Brief Report

Polyploidy Induces Centromere Association

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Abstract. Many species exhibit polyploidy. The presence of more than one diploid set of similar chromosomes in polyploids can affect the assortment of homologous chromosomes, resulting in unbalanced gametes. Therefore, a mechanism is required to ensure the correct assortment and segregation of chromosomes for gamete formation. Ploidy has been shown to affect gene expression. We present in this study an example of a major effect on a phenotype induced by ploidy within the *Triticeae*. We demonstrate that centromeres associate early during anther development in polyploid species. In contrast, centromeres in diploid species only associate at the onset of meiotic prophase. We propose that this mechanism provides a potential route by which chromosomes can start to be sorted before meiosis in polyploids. This explains previous reports indicating that meiotic prophase is shorter in polyploids than in their diploid progenitors. Even artificial polyploids exhibit this phenotype, suggesting that the mechanism must be present in diploids, but only expressed in the presence of more than one diploid set of chromosomes.

Key words: meiosis • pairing • floral development • telomere • nuclear organization

Introduction

Polyploidy is a very wide spread phenomenon in eukaryotes, and may be a fundamental evolutionary mechanism. Known polyploid species include up to 70% of flowering plants (Masterton, 1994) as well as fish and amphibians, and recently even a polyploid rat has been described (Gallardo et al., 1999). Analysis of the genome sequence of the yeast *Saccharomyces cerevisiae* has suggested that it is an ancient tetraploid (Wolfe and Schields, 1997). Polyploidy has been shown to modulate gene expression in yeast (Galitski et al., 1999) and in *Arabidopsis* (Mittelsten Scheid et al., 1996).

Polyploidy can arise either by multiplication of a basic set of chromosomes (autopolyploidy) or as a result of combining related, but not completely homologous genomes (allopolyploidy). Polyploids arise constantly in nature; however, the presence of additional sets of chromosomes possessing a similar structure can create a disruption of chromosome pairing during meiosis. This results in the production of unbalanced gametes, which leads to infertility. To produce viable gametes, polyploids must behave effectively as diploids during meiosis, only true homologous chromosomes pairing. Surprisingly, it has been shown that meiosis is shorter in polyploid plants than in their diploid progenitors (Bennett and Smith, 1972). Apparently the more chromosome sets are present, the shorter the time required to sort them!

A great number of plant species have evolved by means of polyploidization. Among the cereals, the *Triticeae* tribe comprises many polyploid species as well as their diploid progenitors. We have taken advantage of the fact that not only the genome relation and composition of the *Triticeae* polyploids is known (Lilienfield and Kihara, 1951), but their diploid ancestors are still in existence, so that polyploid species can be recreated. This makes the system ideal for studying the effects of polyploidy. Each basic haploid genome (denoted by an uppercase letter) is a set of seven chromosomes (x = 7). Thus, diploid species possess 14 chromosomes (e.g., *Aegilops squarrosa* [DD] 2n = 2x = 14), tetraploids, 28 chromosomes (e.g., *Aegilops cylindrica* [CCDD] 2n = 4x = 28), and hexaploids, 42 chromosomes (e.g., *Triticum aestivum* [AABBDD] 2n = 6x = 42).

We have studied centromere and telomere behavior during anther development in related species with different ploidy levels from the *Triticeae* tribe. Thick anther sections were labeled with centromere and telomere probes by in situ hybridization, and visualized by three-dimensional confocal microscopy. We demonstrate that centromeres associate as pairs early during floral development in all the polyploid species studied, but in diploids, there is an association of centromeres only in the pollen mother cells (PMCs)[1] at meiosis.

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[1]*Abbreviations used in this paper:* DAPI, 4′,6-diamidino-2-phenylindole; PMCs, pollen mother cells.

