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S.J. Armstrong · G.H. Jones

Female meiosis in wild-type *Arabidopsis thaliana* and in two meiotic mutants

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Abstract Female meiosis in *Arabidopsis* has been analysed cytogenetically using an adaptation of a technique previously applied to male meiosis. Meiotic progression was closely correlated with stages of floral development, including the length and morphology of the gynoecium. Meiosis in embryo sac mother cells (EMCs) occurs later in development than male meiosis, in gynoecia that range in size between 0.3 and 0.8 mm. The earliest stages in EMCs coincide with the second division to tetrad stages in pollen mother cells. However, the details of meiotic chromosome behaviour in EMCs correspond closely to the observations we have previously made in male meiosis. In addition, BrdU labelling coupled with an immunolocalisation detection system was used to mark the S phase in cells preceding their entry into prophase I. These techniques allow female meiotic stages of *Arabidopsis* to be analysed in detail, from the S-phase through to the tetrad stage, and are shown to be equally applicable to the analysis of female meiosis in meiotic mutants.

Keywords *Arabidopsis* · Meiosis · Embryo sac mother cells · Megasporogenesis

Introduction

Although considerable progress has been made in understanding many aspects of floral and seed development in *Arabidopsis*, relatively little attention has been given to investigating female meiosis. Earlier studies of megasporogenesis focussed mainly on ovule and embryo sac development, based on section and whole mount preparations, and cytogenetical descriptions of chromosome behaviour during meiosis were necessarily limited (Webb and Gunning 1990, 1994; Robinson-Beers et al. 1992; Schneitz et al. 1995; Bajon et al. 1999).

S.J. Armstrong (✉) · G.H. Jones
School of Biosciences, The University of Birmingham, B15 2TT, Birmingham, UK
e-mail: s.j.armstrong@bham.ac.uk

Generally, cytogenetical investigation of female meiosis in plants has been limited by the technical problems associated with obtaining good-quality preparations of embryo sac mother cells (EMCs) from gynoecia. Early methodology relied on a squashing technique (Darlington and La Cour 1966) which usually proved more successful with large EMCs and large chromosomes, as for example in *Lilium* and *Fritillaria*. Improved methodology using enzyme digestion combined with squashing was introduced by Jongedijk (1987), and has proved useful when applied to species with small chromosomes, such as members of the family Solanaceae (Jongedijk 1987; Havekes et al. 1997). The small genome size, tiny chromosomes and small floral structures of *Arabidopsis* confounded these difficulties. An enzyme digestion, acid hydrolysis and air-drying procedure developed for male meiosis in *Arabidopsis* (Ross et al. 1996, 1997) has been adapted for female meiosis, and applied to wild-type material and to two T-DNA meiotic mutants. To facilitate observations of female meiosis we have linked meiotic stages to key landmarks of floral development and to external features of the gynoecium. To complete the cytological description of female meiosis, we have also investigated the meiotic interphase and S-phase preceding entry into prophase I. Incorporation of the thymidine analogue, bromodeoxyuridine (BrdU) during this S phase has been detected immunologically by means of anti-BrdU antibody.

Material and methods

Plant material

Seeds of *A. thaliana*, accession Wassilewskija (WS) and two meiotic mutants *asy 1* and *dsy 1*, derived from the Feldman T-DNA transformed lines 243 and 2836, respectively (Ross et al. 1997) were sown on a soil-based compost, and grown at 18°C with a 16-h light cycle.

Fixation

Whole inflorescences were taken at the stage when white petals emerged from the largest buds and fixed in Carnoy's fixative (ab-

solute ethanol-chloroform-glacial acetic acid, 6:3:1). The buds were fixed at room temperature overnight and stored at –20°C after replenishing the fixative.

