Mechanistic studies on *Ph1*

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PhD Thesis

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LIST OF FIGURES

Abbreviations

ANOVA	Analysis of variance
APC	Anaphase promoting complex
ARS	Autonomously replicating sequence
ASY1	Asynapsis 1
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BP	Base pair
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
BUB	Budding inhibited by benzimidazole
CAK	CDK-activating kinase
Cdc	Cell division cycle
CDK	Cyclin dependent kinase
CKI	CDK inhibitor protein
CO	Crossover
CS	Chinese spring
DAPI	4',6-Diamino-2-Phenyl-Indole
DDK	Dbf4p-dependent kinase
DSB	Double strand break
dsRNA	Double stranded RNA
EdU	5'-ethynyl-2'-deoxyuridine
EMS	Ethylmethane sulphonate
EtOH	Ethanol
EU	5-ethynyl uridine
FEN1	Flap endonuclease 1
FISH	Fluorescent in situ hybridisation
G-phase	Gap phase
GSK	Glycogen synthase kinase
HAT	Histone acetyl transferase
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HP1	Heterochromatin protein 1
H_2SO_4	Sulphuric acid
LB	Lysogeny broth
LE	Lateral element
LiCl	Lithium chloride
M-phase	Meiotic or mitotic phase
MAD2	Mitotic arrest deficient
MAP-kinase	Mitogen activated protein kinase
MCM	Minichromosome maintenance
MPF	Maturation promoting factor
mRNA	Messenger RNA
MTOC	Microtubule organising centre
NaOH	Sodium hydroxide
NCO	Non-crossover

Abbreviations

NOR	Nuclear organising region
OA	Okadaic acid
ORC	Origin recognition complex
PAM	Plural abnormalities of meiosis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Ph1	Pairing homoeologous 1
PIC	Preinitiation complex
PIPES	2-[4-(2-Sulfoethyl)Piperazin-1-yl]ethanesulfonic Acid
PMC	Pollen mother cell
PPP	Phosphoprotein phosphatase
Pre-RC	Pre-replicative complex
QC	Quality control
RB	Retinoblastoma
RFC	Replication factor C
RISC	RNA-induced silencing complex
RNAi	RNA interference
RPA	Replication protein A
S-phase	Synthesis phase
SC	Synaptonemal complex
SCF complex	Skp, Cullin, F-box containing complex
SIC	Stochiometric inhibitor of CDKs
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SSB	Single strand binding
SSC	Saline sodium citrate
TBS	Tris buffered saline
VIP	Vacuum infiltrator processor
3AT	3 Amino-1,2,4-Triazole
5FOA	5-fluoroorotic acid

Abstract

Bread wheat is the staple diet of over two-thirds of the world's population. The global population is predicted to rise to over 9 billion by 2050 and therefore it is imperative that yields are increased to keep pace with demand. One way of doing this is to introduce beneficial traits from wild relative species, a process that requires the manipulation of chromosome pairing and recombination during meiosis. Studies into wheat meiosis have revealed several chromosome pairing loci which restrict chromosome pairing and recombination during meiosis to true homologues, thus making the introduction of novel traits from wild relatives impossible. The *Ph1* locus is the most extensively studied of these loci. In the presence of *Ph1* pairing (and recombination) during meiosis is restricted to true homologues only, however in its absence, whilst pairing between non-homologues can occur, the resulting genetic instability leads to infertility.

Since the discovery of the *Ph1* locus, studies have been directed at understanding the mechanism by which it functions in the hopes of gaining a good enough understanding to be able to manipulate chromosome pairing during meiosis without affecting genomic stability. The recent discovery that the locus is composed of a series of *Cdk-like* genes with closest homology to mammalian *Cdk2* has been a major development allowing research to progress to an exciting point. This thesis summarises our current understanding of the *Ph1* locus and describes experiments that I have undertaken which have focused on whether the wheat *Cdk-like* genes function in a similar way to *Cdk2*. In the absence of *Ph1*, the activity of these CDK-like proteins increases leading to an increase in histone H1 phosphorylation, which changes chromatin remodelling. *Ph1* also affects pre-meiotic DNA replication, consistent with a CDK2-like function. The implications of this work and the direction of future studies will be discussed.

Chapter 1

Introduction

Chapter organisation

The intention of this chapter is to provide the reader with background information regarding the cell cycle and the wheat *Ph1* locus, in order to enable a clear understanding of the research presented in the subsequent chapters. The process of DNA replication (S-phase) will first be described, followed by an introduction to the more general and basic concepts regarding the cell cycle. First a summary of the mitotic cell cycle and its controls will be given, as this is the most common form of cell division. A summary of meiotic cell division and its regulation will follow, this being the more significant of the two in relation to this project. In this introductory chapter the main focus will be on the DNA replication and M-phases of the cell cycle.

Having provided an introduction to the cell cycle, the process of homologous chromosome pairing will be discussed. The key biological aim of this study is to understand how chromosomes are correctly paired in a polyploid system. Therefore a general overview of polyploidy will first be given, before concentrating on hexaploid wheat, which is the organism upon which this study is focused. Finally a summary of our current understanding of the *Ph1* locus, the dominant genetic locus controlling homologous chromosome pairing in wheat, will be given.

1.1 The Cell Cycle

The cell cycle is the mechanism by which a cell reproduces via a sequence of events during which it duplicates its contents and then divides in two. Although the details of the cell cycle can vary between organisms and at different time points within an organism's life, the fundamental method by which cells pass on their genetic information to the next generation is universal. The DNA must be faithfully replicated, and then must be accurately segregated between the two resulting daughter cells so that each receives a copy of the entire genome. The process can be broken down into four basic stages (see figure 1).

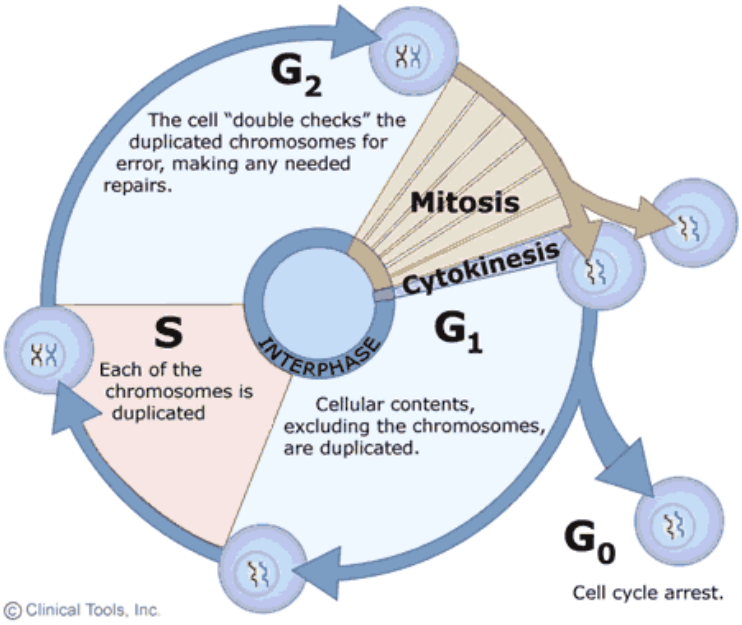


Figure 1:- the phases of the cell cycle. Picture adapted from http://www.le.ac.uk/ge/genie/vgec/he/cellcycle.html

The two key phases are S phase (S for synthesis), during which the DNA is replicated, and M phase (M for mitosis or meiosis), during which chromosome segregation and cell division occur. There are also two gap (G) phases included in most cell cycles, during which growth and synthesis of the proteins and organelles required for DNA replication and

division are completed. G_1 phase is the interval between the completion of the last mitotic division and the onset of the next S phase. G_2 phase is the interval between S phase and M phase. These gap phases also allow the cell to monitor the internal and external environments to ensure that the conditions are favourable for synthesis and division. If the conditions are found to be unfavourable during the G_1 phase the cell can enter a resting state known as G_0 in which it will remain until the conditions improve. Once the cell has completed G_1 it is then committed to the synthesis phase, regardless of extracellular signals. The stages of G_1, S phase and G_2 are known as interphase and generally take up the majority of the time in the cell cycle.

1.2 S-phase

Prior to cell division all organisms must accurately replicate their DNA so that it can be passed on to the daughter cells. As well as ensuring the faithful replication of the genome, strict controls need to be in place to ensure that only one round of replication occurs per cell cycle. The process of DNA replication is best understood in *S.cerevisiae* which serves as a good eukaryotic model for this highly conserved process. Current understanding of the process of replication in eukaryotes shows it to be a highly ordered series of steps, leading to one round of extremely accurate replication in each cell cycle.

1.2.1 Formation of the Pre-Replicative Complex

The first step leading to the process of DNA replication is the formation of the pre-replicative complex (pre-RC) which takes place at the origin of replication during G_1. Although many of the fundamental aspects of DNA replication are conserved between bacteria and eukaryotes, one difference is that bacterial chromosomes only have one origin of replication, whereas eukaryotic chromosomes possess multiple sites. Amongst eukaryotes the sequences defining an origin of replication can vary greatly. In *S.cerevisiae*

three to four sequences of 10 – 15bp spread out over 100 – 150bp are sufficient to act as an origin (Bell and Dutta 2002). These sequences contain a highly conserved sequence known as the autonomously replicating sequence (ARS) as well as less conserved elements termed B elements which are thought to contribute to DNA unwinding at the origin (Bell 1995). In contrast the origins of replication found in *S.pombe* spread over approximately 800-100bp and contain several AT-rich regions (Dubey, Kim et al. 1996). The origin recognition complex (ORC) is a six subunit complex that binds the DNA at the origin of replication. Studies on *S.cerevisiae* and *D.melanogaster* indicate that ATP binding by ORC1 is required for DNA binding (Bell and Stillman 1992), (Austin, Orr-Weaver et al. 1999), (Chesnokov, Remus et al. 2001), (Klemm, Austin et al. 1997). All six ORC proteins have been identified in *Arabidopsis* (Collinge, Spillane et al. 2004), (Gavin, Hidaka et al. 1995), (Masuda, Ramos et al. 2004), whilst genes encoding ORC 1 – 5 have been found in rice and maize (Kimura, Ishibashi et al. 2000), (Li, Yang et al. 2005), (Mori, Yamamoto et al. 2005), (Witmer, Alvarez-Venegas et al. 2003). Interestingly studies on maize and *Arabidopsis* have shown high levels of ORC subunit proteins in post-mitotic cells suggesting that ORC subunits may possess an additional function in plants (Diaz-Trivino, del Mar Castellano et al. 2005), (Witmer, Alvarez-Venegas et al. 2003).

The binding of the proteins making up the ORC is regulated throughout the cell cycle in some species but not all. The ORC of *S.cerevisiae* and *S.pombe* associate constitutively with origin sequences throughout the cell cycle (Ogawa, Takahashi et al. 1999), (Aparicio, Weinstein et al. 1997), (Santocanale and Diffley 1997), (Tanaka, Knapp et al. 1997) and the *Drosophila* ORC has been found to be associated with the chromosomes throughout all of the stages of the cell cycle (Bell and Dutta 2002), (Pak, Pflumm et al. 1997). However, studies carried out on *Xenopus* found that the ORC is removed from the chromosomes during metaphase (Romanowski, Madine et al. 1996), (Carpenter, Mueller et al. 1996). Similarly in mammalian systems it appears that the ORC is removed from the

chromatin during M-phase (Natale, Li et al. 2000), (Kreitz, Ritzi et al. 2001), (Tatsumi, Tsurimoto et al. 2000). There have been several suggestions as to why the ORC is removed in these species. It could be a mechanism used to ensure that there is no pre-RC formation prior to the end of M-phase, or it could be that the process of chromosome condensation either removes or requires the removal of the ORC (Bell and Dutta 2002).

The next protein association required for formation of the pre-RC is CDC6, a member of the large family of AAA+ ATPases. Whilst most eukaryotes possess one copy of the *CDC6* gene, studies on *Arabidopsis* have shown that it has two *CDC6* genes (Ramos, de Almeida Engler et al. 2001), however this is probably not common to all plants as only one gene has been identified in rice (Shultz, Tatineni et al. 2007). CDC6 requires the ORC to successfully bind to the chromatin, and its presence is also required for the binding of other proteins involved in the formation of the pre-RC (Coleman, Carpenter et al. 1996). The abundance of CDC6 is tightly regulated throughout the cell cycle. In yeast cells CDK phosphorylation of CDC6 targets it for ubiquitination by a multi protein E3 ubiquitin ligase complex, termed the SCF complex, which leads to rapid proteolytic degradation of any CDC6 that is not bound to an origin (Drury, Perkins et al. 1997), (Elsasser, Chi et al. 1999), (Perkins, Drury et al. 2001), (Piatti, Böhm et al. 1996). Studies have found that mutations in the yeast CDC6 consensus CDK phosphorylation sites inhibit its degradation (Jallepalli, Tien et al. 1998), (Calzada, Sánchez et al. 2000), (Drury, Perkins et al. 2000). In plants CDC6 is also regulated by the ubiquitin/proteasome pathway (Castellano, del Pozo et al. 2001), whilst in mammalian cells the phosphorylation of CDC6 by CDKs during S-phase targets it for export from the nucleus (Saha, Chen et al. 1998), (Fujita, Yamada et al. 1999), (Jiang, Wells et al. 1999), (Petersen, Wagener et al. 2000). During M-phase ubiquitinylation by the anaphase promoting complex (APC) leads to the degradation of CDC6 (Petersen, Wagener et al. 2000), (Mendez and Stillman 2000).

CDT1 has been identified as another key protein required for pre-RC assembly. When mutated versions of the gene were examined in *S.pombe* DNA replication was found to be blocked, and there appeared to be defects in the S-phase checkpoint (Hofmann and Beach 1994). It has now been shown in *S.pombe* that the association of CDC6 and CDT1 is required to promote the association of the final components of the pre-RC with chromatin; the minichromosome maintenance (MCM) proteins (Nishitani, Lygerou et al. 2000). There are six eukaryotic MCM proteins all of which are important. Each of the six MCM subunits have been identified in *Arabidopsis* (Springer, McCombie et al. 1995), (Stevens, Mariconti et al. 2002), (Masuda, Ramos et al. 2004), (Dresselhaus, Srilunchang et al. 2006), whilst MCM3 and MCM6 have been found in maize (Sabelli, Burgess et al. 1996), (Sabelli, Parker et al. 1999), (Dresselhaus, Srilunchang et al. 2006), MCM3 has been reported in tobacco (Dambrauskas, Aves et al. 2003), and candidates for MCM 2-7 have been reported in rice (Shultz, Tatineni et al. 2007). If any of the MCM genes are deleted in *S.cerevisiae* or *S.pombe* it results in lethality (Dutta and Bell 1997), (Kelly and Brown 2000). In order for the MCM proteins to bind to the chromatin successfully all of the other components of the pre-RC need to be in place, however, once the MCM proteins have been loaded the other components of the pre-RC can be removed without stopping replication. This suggests that the primary function of the pre-RC is to load the MCM proteins (Rowles, Tada et al. 1999), (Hua and Newport 1998). It is thought that the MCM proteins play a role in both replication initiation and elongation, and that the MCM complex acts as a helicase to separate the two strands of the DNA helix so that they can both serve as templates for daughter strand synthesis. Studies carried out on the archaebacteria *Methanobacterium thermoautotrophicum* found that the purified MCM protein formed a large multimeric complex which forms a double hexamer in a similar manner to other DNA helicases. This complex was also found to have strong 3'->5' DNA helicase activity, adding more evidence to suggest that the MCM proteins act as helicases during the process of DNA replication

(Chong, Hayashi et al. 2000), (Kelman, Lee et al. 1999), (Shechter, Ying et al. 2000). In *S.cerevisiae* it has been found that inactivation of any of the MCM proteins halts the progression of the replication fork, whereas in mammals only some of the MCM proteins are required for helicase activity. Electron microscopy carried out on the MCM protein complex found in *S.pombe* showed the complex to have a doughnut like structure with a central cavity (Adachi, Usukura et al. 1997), and later studies showed that the human complex has a similar structure (Sato, Gotow et al. 2000). It is thought that the MCM proteins form a ring around the ssDNA found at the replication fork and that they can oscillate between an open and closed state in order to allow ssDNA loading (Bell and Dutta 2002). Similarly to several of the other components of the pre-RC, the MCM complex has been shown to have robust ATPase activity (Schwacha and Bell 2001). MCM proteins are only found in *S.cerevisiae* nuclei during G_1 and S phase and are exported to the cytoplasm during the other stages of the cell cycle. Only intact complexes are allowed to enter into the nucleus and during S, G2 and M-phases any MCM proteins that are not bound to the chromatin are actively exported from the nucleus (Labib, Diffley et al. 1999), (Nguyen, Co et al. 2000), (Pasion and Forsburg 1999), however, S-phase must be completed before chromatin bound MCM proteins are exported. The nuclear export of MCM proteins seems to be promoted by CDK activity.

Plants seem to be unique amongst eukaryotes in that they have evolved multiple copies of some core DNA replication genes, some of which may have specialized functions (Shultz, Tatineni et al. 2007). Within the pre-RC *ORC1*, *CDC6* and *CDT1* are duplicated in *Arabidopsis,* and may possess distinct functions with respect to mitotic and endocycling cells (Masuda, Ramos et al. 2004), (Diaz-Trivino, del Mar Castellano et al. 2005). Any specialized functions that may have evolved in *Arabidopsis* cannot be essential to plant development, because single copies of *ORC1* and *CDC6* are found in rice (Shultz, Tatineni et al. 2007).

1.2.2 Regulation of pre-RC formation

Tight regulation of the formation of the pre-RC is required to ensure that replication is initiated at the origins only once per cell cycle. Cyclin dependent kinases (CDKs) are of particular importance in this process and will be described in detail later in this chapter. CDK activity plays an important role in the regulation of pre-RC formation in two ways. First, the elevated activity of CDKs is required to activate the origins of replication as the cell enters S-phase. Second, these same elevated levels of activity are required to ensure that the origins do not fire again during S, G_2 and M phases of the cell cycle (Bell and Dutta 2002). The importance of CDKs in the prevention of re-replication was first suggested by studies on *S.cerevisiae* and *S.pombe*, where inactivation of CDK activity in G_2/M cells resulted in re-replication of the genome (Piatti, Böhm et al. 1996), (Dahmann, Diffley et al. 1995), (Nishitani and Nurse 1995). It is thought that these elevated levels of CDK activity during G1 lead to the prevention of further pre-RC formation (Piatti, Böhm et al. 1996), (Dahmann, Diffley et al. 1995), (Detweiler and Li 1998), (Hua, Yan et al. 1997). Further studies have shown that at least three of the components of the pre-RC are phosphorylated by CDKs to prevent re-replication and pre-RC assembly (Bell and Dutta 2002).

The phosphorylation of CDC6 by CDKs is well established (Dutta and Bell 1997). As previously mentioned, this leads to either the degradation (yeast/plants) or the nuclear export (mammals) of CDC6. CDKs are also thought to play a role in MCM phosphorylations, and have been shown to phosphorylate MCM2 and MCM4 both *in vivo* and *in vitro* (Findeisen, El-Denary et al. 1999), (Fujita, Yamada et al. 1998), (Hendrickson, Madine et al. 1996), (Ishimi and Komamura-Kohno 2001), (Pereverzeva, Whitmire et al. 2000). The consequences of the CDK modifications of the MCM complex are still unclear. It has been suggested that it leads to the inhibition of MCM complex activity. In mice CDK

phosphorylation of the MCM complex leads to the inhibition of its helicase activity, and furthermore MCM complexes purified from G1 and G1/S phase cells have helicase activity, but this is lost in M-phase purifications (Ishimi and Komamura-Kohno 2001). Other studies have suggested that the nuclear localization of the MCM proteins could be controlled by CDK phosphorylation. MCM proteins are localized to the nucleus in G_1 and S phases but exported during G_2 and M phases in *S.cerevisiae* (Labib, Diffley et al. 1999), (Nguyen, Co et al. 2000), (Hennessy, Clark et al. 1990). Inactivation of CDK activity leads to the failure of MCM protein export. A third suggestion is that phosphorylation of the MCM proteins leads to their release from the chromatin and the prevention of their reassociation. In *Xenopus*, hyperphosphorylation of the MCM proteins leads to their release from the chromatin (Findeisen, El-Denary et al. 1999), (Hendrickson, Madine et al. 1996), (Pereverzeva, Whitmire et al. 2000), (Coue, Kearsey et al. 1996), and it has also been shown that hyperphosphorylated MCM proteins cannot be assembled onto the chromatin (Pereverzeva, Whitmire et al. 2000).

The ORC is also a substrate for CDK modification, and studies on *S.cerevisiae* have shown that ORC2 and ORC6 are phosphorylated by CDKs *in vivo* (Nguyen, Co et al. 2001), (Vas, Mok et al. 2001). Mutations in the CDK consensus sites of the ORC have been shown to promote re-replication of the genome, possibly because phosphorylation promotes the release of the ORC and CDC6 from the chromatin (Rowles, Tada et al. 1999), (Hua and Newport 1998).

Although CDKs play an important role in the regulation of pre-RC formation they are not the only mechanism by which its formation is controlled. Geminin was first discovered in metazoans and was found to be an inhibitor of pre-RC assembly. Throughout S-phase high levels of geminin are accumulated until late M phase when it is degraded by the APC (McGarry and Kirschner 1998). The mechanism by which geminin acts was found

to be through preventing the loading of the MCM proteins onto chromatin by inhibiting CDT1 function (Wohlschlegel, Dwyer et al. 2000), (McGarry and Kirschner 1998), (Tada, Li et al. 2001). Geminin has not been found in plants, and as yet the mechanisms that lead to CDT1 inhibition in plants are unknown (Shultz, Tatineni et al. 2007).

The final method by which the formation of the pre-RC is regulated is by the chromatin itself. It is thought that the conformation of local chromosome domains has a profound effect on both the activity and timing of origins (Bell and Dutta 2002). For a long time it has been known that heterochromatic regions of DNA are associated with late origins (Diller and Raghuraman 1994) and changes made to the chromatin structure in *Xenopus* and *Drosophila* embryos have been shown to change origin usage (Blumenthal, Kriegstein et al. 1974), (Hyrien, Maric et al. 1995). The influence of chromatin remodelling factors on DNA replication has also been widely reported (Burke, Cook et al. 2001), (Iizuka and Stillman 1999), (Alexiadis, Varga-Weisz et al. 1998), (Wittmeyer, Joss et al. 1999), (Lee, Pascuzzi et al. 2010).

1.2.3 The transition to replication

The formation of the pre-RC marks the sites at which DNA replication could potentially be initiated, however several other proteins are required before DNA synthesis commences. One such protein is MCM10 which has been shown to be required for successful initiation of DNA replication (Merchant, Kawasaki et al. 1997). Mutations to the gene encoding MCM10 result in a pause in replication fork progression, suggesting a role in DNA elongation (Piatti, Böhm et al. 1996), (Kawasaki, Hiraga et al. 2000). MCM10 may also play a part in the initiation of replication as ChIP experiments have shown that it localizes to the origin in *S.cerevisiae* and it has been shown to exhibit weak genetic and biochemical interaction with the ORC (Homesley, Lei et al. 2000), (Kawasaki, Hiraga et al. 2000).

Putative MCM10 homologues have been found in *Arabidopsis*, maize, rice and *Aquilegia formosa* (Shultz, Tatineni et al. 2007).

CDC45 is another protein that is known to be required for the transition to replication. It is essential for the loading of DNA polymerases onto the chromatin. Studies on *Xenopus* have shown that it interacts with DNA polymerase α, and is essential for its loading onto the chromatin (Mimura and Takisawa 1998). Likewise in *S.cerevisiae* CDC45 is necessary for the assembly of DNA polymerases α and ε at the origins (Zou and Stillman 2000), (Aparicio, Stout et al. 1999). Similar interactions have been found in human systems (Kukimoto, Igaki et al. 1999), and in *Arabidopsis*, although interestingly *Arabidopsis* CDC45 also seems to play a role during meiosis, a role not yet discovered in other eukaryotes (Stevens, Grelon et al. 2004). Having assembled at the origin CDC45 is incorporated into the replication fork and appears to colocalize with the polymerases as they replicate the DNA (Zou and Stillman 2000), (Aparicio, Stout et al. 1999). Like many of the proteins required for the process of replication, the loading of CDC45 requires CDK activity (Zou and Stillman 1998), (Walter and Newport 2000). Also essential for the initiation of replication are the four proteins of the GINS complex (PSF1-3 and SLD5) (Shultz, Tatineni et al. 2007), (Bryant and Aves 2011), which are thought to associate with CDC45 and the MCM proteins to function as a helicase (Moyer, Lewis et al. 2006), (Pacek, Tutter et al. 2006).

The primary goal of the steps leading up to the initiation of replication is the loading of DNA polymerases at the origin. As previously mentioned DNA helicases are loaded onto the DNA where they act to separate the two DNA strands by breaking the hydrogen bonds between the bases of the two strands. The process of unwinding is aided by single strand DNA binding (SSB) proteins which straighten the DNA and stop the formation of hairpins. DNA topoisomerases also aid the process by preventing the 'tangling' or 'over-winding' of the double stranded DNA as it is separated. DNA primase

binds to the helicase and synthesises RNA primers which serve as a starting point for DNA synthesis. The association of the helicase and primase is termed the primosome. Synthesis of the daughter strands is carried out by a multienzyme complex which contains DNA polymerase ε, DNA polymerase α (associated with the primase complex) and DNA polymerase δ. DNA polymerase ε forms a complex with DPB11 which seems to be involved in its recruitment to the origin. DPB11 is required for S phase in both *S.cerevisiae* and *S.pombe* and also seems to play an important role in replication checkpoints (Araki, Leem et al. 1995), (McFarlane, Carr et al. 1997), (Wang and Elledge 1999). SLD2 has also been found to interact with DPB11 in *S.cerevisiae* and is required for the initiation of replication (Wang and Elledge 1999). Likewise SLD3 is also required for the initiation of replication due to its association with CDC45 (Kamimura, Tak et al. 2001). DNA polymerases have a tendency to quickly disassociate from the DNA molecule, making the synthesis of long strands of DNA difficult. This problem is solved by the presence of a DNA clamp or sliding clamp which is composed of either a dimer of DNA polymerase III (bacteria) or trimer of proliferating cell nuclear antigen (PCNA) (eukaryotes) which completely encircles the DNA helix and binds to the DNA polymerase, keeping the moving structure associated with the DNA. The loading of the clamp onto the DNA is an ATP dependent mechanism mediated by a protein complex termed the replication factor C (RFC) clamp loader. A detailed summary of the loading of DNA polymerases is shown in figure 2.

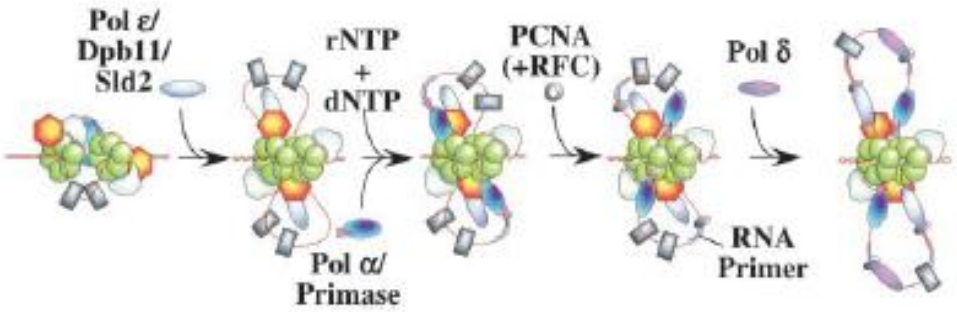


Figure 2: The loading of DNA polymerases during the transition to the replicative stage of the cell cycle. DNA polymerase ε and DNA polymerase α/primase are loaded onto the pre-RC sequentially. Their loading is dependent on CDC45/SLD3. The timing of the association of DNA polymerase δ is still unknown. The synthesis of primers by the primase complex allows the assembly of the DNA clamp (PCNA) by the RFC clamp loader. This allows the binding of DNA polymerases ε and δ to the primer-template junction where they continue the process of DNA synthesis.

Figure and figure legend adapted from (Bell and Dutta 2002).

DNA polymerase works in a 5'->3' direction meaning that due to the antiparallel orientation of the DNA helix, the two daughter strands cannot both be continually synthesised. The 'leading strand' of daughter DNA is synthesised continuously in a 5'->3' direction. The 'lagging strand' is formed from a series of short nucleotide fragments termed Okazaki fragments which are synthesised in a 5'->3' direction and then joined together to create the long DNA strand (Okazaki, Okazaki et al. 1968; Ogawa and Okazaki 1980). The structure of a DNA replication fork showing the leading and lagging strand synthesis is shown in figure 3.

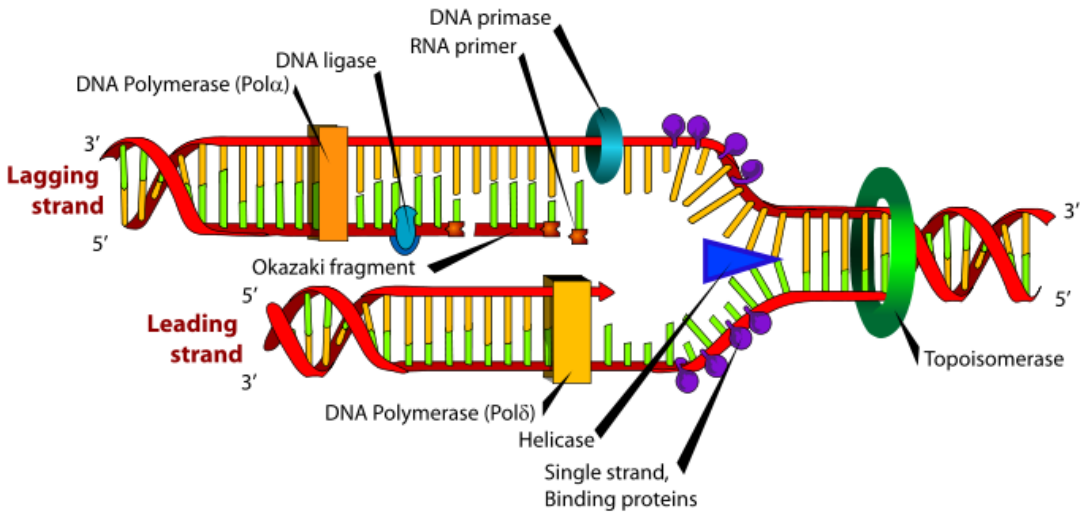


Figure 3: the structure of a DNA replication fork. DNA polymerase synthesises daughter strands of DNA, started from an RNA primer. Due to the 5'->3' action of the polymerase the leading strand is synthesised continuously, whereas the lagging strand is synthesised as a series Okazaki fragments which are later joined together. Figure adapted from http://www.replicationfork.com.

DNA primase creates short RNA primers from which the DNA synthesis is initiated because DNA polymerases can only add nucleotides onto an existing strand. For the leading strand only one primer is needed at the beginning of synthesis as the process is continuous. On the lagging strand a new primer is required at the start of every Okazaki fragment. These primers are then removed by DNA polymerase I (prokaryotes) or DNA polymerase δ (eukaryotes) and the fragments joined by DNA ligase.

DNA synthesis is terminated when the polymerase reaches another replication fork. In eukaryotes with multiple origins of replication this occurs frequently along the length of the chromosome. In bacterial circular chromosomes, replication is terminated when the two replication forks meet. The discontinuous method of DNA replication encounters a problem when it reaches the end of a linear chromosome, because the production of the RNA primer necessary for the synthesis of the last Okazaki fragment is impossible (Olovnikov 1973). Bacteria don't have this problem because of their circular chromosomes. The ends of eukaryotic chromosomes consist of many tandem repeats of

short sequences (e.g. TTAGGG in mammals, TTTAGGG in plants), termed the telomeres. These regions attract an enzyme called telomerase, a reverse transcriptase which carries its own template RNA molecule. The telomerase elongates the telomeric region in a 5'->3' direction (Greider and Blackburn 1985).

Throughout S-phase there is a huge increase in the amount of histone synthesis due to both an increase in the levels of transcription and a decrease in mRNA degradation (Alberts, Johnson et al. 2002). Chromatin assembly factors package the histones and newly synthesised DNA into nucleosomes.

There are several mechanisms in place to ensure that DNA replication occurs accurately. The DNA polymerase itself acts as a 'proofreading' enzyme and ensures the removal of incorrectly paired bases whilst the process of replication is ongoing. The transition to replication is also carefully controlled by the action of at least two kinases.

1.2.4 Control of the transition to replication

CDKs play another important role in controlling the assembly of the DNA synthetic complex, along with another kinase, the DBF4-dependent kinase (DDK). DDK was originally identified as two subunits, CDC7 and DBF4, during screens for cell cycle arrest mutants (McFarlane, Carr et al. 1997). The activity of DDK is dependent on the cyclical appearance of the DBF4 subunit, in a similar manner to the CDKs and their cyclin subunits. Levels of DBF4 reach a maximum during S phase and are controlled by both gene expression and proteolysis (Bell and Dutta 2002). Upon entry into S phase DDK is loaded onto the chromatin (Jares and Blow 2000), (Walter 2000), (Weinreich and Stillman 1999), where it is thought that it phosphorylates the MCM subunits in order to initiate replication (Weinreich and Stillman 1999), (Lei, Kawasaki et al. 1997), (Kihara, Nakai et al. 2000), (Oshiro, Owens et al. 1999). DDK has also been shown to phosphorylate the catalytic subunit of DNA

polymerase α / primase and CDC45 (Weinreich and Stillman 1999), (Nougarede, Della Seta et al. 2000), and it is thought to be involved in recruiting CDC45 to the origins of replication at the initiation of replication.

CDKs are also thought to be involved in origin activation and associate with several components of the pre-RC. As previously mentioned CDKs play a key role in the regulation of CDC6, and it is also thought that CDKs interact with the subunits of the ORC (Vas, Mok et al. 2001), (Romanowski, Marr et al. 2000). Although CDKs are absolutely necessary for DNA replication, the details of how they are required to promote replication are still largely unknown. CDKs target ORC, CDC6 and MCM proteins, but studies on *S.cerevisiae* using mutant forms of these proteins that cannot be modified by CDKs showed that they continue to function during the initiation process, suggesting that if phosphorylation of these proteins is necessary for the activation of replication then it is probably not CDK mediated (Elsasser, Chi et al. 1999), (Drury, Perkins et al. 2000), (Jallepalli, Tien et al. 1998), (Lopez-Girona, Mondesert et al. 1998), (Nguyen, Co et al. 2001), (Vas, Mok et al. 2001), (Pelizon, Madine et al. 2000). Recent work has shown that both SLD2 and SLD3 are essential targets of CDK phosphorylation, which promotes their association with DPB11 (Masumoto, Muramatsu et al. 2002), (Tak, Tanaka et al. 2006), (Tanaka, Umemori et al. 2007), (Zegerman and Diffley 2007). Further work on *S.cerevisiae* has shown that phosphorylation of SLD2 by CDKs strengthens the ability of the components of a 'pre-loading complex' to interact. This pre-loading complex includes DNA polymerase ε, DBP11 and the GINS complex (Kubota, Takase et al. 2003), (Labib 2010), (Takayama, Kamimura et al. 2003). The pre-loading complex is formed as the cells enter S-phase (Muramatsu, Hirai et al. 2010). Further work needs to be undertaken to establish whether CDKs contribute to chromosome replication in other ways. There may well be other important targets for CDK phosphorylation, for example the N-teminal tail of MCM4 can be phosphorylated by CDKs (Devault, Gueydon et al. 2008), (Labib 2010), (Sheu and Stillman 2010).

Hormones also play an important role in the transition to replication in plants. Exposure of *Sinapis alba* to a floral stimulus causes the transition from vegetative to floral growth, a change that is accompanied by the utilization of twice as many origins than used in the vegetative state (Jacqmard and Houssa 1988). Likewise, the application of cytokinin to the shoot apex in *S.alba*, *Lolium temulentum* and to the ovule of the tomato, *Solanum lycopersicum*, causes the utilization of extra origins (Houssa, Jacqmard et al. 1990), (Houssa, Bernier et al. 1994). Conversely, application of abscisic acid to the shoot meristem of *S.alba* halves the number of active origins (Jacqmard, Houssa et al. 1995), and application of trigonelline to lettuce roots also halves the utilization of origins (Mazzuca, Bitonti et al. 2000).

1.2.5 The timing of replication

A number of studies have reported the fact that the timing of replication seems to be related to the chromatin surrounding the origin, and not the origin itself. The level of transcription of the surrounding genes plays an important role, with early replicating origins tending to be associated with regions of active transcription and late replicating origins tending to be associated with transcriptionally repressed genes (Diller and Raghuraman 1994). Consistent with this theory, it has been found that histone acetylation, a feature of transcriptionally active chromatin, is associated with origin activity in mammalian cells (Vogelauer, Rubbi et al. 2002), (Bryant and Aves 2011). Similarly, acetylation of histone H3 at Lys36 has been shown to be associated with early origins in *Arabidopsis* (Lee, Pascuzzi et al. 2010). Studies on *S.cerevisiae* have also shown that the behaviour of origins when moved from early to late loci or vice versa is dependent on the surrounding chromatin (Friedman, Diller et al. 1996). It would appear that these differences are not caused by differences in the formation of the pre-RC as ORC, CDC6 and MCM proteins associate equally with early and late origins throughout G_1 (Zou and

Stillman 2000), (Tanaka and Nasmyth 1998), (Aparicio, Stout et al. 1999), (Santocanale and Diffley 1998). Conversely CDC45, replication protein A and DNA polymerases α and ε all appear to associate with the origins in a manner correlated with their time of initiation (Zou and Stillman 2000), (Tanaka and Nasmyth 1998), (Aparicio, Stout et al. 1999), (Bell and Dutta 2002). Thus, it would appear that the factors controlling replication timing act on the factors involved in the transition to replication rather than pre-RC assembly, thereby ruling out the possibility that the chromatin conformation restricts access to proteins at late origins.

Interestingly, mutations in the RAD53 and MEC1 protein kinases, both of which are involved in the intra-S-phase checkpoint, result in the premature replication of late origins (Santocanale and Diffley 1998), (Shirahige, Hori et al. 1998). CDK function has also been implicated in controlling the timing of replication. CLB5-CDK1 can activate both early and late origins in *S.cerevisiae*, whereas CLB6-CDK1 can only activate early origins (Donaldson, Raghuraman et al. 1998). The differences in the effects of CLB5 and CLB6 could be due to different overall levels of CDK activity, although there does appear to be a differential difference in the ability of the two kinases to activate replication origins (Bell and Dutta 2002), (Donaldson 2000).

Following the successful completion of S-phase most cells move into G_2 phase, a period of rapid cell growth and protein synthesis, during which the DNA is checked for any damage. G_2 phase ends with the onset of M-phase.

1.3 Mitosis

Although M phase lasts for a relatively short time in comparison to interphase, it is one of the key stages in the cell cycle during which nuclear division (mitosis) and cytoplasmic division (cytokinesis) occur. Following replication, the chromosomes are composed of two identical sister chromatids held together by multisubunit protein complexes called cohesins. As M phase commences condensin complexes assemble on the DNA causing the progressive condensation of the chromosomes. This stage is known as prophase and occupies over half of mitosis. During this stage the chromosomes continue to condense until they are clearly individualised. It is also during prophase that the mitotic spindle begins to form. The mitotic spindle is constructed from microtubules and their associated proteins, and is responsible for the segregation of replicated chromosomes. The microtubule organising centre (MTOC) is the structure from which these microtubules emerge. MTOC's have been identified in several diverse evolutionary groups and are termed centrosomes in animals, spindle pole bodies in yeast and basal bodies in algae (Luders and Stearns 2007). In plants it is thought that the nuclear envelope acts as the main MTOC. During the animal cell cycle there is only one centrosome present at the beginning of G_1. Each centrosome consists of two centrioles which are replicated during interphase. As M-phase commences each centriole pair becomes a separate MTOC that nucleates a radial array of microtubules called an aster. The two asters move to opposite sides of the nucleus and form the two poles of the mitotic spindle. As the nuclear envelope breaks down during prometaphase the microtubules of the spindle interact with a specialised protein complex which assembles on the centomeric DNA called the kinetochore. The opposing forces exerted on the chromosomes by the microtubles from opposite poles of the spindle results in them lining up along the spindle equator, forming the metaphase plate. At this point there is an equilibrium between the sister chromatid

cohesion and the forces exerted on the kinetochores by the spindle microtubules. However, when sister chromatid cohesion is lost via proteolytic cleavage of the cohesion complex, anaphase is triggered, and the sister chromatids are drawn to opposite poles of the spindle. The kinetochores then disassemble, releasing the chromosomes from the spindle, and the nuclear envelope begins to re-form (telophase). The chromosomes decondense during this stage.

Cytokinesis is the process by which the cytoplasm is divided following the segregation of the chromosomes during mitosis. The mechanism by which this is achieved is different in plants and animals. In animals a cleavage furrow is formed on the cell surface which progressively deepens and spreads around the cell until it divides it in two. This is achieved by a contractile ring which assembles just below the plasma membrane and contracts to constrict the cell in two. New membrane is synthesised via the fusion of intracellular vesicles to compensate for the increase in surface area that accompanies cytoplasmic division (Alberts, Johnson et al. 2002). The contractile ring is composed of actin filaments, myosin II filaments and several structural and regulatory proteins (Schroeder 1972). The location of the contractile ring and thereby the plane of cell division is dictated by the mitotic spindle. The contractile ring forms at a right angle to the long axis of the mitotic spindle, midway between the microtubule organising centres (termed centrosomes in animal cells). In plants the division plane is marked before mitosis by an arrangement of microtubules and actin filaments termed the preprophase band. This marks the location for the formation of an arrangement of microtubules called the phragmoplast during late anaphase. Vesicles containing cell wall precursors such as polysaccharide and glycoproteins associate with the phragmoplast microtubules and fuse together to produce the early cell plate. The plate expands outwards by further vesicle fusion until it reaches the plasma membrane and original cell wall and divides the cell in two (Alberts, Johnson et al. 2002).

1.4 Control of the cell cycle

Eukaryotic cells have evolved a cell cycle control system to ensure the correct progression through the cell cycle and to guard against uncontrolled cell division. A series of biochemical switches control the main events of the cell cycle for example DNA replication and segregation of chromosomes (Alberts, Johnson et al. 2002). The cell cycle control system monitors the progression of the cell cycle and ensures that the key stages are completed before moving on to the next.

The components of the cell cycle control system have been evolutionarily conserved to a remarkable degree. Much has been learnt about the cell cycle control system from studies on model organisms, notably *Saccharomyces cerevisiae* (budding yeast), *Saccharomyces pombe* (fission yeast), *Xenopus laevis* embryos (frog) and mammalian cell cultures.

Throughout the cell cycle there are several checkpoints at which the cell cycle can be arrested if the previous events have not been correctly completed (Hartwell and Weinert 1989). A simple summary of the cell cycle checkpoints is shown in figure 4.

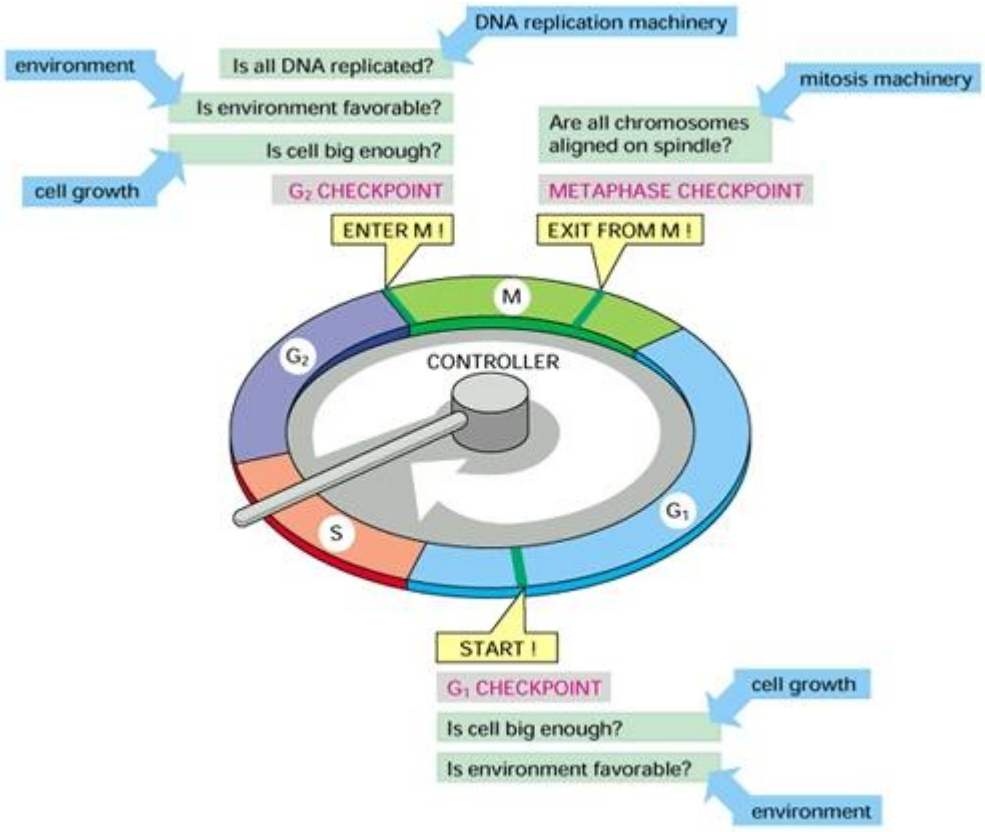


Figure 4:- the most prominent checkpoints within the cell cycle control system. If internal or external signals are unfavourable the cell is not allowed to progress past the checkpoint. Picture adapted from (Alberts, Johnson et al. 2002).

