

The Anaphase-Promoting Complex Is a Dual Integrator That Regulates Both MicroRNA-Mediated Transcriptional Regulation of *Cyclin B1* and Degradation of Cyclin B1 during *Arabidopsis* Male Gametophyte Development

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The anaphase-promoting complex/cyclosome (APC/C), an essential ubiquitin protein ligase, regulates mitotic progression and exit by enhancing degradation of cell cycle regulatory proteins, such as *CYCB1;1*, whose transcripts are upregulated by *DUO POLLEN1* (*DUO1*). *DUO1* is required for cell division in male gametophytes and is a target of microRNA 159 (miR159) in *Arabidopsis thaliana*. Whether APC/C is required for *DUO1*-dependent *CYCB1;1* regulation is unknown. Mutants in both *APC8* and *APC13* had pleiotrophic phenotypes resembling those of mutants affecting microRNA biogenesis. We show that these *apc/c* mutants had reduced miR159 levels and increased *DUO1* and *CYCB1;1* transcript levels and that APC/C is required to recruit RNA polymerase II to *MIR159* promoters. Thus, in addition to its role in degrading *CYCB1;1*, APC/C stimulates production of miR159, which downregulates *DUO1* expression, leading to reduced *CYCB1;1* transcription. Both *MIR159* and APC8–yellow fluorescent protein accumulated in unicellular microspores and bicellular pollen but decreased in tricellular pollen, suggesting that spatial and temporal regulation of miR159 by APC/C ensures mitotic progression. Consistent with this, the percentage of mature pollen with no or single sperm-like cells increased in *apc/c* mutants and plants overexpressing *APC8* partially mimicked the *duo1* phenotype. Thus, APC/C is an integrator that regulates both microRNA-mediated transcriptional regulation of *CYCB1;1* and degradation of *CYCB1;1*.

INTRODUCTION


The gametes of flowering plants are formed within haploid gametophytes (McCormick, 2004). In the male gametophyte, each haploid microspore divides asymmetrically to produce a larger vegetative cell that will form the pollen tube and a smaller generative cell, which divides symmetrically to form twin sperm cells. These sperm cells are delivered to the embryo sac via the pollen tube, where they fuse with an egg and a central cell to produce embryo and endosperm, respectively. The molecular mechanisms underlying the production of male gametes remain largely unknown, although a few required genes have been identified (Okada et al., 2005; Rotman et al., 2005; Iwakawa et al., 2006; Mori et al., 2006; Nowack et al., 2006; Kim et al., 2008; Brownfield et al., 2009b; Gusti et al., 2009; Ron et al., 2010). Among these genes, *DUO POLLEN1* (*DUO1*) encodes a male germ cell-specific R2R3 Myb protein (Rotman et al., 2005) that is


required for expression of the *Arabidopsis thaliana* G2/M regulator CyclinB1;1 (*CYCB1;1*) in the male germline (Brownfield et al., 2009a), suggesting an integrative role for *DUO1* in cell specification and cell cycle progression that is necessary for twin sperm cell production. *DUO1* mRNA is targeted directly by microRNA159 (miR159) (Palatnik et al., 2007), which leads to its degradation.

Cell proliferation in all eukaryotes depends on the ubiquitin ligase (E3) activity of the anaphase-promoting complex/cyclosome (APC/C) (Peters, 2006). The APC/C in *Arabidopsis* contains at least 11 subunits (Capron et al., 2003a). Among them, APC3, APC5, APC6, APC7, and APC8 each contain a tetratricopeptide repeat (TPR) protein–protein interaction domain and are expected to function as receptors that interact with regulatory proteins (Lima et al., 2010). Without APC/C, cells cannot separate their sister chromatids in anaphase, cannot exit from mitosis to divide into two daughter cells, and cannot initiate the steps necessary for DNA replication in S phase (Peters, 2006). The APC/C was initially found to be involved in targeted proteolysis of A- and B-type cyclins, thus facilitating exit from mitosis (Glotzer et al., 1991). The genetic inactivation of APC/C has caused lethality in all species in which it has been investigated so far (Yu et al., 1998; Yamashita et al., 1999; Cullen et al., 2000; Bentley et al., 2002; Garbe et al., 2004; Pál et al., 2007; Jin et al., 2010). For example, in *Caenorhabditis elegans*, an APC8 mutant, *mat-3*,

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is a temperature-sensitive embryonic lethal due to defects in germline meiosis and mitosis (Garbe et al., 2004).

In *Arabidopsis*, single-copy genes encode counterparts of all known vertebrate APC/C subunits, except for APC3/CDC27, which has two genes (Capron et al., 2003a). Plant APC/C is involved in cell cycle regulation. For example, APC2 and APC6 are important for female gametophyte development (Capron et al., 2003b; Kwee and Sundaresan, 2003), as plants with loss-of-function alleles of either of these genes exhibited female gametophytic lethality due to cell cycle arrest at an early stage of embryo sac development. Reciprocal crosses with the *apc6/nomega* mutant suggested that most defects came from the female, although male transmission was somewhat reduced (Kwee and Sundaresan, 2003). Interestingly, a *mir159ab* double mutant (Allen et al., 2007) phenocopied the *apc13* mutant (Saze and Kakutani, 2007) and the *apc6* and *apc10* RNA interference lines (Marrocco et al., 2009) that had pleiotropic morphological defects, including small siliques, curled leaves, and abnormal phylotaxy, suggesting that both APC/C and miR159 might be required for similar developmental processes.

To identify new genes in miRNA biogenesis and function pathways, we performed a forward genetic screen. One mutant had pleiotropic developmental phenotypes expected for mutants in miRNA pathway genes, such as *DICER-LIKE1* (*DCL1*) (Jacobsen et al., 1999), but surprisingly it was a point mutation in *APC8*. This point mutant, *apc8-1*, resulted in reduced *MIR159* transcription and lessened accumulation of mature miR159. This caused an increase in transcripts of both *DUO1* and *CYCB1;1* in pollen. We further showed that APC/C is required for RNA polymerase II (Pol II) recruitment to promoters of *MIR* genes. *CYCB1;1*-green fluorescent protein (GFP) accumulated in both the vegetative cell and sperm cells of tricellular pollen in *apc8-1* but was absent in wild-type tricellular pollen. We also observed an increased incidence of unicellular and bicellular pollen in mature anthers of *apc8-1* and increased ratio of bicellular pollen in mature pollen by overexpressing *APC8* in male germlines, suggesting that disrupted balance of *CYCB1;1* levels prevented efficient transition of male gametophytes through the bicellular and tricellular stages. Similar results were obtained by analyzing a T-DNA insertion allele of *APC13*, another component of the APC/C. Therefore, we propose a dual role for the APC/C both in coordinating miRNA-mediated transcriptional regulation of *CYCB1;1* and in direct protein degradation of *CYCB1;1*.

RESULTS

Isolation and Characterization of a Weak *APC8* Allele

DCL1 plays a critical role in miRNA biogenesis in plants (Jacobsen et al., 1999), and seeds from *dcl1* null alleles cannot be obtained due to embryo lethality. However, a weak allele of *dcl1*, *dcl1-14* (in which the T-DNA insertion is located in the promoter region, SALK_056243; see Supplemental Figure 1A online), showed curly leaves, abnormal reproductive development, and reduced miRNA levels (see Supplemental Figures 1B and 1C online) but is fertile. We therefore performed a genetic screen after ethyl methanesulfonate mutagenesis of *dcl1-14*, aimed at the isolation

of mutants with enhanced or suppressed phenotypes. We isolated a mutant with much more severe developmental phenotypes than those of *dcl1-14*. The pleiotropic phenotypes included distorted leaf shapes, abnormal shoot meristem development, and a delay in the transition from the vegetative to the reproductive stage (Figures 1A and 1B). Moreover, the mutant had severely abnormal flower and silique development, including bushy inflorescences and shorter siliques (Figures 1C and 1D). We showed that these phenotypes were *dcl1-14* independent by backcrossing to wild-type plants. Therefore, the mutant without *dcl1-14* background was used for our subsequent analyses. Collectively, these pleiotropic developmental defects suggested that the mutated gene had roles at multiple stages of plant development.