Morphological markers for meiotic stages

All buds were removed from individual inflorescences and ordered according to their size. Using a Zeiss stereomicroscope (Stemi 2000) gynoecia were carefully removed from individual buds with needles. Each gynoecium was measured with a Zeiss eyepiece graticule from the base of the gynoecium to the tip of the pistil. Meaurements were converted using a slide micrometer to give absolute lengths. Features also noted were initiation and development of papillae on the stigma and presence of immature stamens (white or green, depending on whether fixed or not) or mature stamens (yellow). Ten gynoecia were measured in categories from 0.3–0.9 mm with 0.1 mm increments. Slides were subsequently made from five gynoecia in each category and the slides screened for meiotic stages in EMCs.

Cytology

Fixed gynoecia in the range 0.3–0.9 mm were transferred to a black watchglass containing 3:1 fixative (ethanol:acetic acid). The fixative was changed twice to remove all of the original Carnoy's fixative, which interferes with enzyme digestion. Gynoecia 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. The buffer was changed twice before incubating with an enzyme mixture. This comprised of 0.3% w/v cellulase (C1794 lot 95104025), 0.3% w/v cytohelicase (C8274 lot 070H0867) and 0.3% w/v pectolyase (P5936 lot 481376) (all Sigma) in citrate buffer for 30–60 min (shorter times preserve the organisation of the tissues surrounding the meiocyte). Replacing the enzyme mixture with ice-cold citrate buffer stopped the reaction. Single gynoecia 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 either tapped out in a small volume of buffer, using a fine needle, or left intact to observe the organisation of meiocytes relative to the surrounding tissue. Five microlitres 60% acetic acid was added to the slide, followed by another 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. At this stage the slides can be stored long term, if desired, at –20°C. Slides were stained with DAPI (1 µg/ml) in Vectashield antifade mounting medium (Vector).

Photomicroscopy

Slides were examined with a Nikon Eclipse E600 microscope. Capture and analysis of images was achieved using an image analysis system (Vysis).

Marking the meiocyte S-phase

Stems from well-grown plants were cut under water, using sharp dissecting scissors. Cut ends were quickly transferred to BrdU solution (10^{-2} M) and left for up to 3 h for uptake of BrdU via the transpiration stream and its incorporation into cells in S phase. The buds were fixed after 1 h in BrdU and then at hourly intervals for up to 3 h. Slides of gynoecia in the range 0.3–0.4 mm were made as already described.

Immunofluorescence detection of BrdU

Slides were placed in 2×SSC for 10 min, dehydrated through an alcohol series (70%, 85%, 100%) for 2 min each and dried with a hair drier. They were then placed in a Coplin jar containing 70% formamide in 2×SSC pH 7.0, at 75°C for 2 min, thereby denaturing the DNA and allowing access to the antibodies. After 2 min the slides were snap chilled by briefly placing them in TNT buffer (100 mM Tris-HCL pH 7.5, 150 mM Na Cl, 0.5% Tween) at 4°C. One hundred microlitres monoclonal anti-BrdU (Sigma B2531) concentration 1:500 in TNB buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% BSA) was applied to the slide, covered with parafilm and incubated in a humidified atmosphere at room temperature for 20 min. The slides were washed 3×5 min each in TNT buffer. A second antibody, rabbit anti-mouse FITC (Sigma F7506) 1:1000 in TNB buffer was applied, incubated for 20 min and washed 3×5 min each in TNT buffer. A final antibody, goat anti-rabbit FITC (Sigma F9262) concentration 1:1000 in TNB was applied, incubated for 20 min and washed 3×5 min in TNT buffer. Slides were counterstained with DAPI (1 µg/ml) in Vectashield antifade mounting medium (Vector).

Results and discussion

Each gynoecium of *Arabidopsis* accession WS contains about 40 ovules that develop more or less in synchrony, and each ovule contains a single EMC. On average, between four and six analysable EMCs could be obtained from a single gynoecium, a yield of 10–15%. It is likely that this yield could be improved by refining the technique. In common with many other angiosperm plants, male and female meiosis in *Arabidopsis* do not occur in synchrony. Usually female meiosis is delayed relative to male meiosis (Dickinson 1999) and this was also the case in *Arabidopsis*.

