

# The *DIF1* gene of *Arabidopsis* is required for meiotic chromosome segregation and belongs to the *REC8/RAD21* cohesin gene family

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## Summary

Cohesins are a group of conserved proteins responsible for cohesion between replicated sister chromatids during mitosis and meiosis and which are implicated in double-strand break repair and meiotic recombination. We describe here the identification and characterisation of an *Arabidopsis* gene – *DETERMINATE*, *INFERTILE1* (*DIF1*), which is a homolog of the *Schizosaccharomyces pombe* *REC8/RAD21* cohesin genes, and is essential for meiotic chromosome segregation. Five independent alleles of the *DIF1* gene were isolated by transposon mutagenesis, and the mutants show complete male and female sterility. Pollen mother cells (PMCs) of *dif1* mutants show multiple meiotic defects which are represented by univalent chromosomes and chromosome fragmentation at metaphase I, and acentric fragments and chromatin bridges in meiosis I and II. Consequently, chromosome segregation is strongly affected, resulting in meiotic products of uneven size, shape and of variable ploidy. The similarities in phenotype, and the sequence homology between *DIF1* and the *REC8/RAD21* cohesins suggests that cohesin function is largely conserved between eukaryotes and highlights the essential role cohesins play in plant meiosis.

## Introduction

Unlike the pre-determined germ cell lineage of animals, plants feature an ephemeral 'germ line' involving the development, *de novo*, of numerous microsporocytes in the anther and the megaspore mother cell in the ovule. Within these cell lines, the meiotic processes of chromosome synapsis, recombination and the two consecutive rounds of chromosome segregation take place leading to the formation of male and female haploid gametes. In all organisms the regulation of cohesion between the newly replicated sister chromatids is crucial for accurate chromosome segregation during mitosis and meiosis (Bickel and Orr-Weaver, 1996). Meiotic studies in plants have largely focussed on the isolation of numerous mutants defective in meiosis and the cytogenetic analysis of these mutants (Golubovskaya, 1979; Kaul and Murthy, 1985; Koduru and Rao, 1981). Recently, the versatility of *Arabidopsis* has made it an attractive plant system to study meiosis at the genetic and molecular levels, and numerous mutants of *Arabidopsis* with meiotic defects have been described previously (Chaudhury *et al.*, 1994; Dawson *et al.*, 1993; He *et al.*, 1996; Peirson *et al.*, 1996; Ross *et al.*, 1997; Spielman *et al.*, 1997). With the exceptions of the *AtDMC1* and *MS5/TDM* genes (Glover *et al.*, 1998; Klimyuk and Jones, 1997; Ross *et al.*, 1997), the identity, the expression pattern and implied function of the majority of these genes is unknown.

We describe here the identification of a plant gene, *DIF1*, which is essential for meiosis and is involved in meiotic chromosome segregation. We have cloned *DIF1* by transposon tagging, characterised its function, and demonstrate *DIF1* to be a member of the conserved *REC8/RAD21* cohesin gene family. Cohesins are a group of widely conserved proteins that are responsible for cohesion between replicated sister chromatids, and which have been implicated in double-strand break repair and meiotic recombination (Birkenbihl and Subramani, 1992; Guacci *et al.*, 1997; Losada, *et al.*, 1998; McKay *et al.*, 1996; Michaelis *et al.*, 1997; Molnar *et al.*, 1995). In both budding and fission yeast, two distinct cohesin genes are known to be required during mitosis (*MCD1/RHC21/SCC1* and *RAD21*) and meiosis (*YPR0007* and *REC8*) (Birkenbihl and Subramani, 1992; Chu *et al.*, 1998; Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Molnar *et al.*, 1995), and each is essential for the viability of cells and spores, respectively. We present evidence that the *DIF1* gene of *Arabidopsis*

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encodes a cohesin and is essential for meiotic chromosome segregation.

