ANALYTICAL METHODOLOGY OF MEIOSIS IN POLYPLOID PLANTS

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SUMMARY

Meiosis is the cellular process responsible for producing gametes with half the genetic content of the parent cells. Integral parts of the process in most diploid organisms include the recognition, pairing, synapsis and recombination of homologous chromosomes, which are pre-requisites for balanced segregation of half-bivalents during meiosis I. In polyploids, the presence of more than two sets of chromosomes adds to the basic meiotic program of their diploid progenitors the possibility of interactions between more than two chromosomes and the formation of multivalents, which has implications on chromosome segregations and fertility. The mode of how chromosomes behave in meiosis in competitive situations has been the aim of many studies in polyploid species, some of which are considered here. But polyploids are also of interest in the study of meiosis because some of them tolerate the loss of chromosome segments or complete chromosomes as well as the addition of chromosomes from related species. Deletions allow to assess the effect of specific chromosome segments on meiotic behaviour. Introgression lines are excellent materials to monitor the behaviour of a given chromosome in the genetic background of the recipient species. We focus on this approach here as based on studies carried out in bread wheat, which is commonly used as a model species for meiosis studies. In addition to highlight the relevance of the use of materials derived from polyploids in the study of meiosis, cytogenetics tools such as fluorescence *in situ* hybridization and the immunolabelling of proteins interacting with DNA, are also emphasized.

1 INTRODUCTION

The meiotic program in polyploids

Polyploid organisms are those containing more than two sets of chromosomes. Polyploidy is pervasive and represents a major mechanism of speciation in plants. Some estimates suggest that between 47% and 70% of angiosperms are polyploids (1) and many lineages show evidence of ancient genome duplications (2). Polyploids have been broadly classified into two types: autopolyploids, which have three or more copies of the same genome, and allopolyploids, which originated after interspecific hybridization between related diploid progenitors. Thus, allopolyploids contain two or more distinct subgenomes, whose chromosomes, called homoeologues, preserve some degree of affinity because of their common origin. The polyploid condition creates a competitive situation at the start of meiosis, in which, each chromosome has two or more potential partners to interact physically in order to repair the double-strand DNA breaks (DSBs) produced in early leptotene. Three or more related chromosomes, homologues in autopolyploids and homologues and homoeologues in allopolyploids, usually interact simultaneously, rather with a single partner, and synapse to form complex synaptic multivalents in zygotene and complete homologous/homoelogous recombination. Although competitive chromosome interactions represent the specific meiotic feature of polyploids, meiosis in these organisms takes place following most of the steps that make up the basic meiotic program of the diploid progenitor species. That is, physical interactions leading to chromosome sorting are preceded of the formation of programed DSBs catalyzed by the topoisomerase-like conserved protein, Spo11, in conjunction with a number of additional proteins (3, 4). After a DSB occurs, sections of DNA around the 5' ends of the break are cut away in a process called resection. An overhanging 3' end of the broken DNA molecule then associate with the recombinase RAD51 and/or the meiosis-specific recombinase DMC1 to form a nucleoprotein filament, which invades a similar or identical DNA molecule that is not broken (5). During leptotene, chromosomes decondense and elongate, and form the axial element, a protein-rich backbone that will keep the sister chromatids together until the second division. Proteins of the axis, such as ASY1-ASY3 in *Arabidopsis* or PAIR2-PAIR3 in rice, promote the search of a repair template in the homologous chromosome (6-9). Thus, these early steps of the DSBs repairing are involved in the identification of the homologous partner and are indispensable for homologous alignment and the assembly

of the synaptonemal complex (SC) in zygotene in many organisms (10-13). Homologues are fully synapsed at pachytene. The SC maintains homologues in close juxtaposition along their length and serves as a scaffold to recruit factors of the recombinational repairing machinery (14). In plants and animals, most of DSBs are repaired following a non-crossover (NCO) pathway, only a minor fraction (5%) give rise to crossover COs (15). Thus, the production of DSBs, chromosome sorting, SC assembly and COs formation share similar molecular mechanisms in diploids and polyploids.

Chromosome interactions in early meiosis in autopolyploids

Chromosome interactions leading to pairing, synapsis and COs production in competitive situations are essentially different from interactions in diploids. Assuming that the premeiotic arrangement of homologues is not quite different in polyploids and diploids, that is, they occupy separated nuclear territories, chromosomes have to move at the start of meiosis to find a partner. This movement can be based both in the telomere migration to form the bouquet and in the unfolding of chromatin that increase largely the length of the chromosomes (16, 17). Once the chromosomes have come close enough to interact, a choice has to be made between potential partners when more than two are available. In some polyploids, axial cores of each homologous set are aligned in bundles along much of their length (18-23). In others, pairing is restricted to the distal regions while the rest of the chromosomes remain largely distant (24-26). Differences between these two types of presynaptic alignment fall in the number and distribution of autonomous pairing sites (APSs), sites where pairing can be initiated between any two of three or more homologous chromosomes. Multiple APSs are scattered along the chromosome axes in the extensive presynaptic alignment and two APSs located at both chromosome ends are mainly formed in the distal alignment. Once the first APS is activated, activation of a second APS between two chromosomes, one of which is involved in the first APS, and synapsis extension give rise to a paring partner switch (PPS) in the SC multivalent formed. The number of PPSs in SC multivalents provides an estimates of the number of APSs, APSs = 1,5 PPSs +1 (18). Multivalents formed in autopolyploids in prophase I show stretches of SC that normally involve two homologues at any location of the chromosomes. SCs multivalents formed by homologous sets after distal alignment or extensive pairing differ in the number of PPSs, one PPS is mainly formed in the first case and many in the second.

