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Identification and analysis of *DYAD*: a gene required for meiotic chromosome organisation and female meiotic progression in *Arabidopsis*

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SUMMARY

The *dyad* mutant of *Arabidopsis* was previously identified as being defective in female meiosis. We report here the analysis of the *DYAD* gene. In ovules and anthers *DYAD* RNA is detected specifically in female and male meiocytes respectively, in premeiotic interphase/meiotic prophase. Analysis of chromosome spreads in female meiocytes showed that in the mutant, chromosomes did not undergo synapsis and formed ten univalents instead of five bivalents. Unlike mutations in *AtDMC1* and *AtSPO11* which also affect bivalent formation as the univalent chromosomes segregate randomly, the *dyad* univalents formed an ordered metaphase plate and underwent an equational division. This suggests a requirement for *DYAD* for chromosome synapsis and centromere configuration in female meiosis. The *dyad* mutant showed increased and persistent expression of a meiosis-specific marker, p*AtDMC1::GUS* during female meiosis, indicative of defective meiotic progression. The sequence of the putative protein encoded by *DYAD* did not reveal strong similarity to other proteins. *DYAD* is therefore likely to encode a novel protein required for meiotic chromosome organisation and female meiotic progression.

Key words: Synapsis, Centromere, Cohesion, Polarity, Meiosis, *DYAD*, *Arabidopsis thaliana*

INTRODUCTION

Balanced chromosome segregation at meiosis requires that the cell halves its chromosome number, yet inherit a full complement of genetic information. This is accomplished by ensuring that homologous chromosomes move to opposite poles of the meiotic spindle, and that sister centromeres remain associated at anaphase 1 and separate only at anaphase 2. Following premeiotic DNA synthesis, homologous chromosomes pair and undergo synapsis in most organisms to form bivalents. Genetic recombination leads to the formation of chiasmata which represent crossovers and serve to form stable connections that hold homologues together in the bivalent. Bivalents stay together until anaphase 1 when they dissociate, and each univalent, consisting of a sister chromatid pair, separates from its partner and moves to opposite poles of the meiotic spindle. The controlled union and separation of chromosomes is therefore a multistep process that is central to meiosis.

In higher plants, meiosis is also the transition from a diploid sporophyte to a haploid gametophyte generation. The gametophyte in higher plants consists of a small number of cells surrounded by the sporophyte. In the pathway leading to female gametophyte development in *Arabidopsis*, a single subepidermal cell at the tip of the ovule primordium becomes specified as the archesporial cell and undergoes meiosis to produce a tetrad of four spores. Three of the spores degenerate, and one becomes the functional spore, going on to develop into the female gametophyte (Misra, 1962). Lately there has been a renewed interest and significant advances in our understanding of the molecular mechanisms underlying archesporial cell specification, meiosis and gametophyte development in plants (reviewed by Yang and Sundaresan, 2000; Bhatt et al., 2001).

Recent studies suggest that several of the basic mechanisms underlying meiotic functions are conserved between higher plants and fungi. Analysis of the plant homologues of the yeast *DMC1* and *RAD51* genes, which encode strand exchange proteins indicates expression in meiotic cells and localisation of Dmc1p and Rad51p on meiotic chromosomes (Klimyuk et al., 1997; Anderson et al., 1997; Franklin et al., 1999). The *Arabidopsis dmc1* mutant has been shown to be defective in bivalent formation (Couteau et al., 1999). The phenotype of the *Arabidopsis asy1* mutant defective in chromosome synapsis has been shown to be due to a mutation in an *Arabidopsis* homologue of the yeast *HOP1* gene which is required for homologous pairing (Caryl et al., 2000). The *Arabidopsis SYN1/DIF1* gene encodes a homologue of the yeast REC8 cohesin and is required for chromosome segregation in meiosis (Bai et al., 1999; Bhatt et al., 1999) A mutation in an *Arabidopsis* homologue of the *SPO11* gene, which encodes a type II topoisomerase responsible for generating double strand breaks in meiosis in yeast, has been shown to reduce meiotic recombination and bivalent formation (Grelon et al., 2001).

The molecular analysis of plant mutants has also revealed new information on meiosis. The *STERILE APETALA* gene of *Arabidopsis* codes for a transcription factor required for female meiosis and also plays a role in inflorescence and floral development (Byzova et al., 1999). The *Arabidopsis ASK1* gene encodes a *SKP1* homologue required for separation of homologous chromosomes in male meiosis (Yang et al., 1999). Additional *Arabidopsis* mutants defective in meiosis have been described and several have been characterized at the molecular level (Ross et al., 1997; Hulskamp et al., 1997; Spielman et al., 1997; Glover et al., 1998; He and Mascarenhas, 1998; Sanders et al., 1999). It is likely that the molecular analysis of these as well as meiotic mutants in maize [summarised by Curtis and Doyle (Curtis and Doyle, 1991)] will reveal information on meiotic processes including chromosome dynamics in plants.

The present study was undertaken to understand the role of the *DYAD* gene of *Arabidopsis* in female meiosis and megasporogenesis. The *dyad* mutant was previously identified as being specifically defective in female meiosis (Siddiqi et al., 2000). While this work was in progress a related study appeared on the *SWI1* gene (Mercier et al., 2001) and we note that *DYAD* is identical to *SWI1*. We show here that *DYAD* RNA is expressed in female and male meiocytes. Furthermore, the *dyad* mutant is defective in synapsis and bivalent formation, in centromere organisation and cohesion, as well as in progression through female meiosis.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis strains and growth conditions were as described earlier (Siddiqi et al., 2000). The No-O ecotype was used as the wild-type parent in crosses to *dyad* to generate an F_2 mapping population.

