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The Role of the *Arabidopsis* E2FB Transcription Factor in Regulating Auxin-Dependent Cell Division [W]

Zoltán Magyar,[a,b,c] Lieven De Veylder,[b] Ana Atanassova,[b] László Bakó,[c,d] Dirk Inzé,[b] and László Bögre[a,1]

[a] Royal Holloway University of London, School of Biological Sciences, Egham, TW20 0EX, United Kingdom
[b] Department of Plant Systems Biology, Flanders Interuniversity Institute for Biotechnology, B-9052 Gent, Belgium
[c] Institute of Plant Biology, Biological Research Center, H-6701, Szeged, Hungary
[d] Department of Plant Physiology, Umeå Plant Science Center, Umeå University, S-90187 Umeå, Sweden

The molecular mechanisms by which the phytohormone auxin coordinates cell division with cell growth and differentiation are largely unknown. Here, we show that in *Arabidopsis thaliana* E2FB, accumulation and stability are positively regulated by auxin. Coexpression of E2FB, but not of E2FA, with its dimerization partner A, stimulated cell proliferation in the absence of auxin in tobacco (*Nicotiana tabacum*) Bright Yellow-2 cells. E2FB regulated the entry into both S- and M-phases, the latter corresponding to the activation of a plant-specific mitotic regulator, CDKB1;1. Increased E2FB levels led to shortened cell cycle duration, elevated cell numbers, and extremely small cell sizes. In the absence of auxin, cells elongated with concomitant increase in their ploidy level, but both were strongly inhibited by E2FB. We conclude that E2FB is one of the key targets for auxin to determine whether cells proliferate or whether they exit the cell cycle, enlarge, and endoreduplicate their DNA.

INTRODUCTION

Auxin is a central molecule in plants that coordinates multiple aspects of growth and cell division, such as root growth and the positioning and outgrowth of leaves, lateral buds, and lateral roots. Differential auxin distribution has been proposed to act as a morphogen to set up distinct zones for cell division, cell expansion, and differentiation. Research with both cultured cells and leaf explants has shown that low and high auxin concentrations promote cell expansion and cell division, respectively (Bhalerao and Bennett, 2003). How differences in auxin concentration are read and translated to lead either to division or to elongation is little understood. AUXIN BINDING PROTEIN1 (ABP1) is a long studied putative auxin receptor and is implicated in the regulation of cell elongation primarily and less so of cell division (Chen et al., 2001a). The auxin-dependent degradation of the auxin/indole-3-acetic acid (AUX/IAA) transcriptional regulators is better understood. Auxin regulates the interaction of the AUX/IAA proteins with the ubiquitin ligase SCF^{TIR1} by direct binding to TIR1, a member of a couple of closely related F-box proteins that together fully account for the auxin binding activity in cell extracts (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). How altered gene transcription results in typical auxin responses, such as the induction of cell division, is largely unknown.

[1] To whom correspondence should be addressed. E-mail l.bogre@rhul.ac.uk; fax 44-1784-434326.
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Progression through the cell cycle is driven by conserved heterodimeric kinases constituted of regulatory subunits, designated as cyclins, and catalytic subunits known as cyclin-dependent kinases (CDKs). Plants possess different classes of CDKs and cyclins. A-type CDKs contain a conserved PSTAIRE cyclin binding motif and function throughout the cell cycle, similar to their yeast and mammalian counterparts; B-type CDKs are plant specific, expressed and active in the G2-phase of the cell cycle. The expression of various D-type cyclins often depends on plant hormones, growth conditions, and development. A- and B-type cyclins have a cell cycle–dependent expression pattern, most of them being restricted to the G2- to M-phases (De Veylder et al., 2003; Dewitte and Murray, 2003).

In animal cells, sequential phosphorylation by CDKs of retinoblastoma protein (RB) at multiple sites results in the inactivation of RB and the release of active E2F-DP transcription factors that induce a wave of transcriptional activity essential to proceed through S-and M-phases. Cell cycle–dependent phosphorylation of plant RB-related protein (RBR) by CDKs was also demonstrated (Nakagami et al., 2002; Espinosa-Ruiz et al., 2004). Mutation in *Arabidopsis thaliana RBR1* is gametophytic lethal, producing megagametophytes with excessive nuclear proliferation, showing its function as a suppressor of proliferation by preventing the expression of genes necessary for DNA replication and mitosis (Ebel et al., 2004). Correspondingly, the virus-induced silencing of the tobacco (*Nicotiana tabacum*) RB homologue *RBR1* led to prolonged cell proliferation and surprisingly also induced DNA endoreduplication in tobacco leaf cells (Park et al., 2005). The *Arabidopsis* genome encodes three E2F proteins, E2FA, E2FB, and E2FC, that form heterodimers with one of the two dimerization partner (DP) proteins, DPA or DPB. We follow the nomenclature established by the genome-wide annotation of the core cell cycle genes in *Arabidopsis*, but E2FA

is also known as E2F3, E2FB as E2F1, and E2FC as E2F2 (Shen, 2002; Vandepoele et al., 2002). The overall domain organization of plant and animal E2Fs is similar, with a highly conserved DNA binding domain, a moderately conserved leucine zipper dimerization domain, and a C-terminal transactivation domain that encompasses a conserved RB binding site. The individual *Arabidopsis* E2Fs differ in their function. E2FA in conjunction with DPA promotes cell proliferation (De Veylder et al., 2002). E2FC is likely to be a repressor because it has a shortened C-terminal transactivation domain, its overexpression results in decreased expression of the S-phase genes, and it inhibits cell division leading to enlarged cells (del Pozo et al., 2002). However, the subdivision of E2Fs into activators and repressors is not entirely clear. E2FC was shown to transactivate reporter genes with promoters containing E2F elements (Mariconti et al., 2002), whereas the E2FA-DPA heterodimer could repress M-phase–specific cyclin genes in a concentration-dependent manner (Kosugi and Ohashi, 2003). In addition, the simultaneously elevated expression of E2FA and DPA results in different leaf cell sizes from extremely small to substantially large (De Veylder et al., 2002; Kosugi and Ohashi, 2003). E2FA has been suggested to trigger DNA synthesis in both cell types, and the presence or the absence of an M-phase promoting factor determines whether they proceed into mitosis or endoreduplication (De Veylder et al., 2002).

Similar to yeast and animal cells, cytoplasmic cell growth and cell division in plant cells are regulated independently, but they are coupled so that growth is required for normal proliferation to produce daughter cells with fixed sizes. We know this from several experiments, including treatments with γ-irradiation, overexpression of the dominant-negative form of CDKA, or overexpression of a CDK-inhibitor protein, designated KIP-related protein1, which all block the cell cycle but not growth, leading to enlarged cells (De Veylder et al., 2001; Tsukaya, 2003). By contrast, overexpression of regulators that promote cell division, such as CYCD3;1, CYCA3;2, or E2FA, leads to cells with reduced sizes (De Veylder et al., 2002; Dewitte et al., 2003; Yu et al., 2003). While these genes stimulate cellular proliferation they strongly inhibit differentiation, resulting in larger numbers of small cells with few or no vacuoles. Cells in plants grow either via increase in cytoplasmic mass or via expansion of vacuoles within cells. Cytoplasmic growth is an attribute of rapidly cycling cells, while cell expansion is a feature of cell differentiation and is often accompanied by endoreduplication. Growth through cell-type specific enlargement can result in cells that are hundreds or even thousands of times their original size in the meristem (Sugimoto-Shirasu and Roberts, 2003). Thus, mechanisms that regulate the switch between cell proliferation and endoreduplication are central to determining cell numbers and cell sizes and, thus, the final sizes of organs and plants. It appears, however, that the variation in cell number rather than cell volume is what contributes to the enormous size differences in organs among even such closely related species as *Arabidopsis* and *Brassica* (Mizukami, 2001).

Here, we compared E2FA and E2FB in their ability to promote cell growth and proliferation. We show that E2FB abundance and stability is increased by exogenously applied auxin, while elevated expression of *E2FB* with *DPA*, but not of *E2FA*, was sufficient to support cell proliferation in the absence of auxin. In contrast with E2FA, E2FB does not promote but rather represses cell enlargement and endoreduplication in auxin-free conditions, resulting in cells with extremely small sizes. We demonstrate that E2FB stimulates cell division by promoting both G1-to-S and G2-to-M transitions, leading to shorter duplication times and uncoupling of growth from cell division.

RESULTS

Endogenous E2FB Interacts with DPA and RBR1 Proteins and Is Expressed throughout the Cell Cycle

To be able to follow E2FB protein, we raised a specific antibody against its divergent C-terminal fragment. The antibody specifically detected only the E2FB but not the E2FA and E2FC proteins (Figure 1A). This experiment clearly shows the specificity of the antibody, but the size of the in vitro–translated E2FA was smaller than detected in plant cell extracts, even though the same expression cassette was used. This anomaly might relate to different posttranslational modifications (Figure 3B; see Supplemental Figure 1C online). Only one E2F protein is known from tobacco that appears to be most similar to the *Arabidopsis* E2FB, but this is not recognized by the specific *Arabidopsis* E2FB antibody (Kosugi and Ohashi, 2003). To prove that the endogenous *Arabidopsis* E2FB is able to dimerize with DPA as well as to bind RBR1, we conducted pull-down experiments with glutathione *S*-transferase (GST)-tagged *Arabidopsis* DPA and RBR1 proteins and *Arabidopsis* cell extracts. We found that E2FB could associate with both RBR1 and DPA and therefore has the characteristics of the canonical E2F transcriptional complexes (Figure 1B). In plants, the E2FA, E2FC, and DP genes were found to be transcriptionally regulated during the cell cycle (Magyar et al., 2000; Mariconti et al., 2002). An exception is E2FB mRNA, which appears to be constitutively present throughout the cell cycle (Mariconti et al., 2002). To test the cell cycle–dependent changes in E2FB protein abundance, we synchronized *Arabidopsis* cells in culture by a release from S-phase block induced by the DNA polymerase α-inhibitor, aphidicolin. As a measure of cell cycle phases G1, S, and G2, we determined the percentage of cells with 2C, intermediate, and 4C DNA contents using flow cytometry at time points after the removal of aphidicolin (Figure 1C). Cells were found to move synchronously through the cell cycle. The E2FB protein amounts in these samples were constant throughout the cell cycle progression (Figure 1D). As cells progress toward mitosis, we do find the appearance of a high mobility form that we think is a hypophosphorylated E2FB (Figure 2C).

