Lineage- and stage-specific expressed CYCD7;1 coordinates the single symmetric division that creates stomatal guard cells

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ABSTRACT

Plants, with cells fixed in place by rigid walls, often utilize spatial and temporally distinct cell division programs to organize and maintain organs. This leads to the question of how developmental regulators interact with the cell cycle machinery to link cell division events with particular developmental trajectories. In Arabidopsis leaves, the development of stomata, two-celled epidermal valves that mediate plant-atmosphere gas exchange, relies on a series of oriented stem cell-like asymmetric divisions followed by a single symmetric division. The stomatal lineage is embedded in a tissue in which other cells transition from proliferation to postmitotic differentiation earlier, necessitating stomatal lineage-specific factors to prolong competence to divide. We show that the D-type cyclin, CYCD7;1, is specifically expressed just prior to the symmetric guard cell-forming division, and that it is limiting for this division. Further, we find that CYCD7;1 is capable of promoting divisions in multiple contexts, likely through RBR1-dependent promotion of the G1/S transition, but that CYCD7;1 is regulated at the transcriptional level by cell type-specific transcription factors that confine its expression to the appropriate developmental window.

KEY WORDS: Stomatal development, Cell cycle, Cyclin, Cell division, Differentiation, Guard cell

INTRODUCTION

Development of multicellular organisms requires the coordination and control of cell proliferation with differentiation programs to generate distinct cell types, tissues and organs. Different cell lineages are specified by sets of developmental regulators and display various cell proliferation dynamics, suggesting that the cell cycle machinery might not always be comprised of the same components or controlled in the same way. In Arabidopsis, the mature leaf epidermis contains pavement cells, trichomes and stomata, three different functional cell types with their own developmental trajectories. In the past decade, genetic analyses of these cell types have enabled the discovery of various connections between cell cycle and development. For example, trichome precursors are specified early and patterned via lateral inhibition networks (Schellmann et al., 2002), and their maturation requires a shift from mitotic to endoreplicative programs (Bransiepe et al., 2010). Pavement cells also endoreplicate as they acquire their lobed morphology (Katagiri et al., 2016). Stomata, pivotal for gas exchange between the plant and the environment, are derived from protodermal cells in a process that requires them to first become self-renewing and multipotent, but then to navigate an ordered set of divisions and differentiation programs to create the mature stoma (Matos and Bergmann, 2014). Key transcriptional regulators of the stomatal lineage – the stage-specific, basic-helix-loop-helix (bHLH) transcription factors SPEECHLESS (SPCH), MUTE and FAMA, and their broadly expressed heterodimer partners, SCR/MICE1 and SCR/M2 (Kanaoka et al., 2008) – each have roles in cell division and cell fate (Fig. 1A). SPCH drives asymmetric cell divisions that initiate the lineage, creating meristemoids (M) that can undergo continued self-renewing divisions. Plants lacking SPCH have no stomatal lineage. MUTE is essential to terminate the asymmetric self-renewing divisions and to induce the differentiation of meristemoids into guard mother cells (GMCs) (MacAlister et al., 2007; Pillitteri et al., 2007); loss of MUTE results in excess meristemoids at the expense of GMCs (MacAlister et al., 2007; Pillitteri and Torri, 2007). FAMA is required for the establishment of guard cells (GCs) but also to restrict GMCs to a single division. fama mutants exhibit numerous rounds of symmetric and parallel GMC divisions without acquisition of terminal GC identities (Matos et al., 2014; Ohashi-Ito and Bergmann, 2006). Plants bearing mutations in two R2R3 MYB transcription factor genes, FOUR LIPS (FLP) and MYB88, also exhibit fama-like GMC overproliferation phenotypes (Lai et al., 2005; Xie et al., 2010).

