“A study of the mechanisms of meiosis in wheat”

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PhD Thesis

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∞ The world of the *Ph1* Locus ∞

By Isabelle Colas

This acrylic frame represents the *Ph1* project and its importance in food production. The three approaches of the project, namely Cell biology, Genetics and Proteomics are represented.

Dedication

This thesis is dedicated in loving memory to my friend and colleague Dr Liz Nicholson, who passed away in May 2008. She would ask me every week if I had finished my writing, and I deeply regret that I will never be able to say to her "Yes, I have finished my dear friend."

"Learn as if you were going to live forever.
Live as if you were going to die tomorrow."
Mahatma Gandhi

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Abstract

Breeding programs have the objectives to develop more productive and more stable varieties. Hybridization and selection are frequently employed in plant breeding and the success of introgression of special traits such as disease resistance relies on genetic recombination between the host and alien chromosomes. During meiosis, homologous chromosomes recognized each other, align and pair which ensure their recombination and correct segregation at metaphase. This process controls aberrant chromosome number within the gametes, and ensures that genes are shuffled by recombination. 70% of flowering plants are polyploids including bread (hexaploid) and pasta wheat (tetraploid), and strict homologous pairing in species containing more than one genome is even more important. Wheat homologous chromosomes and their relative homoeologues are genetically close enough to pair during meiosis, however, the *Ph1* locus ensures that only true homologues pair and recombine, stabilizing the wheat genome. Because the pairing is exclusive to homologues, alien chromosomes cannot recombine with wheat chromosome and are eliminated. Deletion lines for the *Ph1* locus, allowing recombination of wheat and its relatives, are used for new wheat variety production. However, these lines have reduced fertility, and inactivity of the *Ph1* locus in hexaploid wheat induces karyotypic instability (unbalanced genome). To be able to control *Ph1* activity, its mechanism has to be understood. Many observations and suggestions about *Ph1* have been raised since the last 50 years but until now it was difficult to link all these funding together. At the onset of meiosis, the chromosomes associate, remodel and pair. *Ph1* is controlling this chromatin remodelling, but is the remodelling essential for pairing and recombination? Moreover, what could the *Ph1* locus be and how does it work at the molecular level? This study, answers to at least two of these questions and proposes a method to elucidate the molecular mechanism of the *Ph1* locus.

Thesis Outline

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Abbreviations

APTES	γ-Aminopropyl Triethoxy Silane
^{14}N or 15N	14-Nitrogen or 15-Nitrogen isotope
2D	2-Dimensionnal
3D	3-Dimensionnal
BAC	Bacterial Artificial Chromosomes
BICP	5-Bromo-4-Chloro-2-Indolyl-Phosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	Bovin Serum Albumin
Ca(NO3)2	CalciumNnitrate
CCD	Charge Couple Device
CDK	Cyclin Dependent Kinase
CID	Collision Cnduced Dissociation
CS	Chinese Spring
Cu(SO4)(H2O)5	Copper Sulphate Pentahydrate
Da	Dalton
DAPI	4',6-Diamino-2-Phenyl-Indole
DNA	Deoxyribo Nucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
dUTP	2'-Deoxyuridine 5'-Triphosphate
DS	Dextran Sulfate
DTT	Dithiothreitol
EDTA	Disodium Ethylene Diamide Tertaacetate
EGTA	Ethylene Glycol Tetraacetic Acid
ESI	Electro Spray Ionization
FA	Formic Acid
FISH	Fluoresecnt in istu hybridization
FITC	Fluorescein isothiocyanate
H3BO3	Boric Acid
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HP1	Heterochromatin Protein 1
HPLC	High Performance Liquid Chromatography
Hx	Histone x

IA	Iodoacetamide
KCl	Potassium Chloride
KNO3	Potassium Nitrate
KOH	Potassium Hydroxide
LC	Liquid Chromatography
LTQ	Linear Trap Quadrupole
m/z	mass-to-charge ratio
MgCl2	Magnesium Chloride
MGF	Mascot Generic Format
MgSO4	Magnesium Sulfate
MnCl(H2O)4	Manganese Chloride Tetrahydrate
MoO3	Molybdic Oxide
MRFA	Methionine–Argenine–Phenylalanine–Alanine
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
MS1	initial mass-to-charge ratio
MS2	tandem MS (m/z and fragment)
MS3	further fragmentation of the fragment ion (MS2)
MW	Molecular Weight
N2	Liquid Nitrogen
NaCl	Sodium Chloride
NaH2PO4	monosodium phosphate
NBT	4-Nitro Blue Tetrazolium Chloride
ºC	Degrees Celcius
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEM Buffer	Pipes, EGTA, MgSO4 buffer
PIPES	2-[4-(2-Sulfoethyl)Piperazin-1-yl]ethanesulfonic Acid
ppm	part per million
PTM	Post Translational Modification
RNA	Ribo Nucleic Acid
SC	Synaptonemal Complex
SDS	Sodium Dodecyl Sulfate
SILAC	Stable Incorporation of Labelled Amino acid in Cell culture
SPtrEMBL	Swiss Prot + trEMBL databases

SSC	Saline Sodium Citrate
TBS	Tris Buffered Saline
TC	Telomere Cluster
TCA	Trichloroacetic Acid
TEAB	Tri-ethyl-ammonium Bicarnonate
TIGR_TA	Institute of Genomic Research Plant Transcript Assemblies
Tris-HCl	Tris Hydrochloride
VIP	Vacuum Infiltration Processor
Zn(SO4)(H2O)7	Zinc Sulfate Heptahydrate
ZSA	Charge State Algorithm
µl	micro litre
µM	micro molar
nm	nano metre
ATP	Adenosine Triphosphate
DSB	Double Strand Break
Ph1	Pairing Homoeologous 1
Ph2	Pairing Homoeologous 2
CDC2	Cell Division Cycle 2
ml	milli litre
1BL	Long arm of the wheat B chromosome 1
1RS	short arm of the rye chromosome 1
F1	Filial 1
F2	Filial 2
µm	micro metre
RFLP	Restriction Fragment Length Polymorphism

Chapter 1

Introduction

Abstract:

"The DNA is highly compacted to fit into the nucleus to compose the chromatin. The building block of the chromatin is made of DNA linked to histones protein and is the main player in chromatin remodelling. Chromatin exists into the cell in a compact or less compact structure called heterochromatin and Euchromatin. Moreover, chromatin remodelling is essential all through the cell cycle as for example in meiosis where the chromosomes have to be further compacted before segregation. Meiosis is a special process combining two rounds of division after one round of DNA replication, producing four haploid cells, in contrast to mitosis which produce two identical cells. Halving the number of chromosomes occurs at metaphase and is possible because of the correct pairing of the homologous chromosomes. Chromosome pairing occurs early in meiosis, and is essential in polyploidy species such as wheat. In wheat, the *Ph1* locus is controlling the chromosomes pairing. This chapter gives an introduction of the chromatin remodelling mechanism, and the wheat chromosome pairing."

1.1 Chromatin and chromosomes

The eukaryotic genome is compacted about 100,000 times to fit in the nucleus in a state called chromatin which is composed of DNA and proteins (Razin et al. 2007). Chromatin can be divided in two forms; euchromatin and heterochromatin. The euchromatin is gene-rich (Hsieh and Fischer 2005, Grewal and Elgin 2007), less condensed during interphase (Hsieh and Fischer 2005), and transcriptionally active (Bender 2004) while heterochromatin is gene-poor but rich in repeat sequences (Hsieh and Fischer 2005, Grewal and Elgin 2007), remains highly condensed throughout the cell cycle, and has a low transcriptionnal activity (Goodrich and Tweedie 2002). The basic repeat element of chromatin is the nucleosome, an octamer of two molecules of the four core histones (H3, H4, H2A and H2B) around which 147 base pairs of DNA are wrapped in 1.65 left-handed superhelix (Goodrich and Tweedie 2002, Kouzarides 2007). This is the building block of chromatin. It is stabilized by a linker histone (H1) which binds outside the core particle interacting with a 20bp of linker DNA (Hsieh and Fischer 2005).

The structure of chromatin varies through the cell cycle and shows different level of organization (Figure 1). The first level of chromatin organization is “beads-on-a-string” appearance making a 10nm wide fibre visualized with the electron microscope (Goodrich and Tweedie 2002). The second level of organization shows a 30 nm condensed chromatin fibre, observed in interphase and is formed by the interaction between nucleosomes. This 30 nm structure allows an easy access of transcription and DNA repair factors to the DNA (Fischle et al. 2003, Razin et al. 2007). The third level of chromatin organization makes up the chromosomes which are at least 10,000 folds shorter than the linear molecules they contain (Goodrich and Tweedie 2002, Bender 2004). This packaging of DNA into chromatin is therefore challenging for mechanisms such as replication, DNA repair, recombination, and transcription (Goodrich and Tweedie 2002, Hsieh and Fischer 2005). Chromatin remodelling is the term used to describe the change of the structure aspect of the chromatin. It is now well established that chromatin remodelling is an important regulatory mechanism (Hsieh and Fischer 2005) and is essential for chromosome condensation during cell division and accurate segregation (Goodrich and Tweedie 2002).

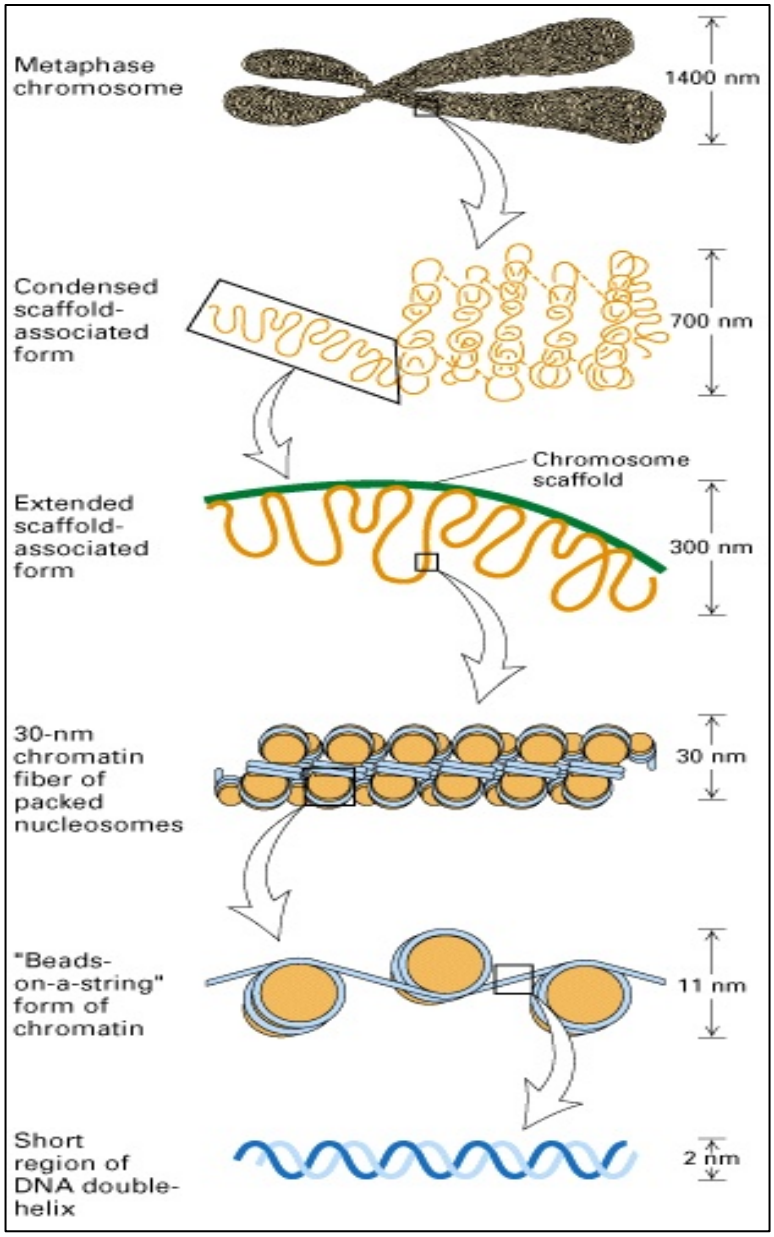


Figure 1: DNA packaging in the nucleus

The DNA is not naked in the nucleus. The beads-on-a-string structure is visualized under electron microscope from chromatin extract, corresponding to a very relaxed chromatin because of the ionic condition. The 30nm chromatin fibre is the state for interphase chromosomes. During cell division, the chromatin is further condensed to make up the chromosome, ensuring an accurate segregation of the genome. (Lodish et al. 2000)

1.2 Changes in chromatin structure

Using an ATP-dependant remodelling complex (Figure 2) is the first way to remodel the chromatin and give access to the DNA to other proteins. They act by sliding the nucleosome along the DNA, transferring the nucleosome to another DNA molecule, or changing the winding up of DNA around the core (Alberts et al. 2002, Brown 2002). The Swi/Snf complex in yeast, disrupts the nucleosome to give access to transcription factors (Brown 2002). There are different classes of Swi/Snf complexes and each one has a specific mechanism. For example, Swi2/Snf2 change the winding of the DNA around the histone core allowing the access of nucleases to the DNA, while Iswi subfamily relocates nucleosomes along the DNA to maintain the chromatin organization (Hsieh and Fischer 2005). Some of the chromatin remodelling complexes are inactivated by phosphorylation during mitosis (Alberts et al. 2002).

A second way to alter chromatin is to introduce histone variants in the nucleosome (Meyer 2001). During the cell cycle, the expression of histone genes is regulated according to the packaging of DNA (Hsieh and Fischer 2005) in various organisms (Wolffe 1998). In tobacco, there are six variants of Histone H1, two major variants (H1A and H1B) and 4 minor variants (H1C, H1D, H1E and H1F).A deficiency of the two major variants in the composition of chromatin leads to chromosomal aberrations and loss of synchrony of the meiotic divisions (Prymakowska-Bosak et al. 1999). In Arabidopsis *thaliana*, an H3-like variant histone has been indentified at the centromere region. By using *in situ* hybridization during meiosis, it seems that the structure of the centromeric region is changing, to give a larger centromere signal suggested as a more “relaxed” chromatin (Talbert et al. 2002). In hexaploid bread wheat, the *Ph1* locus controls both meiotic centromere decondensation and homeologous pairing (Aragón-Alcaide et al., 1997), therefore these larger structures are suggested to help chromosome pairing.

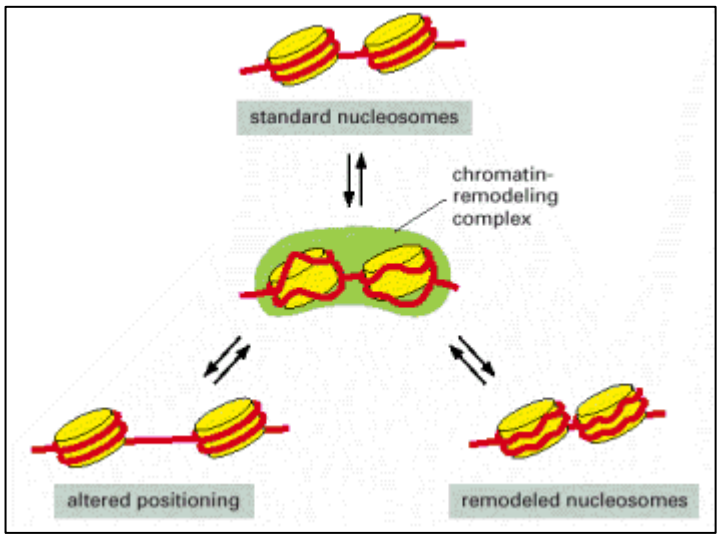


Figure 2: Model for the mechanism of some chromatin remodelling complexes.

The chromatin-remodeling complexes (green) act by disrupting the contact between DNA and nucleosome which results of shifting the nucleosome position or an altered nucleosome. (Alberts et al. 2002)

Finally, histone modifications (Table 1) determine the chromatin structure (Brown 2002). Most of them act to regulate transcription, but also replication, repair, or condensation of chromatin through the cell cycle.

Acetylation of histone tails reduces the affinity of the histones for DNA and possibly also reduces the interaction between individual nucleosomes and destabilized the chromatin structure (Alberts et al. 2002). The fact that, in heterochromatic regions, histones are generally unacetylated and histone acetylation differs according to the cell, suggests that acetylation is associated with gene control during interphase (Lodish et al. 2000). Methylation of H3 and H4 is associated with in gene repression or activation (Iizuka and Smith 2003, Hsieh and Fischer 2005).

Phosphorylation of histone H1 and H3 is known to be involved in transcriptional regulation, and is also associated with chromatin condensation (Gurley et al. 1978, Iizuka and Smith 2003). In mammals, phosphorylation state of Histone H1 and H3 differs between interphase and mitosis. The phosphorylation of H3 and the hyperphosphorylated state of histone H1 occur at mitosis (Gurley et al. 1978). Similarly, in plants, H3 phosphorylation is linked with nuclear division. Moreoever, H3 phosphorylation at serine 10 and serine 28, are distributed at the pericentromeric region during mistosis and meiosis II.. Therefore, H3 phosphorylation does not seem to be directly linked with plant chromosomes condensation as it is in mammals, but is required for cohesion of sister chromatid during metaphase I (Fuchs et al. 2006, Hamant et al. 2006).

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	K-ac	Transcription(+),Repair(+), Replication(+), Condensation (-)
Methylation (lysines)	K-me1 K-me2 K-me3	Transcription (-/+), Repair (+)
Methylation (arginines)	R-me1 R-me2a R-me2s	Transcription(-/+)
Phosphorylation	S-ph T-ph	Transcription(+),Repair (+), Condensation (-)
Ubiquitylation	K-ub	Transcription(-/+), Repair(+)
Sumoylation	K-su	Transcription (-)
ADP ribosylation	E-ar	Transcription(+)
Deimination	R > Cit	Transcription (-)
Proline Isomerization	P-cis > P-trans	Transcription (-)

Table 1: Histones modifications and their potential effects

(+) Activation, (-) Repression, (-/+) Activation or Repression. Acetylation is almost always implicated in genes activation while deacetylation has a role in genes repression. Histone methylation is highly involved in gene activation/repression. Chromatin protein phosphorylation is mostly involved in chromatin condensation and Acetylation is involved in both condensation and replication. (Kouzarides 2007)

1.3 Meiosis: a specialized cell division.

The nuclear division of somatic cells is called mitosis. After DNA replication, one round of division makes two daughter cells, containing the same genetic information (Alberts et al. 2002). Sexual reproduction requires the fusion between two gametes (male and female), and thus implies having only half of the genetic information in each cell to prevent the doubling of the chromosome at each generation. This specialized division to prepare gametes is called meiosis. Meiosis differs from mitosis (Figure 3) in that DNA replication is followed by two rounds of division (meiosis I and meiosis II) which produces four haploid cells from one original diploid cell. Fertilization restores the diploid state. Meiosis has four steps per division; prophase, metaphase, anaphase, and telophase. The first prophase is subdivided into five stages, leptotene, zygotene, pachytene, diplotene, and diakinesis, distinct from each other by the chromatin organization and specific mechanism such as the telomeres mobility.

In both mitosis and meiosis, the chromatin starts to condense at prophase, and chromosomes become visible. While in mitosis, the chromosome align randomly at the metaphasic plate to have the sister chromatid separated, in meiosis, the homologous chromosomes are separated after the first round of division and their sister chromatids are separated in the second round of division (Griffiths 1999a, Lodish et al. 2000, Alberts et al. 2002). Because there is no need for homologues to be pair during the mitosis division, homologous pairing is unique to meiosis. However the mechanism of homologous chromosomes recognition remains unclear (Shaw and Moore 1998).

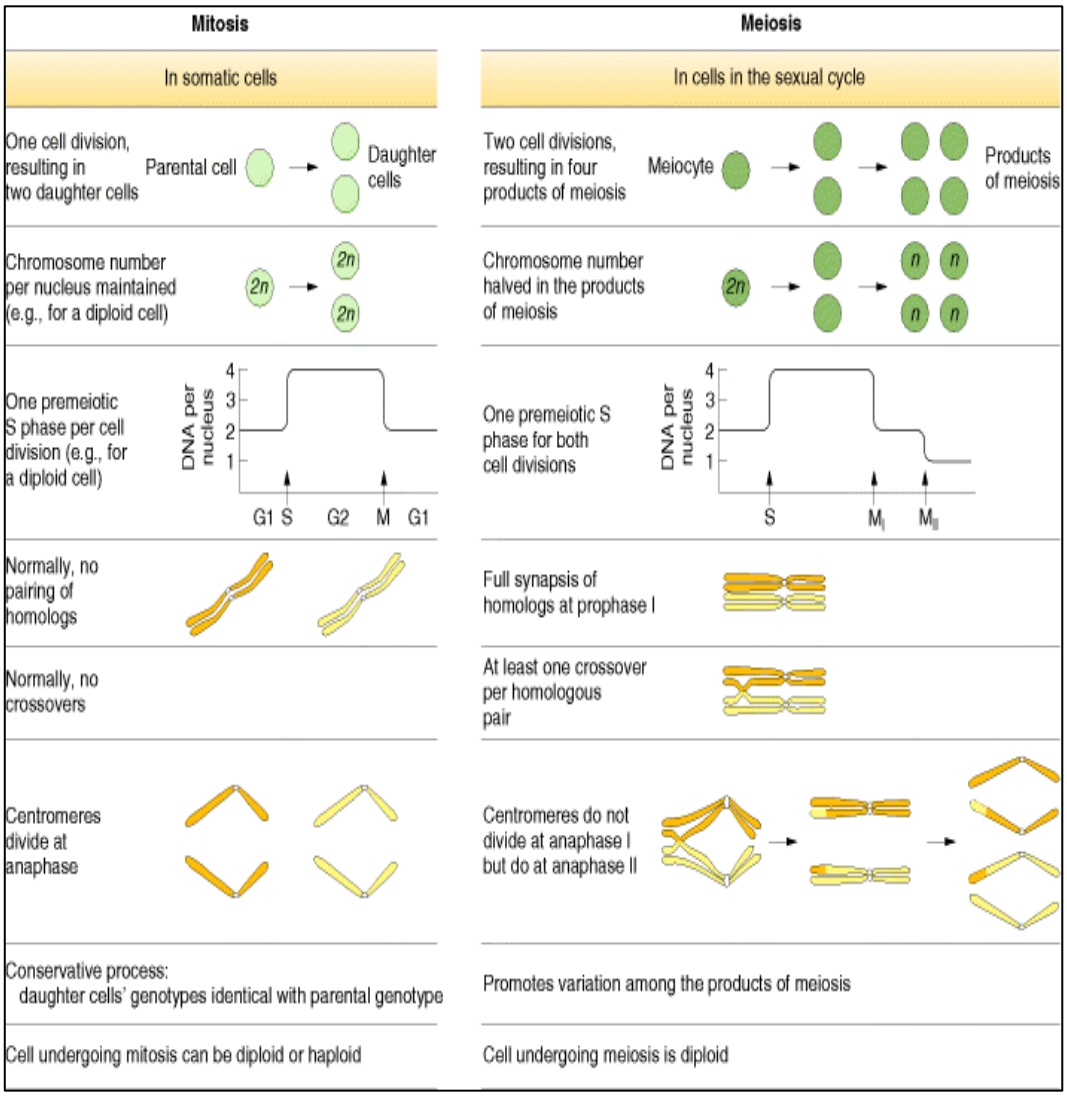


Figure 3: A comparison of Meiosis and Mitosis

In mitosis, one cell gives two daughter cells genetically identical and homologue pairing is not essential. In contrast, in meiosis, one cell gives four cells, genetically different. The chromosomes content is halved, which requires pairing of the homologues prior to division. Genetic exchange happens during cross over which promotes variation of genes content in the meiosis product. (Griffiths 1999b)

1.3.1 Prophase I

This stage can be divided into five steps corresponding to different chromosome organizations and molecular events (Figure 4). It starts at **Leptotene** when the chromatin starts to condense and the chromosomes are visible as long threads with the sister chromatids tightly associated together (Alberts et al. 2002). Moreover, in animals and fungi, the two sister chromatids of each leptotene chromosome are bound to a common protein core known as an axial element (Moens and Pearlman 1988).

The chromosomes have the appearance of a necklace of beads, because of small thick areas called chromomeres developing along each (Griffiths 1999a). Telomeres begin to cluster and the axial element appears to prepare the chromosome for synapsis (Roeder 1997, Dawe 1998, Bass 2003). The chromatids are linked by a protein complex known as an axial element (Dawe 1998, Armstrong and Jones 2003) and the telomere regions of each chromosome are attached to the nuclear envelope with a special structure called the attachment plaque (Alberts et al. 2002). The nucleolus is a large structure which occupies one third of the nuclear volume. At this stage, this structure moves towards the nuclear periphery, where it stays during all of prophase I (Armstrong and Jones 2003).

During **Zygotene**, the chromosomes begin to coil and the sister chromatids become visible for a brief period, then will not become visible again until late prophase (Dawe 1998, Shaw and Moore 1998). The telomeres are clustered in a region of the nuclear envelop to form a structure called the “bouquet”. When the bouquet is complete, chromosome synapsis starts (Bass 2000). The telomere bouquet is thought to anchor the homologues ends in the nuclear membrane and help the homologues to find each other and pair (Griffiths 1999a, Bass 2003). Association of the homologues occurs in a zipper-like manner, resulting in the two homologous chromosomes being side by side and the chromosome pair at this stage is called a bivalent (Roeder 1997, Alberts et al. 2002, Armstrong and Jones 2003).

When the chromosomes are completely synapsed, the cell enters into the **Pachytene** stage. The synaptonemal complex (SC) is fully formed (see paragraph 1.4.4), the chromosomes seem much shorter compared to the beginning of, the prophase and the telomeres disperse (Roeder 1997, Dawe 1998, Shaw and Moore 1998, Armstrong and Jones 2003). Pachytene is the recombination stage (Dawe 1998, Shaw and Moore 1998) and large recombination nodules appear at intervals on the SC (Schwarzacher 2003). The recombination is initiated by the formation of double-stranded breaks (DSBs) (Shaw and Moore 1998) which are resolved as Holliday junctions to make cross over or non cross (Kleckner 1996). In a non crossover situation, the local DNA interacts without exchange of flanking chromosome arms, (Kleckner 1996) while in a cross over situation, an exchange of flanking chromosome arm occurs (Shaw and Moore 1998). Each crossover event produces a connection called a chiasma between the two homologues (Kleckner 1996, Roeder 1997, Dawe 1998, Shaw and Moore 1998, Armstrong and Jones 2003, Schwarzacher 2003). By late pachytene, the bivalents are quite well separated from each other and are often fully apparent (Armstrong and Jones 2003).

The **Diplotene** stage is marked by the gradual and progressive separation of the homologues along their length. Each chromosome of a homologous pair has two sister chromatids linked in a bundle of four homologous chromatids called the tetrad (Griffith, 1999). The SC disappears, and centromeres repeal each other leaving the chromosome linked together by structures called chiasmata (Dawe 1998, Alberts et al. 2002, Armstrong and Jones 2003). Following this, the chromosomes start to condense further (Roeder 1997, Armstrong and Jones 2003).

Diakinesis is the last stage of prophase I, where the chromosomes become shorter and are more condensed by a spiralling process (Dawe 1998). They attach to the spindle as they approach metaphase I (Shaw and Moore 1998). Each bivalent is clearly seen as containing four separate chromatids, with each pair of sister chromatids linked at their centromeres and the non sister chromatids linked by chiasmata (Albert and Redon 1998).

1.3.2 Metaphase I to telophase I

Cells enter into **prometaphase,** where chromosomes are highly condensed, the nuclear envelope disrupts, the microtubules organize into a spindle, and the sister chromatids are linked by a protein complex called the kinetochore (Kleckner 1996, Dawe 1998, Shaw and Moore 1998). These structures are attached to the microtubules and the chromosomes can move and enter into **Metaphase I**. Then, the chromosomes are aligned at the equatorial plate and the kinetochores are co orientated to maintain the chromatids of each chromosome (Dawe 1998). The dissolution of sister chromatid adhesion leads to **Anaphase I** (Kleckner 1996, Dawe 1998, Shaw and Moore 1998), where one chromosome (with two chromatids) from each pair of homologues, moves to each pole of the spindle (Kleckner 1996, Dawe 1998, Armstrong and Jones 2003). At **Telophase I,** only one member of each pair of chromosomes arrives at each pole, and partially decondense (Armstrong and Jones 2003). There is no DNA replication and cells enter immediately into the second division of meiosis (Figure 4)**.**

1.3.3 Second division of meiosis

This is a similar division to mitosis, and the sister chromatids are separated. At **Prophase II**, the DNA is not replicated, and the microtubules organize a spindle perpendicular to the first one (Dawe 1998). The chromosomes move along the microtubules and align at the equatorial plate at **Metaphase II**. The kinetochores are now oriented on opposite sites which leads to the separation of the sister chromatids towards the opposite poles at **Anaphase II** (Dawe 1998). At **Telophase II**, one haploid set of chromosome is at each pole. The nuclear envelope is formed around each haploid number of chromosomes and the cells enter into **Cytokinesis** where the cytoplasm is divided, and leads to four haploid cells (Figure 4). In the anthers of a flower, the four products of meiosis develop into pollen (Griffiths 1999a, Bass 2003).

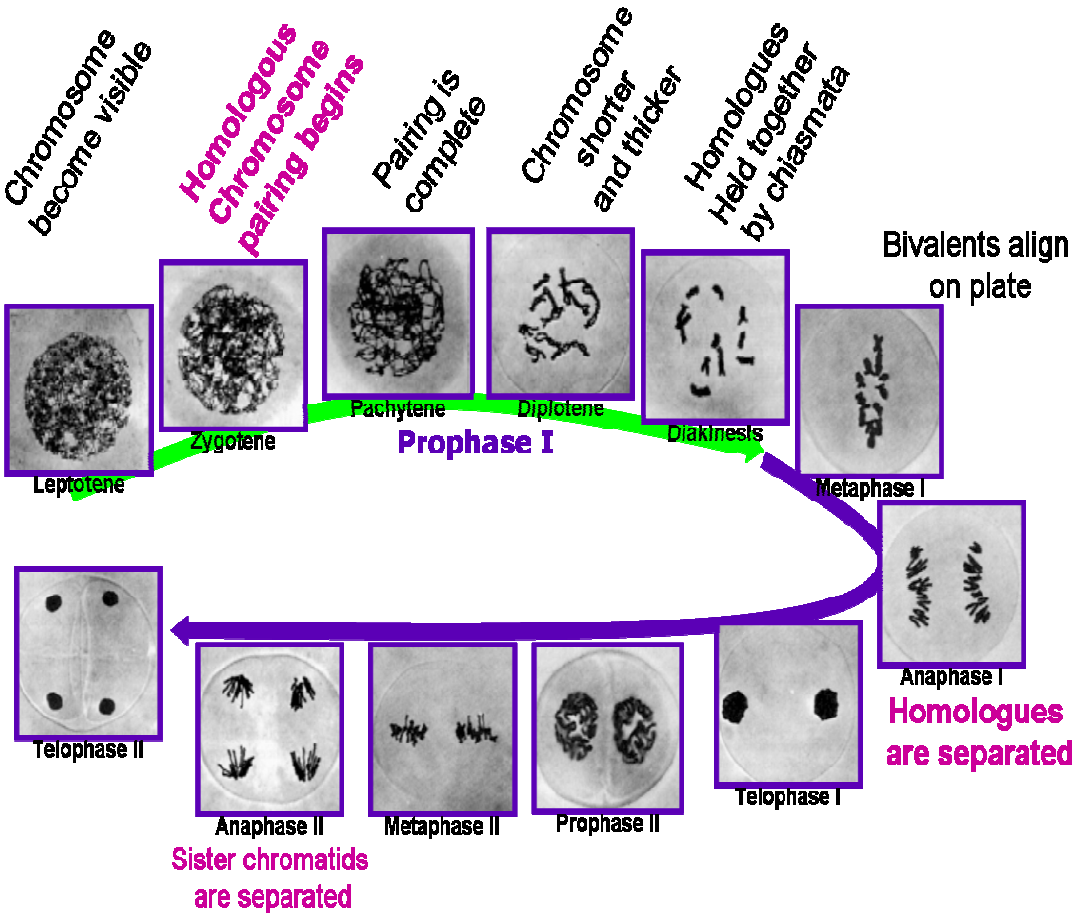


Figure 4: Plant Meiosis

After DNA replication, the chromosomes enter in meiosis. Prophase is substage in 5 stages respectively leptotene, zygotene, pachytene, diplotene and diakinesis. The homologous chromosomes are paired during zygotene to be segregate at metaphase I. The sister chromatids are separated in the second round of division, forming haploid cells. Meiosis pictures scanned from (McLeish and Snoad 1958).

1.4 Control of the Cell Cycle

Vertebrate oocytes (developing eggs) have been particularly useful models for research on the cell cycle. Oocytes can remain arrested at diplotene for long periods of time (up to 40 to 50 years in humans). During this diplotene arrest, the oocyte chromosomes decondense and are actively transcribed. The oocytes of most vertebrates (including frogs, mice, and humans) resume meiosis in response to hormonal stimulation and proceed through meiosis I prior to fertilization. The mature oocyte arrests again at the metaphase II until fertilisation (Alberts et al. 2002, Wolgemuth et al. 2002, Cooper and Hausman 2004). The "meiotic cell cycle" is not strictly speaking a cell cycle because two rounds of division (meiosis I and meiosis II) follow one round of DNA replication to produce four haploid cells which stop until fertilization. However, many of the key regulators of the mitotic cell cycle also control meiosis (Lee and Amon 2001, Marston and Amon 2004).

The cell cycle consists in four phases. The first period of the cell cycle is called interphase and can be divided in three phases namely Gap 1 (G1) phase, Synthesis (S) phase and Gap 2 (G2) phase (Murray and Hunt 1993). The G1 phase is a very active period, where the cell synthesizes proteins needed mainly for replication. The genetic material is replicated during S phase, so at the end of this phase, each chromosome possesses two identical chromatids. The G2 phase is also a growth stage, in which proteins are synthesised, mainly for the following mitosis. The second period consists of cell division (M) phase for mitosis/meiosis (Figure 5).

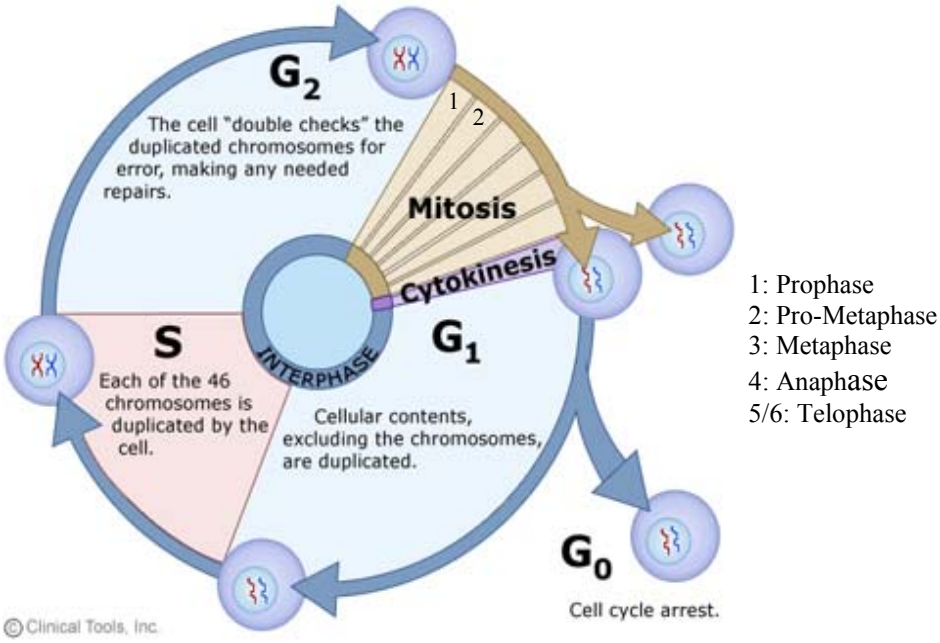


Figure 5: The Cell Cycle

At Interphase, the cells are growing during G1 to prepare for DNA replication occurring at S phase. At G2, the cells prepare the chromosome to enter in Mitosis which consists of the second period of the cell cycle. After mitosis, the daughter cells can either carry on in G1 phase for a new cycle or be arrested in G0, a latent state.

1.4.1 Cell Cycle Checkpoint

Several cell cycle checkpoints function to ensure that incomplete or damaged chromosomes are not replicated and passed on to daughter cells in both mitosis and meiosis (Figure 6). The G1/S and G2/M transitions are major checkpoints of the cell cycles, for both mitosis and meiosis and are strictly regulated (Murray and Hunt 1993). The cells can be arrested at G1 (before S phase) or blocked during S phase, when the DNA is damaged by irradiation with UV light, γ-rays or by chemical modification (Lodish et al. 2000).

In mammalian cells, arrest at the G1 checkpoint is mediated by the action of a protein known as p53, which is rapidly induced in response to damaged DNA (Di Leonardo et al. 1994). Thus, the G1 arrest may allow DNA repair to take place before the cell enters S phase to be replicated (Cooper and Hausman 2004). Only at G1, proteins called MCM bind to DNA, allowing DNA replication to initiate when the cell enters S phase. Once the initiation has occurred, the MCM proteins are displaced from the origin of replication, so replication cannot initiate again until the cell passes through mitosis and enters G1 phase of the next cell cycle (Cooper and Hausman 2004). In yeast the cell decision to enter in either mitosis or meiosis programme is decided at G1.

At the G2/M transition, the cells can be arrested in G2 before cell division until complete DNA replication or DNA repair. This checkpoint involves the recognition of unreplicated DNA and the inhibition of Maturation Promoting Factor (MPF) activation. When the DNA is damaged, arrest in G2 may allow DNA double-stranded breaks to be repaired before mitosis (Lodish et al. 2000). When colchicine (microtubule inhibitor) is added to cultured cells, the cells enter mitosis and arrest with condensed chromosomes. Therefore, this checkpoint "senses" when the mitotic spindle has not assembled properly and prevents activation of complexes that normally lead to degradation of the anaphase inhibitor, required for onset of anaphase (Lodish et al. 2000).

In meiosis, the absence of DNA replication between meiosis I and meiosis II implies a control to stop DNA to be replicated after the first round of division. Moreover, the analysis of mice mutants (Table 2) revealed the possibility of more checkpoints during prophase (Figure 6).

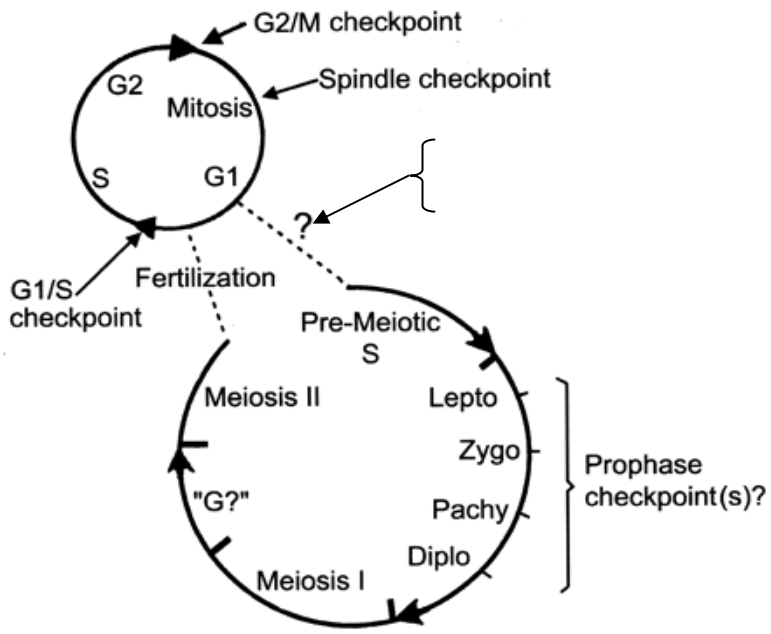


Figure 6: Mitotic and meiotic control points

DNA damage causes arrest of G_1 cells preventing from entering the S phase (G1/S) and G_2 cells from entering mitosis (G2/M). Unreplicated DNA also prevents entry into mitosis (G2/M). Defects in assembly of the mitotic spindle or the attachment of kinetochores to spindle microtubules prevent activation of the APC polyubiquitination system that leads to degradation of the anaphase inhibitor, thus cells do not enter anaphase until all kinetochores are bound to spindle microtubules (spindle checkpoint). Mice mutant for synapsis arrest cells at different prophase stages suggesting the existence of prophase checkpoints. Absence of DNA replication between meiosis I and meiosis II also suggest a restriction point corresponding to an "intermediate" G phase. Yeast cells can divide either by mitosis or meiosis. This decision controlled at G1. Figure adapted from Wolgemuth et al (2008).

Mutation	Arrest point	Synapsis phenotype	Reference
A-myb	Early pachytene	Not reported	Toscani *et al.*, 1997
Atm	Zygotene/early pachytene	Frequent asynapsis	Xu *et al.*, 1996
Dmc1	Zygotene/early pachytene	Asynapsis	Yoshida *et al.*, 1998
Msh4	Zygotene	Frequent asynapsis; Nonhomologous pairing	Kneitz *et al.*, 2000
Msh5	Zygotene/early pachytene	Frequent asynapsis; Nonhomologous pairing	Edelmann *et al.*, 1999
Scp3	Zygotene	Asynapsis	Yuan *et al.*, 2000
Spo11	Zygotene	Asynapsis	Baudat *et al.*, 2000; Romanienko and Camerini-Otero, 2000
XSxr(a)0	Late prophase/meiosis I	Asynapsis of the sex chromosome	Odorisio *et al.*, 1998
Hsp70.2	Late pachytene	Failure to desynapse	Dix *et al.*, 1997
Mlh1	Late pachytene	Failure to desynapse	Edelmann *et al.*, 1996
Cyclin A1	Late pachytene/diplotene	Incomplete desynapsis	D. Liu *et al.*, 1998

Table 2: Genetic Models in Male Mice Exhibiting Meiotic Prophase Arrest and Apoptosis

This table from Wolgemuth et al (2008) shows the mice meiotic mutations leading to arrest in prophase and their effects on homologues synapsis. Zygotene and pachytene are the stages where the synaptonemal complex forms to hold the homologous chromosomes perfectly align along each others. Therefore, it is not surprising that a defect in such a structure leads to arrest at these stages. However, it is not clear how the cells "sense" that the chromosomes are not fully synapsed.

A key discovery in G2/M transition in all eukaryotes is the maturation promoting factor (MPF) or M-phase promoting factor, which consists of serine/threonine protein kinase Cdc2 complexed with cyclin B (Masui and Markert 1971, Nebreda and Ferby 2000, Kishimoto 2003). Cell division for both mitosis and meiosis (figure cdk activity) is regulated by the sequential activation of cyclin-dependent kinases (cdks). Cyclins are the regulatory subunits of the CDK-cyclin complexes, while the protein kinases are the catalytic subunit. The cyclin-Cdk complexes are inactivated by regulated destruction of cyclins occurs by an ubiquitin-dependent mechanism at certain cell cycle stages (Alberts et al. 2002).

Marston and Amon (2004) reviewed how the mitotic cell cycle is modulated to bring about the meiotic programme. In the mitotic cell cycle (figure 7 a), G1-CDK activity induces the destruction of Sic1 (CDK inhinitor sub-unit) and the inactivation of the anaphase-promoting complex/cyclosome (APC/C). The accumulation of S-phase CDK activity initiates DNA replication and Mitotic CDKs 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 G1 (Marston and Amon 2004).

Similarly, in the meiotic cell cycle (figure 7 b), G1-like CDK (Ime2 in budding yeast) promotes the activation of S-phase CDKs (Cdc28–cyclin-B-5/6 (Clb5/6) in budding yeast) by inducing Sic1 destruction and inactivation of the APC/C. Ime2 has a second peak in kinase activity during the meiotic divisions and is required for the execution of the meiotic divisions. It is not known if S-phase CDK activity declines after entry into meiosis I. Meiotic CDKs (Cdc28–Clb1/3/4 in budding yeast) direct chromosome segregation during meiosis I. In the frog (*Xenopus laevis*), meiotic CDKs are partially inactivated between meiosis I and meiosis II, which prevents further DNA replication and chromosome segregation. Meiotic CDK activity rises again to allow entry into meiosis II. Complete inactivation of meiotic CDKs triggers exit from meiosis II (Marston and Amon 2004).

