GIGAS CELL1, a Novel Negative Regulator of the Anaphase-Promoting Complex/Cyclosome, Is Required for Proper Mitotic Progression and Cell Fate Determination in Arabidopsis

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Increased cellular ploidy is widespread during developmental processes of multicellular organisms, especially in plants. Elevated ploidy levels are typically achieved either by endoreplication or endomitosis, which are often regarded as modified cell cycles that lack an M phase either entirely or partially. We identified GIGAS CELL1 (GIG1)/OMISSION OF SECOND DIVISION1 (OSD1) and established that mutation of this gene triggered ectopic endomitosis. On the other hand, it has been reported that a paralog of GIG1/OSD1, UV-INSENSITIVE4 (UVI4), negatively regulates endoreplication onset in Arabidopsis thaliana. We showed that GIG1/OSD1 and UVI4 encode novel plant-specific inhibitors of the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase. These proteins physically interact with APC/C activators, CDC20/FZY and CDH1/FZR, in yeast two-hybrid assays. Overexpression of CDC20.1 and CCS52B/FZR3 differentially promoted ectopic endomitosis in gig1/osd1 and premature occurrence of endoreplication in uvi4. Our data suggest that GIG1/OSD1 and UVI4 may prevent an unscheduled increase in cellular ploidy by preferentially inhibiting APC/C/CDC20 and APC/C/CDH1, respectively. Generation of cells with a mixed identity in gig1/osd1 further suggested that the APC/C may have an unexpected role for cell fate determination in addition to its role for proper mitotic progression.

INTRODUCTION

In multicellular organisms, organ sizes are determined by cell division and cell expansion. The former is conducted by continuous progression of the mitotic cell cycle, whereas the latter is partly achieved by atypical modes of the cell cycle that lead to elevated cellular ploidy (Edgar and Orr-Weaver, 2001). Typically, an increase in ploidy levels is executed by two different strategies, endoreplication and endomitosis, where cells replicate their chromosomes without division (see Supplemental Figure 1 online). Endoreplication, which lacks the entire processes of mitosis, does not affect the number of chromosomes but generates polyploidy chromosome (Edgar and Orr-Weaver, 2001; Lee et al., 2009). On the other hand, in endomitosis, cells enter but do not complete mitosis, most typically proceeding through anaphase but lacking nuclear division and cytokinesis (D’Amato, 1984; Lee et al., 2009). In contrast with endoreplication, endomitosis causes doubling of the chromosome number, yielding cells with a single polyploid nucleus. Endoreplication is widespread especially in plants and is associated with cessation of cell division and onset of cell differentiation during developmental processes in various organs (Beemster et al., 2005; Breuer et al., 2010, De Veylder et al., 2011). Less attention has been paid to endomitosis, but it is also known to occur in various plant species, including Arabidopsis thaliana (Weiss and Maluszynska, 2001), most frequently during development in the tapetum and endosperm (Nagl, 1978; D’Amato, 1984).

Onset of endoreplication typically requires inhibition of mitotic cyclin-dependent kinase (CDK) activities (Lilly and Duronio, 2005; Inzé and De Veylder, 2006), which is often associated with the degradation of mitotic cyclins by the anaphase-promoting complex/cyclosome (APC/C) in insects (Narbonne-Reveau et al., 2008; Zielke et al., 2008) and plants (Cebolla et al., 1999; Larson-Rabin et al., 2009; Eloy et al., 2011). APC/C is a multisubunit protein complex acting as an E3 ubiquitin ligase (Peters, 2006) and is responsible for the transition of key mitotic processes by targeted degradation of numerous cell cycle proteins (Peters, 2006; Marrocro et al., 2010). In contrast with endoreplication, little is known about the mechanisms underlying endomitosis, which, however, may be triggered by the depletion of mitotic cyclins mediated by the APC/C (Zhang et al., 1998).
APC/C activity is generally regulated by both activator and inhibitor proteins (Peters, 2006). APC/C activators, CELL DIVISION CYCLE20 (CDC20)/FIZZY (FZY) and CDC20 HOMOLOG1 (CDH1)/FZY-RELATED (FZR), are evolutionarily conserved, and their binding to APC/C is critical for its ubiquitination activity (Pesin and Orr-Weaver, 2008). Arabidopsis has counterparts of both types of activators, of which CELL CYCLE SWITCH 52A2 (CCS52A2)/FZR1 and CCS52A1/FZR2 are known to positively regulate the onset of endoreplication in different developmental contexts (Lammens et al., 2008; Larson-Rabin et al., 2009). However, there is no Arabidopsis gene that corresponds to the APC/C inhibitors found in metazoa and yeasts, and it remained unclear if plants have such inhibitor proteins at all. Here, we report that GIGAS CELL1 (GIG1) and UV-INSENSITIVE4 (UVI4), a paralog of GIG1, may encode novel plant-specific inhibitors of APC/C. GIG1 may prevent the ectopic occurrence of endomitosis by inhibiting CDC20-dependent APC/C (APC/C\textsubscript{CDC20}), while UVI4 may have negative effects on endoreplication onset by inhibiting FZR-dependent APC/C (APC/C\textsubscript{FZR}) activity. Strikingly, our results suggest that GIG1 may be required not only for proper mitotic progression but also for normal cell fate determination during stomatal development.

