Meiotic telomere clustering is inhibited by colchicine but does not require cytoplasmic microtubules

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Summary

Telomere clustering, the defining feature of the bouquet, is an almost universal feature of meiotic prophase, yet its mechanism remains unknown. The microtubule-depolymerizing agent colchicine was found to inhibit bouquet formation. Telomeres in colchicine-treated cells remained scattered in the nuclear periphery, whereas untreated cells exhibited a prominent telomere cluster. Colchicine administered after the bouquet had formed did not affect telomere dispersal. The effect of colchicine on bouquet formation appeared to be separable from its effect on cytoplasmic microtubules; amiprophos methyl, a highly effective plant microtubule-depolymerizing drug, did not affect telomere clustering. Inhibition of bouquet formation was limited to colchicine and the related drug podophyllotoxin out of the variety of microtubule-depolymerizing drugs tested, suggesting that the target involved in bouquet formation has a structural specificity.

Key words: Bouquet, Telomere cluster, Meiosis, Microtubules, Colchicine, Rye

Introduction

Meiosis is a highly conserved process that occurs in all sexually reproducing organisms. Pairing and synapsis of homologous chromosomes followed by their recombination and subsequent chiasmata formation ensures the bipolar attachment of homologous chromosomes required for the reductional division of meiosis I. Prior to pairing, homologous chromosomes appear to be unpredictably positioned relative to each other and separated by a distance of up to one nuclear diameter. In a large meiotic nucleus, such as the ones in oat, this distance can be over 25 μm (Bass et al., 2000). Homologous pairing culminates in the formation of the synaptonemal complex, which occurs when homologs are separated by approximately 300 nm (Loidl, 1990; Westergaard and von Wettstein, 1972). A mechanism is required to bring about homologous chromosome juxtaposition and alignment, in turn enabling short-range events such as synapsis and recombination to occur.

One feature of the prophase movement of chromosomes is the formation of the meiotic bouquet, which is defined by the aggregation of telomeres on a small region of the nuclear envelope (Cowan et al., 2001; Scherthan et al., 2001; Zickler and Kleckner, 1998). Several hypotheses have been proposed to explain how this clustering contributes to homologous chromosome pairing. For example, the active movement of telomeres might promote the movement of adjacent chromosomal regions, ‘stirring’ (Maguire, 1974) the contents of the meiotic nucleus and increasing the probability of chromosome contacts. Alternatively, telomere clustering might promote homologous interactions by juxtaposing the subtelomeric regions of all chromosomes (Scherthan, 2001)

The mechanism of bouquet formation is not known. The bouquet appears to form in two-steps; telomeres first attach to the nuclear envelope at dispersed sites and subsequently migrate along the nuclear envelope to form the bouquet (Gelei, 1921; Rasmussen and Holm, 1980; Scherthan et al., 1996; Wilson, 1925). Telomeres attach to the inner nuclear envelope via specialized terminal modifications of the axial elements, protein structures that run along the longitudinal axis of unpaired meiotic chromosomes, as revealed by electron microscopic analyses in several animals (Esponda and Gimenez-Martin, 1972; Moens, 1969) and in a few higher plants (Holm, 1977). Mutants that block bouquet formation in either fission or budding yeast appear to affect steps prior to telomere clustering, but their analysis has not yet lead to a dissection of the mechanism of telomere clustering (Cooper et al., 1998; Nimmo et al., 1998; Trelles-Sticken et al., 2000). Recombination proteins are not required for the meiotic bouquet (Trelles-Sticken et al., 1999), and numerous desynaptic mutants display a normal bouquet (Havekes et al., 1994) (I. Golubovskaya and W.Z.C., unpublished). Additionally, bouquet formation occurs in haploid cells (de Jong et al., 1991; Santos et al., 1994; Wang, 1988).

Extensive data accumulated over the last 60 years indicate that the drug colchicine induces marked defects during meiotic prophase. Treatment of meiotic tissues with colchicine reduces the frequency of chiasmata (Barber, 1942; Driscoll and Darvey, 1970; Shepard et al., 1974) and impairs synaptonemal complex formation (Loidl, 1989; Tepperberg et al., 1997). Colchicine interferes with microtubule dynamics, promoting microtubule depolymerization in cells (Wilson and Jordan, 1994). It is not known whether the inhibitory effects of colchicine on meiotic prophase events are caused by microtubule depolymerization in the highly specialized meiotic cell. Experiments attempting to deduce the colchicine-sensitive period have pointed to premeiotic interphase [wheat (Driscoll and Darvey, 1970)],

early leptotene [garlic (Loidl, 1989)] and zygotene [lily (Shepard et al., 1974)].

Because of the proposed role of the bouquet in homolog juxtaposition and the colchicine-sensitive period coincident with the bouquet stage in some organisms, it is possible that the colchicine-sensitive process is bouquet formation. A failure of telomere clustering may result in unpaired chromosomes and consequently reduce both synapsis and recombination. This hypothesis is supported by evidence demonstrating that an isochromosome paired and synapsed normally in the presence of colchicine, although the remainder of chromosomes remained unpaired (Driscoll and Darvey, 1970; Driscoll et al., 1967; Loidl, 1989). The isochromosome, two homologous arms connected at the centromere, was postulated to need no active juxtaposition of homologous regions, as they are physically connected. It was concluded that only the movement of homologs into close proximity is sensitive to colchicine (Driscoll and Darvey, 1970). Although these and other experiments give intriguing hints that colchicine may affect the bouquet (see Zickler and Kleckner, 1998) there is no direct evidence to support this conclusion.

We have developed a new method of culturing *Secale cereale* (rye) anthers, the male reproductive organs of the plant, which allows temporal analysis of meiotic prophase and manipulation of meiotic events by drug treatments. Rye was chosen for our investigation because its chromosomes contain large blocks of heterochromatin in their subtelomeric regions (Lima de Faria, 1952), allowing easy identification of telomeres and thus the bouquet. In addition, *S. cereale* has readily identifiable meiotic stages through its chromatin morphology (Darlington, 1933). We show here that *S. cereale* meiocytes undergo normal bouquet formation in anther culture. To gain further insight into the mechanism of telomere clustering, we treated rye anthers in culture with microtubule-depolymerizing drugs. We demonstrate that the presence of normal cytoplasmic microtubule arrays is neither necessary nor sufficient for bouquet formation. We tested several drugs and found that the clustering of telomeres on the nuclear envelope is uniquely inhibited by colchicine and its close chemical relative, podophyllotoxin. Since colchicine's effect of inhibiting bouquet formation can be separated from its effects on cytoplasmic microtubules, the colchicine-sensitive target associated with telomere clustering may be a novel protein or a tubulin-related protein distinct from β-tubulin.