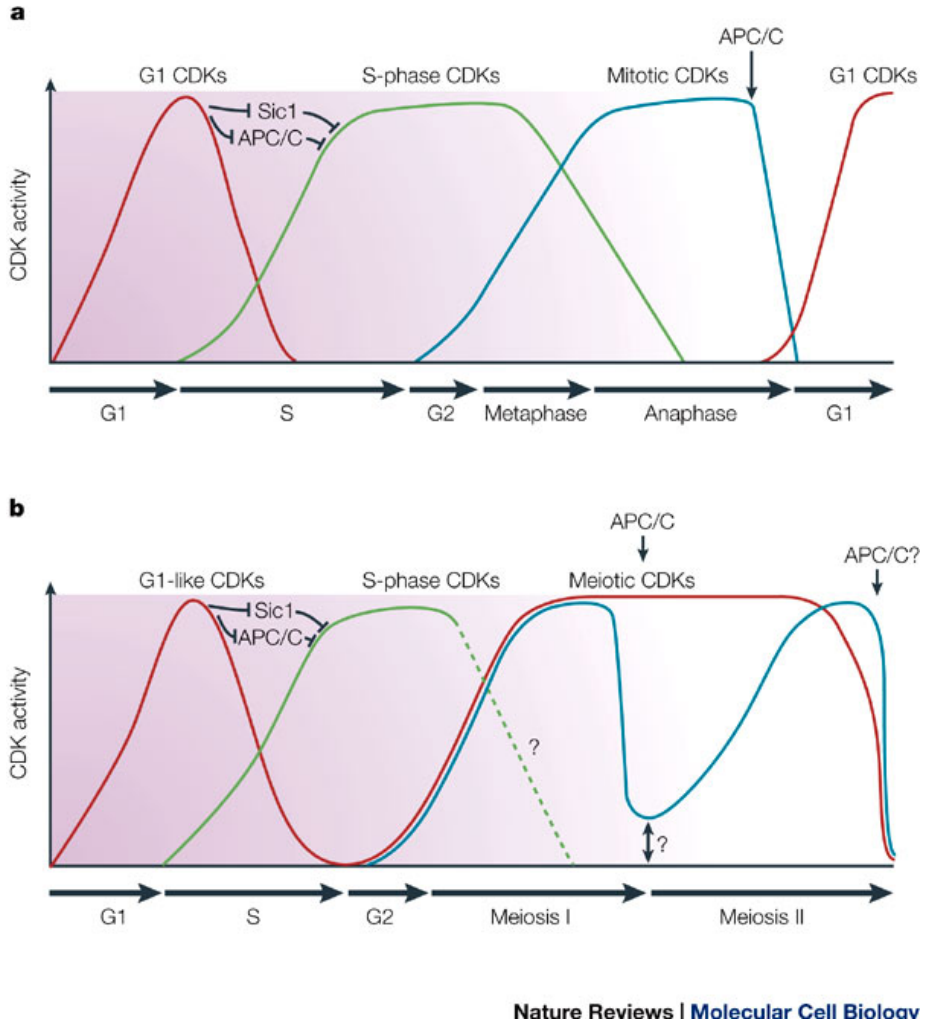


Figure 7: CDKs activity in meiosis and mitosis
(Marston and Amon 2004)

a | 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

b | Meiotic cell cycle: The red line represents the G1-like CDK (Ime2 in budding yeast), the green line represents the S-phase CDKs (Cdc28–cyclin-B-5/6 (Clb5/6) in budding yeast)- the line is dotted to represent the CDK degradation, but there is no evidence for this degradtion yet. The blue line represents the Meiotic CDKs (Cdc28–Clb1/3/4 in budding yeast). In the frog (*Xenopus laevis*), meiotic CDKs are partially inactivated between meiosis I and meiosis II, then rises again to allow entry into meiosis II.

1.4.2 Cyclin Dependant Kinases

First discovered in yeast, the cell division cycle genes *cdc2* (*S. pombe*) and its orthologue *cdc28* (*S. cerevisiae*) control the cell cycle at the G1/S and G2/M checkpoints (Norbory and Nurse, 1992). In animals and plants, these genes have been renamed as cyclin dependent kinases. Human CDKs have been named in the order of their discovery (CDK1, CDK2, etc) and 12 CDKs has been listed (joubes, 2000). In plants, CDKs have been named with letters and numbers and can be sorted in 5 majors classes (A to E) and a distinct class named CDKG (joubes, 2000; Umeda, 2005). For example, the functional orthologue *cdc2* gene from animals is called *CDK1* and from plants *CDKA1*. The CDK belong to the CMGC group (conventional protein kinase group), which also includes the mitogen-activated protein kinases (MAPK), the glycogen synthase kinases (GSK-3) and casein kinase II (CKII) families (Hanks and Quinn 1991). CDKs are activated when a cyclin bind to its "PSTAIRE" motif resulting in significant conformational changes for the kinase and limited modifications for the cyclin (Jeffrey et al. 1995). Cyclins were first identified in sea urchin eggs and similarly to CDKs, a number of cyclins have been identified from various organisms (Evans 2004, Hunt 2004). For example, plants cyclins can be classified in two groups of B-type cyclins (*CYCB1* and *CYCB2* genes) and three groups of A-type (*CYCA1*, *CYCA2* and *CYCA3)* and D-type cyclins (*CYCD1*, *CYCD2* and *CYCD3* genes) (Renaudin *et al.*, 1996).

Eukaryotic cells use several Cdks and cyclins to regulate passage through the cell cycle. For example in mammals (figure …), Cdk4-Cyclin D, Cdk6-Cyclin D and Cdk3–CyclinC function in mid to late G_1 to regulate the G0–G1 transition. Cdk2-Cyclin E functions in G_1 while Cdk2- Cyclin A occurs at S phase. Finaly Cyclin A-Cdk1 and Cyclin B-Cdk1 act in G_2 and M through anaphase (Lodish et al. 2000, Malumbres and Barbacid 2005).

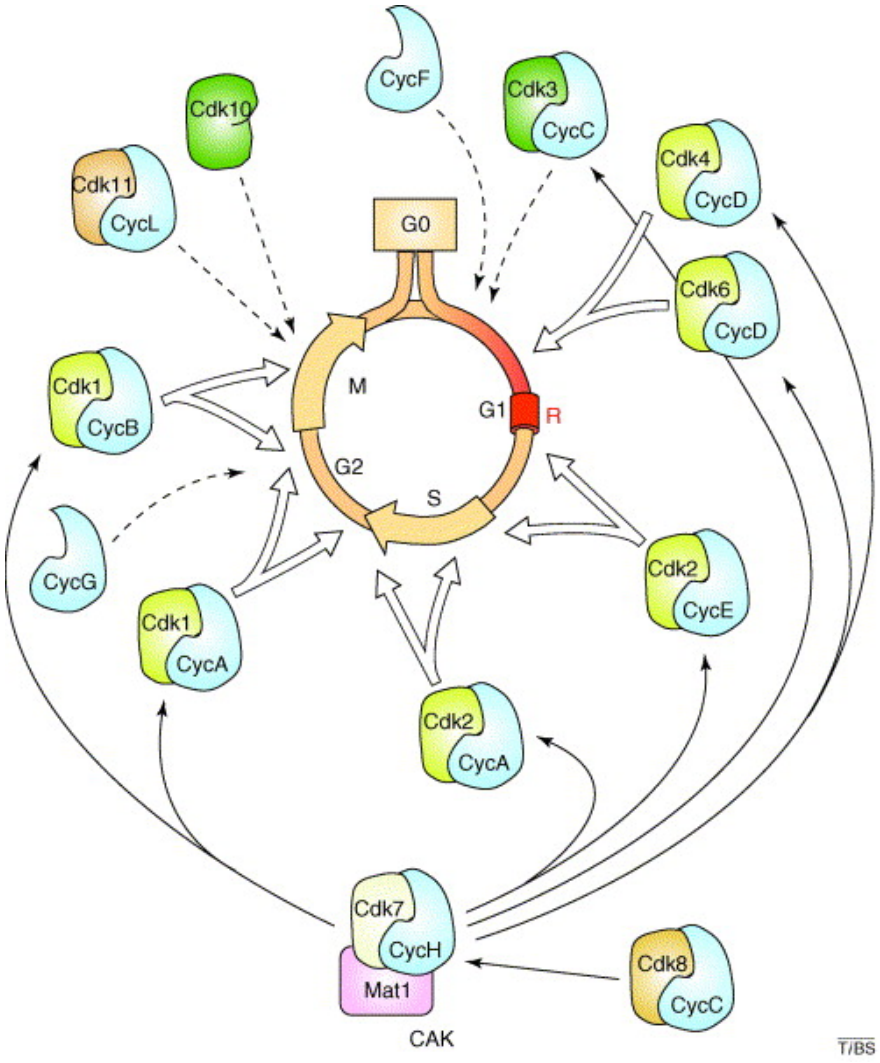


Figure 8: Proposed roles of Cdk–cyclin complexes in the mammalian cell cycle.

From Malunbres and Barbacid (2005)

Central to their functional properties is the positive regulation of CDK by cyclin binding and by phosphorylation (on Thr-160 in human CDK2), and their negative regulation by cyclin-dependent kinase inhibitors (CKI) and by phosphorylation (on Thr-14 and Tyr-15 in human CDK2) (Joubes et al. 2000). Two families of Cdk inhibitors have been described. The first family includes the INK4 proteins ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) which specifically inhibit the catalytic subunits of CDK4 and CDK6. The second family is Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) which binds to Cdk–cyclin complexes (Sherr and Roberts 1999). Binding to Cdk2 and Cdk1 complexes blocks the kinase activity of these complexes.

1.4.3 Cdk2, meiosis and histone phosphorylation

In mammals, both Cdk2 and Cdk4 are expressed in meiosis. However, Cdk4 colocalizes with replication protein A (RPA) on the synaptonemal complexes (SCs) of newly synapsed axes of homologously pairing bivalents, but disappears from these axes by mid-pachynema. In contrast, Cdk2 colocalizes with MLH1, a mismatch repair protein at sites of reciprocal recombination in mid-late pachynema. In addition Cdk2 localizes to the telomeres of chromosomal bivalents throughout meiotic prophase (Ashley et al. 2001). Therefore, Cdk2 and Cdk4 have different role in meiosis, and Cdk2 is involved in recombination events. This is confirmed by Muller-Tidow et al (2004) who shown that the cyclin A1-CDK2 complex regulates DNA double-strand break repair (Muller-Tidow et al. 2004).

Berthet et al (2003) prepared Cdk2 knockout mice. Although Cdk2 is essential for cell proliferation during early embryonic development, the mice are viable, and therefore Cdk2 is not an essential gene in the mouse. However, Cdk2 is required for germ cell development and both male and female Cdk2(-/-) mice are sterile. Immunoprecipitates of cyclin E1 complexes from Cdk2(-/-) spleen extracts displayed no activity toward histone H1 and S phase is delay (Berthet et al. 2003). Thus, Cdk2 is essential for meiosis in mice. If both cdc2 and cdk2 were shown to promote S phase initiation in Xenopus egg extracts(Chevalier et al. 1995), Cdk2 is also implies in timing DNA replication (Berthet et al. 2003).

More recently, Krasinska et al (2008) describe how cyclin-dependent kinase (Cdk) activity controls the efficiency of DNA replication by determining the frequency of origin activation. They find that both Cdk1 and Cdk2 are necessary for efficient DNA replication and that, at low levels, Cdk activity is limiting for the pre-replication complex to pre-initiation complex transition, origin activation and replication efficiency (Krasinska et al. 2008).

The linker histone H1 is involved in maintaining higher-order chromatin structures and displays dynamic nuclear mobility, which may be regulated by posttranslational modifications. It is suggested that Cdk2-directed histone H1 phosphorylation to permit chromatin remodeling (Bhattacharjee et al. 2001). The phosphorylation of histone H1 by Cdk2 results in a more open chromatin structure by destabilizing H1-chromatin interactions (Contreras et al. 2003). Cdc45 is required for initiation of DNA replication and fork progression. In a recent study, Alexandrow et al (2005) suggest that Cdc45 recruits Cdk2 to replication foci, resulting in H1 phosphorylation, chromatin decondensation, and facilitation of fork progression (Alexandrow and Hamlin 2005). Moreover, the heterochromatin protein 1 alpha (HP1alpha) and the linker histone H1 interact in vivo and in vitro through their hinge and C-terminal domains, respectively. The phosphorylation of H1 by CDK2, which is required for efficient cell cycle progression, disrupts this interaction. It is proposed that phosphorylation of H1 provides a signal for the disassembly of higher order chromatin structures during interphase, independent of histone H3-lysine 9 (H3-K9) methylation, by reducing the affinity of HP1alpha for heterochromatin(Hale et al. 2006).

1.5 Chromosome association, pairing and synapsis

1.5.1 Premeiotic chromosome association

In 1985. Carl Rabl hypothesised that the interphase chromosomes adopt a highly regular configuration , with the two chromosome arms lying next to each other and the centromeres and telomeres located at opposite poles of the nuclei. This organization is the result of the chromosome segregation at anaphase which polarizes the chromosomes because sister centromeres are pulled in opposite directions and the rest of the chromosome trails behind. This arrangement of chromosomes persists into the following interphase in some species and is called the Rabl organization (Cowan et al. 2001). In rice, the interphase chromosomes do not show a Rabl configuration in the meiocytes, but show this configuration in endoreduplicated xylem vessel cells between homologous chromosomes. It is suggested that endoreduplication may occur immediately after chromosome segregation in these cells, and that the new chromatin interactions, particularly at the centromeres, in the endoreduplicated chromosomes may stabilize the anaphase chromosome configuration (Prieto et al. 2004b, Santos and Shaw 2004)

It has been established that in many species, chromosomes are not paired before meiosis (Roeder 1997). Homologues are not paired in premeiotic cells of mice and humans for example. In some fungi, homologues pairing is restricted to meiotic cells (Roeder 1997). In diploid plants such as Arabidopsis, maize and diploid progenitors of wheat, no premeiotic association of chromosomes occurs (Prieto et al. 2004b). On the other hand, some species show a premeiotic chromosome association. In *Drosophila* and other Dipterans, homologous chromosomes pair early during embryogenesis and this pairing is not disrupted in meiotic cells (Roeder 1997), which could be a prelude for the establishment of homolog pairing in meiosis (McKee 2004). In mosquitos, homologous pairing is continued from premeiotic cells through to pachytene (McKee 2004). In yeast, it has been shown that homologues associate in non meiotic cells, and that each homologous pair seems to occupy separate territories within the nucleus (Roeder 1997). In *C. elegans*, the pairing is preceded by a reorganization of the nucleus in which the chromosomes cluster at one end. Only one end of each chromosome is in contact with the nuclear membrane (McKee 2004).

1.5.2 Role of the Centromeres

The function of centromeres is highly conserved, but the DNA sequences are not really well known and are very different between species (Schwarzacher 2003). Their regions are composed of heterochromatin containing repetitive DNA sequence like tandem satellite repeats or retroelement-like components (Schwarzacher 2003).

Kinetochores are protein complexes that assemble on centromeric DNA and are important for chromosome segregation. In the first division of meiosis, kinetochores which link the two chromatids have the same orientation and then segregate together, but in the second division, they have opposite orientation and segregate to the opposite pole which results to the sister chromatids separation (Dawe 1998).

In some organisms, clustering of centromeres is an important characteristic of chromosome organization in mitotic cells (McKee 2004). In mitotic Arabidopsis cells, homologous centromeres were found to be paired whenever euchromatic regions of the same chromosome were paired, suggesting that pairing might spread from centromere heterochromatin into the euchromatin, but centromeres are clearly unpaired in early meiotic prophase (McKee 2004), and probably have no specific role in chromosome pairing (Schwarzacher 2003). However, in wheat, premeiotic interphase association of centromeres acts as a prelude to chromosome pairing (Naranjo and Corredor 2004). The role of centromere at meiosis differs between species, and their participation in chromosomes pairing is not very well defined.

1.5.3 Role of the Telomeres

Telomere sequences are highly conserved. In plants, they consist of hundreds of tandem repeats similar to (TTTAGGG)n at each end of chromosomes and the telomerase (enzyme that adds the telomeric sequence) is active in all tissues. The length of the telomere region is species and tissue specific (Schwarzacher 2003). In many organisms, meiotic chromosomes form a bouquet in which telomeres of chromosomes are attached to a small region of the nuclear envelope (Roeder 1997, Dawe 1998, McKee 2004). Telomeres start to cluster at late leptotene, are tighly cluster at zygotene, and disperse during pachytene (Roeder 1997).

In yeast, a disruption in telomere clustering leads to a delay in pairing and reductions in recombination (Roeder 1997, McKee 2004). In the classical picture, the SC is usually initiated by telomere pairing, and completed by the zippering process from each telomere along the chromosomes (Dawe 1998). However, in maize chromosome homologues can pair with a lack of telomere association (McClintock 1941, Schwartz 1958). Therefore, the role of the bouquet could be to initiate synapsis and/or facilitate chromosome pairing (Dawe 1998). Studies in rye and wheat have shown that pairing was only disrupted when colchicines (drug which affects microtubule organization) was applied well before the bouquet stage, and in *Lilium* and *Allium,* colchicine reduced pairing when applied during bouquet formation (Dawe 1998). This suggests a link between telomeres and microtubules.

Bouquet formation and associated chromosome movements have been postulated to promote the homology search process in interstitial as well as distal regions (Roeder 1997, McKee 2004) and is an active process in plants (Roeder 1997, Dawe 1998). The telomere bouquet is nearly universal and specific to meiosis (Bass, 2003).

1.5.4 Chromosome pairing and synapsis

Chromosome pairing is the process in which the homologous chromosomes are closely associated during meiosis, and takes place at Prophase I. This event can be divided in three steps; recognition, presynaptic alignment and synapsis by the **Synaptonemal Complex** (SC) (Zickler 2006).

The SC is a tripartite structure formed between leptotene and zygotene, complete in pachytene and dissolved at diplotene. At leptotene, the two chromatids of each single chromosome develop axial and lateral elements along their entire length. While the axial elements become closely connected to each other along their entire lengths, the lateral elements of each chromosome positioned themselves parallel to the central region. Perpendicular filaments are built in a series of loops to hold the axial and lateral elements (Figure 9). In yeast, the proteins Zip1 (analogue to Scp1 in mammals) and Red1 are associated with the central element and transverse filaments and are specifically expressed in meiosis (Roeder 1997, Schwarzacher 2003). The protein Hop1 in yeast, corresponding to the mammalian proteins cor1 (hamster) and Scp3 (rats) is associated with the lateral elements (Roeder 1997, Schwarzacher 2003).

The composition of SC in plants is still unclear because it is very difficult to isolate the proteins involved in this structure (Schwarzacher 2003). However, model species such as Rice and Arabidopsis have been really useful for meiotic protein identification (Jenkins et al. 2008). In wheat, TaAsy1, orthologue to Asy1 in arabidopsis and PAIR2 in rice, is associated with synapsis and show some similarity with Hop1 in yeast (Schwarzacher 2003, Jenkins et al. 2008). In Rye, Sy1 and Sy9 are linked with the axial elements and are involved in recombination (Jenkins et al. 2008). In Arabidopsis plants lacking SwI1, the homologues do not synapse because their chromatid arms and centromeres are losing their cohesion before metaphase I (Mercier et al. 2001). Moreover, SWI1 is required for the formation of axial elements (Mercier et al. 2003).

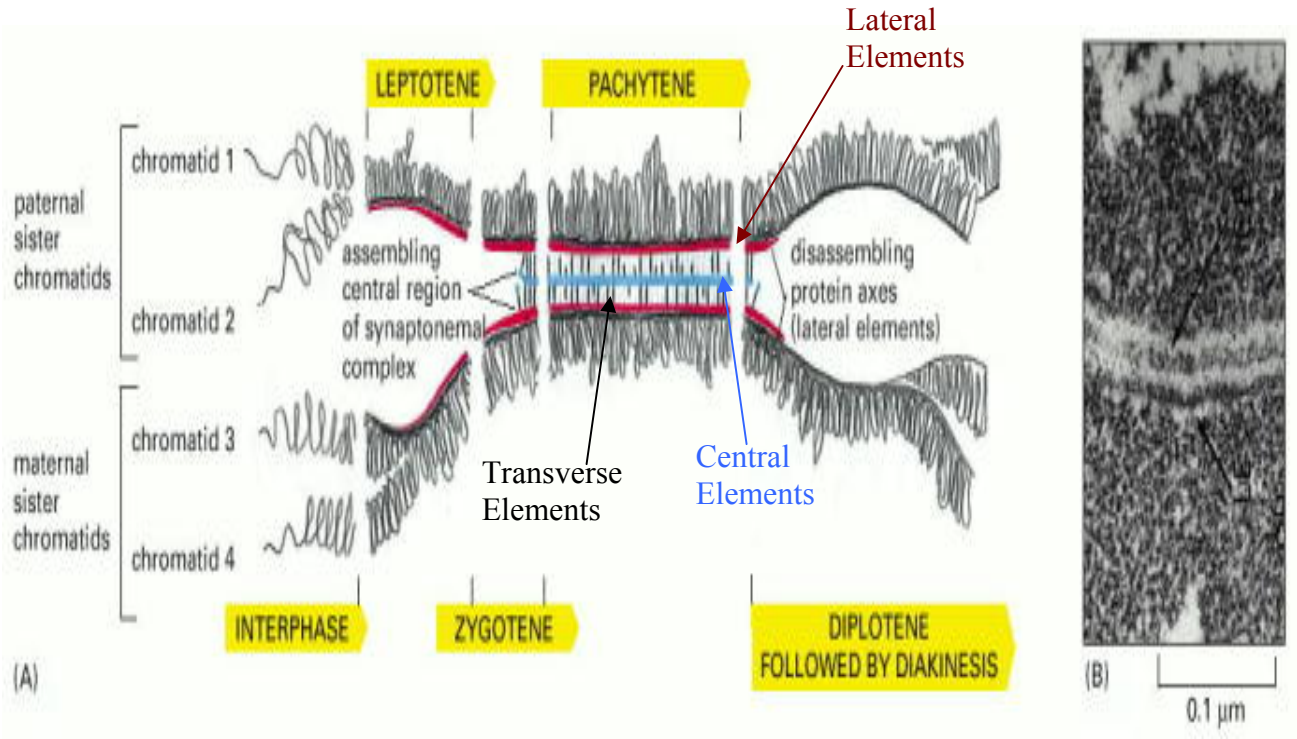


Figure 9: Assembling and disassembling of the synaptonemal complex in prophase I

The synaptonemal complex starts between leptotene and zygotene, is fully formed at pachytene and dissolves at diplotene leaving the two chromosomes linked together via chiasmata. The lateral and central regions are shown is red and blue respectively. The filaments (or transverse elements) hold the central and laterals elements together. (Alberts et al. 2002)

The synaptonemal complex (SC) is found in most eukaryotes to mediate the chromosome pairing during prophase by keeping the homologous chromosomes in a bivalent. It also might be required as a scaffold for recombination and double-stranded breaks (DSBs) formation (Kleckner 1996, Dawe 1998). However this role in recombination does not seem universal. Various studies shows that DSB appears sometimes before SC formation (Kleckner 1996). In yeast deficient in Zip protein, producing no SC or immature SC, recombination events are still visible (Roeder 1997, Shaw and Moore 1998). Finally, S. *pombe* and *Aspergillus nidulans* have high levels of recombination but fail to make SC (Roeder 1997). Based on this last observation, we can hypothesis that DSB formation precedes the SC formation. Therefore, SC may not be necessary for recombination events, but it might have a role in the regulation of these events (Roeder 1997, Dawe 1998, Shaw and Moore 1998) especially in limiting the number of cross-overs formed (Dawe 1998, Shaw and Moore 1998). This phenomenon is called interference (Kleckner 1996, Shaw and Moore 1998). In the last example, these species have no SC, but a high level of recombination which suggest that the recombinations have no limitation because of the lack of a conventional SC (Roeder 1997, Shaw and Moore 1998).

In plants, many studies have been made in *Hypochoeris radicata,* tomato and maize and have observed that the SC and the interference are linked (Dawe 1998). It is not impossible that the SC could be initiated by the occurrence of double-stranded breaks (DSBs) found in early prophase. Because in most organisms, synapsis initiates at a few sites along each chromosome pair, SC initiation may precede chromosomes pairing, contrary to the classical picture. Each location corresponds to a recombination nodule (a protein association link to the axial element of the SC) and it is thought that these early nodules (found at leptotene or zygotene) could initiate the SC formation and it has been shown that they are present on unpaired chromosome and could be involved in the homology search (Dawe 1998). In recent data, it has been shown that the site of these early nodules corresponds to the sites of strand exchange (Roeder 1997).

1.5.5 Chromosome Pairing and Recombination

Recombination is the mechanism by which chromosome exchange can occur and starts by the formation of **Double-stranded breaks** (DSBs), lesions in the arms of chromosomes which initiate gene conversion and crossing (Roeder 1997). The frequency and the distribution of the DSBs throughout the genome seem to correlate with the frequency and distribution of meiotic recombination events (Roeder 1997). The presences of DSBs are not random, and seem to appear at very specific region of the genome. In yeast, the DSBs are often located in the promoter regions of genes, and in maize it is often in retrotransposon regions (Schwarzacher 2003). In wheat and rye, genetic recombination frequency is high near the telomeres, but rare towards the centromeres and in Arabidopsis the recombination seems to be in the region of low chromatin condensation (Schwarzacher 2003). DSBs occur in the region where the DNA is in an accessible chromatin configuration (Kleckner 1996), and in general, recombinations are more frequent in euchromatin that contains active genes (Schwarzacher 2003).

The Archaebacterial topoisomerase VI (**TOP6**) (Schwarzacher 2003), a protein from a novel family of type II topoisomerases, is a homologue of the yeast enzyme Spo11 which generates DSBs. It suggests that DSBs are formed by a topoisomerase-like trans-esterification reaction rather than by endonucleolytic hydrolysis (Roeder 1997). Another protein, Rad51 has an important role in recombination and DNA repair, and seems also implicated in homologous pairing and DSBs (Shaw and Moore 1998). Homolog recognition seems to precede and promote the initiation of recombination (Roeder 1997).

The relationship between pairing, synapsis and DSB formation varies between organisms (McKim 2005), and chromosome pairing and/or synapsis can be DSB independent, or DSB dependant (Shaw and Moore 1998, Zickler 2006). The mutation rad50s, which disrupts processing of DSBs, has a small effect on homologous pairing (McKee 2004). And, in the case of *C. elegans*, and female Drosophila , DSBs are not necessary for homologues associations or synapsis (Shaw and Moore 1998, Zickler 2006).

On the other hand, in yeast, mice and Arabidopsis thaliana, the chromosome pairing (chromosome association) and/or synapsis (SC formation) is DSB dependant (Zickler 2006). It appears that the decision of noncrossingover/crossover is made early in the recombination step and even before synapsis in yeast (Borner et al. 2004) and Arabidopsis *thaliana* (Higgins et al. 2004).

Non Cross-over/Cross-over are the results of the resolution of the Hollidays junction formed in the DSBs repair mechanism (Kleckner 1996, Schwarzacher 2003). The DSBs repair mechanism (Figure 10) predicts two Holliday junctions per chromosome pair (one per DBS), thus one of each side of the region of strand exchange to be randomly resolve (Roeder 1997). In the case of noncross-over, the flanking chromosome arms are not exchanged and only sequences between the two Holliday junctions are affected, while in a cross-over, there is an exchange of the flanking arms of the chromatids (Roeder 1997, Shaw and Moore 1998).

At metaphase I, non sister chromatids of homologous chromosomes are linked by **Chiasmata** (Alberts et al. 2002). Chiasmata hold homologous chromosomes together and many observations suggest that chiasma function depends on sister chromatid cohesion (Roeder 1997). The SC may have a little role in keeping the structures in place (Dawe 1998). The role of chiasmata seems different between species. In insects, chiasmata formation is necessary for the cell to proceed from metaphase to anaphase, and in plants, chiasmata could be required to ensure proper meiotic spindle assembly (Dawe 1998). Chiasmata correspond to the site of DNA breakage and rejoining of two nonsister chromatids (Roeder 1997). Late recombination nodules are found exactly at the site of cross-over, and their number matches the number of chiasmata (Roeder 1997, Dawe 1998). This information leads to the idea that early nodules seem to mark the sites of all strand exchange reactions, whereas late nodules mark those resolve as crossovers (Roeder 1997).

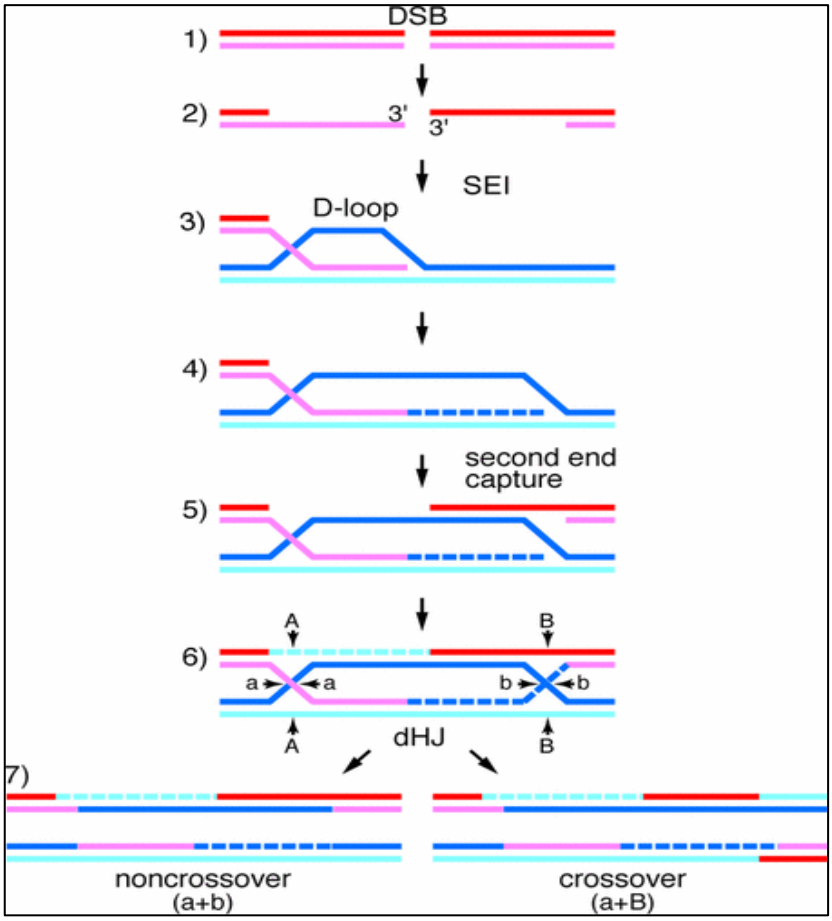


Figure 10: **Meiotic DSB repair.**

In 1983, Szostak et al elaborate a model to explain the DBS resolution. In this model, the resolution of the Holliday junction lead to either exchange (crossover) or non exchanged (noncrossover) of the sister chromatids. Sister chromatids of one chromosome are shown in red and pink, while the sister chromatids of its homologues are shown in dark and light blue. (Szostak et al. 1983)

1.6 Chromosome pairing in wheat

1.6.1 Polyploidy

Polyploidy occurs is some fish, amphibians and insects but is widespread in plants and has a part in evolution (Lokki and Saura 1979, Orr 1990, Otto and Whitton 2000, Venkatesh 2003, Comai 2005). An estimation in angiosperms shows that 50% of them are polyploid, including many important crop species such as wheat (Moore 1998, Shaw and Moore 1998, Martinez-Perez et al. 2000). Autopolyploids are formed by a multiplication of a basic set of chromosomes, and Allopolyploids by a combining of two parental genomes (Comai 2005). Allopolyploids are the result of sexual hybridisation between two species, containing related, but not completely homologous chromosomes, called homoeologues. Homoeologous chromosomes have a similar linear gene sequence, but different repetitive DNA content, while homologous chromosomes have the same linear gene sequence, and the same repetitive DNA content (Moore 2000). Homologous pairing relies on the exact recognition of the chromosomes by each other and their association. This is more difficult in allopolyploids species such as wheat because the homoeologous chromosomes are very similar in composition. Hexaploid wheat (*Triticum aestivum*) contains three closely related genomes (AABBDD) and chromosome pairing is restricted to true homologues, despite the fact of the presence of related (homoeologous) chromosomes (Figure 11).

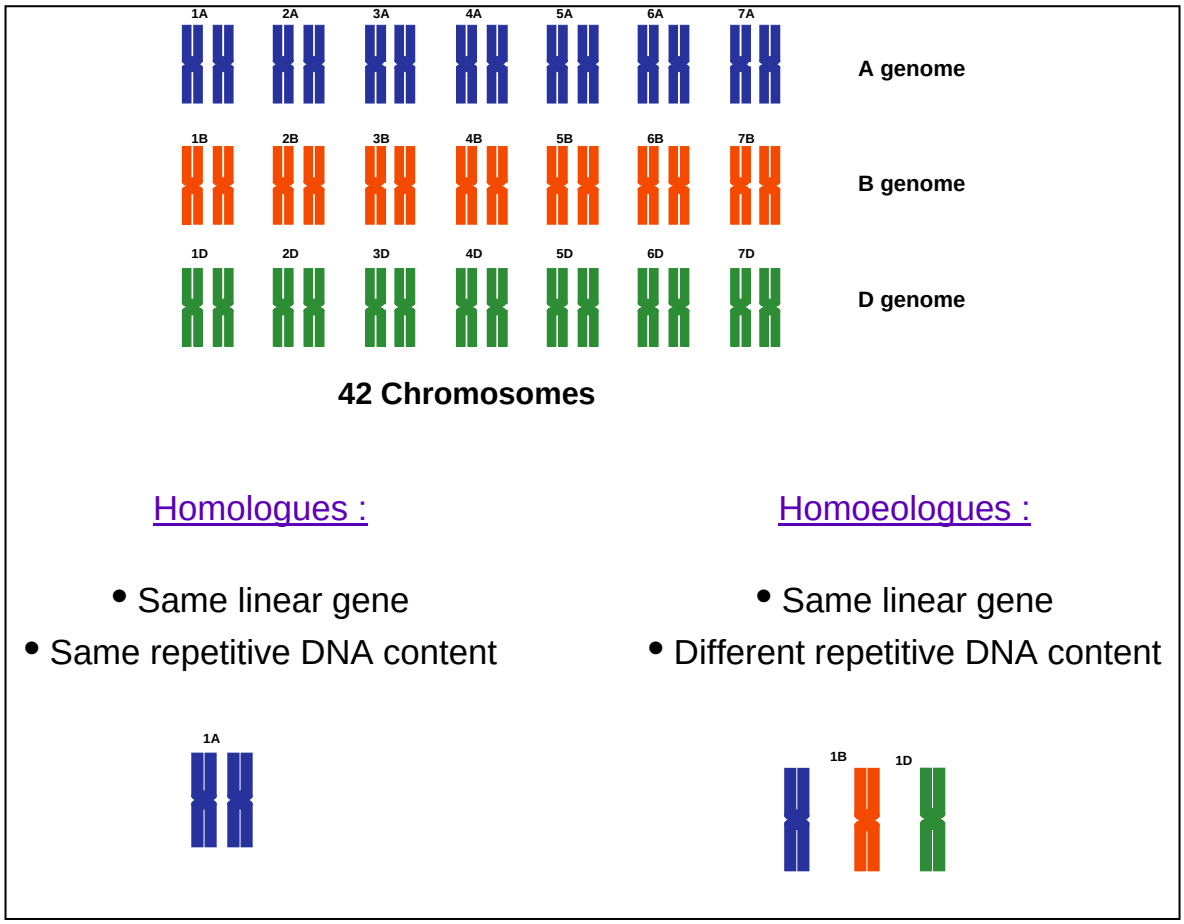


Figure 11: Genetic composition of hexaploid wheat

1.6.2 Wheat Chromosome Pairing

Prior to meiosis, wheat centromeres cluster in 7 groups in a tripartite structure containing each pair of homologues and its relative homoeologues (Martinez-Perez et al. 2003). When the telomere bouquet is fully formed, the sub-telomeric regions of the homologues are brought together and associate (Martinez-Perez et al. 1999). The homologues undergo a change in their conformation from the telomere end and pair in a zipping up process (Prieto et al. 2004a). Then the 7 centromeres cluster are resolved (Martinez-Perez et al. 2003).

Many studies provide observations about a gene control of chromosome pairing. In Arabidopsis, AHP2 is required for bivalent formation and for segregation of homologous chromosomes (Schommer et al. 2003). In rice, PAIR1 encodes a putative coiled-coil protein required for homologous pairing in meiosis (Nonomura et al. 2004). In wheat, chromosome pairing is also genetically controlled ensuring that homologous chromosome exclusively find each other and do not pair with their homoeologues (Sears 1976).

There are several genes involved in chromosomes pairing located on chromosomes 5AL, 5DL, 5AS, 3AS, 3AL, 3BL and 3DL but the two major pairing homoeologous (*Ph*) genes are *Ph1* and *Ph2* (Riley et al. 1959, Sears 1976, Moore 2002). Using the synteny of wheat, rice and barley, candidates for *Ph2* (located on 3DS) have recently been identified (Sutton et al. 2003) but until recently it was unclear if *Ph1* (located on 5BL) was a single gene, several genes or a heterochromatin region with an epigenetic effect (Moore 2002). The locus *Ph1* has the strongest effect on chromosome pairing.

1.6.3 What is known about the *Ph1* locus

The effect of the *Phl* mutant might be ancestral or it is possible that the current 5B allele was not present in the original hybridization but was a mutation that arose and was then selected because it conferred increased fertility (Martinez-Perez et al. 2000). *Ph1* has multiple effects during pre-meiotic interphase, meiosis and chromosome conformations.

Ph1 may have an effect on spindle formation

Ph1 affects the microtubule system (Avivi et al. 1970, Avivi and Feldman 1973, Ceoloni et al. 1984, Gualandi et al. 1984). Cochicine block the microtubules synthesis and therefore distub the spindle formation. In the presence of ATP, the spindle sensitivity to colchicine in plants with two doses of *Ph1* was increased to a greater extent than in plants with zero *Ph1*. (Avivi et al. 1970). Similarly the antitubulin vinblastine disrupts the spindle formation and genotypes lacking *Ph1* were found to be more sensitive to the treatment. Moreover, this sensitivity to vinblastine was in correlation between dosage of *Ph1* (0, 2, and 4) and plants having two or four doses of *Ph1* were found less sensitive to vinblastine (Avivi and Feldman 1973).

Ph1 has an effect on centromere pairing

Following theses observations on the *Ph1* effect on the spindle formation, Vega and Feldman (1977) study the frequency of centromeres misdivisions in plants having different doses (gene copy) of *Ph1*. They showed that in the presence of the normal two doses of *Ph1*, the frequency of transverse breakage (misdivision) of the centromere of univalent chromosomes was high in both first and second meiotic divisions; whereas with zero dose of the gene, this frequency was drastically reduced. The results suggest that *Ph1* is a *trans*-acting gene affecting centromere-microtubules interaction (Vega and Feldman 1998a). In pre-meiotic interphase, pachytene, metaphase I and anaphase I, there is a difference in centromeric structure between wheats carrying different pairing control genes (Aragon-Alcaide et al. 1997a).

In high homoeologous pairing wheats (absence of *Ph1*) the diffuse hybridization sites observed during pre-meiotic interphase remain as diffuse sites at pachytene through to anaphase I. In contrast, in low homoeologous pairing wheat (presence of *Ph1* and *Ph2*), the hybridization sites at premeiotic interphase remain as dense sites at pachytene, metaphase and anaphase I. This diffuse structure may result in increased accessibility of the centromere, exposing regions of the centromere that are more conserved in structure, thus reducing the stringency of pairing at the centromere (Aragon-Alcaide et al. 1997a). It was then hypothesis that *Ph1* regulated MI chromosome pairing by regulating centromere-mediated chromosome alignment before the onset of meiosis. To test this hypothesis, Dvorak and Lukaszewski (2000) made a 2BL.2RL homoeoisochromosome and analysed its MI pairing in the presence and absence of the *Ph1* locus. The two arms were permanently associated in the homoeoisochromosome via a common centromere. However they paired with each other at MI in the absence of *Ph1*, but they never paired in the presence of *Ph1*. This was concluded that it is unlikely that *Ph1* predetermines MI pairing between homoeologous chromosomes solely by controlling premeiotic association of centromeres but determines the chromosome pairing pattern at MI by scrutinizing homology across the entire chromosome (Dvorak and Lukaszewski 2000). Nevertheless, if *Ph1* does not regulate MI chromosome pairing by regulating centromere-mediated chromosome alignment before the onset of meiosis it was shown that *Ph1* acts on centromere association both meiotically and somatically. In this case, non-homologously associated centromeres separate at the beginning of meiosis in the presence, but not the absence, of *Ph1* (Martinez-Perez et al. 2001). Moreover, as the telomeres begin to cluster, the 42 chromosomes of hexaploid wheat associate via the centromeres into seven groups (homologous and homoeologous centromerefrom each of the seven groups of chromosomes). The clusters are resolved in presence of *Ph1*, leaving chromosomes as homologous pairs (Martinez-Perez et al. 2003). *Ph1* may act on premeiotic alignment of homologues and homeologues (Feldman et al. 1966, Feldman 1993, Vega and Feldman 1998b). Because *Ph1* affects the centromere association stability, it might also affect the timing of formation of the telomeric bouquet which occurs after clustering of the centromeres. Because this timing would be delayed, synapsis would be delayed (Martinez-Perez et al. 2000).

Ph1 has an effect on Karyotype stability

In an analysis of wheat hybrids with wild relatives, Vieira et al (1991) have shown evidence of chromosome instability link to the dose of *Ph1* (number of gene copy). In hybrids containing 2n= 41 chromosomes (only one copy of 5BL), all cells consistenly contained 2n= 41 chromosomes, and no difference in morphology was observed during development. In contrast, in the hybrids containing 2n=42 chromosomes (two copies of 5BL), most of the cells were still having 2n=42 chromosomes, but 19% to 40% carried from 2n=6 to 2n=44 chromosomes, and showed disturbances in all phases of the cell cycle leading to final failure in plant development. It is suggested that *Ph1* regulates chromosome stability in the somatic cells of those hybrids (Vieira et al. 1991). More recently, karyotypic analysis of wheat lines with different genotypes for the homoeologous-pairing loci *Ph1* showed chromosomal rearrangements mainly affecting the A and D genomes in all plants of allohexaploid wheat (AABBDD) lacking *Ph1* activity. Thus, inactivity of the *Ph1* locus induces karyotypic instability in wheat (Sanchez-Moran et al. 2001).

Ph1 has an effect on pairing and synapsis

Hexaploid wheat behaves as a diploid with 21 bivalents observed at metaphase 1 (Riley and Chapman 1958) and *Ph1* is controlling that only homologous chromosomes (as opposed to homoeologous chromosomes) paired and form multivalents at metaphase 1 (Sears 1976). The *Ph1* locus may function by actively promoting homologous pairing, and not simply by preventing homoeologous pairing (Martinez-Perez et al. 1999). Synapsis studies of polyploid wheat have revealed that in wild-type wheat, only 5 out of 42 chromosomes engage in multiple associations during the synapsis process, while in the absence of *Ph1*, more than 19 chromosomes can engage in such multiple associations (Holm 1986, 1988). Similarly in the tetraploid wheat *Triticum turgidum* (AABB), the number of synaptonemal complex (SC) bivalents varied through prophase I according to the dose of Ph1. The means of synaptonemal complex (SC) bivalents and of the different SC multivalent associations at mid-zygotene was relatively high in all lines and was similar in two and zero doses of *Ph1*. However, these means changed little with the progression of zygotene but decreased at pachytene because of the transformation of multivalents into bivalents. Multivalent correction was more efficient in the presence than in the absence of *Ph1*. The four

doses of *Ph1* genotype showed a higher number of SC bivalents at mid-zygotene, and the frequency of multivalents decreased progressively throughout zygotene and pachytene (Martinez et al. 2001).