Auxin Increases E2FB Abundance through Stabilization of the Protein

To investigate whether plant growth hormones could have an impact on E2FB protein amounts, we compared E2FB levels in *Arabidopsis* cells cultured with and without the hormones required for the proliferation of this culture, the synthetic auxin 1-naphthalene-acetic acid (NAA) and the cytokinin kinetin.

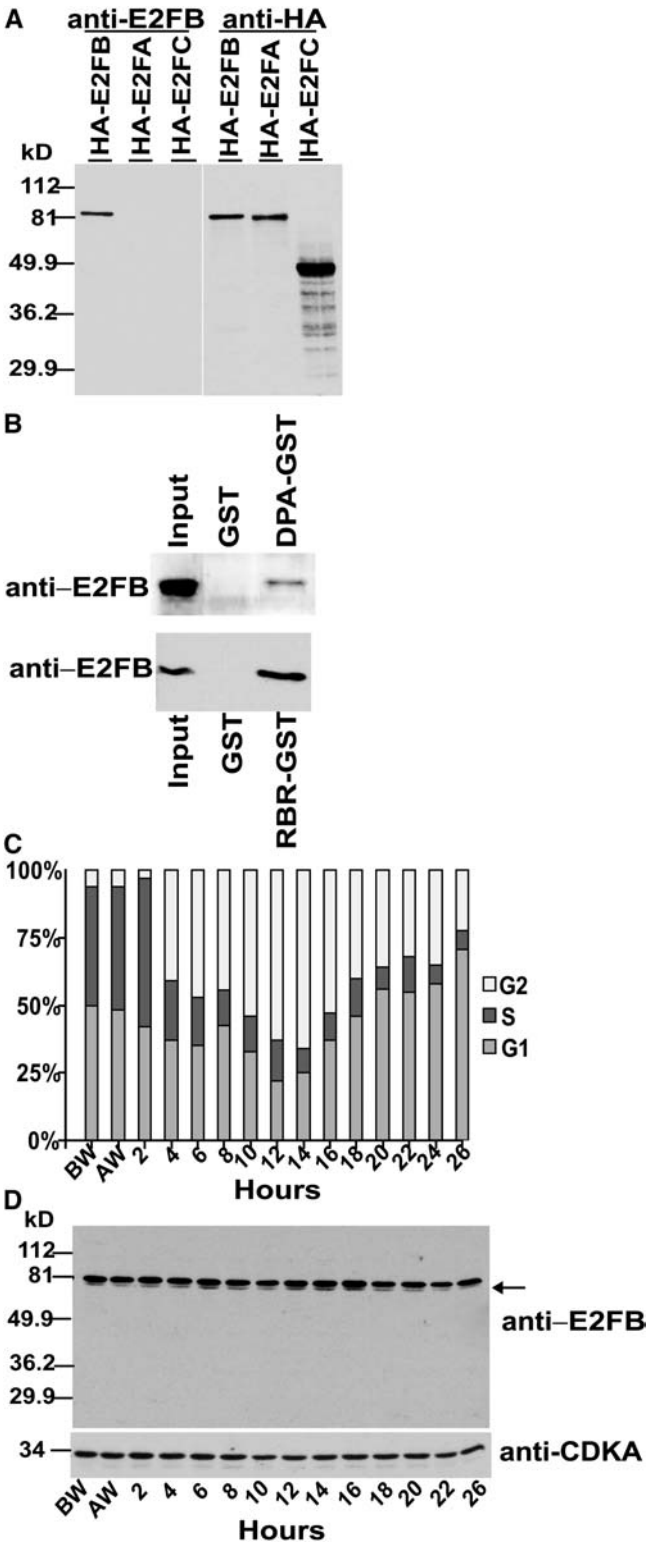

Figure 1. E2FB Interacts with DPA and RBR1 Proteins and Is Constitutively Present throughout the Cell Cycle.

(A) Reaction of anti-E2FB (left three lanes) and anti-HA (right three lanes) antibodies with in vitro–translated hemagglutinin (HA)-tagged E2FA, E2FB, and E2FC proteins.

(B) Protein gel blot analysis with anti-E2FB polyclonal antibody of samples from pull-down experiments from *Arabidopsis* cell extracts with GST, DPA-GST, and RBR1-GST beads and input control, representing one-tenth of the input protein amounts as indicated.

(C) Percentage of cells with G1, S, and G2-M DNA contents as measured by flow cytometry in a cell cycle synchronization experiment from samples before wash of aphidicolin (BW), after wash (AW), and at the indicated time points.

(D) Protein gel blot with anti-E2FB or anti-CDKA (PSTAIRE) antibodies in protein extracts from samples as in **(C)**. Arrow indicates a higher mobility form of the E2FB protein.

Removing these hormones from the medium resulted in a dramatic decrease in the E2FB protein level, whereas readdition of both hormones after 1 d of starvation elevated the E2FB level within an interval as short as 30 min (Figure 2A). No such change was found in the level of CDKA as detected with the anti-PSTAIRE antibody.

Because E2FB levels changed rapidly in response to plant growth hormones, we next asked whether this reflects a post-translational regulation. To test this hypothesis, de novo protein synthesis was blocked by cycloheximide (CHX). In cells cultured without hormones in the presence of CHX, E2FB levels rapidly decreased with an estimated half-life of 10 min. The top band with a size of 80 kD disappeared the most rapidly.

The affinity-purified E2FB antibody could detect three bands in *Arabidopsis* extracts from stationary phase or hormone-starved cultures, while in extracts from logarithmically growing cultures, such as the one used in the synchronization experiments, the 80-kD form is the most prevalent (Figure 1D). Experiments using the phosphatase inhibitor, *p*-nitrophenyl phosphate (pNpp) in the extract indicated that the topmost band was a phosphorylated E2FB form, whereas the bottom band could be a degradation product (Figure 2C).

Addition of the auxin NAA tremendously increased the stability of the topmost 80-kD E2FB form in the presence of CHX, showing unchanged levels for up to 3 h (Figure 2B). In cytokinin, the E2FB protein levels are not maintained to the same extent as in auxin, and the 80-kD form is rapidly disappearing. Interestingly, the addition of cytokinin together with auxin results in the gradual loss of the 80-kD form though not to the extent observed in hormone-free or cytokinin-containing media.

Both E2FA and E2FB Sustain Cell Division under Nutrient-Limited Conditions, but Only E2FB Does so in the Absence of Auxin

As E2FB levels are tightly controlled by auxin, E2F function was studied by controlling the E2F levels experimentally. This work was performed in Bright Yellow-2 (BY-2) tobacco cell cultures rather than in *Arabidopsis* plants or *Arabidopsis* cultured cells because this experimental system allowed us to study auxin-dependent cell growth, cell division, and differentiation in a homogenously responding cell population that has been thoroughly characterized (Nagata and Kumagai, 1999; Geelen and Inzé, 2001). BY-2 cells are grown in the presence of the synthetic auxin 2,4-D and are known to synthesize their own cytokinin pool (Redig et al., 1996). Because of the switch to a different experimental

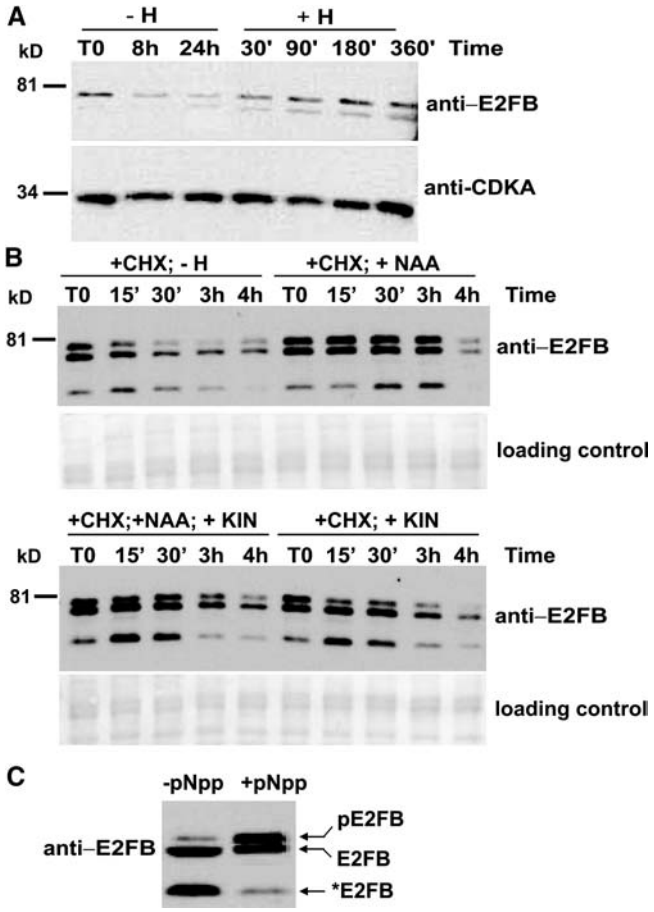

Figure 2. Accumulation and Stability of E2FB Protein Is Regulated by Auxin.

