

# Nucleolus-associated telomere clustering and pairing precede meiotic chromosome synapsis in *Arabidopsis thaliana*

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## SUMMARY

The intranuclear arrangements of centromeres and telomeres during meiotic interphase and early prophase I of meiosis in *Arabidopsis thaliana* were analysed by fluorescent in situ hybridisation to spread pollen mother cells and embryo-sac mother cells. Meiocyte identification, staging and progression were established by spreading and sectioning techniques, including various staining procedures and bromodeoxyuridine labeling of replicating DNA.

Centromere regions of *Arabidopsis* are unpaired, widely dispersed and peripherally located in nuclei during meiotic interphase, and they remain unpaired and unassociated throughout leptotene. Eventually they associate pairwise during zygotene, as part of the nucleus-wide synapsis of homologous chromosomes.

Telomeres, by contrast, show a persistent association with the nucleolus throughout meiotic interphase. Variation in telomere signal number indicates that telomeres undergo pairing during this interval, preceding the onset of general chromosome synapsis. During leptotene the paired telomeres lose their association with the nucleolus and become widely dispersed. As the chromosomes synapse during zygotene, the telomeres reveal a loose clustering within one hemisphere, which may represent a degenerate or relic bouquet configuration. We propose that in *Arabidopsis* the classical leptotene/zygotene bouquet is absent and is replaced functionally by nucleolus-associated telomere clustering.

Key words: Telomeres, Centromeres, Meiosis, *Arabidopsis*

## INTRODUCTION

A defining feature of meiosis I is that homologous chromosomes associate together in pairs. This is an essential prelude to the orderly segregation of homologues at anaphase I, and is also closely associated with the molecular recombination events that generate genetical crossovers and chiasmata (Roeder, 1997). It is currently thought that prior to their intimate synapsis via the synaptonemal complex (SC), homologous chromosomes may, depending on the organism, show some degree of presynaptic association (pairing). This may take the form of rough colocalisation into common nuclear domains or a closer and more obvious alignment (Bass et al., 2000; Walker and Hawley, 2000).

Despite the uncertainty surrounding the function of the SC (Zickler and Kleckner, 1999; Walker and Hawley, 2000) the details of its morphogenesis and structure, including some aspects of its molecular structure, are well established, aided by its obvious and clearly defined ultrastructure. By contrast, the earlier presynaptic events are less well characterised because of the absence of comparable clearly defined and well conserved nuclear structures at these developmental stages. This difficulty has been circumvented in a variety of ways. Some studies have applied fluorescence in situ hybridisation (FISH) to defined chromosome loci (e.g. using cosmid probes) (Weiner and Kleckner, 1994), while others have followed

a chromosome painting approach to highlight entire chromosomes or chromosome arms (Armstrong et al., 1994; Scherthan et al., 1996; Scherthan et al., 1998; Schwarzacher, 1997; Mikhailova et al., 1998; Martinez-Perez et al., 1999; Bass et al., 2000). Other studies have focused on analysing the pairing behaviour of marker structures such as maize heterochromatic knobs (Dawe et al., 1994) or, more commonly, ubiquitous chromosome features such as centromeres and telomeres that can be revealed by applying FISH probes (Scherthan et al., 1996; Bass et al., 1997; Martinez-Perez et al., 1999).

Both centromeres and telomeres have been attributed special roles in the pairing and synapsis of chromosomes during meiosis. Centric heterochromatin has been shown to be particularly important in mediating homologue pairing in *Drosophila* oocytes, where it underlies the special distributive pairing that ensures the segregation of homologues that have failed to cross-over (Dernburg et al., 1996). Studies in hexaploid wheat have established that homologues in pre-meiotic cells are associated via initial centromere contacts (Martinez-Perez et al., 1999). This pattern of homologue pairing has not been widely observed in other species and it is speculated that centromere involvement may be an adaptation to the allopolyploid condition of wheat.

Telomere involvement in pairing/synapsis is much more prevalent and has been widely reported and commented upon

from a diversity of species. In virtually all cases the involvement of telomeres in these events is thought to be closely associated with their binding to the inner surface of the nuclear envelope and their subsequent clustering within a limited area of the inner nuclear surface to produce the so-called bouquet configuration (Dernburg et al., 1995; Zickler and Kleckner, 1998; Cowan et al., 2001). It is generally supposed that the clustering of telomeres facilitates the colocalisation of homologous chromosome ends by bringing them into a common nuclear subregion and making them all roughly codirectional (Cowan et al., 2001). This view is supported by the numerous observations that synapsis, the formation of SCs, often begins at or near chromosome ends (von Wettstein et al., 1984). A secondary role for telomere clustering and pairing is that it may serve to pull other, more internal, homologous chromosome regions into rough alignment (Loidl, 1990; Scherthan et al., 1996).

FISH based analyses of telomere arrangement and movement are not dependent on the presence of distinct chromosome structures or electron-dense axial cores and so can be applied equally to prophase I stages (leptotene onwards) and meiotic interphase nuclei. This approach has been applied to meiotic telomere analysis in fission and budding yeasts (Scherthan et al., 1994; Trelles-Sticken et al., 1999), mice and humans (Scherthan et al., 1996; Scherthan et al., 1998), wheat (Schwarzacher, 1997; Mikhailova et al., 1998, Martinez-Perez et al., 1999) and maize (Bass et al., 1997). Both the timing and degree of telomere clustering during early meiosis show some variation from species to species (see Discussion), but it is universally agreed that this is a fundamentally important aspect of meiotic chromosome behaviour.

The development of improved cytogenetic techniques, combined with the application of molecular genetic methodology, has resulted in a rapid growth of meiosis research in the model dicotyledonous plant *Arabidopsis thaliana*. The structure and behaviour of the chromosomes from early prophase I onwards has been catalogued (Ross et al., 1996) and several *Arabidopsis* meiotic genes have been characterised (Glover et al., 1998; Bai et al., 1999; Caryl et al., 2000). Less is known about events occurring during meiotic interphase (sometimes referred to as premeiotic interphase or preleptotene) and very early prophase I. In this paper we present an analysis of telomere and centromere behaviour during this critical period in *Arabidopsis*. This forms part of a larger study of meiotic chromosome pairing and synapsis in *Arabidopsis*.

## MATERIALS AND METHODS

### Plant material

Seeds of *A. thaliana*, accessions Wassilewskija (WS) and Columbia (Col) as well as the meiotic mutant, *asy1* (derived from the Feldman T-DNA transformed line 243 in the WS background) (Ross et al., 1997) were sown onto a soil based compost, and grown at 18°C with a 16 hour light cycle.

### Preparation of spreads

Whole inflorescences were fixed in acetic alcohol (absolute ethanol-glacial acetic acid, 3:1) or in Carnoy's fixative (absolute ethanol-chloroform-glacial acetic acid, 6:3:1) at room temperature overnight and stored at -20°C after replenishing the fixative.