1.4.1 Cyclin dependent kinases

Progress through the cell cycle is controlled by the sequential activation and inactivation of cyclin dependent kinases (CDKs). These were first discovered in yeast and termed cell division cycle genes or *CDC* genes. *CDC2* (*S.pombe*) and its orthologue *CDC28* (*S.cerevisiae*) were found to play important roles in the G1/S and G2/M checkpoints (Norbury and Nurse 1992). In animals and plants these genes were renamed as cyclin dependent kinases. Human *CDKs* have been numbered in order of their discovery (*CDK1*, *Cdk2* etc.), whereas plant *CDKs* are categorized into five major classes (A-E) with another distinct class termed *CDK G*(Joubès, Chevalier et al. 2000), (Umeda, Shimotohno et al. 2005). Cyclin dependent kinases belong to the CMGC group which include cyclin-

dependent kinases (CDKs), mitogen-activated protein kinases (MAP kinases), glycogen synthase kinases (GSK) and CDK-like kinases. As their name suggests, CDKs are dependent on cyclins for their activity. Their protein kinase activity is dependent on a cyclin binding to a short amino acid sequence known as the PSTAIRE domain (Doerner 1994). Cyclins were first identified in marine invertebrates as proteins whose levels oscillated during the cell cycle and that when injected into frog oocytes could induce meiosis (Doerner 1994). Since then a number of cyclins have been identified in several different organisms (Evans 2004), (Hunt 2004). Interestingly, plants possess many more cyclins than previously described in other organisms (Vandepoele, Raes et al. 2002), (Inze and De Veylder 2006). There are several classes of cyclins, each defined by the stage of the cell cycle at which they bind CDKs and function. It is thought that the binding of a cyclin to its CDK partner not only increases the activity of the complex, but also directs it to specific target proteins depending on the stage of the cell cycle.

1.4.2 Regulation of the activity of cyclin dependent kinases

The transient expression of cyclins is the major determinant of CDK activity. In the absence of a bound cyclin the active site of the CDK is blocked by a large, flexible region of the protein known as the T-loop. Upon binding of a cyclin to the PSTAIRE domain of the CDK the T-loop moves out of the active site resulting in partial activation of the CDK complex. To gain full activity a separate kinase called the CDK-activating kinase (CAK) is required to phosphorylate an amino acid near to the entrance of the active site. This induces a further conformational change that confers full activity to the CDK (Morgan 1997). There are also mechanisms by which the activity of CDK's can be fine tuned throughout the cell cycle. WEE1 inhibits CDK activity by phosphorylating two amino acids (Thr-14 and Tyr-15 in human CDK2) in the roof of the active site (Joubès, Chevalier et al. 2000). Dephosphorylation of these sites by a phosphatase called CDC25 increases CDK

activity. There are also CDK inhibitor proteins (CKIs) which can regulate the activity of CDKs via dramatic rearrangement of the active site (Alberts, Johnson et al. 2002).

Proteolysis of cyclins at certain stages of the cell cycle also serves to inactivate CDK complexes. Multiple copies of ubiquitin are attached to the cyclin which marks the protein for destruction. The transfer of ubiquitin is catalyzed by enzymes known as ubiquitin ligases, two of which are particularly important in the destruction of cyclins and other cell cycle regulators. In G_1 and S phase, an enzyme complex called SCF destroys cyclins and certain CKIs which control S phase initiation. In M phase the anaphase promoting complex destroys M-cyclins and other mitotic regulators (Alberts, Johnson et al. 2002).

1.4.3 CDKs and cell cycle progression

Eukaryotic cells use several CDK complexes to regulate their passage through the cell cycle. There are four classes of cyclins, three of which are needed for cell cycle progression in all eukaryotic cells. The G_1/S cyclins bind CDKs at the end of G_1 and commit the cell to DNA replication. The S cyclins bind CDKs during S-phase and are required for DNA replication. The M-cyclins are required for mitosis. In most cells there is a fourth class of cyclins termed the G_1 cyclins which are required for transition through the G_1 checkpoint or restriction point, after which cells are committed to undergoing cell division. Figure 5 summarises the activity of the CDKs throughout the cell cycle.

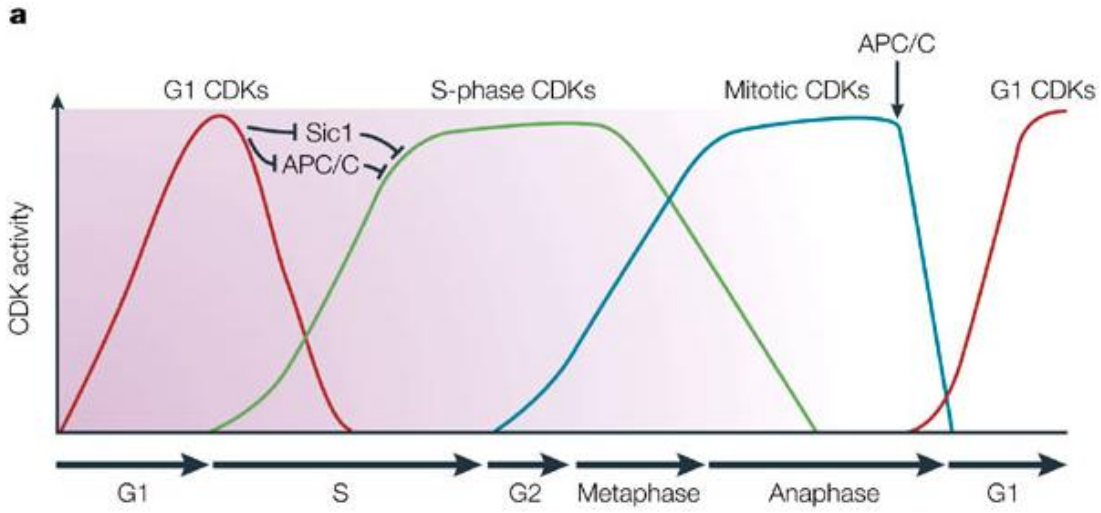


Figure 5: CDK activity during the mitotic cell cycle. The red line represents the G1-CDK activity, the green line represents the S-phase CDK activity and the blue line represents the Mitotic CDKs. Figure adapted from (Marston and Amon 2004).

The increased activity of the G_1 CDKs leads to degradation of the SIC1 protein (stochiometric inhibitor of CDKs) via multiple phosphorylations leading to its destruction by the SCF complex (a multi-protein E3 ubiquitin ligase complex) (Verma, Annan et al. 1997). G_1 CDK activity also leads to the inactivation of the anaphase promoting complex / cyclosome (APC/C), the E3 ubiquitin ligase within the SCF complex, which leads to the degradation of cohesion and the separation of sister chromatids, allowing the onset of anaphase. The APC/C also targets mitotic cyclins for degradation, promoting exit from mitosis. The inactivation of these proteins contributes to the increased activity of S-phase CDKs which leads to the initiation of DNA replication. Mitotic CDKs then promote entry into mitosis. At the end of mitosis, mitotic CDKs are inactivated, which allows for the disassembly of the mitotic spindle and entry into G_1 (Marston and Amon 2004). In yeast there is one CDK protein which binds all classes of cyclin and promotes the different stages of the cell cycle depending on which cyclin it interacts with. In contrast, in the mammalian cell cycle there are several CDK / cyclin complexes used to regulate the different stages of the cell cycle. These are shown in figure 6.

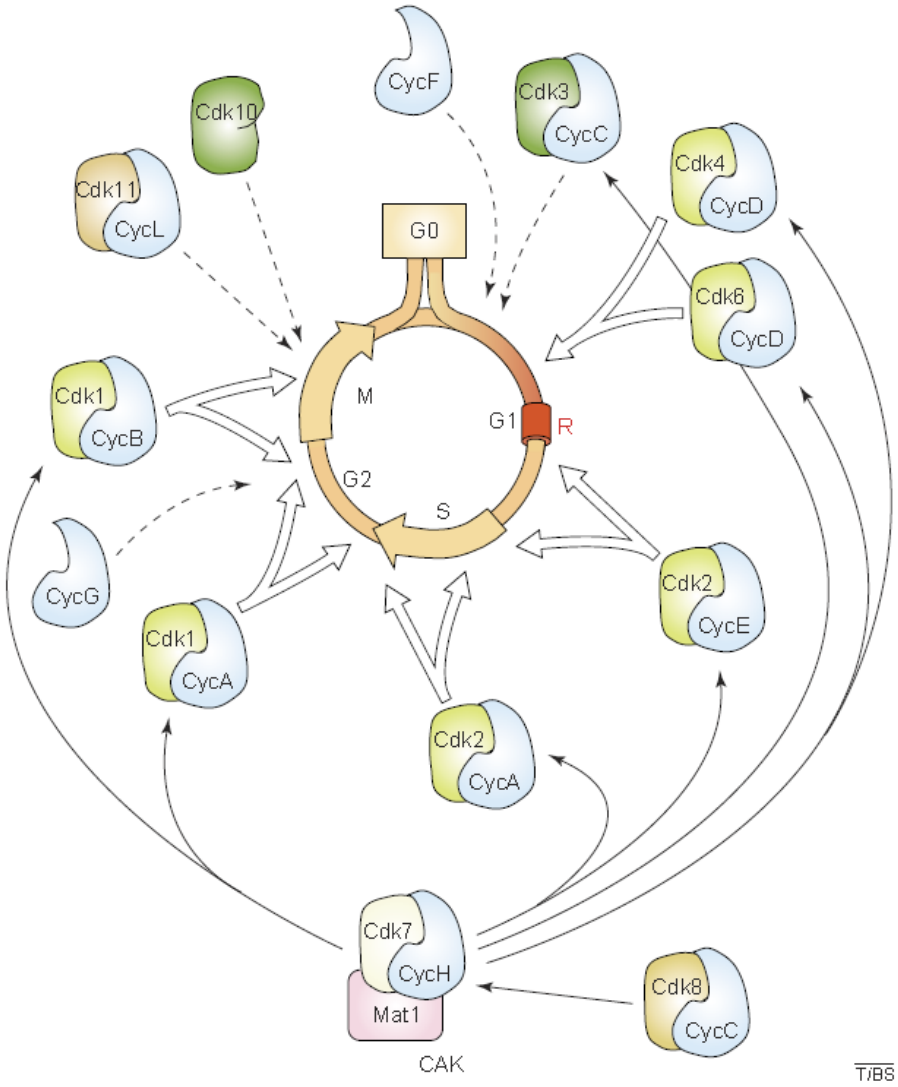


Figure 6: Proposed role of CDK-cyclin complexes in the mammalian cell cycle. Figure taken from (Malumbres and Barbacid 2005).

CDK-cyclin complexes also play a role in passing through the various cell cycle checkpoints. In most organisms there are two main DNA damage checkpoints which ensure that damaged DNA is repaired before the cell attempts to duplicate or segregate them. One is in late G_1, preventing entry into S-phase if DNA is damaged, and one is in late G_2, preventing entry into mitosis (Lodish 2008). The G_1 checkpoint blocks progression into S-phase if DNA damage is detected, by inhibiting the G_1/S-CDK and S-CDK complexes. In mammalian cells this is achieved by the rapid activation of protein 53 (P53), which in turn

stimulates the transcription of several genes including *WAF1*/*CIP1* which encodes P21. P21 binds to the G_1/S-CDK and S-CDK complexes, inhibiting their activity and causing the cell cycle to halt at the G_1 checkpoint until the DNA damage is repaired. The G_1 checkpoint or restriction point (START point in yeast) is also important because after this stage the cells no longer require mitogenic stimuli to undergo cell division (Malumbres and Barbacid 2005). This change is mediated by the interaction between a transcription factor called E2F and retinoblastoma protein (RB). During early G_1 RB binds to E2F, blocking the transcription of proteins required for S-phase entry. The increasing activity of G1-CDK complexes leads to the phosphorylation of RB, decreasing its affinity for E2F, and allowing the transcription of S-phase genes (Sherr and Roberts 1999). RB proteins and other components of the RB pathway have also been discovered in plants, suggesting a conserved mechanism for cell cycle control (de Jager and Murray 1999).

Progression into mitosis can be halted at the G_2 checkpoint if incomplete DNA replication or DNA damage is detected. The inhibition of maturation-promoting factor (MPF) prevents progression into mitosis. MPF is a heterodimeric protein composed of cyclin B and CDK1 (also known as CDC2, CDC28 or P34 kinase). The inhibition and activation of MPF is controlled, as previously described, by the action of WEE1, CAKs and CDC25. When the incomplete DNA synthesis or DNA damage is repaired MPF becomes activated and entry into mitosis is promoted. MPF also triggers the formation of the mitotic spindle, and the breakdown of the nuclear envelope (Masui 2001).

The final cell cycle checkpoint is called the spindle-attachment checkpoint and prevents anaphase onset until all chromosomes are properly attached to the spindle. If any kinetochores are not properly attached to the spindle a negative signal is sent out that prevents cell division cycle protein 20 (CDC20) from activating the anaphase promoting complex (APC). If a kinetochore is not properly attached to the spindle several proteins

including MAD2 (mitotic arrest deficient), MAD3, BUB3 (budding uninhibited by benzimidazole) and CDC20 are recruited to the kinetochores. MAD2 interacts with CDC20 preventing it from activating the APC. When all of the kinetochores are correctly attached CDC20 activates the APC, directing it to ubiquitinate the anaphase inhibitor securin. Securin degradation leads to the release of a protease called separase which cleaves the cohesion complex holding sister chromatids together and triggers anaphase (De Antoni, Pearson et al. 2005), (Musacchio and Salmon 2007). Figure 7 shows a summary of the processes involved in sister chromatid separation by the action of the APC.

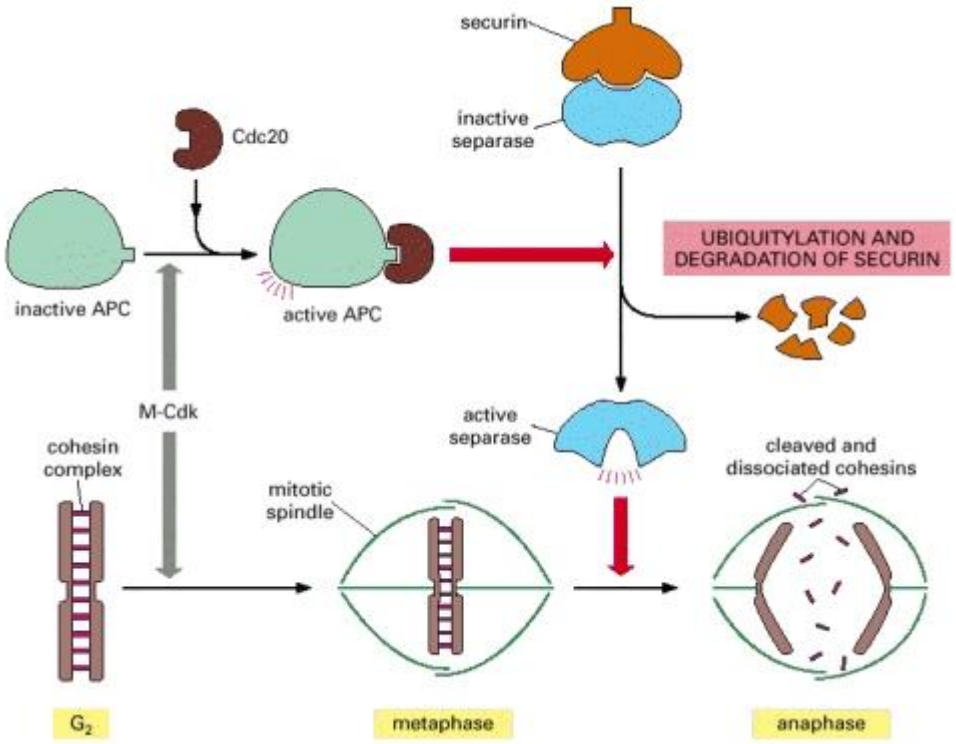


Figure 7: the triggering of sister chromatid separation by the APC. Figure taken from (Alberts, Johnson et al. 2002).

1.5 Meiosis

Meiosis is a specialised form of cell division by which diploid cells halve their chromosome number to produce haploid cells or gametes. During fertilization two haploid gametes are combined to form a diploid zygote in which both maternal and paternal chromosomes are present. The process of meiosis is therefore essential in the life cycle of

all sexually reproducing eukaryotic organisms. A comparison of the processes of mitosis and meiosis is shown in figure 8 below.

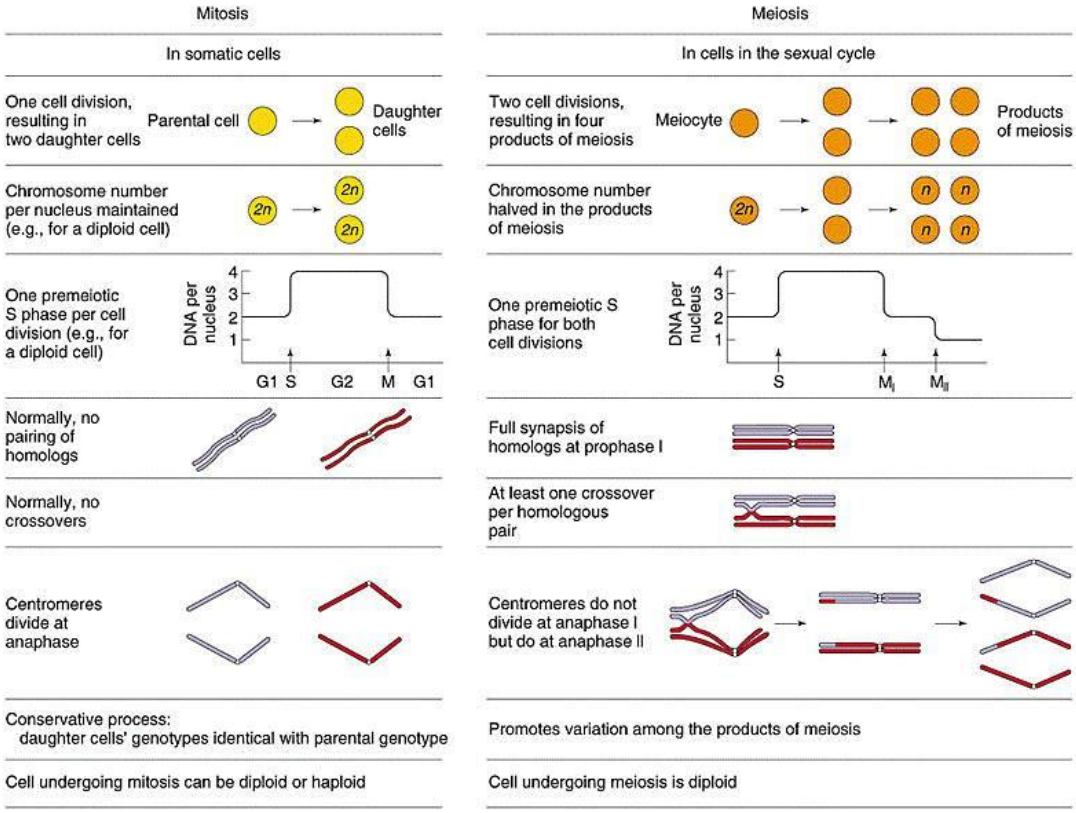


Figure 8: a comparison of meiosis and mitosis. Figure taken from http://www.cbs.dtu.dk/staff/dave/roanoke/fig3_05.jpg

Diploid eukaryotic organisms carry two copies of each chromosome or homologue, one contributed from the paternal gamete and one from the maternal gamete. Before meiosis commences each homologue replicates to form two sister chromatids which remain linked together. The meiotic cell then enters into prophase I during which homologous chromosomes (composed of two sister chromatids) recognise each other and closely align in a process termed synapsis. The homologues are held together by a tripartate proteinaceous structure known as the synaptonemal complex (SC) (Zickler and Kleckner 1999). This consists of two lateral elements which align each homologue and a

central element that holds sister chromatids together and maintains the homologues in a tethered state. Whilst the homologous chromosomes are intimately aligned and held together by the SC, genetic recombination or crossing over occurs, during which homologues exchange genetic material. The sites of crossing over are termed chiasmata, and these remain as physical links between the homologues until anaphase I. This continued association of homologues is critical to their accurate segregation, because it allows them to be correctly oriented by the connection of homologous centromeres to opposite poles. At this point homologous chromosomes separate, leaving the sister chromatids linked together (meiosis I). Meiosis I is then followed by a second division, resembling mitosis, in which sister chromatids are separated (Page and Hawley 2003). This results in the creation of four daughter cells, each of which has a full haploid genome. A simple diagrammatic overview of meiosis is shown in figure 9.

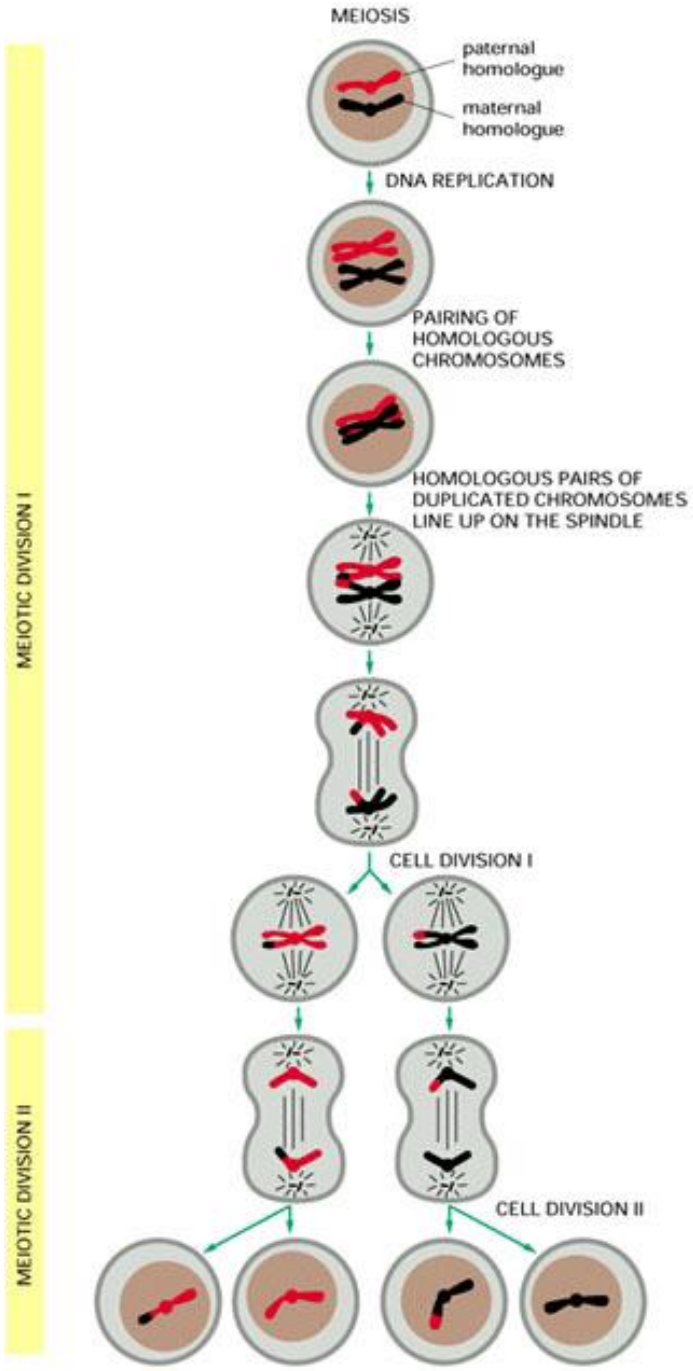


Figure 9:- Summary of meiosis. One round of replication is followed by two cell divisions to produce four haploid daughter cells or gametes. The diploid chromosome number is restored upon fertilization. Figure copied from (Alberts, Johnson et al. 2002).

1.5.1 Pre-meiotic interphase

Similarly to the mitotic cell cycle the cell goes through a period of growth and replication prior to meiosis termed pre-meiotic interphase. It is increasingly thought that many important events occur during this stage, despite the uniform and undifferentiated appearance of the nucleus (Loidl 1990), (Dover and Riley 1977). Interphase consists of two gap phases (G_1 and G_2) during which proteins are made that allow the cell to grow in size, separated by a synthesis phase (S-phase). Taylor and MacMaster (1954) first demonstrated that DNA replication occurs in meiotic S-phase rather than during prophase I as was originally thought (Taylor and MacMaster 1954). During this stage each chromosome duplicates to produce a second identical sister chromatid. The duration of this pre-meiotic synthesis phase (S-phase) is longer than mitotic S-phase (John 1990). Several other important events are thought to occur during this pre-meiotic interphase, including chromatin modification and the expression of many meiotic genes whose products are required for the successful completion of meiosis (Armstrong and Jones 2003).

1.5.2 Prophase I

The key event in meiosis, upon which all others depend, is the pairing of homologous chromosomes. It is during prophase I that this pairing takes place. Meiotic prophase I can be divided into several substages according to the state of synapsis of the chromosomes:- leptotene (chromosomes are separate), zygotene (chromosomes are partially synapsed), pachytene (full synapsis), and diplotene – diakinesis (chromosomes desynapse, but bivalents are held together at chiasmata) (Loidl 1990). A summary of synapsis in relation to prophase I is shown in figure 10.

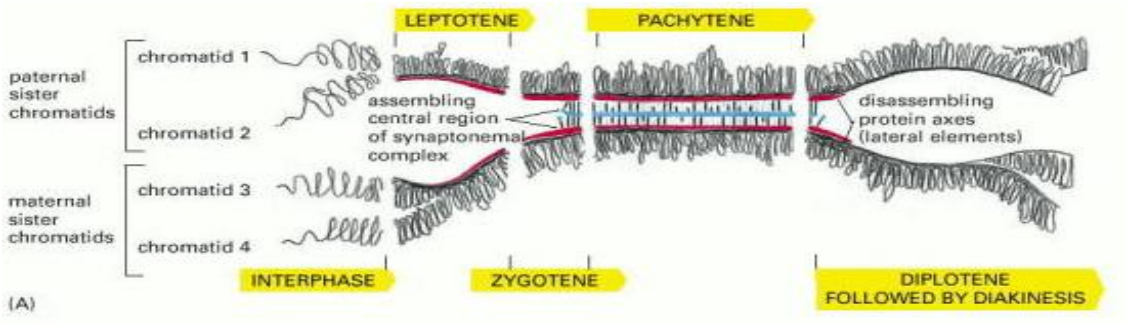


Figure 10:- diagram showing the stages of synapsis of a single bivalent during prophase I. Formation of the synaptonemal complex begins during leptotene and zygotene, is complete by pachytene and disassembles during diplotene leaving the chromosomes linked by chiasmata. The central element is shown in blue whilst the axial or lateral elements are shown in red (Alberts, Johnson et al. 2002).

During leptotene the chromosomes start to condense and are most commonly visible as long threads which consist of two very tightly associated sister chromatids. At this stage a protein core called the axial element is formed between the sister chromatids of each chromosome (Møens and Pearlman 1988). The formation of double strand breaks (DSB's) is first seen during leptotene in some species(Kleckner 1996), (Mahadevaiah, Turner et al. 2001). In many organisms the chromosomes become attached to the nuclear envelope via their telomeres, which begin to cluster. The clustering of the telomeres continues until a tight 'bouquet' is formed on the nuclear envelope during zygotene. At this stage the chromosomes begin to coil and the sister chromatids become briefly visible. When the bouquet is complete the process of synapsis begins. Synapsis initiates at the telomeres, where transverse filaments form and synapsis proceeds via a 'zipping-up' mechanism from these initiation points until homologous chromosomes are connected along their entire length (Loidl 1990). The homologous chromosome pair is termed a bivalent at this point. At the same time a central element is formed between the two homologous axial elements. The association of transverse, central and axial elements is termed the synaptonemal complex (SC) (Zickler and Kleckner 1999). Within the SC, the

axial elements are now termed lateral elements (LE) whilst the central element and transverse filaments are termed the central region. When synapsis is complete the cell enters pachytene. Meiotic recombination occurs during pachytene resulting in the formation of chiasma which provide a physical link between homologous chromosomes. The processes of synapsis and recombination will be discussed in more detail later in this chapter.

There is a meiotic checkpoint which ensures that synapsis and recombination events are complete before the cell can progress towards the first division at metaphase I. This checkpoint is called the pachytene checkpoint (Roeder and Bailis 2000), (Shaw and Moore 1998).

During the diplotene stage of prophase I the SC disassembles leaving the homologous chromosomes joined by chiasmata. The chromosomes continue to condense via a spiralling process (Dawe 1998) throughout diplotene and into diakinesis, and attach to the spindle as they approach metaphase I (Shaw and Moore 1998). At this point each bivalent can clearly be seen as containing four separate chromatids, with sister chromatids joined by their centromeres, and non-sister chromatids linked via chiasmata.

1.5.3 Metaphase I to Telophase I

By metaphase I the nuclear envelope has disappeared allowing the microtubules of the meiotic spindle to interact with a specialised protein complex which assembles on the centomeric DNA called the kinetochore (Dawe 1998). At this point there is a second checkpoint which ensures that all of the chromosomes are correctly attached to the spindle at metaphase I (Shaw and Moore 1998). The sister chromatids are linked via their kinetochores and so behave as a single unit. This means that when the chromosomes line up along the metaphase plate, the kinetochores of each chromosome (composed of two sister chromatids) are oriented towards opposite poles. The connections at the chiasmata

are broken during anaphase I and the maternal and paternal copies of each chromosome are pulled to opposite poles by the meiotic spindle microtubules. At this point the centromeric connections between sister chromatids are maintained (Shaw and Moore 1998). By telophase I the chromosomes have arrived at opposite poles and partially decondense (Armstrong and Jones 2003). The cells then enter into the second meiotic division.

1.5.4 The Second Meiotic Division

This division is similar to a normal mitotic division, except that it does not follow a round of DNA replication. Each chromosome is still composed of two sister chromatids, which are not identical because of the homologous recombination which took place during the first division of meiosis. Cohesion between the sister chromatids is only present at their centromeres. The chromosomes align along the equatorial plate during metaphase II and when the centromeric connections between sister chromatids are broken during anaphase II the microtubules pull each sister chromatid to opposite poles. By telophase II there is one haploid set of chromosomes at each pole. The cell then enters cytokinesis where the cytoplasm divides to produce four haploid gametes.

1.5.5 Control of the meiotic cell cycle

Many of the key regulators of the mitotic cell cycle are also involved in controlling meiosis. Figure 11 summarises the activity of CDKs during the meiotic cell cycle.

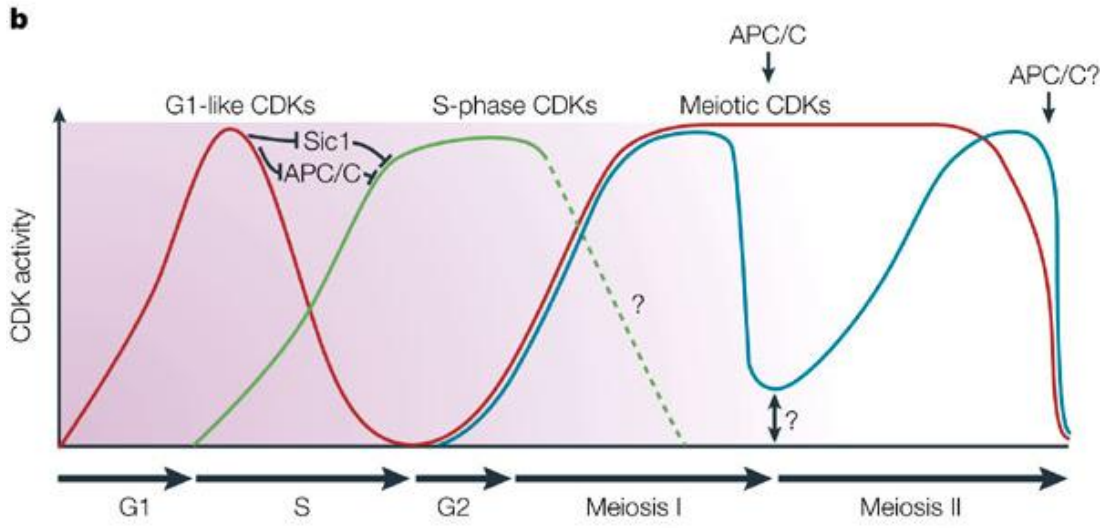


Figure 11: CDK activity during the meiotic cell cycle. The red line represents the G1-like CDKs, the green line represents the S-phase CDKs and the blue line represents the meiotic CDKs. Figure adapted from (Marston and Amon 2004)

The increased activity of the G1-like CDKs (IME2 in budding yeast) leads to the inhibition of SIC1 and the APC/C in the same way as in the mitotic cycle. This promotes the activation of S-phase CDKs which leads to DNA replication after which the meiotic CDKs increase in activity and promote meiosis. Work on *Arabidopsis* has recently identified an SDS gene which is specifically expressed during meiosis and encodes a protein with high levels of similarity to cyclins. This protein is critical for the normal synapsis of homologues and for bivalent formation, suggesting that a CDK-cyclin interaction may play a critical role in these processes (Azumi, Liu et al. 2002). Unlike the mitotic cycle, a second peak in the activity of the G1-like CDKs (IME2) is needed to execute the meiotic divisions. It is not known whether or not the S-phase CDKs decline upon entry into meiosis (hence the dotted line in figure 9). A drop in the activity of meiotic CDKs between meiosis I and II has been seen in the frog *Xenopus laevis*, and is thought that it prevents a second round of DNA replication and segregation. At the end of meiosis the levels of meiotic CDKs decline rapidly (Marston and Amon 2004).

In yeast, cells can either enter the meiotic or mitotic cell cycle during G_1. Therefore a mechanism is required to ensure that cells don't simultaneously enter both cycles. During vegetative growth cyclin-CDK complexes inhibit the inducer of the meiotic cycle, IME1. Conversely, upon entry into the meiotic cycle, cyclin transcription is inhibited leading to reduced activity of CDK-cyclin complexes. IME1 is activated which leads to the transcription of early meiotic genes. The functions that CDK-cyclin complexes perform during the mitotic cycle are either dispensable during the meiotic cycle, or are performed by the meiosis specific protein kinase IME2 (Lee and Amon 2001).

There is some evidence for the presence of multiple checkpoints within the meiotic cell cycle. Yeast cells can arrest during the pachytene stage of meiotic prophase if there is incomplete chromosome synapsis or recombination (Roeder and Bailis 2000). Failure to enter meiosis I has also been observed in mammalian cells when defects occur in prophase (Wolgemuth, Laurion et al. 2002). It seems that there are multiple prophase checkpoints in mammals because cells arrest and undergo apoptosis at several stages during prophase in response to defects in synapsis and recombination (Wolgemuth, Laurion et al. 2002). There is some evidence for a premeiotic DNA damage checkpoint involving P53 in mice (Schwartz, Goldfinger et al. 1999). Studies looking at *Drosophila* meiosis have revealed that the presence of a relatively high number of misaligned chromosomes or a severe disruption of the meiotic spindle results in a significant delay in the time of entry into anaphase, suggesting that a spindle checkpoint may be present (Rebollo and Gonzalez 2000). However, there is still uncertainty as to whether meiotic checkpoints are found in plants. In mammals, *C.elegans* and *Drosophila* there is a pachytene checkpoint which prevents cells from undergoing meiosis if there are defects in pairing, synapsis or recombination (Bhalla and Dernburg 2005), (Ashley, Gaeth et al. 2004), (Roeder and Bailis 2000), (Ghabrial and Schupbach 1999), but most plant meiotic mutants and haploid hybrids complete meiosis and cytokinesis and produce abnormal microspores. In most cases these abnormal

microspores degenerate during pollen development leading to male sterility, but haploid hybrids between related species can be created. Mutations in genes that trigger pachytene arrest in yeast, worms, and mice do not appear to activate a typical meiotic checkpoint in plants, suggesting that plants do not use the same meiotic control systems (Yang, Makaroff et al. 2003).

1.6 Homologous chromosome pairing

As previously mentioned, the success of meiosis is absolutely dependent on the correct recognition and pairing of homologous chromosomes, otherwise segregation would be incorrect. Despite its importance we still have very little understanding of how homologous chromosomes recognise and subsequently pair with one another. There are three stages to homologous chromosome pairing, recognition, alignment and synapsis (Zickler 2006). The problem of how homologous chromosomes recognise one another is the least understood of these processes. Although recognition must ultimately depend on sequence analysis, it cannot simply be attributed to linear searching mechanisms as the process takes a similar length of time across several orders of magnitude of genome size (Moore and Shaw 2009). Even duplicating the genome does not have an effect on the length of time taken for the homology search. Meiosis in polyploid wheat is actually shorter than in its diploid relatives. Much of our understanding of meiotic processes has been provided by studies on diploid model systems, which has caused difficulties in dissecting the mechanics of the homology search. Disruption of chromosome recognition, pairing and recombination in these species results in recombination failure at metaphase I. Therefore studies on diploid species cannot clearly distinguish the processes of recognition, pairing and recombination from the mechanics of pairing and recombination. Furthermore, most diploid models for meiosis have small, non-repetitive genomes, whereas 90% of large genomes are composed of highly repetitive, similar sequences,

raising the question of how these are distinguished during the pairing process. Despite our limited current understanding of the process of homologue recognition, several observations have been made of mechanisms which may contribute to the correct pairing of chromosomes in different organisms. These will be discussed in the following section.

1.6.1 Pre-meiotic chromosome association

In some instances the arrangement of chromosomes prior to meiosis is highly organized. It was first observed by Carl Rabl in 1885 that the segregation of chromosomes at mitotic anaphase results in the chromosomes being arranged with the centromeres clustered in a small region of the nucleus while the telomeres are generally associated with the nuclear envelope on the opposite side. This arrangement of chromosomes persists into the following interphase in some species and is called the Rabl organization (Cowan, Carlton et al. 2001). The presence of Rabl organization varies greatly between species, for example amongst the cereals it has been observed in wheat, rye, barley and oats but not in rice or sorghum. This variation may be a function of genome size and chromosome length (Dong and Jiang 1998). Interestingly, recent work has shown that induced DNA hypomethylation can result in centromeric polarization in rice interphase cells, causing a Rabl-like configuration (Santos, Ferreira et al. 2011). The reasons for this centromeric polarization are unknown, but it has been suggested that the forces that normally move chromosomes away from the Rabl configuration at the end of telophase cannot act on the highly decondensed and presumably flexible chromatin caused by induced hypomethylation (Santos, Ferreira et al. 2011).

It is also thought that the association of homologous chromosomes may commence pre-meiotically, although this has again been shown to differ between organisms. In the budding yeast, *Saccharomyces cerevisiae*, there is evidence of homologue association during the pre-meiotic interphase stage (Weiner and Kleckner

1994), (Loidl 1990). The meiotic cells of some fungi show pre meiotic pairing of homologous chromosomes (Roeder 1997) and in hexaploid wheat, *Triticum aestivum,* associations between homologous chromosomes during pre-meiotic interphase have also been shown (Aragón-Alcaide, Reader et al. 1997). In *Drosophila* and other Dipterans, homologues pair early during embryogenesis and they remain paired throughout subsequent cell cycles (Hiraoka, Dernburg et al. 1993), (Dernburg, Broman et al. 1996) (McKee 2004). This pairing is not disrupted in meiotic cells; instead, chromosomes undergo a gradual transition from somatic pairing to SC formation (Roeder 1997), (Wandall and Svendsen 1985). In mammals, some diploid plants such as *Arabidopsis* and maize, and the diploid progenitors of wheat, homologues do not appear to associate prior to meiotic prophase I (Scherthan, Weich et al. 1996). However, pre-meiotic chromosome pairing is not limited to polyploid plants. Chromosomes have been seen to associate both premeiotically and in the xylem vessel cells of diploid rice (*Oryza sativa*) via their telomeres and centromeres (Prieto, Santos et al. 2004), but not in other cell types (Santos and Shaw 2004).

1.6.2 The role of the centromeres in chromosome pairing

Centromeres are the structures that direct eukaryotic chromosome segregation in mitosis and meiosis. There are two major classes of centromeres. Point centromeres, found in the budding yeasts, are compact loci of about 125 bp. This small region is sufficient to specify centromere function and identity in these organisms. Regional centromeres, best described in the fission yeast , encompass many kilobases of DNA and are packaged into heterochromatin (Pluta, Mackay et al. 1995). In most eukaryotes the centromeric region has no defined sequence, instead consisting of large arrays of repetitive DNA (e.g. satellite DNA) where the sequence within individual repeat elements is similar but not identical. More than a dozen different proteins assemble on the centromeric region, including the

core histones and an H3 variant (CENP-A in most organisms), to create a specialized centromeric nucleosome (Lodish 2008). The functions of this specialized nucleosome are poorly understood but it is thought that it facilitates the binding of the proteins which form the kinetochore. In most organisms, the centromere–kinetochore complex forms at a single point on the chromosome, typically near the middle of the chromosome. The exceptions are holocentric organisms, such as the nematode *Caenorhabditis elegans*, in which the kinetochore forms along almost the entire chromosome arm (Albertson and Thomson 1993), (Pidoux and Allshire 2000). In addition to providing the site for microtubule attachment, centromeres also have an important role in checkpoint regulation during mitosis.

A clustering of centromeres is often a prominent feature of chromosome organization in mitotic cells, although it is difficult to ascertain whether or not these associations are between homologous chromosomes (McKee 2004). In mitotic *Arabidopsis* cells, centromeric associations were originally thought to be almost exclusively homologous with homologous centromeres paired whenever euchromatic regions of the same chromosome were paired, suggesting that pairing might spread from centric heterochromatin into the euchromatin (Fransz, De Jong et al. 2002),(McKee 2004). Further studies on the interphase organisation of *Arabidopsis* cells have disputed this claim, instead suggesting that the arrangement of centromeres relative to one another is predominantly random, with no evidence for homologous associations (Pawlowski 2010), (Pecinka, Schubert et al. 2004), (de Nooijer, Wellink et al. 2009), (Berr and Schubert 2007). However, chromosomes which contain nuclear organising regions (NORs) are seen to associate more often, probably because of their attachment to the nucleolus (Pawlowski 2010), (Pecinka, Schubert et al. 2004), (Berr and Schubert 2007). In *S. pombe*, both somatic and premeiotic pairing frequencies are highest in centromeric regions (Scherthan, Bahler et al. 1994), (Molnar, Doll et al. 2003). Centomere pairing has also been seen in somatic cells of some

cereals such as rice and hexaploid wheat (Prieto, Santos et al. 2004), (Aragón-Alcaide, Reader et al. 1997).

There is little evidence for centromeric associations playing a role in meiotic chromosome pairing in most species. However, it has been shown that wheat and rice chromosomes associate into groups during floral development pre-meiotically via their centromeric regions (Martinez-Perez, Shaw et al. 2000; Martinez-Perez, Shaw et al. 2001; Martinez-Perez, Shaw et al. 2003), (Prieto, Santos et al. 2004). It has been shown that these centromeric associations are not required for the successful pairing of homologous chromosomes (Lukaszewski 1997).

1.6.3 The role of telomeres in chromosome pairing

Telomeres are regions of repetitive DNA found at the ends most eukaryotic and some prokaryotic chromosomes in order to prevent chromosome deterioration due to incomplete replication. Unlike centromeric sequences, telomeric sequences are well conserved between organisms. They consist of many tandem repeats of a short sequence that contains a block of neighbouring G nucleotides. In humans this sequence is (TTAGGG)n, whilst in higher plants it is (TTTAGGG)n. They vary in length considerably between organisms, for example, in yeast they are 300 – 600bp, whilst in humans they can extend for several kilobases (Shampay, Szostak et al. 1984). The repetitive nature of the telomeric sequence has meant that the location of the telomeres during the cell cycle can be fairly easily studied using fluorescent in situ hybridization (FISH) techniques.

There is little evidence to suggest that telomeres play an important role in the pairing of chromosomes during the mitotic cell cycle, however, they do appear to play a prominent role in meiotic chromosome pairing. Studies on *Saccharomyces cerevisiae* (Trelles-Sticken, Loidl et al. 1999), *Schizosaccharomyces pombe* (Chikashige, Ding et al. 1997), (Niwa, Shimanuki et al. 2000), rye (Mikhailova, Sosnikhina et al. 2001),

wheat(Martinez-Perez, Shaw et al. 2001), maize (Bass, Marshall et al. 1997)and mammals(Scherthan, Weich et al. 1996), have shown that the telomeres cluster on the nuclear envelope during early prophase I forming a telomere bouquet. It has been suggested that this 'bouquet' may facilitate the pairing of homologous chromosomes. This is because the formation of the synaptonemal complex initiates at the telomeric region of chromosomes. It has been found that deletion of the telomere region of one homologue results in the reduction or complete prevention of pairing between these chromosomes during metaphase. It is thought that this may be due to a failure of synapsis during prophase I (Lukaszewski 1997). The maize meiotic mutant *pam1* (*plural abnormalities of meiosis 1*) has an extremely irregular telomere bouquet, which is thought to lead to defects in homologous synapsis during prophase I (Golubovskaya, Harper et al. 2002). However, mutations in yeast which disrupt telomere clustering lead to delays in pairing and reductions in recombination but do not lead to the prevention of either (Chua and Roeder 1997), (Conrad, Dominguez et al. 1997). Therefore the role of the bouquet may be to facilitate pairing by bringing the chromosomes into close proximity of one another and / or may initiate synapsis between homologues (Dawe 1998).

1.6.4 Chromosome synapsis and recombination

The association of homologous chromosomes culminates in the formation of the synaptonemal complex (SC), that finally mediates the intimate connection of homologue axis along their lengths at pachytene (Zickler 2006). The SC is found in most organisms (*S.pombe* and *Aspergillus nidulans* being notable exceptions (Heyting 1996)) and so has been studied in great detail.

In budding yeast, seven meiosis specific proteins have been found to be associated with the synaptonemal complex and its axes (HOP1, RED1, MEK1/MRE4, HOP2, PCH2, ZIP1 and ZIP2) (Ajimura, Leem et al. 1993), (Hart and Laemmli 1998), (Hollingsworth, Goetsch et

al. 1990), (Leem and Ogawa 1992), (Leu, Chua et al. 1998), (Smith and Roeder 1997), (Sym, Engebrecht et al. 1993). Corresponding proteins have also been found in mammals, for example SCP1/SYN1 (equivalent to yeast ZIP1) appears to be the major component of the transverse filaments (Schmekel, Meuwissen et al. 1996). Mammalian SCP2 and SCP3/COR1 (equivalent to yeast HOP1 and RED1) localize to the axial elements (Heyting 1996), (Zickler and Kleckner 1999). In plants ASY1 (*Arabidopsis)*, *Ta*ASY1 (hexaploid wheat) and PAIR2 (rice) localize with the axial elements and show some similarity to HOP1 in yeast (Caryl, Armstrong et al. 2000), (Sanchez-Moran, Osman et al. 2008), (Boden, Shadiac et al. 2007), (Nonomura, Nakano et al. 2006). SY1 and SY9 localize with the axial elements and are involved in recombination in rye (Jenkins, Phillips et al. 2008). In *Arabidopsis* SWI1 is required for formation of the axial elements and in *swi1* mutants, synapsis of homologues does not occur (Mercier, Vezon et al. 2001), (Mercier, Armstrong et al. 2003).