To further understand the basis of these heritable pleiotropic phenotypes, we examined their inheritance in F2 progeny from a cross of a mutant plant (Columbia [Col]) to a wild-type Landsberg *erecta* plant. The genotype was determined for 661 F2 plants with obvious mutant phenotypes, which comprised ~10% of the F2 population. Characterization of Col/Landsberg *erecta* polymorphisms throughout the genome showed that all plants with the mutant phenotype were homozygous for the Col haplotype at one region on chromosome 3, suggesting that a disrupted gene in this region was responsible for the mutant phenotype. This region was narrowed down to an interval between BAC clones F18E5 and T8O5 (five recombinants and two recombinants, respectively, out of 1322 chromosomes examined). We sequenced the genes in this region and found a point mutation (G to A) in the coding region of *APC8*. This mutation resulted in the conversion of a highly conserved Asp (Asp-309) to an Asn at a position adjacent to the TPR domain (Figure 1F). To confirm that this mutation was responsible for the phenotype, a genomic fragment was fused to yellow fluorescent protein (YFP) and introduced into the mutant. The morphological phenotypes were fully rescued in 23 of 25 plants (Figure 1E). Therefore, we concluded that the phenotypes were due to the mutation in *APC8* and designated this allele *apc8-1*.

Arabidopsis APC8 Can Complement an *APC8* Mutant in *Schizosaccaromyces pombe*

To determine whether *Arabidopsis APC8* is a functional ortholog of *APC8* found in other eukaryotes, we tested whether *Arabidopsis APC8* was able to functionally complement an *APC8* mutant in fission yeast. We introduced full-length cDNAs of both *APC8* and mutant *apc8-1* (*APC8* D to N) into a yeast expression vector under the control of the Gal promoter. These constructs were then transformed into the temperature-sensitive *cut23-174* mutant, which has a lesion in the *APC8* gene in *S. pombe*. This mutant can grow at 28°C but not at 37°C (Yamashita et al., 1999). Expression of wild-type *APC8* rescued the yeast mutant strain at the restrictive temperature, whereas an empty vector or the mutant *apc8-1* did not (Figures 2A and 2B). Moreover, a construct in which the Asp was changed to Glu (*APC8* D to E) only partially complemented the yeast mutant at 37°C (Figures 2A and 2B), which reinforces the idea that the conserved Asp is important for *APC8* function.

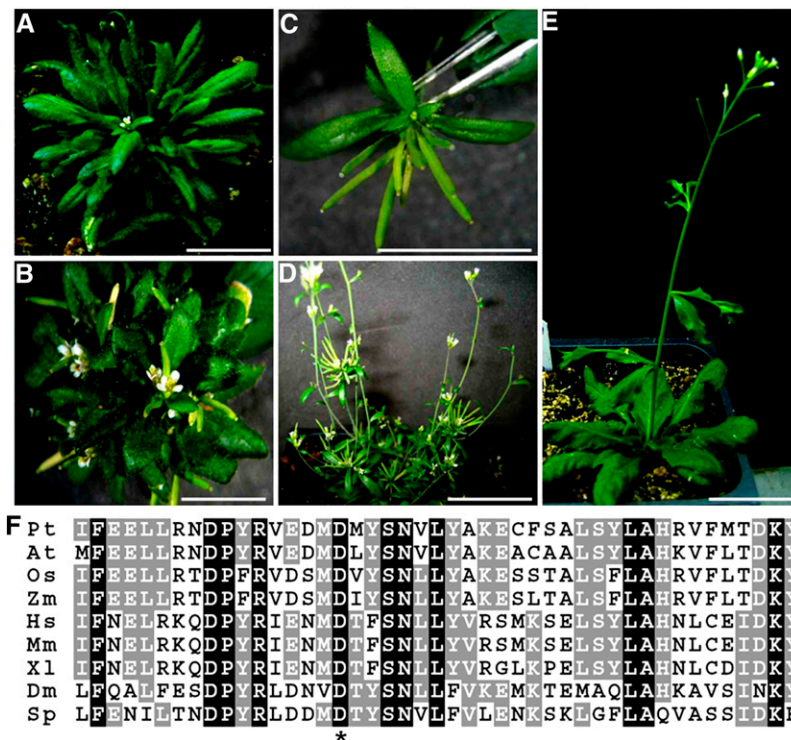


Figure 1. Phenotypes and Complementation of the *apc8-1* Mutant.

(A) A representative 5-week-old seedling of *apc8-1*.

(B) A representative 8-week-old *apc8-1* plant.

(C) Close-up of fasciculate siliques in *apc8-1*.

(D) A representative 12-week-old *apc8-1* plant.

(E) Complementation of *apc8-1*. A representative *apc8-1* plant carrying the *pAPC8:APC8-YFP* transgene.

(F) Partial amino acid sequences of APC8 in various species. Identical residues are white on black background, and conserved residues are white on gray background. Asterisk indicates the Asp (D309) that was mutated to Asn in *apc8-1*. At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*; Os, *Oryza sativa (japonica)*; Zm, *Zea mays*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dm, *Drosophila melanogaster*; Sp, *Schizosaccharomyces pombe*.

Bars = 1 cm in (A), (B), (D), and (E), and 1 mm in (C).

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Subcellular Localization of APC8 Is Regulated during Male Gametophyte Development

Microarray analyses indicated that *APC/C* genes were ubiquitously expressed in *Arabidopsis* tissues (Zimmermann et al., 2004). To validate the expression pattern of *APC8*, we performed RT-PCR analyses using cDNA samples from different *Arabidopsis* tissues. *APC8* expression was detected in a variety of vegetative and reproductive tissues (see Supplemental Figure 2A online). To substantiate the RT-PCR analyses, we constructed different promoter-reporter constructs. The β -glucuronidase (GUS) reporter was expressed in almost all tissues examined, including leaves, shoot apical meristems, inflorescences, and siliques (see Supplemental Figure 2B online). During male gametophyte development, the GFP reporter was detectable at the unicellular and bicellular stages but appeared less intense at the tricellular stage (see Supplemental Figure 2C online). We therefore examined the subcellular localization of a *pAPC8:APC8-YFP* fusion protein during male gametophyte development. This

protein fusion construct complemented the *apc8-1* phenotypes completely (Figure 1E), showing that YFP-tagged APC8 was functional. Foci of APC8-YFP were visible in the nuclei and cytoplasm of unicellular microspores (Figure 3A) and bicellular pollen (Figure 3B), but only a very faint signal was detected in tricellular pollen (Figure 3C), and no signal was detectable in mature pollen (Figure 3D). Moreover, the foci of APC8-YFP varied in different microspores and pollen grains (Figure 3).

APC/C Is Required for Efficient Male Transmission

Microscopy analysis showed that the siliques in *apc8-1* had many (~66%) apparently undeveloped ovules (Figure 4A), which led to obviously reduced seed set (Figure 4B). The mutant was backcrossed to the wild type, and the F1 was allowed to self-pollinate. Only ~10% of the F2 progeny had the mutant phenotypes, instead of the expected 25%. These results suggested that the mutant had transmission problems. Reciprocal crosses

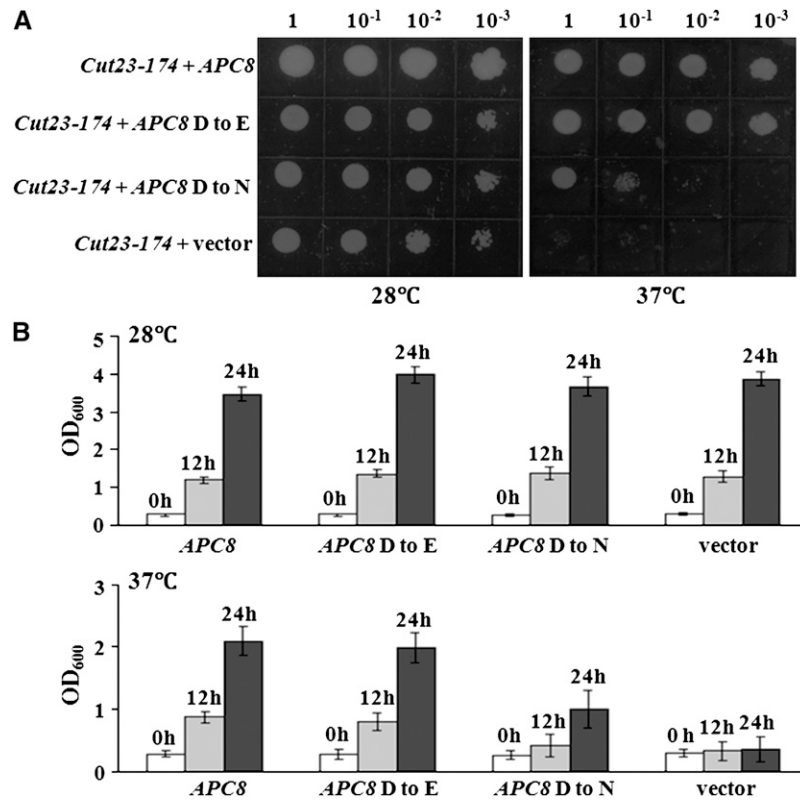


Figure 2. *Arabidopsis* *APC8* Complements the Fission Yeast *APC8* Mutant *cut23-174*.