Meiotic progression and floral development

There is a clear relationship between meiotic progression and gynoecium length and other developmental parameters including the morphology of the gynoecium and maturity of pollen in anthers (Table 1). In gynoecia 0.3–0.4 mm long the end of the pistil is open, and has a distinctly slotted appearance. Anthers in buds at this stage are green and pollen mother cells (PMCs) are at the dyad/tetrad stage. Gynoecia at this stage contain only very early (pre prophase I) meiocytes.

Gynoecia 0.5 mm long are characterised by closure of the pistil end and the first appearance of stigmatic papillae; unfixed anthers are still green at this stage and contain immature pollen. Embryo sac mother cells (EMCs) from gynoecia at this stage show a range of prophase I stages from leptotene to diplotene, with a mode at pachytene. This stage variation reflects both some asynchrony between ovules within gynoecia and developmental variation between gynoecia of approximately the same length. Gynoecia in the size range 0.3–0.5 mm are considered to be from buds at floral stage 10, as defined by Smyth et al. (1990). Gynoecia 0.6 mm long contain EMCs that are also mostly at pachytene, but show a range of stages from leptotene to metaphase I. Gynoecia 0.7 mm long exhibit stages ranging from pachytene to metaphase II with a mode at metaphase I, and 0.8-mm gynoecia contain stages from diakinesis to the tetrad

Table 1 Summary of relationship between floral landmark, developmental stage of gynoecium and female meiotic stage

Floral stage	Floral landmark	Gynoecium morphology	Gynoecium length (mm)	Female meiotic stage
10	Petals level with short stamens, anther green	Cylinder deeply slotted	0.3–0.4	pre-meiosis
		Closure of cylinder, appearance of stigmatic papillae	0.5	Leptotene-diplotene
11	Anthers green, but changing to yellow as gynoecium enlarges	Stigmatic papillae develop	0.6	Leptotene-MI
	Anthers yellow		0.7 0.8	Pachytene-MII Diakinesis-tetrads

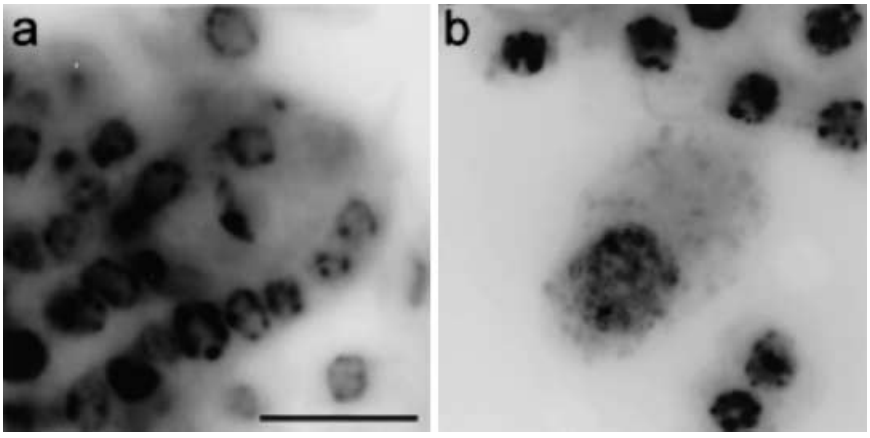

Fig. 1a,b General views of *Arabidopsis* EMCs as seen in sectioned (*a*) and spread (*b*) preparations of *Arabidopsis* ovules to show their relationship to surrounding cells. The EMCs are seen to be distinctive cells, much larger than the surrounding cells of the nucellus, and possessing distinctive nuclear morphology and organelle-rich cytoplasm. The EMCs in these examples are at the pachytene (*a*) and premeiotic interphase (*b*) stages. The preparations were stained with DAPI and the images inverted to give dark-cell structures against a light background. *Bar* = 10 µm

stage. Gynoecia 0.9 mm long contain post-meiotic stages. In the size range 0.6–0.9 mm gynoecia have progressively longer stigmatic papillae and the anthers are yellow, that is they contain mature pollen; they are considered to be from buds at floral stage 11.