The major function of the SC in most eukaryotes is to mediate chromosome pairing during prophase by holding the two bivalents together. However, it was initially hypothesised that it may be required as a scaffold for the double strand break (DSB) formation and genetic recombination that also occurs during these early stages of prophase I (Dawe 1998), (Kleckner 1996). It is now thought that the early events of recombination precede synapsis (Hawley and Arbel 1993), as DSB formation sometimes occurs prior to synthesis of the SC (Kleckner 1996). In *S.cerevisiae* DNA DSBs are seen to occur prior to synapsis (Padmore, Cao et al. 1991), and histone H2A.X phosphorylation and the formation of strand exchange protein complexes indicate that this also occurs in other fungi, higher plants and mammals (Mahadevaiah, Turner et al. 2001), (Barlow, Benson et al. 1997), (Moens, Chen et al. 1997), (Anderson, Offenberg et al. 1997), (Storlazzi, Tesse et al. 2003). In yeast deficient for the ZIP1 protein, which cannot produce a mature SC, recombination events can still be seen (Roeder 1997), (Shaw and Moore 1998), and in *Saccharomyces pombe* and *Aspergillus nidulans* high levels of recombination are seen

despite the lack of SC formation (Roeder 1997), suggesting that recombination is not dependent on SC formation in these organisms. It has been suggested that DSBs may even be a prerequisite to SC formation (Henderson and Keeney 2004). In many organisms mutations in *SPO11*, which encodes the protein required to create DSBs, results in defective SC formation (Dresser and Giroux 1988), (Giroux, Dresser et al. 1989), (Loidl, Klein et al. 1994), (Baudat, Manova et al. 2000), (Celerin, Merino et al. 2000; Romanienko and Camerini-Otero 2000). Furthermore, defects that block the processing of DSBs such as *rad50* and *dmc1* have also been found to cause defects in SC formation (Alani, Padmore et al. 1990), (Bishop, Park et al. 1992), (Pittman, Cobb et al. 1998). Conversely in *Drosophila* and *C.elegans* a normal SC is formed in mutants defective for recombination (Dernburg, McDonald et al. 1998), (McKim, Green-Marroquin et al. 1998), and SC formation always occurs without recombination in the female silk moth *Bombyx mori* (Rasmussen 1976).

Based on these observations the SC may not be necessary for the initiation of recombination events, but may have a role in the regulation of these events, especially in limiting the number of cross-overs formed via a process termed 'interference' (Dawe 1998), (Kleckner 1996), (Roeder 1997), (Shaw and Moore 1998). Although crossover events can occur nearly anywhere along the length of a chromosome they are not distributed uniformly. Instead there appear to be recombination 'hotspots', where DSBs are preferentially induced by a meiotic endonuclease called SPO11. There is also evidence to suggest that the occurrence of one crossover event decreases the probability of a second occurring nearby. This 'interference' seems to ensure that the limited number of crossovers are spread out so that even small chromosomes get one, as required for the homologues to segregate correctly (Alberts, Johnson et al. 2002). Evidence to support a role for the SC in the process of interference comes from species such as *S. pombe* and *A. nidulans* which show very high levels of recombination, perhaps due to the lack of a

conventional SC and therefore no limitation of recombination events (Roeder 1997), (Shaw and Moore 1998).

DSBs play a particularly important role during meiosis because they are repaired using homologous chromosomes rather than sister chromatids. This means that recombination plays an essential role in homologue pairing and hence how chromosomes are segregated and whether the subsequent gametes are viable. It is thought that recombination events in meiosis are catalyzed by large protein complexes that sit at intervals along the SC, called recombination nodules (see figure 12).

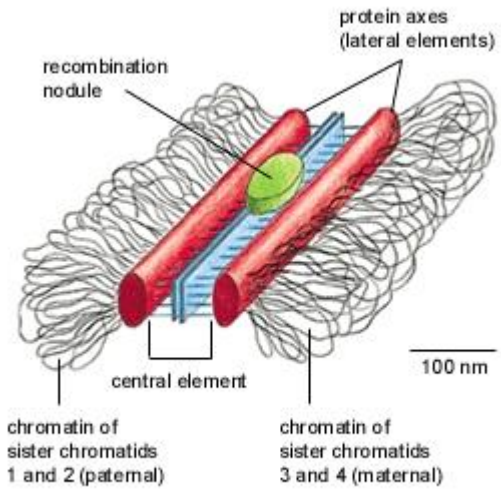


Figure 12:- a short section of a mature synaptonemal complex showing a recombination nodule (Alberts, Johnson et al. 2002).

These recombination nodules contain RAD51, which is the eukaryotic version of the RECA protein that mediates recombination in *E.coli*. There are two types of recombination nodule, early nodules, which are present during leptotene and zygotene, and late nodules which are less numerous and are found during pachytene. Early nodules are thought to mark the sites of the initial DNA strand exchange events of the recombination process (Roeder 1997). It has also been suggested that these early nodules could be the points at which SC formation initiates, and their presence on unpaired chromosomes could suggest a role in the homology search (Dawe 1998).

The process of recombination allows genetic exchange between chromosomes, and starts with the formation of lesions in the chromosome arms termed DSBs. In yeast the enzyme SPO11 catalyzes these breaks, and this protein has been found to be a homologue of the archaebacterial topoisomerase VI (TOP6) (Schwarzacher 2003). This suggests that DSBs are formed by a topoisomerase- like trans-esterification rather than by endonucleolytic hydrolysis (Roeder 1997). In eukaryotes the protein RAD51 is found within the recombination nodules and seems to mediate the process of recombination. RAD51 is also thought to play a role in homolog recognition and pairing (Roeder 1997), (Shaw and Moore 1998), (Pawlowski, Golubovskaya et al. 2003). In fact double strand break formation and strand invasion have long been suggested as a method by which homology searching could take place, with non-homologous regions interacting only transiently, whilst interactions between homologous regions are stabilised (Shaw and Moore 1998), (Kleckner 1996). Whether or not DSB formation impacts on pairing and synapsis seems to vary between organisms (McKim 2005). In some cases pairing and synapsis are DSB dependent, whilst in others they are DSB independent (Shaw and Moore 1998), (Zickler 2006). DSB formation can be disrupted by mutating *rad50*, however this seems to have little impact on homologous pairing (McKee 2004). In *C.elegans* and female *Drosophila*, association of homologous chromosomes and synapsis can occur without DSBs (Shaw and Moore 1998), (Zickler 2006). Conversely in yeast, mice and *Arabidopsis*, DSBs are necessary for chromosome pairing and synapsis (Zickler 2006).

The frequency and distribution of DSBs throughout the genome seems to correlate with the frequency and distribution of recombination events (Roeder 1997). As previously mentioned, the presence of DSBs is not random. They seem to be preferentially induced at recombination hotspots at very specific regions of the genome which vary between organisms. In yeast DSBs are always formed in open chromatin regions, often in the promoter regions of genes. These sites become sensitive to nuclease treatment

immediately before the break occurs. In wheat and rye the frequency of genetic recombination is high near to the telomeres and low in centromeric regions, whilst in *Arabidopsis* areas of low chromatin condensation are favoured (Schwarzacher 2003). In general DSBs are most frequent in areas where the chromatin is in an accessible conformation, and seem to be more frequent in areas of euchromatin that contain actively transcribed genes (Kleckner 1996), (Schwarzacher 2003). Therefore chromosome structure is another factor controlling the location and distribution of double strand breaks.

Studies on *Arabidopsis* have shown that a SPO11 dimer breaks both strands of the DNA, resulting in the formation of a DSB. The 5 prime ends of the DNA surrounding the break are each attached to a SPO11 monomer. For repair of the DSB to occur the SPO11 needs to be removed. Within minutes of the formation of the DSB, histone H2A.X is phosphorylated to form a γ-H2A.X (phosphorylated –H2A.X) domain surrounding the DSB. It is thought that these domains play an important role in recruiting DNA repair factors to the DSB and in changing the chromatin structure to facilitate the repair process. The MRN protein complex which consists of MRE11, RAD50 and NBS1 (Bundock and Hooykaas 2002), (Bleuyard, Gallego et al. 2004), (Puizina, Siroky et al. 2004), (Waterworth, Altun et al. 2007), plays an important role in the repair process. MRE11 possesses nuclease activity suggesting that SPO11 could be removed from the DNA ends surrounding the DSB by MRE11 catalysed cutting of the DNA. It is thought that the SPO11 is released by asymmetrically placed endonuclease cleavages flanking the DSB, a process which establishes asymmetry in the subsequent steps of the meiotic recombination pathway (Keeney and Neale 2006). A 3' ssDNA overhang is generated which is bound by the strand exchange proteins RAD51 and DMC1 which form helical nucleoprotein filaments on opposite sides of the DSB. Homologous recombination is achieved through the single end invasion of one side of the DSB (Bozza and Pawlowski 2008). The replication protein A (RPA) also binds with RAD51 and is involved in the process of strand invasion. Studies of

yeast and mouse show that following single end invasion the meiotic recombination pathway splits into two parallel branches, one which leads to crossovers (COs) and one which leads to non-crossovers (NCOs) (Allers and Lichten 2001), (Hunter and Kleckner 2001), (Guillon, Baudat et al. 2005) Crossovers are reciprocal recombination events that lead to the exchanges of chromosome arms. Non-crossovers (gene conversions) are generated through a non-reciprocal repair of DSBs, without a double Holliday junction intermediate (Bozza and Pawlowski 2008). The current double-strand break repair model for meiotic recombination is shown in figure 13.

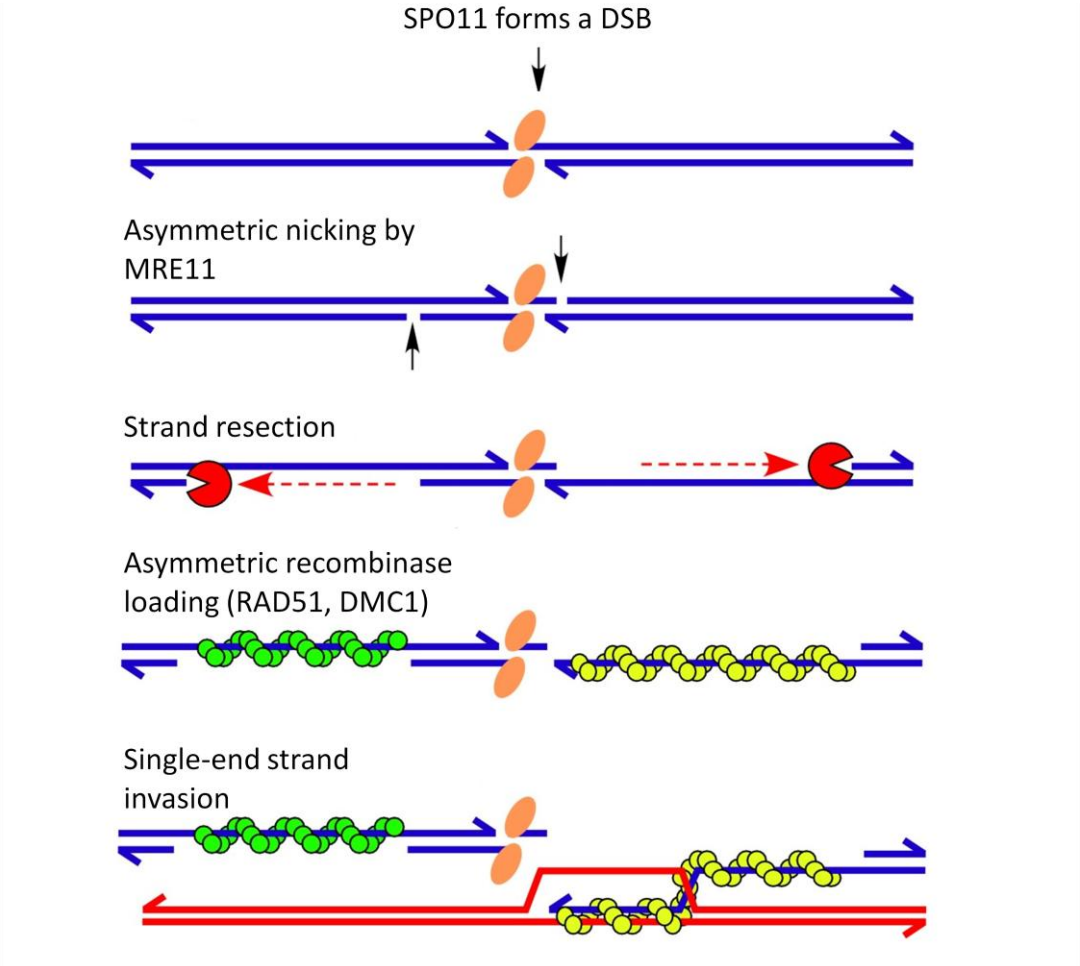


Figure 13:- the current model for meiotic recombination. A SPO11 dimer creates a DSB. The SPO11 is then removed by single-stranded endonucleolytic cleavage releasing SPO11 covalently attached to a short oligonucleotide (Neale, Pan et al. 2005). RAD51 and DMC1 form helical nucleoprotein filaments on opposite sides of the DSB. This is followed by single-end strand invasion leading to a crossover event. Non-crossovers can also occur through the non-reciprocal repair of DSBs.

Figure adapted from (Keeney and Neale 2006)

Each crossover event creates a physical link termed a chiasmata between the homologous chromosomes and in this way recombination not only serves as a mechanism by which DNA is exchanged between homologous chromosomes, which helps to produce genetic diversity, but also provides a physical link between homologous chromosomes when the SC disassembles.

Although it is known that the structure of chromatin is responsible for the distribution of DSBs, little is known about what controls the site at which strand invasion occurs on the donor allele. In diploid species recombination generally occurs between sites at the same chromosomal position on homologous chromosomes, but can also occur between homologous regions on heterologous or homoeologous chromosomes. This leads to ectopic or homoeologous recombination which can result in chromosomal abnormalities which generate unbalanced gametes and therefore infertility. Recent work has suggested that histone modifications could contribute to maintaining genomic stability by preventing recombination between repetitive sequences (Cummings, Yabuki et al. 2007). The mismatch repair system also reduces recombination between non-homologous chromosomes, and the telomere bouquet facilitates the process of homologous recombination by promoting the pairing of homologues. It is now thought that a barrier to sister chromatid repair is created by the meiosis specific kinase, MEK1, in conjunction with two other meiosis specific proteins, HOP1 and RED1 (Hollingsworth, Ponte et al. 1995), (Lao and Hunter 2010), (Niu, Wan et al. 2005). This barrier must be transient because in the absence of homologous chromosomes, or in situations where the homologous partner lacks the corresponding sequence (due to insertions or deletions on one homologue) highly homologous ectopic donor sites on sister chromatids are used for the repair of DSBs (Goldfarb and Lichten 2010). In contrast to diploid organisms, homoeologous or ectopic recombination is prevented in polyploid organisms which contain multiple sets of closely related chromosomes.

1.7 Polyploidy

Polyploidy is a relatively widespread phenomenon in eukaryotes whereby a species possesses two or more sets of closely related chromosomes. There are two ways in which polyploidy can arise. Autopolyploidy occurs as the result of a multiplication of a basic set of

chromosomes whereas allopolyploidy occurs as a result of combining related, but not completely identical genomes (Martinez-Perez, Shaw et al. 2000). Up to 70% of flowering plants have been found to be polyploid (Masterson 1994) as well as some fish, amphibians and even mammals (Gallardo, Bickham et al. 1999). Despite the regular occurrence of polyploidy in nature, a problem is caused by the presence of several sets of similarly structured chromosomes in an organism. These chromosomes must pair and segregate correctly at meiosis or unbalanced gametes will be formed leading to infertility. In effect the polyploid species must behave as a diploid during meiosis with only homologous chromosomes pairing, in order to ensure the production of viable gametes. Maize, a cryptic allopolyploid, has achieved this diploid behaviour during meiosis via extensive rearrangement of its parental genomes so that homology is lost and the polyploid origin of its genomes has been partially hidden.

Hexaploid wheat (*Triticum aestivum)* uses genetically controlled systems which promote homologous chromosome pairing and reduce homoeologouos pairing in order to achieve diploid behaviour during meiosis. Hexaploid, or bread wheat has a genome composed of 42 chromosomes from three homoeologously related ancestral diploid genomes, the A, B and D genomes. Homoeologous chromosomes have very similar gene order and content, varying only in their repetitive DNA content. Therefore during meiosis each chromosome has five very similar potential chromosomes with which to pair. However, pairing is restricted to true homologues only. The lack of homoeologous pairing and recombination cannot be simply attributed to the mismatch repair system because in tetraploid and hexaploid wheat there is less than 1% divergence between the genic regions of related chromosomes.

Many economically important cereals including members of the *Triticeae* tribe are polyploids. In cereals such as wheat, where the economic value comes from the seeds, this process of diploidisation is extremely important as it directly affects the fertility of the

plant. However, this is not the only factor contributing to the importance of the diploidisation process. This mechanism also provides the potential to exploit wild species carrying advantageous traits in breeding programmes. Therefore much research has been directed towards the genomic relationships within the *Triticeae.* The result of sexual hybridisation between a polyploid and a wild species is generally the production of an interspecific hybrid containing a haploid set of polyploid and wild relative chromosomes. However, it is usually found that there are only low levels of pairing and recombination between crop and wild relative chromosomes, despite the fact that subsequent molecular studies have found the two sets of chromosomes to be structurally similar. In cases such as these, pairing and recombination is often prevented by the same mechanisms which prevent homoeologue pairing during the normal meiosis of the polyploid crop. This is a problem for breeding programs as it is difficult to introduce useful traits carried by wild relatives into wheat.

1.8 Chromosome pairing loci

In the case of wheat, diploid behaviour during meiosis is achieved by genetically controlled systems which reduce homoeologous pairing of chromosomes and promote homologous pairing. The major control of this process is provided by a single locus called pairing homoeologous 1 (*Ph1*), located on the long arm of chromosome 5B (Riley and Chapman 1958). However, other loci that contribute to the control of pairing in wheat have also been identified such as *Ph2* on chromosome 3DS, as well as loci on chromosomes 5AL, 5DL, 5AS, 3AL, 3BL and 3DL (Feldman 1993) These loci do not fully compensate for the absence of *Ph1*. The majority of research has been focused on the *Ph1* locus and so the understanding of other loci such as *Ph2* is not as advanced. However, it is thought that *Ph2* affects the synapsis of chromosomes (Martinez, Cuñado et al. 2001). Furthermore a

number of candidate genes have been found to be linked to *Ph2*, including a DNA mismatch repair gene (Dong, Whitford et al. 2002), (Sutton, Whitford et al. 2003).

In the presence of the *Ph1* locus in both tetraploid and hexaploid wheat chromosome pairing during meiosis is restricted to true homologues. Conversely, in deletion mutants lacking the *Ph1* locus the level of homoeologous chromosome pairing during meiosis increases with each generation (Sears 1977), (Roberts, Reader et al. 1999). This eventually leads to sterility as chromosomal rearrangements are accumulated with each new generation (Sánchez, Sánchez-Morán et al. 2001).

1.8.1 The Pairing Homoeologous 1 (*Ph1*) locus

The effect of the *Ph1* locus was first described by Riley and Chapman in 1958. They made squashes of meiocytes at the metaphase I stage of meiosis and observed that in the presence of the 5B chromosome there was no pairing of chromosomes in haploid or interspecific (wheat-rye) hybrid plants (both of which contain no homologous chromosomes). By contrast, when similar squashes were made in the absence of the 5B chromosome pairing was observed between homoeologous chromosomes (Riley and Chapman 1958). Further experiments showed that the long arm of chromosome 5B contained the locus responsible for 'diploidising' the process of meiosis in hexaploid and tetraploid wheat, which was later termed the *Ph1* locus (Riley and Chapman 1958). Subsequently Sears isolated an interstitial deletion (*ph1b* mutant) which further defined the location of the *Ph1* locus to a 70Mb region on the long arm of chromosome 5B (Sears 1977).

The earliest experiments aimed at analysing the mode of action of the wheat *Ph1* locus were based upon classical cytogenetic techniques and the scoring of the pairing configurations seen in metaphase I squashes (Moore 2008). Early studies into how the

Ph1 locus functions showed that the addition of B chromosomes could compensate for the removal of *Ph1* (Dover and Riley 1972). The later observation that B chromosomes increase delayed and asynchronous replication of homoeologous heterochromatin gave some indication as to how the *Ph1* locus might function (Pryor, Faulkner et al. 1980). The implication was that *Ph1* somehow affected heterochromatin and replication (Moore 2008).

Experiments aiming to investigate the effect of the *Ph1* locus on spindle formation revealed some surprising results relating to the dosage effects of *Ph1*. Colchicine and vinblastine treatments were used to disrupt spindle formation in the presence and absence of the *Ph1* locus. Plants lacking the *Ph1* locus were found to have an increased sensitivity to these treatments in comparison to the wild type (Avivi, Feldman et al. 1970; Avivi, Feldman et al. 1970; Avivi and Feldman 1973). Surprisingly, plants with two doses of *Ph1* showed a greater sensitivity to colchicine when treated in conjunction with ATP, than those with no *Ph1* (Avivi, Feldman et al. 1970). An increased dose of the *Ph1* locus has also been shown to contribute to chromosome instability (Vieira, Mello-Sampayo et al. 1991) and disruption of chromosome pairing (Feldman 1966). The fact that duplication of the locus induces a stronger phenotype gives an indication of the complexity of the locus, and also explains why multiple copies have not been preferentially selected for during evolution.

Studies on hexaploid wheat first showed that the chromosomes start synapsing from their telomeres, which are clustered at the start of meiosis. As previously described, this has now been shown to happen in many species. These studies also showed that in wild type hexaploid wheat there are some multiple associations of chromosomes which are corrected at a later stage. In wheat lacking the *Ph1* locus there are far more multiple associations to begin with, and these associations are maintained (Holm 1986; Holm 1988; Holm and Wang 1988). Thus *Ph1* not only reduces the initial level of homoeologous associations, but also affects the stringency at which associations are resolved later in

prophase I (Moore 2008). Several studies have shown that in many species incorrect synapsis of chromosomes triggers a pachytene checkpoint which leads to an elongation of prophase I in order to allow for the correction of any mistakes (Li and Schimenti 2007), (Ghabrial and Schupbach 1999), (Ashley, Gaeth et al. 2004), (Roeder and Bailis 2000), (Bhalla and Dernburg 2005), (San-Segundo and Roeder 1999). This does not appear to happen in the absence of *Ph1* and incorrectly paired chromosomes are allowed to continue into metaphase I. Paradoxically, prophase I in wheat lacking the *Ph1* locus is no longer than that in wild type wheat (Bennett, Dover et al. 1974) and is actually shorter in hybrids between wheat and its wild relatives in the absence of *Ph1* (Gillies 1987). It seems intuitive to expect that the incorrect association of chromosomes should lead to a lengthening of prophase I, as the pachytene checkpoint would prevent progression into metaphase I until incorrect associations had been corrected. The fact that this is not the case suggests that the *Ph1* locus has some function in ensuring mismatch repair. It is possible that in the absence of *Ph1* the pachytene checkpoint is overridden and prophase I becomes shorter as there is no detection or resolution of incorrectly paired chromosomes.

A possible role in the mismatch repair process was again proposed by Dubcovsky and Luo after looking at the effect of *Ph1* on the stringency of recombination following the generation of RFLP genetic maps. The *Ph1* locus was able to block recombination from occurring between similar but distinct chromosome segments located within otherwise identical chromosomes (Dubcovsky, Luo et al. 1995; Luo, Dubcovsky et al. 1996). This implied that *Ph1* could be having an effect on the mismatch repair process which is involved in controlling the stringency at which recombination occurs (Moore 2008).

It was not until the development of techniques allowing the 3D analysis of nuclei that cell biological approaches could be used to accurately investigate the mode of action of the *Ph1* locus, without the possibility of altering the samples during the squashing process (Aragon-Alcaide, Beven et al. 1998). Using these techniques it was shown that

centromeres pair prior to meiosis in both hexaploid and tetraploid wheat (Aragón-Alcaide, Reader et al. 1997), (Martinez-Perez, Shaw et al. 1999; Martinez-Perez, Shaw et al. 2000; Martinez-Perez, Shaw et al. 2001; Martinez-Perez, Shaw et al. 2003). The centromeric associations reduce down to seven sites at the time of the telomere bouquet and then resolve back to their paired state on the nuclear membrane in the presence of *Ph1*. In the absence of *Ph1* they are unpaired and spread throughout the nucleus at this stage. It is impossible to know whether the pairing of centromeres seen pre-meiotically and during the early stages of meiosis are due to homologous or homoeologous associations as there are no probes which can differentiate between homologous and homoeologous wheat centomeres. However, using a wheat-rye hybrid in which there are no homologous chromosomes these associations could be further investigated. In both the presence and absence of *Ph1* the wheat centromeres were seen to cluster into seven groups. It was only when the behaviour of the rye centromeric heterochromatin was visualized that differences could be seen. In the presence of *Ph1* the rye centromeres are associated with the seven wheat centromere clusters. In its absence the rye centromeres remain separate from those of wheat. In the presence of *Ph1* the seven clusters are resolved into 21 wheat foci and seven rye foci (representing 21 wheat homoeologues and seven rye homoeologues) whilst in the absence of *Ph1* the clusters are resolved into 14 groups, seven of which contain rye centromeres, showing that there is pairing between homoeologues both before and during the telomere bouquet in *Ph1*+ and *Ph1*- samples. These homoeologous associations are still seen as meiosis commences in the absence of *Ph1* but are resolved in the presence of *Ph1* (Prieto, Shaw et al. 2004). It is interesting to note that differences in centromere pairing in wheat are not accompanied by a difference in telomere pairing. The telomeres still pair correctly and form the bouquet correctly in both the presence and absence of *Ph1* (Griffiths, Sharp et al. 2006).

Entry into meiosis is accompanied by a remodelling of heterochromatin prior to the intimate pairing and recombination of homologues (Bennett and Stern 1975), (Dawe, Sedat et al. 1994). In the presence of *Ph1* it has been shown that remodelling of homologous heterochromatin sequences occurs synchronously as the cell enters meiosis (Prieto, Shaw et al. 2004), (Colas, Shaw et al. 2008). The homologues then go on to pair via a 'zipping up' process beginning at the telomeres. Pairing and recombination occur successfully and consistently following the synchronous remodelling of homologous sequences. If the sequences are similar but not identical remodelling still occurs but it is no longer synchronous, and pairing occurs via a 'pegging together' of various points along the chromosome before they come together. Successful pairing can be seen in fewer samples, and successful recombination in even fewer. If the sequences are completely different, remodelling does not occur and pairing is only successful in a very limited number of samples, with recombination rarely seen (Colas, Shaw et al. 2008). In contrast pairing and recombination has been observed between homoeologous chromosomes in the absence of *Ph1* (Luo, Dubcovsky et al. 1996). Wheat rye interspecific hybrids have also been used to investigate the remodelling behaviour of heterochromatin (Prieto, Shaw et al. 2004). By using FISH techniques to visualise the subtelomeric heterochromatin it was found that in the presence of *Ph1* homoeologous heterochromatin knobs did not remodel and remained as tight, distinct foci. However, in the absence of *Ph1*, they remodelled synchronously and were seen to subsequently associate with one another. When *Ph1* is present only heterochromatin that is identical can remodel synchronously. Completely identical sequences are only found on homologues meaning that only homologues are remodelled synchronously, at a different time from their homoeologues. Therefore the likelihood of pairing between homologues is increased. In the absence of *Ph1* all similar sequences are remodelled together, meaning that the distinction between homologues and homoeologues is lost. In effect *Ph1* acts to 'diploidise' the remodelling behaviour of

heterochromatin. This helps to explain why the addition of B chromosomes can compensate for the absence of *Ph1* (Dover and Riley 1972). They disrupt the synchronous replication of related but non-homologous heterochromatin (Pryor, Faulkner et al. 1980) which suggests a link between *Ph1's* effect on heterochromatin remodelling and replication.

The *Ph1* locus has also been found to have an effect on one of the earliest expressing meiotic genes, *TaASY1* (Boden, Shadiac et al. 2007; Boden, Langridge et al. 2009), a gene which encodes an axial element associated protein. Experiments done on wheat meiocytes showed that the absence of *Ph1* leads to increased transcription of *TaASY1* and also affects its localisation, causing a more diffuse pattern as it is loaded onto the chromatin, and a spiral- like conformation during zygotene (Boden, Langridge et al. 2009). Interestingly, *Ph1* also failed to prevent pairing between related chromosomes in an *asy1* mutant. These results suggest that the *Ph1* effect has to occur prior to expression of *TaAsy1*, either in premeiotic, or very early meiotic stages.

1.8.2 Defining the *Ph1* locus

The molecular characterisation of the *Ph1* locus has had to overcome many problems. As there is no natural variation of the *Ph1* phenotype, it is only possible to score for its presence and absence. Therefore segregating populations cannot be created, which would be the normal starting point for a positional cloning project. Ethylmethane sulphonate (EMS) treatment fails to generate *Ph1* mutants, which suggests that *Ph1* is not a single gene effect. The fact that duplication of the locus gives a stronger phenotype also indicates that the locus is complex, as otherwise it would have been preferentially selected for during evolution. X-ray and fast neutron irradiation have however managed to create a single deletion of 70Mb within the locus (*ph1b*) (Sears 1977). The *Ph1* locus arose on polyploidisation and so is not thought to have been present in the diploid progenitors of

polyploid wheat. The study of wheat is further complicated by the fact that the wheat genome is 16,000 megabases, five times larger than the human genome. Methods taking advantage of the synteny between cereal genomes and the smaller genomes of rice and *Brachypodium* (Kurata, Moore et al. 1994), (Foote, Griffiths et al. 2004), have become increasingly useful in defining the *Ph1* locus. Recent molecular analyses have made huge progress by using markers from the rice and the *Brachypodium sylvaticum* genomes, which have an extremely similar gene order to that of hexaploid wheat and are much smaller (460Mb and 470Mb respectively). The generation of deletion lines has also allowed the *Ph1* locus to be physically dissected. These studies have defined the *Ph1* locus to a 2.5 megabase structure consisting of a segment of subtelomeric heterochromatin within a series of cyclin dependent kinase (*CDK*) like genes on the long arm of chromosome 5B (Griffiths, Sharp et al. 2006). The identification of additional deletion lines further restricted *Ph1* to a cluster of *Cdk-like* genes (Al-Kaff, Knight et al. 2007). A diagrammatic representation of the *Ph1* region and the deletion lines used to define it are shown in figure 14.

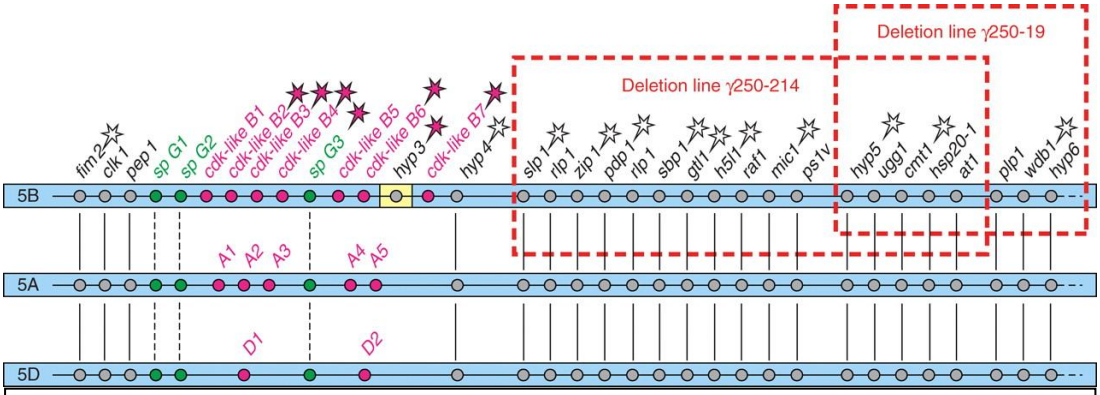


Figure 14:- Schematic diagram of the deletion mutants and annotated genes in the region containing the *Ph1* locus on chromosome 5B compared to the equivalent regions on chromosomes 5A and 5D.

The cluster of *Cdk-like* genes are represented by magenta dots. Grey dots show genes outside of the *Ph1* locus. The yellow box on chromosome 5B represents the inserted sub-telomeric repeats. The stars represent the expression pattern of different genes: uncoloured stars represent genes that are mainly expressed from 5A or 5D, magenta stars represent genes expressed from 5B and genes lacking stars are not expressed Figure and figure legend adapted from (Al-Kaff, Knight et al. 2007).

The insertion of the subtelomeric heterochromatin makes this region distinct from the corresponding *Cdk-like* gene clusters on 5A and 5D. It has also been shown that the *Cdk-like* gene cluster on chromosome 5B is unique compared to the equivalent structures on 5A and 5D (Al-Kaff, Knight et al. 2007).

Transcriptional analysis of the *Cdk- like* genes within the *Ph1* locus has shown that in wild type wheat most of the *Ph1* activity is derived from the cluster of *Cdk-like* genes on chromosome 5B with low levels of transcription of genes on chromosomes 5A or 5D. However, when the *Ph1* locus on chromosome 5B is deleted, the *Cdk-like* genes on chromosomes 5A and 5D increase their expression to compensate for this loss. This suggests that the *Cdk-like* gene complex on chromosome 5B is suppressing the expression of similar gene clusters on chromosomes 5D and 5A (Al-Kaff, Knight et al. 2007). The phenotype produced upon deletion of the 5B *Ph1* locus may be due to the induction and increased expression of the 5A and 5D *Cdk-like* genes.

Detailed bioinformatics has shown that genes within the wheat *Cdk–like* cluster have closest homology to *Cdk2* in humans (Al-Kaff, Knight et al. 2007), (Yousafzai, Al-Kaff et al. 2010). Interestingly, mammalian CDK2 is known to play an important role in meiosis, and has a number of phosphorylation targets (Cohen, Pollack et al. 2006). CDK2–cyclin E complexes are responsible for the complete inactivation of RB and the subsequent progression into S-phase, whilst associations between CDK2 and A type cyclins are also thought to be important for progression through S phase (Ortega, Prieto et al. 2003). Although CDK2 plays an important role within the cell cycle, it has been shown that mitosis can proceed successfully in its absence (or the absence of its partner, cyclin E) (Geng, Yu et al. 2003), (Ortega, Prieto et al. 2003). It does however play an essential role in meiosis, leading to the complete loss of germ cells in mice with mutations in *cdk2* (Ortega, Prieto et al. 2003), (Cohen, Pollack et al. 2006). It is thought that prophase I cannot be completed in

the absence of CDK2 during meiosis (Ortega, Prieto et al. 2003). Further work has shown that CDK2 in humans controls chromatin remodelling at replication (Cohen, Pollack et al. 2006), and is responsible for the remodelling of heterochromatin via the phosphorylation of histone H1 which disrupts the interaction between the histone and heterochromatin protein1 (HP1) (Hale, Contreras et al. 2006). HP1 proteins are the fundamental units of heterochromatin packaging in nearly all eukaryotic organisms, *S. cerevisiae* being a notable exception. The loss of the histone H1 –HP1 interaction causes a decondensation of the heterochromatin, and it is thought that this may be responsible for the disassembly of higher order chromatin structures seen during interphase (Hale, Contreras et al. 2006). It has been suggested that CDK2 plays a role in recruiting mismatch repair proteins to DSBs during the early stages of meiosis (Ward, Reinholdt et al. 2007), which has recently been consolidated by work showing that CDK2 is required during prophase I of male mouse meiosis for the correct processing of programmed DSBs and further recombination, and also for the accuracy of homologous chromosome synapsis and telomere function (Viera, Rufas et al. 2009). Interestingly, it has been shown that human *Cdk2* can compensate for the loss of *Ime2* (a meiosis specific kinase which is necessary for the completion of meiosis) in budding yeast (Szwarcwort-Cohen, Kasulin-Boneh et al. 2009), showing the remarkable degree of evolutionary conservation seen amongst the components of the cell cycle control pathway.

1.9 Investigating the mode of action of the *Ph1* locus in wheat

Over the past decade several exciting advances have been made regarding the *Ph1* locus. The discovery that the locus is composed of a series of cyclin-dependent kinase like genes with closest homology to mammalian *Cdk2*, and that the *ph1* mutant phenotype is probably due to an overexpression of these genes, has prompted a series of experiments to investigate whether the *Ph1 Cdk-like* genes function in a similar way to mammalian *Cdk2*.

The remainder of this thesis will describe the experiments that I have undertaken to further our knowledge in this area.

Wheat-rye hybrid plant material from crosses between rye (*Secale cereale*) cv. Petkus and hexaploid wheat (*Triticum aestivum*) cv. Chinese Spring either carrying or lacking the *Ph1* locus was used for the majority of the experiments described in this thesis. The reason for this is that wheat-rye hybrids often show more pronounced and easily distinguishable effects in the pairing process and in the steps that lead to pairing. This is because they contain a haploid set of both the wheat and rye genome and so contain no homologous chromosomes. In the presence of *Ph1* no pairing occurs between them, whereas in the absence of *Ph1* pairing can be seen to occur. A diagrammatic representation of the wheat rye genome is shown in figure 15.

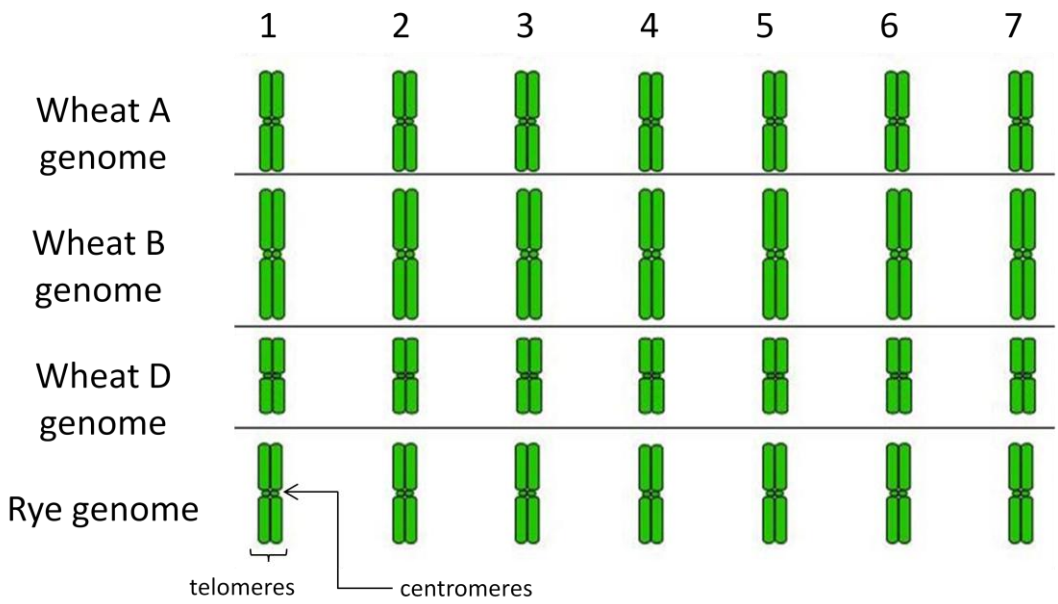


Figure 15: a representation of the wheat-rye hybrid genome which contains a haploid set of the hexaploid wheat genome chromosomes, and a haploid set of the rye genome chromosomes. Therefore there are no homologous chromosomes. The telomeres are located at both ends of each sister chromatid, whilst the centromeres are located at the junction between the long and short arms of the chromosome, at the point where the two sister chromatids are joined together.

Throughout this thesis pre-meiotic refers to the stages after the final mitotic division, but before the formation of the telomere bouquet. Meiosis is considered as starting at the stage of the telomere bouquet.

The primary goal of my PhD project was to establish any differences between meiosis in wheat rye hybrids in the presence and absence *Ph1* that could be contributing to the incorrect pairing seen in mutants lacking the *Ph1* locus. Given the involvement of the CDKs in the *Ph1* story, the first way in which I undertook this was to look at pre-meiotic replication using cell biological techniques. This work is described in chapter 2, which starts with a detailed summary of previous work looking at the process of replication in wheat. The techniques used showed some interesting differences in the progression of replication in *Ph1+* and *Ph1-* meiocytes, consistent with the increase in CDK activity that is associated with the *Ph1* mutant. These results are detailed and discussed in the remainder of chapter 2.

Chapter 3 looks at differences between the two genotypes both pre-meiotically and as they enter meiosis, particularly with regard to chromatin remodelling. Cell biology techniques were again employed to show that the process of chromatin remodelling varies between lines either in the presence or absence of *Ph1*. The potential reasons for these differences are discussed.

Techniques to investigate the levels of expression of *Cdk-like* genes and other early meiotic genes are described in chapter 4. A yeast-2-hybrid experiment aiming to establish the interactions of the wheat *Cdk-like* D2 gene is also described. The results gained from these experiments and the problems involved in carrying out these experiments are discussed.

Finally, chapter 5 gives a brief summary of the work described in this thesis, and also comments on the direction that will be taken in the future to further our knowledge of the *Ph1* locus.

Chapter 2: a study of pre-meiotic replication in relation to centromere and telomere dynamics in the presence and absence of *Ph1*

Abstract

Despite possessing multiple sets of related chromosomes, hexaploid (bread) and tetraploid (pasta) wheat both distinguish between the related chromosomes, behaving as diploids at meiosis. The distinction between homologous and related chromosomes is controlled by a single major locus, *Ph1*. Recently this locus has been defined to a cluster of defective *Cdk-like* genes which show some homology to mammalian *Cdk2*. We reasoned that these defective genes might be suppressing activity of CDKs with functional similarity to CDK2, a hypothesis that has recently been confirmed by proteomics work which shows that histone I phosphorylation is increased at CDK consensus sites in the absence of *Ph1*. CDK2 is known to play an important role in replication and so a change in CDK2 like activity should also be accompanied by a change in replication. Therefore a series of experiments was conducted to examine whether replication is altered when *Ph1* is removed. Cell biological studies reported here do indeed reveal such effects associated with *Ph1.*

The work presented in this chapter is based around a paper that is currently being prepared for publication:-

Emma Greer, Azahara Martin, Alison Pendle, Isabelle Colas, Alex Jones, Graham Moore and Peter Shaw (in preparation). Phosphorylation controls homologue specificity in meiosis.

Chapter 2: a study of pre-meiotic replication in relation to centromere and telomere dynamics in the presence and absence of *Ph1*

2.1 Introduction

The role of CDKs in regulating the process of replication has been widely reported (for reviews see (Bell and Dutta 2002), (Nurse 2000)). Indeed recent studies have shown that altering the activity of CDKs has a significant effect on replication, with a slower replication rate being associated with a lowering of CDK activity, and a higher replication rate being associated with an increase in CDK activity (Thomson, Gillespie et al. 2010). These changes are attributed to a change in the number of active origins rather than a change in the distribution of active origins (Thomson, Gillespie et al. 2010). Likewise, experiments on *V.faba* where CDK activity was artificially increased using okadaic acid showed significant changes in replication (Polit and Kazmierczak 2007). CDK2 is known to be of particular importance for entry into and progression through S-phase (Ortega, Prieto et al. 2003) and is known to activate origins of replication (Krasinska, Besnard et al. 2008). This is of interest with regard to the *Ph1* locus because of the likelihood that the *Ph1* phenotype is caused by increased activity of CDKs with closest homology to mammalian CDK2. Recent work has confirmed the hypothesis that CDK activity is increased in the absence of *Ph1* by showing that histone H1 phosphorylation is increased at CDK consensus sites in *Ph1b* mutants(Greer, Martin et al. In preparation). Given the importance of CDK2 in replication, it is likely that a mutant exhibiting changes in CDK activity will also show changes in replication.

Much work has gone into establishing the relative stages and timings of meiosis and pre-meiosis in wheat (Bennett 1971; Bennett, Chapman et al. 1971; Bennett, Rao et al. 1973; Bennett, Dover et al. 1974). Studies looking at the 48 hours preceding meiosis have shown that following the final premeiotic mitosis of the pollen mother cells (p.m.c.), the cells of the tapetum which surround the meiocytes (tapetal cells) undergo a period of DNA

synthesis, however the tapetal cells are not synchronised in this synthesis, with their DNA content ranging from 2C to 4C. This is followed by a stage at which DNA synthesis cannot be seen in either the tapetal cells or the meiocytes. Interestingly, it has been demonstrated that at this stage temperature changes can affect chromosome pairing and chiasma formation (Bayliss and Riley 1972). A round of synchronous DNA replication is then seen in both the meiocytes and tapetal cells, lasting between 12 and 15 hours prior to the onset of meiosis (Bennett, Rao et al. 1973). Meiosis then commences and has been estimated to last for 24 hours in wheat (Riley and Bennett 1971). The duration of meiosis shows no correlation to the degree of homoeologous pairing, showing that the time available for pairing cannot be the factor which permits homoeologous pairing in the absence of *Ph1* (Bennett, Dover et al. 1974). It has also been suggested that there is no relation between homoeologous pairing and pre-meiotic timing, although further results are required to confirm this (Bennett, Dover et al. 1974).

Several observations have pointed to the importance of events prior to the onset of meiosis as being key in controlling correct homologous pairing during meiosis. A failure in pairing can be seen in wheat samples treated with colchicine, but only if it is applied immediately after the final pre-meiotic mitosis (Dover and Riley 1973). Likewise, the temperature sensitive stage at which pairing and chiasma formation can be affected, occurs before the onset of S-phase (Bayliss and Riley 1972; Bayliss and Riley 1972). Treatment of plants with okadaic acid can also induce non-homologous pairing at metaphase I, but similarly only if the treatment time is prior to the onset of meiosis (Knight, Greer et al. 2010).

