THE ARABIDOPSIS MYB FOUR LIPS CONDITIONALLY RESTRICTS STOMATAL ENDOREDUPLICATION

by

Xuguang Liu

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Abstract

Stomata are two-celled epidermal valves responsible for gas exchange between the shoot and the atmosphere. FOUR LIPS and MYB88 are MYB transcription factors required for correctly patterning *Arabidopsis* stomata. Normally the guard mother cell (GMC), a stomatal precursor cell, divides once symmetrically producing a two-celled stoma. However, when *FLP* function is lost via mutation, excess divisions occur which result in abnormally two (or more) stomata in direct contact. Thus FLP normally represses cell divisions allowing normal stomatal differentiation and patterning. The last division in normal stomatal development, as well as the excess divisions in *flp* mutants, require the activities of *CYCLIN DEPENDENT KINASE B1;1* and *CDKB1;2*. This study reports that the loss of function of all four genes (*i.e.* in a *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant) induces a novel phenotype of endoreduplicated stomata and single guard cells, which are much larger than normal. In addition, chemically blocking mitosis in the *flp-1* mutant or *flp-1 myb88* double mutant, as opposed to in the quadruple mutant, also induces the formation of endoreduplicated stomata and single guard cells. These data suggest that the genetic or chemical blockage of mitosis allows the loss of FLP function, which normally limits division, to instead derepress endoreplication. FLP directly targets and likely represses the expression of a series of core cell cycle genes including those that act during G1-to-S phase (*such as* *CELL DIVISION CONTROL 6A* which promotes DNA replication) and G2-to-M phase (*such as* *CDKB1;1* which promotes mitosis). These data extend previous results showing that FLP is a developmental regulator that coordinates cell cycle and differentiation.
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**Abbreviations**

bp base pair  
CDC6 CELL DIVISION CONTROL 6  
CDK Cyclin Dependent Kinase  
CDKA;1 CDK A type  
CDKB1;1 CDK B type 1;1  
cDNA complementary DNA  
ChIP chromatin immunoprecipitation  
Col-0 *Arabidopsis thaliana*, Columbia-0 ecotype  
Ct cycle threshold value  
DAG days after germination  
DAPI 4’6-diamidino-2-phenylindole  
DNA deoxyribonucleic acid  
dNTP deoxyribonucleotide triphosphate  
DIC differential interference contrast  
DTT dithiothreitol  
DMSO dimethyl sulphoxide  
FLP FOUR LIPS  
GC guard cell  
GFP green fluorescent protein  
GMC guard mother cell  
MIF mitosis inducing factor  
MMC meristemoid mother cell  
mRNA messenger RNA  
MSA M phase specific activator element
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>MYB88</td>
<td>MYB transcription factor 88</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>pre-RC</td>
<td>pre-replication complex</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>real time polymerase chain reaction</td>
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<td>single guard cell</td>
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1. Introduction

1.1. Stomatal development

Stomata enclose small openings in the plant epidermis. Each stoma consists of two highly specialized cells, guard cells, which surround a pore that is conduit for gas and water exchange with the environment. This opening is controlled by guard cell movement in response to changing environmental signals, such as light intensity, humidity, and carbon dioxide concentration. Usually stomata open during the daytime and close in the evening. Stomata are key plant cell types because they enable the influx of carbon dioxide into the shoot where it is used in photosynthetic carbon fixation. In addition, water evaporation through stomata cools plants and the biosphere, and also enables the flow of mineral nutrients and water from roots to shoots (Berry et al., 2010).

Stomatal development in Arabidopsis has been well studied (Bergmann and Sack, 2007). Stomata are produced through a series of cell divisions during leaf development (Fig 1). Stomatal development involves at least one asymmetric division and one symmetric division. In wild type Arabidopsis plants, the asymmetric divisions take place in specialized stem cells, the meristemoids and their mother cells (MMCs). Meristemoids eventually acquire a guard mother cell (GMC) fate, and the GMC subsequently divides symmetrically to produce two daughter cells which later differentiate and form the two guard cells (GC) of mature stomata (Geisler et al., 2000). Many pavement cells are also produced by asymmetric divisions in the stomatal cell lineage. Pavement cells occupy a major area of the leaf epidermis.

Transcriptional regulation plays a significant role in stomatal development. Three bHLH transcription factors SPEECHLESS (SPCH), MUTE, and FAMA are involved in
three successive stages of stomatal development. *SPCH* drives the formation of meristemoid mother cell (MMC) which is the first to divide asymmetrically in the pathway (MacAlister et al., 2007). *MUTE* represses the stem cell fate of the meristemoid cells and promotes their transition to guard mother cells (GMCs) (Pillitteri et al., 2007). *FAMA* is required for newly divided guard mother cells to divide just once symmetrically, and this bHLH is also required for the acquisition of guard cell fate (Ohashi-Ito and Bergmann, 2006). Besides these transcription factors, many other types of genes are involved in stomatal development. For example, *TOO MANY MOUTHS* (*TMM*) is a leucine-rich repeat receptor-like protein which restricts asymmetric divisions in leaves, and it is required to maintain stem cell fate in stems (Geisler et al., 2000; Bhave et al., 2009).

![Stomatal development stages](image)

**Figure 1** Key events in stomatal development

The red cell (meristemoid) forms from an asymmetric division of the meristemoid mother cell.

1.2. *FOUR LIPS* restricts excess guard mother cell divisions

*FOUR LIPS* (*FLP*, *Atlg14350*) encodes a two-repeated MYB transcription factor that prevents excessive symmetric divisions in the *Arabidopsis* stomatal pathway (Yang and Sack, 1995; Lai et al., 2005). *FLP* is specifically expressed around the time of guard mother cell division, and the loss of FLP activity results in a failure of guard mother cells
and their daughter cells to stop dividing. The extent of symmetric division depends upon the strength of different flp loss-of-function alleles. The weaker flp-1 allele results in a cell cluster phenotype that often contains four guard cells which originate from the same guard mother cell (Fig 2) whereas the more severe flp-7 allele displays much larger clusters with more than four guard cells.

![Diagram showing FLPL-1 and FLPL-7 phenotypes](image)

**Figure 2** FLP restricts excess divisions of the guard mother cell

*MYB88* (At2g02820) is a *FLP* paralogue, and their predicted proteins share 71% identity overall. However, *myb88* loss-of-function mutants do not exhibit an abnormal stomatal phenotype and *MYB88* expression appears to be much lower than FLP (Lai et al., 2005). The *flp-1 myb88* double mutant displays an enhanced stomatal phenotype with more and larger stomatal clusters compared to either the *flp-1* or *flp-7* single mutants. In addition, extra copies of the MYB88 genomic region complement the *flp-1* phenotype, suggesting that MYB88 overlaps with FLP in restricting excess divisions (Lai et al., 2005). Both *FLP (MYB124)* and *MYB88* belong to the plant R2R3 MYB transcription factor family. MYB transcription factors are named for their conserved MYB DNA-binding domain, which can contain up to three MYB repeats. Each MYB repeat forms a helix-turn-helix structure of about 53 amino acids, consisting of a hydrophobic core with
three conserved tryptophan residues (Ogata et al., 1992). In plants, the MYB protein can be classified into three subfamilies depending on the number of adjacent MYB repeats in the DNA-binding domain (Jin and Martin, 1999): MYB1R (one repeat), R2R3 MYB (two repeats) and MYB3R (three repeats). R2R3 MYBs make up the largest subfamily in Arabidopsis with 125 members, while the MYB1R and MYB3R subfamilies have relatively few members.

1.3. FLP targets cell cycle genes that act in both G1-to-S and G2-to-M phases

The cell cycle involves an orderly series of events which normally result in the duplication of its contents and the division into two new cells. A cell cycle contains two key events, DNA replication in S phase and mitosis/cytokinesis in M phase. G1 phase and G2 phase separate S phase and M phase, and these periods involve preparation for DNA replication and for mitosis and cytokinesis respectively. During G1 and G2 phases, biosynthesis is highly active, and many proteins required for the next phase are produced. Two pivotal classes of cell cycle proteins, Cyclins and Cyclin Dependent Kinases (CDKs), control progress through the cell cycle.

