraction of DNA is required. For example, those used for preparation of DNA plugs for pulsed field electrophoresis (Guidet *et al.*, 1990). The use of methylation insensitive restriction endonucleases may also resolve puzzle of the occurence of extra DNA bands at meiotic prophase I. *Msp* I is unlikely to reveal any meiosis-specific bands because it cuts pAWJL4.1 and wheat DNA too frequently and no distinct bands could be seen on the Southern blot of wheat DNA hybridised against pAWJL4.1 (data not shown). Therefore, restriction endonucleases with a six bp recognition site will have to be tried.

5.11.4. Prospects

In this investigation a candidate for a partial exon of a *SPO11* homologue has been identified. However, the structure and role of this clone remains confusing. The true identity of the clone cannot be confirmed without the identification of longer homology of amino acid sequences than has been possible. The analysis of the genomic sequence has been complicated by the instability of the lambda clones and the difficulty in determining the sites for intron splicing. The presence of an intron splice site could be confirmed by established procedures (Sambrook *et al.*, 1989), but the possible presence multiple alleles and the difficulty in obtaining large amounts of meiocytes or anther RNA make these experiments extremely difficult.

A new strategy is needed to overcome these problems. The analysis of other WSP clones might provide some more information

on the identity of the clones but this is likely to be a slow and painstaking process. With the identification of a 21 bases perfect homology to the yeast *SPO11* gene, it is now possible to initiate an attack at the RNA level with the aid of PCR, as the region of homolgy is long enough to be used as a primer. From the sequence of the yeast *SPO11* gene, the sequence between the 21 bases conserved region and the whole 3' region would encode the C-terminus of a 264 aa long polypeptide. If this region could be amplified directly from mRNA, the amino acid sequence could be determined quickly by sequencing several random clones of the PCR product. Although the conserved nucleotide sequence at the 3' of the *SPO11* gene is unknown, it may be possible to target the poly(A) tail. A random sequence can be added to the 5' of the oligo(dT) sequence to raise the melting temperature of the 3' primer. When sufficient homology is found, the 5' sequence can be obtained from the genomic clones or by PCR amplification of the dG-tailed cDNA (Belyarsky *et al.*, 1989).

Chapter Six

General Discussion

The transcription of a family of repeat sequences, the EMPRs, has been confirmed in wheat. Two cDNA clones have been isolated with a maize homologue of the EMPR genes. These two clones are only distantly related to the *Lilium* EMPRs since sequence analysis revealed little homology to either the *Lilium* EMPR genes or the soybean small heat shock gene.

The information from yeast has not been as easy to apply to wheat as that from *Lilium*. Nonetheless, the preliminary analysis of a sequence of wheat homologous to a yeast *SPO11* clone has revealed a new family of repetitive sequences that are potentially important during meiosis (WSPs).

The study has provided preliminary evidence that wheat, like *Lilium* and mouse, has special types of DNA replication during meiosis. The DNA band (c.a. 7-8 kb) extractable from anthers at early meiosis, is likely to be the equivalent of the *Lilium* zygDNA. In addition, with the pAWJL4.1 as probe, two extra bands have been detected from Southern hybridisations of digested DNA of anthers from leptotene to diplotene (Fig 5.18). One was observed most clearly at leptotene and the other at the interval between pachytene and diplotene. The result suggests that the genomic clones of pAWJL1 and pAWJL3, and possibly also the WSPs, contain a zygDNA and PDNA element. If this is the case, pAWJL4.1 will be very useful for the analysis of meiotic DNA breaks and the

molecular dissection of the structure and function of the chromatin housing PDNAs and zygDNAs in wheat.

The localisation of PsnRNA in wheat will be an interesting task because, in *Lilium*, this RNA was found to be an important regulator of gene recombination (Hotta and Stern, 1981). One of the discrepancies of recombination between the findings in yeast and *Lilium* relates to the nature of the DNA break for initiation (see Chapter One). Precise localisation of the PsnRNA sequence may help to solve the puzzle of the initiation and termination of recombination.