The idea that the process of chromosome pairing begins prior to meiosis has long been suggested. Furthermore, several early studies have shown that chromosomes are

associated as pairs during pre-meiotic interphase across a variety of species (reviewed by Grell in (Caspari and Ravin 1969)). Studies on the grasshopper *Brachystola magna* showed these associations to be homologous (article reproduced in (Sutton 2009)). In wheat, homologous chromosomes are found to be significantly closer to one another than non-homologous chromosomes in somatic cells (Avivi, Feldman et al. 1982), suggesting that the layout of the somatic nucleus is highly ordered so that homologues and non-homologues occupy distinct territories. More recent work has confirmed that homologous chromosomes associate pre-meiotically in *S.cerevisiae*, some fungi, rice and in hexaploid wheat (Weiner and Kleckner 1994), (Loidl 1990), (Roeder 1997), (Prieto, Santos et al. 2004) (Aragón-Alcaide, Reader et al. 1997). In wheat and rice, centromeric associations are seen pre-meiotically (Prieto, Santos et al. 2004), (Aragón-Alcaide, Reader et al. 1997), however these associations are not required for the successful pairing of homologous chromosomes (Lukaszewski 1997). It has even been suggested that synaptonemal complex formation commences during S-phase in wheat, meaning that homologous chromosomes must be associated to some degree prior to the onset of meiosis (McQuade and Bassett 1977; McQuade and Pickles 1980). Indeed, studies using FISH to monitor the behaviour of subtelomeric heterochromatin have shown that these sites pair prior to the telomere bouquet, and therefore before the onset of meiosis (Colas, Shaw et al. 2008).

The process of replication has been found to be directly linked to DSB formation in *S.cerevisiae* (Borde, Goldman et al. 2000), (Murakami, Borde et al. 2003). If replication is blocked or delayed then DSB formation either fails to occur or is also delayed. The coupling of DSB formation with pre-meiotic replication is particularly interesting because of the role that DSBs have been suggested to play in the homology search (Shaw and Moore 1998),

(Kleckner 1996). This observation again highlights the importance of pre-meiotic events in the pairing process.

Meiotic S-phase is known to be much longer than mitotic S-phase (Williamson, 1983), suggesting that there are some additional processes required at this stage relating to the meiotic cell cycle. Studies on *S.pombe* have revealed that during pre-meiotic S-phase, the association between REC8 and the centromeres must be established to allow the reductional chromosome segregation necessary during meiosis I. REC8 is also thought to promote high levels of recombination (Watanabe, Yokobayashi et al. 2001). The function of REC8 must be linked to DNA replication in some way because S-phase takes longer in *rec8* mutants (Cha, Weiner et al. 2000).

The advent of new techniques with which to study replication allows the process to be studied in a much greater detail. Previously replication was studied using the incorporation of ^{3}H-thymidine into replicating DNA, followed by scintillation counting or autoradiography. This technique is slow and labour intensive and carries with it the risks of handling and disposal of radioactive material. The development of 5'-bromo-2'-deoxyuridine (BrdU) and 5'-ethynyl-2'-deoxyuridine (EdU) allows the easy incorporation of these thymidine analogues into replicating DNA which is then followed by immunocytological detection using fluorescently labelled antibodies.

This chapter focuses on a series of experiments that were undertaken to examine pre-meiotic replication in wheat-rye hybrids using both BrdU and EdU to label proliferating cells. These techniques were combined with fluorescent in situ hybridisation (FISH) to monitor the progression of replication in relation to the organisation of the centromeres and telomeres. These experiments not only show a change in pre-meiotic replication in the

absence of *Ph1*, but also show that the centromeres and telomeres begin to organise themselves and pair much earlier than previously thought, during the replicative stage of the cell cycle.

2.2 Methods

Plant Material

The tillers used for this study came from crosses between rye (*Secale cereale*) cv. Petkus and hexaploid wheat (*Triticum aestivum*) cv. Chinese Spring either carrying or lacking the *Ph1* locus.

BrdU and EdU treatment

Tillers containing an immature pre-meiotic spike were cut immediately after an 8hr period in the dark and transferred immediately to a solution of100mM sucrose and BrdU (1mM) (Merck Chemicals: 203806) or EdU (1mM) (Invitrogen: A10044). The cut tillers in individual tubes were left in the light for four hours, after which the spike was dissected out and fixed in 4% paraformaldehyde solution as described in (Prieto, Shaw et al. 2004) (detailed protocol described in appendices 1, 2 & 3). The fixed samples were placed in biopsy cassettes and run through a Tissue-tek vacuum infiltrator processor (VIP) machine as described in (Schwarzacher and Heslop-Harrison 2000). They were then sectioned using a microtome as described in (Armstrong, Franklin et al. 2001). A detailed protocol for vacuum infiltration processing and sectioning is given in appendix 1.

BrdU detection

Samples were digested using 2% cellulose, 2% pectolyase for 1hr at 37˚C, then washed. A 35minute denaturation treatment in 1.5M HCl was carried out at 37˚C after which samples were immediately placed into ice cold PBS for 5 minutes. The slides were then blocked at R.T. for 1hr using 5% BSA and then incubated with an anti-BrdU antibody (1:1000) for 3hrs at R.T. Samples were then washed and placed to incubate with anti-mouse alexa 488 (1:1000) overnight in the fridge after which they were washed, stained with DAPI and mounted in Vectashield. A detailed protocol is given in appendix 2.

EdU detection

EdU detection was carried out using a Click-iT® EdU Alexa Fluor® 488 Imaging Kit, as per manufacturer's instructions (Invitrogen: C10337) (detailed protocol described in appendix 3). Fluorescence microscopy and image processing techniques used in the EdU detection and fluorescence in situ hybridisation procedures have all been previously described (Prieto, Moore et al. 2007). A generalized linear model with binomial

distribution and a logit link function was used to statistically analyze the number of nuclei in each category of replication.

Fluorescence in situ hybridisation

Protocols for making the telomere, rye heterochromatin knob, and wheat centromere probes have previously been described (Colas, Shaw et al. 2008), (Aragón-Alcaide, Miller et al. 1996). New primers were designed from the *Secale cereale* retrotransposon Bilby sequence, to make the rye centromeric probe (F: gatagaacaccacaccgaactcgtacccgg, R: acttaaattgtgtaagaggtaagcacttgc). In situ hybridisation was carried out as described in (Prieto, Moore et al. 2007) and is outlined in detail in appendices 4 & 5.

Time course experiments

In order to try and better understand the timing of the different stages of meiosis in the presence and absence of *Ph1* some time course experiments were conducted. Two methods were used. The first involved giving the plants a single pulse of EdU and then leaving them for various times to see how far they had progressed within the given time. The second was carried out by giving an initial pulse of BrdU, followed with a pulse of EdU at various points throughout a 24hour period. These methods are described in detail in appendices 2 & 3. The spikelets were then sectioned, and the labelling patterns analysed.

2.3 Results

The pattern of replication in wheat rye anthers was analysed using either BrdU or EdU incorporation. Spikes were pulsed with BrdU or EdU solution for a period of four hours and were then fixed. The full method is explained in detail in the methodology section, however, it is important to emphasise the point that the telomeres and centromeres are fixed at the end of the four hour period throughout which the spikes were pulsed with either BrdU or EdU.

The pattern of replication in the presence and absence of *Ph1* was analysed using BrdU and does not show any differences from previous reports of replication in hexaploid wheat (Bennett 1971; Bennett, Rao et al. 1973; Bennett 1977), in either genotype. Images showing the progression of replication in wheat rye hybrids are shown in figure 1. In short,

prior to meiocyte replication the tapetal cells can be seen to replicate asynchronously (figure 1a). Less than four hours after the tapetal cells have undergone the asynchronous replication stage, the meiocytes begin to replicate (figure 1b). At this point the telomeres are still dispersed. In the four hours preceding the tight telomere bouquet no replication can be seen in the meiocyte or tapetal cells (figure 1c). The tapetal cells go through a synchronised round of replication within four hours of the dispersal of the bouquet (figure 1d).

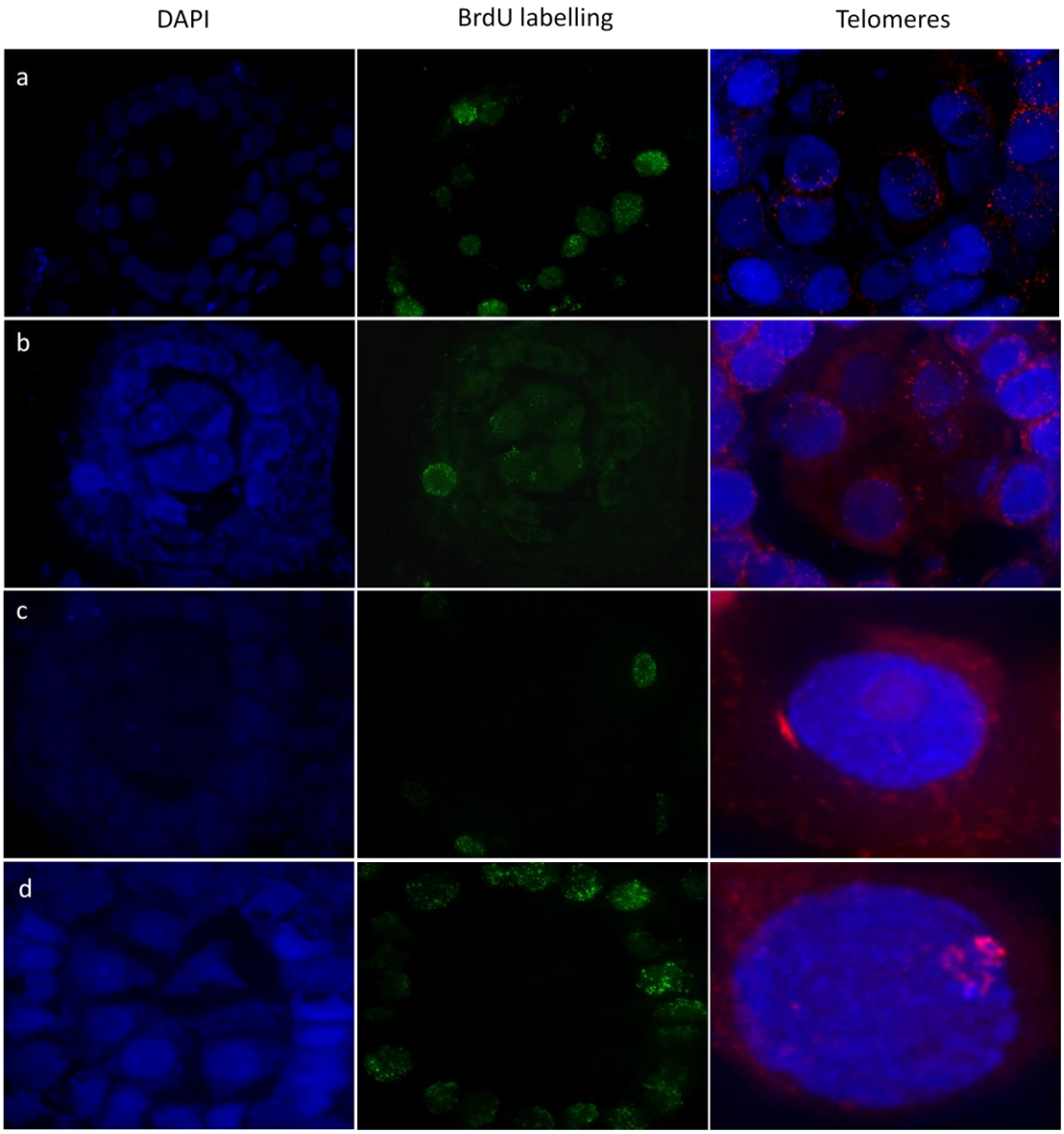


Figure 1: BrdU labelled replication pattern of wheat-rye hybrid anthers in relation to the telomere dynamics. Prior to meiocyte replication the tapetal cells replicate asynchronously whilst the telomeres are still dispersed (a). The meiocytes begin replication within four hours of the tapetal cells undergoing asynchronous replication. At this stage the telomeres are still dispersed (b). In the four hours prior to the telomeres forming the tight bouquet there is no replication (c). Within four hours of the telomere bouquet beginning to disperse, the tapetal cells undergo a synchronous round of division (d).

There were a number of problems with using BrdU to label replication, primarily because the DNA must be denatured in order to allow the large anti-BrdU antibody to access any BrdU that has been incorporated into the DNA. This results in badly DAPI stained sections and means that FISH cannot be carried out on the same section as the BrdU detection. For these reasons, when Invitrogen developed a new label for replicating DNA, EdU, it was decided to try it out. The detection of EdU is based on a click reaction, whereby copper (I) catalyses a reaction between an azide and an alkyne. The EdU contains the alkyne which is reacted with an azide containing detection buffer to form a triazole ring. The major advantage of this is that the detection azide is very small and so detection can take place without a requirement for harsh denaturation or digestion treatments (full details can be found on the Invitrogen website: http://probes.invitrogen.com/media/pis/mp10044.pdf.

Labelling the synthesis of DNA using EdU gave very good results, with nice DAPI labelling and the option of combining EdU detection and FISH on the same sections. For this reason it was decided to use EdU to both confirm the results gained using BrdU and take good pictures of them, and for all further work looking at replication. Figure 2 shows a comparison of BrdU labelling with EdU labelling.

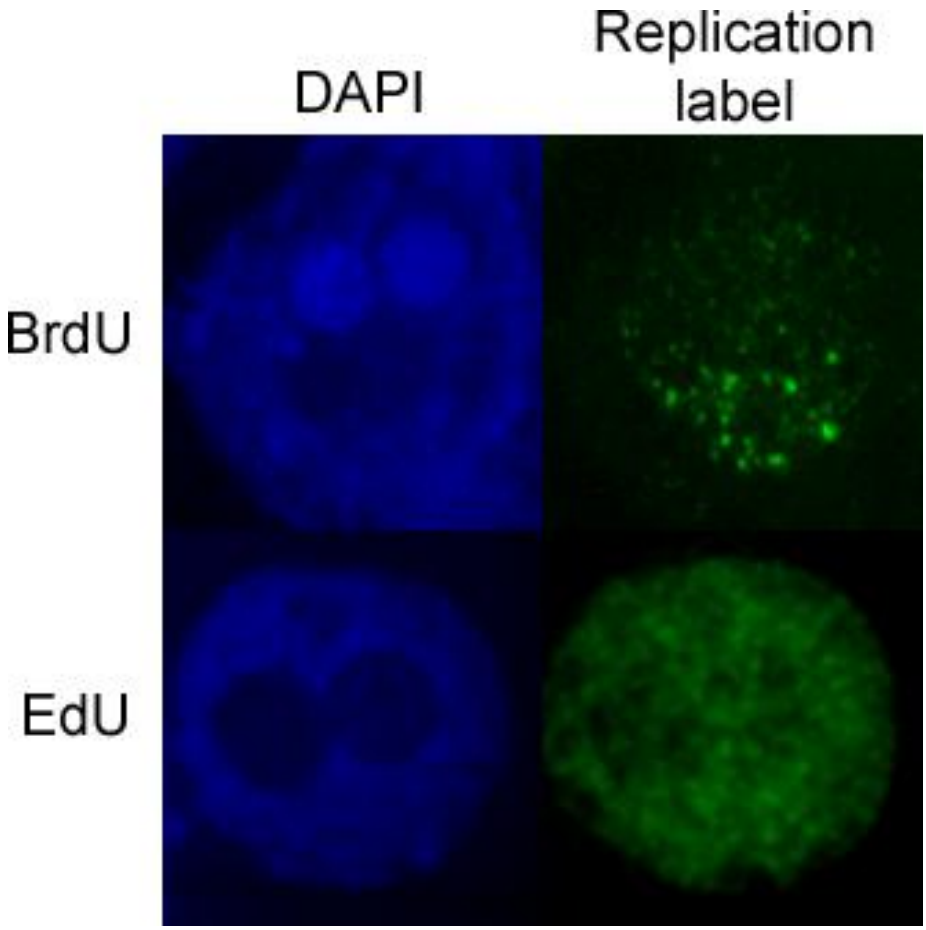


Figure 2: comparison of EdU and BrdU techniques for labelling replicating DNA. The denaturation involved in the BrdU method gives bad DAPI staining and patchy labelling of replication. In comparison the DAPI staining works well, and the replication labelling is not patchy when using EdU.

The time course experiments conducted did not work as well as hoped and so accurate timings for each of the stages of replication could not be established. Limited results were however gained from these experiments which showed that replication commences at approximately the same time in the *Ph1+* and *Ph1-* plants, and that the different stages of replication seem to take the same times in both genotypes. Figure 3 shows some images from this experiment which help to put approximate timings on the stages of meiosis and pre-meiosis.

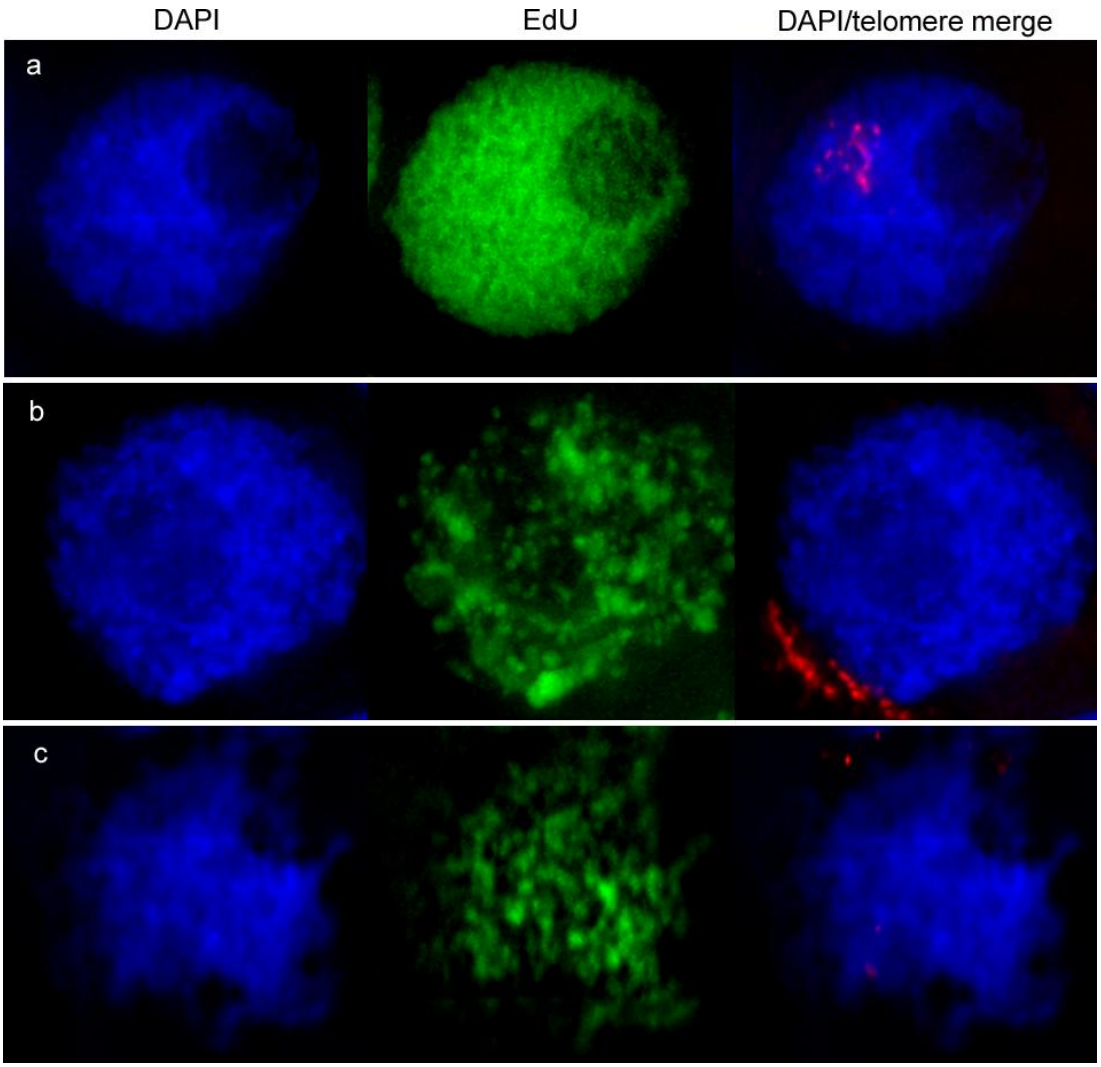


Figure 3: images of meiocytes after they have been pulsed with EdU and left for a certain time. The images in panel a show that the dispersed chromatin replicated approximately 26 hours prior to the telomeres beginning to cluster. Approximately 20 hours before the tight bouquet, the dispersed chromatin and heterochromatin are replicating (panel b). A similar stage of replication, with both dispersed chromatin and heterochromatin replicating is seen 30 hours before pachytene (panel c).

Despite the lack of differences in the overall timing of the stages of replication, differences can be seen in the later stages of replication when the meiocytes are studied in detail. The stages of meiocyte replication were split into three groups:- i)dispersed

chromatin replication only (corresponding to the images in figure 4(b)), ii) dispersed chromatin and rye heterochromatin knob replication (corresponding to the images in figure 4(c)),iii) predominantly rye heterochromatin knob replication (corresponding to the images in figure 4(d)). Over 1000 nuclei (*Ph1+* and *Ph1-*) were sorted into each group based upon visual observations (counts are shown in table 1). The percentage of *Ph1+* and *Ph1-* nuclei in each category of replication is shown in figure 5. Accumulated analysis of deviance demonstrated that there was no difference between the proportion of nuclei in groups i) and iii) in *Ph1+* and *Ph1-* samples ($p > 0.5$ for each group). However, there were significantly fewer *Ph1-* nuclei in group ii) than *Ph1+* nuclei (accumulated analysis of deviance showed $p = 0.005$).

The number of wheat and rye centromeric sites was also examined in relation to replication (images are shown in figure 6). Before replication commences the centromeres are unpaired (figure 6a), but associate as approximately 14 sites as the beginning of meiocyte replication approaches (figure 6b). Throughout the stages of meiocyte replication the number of associations varies between 14 and seven sites (figure 6c-f), including the four hours after replication of the centromeric regions (figure 6 d & e). In the four hours after replication finishes the centromeres disassociate and develop a more diffuse structure consistent with the start of remodelling (figure 6g). Figure seven shows the mean centromere counts in nuclei at the different stages of replication. The mean number of associations reduces throughout the first stages of replication, reaching a minimum as the dispersed chromatin and heterochromatin are replicating. The mean number of associations then begins to increase until they are un-paired as the tapetal cells undergo synchronised replication. Interestingly the number of associations was not constant across the nuclei counted at each stage, with numbers varying from seven to 14. This suggests a

dynamic process of centromeric association in which the centromeres are continually pairing and then further associating into larger groups throughout the process of replication.

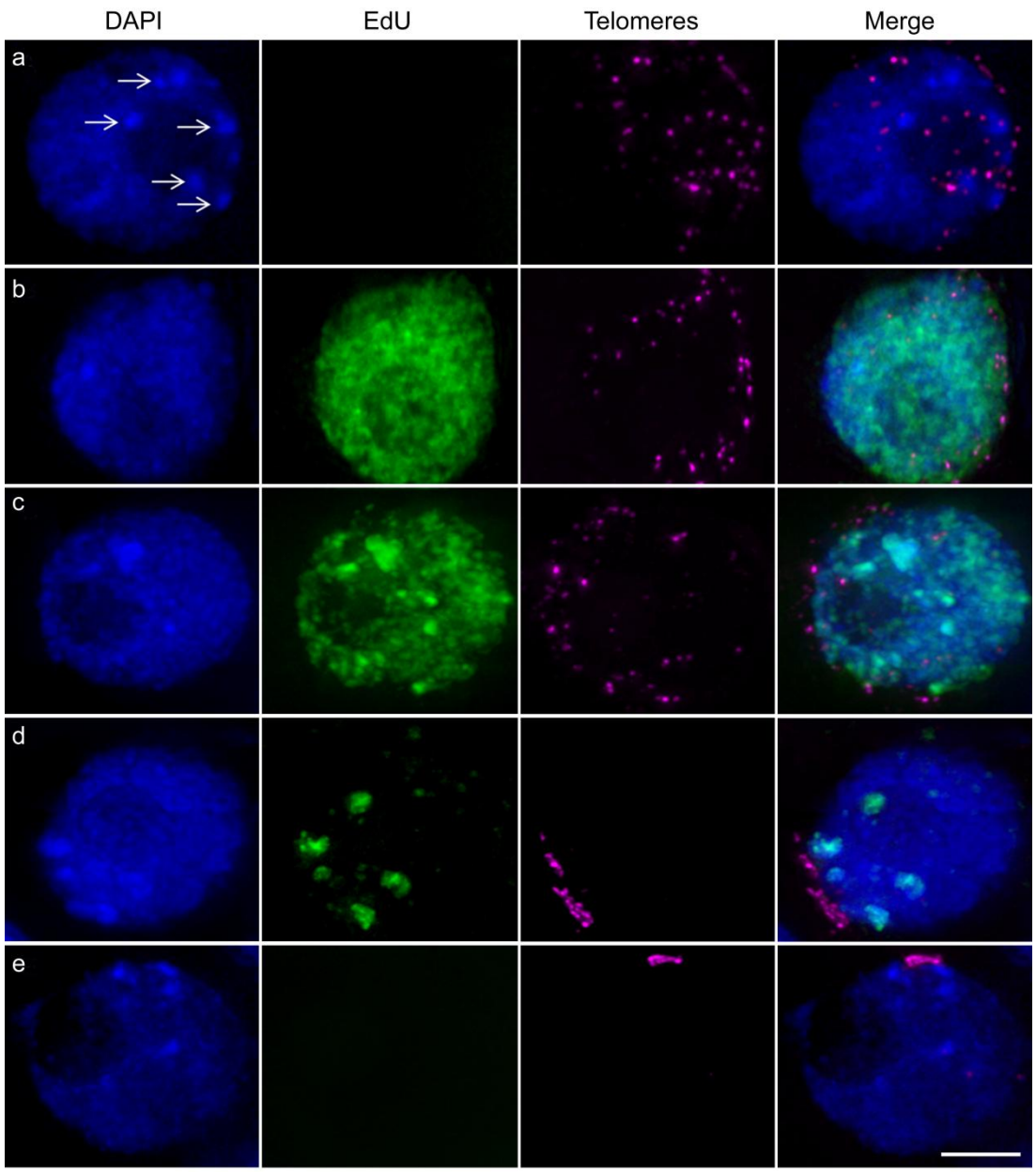


Figure 4: The progression of meiocyte replication in relation to telomere dynamics. (a) prior to meiocyte replication the telomeres are dispersed around approximately half of the nuclear periphery. (b) as meiocyte replication begins the telomeres begin to cluster together on the nuclear membrane. At this point only the dispersed chromatin can be seen replicating. (c) the rye subtelomeric heterochromatin knobs (can be seen as the intensely DAPI stained regions, examples of which are highlighted by arrows in panel a) begin to replicate at the same time as the dispersed chromatin. At this point the telomeres are still aligning on the nuclear membrane. The rye heterochromatin knobs continue to replicate as all other replication finishes until they are the only sites of replication (d). During this time the telomeres are becoming increasingly clustered on the nuclear membrane. (e) the telomeres form a tight cluster or 'bouquet' on the nuclear membrane. At this point all replication has finished. The nuclei shown in this figure are from *Ph1+* samples. *Ph1-* nuclei progress through replication in the same way. Scale bar represents 5um.

	Dispersed chromatin only	Dispersed chromatin and rye heterochromatin knobs	Predominantly rye heterochromatin knobs	Total number of nuclei counted
Ph1+	343	102	166	611
Ph1-	252	8	138	398

Table 1: number of *Ph1+* and *Ph1-* nuclei falling into each category of replication.

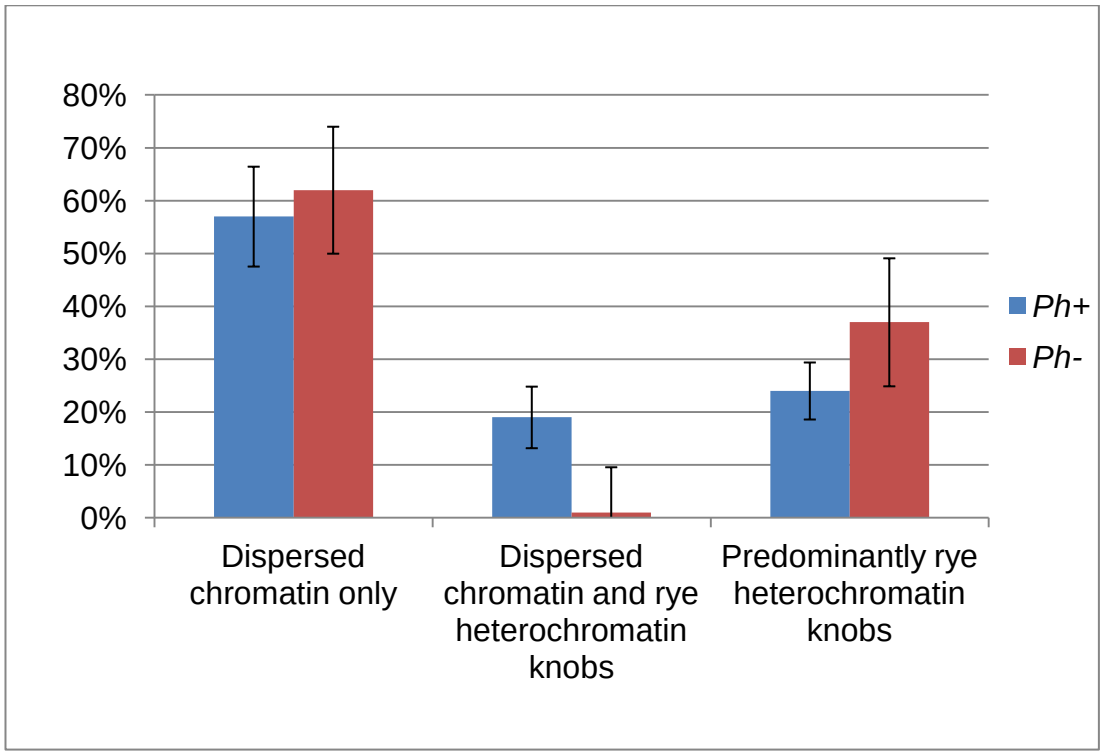


Figure 5: The percentage of *Ph1+* and *Ph1-* nuclei that fall into each category of replication. There are significantly fewer *Ph1-* nuclei in the dispersed chromatin and rye heterochromatin knob replication category, than there are *Ph1+* nuclei. No significant differences were found between the percentages of nuclei in the other categories. Error bars represent the standard error.

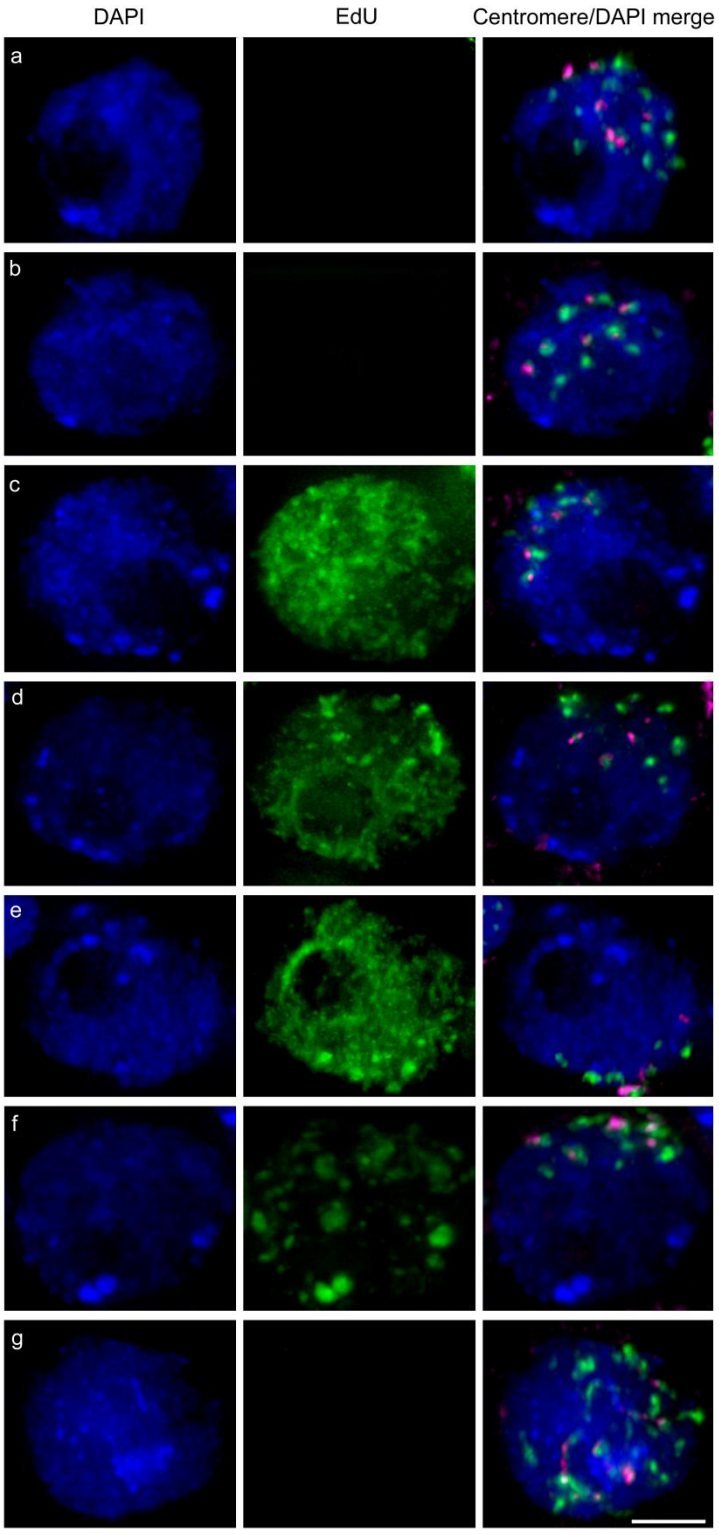

DAPI
EdU
Centromere/DAPI merge
a
b
c
d
e
f
g

Figure 6: (previous page) The dynamics of wheat (green) and rye (magenta) centromeres throughout pre-meiotic replication. Prior to replication the centromeres are unpaired (a). Just before replication begins the centromeres associate as approximately 14 sites (b). As replication commences the centromeric associations persist (c). At this point there are between 14 and seven centromeric sites. The cenromeres replicate within four hours of the dispersed chromatin, at which point they can be visualised as between 14 (d) and seven (e) sites. During the later stages of meiocyte replication, up to four hours following the rye subtelomeric heterochromatin knob replication, the centromeres begin to separate (f). As replication finishes the centromeres are unpaired (g) and begin to remodel. A mixture of *Ph1+* and *Ph1-* nuclei were used to compose this image because the centromere dynamics are the same in both genotypes. Scale bar represents 5um.

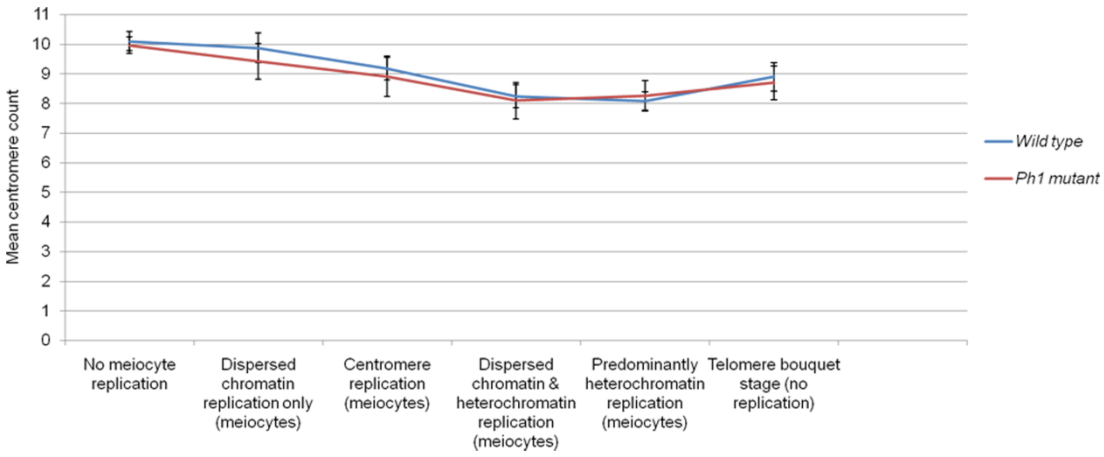


Figure 7: graph showing the mean number of centromere sites at the different stages of replication. Just prior to meiocyte replication beginning the centromeres associate. These associations persist and reduce in number as replication proceeds, reaching a minimum number up to four hours after the dispersed chromatin and heteochromatin replicate. The associations then begin to increase in number until the centromeres are unpaired up to four hours following synchronous tapetal replication. Error bars show the standard error.

2.4 Discussion

This detailed study of pre-meiotic S-phase in wheat-rye hybrid plants in the presence and absence of the *Ph1* locus has shown some interesting results. The general pattern of replication matches previous reports detailing replication in wheat, with the notable addition that the rye subtelomeric heterochromatin knobs are visible in wheat-rye hybrids, and so can be studied. The replication of these heterochromatin knobs begins towards the end of the dispersed chromatin replication period, consistent with previous reports of the timing of heterochromatin replication (Döbel, Schubert et al. 1978).

Although there doesn't seem to be any difference in the timing of the onset of meiosis in *Ph1+* and *Ph1*- plants, an interesting difference can be seen later in S-phase. In the presence of *Ph1,* the replication of dispersed chromatin and the rye subtelomeric heterochromatin knobs are within four hours of one another, and are often seen to both be labelled in the EdU images. The length of time between the two phases increases to over four hours in the absence of *Ph1*, and the two are rarely seen to be both labelled in the EdU images. Considering that replication starts at approximately the same time in the presence and absence of *Ph1*, there are two possibilities which could explain this observation. The first of these is that replication of the dispersed chromatin is shorter in the absence of *Ph1*. The second is that replication of the rye subtelomeric heterochromatin starts later in the absence of *Ph1*. Neither of these hypotheses can be discounted on their own, but a combination of the two would be most in line with our current understanding of the *Ph1* locus. The *Ph1* mutant phenotype is known to be caused by an overexpresison of *Cdk-like* genes, with closest homology to mammalian *Cdk2* (Al-Kaff, Knight et al. 2007), (Yousafzai, Al-Kaff et al. 2010). As CDK2 is known to promote

progression through S-phase and promote the activation of origins of replication it would be no surprise that an overexpression could speed up progress through S-phase. Okadaic acid is a potent protein serine threonine phosphatase inhibitor which activates CDKs and promotes premature chromosome condensation (Yamashita, Yasuda et al. 1990). It has also been shown to be able to induce a *Ph1*- phenotype in treated *Ph1+* wheat-rye tillers (Knight, Greer et al. 2010). Okadaic acid treated *Vicia faba* cells exhibit both an increase in protein kinase activity and an accelerated S-phase (Polit and Kazmierczak 2007). It would be reasonable to assume that a similar mechanism is taking place in the *Ph1*- meiocytes. Polit and Kazmierczak (2007) also noted that initiation of heterochromatin replication is delayed in okadaic acid treated cells. This could also be happening in the *Ph1*- meiocytes, but as the replication of the rye heterochromatin knobs finishes at approximately the same time whether in the presence and absence of *Ph1*, the duration of heterochromatin replication would also have to be accelerated for this hypothesis to stand alone. It is possible that replication is accelerated and the rye heterochromatin knob replication is delayed in the *Ph1*- meiocytes.

Throughout pre-meiotic replication the telomeres begin to associate and cluster, eventually forming a tight bouquet on the nuclear membrane. During this time the centromeres also begin to associate at the opposite pole. The timing of the rye heterochromatin knob replication in relation to the behaviour of the telomeres raises another interesting point. Replication of these areas is still ongoing within four hours of the telomeres beginning to cluster tightly on the nuclear membrane. BrdU labelling of wheat meiocytes has also shown that replication is still occurring as the telomere bouquet is forming. This puts the timing of the beginning of telomere bouquet formation in wheat and wheat-rye hybrids as either during late interphase or very early in meiotic prophase.

This timing is different to that seen in maize (Harper, Golubovskaya et al. 2004), (Sheehan and Pawlowski 2009), and yeast (Joseph and Lustig 2007), where bouquet formation commences during the leptotene-zygotene transition, although it is consistent with observations made in wheat (Martinez-Perez, Shaw et al. 1999). The process of telomere bouquet formation and homologous chromosome pairing seems to be one of the earliest events in meiosis in wheat and wheat-rye hybrids.

The behaviour of the centromeres throughout S-phase also suggests that the process of homology searching and chromosome pairing occurs earlier than previously thought. In fact, the centromeres can first be seen to associate before pre-meiotic replication begins. They then exhibit a dynamic behaviour where they move between pairs and larger clusters throughout S-phase, until they separate again at the end of S-phase. It has previously been shown that early centromeric pairing in wheat occurs between non-homologous chromosomes (Aragón-Alcaide, Reader et al. 1997), (Maestra, de Jong et al. 2002), (Martinez-Perez, Shaw et al. 2001). However, these pairs are seen to further cluster into seven groups (Martinez-Perez, Shaw et al. 2003) before resolving as homologously paired partners (Stewart and Dawson 2008). In a wheat-rye hybrid there are no homologous chromosomes and so the dynamic associations seen between the centromeres could be due to a continual process of forming into larger groups to find a potential partner and then breaking away in pairs to undergo a homology check. This process can be seen taking place throughout pre-meiotic replication, until the centromeres resolve as unpaired sites after replication.

The behaviour of the telomeric and centromeric sites throughout S-phase suggests that the process of homology searching and pairing begins during the replicative stage rather than during meiosis. These observations may explain why treatments of colchicine,

temperature or okadaic acid during S-phase have the ability to disrupt pairing during meiosis (Dover and Riley 1973), (Bayliss and Riley 1972; Bayliss and Riley 1972), (Knight, Greer et al. 2010). The presence of synaptonemal complexes in pre-meiotic interphase also serve to strengthen the argument that the process of pairing begins prior to meiosis (McQuade and Bassett 1977; McQuade and Pickles 1980). It is even thought that the process of replication could be linked to the process of homologous chromosome pairing. For example, in lilies a small portion of the genome goes through delayed replication, coinciding with the beginning of synapsis. This replication is thought to be located near to the SC structures (Hotta and Stern 1971). This raises the interesting question as to whether, in this large genomed species, only a small portion of the genome is used for the homology search, and this is achieved through the late replication of certain sequences. Perhaps a similar mechanism is being used in wheat, and the difference in replication seen in the absence of *Ph1* leads to the disruption of the homology search, and therefore the incorrect pairing seen during meiosis.

Throughout these experiments the behaviour of the telomeres was assumed to be constant in both the *Ph1+* and *Ph1-* genotypes. Therefore, the behaviour of the telomeres was used as a way in which to establish the stages of pre-meiosis and meiosis. Previous studies have shown that telomeres pair correctly, independently of *Ph1* activity (Griffiths, Sharp et al. 2006), and observations of similarly staged *Ph1+* and *Ph1-* spikes show that the telomeres progress to the same stage over a set period of time. Although all of these observations suggest that the behaviour of the telomeres is consistent in *Ph1+* and *Ph1-* samples, without an accurate time course experiment being conducted this cannot be definitively confirmed. Likewise it has been assumed that uptake of BrdU and EdU occurs at the same rate in both genotypes. Similar amounts of solution are taken up by both over

a four hour pulse; however the rate of uptake was not monitored. Both of these factors can be considered as unlikely, but should be taken into account when analysing the results.

This study has provided new insights into the pre-meiotic effects of the *Ph1* locus and is one of a number of studies which indicate that pre-meiotic S-phase is an integral part of meiosis, and is perhaps where the process of homology searching and homologue pairing starts (Baltus, Menke et al. 2006), (Borde, Goldman et al. 2000), (Cha, Weiner et al. 2000), (Jaramillo-Lambert, Ellefson et al. 2007), (Merino, Cummings et al. 2000), (Murakami, Borde et al. 2003), (Watanabe, Yokobayashi et al. 2001).

Chapter 3: a study of pre-meiotic and meiotic chromatin changes in the presence and absence of *Ph1*

Abstract

At the onset of meiosis, chromosomes first decondense and then condense as the process of recognition and intimate pairing occurs between homologous chromosomes. We show here that the dominant locus controlling the pairing of homologous chromosomes in wheat, *Ph1,* has an effect on chromosome condensation both pre-meiotically and meiotically. Furthermore we show that okadaic acid, a drug known to induce chromosome condensation, can be introduced into wheat interspecific hybrids prior to meiosis to induce chromosome pairing. This pairing occurs in the presence of the *Ph1* locus, which usually suppresses pairing of related chromosomes. Thus the timing of chromosome condensation during the onset of meiosis is an important factor in controlling chromosome pairing.

The work presented in this chapter is based around a recently published paper,

Knight, E. Greer, E. Draeger, T. Thole, V. Reader, S. Shaw, P. & Moore, G. (2010) Inducing chromosome pairing through premature condensation: analysis of wheat interspecific hybrids. Functional and Integrative Genomics **10**(4): 603-608.

The majority of work described in this chapter was carried out by me, with the exception of the okadaic acid work which was carried out by Emilie Knight.

Chapter 3: a study of pre-meiotic and meiotic chromatin changes in the presence and absence of *Ph1*

3.1 Introduction

The fully extended DNA which composes the eukaryotic genome needs to be compacted about 10,000 times to fit into the nucleus (Razin, Iarovaia et al. 2007). There are several levels of DNA packaging which allow this compaction to occur. The basic unit of DNA packaging in the eukaryotic cell is the nucleosome, which consists of an octamer of the core histones, H2A, H2B, H3 and H4 (two copies of each), around which 147 base pairs (bp) of DNA are wrapped in two left handed superhelical turns (Luger, Mader et al. 1997). These core particles are connected by up to 80 bp of 'linker DNA'. The organization of DNA around the nucleosome compacts the DNA by five to ten fold and cannot alone explain the level of packaging seen within the nucleus. The repeating nucleosomes and linking DNA form a 10nm fibre which is generally termed 'beads on a string' due to its appearance under the electron microscope (Felsenfeld and Groudine 2003). This structure has been suggested to be further folded into a 30nm fibre, which is stabilised by histone H1 binding the linking DNA as it enters and exits the nucleosome thereby stabilising the zig-zag structure of the fibre (Zhou, Gerchman et al. 1998). The 30nm fibre is thought to be the state found in interphase chromosomes and compacts the DNA by approximately 50 fold. Although the 30nm fibre has been seen *in vitro*, recent studies have cast some doubt as to whether it exists *in vivo* (Maeshima, Hihara et al. 2010). Observations of human mitotic cells have instead suggested that interphase chromosomes exist in a highly disordered, interdigitated and dynamic state. This chromatin fibre is further folded into higher order chromatin structures for cell division and in heterochromatin, although little is known about how this takes place. One suggestion is that the interphase fibre is looped around a central protein scaffold to provide the extra levels of compaction. This process 'disentangles' sister chromatids and protects the fragile DNA molecules, both of which are

important for segregation (Alberts, Johnson et al. 2002). The levels of DNA organization within the chromatin structure are summarized in figure 1.