Several MYB proteins have been shown to directly target cell cycle genes and regulate cell cycle progression in plants, but they primarily regulate the G2-to-M transition. The tobacco MYB3R genes NtMYBA1 and NtMYBA2 bind to the M-phase-specific activator element (MSA) in the promoters of some G2-to-M phase specific genes (Ito et al., 2001). Their Arabidopsis orthologs, MYB3R1 and MYB3R4, positively regulate cytokinesis by activating several genes containing an MSA-like element as well, although the exact binding sites have not yet been defined (Haga et al., 2007).
However, few plant MYB transcription factors are known to regulate target genes that
function during G1-to-S phase. For example, ectopic expression of an Arabidopsis R2R3
MYB gene, AtMYB59, in yeast can alter DNA synthesis, but its known target, CYCB1;1,
mainly functions in G2-to-M phase in plants (Mu et al., 2009).

Several cell cycle genes seem to be direct targets of FLP (Xie et al., 2010), including
CDKA;1 (CYCLIN DEPENDENT KINASE A;1, At3g48750), CDC6a (CELL DIVISION
CONTROL 6A, At2g29680), CDC6b (CELL DIVISION CONTROL 6B, At1g07270), and
CDKB1;1 (CYCLIN DEPENDENT KINASE B 1;1, At3g54180). CDKB1;1 acts during
G2-to-M phase, CDKA;1 acts in both G1-to-S and G2-to-M phases, and CDC6 acts from
late M to G1 phase. Thus, in contrast to plant MYBs that bind MSA sites in G2-to-M
genes, the MYB FLP might regulate cell cycle genes that together act throughout the
whole cell cycle progression.

1.4. CDKB1 is required for the G2-to-M transition that produces stomata

CDKB1;1 is a plant specific B type CDK and belongs to the B1 subgroup. In
Arabidopsis, the B1 CDK subgroup contains two genes, CDKB1;1 (At3g54180) and
CDKB1;2 (At2g38620) (Boudolf et al., 2001; Vandepoele et al., 2002). The expression
of CDKB1;1 overlaps with that of FLP in the stomatal cell lineage, in that it is expressed
before, during, and after guard mother cell symmetric divisions (Xie et al., 2010). The
CDKB1;1 dominant negative mutant N161 exhibits an opposite phenotype to that of flp
mutants in that it has single guard cells, which differentiate from undivided guard mother
cells (Boudolf et al., 2004a). A similar phenotype was found in a cdkb1;1 cdkb1;2
double mutant (Fig 3) but not in either mutant on its own (Xie et al., 2010), indicating
that both CDKB1;1 and CDKB1;2 activities are repressed in N161. Phenotypic analysis of the flp-1 N161 double mutant and of the flp-7 cdkb1;1 cdkb1;2 triple mutant indicates that cdkb1 mutants are genetically epistatic to the flp mutants, since single guard cells but not stomatal cluster phenotype were observed in these lines. In addition, the CDKB1;1 expression level is up-regulated in the absence of FLP (Xie et al., 2010). Therefore FLP, which directly targets CDKB1;1, normally represses CDKB1;1 expression which in turn might block excess guard mother cell divisions.

CDKB1;1 and CDKB1;2 are M phase CDKs which seem to directly promote mitosis late in the stomatal cell lineage. It is likely that the loss of CDKB1 activity initially causes GMCs to arrest at G2-to-M transition. Later these cells acquire a guard cell fate and form single guard cells with doubled DNA content, suggesting that CDKB1 activity primarily controls mitosis (Boudolf et al., 2004a; Xie et al., 2010). Besides CDKB1;1, other data show that FLP targets a set of core cell cycle genes, raising the possibility that these genes also might restrict divisions before stomata formation (Xie et al., 2010).

In addition to controlling mitosis in stomatal development, 35S:CDKB1:N161 also affects the size and ploidy level of pavement cells (the major cell type in the Arabidopsis epidermis) (Boudolf et al., 2004a). Compared to control transformants, pavement cells in N161 are larger and display higher ploidy levels. In addition, the ploidy levels in the whole leaf of N161 also increase.
1.5. CDC6 is key component of the DNA replication licensing system

Cell cycle progression is strictly regulated at G1-to-S and G2-to-M checkpoints, which causes DNA replication (S phase) and mitosis (M phase) to occur sequentially. The occurrence of mitosis resets the entire cell cycle meaning that it affects the ability of a daughter cell to subsequently re-enter the cell cycle. Thus, the initiation of DNA replication depends upon the proper completion of a previous mitosis. A replication licensing system initiates DNA replication in G1 phase and ensures that replication takes place only once after mitosis (within one cell cycle).

This licensing system consists of a protein complex, the pre-replication complex (pre-RC), and its regulators. The pre-RC consists of an ORC (Origin Recognition Complex), CDC6, CDT1, and the MCM (MiniChromosome Maintenance) proteins. The ORC binds to the DNA replication origin sites. In late M phase to early G1 phase, CDC6 and CDT1 bind to the ORC and help load MCM onto replication origins thus forming a complete pre-RC. Replication forks then assemble upon the pre-RC thus licensing replication. Once the replication forks start to move and synthesize new DNA, the pre-RC components (except for the ORC) disassociate from DNA replication origins until
mitosis is completed, after which no further replication will initiate from these replication origins in the same cell cycle.

CDKs are likely the major regulators of pre-RC components. In yeast, CDKs target all pre-RC components thereby inhibiting the pre-RC (Blow and Hodgson, 2002). Phosphorylation of the ORC by some CDKs probably makes it inactive for pre-RC assembly in S phase and G2 phase (Wuarin et al., 2002; Wilmes et al., 2004). The phosphorylation of CDC6 induces its degradation during late G1 (Jallepalli et al., 1997). Pre-RC components are also regulated at the transcriptional level. In Arabidopsis, the E2F-DP transcription factors directly regulate ORC, CDC6 and MCM (Ramirez-Parra et al., 2003; Vandepoele et al., 2005).

1.6. Mitotic cycle and endocycle

The cell cycle is also referred to as the mitotic cycle, in which DNA replication (S phase) and mitosis (M phase) occur subsequently with intervening gap phases G1 and G2. The endocycle (endoreduplication) is a modified cell cycle mode, in which the nuclear DNA content increases via repeated DNA replication without mitosis (Fig 4).

The mitotic cycle and endocycle likely share the same cell cycle components with distinct regulation modes. A plethora of genes affect both the mitotic cycle and the endocycle. As introduced above, the M-phase CDKs, CDKB1;1 and CDKB1;2 are required for a guard mother cell to pass through the G2-to-M transition, and they also restrict endoreduplication in pavement cells (Boudolf et al., 2004b). Constitutive expression of CDC6, which is known to be a component of pre-RC, also increases endoreduplication in the whole leaf of Arabidopsis (Castellano et al., 2001).
In *Drosophila* oogenesis, the follicle cells switch from a mitotic cycle to an endocycle naturally during their development, which is controlled by the Notch signaling pathway. Notch encodes a transmembrane receptor for the ligand Delta. Loss of function of Notch or Delta results in follicle cells that fail to switch from a mitotic cycle to an endocycle (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). The Notch signaling pathway executes the mitotic cycle-to-endocycle switch by regulating a series of cell cycle components involved in different stages. Notch might down-regulate String thereby repressing mitosis, activating Fzr to allow G1 progression, and down-regulating Dacapo to assure entry into S phase (Shcherbata et al., 2004).

In plants the regulation of the cell cycle vs. the endocycle is still not well defined. One possibility is that the activity of a Mitosis-Induce-Factor (MIF) decides the switch between the mitotic cycle and the endocycle, with CDKB1 as part of the MIF (De Veylder et al., 2002; Boudolf et al., 2004a). It has also been suggested that endoreduplication results from a loss of M-phase acting CDKs as well as oscillations in the activities of S phase CDKs (Larkins et al., 2001).

