The DUET gene is necessary for chromosome organization and progression during male meiosis in Arabidopsis and encodes a PHD finger protein

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Summary
Progression through the meiotic cell cycle is an essential part of the developmental program of sporogenesis in plants. The duet mutant of Arabidopsis was identified as a male sterile mutant that lacked pollen and underwent an aberrant male meiosis. Male meiocyte division resulted in the formation of two cells instead of a normal tetrad. In wild type, male meiosis extends across two successive bud positions in an inflorescence whereas in duet, meiotic stages covered three to five bud positions indicating defective progression. Normal microspores were absent in the mutant and the products of the aberrant meiosis were uni- to tri-nucleate cells that later degenerated, resulting in anthers containing largely empty locules. Defects in male meiotic chromosome organization were observed starting from diplotene and extending to subsequent stages of meiosis. There was an accumulation of meiotic structures at metaphase I, suggesting an arrest in cell cycle progression. Double mutant analysis revealed interaction with dyad, a mutation causing chromosome cohesion during female meiosis. Cloning and molecular analysis of DUET indicated that it potentially encodes a PHD-finger protein and shows specific expression in male meiocytes. Taken together these data suggest that DUET is required for male meiotic chromosome organization and progression.

Key words: Chromatin, Male sterility, Checkpoint, Cohesion, Synapsis

Introduction
Meiosis in plants is the transition between the diploid sporophyte and haploid gametophyte generations which in lower plants exist as distinct free-living organisms. During the reproductive phase of development in flowering plants, specialized meiotic cells, the sporocytes, are formed in the anthers and ovules. In plants there is no separate germline as in animals, and the sporocytes are derived from the L2 layer of the meristem (Dawe and Freeling, 1990). The understanding of sporocyte specification and meiosis in plants has advanced significantly in recent years (reviewed by Yang and Sundaresan, 2000; Bhatt et al., 2001). Genetic and molecular approaches in Arabidopsis and maize have led to the isolation of mutants that are altered in sporocyte development or meiosis (Curtis and Doyle, 1991; McCormick, 1993; Chaudhury et al., 1994; Sheridan et al., 1996; Golubovskaya et al., 1997; Ross et al., 1997; Taylor et al., 1998; Sanders et al., 1999) as well as to the identification of several genes that are required for development of the sporocyte or for meiosis. Many of the plant genes that are responsible for basic components of the meiotic machinery that is common to all eukaryotes such as chromosome cohesion, synapsis, recombination and chromosome segregation show conservation with genes in yeast and other organisms (Klimyuk et al., 1997; Couteau et al., 1999; Bai et al., 1999; Yang et al., 1999a; Grelon et al., 2001; Armstrong et al., 2002; Chen et al., 2002). Others appear to be unique to plants and do not have obvious homologues in yeast or animals (Byzova et al., 1999; Mercier et al., 2001; Azumi et al., 2002). The pathway leading to sporocyte specification is found only in plants and the two genes that have been identified in this pathway, SPOROCYTELESS/NOZZLE which encodes a nuclear protein related to MADS box transcription factors and is required for the initiation of sporogenesis (Yang et al., 1999b; Schiefthaler et al., 1999) and EXCESS MICROSPOROCYTES/EXTRA SPOROGENOUS CELLS, which encodes a putative LRR receptor kinase required for tapetum formation and control of male meiocyte number (Canales et al., 2002; Zhao et al., 2002), do not have corresponding homologues in yeast or animals.

The control of meiotic cell cycle progression in yeast is dependent upon checkpoints that monitor morphogenesis of the chromosomes during meiosis. Mutations that affect synapsis and recombination lead to arrest of meiotic progression at the pachytene stage. The identification and analysis of extragenic suppressors of pachytene arrest has led to an understanding of the pachytene checkpoint (Roeder and Bailis, 2000). The pachytene checkpoint has also been found to be unique to plants and do not have obvious homologues in yeast or animals (Byzova et al., 1999; Mercier et al., 2001; Azumi et al., 2002). The pathway leading to sporocyte specification is found only in plants and the two genes that have been identified in this pathway, SPOROCYTELESS/NOZZLE which encodes a nuclear protein related to MADS box transcription factors and is required for the initiation of sporogenesis (Yang et al., 1999b; Schiefthaler et al., 1999) and EXCESS MICROSPOROCYTES/EXTRA SPOROGENOUS CELLS, which encodes a putative LRR receptor kinase required for tapetum formation and control of male meiocyte number (Canales et al., 2002; Zhao et al., 2002), do not have corresponding homologues in yeast or animals.

The DUET gene is necessary for chromosome organization and progression during male meiosis in Arabidopsis and encodes a PHD finger protein.
in plants (Couteau et al., 1999; Garcia et al., 2003). In addition to the pachytene checkpoint, which is specific to meiosis, chromosomal checkpoints that act during mitosis have also been shown to function in meiosis (Lydall et al., 1996). This is consistent with the view that meiosis is a specialized cell cycle based on the mitotic cell cycle (reviewed by Lee and Amon, 2001).

