A CDC45 Homolog in Arabidopsis Is Essential for Meiosis, as Shown by RNA Interference–Induced Gene Silencing

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INTRODUCTION

One of the most important cell cycle checkpoints involves the timing and fidelity of DNA replication during S-phase. The successful passage through the G1/S transition requires the initiation of replication after the formation and activation of the complex of prereplication. Studies in yeast have shown that the formation of this complex is initiated by the binding of cdc6 to origin recognition complexes during G1, allowing the recruitment of the mini chromosome maintenance (MCM) complex of proteins (Toone et al., 1997). S-phase is triggered by the activation of the prereplicative complex by two different Ser-Thr protein kinases: an S-phase cyclin-dependent kinase (S-CDK) and Dbf4-cdc7 kinase (Nougarede et al., 2000).

One of the consequences of S-CDK activity is the loading onto the prereplicative complex of a protein essential for DNA replication: CDC45 (Mimura and Takisawa, 1998; Zou and Stillman, 1998). Evidence suggests that CDC45 may function with the MCM complex, because genetic interactions (Moir et al., 1982; Hardy, 1997; Zou et al., 1997) and biochemical interactions (Hopwood and Dalton, 1996) between the MCMs and CDC45 have been shown in yeast. More recently, CDC45 was shown to bind to Mcm2p at the G1/S transition in an S-CDK-dependent manner (Zou and Stillman, 2000). Also, like the MCMs, CDC45 has been shown to become more distant from a nuclear tether as replication forks progress; therefore, it may be a component of replication forks and thus play a role in DNA elongation (Aparicio et al., 1997). Indeed, inducible cdc45 deletion mutants in yeast that allow the degradation of the CDC45 protein at restrictive temperatures cannot replicate their DNA if CDC45 is depleted in G1. Furthermore, inactivation of CDC45 during S-phase prevents the progression of individual replication forks (Tercero et al., 2000).

CDC45 may function in the switch from the complex of prereplication by recruiting DNA polymerase α to the initiation complex before replication initiation and may link the MCM helicase to DNA polymerase α at the replication fork (Kelly and Brown, 2000). The association of DNA polymerase α with origins is dependent on functional CDC45 (Aparicio et al., 1999), and in Xenopus, immunodepletion of CDC45 prevents DNA polymerase α loading onto chromatin (Mimura and Takisawa, 1998). CDC45 may play both a temporal and a checkpoint-mediated role in the regulation of replication origins, because its binding to late origins has been shown to be delayed relative to early origins (Aparicio et al., 1999).

Intriguingly, some genes with characterized mitotic functions are starting to be identified as having important roles in meiosis. In mouse, CDK2, which recruits CDC45 to replication origins, is essential for prophase I of meiosis but not for mitotic cell divisions (Ortega et al., 2003). Azumi et al. (2000) have shown that a novel cyclin-like protein, SPS, in Arabidopsis is essential for normal homolog synapsis and bivalent formation and suggest that a CDK may play a role in meiotic prophase I. Other plant-specific cases include an Arabidopsis SKP1 homolog.

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that is essential for male meiosis and yeast and human SKP1 genes that regulate the mitotic cell cycle (Yang et al., 1999). Thus, current research aims to compare the mitotic and meiotic cell cycles and to answer questions regarding the functions of genes and checkpoints involved during both mitotic and meiotic DNA replication. Premeiotic S-phase is of particular interest because of its link with recombination and synopsis events of meiosis (Borde et al., 2000; Forsburg, 2002). In plants, we hope to identify the cell cycle–specific checkpoints and regulators that exist using a combination of classic and reverse-genetics approaches.

In plants, many key cell cycle players have been identified (Vandepoele et al., 2002), including the CDKs and their regulatory subunits, the cyclins that are at the core of cell cycle control, activating both the G1/S and G2/M transitions (Mironov et al., 1999). As in other eukaryotes, inhibitory proteins that modulate CDK activity also have been identified in plants (Wang et al., 2000; Jasinski et al., 2002). Other pathways conserved between plants and mammals include the Rb/E2F pathway, which is involved in the repression or activation of genes necessary for the G1/S transition (Helin, 1998; de Jager and Murray, 1999; Maricotti et al., 2002).

To determine a function of the Arabidopsis CDC45 homolog, we first analyzed its cell cycle and developmental expression profiles. AtCDC45 transcripts are upregulated at the G1/S transition and in the early stages of flower bud development. Second, we used RNA interference (RNAi). Using this technique, introduced double-stranded RNA is degraded to produce small RNAs that target homologous mRNAs for destruction (Hamilton and Baulcombe, 1999; Zamore, 2001). Several Arabidopsis plant lines have been generated with reduced CDC45 transcript levels in inflorescences and the presence of 21- to 23-bp RNA fragments specific for the CDC45 gene. Many of these plants are sterile, which led us to analyze the involvement of CDC45 during meiosis. In addition, the spo11 mutant, in which the absence of double strand breaks (DSBs) prevents recombination, was retransformed with the CDC45 RNAi construct to determine whether AtCDC45 function was associated with the events of DSB repair.

RESULTS

Cloning of a CDC45 Homolog from Arabidopsis

A CDC45 homolog was identified in the Arabidopsis database by virtue of its homology with cDNAs from other organisms. Basic Local Alignment Search Tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/blast/) with the Arabidopsis protein sequence showed greatest identity with CDC45 from *Schizosaccharomyces pombe*, human, *Xenopus laevis*, mouse, *Ciona intestinalis*, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, and *Tsd2*, the CDC45 homolog from *Ustilago maydis*. The gene sequence was not predicted to contain any introns; only one copy exists, and no homologs are apparent in the Arabidopsis genome. The *AtCDC45* sequence is 28% identical and 82% similar to the *S. pombe* CDC45 protein sequence, and neither has been identified as containing any recognized or previously identified protein motifs. The identity between human CDC45 and *S. pombe* CDC45 also is 28%. The Arabidopsis CDC45 homolog was cloned from first-strand cDNA using primers as described in Methods and sequenced to check for the absence of errors. The cDNA sequence obtained, of 1791 bp (locus At3g25100), matched exactly the one predicted. AtCDC45 cannot complement the yeast CDC45 temperature-sensitive mutant (data not shown).