![Figure 4 Endocycle and mitotic cycle](image-url)
1.7. Overview of this study

Although tetraploid and higher ploidy guard cells exist in some *Arabidopsis* genotypes and lines, most researchers use diploid plants in which guard cells invariably display 2C levels of nuclear DNA (with C usually representing haploid DNA content). In contrast, the major cell type in the shoot epidermis, so-called pavement cells that surround stomata, undergo varying levels of endoreduplication with ploidy levels often up to 16 and 32C (Melaragno et al., 1993). In *Arabidopsis* endoreduplication research, guard cells are normally used as an internal 2C control for comparison with more endoreduplicated cells.

In this study, we report a novel endoreduplicated stomata phenotype that we found in loss-of-function mutants in *FLP* and *CDKB1* related genes in *Arabidopsis*. In a *flp-1* *myb88 cdkb1;1 cdkb1;2* quadruple mutant, the normally-shaped guard cells and abnormal single guard cells undergo endoreduplication, producing large cells with endoreduplicated nuclei, which are absent from either *flp* or *cdkb1* single or double mutants. Endoreduplicated guard cells also appear in *flp* mutants treated with the mitosis inhibiting chemical oryzalin. Moreover, the expression of G1-to-S genes such as CDC6 is likely up-regulated in the *flp* mutant. FLP has been shown to function in repressing extra symmetric cell divisions before guard mother cells differentiate, and FLP appears to do so in part by repressing the expression of the M-phase CDK *CDKB1;1* (Xie et al., 2010). Here we show that FLP also represses endoreduplication in stomata in a sensitized genetic background, and that it might do so by targeting and down-regulating other G1-to-S genes.
2. Materials and methods

2.1. Plant materials

The Columbia-0 ecotype (Col-0) of Arabidopsis (Arabidopsis thaliana) was used as the wild type control. Loss of function mutations in FLP, MYB88, CDKB1;1 and CDKB1;2 were used (Lai et al., 2005; Xie et al., 2010). Gene structures and the location and types of mutations are shown in Figure 5. Double mutants and higher order mutants were isolated by Dr. EunKyoung Lee.

Seeds were surface sterilized by a mixture of 70% (v/v) ethanol and 6% (v/v) hydrogen peroxide for 1 minute. They were then sown on ½ strength Murashige and Skoog medium containing 2% (w/v) sucrose and 0.8% (w/v) agar in plastic petri plates. Plates were sealed with surgical tape (Micropore, 3M), and the seeds were stratified at 4°C in a cold chamber for two days. Plates were then moved to growth chamber under 16 hours light/8 hours dark at 22°C.

![Diagram of gene structures and mutations](image)

**Figure 5** Loss of function mutant alleles in FLP, MYB88, CDKB1;1 and CDKB1;2
2.2. Measurement of epidermal cell size

To reduce variations in growth, different lines were sown at the same time on ½ MS medium plates for each experiment. Cotyledons were harvested 21 days after germination, one per seedling. For clearing, after rinsing with water, cotyledons were fixed in acidified methanol (containing 20% methanol and 4% concentrated hydrochloric acid) for 15 minutes at 57°C in a water bath. The acidified methanol was then replaced with a basic solution (7% m/v sodium hydroxide in 60% ethanol) for 30 minutes at room temperature. Samples (cotyledons) were then rehydrated in a series of ethanol solutions 40%, 20%, 10%, and were incubated for at least 30mins in each step. Finally, tissues were placed in 5% ethanol and 25% glycerol for storing at room temperature.

The abaxial cotyledon epidermis was visualized using Differential Interference Contrast (DIC) optics in an AX70 light microscope. Digital micrographs were captured using a 20X objective. For sampling, images were captured at two positions along the length of the cotyledon, ¼ and ¾ between the distance from the tip to the base of the cotyledon. Six images were collected from each cotyledon, and six cotyledons were counted for each genotype. Cell areas were then measured by ImageJ software.

2.3. Measurement of stomatal nuclear DNA content

Cotyledons were harvested from seedlings grown on ½ MS medium plates and fixed in 90% ethanol and 10% acetic acid at -20°C for at least one hour. For staining, tissues were rinsed once in TN buffer (Tris-NaCl buffer, 50mM Sodium chloride, 100mM Tris-hydrochloric acid, pH8.0) and then stained in TN buffer containing 0.1µg/ml 4’6-
diamidino-2-phenylindole (DAPI) in the dark for 24 hours. DAPI is a fluorescent dye that can form complexes with double stranded DNA. When DAPI binds to DNA, its fluorescence is enhanced about 20 times. Upon DNA binding, the excitation maximum is 358 nm and the emission maximum is 461 nm.

To measure DAPI fluorescence, a Zeiss Meta 510 two photon Confocal microscope was used, and data were supplemented using an Olympus wide-field microscope. Bright field images were used to show cell shape and position. To standardize image capture, all fluorescence images from different samples were obtained under the same Meta 510 microscope settings. Relative nuclear DNA content was calculated by the total grey values of the nuclear zone using ImageJ software.

2.4. Flow cytometry analysis

Cotyledons were harvested from seedlings grown on ½ MS medium plates, the seeds for these plants were all sown at the same time. The method for isolating nuclei was modified from Suda (Suda and Travnicek, 2006). About 20 mg of cotyledon tissue was gathered and then minced by razor in 600µl OttoI solution (0.1M citric acid monohydrate, 0.5% v/v TWEEN 20, 0.1% v/v β-mercaptoethanol). The solution was subsequently filtered by Miracloth and a 22µm micro-pore membrane to separate nuclei from cell and tissue debris. The supernatant was centrifuged at 3,000 rpm for 3 minutes to spin down nuclei, and the pellet was re-suspended in 150µl OttoI solution. Then 600 µl OttoII solution (0.4 M sodium phosphate dibasic dodecahydrate, 0.05mg/ml propidium iodide, 1mg/ml RNase) was added to the re-suspended nuclei. The solutions were then analyzed
in FACScan flowcytometer (Becton Dickinson). The output graph was processed using flowJo software.

2.5. RNA isolation and quantitative real time PCR

Shoots were harvested from seedlings grown on ½ MS medium plates. Total RNA was prepared using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. Total RNA was dissolved in RNase free water and then stored at -80°C. RNA concentrations and purities were determined by checking absorbance at 260 nm and 280 nm using a spectrophotometer. The concentration was calculated using the formula:

\[ C \text{ (concentration)} = A \text{ (absorbance in 260nm}) \times 40 \times \text{dilution factor} \mu \text{g/ml} \]

The same amounts of total RNA from different samples were used for cDNA synthesis. Reverse transcription was performed by Superscript 3 Reverse Transcriptase (Invitrogen). For 1µg total RNA, 1µl Oligo(dT)20 (50µM), 1µl 10mM dNTPs and distilled water were added to produce a 13µl solution. This mixture was heated at 65°C for 5 minutes and then placed on ice for 1 minute. Then 4µl 5× first strand buffer, 1µl 0.1M DTT, 1µl RNase Inhibitor, and 1µl Superscript 3 Reverse Transcriptase (200U) were added to the 13µl mixture. The resulting 20µl reaction solution was incubated at 25°C for 5 minutes, 50°C for 60 minutes, and then 70°C for 15 minutes. The reaction solutions were used directly as cDNA template.

Before Real time PCR (RT-PCR) was performed, a normal PCR was carried out to verify primer’s specificity and to optimize the annealing temperature. RT-PCR was performed using a BioRad iCycle system. To set up the 25µl reaction volume, 12.5µl of BioRad iQ SYBR Green SuperMix, 2µl of cDNA template, 2µl of primers (10pM, 1µl for
either primer) and 8.5µl of distilled water were added. The expression of a Histone 4 gene (At2g28740) was used as a standard.