There is limited information on the control of meiotic progression in plants. *Arabidopsis* mutants that affect meiotic progression have been described (Siddiqi et al., 2000; Magnard et al., 2001) and several genes have also been characterized at the molecular level. The *ASK1* gene is required for homologue separation during male meiosis (Yang et al., 1999a). The *DYAD/SWII* gene is required for meiotic chromosome organization and meiotic progression (Mercier et al., 2001; Agashe et al., 2002). The Swi1 protein has been shown to be expressed in G1 and S phase of meiosis, and required for axial element formation and initiation of recombination (Mercier et al., 2003). The *SOLO DANCERS* gene encodes a cyclin-like protein that is required for synopsis during meiosis (Azumi et al., 2002). Several of these genes appear to be associated with changes in chromosome organization and dynamics, however the mechanism by which these changes are related to the progression of meiosis in plants remains unknown.

We describe the isolation and characterization of the *duet* mutant of *Arabidopsis* and molecular analysis of the *DUET* gene. We show that the *duet* mutant is defective in chromosome organization and progression during male meiosis. *duet* also shows a synergistic genetic interaction with the *dyad* mutant. The *DUET* gene encodes a plant homeo domain (PHD)-finger protein that is expressed in male meiocytes.

### Materials and methods

#### Plant material and growth conditions

*Arabidopsis* growth conditions were as described earlier (Siddiqi et al., 2000).

#### Light microscopy

Developmental analysis of whole-mount anthers and ovules was done after clearing inflorescences in methyl benzoate as described previously (Siddiqi et al., 2000). The anthers were dissected on a slide under a stereo dissecting microscope, mounted with a coverslip and observed on a Zeiss Axioplan imaging 2 microscope under DIC optics using ×40 and ×100 oil immersion objectives. Photographs were captured on a CCD camera (Axiocam, Carl Zeiss) using the Axiovision program (version 3.1). For a stage-wise comparison of pollen development in the wild type and the *duet* mutant, the ovules from the corresponding pistils were staged and used as a reference for pollen developmental stage. Scoring of ovule stages was based on changes in chromosome organization and dynamics, however the mechanism by which these changes are related to the progression of meiosis in plants remains unknown.

We describe the isolation and characterization of the *duet* mutant of *Arabidopsis* and molecular analysis of the *DUET* gene. We show that the *duet* mutant is defective in chromosome organization and progression during male meiosis. *duet* also shows a synergistic genetic interaction with the *dyad* mutant. The *DUET* gene encodes a plant homeo domain (PHD)-finger protein that is expressed in male meiocytes.

### cDNA isolation and expression analysis

Poly(A)* mRNA was isolated from young flower buds and leaves using the PolyA tract mRNA isolation kit (Promega) according to the manufacturer’s protocol with an inclusion of QI1 DNAase (Promega) treatment before mRNA precipitation. Pistils were dissected and stored in RNA Later (Ambion) and total RNA was isolated using Trizol (Gibco BRL Life Sciences). The cDNA synthesis was carried out with 150 ng of poly(A)* RNA or 5 µg total RNA in the case of dissected pistils, using the Superscript choice system for cDNA synthesis (Gibco-BRL Life Sciences). Amplification of *DUET* cDNA was carried out by using the gene-specific primers SETAF (5*-CGTCTCATCAGGAAGCTAAAATC-3*) and SETR1 (5*-ATCTACA- AAAGTTTGATCAAAAACTGAC-3*). The amplified cDNA was cloned into a pMOsBlue vector (Amersham Pharmacia Biotech) and sequenced. *DUET* expression was examined by PCR using cDNA prepared from poly(A)* mRNA as template and the primers SETF1 (5*-CCAAATCTGAAAACGTTGCTGAAGAG-3*) and SETR13 (5*-TCCTGAGACTTATCACAAGCCATCC-3*). GACP expression was detected using the primers GACP1 (5*-CTTGAGGGGTTGGC- AAGAAGG-3*) and GACP2 (5*-CCGTTGTTGCACCGAAGATT- CAG-3*). DYAD cDNA was amplified with the gene specific primers 3RR1 (5*-CATGGAAGAGACCTATCCAGGTCAATCACA-3*) and g2.2R (5*-AGCTAGTGGATTATGGGAAACCCATTCG-3*).

In situ hybridization was carried out as described previously (Siddiqi et al., 2000). A 1.26 kb coding region of *DUET* cDNA lacking the PHD-finger domain was amplified using the primers STMF1 (5*-GTTGATCATTTGGATGTGCTAAAGAG-3*) and STMR3 (5*-TCAATCTCAGTCATCACAAGATTTCAGAAG-3*) and cloned into pGEM-T (Promega) for synthesis of RNA probes.

