IDENTIFICATION AND CHARACTERISATION OF EARLY MEIOTIC GENES IN WHEAT

by

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ERRATA

Page 5- line 19
(John and Lewis 1984) Add this reference after: "chiasmata have formed."

Page 5- line 24
"chromatids"reach their maximum condensation (not chromosomes)

Page 7- line 2
"This kind of process would function in a systematic way rather than being accidental" (sentence modified).

Page 10- line 3
(von Wettstein "*et al*." 1984)

Page 10- line 24
"which" have developed it (instead of who)

Page 13- line 21
homoeologous "pairing"

Page16 - line 4
"Hobolth"

Page 18- line 9
Section "1.4.1" (instead of 1.4.3)

Page 20- line 9
tranferred "to" a growth

Page 20- line 10
delete: "expositon"

Page 21- line 2
"plasmid"

Page 30- line 16
"Bacterial" transformation

Page 30- line 17
delete: "et al."

Page 41- line 15
Section "2.3.7" (instead of 2.3.6)

Page 41- line17
"glumes" removed (instead of leaves)

Page 51- lines 13 and 14
"47%" and "53%" (instead of 49% and 51%)

Page 56- line 4
replace exposition with "exposure"

Page 56- line 23
a strong "hybridisation" signal

Page 58- line 4
was probably "too" short

Page 60- line 15
of the short "arm" of

Page 60- line 18
homoeologous chromosomes "of" group 3

Page 63- legend of figure 4.5
the areas "covered in" each direction (and delete: "sequencing")

Page 65- line 9
delete: "long"

Page 65- line 16
The sentence beginning by: "In fact, wm5.12-tap..." needs to be modified with the following:

"In fact, wm5.12-tap possesses the main features of the nsLTPs, namely an N-terminal sequence leader and the 8 conserved cysteines residues which include the doublet Cys-Cys and the sequence Cys-Asn-Cys that are highly conserved in plants."

Page 71- line 16
"developing"

Page 71- line 17
"rule"

Page 71- line 26
(Xu "et al" 1995

Page 72- line 24
"solicited"

Page 72- line 25
the suggestion "is" that they all

Page 74- line 18
"developing"

Page 75- legend of figure 5.1
Replace "exposition" with "exposure"

Page 76- line 7
"occurring"

Page 78- line 20
Add: "the Ph genes located on" between the words "for" and "chromosome" in the sentence : "Since our prime interest was for chromosome groups..."

Page 80- line 16
"referring"

Page 82- line 22
reading frame "as" the start codon (replace "of" with "as")

Page 88- line 5; page 89- Fig. 5.8 (line 5); page 90- line 19
"amphipathic"

Page 90- line 16
"displays"

Page 91- line 15
"effects"

Page 92- line 20
"maintained"

A la mémoire de

Paul-Henri et Laurent,

DECLARATION

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

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

Jodelyne Létàrte — Aug 16th, 1996

date

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SUMMARY

Wheat is an allohexaploid consisting of three related genomes called homoeologues. Although homoeologous chromosomes share extensive sequence homology, chromosome pairing at meiosis is restricted to true homologous chromosomes. This confers a diploid-like behaviour to this polyploid species. The strict control of pairing is known to be under the control of several genes (named *Ph* genes) located mainly on chromosome groups 3 and 5. The cloning of the *Ph* genes or other related genes would provide an insight into the control of chromosome pairing in wheat.

In an attempt to identify and isolate genes related to chromosome pairing, a cDNA library was constructed from wheat florets at the premeiotic interphase and prophase I stages of meiosis. The library was differentially screened with cDNA probes made from early meiotic anthers and from leaves, roots and pollen RNA. Clones showing a preferential hybridisation signal with cDNA probes made from the meiotic anthers were selected for further analysis. Out of 34 selected clones, two (wm5.12 and wm2.19) were chosen for complete sequencing and molecular analysis.

The analysis of wm5.12 showed that it was expressed abundantly at meiosis in the tapetal cells of the anthers and weakly in the carpels. The complete sequence revealed that wm5.12 cDNA encoded for a polypeptide similar to non-specific lipid transfer proteins of plants. Chromosomal mapping in Chinese Spring has shown that five genomic bands hybridised to wm5.12 probe: two on chromosome 3AS, one on 3BS and two on 3DS. The two bands located on 3DS were missing in the wheat deletion mutant *ph2a*, indicating that wm5.12 is closely linked to the *Ph2* gene known to be located on chromosome 3DS.

The second clone analysed, wm2.19, had a different pattern of expression. It was found to be highly expressed in anthers and carpels just before and at the beginning of meiosis and also in mitotic tissues such as root tips. The sequencing results showed that wm2.19 was similar to a murine and yeast gene, both involved in the cell cycle. Like wm5.12, the wm2.19 clone was also used as a probe on genomic Southerns and was mapped to the long arms of chromosomes 1A, 1B and 1D of Chinese Spring.

The findings obtained with wm5.12 and wm2.19 are discussed in relation to the roles they could play during meiosis in wheat and we speculate whether they could be involved directly or indirectly in the chromosome pairing process.

CHAPTER I

General introduction.

1.1 Importance of wheat.

The wheat species (*Triticum* spp.) comprises a polyploid series with diploid (2n=2x=14), tetraploid (2n=4x=28) and hexaploid (2n=6x=42) forms. The AABBDD hexaploids originate from amphiploidy between an AABB tetraploid (*T. dicoccum*) and the D genome diploid *Triticum tauschii* (Feldman 1976; Sears 1976).

No wild forms of hexaploid wheat exist (Feldman 1976). It most probably arose from cultivation and archaeological evidence indicates that hexaploid wheat was established by 6000 BC (Jarman 1972). For a comprehensive review on the history of wheat see also Feldman (1976) and Bell (1987).

Today, *T. aestivum*, known as bread wheat or common wheat, is the most widely cultivated wheat in the world together with the macaroni wheat (*T. durum*). It is eaten in various forms by more than 1 billion people and makes a larger contribution to the calories and protein available to people than any other food (FAO 1970).

Wheat not only rates first among crops in feeding mankind, it also has a dominating position in world trade and economy. In 1990, 595 million tonnes of wheat were produced compared with 518 million tonnes of rice and 475 million tonnes of maize (FAO 1991). In Australia, the production of wheat has more than doubled for the past 30 years (ABARE 1993). This tendency of yield increase is also reflected in other world producers such as Canada, China, USA and Russia. This is due not only to better farming practice and pest

control but also results from strong research collaboration between breeders, cytogeneticists, plant physiologists, plant pathologists, biochemists and molecular biologists to release new wheat varieties better adapted to the environment.

In wheat research, the relevance of molecular biology lies first in developing a better understanding of the wheat plant itself. This can lead to a better estimation of means for crop improvement. From chromosome organisation to the introgression of desired alien chromosomal segments, the importance of learning how wheat chromosomes behave during mitotic and meiotic divisions, is fundamental in agricultural research.

The following sections of this introduction focus on a particular research topic in wheat; namely, the study of meiosis and chromosome pairing in wheat at the molecular level. First, a brief morphological description of the wheat anther is given, followed by a cytogenetic account of meiosis. The state of research in chromosome pairing in general will be reviewed with particular emphasis on the *Ph* genes discovered in the fifties in allohexaploid wheat. Finally, the main objectives of this thesis will be presented.

1.2 Meiosis in the allohexaploid wheat.

1.2.1 Development and morphology of the anther at the time of meiosis.

In Chinese Spring (the variety chosen in this study) meiosis occurs when the stamen is about 1 mm long and light yellow in colour (Bennett *et al.* 1973). At this time, the anther comprises four elongated microsporangia arranged in four loculi (see Figure 1.1) and joined by the connective tissue to the filament (Vasil 1967). As with most angiosperms, the anther wall consists of (from the outer to the inner layer) the epidermis, endothecium, middle layer and the tapetum (Bhandari 1984). The tapetum is a secretory type and consists of a single layer of cells completely surrounding the pollen mother cells (Bennett *et al.* 1971). In wheat, about 100 pollen mother cells per loculus will undergo meiosis and form tetrads (Bennett *et al.* 1973). Just before meiosis a callose wall forms around each pollen mother cell and the tapetal cells undergo a dramatic differentiation into binucleated cells.

It is impossible to talk about meiosis without discussing the tapetum. The location and connection of the tapetal cells with the meiocytes makes this tissue of considerable physiological importance to anther development (Vasil 1967). It has long been suggested that the tapetal cells play a nutritive role for the microspores and are involved in the formation of the pollen exine layer (for recent reviews see Mascarenhas 1989, 1990; Scott 1993). Its role in the initiation of meiosis and in the synchrony of the pollen mother cells is unknown.

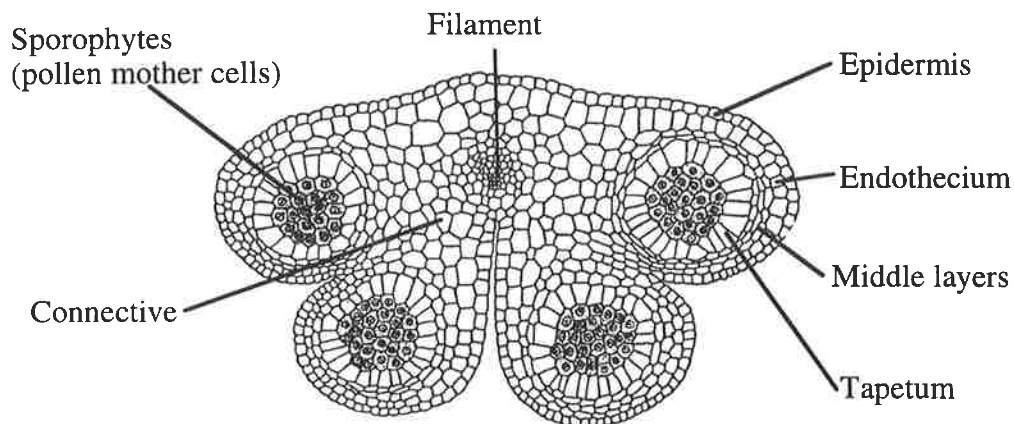


Figure 1.1 **Transverse section of a premeiotic anther from a monocotyledonous plant.**

Adapted from Arthur W. Haupt. Plant Morphology. McGraw-Hill Book Company, Inc. New York. 1953. 464 pp.

The striking synchronisation of hundreds of cells in the same floret has always been thought to be chemically controlled through tapetal secretion (Vasil 1967 and ref. therein). The presence of plasmodesmata between and among the tapetal cells and the meiocytes, creates microscopic channels of communication (Heslop-Harrison 1966) and therefore synchrony could possibly arise in response to a factor that diffuses through these channels (Vasil 1967). Bennett (1971) also believes that the behaviour of the tapetal cells suggests the existence of such a factor. The development of the tapetal cells is asynchronous until

well after the premeiotic mitotic division. However, in wheat and many other species, the tapetal cells undergo a synchronous mitotic division prior or concomitant to the onset of meiosis in the pollen mother cells. It would seem that synchrony in these sporogenous cells happens after a developmental hold has formed in the course of a premeiotic cell cycle (Bennett *et al.* 1973). Although the tapetal cells may have a controlling or intermediate role in the initiation of meiosis, it is now possible to artificially continue meiocyte development *in vitro* after the start of meiosis (mid-leptotene) and without the presence of the tapetal cells (Ito and Stern 1967; Takegami *et al.* 1981). This suggests that once meiocytes are committed to meiosis, the tapetal role is mostly to provide nutrients or hormones to these cells.

1.2.2 Premeiotic interphase and the initiation of meiosis.

According to Bennett and Smith (1972) the duration of premeiotic interphase (that is, the period between the final premeiotic mitosis and beginning of leptotene) lasts around 48 hours when wheat is grown at 20°C. During this period, several cytological changes occur in the pollen mother and tapetal cells. One is the appearance in meiocytes of bundles of fibrillar materials in the nuclei that are attached to the chromatin and/or the inner membrane of the nuclear envelope (Bennett *et al.* 1979). The role and nature of these bundles is unknown but they are probably related to specific changes in the nuclear skeleton (see also Section 1.3).

Also during this time, synchronous DNA synthesis (S phase) starts in meiocytes and tapetal cells. The DNA content increases from 2 to 4 C. Almost immediately after the completion of this phase, the nuclei of all tapetal cells undergo a synchronous mitosis which results in the formation of binucleated cells and concomitantly, leptotene begins in the pollen mother cells (Bennett *et al.*1973). In Chinese Spring, the last mitotic division in the tapetal cells can be used as a cytological marker for the start of meiosis in the pollen mother cells.

1.2.3 Stages of meiosis.

The duration of each meiotic stage in Chinese Spring has been estimated using both autoradiographic and anther sampling methods. At 20°C, the meiotic cycle lasts about 24 hours in *T. aestivum* (Bennett *et al.* 1971).

The first prophase stage, leptotene, is the longest and takes 10.4 hours to complete. Leptotene is characterised by a slight condensation of the chromatin, forming fine loops of thread and also by the disappearance or fusion of the three cell nucleoli into one (Bennett *et al.* 1973). As leptotene proceeds and more chromosome condensation occurs, the cells enter the zygotene stage (3-4 hours) where the homologous chromosomes align and the synaptonemal complex forms. During this period, Bennett and co-workers (1973) have observed that pairing tended to start around the chromosome ends near the nuclear periphery. This suggests that pairing is initiated at the telomeres (see Section 1.3 below). Moreover, interlocking of multivalents (homoeologous chromosome pairing) is frequently observed but this is corrected by pachytene and only strict bivalents are visible at metaphase I (Hobolth 1981).

Once the synaptonemal complex is fully formed along the paired chromosomes, further condensation of the chromatin occurs during pachytene. This stage lasts slightly more than 2 hours and is characterised by the appearance of recombination nodules along the bivalents where chiasmata have formed. Immediately after this, diplotene and diakinesis follow but last only one hour together. During this period, desynapsis begins and becomes visible as a repulsion between paired chromosomes. At this time the synaptonemal complex dissolves and each bivalent remains joined by one or more chiasmata, representing the sites where crossovers have occurred (Alberts *et al.* 1989). Diakinesis emerges subtly. The nuclear envelope disintegrates and the chromosomes reach their maximum condensation and remain linked by their centromeres.

After the long prophase I, two successive nuclear divisions bring meiosis to an end. In wheat cv. Chinese Spring, this takes only 7 hours: only 2.6 hours is needed from metaphase I to telophase I, then 4.4 hours is spent from the dyads to telophase II. These

last stages are perfectly comparable with the mitotic cycle and involve the same type of cellular machinery. Of course, the dramatic distinction with mitosis is that the final tetrads are haploids and the DNA content is reduced to 1 C.

1.3 Chromosome pairing at meiosis.

1.3.1 Homology search and chromosome alignment.

For a eukaryotic organism that has to go through sexual reproduction at some stage in its life cycle, meiosis serves two main functions. First, it ensures the production of a haploid state. Second, it provides for genetic recombination (John and Lewis 1984). One very important prerequisite for the germ cell, before reaching these two goals, is to have the homologous chromosomes find each other, align and pair in an intimate fashion in the presence of a tripartite structure called the synaptonemal complex.

How and when this association of homologues is brought about, has long been one of the major puzzles of meiosis (for detailed reviews see also: Dover and Riley 1977; Maguire 1977, 1983, 1984, 1992; Riley and Flavell 1977; Bennett 1984; Stern 1986; Dickinson 1987; Loidl 1990; Moens 1994).

Loidl (1990) has classified the main hypotheses put forward on homology search and chromosome pairing into three general categories: (i) somatic (premeiotic) chromosome disposition, (ii) specific interaction at prophase, (iii) random contacts at prophase.

In somatic (premeiotic) chromosome disposition, chromosomes would not be completely dispersed within the nucleus (Heslop-Harrison and Bennett 1983; Bennett 1984; Maguire 1984). The haploid genome would tend to be spatially separated, lying side by side or one around the other (Schwarzacher *et al.* 1989). Also, different chromosome types in each haploid set would seem to display secondary association and have relatively fixed positions in relation to each other (Flavell *et al.* 1987). This relationship should not be thought of in terms of direct linkage between homologues, but rather the presence of mechanisms within the nucleus which would permit the "processing" of both haploid sets of chromosomes

such that individual homologues could be paired up when appropriate (Dickinson 1987). This kind of process would be more systematic than accidental and defined structures such as "elastic connectors" (Maguire 1977), "intranuclear fibrillar material" (Bennett *et al.* 1979) or extranuclear microtubules would be required to pair homologous chromosomes (Loidl 1990).

The second category of hypotheses differs from the first one by proposing that long range interactions between homologous chromosomes would start only at the onset of meiosis and not before. This means that chromosomes would probably be randomly positioned in relation to each other but, at the time of meiosis, a systematic movement between the chromosomes (or between chromosomes and the nuclear envelope) would take place and "pull" the homologous chromosomes toward each other (see for example Rhoades 1961; Sybenga 1966; Smithies and Powers 1986).

Finally, chromosomes could be randomly positioned at the time of meiosis (like category two) but some kind of mechanisms could exist that increases the chance of accidental homologous contacts that initiate pairing. For instance, Padmore *et al.* (1991) proposed that the alignment of homologous chromosomes could result from a homology search initiated by site-specific double-stranded DNA breaks in early meiotic prophase (see Section 1.3.2).

These categories proposed by Loidl (1990) differ mainly by the randomness or otherwise of the positioned chromosomes in the nucleus and by the time when the homology search occurs; that is before or after the onset of meiosis.

The frequently observed occurrence of a "bouquet" or clustering of chromosomes at some point in meiotic prophase, seems conceivable in these three categories. Clustering of chromosomes is observed in wheat (Holm and Wang 1988) and several other organisms (see von Wettstein *et al.* 1984; Gillies 1984). When telomeres become attached to the nuclear membrane, intercalary chromosome regions project as a bundle of loops into the nuclear lumen creating a "bouquet" arrangement. This effect could be the consequence of

movement of chromosomes that are already interacting (as in category one or two) or a precondition for the establishment of a random homology search (category three).

As chromosomes without telomeres seem to pair normally (reviewed by Maguire 1984), it is difficult to know if telomere clustering has a direct influence in chromosome pairing. Nonetheless, the recent observations by Chikashige and co-workers (1994) in fission yeast, suggest that the telomeres play an important part in pairing. In these cells, they observed a leading role of the telomeres at meiotic prophase which suggests a role for telomeres in pairing in some eukaryotes.

1.3.2 Possible molecular mechanisms for homology recognition.

The previous section has referred to hypotheses to explain cytological observations of chromosomes alignments. However, the molecular mechanisms that could explain how homologous chromosomes in eukaryotic organisms recognise each other to form a close and stable alignment in the synaptonemal complex, are largely unknown. Most current models suggest direct DNA-DNA interactions or biparental duplex formation to check for homology (Moens 1994).

A popular model for chromosome recognition is the "zyg DNA replication system" (Hotta and Stern 1971; Stern and Hotta 1984). In *Lilium* sp., it is observed that 0.3% of the bulk DNA is not replicated at the premeiotic S phase but delayed until the zygotene stage. Therefore, it was proposed that zygDNA could be involved in the formation of biparental duplexes between homologous chromosomes and play a role in homologous recognition. The occurrence of zygDNA has only been observed in *Lilium* and less convincingly, in mouse. Loidl (1990) has also expressed doubts about the direct role zygDNA could have in homology recognition, mainly because this process can be found well before the zygotene stage (Loidl and Jones 1986; Weiner and Kleckner 1994).

Later, Stern (1986) suggested a new role for zygDNA. It could provide attachment sites for the proteins involved in synaptonemal complex (SC) formation. Again, further

evidence will be needed to verify this model. Alternatives of homology recognition by biparental duplex formation have also been proposed to interpret the increased rate of gene conversion observed at meiosis (for details see Smithies and Powers 1986; Carpenter 1987; Engebrecht *et al.* 1990).

Another approach to investigate the molecular mechanisms involved in homologous recognition was a search for analogs of the *E. coli* recA protein. In this prokaryote, recA is involved in homology searching, homologous pairing and recombination (Radding 1991; Roca and Cox 1990; West 1992). In fact, several genes identified as eukaryotic recA homologs have been found in fungi (eg. *DMC1*: Bishop *et al.* 1992), plants (eg. *LIM15*: Kobayashi *et al.* 1993) and mammals (eg. Morita *et al.* 1993; Shinohara *et al.* 1993). Their involvement in recombination has been recognised but their role in homology recognition needs further investigation, since eukaryotic recA analogues have similarities to mitotic proteins involved in the repair of double strand DNA breaks (DSBs). These function by finding and using the undamaged homologue as a template. There could be a similar mechanism in meiosis to find and recognise homologous pairing (see in particular Bishop *et al.* 1992).

The properties of the *E.coli.* recA protein, also suggest that it is possible to have homologous pairing between two intact DNA duplexes without a single strand nick or a double strand cut (Conley and West 1989). This could be a first step to a genome-wide homology search without a large energy cost and, subsequently, DSBs could provide a means for homology recognition (for details see review by Klekner *et al.* 1991). Can the capacity of recA to form a complex with either ssDNA or dsDNA be extrapolated to the eukaryotic analogues? This is speculative but appealing.

Recently, great advances have been made towards the understanding of the meiotic process in the yeast *Saccharomyces cerevisiae*. Researchers (eg. Hawley and Arbel 1993) are starting to rethink the classical view of meiosis; synapsis-recombination-segregation. This sequence of events is probably not as distinct as first thought. Until data become available, not only from yeast but also from higher eukaryotes, it will be difficult to draw a clear picture of how homology recognition works.

1.3.3 Chromosome pairing and synaptonemal complex formation.

The synaptonemal complex (SC) is morphologically conserved across a wide range of species (von Wettstein 1984). It is a unique chromosome-associated structure which is elaborated in meiotic prophase nuclei and forms the axis of the paired homologues during close physical alignment (synapsis). It is made of two axial elements (or lateral elements) which are closely synapsed, at distances of around 100nm, and a central component which extends longitudinally between the two axial elements (Giroux 1988). Each axial element represents a pair of sister chromatids associated with proteins. The bulk of chromatin is found outside the SC and is arranged in more or less regular loops, each linked at its base to a lateral element (Weith and Traut 1980).

In wheat and most higher eukaryotes, the assembly of this structure starts at the leptotene stage and is fully synapsed by the end of zygotene. At pachytene, the SC appears as a ribbon-like structure along the entire length of each chromosome pair or bivalent. The bulk of the SC is shed at diplotene; once crossovers have occurred.

The role of the SC in meiosis is still far from completely understood. For many years it has been assumed to be involved in meiotic recombination, homologous chromosome pairing and segregation (von Wettstein *et al.* 1984). With recent studies on chromosome pairing in organisms such as *Schizosaccharomyces pombe* (Bähler *et al.* 1992), *Ustaligo maydis* (Fletcher 1981) and *Aspergillus* species (Egel-Mitani *et al.* 1982) in which it is possible to observe pairing and recombination without the presence of the SC, it seems that the proteinaceous structure is not necessarily essential for recombination and proper segregation (Loidl 1993).

However, where it is present, the SC seems to enhance or facilitate the meiotic process and organisms who have developed it, become dependent on it for proper recombination and segregation. Its role is multi-functional but, in the context of this review, only findings related to pairing and synapsis will be mentioned.

An interesting finding has been made regarding the nature of the DNA sequences that are in direct association with the SC proteins. In rat pachytene, Pearlman and co-workers (1992) have purified SCs from which the chromatin had been removed by extensive DNase II digestion. Any remaining DNA had been protected against further digestion by the core of SC associated proteins. Isolation and analysis of the remaining sequences showed that they were not random sequences. An excess of microsatellites was found, mostly GT/CA repeats, along with short and long interspersed repeated elements (SINE/LINE). From this analysis, it has been suggested that a fraction of DNA might interact with the SC and form the basis of the chromatin loops. This may not be true for all organisms forming SC but for higher eukaryotes possessing a considerable amounts of dispersed repetitive DNA sequences, these sequences could serve as secondary sources of homology for pairing (Loidl 1994).

Several other approaches have been taken to identify components of the SC and learn more about chromosome pairing and recombination. The main approaches have been the analysis of meiotic mutants failing to form SC or recombine and the biochemical analysis of protein extracted from purified SC.

In *S. cerevisiae*, several mutants with a defect in SC formation have been identified, for example *SPO11* (Giroux *et al.* 1989), *RAD50* (Alani *et al.* 1990), *MEI4* (Menees *et al.* 1992), *HOP1* (Hollingsworth and Byers 1989), *RED1* (Rockmill and Roeder 1990), *REC102* (Bhargava *et al.* 1992), *DMC1* (Bishop *et al.* 1992), and *ZIP1* (Sym *et al.* 1993). However, for some of those mutants, it has been difficult to identify specific SC functions because the observed defects in SC assembly could be a cause as well as an effect of the disturbance of meiosis (Meuwissen *et al.* 1992).

Nevertheless, in some cases such as *HOP1* and *ZIP1*, there exists a direct link with the assembly of the SC. *HOP1* has a putative zinc finger motif DNA binding domain and is localised in the lateral element of the SC (Hollingsworth *et al.* 1990). No synaptonemal complex is formed in the mutant *hop1* diploid and no proper disjunction of the homologous chromosomes is achieved. This results in a high level of spore death. Similarly, the *ZIP1* gene product is also essential for chromosome synapsis in *S. cerevisiae*. (Sym *et al.* 1993).

The *zip1* mutation aborts meiosis prior to the first division. From sequence analysis and with the help of a specific antibody, the *ZIP1* protein was shown to have a coil-coil protein structure and to be localised in the central element of the SC. Sym and co-workers (1993) have proposed a model in which *ZIP1* would act as a molecular zipper in the intimate pairing of the bivalents.

Apart from the study of yeast mutants, the most common approach used to identify components of the SC is the biochemical analysis of the proteins involved in this structure. The first step is to isolate SCs and then produce monoclonal antibodies against SC. By this method similar structural protein analogs to *ZIP1* were found. *SYN1* in hamsters (Moens 1994) and *SCP1* in rats (Meuwissen *et al.* 1992) seem also to have a coil-coil protein motif indicative of protein-protein interaction. Both are found in the central element of the SC but it is not known if they have a strict structural role in the SC formation or have a controlling effect on chromosome homology testing.

The above discussion would suggest that chromosome pairing, homology recognition and SC formation do not depend on a particular sequence of events or set of mechanisms. In the course of evolution, divergence has probably occurred in respect to the prevalence of one mechanism over another but without affecting the overall outcome of meiosis, the segregation of chromosomes into four distinct haploid cells.

The next section of this review will deal with chromosome pairing in wheat. Because *T. aestivum* is an allohexaploid that contains three sets of related chromosomes, the challenge has been to understand how control of homologous chromosome pairing functions and to establish whether pairing could be modified for breeding purposes.

1.4 Chromosome pairing and the *Ph* genes.

1.4.1 Discovery of the *Ph* genes.

Chromosome pairing is particularly complex in an allopolyploid such as *T. aestivum* (Ceoloni and Donini 1993). The wheat genome is made from three distinct sets of

chromosomes (genomes A, B and D). The three diploid ancestors that donated the genomes show considerable genetic correspondence as shown by Sears (1952, 1954). As a consequence, the corresponding chromosomes between each genome have been referred to as homoeologues in contrast to the truly homologous chromosomes within the same group. Although homoeologues chromosomes have the capacity to pair and undergo crossing-over, they cannot do so in *T. aestivum* where only homologous bivalents are seen at metaphase I (Sears 1976). In fact, this diploid-like behaviour in hexaploid wheat was shown in the 1950s to be under tight genetic control with the discovery of a locus on the long arm of chromosome 5B, suppressor of homoeologous pairing, *Ph1* (Riley and Chapman 1958; Sears and Okamoto 1958; Wall *et al.* 1971b). In nullisomic 5B plants or *ph1b* mutants (Sears 1977), there are no functional copies of the *Ph1* locus, and wheat exhibits an allosyndetic pairing behaviour (Riley 1960). In these plants, multivalents of 3 to 6 chromosomes are formed and remain visible at metaphase I. In contrast, plants trisomic for chromosome 5B (with 6 doses of *Ph1*) are partially asynaptic for homologous chromosomes and allow some pairing between homoeologous chromosomes leading to a reduction of overall chiasma frequency and increased interlocking of bivalents (Feldman 1966).

Although the *Ph1* locus is known to have a strong effect on the control of homoeologous pairing in wheat, researchers for the past three decades have identified other loci that can exert an effect on pairing by either suppressing or promoting it (Sears 1976).