Variation of the meiotic program introduced by autopoliploidy concerns mainly the type, distal or complete, of presynaptic alignment, the number and distribution of synapsis initiation points, the number of partner exchanges and its evolution through zygotene and pachytene, and the existence or not of preferences in the partner election. All these aspects of meiotic prophase I of autopoliploids can be addressed when individual chromosomes can be identified. To date, the behaviour of individual chromosomes in competitive situations in prophase I has been carried out in polyploids of species such as *Crepis capillaris* where homologues sets differ in their length (20, 22) or in primary trisomics as those of rye (*Secale cereale* L.) (27). In the absence of protocols leading to identify individual chromosomes, inferences on pairing and synapsis have been made from metaphase I observations (28). Nevertheless, a reduction of multivalents have been observed both trough prophase I and from prophase I to metaphase I in different polyploids, regardless individual chromosomes were identified (29 and references therein). Some SCs multivalents formed in zygotene are transformed into bivalents in pachytene, but those persisting during pachytene give rise to bivalents at metaphase when not all SC are stabilized by COs formation. The frequency of multivalents at metaphase I has been found to be lower in natural autopolyploids of different species, sourced many generations ago, than in new colchicine induced autopolyploids (30, 31). Such a result suggests the occurrence of partial diploidization of the meiotic behaviour, which may be a common feature of autopolyploid species evolution. Identification of the diploidization mechanism requires extending the analysis to pairing and synapsis.

Chromosome interactions in early meiosis in allopolyploids

Allopolyploids possess homoeologous subgenomes from two or more evolutionary related progenitors. Such subgenomes differentiated by accumulation of changes both in the DNA sequence, which produce homoeoalleles, and in the chromosome architecture and gene order, but their chromosomes retain some degree of genetic affinity. Such a genetic affinity confers homoeologous chromosomes the possibility of competing with homologues in interactions leading to pairing, SC assembly and COs. However, homoeologous subgenome diversification suggests differences in the meiotic behaviour between autopolyploids and allopolyploids. Multivalents are expected to be formed more often in autopolyploids than in allopolyploids. This was confirmed in a survey consisting of 171 reports on neopolyploids (32), which yielded a higher frequency of

multivalents at diakinesis and metaphase I in autopolyploids (28.8%) than in allopolyploids (8.3%). These multivalent frequencies suggest partial diploidization of many autopolyploids and complete diploidization of most allopolyploids. Exclusive bivalent formation at metaphase I is vital to ensure regular homologous disjunction at anaphase I and reproductive stability in allopolyploids. This diploid-like behaviour has been demonstrated to be the result of the emergence of genetic regulatory systems in allopolyploids such as wheat, cotton, *Avena sativa, Festuca arundinacea, Brassica napus,* in amphidiploids related to the diploid species *Lolium perenne, L. multiflorum, and L. rigidum*, and in polyploids in the genera *Aegilops*, *Hordeum, Nicotiana*, and *Coffea* (33).

Common bread wheat, *Triticum aestivum,* is an allohexaploid species with 2n=6x=42 chromosomes (genome formula AABBDD) that is considered a reference model in the analysis of the meiotic behaviour in allopolyploids (34). Genomes A and D are derived from *T. urartu* and *Aegilops tauschii,* respectively, while *Ae. speltoides* or a closely related species to *Ae. speltoides* is, most likely, the progenitor of the B genome (35). Hexaploid wheat arose after two hybridization events, the first hybridization originated the tetraploid wheat (AABB) *T. turgidum* and the second one the hexaploid wheat *T. aestivum*. Relevance of bread wheat in the analysis of meiotic pairing is reinforced by the importance of this species as a crop and because its regulatory system of meiotic pairing is effective both in wheat itself and in interspecific hybrids, which has implications in the transfer to wheat of alien genes controlling important agronomical traits.

Tetraploid and hexaploid wheats form at metaphase I as many bivalents as homologous pairs have, 14 and 21 bivalents, respectively. This meiotic behaviour of polyploid wheats is controlled by the major homoeologous pairing suppressor *Ph1* located on the long arm of chromosome 5B (36-38). *T. aestivum* carries another homoeologous pairing suppressor, called *Ph2*, which is less efficient than *Ph1* and locates on the short arm of chromosome 3D (39, 40). Meiotic phenotype of mutants of *Ph1* is characterized by the presence of multivalents at metaphase I involving homologous and homoeologous chromosomes, as well as by a reduction in the number of ring bivalents due to the increase of the number of univalents and rod bivalents, relative to the wild type. The meiotic phenotype at metaphase I of mutants of *Ph2* is similar to that of the wild type (34). Despite the regular formation of homologous bivalents at metaphase I in the wild type genotypes, 28% of chromosomes of hexaploid

wheat, and 39% of tetraploid wheat, are involved in the formation of SC multivalents during zygotene. A similar SC multivalent frequency is produced in the *Ph* mutants. While SC multivalents are corrected and transformed into pairs of bivalents in the course of pachytene in the wild type and in the *ph2b* mutant, synapsis correction is not completed in the mutants of *Ph1*, *ph1b* in hexaploid wheat and *ph1c* in tetraploid wheat. In these mutants, chiasmata are formed between homologous and between homoeologous chromosomes in some SC multivalents, which persist until metaphase I (34, and references therein).

Unequivocal meiotic phenotypes of wild type and all mutants of *Ph* genes are found when interspecific hybrids of wheat and related species are produced. Wild type, *ph2b*, and *ph1b* hybrids of wheat and related species, such as rye, *Ae. longissima,* and *Ae. sharonensis,* show low (<2), intermediate (5-11) and high (12-18) chiasmata at metaphase I. The number of chiasmata between wheat and related species chromosomes increases with their degree of closeness, it is higher in wheat-*Ae. longissima* and wheat-*Ae. sharonensis* hybrids than in wheat-rye hybrids (34). *Ae. speltoides* is an exception, the three types of hybrids with wheat show a high frequency of chiasmata, since this species carries genes that suppress the effect of *Ph1* (34).