Fixed flower buds from a single inflorescence, in the size range 0.2-0.9 mm were transferred to a black watchglass containing 3:1 fixative (ethanol:acetic acid). The sepals and petals of the buds were carefully removed prior to enzymic digestion. Buds were then washed in 10 mM citrate buffer pH 4.5 (buffer stock 0.1 M citric acid: 0.1 M sodium citrate, diluted 1:10) at room temperature, and the buffer changed twice before incubating with an enzyme mixture comprising 0.3% w/v cytohellicase (C1794), 0.3% w/v pectolyase (C8274) and 0.3% w/v cellulase (P5936) (all Sigma) in citrate buffer for 30 minutes to 1 hour (shorter times preserve the organisation of the tissues surrounding the meiocytes). Replacing the enzyme mixture with ice cold citrate buffer stopped the reaction. For pollen mother cell (PMC) spreads, single buds were transferred by pasteur pipette to clean slides. The digested buds were either tapped out in a small volume of buffer, using a fine needle, or left intact when it was desired to observe the organisation of meiocytes relative to the surrounding tissue. 5 µl 60% acetic acid was added to the slide, followed by a further 5 µl of the same solution. The slide was left on the bench for a few seconds and re-fixed with about 200 µl cold 3:1 fixative. The fixative was drained away and the slide dried with a hair drier.

For embryo-sac mother cell (EMC) spreads, buds were dissected to isolate entire single gynoecia before enzyme digestion. These were transferred by Pasteur pipette to clean slides and their outer walls were carefully removed with dissecting needles leaving two rows of immature ovules attached to the central placenta. The stripped gynoecia were tapped out in a small volume of buffer, using a fine needle before spreading as described above (Armstrong and Jones, 2001).

### Staining of spreads

Some slides were stained with silver nitrate or DAPI for general observations on PMC development. For silver staining, one drop of freshly prepared 50% silver nitrate solution was placed on each slide, covered with a nylon mesh rectangle (Loidl and Jones, 1986) and incubated in a moist chamber at 60°C for one hour or until golden-brown. The nylon mesh and stain were washed off under running tap water, rinsed twice in distilled water, air dried and mounted. For DAPI staining, one or two drops of DAPI (1 µg/ml) in Vectashield (Vector) antifade mounting medium were added to each slide and mounted with a coverslip.

### Wax embedded sections

Flower buds were fixed in a mixture containing 2% paraformaldehyde, 5% glacial acetic acid and 50% ethanol overnight. Fixed buds were dehydrated through an alcohol series to absolute ethanol, followed by two changes of absolute ethanol, then two changes of HistoClear (Agar Scientific) before transferring to molten paraffin wax (BDH) at 56°C for 90 minutes before transferring again to fresh molten wax and leaving overnight to complete the embedding process. Thick sections (10-15 µm) were cut on a microtome and transferred to polylysine or Vectabond (Vector) coated slides. The sections were dewaxed by taking the slides through two changes (5 minutes each) of HistoClear followed by two changes of absolute ethanol. The slides were then dried, stained with DAPI (1 µg/ml) in Vectashield antifade mounting medium and viewed with a fluorescence microscope.

### Resin embedded semi-thin sections

Flower buds were fixed overnight in freshly made 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate. Fixed buds were dehydrated through an alcohol series to 90% ethanol, then 2×2 hours in 1:1 90% ethanol:L.R. White resin (Agar Scientific) before transferring to 100% L.R. White resin for 48 hours. The specimens were then transferred to gelatin capsules, outgassed and polymerised under UV light for 2-3 days. Semi-thin sections were cut on an ultramicrotome and stained with toluidine blue (1% in 1% sodium tetraborate).

### BrdU incorporation and detection

Stems from well grown plants were cut under water, using sharp dissecting scissors. The cut ends were quickly transferred to bromodeoxyuridine (BrdU) solution ( $10^{-2}$  M) and left for up to 3 hours in a growth chamber maintained at 18°C for uptake of BrdU via the transpiration stream and its incorporation into cells in S-phase. The buds were fixed, either for spreading or for wax embedding, after 1 hour in BrdU and then at 4-hourly intervals, for up to 24 hours. Slides of buds/anthers in the range 0.2-0.3 mm were made by the spreading procedure, as already described.

Slides were placed in 2× SSC for 10 minutes, dehydrated through an alcohol series (70%, 85%, 100%) each for 2 minutes and then dried with a hair drier. BrdU was detected immunocytologically in sectioned and spread meiocytes using an anti-BrdU kit (Roche) according to the manufacturer's instructions. Slides were counterstained with DAPI (1 µg/ml) in Vectashield antifade mounting medium.

### Fluorescence in situ hybridisation (FISH)

The following probes were used: (1) pAL1, containing a pericentromeric 180 bp repeat (Martinez-Zapater et al., 1986), obtained from Ohio Arabidopsis Stock Center. (2) The *Arabidopsis* telomere repeat (Richards and Ausubel, 1988). (3) BAC probes F11L15, F17K2, T9J23 (chromosome 2 long arm) and F19K16 (chromosome 1) were obtained from Ohio Arabidopsis Stock Center.

The pAL1 probe was amplified by primary PCR using M13 universal and repeat primers (Pharmacia), and directly labeled by secondary PCR with spectrum green dUTP (Amersham). The telomere probe was amplified by primary PCR using oligonucleotides T1 (TTTAGGG)<sub>5</sub> and T2 (CCCTAAA)<sub>5</sub> corresponding to the *Arabidopsis* telomere repeat sequence (Ijdo et al., 1991). Secondary PCRs were carried out to incorporate biotin-dUTP (Roche). DNA was isolated from BACs by standard methods and labeled with digoxigenin-dUTP by nick-translation (Roche).

The FISH technique used was that previously described (Armstrong et al., 1998; Caryl et al., 2000). Detection of digoxigenin labeled probes was with anti-digoxigenin-fluorescein (Roche) and biotin labeled probes with Cy3-streptavidin (Cambio). Slides were counterstained with DAPI as already described.

### Photomicroscopy

Slides were examined by means of a Nikon Eclipse T300 microscope. Capture and analysis of images was achieved using an image analysis system (Applied Imaging).

## RESULTS

### Experimental approach

In previous studies two distinct methodological approaches have been followed for the application of FISH to the analysis of meiosis. Tissue sections have the advantage of preserving 3D structure and also give a degree of certainty in the identification of meiocytes from other surrounding cells that

may have similar morphology and size (Scherthan et al., 1996; Martinez-Perez et al., 1999). Other studies have been based on applying FISH to intact whole-mounts of meiocytes that are structurally preserved (Bass et al., 1997) or that have been flattened or spread to varying degrees (Weiner and Kleckner, 1994; Trelles-Sticken et al., 1999). These procedures have the advantage that intact cells are analysed, while some degree of 3D information is retained even in partially spread or flattened cells. The loss of some 3D information is compensated by the potential to collect data rapidly from large numbers of cells. Faced with the same dilemma, other investigators have shown that the relative organisation of intact (3D) somatic nuclei from *Drosophila* and humans is not significantly perturbed in 2D preparations (Csink and Henikoff, 1998; Croft et al., 1999). Spread cells and nuclei also present much better accessibility to FISH probes, compared to sections where probe penetration can be restricted. This latter factor was particularly important in the decision to use spreads rather than any other preparative method for the present study. *Arabidopsis* has very small telomeres and it was considered an imperative to maximise the strength of the FISH telomere signals by adopting the spreading procedure. Several trial applications of telomere FISH to wax and resin embedded sections gave unsatisfactory results.