Presumably, among targets and partners of these transcription factors are cell cycle regulators that enable the diverse trajectories and division behaviors of epidermal cells. The components of the core cell cycle machinery are highly conserved among eukaryotes, although there has been a large expansion of genes in plants (Harashima et al., 2013; Inzé and De Veylder, 2006). The plant cell cycle is regulated by five main cyclin-dependent kinases (CDKs): CDKA;1, CDKB1;1, CDKB1;2, CDKB2;1 and CDKB2;2. CDKs require cyclins (CYCs) as binding partners for their kinase activity towards downstream phosphorylation targets. Plants genomes encode much larger families of cyclin genes than animals; for example, Arabidopsis encodes at least 32 cyclins (Vandepoele et al., 2002; Wang et al., 2004), and it has been speculated that this expansion allows plants to specifically regulate their postembryonic development (De Veylder et al., 2007; Harashima et al., 2013; Inzé and De Veylder, 2006). D-type cyclins as partners of CDKA;1 are crucial for the G1/S cell cycle transition and commitment to divide (Dewitte et al., 2007; Harbour and Dean, 2000; Riou-Khamlichi et al., 2000). Eight of 10 plant CYCDs have an RBR1-binding motif...
(LxCxE) (Kono et al., 2007; Menges et al., 2003). RBR1, the Arabidopsis homolog of the human tumor suppressor protein retinoblastoma, is crucial for the negative control of the cell cycle at G1/S transition (Desvoyes et al., 2006; Gutzat et al., 2012; Nowack et al., 2012; Uemukai et al., 2005; Zhao et al., 2012). Phosphorylation of RBR1 by CDKA;1/CYCD complexes inactivates its suppression of E2F transcription factors, allowing entry into S phase and commitment to divide (Fig. 1B) (Harashima et al., 2013; Nakagami et al., 2002; Nowack et al., 2012; Umen and Goodenough, 2001).

Here, we show how the cell cycle and cell fate transition from GMCs to GCs is regulated by the stomatal-lineage specific G1-S phase cell cycle regulator CYCD7;1. We demonstrate that CYCD7;1 activity is that of a typical D-type cyclin, but its expression window is narrowed by stomatal lineage specific transcription factors. By examining how CYCD7;1 works with the core cell cycle machinery and with stomatal regulators, and by revealing the phenotypes upon loss and gain of CYCD7;1 function, we link a core cell cycle regulator with a specific differentiation process and show how a formative division is initiated, but also
restricted, to allow ‘one and only one division’ in GMCs to create a physiologically functional valve structure from its two identical daughters.

**RESULTS**

**CYCD7;1 is expressed prior to the last symmetric division in the stomatal lineage**

Among the ten known D-type cyclins in *Arabidopsis*, CYCD7;1 was uniquely enriched in transcriptional profiles of fluorescence-activated cell sorting (FACS)-isolated cells of the late stomatal lineage (Adrian et al., 2015). We confirmed this predicted expression in GMCs with transcriptional and translational reporters (Fig. 1C-E) and observed that additional copies of *CYCD7;1-YFP* could force ectopic divisions in GCs, suggesting that the protein could play a role in regulating this division (Fig. 1C, white arrowhead). A translational reporter, *pCYCD7;1:CYCD7;1-YFP*, was characterized previously as peaking in GMCs (Adrian et al., 2015); however, the identity of CYCD7;1 expressing cells was only assessed by morphology. To refine the expression pattern, we co-expressed *pCYCD7;1:CYCD7;1-YFP* with CFP reporters for SPCH, MUTE and FAMA (Fig. 1F-N). SPCH-CFP and CYCD7;1-YFP expression appear to be mutually exclusive, suggesting that CYCD7;1 is not expressed in young meristemoids (Fig. 1F-H). MUTE-CFP, a marker of late meristemoids and GMCs, partially overlaps with CYCD7;1-YFP. Closer analysis of cell morphologies indicated that cells expressing MUTE, but not CYCD7;1, were meristemoids, never GMCs, suggesting that MUTE is expressed before CYCD7;1 (Fig. 1I-K). CYCD7;1-YFP is visible before FAMA-CFP in GMCs, and is expressed together with FAMA in newly divided GCs. FAMA, but not CYCD7;1, persists into maturing GCs (Fig. 1L-N) (Ohashi-Ito and Bergmann, 2006). Thus, the expression of CYCD7;1 in the stomatal lineage is temporally and spatially controlled. It commences after MUTE expression and is extinguished before the end of FAMA expression (Fig. 1A).

We did not observe expression of CYCD7;1-YFP in any vegetative tissue from the seedling stage through flowering (data not shown). In adult plants, CYCD7;1-YFP was expressed in pollen sperm cells at anthesis, but not in the vegetative nucleus (Fig. S1A,B). The expression of a D-type cyclin (typically expressed at G1/S) is consistent with the observations that sperm cells undergo an extended S phase in mature pollen grains (Friedman, 1999; Zhao et al., 2012).

Why does CYCD7;1 have such a restricted expression pattern in the stomatal lineage? One possible explanation is that CYCD7;1 has a unique function in GMC divisions. A second possibility is that CYCD7;1 has a canonical role, i.e. it acts like other cyclins in promoting cell divisions, but it is important to be able to tightly control deployment of that role in the stomatal lineage. To distinguish between these models, we characterized plants missing or misexpressing CYCD7;1, tested relationships between CYCD7;1 and other cell cycle regulators, and defined how CYCD7;1 expression was constrained by stomatal lineage transcription factors.