Cell Cycle Regulation of AtCDC45

The *AtCDC45* promoter contains two consensus E2F transcription factor binding sites. In mouse, CDC45 is a transcriptional target of E2F factors that activate genes involved in the G1/S transition (Arata et al., 2000). In yeast, CDC45 has been shown to be involved in the activation of the G1/S transition (Hardy, 1997; Owens et al., 1997; Zou et al., 1997), so we predicted that *AtCDC45* transcripts may be cell cycle regulated. Therefore, RNA samples from an aphidicolin-synchronized Arabidopsis cell suspension culture were used in reverse transcriptase–mediated (RT) PCR to determine the cell cycle regulation of *AtCDC45*. The results are shown in Figure 1. *AtCDC45* transcripts peaked at 6 to 10 h after release from the aphidicolin block, with the increase starting just before the increase in histone H4 transcript levels (an S-phase marker). Thus, *AtCDC45* transcripts appear to be regulated during the cell cycle in agreement with the role of the gene in the activation of DNA synthesis at the G1/S transition.

Developmental Regulation of CDC45

The expression pattern of CDC45 also was analyzed in different plant organs (Figure 2). The expression of CDC45 was increased in tissues demonstrating more S-phase activity, as shown by the level of histone H4 transcripts. Therefore, *AtCDC45* levels were higher in open flowers, young inflorescences, young siliques, stems, and roots and lower in leaves and plantlets (four- to five-leaf stage). We also analyzed the levels of *AtCDC45* expression in different stages of the developing flower. The results are shown in Figure 2B and demonstrate that *CDC45* expression was strongest at the earliest stage of flower bud development (lane 1) compared with histone H4 expression, which increased over the course of flower development, presumably in correlation with an increase in S-phase
activity. The presence of CDC45 transcript in early bud development gave a primary indication that CDC45 may play a role in this tissue (where meiotic divisions occur; buds containing anthers in which the cells are at prophase I of meiosis were ∼0.3 mm long) (Albini, 1994).

**Generation of CDC45 RNAi Plants and Phenotypic Analysis of T1 Plants**

In many organisms, CDC45 is an essential protein in DNA replication. To investigate the role of CDC45 in plants, an 870-bp 5' fragment of the CDC45 cDNA was cloned into the pkAnnibl vector in the sense and antisense orientations (see Methods) for subsequent generation of Arabidopsis RNAi lines. This vector contains a 35S promoter that drives the transcription of a sense and an antisense CDC45 sequence separated by an intron designed so that hairpin RNA structures are formed (Wesley et al., 2001). These double-stranded RNAs are degraded and lead to the production of 21- to 23-bp small RNAs (Hamilton and Baulcombe, 1999; Waterhouse et al., 2001). Therefore, the presence of these small RNA fragments was analyzed in the CDC45 RNAi lines that had decreased levels of CDC45 transcript by RT-PCR. In lines in which sufficient quantities of RNA were available, including the lines T1,7, T1,21, and T1,25 (appearing sterile: siliques of a reduced size, 0 to 5 seeds/silique), T2,23 (appearing partially sterile: siliques of an intermediate size, 5 to 20 seeds/silique), and T8,8 (appearing fertile: siliques of a normal size), polyacrylamide gels were used to detect the presence of these small RNA fragments (Figure 3B). Small RNAs were detectable in three lines (T1,7, T1,21, and T1,25; sterile plants) but not in T1,8 or T2,23 (fertile or only partially sterile plants) or wild-type plants. The detectability of small RNAs specific to CDC45 seemed to correlate with the severity of the sterility. The three lines in which small RNAs were present in sufficient quantities to be detected, T1,7, T1,21, and T1,25, all were sterile, and although lines T1,7 and T1,21 produced enough seeds for analysis of the T2 generation, line T1,25 was completely sterile and produced no seeds.

Plants for analysis in the T2 generation were selected on a phenotypic basis and for the presence of small RNA fragments rather than for lines with only one insertion (because of a “dose” effect).

**Analysis of RNA Levels in T2 Plants**

To confirm that plants of the T2 generation still were silenced and to confirm the role of CDC45 in the sterility observed, RNA was prepared from the inflorescences of both completely sterile (no seeds in siliques) and semisterile (5 to 20 seeds in siliques) plants generated from lines T1,7, T1,21, and T1,25 used in semiquantitative RT-PCR to determine the quantity of CDC45 RNA. In line T1,7, 19 of 30 plants appeared completely sterile; 13 of 17 plants were sterile for T1,21, and 29 of 62 plants were sterile for T1,22. In these lines, sterile plants contained less CDC45 RNA compared to semisterile plants (Figure 4A), and experiments were performed on a minimum of four independent plants per line using an actin constitutive control (first sowing) or an EF1α constitutive control (second sowing). Therefore, the phenotype could be correlated directly with the level of CDC45 transcripts.
The Presence of Small RNAs Correlates with the Severity of the Phenotype Observed

The presence of 21- to 23-bp RNAs was analyzed in plants of the T2 generation. RNAs were extracted from inflorescences from a sterile plant and a semifertile plant in lines T27 and T21. The results are shown in Figure 4B and demonstrate that all plants except the wild type showed the presence of small RNAs of 21 to 23 bp. Furthermore, the quantity of these small RNAs correlated with the severity of the phenotype, so that in plants that were sterile, an increased quantity of small RNAs was seen compared with plants that were semifertile, supporting the link between the decrease of CDC45 transcripts and the effect on the fertility of the plant. The results were repeated using a different sterile and semifertile plant from each line.

The analyses described below were performed on plants of the T2 generation to evaluate two aspects of CDC45’s role in Arabidopsis: first, its possible role in S-phase and mitosis, and second, its role in the sterility apparent in the T1 and T2 generations.