(A) Growth at the permissive (28°C) or restrictive (37°C) temperature of the *cut23-174* yeast strain carrying an empty vector (pYYPGE15), *APC8* (pYYPGE15-*APC8*), *APC8* D to E (pYYPGE15-*APC8*, D was mutated to E), or *APC8* D to N (pYYPGE15-*APC8*, cDNA from the *apc8-1* mutant).

(B) Quantitative analysis of growth (OD_{600} measurements) at the permissive (28°C) or restrictive (37°C) temperature of the *cut23-174* yeast strain carrying each plasmid as in **(A)** at 0, 12, and 24 h growth in liquid YEA media. Three colonies for each construct were analyzed, and all measurements represent the average of three biological replicates with error bars representing the SE.

with wild-type plants showed that transmission through the female was mildly affected but was strongly perturbed through the male (Table 1). To rule out the possibility that disturbed male transmission was specific to *APC8*, we analyzed the T-DNA insertion allele of another APC/C component, *APC13* (SALK_027397, here named *apc13-2*), which was reported to have a *bonsai* phenotype (Saze and Kakutani, 2007) similar to that of *apc8-1*. We found slightly reduced seed set in *apc13-2* (Figure 4B), and reciprocal crosses with wild-type plants indicated that male transmission was more affected than female transmission (Table 1).

Because decreased APC/C function resulted in a reduced seed set phenotype, we next tested whether increased APC/C function had an effect on seed set. We generated transgenic plants overexpressing *APC8* under the control of the *HISTONE THREE RELATED 10* (*HTR10*) promoter. *HTR10* is specifically expressed in the male germ line, and HTR10 accumulates in generative cell nuclei and subsequently in sperm cell nuclei (Ingouff et al., 2007). We obtained 24 independent T1 lines; T2 plants from five T1 lines with single copy insertions were used for further analyses. The T-DNA cassette carrying *APC8* also contained a pollen-specific marker (LAT52-GFP), which we used to

confirm homozygous plants. We measured the relative expression levels of *APC8* RNA in these plants by quantitative RT-PCR (qPCR) and compared the expression levels with those in wild-type plants (see Supplemental Figure 3A online). We also scored the seed set (see Supplemental Figure 3B online). All T2 progenies tested showed higher (3.4- to 15.2-fold) *APC8* expression levels (see Supplemental Figure 3A online). These plants also had, on average, 27% reduced seed set (see Supplemental Figure 3B online), indicating that the higher *APC8* levels in the male germline disrupted seed set.

APC/C Is Necessary for Tricellular Pollen Formation

Given the importance of cell cycle regulation by APC/C (Peters, 2006) and the dynamic expression of *APC8* during male gametophyte development (Figure 3), we suspected that disrupting APC/C function might lead to disorganized cell divisions during male gametophyte development. Indeed, when mature pollen from *apc8-1* plants was stained with 4',6-diamidino-2-phenylindole (DAPI), the proportion of uninucleate cells was 45% and binucleate cells was 37%, whereas in wild-type plants, these proportions were 8 and 9%, respectively (Figures 4C). Introduction

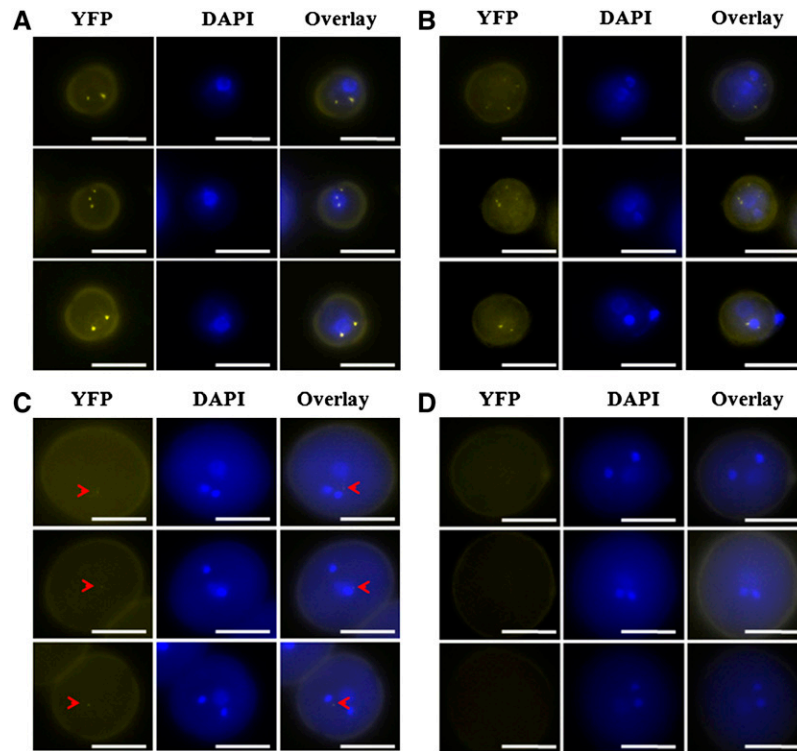


Figure 3. Subcellular Localization of APC8 during Male Gametophyte Development.

Representative fluorescence microscopy images of unicellular pollen (**A**), bicellular pollen (**B**), tricellular pollen (**C**), and mature pollen (**D**) from at least 10 individual T1 *apc8-1* plants harboring the pAPC8:APC8-YFP construct. Three examples for each developmental stage were shown. The left panel for each represents YFP epifluorescence of pAPC8:APC8-YFP. The middle panel for each indicates the developmental stage determined by DAPI staining. The right panel for each shows an overlay of the YFP and DAPI epifluorescence signals. Arrowheads show faint YFP signals. Bars = 10 μ m. [See online article for color version of this figure.]

of wild-type *APC8* into *apc8-1* completely rescued these defects of male gametophyte development (Figure 4C), confirming that *apc8-1* was responsible for these phenotypes. The defects in pollen maturation in *apc13-2* showed a similar trend but were less severe than those in *apc8-1* (Figure 4C). A representative HTR10:APC8 transgenic T2 plant (line 8-2) showed increased incidence of bicellular pollen, but no change in the incidence of unicellular pollen (Figure 4C), consistent with the specific expression of *HTR10* in the male germline.

If the vegetative cell and the generative cell fail to divide in *apc/c* mutants and in *APC8* overexpression lines, the expectation might be that further DNA replication would not occur. We therefore investigated DNA content in the vegetative cell nuclei and the generative cell-like nuclei in *apc8-1* and in the generative cell-like nuclei in an *HTR10:APC8* plant (line 8-2). Relative to the nuclear DNA content in the wild type, nuclear DNA content in both the vegetative cell nuclei and the generative cell-like nuclei in *apc8-1* was significantly increased, to nearly 2C (Figure 4D), suggesting that in *apc8-1*, pollen containing only a vegetative cell nucleus and pollen containing a single sperm cell both finish DNA replication but cannot enter the subsequent mitosis. As expected, the nuclear DNA content in the generative cell-like nuclei in HTR10:APC8-expressing plants increased, also to nearly 2C (Figure 4D). To examine whether pollen cell fate was disturbed,

we introduced pHTR10:HTR10-mRFP (Ingouff et al., 2007) into *apc8-1*. This marker was observed in sperm cell nuclei of *apc8-1* mature pollen (see Supplemental Figure 3C online), indicating that sperm cells in *apc8-1* had not lost their gametic fate. Taken together, our results indicate that APC/C plays an important role in mitotic cell cycle progression during male gametophyte development, promoting tricellular pollen formation.

