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Short title: E2FB-RBR function in leaf development

E2FB interacts with RETINOBLASTOMA RELATED and regulates cell proliferation during leaf development

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One-sentence summary: The main function of the E2FB transcription factor is to restrict cell proliferation and establish quiescence during Arabidopsis leaf development; it acts in a complex with RETINOBLASTOMA-RELATED.

Author contributions

The authors have made the following declarations about their contributions:

Z.M, E.L.J, and L.B conceived the idea and designed the study to analyse the function of E2FB during leaf development. Z.M generated and Z.M and E.Ő. performed the phenotypic analysis and microscopic characterisation of transgenic Arabidopsis lines and mutants; with the exception of the construction of GFP-E2FA$^{\Delta RBR}$, and GFP-E2FB$^{\Delta RBR}$ and transgenic lines were generated by E.M, and the construction of the 3xvYFP-tagged E2FA and E2FB translational fusions and the generation of these transgenic Arabidopsis lines; these were carried out by S.K. and C.G-A. Immunoblottings, immunoprecipitations (IP) and co-IP were carried out by Z.M., E.Ő., and T.L. ChIP with GFP on *CycD3;1*, *CDKB1;1* and *RBR* promoters was performed by Cs.P. The transcriptional level of the cell cycle genes in the transgenic lines, the ploidy measurement by FCM and the measurement of cell size in cotyledonous leaves was performed by B.M. Cell number, cell size and cellular parameters of the various transgenic lines were determined by E.Ő and E.M. The manuscript was written by B.M.H., L.B., E.L.J and Z.M; seen and commented by all authors.

Abstract

Cell cycle entry and quiescence are regulated by the E2F transcription factors in association with RETINOBLASTOMA-RELATED (RBR). E2FB is considered to be a transcriptional activator of cell cycle genes, but its function during development remains poorly understood. Here, by studying E2FB-RBR interaction, E2F target gene expression, and epidermal cell number and shape in *e2fb* mutant and overexpression lines during leaf development in *Arabidopsis thaliana*, we show that E2FB in association with RBR plays a role in the inhibition of cell proliferation to establish quiescence. In young leaves, both RBR and E2FB are abundant and form a repressor complex that is reinforced by an autoregulatory loop. Increased E2FB levels either by expression driven by its own promoter or ectopically together with DIMERISATION PARTNER A, further elevates the amount of this repressor complex, leading to reduced leaf cell number. Cell overproliferation in *e2fb* mutants and in plants overexpressing a truncated form of E2FB lacking the RBR binding domain strongly suggested that RBR repression specifically acts through E2FB. The increased number of small cells below the guard cells and of fully developed stomata indicated that meristemoids preferentially hyperproliferate. As leaf development progresses and cells differentiate, the amount of RBR and E2FB gradually declined. At this stage, elevation of E2FB level can overcome RBR repression leading to the reactivation of cell division in pavement cells. In summary, E2FB in association with RBR is central to regulating cell proliferation during organ development to determine final leaf cell number.

Introduction

The time window for cell proliferation is the most fundamental determinant for meristem size and has the largest impact on final organ size (Gazquez and Beemster, 2017). This is set by the coordination of cell cycle and exit to differentiation that are governed through complex regulatory mechanisms culminating on the evolutionarily conserved Retinoblastoma (Rb) repressor protein and the E2F transcription factor targets (van den Heuvel and Dyson, 2008). According to the textbook model established in animal systems, cell cycle entry is guarded by cyclin dependent kinases (CDKs), which, upon activation by mitogenic signals, phosphorylate and thereby inactivate Rb and other related pocket proteins. When released from Rb repression, the so-called activator E2Fs drive the cell cycle by activating the expression of cell cycle genes required for the G1 to S-phase transition. By contrast, the repressor-type E2Fs function together with Rb to instigate quiescence and to allow differentiation (Morgan, 2007).

In *Arabidopsis thaliana* (Arabidopsis), a single gene codes for the RETINOBLATOMA RELATED (RBR), and this protein acts through three E2F transcription factors, known as E2FA, E2FB, and E2FC. These three E2Fs can only bind to DNA in complex with the DIMERISATION PARTNER A or B (DPA or DPB, De Veylder et al., 2007). Modelling Arabidopsis E2Fs on the animal scenario places E2FA and E2FB as activators and E2FC as a repressor, but similar to animal cells, this subdivision is largely supported by overexpression studies (Magyar et al., 2016). Ectopic co-overexpression of E2FB with DPA allows the continued proliferation of cultured tobacco (*Nicotiana tabacum*) cells in the absence of the plant growth hormone auxin (Magyar et al., 2005). This is reminiscent of the effect of human E2F1 overexpression, which triggers S-phase entry in growth factor-deprived cultured cells (Johnson et al., 1993). Overexpression of E2FB without the DP partner also leads to the upregulation of cell cycle genes and surprisingly a much reduced root growth both in Arabidopsis (Sozzani et al., 2006) and in tomato (*Solanum lycopersicum*; Abraham and del Pozo, 2012), with fruit size increased in the latter. E2FB is expressed throughout the cell cycle phases (Magyar et al., 2000; Mariconti et al., 2002; Magyar et al., 2005) and has the ability to drive both the G1 to S and G2 to M transitions, leading to shortened cell doubling time and reduced cell sizes (Magyar et al., 2005). The accelerated entry into mitosis was correlated with the induced expression of the G2-M specific CDKB1;1, following E2FB overexpression (Magyar et al., 2005; Henriques et al., 2013). The activity of E2FB is tightly

controlled by RBR phosphorylation in response to sucrose availability, overexpression of CYCLIN D3;1 (CYCD3;1), or the counteracting CDK inhibitor KIP-RELATED PROTEIN 2 (KRP2; Magyar et al., 2012).

E2FA differs from E2FB in many respects: (1) E2FA is most abundant in S-phase cells, (2) when overexpressed, it can promote cell proliferation in meristematic cells, whereas in cells that have lost cell division competence, E2FA overexpression supports a modified cell cycle with repeated S-phases, called endoreduplication, and (3) the association of E2FA with RBR is not disrupted, but rather enhanced when cell proliferation is induced by excess sucrose or overexpression of CYCD3;1 (De Veylder et al., 2002; Magyar et al., 2012). Furthermore, E2FA function in endoreduplication does not rely on promoting the transcription of S-phase genes through the trans-activation domain, but rather on the ability of E2FA to associate with RBR and to repress genes regulating the entry into endoreduplication and cell differentiation (Magyar et al., 2012). Therefore, it was suggested that E2FA in association with RBR plays a role in maintaining cell proliferation competence in meristems. In addition, E2FA was shown to play roles in maintaining genome integrity and viability in meristematic cells (Horvath et al., 2017).

E2FA and E2FB appear to be redundantly required for cell proliferation because no viable plants can be generated when predictably null mutants are combined (Li et al., 2017). However, a viable double *e2fab* mutant plant was generated by combining different loss-of-function mutant alleles for E2FA (*e2fa-2*) and E2FB (*e2fb-1*; Heyman et al., 2011), suggesting that at least the C-terminal transactivation function of these E2Fs are dispensable for plant growth and development.

The repressor function of E2FC is supported by its overexpression that suppressed meristematic cell divisions and the expression of mitotic CYCB1;1, and by its silencing that led to the upregulation of both the S-phase associated *HISTONE 4* (*H4*) and *CELL DIVISION CYCLE* 6 (*CDC6*) and the mitotic *CYCB1;1* genes (del Pozo et al., 2006). In mammalian cells, the DP, RB-like, E2F4, and Multi-vulval class B (MuvB) multiprotein complex, known as DREAM, acts as a repressor on cell cycle genes to impose quiescence (Sadasivam and DeCaprio, 2013). In Arabidopsis, E2FC, RBR, and MYB3R3 (a repressor type MYB3R or Rep-MYB3R) are part of the DREAM complex with a repressive function that establishes quiescence (Kobayashi et al., 2015). However, unique to plants is that the activator type E2FB partners the mitosis specific activator MYB3R4 (an Act-MYB3R) in another DREAM

complex (Kobayashi et al., 2015; Harashima and Sugimoto, 2016). This provides additional support for the mitotic role of E2FB.

The leaf is an excellent model to study how the coordinated action between cell proliferation and differentiation is regulated (Andriankaja et al., 2012; Kalve et al., 2014). The leaf has a determinate size, and its growth is the result of two partially overlapping processes: the initial cell proliferation followed by cell expansion, which occurs as cells permanently exit the cell cycle. Cell division is differently regulated in distinct cell populations within the leaf epidermis. The meristematic protodermal cells go through formative cell divisions with a cell proliferation front progressively restricted to the base of the leaf during development. When epidermal leaf cells exit mitosis, they become lobed and enlarged in size, which is coupled with an increase in ploidy through a switch from the mitotic cell cycle to the endoreduplication program (De Veylder et al., 2011). A substantial bulk of pavement cells originate from stomata meristemoids interspersed along the leaf surface, forming a stem cell population that go through several rounds of asymmetric divisions to produce cells that differentiate either into pavement cells or stomata (Andriankaja et al., 2012). The identity of these meristemoid cells are determined by a set of key regulators, such as SCPEECHLESS, but can also be visually recognised by their characteristic round or square shape and a small size of cells below the stomata guard cells, specifically less than 100 μm^2 (Dong et al., 2009). The temporal and spatial regulation of the cell cycle arrest front in the cell populations originating from protodermal cells or meristemoids are different, but the underlying molecular mechanisms are hitherto unknown (White, 2006).

We investigated how E2FB, which is considered to be an activator of cell proliferation, is regulated by RBR interaction to underpin cell proliferation, exit to differentiation, and establishment of quiescence during leaf development. Combined, our biochemical and genetic analyses suggest that E2FB regulates organ development as a corepressor complex with RBR.

Results

Elevated E2FB level inhibits cell proliferation in association with RBR at early stages of leaf development, whereas it perturbs the establishment of quiescence at later leaf developmental stages when RBR levels decline

To follow E2FB protein level in its native context during leaf development, we generated Arabidopsis plants carrying the genomic region of *E2FB* under the control of its own promoter and tagged its C-terminus with 3xVenus YFP, a modified yellow fluorescence protein (pgE2FB-3xvYFP). In young leaves at six days after germination (6 DAG), the E2FB-3xvYFP signal was detected in the nuclei both in the proliferating protodermal and meristemoid cells (Figure 1A, 6 DAG). Interestingly, at a later stage of leaf development, the E2FB-3xvYFP remained present in fully developed stomata as well as in lobbed differentiated pavement cells and vascular cells with characteristic elongated nuclei close to the cell wall (Figure 1A, 10 DAG). By comparing the E2FB-3xvYFP distribution with the localisation of E2FA-3xvYFP and RBR-GFP (Magyar et al., 2012), we found that the E2FA-3xvYFP was largely restricted to proliferating epidermal cells and was not detectable in fully differentiated stomata (Supplemental Figure S1A and B). The RBR-GFP signal was present in the meristemoids and in the proliferating and also in the differentiated pavement cells (Supplemental Figure S1C). RBR was also detectable in fully differentiated stomata, although at lower level (Matos et al., 2014).

To reduce a possible effect of 3xvYFP on the protein function, we also generated transgenic Arabidopsis lines with a single GFP tag (pgE2FB-GFP), and showed that the localisation of both E2FA-GFP and E2FB-GFP was comparable to that observed for E2FA-3xvYFP and E2FB-3xvYFP in the different epidermal cell types (Supplemental Figure S1A to F). Although *E2FB-GFP* expression was driven by the *E2FB* regulatory region, different expression levels of *E2FB-GFP* were identified among the 36 independent transformants (low, medium, and high; pgE2FB-GFP lines 61, 93, and 72, respectively, Supplemental Figure S2A). Despite the difference in the levels, the temporal *E2FB-GFP* expression followed the same declining pattern with leaf development as endogenous *E2FB* in the wild-type (WT) control (Supplemental Figure S2B). The GFP-tagged E2FB was functional in respect to its ability to interact with RBR as well as to dimerise with and to stabilise DPA and DPB proteins (Supplemental Figure S2C to E). Its interaction with RBR protein was also regulated as expected; it did not associate with the phosphorylated RBR form (Supplemental Figure S2C).

Plants of pgE2FB-GFP line 72, with high *E2FB-GFP* expression driven by the *E2FB* promoter, showed reduced growth habit compared to the WT both at seedling stage and as a full-grown plant. As Figure 1B illustrates, the leaf area in pgE2FB-GFP line 72 was smaller

than WT. To investigate the cellular basis underlying the growth retardation, we imaged the epidermal layer of the first leaf pair and quantified the leaf area, total cell number, stomata number, cell size, and cell shape at three equal sections of the base, middle, and tip (Supplemental Table S1-2). We took samples from pgE2FB-GFP lines 72 and 93 at two developmental time points, representing young leaf with abundant cell proliferation (8 DAG) and older leaf when the majority of cells undergo expansion growth (12 DAG, Supplemental Figure S3 and Figure 1D). Surprisingly, this analysis revealed significantly fewer cells in pgE2FB-GFP lines 72 and 93 compared to WT at 8 DAG, whereas this difference became lower at 12 DAG (Figure 1D; Supplemental Table S1-2). In parallel, flow cytometry analysis of DNA content showed an accumulation of 2C nuclei, representing G1 phase in pgE2FB-GFP lines 72 and 93 at an early developmental stage (8 DAG) of the first leaf pair in comparison to the WT, which also indicates a block in cell proliferation (Supplemental Figure S3C). We also observed a shift towards larger cell size in pgE2FB-GFP line 72 compared to WT at 8 DAG (Supplemental Table S1, Supplemental Figure S3B). However, in spite of the enlarged cell size, the entry into endoreduplication was delayed in both pgE2FB-GFP lines 72 and 93 compared to WT, as shown by the reduced 8C nuclei in the first leaf pair at 12 and 15 DAG (Supplemental Figure S3C). Ploidy level of the cotyledons was also behind that of the WT in pgE2FB-GFP line 72, indicated by the reduced 16C and the complete lack of 32C nuclear DNA content (Supplemental Figure S3D). In agreement with this, the circularity index of epidermal cells was higher in pgE2FB-GFP lines than the corresponding WT, suggesting that cells with elevated E2FB level are more round and thus have delayed cell shape differentiation (Supplemental Table S1-2).