### Identification of PMCs and staging

Because centromere and telomere arrangements in this study were determined from spread PMCs (see above) it was necessary to establish clear criteria for their identification and staging. Floral development in *Arabidopsis* has been analysed in detail, based on morphometric characters of flower buds and their component parts (Smyth et al., 1990), and 12 stages of floral development have been recognised. Using a combination of spreading, with two different staining protocols (DAPI and silver nitrate), sectioning of both wax and resin embedded buds and BrdU labeling to mark cells in S-phase we related meiotic development in *Arabidopsis* anthers to these morphometric characters and stages. We also determined the progression and timing of PMCs from the meiotic S-phase through G2 and as far as pachytene of prophase I. In addition we established that the PMCs are distinguishable from other cell types in the anther, especially the tapetal cells, at least from S-phase onwards.

Table 1 summarises the relationships of flower bud morphometric characters, including bud size, to cytologically determined stages of meiotic development in PMCs. The smallest flower buds that could practically be dissected from inflorescences and processed for spreading were about 0.2-0.3 mm long and coincided with late stage 7 or early stage 8

**Table 1. Relationship between floral development, bud size and cytological landmarks**

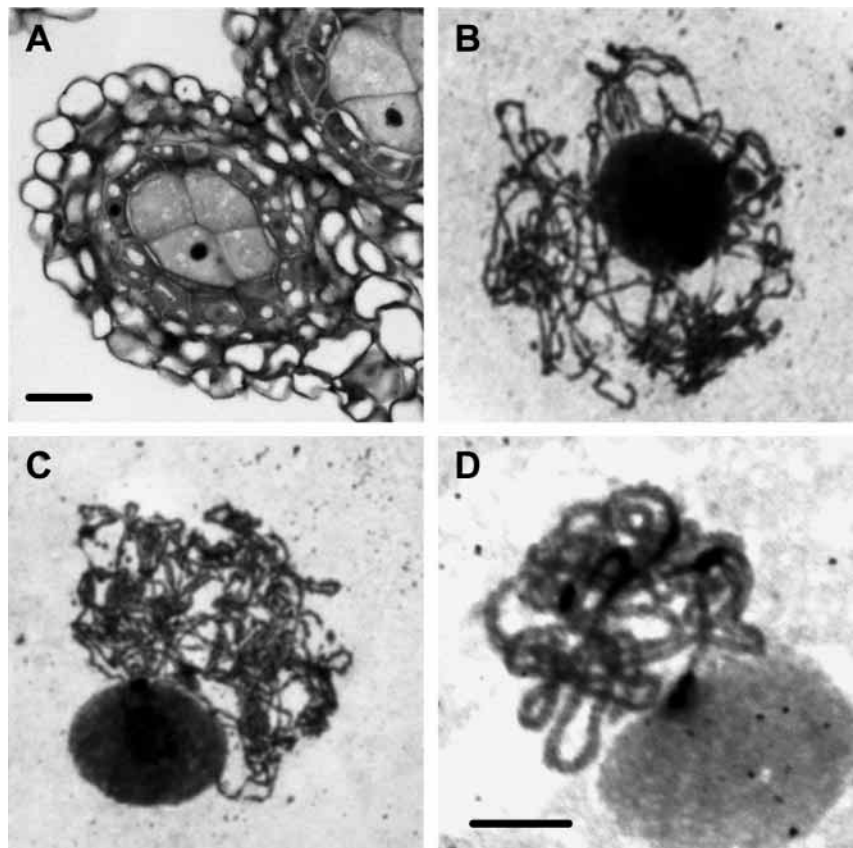
Floral stage*	Bud size (mm)	Cytology
8. Anther and filament regions already separated (stage 7). Locules present in the long stamens. Primordia of petals visible	<0.3	Anthers differentiated into five tissue layers. The innermost cells, the PMCs, are in meiotic interphase, surrounded by the tapetum, the middle cell layer, the endothecium and the epidermis.
9. Petal primordia stalked at base	0.3-0.4	Meiosis in pollen mother cells. Tapetal cells become binucleate at zygotene/early pachytene stages.
10. Petals level with the short stamens; stamens green	<0.5	Microspores

\*As described by Smyth et al., 1990.

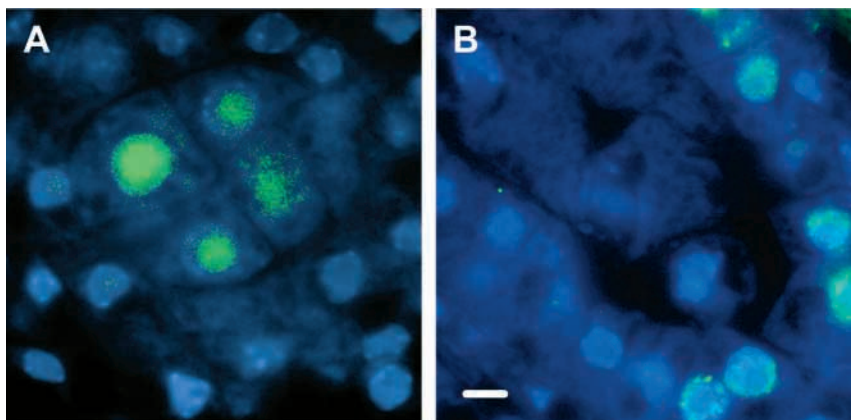
(Smyth et al., 1990). At this stage the anthers have differentiated five tissue layers (Fig. 1A) The innermost cells, the PMCs are surrounded successively by the tapetum, the middle cell layer, the endothecium and the epidermis, each of which is one cell layer deep (Owen and Makaroff, 1995). BrdU labeling and time course experiments allowed us to positively identify S-phase meiocytes and to track their progress through G2 and prophase I. In preliminary experiments, it was shown that BrdU applied to the cut end of flowering stems was rapidly translocated to the flower buds and used in DNA replication within 1 hour. BrdU incorporation into S-phase nuclei was detected by means of an anti-BrdU antibody (Fig. 2; Fig. 3A,B). During S-phase the meiocyte nuclei have characteristically diffuse chromatin, and the usually condensed chromocentres, corresponding to the pericentromeric heterochromatin, assume an extended configuration that gives the impression of multiple, fragmented, signals (Fig. 3B). Each anther locule of *Arabidopsis* contains about 30 PMCs that enter meiosis simultaneously and progress through the S-phase and then the rest of meiosis with a high degree of synchrony. This synchronisation is an aid to identification of meiocytes and allows a clear distinction between the meiotic S-phase and earlier S-phases of the asynchronously cycling (mitotic) pre-meiotic sporogenous cells.