APC/C Is Required for miR159 Accumulation, and APC/C Promotes Pol II Recruitment to *MIR159*

Because *apc8-1* was identified while looking for miRNA accumulation mutants, we hoped to determine the molecular mechanism by which APC8 regulates male gametophyte development by examining whether *apc8-1* was compromised in miRNA accumulation. Of 10 tested miRNAs, miR159 accumulation was obviously reduced (Figure 5A; see Supplemental Figure 4A online), and this molecular phenotype was rescued by wild-type *APC8* (Figure 5A), suggesting that APC8 was involved in the miR159 biogenesis pathway. We also found obvious reductions in accumulation of miR166, miR172, miR173, and miR319, but no detectable changes for miR156, miR164, miR167, miR168, and miR171 (see Supplemental Figure 4A online), suggesting that the role of APC8 in miRNA biogenesis is locus specific. Similar

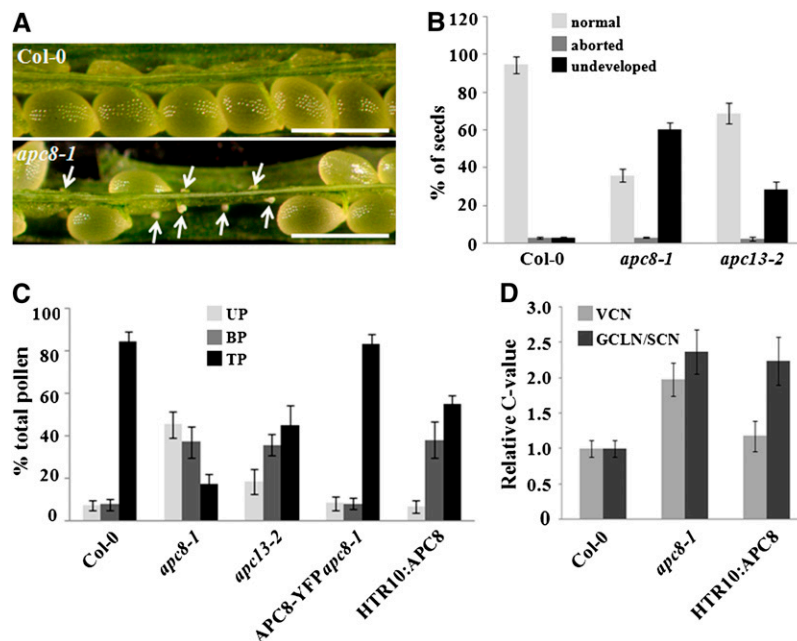


Figure 4. Seed Set Analysis and Pollen Development of *apc8-1*.

(A) Dissected mature siliques of wild-type (Col-0, top) and *apc8-1* plants (bottom). Undeveloped ovules are indicated with arrows. The undeveloped ovules are tiny and white, whereas developing seeds are large and green. Bar = 500 μ m.

(B) Percentage of normal seeds (light gray), aborted seeds (dark gray), and undeveloped ovules (black) from self-pollinated Col-0, *apc8-1*, and *apc13-2* plants. Error bars represent SD from the mean of 10 siliques from each of 20 individual plants.

(C) Distribution of uninucleate microspores (UP; light gray), bicellular pollen (BP; dark gray), and tricellular pollen (TP; black) in mature anthers. Pollen was stained with DAPI, and the percentage of each stage was determined for Col-0, *apc8-1*, *APC8-YFP apc8-1*, and *HTR10:APC8* (line 8-2). All measurements represent the average of three biological replicates with error bars representing the SE. For each independent replicate for each genotype, 600 to 800 pollen grains from 5 to 10 plants were analyzed.

(D) DNA content of vegetative cell nuclei (VCN; light gray), generative cell-like nuclei/sperm cell nuclei (GCLN/SCN; dark gray) in pollen from different genotypes. The relative C values of *apc8-1* ($n = 108$) and *HTR10:APC8* ($n = 126$ from line 8-2) were calculated from DAPI fluorescence values normalized to the mean fluorescence of wild-type cells ($n = 98$ for vegetative cell nuclei; $n = 112$ for sperm cell nuclei). n indicates the number of pollen grains that was measured. All measurements represent the average of three biological replicates, with error bars representing the SE.

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defects in mature miR159 accumulation were observed in *apc13-2* (see Supplemental Figure 4A online). miR159 is encoded by three genes (*MIR159a*, *MIR159b*, and *MIR159c*) (Park et al., 2002; Rhoades et al., 2002).

To investigate which step in miR159 biogenesis was affected, we used qPCR to examine whether primary (pri) miRNA transcription of each *MIR159* was affected in the pollen of *apc/c* mutants. We detected reduced pri-miR159 transcription from all three *MIR159* genes in *apc8-1* and *apc13-2*, most notably an

~70% decrease for *MIR159c* in *apc8-1* (Figure 5B). As expected, overexpression of *APC8* resulted in increased pri-miR159 transcription (Figure 5B). To further confirm these results and to determine the expression patterns of each *MIR159* during male gametophyte development, we generated constructs in which the promoter regions of *MIR159a*, *b*, or *c* were fused to GFP. We introduced these constructs into wild-type and *apc/c* mutants and examined multiple transgenic lines for each construct. In the wild type, *MIR159a-GFP*, *MIR159b-GFP*, and

Table 1. Genetic Analysis of *apc8-1* and *apc13-2*

Parental Genotypes	Genotype of F1 Plants		
$\text{♀} \times \text{♂}$	<i>apc/+</i>	Wild Type	Transmission Efficiency
<i>apc8-1/+</i> \times wild type	136	172	$136/172 \times 100\% = 79.1\%$
Wild type \times <i>apc8-1/+</i>	61	238	$61/238 \times 100\% = 25.2\%$
<i>apc13-2/+</i> \times wild type	158	187	$158/187 \times 100\% = 84.5\%$
Wild type \times <i>apc13-2/+</i>	109	261	$109/261 \times 100\% = 41.8\%$

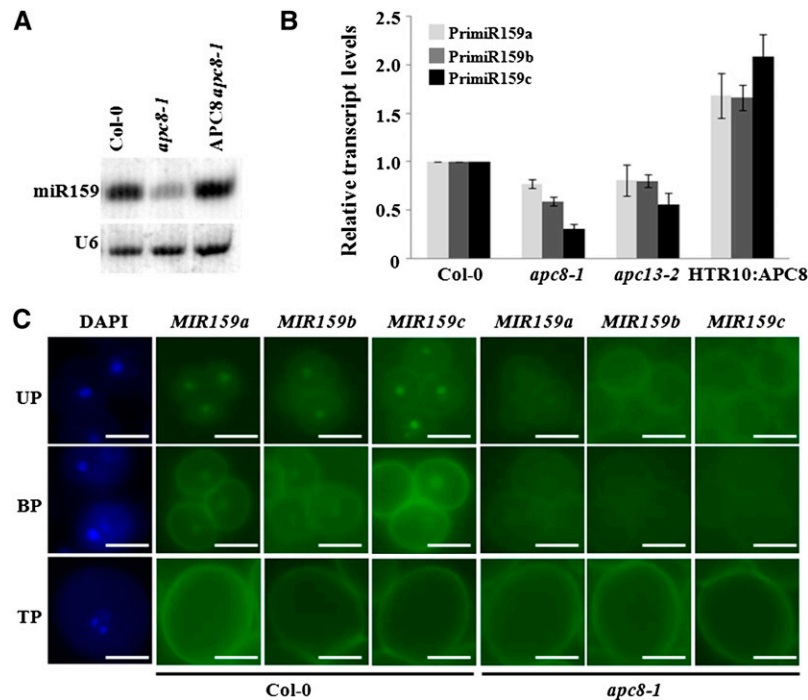


Figure 5. APC8 Is Required for miR159 Biogenesis during Male Gametophyte Development.

(A) miR159 accumulation in Col-0, *apc8-1*, and APC8 *apc8-1* (the *pAPC8:APC8-YFP* construct was introduced into *apc8-1*). Total RNAs were extracted from inflorescences. U6 was the loading control.

(B) Expression of *MIR159a* (light gray), *MIR159b* (dark gray), and *MIR159c* (black) in pollen from Col-0, *apc8-1*, *apc13-2*, and HTR10:APC8 (line 8-2). *UBIQUITIN5 (UBQ5)* was the loading control. All measurements represent the average of three biological replicates with error bars representing the SE.

(C) Expression patterns of *ProMIR159a*, *b*, *c-GFP* during pollen development in wild-type and *apc8-1* plants. Fifteen individual T1 plants in the wild type or the *apc8-1* background were examined. Panels (top to bottom) show three progressive stages: UP, uninucleate microspores; BP, bicellular pollen; TP, tricellular pollen. A representative DAPI-stained image for each stage is shown. Bar = 10 μ m.

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MIR159c-GFP were strongly expressed at the unicellular microspore stage, detectable at the bicellular stage, but undetectable at the tricellular stage; their expression was restricted to vegetative cell nuclei (Figure 5C). By contrast, in *apc8-1*, none of these reporter gene fusions was detectable at any stage of male gametophyte development. Consistent with the much weaker phenotypes in *apc13-2* than in *apc8-1* described above, we observed only a slight reduction of expression of the three *MIR159-GFPs* in *apc13-2* (see Supplemental Figure 4B online). Since GFP signal intensity was not a quantitative measure of reporter gene expression, we performed qPCR to determine the levels of *GFP* mRNA in inflorescences. Indeed, *GFP* mRNA levels were lower in *apc/c* mutants than in the wild type (see Supplemental Figure 4C online). These results indicate that APC/C is required for miR159 biogenesis during male gametophyte development and that it acts at the transcriptional level.