The studies of EMPRs, *SPO11*, zygDNA and PDNA have just scratched the tip of an iceberg. However, it has been shown that the study of meiosis in wheat can exploit probes prepared from other organisms. The three indirect approaches used can all be considered at least partially successful. There are, however, alternative approaches that can be explored. Differential screening of cDNA libraries from anthers at meiosis with cDNAs of leaves, only yields clones specific to anther wall or tapetal cells (Koltunow *et al.*, 1990; McCormick, 1991). It seems, therefore, that differential screening is an inefficient tool for the study of meiosis when pollen mother cells cannot be isolated with ease. One important reason for this is that PMCs compose only a very small proportion of an anther's cell number. Furthermore, meiotic tissue is much less active in gene transcription and polyadenylation (Porter *et al.*, 1983).

6.1. A Theoretical Model for the Function of pAWJL3 Protein and the Evolution of Promoters and Suppressors of *Ph* Genes in Wheat

In Chapter Four the question was asked, i.e., Can the pAWJL3 group of genes be both promoters and suppressors of chromosome pairing? pAWJL3 was predicted to encode a non-basic leucine-zipper-like protein and is unlikely to be a DNA binding protein. However, it is a good candidate for a suppressor of a DNA binding protein if its leucine-zipper-like domain is competent of binding to other proteins via coiled-coil helical interaction. A hetero-dimer of a basic and a non-basic leucine-zipper protein is unlikely to be functional. If the gene for promoter of homoeologous pairing gene encodes a bZip protein, a mutant gene, i.e. encoding the non-basic protein, may retain sufficient homology to allow cross-hybridisation to the original gene. For these reasons, a model of the "normal" *Ph* genes can be proposed based on the assumption that pAWJL3 is a suppressor of homoeologous chromosome pairing, i.e., *Ph2* or other weak suppressor on group 3 chromosomes. The model is illustrated in Fig. 6.1 and explained as followed.

1. The genes promoting homoeologous chromosome pairing are bZip DNA binding proteins. They are possibly regulators of gene transcription or component proteins of the SCs.

2. The genes on group 3 chromosomes suppressing homoeologous chromosome pairing were derived from ~~/~~mutations duplication of the pairing promoter gene on 5BS and

4. It is further proposed that normally (as in the ancestor wheat), an excess of pairing promoter protein is present and this will promote pairing of both homo- and homoeologous chromosome pairs. The evolved *Ph* loci code for proteins that combine with promoter protein to form inactive complexes. The net effect is to reduce the concentration of the pairing promoter protein and therefore only the more specific effects (involving higher affinity binding) are likely to be seen. This results in homoeologous pairing being suppressed.

of the basic amino acid motif of the promoter gene. This would lead to the loss of DNA binding capacity.

3. The strength of a suppressor reflects the strength of the mutant leucine-zipper proteins to compete with the functional basic leucine-zipper proteins to form a defective protein dimer.