**Ectopic expression of CYCD7;1 triggers divisions; cycd7;1 mutants decelerate GMC divisions**

If CYCD7;1 has canonical CYCD activity, it should be able to promote cell divisions outside its normal expression window. To test this, we expressed CYCD7;1 and CYCD7;1-YFP with the pan-epidermal promoter ML1 (Roeder et al., 2010). Ectopic expression of CYCD7;1 (YFP-tagged or untagged) induced cell divisions of pavement cells in the leaf (Fig. 2A-D), indicating that CYCD7;1 can function as a canonical D-type cyclin.

Next, we asked whether mutations of CYCD7;1 result in abnormal phenotypes. We obtained five insertional mutant alleles of *CYCD7;1*: FLAG_369E02 (cycd7;1-1) (Collins et al., 2012), FLAG_498H08 (cycd7;1-2), GK_496G06-019628, SALK_068423 and SALK_068526. Two of these alleles, *cycd7;1-1* and *cycd7;1-2*, were outcrossed twice to Columbia plants (Col-0) and we determined that neither produced transcript by qRT-PCR (Fig. S2B,C) in 6-day-old seedlings. On a whole-plant level, we could not detect any abnormalities in *cycd7;1-1* compared with wild type (Fig. S2D). Because CYCDs promote G1/S transitions and CYCD7;1 is specifically expressed during the GMC divisions, we asked whether *cycd7;1-1* mutants halt the GMC to GC transition by counting the number of GCs in cotyledons. In maturing cotyledons 7 days after germination (dag), we count no difference in GC numbers between *cycd7;1-1* and wild type (Fig. S2E-G). However, when monitored at 4 dag, when the stomatal lineage is still proliferating and GC precursors (meristemoids and GMCs) are abundant, *cycd7;1-1* cotyledons have more GMCs than wild-type cotyledons (Fig. 2E). This suggests that *cycd7;1-1* does not block the development of GCs from GMCs, but might be required to promote timely transition through the GMC stage. Interestingly, the average size of *cycd7;1-1* GMCs is larger than that of wild-type GMCs (Fig. 2F). Plant cells are known to increase in size during G1, so this phenotype also suggests that CYCD7;1 hastens cell cycle progression in the GMC to GC transition. We confirmed that these GMC abundance and size phenotypes were present in plants bearing the *cycd7;1-2* allele (Fig. S2C,H,I). Because *cycd7;1-1* and *cycd7;1-2* were originally in Wassilewskija (Ws) background and outcrossed to Col-0 twice, we tested whether any of the stomatal phenotypes were caused by ecotype background effects. Quantification of GMC number and size revealed no significant differences betweenWs and Col-0, indicating that the phenotype could be attributed to the *cyd7;1* mutations and not to ecotype differences (Fig. S2JK).

We characterized the *cycd7;1-1* mutant in more detail. We introgressed *pCDKB1;1:GUS*, which labels the transition from GMC to GCs (Boudolf et al., 2004), into *cycd7;1-1* mutants. Compared with wild type, *cycd7;1-1* mutants show increased numbers of GUS-positive cells, suggesting that these cells remain longer in GMC fate before they divide into GCs (Fig. 2G-I). To directly test this hypothesis, we labeled S phases with 5-ethyl-2'-deoxyuridine (EdU), a thymidine analogue readily incorporated during DNA replication (Fig. 2J-L). Strikingly, significantly fewer GMCs in *cycd7;1-1* showed EdU labeling (indicating that they were in S phase during the EdU pulse) compared with wild-type GMCs (Fig. 2L). Together, these data suggest that CYCD7;1 is required for GMCs to make a timely entry into S phase before their transition into GCs.

**CYCD7;1 interacts with RBR1**

Typically, CYCDs drive the G1/S transition through inactivation of RBR1, and RBR1 activity was previously shown to be essential for repressing divisions in the stomatal lineage (Borghi et al., 2010; Matos et al., 2014). If CYCD7;1 and RBR1 function together, we would expect these to be co-expressed, to physically interact, and for there to be a phenotypic consequence of disrupting the interaction. Indeed, CYCD7;1 and RBR1 were shown to physically interact in bimolecular fluorescence complementation (BiFC) and yeast two-hybrid (Y2H) assays, dependent on the presence of the RBR1 binding motif LxCxE in CYCD7;1 (Matos et al., 2014). In addition, CYCD7;1 and RBR1 are co-expressed in GMCs (Fig. 3A-C). To test whether this interaction is functionally important, we took
CYCD7;1 needs CDKB1 activity to drive ectopic divisions

