FOUR LIPS and MYB88 conditionally restrict the G1/S transition during stomatal formation

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Abstract

Consistent with their valve-like function in shoot–atmosphere gas exchange, guard cells are smaller than other epidermal cells and usually harbour 2C DNA levels in diploid plants. The paralogous Arabidopsis R2R3 MYB transcription factors, FOUR LIPS and MYB88, ensure that stomata contain just two guard cells by restricting mitosis. The loss of both FLP and MYB88 function in flp myb88 double mutants induces repeated mitotic divisions that lead to the formation of clusters of stomata in direct contact. By contrast, CYCLIN DEPENDENT KINASE B1 function is required for the symmetric division that precedes stomatal maturation. It was found that blocking mitosis by chemically disrupting microtubules or by the combined loss of FLP/MYB88 and CDKB1 function, causes single (undivided) guard cells (sGCs) to enlarge and attain mean DNA levels of up to 10C. The loss of both FLP and CDKB1 function also dramatically increased plastid number, led to the formation of multiple nuclei in GCs, altered GC and stomatal shape, and disrupted the fate of lineage-specific stem cells. Thus, in addition to respectively restricting and promoting symmetric divisions, FLP and CDKB1 together also conditionally restrict the G1/S transition and chloroplast and nuclear number, and normally maintain fate and developmental progression throughout the stomatal cell lineage.

Key words: Endoreplication, FOUR LIPS, microtubules, mitosis, S-phase.

Introduction

Cell differentiation is often associated with defined DNA levels that contribute to the function of specialized cell types (Nagl, 1976; Lee et al., 2009). While many plant cells contain 2C DNA levels throughout their lifespan, others undergo varying degrees of endoreplication meaning DNA replication without mitosis (Lee et al., 2009). Examples of cells and tissues that endoreplicate during development include trichomes, endosperm, epidermal pavement cells in leaves and in the hypocotyl, and suspension cultures (Sugimoto-Shirasu and Roberts, 2003; Lee et al., 2009).

Endoreplication occurs either through endocycling or endomitosis. In endocycling, G and S phases occur but not mitotic entry. The latter does take place in endomitosis, and chromosomes start to condense. However, the chromosomes fail to separate and, instead, the cells revert to a phase resembling G1 (Lee et al., 2009). In addition, endomitosis can lead to nuclear division without cytokinesis, resulting in the formation of several nuclei in one cell (Meserve and Duronio, 2012; Pandit et al., 2012).

DNA levels in endoreplicated cells correlate strongly with cell size (Melaragno et al., 1993; Kondorosi et al., 2000; Sugimoto-Shirasu and Roberts, 2003). Moreover, endoreplication can be essential for cell fate acquisition and maintenance as well as for patterning and distributing specialized types of cells (Bramsiepe et al., 2010; Roeder et al., 2010).
Stomata consist of two guard cells (GCs) around a pore, a configuration central to the regulation of shoot–atmosphere gas exchange. Arabidopsis stomata develop from asymmetric divisions of lineage-specific stem cells with the smaller daughter cell, the merist oid, later developing into a Guard Mother Cell (GMC) precursor (Bergmann and Sack, 2007). The GMC then divides just once which ensures that mature stomata each consist of just two GCs. Diploid Arabidopsis accessions contain GCs that also harbour 2C DNA levels, but adjacent pavement cells in leaves often endoreplicate and reach 16C to 32C DNA levels (Galbraith et al., 1991; Melaragno et al., 1993; see Supplementary Fig. S1E at JXB online).

During Arabidopsis leaf development, a complex containing CDKB1;1 and CYCLIN A2;3 (CYCA2;3) promotes division and restricts endoreplication in many cells (Boudolf et al., 2004b, 2009; Imai et al., 2006). However, excessive endoreduplication takes place in whole organs when cyclin-dependent kinase (CDK) activity is inhibited (De Veylder et al., 2011). During stomatal development, several CDKB1 and CYCA2 genes promote the symmetric division of the GMC precursor, and thus are critical for constructing the mature stomatal valve. The loss-of-function of both CDKB1;1 and CDKB1;2 in double mutants or in a dominant negative form of CDKB1 (pro35S:CDKB1;1 N161) blocks GMC division and induces the formation of abnormal single guard cells (Boudolf et al., 2004a; Xie et al., 2010). Single guard cells (sGCs) also arise when the function of three CYCA2 genes is compromised (cyca2;2, cyca2;3, and cyca2;4) (Vanneste et al., 2011; see Supplementary Fig. S1A-D at JXB online).

The transcription of the CDKB1;1 and CYCA2;3 genes during stomatal development is regulated in part by the FOUR LIPS (FLP) gene which encodes an R2R3 MYB protein (Xie et al., 2010; Vanneste et al., 2011). Loss-of-function mutations in FLP induce ectopic and extra symmetric divisions that produce clusters of GCs and stomata in direct contact (see Supplementary Fig. S1A-D at JXB online). The MYB88 gene, which is an FLP paralogue, shows no loss-of-function phenotype on its own, but acts synergistically in a flp myb88 double mutant by increasing symmetric divisions and stomatal cluster size (Lai et al., 2005). In a f lp myb88 cdkb1;1 cdkb1;2 quadruple mutant, the cdkb1;1 cdkb1;2 phenotype is epistatic to that of f lp-1 myb88 resulting in many sGCs that are oval-shaped in face view and that lack a dividing wall (Xie et al., 2010).