At 12 DAG, the majority of WT epidermal cells exited the cell cycle as indicated by their elongated and lobbed outline. In pgE2FB-GFP line 72, we observed numerous straight and less pronounced cell walls in these puzzle-shaped pavement cells, especially in cells located further towards the leaf-tip area (Figure 1C, Supplemental Table S1-2, Supplemental Figure S3A). The formation of a new division plane across the differentiated pavement cells was even more frequent and pronounced on the cotyledon surface of pgE2FB-GFP line 72 (Supplemental Figure S3E). Some of these elongated pavement cells contained more than a single straight cell wall. Similar divisions of enlarged pavement cells have been previously reported in WT Arabidopsis leaves (Asl et al., 2011), but the frequency of these divisions were dramatically increased in pgE2FB-GFP line 72 (Supplemental Table S1-2). In agreement, in pgE2FB-GFP line 72, the proportion of middle-sized cells ($\leq$ 300–1000 μm^2)

was elevated at 12 DAG at the expense of the number of larger cells (3000–6000 μm^2) as compared to the WT (Supplemental Figure S3B).

To gain insights into the molecular mechanism leading to the altered cell proliferation when E2FB level is elevated during leaf development, we first determined the expression levels of the S-phase related *ORIGIN RECOGNITION COMPLEX 2* (*ORC2*) and the mitotic *CYCLIN-DEPENDENT KINASE B1;1* (*CDKB1;1*). In pgE2FB-GFP line 72, the expression levels of *ORC2* and *CDKB1;1* were comparable to that in WT in young leaves (8 DAG, Figure 2A). At 10 and 12 DAG, the expression of *ORC2* and *CDKB1;1* declined in WT, where expression of these genes in pgE2FB-GFP line 72 remained elevated (Figure 2A). The transcription of *CYCD3;1* and *RBR* also increased in pgE2FB-GFP line 72, most strikingly at the time point of 10 DAG when expression of these genes in WT was significantly reduced control (Figure 2A). The sustained expression of these cell cycle genes correlated well with the division of enlarged pavement cells.

To understand how E2FB activity is regulated during leaf development, we studied both RBR and its phosphorylation level and the interaction between E2FB and RBR. For this, we utilised the human phosphospecific $Rb^{S807/811}$ antibody that was shown to recognize the conserved phosphorylation site of RBR proteins in multiple plant species, specifically at the 911 Serine position in Arabidopsis (P-RBR^{S911}, Abraham et al., 2011; Magyar et al., 2012; Wang et al., 2014). In the WT, both RBR and E2FB protein levels, as well as RBR phosphorylation, were highest at the early stage of leaf development (8 DAG) and displayed a gradual decline afterwards when cells exited proliferation (10–12 DAG, Figure 2B and 2C). By comparing RBR protein and phosphorylation levels in pgE2FB-GFP lines 93 and 72 to that in the WT, we observed clear differences in their kinetics (Figure 2B). The endogenous RBR level was highly elevated throughout the studied developmental stages in both pgE2FB-GFP transgenic lines, indicating a regulatory loop to counteract the excess E2FB level (Figure 2B and 2C). However, whereas RBR phosphorylation remained high at all studied time points in pgE2FB-GFP line 72, it declined in pgE2FB-GFP line 93 to a level similar to WT, indicating that RBR is more active as a repressor in pgE2FB-GFP line 93 than in pgE2FB-GFP line 72 (Figure 2B, quantification in Supplemental Figure S4A and B). In agreement, a considerably greater number of divisions were observed in differentiated epidermal cells at 12 DAG in pgE2FB-GFP line 72 than in pgE2FB-GFP line 93 (Supplemental Table S2).

Next, we compared complex formation between E2FB-GFP and RBR proteins in pgE2FB-GFP lines 93 and 72 (Figure 2C for inputs and 2D for co-IP). Immunoprecipitation of E2FB-GFP showed that the majority of RBR protein was in complex with E2FB-GFP fusion protein throughout leaf development and that the E2FB-RBR complex was the most abundant in young leaves of both pgE2FB-GFP lines, providing an explanation why cell number was decreased in the leaves of these lines (Figure 2D). The level of E2FB and RBR proteins decreased as leaf development progressed, much more in pgE2FB-GFP line 93 than in line 72 (Figure 2C, quantification in Supplemental Figure S4C and D), whereas the level of E2FB-associated RBR was comparable between the pgE2FB-GFP lines (Figure 2D, for quantification see Supplemental Figure S4E). Based on these data, we concluded that more RBR-bound E2FB-GFP is present in pgE2FB-GFP line 93 than in line 72, whereas RBR-free E2FB might be more prevalent in pgE2FB-GFP line 72 and consequently could promote cell proliferation in lobed differentiated leaf pavement cells.

In summary, in young leaves, elevated E2FB level together with RBR present in abundance represses rather than activates cell proliferation. The cellular and molecular data indicate that excess E2FB can only be liberated from RBR repression at later developmental stages when their levels decline, which leads to extra cell divisions in lobed pavement cells.

The *e2fb* mutant has increased number of cells in developing leaves

To investigate the effect of E2FB loss-of-function during leaf development, we analysed two *e2fb* T-DNA insertion mutant alleles, *e2fb-1* (SALK_103138) and *e2fb-2* (SALK_120959) (Berckmans et al., 2011; Kobayashi et al., 2015). The T-DNA insertions in these mutants are located just behind and within the E2FB dimerization domain, respectively (Supplemental Figure S5A). Based on the position of the T-DNA insertion, it is likely that *e2fb-2* is a null mutant as it lacks the dimerization domain required to form a complex with DP proteins, which is a prerequisite for E2Fs to bind to target promoters. Although no full-length E2FB protein could be detected in either of these mutants (Supplemental Figure S5B; and for *e2fb-2* see Berckmans et al., 2011), the size and morphology of both *e2fb-1* and *e2fb-2* seedlings were largely comparable to WT; however, the area of the first leaf pair was moderately, but significantly, larger than that in WT at 8 DAG and 12 DAG (Supplemental Figure S5C, and Supplemental Table S1-2). In young leaves (8 DAG), the cell number in *e2fb* mutants was comparable to WT, but cells were found to be enlarged in size (Supplemental Table S1). Flow cytometry analysis revealed that some *e2fb* mutant leaf cells entered prematurely into

294 the endoreduplication cycle (Supplemental Figure S5D), thus suggesting that certain cells exit
295 cell proliferation earlier. By contrast, at the later developmental stage of 12 DAG, the number
296 of leaf epidermal cells in both *e2fb* mutants was significantly increased in comparison to WT
297 (Figure 3A and Supplemental Table S1-2). By introducing pgE2FB-GFP into the *e2fb-2*
298 mutant background, we could restore *e2fb* leaf epidermis cell number close to that of WT,
299 providing evidence of functional complementation (Figure 3A, and Supplemental Table S1-
300 2).

301 It is known that cells with meristemoid identity have a characteristic round or square
302 shape and a small cell size below the stomata guard cells that is less than 100 μm^2 (Dong et
303 al., 2009). We measured these cell types on the leaf epidermis at 12 DAG and found them to
304 be distributed below 60 μm^2. To reveal whether the increased cell number may result from
305 the overproliferation of meristemoids, we counted cells smaller than 60 μm^2. We indeed
306 found a much larger increase in both *e2fb* mutants within this cell population (Figure 3B). In
307 agreement, the total number of fully developed stomata also increased in the *e2fb* mutant
308 lines (Supplemental Table S1-2). These phenotypes were also complemented by expressing
309 E2FB-GFP in the *e2fb-2* mutant (Figure 3B), indicating that E2FB represses the proliferation
310 of leaf meristemoid cells. The E2FB-GFP protein accumulated to a much higher level in the
311 pgE2FB-GFP-complemented *e2fb-2* lines than that of endogenous E2FB protein in WT,
312 which explains why there was overcompensation (Figure 3D).

313 To study the impact of *e2fb* mutation on the expression of E2F target genes, we
314 selected the S-phase-specific genes *ORC2* and *MINICHROMOSOME MAINTENANCE*
315 *COMPLEX COMPONENT 3* (*MCM3)*, the mitotic *CDKB1;1* and *CYCLIN A2;3* (*CYCA2;3)*,
316 and the two mitosis upstream regulators *CYCD3;1* and *RBR*. The expression levels of all
317 these genes were reduced in the *e2fb* mutants, especially in young leaves (8 DAG). The
318 reduction was stronger in the null-mutant *e2fb-2* than in *e2fb-1* (Figure 3C). We also
319 investigated how the expression levels of the other two *E2F* genes were affected in the *e2fb*
320 mutants. The expression of *E2FA* did not change, whereas the *E2FC* transcript level showed
321 a slight elevation from 10 DAG onwards (Supplemental Figure S5E).

322 To gather further evidence that the mitotic *CDKB1;1*, *CYCD3;1*, and *RBR* genes are
323 directly regulated through the binding of E2FB to their promoters, we performed chromatin
324 immunoprecipitation (ChIP) experiments using the *e2fb-2* mutant complemented with the
325 pgE2FB-GFP construct. There was a significant enrichment of E2FB-GFP protein at the

promoter of these genes, specifically in the regions where consensus E2F binding elements were predicted (Figure 4A and B).

These results show that whereas E2FB is required for the full activation of cell cycle target genes at early stages of leaf development, its absence does not result in compromised cell proliferation. On the contrary, E2FB has a prevalent importance to inhibit cell proliferation, though at a later leaf developmental stage. This effect is most pronounced in cells with a small size that likely belong to the stomata meristemoid linage.

Co-overexpression of *E2FB* with its dimerization partner *DPA* does not lead to hyperproliferation in developing leaves

Co-overexpression of *E2FB* but not *E2FA* with the dimerization partner *DPA* was shown to overcome the requirement of the phytohormone auxin to promote cell proliferation in cultured BY2 tobacco cells (Magyar et al., 2005). In animals, the expression of activator E2Fs is increased in most cancer types and thought to be responsible for uncontrolled cancerous cell proliferations (Chen et al., 2009). To determine whether such overexpression causes cell overproliferation in plants, we studied the Arabidopsis line p35S::HA-E2FB/DPA (E2FB/DPA^{OE}), which overexpresses both *E2FB* and *DPA* (De Veylder et al., 2002; Magyar et al., 2012; Horvath et al., 2017). In contrast to the expected deregulation of cell proliferation and disruption of plant development, we did not observe tumorous growth. Leaf initiation proceeded normally; however, E2FB/DPA^{OE} seedlings were smaller and the total leaf area was reduced to half of that of WT (Figure 5A).

To study the cellular basis behind the retarded leaf growth, we imaged the epidermal cell layer of the E2FB/DPA^{OE} line at 8 and 12 DAG (Figure 5B) and measured cell parameters (Supplemental Table S1-2). At 8 DAG we observed predominantly small-sized and polygonal shaped cells across the entire leaf surface, but the total calculated cell number was less than in WT (Figure 5B and Supplemental Figure S6A and S6D), indicating that both cell proliferation and cell enlargement are inhibited at early stages of leaf development by the overexpression of E2FB together with DPA. By contrast, at 12 DAG the calculated leaf epidermal cell number of E2FB/DPA^{OE} was comparable to WT, whereas cell size remained smaller (Figure 5B, 12 DAG, Supplemental Table 2, Supplemental Figure S6D), suggesting that the transition from proliferation to cell elongation is delayed. The reduced stomatal index and the less complex shape of pavement cells (circularity index) at both time points also

indicated an inhibition of stomata as well as pavement cell differentiation (Supplemental Table S1-2). E2FB/DPA^{OE} seedlings also displayed down-curling cotyledons (Figure 5A). In WT cotyledons at 6 DAG, cell proliferation ceases and all pavement and stomata cells appear differentiated. By contrast, there were a large number of small cells in the cotyledons of E2FB/DPA^{OE} seedlings (Supplemental Figure S6B).

In E2FB/DPA^{OE} seedlings, the level of *E2FB* expression increased from 50 to 100 fold that of the WT level throughout leaf development (Figure 5C). By contrast, the accumulation of E2FB protein did not match the constitutive overexpression of the *E2FB* transcript; its level was highly elevated at the earliest time point only (9 DAG) and showed diminished accumulation reaching levels comparable to the endogenous E2FB protein at later timepoints (Figure 5D). The DPA protein level showed the same kinetics as E2FB (Figure 5D), suggesting their developmental co-regulation at the protein level. The level of the mitotic CDKB1;1 protein was also high in young leaves, but diminished towards the 16 DAG timepoint (Figure 5D). The co-regulation of E2FB and DPA protein with the same kinetics was also observed in cotyledons (Supplemental Figure S6C).