In additon to the *Ph1* locus, suppressors of homoeologous have been found on chromosomes 3AS and 3DS (*Ph2* - Sears 1982) with the *Ph2* locus being the most effective but somewhat weaker than *Ph1* (Mello-Sampoyo 1971; Driscoll 1972; Mello-Sampoyo and Canas 1973; Sears 1984). Other minor suppressors on chromosomes 3BS, 3AL, 3DL, 2DL have also been detected (Miller *et al.* 1983; Mello-Sampoyo and Canas 1973; Ceoloni *et al.* 1986; Cuadrado *et al.* 1990; Sears 1984) and possibly on chromosome 4D and 7A (Driscoll 1973; Mello-Sampoyo 1972; Miller and Reader 1985).

A group of loci promoting pairing has also been identified. They are mainly located on chromosome groups 2, 3 and 5. Those pairing promoters are found on the short arm of

chromosomes 2A, 2B and 2D (Ceoloni *et al.* 1986); the long arm of chromosomes 3A, 3B, 3D (Driscoll, 1973); the short arm of 5B and both arms of chromosomes 5A and 5D (Feldman 1966). In addition, chromosomes 1A and 1D could also produce a slight increase in pairing (Miller and Reader 1985)

Most of those suppressors or promoters of pairing have been detected in wheat haploids, interspecific hybrids or through the use of aneuploids (see Sears 1976). In *T. aestivum* itself, a finely tuned balance seems to exist between all those genes controlling chromosome pairing.

In recent years, mutants such as *ph1b* or *ph2a*, *ph2b* (Sears 1977, 1982, 1984) have been produced to study the effect of pairing (Ceoloni and Donini 1993) and to facilitate the introgression of alien chromosomal segments into wheat (Koebner and Shepherd 1985).

In bread wheat, the *Ph1* locus has been the most studied of all pairing control genes. Researchers have tried to understand when and how its role becomes critical in particular processes such as mitosis and meiosis. The following section will describe various models developed to explain the observed cytological effects of this gene and other *Ph* genes.

1.4.2 Possible models of action of the *Ph* gene(s).

Most hypotheses to explain the mode of action of *Ph1* were established by looking at chromosome pairing in plants containing different doses of the gene (0 to 6 copies). In these aneuploid plants, chromosomes at meiosis (from zygotene to metaphase I) were scored for the frequencies of mono-, bi-, tri-, quadri-, penta- and hexavalents and three-dimensional reconstructions were made from serial sections of nuclei at zygotene/pachytene. The relations between different doses of *Ph1* and anti-microtubule drugs were examined (for review see Gillies 1989).

From these and related approaches, two main categories of models have emerged to explain when and how the *Ph1* gene product could work in meiosis (and to some extent also in mitosis).

The first category of models assumes that *Ph1* exerts its effect at premeiotic interphase. To illustrate this, it is best to refer to the work of Feldman and co-workers (for example Feldman 1966, 1968; Avivi and Feldman 1973; Feldman and Avivi 1984, 1988).

By looking at pairing in plants containing different doses of 5B and of the homoeologues 5A and 5D, Feldman (1966) observed that *Ph1* in normal dosage (2 copies) prevents pairing of homoeologues. However, when present in 4 copies, the gene slightly reduced chiasma frequency between homologues and when in 6 copies, it reduced dramatically synapsis in homologues but at the same time pairing of homoeologous chromosomes was detected. To explain those observations, he suggested that the *Ph1* locus regulates the premeiotic association of homologous and homoeologous chromosomes. High doses of *Ph1* would suppress completely premeiotic association and thus chromosomes would lie randomly in the nucleus. At pairing, the force that attracts homologous chromosomes would fail to bring distant chromosomes together since they would normally have been brought closer together earlier. If by chance, homoeologues chromosomes were lying close to each other, they would pair. This could explain the observed pairing of homoeologues in the presence of 6 copies of *Ph1*.

With these and similar observations in mitotic cells (see review in Feldman 1993 and references therein), Feldman believes that the effect of *Ph1* in premeiotic alignment of chromosomes is evident from its differential effect on intra- and interchromosomal pairing of isochromosomes (Feldman and Avivi 1988).

Feldman and co-workers have also tried to evaluate the effect of colchicine or vinblastine with different doses of *Ph1* (Avivi *et al.* 1970a, 1970b; Ceoloni *et al.* 1984). In the presence of those chemicals, which affect the polymerisation of tubulin, they observed that *Ph1* deficient plants were most affected during chromosome pairing. However, plants treated with a chemical (isopropyl-N-phenylcarbamate) affecting only spindle organisation, showed no differential effect between the plants carrying 0 to 4 copies of *Ph1* (Gualandi *et al.* 1984). From those experiments it was suggested that "*Ph1* acts on the dynamics of microtubule assembly and disassembly, i.e. the exchange of subunits between polymers and a soluble tubulins pool, rather than on spindle organisation" (Feldman 1993). Even if

this is true, it is not known how the *Ph1* gene(s) affects microtubules assembly and subsequently chromosome pairing.

The idea that the level of chromosome organisation in the genome could be partly controlled by the *Ph* genes, is appealing. However many workers disput this hypothesis (Hobolt 1981; Holm 1986; Holm and Wang 1988).

In the second category of hypotheses, it is assumed that *Ph1* operates only at the time of pairing. It would act specifically on the mechanisms involved in synapsis or crossover. This model was originally proposed by Darvey and Driscoll (1971, 1972; Driscoll and Darvey 1970) based on the somatic association of chromosomes in root tip cells. They did not find a stronger or significant correlation between homologous chromosomes lying close together as opposed to homoeologous chromosomes. Sears (1976) believed these negative findings could have been due to experimental errors since the degree of somatic association found by Feldman was low and could be affected by environmental factors.

Later Driscoll and co-workers (1979) suggested another model for *Ph* gene action on the basis of a mathematical analysis of chromosome association in hybrids, aneuploids and colchicine treated wheat. They observed that genes on chromosome groups 3 and 5 did not affect presynaptic associations but had an effect on the number and distribution of chiasmata at prophase I. They suggested that the *Ph* gene product could be an enzyme that determines whether pairing events could lead to crossing-over. If this is true, the *Ph* gene product would prevent completion of homoeologous pairing so no crossing-over would take place. Conversely, crossovers are common between homoeologues 5B aneuploid plants.

Holm and co-workers also believe that the *Ph* gene product suppresses crossovers between homoeologues and also controls the stringency of homologous pairing. They draw this conclusion from electron-microscopic data where they have used the SC spreading technique to investigate pairing in plants containing different doses of *Ph1* (for details see Holm 1986; Holm *et al.* 1988)

Gillies (1987) has also studied the *Ph* gene behaviour in pentaploid hybrids between *T. aestivum* and *T. kotschyi*. His findings also agree with Holm, namely that the *Ph* gene seems to have an effect on the stringency of pairing. However, he has also noticed, in one zygotene *Ph* nucleus, the "bouquet" formation of 18 telomeres with matched ends. This suggests that pairing at telomeres might not be random. This, in turn, could support, to a certain extent, the non-randomness of chromosomes invoked in Feldman's model.

These studies demonstrate that the effect of the *Ph* gene is complex. It probably affects several mechanisms in meiosis: the number of multivalents formed, the suppression of crossing-over between homoeologues, and the chromosome disposition in the nucleus. Even though no agreement has been reached in relation to when and how the *Ph* gene product works during meiosis, there may be some interrelation between all the models presented above. The cloning of the wheat genes encoding the products of these suppressors and promoters of pairing, would provide strong insights into how chromosome pairing in higher eukaryotes is controlled.

1.4.3 Improvement of wheat breeding by the control of the *Ph* genes.

The evolution of polyploid wheat and its outstanding role in our lives is partly due to the presence of a diploidization mechanism. By restricting pairing to completely homologous chromosomes, the *Ph* genes ensure regular segregation of genetic material, high fertility and genetic stability (Feldman 1976). In fact, most of the aneuploid plants lacking *Ph* genes are partially sterile or not as vigorous as their euploid counterparts.

Another influence of the *Ph* genes in *T. aestivum* is the existence of a strong barrier to gene flow between crossable species having homoeologous chromosomal relationships (Ceoloni and Donini 1993). Yet, the introduction of alien traits into *T. aestivum* would prove to be of great benefit as it would enlarge its genetic base.

In response to this limitation, Sears in 1977, produced a mutant (*ph1b*) where a deletion of the *Ph1* locus had occurred. Using this plant in crosses helped breeders to improve the

introgression of alien chromosomal segments into wheat (see review Gale and Miller 1987). Although the effectiveness of the *ph1b* mutant has been demonstrated, it has nonetheless limited utility in some cases. For instance, pairing and recombination between chromosomes from rye and wheat, or barley and wheat are low and desired crosses are laborious to obtain, even in *ph1b* plants (eg. Koebner and Shepherd 1985; Islam and Shepherd 1988). In these and similar cases, it would be a great advantage to pinpoint and rectify the factors that prevent efficient engineering of these crosses.

The first step to resolve this problem would be to understand the mechanisms involved in chromosome pairing at meiosis in *T. aestivum*. As mentioned in Section 1.4.3, the main chromosome loci promoting or suppressing pairing have been identified and their cytological effects well documented. Yet their gene products and the mechanisms of their regulation are still completely unknown. The isolation and characterisation of these genes would help to understand how pairing works. A knowledge of the molecular basis of pairing in wheat would help to acquire the means of controlling pairing and provide an additional tool in wheat breeding.

1.5 Project aims.

The overall goal of this project is to gain a better understanding of the molecular events in meiosis in *T. aestivum*. Most work in meiosis has been pursued in yeast and to a lesser extent in mammals and some plants, notably, lily. Because of the importance of wheat as a crop and the sophistication of its pairing processes, it would be valuable to know the underlying molecular and biochemical mechanisms involved in meiosis.

The *Ph* gene loci, promoting or suppressing homoeologous pairing have only been identified in polyploid plants. Their cytogenetic effects are well documented but their mechanisms of action are unknown and hypotheses for their mode of action are controversial This project has attempted to shed some light on wheat meiosis at the molecular level.

The specific aims of this project were to:

1) Prepare a cDNA library from wheat florets at premeiotic and early meiosis stages.

2) Screen the library differentially against vegetative tissue and mature pollen anthers and select clones showing preferential expression in anthers at early meiosis.

3) Pursue fine analysis of the clones potentially involved in chromosomes pairing or related to the *Ph* genes.

CHAPTER II

Materials and Methods.

2.1 Plant genetic stocks.

Plant stocks of *Triticum aestivum* var. Chinese Spring, *Hordeum vulgare* var. Betzes, *Secale cereale* var. Imperial, and the various Chinese Spring aneuploid lines, wheat/barley addition lines, wheat/rye translocation lines were all obtained from the collection at the Waite Agricultural Research Institute.

All plants were grown in a glasshouse under natural light at 20-25°C. For anther sampling, some Chinese Spring plants were transferred in a growth chamber about 2 weeks before the time of meiosis and kept under the following conditions: 16 hours of light exposition at 20°C and 8 hours of darkness at 16°C.

2.2 Bacterial strains and cloning vectors information.

For the cDNA library construction, *E. coli* strains C600 and C600hfl were provided by Promega with the lambda gt10 packaging kit (Promega 1992).

E. coli C600 genotype: F^-, thi-1, thr-1, leuB6, lacY1, tonA21, supE44.

E. coli C600hfl genotype: F^-, thi-1, thr-1, leuB6, lacY1, tonA21, supE44, hflA150, [chr.Tn10].

For plasmid transformation, *E. coli* strain DH5α from New England Biolab was used with the plasmis vectors: pTZ19U and Bluescript KS.

E. coli DH5α genotype: F^-, lacZΔm15, endA1, recA1, hsR17 (rk^-, mk^-), sup44, thi-1, l^-, gyrA96, Δ(lacZYA-argF).

For the library construction, the vector used was lambda gt10 and for subcloning of the selected clones, the plasmid vectors pTZ19U and Bluescript KS- were used.

Lambda gt10 genotype: imm^{434} b527, EcoR I cloning site (from Promega).

pTZ19U genotype: pUC19 derived, f1(-), ColE1 origin, LacZ, MCS (multiple cloning site), amp^r (from Pharmacia).

Bluescript KS genotype: pUC19 derived, f1(-), ColE1 origin, LacZ, MCS, amp^r, T3/T7 RNA polymerase promoter (from Stratagene).

2.3 Nucleic acid preparation.

2.3.1 Large scale genomic DNA preparation.

Healthy leaf material (~5 g) was collected from 4-6 weeks old plants, frozen in liquid nitrogen and ground into a fine powder using a mortar and pestle. DNA extraction buffer (100 ml) (0.1M Tris-Cl pH 8.5, 0.1M NaCl, 0.01M Na_2EDTA, 4% sarkosyl) was added and mixed to form a homogenised slurry. This mixture was transferred to a 250 ml centrifuge tube and 100 ml of phenol:chloroform:isoamyl alcohol (25:24:1 equilibrated with 10 mM Tris-Cl pH 8.0, 1 mM Na_2EDTA) was added. This was mixed one hour at 4°C on an orbital rotor and subsequently centrifuged for 10 minutes (min) at 5,000 rpm, 4°C (Beckman JA-14 rotor, J2-21M centrifuge). The resulting supernatant was gently collected and filtered through 3 layers of cheesecloth. The volume of this crude extract was measured and one tenth volume (~10 ml) of 3.0 M sodium acetate (pH 4.8) was added. The DNA was then precipitated by adding 2.5 volumes (~250 ml) of 100% ethanol and left at -20°C for a minimum of 2 hours. DNA was pelleted by centrifugation at 10,000 rpm for

10 minutes at 4°C (Beckman rotor JA-14). After centrifugation the DNA was washed twice with 70% ethanol, air dried and resuspended in 7.0 µl of TE buffer (10 mM Tris-Cl pH 8.0, 1 mM Na_2EDTA).

To further purify the DNA from RNA and protein contaminants, a CsCl gradient was run. CsCl (7.5 g) was added to the DNA solution and once the salt was completely dissolved, the mixture was transferred to a 10 ml Nalgene Ultralock centrifuge tube and 0.5 ml of ethidium bromide solution (10 mg/ml) was added. After thorough mixing, the sample was centrifuged to equilibrium at 40,000 rpm for 40 hours at 20°C (Beckman rotor 70.1T1, centrifuge L8-70). It was possible to remove the DNA band from the gradient by piercing gently the tube with an 18 gauge needle attached to a 10 ml syringe and drawing out the DNA. To remove the ethidium bromide from the DNA fraction, 2 volumes of water saturated butanol was added and mixed on an orbital rotor at room temperature for 20 minutes. This butanol wash was repeated 3 to 4 times or until no ethidium bromide (pink colour) was visible in the DNA phase. Finally, to remove the CsCl salts in this DNA sample, a dialysis was performed against 2 changes of TE buffer, overnight at 4°C.

The DNA concentration was determined by spectrophotometry. A 1:50 dilution of the sample was made and its optical density (OD) measured at a wavelength of 260 nm.

2.3.2 Small scale genomic DNA preparation.

A young leaf (~10 cm long) was placed in a 2 ml eppendorf tube and frozen in liquid nitrogen. With the help of a knitting needle, the leaf was finely ground directly in the eppendorf tube and 600 µl of DNA extraction buffer was added to homogenise. To the resulting slurry, 600 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added and mixed thoroughly on an orbital rotor for 15 minutes at room temperature. The aqueous and organic phases were separated by centrifugation at 12,000 rpm for 10 min in an eppendorf centrifuge. The upper phase was collected to a fresh tube and the phenol extraction step was repeated. After the second extraction, the DNA contained in the aqueous phase was precipitated by adding 60 µl of 3 M sodium acetate pH 4.8 and 600 µl of isopropanol. This

was mixed by manual shaking (30 to 60 sec.) and left at -20°C for one hour. The precipitated DNA was then pelleted at 12,000 rpm for 15 min and washed twice with 70% ethanol. The DNA pellet was dried and resuspended in 50 µl TE buffer containing 40 µg/ml ribonuclease A (Sigma).

2.3.3 Plasmid DNA extraction.

The alkaline lysis method for mini-scale plasmid preparation was used as described by Sambrook *et al.* (1989). A sterile culture tube containing 4 ml of LB medium (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl, pH 7.5) with 50 µg/ml ampicillin was inoculated with a single transformed bacteria colony and grown overnight in a 37°C shaker. Cells were pelleted by centrifugation at 3,000 rpm for 10 min and the supernatant discarded. The cells were resuspended in 100 µl of plasmid I solution (50 mM glucose, 25 mM Tris-Cl pH 8.0 , 10 mM Na_2EDTA) and left on ice for 10 minutes. Cells were lysed by an addition of 200 µl of plasmid II solution (0.2 N NaOH, 1% SDS), freshly made, and placed again on ice for a further 10 minutes. To precipitate the bacterial DNA and other cell debris, 150 µl of 3 M sodium acetate pH 4.8 was added, mixed thoroughly and placed at -20°C for 15 minutes. The cell debris were subsequently discarded after centrifugation at 12,000 rpm for 10 minutes. The supernatant containing the plasmid DNA was transferred into a fresh tube and extracted by an equal volume of phenol solution. The plasmid DNA was precipitated with one tenth volume of salt (3 M sodium acetate pH 4.8) and 2.5 volumes of 100% ethanol and left at -20°C for 2 hours or -80°C for 30 minutes. The DNA was recovered by centrifugation at 12,000 rpm for 15 min, washed twice with 70% ethanol, dried under vacuum and resuspended in 100 µl of autoclaved nanopure water.

2.3.4 Lambda phage DNA purification.

An isolated phage plaque from a plate (Section 2.4) was picked and left to diffuse in 100 µl of SM buffer (20 mM Tris-Cl pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$, 0.1% gelatin) at

4°C. From this phage stock 25 to 50 μl was taken to inoculate 0.5 ml of *E. coli* C600hfl grown previously to a bacterial density of 0.6 (absorbance at 600 nm). The phage was left to adsorb on bacteria for 20 min at 37°C then 5 ml of LB medium containing 5 mM $CaCl_2$ was added. The phage mixture was then incubated in a 37°C shaker for about 5 hours or until the culture became clear (complete lysis). The resulting lysate was then mixed with 0.5 ml of chloroform and centrifuged at 7,000 rpm (Beckman JA-20.1 rotor) for 20 min at 4°C, to remove bacterial cells debris. To the collected supernatant, 10 μl of Dnase I and Rnase A were added to a final concentration of 5μg/ml each, and incubated at 37°C for 30 minutes. An equal volume of phage precipitating buffer (20% PEG 8,000, 2 M NaCl, 10 mM Tris-Cl pH 7.5) was added and left on ice at 4°C, overnight. This solution was centrifuged at 10,000 rpm (Beckman rotor JA-20.1) for 20 min at 4°C and the supernatant was discarded. The tube containing the phage pellet was turned upside down and left to drain for 15 minutes. The pellet was resuspended in 700 μl of LB medium, transferred in a 2 ml eppendorf tube which contained 700 μl of DEAE-cellulose (Whatman DE52) LB suspension (Ausubel *et al.* 1987). After the sample had been inverted 20 to 30 times, it was centrifuged at 10,000 rpm for 5 min. The supernatant containing the phage was kept and transferred to a fresh tube and 13 μl of proteinase K (0.1 μg/μl), 32 μl of 10% SDS (sodium dodecyl sulphate) were added and mixed. After 5 min incubation at room temperature, 130 μl of 3 M potassium acetate was added. This phage mixture was mixed and left at 88°C for 20 minutes and then placed immediately on ice for 10 minutes. It was centrifuged at 10,000 rpm for 5 min to remove phage debris and the precipitated SDS. Finally, the phage DNA in the supernatant was precipitated with an equal volume of isopropanol and left at -20°C for one hour or more. The DNA was later pelleted by centrifugation for 15 min at 12,000 rpm, washed twice with 70% ethanol and resuspended in 100 μl autoclaved nanopure water.

2.3.5 DNA fragments isolation from agarose gel.

When DNA fragments from plasmids or lambda vectors needed to be used as probes or other subcloning reasons, the DNA was isolated by the Geneclean method (Bio101 from

Bresatec). First, the plasmid DNA was digested with an appropriate restriction enzyme to release the DNA insert. Secondly, the digested sample was run in an agarose gel to separate the insert from the vector. After staining the gel with ethidium bromide, the visible DNA band under UV light was cut from the gel, put in a tube containing 6 M sodium iodide and processed according to the Bio101 Geneclean instructions. The eluted DNA was resuspended in 50 to 100 µl water and ready to use.

2.3.6 Total RNA extraction.

For the cDNA library construction or the preparation of RNA blots, total RNA was extracted from different wheat parts (stems, leaves, roots, root tips, anthers, etc.) by the following method. Note that before starting any RNA extraction, glassware to be used was baked at 160°C, 6 hours and plasticware was treated with 0.1 N NaOH for 15 min and rinsed thoroughly with nanopure water.

Frozen plant material (kept at -80°C) was put in a small Rnase-free mortar and crushed carefully with a pestle. Once a fine powder was obtained, 4 ml of REB buffer (100 mM Tris-Cl pH 8.4, 10 mM Na_2EDTA, 4% sarkosyl) was added and the powder homogenised into a slurry. This was then transferred in a 15 ml Corex tube and allowed to thaw. The sample was centrifuged at 4°C, 5,000 rpm (Beckman rotor JA-20) for 15 min to remove cell debris. The supernatant was measured and transferred directly into a Beckman ultracentrifuge tube. If it was needed, the sample volume was adjusted to 4.5 ml with REB buffer and 4.5 g of CsCl was added and dissolved carefully by gentle mixing of the tube. Following this, 3 ml of CsCl cushion solution (0.965 g/ml CsCl in TE buffer) was laid slowly at the bottom of the tube with a Pasteur pipette. This gradient was centrifuged 16 hours at 30,000 rpm (Beckman rotor 70.1 T1) at 4°C. After the run, the top protein layer was removed with sterile cotton buds and the supernatant poured out. The remaining RNA pellet was then resuspended with 0.4 ml of REB buffer and transferred in a fresh eppendorf tube. An equal volume of the phenol solution (as described in Section 2.3.1) was added and the sample vortex for 30 seconds. This was centrifuged for 5 min at 12,000 rpm and

the upper phase was transferred to a fresh eppendorf. The RNA was precipitated by adding 40 µl of 3 M sodium acetate pH 4.8, 1 ml of ethanol and left at -20°C for 2 hours or more. The RNA was pelleted by centrifugation at 12,000 rpm for 15 min, 4°C and washed twice with 70% ethanol. The RNA pellet was dried under vacuum and resuspended in 100 to 200 µl of Rnase-free water. The amount of RNA obtained was measured by optical density at a wavelength of 260 nm. For the cDNA library construction, the RNA used was ethanol precipitated twice.

2.3.7 PolyA$^+$ RNA purification.

For the library construction, polyA$^+$ RNA was purified from 200 µg total RNA from wheat meiotic florets and as a control for the yield estimation, polyA$^+$ RNA was also purified from 100 µg leaf total RNA by the streptavidin-paramagnetic beads method. The method used is described in the Promega technical manual "PolyA tract mRNA isolation systems". Their instructions were followed exactly in the protocol for small scale mRNA isolation. After purification the polyA$^+$ RNA from wheat meiotic tissue was ethanol precipitated and the polyA$^+$ RNA sample obtained from leaves was measured by optical density to estimate the yield obtained by this method.

2.4 Library preparation and screening.

2.4.1 cDNA library construction.

The mRNA purified in Section 2.3.7 was resuspended after ethanol precipitation in 20 µl Rnase-free water. The Pharmacia cDNA synthesis kit was used to make the library and their instructions were followed exactly. To monitor the quality of the first and second strand reactions, radioactivity was used in small reaction aliquots. After mixing the mRNA solution to the Pharmacia first strand reaction mix, 5 µl was taken out and placed in a fresh tube with 0.5 µl α-^{32}P-dCTP. This sample was then incubated at 37°C for 60 minutes along with the main RNA mix. After the incubation, the radioactive sample was put aside

and the main mix was added to the Pharmacia second strand mix. From this main solution, a second aliquot of 10 μl was taken out and put in a fresh tube with 1 μl of α-^{32}P-dCTP. The tubes were incubated at 12°C for one hour and then at 22°C for another hour. After the last incubation, the main mix containing the non-radioactive double strand cDNA molecules was purified and EcoR I/Not I adaptors were ligated according to the Pharmacia instructions booklet.

The two tubes containing ^{32}P for the monitoring of the first and second strand cDNA synthesis were prepared for loading in an alkaline agarose gel. To these samples 1 μl of 10 mM Na_2EDTA and 1 μl of 0.75 N NaOH were added and the volumes were adjusted to 15 μl with water. The samples were mixed and left standing 5 min to denature and 1X loading buffer (10X: 30% ficoll 400, 0.25% bromocresol green, 0.25% xylene cyanol FF) was added just prior to loading. A 1.2% alkaline agarose gel was prepared following the protocol described by Odgen and Adams (1987). Once the gel was run in denaturing conditions (running buffer: 50 mM NaOH, 1 mM Na_2EDTA), it was soaked in several volumes of 7% of trichloroacetic acid for 30 min and dried overnight on a piece of Whatman 3MM paper. The dried gel was finally covered with plastic wrap and exposed to Fuji X-ray film at 4°C.

To estimate the best ratio of cDNA molecules to be ligated to dephosphorylated EcoR I lambda arms (Promega), three different ligation reactions were set up. In three separate 1.5 ml eppendorf tubes, 1 μg of EcoR I lambda arms DNA was ethanol precipitated with 5, 10 and 30 μl of the cDNA solution. After centrifugation, the resulting pellets were dried and resuspended in 8 μl ligation buffer (66 mM Tris-Cl pH 7.6, 1 mM spermidine, 10 mM $MgCl_2$, 15 mM dithiothreitol, 0.2 mg/ml Dnase-free bovine serum albumin, BSA), 1 μl of 0.5 mM ATP and 1 μl (1 unit Weiss/μl) of T4 DNA ligase (Pharmacia) were added. The three DNA samples were ligated overnight at 12°C and subsequently encapsidated according to the instructions of the Promega Packagene System.

2.4.2 Library plating and amplification.

Titres of the packaged phage preparations were done by dilution series from 1:10 to 1:1000 in SM buffer. To 100 µl of each dilution mix, 100 µl of *E. coli* strain C600 or C600hfl was added. Note that bacterial cells were first grown to an optical density of 0.6 (A_{600} nm) in LB medium (see Section 2.3.3) containing 0.2% maltose and 10 mM $MgSO_4$. The phage bacteria mix was added to 42°C melted top agarose (LB medium containing 0.8% agarose) and poured on LB agar plates (LB medium containing 1.5% agar). Phage were grown for 7 to 8 hours at 37°C and the titres of the original phage libraries were calculated:

1) 1 µg of EcoR I lambda arms + 5 µl cDNA solution gave 35,000 pfu.

2) 1 µg of EcoR I lambda arms + 15 µl cDNA solution gave 150,000 pfu.

3) 1 µg of EcoR I lambda arms + 30 µl cDNA solution gave less than 100 pfu.

Sample 3 was done with a Packagene tube that was expired and probably not efficient any more. Samples 2 had the best ratio of lambda arms versus cDNA molecules. It was combined with sample 1 and formed the basis of the cDNA library.

For the differential screening purpose, 30,000 to 35,000 plaque forming units (pfu) from the primary packaging library were grown on 15 cm diameter LB agar plates at around 2,000 pfu/plate as described above. The remainder of the library (~150,000 pfu) was also plated to amplify the phage. These were collected in SM buffer for storage at 4°C (Sambrook *et al.* 1989).

2.4.3 Preparation of DNA phage replica filters.

To screen the phage, 15 cm diameter nylon membranes (Amersham Hybond N^+) were gently laid over the plaques on the LB plates. Membranes were left 1 min and marked to show their orientation in relation to the phage plaques. Then, they were lifted carefully off and placed on 3MM Whatman papers pre-soaked in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 minutes. Following denaturation, they were immediately transferred on

Whatman 3MM papers pre-soaked in neutralising solution (0.5 M Tris-Cl pH 7.2, 1.5 M NaCl, 10 mM Na_2EDTA) for another 5 minutes. Membranes were rinsed quickly in 2X SSC (20X SSC: 3 M NaCl, 0.3 M trisodium citrate), left to dry and the DNA was cross linked under UV for 7 minutes (details in Section 2.9.2.1). Finally, membranes were washed in 1X SSC, 0.1% SDS at 65°C for 5 min to remove bacterial debris. Phage DNA membranes were prepared in duplicate for each LB plate containing ~2,000 pfu.