SSLP and CAPS marker analysis

A set of 7 SSLP and 9 CAPS (Konieczny and Ausubel, 1993) markers were developed based on the sequence of the genomic DNA for the region south of the nga129 marker on chromosome 5 where *dyad* maps. Details of markers are available on request. In the case of CAPS markers, primers were designed to PCR amplify a 1-2 kb region of genomic DNA which was then digested with a panel of restriction enzymes, and electrophoresed on a gel to identify polymorphisms between the L*er* and No-O ecotypes and in some cases between L*er* and Col-O. Recombinants north and south of *dyad* were first screened and identified with respect to the markers nga129 (north of *dyad*) and KMR (south of *dyad*). DNA from 1-2 inflorescences per *dyad* plant from the F_2 mapping population was isolated using the Nucleon Phytopure kit (Amersham) and typed with respect to the markers. For mapping the position of crossovers between the markers and the *dyad* locus, DNA from the recombinants was typed with respect to one or more of the markers between nga129 and KMR.

Transformation and complementation analysis

DNA from the P1 clone MFG13 which contained the *dyad* locus was extracted by alkaline lysis. The region from 3-25 kb containing 6 complete genes as predicted by the GENSCAN 1.0 program (Burge and Karlin, 1997) was subcloned into pBSII(KS+) as a set of 2 *Sal*I and 2 *Xba*I fragments: S1 (3878-9679), X1 (7114-15855), S2 (11076-24875) and X2 (16344-24659). These constituted an overlapping set which contained each of the 6 predicted genes in intact form in at least one clone. For transformation, each fragment was then subcloned into the binary vector pBINPLUS (van Engelen et al., 1995) and introduced into the *Agrobacterium* strain AGL1 by triparental mating using *E. coli* HB101(pRK2013) as a helper. To test for complementation, F_1 plants from a cross of *dyad* (L*er* at the *dyad* locus) to wild-type Col-O were transformed with the respective clones by in planta transformation (Bechtold et al., 1998). Transformants were selected on MS (Murashige-Skoog) plates containing 2% sucrose and kanamycin at 50 μg/ml. 20-50 transformants for each clone were selected and characterized with respect to their phenotype (mutant or wild type) and genotype at the *dyad* chromosomal locus. The genotype at the *DYAD* locus was assigned based on the markers KKL and KNE, which are closely linked to *DYAD* and on either side of it, and are outside the 25 kb genomic region being tested for complementation.

Light microscopy

Developmental analysis of cleared ovules and of p*AtDMC1::GUS* expression was as described earlier (Siddiqi et al., 2000). A rapid method for scoring of the defective meiosis phenotype seen in *dyad* was employed to confirm *dyad* mutant plants when screening the mapping population. Pistils from young unopened buds were dissected in a droplet of 3 N NaOH on a slide to reveal ovules, followed by mounting with a coverslip and observation under DIC optics at 40× magnification using an Olympus BX60 microscope. Although the method did not give details of intracellular structure, the 2-4 enlarged cells characteristic of the *dyad* mutant could be unambiguously distinguished from wild type.

Fluorescence microscopy of meiotic chromosomes

Analysis of meiotic chromosome spreads of female meiocytes was carried out according to the method of Armstrong et al. (Armstrong et al., 2001) with minor modifications, which were based on availability of materials. The enzyme digestion mixture contained cellulase/pectinase/driselase all at 0.3%. 3% stock solutions of cellulase (C9422, Sigma), and driselase (D9515, Sigma) were prepared in 10 mM citrate pH 4.5/45% glycerol and stored at –20°C as was pectinase (P4716, Sigma). Staining of chromosomes was done using 1 μM Hoechst 33342 in PBS/40% glycerol. Chromosomes were observed on a Zeiss Axioskop microscope using a 365 nm excitation, 420 nm long-pass emission filter and photographed using 50 ASA Kopex Rapid film with exposure times of 4 seconds to 30 seconds. Negatives were scanned and images inverted and edited using Adobe Photoshop.

cDNA isolation and expression analysis

Total RNA was isolated from inflorescences using the RNEasy plant RNA isolation kit (Qiagen). After treatment with RQ1 DNAase (Promega), 5 μg of RNA was used for cDNA synthesis using the One-step RT-PCR kit (Qiagen) and the primers ismf2 (5′TGGTACTTTTAAATACCTGCTCGCTTGT3′; 5211-5238 of MFG13) and 5rf1 (5′GGAGGAACGAAGATTATCGAGAGCA3′; 8294-8270 of MFG13) for the primary PCR. A secondary PCR was then performed using the primers 5rf1 and 3rr1 (5′CATGGAAGAGACCTTACCAGTTCACATCA3′; 5255-5283 of MFG13). The amplified cDNA was directly sequenced and also cloned into a pGEM-T vector (Promega). Analysis of *DYAD* expression was carried out by PCR on cDNA synthesised using the gene-specific primers ismf2 for *DYAD* and gapc2 (5′CCTGTTGTCGCCAACGAAGTCAG3′) corresponding to the cytosolic *GAPDH* gene for normalisation. 1-5 μg of total RNA was used for cDNA synthesis in a volume of 40 μl. 0.5-2.0 μl of the cDNA synthesis reaction was used for PCR in a volume of 40 μl. The primers used for detecting *DYAD* expression were, ismr1 (5′GGCAAAGGAGATAACTAATGGAAATCGTA3′; 7026-6998 of MFG13) and 3rr1, which gave a 1.26 kb product corresponding to the 3′ portion of the coding region plus 80 bp of 3′ untranslated region. *GAPDH* expression was detected using the primers gapc1 (5′CTTGAAGGGTGGTGCCAAGAAGG3′) and gapc2. The

products were electrophoresed on a 1% agarose gel, blotted on Hybond N+ membrane (Amersham) and probed with the respective probes which were labelled with ^{32}P.

In situ hybridisation was carried out as described earlier (Siddiqi et al., 2000) using antisense riboprobe synthesised from the complete coding region of the *DYAD* cDNA. Control experiments using sense RNA gave no signal (data not shown).