Ph1 has an effect on recombination

Ph1 could affect recombination (Martinez-Perez et al. 2000) and chiasmata pairing between homoeologous chromosomes at metaphase I (Dvorak and Lukaszewski 2000). Chromosome 1A (m) of *Triticum monococcum* is closely homeologous to *T. aestivum* chromosome 1A but recombines with it little in the presence of *Ph1*. In the absence of *Ph1*, the two chromosomes recombine as if they were completely homologous (Luo et al. 1996). *Ph1* ensures that pairing and recombination are restricted to true homologues, and it acts both meiotically and somatically (Aragon-Alcaide et al. 1996, Martinez-Perez et al. 2001).

***Ph1* has an effect on chromosome organization**

Ph1 affects the chromatin organization of homologous chromosomes which leads to their intimate alignment (Dvorak and Lukaszewski 2000, Prieto et al. 2004b). *Ph1* affects the centromere structure and their association. In presence of *Ph1*, the centromeres show a diffuse site while in absence of *Ph1*, the centromere remains as densed site. Thus, *Ph1* is affecting the centomere structure (Aragon-Alcaide et al. 1997a). *Ph1* affects the telomere region structure. Rye subtelomeric regions behaved differently in wheat/rye hybrids lines, containing 28 homoeologues consisting of 21 wheat chromosomes and 7 rye chromosomes. In the presence of *Ph1*, 7 groups of associated wheat and rye centromeres are formed as a prelude to meiosis. At the telomere bouquet the 7 rye subtelomeric regions are compacted. In contrast, in the absence of *Ph1*, 7 groups of wheat centromeres and 7 distinct groups of rye centromeres are formed prior to meiosis. At the telomere bouquet the 7 rye subtelomeric regions are elongated (Aragon-Alcaide et al. 1997a, Prieto et al. 2004a). *Ph1* affects the chromosome structure. By analysing the behaviour of a wheat line in which a rye segment that covers 15% of the distal chromosome arm has been substituted for the equivalent region in the 1D pair of wheat chromosomes in presence and absence of Ph1. Prieto et al (2004) showed that in presence of Ph1, both honologues elongated synchronously at the telomere bouquet, while in absence of Ph1, elongation of the two homologues is not syncrhonized.

The transfer of useful traits such as disease, drought and salt tolerance from wild species into wheat is a difficult and complex process. Plant breeding crossing techniques rely on the recombination process of the chromosomes, to introduce the gene of interest in the genotype. When wide crossing between wheat and its wild relatives, the chromosome carrying the interresting gene does not recombine with the hosting homologue, thus there is no introduction of the resistance gene into the hosting genome. This is particularly true in wheat, thus understanding of the mechanism of *Ph1* could provide solutions for creating new wheat varieties by plant breeders.

1.7 Investigation of chromosome pairing in wheat

Chapter 2 will discuss the mapping of the *Ph1* locus and how cell biology was used to visualized the genomic information and confirm the new map.

In chapter 3, I will explain how I investigated the *Ph1* effect on "chromatin remodelling" in experiments using wheat-rye translocation lines. For this study different wheat-rye translocation lines, well known in plant breeding to behave as normal wheat, were analysed to define the behaviour and remodelling of the rye heterochromatin knobs, to understand the effect remodelling might have on pairing and recombination in wheat in the presence of the *Ph1* locus. Chomosomes are composed of chromatin and change their conformation during meiosis showing a structure more relaxed or more condensed. Although the term "chromatin remodeling" is used for the regulated alteration of chromatin structure (covalent modification of histones or by the action of ATP-dependent remodeling complexes) at the nucleosome level, in this study, I will use the term chromatin remodelling for the global changes in the chromosomes. In this study, chromatin remodelling refers to chromosome remodelling or the change of the global structure of the chromosome during meiosis.

In chapter 4, I will describe a new possible approach for the *Ph1* project. Based on the new information from genomics and cytology, I will discuss how proteomics can help to understand the molecular mechanism of *Ph1* and more generally of meiosis.

In conclusion a model for the *Ph1* mechanism and investigation methods for its understanding will be discussed.

Chapter 2

Molecular characterization of *Ph1:*

A major chromosome pairing locus in polyploid wheat

Abstract

"The foundation of western civilization owes much to the high fertility of bread wheat, which results from the stability of its polyploid genome. Despite possessing multiple sets of related chromosomes, hexaploid (bread) and tetraploid (pasta) wheat both behave as diploids at meiosis. Correct pairing of homologous chromosomes is controlled by the *Ph1* locus. In wheat hybrids, *Ph1* prevents pairing between related chromosomes. Lack of *Ph1* activity in diploid relatives of wheat suggests that *Ph1* arose on polyploidization. Absence of phenotypic variation, apart from dosage effects, and the failure of ethylmethane sulphonate treatment to yield mutants, indicates that *Ph1* has a complex structure. Here we have localized *Ph1* to a 2.5 megabase interstitial region of wheat chromosome 5B containing a structure consisting of a segment of subtelomeric heterochromatin that inserted into a cluster of cdc2-related genes after polyploidization. The correlation of the presence of this structure with *Ph1* activity in related species, and the involvement of heterochromatin with *Ph1* and cdc2 genes with meiosis, makes the structure a good candidate for the *Ph1* locus."

Simon Griffiths, Rebecca Sharp, Tracie N. Foote, Isabelle Bertin, Michael Wanous, Steve Reader, Isabelle Colas & Graham Moore
Nature 439(7077): 749-52.

My contribution in this work was to use *in situ* hybridization to confirm the genes position expected from the mapping analysis.

2.1 Introduction

Hexaploid wheat (*Triticum aestivum*) contains three genetically related genomes (AABBDD) and the tetraploid wheat (*Triticum durum*) contains two related genomes (AABB). In these polyploids, despite the close homology between the genomes, only true homologues are paired during meiosis, which is essential for successful segregation and maintenance of a stable genome. *Ph1* (Pairing homoeologous 1), located on the chromosome 5BL, is the major locus for genetic control of pairing specificity, ensuring that only homologues pair during meiosis (Riley et al. 1959).

Two mutants permiting the pairing of homoeologous chromosomes have been characterized, and consist of a deletion on 5B in hexaploid wheat (*Ph1b*) (Sears 1977) and tetraploid wheat (*Ph1c*) (Giorgi 1978). Homoeologues 5A, 5B, and 5D contain a very similar gene order but *Ph1* activity is specific to 5B. The effect of *Phl* might be ancestral or alternatively it is possible that the current 5B allele was not present in the original hybridization event but was a mutation that arose and was then selected because it conferred increased fertility (Martinez-Perez et al. 2000). It was possible to produce an interstitial deletion mutant of *Ph1* by X-ray irradiation (*Ph1b)* (Sears 1977, Giorgi 1978), but it has not been possible to make a *Ph1* mutant by point mutation by, for example, ethylmethane sulphonate (EMS) treatment (Wall et al. 1971). This suggests that this locus is likely to be complex and to be the result of chromosome arms re-organization after polyploidization (Sanchez-Moran et al. 2001, Sutton et al. 2003).

Increasing the dose of *Ph1* by a factor of two (two doses of chromosome 5B) leads to a stronger phenotype (Feldman 1966); therefore if *Ph1* was a single gene effect, this solution would probably have been selected during wheat evolution. *Ph1* may affect chromatin organization of homologous chromosomes which leads to their intimate alignment (Prieto et al. 2005), and this mechanism may affect both euchromatin and heterochromatin (Martinez-Perez et al. 2001). The *Ph1* effect is well described but little is known about its molecular mechanism.

2.2 Molecular characterization of *Ph1*

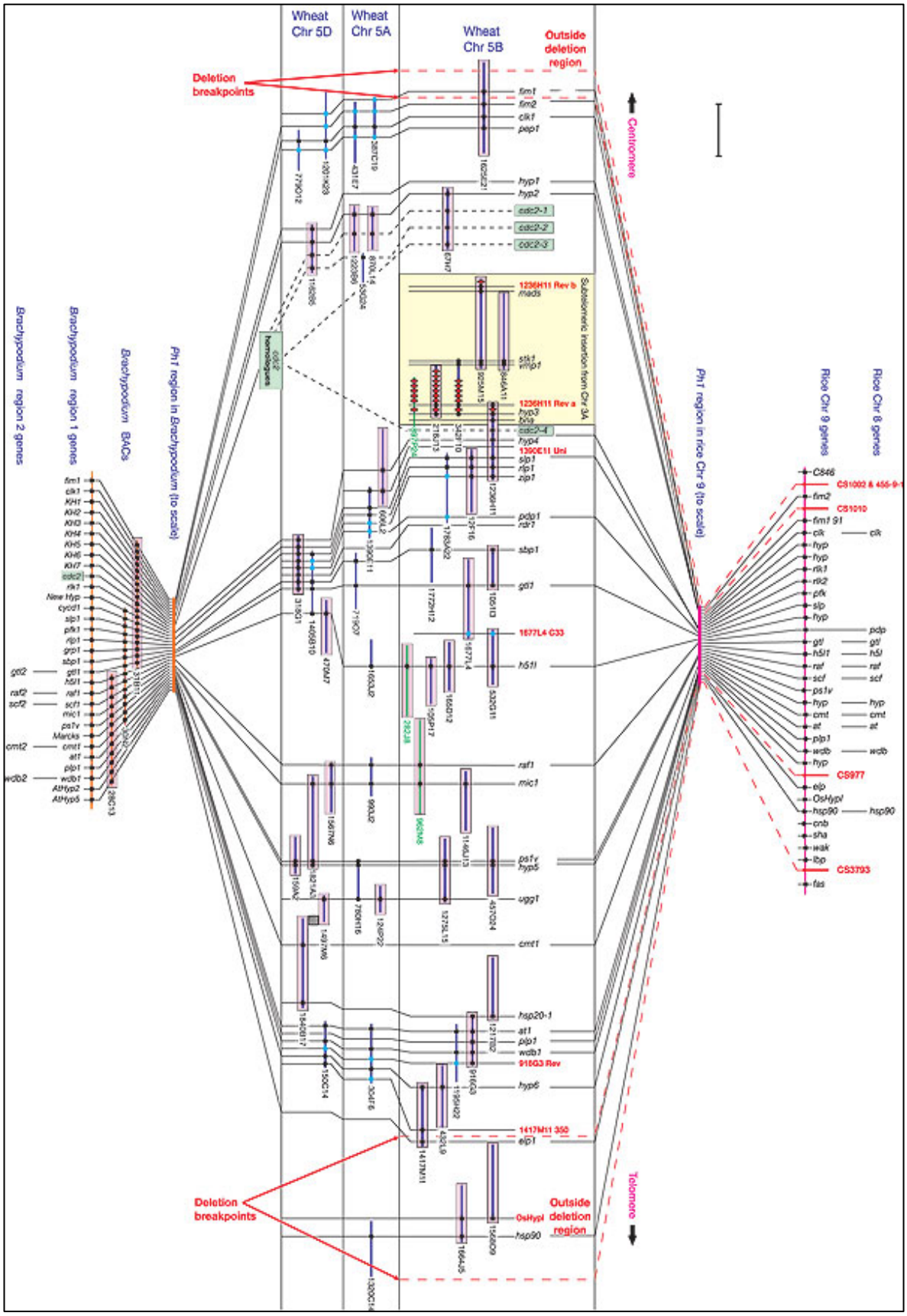
The Moore group have been working for many years on *Ph1* mapping, and devised a two part strategy to map this locus (Moore et al. 1993, Kurata et al. 1994, Sasaki 2005).. The first part was based on synteny comparison of the hexaploid wheat genome with rice and *Brachypodium sylvaticum* (Foote et al. 2004). This revealed the gene content of the *Ph1* locus, and provided enough makers to maximum cover the wheat region. The second part of this study was based on deletion lines to dissect physically the *Ph1* locus, using 5 new deletion lines which overlapped the *Ph1b* deletion (Roberts et al. 1999). This defined a smaller candidate region for this locus, equivalent to a 140-kb region of the rice chromosome 9 (Griffiths et al. 2006).

42 markers were used to screen a wheat BAC library of hexaploid (Allouis et al. 2003) and tetraploid wheat (Cenci 2003). 36 genes were found within the smallest *Ph1* deletion, of which 25 genes were collinear with their orthologues on rice chromosome 9 and/or *Brachypodium* region 1. A segment unique to this region was found to contain a cluster of cdk (cyclin-dependent kinase) related genes (Figure 12).

Figure 12: The Full *Ph1* region annotation

BAC tiling path and annotated gene content of the *Ph1* region and its equivalent region in rice chromosomes 9 and 8 and Brachypodium regions 1 and 2

Pink vertical lines represent part of rice chromosomes 9; orange vertical lines represent Brachypodium chromosome region 1. Co-linearity of rice and Bracypodium markers with markers on a part of wheat chromosomes 5B, 5A and 5D is shown with horizontal and diagonal black lines; red dashed lines show the location of the fast neutron-induced breakpoints on chromosome 5B. Blues bars represent hexaploid wheat BACs; green bars show tetraploid wheat BACs (*T.durum*). Pink boxes indicate sequenced BACs. Gene and BACs names are indicated in black, whereas other marker names are in red. Black circles indicate the presence of markers from sequence data or by Southern analysis; blue circles show presences of marker by colony hybridization alone; no circle indicates not tested. The yellow box represents the subtelomeric insertion from wheat chromosome 3A. Red diamonds represent tandem repeats. The high level of homology between cdc2-type genes on different BACs means that homologues cannot be distinguished from paralogues; they are therefore connected by black dashed lines. The hashed box indicates overlap between BACs based on fingerprint only. Centromeres and telomeres are represented with a little arrow indicating the orientation of the chromosome arm.


Wheat Chr 5D
Wheat Chr 5A
Wheat Chr 5B
Outside deletion region
Deletion breakpoints
Centromere
Telomere
Brachypodium region 2 genes
Brachypodium region 1 genes
Brachypodium BACs
Ph1 region in Brachypodium (to scale)
Ph1 region in rice Chr 9 (to scale)
Rice Chr 9 genes
Rice Chr 8 genes
fim1
fim2
clk1
pap1
hyp1
hyp2
cdc2-1
cdc2-2
cdc2-3
1236H11 Rev b
mads
stk1
vrn1
1236H11 Rev a
hyp3
hyp4
cdc2-4
1300E11 Uni
slp1
rip1
zip1
pdp1
rdr1
sbp1
gti
1677L4 C33
h5l1
raf1
mic1
ps1v
hyp5
cmt1
hsp20-1
at1
plp1
wdb1
916G3 Rev
hyp6
1417M11 352
elp1
OsHypI
hsp90
cmt2
wdb2
gti2
raf2
scf2
CS977
CS3793

Based on sequence homology and function, it seems that cdc2 is the most closely related cdk to those at the *Ph1 l*ocus (Figure 13), and it has been found that at least one of its members (cdc2-4) is 5B specific. Cdc2 coupled with cyclin B form a complex called M-Phase Promoting Factor (MPF) which regulates G1/S and G2/M transition during mitotic and meiotic division (Doree and Hunt 2002). Cdc2 is recruited by Topoisomerase II leading to extensive chromatin remodelling (early prophase chromosome structure) (Escargueil et al. 2001). Similarly, *Ph1* is involve in chromatin remodelling (Prieto et al. 2004a), thus, Cdc2 genes might be good candidates for *Ph1*.

The cluster has an insertion of a large region which has no equivalent on 5A and 5D. This additional segment is present in a sub-telomeric region of 3AL, and was probably inserted during the *Ph1* locus evolution. The locus also contains two tandem arrays of a 2.3kb repeating sequences. These sequences are not found on 5A and 5D, but the Southern analysis found a number of similar tandem array of those sequences on the chromosome 3B. To confirm this location, I decided to use in situ hybridization to investigate the structure of the heterochromatin region (Figure 13), by making probes to this repeat sequence.

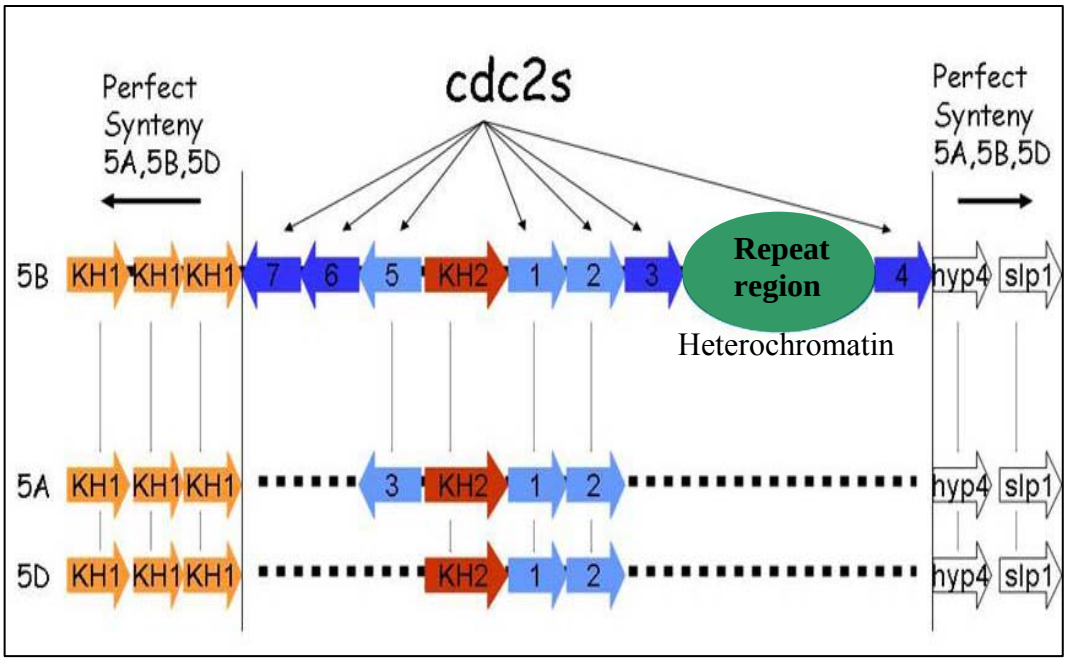


Figure 13: *Ph1* Region

There is good synteny between the region of 5A, 5B and 5D. However, one region is specific to 5B and is assigned as the *Ph1* Locus. The region consists of a cluster of Cdc2-like genes, interrupted by a large region which has no equivalent on 5A and 5D. This heterochromatin region consists of two tandem arrays of a 2.3kb repeating sequences which has an equivalent on the subtelomeric region of 3B. The heterochromatin motif might have been translocated to 5B chromosome from the 3A chromosome during polyploidization

2.3 *In situ* Hybridization of the *Ph1* Locus

2.3.1 Materials and Methods

Material preparation

Seeds of Chinese Spring cultivar were germinated for 3 days on wet filter paper in the dark at room temperature. Root tips were cut and placed overnight in the fixative Ethanol/Acetic acid (3:1) in a 1.5ml tube (Stack S.M. et al. 1977). Fixed root tips can be stored for 2 weeks in the fixative solution in the fridge. They were transferred into 0.2M HCl for 1 hour, followed by a few minutes in 45% acetic acid before use. On a clean polylysine-coated slide (or APTES coated), the material was dissected by scraping the tissue to make a longitudinal incision with a sharp knife. The tissue was macerated for a few minutes and a coverslip is placed on top of it and squashed. Slides were placed in liquid nitrogen, and coverslips are removed (popped off). Slides were dried overnight at room temperature, and stored at -20°C until use.

Probe preparation

The product was a heterochromatin sequence (Griffiths et al. 2006), amplified by PCR from the BAC 218J13 (Appendix 8) under the following conditions: 35 cycles of 1 minute at 94ºC, 30 seconds at 55ºC and 1 minute at 72ºC, followed by 5 minutes at 72ºC (Aragon-Alcaide et al. 1996). Probes were labelled either with digoxygenin-11-dUTP (Boeringer Mannheim) or biotin-16-dUTP (Boeringer Mannheim) by nick translation (Schwarzacher T. and P. 2000) (Appendix 7). All PCR products and probes were monitored on 1% Agarose gels, to assess the quality and the size of the product. Incorporation of the label was checked by dot blots (Schwarzacher and Heslop-Harrison 2000) using the following protocol. The probes were transferred to a hybond N+ Membrane previously incubated in TBS (0,1M Tris-HCl pH 7,5, 0,15M NaCl) and air dried. The membrane was incubated in a blocking solution (3% of BSA in TBS) for 15 minutes. The antibody solution was applied for 30 minutes at 37°C with the appropriate antibody (anti-biotin/alkaline phosphatase and/or anti-digoxigenin/alkaline phosphatase), then the membrane was rinsed, first in TBS and then in the following Tris buffer (0,1M Tris-HCl pH 9,5; 0,01M NaCl; 0,05M $MgCl_2$).

It is developed in the Tris buffer containing 2µl NBT (4-nitro blue tetrazolium chloride) and 1.5 µl BICP (5-bromo-4-chloro-2-indolyl-phosphate) per ml in the dark.

In situ hybridization

The protocol is adapted from Aragon-Alcaide et al (1997a) and Schwarzacher and Heslop-Harrison (2000) (Appendix 9). Dry sections were dehydrated in a series of 30, 50, 70 and 100% methanol. Then, tissues were treated with a mixture of 1% cellulase, 0.5% pectolyase in 1X TBS (0.1M Tris-HCl, pH 7.5; 0.15M NaCl) for 30 min at room temperature, washed in 1X TBS for 10 min, and dehydrated again in a series of 30, 50, 70 and 100% methanol. Hybridization mix, composed of 50% deionised formamide, 20% dextran sulfate, 1X PIPES/EDTA buffer (100:10), 0,3M NaCl, 500ng of salmon sperm blocking DNA, and 50 ng of each probe, was denatured for 8 min at 100°C and immediately placed on ice to cool down for 5 minutes. The probe mixture was applied onto the tissue, covered with a plastic coverslip and put in a modified Omnislide thermal-cycler (Hybaid Ltd). Chromosome DNA was denatured at 78°C and hybridisation is performed at 37 °C overnight. Slides were washed at 42°C in 20% formamide, 0.1 X SSC for 10 min, then in 2 X SSC for 10min. This was followed by a wash at room temperature in 2 X SSC for 10 min, and in 4 X SCC, 0.2% Tween 20 for 10 min. A protein blocking solution, consisting of 5% BSA in 4 XSSC, 0,2% Tween 20 was applied for 5min in a humidity chamber at room temperature. Digoxygenin labelled probes were detected by an anti-digoxygenin FITC-conjugated antibody and biotin labelled probes were detected with extravidin-cy3. Slides were counterstained in 1µg/ml DAPI (4',6-diamino-2-phenyl-indole) and mounted in Vectashield (H-1000) medium.

Microscopy

Images of the chromosome spreads were taken with a Nikon Eclipse E600 fluorescent microscope connected to a Hamamatsu CCD camera, and processed with the public domain program ImageJ. (Project developer Wayne Rasband, http://rsbweb.nih.gov/ij/index.html)

2.3.2 Results and discussion

The picture of the chromosome spread clearly shows two strongly hybridized interstitial sites on a pair of homologues and two weaker hybridised sites in the subtelomeric regions of another pair of homologues. When paired as a single dot, the two weaker sites were closer to the telomere bouquet and at constant distance. It is concluded that the two stronger hybridized sites correspond to the tandem repeat within the locus on 5B, while the two weaker hybridized sites correspond to the array of the subtelomeric region on 3B (Figure 14a). In meicoytes (Figure 14b-f), the four dots are clearly visible before pairing and at the telomere bouquet. However, in the 5B deletion lines, only two dots from the subtelomeric region on 3B are visibles. The telomere cluster formation is not affected in the deletion lines, thus, *Ph1* does not affect the telomere bouquet.

The hexaploid wheat is originated from two independent polyploidization events (Gu et al. 2006). The first event involved the hybridization of *Triticum urartu* (A genome) and *Aegilops speltoides* (S genome, ancestor of the B genome). This event resulted in the tetraploid wheat (*T. turgidum*, genomes AABB) (Dvorak et al. 1993, Blake et al. 1999). The second event, involved the hybridization of the diploid *Aegilops tauschii* (D genome) with the allotetraploid (AABB) to form the hexaploid wheat (AABBDD) (Feldman et al. 1997). The S genome is also the ancestor the G genomes of *Triticum timopheevi* (AAGG) and *Triticum araraticum* (AAGG), which appear after the *T. dicoccoide* (AABB). If the identified region is the *Ph1* locus, it should be present in all related genomes having *Ph1* activity.

Southern analysis of this tandem repeat on the tetraploid wheat and on the *Ph1c* associated deletion has shown that the segment containing the repeat sequences and the cdc2-4 pseudogene (5B specific) is present on 5B (Griffiths et al. 2006). The restriction profiles of the tetraploid and hexaploid wheat also reveals a high level of conservation of the BACs carrying the tandem repeat, in both polyploids. Additionally, the S genome does not possess *Ph1* activity, but the B and G genomes of these tetraploids do. PCR assays that are 5B specific for the cdc2-4 pseudogene and 2.3-kb repeat confirm the presence of the pseudogene sequence in all the tetraploids, and its absence in the in the putative B genome progenitor. All together may suggest that *Ph1* may arise with polyploidisation.

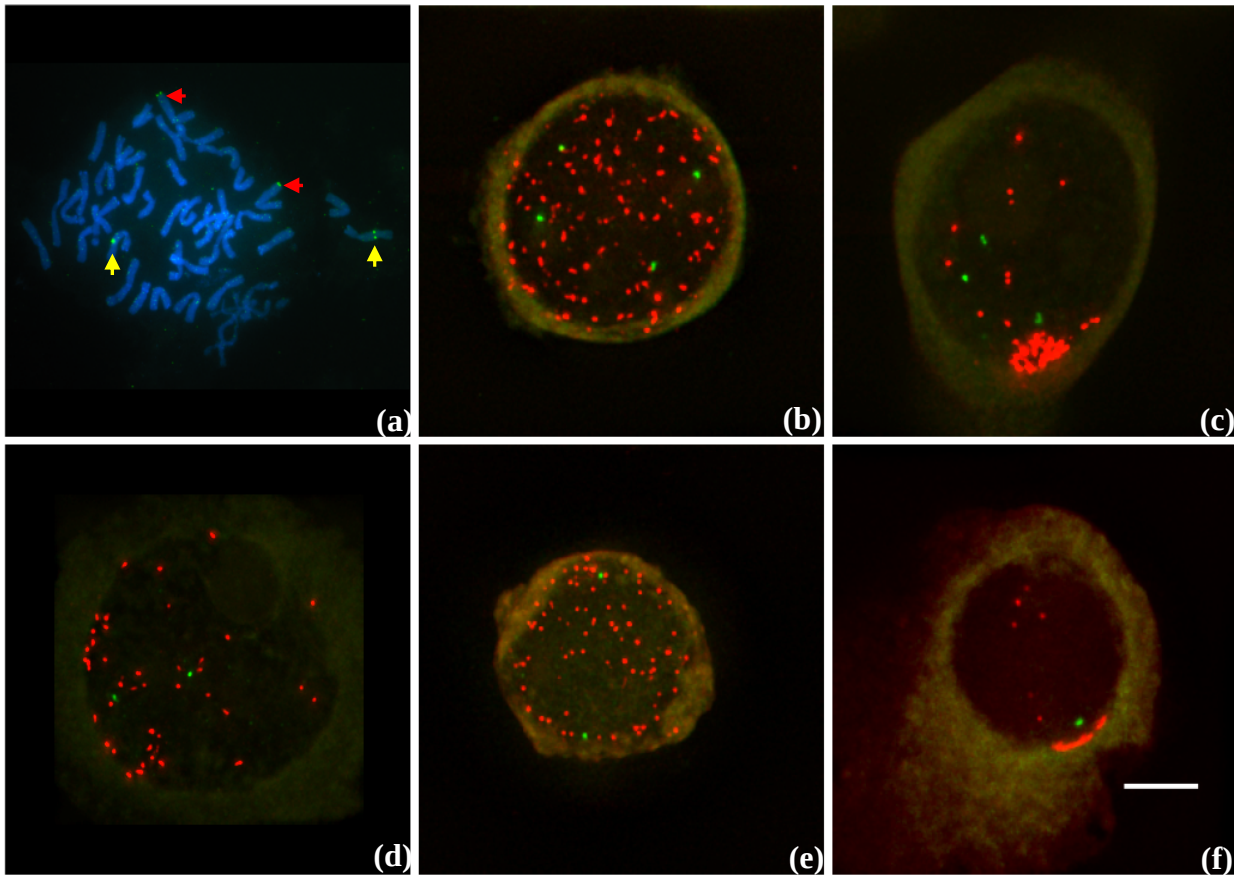


Figure 14: *In situ* hybridization of the tandem repeat on mitotic chromosome and in meiocytes (Griffiths et al. 2006)

Telomeric repeat probe labelled red; *Ph1* repeat probe labelled green. *(a)* Spreads from hexaploid wheat root meristems reveal two strongly hybridizing interstitial (Yellow arrows) and two weaker hybridizing subtelomeric locations (red arrows). (b, c, d) Line carrying 5B chromosome. The four dots are clearly visible in the meiocytes before pairing (b). At the telomere bouquet stage (c) the two 3BL subtelomeric repeat sites are paired as a single dot always close to the telomere bouquet; the two 5B repeat sites are unpaired, the fours dots are paired two by two after the telomere bouquet is dissolved (d). (e,f) 5B deletion line (Ph1b). Only two dots are visibles (3B subtelomeric repeat) in the meiocytes before pairing (e). At the telomere cluster stage, the 3B subtelomeric repeats are paired (f). Scale represents 10-15 µM.

2.4 Conclusions

The *Ph1* locus is a cluster of Cdc2-like genes, interrupted by a large region which has no equivalent on 5A and 5D. This heterochromatin region consists of two tandem arrays of a 2.3kb repeating sequences which has an equivalent on the subtelomeric region of 3BL. (Griffiths et al. 2006). Al-Kaff et al have further restricted the locus to a cluster of cdk-like genes and confirmed the presence of the 3B sub-telomeric heterochromatin segment inserted within this cdk-like cluster on 5B (Al-Kaff et al. 2008). Morever, 5B contains the heterochromatin insertion, but also contains seven copies of cdk-like gene whereas 5A and 5D contain five and two cdk-like copies respectively. Furthermore, this analysis shows that in the presence of the *Ph1* locus, fives genes (including two pseudogenes) are expressed from the 5B locus, when 5A and 5D are only slightly expressed. In the *Ph1* deletion line, when 5B can not be expressed, the level of expression of cdk-like genes increased from 5A and 5D. Moreover, the two pseudogenes *cdk-like B6* and *cdk-like B7* are also expressed. One explanation would be that the pseudogenes transcripts may be non-coding and involve in a fine regulation by small RNA interference repressing the expression from 5A and 5D. Interrestingly, the heterochromatin region is inserted between the two pseudogenes, thus, are the pseudogene expressions, a consequence of this insertion, and in this case, what could be the role of the heterochromatin insertion in the locus? A further analysis can be done by RNA in situ of the heterochromatin region to visualize if there is any transcript from this region before and during meiosis.

Ph1 is acting on the chromosome by changing the chromatin conformation at the onset of meiosis, allowing the pairing of the homologues from the telomeres. Thus it seems intuitively possible that a failure in remodelling would lead to incorrect pairing and therefore low recombination. However there was no previous evidence for this hypothesis. In the next chapter, I will show how I answered this question by using in situ methods to visualize the behaviour of different wheat-rye translocation lines

Chapter 3

Effective chromosome pairing and recombination

requires chromatin remodelling at the onset of meiosis

Abstract

"During meiosis, homologous chromosomes (homologues) recognize each other and then intimately associate. Studies exploiting species with large chromosomes reveal that chromatin is remodelled at the onset of meiosis before this intimate association. However, little is known about the effect the remodeling has on pairing. We show here in wheat that chromatin remodeling of homologues can only occur if they are identical or nearly identical. Moreover, a failure to undergo remodeling results in reduced pairing between the homologues. Thus, chromatin remodeling at the onset of meiosis enables the chromosomes to become competent to pair and recombine efficiently**."**

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My contribution to this work represents most of the work presented in this chapter which includes the *in situ* of the wheat-rye translocated lines, their analysis and interpretation. I also made the figures and wrote the paper in collaboration with Graham Moore and Peter Shaw. The work on the wheat-rye hybrids was previously published by Pilar Prieto. The recombination work was previously published by Wolfgang Spielmeyer and Rohit Mago. Mike Wanous contributed in crossing the lines provided by Wolfgang Spielmeyer'group.

3.1 Introduction

During meiosis, homologous chromosomes (homologues) recognise each other and then intimately associate (Zickler and Kleckner 1998). Synapsis is a process in which the two homologues align with each other, and the synaptonemal complex (SC) is generated between them (Kleckner 1995). When synapsis is completed, the two homologues can exchange DNA strands during meiotic recombination and subsequently remain physically linked via chiasmata after the SC disassembles. At Prophase I, the telomeres cluster at the periphery of the nucleus to form the telomere bouquet (Bass et al. 1997) to bring the sub-telomeric regions of homologous chromosomes together and initiate homologue pairing and synapsis (Bass 2000).

The remodelling of the chromatin is an important regulatory system through the cell cycle (Meyer 2001, Hsieh and Fischer 2005, Falbo and Shen 2006). Studies of species with large chromosomes reveal that chromatin is remodelled at the onset of meiosis prior to this intimate association (Dawe et al. 1994, Dawe 1998). These changes of chromatin structures (remodelling events) were visualized on subtelomeric heterochromatin knobs in Lilium and maize (Bass et al. 1997, Dawe 1998). In wheat, the centromeres change conformation at the beginning of meiosis (Aragon-Alcaide et al. 1997a, Prieto et al. 2004a).

In wheat, at the telomere cluster, homologues undergo a synchronized chromatin remodelling from their telomeres, and intimately align with each other by a "zipping up" process (Maestra et al. 2002, Prieto et al. 2004a). *Ph1* is known to be a locus controlling the specificity of homologue pairing in wheat, ensuring that only true homologues as opposed to homoeologues pair (Riley et al. 1959). Thus, the lack of chromosome synapsis in the *Ph1b* mutant could result from abnormal chromatin decondensation and organization (Maestra et al. 2002). More recently, a study of a wheat-rye hybrid line (in which there are no homologues) showed that in the presence of *Ph1*, there is no remodelling of the heterochromatin. However, in absence of *Ph1*, the homoeologous chromosomes remodel (Prieto et al. 2004a). Despite these observations, little is known about the effect the remodelling has on pairing and recombination.

To address this question, I used a number of lines (in presence of *Ph1*) generated by Wolfgang Spielmeyer (CSIRO, Canberra, Australia), in which the long arms of a pair of wheat chromosome 1B (1BL) have been substituted by the small arms of the rye chromosome 1 (1RS) (Berzonsky and Francki 1999). Wheat cultivars containing a 1BL.1RS wheat-rye translocation have been used for many years in plant breeding program as they usually carry important resistances genes (Berzonsky and Francki 1999, Ko et al. 2002). Therefore, it is reasonable to assume that the translocated chromosomes behave like the rest of the wheat chromosome. Rye chromosome can be identified by heterochromatin banding pattern (Verma and Rees 1974, Darvey and Gustafson 1975, Weimarck 1975). In wheat rye translocation line 1BL.1RS, the chromosome 1RS of 'King II' rye and 'Petkus' rye were easily distinguished from the wheat chromosomes by the presence of prominent telomeric C-bands on each of the rye chromosome arms (Singh et al. 1990). However, the the chromosome 1Rs from imperial did not show a major C-band in the sub-telomeric region suggesting a smaller heterochromatin region in comparison to its homologues King II and Petkus (Singh et al. 1990).

In this study, the rye segments are called identical, similar or distinct in sequence and lenght in the sub-telomeric heterochromatin. Two lines (identical) carry homologues with identical heterochromatin regions, both arms being derived from the same rye variety, either King II or Petkus (Figure 12e) (Lawrence 1969, Mettin 1973). The third wheat line (distinct) carries a pair of homologues that differ in the size of their subtelomeric heterochromatin regions, one arm being derived from the rye variety Petkus and the other from Imperial (Figure 12a) (Merker 1982, Koebner et al. 1986).The fourth line (similar) is the crossing result between the line one and two, and carries homologues with similarly sized (same 1RS length) but different heterochromatin regions, one arm from the variety King II and the other from Petkus (Figure 12i).

The chromatin remodelling was visualized by 3D fluorescence in situ hybridization using a probe to the rye heterochromatin in meiocytes of these three lines at different stages of meiosis I.

3.2 Materials and methods

Plant materials

The following wheat-rye translocation lines were exploited in the present study: Gabo 1BL-1RS (Imperial 1RS) x Veery 3 1BL-1RS (Petkus1RS), CS/Holdfast 1BL-1RS (King II 1RS) x Federation/Kavkas 1BL-1RS (Petkus 1RS), CS/Holdfast 1BL-1RS (King II 1RS), Federation/Kavkas 1BL-1RS (Petkus 1RS) in a *Ph1* background and Chinese Spring/*Secale cereale* cv.Petkus F1 hybrids with and without the *Ph1* locus (*ph1b* deficiency). After 2-5 days of germination, seedlings were vernalized for 3 weeks at 4°C. Plants were transferred in a controlled environment room and have been grown under the following conditions: 16 hours under light at +20°C; 8 hours in the dark at +15°C; Constant humidity level 85%.

Meiosis staging

Meiosis of harvested plants (Appendix 1) was monitored in anther squashes by aceto-carmine staining using light microscopy (Stack S.M. et al. 1977). Wheat spikes were collected by removing the covering leaves. One anther from the middle spikelet (later stage of meiosis), was spread onto a slide, stained with aceto-carmine, squashed and then checked on a microscope (10X, and 40X magnification) to monitor the stage of meiosis. Earlier stages of meiosis will then be found in spikelets further away from the middle spikelet.

Tissue fixation and wax embedding

Material was fixed (Appendix 2) by vacuum infiltration in freshly prepared 4% paraformaldehyde in 2X PEM (50mM PIPES/KHO (pH 6.9); 5 mM EGTA; 5 mN MgSO4) for 1 hour, and washed 15 min in 1X TBS (Prieto et al. 2004a). The samples were kept at 4^{o}C for 1 or 2 days if used for Vibratome sectioning, or up to two weeks for wax embedding preparation and Microtome sectioning. For tissue embedding, fixed samples were placed in a biopsy cassette (Tissue Tek III, Sakura). Tissues were processed in a Tissue-Tek Vacuum Infiltration Processor (Appendix 3) though a dehydration series (70%, 80%, 90%, and 100% Ethanol), and Xylene steps for wax embedding (Schwarzacher and Heslop-Harrison 2000, Armstrong et al. 2001).

Samples were then removed from the VIP and transferred to the wax basin of the Tissue-Tek Embedding Station. A thin layer of wax was poured into a warm mould, the tissue was orientated and when the first layer started to solidify, more wax was added until the mould was full. A plastic backing was applied and allowed to solidify on a cold-plate. Wax blocks were stored in the refrigerator after the wax has cooled until required for sectioning. The material can be stored in the fridge for one or two years.

Vibratome Sectioning

This method was used to prepare section of a freshly fixed material (Aragon-Alcaide et al. 1998, Schwarzacher and Heslop-Harrison 2000). Florets were removed from the spikelet, cut on their bases and put onto the vibratome plate with superglue (1000plus sectioning system, TAAB laboratories equipment ltd). Sections were made in water, with a razor blade (Wilkinson sword) which is rapidly vibrated. Thickness was approximately 50-100µm containing 2-3 layers of cells and the angle of the machine was set up at 10-15 degrees (adjusted if necessary to obtain good sections). Each section was placed onto a γ-aminopropyl triethoxy silane (APTES) coated slide (Appendix 4) which has been activated with glutaraldehyde (2.5%) and then allowed to air dry (Schwarzacher T. and P. 2000). Quality of the section was checked under light microscope (Figure 15a,b).

Microtome sectioning

This method was used to prepare sections of fixed material embedded in wax when unembedded tissues are too soft for using the vibratome (Armstrong et al. 2001). In a cool environment (18-22^{o}C), the wax block was cut to a small trapezoid shape. The block was mounted so that the longer side is to the bottom and therefore hits the blade first. The ribbons of sections were cut at about 10-20µm thickness and a fine paint brush was used to move ribbons to clean paper towel. Two ribbons of five or six sections were placed onto pre-coated polylysine slides (BDH) and the quality of the sections is checked under the microscope (Figure 15d, e). After adding water to soak the ribbons, the slides were placed in an oven (42^{o}C) to unwrinkle the wax. After one hour, the water was removed and the slides were dried overnight. Immediately before the in situ hybridization, wax was removed with two Histoclear baths of 30 minutes. Tissues were rehydrated in a series of Ethanol (100%, 85%, 70%, 50%, and 30%) and placed in 1xPBS for at least 10 minutes.

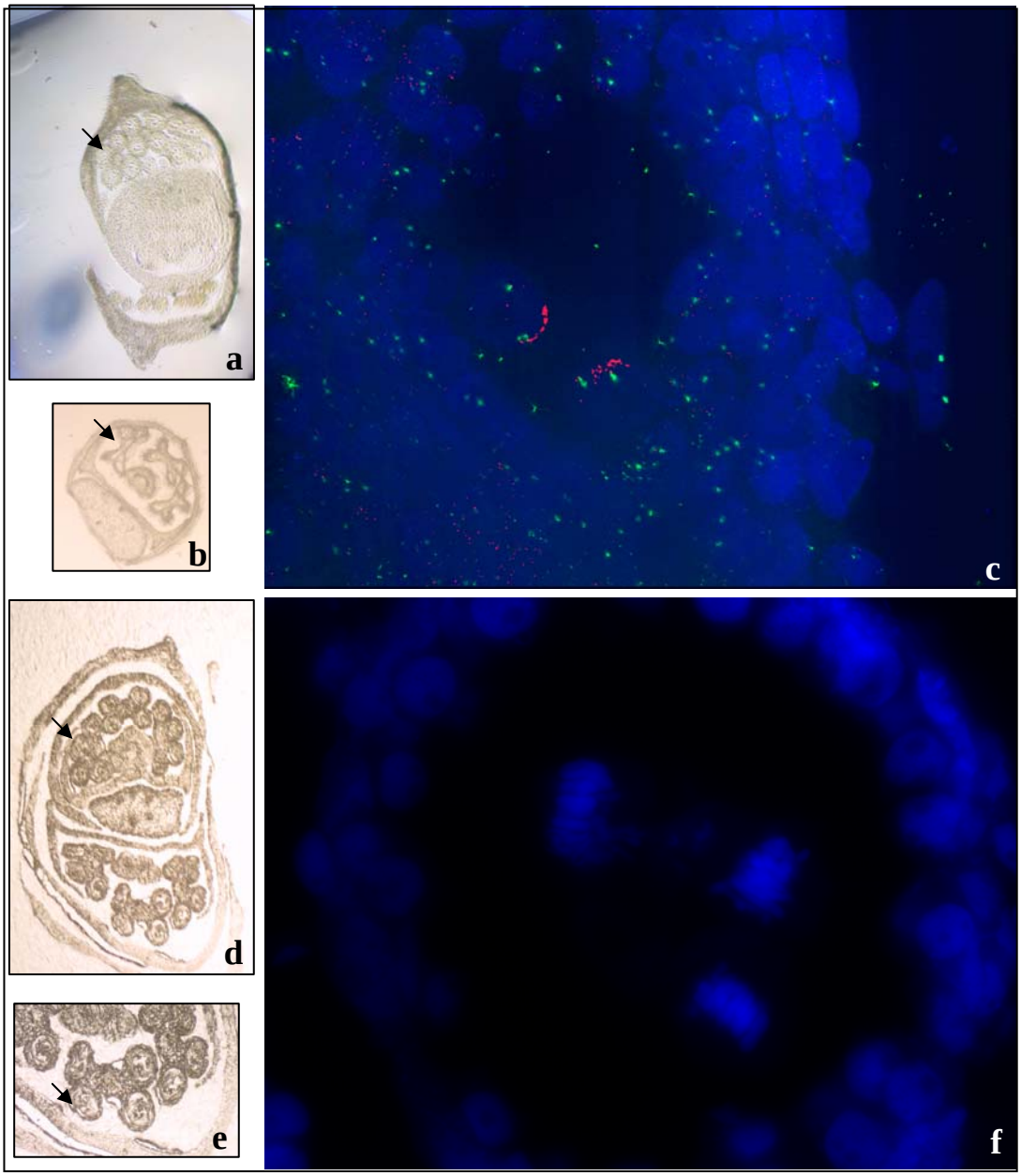


Figure 15: Wheat Floret Sections for in situ hybridization.