Although we showed that in the *apc/c* mutants the levels of pri-miR159 and the activities of the *MIR159* promoters were compromised, the defects in *MIR* gene transcription in *apc/c* mutants could be an indirect effect of reduced expression of certain known genes in the miRNA biogenesis pathway. To evaluate this possibility, we first examined the expression of several miRNA

biogenesis pathway genes in *apc8-1* by qPCR. None of these genes was affected in *apc8-1* (see Supplemental Figure 5A online). Furthermore, the protein levels of these genes were comparable to those in the wild type (see Supplemental Figures 5B and 5C online). Therefore, the reduced pri-miRNA and mature miRNA levels in the *apc/c* mutants are unlikely due to reduced expression of miRNA biogenesis genes. This result prompted us to examine whether APC/C plays a direct role in the transcription of *MIR* genes. Pol II is responsible for *MIR* transcription in animals and plants (Lee et al., 2004; Xie et al., 2005; Kim et al., 2011). Therefore, we next examined Pol II occupancy at promoters of several *MIR* genes by chromatin immunoprecipitation (ChIP) using an antibody against the largest subunit of Pol II (RPB1). We monitored *MIR159a*, *MIR159b*, *MIR159c*, *MIR166a*, *MIR167a*, and *MIR171a* using Actin2 as a positive control (Figures 6A and 6B). Pol II occupancy was enriched at regions encompassing the transcription start sites at these loci (*MIR159b_1* and *MIR159b_2* represent two regions flanking two transcription start sites in *MIR159b* loci, respectively), over the no antibody ChIP that served as a negative control (Figures 6A and 6B). Pol II occupancy at these loci was significantly reduced in *apc8-1* (Figure 6B). These results indicate that APC/C promotes Pol II recruitment to *MIR* genes.

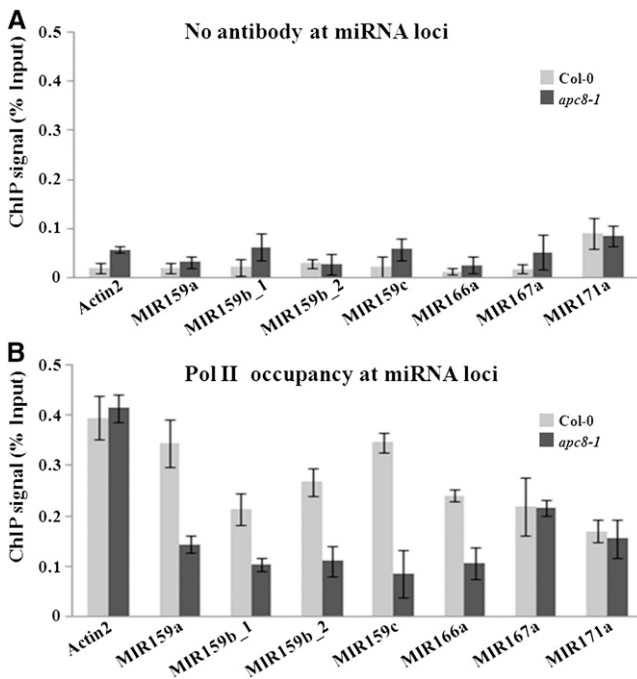


Figure 6. Pol II Occupancy at miRNA Loci.

(A) ChIP performed with Col-0 (light gray bars) or *apc8* (dark gray bars) with no antibody as the negative control.

(B) ChIP performed with anti-RPB1 antibody. The results were reproducible in two biological replicates. The mean and SD were determined from one representative biological replicate.

Error bars in **(A)** and **(B)** show SD calculated from three technical replicates.

APC/C-Mediated miR159 Accumulation Led to *DUO1* Repression

Because miR159 biogenesis was perturbed in these *apc/c* mutants, we next asked whether this perturbation had biological significance. Since *DUO1*, an miR159 target (Palatnik et al., 2007), was reported to play a role in twin sperm cell formation (Brownfield et al., 2009a), we first used qPCR to examine whether *DUO1* expression was affected in the pollen of *apc/c* mutants. The mRNA level of *DUO1* in *apc8-1* was increased ~2-fold (Figure 7A), consistent with the notion that *DUO1* is negatively regulated by miR159. By contrast, *DUO3*, which has a function similar to that of *DUO1* but is not a miR159 target, had similar expression levels in *apc8-1* and wild-type pollen (Figure 7A). A similar trend was observed for *apc13-2* (Figure 7A). As expected, *DUO1* levels were reduced in HTR10:APC8 pollen (Figure 7A). To substantiate the above result, we compared the expression level of DUO1-mRFP at different stages during pollen development in wild-type or *apc8-1* plants containing the *pDUO1:DUO1-mRFP* construct. We consistently observed a slightly increased intensity of DUO1-mRFP signals through early bicellular stage to mature pollen in *apc8-1* (Figure 7B). Therefore, our results demonstrate that APC/C plays a role in miR159 biogenesis that is important for miR159-dependent *DUO1* repression.

APC/C Regulates *CYCB1;1* mRNA Levels

As increased *DUO1* expression was observed in *apc/c* mutants (Figure 7) and *CYCB1;1* expression was reported to be promoted by *DUO1* (Brownfield et al., 2009a), we speculated that APC/C might affect not only the stability of *CYCB1;1* but also expression of *CYCB1;1*. To test this hypothesis, we compared *CYCB1;1* mRNA levels in wild-type and *apc8-1* pollen by qPCR. There was significantly more *CYCB1;1* mRNA in *apc8-1* than in the wild type (Figure 8A). Similarly, increased *CYCB1;1* mRNA was also observed in *apc13-2* (Figure 8A). Taken together, these results indicate that APC/C can regulate *CYCB1;1* expression.

To determine if *CYCB1;1* accumulates during male gametogenesis in these *apc/c* mutants, we introduced *pCYCB1;1:CYCB1;1-GFP* into wild-type, *apc8-1*, and *apc13-2* plants. In the wild type, *CYCB1;1-GFP* was detected only in the nucleus of unicellular microspores and in the vegetative nucleus of bicellular pollen, but not in tricellular or mature pollen (Figure 8B), consistent with microarray results (Hony and Twell, 2004). By contrast, *CYCB1;1-GFP* accumulated in nuclei of both the vegetative cell and sperm cells of tricellular and mature pollen from *apc8-1* (Figure 8B) and *apc13-2* (see Supplemental Figure 6A online). The *CYCB1;1-GFP* accumulation in vegetative nuclei and expanded expression in sperm cell nuclei at the tricellular stage in these *apc/c* mutants suggest that APC/C is necessary for removal of *CYCB1;1* during male gametophyte development.

To further support the observation of accumulated *CYCB1;1-GFP* in *apc8-1*, we used immunoblots to examine the total protein levels of *CYCB1;1-GFP* in inflorescences from multiple individual wild-type and *apc8-1* transgenic plants expressing *pCYCB1;1:CYCB1;1-GFP*. *CYCB1;1-GFP* was greatly increased in *apc8-1* (see Supplemental Figure 6B online), further indicating that disruption of APC8 function caused the failure of *CYCB1;1* degradation. Taken together, our data demonstrate that APC/C, in addition to its known role in regulating *CYCB1;1* degradation, can regulate *CYCB1;1* at the mRNA level.

DISCUSSION

We obtained evidence for a mechanism by which APC/C regulates *CYCB1;1* at the transcriptional level and directly degrades *CYCB1;1*. To explore this dual regulation in cell cycle progression, we focused on the well-defined cell lineages in male gametophyte development and showed that disruption of APC/C resulted in increased incidence of uninucleate or binucleate pollen (Figure 4C).