The mode of action of the *Ph1* locus has been the focus of numerous studies since its discovery 60 years ago. A number of hypothesis (reviewed in 34) were advances to explain how *Ph1* restricts recombination to homologous chromosomes. *Ph1* seems to exert its action on two different steps of the process of bivalent formation: the first impedes consolidation of crossover that could be initiated between homoeologous chromosomes; the second promotes the correction of SC multivalents, which are transformed into bivalents. The fact that the number (19.5) of ring bivalents at metaphase I is higher than the number (17.8) of SC bivalents at late zygotene suggests that some corrected SC bivalents form chiasmata in resynapsed stretches, which implies that the recombination machinery can be loaded after multivalent correction at pachytene. The lack of an accurate identification of *Ph1* itself has represented a great obstacle in the clarification of its mode of action. The *Ph1* locus is confined to a region of 3Mb in the middle of chromosome arm 5BL (41). This region contains 36 genes and the *Ph1* locus was assigned to a subregion carrying a subtelomeric heterochromatin segment inserted into an array of seven *CDK2*-like copies (42). Included in the heterochromatin segment is the *TAZYP4-B2* gene. This gene was proposed to be *Ph1* as supported by the high level of homoeologous pairing found in hybrids of two *Tazyp4-*

B2 mutants of wheat with *Ae. variabilis* (43). An alternative approach based on gene silencing identified another gene located in a 450-Kb segment of the *Ph1* region, which was termed *C-Ph1* (candidate *Ph1*). Silencing of *C-Ph1* results the same meiotic phenotype as the *ph1b* mutant (44).

The *Ph2* gene has not yet been identified. This gene resides in a segment of 80 Mb of 3DS, which is deleted in the *ph2a* mutant; *ph2b* was suggested to be a point mutation (45). The mismatch repair *TaMSH7* gene, which shows highest similarity to *MSH7* of *Arabidopsis* and maize, is one of the genes located in this 80 Mb sub-region and was postulated a possible candidate for *Ph2* (46). Some role of *MSH7* on recombination in cereals was suggested after the reduction of fertility caused by RNAi-induced silencing of this gene in barley (47). Other minor suppressor genes located on 3AS, 2DL and 4D as well as promoters of meiotic pairing located on group 2, 3 and 5 chromosomes have been reported (reviewed in 34).

Brassica napus is an allotetraploid species (2n=4x=38, genomes AACC) in which a homoeologous pairing suppressor gene, termed *PrBn*, has been identified (48). The diploid progenitors of *B. napus* are *B. rapa* (2n=18, AA) and *B. oleracea* (2n=20, CC), which underwent recurrent hybridizations to originate the current tetraploid. *B. napus* shows 19 bivalents at metaphase I, but haploids from different varieties show a low (~10 univalents) or high (~4 univalents) level of homoeologous pairing at metaphase I, which are controlled by different alleles of *PrBn* (49). This locus maps within an interval of 10-20 cM in the linkage group C9. Thus, *PrBn* resembles *Ph2* of wheat, since its effect on meiosis is manifested in the allohaploid genotypes. SC analysis of euploid plants of varieties with different *PrBn* alleles revealed no difference in their synaptic behaviour. Although some multivalents appeared at pachytene, most homologous pairs formed SC bivalents (50). Allohaploids with different genotype showed also a similar synaptic pattern, characterized by the presence of incomplete synapsis between homoeologous chromosomes. Differences between genotypes concerned the number of class I COs and were only manifested in the allohaploids (50).

2 PLANT MATERIALS DERIVED FROM POLYPLOIDS USED IN MEIOTIC STUDIES

Introgression lines

In addition to natural and synthetic polyploids and interspecific hybrids mentioned above, other materials, such as introgression lines derived from allopolyploids, emerged in the last two decades as powerful tools in the study of meiosis in polyploids. The addition of a pair of alien chromosomes to the full genome complement of a crop species is frequently used as a first step for accessing genetic variation from the secondary gene pool. However, introgression lines can be also used in the study of interspecific genetic interactions, in the chromosomal location of genetic markers and in the study of chromosome structure and behaviour in somatic and meiotic cells.
Many studies on meiosis, both in diploids and polyploids, are focused on the isolation of meiotic mutants, identification of the mutant phenotype and molecular characterization of the affected meiotic step. An alternative approach consists in the study of the behaviour of chromosomes introgressed in the genetic background of a different species. Such an approach can be used in polyploid plants such as bread wheat, which tolerates the introgression of chromosomes from related species. Introgression lines of a number of triticeae species into wheat are available. The study of meiosis in the complete set of introgression lines of rye into wheat has shown that individual chromosomes of rye behave different when the rye genetic background is changed by that of wheat. Differences in the meiotic behaviour or rye chromosomes concern chromatin remodeling produced at leptotene, synapsis and chiasma formation (51-53). The identification of a specific chromosome or chromosome segments from one species in another genetic pool strongly depends on the phylogenetic proximity between both species. In the case of rye, introgressed chromosomes or segments are identified in fluorescence *in situ* hybridization (FISH) studies using rye specific repetitive DNA probes, which label almost completely the chromosomes (Figure 1). Alternatively, total genomic DNA can be used as a probe in genomic *in situ* hybridization (GISH) experiments (Figure 1) (54). In the case of barley, where two sets of cultivated (*Hordeum vulgare*) and wild (*H. chilense*) barley additions were developed in wheat (55-57), GISH protocols has been used in the analysis of meiosis and identification of chromosome rearrangements (58, 59). Double monosomic wheat-barley addition lines containing one chromosome from *H. vulgare* and the other from *H. chilense*, belonging or not to the same homoeologous group have been also developed to study chromosome recognition and pairing during meiosis in the absence of a homologous partner (59). Observations confirmed that *Hordeum* homoeologous chromosomes can recognize each other and associate correctly in pairs in prophase I, even in the presence of the *Ph1*

locus of wheat, but no cross-over was formed as they appeared as univalents at metaphase I (59). Sister chromatids segregation and misdivision of univalents at anaphase I was also analyzed in double monosomic additions (60, 61). Chromosome segregation can occur independently of whether or not related chromosomes associate in pairs in early meiosis in the wheat background.