(A) Protein gel blots with anti-E2FB and anti-CDKA (PSTAIRE) antibodies on samples from *Arabidopsis* cultured cells (7 d old; T0) were washed and maintained in hormone-free medium (−H) for 8 and 24 h, when hormones were re-added and cells further incubated for the indicated times (+H).

(B) Protein gel blot with anti-E2FB antibody on samples from *Arabidopsis* cells subcultured with media containing 100 μM CHX and either no hormones (+CHX; −H) or with 0.5 mg/L NAA (+CHX; +NAA), 0.05 mg/L kinetin (+CHX; +KIN), or 0.5 mg/L NAA and 0.05 mg/L kinetin (+CHX; +NAA; +KIN) for the indicated times.

(C) *Arabidopsis* cells at day 7 after subculturing were washed with media without hormones (T0). As a loading control, amido black staining of the same membrane is shown. Molecular mass is indicated on the side. Protein gel blot with anti-E2FB antibody on protein samples extracted from *Arabidopsis* cells at day 7 after subculturing in the presence or in the absence of 15 mM phosphatase inhibitor pNpp. pE2FB indicates the phosphorylated form, and *E2FB is a possible degradation product.

system and to a different synthetic auxin, we also determined the changes of E2FB levels in response to 2,4-D both in BY-2 cells and in *Arabidopsis* (see Supplemental Figure 1 online). The epitope-tagged E2FB expressed under the control of the 35S promoter accumulated to much higher levels in the presence than in the absence of 2,4-D both in stably transformed tobacco cells and in transfected *Arabidopsis* protoplasts.

Previous studies demonstrated that elevated levels of E2F proteins only become potent and fully active when coexpressed with their dimerization partners (DPs) (De Veylder et al., 2002; Kosugi and Ohashi, 2002; Mariconti et al., 2002). As both E2FA and E2FB interact with DPA, we generated stable transgenic BY-2 cell lines coexpressing either epitope-tagged E2FA or E2FB with DPA. As a control, a transgenic BY-2 cell line with the empty vector was used. Out of the 12 independent transformants obtained for the E2FB construct, two lines (line 11 and line 12) that had elevated levels of both E2FB and DPA were selected (Figure 3A). Though a large number of E2FA transformed calli were generated, only in two could we detect the E2FA protein, albeit at lower levels than for E2FB (Figure 3B). The transformed lines differed in their DPA contents; line 3 had a lower level of DPA than line 5. It has been suggested that elevated E2FA activity might interfere with the cell cycle (Kosugi and Ohashi, 2003).

The epitope-tagged E2FB and DPA proteins are likely to be functional in BY-2 cells because they form heterodimers (Figure 3C) and interact with the bacterially purified *Arabidopsis* RBR1 (Figure 3D).

First, we analyzed cell cycle parameters of the chosen E2FA/DPA and E2FB/DPA lines cultured in normal or auxin-free media. As reported previously, under normal growth conditions, the mitotic index peaked at days 2 and 3 and then declined rapidly when cells entered the stationary phase (Figure 4A; Sorrell et al., 2001). On the contrary, in both lines with elevated E2FB levels, the period during which mitotic figures were observed was prolonged (Figure 4A). A similarly extended period of mitotic activity was seen in the culture with elevated E2FA levels, but only in line 5, with a higher DPA level than in line 3 (Figure 4B).

Because we found E2FB levels to be significantly modulated by auxin both in *Arabidopsis* cultured cells, as well as when expressed in BY-2 cells (Figure 2; see Supplemental Figure 1 online), we tested whether elevated E2F levels could abrogate the cell cycle block in BY-2 cells cultured in hormone-free medium. In auxin-starved control cultures, the mitotic index slightly increased up to day 2 and dropped from day 3 (Figure 4C). On the contrary, in the two BY-2 lines with elevated E2FB levels, cells carried on dividing without auxin at a rate similar to control cells in normal hormone-containing medium (Figure 4C). Surprisingly, elevated E2FA levels did not have this capacity to promote cell division without auxin because the E2FA/DPA lines had a mitotic index curve similar to that of the control culture without auxin (Figure 4D). To further confirm that cells keep dividing in hormone-free medium when E2FB is expressed, cells were counted. As shown in Figure 4E, cell numbers continuously increased in the E2FB- but not the E2FA-expressing line.

Next, we investigated cell cycle parameters and ploidy levels by flow cytometry of stationary phase cultures (day 7) of the control, E2FA/DPA, and E2FB/DPA lines. As expected, control cells predominantly displayed a 2C DNA content, suggesting that cells left the cell cycle in G1-phase under normal conditions. By contrast, in the lines with increased E2FA and E2FB levels, some cells were still in S-phase, with sustained mitotic figures in these cultures (Figure 4F). The increase in the proportion of S-phase cells was more pronounced in the E2FB/DPA line. Under hormone-free conditions, endoreduplication was stimulated, leading to 4C and 8C DNA content in control BY-2 cells as

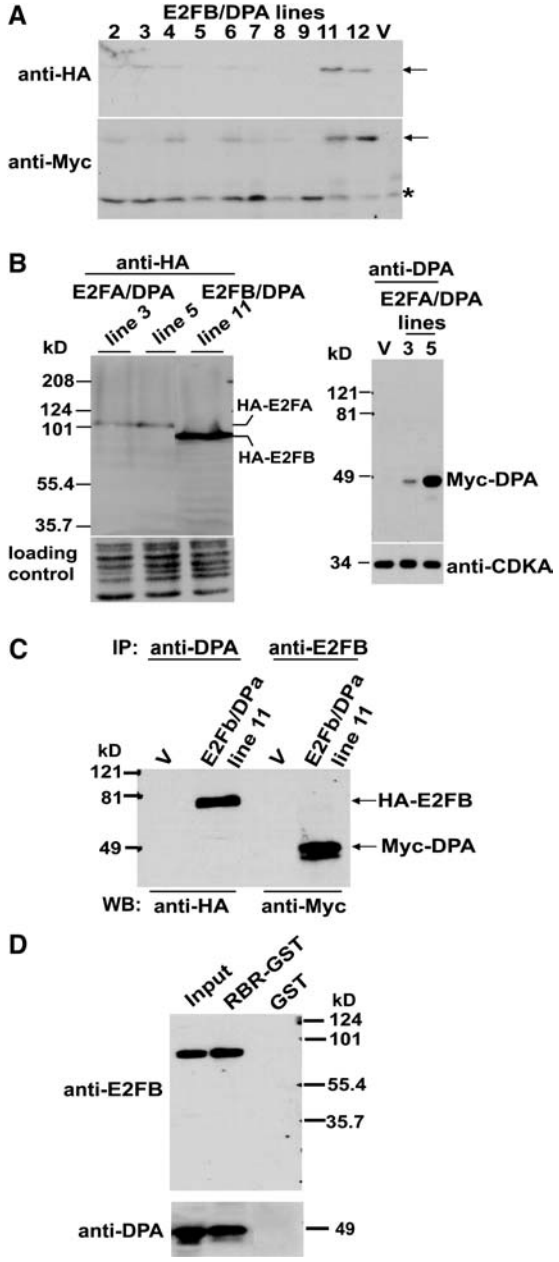

Figure 3. Generation of Transgenic BY-2 Cell Lines Cotransformed with E2FA or E2FB and DPA.

(A) Protein gel blot with anti-HA and anti-Myc antibodies on samples from BY-2 cell lines (numbered) cotransformed with HA-tagged E2FB and c-myc-tagged DPA or only with the empty vector (V). Arrows indicate specific protein bands, and the asterisk marks nonspecific cross-reacting bands with the c-myc antibody as an internal loading control.

(B) Protein gel blot with anti-HA antibody (left panel) using samples from BY-2 cell lines cotransformed with HA-tagged E2FA (line 3 and line 5) or E2FB (line 11) and c-myc-tagged DPA. Specific HA-E2FA and HA-E2FB bands are indicated, and a Coomassie blue–stained portion of a gel is included as loading control. Protein gel blot with anti-DPA antibody (right panel) with samples of E2FA- and DPA-transformed cells (line 3 and line 5) or transformed with the empty vector as a control (V). As a loading control, the same membrane was reblotted with the anti-CDKA (PSTAIRE) antibody. Molecular masses are indicated at the left.

(C) Protein gel blot with anti-HA or anti-Myc antibodies was done to demonstrate in vivo interaction between HA-E2FB and Myc-DPA. Immunocomplexes were purified from extracts of HA-E2FB and Myc-DPA cotransformed BY-2 cells (line 11) by the rabbit polyclonal antibody, anti-DPA (left), or anti-E2FB (right). Control protein samples (V) were taken from BY-2 cells transformed with the empty vector.

(D) Total protein extracts derived from the same line 11 were incubated with *Arabidopsis* RBR1-GST beads, and interacting HA-E2FB and Myc-DPA proteins were detected by anti-E2FB and anti-DPA antibodies. In the pull-down assay, one-tenth of the protein amount was loaded onto the same gel representing the input as labeled. Molecular masses are indicated on the side.

reported before (Quelo et al., 2002). E2FB was a much more potent inhibitor of endoreduplication than E2FA in the absence of auxin, confirming its function in specifically promoting cell division in this condition (Figure 4F).