Cyclins bind to CDKs to ensure kinase activity and completion of cell division. It was previously shown that CDKB1;1 is enriched in late stomatal lineage cells (Boudolf et al., 2004) and that CDKA;1 is expressed in all dividing cells (Adachi et al., 2009), making these CDKs candidates for regulating CDKB1;1 activity. It was previously shown that CDKB1;1 is expressed in GMCs and young GCs (Lai et al., 2005; Lee et al., 2013; Vanneste et al., 2011; Xie et al., 2010). Given these patterns, we tested whether MUTE preceded CYCD7;1, while FAMA persisted longer. In addition, the R2R3 MYB transcription factor, FOUR LIPS (FLP), partially overlapped CYCD7;1 expression (Fig. 1I–N), but CYCD7;1 expression domain is constrained by stomatal lineage transcription factors

Our evidence points to CYCD7;1 acting like a canonical CYCD; therefore, we turned our attention to regulation of its highly restricted expression pattern. Transcription factors MUTE and FAMA partially overlap CYCD7;1 expression (Fig. 1I–N), but MUTE precedes CYCD7;1, while FAMA persists longer. In addition, the R2R3 MYB transcription factor, FOUR LIPS (FLP), is expressed in GMCs and young GCs (Lai et al., 2005; Lee et al., 2014; Xie et al., 2010), and has been associated with cell cycle control in GCs through its repression of CDKB1;1 (Lee et al., 2013; Vanneste et al., 2011; Xie et al., 2010). Given these patterns, we tested whether MUTE was necessary to induce CYCD7;1 expression and whether FLP and FAMA repressed it. When pCYCD7;1:CYCD7;1-YFP was crossed into the mute mutant, we could observe the typical mute phenotype of many small meristemoid-like cells that fail to differentiate into GMCs (Pillitteri et al., 2007). In a few of these meristemoid-like cells, we detected weak CYCD7;1-YFP signal (Fig. 4A,B). Fluorescence intensity measurements showed that CYCD7;1-YFP signals in mute...
are ∼50% reduced (Fig. 4C-E), indicating that MUTE promotes CYCD7;1 expression, although it is not absolutely essential for it. In none of these images did we observe any ectopic divisions of the meristemoid-like cells. Mutations in FLP and its redundantly acting homolog MYB88 result in GMCs dividing multiple times before transitioning into GCs (Fig. 4F,I). CYCD7;1-YFP (and CYCD7;1LGK-YFP) translational reporters are highly expressed in flp/myb88, a result consistent with FLP/MYB88 repressing CYCD7;1 (Fig. 4F-L). Interestingly CYCD7;1-YFP, but not CYCD7;1LGK-YFP, induces ectopic divisions in the flp/myb88 GMCs and GCs (Fig. 4F-L).

The elevation of CYCD7;1 levels in flp/myb88 suggests that CYCD7;1 is repressed to limit its expression domain. We next tested whether this was true for FAMA. Additionally, because FAMA is a master transcriptional regulator of stomatal division and differentiation, we tested whether its regulation of CYCD7;1 was direct. In fama mutants, GMCs divide repeatedly without attaining GC fate (Fig. 5A-E). We found that these ‘tumors’ express CYCD7;1-YFP (Fig. 5B,C), as would be expected if FAMA acted to repress CYCD7;1. It is important to note, however, that after its initial upregulation, CYCD7;1-YFP expression fades in the fama ‘tumors’ in older leaves, suggesting that CYCD7;1 is also subject to post-translational regulation (Fig. 5D,E). In the fama ‘tumors’, pCYCD7;1:CYCD7;1-YFP also drives ectopic divisions (Fig. 5B,D,F, white arrowheads), but the CYCD7;1LGK version that cannot bind RBR1 does not (Fig. 5C,E,F). To test whether
FAMA might directly regulate CYCD7;1, we extracted reads from a FAMA chromatin immunoprecipitation with sequencing (ChIP-seq) experiment, performed under similar conditions as in Lau and Bergmann (2015) and Lau et al. (2014), and found FAMA associated with the promoter region and gene body of CYCD7;1 (Fig. 5G, Fig. S3).

Because CYCD7;1-YFP expression is extinguished before FAMA-YFP (Fig. 1L-N) and prolonged in fama, FAMA is expected to repress CYCD7;1 expression in GMCs. To test this, we transformed an estradiol-inducible version of FAMA (Hachez et al., 2011; Ohashi-Ito and Bergmann, 2006) into plants harboring the pCYCD7;1:YFP reporter line. This enabled us to provide a pulse of FAMA overexpression and follow CYCD7;1 response in the appropriate cells over time in intact leaves. We observed a significant reduction of YFP fluorescence in GMCs during an 8 h time course following estradiol (but not mock) induction of FAMA (Fig. S4 and supplementary Materials and Methods).

