Interplay between Arabidopsis Activating Factors E2Fb and E2Fa in Cell Cycle Progression and Development[1][W]

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Eukaryotic E2Fs are conserved transcription factors playing crucial and antagonistic roles in several pathways related to cell division, DNA repair, and differentiation. In plants, these processes are strictly intermingled at the growing zone to produce postembryonic development in response to internal signals and environmental cues. Of the six AtE2F proteins found in Arabidopsis (*Arabidopsis thaliana*), only AtE2Fa and AtE2Fb have been clearly indicated as activators of E2F-responsive genes. AtE2Fa activity was shown to induce S phase and endoreduplication, whereas the function of AtE2Fb and the interrelationship between these two transcription factors was unclear. We have investigated the role played by the *AtE2Fb* gene during cell cycle and development performing in situ RNA hybridization, immunolocalization of the AtE2Fb protein in planta, and analysis of *AtE2Fb* promoter activity in transgenic plants. Overexpression of *AtE2Fb* in transgenic Arabidopsis plants led to striking modifications of the morphology of roots, cotyledons, and leaves that can be ascribed to stimulation of cell division. The accumulation of the AtE2Fb protein in these lines was paralleled by an increased expression of E2F-responsive G1/S and G2/M marker genes. These results suggest that *AtE2Fa* and *AtE2Fb* have specific expression patterns and play similar but distinct roles during cell cycle progression.

The identification of various components of the plant cell cycle machinery has revealed remarkable similarities with the regulatory pathways found in animal cells, for which a key role is exerted by the E2F/DP family of transcription factors. The genome of the model plant Arabidopsis (*Arabidopsis thaliana*) contains eight genes of this family (six *E2F*s and two *DP*s), whereas in mammalian cells 10 E2F/DP members have been discovered (eight *E2F*s and two *DP*s; Attwooll et al., 2004; Christensen et al., 2005; Dimova and Dyson, 2005; Maiti et al., 2005). Most mammalian E2F proteins (E2F1–5) and three of the Arabidopsis members (AtE2Fa–c) show a similar domain organization, characterized by a highly conserved DNA-binding domain followed by a DP heterodimerization domain and a C-terminal transactivating domain, containing the pocket protein-binding region. The mammalian E2F6 lacks the carboxy-terminal transactivating region. Six mammalian E2Fs (E2F1–6) and three Arabidopsis E2F proteins (AtE2Fa–c) bind DNA by forming heterodimers with the distantly related DP proteins that contribute a second DNA-binding domain for binding to the consensus E2F cis-elements found in several E2F-responsive promoters. The remaining Arabidopsis E2Fs (AtE2Fd, e, and f/DEL2, 1, and 3) and the E2F7 and E2F8 proteins of mammalian cells only contain conserved duplicated DNA-binding domains. They cannot form heterodimers with DP proteins, but their duplicated DNA-binding domains allow autonomous binding to the consensus E2F sites (Mariconti et al., 2002; Kosugi and Ohashi, 2002a; de Bruin et al., 2003; Di Stefano et al., 2003; Christensen et al., 2005; Maiti et al., 2005).

E2F transcriptional regulation relies on activating or repressing functions that depend, in part, on the interaction between some E2Fs and the pocket proteins, known as the pRB/E2F pathway (Stevens and La Thangue, 2003). The mammalian E2F1 to 5 proteins can interact with hypophosphorylated pocket proteins and have been divided in two subclasses of activating and repressive factors, playing crucial and antagonistic roles in the regulation of several genes involved in DNA replication and expressed during late G1 and near the G1/S boundary (Trimarchi and Lees, 2002). E2F1 to 3 are potent activators of E2F-responsive genes and their overexpression can induce quiescent cells to reenter the cell cycle (Johnson et al., 1994; Shan and Lee, 1994; Singh et al., 1994; Xu et al., 1995). As judged by the changes in global gene expression induced by their overexpression, E2F1 to 3 play different activating roles during differentiation and development. This

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observation is supported by the analysis of mouse mutant strains characterized by the knocking out of these *E2F* genes (Field et al., 1996; Yamasaki et al., 1996; Humbert et al., 2000). In contrast, E2F4 and E2F5 expressed predominantly in quiescent cells and hence are thought to act mainly as repressors of cell cycle genes (Trimarchi and Lees, 2002). E2F6 has been shown to be a transcriptional repressor, whereas the E2F7 and E2F8 factors are believed to act as inhibitors of E2F transcriptional activity (Trimarchi et al., 2001; de Bruin et al., 2003; Di Stefano et al., 2003; Maiti et al., 2005). Similar to human E2F1 to 5, the homologous Arabidopsis AtE2Fa to c proteins have been classified as activating (AtE2Fa and b) or repressive factors (AtE2Fc) and shown to interact with plant pocket proteins (pRBR) in yeast two-hybrid and in vitro pull-down experiments (de Jager et al., 2001; del Pozo et al., 2002).

The physiological roles of AtE2Fa and AtE2Fc have been examined at the cellular and organism levels. Transient overexpression of *AtE2Fa* in Arabidopsis protoplasts from mature leaves induces these quiescent cells to progress into S phase (Rossignol et al., 2002). In transgenic Arabidopsis plants, *AtE2Fa* overexpression induces ectopic cell division, while overexpression of *AtE2Fa* in combination with *AtDPa* can either induce endoreduplication or cell proliferation depending on the cellular or developmental context, resulting in delayed differentiation and a striking block in development (De Veylder et al., 2002). Plants ectopically overexpressing *AtE2Fa* and *AtDPa* also up-regulate S-phase-specific genes, such as DNA polymerase α, cell division cycle 6 (*AtCDC6*), origin recognition complex 1 (*AtORC1*), and minichromosome maintenance 5 (*AtMCM5*). Similar results were obtained when *AtE2Fa* and *AtDPa* cDNAs were overexpressed in transgenic tobacco (*Nicotiana tabacum*) plants (Kosugi and Ohashi, 2003). Consistent with its role as an S-phase inducer, *AtE2Fa* is highly expressed in the shoot apical meristem (SAM), emerging leaf primordia, and vascular tissues of young leaf primordia (De Veylder et al., 2002). *AtE2Fa* is also expressed in the epidermis and cortex of the hypocotyls, which show a high level of endoreduplication (De Veylder et al., 2002). These observations are in agreement with reverse transcription (RT)-PCR results showing that *AtE2Fa* is maximally expressed in late G1 and early S phase (Mariconti et al., 2002). In contrast, AtE2Fc, which possesses all the features of activating factors but a truncated transactivation domain, is a poor transcriptional activator (Kosugi and Ohashi, 2002b) and down-regulates the early S-phase gene *AtCDC6* through its interactions with pRBR, thereby acting as a repressor of cell proliferation (del Pozo et al., 2002).

Although structural features and transient expression data suggest a strong activating role for AtE2Fb, this factor has not been as thoroughly investigated as AtE2Fa and AtE2Fc. Only recently, it was reported that *AtE2Fb* overexpression in tobacco Bright Yellow-2 (BY-2) cells increases cell cycle rate and promotes cell division in the absence of auxin (Magyar et al., 2005). In this work, we analyzed the role played by *AtE2Fb* during cell cycle progression and development. Our results show that AtE2Fb is an activator of E2F-responsive G1/S and G2/M marker genes and suggest that, as in mammals, plant activating E2Fs play similar but distinct roles during cell cycle and development.

RESULTS

Expression of *AtE2Fb* during Development

It was previously reported that *AtE2Fb* is poorly transcribed in quiescent Arabidopsis suspension cells and is expressed in proliferating cells, with its RNA accumulating to slightly higher levels at the G1/S transition (de Jager et al., 2001; Mariconti et al., 2002). We used two different strategies to analyze the expression pattern of *AtE2Fb* during plant development. The first approach relied on the generation of transgenic Arabidopsis lines expressing the *uidA* (*β-glucuronidase* [*gus*]) reporter gene under the control of the putative *AtE2Fb* promoter (*AtE2Fb*::*uidA*), while the second was the analysis of *AtE2Fb* transcript accumulation by in situ hybridization.