The relative expression level was calculated as follows:

\[
\text{Relative expression} = \frac{2^{\Delta Ct \text{ target (control-sample)}}}{2^{\Delta Ct \text{ ref (control-sample)}}}
\]

The primers used are listed below:

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<th>Target gene</th>
<th>No.</th>
<th>Primer</th>
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<td>Forward AGTCTCTCTCTCAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse CTACTCTCAGCTACTTC</td>
</tr>
<tr>
<td>ORC2</td>
<td>At2g37560</td>
<td>Forward GTGGGACAAGAAAATGGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse TCTGGGTGGAAGGTG</td>
</tr>
<tr>
<td>ORC3</td>
<td>At5g16690</td>
<td>Forward AAGCCAGTTGGAGGAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse ATGGAGGCGAGATG</td>
</tr>
<tr>
<td>MCM2</td>
<td>At1g44900</td>
<td>Forward CTCGTTGCTCTGGAAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse ATCCCTGAATGCCAT</td>
</tr>
</tbody>
</table>

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2.6. Oryzalin treatment

Oryzalin (4-(dipropylamino)-3,5-dinitrobenzenesulfonamide) is an anti-tubulin drug (structure shown below). It has specific binding affinity to plant tubulins, an affinity that is 1000 times stronger than colchicines which is an older anti-tubulin drug. Oryzalin inhibits the polymerization of plant microtubules. Low concentrations of oryzalin are routinely used in studies of microtubule dynamics. Since high oryzalin concentrations induce cell cycle arrest at mitosis, this concentration level was used in this study.

<chemical structure image>

To make the oryzalin stock solution (25mM), powder was dissolved in pure DMSO and then filtered using a micropore membrane for sterilization. The stock solution was stored in -20°C for up to a few months. The working solution (25µM) was freshly made by diluting the stock solution 1000 times with sterilized water.

Arabidopsis seeds were grown on ½ MS medium in multi-well plates or small petri dishes. Three days after seed germination, the oryzalin working solution was added to petri dishes containing seedlings in the growth chamber. One day later the oryzalin
solution was removed and the plants and plate were rinsed with sterilized water. Seedlings were then grown for several days in the growth chamber. As oryzalin is toxic to seedlings (which start to turn yellow after one week, and die after two weeks) DNA ploidy was measured three days after treatment, when most seedlings were still healthy.

2.7. Transgenic plants harbouring proCDC6a: GFP

For expression analysis, the 2734bp promoter fragment of CDC6a (At2g29680) was isolated from Arabidopsis genomic DNA using a forward primer, caccTTGTAAACACAGACGATTGGCC, and a reverse primer, AGATATCTGTAACAACTATTTT. This fragment includes the entire region between the stop codon of the previous gene and the start codon of CDC6a. This upstream region was subsequently integrated into a pENTR/TOPO entry vector (Invitrogen) and into a binary vector pGWB4 (no promoter, C terminal fused GFP) (Karimi et al., 2007). The construct proCDC6: GFP was transformed into Col-0 and flp-1 mutant plants (Agrobacterium strain GV3101). Transgenic lines were selected on ½ MS medium containing 25 µg/ml hygromycin. In T2 lines, reporter expression was verified 3 days after cotyledon germination.
3. Results

3.1. Combined loss of FLP/MYB88 and CDKB1 function induces larger stomata

The genetic relationships among two pairs of closely related genes, the R2R3 MYBs, *FOUR LIPS* and *MYB88*, and *CYCLIN-DEPENDENT KINASE CDKB1;1* and *CDKB1;2* were previously established (Xie et al., 2010). This work showed that the single guard cell phenotype (lack of symmetric division of the GMC) found in *cdkb1;1 cdkb1;2* double mutant was epistatic to the stomatal cluster phenotype of *flp-1 myb88* mutant in the quadruple mutant. These data are consistent with the finding that FLP/MYB88 directly target the promoter of *CDKB1;1*, and that *CDKB1;1* is genetically downstream of *FLP* and perhaps *MYB88* as well (which displays no abnormal stomatal phenotype on its own).

Further examination of the quadruple mutant, *flp-1 myb88 cdkb1;1 cdkb1;2*, revealed a novel phenotype of enlarged stomata (Fig 6 A and B). First, some normally-shaped stomata (with paired guard cells) appeared to be much larger in the quadruple mutant (Fig 6 G) than in the *cdkb1;1 cdkb1;2* double mutant (Fig 6 F), in the *flp-1* mutant (Fig 6 D) or in the wild-type (Fig 6 C). Second, some of the single guard cells in the quadruple mutant (Fig 6 H) also appeared much larger than single guard cells in the *cdkb1;1 cdkb1;2* double mutant (Fig 6 F).

To determine whether these differences were significant, the sizes of these stomatal cell types were quantified in 21 DAG cotyledons. By this age and stage, most epidermal cells have stopped dividing and enlarging in the cotyledon (Boudolf et al., 2004a). Stomata with a normal morphology were found to be roughly consistent in size in the wild-type, in the *flp-1* mutant and in the *cdkb1;1 cdkb1;2* double mutant. By contrast,
many normally-shaped stomata in the quadruple mutant, were much larger than in the above three genotypes (wild-type, flp-1 and cdkb1;1 cdkb1;2). Also, single guard cells in the quadruple mutant were much larger than those cells in the cdkb1;1 cdkb1;2 double mutant. Leaves of the quadruple mutant also showed enlarged stomata and single guard cells which were identical to those found in the cotyledons, suggesting that enlarged stomata are not related to just one stage of cotyledon or leaf development. Note that stomatal size was not quantified in the flp-1 myb88 double mutant, because this genotype shows relatively few normal (non-clustered) stomata.

Beside the enlarged guard cell phenotype, guard cells in the quadruple mutant flp-1 myb88 cdkb1;1 cdkb1;2 were almost filled with chloroplasts (Fig 5 G and H). By contrast, the number of chloroplasts in stomata in flp-1, flp-1 myb88, and in cdkb1;1 cdkb1;2 seemed to be equivalent to those in the wild-type (Fig 6 C to F). High numbers of chloroplasts were present in normally-shaped stomata as well as in single guard cells in the quadruple mutant. Normally-shaped stomata (with paired guard cells) in Col-0, flp-1, and in cdkb1;1 cdkb1;2 contain about 10 chloroplasts, a number consistent with previous results for chloroplasts in stomata of wild-type Arabidopsis plants (Pyke and Leech, 1994). By contrast, the mean number of the chloroplasts in stomata (with paired guard cells) in the quadruple mutant flp-1 myb88 cdkb1;1 cdkb1;2 was 25.4 (Fig 5 H).
Figure 6 Combined Loss of FLP/MYB and CDKB1 function increases stomatal size and chloroplast number

(A) to (H) shows stomata in the abaxial epidermis of 21 DAG cotyledon imaged from different genotypes. (A) Tracing of cotyledon epidermis in cdkb1;1 cdkb1;2 double mutant with normally-shaped stomata and single guard cells shown in green. (B) Tracing of cotyledon epidermis in flp1 myb88 cdkb1;1 cdkb1;2 quadruple mutant. Scale bar in (B) represents 25 µm for both (A) and (B). (C) Normally-shaped and patterned stomata in wild-type from fixed and dehydrated cotyledons. (D) Normally-shaped stomata and stomatal cluster with four guard cells in flp-1. (E) Stomatal cluster in flp-1 myb88 contains more guard cells than in flp-1. (F) Normally-shaped stomata and a single guard cell (upper right) in cdkb1;1 cdkb1;2. Note that the closeness between the single GC and the nearby stomata is an artefact of tissue clearing and dehydration. (G) and (H) Enlarged normally-shaped stomata and a single guard cell in flp-1 myb88 cdkb1;1 cdkb1;2 (quadruple mutant). Scale bar in (H) represents 25µm for (C) to (H). (I) The mean areas of guard cells (in stomata of normal morphology) and single guard cells in 21 DAG cotyledons. Both types of guard cells in the quadruple mutant were larger than guard cells in other genotypes. Error bars represent standard error, p-value<0.001. The number of stomata with normal morphology scored in each genotype was 225. The numbers of single guard cells scored was 164 in cdkb1;1 cdkb1;2, and 182 in flp-1 myb88 cdkb1;1 cdkb1;2. Six cotyledons were scored for each genotype. (J) Average chloroplast number in normal-shaped stomata (two guard cells) of different genotypes. Stomata in in flp-1 myb88 cdkb1;1 cdkb1;2 contain more chloroplasts than those in Col-0, flp-1, cdkb1;1 cdkb1;2. Error bars represent standard deviation, p-value<0.001. Ten stomata were scored for each genotype.