Surprisingly, there was no excess of cell proliferation in the E2FB/DPA^{OE} line, and so we looked to see whether there was any deregulation of E2F target genes in this line. We analyzed the expression of two S-phase specific genes, *ORC2* and *MCM3*, and the mitotic *CDKB1;1* (Figure 6A). These E2F target genes were greatly upregulated throughout leaf development in the E2FB/DPA^{OE} line, although they declined in parallel with the diminishing E2FB and DPA protein levels as leaf development progressed (Figure 6A-B and Figure 5D). Two other cell cycle genes were tested, namely the CDK inhibitor *KIP-RELATED PROTEIN 4* (*KRP4*) and *CYCLINA3;1* (*CYCA3;1*), which were also upregulated but not to the same extent and their upregulated expression was not observed at every time point (Supplemental Figure S6E). Expression of the upstream positive and negative regulators of *E2FB*, *CYCD3;1* and *RBR*, respectively, were also upregulated in the E2FB/DPA^{OE} line (Figure 6A), indicating the presence of a regulatory feedback loop. In accordance, we also found an elevated RBR protein level and RBR phosphorylation (P-RBR^{S911}) in E2FB/DPA^{OE} leaves compared to WT (Figure 6B, for quantification see Supplemental Figure S6F and G). RBR was also strongly upregulated in E2FB/DPA^{OE} cotyledons (Supplemental Figure S6C).

To explore how the overexpression of *E2FB/DPA* and the consequent change in RBR level and its phosphorylation affected the amount of RBR-associated E2FB, we performed

co-immunoprecipitation experiments (Figure 6C and D). Utilising the HA-tag on E2FB in the E2FB/DPA^{OE} line, we immunoprecipitated HA-E2FB from seedlings (7 DAG). As Figure 6C shows, only a relatively small amount of DPA was associated with HA-E2FB, and RBR was also not enriched in the complex. However, using the DPA antibody in young leaves (8 DAG), we detected a higher level of immunoprecipitated E2FB as well as RBR compared to those levels observed in seedlings (Figure 6C and D). This shows that RBR effectively binds to the overexpressed E2FB-DPA heterodimer in young leaves, which explains the repression of cell proliferation. However, in some cells or at some cell cycle stages, active RBR-free E2FB-DPA heterodimer must also be present to cause the high upregulation of E2F target genes.

RBR recruitment through E2FB is important to halt cell proliferation in developing leaves

To address how the function of E2FB is dependent on its ability to bind RBR, we constructed a truncated E2FB where we deleted the C-terminal 84 amino-acid region containing the conserved RBR-binding and the overlapping transactivation domains, as we previously did for E2FA (Magyar et al., 2012), and co-overexpressed this HA-tagged E2FB$^{\Delta RBR}$ with DPA (Supplemental Figure S7A), as we did for the full-length E2FA earlier. Two independent HA-E2FB$^{\Delta RBR}$/DPA lines (1 and 10) showed identical developmental abnormalities; their growth was arrested both *in vitro* and on soil (Figure 7A, Supplemental Figure S7B-C). With high frequency (10–15%), we observed abnormally developing seedlings that had three cotyledons and missing or fused organs, indicating abnormal embryo development (Supplemental Figure S7B). In the HA-E2FB$^{\Delta RBR}$/DPA line, we observed clusters of small cells on the leaf epidermis interspersed among large lobbed pavement cells (Figure 7B, and Supplemental Figure S8A and F). Quantifying epidermal cell sizes over a developmental time series (8, 10, and 12 DAG, Supplemental Figure S8B, and Supplemental Table S1-2) showed that the ratio of small-sized cells ($\leq 300\ \mu m^2$) diminished gradually in WT, but remained high in both independent HA-E2FB$^{\Delta RBR}$/DPA lines. On the other hand, large cells (1000–3000 μm^2) formed earlier in the HA-E2FB$^{\Delta RBR}$/DPA lines than in WT, and at 8 DAG the large cells were also more prominent in the middle and the tip region of the leaf (Supplemental Figure S8C). In agreement, the total cell number in the leaf was also higher in the E2FB$^{\Delta RBR}$/DPA lines compared to WT at the later developmental stage of 12 DAG (Supplemental Table S1-2). To reveal the proportion of possible stomata meristemoids

among the small cells that are prominent at the late leaf developmental stage of 12 DAG, we quantified the number of cells with ≤ 60 μm^2. This cell population showed an even larger increase, specifically more than four-fold greater in the HA-E2FB$^{\Delta RBR}$/DPA lines compared to WT (Supplemental Figure S8D).

To reveal whether cell size relates to ploidy changes, we measured the DNA content in the first leaf pairs of HA-E2FB$^{\Delta RBR}$/DPA, but found no difference compared to WT (Supplemental Figure S8E). Thus, the observed phenotypes of HA-E2FB$^{\Delta RBR}$/DPA lines were markedly different from what was observed previously for the HA-E2FA$^{\Delta RBR}$/DPA line, which showed a dramatically elevated extent of endoreduplication (Magyar et al., 2012).

To gather molecular evidence behind the sustained proliferation in the cell clusters observed in the HA-E2FB$^{\Delta RBR}$/DPA line, we determined CDK activity using p13^{Suc1} affinity chromatography that pulls down both A- and B-type CDKs (Magyar et al., 2005). As expected, CDK activity declined in WT, whereas it remained high throughout leaf development in the HA-E2FB$^{\Delta RBR}$/DPA line (Figure 7C), further supporting the persistence of cell proliferation in this line. To demonstrate that the C-terminally truncated E2FB cannot bind RBR, we utilised transgenic lines where we tagged at the N-termini of both E2FA and E2FB deletion constructs with GFP for efficient pull down (Figure 7D and see details in Materials and Methods). By using these transgenic lines in co-immunoprecipitation experiments, we confirmed that neither E2FA nor E2FB could pull RBR down in the absence of the C-terminal RBR-binding domain, but both associated with the DPB protein (Figure 7D).

We also determined the expression of cell cycle E2F target genes (*ORC2, CDKB1;1, CYCD3;1*, and *RBR*) in both HA-E2FB$^{\Delta RBR}$/DPA lines during leaf development (Figure 7E). The transcript levels of all examined genes were upregulated at 8 and 10 DAG compared to WT (Figure 7E). Since HA-E2FB$^{\Delta RBR}$ lacks the transactivation domain, this upregulation is likely due to the lack of RBR repression on these genes.

In summary, whereas the deletion of the RBR-binding domain in the HA-E2FA$^{\Delta RBR}$/DPA lines leads to dramatic over-endoreduplication (Magyar et al., 2012), the same manipulation made to E2FB in HA-E2FB$^{\Delta RBR}$/DPA lines results in overproliferation of cell clusters during leaf development.

Discussion

Plant growth is centred on meristem activity, yet surprisingly little is known about how cell proliferation is regulated at the molecular level in a developmental context. E2F transcription factors are the prime candidates for regulating meristematic function in close association with RBR. Previously, we showed that E2FA in complex with RBR is involved in meristem maintenance (Magyar et al., 2012). E2FB was considered as a canonical transcriptional activator, and indeed we found that its overexpression can activate the expression of cell cycle genes, whereas *e2fb* mutations compromise expression of these same genes. However, the cell proliferation outcome does not follow these molecular changes in the developing leaves. On one hand, elevated or ectopic overexpression of E2FB (pgE2FB-GFP or p35S:HA-E2FB/DPA) causes a decrease in total cell number rather than an increase. On the other hand, the *e2fb* mutant lines produce more cells during leaf development in comparison to the control WT. Furthermore, we demonstrated both biochemically and genetically that the repressor function of E2FB on cell proliferation relies on the RBR association, which is reinforced by autoregulatory loops.

In animal cells, Rb level and activity increases as cells exit proliferation and enter differentiation (Zacksenhaus et al., 1996). By contrast, RBR in plants is most abundant in meristematic cells, and its level diminishes as development proceeds (Borghi et al., 2010; Magyar et al., 2012). Thus, RBR co-expresses with E2FA and E2FB in proliferating plant cells and forms repressor complexes. Moreover, we found that elevated and ectopic overexpression of E2FB leads to increased RBR level. This autoregulatory loop enforces the repression, which ensures that cell proliferation is kept under control and thus increased E2FB level does not lead to tumorous growth. RBR repression on cell proliferation through inhibiting E2FB is suppressed by RBR phosphorylation, and E2FB positively regulates the regulatory cyclin subunit (CYCD3;1) of the RBR-kinase (CDKA;1) as well. It is known that Rb phosphorylation and thus repressor activity is cell-cycle regulated; dephosphorylated Rb is active in G1 phase and as cells pass through the G1/S control point the hyperphosphorylated Rb becomes inactive, leading to the expression of cell cycle genes (Morgan, 2007). It is feasible that in plants the elevated E2FB and consequent RBR levels in G1 leads to overabundance of E2FB-RBR repressor complex and thereby inhibition of cell proliferation, whereas after cells pass through the control point, when RBR becomes hyperphosphorylated, the overexpressed and now free E2FB hyperactivates cell cycle target

genes. A block in cell proliferation is consistent with increased 2C DNA content when E2FB is elevated.

Both the protein levels of E2FB and RBR decline as leaf development proceeds. During this transition phase from cell proliferation to differentiation, the E2FB-RBR complex is important to exit cell proliferation and to establish quiescence. When E2FB escapes from RBR repression after the transition phase, differentiated cells re-enter cell division, which is the case when E2FB level is elevated with expression driven by its own promoter. When E2FB is ectopically overexpressed together with DPA, these extra cell divisions of differentiated pavement cells were not present. Instead, cells are arrested in an undifferentiated state, as indicated by their small size without lobbed shape and decreased number of stomata. This suggests that overexpression of E2FB together with DPA prevents the transition from proliferation to differentiation. Thus, the ectopic co-overexpression of E2FB with DPA or elevation of E2FB with expression driven by its own promoter have very different consequences. In the first case, a large amount of E2FB-DPA heterodimer is present that is still kept under control of RBR to inhibit both cell proliferation and differentiation, leading to growth arrest. The destabilisation of E2FB and DPA during leaf development may allow an escape mechanism from this block. By contrast, elevated E2FB with expression driven by its own promoter can form heterodimers either with the endogenously available DPA or DPB. It was suggested that the interaction of DPA with activator E2Fs stimulates nuclear translocation and mediates a higher level of transactivation than interaction with DPB (Kosugi and Ohashi, 2002). This might explain why there is less pronounced growth arrest and cells can exit proliferation when the E2FB level is elevated on its own.

We show that E2FB is required and sufficient to restrain cell proliferation in developing leaves by demonstrating that leaves produce fewer cells when *E2FB* is overexpressed and more cells when it is mutated. We also show biochemically that E2FB has strong affinity to associate with RBR in young leaves enriched with proliferating cells. To provide further evidence that RBR acts through E2FB to inhibit cell proliferation, we deleted the C-terminal RBR binding domain of E2FB and overexpressed this mutant form with DPA. Indeed, we observed overproliferation of cells in developing leaves that strongly suggests that the formation of RBR-E2FB repressor complex is important to control cell proliferation during leaf development. Based on their small size and shape, proliferation in clusters, and the increased number of fully developed stomata at a later stage, the cell overproliferation is

likely within the stomata meristemoid linage, but this has to be confirmed by cell type specific markers, such as the expression of *SPEECHLESS*. Because the C-terminal deletion on E2FB also removed the transactivation domain, the overproliferation of meristemoids must be a consequence of derepression from RBR control. The presence of meristemoid overproliferation in two independent *e2fb* mutants strongly suggests that this phenotype is E2FB specific.

RBR silencing was shown to upregulate the expression of *TOO MANY MOUTH* (*TMM*), the key regulator of stomata meristemoid divisions, leading to their overproliferation (Borghi et al., 2010). At later developmental stages in the stomata linage, RBR silencing can also interfere with the division arrest of the fully developed guard cells (Borghi et al., 2010; Yang et al., 2014). We did not observe such phenotypes when the truncated E2FB was overexpressed, suggesting that RBR does not regulate these later steps in stomata differentiation through E2FB association, but likely through binding and repression of other transcription factors, as it was shown in the case of FAMA (Xie et al., 2010). Interestingly, SOL1 and SOL2, two Arabidopsis homologues of LIN54, a component with DNA binding activity within the mammalian DREAM complex, were shown to regulate cell fate and division in the stomatal lineage (Simmons et al., 2019). Both SOL1 and SOL2 were found to be upregulated in the E2FA/DPA overexpression line, but only SOL2 was hyper-activated in RBR-silenced RBR-RNAi plants and has the consensus E2F-binding element in its promoter region (Borghi et al., 2010). Accordingly, the E2F-RBR pathway could regulate these transcription factors, but whether these DREAM-related components function in complex with E2Fs and RBR to control cell proliferation in the stomatal lineage is not yet known.

Using GFP-tagged constructs, we found important differences in the expression pattern of these two E2Fs; E2FA is largely restricted to proliferating cells whereas E2FB and RBR are also present in differentiated pavement and fully developed stomata guard cells. The co-occurrence of E2FB but not E2FA with RBR in these differentiated cell types is consistent with the idea that E2FB with RBR is required to repress cell proliferation and impose quiescence to allow differentiation, whereas E2FA acts with RBR to maintain proliferation competence (Magyar et al., 2012). E2FA and E2FB are also distinctly regulated by RBR; excess sucrose or overexpression of *CYCD3;1* promotes E2FA-RBR interaction whereas these factors disrupt E2FB-RBR interaction (Magyar et al., 2012). The distinct cellular phenotypes upon the overexpression of C-terminally truncated dominant-negative forms of

E2FA or E2FB further underlines the difference in the mode of their action in relation to RBR-repression and transactivation of target genes. The overexpression of $E2FA^{\Delta RBR}$ resulted in over-endoreduplication due to the inability to repress the expression of endoreduplication genes (Magyar et al., 2012), whereas $E2FB^{\Delta RBR}$ overexpression had no effect on endoreduplication, but led to the early formation of large pavement cells and clusters of small cells. The fact that overexpression of both the full-length and the truncated forms of E2FA and E2FB have specific phenotypic outcomes suggest that they might have distinct sets of target genes. In agreement, overexpression of E2FA and E2FC also caused very different genes to be deregulated (de Jager et al., 2009).