The phenotypes of loss and gain of CYCD7;1 activity suggest that its narrow window of expression is essential to guarantee a two-celled stomatal complex. Using the FAMA promoter in wild type, thus driving CYCD7;1 slightly later than under its endogenous cis-regulatory control, we find a dramatic enhancement of ectopic divisions (Fig. 5H). This directly links the activity of FAMA as a lineage specific transcription factor with the cell cycle regulator CYCD7;1 to ensure ‘one and only one division’ to create a pair of GCs.

**DISCUSSION**

We have shown that CYCD7;1 is specifically expressed in GMCs prior to the last symmetric cell division that forms the two-celled stomatal complex. Depletion of CYCD7;1 slows down this cell division, whereas ectopic expression of CYCD7;1 can trigger cell divisions in GCs. Mutation of the RBR1 binding motif in CYCD7;1 disrupts its interaction with RBR1 and renders CYCD7;1 incapable of driving ectopic division. The connection to RBR1 fits with previous work showing that CYCD7;1 interacts with CDKA;1 (Van Leene et al., 2010), together supporting a role for CYCD7;1 in the canonical regulatory complex for G1/S transitions and the commitment to divide. CYCD7;1 activity in cell cycles, however, is directly repressed by the lineage specific transcription factor FAMA to ensure a coupling between the cell division, which terminates the stomatal lineage, and the formation of terminally fated GCs. This interconnection represents a direct link between cell cycle regulators and developmental decisions (Fig. 6).

CYCDs are crucial for the G1/S transition and commitment to divide, and are therefore interesting candidate hubs for the...
Fig. 5. CYCD7;1 expression is regulated by FAMA, which serves to constrain CYCD7;1 activity. (A–E) Confocal images of fama at 12 dag, and pCYCD7;1:CYCD7;1-YFP in fama mutant background and pCYCD7;1:CYCD7;1-YFP in fama mutant background at 12 dag and 16 dag. (F) Quantification of ectopic divisions in tumors of fama mutants, pCYCD7;1:CYCD7;1-YFP in fama mutant background and pCYCD7;1:CYCD7;1-YFP in fama mutant background. *P<0.001, Student’s t-test. (G) ChIP-seq profile of FAMA binding to the promoter and gene body of CYCD7;1. Black arrow indicates gene orientation and transcriptional start sites. (H) Confocal image of pFAMA:CYCD7;1-YFP at 5 dag. White arrowheads show ectopic division and prolonged CYCD7;1-YFP presence. (I–K) DIC images of abaxial cotyledon epidermis of wild type, pCYCD7;1:CYCD7;1 and pFAMA:CYCD7;1:YFP at 12 dag. White arrowheads point at ectopic cell divisions. (L) Quantification of ectopic cell divisions in wild type (n=142) and pFAMA:CYCD7;1-YFP (n=237) at 5 dag (P=1.4371×10−58, Mann–Whitney U-test), and in wild type (n=125) and pFAMA:CYCD7;1-YFP (n=1153) at 12 dag (P=1.3625×10−37, Mann–Whitney U-test). Differences between pFAMA:CYCD7;1-YFP at 5 dag and 12 dag are significant (P=6.4773×10−54, Mann–Whitney U-test). Differences between pFAMA:CYCD7;1-YFP at 5 dag and pCYCD7;1:CYCD7;1-YFP at 5 dag are also significant (Fig. 3G; P=1.0473×10−37, Mann–Whitney U-test). Confocal images show cell outlines (magenta) stained with propidium iodide. Scale bars: 10 µm.

Integration of developmental control with the cell cycle machinery. In *Arabidopsis*, there are 10 D-type cyclins, some active in multiple tissues (CYCD3s, CYCD4s, CYCD2;1), and others for which activity is linked to specific cell types (CYCD6;1 and CYCD7;1) or cell cycle behaviors (CYCD5;1 in endoreplication) (Dewitte et al., 2007; Kono et al., 2007; Sanz et al., 2011; Sterken et al., 2012; Adrian et al., 2015; Sozzani et al., 2010). Phylogenetic analyses showed that CYCD6;1 and CYCD7;1 proteins diverge from other D-type cyclins in *Arabidopsis* (Wang et al., 2004), but also that CYCD7;1 most closely resembles the single D-type cyclin in *Physcomitrella* (Menges et al., 2007), consistent with our observation that it could promote G1/S transitions (a core D-type activity) in multiple cell types.