Finally, we looked at the cell morphology of these lines in the presence or absence of auxin in the medium. In the control BY-2 line close to stationary phase, cells were organized in typical filamentous structures and had nuclei close to the cell wall, indicative of cell cycle arrest (Figure 4G). In the absence of auxin, these filamentous structures were disrupted, with cells enlarged in all directions, as well as larger nuclei. The coexpression of *E2FA* or *E2FB* with *DPA* in hormone-containing medium resulted in a dramatic reduction in the cell size. The nuclei of these cells remained small and centrally positioned, indicative of active cell proliferation. E2FB appeared to be more potent in triggering the small cell phenotype, and we often observed cells with the cell division plane transverse to the filaments (Figure 4G, arrow). There was some heterogeneity in cell size in both the E2FA/DPA and E2FB/DPA lines, which we believe is due to varying expression levels of the transgene in individual cells. Confirming our results on cell cycle parameters, only *E2FB* but not *E2FA* expression could suppress the cell enlargement phenotype of BY-2 cells under the hormone-free condition. Cells of both E2FB/DPA lines became abnormally small to a degree similar to that in hormone-containing medium. The observed cell cycle parameters together with these morphological characteristics strongly support the notion that elevated expression of both *E2FA* and *E2FB* uncouples growth from cell division, but only expression of *E2FB* could overcome cell cycle arrest and inhibit cell enlargement and endoreduplication in the absence of auxin.

Mitotic CDK Levels and Activities Are Largely Elevated in Cells Expressing E2FA and E2FB

How E2FA and E2FB keep cells cycling and lead to such a dramatic decrease in cell size can only be explained if they trigger not only the G1-to-S-phase transition but also the transition to mitosis. To examine this hypothesis at the molecular level, we followed the accumulation and activity of two mitotic regulators, the PSTAIRE containing CDKA;1 and the mitosis-specific CDKB1;1, both of which are known to be downregulated

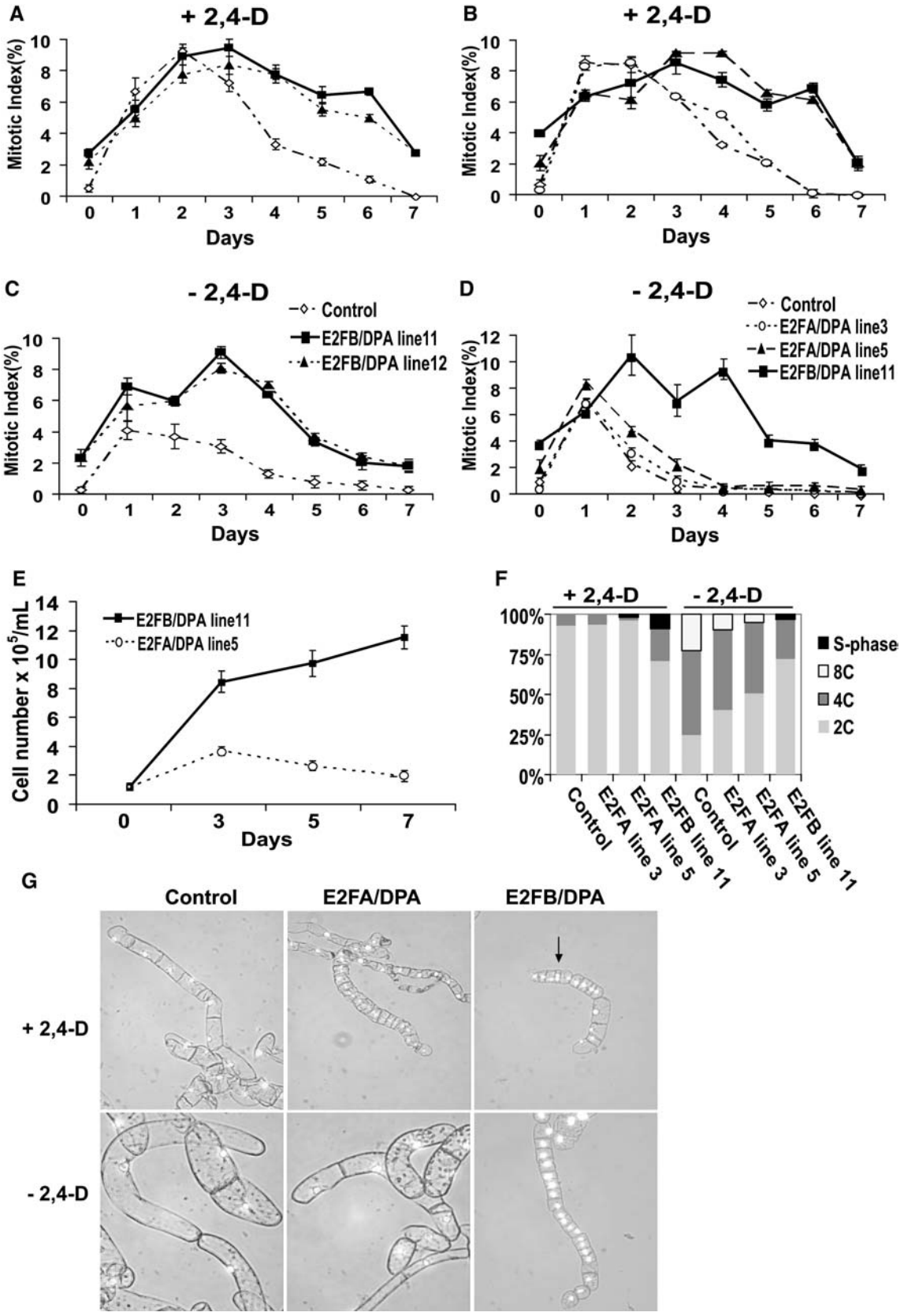

Figure 4. Growth, Cell Cycle, Ploidy, and Morphology of E2FA- and E2FB-Transformed BY-2 Cells Cultured with and without Auxin.

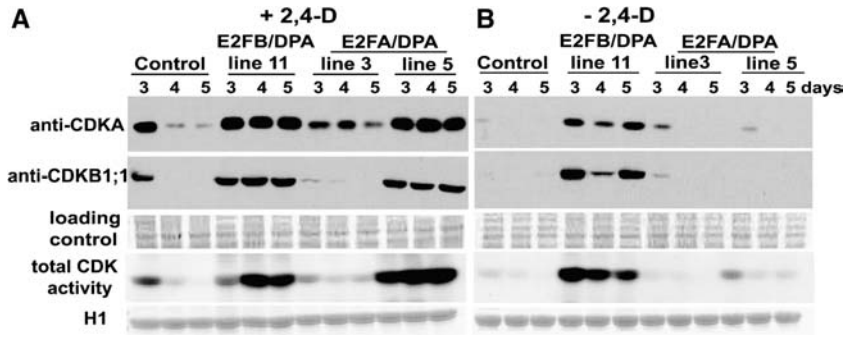

Figure 5. CDK Protein Levels and Histone H1 Kinase Activities in E2FA- and E2FB-Transformed BY-2 Cells in the Presence or Absence of Auxin.

Protein gel blots of samples at 3, 4, and 5 d after subculturing in auxin (+2,4-D; **[A]**) and auxin-free (−2,4-D; **[B]**) mediums from control, E2FB/DPA (line 11), and E2FA/DPA (lines 3 and 5) double-transformed transgenic BY-2 cells with anti-CDK (PSTAIRE) to detect CDKA levels and with anti-CDKB1;1-specific antibodies (top two panels). Coomassie blue–stained gel with these samples as loading control (middle panel). Autoradiogram detecting H1 phosphorylation by total CDK activities purified on $p13^{suc1}$-Sepharose beads from the same samples as used for protein gel blots and the corresponding image of Coomassie blue–stained H1 protein on the gel.

when BY-2 cells enter the stationary phase (Sorrell et al., 2001). We analyzed samples from control, E2FB, and E2FA transgenic cells using PSTAIRE- and CDKB1;1-specific antibodies and measuring total CDK activity by affinity purification of CDKs bound to $p13^{suc1}$-Sepharose beads from day 3 to day 5, with (Figure 5A) and without auxin (Figure 5B). Both the CDKA;1 and CDKB1;1 levels and the total CDK activity perfectly mirrored the mitotic indexes (Figures 4B and 4D). In control cells with auxin (Figure 5A), CDKA;1 was hardly detectable, while the CDKB1;1 protein completely disappeared as cells progressed to stationary phase, similar to control cells cultured without auxin (Figure 5B). On the contrary, no decrease in CDKA;1 or CDKB1;1 occurred up to 5 d in hormone-containing medium in the E2FB/DPA line 11 and in the E2FA/DPA line 5 that coexpress high DPA levels (Figure 5A). Interestingly, the corresponding total CDK activity increased to levels even higher than those in the control line at day 3 in auxin-containing medium (Figure 5A). These experiments confirmed that increased E2FA and E2FB expression could sustain both CDK levels in nutrient-limited stationary phase cultures and possibly also induced factors required for CDK activity, such as cyclins.

The results were quite different when these cell lines were cultured without hormone (Figure 5B). Elevated CDKA;1 and CDKB1;1 levels and increased CDK activity were found only in the E2FB/DPA line 11 (Figure 5B). Thus, under normal growth conditions, both E2FA and E2FB elevate CDK levels and activity either directly or as a consequence of high mitotic activity, but only E2FB does so under hormone-free conditions.