For the promoter expression analysis, histochemical staining for GUS activity was investigated in the T_2 progeny of 19 *AtE2Fb*::*uidA* transgenic plants using 4-, 7-, 18-d-old seedlings and flowering plants. In 4-d-old seedlings, GUS staining was observed in the SAM and in cotyledonary vascular tissues (Fig. 1A). In older plantlets (7 and 18 d old), GUS staining was intense and generalized in young leaves, while it was weaker or limited to tips in old leaves and cotyledons (Fig. 1, B, C, and C1). GUS staining was detected also in cells other than the vascular tissue, and in young leaves a strong signal was found at the base of trichomes (Fig. 1B). The *AtE2Fb* promoter appeared to be highly active in the central cylinder of both primary and secondary roots (Fig. 1, B, C2, D, and E). In 18-d-old primary roots, GUS activity was strong in the root tip, particularly at the elongation zone (Fig. 1D), whereas in secondary roots the staining appeared associated with the development of lateral root primordia (Fig. 1E). In young inflorescence meristems, GUS staining was widespread (Fig. 1F) while in a maturing inflorescence was maintained at different extents in sepals, petals, and styles (Fig. 1G). The staining of pistils and stamens clearly showed differences between immature and mature florets. In the immature flowers, the pistils were entirely blue while the anthers were unstained (Fig. 1H), whereas GUS staining of pistils disappeared and a strong GUS activity was observed in anthers of mature flowers (Fig. 1I). GUS staining of anthers appears to be due to the expression of *AtE2Fb* in maturing pollen grains (Fig. 1, J and K).

For in situ hybridization, a fragment corresponding to the 3′ untranslated region of the *AtE2Fb* mRNA was used as a probe to minimize cross-hybridization with

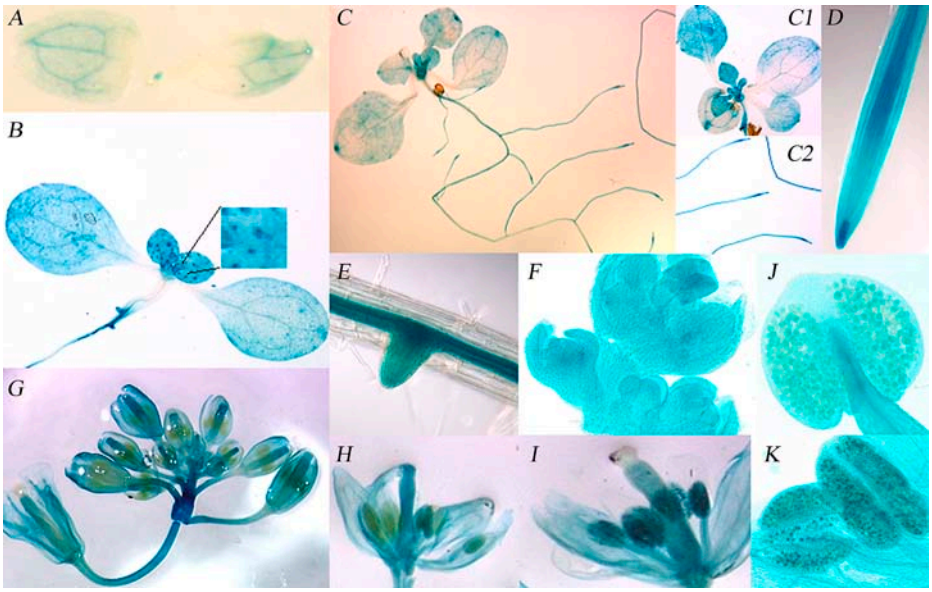

Figure 1. Histochemical localization of GUS activity in transgenic Arabidopsis plants carrying the chimeric *AtE2Fb*::*uidA* gene. A, Four-day-old seedling. B, Seven-day-old seedling. Inset in B, Magnification of young leaves, which shows GUS staining at the base of trichome cells and primary root tip. C, Eighteen-day-old Arabidopsis plants. Leaves at different stages of development and root tips are shown in C1 and C2, respectively. D, Primary root tip and elongation zone of an 18-d-old Arabidopsis plant. E, Eighteen-day-old seedling with developing lateral root primordia. F, Flower meristems. G, Developing and mature flowers. H, Pistils and stamens in immature florets. Note the strong staining of the stigma that disappears in mature florets (see I). I, Staining of pistils and stamens in mature florets. Note the strong staining of anthers that is not present in immature florets (see H). J, Anthers in immature florets. K, Anthers with mature pollen grains.

other *AtE2F* transcripts. The shoot apex of a 10-d-old Arabidopsis plant continuously generates organs at its flanks, and, consequently, the SAM exhibits various kinds of tissues at different proliferating and differentiating stages. *AtE2Fb* transcripts accumulated at high levels in cells of the shoot apex and leaf primordia (Fig. 2, A and B). In the apex of 20-d-old plants, the hybridization signal was also visible in the main veins of the leaves and in the axillary meristems (Fig. 2, C and D). The signal was particularly strong in actively dividing tissues such as leaf primordia (Fig. 2, B and D) and the tips of young leaves (Fig. 2B). Interestingly, a very strong signal was also observed in trichomes of young leaves (Fig. 2B). In Arabidopsis inflorescences, *AtE2Fb* mRNAs were localized in floret primordia (Fig. 2F) and in petals, stigma, styles, and ovaries upon complete flower differentiation (Fig. 2, H and I). The hybridization signal was also detectable in the stamens and the flower pedicels (Fig. 2, G and H). No signal was detected in microspores or anther locules of flower buds.

Accumulation and Localization of the AtE2Fb Protein

To evaluate the in vivo accumulation and subcellular localization of AtE2Fb protein, immunolocalization experiments were carried out using longitudinal sections of the shoot apex of 15-d-old Arabidopsis plants. Results of these experiments showed that the AtE2Fb is localized primarily in the SAM and leaf primordia. This pattern of accumulation is very similar to that of the relevant transcripts (Fig. 2J). In the cells of young leaves, the protein was located in both the nucleus and cytoplasm (Fig. 2L). Interestingly, a strong signal was observed in leaf trichomes (Fig. 2J) also in both the nucleus and cytoplasm. Transient expression assay using the chimeric fusion construct *Cauliflower mosaic virus 35S* (*CaMV35S*)::*AtE2Fb-green fluorescent protein* (*GFP*) in BY-2 protoplasts confirmed both cytoplasmic and nuclear localizations of the AtE2Fb-GFP fusion protein as previously reported (Kosugi and Ohashi, 2002b). Remarkably, all the cells containing AtE2Fb-GFP featured two nuclei, suggesting that expression of the fusion protein stimulated cell cycle progression toward mitosis. In contrast, control cells that accumulated the truncated inactive NtKIS1b fused to the GFP as a nuclear marker (Jasinski et al., 2002) and mock transfected cells were characterized by the presence of only one nucleus (Supplemental Fig. 1).

AtE2Fb-Overexpressing Plants Are Characterized by an Altered Phenotype and Up-Regulation of S-Phase Genes

To assess the effect of an increased level of AtE2Fb on cell cycle and development, we generated transgenic Arabidopsis plants carrying the *AtE2Fb* cDNA under the control of the double CaMV 35S promoter. Of the 15 transgenic lines obtained, most showed striking morphological effects. Compared to untransformed control plants, the most evident alteration in