#### Molecular analysis of the *duet* mutant locus

Southern hybridization experiments using a Ds transposon-specific probe derived from pWS31 (Sundaesran et al., 1995) was used to establish copy number of the insertion. TAIL-PCR was used to amplify sequences flanking the transposon insertion (Parinov et al., 1999). The amplified product was sequenced. The site of insertion was confirmed by Southern analysis using as the probe a genomic fragment amplified using the primers Set3 (5*-TCTCGAGGAGCAAGTTGAAAGG-3*) and R16 (5*-AAAGTTTGAATCCCAAATCCGAC-3*) that were specific to At1g66170. To obtain genomic sequences flanking the Ds element, Ds-specific primers from both the 5` and 3` ends, Ds5-2 (5*-CGTCTCATTCCGTTCCGAAAATGTG-3*) and Ds3-2 (5*-CCGTTATCCGCTTTGCGCTC-3*) were used in combination with gene-specific primers set5 (5*-GTAACCTCAGGTACCGGTTA-3*) and Set3 respectively.

#### Double mutant analysis

*duet* (Ler) as the female parent was crossed to *dyad* (Col) as the male parent. F1 and F2 were selected on MS plates containing kanamycin at 50 µg/ml. 96 F2 plants were transferred to soil; 49 of these were sterile. Plants homozygous for *dyad* were identified by examination of ovules as described previously (Agashe et al., 2002). The presence of the insertion and wild-type alleles at the *duet* locus was examined by PCR using a gene-specific primer R12 (5*-ATCTCCTGAAGCTGAAACTCATCATTTG-3*) in combination with Ds5-2 for the insertion allele, and two gene-specific primers Ds5-2 (5*-GTA- GTAGATCAGCCTGAGAGATCAA-3*) and STR5 (5*-TCTGC-
AAATTCTTCAGCAATTCG-3') on either side of the insertion site for the wild-type allele. DNA was isolated from one or two rosette leaves using the Nucleon Phytopure kit (Amersham). Pollen viability was measured using fluorescein diacetate according to the method of Tsyul’ko et al. (Tsyul’ko et al., 1995) with minor modifications (Agashe et al., 2002). Chromosomes were observed on a Zeiss Axioplan2 imaging microscope using a 365 nm excitation, 420 nm long-pass emission filter and a 100x oil objective. The photographs were captured on an Axiocam CCD camera (Carl Zeiss) using the Axiovision program (version 3.1) and were edited with Adobe Photoshop 5.0.

Results
The duet mutant is male sterile because of a Ds transposon insertion
The duet mutant was identified as a sterile line, SET8286, carrying a Ds enhancer trap insertion (Parinov et al., 1999). An examination of flowers showed that the mutant anthers lacked pollen. To determine the reason for sterility, reciprocal crosses were carried out to wild type. The results indicated that the mutant was male sterile and that female fertility was normal (Table 1). The anther filament did not fully elongate and remained below the level of the stigma (Fig. 1E), a feature that has also been observed in other male sterile lines (Sanders et al., 1999). Southern analysis indicated the presence of a single Ds insertion in the duet mutant (data not shown). DNA flanking the insertion site was isolated using TAIL PCR (Liu et al., 1995) and sequenced. The sequence indicated the insertion to be within the putative gene At1g66170. Three out of the seven sectors lacking Ds indicated that excision had occurred from both chromosomes and was associated with the reversion to fertility. Genomic DNA flanking the site of excision was amplified by PCR and sequenced for multiple plants from each of the three sectors lacking Ds (Fig. 1F). Two out of the three sectors contained one wild-type and one mutant allele carrying the same 7 bp footprint at the excision site. The third sector contained one

Table 1. The duet mutation causes male sterility

<table>
<thead>
<tr>
<th>Female parent</th>
<th>Male parent</th>
<th>Number of seeds per silique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>duet</td>
<td>0</td>
</tr>
<tr>
<td>duet</td>
<td>Wild type</td>
<td>25.8±2.8</td>
</tr>
<tr>
<td>Wild type</td>
<td>Wild type</td>
<td>27.2±1.8</td>
</tr>
</tbody>
</table>

Reciprocal crosses between duet and wild type, both in the Ler background were conducted to measure the seed yield. The results represent the mean and standard deviation from a minimum of eight crosses.

Table 2. Segregation of the duet phenotype

<table>
<thead>
<tr>
<th>Observed</th>
<th>Expected</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>194:51</td>
<td>183.75:61.25 (3:1)*</td>
<td>2.18</td>
<td>$&gt;0.01$</td>
</tr>
<tr>
<td>228.66:16.33 (15:1)†</td>
<td>70.7</td>
<td>$&lt;0.001$</td>
<td></td>
</tr>
</tbody>
</table>

*Mutant phenotype results from a single gene recessive mutation.
†Two unlinked mutations are responsible for the mutant phenotype.

Fig. 1. Wild-type (Ler), duet and revertant plants. (A) Wild-type plant showing normal elongating siliques. (B) duet mutant plant with short siliques. (C) Mutant plant with revertant sector showing elongating siliques (arrow). (D) Wild-type flower with long anther filaments and plentiful pollen. (E) duet flower with short filaments and anthers lacking pollen. (F) Wild-type cDNA and derived amino acid sequence of DUET near the site of the Ds insertion. The duet mutant sequence shows a 8 bp duplication (bold underlined). The two excision alleles have a 7 and 8 bp footprint respectively (bold).
wild-type and two mutant alleles carrying the 7 bp and an 8 bp footprint, respectively. We found one plant that was heteroallelic for the two mutant alleles and was sterile. This is consistent with the development of the pollen that is carrying the mutant excision allele being associated with a multiple event that gave rise to the wild-type allele together with the mutant allele in the sporophyte. These data show that the duet mutant phenotype is due to the Ds transposon insertion.