Role in Mitosis

Role of CDC45 in DNA Synthesis in T2 Plantlets

The first aim was to evaluate the role of CDC45 at the G1/S-phase of the cell cycle in plants. In many organisms, CDC45 plays a role in DNA replication during the mitotic cell cycle, although it is not essential for the transition to mitosis (Tercero et al., 2000). An efficient way to test for cell cycle or DNA synthesis defects is to analyze the incorporation of the thymidine analog 5-bromodeoxyuridine (BrdU) during S-phase. Therefore, the synthesis of DNA was measured in CDC45 RNAi plantlets (T24, T25, and T27, lines for which seeds were available, which had decreased CDC45 transcript levels, and which had appeared sterile in the first generation) and wild-type plantlets. The incorporation of BrdU during S-phase resulted in Hoechst fluorescence being quenched and therefore no longer proportional to cellular DNA content, enabling the effects to be followed from one cycle to the next. Single-parameter staining with Hoechst 33258 yielded no information about the cell’s location within...
the cell cycle. This additional information can be obtained by staining nuclei simultaneously with a DNA-specific dye, such as propidium iodide, that is not sensitive to the BrdU-induced quenching. A biparametric analysis based on dual staining with Hoechst 33258 and propidium iodide can provide complete information about cell cycle progression within the time interval between the beginning of BrdU incorporation and cell harvest (Trehin et al., 1997; Perennes et al., 1999). A double-parameter analysis allows for each quantity of DNA (2C, 4C, and 8C) to distinguish nuclei that had incorporated BrdU from those that had not. Nuclei that had incorporated BrdU were easily distinguishable by their Hoechst 33258 fluorescence decrease (denoted with asterisks throughout).

In the absence of BrdU, roots of 8-day-old plantlets had two populations of nuclei with 2C and 4C DNA content (data not shown; note that nuclei with 4C DNA content are either G2 nuclei or may result from endoreduplication). On the addition of BrdU, a third common population was seen, 2C′, nuclei that had synthesized DNA and incorporated BrdU with a resulting decrease in Hoechst fluorescence. BrdU incorporation was seen for both wild-type and CDC45 RNAi roots (Figure 5A, left), 2C nuclei incorporating BrdU and dividing to become 2C′ nuclei. Quantification revealed that incorporation efficiency was not altered significantly between the two wild-type samples and the three CDC45 RNAi lines, with 20 to 30% of nuclei having incorporated BrdU (Figure 5B, right). Similar results were obtained from the leaves of young plantlets, and again, no difference was seen between CDC45 RNAi and wild-type leaves (data not shown).

In an attempt to increase the level of cell divisions and thus make the effect of reduced CDC45 RNA levels more obvious, plantlets also were incubated in the presence of exogenous auxin, naphthylene acetic acid (NAA). This hormone increases the divisions by reactivating the cell cycle in the xylem pericycle of roots, which is necessary for lateral root formation (Himanen et al., 2002). The induction of cell division was seen clearly by the higher proportion of nuclei having incorporated BrdU in the root samples and the lower proportion of 4C nuclei (Figure 5B, left); quantification of the data from the cytograms revealed that a population of 2C′′ nuclei (i.e., nuclei that had been through two rounds of division and incorporated BrdU twice; ~40% of nuclei) was obtained but that CDC45 RNAi lines were no more or less efficient at this incorporation than was the wild type (Figure 5B, right). The effects of NAA also were made obvious by the decreased proportion of 4C nuclei (nuclei that must have been in G2, because endoreduplicated nuclei would not generate increased populations of 2C nuclei) to <10% compared with 25% for plantlets incubated without NAA. Therefore, NAA acted as an efficient activator of division in roots, and although this effect was obvious, the efficiency of DNA synthesis and cell cycling was not obviously impaired in CDC45 RNAi lines compared with wild-type lines.

Cell Cycle Genes Are Upregulated or Downregulated in CDC45 RNAi T2 Lines

To investigate the effects on gene regulation of reducing CDC45 levels in plants, small-scale microarray analysis was performed on RNA prepared from inflorescences of sterile CDC45 RNAi lines (T7; sterile in T1, reduced CDC45 transcripts, presence of small RNA fragments) compared with wild-type RNA. A mini chip was constructed from clones acquired from partners in the European Cell Cycle Consortium. A total of 107 cell cycle–related clones were available on the chip, including 5 non-cell cycle–related controls. RNA prepared from inflorescences of sterile CDC45 RNAi plants was compared with a control sample of RNA prepared from inflorescences of Wassilewskija plants at the same age. The results from two experiments are shown in Table 1. The limited number of genes available on the chip did not allow for an extensive analysis of gene regulation in CDC45 RNAi plants; however, taking a broad view, the data show that 5 of the 12 clones that were downregulated are cyclin-dependent kinase inhibitors (KRP). Other clones that were downregulated include CDC6 of the prereplication complex, DP1a and E2F-like factors of transcription involved in activating genes of the G1/S transition, and a wee1 homolog, a negative regulator of entry into mitosis. Genes that were upregulated include HAP transcription factors (homologs of yeast transcription factors that bind the CCAAT box in many eukaryotic promoters and that exists in multiple forms in Arabidopsis [Edwards et al., 1998]) and genes involved in the G1/S transition or S-phase, such as CDC2 or histone H4, respectively. The levels of the five non-cell cycle control genes in CDC45 RNAi plants were not altered significantly compared with those in the wild type.

