Chromosome Research (in press)

Effect of colchicine and telocentric chromosome conformation on centromere and telomere dynamics at meiotic prophase I in wheat-rye additions

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Running title: Chromosome dynamics at early meiosis

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Abstract

Association of telomeres in a bouquet and clustering of centromere regions have been proposed to be involved in the search and recognition of homologous partners. We have analysed the role of these structures in meiotic chromosome pairing in wheat-rye addition lines by applying colchicine for disturbing presynaptic telomere movements and by modifying the centromere position from submetacentric to telocentric for studying centromere effects. Rye chromosomes, wheat and rye centromeres, and telomeres were identified by fluorescence *in situ* hybridization. Presynaptic association of centromeres in pairs or in more complex structures involved mainly non-homologous chromosomes as deduced from the behaviour of rye centromeres. While centromere association was not affected by colchicine, colchicine inhibited bouquet formation, which caused failure of homologous synapsis. Homologous centromeres of rye telocentrics associated earlier than those of rye submetacentric chromosomes, indicating that migration of the telocentrics' centromeres to the telomere pole during bouquet formation facilitated their association. Homologous chromosomes associated in premeiotic interphase can recognize each other and initiate synapsis at zygotene. However, telomere convergence is needed for bringing together the majority of homologous pairs that normally occupy separate territories in premeiotic nuclei.

Key words: Bouquet, telomeres, centromeres, meiotic pairing, colchicine, wheat.

Introduction

Regular bivalent pairing of homologous chromosomes at the first meiotic division is required for both recombination and chromosome number reduction. Synapsis between homologues must be preceded by a search and recognition of homology. How homologues find each other represents one of the least understood mechanisms of the meiotic process (Roeder 1997, Zickler and Kleckner 1998, Page and Hawley 2003, Pawlowski *et al.* 2003). In most organisms, telomeres congregate in a small region of the nuclear envelope forming the so-called bouquet configuration, which is thought to facilitate homologous recognition (Bass *et al.* 2000, Niwa *et al.* 2000, Trelles-Sticken *et al.* 2000, Cowan *et al.* 2001, Scherthan 2001, Harper *et al.* 2004).

The bouquet appears to arise in two steps: at early leptotene, telomeres attach to the inner surface of the nuclear envelope at dispersed sites and, subsequently, they coalesce into a single cluster (von Wettstein *et al.* 1984, Bass *et al.*, 2000, Scherthan *et al.* 2000). A small number of genes has been shown to be involved in the bouquet formation; *kms1*, *taz1, lot2-s17, bkt1, bkt2*, and four *dot* genes in fission yeast (Shimanuki *et al.* 1997, Cooper *et al.* 1998, Nimo *et al.* 1998, Jin *et al.* 2002, Chikashige *et al.*, 2006), *ndj1* in budding yeast (Trelles-Sticken *et al.* 2000), *pam1* in maize (Golubovskaya *et al.* 2002), and *sy1* in rye (Mikhailova *et al.* 2001).

Colchicine is known to interfere with the dynamics of microtubules and to destroy karyokinetic spindle in cells (Dustin 1978). Applied premeiotically, it causes the formation of univalents at metaphase I in such plant genera as *Tradescantia* (Walker 1938), *Rhoeo* (Derman 1938), *Allium* (Levan 1939), *Fritillaria* (Barber 1942), *Lilium* (Shepard *et al.* 1974), *Secale* (Bowman and Rajhathy 1977), *Triticale* (Thomas and Kaltsikes 1977), and *Triticum* (Driscoll *et al.* 1967). The colchicine-sensitive period stretched over a range of premeiotic and meiotic stages in different species (Loidl 1990). Premeiotic chromosome arrangement, presynaptic alignment, SC formation, and crossing over are all possible colchicine targets (Loidl 1989, Tepperberg *et al.* 1997).

The colchicine-sensitive period in bread wheat was placed at premeiotic interphase (Driscoll *et al.* 1967, Dover and Riley 1973, 1977). Colchicine causes a reduction of chiasma formation between homologous arms of conventional chromosomes but not between the homologous arms of isochromosomes (Driscoll and Darvey 1970, Vega and Feldman 1998). In an isochomosome, the two arms are connected by a common centromere, hence they remain closely associated through the entire cell cycle. This suggests that colchicine affects the presynaptic alignment of homologues, but does not affect synapsis and chiasma formation. Consistent with this, Cowan and Cande (2002) demonstrated that colchicine impeded the bouquet organization in cultured anthers of rye.

Bread wheat, *Triticum aestivum*, is an allohexaploid species (2n = 6x = 42) with three genomes, A, B and D, from three related diploid species. The allopolyploid condition adds another complication to the homologous recognition, *i.e.*, discrimination of genetically related (homoeologous) chromosomes from homologous chromosomes. In polyploid wheats, the *Ph1* (Pairing homoeologous) locus controls the exclusive formation of bivalents at metaphase I (Riley and Chapman 1958, Sears and Okamoto 1958). Feldman and co-workers (Feldman 1966, 1993, Feldman and Avivi 1988, Vega and Feldman 1998) argued that *Ph1* governs the spatial arrangement of chromosomes at premeiotic interphase in such a way that homologues, but not homoeologues, become associated. They suggests that *Ph1* exerts its effect through the centromeres.

Studies using fluorescence *in situ* hybridisation (FISH) on the behaviour of alien chromosomes added to wheat suggested that homologous recognition starts at the centromere since, in premeiotic interphase, centromeres are associated in pairs (Aragón-Alcaide *et al.* 1997, Martínez-Pérez *et al.* 1999, 2001). The *Ph1* locus was proposed to suppress homoeologous pairing through the control of the specificity of premeiotic association of centromeres. However, Maestra *et al.* (2002) reported that in a majority of cells analysed, rye homologous chromosomes added to wheat were separated in the premeiotic interphase and in leptotene. A further study by Martínez-Pérez *et al.* (2003) demonstrated the formation of multicentromere structures in leptotene, and proposed that these structures were involved in the mechanism of chromosome sorting. Based on the number of such centromere clusters, each cluster was assumed to include the three centromere pairs from homoeologous A-, B-, and D-genomes and homologous centromeres would remain associated in pairs after the resolution of the six centromere clusters. However no evidence was ever presented that pairs of homologous centromeres were regularly included in the same clusters (Naranjo and Corredor 2004) and, it has been shown in wheat that distal, but not proximal, chromosome regions were critical in the initiation of synapsis and chiasmate pairing (Lukaszewski 1997, Jones *et al.* 2002).