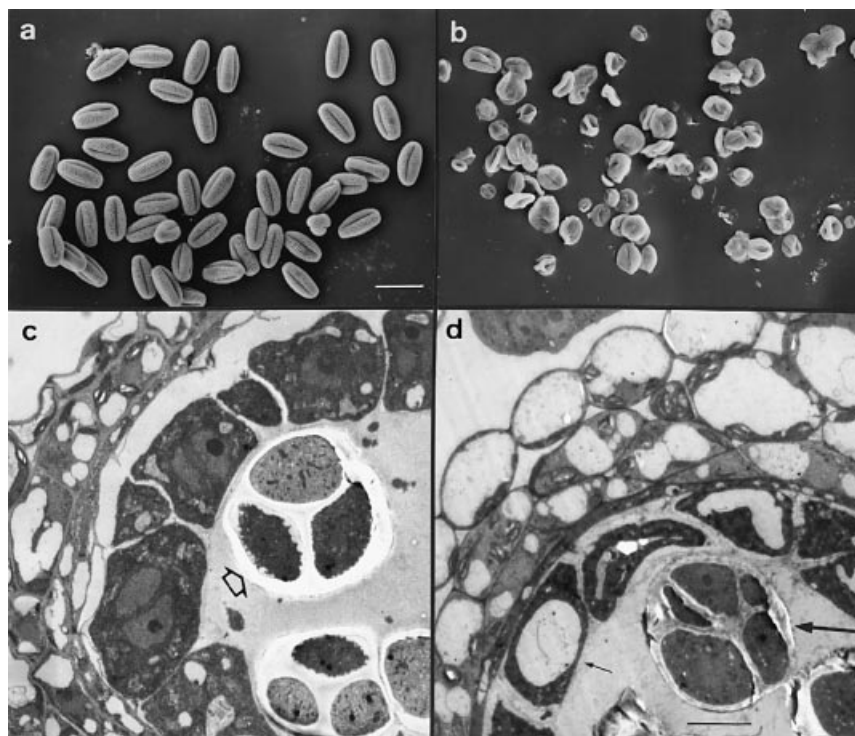
## Results

### *dif1-1* mutant phenotype

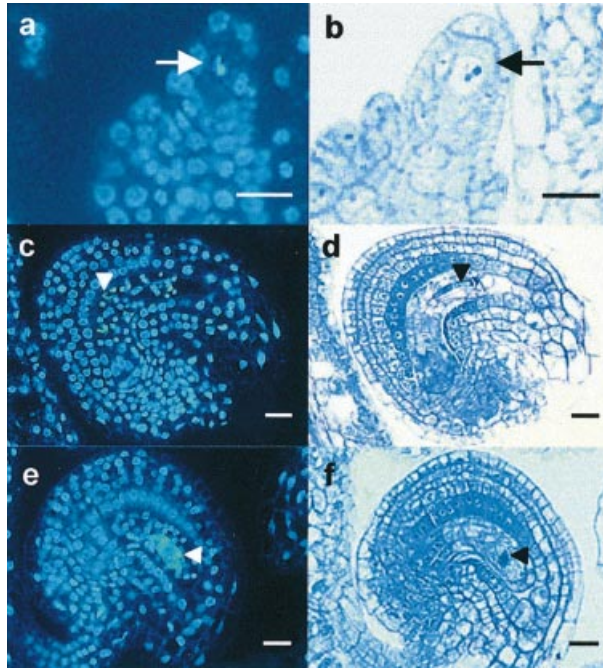
Mutations in the *DIF1* gene of *Arabidopsis* exclusively result in male and female sterility. Mutant plants have no other vegetative defects besides their sterility, but do flower more profusely than the wild type – a trait common to *Arabidopsis* male sterile mutants (Chaudhury *et al.*, 1994). We have cloned *DIF1* by transposon tagging (Bhatt *et al.*, 1996) and analysed its expression pattern and mutant phenotype. Five independent *Ac* insertion alleles of *DIF1* were generated from the same donor T-DNA, and their molecular analysis showed that the *Ac* element had inserted in the same *SacI* genomic fragment, which contains most of the *DIF1* gene (data not shown). Examination of *dif1-1/dif1-1* anthers under the scanning electron microscope showed limited anther dehiscence, and the few pollen grains released from such anthers to be collapsed and of differing sizes (Figure 1b). Analysis of sections of anthers from *dif1-1/dif1-1* buds at different stages of development revealed that cellular differentiation and commitment to meiosis were normal in the mutant anthers, and that there were no defects in the tapetal tissue. However, the pollen mother cells (PMC) of

*dif1-1* plants often produced extra meiotic products; with the callose wall of the 'tetrad' enclosing five cells, rather than the usual four equal meiotic products (Figure 1d). Furthermore, some of the *dif1-1* meiotic products also featured additional micronuclei. The subsequent development of these abnormal meiotic products results in collapsed microspheres of uneven size and shape (Figure 1b). As no defects were evident in the peripheral mitotic tissue of the anther locule, *DIF1* is only essential for male meiosis.

In order to define the cause of female sterility in the *dif1* mutant, we examined serial sections of *dif1-5* mutant ovules, first staining them with DAPI and aniline blue to detect DNA and callose, and subsequently with toluidine blue. The development of the sporophyte appears normal and meiosis is clearly initiated in the *dif1-5* ovules (Figure 2a,b), but we were unable to determine if meiotic chromosome segregation is defective in the *dif1-5* ovules. The majority of the mature *dif1-5* ovules (floral stage 12) examined lacked a distinct embryo sac, and callose deposition was often observed in cells around the gametophyte (Figure 2c,e); an extreme example of which is shown in Figure 2(e). A few ovules with a mature differentiated female gametophyte were produced by *dif1-5* mutants, but these were in a minority and were likely to be the outcome of somatic *Ac* excision events. Thus, the loss of *DIF1* adversely affects embryo sac development which subsequently results in female sterility.



**Figure 1.** Phenotypic defects seen during anther development in *dif1* mutants. (a) Scanning electron micrograph of wild-type pollen grains; the trilobular pollen grains are plump and are even in size and shape. Bar = 30  $\mu$ m. (b) Scanning electron micrograph of mutant pollen grains extruded from *dif1* anthers; the pollen grains differ in size, shape and are collapsed. Bar = 30  $\mu$ m. Transmission electron micrographs of transverse sections of anthers from (c) wild-type and (d) *dif1-1* mutant buds showing the tetrad stage of meiosis. The meiotic products are enclosed in a callose wall (open arrow) and *dif1-1* mutant anther has extra meiotic products (five cells) enclosed within the callose wall (large arrow). The surrounding tapetal tissue (small arrow) is normal and unaffected in the *dif1-1* mutant. Bar = 5  $\mu$ m.



**Figure 2.** *dif1* ovules have a defective female gametophyte. Ten microgram sections of *dif1-5* ovules were stained with DAPI and aniline blue (a,c,e), and later with toluidine blue (b,d,f). The early stages of megaspore development proceed normally in *dif1-5* (a,b) and the MMC is indicated by an arrow. However, mature *dif1-5* ovules lack a developed embryo sac (c–f). The black arrowheads indicate the position of the female gametophyte, and white arrowheads indicate callose deposition. Bar = 10  $\mu$ m.