RESULTS

Loss of GIG1 Generates Giant Guard Cells

The two allelic recessive mutants gig1-1 and gig1-2 were obtained in a forward genetic screen to identify enhancers of the myb3r4 mutant phenotype (Haga et al., 2011). MYB3R4 belongs to the Myb family of transcriptional regulators that positively regulate mitotic progression in Arabidopsis. These mutants displayed giant cells, which were also observed in gig1 plants without the myb3r4 mutation, although this phenotype was strongly enhanced when MYB3R4 was simultaneously mutated (see Supplemental Figure 2 online). The giant cells in gig1 cotyledons, herein designated as gigas cells, showed guard cell–like appearance in differential interference contrast (DIC) images, suggesting that their cell walls may be biochemically similar to each other (Figures 1A and 1B). These cells showed some other guard cell–like characteristics, including possession of structures similar to stomatal pores (Figure 1C) and expression of guard cell–specific markers, E994, E1728, and KAT1:β-glucuronidase (GUS) (Figures 1D and 1E; see Supplemental Figure 3 online) (Ohashi-Ito and Bergmann, 2006; Pillitteri et al., 2007). We also showed that generation of gigas cells is

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Figure 1. Loss of GIG1 Causes the Occurrence of Giant Cells with Guard Cell–Like Characteristics.

(A) and (B) Giant guard cell–like cells in cotyledons (A) and leaves (B) in gig1-2 seedlings observed by DIC microscopy. These cells are herein called gigas cells. Outline of an example of a gigas cell is marked by the white solid line in each image. Asterisks indicate gigas cells in (B).

(C) Gigas cells are occasionally accompanied by structures similar to stomatal pores as observed by scanning electron microscopy.

(D) and (E) Expression of guard cell–specific markers, E1728 (D) and KAT1:GUS (E), in gigas cells. Wild-type cotyledon, left; gig1-2 cotyledon, right.

(F) and (G) Expression of TMM:GUS-GFP in wild-type (F) and gig1-1 myb3r4 (G) cotyledons. Cell outlines were visualized by counterstaining with FM4-64 (shown in red).

(H) and (I) Mutation in spch eliminates not only guard cells but also gigas cells in cotyledons of gig1-1 myb3r4 seedlings. Cleared cotyledons from double gig1-1 myb3r4 mutants (H) and triple gig1-1 myb3r4 spch mutants (I) were observed by DIC.

(J) Enlarged nuclei in gigas cells. Cotyledons of gig1-1 myb3r4 seedlings were stained with DAPI (shown in red). A gigas cell expressing TMM:GUS-GFP (shown in green) contains an enlarged nucleus (arrowhead) in comparison with dividing stomatal lineage cells (asterisks). Note that the nuclear size of the gigas cell is equivalent to that of the adjacent endoreplicated pavement cell (arrow).

Bars = 50 μm in (A) to (C) and (F) to (I) and 20 μm in (J).
associated with TOO MANY MOUTHS (TMM); green fluorescent protein (GFP) expression, a marker for stomatal precursor cells (Nadeau and Sack, 2002) (Figures 1F and 1G), and requires SPEECHLESS (SPCH) function, which is essential for stomatal development (Pillitteri et al., 2007) (cf. Figures 1H and 1I). These results suggest that gigas cells may have a guard cell–like identity, which may be generated through a similar developmental pathway that generates stomata. However, the gigas cells are more similar to jigsaw puzzle–shaped pavement cells in terms of size and morphology and are not paired, in contrast with guard cells in normal stomata. Furthermore, their nuclei are larger than those in normal guard cells and their precursors and are equivalent in size to endoreplicated nuclei in pavement cells (Figure 1J; see Supplemental Figure 5 online).

In addition to the gigas cells, the gig1 cotyledons have two other types of abnormal cells: large guard cells and round cells (see Supplemental Figure 5 online). The former is characterized by abnormally enlarged guard cells, a pair of which forms normal-shaped giant stomata. The latter is reminiscent of single-celled stomata, which are typically generated when guard mother cells fail to undergo cytokinesis or are arrested at the G2 phase, but achieved differentiation into guard cells (Falbel et al., 2003; Boudolf et al., 2004). This notion is consistent with our observation that guard cell–specific markers, E994 and KAT1:GUS, were expressed in the round cells (see Supplemental Figures 3 and 5 online).

All of these abnormal cell types were observed both in gig1-1 and gig1-2, irrespective of the presence of the myb3r4 mutation. There were no qualitative differences in epidermal phenotypes among the mutant combinations, which only affect the frequency of such abnormal cells. In the following experiments, we used both gig1-1 and gig1-2 alleles, which were, in some cases, combined with the myb3r4 mutation, especially when the weak gig1-1 allele was used.