Telomere clustering and centromere association have been proposed as the major forces in preparing for presynaptic alignment in wheat. They appear to play crucial roles in meiotic prophase I of organisms such as fission yeast (Scherthan *et al.* 1994). Because, in most cases, homologues occupy separate nuclear territories in the premeiotic interphase (Maestra *et al.* 2002) colchicine is expected to affect some component of the mechanism that brings them together during leptotene. Thus, telomere convergence and centromere clustering are two possible targets of colchicine. On the other hand, in a telocentric chromosome, the centromere is located close to one of the telomeres. By virtue of its proximity to the telomere, such a centromere migrates to the telomere pole in the meiotic bouquet while centromeres of bi-armed chromosomes remain at the centromere pole. (Maestra *et al.* 2002). In this work, we take advantage of this phenomenon to assess the roles of centromeres and telomeres in homologous recognition by studying the effects of colchicine and centromere position on the dynamics of centromeres and telomeres during leptotene and zygotene in wheat.

Material and methods

Plant material

The observations were made on disomic additions of bi-armed chromosome 5R of rye (*Secale cereale* cv. “Imperial”) and of its telocentric long arm 5RL to hexaploid wheat (*Triticum aestivum* cv. “Chinese Spring”, 2n = 6x = 42) (Driscoll and Sears 1971, Mikhailova *et al.* 1998). Plants with the disomic addition of chromosome 5R (2n = 44) were selected by C-banding of root tip mitotic metaphases (Giráldez *et al.* 1979). Monotelosomic (2n = 42 + t) and ditelosomic (2n = 42 + 2t) additions were selected among the offspring of ditelosomic additions of wheat-5RL by routine acetocarmine squashes of root tips.

Colchicine treatment

The colchicine treatment was carried out in immature spikes of monotelosomic and ditelosomic wheat-5RL plants. About 0.50 ml of 2×10^{-4} M (0.008%) colchicine solution was injected with a hypodermic syringe through the leaf sheaths into the space surrounding the developing spike when it was at the height of the second leaf node. This concentration used has been reported to produce univalents at metaphase I (Vega and Feldman 1998). Spikes of monotelosomic and ditelosomic wheat-5RL plants injected with 0.50 ml of distilled water were used as a control. Because of the developmental gradient in the spike (Maestra *et al.* 2002), both florets with anthers containing premeiotic cells and florets with anthers at early meiosis could be exposed to colchicine in the same application. Anthers were collected 48 h, 72 h or 96 h after colchicine administration. One of the three anthers from each flower was checked to establish the meiotic stage and the other two were fixed in 3:1 ethanol-acetic acid, and stored at 4ºC.

Four colchicine treatments covering different development stages of germinal cells from premeiotic interphase to pachytene were carried out. Their durations and positions according to the timing of meiosis (Bennett *et al.* 1973, Maestra *et al.* 2002) are indicated in Figure 1. Condensation of chromosomes in adjacent somatic tissues of the anthers and formation of 4N nuclei in tapetal cells after the last synchronous division indicated the action of colchicine.

Fluorescence in situ hybridization

Fixed anthers were digested in a pectolytic enzyme mixture, transferred to a clean slide, and spread according to Zhong *et al.* (1996). This procedure involves no mechanical pressure to spread the cells on the slide and the three-dimensional information is largely preserved. Preparations were pretreated as previously described (Maestra *et al.* 2002).

For the analysis of the centromere and telomere dynamics the following three repeat DNA probes were used in the hybridisation mix: 5 ng/µl pAtT4 containing the *Arabidopsis* telomeric tandem repeat (Richards and Ausubel 1988), 10 ng/µl CCS1 containing a cereal-specific centromere repeat (Abbo *et al.* 1995, Aragón-Alcaide *et al.* 1996), and 10 ng/µl pAWRC.1 containing a rye-specific centromere repeat (Langridge *et al.* 1998, Franki 2001). DNA probes were labelled by nick-translation with biotin-16-dUTP, for pAtT4 and pAWRC.1, and digoxigenein-11-dUTP, for CCS1.

Whole genome DNA probe was used for rye DNA painting in the analysis of synapsis. The hybridisation mix contained 2.2 ng/µl sonicated rye genomic DNA (fragment size less than 2 kb), 5 ng/µl pAtT4, and 10 ng/µl pAWRC.1. Rye genomic DNA was random primed labelled with fluorescein-12-dUTP (Roche) while pAtT4 and pAWRC.1 were labelled with biotin-16-dUTP. For each slide, the probes and wheat blocking DNA were diluted in 20 µl of hybridisation buffer (10% dextran-sulfate, 50% formamide, 2 x SSC, in 0.25 M sodium phosphate buffer pH 7).

Hybridisation was carried out according to Maestra *et al.* (2002). The digoxigenin-labelled probe was detected with 6-8 ng/µl of the FITC-conjugated antidigoxigenin antibody (Sigma) in 4B (0.5% blocking reagent in 4 x SSC) and biotin-labelled probes with 10-15 ng/µl of the Cy3-conjugated avidine (Sigma) in 4B.

Fluorescence microscopy and image processing

Images of cells were studied under an Axioplan-2 (Carl-Zeiss GmbH) fluorescence microscope equipped with a Spot-2 CCD camera (Diagnostic Instruments, Sterling Heights, MI). Stacks of five to ten different focal planes in the Z-axis per nucleus were captured with MetaMorph software (MetaMorph Imagin System, Universal Imagin Corp., PA). Images of consecutive sections were processed in a PC computer using Adobe Photoshop 7.0 (Adobe System Incorporated). The entire data set were merged with Image Tool 3.0 (UTHSCSA). This software was also used to measure the length of rye chromosome domains.

Results

Identification of stages in the germinal cells' development

We examined the arrangement of centromeres and telomeres from premeiotic interphase to mid zygotene in spread preparations of anthers from the monotelocentric and ditelocentric wheat-5RL addition (2n = 42 + t and 2n = 42 + 2t, respectively) and from the disomic wheat-5R addition (2n = 44). The CCS1 cereal centromere DNA probe was used to label all centromeres, rye centromeres were labelled with the pAWRC.1 DNA probe, and telomeres were labelled with the pAtT4 DNA probe. The developmental stages of germinal cells were established according to Maestra *et al.* (2002). Figure 2 shows diagnostic features concerning the number and position of nucleoli and the arrangement of centromeres and telomeres in nuclei at premeiotic interphase, leptotene and mid zygotene. In colchicine-treated cells, where normal telomere convergence was inhibited, identification of the leptotene and zygotene stages was based on the chromatin appearance and the number and the position of nucleoli. Figure 3 shows a colchicine-treated cell at leptotene with compact centromere structures and groups of telomeres spread through the nuclear membrane. Five 3D consecutive sections show the signal distribution in the Z axis.

Colchicine does not disturb the presynaptic centromere clustering

The stacks of consecutive focal plains contained sufficient spatial information to count the number of centromere sites in untreated and colchicine-treated cells from premeiotic interphase to mid zygotene (Table 1). As expected, cells at premeiotic interphase of untreated anthers (Fig 4A, B) yielded a mean number of signals close to the haploid chromosome number. Because there were no statistically significant differences between cells exposed to colchicine for 48 h and the controls (Student $t = 1.14$, $p > 0.20$) we concluded that the pair-wise premeiotic centromere association was not affected by colchicine.