Inducible Expression of E2FB Leads to the Production of More Cells with a Reduced Total Fresh Weight

Constitutively elevated expression of *E2FB* did not allow the maintenance of cell cultures longer than a few months, possibly because of the counterselection of cells with high expression levels as a result of cell division abnormalities. To overcome this problem, *E2FB* expression was controlled by the β-estradiol–inducible expression system, while *DPA* was constitutively expressed (Zuo et al., 2000). We identified nine independent double transformed BY-2 cell lines in which the level of E2FB was tightly controlled and only detectable after treatment with 5 μM β-estradiol (Figure 6A). Interestingly, the level of DPA protein also increased when E2FB expression was induced by β-estradiol, perhaps indicating that the stability of DPA is influenced by the stochiometry of the E2FB DPA proteins (Figure 6A).

In the E2FB/DPA line treated with β-estradiol, more cells were produced than in control, indicating a shorter cell doubling time (Figure 6B). Consistently, the percentage of mitotic cells more than doubled when E2FB expression was induced as compared with the uninduced sample, while no such effect was observed in the culture transformed with the empty vector (Figures 6C and 6D). Surprisingly, although more cells were produced upon

Figure 4. (continued).

(A) to **(D)** Mitotic index of control, E2FB/DPA line 11, E2FB/DPA line 12, and control (**[A]** and **[C]**), E2FA/DPA line 3, E2FA/DPA line 5, and E2FB/DPA line 11 (**[B]** and **[D]**) subcultured to auxin-containing (+2,4-D) (**[A]** and **[B]**) and auxin-free (−2,4-D) medium (**[C]** and **[D]**). Samples were taken daily for 7 d.
(E) Growth curve of BY-2 cells cotransformed with E2FA (line 5) and E2FB (line 11) with DPA in suspension cultures. The mean cell number of triplicates was determined at the indicated time points after subculturing in the absence of auxin.
(F) Flow cytometric analysis of the DNA content of the indicated BY-2 lines at day 7, cultured with (+2,4-D) or without auxin (−2,4-D). The percentage of 2C, 4C, and 8C as well as the S-phase cells are indicated.
(G) Micrograph images of 4′,6-diamidino-2-phenylindole (DAPI)-stained control, E2FA/DPA line 5, and E2FB/DPA line 11 transgenic BY-2 cells are shown 5 d after subculturing into fresh medium supplemented with (+2,4-D) or without auxin (−2,4-D) as indicated.

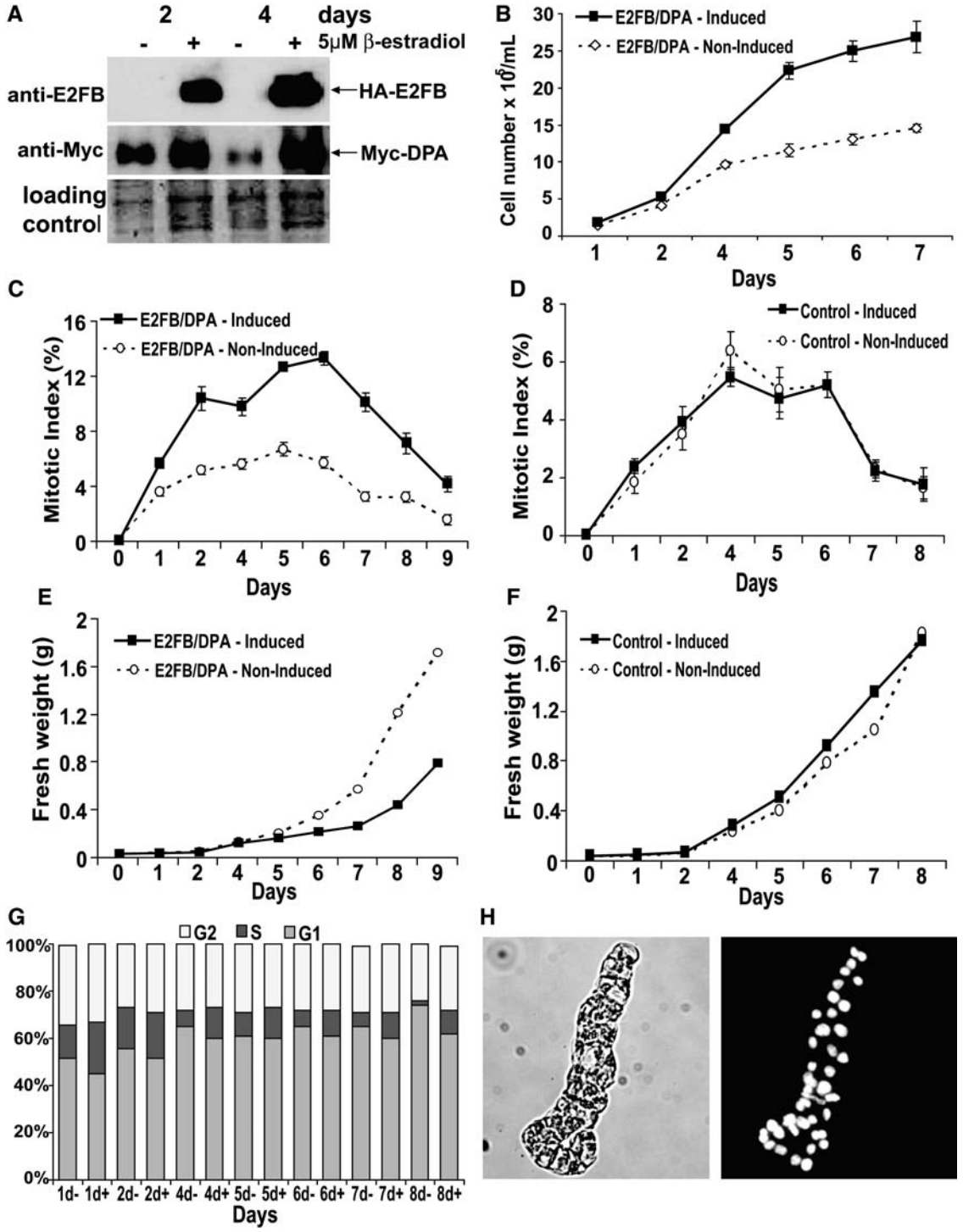

Figure 6. Conditional E2FB Expression Stimulates Both S- and M-Phases and Represses Cell Growth.

(A) Immunoblot analysis of HA-E2FB and Myc-DPA protein levels in transgenic BY-2 cells doubly transformed with estradiol-inducible pER8-E2FB and constitutive DPA constructs. Time points after subculture in the presence or absence of 5 μM β-estradiol is indicated. Amido black staining of the same membrane shows equal loading.

(B) Mean cell numbers of triplicates from pER8-E2FB transgenic BY-2 line was determined in the presence or absence of 5 μM β-estradiol at the indicated time points.

(C) and **(D)** Time course of mitotic index after subculturing pER8-E2FB/DPA line **(C)** or control pER8-BY-2 transformed with the empty vector **(D)** in the presence or absence of 5 μM β-estradiol as indicated.

induction of *E2FB* expression, the total fresh weight of the cells was significantly lower. As expected, this effect was not observed in the β-estradiol–treated control culture (Figures 6E and 6F). Thus, elevated *E2FB* expression promotes cell division and shortens cell doubling time but inhibits cell growth.

In addition, we measured by flow cytometry the DNA content of E2FB-transformed cells untreated or treated with β-estradiol for 8 d of the culture period (Figure 6G). Unexpectedly, the proportion of cells with 4C DNA content, indicative of the G2-phase, was elevated from the typical 5% of a BY-2 control culture (Figures 4C and 7A) to close to 40% irrespective to the induction of E2FB expression. A possible explanation for this is that the constitutive DPA expression in complex with an endogenous E2F promotes the G1-to-S but not the G2-to-M transition. Cells with DNA contents indicative of S-phase significantly increased in the β-estradiol–induced samples throughout the time course. The fact that the percentages of both the S-phase and mitotic phase are increased further supports a role for E2FB at both transitions and explains why the cell doubling time becomes shorter leading to more cells with progressively smaller cell sizes.

We also observed the morphology of cells conditionally expressing *E2FB* after β-estradiol induction. Similarly to the lines constitutively expressing *E2FB*, we found cells with extremely small size. The filamentous growth of the culture was completely disrupted, and longitudinal division planes established new cell files that remained attached to each other and created bizarre structures (Figure 6H). Induction of E2FB expression by β-estradiol treatment in the hormone-free condition also led to increased cell division rate along with the characteristic small cell size but with a lower frequency than in the cell line with constitutive expression of E2FB. The morphological heterogeneity precluded the analysis of cell cycle and molecular parameters in hormone-free conditions in the cell line with inducible E2FB expression.