It is shown here that guard cells exhibit high levels of endoreduplication when the loss of FLP and MYB88 function in restraining Guard Mother Cell division is combined with blocked mitosis. Therefore, these MYB proteins can limit S-phase entry as well as mitosis. Moreover, the loss of these combined functions leads to the fate disruption of several types of epidermal cells and induces the abnormal expression of a stomatal lineage stem cell gene.

Materials and methods

Plant materials

All the Arabidopsis lines used were in the Columbia (Col-0) ecotype including the f lp-1 myb88 and cdkb1;1 cdkb1;2 double mutants, and the f lp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant (Lai et al., 2005; Xie et al., 2010). Plants were grown on sterile agar or soil (Promix, Premier Brands) at 22 °C with a 16/8 h light/dark cycle.

Flow cytometry

First leaves from plants grown for 21 d after germination were harvested from seedlings grown on half-strength MS medium plates.
Seeds were sown simultaneously. Nuclei were isolated according to Suda and Travnick (2006) with only slight modifications. About 20 mg of cotyledon tissue was minced with a razor in 600 µl OttoI solution (0.1 M citric acid monohydrate, 0.5% v/v TWEEN 20, 0.1% v/v β-mercaptoethanol). The solution was filtered with Miracloth and a 22 µm micro porous membrane to separate nuclei from cell and tissue debris. The supernatant was centrifuged at 3000 rpm for 3 min to spin down nuclei and the pellet was re-suspended in 150 µl OttoI solution. Then 600 µl of OttoII solution (0.4 M sodium phosphate dibasic dodecahydrate, 0.05 mg ml⁻¹ propidium iodide, 1 mg ml⁻¹ RNase) was added to the re-suspended nuclei. Suspensions were analysed using a FACScan flow cytometer (Becton Dickinson). The experiments were repeated independently two or three times with comparable results. The output graph was processed using flowJo software.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from 15–18-d-old Col-0, flp-1 myb88, cdkb1;1 cdkb1;2, and flp-1 myb88 cdkb1;1 cdkb1;2 cotyledons to quantify the expression of cell cycle-related genes using a Plant RNAeasy Kit (Qiagen). CDNA was synthesized from 1 µg of DNase-treated total RNA using an oligo (dT)₁₀ primer and superscript III reverse transcriptase enzyme (Invitrogen, Carlsbad, CA, USA). One microlitre of RT product was used for quantitative real-time PCR that used an iQ SYBR Green Supermix (Bio-Rad), and then was analysed using an iQ5 real-time PCR machine (Bio-Rad). The Arabidopsis ACTIN2 gene was used to approximate relative levels of constitutive and normalized control expression. Relative changes in gene expression were quantified using the 2⁻ΔΔCT method (Livak and Schmittgen, 2001) with data obtained from at least two biological replicates. Gene-specific primers are shown in Supplementary Table S1 at JXB online.

Oryzalin treatment

Oryzalin (Sigma) was dissolved in dimethylsulphoxide (DMSO) at 25 mM (stock solution), stored at −20 °C, and diluted with sterilized water to 25 µM. Cotyledons from 3-d-old seedlings were submerged in this oryzalin solution for 24 h and then removed 1 d later. Plants were then rinsed three times with sterilized water and then cultivated in a high-nutrient solution for 3–5 d. DMSO was used as a control. These experiments were repeated independently three times with similar results.

Results

Chemical disruption of microtubules induces GC endoreplication in a myb double mutant

To probe the role of microtubule (MT) function in stomatal development, MT depolymerizing drugs were applied to developing Arabidopsis cotyledons and leaves. Oryzalin is a dinitroaniline herbicide that depolymerizes microtubules, a disruption that can arrest division in the M-phase (Morejohn et al., 1987).

While many stomata appear unaffected by oryzalin treatment, a fraction formed undivided, round-to-oval-shaped single guard cells (sGCs). Thus, GMC precursor cells exposed to oryzalin eventually acquire a mature stomatal fate, as shown by the expression of the guard cell fate markers E1728 and proFAMA:GFP (Fig. 1C, D, F). This sGC phenotype closely resembles that of the cdkb1;1 cdkb1;2 double mutants (Fig. 1B).

Since FLP and MYB88 restrict GMCs to a single mitotic cell cycle, the effects of oryzalin application were analysed in various flp and myb88 mutant backgrounds. Treatment with oryzalin of flp-1 and flp-7 (a stronger allele) single mutants and of flp-1 myb88 and flp-7 myb88 double mutant plants suppressed the excess division (cluster) phenotype and instead induced the formation of sGCs. Unlike the stomatal clusters found in mutant plants, these sGCs are correctly patterned.