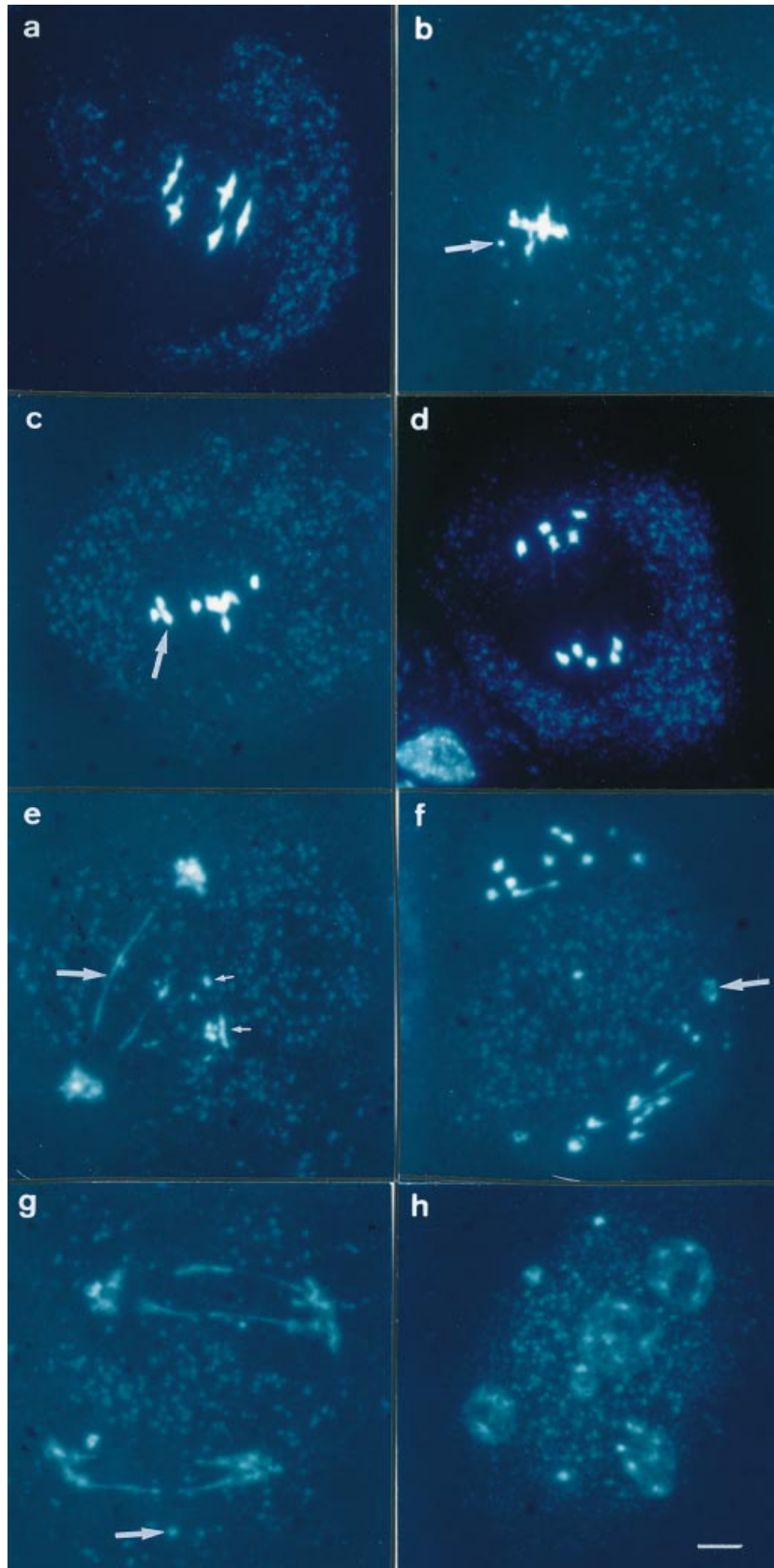
#### *dif1-1* is defective in meiotic chromosome segregation

We analysed chromosome segregation in *dif1-1* pollen mother cells (PMCs) during meiosis using light microscopy. Although *dif1* mutants are completely male and female sterile, a minority of the *dif1-1* PMCs had normal cytology, possibly due to somatic *Ac* excision events. The early prophase I stages of leptotene and zygotene proved difficult to analyse in *dif1-1* PMCs as the chromosomes were often resistant to the spreading method used. However, pachytene appears to be substantially normal with little evidence of chromosome fragmentation. Numerous meiotic defects seen in *dif1-1* PMCs were seen in the later meiotic stages from metaphase I onwards (see Figure 3c–h), when the condensed chromosome state facilitates their analysis. However, some metaphase I figures proved difficult to analyse because of chromosome aggregation. The PMC spreads revealed two distinct defects, univalence and chromosome fragmentation. During meiosis I, chromosome fragments of different sizes lagged behind bivalents as they congressed to the metaphase plate (Figure 3b) and, in cells where less fragmentation was occurring, univalents could clearly be seen (Figure 3c). The six univalents in Figure 3(c) could have arisen either through asynapsis or desynapsis. The

presence of univalents indicates that the association of homologs is clearly impaired in *dif1-1* mutants. Therefore, the subsequent segregation of univalent homologs cannot always be reductional as expected, but will also involve random segregation, potentially generating aneuploid gametes. At anaphase I and telophase I, many acentric fragments become evident lying at, or around, the spindle equator; these fragments vary in size from almost chromosome size units to minute fragments (Figure 3e). At this stage chromatin bridges are also present which stretch between the polar group of chromosomes. Chromosome segregation defects are also evident during meiosis II, with acentric fragments and chromatin bridges being generated in second division nuclei of *dif1-1* mutants (Figure 3f,g).