Occurrence of Endomitosis in gig1 Cotyledons

All three types of abnormal cells in gig1 cotyledons (gigas cells, large guard cells, and round cells) contained enlarged nuclei in comparison with those of wild-type guard cells (Figure 1J; see Supplemental Figure 5 online), suggesting increased ploidy levels of these cells. To quantitatively analyze the polyploidy, we estimated nuclear DNA content by measuring relative nuclear sizes and determined the number of chromosomes in each nucleus using a kinetochore-specific marker, tdTomato-CENH3 (Figures 2A to 2D) (Kurihara et al., 2008). The normal guard cells, containing 2C nuclei with relative nuclear sizes of 2.0, showed 10 spots marked by tdTomato-CENH3, which are equivalent to diploid chromosome number (2n) of Arabidopsis. By contrast, the round cells exhibited nuclei with relative sizes of around 4.0 containing 10 chromosomes, which can be explained by G2 cell cycle arrest. The large guard cells also displayed nuclei with relative sizes of around 4.0 but contained 20 chromosomes, which corresponds to a tetraploid chromosome number (4n). This indicates that endomitosis, but not endoreplication, had occurred in the developmental processes of the large guard cells and that endomitosis had occurred only once in such processes. The nuclei of gigas cells also contained 20 chromosomes, but their sizes were distributed discretely from 8 to 32 (Figure 2D).

One explanation is that endomitosis had occurred just one time prior to one to three rounds of endoreplication during the production of gigas cells.

The occurrence of endomitosis was confirmed by live-cell imaging of cotyledon epidermis in which microtubule arrays and chromosomes were labeled by GFP-TUA6 and H2B-tdTomato, respectively. We observed epidermal cells undergoing normal progression of mitosis and cytokinesis in wild-type cotyledons.
In gig1-1 myb3r4 cotyledons, however, we occasionally observed that condensed chromosomes failed to transition into anaphase in a timely fashion and remained in a condensed state for 1.5 to 2.0 h before separation of sister chromatids took place (Figure 3B; see Supplemental Movie 2 online), which only takes 10 to 15 min in the wild type (Figure 3A; see Supplemental Movie 1 online). After separation, sister chromatids could not further move toward spindle poles; instead, they merged into a single entity, resulting in the generation of a single polyplid nucleus. Moreover, in those cells, phragmoplasts were not properly formed between separated sister chromatids; instead, microtubules accumulated in the cytoplasm at one edge of the cell, resulting in the failure of the entire process of cytokinesis (Figure 3B; see Supplemental Movie 2 online).

**Endomitosis Occurs Early in Stomatal Development in gig1 Mutants**

To discern the developmental processes of the large guard cells and the gigas cells, we analyzed cell division history using TMM:GUS-GFP, which marks dividing and recently divided stomatal precursor cells (Figures 4A and 4B). The large guard cells containing 20 chromosomes are typically surrounded by a group of small cells expressing TMM:GUS-GFP, and these cells contained 20 chromosomes, which are further surrounded by cells with 10 chromosomes. This suggests that the occurrence of endomitosis in stomatal lineage cells might have produced a tetraploid cell that then divided several times to produce the tetraploid guard cells. By contrast, cells surrounding a gigas cell carried 10 chromosomes and did not express TMM:GUS-GFP, suggesting that a tetraploid cell that had been produced by endomitosis underwent endoreplication, but not cell division, to generate the gigas cells. Next, we showed that cells expressing SPCH-GFP and EPF2:GFP, which mark precursor cells characteristic of the initial stage of stomatal development (i.e., meristemoid mother cells and the meristemoid) (Pillitteri et al., 2007; Hara et al., 2009), had already enlarged or contained enlarged nuclei (Figures 4C to 4F). Therefore, it can be speculated that endomitosis had occurred in the early stages of stomatal development and that the resulting tetraploid cells later produced either the large guard cells or the gigas cells. The gigas cells, possessing characteristics of both guard cells and pavement cells, might have originated by the occurrence of endomitosis in place of asymmetric division, which would normally segregate stomatal and pavement cell fates (Ten Hove and Heidstra, 2008), and such failed asymmetric division might produce cells with a mixed fate.

**Molecular Identification of GIG1**

We identified GIG1 via the map-based cloning and sequencing of the mutant genome. We found base substitutions in At3g57860 in both gig1-1 and gig1-2 (Figure 5A). The gig1-1 mutation changes Val at position 42 into Met, while, in gig1-2, the G-to-A substitution at the 3’ splice site of intron 2 results in splicing variants encoding truncated GIG1 (see Supplemental Figure 6 online). This suggests that gig1-2 is a null allele of GIG1, which is consistent with the stronger phenotype in gig1-2 in comparison with gig1-1. An additional mutant allele, gig1-3, was identified in the collection of RIKEN transposon insertion lines (Kuromori et al., 2004), and homozygous gig1-3 mutation resulted in a similar phenotype in cotyledons. Furthermore, introduction of a genomic fragment of At3g57860 into gig1-2 homozygotes completely abolished the gig1 phenotype in cotyledons, confirming that GIG1 was At3g57860 (see Supplemental Figure 7 online).