Single GCs in various combinations of flp/myb88 alleles and mutants were about 50% larger compared with those in oryzalin-treated wild-type plants with respect to sGC area and diameter (Fig. 1F–I). Similarly, the largest sGCs (maximal diameter) were twice as large (72 versus 36 µm) in flp-1 myb88 compared with the wild type (Fig. 1H).

To analyse the relationship between GC size and nuclear DNA content, gray-scale values derived from DAPI (4′,6-diamidino-2-phenylindole) fluorescence were measured using wide-field microscopy. Although pavement cells in the Arabidopsis epidermis undergo varying degrees of endoreplication, stomata in the Col-0 ecotype harbour 2C levels of DNA (Melaragno et al., 1993). As expected, many single GCs induced by oryzalin in wild-type plants displayed roughly double the DNA content of normal GCs (Fig. 1J–N). This doubling is consistent with oryzalin blocking the G2-to-M transition but not S-phase progression. These 4C DNA levels after oryzalin treatment are comparable with those in sGCs induced by transformation with a CDKB1;1 N161 dominant negative construct (Boudolf et al., 2004a) (Fig. 1N).

In contrast to treatment of N161 plants, oryzalin dramatically increased nuclear DNA levels in sGCs in a various flp myb88 mutant combinations. Whereas the Col-0 wild-type exhibited mostly 4C levels, sGCs in a flp1 myb88 double mutant harboured a mean DNA content of 10C when treated with oryzalin (Fig. 1N). By contrast, oryzalin did not affect GC size in cdkb1;1 cdkb1;2 double mutants (see Supplementary Fig. S2 at JXB online). Thus, the substantive enlargement of drug-induced sGCs depends primarily upon the combined loss of FLP/MYB88 function.

Combined loss of FLP/CDKB1 function induces guard cell and nuclear enlargement

To explore the effects of the loss of both MYB and CDKB1 function on stomatal size and morphology, a flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant was used. In addition to exhibiting many relative large sGCs, this combined loss-of-function background also induced some stomata to enlarge considerably in mature leaves and cotyledons (Fig. 2). By contrast, guard cells in wild-type, flp-1, and in cdkb1;1 cdkb1;2 double mutant plants were much smaller than in the quadruple mutant (Fig. 2H).
Fig. 1. Chemical disruption of mitosis induces enlarged and highly endoreplicated single guard cells (sGCs) in flp myb88 mutants. (A) Wild-type stomata expressing the mature guard cell fate marker, E1728. (B) cdkb1;1 cdkb1;2 sGCs expressing E1728 (arrows). (C) Oryzalin-induced sGCs in wild-type also express E1728. (D–G) proFAMA:GFP expression which also marks GC fate. (D) Wild-type control (DMSO treatment). (E) flp-1 mutant background (DMSO). (F) Wild-type stomata treated with oryzalin for 24 h showing an undivided
These size differences extend to nuclei. Single GCs in a *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant harbour abnormally large nuclei with a mean DNA level of 6C (Fig. 3A–E). Because high GC ploidies were only detected in the *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant, endoreduplication is only likely to ensue when both sets of genes (*FLP/MYB88* and *CDKB1;1/CDKB1;2*) are non-functional. These data are consistent with genetically blocked mitosis (loss of *CDKB1* function) inducing cell-type specific endoreplication when excess mitotic divisions (loss of MYB function) are also precluded.

This increase in nuclear size in large GCs of the quadruple mutant was also visualized using histone H2B fused to fluorescent GFP, which marks nuclei (Fig. 3F–I). Surprisingly, fluorescence from this construct revealed the occasional presence of multiple (2–3) nuclei in large but normally shaped guard cells (Fig. 3H–K). This finding is consistent with the presence of endomitosis, as well as endocycling, during GC enlargement in *flp-1 myb88 cdkb1;1 cdkb1;2*.

**Reduced stomatal production in the quadruple mutant**

The total number of stomata or stomatal ‘units’, which here refers to normal stomata as well as sGCs, was significantly lower in the *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant than in the wild type (Fig. 4A). Stomatal number in *Arabidopsis* correlates closely with the extent of expression of the *TOO MANY MOUTHS (TMM)* gene. *TMM* encodes a receptor-like protein, and its expression specifically marks the stomatal stem cell compartment which includes meristemoids and their sister cells produced by divisions that are asymmetric in size and cell fate (Fig. 4B; Nadeau and Sack, 2002). Consistent with a decreased number of mature stomata formed in the quadruple mutant, the number of stem cells, as marked by *TMM* expression, was considerably reduced (Fig. 4A, B; see Supplementary Fig. S3 at *JXB* online). Thus the combined loss of *FLP/MYB88* and *CDKB1;1/CDKB1;2* function inhibits the number of stomatal lineage-specific stem cells that form.