Interestingly, both CYCD6;1 and CYCD7;1 are limiting for essential formative divisions during development. In the root, CYCD6;1 is important for the cortex endodermis initial daughter (CEID) cell divisions (Sozzani et al., 2010; Weimer et al., 2012). Here, SHORTROOT (SHR) directly activates expression of CYCD6;1, which works in concert with CDKA;1 to trigger the formative division of the CEID (Cruz-Ramirez et al., 2012; Sozzani et al., 2010; Weimer et al., 2012). This interaction promotes the initiation of an asymmetric stem-cell division program. By contrast, CYCD7;1 expression marks the boundary between two types of divisions: the continual asymmetric divisions of meristemoids versus the single symmetric division of a GMC. Here, we find a quantitative requirement for MUTE to promote full CYCD7;1 expression, but a clear requirement for FAMA and FLP/MYB88 to repress CYCD7;1 after GMC division. The low expression level of CYCD7;1 in the absence of MUTE might point to a direct role for MUTE in activating CYCD7;1 expression. MUTE is structurally similar to FAMA, and therefore might be able to interact with CYCD7;1 regulatory sequences. Alternatively, as meristemoid cells in *mute* never transition into GMCs, low CYCD7;1 levels might be an indirect consequence of altered cell fate. In either case, it is notable that the introduction of CYCD7;1-YFP in *mute* did not drive additional meristemoid cell divisions, suggesting that CYCD7;1 division-promoting behavior requires a threshold level not reached in this genetic background.

It is tempting to speculate that spatiotemporal restriction of CYCDs could be a mechanism to control the cell cycle machinery more efficiently, and to cope with different developmental programs. For example, leaves lose overall division competency and general cell cycle gene expression as they mature, leading to a situation in which GMCs are caught in a largely postmitotic zone. Formation of functional stomata, however, requires that the GMC divides again, though only once, as there have yet to be found plants in which stomatal pores are flanked by more than two GCs (McElwain et al., 2016). The importance of specialized CYCDs, however, must be squared with the relatively minor phenotypes associated with their loss; neither CYCD7;1 nor CYCD6;1 mutants ablish the production of specialized cells or tissue layers (Fig. 2) (Sozzani et al., 2010). Most likely, CYCD6;1 and CYCD7;1 assist other, more general, cyclins in executing cell division programs, or they might ensure particularly high cell cycle kinase activity. For example, in leaves, CYCD3;1 and CYCD3;2, despite being considered general G1/S cyclins (Dewitte et al., 2007, 2003; Menges et al., 2006), show high expression in the stomatal lineage (Adrian et al., 2015) and could be partially redundant with CYCD7;1. It is also important to recognize that CYCD/CDKA complexes likely have various downstream targets, and that increased kinase activity could induce different downstream processes, either in a feedback loop or for differentiation.
processes. In plants, specific CDK/cyclin complexes can have differential activity toward individual substrates. Both CDK and cyclin proteins contribute to substrate recognition (Harashima and Schnittger, 2012), but there is evidence that the cyclin plays the more prominent role (Weimer et al., 2016). Specific expression of individual cyclins, such as CYCD7;1, in the stomatal lineage could, therefore, contribute to fine-tuning of cell division control and downstream substrate recognition.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

*Arabidopsis thaliana* Columbia (Col-0) was used as wild type in all experiments except as noted in Fig. S2. Seedlings were grown on half-strength Murashige and Skoog (MS) medium (Caisson Labs) at 22°C under 16 h-light/8 h-dark cycles and were examined at the indicated times. The following previously described mutants and reporter lines were used in this study: *mute* (Pillitteri et al., 2007); *fama-1* (Ohashi-Ito and Bergmann, 2006); *flp;myb88* (Lai et al., 2005); *proSPCH:SPCH:CFP* and *proMUTE: MUTE-YFP* (Davies and Bergmann, 2014); *proRBR1:RBR1-CFP* (Cruz-Ramirez et al., 2012); *pro35S:CDKB1;1:N161* (Boudolf et al., 2004); and *proCDK1:1:GUS* (Boudolf et al., 2004).

**CYCD7;1 mutants**

CYCD7;1 mutants FLAG_369E02 (cycd7;1-1) and FLAG_498H08 (cycd7;1-2) were derived from the INRA/Versailles collection (Versailles, France) and both lines were outcrossed to Col-0 twice. GK_496G06-019628 was derived from the GABI-Kat collection (Cologne, Germany). SALK_068423 and SALK_068526 were obtained from the Arabidopsis Biological Resource Center (Columbus, USA).