Telosomic addition lines derived from wheat-rye standard disomic additions are also available and have been used to assess the effect of the *Ph1* locus on chromosome association and synapsis (17) or the effect of colchicine on meiotic chromosome behaviour (51). Some deletion lines lacking a terminal segment of the alien chromosome added to wheat has been also isolated from wheat-rye or wheat-barley additions (61, 62). Such lines are informative on the role of subtelomeres on chromosome recognition/pairing at early meiosis.

In polyploids, the replacement of a chromosome by its homoeologue is called chromosome substitution. The development of substitution lines involves the replacement of a pair of chromosomes in one variety or species, the recipient, by the homologous pair from another variety or species, the donor. They can be easily obtained after crossing between addition lines and monosomics of the recipient species. Substitution lines are available in different polyploids, but their development may differ according to the ploidy level. In addition to their possible use in the study of meiosis, a considerable amount of genetic information can be obtained from the genomes concerned. In fact, an important advance in understanding the genetic architecture of hexaploid wheat was based in compensation-substitution tests using nulli-tetrasomic lines. These tests demonstrated the existence of seven homoeologous groups with three chromosomes each (63). Later, two series of D-genome chromosome substitution lines in tetraploid durum wheat were developed by replacement of one chromosome from the A or B genome by its homeologue from the D-genome (64). Examples of substitution lines in hexaploid wheat involving chromosomes from other species are: i) a set of disomic substitutions (DS) of S-genome chromosomes from *Ae. speltoides* for the wheat B-genome chromosomes, which were studied to unzip the effect of such chromosome substitution during meiosis (65), ii) the substitution lines in hexaploid wheat for all barley chromosomes except 1H and 5H (66-68), iii) the wheat-rye substitution lines (69).

Other alien introgressions in wheat are chromosome translocations. Robertsonian translocations are the result of fusion of two misdivision products

originated in the same meiotic cell. Intergenomic translocations can be also originated by meiotic recombination between homoeologous chromosomes. Some wheat-rye translocations have been developed for breeding purposes to introgress genetic traits, such as resistance to diseases, from rye into wheat (70-72). Others have been used in the identification of crossover-rich regions and their effect on homologous interactions at early meiosis (73). A list of wheat lines carrying rye genetic introgressions for breeding purposes is available on line (74). Translocations between chromosomes of wheat, *H. chilense* and *H. vulgare* have been obtained from plants carrying chromosomes of these three species, which can be used in breeding related programs and in studies on meiosis (75).

Chromosome rearrangements such as inversions have been also identified in wheat-rye additions. They provide valuable information about chiasma location and chromosome dynamics in the search of the homologous partner, especially when homologous regions are located in opposite poles of the nucleus as in heterozygotes for large paracentric inversions (76, 77).

Deletion lines

Finally, polyploids such as bread wheat tolerate the presence of deletions even in homozygous condition. The deletion stocks of wheat obtained after chromosome truncation induced by the action of gametocidal chromosomes (78) are widely used in genetic and genomic studies. They represent also an excellent material in studies on meiosis. For example, they showed that the CO events occur mainly in the distal regions of the chromosomes, with a gradient from the centromeres to the telomeres, as well as that recombination frequency can increase in intercalary regions when the chromosome truncation forces the first crossover to move toward the centromere regions (79). Nevertheless, recombination is highly restricted in the proximity of the centromere.

3 CYTOGENETIC TOOLS IN THE STUDY OF MEIOSIS IN POLYPLOIDS AND THEIR DERIVATIVES

Chromosome content screening in somatic cells

Cytological procedures such as *in situ* hybridization, which are capable of identifying alien chromatin introgressed in a given polyploid, are extensively used to confirm the presence of the alien genetic material in the introgression line, or in derivatives obtained

in breeding related programs based on marker-assisted selection. Routinely, GISH and FISH are accomplished on chromosomes recovered either from rapidly dividing root tips that have a high mitotic index (Figure 1). Root tip sampling can be performed on seedlings that are only a few days old, which does not destroy the individual. The root tips of more mature plants can also be sampled; the plant is removed from its pot, the ends of the roots are clipped, and ample water is applied. In a few days, newly grown root tips emerge that can be used for cytological analysis. A variety of treatments, including colchicine, cold, hydroxyquinoline and nitrous-oxide, have been used to arrest somatic cells in metaphase. Such treatments, in addition to accumulate a large number of metaphase-arrested cells, facilitate chromatin condensation and visualization of the morphology of the chromosomes (80-85). After this treatment, root tips are fixed and stored at 4ºC, even for months, until perform the FISH or GISH experiment. Most of the protocols are focused on somatic chromosome spreads, but equipment, chemicals and solutions are quite similar for studying meiosis in FISH experiments.

Sampling of meiotic cells

The sampling of cells at prophase I or metaphase I requires growing plants to near the flowering stage and some knowledge of the best sampling time for each species. Anthers are the preferred source of meiocytes because of their abundance compared to ovaries. Here we summarize the whole procedure for studying meiosis in polyploid cereals, although this protocol can be adapted in other species. The outline of the procedure is represented in Figure 2. An important issue is the identification of the maturity stage when a plant is entering meiosis. Previously to anther excision, the developmental stage of each main shoot or tiller should be precisely estimated. Taking wheat as an example, meiosis occurs when the immature spike is still inside the tiller (Figure 2). External features of the tiller help to recognize when the spike is in meiosis. Briefly, the flag leaf should be emerged and the spike enclosed within the sheaths of the leaves just in the internode between the leaves that precede the flag leaf. The spike length inside the tiller can be estimated by feeling gently with the fingers its bottom and top end. The immature spike is considered to be in meiosis when its length is around 5 cm. Anthers are fixed without pre-treatment and stored at 4ºC. Materials, reagents and equipment for anthers sampling and staging of PMCs (pollen mother cells) in FISH and GISH experiments have been described (86-89). Given that meiosis may be affected by temperature, humidity or day-length, considerations to grow polyploid plants to

fruitfully isolate meiocytes for FISH or GISH experiments should be also taken into account (86).