The black arrows indicates the anthers section.(a) Fresh vibratome transverse section of a wheat floret showing the ovary in the centre, surrounded by three anthers with four loculi. (b) Dry vibratome transverse section. (C) DNA *in situ* bybridization on vibratome section showing one anther chamber containing two meiocytes at the telomere (red label) cluster stage where two rye segments are labeled in green. (d) Microtome transverse section of wax embedded wheat floret at metaphase I. (e) Transverse section of a wax embedded anther at metaphase I, the black dots inside the loculi are metaphase meiocytes. (f) DAPI staining on microtome section showing one anther chamber containing 3 meiocytes at metaphase I.

Probe making

The telomere probes (Appendix 5) are labelled with biotin-16-dUTP (Boeringer Mannheim) by nick translation (Schwarzacher and Heslop-Harrison 2000) of the PCR product obtained by amplification of the oligomer primers (5'-TTTAGGG-3') and (5'-CCCTAAA-3') in the absence of template DNA (Aragon-Alcaide et al. 1996). The rye probes (Appendix 6) are labelled with digoxygenin-11-dUTP (Boeringer Mannheim) by nick translation (Appendix 7) of the pSc250 rye sequence (Vershinin et al. 1995). PCR products and probes are monitored on 1% Agarose gels, to assess the quality and the size of the product. Incorporation of the label is checked by dot blots (Schwarzacher and Heslop-Harrison 2000).

Fluorescence *in situ* hybridization

The protocol is adapted from Aragon-Alcaide et al (1997a) and Schwarzacher et al (2000) (Appendix 9). Dry sections are dehydrated in a series of 30, 50, 70 and 100% methanol. Then, tissues are treated with a mixture of 1% cellulase, 0, 5% pectolyase in TBS (0.1M Tris-HCl, pH 7.5; 0.15M NaCl) for 30 min at room temperature, washed in TBS for 10 min, and dehydrated again in a series of 30, 50, 70 and 100% methanol. Hybridization mix, composed of 50% deionised formamide, 20% dextran sulfate, 1X PIPES/EDTA buffer (100:10), 0.3M NaCl, 500ng of salmon sperm blocking DNA, and 50 ng of each probe, is denatured for 8min at 100°C and immediately placed on ice to cool down for 5 minutes. The probe mixture is applied onto the tissue, covered with a plastic coverslip and put in a modified Omnislide thermal-cycler (Hybaid Ltd).

Chromosome DNA is denatured at 78°C and hybridisation is performed at 37 °C overnight. Slides are washed at 42°C in 20% formamide, 0.1 X SSC for 10 min, then in 2 X SSC for 10min. This is followed by a wash at room temperature in 2 X SSC for 10 min, and in 4 X SCC, 0.2% Tween 20 for 10 min. A blocking solution, consisting of 5% BSA in 4 XSSC, 0.2% Tween 20 is applied for 5min in a humidity chamber at room temperature. Digoxygenin labelled probes are detected by an anti-digoxygenin FITC-conjugated antibody and biotin labelled probes are detected with extravidin-cy3. Slides are counter stained in 1µg/ml DAPI (4',6-diamino-2-phenyl-indole) and mounted in Vectashield (H-1000) medium.

Microscopy and Imaging

Meiocytes were visualized using a Nikon Eclipse E600 epifluorescence microscope equipped with a Hamamatsu Orca-ER cooled CCD camera and a Prior Proscan x,y,z stage (Griffiths et al. 2006). Stack images of individual cells were collected using MetaMorph (Universal Imaging Corp.) software. Deconvolutions of images were processed with AutoDeblur (AutoQuant Imaging). Projections of 3D picture were performed with the public domain program ImageJ written by Wayne Rasband and obtainable from http://rsb.info.nih.gov/ij/.

Statistics

The two-tailed t-test probability was performed with Genstat 9th software to evaluate the differences in the means between the line with identical heterochromatin and the line with similar heterochromatin. We tested against the null hypothesis that the mean length of segment 1 is equal to the mean length of segment 2 for each genotype and that the two sets of data were independent. The null hypothesis was tested for 95% confidence interval for the difference in means where the cutoff point (alpha level) is equal to 0.05 (). The *p*-value represents the probability of error involved in rejecting the null hypothesis. If *p*-value > alpha level, the null hypothesis is not rejected, and the mean length of segment 1 is not significantly different from the mean length of segment 2. However, if *p*-value < alpha level, the null hypothesis is rejected and the mean length of segment 1 is not equal to mean length of segment 2.

3.3 Results and discussion

Prior to meiosis, the telomeres were dispersed around the nuclear periphery in all lines. In these premeiotic cells, no change in conformation of the subtelomeric heterochromatin was seen and the chromatin of these regions remained compact in all of the meiocytes examined (Figure 16*b,f,j*). When the telomeres began to cluster, the chromosome remodelling events differed between the lines in these subtelomeric heterochromatin regions.

When the subtelomeric heterochromatin regions were identical on the two homologues (King II/King II), they were localised together prior to the telomere bouquet formation in 50% of the meiocytes examined (Figure 17c,d). During the telomere bouquet stage, these regions then underwent extensive remodelling in all the meiocytes examined from this line (Figure 16c,d) The remodelled (or elongated) subtelomeric heterochromatin regions on the homologues extended up to 5µm in length but differed by no more than 30% in length from each other (Table 3). The extended subtelomeric heterochromatin then formed a V-shaped paired structure with the telomere sites at the apex before "zipping up" (Figure 17d and Figure 17f). The subtelomeric heterochromatin regions were paired in 98% of the meiocytes examined from diplotene to metaphase I (Table 4). Similar results were obtained for the Petkus/Petkus line.

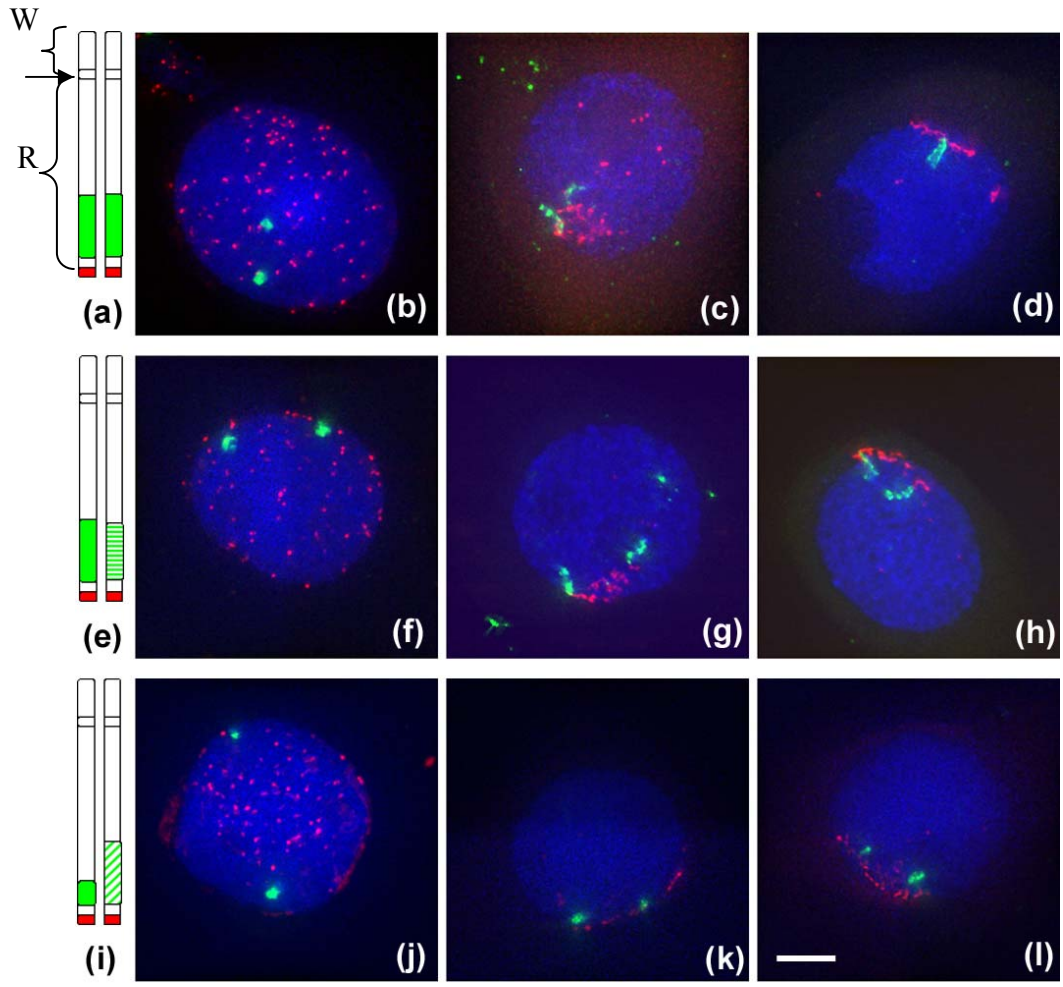


Figure 16: Heterochromatin remodelling at meiosis.

(W) small arm of the wheat chromosome 1B: (R) translocated short arm of the rye chromosome 1; (Arrow) centromere: (green rectangle) Subtelomeric region: (red rectangle) telomere. The subtelomeric heterochromatin is labelled in green using the pSc250 rye sequence as a probe and telomeres are labelled in red using a PCR product derived from primers $(5'\text{-TTTAGGG-}3')_5$ and $(5'\text{-CCCTAAA-}3')_5$ as the probe. In premeiotic nuclei (*b*,*f*,*j*), the rye segments are condensed in all lines; The behaviour of the rye segments have been analysed during telomere bouquet (*c*,*d*,*g*,*h*,*k*,*l*). In the line King II/King II (*a*) with identical heterochromatin, the rye heterochromatin elongates before the full formation of the telomere cluster (*c*), and the homologues align in most of the cells (*d*); In the line Petkus/King II (*e*) with similar heterochromatin, the rye heterochromatin elongates before the full formation of the telomere cluster (*g*) and homologue alignment is slightly delayed (*h*); In the line Petkus/Imperial (*i*) with different heterochromatin, the rye heterochromatin does not elongate even at the telomere cluster (*k*,*l*). The scale bar represents ~10µm.

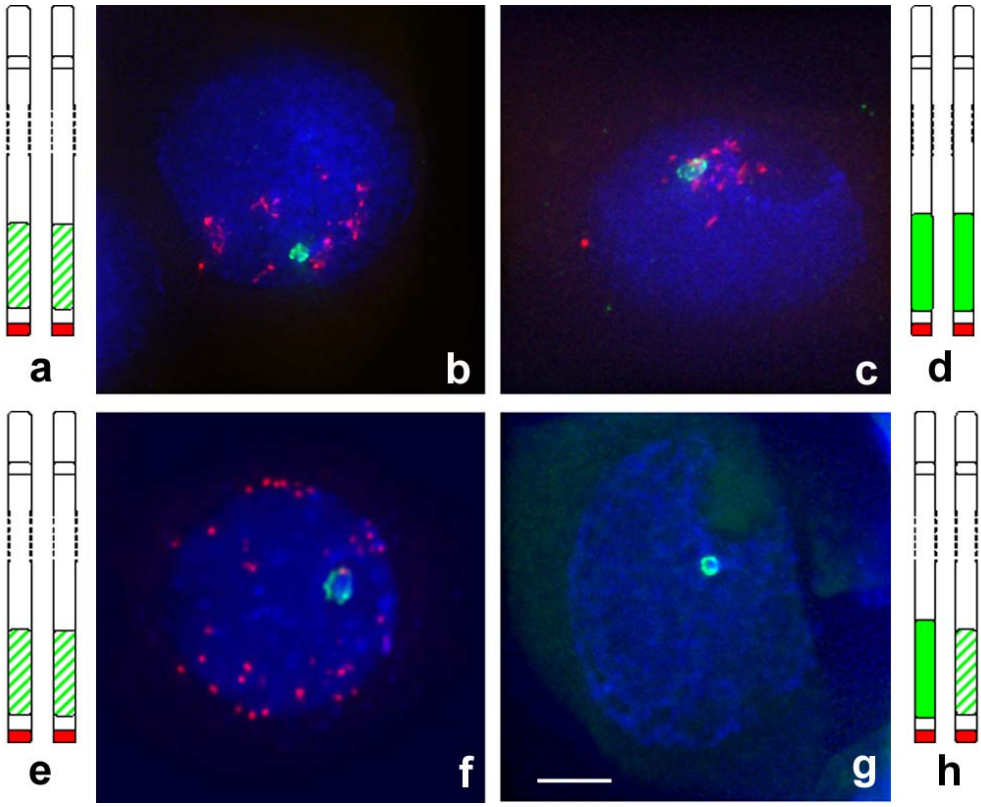


Figure 17: Heterochromatin colocalisation and association at meiosis.

Subtelomeric heterochromatin is labelled in green using the pSc250 rye sequence as a probe and telomeres in red using a PCR product derived from primers (5'-TTTAGGG-3')$_5$ and (5'-CCCTAAA-3')$_5$ as the probe. In the lines Petkus/Petkus (*a*,*e*) and King II/King II (*d*), with identical heterochromatin, the chromosomes can colocalize before the telomere bouquet (*b*,*c*) and can associate as a fork after telomere bouquet (*f*). In the line Petkus/King II (*g*,*h*), with similar sized heterochromatin, the segments associate as a ring structure at late zygotene (single probe used). Scale bar represents ~10µm.

In the line carrying similar sized but slightly different subtelomeric heterochromatin (Petkus/King II), at the telomere bouquet stage both the subtelomeric heterochromatin regions did undergo chromatin remodelling (Figure 16g and h), but the remodelled regions differed from each other by up to 2-fold in length (Table 3). The remodelling of the rye segments is not synchronized. Moreover, the extended heterochromatin regions then did not ‘‘zip up’’ as in the parental lines but paired at either end of the heterochromatin regions forming a loop structure (Figure 17g). The loop structure then coalesced so that the remodeled heterochromatin regions were paired in 79% of the meiocytes at diplotene and 56% at metaphase I (Table 4). In this case, recombination has been observed between markers on the Petkus/King II chromosome arms in an F2 mapping population (Singh NK et al. 1990, Mago et al. 2005). In contrast to these observations, the subtelomeric heterochromatin remained compact during the telomere clustering and bouquet formation in the wheat line carrying homologues with differently sized subtelomeric heterochromatin regions (Petkus/Imperial), and these regions remained unassociated (Figure 16k–l). Subsequently, the subtelomeric heterochromatin regions were only paired with each other in 30% of the meiocytes at diplotene, which then reduced further to 16% of the meiocytes by metaphase I (Table 4).

The low degree of pairing was consistent with a lack of recombination between the markers on Petkus/Imperial chromosome arms in F2 mapping population. Recombination has been assessed in these lines using RFLP markers rga5.2 and iag95 and the rye seed storage protein locus Sec-1, which are predicted to span approximately 50% of the physical rye arm. However, these markers cosegregated in a wheat mapping population of 120 F2 lines derived from a 1BL-1RS (Petkus) and 1BL-1RS (Imperial) heterozygote, showing a lack of recombination between the Petkus and Imperial chromosome arms (Spielmeyer W. et al. 2000, Ko JM et al. 2002, Mago et al. 2002). These results suggest that this lack of recombination is due to the failure of chromosome remodelling and pairing.

Table 3 Heterochromatin remodelling during the telomere bouquet formation.

The length of the remodelled heterochromatin was measured in 3D stacks of meiocytes exhibiting telomere clustering. Segment 1 is shown as the longest of the pair. The *t* test on the samples was performed by using Genstat 9th for the following null hypothesis that the mean length of segment 1 is equal to the mean length of segment 2 for each genotype. The hypothesis was tested under a 95% confidence interval for difference in means (alpha level = 0.05). For the line with two identical segments, the probability is $P = 0.279$ (P value > alpha level). Therefore, the null hypothesis is not rejected and the mean length of segment 1 is equal to mean length of segment 2. The two segments elongate at the same time. For the line with two similar segments, the probability is $P < 0.001$ (P value < alpha level). Therefore, the null hypothesis is rejected and the mean length of segment 1 is not equal to the mean length of segment 2. The two segments do not elongate at the same time.

	Identical Heterochromatin King II / King II (Figure 16a)			**Similar Heterochromatin King II / Petkus (Figure 16e)**		
	Segment1	Segment2	Ratio	Segment1	Segment2	Ratio
Segment Elongation	5.269	4.509	1.2	4.425	4.093	1.1
	4.217	3.948	1.1	3.893	2.652	1.5
	4.676	3.470	1.3	4.952	3.298	1.5
	5.023	4.899	1.0	5.040	3.938	1.3
	4.053	3.797	1.1	4.106	3.302	1.2
	4.021	3.195	1.3	3.823	3.567	1.1
	3.678	2.896	1.3	3.773	3.752	1.0
	4.333	3.809	1.1	3.034	2.539	1.2
	3.942	3.064	1.3	3.865	3.098	1.2
	3.451	3.451	1.0	5.313	3.524	1.5
	3.936	3.936	1.0	3.463	3.431	1.0
	3.677	3.677	1.0	3.175	2.965	1.1
	2.515	2.515	1.0	2.512	2.155	1.2
	2.143	2.143	1.0	4.359	3.558	1.2
	1.692	1.692	1.0	3.662	0.891	4.1
				4.082	2.263	1.8
				4.796	2.450	2.0
				3.599	3.238	1.1
				3.143	2.236	1.4
				2.956	1.766	1.7
				3.466	3.425	1.0
				4.915	3.509	1.4
				3.953	2.474	1.6
				4.424	3.941	1.1
				3.140	2.736	1.1
				4.115	3.022	1.4
				5.041	3.913	1.3
				4.100	1.843	2.2
				1.997	1.586	1.3
				2.390	1.803	1.3
				2.508	1.655	1.5
				3.697	3.799	1.0
				3.653	2.724	1.3
Mean	3.775	3.400		3.799	2.883	
Difference of means	0.375			0.916		
Standard error of difference	0.339			0.203		
F Test	1.38 on 14 .f.			1.00 on 32 d.f.		
Probability *t* test	0.279			< 0.001		

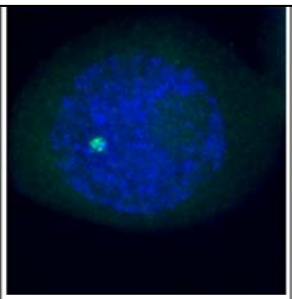
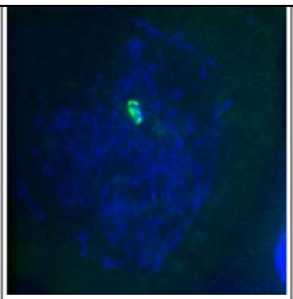
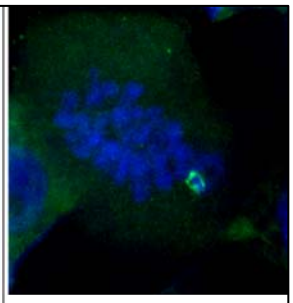
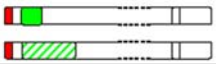
Homologous pairing during meiosis	 Late Zygotene	 Diplotene	 Metaphase I
(a) 	98%	98%	98%
(b) 	82%	79%	56%
(c) 	44%	30%	16%

Table 4: Percentage of meiocytes with paired heterochromatin sites during prophase

(d) King II/King II or Petkus/Petkus lines, (e) Petkus/King II line and (f), Petkus/Imperial line. Homologous pairing site at late Zygotene (a), diplotene (b) and Metaphase I (c). In table 3, 36% of cells for Petkus/King II have a bigger ratio that the identical lines which would suggest 63% pairing at metaphase. However, only 56% was observed in this experiment. 100 meiocytes were analysed at metaphase for Petkus/King II (b) and Petkus/Imperial (c) which clearly shown differences in pairing between the two lines. However, this population may be too small to represent the theoretical result.

3.4 Discussion

The results show that homologous chromosomes can be associated before the telomere bouquet but this association only happens where the homologues are identical in their subtelomeric regions. If the homologues are identical in length and sequence they behave as observed previously. They can intimately align in a "zipping up process" from the telomeres (Prieto et al. 2004a). If these regions are similar but not identical (same heterochromatin region size, but different cultivar), as in Petkus/King II, then homologue association occurs within the telomere bouquet, but only after the chromatin remodelling has occurred. In this case, the chromatin is remodelled to different lengths in the two homologues. Then both ends of the subtelomeric regions are linked together, and their alignment occurs through "a pegging together and coalescing" process. In this line (Petkus/King II) there is a reduction in the overall level of pairing and subsequent recombination which can be correlated with the observation that the remodelled regions differed from each other by up to 2-fold in length when elongated. This hypothesis is confirmed with the line carrying segments of different sizes and sequence, where there is no remodelling, and subsequently the meiocytes have a lower level of pairing and recombination. If the rye arms are really diverged, there is a failure of remodelling and recombination. Therefore we conclude that the ability to remodel chromatin (Figure 14), which affects subsequent pairing and recombination, is dependent on overall relatedness of chromosomes.

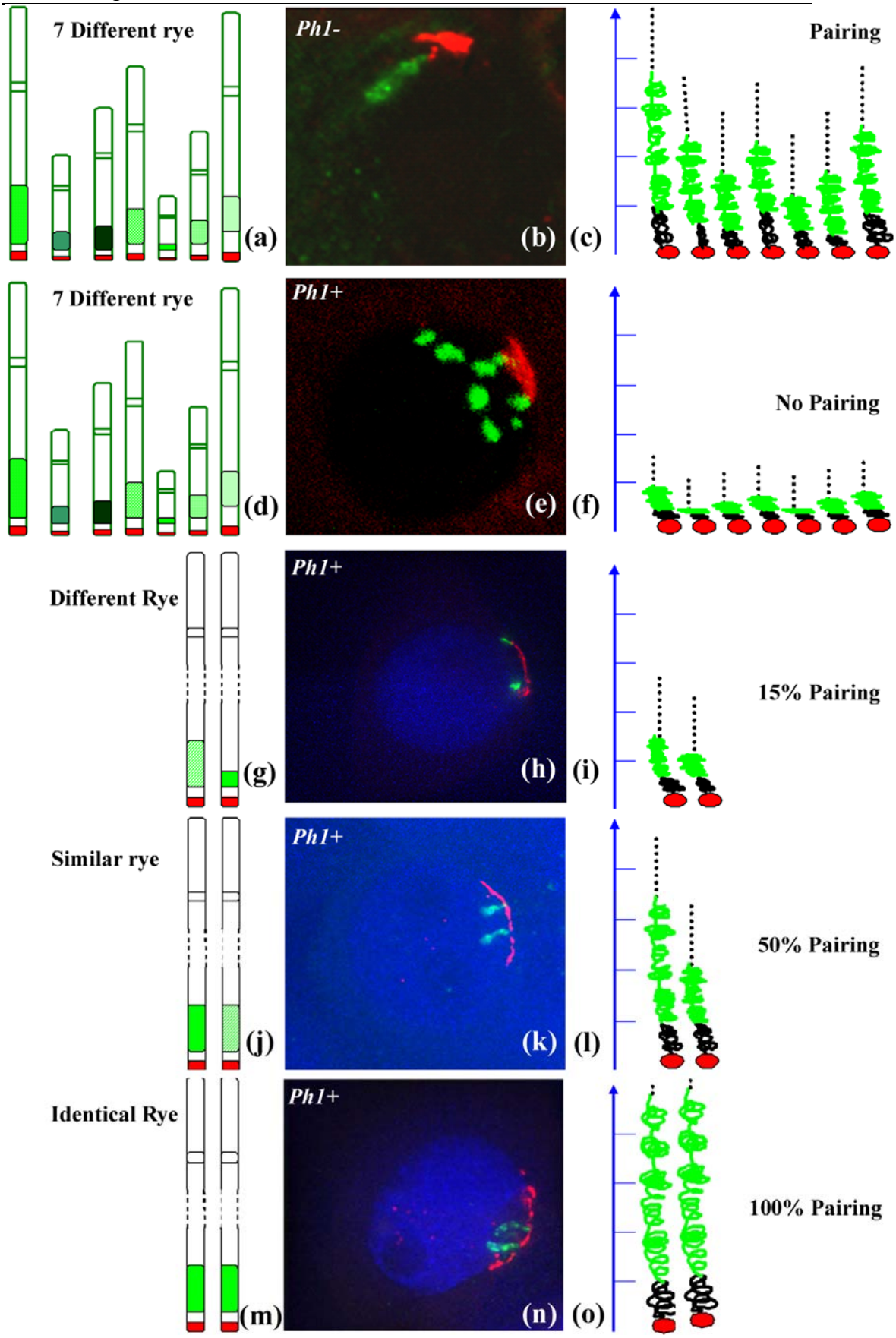
The data in the present study are complementary to studies exploiting wheat-rye hybrids (Figure 18) (Prieto et al. 2004a, Prieto et al. 2005). In wheat-rye hybrids, where there are no true homologues, in the absence of the *Ph1* locus, the subtelomeric heterochromatin remodels (Prieto et al. 2004a), and then associates. In contrast, in the presence of the *Ph1* locus with no true homologues present, the subtelomeric heterochromatin cannot remodel and thus pairing and recombination do not occur. The remodeled subtelomeric heterochromatin can colocalize together as a single diffuse structure (Figure 18*b*). Recombination can occur in the absence of *Ph1* between the wheat and rye chromosomes (Benavente et al. 1996, Naranjo and Fernandez-Rueda 1996).

In contrast, in the presence of the *Ph1* locus with no true homologues present, the subtelomeric heterochromatin cannot remodel (Figure 18d–f). Recombination does not occur between the wheat and rye (Benavente et al. 1996, Naranjo and Fernandez-Rueda 1996). Even possessing two homologous chromosomes is in itself not sufficient to induce chromatin remodeling of both homologues in the presence of *Ph1* (Figure 18g–i). Both homologues need to be identical or near identical for remodeling to occur (Figure 18 j-o). Thus, *Ph1* in wheat affects the ability to coordinate and control chromatin remodeling at meiosis (Prieto et al. 2005). The chromatin remodelling enables chromosomes to become competent to pair and recombine. Moreover, the *Ph1* locus in wheat is also able to block recombination from occurring between similar but distinct chromosome segments located within otherwise identical chromosomes (Dubcovsky et al. 1995, Luo et al. 1996). Thus, the present study suggests that in the presence of *Ph1,* the control of the chromosome remodelling at the onset of meiosis is more stringent allowing only identical or nearly identical chromosomes to remodel at the same rate and therefore recombine.

The subtelomeric heterochromatin regions were localized together prior to the telomere bouquet formation in 50%-60% of meiocytes in the line carrying two similar segment in size and sequence. This colocalization is not seen in the others lines where chromosomes are not completely homologous. This is interesting because there was some evidence of formation of synaptonemal complexes in premeiotic interphase of wheat (McQuade and Pickles 1980) which suggests a possibility of pairing initiation prior to meiosis. It is also remarkable that an increasing dose of *Ph1* leads to the suppression of premeiotic associations resulting in a reduction of pairing and recombination (Feldman 1966). These observations together suggest that *Ph1* regulates events prior to meiosis and that this mechanism is linked to the homology.

Figure 18: Summary of the ability to chromatin remodel at meiosis

Subtelomeric heterochromatin is labelled in green using the pSc250 rye sequence as a probe and telomeres in red using a PCR product derived from primers (5'-TTTAGGG-3')$_5$ and (5'-CCCTAAA-3')$_5$ as the probe. In wheat-rye hybrids carrying 7 rye chromosomes in the absence of *Ph1* (*a*), the rye heterochromatin can elongate (*b*, *c*) and the chromosomes can pair. In a wheat-rye hybrid carrying 7 rye chromosomes (*d*), the rye heterochromatin does not elongate (*e*,*f*) and there is no pairing. In the presence of *Ph1*, when homologues carry different rye subtelomeric heterochromatin (*g*), the heterochromatin does not elongate at the telomere cluster (*h*,*i*). When homologues carry similar rye subtelomeric heterochromatin (*j*), the heterochromatin elongates at the telomere cluster (*k*) and the remodelled regions can differ from each other by up to 2-fold in length (*l*). When homologues carry identical rye subtelomeric heterochromatin (*m)*, heterochromatin elongates synchronously at the telomere cluster (*n*,*o*) up to 5 µm in length. Scale bar represents ~10µm for (*b*,*e*) and 5 µm for (*h*,*k*,*n*).


7 Different rye
(a)
Ph1-
(b)
(c)
Pairing
7 Different rye
(d)
Ph1+
(e)
(f)
No Pairing
Different Rye
(g)
Ph1+
(h)
(i)
15% Pairing
Similar rye
(j)
Ph1+
(k)
(l)
50% Pairing
Identical Rye
(m)
Ph1+
(n)
(o)
100% Pairing

3.5 Conclusions

The present study suggests that the recombination between the rye regions in the presence of *Ph1* is based on the ability to remodel the regions at the onset of meiosis. By visualising the behaviour of homologues which are distinct in their subtelomeric heterochromatin, we have assessed the effect that varying homology has on the ability to remodel chromatin and the consequent effect that this has on pairing and recombination.

In the chapter 2, the *Ph1* locus was defined as a cluster of *cdk*-like genes (Griffiths et al. 2006), to which *Cdk2* from humans and mice is the closest apparent homologue (Al-Kaff et al. 2008). Centromere pairing occurs during S-phase (Jasencakova et al. 2001) and *Ph1* affects this association meiotically and somatically (Martinez-Perez et al. 2001). In the case of the wheat-rye hybrids (Figure 14), in the absence of *Ph1*, there are 7 sites of rye centromeres and 7 sites of wheat centromeres, while in the presence of *Ph1*; there are 7 sites of associated wheat and rye centromeres at the onset of meiosis. B chromosomes (heterochromatin), which delay replication, compensate for the absence of *Ph1* in wheat hybrids. Threfore, we can suggest that the differences observed in centromere associations between lines carrying *Ph1* and the lines lacking *Ph1*, might be the result of different timing of replication of the wheat and rye centromeres in the presence and absence of *Ph1*. Interestingly, Cdk2 knockout mice are viable, and the loss of Cdk2 affects the timing of S phase (Berthet et al. 2003). CDK2 (and CDK1) are controlling the efficiency of DNA replication by determining the frequency of origin activation (Krasinska et al. 2008).

The cyclinA1-CDK2 complex regulates DNA Double Strand break repair (Muller-Tidow et al. 2004). Ashley et al (1980) shown that Cdk2 colocalizes with MLH1, a mismatch repair protein at sites of reciprocal recombination in mid-late pachynema. In addition Cdk2 localizes to the telomeres of chromosomal bivalents throughout meiotic prophase (Ashley et al. 2001). In CDK2 knockout mice SC proteins fail to assemble on the homologous chromosome pair in absence of CDK2, resulting in desynapsis and nonhomologous pairing (Cohen et al. 2006). All these observations together suggest that CDK2 is implicated in recombination and synapsis.

The *Ph1* locus must be suppressing the expression of *Cdk2* loci on other chromosomes because the loss of the *Ph1* locus increases their expression level (Al-Kaff et al. 2008), which then results in homoeologous pairing.Because both *Cdk2* and *Ph1* affect replication we hypothesize that the sensing mechanism occurs during premeiotic replication. Thus the ability to initiate both replication and the onset of meiosis may be intimately linked to the ability to remodel chromatin which is required for the chromosomes to become competent to pair.

Furthermore, in the hypothesis that *Ph1* has a CDK-like mechnism, it may be possible to regulate the *Ph1* activity by using drugs known to regulate the CDK activity (Sherr and Roberts 1999) or the phosphorylation state (Keller and Krude 2000). If such system is working in the whole plant, plant breeding could "switch off" the *Ph1* activity during crossing by applying the drug, which will disappears at the next generation. Then the new genome will be stabilized with the *Ph1* activity "switched on" again.

In conclusion, *Ph1* is controlling the chromosome pairing by changing the chromatin conformation. The remodelling of the chromosomes occurs only if the sub-telomerique regions are identical or nearly identical, enables the chromosomes to become competent to pair and recombine. The linker histone H1 is involved in maintaining higher-order chromatin structures and is phopsphorylated by CDK2 in vivo, resulting in a more open chromatin structure by destabilizing H1-chromatin interactions (Contreras et al. 2003). If the *Ph1* locus is a CDK-like mechanism, we would expect a change in proteins such as histone in presence or in absence of *Ph1*. Therefore, proteomic studies are now necessary to unveil the *Ph1* mystery, and mass spectrometry is one of the possibilities.

Chapter 4

Investigation of wheat meiosis using proteomics:

A pilot study

Abstract

"Since Sir Ralph Riley discovered *Ph1* 50 years ago, many effects of the locus have been described but the puzzle has always missed some clues to unveil its mechanism. The cell biology of the *Ph1* effect on chromosome pairing is now well established. The major breakthrough was the fine mapping of the locus: Characterized as a cluster of Cdk-like genes, it is likely that the *Ph1* acts in the same way of Cdk2, its closest homologues from human and mice. Both Cdk2 and *Ph1* act on chromatin remodelling, have effects on recombination and DNA replication. Therefore, we now have a good candidate for a proteomic study of *Ph1* and more generally of wheat meiosis. Mass spectrometry is one of the best tools for protein identification of complex mixtures. Moreover, mass spectrometers are now very accurate which enables the detection of post-translational modification involved in protein pathways such as phosphorylation. Therefore, mass spectrometry can be used to characterize the protein content of wheat meiocytes at different stage of meiosis. Furthermore, comparison of proteins from plants carrying the *Ph1* locus and plants deficient for the *Ph1* locus might reveal changes in protein content which could explain the *Ph1* activity on chromosome pairing. This chapter describes a pilot study for the use of mass spectrometry on wheat meiocytes."

4-1 –Introduction

The proteome generally signifies the complete set of **prote**ins expressed by a gen**ome,** cell, tissue or organism at a certain time of development and is the functional representation of the genome (Wilkins et al. 1997). The proteome size is much bigger than the genome size of an organism. For example, the size of the human proteome is estimated at a million of proteins for a range of 20,000-40,000 genes only, and this proportion differs from one genome to another (yeast, fly or worm) which is assumed to be essentially due to the different splicing events (Harrison et al. 2002). The proteome is also highly dynamic because of tissue/development specific mRNA expression and many post-translational modifications (Wilkins et al. 1997). Therefore, one of the major challenges in proteomics is to analyse thousands of proteins in a single sample, and mass spectrometry is the most powerful tool to achieve this with accuracy (Pandey and Mann 2000).

Mass spectrometry (MS) is a tool that analyses the components of a sample by calculating the mass-to-charge ratio (m/z) of positive (or negative) ions and provides structural information by fragmentation analysis. The most basic mass spectrometer possesses an ionization source, an analyser and a detector. MALDI (Matrix Assisted Laser Disorption Ionization) and ESI (Electro Spray Ionization) are the two major ion sources used in MS (Karas 1985, Tanaka 1988, Fenn et al. 1989). With MALDI, the sample is mixed with a matrix consisting of very small crystals and placed on a MALDI plate. The solvent evaporates leaving the crystals and the sample linked together. The laser is directed onto the matrix and excites the crystals, which release the ions.

With ESI, the liquid sample is injected through a capillary into the ion source. The droplets are ionized under a strong electric field applied in the chamber. ESI is a very gentle ionization and can be coupled with liquid chromatography (LC), which is useful to reduce the complexity of the sample before MS for better resolution. Furthermore, because proteins are very large and complex organic molecules (3D structures) and have different physical and chemical properties (solubility, isoelectric point), they are not usually analysed intact for protein identification analysis but are first digested by specific proteolytic enzyme such as trypsin.

Tandem MS, such as the LTQ (ThermoFisher) are capable of sequential MS analyses and use a linear ion trap (based on a quadrupole) which generates a strong oscillating electric field for the ions to move in. When a particle exits the ion trap, the mass and charge of this precursor ion are calculated by the instrument (MS1). The precursor ion is also fragmented by one or more collisions created by the electric field and the presence of gas molecules, such as helium, which will generate the peptide sequence (MS2). The generated data are submitted to a search engine, such as Mascot, for database searching. Mass spectrometry allows identification of thousands of proteins in the same mixture and their modifications, but it also can be used for quantification (Mann 1999, Mann et al. 2001, Ong and Mann 2005). For protein identification and quantification of complex mixtures, relative quantification, based on the use of isotopes, is the most accurate method (Steen and Mann 2004). For example, SILAC (stable isotope labelling with amino acids in cell culture) is a method based on the incorporation of amino acids containing substituted stable isotopes (e.g. deuterium, ^{13}C, ^{15}N).

The "heavy" or the "light" amino acid is incorporated in vivo via the normal metabolism of the cell, and generates proteins of altered molecular mass, whose peptides can be easily differentiated in the mass spectrometer. Two or more samples can therefore be analysed in a same mass spectrometer run, and compared to each other (Wisniewski et al. 2008). SILAC is a very straightforward method to analyse cell culture samples, but is very hard to use on whole organisms. Moreover, plants can interconvert different amino acids much more easily than animals, which complicates the use of SILAC in plants. An alternative is to grow the plants hydroponically to label the proteins by incorporation of ^{15}N from supplied $^{15}NO_3$, which can then be compared to the normal ^{14}N proteins.

In species such as human or yeast, the chromosomes are brought together at the telomere bouquet and pair (Bass 2003). While meiosis events such as chromosome pairing have been described in various species (Human: (Vallente et al. 2006); mammals: (Cohen et al. 2006); plants: (Hamant et al. 2006); yeast: (Wells et al. 2006)), still little is known about their mechanisms and how the homologues recognize each other for pairing.

In wheat, at the telomere bouquet, homologues undergo a synchronized chromatin remodelling from their telomeres, and intimately align with each other by a "zipping up" process (Maestra et al. 2002, Prieto et al. 2004a) which enables the chromosomes to become competent to pair and recombine efficiently (Colas et al. 2008). This is controlled by the *Ph1* locus, which ensures that only homologues pair during meiosis. In the absence of *Ph1* (*Ph1* mutant), there is random remodelling of the chromosomes, which allows non-homologue chromosomes to pair (Prieto et al., 2004).

Because *Ph1* "senses" the homologues for remodelling and pairing, this is a good model to understand homologue recognition. Furthermore, *Ph1* has recently been defined to a cluster of cdk-like genes (Griffiths et al. 2006) and Cdk2 from humans shows the closest homology to these wheat cdk-like genes (Al-Kaff et al. 2008). Several studies revealed that Cdk2 controls histone H1 phosphorylation to decondense the chromatin during S- phase (Alexandrow and Hamlin 2005) or to modulates mouse mammary tumor virus transcription through chromatin remodelling (Bhattacharjee et al. 2001) or even to recrut chromatin assembly factor 1-dependent chromatin assembly during DNA synthesis (Keller and Krude 2000). Therefore CDK2 is implicated in chromatin remodelling, at replication (Cobb et al. 1999, Alexandrow and Hamlin 2005) and the initiation of meiosis (Dutta and Stillman 1992, Murakami and Nurse 1999). Similarly, *Ph1* is involved in chromatin remodelling (Prieto et al. 2004a, Colas et al. 2008) and may affect replication (Aragon-Alcaide et al. 1997b, Martinez-Perez et al. 2003, Prieto et al. 2004a). If *Ph1* is a cdk-like mechanism, there is a possibility to dissect the mechanism at the protein level based on the mechanism of its homologues Cdk2. Large scale tudy of proteins (structures, functions) or proteomics is likely to provide precious information on meiosis and homologous pairing.

LC-ESI-MS/MS was used in this study and samples were run on an Ion Trap (Linear Trap Quadrupole), and/or the LTQ-Orbitrap mass spectrometer. The LTQ possess a single sophisticated quadrupole based linear ion trap. The ions are alternatively trapped and scanned out to the detectors for analysis. This machine has the advantage of being very quick but has a poor mass accuracy. The LTQ-Orbitrap (Thermo Electron Corporation, 2006) is a hybrid composed of a fast linear ion trap and a new electrostatic analyzer.

Instead of moving straight (like in the Ion trap), the ions osccilate and the oscillation pathway are measured by the analyser to determine m/z by Fourier transform giving better mass accuracy. Although the Orbitrap is slower that the Ion Trap, its better mass accuracy makes it very powerful for protein identification and quantification.

The first part of this study will present the different methods used to prepare the samples for mass spectrometry. Briefly, the wheat meiocytes were isolated for each stage of meiosis, and the proteins extracted and trypsin digested for mass spectrometry. Different extraction methods were tested and are discussed. The samples were analysed by LC-ESI-MS/MS which has enabled me to analyse proteins involved in chromatin organization. I have also shown some evidence of protein modification detection, which confirms the quality of the samples for further analysis. Protein validation will be discussed. To dissect the *Ph1* CDK-like protein pathway, it is important to be able to compare the protein contents of sample containing the *Ph1* locus (and then expressing the associated proteins) and sample without the *Ph1* locus (*Ph1b* deletion mutant). For this purpose a ^{15}N quantification method had to be developed and in this chapter, I will describe how I have grown plants in a hydroponic solution containing either light nitrogen (^{14}N), or heavy nitrogen (^{15}N) and compare the peptide ratio of the MS data generated.

4-2 -Materials and methods

4-2-1 Plant material

Chinese Spring (CS) cultivar plants were vernalized for 3 weeks to synchronize the plants, and then transferred to a controlled environmental room until meiosis, with the following growth conditions: 16 hours at +20°C, and 8 hours at +15°C, 85 % humidity. Meiosis starts after 6-7 weeks under these conditions.

4-2-2 Heavy Nitrogen incorporation

Chinese spring (CS) cultivar and *Ph1b* deletion line in Chinese Spring background (CS-*Ph1b*) were germinated for 5 days at room temperature on a wet filter paper. Seedlings were vernalized for 3 weeks either in ^{14}N Hoagland solution (6mM KNO_3, 4mM $Ca(NO_3)_2$, 1mM NaH_2PO_4, 2mM $MgSO_4$, 50µM H_3BO_3, 1µM $MnCl(H_2O)_4$, 0.6µM $Zn(SO_4)(H_2O)_7$, 0.3µM $Cu(SO_4)(H_2O)_5$, 0.12µM MoO_3 and 25µM Iron chelate) or ^{15}N Hoagland solution in which the potassium nitrate and calcium nitrate were substitute with the heavy (98-99% ^{15}N) labelled components (KNO_3, and $Ca(NO_3)_2$). The seedlings were grown in 50ml falcon tubes in which a hole was made to allow the roots to soak in the solution (Figure 19A). After the vernalization period, the plants were transferred to pots containing vermiculite to simulate the Ebb & Flow (or Flood & Drain) hydroponic system (Figure 19B), where the nutrients are automatically pumped into the top tray to fill the reservoir and feed the roots and the solution go back to the reservoir for the next time. The vermiculite has the advantage of retaining the medium to feed the roots, and allowing enough oxygen for the plants to develop and grow properly. Leaves were collected at intervals to check the ^{15}N incorporation in the proteins by mass spectrometry.