3.2. Enlarged guard cells are endoreduplicated

Stomatal size and chloroplast number have long been used as morphological markers to quickly estimate ploidy levels such as in polyploidy plants. For example, natural octoploid lines in Coffea have larger guard cells than diploid lines, and commercially bred tetraploid lines have guard cells of intermediate size between guard cells in
octoploid and diploid lines (M.K. Mishra, 1997). Similarly in alfalfa, diploid, triploid, tetraploid and hexaploid plants averaged 9.2, 9.4, 12.8 and 15.2 chloroplasts respectively per paired guard cells (E.T. Bingham, 1968). *Arabidopsis* Col-0 plants are considered to be diploid with guard cells which are roughly consistent in cell size and chloroplast number (Melaragno et al., 1993; Pyke and Leech, 1994).

To determine whether enlarged guard cells in the quadruple mutant also show abnormal DNA levels and whether FLP/MYB88 influence stomatal nuclear ploidy, relative DNA content was measured using the DNA-specific fluorochrome 4’, 6-diamidino-2-phenylindole (DAPI). Data were collected from stomata of all four genotypes (*Col-0, flp-1 myb88, cdkb1;1 cdkb1;2* and *flp-1 myb88 cdkb1;1 cdkb1;2*) (Fig 7). All guard cells sampled in *Col-0* and in clusters of *flp-1 myb88* displayed similar DNA levels, suggesting that they all contain 2C nuclei. In the *cdkb1;1 cdkb1;2* double mutant, guard cells are also 2C, but single guard cells shows fluorescence intensities that were approximately double that of guard cells, suggesting that these single guard cells contained 4C levels of DNA. A identical result was reported for single guard cells produced in wild type plants transformed with a *pro35S:CDKB1* dominant negative construct (*N161*) (Boudolf et al., 2004a). This construct has been shown to produce single guard cells by interfering with the activities of both the *CDKB1;1* and *CDKB1;2* genes (Xie et al., 2010). Because these genes promote the symmetric division of guard mother cells, it is likely that the single guard cells (whether produced by *N161* or the *cdkb1;1 cdkb1;2* double mutant) each contains a single 4C nucleus as a default when post-S-phase cells fail to progress through the G2-to-M transition (Boudolf et al., 2004a;
Xie et al., 2010). Thus these 4C single guard cells do not appear to represent endoreduplication but rather a failure to pass through G2-to-M transition.

By contrast, single guard cells, as well as many normally-shaped guard cells in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant display ploidy levels above 4C (Fig 6 E and F).

Therefore, the loss of function of either FLP and MYB88 or CDKB1;1 and CDKB1;2 does not appear to induce endoreduplication in the stomatal lineage. Instead, the loss of FLP and MYB88 function induces excessive mitotic cell cycles in guard mother cells and their GMC-like progeny. The very large and high ploidy normally-shaped guard cells as well as single guard cells appear to form only when both sets of functions (FLP/MYB88 and CDKB1;1/CDK B1;2) are lost.
Figure 7 Loss of FLP and MYB88 function increases guard cell ploidy levels in a sensitized mutant background (cdkb1;1 cdkb1;2) (A) to (F) show nuclear staining (DAPI) of guard cells in different genotypes. White dashes indicate stomatal outline. (A) Normal stoma in wild-type plant. (B) Normal-appearing stoma in a cdkb1;1 cdkb1;2 double mutant. (C) Single guard cell in a cdkb1;1 cdkb1;2 double mutant, with slightly larger nucleus (likely 4C). (D) Stomatal cluster in flp-1 myb88 contains 4 stomata (mature and developing) and one unpaired guard cell (third from left). Nuclear size appears roughly similar in all cells in this cluster. (E) A larger normally-shaped stoma with a larger nucleus in a flp-1 myb88 cdkb1;2 cdkb1;2 quadruple mutant. (F) A larger single guard cell in flp-1 myb88 cdkb1;2 cdkb1;2 quadruple mutant, with a larger nucleus than in a cdkb1;1 cdkb1;2 double mutant. Scale bar in (F) represents 25µm for (A) to (F). (G) Comparison of relative DNA content in guard cells of different genotypes. Guard cells in Col-0 and flp-1 myb88 contain similar amounts of DNA (2C). Single guard cells in cdkb1;1 cdkb1;2 show 4C DNA levels. The mean DNA content in single guard cells of the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant is higher than those in the cdkb1;1 cdkb1;2 double mutant. Bars indicate standard error, p-value < 0.001. 216 guard cells were scored in each of Col-0 and flp-1 myb88 background. Fifty single guard cells were scored in cdkb1;1 cdkb1;2, and 68 in the flp-1 myb88 cdkb1;2 cdkb1;2 quadruple mutant. Six cotyledons were scored for each genotype.

3.3. FLP and MYB88 do not affect endoreduplication in whole organs

It was previously shown that the CDKB1;1 dominant negative mutant N161 (pro35S:CDKB1;1-N161) promotes endoreduplication in pavement cells and also shows an increase in the ploidy level in the entire cotyledon (Boudolf et al., 2004a). Because Loss of FLP/MYB88 function promotes endoreduplication in the stomatal lineage in a sensitized background, we asked whether various mutant combinations also promote endoreduplication at the level of whole organ. Using nuclei isolated from whole first leaves we analyzed the distribution of ploidy by flow cytometry in Col-0, flp-1 myb88,
cdkb1;1 cdkb1;2 and flp-1 myb88 cdkb1;1 cdkb1;2. The ploidy distributions between Col-0 and flp-1 myb88, or between cdkb1;1 cdkb1;2 and flp-1 myb88 cdkb1;1 cdkb1;2 were highly similar (Fig 8), indicating that FLP/MYB88 do not normally influence endoreduplication in whole leaves. Here we used first leaves instead of cotyledons, because cotyledons are smaller in the quadruple mutant than in other genotypes and it’s hard to harvest enough tissues for flow cytometry.

Figure 8 Flow cytometry of whole leaves in four different genotypes.

Ploidy distributions were analyzed by flow cytometry in 21 DAG first leaves of four genotypes: (1) Col-0, (2) flp-1 myb88 (3) cdkb1;1 cdkb1;2 (4) flp-1 myb88 cdkb1;1 cdkb1;2. No obvious differences were detected between Col-0 and flp-1myb88, or between cdkb1;1 cdkb1;2 and flp-1 myb88 cdkb1;2 cdkb1;2.
3.4. Chemically induced mitotic inhibition causes stomatal endoreduplication in flp mutants

As shown above, FLP and MYB88 normally prevent excessive mitotic cycles in guard mother cells and their progeny. Also, flp-1 and flp-1 myb88 mutants do not show large, and presumably endoreduplicated stomata. Nor do they show single guard cells that result from a failure of GMC division. Similarly, the cdkb1;1 cdkb1;2 double mutant lacks very large single guard cells compared to the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant. It is only in the quadruple mutant that endoreduplicated single guard cells and endoreduplicated normally-shaped stomata appear.