Probes and equipment used for GISH and FISH analyses

Detailed protocols for GISH and multicolour FISH analysis, including materials and the necessary equipment for plant cytogenetic analysis, have been extensively described. DNA or RNA can be used to obtain the probe for *in situ* hybridization experiments. Total genomic DNA, cloned DNA sequences, PCR products and synthetic oligonucleotides are commonly used as probes (88) (Table 1). Molecular information concerning the DNA sequence, number of copies, genomic organization and species specificity of the probes is of interest in the design of the *in situ* hybridization experiment. Most of this information is often known, however additional DNA *in situ* hybridization experiments may be performed in order to unravel long-range evolutionary changes involving the probe and its interspersion with other sequences.

Technical advances in DNA synthesis have allowed a large-scale de novo production of thousands of independent oligonucleotides (oligos). These massively synthesized oligos have been successfully labelled to be used as FISH probes and has paved the way for the development of chromosome-specific painting probes in plants, which also have the potential to be used in meiosis studies in polyploids (90-92).

RNA *in situ* hybridization gives information about the location and amount of expression of particular genes. Detailed information on probes (total genomic DNA, cloned DNA sequences, PCR fragments, and RNA) preparation and *in situ* hybridization protocols, equipment, supplies, chemical and solutions has been described (88, 89).

Antibodies and equipment in immunolocalization

The use of immunolocalization of chromosome-associated proteins, alone or combined with FISH and GISH, is an indispensable tool that has greatly contributed to the study of meiosis in plants (93, 94). The immunolocalization technique is based on the principles of antigen-antibody reactions to find highly specific molecules in a cell or tissue. The common reagent for immunolocalization are polyclonal or monoclonal antibodies, both produced in animals such as rabbits, goats, rats, mice, among others by specific procedures (95). There are many commercially available antibodies to detect chromosomal proteins in plants. Some of them, for example, the anti-centromeric H3

histone, are of universal use (96). For detection of proteins by immunolocalization, cells must be fixed and permeabilized to facilitate the access of antibodies to the nucleus. Different procedures for immunodetection of proteins associated to chromosomes to study meiosis in plants have been recently reviewed (87, 97).

Identification of alien chromatin in a polyploid background in somatic and meiotic cells by FISH or GISH

In situ hybridization is routinely used in the identification of alien chromatin introgressed in a given polyploid. *GISH*, which was first applied to plant tissues in the latest eighties (98), and FISH are well-established methodologies to study meiosis. Highly efficient protocols for cytological identification of specific sequences in plants have been deeply described (88, 89). These protocols were carried out in somatic or meiotic chromosome spreads, but can be also adapted with little modifications to 3D vibratome plant tissues, which allows the analysis in intact cells (99).

GISH is used to differentiate chromosomes from different genomes in interspecific hybrids and allopolyploids (98, 100). Homologous and homoeologous chromosome associations can be identified using GISH in meiosis across a wide range of triticeae species (Figure 1) (59, 77). Protocols referred above can be somehow customized with few modifications according to the types of cells and plant species concerned. For example, in *Brassica*, GISH is routinely performed to study genome stability and interspecific chromosome associations during metaphase I in hybrids between *Brassica* and related species (101). In banana (*Musa* sp.), GISH has been revealed very useful to investigate meiotic chromosome pairing of different chromosome in the allotetraploid (AABB) of *M. acuminata* and *M. balbisiana* (102).

In wheat-rye introgression lines, FISH has been used to visualize the complete rye chromosomes through all meiotic stages (Figure 1) (62).

FISH analysis of chromosome dynamics in meiosis

Multicolour FISH is performed as a routine tool to study meiosis in numerous plant cytogenetics laboratories due to the development of versatile probe-labelling reagents and techniques, signal detection systems, optical filter technology and digital image analysis systems for pseudo-colouring and merging of images. This methodology is usually applied to visualize chromosome structures such as centromeres or telomeres,

which play a relevant role on chromosome dynamics during meiosis. Many efforts have been focused on centromeres and telomeres behaviour, especially in hexaploid wheat (51-53, 103-107). A list of useful DNA probes and antibodies to target chromosome dynamics during meiosis and specific meiotic events in polyploids is shown in Table 1.

In most plants, centromeres are usually composed of a small DNA repeat that is tandemly arranged thousands of times at each primary constriction (108). A cereal centromeric sequence (CCS1 sequence), identified within a clone (Hi-10) from *Brachypodium sylvaticum*, hybridizes to the centromeres of wheat and some other triticeae species (109). This CCS1 and the rye centromere-specific repetitive sequence pAWRC.1 (110) have been used in the analysis of centromere dynamics during early meiosis (103-107) as well as to investigate the neocentric activity of 5RL chromosome in wheat (111) or the centromeric structure of wheat–rye 1BL.1RS translocation lines (112). Synthetic oligonucleotides can replace DNA probes such as CCS1 or pAWRC.1 in the identification of centromeres in FISH analysis in somatic cells (90, 91) but it is possible to use them also for meiosis studies. In fact, oligo-based probes have been employed to monitor pairing of a specific homeologous chromosome pair throughout meiosis in *Cucumis hystrix* × cucumber hybrids (92).