RESULTS

Positional cloning of the *DYAD* gene

The *dyad* mutant shows female sterility due to a defect in meiosis. Mutant ovules contain two enlarged cells in place of an embryo sac (Fig. 1A,B) and these are the products of a single division of the megaspore mother cell followed by an arrest in further development in the majority of ovules. The phenotype of the *dyad* mutant is caused by a single recessive gene that maps 3 cM south of the nga129 marker on chromosome 5 (Siddiqi et al., 2000). A positional cloning approach was adopted to identify the gene. An F_2 mapping population was generated by crossing the mutant in L*er* background to wild-type No-O. The *DYAD* gene was further localised with respect to the SSLP marker KMR and found to map 1.2 cM north of KMR and 2.3 cM south of nga129 based on analysis of 956 F_2 mutant plants from the mapping population (Fig. 1C). Forty-five recombinants were obtained between *dyad* and nga129 and 23 recombinants between *dyad* and KMR. SSLP markers were developed for this region using the available Columbia sequence information and used to identify polymorphisms between L*er* and No-O ecotypes. In addition CAPS were developed and used to map the position of crossovers in the recombinants. In all, 7 SSLPs and 9 CAPS markers were developed. Mapping of the recombinant crossovers points between nga129 and KMR led to narrowing the *DYAD* gene to a P1 clone MFG13. Only 1 plant was heterozygous for the MDL marker whereas 2 plants were heterozygous for Mf6 further localising the gene to a 25 kb region on MFG13. Annotation of the region using GENSCAN 1.0 (Burge and Karlin, 1997) identified 6 putative genes.

To test for complementation, DNA from the 25 kb region of the MFG13 P1 clone was subcloned into a binary transformation vector. The 6 genes were represented as 4 overlapping fragments. Plants heterozygous for the *dyad* mutant (L*er*) and the wild-type Col-O allele were transformed with each clone by in planta transformation (Bechtold et al., 1998). Out of the 4 clones, the S1 clone, containing the region from 3878 to 9679 bp of MFG13 as a *Sal*I fragment showed complementation. 50 transformants were typed using CAPS markers KKL and KNE that closely flank *DYAD*. Out of the 12 plants that were homozygous for the L*er* allele for both markers, 10 were fertile, indicating complementation. Seeds from these transformants segregated into fertile and sterile plants in the next generation. This suggests that the 5.8 kb clone has all the regulatory and structural regions required for *DYAD* function. 6 and 4 transformants that were heterozygous and homozygous for the wild-type Col-O respectively, were sterile, presumably as a result of co-suppression. Likewise, the X1 *Xba* 8.7 kb clone did not show any complementation whereas it did show co-suppression.

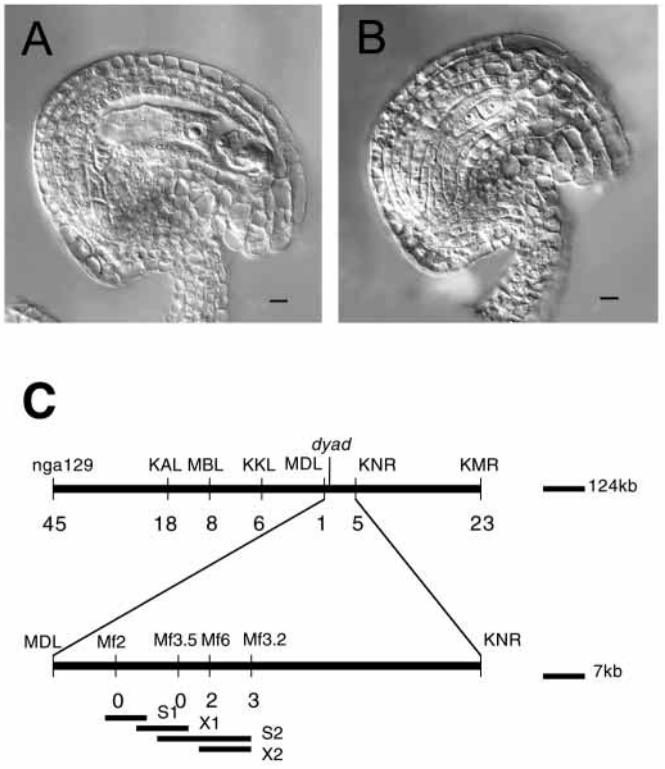

Fig. 1. (A,B) Mature ovule of wild type (A) and *dyad* (B) plants. Bar: 10 μm. (C) Localisation of *dyad* on chromosome 5. The region between the markers nga129 and KMR gives the positions of DNA markers and number of crossovers in the respective intervals that were used to localise *dyad*. The portion of P1 clone MFG13 in which *dyad* was mapped to a 25 kb segment between the markers MDL and Mf3.2 is indicated below. The 25 kb region was subcloned as 2 *Sal*I fragments (S1,S2) and 2 *Xba*I fragments (X1,X2) each of which was tested for complementation.

Sequence analysis of the *DYAD* gene

The 5.8 kb complementing clone contained a single predicted gene. Based on the predicted cDNA sequence obtained using NetPlantGene (Hebsgaard et al., 1996), oligonucleotide primers were designed and used to amplify by RT-PCR, a 2.2 kb cDNA. The sequence of the cDNA obtained (GenBank accession no. AF466153) indicated the presence of 7 exons potentially encoding a protein of 635 amino acids and having a mass of 72 kDa (Fig. 2A) in close agreement with that predicted by the annotation (GenBank accession no. AB025621). The sequence contained a high proportion of charged amino acids (17.3% positively charged; 13.9% negatively charged). A potential nuclear localisation signal RKRK was also observed (aa residues 250-253). The region comprising amino acids 407-441 is predicted to adopt a coiled-coil conformation. Homology searches with the Dyad protein sequence filtered for low complexity regions using BLASTP 2.2.1 revealed no strong relatedness to any other known protein. However a low degree of similarity was observed to several proteins including the recently identified Male sterility 1 (Ms1) protein (Wilson et al., 2001) (E=0.008) which has been proposed to be a transcriptional regulator of male gametogenesis in *Arabidopsis*. The similarity to Ms1 is in the region of aa residues 291-349. Using BLASTP 2.1.2