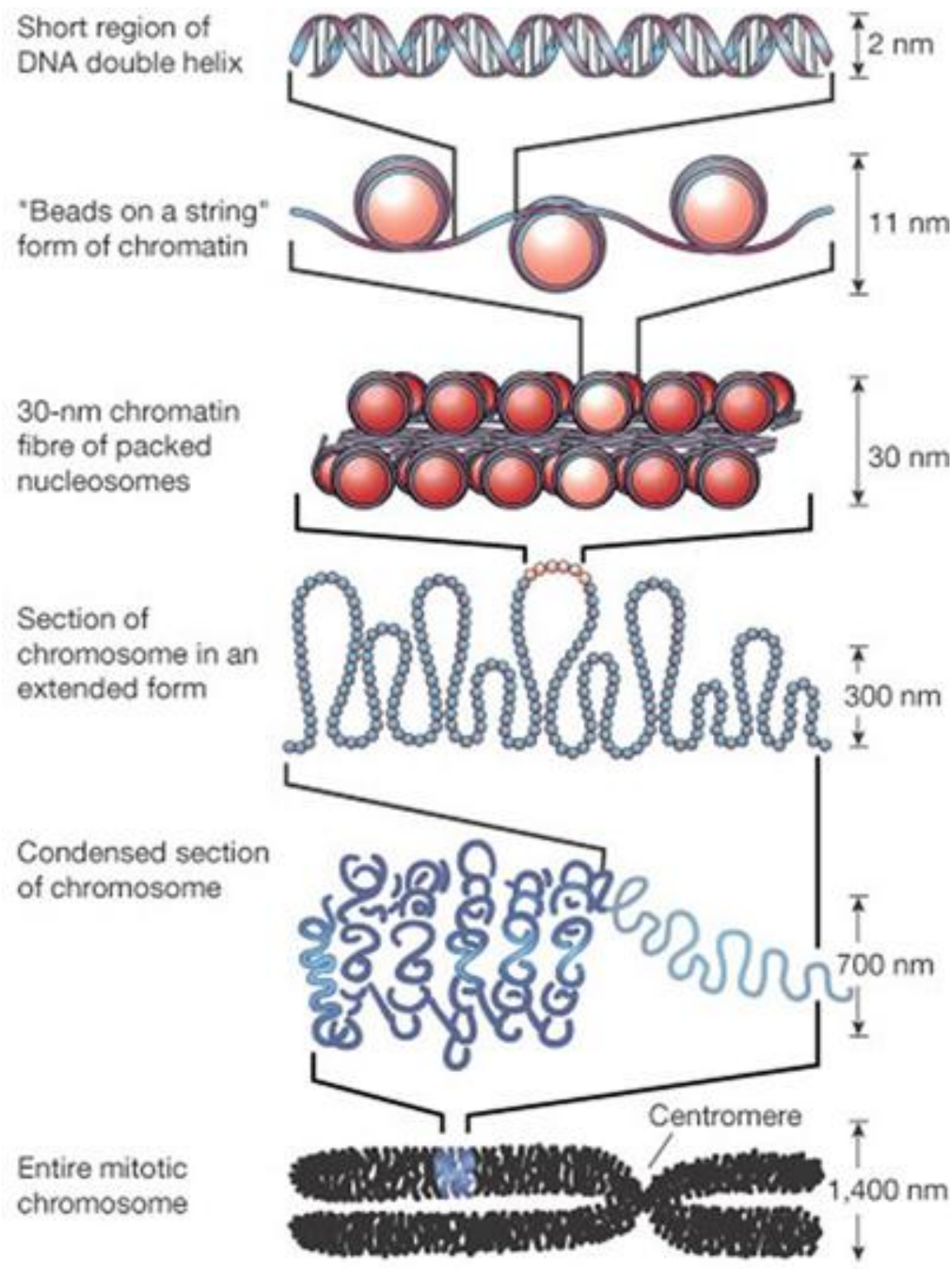


Figure 1:- the levels of DNA organization within the chromatin fibre. The lowest level of organisation is the 10nm 'beads on a string structure' formed by the DNA wrapping around the nucleosome. This is further compacted into the '30nm' fibre with the addition of histone H1. These fibres are further folded into higher order chromatin structures. Figure adapted from (Felsenfeld and Groudine 2003).

Chapter 3: a study of pre-meiotic and meiotic chromatin changes in the presence and absence of *Ph1*

The organisation of DNA into chromatin presents immediate challenges for processes such as replication, transcription, recombination and DNA repair, all of which require the chromatin to partially unravel (Hsieh and Fischer 2005). Therefore the structure of chromatin varies throughout the cell cycle in a process termed 'chromatin remodelling'. Chromatin exists in two distinct forms, heterochromatin, which remains tightly condensed throughout the cell cycle and euchromatin, which is decondensed during interphase.

There are several ways in which chromatin structure can be altered or reconfigured to change its accessibility to a variety of enzymatic or chemical probes (Aalfs and Kingston 2000). The first of these uses various ATP-dependent remodelling complexes to move the position of the nucleosomes relative to the DNA leading to either condensing or decondensing of the chromatin. This leads to either a repressed or transcriptionally active chromatin state in which the binding of transcription factors to their target sites is facilitated (Gavin and Simpson 1997), (Kingston and Narlikar 1999), (Wu and Winston 1997). The SWI/SNF ATP dependent remodelling complex was first discovered in *S. cerevisiae* (Winston and Carlson 1992). Since then several subfamilies of ATP-dependent remodelers have been defined, SWI2/SNF2, ISWI and CHD, and the 'split ATPases' (which bear an insertion within their ATPase domains) INO80 and SWR1 (Cairns 2007), (Saha, Wittmeyer et al. 2006). It has also been suggested that the protein RAD54 might act as a remodeler as it can alter chromatin structure *in vitro* and may reposition nucleosomes during DNA repair, although it seems to lack any specific nucleosome interacting domains (Alexeev, Mazin et al. 2003), (Durr, Korner et al. 2005), (Jaskelioff, Van Komen et al. 2003). The division into subfamilies is based on their structure and the biochemical mechanism that each family uses to remodel the chromatin (Hsieh and Fischer 2005 1019), (Langst and

Becker 2004), (Lusser and Kadonaga 2003). There are at least four ways in which remodelers can change the conformation of chromatin. The core histones can either be slid or moved to a new position in order to expose the DNA, the core histone octamer can be ejected or displaced to expose the DNA, the H2A-H2B dimers can be removed to leave only the H3-H4 tetramer which exposes the DNA and destabilises the nucleosome, and histone variants can be exchanged, for example replacing the H2A-H2B dimers with dimers containing the H2A variant H2A.X (see Cairns (2007) and the references therein). Specialised histone variants are also found at the centromeres (CENP-A histone H3-like variants)(Smith 2002), and genes which have been silenced by modifications to histone H3 can quickly be activated by exchanging H3 for the variant H3.3 (Ahmad and Henikoff 2002)

A second way of inducing chromatin remodelling is via the modification of histone proteins by processes such as phosphorylation, acetylation, methylation, ADP-ribosylation glycosylation, sumoylation and ubiquitination. These modifications affect the interactions between the histone tails and the DNA and surrounding nucleosomes (Meyer 2001). Each histone tail has multiple target sites for modifications, and so there is the potential for endless combinations of modifications. Histone acetylation and deacetylation play an important role in chromatin remodelling. Acetylation of histones causes a charge neutralization, which leads to weakened interactions between the histones and the DNA, leading to a decondensed structure which results in transcriptional competence (Fransz and de Jong 2002), (Kurdistani and Grunstein 2003), (Marmorstein and Roth 2001), (Roth, Denu et al. 2001). Histone acetylation patterns are controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs).

Methylation of histones H3 and H4 have been shown to be associated with several chromatin functions, including both the repression and activation of genes (Iizuka and

Smith 2003). DNA methylation is catalyzed by DNA methyltransferases, which act to transfer a methyl group from S-adenosyl-L-methionine, to the 5-carbon position of cytosine (Robertson 2002). It is thought that both the location and the number of methyl groups in each modification contribute to the functional consequences of the histone methylation. For example, early experiments indicated that H3 K4 methylation was linked to active genes, whilst H3 K9 methylation was linked to inactive genes (Lachner and Jenuwein 2002). This was disputed after experiments carried out on *S.cerevisiae* showing that H3 K4 methylation was linked to silenced regions (Briggs, Bryk et al. 2001), (Bryk, Briggs et al. 2002). It is now thought that tri-methylation of H3 K4 is linked to transcriptionally active regions, whilst H3 K4 di-methylation can be seen in both active and repressed genes (Santos-Rosa, Schneider et al. 2002).

The phosphorylation of histones is another modification which plays an important role in chromatin remodelling. The phosphorylation of histone H1 is thought to control the levels of chromatin condensation throughout the cell cycle (Roth and Allis 1992). Phosphorylation of histone H1 disrupts the H1-chromatin interactions and leads to a more relaxed chromatin structure (Contreras, Hale et al. 2003), (Herrera, Chen et al. 1996). Further investigations into this subject have revealed that phosphorylation of histone H1 reduces its affinity for heterochromatin protein 1 α (HP1α), one of the fundamental units of heterochromatin packaging. It is thought that this could be responsible for the disassembly of higher order chromatin structure seen during interphase (Hale, Contreras et al. 2006). The role of histone H1 phosphoryaltion in chromosome condensation is still a much debated topic. The phosphorylation state of histone H1 reaches its maximum during G2 and M-phase and has been shown to play an important role in the structure and function of chromatin during mitosis (Maresca, Freedman et al. 2005). In fact, blocking histone H1

phosphorylation has been shown to prevent entry into mitosis in mammalian cells (Gurley, Valdez et al. 1995), (Th'ng, Guo et al. 1994). It has been suggested that weakening the interaction between histone H1 and the chromatin is necessary to allow condensing factors to interact with the chromatin and cause subsequent condensation (Roth and Allis 1992). Others have suggested that the partial phosphorylation of histone H1 leads to the decondensed chromatin structure seen during interphase, whereas hyperphosphorylation leads to the condensed structure seen later in mitosis and meiosis (Roque, Ponte et al. 2008).

The phosphorylation of histone H3 is also thought to play a role in two structurally opposing processes throughout the cell cycle. It is thought to be involved in both the chromosome decondensation seen during gene transcription in interphase, and in chromosome condensation seen during mitosis and meiosis (Perez-Cadahia, Drobic et al. 2009). In animal cells highly condensed metaphase chromosomes are found to have high levels of histone H3 phosphorylation at the Ser10 residue. Exit from meiosis or mitosis is accompanied by a global dephosphorylation of this site (Hans and Dimitrov 2001). Chromosome condensation has been shown to occur in the absence of H3 Ser10 phosphorylation in maize, and seems to have a role in the maintenance of sister chromatid cohesion rather than condensation (Kaszas and Cande 2000). In most organisms H3 Ser28 is also phosphorylated during meiosis and mitosis and the pattern seems to correlate with that of Ser10 phosphorylation (Goto, Tomono et al. 1999), (Perez-Cadahia, Drobic et al. 2009). Both Ser10 and Ser28 phosphorylations have been seen to occur along the same chromosome during mitosis, although it is not known whether they occur on the same histone H3 tails (Goto, Tomono et al. 1999), (Perez-Cadahia, Drobic et al. 2009). It has been

suggested that in plants mitotic and meiotic condensation could correlate with phosphorylation of H3 residues Thr 3 and Thr 11 rather than the phospho-H3 Ser10 and Ser28 seen in animals (Houben, Demidov et al. 2005), (Houben, Demidov et al. 2007), (Perez-Cadahia, Drobic et al. 2009).

Phosphorylation of H3 Ser10 and Ser28 is also seen during interphase and is thought to be linked with gene expression (Goto, Yasui et al. 2002), (Cerutti and Casas-Mollano 2009). These phosphorylations are thought to occur on a smaller number of nucleosomes than mitotic and meiotic phosphorylations, and the two phosphorylation events occur independently at distinct chromatin regions (Goto, Yasui et al. 2002), (Dunn and Davie 2005). The precise mechanism by which phosphorylation of H3 residues could contribute to interphase chromosome decondensation is unknown, although it has been suggested that phosphorylation of H3 could lead to the ejection of HP1γ (Vicent, Ballare et al. 2006), (Winter, Simboeck et al. 2008), one of the three mammalian heterochromatin proteins (HP1α, HP1β and HP1γ) (Minc, Allory et al. 1999).

The phosphorylation of histones is of particular interest with regard to the wheat *Ph1* locus because of the involvement of CDKs. The CDK2/cyclin E complex is responsible for the phosphorylation of H1 (Roth and Allis 1992), (Hale, Contreras et al. 2006), and it has been shown that increased CDK2 activity can lead to both a more relaxed chromatin structure during interphase (Herrera, Chen et al. 1996), (Hale, Contreras et al. 2006), and premature chromosome condensation during mitosis (Furuno, den Elzen et al. 1999).

The phosphorylation of histone H3 is dependent on a family of Aurora (*Drosophila*)/IPL1 (*S. cerevisiae*) like protein kinases (Glover, Leibowitz et al. 1995), (Francisco, Wang et al. 1994). It is thought that Aurora B is responsible for the

phosphorylation of H3 Ser10 and Ser28 residues (Goto, Yasui et al. 2002). The activity of phosphoprotein phosphatase I (PPP1) inhibits Aurora B kinase activity. It has been proposed that prior to mitosis CDC2 (human CDK1) inhibits the phosphatise activity of PPP1, thereby increasing the activity of Aurora B and leading to H3 phosphorylation (Goto, Yasui et al. 2002), (Swain, Ding et al. 2007).

The wheat *Ph1* mutant phenotype is caused by the increased activity of *Cdk2-like* genes (Al-Kaff, Knight et al. 2007). Given the involvement of CDKs in histone phosphorylations which contribute to changes in chromatin structure, it is likely that the *Ph1* locus also plays a role in chromatin remodelling. Previous studies on maize have identified a prezygotene stage at which the chromosomes undergo a dramatic remodelling, during which there is a partial separation of sister chromatids, an elongation of knob heterochromatin, an increase in surface complexity, and a 50% increase in total chromosome volume (Dawe, Sedat et al. 1994). This change in morphology is thought to facilitate homologue recognition. An open chromatin conformation has been found to be associated with DSB hotspots in *S.cerevisiae* (Berchowitz, Hanlon et al. 2009), perhaps suggesting a way in which chromatin remodelling at this stage could facilitate the homology search. This stage has also been identified in wheat in studies using FISH to visualize the behaviour of specific chromosomal regions. When comparing this stage in plants either carrying or lacking the *Ph1* locus, differences in the timing of both centromeric remodelling (Aragón-Alcaide, Reader et al. 1997) and subtelomeric heterochromatin remodelling (Prieto, Shaw et al. 2004), can be seen, with *Ph1* mutant plants undergoing earlier remodelling. Similar results were reported by (Mikhailova, Naranjo et al. 1998) in studies using squashed meiocyte preparations. Further work using FISH to visualize the behaviour of subtelomeric heterochromatin regions has given some understanding as to

why these differences in the timing of remodelling are seen. In the presence of *Ph1*, homologous regions of subtelomeric heterochromatin remodel synchronously, however if the regions of subtelomeric heterochromatin are not identical remodelling is delayed (Colas, Shaw et al. 2008). In the absence of *Ph1* these regions elongate synchronously regardless of the degree of identity between them (Colas, Shaw et al. 2008), (Prieto, Shaw et al. 2004). It is likely that in the presence of *Ph1*, remodelling is delayed until a homologous partner is found, whereas in the absence of *Ph1*, this delay does not occur.

Wheat-rye hybrids provide a particularly good background in which to study this effect, as they contain no homologous chromosomes, therefore a variety of methods were employed to look at the condensation state of the chromatin in wheat-rye hybrid meiocytes both pre-meiotically and during meiosis. Pre-meiotically there is a stage of decondensation, corresponding to the prezygotene chromatin remodelling seen in maize and wheat. As the cell enters meiosis the chromosomes begin to condense, until they reach a state of full condensation at metaphase I.

The results of these experiments add further evidence to the hypothesis that *Ph1* does play a role in controlling chromatin remodelling during the meiotic cell cycle. Furthermore, by changing the chromatin condensation state in a wild type wheat-rye plant using okadaic acid, a protein serine/threonine phosphatise inhibitor known to induce premature chromosome condensation (Yamashita, Yasuda et al. 1990), a *Ph1* mutant phenotype can be induced (Knight, Greer et al. 2010).

3.2 Methods

Plant material

The tillers used for this study came from hexaploid wheat, *Triticum aestivum* cv. Beaver, crosses between hexaploid wheat cv. Hobbit sib and rye (*Secale cereale*) cv. Petkus and also between *T. aestivum* cv. Chinese Spring (either carrying or lacking the *Ph1* locus) and rye cv. Petkus.

Hydrochloric acid denaturation of sections

10um sections were made through wheat rye spikelets both in the presence and absence of *Ph1* (described in detail in appendix 1). These were exposed to a 40 minute denaturation treatment using 1.5M HCl at 37˚C, and then immediately transferred to ice cold PBS for 5 minutes. These were then stained with DAPI and mounted using Vectashield (H-1000) medium.

Sectioning, fluorescence in situ hybridisation, immunolocalization, microscopy and image processing

The tissue sectioning, specimen preparation, in situ hybridisation, fluorescence microscopy and image processing have all been described previously (Prieto, Moore et al. 2007), and are described in detail in appendices 1, 4, 5 & 6. Axial element lengths were determined using ImageJ by counting the total pixels of skeletonised Asy1-labelled elements (examples of which are shown in figure 5). Analysis of deviance was carried out using Genstat 11th edition. The TaAsy1 antibody was kindly provided by Dr. Jason Able and the fluorescence immuno-localisation was performed as described in Boden et al., using the rabbit anti-TaAsy1 antibody and AlexFluor 568 conjugated donkey anti-rabbit antibody (Boden, Langridge et al. 2009).

Okadaic acid treatment

Tillers containing an immature pre-meiotic spike were detached immediately after an 8-h dark period and transferred immediately to a solution of 100 mM sucrose solution containing dilutions of a 10 mM OA (Sigma) stock solution to give a concentration range from 100 nM to 1 µM. These were incubated for 24 hours in the dark. Each treatment was repeated twice to nine times in separate experiments. Analysis of variance (ANOVA) was carried out using generalised linear models in Genstat 11th Edition.

3.3 Results

Simple 3D analysis of the DAPI labelling of pre-meiotic cells revealed no obvious differences between *Ph1+* and *Ph1*- plants at this stage. The first indications that *Ph1* has an involvement in chromatin condensation state at this early stage were demonstrated by using hydrochloric acid (HCl) to denature the DNA in pre-meiotic sections of wheat-rye hybrid anthers in the presence and absence of *Ph1*. Figure 2 shows sections through wild type and *Ph1* mutant wheat-rye anther sections at the same stage of meiosis, which have undergone a 40 and 45 minute denaturation treatment. In the presence of *Ph1* the rye heterochromatin knobs can still clearly be seen after 40 minutes, but in the *Ph1* mutant the DAPI labelling has been destroyed by the denaturation treatment with the labelling seeming more prominent in the cytoplasm and nucleolus and not so prominent in the nucleus, and the rye heterochromatin knobs can no longer be seen. This could be because the DNA is somehow fragmenting and moving around, although the definite reason for this change in labelling pattern remains to be discovered. It takes 45 minutes of denaturation for the *Ph1+* sample to reach a similar level. This suggests that there is a difference in chromatin structure during the pre-meiotic stages.

As the cells enter meiosis the chromosomes go through a change in morphology, becoming more decondensed in structure. Observations of the remodelling behaviour of the rye subtelomeric heterochromatin knobs and centromeres at this stage served to confirm this difference between *Ph1+* and *Ph1*- plants (as shown in figure 3). At the time of the telomere bouquet the rye subtelomeric heterochromatin knobs are still as discrete, tight foci in the presence of *Ph1* (figure 3a). However in the absence of *Ph1* at the same stage they have grouped together and started the process of remodelling (figure 3b).

Similar behaviour can also be seen at the centromeric sites. At the beginning of the telomere bouquet dispersal, the wheat and rye centromeres are discrete, tight foci in the presence of *Ph1* (figure 3c) but have begun the process of remodelling in the absence of *Ph1* (figure 3d).

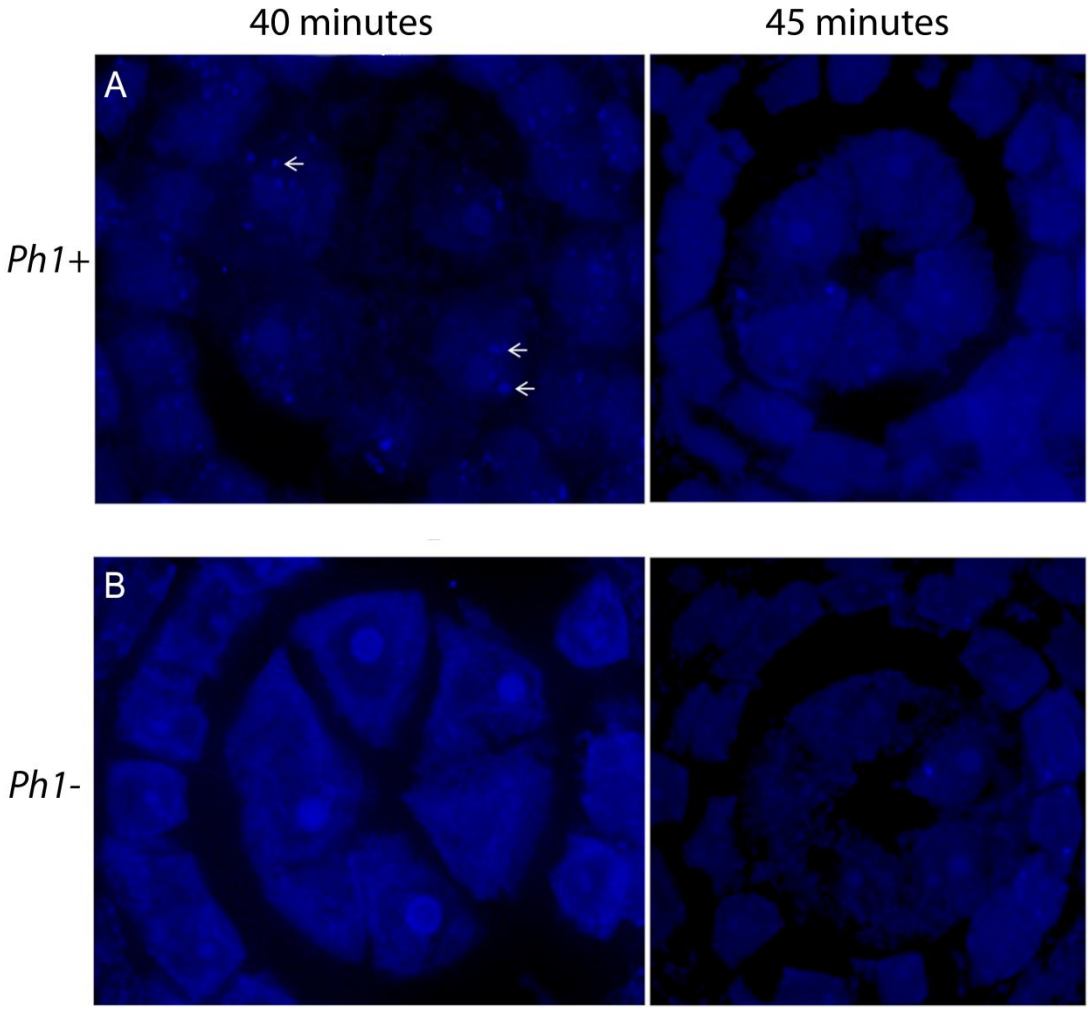


Figure 2:- DAPI stained sections through wild type and *Ph1* mutant wheat-rye anthers following a 40 minute and 45 minute denaturation in 1.5M HCl. In the presence of *Ph1* (A) the rye heterochromatin knobs are still visible (the intensely stained DAPI regions, examples of which are highlighted by arrows in panel A) after a 40 minute denaturation and it takes 45 minutes of denaturation for the DAPI labelling to be destroyed. In the absence of *Ph1* (B) the rye heterochromatin knobs can no longer be seen and the DAPI labelling is destroyed after only 40 minutes of denaturation.

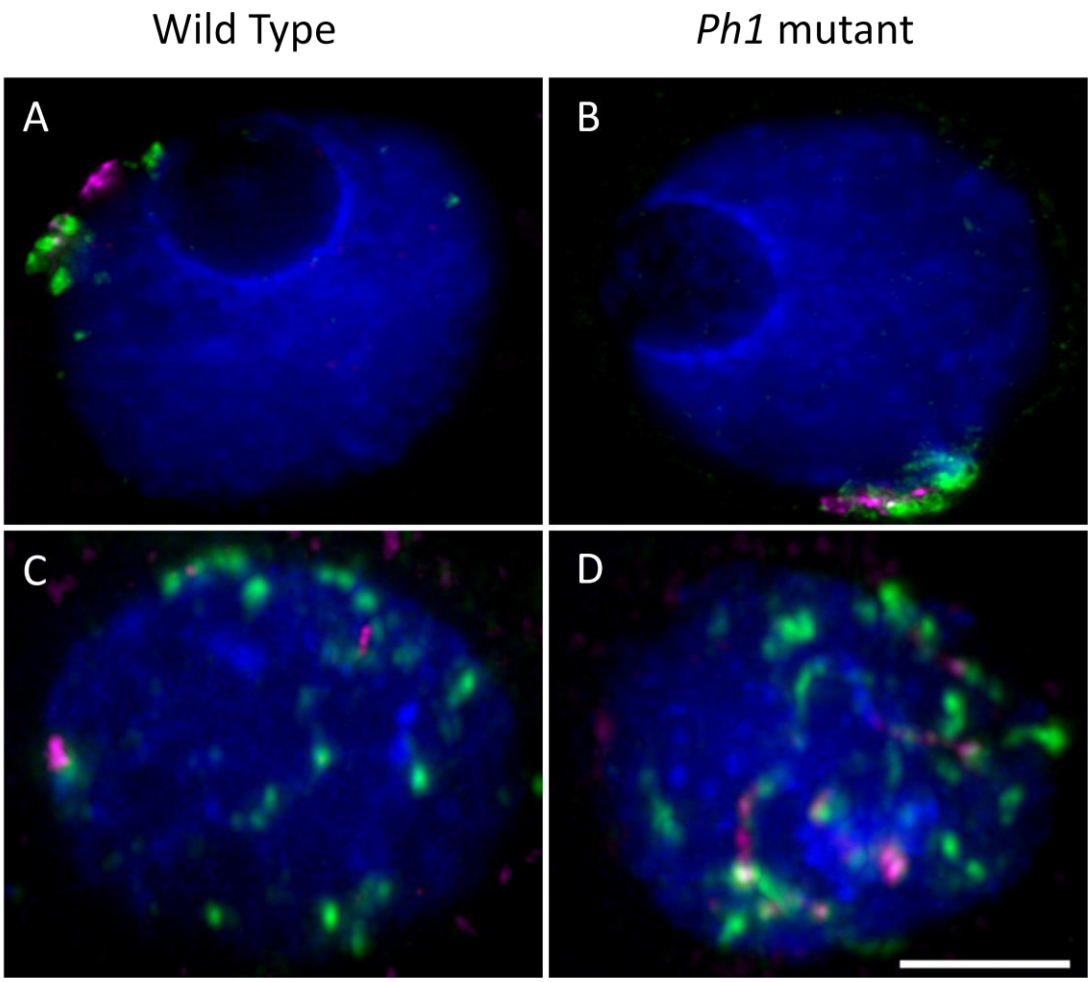


Figure 3:- the dynamics of rye subtelomeric heterochromatin (A&B) and centromeric (C&D) remodelling. At the timing of the telomere bouquet (telomeres are shown in magenta in panels A & B) the rye heterochromatin knobs are still as discrete foci in the *Ph1+* sample (A). However, they have started to remodel in the *Ph1-* sample (B). Similarly the wheat (green) and rye (magenta) centromeres remain condensed in the *Ph1+* sample (C), whilst at the same stage they have started to remodel in the *Ph1-* sample (D). Scale bar represents 5um.

At the onset of meiosis, as the telomeres cluster to form the bouquet, the process of chromosome synapsis begins. A number of genes have been found to be essential for the correct formation of the synaptonemal complex to occur, including *ASY1* (*Asynapsis 1*), a functional homologue of the *S. cerevisiae HOP1* gene. *ASY1* encodes an axial element associated protein and can be seen to localise along the chromosome axes during prophase I. Due to its close association with the chromosome axes the localisation of ASY1 provides a useful tool to follow chromosome condensation during meiosis. Figure 4 shows wheat and wheat-rye nuclei following incubation with an anti-ASY1 antibody. Labelling can first be seen at the time of the telomere bouquet, just after DNA replication in the meiocytes has been completed. The ASY1 can be seen to delineate the chromosomes and the brightness of labelling seems to be most intense at the regions where the telomeres are clustering. This could be because the chromosomes are most concentrated at this region, or could be that ASY1 is more prevalent in these regions. It is interesting to note that pairing can be seen occurring in the wheat-rye *Ph1*- sample, starting from the bouquet area.

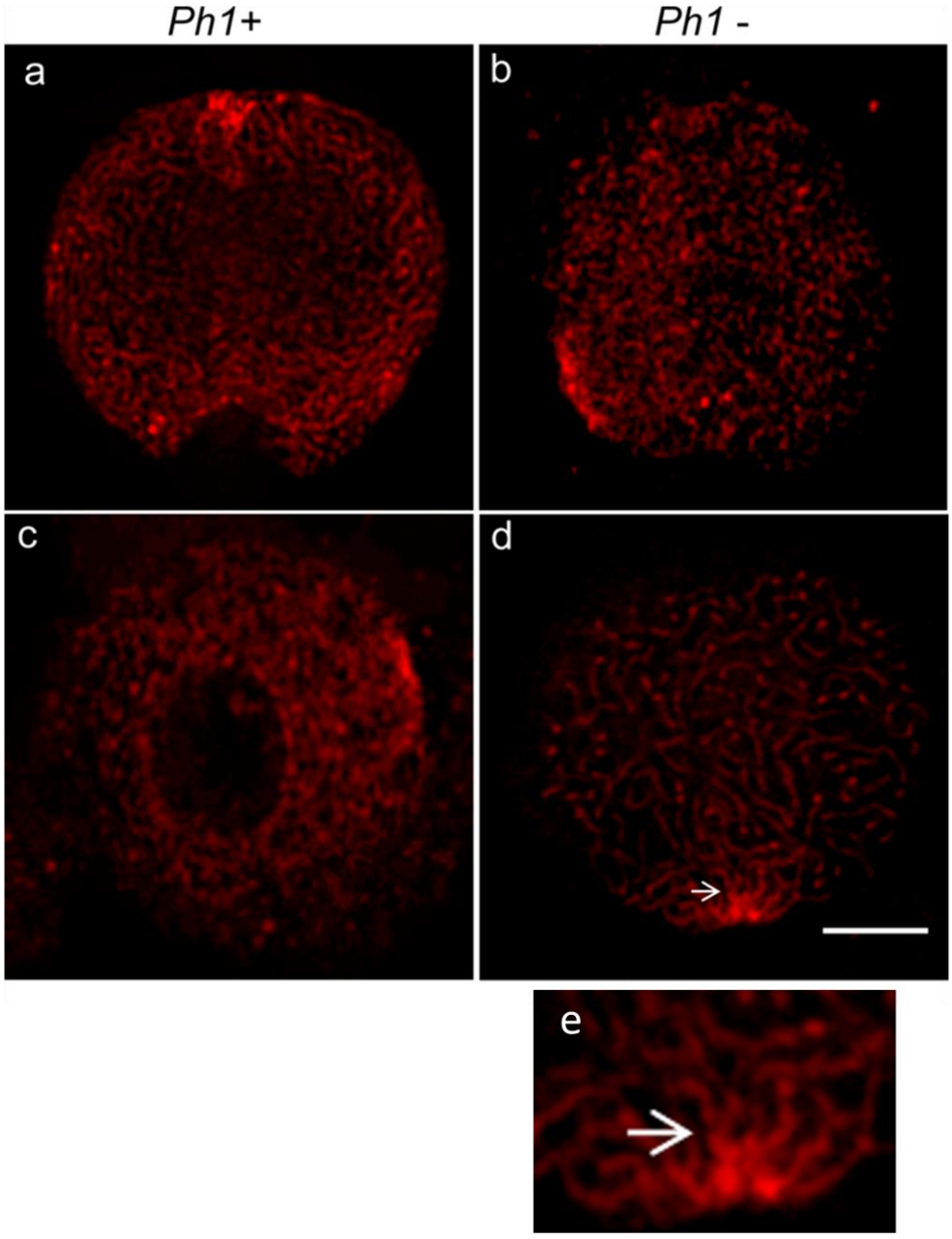


Figure 4: *Ta*ASY1 labelling of wheat (a&b) and wheat-rye (c&d) meiocytes at the time of the telomere bouquet. The ASY1 clearly delineates the chromosomes, and can be seen to more intensely label the telomere bouquet regions. Some chromosomes have started to pair in the wheat-rye *Ph1*- sample, as highlighted by an arrow in panel d (image enlarged in panel e). Scale bar represents 5um.

3D immunofluorescence images of meiocytes labelled with anti-ASY1 revealed that in wheat-rye interspecific hybrids chromosomes were less condensed in the presence of *Ph1* than when it was absent at both the late bouquet stage and as the bouquet disperses (figure 5, figure 6). At both stages there were significant differences ($p<0.05$) in the lengths of the labelled axial elements in the presence and absence of *Ph1*.

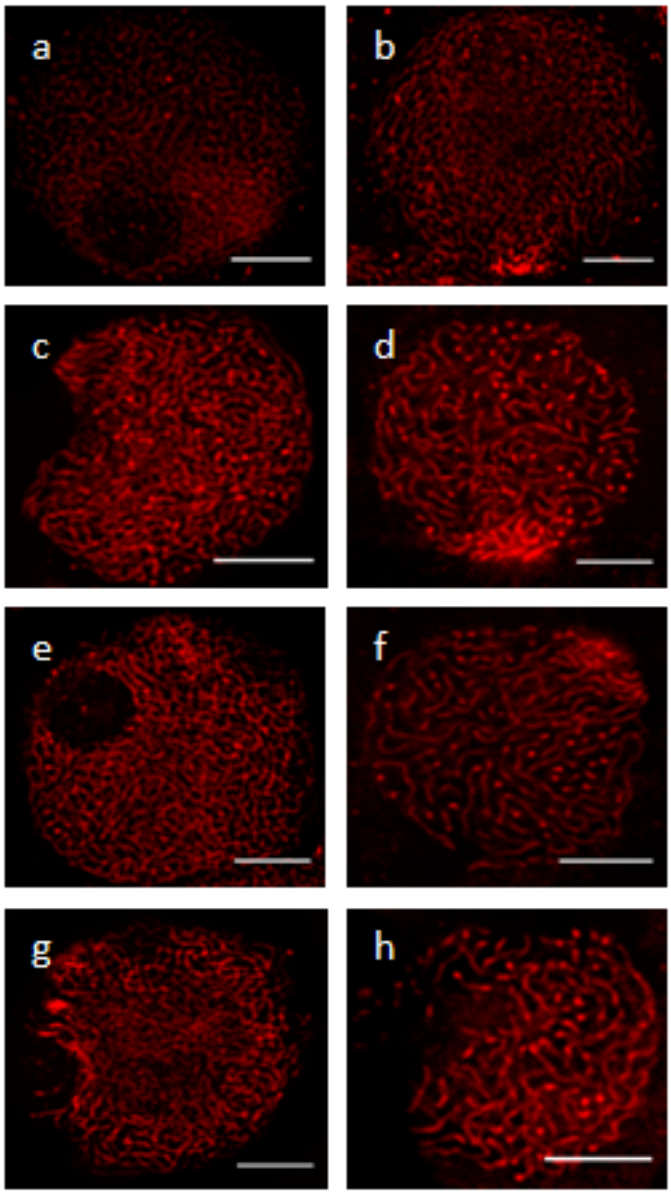
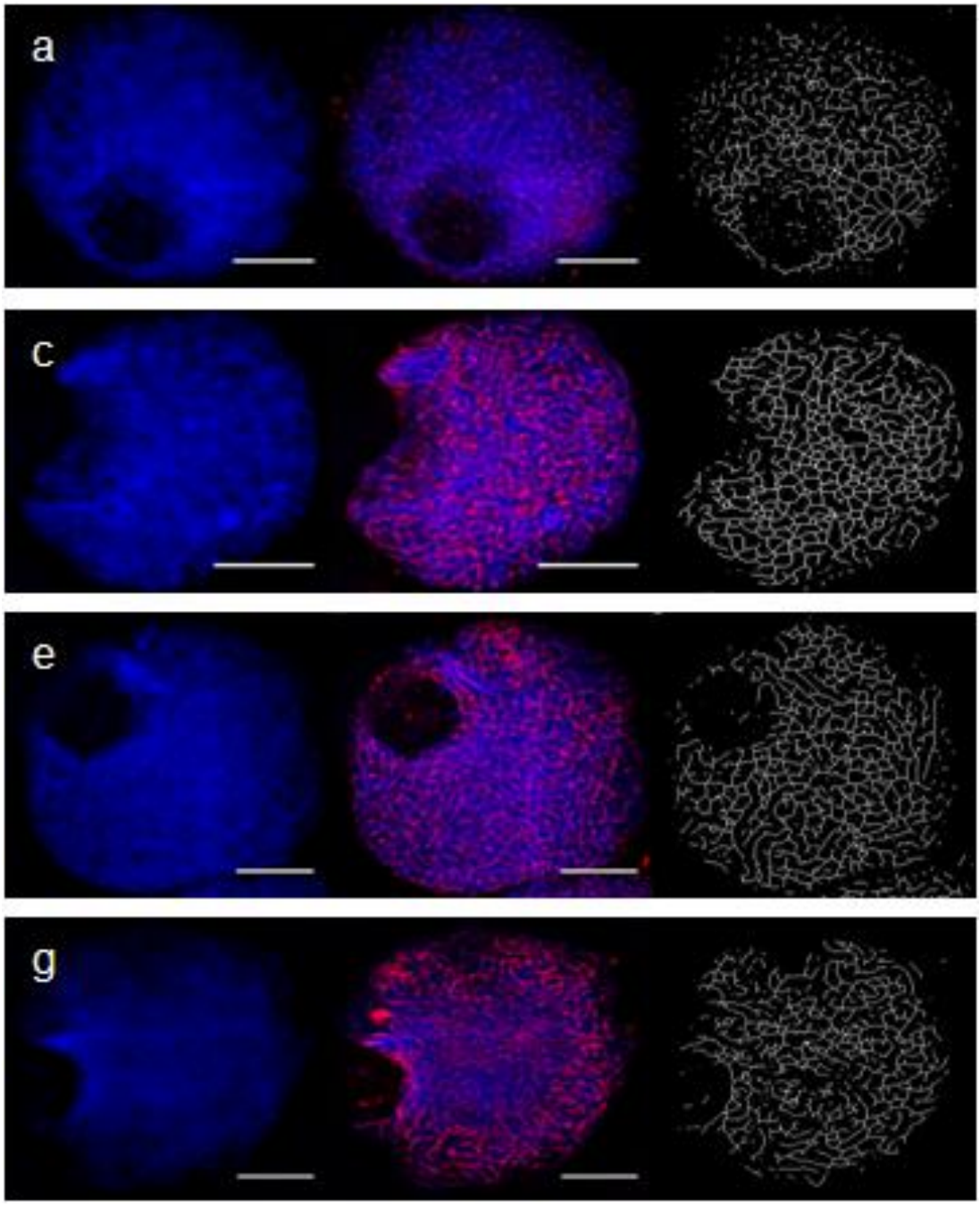


Figure 5:- *Ta*ASY1 localisation (red) at early meiosis in wildtype (a, c, e, g) and *Ph1* mutant wheat-rye F1 wheat (b, d,f, h). Meiocytes at pre-telomere bouquet (a, b), early bouquet (c, d), late bouquet (e, f), as bouquet disperses (g, h). Scale bars 5 µm


a
c
e
g

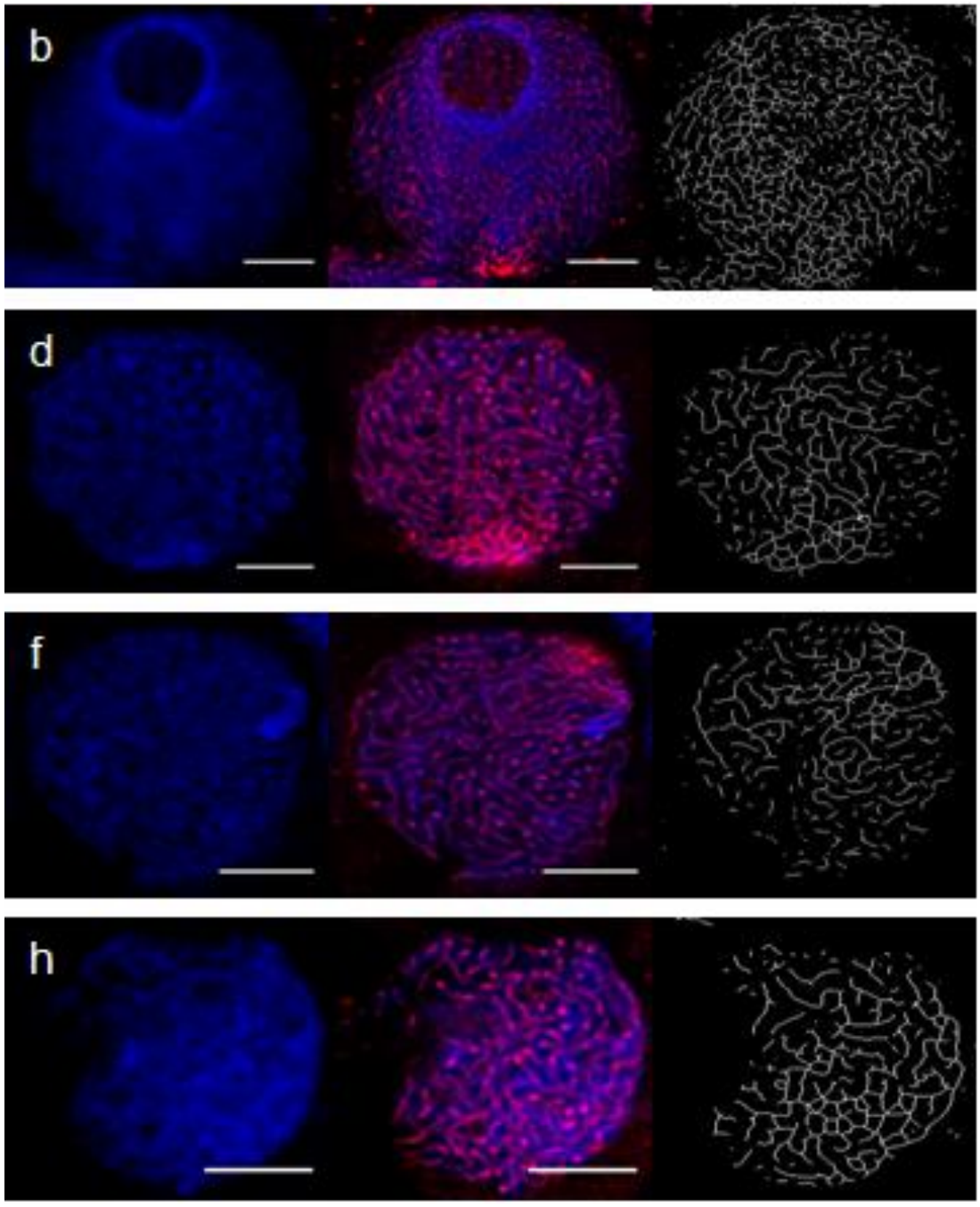


Figure 6: *Ta*ASY1 localisation at early meiosis in wild type (a, c, e, g) and *Ph1* mutant wheat-rye F_1 hybrids (b, d, f, h). DAPI (blue), DAPI and ASY1 labelled (blue and red) meiocytes and skeletonised axial element images for pixel counting at pre telomere bouquet (a, b), early bouquet (c, d), late bouquet (e, f), as the bouquet disperses (g,h). Scale bars represent 5 µm.

Having established that *Ph1* plays a role in the timing of chromosome condensation, the next question was whether altered chromosome condensation could be induced during meiosis to give a *Ph1* phenotype. Okadaic acid (OA) was used to affect phosphorylation, and to induce chromosome condensation. Wheat-rye tillers were incubated in a sucrose solution with and without OA for 24 hours. Analysis of chromosome spreads at metaphase I revealed that OA affected chromosome pairing in a dose-dependent manner. There were very few chromosome arm associations observed in the sucrose control with most chromosomes visualised as univalents. However, as the OA concentration was increased from 100 nM to 1 µM, the number of chromosome associations also increased, with the mean number of univalents per cell decreasing from 10 down to four for treatments with 1 µM OA and the mean number of ungrouped chromosomes (a group being defined as three or more associated chromosomes) also decreasing from 15.3 at 100 nM to 6.9 with 1 µM OA (figure 7). ANOVA revealed that the effect of OA on the number of univalents and ungrouped chromosomes per cell was highly significant ($P<0.001$). However this analysis also revealed that treatments with concentrations greater than 200 nM OA produce few rod bivalents suggesting little potential for genetic exchange. In contrast, treatment with 100 nM OA resulted in a less marked chromosome association, but more importantly a strong increase in the number of rod bivalents (figure 8). Rod bivalent pairing is induced by timing the 100 nM treatment to a period within 12 h of the onset of meiosis during pre-meiotic S phase. However, this pairing is not observed in every treated anther within the spike, which suggests that the window for effective treatment is quite narrow.