*CDKB1;1* and *CDKB1;2* together are required for guard mother cells to pass through the G2-to-M phase transition in the *Col-0* wild type. The loss of these gene functions causes single guard cells to form in *N161* and in *cdkb1;1 cdkb1;2* (Boudolf et al., 2004a; Xie et al., 2010). In addition to forming via genetic deficiencies, single guard cells can also develop after microtubule depolymerization such as after colchicine treatment (Reese, 1950; Dehnel, 1961; B. Galatis, 1977). However, the effects of cytoskeletal disruption on possible endoreduplication do not appear to have been reported in plants. To test whether microtubule polymerization inhibitors might also induce endoreduplication in stomata, oryzalin was applied to developing cotyledons in different genetic backgrounds. Oryzalin is a dinitroaniline herbicide, which has 1000 times the binding affinity to plant microtubules as colchicine. Oryzalin depolymerises microtubules and prevents the polymerization of new microtubules thus disrupting the organization of the mitotic spindle, the separation and movement of chromosomes, and the formation of the cell plate during mitosis (L.C.Morejohn et al., 1987).
We determined the effect of oryzalin in Col-0 and flp-1 backgrounds which were transformed with proFAMA:GFP, a transcriptional fusion that marks GMC and guard cell fates in normal Arabidopsis plants (Fig 9 A and C). As expected, oryzalin application to wild type plants blocked GMC symmetric divisions and produced undivided, round to oval-shaped cells that expressed proFAMA:GFP (Fig 9 B). Single guard cells (confirmed in identity by proFAMA:GFP expression) also formed in flp-1 plants treated with oryzalin (Fig 9 D). Although not quantified, some of the single guard cells in flp-1 (Fig 9 D upper right) appeared to be larger than those in Col-0 (Fig 9 C).

**Figure 9** Oryzalin treatment blocks GMC mitosis and induces single guard cells

(A) and (B) show control stomata in the abaxial epidermis of a 7DAG cotyledons in Col-0 and in flp-1, which were treated with 25µM DMSO for 24 hours beginning at the fourth day after germination. (C) and (D) show stomata in the abaxial epidermis of 7 DAG cotyledons in Col-0 and flp-1, which were treated with 25µM oryzalin for 24 hours beginning from the fourth day after germination. Scale bar in (D)
represents 25µm for all figures. *proFAMA:GFP* expression indicates a likely guard cell fate (it can also mark the GMC). *proFAMA:GFP* is expressed in all normally-shaped stomata, in guard cells in a *flp-1* cluster (B, left), and in single guard cells (starred) of oryzalin treated *Col-0* and *flp-1*(*C* and *D*). The application of oryzalin treatment for 24 hours appears to affect developing stomata (e.g. GMCs) but not mature stomata.

DAPI staining was then used to estimate nuclear DNA content in the stomatal cell lineage in wild-type plants treated with oryzalin. These plants showed single guard cells that appeared to have a doubled DNA content (4C) (Fig 10 *A*), whereas guard cells in normal stomata were 2C. In *flp-1 myb88*, some guard cells in stomatal clusters displayed 2C DNA levels (Fig 10 *B*). This lack of an oryzalin effect might be due to the timing of the chemical treatment e.g. after the guard cells had differentiated. Some single guard cells as well as normally-shaped stomata showed ploidy levels higher than 4C (Fig 10 *C* and *D*). The relative DNA content was then compared between single guard cells in oryzalin-treated *Col-0*, *flp-1* and *flp-1 myb88* plants (Fig 10 *E*). In *Col-0*, the relative DNA content in single guard cells was approximately doubled compared to paired guard cells, results which are similar to those found in the dominant negative mutant (*pro35S:CDKB1;1 N161*) (Boudolf et al., 2004a). Presumably the DNA was replicated in these single guard cells that were blocked at the G2-to-M transition by oryzalin. In contrast, the relative nuclear DNA content in *flp-1* and *flp-1 myb88* single guard cells was significantly higher than those in *Col-0*, with the value for *flp-1 myb88* being the highest. Oryzalin application induced the strongest endoreplication phenotype in the more severe *flp-1 myb88* background, whereas a lower endoreduplication phenotype was detected in the less severe *flp-1* background. Thus it is likely that *flp* mutants provide a
sensitized background, and when both FLP and MYB88 functions are lost, extra rounds of S-phase occur without cell or nuclear division.

Together these data suggest that this endoreduplication phenotype results from a division block, induced by the loss of the mitosis promoting functions of CDKB1;1 and CDKB1;2 or by a cytoskeletal failure of nuclear and cell division. These results show again that stomatal endoreduplication only occurs when FLP and MYB88 functions are compromised and thus provide a sensitized background allowing endoreduplication.

Therefore oryzalin treatment induces a novel effect in dividing and sensitized cells. In GMCs in wild type plants, oryzalin arrests the cell cycle at G2/M. However, in flp mutants, oryzalin switches the mitotic cycling of GMCs into an endocycling mode.

![Figure 10](image)

**Figure 10** Oryzalin treatment induces endoreplication in guard cells of flp mutants

(A) Paired 2C guard cells (left) and a single 4C guard cell (right) in Col-0 in plants treated with oryzalin.

(B) Stomatal cluster of 2C guard cells in flp-1 myb88, which probably formed before oryzalin was added,
suggesting that oryzalin only acts when the cells are still cycling. (C) Likely endoreduplicated guard cells with pore either not yet developed or arrested in flp-1 myb88. (D) Likely endoreplicated single guard cell in flp-1 myb88. (B) to (D) display different stomatal types from the same cotyledon, it is likely the DAPI fluorescence levels, and thus the DNA content, can be directly compared. Scale bar in (D) represents 25µm for all DAPI pictures. (E) Relative DNA content in guard cells measured from total grey value of DAPI stained nuclei. Single guard cells have approximately double the DNA content compared to normal guard cells in Col-0. However, many oryzalin-treated single guard cells in flp-1 and flp-1 myb88 show a higher DNA content than those in Col-0. Bars indicate standard error, p-value <0.001. Sample sizes: (1) wild-type: 147 normal guard cells and 63 single guard cells. (2) flp-1: 59 single guard cells. (3) flp-1 myb88: 48 single guard cells. Six cotyledons were scored for each genotype.

3.5. CDC6 is likely up-regulated in flp mutant

In the flp-1 myb88 mutant, repeated symmetric divisions take place in each new pair of daughter cells thus forming a stomatal cluster. But when mitosis is blocked genetically or by oryzalin treatment, endoreplication takes place instead of repeated symmetric divisions, indicating that DNA replication is repeated persistently licensed.

Because both a genetic background and chemical treatment appear to switch GMCs from excess mitotic cycling to endocycling (repeated DNA replication), the potential relationship between FLP/MYB88 and core G1-to-S cell cycle genes was examined.

FLP and MYB88 have been shown to directly target several G1-to-S cell cycle genes including CELL DIVISION CYCLE6a and 6b (CDC6a At2g29680 and CDC6b At1g07270) (Xie et al., 2010), which play central roles in DNA replication licensing. CDC6 activity normally oscillates in the cell cycle and peaks from late M phase to early G1 phase when it licenses the DNA replication, an event which is highly regulated at both the transcriptional and post translational levels. Constitutively expressed CDC6 has
been shown to induce endoreduplication in yeast (Blow and Dutta, 2005), and the expression level and protein stability of CDC6a are also associated with endoreduplication in *Arabidopsis* (Castellano et al., 2001).

To investigate whether CDC6a expression is altered in *flp* mutants, a transcriptional *proCDC6a: GFP* reporter was constructed and then stably transformed into *Col-0* and *flp-1* backgrounds. In both genotypes, *CDC6a* is expressed in the stomatal cell lineage and in pavement cells (Fig 11). Both developing stomata (which normally undergo mitotic cycling) and pavement cells (which normally undergo endocycling) probably have DNA replication in common and require replication licensing.

**Figure 11** CDC6 expression pattern in cotyledon epidermis

*proCDC6a: GFP* is expressed in the stomata cell lineage in *Col-0* wild type and *flp-1* mutant plants. Scale bar represents 25µm.