Centromere association continued through leptotene. In anthers containing both premeiotic and meiotic cells, some nuclei at the premeiotic interphase showed pairs of close centromere signals, which may represent a transient step in the formation of complex centromere structures (Fig 4B). Large centromere signals, denoting the formation of multicentromere structures, became apparent at the time of telomere convergence in control cells (Fig 4C). Large multicentromere signals were accompanied by smaller signals produced by one or two centromeres, but the mean number of centromere signals decreased relative to the premeiotic interphase (Table 1, Student $t = 9.34$, $p < 0.001$). Complex centromere structures were also observed in cells at leptotene after the 72 h colchicine treatment (Fig 4D). The mean number of centromere sites showed a reduction similar to that observed in the controls (Table 1). Differences between control and colchicine-treated cells at early leptotene were significant in the disomic (Student $t = 2.38$, $p < 0.05$) but not in the monosomic addition 5RL (Student $t = 0.37$, $p > 0.5$). Multicentromere structures resolved into pairs of centromeres concomitant with telomere dispersal during zygotene (Fig 4E, F; Table 1). This was observed in both the control and colchicine-treated lines, which showed similar number of signals (Student $t = 0.86$, $p > 0.20$). Thus, neither the formation of the multicentromere structures nor their resolution was affected by colchicine.

Colchicine impedes the migration but not the association of telomeres

The number of telomere signals at the premeiotic interphase approximated twice the chromosome number in control cells (Table 2). Telomere signals were scattered throughout a considerable portion of the nuclear surface. The fraction of the projected area of the nucleus that included telomere signals was above 2/3 in 52.9% of premeiotic cells, and between 1/3 and 2/3 in the remainder. At the transition to meiosis, many telomere signals were arranged in pairs, which probably represented a prelude of their association (Fig 4B). At early leptotene, most telomeres were associated in clumps, probably with unequal numbers, while only a minor fraction appeared as weak signals formed by one or two units (Fig 4C; Table 2). The telomere clusters concentrated in the region of the nuclear periphery opposite the centromeres, with a projected area less than 1/3 of the nuclear surface. Grouping of telomeres coincided with centromere clustering.

Colchicine treatment did not affect the arrangement of telomeres at the premeiotic interphase. At early leptotene, telomeres associated in groups although a number of weak signals, probably from single telomeres, were present (Fig 4D; Table 2). The total number of telomere aggregates was higher relative to the control (Student t = 3.74, $p < 0.005$) and telomere signals were scattered through a much higher proportion of the nuclear surface than in control cells. Only a minor fraction of the colchicine-treated cells (13.8%) showed telomere clustering resembling the bouquet configuration. Spatial separation of telomere aggregates demonstrated that colchicine impeded telomere migration. The centromeric end of the rye telocentrics migrated to the telomere cluster in control cells but remained at the centromere pole in colchicine-treated cells (Fig 5). The fact that, in the absence of colchicine, the rye centromere of a monosomic telocentric reached the telomere pole indicates that telomere association is not required for telomere migration. That is to say, the association and migration of telomeres are two distinct steps of the bouquet organization.

The effect of centromere position on the homologous centromere association

Labelling of the rye centromeres in the disomic addition 5RL with the pAWRC.1 DNA probe revealed their spatial arrangement, associated in the same cluster or positioned in separated clusters (Fig 6), in premeiotic and early meiotic cells. At the premeiotic interphase, homologous centromeres were associated in a relatively low proportion (13.1%) of untreated nuclei (Fig 7). The proportion of nuclei showing homologous centromeres in tight association increased at leptotene, and reached 33 % at the leptotene-zygotene transition, and 68.7% at mid zygotene.

The colchicine treatment did not essentially change the arrangement of rye centromeres at premeiotic interphase, but remarkable differences were observed in the subsequent meiotic stages relative to the control (Fig 7). In the treated material, homologous centromeres did not associate during leptotene and early zygotene, and, at mid zygotene, showed a lower level of association (33.3%) than in the untreated control. This delay in the association of centromeres was most likely a result of the suppression of telomere migration by colchicine. In control cells at mid zygotene, 60% of the rye centromeres had already reached the telomere pole (Table 3). The fact that the level of association was similar for both centromeres not incorporated and incorporated to the telomere pole (Contingency $X^2 = 1.73$, $p > 0.20$) suggests that the oriented movement of centromeres facilitated their association before reaching the telomere pole.

Whether migration of homologous centromeres accelerated their association was tested studying the behaviour of centromeres of submetacentric chromosome pair 5R added to wheat. Such centromeres, and those of wheat, remained stationary at the centromere pole during the bouquet stage. Association of the centromeres of chromosome pair 5R was delayed relative to the migrating centromeres of 5RL telosomes. Up to the early zygotene, centromeres of the chromosome pair 5R retained their premeiotic non-associated positions (Fig 7) and, only at mid zygotene, the frequency of association underwent a significant increase (43.8%). Because at mid zygotene synapsis has already been in progress, this increase can be interpreted as a result of synaptonemal complex expansion. Thus, the observations made here indicate that subtelomeric centromeres find their homologous partners earlier than centromeres located centrally in bi-armed chromosomes, probably because of their telomere-driven ability to migrate into the telomere cluster.

Disturbance of the bouquet formation affects synapsis

The total rye genomic DNA probe was used to study the position of the chromosome pair 5RL in germ cells of the disomic addition (Fig 8). We observed nuclei in premeiotic interphase both with the rye chromosome domains completely separated and with the rye chromosome domains occupying adjacent nuclear territories. Rye chromosomes occupied adjacent territories in about 20% of both control and the colchicine-treated cells (Fig 9), confirming that colchicine did not affect premeiotic chromosome arrangement. Physical separation of homologues in premeiotic cells (Fig 8A) implies that they have to approach each other to initiate synapsis. Prior to synapsis, chromosomes increase their length (Fig 8B). The mean lengths of rye chromosome domains at the premeiotic interphase and at pachytene were 29 μm and 78 μm, in the untreated cells, and 30 μm and 90 μm, in the colchicine-treated cells, respectively (Table 4). The differences between the control and colchicine-treated cells were not significant (for premeiotic nuclei, Student $t = 0.41$, $p > 0.50$; for nuclei at pachytene, Student $t = 1.30$, $p > 0.20$,) showing that chromosome elongation was not affected by the colchicine treatment.