Materials and Methods

Growth of *S. cereale* plants

Secale cereale cv. 'Blanco' plants were grown in the greenhouse (Berkeley, CA). Spikes containing flowers were used within 1.5 hours of harvesting. Anthers were dissected out of their florets on a dry glass microscope slide and transferred into culture medium within 1 hour. Only the pedicilate floret was used in all experiments.

Anther culture

Preliminary anther culture experiments were performed on intact anthers. We were able to bisect the anthers longitudinally prior to placing them in culture medium, thereby increasing the starting number of synchronous meiocyte populations to six. There was no difference in the success rate of anther cultures performed with whole or halved anthers, and all subsequent experiments were performed on anther halves.

Upon removing anthers from the floret, the three anthers were sequentially cut down the connective tissue joining the locules, giving rise to two anther halves, each consisting of two locules. Upon bisecting an anther, the two halves were immediately placed into culture medium. Anther culture and fixations were performed in flat-bottom 96-well plates, using 50 μl solution per well, and one anther half per well. After all anther sets had been placed into wells, 96-well plates were placed on a rotary shaker at 80 rpm and covered to keep the dishes dark. After the specified culture time at room temperature, anthers were fixed as described below. Anther culture medium consisted of 1× Murashige and Skoog medium (GIBCO), 3% sucrose, and 1× Murashige and Skoog vitamins (Sigma) at pH 7.

Duration of meiotic stages in vivo

Bennett et al. determined the duration of meiotic stages for *S. cereale* anthers in vivo (Bennett et al., 1971). Anther samples were excised from the plant over a time course and the meiotic stage determined cytologically. These experiments, however, were performed on plants growing at 20°C, whereas our anther culture experiments were conducted at room temperature (25°C) and are thus not directly comparable. In earlier work, Bennett et al. determined the duration of meiosis for *S. cereale*, although not the substages, at several temperatures (Bennett et al., 1972). Additionally, Bennett reported that in the angiosperms, changes in the total duration of meiosis generally result from proportional changes in all of the meiotic substages (Bennett, 1977). To compare our timing of leptotene with the work of Bennett et al. (Bennett, 1971), we made the following calculations on the basis of the above data. At 20°C, *S. cereale* meiosis lasted 2.12 days, whereas at 25°C, meiosis lasted 1.63 days (Bennett et al., 1972); thus meiosis proceeds 1.3 times slower at the lower temperature. Bennett et al. report that leptotene occurs over 20 hours at 20°C (Bennett et al., 1971). By dividing this value by the temperature-dependence ratio above (1.3), we would expect leptotene to last roughly 15.4 hours at 25°C.

Drug treatments

Initial drug concentrations were determined from values reported in the literature. Concentrations were tested in a spindle-function assay in cultured anthers. Either tapetal cell nuclear divisions (which occur when pairing is near completion) or meiosis I or II divisions were observed for accurate chromosome alignment (metaphase) and segregation (anaphase/telophase) by DAPI staining. Spindle function was used to determine the time required for drugs to exert their effects. Colchicine and amiprophos methyl (APM) resulted in failed mitosis after 3 hours of treatment (Table 1); all treatments were performed for a minimum of 6 hours.

All drugs were stored as 100× stocks in DMSO at –20°C. Colchicine (Sigma) stocks ranged from 1 mM to 500 mM. For inhibition of telomere clustering, 100 μM was consistently used. Efficient MT depolymerization required at least 1 mM colchicine. β- and γ-lumicolchicine (Sigma), vinblastine sulfate (CalBiochem), podophyllotoxin (Sigma), trimethoxybenzene (ACROS) and 2-methoxy estradiol (Sigma) were prepared as 10 mM stocks. APM was a gift of L. Morejohn (University of Texas, Austin) and stored as a 2.5 mM stock. Drugs were diluted 1:100 in anther culture medium immediately prior to use. Control medium contained 1% DMSO.

Fixation

For fluorescent in situ hybridization (FISH) experiments, anthers were fixed for 1 hour in 4% paraformaldehyde in 1× buffer A (80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 15 mM PIPES, 0.35 M

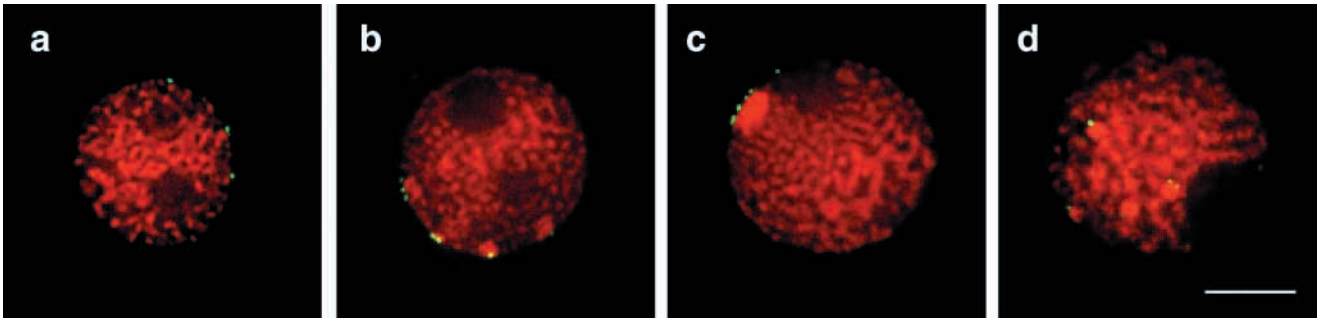

Fig. 1. Meiotic prophase stages and timing of the bouquet in *Secale cereale* cv. 'Blanco'. Telomeres (green) were detected by FISH using a probe to the telomere repeat, and chromatin (red) was stained with DAPI. Single optical sections of meiotic nuclei are shown. (a) Premeiotic interphase, (b) leptotene and (c) zygotene. The bouquet is evidenced by the close spacing of the telomere FISH signals. (d) Pachytene. Bar, 10 μm.

sorbitol, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, pH 7.0) at room temperature. Anthers were subsequently washed in 1× buffer A and stored at 4°C.

For MT fixation, anthers were fixed in 8% paraformaldehyde in 1× PHEMS (60 mM PIPES, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, 0.32 M sorbitol, pH 6.8) for 2 hours at room temperature (Chan and Cande, 1998). Anthers were subsequently washed in 1× PHEMS and stored at 4°C.