The progress of meiocytes from the S-phase through G2 and into prophase I was followed by means of time course experiments. One hour pulses of BrdU were administered, the flowering stems returned to water and flower buds fixed at 4 hour intervals. The minimum duration of G2 interphase is 16 hours, since this was the shortest time taken for BrdU labeled cells to progress to early leptotene, defined as the earliest appearance of, as yet incomplete, distinct chromosome axes. The average duration of this interval, however, is closer to 20 hours. Labeled late zygotenes and early pachytenes appeared at 24 hours. Division stages having condensed chromosomes (i.e. metaphase/anaphase I and II) were not seen labeled in the samples taken, but this probably reflects their relatively brief duration compared with prophase I. The meiotic stage durations derived from these experiments of course assume that meiosis progresses normally in detached flower-bearing stems maintained in water.

In the absence of BrdU labeling, S-phase meiocytes are readily identified by the characteristic diffuseness of their chromatin and chromocentre extension. G2 nuclei,



**Fig. 1.** (A) Semi-thin section through a <math><0.3\text{ mm}</math> flower bud, stained with toluidine blue, showing one complete anther locule. Each locule contains a group of PMCs in meiotic interphase with prominent nucleoli, surrounded by four cell layers. It can be seen that the tapetal cells, adjacent to PMCs, are much smaller than the PMCs. (B-D) Spread and silver stained PMCs to show the typical progression of nuclear and nucleolar morphology through early meiosis. (B) Leptotene; chromosome axes are fully developed and the nucleolus is slightly off-centre. (C) Zygotene; chromosome axes beginning to synapse and showing some aggregation; the nucleolus is shifted to a peripheral location. (D) Pachytene; the chromosomes are fully synapsed; the nucleolus remains peripherally located. Bar, 5  $\mu\text{m}$  (B,C,D); 10  $\mu\text{m}$  (A).



**Fig. 2.** Sections through wax-embedded anthers following a 1 hour pulse label of BrdU and fixation 3 hours later. BrdU is detected by anti-BrdU antibody labeled with FITC (green). (A) BrdU labelled PMC nuclei at S-phase of meiotic interphase. (B) BrdU labeled tapetal cell nuclei; the PMCs in this anther are at leptotene/zygotene. Sections are counterstained with DAPI. Bar, 10  $\mu\text{m}$ .

by contrast, present a rather uniform appearance with characteristic condensed and peripherally located chromocentres, but other cellular changes combined with BrdU time course data allow us to subdivide G2 into two intervals, early and late. In S and early G2 the PMCs are already large cells compared with the surrounding cell layers and have large nuclei with large centrally located nucleoli, abundant cytoplasm and prominent cytoplasmic organelles. At this stage the cell walls are thin and callose deposition has not commenced (Fig. 1A). This appears to correspond to the stage defined as 'premeiosis I' (Owen and Makaroff, 1995). In late G2 callose deposition has commenced and the PMCs are separated from each other and from the tapetum by a growing layer of callose. This corresponds to the stage defined as 'premeiosis II' (Owen and Makaroff, 1995). The consistent identification of G1 PMCs presents more difficulties. In theory we would expect these cells to be smaller, with smaller nuclei, and they should never appear synchronously labeled by BrdU in time course experiments. We were occasionally able to identify such groups of cells, but only in very small and early buds that were close to the limit of our handling practicality.

In meiotic S and G2 the PMCs, seen in sections, are distinctive cells that are obviously different in terms of overall size and nuclear size from the adjacent tapetal cells (Fig. 1A; Fig. 2). We are therefore confident of being able to distinguish these cell types in anther spreads. We have confirmed this in very gently spread anther preparations in which the PMCs and tapetal cells are slightly dispersed but retain their original central and peripheral relative positions; again the two cell types are obviously different in size (not shown).

The onset of leptotene, the initial stage of prophase I, is difficult to identify and define precisely. Traces of fine chromosome threads/axes are evident in early leptotene, both in DAPI stained and silver stained spread preparations. These extend progressively through leptotene until eventually the full complement of complete chromosome axes is fully formed (Fig. 1B). The nucleolus is a large structure in these leptotene nuclei, occupying perhaps as much as one third of the nuclear volume.

In early leptotene the nucleolus occupies a central position in the nucleus, but towards the end of leptotene it moves progressively towards the nuclear periphery, where it remains

throughout prophase I. The progression of *Arabidopsis* PMC through prophase I, has been thoroughly described previously from DAPI or haematoxylin stained spread preparations (Ross et al., 1996), but is particularly clearly seen in silver stained spreads (Fig. 1B-D). During mid-prophase I, coinciding with the zygotene stage, the pericentric heterochromatin regions appear aggregated into a variable number of clumps. This may be a manifestation of a nucleus-wide change in chromosome organisation, characteristic of this stage, involving a reduction in nuclear and chromosome volume (Dawe et al., 1994; Zickler and Kleckner, 1999). This may also be the origin of the tendency of zygotene chromosomes in many plants, including *Arabidopsis*, to form a dense tangled knot in acid-alcohol fixed preparations, the so-called synizetic knot. At pachytene the chromosomes are fully synapsed into five bivalents and the nucleolus remains at the nuclear periphery (Fig. 1D). Pachytene is followed by a diffuse early diplotene during which the homologues progressively desynapse as the synaptonemal complex (SC) degrades. The bivalents, maintained by chiasmata linking the homologous chromosomes, then condense progressively through diplotene and diakinesis and reach their fullest condensation at metaphase I.