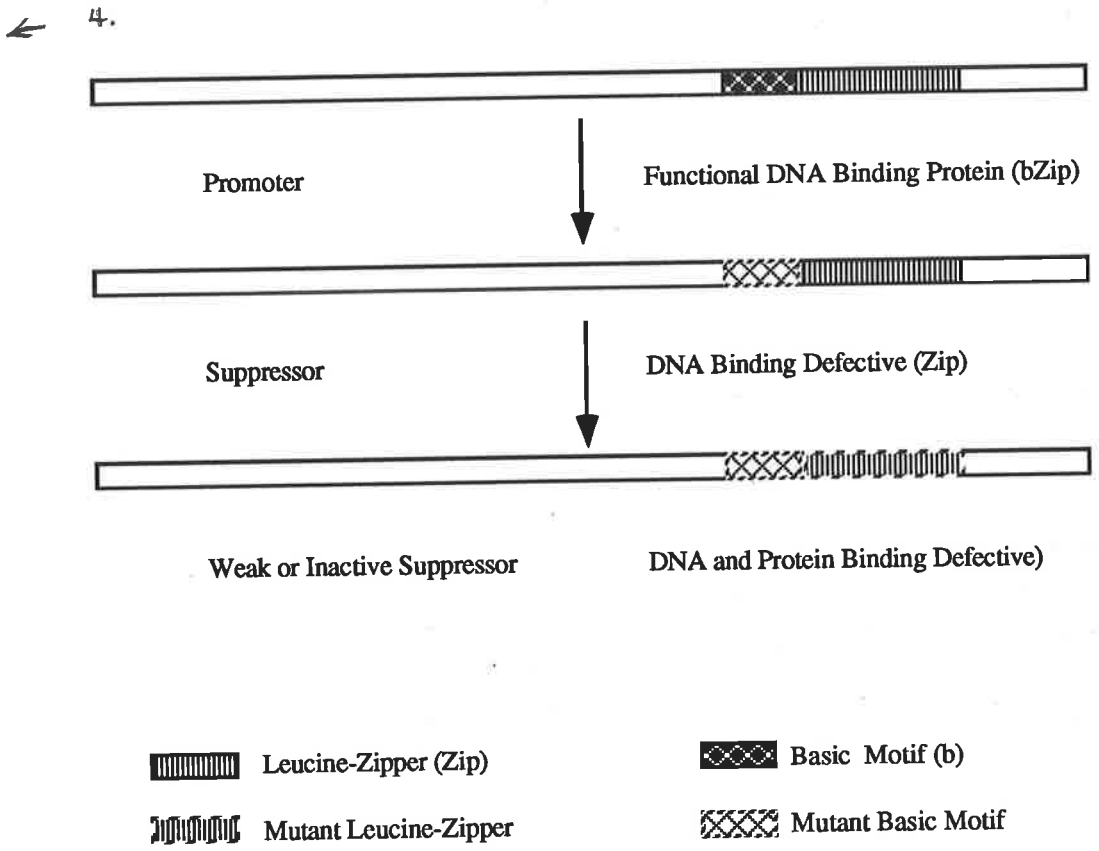


Fig. 6.1 A diagrammatic illustration on the evolution of suppressor and promoter *Ph* genes.

To date, there has been no report of suppressors of this type. However, a recently isolated *Drosophila* protein, Id, which is a helix-loop-helix (HLH) protein lacking the basic region adjacent to the HLH domain, is an analogous example. The protein

has been shown to associate with three other HLH proteins (MyoD, E12 and E47) and inhibit MyoD-dependant activation of the MCK (muscle-specific cyclic AMP kinase) enhancer (Bebezra *et al.*, 1990).

It can be inferred from the model that, in the absence of *Ph3p*, the effect of pairing suppressor on 3DS can not be observed since it has no target to interact with. This is consistent with cytological observation. The level of homoeologous chromosome pairing in 5B-3D double deficient plants was shown to be similar to that in 5B deficient plants (Kempanna and Riley, 1962; Mello-Sampayo and Canas, 1973). However, experiment with *Ph3p* mutant will be needed to further support this model.

This model is similar to an early hypothesis on the origin of the *Ph1*. Both propose that the suppressor derives from a promoter gene and that the pairing suppressor gene product forms defective enzyme dimers or multimers (Driscoll, 1972; Sears, 1976). However, it must be mentioned that both are purely theoretical models and neither has much experimental support. The model described here is based on the assumption that the leucine-zipper-like domain is the functional motif of the pAWJL3 protein. The picture changes completely if the protein's functional site is the 24 aa repeats, which might be able to interact with similar repeats, such as those of adenylate cyclase or protein phosphatase. Alternatively, this protein may be a structural protein required during meiosis.