2.4.4 cDNA phage library differential screening.

To screen each set of filters, ^{32}P labelled cDNA probes were used (Section 2.7). The conditions of hybridisation and washing were the same as for Southern DNA hybridisation (see Section 2.8.3). After autoradiography, corresponding plaques showing a strong signal when probed with cDNAs from wheat meiotic anthers in comparison with cDNAs from leaf, roots and pollen were isolated using a sterile Pasteur pipette and placed in 500 µl SM buffer at 4°C to let the phages diffuse out of the agar plug. A total of three rounds of screening was done by repeated plating, phage DNA transfer and hybridisation.

2.5 DNA ligation.

Several selected cDNA inserts had to be subcloned in a suitable vector for sequencing and *in situ* work. The desired DNA inserts were first isolated by the Geneclean method (Section 2.3.5) and the vector (Bluescript KS- or pTZ19U) was prepared by the following way. DNA vector was cut with an appropriate restriction enzyme compatible with the two ends of the digested DNA insert. The reaction contained 2 µg vector, 2 µl of 10X buffer (supplied by the manufacturer), 1 µl of a suitable restriction enzyme (8-12 units (u) from Boehringer Mannheim) and water to 20 µl. The reactions were incubated at 37°C for 90 minutes and the volume increased to 100 µl with water. A phenol:chloroform:isoamyl alcohol (25:24:1) extraction was performed and the DNA was ethanol precipitated (one tenth volume of 3 M sodium acetate, 2.5 volumes of ethanol) at -80°C for 30 minutes.

After centrifugation to recover the digested DNA, the pellet was washed with 70% ethanol, dried and resuspended in 20 μl of water. Half of the cut vector (1 μg) was then dephosphorylated by adding 5 μl of 10X calf intestine phosphatase buffer (CIP buffer, supplied by the manufacturer), 1 μl (1 u) of CIP (Boehringer Mannheim) and water to 50 μl. The incubation was done at 37°C and after 30 min, 1 additional unit of enzyme was added for another 30 min incubation. The reaction was stopped by adding 1 μl of 0.5 M Na_2EDTA and heating the sample at 65°C for 10 minutes. An extraction with phenol was done to remove any traces of the enzyme and the DNA was ethanol precipitated to be finally resuspended in 20 μl of water.

For the ligation two reactions were set up with ratios of vector to insert of either 1:1 or 1:3. The tubes contained 50 ng of cut/dephosphorylated vector, 50 or 150 ng insert, 1 μl of 10X ligation buffer (supplied by the manufacturer) 0.5 μl of 10 mM ATP pH 7.0, 0.5 μl (0.5 u) of T4 DNA ligase (Boehringer Mannheim) in a total volume of 10 μl. Reactions were incubated at 12°C overnight and they were used directly for the transformation of the competent cells.

2.6 Bacteria transformation.

The method follows the protocol by Hanahan *et al.* (1983) with minor modifications. A small overnight culture of *E. coli*, strain DH5α was prepared by inoculating 5 ml of LB medium with a small loop of the bacteria stock. The following day, 0.5 ml of this overnight culture was added to 25 ml of SOB medium (2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and grown in a 37°C shaker until the culture reached the optical density of 0.4-0.5 (absorbance at 600 nm). The cells were then kept on ice for 10 min and transferred to a sterile tube to be centrifuged at 2,500 rpm for 15 min at 4°C (Beckman rotor JA-20). The supernatant was discarded and the cell pellet resuspended in 8.5 ml of TFB buffer (10 mM MES, 45 mM $MnCl_2$, 100 mM RbCl, 10 mM $CaCl_2$, 3 mM hexamine cobalt chloride) and chilled on ice for 10 minutes. The centrifugation was repeated and the cells were gently resuspended in 2 ml of TFB

buffer with 70 μl of redistilled DMSO (dimethylsulphoxide). Cells were left on ice for 5 min and 158 μl of 1 M dithiothreitol (DTT) was mixed gently with the cells. After 10 min on ice, 75 μl of DMSO was added and the cells were left on ice for 15 more minutes. As soon as these competent cells were ready, the 10 μl ligation mix (Section 2.5) was added to 100 μl of the cell suspension. After gentle mixing, it was left on ice for 30 min and then heat shocked for 2 min at 42°C. Immediately after this heat treatment, 400 μl of SOC medium (SOB medium containing 0.35% (w/v) glucose) was added and the transformed cells incubated 60 min in a 37°C shaker.

Cells were plated out as 100 μl aliquots onto LB agar petri dishes containing ampicillin (50 μg/ml), X-gal (0.004% w/v), IPTG (0.1 mM) and incubated overnight at 37°C. Recombinant pTZ19U or Bluescript KS- were identified as white colonies. Plasmid DNA was purified as described in Section 2.3.3.

2.7 Probe labelling.

dsDNA probes: Approximately 50 ng of isolated DNA fragments was mixed with 0.3 μg of 9-mers random primers in 8.5 μl water, boiled 5 min and chilled on ice. To this mix, 12.5 μl of labelling buffer (60 μM dATP, dGTP, dTTP, 150 mM NaCl, 150 mM Tris-Cl pH 7.6, 30 mM $MgCl_2$, 300 μg/ml BSA), 3 μl α-^{32}P-dCTP (10 μCi/μl, 3,000 Ci/mmole, Amersham) and 1 μl (2 u) of DNA polymerase (Klenow fragment, Boehringer Mannheim) were added, followed by 90 min incubation at 37°C. The labelled DNA was separated from unincorporated nucleotides on G-100 Sephadex columns (3% w/v in TE buffer containing 0.1% SDS). The probe was boiled 5 min before adding it to the hybridisation mix.

cDNA probes: Ten micrograms of total RNA was mixed with 1 μg of 18-mers oligo dT primers, incubated 10 min at 70°C and chilled on ice. Then 6 μl of 5X Gibco-BRL Superscript buffer, 3 μl of 0.1 M DTT, 1 μl (20 u) Rnasin (Promega), 1 μl of 10 mM dATP, dGTP, dTTP, 1 μl of 0.1 mM dCTP, 6 μl of α-^{32}P-dCTP, 1.5 μl (200 u/μl) of Gibco-BRL Superscript reverse transcriptase and water to 30 μl were added. The sample was incubated at 37°C for 2 hours. The RNA was then hydrolysed by adding 1 μl of 10%

SDS and 3 µl of 3 N NaOH followed by 30 min incubation at 65°C. To neutralise the sample, 10 µl of 1 M Tris-Cl pH 7.4, 3 µl of 2 N HCl were added and mixed carefully. The unincorporated nucleotides were removed by eluting the sample through a G-100 Sephadex column.

2.8 DNA Southern blot analysis.

2.8.1 DNA fractionation on agarose gel.

Approximately 5 µg of plant genomic DNA was first digested for 5 hours with 20 to 30 units of restriction enzyme (Promega) in 20 µl reaction volume containing 1X restriction buffer (supplied by the manufacturer), 4 mM spermidine, 100 µg/ml Dnase-free BSA. The most frequently used restriction enzymes were BamH I, Dra I, EcoR I and Hind III.

The digested DNA was mixed with 2 µl of 10X ficoll dye (0.1 M Tris-Cl pH 8.0, 0.2 M Na_2EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol, 30% Ficoll 400) and fractionated on 1% agarose gel in TAE buffer (0.04 M Tris-Cl pH 8.0, 1 mM Na_2EDTA) at 15 mA overnight. After electrophoresis, the gel was stained in ethidium bromide (5 µg/ml) and photographed under UV light with a Polaroid 667 black & white film.

2.8.2 DNA transfer to a nylon membrane.

Before transfer, the gel was soaked in denaturing solution (0.5 N NaOH, 1.5.M NaCl) for 30 minutes, followed by 20 minutes in neutralising buffer (0.5 M Tris-Cl pH 7.2, 1.5 M NaCl, 10 mM Na_2EDTA). The gel was placed inverted on 3 Whatman 3MM papers supported by a thick sponge soaked in 20X SSC (3 M NaCl, 0.3 M trisodium citrate) and the Hybond N^+ membrane (Amersham) was in turn placed on the gel followed by 2 Whatman papers and 5 cm stack of paper towels placed on top. The transfer was done for 6 to 7 hours by capillary blotting (Southern 1975). After a complete transfer, the membrane was briefly rinsed in 2X SSC, blot dried and the DNA was fixed by placing the membrane

(DNA side up) on a 3MM Whatman paper pre-soaked in 0.4 N NaOH for 20 minutes. Finally, the membrane was rinsed sequentially in neutralising buffer and 4X SSC and left to dry until ready to use.

2.8.3 DNA hybridisation and detection.

Membranes were pre-hybridised in hybridisation bottles (Hybaid) at 65°C for 5 hours in a hybridisation buffer containing 4.5 ml of 5X HSB (3 M NaCl, 0.1 M PIPES pH 6.8, 25 mM Na_2EDTA), 4 ml of 25% (w/v) dextran sulphate, 3 ml of Denhardt's III (2% BSA, 2% polyvinyl pyrrolidine, 2% ficoll 400, 10% SDS) 0.4 ml of denatured salmon sperm DNA (5mg/ml) and 3 ml of water.

After the pre-hybridisation step, the labelled probe was boiled with 0.2 ml of salmon sperm DNA (5 mg/ml), chilled on ice and added directly in the bottle containing the membranes. The hybridisation was done overnight (16 hours) at 65°C.

Membranes were washed at increasingly stringent conditions at 65°C: 2X SSC, 0.1% SDS, 15 min; 1X SSC, 0.1% SDS, 15 min; 0.5X SSC, 0.1% SDS, 15 min. The membranes were then sealed in plastic wrap, placed between intensifying screens and exposed to X-ray film (Fuji HR-G) for 2 to 7 days (depending on signal intensity) at -80°C.

2.9 RNA blot analysis.

2.9.1 Total RNA fractionation in denaturing gel.

The RNA was fractionated in an agarose gel by the formamide-formaldehyde method (Gerard and Miller 1986). The 1.5% agarose gel was made in 1X MOPS buffer [20X: 0.4 M 3-(N-morpholino)propanesulfonic acid, 0.1 M sodium acetate, 0.02 M Na_2EDTA, pH 7.0] containing 2.2 M formaldehyde and pre-run 30 minutes in 1X MOPS buffer at 50 volts before loading the samples.

Each lane was loaded with 10 μg of total RNA prepared the following way. To 4 μl (10 μg) of RNA, 7.5 μl ultra pure deionised formamide, 2.4 μl of 37% (v/v) formaldehyde, 0.75 μl of 20X MOPS were added and heated at 65°C for 10 minutes. Just prior to running the samples, 1X loading buffer was added. The samples were run at 70 volts for 3 to 4 hours or until the bromophenol blue dye had run 2/3 of the gel length. Finally, the gel was washed 10 minutes in water to remove excess of formaldehyde.

2.9.2 RNA transfer to a nylon membrane.

2.9.2.1 Northern blot.

The RNA gel was soaked in 20X SSC for 15 minutes and the RNA was transferred overnight by capillary blotting as described in Section 2.8.2. Upon completion of the transfer, the membrane was rinsed briefly in 4X SSC and blot dried between 3MM Whatman papers. The RNA was fixed to the membrane by irradiation under UV light (15 Watt, 254 nm, 24 cm above the filter) for 7 minutes.

2.9.2.2 Dot blot.

For the selection of the lambda cDNA clones, RNA dot blots were prepared by spotting 5 μg of total RNA on nylon membranes with the help of a Minifold II apparatus (Shleicher and Schuell). This was done by denaturing the RNA samples with formamide-formaldehyde (see above), mixing them with an equal volume of cold 20X SSC and then applying them directly onto the membrane pre-soaked in 10X SSC (and secured in the blotting apparatus with the vacuum on). The membranes were rinsed in 4X SSC and the RNA dot blots were fixed as described above.

2.9.3 RNA hybridisation and detection.

Membranes were pre-hybridised in hybridisation bottles (Hybaid) for 24 hours at 42°C in a solution containing 50% formamide, 5X SSC, 1X blocking solution (5X: 10% casein, 100 mM maleic acid pH 7.5, 150 mM NaCl), 50 mM phosphate buffer pH 7.0, 100 µg/ml denatured yeast total RNA, 0.1% sarkosyl and 7% SDS.

The hybridisation step was as described above (2.8.3) except the denatured labelled probe was added to the mix. Membranes were washed sequentially at 65°C in 2X SSC, 0.1% SDS, 10 min; 0.5X SSC, 0.1% SDS, 15 min; 0.2X SSC, 0.1% SDS, 10 min. Finally, they were sealed in plastic wrap, put between intensifying screens with an X-ray film and exposed from a few hours to several days at -80°C.

2.10 Sequencing.

Sequences were determined using an Applied Biosystems Inc. (California, USA) automated sequencer. DNA was prepared according to the sequencer manufacturer's instructions and involved the use of universal or reverse dye primers, a mixture of deoxynucleotides with dideoxynucleotides and, the PCR amplification technique.

2.11 *In situ* hybridisation.

2.11.1 Plant material embedding.

Wheat florets at the time of meiosis were dissected and put in freshly made fixation buffer (4% paraformaldehyde, 0.25% glutaraldehyde, 50 mM PIPES pH 7.0) for 16 hours on an orbital rotor at 4°C. The samples were then washed 3 times, 15 min each, in 50 mM PIPES pH 7.0 to remove excess aldehydes. This was followed by dehydration of the samples by soaking for 20 minutes each time in: 20% ethanol, 25 mM PIPES; 30% ethanol, 10 mM PIPES and 50 to 70% ethanol only. After these steps, samples were placed in plastic cassettes and were processed automatically in 70% ethanol, 1 hour; 80% ethanol, 1 hour;

95% ethanol, 90 min; 100% ethanol, 4 hours; histoclear:ethanol (50:50), 1 hour; histoclear, 4 hours; liquid paraffin (DIFCO lab), 4 hours.

At the end of the fixation process, samples were ready to be embedded in wax (Polywax, DIFCO lab). The florets were transferred in metal moulds containing melted wax (57°C) and cooled rapidly to embed them. The paraffin block of floret tissue was cut with metal blades on a rotary microtome (Leitz 1512) to produce sections of 6 to 10 µm. The ribbons were floated and spread on a 45°C waterbath before transferring them on microscope slides coated with 2% aminopropylethoxysilane. Slides were left to dry at room temperature overnight.

2.11.2 Riboprobes preparation.

Digoxigenin-labelled sense and antisense cRNA probes were synthesised by *in vitro* transcription from Bluescript KS- template containing the desired cDNA insert. All cDNA inserts were sequenced to ensure identity and orientation of the probes.

Plasmids were first linearized downstream of the insert in order to prevent run-through transcripts: 2 to 5 µg of recombinant plasmid DNA, 10 to 20 units of an appropriate restriction enzyme (Boehringer Mannheim), 1X restriction buffer (supplied by manufacturer) were mixed in 20 µl volume reaction and incubated 90 min or more at 37°C. The linearized plasmid was run on a gel and purified by the Geneclean method.

In Rnase-free conditions, 20 µl reactions were incubated at 37°C for at least 2 hours and consisted of 1X transcription buffer (supplied in the Promega *in vitro* transcription kit), 10 mM DTT, 20 u Rnasin, 1 mM each of ATP, CTP, GTP, 0.65 mM UTP, 0.35 mM DIG-UTP (digoxigenin-11-UTP from Boehringer Mannheim), 1.0 µg linearized template and 50 units of T3 or T7 RNA polymerase to give either sense or antisense probe. To stop the reaction, 2 µl of 0.2 M Na_2EDTA was added, followed by 2.5 µl of 4 M LiCl and 75 µl cold ethanol to precipitate the RNA transcripts and remove unincorporated nucleotides. The samples were left at -80°C at least 30 minutes. To recover the RNA, samples were

centrifuged at 12,000 rpm for 15 min at 4°C, washed with 70% ethanol and dried under vacuum. The samples were finally resuspended in 100 µl Rnase-free water.

The quality of the probe was assessed on a 1.5% agarose, 2.2 M formaldehyde gel (see Section 2.9.1) and the concentration of the labelled probes was estimated by RNA dot blots in parallel with a digoxigenin labelled standard (Boehringer Mannheim). First, probes and standard were spotted and fixed on nylon membranes (Section 2.9.2.2) at different dilutions (1:10 to 1:10,000). Then, membranes were washed 5 min in buffer I (150 mM NaCl, 100 mM Tris-Cl pH 7.5) and incubated 60 min at 37°C in buffer II (2% BSA in buffer I). Following this blocking step, membranes were incubated with anti-DIG-AP (75 mU/ml anti-digoxigenin alkaline phosphatase from Boehringer Mannheim) diluted 1:10,000 in buffer II for 30 min at 37°C. Membranes were subsequently washed 15 min each time, in buffer I containing 0.3% Tween 20. To detect antibodies, a chemiluminescence substrate (Lumigen™ PPD from Boehringer Mannheim) was applied according to the manufacturer instructions. Finally, an X-ray film was placed on the membrane and exposed 30 to 60 min at room temperature. From comparison of the signal intensities between probes and standard, concentrations of the probes were estimated.

Just prior to the hybridisation step, the length of the probes was reduced to 150 bases by partial alkaline RNA hydrolysis using the method described in Cox *et al.* (1984).

2.11.3 Pre-hybridisation treatments.

Wax was removed from tissue sections by submerging 5 min in xylene and washing 5 min each time in 100% ethanol, 70% ethanol and then air drying. A proteinase K treatment (1 µg/ml in 0.1 M Tris-Cl pH 7.5, 0.05 M Na_2EDTA) was done at 37°C for 15 min followed by three washes of 5 min each in 2X SSC and finally water. Once the excess of water was wiped out, the sections were acetylated with 0.25% acetic anhydride, 0.1 M triethanolamine pH 8 for 10 min at room temperature. The sections were rinsed several times in 2X SSC, followed by water and dehydrated as quickly as possible in increasing concentrations of ethanol (25, 40, 55, 70 and 100%). Once the sections were dried, the hybridisation

solution (50% ultra pure deionised formamide, 4X SSC, 1X Denhardt's, 200 µg/ml tRNA, 0.1 M DTT, 5% dextran sulphate) was heated at 80°C for 5 min and applied with coverslips on the slides (~40 µl/section). Sections were pre-hybridised for 2 hours at 45°C.

2.11.4 Hybridisation and washes.

After the pre-hybridisation, slides were dipped quickly in 2X SSC to remove coverslips and residual solution was gently removed around the sections with a soft paper towel. The RNA probes were then added to a freshly prepared hybridisation mix at about 8-10 ng/µl, heated at 80°C for 5 min and applied to the sections. Slides were incubated with coverslips in a humid chamber for 16 hours at 45°C. The next day, coverslips were removed and sections were washed sequentially in the following solutions: 2X SSC, 5 min at room temperature; Rnase A solution (40 µg/ml in 0.5 M NaCl, 10 mM Tris-Cl pH 8, 1 mM Na_2EDTA) for 15 min at 37°C; 2X SSC, 45 min at 42°C; 1X SSC, 45 min at 42°C and 0.5X SSC, 30 min at room temperature.

2.11.5 Immunological detection.

The slides were incubated 5 min in buffer I followed by 60 min incubation in buffer II (2% BSA in buffer I). Then anti-DIG-AP was diluted (1:500) in buffer II and applied in 100 µl aliquots onto the sections for 2 hours at room temperature. Unbound antibody conjugate was removed by washing the slides twice in buffer I containing 0.3% Tween 20, 15 min each wash and rinsing in buffer III (0.1 M Tris-Cl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 2 min.

Any excess solution was gently removed with a soft paper towel and the substrate solution (300 µg/ml nitro-blue tetrazolium salt, 150 µg/ml 5-bromo-4-chloro-3-indolylphosphate in buffer III) was carefully added on the sections. Slides were kept in the dark from 4 hours to overnight depending on the rapidity of the colour development. Once the signal was strong enough against a low background, the detection was stopped by dipping the slides in

several volumes of TE buffer and then water. The sections were dehydrated by rinsing in increasing concentrations of ethanol (10, 20, 30, 50, 70, 90 and 100%), 30 seconds each time. Finally they were left to dry and were mounted with coverslips in DPX mountant (BDH, England). The sections were photographed under bright field at different magnifications using an automatic camera attached to a Zeiss Axioscope microscope.

CHAPTER III

Differential screening of a wheat meiosis cDNA library.

3.1 Introduction.

Most studies on meiosis in plants have been conducted on the male reproductive organ namely, the anther. This is largely due to the fact that several cells undergo meiosis in a single anther compared to one cell in a single female ovary. In wheat, there are three anthers in each floret and these are physically easier to excise and collect than the female ovule. Moreover, within each floret, meiosis in pollen mother cells is synchronous with, and has the same duration, as meiosis in the ovule (Bennett *et al.* 1973). The fine study of cell development in the Chinese Spring anther and ovule by Bennett and co-workers (1971, 1973) has also shown that there is a gradient of development within the flower spike. Spikelets in the middle of the spike are the most advanced in development and progressively less developed spikelets appear acropetally and basipetally. Also, within the same spikelet, the first floret is the most developed and is around 12 hours more advanced that the second floret. However, if the development is compared between the first or second florets and those florets in the adjacent spikelet, the time difference is 5 hours or less.

These observations were taken into account when tissue was collected to prepare the cDNA library and only florets at about the same stage of development were dissected and pooled. The aim of the work reported in this section was to screen a cDNA library for meiosis specific clones. The meiotic stages of all material collected was assessed by chromosome squashing.

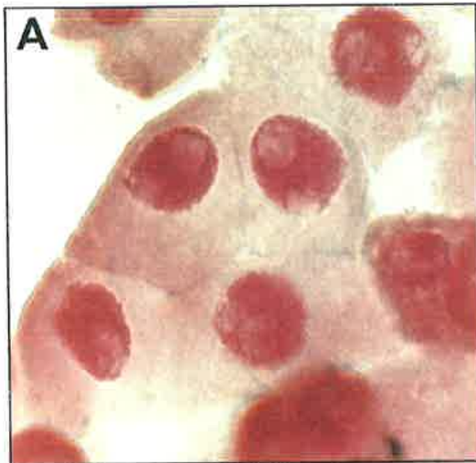
3.2 Construction and screening of the wheat meiotic cDNA library.

To collect the tissue for the RNA extraction, spikes were cut from plants believed to be undergoing meiosis and from each spike, 5 spikelets were excised in the region most advanced in development. In the 5 spikelets excised, the one that was located in the middle was transferred in a solution of 1:3 acetic ethanol for fixation and the 4 remaining spikelets were immediately frozen and kept at -80°C. The spikelet fixed in the acetic ethanol solution was later dissected and anthers from the first and second florets were squashed and stained in aceto-orcein for microscopic examination. Typical chromosome squashes of those selected anthers at different stages of meiosis are shown in Figure 3.1. The pollen mother cells in premeiotic interphase, **A**; leptotene, **B**; mid-zygotene, **C**; pachytene, **D**; diplotene-diakinesis, **E**; and from **F** to **H**; metaphase I to dyads (prophase II), were stained with aceto-orcein. The remaining stages (metaphase II through tetrads) are not shown but were also collected for later use in Northern blots.

For the cDNA library construction, polyA$^+$ RNA was purified from 200 μg of total RNA by the streptavidin-paramagnetic bead method (see details in Section 2.3.6). This RNA was extracted from 150 dissected florets (i.e. 3 anthers, one carpel/floret with the protecting leaves removed). The proportion of the early meiosis stages represented in this RNA extract were 17% premeiotic interphase anthers, 30% leptotene, 38% zygotene and 15% pachytene. To demonstrate that the poly A$^+$ RNA was of adequate quality for reverse transcription into cDNA molecules, the first and second strand cDNA synthesis was monitored with α-^{32}P-dCTP (refer to Section 2.4 for details). The result of the first and second strand radioactive samples ran in an alkaline agarose gel and exposed for 2.5 hours is shown in Figure 3.2. The first and second strands were both of good quality. According to Sambrook *et al.* (1989) undegraded mRNA should yield first strand cDNA molecules that range in size from 500 bases and more with the bulk of radioactivity found between 1 and 2 kb. The radioactivity from the second strand molecules is also expected to be of similar size. The remaining steps followed the normal procedure for library construction in

<u>Figure 3.1</u> Bright-field photographs of wheat pollen mother cells before, during and after meiosis I.

Anthers were fixed in ethanol:acetic acid (3:1) and crushed to expose the cells. Squashed chromatin was stained with aceto-orcein. Photographs were taken using a 100X objective. **A**. Premeiotic interphase; **B**. Leptotene; **C**. Zygotene; **D**. Pachytene; **E**. Diplotene-diakinesis; **F**. Metaphase I and Anaphase I; **G**. Telophase I; **H**. Dyads (prophase II).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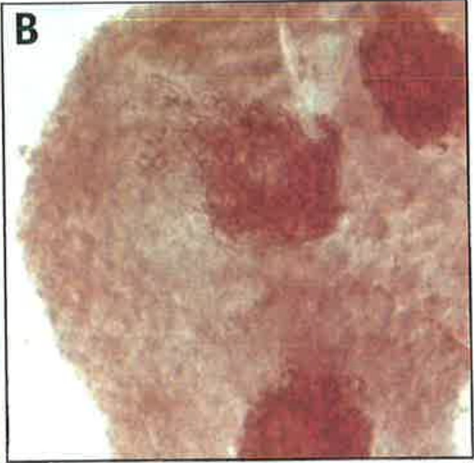
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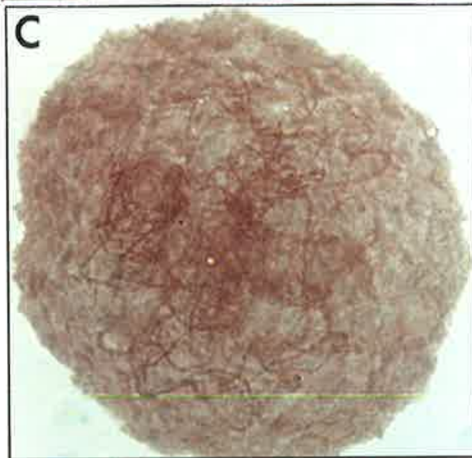
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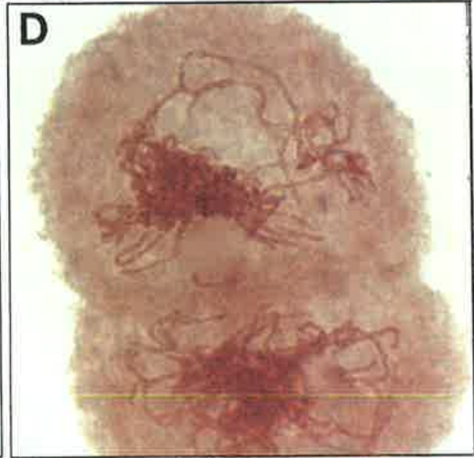
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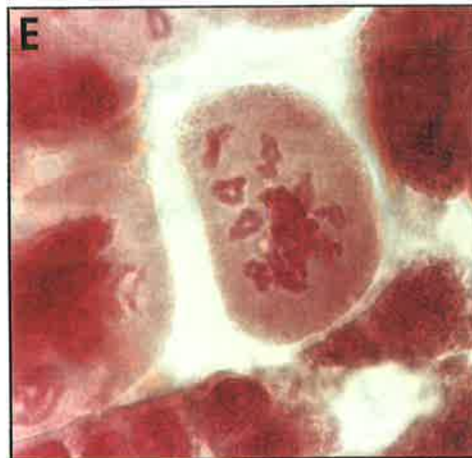
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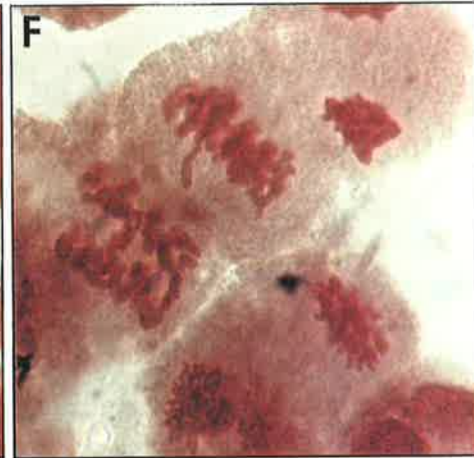
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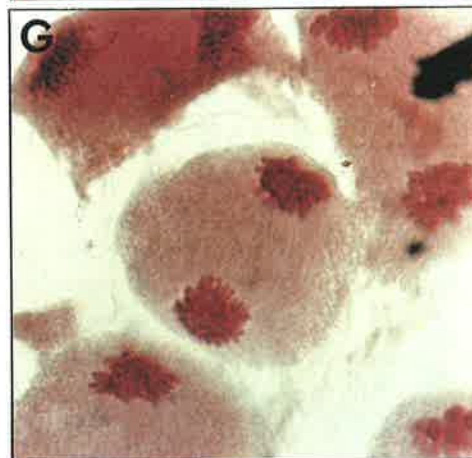
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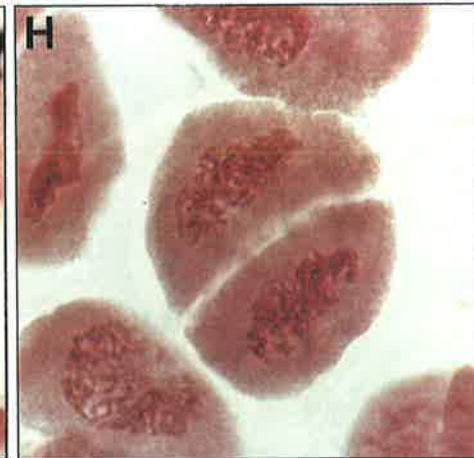
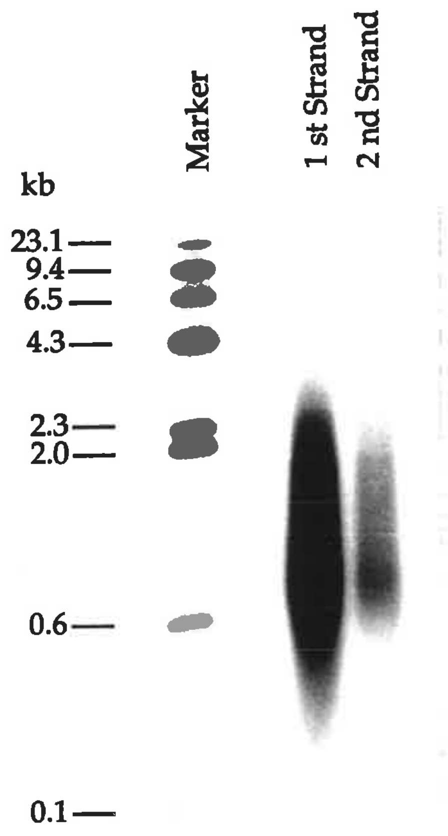

H

Figure 3.2 Monitoring the quality of cDNA molecules synthesis.