The functional difference between E2FA and E2FB may rely on their interaction with distinct sets of proteins. As we previously showed, E2FB and E2FC can associate with proteins that are known to be conserved components of the so-called DREAM complex (Kobayashi et al., 2015). By contrast, though E2FA can interact with RBR and DPs, none of the DREAM components were found in complex with E2FA (Horvath et al., 2017). Both E2FB and E2FC, as part of the DREAM complex, function to repress cell proliferation. However, our results suggest that E2FB acts at an earlier stage during the transition from proliferation to differentiation as well as in the immediate establishment of quiescence, possibly as part of the activator MYB3R1/4 complex (Kobayashi et al., 2015), whereas E2FC might be required at a later stage to permanently maintain the cell cycle repression (del Pozo et al., 2006), as part of the repressor MYB3R1/3/5 complex (Kobayashi et al., 2015).

Plants are remarkably resistant to cancerous transformation, but this ability is poorly understood (Doonan and Hunt, 1996). In animals, the activator E2Fs are found to be increased in most cancer types and they contribute to the uncontrolled proliferations (Chen et al., 2009). Here, we show that E2FB, the canonical activator E2F in Arabidopsis, could not drive cancerous divisions even when its level was elevated fifty fold. A potential reason why the large amount of E2FB does not activate tumorous growth is the direct activation of RBR by E2FB and the accumulation of RBR/E2FB repressor complex in proliferating cells. However, CYCD3;1 is also a direct target of E2FB leading to increased RBR phosphorylation and inactivation of RBR repression. It is likely that the simultaneous activation of positive and negative upstream regulators to E2FB is important to keep cell proliferation under tight control in plant cells.

In summary, E2FB-RBR relays meristematic activities to differentiation through the regulation of (1) cell cycle transitions by transcriptional activation of cell cycle genes, (2) cell cycle exit and establishment of quiescence through the repression of cell cycle genes when associated with RBR, and (3) stem cell amplifying divisions through an active repression mechanism together with RBR (Figure 8). Plant growth is fundamentally determined by the number of cells kept in proliferation in the meristem (Bogre et al., 2008). Meristem size is sensitively responsive to environmental conditions and we suggest that the interconnected action of the three E2Fs plays a central role in meristem activities, thus providing an entry point to understand and manipulate the growth potential of plants and crops.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia wild-type (WT) and transgenic seeds were sterilized in commercial bleach, re-suspended in sterile water, and cold-treated at 4°C in darkness for 2 days (Clough and Bent, 1998). Unless otherwise stated, plants were grown under a 16-h light/8-h dark photoperiod at 22°C *in vitro* on half-strength germination medium (1/2GM) with 100 μEm^{-2} s^{-1} light intensity or on soil mixture of decomposed raised bog peat (Plantobalt; Plantaflor Humus Verkaufs-GmbH) under long-day conditions (16-h light/8-h dark) with 100 $\mu Em^{-2}s^{-1}$ light intensity. The cotyledons and the first leaf pairs of WT or the transgenic Arabidopsis lines (p35S:HA-E2FB/DPA, pgE2FB-GFP, and p35S:HA-$E2FB^{\Delta RBR}$/DPA) grown *in vitro* were harvested 8–15 DAG, flash frozen, and stored at -80°C. The T-DNA insertion mutants of E2FB were previously reported (*e2fb-1* - SALK_103138 and *e2fb-2* - SALK_120959; Berckmans et al., 2011; Heyman et al., 2011; Horvath et al., 2017).

Plasmid construction and generation of transgenic Arabidopsis plants

The construct of the pE2FB:gE2FB-GFP (pgE2FB-GFP) and the pE2FA:gE2FA-GFP (pgE2FA-GFP) translational fusion has been described before (Berckmans et al., 2011; Magyar et al., 2012). Using the pgE2FB-GFP construct, transgenic Arabidopsis lines were generated by Agrobacterium-mediated transformation in the WT (Col-0) background and 36 independent T1 Arabidopsis lines were identified on selection medium containing norflurazon. The pgE2FB-GFP construct was also introduced into the *e2fb-2* mutant by Agrobacterium-mediated transformation and homozygous T2 lines were generated afterwards. The genomic sequence of E2FB or E2FA was also fused in frame with triple Venus YFP (3xvYFP) in a pGreenII-based pGII0125 destination vector (Galinha et al., 2007) by using the Invitrogen 3way Gateway System (Invitrogen, USA). The previously described HA epitope-tagged full length E2FB and its C-terminal deletion mutant form (HA-$E2FB^{\Delta RBR}$) missing an 84 amino acid-long region containing the conserved RBR-binding motif (Magyar et al., 2000) were placed under the control of the constitutive cauliflower mosaic virus 35S promoter in the Gateway vector pK7WG2 (Karimi et al., 2002). These constructs were introduced into the previously established p35S:DPA transgenic Arabidopsis line (De Veylder et al., 2002) using the floral-dip method for Agrobacterium-mediated transformation

as described (Zhang et al., 2006). Thirteen p35S:HA-E2FB/DPA co-overexpression transgenic T1 lines were selected based on the presence of the appropriate antibiotic resistance (kanamycin). A strong HA-E2FB expressing single copy T-DNA insertion line was identified and homozygous T2 segregation was selected on kanamycin-containing medium. Twelve p35S:HA-E2FB$^{\Delta RBR}$/DPA primary transgenic lines were identified and two homozygous T2 segregations (named as 1/10 and 10/X) were selected on medium containing kanamycin for further studies. We generated the GFP-tagged version of E2FA$^{\Delta RBR}$ and E2FB$^{\Delta RBR}$ where we cloned the C-terminal deleted version (missing the entire transactivation domains until the Marked box region; deletion of 135- and 160 amino acid-long regions from the C-terminus of E2FA and E2FB, respectively) into the pK7WGF2 gateway vector adding the GFP tag to the N-terminal position. In each case, 15 independent single copy T-DNA insertion lines were identified on kanamycin-containing medium.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

RNA was extracted from leaf samples using the RNeasy Plant Mini Kit (Qiagen, UK). cDNA was synthesized using 1 μg of RNA using the QuantiTect Reverse Transcription Kit (Qiagen). Reverse transcription quantitative PCR (RT-qPCR) in the presence of SYBR Green was carried out using a BioScript PCR kit (Bioline, UK) according to the manufacturer's instructions in a Rotor-Gene 6000 apparatus (Corbert Life Science, Australia). All the data was normalized to housekeeping genes (*ACTIN* and/or *UBIQUITIN*) and the calculated efficiency was added to the analysis. Primer sequences are summarised in Supplemental. Table S3. All reactions were carried out in triplicate.

Image and flow cytometry analysis, determining cellular parameters of leaf samples

To visualize the leaf or cotyledon epidermis, a gel cast was made of the leaf surface, specifically the adaxial side of the first leaf pair, which was then observed under a DIC light microscope Nikon Optiphot 2 as described (Horiguchi et al., 2006).

First true leaf pairs of WT and of various transgenic lines were dissected from seedlings at 8 or 12 DAG. Leaves were stained with propidium iodide (PI, 20 mg/ml) and images on the abaxial side of three different zones (the basal, middle and tip part) of the leaf were taken and

analyzed by confocal laser microscopy (Leica SP5, Germany). Across the three zones, approximately 600 cells were counted and measured per leaf sample, n≥3 were studied for each transgenic line and the control using the Image J software. Average cell size was calculated and the total cell number was extrapolated to the whole leaf according to previously described methods (Asl et al., 2011). The stomata number and stomatal index was calculated in a similar way. For determining the circularity of epidermal cells by using Image J software, guard cells were extracted (Andriankaja et al., 2012). To visualize the distributions of the cell area, only non-guard epidermal cells from the three zones were pooled together and used for calculation at a given time point, unless described otherwise (Asl et al., 2011). The number of elongated pavement cells with newly formed cell wall (described as extra cell division) was counted in all three zones and extrapolated to the whole leaf.

For flow cytometry measurements, the first leaf pairs were collected and chopped with razor blades in nuclei extraction buffer and stained with DAPI as described before (Magyar et al., 2005). Flow cytometry data were obtained using a Partec PAS2 Particle Analysing system (Partec, Germany).

Immunoprecipitation, immunoblotting, and kinase assays

Immunoprecipitation (IP) and immunoblotting assays were carried out as described (Henriques et al., 2010). Briefly, total proteins were extracted from dissected leaves or seedlings in extraction buffer (25 mM Tris-HCl pH 7.5, 75 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 15 mM p-nitrophenylphosphate, 60 mM ß-glycerophosphate, 1 mM DTT, 0.1 % IgePal CA630, 0.5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 × protease inhibitor cocktail-Sigma P9599). Equal amount of proteins were loaded to SDS-Polyacrylamide (PAGE) gel (10% or 12%), and proteins were transferred onto polyvinylidene difluoride (Millipore, Bedford) membranes. The membranes were blocked in 5% (w/v) milk powder with 0.05% (v/v) Tween-20 in TBS (25mM Tris-Cl, pH 8.0, 150mM NaCl; TBST) buffer for one hour at room temperature. The membrane was incubated with 5% (w/v) milk-powder TBST containing the primary antibodies and agitated overnight at 4^0C. Primary antibodies used in immunoblotting experiments: chicken anti-RBR antibody (1:2000 dilution, Agrisera, Sweden), mouse monoclonal anti-PSTAIRE (1:40000 dilution, CDKA;1 specific; Sigma),

rabbit polyclonal antibody anti-CDKB1;1 (1:2000 dilution; Magyar et al., 2005), anti-phospho-specific Rb (Ser807/811) rabbit polyclonal antibody (1:500 dilution, Cell Signaling Tech), anti-E2FB polyclonal rabbit antibody (1:400 dilution, Magyar et al., 2005). After the primary antibody reaction, the membrane was washed three times with TBST, and incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (HRP) for another hour at room temperature, followed by three washing steps (TBST) and afterwards chemiluminescence substrate was applied according to the manufacturer description (SuperSignal West Pico Plus – Thermo Scientific, USA or Immobilon Western HRP – Millipore, USA). For immunoprecipitation equal amount of protein samples (between 500–800 μg) in extraction buffer (see above) were incubated with antibodies or GFP-trap magnetic agarose beads (8–10 μL – ChromoTek, Germany) for 40 minutes to 1 hour at 4°C. The following antibodies have been used in co-IP experiments: anti-DPA (Magyar et al., 2005) and anti-DPB (Umbrasaite et al., 2010), and anti-GFP monoclonal mouse antibody (Roche) or GFP-Trap coupled to magnetic agarose beads (ChromoTek). Protein A or protein G-Sepharose were used to pulldown polyclonal or monoclonal antibodies, respectively, and then the beads were washed three times with extraction buffer and proteins were eluted by adding SDS-sample buffer followed by 5 minutes boiling. Eluted proteins were loaded on SDS-PAGE (10% or 12%) and after protein gel-electrophoresis they were immunoblotted as described above.

The kinase assay was carried out as described earlier (Magyar et al., 1997). Briefly, total proteins were extracted from frozen leaf samples harvested 8–15 DAG and equal protein amounts were incubated with $p13^{Suc1}$-Sepharose beads for an hour at 4°C on rotary shaker. Kinase reaction was initiated by the addition of 1 mg/mL histone H1 substrate and 2.5 μCi of γ-^{32}P-ATP.

Chromatin immunoprecipitations (ChIP)

Chromatin immunoprecipitation (ChIP) assay was carried out as described previously (Saleh et al., 2008). Four grams of E2FB-GFP-, E2FA-GFP-, and GFP-expressing seedlings, the latter from a 35S:GFP line, were crosslinked with 1% (w/v) formaldehyde solution at 6 DAG. Chromatin was precipitated using anti-GFP polyclonal rabbit antibody (Invitrogen) and were collected with salmon sperm DNA/protein A-agarose (Sigma). The purified DNA was used in RT-qPCR reactions to amplify promoter regions with specific primers listed in

Supplemental Table 3. Fold DNA enrichment was calculated by dividing the antibody immunoprecipitation signals with the no-antibody signals.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: ATE2FB, AT5G22220; ATE2FA, AT3G36010; ATE2FC, AT1G47870; ATDPA, AT5G02470; ATDPB, AT5G03415; ATRBR, AT3G12280; ATCDKA;1, AT3G48750, ATCDKB1;1, AT5G54180; ATCYCD3;1, AT4G34160; ATCYCA2;3, AT1G15570; ATCYCA3;1, AT5G34080; KRP4, AT2G32710; MCM3, AT5G46280; ORC2, AT2G37560.

Supplemental Data

Supplemental Figure S1. E2FB and RBR, but not E2FA, are present in differentiated pavement and fully developed stomata guard cells.

Supplemental Figure S2. The E2FB-GFP protein could make complex with DPs, and the non-phosphorylated form of RBR, with these well-known, major interactors of E2FB.

Supplemental Figure S3. Elevated expression of *E2FB* with expression driven by its own promoter inhibits cell proliferation in young leaves and disturbs quiescence in older leaves.

Supplemental Figure S4. E2FB-GFP binds less RBR in older leaves of pgE2FB-GFP line 72 than in line 93.

Supplemental Figure S5. Lack of E2FB function prematurely switches mitosis to endocycle.

Supplemental Figure S6. Elevated HA-E2FB/DPA heterodimer stimulates the accumulation of RBR and its phosphorylated form, RBR^{S911}.

Supplemental Figure S7. Mutant E2FB protein ($HA\text{-}E2FB^{\Delta RBR}$) in conjunction with DPA causes drastic phenotypic changes during development.

Supplemental Figure S8. Expression of $HA\text{-}E2FB^{\Delta RBR}$/DPA hyper-activates cell proliferation of meristemoid cells.