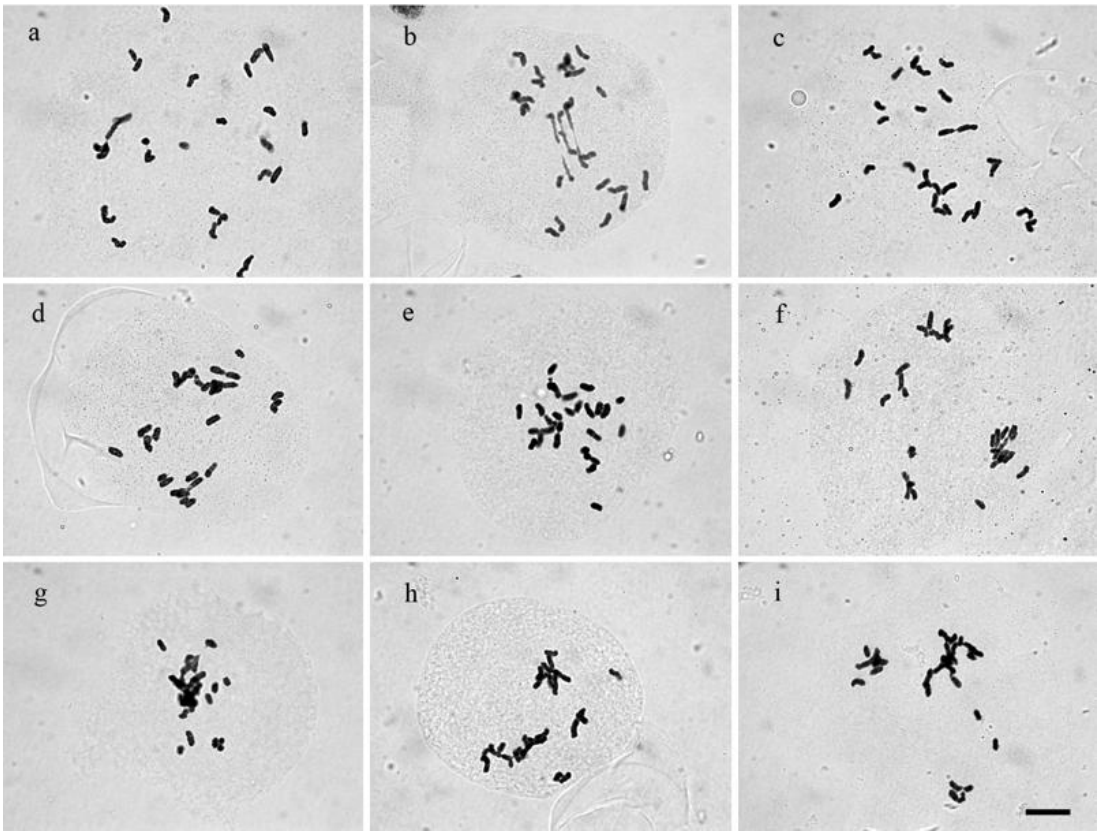


Figure 7: Induction of chromosome associations in wheat rye F_1 hybrids in the presence of *Ph1* with increasing OA concentrations. Metaphase I chromosome spread from a detached tiller treated with sucrose only (a), 100nM OA (b), 200nM OA (c), 300nM OA (d), 400nM OA (e), 500nm OA (f), 600nM OA (g), 750nM OA (h), 1 µM OA (i). Scale bar

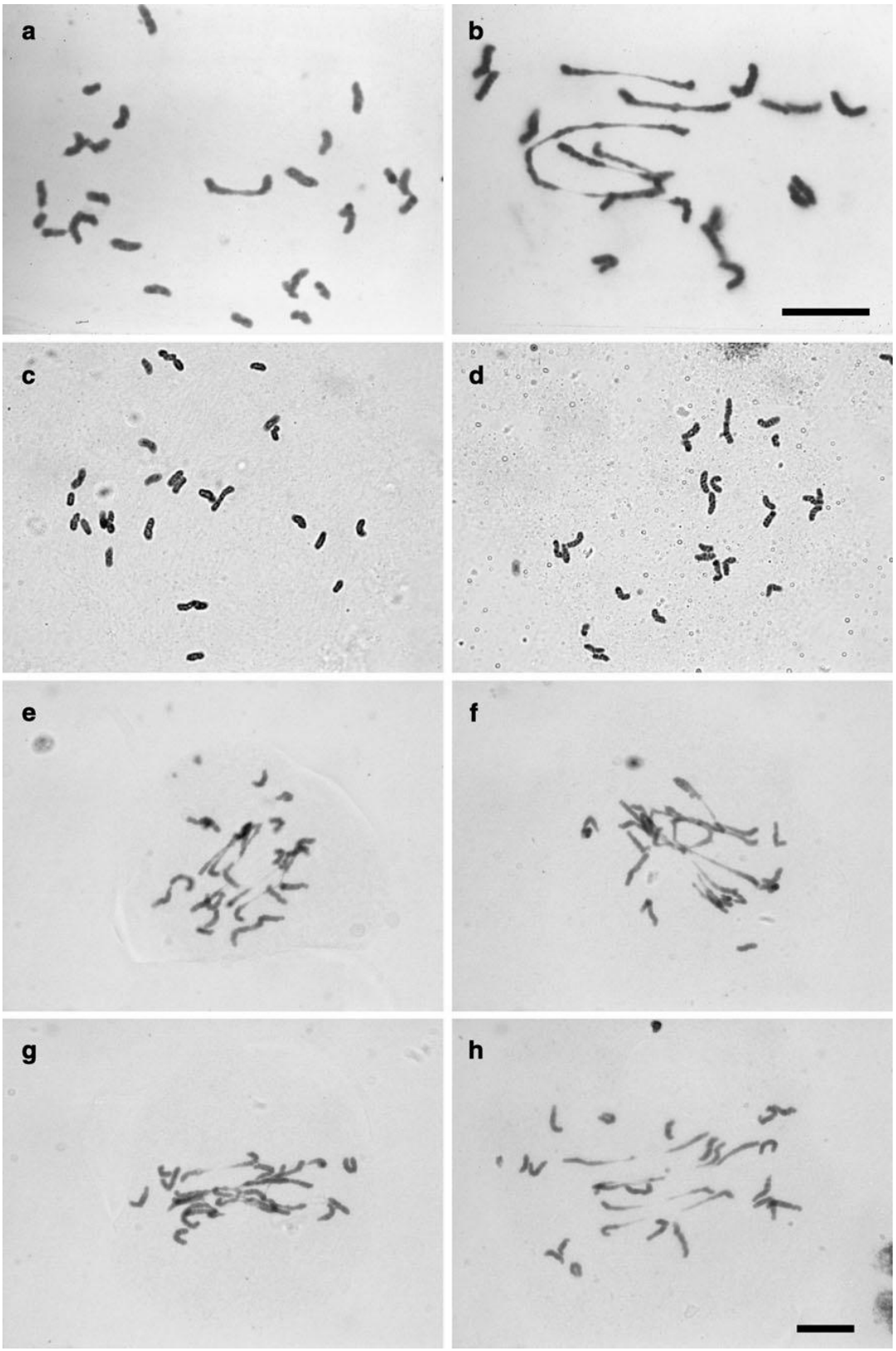

a
b
c
d
e
f
g
h

Figure 8: Induction of chromosome pairing in wheat-rye hybrids. Metaphase I chromosome spread from the hybrid with *Ph1* (a) and without *Ph1* (b). Metaphase I chromosome spread from *Ph1+* detached tillers treated with sucrose (c, d) and with 100 nM OA (e, f, g, h). Scale bar 10 µm

3.4 Discussion

The *Ph1* locus on the long arm of chromosome 5B has recently been defined to a series of aberrant *Cdk-like* genes with closest homology to mammalian *Cdk2* (Griffiths, Sharp et al. 2006; Al-Kaff, Knight et al. 2007). We propose that these defective genes lead to a suppression of the activity of *Cdk2- like* genes on related genomes. Previous work has shown that in *Ph1* mutants, transcription of *Cdk2- like* genes is increased (Al-Kaff, Knight et al. 2007), which leads to an increase in CDK activity as evidenced by an increase in histone H1 phoshorylation at CDK consensus sites in the absence of *Ph1* (Greer, Martin et al. In preparation). Furthermore, changes in CDK activity have been shown to affect chromatin condensation in mammalian systems (Furuno, den Elzen et al. 1999).

The results presented in this chapter show that chromosome condensation is also affected in wheat-rye hybrids exhibiting increased CDK activity, both pre-meiotically and meiotically. The exact mechanism by which condensation is altered cannot be pinpointed at this time, however there are several plausible suggestions which could explain the changes that we see. The first of these is that the increased activity of *Cdk2-like* genes in the *Ph1* mutant could lead to differences in histone modifications, which in turn affect chromatin remodelling. Recent work showing that phosphorylation of histone H1 is increased in the absence of *Ph1* (Greer, Martin et al. In preparation), is of particular interest because of the role of histone H1 phosphorylation in controlling chromatin remodelling throughout the cell cycle (Roth and Allis 1992). This modification could also explain why pre-meiotically chromatin appears more decondensed in the *Ph1* mutant, whereas meiotically it undergoes earlier condensation. It is well known that histone H1

phosphorylation can lead to a relaxed chromatin structure via a reduction in its affinity for HP1α, which has been suggested to lead to the disassembly of higher order chromatin structure that is seen during interphase (Contreras, Hale et al. 2003), (Hale, Contreras et al. 2006), (Herrera, Chen et al. 1996). The role of H1 phosphorylation in chromatin condensation is still unclear, with some suggesting that the relaxed structure of chromatin caused by H1 phosphorylation could allow access to the condensing factors that are required for condensation in mitosis and meiosis (Roth and Allis 1992). It has also been suggested that partial phosphorylation of histone H1 is responsible for the decondensed chromatin structure during interphase, whereas hyperphosphorylation causes the more condensed structure seen in M-phase (Roque, Ponte et al. 2008).

The increased histone H1 phosphorylation in the *Ph1* mutant may cause the more decondensed pre-meiotic chromosomes, which then allows access to condensing factors such as condensins or allows histone H3 phosphorylation, which causes the earlier condensation that is seen as the cell enters meiosis. Studies on maize and wheat have shown that prior to pairing there is a stage at which the chromosomes decondense giving an increase in chromosome volume and an elongation of the heterochromatic regions (Dawe, Sedat et al. 1994), (Colas, Shaw et al. 2008). At this stage there is also a partial separation of sister chromatids, which may contribute to providing a barrier to sister chromatid DSB repair. The later decondensation seen in the presence of *Ph1* may prevent the access to condensing factors and this could explain why delayed meiotic condensation is seen. It also raises the possibility that the barrier to sister chromatid DSB repair is unable to form at the correct stage and is therefore ineffective. Work is currently underway to visualise DSBs in *Ph1+* and *Ph1-* meiocytes which may provide answers to some of these questions. This work is discussed further in chapter 5.

As previously mentioned the phosphorylation of histone H3 has also been shown to play a role in both decondensation of chromatin during interphase and M-phase condensation (Perez-Cadahia, Drobic et al. 2009). Increased CDK activity has been linked to histone H3 phosphorylation (Goto, Yasui et al. 2002), (Swain, Ding et al. 2007), making this an interesting area for future proteomics work.

Although histone modifications provide plausible explanations as to how *Ph1* could affect chromosome condensation it is important to remember that CDKs have multiple phosphorylation targets which could account for the differences that we see in chromatin remodelling. For example, CDK2 phosphorylates the retinoblastoma protein (RB), which is known to be a key regulator of the G1/S transition via the suppression of E2F transcription factors. Recent work on *Arabidopsis* has shown that male meiocytes deficient for RBR (retinoblastoma related), the *Arabidopsis* homologue of the tumor suppressor RB, show strongly reduced fertility due to a reduction in the expression of several meiotic genes, leading to a failure in synapsis (Chen, Higgins et al. 2011). Interestingly, RBF1 (one of the *Drosophila* retinoblastoma proteins) has been shown to promote chromosome condensation through its interaction with the condensin II protein dCAP-D_3 (Longworth, Herr et al. 2008). Mutants in *rbf1* are seen to exhibit extensive defects in mitotic chromosome condensation. The condensation defects seen in the *Ph1* mutant could be explained by increased inactivation of RB through the increased activity of CDK/cyclin complexes. In *Vicia faba* cells treated with okadaic acid, a phosphatase inhibitor known to induce chromosome condensation and increase levels of CDK activity, show higher levels of phosphorylated RB than the untreated controls (Polit and Kazmierczak 2007).

Regardless of the specific mechanism by which these differences in chromatin remodelling occur, the next step in elucidating the mode of action of the *Ph1* locus was to

ask whether inducing early chromosome condensation during meiosis could phenocopy the *Ph1* effect. Treatment of wheat-rye tillers in the presence of *Ph1* with OA did induce chromosome associations to a significant extent, suggesting that the *Ph1* effect can be phenocopied using drug treatments. In particular treatment with 100nm OA resulted in a strong increase in rod bivalents, providing the potential for genetic exchange between related chromosomes. The practical implications of this discovery could be particularly useful in breeding programs aiming to introgress beneficial traits from related species.

The mechanism by which changes in chromosome condensation induce the pairing between related chromosomes that is seen in the *Ph1* mutant is still unknown, although hypotheses have been suggested which could explain it. It is possible that the chromosome condensation in the *Ph1* mutant is not premature, but occurs at the normal time, and is instead delayed in the presence of *Ph1*. Previous work has shown that when chromosomes are not-identical, chromosome remodelling is delayed, and the level of pairing between these chromosomes is severely reduced (Colas, Shaw et al. 2008). In a wheat-rye hybrid where there are no homologous chromosomes, it is likely that remodelling is delayed, providing a barrier to non-homologous pairing. In the absence of *Ph1* remodelling does not appear to be delayed and so there is no barrier to non-homologous pairing.

Related chromosomes within the wheat genome share high levels of similarity in their gene order and content, varying only in their repetitive DNA content. Most recombination occurs within the genic regions of the chromosomes. Recombination between non-homologous chromosomes is prevented in plants containing the *Ph1* locus because of the size difference of non-homologous chromosomes. The earlier condensation seen in the absence of *Ph1* may allow the related chromosomes of different sizes to pair. This has previously been reported in hybrids between *Lolium* species and in interspecific

crosses between diploid species of *Lolium* and *Festuca*. Although the homoeologous chromosomes can be markedly different in size, they condense to a similar length at pachytene, allowing pairing and recombination to occur (Jenkins 1985), (King, Armstead et al. 2002). Similarly, in haploids derived from allotetraploid cotton the A and D genome chromosomes pair at pachytene despite the fact that the A genome chromosomes are approximately twice the size of the D genome chromosomes. Again, this can be attributed to the fact that they condense to a similar length prior to pairing (Jack Mursal and Endrizzi 1976). However, at metaphase I these chromosomes appear as univalents showing that recombination between the genic regions is prevented. In the absence of *Ph1* recombination can occur between the genic regions of related chromosomes in wheat. It is possible that the premature condensation seen in the absence of *Ph1* could allow the related chromosomes of different sizes to pair successfully, but that the other changes seen during pre-meiotic replication contribute to the ability of related chromosomes to recombine.

In the presence of *Ph1* there is no pairing between non-homologous chromosomes and so it is likely that DSBs are repaired using sister chromatids. In the absence of *Ph1*, there is some pairing between non-homologous chromosomes, and these non-homologous partners could be used for the repair of DSBs. Preliminary work using an antibody to RAD51 to visualise the DSB sites have shown that RAD51 labelling is more widespread and persists for longer in the absence of *Ph1*, suggesting that there are differences relating to DSBs in the presence and absence of *Ph1* (Martin, unpublished data). This could be due to an increased number of DSBs in the absence of *Ph1*, because of the differences in chromatin remodelling, or could suggest that there is a difference in their mechanism of repair. Further work will need to be done to investigate this further.

Chapter 4: the expression and interactions of early meiotic genes in the presence and absence of *Ph1*

Abstract

The *Ph1* locus is the dominant genetic locus controlling the correct pairing of homologous chromosomes during meiosis in wheat. It has recently been defined to a series of truncated and frame shifted *Cdk-like* genes on the long arm of chromosome 5B, with closest homology to human *Cdk2*. When the *Ph1* locus is deleted, expression and activity of *Cdk-like* genes on the homoeologous chromosomes increases. Previous studies have been unable to determine the exact expression profiles of the *Cdk-like* genes because of their extremely high levels of similarity to one another. This chapter describes a new method enabling the accurate expression profiling of these genes. The expression profiles of other early meiotic genes are also examined. Finally, the interactions of the CDK-like proteins are studied using a yeast-2-hybrid system to see whether the interactions are similar to those of mammalian CDK2.

4.1 Introduction

The *Ph1* locus has been defined to a series of *Cdk like* genes on the long arm of chromosome 5B disrupted by a piece of subtelomeric heterochromatin, (Griffiths, Sharp et al. 2006), (Al-Kaff, Knight et al. 2007). The homoeologous regions on chromosomes 5A and 5D contain five and two *Cdk-like* genes respectively. Protein modelling studies have shown that the *Cdk-like* genes within the *Ph1* locus bear closest homology to mammalian *Cdk2* (Yousafzai, Al-Kaff et al. 2010). Previous work involving BAC and genomic analyses has provided the sequences of the seven *Cdk-like* genes on chromosome 5B, the 5 *Cdk-like*

genes on chromosome 5A, and the 2 *Cdk-like* genes on chromosome 5D (Al-Kaff, Knight et al. 2007). Examination of these sequences shows them to be highly homologous, each varying by only a few single nucleotide polymorphisms (SNPs). These high levels of homology have caused problems for further work attempting to look at the expression profiles of each *Cdk-like* gene, as the techniques were not sensitive enough to distinguish between the highly homologous genes. Methods involving the use of conserved *Cdk-like* primers to examine RT-PCR products, and identify each *Cdk-like* gene based on their unique SNPs gave inconclusive results, however further work showed that in the presence of the 5B *Ph1* locus expression from the *Cdk-like* homoeologues on other genomes is suppressed, whilst in its absence transcription is activated on the other genomes to compensate (Al-Kaff, Knight et al. 2007). Until recently it was not known whether this increase in expression of C*dk-like* genes from homoeologous regions in the absence of the *Ph1* locus corresponded to an increase in CDK activity. Studies carried out using phosphorylation of histone H1 as a measure of CDK activity have shown that histone H1 phosphorylation is increased at CDK consensus sites in the absence of *Ph1*, suggesting that CDK-like activity is indeed increased (Greer, Martin et al. In preparation).

Examination of the protein sequences encoded by these genes gives us some idea as to how this could be occurring. Many of the *Cdk-like* genes, including all of those found on the 5B genome, contain frame shifts or stop codons within their sequences. There are two mechanisms by which these truncated or frame shifted genes could be suppressing the activity of homoeologous CDKs from the other genomes. The first is by RNA interference (RNAi) whereby small pieces of RNA (siRNA) are generated by one of two methods. Either transcription of the 5B *Cdk-like* genes could produce short fragments of 20-25 nucleotide long RNA which are homologous to the transcripts of the *Cdk-like* genes on the

homoeologous genomes, or transcription of the 5B *Cdk-like* genes could produce sequences which can interact with themselves, forming a 'hairpin' structure of double stranded RNA (dsRNA) This dsRNA would be targeted for cleavage by the ribonuclease enzyme Dicer, which cleaves the dsRNA into short sequences and separates it into single strands (Bernstein, Caudy et al. 2001). The siRNA molecules produced by either of these methods serve as guide sequences, which bind to complementary regions of mRNA, and induce cleavage by Argonaute (Liu, Carmell et al. 2004), (Song, Smith et al. 2004), the catalytic component of the RNA-induced silencing complex (RISC) (Hammond, Bernstein et al. 2000), (Zamore, Tuschl et al. 2000), (Tuschl, Zamore et al. 1999). This then prevents use of the mRNA as a translation template (Ahlquist 2002).

The second possibility is that the products of the 5B *Cdk-like* genes act in a dominant negative manner towards the products of the *Cdk-like* genes on the other genomes. The CDKs encoded by the 5B genome may still be able to interact with the same substrates as the full length copies, but the function of the interaction could be inhibited or changed in the truncated or frame shifted copies (Herskowitz 1987).

As yet little is known about the interactions of the products of the wheat *Cdk-like* genes, although suggestions can be made based on the interactions of mammalian CDK2, the closest homologue of the wheat *Cdk-like* genes. The interaction between CDK2 and cyclin E is known to be important at the G_1 – S transition, where the complex acts to inactivate the retinoblastoma protein, thereby allowing progression into S-phase (Dulic, Lees et al. 1992). Progression through S-phase is also thought to be dependent on the interaction between CDK2 and A type cyclins (Heichman and Roberts 1994), (Pines and Hunter 1990). PCNA has also been found to form complexes with many CDKs, and it has been shown that CDK2 can directly interact with PCNA (Koundrioukoff, Jonsson et al. 2000).

It has been proposed that PCNA brings CDK2 to proteins involved in DNA replication and possibly might act as an "adaptor" for CDK2-cyclin A to PCNA-binding DNA replication proteins, in particular DNA ligase I (Koundrioukoff, Jonsson et al. 2000). Similarly, an interaction has been shown between CDK2 and human flap endonuclease I (FEN1) (Henneke, Koundrioukoff et al. 2003), another enzyme involved in the processing of Okazaki fragments (Henricksen, Veeraraghavan et al. 2002). CDK2 is also involved in the regulation of the replication process. CDK2-cyclin A complexes have been shown to phosphorylate human ORC1, the largest subunit of the human origin recognition complex, providing a common signal for ubiquitin mediated proteolysis (Méndez, Zou-Yang et al. 2002).

CDK, cyclin and PCNA complexes have been shown to associate with p21to form a quaternary complex, allowing p21 to inhibit CDK-cyclin complex activity at checkpoints if DNA damage has been detected (Xiong, Zhang et al. 1992). $p27^{KIP1}$ is closely related to p21, and inhibits the activity of CDK2/cyclin A and cyclin E complexes (Polyak, Lee et al. 1994), (Toyoshima and Hunter 1994). $p12^{DOC-1}$ specifically interacts with CDK2 to lower its levels of activity, probably by targeting it for proteolysis (Shintani, Ohyama et al. 2000), and WEE1 is yet another cyclin inhibitor protein that is known to interact with CDK2 (Wroble, Finkielstein et al. 2007). The reactivation of CDKs after inhibition by CKIs is known to occur through the phosphatase activity of CDC25, which has also been shown to interact directly with CDK2 (Sohn, Parks et al. 2005). In order to gain full function, CDKs require an activating phosphorylation by CDK-activating kinases (CAKs), in higher eukaryotes known to be $p40^{MO15}$/CDK7, cyclin H, and an assembly factor, MAT1 (Cheng, Kaldis et al. 2000). A human protein phosphatase known as KAP is also known to reverse the activating phosphorylation on CDK2 (Poon and Hunter 1995), and it has also been shown that the

serine/threonine protein phosphatase 2C is capable of dephosphorylating human CDK2 (Cheng, Kaldis et al. 2000).

These are amongst many of the interactions described for CDK2 which regulate and contribute to its ability to regulate replication and control chromatin structure in mammalian cells (Yousafzai, Al-Kaff et al. 2010(a); Yousafzai, Al-Kaff et al. 2010(b)). Similar functions involving replication and chromatin remodelling have been reported for the *Ph1 Cdk-like* genes (Knight, Greer et al. 2010),(Greer, Martin et al. In preparation). Therefore it would be of little surprise to find that many of the interactions documented for mammalian CDK2, are also common to the wheat *Cdk-like* gene products.

The involvement of the *Ph1* locus in the control of chromatin remodelling would also suggest that the expression of pre-meiotic and meiotic genes is altered in plants lacking the *Ph1* locus. As reported in chapter three, the chromatin of meiocytes is more decondensed pre-meiotically, and more condensed meiotically in plants lacking the *Ph1* locus when compared to the wild type. The link between chromatin state and gene expression has long been reported. Gene expression can be silenced by inducing the methylation and subsequent condensation of DNA using carcinogenic nickel treatment (Lee, Klein et al. 1995). Studies on plants, animals and fungi now leave little doubt that gene silencing is a major biological consequence of DNA condensation caused by methylation (Bird and Wolffe 1999), (Colot and Rossignol 1999). Chromatin remodelling regulates nearly every step of the pathway that leads to gene transcription (Fry and Peterson 2002), (Soutoglou and Talianidis 2002). It is thought that prior to the activation of transcription, chromatin remodelling enzymes must first be recruited to decondense the local chromatin in order to allow the recruitment of the preinitiation complex (PIC) which is composed of RNA polymerase II and other transcription factors (Fry and Peterson 2002).

Chapter 4: the expression and interaction of early meiotic genes in the presence and absence of *Ph1*

Studies on mammals have shown that the degree of transcription of a gene is proportional to the level of chromatin decondensation (Müller, Walker et al. 2001). Likewise, the expression of gene clusters in plants has been shown to be associated with chromatin decondensation (Wegel, Koumproglou et al. 2009). These results would point to a hypothesis whereby in the pre-meiotic *Ph1*- cells there may be an increase in gene transcription when compared to wild type, due to the increased decondensation of the chromatin, whereas meiotically there may be less transcription due to the earlier condensation of the chromatin.

Indeed, quantitative PCR analysis of wheat plants either carrying or lacking the *Ph1* locus has suggested that the removal of *Ph1* causes a twenty fold increase in *TaASY1* transcripts (Boden, Langridge et al. 2009). Although a comprehensive study has investigated the transcript levels of several early meiotic genes in the presence of *Ph1*, little is known about how this compares to the *Ph1b* mutant (Crismani, Baumann et al. 2006).

This chapter details experiments that were undertaken to investigate the expression profiles of the wheat *Cdk-like* genes in the presence and absence of *Ph1*, using a method with increased fidelity to allow each *Cdk-like* gene to be accurately sequenced and distinguished. Experiments were then carried out to attempt to elucidate the interactions of one of the wheat CDK-like proteins using a yeast-2-hybrid method. Finally, quantitative PCR methods were used to investigate the expression of several early meiotic genes in the presence and absence of *Ph1*.

4.2 Methods

Examining the expression profiles of the cdk-like genes

100 anthers were staged and collected from pre-meiotic wheat *Ph1+* and *Ph1-* spikes. Upon collection the anthers were immediately frozen on dry ice. RNA was extracted from the anthers using a Qiagen RNeasy Plant Mini Kit as per manufacturer's instructions. The RNA was run on a gel to check that it was good quality and that there was no DNA contamination. Reverse transcription of the RNA was carried out using Invitrogen's Superscript II RT kit as per manufacturer's instructions. A PCR was then carried out using GAPdH primers to confirm that there was no DNA contamination. PCR was then carried out using primers to conserved regions of the cdk-like genes (F: CCCGACTTCAAGGTGGAC; R: GACACCGCGAGCCCTAGGTC) using Phusion High Fidelity DNA Polymerase (Finnzymes: F-530S). The PCR product was used to transform chemically competent *E.coli* cells using Invitrogen's Zero Blunt TOPO PCR Cloning Kit as per manufacturer's instructions. Colonies were then grown overnight on L.B. plates containing 50ug/ml ampicillin. 48 colonies were selected for each genotype and grown up overnight in LB containing ampicillin. Plasmid DNA was then isolated using a QIAprep Miniprep kit as per manufacturer's instructions. To check that each sample contained the correct insert a PCR was carried out using M13 primers and any samples with inserts of an incorrect size were discarded. The samples were then purified using a Qiagen MinElute 96 UF PCR purification kit as per manufacturer's instructions. Samples were then prepared for sequencing using Big Dye Terminator v.3.1 Cycle Sequencing kit, and then sent for sequencing at the genome centre. The results were individually aligned to the known *Cdk-like* sequences provided by Al-Kaff, Knight et al. (2007) using the Vector NTI AlignX program. A detailed protocol is given in appendix 7.

Yeast-2-Hybrid

Ph1+ wheat anthers were staged as pre-meiotic and collected on dry ice and liquid nitrogen until 1g of material was obtained. These were sent to Invitrogen for library construction. It was decided to use CDK-like D2 as the bait for the yeast 2 hybrid, as this is one of the *Cdk-like* genes that is a full open reading frame, and because previous results have suggested that expression is increased in the *Ph1* mutant, suggesting that it may play a role in producing the mutant phenotype. 5ug of D2 plasmid was also sent to Invitrogen where it was recloned into a pDEST32 vector for use as bait. Invitrogen performed a 3 Amino-1,2,4-Triazole (3AT) optimization test to establish which concentration of 3AT should be used in order to reduce false positives. It was decided to use a 20mM concentration of 3AT as this was the lowest concentration at which self-activation failed to occur. The bait and prey plasmids were then introduced into MaV203 yeast cells using co-transformation, and were incubated for 5 days. From this 90 colonies were identified as candidates. A second screening was then carried out using an SD-LT plate (lacking leucine and tryptophan), an SD-LTH plate (lacking leucine, tryptophan and histidine) with 20mM 3AT, and a nylon filter membrane on a YPD plate which allows for the use of a β-

galactosidase assay to identify positive interactions. The secondary screen was repeated using 25mM 3AT to ensure that the background was properly suppressed.

Real Time RT PCR

Real time RT PCR was carried out using a protocol developed by Ruoyu Wen which is detailed in full in appendix 8. Anthers from wheat and wheat-rye plants were collected at the pre-meiotic stage on dry ice, both in the presence and absence of *Ph1*. RNA was extracted from the anthers by adding TRI reagent to ground anthers, followed by separation of the solution into phases using BCP. The aqueous phase (containing the RNA) was removed, and the RNA pelleted, washed and re-suspended in water. At this stage the RNA concentration was measured using a Nanodrop spectrophotometer. Any contaminating DNA was removed using DNAse I, and acid-phenol:chloroform added to separate the mixture into phases from which the aqueous phase was removed. RNA was then precipitated using LiCl treatment. The RNA was then pelleted and resuspended in water. Reverse transcription was then carried out using Superscript III (Invitrogen). Primers were used for several early meiotic genes (*Asy1, Dmc1, Msh4, Msh6, Phs1, Rad51B, Rad51C, Rad54, Rpa*) as well as primers designed to a conserved region of the *Cdk-like* genes, and primers designed to the full length of the *Cdk-like* genes. In this way it was hoped to establish what proportion of *Cdk-like* expression could be attributed to full-length copies. *Actin* and *GAPdH* were used as reference genes. All primer sequences apart from those designed from the *Cdk-like* genes are listed in Crismani, Baumann et al. (2006). *Cdk-like* primer sequences are as follows (*Cdk* full length 1, F:ACCCCGACTTCAAGGTGGAC, R:GTGAGGTGGCGCGCCTCCACG, *Cdk* full length 2, F:TGGAGGCGCGCCACCTCAC, R:AGCGCCCGGAGCTCGACGATG, *Cdk* conserved, F:TTCGAAGTCCTCAAGGGGCTC, R:GGCGTTGGCGAACCATGGTCG). Real time PCR was carried out using a Lightcycler 480 machine. Data was analysed using LinRegPCR software as described in (Ruijter, Ramakers et al. 2009).

4.3 Results

The PCR product obtained by using conserved *Cdk-like* gene primers and the cDNA as a template was transformed successfully into most of the colonies picked for sequencing. Figure 1 shows a gel of a PCR using the plasmid DNA with M13 primers to check that the insert was of the correct size. Only a few were incorrect, and these were re-sampled so that in total 48 *Ph1+* samples and 47 *Ph1-* samples were sent for sequencing.

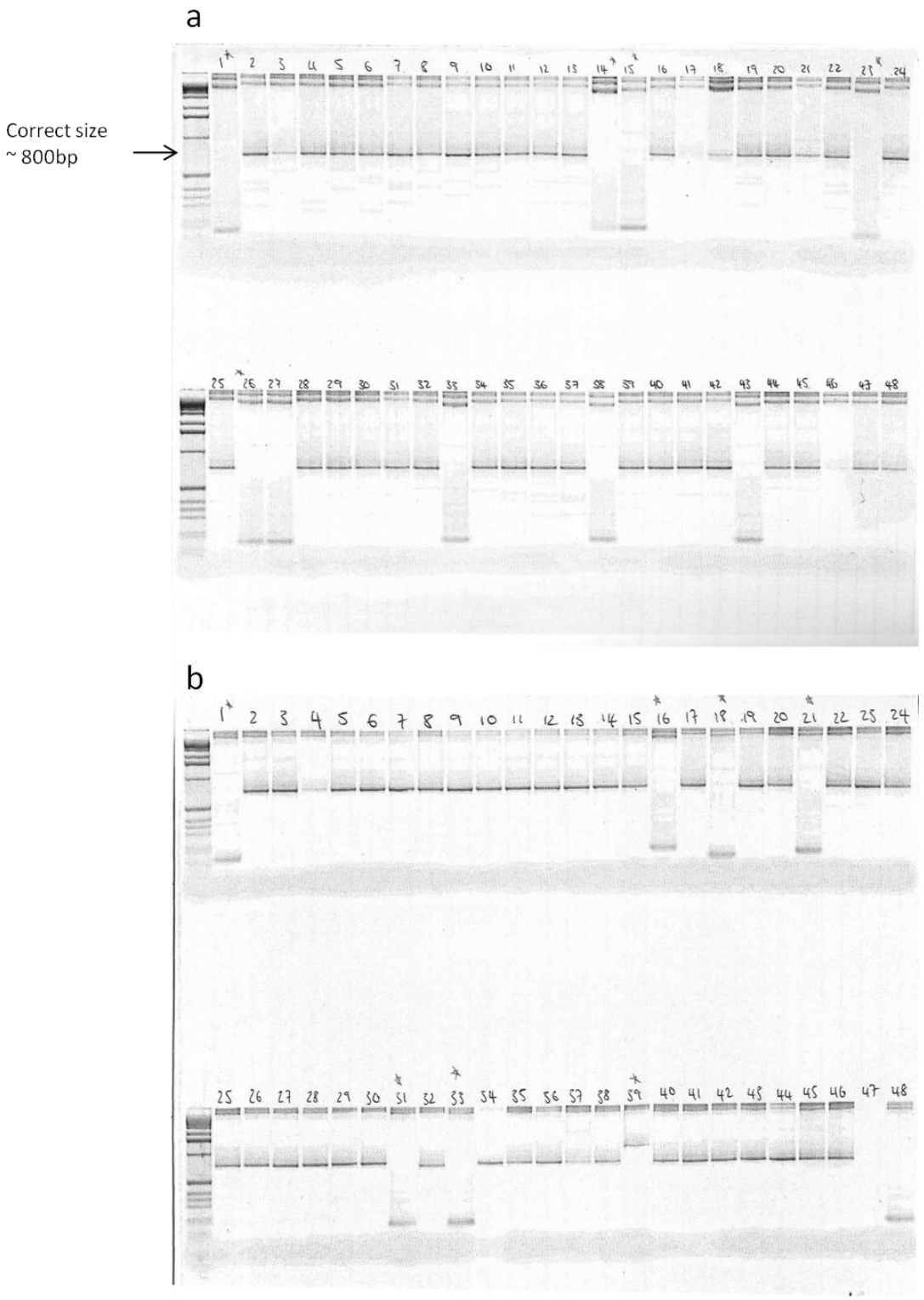


Figure 1: gel of a PCR carried out using M13 primers to check that plasmids contained the correct insert. *Ph1+* samples are shown in panel a, *Ph1-* samples in panel b. Most samples contained an insert of the correct size and so were sent for sequencing.

Vector NTI analysis of the sequence data allowed the majority of samples to be assigned to a single *Cdk-like* gene. Due to the high levels of similarity between the A2 and A5 *Cdk-like* sequences these two genes could not be distinguished using this method and so were counted together. Interestingly, a number of 'unknown' or previously unidentified sequences were found during the analysis, all of which were identical to one another. The translation of this sequence showed that it contains the DARTL motif, which is thought to be the binding motif in the wheat CDK-like proteins, instead of the PSTAIRE domain which is required for cyclin binding in mammalian CDK2 and other CDKs (Yousafzai, Al-Kaff et al. 2010). Figure 2 shows the percentage expression of *Cdk-like* genes in the presence and absence of *Ph1*.

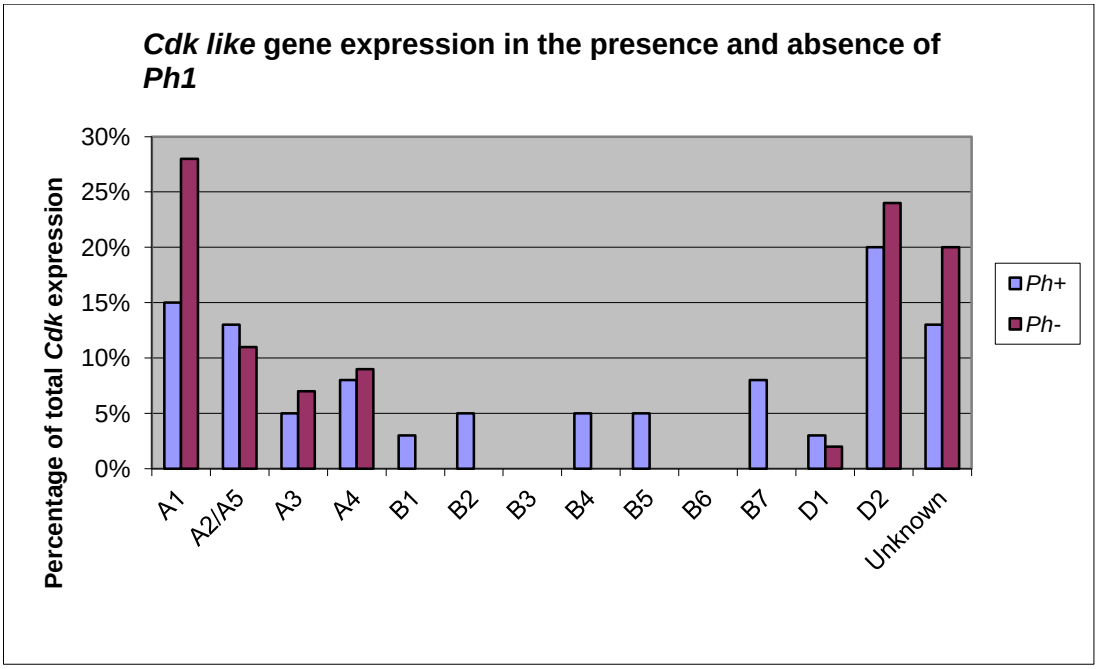
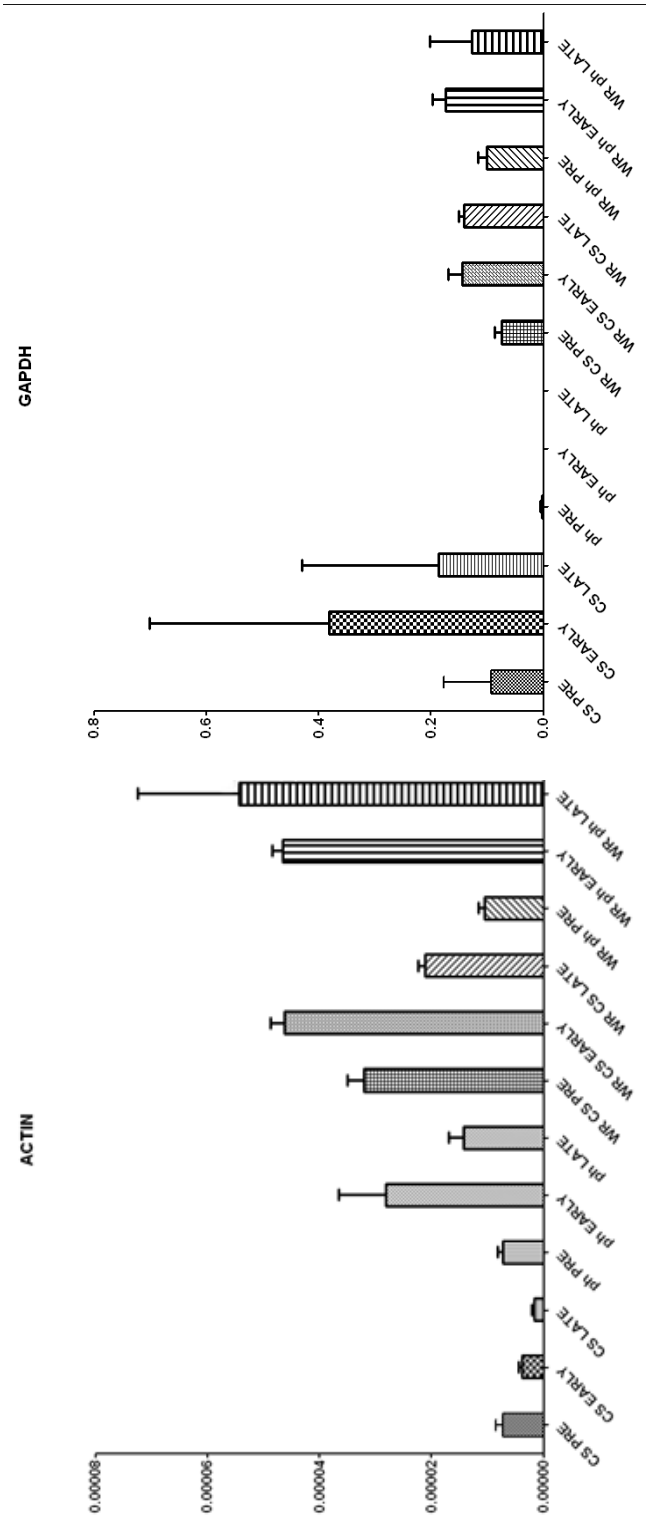


Figure 2: Expression patterns of the *Cdk-like* genes in the presence and absence of *Ph1*. There is no expression of the B genome *Cdk-like* genes in the absence of *Ph1* as this region has been deleted in the *Ph1b* mutant.

The most obvious difference in expression pattern between the two genotypes is that there is no expression of B-genome *Cdk-like* genes in the absence of *Ph1* because this region has been deleted.