Our results showed that the transcription of all three *MIR159* genes was reduced in *apc/c* mutants (Figures 5B and 5C; see Supplemental Figures 4B and 4C online), which is consistent with the functional redundancy shown among *MIR159a*, *MIR159b*, and *MIR159c* (Allen et al., 2007). Interestingly, although these three genes had nearly identical expression patterns during male gametophyte development, the *MIR159c* expression level was higher than the expression levels of *MIR159a* and *MIR159b*, and a more obvious reduction of *MIR159c* transcription was seen in *apc/c* mutants (Figures 5B and 5C; see Supplemental Figures 4B and 4C online). Deep sequencing showed that miR159c was much less abundant in leaves, seedlings (Rajagopalan et al.,

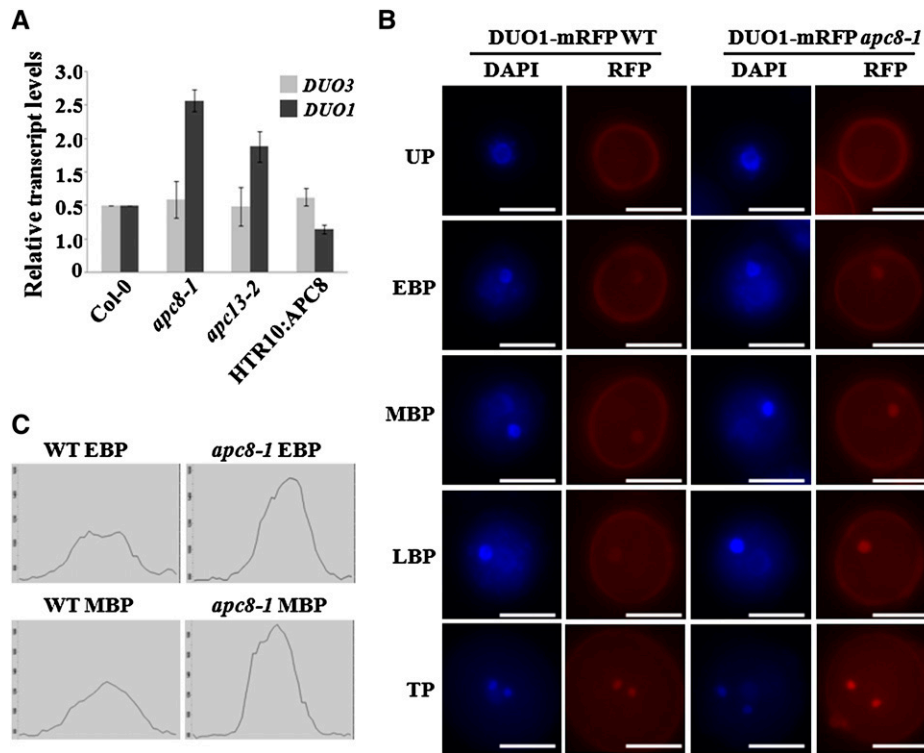


Figure 7. Increased *DUO1* Expression in *apc8-1* during Male Gametophyte Development.

(A) Expression of *DUO1* and *DUO3* in mature pollen from Col-0, *apc8-1*, *apc13-2*, and HTR10:APC8 as determined by qPCR. *UBIQUITIN5* (*UBQ5*) was the loading control. All measurements represent the average of three biological replicates with error bars representing the SE.

(B) Expression of pDUO1:DUO1-mRFP in the wild type and *apc8-1*. Plants homozygous for pDUO1:DUO1-mRFP were crossed into *apc8-1* and then F3 plants homozygous for both *apc8-1* and pDUO1:DUO1-mRFP were analyzed. One example for each developmental stage is shown. All images were acquired using the same exposure times. UP, unicellular pollen; EBP, early bicellular pollen; MBP, middle bicellular pollen; LBP, late bicellular pollen; TP, tricellular pollen. Bar = 10 μ m.

(C) Quantitative data of RFP signal intensity at the early bicellular pollen and middle bicellular pollen stages in **(B)** is shown. Each x axis represents a 5- μ m distance centered on the fluorescent focus. WT, wild type.

[See online article for color version of this figure.]

2006), and inflorescences (Fahlgren et al., 2007), consistent with the hypothesis that miR159c is pollen specific and accordingly plays a predominant role during male gametophyte development.

The temporal expression patterns of *APC8* (Figure 3), *MIR159* (Figure 5C), and *CYCB1;1* (Brownfield et al., 2009a; Figure 8B) are similar during male gametophyte development and are reciprocal to the temporal expression pattern of *DUO1*. Why is it necessary that expression patterns of these genes are regulated? One of the well-known functions of the APC/C complex is to degrade cyclins to allow progression through the cell cycle. Enrichment of APC/C at the early stages of male gametophyte development correlates precisely with the period of active and continuous cell division. The most probable consequence of *MIR159* expression is to restrict the expression of its target genes. *DUO1*, one of its target genes, functions in the formation and cell specification of male germline cells (Rotman et al., 2005). Thus, the abundance of miR159 at early stages of male gametophyte development might be mainly to limit the expression of *DUO1*, which is also consistent with reduced seed set of

mir159ab double mutant (Allen et al., 2007). However, the expression of *MIR159* and *DUO1* overlaps at the bicellular stage (Brownfield et al., 2009a; Figure 5C). Other miR159 targets might play a role in male gametophyte development when their expression patterns differ from that of *DUO1* (Schwab et al., 2005). Another possibility is that *DUO1* expression is regulated by factors other than miR159.

DUO1 expression is initiated at late stages (Rotman et al., 2005), and *DUO1* can promote *CYCB1;1* expression by an unknown mechanism (Brownfield et al., 2009a), which suggests that the relationship between *DUO1* and *CYCB1;1* might be important and specific to the second mitosis. Moreover, overexpression of *CYCB1;1* in male germlines rescued only the cell division defects of *duo1/+* heterozygous plants (Brownfield et al., 2009a), indicating that *CYCB1;1* is the output of a *DUO1*-dependent pathway during male gametophyte development. The dynamic requirements for *CYCB1;1* expression in the second mitosis can be accounted for, as *CYCB1;1* was expressed in the generative cell at the early bicellular stage and the late bicellular stage, but not at the middle bicellular stage or at the tricellular stage

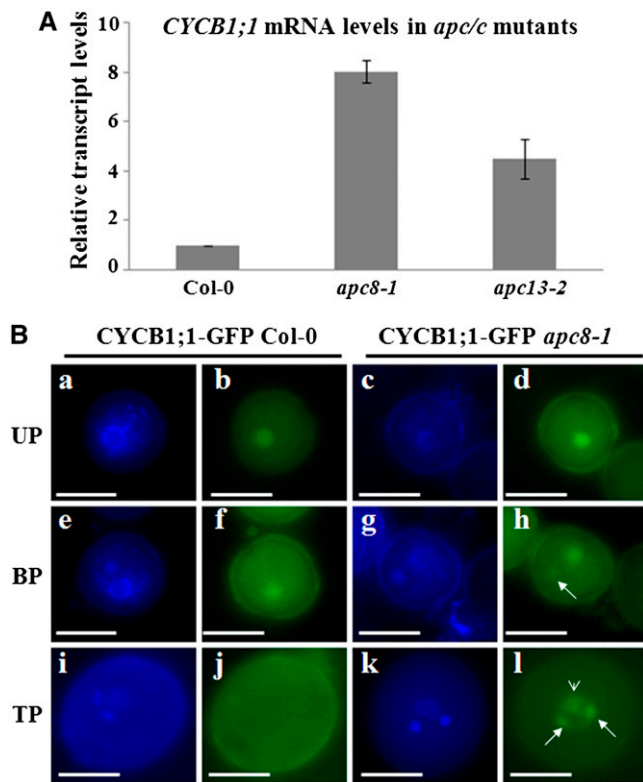


Figure 8. *CYCB1;1* Expression Is Increased in Male Gametophytes of *apc/c* Mutants.

(A) Expression of *CYCB1;1* in mature pollen from Col-0, *apc8-1*, and *apc13-2*. *UBIQUITIN5 (UBQ5)* was the loading control. All measurements represent the average of three biological replicates with error bars representing the SE.

(B) Subcellular localization of *CYCB1*-GFP during pollen development in wild-type and *apc8-1* plants harboring *pCYCB1;1::CYCB1-GFP*. Fifteen individual T1 plants in the wild type or the *apc8-1* background were examined. DAPI staining of uninucleate microspores (UP; [a] and [c]), bicellular pollen (BP; [e] and [g]), and tricellular pollen (TP; [i] and [k]). GFP was examined in uninucleate microspores ([b] and [d]), bicellular pollen ([f] and [h]), and tricellular pollen ([j] and [l]). *CYCB1;1*-GFP in *apc8-1* was detected weakly in generative cell nuclei (arrow) of bicellular pollen (h) and obviously accumulated in both the vegetative nucleus (arrowhead) and sperm cell nuclei (arrow) of tricellular pollen (l). Bar = 10 μ m. [See online article for color version of this figure.]