The presence of cells at mid zygotene with rye homologues associated intimately only in their terminal regions (Fig 8C) is indicative of the bouquet's role in bringing the homologues together. To verify whether a disruption of telomere clustering caused by colchicine was the main reason of pairing failure at metaphase I (Driscoll *et al.* 1967), we have quantified the levels of synapsis at pachytene between the 5RL chromosomes pair in three samples of anthers: with colchicine treatments at 0 h (control), 48 h, and 72-96 h, respectively. Synapsis was considered to occur in regions showing tight association of the two homologues since at pachytene, synaptonemal complexes are completely, or almost completely, formed (Holm and Wang 1988, Martínez *et al.* 2001a,b) and two painted chromosomes appear intimately associated along their length (Mikhailova *et al.* 1998, Maestra *et al.* 2002). Five different types of cells were scored: cells with no synapsis, cells with partial synapsis at the telomeric end, cells with partial synapsis at the centromeric end, cells with partial synapsis at both chromosome ends, and cells with complete synapsis (Fig 8D-F; Table 5). Rye chromosomes were completely synapsed in 68.9% of control meiocytes, and showed partial synapsis of less than 80% of the chromosome length in the remaining 31.1% of cells. At 48 h after the treatment, meiocytes were exposed to colchicine during synaptonemal complexes formation, but no significant variation in the level of synapsis relative to the control was observed (Contingency $\chi^2 = 1.04$, $p > 0.30$). By contrast, meiocytes that were exposed to colchicine before bouquet formation started (t = 72-96 h) showed complete synapsis, partial synapsis, and no synapsis with frequencies of 40.9%, 55.3%, and 3.8%, respectively. This is a significant reduction of the level of synapsis relative to the control (Contingency $\chi^2 = 13.61$, $p < 0.001$), and it is reasonable to assume that the reduction is a consequence of the inhibition of telomere movement caused by colchicine.

Discussion

Meiotic chromosome pairing has been extensively studied in wheat. The development of nulli-tetrasomic and ditelocentric series, and other chromosome aberrations such as isochromosomes allowed the identification of genetic loci that control chromosome pairing (Sears 1976), and the effect of colchicine on chromosome pairing has been studied by Driscoll and others (Driscoll *et al.* 1967, Dover and Riley 1973, 1977, Driscoll and Darvey 1970, Vega and Feldman 1998). Chromosome painting is another useful method for studying chromosome pairing, but development of chromosome painting tools is more difficult in hexaploid wheat than in most other plant species because of the presence of homoeologous chromosomes and the huge size of the wheat genome: 17,000 Mb compared to the 110 Mb genome of *Arabidopsis thaliana* or 430 Mb genome of rice. Also labelling specific segments of individual wheat chromosomes is not yet possible. However, wheat has a wealth of cytogenetic stocks including a range of various additions from chromosome variants of related species. These chromosomes can be visualized with total genomic DNA probes. We decided to carry out our study in the wheat-5RL addition because pairing at metaphase I of chromosomes 5RL is comparable to that of wheat chromosomes (Mikhailova *et al.* 1998) and because this is the same line that led to the original discovery that telomeric centromeres migrate to the telomere bouquet (Maestra *et al.* 2002). This line also permitted investigation of the effect of colchicine on synapsis and centromere and telomere dynamics. Although the possibility exists that an alien chromosome in wheat may behave somewhat differently than wheat chromosomes themselves, the results obtained are consistent with the known effects of colchicine on meiosis.

Bouquet organization is needed for synapsis

In experiments using cultured anthers of rye, colchicine was found to inhibit bouquet formation (Cowan and Cande, 2002). Colchicine administered after the bouquet was formed did not affect telomere dispersal. The in vitro culture system used restricted the analysis to colchicine treatments of 24 h or less. This made it difficult to determine the consequences of bouquet inhibition in subsequent meiotic stages. In earlier studies in wheat (Driscoll *et al.* 1967, Dover and Riley 1973, 1977, Driscoll and Darvey 1970), colchicine was administered by injection into immature spikes. This allowed analysis of the effect of colchicine for several days. Meiocytes at metaphase I showed pairing failure when exposed to colchicine for 3-6 days, but neither bouquet formation nor synapsis was analysed. We have examined the effect of colchicine applied to immature spikes at different intervals during premeiosis and meiosis. The results demonstrated that inhibition of bouquet formation caused by colchicine leads to failure of synapsis.

Only treatments of 72-96 h in duration that were initiated before bouquet organization induced failure of synapsis; while cells at pachytene exposed to colchicine for 48 h showed normal synapsis like the control. This is consistent with the conclusion that the colchicine sensitive time immediately follows the last premeiotic mitosis (Dover and Riley 1973, 1977).

Telomere movement that is inhibited by colchicine is not mediated by cytoplasmic microtubules (Cowan and Cande 2002). A tubulin not involved in microtubules, such as membrane-associated tubulin, as well as tubulin-related proteins and/or non-tubulin proteins were suggested as the target of colchicine. Trelles-Sticken *et al.* (2005) reported that administration of latrunculin B, which prevents the G-actin polymerisation, inhibits the telomere cluster formation in *Saccharomyces cerevisiae*. These authors suggest that nuclear, but not cytoplasmic, actin is responsible for telomere clustering. This is consistent with the presence of intranuclear bundles of fibrillar material in the nuclei of wheat at the time of bouquet organization, and the distribution and assembly of which are affected by colchicine treatment (Bennett *et al.* 1979, Bennett and Smith 1979).

Centromere clustering plays no major role in chromosome sorting

Premeiotic association of centromeres was first detected in three-dimensional reconstructions from electron micrographs of serial thin sections across microsporocyte nuclei of *Allium fistulosum* (Church and Moens 1976) wheat, rye and triticale (Bennett 1979, Bennett *et al.* 1979), and *Lilium speciosum* (Del Fosse and Church 1981). Clustering of more than two centromeres in such associations indicated that non-homologous chromosomes had to be involved. Although centromeres associate in pairs at premeiotic interphase in hexaploid wheat and related polyploids (Martínez-Pérez *et* *al.* 1999, 2000) as well as in diploids such as rice or *Luzula* (Prieto et. al. 2004, Haizel *et al.* 2005), the behaviour of the rye centromeres studied here suggests that premeiotic centromere association involves mainly non-homologous chromosomes. The formation of multicentromere structures in leptotene of tetraploid and hexaploid wheats was proposed as an important component of the chromosome sorting mechanism and a feature of the polyploid condition (Martínez-Perez *et al.* 2003). Such a proposal assumes a directed association of homologous and non-homologous centromeres. After homology recognition, the multimeric structure would be resolved in pairs of homologous centromeres. This hypothesis might seem feasible in the light of the dynamics of centromeres at meiosis in yeast (Tsubouchi and Roeder 2005). Centromeres of yeast chromosomes associate in pairs at early meiosis. Most pairs initially are non-homologous but undergo switching until all of them involve homologues. However, the role proposed for the centromere clusters in wheat implies a strong increase of homologous centromere association prior to synapsis. Among the the rye centromeres, the frequency of association increases at leptotene only for those of telocentrics, and in cells not treated with colchicine. No increase is evident for the same centromeres under the effect of colchicine or when located in bi-armed chromosomes. Close proximity between centromere and telomere in telocentrics confers on centromeres the ability to migrate to the telomere pole. Movement of centromeres of telocentrics facilitate their association at leptotene while centromeres of the submetacentric chromosome pair 5R and telocentric 5RL under the effect of colchicine remain stationary at the centromere pole and do not associate. Because wheat chromosomes are metacentric or submetacentric, our results contradict the notion that centromere clustering plays a role in early meiotic sorting homologoues. The increased frequency of associated centromeres at the centromere pole at mid zygotene is most likely the result of the synapsis extension.