In the stomatal lineage of wild-type plants, *CDC6a* is expressed from the meristemoid stage to young guard cells (Fig 12 A to D), and expression appears to peak at the late guard mother cell stage (Fig 12 B). No expression was detected in mature
guard cells (Fig 12 E), which might reflect a cell cycle exit in these highly differentiated cells. The expression of \textit{CDC6a} in \textit{flp-1} was similar in pattern to the wild-type except that expression was also found in young guard cells in developing clusters (Fig 12 F to I). As in the wild-type, CDC6a expression was no longer detectible in mature stomatal clusters in \textit{flp-1} (Fig 12 I). Thus CDC6 transcription likely decreases after GMC division in wild-type plants, which is consistent with the observation that guard cells appear to exit from cell cycle. However in the \textit{flp-1} mutant, the duration of \textit{CDC6a} transcription is extended into young stomatal clusters, when additional DNA replication licensing occurs and is coupled with one or more extra mitotic cycles.

![Figure 12](image)

**Figure 12** \textit{proCDC6a: GFP} expression during stomata development

(A) to (E) show \textit{proCDC6a: GFP} expression in different stages of stomatal development in \textit{Col-0}. (A) Meristemoid. (B) Guard mother cell. (C) Young guard cell (newly divided GMC). (D) Developing guard cell (start of stomatal pore formation). (E) Mature stoma. (F) to (J) show \textit{proCDC6a: GFP} expression in different stages of stomatal development in \textit{flp-1}. (F) Meristemoid. (G) Newly divided GMC, which might either differentiate directly into a mature guard cell or undergo a second division to produce a stomatal cluster. (H) Developing stomatal cluster, in which only one daughter has undergone a second round of symmetric division. (I) Young stomatal cluster. (J) Mature stomatal cluster. Scale bar in (J) represents 25\(\mu\)m for all pictures.
Besides the target genes previously verified by ChIP, it is possible that FLP might regulate other core G1-to-S genes such as ORC and MCM. E2F, the major transcriptional regulator of the cell cycle, binds to the same or overlapping cis-regulatory elements as FLP in the \textit{CDKB1;1} promoter (Xie et al., 2010). In addition to being a FLP target, \textit{CDC6a} might also be targeted by E2F, since the \textit{CDC6a} promoter has the same TTTCCCGC E2F binding site as \textit{proCDKB1;1}. \textit{Drosophila} has a MYB, dMYB, which along with E2F, DP, and several other interacting proteins, assembles to form a transcriptional complex. This complex likely serves the dual functions of activation and repression of transcription, with the presence or absence of other factors determining the role of the complex (Beall et al., 2002; Lewis et al., 2004). We doubt that FLP may interact with E2F thus taking part in regulating series E2F targets during the stomata development. In \textit{Arabidopsis}, E2F targets that are involved in G1-to-S phases include \textit{CDC6a}, \textit{CDT1a(At2g31270)}, \textit{ORC1a(At4G14700)}, \textit{ORC1b(At4g12620)}, \textit{ORC2(At2g37560)}, \textit{ORC3(At5g16690)}, \textit{ORC4(at2g01120)}, \textit{ORC6(At1g26840)} and \textit{MCM3 (At5g46280)} (de Jager et al., 2001; Stevens et al., 2002; Castellano Mdel et al., 2004; Diaz-Trivino et al., 2005). All these promoters contain the TTTSSSSSS (S being C or G) consensus E2F binding site.

To explore whether FLP function affects the expression levels of G1-to-S genes, quantitative Real Time PCR was used to compare the levels of \textit{CDKA;1, CDC6a, CDC6b, ORC2, ORC3} and \textit{MCM2} genes in developing seedlings of Col-0 and \textit{flp} mutants. However after three repeats, no significant differences were found in expression level in developing shoot between \textit{Col-0} and \textit{flp-1} or \textit{flp-1 myb88} (data not shown). This result might be due to the low expression level of \textit{FLP} which is also highly cell-type specific.
In addition to developing stomata, \textit{CDC6a} and other G1-to-S genes are also expressed in developing pavement cells, an expression that might swamp any possible differences using qPCR between \textit{flp} mutants and wild-type plants in stomata development.
4. Discussion

The loss of FLP and MYB88 function induces excessive divisions at the end of the stomatal cell lineage, indicating that both MYBs play an essential role in restricting symmetric divisions to form functional and normally patterned stomata (Lai et al. 2005). Here, we show that these two MYB proteins are also required to restrict endoreduplication when the G2-to-M progression is inhibited, either by knocking out Mitosis Induce Factor (MIF) CDKB1 or the chemical disruption of mitosis spindle. In these backgrounds, a concomitant loss in FLP and MYB88 function can result in repeated DNA replication, cell enlargement, and enhanced chloroplast biogenesis in cells that retain a guard cell identity.

![Figure 13](image)

**Figure 13** Scenario for development of larger endoreduplicated stomata
4.1. *Arabidopsis* guard cell size and chloroplast number correlate with ploidy

The majority of cells in the *Arabidopsis* leaf epidermis undergo endoreduplication including most trichomes and pavement cells. In endoreduplicating cells, chromosomal DNA is repeatedly replicated without an intervening mitosis, a process that is usually coupled with an increase in cell size. Final cell area is related to, but not directly proportional to the level of endoreplication or ploidy.

*Arabidopsis* Col-0 plants are diploid with 2C guard cells that vary relatively little in cell size and in chloroplast number (Melaragno et al., 1993; Pyke and Leech, 1994). This might be adaptive since a smaller size might allow more rapid changes in turgor and hence in stomatal pore size.

Here we found that guard cells uncharacteristically undergo endoreduplication in a specific background with the loss of function of four genes (*flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant) as well as in oryzalin treated *flp* mutants. Compared to normal guard cells, endoreduplicated guard cells are larger and contain more chloroplasts. Although stomatal endoreduplication does not appear to occur in the wild type *Arabidopsis* normally, mature guard cell size and chloroplast number also increase as the ploidy level elevates. Thus, the coordination between cell size and ploidy level exists in all *Arabidopsis* epidermal cell types. And identical to that in polyploid plants, stomatal chloroplast number also coordinates with ploidy level in diploid plants.

4.2. *flp* mutants provide a sensitized background for stomatal endoreduplication

The formation of stomata depends upon a single, equal division of the GMC precursor cell, a division that is positively and jointly regulated by the *CDKB1;1* and
CDKB1;2 genes (Xie et al., 2010). MYB124 (FLP) as well as MYB88 activity are required to repress excess mitotic cycles in the newly formed daughter cells of GMC divisions (young guard cells). We show here that when both FLP and CDKB1 activities are lost, many GMCs stop mitotic cycling. Instead, endoreduplication occurs resulting in larger unpaired guard cells with higher ploidy levels. Thus both CDKB1 genes are required for mitotic cycling in GMCs of the flp mutants.

This result is consistent with the hypothesis that the activity of a Mitosis-Induce-Factor (MIF) regulates the switch between mitotic cycling and endocycling in plants (De Veylder et al., 2002). Altering the activities of some G1-to-S cell cycle related genes might cause cell type specific effects depending on the presence of MIF. In Arabidopsis, the co-overexpression of E2Fa-DPa (pro35S:E2Fa × pro35S:DPa) induces ectopic division in cells that have the potential to divide, and that probably contain an MIF. By contrast, in cells that lack an MIF, S phase re-entry occurs which bypasses mitosis thus causing endoreduplication. An imbalance in the expression of cell cycle genes can induce similar cell type specific effects in animal cells. For example, the co-overexpression of a cycling (CycD) and a cyclin dependent kinase (Cdk4) in Drosophila causes dividing cells to undergo extra mitotic cycling, and also induces endoreduplicating cells to undergo further DNA replication (Datar et al., 2000).

It is hypothesized that CYCLIN DEPENDENT KINASE B1;1 is part of the MIF in Arabidopsis. When the key division regulatory proteins, E2Fa-DPa, are constitutively expressed in Arabidopsis, the loss of CDKB1 activity rescues the E2Fa-DPa induced ectopic cell division phenotype while enhancing the concomitant endoreduplication phenotype (Boudolf et al., 2004b).
As discussed, *FLP* targets, regulates, and limits the expression of the *CDKB1;1* gene. In *flp* mutants, when *CDKB1;1* expression is de-repressed, there is an excess of mitotic division resulting in stomatal clusters. However, the expression of both the *CDKB1;1* and *CDKB1;2* genes is required for promoting the symmetric (and terminal) division in the stomatal pathway (divisions are normal in either single mutant). This suggests that *CDKB1;1* and *CDKB1;2* together (referred as *CDKB1*) act as a Mitosis Induce Factor in the stomatal cell lineage (Figure 14). *flp* mutants only undergo additional mitotic cycles in GMCs when both *CDKB1;1* and *CDKB1;2* are active (Xie et al., 2010).