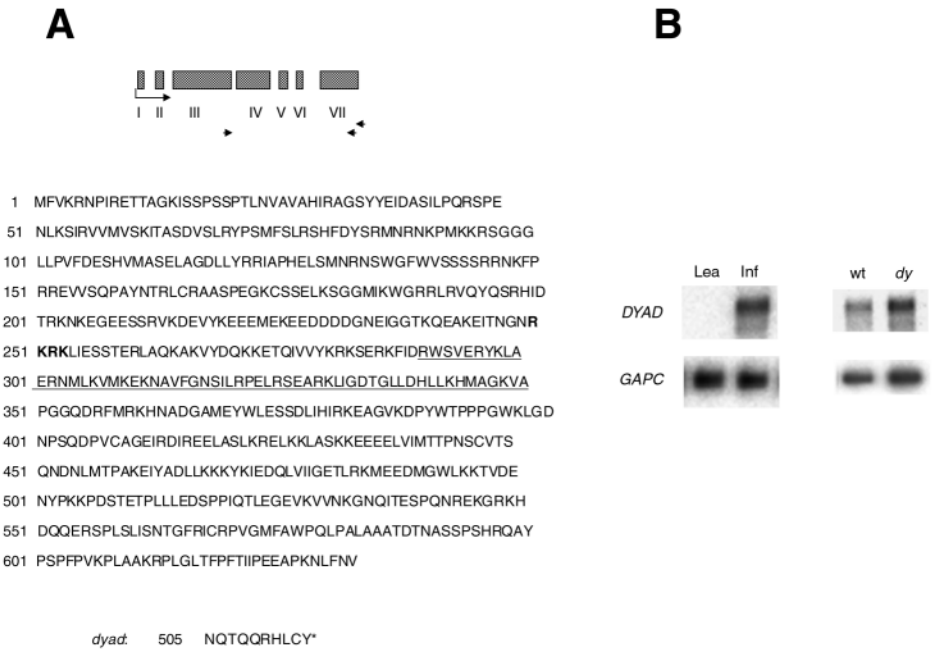

Fig. 2. (A) Sequence analysis of the *DYAD* gene. The top panel is a box diagram representation of *DYAD* showing the positions of the exons. Arrowheads indicate positions of primers used for examination of expression by RT-PCR. The predicted sequence of the DYAD protein is given below, and the region showing similarity to MS1 is underlined. The putative nuclear localisation signal is in bold. The *dyad* mutation causes a frameshift at aa position 505. (B) Analysis of *DYAD* expression by RT-PCR. The left panel compares *DYAD* expression in leaves (Lea) and inflorescence (Inf) of wild type. The right panel examines expression of *DYAD* in the inflorescence of wild type (wt) and mutant (*dy*) plants.

weaker similarity was detected to several other proteins, among them SMC family proteins (Smc3 protein from Bos Taurus, and Basement membrane-associated chondroitin proteoglycan proteins from human, rat and mouse) with an E value of 1.4. The SMC homology was in the region from aa 187 to 332.

Analysis of the *dyad* mutant allele

The 5.8 kb genomic region containing the *DYAD* gene was sequenced after PCR-amplification of DNA from the *dyad* mutant as well as from the wild-type L*er* allele. A comparison of the two sequences revealed that the mutant carried a single base deletion of a C residue at position 5728 with respect to the MFG13 coordinate. The effect of the mutation would be to cause a frameshift starting at amino acid position 505 leading to premature termination after 9 additional amino acids (Fig. 2A). Expression of the *DYAD* gene was compared by RT-PCR using RNA isolated from inflorescences and from leaves. The presence of the transcript was detected in the inflorescence but not in leaves in the experiment shown in Fig. 2B. However using 5- to 10-fold higher amounts of cDNA we were able to detect expression of *DYAD* in leaves (data not shown). This would suggest a low basal level of expression in vegetative tissue compared to that seen in the inflorescence. The mutant showed a level of expression comparable to that in wild type in the inflorescences indicating that it is not a null allele.

DYAD is expressed in female and male meiocytes

To determine the expression pattern of *DYAD* at the cellular level we carried out RNA in situ hybridisation to sections of

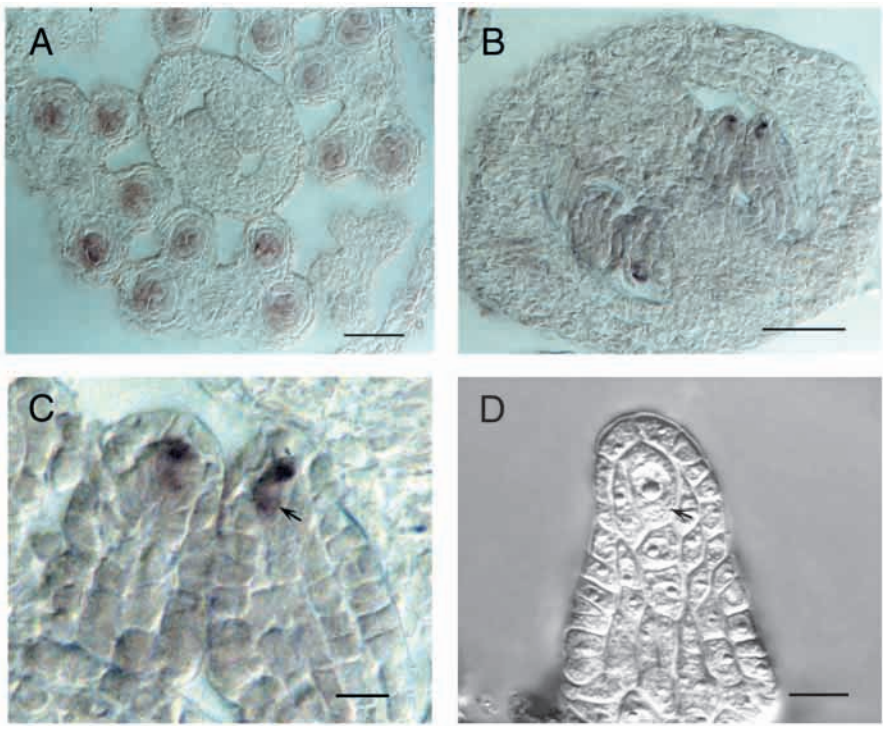

Fig. 3. Expression of *DYAD* in male and female meiocytes. RNA in situ hybridisation of *DYAD* antisense RNA to sections of flower buds. Expression is seen (A) in pollen mother cells and (B) in megaspore mother cells at an early stage of ovule development at the time of integument initiation. (C) Magnification of the distal portion of the upper two ovules shown in B. The signal appears localised towards the apical end of the MMC in both ovules. (D) Whole- mount optical section of a cleared ovule at the same developmental stage as in B,C. Bar: 100 μm (A,B) and 20 μm (C,D).