Meiotic cytology

In female meiosis of *Arabidopsis*, the single-celled archesporangium functions directly as the EMC and forms a distinctive cell even in the early meiotic and premeiotic stages. In sections the EMC is seen to be more than 3 times larger than surrounding cells (Fig. 1a; also see Webb and Gunning 1990; Bajon et al. 1999). Similarly, in spread preparations the EMC is clearly larger than any of the surrounding cells of the ovule (Fig. 1b) and consists of a large nucleus with a prominent nucleolus and a large volume of cytoplasm having prominent organelles. The spreading technique is distinct from the squashing procedure previously applied to ovules, and yields very clear images of EMC meiosis. This method is transferable, and we have successfully applied it to *Brassica oleracea* and tomato EMCs. A great advantage of this system for analysing *Arabidopsis* meiosis is that the EMCs are such distinctive cells that there is no possibility of confusing them with other cells of the ovule, whereas in anther preparations PMCs in early stages of development may be confused with tapetal cells (Martinez-Perez et al. 1999).

Meiotic S-phase

For identification of the meiotic S-phase, anti-BrdU antibody was applied to preparations of 0.3–0.4 mm gynoecia. During preparation the gynoecia were not physically disrupted, in order to preserve the ovule tissues surrounding the EMCs. After applying the FITC-tagged detection molecules and viewing with a fluorescence microscope, cells that had incorporated BrdU, and were therefore in S phase at the time of labeling, showed bright nuclear fluorescence (Fig. 2a). Labeled EMC nuclei were seen at all sampling times, including the shortest period of 1 h, and showed that the BrdU was rapidly translocated to the meiocytes and utilised by cells that were replicating their DNA. Although it was clear that some somatic cells from supporting tissues were also labeled, they could be distinguished from meiocytes by their smaller nuclear size and absence of large amounts of cytoplasm. From these current observations we cannot estimate the length of the S phase in the meiotic pathway of *Arabidopsis*, but we can be confident that the marked cells seen after 1 h labeling were almost exclusively