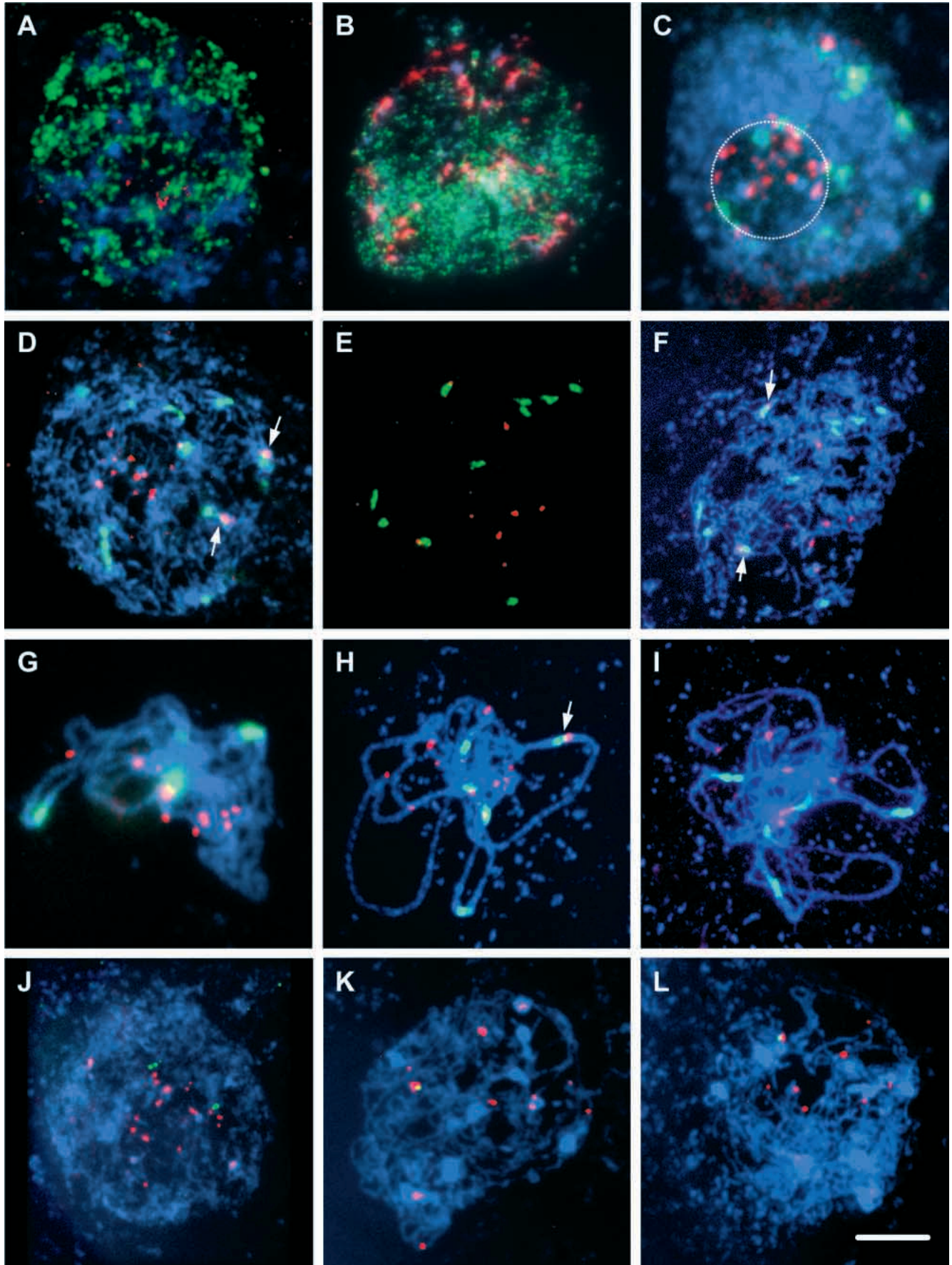
### Centromere arrangement in PMCs

Centromere (CEN) regions were identified and located by FISH using the pAL1 DNA sequence as a probe. pAL1 is a 180 bp tandemly repeated sequence that localises to the central domain of the pericentromeric heterochromatin of all ten *Arabidopsis* chromosomes and is not present elsewhere in the genome (Fransz et al., 1998). The CEN signals invariably colocalise with DAPI-bright chromocentres. One pair of CENs (chromosome 1) is further marked by having internal telomere sequences located immediately adjoining the CEN region. These give a pair of bright CEN-associated telomere signals in addition to the terminal signals (Fig. 3D,F,H, arrowed). There is no indication of CEN pairing throughout meiotic interphase from G1 through S and G2 to leptotene. The CEN signals always appear dispersed in spread interphase PMCs (Fig. 3C) and observations on sections indicate that they are located peripherally in the nuclei. Ten CEN signals are seen in all nuclei at this stage (Table 2), indicating that centromere regions do not pair during the meiotic interphase in *Arabidopsis*. This is confirmed by the consistent appearance of two unpaired

**Table 2. Telomere and centromere FISH signals at different meiotic stages of wild-type and *asy1* PMCs\***

	Meiotic interphase	Leptotene(I)	Leptotene(II)	Zygotene	Pachytene	Diplotene
Wild-type	<i>n</i> =51	<i>n</i> =17	<i>n</i> =29	<i>n</i> =43	<i>n</i> =66	<i>n</i> =40
Telomeres	15.3 (10-20)	11.2 (10-15)	10.0	10.0	10.0	10.1 (10-12)
Centromeres	10.0	10.0	9.6 (8-10)	2.4 (1-5)	5.0	5.1 (5-6)
<i>asy1</i>	<i>n</i> =22	<i>n</i> =8	<i>n</i> =16	Post leptotene <i>n</i> =49		
Telomeres	14.8 (12-18)	11.0 (8-16)	10.4 (8-13)	11.4 (8-20)		
Centromeres	10.0	10.0	9.1 (6-10)	3.0 (1-7)		

\*Mean numbers and ranges (in brackets) of telomere and centromere FISH signals at different meiotic stages of wild-type and *asy1* PMCs. Leptotene (I) and (II) substages refer to nuclei having clustered (I) and distributed (II) telomeres. *n*, number of cells analysed.



**Fig. 3.** FISH of telomere, centromere (CEN=pAL1) and BAC probes to wild-type *Arabidopsis* nuclei. (A,B) Combined BrdU labelling and FISH following a 1 hour BrdU pulse +3 hours to show clustered telomere signals (A) and dispersed and extended CEN signals (B). BrdU is detected by anti-BrdU antibody labeled with FITC (green); both telomere and CEN signals are red. (C-I) Dual FISH of telomere (red) and CEN (green) probes to *Arabidopsis* PMC. (C) Nucleolus-associated clustering of telomeres and dispersal of CENs seen in meiotic interphase; the white dotted circle indicates the approximate boundary of the nucleolus (D). (D-I) Progressive changes in telomere and CEN signal numbers and distributions during meiotic prophase I; (D) early leptotene showing 15 clustered telomere signals and ten dispersed CEN signals, arrows here and elsewhere indicate the chromosome 1 CEN-associated telomere signals; (E,F) dual (E) and triple (F) filter images of the same leptotene nucleus showing ten dispersed CEN signals and eight semi-dispersed telomere signals, indicating colocalisation of some telomeres; (G) zygotene, showing nine telomere signals (two colocalised) and four CEN signals resulting from stage-specific chromatin clumping; (H) pachytene, showing nine telomere signals (two colocalised) and five CEN signals; (I) early diplotene showing early stage of desynapsis; note that telomeres are still paired at this stage. (J-L) Dual FISH with telomere (red) and BAC (green) probes to demonstrate that telomere pairing involves homologous chromosomes; (J) meiotic interphase showing clustered unpaired telomeres and BACs; (K,L) leptotenes, showing paired telomeres and BACs T9J23 (K) and F19K16 (L). All nuclei are counterstained with DAPI. Bar, 10  $\mu$ m.

chromosome 1 CENs marked by telomere signals. In S-phase nuclei the CEN signals are typically very extended (Fig. 3B), presumably reflecting the generally extended and diffuse condition of chromatin during DNA replication, but they recondense during G2. The replicated state of G2 chromosomes is often, but not always, visibly expressed as doublet CEN structures.

The interphase appearance and arrangement of CENs persists, more or less unchanged into early prophase I (Fig. 3D-F). In almost all cases, ten unpaired and widely dispersed CEN signals were observed throughout leptotene (Table 2). Very occasionally, fewer than ten CEN signals are present in interphase nuclei, but so infrequently that these cases could be ascribed to chance juxtaposition or overlap of unpaired centromere regions. During zygotene however, the number of CEN signals abruptly reduces to a maximum of five, reflecting the synapsis of homologous chromosomes into bivalents (Table 2; Fig. 5). Chromosome 1 CENS, marked by telomere sequences, reduce to a single signal in zygotene nuclei. Some further reduction of CEN signal number to four, three or less is due to the clustering and aggregation of chromocentres typical of this stage (Fig. 3G). Pachytene bivalents typically have more extended and non-aggregated CENs (Fig. 3H). The separation of homologues (desynapsis) during early diplotene (Fig. 3I) does not extend to the CENs, which remain associated until mid-late diplotene, when they finally separate.