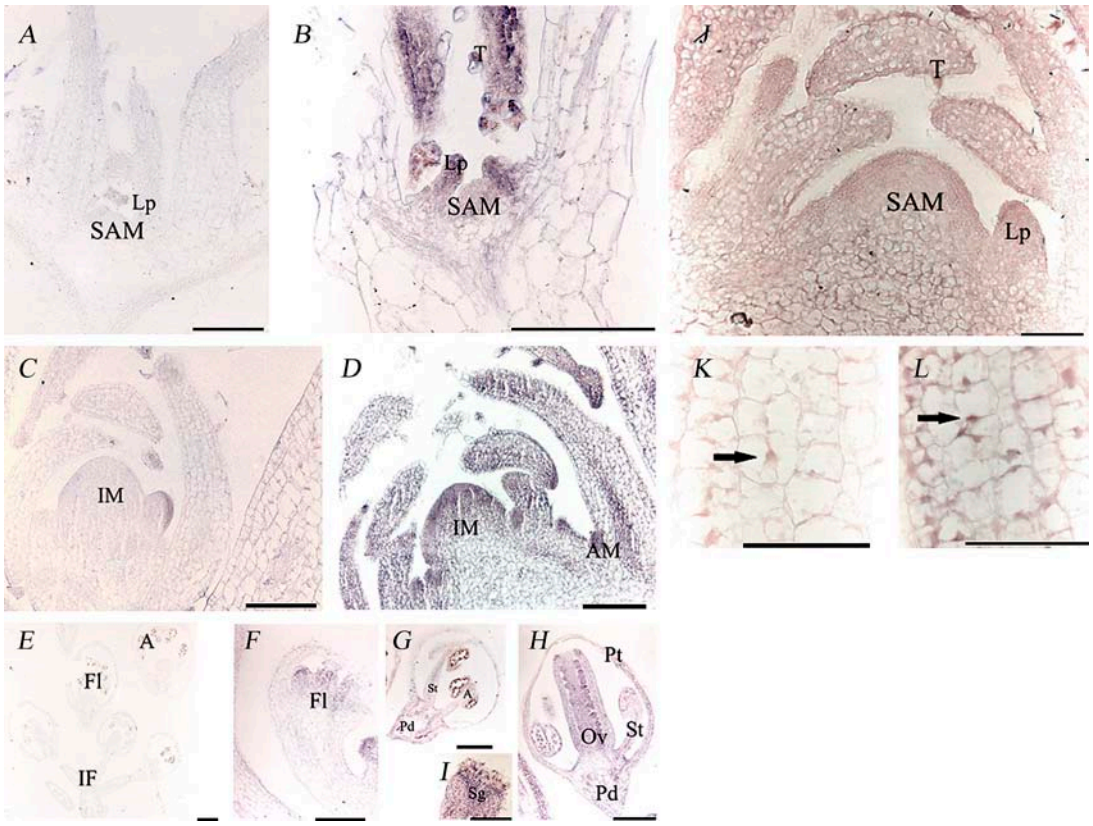

Figure 2. A to I, Localization of *AtE2Fb* transcripts by in situ hybridization in Arabidopsis plantlet and inflorescence sections. The hybridization signal is represented by the purple to blue staining. A, C, and E show sections hybridized with the sense probe (negative controls); B, D, F, G, H, and I show sections hybridized with the antisense probe. In A and B, longitudinal sections through the plantlets were obtained from plants 10 d after germination; in C and D, from plants 20 d after germination. In E to I, longitudinal sections were obtained from inflorescences. Bars = 100 μm. J to L, Immunolocalization of AtE2Fb in Arabidopsis plantlet longitudinal sections, 20 d after germination. J, A longitudinal section through a plant apex and leaf primordia. K and L show a higher magnification of a leaf primordium. In K, the treatment with the secondary antibody was omitted (negative control). Bars = 50 μm. Arrows indicate nuclei. A, Anther; AM, axillary meristem; IM, inflorescence meristem; IF, inflorescence; Fl, flower; Lp, leaf primordium; Ov, ovary; Pd, pedicel; Pt, petal; Sg, stigma; St; stamen; T, trichome.

16-d-old *AtE2Fb*^{OE} (''OE'' for overexpressing) seedlings was a shortening of the primary root, which featured an almost 3-fold reduction in length (Fig. 3A) combined with a closeness of the lateral root primordia and a higher density of thicker root hairs (Fig. 3, B–D). Hypocotyls were also shorter compared to untransformed controls (data not shown). A reduced size of *AtE2Fb*^{OE} plants was particularly evident in 4-d-old seedlings germinated in the dark. In these plants, hypocotyls were about 60% the size of those of untransformed controls, while roots were reduced in length to less than one-third (Supplemental Fig. 2).

AtE2Fb^{OE} seedlings were also characterized by young leaves essentially lacking trichomes (Fig. 3, insets A1 and A2). Like *AtE2Fa*^{OE} plants (De Veylder et al., 2002), *AtE2Fb*^{OE} cotyledons were slightly larger than controls, although a scanning electron microscope (SEM) analysis revealed a clear reduction of the size of cotyledonary epidermal cells (Table I) compensated by an increase in their number (Fig. 3, E–G). These data suggest a positive influence of AtE2Fb on cell proliferation. The microscopic analysis of root sections showed the presence of shorter isodiametric cortex cells in *AtE2Fb*^{OE} plants (Fig. 3, H and J) compared to elongated cells in untransformed controls (Fig. 3, I and K). Remarkably, this phenotype is very similar to that of *AtE2Fa*^{OE} plants that showed a marked radial growth (De Veylder et al., 2002). To evaluate any possible effect of AtE2Fb accumulation on endoreduplication, we measured the ploidy levels of nuclei extracted from leaves and hypocotyls/roots of wild-type and *AtE2Fb*^{OE} plants. Results of this analysis showed a modest increase of endoreduplicated cells in transgenic plants as compared to untransformed controls (Supplemental Fig. 3).

Semiquantitative RT-PCR of three of the most affected transgenic lines showed an increased steady-state level of *AtE2Fb* transcripts (Fig. 4A). A paralleled increase in the content of AtE2Fb protein was also revealed by immunoblot analyses performed using

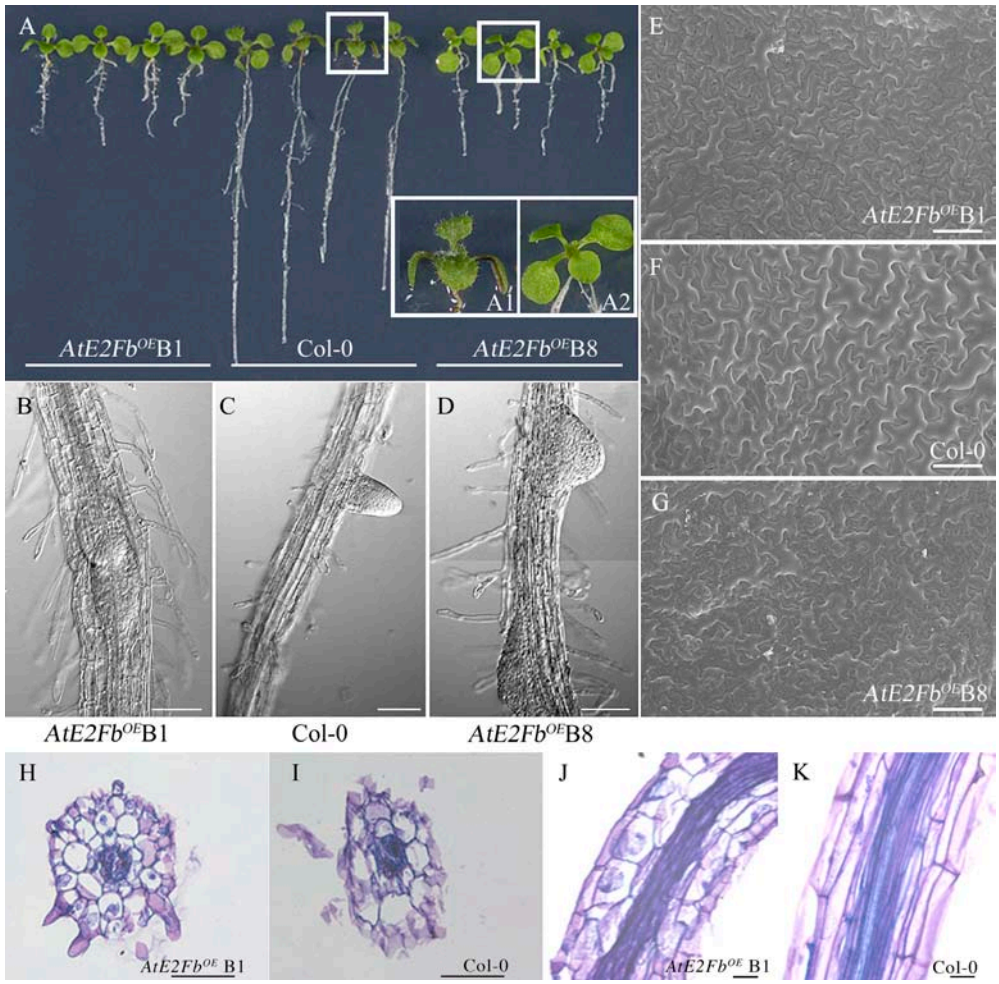

Figure 3. Phenotype of *AtE2Fb*^{OE} 16-d-old seedlings. A, *AtE2Fb*^{OE} line B1, left; untransformed control (Col-0), middle; and *AtE2Fb*^{OE} line B8, right. B to D, Scanning electron micrographs of roots: B and D, *AtE2Fb*^{OE} lines; and C, Col-0. E to G, Scanning electron micrographs of cotyledons: E and G, *AtE2Fb*^{OE} lines; and F, Col-0. H to K, Microscopic analysis of root sections of *AtE2Fb*^{OE} plants, line B1 and Col-0: H and I, same magnification, bar = 20 μm, radial sections; and J and K, same magnification, bar = 100 μm, longitudinal sections.

monospecific antibodies raised against the N terminus of AtE2Fb (Fig. 4B; see Supplemental Fig. 4 for the specificity of antibodies).