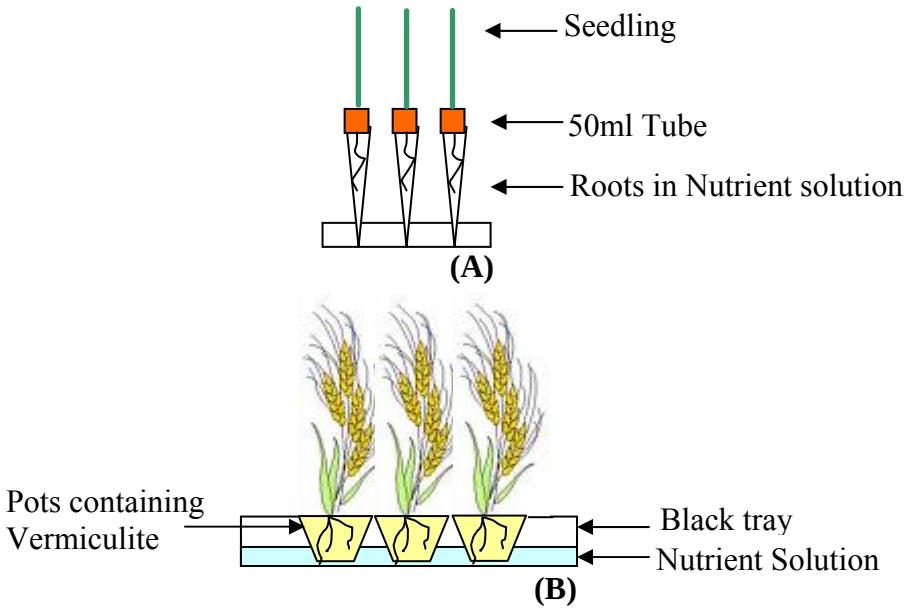


Figure 19: Hydroponic Setup for wheat plants.

The 5 day old seedlings were grown in a hydroponic solution with the system (A) for 3 weeks in a vernalization room. Then, they were transferred to pots containing vermiculite to oxygenate the roots with system (B) until flowering. The nutrient solution is poured on the tray to soak the end of the roots in solution. The level of the nutrient solution has to be checked every two days and must be all the time at least 2cm deep for the plants.

4-2-3 Meiosis staging

Plants were harvested and kept in cold water for analysis within one day. Wheat spikes were collected by removing the covering leaves. One anther from the middle spikelet (which is at the latest developmental stage), was spread onto a slide, stained with aceto-carmine (Stack et al. 1977), squashed and then checked on a microscope (10X, and 40X magnification) to monitor the stage of meiosis. The two remaining anthers were kept on ice.

4-2-4 Meiocytes Isolation

Anthers collected from the same meiosis stages were then transferred to a 1.5ml tube containing 50µl of phosphate buffer (1XPBS) and protease inhibitor (Protease Inhibitor Cocktail Set 1 (1VL), calbiochem, cat#539131). The tubes were stored at 4°C, and meiocytes were extracted from the anthers after one hour (up to overnight). Under a stereomicroscope, the anthers were cut in half, and the meiocyte columns were removed by gently rolling them in a drop of buffer. The meiocytes were transferred to an Eppendorf tube using fine needles and kept at -80°C until protein extraction. Root tips for determination of ^{15}N incorporation were collected after 3 days of germination, and immediately frozen until required.

4-2-5 Protein Extraction of Root tips

Roots tips of 20 seedlings were ground in liquid nitrogen with mortar and pestle and kept frozen until extraction. The grind tissues were extracted with 500µl of TEAB buffer, adapted from Dunkley et al. (2006), consisting of 25mM triethylammonium bicarbonate (TEAB)/ 8 M urea/ 2% Triton X-100/0.1% SDS and protease inhibitor (Dunkley et al. 2006). After 8 minutes incubation on ice, the solution was centrifuged at 14000 x g (4°C), for 20 minutes. The supernatant, containing all the proteins was transferred in a new tube and the concentration of the proteins was evaluated by Bradford reaction (Bradford Reagent, Sigma B6916).

4-2-6 Protein Extraction of Meiocytes

Meiocytes suspensions were extracted with three protocols, respectively called in this study TEAB, TCA protocol (Trichloroacetic acid) and Chromatin isolation. The concentration of the proteins was evaluated by Bradford reaction (Bradford Reagent, Sigma B6916).

TCA protocol (Appendix 12)

Meiocytes suspension from one hundred anthers in meiosis in 1X PBS buffer (containing protease inhibitor) were extracted with an equal volume of the 2X extraction buffer (100mM Tris-HCl pH7.6, 300mM Nacl, 0.2%Tween20, 5mM EDTA, 2mM DTT and Roche protease inhibitor cocktail) on ice. The solution was centrifuged at 14000 x g (4°C), for 20 minutes and the supernatant transferred into a new tube. The proteins were precipitated overnight at 4°C by adding TCA in order to have 20% of TCA in final concentration. The tubes were centrifuged at 14000 x g (4°C), for 15 minutes and the supernatant discarded. The pellet was washed twice in cold acetone, and allowed to air dry. The pellet was either resuspended in SDS buffer for running on a gel, or in water for use in mass spectrometry.

TEAB Extraction (Appendix 10)

Meiocytes from one hundred anthers in meiosis in 1X PBS buffer (containing protease inhibitor) were centrifuged at 14000 x g (4°C) for 2 minutes. The supernatant (S1) was transferred to a new tube and precipitated with 80% cold acetone overnight at 4°C, centrifuged and washed in cold 70% ethanol to keep the protein pellet (P2). The P2 pellet was allowed to air dry in the cold room and re-suspended in 50µl of TEAB buffer. The first pellet (P1), was re-suspended in 100µl of TEAB buffer, incubated for 8 minutes on ice and centrifuged at 14000 x g (4°C), for 20 minutes. The supernatant (S2), containing this time all the proteins was transferred in a new tube.

Chromatin isolation (Appendix 11- Wysocka et al., 2001)

Meiocytes from one hundred anthers in meiosis in 1X PBS buffer (containing protease inhibitor) were centrifuged at 14000 x g (4°C) for 2 minutes. The pellet was extracted in 200µl of buffer A (10mM HEPES (pH 7.9), 10mM KCl, 1.5mM $MgCl_2$, 0.34M Sucrose, 10% Glycerol, 1mM DTT, 0.1% Triton X100 and protease inhibitor cocktail (Roche) on ice and centrifuged at 1300 x g for 5 minutes (4°C). The supernatant was further centrifuged for 20 minutes at 14000 x g (4°C). The resulting supernatant (cytosolic proteins) was precipitated for 1 hour in 80% cold acetone at -20°C, washed twice in cold 70% ethanol, dried, and resuspended in water.

The pellet from the first centrifugation was re-suspended in 100 µl of buffer B (3mM EDTA, 0.2mM EGTA, 1mM DTT and protease inhibitor cocktail (Roche)) and incubated for 30 minutes on ice, then centrifuged at 17000 X g for 5 minutes (4°C). The supernatant was discarded, the pellet resuspended in 100 µl of buffer B, and centrifuged at 14000 x g for 5 minutes (4°C). The last pellet was the insoluble enriched chromatin, and the supernatant was the soluble nuclear proteins. The pellet fraction was resuspended in 100 µl of TEAB buffer before use.

4-2-7 Peptide preparation for mass spectrometry

Proteins in extraction buffer or water were mixed with sample buffer (4X Tris-Glycine SDS, Coomassie G250 and Phenol Red), reducing agent (10X 500 mM dithiothreitol (DTT)) and run on 1D Polyacrylamide Gel Electrophoresis (NuPAGE 4-12% Bis Tris gel, from Invitrogen) for 30 minutes. The gel was stained with colloidal coomassie blue for 1 hour to reveal the proteins and rinsed overnight in distilled water. Protein bands were excised from the gel, and diced into 1mm pieces for in gel digestion (Shevchenko et al. 2006) by trypsin (Promega, Madison, WI). The coomassie blue was destained with 100% Acetonitrile. The samples were reduced in 100 µl of 50mM ammonium bicarbonate containing 10mM DTT (Melford, MB1015) and incubated for half an hour at 57°C. An equal volume of 50mM iodoacetamide (Sigma, 1M49-25g) for alkylation was added to the sample and incubated in the dark at room temperature for 15 minutes. After removing the solution, the gel pieces were rinsed with 50% acetonitrile to further de-stain the gel (at room temperature or 45°C) and washed in 100% acetonitrile to dehydrate the slices.

Proteins were digested by trypsin (Promega Gold, V511A) at 37°C overnight by adding 20 µl of trypsin directly onto the gel pieces and 1ml of 50mM ammonium bicarbonate buffer. The supernatant (containing the peptides) was transferred to a new tube. 100 µl of 100% acetonitrile was added to the gel pieces to extract more peptides and pooled with the supernatant. Peptides were lyophilised and re-dissolved in 20 µl 1% acetonitrile containing 0.1% formic acid before analysis by LC-MS/MS.

4-2-8 Tandem Mass Spectrometry

For the samples run with the LTQ, a surveyor HPLC system was used to deliver a flow rate of approximately 250 nL min^{-1} to the mass spectrometer (LTQ, Thermo Electron Corp.). Peptides were desalted using a precolumn (C18 pepmap100, LC Packings), which was switched in line to the analytical self-packed C18, 8-cm analytical column (Picotip 75 µm id, 15 µm tip, New Objective.). Peptides were eluted by a gradient of 2 to 30% acetonitrile over 40 min. The mass spectrometer was operated in positive ion mode with a nano-spray source and a capillary temperature of 200 ºC, and no sheath gas was employed. The source voltage and focusing voltages were optimised for the transmission of angiotensin. Data dependent analysis consisted of the six most abundant ions in each cycle: MS mass-to-charge ratio (m/z) 300 to 2000, minimum signal 1000, collision energy 25, 5 repeat hits, 300 sec exclusion. Isolation width for MS2 analysis was 2 *m/z*. MS3 was triggered by neutral loss of 32 or 49 from the parent ion. One sample (Cp) was analysed for a second time using a longer LC gradient of 90 minutes and the dynamic exclusion parameters were changed to allow for two repeat hits for the top five parent ions with a 30 second exclusion time using MS2 only. All other parameters remained as before.

For the samples run with the LTQ Orbitrap, a surveyor HPLC system was used to deliver a flow rate of approximately 250 nL min^{-1} to the mass spectrometer (LTQ_Orbitrap, Thermo Electron Corp.). Peptides were desalted using a precolumn (C18 pepmap100, LC Packings) which was switched in line to the analytical self-packed C18, 10-cm analytical column (Picotip 75 µm id, 15 µm tip, New Objective.) after a wash time of 8 min. Peptides were eluted by a gradient of 10 to 50% acetonitrile over 45 min. The mass spectrometer was operated in positive ion mode with a nano-spray source and a capillary temperature of 175 ºC; no sheath gas was employed. The source voltage and focusing voltages were optimised for the transmission of MRFA (methionine–argenine–phenylalanine–alanine) peptide (m/z 524). Data dependent analysis was carried out in orbitrap-IT parallel mode (CID fragmentation) on the 4 most abundant ions in each cycle. The orbitrap was run with a resolution of 30,000 over the MS range from m/z 300 to m/z 2000 and an MS target of $1e^6$ and 1 s maximum scan time. The MS2 was triggered by a minimal signal of 2000 with a target of $2e^4$ and 100 ms scan time. For selection of 2+ and 3+ charged precursors, charge state and monoisotopic precursor selection was used.

Collision energy was 35, and an isolation width of 2 was used. Dynamic exclusion was set to 1 count and 200 s exclusion with an exclusion mass window of -0.5 to +1.5.

4-2-7 Spectrum Analysis

The raw data from the LTQ were exported as a peak list in the Mascot generic format (mgf). All MS/MS data were processed with Mascot 2.2 (Matrix-Science) search engine against the followings databases; TIGR_TA_Triticum 20070918 (2008812 sequences; 411297010 residues) TIGR_TA_Oryzasativa sativa_20060605 (1485096 sequences; 315905038 residues), Triticum aestivum (bread wheat) (2875 sequences), and Oryza sativa (rice) (143022 sequences) both sections of SPtrEMBL sptrembl20070911 (2988110 sequences; 999295482 residues). For all searches, carbamidomethylation (alkylation of the proteins with iodoacetimide) was selected as a fixed modification on cysteine residues. Oxidation of methionine was allowed as a variable modification. The search included a maximum of one mis-cleavage, peptide and fragment Mass Tolerance of ± 1.6 Da and ± 0.4 Da respectively and unrestricted protein mass.

The raw files from the LTQ Orbitrap were processed in Bioworks to generate dta files (group scan 300, 10 ppm, ZSA on (Charge State Algorithm), with and without spectral quality on). The merged dta files were used to search TIGR_TA_Triticum 20070918 (2008812 sequences; 411297010 residues) TIGR_TA_Oryzasativa sativa_20060605 (1485096 sequences; 315905038 residues), Triticum aestivum (bread wheat) (2875 sequences), and Oryza sativa (rice) (143022 sequences) both sections of SPtrEMBL sptrembl20070911 (2988110 sequences; 999295482 residues) database with Mascot 2.2 (Matrix science). The search included a maximum of three mis-cleavages, with a peptide tolerance of 5 or 3 ppm (part per million) and a fragment tolerance of 0.6 Da (one ^{13}C isotope).

Data with score higher to 20 and with at least two different peptides appearing for the first time in the search with a high top ranking peptide were validated as potentially good protein hits. Results were compared between databases to remove the last redundancy results, and validate the final proteins of the sample. The whole process is summarized in the figure 21.

4-3 -Results and discussions

4-3-1 Protein preparation

In wheat, meiosis takes only 24 hours to complete and prophase I takes about 17 hours to complete (Bennett 1972). Therefore, the plants were collected mostly in the morning for early stages of meiosis, and in the afternoon for later stages of meiosis. This enabled us to have anthers at different stages of meiosis in the same day, and prepare an adequate amount of meiocytes within a week or two. In one week, 100 anthers could be collected at early prophase, which was enough to extract proteins for one or two runs with the mass spectrometer. The aceto-carmine staining shows that the meiocytes are synchronized (Figure 20) in the anther. Meiosis is temperature sensitive (Bennett 1977, Lu and Chiu 1978, Gabara 1980), and storing the anther on ice for a few hours is reported to slow down dramatically or arrest the mechanism; this helps to extract meiocytes at the desired meiosis stage. To verify this, a few anthers at different stages of meiosis were kept at 4ºC in the dark on a wet filter paper for one or two days. After squashes, the anthers were still at the same stage of meiosis which suggests that the chromatin proteins are still bound to the chromatin. The anther sizes and meiosis stages are usually correlated (Sanchez-Moran et al. 2005, Crismani et al. 2006), and the size of the anther is different between prophase and metaphase. However, in order to dissect the sub stage of prophase, it is necessary to evaluate the chromosome conformation. The meiosis stage of one of the three anthers of the flowers was checked, and the two remaining anthers were kept on ice before meiocyte isolation, which ensured a homogeneous preparation of meiocytes at a single stage of meiosis.

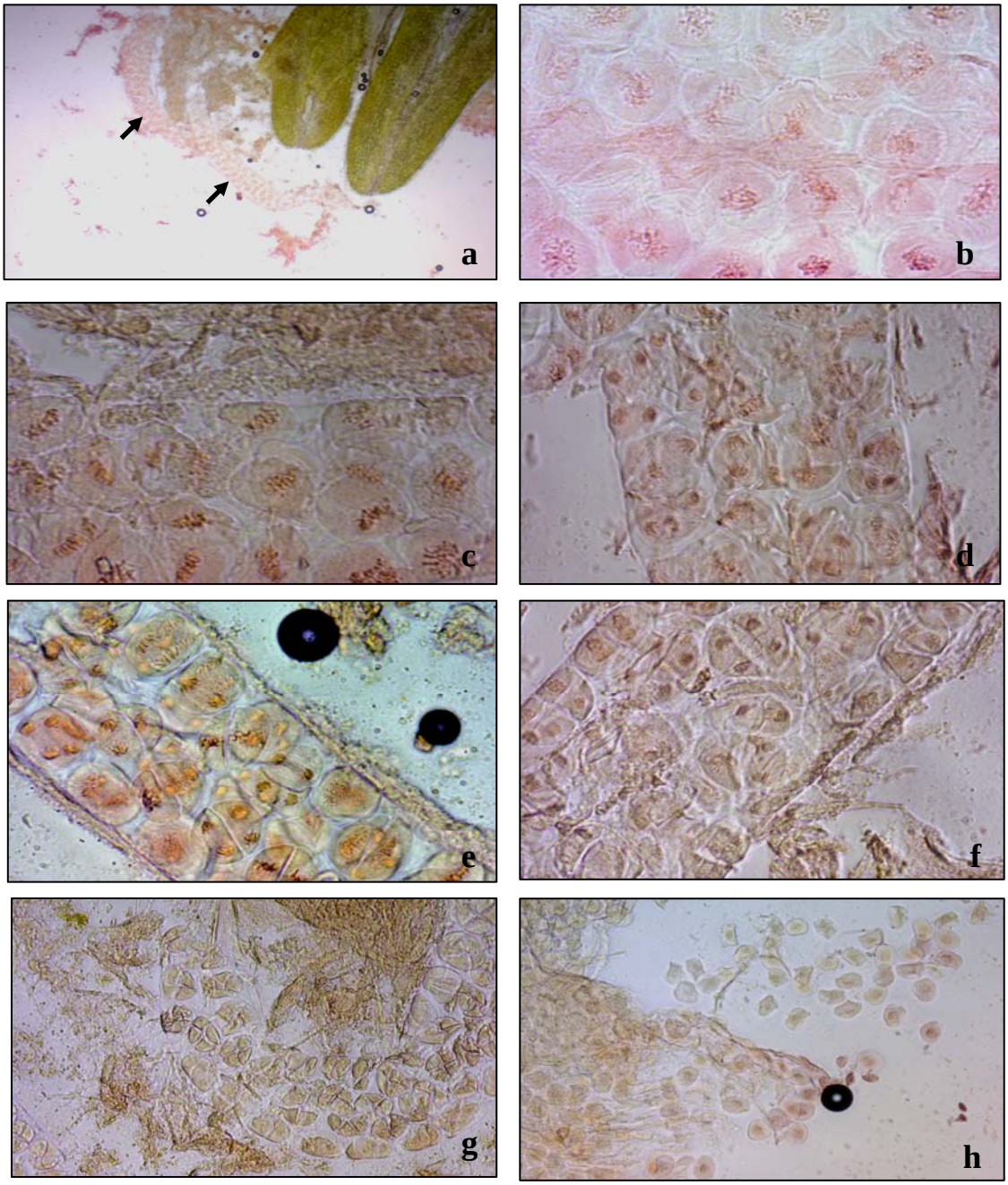


Figure 20: Meiosis staging in hexaploid wheat (Triticum aestivum).

(a) Spread of the whole anther at 10X magnification. The arrows indicate the meiocyte column extracted from the anther loculi when gently squashed. The other pictures are 40X magnification of meiocyte columns at various stage of meiosis: (b) Diakinesis, (c) Metaphase I, (d) Anaphase I, (e) Metaphase II-Anaphase II, (f) Telophase II, (g) Tetrads, (h) Young pollen.

The first question to answer for this study was to choose the starting material, and the quantity needed to extract enough protein to run onto the mass spectrometer. After extraction of meiocytes from formaldehyde fixed anthers, it is possible to count the number of meiocytes per loculus. This was approximately 100 meiocytes for one locule wich corresponds to 400 meiocytes for one anther. Therefore, for 100 anthers, I estimated a total of 40,000 meiocytes per sample, which is large enough for protein extraction if we are able to extract proteins from all of them. Therefore, 100 anthers were soaked in 1X PBS (containing protease inhibitor) for a few hours and the meiocyte purification was performed under a stereomicroscope. After PBS treatment, the structure of the meiocyte bags is still visible on the slides (Figure 21a), all the meiocytes remain at the same stage (Figure 21b), and chromatin is visible (Figure 21c).

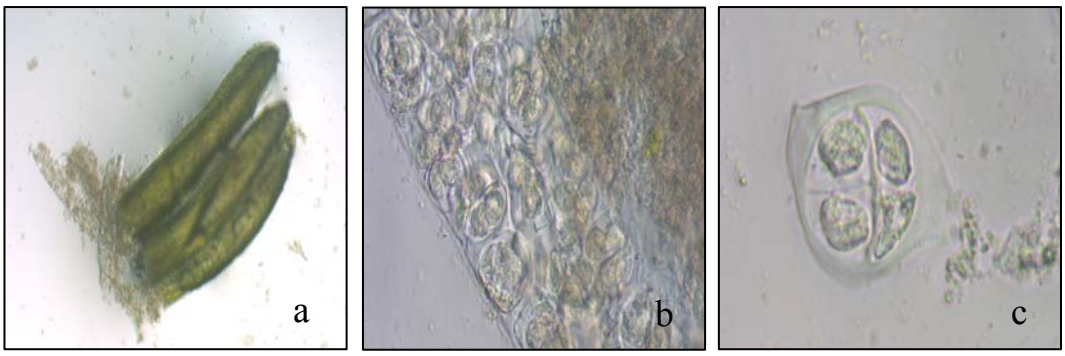


Figure 21 : Meiocyte Isolation after PBS treatment.

The anthers have four loculi, each containing a "bag" of synchronized meiocytes. Arrows show the meiocyte "bag" isolated from the anther. (a) Extraction of the meiocyte column after PBS (Mag.10X). (b) Meiocyte column after PBS treatment, extruded form the anther (Mag.40X), the meiocytes are still visible. (c) Isolated meiocyte at tetrad stage after PBS treatment, chromatin is still visible.

In early stages of meiosis, the meiocytes seem to be surrounded by a "jelly" substance that sticks them together (Figure 22A, c,d). This mass of cells also contains tapetal cells and some other somatic cells, which are impossible to remove. Isolation of meiocytes is much easier when the anthers are fixed in 4% formaldehyde. The structure of the meiocytes bag and each individual meiocytes remained intact and well stained with DAPI (Figure 22A, a,b). Thus, three samples were compared together. The first sample is meiocytes suspension from 6 fixed anthers in para-formaldehyde (Figure 22B, gel line 1), the second sample is meiocytes suspension from 100 anthers fixed in para-formaldehyde fixed anthers (Figure 22B, gel line 2) and the third samples is meiocytes from 100 anthers soaked 1h in 1X PBS (Figure 22B, gel line 3).

All the three samples were denatured by SDS and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel under denaturing conditions. When stained with Coomassie blue, down to 0.2 μg of protein can be detected (Berg 2002). An aliquot of 10μl of each mixture was run onto an SDS gel for 1 hour. No proteins were visible on the gel for the two samples of fixed anther (Figure 22B, gel line 1 and 2), which means the extraction of proteins from fixed material might be not possible. This is likely because formaldehyde fixes the tissue by cross linking between proteins. In comparison, a large number of proteins were visible on the gel after a simple isolation of unfixed anthers from PBS buffer (Figure 22B, gel line 3).

TCA has already been used to extract or more recently to concentrate proteins from plant meiocytes (Sanchez-Moran et al. 2005, Phillips et al. 2008). However, I found that precipitating the proteins by TCA resulted in a pellet which was very hard to resolubilise, and then digested poorly with trypsin. Therefore, the extracted proteins were kept in the extraction buffer to avoid the TCA precipitation step, and a small aliquot run onto a 1D SDS gel. Bands of proteins were cut out and the trypsin digestion was performed in gel (Speicher 2000). This method has the advantage of cleaning the proteins mixtures from any detergent or chemicals that could interfere with the mass spectrometer, and also to determine the quality of the proteins and concentrate the sample.

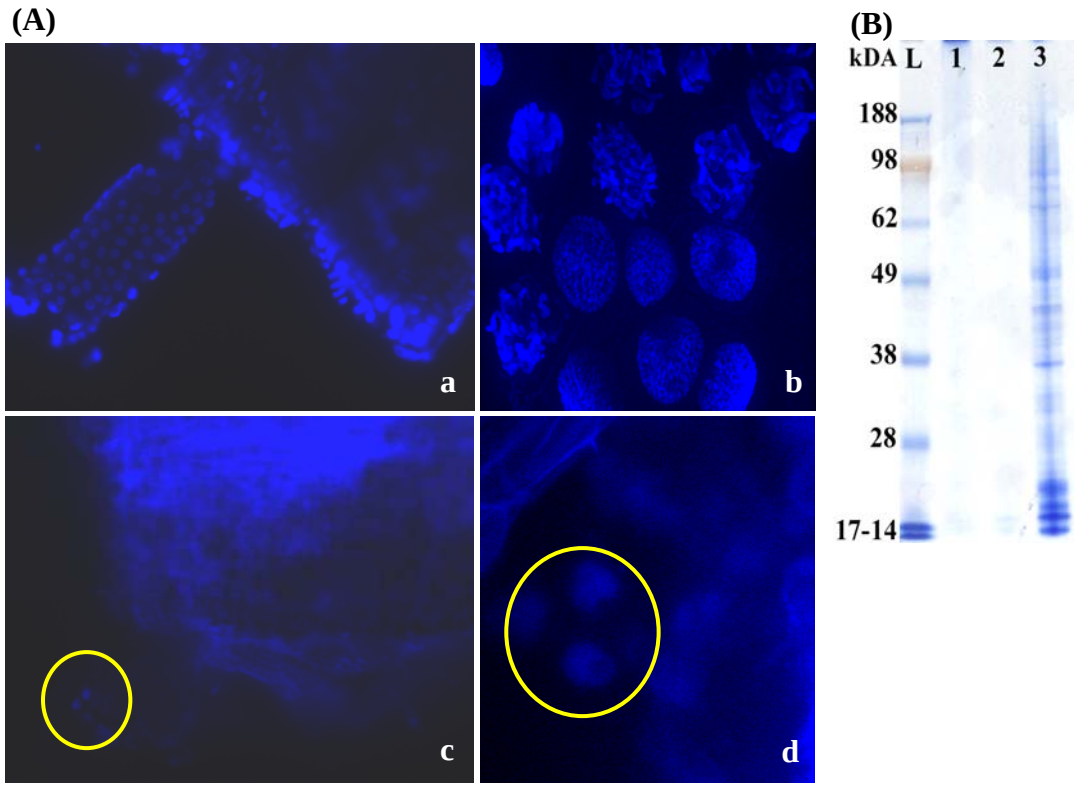


Figure 22: Extraction of wheat meiocytes.

Meiocyte bag (column) from a para-formaldehyde fixed anther stained with DAPI (a), the chromatin structure is well preserved (b). After PBS treatment (c,d), in young anther, the meiocytes (yellow circles) are embedded in a jelly, which is difficult to remove. Ladder: Invitrogen Seeblue Plus 2 (L); Proteins of isolated meiocytes from 6 para-formaldehyde fixed anthers (Line 1), proteins of 100 para-formaldehyde fixed anthers (line 2) and proteins from 100 anthers squashes in 1xPBS (line 3). The samples were simply denatured and loaded (5µl) onto the gel (Nu PAGE 4-12% Invitrogen). The gel was stained with coomassie blue overnight.

The chromatin extraction method was used to isolate proteins associated with chromatin. In this test, the chromatin of meiocytes from 100 anthers was isolated by small-scale biochemical fractionation leading to three protein mixtures: a soluble cytosolic fraction, a soluble nuclear fraction and a chromatin enriched fraction (Wysocka et al. 2001). The proteins from the soluble nuclear fraction (Figure 23, line 5) and the chromatin fraction (Figure 23, line 6) were run onto a gel to evaluate the quality of the proteins. The quantity of protein obtained from the soluble nuclear fraction remained very low as judged by coomassie staining, and very few proteins (5 matches) were identified after mass spectrometry analysis. The quantity of the starting material might be the reason for this low level of proteins. On the other hand, quality of the fraction of the chromatin purification seems enough to visualize a reasonable number of proteins.

Many proteins, particularly proteins bound to other structures such as chromatin, are not easily soluble and the use of a chaotropic agent (urea) and detergent (like SDS, etc…) are essential in the extraction to solubilise the maximum number of proteins. The sample in Figure 23, line 1 corresponds to the proteins from 100 anthers ground in liquid nitrogen and extracted in 8M Urea + detergent (0.1% SDS) in a phosphate buffer. Sample on Figure 23, line 2 correspond to the proteins from 100 anthers ground in PBS solution and extracted in 8M Urea + detergent (0.1% SDS) in a phosphate buffer. Samples on Figure 23, line 3 and line 4 correspond to the proteins from a meiocyte suspension from 100 anthers after extraction by 8M Urea + detergent (0.1% SDS) in a phosphate buffer. However, the meiocytes suspension was centrifuged at low speed before extraction to pull down the cells and allow work separately on the pellet and the supernatant.

The cell pellets (Figure 23, line 4) containing the non solubilised proteins or chromatin proteins was extracted in 8M Urea + detergent (0.1% SDS) in a phosphate buffer, while the supernatant was collected in a new tube and precipitated by acetone to concentrate the proteins already solubilised in phosphate buffer (Figure 23, line 3). Fractionating the sample before extraction by centrifugation ensured that most of the proteins will be extracted from the tissue and helped to concentrate the sample (Figure 23, line 4). Therefore, TEAB extraction was my extraction of choice for this study.

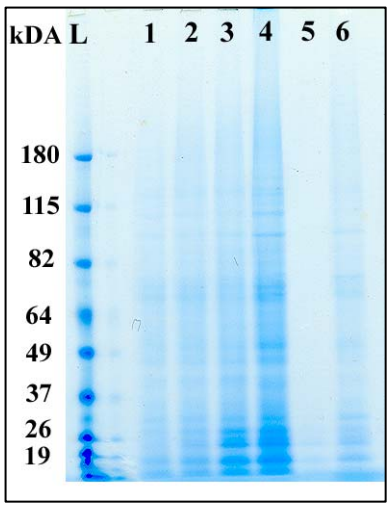


Figure 23: Protein extraction of wheat meiocytes from 100 anthers in late meiosis (from MI to tetrads) for mass spectrometry analysis.

Ladder: Invitrogen BenchMark™ Pre-Stained Protein (L); TEAB Extraction of 200 µf of meiocytes suspension from 100 anthers (1), TEAB Extraction of 20 whole anthers (2), TEAB Extraction of meiocytes pellet from 100 anthers in PBS buffer –fraction S2 (3), TEAB Extraction of meiocytes supernatant from 100 anthers in PBS buffer –fraction S1 (4). Chromatin Protocol Extraction (5,6), Pellet from extraction of 100 anthers (5), supernatant of extraction of 100 anthers (6). Samples 3 and 4 gave the best results in Mass Spectrometry (LTQ) for the analysis of the total protein mixture.

In a parallel experiment, despite the different size of anthers between species, I have shown the possibility of rice and brachypodium meiosis staging, which could lead to a similar study in these two species (Figure 24). Because the anthers are much smaller, extraction of meiocytes might not be possible as it is in wheat, and in this case, the whole anther will have to be used for analysis. However, as these two genomes are sequenced, the analysis might result in better protein identification. Although little is known about genetic control of rice meiosis, rice is still the best model for monocot plants, thus information for meiotic proteins in rice is already available (Jenkins et al. 2008). The big size of the wheat nucleus is an advantage in microscopy; here I show that it is also an advantage obtaining purified proteins for analysis.

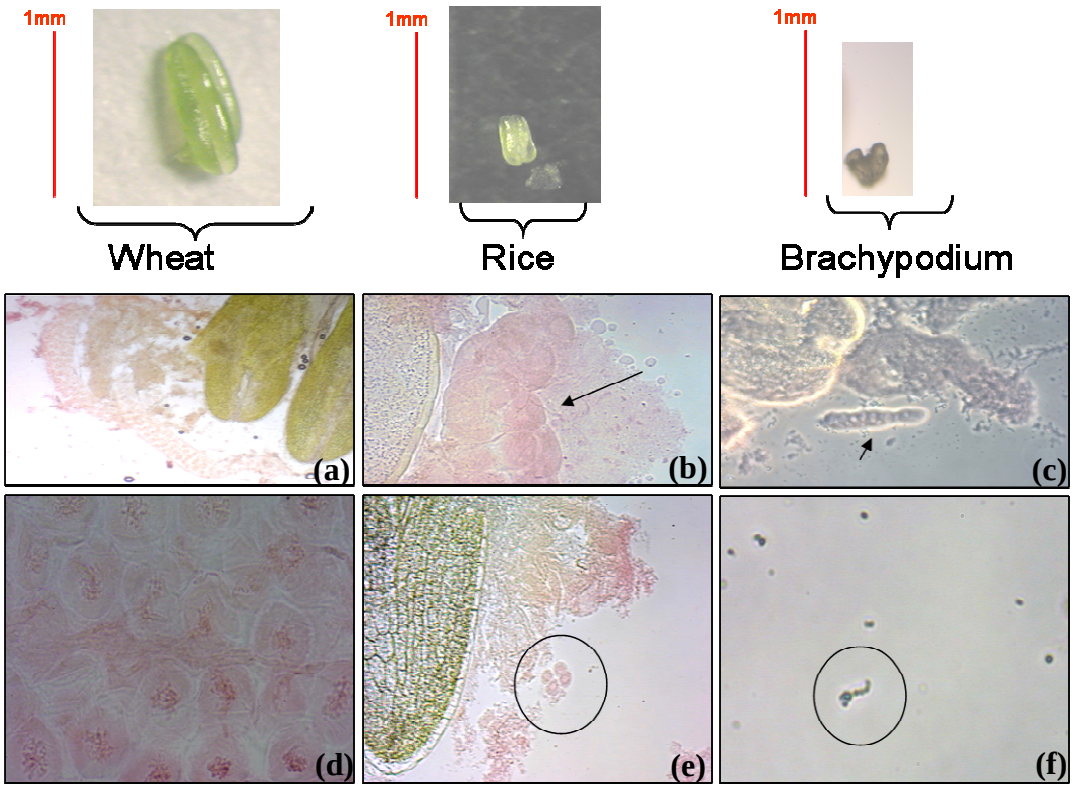


Figure 24: Anthers from wheat, rice and brachypodium

Anthers from wheat were squashed to reveal the meiocytes in a "bag" (a) at prophase (d). Anthers from rice were squashed to reveal the meiocytes in a "bag" at before meiosis (b) and tetrad stage (e). Anthers from brachypodium were squashed to reveal the meiocytes in a "bag" at before meiosis (c) and metaphase I stage (f).

4-3-2 Mass spectrometric analysis of complex mixtures

Before LC-ESI-MS/MS, the proteins were first separated on a polyacrylamide gel (SDS or 2D –gel), which has the advantage of cleaning the sample from salt or detergent, which can interfere with the mass spectrometer, concentrating the proteins, and reducing the complexity of the mixture. The proteins are then digested by enzyme to generate peptides for the analysis. Trypsin is the most used because it has the advantage of being very stable and specific which is valuable for protein identification. It cuts after arginine and lysine, two of the basic amino acids to generate a population of peptides carrying 2 or 3 positives charges which fragments separate well in the MS (Kuster et al. 2001).

The peptides are injected into a High Performance Liquid Chromatography (HPLC or LC) C18 reverse phase column to be separated by hydrophobicity under a solvent gradient. At the end of the column, the peptide fractions go through a fine needle to be converted to a spray by the ionization source (ESI) of the mass spectrometer to be ionized under the strong electric field applied in the chamber. The ion trap samples the positive ions (positive droplets which correspond to the charged fragment) and sequentially performs either intact mass analysis (MS1) or collects and then fragments a specific ion/peptide (MS2). Each time the trap is empty from the previous collected ions the mass spectrometer refills with ions from the spray. Figure 25A summarizes the steps in the analysis.

The proteome of a tissue or organelle generates a very complex protein mixture of different sizes. In addition, it contains abundant and less abundant proteins, which is another challenge for protein analysis of the mixture. Therefore, fractionation steps are crucial for a successful analysis and this can be done at the protein level with an electrophoresis gel, and/or at the peptide level with HPLC. Figure 25B summarizes the steps from the material preparation to the protein analysis used in this study.

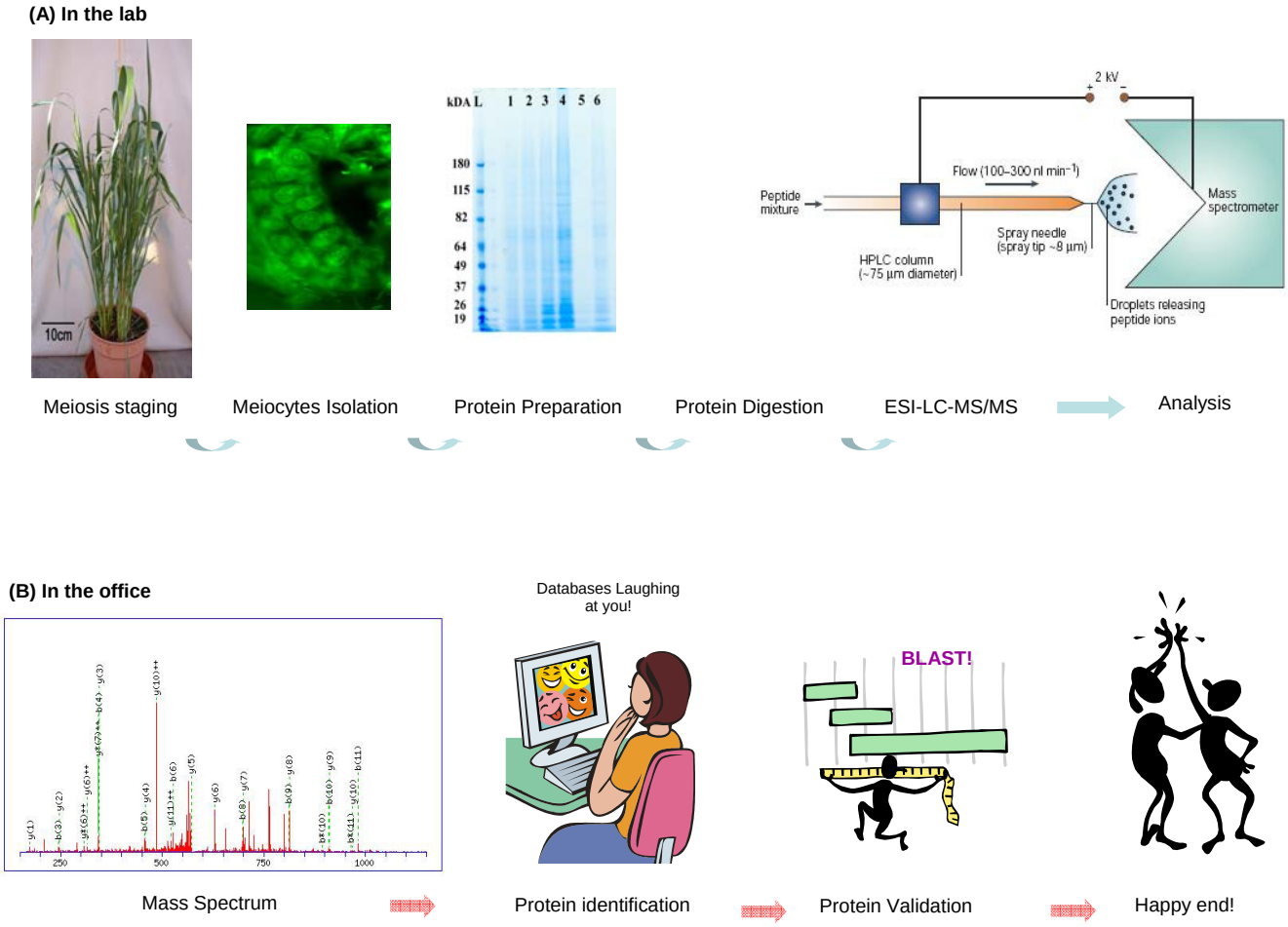


Figure 25: From plant to meiocyte proteome with mass spectrometry in wheat

The anthers are collected for different stages of meiosis and the meiocytes are isolated. The protein extract is separated on a polyacrylamide gel in 1 dimension, and digested by a proteolytic enzyme (trypsin). The resulting peptide is process by ESI-LC-MS/MS and the resulting mass spectrum is analysed by computer. Proteins are identified by using search engines such as Mascot. Peptides/proteins of interest can be further analysed by the BLAST tool

The gradient time is calculated based on the diameter/length of the C18 reverse phase column and at a flow rate of 250 nL min^{-1} delivered by the HPLC system, so 40-45 minutes was previously estimated as a standard gradient for moderately complex samples. However, a complex mixture contains thousands of peptides that are sometimes eluted around the same times. Therefore increasing the LC runtime gives a better petides separation, and thus a better identification.

The meiocyte proteins from 100 anthers (Figure 23, lane 3) were separated for 40 minutes (short run) by HPLC before running on the linear ion trap LTQ (LTQ, Thermo Electron Corp) using the parameters described in the methods. The MS raw data showed the presence of a very large number of peaks with high intensity (and small background), which is likely to indicate the presence of good quality peptides in sufficient amounts. The LTQ data contained 1391 MS2, of which we could unequivocally identify about 134 peptides in a wheat EST database, giving about 39 confident protein identifications (Table 5). The low amount of protein in the starting sample is therefore not a problem for the mass spectrometer to detect the presence of proteins in the complex mixture.

The same sample (Figure 23, line 3) was analyzed again using a longer LC gradient of 90 min aiming to reduce the complexity of the sample by allowing more time for the peptides to be separated. The raw data spectrum gave a similar result to the shorter run, but the LTQ data contained this time 1493 MS2, of which we could unequivocally identify about 249 peptides in the wheat EST database, giving about 85 confident protein identifications (Table 5). These results confirm that there were a high number of peptides running at the same time due to the complexity of the sample, which can affect the detection of low abundance peptides (and therefore proteins). However, a longer elution run from the LC column improved the separation of the peptides before MS analysis, leading to better protein identification.

Similarly, peptides prepared from root tip proteins were separated with 1 and 6 hrs LC runs and analysed with an ion trap MS. A total of 914 and 3315 MS2 respectively were analysed with Mascot resulting in about 50 proteins validated from the one hour run and 149 proteins from the 6h runs. This result shows again that the number of identified proteins is three times more than a shorter run, confirming that a longer LC gradient leads to a better peptide separation and detection. More interestingly, no histones could be indentified within the 149 proteins from the root sample in either after the short or long LC run (Appendix 13). However, in the meiocyte sample searched against the same database (SPtrEMBL), the histones H4, H2A and H2B were identified in the first 20 proteins hits even with a relatively short run.

Meiocyte proteins of one hundred anthers in prophase were separated on a 1D-SDS gel under denaturing conditions. To further reduce the complexity of the sample, the gel was cut into 5 pieces. The resulting peptides from each fraction were separated for 45 minutes on a LC gradient (short run) and analysed with the LTQ_Orbitrap. The LTQ_Orbitrap data contained 4094 MS2, from which we could unequivocally identify about 623 from the wheat EST database, giving about 277 confident protein identifications (Table 5). Thus, four times more peptides were separated on the Orbitrap after pre-fractionation, resulting in three times more validated proteins with a better mass accuracy. Although the percentage of peptides assigned to a protein compared to those not assigned was similar for the two mass spectrometers (15%), we can be more confident about the proteins identification with the Orbitrap.

Run	Total Queries	Peptide not assigned	Peptide assigned	% identification	Validated Proteins	Protein range
LTQ						
1 H	1391	1257	134	11	39	6-200kDa
2 H	1493	1244	249	17	85	6-200kDa
LTQ_Orbitrap						
1	1151	1064	87	8	*	6-20kDa
2	607	501	106	17	*	20-40kDa
3	1024	860	164	16	*	40-70kDa
4	935	751	184	20	*	70-120kDa
5	377	295	82	22	*	120-200kDa
Total	**4094**	**3471**	**623**	**15**	**277**	

Table 5: Comparison of the analysis of complex protein mixtures on LTQ and LTQ_orbitrap instruments

A similar percentage of peptides were identified on the both instruments. However, a pre-fractionation of the proteins by SDS gel, followed by a fractionation of the peptides by reverse phase liquid chromatography resulted in more peptides analysed by MS, thus more proteins identified.

Peptides separated with a 40 minute LC gradient and searched against the EST Triticum databases gave around 20-25% of protein coverage for an histone-like protein (human) with less than 10 peptides assigned to the protein hit. However, a 90 minute LC gradient with the same MS conditions led to 37% coverage and 3 times more peptides assigned to the protein (Table 6). Similar results are found for histones H2A, H2B and H4 and this is true whatever the database (EST Triticum and SPtrEMBL). More interestingly, in the chromatin fraction run for 1h, histone H1, H2A, H2B and H4 were identified, but Histone H3 was missing. In the same fraction run for twice as long, Histone H3 became detectable, which means that the peptides of this protein were obscured by more abundant or co-eluting peptides. Table 7 also shows the detection of phosphorylation sites of H2B and H3, even with low protein coverage indicating that the extraction method is suitable for detection of post-translational such as phosphorylation. Running the sample on a longer LC gradient not only has the advantage of reducing the sample complexity for protein identification but also has the advantage of increasing the percentage of protein coverage for each identified protein. This is important for post-translational modification, which requires the best coverage possible of a protein to be able to detect all the possible modifications of this particular protein and fractionation is one of the ways to increase the coverage.