### Telomere arrangement in PMCs

Telomeres were located and identified by FISH using the *Arabidopsis* telomere DNA repeat sequence as a probe. Throughout premeiotic interphase, up to 20 separate telomere signals were observed to be clustered in the centre of the nucleus and closely associated with the nucleolus (Fig. 3C,J). In DAPI stained PMC nuclei the single nucleolus appears as a more or less spherical hole or 'ghost' surrounded by DAPI

positive chromatin. The size, shape and intranuclear location of this 'ghost' concur with identical features of the nucleolus in silver-stained preparations. Furthermore, dual FISH with telomere and 45S rDNA probes confirms that the telomeres are indeed associated with the nucleolus (not shown). The precise nature of this association has not been determined; however, it appears that the telomeres are most likely attached to the periphery of the nucleolus.

This arrangement of telomeres is not a uniquely meiotic phenomenon. It is seen, for example, in tapetal cell nuclei and appears to be consistently present in *Arabidopsis* interphase nuclei of all categories and types, certainly within flower buds and leaves, and probably more generally (P. Fransz, personal communication). The numbers of discernable separate telomere signals associated with the nucleoli of meiotic interphase cells varied considerably (Table 2). The full complement of 20 telomere signals, disregarding the two chromosome 1 CEN-associated signals, was visible in 12 out of 51 interphase cells that were recorded; the remainder showed a wide and continuous range of signals between 10 and 19. Some of this variation is undoubtedly due to merging and overlap of adjoining signals in the nucleolus-associated telomere cluster. However, the steep and continuous gradient in signal number to a minimum of ten suggests that telomeres are involved in progressive pairwise association during interphase while still associated with the nucleolus. This conclusion is supported by the observation of ten telomere signals in the majority of early leptotene nuclei (Table 2).

The transition from interphase to leptotene is, as noted earlier, not especially abrupt. Chromosome axes emerge progressively and gradually extend until each chromosome has a fully developed axis running from end to end. In the earliest recognisable leptotene nuclei, the telomeres remain clustered around the nucleolus (Fig. 3D), and the number of telomere signals continues to diminish towards the reduced number of ten. By mid-late leptotene all nuclei have ten, or slightly fewer, telomere signals (Fig. 3E,F) and it remains at this number throughout almost the whole remainder of prophase I (Table 2). As leptotene proceeds, a dramatic change in telomere arrangement and location occurs. The paired telomeres abandon their association with the nucleolus and by late leptotene they are widely dispersed.

During zygotene, coinciding with nuclear reorganisation, telomere distribution changes again. The widespread distribution typical of leptotene is lost and instead they exhibit a semi-polarised non-clustered distribution such that they are loosely confined to one hemisphere of the nucleus (Fig. 3G). This is the nearest approximation to a bouquet arrangement that we have observed in *Arabidopsis*. During pachytene the paired telomeres are once again widely distributed (Fig. 3H). They retain this distribution and their paired status into early diplotene (Fig. 3I) and it is only gradually, as diplotene progresses and homologous chromosomes desynapse that the paired telomeres separate and the number of signals increases to 20.

### Telomere pairing is homologous

An important and obvious question is whether the paired telomeres (ten signals) represent the pairing of homologous chromosome ends, or just random pairwise association? The fact that telomere signal number remains at ten from leptotene,

**Table 3. Single and double BAC signals detected in early meiotic stages of wild-type PMCs\***

Telomere no. BAC signal no.	Meiotic interphase		Leptotene(I)		Leptotene(II)	
	10-20		10-15		10	
F19K16 (st-1)	0	4	6	7	9	0
T9J23 (st-2)	0	5	1	8	23	1
F11L15 (st-2)	0	2	3	4	8	0
F17K2 (i-2)	0	9	1	12	7	34

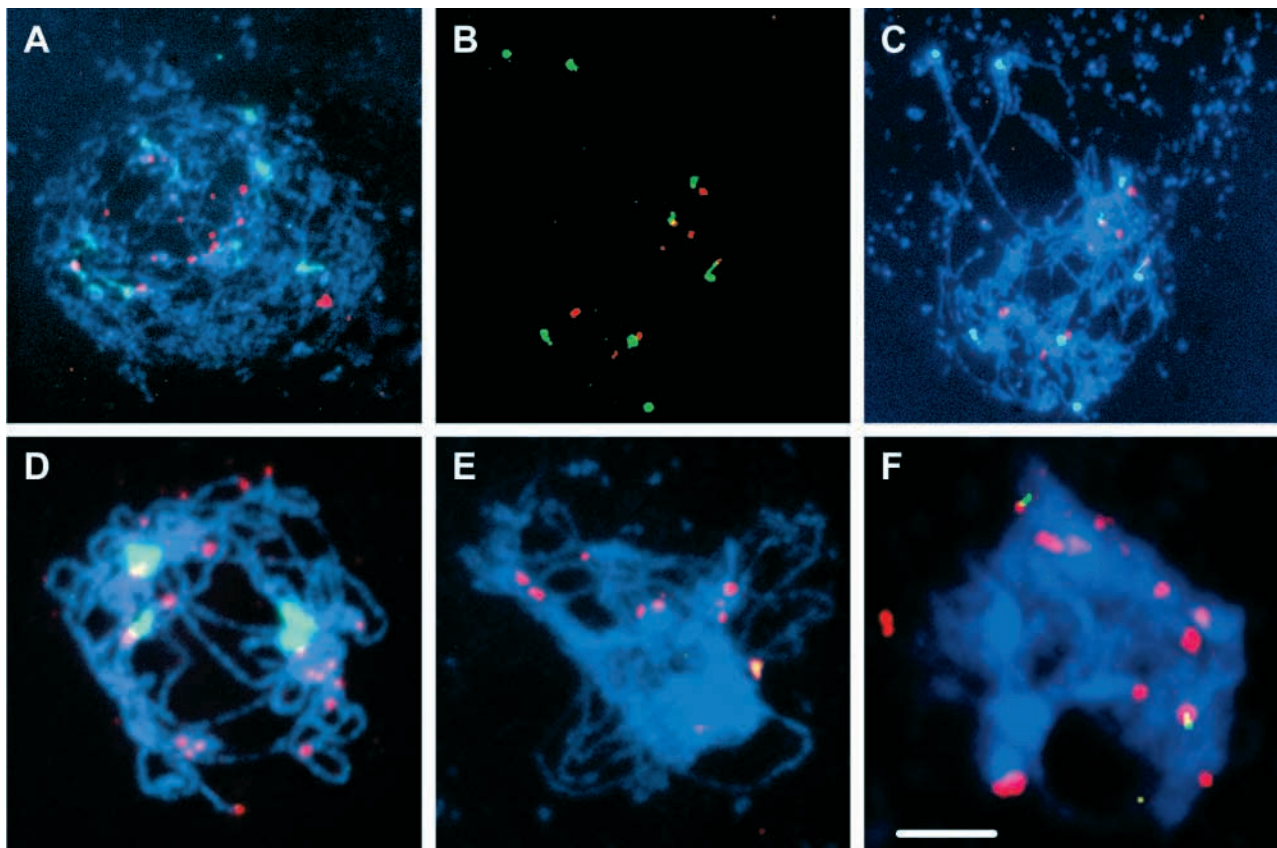
\*Numbers of single and double BAC signals detected in early meiotic stages of wild-type PMCs. Three BACs are subtelomeric (st) on either chromosomes 1 or chromosome 2. The fourth BAC is interstitially located (i) on the long arm of chromosome 2.