FISH procedure

Meiocytes and associated cells from a single anther or anther half were embedded in 5% acrylamide polymerized between two coverslips. FISH protocols were essentially as described previously (Bass et al., 1997): prehybe I (1×SSC, 1×buffer A, 20% formamide), prehybe II (2×SSC, 35% formamide), prehybe III (2×SSC, 50% formamide). Coverslips were then incubated in hybridization solution (prehybe III plus approximately 200 ng probe) for at least 30 minutes (Bass et al., 1997). Coverslips were heated 95°C for 5.5 minutes, transferred to a humid chamber for incubation overnight at room temperature. Coverslips were washed in 1×PBS. Chromatin was stained with 3 μg/ml DAPI in 1×PBS. Coverslips were mounted in glycerol. Telomeres were detected using a probe to the telomere repeat (CCCTAAACCCTAAACCCTAAACCCTAAA) with either 5′ Cy-5 or FITC label (Genset, Paris).

Microtubule immunofluorescence

Meiocytes and associated cells were embedded in 5% acrylamide polymerized between two coverslips. Cell walls were then digested with 1.5% β-glucuronidase (from *Helix promatia*, Sigma) in 1×PBS for 15 minutes at 36°C. Coverslips were washed thoroughly with 1×PBS. A monoclonal antibody against sea urchin α-tubulin (a gift of D. Asai, Purdue University) was applied in 1×PBS at 1:500 dilution and incubated at room temperature overnight. Coverslips were washed in 1×PBS. Secondary antibody, Alexa488-conjugated goat anti-mouse IgG (Molecular Probes) at 1:50 dilution, was applied in 1×PBS and incubated overnight at room temperature. Coverslips were washed in 1×PBS. Chromatin was stained with 3 μg/ml DAPI, and samples were mounted in glycerol.

Microscopy

All images were acquired with an Applied Precision DeltaVision microscope system equipped with an Olympus IX70 inverted microscope. A 40× 1.35 NA UApo oil immersion lens was used for all experiments. Cells were imaged in three dimensions (xyz); z-axis sections were collected at 0.2 μm spacing. Images were deconvolved using a standard conservative algorithm (Chen et al., 1995).

Modeling and quantification

Models of nuclei were created using the DeltaVision/softWoRx 3DModel program. The nuclear periphery was modeled on the outer edge of DAPI-stained chromatin. A single point was picked for each telomere detected by FISH; we attempted to pick the peak intensity of the signal. 3DModel data were imported into MATLAB (version 5.1.0.420, The MathWorks, Inc.) for analyses. Two measurements were calculated to determine the degree of telomere clustering: (1) all pairwise telomere-to-telomere distances, which are referred to as telomere distances; and (2) the minimum of all possible telomere-to-telomere distances for each telomere, which are referred to as nearest neighbor distances. Nearest neighbor distances were used to judge small changes in telomere distributions. All distance measurements were normalized to the nuclear radius.

Telomere and nearest neighbor distances are presented as the distribution of means from individual nuclei in a given sample. All possible distances were calculated for a single nucleus, the mean of the distances for a single nucleus was obtained, and the mean distances from all nuclei in a sample were combined. Distributions (box-whisker plots) or means (*t*-test) are presented. Sample means (the mean of the mean distances) are described plus or minus the standard deviation. Differences were assessed at 99.9% confidence (P=0.001) using an unequal variance Student's *t*-test, unless indicated otherwise.

Results

Telomeres are tightly clustered in the bouquet during zygotene in *Secale cereale* cv. 'Blanco'

We first established guidelines for classifying the early meiotic stages in *S. cereale* cv. 'Blanco' using our fixation, staining and imaging procedures. Meiotic stages were defined by chromatin appearance in accordance with classic definitions (Wilson, 1925; Zickler and Kleckner, 1998) (Fig. 1). We found that chromatin morphology correlated closely with telomere distribution, nucleolar number and nuclear diameter throughout meiotic prophase. During the stages we examined, cells from a single anther were synchronous with respect to meiotic stage (Fig. 2b), as is true of most grasses, and thus the general descriptions provided are representative of all of the meiotic cells observed.

Premeiotic interphase nuclei (Fig. 1a) have uncondensed chromatin, unfused nucleoli and polarized but dispersed telomeres. Telomere polarization, perhaps indicative of a Rabl chromosome configuration, was suggested by telomeres that resided in one nuclear hemisphere and were located in the

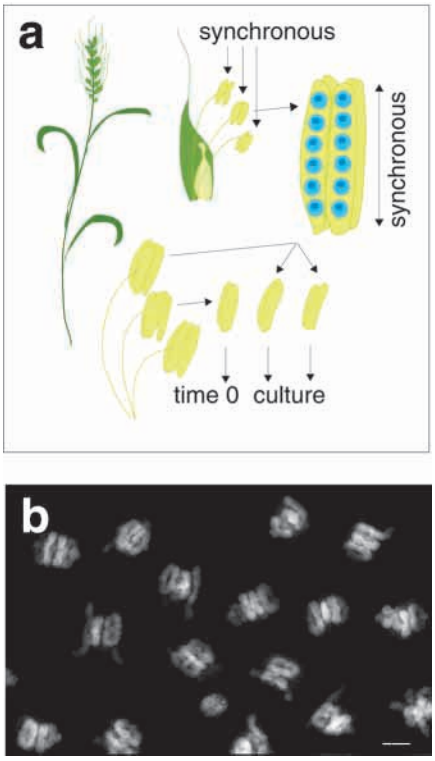

Fig. 2. (a) Diagram of rye plant and meiotic development and anther culture methodology. A single rye floret contains three anthers at an equivalent developmental stage. Meiosis occurs synchronously within a single anther and among the three anthers from one floret. The anthers are shown in yellow-green, and meiotic cells are depicted in blue. The meiotic cells cannot be observed through the anther wall; the diagram merely suggests their location. One anther half provides the time 0 control, whereas the halves of a second anther are put into culture. (b) Synchrony of meiosis in rye anthers. A projection of a 3D image of meiotic cells from a single anther. All cells appear to be in metaphase of the first meiotic division, as indicated by the presence of seven bivalents. Bar, 10 μm.

nuclear periphery. Premeiotic nuclei could be distinguished from somatic nuclei in the anther because the diameter of the premeiotic nuclei was several micrometers greater than the diameter of somatic nuclei. Nuclei were defined as leptotene if they contained visible chromatin threads (Fig. 1b) of irregular widths. Telomeres were scattered in one half of the nuclear periphery in leptotene nuclei and nucleoli were unfused.