Role in Sterility

Sterility Phenotype in T2 Plantlets

For the analyses of the role of CDC45 in sterility in the T2 generation, two lines were used: T27 and T221 (partially sterile in the T1 generation, presence of small RNAs). In parallel, for initial analyses, other lines, including T12, T19, T23, T35, T37, and T40, also were analyzed for phenotype in the T2 generation (lines T35, T37, and T40 also were sterile). In the T2 generation of all lines, the phenotype observed in the CDC45 RNAi lines was partial to complete sterility, even if the plant had not appeared sterile or was only slightly sterile in the T1 generation (as was the case for plants T19 and T23, respectively). The descendants of each line showed different levels of sterility, varying from plants that were completely sterile (defined as containing no properly formed seeds in the siliques) to those that appeared fertile as judged by silique size but that in fact contained fewer seeds than a wild-type silique (Figures 6A and 6B). When they finally formed, siliques were considerably smaller than those from nonsterile and wild-type plants (Figure 6C). Flowers on sterile plants were open for considerably longer periods than those on wild-type plants and remained unfertilized (Figures 6D and 6E).

Pollen Development and Female Fertility of CDC45 RNAi Plants Are Severely Affected

To establish the reasons for the reduction in fertility of CDC45 RNAi plants, analyses were performed on the development of
Figure 5. Cytometric Analyses of BrdU Incorporation in Roots of CDC45 RNAi Plantlets Show That Cell Cycle Progression Is Similar to That in the Wild Type.

Graphs show the percentage of nuclei at each cell cycle phase. 2C nuclei have not incorporated BrdU, 2C* nuclei have been through one round of incorporation, and 2C** nuclei have been through two rounds. Results from two wild-type samples and three CDC45 RNAi lines (T27, T24, and T25) are shown. Sample cytograms are shown at left, one for a wild-type (WT) sample and one for a typical CDC45 RNAi line (arrowheads indicate the position of each population of nuclei). Note that the density of spots is proportional to the number of nuclei counted. Spots on the top cytogram are fainter because the sample contained fewer nuclei.

(A) In the absence of NAA.

(B) In the presence of NAA to induce additional divisions.
the pollen in the two lines mentioned previously, T27 and T221. Differential interference contrast (DIC) microscopy of wild-type and CDC45 RNAi anthers from sterile plants was performed on anthers cleared in Herr’s solution and showed that although wild-type anthers were filled with regularly sized, well-formed pollen grains, CDC45 RNAi anthers from sterile plants contained shrunk and malformed grains and the anthers were partially empty (Figure 7A). Plants in the T2 generation that appeared fertile in fact showed an intermediate phenotype between that of the sterile CDC45 RNAi plants and the wild type (data not shown). To determine whether the malformed pollen grains were living, staining with Alexander solution was performed. These analyses showed that wild-type anthers appeared full of purple-stained (viable) pollen grains, unlike CDC45 RNAi plants, in which anthers were partially empty and the majority of pollen grains were nonviable (green grains) (Figure 7B). Although anthers from sterile CDC45 RNAi plants were smaller than the anthers from wild-type flowers at an equivalent stage of development, they did not appear altered in their morphology. Transversal flower sections confirmed that although another structure was not altered, the only defect was that most pollen grains were shrunken, with anthers still appearing partially dehiscent (Figure 7C). Defects in female fertility were analyzed by crosses of CDC45 RNAi plants with wild-type pollen. The following average numbers of seeds per silique were recovered: T27, 12; T221, 14; T2, 12. Very few abnormal seeds were observed, and the wild-type control gave 41 seeds per silique.

**Male and Female Gametophyte Development Proceeds Abnormally in CDC45 RNAi Flowers**

The defects in pollen formation and female fertility led us to analyze in more detail the development of male and female gametophytes. These analyses were performed for sterile plants in both CDC45 RNAi lines T27 and T221 and the wild type (Figure 8). Uniformly sized meiocytes appeared in wild-type as well as CDC45 RNAi anthers (Figures 8A and 8B). However, the development of the tetrads that form subsequently and contain the four products of meiosis was disrupted severely. Compared with the wild type, in which tetrads appeared with a regular tetrahedral structure, in CDC45 RNAi anthers (35S or dmc1 promoter), the tetrads were replaced by asymmetric polyads of two to six cells (Figures 8C and 8D). Although the megasporocyte...
In CDC45 RNAi ovules, the embryo sac was not visible (Figure 8H); thus, the divisions necessary for the development of the mature gametophyte had not occurred. The degree of curvature varies with the stage of ovule development (Reiser and Fischer, 1993), enabling ovules at a similar stage of development to be compared.

**Male Meiosis Is Disrupted in CDC45 RNAi Plants**

The defects observed in the formation of polyads in place of tetrads indicated a possible malfunctioning of meiosis leading to an incorrect distribution of meiotic products. Therefore, male meiosis was investigated by staining chromosomes with 4'-6-diamidino-2-phenylindole (DAPI). The stages in wild-type meiosis have already been described (Ross et al., 1996). Meiotic prophase is a long and complicated phase that differs from mitotic prophase and allows synapsis and recombination between homologous chromosomes (Figure 9A). The appearance of five bivalents (Figure 9B) preceded the separation of homologous chromosomes at the first meiotic division (Figures 9C and 9D) and sister chromatids at the second meiotic division (Figures 9E and 9F). In CDC45 RNAi plants (T27 and T221), chromosome synapsis can occur in meiocytes undergoing meiotic prophase as in the wild type (Figures 9G and 9H), which in less severely affected cells allowed the formation of bivalents (Figure 9I), although some fragmentation was seen. Meiocytes at metaphase I often showed severe chromosome fragmentation (Figure 9J), and although the chromosomes aligned before anaphase I, the first meiotic division did not proceed correctly, with fragments of chromosomes being distributed unequally (Figure 9K). The resulting fragmented chromosomes (Figures 9J and 9L) led to an unequal partitioning of chromosomes compared with the wild type and the formation of irregular polyads. It also is notable that the phenotype appeared more or less severe in different cells and that some meiocytes were seen in which meiosis proceeded more or less normally (Figures 9G to 9I).