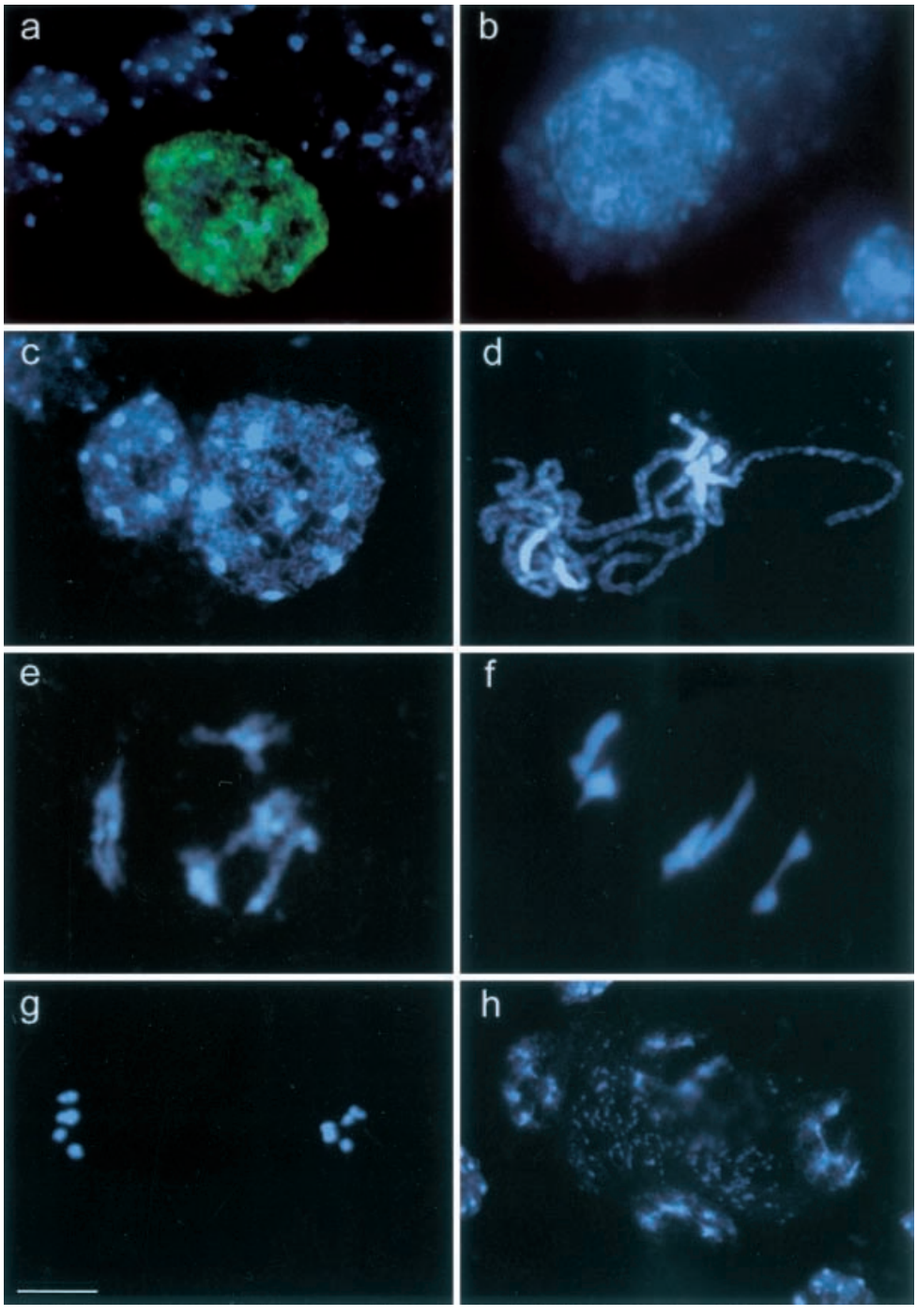

Fig. 2a–h Female meiosis in wild-type EMCs of *Arabidopsis* prepared by the spreading technique and stained with DAPI. **a** Meiotic S-phase identified by anti-BrdU antibody staining (*green*). **b** Meiotic S-phase, without antibody staining, showing the characteristic extension of pericentromeric heterochromatin. **c** Early leptotene showing thin extended chromosome axes and condensed heterochromatin (*right* nucleus). **d** Pachytene showing fully synapsed homologues. **e** Diakinesis showing five moderately condensed but unaligned bivalents, each with a pair of pericentromeric heterochromatin regions. **f** Metaphase I showing five fully condensed and aligned bivalents. **g** Metaphase II showing two groups of five chromosomes. **h** Tetrad stage showing a group of four haploid nuclei at the termination of meiosis. *Bar* = 10 µm

S-phase cells. It is likely that a proportion of cells that had been exposed to BrdU for 3 h would have progressed to G2/early leptotene. In meiocytes labeled for 1 h only, and therefore presumed to be in S phase, the pericentromeric heterochromatin regions were extended (Fig. 2b). This decondensation of the heterochromatin appeared to be characteristic of the pre-meiotic S phase. Somatic S-phase cells also appeared to show some decondensation of heterochromatin but not to the same extent as pre-meiotic S-phase cells (Fig. 2b). A proportion of labeled meiocytes from the 3-h treatment had condensed pericentromeric heterochromatin, suggesting that as meiocytes leave the S-phase and enter G2/leptotene there is a programmed change in heterochromatin condensation. To our knowledge this is the first demonstration of BrdU labelling for the identification of meiotic S-phase in plants.

Meiosis

The progression of meiosis in EMCs of the WS accession closely resembles that seen in PMCs (Ross et al.

1996) and therefore is described only briefly. However, the large size of EMCs confers certain advantages since the meiotic chromosomes are often particularly clear, and it may be that this system will be preferable to PMCs for some experimental and analytical purposes.