Alkaline agarose gel electrophoresis of radiolabelled first and second strands of cDNA synthesized from polyA^{+} RNA extracted from wheat florets at premeiotic interphase and early meiosis. The marker is lambda DNA digested with Hind III and end labelled with ^{32}P-dCTP. Size of the fragments is given on the left. The dried gel was exposed to X-ray film for 2.5 hours at room temperarure.



kb
Marker
1 st Strand
2 nd Strand
23.1
9.4
6.5
4.3
2.3
2.0
0.6
0.1

lambda gt10. A total of 185,000 plaque forming units (pfu) resulted from the pooled libraries (refer to Section 2.4.2) and about 30 to 35,000 pfu from the primary packaging of the cDNA library were plated on 15 cm diameter petri dishes at around 2,000 pfu/plate. The remainder of the library was also plated and the amplified phages were collected for storage. The size quality of the cDNA inserts was verified by running a gel with DNA extracted from 12 randomly picked phages. The inserts were between 200 to 1,000 bp, with most (7 out of 12) around 500 bases (data not shown).

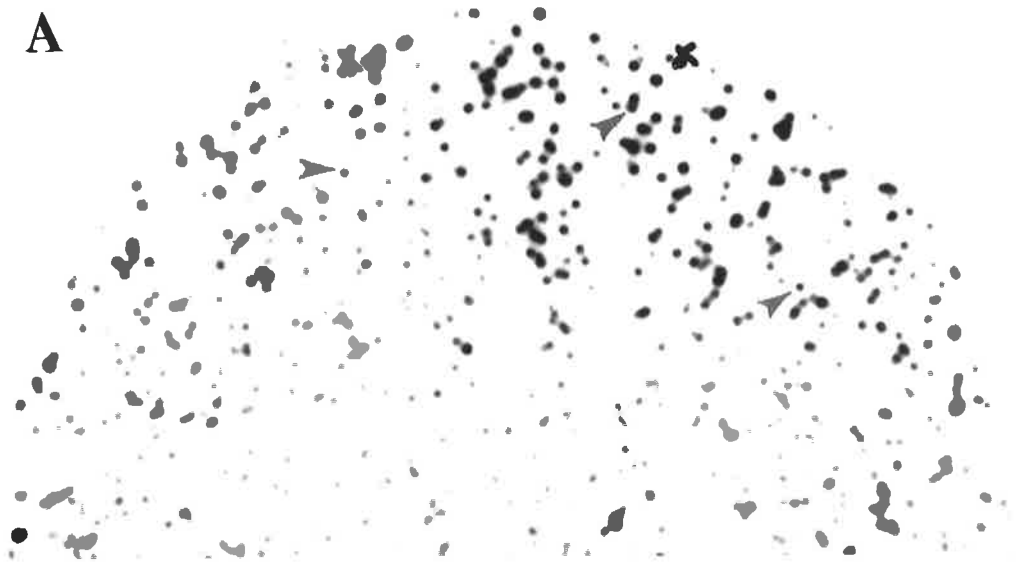
For the differential screening of the phages, filters were prepared in duplicate for each plate. Figure 3.3 illustrates a typical result obtained from one duplicate plate. The membrane from Figure 3.3.A was hybridised with ^{32}P-cDNA probes made from RNA of early meiotic anthers and the membrane in Figure 3.3.B, with ^{32}P-cDNA probes made from leaf, root and pollen RNA. When a difference in the signal intensity was seen after comparing each plaque dot, the corresponding phage was collected and placed in a buffer for diffusion. A phage was regarded as interesting when there was a strong signal with the meiotic anther probes and no signal (or a weaker one) with the leaves-roots-pollen probes. A total of three rounds of screening was done and the results are shown in the Table 3.1.

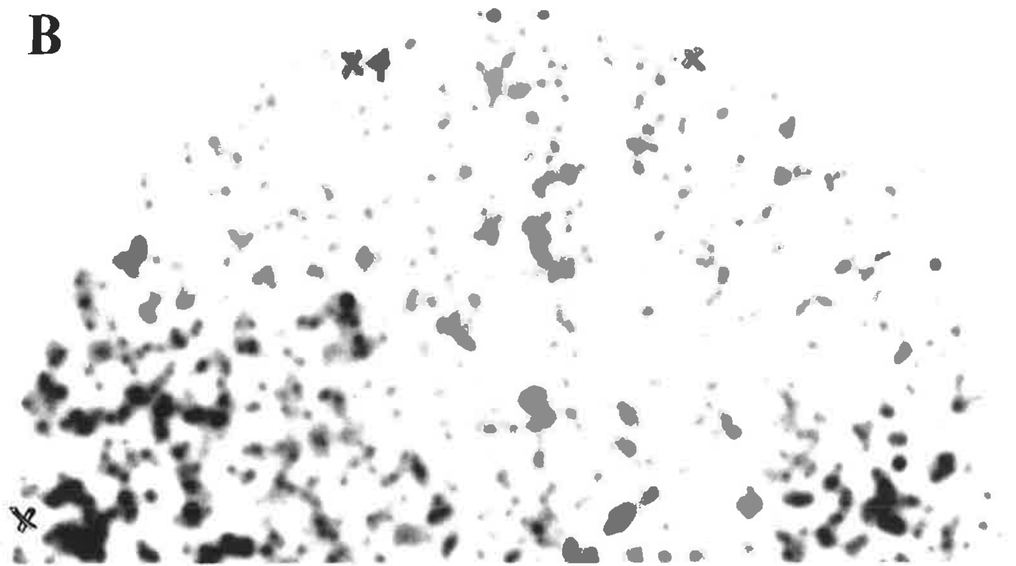
Table 3.1 Differential screening results.

	Screening Rounds (number of clones identified as early meiosis specific)		
Clones Screened	1 st	2 nd	3 rd
~30,000	251	92	34

<u>Figure 3.3</u> First round of differential screening of the wheat meiotic cDNA library.

Representation of a typical set of replica filters of lambda gt10 recombinants screened with either radioactive cDNA probes made from RNA of (**A**) meiotic anthers or (**B**) leaves, roots and pollen using the procedure described in Section 2.4.4. Membranes were exposed to X-ray film for 24 hours at -80°C. As an example, the 3 arrows indicate clones identified as potentially meiosis specific.


A


B

3.3 Partial analysis of the selected clones.

The 34 final isolates were grown in culture medium to extract the DNA and analyse the size of the inserts. Figures 3.4 A and B show the 34 clones digested with EcoR I, transferred to a nylon membrane and hybridised with cDNA probes made from RNA of anthers at early meiosis stages (leptotene, zygotene, pachytene). The size of the inserts is between 0.3 and 2.4 kb with the majority around 600 bp. Differences in signal intensity are due to the uneven loading of phage DNA since yield varied between extracts. The signal do not reflect differences in the abundance of the cDNA probes hybridising to the clones.

Some clones were assessed for sequence similarity by cross-hybridisation, they were also used as probes on RNA dot blots to evaluate the specificity of their expression in wheat. Figure 3.5 shows 7 identical RNA dot blots screened with different probes. These results, along with the cross-hybridisation data have been compiled in Table 3.2, which shows a preliminary analysis of 29 clones of the 34 selected. Two main patterns of expression were observed: one group of clones (wm5.12 and wm9.10) is characterised by expression mainly in the meiotic anthers while another group (wm1.18, wm2.19, wm4.09, wm11.29) shows expression in meiotic and mitotic tissues (root tips). Most of the final isolates were picked out more than once when the screening was carried out, for instance, wm5.12 cross-hybridised with 5 other separately selected clones. This is probably due to the initial screening which was biased for intensely expressed transcripts that gave the strongest signals. In addition, 14 clones out of 34 (41%) were members of the histone gene family (see below).

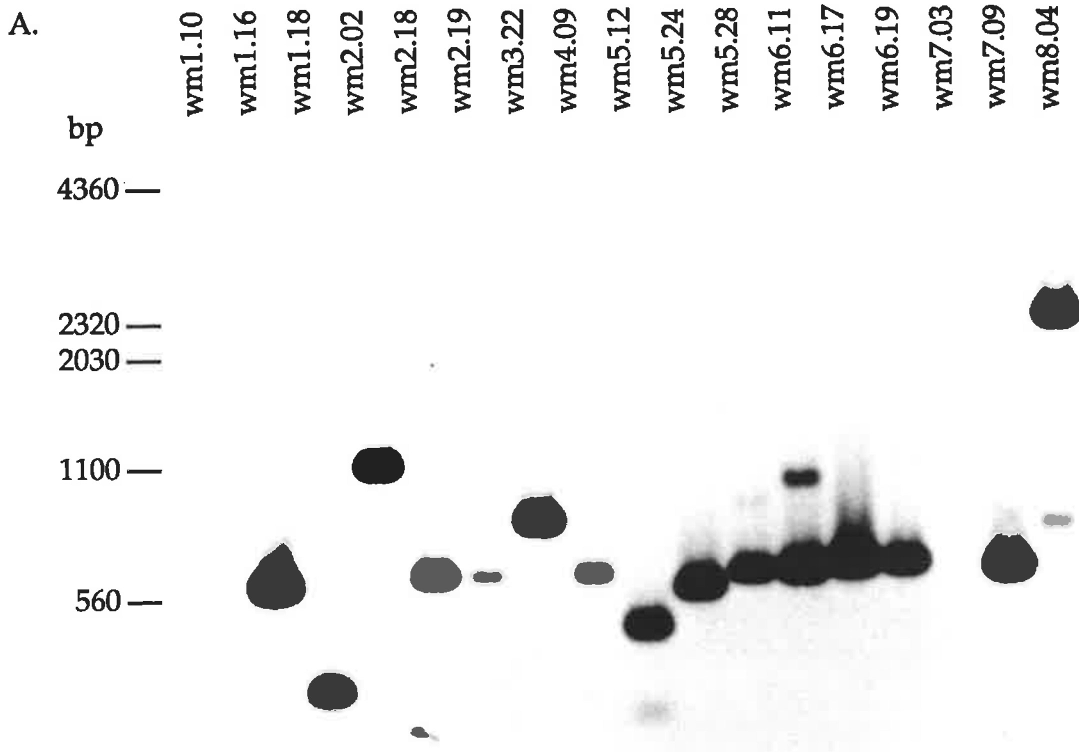
3.4 Discussion.

Overall strategy of cloning early meiotic genes.

Over the years, the main approach used to isolate specific meiotic genes was to produce mutants defective in one or several stages of meiosis. To do so, the organism of choice was

Figure 3.4 Final isolates after the third differential screening.

Lambda cDNA clones were digested with EcoR I, electrophoresed, transferred to membranes and hybridised with ^{32}P labelled cDNA probes made from RNA of meiotic anthers. Membranes were exposed to X-ray film for 16 hours at -80°C. **A**. Digested clones wm1.10 to wm8.04; **B**. Digested clones wm8.06 to wm7.18. DNA molecular size markers are given on the left.


A.
bp
4360
2320
2030
1100
560
wm1.10
wm1.16
wm1.18
wm2.02
wm2.18
wm2.19
wm3.22
wm4.09
wm5.12
wm5.24
wm5.28
wm6.11
wm6.17
wm6.19
wm7.03
wm7.09
wm8.04

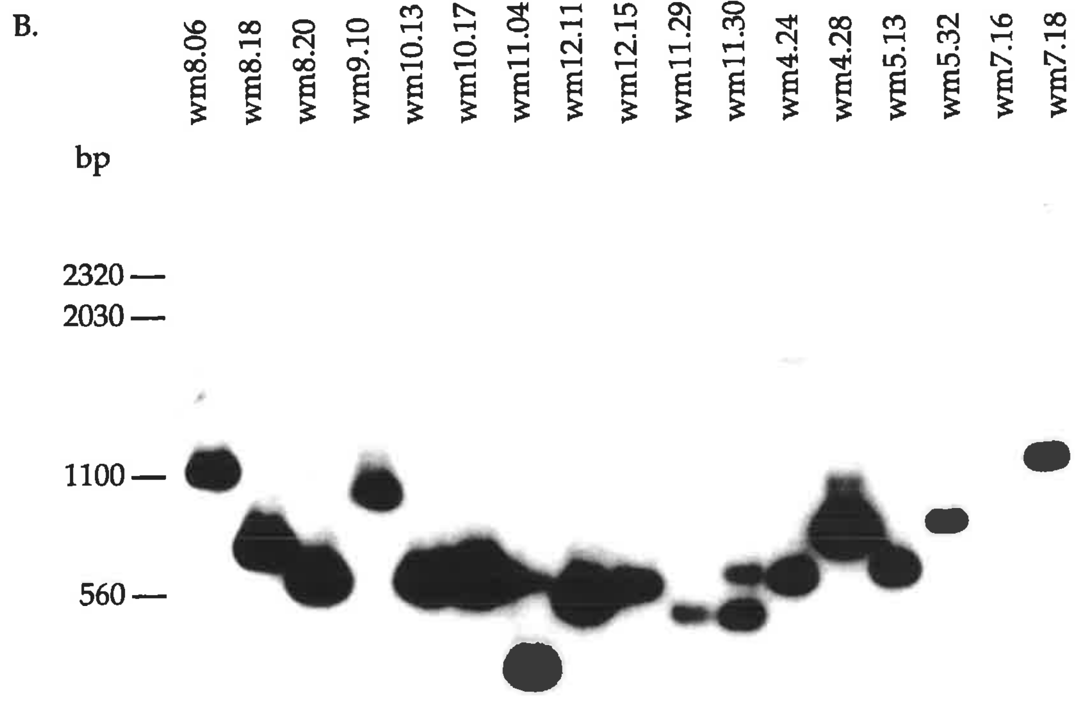

B.
bp
2320
2030
1100
560
wm8.06
wm8.18
wm8.20
wm9.10
wm10.13
wm10.17
wm11.04
wm12.11
wm12.15
wm11.29
wm11.30
wm4.24
wm4.28
wm5.13
wm5.32
wm7.16
wm7.18

Figure 3.5 **Representation of anther-specific mRNAs in different wheat tissues.**

Five micrograms of RNA from stems, leaves, premeiotic interphase anthers (P.M.Int.), prophase I anthers, tetrad anthers, mature pollen anthers, roots and root tips (R. tips) were spotted on a Hybond$^+$ membrane and hybridised with radioactively labelled. Each RNA blot was hybridised with the cDNA probe identified on the left hand side of the autoradiograph. Film exposure times differed for each RNA blot and varied from 2 hours to several days.

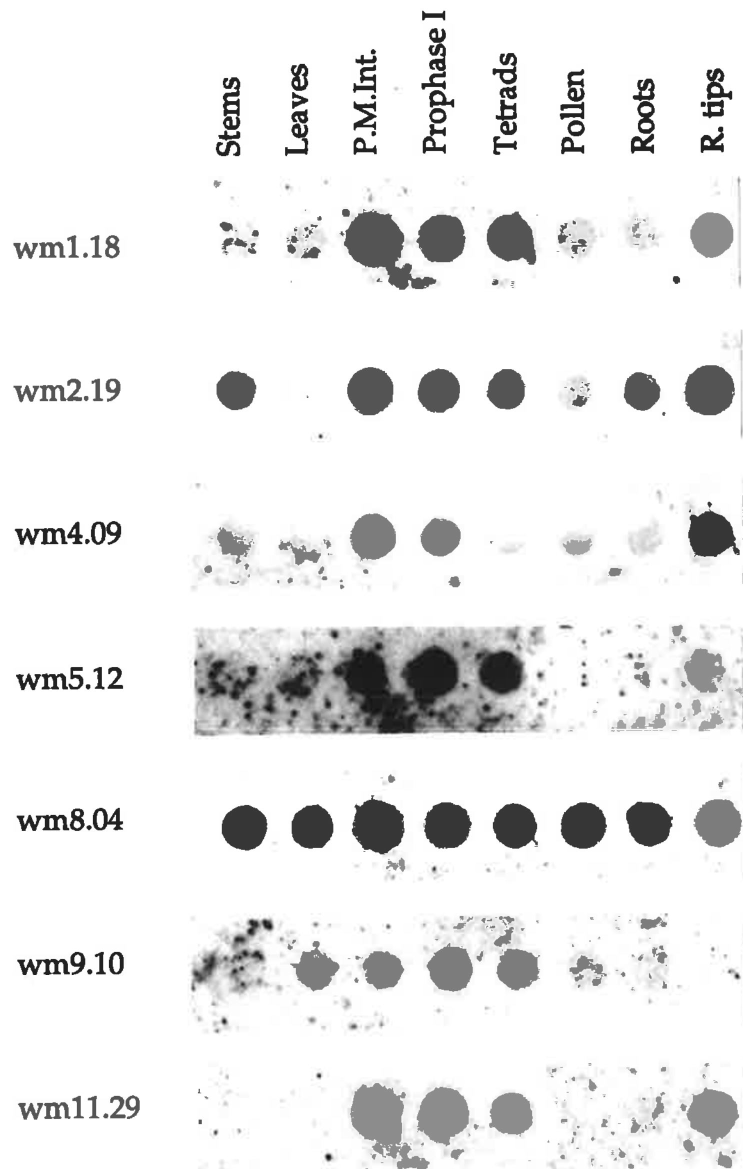

Stems
Leaves
P.M.Int.
Prophase I
Tetrads
Pollen
Roots
R. tips
wm1.18
wm2.19
wm4.09
wm5.12
wm8.04
wm9.10
wm11.29

Table 3.2 **Summary of RNA dot blots and sequence similarity analysis of the selected cDNA clones.**

cDNA clone[a]	No. of cross-hybridising clones	Size[b] (bp)	Tissue specificity[c]				Partial sequencing analysis[d]
			Vegetative parts	Root tips	Pollen	Meiotic anthers	
wm1.18	5	300	+	++++	+	++++	n.a.
wm2.19	2	800	+	++++	+	++++	unknown
wm4.09	8	500	+	++++	++	++++	Histones H2
wm5.12	6	300	-	-	-	++++	Lipid transfer protein
wm8.04	1	700	+++	++++	+++	++++	n.a.
wm9.10	1	1000	+	-	-	+++	n.a
wm11.29	6	500	+	++++	++	++++	Histones H4

[a]These clones were used as probes to check for cross-hybridisation among them. Of the 34 clones selected, 5 have not been analysed.

[b] This represents the size of the clone analysed and used as a probe.

[c] Specificity; + to ++++ represent very weak to strong expression, respectively; - represents not detectable.

[d] According to search in Genebank; n.a.: not available.

(and still is today) the budding yeast *Saccharomyces cerevisiae* because of the ease of mutant production, screening and selection. However, when more complex organisms are used to study meiosis, this approach becomes difficult to use, particularly in higher eukaryotes such as plants, where large genomes are common and mutant genes are not easy to pinpoint. Instead, biochemical or molecular approaches tend to be favoured to identify proteins, DNA or RNA sequences that appeared to be specific for meiosis. For instance, Hotta, Stern and colleagues (for a review see Stern and Hotta 1987) were able to isolate meiosis specific proteins by culturing lily meiocytes. In our case, working with wheat made a similar approach very difficult, mainly because wheat anthers at the beginning of meiosis are much smaller than those of lily and the meiocytes cannot be easily isolated and cultured (Takegami *et al.* 1981). Therefore, a more direct molecular approach was chosen to isolate potential meiosis specific clones.

We chose to conduct a differential screening of a cDNA library made from wheat florets at the desired stage of development because cellular differentiation and development are, in general, reflected by the corresponding changes in messenger population. Therefore, messengers relevant for a specific development process will tend to vary in abundance due to induction or repression of transcription. Thus, targeting gene expression in a specific tissue and developmental stage offered us the possibility of cloning meiosis related genes. Moreover, the pooling of wheat florets at either premeiotic interphase, leptotene, zygotene and pachytene stages has helped us to circumvent the problems of tissue amount and the restricted time frame of these finely tuned events.

The main disadvantage of the differential screening method is that selected cDNA clones fall usually into the category of medium to highly expressed genes and low expression mRNAs tend to remain undetected (Scott *et al.* 1991). When this project was initiated molecular techniques such as mRNAs differential display (Liang and Pardee 1992; Liang *et al.* 1993) or RT-PCR libraries from a few cells (Don *et al.* 1993; Dresselhaus *et al.* 1994) were not available. Compared to the differential screening technique, these methods would present the advantage of requiring less tissue and be less laborious. Also, one is more likely to identify weakly expressed genes by these new methods. Nevertheless, these methods can

give problems with false positive clones that tend to appear when several PCR cycles are involved and with the isolation of short truncated cDNA clones.

Selection of the clones.

The overall quality of the cDNA library was good in terms of size of the inserts and number of clones obtained. Our approach, when doing the differential screening, favoured clones that showed a strong signal with the appropriate set of probes (meiotic anthers). The diversity of the clones obtained after a partial analysis (Table 3.2) could be classified into 3 categories: i) histone clones; ii) cell division clones but not histones; iii) meiotic clones.

The first category, histone clones, were all members of the histone H2 and H4 family. Histones genes are subdivided into four major classes H1, H2, H3 and H4. Their expression is mainly cell cycle dependent and is closely linked to DNA replication (Wu and Bonner 1981). Consequently, histone mRNAs are highly accumulated during the S phase of the cell cycle (Nakayama and Iwabuchi 1993). Since 49% of the florets used for the cDNA library construction were at premeiotic interphase and leptotene (the remaining 51% were from zygotene and pachytene), the bulk of DNA had in fact just been replicated and therefore histone genes were highly expressed. We therefore expected some clones of these category to be selected and in fact their selection has demonstrated indirectly that the screening method was efficient since those clones fit into the category of genes involved in meiosis. Nevertheless, the number of histone clones selected could have been reduced by screening the library with histone probes. However, this may have some risks since some mRNAs (for example, meiotin-1) have been found to be partially related to certain types of histones and are specifically expressed during meiosis (Riggs 1994).

The second category of messengers is cell division clones present not only in meiotic tissues but also in mitotic tissues such as root tips (for example, wm1.18, wm8.04). These clones probably come from genes that play a role in both mitosis and meiosis. They can be of several types such as genes coding for proteins involved in DNA replication, DNA repair, chromatid segregation, chromosome movement or other processes. Moreover, it

has also been suggested that some genes expressed both in mitosis and meiosis could be somehow multifunctional. For instance, several genes (*CDC40, MRE11, RAD6, RAD50-57, XRS2*) identified in *S. cerevisiae* are involved in recombination during meiosis have been found to have additional mutant phenotypes during mitosis such as cell growth deficiency, DNA repair deficiency, hypo- or hyper-mitotic recombination (Kassir and Simchen 1978; Ajimura *et al.* 1993; Game *et al.* 1980; Baker *et al.* 1976; Petes *et al.* 1991; Ivanov *et al.* 1992). The selection of clones which are expressed in both meiosis and mitosis did not come as a surprise. It is well accepted that meiosis has evolved from mitosis and some molecular mechanisms would have been conserved in both events (Maguire 1992). In plants and other eukaryotes, it is also important not to neglect this category of clones since mechanisms essential for meiosis such as synapsis and homologous pairing are not well understood and genes involved may not be invariably meiosis specific.

The third category of clones isolated in the course of the library screening, was called meiotic clones. According to the partial analysis done with RNA dot blots, these clones can represent genes specifically expressed either in the meiocytes or the tapetal cells of the anthers since both are metabolically very active tissue during meiosis. For instance, wm5.12 or wm9.10 gave a strong signal when hybridised to total RNA of meiotic anthers but no detectable signal with root tips or pollen RNA (Figure 3.5). Such clones are expected to be related to the meiotic process but not involved in the mitotic cell cycle. In *S. cerevisiae*, several clones related specifically to meiosis have been characterized: *HOP1* (Hollingworth and Byers 1989); *MEI4* (Rockmill and Roeder 1991); *MER1*, *MER2* (Engebrecht and Roeder 1989; Engebrecht *et al.* 1991); *REC102*, *104*, *107*, *114* (Malone *et al.* 1991), *SPO11* (Atcheson *et al.* 1987); *RED1* (Rockmill and Roeder 1988); *DMC1* (Bishop *et al.* 1992); *ZIP1* (Sym *et al.* 1993); etc. Most affect crucial molecular events at the early stages of meiosis like recombination, pairing and synaptonemal complex formation. With the exception of lily, few clones have been characterised in plants that are specifically expressed in meiocytes. The main reason for this, is the tapetal dominance during sporogenesis and early microspore development (Scott *et al.* 1991). In libraries prepared from young anthers (before or during meiosis), the tapetum seems to be the

transcriptionally dominant cell-type either in the diversity or the amount of mRNAs produced or both (Scott *et al.* 1991). This fact makes it even more difficult to clone meiocyte specific mRNAs in plants. It is only by doing *in situ* localisation of clones that one can say whether expression comes from tapetal or pollen mother cells or both. In the case of tapetum specificity, the function of such genes in meiosis can still be essential due to the very close interdependance existing between meiocytes and tapetal cells. This is especially relevant when we consider that the transcriptional machinery is partially turned off in the meiocytes around the time of meiosis (Porter *et al.* 1984).

In the next chapters, the results of the molecular analysis of wm5.12 and wm2.19 are presented. These clones were chosen because they represented each expression class (other than histones) described above and the results from their partial analysis had shown no significant homology with sequences from Genebank. Chapter IV will provide the results from the analysis of clone wm5.12 and Chapter V for clone wm2.19.

CHAPTER IV

Molecular analysis of wm5.12 cDNA clone.

4.1 Introduction.

In this chapter, a detailed analysis of the clone wm5.12 is presented. As described previously, results from the RNA dot blots have shown that wm5.12 is expressed preferentially in the wheat anther. In order to characterise this clone and learn more about its nature, the spatial and temporal expression of wm5.12 was studied and the full length nucleic acid sequence determined. As a possible indication of wm5.12 being related to any known *Ph* genes in wheat, which are mainly located on chromosome groups 3 and 5, the cDNA clone was mapped to specific wheat chromosome arms. From the analysis of the results, a possible correlation between this clone and the chromosome pairing process will be discussed.