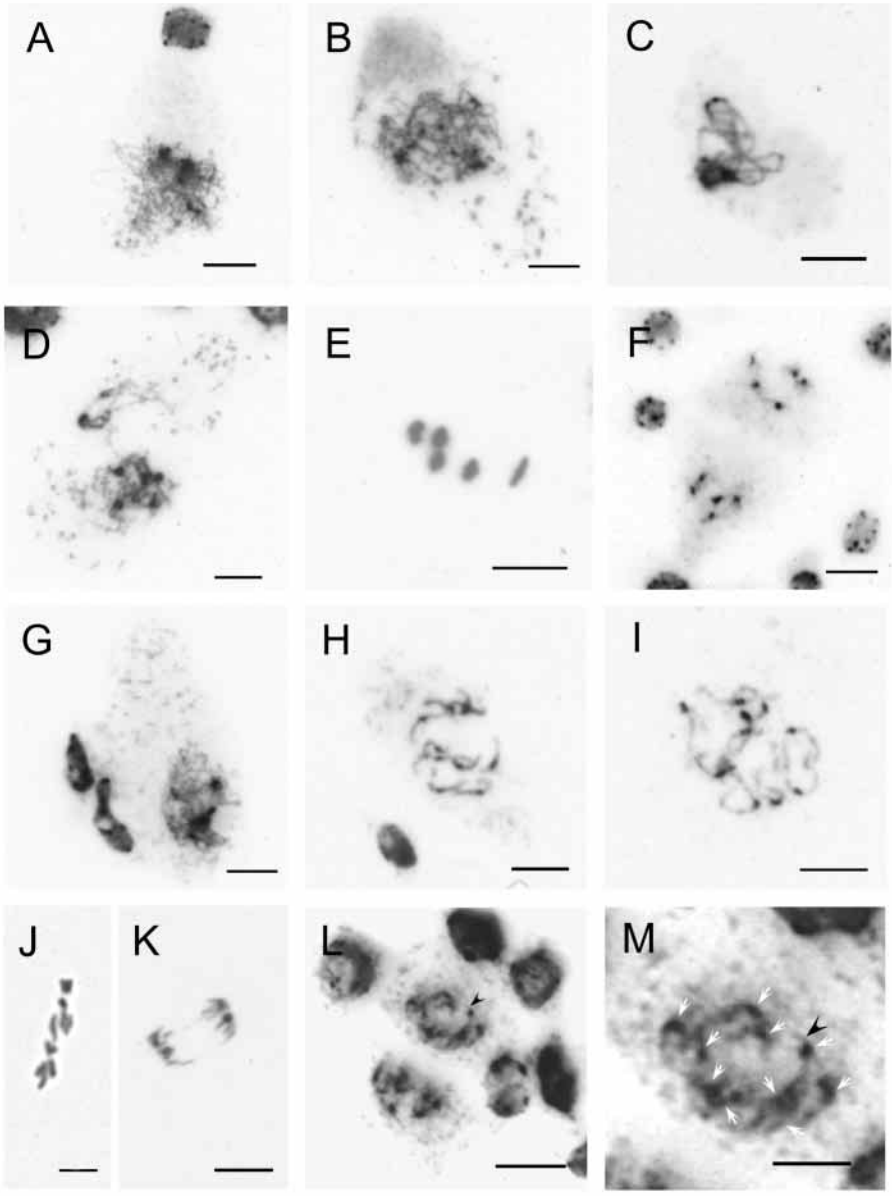

Fig. 4. Chromosome analysis in various stages of female meiosis in wild type and *dyad* plants. (A-F) Wild type, (G-M) *dyad*. (A,G) Chromosomes first become visible as elongated strands during leptotene. (B) Synapsis takes place during zygotene and synapsed regions can be detected as thicker segments. (C) Synapsis is complete at pachytene and chromosomes have a shorter and thicker appearance. (D) Late diplotene stage, when bivalents have undergone partial decondensation of the arms but not centromeric regions and the two halves of bivalents can be seen. (E) Metaphase stage showing five condensed bivalents. (F) Dyad stage after completion of meiosis 1. Each of the two cells contains 5 univalents at prometaphase 2. (H) In *dyad*, 10 univalents are observed instead of 5 bivalents indicating failure to undergo synapsis. (I) The univalents undergo partial decondensation of the arms. (J) Metaphase plate containing 10 chromosomes. (K) Anaphase 1 resembles an equational division. (L) Dyad stage at prometaphase 2 after completion of meiosis 1. (M) Magnification of the upper cell of the dyad in L. The cell is estimated to contain 10 chromosomes (white arrows). Black arrowhead indicates a chromosome with a pair of sister chromatids suggesting an additional round of replication has occurred. Bar: 5 μm (J,M) and 10 μm (A-I,K,L).

inflorescence using antisense RNA complementary to the *DYAD* cDNA as a probe. Expression was seen in male and female sporocytes (Fig. 3). In the megaspore mother cell (MMC), expression was detected in stage 2-1 ovules prior to or at the time of integument initiation and up to stage 2-3. This stage corresponds to premeiotic interphase/meiotic prophase (Schneitz et al., 1995). Expression at later stages was not observed (0 out of 33 ovules showing signal). In anthers, expression was detected in pollen mother cells at an early stage corresponding to premeiotic interphase/meiotic prophase.