**Double Mutation of GIG1 and UVII4 Is Lethal**

At3g57860 was previously identified as OMISSION OF SECOND DIVISION1 (OSD1) in a reverse genetic approach, and its
and ses overbranched trichomes that were associated with the POLYCHOME encodes a gene (At2g42260) that is paralogous to GIG1 (see Supplemental Figure 8 online). The Arabidopsis gets of the strong et al., 2009). The diploid pollen was also observed in homozygous mutation generates diploid pollen due to the absence of meiosis II in male gametophyte development (d’Erfurth et al., 2009). The diploid pollen was also observed in homoygotes of the strong gig1-2 allele but not of the weak gig1-1 allele (see Supplemental Figure 8 online). The Arabidopsis genome encodes a gene (At2g42260) that is paralogous to GIG1 (d’Erfurth et al., 2009), which was previously identified as POLYCHOME/UVI4 in two independent forward genetic screens (Figure 5A) (Perazza et al., 1999; Hase et al., 2006). It has been reported that a homozygous recessive pym/uvi4 mutation causes overbranched trichomes that were associated with the promotion of endoreplication (Perazza et al., 1999; Hase et al., 2006). To test the functional redundancy between GIG1 and UVI4, we made crosses between null alleles of GIG1 (gig1-2) and UVI4. We could not obtain double gig1-2 uvi4 mutants in the F2 generation, suggesting that the double mutation was lethal. Reciprocal cross experiments suggested that transmission of double mutant gametes was significantly reduced by ~45% on the female side (P value = 0.018, χ² test), whereas it was normally transmitted on the male side (P value = 0.53, χ² test). Consistent with these results, we observed a reduced number of enlarged nuclei in the female, but not male, gametophyte in gig1-2/+ uvi4/ uvi4 plants (Figures 5B to 5D). We also found developing seeds with a reduced number of abnormally enlarged nuclei in the endosperm (Figures 5E and 5F). Mitotic figures of nuclei in such malformed endosperms contained an increased number of chromosomes (Figures 5G and 5H), suggesting the occurrence of endomitosis. Our results showed that functions of GIG1 and UVI4 may at least be partially redundant and support essential roles of these genes in nuclear division during gametogenesis and endosperm development.

**Expression of GIG1**

Expression of GIG1 was analyzed in plants transformed with the upstream region of GIG1 (1.6 kb) fused to the reporter gene, GUS, or nuclear-localized yellow fluorescent protein (YFP). GUS expression was observed in the shoot apical meristem and young leaves, which are rich in rapidly dividing cells (Figures 5I and 5J). In the epidermis of cotyledons and leaves, expression of YFP was observed in dividing and recently divided stomatal precursor cells, especially in dividing guard mother cells and young guard cells (Figure 5K). Consistent with the mixed-fate nature of gigas cells, we also observed YFP expression in asymmetrically dividing meristemoid mother cells and meriste-monds (Figure 5L). In roots, YFP was expressed preferentially in the division zones of root tips (Figures 5M). These data suggest that GIG1 expression is associated with cell division but also with specific cell types.

**GIG1 and UVI4 Physically Interact with APC/C Activators**

It was recently shown by proteomic studies that GIG1 and UVI4 associate in vivo with the APC/C in cultured Arabidopsis cells (Van Leene et al., 2010). The functional relevance of this physical interaction between GIG1/UVI4 and APC/C remained unknown. Interestingly, we observed that GIG1 overexpression leads to a similar dwarf phenotype that is caused by knockdown of the APC/C core subunit genes (Figures 6A and 6B) (Saze and Kakutani, 2007; Marrocco et al., 2009). This suggests that GIG1 might inhibit the activity of APC/C through protein–protein interaction. We examined which components of APC/C physically bind with GIG1 and UVI4 by yeast two-hybrid assays (Figure 6C) and showed that both GIG1 and UVI4 interacted with all APC/C activators tested (CCS52A1/FZR2, CCS52B/FZR3, CDC20.1, and CDC20.5), whereas no obvious interaction was observed for core subunits of APC/C (APC2, APC7, APC10, CDC27a, and HBT). This suggests that GIG1 and UVI4 may inhibit APC/C
through preventing the APC/C activation mediated by FZR/CDH1 and FZY/CDC20.

**Genetic Interaction of GIG1 and UVI4 with APC/C**

We further analyzed the genetic interactions between GIG1 and the APC/C using the 35S::APC10 cosuppression line with decreased expression of APC10, a gene encoding a core subunit of APC/C (Marrocco et al., 2009). Our genetic analysis showed that the gigas cell phenotype was suppressed in gig1-2/gig1-2 cotyledons that retained the 35S::APC10 transgene, while such suppression was not observed in the segregants that had lost the 35S::APC10 transgene (Figure 6D). Our interpretation is that the gig1-2 mutant may have increased APC/C activity, causing endomitosis; however, when elevated APC/C activity is down-regulated by decreased expression of APC10, endomitosis is suppressed.