6.2. Other Possible Approaches to the Isolation of Meiosis-specific Sequences

6.3.1. Gene Complementation in Yeast

Gene complementation is a promising new approach to the isolation of meiosis-specific genes already identified in yeast. During the past few years, several cell division control (*cdc*) genes have been shown to be sufficiently conserved among eukaryotes to allow their isolation by complementation in yeast. For example, the human *CCG2* gene (Watanabe *et al.*, 1991), the human *CDC2* kinase gene (Lee and Nurse, 1987), the yeast *CDC2/CDC28* anologue of *Xenopus* (Paris *et al.*, 1991) and human (Ninomiya-Tsuji *et al.*, 1991) and the *Xenopus RCC1* gene (Nishitani *et al.*, 1990) have all been isolated by complementation of yeast mutants.

Surprisingly, no genes required for meiosis have been isolated by this procedure. Genes controlling meiotic division are likely to be as highly conserved as the mitosis-specific sequences. A large number of meiotic mutants have been isolated in yeast (see Chapter One), and these would be powerful tools for the study of meiosis in higher eukaryotes. The cDNAs of anthers or meiocytes can be inserted into yeast expression vectors and the clone library used to transform the appropriate meiotic mutant (see references described above). Any gene that is able to complement the mutant should restore the wildtype phenotype.

6.3.2. Polymerase Chain Reaction

The shortage of materials for the study of meiosis has become a less serious problem than previously with the development of PCR techniques. Today, a cDNA library can be made from a small number of cells (Akowitz and Manuelidis, 1989; Tam *et al.*, 1989) and mixed cDNA probes can be prepared with ease without the need for cloning or preparation of a large amount of material.

Despite the improvement in the cloning protocols, the screening of a cDNA library will remain a laborious task, although subtraction techniques can reduce the number of clones needed to be screened (Palzzolo and Meyerowitz, 1991). However, the most serious problem facing differential screening is our inability to isolate low abundance genes. An appealing shortcut to overcome this problem is again to borrow information on meiosis and cell cycle regulation from yeast and other eukaryotes. The molecular control of tissue-specific gene expression relies largely on two classes of proteins; one involved in the protein phosphorylation-dephosphorylation pathway and the other *cis*-acting or *trans*-acting action of transcriptional regulation (Kelly and Darlington, 1985; Hanks *et al.*, 1988; Martha and Thorner, 1989; Hoekstra *et al.*, 1991). Although the overall sequences of a class or a family of proteins may vary enormously, often there is a motif that is highly conserved. This will be useful information for PCR. For example, degenerate primers derived from the nucleotide binding motif of protein kinases, GXGXXGXV (Hanks *et al.*, 1988) in conjunction with a tailed-oligo(dT) primer can be used to

preferentially amplify genes with a nucleotide binding site either from a cDNA library or directly from single-stranded cDNA. By this method the scope of targets could be narrowed by several thousand fold. The screening process will be straight forward. By using similar mixed cDNA probes from somatic tissue and high stringency hybridisation, meiosis-specific kinases could be isolated. To date no such protein gene has been found nor has the method been tried.

6.3. Concluding Comment

The molecular events of meiosis in plants have been poorly studied partly due to the small amounts of tissues available for study and the short time interval of developmental stages. However, this process is of such importance that special effort is justified. The research in lily and yeast has lead the way in elucidating the biochemistry and molecular genetics of meiosis. It is now clear that this work can be extended to other organisms. The understanding of the molecular genetics of chromosome pairing in wheat may assist in work to engineer and exploit alien chromosomes in this important crop plant.

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Appendix A

E. coli Strains and Cloning Vectors

1. *E. coli* Strains.

E.coli JM103:

endA1, hsdR, supE, sbcB15, thi-1, strA, Δ(*Lac-pro*), [F', *traD36, proAB, lacI^{q}ZΔm15*] (Hanahan, 1983)

E. coli JM109:

endA1, recA1,syrA96, thi, hsR17, (*rk$^-$,mk$^+$*), relA1, supE44, λ$^-$, Δ(lac$^-$proAB), [F', traD36, *proAB, lacI^{q}ZΔm15*] (Yanisch-Perron *et al*, 1985)

E. coli ER1647:

Δ(*mrr$^-$hsRMS$^-$mcrB*), *recD, mcrA1272, serB28* (Woodcock *et al.*, 1989).