Materials and Methods

Species

All plants used in this study came from the John Innes Centre seed collection. The species used were the following (the John Innes Centre seed collection accession number is given in parenthesis): *Triticum monococcum* (1040005), *Aegilops squarrosa* (2220007), *Aegilops speltoides* (2140008), *Aegilops bicornis* (2190001), induced autotetraploid *Aegilops bicornis* (2200001), *Triticum durum* (1180351), artificial AADD allotetraploid (*Triticum aegilopoides* × *Aegilops squarrosa*, 7010071), *Aegilops cylindrica* (2100001), *Triticum aestivum* (1190830), and *Aegilops vavilovi* (2260001). Ploidy and genome composition of each species is given in Table I.

Anther Sections

All spikes were harvested between late April and early September. Spikes at different developmental stages were fixed for 1–2 h in 4% formaldehyde in PEM (50 mM Pipes, 5 mM EGTA, 5 mM $MgSO_4$, pH 6.9). Single spikelets were detached from the spike and sectioned (50–100-μm-thick sections) under water using a Vibratome Series 1000 (TAAB Laboratories Equipment Ltd.). Spikelet sections were placed on multiwell slides (ICN Biomedicals Inc.) coated with 2% (vol/vol) γ-aminopropyl triethoxy silane (APTES; Sigma Chemical Co.) and dried overnight at 37°C.

Seeds were germinated and grown for 3–4 d before the root tips were excised, and then fixed in 4% formaldehyde in PEM. The sectioning was carried out in the same way as the anthers.

Fluorescence In Situ Hybridization

Spikelet sections on multiwell slides were dehydrated and rehydrated in a methanol series (30, 50, 70, 100, 70, 50, and 30%) for 5 min each. Sections were treated with 2% (wt/vol) cellulase at 37°C for 1 h. The sections were dehydrated in a series of steps in 70, 90, and 100% ethanol, and air dried.

Table I. Counting of Centromeres at Early Anther Development and the Beginning of Meiotic Prophase in Species with Different Ploidies

Diploids 2n = 2x = 14	*A. bicornis* SbSb	*A. speltoides* SS	*A. squarrosa* DD	*T. monococc.* AA
Early anther development	13.54 (0.68)	12.43 (1.22)	12.15 (0.87)	12.29 (1.40)
Early meiotic prophase	6.64 (1.01)	6.06 (1.32)	6.83 (0.71)	6.54 (1.10)
Tetraploids 2n = 4x = 28	***T. durum* AABB**	***A. cylindrica* CCDD**	***A. bicornisx4* SbSbSbSb**	***Artificial AD* AADD**
Early anther development	13.78 (1.84)	14.58 (1.16)	16.6 (2.9)	9–18*
Early meiotic prophase	12 (1.9)	12.90 (1.04)	8.13 (0.91)	6–17*
Hexaploids 2n = 6x = 42	***A. vavilovi* DDMMSpSp**	***T. aestivum* AABBDD**		
Early anther development	21.53 (2.14)	19.55 (1.82)		
Early meiotic prophase	19.55 (1.74)	19.2 (1.47)		

The genome composition (A,B,C,D,M,Sb,Sp,S) is given under each species. In each case the average value of the number of centromeres is given with the SD in brackets.

For each species at least two plants were used, and at least five spikes from each plant were examined, making a total of >2,000 sections for this study. Nuclei were included in the statistics shown only if they were at the correct developmental stage, and if they were clearly and unambiguously visible throughout their entire volume. The total number of nuclei included was 334, and the minimum number for any mean value shown was 10 nuclei.

*In the artificial AADD line (last column of tetraploids), the range of number of centromeres found is given, as the values were very variable and, in most cells, the counting was impossible due to the irregular associations of the centromeres.

The hybridization mixes with the probes for centromere (CCS1) and telomere (TTTAGGG repeats) were prepared as described in Martinez-Perez et al. (1999). The slides with the hybridization mix were placed in a modified thermocycler (Omnislide; Hybaid Ltd.). Denaturation was carried out at 77°C for 10 min, and then hybridization overnight at 37°C. Posthybridization washes were carried out at 42°C with 20% formamide in 0.1× SSC for 10 min.

Probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim Corp.) and biotin-16-dUTP (Boehringer Mannheim). Probes were detected using FITC-conjugated sheep antidigoxigenin antibody (Boehringer Mannheim) and extravidin-Cy3 (Sigma Chemical Co.). Both antibodies were prepared in 4× SSC, 0.1% Tween 20, and 5% BSA. Antibody incubations were carried out for 1 h in a humid chamber at 37°C. After three washes with 4× SSC and 0.1% Tween 20, slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.), and then mounted in antifade solution (Vectashield; Vector Laboratories Inc.).

Microscopy and Imaging Processing

Confocal optical section stacks were collected using a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg GmbH) equipped with a krypton and an argon laser. All the DAPI confocal images were collected using a confocal microscope (model MRC-1000; Bio-Rad Laboratories) equipped with a UV laser. Low magnification DAPI images (see Fig. 1, a, c, and e) were acquired using a series 300 CCD camera (Photometrics) attached to a Nikon microphot-SA. Images were processed on a Macintosh computer using Adobe Photoshop and printed on a Fuji Pictrography P3000 printer.

Results

The floral anatomy of all the studied species is very similar. The flower contains three anthers, each one having four loculi (Fig. 1 a). In the center of the loculi, the PMCs can be found surrounded by a layer of tapetal cells (Fig. 1 b). A clear marker for the onset of meiosis is the presence of a telomere cluster in the PMCs (Martinez-Perez et al., 1999). At that point, PMCs display only one or two nucleoli as opposed to up to six earlier in anther development. The same loculus as in Fig. 1 b is shown in Fig. 1 c labeled with the centromere (CCS1) and telomere probes. The telomere cluster can be easily seen in the PMCs, whereas the tapetal cells still have their telomeres dispersed. Before the telomeres form a cluster, PMCs and tapetal cells are not morphologically distinguishable, and can be only classified by their position in the anther. For the unequivocal identification and staging of the cells, we have used the general morphology of the flower, the number of nucleoli present in the nucleus, the morphology of the nucleus, the position of the cell in the anther, and the disposition of the telomeres.

Using three-dimensional confocal image stacks from anther sections labeled with the centromere and telomere probes, we counted the number of centromeres at different stages in species with different ploidy levels. These numbers are presented in Table I. For each species, the number of centromeric sites is given at an early anther developmental stage (before PMCs and tapetal cells are morphologically differentiated) and at the beginning of the meiotic prophase as judged by clustered telomeres. All the diploid species (2n = 14) analyzed (*T. monococcum*, *A. speltoides*, *A. bicornis*, and *A. squarrosa*) at the early development stage displayed a number of centromeric sites very close to 14, indicating that the centromeres are unassociated at this stage (Fig. 2 a). However, at the beginning of meiotic prophase, all the diploid