**The loss of MYB/CDKB1 function also disrupts GC shape and fate**

Strikingly, guard cell shape and size were severely disrupted in this quadruple mutant (Fig. 2E–G). Many normally shaped guard cells later lost their shape and grew over the original edges of the GCs. No such shape changes were found in *flp myb88* or in *cdkb1;1 cdkb1;2* double mutants (Fig. 2A–C), or following oryzalin treatment (Fig. 1). These abnormally shaped and enlarged cells still maintained a guard cell fate as shown by their expression of stomatal fate markers (Fig. 2E). However, this distortion of guard cell morphology probably impairs stomatal function since overall bilateral symmetry is compromised, and pore apertures and gaseous diffusion pathways become contorted, restricted or blocked. Consistent with these defects, shoot growth slowed and seed production was reduced during ageing (see Supplementary Fig. S4 at *JXB* online). Root growth was also severely reduced in the quadruple mutant (see Supplementary Fig. S4 at *JXB* online).

Cell fate and gene expression were also at least partially altered in the *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant (Fig. 4). The majority (60.7 ± 7.08% SE, n=150) of smaller daughter cells produced after asymmetric division failed to express *TMM* in 9-d-old cotyledons (Fig. 4C–E), consistent with a loss in meristemoid cell fate. In addition, expression from a *proAMA:GFP* transcriptional fusion was absent from some mature single guard cells in the quadruple mutant (Fig. 4F–H), indicating that some sGCs lose their fate. These findings suggest that abnormal endoreplication is associated with a disruption in cell fate as well as a decrease in epidermal cell number.

**Increased GC size correlates with extensive chloroplast replication**

In addition to the larger size and abnormal shape of guard cells in the *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant,

sGC (arrow). (G) *flp-1* treated with oryzalin showing a sGC (arrow). (H) Mean GC diameter 6 d after treatment with 25 µM oryzalin for 24 h as a function of genotype. The diameters of normal GCs as well as abnormal sGCs were scored. Genotypes ranked from lowest to highest diameters (bars). (I) Mean GC area after oryzalin treatment. Three-day-old Col-0, *flp-1*, and *flp1 myb88* seedlings treated and scored as in (H). Genotypes ranked from lowest to highest values (bars). Data in (H) and (I) presented as box plots, in which the box encompasses data for the 25th to 75th percentiles. The horizontal line within each box is the median (50%). Error bars represent the 5th (lower bar) and the 95th (upper bar) percentiles. (A–G) Scale bars=25 µm. Stars (*) denote means significantly different from the wild type (*P*<0.005). (J–N) Cotyledons from Col-0, *flp-1*, and *flp1 myb88* plants were treated with oryzalin (or DMSO control) for 24 h after staining with 4′,6-diamidino-2-phenylindole (DAPI). Nuclear fluorescence was imaged using wide-field microscopy. Whereas sGCs that form after oryzalin treatment display mostly 4C levels in wild-type plants, in *flp1 myb88* this mean was 10C. (J–M) Micrographs showing examples of normal and enlarged guard cell nuclei. Stomatal outlines indicated by dashed lines. Arrows indicate nuclei in guard cells that are enlarged compared with other nuclei shown and that probably contain 4C DNA levels. (J–M) Bars=25 µm. (J) Stomata from wild-type (Col-0) plants treated with oryzalin. Left: probably 2C guard cells in a normal stoma. Right: a GC with a larger nucleus likely to be 4C. (K) Stomatal cluster in a *flp-1 myb88* cotyledon that was not treated with oryzalin containing 2C DNA levels in guard cells. (L, M) *flp-1 myb88* cotyledons treated with oryzalin. Arrows indicate enlarged nuclei. (L) Nuclei are enlarged in both GC-like cells. (M) Enlarged nucleus in sGC. Stomatal morphogenesis appears to be blocked or delayed. (N) Graph showing nuclear sizes in Col-0, *flp-1*, and *flp1 myb88* plants treated with oryzalin. Relative DNA levels quantified by the areas of nuclei/gray values derived from DAPI-fluorescence. Only single guard cells were sampled in *flp-1* and *flp1 myb88*. Sample sizes: Col-0: 145 paired GCs and 65 sGCs; *flp-1*: 59 sGCs; 48 *flp1 myb88* sGCs. Bars, means ±SE. Stars (*) denote means significantly different from the wild type (*P*<0.001). (This figure is available in colour at *JXB* online.)
Fig. 2. Combined loss of FLP/MYB88 and CDKB1;1/1;2 function increases stomatal size and disrupts GC and stomatal shape. (A) Wild-type (WT) stomata. (B) flp-1 myb88 stomatal clusters. (C) cdkb1;1 cdkb1;2 stomata. Single, oval-shaped GC shown at upper left. Lower right: kidney-shaped single GC. (D) flp-1 myb88 cdkb1;1 cdkb1;2 background showing enlarged GCs in an otherwise normal stoma (*) as well as an enlarged sGC upper right (red arrow). (E) proFAMA:GFP expression indicates stomatal identity in sGC (arrow, lower left) as well as in an abnormally shaped stoma (*) in flp-1 myb88 cdkb1;1 cdkb1;2. First leaf from a 15-d-old plant. Inset: normal stoma in a wild-type plant. (F) Abnormal guard cell enlargement, twisting (red arrows), and loss of kidney shape. From a 20-d-old flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant. (G) Four successive optical sections of the same abnormal stoma. Guard cells 1 and 2 are labelled in red. Star marks an abnormal and twisted stomatal pore. From a 20-d-old flp-1 myb88 cdkb1;1 cdkb1;2 cotyledon. (H) Quantification of guard cell areas measured from six, 21-d-old cotyledons. Sample sizes: Col-0 (225), flp-1 (225), cdkb1;1/1;2 (225 paired GCs and 164 sGCs), and flp-1 myb88 cdkb1;1 cdkb1;2 (225 paired GCs and 182 sGCs). Bars, means ±SE. Stars indicate values significantly different from the wild type (P <0.001). All figures in (A) to (E) are at same magnification and from 12-d-old first leaves. Cell walls in all figures (except F) were visualized with propidium iodide fluorescence. All scale bars=20 µm. (This figure is available in colour at JXB online.)
this genotype also displayed high numbers of chloroplasts in stomata (Fig. 5). *Arabidopsis* stomata normally contain about 10 chloroplasts per guard cell (Pyke and Leech, 1994). Comparable numbers were present in the *flp-1, flp-1 myb88*, and *cdkb1;1 cdkb1;2* mutant backgrounds (Fig. 5A–C, F). By contrast, mean chloroplast number in stomata with paired guard cells in the *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant was more than double that of the wild type.
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Fig. 4. Cell fate is disrupted in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant. (A) Numbers of stomata or sGCs (both referred to as 'stomatal units') in wild type, quadruple mutant, flp-1 myb88, and cdkb1;1 cdkb1;2. Bars, mean ±SE. Scored from six independent samples per genotype, 15-d-old cotyledons. (B) Number of cells expressing proTMM:TMM-GFP (TMM-GFP) scored from a 320 µm² region of the wild type (n=97) and the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant (n=234). Bars, means ±SE. Scored from 9-d-old cotyledons, six different samples per genotype. (C) TMM-GFP in the wild type showing normal expression in an asymmetric division (upper right). (D, E) Cells that are comparable in size to a meristemoid and that appear to have formed in an asymmetric division vary in whether they express TMM-GFP. Arrowheads, meristemoid-like cells that lack GFP signal. Other small cells in the same fields display fluorescence. However, few meristemoid-like cells are normal in shape. TMM-GFP in 9 and 13-d-old cotyledons in the quadruple mutant. Note the abnormal shape and enlargement of stoma at the lower left in (E). (F) Wild type showing proFAMA:GFP fluorescence in mature stomata from a 14-d-old cotyledon. (G, H) As in (F), except in a quadruple mutant. Arrows indicate large cells that likely developed from single guard cells but that mostly (except, for example, H lower right) do not express proFAMA:GFP. All scale bars=20 µm. (This figure is available in colour at JXB online.)