Early prophase I

The earliest prophase I chromosomes stained with DAPI showed the chromosomes as unsynapsed threads with evidence of a chromomere pattern (Fig. 2c). Up to 14 relatively large DAPI-bright regions could be seen due to condensation of the pericentromeric and NOR-associated heterochromatin. Two small DAPI-bright regions were observed in some cells and were interpreted as the chromomere on the short arm of chromosome 4 that is known to be present in accession WS (Fransz et al. 1998).

Early zygotene cells demonstrated the first signs of synapsis, and as zygotene progressed the proportion of synapsed chromosome regions increased. The pericentric heterochromatin could be either synapsed or unsynapsed, depending on how advanced in zygotene the cells were, and consequently the number of condensed heterochromatin regions was variable. In some cells the pericentric heterochromatin was restricted to a small region of the nucleus; this was presumed to reflect the nuclear organisation that is referred to as the synizetic knot, a feature that is typical of meiotic prophase I in plants. The asymmetric distribution of organelles that characterises the equivalent stage of male meiosis (Ross et al. 1996) was not seen. Rather, the organelles appeared to be distributed evenly throughout the cytoplasm.

In pachytene nuclei the homologous chromosomes were fully synapsed and therefore showed thicker and more heavily staining chromosome threads, with obvious chromomere differentiation (Fig. 2d). The two NORs on the short arms of chromosomes 2 and 4 were usually joined due to their association with the single nucleolus.

Late prophase I-diplotene and diakinesis

Late diplotene-diakinesis cells contained five compact bivalents that were differentiated by DAPI staining (Fig. 2e). The pericentromeric heterochromatin was characteristically DAPI bright at this stage and marked the centromeric region of each chromosome. The three longer bivalents, 1, 3 and 5, are metacentric/submetacentric, whilst the acrocentric chromosomes (2 and 4) could be easily identified by their relatively smaller size, their morphology and their association with the nucleolus. The bivalents in EMCs had either a single chiasma (typical of the acrocentric chromosomes) or two chiasmata, generally either both distal or an interstital and a distal chiasma, forming a ring bivalent. No bivalents with three chiasmata were seen in the small sample of EMCs scored. The mean chiasma frequency of EMCs was estimated to be 8.5 from a sample of ten diakinesis and metaphase I cells. This sample was necessarily rather small, and chiasma scoring in *Arabidopsis* is possibly imprecise due to its extremely small chromosomes. Nevertheless, this value is distinctly lower than our estimate of 9.7 for PMC mean chiasma frequency (based on 14 diakinesis and 14 metaphase I cells). Albini et al. (1992) reported a mean chiasma frequency of 10.1 for PMCs. Although these sets of data were collected at different times, the trend is in agreement with genetic recombination differences observed by Vizir and Korol (1990) between male and female *Arabidopsis* meiosis.

Metaphase I and the second division

These stages were seen infrequently. At metaphase I the five bivalents were maximally condensed, and it was difficult to differentiate the heterochromatin and euchromatin (Fig. 2f). The bivalents were co-orientated with homologous centromeres directed towards opposite poles. Anaphase I was characterised by separation of homologues to opposite poles. At metaphase II (Fig. 2g) the individual chromosomes were aligned on the equator and they separated to the poles during anaphase II. Chromosome orientation suggested that the spindles of meiosis I and II were at right angles to one another. These divisions resulted in four nuclei in a common cytoplasm (Fig. 2h), which has been termed the ‘promegasporotic cell’ by Webb and Gunning (1990).