Nuclei were classified as zygotene if they contained two types of chromatin fibers (Fig. 1c): one regular chromatin thread with clearly visible edges, which is likely to represent the paired homologous chromosomes and a second fuzzy-edged chromatin thread of irregular width, similar to leptotene chromosomes. Fully clustered telomeres were found predominantly during zygotene. The delineated chromosome threads were located in the nuclear hemisphere containing the bouquet, whereas the leptotene-like chromatin threads were found away from the telomere cluster site. This is a classic example of zygotene (Wilson, 1925), the chromatin thread morphologies most probably representing paired and unpaired chromosome territories. Nucleolar fusion appeared to take place during zygotene; nucleolar number varied during this stage even among meiotic cells from a single anther.

In pachytene nuclei (Fig. 1d), the chromosome threads were distinct throughout the nucleus, being both of equal width and regularly spaced. The telomere cluster was dispersed in pachytene, and telomeric heterochromatin was barely distinguishable from the rest of the chromosome, probably due to continued chromatin condensation.

In our analyses of meiosis in *S. cereale* cv. 'Blanco', we established that the bouquet is present during zygotene (Fig. 1c), as defined by the appearance of the chromosome fibers. Classic examples of the bouquet in organisms such as lily and salamander as well as more recent studies using model genetic organisms such as *Saccharomyces cerevisiae* and maize demonstrate that the telomere cluster is generally seen during zygotene (Scherthan, 2001). However, Mikhailova et al., studying a different inbred line of rye, place the formation of the telomere cluster in premeiotic interphase (Mikhailova, 2001). It is possible that either strain variations or the different fixation methods employed could account for this temporal discrepancy within *S. cereale*.

Telomere clustering can proceed in an in vitro anther culture system

There are three anthers in each *S. cereale* floret (Fig. 2a), and each single anther contains several hundred meiotic cells. In rye, as in many grasses, the meiotic cells of a single anther are developmentally synchronous throughout meiosis (Fig. 2b); the three anthers of one floret are also meiotically synchronous (Fig. 2a). To establish the starting stage, one anther half was fixed immediately (time 0). The remaining anther halves were allowed to progress through meiosis in culture.

Experiments were initially evaluated for success by examining the chromatin morphology at time 0 and after various time periods of in vitro culture. Experiments were assessed for progression in meiotic stage between time 0 and the end of the culture period. Nuclei were scored for chromatin appearance, nucleolar number and relative nuclear diameter. Additionally, mitosis in the tapetal cells was monitored.

Indicators of unsuccessful anther culture were micronuclei, collapsed or shrunken nuclei, burst nuclei or aberrant chromatin appearance, most often decondensed chromatin. Distinguishing successful anther culture experiments was straightforward, as gross abnormalities were easily detected at the light microscope level. Anthers that were cultured for longer than 36 hours very rarely yielded normal-appearing nuclei in preliminary experiments; hence all experimental results described here were from experiments of 24 hours or less in duration. Since meiosis in *S. cereale* is more than one and a half days in duration at 25°C (Bennett et al., 1971), we were unable to obtain a complete meiotic sequence in any one experiment. However, the interval from premeiotic interphase to zygotene could be completed within 10-16 hours in our in vitro culture system (for example, Figs 3 and 6) (C.R.C. and W.Z.C., unpublished), and thus bouquet formation could be studied readily in cultured anthers.

Over the course of 95 experiments using optimized medium conditions and culture periods less than 24 hours, 29 successful experiments were performed, giving a success rate of approximately 30%. Successful anther culture experiments accurately approximated in vivo meiotic progression. Meiotic chromosome morphology of cells from cultured anthers

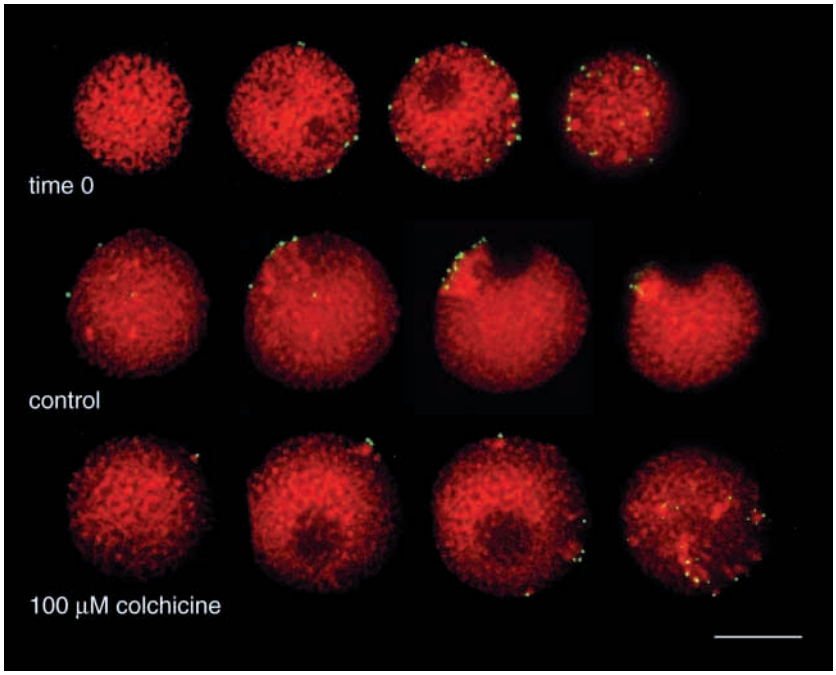

Fig. 3. The effect of colchicine on telomere clustering. To convey the 3D architecture of the cell, the data corresponding to the entire nuclear volume (approximately 100 z-sections) was divided into quarters, and each quarter was projected into a single image (Bass et al., 1997). The resulting images are displayed sequentially. Control and 100 μM colchicine-treated anthers were cultured for 16 hours. Telomeres (green) were detected by FISH using a probe to the telomere repeat, and chromatin (red) was stained with DAPI. Bar, 10 μm.

appeared to be identical to that of meiotic cells from anthers taken directly from the plant (compare Fig. 3 control with Fig. 5 time 0). Meiotic cells of a single cultured anther (or anther half) remained synchronous throughout the culture period, evidenced both cytologically (data not shown) and by the small standard deviations for telomere distance measurements (discussed below). Failed anther culture experiments may result from mishandling of the tissue, leading to damage and general necrosis, or may be correlated with some unknown environmental factor, such as growth conditions in the greenhouse that affect general anther health. The failure of meiotic progression in such cases appears to be an all or nothing response, affecting the entire meiotic cell population of an anther as opposed to a subset of cells. Our anther culture experiments suggested that the interval between premeiotic interphase and the bouquet stage, corresponding to leptotene, could be completed in 10-16 hours (see above). Importantly, the duration of leptotene we determined is in agreement with that obtained previously (Bennett et al., 1971) for the duration of *S. cereale* meiotic stages in vivo (15.4 hours; see Materials and Methods).

