Chapter 15
Exploiting Comparative Biology and Genomics to Understand a Trait in Wheat, *Ph1*

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Abstract For hexaploid wheat to be highly fertile, only true homologues must pair at meiosis, rather than the highly related chromosomes present. The mechanism, which restricts this pairing, must have arisen rapidly on wheat's polyploidisation, to ensure stability and fertility. From the analysis of *Ph1*, which is the major locus restricts this pairing, tweaking Cdk-type phosphorylation levels is one way to provide such a control.

Introduction

Meiosis is a cell division process that ensures gametes carry the correct number of chromosomes, without a doubling of chromosome number. During meiosis, chromosome numbers are halved, leading to haploid gametes, a process that is crucial for the maintenance of a stable genome through successive generations. The process to achieve an accurate segregation of the homologous chromosomes (homologues) starts in pre-meiosis as each homologue is replicated and the respective products, sister chromatids are held together as via specific cohesion proteins. Then at the start of meiosis, each chromosome must recognize its homologue from amongst all the chromosomes present in the nucleus and associate or pair, and then recombine with that homologue. The homologues are observed as paired at Metaphase I. The homologues are then separated and segregated to two different daughter cells. In the next round of division, the sister chromatids are then separated, moving to one of four haploid cells. The accuracy of recognition and segregation of the homologues has a profound effect on overall fertility. This is more complicated in polyploids because of the greater number of related chromosomes. Polyploid fertility depends on the efficiency by which they behave as diploids during meiosis by restricting pairing to true homologues, despite the presence of related chromosomes (homoeologues). The mechanism, which restricts this pairing, must arise rapidly on polyploidisation

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to ensure fertility of the new polyploid plant. So what controls the difference between diploid and polyploid pairing in a polyploid species such as wheat?

The hexaploid wheat (AABBDD, 2n = 42) carries a diploid set of 42 chromosomes composed of three ancestral genomes, A, B and D. The 42 chromosomes can be divided into seven groups of six chromosomes (two chromosomes from each of the ancestral genomes). In hexaploid wheat, chromosome 1A must pair with 1A at meiosis and not with 1B or 1D. In wheat, a single locus, *Ph1*, (*P*airing *h*omoeologous *1*) on the long arm of chromosome 5B has a major controlling effect (Okamoto 1957; Riley and Chapman 1958; Sears and Okamoto 1958). Both Riley and Sears experimented with both haploids and wheat-wild relative hybrids, which lack homologues and only possess homoeologues. They observed that, when chromosome 5B was deleted in both haploids and wheat-wild relative hybrids, that there was a level of pairing between the related chromosomes at Metaphase I. They recognized that a locus on chromosome 5B was responsible for the major effect on polyploid pairing in wheat. Using deletions, Sears further defined the effect to the long arm of wheat chromosome 5B (Sears 1977). Thus *Ph1* is defined as a deletion phenotype.

The Basic Chromosome Pairing and Recombination Process

In many species, there are chromosome movements at the onset of meiosis, which enable the telomeres of the chromosomes to cluster as a telomere bouquet. Within this cluster of telomeres, the terminal regions of homologous chromosomes find their correct homologous partner. The two homologues then "zip up" from the telomere regions. This process of "zipping up" or synapsis involves the placement of a protein complex, a synaptonemal complex, between the homologues. Essentially this is equivalent to "gluing" the chromosomes together. Within this synaptonemal complex structure, double strand breaks can be repaired. Meiotic recombination occurs through the generation and repair of double strand breaks (DSBs) using the homologues or homoeologues. Essentially the double strand break is formed early during meiosis and is then resectioned to generate a single strand end. The pairing process involves the single strand end finding and invading the homologous region of the corresponding homologue or homoeologue. Successful invasion results in strand displacement in this region, generation of a Double Holiday Junction, a cross-over event (chiasmata) leading to recombination. The single strand invasion occurs during late zygotene, and double holiday formation occurs during pachytene. The synaptonemal complex (SC) starts to be disassembled during pachytene. The formation of chiasmata, physical links which together with sister chromatid cohesion, still hold the homologues together after the disassembly of the SC, so the chromosomes are visualized as paired at metaphase I. If single strand invasion is unsuccessful, the double strand break can be repaired through using its own sister chromatid, leading to the chromosomes being visualized as unpaired at metaphase I.