Elevated E2FB Expression Advances Cells into Both S-Phase and Mitosis, thereby Shortening Cell Cycle Duration

Data from asynchronously growing cell cultures already indicated that elevated E2FB levels speed up the cell cycle by affecting the entry into both the S- and M-phases. Therefore, we conducted a cell synchronization experiment whereby we blocked cells in S-phase with the DNA polymerase α-inhibitor aphidicolin. We determined cell cycle phase parameters by flow cytometry and by counting the mitotic indexes in the cultures released from the block. Our control, a BY-2 cell line carrying an empty vector, did not show any significant differences in the cell cycle progression whether or not cells were treated with β-estradiol (Figures 7A and 7B). Similar to what we observed in the asynchronous E2FB-expressing culture, there was an increased proportion of cells with 4C DNA content both with and without β-estradiol induction (Figures 7C and 7D). Moreover, the proportion of S-phase cells considerably increased during the aphidicolin arrest, but this was approximately twofold greater when E2FB expression was induced. DNA polymerase α is the target of the aphidicolin drug, and the promoter of this gene is known to contain an E2F binding element. Thus, elevated E2Fb level might increase the DNA polymerase α levels to an extent that makes cells escape the aphidicolin block (de Jager et al., 2001). Furthermore, remarkable differences were observed in cell cycle progression when we compared the E2FB-expressing cultures with and without the β-estradiol induction (Figure 7A). The ratio of cells entering the S-phase and subsequently the G2- and M-phases at 2, 6, and 12 h, respectively, were still comparable between the induced and noninduced cells, though mitoses were already observed in aphidicolin-treated samples, and the mitotic index peak was ~2 h earlier (Figure 7E). Remarkably, shortly after the E2FB-expressing cells exited mitosis, they entered into S-phase and then rapidly went through G2 and entered again into mitosis, which did not happen during this time interval in the untreated cells (Figure 7E). Reentering the second mitosis within the time interval of 26 h occurred only in the culture with induced E2FB expression, which we interpret as a shortened cell cycle. We found some heterogeneity in cell cycle progression as well as the simultaneous appearance of both very small and normal sized cells during the growth cycle in the induced culture, reflecting varying levels of *E2FB* expression.

Cells at a defined stage of the cell cycle in a synchronous culture also allowed us to ask whether the elevated CDKA;1 and CDKB1;1 protein levels and kinase activity are related to a particular phase of the cell cycle or a direct consequence of *E2FB* expression. We followed the E2FB and DPA levels together with the accumulation of CDKA;1 and CDKB1;1 proteins and their activity throughout the synchronization. As shown in Figure 7F, the CDKA;1 levels detected by the PSTAIRE antibody were not influenced by the elevated *E2FB* expression in the β-estradiol-treated cells, while a correlation was found between E2FB and CDKB1;1 proteins levels. Normally, CDKB1;1 shows a cell cycle–dependent accumulation becoming abundant during the G2-M-phases. By contrast, the CDKB1;1 protein level was at a constitutively high level in the treated cells, supporting a direct regulation of *CDKB1;1* by the E2FB transcription factor. The CDK activity still fluctuated with three peaks, two of which mirrored the mitotic indexes at 10 to 12 and at 24 to 28 h, while the one at 4 h did not correlate with a high percentage of cells in mitosis (Figure 7G). One possibility is that elevated E2FB levels directly induce some positive regulators of CDK activity but that high CDK activity does not directly lead to mitosis after aphidicolin release.

Figure 6. (continued).

(E) and **(F)** Fresh weight of cells from pER8-E2FB/DPA line **(E)** or from pER8-BY-2 **(F)** at the indicated time points after subculture in the presence or absence of 5 μM β-estradiol.

(G) Cell cycle phase distribution in the pER8-E2FB/DPA line at the indicated time points after subculturing with (+) and without (−) 5 μM β-estradiol.

(H) Phase contrast (left) and fluorescence (right) image of DAPI-stained cells from the pER8-E2FB/DPA line 4 d after subculturing with 5 μM β-estradiol.

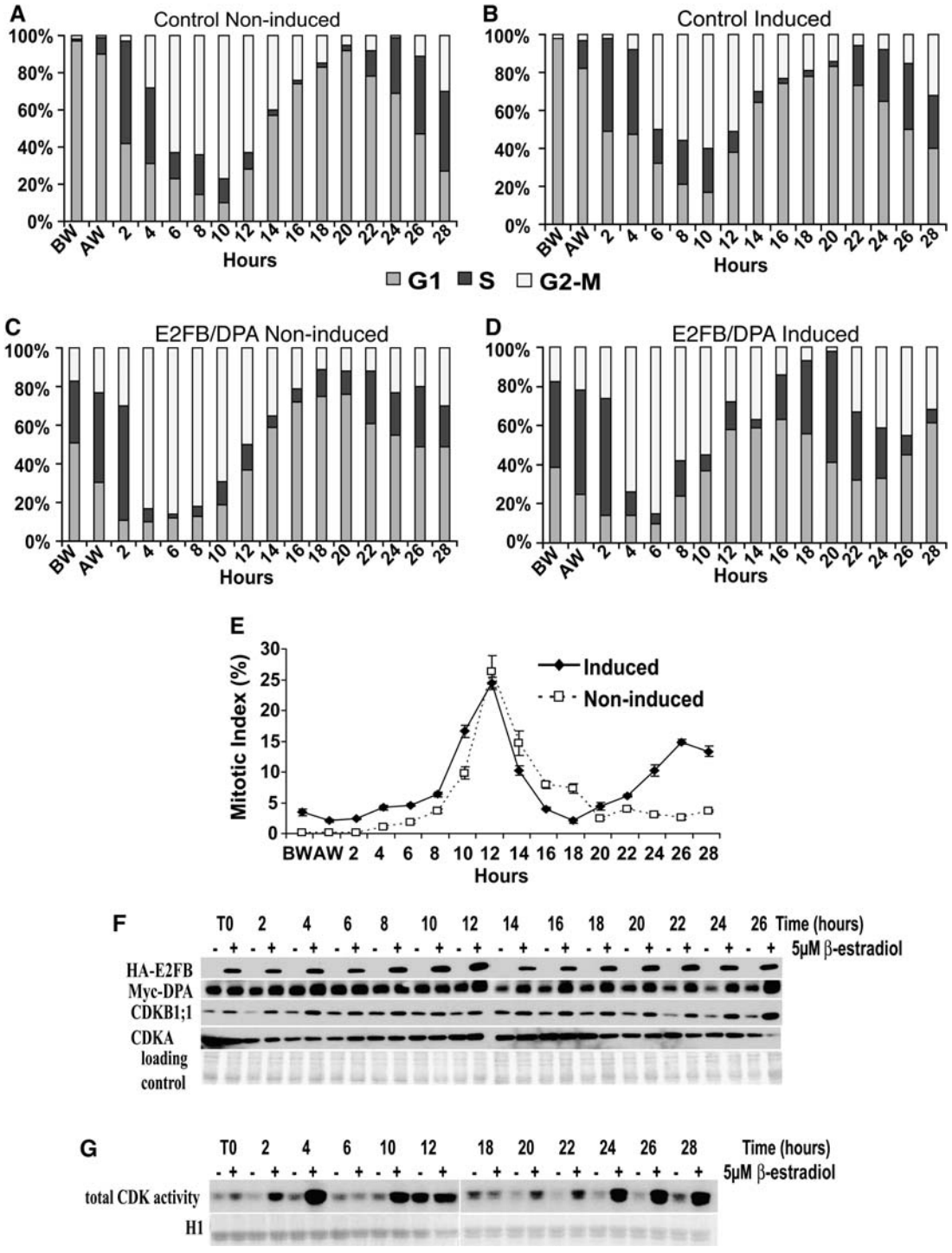

Figure 7. Cell Cycle Synchronization of pER8-E2FB and pER8-BY-2 Cultures.

(A) to **(D)** pER8-BY-2 control culture was synchronized in S-phase with aphidicolin (5 μg/mL) in the absence **(A)** or presence **(B)** of 5 μM β-estradiol. Similarily, the pER8-E2FB culture was synchronized in S-phase with aphidicolin (5 μg/mL) in the absence **(C)** or presence **(D)** of 5 μM β-estradiol. Progress of cells through the cell cycle was monitored by determining the proportion of G1-, S-, and G2-M-phase cells using flow cytometry in samples before wash (BW), after wash (AW), and at the indicated time points.

(E) Time course of mitotic index of aphidicolin-synchronized pER8-E2FB culture in the presence or the absence of 5 μM β-estradiol.

(F) Protein gel blot analyses of HA-E2FB and Myc-DPA as well as CDKB1;1 and CDKA;1 protein levels at time points indicated after washing out

A second possibility is that a minor subpopulation of cells expresses particularly high levels of E2FB and that in these cells the CDK is superactivated. The presence of mitotic figures in aphidicolin-arrested samples and the severity of the small cell phenotype in a small portion of cells support the second possibility.

DISCUSSION

The E2F transcription factors are traditionally grouped into two main categories depending on whether they activate or repress the expression of positive regulators of cell cycle and control a battery of genes involved in differentiation through the recruitment of chromatin-remodeling proteins (Trimarchi and Lees, 2002). We compared the potency of elevated E2FA and E2FB to promote cell division in the model BY-2 tobacco cell line and found that both are positive regulators of cell cycle progression in nutrient-limiting conditions, but only E2FB allows auxin-independent proliferation while inhibiting cell growth and endoreduplication. These experiments confirmed previous results in intact plants, namely that E2FA is a positive regulator of cell cycle progression, depending on cell type and expression level (De Veylder et al., 2002; Kosugi and Ohashi, 2003). We established that E2FB is a strong positive regulator able to promote cell division in a number of physiological conditions.