To summarize, extraction of the proteins from purified meiocytes gave enough material to identify proteins by mass spectrometry. A pre-fractionation of the proteins mixtures by electrophoresis (SDS gel) and a longer LC gradient to separate the peptides before MS/MS gave the best results. About 70% of the identified peptides were from proteins that are clearly nuclear and chromatin components. The identification of the histones indicates a good enrichment of chromatin.

EST Triticum		
	Supernatant 1H LC	
Protein description	**Coverage**	**Peptide assigned**
CK154925 Homolog of Homo sapiens ~~Histone 1	25.1	7
TA179_4568 Histone H2A.2.1 related cluster	14.8	2
DR737114 Histone H2B.2 [Triticum aestivum (Wheat)]	4	1
	Chromatin fraction 1H LC	
Protein description	**Coverage**	**Peptide assigned**
TA60832_4565 Homolog of Homo sapiens ~~Histone 1	21.1	6
TA179_4568 Histone H2A.2.1 related cluster	14.8	4
CJ663026 Histone H2B.2 [Triticum aestivum (Wheat)]	10.2	5
	Chromatin fraction 2H LC	
Protein description	**Coverage**	**Peptide assigned**
BJ235270 Homolog of Homo sapiens ~~Histone 1	37.4	34
Histone H2A.2.1 [Triticum aestivum (Wheat)]	16.1	3
CA631173 Histone H2B.2 [Triticum aestivum (Wheat)]	13.4	3
CV779037 Histone H3 [Triticum aestivum (Wheat)]	12.9	4
SptrEMBL		
	Supernatant 1H LC	
Protein description	**Coverage**	**Peptide assigned**
Q1RU62 Histone H4.	47.6	7
A5AFR3 Histone H2A.	21.5	1
	Chromatin fraction 1H LC	
Protein description	**Coverage**	**Peptide assigned**
A8MRV1 Histone H4.	50	8
A5AFR3 Histone H2A.	21.5	3
A8JJQ6 Histone H2B variant (Fragment).	9.9	7
	Chromatin fraction 2H LC	
Protein description	**Coverage**	**Peptide assigned**
A6N0V4 Histone h4 (Fragment).	65.9	16
A8JJQ6 Histone H2B variant (Fragment).	15.8	7

Table 6: Histone coverage in a protein mixture of meiocytes analysed with the Ion Trap.

Run Time	Protein	Coverage	Peptide mass experimental	Peptide mass calculated	Peptide delta	Peptide sequence	Peptide Variable modification
2 Hours	Histone H2B.2	10.7	938.94	938.58	0.36	LVLPGELAK	
			1808.08	1808.81	-0.73	AMSITNSFINDIFEK	Phospho (ST)
2 Hours	Histone H3	12.9	1016.19	1015.59	0.6	FRPGTVALR	
			1016.33	1015.59	0.74	FRPGTVALR	
			1016.51	1015.59	0.92	FRPGTVALR	
			1335.41	1334.68	0.73	EIAQDFKTDLR	
			1383.5	1382.67	0.83	ESLGAARPFISR	Phospho (ST)

Table 7: Peptides Phosphorylation site detection

4-3-3 Analysis and Protein Validation

When digested, the protein backbone can break at different places giving neutral fragments and charged fragments and the mass analyser can only detect the charged one. There are six possible fragment ions detectable by the mass analyser for each amino acid residue, named a, b, and c" ions if the charge is retained on the N-terminal fragment, and the x, y", and z ions if the charge is retained on the C-terminal fragment (Figure 26) according to the fragment ions nomenclature established by Roepstorff and Fohlman (Roepstorff and Fohlman 1984, Biemann 1992).

The ion trap instrument provides a low energy collision into peptides which mainly cleaves the amide bond generating mostly b and y ions. The b and y ion information usually provides enough information to deduce the peptide sequence by calculating the difference between two adjacent b or y ions (Figure 27) to determine each amino acid. The MS2 data, which contain the fragment ion information, are analyzed by computer and compared against a database of previously sequenced proteins (or a translated nucleotide sequence database) in order to determine the sequences of the fragments.

SEQUEST (Eng et al. 1994, MacCoss et al. 2002) is the first algorithm developed to identify each tandem mass spectrum individually. It artificially generates a peptide and its theoretical tandem mass spectrum from the database candidate sequence which has the same mass as the precursor ion, compares each theoretical spectrum to the observed tandem mass spectrum and estimates the best match of the two sequences. There are several other software packages to analyse MS/MS data such as Mascot (Perkins et al. 1999), X!Tandem (Craig et al. 2004), OMSSA (Geer et al. 2004) or PEAKS (Apolinarska 2003) for *de novo* sequencing. This chapter will focus on tandem mass spectrum analysis by Mascot.

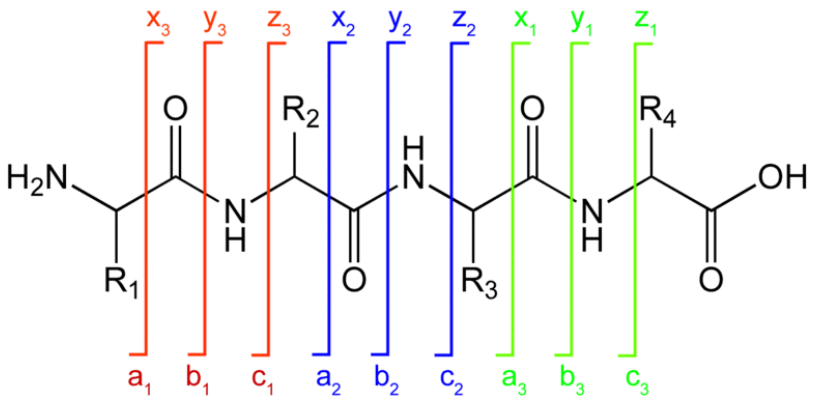


Figure 26: Types of ions generated after peptide fragmentation

This peptide of 4 amino acids can be broken at 9 sites and results in a series of 3 sets of a,b,c ions on the N-terminal side and x,y, and z ions on the c-terminal side.

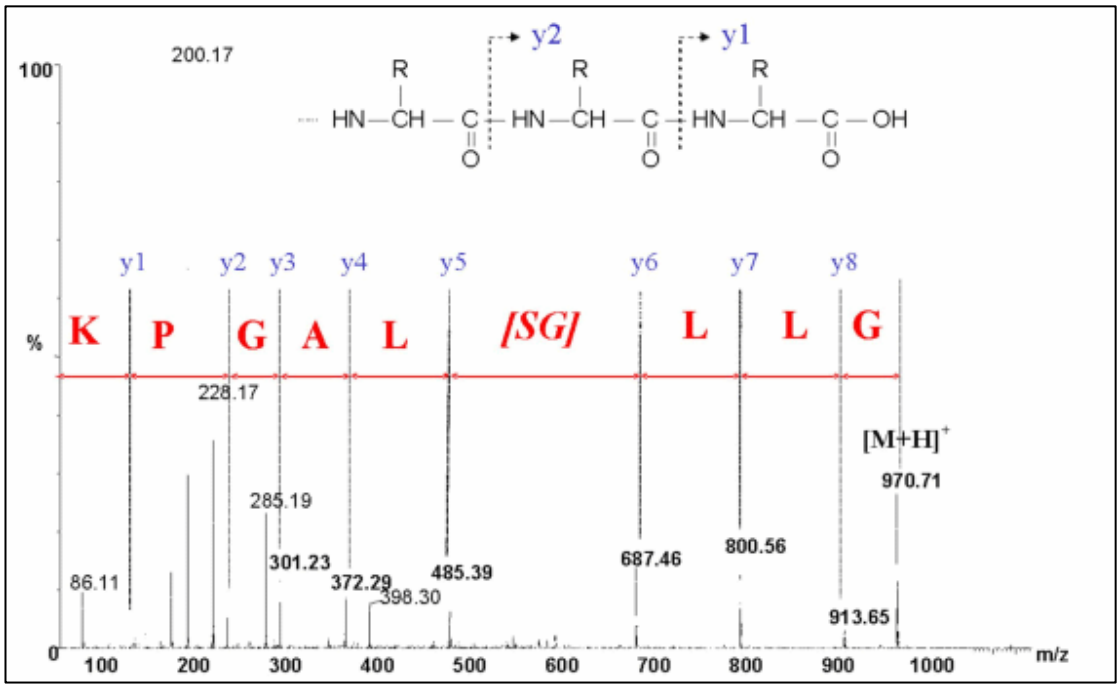


Figure 27: Mass spectrum of the peptide fragment GLLGSLAGPK.

The mass difference between y ions determines the sequence from C-Terminal to N-terminal side of the peptide GLLGSLAGPK. (*Image from www-helix.inrialpes.fr)*

The MS2 data are submitted to Mascot in a form of peak list, which contains, for each peptide, the mass of the precursor ion (MS1) and the mass of its fragment ion (MS2). Like SEQUEST, Mascot selects peptides in the database with a similar mass to the precursor ion, compares the theoretical spectrum of the peptide sequence to the observed tandem mass spectrum and determines the best matching sequence.

Protein identification of unsequenced organisms is a difficult task, but not impossible, especially for cereal genomes in which there is good conservation of genes and gene orders (Moore et al. 1995). The *Ph1* locus was mapped using alignment of brachypodium (sequenced genome available in few months), rice (sequenced genome) and wheat (Griffiths et al. 2006). Thus, peptides were searched against SPtrEMBL database, which is a fusion between Swiss-Prot and TrEMBL (**Tr**anslation of **EMBL** nucleotides) and TIGR Plant Transcript Assemblies (TA) databases of wheat, rice and brachypodium.

Swiss-Prot was developed in collaboration between the University of Geneva and the EMBL Data Library (the European Bioinformatics Institute). This is an annotated universal database which provides a high level of manual annotation (description of the function of the protein, domain structure, post-translational modifications and variants), a minimal level of redundancy, a high level of integration with other biomolecular databases and extensive external documentation (Bairoch and Apweiler 1997).

TrEMBL is a protein sequence database supplementing Swiss-Prot, consisting of computer-annotated entries in Swiss-Prot-like format, derived from the translation of coding sequences in the EMBL nucleotide sequence database (Bairoch and Apweiler 1997). In addition to the SPtrEMBL database, protein identification of complex mixture will benefit from the use of DNA-derived databases such as Expressed Sequence Tags (EST) databases or cDNA databases even if they usually are highly redundant.

The TIGR plant transcript assemblies (TAs) (Childs et al. 2007), contain the sequences of expressed transcripts collected from EST and the NCBI GenBank nucleotide database (full length and partial cDNAs) . Each nucleotide sequence is automatically annotated with the best protein alignment, which is also the reason of the redundancy in such databases.

EST databases contain the proteins predicted from mRNA (cDNA) sequences. However, you can have a protein in the cell when its mRNA is no longer present or presence of mRNA but no translation into protein. Therefore, most of the ESTs don't give protein sequence. On the top of this, for low abundance proteins, mRNA is often not detected; thus the predicted protein will be missing, which does not make EST databases perfect for protein identification. Nevertheless, using ESTs is still a powerful approach for organisms lacking genome sequences, as already demonstrated in maize, ginseng (Panax ginseng C. A. Meyer) and other species such as wheat (Porubleva et al. 2001, Nam et al. 2003, Song et al. 2007).

Twenty anthers were ground in liquid nitrogen, the protein extracted with TEAB (figure 23, line 2) and digested by trypsin. The resulting peptides were analysed on the LTQ after a short LC gradient (40 minutes). The MS2 were searched against the wheat, rice and brachypodium TIGR_TA databases and against SPtrEMBL for wheat and rice species (Table 8). 100 proteins were identified for which 40 were the best match with 2 or more different peptides with very high score. Of the remaining 38 proteins, 33 were identified using TIGR_TA *Triticum* database and 5 with TIGR_TA *Oriza*, TIGR_TA *Brachypodium* and SPtrEMBL. Nine of the proteins were identified in common when searching against TIGR_TA *Triticum* and SPtrEMBL. Therefore, TIGR_TA *Triticum* database is our best choice of database at the moment although protein identification benefit from a search against several databases.

	Protein	TIGR_TA Triticum	SPtrEMBL Triticum	TIGR_TA Oriza	SPtrEMBL Oriza	TIGR_TA Brachypodium
1	Histone H2B	CJ663026	*	AU031294	*	*
2	Histone H2A.2.1.	*	P02276	*	*	*
3	Phosphoglycerate kinase, cytosolic	TA2655_4568	Q850M3	*	*	*
4	Eukaryotic initiation factor 4A	TA280_4571	*	*	*	TA374_15368
5	ATP synthase beta subunit	TA64894_4565	Q41534	TA32252_4530	Q0DG48	TA668_15368
6	ATP synthase CF1 alpha chain	TA88968_4565	Q332R4	TA36343_4530	P0C520	*
7	40 kDa PI 8.5 ABSCISSIC acid-induced protein	TA121_4571	*	*	*	*
8	ADP,ATP carrier protein, mitochondrial precursor	TA127_4571	Q41628	TA35896_4530	*	TA87_15368
9	Mitochondrial prohibitin complex protein 2	TA3010_4568	*	*	*	*
10	DnaK protein	TA62916_4565	*	TA52186_4530	*	TA59_15368
11	Anther-specific protein YY2	TA68614_4565	*	TA41135_4530	*	TA1044_15368
12	ADP-ribosylation factor	TA180_4571	Q5XUV1	AU030148	*	TA490_15368
13	Chlorophyll a/b-binding protein CP26	CA681670	*	*	*	*
14	Putative elongation factor 2	TA59983_4565	Q7XYB6	*	*	TA385_15368
15	Putative 40S ribosomal protein S2	TA58189_4565	*	*	*	DV486964
16	Mitochondrial chaperonin-60	TA65072_4565	*	*	*	*
17	Proliferating cell nuclear antigen	TA74309_4565	*	TA36406_4530	*	*
18	Putative dihydroflavonol reductase	TA76131_4565	*	*	*	*
19	Putative prohibitin	TA66637_4565	*	*	*	*
20	ADP,ATP carrier protein, mitochondrial precursor	TA126_4571	*	*	*	*
21	Putative chaperonin 60 beta	TA668_4571	*	*	*	TA7_15368
22	Putative rubisco subunit binding-protein alpha subunit	TA68359_4565	*	CF955904	*	*
23	5-methyltetrahydropteroyltriglutamate --homocysteine S- methyltransferase	TA51576_4565	*	*	*	DV485908
24	Heat shock protein 70	TA51525_4565	Q9SAU8	*	*	*
25	Cytosolic heat shock protein 90	TA1868_4568	Q0Q0I7	*	*	*
26	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	TA1831_4568	A5YVV3	*	*	*
27	Mitochondrial aldehyde dehydrogenase	TA54149_4565	*	*	*	*
28	Photosystem II 44 kDa reaction center protein	TA65639_4565	*	TA35050_4530	P0C362	*
29	Vacuolar H+-pyrophosphatase	TA63572_4565	*	*	*	*
30	Alpha tubulin-2A (Alpha tubulin-2D) (Alpha tubulin-2B).	*	A4K4Y1	TA35013_4530	*	*
31	ATP citrate lyase a-subunit	TA52076_4565	*	*	*	*
32	Calcium-binding protein precursor	TA50055_4565	*	*	*	*
33	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	TA1831_4568	*	TA35281_4530	*	*
34	Mitochondrial aldehyde dehydrogenase	TA53952_4565	*		*	*
35	Fructose 1,6-bisphosphate aldolase	TA130_4571	*	AK100371	*	*
36	Photosystem II P680	*	P24065	*	*	*
37	Protein disulfide isomerase (EC 5.3.4.1) (Fragment).	*	Q6JAB5	*	*	*
38	Heat shock protein 81-3	*	*	TA32803_4530	*	TA85_15368

Table 8: Database comparison

Most of the proteins were identified using the TIGR_TA Triticum database. Proteins number 2, 30, 36, 37 and 38 were identified by other database such as SPtrEMBL in addition to the EST database identification. The use of several databases increases the chances of protein identification, and their validation.

Histone proteins were identified in the top matches, indicating that isolating the meiocytes might be not necessary to study wheat meiosis. It might be possible with frozen anthers as a starting material which could have the advantage of being easier to handle, and less time consuming as 20 anthers were enough to get proteins compared to the 100 anthers necessary for meiocyte isolation. However, proteins involved in photosynthesis (Table 8, lines 13, 22, 28 and 36) or in mitochondria (Table 8, lines 8, 9, 16, 20, 27, 34) were also detected in large amounts and thus may hide other interesting protein involve in chromatin organization. Indeed, in meiocyte samples (LTQ-Orbitrap), we could identify more proteins involved in chromatin organization (Table 9). Therefore, this demonstrates again a good nuclear enrichment, and supports the choice of isolating meiocytes despite the time and the difficulty of the task. As *Ph1* is involved in chromatin remodelling, the identification of chromatin organization proteins is encouraging.

Redundant proteins hits can be very confusing and applying a Mascot filter by score (request a score > 20) and peptide matches can remove the results with poor probability matching and hide the records which already matched the peptides. The first time a peptide match to a query appears in the Mascot report, it is shown in bold face. Whenever the top ranking peptide match appears, it is shown in red in the Mascot report. This means that protein hits with peptide matches that are both bold and red are the most likely assignments. Using those filters helps for a first screening to get rid of the redundant proteins hits, but because mascot is only a probability test calculating the match between a peptide and a protein, the top protein hits are not necessarily true. Nevertheless, having more than one hit for the same peptide could signify that the peptide is abundant, and thus that the protein from which it comes is also abundant (histones are likely to be abundant in a chromatin fraction), unless the peptide belongs to more than one protein.

Accession Number	Protein	Function
BJ235270	Homolog of Homo sapiens ~~Histone 1, H2ai	chromatin organization
CK212468	Homolog of Homo sapiens ~~Histone 1, H2ai	chromatin organization
TA56291_4565	Homolog of Homo sapiens ~Histone 1, H2ai	chromatin organization
TA57743_4565	Histone H2B.2 [Triticum aestivum (Wheat)]	chromatin organization
TA49912_4565	Histone H2A [Hyacinthus orientalis	chromatin organization
TA58710_4565	Histone H3	chromatin organization
TA18_4568	Low temperature-responsive RNA-binding protein related cluster	RNA splicing, zwille protein, argonaute…
TA143_4568	14-3-3-like protein A related cluster	Nuclear export complex, kinase regulation
TA61169_4565	AHM1	Type of Nuclear Matrix-Localized, MAR Binding ...
TA65029_4565	RNA binding protein Rp120	RNA binding
CV767060	Low temperature-responsive RNA-binding protein	RNA binding
TA50577_4565	TaWIN1	*Triticum aestivum* WPK4-interacting factor 1
TA62488_4565	TaWIN2	*Triticum aestivum* WPK4-interacting factor 2
TA418_4571	Putative TCP-1/cpn60 chaperonin family protein	protein folding function
TA50173_4565	Nucleosome/chromatin assembly factor A	chromatin organization
BT009475	Prolyl-tRNA synthetase	RNA
TA550_4571	Putative MA3 domain-containing protein	RNA binding
TA63_4571	T6D22.2 [Arabidopsis thaliana (Mouse-ear cress)]	Elongation factor
TA63905_4565	AAA family ATPase, CDC48 subfamily	ATPase family
TA53637_4565	Putative zwille protein	Argaunote-like protein, splicing
TA54595_4565	Putative transketolase	Calvin cycle of photosynthesis
TA66211_4565	Ubiquitin-activating enzyme E1 1	Post translationnal modification
TA52987_4565	CUL1 [Oryza sativa (japonica cultivar-group)]	Ubiquitin pathway
TA61661_4565	Methionine synthase 1 enzyme	
TA69168_4565	Replication origin activator 2	
TA50309_4565	Methionine synthase 2 enzyme	
TA68955_4565	Chromomethylase 1	
TA65374_4565	Eukaryotic initiation factor subunit, putative	
TA52757_4565	GF14-b protein	GF14b interacted with a leucine zipper protein
TA65016_4565	RNA binding protein Rp120	

Table 9: Protein identification from a meiocytes sample run onto LTQ-Orbitrap

Severals wheat histones proteins or variants from each class of histones have been identified from cDNAs library. From NCBI database, 7, 5, 2, 3 and 7 sequences (variants) have been downloaded for histone H2A, H2B, H3, H4 and H1 respectively. Similarly, from ChromDB website (chromatin protein database), 2, 7, 1, 3 and 10 proteins sequences (variants) can be downloaded for H2A, H2B, H3, H4 and H1/H5 respectively. Although several severals histones protein sequences (variants) have been identified, the histone genes composition is still unclear in wheat.

A peptide is a small sequence of amino acids, derived from the protein sequence itself, and one peptide can be conserved between more than one proteins. For this reason, the identification of a protein in the mixture has to be confirmed at least by the presence of two or more than 2 different peptides (with high score) per identified protein. Some homologous proteins, such as histones have identical peptide fragments meaning that we cannot tell if only one or both proteins in question were present in the mixture. The BLAST of these peptides helps to identify these conserved rejoins and also helps to verify their position and coverage in the protein sequence.For example, Table 10 lists the best peptide protein matches for histone proteins identified by Mascot when searching against the TIGR_TA Triticum database. Figure 28 represents the alignment of the sequences for H2A, H2B and H3 and the position of identified peptides.

Of the 5 different peptides assigned to H2A in TIGR-EST results, only 4 are found in the wheat protein sequences from NCBI. The peptide AGLQFPVGR is located in the conserved region of the 7 wheat variants, while LLSGVTIAHGGVIPNINPVLLPK and HLLLAIR are unique to H2A2, and HLLLAVR is common to H2A1, H2A3 and H2A6. In addition, the peptide AVVQVFEGTSGIDNK matches only on 3 amino acid in all the sequences and is likely to belong to another protein.

Similarly for H2B, of the 4 peptides assigned to the protein, only LVLPGELAK is in the conserved region of all the sequences and AMSIMNSFINDIFEK belongs to H2B1, H2B2, H2B3 and H2B5. The peptides SSSSSHAPQGR and VALEACVQAR are not assigned to H2B and are likely to belong to another protein.

Finally for histone H3, only 2 of the 3 peptides belong to the H3 sequence and FRPGTVALR is specific to one H3 variant. None of the listed peptides were found in the H1 sequences and no H4 were identified.

Triticum EST	
Protein Identification	**Corresponding Peptides**
Histone H2A.2.1 related cluster	**AGLQFPVGR**
	LLSGVTIAHGGVIPNINPVLLPK
	AVVQVFEGTSGIDNK
	HLLLAIR
Histone H2A.1 related cluster	**HLLLAVR**
	AGLQFPVGR
Histone H2B.2 [Triticum aestivum (Wheat)]	VALEACVQAR
	LVLPGELAK
	SSSSSHAPQGR
	AMSIMNSFINDIFEK
TA56299_4565 Homolog of Homo sapiens ~~Histone 1, H2ai [Takifugu rubripes]	TLYGFGG
	HSLSPPADSR
	ISGLIYEETR
	TVTAMDVVYALK
	TVTAMDVVYALKR
	ISGLIYEETRGVLK
	VLRDNIQGITKPAIR
	HRNMLRDTIQGITKPAIR
	GISGLIYEETR
	KVLGEKIQGITKPAIR
	KTVTAMDVVYALKR
	IFLENVIR
	DNIQGITKPAIR
	RISGLIYEETR
	QGITKPAIR
	LENVIR
CV779037 Histone H3 [Triticum aestivum (Wheat)]	**FRPGTVALR**
	EIAQDFKTDLR
	ESLGAARPFISR

Table 10: Histone idenfication with TIGR_TA Triticum

List of peptides assigned to histones proteins H2A, H2B, H3 and homolog to H1 when searching against TIGR_TA Triticum. Only the peptides in bold green are found in the proteins sequences of the wheat histones from NCBI. Only the 3 last amino acid (in blue) of the peptide AVVQVFEGTSGIDNK matched in the H2A sequence which is not enough to prove that the peptides belongs to H2A. The peptides in black are wrongly assigned.

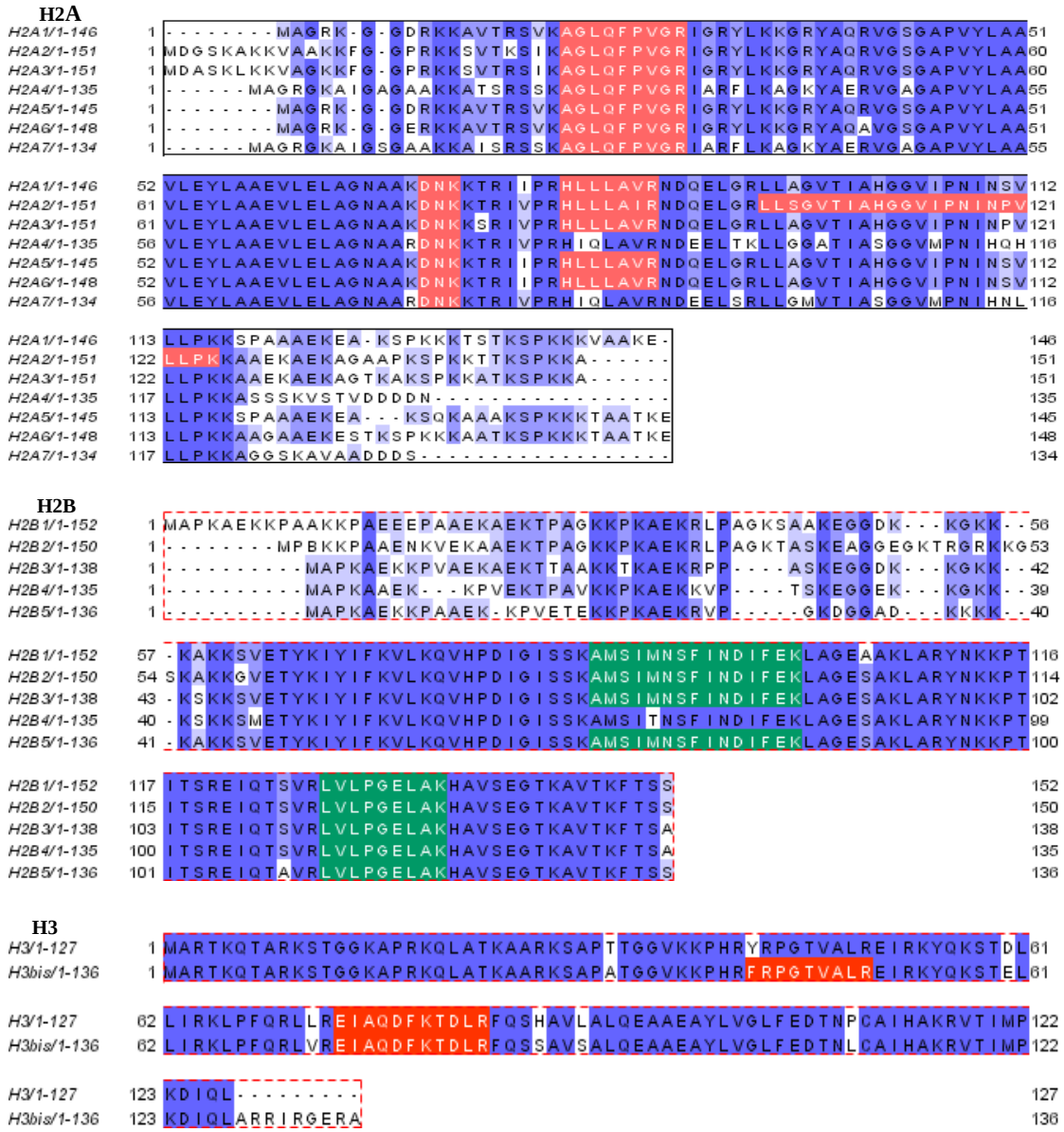


Figure 28: Sequence alignments of H2A, H2B and H3 variants – TIGR_TA Triticum matches

Protein sequences were downloaded from NCBI and aligned with Jalview (Clamp et al. 2004).Peptide sequences correctly matched after search against TIGR_TA *Triticum* are in colour in the sequences: in pink for H2A, green for H2B and red for H3.

Table 11 lists the best peptide protein matches for histone proteins by Mascot when searching against the SPtrEMBL.

Of the 3 different peptides assigned to the protein H2A, only 1 is perfectly aligned with the protein sequences (Figure 29). The peptide AGLQFPVGR is located in the conserved region of the 7 wheat variants, and was also successfully assigned to H2A when searching with the EST database. In this case, the information from the EST database was much more helpful than the SPtrEMBL alone.

All the peptides assigned to H2B and H4 are in the sequences (Figure 29). The peptide AMSIMNSFINDIFEK for H2B is present in the variants 1, 2 3 and 5 but not in variant 4, indicating that the protein could be one of those variants without defining which one.

None of the peptides was specific for H4 "variants", although the 3 sequences differ only by 1 amino acid. No H1 and H3 were identified. Moreover, the peptides assigned to H1 when searching against TIGR_TA triticum are the same peptides assigned to H4 when searching against SPtrEMBL. I first hypothesised the peptides belong to the conserved regions of H1 and H4, however Histone H4 and H1 sequences are very different as shown on the Figure 30 and the peptides really belong to H4 as shown in the Figure 25. So the second hypothesis is that the EST database might be wrongly annotated for this protein. The TIGR is an assembly of EST sequences, and is mostly "automatically" annotated in contrast to SPtrEMBL which is mostly "manually" annotated, and therefore should contain less error. This EST entry should therefore be corrected and my study would help to do it.

Protein Identification	**Corresponding Peptides**
Histone H2A.	**AGLQFPVGR**
	IVTVCLEGLENILK
	SPPIEEVIQSGVVPR
Histone H2B variant (Fragment).	**LVLPGELAK**
	AMSIMNSFINDIFEK
Histone H2B.	**LVLPGELAK**
	AMSIMNSFINDIFEK
Histone H2B.3.	**LVLPGELAK**
	QVHPDIGISSK
	AMSIMNSFINDIFEK
Histone H4 TH011 variant.	**TLYGFGG**
	IFLENVIR
	ISGLIYEETR
	TVTAMDVVYALK
	DNIQGITKPAIR
	TVTAMDVVYALK
	DNIQGITKPAIR
	RISGLIYEETR
	KTVTAMDVVYALK
	TVTAMDVVYALKR
	LENVIR

Table 11: Histone identification with SPtrEMBL

List of peptides assigned to histones proteins H2A, H2B, H4 when searching against StrrEMBL. Only the peptides in green are found in the proteins sequences of the wheat histones from NCBI. The peptides in black are wrongly assigned.

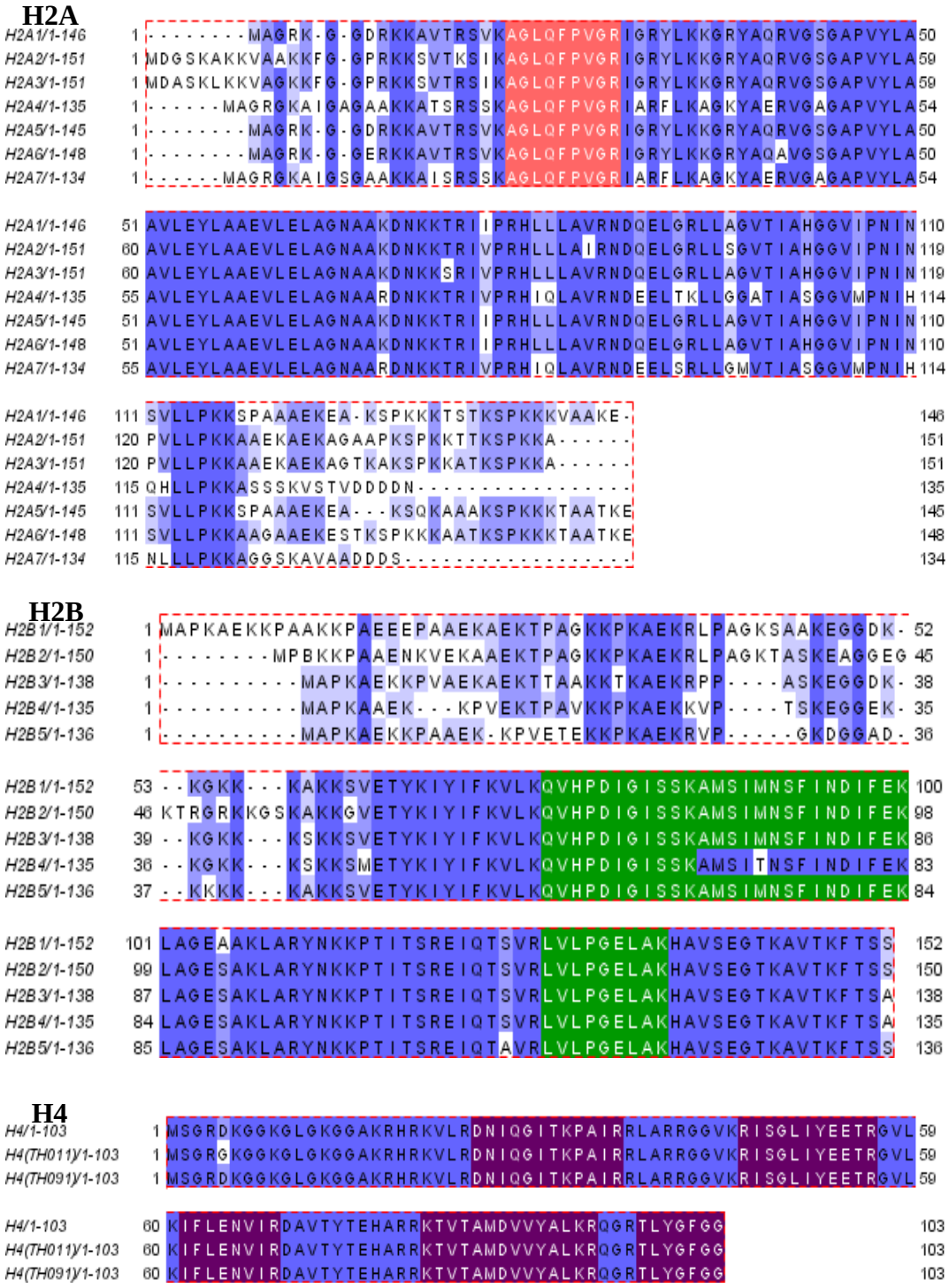


Figure 29: Sequence alignments of H2A, H2B and H4 variants – SPtrEMBL matches

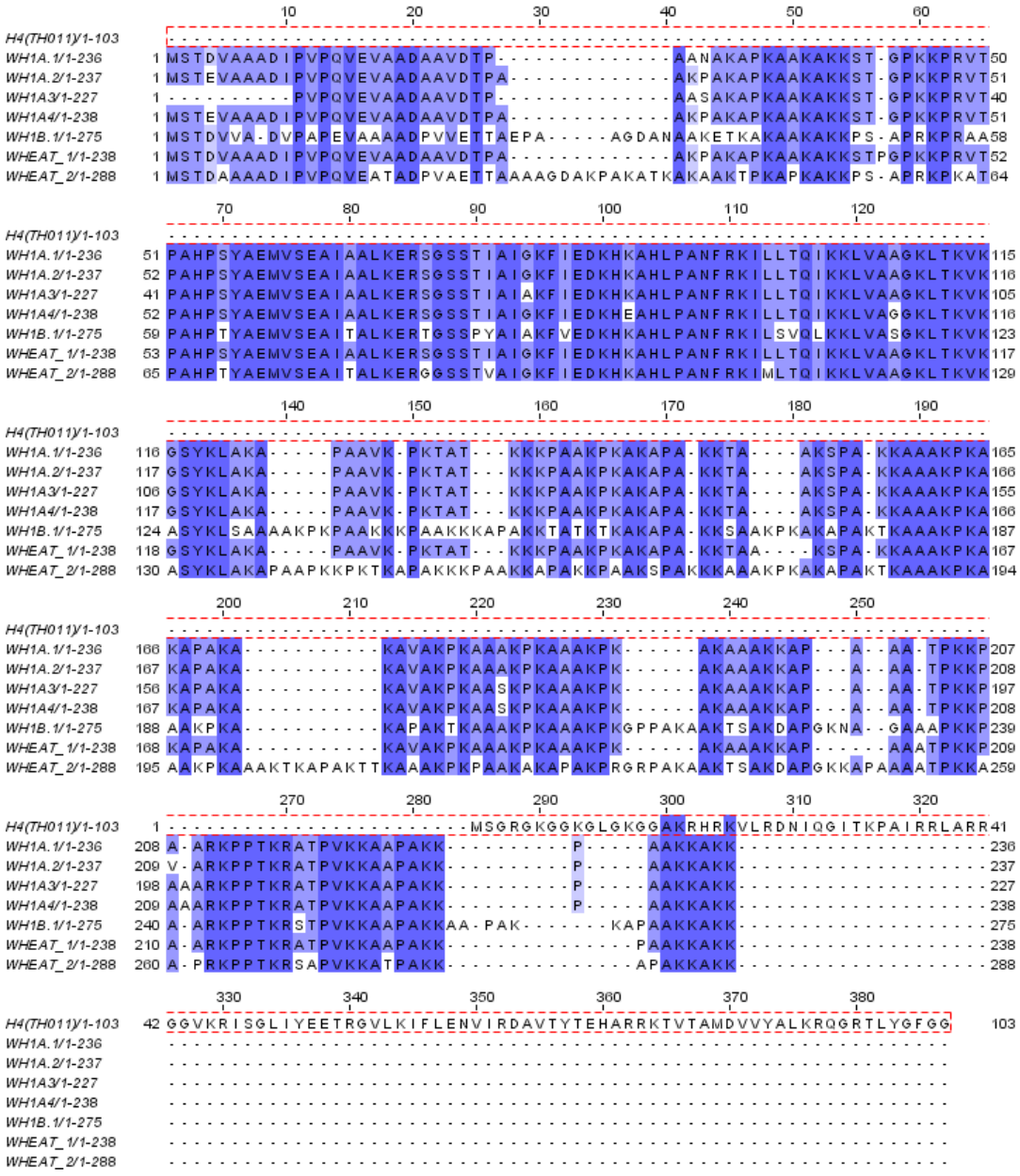


Figure 30: Histone H1 variants and H4 alignment

In summary, software such as Mascot is a very useful tool for automatic protein identification from MS2 data extracted from the mass spectrometer. Protein-peptide matches are validated on the basis of probability scores calculated by the algorithm, but this does not necessary mean that the protein identifications are correct. Each peptide can be blasted to find its position in the sequence – if it belongs to the assigned protein - or to show which other protein sequences match this peptide. Protein identification also benefits from the use of several databases. Other algorithm like X!Tandem (http://www.thegpm.org/TANDEM/) or OMSSA (http://pubchem.ncbi.nlm.nih.gov/omssa/) should be used in a further analysis in addition to Mascot in order to analyze the peptides as these methods do not use the same method of calculation or even de novo sequencing methods such as PEAKS (http://www.bioinformaticssolutions.com/products/peaks/).

4-3-4 Comparative Proteomics of wheat lines using stable isotope labelling

The *Ph1* effect on chromatin remodelling is very subtle in wheat homoeologues (Colas et al. 2008). However, in wheat-rye hybrids, the situation is much clearer. In absence of *Ph1*, the rye heterochromatin elongated randomly at the telomere bouquet, whereas in presence of *Ph1*, the rye heterochromatins remained tight at the telomere bouquet (Prieto et al. 2004a, Colas et al. 2008). I hypothesis that this dramatic change might be related to dramatic protein regulation, and therefore, Wheat/rye hybrid lines containing the *Ph1* locus and wheat/rye hybrids lines lacking the *Ph1* locus will be excellent material for a comparative study analysing protein and phospho-protein profiles in the presence or absence of *Ph1*. A powerful approach to performing this comparison is to "label" the proteins so that the source of each peptide in the whole complex mixture can be identified after mass spectrometry of the two samples mixed together. SILAC (stable isotope labelling with amino acids in cell culture) is a method based on the incorporation of amino acids containing a substituted stable isotope (e.g. deuterium, 13C, 15N). The "heavy" or the "light" amino acid is incorporated in vivo via the normal metabolism of the cell, and generates proteins of altered molecular mass, whose peptides can be easily differentiated in the mass spectrometer. The two samples can therefore be analysed in a same mass spectrometer run (Figure 31), and compared to each other (Gruhler et al. 2005, Wisniewski et al. 2008).

SILAC is a very straightforward method to analyse cell culture samples, but is very hard to use on whole organisms. Moreover, plants can interconvert different amino acids much more easily than animals, which complicate the use of SILAC in plants. An alternative is to label all the proteins by incorporation of ^{15}N from supplied $^{15}NO_3$, which can then be compared to the normal ^{14}N proteins. However, hydroponic culture must be used to grow the whole plant in a medium containing the "heavy" isotope, and thus label the total protein content. In this study, I have shown that it is possible to use a hydroponic-based system to completely label the proteins of a wheat plant so as to enable comparative mass spectrometry.

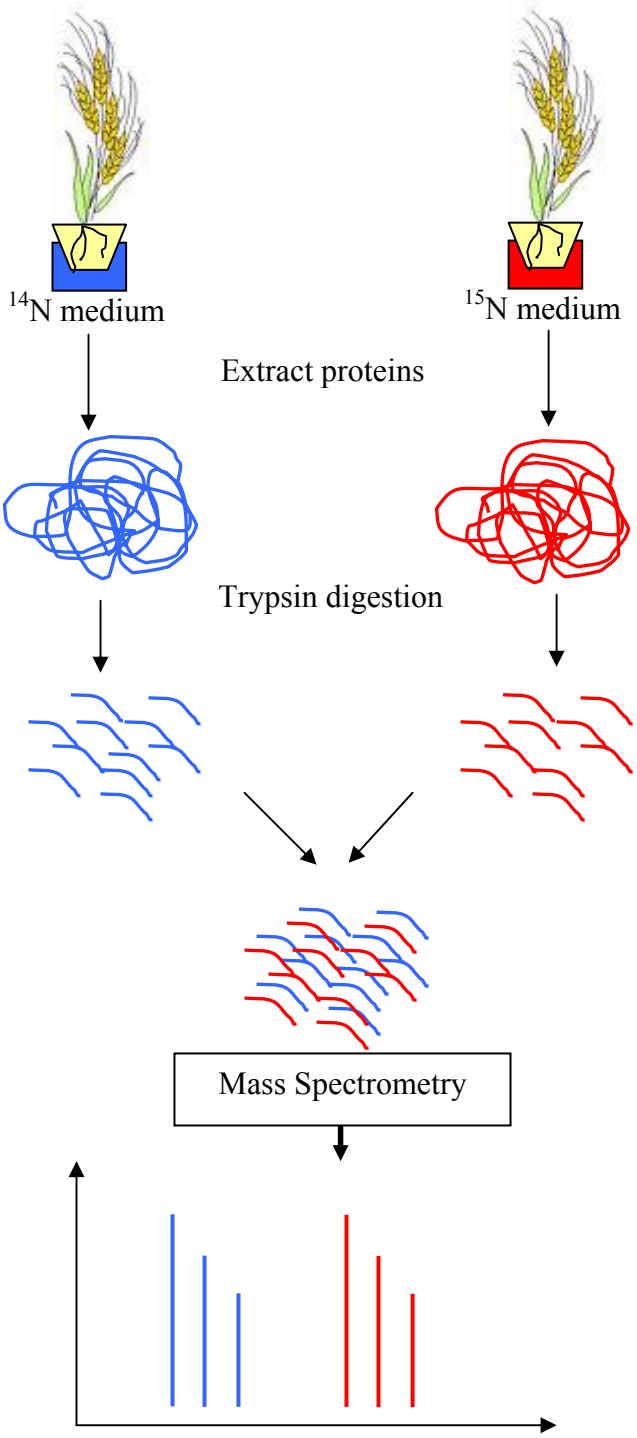


Figure 31: Principle of the quantitative proteomic using stable isotope

Hydroponic culture is widely used in agriculture since Hoagland and Arnon verified the work of Gericke on growing plants without soil and developed different growing media named Hoagland's solutions (Gericke 1937, Hoagland and Arnon 1938). Scientists also grow plants in hydroponic media such as Hoagland's for various studies. For examples, Raab and Terry (1994) used the Hoagland media to grow sugar beets to study nitrogen metabolism (Raab and Terry 1994). Christ et Korner (1995) studied the short term effect of elevated CO_2 during the early life phase of hydroponically grown wheat seedlings (Christ 1995). Using Hoagland media, uniform labelling with ^{15}N isotope was first used successfully in potato plants (Ippel et al. 2004) where 98% of the total proteins were 15N labeled. A more recent study labelled the whole Arabidopsis plant and showed that the labeling did not affect plant development (Hebeler et al. 2008).