Female meiosis in meiotic mutants

Previous observations of female meiosis in meiotic mutants of *Arabidopsis* are rather scarce, and rely upon sectioning (Bhatt et al. 1999; Couteau et al. 1999) or cleared whole-mount preparations (Couteau et al. 1999; Motamayor et al. 2000). While these methods permit an overview of megasporogenesis, they are not ideal for the cytogenetic analysis of meiosis in *Arabidopsis.*

The meiotic gene *ASY1* has been recently characterised (Caryl et al. 2000) and has been shown to be a homologue of the *HOP1* gene of S*accharomyces cerevisiae*. The original cytogenetic description of meiosis in *asy1* and other mutants focused on male meiosis in PMCs (Ross et al. 1997). A brief description establishes that the same defects are present in female meiosis of this mutant.

At early leptotene (Fig. 3a), faint thread-like chromosomes were seen that resembled those seen in the wild-type leptotene stage. In *asy1* male meiosis, at a stage equivalent to zygotene, some very short stretches of possible homologous synapsis were observed. However, there was no indication of this in female meiosis (Fig. 3b), but there was some evidence of homologue alignment (not shown). As in male meiosis of this mutant, progression to the typical pachytene stage, with fully synapsed bivalents, was never seen. By diakinesis, the discrete chromosomes were clearly seen to be uncon-