(25.4±3.37 SD) (Fig. 5D–F). Single GCs in the quadruple mutant also contained many chloroplasts (30.2±7.97 SD, n=25) (Fig. 5D). Thus abnormal guard cell endoreplication increases plastid number.

FLP and MYB88 do not affect leaf endoreduplication overall

The CDKB1;1 dominant negative mutant N161 (pro35S: CDKB1;1 N161) promotes endoreduplication in pavement
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... in other cells in the cotyledon (Boudolf et al., 2004b). Because the loss of FLP/MYB88 function promotes endoreduplication in the stomatal lineage in a sensitized background, it was probed whether various mutant combinations also affect the degree of endoreduplication at the level of the whole organ. Using nuclei isolated from first leaves harvested 21 d after germination, ploidy distributions were analysed by flow cytometry in control (Col-0) as well as in different mutant backgrounds, i.e. in the flp-1 myb88 and cdkb1;1 cdkb1;2 double mutants, and in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant. Ploidy levels and their distributions were mostly similar between all four genotypes (see Supplementary Fig. S5 at JXB online). However, genotypes lacking CDKB1 function (i.e. cdkb1 double mutants as well as the quadruple mutant), showed 32C peaks, albeit small, and also had 16C peaks (see Supplementary Fig. S5C, D at JXB online).

Although it is likely that endoreplicated nuclei from single guard cells were present in selected flow cytometry samples, presumably this fraction was small compared with the number of nuclei present throughout the leaf (including pavement cells which are highly endoreplicated). Thus FLP and MYB88 do not normally influence endoreduplication in whole leaves, a result consistent with the prominent expression of both MYBs only in the stomatal cell lineage in the cotyledon epidermis, as well as with the lack of any obvious increase in plastid number in other epidermal cells.