Given the differences in chromatin remodelling seen in the presence and absence of *Ph1,* and its link to gene expression it was decided to see whether the expression profiles of several early meiotic genes changed in different genotypes. To do this real time RT PCR was used, but unfortunately gave inconclusive results. The expression patterns of each of the genes are shown in figure 3.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0.4
0.2
0.0
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CS LATE
ph PRE
ph EARLY
ph LATE
WR CS PRE
WR CS EARLY
WR CS LATE
WR ph PRE
WR ph EARLY
WR ph LATE
ACTIN
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0.00006
0.00004
0.00002
0.00000
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ph LATE
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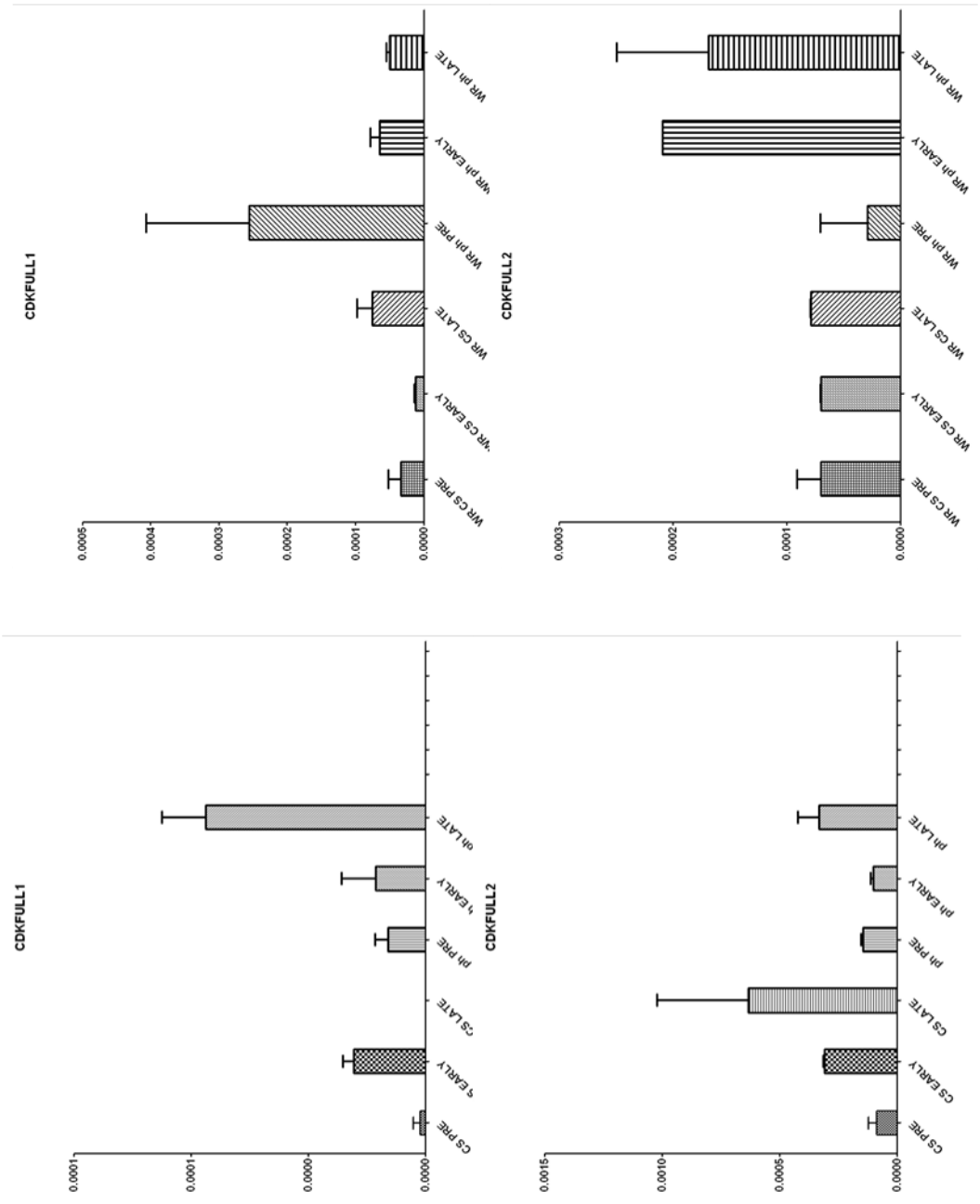
WR CS LATE
WR ph PRE
WR ph EARLY
WR ph LATE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ph PRE
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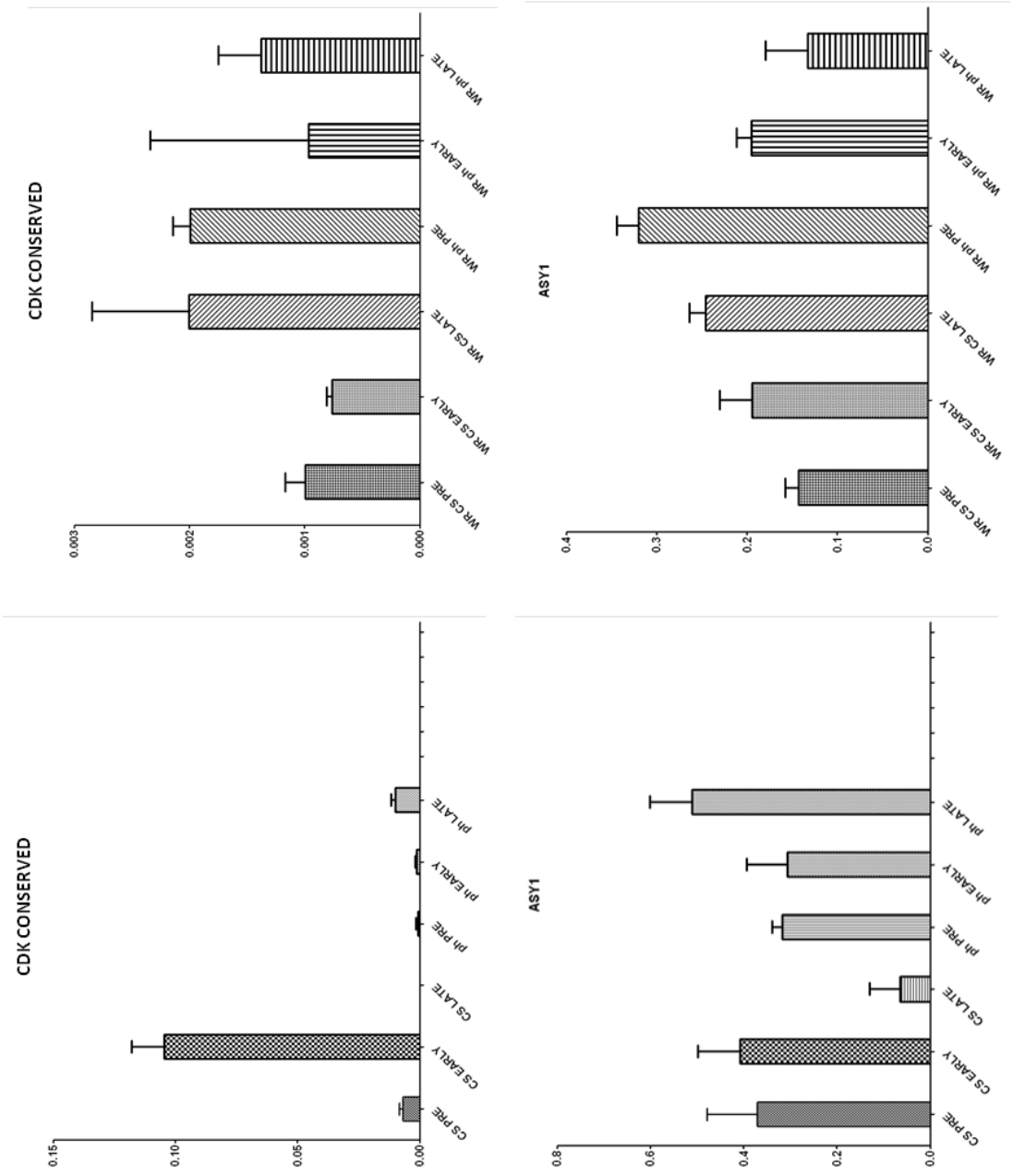
ph LATE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ASY1
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0.1
0.2
0.3
0.4
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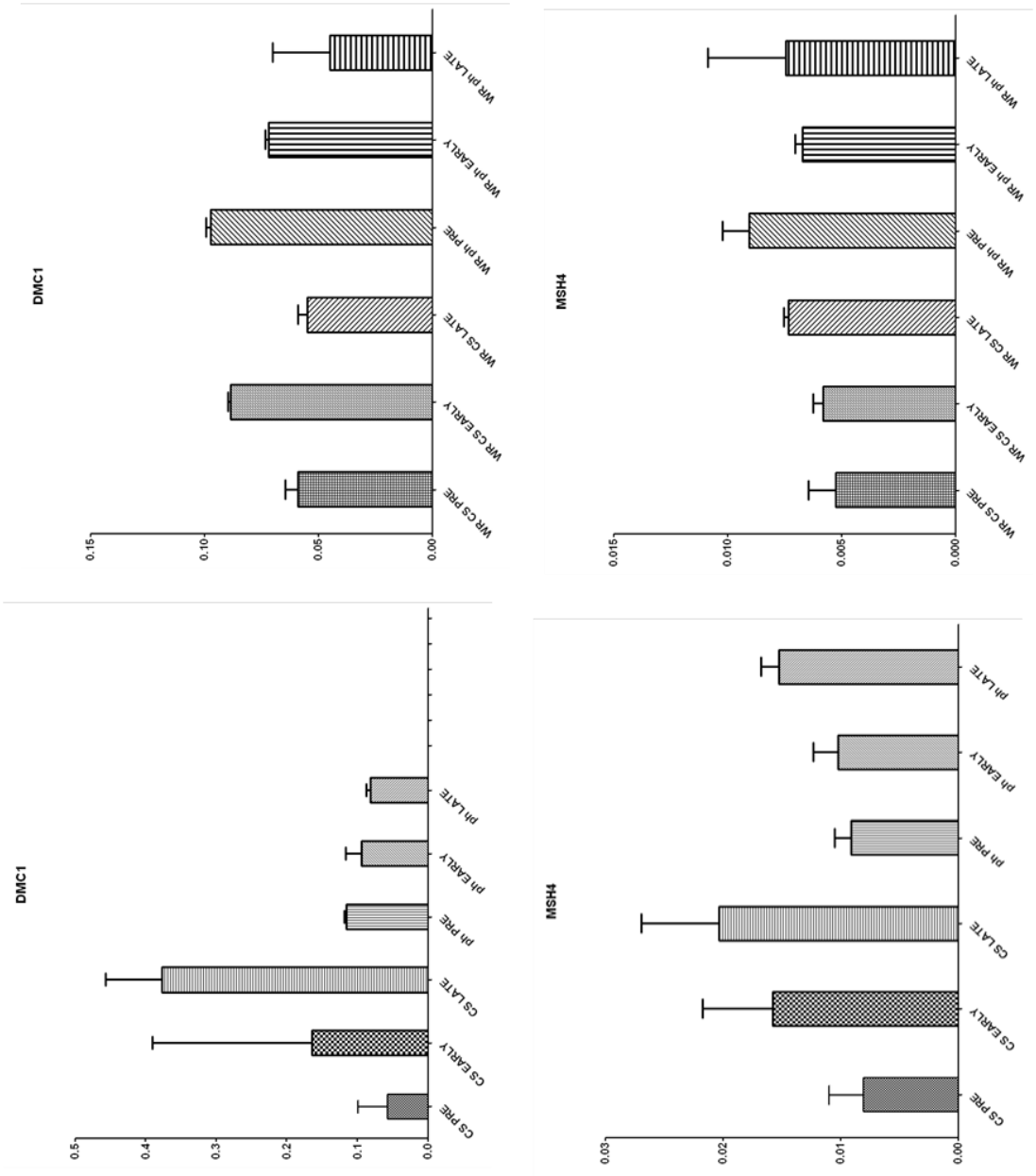
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MSH4
0.015
0.010
0.005
0.000
DMC1
CS PRE
CS EARLY
CS LATE
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ph EARLY
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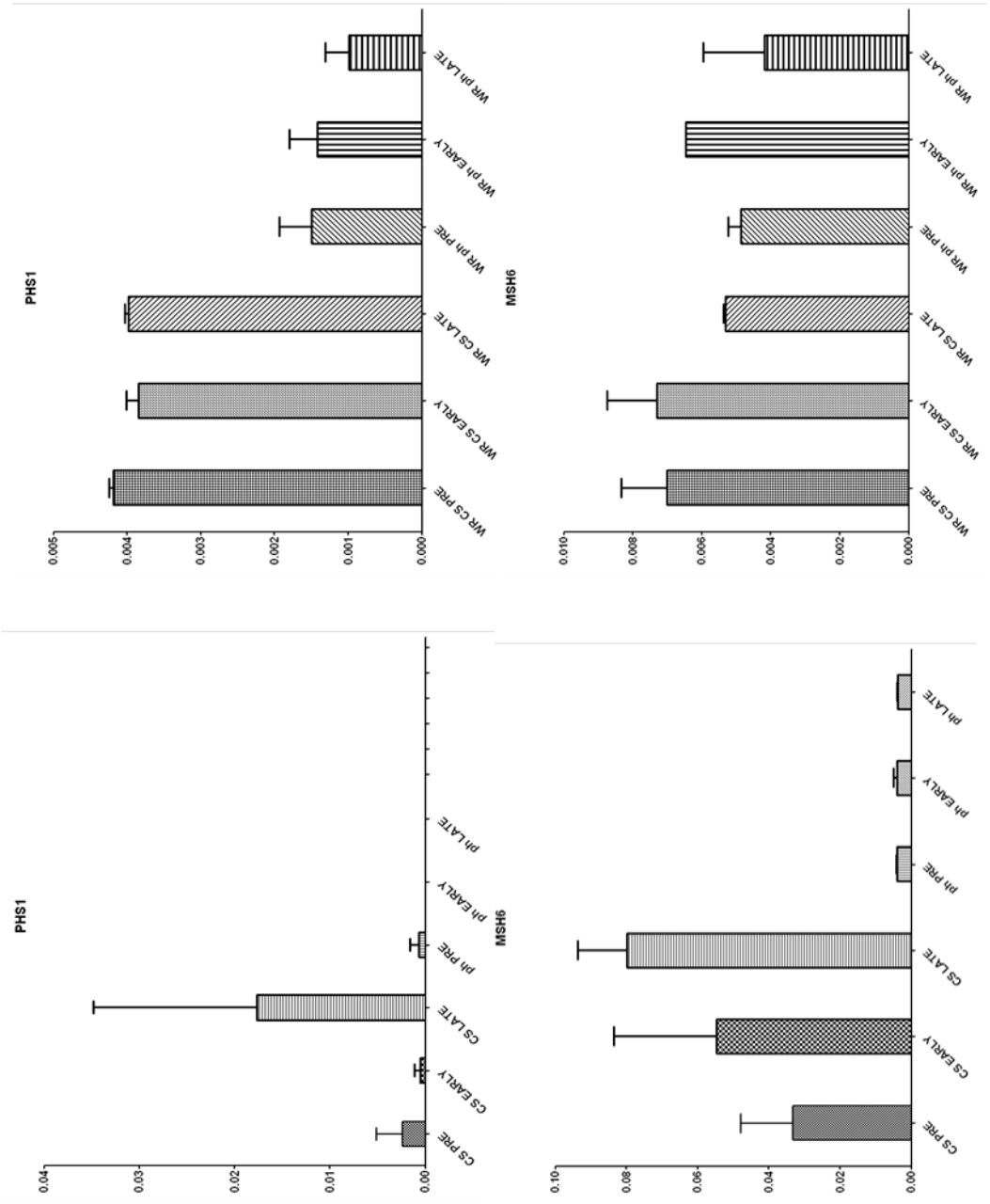
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WR ph PRE
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WR CS PRE
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0.006
0.004
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0.03
0.02
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0.00
MSH6
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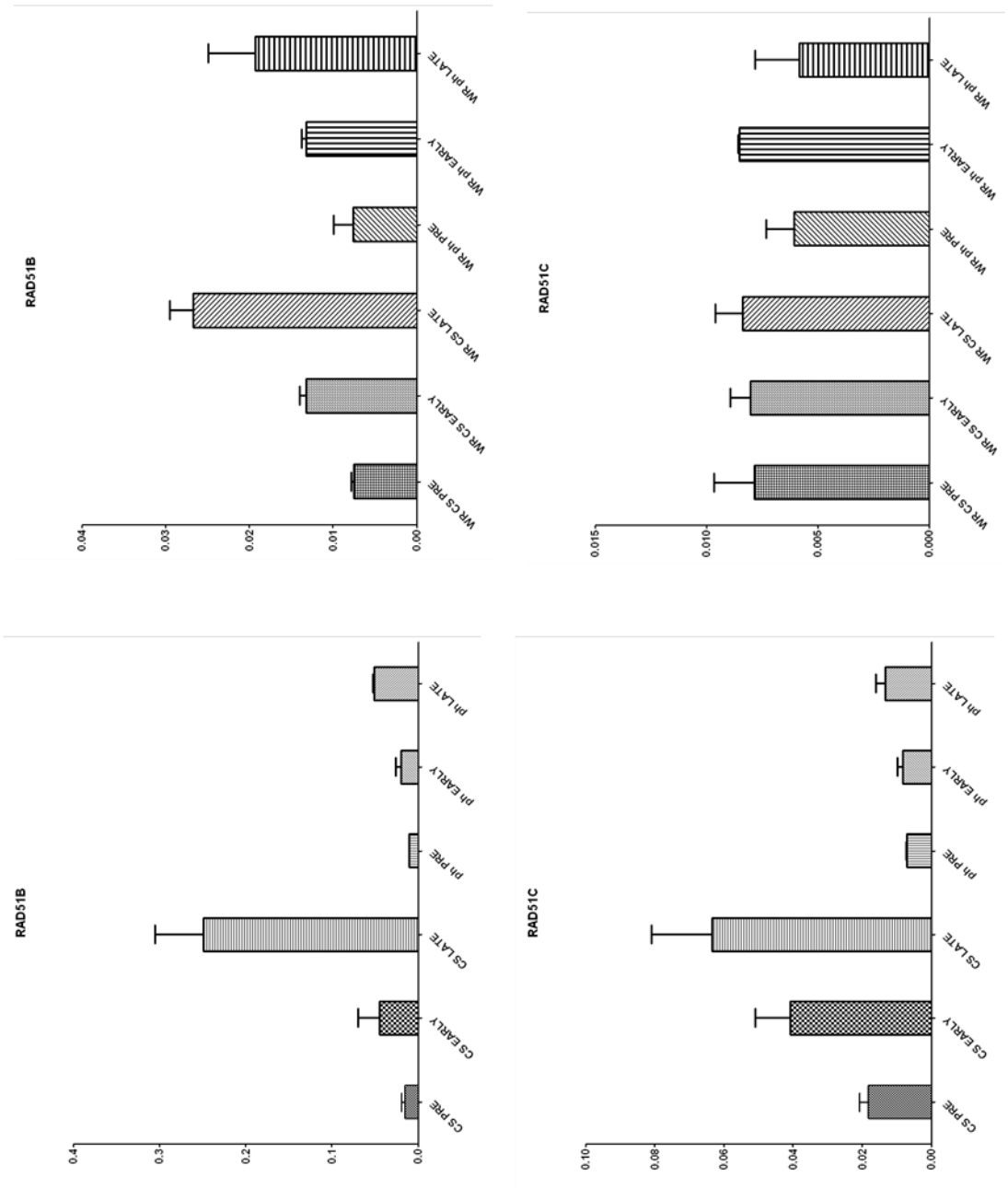
0.00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0.03
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0.01
0.00
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WR Ph EARLY
WR Ph PRE
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WR CS EARLY
WR CS PRE
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0.005
0.000
RAD51B
Ph LATE
Ph EARLY
Ph PRE
CS LATE
CS EARLY
CS PRE
0.4
0.3
0.2
0.1
0.0
RAD51C
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0.02
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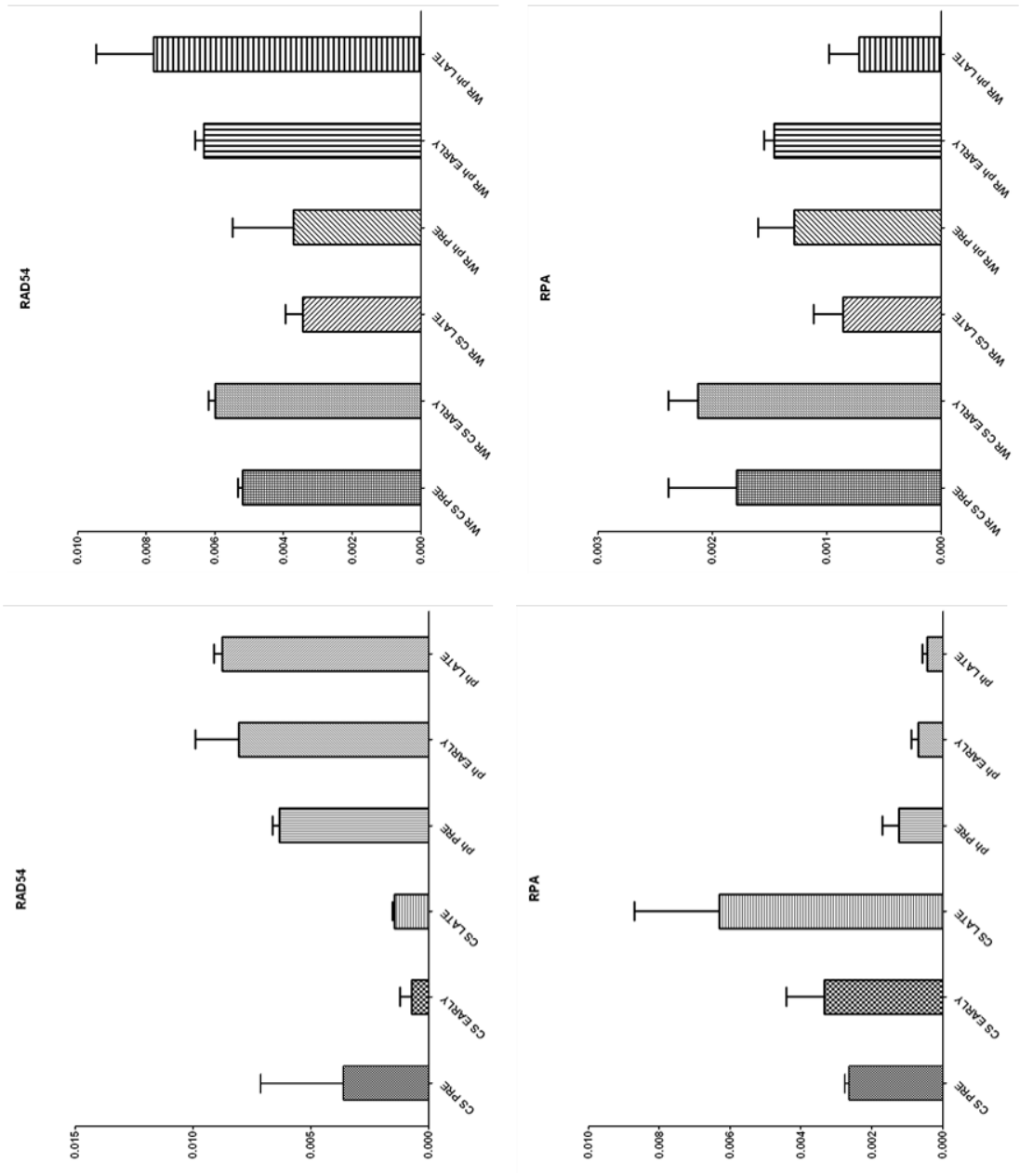


Figure 3: real time RT PCR results for wild type wheat (CS), *Ph1b* mutant wheat (Ph) and wheat-rye hybrid crosses in the presence (WR CS) and absence (WR ph) of *Ph1*. Samples were collected from three meiotic stages; pre-meiotic (pre), early meiosis (early) and late meiosis (late). The y-axis shows the ratio of total expression / actin reference gene expression. Three technical replicates were carried out for each sample. Error bars show S.E.M.

The expression patterns of these early meiotic genes do not seem to follow a particular pattern. In some cases expression is high during the pre-meiotic time point and decreases as meiosis progresses. However, in other cases the reverse can be observed. This doesn't necessarily mean that there is no pattern in early meiotic gene expression, as the reference gene readings indicated that the results could be unreliable. It is unusual to see so much variance in the reference gene readings, especially in samples where the initial concentration of starting RNA should have been the same. There is also a lot of variance between the wheat-rye and wheat readings, which is again unexpected and indicates that there was a problem with the experiment. For example, the wheat and wheat-rye readings vary by as much as ten fold for genes such as *Phs1*, *Msh6*, *Rad51B* and *Rad51C*. Although a small degree of variation between wheat and wheat-rye samples might be expected, this amount seems unlikely and is another factor pointing towards the unreliability of the results. This experiment would have to be repeated to allow a full analysis of early meiotic gene expression patterns, perhaps using additional or different reference genes that may be more reliable.

The yeast-2-hybrid experiment also gave disappointing results. Several controls were used throughout these experiments with a range of interaction strengths. Control A contains both the bait and prey vectors but with no inserts, this should show no interaction. Control B uses human RB as the bait and human E2F as the prey, which should display a weak interaction. Control C is composed of *Drosophila* DP as the bait and *Drosophila* E2F as the prey, and shows a moderately strong interaction. Control D consists of rat cFos as the bait and mouse cJun as the prey and should display a strong interaction. Finally, control E uses GAL4 as the bait whilst the prey contains no insert, which should produce a very strong interaction.

When grown on SD-LT plates only yeast that contains both the bait and prey vectors (which contain genes enabling the synthesis of leucine and tryptophan respectively) should be able to grow. Figure 4 shows the results from growth on SD-LT plates.

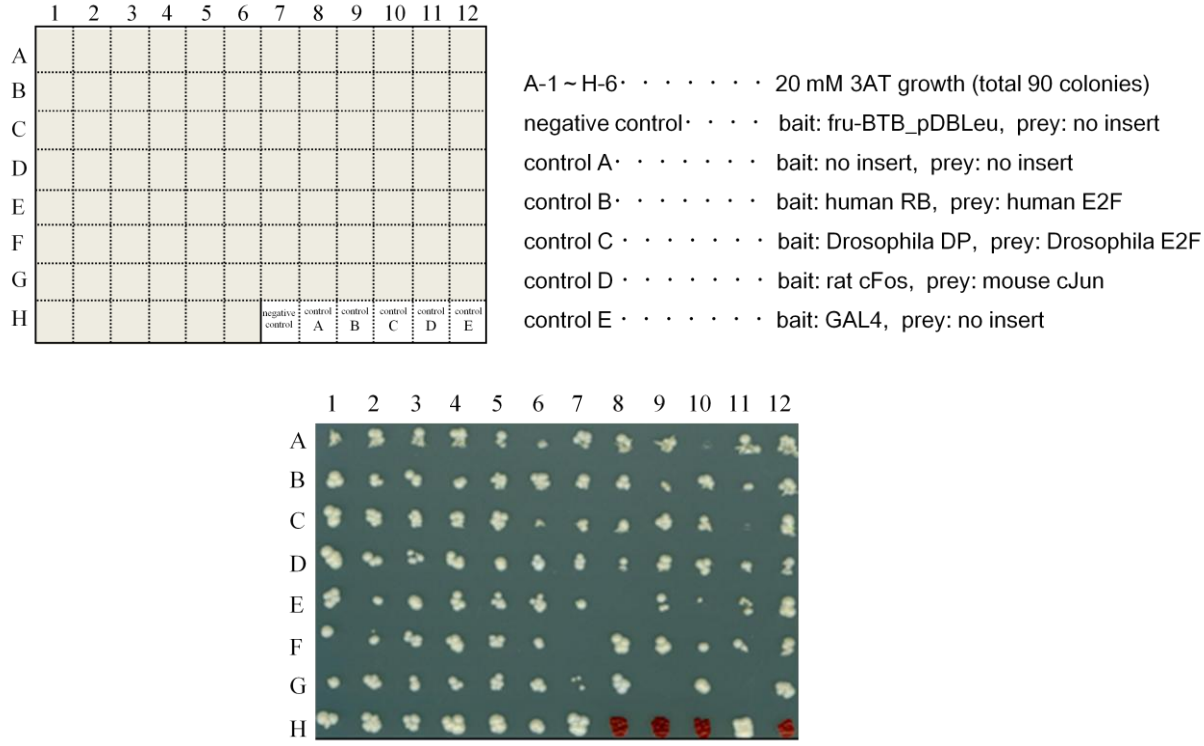
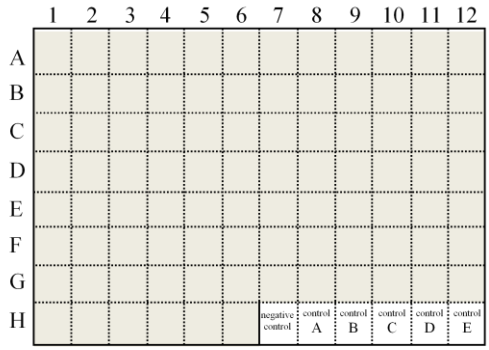


Figure 4: growth of yeast colonies on SD-LT plates. As expected almost all colonies grew well showing that they contain both the bait and prey vectors.

The majority of colonies grew well on this plate showing that the bait and prey vectors have both been incorporated successfully. The clones of A10, C11, E8, E10, F7, G9, and G11 showed low growth which is a typical feature of pseudo clones.

When grown on SD-LTH only yeast colonies containing both vectors with a successful interaction between them should be able to grow. To prevent the growth of pseudo-clones 20mM 3AT was included. Figure 5 shows the results from growth on SD-LTH plates.



A-1 ~ H-6 · · · · · · · 20 mM 3AT growth (total 90 colonies)
negative control · · · · bait: fru-BTB_pDBLeu, prey: no insert
control A · · · · · · · bait: no insert, prey: no insert
control B · · · · · · · bait: human RB, prey: human E2F
control C · · · · · · · · bait: Drosophila DP, prey: Drosophila E2F
control D · · · · · · · · bait: rat cFos, prey: mouse cJun
control E · · · · · · · · bait: GAL4, prey: no insert

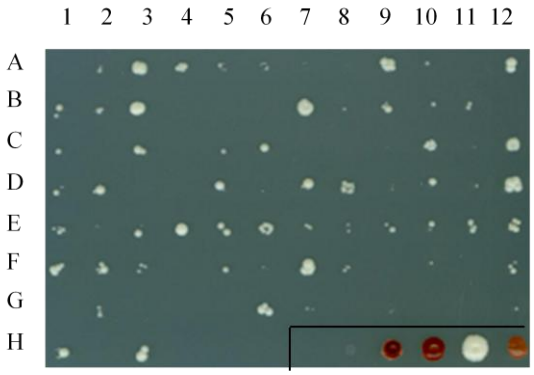
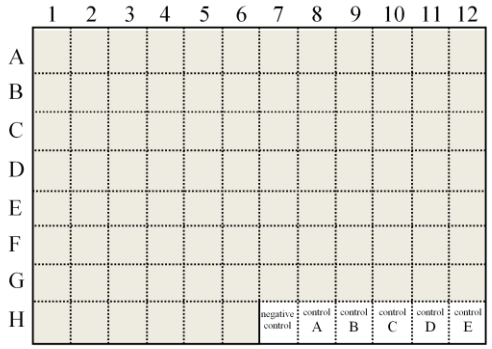


Figure 5: growth of clones on SD-LTH + 20mM 3AT plates.

Although there appears to be some colony growth, when compared to the controls it doesn't seem to be a significant amount. Most colonies are smaller than even the weakly interacting control. This suggests that any interactions seen are likely to be very weak or could be attributed to the growth of pseudo clones due to the concentration of 3AT being too low. In order to confirm whether or not any clones growing on the SD-LTH plate contain true interactions an X-gal assay was carried out, whereby if there are interactions the clones express a lacZ gene and turn blue in colour.



A-1 ~ H-6 · · · · · · · 20 mM 3AT growth (total 90 colonies)
negative control · · · · bait: fru-BTB_pDBLeu, prey: no insert
control A · · · · · · · bait: no insert, prey: no insert
control B · · · · · · · bait: human RB, prey: human E2F
control C · · · · · · · bait: Drosophila DP, prey: Drosophila E2F
control D · · · · · · · bait: rat cFos, prey: mouse cJun
control E · · · · · · · bait: GAL4, prey: no insert

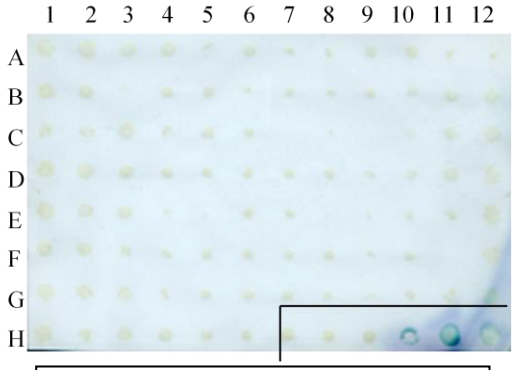


Figure 6: X-gal assay at 20mM 3AT

In the X-gal assay all of the clones remained white indicating that none of them expressed the lacZ gene (see figure 6). Controls C, D and E turned blue showing that the assay worked correctly. However, this test does not exclude the possibility of weak interactors like control B. Therefore each of the screens was repeated using a higher concentration of 3AT (25mM) to reduce the appearance of pseudo clones. The results are shown in figure 7.

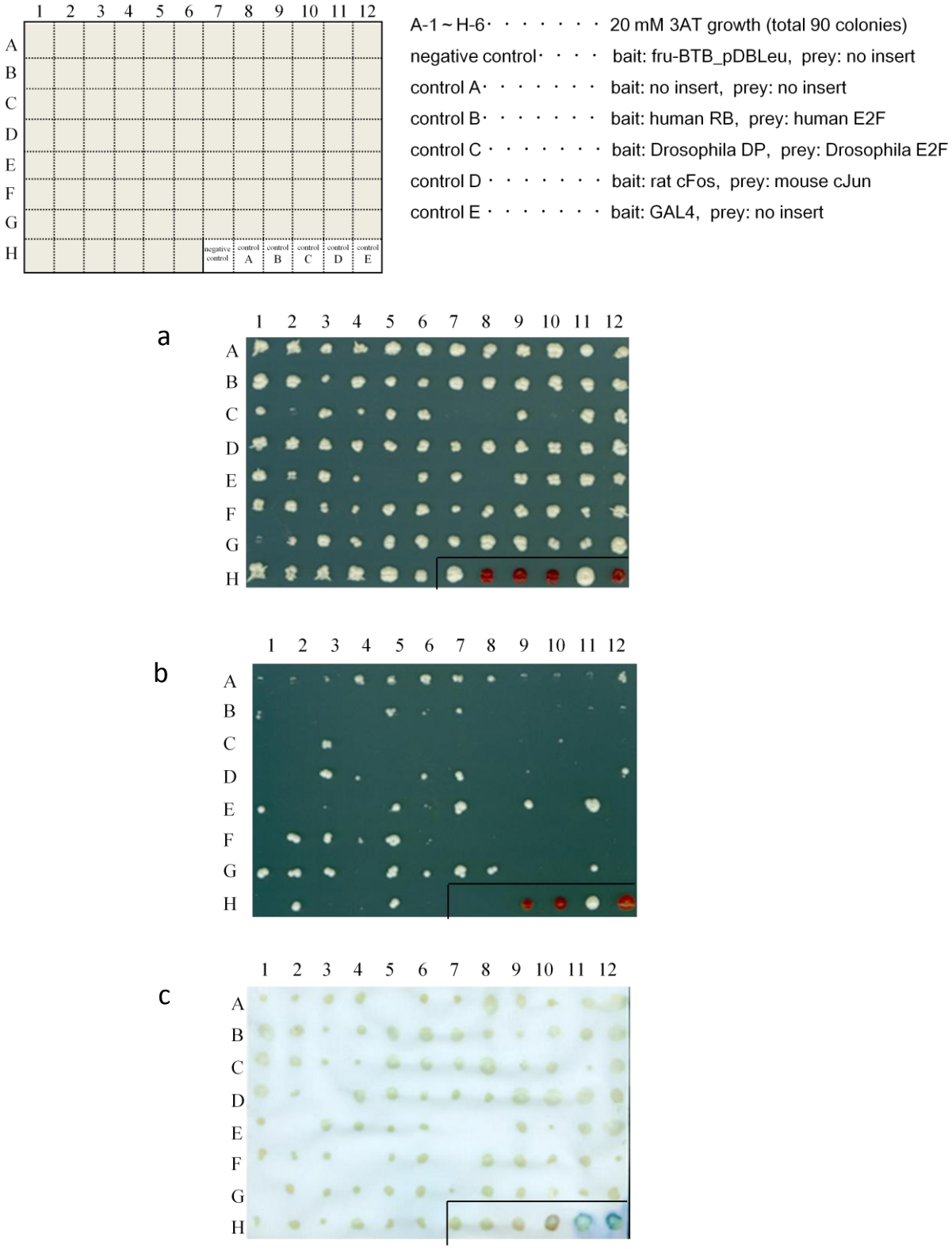


Figure 7: growth of yeast colonies on a) SD-LT plates + 25mM 3AT, b) SD-LTH plates + 25mM 3AT and c) an X-gal assay to look for interactions between the bait and prey.

As expected most colonies grew well on the SD-LT plates showing that the bait and prey vectors were present in most. The growth on the SD-LTH plates was limited, with few samples growing to the same extent as the weak interacting control. The X-gal assay confirms that any interactions must be very weak, as none of the samples turns blue, whilst controls C, D and E all do. Again this series of tests suggest that there are no strong interactions, however, the possibility of weak interactions still exists.

As well as HIS3 and lacZ, URA3 can also be used as a reporter gene in this yeast strain, allowing growth on plates lacking uracil if there is an interaction. SD-LT-Ura plates lack leucine, tryptophan and uracil and so only yeasts containing both the bait and prey vectors and with an interaction between the two can grow. Samples were plated onto SD-LT-Ura plates containing both 20mM and 25mM concentrations of 3AT. The results are shown in figure 8.

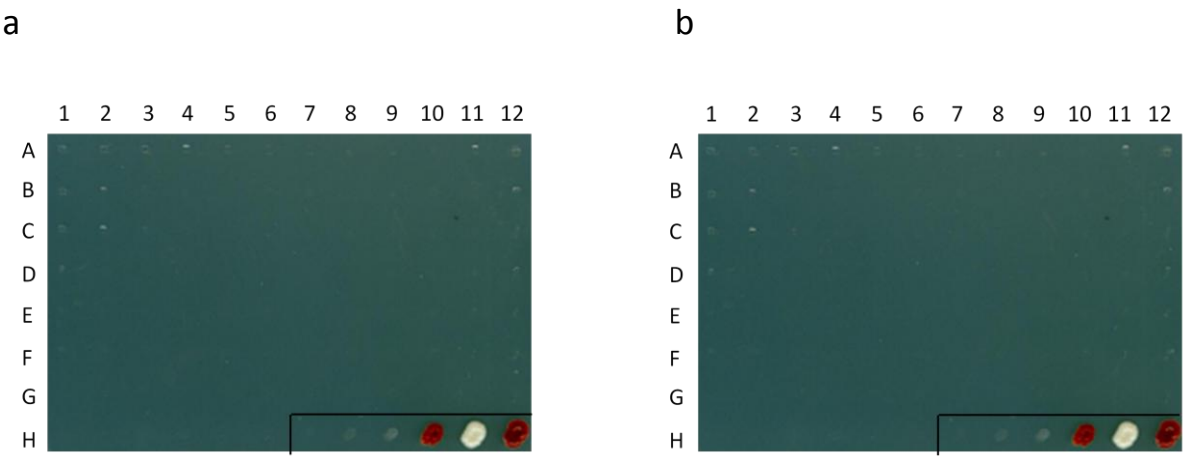


Figure 8: growth on SD-LT-Ura plates containing 20mM (a) and 25mM (b) 3AT.

No growth could be seen on these plates suggesting that there are no strong interactions. Controls C, D and E all grew well.

Induction of *URA3* leads to the conversion of 5-fluoroorotic acid (5FOA) to 5-fluorouracil, which is toxic. Hence, cells containing interacting proteins grow when plated on medium lacking uracil, but growth is inhibited when plated on medium containing 5FOA. SD-LT-5FOA plates were made and the samples plated out. Only yeasts which contain both the bait and prey vectors but in which there is no interaction between the two should grow. The results are shown in figure 9.

a b

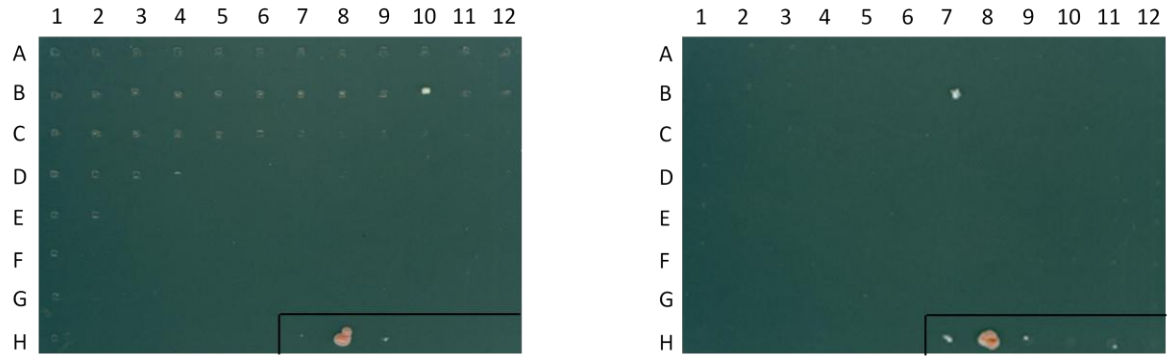


Figure 9: growth on SD-LT-5FOA plates at 20mM (a) and 25mM (b) concentrations of 3AT.

No colonies were obtained on SD-LT-5FOA plates and control A grew as expected, showing that the test functioned correctly.

The overall results from this group of experiments suggest that there are no strong interactions observed within this yeast-2-hybrid assay. However, the possibility of weak interactors cannot be ruled out. As observed with Yeast Control Strain B (DB-Rb/AD-E2F1), intermediate levels of *URA*3 expression can result in both cell growth inhibition on plates containing 5FOA and insufficient *URA*3 gene product to allow growth on plates lacking uracil. The 5FOAS/Ura- phenotype is apparently often indicative of protein pairs that interact weakly in the ProQuest™ Two-Hybrid System.

4.4 Discussion

The results gained from analysis of the expression of the wheat *Cdk-like* genes have given greater clarity to which genes are expressed in the presence and absence of *Ph1*. Of the *Cdk-like* genes found on the 5A, 5B and 5D genomes only two are complete open reading frames; A3 and D2. Although further results will be required to confirm this, it is likely that in the absence of *Ph1* expression of both of these genes is increased, which would explain why an increase in CDK-like activity is seen. The effects of increasing expression of *Cdk-like* genes A3 and D2 have been demonstrated during studies on *Brachypodium distachyon* and *Arabidopsis*. The overexpression of A3 in these model systems causes meiotic defects, whilst overexpression of D2 causes an even more extreme phenotype, with most plants dying in the early stages of growth (Ruoyu Wen, unpublished data). This suggests that the hypothesised increase in expression of both A3 and D2 contribute to the *Ph1* mutant phenotype, but that D2 probably plays a more prominent role.

Interestingly, expression of an 'unknown' *Cdk-like* sequence was detected using this method. It would appear that this is not a full length *Cdk-like* gene as two premature stop codons are found, even in the short fragment used for the cloning in this experiment. It does contain the cyclin binding DARTL site, which suggests that it may still bind cyclins but it may not be able to carry out the subsequent functions of the CDK:cyclin complex. Synteny studies on *Brachpodium distachyon* have identified two *Cdk-like* genes which correspond to the *Ph1* region, but have also identified a third *Cdk-like* gene in a different area of the genome (Ruoyu Wen, personal communication). It is possible that this 'unknown' wheat *Cdk* could correspond to the third *Brachypodium Cdk-like* gene, although

the possibility that it is a previously unidentified *Cdk* from the cluster within the *Ph1* and corresponding homoeologous regions cannot be discounted. This previously unidentified gene could also be an artefact of the PCR process. Given the high degree of similarity of the *Cdk-like* genes and their high GC base content it is possible that splicing occurred during the PCR, whereby synthesis began on one *Cdk-like* gene, but then somehow switched to another partway through. This was a common problem during previous studies on the *Cdk-like* genes (Griffiths, Al-Kaff, personal communication), and although it was hoped that the use of the Phusion PCR kit would eradicate it, it is possible that in this case it still happened.

Studies have shown that in the absence of *Ph1* CDK activity increases (Greer, Martin et al. In preparation). As discussed in the introduction two mechanisms have been proposed which could account for this increase in activity; RNA interference whereby transcripts of the truncated or frame shifted genes inhibit the translation of functional sequences, or a dominant negative mechanism whereby the translated products of the truncated or altered gene sequences can still interact with the correct substrates, but the function of the interaction is inhibited. The results generated from these experiments do not allow either of these hypotheses to be discounted. Although this experiment will need to be repeated with a larger sample size before definite results can be gained, these results give a preliminary indication of expression levels. In the wild type 78% of total *Cdk* expression can be attributed to truncated or altered genes, whilst in the *Ph1* mutant this figure falls to 70%. It could be that the reduced expression of the altered genes leads to a less effective RNAi mechanism, allowing an increase in the translation of the functional CDKs. Likewise, when considering the dominant negative mechanism in relation to these results, the reduced expression of the altered genes may lead to less non-functional interactions, whilst the increased expression of complete *Cdk-like* genes would increase the

number of functional interactions. Further work will need to be undertaken to establish which of these mechanisms accounts for the increase in CDK activity.

Given the differences in chromatin remodelling shown in chapter 4, it is likely that the expression patterns of early meiotic genes are also affected in the absence of *Ph1*. However, the results of the real time RT PCR which was used to investigate this gave unsatisfactory and unreliable results. No pattern could be seen in the expression of early meiotic genes throughout the early stages of meiosis. It should not be assumed that there is no pattern in expression levels. The degree of variation seen within the reference gene readings suggests that there may have been a problem with the sample quality. There is also a lot of variance in the readings between the wheat and wheat-rye samples. Although a small degree could be expected, the levels seen in these experiments suggest that the results are unreliable. Similarly, these results do not match previous reports of early meiotic gene expression in hexaploid wheat (Crismani, Baumann et al. 2006). There is one obvious point at which this experiment could have gone wrong. Following the preparation of the plates for the PCR, they should be transferred immediately to the lightcycler 480 machine. However, there were technical problems with the machine which meant that the plates had to be stored at -80˚C for almost a week until the machine could be fixed. It is likely that the samples could have been damaged or degraded during this storage time, leading to the unreliable results generated by this experiment. Following the announcement that the wheat genome would soon be fully sequenced, it was decided not to continue using this method to investigate expression levels of early meiotic genes. The full wheat genome sequence will allow the use of high throughput sequencing techniques that should be more suitable to investigate large scale gene expression over the course of meiosis. Future experiments should soon give us a comprehensive assessment of gene

expression patterns throughout meiosis, and differences in those patterns in plants either lacking or carrying the *Ph1* locus.

The yeast-2-hybrid experiments provide some puzzling results. Yeast-2-hybrid experiments are commonly used to identify protein-protein interactions, and have successfully been used to identify interactions between mammalian CDK2 and other proteins (Hannon, Demetrick et al. 1993), (Hannon, Casso et al. 1994). In these experiments however, the yeast-2-hybrid system failed to identify any strong interactions involving wheat CDK-like D2. Although this could simply mean that the D2 protein does not undergo any strong interactions, this is unlikely given its structural similarity to mammalian CDK2 which is known to interact with several proteins. A more likely explanation as to why no strong interactions were found is that there was a problem with the experiment. Invitrogen did initially have some problems with transferring the bait sequence to the vector necessary for the screen, leading to it failing the first Q.C. test. The transfer was repeated and upon the second attempt the Q.C. test was passed. The D2 bait construct was sequenced prior to its use and found to match the known sequence, and so it is unlikely that this was the source of the problem. Furthermore, both the bait and prey vectors were present, as evidenced by yeast growth on SD-LT plates. This suggests that the problem may have been due to the bait sequence being out of frame, or may have arisen upon the construction of the prey library. RNA isolation was successful, and the subsequent cDNA library passed the Q.C. tests, leaving the transfer of the prey library to the required vector as the only point at which this process could have gone wrong. Regardless of the reason for the lack of strong interactions, further investigation into the issue could not have been undertaken without significant expenditure and so it was decided to not pursue it any further. Following reports of the ability of mammalian *Cdk2* to partially compensate for the

loss of *Ime2* in yeast (Szwarcwort-Cohen, Kasulin-Boneh et al. 2009), experiments were undertaken to attempt complement the yeast *ime2* mutant with the wheat *Cdk-like D2* gene. The results from these experiments indicated that wheat *Cdk-like D2* could not compensate for the loss of *Ime2* (a yeast meiosis specific CDK-like kinase)(Ruoyu Wen, unpublished data). This is most likely attributable to the fact that the cyclin binding motif of the wheat CDKs is different to that of mammalian CDK2, a change that is mirrored by a corresponding change in plant cyclins(Yousafzai, Al-Kaff et al. 2010). This is probably why it cannot compensate for the loss of *Ime2*, although it could be that CDK-like D2 doesn't undergo any significant interactions with other proteins (as suggested by the yeast-2-hybrid experiments), therefore making it unable to functionally compensate for the loss of *Ime2*. It is also possible that the wheat CDKs need to undergo specific modifications such as phosphorylations to become functional. These may not be happening in the yeast system and this may be why experiments involving yeast do not seem to work.

The work presented in this chapter has brought us a step closer to understanding why we see an increase in CDK activity in the absence of *Ph1*. There may be an increase in the expression of functional *Cdk-like* genes, which explains the differences in replication and chromatin remodelling that have been reported in the preceding chapters. However, further work needs to be undertaken to allow an understanding of the interactions of the wheat CDK-like proteins, and the expression of meiotic genes throughout the stages of meiosis. The advent of the complete wheat genome sequence will allow the use of more large-scale and reliable methods with which to investigate several unresolved questions in this area.

Chapter 5: General conclusions

The major genetic locus responsible for controlling homologous chromosome pairing during meiosis, the *Ph1* locus, is known to consist of a series of defective *Cdk-like* genes containing a segment of heterochromatin on the long arm of chromosome 5B (Griffiths, Sharp et al. 2006), (Al-Kaff, Knight et al. 2007). *Cdk-like* genes are also found on the 5A and 5D chromosomes, with each chromosome containing one full-length, functional copy along with defective copies. It has been suggested that in the presence of *Ph1*, the aberrant genes on 5B suppress the expression of the 5A and 5D copies (Al-Kaff, Knight et al. 2007) and that this results in an increase of CDK-like activity when the *Ph1* locus is deleted (Greer, Martin et al. In preparation).