E. coli ER1648:

as ER1647, but *recD$^+$* (Woodcock *et al.*,1989)

E. coli NW2:

Δ(*mrr$^-$hsRMS$^-$mcrB*), *mcrA* (Woodcock *et al.*,

1989).

E.coli NM514:

HsdR514(*r$^-$m$^-$*), *ArgH, galE, strA, lycB7*(*Hfl$^+$*) (Murray, unpublished. Provided by Amersham).

E.coli L87:

supE, upF, hsdR, r-m+ (Brammar and Leicester, unpublished. Provided by Amersham)

E.coli DH5α:

F-, *lacZΔm15, endA1, recA1, hsR17* (*rk-, mk-*), *sup44, thi-1, gyrA96*, Δ(*lacZYA-argF*), *U169*. (Hanahan, 1983)

2. Cloning Vectors.

pTZ19R:

E.coli ori, f1 ori, MCS (multiple cloning sites), lacZ, Amp^R. (Pharmacia)

pTZ18U:

E.coli ori, f1 ori, MCS(multiple cloning sites), lacZ Amp^R. (Pharmacia)

pBluescribe:

pUC19, T3 and T7 RNA promoter (Stratagene)

λgt10:

Eco RI cloning site, *imm434, b527* (Huynh *et al.*, 1985)

λEMBL4:

Eco RI, *Bam* HI and *Sal* I cloning sites (Frischauf *et al.*, 1983)

M13 K07 (helper phage):

Kan^R, gene 2 mutation (Vieira and Messing, 1987)

Appendix B

Buffers, Stock Solutions and Media

1. Buffers and Stock Solutions.

1X Oligo(dT) cellulose binding buffer: 20 mM Tris-HCl (pH7.5), 0.5 M NaCl, 1 mM Na_2EDTA (pH8.0), 0.2% SDS.

1X TE buffer: 10 mM Tris-HCl (pH7.5), 1 mM Na_2EDTA.

2X Oligo(dT) cellulose binding buffer: 20 mM Tris-HCl (pH7.5), 1 M NaCl, 1 mM Na_2EDTA (pH8.0), 0.2% SDS.

5X M-MLV reverse transcriptase buffer: 250 mM Tris-HCl (pH8.3), 375 mM KCl,15 mM, $MgCl_2$, 50 mM DTT (DTT was stored separately as a 100 mM stock).

10X Calf intestinal alkaline phosphotase buffer: 500 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine, pH9.0.

10X DNase buffer: 20 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM DTT, pH7.6.

10X MOPS/EDTA Buffer: 500 mM MOPS (pH7.0), 10 mM Na_2EDTA.

10X Nick translation buffer: 500 mM Tris-HCl (pH 7.5), 100 mM $MgSO_4$, 1 mM DTT, 500 μg/ml BSA.

10X PCR buffer: 500 mM KCl, 200 mM Tris-HCl, 25 mM $MgCl_2$, 1 mg/ml BSA, pH 8.4.

10X Restriction endonuclease buffer B: 10 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl,1 mM 2-mercaptoethanol, pH8.0.

10X Restriction endonuclease buffer H: 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTE, pH7.5.

10X Restriction endonuclease buffer L: 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTE, pH7.5.

10X Restriction endonuclease buffer M: 10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTE, pH7.5.

10X Restriction endonuclease buffer: A: 33 mM Tris-acetate 10 mM MgAcetate, 66 mM KAcetate, 0.5 mM DTT, pH7.9.

10X TAE buffer: 400 mM Tris-HCl, 30 mM sodium acetate, 10 mM Na_2EDTA, pH7.8.

10X TBE buffer: 1 M Tris base, 10 mM Na_2EDTA, 0.863 M boric acid (pH8.3).