Variable expression of sets of G1-to-S phase genes in the quadruple mutant

Since the quadruple mutant blocks mitosis, the expression of Arabidopsis genes known to act in the G1-to-S phase and during endoreplication were analysed in this loss-of-function background. Many G1-to-S phase genes in the quadruple mutant showed no difference in expression levels compared with the wild type. These included CELL DIVISION CYCLE6 genes (CDC6s), CDC10 TARGET1 genes (CDT1s), CDKA;1, as well as the CCS52A2/FZR1 and CCS52A1/FZR2 genes which are required for endoreplication as activators of the Anaphase-Promoting Complex/Cyclosome (APC/C) (see Supplementary Fig. S6 at JXB online; Lammens et al., 2008; Larson-Rabin et al., 2009). However, another set of genes was much more highly expressed in the quadruple mutant compared with the wild type. This set included six G1-to-S phase entry genes: (i) MINI-CHROMOSOME MAINTENANCE 2 (MCM2), (ii) MCM5, (iii–v) ORIGIN RECOGNITION COMPLEX 1a (ORC1a), ORC2, ORC3, and (vi) PROLIFERATING CELL NUCLEAR ANTIGEN1 (PCNA1) (see Supplementary Fig. S6 at JXB online).

Fig. 5. Combined loss of FLP/MYB88 and CDKB1;1/1;2 function increases chloroplast number in GCs. (A–E) Comparison of chloroplast number in guard cells as a function of genotype. Propidium iodide staining, confocal microscopy. (A) Wild type. (B) flp-1 myb88 double mutant. (C) cdkb1;1 cdkb1;2 double mutant. (D) Single guard cell of the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant. (E) Abnormally-shaped stoma in the quadruple mutant. (F) Mean chloroplast number in paired guard cells of different genotypes (n=10 guard cells per genotype). Bars, means ±SD. Star shows value significantly different from the wild type (P<0.001). 20-d-old cotyledons. (A–E) Scale bars=20 μm.
Endoreplication initiation is also regulated by atypical E2F repressors, which are characterized by two DNA binding domains, a lack of binding to DIMERIZATION PARTNER (DP) proteins, and the absence of a RETINOBLASTOMA (RB) protein-binding domain (Lammens et al., 2009). Gene expression levels corresponding to a set of E2F related proteins that included typical E2Fs (E2Fa, E2Fb, E2Fc) as well as atypical E2Fs (E2Fδ/DEL2, E2Fε/DEL1, E2Fθ/DEL3) were therefore quantified in 15-d-old wild-type, flp-1 myb88, cdkb1;1 cdkb1;2 double mutants, and in the quadruple mutant cotyledons. While five of these E2Fs showed no significant differences in expression in the four genotypes, the atypical E2Fθ/DDEL3 was strongly expressed in the quadruple mutant (see Supplementary Fig. S7 at JXB online). Thus E2Fθ probably restricts endoreplication, at least in some cell types in the epidermis of wild-type cotyledons.

Discussion

**FLP conditionally limits guard cell size**

The relatively small size of stomata is likely to be adaptive in regulating shoot–atmosphere gas exchange by allowing rapid pore opening and closing. This facilitates rapid stomatal responses to environmental parameters such as local and transient fluctuations in light intensity.

Stomatal size directly correlates with ploidy levels of whole plants. For example, mean stomatal length in natural hexaploid and octoploid Coffea plants were more than 50% longer than in diploids (Mishra, 1997). Stomatal size also increases with genome dosage in diploid versus triploid and tetraploid plants were more than 50% longer (Mishra, 1997). Stomatal size also increases with genome dosage in diploid versus triploid and tetraploid

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**FLP restricts divisions and enforces patterning in Arabidopsis stomatal development by ensuring that only one symmetric division occurs before stomata form (Lai et al., 2005). It is shown here that the paralogous FLP and MYB88 transcription factors can also limit guard cell endoreplication, and thus size but only when the ability of GMCs to divide is blocked.**

Whereas oryzalin only arrests GMCs in G2/M in wild-type plants, in flp mutants, oryzalin switches GMCs from mitotic to endocycling. These data indicate that GC endoreplication results from the disruption of microtubule arrays that control both nuclear and cell division.

A similar phenotype arises when CDKB1;1 function is lost. Normally, in addition to promoting the symmetric division of guard mother cells, CDKB1;1 negatively regulates when endocycling starts (Boudolf et al., 2009). While endocycling is pervasive in many tissues and organs in cdkb1 mutants, it is notably absent from guard cells, even though CDKB1;1 is expressed late in the stomatal cell lineage (Boudolf et al., 2004b). Thus, the lack of endoreplication in cdkb1;1 cdkb1;2 guard cells, but its presence when FLP function is lost, shows that FLP can conditionally restrict stomatal endocycling and enlargement.

**Direct versus indirect regulation of endoreplication**

Mutations in mitotic CYCLINA2 (CYCA2) genes precociously induce and extend the duration of endocycling throughout the shoot (Imai et al., 2006; Vanneste et al., 2011). Endocycling also occurs in Arabidopsis cotyledons when the CDKB1;1-CYCA2;3 mitotic complex is inactivated by the APC/C complex (Boudolf et al., 2009). In addition to CDKB1, CYCA2 genes are also expressed late in stomatal development. CYCA2;3 can also promote the last mitosis in stomatal development and its loss of function also induces the formation of single guard cells, but not guard cell endoreplication (Boudolf et al., 2004a; Vanneste et al., 2011). Both the CDKB1;1 and CYCA2;3 genes are direct transcriptional targets of FLP/MYB88 (Xie et al., 2010; Vanneste et al., 2011). It remains to be seen whether the loss of CYCA2 function might also ectopically activate endoreplication, such as in a flp myb88 double mutant background.