**spo11 CDC45 RNAi Double-Mutant Phenotype**

The chromosome fragmentation seen in the CDC45 RNAi plants could indicate that CDC45 is involved in repairing meiosis-specific DSBs. The formation of DSBs is the major event specific to the meiotic cycle that requires DNA repair. The DSBs are necessary for recombination, the 5' ends being resected, leaving 3' overhangs for strand invasion and polymerase extension. Because CDC45 is necessary for polymerase recruitment during normal DNA replication, a role for CDC45 could be envisaged in this polymerase extension. DSBs are catalyzed by the protein SPO11 (Bergerat et al., 1997; Keeney et al., 1997; Grelon et al., 2001), and the Arabidopsis spo11 phenotype has been described in detail (Grelon et al., 2001, 2003). The absence of DSBs prevents recombination between homologous chromosomes; therefore, at metaphase I, 10 univalents were seen instead of 5 bivalents. The univalents then lead to the eight nuclei contained in the mature gametophyte.

**Figure 6. CDC45 RNAi Lines Show Reduced Fertility.**

(A) Semifertile (left) and sterile (right) CDC45 RNAi plants from line T27.
(B) Siliques decolored in Herr’s solution from a wild-type plant (top), a T27 semifertile plant (middle), and a T27 sterile plant (bottom). Bar = 2 mm.
(C) Comparison of mature siliques size from a T27 sterile plant (left) and a wild-type plant (right). Bar = 1 mm.
(D) and (E) Flower developmental sequences from a T27 sterile plant (D) and a wild-type plant (E). Bar = 0.5 mm.
segregated randomly at anaphase I. To analyze in more detail the role of CDC45 in the repair of meiotic DSBs, we introduced the CDC45 RNAi transcript into a spo11 mutant background (see Methods). Four plant lines homozygous for the spo11 mutation and containing the CDC45 RNAi insert were analyzed for their meiotic behavior. The lines showed the phenotype illustrated in Figure 10. As in the CDC45 RNAi plants, meiocytes were more or less severely affected, with a few meiocytes resembling the spo11 phenotype (Figure 10A), others showing an intermediate phenotype with fragmentation of some univalents (Figure 10B), and the majority showing highly fragmented chromosomes that could not be distinguished from the CDC45 RNAi phenotype (Figures 10C and 10D). Therefore, the fragmentation seen in CDC45 RNAi plants was not attributable to a defect in the repair of SPO11-induced DSBs, which is compatible with the role of the CDC45 protein in premeiotic DNA replication.

**DISCUSSION**

**CDC45 and Meiosis**

In both yeast and mammals, CDC45 has been shown to be necessary for DNA replication during the mitotic cell cycle, but a role in meiosis has not yet been demonstrated. A CDC45 homolog has been cloned from Arabidopsis that is highly expressed in young flower buds (where meiotic divisions occur) and has been shown to be necessary for the correct progression of meiotic divisions required for gametogenesis and thus plant fertility. These results are supported by the observation that using the dmc1 promoter (a meiosis-specific promoter), the number of sterile plants generated was increased. This is a novel finding in that CDC45 had not been shown previously to play a role in meiosis in any organism. Supporting evidence for our observations of CDC45’s role in meiosis come from two re-

![Figure 7](image.png)

**Figure 7.** Pollen Development Is Affected Severely in CDC45 RNAi Lines.

(A) DIC microscopy of anthers cleared in Herr’s solution. The wild type (left) shows regularly sized pollen grains, and CDC45 RNAi anthers (right) show partially empty anthers and malformed, irregularly sized pollen grains. Bars = 50 μm.

(B) Alexander staining of anthers to show pollen grain viability. The wild type (left) shows viable, purple-stained pollen grains, and CDC45 RNAi anthers (right) show few viable pollen grains (purple) and many nonviable pollen grains (green). Bars = 50 μm.

(C) Transverse sections from wild-type (left) and CDC45 RNAi (right) anthers. Wild-type anthers are dehiscent and contain living pollen grains. CDC45 RNAi anthers are smaller and contain many nonviable pollen grains but still are dehiscent and do not show an altered structure compared with the wild type. Bars = 100 μm.
The authors suggest the existence of specific meiotic sub-
targets for CDK2. Second, Cut5 in fission yeast and
necessary for the recruitment of CDC45 to origins, has been shown
to have an unanticipated role in prophase I of meiosis but is not
required for mitotic cell divisions in mouse (Ortega et al., 2003). We addressed the question of whether the meiotic chromo-
some fragmentation found in CDC45 RNAi plants reflects a de-
fect in the repair of these breaks by retransforming spo11 heter-
zygote plants with the CDC45 RNAi construct. Analysis of the double mutant spo11 CDC45 RNAi showed that in the absence
of SPO11-induced DSBs, chromosomes remain fragment-
ated. This finding suggests that the fragmentation is not the result of a defect in the repair of the meiosis-specific SPO11-
induced DSBs, as in MEI1 (Grelon et al., 2003), and therefore
may arise from events that occur earlier in meiosis. Thus, it is
likely that CDC45 plays a role in the round of DNA replication
that occurs before meiosis, a premise that is compatible with
its conserved function in other organisms. In yeast cells,
CDC45 appears to have dual roles: not only is this protein nec-
ecessary for the switch from a prereplicative to a postreplicative
complex, but it also forms part of the complex of proteins associ-
ated with the replication fork and is necessary for elongation
(Tercero et al., 2000). Thus, defects in replication caused by
low CDC45 levels, which result from the lack of initiation of rep-
lication or stalling of replication forks, could change the struc-
ture of the meiotic chromosomes, making them “stickier” or lia-
ble to form interchromosomal bridges (e.g., if unreplicated DNA
leads to inappropriate associations). Bridges and chromo-
somes with a sticky appearance are characteristic of the
CDC45 RNAi phenotype. The fragile chromosomes then frag-
ment at metaphase as a result of the forces applied by the
spindle apparatus. Moreover, the stalled replication forks cre-
ate additional double-strand breaks (Haber, 1999). If these re-
main unrepaired while the cell cycle continues, they will lead to
incomplete replication, possible inversions and subsequent in-
appropriate associations, and the fragmentation seen later in meiosis.