To further characterize these *AtE2Fb*^{OE} lines, we analyzed expression of the E2F-responsive genes encoding *AtMCM3* and the ortholog of tobacco ribonucleotide reductase 1b (*AtRNR1*; Chabouté et al., 2002; Stevens et al., 2002). Semiquantitative RT-PCR analysis showed that the overproduction of AtE2Fb results in up-regulation of these S-phase-specific genes, whereas the transcription of a gene encoding a dicer-related helicase (*AtDRH1*), which is not expected to be E2F responsive, was unchanged in the *AtE2Fb*^{OE} lines (Fig. 4C). Similarly, the *AtCYCD3*, *AtPCNA* (proliferating cell nuclear antigen), and *AtCDC6* genes, which are also S phase specific and E2F responsive, were up-regulated in *AtE2Fb*^{OE} lines, while the expression of *AtORC1*, another E2F-responsive gene, was unchanged (Supplemental Fig. 5). The up-regulation of *AtCYCD3*, which is induced before the onset of S phase and does not contain E2F sites, could depend on the cellular context rather than on the accumulation of AtE2Fb.

Given the phenotypic similarities between *AtE2Fb*^{OE} and *AtE2Fa*^{OE} plants (De Veylder et al., 2002), we asked whether *AtE2Fb* overexpression results in an increase

Table I. *Adaxial epidermal cell size in cotyledons of AtE2Fb*^{OE} *plants*

Mean values are ±SE. To warrant homogeneous growth, control and transgenic plants were grown on semisolid medium in petri dishes in a growth chamber. The reported results are the average of at least three different experiments.

Line	Cotyledons 16 d after Sowing	Adaxial Epidermal Cells 16 d after Sowing
	mm^2	μm^2
Col-0	4.80 ± 0.49	4,761 ± 2,636
AtE2Fb^{OE} line B1	5.22 ± 0.63	1,257 ± 693
AtE2Fb^{OE} line B8	5.81 ± 1.19	1,861 ± 1,297

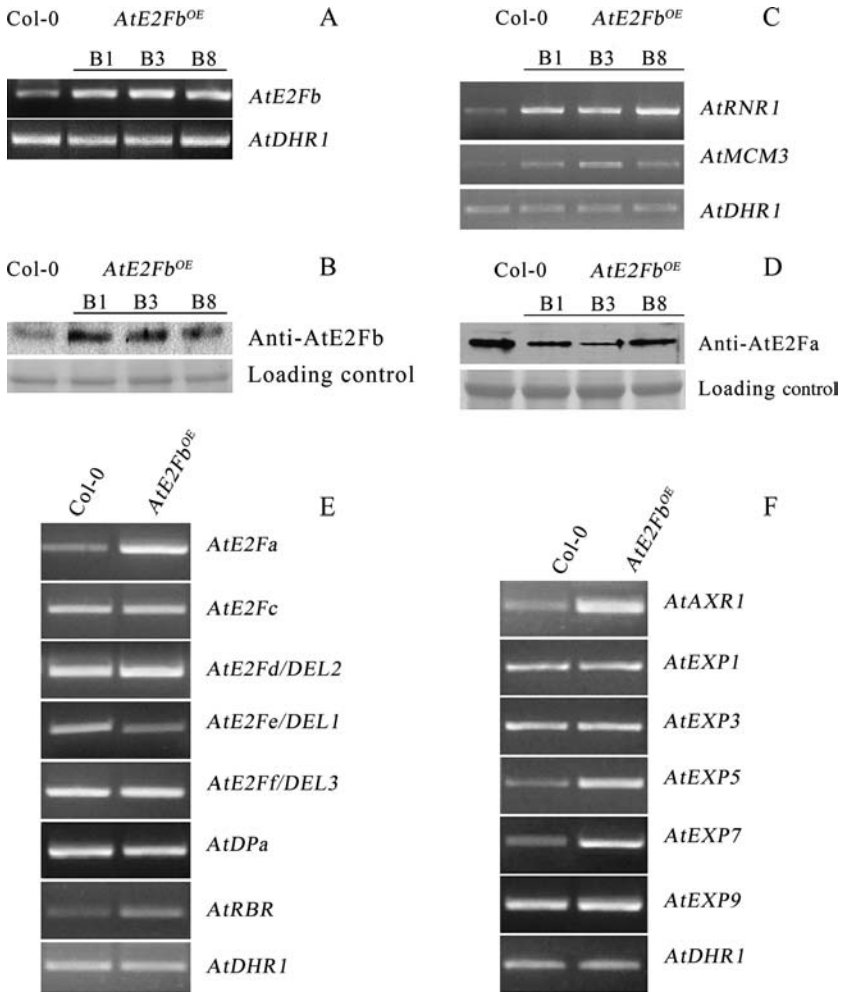

Figure 4. Levels of activating *AtE2F* transcripts and relevant protein in *AtE2Fb*^{OE} plants. A, RT-PCR analysis using primers specific for *AtE2Fb* of 7-d-old seedlings of wild type (Col-0) and three *AtE2Fb*^{OE} lines (B1, B3, and B8). The level of *AtDHR1* transcripts was used as the loading control. B, Immunoblot analysis of 15-d-old plants using antibodies against AtE2Fb. Wild type and *AtE2Fb*^{OE} lines are as in A. The molecular mass of the recognized AtE2Fb protein is 52 kD. A region of the filter stained with the Ponceau S is shown as the loading control. C, Semiquantitative RT-PCR of *AtMCM3* and *AtRNR1* using RNA extracted from 7-d-old *AtE2Fb*^{OE} seedlings. D, Immunoblot analysis of 15-d-old plants using antibodies against AtE2Fa. The molecular mass of the recognized AtE2Fa protein is 66 kD. E, RT-PCR analysis of wild type (Col-0) and *AtE2Fb*^{OE} line B8 using primers specific for genes of the *AtE2F* family and for *AtRBR*. F, RT-PCR analysis of wild type (Col-0) and *AtE2Fb*^{OE} line B8 using primers specific for *AtAXR1* and for *AtEXP* genes.

in AtE2Fa protein levels. Surprisingly, immunoblot analysis using monospecific antibodies raised against AtE2Fa revealed that the amount of AtE2Fa protein was reduced in plants overexpressing *AtE2Fb* (Fig. 4D). We then used RT-PCR to evaluate the steady-state mRNA levels of the other five *AtE2F* genes in *AtE2Fb*^{OE} plants. The promoters of the *AtE2Fc*, *AtE2Fe/DEL1*, *AtE2Ff/DEL3*, and *AtRBR* genes contain E2F cis-elements, while *AtE2Fa* and *AtE2Fd/DEL2* promoters lack recognizable E2F consensus motifs. This experiment (Fig. 4E) showed that *AtE2Fc*, *AtE2Fd/DEL2*, *AtE2Ff/DEL3*, and *AtDPa* mRNA levels did not change in the *AtE2Fb*^{OE} plants compared to control plants. In contrast, *AtE2Fe/DEL1* mRNA was reduced, while AtE2Fa and *AtRBR* were up-regulated. The up-regulation of *AtE2Fa* was unexpected because its promoter lacks E2F sites and AtE2Fa protein content decreased in *AtE2Fb*^{OE} plants. Together, these results strongly suggest that the expression of *AtE2F* genes is regulated by both transcriptional and posttranscriptional mechanisms. Previous studies have shown a strong influence of auxin over the regulation of AtE2F stability (del Pozo et al., 2002; Magyar et al., 2005). The presence of an E2F cis-element in the promoter of *AXR1*, the product of which is required for auxin response (del Pozo et al., 1998), prompted us to evaluate the level of *AXR1* transcripts in an *AtE2Fb*^{OE} line. Results of RT-PCR showed that *AXR1* is indeed up-regulated, thus indicating a possible link between AtE2Fb and auxin action.