We also tested for genetic interactions between the gig1 mutation and APC/C activators. Among the four APC/C activators tested in yeast two-hybrid assays, we selected CDC20.1

![Image of genetic interaction](image-url)
and CCS52B/FZR3 for our genetic analysis because these two genes are known to be preferentially expressed in dividing cells, in which GIG1 is also strongly expressed (Larson-Rabin et al., 2009; Kevei et al., 2011). To obtain a high expression of these genes in dividing cells, we used the CDKA;1 promoter, whose activity is associated with dividing Arabidopsis cells (Hemerly et al., 1993). When an overexpression construct, CDKA;1: CDC20.1, was introduced into gig1-1 myb3r4 plants, the gig1 phenotype was markedly enhanced, increasing the frequency of gigas cells and, more dramatically, that of the round cells (Figures 7A and 7B), whereas the introduction of CDKA;1:CCS52B showed no such effects (Figure 7C). Even greater enhancing effects were observed when CDC20.1 was overexpressed under the promoter of EPF2 (Figure 7D). Conversely, CDKA;1:CCS52B caused a dramatic enhancement of endoreplication in uvi4 root tips, whereas no such effect was observed in the case of CDKA;1:CDC20.1 (Figures 7E to 7G; see Supplemental Figure 9 online). Similar differential effects were observed for trichome overbranching and dwarf phenotypes in uvi4 (see Supplemental Figure 10 online). All our data are generally consistent with the idea that GIG1 and UVI4 may act as inhibitors of the APC/C. It is also noted that there may be some functional differences between GIG1 and UVI4 because their loss-of-function causes different phenotypes, which are affected differently by the overexpression of CCS52B/FZR3 and CDC20.1.

**DISCUSSION**

**GIG1 Is a Novel Plant-Specific Inhibitor of APC/C**

Activity of APC/C is generally regulated by both activator and inhibitor proteins. The APC/C activators CDC20/FZY and CDH1/FZR activate APC/C at different points in the cell cycle and also bind to the target proteins of APC/C for selective substrate recognition (Pesin and Orr-Weaver, 2008). CDC20/FZY and CDH1/FZR are related to each other and conserved in all plant species, whereas APC/C inhibitors are species-specific. GIG1 and UVI4 are two such plant-specific APC/C inhibitors, and their overexpression causes a dramatic enhancement of endoreplication in dividing cells (Figures 7A and 7B). The overexpression of GIG1 and UVI4 also causes a dramatic enhancement of the gigas phenotype (Figure 7C). Conversely, the overexpression of CDC20.1 and CCS52B/FZR3 caused a dramatic enhancement of the uvi4 phenotype (Figures 7E to 7G; see Supplemental Figure 9 online). Our data are generally consistent with the idea that GIG1 and UVI4 may act as inhibitors of the APC/C. It is also noted that there may be some functional differences between GIG1 and UVI4 because their loss-of-function causes different phenotypes, which are affected differently by the overexpression of CCS52B/FZR3 and CDC20.1.

**Modified GIG1 Function Affects the Levels of Mitotic Cyclins**

To test if decreased expression of mitotic cyclins, well-known substrates of APC/C, is critical for the endomitosis phenotype in gig1 cotyledons, we examined the effects of cycb2;2 mutations in the gig1 mutant background (Figures 7H and 7I). We found that the additional cycb2;2 mutation significantly enhanced endomitosis phenotype in gig1-1 plants. To examine the actual effects on cyclin expression, we made a CYCB1;2-YFP construct in which YFP fused to the N-terminal region of CYCB1;2, which contained the destruction box motif, was placed under its own promoter (proCYCB1;2:dBox-YFP). We observed that expression of CYCB1;2-YFP was dramatically increased by GIG1 overexpression under the dexamethasone-inducible promoter, causing strong and uniform YFP expression in root tips (Figure 7J, right), which otherwise shows patchy expression pattern (Figure 7J, left). Overexpression of UVI4 caused similar but less prominent effects (see Supplemental Figure 12 online). Our quantitative RT-PCR analysis indicated that overexpression of neither GIG1 nor UVI4 affects significantly the transcription of mitotic cyclins, supporting our idea that these proteins affect mitotic cyclin stability (see Supplemental Figure 12 on line).
eukaryotic species. In vertebrates, the negative regulation of APC/C is typically achieved by two related proteins, Emi1 and Emi2, which bind to APC/C activators and the core complex (Barford, 2011). The primary function of Emi1 is to inhibit APC/CFZR and thereby terminate DNA replication in a timely fashion and enable the transition from the G2 to M phases (Grosskortenhaus and Sprenger, 2002; Machida and Dutta, 2007), while Emi2 inhibits APC/CCDC20 and thereby enables the proper progression through meiosis (Madgwick et al., 2006). Plants do not have counterparts of vertebrate Emi1 and Emi2, and it remains unclear if plants also have a negative regulator of APC/C. Our results suggest that GIG1 and UVI4 may correspond to such negative regulators of APC/C. This is supported by the fact that these proteins both interact with APC/C activators. Moreover, GIG1 overexpression phenocopies the downregulation of APC/C. Our genetic interaction studies also support a model in which GIG1 and UVI4 negatively regulate APC/C activity. For instance, the gig1-2 mutant phenotype that may result from increased APC/C activity can indeed be suppressed by decreasing APC/C activity. Finally, the overaccumulation of CYCB1;2-YFP in GIG1-overexpressing plants also fits this model. We showed that GIG1 and UVI4 may have partially overlapping functions that are essential for female gametogenesis and endosperm development. In these developmental processes, GIG1 and UVI4 may be required for the accumulation of still unknown mitotic regulators by inhibiting their degradation.