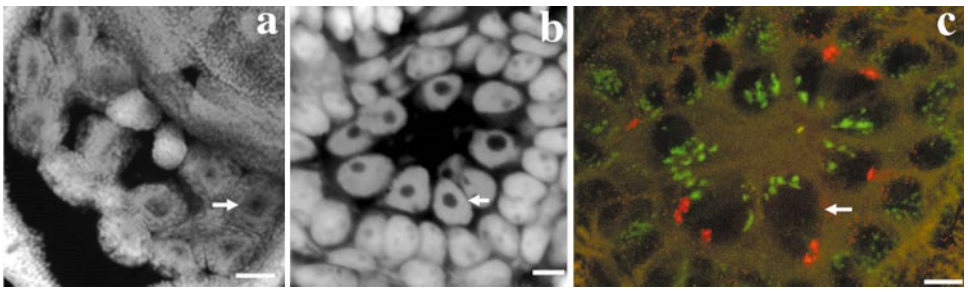

Figure 1. DAPI and fluorescence in situ confocal images of spikelet sections from *T. durum* (2n = 4x = 28). (a) A whole flower with three anthers, each one containing four loculi, is shown. The image was collected with a CCD camera. (b) A single confocal optical section of the arrowed loculus in a is shown in which the PMCs can be seen in the middle surrounded by a layer of tapetal cells. The arrowed cell is the same one as that arrowed in c. (c) Fluorescence in situ confocal image from the anther section shown in b labeled with telomere (red) and centromere (green) probes. The image is a projection of four consecutive confocal sections spaced 1 μm apart. The z section range for projection has been chosen to display the telomere clusters, but does not show all the centromeres. The intense red signals correspond to telomere clusters in the PMCs, clearly diagnostic of early meiotic prophase. In the tapetal cells surrounding the PMCs, the telomeres remain dispersed. Bars: (a) 50 μm; (b and c) 5 μm.

species show close to 7 centromere sites, indicating that the centromeres are associated in pairs (Fig. 2 b). In the case of all the polyploid species studied, the number of centromere sites during early anther development was reduced to a number close to half the number of chromosomes present, indicating that the centromeres are mostly associated in pairs. Tetraploid species (2n = 4x = 28) display a number of centromeric sites close to 14 (Fig. 2 c), and hexaploid species (2n = 6x = 42) display a number of centromeric sites close to 21 (Fig. 2 e). By the beginning