DYAD is required for synapsis and reductional chromosome segregation at meiosis 1

The meiotic defect seen in the *dyad* mutant described previously is restricted to the MMC, pollen meiosis being normal. To examine chromosome organization and segregation during meiosis we carried out chromosome spreads of female meiocytes present in early stage ovules using the method of Armstrong et al. (Armstrong et al., 2001) with minor modifications (Fig. 4). Although each ovule contains only one meiocyte, these can be readily distinguished from other cells as they are larger and have enlarged nuclei. In addition they contain a higher number of organelles which give the cells a denser, more granular appearance. Wild-type chromosomes first appeared as thread-like structures at leptotene (Fig. 4A). Synapsis occurs during zygotene and synapsed regions could be detected at this stage (Fig. 4B). The completion of synapsis could be seen at the pachytene stage when chromosomes appeared thicker and shorter (Fig. 4C). After completion of synapsis, chromosomes underwent partial decondensation concomitant with disassembly of the synaptonemal complex during the diplotene stage. The centromeric regions remained condensed and pairs of pericentromeric heterochromatin spots could be seen to mark the five bivalents which can be distinguished at this stage (Fig. 4D). Following shortening and thickening of the

chromosomes during diakinesis, bivalents became aligned on an equatorial plane at metaphase 1 (Fig. 4E). Reductional segregation of the chromosome gave rise to two cells each of which contains five univalents consisting of a pair of sister chromatids. Fig. 4F shows a dyad with each cell containing five univalents at prometaphase 2. In the *dyad* mutant, the leptotene stage of meiosis could be observed (Fig. 4G), however later stages appeared different from wild type. Chromosome synapsis did not take place and the zygotene and pachytene stages were missing. Instead chromosomes were seen to condense out as 10 univalents instead of the normal 5 bivalents at what probably corresponds to pachytene for wild type (Fig. 4H). Partial decondensation of the univalents was observed, and pericentromeric heterochromatin regions were visible as characteristic twin knobs (Ross et al., 1997) similar to the appearance of wild-type chromosomes at late pachytene and diplotene stages (Fig. 4I). The univalents underwent condensation and formed an organised metaphase consisting of 10 chromosomes (Fig. 4J) resembling a mitotic metaphase, however the size of the metaphase plate is much larger than that of the other mitotic cells in the ovule (data not shown). Although individual chromosomes cannot be clearly distinguished, chromosome segregation at anaphase appeared to be equational with separation of sister chromatids (Fig. 4K). Fig. 4L shows a dyad at prometaphase 2, and in proximity to other non-sporogenic cells of the ovule. The upper cell (enlarged in Fig. 4M) contains approximately 10 chromosomes which also suggests that anaphase 1 is equational. We have previously reported that in about 50% of mature ovules of the *dyad* mutant, the meiotic products are seen to consist of a triad or a tetrad of cells that are of similar size and contain similar-sized nuclei, which appear equilibrated, indicating the occurrence of a second division in one or both cells of the dyad (Siddiqi et al., 2000). Since sister chromatids have separated in the first division, the question arises as to whether the second division is accompanied by replication of the chromosomes, which is not the case for a normal meiosis 2 division. The black arrowhead in Fig. 4M marks a chromosome that has undergone one more round of replication and two sister chromatids can be distinguished. Hence it appears that at least some of the chromosomes do undergo two rounds of replication in proceeding to the second division. However, we cannot rule out the possibility that although the majority of chromosomes segregate equationally, a small minority of chromosomes may segregate reductionally and appear as univalents after the first meiotic division.

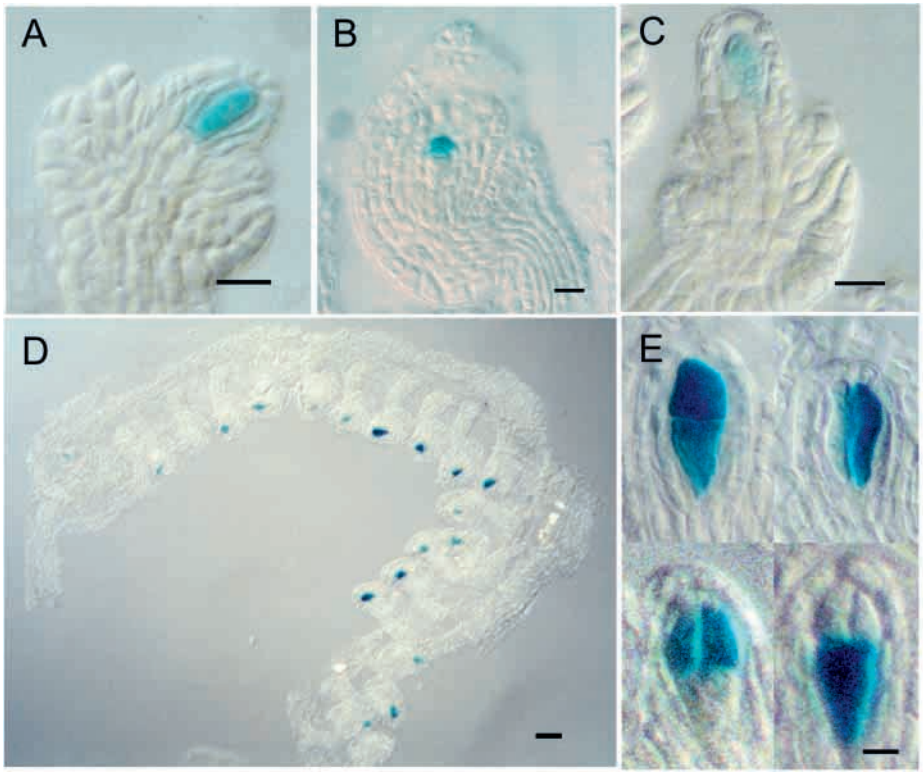

Fig. 5. Expression of p*AtDMC1::GUS* in wild-type and *dyad* plants. (A,B) Wild type. (C-E) dyad. (A,C) GUS expression is specific to the MMC in stage 2-4 ovules. (B) After meiosis expression is seen in degenerating spores but not in the developing embryo sac at ovule stage 3-2. (D) Pistil containing ovules from stages 2-5 onwards. Ovules towards the basal end of the pistil (right) are slightly more advanced in development than those at the stigmatic end (left). Several of the basal ovules show intense GUS expression in the division products of the MMC consistent with defective meiotic progression. (E) Four examples of division products of the MMC where persistent expression is seen in one or both of the cells. Bar: 20 μm (A-C); 50 μm (D); 10 μm (E).