The rare presence of homologues in the same centromere cluster suggests that spatial proximity, first between individual centromeres and later between structures formed by two or more centromeres, conditions their grouping. Paired probe signals at the onset of meiosis, and their variable number and size during progression through leptotene, support the notion of random centromere associations and groupings. If centromere clusters are not involved in chromosome sorting at early meiosis, they might represent a chromosome anchoring mechanism that maintains the centromere pole in a stable position while telomeres migrate at the opposite pole of the nucleus and chromosomes decondense and elongate.

Finding the homologous partner

Painting of entire chromosomes 5RL with total genomic DNA indicated that, at the onset of meiosis, the two homologues under study occupied separate nuclear territories in about 80 % of the cells. Assuming a comparable arrangement for wheat chromosomes, nuclei in premeiotic interphase should contain many pairs of non-homologous chromosomes and some pairs of homologous chromosomes associated at the centromeres. The evolution of these two types of chromosome pairs is illustrated in Figure 10. Grouping of centromeres and telomeres conditioned only by proximity of chromosome territories at early leptotene would maintain the positioning of homologues that are premeiotically associated. Such chromosomes can initiate synapsis as the two arms of an isochromosome do in cells exposed to colchicine (Driscoll and Darvey 1970). By contrast, homologues that occupy separate territories in premeiotic nuclei will likely remain separated at early leptotene. Such chromosomes will find their homologues after the telomere convergence brings them together. Inhibition by colchicine of the forces that cluster telomeres would affect mainly synapsis of homologues that are separated in premeiosis. Identification of the homologous partner in the meiotic bouquet is probably accomplished after failed interactions between non-homologous chromosomes in a close proximity. Redistribution of chromosomes at the tight bouquet configuration may facilitate encounters between unmatched homologues. Accordingly, recurrent bouquet re-formation has been reported in living yeast meiocytes (Trelles-Sticken, *et al.* 2005).

Acknowledgments

We thank G Moore and P Landgridge for kindly supplying centromere DNA probes CCS1 and pAWRC.1, respectively, B. Maestra for her technical assistance and A.J. Lukaszewski for his help in writing the manuscript. This work has been supported by grant BFU2004-02261 from Dirección General de Investigación, Ministerio de Educación y Ciencia of Spain.

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Figure legends

Figure 1. Durations and positions of four colchicine treatments in anthers of wheat-5RL additions relative to meiotic time course and morphological landmarks described by Bennett *et al.* (1973) and Maestra *et al.* (2002). Arrow heads mark the points of fixation.

Figure 2. Nuclear morphology (DAPI images) and arrangement of centromeres (green) and telomeres (magenta) in premeiotic interphase and early meiosis of wheat-rye additions**. (A-B)** Nucleus at premeiotic interphase with two internal nucleoli (n) and centromeres and telomeres arranged in the Rabl's configuration. **(C-D)** Nucleus at late leptotene with three internal nucleoli (n), and an almost consolidated bouquet. **(E-F)** Nucleus at mid zygotene with only one peripheral nucleolus and partial disorganization of the bouquet. Scale bars represent 10 µm.

Figure 3. 3D stack of consecutive focal planes showing the arrangement of telomeres and centromeres in a colchicine-treated wheat-5RL nucleus at leptotene. **(A)** Telomere and rye centromere (arrows) signals. **(B)** Centromere signals of wheat and rye (arrows) chromosomes. **(C)** Merge of **A** (magenta) and **B** (green)**.** The last panel of each series is the projection of the whole stack. Scale bars represent 10 µm.

Figure 4. Centromere (green) and telomere (magenta) dynamics at early meiosis in control and colchicine-treated cells of wheat-5RL additions. **(A)** Premeiotic colchicine-treated cell. **(B)** Untreated premeiotic nucleus with pairs of centromere and telomere signals (grey scale in the insert) closely positioned (arrows). **(C-D)** Centromere and telomere clumps at early leptotene of control **(C)** and colchicine-treated **(D)** cells. **(E-F)** Resolution of mutlticentromere structures in centromere pairs in control **(E)** and colchicine-treated **(F)** cells at mid zygotene**.** Scale bars represent 10 µm.

Figure 5. The position of the rye centromere in the leptotene of the monosomic wheat-5RL addition. **(A-B)** Cells with almost consolidated bouquet **(A)** and bouquet inhibited

by colchicine **(B)** showing migration or immobility, respectively, of the centromeric end of chromosome 5RL (arrowheads). **(C-F)** Magnifications of **(A)** and **(B)** showing the green signal of the CCS1 probe **(C, E)** and the merge **(D, F)** of probes CCS1 and pAWRC.1 signals (arrowheads). Scale bars represent 10 µm.

Figure 6. Relative position of homologous centromeres at early meiosis. **(A)** Disomic wheat-5RL nucleus at late leptotene showing an almost consolidated bouquet and association of the two rye centromeres detected with the pAWRC.1 probe (arrow). **(B)** The same nucleus in **A** showing also the green signal of the centromere CCS1 probe. **(C-D)** Disomic wheat-5R nucleus at early zygotene with the two rye centromeres (arrows) separated. Scale bars represent 10 µm.

Figure 7. Frequency (%) of association of the rye centromeres at premeiotic interphase (PI), early leptotene (EL), late leptotene-early zygotene (LLEZ) and mid zygotene (MZ) in disomic untreated wheat-5R, colchicine-treated wheat-5RL, and untreated wheat-5RL additions. On average 57 nuclei per line were analysed in each stage.

Figure 8. Arrangement of two 5RL telocentrics added to wheat in control **(A, B, C, F)** and colchicine-treated **(D, E)** germ cells. **(A)** Rye chromosomes occupying separate territories in a nucleus at premeiotic interphase. **(B)** Elongated and intermingled chromosome domains in the leptotene-zygotene transition versus the condensed state of a somatic cell. **(C)** Both chromosome ends are at the bouquet pole and have started synapsis while some intercalary regions are aligned. **(D)** Rye chromosomes are separated in a nucleus at pachytene. **(E)** Y-shaped rye bivalent with unmatched centromeric ends (arrowheads). **(F)** Complete synapsis. Scale bars represent 10 µm.