![Figure 14](image)

**Figure 14** Loss of *CDKB1* activity switches GMCs from mitotic cycling to endocycling when FLP and MYB88 functions are lost.

The inhibition of mitosis by oryzalin, as well as the genetic loss of *CDKB1* function, switches mitotic cycling to endocycling in GMCs of *flp* mutants. However, this switch to endocycling is in some ways unexpected since oryzalin treatment might just arrest the mitotic cycle at G2/M. This is because cells usually pass through the G1/S and G2/M checkpoints sequentially in the mitotic cycle, in addition the progression to S phase
usually depends upon the completion of previous M phase (Hartwell and Weinert, 1989). Thus endoredupliated guard cells appear to be a novel effect of oryzalin treatment that differs from previous reports of microtubule disruption (H. A. Verhoeven, et al., 1990; Shaul et al., 1996; Planchais et al., 1997). Therefore, mitotic cycling GMCs in flp mutants differ from normal mitotic cycling cells. Presumably it is both the loss of FLP function combined with oryzalin-blocked mitosis which results in stomatal endoreduplication.

4.3. The stomatal mitotic to endocycle switch might require the coordinated regulations of DNA replication and mitosis

The mitotic cycle and endocycle likely share similar cell cycle related components and have DNA replication in common. Indeed the endocycle has also been described as a modified mode of mitotic cycling (Inze and De Veylder, 2006).

The Notch-Delta signaling pathway regulates a natural transition from mitotic cycling to endocycling in follicle cells in Drosophila. This transition involves the Notch-Delta regulations of series of cell cycle related genes that act in both G1-to-S and G2-to-M phases. During this transition, Notch-Delta appear to repress String which blocks M phase, activate Fzr to allow G1 progression and represses Dacapo to allow entry into S phase. Similarly, the chemical mitosis inhibition (spindle disruption) induces a switch from mitotic cycling to endocycling in animal cells which lacks p53, pRb, and the CDK inhibitor p16, p21 (Cross et al., 1995; Minn et al., 1996; Harrington et al., 1998; Lanni and Jacks, 1998; Stewart et al., 1999). In these cell types, the phosphorylated form of pRb relieves E2F repression and subsequently activates series of G1-to-S genes which are
essential for S phase entry. Thus when mitosis is chemically inhibited, formerly dividing cells are capable of re-entering S phase and switch to an endoreduplication program. Together these findings suggests that mitotic cycle to endocycle switch (natural switch in *Drosophila* follicle cells or artificial switch in cells with disturbed E2F/DP pathway) requires coordinated modifications to DNA replication and mitosis in the mitotic cycle in animals. According to the MIF theory in *Arabidopsis*, this switch could be controlled by a simple type of factor such as CDKB1. Besides prompting mitosis, it’s unclear whether CDKB1 can influence DNA replication. However, based on our results, CDKB1 only acts in G2-to-M transition in the stomatal cell lineage. Considering the major findings that support MIF theory are from transgenic lines with over expressed E2F/DP (De Veylder et al., 2002; Boudolf et al., 2004b), it’s possible that the MIF effect on mitotic cycle to endocycle switch depends on the E2F/DP overexpression background, in which core G1-to-S genes are up-regulated so DNA replication is also modified.

A comparable modification to mitotic cycle may also exist in plant endoreduplication onset. In plants, the degree of endoreduplication has been shown to increase either when DNA replication is stimulated or when the activities of some G2-to-M CYCLIN DEPENDENT KINASEs are down-regulated (Inze and De Veylder, 2006). However, to our knowledge, the molecular mechanism that controls the switch from mitotic cycling to endocycling has not been clearly shown in any plant cell types.

In addition to targeting *CDKB1;1*, a G2-to-M gene, FLP targets *CDC6a* which is also E2F/DP targets that involve in G1-to-S phase. Since FLP normally restricts cell divisions and represses *CDKB1;1* expression, FLP might behave as a transcriptional repressor of these G1-to-S genes as well. Although CDC6a protein activity levels have
not been determined, the window of \( CDC6a \) transcription is extended in \( flp-1 \) mutant (Fig 11). Clearly based upon phenotype, excess divisions in \( flp \) require the extended of many cell cycle related genes in both G1-to-S and G2-to-M phases. Indeed, FLP and MYB88 appear to be developmental regulators that normally coordinate the timing of the expression of core cell cycle genes with the onset of stomatal differentiation. One scenario to explain the mutant phenotype is that the loss of FLP function (1) promotes \( CDC6a \) and \( CDC6b \) expression which in turn licenses DNA replication, (2) activates \( CDKA1;1 \) to allow DNA replication take place, and (3) promotes \( CDKA;1 \) and \( CDKB1;1 \) expression which leads to mitosis. The excess symmetric divisions in \( flp \) might be promoted by the absence of repression to direct FLP target genes. Perhaps the deregulation of these target genes dampens normal oscillations in gene activity and allows these genes to continue to function throughout the entire cell cycle. With respect to oryzalin treatment, which chemically blocks mitosis, DNA replication in \( flp \) mutants still occurs, especially if signals that normally block continued replication, such as those that depend upon the completion of mitosis are absent.

As discussed, FAMA is a bHLH protein that blocks excess symmetric divisions like FLP, but unlike FLP, FAMA is also required for a guard cell fate. The application of oryzalin to \( fama \) mutants reduced the number of extra divisions as would be expected since microtubules are required for division. However, oryzalin treated \( fama \) plants failed to display large guard cells suggesting that the loss of FLP and MYB88 function provides a more sensitized background than \( fama \). This might result if FAMA regulates a different subset of cell cycle genes than FLP, e.g. FAMA does not repress as many G1-to-S genes or that \( fama \) allows continued oscillations in the activities of cell cycle genes.
Since FLP, MYB88, as well as FAMA and other bHLHs, can be required to restrict excessive GMC divisions, the matrix of cell cycle gene regulation is likely to be coordinated overall, but complex in specific gene classes.

Together these data suggest that the loss of FLP function regulates DNA replication and mitosis at least somewhat independently. In flp mutants, DNA replication and mitosis still occur, but it is not known whether these phases become less coordinated or whether the duration of cell cycle phases are abnormal in flp. When mitosis is inhibited genetically or chemically, DNA replication still occurs thus switching mitotic cycling to endocycling. The onset of stomatal endoreduplication might require the coordinated stimulation of DNA replication and the repression of mitosis.

4.4. Conclusion

This work reveals a novel endoreduplicated stomata phenotype in diploid Arabidopsis. Guard cell size and chloroplast number are increased by endoreduplication, identical to that reported in polyploidy plants.

The loss activity of CDKB1, a potential Mitosis Induce Factor (MIF) switches GMCs from mitotic cycling to endocycling when combined with the loss of FLP function. The chemical inhibition of mitosis by oryzalin in flp mutants roughly mimicks the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant phenotype. Oryzalin treatment displays a novel effect by inducing stomatal endoreduplication instead of arresting mitotic cycle at G2/M, suggesting that these mitotic cycling GMCs in flp mutants are somehow different from the normal mode, in which replication initiation depends upon the completion of a previous mitosis. This defect is likely to involve the direct up-regulation of core G1-to-S
genes by relieving transcriptional repression by FLP. In flp mutants these genes might lose their normal oscillatory in activity and resulting in additional DNA replications. Thus the onset of stomatal endoreduplication might involve a stimulation of DNA replication induced by the loss of FLP function, as well as repression of mitosis by either loss of CDKB1 function or chemical spindle disruption.
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