Persistent expression of a meiotic marker in *dyad* female meiocytes

Ovules of the *dyad* mutant contain two enlarged cells resembling MMCs, that are the products of a single division of the MMC. It has been proposed by Mercier et al. (Mercier et al., 2001) that *swi1* mutants, which are allelic to *dyad* and show a similar phenotype, undergo a switch from a meiotic to a mitotic programme in the female meiocyte. To address the basis of the enlarged cell phenotype we compared the expression of the *GUS* reporter gene driven by the promoter of the meiosis-specific gene *AtDMC1* (Klimyuk et al., 1997) in wild type and *dyad*. In wild type, p*AtDMC1::GUS* is expressed in the MMC and earliest expression is detected at ovule stage 2-2 (Fig. 5A; Table 1). Staining in the MMC is seen in ovules from stages 2-2 to 2-5. Following meiosis, later expression is seen in the degenerating megaspores (Fig. 5B) until stage 3-4 by which time material from the degenerating spores has been removed. In *dyad* mutant ovules, expression is seen in the MMC and in one or more of the products of MMC division (Fig. 5C,E; Table 1). The proportion of stage 2-2 through stage 2-5 ovules showing expression of the marker is similar though somewhat less for the *dyad* mutant (155/346=48%) than for the wild type (268/364=74%). In the case of the mutant, of those ovules

Table 1. Expression of the meiosis-specific marker p*AtDMC1::GUS* in ovules of wild type and *dyad*

	Wild type		*dyad*	
Ovule stage	GUS+	GUS–	GUS+	GUS––
2-1	0	8	1 (1)	51 (51)
2-2	2	16	13 (9)	44 (44)
2-3	261	70	129 (25)	143 (61)
2-5	5	10	13 (0)	4 (0)
3-1	18	20	73 (5)	33 (1)
3-2	11	14	76 (4)	36 (0)
>3-2 to 3-6	76	199	192 (6)	50 (1)

Figures in brackets indicate the number of ovules containing an undivided MMC in each category. Ovule stages are according to Schneitz et al. (Schneitz et al., 1995).

(stages 2-2 to 2-5) containing an undivided MMC, expression of p*AtDMC1::GUS* could be detected in 24% (34/139) indicating that the MMC enters meiosis in the *dyad* mutant. Expression seen in the division products of the MMC in the *dyad* mutant, is frequently stronger than that observed in the undivided MMC or seen in degenerating spores of wild type. In addition the percentage of post stage 3-2 ovules showing expression of the marker is greater for the mutant (192/250=79%) than for the wild type (76/275=27%). The greater intensity of expression as well as the higher proportion of older ovules showing expression is consistent with the mutant defect being due to defective meiotic progression resulting in persistent expression of the meiotic marker. The enlarged size of the MMC division products (spores) may possibly be due to defective meiotic progression resulting in delayed cell division, and a corresponding increase in size of the cell. A quantitative comparison of the p*AtDMC1::GUS* meiotic marker expression therefore indicates that in the *dyad* mutant the MMC enters the meiotic programme but is defective in progressing through both meiotic divisions, and does not support the idea that the MMCs regress into a mitotic programme.

DISCUSSION

Three aspects of chromosomal processes that are specific to meiosis and necessary for reductional division are synapsis of homologous chromosomes, monopolar attachment of sister centromeres to the meiotic spindle, and maintenance of sister centromere cohesion through anaphase 1. Properties of the *dyad* mutant described above indicated that the *DYAD* gene is required for all three functions in female meiosis. The *DYAD* gene was identified by positional cloning, and analysis of the sequence indicated no strong similarity to any other known protein. Limited similarity was found to the *Arabidopsis MS1* gene, which has been recently identified as a proposed transcriptional regulator of male gametogenesis (Wilson et al., 2001). Weaker relatedness to several SMC proteins from mammals was also observed.

Cohesion between sister chromatids plays a key role in chromosome organisation and segregation during meiosis as in mitosis (reviewed by Nasmyth, 2001). Sister chromatid cohesion is mediated by a multisubunit complex of proteins called the cohesin complex (reviewed by Hirano, 2000). In *Saccharomyces cerevisiae*, the cohesin complex consists of at least four proteins, three of which (Smc1, Smc3, and Scc3) are required for both mitosis and meiosis while the fourth, Scc1, is required for mitosis and is also present at reduced levels in meiosis (Michaelis et al., 1997; Guacci et al., 1997; Klein et al., 1999). In meiosis, Scc1 is largely replaced by a meiosis-specific homologue called Rec8 (Molnar et al., 1995; Watanabe and Nurse, 1999; Klein et al., 1999). Scc1 and Rec8 occupy a key position in the cohesin complex as their cleavage at the end of metaphase is necessary for loss of cohesion and separation of chromosomes at anaphase in mitosis and meiosis respectively (Uhlmann et al., 2000; Buonomo et al., 2000). The *Arabidopsis SYN1*/*DIF1* gene is homologous to *REC8* and when mutated causes chromosome fragmentation and defects in chromosome segregation at meiosis (Bai et al., 1999; Bhatt et al., 1999). Homologues of *REC8* have also been identified and analysed in *C. elegans* and humans, suggesting conservation in the mechanism of cohesion between sister chromatids in meiosis (Parisi et al., 1999; Pasierbek et al., 2001).

Studies in yeast and mammalian cells have indicated that cohesion takes place during S phase and is closely connected to DNA replication (reviewed by Carson and Christman, 2001). There is also evidence that in meiosis, cohesins are important for synapsis. In *S. pombe* meiosis, Rec8 acts during S phase to establish sister chromatid cohesion (Watanabe et al., 2001). *S. cerevisiae rec8* and *smc3* mutants are defective in formation of the synaptonemal complex (SC) (Klein et al., 1999) and in rat spermatocytes, Smc1 and Smc3 proteins localise along the axial elements of the SC (Eijpe et al., 2000). It has been proposed that the assembly of the SC and synapsis is mediated through cohesins and that establishment of cohesion is required for the formation of axial elements of the SC (van Heemst et al., 2000). Taken together these studies point to a close connection between the establishment of sister chromatid cohesion, which takes place in premeiotic S phase, and synapsis, which occurs later.