4.2 Spatial and temporal analysis of wm5.12 messenger.

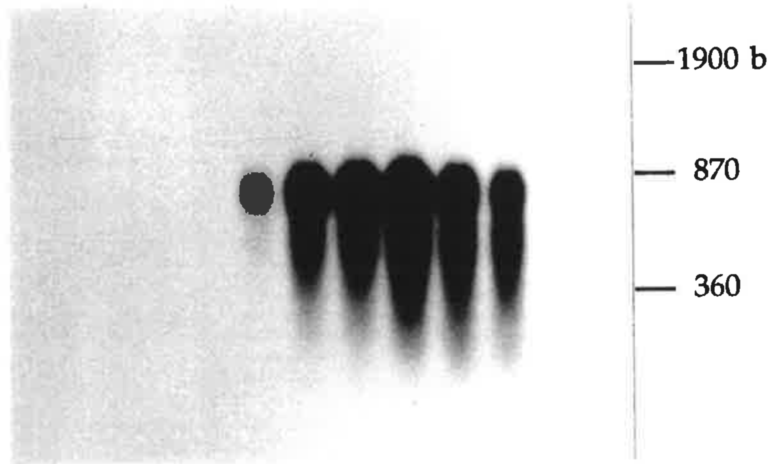
The levels of expression of wm5.12 mRNA were determined by Northern blot analysis. This revealed which tissue organs in wheat expressed the messenger strongly and/or weakly. A Northern blot was prepared containing equal amounts of total RNA extracted from vegetative and flower organs at several developmental stages (Figure 4.1). After hybridisation of the radioactive wm5.12 cDNA insert, a short exposure time of 4 hours showed (Figure 4.1 A) that the transcripts were absent in stems, leaves, roots, root tips, carpels and mature pollen. However in the anthers, strong expression occurred throughout

Figure 4.1 **Northern blot analysis of the expression of the gene corresponding to the wm5.12 wheat cDNA clone.**

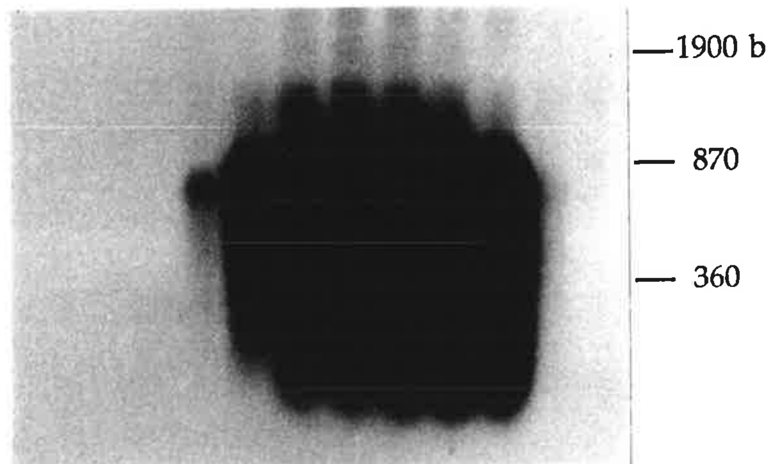
The radioactive wm5.12 probe was hybridized to 10 µg of total RNA in each lane extracted from different plant organs and several developmental stages of the anther. The blot was exposed to an X-ray film for 4 hours (**A**) or 3 days (**B**). To verify an even loading of the samples in each lane, the blot was subsequently hybridised with a radioactive 18S ribosomal probe from pea (**C**). Exposition time: 1 hour. **P. M. Int.**: RNA from anthers at premeiotic interphase; **Pac.-Dip.**: RNA from anthers at pachytene and diplotene; **Met.-Dyads**: RNA from a mixture of anthers at metaphase I, anaphase I, telophase I and Dyads; **R. tips**: RNA from root tips. RNA size markers are indicated on the right.

Stems Leaves Roots Carpels P.M.Int. Leptotene Zygotene Pac.-Dip. Met.-Dyads Tetrads Pollen R. tips

A.



B.



C.

the different meiosis stages (from leptotene to tetrads). It was also detectable in premeiotic interphase anthers but at a much lower level. To confirm the absence of the wm5.12 transcript in the vegetative parts, pollen and carpels, the same RNA blot was exposed for a further 3 days (Figure 4.1 B). After this exposition time a low level of expression became apparent in the wheat carpels but nothing was detected in the pollen and vegetative parts. Moreover, even after 3 weeks of exposure (data not shown), these results remained unchanged, indicating that the gene encoding the wm5.12 transcript is specifically expressed in wheat floral tissue (carpels and anthers), preferentially in meiotic anthers.

The size of the transcript on the blot that hybridised with wm5.12 (Figures 4.1A and B) is larger than the size of the cDNA insert. In fact, wm5.12 is about 300 bp and the corresponding messenger seems around 800 bases according to the RNA markers shown on the right side of Figure 4.1. Therefore, the clone selected is most probably a partial cDNA clone and the library would need to be re-screened to obtain a full length cDNA.

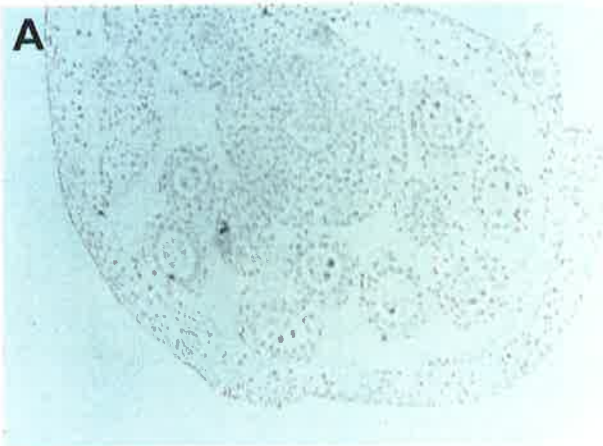
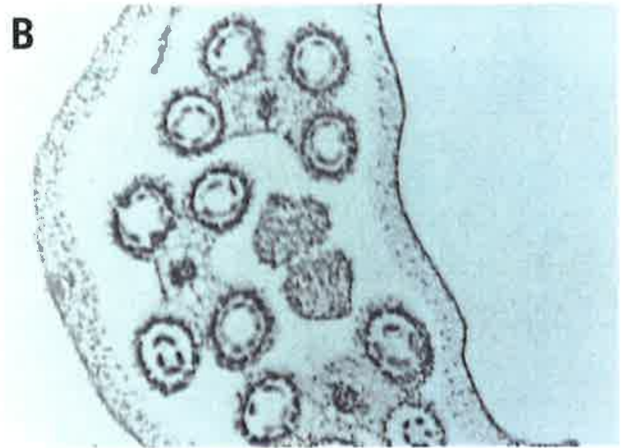
To monitor the quality of the RNA and the even loading of the samples, the blot was subsequently hybridised with an 18S ribosomal probe from pea (McFadden *et al.* 1988). Figure 4.1 C shows this result and confirms that the variation observed in wm5.12 expression is not caused by variation of the amount of RNA loaded.

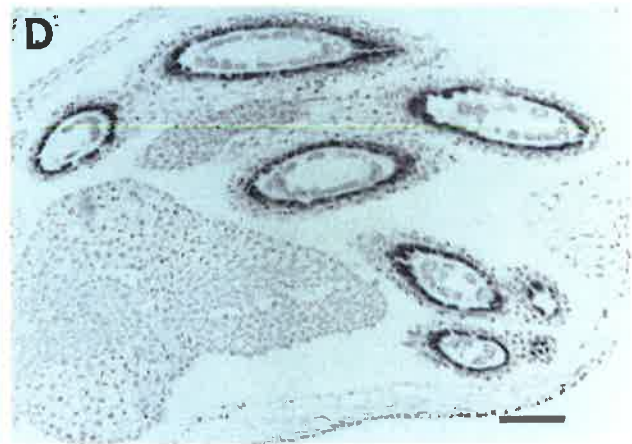
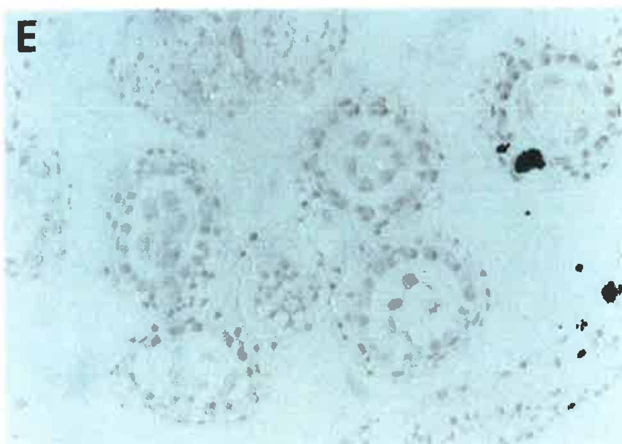
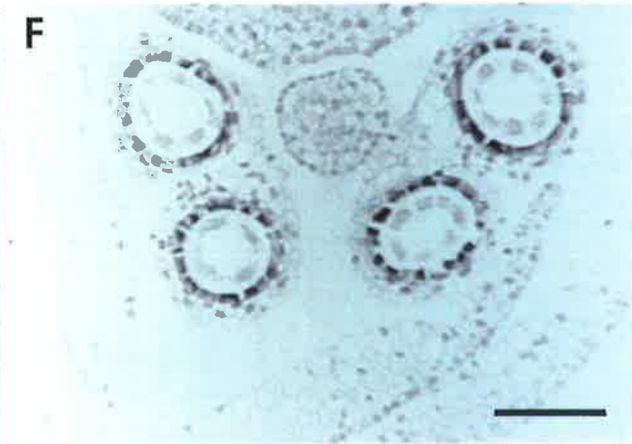
4.3 Cell type specific expression of wm5.12 messenger.

In situ hybridisation was used to address the cellular specificity of wm5.12 expression. Cross and longitudinal sections of wheat florets (containing carpels and anthers) at the early stages of meiosis were examined. Hybridisation of the sections with a DIG-labelled sense-strand probe resulted in no specific purple-dark brown precipitates (Figures 4.2 C and E). In contrast, a strong hybridszation signal with the antisense-strand probe was detected in the anthers (Figures 4.2 D and F). This positive signal (dark brown precipitates) was seen specifically over a single cell layer (namely the tapetum) that surrounds the locule containing the developing microspores. In the sections used for the *in situ* experiment no

Figure 4.2 Location of wm5.12 mRNAs in wheat cv. Chinese Spring florets.

Longitudinal and cross sections of florets at the prophase I stage of meiosis were hybridised with digoxigenin labelled sense and antisense cRNA probes. Sections (**A**) and (**B**) were used as a control with a cRNA 18S ribosomal probe sense and antisense, respectively. Sections (**C**) and (**E**) hybridised with sense cRNA of wm5.12. Sections (**D**) and (**F**) hybridized with antisense cRNA of wm5.12. The photographs were taken using brightfield microscopy. Bars = 0.1 mm.


A

B

C

D

E

F

signal was detected in the wheat carpels. This could be explained by the way the DIG signal was monitored. To avoid a high background, the development of the reaction precipitate was usually stopped as soon as it was easily detectable, so the development time was probably to short to obtain a visible signal in the carpels. Furthermore, another possibility could be the quality of the carpel section. Since we were primarily screening for tissue showing good anther morphology with meiocytes remaining in the section, it is possible that the quality of the carpel tissue was not adequate to obtain a clear signal.

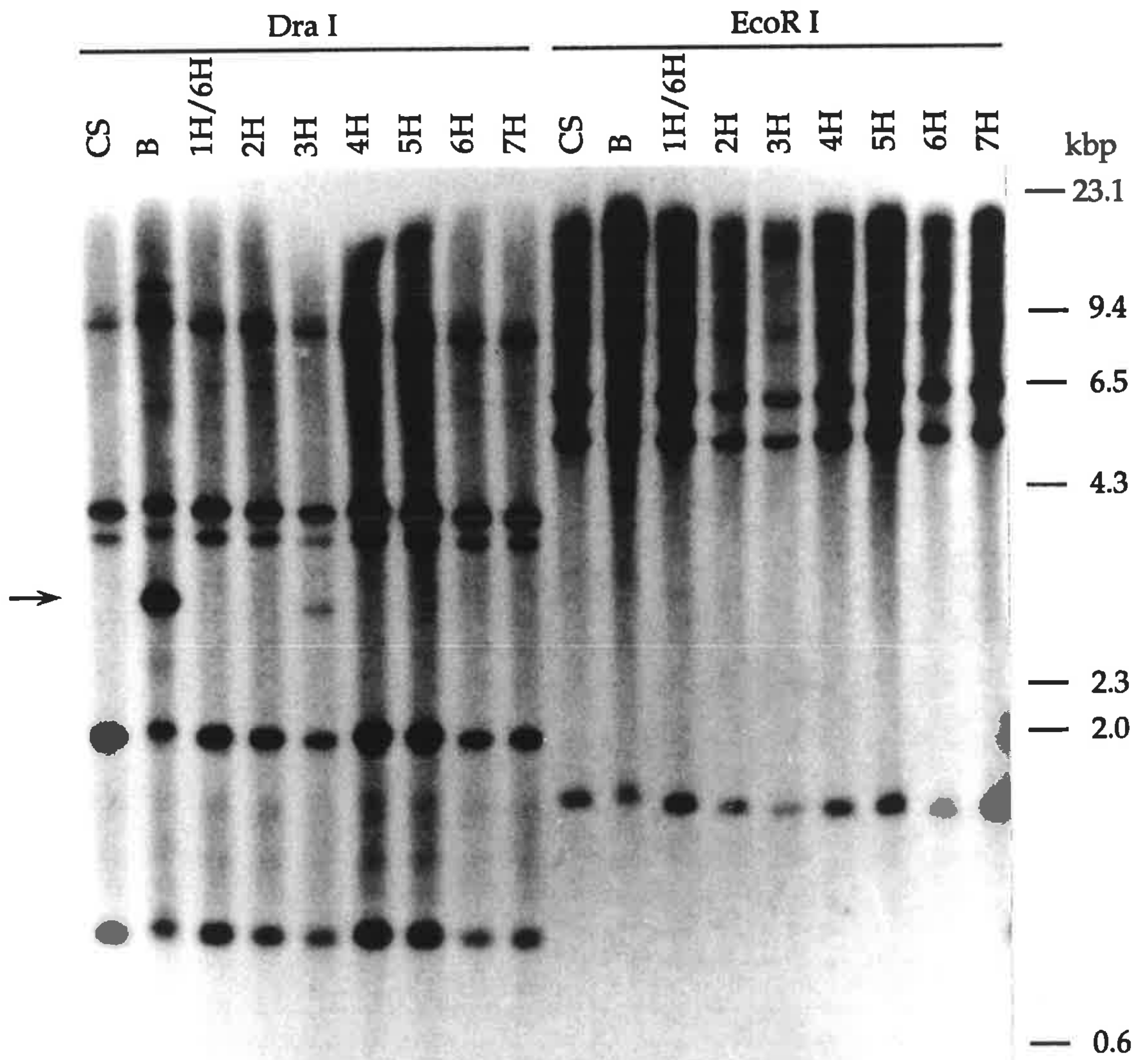
To compare these results with a more ubiquitously expressed messenger, a positive control was performed with sense and antisense 18S ribosomal probe from pea (McFadden *et al.* 1988). Figures 4.2 A and B show the results for the sense and antisense probe, respectively. The strong signal in Figure 4.2 B is not only confined in the tapetal cells but it is also equally abundant in all cell types present in the whole wheat floret. This is in contrast with the specificity of signal found with the wm5.12 probe.

4.4. Chromosomal location of the wm5.12 cDNA clone in Chinese Spring.

To facilitate the assignment of wm5.12 to a specific chromosomal location in wheat, a Southern hybridisation was first performed on the DNA from the wheat/barley addition lines. In these genomic lines, each pair of barley (var. Betzes) chromosomes has been added to the wheat Chinese Spring genome (Islam and Shepherd 1981). Therefore with the occurrence of an alien hybridising fragment to a particular line, it becomes possible to assign bands hybridising to wm5.12 to a specific barley chromosome. The wheat/barley addition lines genomes were digested either with Dra I or EcoR I and probed with wm5.12. In the EcoR I digest, no difference in the hybridisation pattern was found between Chinese Spring and Betzes genomes (Figure 4.3). However, in the Dra I digest the presence of an extra band in Betzes relative to Chinese Spring permits this particular band (indicated by an arrow) to be assigned to chromosome 3 of barley since this additional Betzes band is present in the lane that corresponds to the Chinese Spring genome containing an additional pair of barley chromosome 3.

Figure 4.3 Southern hybridisation to genomic DNA from wheat single chromosome addition lines of barley.

EcoR I or Dra I restricted DNA were hybridised to wm5.12 cDNA insert. The *arrow* indicates the polymorphic band on chromosome 3 H of barley digested in the Dra I digest. **CS**, wheat cv. Chinese Spring; **B**, barley cv. Betzes; **1H/6H**, barley chromosomes 1H and 6H added to Chinese Spring; **2H** to **7H** barley chromosomes 2H to 7H added to Chinese Spring. Hybridisation conditions are as described in Materials and Methods. Membrane was exposed for 5 days at -80°C.



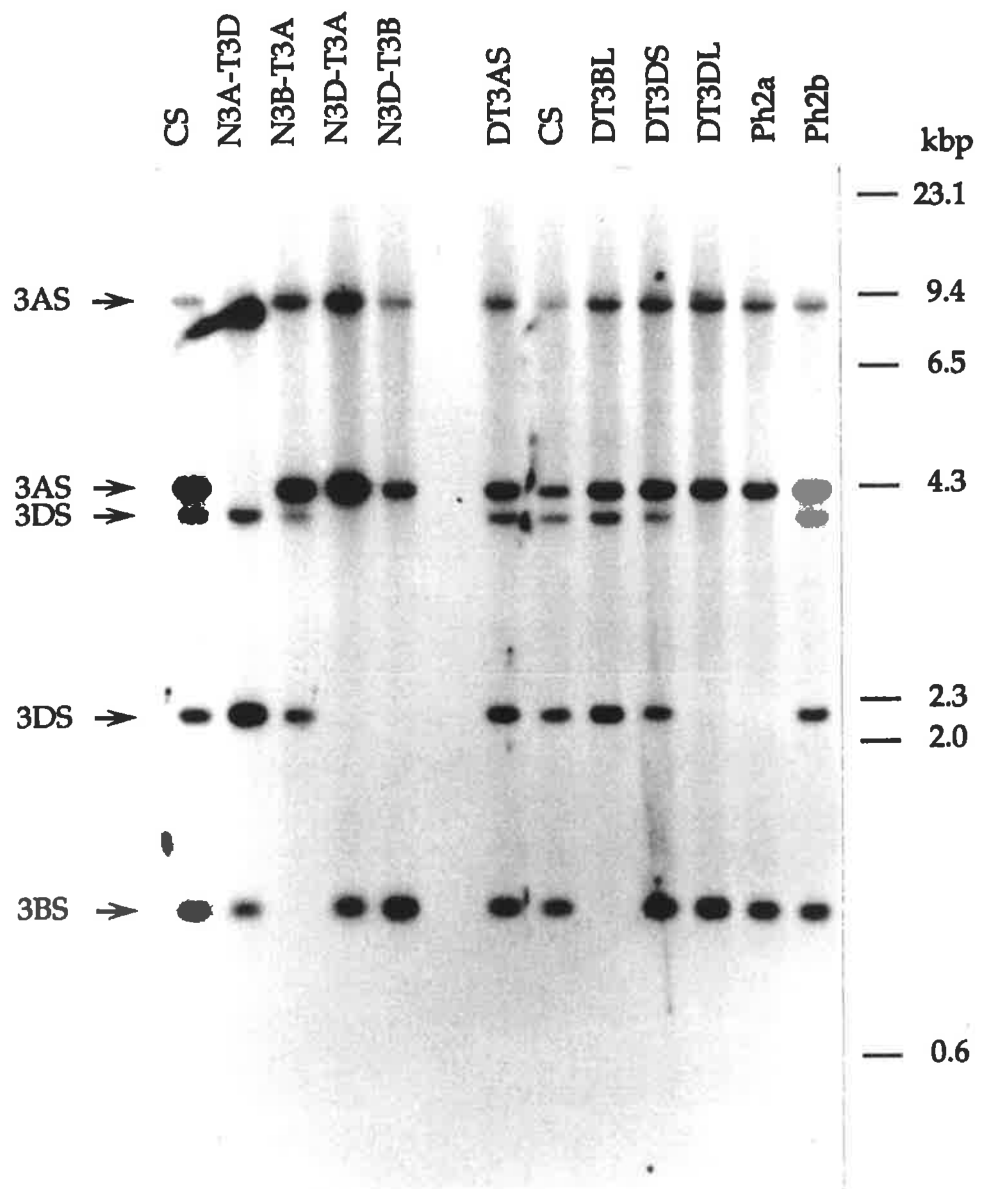
Dra I
EcoR I
CS
B
1H/6H
2H
3H
4H
5H
6H
7H
CS
B
1H/6H
2H
3H
4H
5H
6H
7H
kbp
23.1
9.4
6.5
4.3
2.3
2.0
0.6

Barley and wheat have closely related genomes (Devos *et al.* 1993; Van Deynze *et al.* 1995) and this advantage was used to extrapolate the chromosomal location of wm5.12 cDNA clone found in barley into wheat. A finer chromosomal assignment of the bands hybridising to wm5.12 probe was conducted by using the nullisomic-tetrasomic (NT) and ditelosomic (DT) lines available in Chinese Spring. Dra I digests of the wheat Chinese Spring genome and the homoeologous group 3 nullisomic-tetrasomic and ditelocentric lines were hybridised with wm5.12 insert (Figure 4.4.). The first lane (CS) was used as a control and indicates that the wm5.12 corresponding gene is in low copy number with 5 bands appearing after hybridisation. The next 4 lanes are DNA from the homoeologous group 3 nullisomic-tetrasomic lines. For instance, lane N3A-T3D indicates that the chromosome 3A is absent and has been replaced by an extra pair of chromosome 3D. Thus bands that are missing in the Southern hybridisation pattern of this lane can be assigned to chromosome 3A. Furthermore, Southern bands in the Chinese Spring genome can also be mapped to chromosome arms through the use of ditelosomic lines. For instance, lane DT3AS (Figure 4.4) means that this plant DNA has 2 copies of the short arms of chromosome 3A but is completely lacking the long arm. All of the five bands hybridising to the wm5.12 probe are localised on the short arms of the homoeologous chromosomes group 3: two bands on 3AS, one on 3BS and two on 3DS (indicated by arrows in Figure 4.4).

As explained in the introduction, the most studied genes involved in the control of chromosome pairing in wheat are *Ph1* on 5BL and *Ph2* on 3DS. Mutants are available for both of these genes. To determine whether the two Dra I bands hybridising to 3DS were linked to the *Ph2* gene, DNA was prepared from *ph2a* (deletion mutant, Sears 1977) and *ph2b* plants (point mutation, Wall *et al.* 1971a; Sears 1984). The lanes *ph2a* and *ph2b* (Figure 4.4) show the results when these two mutants are hybridised with wm5.12. In the deletion mutant (*ph2a*), the two 3DS bands are missing but remain present in the point mutation mutant (*ph2b*). This is an indication that wm5.12 could be related to the *Ph2* gene or at least closely linked to it.

Figure 4.4 Chromosomal assignment of wm5.12 in wheat.

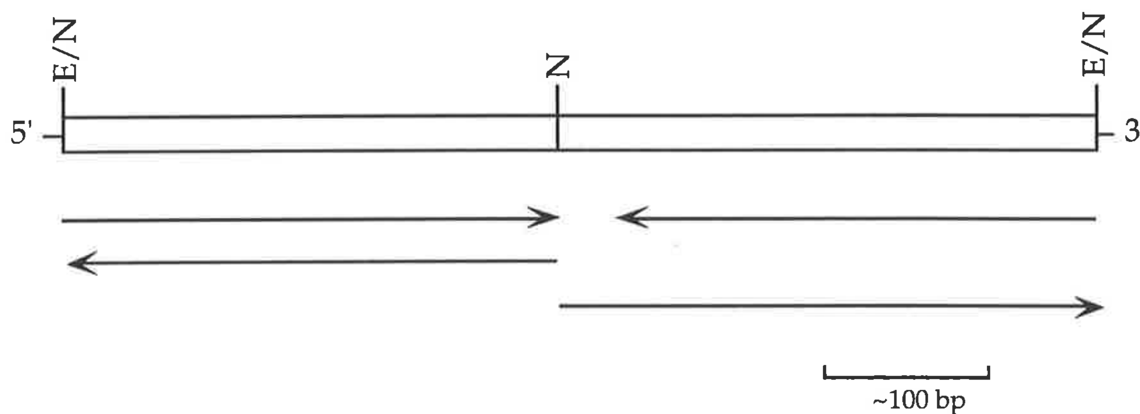
Southern blot analysis of Dra I-restricted genomic DNA from Chinese Spring (**CS**) and homoeologous group 3 nullisomic-tetrasomic (**NT**) and ditelosomic (**DT**) lines and two *Ph* mutants using wm5.12 cDNA insert as a radioactive probe. Hybridisation conditions are as described in Materials and Methods. Membrane was exposed for 5 days at -80°C. The assignments for the various bands are indicated on the left with *arrows*. Molecular size standards are shown on the right.


CS
N3A-T3D
N3B-T3A
N3D-T3A
N3D-T3B
DT3AS
CS
DT3BL
DT3DS
DT3DL
Ph2a
Ph2b
kbp
23.1
9.4
6.5
4.3
2.3
2.0
0.6
3AS
3AS
3DS
3DS
3BS

4.5 Nucleotide sequence analysis of wm5.12 and the putative translated product.

The estimation of the messenger size of wm5.12 in the Northern blot (Figure 4.1) has revealed that wm5.12 was probably a partial clone. In the hope of obtaining a full length one, the amplified cDNA library was screened with the wm5.12 probe. Several lambda clones were positive but one was about 700 bp and was chosen for sequencing since the estimated length of the messenger was approximately this size (Figure 4.1). The 700 bp lambda clone was subcloned into the bluescript plasmid and named wm5.12-*tap*. Both clones, that is wm5.12 and wm5.12-*tap* were sequenced and the sequencing strategy used for wm5.12-*tap* has been illustrated in Figure 4.5. The complete nucleotide sequence of wm5.12-*tap* cDNA clone is 653 bp and is presented with the derived amino acid sequence in Figure 4.6. The sequence obtained with wm5.12 was identical with wm5.12-*tap* but shorter (only 300 bp).

The wm5.12-*tap* cDNA clone is G-C rich (60%) and has an open reading frame (ORF) of 372 bp. The translational start codon deduced from the cDNA sequence at base number 65, is flanked by the nucleotides CAAAATGGG with an A at position -3 and a G at position +4 and therefore this environment qualifies as a strong initiator codon (Kozak 1989) even if it is slightly different than the accepted plant consensus sequence AACAATGGC (Lutcke *et al.* 1987). Furthermore, the clone contains an in frame termination codon upstream of the ATG start codon indicating that wm5.12-*tap* possesses the complete ORF.



<u>Figure 4.5</u> Sequencing strategy of <u>wm5.12-*tap*</u> cDNA clone.