The Power of a Cell Biological Experiment

After 50 years, the cell biological tools (antibodies to key meiotic proteins) finally became available to answer two fundamental questions about *Ph1*.

Firstly, the question as to whether *Ph1* actually blocks chromosome pairing between related chromosomes (homoeologues). The locus is named homoeologous pairing 1 (*Ph1*) because it is always assumed that it reduces pairing or synapsis between homoeologues, and that this then subsequently affects the levels of recombination between such chromosomes. However, our recent cell biology data reveals that in wheat-rye hybrids, where there are no homologues, only homoeologues, the related chromosomes pair or synapse to a similar level, whether *Ph1* is present or absent (Martin et al. 2014). Therefore *Ph1* doesn't suppress homoeologues pairing in the hybrid. This implies that in wheat itself where there are both homologues and homoeologues, the overall effect of *Ph1* on chromosome pairing as distinct from recombination must be the promotion of homologue pairing rather than specifically suppressing pairing between the related chromosomes.

Secondly at what stage does *Ph1* block recombination between homoeologues, a process that occurs on both the male and female sides from leptotene to diplotene? The major surprise of our recent study is that double strand breaks are formed at similar levels, and are processed with similar kinetics into Double Holliday Junctions between the paired homoeologues whether *Ph1* is present or absent (Martin et al. 2014). This results in a similar number of Double Holliday Junctions at diplotene in the hybrid as revealed by immunolabelling with the MLH1 antibody. In all other species so far studied, MLH1 marks sites on paired chromosomes that will become crossovers. However, these studies have been performed on paired homologues rather than paired homoeologues as in the case of the wheat-rye hybrid. Based on the number of MLH1 sites, 21 crossovers would be expected between the paired homoeologues in the wheat-rye hybrid whether *Ph1* is present or absent, yet only seven crossovers on average occur in the absence of *Ph1,* and one or none in the presence of *Ph1*. Therefore the resolution of Double Holliday Junctions to crossovers between the paired homoeologues fails in both the presence and absence of *Ph1*, but this failure is partially alleviated by deleting *Ph1* or increasing Cdk2-type activity. Thus *Ph1* suppresses recombination between homoeologues by preventing the resolution of Double Holliday Junctions as crossovers (Martin et al. 2014).

The MLH1 protein complex involved in this resolution has been characterized in other species. It contains two mismatch repair proteins, MLH1 and MLH3, EXO1 (a nuclease), CDK2 (which is activated by a meiotic specific cyclin), and finally an E3 Ubiquitin ligase (HEI10), which may be involved in the degradation of other cyclins, enabling the meiotic cyclin to activate CDK2. Various recent reports have provided indirect evidence that CDK2 regulates the activity of the MLH1 complex and crossovers (Martin et al. 2014).

Thus this cell biological study reveals that *Ph1* has two distinct effects on chromosome pairing and recombination. Firstly it promotes homologue pairing rather

than prevent homoeologue pairing, and secondly it prevents recombination between paired homoeologues by stalling Double Holliday Junctions from being resolved as crossovers (Martin et al 2014). Interestingly subsequently a number of Cdk2 studies have reported that it has two distinct effects on chromosome pairing and recombination. It affects chromosome pairing through altering the function of the telomere bouquet, and recombination via crossover resolution (Liu et al. 2014; Viera et al. 2015). So what is *Ph1* locus?