Unlike FLP/MYB88, some transcription factors directly regulate endocycling. For example, the loss of function of GTL1, which encodes an Arabidopsis trihelix transcription factor, induces extra rounds of endocycling that prolong trichome growth (Breuer et al., 2009). In Drosophila, the ESCARGOT gene, which encodes a zinc finger transcription factor, induces diploid larval histoblasts to undergo endoreplication at the appropriate developmental stage (Hayashi, 1996). This comparison highlights the relatively indirect role that FLP plays in only conditionally restricting endoreplication.

**FLP and broader regulation of balance between mitosis and endocycling**

Although FLP directly targets the DNA replication licensing factor genes, CDC6a and CDC6b, no changes in their expression levels were detected in flp myb88, a result that might reflect the ubiquitous functions of CDC6 genes. However, the expression of other genes that regulate replication, such as PCNA1 and some ORCs and MCMs, was significantly up-regulated in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant, but not in either double mutant (flp-1 myb88 or cdkb1;1 cdkb1;2), profiles consistent with guard cell endoreplication only occurring in flp myb88 after a mitotic block.

Notably, FLP/MYB88 bind specifically to a cis-element present in the promoter regions of some potential target genes that overlaps with E2F. For example, FLP/MYB88 directly target and regulate the expression of G1-to-S phase factor, induces diploid larval histoblasts to undergo endoreplication at the appropriate developmental stage (Hayashi, 1996). This comparison highlights the relatively indirect role that FLP plays in only conditionally restricting endoreplication.

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development. However, it cannot be ruled out that high expression levels of some G1-to-S phase genes might only be an indirect effect with respect to FLP/MYB88. In addition, FLP/MYB88 might only act conditionally in limiting cell cycling when mitosis itself is restricted.

The *Drosophila* MYB protein, DmMYB, is a component of a multisubunit transcriptional repressive complex termed dREAM/MMB that includes the E2F and RETINOBLASTOMA proteins (Korenjak et al., 2004). The dREAM complex targets E2F promoter binding sites and represses the transcription of genes regulated by E2F (Georlette et al., 2007; van den Heuvel and Dyson, 2008). Thus, FLP/MYB88 and E2F might act in a similar complex in Arabidopsis or might regulate common cell cycle target genes. These relationships might also explain why transcription levels of S-phase entry genes increase in a *flp myb88 cdkb1;1 cdkb1;2* background even though many of these genes are direct targets of E2F but not FLP.

The high expression of E2Ff (an ‘atypical’ E2F) in the quadruple mutant is interesting since the mammalian atypical E2Fs, 7 and 8, promote endoreplication, whereas the Arabidopsis E2Fe inhibits endocycling (Lammens et al., 2008; Chen et al., 2012; Pandit et al., 2012). This raises the possibility that E2Ff might contribute to abnormally high levels of endoreplication when both FLP and CDKB1 functions are lost. To date, E2Ff has only been shown to function in restricting cell expansion in Arabidopsis by directly targeting cell wall biosynthesis genes, a role that might be consistent with E2Ff contributing to normal guard cell formation (Ramirez-Parra et al., 2004).

A well-studied endoreplication pathway involves the Anaphase-Promoting Complex/Cyclosome (APC/C). Constitutive expression of *CCS52A1/FZR2*, an APC/C activator, is known to result in abnormally large stomata, as does perturbation of APC/C activity (Larson-Rabin et al., 2009; Iwata et al., 2011; Komaki and Sugimoto, 2012). APC/C activity is required to initiate endoreduplication. APC/C activator genes, such as CDH1 (also referred to as FZR or CCS52A) promote endocycle onset and progression (Larson-Rabin et al., 2009). The initiation of endocycling can also be induced when mitotic cyclins are degraded (Shcherbata et al., 2004; Narbonne-Reveau et al., 2008; Boudolf et al., 2009; Eloy et al., 2012). The activation of APC/C from late G2 to early mitosis requires its interaction with CELL DIVISION CYCLE 20 (CDC20) (also known as FIZZY (FZY)). Later during anaphase to S phase, APC/C remains active by interacting with CELL CYCLE SWITCH 52 (CCS52) (also known as CDC20 HOMOLOG 1 (CDH1)/FIZZY-RELATED (FZR)) (Pesin and Orr-Weaver, 2008).

The APC/C activator CCS52A1 has been shown to regulate the stability of the CYCA2;3 protein which restricts leaf endoreplication (Boudolf et al., 2009). Since FLP/MYB88 also directly limit the expression of CYCA2;3 and CDKB1;1, their combined control is also likely to restrict endoreplication in the stomatal pathway (Boudolf et al., 2009; Xie et al., 2010; Vanneste et al., 2011).