Other Mitotic Genes Involved in Meiosis

Genes involved in the regulation of the mitotic cell cycle often
have roles in meiosis. Genes involved in the prereplicative com-
plex of mitotic S-phase also seem to be necessary for premei-
otic S-phase. A degon mcm4 mutant in fission yeast cannot
perform meiosis (Lindner et al., 2002). Furthermore, reduced activity of the S. pombe equivalents of cdc6p, mcm2p, mcm4p,
and orc1p prevents the completion of DNA replication but not
the formation of DSBs (Murakami and Nurse, 2001). How-
ever, in S. cerevisiae, mutations in CLB5 and CLB6 (encoding
B-type cyclins) prevent premeiotic DNA replication (Stuart and
Wittenberg, 1998; Smith et al., 2001), and the mutants clb5
and clb6 are defective in DSB induction, recombination,
and synaptonemal complex formation (Smith et al., 2001). The latter experiments show that the regulators of mitosis, as well as the proteins involved in the prereplication complex, have roles in meiosis. Similarly, in *S. pombe*, cdc2, which is required for the initiation of both mitotic S-phase and M-phase, is essential for premeiotic DNA synthesis (Iino et al., 1995). Other enzymes, such as DNA polymerase I, are required for premeiotic DNA replication and sporulation in *S. cerevisiae*. Thus, CDC45, either as a component of the prereplication complex or the elongation complex or as the recruiter of DNA polymerase, could play an essential role in premeiotic S-phase.

**CDC45 in Mitosis**

The transcripts for *CDC45* are upregulated at the G1/S transition, as has been shown in other organisms (Saha et al., 1998). Although this finding does not give an indication of the level of protein activity during the cell cycle, it indicates that *CDC45* may be one of a number of genes important in the G1/S transition. We also have shown here that *CDC45* is upregulated in many plant tissues, in agreement with the S-phase activity found in these tissues. *CDC45* probably plays a role in mitosis in Arabidopsis. However, using RNAi, we were unable to detect an obvious mitotic phenotype. The reasons for this may be twofold. First, because the RNAi technique gives rise to plants differing in the amount by which the transcript levels have been reduced, plants with severe mitotic phenotypes resulting from a severe reduction in transcript levels would not be selected for. Second, as indicated by the microarray analyses, the plant may be able to compensate for the reduced levels of *CDC45* by regulating other cell cycle genes, such as reducing the activity of cyclin-dependent kinase inhibitors, which are negative cell cycle regulators. Thus, a reduction in a negative regulator could compensate for a possible slowing of S-phase or the G1/S transition caused by reduced *CDC45* levels, allowing a certain plasticity of the cycle. Increased levels of feedback

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**Figure 9.** DAPI Staining of Wild-Type and CDC45 RNAi Pollen Mother Cells Shows That CDC45 RNAi Plants Undergo Abnormal Meiosis.

(A) to (F) Wild-type meiosis showing meiotic prophase I, during which chromosome condensation and homologous chromosome synopsis occur (A) before the five bivalents appear (B), which are separated at metaphase I to anaphase I ([C] and [D]) of the first meiotic division. Sister chromatids then are segregated, giving rise to the four meiotic products in the second meiotic division ([E] and [F]).

(G) to (L) Meiosis in *CDC45* RNAi plants showing prophase events such as homologous chromosome synopsis ([G] and [H]) and bivalent formation ([I]) with the presence of some fragmentation. Metaphase I and anaphase I occur in less affected meiocytes, but chromosome fragmentation is seen already ([J] and [K]) and meiocytes show phenotypes varying in severity. Many meiocytes show severe chromosome fragmentation (>20 fragments; [J] and [L]) and many interchromosome bridges ([L]).

Bar in (A) = 5 μm for (A) to (L).
regulation and checkpoints may exist during mitosis that enable mitosis to proceed normally even when the level of CDC45 transcripts is reduced. Indeed, in yeast, CDC45 is not necessary for the mitotic transition (Tercero et al., 2000). Therefore, we suggest that premeiotic DNA replication requires a higher level of CDC45 than mitotic DNA replication, the high levels of CDC45 transcript in early flowers being consistent with this theory. Premeiotic S-phase is longer than mitotic S-phase; thus, the requirements for proteins such as CDC45 may be different. In yeast, the cyclin Bs CLB5 and CLB6 are indispensable for meiosis, whereas their loss results in only a slight delay in the initiation of DNA replication (Stuart and Wittenberg, 1998). Premeiotic replication may differ from the round of replication associated with mitosis, based on suggested links with recombination and chromosomal interactions required for interhomolog interactions (Borde et al., 2000; Cha et al., 2000). These observations highlight the differences between the mitotic and the meiotic cell cycles that constitute fascinating areas of future study.

METHODS

Plant Material

Plants of Arabidopsis thaliana ecotype Wassilewskija were grown under long-day conditions (16 h light) at 19.5°C (day) and 17.5°C (night). The Arabidopsis cell suspension culture derived from Arabidopsis ecotype Columbia was grown at 21°C in 16 h light and subcultured every 7 days in Gamborg’s B5 medium (Sigma) supplemented with 0.2 mg/L α-naphthalene acetic acid. Suspension cells were synchronized by a 24-h treatment with 20 μg/μL aphidicolin as described previously (Doutreux et al., 1998). At different times, the cells were harvested and quickly frozen in liquid nitrogen before isolation of RNA.

RNA Extractions and Reverse Transcriptase–Mediated PCR

Total RNA was isolated by grinding tissue in liquid nitrogen in the presence of Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 5 μg of total RNA using Superscript II reverse transcriptase (Life Technologies) according to the manufacturer’s instructions. For semiquantitative reverse transcriptase–mediated (RT) PCR analysis, 2 μL was used for PCR in a final volume of 20 μL ensuring that, for the control, the amount of amplified product produced was proportional to the initial concentration of template present in the reaction, in this case 20 cycles. After electrophoresis on a 1% agarose gel and blotting onto Hybond N+ membranes (Amersham), hybridization was performed with 32P-labeled probes labeled using the random primer method (Qiagen, Carlsbad, CA). Hybridizations were performed at 62°C (Church and Gilbert, 1984). CDC45, histone H4, Actin2, and EF1α probes correspond to the respective coding sequences.