In my study, wheat plants (cultivar Chinese Spring - CS) were vernalized for three weeks in Hoagland media and then transferred into a controlled environment room until flowering. In these conditions, the plants showed normal development and had synchronized meiocytes. The hydroponically grown plants were generally healthy (Figure 32). However, I observed the presence of nutrient deficiency spots (Figure 32b) on the leaves after five weeks of growing in the same medium composition, despite the fact that the solution was changed every week for the first three weeks and then every two or three days until maturity of the plants. Addition of iron solution and micronutrient into the initial growing medium solved the problem. CS plants were grown in the medium containing $K^{15}NO_3$ and $Ca(^{15}NO_3)_2$ (Figure 32c), while the *Ph1* plants (CS-*Ph1*) were grown in the medium containing the natural isotope (Figure 32a).

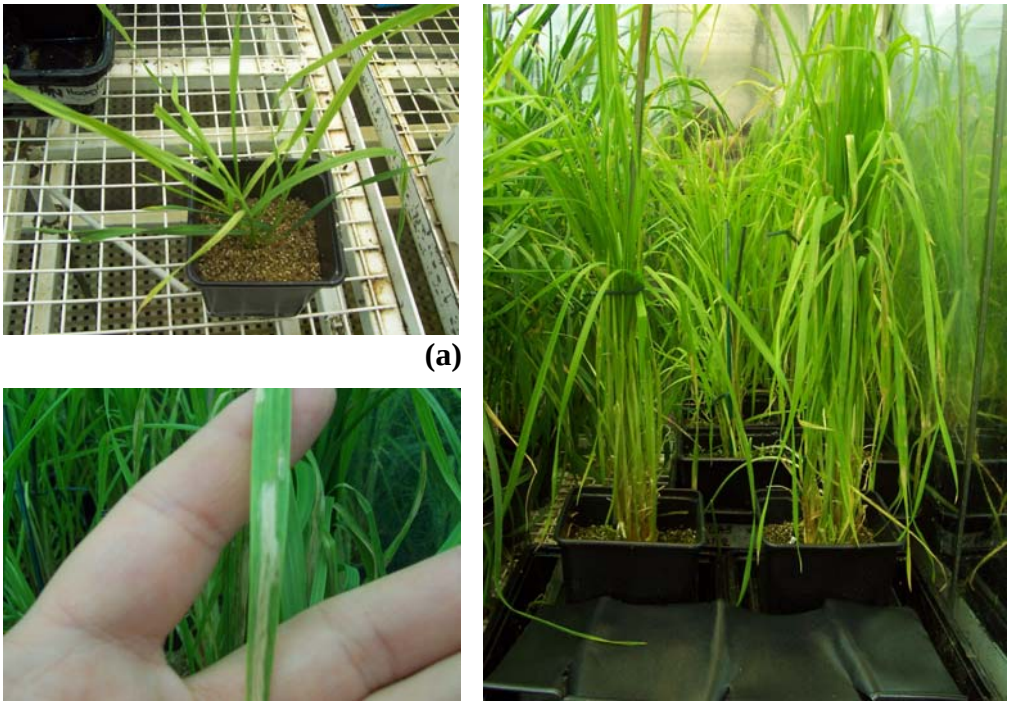


Figure 32: Wheat plants growing in ^{14}N or ^{15}N solution

(a) CS-*Ph1* plants after 14 days of growing in vermiculite and modified Hoagland's solution. The wild type CS and the mutant CS-*Ph1*show normal development under hydroponic conditions either with the presence of the natural isotope (^{14}N) or the heavy isotope (^{15}N). (b) Nutrient deficiency spot visible on the new leaves of the both genotypes after 5 weeks of growth in the medium. Addition of iron to the medium reduced the discolouration of the leaves. (c) CS-Ph1 plants after 10 weeks growing in hydroponics medium. The floral development is normal in both genotypes.

Samples from the leaves were taken after 14 and 24 days of growth in the nutrient solution. The leaf samples were ground in liquid nitrogen, and proteins were extracted with TEAB buffer. The proteins were digested by trypsin and run onto the LTQ-orbitrap to check the incorporation of the ^{15}N into the proteins. At two weeks, analysis of the peptides showed the presence of three types of peptides, easily identified by their mass. The first category is the unlabelled peptide, in which the entire nitrogen isotope is "light" (^{14}N). The second category is the partly labelled peptide showing various amount of ^{14}N and ^{15}N isotope in the same molecule. The last category is the fully labelled peptide in which all the nitrogen atoms are the "heavy" isotope (^{15}N). To illustrate the heavy isotope incorporation, the spectrum of one peptide from an abundant protein (Rubisco) is presented.

After 14 days of growth in the ^{15}N solution, mass spectrometry and Mascot (Palmblad et al. 2007) analysis identified only a majority of "light" peptides (Figure 33a), which meant the plant proteins were not fully labelled. In this sample, only a small proportion of peptides were fully ^{15}N labelled (Figure 33c) and by directly looking at the MS1 spectra we could detect the presence of fairly abundant $^{14}N/^{15}N$ peptide mixture (Figure 33b). The presence of the mixed and heavy peptides showed that the plants had taken up the nitrogen from the medium to synthesise the new proteins. However, the proteins were not fully labelled with ^{15}N after two weeks, which suggested that the plants were probably using the seed nitrogen for at least 14 days.

After 24 days of growth in the ^{15}N solution, mass spectrometric analysis identified the presence almost exclusively "heavy" peptides (Figure 34), which means the plant proteins are close to fully labelled. In this sample, there was a trace of the mixed and light peptides in the protein mixture (Figure 34). If we assume that the medium solution contains 98% heavy nitrogen, we expect about 2% of the nitrogen atoms in the peptides to be the light isotope. Indeed, comparing the intensity of the mass spectrum of the ^{15}N peak and the ^{14}N and/or $^{14}N/^{15}N$ peaks, there is a ratio of between 2% and 3%. Therefore we can deduce that the proteome will be 98% labelled with the heavy isotope, which is the maximum labelling to be expected for this type of method (Ippel et al. 2004).

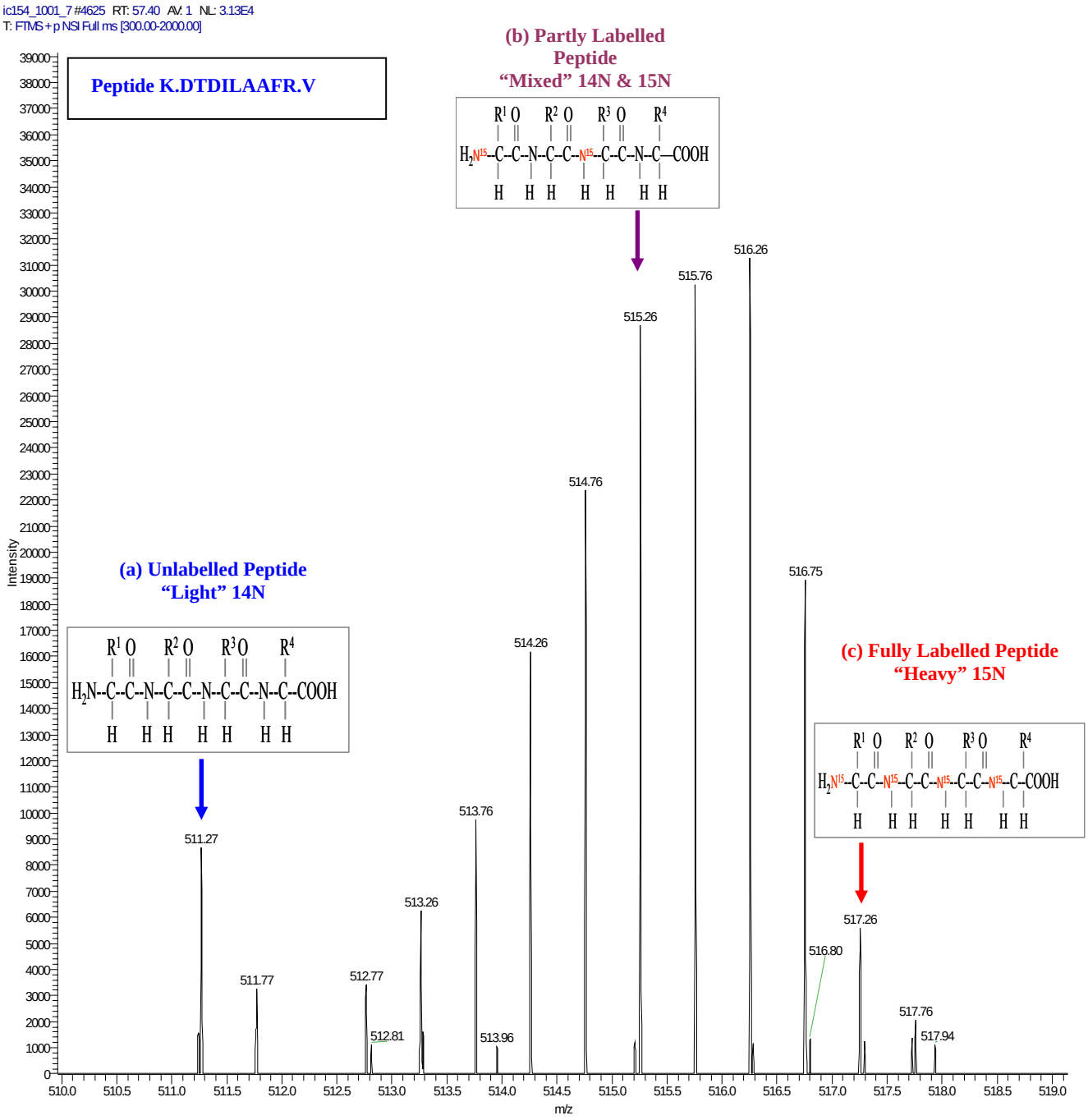


Figure 33: Peak analysis of a rubisco peptide in a 14 day leaf sample

"Light" peptide contains only 14N isotope (a), "mixed" peptides contain 14Nand 15N isotope and (b) "heavy" peptide contains only 15N isotope (c). After 14 days of growth in heavy medium, most of the proteins are still not fully labelled and contain the both isotopes.

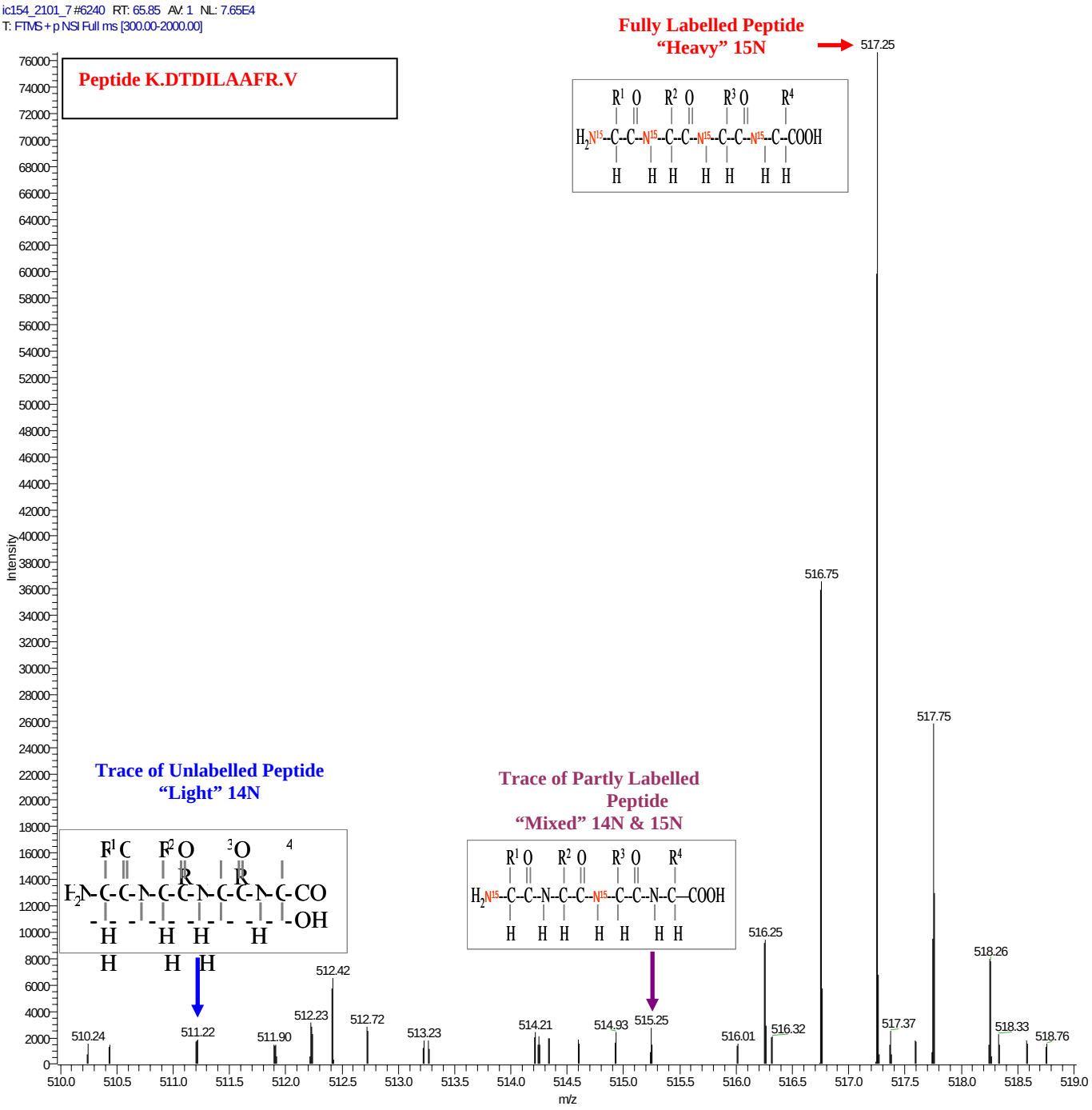


Figure 34: Peak analysis of a rubisco peptide in the 24 days leaf sample

"Light" peptides contain only ^{14}N isotope (a), "mixed" peptides contain $^{14}N$and ^{15}N isotope and (b) "heavy" peptides contain only ^{15}N isotope (c). After 24 days of growing in the heavy medium, the proteins of the leaves are identified as fully ^{15}N labelled. The trace of the light and mixed peptides is not a problem.

The ^{14}N and ^{15}N samples were mixed in different ratios to check if the mass spectrometer was able to measure the same light/heavy ($^{14}N/^{15}N$) ratio for each peptide. We looked at the peptides in different ratios (1:1, 2:1, 5:1 & 10:1) and showed that the relative peak heights of the light and heavy peptides measured the ratios of the mixtures. The Figure 35 shows the example of the same rubisco peptide previously described. This result was consistent for the other peptides detected by the mass spectrometer, thus validating this as a reliable method for comparative quantitation of plant material during mass spectrometry. The ^{14}N peak always seemed "lower" in the 1:1 ratio, which is probably due to differences in peptide concentration when the samples were mixed. Measuring peptide concentration before running the sample might be not accurate, but this problem can be mitigated by evaluation of the consistent difference between peptides from abundant proteins in the 1:1 mixture.

Wheat seed proteome was analysed in several wheat chromosome deletion lines using an analytical approach based on isotope coded affinity tag labelling (ICAT) of peptides in tryptic digests followed by electrospray ionization mass spectrometry (Islam et al. 2002, Islam and Hirano 2004) This approach showed some evidence of down-regulated or up-regulated peptides measuring the relative peak intensity for selected peptides. The proteome patterns of diploid, tetraploid and hexaploid wheat were analyzed to explore the genome interaction in protein expression by two-dimensional electrophoresis (2-DE) (Islam et al. 2003). In this study, the authors shown that the expression of nonstorage proteins was affected substantially due to the removal of the D genome from hexaploid constitution and that the location of the structural gene controlling one of the alpha amylase inhibitor proteins in the nonstorage protein region was identified in the short arm of chromosome 3D. In my study, the plant grown in ^{14}N solution was the *Ph1* mutant, while the plant grown in the ^{15}N solution was the wild type. Therefore, the difference of the peak intensity observed in the *Ph1* sample might also be the result of the difference between the two proteomes affected by the deletion. This hypothesis can be tested with reciprocal labelling experiments for the two genotypes.

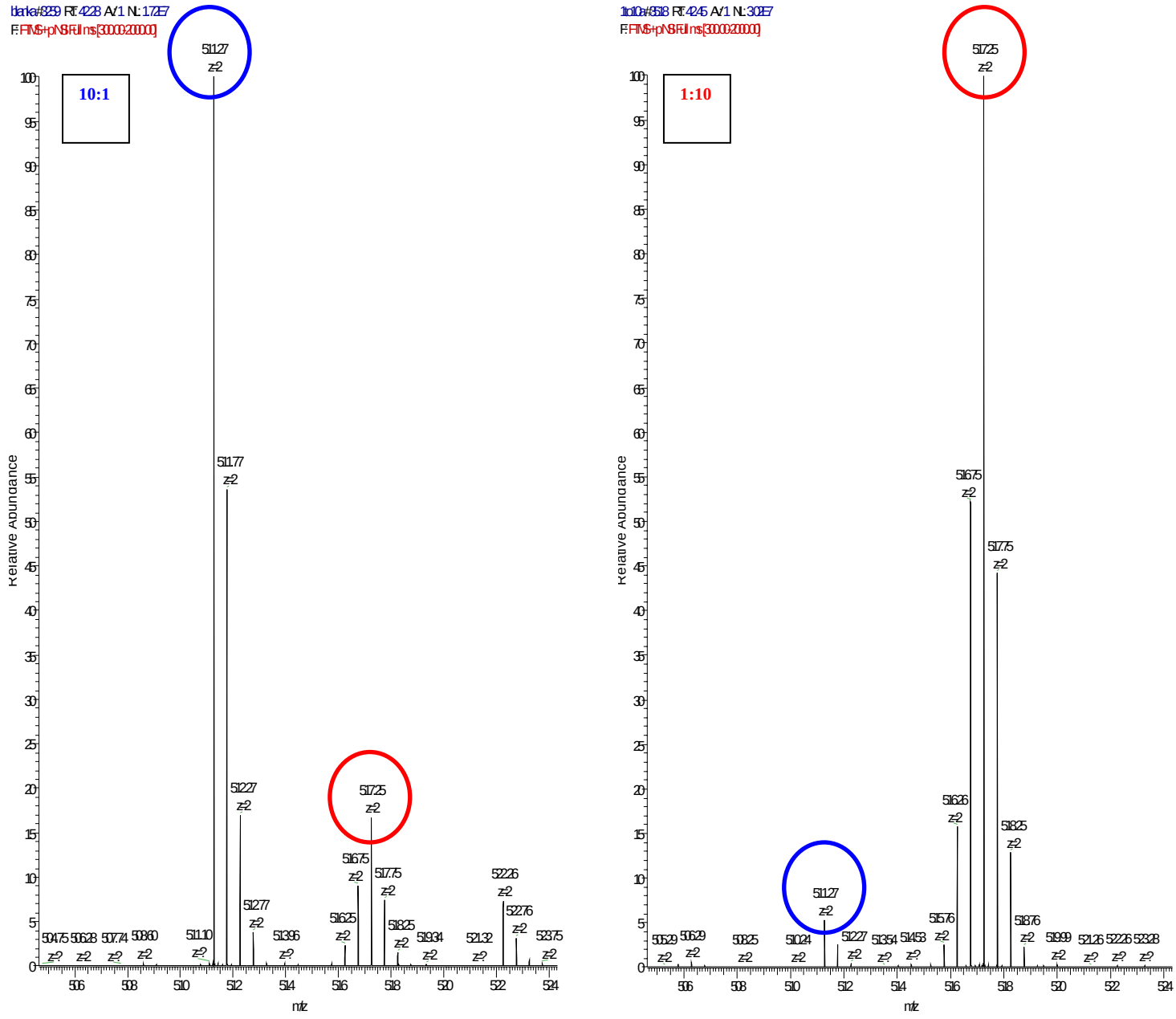


Figure 35: $^{14}N/^{15}N$ ratios for the same peptide unlabelled or fully labelled

Left figure: In the 10:1 ratio, where light peptides (^{14}N) should be 10x more abundant than the heavy peptides (^{15}N), the peak at 511.27 (light peptide) is 10x higher than the peak at 517.25 (heavy peptide). Right figure: In the 1:10 ratio, where light peptide (^{14}N) should be 10x less abundant than the heavy peptides (^{15}N), the peak at 511.27 (light peptide) is 10x lower than the peak at 517.25 (heavy peptide)

4-4 Conclusions and future work

One of the more serious problems is that the wheat genome is as yet unsequenced. However, I proved that wheat EST sequences can be used for analysis and many peptides could be assigned from this database. Furthermore, the rice genome is complete and importantly a genomic sequence is currently being determined for brachypodium, a species much more closely related to wheat than rice. Some of the databases used are updated at least twice a year, so it is important to archive the data carefully in order to enable repetition of searches. Wheat is a particularly important crop, and there is no doubt that the genome sequence will be completed in the next few years. Thus again, archiving the MS data will be useful to search in future against the complete genome.

Nevertheless, I have shown the feasibility of extracting proteins from a purified meiocyte fraction for mass spectrometry analysis. Judged by the abundance of histones and chromatin binding proteins, I conclude that I obtained a good enrichment of nuclear proteins. However, instead of having all the histones (H1, H2A, H2B, H3 and H4) identified as I thought at first, I discovered that histone H1 was not detected in my mixture. Therefore, none of the Histone 1 peptides could be detected by the mass spectrometer and I need to fractionate much more the starting gel, in order to reduce the complexity of the sample.

I have already shown that cutting the gel in five pieces reduced the complexity and increased the number of proteins identified. Therefore, the gel can be further fractionating into 10 or even 20 pieces to reduce the complexity of the protein mixture, and the peptides can be separate by a longer LC gradient. Moreover, two chromatography steps can be used as in Mudpit (Maor et al. 2007). In this approach, the peptides are first separated by strong cation exchange (SCX) chromatography before the reversed-phase (C18) chromatography. In a recent study, Delmotte et al developed a two-dimensional separation employing high-pH reversed phase HPLC in the first and low-pH ion-pair reversed-phase HPLC in the second dimension. The study revealed that combining the identification results of this method and the classical separation by SCX followed by RP chromatography increased the number of proteins identified (Delmotte et al. 2007).

Immobilized metal affinity chromatography (IMAC) columns can be used prior to SCX column and reverse phase chromatography to enrich for phosphopeptides (Gruhler et al. 2005). TiO_2 chromatography is an alternative to IMAC for phosphopeptide enrichment and was proved to be superior in performance than IMAC (Larsen et al. 2005, Cantin et al. 2007). If *Ph1* mechanism is related to Cdk2 mechansim, which is known to affect histone H1 phosphorylation state, I would expect differences in histone H1 phosphorylation state in presence or absence of *Ph1*. Thus, enrichment in phospho-peptides is particularly important for the *Ph1* study.

The linear ion trap (LTQ) has great sensitivity due to its fast scan speed but poor mass accuracy (Zubarev and Mann 2007) which means that confidence levels in peptide assignment are reduced. This also means that this machine is very good for the analysis of complex mixtures, but the low mass accuracy makes identification of peptides from non sequence genomes prone to a high level of false positives. However, the LTQ-Orbitrap is an ion trap with a better mass accuracy and gives us more confidence on the protein identification. This feature is particularly important when studying post-translational modification (PTM) analysis (especially phosphorylation) and in quantification using isotopes. Therefore, multiple peptide fractionation, coupled with LTQ-Orbitrap analysis is likely to give high confidence in the protein identified from wheat. Moreover, quantitative proteomics is another important approach to solve the *Ph1* mechanism.

In chapter 3, I showed that chromatin remodelling is essential for correct pairing and recombination, and this occurs when the chromosomes are identical or nearly identical. Moreover, in a wheat/rye hybrid, containing only homoeologues, the absence of *Ph1* leads to an asynchronous remodelling of the rye heterochromatin, allowing association of the wheat and rye chromosome. In the presence of the *Ph1* locus, there is no remodelling and no pairing between wheat and rye chromosomes (Prieto et al. 2004a, Prieto et al. 2005, Colas et al. 2008).

I successfully set up a method to label the whole plant with ^{15}N isotope. Each peptide can then be assigned to one or other genotype on the basis of ^{15}N content, and thus I will be able to determine relative levels of each protein, or post-translational modifications in the two genotypes. I have also pointed out that the reciprocal ^{14}N and ^{15}N experiments will be important to obtain reliably controlled data. Therefore labelling of wheat/rye hybrid lines containing the *Ph1* locus and wheat/rye hybrid lines lacking the *Ph1* locus will be excellent material to study to protein profiles in the absence of *Ph1*.

In conclusion, despite the fact that the wheat genome is as yet unsequenced, wheat is an excellent system to study meiosis in a monocot because the meiocytes are easy to handle in comparison to plant models such as rice or brachypodium. I have shown the possibility of using mass spectrometry technology to identified proteins from complex mixtures from wheat meiocytes and shown already some evidence for phosphorylation site detection in histones. I also showed the feasibility of a labelling technique using stable isotopes for a comparative study of plants containing or lacking the *Ph1* locus. The *Ph1/CDK2* protein might control the remodelling by phosphorylation of histone H1 or other components of the chromatin, leading to synchronous remodelling of the homologues. Therefore, mass spectrometry might be a very useful tool to further study wheat meiosis and give clues about the *Ph1* mechanism.

Chapter 5

General Conclusions

5.1 What is the *Ph1* Locus and what does it involve?

Located on chromosome 5B, the locus consists of a cluster of Cdk2-like genes, interrupted by a large region which has no equivalent on 5A and 5D.

Cdk2 is involved in chromosome synapsis and recombiantion. Mice lacking Cdk2 show severe defects in synapsis (Berthet et al. 2003). Furthermore, without Cdk2 action, SC proteins fail to assemble on the homologous chromosome pair, resulting in desynapsis and nonhomologous pairing (Cohen et al. 2006). In mammals, Cdk2 colocalizes with MLH1, a mismatch repair protein at sites of reciprocal recombination in mid-late pachynema.(Ashley et al. 2001). V(D)J recombination (the process by which antigen receptor genes are assembled) is initiated by the recombination activating proteins RAG-1 and RAG-2. Suppression of cyclinA/Cdk2 activity allows the prolongation of G1 phase, the accumulation of RAG-2 stimulating the V(D)J recombination (Lee and Desiderio 1999).

Cdk2 is involved in the timing of replication. Cdk2–cyclin E (or A) can negatively regulate DNA replication. In this case a high concentration of cyclinE/Cdk2 prevents association of the protein complex ORC and MCM3, a protein essential for replication (Hua et al. 1997). Indeed, loss of Cdk2 delay S phase in mice (Berthet et al. 2003).

Cdk2 is involved in chromatin remodelling. Chromatin decondensation in S-phase involves recruitment of Cdk2 by Cdc45 and histone H1 phosphorylation (Bhattacharjee et al. 2001, Contreras et al. 2003, Alexandrow and Hamlin 2005). Phosphorylation of H1 by Cdk2 provides a signal for the disassembly of higher order chromatin structures during interphase, by reducing the affinity of HP1α for heterochromatin. (Hale et al. 2006)

If the function of *Ph1C*DK protein is similar to CDK2, then *Ph1*CDK should also be involved in controlling the synapsis of non homologous chromosomes, in chromatin remodelling, replication, and in recombination. Holm (1986 and 1988) showed from synaptonemal complex studies that *Ph1* affected the synapsis of chromosomes (Holm 1986, 1988). In the absence of *Ph1*, there was more multivalent formation in early stages of meiosis and such associations fail to resolve at later stages of meiosis. Therefore, this suggests that *Ph1*CDK is indeed involved in non-homologous synapsis as its CDK2 homologues.
Is there evidence for *Ph1*CDK involvement on chromatin remodelling and recombination?

5.2 Ph1CDK involvement on chromatin remodelling and recombination.

In wheat, as the telomeres cluster, the chromosomes are brought together and associate via their sub-telomeric regions (Martinez-Perez et al. 1999, Prieto et al. 2004a). A change of the chromatin conformation occurs in the two homologues which enables them to align with each other and pair (Prieto et al. 2004a). Moreover, the synchronization of this remodelling is based on the chromosome homology, allowing identical or nearly identical homologues to remodel, to pair and efficiently recombine. If no remodelling occurs at the subtelomeric regions, the chromosomes fail to pair and recombine (Colas et al. 2008). If they all remodel, like in the wheat-rye hybrids in absence of *Ph1*, the homoeologous chromosomes become competent to pair to each others (Prieto et al. 2004a).

Although the situation is very subtle in wheat, the events are much clearer in wheat/rye hybrids lines, containing 28 homoeologues consisting of 21 wheat chromosomes and 7 rye chromosomes. In the presence of *Ph1*, 7 groups of associated wheat and rye centromeres are formed as a prelude to meiosis. At the telomere bouquet there is no remodelling of the 7 rye subtelomeric regions. In contrast, in the absence of *Ph1*, 7 groups of wheat centromeres and 7 distinct groups of rye centromeres are formed prior to meiosis (Aragon-Alcaide et al. 1997a, Prieto et al. 2004a).

Moreover, the rye centromeres show elongated structures prior to meiosis (Aragon-Alcaide et al. 1997a). In this line, the 7 rye subtelomeric regions can remodel, but in fact often pair with each other illegitimately to form a mass of elongated rye chromosome regions, rather than pairing with their wheat homoeologues. This may explain the low frequency of recombination between wheat and rye (Prieto et al. 2004a).

Therefore *Ph1* controls the remodelling activity at the sub-telomeric regions (Colas et al. 2008), at centromere regions (Martinez-Perez et al. 2003) and the rest of the chromosomes (Prieto et al. 2005). Moreover, the effect of *Ph1* on pairing and recombination is linked to the chromatin remodelling. Taken all together this suggests that like its Cdk2 homologue, the *Ph1*CDK is involved in chromatin remodelling and recombination. Is there any evidence for involvement of *Ph1* at replication?

5.3 *Ph1*CDK involvement at replication

I have shown that identical subtelomeric heterochromatin regions localized together prior to the telomere bouquet formation. This is consistent with previous studies, showing association of homologues in both pre meiotic and somatic cells suggesting that *Ph1* regulates events prior to meiosis such as replication.

A maize study has shown that homologues were synchronously replicated. However, the addition of heterochromatin in the form of a B chromosome or an abnormal 10 chromosome increased asynchrony twofold (Douglas and Walden 1974). Moreover, B chromosomes (heterochromatin), which delay replication, compensate for the absence of *Ph1* in wheat hybrids with other species. In the presence of *Ph1*, in wheat-rye hybrids, the subtelomeric heterochromatin regions do eventually remodel but only after the telomere bouquet has resolved. If remodelling is linked to replication, then the replication of the subtelomeric regions may occur after the telomere bouquet stage. We can hypothesis that such late replication may then explain why these regions don't pair as they will miss the pairing stage.

In the case of the wheat-rye hybrids described previously, in the absence of *Ph1*, there are 7 sites of rye centromeres and 7 sites of wheat centromeres, while in the presence of *Ph1*; there are 7 sites of associated wheat and rye centromeres at the onset of meiosis. The centromere pairing occurs during S-phase (Jasencakova et al. 2001) and *Ph1* affects this association meiotically and somatically (Martinez-Perez et al. 2001). Thus we can suggest that the differences observed in centromere associations between lines carrying *Ph1* and the lines lacking *Ph1*, might be the result of different timing of replication of the wheat and rye centromeres in the presence and absence of *Ph1*.

If replication is important to understanding the *Ph1* effect, then markers for replication are required. Replication timing experiments can involve BrdU labelling or the immunofluorescence detection of proteins involved in replication such as Proliferating Cell Nuclear Antigen (PCNA) or Replication Protein A.

BrdU has been recently successfully used on barley, Arabidopsis and rice, but initial experiments suggest that it might be more difficult to detect BrdU labelled DNA in wheat meiocytes (Jasencakova et al. 2001, Armstrong and Jones 2003, Nonomura et al. 2006).

A PCNA antibody is available which may work on wheat. RPA has been characterised in rice and its DNA sequence is available. BLAST searches identify the corresponding wheat sequences for this protein which can be exploited to raise antibodies against wheat RPA. Moreover, TaASY1 provides a specific antibody for the synaptonemal complex and can be used to determine whether the cell is in S-phase or meiosis. Finally, RNA in situ can be performed for the various histones, which are highly expressed in S phase to reveal any differences in the timing of replication between wheat-rye hybrids lacking *Ph1* and those with *Ph1*.

5.4 Towards the molecular mechanism of *Ph1*

The initial indications are that as with CDK2, *Ph1*CDK may be involved in non homologous synapsis, chromatin remodelling, replication and recombination. If this is the case, then *Ph1*CDK may interact with the same proteins as CDK2 such as Cyclin A, Cyclin E to regulate the cell cycle for example (Kaldis and Aleem 2005). *Ph1*CDK may collocolize the mismatch repair protein MLH1 (Ashley et al. 2001). I also would expect to see the *Ph1*CDK recruited by CDC45 to phosphorylate histone H1 (Alexandrow and Hamlin 2005). If *Ph1*CDK is a Cdk2 mechanism, involving protein phosphorylation (such as histone H1 for example), then we should see an effect by using phosphatase inhibitor in wheat plants.

Proteomics approaches are now important to further understand *Ph1* function. Immunological experiments, yeast 2-hybrid screening, and direct proteomic analysis of meiocytes chromatin are three complementary approaches that can be used to explore further the function of *Ph1*CDK. The CDK gene open reading frame can be cloned into a vector system to express the protein. This protein can be purified and use to prepare an antiserum which can be used to quantify the level of this protein by western blotting and for immunofluoresecence detection on pre-meiotic and early meiotic cells. A custom yeast 2 hybrid system can be made with a cDNA library from pre-meiotic and early meiotic tissues. All positives candidates can be sequenced to determine if they correspond or not to the potential candidates for CDK2 interaction.

In this study I set up a protocol using mass spectrometry which is likely to give us good candidates for meiotic proteins and their modifications. Protein extraction of purified meiocytes generates good samples enriched in nuclear proteins in enough quantity and quality for detection by the mass spectrometer.

Separation methods such as SDS gel (protein separation) or chromatography (peptide separation) prior to mass spectrometry are essential to reduce the complexity of the sample resulting in more protein indentification. Despite the fact that the wheat genome is not yet sequenced, wheat EST sequences can be used in addition to the more general databases for analysis and many nuclear proteins could be indentified. A successful method was developed to label the whole plant with ^{15}N isotope for comparative proteomic. Each peptide can then be assigned to one or other genotype on the basis of ^{15}N content, and thus will be easily identified, quantified and compared. In wheat-rye hybrids lacking *Ph1*, the rye heterochromatin region can remodel, whereas in wheat-rye hybrids containing *Ph1* the rye heterochromatin region remain condensed at the telomere bouquet. In this case, theses lines can be used as a positive/negative control of the *Ph1* effect, and might have a dramatic change in the protein levels. Therfore, wheat/rye hybrids lines containing the *Ph1* locus and wheat/rye hybrids lines lacking the *Ph1* locus will be excellent material to study the protein profiles.

A recent study showed that phosphorylation of Histone 1 by CDK2 regulates its binding to HP1 (heterochromatin protein 1) and hence regulates heterochromatin conformation (Hale et al. 2006). Therefore, future work would include enriching the protein fraction for phosphopeptides. This would require the exploitation of chromatography methods, such as IMAC or Ti O_2.

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Appendices

Appendix 1 - Material Harvest

1. Collect the spike in the morning after at least 2 hours of light to get early stage of meiosis. Can be collected later in the afternoon for stages after metaphase I.
2. Feel the spike with your finger, and cut at the base of the plant to collect and place the stem in water.
3. A spike has between 15 and 22 spikelets, and the spikelet in the middle (7-10) is at the later stage of meiosis.

Prophase I

- The base of the 1st leaf at the top is still hidden in the plant.
- There is no "gap" between the nod below the spike and the base of the spike.
- You can't feel individual spikelets on the spike

Metaphase I

- The base of the 1st leaf at the top came out of the plant and ~1cm of stem is visible.
- There is ~1cm "gap" between the nod below the spike and the base of the spike.
- You can sometimes feel individual spikelets on the spike

Appendix 2 - Material Fixation

1. Prepare a freshly fixative 4% formaldehyde in 1 X PEM;

For 25 ml fixative solution:

   1. 1g Para formaldehyde + 12,5ml H2Od
   2. Add one drop of NaOH 1M
   3. Heat at 100ºC at least 10-15 minutes, the solution must be transparent.
   4. Cool down the solution on Ice.
   5. Add 12, 5 ml of 2 X PEM (50mM PIPES/KOH, 5mM EGTA, 5mM MgSO4, pH 6.9).

2. Gently infiltrate the tissues with vacuum to remove air from the spikelet for 10 or 15 minutes at least. The spike must fell at the bottom of the tube, when tin is well infiltrated.
3. Fix for one hour at room temperature
4. Empty the fixation and wash with 1 X TBS (10mM Tris HCL, 140 mM NaCl, pH 7.4) for 15 minutes on a shaker.
5. Store the sample in the fridge no more than two day for sectioning, or up to two weeks for wax embending, of meiocyte spread. It is recommended to discard any sample older than 2 weeks as the Formaldehyde fixation is reversible.

Appendix 3 - Tissue-Tek Vacuum Infiltration cycle

- Tissue should not be older than 2 weeks after being fixed.
- Place tissues in cages and place all in the Tissue-Tek Vacuum Infiltration Processor and initiate the following fix 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
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

Appendix 4 - APTES Slide Preparation

1. Wash the slides in H2Od with decon90 for at least 1 hour or overnight shaking.
2. Rinse the slide in H2Od 2 or 3 times.
3. Let to air dry.
4. Soak the slide in a freshly prepared 2% of 3-aminopropyltriethoxyl – silane (APTES, Sigma) in Acetone for 10 seconds. More than 10s can give a lot of background on the slide.
5. Transfer the slides briefly in acetone alone.
6. Let them to air dry.
7. Keep them away from dust, and store at room temperature.
8. Befor using, soak the slide in a 2.5% solution of glutaraldehyde in phosphate buffer for at least 30 minutes.
9. Rinse the slide in H2Od.
10. Air dry the slide.

The glutaraldehyde solution can be reused and store at 4ºC for 1 month.

Appendix 5 - Telomere Amplification

Primers

TelomF (5'-TTTAGGG-3')

TelomR (5'-CCCTAAA-3')

Reaction mix

100 µM TelomF	1 µl
100 µM TelomR	1 µl
2X Qiagen master mix	12.5 µl
DNA	No template DNA
Water	10.5 µl
Total Volume	25µl

Cycle

94°C, 1 minute

94°C, 1 minute
60°C, 1 minute
72°C, 1 minute
} 10 x

94°C, 1 minute
60°C, 1 minutes
72°C, 1 minute 30 seconds
} 30X

8°C, for ever

Appendix 6 - Rye Heterochromatin Amplification

Primers

1pSc250 (5'GAGCAAGTTACCTTGGA3')

2pSc250 (5'TTTTGCGCCACTCGAAAT3')

Reaction mix

100 µM 1pSc250	1 µl
100 µM 2pSc250	1 µl
2X Qiagen master mix	12.5 µl
1mg/ml genomic DNA	2 µl
Water	8.5 µl
Total Volume	25µl

Cycle

94°C, 1 minute

94°C, 1 minute
55°C, 1 minute
72°C, 1 minute
} 35X

72°C, 5 minutes

8°C, for ever

Appendix 7 - Nick Translation

Reaction Mix

10X NTB	5 µl
DTT (100mM, Promega)	1 µl
Unlabeled dNTPs[b]	5 µl
dUTP-biotin[c]	1 µl
DNA template (1µg)	3 (varies) µl
DNAPI/DNaseI[d]	5µl
Sterile water[a]	30 (varies)
Final Volume	50 ul

- Mix up and incubate at 15 °C for 90 min.
- Stop the reaction by adding 5 µl of 0.5 M EDTA, pH 8.

NOTES:

a. Adjust volume to 50 µl with sterile pure water.

b. Unlabeled dNTP mixture: 2µl of each 100mM (dATP, dCTP, dGTP) + 394 µl NTB

c. Labeled nucleotide: 1mM of d-UTP labeled with Digoxygenin or biotin

d. Invitrogen 18162-016.

10X Nick Translation Buffer (NTB)

1M Tris-HCl pH 7.5	250µl
1M $MgCl_2$	25µl
20mg/ml BSA (Sigma B8667)	125µl
Water	100µl
	500µl

Appendix 8 - The *Ph1* repeats Amplification

Primers

F1(5'TAATGTACGTAGCGAAGCGAAG3')

R17(5'CTTATACCACCGCACTAGATCTG3')

Reaction mix

10 µM F1	1 µl
10 µM R17	1 µl
10 mM dNTP	0.5 µl
50mM MgCl2	1 µl
10X PCR Buffer	2.5 µl
Tag Gold	0.5 µl
1mg/ml BAC Ta218J13	1 µl
Water	18.75 µl
Total Volume	25µl

Cycle

95°C, 5 minutes

95°C, 30 secondes

60°C, 1 minute } 35X

72°C, 2 minute

72°C, 5 minutes

8°C, for ever

Appendix 9 - In situ hybridization

DAY 1

1. Process slides through a dehydration series, 2 min., at room temperature: 30% methanol, 50% methanol, 70% methanol and 100% methanol
2. Air-dry the slides.
3. Apply 20 ul of 0.5% pectolyase/1% cellulase solution (Prepare 10 ml at a time in TBS, aliquot and freeze, double the concentration for section) to wells of slide and put small cover slip on each well. Incubate 1 hr at 37C.
4. Wash slides in 1XTBS 10 min., at room temperature, on shaker.
5. Repeat dehydration series (step 1).
6. Air-dry the slides.
7. Make the hybridization mix (multiply by the number of wells).

Deionized formamide	5 µl
100 mM PIPES, 10 mM EDTA, pH 8	1 µl
50% dextran sulfate	2 µl
3M NaCl	1 µl
Salmon sperm blocking DNA (1 mg/ml)	0.5 µl
Probe	1 µl of each
	~10.4 µl

8. Denature the hybridization mix for 8 min in boiling water or at 100 °C in a dry heat blocks. Immediately place probe on ice and cool down at least 5 minutes.
9. Add 9 µl of hybridization mix to each wells of the slide, placed onto the OMNISLIDE thermal cycler, and apply a soft plastic coverslips for each well.
10. Denatures the DNA in the chromosomes and gradually takes the temperature down to 37 °C. It Takes about 45 min.

Program Setting:

75°C 7 min ramp 0.1

55°C 3 min ramp 0.5

50°C 5 min ramp 0.5

45°C 5 min ramp 0.5

42°C 5 min ramp 0.5

40°C 5 min ramp 0.5

38°C 5 min ramp 0.5

37°C infiny ramp 0.5

11. Move slides to the humidity chamber and incubate at 37 °C overnight or let the sample in the machine chamber overnight at 37 °C. It is very important that the samples never dry.
12. Place the required wash solutions at 42 °C for the next morning.

DAY 2

Important: For all steps, remove the maximum of solution from the slide but never dry the sample to avoid background.

42 °C washes

1. 3 min in 2XSSC, to remove the cover slips.
2. 10 min shaking in formamide (Mix up 20 ml formamide + 80 ml 0.1XSSC).
3. 10 min in 2XSSC, shaking.

Room Temperature washes

4. 10 min in 2XSSC.
5. 10 min in 4XSSC, 0.2% Tween 20 (2 ml Tween 20 in 1L 4XSSC).
6. Add 20 µl per well of Blocking solution (3 ml 4XSSC, 0.2% Tween 20 + 0.15 g BSA.) for 5 min in humidity chamber (no coverslips required).