The phenotype of *AtE2Fb*^{OE} plants also resembled *AtE2Ff*^{OE} plants (Ramirez-Parra et al., 2004) with respect to the reduced length of roots and hypocotyls compared to controls. However, *AtE2Ff/DEL3* transcripts levels were similar in *AtE2Fb*^{OE} and untransformed control plants, suggesting that the *AtE2Fb*^{OE} morphology is not due to increased AtE2Ff/DEL3 expression. This hypothesis is consistent with the observation that *AtEXP* mRNA levels (*AtEXP1*, *3*, *5*, *7*, and *9*) were either unchanged (*AtEXP1*, *3*, and *9*) or up-regulated (*AtEXP5* and *7*; Fig. 4F). Thus, this situation differs sharply from that of *AtE2Ff*^{OE} plants, in which the down-regulation of *AtEXP3*, *7*, and *9* was deemed responsible for the phenotype characterized by short roots and hypocotyls (Ramirez-Parra et al., 2004).

The *AtE2Fb* Promoter Is Regulated by an Activating AtE2F Factor

E2F consensus binding sites have been found in one or more copies in close proximity to the transcription start sites of several plant cell cycle-specific promoters. Using in silico analysis, we have identified three putative E2F cis-elements in the *AtE2Fb* promoter. To verify whether any of these E2F sites could be actually recognized by activating AtE2F factors, we applied the chromatin immunoprecipitation (ChIP) technique using polyclonal antibodies against the carrot (*Daucus carota*) DcE2F protein (Albani et al., 2000). These antibodies recognize only activating AtE2Fs with a greater preference for AtE2Fa (Supplemental Fig. 4). As a positive control to assess the efficiency of the ChIP assay, PCR reactions were performed on immunoprecipitated genomic fragments using primers specific for *AtRNR1* and the *AtPCNA* promoters, which contain E2F sites and are well known E2F targets (Chabouté et al., 2002; Egelkrout et al., 2002). A mock reaction with no antibody was used as a negative control. To rule out nonspecific interactions, PCR was also performed using primers specific for the Glu dehydrogenase (*AtGDH*) promoter that is not predicted to be an E2F target. The results of this experiment (Fig. 5) revealed a positive amplification with the *AtE2Fb* primer set, indicating that the *AtE2Fb* promoter, like the *AtRNR1* and *AtPCNA* promoters, is bound in vivo by an activating AtE2F and can be immunoprecipitated by antibodies against DcE2F. The specificity of this recognition was confirmed by the negative result obtained in a ChIP reaction performed using nonspecific antibodies against the epitope Flag 7 (data not shown).

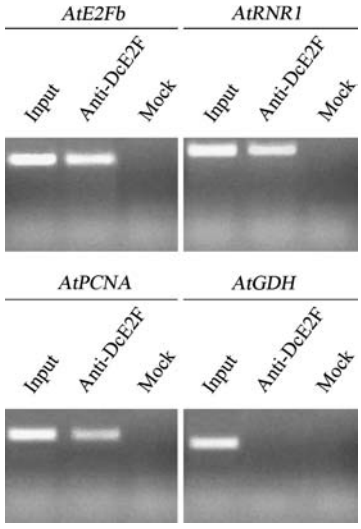

Figure 5. ChIP of the *AtE2Fb* promoter. The immunoprecipitation assay was performed using antibodies against DcE2F. Immunoprecipitated genomic DNA was amplified with primers specific for *AtE2Fb* and for two known E2F-responsive promoters of *AtRNR1* and *AtPCNA* (positive controls). The mock reactions and the PCR on immunoprecipitated fragments, using primers specific for the E2F-nonresponsive promoter (*AtGDH*), were used as negative controls. As a further control, an aliquot of the input was examined by PCR using *AtE2Fb*-, *AtRNR1*-, *AtPCNA*-, and *AtGDH*-specific primers.

These results point to a positive regulation of the *AtE2Fb* promoter by an activating AtE2F, possibly AtE2Fa. To test this hypothesis, expression of *AtE2Fb* and the accumulation of the corresponding protein in *AtE2Fa*^{OE} plants versus untransformed controls were compared by semiquantitative RT-PCR and immunoblot analyses. These experiments (Fig. 6A) revealed that *AtE2Fa*^{OE} plants contain an increased level of *AtE2Fb* transcripts that is paralleled by an increase in the amount of the AtE2Fb protein (Fig. 6B), suggesting that *AtE2Fb* expression might actually be up-regulated by the AtE2Fa transcription factor.

AtE2Fb^{OE} Plants Also Up-Regulate Some G2/M Marker Genes

To evaluate whether overproduction of AtE2Fb affects the expression of cell cycle genes acting at a stage different from G1/S, the steady-state level of transcripts of selected G2/M marker genes was investigated in the *AtE2Fb*^{OE} plants. For this purpose, the expression of *AtCYCA2;1* and *AtCDKB1;1*, both containing a E2F consensus site in their promoters, and *AtKRP1*, which lacks E2F sites (Mironov et al., 1999; Menges and Murray, 2002), was analyzed by RT-PCR. Results of this analysis (Fig. 7A) show that the steady-state level of *AtCYCA2;1* transcripts and, to a lesser extent, of *AtCDKB1;1* transcripts increased compared to control plants. In contrast, the transcript level of *AtKRP1* was unchanged. Remarkably, when this analysis was conducted on plants overexpressing *AtE2Fa*, an increase in the expression of the two E2F-containing G2/M marker genes was again observed (Fig. 7B). To establish whether the up-regulation of these genes is due to a stimulation of cell cycle progression or a direct involvement of activating AtE2Fs, ChIP was performed using antibodies anti-DcE2Fs. Results of this analysis showed that the E2F sites present in these promoters were occupied in vivo by activating AtE2Fs (Fig. 7C). Another G2/M-specific marker, such as *AtKRP2*, was up-regulated in *AtE2Fb*^{OE} plants, while the expression of *AtKRP3* was unchanged (Supplemental Fig. 5). It is worth noting that the promoter of *AtKRP2* does not contain consensus E2F sites and its up-regulation might not be directly dependent on AtE2Fb overaccumulation.

DISCUSSION

Of the six E2Fs of Arabidopsis, only two (AtE2Fa and AtE2Fb) possess all the structural and functional features of typical activating animal and plant E2Fs (del Pozo et al., 2002; Kosugi and Ohashi, 2002b; Mariconti et al., 2002). AtE2Fa is an activator of E2F-responsive genes (Kosugi and Ohashi, 2002b; Mariconti et al., 2002) that induces quiescent leaf cells to enter S phase (Rossignol et al., 2002). The ectopic *AtE2Fa*

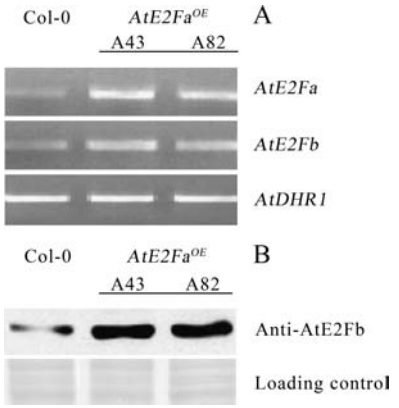

Figure 6. Levels of *AtE2Fb* transcripts and the relevant protein in *AtE2Fa*^{OE} plants. A, RT-PCR of *AtE2Fb* using RNA extracted from 7-d-old wild-type (Col-0) and *AtE2Fa*^{OE} (lines A43 and A82) seedlings. The level of *AtDHR1* transcripts was used as the loading control. B, Immunoblot analysis of 15-d-old plants of control (Col-0) and *AtE2Fa*^{OE} (lines A43 and A82) using antibodies against AtE2Fb. The molecular mass of the recognized AtE2Fb protein is 52 kD. A region of the nitrocellulose filter stained with Ponceau S was used as the loading control.

overexpression in Arabidopsis plants stimulates cotyledonary cells to proliferate and delays differentiation (De Veylder et al., 2002). In *AtE2Fa-DPa*^{OE} plants, the decision to engage in proliferation or endoreduplication depends on the cellular context through the action of other cell cycle regulators, such as AtCDKB1;1 and the repressing E2F factor AtE2Fe/DEL1 (Boudolf et al., 2004; Vlieghe et al., 2005). In this work, we have demonstrated that AtE2Fb also is a stimulator of cell cycle progression and that its overexpression affects plant morphology.