E2FB Regulates Both the G1-to-S and G2-to-M Transitions

In BY-2 cultures, cells grow and divide in filaments with fairly uniform size and morphology. In contrast with the control culture, in the *E2FA* and *E2FB* overexpression lines, we observed heterogeneity in both size and morphology, with some cells much reduced in size. The proportion of small cells was much higher for *E2FB* overexpression than for that of *E2FA*, suggesting that E2FB might be more potent in stimulating cell division. The production of more and smaller cells, the equal increase of the percentage of cells in S-phase and in mitosis, and the unaltered proportion of cells in G1- and G2-phases all suggest that E2FB promotes both the G1-to-S and G2-to-M transitions, leading to shorter cell cycle length. We further confirmed this result by constructing cell lines with conditional expression of *E2FB*. The inducible expresssion of *E2FB* also led to larger numbers of cells with smaller sizes. Synchronizing cells for a cell cycle that progressed through two waves of S-phase and of mitosis further confirmed the shortened cell doubling times. Previous studies have shown that overexpression of *CYCD3;1* led to a shortened G1-phase that was compensated with a longer G2 (Dewitte et al., 2003), while overexpression of *CYCB2;1* reduced G2 length but resulted in a longer G1, leading to unaltered cell cycle lengths (Weingartner et al., 2003). A similar compensation mechanism that relies on the ability of E2F1 to regulate the expression of Cyclin E and the *string* gene, encoding a CDC25 homologue, was found in *Drosophila* (Reis and Edgar, 2004). E2FB might have a function in plant cells comparable to that of the *Drosophila* E2F1 to simultaneously increase the expression of critical S- and M-phase regulators. Indeed, our recent data show that E2FB can directly induce the promoter of the *Arabidopsis CDKB1;1* gene (Z. Magyar, unpublished results). Moreover, we found that in the cell lines with elevated E2FA and E2FB levels, the CDKA;1 and CDKB1;1 levels and activities were also higher and sustained during the growth period of the culture. Elevated levels of mitotic regulators provide a molecular basis for the reduced cell size and increased cell number in the E2FA and E2FB cultures.

It is not clear how E2FA could promote the expression of *CDKB1;1*, which has a separate expression window in S- and M-phases (Magyar et al., 1997, 2000). Moreover, high CDK activity in G2- and M-phases should lead to the phosphorylation of E2FA and thus inhibit its binding to DNA (Espinosa-Ruiz et al., 2004). We also found that E2FA has a high turnover rate that depends on an N-terminal part of the protein with a number of putative CDK phosphorylation sites, and so the E2FA protein levels should be extremely low in cells with high CDK activity (Z. Magyar, unpublished results). It is possible that E2FA indirectly increases CDKB1;1 levels, perhaps through E2FB. Correspondingly, E2FB mRNA and protein levels were upregulated in the E2FA-DPA plants (L. De Veylder and Z. Magyar, unpublished results). The promoter of *E2FB* and of *CDKB1;1* genes contains E2F binding sites, and *CDKB1;1* is shown to be regulated by E2FA together with DPA (Boudolf et al., 2004).

One possible explanation of how elevated expression of *E2FA* and *E2FB* promotes cell division is that they exceed the level of the endogenous RB protein and thus escape from suppression. When CYCD3;1 was ectopically expressed in trichomes, these cells also went through mitosis rather than endoreduplication and growth, which would happen normally. A knockout in *RBR1*, the single *Arabidopsis* RB-related gene, also abrogated the arrest of megagametophytes in mitosis, leading to their overproliferation (Ebel et al., 2004). Collectively, these data indicate that neither RBR1 nor the E2F proteins are exclusive G1-phase controllers in plants, as was previously thought, but regulate cell cycle at both G1-to-S and G2-to-M transitions and provide a link between cell growth and cell division. The ability of E2F to promote G2-to-M-phase transition is well documented in *Drosophila* (Neufeld et al., 1998) and has been suggested by genome-wide expression studies in mammalian cells (Ishida et al., 2001). Mutation in the RB-related gene *mat3* in the unicellular green alga *Chlamydomonas* does not abrogate G1 length at all but impairs size control and leads to extra rounds of S-phase and mitosis (Umen and Goodenough, 2001). In mammalian cells, E2Fs regulate the expression of the mitotic

Figure 7. (continued).

aphidicolin (T0) in the pER8-E2FB/DPA line with (+) or without (−) 5 μM β-estradiol. As a loading control, amido black staining of the same membrane is shown.

(G) Total CDK H1 kinase activity purified by $p13^{suc1}$-Sepharose beads from samples as in **(C)** and shown on autoradiographs of histone H1 (top panel) together with the corresponding images of Coomassie blue–stained gels (bottom panel).

checkpoint regulator, mad2, and inactivation of RB by mutation leads to uncontrolled cell cycle progression because of abrogated mitotic checkpoints (Hernando et al., 2004). We also observed mitotic abnormalities or cells with multiple nuclei in the E2FB transgenic lines possibly explaining why the constitutively expressed E2FB gene was rapidly silenced in the BY-2 cells (Z. Magyar, unpublished results).

Elevated E2FB Expression Represses Cell Growth Driven by Endoreduplication and Vacuolization

Overproliferation with elevated E2FA and E2FB levels resulted in higher numbers of cells with smaller sizes. More cells, however, did not lead to an increased cell mass, but rather the opposite, to reduced fresh weight of the culture. This observation indicates that elevated E2FB levels do not merely uncouple cell growth from the cell cycle, but also actively repress growth. Most plant cells enlarge during differentiation, a process that is often accompanied by endoreduplication (Sugimoto-Shirasu and Roberts, 2003). We suggest that the reduced cell growth reflects the inhibition of cell enlargement and endoreduplication, similarly to what was observed in *Arabidopsis* plants overexpressing *CYCD3;1* or *CYCA3;2* (Dewitte et al., 2003; Yu et al., 2003).

Auxin Regulates the Balance of Growth, Cell Division, and Differentiation by Altering E2FB Levels

The expression of the mammalian E2F1 transcription factor was shown to stimulate DNA synthesis in cells that would otherwise arrest in the absence of growth factors (Johnson et al., 1993). Our present data show that *Arabidopsis* E2FB but not E2FA is able to activate cell division in a hormone-free condition in BY-2 cells when it is coexpressed with *DPA.* The CDKA;1 and CDKB1;1 proteins rapidly disappear when cells are transferred to hormone-free medium (Sorrell et al., 2001) but not in cells with elevated E2FB levels, underpinning the sustained proliferation under hormone-free conditions.

In the BY-2 cell culture model for auxin-dependent growth, the synthetic auxin 2,4-D stimulates cell division, while the hormone-free condition, low levels of 2,4-D, or replacement of 2,4-D with another synthetic auxin, NAA, induce cell elongation with simultaneous endoreduplication (Campanoni et al., 2003; Campanoni and Nick, 2005). While E2FB expression promoted cell division under auxin-free conditions, it completely inhibited cell elongation and endoreduplication. Reduction of ABP1 levels by antisense expression similarly inhibited cell elongation and disrupted the orientation of cell division but did not affect cell proliferation (Chen et al., 2001b). On the other hand, overexpression of ABP1 induced cell elongation both in tobacco plants and in cultured tobacco BY-2 cells (Chen et al., 2001a). Interestingly, this cell enlargement was concomitant with an increased percentage of cells with 4C DNA content. It is not clear whether this reflects an enrichment of cells in G2-phase or endoreduplication of their DNA. If it is the latter scenario, it might be that ABP1 negatively regulates E2FB function, thereby promoting cell elongation but not cell division.

Because neither CDKA;1 and CDKB1;1 proteins nor CDK activity are detectable in BY-2 cells in hormone-free conditions, endoreduplication could be a modified cell cycle that proceeds in the absence of these CDKs. Moreover, the DNA polymerase α inhibitor aphidicolin can fully arrest DNA synthesis in the mitotic cell cycle but stimulates the endocycle in BY-2 cells (Quelo et al., 2002). This led to the idea that DNA synthesis during the endocycle is maintained by the aphidicolin-resistant DNA polymerase β-enzyme. E2FB appears to operate the switch that decides whether cells proliferate or endoreduplicate their DNA, enlarge, and differentiate.

Leaf cells reacted very differently to the elevated coexpression of E2FA and DPA transcription factors: some became extremely small, while others grew to abnormally large sizes (De Veylder et al., 2002; Kosugi and Ohashi, 2003). We do not know why cells in close proximity behave differently. One possible explanation is that they differ in their hormonal (i.e., their auxin) content. Based on this model, the mitosis- or endoreduplication-stimulating functions of E2FA would be selected based on the hormonal content of the cells. This is supported by our results that E2FA only stimulates cell division in BY-2 cells in the presence of auxin. Because E2FA increases E2FB levels, it is tempting to speculate that the cell division promoting activity of E2FA is indirect and happens through E2FB. By contrast, E2FB is a major target for auxin; we found that its turnover is tightly regulated by auxin, stabilized at high but degraded at low auxin concentrations. We do not yet fully understand the regulation of E2FB degradation by auxin, but our preliminary data show that it does not depend on AXR1 or TIR1 functions, as E2FB protein levels do not accumulate in plants that are mutants for these genes (Z. Magyar, unpublished results). Contrary to this, auxin appears to modulate E2FB protein levels through the involvement of the COP9 signalosome, as E2FB stability is increased when the CSN5 subunit of this complex is reduced through RNA interference (Z. Magyar, unpublished results). E2FC stability was shown to be oppositely regulated, destabilized in growth-promoting physiological conditions, for example, in plants grown in light, and regulated by the ubiquitin-SCF pathway (del Pozo et al., 2002). Experimentally increased E2FB levels could specifically keep cells dividing in the absence of auxin, while elevated E2FC levels inhibited cell division.