through zygotene and pachytene, when the homologous chromosomes are known to be synapsing or fully synapsed, suggests very strongly that the initial pairwise contacts in interphase and leptotene are homologous. To investigate this further, dual FISH was performed using the telomere repeat probe and three different sub-telomeric BAC probes (Table 3). BACs F11L15 and T9J23 are the ultimate (most distal) and penultimate BACs, respectively, on the long arm of chromosome 2, while BAC F19K16 occupies a distal location on chromosome 1. Dual FISH applied to mitotic metaphase

and pachytene chromosomes (not shown) confirmed that all three BACs are located immediately adjacent to their respective telomeres. All three BACs showed similar patterns of pairing. In premeiotic nuclei, where telomeres are mostly unpaired, we observed only unpaired BAC signals (Fig. 3J), whereas in early leptotene (defined as having from 10-15 telomere signals) about one third of BAC signals were paired while the remainder were unpaired (Table 3). Mid-late leptotene, having fully paired telomeres (10 signals) in most cases showed single BAC signals immediately adjoining one of the telomere signals (Fig. 3K,L), thus confirming that telomere pairing does indeed involve the pairing of homologous telomeres. As a control, this experiment was repeated using an interstitial BAC (F17K2) located near the middle of chromosome 2 long arm. In this case the BAC signals remained largely unpaired throughout leptotene (Table 3).

### Observations on female meiosis in embryo-sac mother cells

Meiosis in embryo-sac mother cells (EMC) occurs later than male meiosis, coinciding with floral stages 10-11 when the developing gynoeceum is about 0.6-0.8 mm long (Armstrong and Jones, 2001). EMCs are large and prominent cells, easily distinguished in sections and spreads from the surrounding ovular tissues. FISH using the CEN and telomere probes was



**Fig. 4.** (A-F) FISH with telomere, CEN and BAC probes applied to meiotic prophase of the *asy1* meiotic mutant. (A-D) FISH with telomere (red) and CEN (green) probes to show unpaired telomeres in meiotic interphase (A), paired telomeres in leptotene (B,C) and unpaired (dissociated) telomeres in the asynaptic post-leptotene stage (D). B and C show dual and triple filter images of the same leptotene nucleus. (E,F) Dual FISH with telomere (red) and BAC T9J23 (green) probes to show variable pairing (E) or unpairing (F) of the BAC in post-leptotene asynaptic nuclei. Bar, 10  $\mu$ m.



applied to spread preparations of gynaecia, but the yield of analysable EMCs, especially from the earlier stages, was low and so the results obtained will be only briefly reported. Essentially the same patterns of centromere and telomere arrangement, and rearrangement, were observed in EMCs as in PMCs.

### Centromere and telomere behaviour in PMCs of the *asy1* meiotic mutant

The *asy1* mutant of *Arabidopsis* is defective in meiotic chromosome synapsis (Ross et al., 1997). The mutation results from a T-DNA insertion, and a simultaneous deletion of plant DNA, in the 5' UTR region of the *ASY1* gene (Caryl et al., 2000). At mid prophase I, corresponding to the zygotene/pachytene interval in wild-type, the chromosomes appear to be entirely unsynapsed. At metaphase I the chromosomes are predominantly univalent, but there is a low level (mean=1.5 per cell) of residual bivalent formation, associated by mainly near-terminal chiasmata (Sanchez-Moran et al., 2001). Because of the widespread presumption that telomeres play a key role in chromosome synapsis, the FISH analysis of telomeres and centromeres was extended to this mutant.

Just as in wild-type *Arabidopsis*, telomeres are clustered around the nucleolus in meiotic interphase of *asy1* (Fig. 4A). Telomere signal number is again variable and shows a wide range, as seen in wild-type, between 18 and 12 (Table 2). The gradient in signal number implies that telomere pairwise association is occurring in the mutant, as in the wild-type. This conclusion is supported by the leptotene observations (Fig. 4B,C). However, while the leptotene telomere signal number in wild-type is consistently 10, in *asy1* it ranges from 8 to 13 and only 50% of cells have 10 or fewer telomere signals, and this applies equally to early leptotene (clustered telomeres) and late leptotene (dispersed telomeres) (Table 2). However, it is clear that telomere pairing does happen in the *asy1* mutant.

Post leptotene stages in *asy1* are atypical, since no synapsis occurs and hence conventional zygotene and pachytene stages are absent (Fig. 4D). Telomere signal number in these nuclei is extremely variable, and ranges between 10 and 20. This is probably due to two different factors. First, telomere pairing during the preceding stages may have been incomplete in some nuclei. Second, in the absence of synapsis and SC formation, the pairing of telomeres during the preceding stages is not stabilised and those telomeres that did pair eventually disjoin. Dual FISH using the telomere probe and the sub-telomeric BAC T9J23 on chromosome 2 confirmed that, as in wild-type, telomere pairing involved homologous ends (Fig. 4E,F).

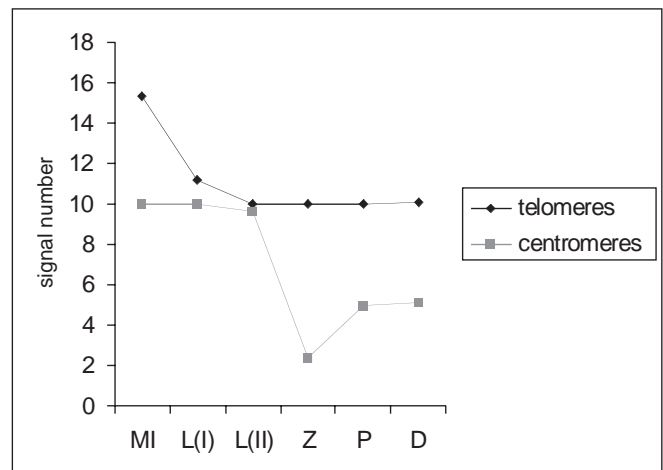
Centromere arrangement during meiotic interphase and leptotene in *asy1* is very similar to that seen in wild-type. Ten widely dispersed CEN signals are present throughout these intervals (Fig. 4A-C) although some reduction in signal number is found occasionally due to colocalisation or overlap of CENs. We would expect that CENs would remain unsynapsed throughout the following post-leptotene period in *asy1*. However, this is masked by a strong tendency of the behaviour of these regions during zygotene in wild-type. Nevertheless, it is clear from the FISH signals located adjacent to the chromosome 1 CENs, that homologous centromere regions remain unsynapsed in *asy1*.