According to in situ hybridization analyses, immunolocalizations, and the analysis of promoter activity in transgenic plants, *AtE2Fb* appears to be mainly expressed in proliferating cells. However, there is also a clear expression at the base of trichomes and in several differentiated tissues. Altogether, these data point to a relationship between *AtE2Fb* expression and cell division without excluding a possible involvement of this transcription factor in endoreduplication and differentiation.

Experiments of transient expression of a chimeric *AtE2Fb*::*GFP* construct in BY-2 tobacco suspension cells are also supporting a role of this factor in cell proliferation as judged by the presence of two nuclei in cells accumulating the AtE2Fb::GFP protein. Remarkably, the analysis of Arabidopsis plants ectopically overexpressing *AtE2Fb* showed slightly enlarged cotyledons containing almost twice the number of smaller epidermal cells. These plants also showed up-regulation of the E2F-responsive S-phase genes *AtRNR1* and *AtMCM3* (Chabouté et al., 2002; Stevens et al., 2002) as well as the G2/M marker genes *AtCYCA2;1* and *AtCDKB1;1*. Altogether, these results indicate that *AtE2Fb* overexpression induces G1/S transition and cell division similar to what was observed following overexpression of *AtE2Fa* (De Veylder et al., 2002; Rossignol et al., 2002; Kosugi and Ohashi, 2003). This observation agrees with the experiments conducted very recently by Magyar et al. (2005) in tobacco BY-2 cells, in which accumulation of either AtE2Fa or AtE2Fb, together with their partner AtDPa, could sustain cell division, and AtE2Fb also could be effective in the absence of 2,4-dichlorophenoxyacetic acid.

The overexpression of *AtE2Fb* in transgenic plants led to an up-regulation of *AtE2Fa* and *AtRBR* and a down-regulation of *AtE2Fe/DEL1*, whereas the transcript levels of the other *AtE2Fs* and of the *AtDPa* gene remained unchanged. However, contrary to the increased level of *AtE2Fa* transcripts, the accumulation of AtE2Fa protein was reduced in *AtE2Fb*^{OE} plants. Since the *AtE2Fa* promoter lacks E2F-binding sites, its up-regulation could be ascribable to an overall stimulation of cell cycle progression. In contrast, the low

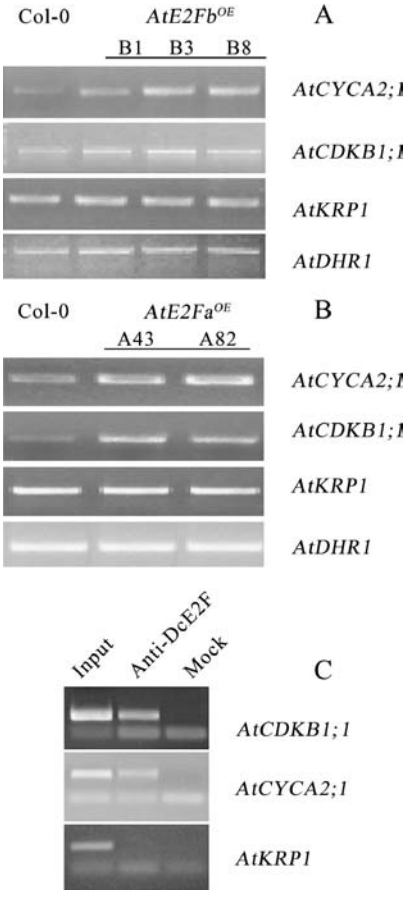

Figure 7. Levels of G2/M marker gene transcripts in plants overexpressing activating AtE2Fs. A, RT-PCR of *AtCYCA2;1*, *AtCDKB1;1*, and *AtKRP1* using RNA extracted from 7-d-old wild type (Col-0) and *AtE2Fb*^{OE} lines (B1, B3, and B8). B, RT-PCR of *AtCYCA2;1*, *AtCDKB1;1*, and *AtKRP1* using RNA extracted from 7-d-old wild type (Col-0) and *AtE2Fa*^{OE} lines (A43 and A82). The level of *AtDHR1* transcripts was used as the loading control. C, ChIP of *AtCDKB1;1*, *AtCYCA2;1*, and *AtKRP1* promoters. Experimental conditions were as described in the legend for Figure 5.

level of AtE2Fa could be due to its higher turnover rate as a result of hyperphosphorylation by AtCDKB1;1 as suggested by Magyar et al. (2005). It is worth noting that the *AtE2Fe/DEL1* and *AtRBR* promoters contain E2F cis-elements and could be direct targets of AtE2Fb. On the other hand, *AtE2Fc* and *AtE2Ff/DEL3*, which contain E2F cis-elements in their promoters, were not up-regulated in $AtE2Fb^{OE}$ plants, whereas they were strongly up-regulated in $AtE2Fa\text{-}DPa^{OE}$ plants (Vandepoele et al., 2005). This suggests that the activating AtE2Fs are likely to have different DNA-binding specificity. In this respect, it was previously observed that E2F sites could be differentially recognized by different E2F factors (Egelkrout et al., 2002; Ramirez-Parra et al., 2004).

The presence of putative E2F cis-elements in the *AtE2Fb* promoter suggests that *AtE2Fb* gene expression could be regulated by other AtE2F factors. In fact, the up-regulation of *AtE2Fb* and the overaccumulation of the relevant protein in $AtE2Fa^{OE}$ plants indicate that AtE2Fa might up-regulate the *AtE2Fb* gene. This is also supported by the results of ChIP experiments showing that one or more putative E2F cis-elements of the *AtE2Fb* promoter are actually occupied in vivo by an activating AtE2F. Up-regulation of *AtE2Fb* in plants overexpressing *AtE2Fa* and *AtDPa* also has been detected recently by microarray analysis (Vandepoele et al., 2005). Nevertheless, the fact that $AtE2Fb^{OE}$ plants showed a reduction of the AtE2Fa protein content suggests a complex interplay between these two transcription factors and/or a sequential scheduling of these AtE2Fs during cell cycle progression. Whether the expression of *AtE2Fb* might actively contribute to reduce the accumulation of AtE2Fa or rather enhance a scheduled physiological change in the stability of the two proteins remains to be established.

A remarkable result concerning the phenotype of $AtE2Fb^{OE}$ seedlings was the considerably shorter length of the primary roots (about 3-fold shorter than controls) that appeared to correlate with a reduction of the length of root cells. This suggests that accumulation of AtE2Fb antagonizes cell elongation and might delay root cell differentiation. A similar phenotype also was seen in plants overexpressing *AtE2Ff/DEL3*. However, overexpression of *AtE2Fb* led to increased content of *AtEXP5* and *AtEXP7* transcripts and did not decrease the expression of other *AtEXP* genes (*AtEXP3* and *AtEXP9*) shown to be down-regulated in $AtE2Ff^{OE}$ plants (Ramirez-Parra et al., 2004). Therefore, the altered root phenotype of $AtE2Fb^{OE}$ and $AtE2Ff^{OE}$ plants is likely to depend on different regulatory mechanisms.

Another striking feature of $AtE2Fb^{OE}$ seedlings was the absence (or almost complete absence) of trichomes in leaves. Remarkably, this phenotype is not seen in $AtE2Fa^{OE}$ plants that in absence of a concomitant overexpression of the AtDPa partner did not show major developmental abnormalities (De Veylder et al., 2002). A strong inhibition of trichome development also has been found recently in *Nicotiana benthamiana* plants upon disruption of RBR function by virus-induced gene silencing. Confirming the expected role of RBR as a negative regulator of activating plant E2Fs, the reduction of RBR levels in these plants also resulted in induction of ectopic cell division, increased endoreduplication, and delay in cell differentiation (Park et al., 2005). Thus, it appears that in particular cellular contexts, overexpression of *AtE2Fb* could lead to inhibition of cell differentiation.