Ph1 Locus at a Molecular Level

Given *Ph1* is a deletion phenotype effect, its mapping required the screening of mutagenised hexaploid and tetraploid wheat populations to identify a set of overlapping deletions covering chromosome 5B. Over a 15-year period ten mutagenised populations were developed and screened in hexaploid wheat (Roberts et al. 1999), and one mutagenised population in tetraploid wheat with the help of Shrahryar Kianian. The deletion breakpoints needed to be located and the gene content of the regions covered by these deletions revealed. The hexaploid wheat genome is 5x larger than the human genome and was unsequenced. To solve this problem, the use of the small rice genome, as a model for the larger wheat genome was postulated, should there be conservation of gene order (Moore et al. 1993). This needed to be confirmed before the *Ph1* cloning strategy could be implemented (and funded). Conservation in gene order, was confirmed first at the genetic and then at the physical level (Moore et al. 1993; Kurata et al. 1994; Foote et al. 1997; Griffiths et al. 2006). *Brachypodium* with its small genome was also added to this concept (Moore et al. 1993; Foote et al. 2004; Griffiths et al. 2006). Taking this approach further, it should be possible to reconstruct the ancestral genome from which the genomes of present day cereals and grasses have evolved (Moore et al. 1993). To this end, mapping data for rice from Japan, for maize and sorghum from North America, for sugarcane from France and finally for rye, wheat and millets from the UK was used. From these datasets, there was indeed a pattern of genomic building blocks or groups of genes within the rice genome, which could be used to describe the structure of all the other cereal chromosomes (Moore et al. 1995). The comparison of the order of blocks within the different cereal chromosomes, revealed that they could all be derived from the cleavage of a single structure, a hypothetical 'ancestral' genome, formed from the blocks, and a diagrammatical framework for comparing the order of all the major cereal genomes unified cereal genetics (Moore et al. 1995). This concept was purposefully termed "Synteny", which in classical genetics had been used in a different context, but which is today the widely used term for the concept, indicating its cereal origin (see Encyclopaedia Britannica). With the development of this concept, funding was made available from BBSRC for the *Ph1* cloning strategy, in particular the development of genomic libraries for wheat and *Brachypodium* in order to generate a physical contig of the *Ph1* locus. A 1.2 million clone BAC library was constructed with INRA (Allouis et al. 2003). The *Ph1* deletion effect

region was delineated by phenotyping the deletion lines and mapping the deletion breakpoints using Synteny. The breakpoints of deletions lacking *Ph1* clustered non-randomly either side of a 2.5 Mb region carrying a large segment of satellite DNA, located within an amplified Cdk locus (Griffiths et al. 2006). There is also an anther specific gene within the delimited *Ph1* region, which is the homologue of RA8 in rice, now named Raftin1 protein. We initially named the annotated wheat gene, as RA8, then Raf1 in subsequent analyses (Griffiths et al. 2006; Al-Kaff et al. 2008). The Raftin genes are anther specific and have been extensively characterized in rice, and now in wheat (Jeon et al. 1999; Wang et al. 2003; Sheng et al. 2011). They are mainly expressed in tapetal cells and are responsible for transporting lipids and cell wall proteins to the developing meiocytes. Mutation or deletion of the genes produces male steriles, as a result of the microspores becoming stressed (dehydrated). Stressed meiocytes exhibit chromosome clumping or clustering at metaphase I. Thus the genes have been patented in rice, maize and wheat for making male steriles in hybrid production. We excluded this gene as being responsible for the *Ph1* effect because: it is only expressed on the male side and not on the female side; it is not expressed during the stages when recombination occurred on the male side; and finally if the 5B copy is functional, its deletion would result in male steriles, which are not observed with deletion of the *Ph1* region. Consistent with this observation, the 5B copy of RA8/Raf1 carries an early stop codon, and the transcripts derived from this copy are antisense and not sense. The transcripts run into the promoter regions and contain exonintron junctions in the incorrect orientation.