In other species, a particular type of retrotransposon is present in centromeres, such as the tandem repeats of rice (CentO, previously named RCS2) and maize (CentC) centromeres, which are interspersed with a particular family of retrotransposons called Centromeric Retrotransposons of Rice (CRR) and Maize (CRM) (113-116). A centromere-specific retrotransposon in *Brassica* (CRB) has been also detected by *in situ* hybridization as a major component of all centromeres in three diploid *Brassica* species and their allotetraploid relatives (117).

Telomeres are highly conserved structures among plants. An *Arabidopsis* telomere clone has been used for *in situ* hybridisation to chromosomes of several monocots and dicots species. In all of them, the sequence hybridize exclusively to the ends of chromosomes indicating a high level of conservation of the telomeric repeats (118, 119). This telomeric repeat has been used to investigate telomeres dynamics during meiosis in polyploids (53).

In species with large genomes, as hexaploid wheat, chromosomes enter in meiosis arranged in the Rabl orientation, that is, centromeres are concentrated in one pole of the nucleus and telomeres fanned out the opposite hemisphere. This arrangement facilitates a codirectional orientation of homologous chromosome segments (120).

Then, telomeres undergo an oriented migration and associate in a bouquet (121). The bouquet configuration brings homologues together, which is considered an initial step in the search and recognition of the homologous partner. In hexaploid wheat, telomere convergence is affected by the *Ph1* locus (122). Concomitant with telomere clustering, a conformational change in the chromatin is triggered, which facilitates physical interaction between homologues and the initiation of pairing and synapsis in distal positions (53, 123). Later, pairing and synapsis extend from both ends to the chromosome centre.

Since the DNA sequence of the telomeres is largely conserved, it remains poorly understood how chromosomes identify their homologous partners to correctly associate in pairs. Further FISH and GISH experiments may shed light on homologous recognition. Some works are focused on telomeres-associated sequences (TAS). These sequences are located on the ends of the chromosomes, can be visualized using FISH and their role on chromosome recognition and pairing is being assessed (61). TAS are organised in long subtelomeric arrays of hypervariable tandemly repeat units. In rye, TAS are highly repeated and form subtelomeric heterochromatin chromomeres of variable size (124), which reveals the position of the adjacent telomere in the prophase I nucleus (53). The probability of inclusion of the rye telomeres in the telomere cluster was found to be chromosome conformation dependent in wheat-rye additions, which has implication in the subsequent development of synapsis. The behaviour of the polymorphic nature of subtelomeres is an exciting area for study, but also presents a difficult challenge from the technical perspective. Gene-rich regions, less evolutionary conserved than telomeres, are also distally located and represent hot spots of recombination (125). The presence of repeated and single copy DNA sequences in subtelomeric regions most likely confer an increased difficulty when it comes to discerning what type of sequence play a critical role on chromosome recognition. The subtelomeric repeat sequences are much more divergent than evolutionary conserved telomeric sequences and they have been cloned and used as molecular markers to cap for example genetic maps in hexaploid wheat (126).

Bacterial artificial chromosomes probes (BAC-FISH) represent an additional tool in FISH experiments aimed to study the role of chromosomal regions different from centromeres or telomeres on chromosome pairing in meiosis of polyploids and interspecific hybrids. For example, BAC-FISH has allowed tracing the meiotic behaviour of specific chromosome pairs in allotriploid hybrids between cultivated

potato, *Solanum tuberosum*, and its wild relatives *S. commersonii* (127). BAC-FISH has contributed also to predict, from meiotic homoeologous recombination, the possibility to transfer some resistant traits coded by genes of the RR subgenome of allotetraploid *Brassicoraphanus* (RRCC, 2n = 4x = 36) into *Brassica napus* (AACC, 2n = 4x = 38) (128).

Immuno-FISH

Immuno-FISH combines immunolabeling with FISH in order to visualize simultaneously the nuclear distribution of proteins and specific DNA sequences on the chromosomes (Figure 3). This methodology is particularly interesting in studies of meiosis, because morphogenesis and function of meiotic proteins are associated to stage-specific conformational changes of chromatin.

Different procedures for immunodetection of chromosome associated proteins in plant meiosis have been reviewed (87, 97). Several proteins are associated with the DNA of the meiotic chromosomes, centromeric proteins associate constitutively, others transiently. Centromeric histone H3 (CENH3) is extensively used for labelling of centromeres, also in studies on meiosis in polyploids (129). Some proteins involved in the synaptonemal complex assembly during zygotene can be also inmunolabelled. For example, ASY1 was reported as an axial element (AE)-associated protein in plants that is loaded onto the unsynapsed chromosome axes before synapsis (94). ZYP1 is a protein that polymerizes between the unsynapsed axial cores of homologous chromosomes during zygotene until synapsis is complete at pachytene (130). Antibodies raised against the *Arabidopsis* ASY1 and ZYP1 have been used to detect their homologous proteins in barley and rye, where they conserve the same localization in the synaptonemal complex (131,132). ASY1, ZYP1 and CENH3 have been combined with the telomeric repeat DNA probe in immune-FISH experiments to analyse centromere dynamics and the timing of chromosome synapsis in wheat prophase I (129,133). In addition, dynamics during plant meiosis of other proteins such as cohesins SCC3 and SYN1, histone HTR12 (the histone H3 variant of *A. thaliana*) or recombination proteins REC8, RAD51, and MLH1 has been deeply studied by immunolabeling (97, 134). All of these proteins are useful tools to unzip chromosome dynamics during meiosis in polyploids (Table 1, 133, 135, 136).