Whereas ectopic overexpression of *AtE2Fa-DPa* or *AtE2Fe/DEL1* leads to changes in ploidy levels in several plant organs (De Veylder et al., 2002; Vlieghe et al., 2005), *AtE2Fb* overexpression did not induce major changes in ploidy levels in $AtE2Fb^{OE}$ plants. This suggests that activation of S phase by AtE2Fb is almost

Table II. *Primers used for RT-PCR analyses*

Name	Target	Sequence
A	*AtE2Fa*	TCTTTAGGTCTCCTTACAAAA
		AGAAGTACAATGGGACCTAT
B	*AtE2Fb*	TCAACATCTGGTCTCCCTGA
		AGCGTGGTCTTGATCAATG
C	*AtE2Fc*	CAGGCGAAGAT CCGACTC
		GCCATTCGCCATTCGTT
D	*AtE2Fd/DEL2*	ACCGGACGTGAAGAATTTTG
		TCGTTGTAATGCGCAAAAAG
E	*AtE2Fe/DEL1*	AGTGAGGCGGCTTTATGA
		TCCAGATTCTCAACATCAAAAG
F	*AtE2Ff/DEL3*	GTTAGAAGACTTTACGACATTGC
		CCTCGATCTCTAGTAACCTTCC
RB	*AtRBR*	AAGGTGTAGACTTGGTTGCAT
		TTGTCATTGCTGTGCTCACT
DP	*AtDPa*	AACCCTCACGCAGTAGTC
		GCGAGTATCAATGGATCC
RN	*AtRNR1*	GGGCTTAGCAGTGACCATTGTGA
		TTCTGGTACCATGGAGCCGCCAC-AGCATCAG
M	*AtMCM3*	TTCTGGTACCATGGAGCCGCCAC-AGCATCAG
		TCTTGGAGCTCCTAGTTCAGACG-TAGCTCAAG
CY	*AtCYCA2;1*	ATTCTCGATTCCGGTTTA
		AACGTAGTTTACTGCCAAAT
CD	*AtCDKB1;1*	GGTGGTGACATGTGGTCTGTT
		CGCAGTGTGGAAACACC
KRa	*AtKRP1*	AGCTAAAGGAATTGTAGAAGC
		ACTTTACCCATTCGTAACG
AX	*AtAXR1*	GATTTGGGGGGAGGTAGG
		CTTTACAGAGATGCGAACAAACC
EXa	*AtEXP1*	ATGGTCTAAGT TGTGGTGCTT
		AAAGACCAGCCTGCGTT
EXb	*AtEXP3*	TATACCGTGCAGGCATTGTC
		AGTGATTGGCCCGATGAGAAC
EXc	*AtEXP5*	AGGACTTAGT TGTGGCGC
		GTGGGTGGTGCAACATTA
EXd	*AtEXP7*	ACGCCACTTTCTACGGTGAC
		TAGGAGGGCAAAGATTGGTG
EXe	*AtEXP9*	TCAAGCTAGCGACAATGGTG
		AGCTCCGGCTACGTTAGTGA
DR	*AtDRH1*	AAGAGGAGCAGATATCGTGGTTG
		CGACGAGATATGTACTCTTGTT

completely counterbalanced by a comparable activation of M phase. This latter effect is likely ascribable to the up-regulation of *AtCDKB1;1*, the product of which was shown to inhibit the endocycle, thus acting as the mitosis-inducing factor (De Veylder et al., 2002; Boudolf et al., 2004).

In summary, our results suggest an interplay between the activating factors AtE2Fa and AtE2Fb in controlling the balance of cell division, endoreduplication, and differentiation in Arabidopsis plants. Both factors appear to stimulate cell proliferation and inhibit differentiation. This similarity of roles most likely results from activation of *AtE2Fb* expression by the AtE2Fa protein. Although in different contexts these two factors could act independently from each other and exert specific roles, the emerging framework of cell cycle progression in plants suggests a hierarchical organization of the E2F players. AtE2Fa is likely to be implicated in the control of early cell cycle genes and activates the expression of *AtE2Fb*, which in turn could be the primary E2F factor responsible for direct stimulation of cell proliferation as also suggested by Magyar et al. (2005) in a heterologous system.

MATERIALS AND METHODS

Plant Material and Cell Suspension Lines

All Arabidopsis (*Arabidopsis thaliana*) transgenic lines in this study were generated by the floral dip method (Clough and Bent, 1998) using *Agrobacterium tumefaciens* GV3101/pMP90 and HBA105 strains. The transgenic T_1 seeds were selected on 0.5× Murashige and Skoog medium (Duchefa) containing kanamycin (50 mg/L) or hygromycin (40 mg/L) as required. Wild-type and transformed plants were transferred to soil and grown to maturity in a greenhouse or in a growth chamber. In both cases, the growing conditions were 16 h of light (23°C ± 3°C) and 8 h of dark (18°C ± 3°C) with 70% relative humidity. For phenotypic analysis, *AtE2Fb*^{OE} T_2 seeds were germinated on semisolid Murashige and Skoog salt medium without kanamycin.

The Arabidopsis cell line T87 (Axelos et al., 1992) was grown under dim light conditions at 23°C on a rotary shaker (130 rpm) in B5 Gamborg's medium (Duchefa), pH 5.8, supplemented with 30 g/L Suc and 1 μM naphthylacetic acid. Suspension cells were subcultured weekly transferring 5 mL into 100 mL of fresh medium.

In Situ Hybridization and Immunolocalization

In situ hybridization experiments were carried out as described (Varotto et al., 2003). In brief, plant materials (seedlings and plantlets 10, 15, and 20 d after germination) were fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 16 h at 4°C and embedded in Paraplast Plus (Sigma-Aldrich). Sections (7–10 μm) were cut using a microtome (RM 2135; Leica) and collected on xylane-coated slides. Slides were deparaffinized, treated with 5 μg/mL Proteinase K, and hybridized with sense and antisense riboprobes in 50% formamide at 50°C overnight. The sections were hybridized with an *AtE2Fb*-specific, digoxigenin-labeled probe corresponding to 380 bp of the 3′ untranslated region cloned into the pBS II KS plasmid (Stratagene). T7 and Sp6 polymerases were used for the synthesis of sense and antisense labeled transcripts. Sense transcripts were used as negative control in the hybridization experiments. After hybridization, the slides were washed extensively in 2× SSC at 50°C and treated with 20 μg/mL RNaseA (Roche). Digoxigenin detection and signal visualization were carried out using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine (Roche) following the manufacturer's instructions. Images were acquired using a Leica DC 300F camera.

For immunolocalization experiments, sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, and finally rinsed in Tris-buffered saline (Sambrook et al., 1989). Before the antibody reactions, sections were briefly treated with 5 μm/mL of proteinase K. Primary antibodies against AtE2Fb were used at a 1:200 dilution. Slides were incubated for 2 h at 37°C with primary antibodies as described below and with mouse monoclonal anti-rabbit IgGs conjugated with alkaline phosphatase (Sigma) for 1 h at room temperature. Digoxigenin detection and signal visualization were carried out as described above for in situ hybridization experiments.

Construction of the *CaMV35S*::*AtE2Fb*::*GFP* Fusion Plasmid

A full-length *AtE2Fb* cDNA (1.4 kb; GenBank accession no. AF242580) was amplified by PCR. The primers were designed to mutate the stop codon as well as to introduce *Bam*HI and *Sal*I cloning sites. The primer sequences were MWD9-5 (5′-GGGGATCCTTATGTCTGAAGAAGT) and MWD9-3 (5′-TTGTCGACGCTACCTGTAGGTGATCT). The 1.4-kb PCR product was digested with *Bam*HI-*Sal*I, purified by gel electrophoresis, and inserted upstream of the GFP sequence in a modified pBI121 vector (CLONTECH) in which the *uidA* sequence was replaced with GFP coding sequence. AtE2Fb-GFP fusion protein localization was determined by confocal microscopy (Leica TCS SP2) at a wavelength between 504 and 530 nm.

Isolation of the *AtE2Fb* Promoter Region and Construction of the *AtE2Fb*::*uidA* Chimeric Plasmid

The promoter region of *AtE2Fb* was amplified from Arabidopsis genomic DNA using the primers Pro-5B (5′-GGGGTTCTTCTATTGTTGTCTC) and Pro-3B (5′-CAGCTGCCAATAAAGTCACCAA), which amplified the region spanning the stop codon of the upstream gene to the first intron of the *AtE2Fb* gene. The PCR fragment was cloned into pCR-Blunt (Invitrogen), checked by sequencing, and subcloned as a *Hin*dIII-*Xba*I fragment upstream the region encoding the *gus* (*uidA*) into the vector pTAK to create *AtE2Fb*::*uidA*. The *Hin*dIII-*Eco*RI fragment of the *AtE2Fb*::*uidA* plasmid containing the *AtE2Fb* promoter, the *uidA* coding region, and the terminator was then inserted into the binary vector pPZP111 (Hajdukiewicz et al., 1994) for the stable transformation of Arabidopsis plants.