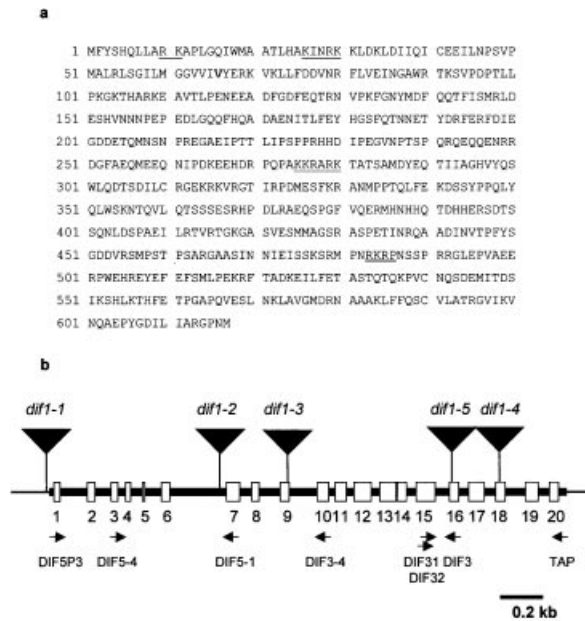
#### DIF1 is a homolog of the *REC8/RAD21* cohesin genes

Plant DNA flanking the *Ac* insertion in *dif1-1* was cloned by inverse PCR (IPCR) and the IPCR fragment was used to isolate the *DIF1* gene. A *SacI* fragment that was disrupted in all the insertion alleles was judged likely to contain the *DIF1* gene, it was sequenced and several potential exons were identified. As the *SacI* fragment did not identify any cDNA clones from a floral cDNA library (data not shown), we amplified the *DIF1* cDNA by RT-PCR and 3'RACE. The *DIF1* cDNA is 2052 bp long, is interrupted by 19 introns, has at least 56 bp of UTR at the 5' end and a 145 bp long 3'UTR; the largest open reading frame encodes a predicted protein of 617 amino acids with a net negative charge (Figure 4a). A BLAST search (Altschul *et al.*, 1990) of the sequence database revealed that the ORF encoding DIF1 has significant homology to proteins belonging to the cohesin gene family, which include the meiotic cohesins *REC8* and *YPR007*, and their mitotic counterparts, the *RAD21* gene family (Figure 5) (Birkenbihl and Subramani, 1992; Guacci *et al.*, 1997; Losada *et al.*, 1998; McKay *et al.*, 1996; Michaelis *et al.*, 1997; Molnar *et al.*, 1995). The Columbia allele of *DIF1* was also identified in the database as part of a P1 clone sequenced on chromosome five of Arabidopsis (EMBL accession number Ab005241; <http://www.kazusa.or.jp/arabi/>). Cohesins show the greatest amino acid sequence conservation amongst the N and C terminal domains (Guacci *et al.*, 1997), where sequence identity is about 50%. Overall, cohesins show 20–26% amino acid sequence identity and 41–51% amino acid sequence similarity with DIF1 throughout their protein sequences. Visual examination of the DIF1 sequence also revealed the presence of a putative bipartite nuclear localisation signal sequence near its N-terminus (Figure 4a). DIF1 also has several potential phosphorylation sites (not shown). In addition, a highly conserved isoleucine residue, functionally important for the RAD21 protein, and conserved in a similar position in the majority of cohesins



**Figure 3.** Meiosis in *dif1-1* pollen mother cells from metaphase I onwards to the microspore 'tetrad' stage.

(a) Metaphase I in wild-type PMC showing five bivalents at the metaphase plate. (b) Metaphase I defect of *dif1-1* showing a chromosome fragment (arrow) and a set of bivalents at the metaphase plate in *dif1-1* PMC. (c) Metaphase defect of *dif1-1* showing univalent chromosomes at an equatorial position during metaphase I. One of the two bivalents in the picture is indicated by the arrow. (d) Anaphase I in wild-type PMC showing five pairs of segregated homologs. (e) Late anaphase I/ telophase I defect of *dif1-1* showing many acentric fragments (small arrow) and chromatin bridges (big arrow). (f) Metaphase II and (g) anaphase II in *dif1-1* PMCs show micronuclei that are relics of the first division defects (arrows), and a new crop of acentric fragments and chromatin bridges generated during these stages. (h) The net effect of such meiosis I and II defects produces microspores of varying sizes together with micronuclei.



**Figure 4.** Predicted amino acid sequence of DIF1 and schematic representation of the DIF1 gene.

(a) The longest ORF encoded by the *DIF1* cDNA encodes a 617 amino acid protein and its sequence is shown here. Several potential nuclear localisation signals identified are shown, those identified using PSORT are underlined twice, and the putative bipartite nuclear localisation signal is underlined once. The conserved isoleucine residue present at equivalent positions in most of the identified RAD21 cohesins is substituted by a conserved valine residue at position 66 in DIF1 and is shown in bold. (b) The 19 introns and 20 exons of the *DIF1* gene are shown, the numbered open boxes represent the exons, and the thick line represents the introns and the 5' and 3' non-coding sequence, the position of the *Ac* transposon insertion in the different alleles is denoted by triangles. The position and orientation of primers used for RT-PCR analysis and for cloning the *DIF1* cDNA DIF5P3, DIF5-4, DIF5-1, DIF3-4, DIF31, DIF32, DIF3 and TAP is shown by arrows that are not drawn to scale. Bar = 0.2 kb.

identified to date, is also present as a conserved valine substitution in DIF1 (Figure 4a). On the basis of its sequence homology and mutant phenotype, DIF1 is likely to be a cohesin.

#### DIF1 is expressed in vegetative and reproductive tissue

As the phenotypic defects in *dif1* mutants were restricted to PMCs, the embryo sac and pollen grains, we analysed the *DIF1* expression pattern to determine if *DIF1* transcripts were restricted to reproductive tissues. Due to the low abundance of the *DIF1* transcript, RT-PCR analysis was used to study *DIF1* expression in the tissues of wild-type plants (Figure 6), and in young floral buds of the agamous mutant (Yanofsky *et al.*, 1990). *DIF1* mRNA was most abundant in young floral buds but was consistently detectable, albeit at lower levels, in the different vegetative tissues (Figure 6). Amongst the vegetative material investigated, 7-day-old seedlings which contain actively