**Difference in GIG1 and UVI4 Functions**

Besides having overlapping functions, GIG1 and UVI4 also have specific roles in mitotic regulation. We showed that mutations in GIG1 and UVI4 preferentially affect the increase in cellular ploidy caused by endomitosis and endoreplication, respectively. Whereas endoreplication results from arresting cells at the G2 phase before they enter mitosis, endomitosis results from arresting cells within the M phase before they complete mitosis (Edgar and Orr-Weaver, 2001). This suggests that UVI4 may function earlier than GIG1 in mitotic cell cycle. Probably, UVI4 may act at the G2-to-M transition by promoting the accumulation of specific mitotic cyclins, such as CYCA2;3, which has been identified as a negative regulator of endoreplication (Imai et al., 2006). Loss of UVI4 may thus result in a reduced amount of such cyclins, thereby accelerating the onset of endoreplication. On the other hand, GIG1 may function later in the cell cycle, negatively regulating APC/C activity to control DNA replication and cell division. This dual role of GIG1 and UVI4 in mitotic regulation suggests a complex interplay between these two proteins and their respective partners to ensure proper cell cycle progression and ploidy control.

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**Figure 7.** GIG1 and UVI4 May Inhibit APC/C Activation and Stabilize Mitotic Cyclins.

(A) to (C) Effects of overexpression of CCS52B/FZR3 and CDC20.1 in leaves of gig1-1 myb3r4 seedlings. DIC images of gig1-1 myb3r4 seedlings (A) and of gig1-1 myb3r4 seedlings transformed with CDKA1:CDC20.1 (B) and CDKA1:CCS52B (C).

(D) Enhanced gigas cell phenotype in leaves of gig1-1 myb3r4 seedlings transformed with EPF2:CDC20.1. TMM:GUS-GFP signals are shown in green.

(E) to (G) Effects of overexpression of CCS52B/FZR3 and CDC20.1 on the roots of uvi4 seedlings. Roots of uvi4 seedlings (E) and of those transformed with CDKA1:CDC20.1 (F) and CDKA1:CCS52B (G) were stained with DAPI.

(H) and (I) The cycb2;2 mutation enhanced the gigas cell phenotype in gig1-1 seedlings. Developing cotyledons from gig1-1 single mutants (H) and double gig1-1 cycb2;2 mutants (I). TMM:GUS-GFP signals are shown in green.

(J) Overexpression of GIG1 causes accumulation of CYCB1;2-YFP. Seedlings transformed with the proCYCB1;2:dBox-YFP construct are cultured with 10 µM dexamethasone (DEX) for induction of GIG1 overexpression (right) or without dexamethasone as a control (left). YFP signals are shown in green. Bars = 40 µm in (A) to (D) and 100 µm in (E) to (J).
other hand, GIG1 may act later during mitosis for the scheduled degradation of mitotic regulators, such as CYCB2;2, whose amount was critical for ectopic occurrence of endomitosis in gig1. In this respect, it has been shown that tobacco (Nicotiana tabacum) cells expressing nondestructible cyclin B1 exhibit doubled DNA content as a result of endomitosis (Weingartner et al., 2004).

Our data also suggested that GIG1 and UVI4 may preferentially affect the activities of APC/C\textsubscript{CDC20} and APC/C\textsubscript{FZR}, respectively. Overexpression of CDC20.1 caused a severe endomitosis phenotype in gig1 but not in uvi4, while overexpression of CCS2B/FZR3 resulted in enhanced endoreplication in uvi4 but less efficient enhancement in gig1. It is generally known that CDC20-type APC/C activators execute their function specifically during mitosis, while FZR-type activators can function during interphase (Pesin and Orr-Weaver, 2008). This is consistent with our idea that GIG1 and UVI4 have temporally different activities in the cell cycle. GIG1 may prevent the ectopic occurrence of endomitosis by selective inhibition of APC/C\textsubscript{CDC20} during mitosis, while UVI4 may negatively regulate endoreplication onset by inhibiting APC/C\textsubscript{FZR} at the G2 to M transition.

A Role for APC/C in Cell Fate Determination?