Figure 9. Frequency (%) of nuclei at premeiotic interphase with the rye chromosomes associated or separated in control (N = 53) and colchicine-treated (N = 76) cells of the disomic wheat-5RL addition.

Figure 10. Centromere and telomere dynamics in the search of the homologous partner. Chromosome arrangement at premeiotic interphase showing two homologous pairs occupying separate (pair 1) and adjacent (pair 2) territories. Simultaneous clustering of centromeres and telomeres in leptotene brings about the formation of bundles of chromosomes. Homologues premeiotically associated maintain the position and can initiate synapsis. Telomere convergence facilitates encounters and synapsis between separated homologues.

Table 1. Mean number of centromere signals at premeitoic interphase (PI), early leptotene (EL), late leptotene-early zygotene (LLEZ), and mid zygotene (MZ) in untreated and colchicine-treated disomic and monosomic wheat-5RL additions.

Stage	Addition	Treatment	Centromere signals (range)	No. of cells
PI	disomic	control	19.87±0.34 (13-28)	75
	disomic	colchicine	19.32±0.34 (12-33)	110
EL	monosomic	control	16.30±0.38 (11-22)	50
	monosomic	colchicine	16.50±0.38 (10-26)	103
	disomic	control	15.09±0.38 (11-20)	75
	disomic	colchicine	16.38±0.42 (8-26)	68
LLEZ	monosomic	control	17.90±0.39 (13-22)	42
	monosomic	colchicine	17.55±0.39 (9-29)	56
	disomic	control	17.76±0.35 (12-23)	48
	disomic	colchicine	17.75±0.66 (11-29)	19
MZ	disomic	control	19.64±0.62 (16-27)	48
	disomic	colchicine	18.89±0.62 (15-24)	45

Table 2. Mean number of telomere aggregates and small signals with one or two telomeres and their area of distribution (<1/3, 1/3-2/3 and >2/3 of the projected nucleus) in cells at premeiotic interphase and early leptotene of monosomic and disomic wheat-5RL additions.

Addition	Treatment	Telomere aggregates	Small signals	Nuclei (%) with signals in surface			No. of cells
				<1/3	1/3-2/3	>2/3	
Premeiotic interphase							
Disomic	control	0	80.0±3.3	0	47.1	52.9	75
Disomic	colchicine	0	69.4±2.7	0	47.3	52.7	110
Early leptotene							
Monosomic	control	3.7±0.6	10.7±1.2	80.4	19.6	0	46
Monosomic	colchicine	6.4±0.4	21.5±1.2	13.8	56.4	29.7	101

Table 3. Number of cells with 0, 1 or 2 rye telocentromeres in the telomere cluster and their association frequency (%) at early leptotene (EL), late leptotene-early zygotene (LLEZ), and mid zygotene (MZ) in untreated cells of the disomic wheat-5RL addition.

Stage	Rye centromeres at the telomere pole					
	0	(% associated)	1	(% associated)	2	(% associated)
EL	60	(28.3)	10	(0.0)[a]	5	(40.0)
LLEZ	19	(31.6)	9	(0.0)[a]	20	(50.0)
MZ	19	(63.2)	3	(0.0)[a]	26	(80.8)

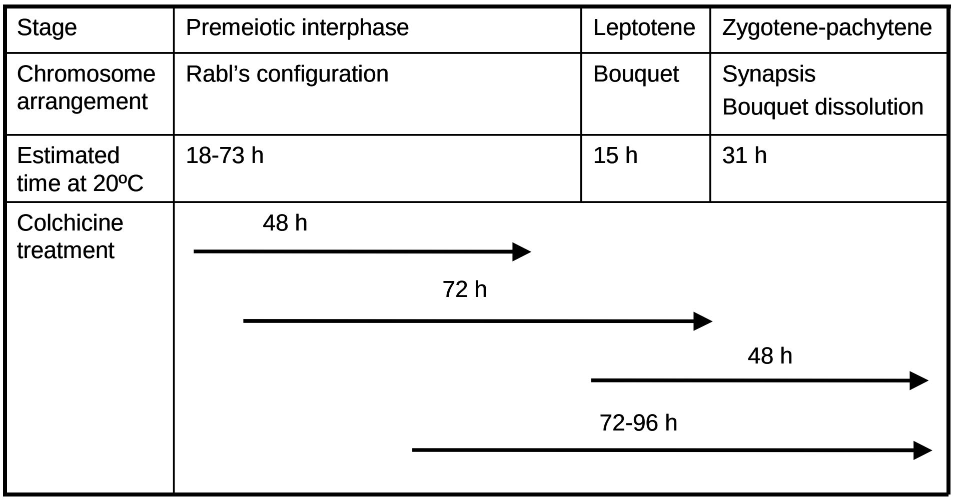
[a] The two rye centromeres were separated since only one had reached the telomere pole.

Table 4. Mean length in µm of chromosome domain 5RL at premeiotic interphase and pachytene in control and colchicine-treated cells of the wheat-5RL addition. Number of cells analysed in parentheses.

Treatment	Premeiotic interphase	Pachytene
Control	29.18±1.97 (21)	76.97±6.91 (17)
Colchicine	30.40±2.23 (15)	89.99±7.30 (15)

Table 5. Number of cells (%) at pachytene with asynapsis, partial synapsis, or complete synapsis between the rye homologues in a disomic wheat-5RL addition.

Configuration	Time of colchicine treatment prior to pachytene		
	0 h	48 h	72-96 h
Asynapsis	0 (0.0)	0 (0.0)	4 (3.8)
Partial synapsis	23 (31.1)	37 (24.7)	58 (55.2)
Complete synapsis	51 (68.9)	113 (75.3)	43 (40.9)
Total	74	150	105



Stage	Premeiotic interphase	Leptotene	Zygotene-pachytene
Chromosome arrangement	Rabl’s configuration	Bouquet	Synapsis Bouquet dissolution
Estimated time at 20ºC	18-73 h	15 h	31 h
Colchicine treatment	48 h 72 h 48 h 72-96 h		