The <u>wm5.12-*tap*</u> cDNA insert was subcloned in Bluescript KS(-) and sequenced as described in the Materials and Methods. Restriction enzyme sites indicate where the cDNA was digested and subcloned in smaller fragments. Arrows show the areas cover for each sequencing direction. Restriction sites are E: EcoR I; N: Not I.

```
1          GAGCTCACCTCAGAGAGCTCCAAGCCTGCTAGCGCGTGTCCGTTCACCCTAG    52

53    ACCAAGAACAAAATGGGCATCCAGGCGAGCCGCGCCCTCGCCGCGCTCCTTCCTGGC   109
1                 M   G   I   Q   A   S   R   A   L   A   A   L   L   P   G    15

110   GGCGCTGGCCGCGCGCGACTGCGGGGCGCCTCGGCGGTGGTGCAGTGCGGCCAGGTG   166
17    G   A   G   R   A   R   L   R   G   A   S   A   V   V   Q   C   G   Q   V    34

167   ACGCAGCTGATGGCGCCCTGCATGCCGTACCTCAGCGGCGCCCCCGGGATGACGCCC   223
36    T   Q   L   M   A   P   C   M   P   Y   L   S   G   A   P   G   M   T   P    53

224   TACGGCATCTGCTGCAACAGCCTCGGGGTGCTCAACCAGCTCGCCGCCAGCACCGCC   280
55    Y   G   I   C   C   N   S   L   G   V   L   N   Q   L   A   A   S   T   A    72

281   GACCGCGTCGCCGCCTGCAACTGCGTCAAGGCGGCCGCCAGCGGGTTCCCGGCCGTC   337
74    D   R   V   A   A   C   N   C   V   K   A   A   A   S   G   F   P   A   V    91

338   GATTTCAGCCGCGCCGCAGCTCTCCCCGCCGCCTGCGGCTCGCCAATCAACTTCGCC   394
93    D   F   S   R   A   A   A   L   P   A   A   C   G   S   P   I   N   F   A   110

395   GTCACCCCCAACATGGACTGCAACCAGGTTACGGATGAACCCTGAGATCGGACTGGA   451
112   V   T   P   N   M   D   C   N   Q   V   T   D   E   P   *                   124

452   GAACCACACGCACGCAACGTACACAGAGGAAGAGCTTAGGTGTTGGACTGACCAAAA   508

509   TAAAATCAGCAAAGATAGTATAAGATTGAATCAAGGGCCTTCAATCATGCATGTGCC   565

566   GAACTGTTTAGTTGATTAATATGTAATTCAATTGTTATCCCGATGTATTATTTCTGT   622

623   GCTTATATTGGAATAAAGTATATGCATCTAC                             653
```

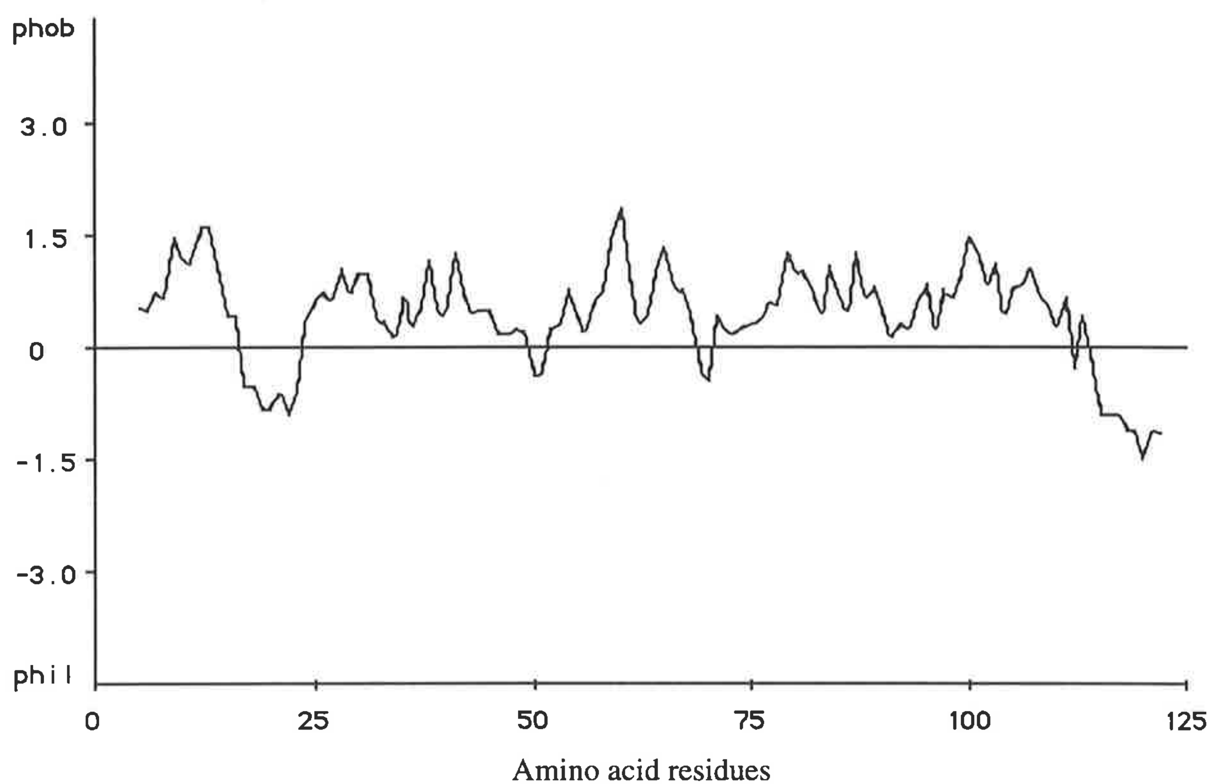
Figure 4.6 Nucleotide sequence of wm5.12-*tap* cDNA and the deduced amino acid sequence.

Solid underlines indicate the putative positions of the polyadenylation signal. Dashed underline identifies the putative signal peptide of the N-terminus of the wm5.12-*tap* peptide. The asterisk indicates the stop codon. The upper numbers indicate the DNA sequence base number, while the lower numbers refer to the position of the amino acids of the protein sequence.

After the stop codon at position 439, two possible polyadenylation sites (Joshi 1987) are located in the 3'-untranslated region. The first one is at position 506 and the second one, at position 633. Surprisingly, no polyA tail was found in clones wm5.12 or wm5.12-*tap*. Therefore, the actual length of the 3' non coding region could be slightly longer than the recorded 214 bases in wm5.12-*tap*.

The polypeptide sequence derived from wm5.12-*tap* has 124 amino acids. After the ATG codon, there is a stretch of 27 amino acids which possesses many features of a signal peptide (von Heijne 1986). If this is the case the mature protein would have 97 residues long corresponding to a molecular mass of 9.8 kDa and a basic pI (isoelectric point) of 7.7. The mature polypeptide does not contain any tryptophan and histidine but is rich in alanine, valine, proline and leucine which contribute 43% of the amino acid content. The hydropathy plot of the protein (Figure 4.7) shows that it is mainly hydrophobic except for the C-terminal region which tends to be hydrophilic.

Databases searches using the BLAST program (Altschul *et al.* 1990) have revealed that the deduced wm5.12-*tap* protein has significant sequence similarity with non-specific lipid transfer proteins (nsLTP). In fact, wm5.12-*tap* possesses the main features of the nsLTPs, namely the 8 conserved cysteines residues including a doublet Cys-Cys and the sequence Cys-Asn-Cys which are highly conserved in all plants including the N-terminal sequence leader. Figure 4.8 shows the alignment of the wm5.12-*tap* protein sequence with other known non-specific lipid transfer proteins in plants. High homologies were found with nsLTPs from *Nicotiana*, *Brassica*, *Gerbera*, etc. Multiple alignment analysis of the wm5.12-*tap* product with 13 selected nsLTP sequences was performed with the GAP comparison program (Wisconsin GCG package, Version 8.0, 1994) (Table 4.1). Most of the identity percentages calculated were between 29 and 40%. However, when conserved amino acid changes were taken into account, the similarity percentages found were much higher (47-61%). Furthermore, other wheat nsLTPs have been identified in embryos (Neumann *et al.* 1994) and seeds (Dieryck *et al.* 1992; Desormeaux *et al.* 1992)) but wm5.12-*tap* is clearly different. When the amino acid sequences were compared only 39% and 35% identities were obtained, confirming that they originate from different genes.



<u>Figure 4.7</u> Hydropathy plot of the deduced <u>wm5.12-*tap*</u> protein.

The hydropathy was calculated according to Kyte and Doolittle (1982). Window size: 9 residues. Negative values indicate hydrophilicity.

Figure 4.8 Alignment of the 12.3 kDa product of wm5.12-*tap* with members of the plant non-specific lipid transfer proteins.

Amino acid residues identical to wm5.12-*tap* sequence are shown in shaded boxes. Gaps are shown with *dots*. Termination codons are indicated by (*). The sequences compared are refered to Table 4.1.

```
Nicotiana        ........MA RFLALALVVI ALSNDALGAP PSCQTVTTQL
Brassica         .....MAFAS KIITCLLILT IYIAAPTESH ITCGTVTSTM
Gerbera          ...MASMVMN VLCVAVACMV FS.ASYADA. ISCGQVTSGL
Ricinus          .......... .......... .......... VDCGQVNSSL
Daucus           ...MGVLRSS FVAMMVMYMV LATTPNAEAV LTCGQVTGAL
Lycopersicon     ....MEMVSK IACFVLLCMV VV.APHAEA. LTCGQVTAGL
Spinacia         ..MASSAVIK LACAVLLCIV VA.APYAEAG ITCGMVSSKL
Oryza            .......... .......... .......... ITCGQVNSAV
Sorghum          MAR....LAV AIAVVAAVVV VLAATTSEAA ISCGQVSSAI
Zea              MARTQQLAVV ATAVVA..LV LLAAATSEAA ISCGQVASAI
Hordeum          ....MARAQV LLMAAALVLM LTAAPRAAVA LNCGQVDSKM
Triticum-A       .......AQV MLMAVALVLM LAAVPRAAVA IDCGHVDSLV
Triticum-B       .......... .......... ........AV ANCGQVVSYL
wm512-tap        ..MGIQASRA LAALLPGGAG RARLRGASAV VQCGQVTQLM

Nicotiana        APCLSYIQNR VKGGGNPSVP CCTGINNIYE LAKTKEDRVA
Brassica         TQCISYLTNG ....GPLPSS CCVAVKSLNQ MAQTTPDRRQ
Gerbera          VPCFGYLAAG ....GPVPPA CCNGVRGLNN AAKTTPDRQT
Ricinus          ASCIPFLTGG ..VASP.SAS CCAGVQNLKT LAPTSADRRA
Daucus           APCLGYLRSQ ..VNVPVPLT CCNVVRGLNN AARTTLDKRT
Lycopersicon     APCLPYLQGR .....GPLGG CCGGVKNLLG SAKTTADRKT
Spinacia         APCIGYLKGG .....PLGGG CCGGIKALNA AAATTPDRKT
Oryza            GPCLTYARG. ..GAGP.SAA CCSGVRSLKA AASTTADRRT
Sorghum          ALCLSYARGQ ..GFAP.SAG CCSGVRSLNS AARTTADRRA
Zea              APCISYARGQ ..GSGP.SAG CCSGVRSLNN AARTTADRRA
Hordeum          KPCLTYVQG. ..GPGP.SGE CCNGVRDLHN QAQSSGDRQT
Triticum-A       RPCLSYVQG. ..GPGP.SGQ CCDGVKNLHN QARSQSDRQS
Triticum-B       APCISYAMGR ..VSAP.GGG CCSGVRGLNA AAATPADRKT
wm512-tap        APCMPYLSGA ..PGMTPYGI CCNSLGVLNQ LAASTADRVA

Nicotiana        ICNCLKNAFI HAGNVNPTLV AELPKKCGIS FNMPPIDKNY
Brassica         VCECLKSAGK EIKGLNIDLV AALPTTCGVS LSY.PIGFNT
Gerbera          ACGCLKGILA ANTRINLNNA NSLPGKCGIS IGY.KITPNI
Ricinus          ACECIKAAAA RFPTIKQDAA SSLPKKCGVD INI.PISKTT
Daucus           ACGCLKQTAN AVTGLNLNAA AGLPARCGVN IPY.KISPTT
Lycopersicon     ACTCLKSAAN AIKGIDLNKA AGIPSVCKVN IPY.KISPST
Spinacia         ACNCLKSAAN AIKGINYGKA AGLPGMCGVH IPY.AISPST
Oryza            ACNCLKNAAR GIKGLNAGNA ASIPSKCGVS VPY.TISASI
Sorghum          ACNCLKNAAR GISGLNAGNA ASIPSKCGVS VPY.TISTST
Zea              ACNCLKNAAA GVSGLNAGNA ASIPSKCGVS IPY.TISTST
Hordeum          VCNCLKGIAR GIHNLNLNNA ASIPSKCNVN VPY.TISPDI
Triticum-A       ACNCLKGIAR GIHNLNEDNA RSIPPKCGVN LPY.TISLNI
Triticum-B       TCTCLKQQAS GIGGIKPNLV AGIPGKCGVN IPY.AISQGT
wm512-tap        ACNCVKAAAS GFPAVDFSRA AALPAACGSP INF.AVTPNM

Nicotiana        DCNTISMY*
Brassica         NCDSISIAV*
Gerbera          DCSKIH*
Ricinus          NCQAIN*
Daucus           DCNRVV*
Lycopersicon     DCSTVQ*
Spinacia         NCNAVH*
Oryza            DCSRVS*
Sorghum          DCSRVS*
Zea              DCSRVN*
Hordeum          DCSRIY*
Triticum-A       DCSRV*
Triticum-B       DCSKVR*
wm512-tap        DCNQVTDEP*
```

Table 4.1 Percentage identity and similarity between amino acid residues of plant non-specific lipid transfer proteins and the product of *wm5.12-tap*.

The Wisconsin GCG (1994) program GAP was used for sequence comparison.

Species	Tissue	Identity (%)	Similarity (%)	References
Brassica napus	anthers	29	53	Foster *et al.* 1992
Daucus carota	embryos	37	53	Sterk *et al.* 1991
Gerbera hybrida	corollas/carpels	33	52	Kotilainen *et al.* 1994
Hordeum vulgare	seeds	36	58	Shriver *et al.* 1992
Lycopersicon esculentum	seeds	36	58	Torres-Schumann *et al.* 1992
Nicotiana tabacum	anthers	34	47	Crossland & Tuttle 1994
Oryza sativa	seeds	37	60	Yu *et al.* 1988
Ricinus communis	seeds	40	60	Takishima *et al.* 1988
Sorghum vulgare	seeds	35	53	Peles-Siebenbourg *et al.* 1994
Spinacia oleracea	leaves	41	61	Bernhard *et al.* 1991
Triticum aestivum-A	seeds	35	57	Dieryck *et al.* 1992; Désormeaux et al. 1992
Triticum aestivum-B	embryos	39	54	Neumann *et al.* 1994
Zea mays	seeds	38	56	Tchang *et al.* 1988

4.6 Discussion.

In this chapter, the analysis of the clone wm5.12 was described. The aim was to learn more about the nature of this wheat cDNA clone and its role, if any, in meiocyte development. Northern blot analysis has demonstrated that wm5.12 mRNA is strongly expressed in the anther throughout meiosis and very weakly in the carpels. This much weaker signal could be due to the type of RNA used. The RNA extracted from the wheat carpels were from a mixture of premeiotic to fully mature ovaries. The result in the carpel lane of the Northern blot does not represent a specific time of development of the wheat carpels, as was done for the anthers, but by pooling premeiotic to postmeiotic female tissue. The weak signal could be due to a small proportion of the RNA being at the specific stage when wm5.12 is expressed. Alternatively, wm5.12 may not be as abundant in the female tissue as in the anthers. From *in situ* hybridisation experiments, it was possible to localise the specific expression in the wheat anther to the tapetal cells. Knowing that wm5.12 most probably encodes for a nsLTP, the results need first to be discussed in relation to what has been found so far for the role or function of these proteins in other plants.

To date, more than twenty cDNA/genes encoding LTPs have been found in both mono- and dicotyledons. Lipid transfer proteins are characterised by their capacity *in vitro* to exchange or transfer amphiphilic lipids between natural or synthetic membranes (Arundel and Kader 1990). So far, only non-specific lipid transfer proteins have been identified in plants (Gausing 1994). They are synthesised as high molecular weight precursors with N-terminal signal peptide and, where tested, this signal peptide participates in co-translational membrane transport *in vitro* (Bernhard *et al.* 1991; Madrid 1991). Early findings have led to the suggestion that nsLTP in plants could be involved *in vivo* in the transfer of lipids from the endoplasmic reticulum (ER) to the mitochondria (Kader *et al.* 1984). However, this contradicts the finding of a secretory signal in the plant peptide and the fact they all lack the ER retention signal H/KDEL (Thoma *et al.* 1994). Indeed, evidence is accumulating that nsLTPs are secreted extracellularly suggesting a role *in vivo* that could be different from the *in vitro* findings. For instance, a carrot LTP was found to be secreted by embryogenic cells in culture (Sterk *et al.* 1991; Meijer *et al.* 1993). Similarly, Thoma *et al.*

(1994) showed an *Arabidopsis* LTP localised in the cell wall of leaves, stems, meristems, flower organs, etc. In addition, a barley aleurone protein, which was later re-classified as a LTP (Breu *et al.* 1989), has been reported to be secreted into the medium of aleurone cell cultures (Mundy and Rogers 1986).

Gausing (1994) also believes that if LTPs were involved in the transfer of phospholipids between the endoplasmic reticulum and mitochondria, it would imply that they are present in all cells. This is not the case. In fact, plant nsLTP identified thus far have specific spatial patterns of expression. For instance, clones encoding LTPs have been isolated in maize coleoptiles (Tchang *et al.* 1988), spinach leaves (Bernhard *et al.* 1991), tobacco flowers (Masuta *et al.* 1992), castor bean cotyledons (Tsuboi *et al.* 1991), barley coleoptiles and leaves (Gausing 1994), *Gerbera* corolla and carpels (Kotilainen *et al.* 1994) and several aerial organs of tomatoes (Torres-Schumann *et al.* 1992) and *Arabidopsis thaliana* (Thoma *et al.* 1994).

Interestingly, two other cDNA clones encoding lipid transfer proteins have also been localised in the anthers. One clone was isolated from *Brassica napus* (Foster *et al.* 1992) and the other one from *Nicotiana* (Crossland and Tuttle 1994). In tobacco, the results have not been published yet. Only the sequence has been reported in the EMBL database with a mention of the clone being expressed exclusively in the anthers. In *Brassica*, Foster and co-workers (1992) have shown results showing their LTP clone being expressed not only in the tapetal cells but also in immature microspores. In the case of wm5.12 mRNA, no expression was detected in wheat mature pollen but it is possible that the transcript could be present in the immature microspores. However, this will need to be tested. Foster *et al.* (1992) did not mention any evidence of the *Brassica* LTP mRNA being present in other flower parts, unlike wm5.12 mRNA which was detected in wheat carpels RNA on Northerns (Figure 4.1). However, it is quite probable that Foster *et al.* (1992) did not assay this tissue.

With more observations being made that nsLTPs are secreted extracellularly, some hypotheses have been brought forward to explain their possible roles. Sterk *et al.* (1991) and Meijer *et al.* (1993) have suggested that nsLTP in carrot embryos could be involved in

the transport of cutin monomers from their site of synthesis through the cell wall of epidermal cells to sites of cutin polymerization. Thoma *et al.* (1994) supports this proposed role. They have examined ß-glucoronidase (GUS) activity in *Arabidopsis* transgenic plants containing LTP promoter-GUS fusions and confirmed the GUS activity in epidermal cells of young leaves, stem, flower, inflorescences, cotyledons, embryos, stigmas, ovule walls, petal tips, etc. Other recent *in vitro* observations have also shown that LTPs from barley and maize leaves possess the ability to inhibit growth of bacterial and fungal pathogens (Molina *et al.* 1993). This role could possibly be present *in vivo* but remains to be demonstrated.

The fact that wm5.12 encodes a probable nsLTP secreted extracellularly, fits well with the physiological and development role of the tapetum. From early meiosis to the maturation of the microspores, the tapetal cells secret a multitude of enzymes, nutrients, etc., to support meiocyte development (Scott *et al.* 1991). Foster and co-workers (1992) have suggested that the *Brassica napus* LTP clone could be involved in sporopollenin production since the pattern of expression of the clone correlates with the process of exine wall formation in the developping microspores. This could also be the case for wm5.12 in wheat. However, this does not ruled out other possible roles.

On a genomic Southern (Figure 4.4), five bands hybridised to wm5.12 when Chinese Spring DNA was digested with Dra I. Among these bands, two are located on chromosome 3D. At this stage, it is not known which band encodes the wm5.12 mRNA but it has been observed that plants nullisomic for 3DS or containing the deletion *ph2a* mutant have significantly reduced male fertility (Sears 1982). At this point, it is not known if this reduced fertility is a direct consequence of the absence of the gene coding for wm5.12 but this assumption is appealing since the mRNA is very abundant in the tapetal cells and it has been documented that the tapetum plays a crucial role in male fertility in higher plants (Xu 1995; Kaul 1988).

The absence of two 3DS bands in the *ph2a* mutant is not sufficient to prove that wm5.12 corresponds to the *Ph2* gene. As *ph2a* is a mutant with a fairly large deletion (Sears 1977; Ceoloni and Feldman 1987), wm5.12 may only be linked to the *Ph2* gene. The presence of

the bands in the point mutation (*ph2b*) does not diminish the link to *Ph2* since Southern hybridisation may not detect this mutation. Moreover, other wheat genes related to meiosis have been mapped on chromosome 3DS and are also absent from the *ph2a* mutant (Ji and Langridge 1994). This could be an indication that chromosome group 3 in *Triticum aestivum* is a major site for the location of genes related to meiosis.

Other nsLTPs have been isolated from wheat. They were either purified from *T. aestivum* seeds (Désormeaux *et al.* 1992) or embryos (Neumann *et al.* 1994). Their amino acid sequences were compared with wm5.12 and the similarity found was between 54 and 57% indicating that they are different proteins. The chromosomal localisation of these wheat LTPs has not been reported but it will be interesting to see if they also map to chromosome group 3. The work with the embryo nsLTP family, which comprises four characterised proteins, was to demonstrate that they are phosphorylated *in vitro* by a calcium dependent protein kinase (Neumann *et al.* 1994). Whether or not phosphorylation also occurs on wm5.12 protein is unknown but the consequence of phosphorylation of wm5.12 adds an extra dimension to the regulation and function of this protein.

With the data analysed so far, it is difficult to say if wm5.12 gene has a direct effect on the control of chromosome pairing in wheat. We would expect genes involved in such a process to be expressed very early in meiosis as has been observed with wm5.12. However, the expectation was that these types of genes would be expressed in the meiocytes (pollen mother cells), since this is where chromosome pairing takes place. It has been shown that transcription is greatly reduced in meiocytes during meiosis (Porter *et al.* 1984). Since the cellular machinery is partially turned off to simplify chromosome movements and recognition and to allow the massive chromatin condensation of meiosis, it could be possible that the tapetum is sollicited to produce and export factors directly or indirectly involved in chromosome pairing.

The true function of the nsLTP has not been elucidated. It is somewhat puzzling that there are so many different nsLTPs in a given plant and yet the suggestion that they all perform similar molecular functions, that is the transfer of lipidic materials extracellularly. Why

would plants not have one type of nsLTP that is regulated differently according to where and when the protein is required?

The results of this chapter open several doors toward an investigation on the role(s) of nsLTP in meiocytes/microspores development. Care has to be taken when interpreting data about what has been found *in vitro* for other nsLTPs. Until a detail analysis of their function *in vivo* becomes available, it remains possible that nsLTP, such as wm5.12, may play a key role to support the meiotic process. The close relationship existing between the tapetum and the meiocytes prior and during the meiotic event show that several aspects of the tapetum have not been explored yet. Following the results described in this chapter, the next experiments would be to establish how wm5.12 functions in relation to the meiocyte development and male fertility in general.

CHAPTER V

Molecular analysis of wm2.19 cDNA clone.

5.1 Introduction.

This chapter describes the molecular analysis of the cDNA clone wm2.19. The results of this analysis are presented in a similar manner to Chapter IV where the cDNA clone wm5.12 is described. As mentioned previously, wm2.19 was selected following differential screening of the wheat cDNA library and the RNA dot blots analysis presented in Chapter III.

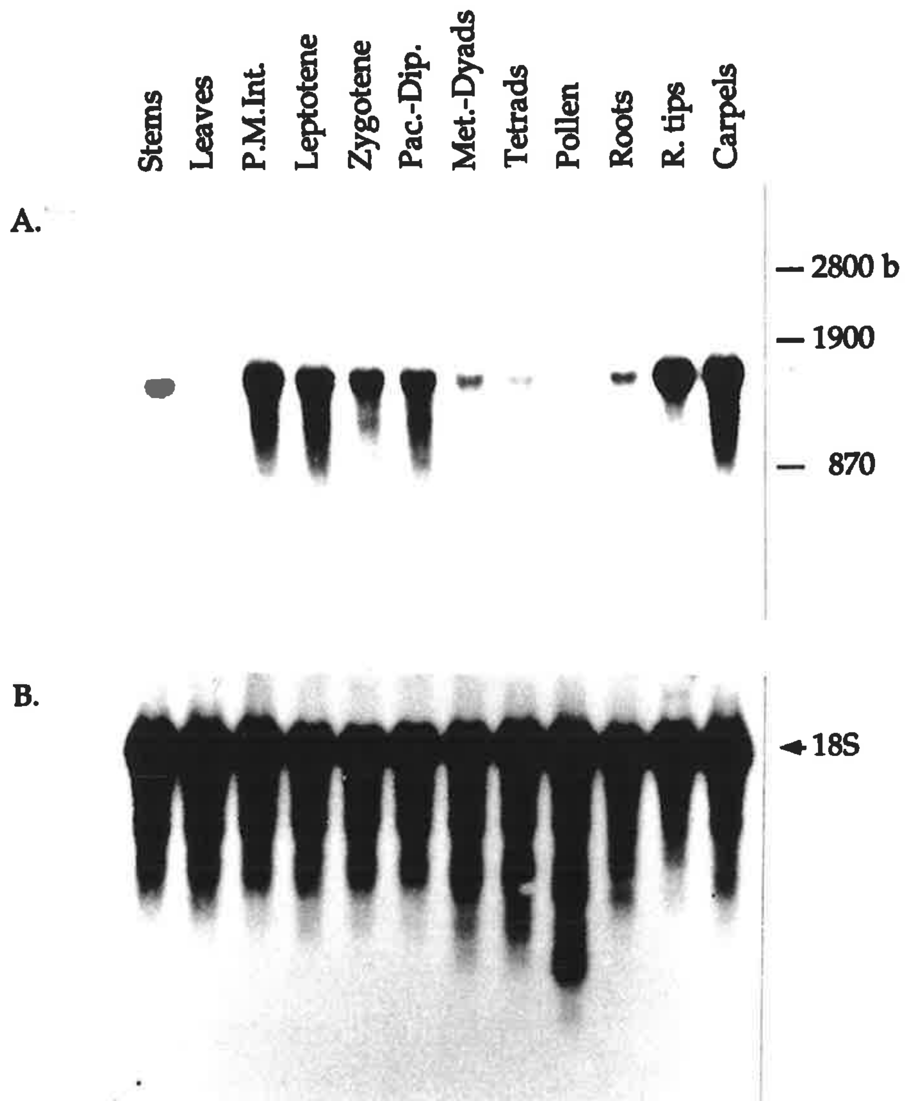
The first sections have focused on the expression of gene represented by wm2.19. The chromosomal location of this gene encoding the cDNA clone was found by RFLP analysis of wheat and barley and the probing of the appropriate Chinese Spring nullisomic-tetrasomic (NT) lines with wm2.19. Finally, the complete nucleic acid sequence of the clone is given to help us determine the possible function of wm2.19 corresponding protein. The overall results are discussed and examined in relation to meiosis and cell division in general.

5.2 Spatial and temporal analysis of wm2.19 messenger.

To examine the spatial and temporal expression pattern of wm2.19, Northern blot analysis were performed on different Chinese Spring tissues and on developping anthers at several stages of meiosis (Figure 5.1 A). Transcripts were detected in anthers with maximum

Figure 5.1 Northern blot analysis of the expression of the gene corresponding to wm2.19 cDNA clone.

The radioactive wm2.19 probe was hybridised to 10 µg of total RNA loaded in each lane and extracted from different plant organs and several developmental stages of the anther. The blot was exposed to an X-ray film for 5 days (**A**). To verify an even loading of the samples in each lane, the blot was subsequently hybridized with a radioactive 18 S ribosomal probe from pea (**B**). Exposition time: 1 hour. **P. M. Int.**: RNA from anthers at premeiotic interphase; **Pac.-Dip.**: RNA from anthers at pachytene and diplotene; **Met.-Dyads**: RNA from a mixture of anthers at metaphase I, anaphase I, telophase I and Dyads; **R. tips**: RNA from root tips. RNA size markers are indicated on the right.