**Vector construction and plant transformation**

Constructs were generated using the Gateway system (Invitrogen). Appropriate genome sequences (PCR amplified from Col-0 or from entry clones) were cloned into Gateway-compatible entry vectors, typically pENTR/D-TOPO (Life Technologies), to facilitate subsequent cloning into the plant binary vector series R4pGWB (Nakagawa et al., 2008; Tanaka et al., 2011), with the Gateway entry clones of the promoters and coding sequences compatible with the binary R4pGWB destination vector system. Primer sequences used for entry clones are provided in Table S1. Transgenic plants were generated by Agrobacterium-mediated transformation (Clough, 2005), and transgenic seedlings were selected by growth on half-strength MS plates supplemented with 50 mg/l Hygromycin (pHGY, p35HGY-; pGWB1-; pGWBV540-based constructs), 100 mg/l Kanamycin (pGWB440- and pGWB401-based constructs) or 12 mg/l Basta (pGWB640-based constructs).

**Confocal and differential interference contrast microscopy**

For confocal microscopy, images were taken with a Leica SP5 microscope and processed in ImageJ. Cell outlines were visualized by 0.1 mg/ml propidium iodide in water (Molecular Probes). Seedlings were incubated for 10 min in the staining solution and then rinsed once in H2O. For differential interference contrast (DIC) microscopy, samples were cleared in 7:1 ethanol:acetic acid, treated for 30 min with 1N potassium hydroxide, rinsed in water and mounted in Hoyer’s medium. DIC images were obtained from the middle region of adaxial epidermis of cotyledons on a Leica DM2500 microscope or a Leica DM6 B microscope.

**Quantification of fluorescence intensity**

Images of GMCs in cotyledons were taken at 4 dag with identical settings between *mute* mutants and their sister plants from a segregating population and processed in ImageJ. Fluorescence intensity was measured as mean gray value in the nucleus, subtracted by the background. Measurements were averaged for mutant and control experiments, and Student’s t-test was used to determine the statistical significance.

**GUS staining**

The *cyd7;1-1* mutant (which had been outcrossed two times to Col-0), was introgressed into CDKB1;1-GUS marker lines. Five-day-old seedlings were incubated in staining solution for 12 h and destained in 70% ethanol at 60-70°C for 4 h. The staining solution (5 ml) contained 100 µl 10% Triton X-100, 250 µl 1 M NaPO₄ (pH 7.2), 100 µl 100 mM potassium ferrocyanide, 100 µl potassium ferricyanide, 400 µl 25 mM X-Gluc and 4050 µl dH₂O. Images were taken with a Leica DM6 B microscope.

**EdU labeling**

EdU labeling was performed using the Click-IT EdU Alexa Fluor 488 Imaging Kit (ThermoFisher Scientific). Four-day-old seedlings were incubated in 20 µM EdU solution in half-strength MS for 90 min at room temperature. Seedlings were transferred to new tubes and washed three times.
with wash buffer (1% BSA in PBS). Wash buffer was removed and fixation buffer was added (3% formaldehyde in PBS) for 30 min at room temperature. Seedlings were transferred to new tubes and washed twice with permeabilization buffer (0.5% Triton X-100 in PBS) for 10 min each, protected from light on a slow rocking platform. Plants were transferred to new tubes and incubated in reaction cocktail (455 µl Click-IT reaction buffer, 20 µl CuSO₄, 2 µl Alexa Fluor Azide 488, 25 µl 1× Click-IT Edu additive) for 1 h at room temperature, protected from light, without agitation. Seedlings were transferred to new tubes and washed twice for 10 min at room temperature with wash buffer on slow rocking platforms, protected from light. Cotyledons were imaged using a Leica SP5 microscope.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions


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### Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.160671.supplemental

### References


McElwain, J. C., Yiotis, C. and Lawson, T. (2016). Using modern plant trait relationships between observed and theoretical maximum stomatal conductance...
and vein density to examine patterns of plant macroevolution. New Phytol. 209, 94-103.


Supplementary Data

Figure S1: Additional CYCD7;1 expression patterns outside of the stomatal lineage

(A, B) CYCD7;1 (yellow) is expressed in sperm cells during pollen anthesis. (C) Intensity measurements of fluorescent nuclei were 179 a.u. +/-10 S.E.M. for proCYCD7;1:CYCD7;1-YFP vs 176 a.u. +/-8 S.E.M. for proCYCD7;1:CYCD7;1^{LGK}-YFP (N=15 nuclei/line; p> 0.05; Student’s t-test) and 166 a.u. +/-11 S.E.M. for proFAMA:CYCD7;1-YFP (N=15 nuclei/line; p> 0.05; Student’s t-test). Error bars show standard error. a.u., arbitrary units; n.s. non-significant; S.E.M. standard error of measurement.
Figure S2: Additional CYCD7;1 T-DNA insertional alleles and mutant phenotypes