Figure 1

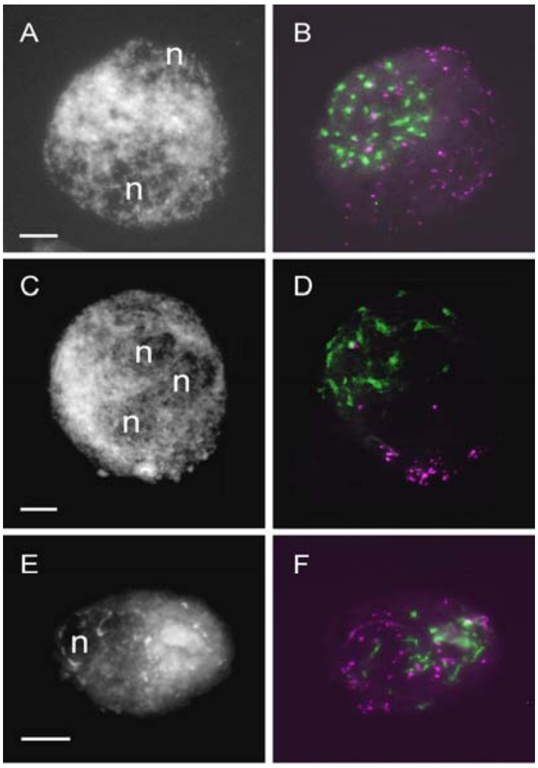


Figure 2

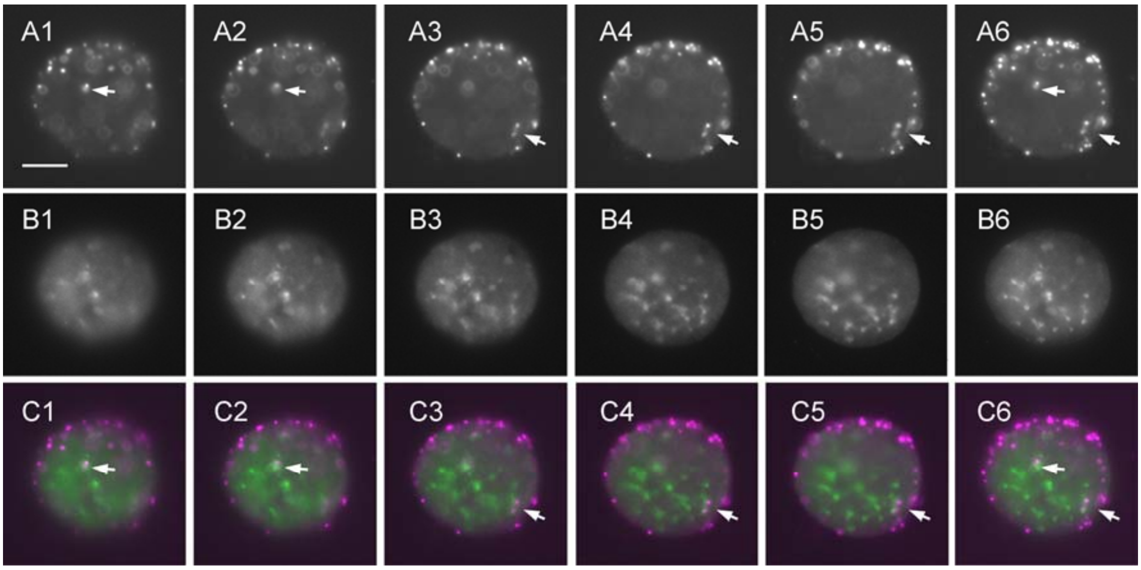


Figure 3

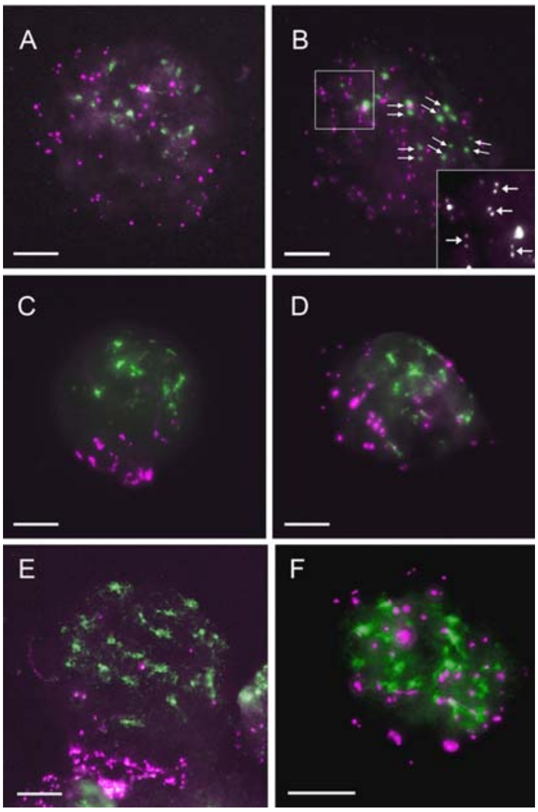


Figure 4

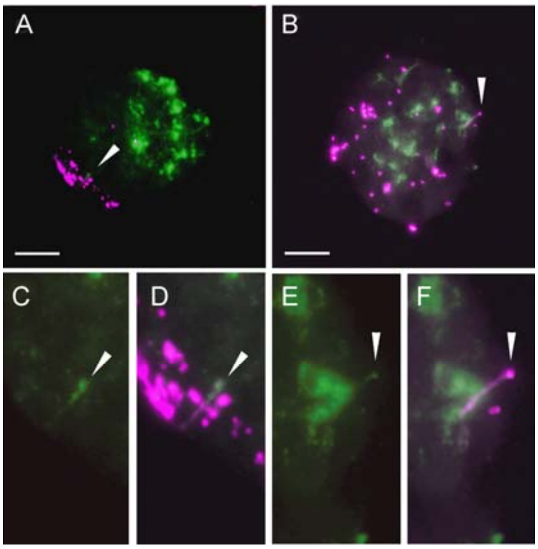


Figure 5

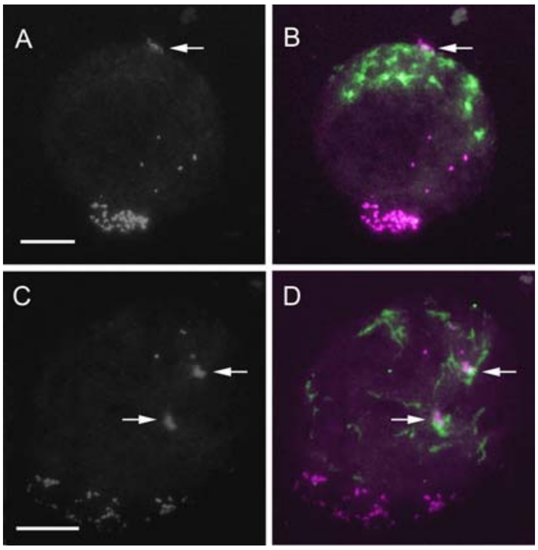


Figure 6

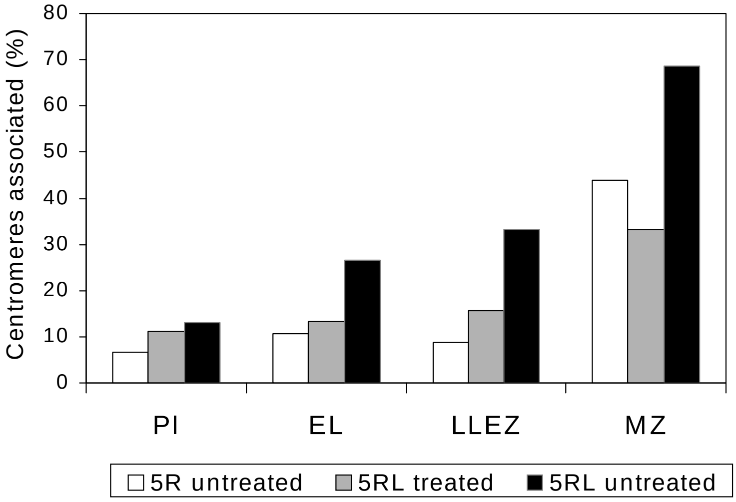

Centromeres associated (%)
0
10
20
30
40
50
60
70
80
PI
EL
LLEZ
MZ
□ 5R untreated
▨ 5RL treated
■ 5RL untreated