Stems
Leaves
P.M.Int.
Leptotene
Zygotene
Pac.-Dip.
Met.-Dyads
Tetrads
Pollen
Roots
R. tips
Carpels
A.
2800 b
1900
870
B.
18S

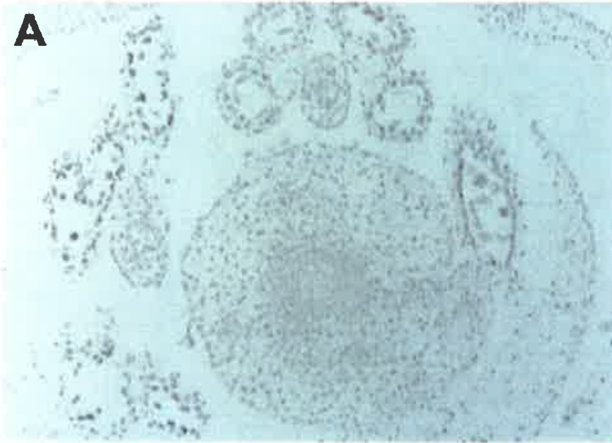
expression observed at premeiotic interphase, leptotene, zygotene and pachytene/diplotene. A much weaker hybridisation signal was detected in the later stages of meiosis and also in pollen, stems, leaves and roots. However, total RNA from root tips and carpel gave an equally strong signal when hybridised with the radioactive wm2.19 cDNA insert. This indicates that wm2.19 mRNA is expressed not only in meiotic tissue such as the anthers and carpels but also in young dividing tissue such as root tips and stems where a high level of mitotic activity is occuring. Since the size of wm2.19 cDNA insert was estimated to be close to 800 bases and the size of the transcripts calculated on the Northern blot seemed to be about 1,500 bases, there is little doubt that wm2.19 is a partial cDNA clone. Therefore the cDNA library was re-screened to obtain the full length clone. To control the quality and the even loading of the RNA in each lane, the same blot was stripped from its former probe and re-hybridised with a ^{32}P-labelled 18S ribosomal probe DNA insert from pea (McFadden *et al.* 1988). The result is shown in Figure 5.1 B.

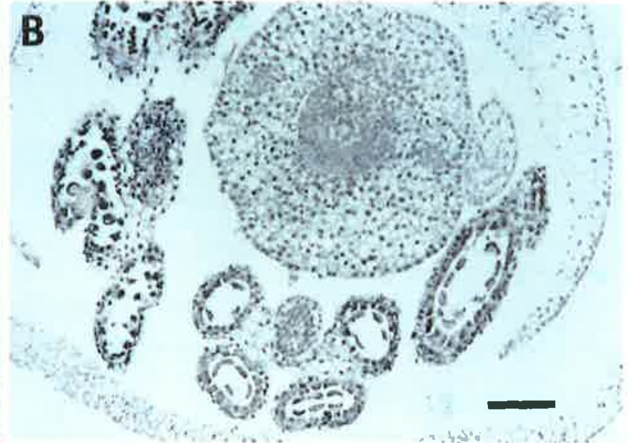
5.3 *In situ* localisation of wm2.19 messenger in Chinese Spring anthers.

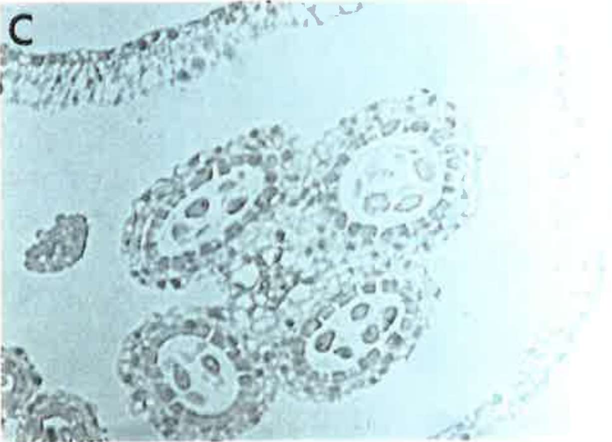
In order to determine the cellular specificity of the transcripts of wm2.19 within the wheat anther, *in situ* hybridisation was carried out using sense and antisense RNA probes labelled with digoxigenin (DIG)-11-UTP as described in the Materials and Methods section. Cross and longitudinal sections were made from wheat florets containing anthers at the early stages of meiosis (either leptotene, zygotene or pachytene). Because the Northern blot results showed that the mRNA seemed abundant in young dividing tissue, careful selection was made to choose anthers containing sufficient meiocytes remaining in the section. As shown in Figures 5.2 B and 5.2 D, the antisense wm2.19 probe gave a strong signal with the meiocytes and the tapetal cells of the anther. Figure 5.2 D is at a higher magnification than 5.2 B and shows clearly the purple-brownish precipitates in both cell types. In Figure 5.2 B, where part of the carpel was also sectioned, a signal slightly stronger than the background can also be seen. As a control and for comparison, a sense wm2.19 probe

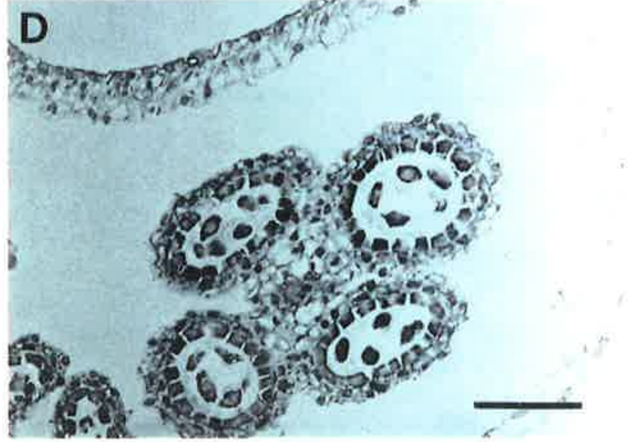
Figure 5.2 Location of wm2.19 mRNAs in wheat cv. Chinese Spring florets.

Cross sections of florets at the prophase I stage of meiosis were hybridised with digoxigenin labelled sense and antisense cRNA probes. Sections (**A**) and (**C**) were used as a control with wm2.19 sense cRNA. Sections (**B**) and (**D**) were hybridised with wm2.19 antisense cRNA. The photographs were taken using bright field microscopy. Bars = 0.1 mm.


A


B


C


D

was also made and hybridised under the same conditions as the antisense probe. Figures 5.2 A and 5.2 C show the results when no specific hybridisation is present. We observed in this case, an unspecific light brown background all over the sectioned tissue.

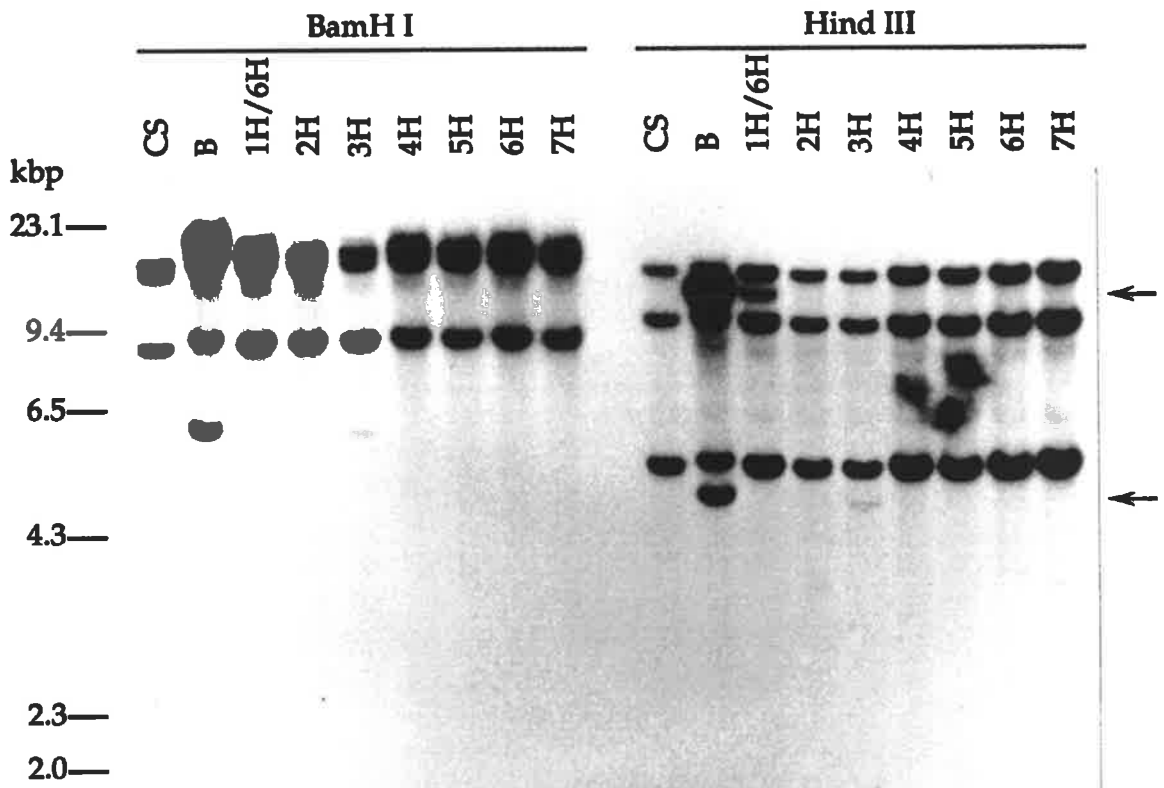
5.4 Chromosomal location of the wm2.19 cDNA clone in Chinese Spring.

To assign the cDNA clone wm2.19 to a particular chromosomal location in wheat, a similar approach was conducted as used for the clone wm5.12 in the previous chapter. First, wm2.19 probe was used on a Southern blot against plant genomic DNA from the wheat-barley addition lines. By refering to Figure 5.3, the DNA from the plants was either digested with BamH I or Hind III and hybridised with the radioactive cDNA insert wm2.19. After 5 days exposition, the result showed three hybridising bands in wheat (CS lane digested BamH I or Hind III) and four to five bands in barley (B lane digested BamH I or Hind III). Due to the different hybridising pattern between the two genomes, it was possible to localise the two extra bands present in barley (Hind III digest) to chromosomes 1H and 3H. The presence of one of these extra bands in the lane 1H/6H (Hind III digest) containing both chromosomes 1H and 6H of barley and its absence in lane 6H, signifies that the band is strictly located on chromosome 1H (first *arrow* in Figure 5.3) and not on 6H. This preliminary result suggested that in Chinese Spring, the three bands hybridising to wm2.19 could be located on chromosomes groups 1 and/or 3 due to the familial relationship between barley and wheat (Devos *et al.* 1993; Van Deynze *et al.* 1995).

Since our prime interest was for chromosome groups 3 and 5 of Chinese Spring, we first checked the hybridising pattern of probe wm2.19 on a genomic Southern containing the aneuploid plants DNA for chromosome 3. The radioactive wm2.19 probe was hybridised to the filter to determine if any of the three bands on Chinese Spring DNA (cut with BamH I or Hind III) could be mapped to either chromosomes 3A, 3B or 3D. However, none of

Figure 5.3 Southern hybridisation of plants genomic DNA from wheat-barley single chromosome addition lines.

BamH I or Hind III restricted DNA were hybridised to wm2.19 cDNA insert. The *arrows* indicates the polymorphic bands on chromosomes 1H and 3H of barley digested with Hind III. **CS**, wheat cv. Chinese Spring; **B**, barley cv. Betzes; **1H/6H**, barley chromosomes 1H and 6H added to Chinese Spring; **2H** to **7H**, barley chromosomes 2H to 7H added to Chinese Spring. Hybridisation conditions are as described in Materials and Methods. Membrane was exposed for 6 days at -80°C.


BamH I
Hind III
CS
B
1H/6H
2H
3H
4H
5H
6H
7H
kbp
23.1
9.4
6.5
4.3
2.3
2.0

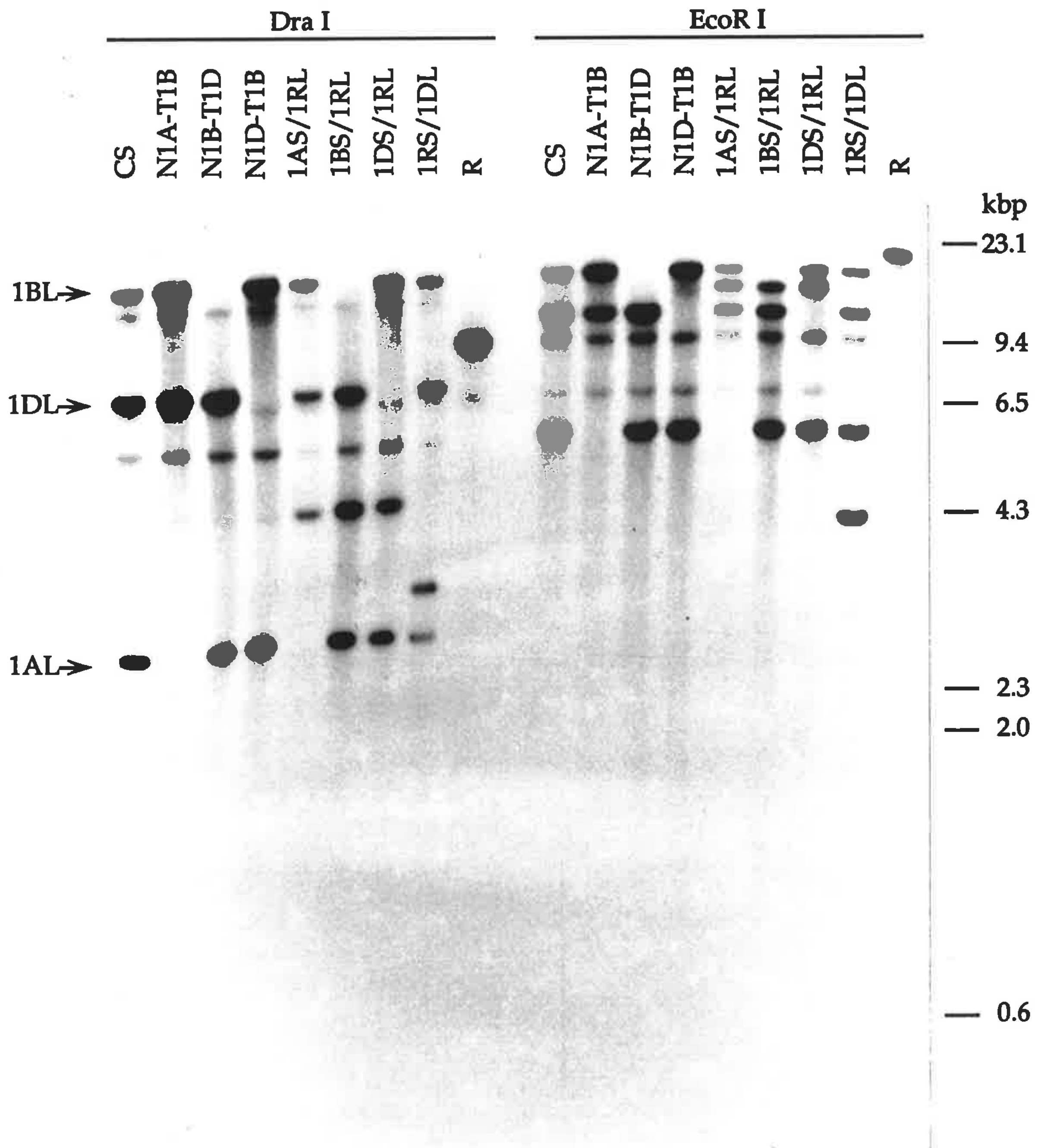
the three bands could be assigned to the homoelogous group 3 of Chinese Spring (data not shown).

Therefore, the second possibility was to verify if the bands hybridising with wm2.19 could be located on group 1 chromosomes. A Southern blot was prepared with the nullisomic-tetrasomic (Sears 1966) and ditelocentric (Sears and Sears 1978) plant DNA of homoelogous group 1 and the wheat-rye substitution lines where single arms of chromosomes 1A, 1B and 1D have been substituted with the short or long arm of chromosome 1 of rye. Figure 5.4 shows the genomic plant DNA digested with the restriction enzyme Dra I or EcoR I and hybridised with wm2.19 cDNA insert. Chromosomal assignment of the genes encoding the mRNA represented by clone wm2.19 was possible by observing if any of the 3 strong bands present in CS (Dra I or EcoR I digest) were absent in the following DNA digests. For instance, in lane N1A-T1B (Dra I or EcoR I), where chromosome 1A is absent and is replaced by an extra pair of chromosome 1B, we can see in the hybridising pattern that a band is missing when compared to the Chinese Spring lane (CS). Consequently, this missing band can be assigned to chromosome 1A of Chinese Spring. Also by refering to lanes 1AS/1RL, 1BS/1RL and 1DS/1RL in the same Figure, we can further localise the CS Southern bands to specific chromosome arms. In these lanes, the long arm of chromosomes 1A, 1B and 1D respectively, are absent and substituted by the long arm of chromosome 1 of rye. Again, by looking for the presence or absence of any of the CS bands, it is possible to demonstrate that wm2.19 corresponding genes are located on the long arms of chromosomes 1A, 1B, and 1D (as indicated by the *arrows* on the left).

Interestingly, the two weaker bands present in CS are probably related partially to wm2.19 since under a more stringent post-hybridisation wash they disappear and only the three bands located on homeologous chromosomes 1 remain (see figure 5.3).

Figure 5.4 Chromosomal assignment of wm2.19 in wheat.

Southern blot analysis of Dra I or EcoR I-restricted genomic DNA from Chinese Spring (**CS**), Imperial rye (**R**), homoeologous group 1 nullisomic-tetrasomic (**NT**) lines and wheat-rye translocation lines of chromosome 1 using wm2.19 cDNA insert as a radioactive probe. Hybridisation conditions are as described in Materials and Methods. Membrane was exposed for 7 days at -80°C. The assignments for the various bands are indicated on the left with *arrows*. Molecular size standards are shown on the right.


Dra I
EcoR I
CS
N1A-T1B
N1B-T1D
N1D-T1B
1AS/1RL
1BS/1RL
1DS/1RL
1RS/1DL
R
kbp
23.1
9.4
6.5
4.3
2.3
2.0
0.6
1BL
1DL
1AL

5.5 Nucleotide sequence analysis of wm2.19 and the putative translated product.

Following the re-screening of the amplified cDNA library with probe wm2.19, several positive lambda clones were selected and checked for the size of their inserts (data not shown). From this analysis, one clone had the expected size of the transcripts hybridising to probe wm2.19 on the RNA gel blot (Figure 5.1). To facilitate sequencing, it was subcloned in a Bluescript plasmid and named wm2.19-*fl* (for full length). Both clones (wm2.19 and wm2.19-*fl*) were sequenced and the sequencing strategy used for the longest cDNA (i.e. wm2.19-*fl*) is illustrated in Figure 5.5. The sequencing result revealed that wm2.19 was indeed a partial cDNA clone and contained the last 956 bases of the 3' end of clone wm2.19-*fl*.

In Figure 5.6, the nucleotide acid sequence of wm2.19-*fl* is shown along with the corresponding amino acid residues. The longest ORF of wm2.19-*fl* has 1,182 bases; starting at base number 136 and finishing at base 1,318. The sequence context of the ATG start codon is slightly different from the usual plant consensus sequence at the start site (AACAATGGC, Lutke *et al.* 1987). There is a G at base 135 instead of an A and a T at base 139 instead of a G. Despite these differences, the ATG codon still qualifies as a functional start site for protein translation. The fact that it is the first ATG codon that the 40S ribosomal subunit would encounter in the scanning process and the presence of an adenine at base 133 would be sufficient, according to Kozak (1989) to believe it is a good initiator site. The untranslated 5' end possesses a stop codon at base 130 in the same reading frame of the start codon. This suggests that the complete ORF has been sequenced. After the stop codon (at base 1318), there is an untranslated 3' end of 164 bases which contains a poly A tail of 21 nucleotides. In this important region for messenger processing, no typical polyadenylation signal (AATAAA, Joshi 1987) could be identified. However, the presence of a sequence, AATGAA, known as a "near upstream element" (Hunt 1994) was found at base 1434. Several near upstream elements (NUE) have been characterized so far in plants but their exact function is still unknown and they are thougth to be *cis* elements able to affect messengers stability and processing (Hunt 1994).

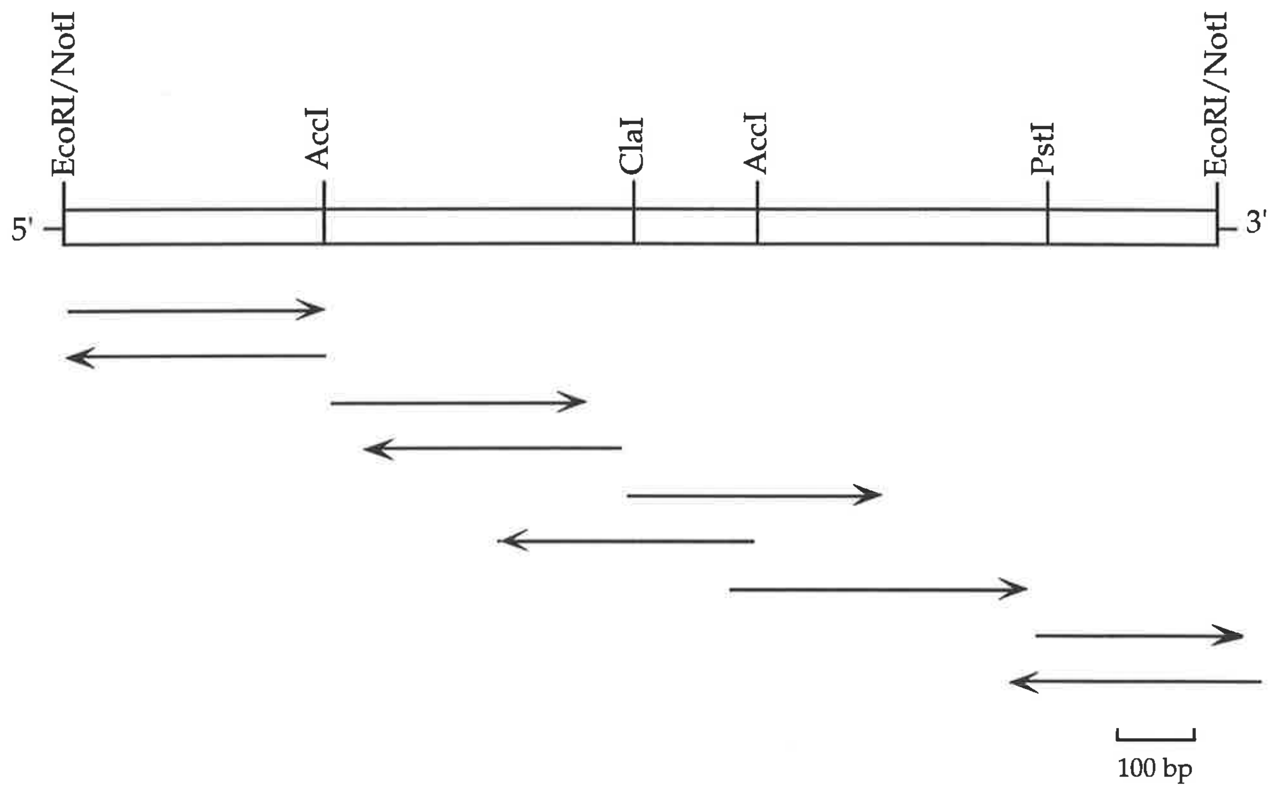


Figure 5.5 **Sequencing strategy of wm2.19-*fl* clone.**

The wm2.19-*fl* cDNA insert was subcloned in Bluescript KS(-) and sequenced as described in the Materials and Methods. Restriction enzyme sites indicate where the cDNA was digested and subcloned in smaller fragments. Arrows show the areas covered for each sequencing direction.

Figure 5.6 Nucleotide sequence and the derived amino acid sequence of *wm2.19-fl* cDNA clone.

Underlined with dashes is the putative "near upstream element" signal (Hunt 1994). Double underlines show the potential nuclear localisation signal. The asterisk indicates the stop codon. The upper numbers indicate the DNA sequence base number, while the lower numbers refer to the position of the amino acids of the protein sequence.

```
1           TCC GCC CCC TCC CGA GAC GCC AAA GCC CCA AAC CCC TTG CCC TCC    45

46    CAA ACC CTA ACC CTA AAA CCT CCG CCC TCC CGC CGC CGC CGC CGC CCG ATT    96

97    CCC CTC CCC AGC CTC CGC ATC CCG CCT ACG GCT TGA ACG ATG TCG TCC GAC   147
1                                                         M   S   S   D     4

148   GAG GAG GTC AGG GAG GAG AAG GAG CTC GAC CTC TCC TCC AAC GAG GTC GTC   198
5     E   E   V   R   E   E   K   E   L   D   L   S   S   N   E   V   V      21

199   ACC AAG TAC AAG ACC GCC GTC GAG ATC ATT AAC AAG GCT CTG AAG TTG GTA   249
22    T   K   Y   K   T   A   V   E   I   I   N   K   A   L   K   L   V      38

250   TTG TCG GAG TGC AAG CCG AAA GCC AAG ATT GTT GAC ATT TGT GAG AAG GGT   300
39    L   S   E   C   K   P   K   A   K   I   V   D   I   C   E   K   G      55

301   GAC AAT TTC ATA ACA GAG CAA ACT GGG AAT GTC TAC AAG AAC GTG AAG AGG   351
56    D   N   F   I   T   E   Q   T   G   N   V   Y   K   N   V   K   R      72

352   AAG ATT GAA AGG GGC ATT GCT TTC CCG ACA TGT GTA TCT GTG AAC AAC ACC   402
73    K   I   E   R   G   I   A   F   P   T   C   V   S   V   N   N   T      89

403   GTC TGT CAT TTC TCC CCA CTG GCA ACT GAC GAT TCT GTA CTC GAA GAA AAT   453
90    V   C   H   F   S   P   L   A   T   D   D   S   V   L   E   E   N     106

454   GAC ATG GTG AAG ATC GAT ATG GCT TGC CAT ATT GAT GGT TTT ATT GCT GTG   504
107   D   M   V   K   I   D   M   A   C   H   I   D   G   F   I   A   V     123

505   GTG GCT CAT ACA CAT GTA ATC AAA GCT GGG CCG GTT ACT GGA AGA GCA GCT   555
124   V   A   H   T   H   V   I   K   A   G   P   V   T   G   R   A   A     140

556   AAT GTT CTT GCT GCT GCA AAC ACA GCA GCA GAA GTT GCA ATG AGG CTT GTT   606
141   N   V   L   A   A   A   N   T   A   A   E   V   A   M   R   L   V     157

607   AGA CCT GGC AAG AAG AAT AAG GAT GTC ACT GAA GCA ATT CAG AAA GTT GCT   657
158   R   P   G   K   K   N   K   D   V   T   E   A   I   Q   K   V   A     174

658   GCT GCT TAT GAT TGT AAA ATT GTT GAA GGA GTT CTT AGC CAT CAG CTG AAA   708
175   A   A   Y   D   C   K   I   V   E   G   V   L   S   H   Q   L   K     191

709   CAA TTT GTC ATC GAT GGT AAC AAA GTG GTA CTT AGT GTT TCA AAC GCG GAC   759
192   Q   F   V   I   D   G   N   K   V   V   L   S   V   S   N   A   D     208

760   ACA AAG GTG GAT GAT GCT GAA TTT GAA GAA AAT GAA GTG TAT GCA ATT GAT   810
209   T   K   V   D   D   A   E   F   E   E   N   E   V   Y   A   I   D     225

811   ATT GTC ACC AGC ACT GGC GAG GGA AAG CCG AAG CTA CTG GAT GAG AAG CAG   861
226   I   V   T   S   T   G   E   G   K   P   K   L   L   D   E   K   Q     242

862   ACC ACT ATT TAC AAG AGA GCT GTA GAC AAG AAC TAT CAC TTG AAG ATG AAG   912
243   T   T   I   Y   K   R   A   V   D   K   N   Y   H   L   K   M   K     259

913   GCA TCA AGG TTC ATC TTC AGT GAG ATT AGC CAG AAG TTC CCA ATC ATG CCA   963
260   A   S   R   F   I   F   S   E   I   S   Q   K   F   P   I   M   P     276

964   TTC ACT GCT AGG GCG CTG GAG GAG AAG CGT GCA CGC TTA GGT TTG GTA GAA  1014
277   F   T   A   R   A   L   E   E   K   R   A   R   L   G   L   V   E     293

1015  TGC ATG AAT CAT GAG CTG TTG CAG CCA TAC CCT GTT CTA CAT GAG AAG CAA  1065
294   C   M   N   H   E   L   L   Q   P   Y   P   V   L   H   E   K   Q     310

1066  GGC GAC CTG GTT GCC CAC ATC AAG TTC ACC GTG TTG TTG ATG CCA AAT GGG  1116
311   G   D   L   V   A   H   I   K   F   T   V   L   L   M   P   N   G     327

1117  TCT GAC AAG ATA ACT TCA CAT CCA CTA CAG CAA CTG GAG CCC TCA AAA TCC  1167
328   S   D   K   I   T   S   H   P   L   Q   Q   L   E   P   S   K   S     344

1168  ATC GAG GGC GAT GCT GAG ATT AAG GCT TGG CTT GCT TTG GGC ACA AAG TCA  1218
```

```
 345 I   E   G   D   A   E   I   K   A   W   L   A   L   G   T   K   S     361

1219 AAG AAG AAG GGT GGT GGC AAG AAA AAG AAA GGC AAG AAA GGA GAT GCT GCA  1269
 362 K   K   K   G   G   G   K   K   K   K   G   K   K   G   D   A   A     378

1270 GAA GTA GCA GAG CCC ATG GAG GTT TCT AAG GAC GCA CCA AGC CAG GAA TAA  1320
 379 E   V   A   E   P   M   E   V   S   K   D   A   P   S   Q   E   *     394

1321 ATC TCA TTT TTT TTG AAT TGC TTC CTT TAC GTT TGT ACC TGC TCT TTG CTG  1371

1372 CAT CTG ATT GTG TTA TAT TAC ATT CAG CTG TGG GGA TTT GAG TAC CTT TTT  1422

1423 TTG AAG TGT CTA ATG AAA ATG GTT GAT CCT GGT GCA CAT CAA AAA AAA AAA  1473

1474 AAA AAA AAA AAA                                                      1485
```

The deduced protein product with 394 amino acid residues (Figure 5.6) has a calculated total molecular mass of 43 kDa and a predicted isoelectric point (pI) of 7.5. The amino acid content is high in lysine (12%), valine (10%), alanine (9%) and glutamic acid (9%). Several putative casein kinase II and protein kinase C phosphorylation sites have been found (Pinna 1990; Woodget *et al.* 1986). The sequence exhibits also two potential N-linked glycosylation sites (N-X-[S, T]) at the amino acid residues 87 and 326 (Wagh and Bahl 1981). The hydropathy plot (Kyte and Doolittle 1982) shows that wm2.19-*fl* protein is essentially hydrophilic with increases in hydrophobicity occurring at fairly regular intervals (Figure 5.7).