First Antibody Incubation.

Antibody are diluted in blocking solution

7. Apply 10 µl of Primary mouse anti-dig antibody (1:5000) per well, apply a coverslip and incubate 1h at 37°C in humidity chamber.
8. Wash slides at Room temperature in 4XSSC, 0.2% Tween 20, 15 min, shaking. Make sure that the coverslips have been removed in the solution.

Second Antibody Incubation.

9. Apply 10 µl of Secondary anti-mouse-Alexa 488 (1:500) per well and anti-biotin-Cy3 (ExtrAvidin-Cy3) (1:300). Incubate for 1h at 37°C (use coverslip).

FOLLOWING STEPS MUST BE DONE IN DARKNESS, USE AL FOIL

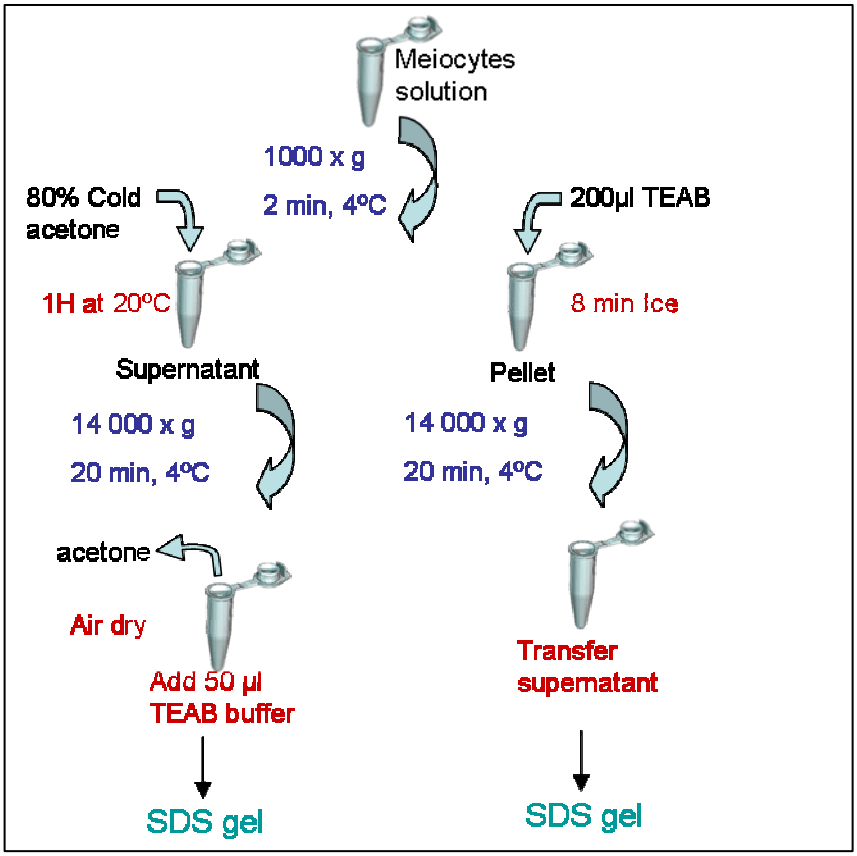
10. Repeat step 8.
11. Incubate 10 min. in DAPI (1µg/ml), 20 µl per well (optionnal coverslips).
12. Briefly wash slides at Room temperature in 4XSSC, 0.2% Tween several seconds.
13. Mount slides in Vectashield (mounting medium H-1000). Add 1 small drop on each well, and cover with a full-length glass cover slip (CoverGlass, 22x50 mm, thickness No 1.5), press upside down on paper towel, and Seal ends with nail polish. Store in slide box at room temperature to dry, and at 4°C for longer strorage.

Appendix 10 – TEAB Proteins Extraction

TEAB Extraction Buffer

TEAB Buffer 1M	1ml (25Mm)
Urea	19.2g (8M)
20%SDS	0.2ml (0.1%)
Triton X-100	0.8ml (2%)
Distilled Water	to 40ml

Dissolve Urea in 30ml of distilled water, add the other reagent and adjust the volume to 40ml.

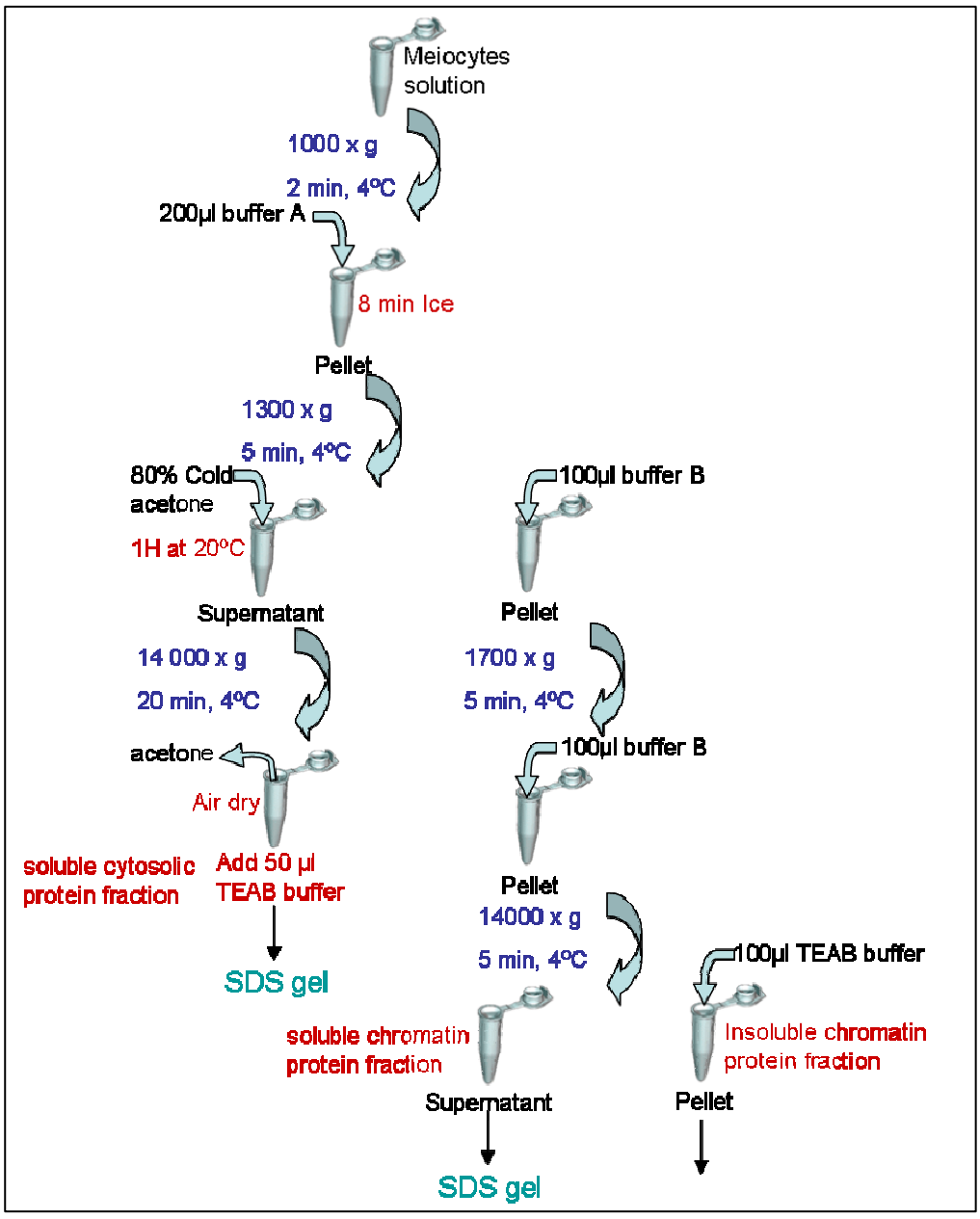


Appendix 11 - Chromatin isolation

Buffer A *	
100mM HEPES	1ml
100mM KCl	1ml
15mM $MgCl_2$	1ml
0.68M Sucrose	5ml
100% Glycerol	1ml
0.5M DTT	20µl
H_2O	980 µl

* Add 0.1% of TritonX100 before extraction

Buffer B - Chromatin isolation	
500mM EDTA	60µl
100mM EGTA	20µl
0.5M DTT	20µl
Dilute in H_2O for 10ml of solution	



Appendix 12 - TCA protocol

Base Buffer 1

1M Tris-HCl pH7.6	10ml
5M NaCl	6ml
Tween20	2ml

Dilute in H_2O for 100ml of solution and keep in the fridge

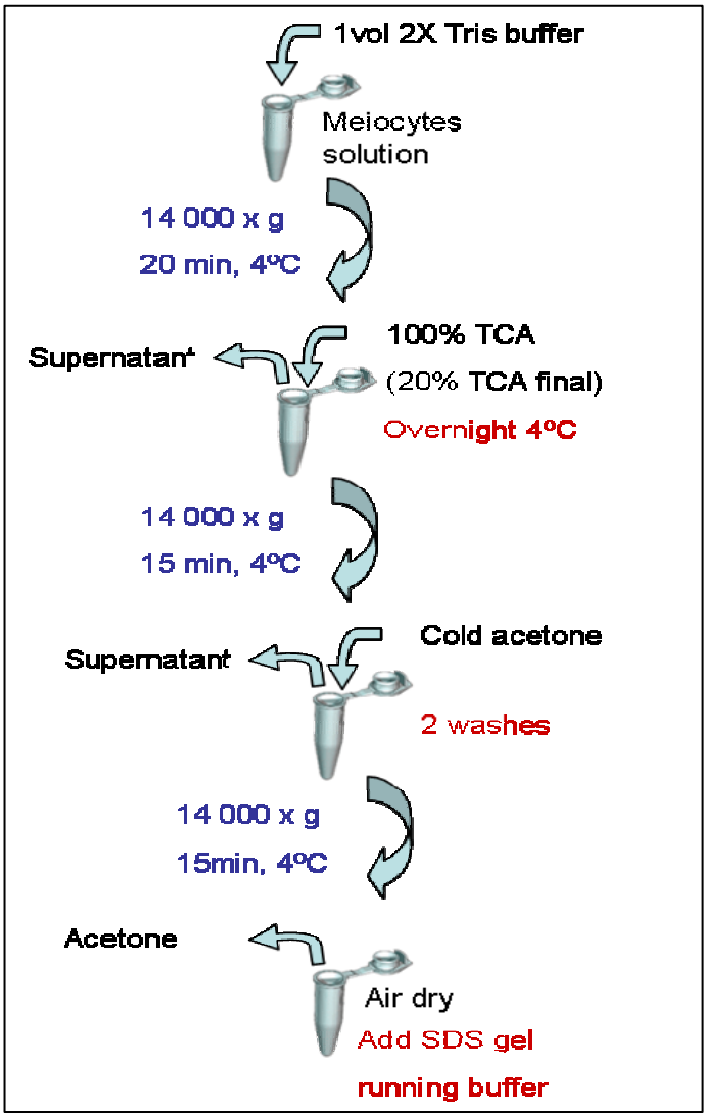
2X Extraction Buffer

Add to 10ml of base buffer 1

100 µl of 0.5M EDTA

20 µl of 1M DTT

Roche protease inhibitor



Appendix 13 - Protein list from Long run of Roots Tips

prot_desc	prot_cover	pep_exp_mz	pep_exp_mr	pep_calc_mr	pep_delta	pep_seq
TA61661_4565 Methionine synthase 1 enzyme [Hordeum vulgare (Barley)]	10.6	876.05	1750.09	1749.99	0.09	SFSPLSLLSSILPVYK
TA60000_4565 Putative elongation factor 2 [Oryza sativa (japonica cultivar-group)]	11.1	857.44	1712.87	1712.9	-0.03	ALLEMMIFHLPSPSK
TA64426_4565 Glutathione transferase [Triticum aestivum (Wheat)]	12.3	578.7	1733.08	1732.93	0.15	GLVLLDFWVSPFGQR
TA125_4571 Malate dehydrogenase, cytoplasmic [Zea mays (Maize)]	13.1	680.87	1359.73	1359.75	-0.02	MELIDAAFPLLK
TA61175_4565 Eukaryotic initiation factor 4A [Oryza sativa (japonica cultivar-group)]	12.5	778.34	1554.67	1554.71	-0.04	MFVLDEADEMLSR
TA61180_4565 Eukaryotic initiation factor 4A [Triticum aestivum (Wheat)]	8.3	778.34	1554.67	1554.71	-0.04	MFVLDEADEMLSR
CV065259 Malate dehydrogenase [Vitis vinifera (Grape)]	19.8	897.08	1792.15	1792.06	0.08	VAILGAAGGIGQPLALLMK
TA136_4571 Putative actin depolymerizing factor [Oryza sativa (japonica cultivar-group)]	12.5	744.36	1486.71	1486.75	-0.04	IFYIFWSPDTAK
TA64396_4565 Beta-glucosidase [Triticum aestivum (Wheat)]	7.4	558.82	1115.63	1115.66	-0.04	GLTDLLLIMK
TA59590_4565 Tubulin beta-1 chain [Zea mays (Maize)]	5.8	812.02	1622.03	1621.84	0.18	LHFFMVGFAPLTSR
TA64777_4565 Putative Aconitate hydratase [Oryza sativa (japonica cultivar-group)]	13.5	611.38	1831.12	1830.99	0.13	NGVTATDLVLTVTQMLR
TA536_4571 putative 40S ribosomal protein S15 [Oryza sativa (japonica cultivar-group)]	16.9	683.38	2047.12	2047.08	0.03	NMIIVPEMIGSLIGVYNGK
TA123_4568 Cell division control protein 48 homolog E related cluster	8	716.44	1430.87	1429.82	1.04	IVSQLLTLMDGLK
TA52980_4565 H0212B02.14 protein [Oryza sativa (Rice)]	11.4	859.53	1717.05	1716.97	0.08	IGEIPAIEEFVFLKL
TA56700_4565 Immature spike ubiquitin-conjugating enzyme 2 [Triticum aestivum (Wheat)]	9.5	881.42	2641.24	2641.27	-0.03	EQVFSTYSDNQPGVLIQVFEGER
TA49777_4565 Unidentified 6.3/40K protein [Oryza sativa (Rice)]	9.7	667.84	1333.67	1333.67	-0.01	TNIFSYFLMAK
TA64894_4565 ATP synthase beta subunit [Triticum aestivum (Wheat)]	5.3	729.45	1456.89	1456.83	0.05	TVLIMELINNVAK
TA131_4568 Nucleoside diphosphate kinase related cluster	6.5	858.91	1715.81	1715.87	-0.06	EIALWFPEGIAEWR
BE585841 Tubulin beta-1 chain [Zea mays (Maize)]	12.3	658.42	1314.83	1313.72	1.11	GELIDSVLDVVR
Enolase 2 related cluster	7	619.04	1854.1	1853.94	0.16	LAMQEFMILPTGAASFK
TA596_4571 Cytosolic 6-phosphogluconate dehydrogenase [Oryza sativa (Rice)]	13.8	666.09	1995.25	1995.12	0.13	IGLAGLAVMGQNLALNIAEK
CV066141 T6D22.2 [Arabidopsis thaliana (Mouse-ear cress)]	15.6	631.67	630.66	630.34	0.32	GTQAVR
TA27_4571 Glyceraldehyde-3-phosphate dehydrogenase, cytosolic [Hordeum vulgare (Barley)]	9.9	730.39	2188.15	2188	0.15	GIMGYVEEDLVSTDFVGDSR
OSJNBa0035M09.3 protein [Oryza sativa (japonica cultivar-group)]	10.1	784.06	783.05	782.5	0.55	APLGGLKK
TA50950_4565 Poly(A)-binding protein [Triticum aestivum (Wheat)]	5.3	636.46	1906.36	1906.9	-0.54	ALYDTFCVFGNILSCK

TA58321_4565 DnaK protein, putative [Oryza sativa (japonica cultivar-group)]	15.6	608.31	1214.61	1214.64	-0.04	DAGVIAGLNVMR
TA62920_4565 DnaK protein, putative [Oryza sativa (japonica cultivar-group)]	14.7	585.86	1169.71	1168.66	1.05	DAGAIAGLNVLR
TA58286_4565 DnaK protein, putative [Oryza sativa (japonica cultivar-group)]	15.7	599.35	1196.69	1196.69	0	DAGVIAGINVLR
TA78_4568 Heat shock cognate 70 kDa protein 3 related cluster	14.8	608.31	1214.61	1214.64	-0.04	DAGVIAGLNVMR
TA58438_4565 Cyclophilin A-3 [Triticum aestivum (Wheat)]	24.5	551.77	1101.53	1101.55	-0.02	QVVIADCGQL
TA61755_4565 Phosphoglycerate kinase, cytosolic [Triticum aestivum (Wheat)]	19.3	694.84	1387.67	1387.73	-0.07	ELDYLVGAVANPK
TA38_4568 Heat shock protein 81-3 related cluster	10.1	636.38	1270.75	1271.68	-0.94	SDLVNNLGTIAR
TA76_4571 Cyclophilin A-2 [Triticum aestivum (Wheat)]	22.6	551.77	1101.53	1101.55	-0.02	QVVIADCGQL
TA51577_4565 Tubulin beta-1 chain [Triticum aestivum (Wheat)]	12.5	530.26	1058.51	1058.52	-0.02	YLTASAMFR
TA64208_4565 Putative nucleolin [Oryza sativa (indica cultivar-group)]	15.1	754.18	1506.35	1506.78	-0.44	QFFAQIGEVVDVR
TA5_4568 Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3 related cluster	12.4	581.31	1160.61	1160.62	-0.01	AGIALNDNFVK
TA61783_4565 40 kDa PI 8.5 ABSCISSIC acid-induced protein [Oryza sativa (Rice)]	17.2	509.76	1017.51	1017.55	-0.04	DGTSLVLWK
TA55_4568 Tubulin alpha-1 chain related cluster	12.5	566.8	1131.59	1131.56	0.03	EIVDLCLDR
TA57592_4565 Enolase 2 [Zea mays (Maize)]	8	787.4	1572.79	1572.84	-0.05	VNQIGSVTESIEAVK
TA8_4568 Elongation factor 1-alpha related cluster	14.8	649.9	1297.79	1297.74	0.05	EHALLAFTLGVK
TA11_4568 Elongation factor 1-alpha related cluster	17.4	649.9	1297.79	1297.74	0.05	EHALLAFTLGVK
TA59983_4565 Putative elongation factor 2 [Oryza sativa (japonica cultivar-group)]	8	594.27	1186.53	1187.61	-1.09	VMQTWLPASR
TA61661_4565 Methionine synthase 1 enzyme [Hordeum vulgare (Barley)]	7.3	548.8	1095.59	1095.57	0.01	YLFAGVVDGR
TA61796_4565 40 kDa PI 8.5 ABSCISSIC acid-induced protein [Oryza sativa (Rice)]	19.6	565.65	1693.93	1692.87	1.06	IKDEEGYPAFALVNK
TA63746_4565 Elongation factor 1-beta [Triticum aestivum (Wheat)]	17.5	660.32	1318.63	1318.67	-0.04	WYDTVAAAVAPR
TA64421_4565 Glutathione transferase [Triticum aestivum (Wheat)]	10.2	473.75	945.49	945.52	-0.04	AEMLEILK
CJ646641 NME2 protein [Homo sapiens (Human)]	24.3	472.28	942.55	942.55	0	GLIGEVISR
TA86_4568 Fructose-bisphosphate aldolase, cytoplasmic isozyme related cluster	11.8	673.87	1345.73	1345.72	0	VAPEVIAEYTVR
TA50622_4565 Heat shock protein 70 [Cucumis sativus (Cucumber)]	6.8	578.36	1154.71	1154.64	0.06	DAGVIAGLNVAR
TA139_4571 UTP--glucose-1-phosphate uridylyltransferase [Hordeum vulgare (Barley)]	10.6	679.86	1357.71	1357.75	-0.04	IVTEDFLPLPSK
CK207978 Putative nucleolin [Oryza sativa (indica cultivar-group)]	19.1	754.18	1506.35	1506.78	-0.44	QFFAQIGEVVDVR
TA339_4568 60S ribosomal protein L24 related cluster	11.5	629.39	1256.77	1256.73	0.03	SIVGATLEVIQK
TA620_4568 60S ribosomal protein L9 related cluster	19.7	679.87	1357.73	1358.76	-1.03	KVDMLDGVTILR

TA1696_4568 Nascent polypeptide associated complex alpha chain related cluster	18.5	742.95	1483.89	1483.72	0.17	SPASDTYVIFGEAK
TA73_4568 40S ribosomal protein S8 related cluster	9.5	867.94	1733.87	1733.89	-0.03	LLDVVYNSSNNELVR
TA55294_4565 UDP-glucose/GDP-mannose dehydrogenase family	5.9	600.37	1198.73	1198.59	0.14	NLFFSTDVEK
CJ679170 Cyclophilin A-3 [Triticum aestivum (Wheat)]	14.8	710.9	1419.79	1419.66	0.12	VFFXMTVGGAPAGR
BJ277510 Probable elongation factor 1-gamma 2 [Arabidopsis thaliana (Mouse-ear cress)]	22.9	547.34	1092.67	1092.62	0.05	NPLDLLPPSK
TA58132_4565 Triosephosphat-isomerase [Triticum aestivum (Wheat)]	5.5	684.58	1367.15	1367.78	-0.63	LVVIVDVVDQNR
TA62000_4565 Hypothetical protein [Solanum tuberosum (Potato)]	10.2	547.34	1092.67	1092.62	0.05	NPLDLLPPSK
TA222_4568 Putative acidic ribosomal protein P3a related cluster	17.3	493.23	984.45	984.49	-0.04	GVFTFVCR
TA143_4568 14-3-3-like protein A related cluster	8.4	607.03	1818.07	1817.96	0.1	SAQDIALADLPTTHPIR
CV766197 Low temperature-responsive RNA-binding protein [Hordeum vulgare (Barley)]	7.6	586.33	585.32	586.38	-1.06	GGKVVK
CK216562 Putative 40S ribosomal protein S2 [Oryza sativa (japonica cultivar-group)]	13.2	686.35	1370.69	1370.71	-0.03	ALMLDAPAEKIEA
TA58210_4565 Putative 40S ribosomal protein S2 [Oryza sativa (japonica cultivar-group)]	16	678.81	1355.61	1356.7	-1.09	ALMLDAPAEKVEA
CJ565957 PREDICTED: similar to tubulin, alpha 2; tubulin alpha 2 [Gallus gallus]	9.5	634.25	1266.49	1266.5	-0.02	YMACCLMYR
TA61177_4565 Eukaryotic initiation factor 4A [Triticum aestivum (Wheat)]	5.8	557.29	1112.57	1113.68	-1.11	VLITTDLLAR
TA328_4571 Elongation factor 1-gamma 3 [Oryza sativa (Rice)]	13.3	618.34	1852	1850.95	1.05	SKGDSLLWGGSLIEYAR
TA64426_4565 Glutathione transferase [Triticum aestivum (Wheat)]	4.8	466.74	931.47	931.5	-0.04	AEMLDILK
TA194_4568 Guanine nucleotide-binding protein beta subunit-like protein related cluster	8.8	624.83	1247.65	1247.65	-0.01	LWDLSTGVTTR
TA316_4568 60S acidic ribosomal protein P0 related cluster	5.3	648.81	1295.61	1295.64	-0.04	LCQLLEEYTK
CK211262 Ribosomal protein L7 [Triticum aestivum (Wheat)]	6.9	633.35	1264.69	1265.61	-0.92	EADNFLWPFK
TA63905_4565 AAA family ATPase, CDC48 subfamily [Oryza sativa (japonica cultivar-group)]	3.8	572.31	1142.61	1141.65	0.96	GILLFGPPGSGK
TA231_4568 40S ribosomal protein S12 related cluster	11.6	567.8	1133.59	1133.55	0.03	TLGEWAGLCK
TA61148_4565 Adenosine kinase-like protein [Oryza sativa (japonica cultivar-group)]	6.6	712.85	1423.69	1423.71	-0.03	SLIANLSAANCYK
BG314470 40S ribosomal protein S9 related cluster	11.3	495.78	989.55	988.57	0.97	IFEGAALLR
TA1313_4568 60S ribosomal protein L7a related cluster	8.6	689.39	1376.77	1376.77	0	TLDKNLATNLFK
TA63975_4565 Ascorbate peroxidase [Hordeum vulgare (Barley)]	9	578.36	1154.71	1154.57	0.14	WGVALFCFR
TA136_4568 14-3-3 protein related cluster	8.9	596.29	1785.85	1785.97	-0.13	AAQDIALAELAPTHPIR
TA51202_4565 Putative 60S ribosomal protein L1 [Oryza sativa (japonica cultivar-group)]	6.7	482.61	1444.81	1444.82	-0.01	LNLLDLAPGGHLGR
CA669031 Wound-induced basic protein [Phaseolus vulgaris (Kidney bean) (French bean)]	18.6	472.28	942.55	943.55	-1	LGLAGTGLSR

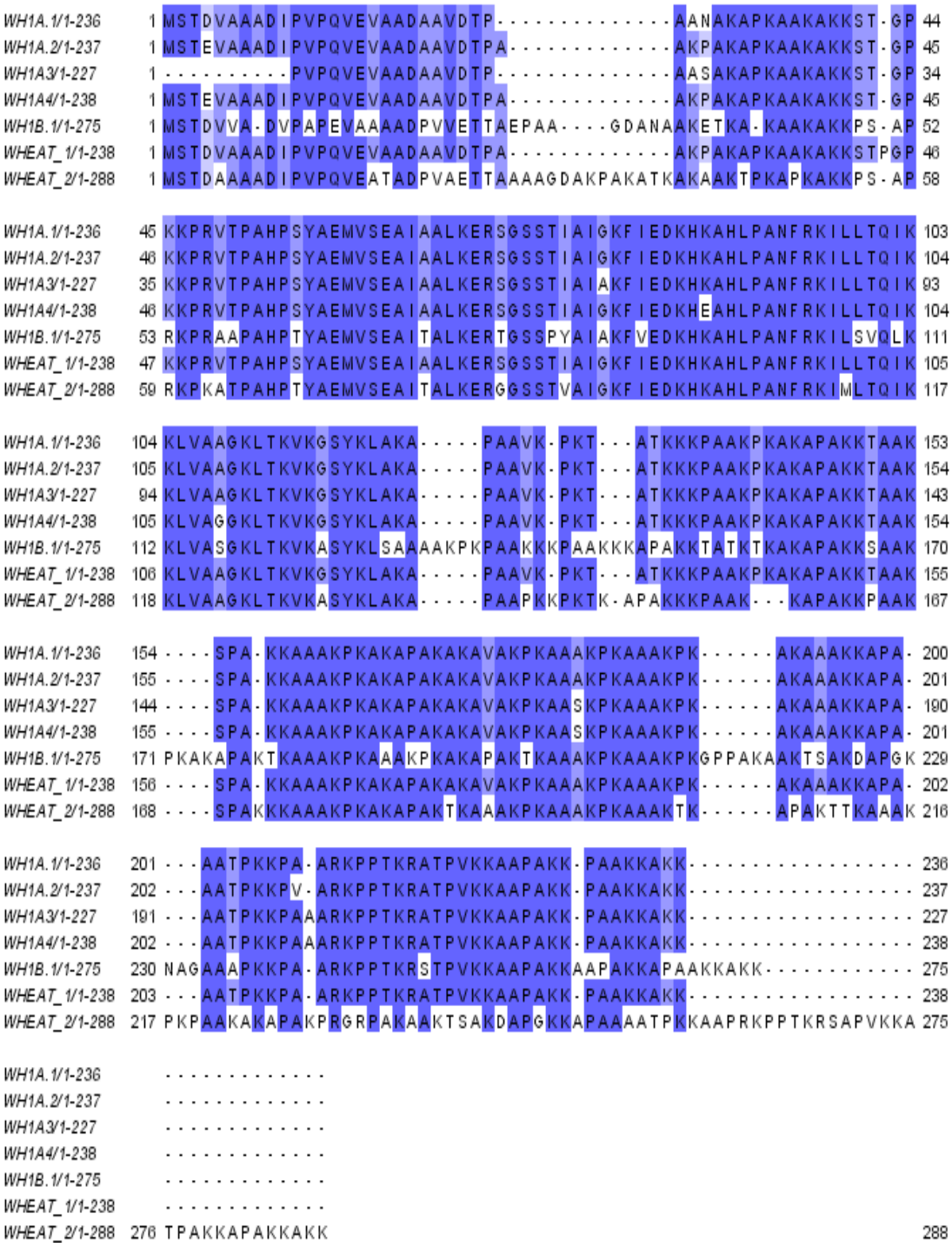
TA58414_4565 Fructose-bisphosphate aldolase [Persea americana (Avocado)]	10	444.74	887.47	887.51	-0.04	ALQQSTLK
TA50407_4565 Unidentified 6.3/40K protein [Oryza sativa (Rice)]	19	495.77	989.53	989.52	0	ALSMQLAEK
TA50411_4565 Unidentified 6.3/40K protein [Oryza sativa (Rice)]	25	495.77	989.53	989.52	0	ALSMQLAEK
TA86_4568 Fructose-bisphosphate aldolase, cytoplasmic isozyme related cluster	16.7	444.74	887.47	887.51	-0.04	ALQQSTLK
CK162234 Fructose-biphosphate aldolase [Mesembryanthemum crystallinum]	10	442.64	883.27	883.49	-0.22	TTGTHLVR
TA60452_4565 60S acidic ribosomal protein P0 [Zea mays (Maize)]	9.3	531.28	1060.55	1060.58	-0.03	VGSSESALLAK
TA55987_4565 Low temperature-responsive RNA-binding protein [Hordeum vulgare (Barley)]	27.9	448.71	895.41	895.43	-0.02	YGDVIDSK
CK170672 Adenosylhomocysteinase [Triticum aestivum (Wheat)]	14.6	423.74	845.47	845.54	-0.07	KVYVLPK
TA54977_4565 T6D22.2 [Arabidopsis thaliana (Mouse-ear cress)]	18.5	390.72	779.43	779.41	0.02	YEEIVK
TA61177_4565 Eukaryotic initiation factor 4A [Triticum aestivum (Wheat)]	8.7	552.75	1103.49	1103.65	-0.16	KGVAINFVTR
TA59267_4565 60S ribosomal protein L5-1 [Oryza sativa (Rice)]	5.9	649.78	1297.55	1297.59	-0.04	GISADDMEAVYK
TA55964_4565 Low temperature-responsive RNA-binding protein [Hordeum vulgare (Barley)]	25.6	448.71	895.41	895.43	-0.02	YGDVIDSK
TA51202_4565 Putative 60S ribosomal protein L1 [Oryza sativa (japonica cultivar-group)]	20.2	439.18	876.35	876.41	-0.07	MTNADLGR
TA63551_4565 Putative 40S ribosomal protein [Oryza sativa (japonica cultivar-group)]	14.6	389.18	776.35	776.4	-0.06	MVLQMR
TA59227_4565 14-3-3 e-2 protein [Nicotiana tabacum (Common tobacco)]	13	409.73	817.45	817.44	0.01	ICDGILK
TA56748_4565 OSJNBa0085I10.12 protein [Oryza sativa (japonica cultivar-group)]	16.5	561.24	1120.47	1120.51	-0.04	TAEVAEGAMDK
TA347_4568 Triosephosphate isomerase, cytosolic related cluster	15.4	645.21	1288.41	1288.63	-0.22	TNVSPEVAESTR
TA60196_4565 Putative 60S ribosomal protein L13E [Oryza sativa (japonica cultivar-group)]	7.2	631.77	1261.53	1261.61	-0.08	SLEGMQSNIQR
CK163602	6.8	602.26	1202.51	1203.57	-1.07	DAAAGATQTAAEK
CA704455 14-3-3 e-2 protein [Nicotiana tabacum (Common tobacco)]	13.5	536.89	1071.77	1071.55	0.22	TKIETELXK
TA56734_4565 OSJNBa0085I10.12 protein [Oryza sativa (japonica cultivar-group)]	7.7	558.78	1115.55	1115.58	-0.04	VAETAQALGEK
TA51577_4565 Tubulin beta-1 chain [Triticum aestivum (Wheat)]	9.3	533.21	1064.41	1064.42	-0.01	NMMCAADPR
TA57568_4565 LEA3 protein [Triticum aestivum (Wheat)]	14.5	499.66	997.31	997.47	-0.17	ASETAQYTK
TA78_4568 Heat shock cognate 70 kDa protein 3 related cluster	10.7	446.2	890.39	891.38	-1	CMEPVEK
TA60000_4565 Putative elongation factor 2 [Oryza sativa (japonica cultivar-group)]	12	365.19	728.37	728.41	-0.04	VASDLPK
TA58286_4565 DnaK protein, putative [Oryza sativa (japonica cultivar-group)]	11.4	446.2	890.39	891.38	-1	CMEPVEK
TA71867_4565 Peroxidase precursor [Triticum aestivum (Wheat)]	7	457.73	913.45	913.52	-0.08	VALDLIDR
TA56768_4565 DnaK protein, putative [Oryza sativa (japonica cultivar-group)]	16.1	446.2	890.39	891.38	-1	CMEPVEK

CV759706 Low temperature-responsive RNA-binding protein [Hordeum vulgare (Barley)]	13.2	448.71	895.41	895.43	-0.02	YGDVIDSK
TA411_4571 Actin-1 [Oryza sativa (Rice)]	13.1	488.24	974.47	975.44	-0.98	AGFAGDDAPR
TA61777_4565 40 kDa PI 8.5 ABSCISSIC acid-induced protein [Oryza sativa (Rice)]	12.8	373.69	745.37	745.39	-0.03	MVNNIR
TA55984_4565 Low temperature-responsive RNA-binding protein [Hordeum vulgare (Barley)]	20	448.71	895.41	895.43	-0.02	YGDVIDSK
TA61781_4565 40 kDa PI 8.5 ABSCISSIC acid-induced protein [Oryza sativa (Rice)]	15.7	373.69	745.37	745.39	-0.03	MVNNIR
TA52757_4565 GF14-b protein [Oryza sativa (Rice)]	6.8	409.73	817.45	817.44	0.01	ICDGILK
AL817087 OSJNBa0085I10.12 protein [Oryza sativa (japonica cultivar-group)]	13.8	561.24	1120.47	1120.51	-0.04	TAEVAEGAMDK
CK216055 40S ribosomal protein S14 [Ictalurus punctatus (Channel catfish)]	11.4	519.78	1037.55	1037.56	-0.02	TPGPGAQAALR
TA125_4571 Malate dehydrogenase, cytoplasmic [Zea mays (Maize)]	10.1	437.22	872.43	872.47	-0.05	ALGQISER
AB003682 Alpha-amylase inhibitor [Triticum urartu]	20.2	581.77	1161.53	1161.62	-0.09	LTAASITAVCR
BE427364 Eukaryotic initiation factor 4A [Triticum aestivum (Wheat)]	7.3	530.76	1059.51	1060.46	-0.95	FPGCRXSAR
WHTAGGTD Agglutinin isolectin 3 precursor [Triticum aestivum (Wheat)]	7.4	634.71	1267.41	1267.51	-0.1	GCQNGACWTSK
TA38_4568 Heat shock protein 81-3 related cluster	6.7	357.74	713.47	713.44	0.02	IAELLR
TA57670_4565 Putative ribosomal protein S18 [Triticum aestivum (Wheat)]	9.9	458.72	915.43	915.48	-0.05	LRDDLER
TA62522_4565 NME2 protein [Homo sapiens (Human)]	14.2	384.13	766.25	766.36	-0.12	SSLMASR
TA58148_4565 Triosephosphat-isomerase [Triticum aestivum (Wheat)]	15.7	616.24	1230.47	1230.62	-0.15	TNVSPEVAASTR
TA58132_4565 Triosephosphat-isomerase [Triticum aestivum (Wheat)]	11.9	521.74	1041.47	1041.59	-0.13	GGAIRQELAK
TA64396_4565 Beta-glucosidase [Triticum aestivum (Wheat)]	4.3	482.24	962.47	962.53	-0.06	RLDYLQR
TA59632_4565 Tubulin beta-1 chain [Zea mays (Maize)]	9.1	533.21	1064.41	1064.42	-0.01	NMMCAADPR
TA65088_4565 Mitochondrial chaperonin-60 [Oryza sativa (japonica cultivar-group)]	4.8	462.76	923.51	924.44	-0.93	AIFTEGCK
BQ837967 40S ribosomal protein S20 [Oryza sativa (Rice)]	7.1	408.21	814.41	815.49	-1.08	VLNITTR
TA59625_4565 Tubulin beta-1 chain [Zea mays (Maize)]	11.2	533.21	1064.41	1064.42	-0.01	NMMCAADPR
CK209102 OSJNBa0058K23.21 protein [Oryza sativa (japonica cultivar-group)]	6.4	676.78	1351.55	1351.6	-0.05	GFDSSQQEDAIR
TA79_4568 Putative nucleolin related cluster	9.8	531.79	1061.57	1061.51	0.05	SSLQEHFSK
TA150_4568 Ribosomal protein L6 related cluster	10.6	436.72	871.43	871.5	-0.08	AIDAELIK
TA1174_4568 Adenosylhomocysteinase 1 related cluster	6.5	558.57	557.56	557.32	0.25	SKVQP
CJ724275 DnaK protein, putative [Oryza sativa (japonica cultivar-group)]	14.4	509.24	1016.47	1016.56	-0.1	ITITNDKGR
TA64451_4565 Putative 40S Ribosomal protein [Oryza sativa (Rice)]	12.9	389.18	776.35	776.4	-0.06	MVLQMR

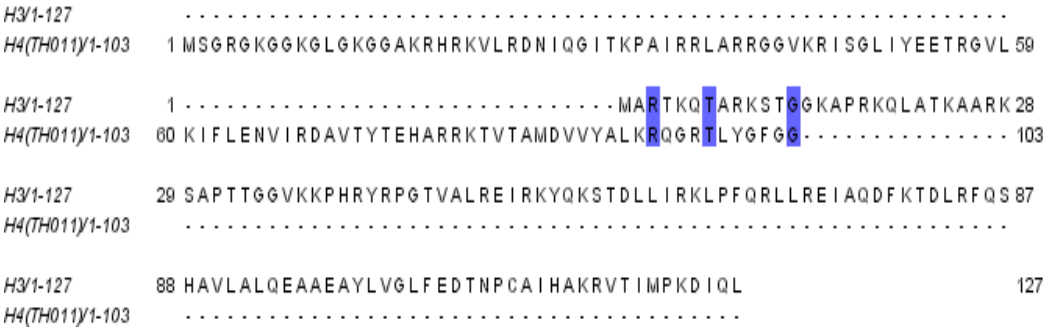
TA329_4568 BRI1-KD interacting protein 108 related cluster	12.8	595.31	1188.61	1188.61	0	AGNLGDSVTVTR
TA70059_4565 Hypothetical protein OSJNBb0006H05.31 [Oryza sativa]	23.9	540.21	1078.41	1078.54	-0.14	KVDDSAFAAR
TA63905_4565 AAA family ATPase, CDC48 subfamily [Oryza sativa]	3.9	538.27	1074.53	1074.53	-0.01	LAGESESNLR
TA152_4568 60S ribosomal protein L2 related cluster	7.2	508.76	1015.51	1015.52	-0.02	AMIGQVAGGGR
CJ525437 60S ribosomal protein L2 [Arabidopsis thaliana]	14.3	508.76	1015.51	1015.52	-0.02	AMIGQVAGGGR
TA63860_4565 15.5 kDa oleosin [Sesamum indicum]	8.5	507.31	1012.61	1012.48	0.13	MADAAAAAGHK
TA315_4571 Ascorbate peroxidase [Hordeum vulgare (Barley)]	10.4	487.72	973.43	973.48	-0.06	NCAPLMLR
CD901129 Ribosomal protein L18 [Triticum aestivum (Wheat)]	14	429.23	856.45	856.48	-0.03	GIDLVAGGR
TA281_4568 40S ribosomal protein S20 related cluster	10.8	408.21	814.41	815.49	-1.08	VLNITTR
TA118_4568 60S ribosomal protein L12 related cluster	8.2	441.23	880.45	880.54	-0.09	IGPLGLSPK
TA64894_4565 ATP synthase beta subunit [Triticum aestivum (Wheat)]	5.2	933.04	1864.07	1863.94	0.13	DAEGQDVLLFIDNIFR
TA65300_4565 Acetyl-coenzyme A carboxylase [Triticum aestivum (Wheat)]	1.3	822.24	1642.47	1642.93	-0.47	LLSSQMILLLELDR

Appendix 14 - Wheat Histone Sequences alignment

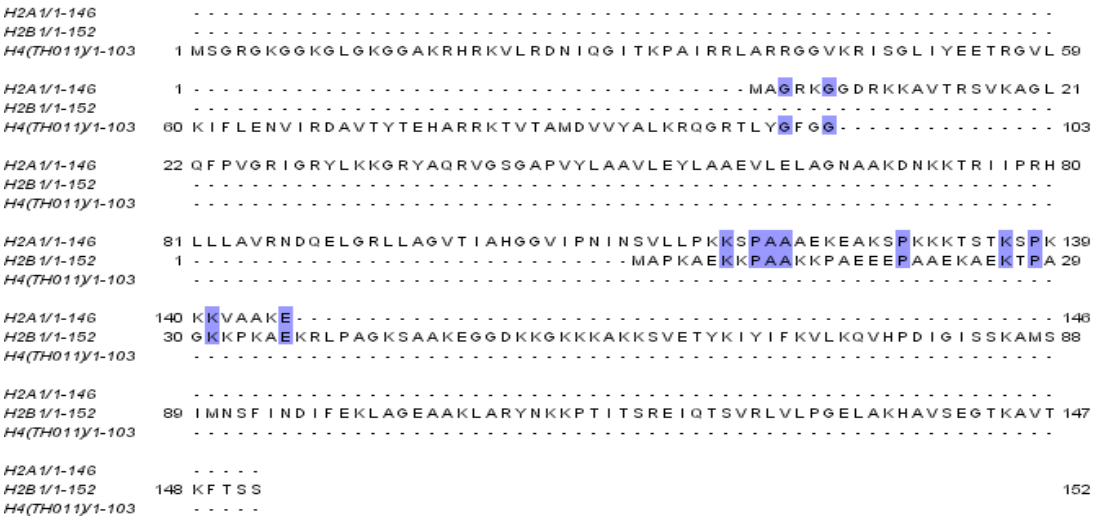
Wheat Histone H1 variants



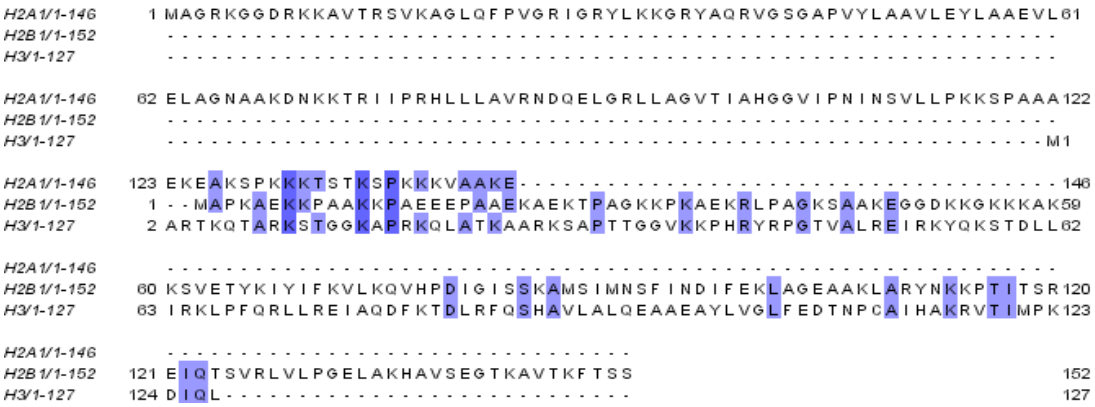
Wheat Histone H3/H4 variants



Histone H2/ Histone H4 alignment



Histone H2/ Histone H3 alignment



Histone H2A/ Histone H2B alignment