Figure 7

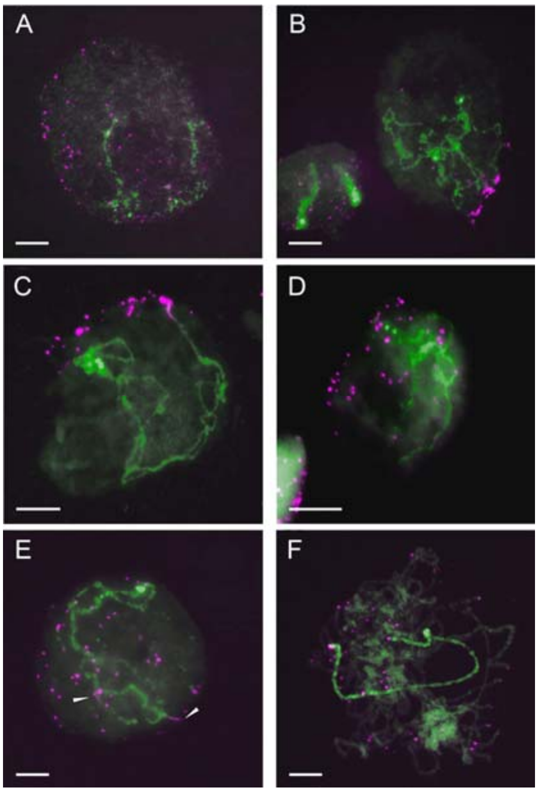


Figure 8

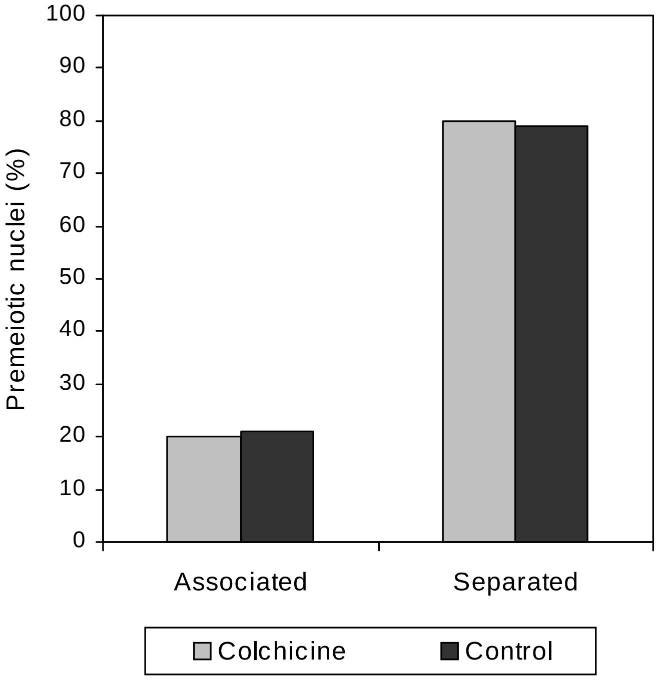

100
90
80
70
60
50
40
30
20
10
0
Premeiotic nuclei (%)
Associated
Separated
Colchicine
Control

Figure 9

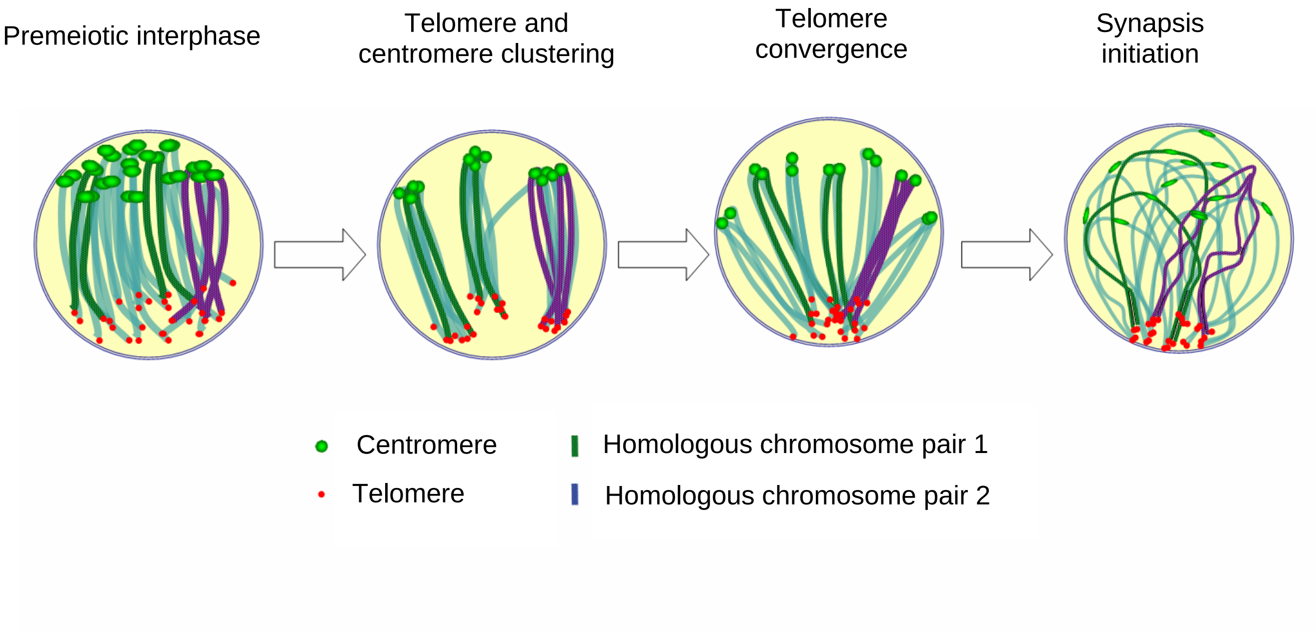

Premeiotic interphase
Telomere and centromere clustering
Telomere convergence
Synapsis initiation
● Centromere
• Telomere
▮ Homologous chromosome pair 1
▮ Homologous chromosome pair 2

Figure 10