## DISCUSSION

The patterns of change in telomere and centromere numbers and arrangement through meiotic interphase and prophase I of wild-type *Arabidopsis* are summarised in Fig. 5 and Fig. 6.

*Arabidopsis* telomeres are involved in directing homologue association, but in an unusual and apparently novel manner. The tight association of telomeres with the nucleolus during meiotic interphase, as well as in mitotic interphase nuclei (P. Fransz, personal communication), is highly unusual. Since only 4 of the 20 chromosome termini in diploid nuclei of *Arabidopsis* carry nucleolus organising regions (NORs), this general association of telomeres with the nucleolus must have some other organisational basis. Colocalisation of the telomeres with the nucleolus has been reported at the zygotene stage in maize (Bass et al., 1997), but this is a quite different and less intimate association; the telomeres are merely alongside and in the same general area as the nucleolus. *Arabidopsis* centromeres are widely dispersed and unpolarised during meiotic interphase. Taken together, these features indicate clearly that Rab1 polarisation is not present in meiotic interphase, or indeed in mitotic interphases, of *Arabidopsis*. This in itself is not particularly surprising since comparative studies have shown that a diversity of interphase chromosome organisation is found among different plant species, including both Rab1 and non-Rab1 patterns (Dong and Jiang, 1998), and furthermore non-Rab1 patterns are characteristic of relatively small genomes. The specific and tight association of telomeres with the nucleolus in *Arabidopsis* is evidence of yet further diversity in interphase nuclear organisation.

Telomere association with the nucleolus appears to be a universal feature of mitotic and meiotic cells in *Arabidopsis*. This implies that telomeres are already clustered around the nucleolus at the beginning of meiotic interphase, and that they remain in this configuration until early-mid leptotene. Significantly, telomere pairing takes place during meiotic interphase while they are still clustered around the nucleolus. This raises the interesting possibility that nucleolus-associated



**Fig. 5.** Changes in telomere and CEN FISH signal number during meiotic interphase and prophase I of wild-type *Arabidopsis*. MI, premeiosis; L(I), early leptotene (clustered telomeres); L(II), late leptotene (dispersed telomeres); Z, zygotene; P, pachytene; D, diplotene.



**Fig. 6.** The changes in telomere and CEN number and intranuclear distribution through meiotic interphase and prophase I. (A) Meiotic S-phase; telomeres (red) are clustered around the nucleolus and unpaired; pericentric heterochromatin (green) regions (CENs) are unpaired, extended and dispersed. (B) Meiotic interphase-G2; similar to preceding S-phase except that CENs are condensed. (C) Early leptotene; telomere number variable, between 10 and 20, indicating that telomere pairing is occurring; CENs remain unpaired and widely dispersed. (D) Leptotene; telomeres fully paired (10 signals) and widely dispersed; CENs unpaired and dispersed. (E) Zygotene; telomeres paired and loosely confined to one hemisphere of the nucleus; CEN signal number variable (1-5) due to homologous synapsis and aggregation. (F) Pachytene; telomeres and CENs fully paired and widely dispersed through nucleus.

clustering seen in *Arabidopsis* is equivalent to and replaces the bouquet clustering seen in other species, as the basis for telomere pairing. If this is so, telomere pairing can be regarded as exploiting a pre-existing arrangement rather than engaging in de novo clustering of the bouquet type. This might explain why telomere pairing occurs so much earlier in *Arabidopsis* than in many other species. Telomere clustering, which is generally regarded to precede telomere pairing, has been reported to occur at early leptotene (wheat), late 'pre-leptotene'/zygotene (mouse), leptotene/zygotene equivalent (yeast) and late leptotene/pre-zygotene (maize) (Martinez-Perez et al., 1999; Scherthan et al., 1996; Trelles-Sticken et al., 1999; Bass et al., 1997). The timing of telomere pairing, deduced from the halving of telomere signal number, is less precisely given in these publications, or not stated. In wheat the telomeres remain unpaired throughout meiotic interphase, but pairing can be only imprecisely timed, from the data given, to early prophase I; not surprisingly, telomeres are fully paired by late zygotene (Martinez-Perez et al., 1999). In maize, telomere pairing may occur relatively early, during meiotic interphase and preceding bouquet formation, based on the reported numbers of telomere signals (Table 1) (Bass et al., 1997).

Although telomere pairing in *Arabidopsis* appears not to require bouquet clustering, a loose bouquet arrangement does nevertheless appear during zygotene, coinciding with the synzytic knot. Several commentators have remarked that the extent of bouquet formation is variable, especially in plants, and that in some species it is no more than a loose polarisation or clustering of telomeres (Zickler and Kleckner, 1998; Cowan et al., 2001).

The centromere regions of *Arabidopsis* do not appear to be involved in directing homologue association or synapsis. They remain unpaired and widely dispersed throughout meiotic interphase and early prophase I and only associate pairwise as a consequence of the general synapsis of homologous chromosomes during zygotene. This resembles the situation seen in mammals (Scherthan et al., 1996) and probably in maize, where chromosome pairing and synapsis are coincident telomere-led events (Bass et al., 1997; Bass et al., 2000). However, in wheat and yeast, there is evidence of extensive

interphase pairing of homologues, preceding prophase I and, in the case of wheat, at least, this is driven by the initial pairing of homologous centromeres (Martinez-Perez et al., 1999). It is noteworthy in this context that wheat exhibits persistent Rabl orientation of chromosomes through interphase, including meiotic interphase, and indeed the bouquet orientation in this species is regarded as an intensification of the pre-existing Rabl configuration (Aragon-Alcaide et al., 1997).

The consequent polarised clustering of centromeres may provide the physical basis for meiotic interphase centromere pairing in wheat. It has recently been proposed that variation in Rabl orientation persistence among plant species may be related to genome size and chromosome length (Dong and Jiang, 1998; Cowan et al., 2001). Species with relatively large chromosomes, including wheat, exhibit persistent Rabl orientation, whereas it is lacking from relatively smaller genome plants such as rice, maize, *Sorghum* and *Arabidopsis*. This may also account for the observation of centromere pairing during meiotic interphase in *Lilium* (Suzuki et al., 1997), a plant genus with notably large chromosomes.

The *asy1* mutant is defective in chromosome synapsis, but nevertheless exhibits near normal telomere pairing during meiotic interphase and early prophase I. This clearly indicates that these are two distinct and separable functions. The defect in synapsis, attributable to the absence of *Asy1* protein, does not prevent telomere pairing functions.

The apparent functional separation of telomere pairing and homologous chromosome synapsis raises intriguing questions concerning the basis for homologous telomere pairing. Since all telomeres consist of the same repeated DNA sequence, specificity cannot reside in the sequence of bases at chromosome ends. It may, however, depend on other features of the telomere DNA-protein complex (Scherthan et al., 2000). Alternatively the pairing of telomeres could be a homology-dependent process involving immediately sub-telomeric DNA sequences.

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