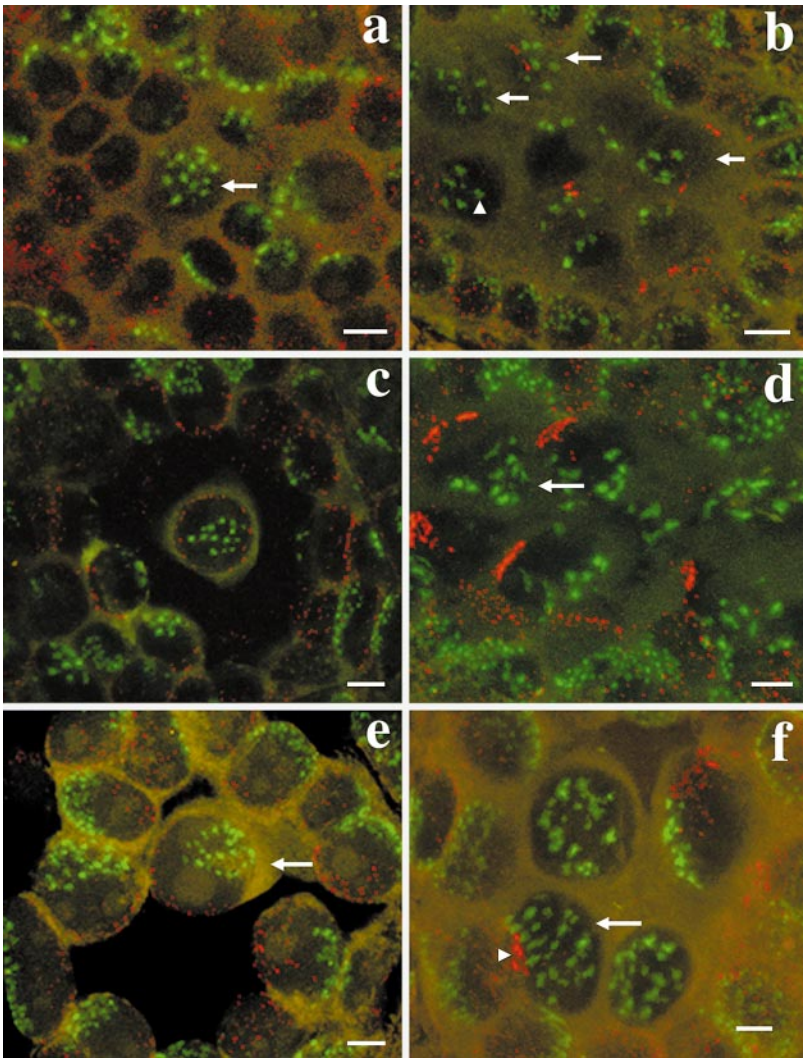

Figure 2. Projections of confocal sections from anthers from different species labeled with the centromere (green) and the telomere (red) probes. In each, two developmental stages are presented from the same species. In the first column, all the anthers are in early floral development in which PMCs and tapetal cells are still not morphologically differentiated. In the second column, all the anthers are at the beginning of meiotic prophase as shown by the presence of the telomere cluster. Because of the three-dimensional cellular organization, it is usually necessary to analyze the three-dimensional data set to count the centromeres in each nucleus. However, for the purpose of this figure, we have made partial projections that display all the centromeres of the arrowed nuclei only. (a) *A. squarrosa* (2n = 2x = 14) in early development. In the arrowed PMC, 13 centromere signals can be clearly counted. (b) *A. squarrosa* (2n = 2x = 14) at the beginning of meiotic prophase. The telomere clusters lie within the z range of the projection in four PMCs. In the arrowed cells, six or seven centromeres can be counted, indicating that the centromeres are present as pairs. Some of the centromeric signals, when analyzed in detail, are composed of two smaller signals together (arrowhead). (c) *T. durum* (2n = 4x = 28) at an early developmental stage. In the cell in the middle of the loculus, 14 centromeres can be seen, indicating association of the centromeres. (d) *T. durum* (2n = 4x = 28) at the beginning of meiotic prophase. Several PMCs display telomere clusters. In the arrowed cell, 12 centromeric signals can be counted. (e) *A. vavilovi* (2n = 6x = 42) at an early developmental stage. The arrowed cell displays 23 centromeric signals, showing the centromeres are associated. (f) *A. vavilovi* (2n = 6x = 42) at the beginning of meiotic prophase. The arrowed PMC shows 21 centromeric sites, and the arrowhead indicates its telomere cluster. Bars, 5 μm.

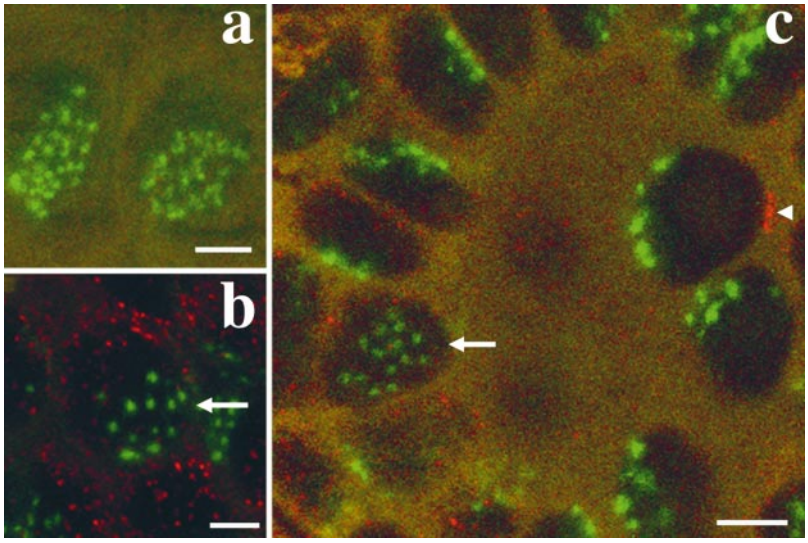

Figure 3. Number of centromeres in different cell types at different stages. All images are projections from confocal sections. The projections have been produced to show the number of centromeres in the indicated cell. (a) Root tip section from *T. durum* (2n = 4x = 28) labeled with the centromeric probe. In the left cell, 28 sites are present, indicating that the centromeres are not associated. Not all the sites can be clearly identified in the projection because of overlapping of the signals. (b) Early development anther section from *T. durum* (2n = 4x = 28) labeled with the centromere (green) and telomere (red) probes. The cell shown is a tapetal cell where 13 centromere sites can be counted, indicating association of the centromeres. (c) Anther section from *A. squarrosa* at the beginning of meiotic prophase, as is demonstrated by the telomere cluster (arrowhead) in the PMC. In the indicated tapetal cell (arrowed), 14 centromeric (green) sites are present, showing the centromeres are not associated. Bars, 5 μm.