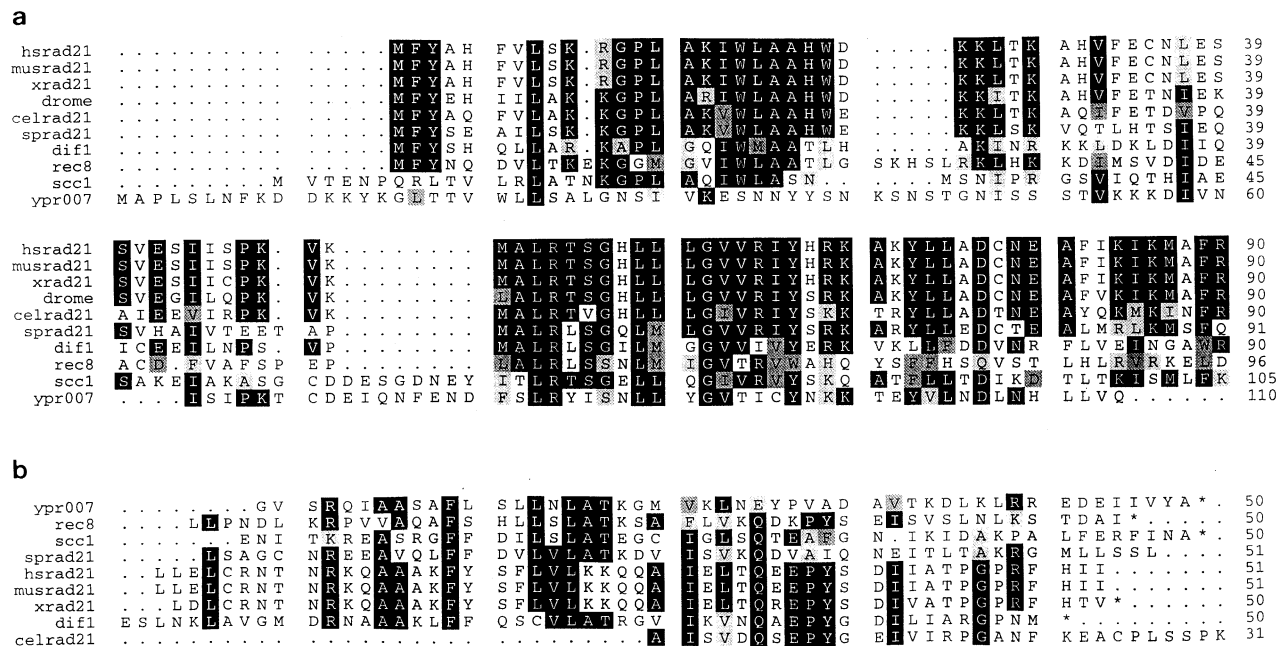
dividing cells had the greatest abundance of *DIF1* transcript. The low abundance of the *DIF1* mRNA in mutant buds from *agamous* plants indicates that the high level of expression in wild-type floral buds correlates with the presence of anthers and carpels. Young mutant floral buds from five of the *dif1* alleles were also analysed for the expression of *DIF1* by RT-PCR (Figure 6), and all but one of the five mutant alleles exhibited significantly reduced levels of *DIF1* transcript compared with wild-type floral buds, demonstrating that the *Ac* insertion reduced levels of the *DIF1* mRNA. The fifth allele (*dif1-5*) also had lower levels of *DIF1* transcript, but the decrease was not as significant as that seen for the four other alleles. In conclusion, the expression of the *DIF1* gene is clearly not meiosis specific, although its phenotype is limited to reproductive organs.

#### Molecular characterisation of *dif1* alleles

We cloned and sequenced Arabidopsis genomic DNA flanking the *Ac* insertion sites in all five *dif1* insertion alleles (Figure 4b). Transposon insertions were scattered throughout the length of the *DIF1* gene. The *dif1-1* allele was generated by an insertion upstream of the *DIF1* transcript, very near the likely transcription start site of *DIF1*, and is expected to block transcription initiation. The remaining four *dif1* alleles are caused by insertions in the transcribed regions of the *DIF1* gene. *dif1-2* is caused by an *Ac* insertion in the sixth and also the largest intron of the *DIF1* gene. Consequently, the *dif1-2* allele is the most unstable amongst the five *dif1* alleles and phenotypically often reverts to produce fertile sectors (data not shown). The remaining three alleles all have insertions in *DIF1* exons; *dif1-3* has an *Ac* insertion in exon 9, *dif1-4* has an *Ac* insertion in exon 18, whereas the last allele *dif1-5* is generated by an *Ac* insertion in exon 16. For alleles *dif1-2*, *dif1-3*, *dif1-4* and *dif1-5*, the *Ac* insertions could have two possible effects; they could affect the abundance of the *DIF1* mRNA (Figure 6), as observed, in addition to generating prematurely truncated DIF1 proteins.

#### Relationship between DIF1 and other cohesins

A phylogenetic analysis utilising parsimony (PAUP; Swofford, 1993) (Figure 7) was also carried out to evaluate the relationship between DIF1 and the different mitotic and meiotic cohesin sequences, using the more conserved N-terminal domains of the cohesins (110 amino acids) for the analysis. The strict consensus tree generated from the analysis is shown in Figure 7. When the N-terminal regions of cohesin sequences were compared, two trees 343 steps long were generated. The strict consensus data show that DIF1, the two meiotic cohesins of yeast (REC8 and YPR007), and the *S. cerevisiae* mitotic cohesin SCC1 are more closely



**Figure 5.** Domains conserved between DIF1 and the REC8/RAD21 cohesin proteins. Prettybox alignments of the deduced amino acid sequence of DIF1 (*dif1*) and cohesin sequences derived from human (*hsrad21*), mouse (*musrad21*), *Xenopus* (*xrad21*), *C. elegans* (*celrad21*), *S. pombe* (*sprad21*), *S. pombe* (*rec8*), *S. cerevisiae* (*scc1*), *S. cerevisiae* (*ypr007*) coding regions, and a *Drosophila* EST (*drome*) identified in the database are shown. Boxed amino acids indicate similarity between these sequences, gaps introduced in the sequence for optimal alignment are shown as dots. The N-terminal domains represented by 100 amino acids are shown in (a), and alignments between the 50 amino acids at the C-termini of all cohesins are shown in (b).

related to each other than to the human, mouse, *Drosophila*, *Xenopus* or *C. elegans* cohesins (Figure 7).