(Brownfield et al., 2009a). By contrast, we showed that *CYCB1;1*-GFP was detected only in the vegetative nuclei of bicellular pollen and not in tricellular or mature pollen (Figure 8B). The differences in these two studies might be due to the different constructs used: Brownfield et al. (2009a) used a possible transcriptional reporter (Glotzer et al., 1991), which included the mitotic destruction box in the first exon fused to GUS, whereas we used a fusion protein construct that encodes the entire protein. This might indicate that the remaining coding region of *CYCB1;1* contains posttranscriptional information that regulates translation or protein stability. Alternatively, the possible transcriptional reporter might be more sensitive than the protein fusion construct.

Mitotic cyclins were the first demonstrated APC/C substrates (Glotzer et al., 1991), and *CYCB1;1* was shown to accumulate in several *apc/c* mutants (this study; Capron et al., 2003b; Kwee and Sundaresan, 2003; Pérez-Pérez et al., 2008). *CYCB1;1* expression was reported to be promoted by *DUO1* (Brownfield et al., 2009a). What is the relationship between APC/C-directed *CYCB1;1* degradation and *DUO1*-mediated *CYCB1;1* regulation? We propose a model in Figure 9 to explain the dual regulation of *CYCB1;1* by APC/C during male gametophyte development. We showed that two pathways, integrated by APC/C, regulate *CYCB1;1* expression and *CYCB1;1* degradation. One outcome is that disrupted APC/C function directly causes *CYCB1;1* accumulation, as *CYCB1;1* is a direct substrate of APC/C (Glotzer et al., 1991). The other outcome is that reduced miR159 accumulation in *apc/c* mutants, accompanied with increased *DUO1* expression, promotes *CYCB1;1* upregulation in a direct or indirect manner because *DUO1* is a transcription factor (Rotman et al., 2005), and reduced *CYCB1;1* transcription was observed in *duo1* heterozygous plants (Brownfield et al., 2009a). In this model, we propose that the *CYCB1;1* expression promoted by *DUO1* is transient, which only ensures a proper G2/M transition during pollen maturation; once that is fulfilled, *CYCB1;1* must be removed immediately by APC/C. This hypothesis is consistent with the observation of dynamic expression of *CYCB1;1* (Brownfield et al., 2009a). The existence of *CYCB1;1* transcripts at the early bicellular stage implies that *CYCB1;1* from the first mitosis has not been completely degraded by APC/C. The absence of *CYCB1;1* transcripts at the middle bicellular stage implies that APC/C has

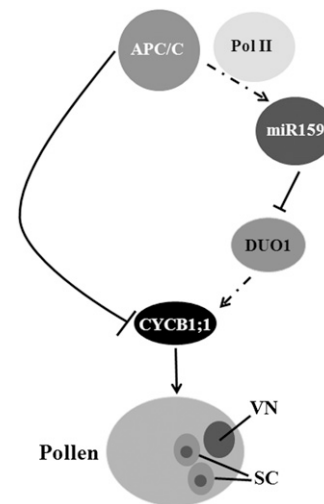


Figure 9. A Model for the Dual Roles of APC/C in Regulating Cyclin B1;1 during Male Gametophyte Development.

APC/C not only degrades *CYCB1;1* directly but also directly or indirectly regulates miR159 transcription by recruiting Pol II to promoter regions. The positive regulation of miR159 by APC/C causes the correspondingly negative regulation of a miR159 target, *DUO1*, which ultimately leads to reduced *CYCB1;1* expression. The dual role of APC/C in *CYCB1;1* function ensures proper progression of the two mitoses. VN, vegetative nucleus; SC, sperm cells.

degraded all the *CYCB1;1* that was required for the first mitosis. The reappearance of *CYCB1;1* transcripts at the late bicellular stage is presumably due to the miR159-mediated *DUO1* repression, which facilitates progression of the second mitosis.

Regulation of *CYCB1;1* by APC/C at the transcriptional level was unexpected. Based on the observation that APC/C is necessary for *MIR159* transcription (Figure 5C), perhaps APC/C, as an ubiquitin ligase, is recruited to the transcriptional complex and acts to degrade a component specific for *MIR* gene transcription. It will be interesting to determine how APC/C affects the transcription of *MIR* genes and especially to examine whether the transcriptional machinery for *MIR* genes is different from that of most coding genes, although *MIR* genes are also transcribed by Pol II (Lee et al., 2004; Xie et al., 2005; Kim et al., 2011). Recent studies showed that a human mediator of DNA damage checkpoint protein 1 (hMDC1), which is associated with one of the core subunits of APC/C (Townsend et al., 2009), is required for normal metaphase-to-anaphase transition (Coster et al., 2007). hMDC1 is a transcription factor that belongs to the BRAC1 complex (Goldberg et al., 2003; Lou et al., 2003; Stewart et al., 2003). BRAC1 was shown to be a component of the Pol II holoenzyme (Scully et al., 1997). These relationships suggest that APC/C could be directly or indirectly involved in the transcription of *MIR* genes and that *Arabidopsis* orthologs of hMDC1 might be a bridge to recruit APC/C to transcriptional sites.

Considering that APC/C degradation of *CYCB1;1* is conserved from fungi to mammals, we suspect that the dual regulation of *CYCB1;1* expression by APC/C and the involvement of miRNAs in this process could also be widely conserved. A recent study showed that human immunodeficiency virus (HIV)-encoded Tat protein regulates cyclin B1 by promoting both its expression and degradation in HIV-infected lymphocytes (Zhang et al., 2010). In this case, the Tat protein appears to act as APC/C does in plants; however, the mechanism remains unknown. It will be important to understand how APC/C coordinates its proteasome activity and its role in regulating a miRNA pathway.

We showed that the *Arabidopsis* APC/C is a key regulatory factor in male gametophyte development, in addition to its documented role in female gametophyte development (Capron et al., 2003b; Kwee and Sundaresan, 2003). Given that APC/C is a protein complex, it is unknown why mutations in different APC/C subunits can affect one sex more profoundly than the other, and this phenomenon appears to be conserved in yeast and animals (Garbe et al., 2004; Pál et al., 2007; Jin et al., 2010). Perhaps this can be explained by target specificity of the APC/C subunits. APC/C is an E3 ubiquitin ligase, but it can only ubiquitinate substrates with the help of two cofactors, the ubiquitin-activating (E1) enzyme and a ubiquitin-conjugating (E2) enzyme. The impact of a particular mutation on male or female transmission might be related to structural similarities among subunits. For example, APC6 and APC8 both have a TPR domain, while APC2 does not; mutants of APC6 and APC8 both showed defects in male transmission (Kwee and Sundaresan, 2003; Table 1), but the *apc2* mutant did not (Capron et al., 2003b). These results indicate that while the overall structure of the APC/C is conserved among eukaryotes, this E3 ligase might have assumed specialized functions in different kingdoms (Lima et al., 2010). Another important layer of APC/C regulation in

plants could be through subunit availability in specific tissues and/or cellular compartment, as it is known that APC subunits are differentially expressed in *Arabidopsis* organs (Eloy et al., 2006). Thus, in plants, the complex might be regulated by subunit availability and the different subunits could play unique regulatory roles (Eloy et al. 2006).

METHODS

Seeds, Strains, and *apc8-1* Genotyping

The pDUO1:DUO1-mRFP and pHTR10:HTR10-mRFP seeds were kindly provided by David Twell and Fred Berger, respectively. The pDCL1:DCL1-YFP and pHYL1:HYL1-YFP plasmids were kindly provided by David L. Spector. T-DNA insertion mutants (SALK_027397, *apc13-2*; SALK_056243, *dcl1-14*) were obtained from the ABRC. The *Schizosaccharomyces pombe* temperature-sensitive *apc8* strain (*Cut23-174*, FY9688) was obtained from the Yeast Genetic Resource Center in Japan (YGRC/NBRP). The *Arabidopsis thaliana apc8-1* mutant was genotyped by *ApoI* digestion of the PCR fragment generated using primers APC8F2 and APC8R2; the fragment in the mutant is 25 bp smaller than that in the wild type, which is detectable when separated on a 3% agarose gel.