The most striking phenotype of gig1 is the generation of gigas cells, which display characteristics of both guard cells and pavement cells. During epidermal development in Arabidopsis, meristemoid mother cells divide asymmetrically to generate two daughter cells that follow either one of the two developmental pathways, ultimately generating guard cells or pavement cells (Nadeau, 2009). Our examination of gigas cells showed that they have guard cell–like identities and share a developmental pathway with stomatal lineage cells. At the same time, these cells are similar to pavement cells in terms of size and morphology. The occurrence of endoreduplication in developing gigas cells, which is absent in the developmental pathway that generates guard cells, may also be explained by pavement cell–like characteristics in the mixed-fate cells. The occurrence of endomitosis in place of asymmetric division of either meristemoid mother cells or meristemoids may produce single cells with identities of both daughter cells during the generation of gigas cells.

However, failure of cell division alone may not account for the generation of mixed-fate cells in the epidermis. Many mutations are known in Arabidopsis that cause incomplete cytokinesis in epidermal cells; none of them, however, was reported to produce cells with such a mixed fate (Jürgens, 2005). Similarly, it is well known that the application of antimicrotubule drugs, such as colchicine, causes transient arrest at mitosis and generates polyploid somatic cells (Eigsti, 1938; Levan, 1938). Even though colchicine has long been used to produce polyploid plants (Eigsti, 1938; Levan, 1938), it is generally known that the application of antimicrotubule drugs, such as colchicine, causes transient arrest at mitosis and generates polyploid somatic cells (Eigsti, 1938; Levan, 1938). Even though colchicine has long been used to produce polyploid plants, the occurrence of such mixed-fate cells has not been reported upon application of the drug. These observations led us to hypothesize that APC/C may be involved in the determination of epidermal cell fate in addition to its role in mitotic progression. The unexpected roles of APC/C in the determination of cell identity may not be specific for stomatal development, since recent findings have suggested similar nonmitotic roles of APC/C in some different contexts, which include differentiation of the lens and axons in animals (Wu et al., 2007; Yang et al., 2009), as well as vascular development and maintenance of stem cell identity in plants (Marrocco et al., 2009; Vanstraelen et al., 2009).

In summary, we identified novel APC/C inhibitors in Arabidopsis, which may have a role in cell fate determination. Future genetic and biochemical studies will uncover the mechanisms that link the cell cycle to asymmetric cell fate determination, as well as the upstream and downstream signaling pathways of these inhibitors.

METHODS

Plant Materials

Arabidopsis thaliana Columbia (Col) was used as the wild type. All mutants and transgenic lines are in the Col background, except for gig1-3 and KAT1:GUS, which are in the Nossen and RLD1 background, respectively. The gig1 mutant lines, gig1-1 and gig1-2, were derived from an ethyl methane sulfonate–mutagenized myb3r4-1 population and backcrossed four times before analysis. Another allele of GIG1 (gig1-3, pst15307), which has a Ds transposon insertion in exon 2, was obtained from the Plant Functional Genomics Research Group of RIKEN Genomic Sciences Center (Kumori et al., 2004). Other mutants and transgenic plants, enhancer trap lines E994 and E1728, TMM:GUS-GFP, KAT1:GUS, SPCH-GFP, EPF2:GFP, GFP-TUA6, H2B-tomato, and spch-3 were described previously (R.L. Nakamura et al., 1995; Nadeau and Sack, 2002; M. Nakamura et al., 2004; Ohashi-Ito and Bergmann, 2006; Piliatti et al., 2007; Har a et al., 2009; Adachi et al., 2011).

Map-Based Cloning of GIG1

The genetic screen was conducted by mutagenizing ~20,000 myb3r4-1 seeds with 0.3% ethyl methane sulfonate (Sigma-Aldrich) for 10 h. The cotyledon was removed from each M2 plant, mounted after clearing, and visually screened for abnormality in stomatal shape with DIC microscopy. From a screen of 5000 M2 seedlings, two alleles of GIG1 were identified. A mapping population was generated by outcrossing to Landsberg erecta ( Ler). DNA markers were used to detect polymorphisms between Col and Ler. The markers were designed based on the information available in the Monsanto Arabidopsis Polymorphism and Ler Sequence Collection (http://www.Arabidopsis.org/browse/Cereon/index.jsp).

Microscopy

Microscopy observations with DIC and fluorescent optics were done as described previously (Haga et al., 2007). For images of epidermal cell patterns, live tissues were mounted in water and visualized using an Olympus FV1000 confocal microscope. For counterstaining of cell outlines, tissues were placed in 10 μM solution of FM4-64 ( Molecular Probes) for 2 min. GFP was excited at 473 nm, and fluorescence was detected at 485 to 545 nm, FM4-64 was excited at 559 nm, and fluorescence was detected at 570 to 670 nm.

Clearing of plant materials, histochemical GUS assay, and 4,6-diamidino-2-phenylindole (DAPI) staining were performed as described previously (Haga et al., 2007, 2011). Scanning electron microscopy was performed as described previously (Semiarti et al., 2001).