Supplemental Table S1. Cellular parameters quantified from the first leaf pair of WT and E2FB-related transgenic lines of leaf development at 8 DAG.

Supplemental Table S2. Cellular parameters quantified from the first leaf pair of WT and E2FB-related transgenic lines of leaf development at 12 DAG.

Supplemental Table S3. List of primers and their sequences used for RT-qPCR analysis and in ChIP assays.

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure Legends

Figure 1. Elevated E2FB level in its own expression domain inhibits cell proliferation in young leaves and disturbs quiescence in older leaves.

(**A**) Representative confocal laser scanning microscopy (CM) images of the abaxial leaf surface from the first leaf pair of the transgenic line pgE2FB-3xvYFP at 6 and 10 days after germination (DAG; top panels), and localisation in the epidermis and vascular tissues of the same transgenic line at 10 DAG (bottom panels). YFP signal (green) is counterstained for cell

membrane with propidium-iodide (PI, red). Yellow arrows point towards dividing protodermal cells, yellow arrowheads indicate stomatal meristemoids, green arrowheads label fully developed stomata guard cells, blue arrowheads mark elongated pavement cells, and red arrowheads show elongated vascular cells with GFP signal in their nucleus. Scale bars = 20 µm (top panels) and 25 µm (bottom panels).

(**B**) Images of wild type (WT) and the transgenic line with high E2FB expression (pgE2FB-GFP line 72) grown for 9 DAG *in vitro* and for 20 DAG on soil. Scale bars = 0.5 cm.

(**C**) Representative images of the abaxial epidermal cell layer of the first leaf pair from WT and pgE2FB-GFP line 72 seedlings (12 DAG) taken by differential interference contrast microscopy (DIC) for which the imprints were made by the gel casting method. An example of elongated puzzle-formed pavement cell is outlined by red colour. Arrows indicate straight cell walls inside the cell, whereas arrowheads mark newly formed cell walls inside the elongated pavement cells. Scale bars = 20 µm.

(**D**) Quantification of the total number of epidermal cells from first leaf pair of the WT and two pgE2FB-GFP transgenic lines (lines 72 and 93). Values represent means and error bars indicate standard deviation (SD). Significance was determined by Student's *t*-test, *a*: *p*-value <0.05. n= 3 and N > 600. The quantifications of cellular parameters are summarised in Supplemental Table S1 and S2 from 8 DAG and 12 DAG leaves, respectively.

Data information n= biological repeat, N= samples per biological repeat, here and in following Figure legends.

Figure 2. RBR efficiently counteracts the excess of E2FB accumulation in proliferating but not in differentiating first leaf pairs.

(**A**) Relative expression level of *ORC2*, *CDKB1;1*, *CYCD3;1*, and *RBR* in wild type (WT) and pgE2FB-GFP line 72 from the developing first leaf pair of seedlings 8, 10, 12, and 15 days after germination (DAG). Values represent mean of fold change, normalised to the value of the relevant transcript of the WT at 8 DAG, which was set arbitrarily at 1. Error bars: SD. *a*: $p<0.05$, statistical significance determined using Student's *t*-test between WT and the transgenic line at a given time point (n=3, N>50). Abbreviations of genes and primer sequences are listed in Supplemental Table S3.

(**B**) The phosphorylation level of RBR on the conserved Serine site at 911 position (P-RBR^{S911}) was followed in the developing first leaf pair of two independent pgE2FB-GFP-expressing lines (lines 93 and 72), each with different E2FB protein level, and compared to WT at the indicated time points (DAG) using anti-RBR and P-RBR^{S911}-specific antibody (anti-P-Rb$^{807/811}$) in immunoblot analysis.

(**C**) To follow RBR accumulation in conjunction to E2FB level, anti-RBR, anti-E2FB, and anti-GFP antibodies were used in immunoblot analysis of proteins in the developing first leaf pair in the same transgenic lines as in (B). In the first panel, the antibody labels RBR (arrow), in the second panel the anti-E2FB antibody labels both the E2FB-GFP (arrow) and the endogenous E2FB (arrowhead), whereas in the third panel the anti-GFP antibody marks the accumulation of the E2FB-GFP fusion protein (arrow).

(**D**) Co-IP of RBR in the E2FB-GFP pull-down was labelled on the immunoblot with anti-RBR. On the same gel, 1/80 of the IP from the extract of the pgE2FB-GFP 72 line was loaded as input. For comparison, in (**C**) 1/20 of IP was loaded for all genotypes.

Non-specific membrane-bound proteins stained by Coomassie-blue were used as loading control (**C-D**). Note, the quantitation of relative intensities of the protein bands in (B) are shown in Supplemental Figure 4A and 4B, (**C**) in Supplemental Figure 4C and 4D, whereas the measurement related to proteins in (**C** and **D**) are given in Supplemental Figure 4E.

Figure 3. **E2FB restricts cell proliferation in developing first leaf pair.**

(**A**) Total cell number and (**B**) the ratio of small-sized cells (<60 μm^2) in the epidermis of the first leaf pair from wild type (WT), the *e2fb-1* and *e2fb-2* mutant, and from the *e2fb-2* mutant expressing E2FB-GFP under its own promoter (*e2fb-2* E2FB-GFP lines 1 and 2) at 12 days after germination (DAG) (n=3, N>600). Error bars: SD. *a*: $p<0.05$ statistical significance determined using Student's *t*-test between WT and the two *e2fb* mutants, whereas *b*: $p<0.05$ statistical significance between the complemented lines and *e2fb* mutants.

(**C**) Comparison of the *ORC2*, *MCM3*, *CDKB1;1*, *CYCA2;3*, *CYCD3;1*, and *RBR* transcript levels in the first leaf pair of seedlings of the *e2fb-2* and *e2fb-1* mutants and WT at 8, 10, 12, and 15 DAG. Values represent mean of fold change, normalised to the value of the relevant transcript of the WT at 8 DAG which was arbitrarily set at 1 (n=3, N>50). *a*: $p<0.05$ statistical significance determined using Student's *t*-test between WT and the mutant lines. Error bars: SD. Abbreviations of genes and primer sequences are listed in Supplemental Table S3.

(**D**) Endogenous E2FB and transgenic E2FB-GFP proteins were detected in 1-week-old seedlings from WT and from the two complemented *e2fb-2* E2FB-GFP lines (1 and 2). The arrow indicates the position of E2FB, whereas the arrowhead indicates E2FB-GFP. Non-specific, cross-reacting proteins are used as loading control.

Figure 4. **E2FB directly binds to *CYCD3;1*, *CDKB1;1*, and *RBR* promoters.**

(**A**) Schematic representation of the *CYCD3;1*, *CDKB1;1*, and *RBR* promoters; arrows labelled p1, p2, or p3 indicate the position of the primer pairs used for qPCR analysis. The

position of the canonical E2F elements (white arrowheads) and their distance from the start codon (ATG) are depicted. Primer sequences are listed in Supplemental Table S3.

(**B**) Chromatin immunoprecipitation (ChIP) followed by qPCR was carried out on chromatin isolated from complemented *e2fb-2* E2FB-GFP seedlings (7 days after germination; DAG) using polyclonal anti-rabbit GFP antibody; the graph shows fold enrichment calculated as a ratio of chromatin bound to the numbered section of the *CYCD3;1*, *CDKB1;1*, and *RBR* promoters with or without antibody. Shown is a representative experiment of three biological replicates. *a,b*: $p<0.01$, statistically significant enrichment (*a*) between the relevant fragment and the neighbouring fragments and (*b*) between the relevant regulatory region and the negative control (Actin2) determined by Student's *t*-test. The values represent the means of three technical replicates. Error bars: SD. The enrichment on the Actin2 promoter was arbitrarily set to 1. The labels p1, p2, and p3 on the x-axis refer to the regions indicated in (**A**).

Figure 5. Co-overexpression of *E2FB* and *DPA* results in reduced leaf and cell size.

(**A**) Representative images of wild-type (WT) and p35S::HA-E2FB/DPA^{OE} (HA-E2FB/DPA^{OE}) seedlings 8 and 12 days after germination (DAG) grown *in vitro* and 21 DAG grown on soil. Scale bars: 0.5 cm at 8 and 12 DAG; 1 cm at 21 DAG.

(**B**) Representative confocal microscopy images of PI-stained abaxial leaf surfaces taken from tip to base of the first leaf pair from WT and HA-E2FB/DPA^{OE} seedlings (8 and 12 DAG). Scale bars: 20 μm.

(**C**) Comparison of *E2FB* expression levels in the developing first leaf pair of HA-E2FB/DPA^{OE} and WT seedlings at 8, 10, 12, and 15 DAG, where the expression of *E2FB* was set arbitrarily at 1 at each timepoint. Values represent fold change. Error bars: SD referring to technical repeats. The data is from one biological replicate ($N<50$), the transcript level correlates well with the HA-E2FB protein accumulation illustrated in (D).

(**D**) Detection of protein levels of epitope-tagged (HA-E2FB) and endogenous E2FB, DPA, and CDKB1;1 in the first leaf pair of WT and HA-E2FB/DPA^{OE} seedlings at the indicated time points (DAG) using anti-HA, anti-E2FB, anti-DPA, and anti-CDKB1;1 antibodies. The arrowhead indicates the position of HA-tagged E2FB, whereas arrows indicate endogenous E2FB and CDKB1;1 proteins. The asterisk indicates a non-specific protein cross-reaction with the anti-CDKB1;1 antibody. Non-specific membrane-bound proteins stained by Coomassie-blue were used as loading control.

Figure 6. Ectopic E2FB/DPA functions as transcriptional activator on cell cycle genes.

(**A**) The expression levels of *ORC2*, *MCM3*, *CDKB1;1*, *CYCD3;1*, and *RBR* were determined in wild-type (WT) and HA-E2FB/DPA^{OE} seedlings by RT-qPCR. Developing first leaf pair was analysed at each time point as indicated. Values represent mean of fold change normalised to values of the relevant transcript from WT at 8 days after germination (DAG) which was set arbitrarily at 1. Error bars: SD, *a*: $p<0.05$ statistical significance between WT and the transgenic line at a given timepoint, whereas *b*: $p<0.05$ significance between two consecutive timepoints determined using Student's *t*-test (n=3, N>100). Abbreviations of genes and the list of primers used in this study is listed in Supplemental Table S3.

(**B**) Protein level of RBR, P-RBR^{S911}, HA-E2FB, and endogenous E2FB in the developing first leaf pair of WT and HA-E2FB/DPA^{OE} seedlings at 8, 9, and 12 DAG detected using anti-RBR, anti-P-RBR^{S911} (anti-P-Rb$^{807/811}$), anti-E2FB, and anti-CDKA;1 antibodies in immunoblot assays. Note, the relative intensities of the RBR and P-RBR^{S911} protein bands are quantified in Supplemental Figure S6F and G.

(**C** and **D**) Co-immunoprecipitation (co-IP) of HA-E2FB with RBR and DPA proteins in WT and HA-E2FB/DPA^{OE} in seedlings at 7 DAG **(C)** and in first leaf pair at 8 DAG **(D)**. Co-IP of RBR or HA-E2FB proteins with DPA was determined through immunoblot analysis with anti-RBR or anti-E2FB antibodies. 1/25 of the IP from the extract was loaded as input. Asterisk indicates a non-specific protein cross-reaction with the anti-DPA antibody in the input.

In panels **B** and **D**, anti-CDKA;1 antibody was used as control. In panel C, non-specific membrane-bound proteins stained by Coomassie-blue were used as loading control. Arrowhead in panel **B** indicates HA-E2FB and arrows mark the positions of endogenous E2FB, DPA, and CDKA;1 in **B**, **C** and **D**, respectively.

Figure 7. Co-expression of the mutant HA-E2FB$^{\Delta RBR}$ with DPA, which is unable to transactivate and bind to RBR, hyper-activates meristematic cell divisions in leaf epidermis.

(**A**) Representative images of p35S::HA-E2FB$^{\Delta RBR}$/DPA (HA-E2FB$^{\Delta RBR}$/DPA), wild type (WT), and p35S::HA-E2FB/DPA (HA-E2FB/DPA^{OE}) plants grown for 20 days on soil. Scale bar: 1 cm.

(**B**) CM images of PI-stained abaxial leaf surfaces from the first leaf pair of WT and HA-E2FB$^{\Delta RBR}$/DPA seedlings at 10 days after germination (DAG). White outline shows a typical puzzle formed pavement cell. Arrowheads in both images indicate normally dividing meristemoid cells, whereas white circles illustrate clusters of overproliferated meristemoid cells. Scale bars: 20 μm.

(**C**) Total CDK histone H1 kinase activity purified by p13suc1-Sepharose beads is shown and compared to Histone H1 from the first leaf pair at four different developmental time points (8, 10, 12, and 15 DAG). For comparison, CDKA;1 protein level is also shown in the same

leaf samples. Commassie-stained non-specific membrane-bound proteins in the range of 50–60 kDa were used as loading controls.

(**D**) Co-IP of RBR and DPB proteins in the GFP-E2FB$^{\Delta RBR}$ and GFP-E2FA$^{\Delta RBR}$ pull-down was labelled with anti-RBR and anti-DPB antibodies. On the same gel, 1/12th of the IP from the extract of the GFP-E2FB$^{\Delta RBR}$ and GFP-E2FA$^{\Delta RBR}$ lines were loaded as input. Arrows point towards the specific proteins as indicated. The arrowhead indicates a faster migrating DPB protein. Molecular weight markers are indicated on the left.