Searches of nucleic acid and protein databases with wm2.19-*fl* sequence has revealed significant similarities with cDNAs/genes clones from *Arabidopsis thaliana*, yeast and mouse. In Table 5.1 the most significant alignments found with wm2.19-*fl* have been compiled. The highest score was found with an *A. thaliana* cDNA clone (Philipps and Gigot 1992). This partial clone of only 292 bases shared 75.1% identity with the 3' end of wm2.19-*fl*. Another *A. thaliana* clone of 446 bases, which is also a partial cDNA (Newman *et al.* 1994), had 68.9% identity with wm2.19-*fl* at the nucleotide level. Those two partial clones were sequenced in the course of the large scale sequencing program of anonymous *Arabidopsis* cDNA clones. Sequence data have been compiled in Genebank but information about the location and function of these clones have not been documented.

Besides these *Arabidopsis* clones, we also found interesting homology with a mouse (p38-2G4) and a yeast (42K) clone (58.6% and 54.3% identity, respectively). Considering the phylogenetic distance between wheat, mouse and yeast, these high scores at the nucleotide level are clearly significant. After a comparison of the protein product of wm2.19-*fl* and those of p38-2G4 (Radomski and Jost 1995) and 42K (Yamada *et al.* 1994), the similarities reached 71% with p38-2G4 and 60% with the 42K protein. This is a strong indication that we have probably cloned the plant homologue of these two proteins. Moreover, after a

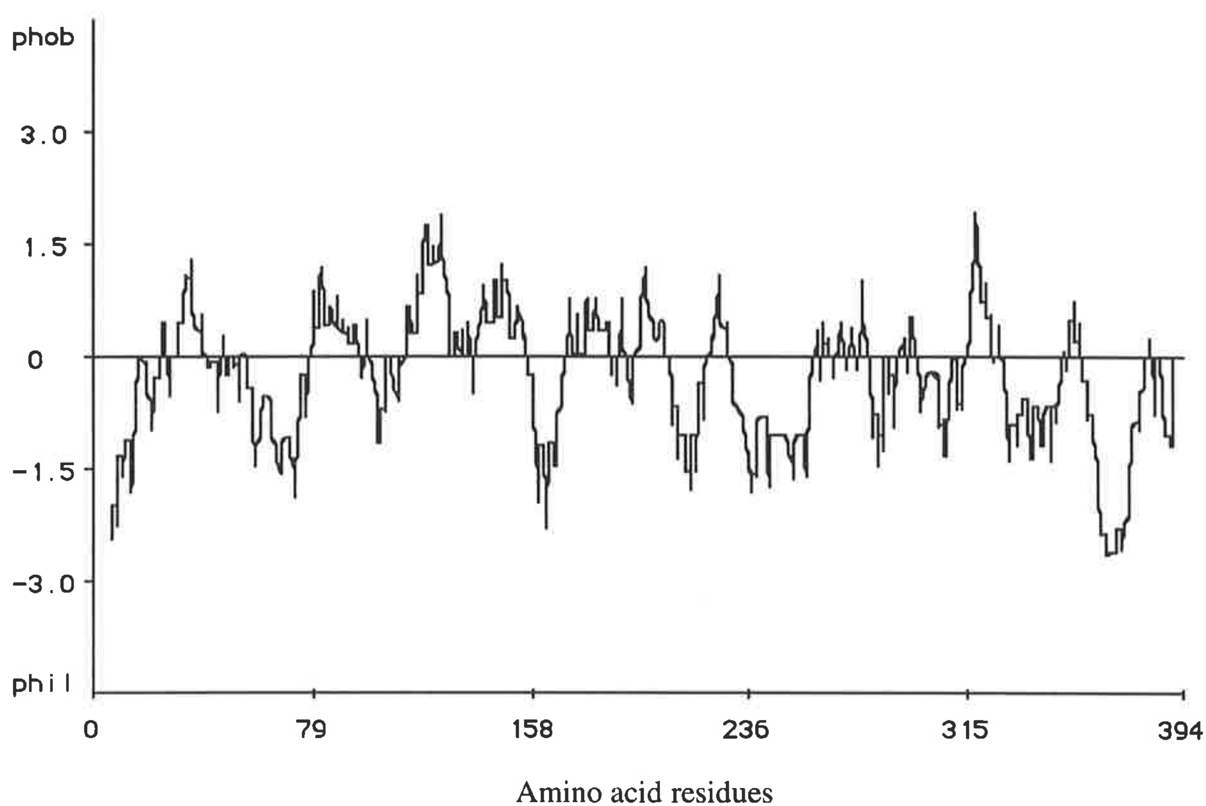


Figure 5.7 Hydropathy plot of the deduced wm2.19-*fl* protein.

The hydropathy was calculated according to Kyte and Doolittle (1982). Window size: 11 residues. Negative values indicate hydrophilicity.

Table 5.1 Comparison of the wm2.19-*fl* cDNA sequence to nucleic acid sequences from Genebank.

The NCBI Fasta Search program was used. [a] nt: number of overlapped nucleotides in the sequence comparison.

Source	Clone	Size (bp)	Identity %/nt[a]	References
A. thaliana	147J24T7	446	68.9/428	Newman *et al.* (1994)
A. thaliana	TASG018	292	75.1/292	Philips and Gigot (1992)
Mouse	p38-2G4	1639	58.6/1183	Radomski and Jost (1995)
Yeast	42K	1905	54.2/1087	Yamada *et al.* (1994)

multiple alignment of the three proteins (Figure 5.8) it is apparent that the C-terminal region has a common block containing several lysine residues. This could possibly be a nuclear localisation signal (Dingwall and Laskey 1991; Garcia-Butos *et al.* 1991). Finally, the presence in all three proteins of repetitive charged amino acids (Figure 5.8) at a regular interval suggest they share a common structural domain resembling an amphipatic helical domain which could either bind to DNA or other proteins (Darnell *et al.* 1990)

5.6 Discussion.

The wheat cDNA clone reported here appears to represent a gene involved in the cell cycle. From the RNA blots and *in situ* analysis, wm2.19 mRNA is shown to be abundantly expressed in young dividing tissues such as the meiotic anthers, carpels and root tips. It is also expressed in other vegetative tissues where cell division is active such as during the elongation of the plant stem and the growth of the young roots. When RNA is extracted from tissue that is more mature, such as the leaves used for the Northern blot in Figure 5.1, the mRNA is only weakly expressed. *In situ* experiments have also allowed the localisation of wm2.19 transcripts in wheat florets. The results show that wm2.19 expression is not only confined to the meiocytes of the anther but also to any cell types actively dividing. This is particularly true for the tapetal cells. This pattern of expression is typical of well characterised cell cycle linked genes. For instance, the histones H2B and H4, that were isolated in the course of the library differential screening (Chapter III), had a similar pattern of expression, that is abundant in tissue where cell division was in progress and present at a much lower level where tissue maturity was reached and cell division scarce.

Structural analysis of wm2.19-*fl* cDNA and its deduced amino acid sequence displays striking homology to a murine cDNA encoding a protein involved in the cell cycle (Radomski and Jost 1995). With 71% similarity between the proteins despite the considerable phylogenetic distance of the source organisms, it is almost certain that we have

<u>Figure 5.8</u> Comparison of the deduced amino acid sequence of the putative <u>wm2.19-*fl*</u> protein and the mouse p38-2G4 and yeast 42K proteins.

The degree of similarity is indicated below the sequence alignment (*: 3 out of 3; +: 2 out of 3). The shaded boxes represent the potential nuclear localisation signal of the proteins. Charged amino acids in a potential amphipatic helical domain are in bold letters. Gaps are shown with dots. See Table 5.1 for references.

```
            1                                                    50
wm2.19-fl   MSSDEEVRE. EKELDLSSNE VVTKYKTAVE IINKALKLVL SECKPKAKIV
p38-2G4     .......... .......... .......... .......... ..........
42K         MSTKEATSET AVDYSLSNPE TVNKYKIAGE VSQNVIKKVV ELCQPGAKIY
            ++  +   +       ++  +  + +++ + +       + +    + + +++

            51                                                  100
wm2.19-fl   DICEKGDNFI TEQTGNVYKN VKRKIERGIA FPTCVSVNNT VCHFSPLATD
p38-2G4     ........MI MEETGKIFKK EK.EMKKGIA FPTSISVNNC VCHFSPLKSD
42K         DICVRGDELL NEAIKKVYR. .TKDAYKGIA FPTAVSPNDM AAHLSPLKSD
            +++  ++  +  * ++++++   +    +*** *** +*+*+  ++*+***++*

            101                                                 150
wm2.19-fl   .DS..VLEEN DMVKIDMACH IDGFIAVVAH THVI...KAG PVTGRAANVL
p38-2G4     QDY..ILKEG DLVKIDLGVH VDGFIANVAH TFVIGVAQGT QVTGRKADVI
42K         PEANLALKSG DVVKILLGAH IDGFASLVAT TTVVS...EE PVTGPAADVI
             +    *+++ * ***+++ * +***++ **+ * *+       +***++*+*+

            151                                                 200
wm2.19-fl   AAANTAAEVA MRLVRPGKKN KDVTEAIQKV AAAYDCKIVE GVLSHQLKQF
p38-2G4     KAAHLCAEAA LRLVKPGNQN TQVTEAWNKV AHSFNCTPIE GMLSHQLKQH
42K         AAASAALKAA QRTIKPGNTN WQVTDIVDKI ATSYGCKPVA GMLSHQQERE
            +**  ++++*  *+++**+ *  +**++  *+ * ++ *++++ *+****+++

            201                                                 250
wm2.19-fl   VIDGNKVVLS VSNADTKVDD ..AEFEENEV YAIDIVTSTG EGKPKLLDEK
p38-2G4     VIDGEKTIIQ NPTDQQKKDH EKAEFEVHEV YAVDVLVSSG EGKAKDAGQR
42K         VIDGKKQVIL NPSDSQRSEM DTFTFEEGEV YGVDILVSTS PSGKVKRSDI
            **** * ++  ++ + ++ +    ++**+ ** *++*+++*++ +++ +

            251                                                 300
wm2.19-fl   QTTIYKRAVD KNYHLKMKAS RFIFSEISQK FPIMPFTARA LE.EKRARLG
p38-2G4     .TTIYKRDPS KQYGLKMKTS RAFFSEVERR FDAMPFTLRA FEDEKKARMG
42K         ATRIYKKT.D TTYMLKLQAS RKVYSEIQTK FGPFPFSTRN ISFDSRTNMG
             *+***+  + + * **+++* *  +**+  + *  +**+ *+  + ++++++*

            301                                                 350
wm2.19-fl   LVECMNHELL QPYPVLHEKQ GDLVAHIKFT VLLMPNGSDK ITSHPLQ.QL
p38-2G4     VVECAKHELL QPFNVLYEKE GEFVAQFKFT VLLMPNGPMR ITSGPFEPDL
42K         LNECTSHKLL FPYEVLLDKD GGIVAEFYST IALTKKGTII LSDSEPKEDF
            ++**  *+** +*+ ** +*  *  ** +++* ++*+++*    +++ +   ++

            351                                                 400
wm2.19-fl   EPSKSIEGDA EIKAWLALGT KSKKKGGGKK KKGKKGDAAE VAEPMEVSKD
p38-2G4     YKSEMEVQDA ELKALLQSSA SRKTQKKKKK KASKTVENAT SGETLEENGA
42K         IKSDKKVEDP EIVALLETPI KVTKNKKKSK KPSKANE... ..........
             +*   +  *+ *++*+*     + ++ ++++* * +*  +  +    +  +

            401
wm2.19-fl   APSQE
p38-2G4     GD...
42K
```

cloned the plant homologue of this murine gene. Radomski and Jost (1995) have checked the protein expression and localisation of p38-2G4 during the cell cycle of cultured HeLa cells. They found that p38-2G4 is mainly localised in the nucleus, binds to DNA and partially to the nuclear membrane. It also fluctuates according to the stage of the cell cycle and is most abundant during G_1/S transition phase when DNA synthesis starts. This variation of expression linked to DNA synthesis could possibly be applied to wm2.19 protein. Since wm2.19 is strongly expressed at premeiotic interphase and its expression tends to decrease as cells go further into meiosis, we could hypothesise that the protein is somehow related to DNA synthesis which occurs before the start of meiosis. The role of wm2.19 during this crucial time is not solely related to meiosis but also to mitosis, hence the expression in root tips (Figure 5.1). *In situ* experiments on wheat root tips have not been conducted yet but we would expect wm2.19 to be specifically expressed in root meristem cells. Moreover, with its putative nuclear localisation signal (Dingwall and Laskey 1991; Garcia-Butos *et al.* 1991) similar to p38-2G4, the product of wm2.19 is probably also localised in the nucleus. Whether or not wm2.19 would bind directly to DNA or indirectly via a protein complex is still unknown. The protein does not diplay any significant similarity to known DNA binding proteins. Motifs such as helix-turn-helix, zinc finger or b-ZIP (Baxevanis and Vinson 1993) are missing. However, the presence of the amphipatic helical domain mentioned in Section 4.5 would indicate a capacity of wm2.19 to interact with DNA or other proteins.

Apart from the similarity found with the mouse p38-2G4 clone, another significant homology (60% similarity) was found with 42K, a clone isolated from *Schizosaccharomyces pombe* (Yamada *et al.* 1994). In their study, Yamada and co-workers demonstrated that 42K was localised in the cell nucleus and tended to bind preferentially to a synthetic curved DNA sequence *in vitro*. The biological significance of naturally occurring curved DNA (Hagerman 1990; Crothers *et al.* 1990) is far from understood. However reports on this subject have suggested a potential function of curved DNA (and indirectly curved DNA-associated proteins) in cellular processes such as replication, recombination and transcription (Hagerman 1990; Travers 1989). The ability

of the wm2.19 protein to bind to curved DNA remains to be tested but the homology to 42K suggests again that wm2.19 might interact with DNA or chromatin.

Chromosomal assignment of wm2.19 on the hexaploid wheat genome has revealed three hybridising fragments located on the long arm of chromosomes arms 1A, 1B and 1D, indicating that the gene is present as a single copy gene in each of the three wheat progenitor genomes (A, B and D). Surprisingly, in diploid barley, more fragments hybridising to wm2.19 were found. Beside chromosome 1H, fragments were also localised on chromosome 3H. A possible explanation could be that wm2.19 loci were duplicated in the course of barley evolution or that wm2.19 probe binds to related genes present in the barley genome but absent in wheat.

The location of wm2.19 on homoeologous group 1 in wheat indicates that wm2.19 does not correspond to either *Ph1* or *Ph2* genes located on chromosomes 5BL and 3DS, respectively. As described in the General Introduction, most of the gene loci known to have a strong effect on chromosome pairing are located on homoeologous groups 3 and 5. However, promoting or suppressing effect of chromosome pairing have also been observed in hybrids such as Chinese Spring X rye, containing extra dosage of other wheat chromosomes. Miller and Reader (1985) found that homoeologous group 1 were among the chromosomes that have an effect on pairing. They found a slight but significant increase in pairing with extra dose of chromosome 1A and 1D but no effect was reported with chromosome 1B.

Chromosome group 1 in wheat is known to contain many important genes valuable for crop improvement such as those controlling end-use quality, rust and pest resistance (Worland et al. 1987; Chao et al. 1989; Vandeyze et al. 1995). However, little is known about genes involved in meiosis or the cell cycle on this chromosome. Miller and Reader (1985) did not document to which arms of group 1 chromosomes, the effect of pairing was due. From the mapping described here, the wm2.19 cDNA was shown to hybridise to three bands located on the long arms of homeologous group 1. It will be interesting to establish which one or more of the three bands contains the active gene encoding the messenger. If all the three genomic bands represent active wm2.19 genes or if the band from chromosome 1BL is

active, it would be unlikely that the increase of chromosome pairing due to chromosome group 1 is related to wm2.19 since Miller and Reader (1985) did not report any significant effect with an extra dose of chromomose 1B in wheat X rye hybrids.

Beside isolating the gene encoding wm2.19, several experiments are needed to establish if the wm2.19 protein behaves in a similar manner to p38-2G4 in the cell cycle. If this is the case and wm2.19 accumulates also at the G1/S boundary phase and remains present during DNA synthesis, it will be interesting to find out how wm2.19 is regulated. Since the wm2.19 protein product contains several putative phosphorylation sites, it could possibly be under the control of protein kinases which are known to play a key role in the regulation of many cell cycle linked proteins (Lewin 1990; Deckert *et al.* 1994; Francis and Halford 1995). Moreover, in plants, phytohormones have been found to control some cell cycle genes (Miao *et al.* 1993; John *et al.* 1990). It would be important to check this aspect in relation to wm2.19 expression.

Once it is demonstrated that wm2.19 behaves similarly during the cell cycle to its murine counterpart, it would be important to transform plants with a construct of wm2.19 cDNA linked to the CaMV 35S gene promoter. This would clarify the role of wm2.19 in the cell cycle and its relationship to plant development. However, it is possible that such construct will be lethal. We would expect this novel plant messenger to play an essential role in the cell cycle, given the alignment between wm2.19 and p38-2G4 and their extraordinary degree of conservation that has been maintain in the course of evolution.

CHAPTER VI

General discussion and conclusion.

In the introduction we have seen the importance of wheat as a crop and how the knowledge of the molecular mechanisms involved in meiosis could facilitate wheat breeding and the introgression of desired alien chromosomal segments. Because meiosis is a complex sequence of molecular events leading to four genetically unique haploid cells, this study was limited to a particular aspect of the meiotic process. The prime interest was the identification of genes related to the very early stages of meiosis when homologous pairing occurs. In wheat, this process is governed mainly by genes (*Ph1*, *Ph2*) located on chromosome groups 3 and 5. The identification of these genes, their corresponding mRNAs or proteins has not been accomplished. In an attempt to shed some light on wheat meiosis and identify genes potentially related to chromosome pairing in wheat, a cDNA library was prepared at the premeiotic interphase and prophase stages of meiosis. Differential screening was used to identify and select clones showing preferential expression in anthers at early meiosis. Among 34 clones selected, two were chosen for further analysis and to investigate a possible role in chromosome pairing.

Several important results were obtained. First, the quality of the cDNA library constructed was good in terms of diversity of the clones present and size of the cDNA inserts. This was important in order to successfully select good clones by the differential screening procedure and also for the use of the library as a base material for other studies on meiosis. Only two clones would be analysed in detail: wm5.12 and wm2.19. These were chosen

because of their different patterns of expression and also because partial sequencing results had shown no similarities with other genes in the databases.

The molecular analysis of wm5.12 has indicated that the messenger is strongly expressed in the tapetal cells and weakly in the carpels during meiosis. The complete sequence of the cDNA has revealed that it was similar to other non-specific plant lipid transfer proteins (LTPs). This group of proteins has been found in several aerial organs of diverse plant species and more specifically in the tapetum of *Nicotiana* sp. and *Brassica* sp. (see Section 4.6). Most plant LTPs are known to be secreted extracellularly. The protein encoded by wm5.12 also possesses an N-terminal signal peptide for extracellular transport. A noteworthy feature of wm5.12 and other cloned LTPs is that even if these proteins seem to have preserved a common structure, significant divergence still exists both in their protein sequences and their specificity of expression in plant organs. It could be possible that several genes were present in angiosperm genomes over a long evolutionary period. With time these genes could have diverged depending when and where they were required in plants, leading to related proteins with different functions and specificity of expression. Only wm5.12 LTP has been mapped thus far to specific chromosome arms (3AS, 3BS and 3DS). It would be important to discover where other LTPs are present in the genomes of wheat, barley, rice and other grasses. In addition, it was found that among the five genomic bands hybridising to wm5.12 probe, two bands were absent in the deletion mutant, *ph2a*. This implies that wm5.12 is closely linked to the *Ph2* gene known to be located on chromosome 3DS.

The findings obtained with the analysis of our second clone, wm2.19, show that it is involved in meiosis and mitosis. The results have demonstrated that the messenger is strongly expressed in anthers and carpels during meiosis and also in meristematic tissues such as root tips, where cells are actively dividing (Chapter V). In anthers, the expression of wm2.19 was localised to both meiocytes and tapetal cells. Furthermore, the deduced amino acid sequence showed strong homology with a yeast and a murine clone known to be involved in the cell cycle (Yamada *et al.* 1994; Radomski and Jost 1995). Wm2.19 is a novel plant gene, homologous to the mouse gene, p38-2G4 (see Section 5.6). The

presence of a nuclear localisation signal in the wm2.19 corresponding protein could signify that wm2.19 plays an important role in the nucleus and possibly binds to DNA. Protein expression studies in plants will help to determine the role of wm2.19 and if the protein has the same role in meiosis and mitosis. The gene encoding wm2.19 cDNA was mapped in wheat to the long arms of chromosome 1A, 1B and 1D. Although, *Ph* genes with strong effects have not been identified on these chromosomes, it has been suggested that in wheat hybrids, chromosomes of group 1 have an influence on chromosome pairing (Miller and Reader 1985). To date no genes involved in the cell cycle have been mapped to chromosome group 1 in wheat or other grasses. Taking into account the high degree of conservation maintained between wm2.19 and the mouse clone p38-2G4 despite the considerable phylogenetic distance between the organisms, it is expected that these proteins play an essential role during meiosis and mitosis.

As mentioned in the discussion of Chapter III, the most laborious part of the project was to obtain wheat anthers at the required stages of meiosis. To obtain sufficient RNA to construct the cDNA library and prepare the cDNA probes, hundreds of florets were assessed in order to establish the stage of anther development. In the course of the differential screening of the library, we also noticed that most of the selected clones corresponded to strongly expressed messengers. This could mean they were mostly from the tapetum since it is known to be the dominant tissue during early meiosis in terms of transcripts produced, concentration of individual mRNAs species or both (Scott *et al.* 1991). This situation would explain why both clones selected were either expressed specifically (wm5.12) or partly (wm2.19) in the tapetal cells. The difficulty with the differential screening of a cDNA library, is that weakly expressed clones do not give a clear signal with either of the cDNA probes made from meiotic anthers RNA or leaves, roots and pollen RNA. Using an alternative method such as mRNA differential display (Liang and Pardee 1992) could have helped to increase the chances of obtaining more meiocyte specific messengers belonging to the low expression class of transcripts.

The difficulties encountered in trying to isolate wheat clones specific to meiosis or related to the diploid-like behavior of the plant during chromosome pairing bring up the question of

what other methods could be used to isolate genes such as *Ph1* and *Ph2*. Gill and Gill (1991) have identified a DNA fragment mapping within the submicroscopic deletion of the *ph1b* mutant. This fragment could be used as a starting point to perform "chromosome walking" towards the deleted region. However, to facilitate this "walk" more markers need to be produced to limit as much as possible the region containing the *Ph1* gene. Similarly, Clarke and co-workers (1992) have attempted by modifying the "Phenol Emulsion Reassociation Technique" (Kohn *et al.* 1977) to isolate low copy-number sequences present in euploid wheat Chinese Spring but absent in the *ph1b* mutant. They obtained a clone closely linked to the gene controlling chromosome pairing. This marker, in addition to the marker found by Gill and Gill (1991) could be useful for "walking" towards the *Ph1* gene. The chromosome walking method has been successful with the identification of defective genes in human such as the Duchenne muscular dystrophy (Kunkel *et al.* 1985, 1986). Until more markers become available to limit the region containing the *Ph1* gene, it will be difficult to isolate the gene by this method since the wheat genome is even larger than the human genome and 83% is comprised of repetitive sequences. A possible way to produce RFLP markers specific for a particular chromosome would be to construct a DNA library from a single chromosome (Chen and Armstrong 1995; Albani *et al.* 1993). This would allow the production of a high density map. Moreover, the use of the amplified restriction fragment polymorphism (AFLP) technique (Vos *et al.* 1995; Zabeau and Vos 1993; Thomas *et al.* 1995) would further increase the number of specific markers around the *Ph1* gene area. Another alternative could be the use of the rice genome for chromosome walking. It has been demonstrated that, despite large differences in DNA content and basic chromosomes number, the genome of wheat and rice are highly collinear (Kurata *et al.* 1994; Devos *et al.* 1995). This conservation of genome synteny could have a direct application by using the smaller genome of rice (about 40 times smaller than the wheat genome) as a model species for map-based cloning of genes from wheat.

Other possible research avenues could also be the use of yeast genes known to be involved in homologous pairing as probes to screen the cDNA library described here. Important genes such as *DMC1* (Bishop *et al.* 1992) are likely to have been conserved in the course of evolution and could have a plant homologue in Chinese Spring. By identifying as many

genes directly or indirectly involved in the chromosome pairing process and by using mutants such as *ph1b*, we could increase our chances of encountering proteins encoded by *Ph1*, *Ph2* or related genes.

This project has shown that the elucidation of the molecular mechanisms controlling chromosome pairing in wheat is not a simple task. As a next step, it would be important to investigate the role of the two clones described here, wm5.12 and wm2.19, in the meiotic anthers. Antibody preparations, protein expression studies and plant transformation would be excellent experiments to perform in order to discover how the corresponding proteins function and how they affect the meiotic process. Moreover, other clones selected during the screening process such as wm9.10 or wm1.18 (see Chapter II) would be worth further characterisation to determine if they are novel genes related specifically to meiosis. To isolate more candidates related to meiosis in wheat, it would also be worth trying mRNA differential display and a comparison of the amplified cDNAs between Chinese Spring anthers and mutants anthers from *ph1b* or from nullisomic-5B plants. This could give an indication of the number of meiotic genes whose expression is affected in these mutant plants. The cloning of the gene(s) in wheat controlling the chromosome pairing process is a long quest worth pursuing as it would not only have a crucial impact in wheat breeding and the genetic improvement of this crop but it would also provide an important insight into the pairing process essential to the diversity of all higher eukaryotes.

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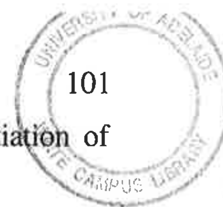

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