of meiotic prophase, the number of centromeric sites is still close to half the number of the chromosomes present, indicating in both cases that the centromeres remain associated in pairs. Thus, for allotetraploids, 14 centromeric sites (*T. durum* and *A. cylindrica*) and, for allohexaploids, 21 centromeric sites (*A. vavilovi* and *T. aestivum*) are observed (Fig. 2, d and f). However, the situation is different in the case of autotetraploids. The autotetraploid *A. bicornis* line was induced by colchicine treatment, and has been grown for ~10 generations. Although the autotetraploid displays close to 14 centromeric sites at early anther development, by the beginning of meiotic prophase, the number is reduced further to ~7 sites, suggesting that the centromeres are associated in groups of four (Table I).

In all the polyploid species studied, the centromeres associate at an early stage of anther development, and both PMCs and tapetal cells display associated centromeres (Fig. 3 b). In diploids, centromeres associate only in the PMCs and not until the beginning of meiotic prophase, whereas tapetal cells at this stage still display unassociated centromeres (Fig. 3 c). As a control for this study, in situ hybridization using the centromere probe was carried out on root tip sections. The centromeres were unassociated (Fig. 3 a).

An artificial allotetraploid containing the A and D genomes (*Triticum aegilopoides* × *Aegilops squarrosa*) was also analyzed. This line has not been reported to occur naturally, and has only been grown for six generations since it was created. The fertility of this line is low. As in the case of the other polyploids, the centromeres also associated during early anther development before the PMCs and the tapetal cells were morphologically distinct. However, the number and size of the centromeric sites was very irregular (Fig. 4). The irregular associations were not corrected to pairs at the beginning of meiotic prophase (Fig. 4, c and d). This contrasts with the regularity in size and number of the centromeric sites found in other polyploids studied (Fig. 2, c–f).

Discussion

We have demonstrated that in a wide range of cereal species polyploidy results in the early association of cen-

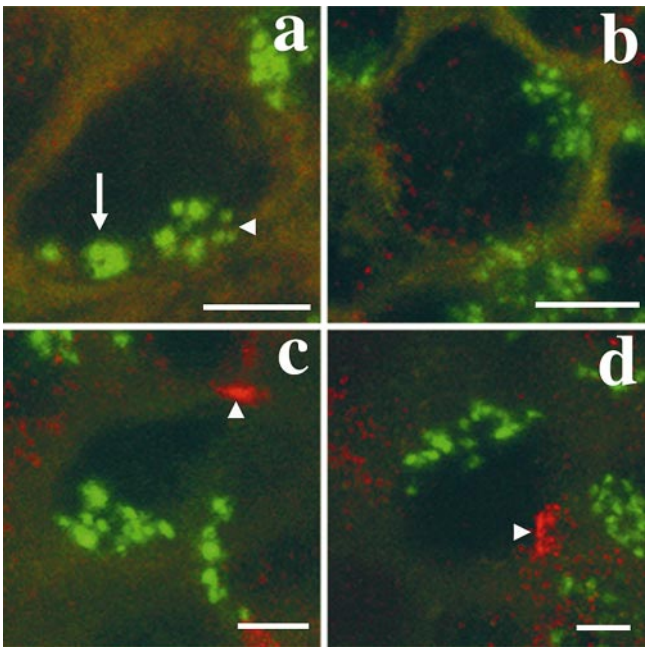

Figure 4. Confocal projections of PMCs from *T. aegilopoides* × *A. squarrosa* (artificial AADD) anther sections labeled with probes for centromeres (green) and telomeres (red). (a and b) A cell from early anther development stages. The centromeres are associating in groups of varying size, ranging from single centromeric sites to large and irregular clusters, and in many cases making it impossible to count the centromeres. In a, one such cluster is shown (arrow) and, in the right side of the same cell, three small signals can also be seen (arrowhead). (c and d) PMCs at the beginning of meiotic prophase. In both cells, the telomere cluster is indicated (arrowheads). The centromeres are still associated in irregular groups. Bars, 5 μm.