(**E**) The expression level of *ORC2*, *CDKB1;1*, *CYCD3;1*, and *RBR* was followed in two independent HA-E2FB*ΔRBR*/DPA lines (lines 10 and 1) using RT-qPCR. The developing first leaf pair was analysed at each time point as indicated. Values represent fold change normalised to values of the relevant transcript from WT at 8 DAG, which was set arbitrarily at 1. As the two independent lines show the same tendencies, here n=2, N>50. *a*: $p<0.05$ statistical significance between WT and the transgenic line at a given timepoint determined using Student's *t*-test.

Figure 8. Model explaining the functions of E2FB during leaf development.

E2FB has three different activities, each is being dominant (**A**) at different leaf developmental stage or (**B**) in different cell types.

(**A**) Activator E2FB is in its RBR-free form, characteristic of that in young leaves consisting of mostly proliferating cells. The young meristematic leaf is a nutrient-rich sink-tissue where E2FB is released from the repression of RBR by the CYCD3;1-regulated RBR kinase in a sucrose-dependent manner. E2FB controls the activity of RBR by regulating both its transcriptional and protein level as well as its phosphorylation status by controlling CYCD3;1 activity.

In leaf cells where the growth-promoting signal is weakened, the protein level of both E2FB and RBR decreases and RBR becomes more active (less phosphorylated) to bind and inhibit E2FB. This repression is important to establish quiescence in leaf cells committed to differentiate.

(**B**) In developing leaves, E2FB also forms a repressor complex with RBR in meristemoid leaf cells to co-repress their divisions. How this repression is regulated by up-stream signal(s) is hitherto unknown.

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Figure 1

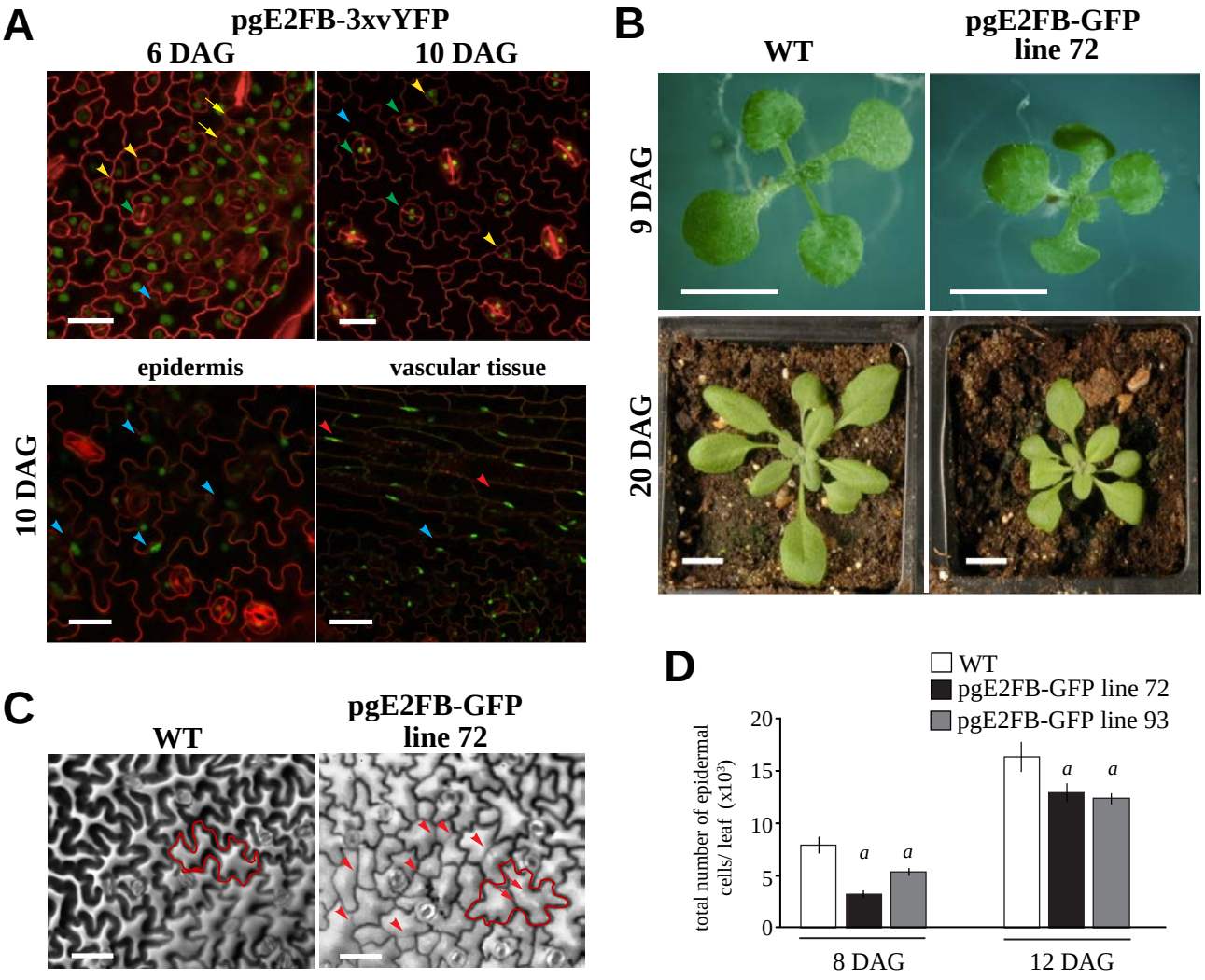
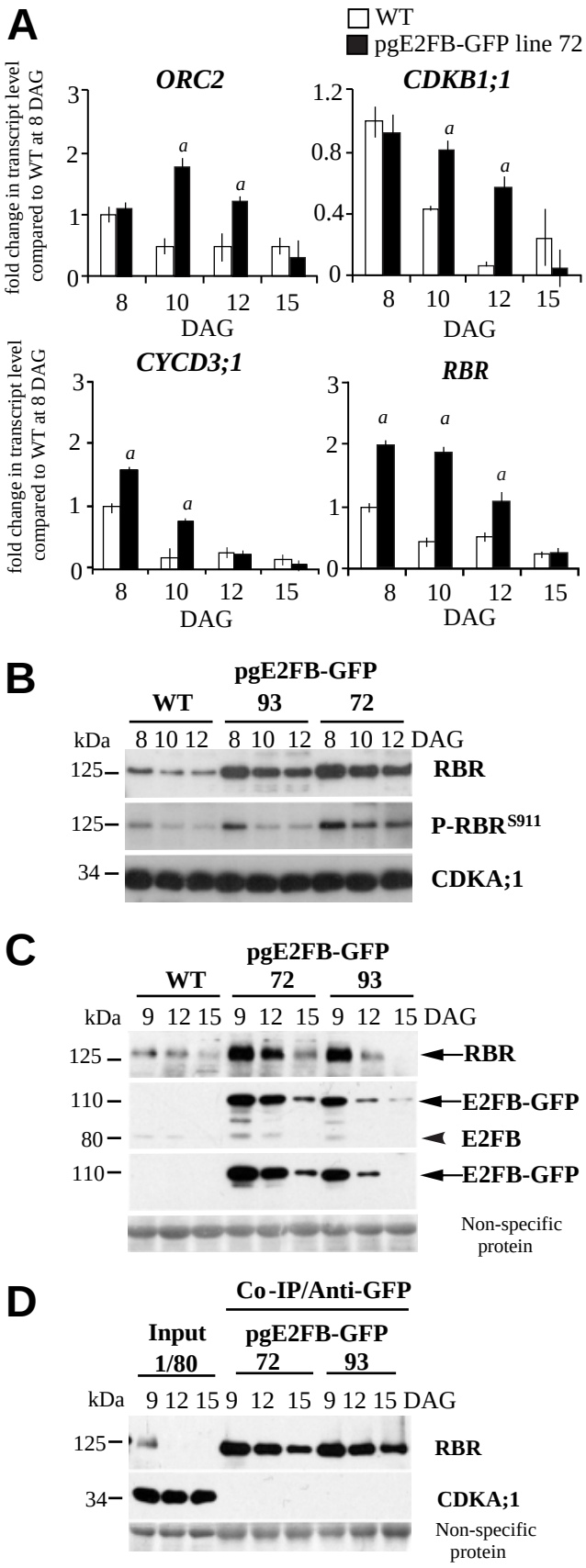


Figure 1. Elevated E2FB level in its own expression domain inhibits cell proliferation in young leaves and disturbs quiescence in older leaves.

(**A**) Representative confocal laser scanning microscopy (CM) images of the abaxial leaf surface from the first leaf pair of the transgenic line pgE2FB-3xvYFP at 6 and 10 days after germination (DAG; top panels), and localisation in the epidermis and vascular tissues of the same transgenic line at 10 DAG (bottom panels). YFP signal (green) is counterstained for cell membrane with propidium-iodide (PI, red). Yellow arrows point towards dividing protodermal cells, yellow arrowheads indicate stomatal meristemoids, green arrowheads label fully developed stomata guard cells, blue arrowheads mark elongated pavement cells, and red arrowheads show elongated vascular cells with GFP signal in their nucleus. Scale bars = 20 µm (top panels) and 25 µm (bottom panels).
(**B**) Images of wild type (WT) and the transgenic line with high E2FB expression (pgE2FB-GFP line 72) grown for 9 DAG *in vitro* and for 20 DAG on soil. Scale bars = 0.5 cm.
(**C**) Representative images of the abaxial epidermal cell layer of the first leaf pair from WT and pgE2FB-GFP line 72 seedlings (12 DAG) taken by differential interference contrast microscopy (DIC) for which the imprints were made by the gel casting method. An example of elongated puzzle-formed pavement cell is outlined by red colour. Arrows indicate straight cell walls inside the cell, whereas arrowheads mark newly formed cell walls inside the elongated pavement cells. Scale bars = 20 µm.
(**D**) Quantification of the total number of epidermal cells from first leaf pair of the WT and two pgE2FB-GFP transgenic lines (lines 72 and 93). Values represent means and error bars indicate standard deviation (SD). Significance was determined by Student's *t*-test, *a*: *p*-value <0.05. n= 3 and N > 600. The quantifications of cellular parameters are summarised in Supplemental Table S1 and S2 from 8 DAG and 12 DAG leaves, respectively.
Data information n= biological repeat, N= samples per biological repeat, here and in following Figure legends.

Figure 2



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(**A**) Relative expression level of *ORC2*, *CDKB1;1*, *CYCD3;1*, and *RBR* in wild type (WT) and pgE2FB-GFP line 72 from the developing first leaf pair of seedlings 8, 10, 12, and 15 days after germination (DAG). Values represent mean of fold change, normalised to the value of the relevant transcript of the WT at 8 DAG, which was set arbitrarily at 1. Error bars: SD. *a*: $p<0.05$, statistical significance determined using Student's *t*-test between WT and the transgenic line at a given time point (n=3, N>50). Abbreviations of genes and primer sequences are listed in Supplemental Table S3.

(**B**) The phosphorylation level of RBR on the conserved Serine site at 911 position (P-RBR^{S911}) was followed in the developing first leaf pair of two independent pgE2FB-GFP-expressing lines (lines 93 and 72), each with different E2FB protein level, and compared to WT at the indicated time points (DAG) using anti-RBR and P-RBR^{S911}-specific antibody (anti-P-Rb$^{807/811}$) in immunoblot analysis.

(**C**) To follow RBR accumulation in conjunction to E2FB level, anti-RBR, anti-E2FB, and anti-GFP antibodies were used in immunoblot analysis of proteins in the developing first leaf pair in the same transgenic lines as in (B). In the first panel, the antibody labels RBR (arrow), in the second panel the anti-E2FB antibody labels both the E2FB-GFP (arrow) and the endogenous E2FB (arrowhead), whereas in the third panel the anti-GFP antibody marks the accumulation of the E2FB-GFP fusion protein (arrow).

(**D**) Co-IP of RBR in the E2FB-GFP pull-down was labelled on the immunoblot with anti-RBR. On the same gel, 1/80 of the IP from the extract of the pgE2FB-GFP 72 line was loaded as input. For comparison, in (**C**) 1/20 of IP was loaded for all genotypes.

Non-specific membrane-bound proteins stained by Coomassie-blue were used as loading control (**C**-**D**). Note, the quantitation of relative intensities of the protein bands in (B) are shown in Supplemental Figure 4A and 4B, (**C**) in Supplemental Figure 4C and 4D, whereas the measurement related to proteins in (**C** and **D**) are given in Supplemental Figure 4E.