CDC45 Cloning

The CDC45 cDNA was cloned by RT-PCR from first-strand cDNA prepared from Arabidopsis RNA, ecotype Columbia, using the Platinum Pfx DNA polymerase (Invitrogen). The position of the 5′ end was determined according to alignments with cDNAs from other organisms and the position of an in-frame stop codon upstream of the ATG. The complete AtCDC45 cDNA was cloned as two fragments: the 5′ fragment using primers 5′-CGGAAATGTTGAGGATTAG-3′ and 5′-GCTTAACCATG-AAAGCCGAGAAATCCTGCG-3′ and the 3′ fragment using primers 5′-TGTTCATGGAGGTGACTGAGAAATA-3′ and 5′-GACTATGTCAGAGTCCTCG-3′. After sequencing to ensure that no errors were present, the two fragments were assembled in pUC18 to create the full-length cDNA using a unique SpeI site in the CDC45 coding sequence and present in the overlapping regions of the 3′ end of the 5′ clone and the 5′ end of the 3′ clone.

RNA Interference Constructs

An 870-bp fragment of CDC45 was amplified from the CDC45 coding sequence (between 33 and 902 bp relative to the ATG at +1 bp) using the following primers containing restriction sites as indicated in boldface: 5′-CGGAAAGCCTTCTAGAGTCAGCTACTTTATC-3′ (XbaI), 5′-GAACAGTCGTCGAGGATTAAG-3′ (BamHI), 5′-CGGAAAGCCTCAGAGTCCTACCTTATC-3′ (XhoI), and 5′-GAGACTATGTCTGTCGTTTTCTC-3′ (EcoRI). The PCR fragment generated with the Xba-Bam pair of primers (to form the sense fragment) was cloned first into pGEM-T and then the XbaI-EcoRI–digested (antisense fragment) was also cloned first into pGEM-T and then the XhoI-EcoRI–digested CDC45 fragment was cloned into the same restriction enzyme sites present in pKannibal (Wesley et al., 2001) to create pKannibal 45XB. The PCR fragment generated with the Xho-Eco pair of primers (to form the antisense fragment) was also cloned first into pGEM-T, and then the XhoI-EcoRI–digested CDC45 fragment was cloned into the same sites in pKannibal 45XB to create pKannibal (45S). The Xba-Xhol RNA interference (RNAi) fragment containing the two CDC45 sequences in opposing orientations separated by an 800-bp intron sequence was cloned into the binary vector p2P111 containing the 35S promoter and either the gene for kanamycin or gentamycin resistance and cut with XbaI and Sail.
to create pPZP111(45). The same XbaI-XhoI RNAi fragment was cloned downstream of the actin1 promoter (Klimyk and Jones, 1997) in the pPF111 vector (Basta resistant).

**Plant Transformation**

The plasmid pPZP111(45) was introduced into Agrobacterium tumefaciens (HBA10S), and Arabidopsis plants, ecotype Wassilewskija, were transformed using floral-dip method (Clough and Bent, 1998). Seeds from the A. tumefaciens–treated plants were selected on 0.5× Murashige and Skoog (1962) medium containing 100 mg/L kanamycin (MS-Km). Kanamycin-resistant plantlets (T1) were transferred to soil and grown in the greenhouse under long-day conditions (16 h of light). The presence of the CDC45 insert was checked by PCR on DNA extracted from plantlets. Seeds from mature plants were collected and plated onto MS-Km (T2 generation).

**Visualization of Small RNA Fragments**

Twenty-one- to 23-nucleotide RNA fragments were detected using a previously described method (Hutvagner et al., 2000). Total RNA (25 μg) was separated on 6% polyacrylamide 8 M urea gels in the Protean apparatus (Bio-Rad). After electrophoresis in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA, pH 8), RNA was electroblotted onto a Hybond-N membrane (Amersham) for 1 h at 340 mA at room temperature. RNA was fixed on the membrane using a UV cross-linker. Prehybridization and hybridization were performed as described above except at 47.5 or 50°C, and membranes were rinsed in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) followed by two washes of 20 min in 2× SSC and 1% SDS before exposure to film.

**5-Bromodeoxyuridine Incorporation and Flow Cytometry Analyses**

Eight-day-old Arabidopsis plantlets, having been selected on kanamycin-containing medium, were incubated in the dark in 0.5× Murashige and Skoog (1962) medium in the presence of 30 μM freshly prepared BrdU (a thymidine analog) with or without the addition of 10 μM exogenous auxin (naphthalene acetic acid; NAA) to induce root pericycle divisions (Himanen et al., 2002). A minimum of eight plantlets were used for each sample. After 4 days, plantlets were harvested by separating roots and leaves and chopping each tissue in 0.5 mL of Gailbraith buffer (45 mM MgCl, 30 mM sodium citrate, and 20 mM 4-morpholinepropane sulfonate supplemented with 1% [w/v] Triton X-100, pH 7) with a razor blade. The chopped sample containing liberated nuclei was stored at 4°C after the addition of 1% formaldehyde. The samples were filtered through nylon (30-μm pore size). Nuclei were treated with 10 μg/mL RNase before being stained for 10 min with 1 μg/mL Hoechst 33258 (HO; Sigma), and 3 μg/mL propidium iodide (PI; Sigma) was added for another 10 min. Cytometric analysis was performed on 104 nuclei with an Elite E.S.P. Beckman-Coulter flow cytometer according to the conditions described by Perennes et al. (1999). Biparametric analysis was performed according to Glab et al. (1994): the nuclei were excited with UV light (351 to 364 nm), and a bivariate cytogram of red (PI > 610 nm) versus blue (408 < HO < 500 nm) fluorescence was recorded. Care was taken to eliminate both debris and doublets through light-scatter and pulse-shape analysis. Quantification of spots was performed using the Beckman-Coulter software.