Finally, we often observed the disruption of filamentous growth of BY-2 cells because of a change in division plane from longitudinal to transverse, thus creating new cell files. This might merely reflect selection of a cell division plane across the shortest width of the cell, which, with reduced cell size, becomes oblique. A similar change in division plane was observed in a tobacco culture when cell division was stimulated by switching to a medium containing only 2,4-D (Campanoni and Nick, 2005) or by the inhibition of cell enlargement through the reduction of ABP1 levels (Chen et al., 2001a). In summary, our data identified E2FB as a potential mediator for the action of auxin in regulating cell growth and division.

METHODS

Generation of Plasmid Constructs and Transgenic Lines

The influenza HA-tagged E2FA and E2FB as well as the c-myc-tagged DPA had been constructed previously (Magyar et al., 2000). The coding

sequence of E2FC was amplified by PCR and cloned into *Nde*I and *Spe*I sites of the pBluescript SK− plasmid (Stratagene) containing a single HA-tag (HA-SK). The HA-tagged E2FA and E2FB and the c-myc-tagged DPA were placed under the control of the constitutive cauliflower mosaic virus 35S promoter in the Gateway vector pK7WG2 or pH7WG2 (Karimi et al., 2002). By gateway cloning, we also generated an inducible HA-tagged E2FB construct with the estrogen receptor-based chemical-inducible system modified for gateway cloning, pER8, kindly provided by N.-H. Chua and B. Ulker (Zuo et al., 2000).

Transformation of BY-2 Cell Cultures and Transfection of *Arabidopsis thaliana* Protoplasts

BY-2 tobacco (*Nicotiana tabaccum*) cells were maintained as previously described (Nagata and Kumagai, 1999). The tobacco BY-2 cells were transformed by *Agrobacterium tumefaciens*–mediated transformation (An, 1985) with a hypervirulent strain of *Agrobacterium* (LBA 4404; van der Fits et al., 2000). To generate double transgenic BY-2 cell lines, we mixed equal amounts of *Agrobacterium* strains containing the HA-E2FA or HA-E2FB in pK7WG2 or pER8 vectors and c-myc-DPA in pH7WG2 or pK7WG2 constructs. The transformation was performed according to the protocol. As control transgenic cell lines, BY-2 cells were transformed with the empty pK7WG2 or pER8 constructs.

Transgenic BY-2 cells were selected, first by screening for antibiotic resistance (kanamycin, hygromycin, or both) and then by immunoscreening the BY-2 callus samples with antibodies specific for the HA or c-myc epitopes (HA.11 or 9E10 c-myc; Roche Diagnostics). In the case of the inducible cell lines, the antibiotic-resistant BY-2 calli were transferred onto agar plates containing 5 μM β-estradiol and incubated for 1 week at 28°C in the dark. The HA-E2FB protein production was measured by immunoblot analyses with monoclonal HA antibodies. Suspension cultures were generated from the positive calli and maintained in Murashige and Skoog (MS) medium supplemented with the appropriate antibiotics.

Protoplast isolation from *Arabidopsis* suspension cells (Columbia ecotype; maintained in MS medium supplemented with 1 μM 2,4-D) and the polyethylene glycol–mediated transfection were performed as described before with some modifications (Anthony et al., 2004). For each transfection, 5 μg of plasmid DNA was transfected into 5×10^5 cells, and the cells were cultured for 24 h in the presence or absence of 2,4-D before harvesting.

Cell Synchronization and Hormone Starvation Experiments

The synchronization of *Arabidopsis* MM2d cells was conducted according to Menges and Murray (2002), with some modifications. Four days after subculturing, 20 mL of logarithmically growing cell culture was diluted with fresh MS medium to 100 mL containing 5 μg/mL aphidicolin (Sigma-Aldrich) and incubated for 24 h. To release the cells from the aphidicolin block, cells were pelleted and washed with 1 liter of MS medium and subsequently resuspended in 100 mL MS. Samples were taken every 2 h.

The synchronization of inducible E2FB or control transgenic BY-2 cells using aphidicolin was performed according to Nagata and Kumagai (1999) in the presence or the absence of 5 μM β-estradiol.

The hormone starvation-readdition experiment was conducted with a 7-d-old *Arabidopsis* MM2d cell culture (Menges and Murray, 2002). After extensive washing of cells without hormones, they were further incubated for 24 h under hormone-free conditions. Hormones (0.5 mg/L NAA and 0.05 mg/L kinetin) were re-added afterwards, and samples were taken at different time points. In a separate experiment, 7-d-old *Arabidopsis* cells were washed extensively with hormone-free medium and afterwards treated with CHX (100 μM final concentration) to block protein synthesis in the absence of any hormones or in the presence of NAA or kinetin, or NAA and kinetin. For the phosphatase treatment, total protein was extracted from 7-d-old *Arabidopsis* MM2d cell culture according to Magyar et al. (1997) in the absence or the presence of 15 mM phosphatase inhibitor pNpp.

The auxin starvation experiments in transgenic BY-2 cell cultures were performed by briefly centrifuging (3 min at 1000 rpm) 2.5 mL of 7-d-old transgenic BY-2 cell suspension, washing the cells four times with 200 mL of MS medium prepared without 2,4-D, and resuspending the cells in 100 mL of auxin-free MS medium supplemented with the appropriate antibiotics in a 500-mL flask.

Flow Cytometry Analysis, Determination of Mitotic Index, and Microscopy Analysis

Samples of 0.5 mL were taken from suspension cultures of *Arabidopsis* or transgenic BY-2 cells for flow cytometry. After a brief centrifugation, the pellets were frozen in liquid nitrogen. Samples were stained with DAPI with the CyStain UV Precise P kit and analyzed on a Partec PAS II flow cytometer (Partec). Cell cycle data were analyzed with Flomax software (Partec). The mitotic index was determined by fixing the samples in an ethanol:acetic acid (3:1; v/v) solution. Samples were stained with 1 μg/mL of DAPI and observed under a fluorescence microscope. Cells (500) were scored in triplicate for each sample.

Preparation of Antibodies, Immunoblotting, $p13^{suc1}$-Affinity Binding, and Histone H1 Kinase Assays

A 249-bp long PCR fragment with extensions containing the restriction sites *Bam*HI and *Sal*I of the *Arabidopsis* E2FB cDNA that encodes the C-terminal part of the deduced protein sequence was cloned in the pQE-30 expression vector in frame with the 6-histidine tag (Qiaexpressionist; Qiagen). The full-length open reading frame (ORF) of DPA cDNA was amplified by PCR and cloned into the pQE-30 expression vector at the *Sph*I and *Sal*I sites. The recombinant proteins were produced in *Escherichia coli* and purified under denaturing conditions according to manufacturer's protocol (Qiaexpressionist). Two rabbits were immunized with each antigen (Biotrend Chemikalien). For the affinity purification of the E2FB and DPA antibodies, the C-terminal fragment of E2FB and the full-length ORF of DPA were further cloned into pGEX-4T1 (Amersham Biosciences) in frame with the N-terminal GST protein. Expression and purification of these recombinant proteins were performed according to the manufacturer's protocol. Pure GST fused antigen (5 to 10 μg) was separated by SDS-PAGE and blotted onto nitrocellulose membranes. After a brief staining of the membrane by Ponceau S, a strip containing the antigen was cut out and incubated with 500 μL of serum overnight in a cold room. The bound antibody was eluted from the strip by adding 0.5 mL of Gly elution buffer (0.1 M Gly, 0.5 M NaCl, and 0.05% Tween 20, pH 2.6), and the elution was repeated once more. The pooled antibody solution was immediately neutralized by adding 60 μL of 1 M Tris, pH 8.0. The eluted antibody was dialyzed in TBS buffer before the immunoblot assay tests.

Immunoblotting, $p13^{suc1}$-affinity binding, and histone H1 kinase assays were performed as described previously (Magyar et al., 1997). The monoclonal anti-PSTAIRE antibodies were purchased from Sigma-Aldrich, and the monoclonal anti-HA antibodies (HA.11) and the monoclonal c-myc-antibodies (9E10) were purchased from Roche Diagnostics. The polyclonal anti-AtCDKB1;1 antibodies were used at a 1:2000 dilution in immunoblot assays.

Immunoprecipitations and GST Pull-Down Experiments

Immunoprecipitations were conducted as described previously with 2 μL of affinity-purified polyclonal anti-E2FB or anti-DPA antibodies (Magyar et al., 1997). The immunopurified complexes were resolved by SDS-PAGE and protein gel blots were performed. The cDNA clone of the *RBR1* gene was kindly provided by W. Gruissem. The full-length ORF of *RBR1* was PCR amplified and cloned into the pGEX-4T-1 expression vector at

the *Bam*HI and *Xho*I sites. The recombinant protein was produced bacterially in BL21 (DE3) *E. coli* strains at 28°C and purified according to the manufacturer's protocol (Amersham Biosciences). Total protein was extracted from *Arabidopsis* or BY-2 cell suspensions (Magyar et al., 1997): 250 μg of total protein was incubated with GST, GST-DPA, or GST-RBR1 (1 μg bound to gluthatione-Sepharose beads) for 2 h at 4°C. The beads were washed four times with Nonidet P-40 buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, and 1 mM PMSF). Proteins were released by boiling in SDS sample buffer and fractionated by SDS-PAGE.

In Vitro Translation

The in vitro translation of the HA-tagged version of E2FA, E2FB, and E2FC proteins was conducted as described before (Magyar et al., 2000) using the TNT T7-coupled wheat germ extract kit (Promega).

Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for genes described are as follows: E2Fa (also known as E2F3; At2g36010), E2Fb (also known as E2F1; At5g2220), E2FC (also known as E2F2; At1g47840), DPA (At5g02470), and DPB (AT5G03415).

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