12X DNA sample loading buffer: 0.5% bromophenol blue, 0.5% xylene cyanol, 30% ficoll, in water.

20% Acylamide-urea solution: 19.3% acrylamide, 0.67% N,N'-methylene bisacrylamide, 46.7% urea. Deionized with 0.2% (W/V) AG 501-X8 ion exchange resin (Bio-Rad) and filtered with Whatman 541 paper.

20X SSC: 3 M NaCl, 0.3 M $Na_3Citrate$.

20X SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02M Na_2EDTA, pH7.4.

46% Urea solution: 46% urea in water, deionized with 0.2% (W/V)AG 501-X8 for 30 minutes and stored above the resin.

Buffer A: 294 µl 10X MOPS/EDTA Buffer, 706 µl water.

DEAE-cellulose LB suspension: 100 g Whatman DE52 was suspended in 500 ml 0.0 5M HCl. 2 M NaOH was slowly added until pH7.5. The resin was washed several times with LB until pH reached equilibrium. Finally, the resin was made to about 75% (v/v) with LB.

Denaturing solution: 1.5 M NaCl, 0.5 M NaOH.

DNA extraction buffer: 4% sarkosyl, 0.1 M Tris-HCl (pH8.4), 0.01 M Na_2EDTA.

Neutralising solution: 1.5 M NaCl, 0.5 M Tris-HCl, 0.01 M

Na_2EDTA, pH7.0.

Oligo(dT) elution buffer: 20 mM Tris-HCl (pH7.5),1 mM Na_2EDTA (pH8.0), 0.2% SDS.

Phage precipitating buffer: 20% PEG, 2 M NaCl, 5 mM Tris-HCl (pH7.5), 0.01% gelatine, 10 mM $MgSO_4$.

Phenol/chloroform/iso-amyl alcohol(25:24:1): redistilled phenol was saturated with 0.5 M Tris-HCl (pH8.0) and then mixed with chloroform and iso-amyl alcohol as indicated.

R40: 40 µg/ml DNase-free RNaseA in 1X TE buffer.

RNA loading buffer: 322 µl buffer A, 5 mg xylene cyanol, 5 mg bromocresol green, 400 mg sucrose, 178 µl 37% formadehyde, 500 µl formamide.

Second strand cDNA synthesis buffer: 125 mM Tris-HCl (pH8.3), 812.5 mM KCl, 42.5 mM $MgCl_2$, 25 mM DTT.

SM: 0.1 M NaCl, 10 mM MgSO4, 5 mM Tris-HCl pH7.5, 0.01% gelatine.

Sucrose solution H: 34% sucrose, 1 M NaCl, 5 mM Na_2EDTA, 20 mM Tris-HCl, pH 8.0.

Sucrose solution L: 10% sucrose, 1 M NaCl, 5 mM Na_2EDTA, 20 mM Tris-HCl, pH 8.0.

T4 DNA ligase buffer: 660 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 10 mM DTE, 10 mM ATP.

Transformation and storage buffer (TSB): LB (pH6.1) medium supplemented with 10% PEG 4000, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 5% DMSO. Filter sterilised. (DMSO was added immediately before use)

2. Media.

LB (per litre):10 g Bacto-Trytone, 5 g yeast extract, 10 g NaCl, pH7.0

LB-agar: 15 g agar per litre LB

TYP (per litre): 16 g Bacto-Trytone, 16 g yeast extract, 5 g NaCl, 2.5 g K_2HPO_4

Top Agar (per litre): 10 g Bacto-Tryptone, 5 g NaCl, 2.5 g $MgSO_4 \cdot 7H_2O$.

M9 (per litre): 6 g Na_2HPO_4, 3 g $KHPO_4$, 0.5 g NaCl, 1 g NH_4Cl, pH7.4. Immediately before use the following were added to one litr: 2 ml 1 M $MgSO_4$, 10 ml 20% glucose, 100 µl $CaCl_2$ and 10 µl 1 mg/ml thiamine.