CDKB1 promotes pavement cell formation but restricts their size

The combined loss of CDKB1 and FLP functions leads to smaller leaves and cotyledons as well as an increase in epidermal cell size, especially in non-stomatal epidermal cells, (NSECs), which collectively occupy the greatest share of leaf area. However, the loss of just CDKB1;1 and CDKB1;2 function, whether in a double mutant or in a CDKB1;1, N161 dominant negative construct, almost halves the number of pavement cells/NSECs (although the N161 construct does not affect the size of cotyledons, as opposed to leaves) (Boudolf et al., 2004b). By contrast, the size of leaves and cotyledons appears to be unaffected in *flp myb88*. Our results are consistent with previous findings that the loss of CDKB1 function is largely responsible for increased epidermal cell size. However, mechanisms underlying the relationship between increased pavement cell size and a reduction in the number of stomatal lineage stem cells formed in *cdkb1* mutants remains to be determined.

Endoreplication, cell shape, and cell fate

Stomatal endoreplication in a *flp myb88 cdkb1;1 cdkb1;2* quadruple mutant background is accompanied by abnormal changes in guard cell shape as well as size. Pavement cells appear to maintain a normal morphology consistent with the confinement of FLP/MYB88 expression to developing stomata in the leaf epidermis. However, many guard cells in this quadruple mutant expand out of the plane of the epidermis and grow and twist over each other resulting in the loss of stomatal bilateral symmetry.

Abnormal and excessive endoreplication has been shown to be associated with the formation of exceptionally large and irregularly shaped cells that harbour mixed guard cell and pavement cell identity but that bear little resemblance to stomata (Iwata et al., 2011). These cells arise when the function of the *OMISSION OF SECOND DIVISION 1* (*OSD1*) gene, also known as *GIGAS CELL1*, is also lost (Iwata et al., 2011). OSD1, which encodes a novel protein, as well as related genes, probably inhibit APC/C function. Thus while both the *osd1* and the *flp* quadruple mutant phenotypes involve endoreplication, the former induces a much more severe shape defect. In the *flp-1 myb88 cdkb1;1 cdkb1;2* quadruple mutant, cells that resemble mature single GCs later fail to express stomatal fate markers. In addition, many meristemoids, which are the smaller daughter cells of asymmetric division in the lineage and whose expression is normally marked by *TMM*, later lose *TMM* expression. In turn, this reduces the total number of stomata that form in the quadruple mutant (compared with the wild-type).

Cell fate disruption in the quadruple mutant is most prominent in mature cotyledons when endoreplication is more extensive. The close relationship between abnormal endoreplication, altered cell shape, and cell fate disruption, highlights the importance of co-ordinating DNA levels during normal cell morphogenesis and fate determination.
Guard cell chloroplast number and endoreplication

Whereas diploid Arabidopsis Col-0 plants have 2C guard cells that exhibit a relatively small range of sizes and number of chloroplasts (Melaragno et al., 1993; Pyke and Leech, 1994), endoreplicated guard cells in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant display more than twice as many chloroplasts compared with the wild-type or to flp-1 myb88 or cdkb1;1 cdkb1;2 double mutants. Thus, in addition to restricting GMC symmetric division, FLP and MYB88 also conditionally limit plastid replication.

While a relationship between cell size and plastid number has been questioned based upon comparisons of mesophyll and bundle sheath cells in different taxa (Olszewska et al., 1983), numerous data show that chloroplast number increases relative to nuclear DNA levels (Butterfass, 1973). This relationship has been especially well studied in guard cells in plants that have undergone polyploidization (Butterfass, 1973). Our results show that local and cell-type-specific endoreplication can also induce excessive but proportional chloroplast replication. The abundance of chloroplasts in guard cells in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant is likely to be a direct consequence of endoreplication since the combined loss of FLP and MYB88 function on its own does not increase plastid number. However, it cannot be ruled out that the enhanced number of chloroplasts in the quadruple mutant might result from unknown factors in addition to or instead of endoreplication.

Overall, this work has shown that two MYB transcription factors that enforce a single symmetric division essential for normal stomatal formation can also conditionally restrict endoreplication and chloroplast and nuclear number, as well normally maintain fate and developmental progression throughout the stomatal cell lineage.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Fig. S1. Stomatal development in the wild type and selected mutants as well as Histone 2B-YFP expression.

Supplementary Fig. S2. Oryzalin treatment does not appear to affect sGC diameter in cdkb1;1 cdkb1;2 double mutants.

Supplementary Fig. S3. Expression of TOO MANY MOUTHS during stomatal and cotyledon differentiation.

Supplementary Fig. S4. Seedling growth defects in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant compared with the wild-type as well as to the flp-1 myb88 and cdkb1;1 cdkb1;2 double mutants.

Supplementary Fig. S5. Flow cytometry profiles from PI-stained leaves.

Supplementary Fig. S6. Expression levels of cell cycle-related genes in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant as well as other mutants.

Supplementary Fig. S7. Expression levels of E2F genes in the flp-1 myb88 cdkb1;1 cdkb1;2 quadruple mutant as well as in other mutants.

Supplementary Table S1. Primers used.

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