Microsporogenesis is defective in duet

To examine the basis of male sterility and lack of pollen in the duet mutant we carried out a stagewise analysis of anther and pollen development by examining cleared anthers as well as plastic sections. Early stages of pollen development corresponding to anther stage 5 (Sanders et al., 1999) were normal (Fig. 2). The endothecium and internal layers surrounding the microsporocyte were indistinguishable from wild type as was the appearance of the microsporocyte prior to meiosis. We compared the time course of pollen development in wild type and duet by examination of cleared anthers and ovules from successive buds of inflorescences. Meiocyte division was prolonged (Table 3) and extended across three to four successive bud positions in the mutant inflorescence whereas in wild type it covered no more than two successive bud positions. The major product of division of the meiocyte was a pair of cells instead of a normal tetrad that is observed in wild type (Fig. 3). Tetrads were seen only rarely (Fig. 2E). The pair of cells separated and formed enlarged cells that were uninucleate (Fig. 2F, 3I). Subsequently nuclear division took place and cells were seen to contain one to three nuclei. Normal microspores were not observed and the exine was not formed. The enlarged cells later degenerated, leaving an empty locule (Fig. 2I). Mutant meiocytes at the time of division had a prominent callose wall (Fig. 2C).

<table>
<thead>
<tr>
<th>Ovule stage</th>
<th>Wild type</th>
<th>duet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2</td>
<td>Microsporocytes</td>
<td>Microsporocytes</td>
</tr>
<tr>
<td>2-1</td>
<td>Tetrads</td>
<td>Separated microsporocytes</td>
</tr>
<tr>
<td>2-2</td>
<td>Free microspores (expanding)</td>
<td>Dyads</td>
</tr>
<tr>
<td>2-3</td>
<td>Microspores with exine</td>
<td>Dyads (separating)</td>
</tr>
<tr>
<td>2-4</td>
<td>Vacuolated microspores</td>
<td>Uninucleate spores</td>
</tr>
<tr>
<td>2-5</td>
<td>Mature pollen</td>
<td>Uni, binucleate spores</td>
</tr>
<tr>
<td>3-1</td>
<td>Multinucleate spores</td>
<td>Spores degenerate</td>
</tr>
<tr>
<td>3-2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Three inflorescences each for wild type and duet were analyzed. Ovule stages are according to Schneitz et al., (Schneitz et al., 1995).
whereas the pair of cells formed by division of the meiocyte lacked a thick callose wall and separated into single cells. This probably corresponds to loss of the callose wall from tetrads at the time of microspore release in wild type. The stage at which the pair of cells separated in the duet mutant corresponded to ovule stage 2–4 which is later than the time of microspore release in wild type (ovule stage 2–2; Table 3). Taken together these data indicate that duet is defective in male meiotic progression.

**DUET potentially encodes a PHD finger protein**

Based on the predicted cDNA sequence of At1g66170, PCR primers were designed to span the coding region. RT-PCR was carried out using cDNA prepared from inflorescence mRNA. The sequence of the cDNA obtained (GenBank accession no. AY305007) indicated the presence of 3 exons potentially encoding a protein of 704 amino acids and having a mass of 80.8 kDa (Fig. 4). The Ds insertion is in the third exon at a position corresponding to aa 550 and hence likely to create a null allele. The putative protein was similar to that of the AGI annotation but contained an additional exon that was not present in the annotated sequence. A potential nuclear localization signal was found at amino acid residues 10–15.

A homology search using BLASTP 2.2.6 revealed the presence of a PHD finger domain towards the C-terminal portion of the predicted DUET protein, from aa 609-656. The PHD domain is a modified zinc finger thought to be involved in transcriptional regulation and chromatin organization (Aasland et al., 1995). The closest known protein to DUET was the MALE STERILITY 1 (MS1) protein (expectation value, $E=4\times10^{-83}$), which has been proposed to be a transcriptional regulator of male gametogenesis and also contains a C-terminal PHD finger domain (Wilson et al., 2001). In addition, two predicted genes from *Arabidopsis* (At1g33420, and At2g01810) and one from rice (GenBank ID AC090882) showed similarity to DUET. All three predicted proteins have a PHD domain towards the C-terminal end. All five genes show similarity throughout their length and hence constitute a gene family. Apart from the above genes, which showed strong similarity to DUET, weak similarity was detected to other *Arabidopsis* genes including SWI1/DYAD ($E=1\times10^{-4}$). The SWI1/DYAD gene has been shown to be required for chromosome cohesion in meiosis and for female meiotic progression (Mercier et al., 2001; Agashe et al., 2002). The SWI1/DYAD homology resides towards the middle portion of the gene from aa 309 to 395.