The drugs we initially tested in our investigation are known MT-depolymerizing agents, and monitoring spindle function provided a straightforward means of testing drug effectiveness. Anthers were split and the halves were placed in control or drug-containing medium. After 0.5, 1, 2, 3, 4 or 5 hours in culture, anthers were fixed and examined for indications of MT function. Colchicine and APM inhibited cell division after 3 hours of culture, as indicated by the failure of metaphase chromosomes to align in the spindle midzone or undergo anaphase chromosome separation in the drug-treated cells (Table 1). Control cells either exhibited normal metaphase alignment or had already completed division (Table 1).

Colchicine inhibits meiotic telomere clustering

We investigated the effect of colchicine on bouquet formation. Colchicine is an effective MT depolymerizing agent in plants at millimolar or greater concentrations (Morejohn, 1991), while at 100 μM, its effects on MTs are less pronounced or undetectable (Liu et al., 1995). Previous experiments examining the effect of colchicine on meiotic prophase have used high colchicine concentrations (1-25 mM), which have the potential of toxic side effects. We assessed the concentration dependence of the effect of colchicine on bouquet formation in the range from 25 μM-5 mM (data not shown). Colchicine was effective in inhibiting telomere clustering at concentrations as low as 25 μM in anther culture, although only a subset of cells were affected. All meiotic cells in an anther were affected at concentrations greater than 75 μM, and thus 100 μM colchicine was used in all subsequent experiments examining bouquet formation. Anthers containing premeiotic interphase or early leptotene cells were cultured in colchicine for 10-16 hours, allowing sufficient time for

Table 1. Time required for drug effectiveness in untreated and treated anther co-culture

Time (hours)	Control (meiotic stage)	5 mM colchicine (meiotic stage)
0.5	Telophase II	Telophase II
1	Telophase I	Anaphase/telophase I
2	Dyads	Dyads
3	Metaphase I	Disorganized metaphase
4	Tetrads	Disorganized metaphase
5	Metaphase I	Disorganized metaphase
Time (hours)	**Control (meiotic stage)**	**25 μM APM (meiotic stage)**
0.5	Dyads	Dyads
1	Dyads	Dyads
2	Metaphase II	Dyads
3	Metaphase I	Disorganized metaphase
4	Tetrads	Dyads
5	Tetrads	Dyads

The effect of microtubule-depolymerizing drugs on the meiotic divisions was used to assess the time required for the drugs to exert an effect on anther cultures. Single anthers were split longitudinally and the two resultant halves were placed into either control or drug-containing medium; anthers were co-cultured for 0.5, 1, 2, 3, 4 and 5 hours. After fixation, chromatin was stained with DAPI, and the nuclei were viewed by fluorescence microscopy. The meiotic division stage was assigned on the basis of chromosome configurations and cellular architecture. Only one example is described for each time point, although several examples were obtained. Dyads are the products of the first meiotic division; tetrads are the products of the second meiotic division. Disorganized metaphase refers to metaphase-appearing chromosomes that are not aligned on a metaphase plate, a C-meiosis.

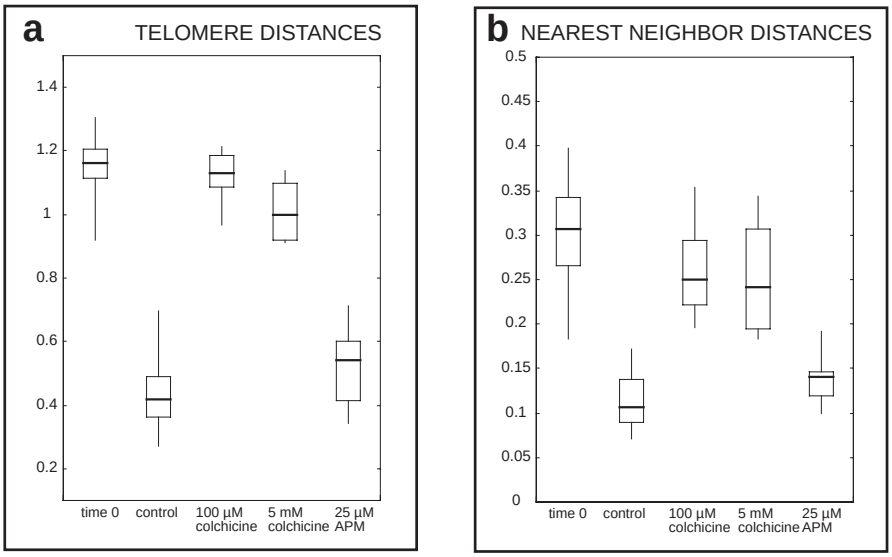

Fig. 4. Telomere distributions in colchicine-treated nuclei. Box-whisker plots of the (a) mean telomere distances and (b) nearest neighbor distances are shown. Time 0, *n*=26; control, *n*=37; 100 μM colchicine, *n*=20. Boxes represent the second and third quartiles (50th and 75th percentiles); the bold line through the box is the median, and whiskers extend to the range.

bouquet formation (Fig. 3). At the beginning of these experiments, all meiotic cells exhibited dispersed telomeres. The telomeres were associated with the nuclear periphery and were polarized within the nucleus. At the end of the culture period, untreated nuclei showed a prominent telomere cluster, indicated by closely associated telomere FISH signals. Nuclei treated with either 5 mM or 100 μM colchicine did not form a bouquet; telomeres remained dispersed. The telomere distribution in colchicine-treated cells was similar to that observed in time 0 cells; the peripheral localization and polarization of telomeres within the nucleus were retained after colchicine treatment. The results were similar for both colchicine concentrations.

Telomere distributions in colchicine-treated nuclei were analyzed by calculating mean telomere and nearest neighbor distances (see Materials and Methods) (Fig. 4a,b). The telomere distance (Fig. 4a) in nuclei treated with 100 μM colchicine for 10-16 hours (1.13±0.07, *n*=20) was not significantly different from telomere distances in time 0 nuclei (1.15±0.09, *n*=26). The mean nearest neighbor distance (Fig. 4b), however, differed slightly but significantly between time 0 (0.30±0.05, *n*=26) and 100 μM colchicine-treated (0.26±0.05, *n*=20) nuclei after 10-16 hours, indicating that limited telomere juxtaposition occurred in colchicine-treated cells.