The wheat *Cdk-like* genes bear closest homology to mammalian *Cdk2* (Yousafzai, Al-Kaff et al. 2010), a gene known to be essential for the successful progression though meiosis. Severe defects in synapsis are seen in mice lacking *Cdk2* (Berthet, Aleem et al. 2003) due to the failure of SC assembly between homologous chromosome pairs (Cohen, Pollack et al. 2006). Non-homologous pairing is common in *cdk2* mutant mice, which causes incorrect chromosome segregation and therefore infertility (Cohen, Pollack et al. 2006). Specifically, *Cdk2* is known to associate with cyclins E and A in order to control the timing and progression through pre-meiotic S-phase. It is also known to regulate chromatin remodeling, primarily through the phosphorylation of histone H1 (Hale, Contreras et al. 2006), (Roth and Allis 1992), (Roque, Ponte et al. 2008), although CDKs are

also known to play a role in histone H3 phosphorylation (Goto, Yasui et al. 2002), (Swain, Ding et al. 2007).

Given the close homology of the wheat *Cdk-like* genes and mammalian *Cdk2*, it was predicted that the increased activity of CDKs seen in the absence of *Ph1* would have effects on both pre-meiotic replication and chromatin remodeling, in line with our understanding of the roles of mammalian CDK2. Indeed, the work presented in this thesis has shown that changes can be seen in pre-meiotic S-phase and in chromatin remodeling when the *Ph1* locus is absent. In terms of replication, there appears to be less of an overlap between the replication of dispersed chromatin and the replication of heterochromatin in wheat rye hybrids lacking the *Ph1* locus. It is likely that this can be attributed to faster replication of the dispersed chromatin due to an increase in CDK2-like mediated activation of origins of replication. However, a delay in the onset of heterochromatin replication cannot be ruled out. A change in chromatin remodeling is also seen in wheat-rye hybrids lacking the *Ph1* locus. The chromatin appears to be more decondensed in pre-meiotic cells, whilst upon entry into meiosis premature condensation is seen. Again, this result can be most simply attributed to an increase in histone phosphorylations due to the increase in CDK-like activity. It has already been shown that the phosphoryaltion of histone H1 is increased in the absence of *Ph1* (Greer, Martin et al. In preparation), which could alone cause differences in chromatin remodeling, although other histone modifications need to be investigated before ruling them out.

Changes in replication and chromatin remodeling, consistent with an enhancement of CDK2-like activity can be seen in plants lacking the *Ph1* locus, but could either of these changes contribute to the non-homologous pairing that is indicative of *Ph1* mutant plants? The importance of events prior to the onset of meiosis in determining correct chromosome

pairing is becoming increasingly apparent. There have been several reports of a pre-meiotic stage at which plants are sensitive to drug or temperature treatments causing incorrect pairing during meiosis (Knight, Greer et al. 2010), (Bayliss and Riley 1972), (Bayliss and Riley 1972), (Dover and Riley 1973). Wheat centromeres, and regions of subtelomeric heterochromatin have also been shown to associate prior to meiosis (Martinez-Perez, Shaw et al. 2003), (Prieto, Shaw et al. 2004), (Colas, Shaw et al. 2008). The behavior of telomeres and centromeres throughout S-phase certainly suggests that pairing is occurring before the onset of meiosis and even before replication has been fully completed. The replication of the rye heterochromatin knobs can be seen occurring as the telomeres are beginning to cluster and after pairing has already been initiated. In wheat, where a large proportion of the genome is composed of repetitive sequences that are highly conserved across the three genomes, the late replication of heterochromatin may serve as a mechanism by which the repetitive sequences are omitted from the initial homology search. If this is indeed the stage at which homology searching and pairing takes place, then any changes during this stage may contribute to incorrect pairing later on. The differences in replication seen when the *Ph1* locus is absent could result in certain sequences either being omitted from, or being allowed to take part in the homology search, leading to aberrant pairing at later stages.

Likewise, changes in chromatin remodeling could also contribute to the non-homologous pairing that is seen in the absence of *Ph1*. One obvious barrier to non-homologous pairing in the wild type system is that the chromosomes from the three homoeologous genomes are different sizes. Altered chromatin remodeling may disrupt the barrier to pairing created by size differences, by allowing the homoeologous chromosomes to condense or decondense to similar lengths. A similar mechanism has previously been reported in *Lolium* species, *Lolium* x *Festuca* interspecific crosses, and in haploid lines

derived from allotetraploid cottom (Jenkins 1985), (King, Armstead et al. 2002), (Jack Mursal and Endrizzi 1976).

A key biological aim of studies into the *Ph1* locus is to gain enough understanding of the mechanisms which prevent non-homologous pairing so that it can be manipulated to allow the introgression of beneficial traits from wild relatives of wheat into the genome. In the presence of *Ph1* there is no pairing or recombination between non-homologous chromosomes and so any introduced genes are not incorporated into the genome. However, in the absence of *Ph1*, although pairing can occur between non-homologous chromosomes, the aberrations caused by this incorrect pairing eventually lead to sterility making these plants unsuitable as a crop. The ultimate goal of research into *Ph1* is to be able to switch the locus off in order to allow the incorporation of beneficial traits from wild relatives, but then to switch it back on again so that there are no subsequent problems with sterility. The work presented in this thesis has allowed us to progress a little further towards this goal. By targeting pre-meiotic stages for treatment with okadaic acid, a phosphatase inhibitor which alters chromatin remodeling and increases the activity of CDKs, pairing could be induced between non-homologous chromosomes of a wheat-rye interspecific hybrid in the presence of *Ph1*. Although a lot more work will have to be carried out before this technique can be reliably used to introduce novel traits into wheat, it has at least shown the potential for the use of drug treatments to induce non-homologous pairing in a *Ph1+* background. The mechanism by which OA treatment induces non-homologous associations remains to be discovered as OA has a broad target range. Recent work however has shown that OA treated spikes show an increase in histone H1 phosphorylation at the same site at which phosphorylation is increased in the absence of *Ph1* (Martin, unpublished data),(Greer, Martin et al. In preparation). This would indicate

that OA treatment is causing similar changes in replication and condensation to those seen in the *Ph1b* mutant, leading to the non-homologous pairing that is observed.

There still remain several unanswered questions regarding the *Ph1* locus that future studies will attempt to answer. The complete sequencing of the wheat genome will allow the use of novel techniques to look at the expression levels of genes throughout meiosis, including the *Cdk-like* genes. Although the study presented in this thesis has given us a preliminary idea of the expression patterns of the *Cdk-like* genes, a much larger sample would be necessary to provide a definitive picture of these levels. The work required using the techniques described in chapter 4 would be both time consuming and costly. High throughput sequencing technologies will allow expression patterns to be analyzed much more reliably. Likewise, the expression patterns of the early meiotic genes used in the real time RT-PCR analysis in chapter 4 will be prime targets for the use of high throughput sequencing. Given the link between chromatin remodeling and gene expression it would be unsurprising to find that gene expression patterns also change when *Ph1* is removed. Another promising technique that could be used to see if the chromatin remodeling differences seen in the absence of *Ph1* contribute to changes in gene expression is 5-ethynyl uridine (EU). This can be incorporated into RNA and then detected using a Click-iT reaction similarly to that described for EdU in chapter 2. Using this technique the patterns of RNA transcription could be monitored in the presence and absence of *Ph1*, giving an indication of the global gene expression patterns in these genotypes.

Another interesting avenue for future investigation is whether or not the changes in chromatin remodeling have an effect on DSB formation. Chromatin structure is known to be an important factor in determining the distribution of DSBs with formation generally occurring in areas in which the chromatin is accessible (Kleckner 1996), (Schwarzacher

2003). In yeast DSBs are formed in regions of open chromatin which become sensitive to nuclease digestion before the formation of the break showing that the chromatin is decondensed (Berchowitz, Hanlon et al. 2009). Similarly in *Arabidopsis* DSB formation occurs in areas of low chromatin condensation (Schwarzacher 2003). The genic regions of the genome are generally areas open chromatin associated with active gene transcription, meaning that the majority of DSBs are formed in these regions. The differences in chromatin remodeling, and potentially in DSB formation seen in the absence of *Ph1* may mean that transcription from the genomes is altered in some way. For example, in the presence of *Ph1*, transcription from the homoeologous genomes may be different and therefore provide a barrier to pairing and recombination. If these differences in transcription were 'normalized' across the genomes in the absence of *Ph1* it may give an indication as to why non-homologous pairing and recombination is occurring.

Wheat-rye hybrids would also provide a very interesting system in which to study DSBs. During meiosis DSBs would normally be repaired using recombination with the homologous partner, which promotes genetic diversity and also holds the chromosomes together when the SC disassembles. In a wheat-rye hybrid however there are no homologous chromosomes and so DSB repair cannot occur in this manner. There is growing evidence to suggest that in the absence of a homologous partner the sister chromatid can be used for DSB repair (Goldfarb and Lichten 2010). This mechanism of repair has been seen in rye haploid plants (Neijzing 1982), and it is therefore likely that a similar mechanism could take place in wheat-rye hybrids in the presence of *Ph1*. In the absence of *Ph1* however, pairing does take place between non-homologous chromosomes and so it would be interesting to see whether DSBs are repaired using recombination with the non-homologous partner, or by recombination with the sister chromatid. The phosphoryaltion of ASY1 may provide a mechanism by which sister chromatid repair is

prevented. Studies on yeast have shown that phosphorylation of HOP1 (the yeast ASY1 homologue) by MEC1 and TEL1 leads to the prevention of sister chromatid DSB repair (Carballo, Johnson et al. 2008). It is plausible to think that the conformational change seen in the chromatin and the partial separation of sister chromatids as the cell enters meiosis corresponds with this phosphorylation. Previous work has shown that chromosomes have to be identical or near-identical for remodeling to occur, in instances where it is different remodeling is delayed (Colas, Shaw et al. 2008). This observation provides a good explanation as to why delayed condensation is seen in wheat rye hybrids in the presence of *Ph1*; there are no identical chromosomes and so the remodeling process is postponed. In the absence of *Ph1* however, remodeling occurs as normal despite the absence of homologous chromosomes. We hypothesize that the delayed remodeling seen in the *Ph1+* plants, could lead to the prevention of ASY1 phosphorylation, leading to a failure in the barrier to sister chromatid DSB repair. In the wheat system, there are always homologous chromosomes and so remodeling should occur at the correct time whether *Ph1* is absent or not. Therefore ASY1 phosphorylation should occur as normal, and DSBs should be repaired using the homologous (or sometimes non-homologous in the absence of *Ph1*) partner. A summary of this hypothesis is shown in figure 1.

	Wheat	Wheat rye hybrid
Ph1+	•Chromatin remodelling occurs as normal •ASY1 phosphorylation can occur •DSBs are repaired using homologous partner	•Chromatin remodelling is delayed •ASY1 phosphorylation does not take place •There is no barrier to sister chromatid DSB repair, and as there is no pairing, this is the method by which DSBs are repaired
Ph1-	•Chromatin remodelling occurs as normal •ASY1 phosphorylation can occur •DSBs are repaired using the homologous (or occasionally non-homologous) partner	•Chromatin remodelling occurs as normal •ASY1 phosphorylation can occur •DSBs are repaired using the non-homologous partner

Figure 1: a summary of the factors involved in the hypothesised ASY1 mediated formation of a barrier to sister chromatid DSB repair.

There are a number of cytological ways in which the DSBs could be monitored, primarily through the use of antibodies. Antibodies against phosphorylated histone H2A.X, which accumulates at DSB sites, and the strand exchange protein RAD51 have been successfully used in the past to visualize DSBs within the nucleus (Mahadevaiah, Turner et al. 2001), (Rogakou, Pilch et al. 1998), (Shinohara, Ogawa et al. 1992). The production of an antibody against replication protein A (RPA), which binds along with RAD51, would also provide a useful marker for DSBs. Preliminary results have shown that DSBs can be labeled using a RAD51 antibody in wheat-rye hybrids, and it would appear that DSBs are more widespread and persist for longer in the absence of *Ph1* (Azahara Martin, unpublished data). This observation could indicate that non-homologous recombination is occurring in these meiocytes. It could also be a consequence of the earlier decondensation prior to meiosis that is seen in the absence of *Ph1*.

The ultimate experiment to investigate whether or not ASY1 phosphorylation is seen as described in figure 1 would be to develop an antibody to phospho-ASY1. This process has already been initiated, and so before long we should know whether this hypothesis is correct or not.

The remodeling upon which we hypothesize the phosphorylation of ASY1 could be dependent is thought to correspond with the onset of pairing. The telomeric regions of the chromosomes are known to be essential for the successful pairing of chromosomes, with a reduction or complete prevention of pairing between homologues in which the telomeric region has been deleted (Lukaszewski 1997). The sub-telomeric regions are known to remodel synchronously in *Ph1+* plants, only if the regions are identical. If there are differences in this region remodeling is delayed(Colas, Shaw et al. 2008), (Knight, Greer et al. 2010). This does not seem to occur in the absence of *Ph1*, with remodeling occurring

regardless of similarity (Knight, Greer et al. 2010). This then allows the pairing of non-homologous chromosomes as shown in figure 2.

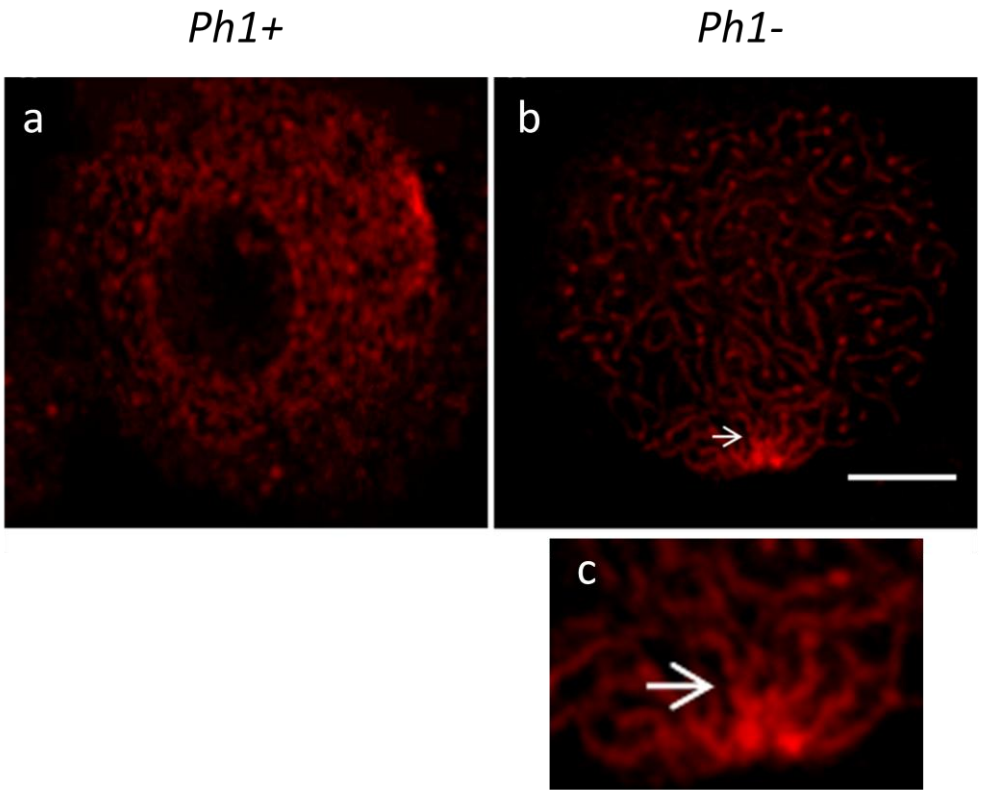


Figure 2: ASY1 labelling of *Ph1+* and *Ph1-* meiocytes at the time of the telomere bouquet. The *Ph1+* chromosomes are less condensed and no pairing can be seen (a). The *Ph1-* chromosomes are more condensed and some pairing can be seen between the non-homologous chromosomes (b and enlarged in c). Scale bar represents 5um

It may be that the telomeric region of chromosomes in the absence of *Ph1* is somehow altered, resulting in the homoeologous pairing that is seen. Work is currently being undertaken to map this region of the chromosomes in wheat, and this should give us some indication as to whether there are any differences in this region in *Ph1+* and *Ph1-* plants.

Antibodies would also provide a useful tool to study other proteins involved in meiosis. Antibodies against phosphorylated retinoblastoma protein have been successfully used in *V.faba* (Polit and Kazmierczak 2007) and may provide interesting results regarding the *Ph1* locus given the involvement of CDK2-complexes in RB phosphorylation. It may even be possible to produce peptide antibodies against specific CDK-like proteins. If so these would provide a valuable insight into their expression patterns and timings.

Ultimately, a detailed study of the mechanisms by which the *Ph1* locus functions may not be possible to determine in wheat. The extremely large, highly repetitive, hexaploid genome of wheat makes molecular techniques extremely difficult to undertake. Generating and identifying mutations in an organism where there are six copies of every gene is extremely complicated, although advances have recently been made allowing the generation of point mutations across all of the chromosomes carrying a particular gene (Weil 2005). Positional cloning is also extremely complicated. It is likely that future work on the *Ph1* locus will have to move into less complicated model organisms such as *Brachypodium*, which shares a high degree of synteny with wheat, and *Arabidopsis*. The wheat *Cdk-like* genes have already been successfully overexpressed in *Brachpodium*, and studies on *CdkG*, the closest homologue of the wheat *Cdk-like* genes in *Arabidopsis* have also provided interesting results (Ruoyu Wen, personal communication). Research into the *Ph1* locus has reached an exciting period, and by utilizing the techniques outlined in this chapter we will become closer to gaining a full understanding of the mechanisms by which it functions, ultimately allowing it to be effectively exploited in breeding programs.

Appendix 1: preparation of material for wax embedding and microtome sectioning

1) Prepare a fresh solution of 4% paraformaldhyde in 1 x PBS for the fixation of materials via the cross linking of proteins
The first step is to make up an 8% solution of formaldehyde in water. Parformaldehyde must be weighed out in the fume cupboard whilst wearing the appropriate safety clothing (lab coat, gloves, eye protection). Place the paraformaldehyde into a flask and then add the required amount of dH_20.

NB. Formaldehyde may be carcinogenic so all steps must be carried out in a fume hood

Heat the solution to approximately 60˚C whilst stirring with a magnetic stirrer. The solution must not be allowed to boil. Drops of 1M NaOH should be added until the parafomaldehyde dissolves. If more than 5 – 6 drops are required then this could indicate a deterioration of the paraformaldehyde powder.

Add the required amount of 2 x PBS and place on ice to cool. Check the pH with pH indicator strips (should be pH7). If adjustments are needed use dilute H_2SO_4

Anything that has come into contact with the paraformaldehyde should be decontaminated by soaking overnight in a solution of sodium metabisulphite in the fume cupboard. Items should then be rinsed with water and sent for washing up.

Formaldehyde solution should be disposed of into the waste formaldehyde bottle. When full this should be placed in the 'waste not suitable for bulking up' section of the chemical waste store.

2) Carefully remove the spike from the tiller using a razor blade. Place in a falcon tube and cover with the ice cold paraformaldehyde solution.

3) Gently infiltrate the tissue using a vacuum to remove air from the spike. This is complete when the spike falls to the bottom of the tube upon removal of the vacuum (10 – 20 minutes).

4) Fix for at least one hour in the fridge. Then dispose of the paraformaldehyde solution and wash the spike with 1 X TBS for at least 15 minutes. The sample can then be stored in the fridge for up to two weeks.

5) Carefully remove each spikelet using a razor blade and place in a Tissue-Tek biopsy cassette (store in 70% ethanol until ready to load into the VIP machine).

6) Place the samples into the Tissue-Tek Vacuum Infiltration Processor and run on the following cycle.

70% Ethanol 1hr 35°C
80% Ethanol 1.5hr 35°C
90% Ethanol 2hr 35°C
100% Ethanol 1hr 35°C

100% Ethanol 1.5hr 35°C (0.01% Eosin Y can be added to this step to make the samples easily visible for wax embedding)
100% Ethanol 2hr 35°C
Xylene 0.5hr 35°C
Xylene 1 hr 35°C
Xylene 1.5hr 35°C
Wax 1hr 60°C
Wax 1hr 60°C
Wax 2hr 60°C
Wax 2hr 60°C

7) Drain wax from the chamber and quickly move samples to the embedding station.

8) Orientate the sample as required in the metal mould and then cover with liquid paraffin wax. Place the bottom half of the biopsy mesh cage on top of the mould and fill with liquid wax. Leave to cool until solidified and then remove the metal mould.

9) Cut the area around the sample into a trapezium shape, keeping the sides angled. Tightly secure the sample into the microtome and make any adjustments necessary. Put the blade into position and set the thickness to the required distance (for all of the experiments described in this thesis a thickness of 10um was used).

10) Cut the required sections, gently guiding with a small paintbrush. Place onto a polysine slide and pipette water underneath the sections until they are floating. Leave at 37˚C overnight to straighten the sections and evaporate the remaining water.

11) Wax should be removed from around the sample by incubating for 2 x 10 minutes in histoclear solution, followed by a wash in 100% EtOH.

Appendix 2: BrdU pulsing and detection

1) Prepare a solution of 1mM BrdU (Merck Chemicals: 203806) dissolved in 100mM sucrose.
2) Select a tiller and cut at the second node from the spike, underwater, making sure that no air is allowed in. This should be done as the plants are coming out of the dark phase.
3) Slip a falcon tube underneath the tiller allowing it to fill with water, again being careful not to allow any air in.
4) Remove as much water as possible using a pipette, leaving a small amount in the tube so that air cannot get into the tiller.
5) Place approximately 3mls of BrdU/sucrose solution into the tube. Cover the opening with parafilm and leave in the CER for 4hrs.
6) At the end of the four hour period fix and process the spike as described in appendix 1.
7) Dehydrate slides in a methanol series, 2mins in 30% methanol, 50% methanol, 70% methanol and 100% methanol. Leave to air dry.
8) Digest samples for 1hr at 37˚C in 2% pectolyase, 2% cellulose in 1 x TBS (use a plastic coverslip).
9) Wash in 1 x TBS for 20 minutes at room temperature
10) Denature in 1.5M hydrochloric acid at 37˚C for 35 minutes.
11) Immediately place in ice cold PBS for 5 minutes
12) Block for 1hr at room temperature in 5% bovine serum albumin (BSA) in TBS
13) Apply anti-BrdU (1:1000) for 3hrs at room temperature (Sigma B2531)
14) Wash in 1 x TBS at room temperature for 20 minutes
15) Apply anti-mouse alexa 488 (1:1000) overnight in the fridge
16) Wash in 1 x TBS at room temperature for 20 minutes
17) Incubate in DAPI (1ug/ml) for 10 minutes at room temperature
18) Briefly wash in H_2O and then mount in Vectashield mounting medium (Vector laboratories: h1000).

Appendix 3: EdU pulsing and detection

1) Prepare a 1mM solution of EdU dissolved in 100uM sucrose solution
2) Cut wheat tillers at the required stage under the second node from the spike. This must be done underwater to prevent air from getting in.
3) Remove water (leaving a very small amount) and pipette at least 2mls of EdU sucrose solution into each tube.
4) Cover the top of the tube with parafilm and leave for 4 hrs.
5) Fix and process the spike as described in appendix 1.
6) Prepare all stock solutions as per manufacturers instructions
7) Draw around samples using a pap pen
8) Digest in 2% cellulose, 2% pectolyase for 3hrs at 37˚C
9) Wash for 10 minutes in 1 x PBS on shaker
10) Block in 3% BSA in 1 x PBS for 10 minutes at room tempertaure
11) Prepare 1 x Click-iT® EdU buffer additive (from 10 x stock solution). This must be used fresh.
12) Prepare Click-iT® reaction cocktail according to the table. Note:- these components must be added in the order listed. The reaction cocktail should be used within 15 minutes of mixing.

Reaction components	Total volume						
	500 µL	1ml	2ml	2.5ml	5ml	12.5ml	25ml
1X Click-iT® reaction buffer	430 µL	860 µL	1.8 mL	2.2 mL	4.3 mL	10.7 mL	21.4 mL
CuSO4	20 µL	40 µL	80 µL	100 µL	200 µL	500 µL	1 mL
Alexa Fluor® azide	1.2 µL	2.5 µL	5 µL	6 µL	12.5 µL	31 µL	62 µL
Reaction buffer additive	50uL	100ul	200ul	250 ul	500ul	1.25 mL	2.5 mL

13) Place 100ul of the reaction cocktail mix onto each slide
14) Incubate for 30 minutes at room temperature in the dark
15) Wash with 1 x PBS
16) Incubate with DAPI (1ug/ml) for 10 minutes at room temperature
17) Mount slides in vectashield

If wanting to do dual labelling with an in situ probe hybridise the probe to the sample after the digestion step, then go through the required washes until the blocking stage. After blocking first go through the EdU detection protocol and then before incubating with DAPI incubate with the required antibodies for your probe (in situ protocol is described in appendix 4).

Appendix 4: Fluorescent in situ hybridisation

1) Collect tillers at the required stage and fix and process them as described in appendix 1.
2) Draw around the samples using a pap pen
3) Process the slides through a dehydration series; 30%, 50%, 70%, 100% methanol at room temperature. Allow to air dry.
4) Digest in 2% cellulose, 2% pectolyase in 1xTBS for 3hrs at 37˚C
5) Wash in 1 x TBS at room temperature for 20 minutes
6) Repeat the dehydration series
7) Make hybridisation mix (for a multi-well slide you will need ~ 10ul per well, for a polysine slide at least 50ul for a whole slide)

Deionized formamide	5ul
100mM PIPES, 10mM EDTA, pH 8	1ul
50% Dextran sulphate	2ul
3M NaCl	1ul
Salmon sperm, blocking DNA (1mg/ml)	0.5ul
Probe	1ul (of each probe)
Total	10.5ul

8) Denature the hybridisation mix for 8 minutes at 100˚C. Immediately place on ice for at least 5 minutes.
9) Apply the hybridisation mix to the slides and cover with a plastic coverslip.
10) Place slides in the OMNISLIDE machine and run on program 7

Temperature	Time	Ramp
75˚C	7 mins	0.1
55˚C	3 mins	0.5
50˚C	5 mins	0.5
45˚C	5 mins	0.5
42˚C	5 mins	0.5
40˚C	5 mins	0.5
38˚C	5 mins	0.5
37˚C	Infinite	/

It is very important that the samples do not dry out at this stage so make sure that there is a lot of water in the OMNISLIDE to keep the humidity high, or move to a humidity chamber at 37˚C.

11) Leave overnight at 37˚C
12) Remove coverslips in 2 x SSC at 42˚C
13) Wash in 20% formamide, 80% 0.1 x SSC for 10 minutes at 42˚C
14) Wash in 2 x SSC for 10 minutes at 42˚C
15) Wash in 2 x SSC for 10 minutes at room temperature
16) Wash in 4 x SSC, 0.2% Tween 20 for 10 minutes at room temperature
17) Block for 10 minutes in 5% BSA dissolved in 4 x SSC, 0.2% Tween 20.

18) Apply the first antibody diluted to the correct concentration in blocking solution. Incubate for 1hr at 37˚C. Use plastic coverslips and a humidity chamber.
19) Wash for 15 minutes at room temperature in 4 x SSC, 0.2% Tween 20.
20) Apply the secondary antibody, diluted in blocking solution. Leave for 1hr at 37˚C. Use plastic coverslips and a humidity chamber.
21) Wash the slides in 4 x SSC, 0.2% Tween 20 for 10 minutes at room temperature. Wrap container in tin foil so that they are protected from the light.
22) Incubate for 10 minutes in DAPI (1mg/ml)
23) Briefly wash slides in H_20
24) Mount slides in Vectashield mounting medium.

Appendix 5: Probe preparation

The probes used in these experiments were prepared in one of two ways. Either a probe was directly labelled using PCR, or a template was produced by PCR and then labelled using Nick Translation. The protocol for Nick translation will first be given followed by a detailed description of how to make each of the probes that were used for these experiments.

Nick Translation

10 x Nick Translation Buffer (NTB)	5ul
Unlabelled dNTP's (G,C,A)	5ul
dTTP	1.7ul
DNA template	5ul
DTT (Promega P117A 100mM)	1ul
dUTP (biotin, digoxigenin etc)	1ul
DNA polymeraseI / DNaseI (Invitrogen 18162-016)	5ul
H_2O	26.3ul

(Biotin: Roche 11093070910, Digoxigenin: Roche 1093088)

10 x Nick Translation Buffer

1M Tris-HCl pH 7.5	250ul
1M $MgCl_2$	25ul
20mg/ml BSA (Sigma B8667)	125ul
H_2O	100ul

Telomere probe preparation

Primers: Telomere F: 5'-TTTAGGG-3'
Telomere R: 5'-CCCTAAA-3'

Amplification PCR

Primers (10uM)	1ul of each
Phusion (Finnzymes: F-530S) DNA polymerase	0.2ul
dNTP mix (10mM each) (New England Biolabs: N0447S)	0.4ul
High Fidelity Buffer	4ul
DNA template	None necessary
H_2O	13.4ul

PCR Cycle

99˚C	3 minutes	
99˚C	1 minute	x10
60˚C	1 minute	
72˚C	15 seconds	
99˚C	1 minute	x30
60˚C	1 minute	
72˚C	15 seconds	

8˚C Infinite

The product should appear as a smear when run on a gel. Purify, and then label with biotin using Nick translation for 1 ½ hours at 15˚C. At the end of this time heat at 70˚C for 15 minutes and then purify. Store at -20˚C.

Wheat Centromere Probe

Primers: CCS1A: 5'TGCATCTATATTCTTGCTTGTT3'
CCS1D: 5'GGTGCCCGATCTTTCGATGAGA3'

Amplification PCR

Primers (10uM)	1ul of each
High fidelity buffer	4ul
Phusion DNA polymerase	0.2ul
dNTPs	0.4ul
Hi10 plasmid DNA (1/100 dilution)	1ul
H_2O	12.4ul

Labelling PCR

Primers (10uM)	1ul of each
High fidelity buffer	4ul
Phusion DNA polymerase	0.2ul
dNTPs	0.4ul
PCR product (1/10 dilution)	2ul
Biotin/digoxigenin	1ul
H_2O	10.4ul

Purify the product after both PCR's.

PCR Cycle

99˚C	3 minutes	
99˚C	1 minute	x35
55˚C	1 minute	
72˚C	15 seconds	

72˚C 10 minutes
8˚C Infinite

Rye Centromere Probe

Primers: Secalecent 2F: 5'GATAGAACACCACACCGAACTCGTACCCGG3'
Secalecent 2R: 5'ACTTAAATTGTGTAAGAGGTAAGCACTTGC3'

Amplification PCR

Primers (10uM)	1ul of each
High fidelity buffer	4ul
Phusion DNA polymerase	0.2ul
dNTPs	0.4ul
Rye genomic DNA	1ul
H_2O	12.4ul

Labelling PCR

Primers (10uM)	1ul of each
High Fidelity buffer	4ul
Phusion DNA polymerase	0.2ul
dNTPs	0.4ul
PCR product form amplification step	1ul
Biotin / digoxigenin	1ul
H_2O	11.4ul

Purify the product after both PCR's.

PCR Cycle

99˚C 3 minutes
99˚C 1 minute
64˚C 1 minute } x35
72˚C 15 seconds
72˚C 10 minutes
8˚C Infinite

Rye Subtelomeric Heterochromatin Probe

Primers: 1pSc250: 5'GAGCAAGTTACCTTGGA3')
2pSc250: 5'TTTTGCGCCACTCGAAAT3')

Amplification PCR

Primers (10uM)	1ul of each
Rye genomic DNA	2ul
High fidelity buffer	4ul
dNTPs	0.4ul
Phusion DNA polymerase	0.2ul
H_2O	11.4ul

PCR Cycle

99˚C	3 minutes	
99˚C	1 minute	x35
51˚C	1 minute	
72˚C	15 seconds	
72˚C	10 minutes	
8˚C	Infinite	

Purify the product and label with biotin or digoxigenin using Nick translation for 1 ½ hours at 15˚C. At the end of this time heat at 70˚C for 15 minutes and then purify. Store at -20˚C.

Appendix 6: ASY1 immunolocalization on sections

1) Material should be pre-fixed in 4% paraformaldehyde as described in appendix 1.
2) Draw around samples using a pap pen
3) Digest samples in 2% cellulose, 2% pectolyase for 2 – 4 hours depending on the enzyme mixture (each new enzyme mixture should be tested to calculate the optimum digestion time)
4) Wash in 1 x TBS at room temperature for 10 minutes on a shaker
5) Fix in 4% paraformaldehyde for 10 minutes
6) Wash in 1 x TBS for 10 minutes on a shaker (if background levels are high this wash can be followed by 10 minutes in 2 x SSC and 10 minutes in 4 x SSC, 0.2% Tween 20. 4 x SSC, 0.2% Tween 20 should then be used instead of 1 x TBS in all following steps).
7) Block in 5% BSA (in either 1 x TBS or 4 x SSC, 0.2% Tween 20) for 1 hr at room temperature
8) Remove BSA and apply the primary antibody at the required dilution (for ASY1 use 1:100). Leave at 4˚C overnight in a humidity chamber
9) Wash for 20 minutes in 1 x TBS (or 4 x SSC, 0.2% Tween 20) on a shaker at room temperature.
10) Apply the secondary antibody at required dilution (for ASY1 I used α-Rabbit FITC (Sigma: F7512) at 1:50 dilution) and leave at 37˚C for 1 hr in a humidity chamber (can be left for longer at room temperature).
11) Wash for 20 minutes in 1 x TBS (or 4 x SSC, 0.2% Tween 20) on a shaker at room temperature (THIS STEP SHOULD BE PERFORMED IN THE DARK – USE TIN FOIL)
12) Apply DAPI (1µg/ml) and leave for 5 – 10 minutes
13) Wash briefly in 1 x TBS (or 4 x SSC, 0.2% Tween 20)
14) Mount slides in Vectashield

Appendix 7: Expression profiling of the *Cdk-like* genes

Using aceto-carmine, stage one anther from each floret, and if at the correct stage collect the remaining two in a tube placed in liquid nitrogen.

1) Grind the collected anthers in an eppendorf tube using a blue pestle. Make sure that the material remains frozen throughout this time.

RNA extraction using Qiagen Rneasy Plant Mini Kit

2) Add 450ul of buffer RLT (containing β-mercaptoethanol) and vortex vigorously.
3) Transfer to a QIAshredder spin column and centrifuge for 2 minutes at full speed. Remove the supernatant carefully and transfer to a new tube.
4) Add 0.5 volume of 96 – 100% ethanol and mix immediately by pipetting.
5) Transfer the sample to an Rneasy spin column and centifuge for 15s at ≥ 8000g. Discard the flow though.
6) Add 700ul of buffer RW1 to the Rneasy spin colum and centifuge for 15s at ≥8000g. Discard the flow through.
7) Add 500ul of buffer RPE to the Rneasy spin colum and centifuge for 15s at 8000g. Discard the flow through.
8) Add 500ul of buffer RPE to the Rneasy spin colum and centifuge for 2min at 8000g.
9) Place the Rneasy spin colum into a new 2ml collection tube and centrifuge at full speed for 1 minute.
10) Place the Rneasy spin column in a new 1.5ml collection tube and add 30ul Rnase-free water to the membrane. Centifuge for 1 minute at ≥8000g to elute the RNA.

(Run the RNA on a gel to see if there is any DNA contamination. If there is this can be removed using Dnase treatment).

Reverse Transcription using Invitrogen Suoerscript II RT kit

11) Mix together the following components: 1ul Oligo$(dT)_{12\text{-}18}$ (500ug/ml) primers, 5ul RNA, 1ul dNTP mix (25mM), 5ul H_20d.
12) Heat mixture at 65^{o}C for 5 minutes and then quick chill on ice. Collect contents by centrifugation, then add: 4ul 5xFirst strand buffer, 2ul 0.1M DTT, 1ul RNase out.
13) Mix contents gently and heat at 42°C for 2 minutes
14) Add 1ul (200 units) of superscript II RT and mix by pipetting.
15) Incubate at 42°C for 50 minutes.
16) Inactivate the reaction by heating at 70°C for 15 minutes.

PCR of conserved cdk fragment

17) Using Phusion high fidelity DNA polymerase run a PCR using the cDNA as the template. Primers are as follows; F: cccgacttcaaggtggac, R: gacaccgcgagccctaggtc.
18) Run the product on a gel to check that it is the correct size.

Cloning using Invitrogen Zero Blunt TOPO PCR cloning kit

19) Mix together: 4ul fresh PCR product, 1ul salt solution, 1ul TOPO vector. Incubate for 5 minutes at room temperature. Then place on ice.
20) Heat water bath to 42°C and thaw one shot cells on ice
21) Add 2ul of TOPO cloning reaction into a vial of one shot TOP10 chemically competent *E.coli* and mix gently. Incubate on ice for 20 minutes.
22) Heat shock for 30 seconds at 42°C. Immediately transfer to ice.
23) Add 250ul of room temperature SOC medium. Shake the tube horizontally at 200rpm at 37°C for 1hr.
24) Spread 10 – 50ul from each transformation onto pre-warmed LB plates containing 50ug/ml ampicillin. Incubate overnight at 37°C.
25) Pick individual colonies and grow overnight in LB media with ampicillin at 37°C.

Miniprep using Qiaprep spin miniprep kit

26) Pellet the *E.coli* by spinning at 13,000 rpm for 10 minutes. Discard supernatant.
27) Resuspend the pelleted bacterial cells in 250ul buffer P1 and transfer to a microcentrifuge tube.
28) Add 250ul buffer P2 and mix thoroughly by inverting the tube.
29) Add 350ul buffer N3 and mix thoroughly by inverting the tube.
30) Centrifuge for 10 minutes at 13,000rpm.
31) Place the supernatant into a QIAprep spin column and centrifuge for 60s. Discard the flow through.
32) Add 0.75ml buffer PE and centrifuge for 60s. Discard the flow through and centrifuge for an additional minute.
33) Place the QIA prep column in a clean 1.5ml microcentrifuge tube and add 50ul H_2O. Let it stand for 1 minute and then centrifuge for 1 minute to elute the DNA.

(At this point a PCR should be run using M13 primers to check that the correct insert is present. M13F: GTAAAACGACGGCCAG, M13R: CAGGAAACAGCTATGAC)

Preparing the samples for sequencing using Big Dye 3.1

34) Purify the miniprepped DNA samples
35) Run big dye PCR: 1ul Big Dye 3.1, 1.5ul Big Dye Buffer, 1ul M13 forward OR reverse, 1ul purified DNA, 5.5ul H_2O.

96°C	1 minute
96°C	10 sec
50°C	5 sec
60°C	4 min
4°C	Infinite
(29 cycles)	

36) Samples then submitted to the genome centre for sequencing.

Analysis of sequences

37) Using the Vector NTI AlignX function each of the returned sequences should be individually aligned with the known sequences of the *cdk-like* genes. Check for any SNPs. If there is more than one SNP discount.

Appendix 8: Real time RT PCR

This protocol was designed and optimized by Ruoyu Wen. The protocol detailed here is a copy of his original.

RNA extraction

- Using aceto-carmine, stage one anther from each floret, and if at the correct stage collect the remaining two in a tube placed in liquid nitrogen.
- Grind the plant tissue thoroughly with a mortar and pestle in a 2 ml tube with liquid nitrogen. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.
- Add 1 ml TRI Reagent, incubate 10' at RT, then transfer the lysate to a new tube, spin at 12,000g x 10', 4 °C to remove the debris and/or high molecular mass DNA.
- Add 0.1 ml of 1-bromo-3-chloropropane (BCP). Cover the sample tightly, shake vigorously for 15s, incubate 10' at RT, and spin at 12,000g x 10', 4 °C to separate the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA).
  1-Bromo-3-chloropropane is less toxic than chloroform and its use for phase separation decreases the possibility of contaminating RNA with DNA. The chloroform used for phase separation should not contain isoamyl alcohol or other additives
- Transfer the aqueous phase (about 430 µl) carefully to a fresh tube and add 0.5 ml 2-propanol (Sigma, I9516, or ACS grade) and mix, incubate 10' at RT, and spin at 12,000g x 15', 4 °C to form the RNA pellet.
  Note: Store the interphase and organic phase at 2–8 °C for subsequent isolation of the DNA and proteins.
- Remove the supernatant and add 0.9 ml of 75% ethanol. Vortex gently to wash the RNA, then spin at 12,000g x 5', 4 °C to re-settle the pellet.
  Note: Store the interphase and organic phase at 2–8 °C for subsequent isolation of the DNA and proteins.
- Remove most of the supernatant with 1 ml tips, spin at 12,000g x 5', 4 °C, and then remove the remaining trace liquid carefully with 10 µl tips. Briefly air-dry the RNA pellet for 5'. Resolving the pellet with 60 µl super-water for exactly 2' at 55°C, then put on ice for Nano drop.
  Remaining ethanol may interfere with the downstream applications, so make sure all the ethanol is gone before resolving the RNA. However, over dry the RNA pellet will greatly decrease its solubility.
- Measure the RNA concentration by Nanodrop.

gDNA contamination digestion

10x buffer	6µl
DNAse I (Roche, 04716728001, stores item or Ambion, AM2238)	6µl
Total RNA	12µg
H_2O	to 60µl

- RT for 25', then add 50µl Acid-Phenol:Chloroform (Ambion, AM9720), vortex for 15'' and incubate 10' at RT, and spin at 12,000g x 10', 4 °C to separate the mixture into 3 phases,
- Transfer the aqueous phase carefully to a fresh tube, mix with 17µl 8M LiCl (Sigma, L7026) and let RNA precipitate overnight at 4 ^{o}C.
- Spin the mixture above at 12,000g x 20', 4 °C to form the RNA pellet.
- Remove the supernatant and add 1 ml 75% ethanol (ACS grade ethanol mix with super water). Vortex gently to wash the RNA pellet, then spin at 12,000g x 5', 4 °C to re-settle the pellet.
- Remove most of the supernatant with 200 µl tips, spin at 12,000g x 5', 4 °C, and then remove the remaining trace liquid carefully with 10 µl tips. Briefly air dry the RNA pellet for 5'. Resolving the pellet with 25 µl super-water for 2' at 55°C, then put on ice for Nano drop.

  Remaining ethanol may interfere with the downstream applications, so make sure all the ethanol is gone before resolving the RNA. However, over dry the RNA pellet will greatly decrease its solubility.
- Measure the RNA concentration by Nanodrop, also run samples on agar gel.

cDNA synthesis

Primer annealing

DNase treated RNA, from above	2µg
Primer Mix (see below)	1µl
10mM dNTPs	1µl
H_2O	to 12µl

- 65 ^{o}C 5' then on ice whilst you prepare the reverse transcription mastermix

  Primer mix: 1:2 of Oligo-dT primer, Invitrogen, 0.5ug/ul, stores item, plus 1:20 of Random Primers, Invitrogen, 3ug/ul e.g. 10ul dT +1ul RP + 9ul H_2O (RNase free)

Reverse Transcription

Primer-annealed RNA from above	12µl

5x First Strand Buffer	4µl
0.1M DTT	2µl
RNA-guard*	1µl

- Divide the mixture into two tubes, each 9.4µl, then add 0.45µl Superscript III (Invitrogen, stores item) for the RT+ tubes, and 0.45µl water for the RT- tubes. Incubate at 42°C for 50', then 70°C for 15' (use PCR machine). Use directly for Real-time PCR or store at -80°C immediately.

Real-time PCR

PCR mixture

Master Mix	2.5µl
Primers (15µM pair)	0.2µl

- Mix the same primer sets in one 1.5ml tube then add 2.7µl mixture to the plates by 2-20µl electronic pipette (StarlabE).
- Then add 2.3µl cDNA (1:3 diluted), and seal the plate with film applicator.
- Spin at 1500g for 2' before putting the plate in the LC480 machine.
- Run cycles at below:

Program Name	Cycles	Analysis Mode
Preincubation	1	None
Amplification	45	Quantification
Melting curve	1	Melting Curves

Preincubation

Target (°C)	Acq Mode	Time	Ramp	Acq (per °C)	Sec Target	Step Size	Step Delay
95	None	00:02:00	4.8	5	0	0	0

Amplification

95	None	00:00:25	4.8	5	0	0	0

58	None	00:00:25	2.5	5	0	0	0
72	Single	00:00:40	4.8	5	0	0	0

Melting curve

95	None	00:00:05	4.8	5	0	0	0
65	None	00:01:00	2.5	5	0	0	0
97	Cons	00:00:00	0.11	5	0	0	0

Primers used

cdk full length1 F	ACCCCGACTTCAAGGTGGAC
cdk full length1 R	GTGAGGTGGCGCGCCTCCACG
cdk full length2 F	TGGAGGCGCGCCACCTCAC
cdk full length2 R	AGCGCCCGGAGCTCGACGATG
cdk conserved F	TTCGAAGTCCTCAAGGGGCTC
cdk conserved R	GGCGTTGGCGAACCATGGTCG
Asy1 F	AGGACTCCCACAAGCAATCG
Asy1 R	ACCTGCTGGAGGATCGGCTC
DMC1 F	GCTGAGGAAAGGCAAAGGCG
DMC1 R	CGGTGCAGTAGCCTTTGTCGATTT
MSH4 F	CTGAAGGATGGTGTCCGACG
MSH4 R	AGGTCCTGCAATGCTTCACG
MSH6 F	CATAATATTGGCACAGATTGGAG
MSH6 R	CTGACGAAAGCACGGAAGC
PHS1 F	TGATGCTGCTGGTGGAAATTCG
PHS1 R	CGGACACTAGGCATGATAGGCG
RAD51B F	CTGCCTGGCTGAAGCTGAAG
RAD51B R	GATAGAACAAGCAAACTATGGGAG
RAD51C F	TATTGATACAGAGGGCAGTTTC
RAD51C R	AGCTGCATATTCGGAAGTAG
RAD54 F	GCTGTCGCAAACCCTTTCG
RAD54 R	GTCGTAGGCACCAATCATCCATC
RPA F	GAATGTCTTCCGTGAACCG
RPA R	CATCTAAGGACGGGTGCTAG
GAPdH F	TTCAACATCATTCCAAGCAGCA
GAPdH R	CGTAACCCAAAATGCCCTTG
Actin F	GACAATGGAACCGGAATGGTC
Actin R	GTGTGATGCCAGATTTTCTCCAT

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