The complete absence of zygotene and pachytene stages and the observation of ten univalents being the major class in female meiocytes clearly indicates a defect in synapsis in the *dyad* mutant. The timing of *DYAD* expression corresponds to premeiotic interphase or early meiotic prophase. Expression is seen in early ovules prior to and up to the time of integument initiation (stage 2-1 to 2-3); expression was not observed at later stages. In comparison, expression of the meiotic-specific *AtDMC1* marker both as a promoter-*GUS* fusion and in RNA in situ hybridisation (Siddiqi et al., 2000) (data not shown) was observed in the meiocyte in ovules that were 1 to 2 stages older where the integuments could be seen to have extended beyond the primordial stage. Since *AtDMC1* most likely acts at zygotene to promote synapsis, this would suggest the timing of expression of *DYAD* to be prior to zygotene, possibly in premeiotic interphase. Hence *DYAD* appears to have an early function in centromere configuration and promoting synapsis.

The establishment of centromere cohesion in meiosis probably also takes place at S phase although the possibility that Rec8 is modified later at the centromeres to make it resistant to cleavage at anaphase 1, has not been ruled out. The timing of monopolar attachment, based on studies in *S. cerevisiae* involving return to mitotic growth experiments, is thought to be at pachytene at the time recombination takes place (Zenvirth et al., 1997). Mutations in *AtDMC1* (Couteau

et al., 1999) and *AtSPO11-1* (Grelon et al., 2001) result in absence of synapsis followed by random segregation of univalent chromosomes at meiosis 1. This indicates that monopolar attachment is retained in both mutants. However, the *dyad* mutant undergoes an equational separation of chromosomes instead of a reductional one in the first division of female meiosis, indicating that monopolar attachment and centromere cohesion are both affected. The meiotic chromosome spreading technique offers improved resolution and detail compared to the use of confocal methods in whole mounts, which we had employed earlier to examine chromosome segregation in *dyad* (Siddiqi et al., 2000). We therefore reassessed this issue and our revised conclusions differ from what we reported earlier. The change from a reductional to an equational division in *dyad* is similar to what has been described for the *rec8* mutant of *S. pombe* (Watanabe and Nurse, 1999) and for *spo13* and *slk19* mutants of *S. cerevisiae* (Klapholz and Esposito, 1980; Kamieniecki et al., 2000; Zeng and Saunders, 2000). Spo13 and Slk19 have been implicated in delaying removal of Rec8 from the centromere region thereby allowing sister centromere cohesion to persist through anaphase 1 (Klein et al., 1999; Kamieniecki et al., 2000). It is therefore possible that all three chromosomal defects in the *dyad* mutant trace back to a requirement for *DYAD* in cohesion establishment and/or maintenance. Further work is necessary to determine which of these is the case.

Our observations on the chromosomal defects in the *dyad* mutant are in general agreement with those reported recently for *swi1.1* and *swi1.2* which are allelic to *dyad* and have similar phenotypes in female meiosis (Mercier et al., 2001). The *swi1.1* allele is similar to *dyad* in that the phenotype is female-specific. The *swi1.1* mutation is caused by a T-DNA insertion in the 5′ untranslated region of the gene and this has been inferred to result in a low level of production of the normal SWI1 protein by reinitiation of translation from a fusion transcript. The *swi1.2* allele is stronger and also causes male sterility with defects in sister chromatid cohesion in male meiosis. The *swi1.2* mutation is a single base change that introduces a stop codon at position 390. The *dyad* allele also causes premature truncation of the protein but 115 codons further down at position 505. Since the phenotype of *dyad* is less severe than that of *swi1.2*, this would imply that the mutant protein produced by *dyad* retains some biological activity and that the region between amino acids 390 and 505 contributes to its function.

Expression of the *DYAD* gene in the inflorescence is specific to the female and male meiocytes as detected by RNA in situ hybridisation. In several cases we observed what appears to be a concentration of the in situ hybrdisation signal towards the apical end of the MMC as in Fig. 3C. We found this effect to be variable with respect to both the degree of polarity and the proportion of ovules showing polarity of the signal in different experiments. Further investigation is therefore required to establish whether this effect is significant.

A comparison of the three mutant alleles suggests interesting differences in meiotic chromosome organisation between male and female meiocytes. The female phenotype is very similar for all three alleles and appears to be largely due to loss of synapsis and a change in centromere configuration leading to bipolar attachment and loss of centromere cohesion at anaphase 1. The two weaker alleles *swi1.1* and *dyad*, do not have a male phenotype indicating that centromere configuration and synapsis in the male are less sensitive to a reduction in dosage/activity of the gene product than in the female. The stronger allele *swi1-2* however, in addition to the female phenotype has a more drastic effect in the male in which both centromere as well as chromatid arm cohesion are lost. Whether some level of SWI1/DYAD is necessary for arm cohesion in the female is not certain since all three alleles retain arm cohesion in the female and *swi1-2* which is the strongest may still have some activity. Hence the different aspects of chromosome organisation are differentially sensitive to a reduction in SWI1/DYAD activity, and there are differences in the relative sensitivities between the two sexes.

Examination of p*AtDMC1::GUS* expression in the *dyad* mutant indicated that the MMC enters meiosis in large part, but is defective in progression through both meiotic divisions. Expression of p*AtDMC1::GUS* persists for longer in the *dyad* mutant than it does in wild type. The reason for the progression defect is unclear. Defective progression has also been observed in the case *of S. cerevisiae spo13* mutants, which fail to undergo a second meiotic division (Klapholz and Esposito, 1980).

An unexpected observation was the occurrence of a round of replication during the second meiotic division in the female meiocyte in the *dyad* mutant. This could mean that the mechanism by which replication is bypassed in meiosis 2 is connected to cohesion during meiosis 1. It is unlikely to be cohesion per se that blocks replication, since that is destroyed at anaphase 1 (except at the centromeres). One possibility is that a cohesion-related block is established on the chromosome during meiosis 1, and prevents replication during meiosis 2. This would be formally analogous to the persistence of centromere cohesion preventing separation of chromatids after anaphase 1, and its dissolution at anaphase 2.

In summary, the analysis of the *DYAD* gene suggests that it acts specifically in meiosis where it functions in chromatid cohesion. In addition we have shown on the basis of a quantitative comparison with wild type, that the *dyad* mutant is defective, not in entry into, but in progression through female meiosis.

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