Figure 3

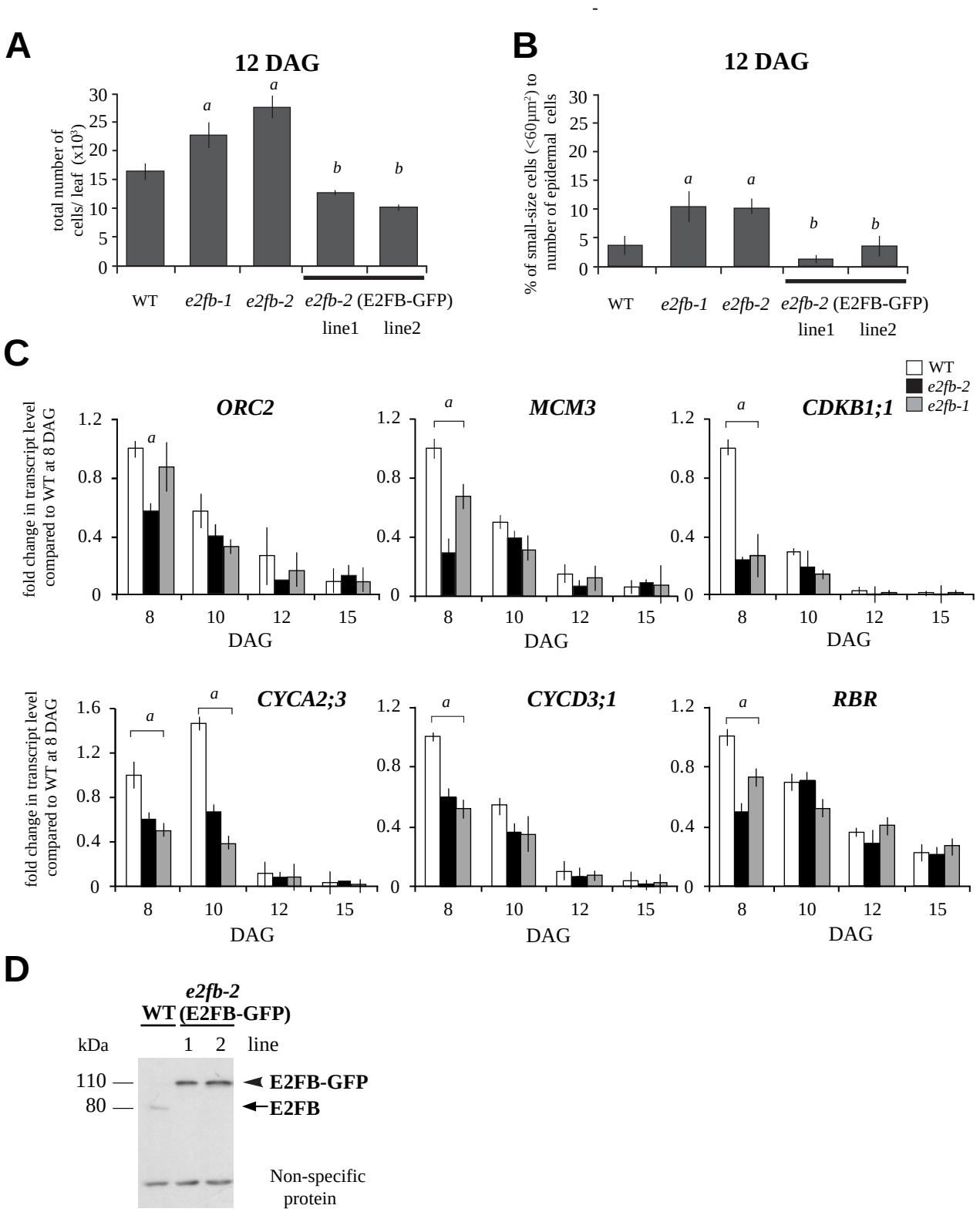

Figure 3. **E2FB restricts cell proliferation in developing first leaf pair.**

(**A**) Total cell number and (**B**) the ratio of small-sized cells (<60 μm^2) in the epidermis of the first leaf pair from wild type (WT), the *e2fb-1* and *e2fb-2* mutant, and from the *e2fb-2* mutant expressing E2FB-GFP under its own promoter (*e2fb-2* E2FB-GFP lines 1 and 2) at 12 days after germination (DAG) (n=3, N>600). Error bars: SD. *a*: $p<0.05$ statistical significance determined using Student's *t*-test between WT and the two *e2fb* mutants, whereas *b*: $p<0.05$ statistical significance between the complemented lines and *e2fb* mutants.
(**C**) Comparison of the *ORC2*, *MCM3*, *CDKB1;1*, *CYCA2;3*, *CYCD3;1*, and *RBR* transcript levels in the first leaf pair of seedlings of the *e2fb-2* and *e2fb-1* mutants and WT at 8, 10, 12, and 15 DAG. Values represent mean of fold change, normalised to the value of the relevant transcript of the WT at 8 DAG which was arbitrarily set at 1 (n=3, N>50). *a*: $p<0.05$ statistical significance determined using Student's *t*-test between WT and the mutant lines. Error bars: SD. Abbreviations of genes and primer sequences are listed in Supplemental Table S3.
(**D**) Endogenous E2FB and transgenic E2FB-GFP proteins were detected in 1-week-old seedlings from WT and from the two complemented *e2fb-2* E2FB-GFP lines (1 and 2). The arrow indicates the position of E2FB, whereas the arrowhead indicates E2FB-GFP. Non-specific, cross-reacting proteins are used as loading control.

Figure 4

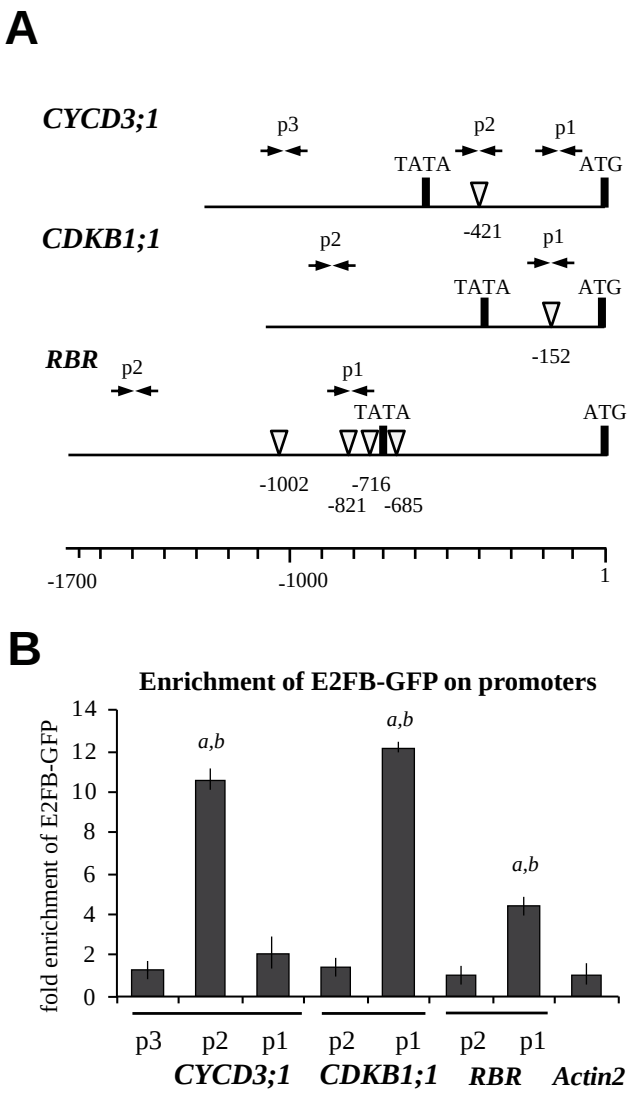

Figure 4. **E2FB directly binds to *CYCD3;1*, *CDKB1;1*, and *RBR* promoters.**

(**A**) Schematic representation of the *CYCD3;1*, *CDKB1;1*, and *RBR* promoters; arrows labelled p1, p2, or p3 indicate the position of the primer pairs used for qPCR analysis. The position of the canonical E2F elements (white arrowheads) and their distance from the start codon (ATG) are depicted. Primer sequences are listed in Supplemental Table S3.

(**B**) Chromatin immunoprecipitation (ChIP) followed by qPCR was carried out on chromatin isolated from complemented *e2fb-2* E2FB-GFP seedlings (7 days after germination; DAG) using polyclonal anti-rabbit GFP antibody; the graph shows fold enrichment calculated as a ratio of chromatin bound to the numbered section of the *CYCD3;1*, *CDKB1;1*, and *RBR* promoters with or without antibody. Shown is a representative experiment of three biological replicates. *a*,*b*: $p<0.01$, statistically significant enrichment (*a*) between the relevant fragment and the neighbouring fragments and (*b*) between the relevant regulatory region and the negative control (Actin2) determined by Student's *t*-test. The values represent the means of three technical replicates. Error bars: SD. The enrichment on the Actin2 promoter was arbitrarily set to 1. The labels p1, p2, and p3 on the x-axis refer to the regions indicated in (**A**).

Figure 5

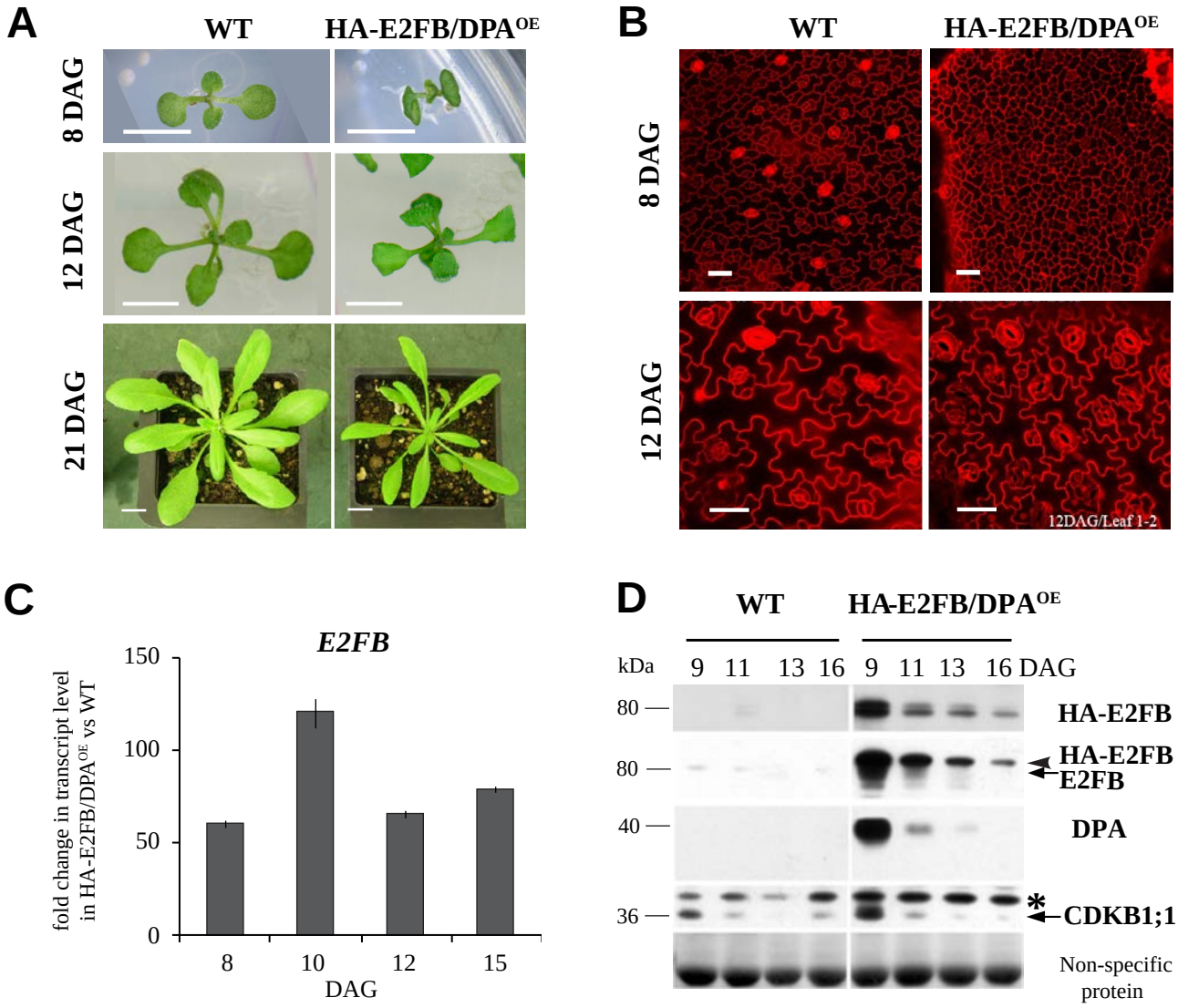

Figure 5. Co-overexpression of *E2FB* and *DPA* results in reduced leaf and cell size.

(**A**) Representative images of wild-type (WT) and p35S::HA-E2FB/DPA^{OE} (HA-E2FB/DPA^{OE}) seedlings 8 and 12 days after germination (DAG) grown *in vitro* and 21 DAG grown on soil. Scale bars: 0.5 cm at 8 and 12 DAG; 1 cm at 21 DAG.

(**B**) Representative confocal microscopy images of PI-stained abaxial leaf surfaces taken from tip to base of the first leaf pair from WT and HA-E2FB/DPA^{OE} seedlings (8 and 12 DAG). Scale bars: 20 µm.

(**C**) Comparison of *E2FB* expression levels in the developing first leaf pair of HA-E2FB/DPA^{OE} and WT seedlings at 8, 10, 12, and 15 DAG, where the expression of *E2FB* was set arbitrarily at 1 at each timepoint. Values represent fold change. Error bars: SD referring to technical repeats. The data is from one biological replicate (N<50), the transcript level correlates well with the HA-E2FB protein accumulation illustrated in (D).

(**D**) Detection of protein levels of epitope-tagged (HA-E2FB) and endogenous E2FB, DPA, and CDKB1;1 in the first leaf pair of WT and HA-E2FB/DPA^{OE} seedlings at the indicated time points (DAG) using anti-HA, anti-E2FB, anti-DPA, and anti-CDKB1;1 antibodies. The arrowhead indicates the position of HA-tagged E2FB, whereas arrows indicate endogenous E2FB and CDKB1;1 proteins. The asterisk indicates a non-specific protein cross-reaction with the anti-CDKB1;1 antibody. Non-specific membrane-bound proteins stained by Coomassie-blue were used as loading control.