Subsequently, we identified two additional deletion mutants which possessed wild type pairing in wheat itself, and therefore both retained the *Ph1* locus (Al-Kaff et al. 2008). One of these deletions encompassed the RA8/Raf1 gene. Thus by a process of exclusion, the analysis delineated the *Ph1* locus to a region where nearly half the genes are a cluster of kinases, including Cdk2-like genes. Expression analysis revealed that many of these Cdk2-like genes are expressed during meiotic prophase I, where the processes of pairing and recombination occur. To take the molecular study further required a working hypothesis for *Ph1's* mode of action. Given nearly half the genes in the delimited region are kinases, our hypothesis is that *Ph1* affected kinase activity and hence overall phosphorylation levels. Amongst these kinases is a cluster of defective kinase genes (Cdk-like), with similarity to Cdk2 (Griffiths et al. 2006; Al-Kaff et al. 2008; Yousafzai et al. 2010). Therefore the deletion of *Ph1* region could result in either an increase or decrease Cdk activity and phosphorylation levels, and that this altered phosphorylation levels could induce pairing between related chromosomes. We were able to test whether increasing Cdk-type activity phenocopies the effect of deleting the *Ph1* locus. Treatment with okadaic acid, a serine-theonine phosphatase inhibitor increases Cdk-type activity. Treatment of detached tillers from *Ph1* wheat-rye hybrids with okadaic acid from the onset of meiosis, does indeed phenocopy the effect of *Ph1* deletion by inducing metaphase I pairing between related chromosomes (Knight et al. 2010). Thus increased phosphorylation levels overcomes the stalling of MLH1 sites on paired homoeologues in the presence of *Ph1*, enabling some of the sites to progress to crossovers which are visualized as pairing between related chromosomes at meta-

phase I. However does deleting the *Ph1* region actually increase phosphorylation levels during meiosis? Our mapping of *Ph1* region reveals the presence of a defective Cdk2-like kinase complex, which therefore could suppress active Cdk2-like genes via a dominant negative effect. Consistent with this proposal, phosphoproteomics revealed that phosphorylation at Cdk2 consensus sites on Histone H1 is increased in the absence of *Ph1* (Greer et al. 2012). As indicated previously, phosphatases directly dephosphorylate proteins including Cdks, and are inhibited by Okadaic acid, which therefore can increase Cdk2 type activity and hence Cdk2-type phosphorylation. Okadaic acid treatment during meiosis mimics the effect of deleting *Ph1* by inducing pairing and recombination between homoeologues even in the presence of *Ph1* (Knight et al. 2010). This treatment also increases phosphorylation of the same Cdk2 consensus sites on Histone H1 as deleting *Ph1* (Greer et al. 2012). Thus the reduced phosphorylation levels at Cdk2 consensus sites (hence Cdk2-type activity) in the presence of *Ph1* and the stalling of MLH1 complex (which in other species has been shown to contain CDK2) on Double Holliday Junctions between paired homoeologues, are all entirely consistent. The *Ph1* data implies that the MLH1 complex needs to be more active to resolve junctions on paired homoeologues than it does for junctions between paired homologues. This is consistent with the observations of Dvorak and colleagues. They found that in the absence of *Ph1*, recombination occurred between a pair of wheat chromosomes composed of combinations of homoeologous and homologous segments, but in the presence of *Ph1*, recombination was restricted to homologous segments (Dubcovsky et al. 1995). Interestingly mutating the *Ph1* Cdk homologue in *Arabidopsis* also affects meiotic chromosome pairing (see Wen 2011 for initial studies on models).

What Pairing in Euploid Wheat Itself Tells Us?

Is the effect on chromosome exchange the whole explanation of *Ph1's* action? As stated previously from analysis of hybrids, *Ph1* can't prevent related chromosomes synapsing (Martin et al. 2014), however *Ph1* does prevent related chromosomes from synapsing in wheat itself. Wheat synapsis studies reveal that the chromosomes are essentially synapsed as bivalents at pachytene when *Ph1* is the present, but synapse as multivalents when *Ph1* is absent (Holm 1986; Holm and Wang 1988). This implies that in wheat itself where there are both homologues and homoeologues, the overall effect of *Ph1* on chromosome pairing as distinct from recombination must be the promotion of homologue pairing rather than specifically suppressing pairing between the related chromosomes. How does this work? Euploid wheat homologues synchronously elongate prior to pairing at the onset of meiosis (Prieto et al. 2004; Colas et al. 2008). The degree of homologue elongation reflects the level of homology between the two parental chromosomes (Colas et al. 2008). This promotes homologue pairing which is disrupted in the absence of *Ph1* leading to incorrect pairing. Thus the level of chromosome homology in the presence of *Ph1* influences conformational changes required for initial pairing, which determines the extent of