Imaging Analysis

To determine kinetochore number, fluorescent signals of tdTomato-CENH3 in epidermis were counted using Metamorph version 7.5 ( Molecular Devices). Imaging was performed using a fluorescence microscope (IX-81; Olympus) equipped with a confocal laser scanner unit.
Supplemental Data

KAT1: At2g42260; EPF2: At4g35620; CYCA2;3: AT3G16320; article are as follows:
Arabidopsis Genome Initiative numbers for the genes discussed in this
Accession Numbers

PLasmid Construction and Transformation

See Supplemental Table 1 online for a list of plasmid constructs generated
in this study and Supplemental Table 2 online for a list of primer DNA
sequences used for generating these plasmids. Generation and selection
of transgenic plants were done as described previously (Haga et al.,
2007). Binary vectors, pPZP211, pTA7001, pBGGUS, and pBGYN, were
previously described (Hajdukiewicz et al., 1994; Aoyama and Chua,
1997; Kubo et al., 2000)

Yeast Two-Hybrid Assays

Yeast two-hybrid assays were performed as described by Soyano et al.
(2003). Saccharomyces cerevisiae strain L40 was cotransformed with
pBTM116- and pVP16-based plasmids carrying different cDNAs. A single
colony was diluted with water and spotted on medium lacking His that was
supplemented with 5 mM 3-amino-1,2,4-triazole and cultured at 30°C for 2 d.

Quantitative Real-Time PCR

Extraction of total RNA and synthesis of first-strand cDNA were
performed as described previously (Haga et al., 2007). Quantitative real-time
PCR was performed using the SYBR PremixEx Taq (Perfect Real Time) kit
(TaKaRa Biomedical) on the LightCycler480 machine (Roche Diagnostics).
See Supplemental Table 3 online for a list of primer DNA sequences
used for real-time PCR.

Accession Numbers

Arabidopsis Genome Initiative numbers for the genes discussed in this article are as follows: APC2, AT2G04660; APC7, At2g39090; APC10, At2g18290; CDC20.1, At4g33270; CDC20.5, At1g27570; CDC27a, AT3G16320; CYCA2;3, At1g15570; CYCB1;2, At5g46240; CYCB1;2, At5g27570; CDC27a, AT3G16320; CYCA2;3, At1g15570; CYCB1;2, At5g46240; CYCB2;2, At4g35620; ERF2, At1g34245; CCS52A1/FZR2, At4g22910; CCS52B/FZR3, At5g13840; GIG1/OSD1, At3g57860; HBT, At2g18290; UV4, At2g42260; KAT1, At5g42240; SPCh, At5g33210; and TMM, At1g80080.

Supplemental Data

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

Supplemental Figure 1. Schematic Representation of Endoreplication
and Endomitosis.

Supplemental Figure 2. Abnormalities of Epidermal Cells in gig1
Cotyledon Are Enhanced by myb3r4 and Double myb3r1 myb3r4
Mutations.

Supplemental Figure 3. Expression of Guard Cell–Specific Markers
in gig1 Cotyledons.

Supplemental Figure 4. Gigas Cells Contain Enlarged Nuclei with
Abnormal Shapes.

Supplemental Figure 5. Abnormal Guard Cells in gig1 Epidermis.

Supplemental Figure 6. Splice Variants Generated from the gig1-2
Allele.

Supplemental Figure 7. Complementation of gigas Cell Phenotype in
gig1 Epidermis.

Supplemental Figure 8. gig1-2 but Not gig1-1 Plants Produce
Enlarged Pollen Grains with Increased Sizes of Nuclei.

Supplemental Figure 9. Overexpression of CCS52B/FZR3 Severely
Affects Cell Patterns in uv4 Roots.

Supplemental Figure 10. Phenotypes of uv4 Are Enhanced by
Overexpression of CCS52B/FZR3.

Supplemental Figure 11. DAPI-Stained Root Tips in CDC20.1- and
CCS52B/FZR3-Overexpressing Plants in the Wild-Type Background.

Supplemental Figure 12. Overexpression of GIG1 and UV4 Causes
Stabilization of CYCB1;2-YFP.

Supplemental Table 1. List of Plasmid Constructs Used in This Study
and Their Description.

Supplemental Table 2. List of Primers and Their DNA Sequences
Used for Plasmid Construction.

Supplemental Table 3. List of Primers and Their DNA Sequences
Used for Real-Time PCR.

Supplemental Movie 1. Live-Cell Imaging of a Wild-Type Epidermal
Cell Expressing GFP-TUA6 and H2B-tdTomato.

Supplemental Movie 2. Live-Cell Imaging of a gig1 myb3r4 Epidermal
Cell Expressing GFP-TUA6 and H2B-tdTomato.

Supplemental Movie Legends. Legends for Supplemental Movies
1 and 2.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS


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605–612.


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GIGAS CELL1, a Novel Negative Regulator of the Anaphase-Promoting Complex/Cyclosome, Is Required for Proper Mitotic Progression and Cell Fate Determination in *Arabidopsis*

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