**DUET is expressed in male meiocytes**

Expression of the DUET gene was examined using RT-PCR. The presence of the transcript could be detected in the
inflorescence but not in leaves (Fig. 4C). The mutant did not show expression under these conditions and hence is likely to be a null allele. We have previously demonstrated the presence of DYAD mRNA specifically in male and female meiocytes (Agashe et al., 2001). To test whether DUET expression is male specific, we compared the levels of DUET message with that of DYAD in dissected pistils using RT-PCR. We did not observe expression of DUET whereas DYAD expression could be detected under the same conditions (Fig. 4D). To determine the DUET expression pattern in the inflorescence at the cellular level we carried out RNA in-situ hybridization using antisense RNA complementary to DUET cDNA excluding the PHD domain as a probe. Expression was first observed in sporogenous cells at late anther stage 4 (Sanders et al., 1999) (Fig. 5A), reached a maximum in male meiocytes at anther stage 5, prior to meiosis (Fig. 5C). Lower expression was observed at anther stage 6, during meiosis (Fig. 5D) and subsequently declined. A weak signal could be seen in very young pistils in the placenta, corresponding to the presumptive site of ovule initiation (Fig. 5B,C). We did not see expression in female meiocytes or in ovules (Fig. 5E,F). The lack of female meiocyte expression as well as a phenotype in what appears to be a null allele, would suggest that DUET does not have a function in the female meiocyte. We also examined GUS reporter gene expression in plants hemizygous and homozygous for the insertion but did not observe expression in anthers at stages 5 to 6.

Aberrant meiotic chromosome organization and metaphase 1 arrest in the duet mutant

Meiotic chromosome stages in wild type and duet were analyzed in spread chromosome preparations from anthers of...
meiotic stage buds using the method of Ross et al. (Ross et al., 1996). Chromosome development in duet appeared normal through early stages of meiotic prophase up to pachytene. Abnormalities first became noticeable at diplotene when duet chromosomes started to appear somewhat diffuse in comparison to wild type (Fig. 6D,N). During diplotene and diakinesis, duet chromosomes were observed to desynapse along much of their length including centromeres, to a greater extent than was observed for wild type (Fig. 6N-P). At diakinesis, duet chromosomes were irregular and less distinct than wild type (Fig. 6P,Q). Metaphase 1 in duet varied from nearly normal (Fig. 6R) to a diffuse mass where individual chromosomes could not be clearly distinguished (Fig. 6T). In wild type, male meiotic stages extended across no more than two successive bud positions in an inflorescence whereas in duet, meiotic stages covered three to five bud positions. The total number of meiotic stages in an inflorescence was also several times greater for duet than for wild type. This could be approximately gauged from the fact that 364 meiotic stages were counted from six inflorescences in the case of wild type and 602 from three

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**Fig. 6.** Chromosome analysis in spreads of male meiocytes in wild type and duet. (A-J) Wild type; (K-W) duet. (A,K) Chromosomes first become visible as elongated strands during leptotene. (B,L) Synapsis takes place during zygotene. (C,M) Synapsis is complete at pachytene and chromosomes have a shorter and thicker appearance. (D) Diplotene stage, when bivalents have undergone partial decondensation. (E) Late diakinesis showing five pairs of chromosomes with chiasmata at their ends. (F) Metaphase I stage showing five condensed bivalents arranged on a metaphase plate. (G) Telophase I: five chromosomes at each end are separated by an organelle band. (H) Metaphase II. (I) Anaphase II. (G-I) Arrows indicate the densely compacted organelle band. (J) Telophase II where four groups of five chromosomes each have separated. (N) First apparent visible defect in duet at diplotene. Chromosomes start to look diffuse and two bivalents have undergone partial desynapsis (arrowheads). (O) A more severe form of desynapsis can be observed in the majority of bivalents. (P,Q) Disorganized diakinesis in duet with diffuse chromosomes including the centromeric region. (R,S,T) Metaphase I. (U) Anaphase I. (V) Defective anaphase I stage with fragmented chromosome and laggards. (W) Telophase I. The organelle band is absent. Scale bars: 12.5 μm.
inflorescences for *duet*. In most of the buds there was an excess of metaphase 1 stages in the *duet* mutant (Fig. 7), whereas these were relatively few in wild type. The fraction of anaphase 1 meiocytes was also greater for *duet*. A clear feature of *duet* meiocytes was the absence of a band of organelles characteristic of wild type and prominently visible starting at telophase 1 in the central portion of the meiocyte (Fig. 6G). Instead organelles were dispersed throughout the cytoplasmic region of the male meiocyte in the case of *duet* (Fig. 6W).

These data confirm and extend the analysis of male meiocyte division in the *duet* mutant. The results indicate that *duet* has a defect in chromosome organization during male meiosis and is also defective in male meiotic progression. Many of the meiocytes arrest at metaphase 1.

**The *duet* mutation interacts with *dyad***

The *duet* mutant phenotype is similar to what is seen for female meiosis in the *dyad* mutant (Siddiqui et al., 2000). In each case a pair of cells is formed after meiosis instead of a tetrad. The pair of cells do not develop further into functional gametes. For both *duet* and *dyad*, the two-cell phenotype correlates with defective progression through meiosis and the underlying cause appears to be altered chromosome organization in both cases. The *DYAD/SW11* gene is required for female meiotic progression, and for chromosome cohesion in male and female meiosis (Mercier et al., 2001; Agashe et al., 2002). However, the *dyad* mutant allele is female specific and shows normal pollen development and male fertility.