Colchicine does not inhibit telomere dispersal from the bouquet

To determine whether colchicine affected telomere dispersal as cells enter pachytene, anthers containing bouquet-stage cells were cultured with 100 μM colchicine for 14-18 hours, allowing sufficient time for telomere migration out of the bouquet (Fig. 5). Time 0 anthers showed a prominent telomere cluster. At the end of the culture period, both untreated and colchicine-treated nuclei were marked by dispersed telomeres. Thus, telomere movement resulting in bouquet dissolution did not appear to be affected by colchicine.

Depolymerization of cytoplasmic microtubules does not inhibit telomere clustering

We wished to determine whether colchicine's inhibitory effect on bouquet formation was a result of cytoplasmic MT depolymerization. The concentration of colchicine that exerts a maximal effect on bouquet formation (100 μM) was much lower than the concentrations commonly employed to affect MTs in plants (Morejohn, 1991), and cytoplasmic MT arrays were present in the meiocytes. However it was possible that these arrays were subtlety disturbed. Thus, we used APM, a highly effective MT depolymerizing agent in higher plants (Bajer and Mole-Bajer, 1986) and green algae (Collis and Weeks, 1978), to assess whether cytoplasmic microtubules were required for bouquet formation.

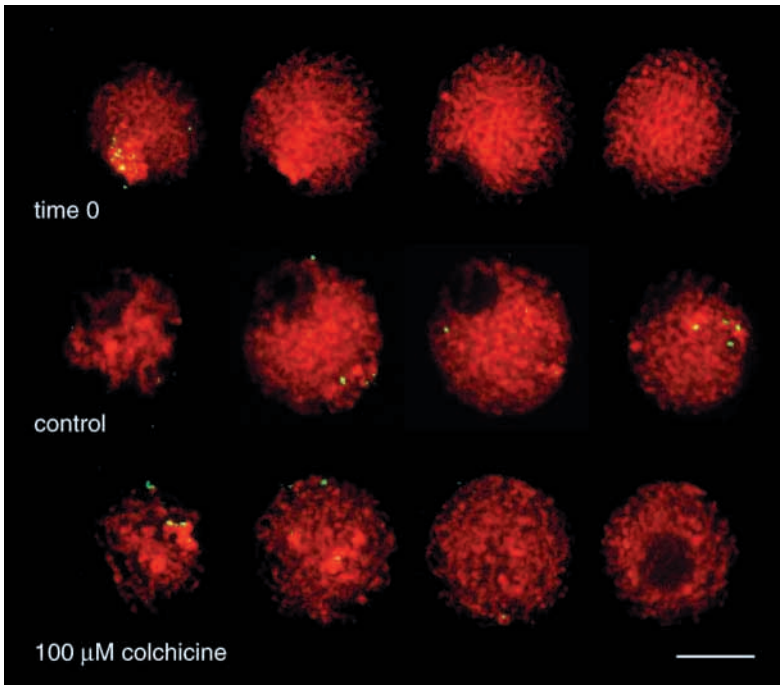

Fig. 5. The effect of colchicine on bouquet dispersal. Representative nuclei are displayed as sequential projections of the 3D nucleus as in Fig. 3. Control and 100 μM colchicine-treated anthers were cultured for 18 hours. Telomeres (green) were detected by FISH using a probe for the telomere repeat, and chromatin (red) was stained with DAPI. Bar, 10 μm.

To investigate the effect of MT depolymerization on telomere clustering, anthers in early meiotic prophase were cultured with 25 μM APM for 12-16 hours, allowing for meiotic progression (Fig. 6). Time 0 nuclei displayed unclustered telomeres; telomeres were distributed over approximately half of the nuclear surface. Telomeres were fully clustered in both untreated and APM-treated nuclei after culture (Fig. 6). To determine whether colchicine and APM depolymerized cytoplasmic MTs during meiotic prophase at the concentrations used, we observed the organization of MTs after 15 hours of treatment with colchicine or APM (Fig. 7). Untreated meiotic cells exhibited an array of MTs asymmetrically distributed around the nucleus (Fig. 7a). Cells treated with 5 mM colchicine (Fig. 7c) or 25 uM APM did not have visible MTs (Fig. 7b). Cells treated with 100 μM colchicine (Fig. 7d) appeared to contain a normal MT array, although a few cells within the sample (less than 10%) appeared to have aberrant MT distributions (data not shown). We conclude that cytoplasmic MT arrays are not required for telomere clustering. This suggests that the inhibitory effects of colchicine on bouquet formation may not be mediated through cytoplasmic MT depolymerization.

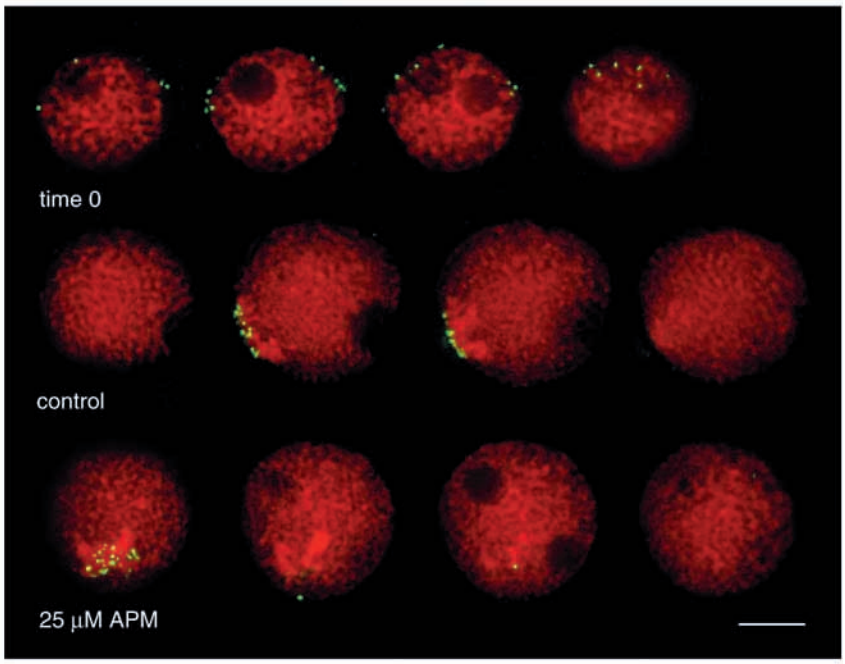

Fig. 6. The effect of APM, a plant-specific microtubule-depolymerizing agent, on telomere clustering. Representative nuclei are displayed as sequential projections of the three-dimensional nucleus, as in Fig. 3a. Control and 25 μM APM-treated anthers were cultured for 14 hours. Telomeres (green) were detected by FISH using a probe to the telomere repeat, and chromatin (red) was stained with DAPI. Bar, 10 μm.