Vibratome sectioning for 3D meiosis study in intact cells in FISH and immune-FISH experiments

It is worthy to mention here, that most interesting process of plant cell biology and development such as meiosis, occur in cells deep within tissues of the plant. These cells are difficult to image, even when confocal microscopy is used. Several studies on wheat and maize using FISH have all shown the importance of examining well-preserved material, to be confident of both the 3-D preservation of delicate nuclear structure and the cell types that are being analysed (137). In squashed preparations of anthers, cell types could be misclassified, or polarised centromeres and telomere clusters produced during early meiosis can easily be displaced or brought together artificially. Several procedures have been determined for undertaking 3D *in situ* approaches on rice and *Arabidopsis*. In wheat, FISH was applied on three-dimensionally preserved tissue sections derived from intact plant structures such as florets (99). This method combines vibratome sectioning with *in situ* hybridization and confocal microscopy, does not require embedding of tissue wax or resin, and can be applied to fixed or unfixed tissues. Vibratome tissue sectioning produces relatively thick sections (20-50µm) which can be imaged to reveal the three-dimensional structure of the underlying tissues. It has the advantage of preserving well the 3D structure, so that subcellular structures can be reliably imaged. Furthermore, reliable identification of cell types often requires an accurate assessment of the tissue context, which is lost when cytological squash preparations are made. For example meiocytes can be clearly identified at all stages of meiosis and can be imaged in the context of their surrounding maternal tissue. FISH can be used to localize centromeres, telomeres and sub-telomeric regions, and total genomic DNA can be used as probes to visualize introgressed chromosomes or chromosome segments. Vibratome sections can be used in other labelling experiments, such as RNA-FISH to detect transcripts, in antibody immunofluorescence labelling, in labelling of nascent transcripts by BrUTP incorporation in unfixed tissue, and in combined antibody and BrUTP labelling with FISH (e.g 138). Vibratome sectioning, which has been used to study meiosis in florets of wheat and related species (103, 104, 137) and in polyploid rice (*Oryza sativa* L.) (139), is a reliable tool for investigating meiotic events in plants, over a wide range of genome and taxa.

Diagnostic meiotic configurations

Meiotic configurations of polyploids may include the presence of univalents, bivalents and multivalents. In the absence of chromosome translocations, the types of multivalents that can be found in polyploids depend on the ploidy degree: trivalents are expected in triploids, trivalents and quadrivalents in tetraploids, trivalents, quadrivalents, pentavalents and hexavalents in hexaploids, and so one. Triploids show a very low fertility since they form genetically balanced gametes (n or 2n) with a probability of $(1/2)^{n-1}$, where n is the haploid chromosome number. Thus, this ploidy level is barely transmitted by sexual reproduction. In contrast, tetraploids produce diploid gametes (2n) with a relatively high frequency, which ensures the transmission of the tetraploid condition. Evolutionarily, tetraploids represent the first step in the formation of polyploids species either in autoplolyploids or allopolyploids. By this reason we will concern mainly meiotic configurations of tetraploids. Studies of pairing and synapsis have been usually carried out in nuclei at early and mead prophase I in the absence of chromosome-specific markers. This methodology make it difficult to establish whether there are differences between chromosomes or not. The existence of a genome reference sequence in many species offers the possibility of generating chromosome specific molecular tools to identify individual chromosomes in prophase I. In the meantime these genomic tools are designed, information about specific chromosomes can be obtained from metaphase I observations.

Sybenga (28) developed a model that allow to estimate the frequency of the two modes of synapsis, one quadrivalent or two bivalents, involving four homologous chromosomes with two distal pairing initiation sites per chromosome, as well as the frequency of chiasmate bonds in each chromosome arm in both types of SCs configurations. The model concerns metacentric chromosomes with undistinguishable arms in organisms with exclusive localization of chiasmata in distal regions. Under this assumption, the following metaphase I configurations can be produced: ring quadrivalents (frequency *rq*); chain quadrivalent (frequency *cq*); trivalent plus univalents (frequency *t*); ring bivalent (frequency *r*); open bivalent (frequency *o*) and univalent pair (frequency *u*). All multivalents are obviously derived from the SC quadrivalent, ring bivalents are derived from two SC bivalents and the open bivalents and univalents pairs can be derived from both SC configurations. The frequency *f* of quadrivalents resulted to be:

$$f = \frac{(t+2cq+4rq)^2}{16rq}$$

Frequencies *a' and b'* of chiasmate bond of the two chromosome arms in the SC quadrivalent type can be deduced from the following equations:

$$rq = fa'^2b'^2$$

$$cq = 2f(a'b'^2 + a'^2b' - 2a'2b'^2)$$

Frequencies *a* and *b* of chiasmate bond of the two arms in the two bivalents synaptic type can be derived from *a'* and *b'* and the mean frequencies *A* and *B* of association of each chromosome arm obtained from all metaphase I configurations.

$$f\,x\,a' + (1\text{-}f)\,x\,a = A$$

$$f\,x\,b' + (1\text{-}f)\,x\,b = B$$

In tetraploid *Tradescantia virginiana* (4n = 24) *f* was estimated to be 0.658, close to 0,667 (2/3), the expected frequency of SC quadrivalents in random pairing. The means of chiasmate association of the two arms were *A* = 0.946 and *B*= 0.683. The estimates of *a', b', a,* and *b* were: *a'* = 0.950, *b'* = 0.605, *a* = 0.938, and *b* = 0.830. This model was later refined and adapted to individual rye chromosomes 1R, 2R and 5R, where interstitial chiasmata in addition to distal chiasmata were formed and distinction between chromosome arms was possible (140). The frequencies estimated for one quadrivalent and two bivalents differed from those expected for random pairing in the three chromosomes. An increased bivalent pairing was found, which was not produced by preferential pairing. Either spatial separation of the four homologues in two groups of two chromosomes in premeiotic cells, or correction of SC multivalents, or both, were suggested to be responsible of the increase in the frequency of two bivalents. Estimates of chiasma frequency of each chromosome arm were different after quadrivalent and bivalent pairing. This model may be extended to other tetraploids.