To test if both mutants are affected in a related aspect of chromosome organization during meiosis, we intercrossed *duet* and *dyad* as female and male parents respectively to test for genetic interaction. The F₁ were fully fertile and showed normal pollen and embryo sac development (data not shown). F₂ plants were genotyped with respect to the alleles present at the *duet* locus (*duet/duet*, *duet/+*, and +/+ ) and at the *dyad* locus (*dyad/dyad*, and +/-), and various dose combinations of *duet* and *dyad* were examined for evidence of genetic interaction.

The *duet dyad* double mutant flowers lacked viable pollen. Stagewise analysis of pollen development in cleared anthers (Fig. 8) showed meiocytes followed by enlarged multinucleate cells that underwent further enlargement to produce binucleate cells. We did not observe clear cytological evidence of meiotic divisions. At a later stage anthers contained irregular enlarged cells containing multiple nuclei and surrounded by an irregular cell wall that resembled the exine. The exine-like structure was not observed in the *duet* single mutant. Buds from plants that were *duet/+ dyad/dyad* showed reduced numbers of pollen grains compared to the corresponding single mutants. Anthers from freshly opened flowers were dissected and tested for pollen viability. The mean pollen viability for *duet/+ dyad/dyad* was 34±12% whereas for plants that were *duet/+ +/-* the pollen viability was 75±13%. The total number of pollen grains was also lower for the interaction genotype. A rough indication of this could be obtained from the total amount of pollen on the slide. For the *duet/+ dyad/dyad* genotype the mean count of total pollen grains per flower was 131±50 whereas for sibling plants that were *duet/+ +/-* the mean pollen count was 326±171. Each individual genotype *duet/+* and *dyad/dyad* produced numbers of viable pollen that were comparable to wild type (Siddiqui et al., 2000; data not shown). Analysis of pollen development in cleared anthers showed that male meiocytes in *duet/+ dyad/dyad* plants underwent an aberrant division to produce mostly dyads and triads, as well as some tetrads (Fig. 8). The majority of spores produced were defective and formed enlarged cells containing 1-3 nuclei and that subsequently degenerated. The phenotype therefore broadly resembled that of homozygous *duet* plants though it was less severe.

Examination of meiotic chromosome behavior revealed differences in *duet/+ dyad/dyad* plants from that observed in homozygous *duet* plants. The early stages of meiotic prophase were detected though aberrant chromosome morphology was apparent at the pachytene stage (Fig. 9B). Diakinesis was variable with some being nearly normal and having all or a majority of the chromosomes retaining their bivalent structure (Fig. 9C). In others, many of the chromosomes dissociated into univalents (Fig. 9D). A more extreme phenotype observed at diakinesis was the loss of sister chromatid cohesion resulting in dissociation of chromosomes into isolated chromatids (Fig. 9E). The ensuing meiosis 1 divisions spanned the range from a normal reductional division (these were relatively rare and not observed, but their existence could be inferred from the production of tetrads and viable pollen) to an equational mitotic-like division in which univalents separated into sister chromatids (Fig. 9G,H), to an unequal division arising from random segregation of univalents and single chromatids (Fig. 9IJ). We did not observe a high proportion of metaphase 1 arrest which correlated with a reduction in the number of cell pairs that were seen compared to the *duet* single mutant. The homozygous double mutant combination was also examined. Here too bivalents were observed to separate into univalents at diakinesis (Fig. 9L) and in some meiocytes chromosome cohesion was lost.

**Fig. 7.** Quantitation of different stages of male meiosis in wild type and *duet*. (White bars) Wild type; (Black bars) *duet*: leptotene (L), zygote (Z), pachytene (P), diplotene (DP), diakinesis (DI), metaphase I (M-I), anaphase I (A-I), telophase I (T-I), metaphase II (M II), anaphase II (A-II), telophase (T-II) and tetrads (TD). Note the large increase in the proportion of metaphase I cells in *duet*. 
resulting in isolated chromatids (Fig. 9M). At prometaphase between eight and ten thick and diffuse chromosomes could generally be counted, most probably they were a mixture of univalents and bivalents (Fig. 9N). Male meiotic chromosomes in the dyad mutant showed no apparent differences from wild type. In the case of duet/+ also, the vast majority of meiotic structures observed were normal, in agreement with the essentially wild-type levels of viable pollen that were measured in freshly opened flowers. However, in one inflorescence out of four examined we observed a single bud in which a minority (<10%) of meioses were aberrant. Chromosomes in this bud showed defects in diakinesis that were similar to those observed in homozygous duet, and some meiocytes were arrested at metaphase 1 (data not shown). Separated microspores were the major species present in this bud indicating that the majority of meiocytes had completed meiosis, and that the minority, showing defective meiotic structures, were defective in progression.

The increased severity of defects in pollen development resulting from defects in male meiosis that were observed in duet/+ dyad/dyad plants, coupled with the loss of chromosome cohesion provide evidence of a synergistic interaction between the duet and dyad mutant alleles.