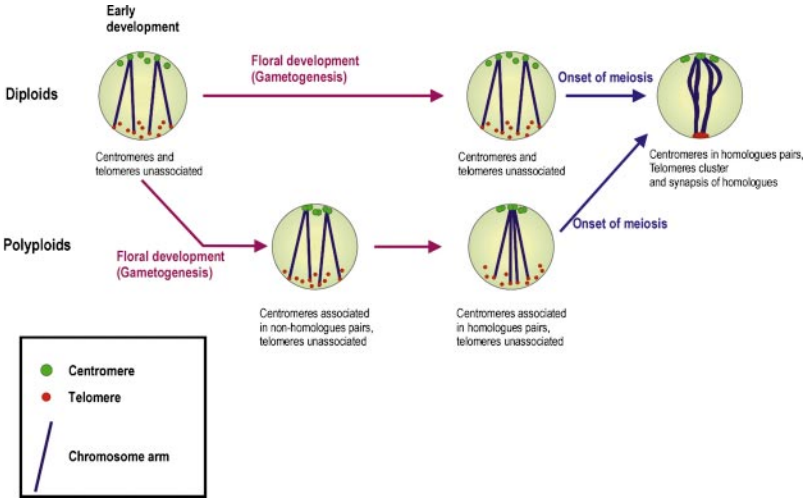

Figure 5. Model for centromere association and homologue pairing in diploid and polyploid species. In both diploids and polyploids, the centromeres (green) and telomeres (red) of three pairs of homologues are represented. Only the chromosome arms of one pair of homologues are shown for more clarity. In both cases, in early development, the centromeres are present as single units, so the number of centromeres equals the number of chromosomes present in the species. In diploids, this organization remains throughout floral development (purple arrow) until the onset of meiosis (blue arrow), when centromeres are associated in homologous pairs and telomeres form a cluster. In polyploids, the centromeres associate in pairs during anther development, so the number of centromeres is half the number of chromosomes present in the species. These associations are initially nonhomologous, but are later corrected to homologous associations. Before the onset of meiosis (blue arrow) in polyploids, the homologues are associated at their centromeres. As in diploids, meiosis begins with a cluster of telomeres that is followed by synapsis of the homologues.

tromeres during anther development, whereas in diploids centromeres only associate at the beginning of meiotic prophase. The large nuclei and centromeres in these species makes them more amenable to study than, for example, yeast. Although the cereal centromere sites are located at one pole, as in the yeast nucleus, they are visualized as individual structures rather than the single cluster of centromeres seen in yeast nuclei (Jin et al., 1998).

The early centromeric associations during anther development are mainly observed as pairs since the number of centromere signals is close to half the number of chromosomes present. The only exception was the artificial AD allotetraploid, in which we saw associations involving >2 centromeres. In polyploids, the association of centromeres is initiated before the differentiation of tapetal cells and PMCs; therefore, on differentiation, both cell types exhibit centromere association. Although the early association of the centromeres is only triggered when more than one complement of chromosomes is present, the potential for this somatic centromere association must be present in the diploid species since it is expressed during anther development in recently created polyploids (AD artificial allotetraploid line and the induced autotetraploid). Although it is possible, but unlikely, that the association mechanism could have evolved independently de novo for each of the naturally occurring polyploid species, it would be very difficult to explain its immediate expression in newly created polyploid lines. For example, the AD artificial line has only been grown for six generations, which is not enough time for such a mechanism to be generated.