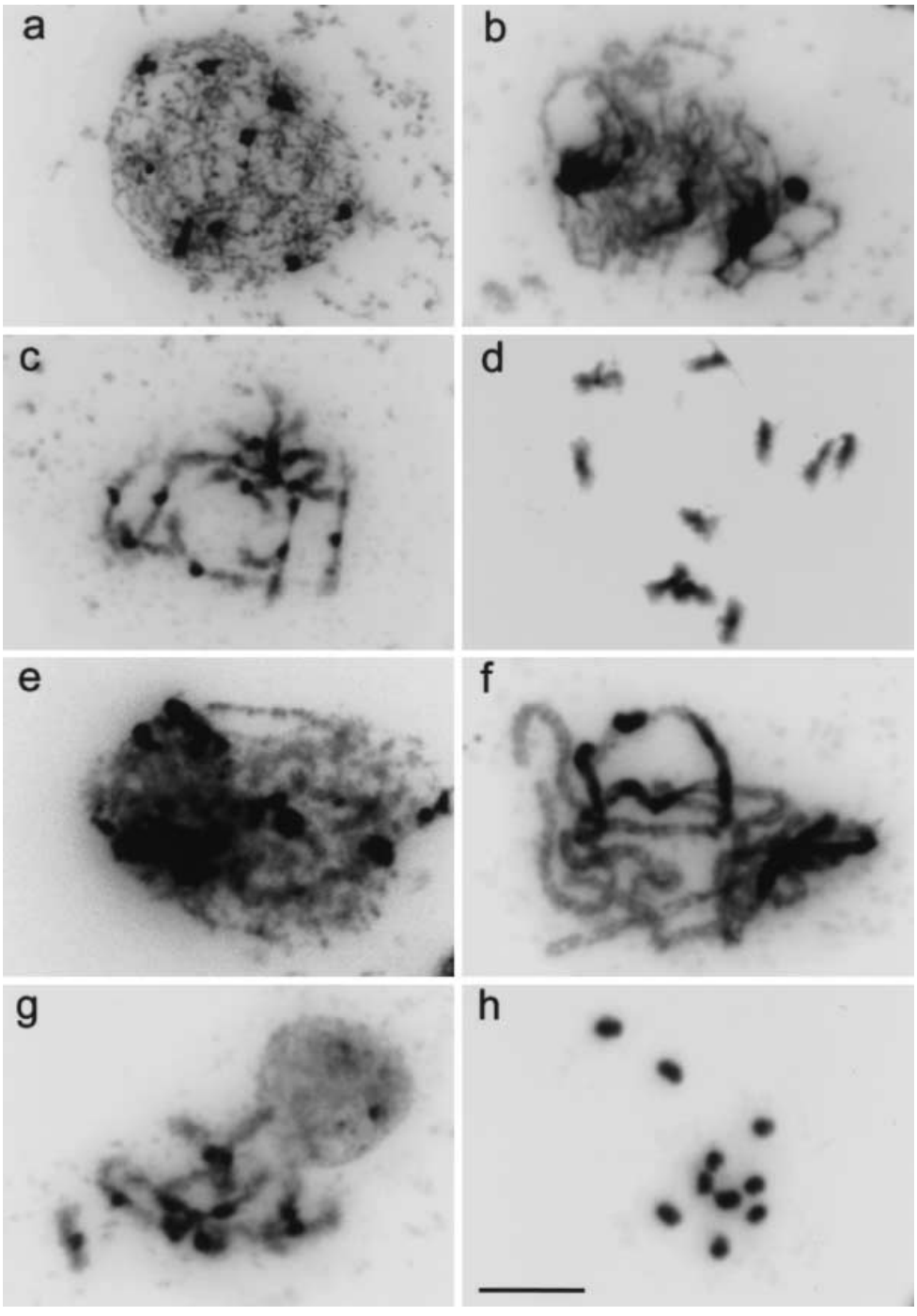

Fig. 3a–h Female meiosis I in asynaptic (*asy1*) and desynaptic (*dsy1*) meiotic mutants of *Arabidopsis* prepared by the spreading technique and stained with DAPI. Images inverted to give dark cellular structures against a light background. **a** Leptotene in *asy1* showing extended unsynapsed chromosome threads. **b** Mid-prophase I (corresponding to zygotene in wild-type) in *asy1* showing failure of synapsis. **c** Diplotene in *asy1* showing unassociated univalents, except for the NOR bearing chromosomes 2 and 4 clustered around the nucleolus. **d** Diakinesis in *asy1* showing ten separate univalents. **e** Leptotene in *dsy1* showing extended unsynapsed chromosome threads alongside a somatic nucleus of the nucellus. **f** Pachytene in *dsy1* showing fully synapsed homologues. **g** Diplotene/diakinesis in *dsy1* showing the five chromosome pairs still loosely associated. **h** Metaphase I in *dsy1*showing ten fully condensed univalents

nected to their homologues (Fig. 3c), with the exception of nucleous organising chromosomes 2 and 4 which appeared to be associated, but this is likely to reflect attachment to the nucleolus. At metaphase I (Fig. 3d) the chromosomes were all univalent reflecting the earlier failure of synapsis. In our sample of EMCs, no bivalents were seen. Because of the failure of synapsis, subsequent stages, anaphase I and division II, exhibited irregularities associated with non-disjunction.

Meiosis in EMCs of the desynaptic mutant *dsy1* (Fig. 3e–h) also appeared to correspond to that seen previously in PMCs (Ross et al. 1997) and is therefore described only briefly. Early meiotic prophase up to and including pachytene (Fig. 3e,f) appeared to be normal. However, at diakinesis it was obvious that although the chromosomes were aligned, they did not form bivalents (Fig. 3g). As in *asy1*, no chiasmata were detectable in EMCs and all chromosomes appeared as univalents at metaphase I (Fig. 3h). Subsequent chromosome behaviour resembled that described for *asy1*.

It is not surprising to find that the *asy1* and *dsy1* mutants are defective in both male and female meiosis,

since it must be assumed that they share many gene-regulated processes that are fundamental to the efficient progression of meiosis. This reflects the situation that is found generally in plants. Although sex-specific (mostly male-sterile) mutants have been described, most of them appear to be defective in either premeiotic developmental events or the cellular milieu of the meiocytes, both of which differ between male and female reproduction in plants (Baker et al. 1976).

An interesting exception is the recently described *swi1* mutant of *Arabidopsis*, which appears to be defective in female meiosis only (Motamayor et al. 2000). However, this appears to be an allele-specific effect since another mutant allele of the same gene (*swi1-2*) is defective in both male and female meiosis (R. Mercier and C. Horlow personal communication).

The overall conclusion from this study is that female meiosis in *Arabidopsis* is amenable to cytogenetical analysis, both in wild-type and in mutant plants. Furthermore, *Arabidopsis* EMCs constitute a potentially valuable system for the experimental analysis of meiosis.

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