To test for possible effects of duet on female meiosis we examined cleared ovules as well as female meiosis in duet dyad double mutant plants (Fig. 9U-W). The ovule phenotype was identical to that of the dyad mutant. In both cases the majority of ovules showed a single division meiosis and the presence of two enlarged cells in place of an embryo sac. Cytogenetic analysis of female meiosis in duet dyad plants also indicated that chromosome behavior was the same as that described earlier for dyad, which was shown to undergo an equational meiosis 1 division (Agashe et al., 2002). No additional effects were observed from the presence of the duet mutant allele in a dyad background, on the integrity or appearance of chromosomes. We also examined female fertility of duet/duet
dyad/+ plants and did not observe any reduction in fertility (data not shown). We therefore did not find any evidence to support a role for DUET in female meiosis.

**Discussion**

Progression through the meiotic cell cycle requires orchestration of a set of events that includes the assembly of chromosomes for reductional division, pairing of homologous chromosomes, recombination and segregation of homologues to opposite poles of the meiosis 1 spindle (reviewed by Dawe, 1998). Defects in meiotic chromosome organization can result in delayed progression through the meiotic cell cycle. The role of chromosomal checkpoints in monitoring and ordering the phases of the mitotic and meiotic cell cycle is well documented in yeast and animals (Roeder and Bailis, 2000; Handel et al., 1999). In plants there is little information on the control of meiotic progression and the role of chromosomal checkpoints in meiosis (Garcia et al., 2003). Mutations in the yeast DMC1 gene cause arrest as a result of activation of the pachytene sister chromatid cohesion have been lost to yield single chromatids. (F) Early anaphase 1 undergoing mixed segregation in which both univalents and bivalents are involved. (G) Late anaphase 1 showing approximately equal separation of chromosomes. Eight to ten chromosomes are present at each pole indicating an equal division. (H) Telophase 1. Equal division. Ten chromosomes are present at each pole. (I) Telophase 1. Unequal division. (J) Dyad formed after unequal division. (K) Zygotene. (L) Diakinesis involving 2 bivalents and 6 univalents. (M) Extreme diakinesis containing mostly single chromatids. (N) Prometaphase 1 having eight to ten thick diffuse chromosomes. (O,R) Normal diakinesis. (P,S) Metaphase 1. (Q,T) Telophase 1. (U,V) Cleared ovules of dyad (U) and duet dyad (V). (W) Metaphase 1. Scale bars: 12.5 μm (A-T,W) and 25 μm (U,V).
checkpoint (Bishop et al., 1992; Lydall et al., 1996). However, a mutation in AtDMC1, the Arabidopsis homolog of DMC1 does not cause arrest. Instead it leads to random chromosome segregation in meiosis and the production of defective spores (Couteau et al., 1999). Hence the control of meiotic progression in plants may differ from that in yeast.

The properties of the DUET gene described above indicate that it is required for male meiotic chromosome organization and suggest that in the absence of DUET function, male meiocytes undergo defective progression through the meiotic cell cycle. In the duet mutant, male meiocytes went through a single division to produce a pair of cells instead of a normal tetrad. The defective spores produced did not complete gametogenesis and degenerated. The phenotype resembled that observed in the dyad mutant of Arabidopsis (Wilson et al., 2001), to two other putative Arabidopsis genes, At1g33420 and At2g01810, and so a putative rice gene (GenBank ID AC090882). The PHD finger is a modified zinc finger and is found in a number of proteins that play a role in chromatin organization and transcriptional regulation and include members of the Trithorax and Polycomb groups (reviewed by Aasland et al., 1995). In plants the PHD finger has been found in a transcriptional regulator of genes involved in defense against pathogens (Korfhage et al., 1994) and in genes that are required for reproductive development and fertility: the PICKLE gene is required to prevent re-expression of embryonic traits in germinated seedlings and encodes a CHD3 domain protein that has been proposed to act as a regulator to promote the transition from embryonic to postembryonic development (Ogas et al., 1999); overexpression of the SHL gene has been show to lead to early flowering and defective reproductive development whereas antisense inhibition caused dwarfism and delayed growth (Musig et al., 2000). Hence, in both plants and animals PHD finger genes play a role in developmental transitions. Recently the PHD finger domain has also been found in proteins that act as E3 ubiquitin ligases (Cosoy and Ganem, 2003). This latter class of PHD finger proteins are localized to the membrane or cytoplasm. The PHD finger in DUET is more similar to that found in proteins that act as chromatin remodeling factors or transcriptional regulators. The DUET gene also showed limited similarity to SWI1/DYAD a gene that has been demonstrated to be required for chromosome cohesion during meiosis in Arabidopsis (Mercier et al., 2001; Agashe et al., 2002).

Expression of the DUET gene in the florescence appeared to be specific to the male meiocyte. Earliest expression was detected in stage 4 anthers at a time that corresponds to the presence of sporogenous cells. Maximal expression was seen at anther stage 5 prior to meiosis, after which the signal declined. Since the expression and phenotype for DUET and the related MSI1 gene appears to be sex specific (Wilson et al., 2001; Ito and Shinozaki, 2002), it is possible that along with the other two closely related putative genes At1g33420 and At2g01810, they define a family of transcriptional regulators that function during male meiosis and gametogenesis.