**Microarray Analysis**

Cell cycle–related cDNA clones were obtained from the partner laboratories of the European Cell Cycle Consortium. Clones were amplified using standard PCR techniques, and products were precipitated using isopropanol and resuspended in nuclease-free water to a final concentration of at least 400 ng/μL. Product concentration and purity were confirmed by electrophoresis. A total of 12.5 μL of PCR product was mixed with 12.5 μL of DMSO and printed onto CMT-GAPS–coated slides (Acton, MA) using a GMS 417 Arrayer (Genetic MicroSystems, Woburn, MA) according to the manufacturer’s instructions with 10 replicas. The printed slides were dried for 3 h at room temperature and subjected to UV cross-linking (65 mJ).

For hybridization, 5 μg of total RNA was used for amplification using the MessageAmp aRNA kit (Ambion, Austin, TX) according to the manufacturer’s instructions. Five micrograms of amplified RNA was reverse-transcribed with Cy3-dUTP or Cy5-dUTP (Amersham Biosciences, Bucks, UK): 5 μg of amplified RNA and 6 μg of random primer (Roche Diagnostics, Mannheim, Germany) were dissolved in a final volume of 27 μL of nuclease-free water, heated at 70°C for 10 min to denature the RNA, and cooled to room temperature for 10 min. A master mix containing 10 μL of First-Strand Buffer (Invitrogen, Paisley, UK), 5 μL of 0.1 M DTT, 1 μL of RNase inhibitor (Invitrogen), 1 μL of deoxyribonucleotide triphosphate mix (5 mM each of dATP, dGTP, and dCTP), 2 μL of 1 mM dUTP, 2 μL of 25 mM Cy3-dUTP or Cy5-dUTP (Amersham Biosciences), and 2 μL (400 units) of Superscript II reverse transcriptase (Invitrogen) was added. The reaction sample was incubated at 42°C for 2 h, and 1 μL (2 units) of RNase H (Invitrogen) was added and incubated at 37°C for 15 min to degrade the RNA template. The labeled cDNA was purified using the QIAquick PCR Purification Kit (Qiagen, West Sussex, UK) and dried. The pellet was resuspended in 20 μL of hybridization buffer (50% formamide, 5× SSC, and 0.1% SDS), and the Cy3- and Cy5-labeled cDNAs were combined. The labeled cDNA was heated at 95°C for 3 min and centrifuged at 13,000 rpm for 1 min. Microarray slides were incubated in prehybridization buffer (5× SSC, 0.1% SDS, and 1% BSA) for 1 h at 42°C. The slides were washed in sterile water and isopropanol and dried using compressed air. For hybridization, the labeled cDNA solution was added to the slide and covered with the cover slip. The slide was placed in a sealed ArrayIt hybridization cassette (TeleChem International, Sunnyvale, CA) and submerged in a 42°C water bath for 16 to 20 h. After hybridization, slides were washed in 1× SSC and 0.2% SDS for 5 min, then in 0.1× SSC and 0.2% SDS for 5 min, and finally in 0.1× SSC for 5 min. Then, the slides were dried immediately. The slides were scanned in a GMS418 Array Scanner (Genetic MicroSystems).

For microarray data analysis, image analysis and signal quantification were performed with Image software version 4.0 (BioDiscovery, Los Angeles, CA). After scanning and quantification of signals, the data were imported into Microsoft Excel (Redmond, WA) for further analysis. The median of all background fluorescence values was calculated and subtracted from all samples. The resulting values were divided by the average signal intensity of all sample values to obtain a proportional value for each sample. Ratios of the proportional values for Cy3 and Cy5 signals were calculated for each clone. The 10 replicates were used to calculate means and standard deviations of these ratio values for each clone.

**Analyses by Light Microscopy**

Anthers were stained in Alexander solution to stain pollen grains and observed by light microscopy (Alexander, 1969). Differential interference contrast microscopy was used to observe anthers or ovules that had been cleared using Herr’s solution (phenol:chloral hydrate:85% lactic acid:xylene:oil of clove [1:1:0.5:1]).

Wild-type and CDC45 RNAi flowers were fixed with FAA (50% ethanol, 5% acetic acid, and 10% formaldeyde), dehydrated in increasing ethanol concentrations and eosin, and embedded in Historesin (Leica, Wetzlar, Germany) overnight at room temperature. Transversal sections of 1.5 μm were cut using an LKB Ultratome III ultramicrotome, colored with
Creation of spo11 CDC45 RNAI Lines

Plants heterozygous for the spo11 mutation (kanamycin resistant) (Grelon et al., 2001) were transformed with the CDC45 RNAI construct (now associated with gentamycin resistance), and transformants were selected for kanamycin and gentamycin resistance. The resulting transformants (140 lines) were screened by PCR for the presence of the CDC45 RNAI insertion (primers 5′-TGTTTATGAGGTGGTGAGTA-GAAAAGATA-3′ and 5′-CTTCCAAGCTGGGTACCCGTAATCC-3′) and the spo11 mutant and SPO11 wild-type alleles using oligonucleotides as described previously (Grelon et al., 2003). Plants homozygous for the spo11 mutation and transformed with CDC45 RNAI were selected. At the same time, heterozygous spo11 mutant plants transformed with the CDC45 RNAI transcript were self-pollinated, and the resulting spo11 homozygous mutants were selected. (The transformation technique was used in preference to crossing two heterozygous lines because of the high level of sterility in the T1 progeny resulting from a cross that made obtaining T2 lines homozygous for the spo11 mutation very difficult.)

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Catherine Bergounioux, bergouni@bpb.u-psud.fr.

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A *CDC45* Homolog in Arabidopsis Is Essential for Meiosis, as Shown by RNA Interference—Induced Gene Silencing
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