Bouquet inhibition is specific to colchicine and podophyllotoxin

We tested compounds structurally related to colchicine and additional MT-depolymerizing drugs for effects on the bouquet. The selected drugs (Table 2) were used to address three questions. (1) Are the bouquet target and tubulin pharmacologically identical? The β-tubulin molecule contains several distinct binding sites for anti-mitotic drugs. Colchicine and podophyllotoxin bind to one site, whereas vinblastine binds to another site (Wilson and Jordan, 1994; Hamel, 1996). (2) Is the colchicine A-ring sufficient for bouquet inhibition? Colchicine and podophyllotoxin contain identical methoxybenzene rings, and we wished to determine whether this ring was the only requirement for bouquet inhibition. 3-methoxy estradiol and trimethoxybenzene share the conserved ring, and 3-methoxy estradiol binds weakly to the colchicine-binding site on β-tubulin (Hamel, 1996). (3) Do toxic colchicine side effects, for example, interactions with membranes, result in a failure of bouquet formation? Lumicolchicine is a non-MT binding derivative of colchicine, with similar lipophilic properties, which should act as a control for the potential membrane-disrupting actions of colchicine.

Anthers containing meiotic cells in early prophase were treated separately with the various drugs and cultured for 12-18 hours. Telomere distribution was determined by heterochromatin; MTs were judged by immunofluorescence, as described in Fig. 7. Time 0 anthers contained meiotic nuclei with dispersed telomeres. Untreated nuclei that exhibited clustered telomeres were compared with the drug-treated nuclei to determine the presence or absence of the bouquet and cytoplasmic microtubules. Of the drugs tested, we found that only colchicine and the related compound podophyllotoxin inhibited telomere clustering. The remaining drugs had no obvious effects on bouquet formation even though several of the drugs disrupted cytoplasmic microtubules (Table 2). Importantly, the non-MT depolymerizing derivatives of colchicine, β- and γ-lumicolchicine, did not affect telomere clustering, suggesting that lipophylic-based side effects are an unlikely cause of bouquet inhibition.

Discussion

Studies of bouquet formation have been hindered by the lack of an ideal experimental system. Mutants defective in telomere clustering are rare, and the organisms suited for in vitro studies are limited (Ito and Stern, 1967; Parvinen and Soderstrom, 1976). Isolated meiocytes have proven difficult to culture (Chan and Cande, 1998). There are difficulties in obtaining synchronous meiotic populations of cells, observing chromosome morphology in early meiotic prophase and/or detecting the bouquet. To avoid several of these complications, we have developed simple and effective in vitro conditions in which meiotic cells of explanted *S. cereale* anthers undergo normal meiotic development. Most previously described anther culture methods support haploid embryo development rather than maintenance of meiosis (Bhojwani, 1996). In our system, chromosome morphology and nuclear organization appeared normal after successful anther culture. Drugs elicited an effect after as few as three hours in culture, providing a means of manipulating meiotic prophase.

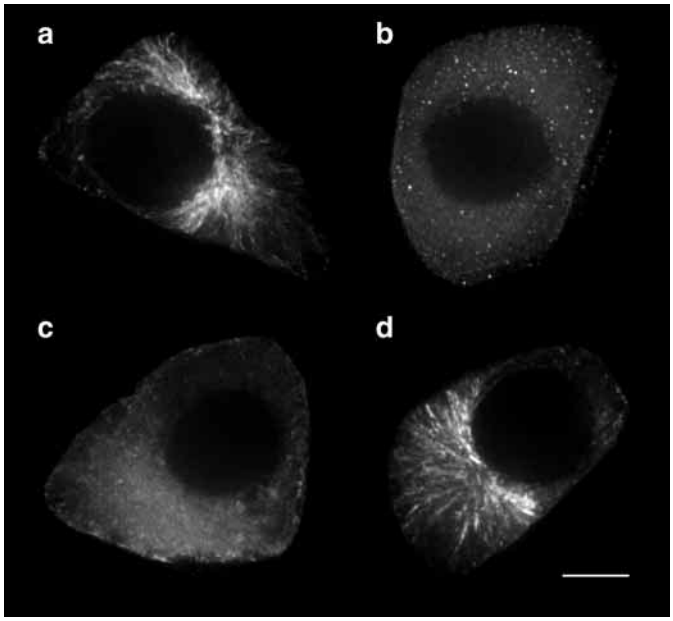

Fig. 7. The effect of colchicine and APM on cytoplasmic microtubule organization. MTs were detected using an antibody against sea urchin α-tubulin. The cells shown are of roughly equivalent meiotic stages. (a) Control, (b) 25 μM APM, (c) 5 mM colchicine and (d) 100 μM colchicine. Bar, 10 μm.

Our experiments demonstrate that telomere clustering is sensitive to colchicine, although the failure of telomere clustering cannot be a direct consequence of the drug's effects on the cytoplasmic MTs. MT depolymerization and bouquet inhibition showed distinct differences in their concentration-dependent response to colchicine. Bouquet inhibition was observed with concentrations of colchicine (100 μM) that did not appear to affect the cytoplasmic MT organization in the meiotic cell. This distinction is only possible because of the relative insensitivity of plant MTs to colchicine (Morejohn, 1991), and thus experiments in animals would be unlikely to reveal this important detail. Further evidence of the independence of telomere clustering and the MT cytoskeleton was revealed by the finding that APM and vinblastine sulfate did not inhibit telomere clustering at concentrations that lead to microtubule deploymerization.

Colcemid, a colchicine derivative, inhibits meiotic prophase chromosome movements (Salonen et al., 1982). Zygotene chromosomes in rat spermatocytes exhibit oscillatory and rotational movements (Parvinen and Soderstrom, 1976); however, these movements do not occur in the presence of colcemid (Salonen et al., 1982). The chromosome movement was unaffected by vinblastine. The drug sensitivity of zygotene chromosome movements in the rat is similar to our observations regarding the specificity of the inhibition of telomere clustering by colchicine-related drugs. This observation suggests that the colcemid-sensitive chromosome movements in rat spermatocytes at zygotene are likely to be related to the chromosome movements associated with telomere clustering.