There are two possibilities for this association mechanism: (1) either it is a purely somatic mechanism, which is not expressed in diploid species, but which is triggered during floral development in polyploids; or (2) it is the same mechanism that leads to homologous centromeres associating during meiosis, which is brought forward to start earlier during development in polyploids. Whichever of these two possibilities, we have to explain why the presence of more than one complement of chromosomes induces the phenotype. Possible explanations include gene dosage effects, homology-dependent cosuppression, or purely physical effects due to the consequences of changes in nuclear volume.

The autotetraploid line also showed association of centromeres in pairs during anther development. At the beginning of meiotic prophase, the number of centromere sites reduced to seven, suggesting that these signals must each be composed of four centromeres. This is consistent with previous studies reporting the formation of quadrivalents during meiotic prophase in autotetraploid cereals (Gillies et al., 1987) and it is likely that the quadrivalents are formed through the linking of four identical chromosomes at their centromeres.

Bennett and Smith (1972) showed that the duration of meiosis is shorter in polyploids when compared with their diploid progenitors. Thus, apparently the more sets of chromosomes that are present, the shorter the time necessary to sort them. We suggest that our data resolves this paradox; in polyploids, the centromere associations begin the process of sorting of chromosomes early during anther development. Our previous data (Aragon-Alcaide et al., 1997; Martinez-Perez et al., 1999) showed that in wild-type hexaploid wheat (*T. aestivum*) the early centromere associations finally lead to homologous chromosomes being associated at their centromeres before meiotic prophase. In the other polyploids studied here, it is not possible at present to assess whether the centromere associations occur between homologous or nonhomologous chromosomes since we currently do not have chromosome-specific centromeric probes. Nevertheless, we propose that as in *T. aestivum*, in the other polyploids the early centromere associations provide a mechanism for the early initiation of homologue sorting before meiosis. The extension of the homologue searching mechanism back into anther development can explain how multiple sets of chro-

mosomes can be sorted while reducing the time needed for meiotic prophase. Fig. 5 shows a schematic model describing chromosome pairing in diploids and polyploids based on our current knowledge.

The one exception to regular centromere pairing in the polyploids we have studied is the AD artificial allotetraploid. This line shows irregular multiple centromere associations rather than strict pairing. Thus, not all possible genome combinations can lead to regular centromere associations, and irregular association of centromeres may lead to low fertility (as in the AD line); this may have been a factor in determining which genome combinations have evolved naturally.

Control of centromere association operates at least at three different phenotypic levels: (1) association, (2) association as pairs, and (3) association as homologous pairs. In hexaploid wheat, the *Ph1* locus promotes pairing between the homologous chromosomes. A single line carrying a deletion of this locus has been important for breeding purposes during the last 20 yr because wheat chromosomes are able either to pair with their homologous chromosomes or with chromosomes from related species. In wheat lacking the *Ph1* locus, the centromeres associate as nonhomologous pairs during anther development, but the sorting to homologous associations before meiosis is disrupted (Aragon-Alcaide et al., 1997; Martinez-Perez et al., 1999). So the *Ph1* locus is a clear candidate for controlling the nature of the centromeric associations. The cloning of the *Ph1* locus will help elucidate the mechanism described above (Roberts et al., 1999).

By polyploidization, new species can be produced within just one generation. But for the new species to be established, they need to produce viable and balanced gametes. The early developmental association of centromeres may be important in achieving that goal. Many species are polyploid, particularly among plants but also in other kingdoms. It will now be interesting to determine whether a similar mechanism operates in other polyploids.

We thank Steve Reader and Dr. Terry Miller (John Innes Centre) for providing all the seeds used in this study.

This work was funded by the Biotechnology and Biological Research Council of the United Kingdom, and the John Innes Foundation (to E. Martinez-Perez).

Submitted: 12 October 1999
Revised: 3 December 1999
Accepted: 8 December 1999

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