(A) Schematic drawing of CYCD7;1 gene structure with available T-DNA insertion lines and their insertion sites. Black boxes indicate exons. Gray arrowheads marked with fwd and rev show primer binding sites for qPCR. (B) qPCR of CYCD7;1 expression in wild type (Col-0 and Ws) and the cycd7;1-1 mutant. Primer binding sites are shown in (A). (C) qPCR of CYCD7;1 expression in wild type (Col-0 and Ws) and the cycd7;1-2 mutant. Primer binding sites are shown in (A). (D) Wild type and cycd7;1-1 mutant seedlings at 14 dag. (E) Wild type cotyledon with mature GCs, labeled with black asterisks at 7 dag. (F) Cotyledon of cycd7;1-1 mutant with mature GCs, labeled with black asterisks, images were taken at 7 dag. (G) Quantification of GCs in wild type and cycd7;1-1 mutants at 5 dag on the abaxial side of cotyledons (N=12 cotyledons for each genotype). Difference between the wild type and cycd7;1-1 is not significant (p-value = 0.8169; Mann-Whitney U test). (H) Quantification of the number of GMCs in wild type and cycd7;1-2 cotyledons at 4 dag. Asterisk indicates significant difference (p-value = 0.0031; Mann-Whitney U test). (I) Quantification of GMC area in wild type (N=29) and cycd7;1-2 (N=46) cotyledons at 4 dag. Asterisk indicates significant difference (p-value = 0.0053; Mann-Whitney U test). (J) Quantification of the number of GMCs in Col-0 wild type and Ws wild type cotyledons at 4 dag. Difference is not significant (p-value = 0.6970; Mann-Whitney U test). (K) Quantification of GMC area in Col-0 wild type (N=22) and Ws wild type (N=45) cotyledons at 4 dag. Difference is not significant (p-value = 0.2295; Mann-Whitney U test).
Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 2.5 times the interquartile range from the 97.5th percentile. Scale bar 1 cm in (C) and 20 µM in (E and F). Note that stomatal production is dynamic and is sensitive to exact age and growth conditions (e.g. media, light, temperature). Therefore, all quantitative measurements were performed with wildtype controls grown side-by-side with mutants under the exact same conditions to enable comparisons.
**Figure S3: ChIP-seq profiles of FAMA and SPCH on selected loci**

ChIP-Seq profile of SPCH (green) and FAMA (blue) binding to the promoter and gene body of ICE1 and CYCD7;1, respectively. The y-axis is the output peak score from MACS2 (in arbitrary units). ICE1 was previously demonstrated to be a direct SPCH target (Lau et al., 2014) and serves as a reference to provide intuition about the meaning of these peak score values. Black arrow indicates gene orientation and transcriptional start sites. The profile in (D) is replicated from Fig. 5G to enable a more convenient comparison among transcription factors and targets.
Figure S4: Evidence that FAMA represses CYCD7;1 in GMCs

(A) Transgenic plants expressing proCYCD7;1:YFP were subjected to FAMA induction and the fluorescence intensity of YFP was monitored over time in cotyledon GMCs. Four-day old plants treated with 5µM β-estradiol show ~25% reduction in YFP fluorescence as compared to the control treatment in an 8-hour imaging period suggesting that FAMA represses CYCD7;1. Error bars represent standard error.

(B) DIC image of the epidermis of a 10 dag cotyledon from genotypically identical siblings of the plants monitored in (A) grown on media supplemented with 50µM β-estradiol. The plant demonstrates the typical FAMA overexpression phenotype of ectopic unpaired (kidney shaped) GCs. Scale bar is 25µm.
### Table S1: Primers used in this study.

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<th>CYCD7 genomic region (promoter + CDS)</th>
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### Supplemental Material and Methods

#### Time-lapse of estradiol inducible constructs

Estradiol inducible FAMA (*proEST:FAMA*) was transformed into plants harboring *proCYCD7;1:YFP* and 4 dag T3 plants were used for time-lapse experiments following general protocols described in (Davies and Bergmann, 2014), except that that normal media (1/4 strengths MS, 0.75% Sucrose) was supplemented with 5µM β-estradiol (25µL of 10mM β-estradiol dissolved in 95% ethanol for 50mL media) or ethanol alone (25µL 95% ethanol for 50mL media), and pumped through the chamber with a constant flow at 2mL/hour using a syringe pump. Z-stacks through the epidermis were captured on a confocal microscope with Leica software every 30 min for 8 hours. Fiji software was used to measure Integrated Density (total fluorescence) of 36 (control) or 28 (estradiol-treated) GMC nuclei. % fluorescence per nucleus was calculated with respect to “initiation” time point (T0). Three independent replicates of the time course were performed, each on a separate plant, for both control and induced lines, and the averaged data plotted in Fig S4.