The appearance of chromosomes during early stages of meiotic prophase in the duet mutant was normal up to pachytene. Differences from wild type first became noticeable at diplotene with chromosomes appearing more irregular and diffuse in the mutant. This would suggest that either the timing of DUET action is at the onset of diplotene or else that DUET may act earlier, but its absence may lead to visible changes in chromosome structure only at a later stage when the synaptonemal complex is disassembled and most of the sister chromatid cohesion is removed at diplotene. The difference between duet and wild type was accentuated at diakinesis and culminated in a high proportion of meiocytes showing arrest at metaphase 1. The appearance of chromosomes at metaphase 1 was variable and distinctly different from wild type. The metaphase 1 phenotype ranged from nearly normal looking structures to ones in which the chromosomes appeared an irregular mass towards the center of the cell in which individual chromosomes could not be clearly distinguished. A distinct characteristic of the mutant meiocytes was the absence of the organelle band which is a prominent feature found at the center of the cell of wild-type meiocytes at telophase 1. Instead, the organelles in the mutant meiocytes appeared more evenly distributed throughout the cytoplasm. The reason for this could be that the localization of mitochondria and plastids in the dividing meiocyte requires expression of specific genes during meiosis, and that the expression of these genes is adversely affected in the mutant. Alternatively the cause could be a more general disruption of cytoplasmic and cytoskeletal organization that affects organelle transport and localization in the meiocyte. The finding of defects in chromosome organization in meiosis caused by disruption of the DUET gene extends the role of PHD finger proteins to include functions that are specific to meiosis.

Double mutant combinations of duet with dyad revealed genetic interaction manifesting in defects during male meiosis. The effect was most apparent in plants that were duet+/dyad/dyad. Whereas the individual dyad/dyad and duet/+ plants showed no or very weak effects on pollen development, the combination resulted in strong defects in male meiosis. Microsporocytes showed defective division patterns and the products were dyads, triads and tetrads. Most of the microspores produced were defective and degenerated. However a minority of spores did develop into viable pollen. At the cellular level, the defective divisions of the male meiocyte were similar to those in duet/duet, although less severe. The duet dyad double mutant showed a progression defect that was more severe than in the duet single mutant as the male meiocytes failed to divide. At the chromosomal level there were additional defects that were not observed in either single mutant. In duet+/dyad/dyad meiocytes, chromosomes lost synopsis or cohesion prior to meiosis 1 and segregated unequally in many cases. Loss of cohesion was not observed in either the dyad or duet single mutants. dyad is a female-specific allele and shows normal male meiosis and pollen development (Siddiqui et al., 2000). The stronger allele swi1-2 is male sterile and shows loss of sister chromatid cohesion.
during prophase of male meiosis (Mercier et al., 2001). Hence the loss in sister chromatid cohesion as a result of the interaction may be interpreted to mean that hemizygous duet/+ enhances the dyad mutant phenotype. The genetic interaction between duet and dyad could be specific and the genes may act at the same level of chromosome architecture. The presence of the PHD finger in DUET implicates it as functioning in the control of transcription at the level of chromatin organization whereas DYAD/SW11 has been shown to function in chromosome organization and cohesion. If DUET does in fact function as a transcriptional regulator, this would point to a close connection between cohesion and the control of transcription at the chromatin level during meiosis. The formation of dyads and the fact that meiosis is more extended in the duet mutant clearly suggests a defect in meiotic progression. Analysis of single division meiosis in the spo13 mutant of yeast has shown that the basis for the progression defect is the activation of the spindle checkpoint. In the absence of the spindle checkpoint, spo13 mutants undergo normal meiotic progression and form four spores (Shonn et al., 2002; Lee et al., 2002). There is at present limited information on the control of meiotic progression in plants. Immunolocalization of a maize homologue of the yeast spindle checkpoint protein MAD2 has shown that it is expressed in meiosis and localized to the kinetochore where it functions through a tension-dependent mechanism (Yu et al., 1999). Hence the basic apparatus for the spindle checkpoint protein MAD2 has shown that it is expressed in meiosis and localized to the kinetochore where it functions through a tension-dependent mechanism (Yu et al., 1999).

The further analysis of the role of the maize homologue of the yeast spindle checkpoint protein MAD2 has shown that it is expressed in meiosis and localized to the kinetochore where it functions through a tension-dependent mechanism (Yu et al., 1999). Hence the basic apparatus for the spindle checkpoint protein MAD2 has shown that it is expressed in meiosis and localized to the kinetochore where it functions through a tension-dependent mechanism (Yu et al., 1999). Hence the basic apparatus for the spindle checkpoint protein MAD2 has shown that it is expressed in meiosis and localized to the kinetochore where it functions through a tension-dependent mechanism (Yu et al., 1999).

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Note added in proof

While the manuscript was under review, Yang et al. reported the cloning and expression analysis of the MMD gene, and analysis of the mmd mutant. The MMD gene is identical to DUET (Yang, X. et al., 2003).

References