chromosome pairing in wheat. Thus if two segments within homologous chromosomes are too distinct with respect to each other, they can't associate or synapse at all and if they are related, they can partially pair or synapse. Recent studies have reported a similar effect in *C. elegans* suggesting that this is a general meiotic phenomenon (Nabeshinia et al. 2011). They are identified some of the proteins involved, whose activity will depend on phosphorylation levels. The data suggests that these proteins bind along the chromosome triggering the conformational changes at a regional level. The increased Cdk2 activity through deleting *Ph1* will also affect the phosphorylation of the protein SUN1 early in meiosis. This may well affect the functioning of the telomere bouquet, and therefore pairing of chromosomes via the telomeres early in meiosis. Reduced shaking of chromosomes by the telomere bouquet will enable homoeologous associations to be maintained, while more rigorous shaking will rip such associations apart leaving just homologous associations between the chromosomes. We are currently studying the effect of *Ph1* on telomere bouquet function, and its subsequent effect on chromosome pairing early in meiosis (Richards et al. 2012).

Independent Centromere Pairing

Finally proper segregation of chromosomes to daughter cells requires that the paired chromosomes correctly orientate themselves so that the spindle fibres attach to the centromeres and pull the chromosomes in opposite directions. To achieve balanced gametes, the homologous centromeres must be correctly paired. We isolated an element Hi-10, which is found at the centromeres of all cereals (Aragon-Alcaide et al. 1996) and exploited it as an in situ marker for studying centromere pairing behaviour during meiosis. We reported that wheat centromeres pair independently from the rest of the chromosome, which associate and synapse from the telomeres during the telomere bouquet stage at the onset of meiosis. Our studies were controversial at the time, however it has been since reported that centromeres pair independently from the rest of the chromosome for meiosis in *Arabidopsis*, rice, *Brachypodium* and maize. It has been elegantly demonstrated in some of these systems that the centromeres synapse homologously, and independently of the rest of the chromosome (Da Ines et al. 2012). Tetraploid and hexaploid wheat possess 28 and 42 chromosomes respectively, or two and three copies of seven sets of chromosomes. During anther development in wheat-wild relative hybrids, tetraploid and hexaploid wheat, the centromeres associate as pairs (Aragon-Alcaide et al. 1997; Martinez-Perez et al. 1999; Martinez-Perez et al. 2003). During premeiotic replication, the pairs engage in a sorting process reducing to seven centromere sites at the onset of meiosis, again as the telomeres cluster to form a telomere bouquet (Martinez-Perez et al. 2003; Greer et al. 2012). *Ph1* increases the stringency of this independent centromere pairing process (Martinez-Perez et al. 2001), and therefore will affect the correct segregation of chromosomes, and the production of balanced gametes.

Summary

We have shown that *Ph1* has two distinct effects on chromosome pairing and recombination. It promotes homologous pairing through: influencing conformational changes required for initial pairing, increasing the stringency of independent centromere pairing, and finally altering telomere bouquet formation (and possibly function, we are currently studying this). *Ph1* also stalls Double Holliday Junctions from resolving as crossovers on paired homoeologues. The *Ph1* locus has been delineated to a region containing a cluster of Cdk2-like genes containing a large segment of heterochromatin. Interestingly recent mouse studies also reveal that Cdk2 has two distinct effects on this system, one on telomere bouquet function, and one at the stage when Double Holliday Junctions are being resolved.

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