**Discussion**

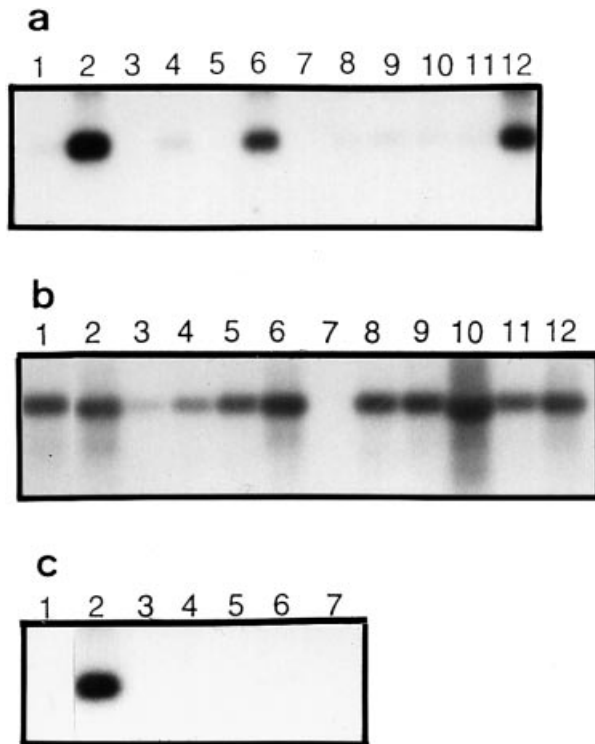
*dif1* shows meiosis specific defects

The severe meiotic defect seen in *dif1-1* anthers indicates that *DIF1* is essential for both stages of meiosis. The meiotic defect does not affect progression through meiosis, as meiotic products differentiate into non-functional microspores of uneven sizes and shapes. It is not clear if *DIF1* has a similar function during female meiosis, but *DIF1* is clearly required for the development of a mature embryo sac. The mutant phenotype of *dif1* ovules most resembles the phenotype of ovules seen in the *msd* or *emd* class of mutants described by Schneitz *et al.* (1997) which, like *dif1*, lack a distinct embryo sac. It is also possible, but not proven here, that aberrant meiotic chromosome segregation in the Megaspore Mother Cell could lead to defective gametogenesis, as observed in *dif1* anthers.

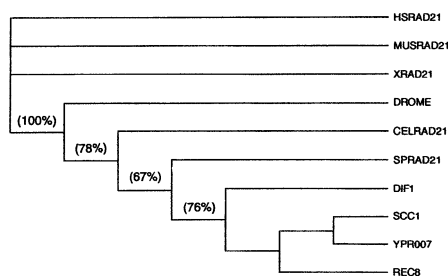
*The cohesin DIF1 is required for meiotic chromosome segregation*

The cytological investigation was hampered by the fact that *dif1-1* chromosomes were inherently more 'sticky'

than those of wild type. Nevertheless, the clear premature separation of bivalents during metaphase I in *dif1-1* pollen mother cells indicates that *DIF1* is required for maintaining the association between homologs. Unfortunately, technical difficulties have prevented us from obtaining detailed cytological data on the *dif1* mutant which demonstrate or exclude a role for *DIF1* in the pairing of homologous chromosomes, or in sister chromatid cohesion, as is the case for its counterparts in yeast and *Xenopus*. *DIF1* may also play a role in recombination, like its *S. pombe* mitotic counterpart *RAD21*, as chromosome fragments accumulate in *dif1-1* PMCs during meiosis. Another common feature, indicative of their similar functions, is the presence of chromatin bridges at the metaphase-anaphase transition in both the *dif1-1* and *rad21<sup>ts</sup>* mutants (Tatebayashi *et al.*, 1998). However, unlike the *S. pombe* *RAD21* gene, *DIF1* is not essential for mitosis or for mitotic DNA repair (as *DIF1/dif1* progeny are uniformly insensitive to sublethal concentrations of methyl-methanesulfonate; Bhatt and Dickinson, preliminary data). Therefore, it is reasonable to assume that *DIF1* belongs to the functional class of meiotic cohesins (*REC8* and *YPR007*), especially as the *dif1* mutation affects meiosis and has no vegetative phenotype. Two features of the *dif1-1* phenotype (the acentric fragments and chromatin bridges seen during meiosis I and II) are not described for the *S. pombe* meiotic cohesin



**Figure 6.** Expression analysis of *DIF1*. RT-PCR products were amplified using primers specific for (a) *DIF1*, (b) all the members of the  $\beta$ -tubulin gene family, and (c) *AtDMC1*, 20  $\mu$ l of each product was subsequently used for Southern blot analysis, hybridised to the corresponding probes, and the representative autoradiographs are shown. RT-PCR products amplified from (1) agamous (*ag/ag*) buds, wild-type tissue representing (2) closed floral buds, (3) open flowers, (4) expanded rosette leaves, (5) cauline leaves, (6) 7-day-old seedlings, and (7) control RT-PCR without any cDNA input. *DIF1* and  $\beta$ -tubulin RT-PCR products were also amplified from floral buds of the five *dif1* mutant alleles, all at a comparable stage of development to the sample in lane 2, and included (8) *dif1-1*, (9) *dif1-2*, (10) *dif1-3*, (11) *dif1-4* and (12) *dif1-5* mutants. The RT-PCR products indicate the relative abundance of the corresponding transcripts in the different samples.