Prior to our study, there was intriguing evidence that colchicine inhibited *premeiotic* telomere clustering in *S. cereale* (Bowman and Rajhathy, 1976). The fusion of large subtelomeric heterochromatic regions of the rye chromosomes was blocked in early premeiotic interphase by 5 mM colchicine treatment. The authors found, however, that bouquet formation was unaffected (Bowman and Rajhathy, 1976). The different fixation methods employed or alternative staging criteria might have lead to this discrepency. We detected no evidence of premeiotic telomere clustering in *S. cereale* (other than a persistent telomere polarization; Fig. 1) and conclude that colchicine exclusively blocked formation of the zygotene bouquet in our analyses.

Only colchicine and the related compound podophyllotoxin appeared to inhibit bouquet formation, whereas vinblastine and APM caused MT depolymerization but did not affect telomere clustering. Colchicine and podophyllotoxin have been shown to bind to the same site on β-tubulin, whereas vinblastine and APM bind elsewhere on the tubulin dimer (Morejohn, 1991; Wilson and Jordan, 1994). The non-MT binding derivatives of colchicine, β- and γ-lumicolchicine, and 2-methoxy estradiol and trimethoxybenzene, which share part of the ring structure of colchicine and podophyllotoxin, did not affect telomere clustering, suggesting that a specific colchicine-binding target with similarities to β-tubulin is required.

The results presented suggest the colchicine target involved in telomere clustering is not the cytoplasmic MT cytoskeleton. It is necessary to consider other potential targets. The similarity in specificity of colchicine for β-tubulin and the bouquet target suggests that the bouquet target may be a specialized non-MT tubulin, such as membrane-associated tubulin (Stephens, 1986) or a tubulin-related protein. Palmitylation of tubulin promotes tubulin association with membranes (Stephens, 1986); however, this process in vitro is inhibited by many drugs that promote microtubule depolymerization, including not only colchicine but also vinblastine and nocodazole (Caron, 1997). The drug sensitivity and the roles of the recently identified

Table 2. The effects of microtubule-depolymerizing drugs on telomere clustering and microtubule integrity

	Bouquet	Microtubules
Colchicine and structurally related compounds		
5 mM colchicine	–	–
100 μM colchicine	–	+
100 μM lumicolchicine	+	+
100 μM podophyllotoxin	-	+
100 μM trimethoxybenzene	+	+
100 μM 2-methoxy estradiol	+	nd
Other MT-depolymerizing drugs		
100 μM vinblastine	+	–
25 μM APM	+	–

A number of microtubule-depolymerizing drugs were tested for their ability to affect the telomere cluster. The effectiveness of many of the included drugs has not been tested in plants and thus concentrations employed were estimates on the basis of related compounds. + indicates that the telomeres clustered or that intact MTs were present and/or functional during chromosome segregation; – indicates that the bouquet was not observed or that MTs were depolymerized and/or non-functional during chromosome segregation. MTs were not investigated in 2-methoxy-estradiol-treated cells, indicated by 'nd'. The results of β- and γ-lumicolchicine treatments were the same and thus are described as 'lumicolchicine' in the table. For all drugs, at least 25 cells were assessed. The drug was scored as + or – if greater than 90% of the population showed the phenotype. Drugs were tested in a minimum of 10 experiments, with the exceptions of trimethoxybenzene and 2-methoxyestradiol, for which only two experiments were performed.

δ-, ε-, ζ- and η-tubulins (Dutcher, 2001) remain to be elucidated, especially in the context of the meiotic cell cycle. One of these proteins may be involved in bouquet formation and be sensitive to colchcine. Finally, it is possible that the target is not related to tubulin. Although colchicine disruption of non-microtubule targets and processes are usually also affected by lumicolchicine, Weiner et al. have recently demonstrated that colchicine but not lumicolchicine is an antagonist of gamma-amino-butyric acid (GABA) A receptor function (Weiner et al., 1998). This is an example of a non-tubulin target with drug-binding specificity similar to that of bouquet inhibition.

Colchicine-induced non-specific membrane damage or changes in nuclear architecture are unlikely to be the cause of bouquet inhibition. Although colchicine binds to cellular membranes at high concentrations (Stadler and Franke, 1974), the bouquet formed normally in lumicolchicine-treated cells, suggesting that the effect of colchicine was specific and not a result of its lipophilic properties. Bouquet dispersal proceeded normally in colchicine-treated cells, demonstrating that colchicine does not affect all telomere movements on the nuclear envelope. By light microcopy, there were no cytological indications of damage after colchicine treatment.

An early step in bouquet formation is a rearrangement of nuclear pores around the nuclear envelope. This has been demonstrated in a variety of meiotic cells, including mouse and human (Scherthan et al., 2000) and lily (Holm, 1977) cells. We have shown that the spatial redistribution of nuclear pores around the nuclear envelope that accompanies bouquet formation in *S. cereale* occurs normally in meiotic cells treated with 100 μM colchicine (Cowan et al., 2002). It appears, therefore, that colchicine targets a specific step in telomere clustering rather than other changes in nuclear envelope architecture that may accompany or be required for telomere movement

Telomeres in colchicine-treated nuclei were located in the nuclear periphery, and thus colchicine appeared to block the lateral movement of telomeres on the nuclear envelope rather than alter their association with the nuclear envelope. However, the persistent Rabl organization of *S. cereale* chromosomes and the large blocks of subtelomeric heterochromatin may predispose rye telomeres to reside near or on the nuclear envelope even in the presence of colchicine (Hochstrasser et al., 1986). Our finding that minimal telomere clustering appeared in colchicine-treated nuclei, as shown by the formation of telomere miniclusters and by the shift to smaller nearest neighbor distances (Fig. 4b), suggests that some telomere movement occurs despite the ultimate failure of bouquet formation. The formation of miniclusters, small groups of telomeres, was also observed in untreated leptotene nuclei (Fig. 1b). We suspect that minicluster forrmation observed in colchicine-treated cells represents an intermediate in bouquet formation. We have not determined whether the miniclusters are random telomere associations or reflect a step in homologous chromosome recognition.

In summary, we have demonstrated that colchicine specifically inhibits bouquet formation. Since colchicine has been shown in many different meitoic cells to disrupt the pairing of homologous chromosomes and their subsequent synapsis, our results strongly suggest that the clustering of telomeres on the nuclear envelope during meiotic prophase plays an essential role in homologous chromosome pairing. By bringing homologs into proximity with an appropriate alignment, the bouquet is likely to promote timely synapsis. The mechanism of telomere movement on the nuclear envelope during bouquet formation is unknown; however, our analyses demonstrate that bouquet formation can occur in the absence of cytoplasmic microtubules. Our observations suggest that the mechanism for generating the chromosome movements associated with bouquet formation may be autonomous to the nucleus rather than being generated by the cytoplasmic cytoskeleton.

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