The occurrence of preferential pairing can be investigated when a set of homologous chromosomes is polymorphic for a chromosome marker. This is the case of

the homologues set indicated in Figures 4 and 5. The tetrasome consists of four submetacentrics, two with and two without a distal marker in the short arm. Such a situation can be generated when chromosome duplication is produced in a diploid heterozygote for the chromosome marker. Because of the difference in length between arms, the pairing partner switch in the quadrivalent is assumed to locate in the long arm. In such a situation, interstitial chiasmata in addition to distal chiasmata can be formed in this arm. Two pairing types are expected: i) homomorphic pairing (Figure 4), when the short arms carrying the marker, as well as those unlabelled, synapse either in the mode of one quadrivalent or two bivalents; ii) heteromorphic pairing (Figure 5), when synapsis occurs between arms carrying and lacking the marker. Configurations at metaphase I assuming a minimum of one COs per chromosome pair are shown in both pairing types (Figures 4 and 5). Markers positioning identifies each pairing type, which allows to assess whether chromosomes pair or not at random. Metaphase I configurations provide also information of the frequency of two COs, intercalary and distally located, respectively. Such situation is apparent in trivalents and quadrivalents with COs formed in the interstitial segment 5 or 6 of Figures 4 and 5. Of course, estimates of *f*, *a*, *b*, *a'* and *b'* can be derived as indicated above. Similar models are possible for chromosomes with a different conformation or carrying markers in both arms.

4. CONCLUDING REMARKS

We have presented here a summary of achievements reached in the study of meiosis in polyploids. We have mainly emphasized the development of materials and methodologies that have favored the most relevant advances, which can be applied in future research. The main feature of the meiotic chromosome behaviour in polyploids concerns the presence of more than two potential partners and their physical interactions leading to pairing, synapsis and recombination. Although the mode of how chromosome sorting is produced remains still poorly understood, polyploids, especially allopolyploids, can shed light on this issue when interactions between alternative combinations of potential partners are identified. Introgression lines can help to obtain such chromosome constructions and to identify the different pairs that can be generated. Advances produced on chromosome painting technologies can help also in this attempt. More difficult may be this analysis in autopolyploids given that all chromosome sets

they have are homologous. Perhaps, future research works should be focused on the identification of individual sets of homologues and the comparative analysis of their behaviour especially in the first meiotic division.

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Table 1. List of DNA probes and antibodies used for targeting specific meiotic events in polyploid species

DNA probes (FISH and GISH)			
Target	**DNA probes**	**Application in meiosis**	**References**
Alien genetic introgressions	Total genomic DNA	Homologous chromosome pairing and segregation	51-53,59,60
Centromeres	CCS1 CentO CentC CRB Bilby	Chromosome dynamics, role in chromosome recognition, association and segregation	103-105, 108,110,114,116,117
Telomeres	pAtT4	Chromosome dynamics, role in chromosome recognition, association and segregation	53,118, 119
Subtelomeres	HvT01 pSc119.2 pSc74	Role of subtelomeric sequences in homologous recognition and pairing	59,61,62
Several DNA sequences	Synthetic oligonucleotides	Diverse specific meiotic events	90-92
Antibodies (Immunolocalization)			
Target	**Antibody**	**Application in meiosis**	**References**
Centromeres	anti-CEN-H3	Chromosome dynamics, role of functional centromeric sequences in chromosome associations and segregation	96, 129
Sinaptonemal complex:			
axial element proteins	anti-ASY1	Synapsis	6, 94,131
central element proteins	anti-ZYP1	Formation of sinaptonemal complex	130,131,133
Crossing overs	anti-MLH1 anti-RAD51	Crossing over Early recombination nodules	97, 134 97, 135
Cohesins and cohesins-associated proteins	anti-SMC1 anti-SMC3 anti-SCC3 anti-REC8/SYN1	Sister chromatid cohesion Sister chromatid cohesion Sister chromatid cohesion Chromosome condensation and pairing	97 97 97, 134 97

FIGURE LEGENDS

Figure 1. Painting of alien chromosomes in mitotic and meiotic cells of wheat-barley and wheat-rye addition lines. Total genomic DNA from *H. chilense* (green in a, d) and DNA probes pUCM600 (for most rye chromatin, red), pSc74 (for subtelomeric heterochromatin, red in b, c; green in e, f), pAWRC1 (for rye centromeres, red in b, c, e, f) and pAtT4 (for wheat and rye telomeres, green in b, c, e) were used. **a)** Somatic metaphase of a wheat-*H. chilense* disomic addition line for chromosome 5H. **b)** Nucleus at the leptotene-zygotene transition, rye chromosomes (4R) occupy separate territories, but their ends converge in the telomere cluster. **c)** Nucleus at the leptotene-zygotene transition with less packaged rye chromatin (4R) than **a). d)** Nucleus at zigotene with a pair of *H. chilense* chromosomes (3H) almost fully associated. **e)** Complete synapsis of rye chromosomes (3R) at pachytene. **f)** Cell at metaphase I showing a ring bivalent formed by chromosome pair 7R. tel, telomeres; c, centromeres. Scale bars represent 10 μm in all panels.

Figure 2. Summary of the procedure for the development of *in situ* hybridization experiments in polyploid plants. Wheat is taken as an example. Numbers in brackets correspond to the references cited in the references list.

Figure 3. Immunolocalization and immuno-FISH experiments on wheat and a wheat-barley addition line, respectively, during pachytene. a) Simultaneous immunolocalization of ZYP1 (green) and CENH3 (red) on a wheat nucleus. ZYP1 formed long thick tracts along the synapsed homologous chromosomes. (**b**) Immuno-FISH on a wheat-*H. chilense* addition line using the CENH3 antibody to detect centromeres (red) and total genomic DNA from *H. chilense* as a probe to label the synapsed barley chromosomes (green). Scale bar represents 10 μm.

Figure 4. Configurations at pachytene and metaphase I of four homologoues showing homomorphic pairing. Numbers indicate chromosome segments where COs locate.

Figure 5. Configurations at pachytene and metaphase I of four homologoues showing heteromorphic pairing. Numbers indicate chromosome segments where COs locate.