**Figure 7.** Phylogenetic analysis of *DIF1* and different cohesins. Alignments of the highly conserved N terminal domains (110 amino acids) of the amino acid sequence of *DIF1* and the different mitotic and meiotic cohesins found in the database were used for PAUP analysis. The analysis included the Arabidopsis cohesin (*DIF1*), human (*HSRAD21*), mouse (*MUSRAD21*), *Xenopus* (*XRAD21*), *C. elegans* (*CELRAD21*), a partially sequenced *Drosophila* EST (*DROME*), *S. pombe* (*SPRAD21*), *S. pombe* (*REC8*), *S. cerevisiae* (*SCC1*), and *S. cerevisiae* (*YPR007*) sequences. Two unrooted trees 343 steps long were generated, and the strict consensus of the data is shown. Bootstrap values for the confidence limits are given in parentheses.

mutant *rec8-110* (Molnar *et al.*, 1995). Such defects could be the outcome of aberrant or incomplete recombination events occurring in meiosis I, which consequently would result in the production of 'illegitimate' recombinant chromosomes that fail to segregate correctly. Importantly, this would indicate a role for *DIF1* in meiotic recombination.

The lack of a mitotic phenotype in *dif1* mutants suggests that either the loss of *DIF1* function may be compensated by other gene(s) during mitosis, or that *DIF1* may not be essential for mitosis. The possibility that post-translational factors could confer the meiosis-specific phenotype of *DIF1* is feasible but less likely, especially since a second cohesin gene *AtRAD21* has been identified (MHK7; <http://www.kasuzo.or.jp/arabi/>, and Costa-Nunes, Bhatt and Dickinson, unpublished data) which may also provide cohesin function in Arabidopsis.

In conclusion, *DIF1* belongs to a conserved family of proteins, the cohesins, and is clearly important for maintaining the association between homologs. The generation of univalent chromosomes at metaphase I suggests that synapsis could be affected in the *dif1* mutant, but does not prove that synaptic defects occur during meiosis in *dif1*. In addition, the meiotic phenotype of *dif1*, and the function of cohesins in meiotic recombination, supports the view that *DIF1* may also play a role in meiotic recombination.

## Experimental procedures

### General molecular techniques

Plant genomic DNA was prepared essentially as described by Dean *et al.* (1992). Total RNA was extracted from plants as described by Logemann *et al.* (1987) but with the following modifications; the nucleic acid pellet was dissolved in sterile water, and RNA was selectively precipitated with an equal volume of 4M Lithium chloride at 4°C overnight. The RNA pellet was washed with 70% ethanol, dissolved in water, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and RNA was precipitated with sodium acetate and ethanol at -20°C overnight. Standard techniques were used for cloning DNA, electrophoresis of RNA and DNA as described in Sambrook *et al.* (1989). PCR amplifications were carried out in 1 $\times$  PCR reaction buffer (Life Technologies, USA), 200  $\mu$ M dNTP, 1 ng  $\mu$ l<sup>-1</sup> of each primer and 1.5 mM MgCl<sub>2</sub>, in a final volume of 100  $\mu$ l. DNA probes were labelled with  $\alpha$ -<sup>32</sup>P-dCTP by random priming using the Prime-It II Random Primer labelling kit (Promega, USA). Radiolabelled DNA was separated from unincorporated isotope using Sephadex G-50 microspin columns (Pharmacia, USA), and hybridisation was carried out as described previously (Sambrook *et al.*, 1989).

### *DIF1* cloning

IPCR fragments representing insertions of *Ac* into the *DIF1* gene were used to screen a Landsberg *erecta* cosmid library (see Macknight *et al.*, 1997), and two overlapping cosmid clones 64O15 and 17M5 were isolated. Cosmid DNA was prepared from 17M5

and 64O15 by a standard alkaline lysis method (Sambrook *et al.*, 1989) and digested with *SacI*. After electrophoresis and Southern blotting onto a nylon membrane the DNA was hybridised with the *dif1-1::Ac* IPCR fragments. The 4637 bp *SacI* fragment present in 17M5 was subcloned into pBluescript SK+ and sequenced completely; 64O15 had a truncated *SacI* fragment. X-GRAIL analysis (Mural *et al.*, 1992) was used to predict potential open reading frames encoded by the 4637 bp of genomic sequence. Primer combinations were designed for the different exons predicted by X-GRAIL, and cDNA reverse transcribed from Landsberg *erecta* inflorescence RNA was used as a template for PCR amplification. Thirty-five to forty cycles of PCR amplification were carried out using Taq DNA polymerase, and the amplified cDNA products were cloned and sequenced on both strands. The internal exons of *DIF1* were amplified as two overlapping cDNAs using primers DIF5-4 (5'-CCGATGGCTCTTAGACTCTC-3') and DIF3-4 (5'-GCTCAGCAAATCCGCTCCCTAC-3'), and DIF5-4 and DIF3 (5'-CTAGGCATGGATCTCACATC-3'). The 3' end of the transcript was cloned by 3'RACE as follows; cDNA was synthesised with the primer TAP (5'-TCGAGGTCGACGGTATCTTTTTTTTTTTTTTTTTT-3') and was used in PCR amplification with primers DIF3-1, 5'-TGCTTCAGTAGAAAGCATGATG-3' in the first round. An aliquot of the primary PCR reaction was reamplified with a nested primer DIF3-2, 5'-TGATATTAATGTCACGCCATT-3' and the TAP primer. Several attempts to amplify the 5' end of the *DIF1* cDNA by conventional 5'RACE were unsuccessful. Visual examination of the genomic sequence had previously identified putative N-terminal motifs conserved amongst the *REC8/RAD21* cohesin gene family, several overlapping primers were designed upstream of this genomic sequence. One primer combination DIF5P3, 5'-CTCTCTTCTCTGTTTTCTCTTC-3', and DIF5-1, 5'-CTTCGTTCTCAGGCAATGTAAC-3', was successfully used to amplify the 5' end of *DIF1*, which includes 56 bp of the 5'UTR, and the PCR product was cloned and sequenced. The entire sequence of the *DIF1* cDNA was compiled from the 3'RACE and RT-PCR products and checked for accuracy with the genomic sequence.