Figure 6

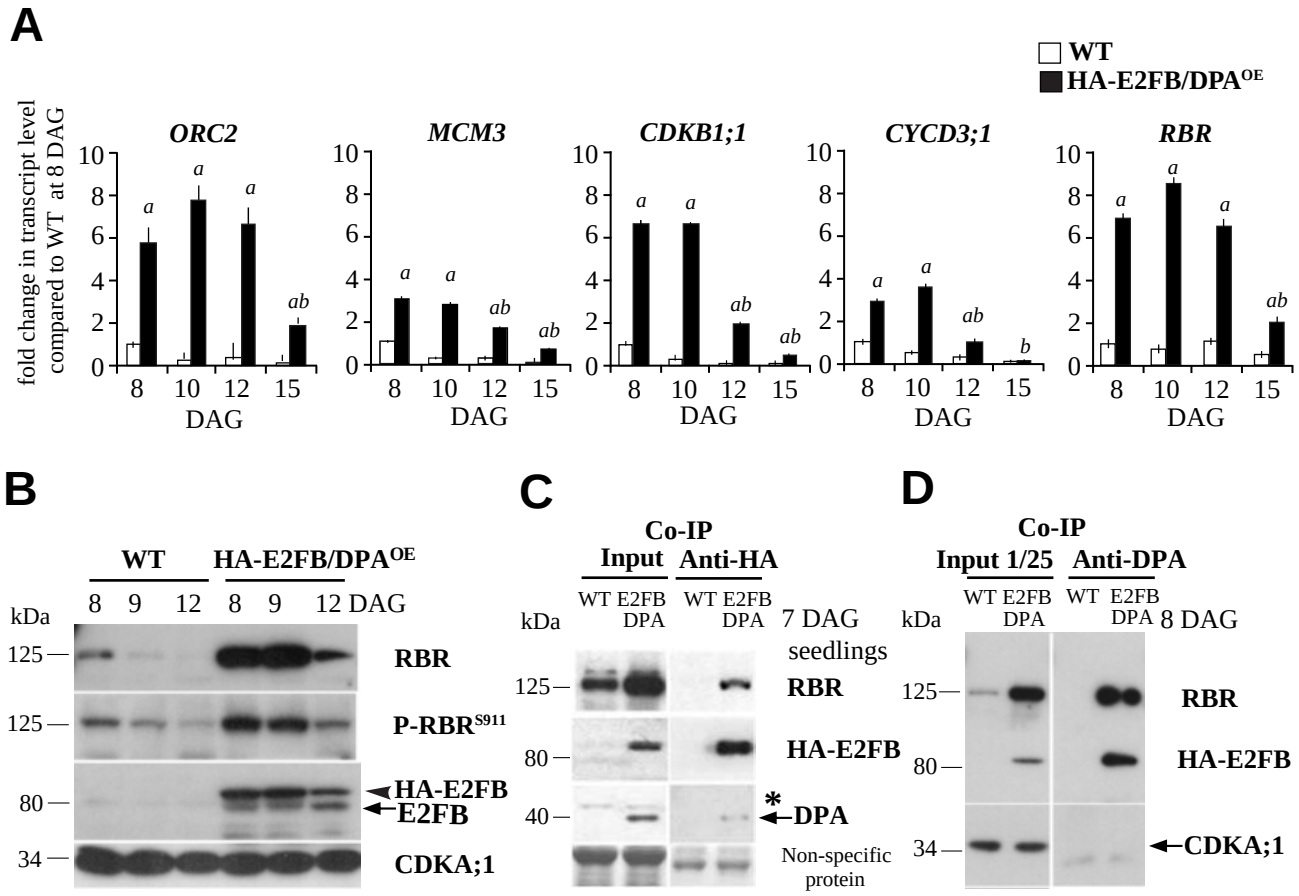

Figure 6. Ectopic E2FB/DPA functions as transcriptional activator on cell cycle genes.

(**A**) The expression levels of *ORC2*, *MCM3*, *CDKB1;1*, *CYCD3;1*, and *RBR* were determined in wild-type (WT) and HA-E2FB/DPA^{OE} seedlings by RT-qPCR. Developing first leaf pair was analysed at each time point as indicated. Values represent mean of fold change normalised to values of the relevant transcript from WT at 8 days after germination (DAG) which was set arbitrarily at 1. Error bars: SD, *a*: $p<0.05$ statistical significance between WT and the transgenic line at a given timepoint, whereas *b*: $p<0.05$ significance between two consecutive timepoints determined using Student's *t*-test (n=3, N>100). Abbreviations of genes and the list of primers used in this study is listed in Supplemental Table S3.

(**B**) Protein level of RBR, P-RBR^{S911}, HA-E2FB, and endogenous E2FB in the developing first leaf pair of WT and HA-E2FB/DPA^{OE} seedlings at 8, 9, and 12 DAG detected using anti-RBR, anti-P-RBR^{S911} (anti-P-Rb$^{807/811}$), anti-E2FB, and anti-CDKA;1 antibodies in immunoblot assays. Note, the relative intensities of the RBR and P-RBR^{S911} protein bands are quantified in Supplemental Figure S6F and G.

(**C** and **D**) Co-immunoprecipitation (co-IP) of HA-E2FB with RBR and DPA proteins in WT and HA-E2FB/DPA^{OE} in seedlings at 7 DAG **(C)** and in first leaf pair at 8 DAG **(D)**. Co-IP of RBR or HA-E2FB proteins with DPA was determined through immunoblot analysis with anti-RBR or anti-E2FB antibodies. 1/25 of the IP from the extract was loaded as input. Asterisk indicates a non-specific protein cross-reaction with the anti-DPA antibody in the input.

In panels **B** and **D**, anti-CDKA;1 antibody was used as control. In panel C, non-specific membrane-bound proteins stained by Coomassie-blue were used as loading control. Arrowhead in panel **B** indicates HA-E2FB and arrows mark the positions of endogenous E2FB, DPA, and CDKA;1 in **B**, **C** and **D**, respectively

Figure 7

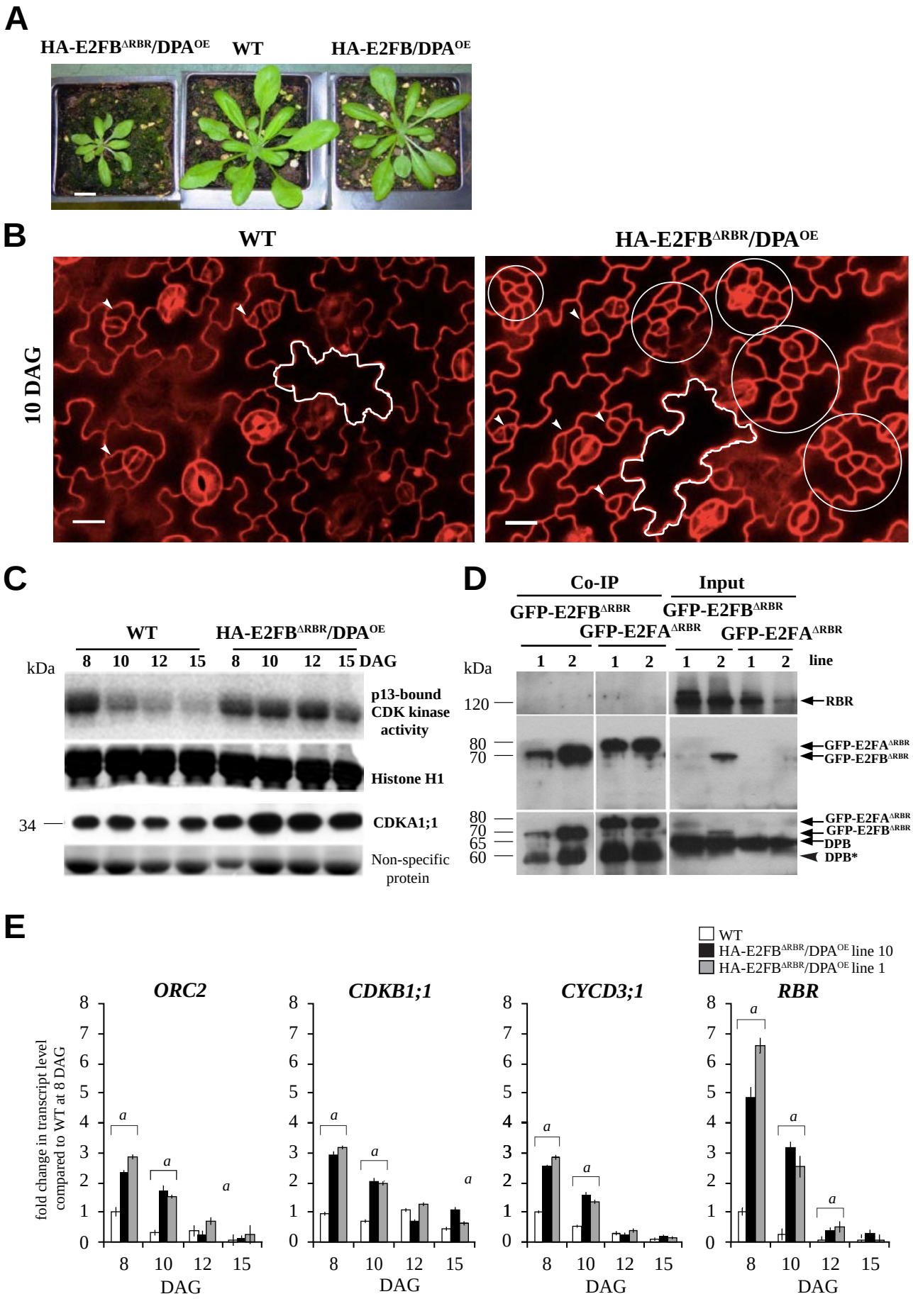

Figure 7. Co-expression of the mutant HA-E2FB$^{\Delta RBR}$ with DPA, which is unable to transactivate and bind to RBR, hyper-activates meristematic cell divisions in leaf epidermis.

(**A**) Representative images of p35S::HA-E2FB$^{\Delta RBR}$/DPA (HA-E2FB$^{\Delta RBR}$/DPA), wild type (WT), and p35S::HA-E2FB/DPA (HA-E2FB/DPA^{OE}) plants grown for 20 days on soil. Scale bar: 1 cm.
(**B**) CM images of PI-stained abaxial leaf surfaces from the first leaf pair of WT and HA-E2FB$^{\Delta RBR}$/DPA seedlings at 10 days after germination (DAG). White outline shows a typical puzzle formed pavement cell. Arrowheads in both images indicate normally dividing meristemoid cells, whereas white circles illustrate clusters of overproliferated meristemoid cells. Scale bars: 20 µm.
(**C**) Total CDK histone H1 kinase activity purified by p13suc1-Sepharose beads is shown and compared to Histone H1 from the first leaf pair at four different developmental time points (8, 10, 12, and 15 DAG). For comparison, CDKA;1 protein level is also shown in the same leaf samples. Commassie-stained non-specific membrane-bound proteins in the range of 50–60 kDa were used as loading controls.
(**D**) Co-IP of RBR and DPB proteins in the GFP-E2FB$^{\Delta RBR}$ and GFP-E2FA$^{\Delta RBR}$ pull-down was labelled with anti-RBR and anti-DPB antibodies. On the same gel, 1/12th of the IP from the extract of the GFP-E2FB$^{\Delta RBR}$ and GFP-E2FA$^{\Delta RBR}$ lines were loaded as input. Arrows point towards the specific proteins as indicated. The arrowhead indicates a faster migrating DPB protein. Molecular weight markers are indicated on the left.
(**E**) The expression level of *ORC2*, *CDKB1;1*, *CYCD3;1*, and *RBR* was followed in two independent HA-E2FB*ΔRBR*/DPA lines (lines 10 and 1) using RT-qPCR. The developing first leaf pair was analysed at each time point as indicated. Values represent fold change normalised to values of the relevant transcript from WT at 8 DAG, which was set arbitrarily at 1. As the two independent lines show the same tendencies, here n=2, N>50. *a*: $p<0.05$ statistical significance between WT and the transgenic line at a given timepoint determined using Student's *t*-test.

Figure 8

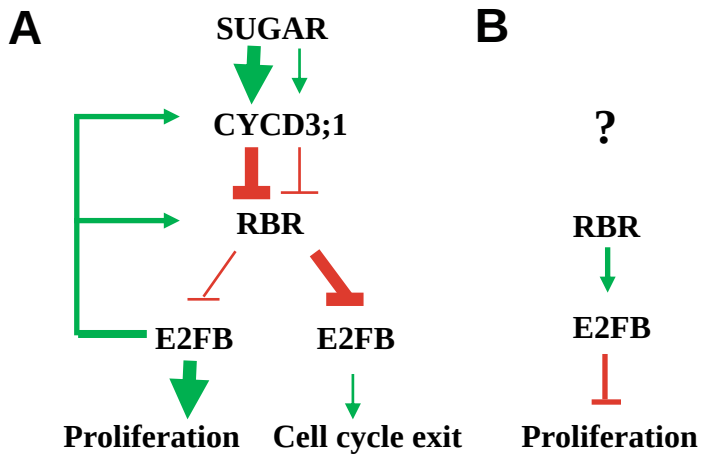

Figure 8. Model explaining the functions of E2FB during leaf development.

E2FB has three different activities, each is being dominant (**A**) at different leaf developmental stage or (**B**) in different cell types.
(**A**) Activator E2FB is in its RBR-free form, characteristic of that in young leaves consisting of mostly proliferating cells. The young meristematic leaf is a nutrient-rich sink-tissue where E2FB is released from the repression of RBR by the CYCD3;1-regulated RBR kinase in a sucrose-dependent manner. E2FB controls the activity of RBR by regulating both its transcriptional and protein level as well as its phosphorylation status by controlling CYCD3;1 activity.
In leaf cells where the growth-promoting signal is weakened, the protein level of both E2FB and RBR decreases and RBR becomes more active (less phosphorylated) to bind and inhibit E2FB. This repression is important to establish quiescence in leaf cells committed to differentiate.
(**B**) In developing leaves, E2FB also forms a repressor complex with RBR in meristemoid leaf cells to co-repress their divisions. How this repression is regulated by up-stream signal(s) is hitherto unknown.

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