Plasmid Construction

APC8 was amplified from Col-0 genomic DNA with primers APC8F1 and APC8R1 (see Supplemental Table 1 online), cloned into pENTR-D/TOPO (Invitrogen), and then transferred into the plant expression destination vector pGWB40 (Nakagawa et al., 2007). The resulting plasmid was introduced into *apc8-1* plants by agroinfiltration for complementation (Clough and Bent, 1998). For *APC8* promoter analysis, a 2.4-kb fragment upstream of the ATG was amplified using primers APC8F1 and APC8R7 and was subcloned into pENTR-D/TOPO and then transferred into the plant expression vectors pGII-NLS3XGFP (Hellens et al., 2000) and pMDC164 (Curtis and Grossniklaus, 2003). For yeast complementation, full-length cDNAs of *APC8* and *APC8-1* were amplified from leaves of Col-0 and *apc8-1* plants, respectively, using RT-PCR and primers APC8F3 and APC8R3, and then cloned into the pYPGE15 yeast expression vector (Brunelli and Pall, 1993). The amino acid Asp was changed to Glu by site-directed mutagenesis using primers APC8F11 and APC8R11. These plasmids were introduced into the *S. pombe* temperature-sensitive *Cut23-174* mutant (Cullen et al., 2000). For the HTR10:APC8 overexpression construct, we first generated a modified vector pB7WG2*. The vector pB7WG2 (Karimi et al., 2002) was digested with *SacI* and *SpeI* to replace the 35S promoter with the HTR10 promoter and then digested with *KpnI* and *Apal* to insert the LAT52-GFP cassette. A LR reaction was performed between the TOPO vector containing the full-length *APC8* cDNA and pGWB7*.

For *CYCB1;1* expression analysis, a genomic fragment of *CYCB1;1*, including a 2.5-kb fragment upstream of the ATG and downstream sequences to the stop codon, was amplified with primers *CYCB1;1*F1 and *CYCB1;1*R1 and subcloned into pENTR-D/TOPO and then transferred into the plant expression destination vector pMDC107 (Curtis and Grossniklaus, 2003). For *MIR159* expression analysis, fragments of 1.9, 2.4, or 1.6 kb upstream of the first nucleotide of *MIR159a*, *MIR159b*, and *MIR159c*, respectively, were subcloned into pENTR-D/TOPO and then transferred into the plant expression vector pGIINLS3XGFP (Hellens et al., 2000). The primer sequences are listed in Supplemental Table 1 online.

Yeast Complementation

A single colony of *cut23-174* was grown in YEA medium (5 g/L yeast extract, 30 g/L Glc, 225 mg/L adenine, 225 mg/L His, 225 mg/L Leu,

225 mg/L Lys hydrochloride) for 2 to 3 d at 28°C. One microgram of DNA of pYPGE15, pYPGE15-APC8, pYPGE15-APC8 Δ DE, or pYPGE15-APC8 Δ DN was transformed into 100 μ L freshly made competent cells. After incubation at 28°C for 2 h, the cell suspension with each plasmid was divided and plated on YEA plates, one incubated at 28°C and the other at 37°C. Colonies were observed after 2 to 3 d. A single colony from each plate was diluted 1:10, 1:100, or 1:1000 and spotted onto YEA plates for 2 to 3 d of growth. Photographs were taken after 2 to 3 d. For quantitation, a single colony from each plate was cultured in YEA liquid medium for 2 to 3 d at 28°C and then inoculated into fresh YEA liquid medium to a starting OD₆₀₀ of 0.2 to 0.3 and then OD₆₀₀ was measured after growth for 12 or 24 h. Three colonies were analyzed from each genotype, with two biological replicates.

Microscopy Analysis and Seed Set Analysis

Pollen dissected from closed buds and open flowers from 10 to 15 individual transgenic T1 plants was stained with DAPI for 30 min at room temperature in the dark. Images were acquired with an Axiovert microscope (Zeiss) under the epifluorescence channel (the excitation/emission wavelengths are 365 nm/397 nm [DAPI], 470 nm/505 to 530 nm [GFP], 500 nm/535 nm [YFP], and 545 nm/605 nm [RFP]) using an AxioCamRM camera and AxioVision 4.8.1 software. Images were processed using Adobe Photoshop CS2 (Adobe). Seed set analysis was according to Ron et al. (2010).

Analysis of Nuclear DNA Content

DNA content was determined as described by Rotman et al. (2005) with modifications. Mature pollen from various genotypes was stained by DAPI. Relative DNA content of vegetative cell nuclei (Col-0 plants), vegetative cell-like nuclei (*apc8-1*), sperm cell nuclei (Col-0 plants), and generative cell-like nuclei (*apc8-1* and HTR10:APC8 plants) were measured by DAPI fluorescence. Images were analyzed for fluorescence with AxioVision 4.8.1 software. A net value for each nucleus was obtained by subtracting a corresponding background reading from the cytoplasm. To standardize the relative fluorescence per C value of DNA, we first set the average net value of vegetative cell nuclei and sperm cell nuclei using wild-type mature pollen as 1C.

miRNA RNA Gel Blotting

Total RNA was isolated from inflorescences of Col-0, *apc8-1*, *apc13-2*, and APC8-YFP *apc8-1*. Small RNA hybridization for miRNAs was performed as described (Park et al., 2002). The 5' end-labeled (³²P) antisense oligonucleotides were used to detect U6 and miRNAs. Radioactive signals were detected with a phosphor imager.

qPCR

Total RNA was extracted using a RNeasy plant mini kit (Qiagen) and reverse transcribed with the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. Real-time qPCR was performed with three technical replicates for each of three biological replicates using a MyiQ Real-Time PCR detection system (Bio-Rad) with SYBR Green PCR Master Mix (Bio-Rad). The difference between the cycle threshold (Ct) of target genes and the Ct of control primers (Δ Ct = Ct_{target gene} - Ct_{control}) was used to obtain the normalized expression of target genes. Primers are listed in Supplemental Table 1 online.

ChIP

ChIP was performed according to Zheng et al. (2009). Pol II occupancy at several miRNA loci was determined by ChIP using anti-RPB1 antibody

and 2-week-old seedlings from Col-0 and *apc8-2*, respectively. DNA present in the immunoprecipitates was quantified by qPCR relative to total input DNA. The results shown were consistent in two biological replicates. Commercial anti-RPB1 (Abcam) was used. The primer sets used for the PCR are listed in Supplemental Table 1 online.

GUS Staining

Tissue from 10 to 15 individual *PAPC8-GUS* transgenic T1 plants was fixed in 90% acetone for 2 to 3 h and then stained for 12 h in 50 mM sodium phosphate buffer, pH 7.0, containing 0.2% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 1 mM X-Gluc, then washed in 70% ethanol three times. Images were taken with a Nikon SMZ800 stereoscope. Images were processed using Adobe Photoshop CS2 (Adobe).

Immunoblotting

Inflorescences were ground into powder in liquid nitrogen, agitated vigorously after adding 2 \times SDS-PAGE loading buffer, boiled for 5 min, and centrifuged for 5 min at 4°C. The supernatants were loaded onto 10% SDS-PAGE gels, electrophoresed for 2 to 3 h at 80 V, and transferred to nitrocellulose membrane. Mouse anti-GFP (Clontech) and anti-Hsc70 (Stressgen) antibodies were used at 1:2000 and 1:10,000 dilutions, respectively. HEN1 antibody and AGO1 antibody were made by X.C.'s lab. Detection was with an ECL plus detection kit (GE).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *APC8* (At3g48150), *APC13* (At1g73177), *MIR159a* (At1g73687), *MIR159b* (At1g8075), *MIR159c* (At2g46255), *CYCB1;1* (At4g37490), *DUO1* (At3g60460), *DUO3* (At1g64570), *UBQ5* (At3g62250), *DCL1* (At1g01040), *HYL1* (At1g09700), *HEN1* (At4g20910), *AGO1* (At1g48410), *MIR166a* (At2g46685), *MIR167a* (At3g22886), *MIR171a* (At3g51375), and *Actin2* (At3g18780). T-DNA insertion mutants used were *dcl1-14* (SALK_056243) and *apc13-2* (SALK_027397).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Isolation and Characterization of *dcl1-14*.

Supplemental Figure 2. Expression Analysis of *APC8*.

Supplemental Figure 3. Overexpression of *APC8* in Male Germline Disrupts Seed Set Development and HTR10 Expression in *apc8* Mature Pollen.

Supplemental Figure 4. Mature miRNA Levels and miRNA Transcription in *apc/c* Mutants.

Supplemental Figure 5. Expression of Known miRNA Biogenesis Genes in *apc8-1*.

Supplemental Figure 6. Increased *CYCB1;1* Expression in *apc8-1* and *apc13-2*.

Supplemental Table 1. Primers Used for This Study.

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MicroRNA-Mediated Transcriptional Regulation of *Cyclin B1* and Degradation of Cyclin B1
during *Arabidopsis* Male Gametophyte Development**

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