#### DNA sequencing and analysis

Cloned DNA fragments were sequenced using protocols and primers recommended in the ABI Sequencing protocols (ABI, Perkin-Elmer/Roche Molecular, NJ, USA). DNA sequence manipulation and analysis was carried out using the GCG and STADEN sequencing packages. Exon identification was done with X-GRAIL. The PSORT program was used to scan *DIF1* for potential motifs (<http://psort.nibb.ac.jp:8800/>).

#### Mapping of *Ac* insertion sites in *dif1* alleles

Plant DNA flanking *Ac* insertions in the different *dif1* alleles were amplified with Taq DNA polymerase, using IPCR as described previously (Bhatt *et al.*, 1996), or by direct PCR amplification using primers specific for *DIF1*, DIF3A 5'-CTATGGATAATCGGTTCCACAG-3', DIF3AR 5'-CTGGTGAACCGATTATCCATAG-3', and for *Ac*, A3 5'ATACGATAACGGTTCGGTACGGG-3' and D71 5'-CCGTTA-CCGACCGTTTTTCATCCCTA-3'. The amplified PCR products were purified after electrophoresis on an agarose gel, cloned into pGEM-T Easy (Promega, USA) and sequenced to determine the *Ac* insertion site.

#### Expression analysis

One microgram of total RNA was used for cDNA synthesis using Superscript II (Life Technologies, USA) as per the manufacturer's

protocol. In addition to spectrophotometric quantitation of the RNA samples, all RNA samples were also analysed on denaturing agarose gels to confirm that equal amounts were used for reverse transcription. One microlitre of the cDNA was used for 25 cycles of PCR amplification with 2.5 units of Taq polymerase (Life Technologies, USA) and the following primers. *DIF1* cDNA was amplified using primers DIF5-4 and DIF3-4;  $\beta$ -tubulin cDNAs were amplified using primers TUB-R1000 (5'-GTGAACTCCATCTC-GTCCAT-3') and TUB-L (5'-CCTGATAACTTCGCTTTGG-3'), and *AtDMC1* cDNA was amplified with primers mei1U and mei4U (Klimyuk and Jones, 1997). Twenty microlitres of each PCR reaction was used for Southern blot analysis, and the filters were hybridised separately to the following radiolabelled probes: *AtDMC1* cDNA,  $\beta$ -tubulin cDNA and *DIF1* cDNA. All filters were subjected to a final high stringency wash of 0.5 $\times$  SSC/0.1% SDS (1 $\times$  SSC is 1.5M NaCl and 0.15M Sodium citrate), at 65°C for 20 min, and exposed to X-RAY film.

#### Pollen mother cell cytology

*DIF1* and *dif1* mutant buds were used to study male meiosis. The meiotic cytology of wild-type and *dif1-1/dif1-1* PMCs was analysed by the DAPI staining of chromosome spreads as described by Ross *et al.* (1996).

#### Scanning electron microscopy

Gynoecia and anthers from *dif1* and *DIF1* plants were quickly dissected by hand under a light microscope and rapidly frozen in liquid nitrogen slush at -210°C. Tissue was sputter coated with gold and then observed whilst being maintained in its frozen state on the cold stage of the SEM (Gresham CamScan mark IV, Cambridge, fitted with a Hexland CT1000 cryo-transfer system, Oxford Instruments, Oxford, UK). Images were recorded at 16 kV using Ilford FP120 roll film.

#### Transmission electron microscopy

Samples for TEM were fixed by placing them in 2% (v/v) glutaraldehyde, 0.2% (v/v) saturated aqueous picric acid and 0.05% non-idet P-40, and vacuum infiltrated gently for a few minutes. The solution was replaced by fresh fixative and the samples were left at room temperature for 2-4 h. Fixed material was washed twice for 10 min in phosphate buffer pH 6.9, and dehydrated in an ethanol series of 10%, 20%, 30%, 50%, 70%, 90% and a 100% ethanol for 10 min each. The 100% ethanol step was repeated and the samples were gradually infiltrated with LR white resin (London Resin Company) at room temperature using the following ratios of resin:ethanol 1:3 for 30 min, 1:1 for 30 min, 3:1 for 30 min, and finally in 100% LR white overnight. The resin was replaced with fresh resin every morning and evening each day for a further 3 days after which the resin was polymerised at 65°C for 48 h.

Ultra thin, 90 nm sections were cut using a Leica Ultracut E (Leica, Cambridge, UK) and picked up on carbon-coated, pyroxilin-coated copper grids and then stained with uranyl acetate and lead citrate. The sections were examined in a Jeol 1200EX TEM at 80 kV and photographs were taken using Kodak film.

#### Light microscopy

Floral tissue was fixed in ethanol:acetic acid (3:1) overnight and stored in 70% ethanol at -20°C until required. Tissue was

transferred to 95% ethanol at room temperature and left overnight. Samples were embedded in methacrylate resin using the JB4 Resin kit (Agar Scientific, UK), initially for 12 h in increasing proportions of JB4 resin diluted in 95% ethanol; first with 1 volume of resin: 3 volumes of ethanol, then 1:1, and finally with 3:1 volumes of resin:ethanol. Samples were infiltrated with 100% resin with three changes over 3 days, transferred to moulds and polymerised under a steam of nitrogen according to the manufacturer's instructions. Ten µm thick sections were cut with glass knives using a Reichert-Jung microtome. Serial sections were stained with a mix containing 2.5 µg ml<sup>-1</sup> DAPI, 0.1% aniline blue, 0.5% Triton X100 and 0.5 M Tris (pH 7.0) in vectashield mounting medium (Vector Laboratories, USA), and photographed using a Zeiss Axiophot microscope. Slides were washed in a large volume of distilled water to remove DAPI and aniline blue, stained with toluidine blue for 1 min, and excess stain was washed off in distilled water. Sections were coated with DPX mountant (Agar Scientific, UK) and dried at 40°C overnight.

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