Histochemical Determination of GUS Activity

Histochemical detection of GUS activity was performed on Arabidopsis transgenic plants at different developmental stages using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Jefferson et al., 1987). Plants were incubated in the GUS staining solution (100 mM phosphate buffer, pH 7, 1 mg/mL X-Gluc A, 1 mM potassium ferricyanide) for at least 1 h at 37°C. After staining, the samples were transferred in 70% ethanol to remove the chlorophyll. Images were captured with a Zeiss (SV11) equipped with a Sony PowerHAD camera and AxioVision 1.01 software (Zeiss).

Construction of *CaMV2x35S*::*AtE2Fb* and *CaMV35S*::*AtE2Fa*

For the overexpression of *AtE2Fb* in transgenic plants, the corresponding cDNA (Mariconti et al., 2002) was inserted as a *Bam*HI-*Xho*I fragment into the *Bam*HI-*Sal*I digested polylinker of the binary vector pGusNpt/FF19 that carries a T-DNA region containing the expression cassette of the pFF19 plasmid (Timmermans et al., 1990) and a bifunctional GUS/neomycin phosphotransferase marker gene (Datla et al., 1991). The resulting *2x35S*::*AtE2Fb* vector was introduced into the *A. tumefaciens* GV3101/pMP90 strain for plant transformation. The *35S*::*AtE2Fa* vector (Rossignol et al., 2002) was introduced into *A. tumefaciens* HBA105.

Optical and SEM Analyses

For SEM analyses, samples were slowly frozen at −18°C under a partial vacuum on the Peltier stage before observation under the environmental secondary electron detector mode (Hitachi 3-3000). Differential interference contrast microscopy was used to observe plantlets that had been fixed with FAA (50% ethanol, 5% acetic acid, and 10% formaldehyde) for 6 h, depigmented in increasing ethanol concentration, and cleared in chloral hydrate (8 g chloral hydrate, 1 mL glycerol, 2 mL water). Images were elaborated using the

Olympus DP-Soft software (Olympus). For root histology, tissues were fixed and embedded as described for in situ hybridization. Deparaffinized sections were stained with toluidine blue, and pictures were taken with a Leica DC 300F camera.

Gene Expression Analysis

Total RNA was extracted from leaves, stems, roots, and flowers of wild-type (Col-0), *AtE2Fb*^{OE} (*2x35S::AtE2Fb*), and *AtE2Fa*^{OE} (*35S::AtE2Fa*) transgenic Arabidopsis plants using the RNeasy Plant Mini kit (Qiagen). cDNA reactions were performed using the SuperScript first-strand synthesis system (Invitrogen). The level of gene expression was determined by semiquantitative RT-PCR using 1 μg of total RNA in each reaction. RT-PCR was performed using the primers listed in Table II. All primers were designed on the basis of the relevant cDNA sequences.

Production of a Recombinant Polypeptide Corresponding to the N-Terminal Domain of the AtE2Fb Protein

To raise AtE2Fa- and AtE2Fb-specific antibodies, the cDNA sequences corresponding to the divergent N-terminal 165 and 127 amino acids of AtE2Fa and AtE2Fb, respectively, were expressed in *Escherichia coli*. The corresponding region of the *AtE2Fa* and *AtE2Fb* cDNA were amplified by PCR using primers AtE2Fa/5Bam and AtE2Fb/5Bam (Mariconti et al., 2002), and ΔF11F19 (5′-TAACTGCAGCTTCCTGATGGAGTAAGT) and ΔMWD9 (5′-ATCCTG-CAGTACCGGCCTGTGCAAAG). The PCR fragments were digested with *Bam*HI and *Pst*I and cloned in pRSET (A and B, respectively, for *AtE2Fa* and *AtE2Fb*; Invitrogen) digested with the same restriction enzymes. The resulting plasmids were then introduced into *E. coli* BL21 (DE3) pLysE for the production of the recombinant N-terminal portion of the two AtE2Fs. The HIS-AtE2Fa-(1-165) and HIS-AtE2Fb-(1-127) polypeptides were purified by metal-affinity chromatography on nickel-nitrilotriacetic acid resin (Qiagen) using phosphate buffer containing 8 M urea. The analysis by SDS-PAGE of the eluted protein revealed a single polypeptide of the expected dimensions.

Antisera and Immunoblotting

Four rabbit polyclonal antisera were used in this study. The first antiserum was raised against the carrot (*Daucus carota*) DcE2F, which recognizes AtE2Fa and, to a much lesser extent, AtE2Fb (Albani et al., 2000; Supplemental Fig. 2). The monospecific antisera against AtE2Fa and AtE2Fb were obtained by the immunization of rabbits using the dialyzed HIS-AtE2Fa-(1-165) and His-AtE2Fb-(1-127) polypeptides eluted under denaturing conditions. These antibodies showed a low cross-reactivity. The fourth antiserum was against the AtE2Fb C-terminal oligopolypeptide DQDHAGPSDNKILE conjugated to a carrier protein. To determine the amount of AtE2F protein in suspension cell cultures and transgenic plants, a 100-mg aliquot of each sample was frozen in liquid nitrogen, ground in a mortar to a fine powder, which was dissolved in 500 μL of extraction buffer containing 100 mM Tris-HCl, pH 7.8, 1 mM EDTA, 200 mM NaCl, 0.2% Triton X-100, and 1 × Complete Mini protein inhibitors (Roche), and centrifuged for 10 min to remove debris. Protein concentration was estimated by the Bradford assay. Protein extracts (30 μg) were subjected to SDS-PAGE in a 12% polyacrylamide gel and transferred to a nitrocellulose membrane using standard techniques (Sambrook et al., 1989). Immunodetection was performed using the indicated polyclonal antibodies at 1:1,000 (v/v) dilutions. Goat anti-rabbit alkaline phosphatase-conjugated secondary antibody was used at 1:15,000 (v/v) dilutions and detection was performed using ECL chemiluminescence detection reagents (Amersham).

ChIP

ChIP assays were performed using nuclei extracted from suspension-cultured Arabidopsis T87 cells. Nuclei, extracted as described previously (Albani et al., 2000), were treated with 1% formaldehyde at 22°C for 10 min, and the cross-linking was stopped by the addition of 0.125 M Gly. Fixed nuclei were resuspended in SDS buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 1% SDS) and sonicated to shear DNA to 600- to 1,000-bp fragment size. To reduce false positives, sonicated chromatin samples were preincubated with 20 μL of preimmune serum for 1 h at 4°C with gentle mixing, transferred to a new tube with 20 μL of protein A-Sepharose (50% slurry in 15 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100), and incubated with gentle mixing for 1 h at 4°C. Samples were centrifuged at 13,000 rpm for 2 min at 4°C. The resulting supernatant was specifically immunoprecipitated with 20 μL of anti-DcE2F serum and further incubated for 2 h at 4°C with gentle mixing. Immunocomplexes were recovered using 20 μL of protein A-Sepharose (50% slurry) for 2 h at room temperature with gentle mixing, extensively washed, and eluted from beads. The immunoprecipitated chromatin was incubated for 5 h at 65°C, added with two volumes of ethanol, and centrifuged. The resulting pellet was incubated with proteinase K (18.5 mg/mL) for 2 h at 42°C and extracted with phenol/chloroform. Upon ethanol precipitation, DNA was resuspended in 10 μL of water, and 1 μL was used for PCR analysis using the following primers adjacent to promoter E2F cis-elements: B5 (5′-TCCCCTCAATCTCAAGGAAA-3′) and B3 (5′-AAGAACGAATCTC-GATAAAA-3′) for *AtE2Fb*; P5 (5′-GAGACAAGACTCACAGATGA-3′) and P3 (5′-GGTTAGAGTGTGAATCGA-3′) for *AtPCNA*; R5 (5′-AATGGGCTT-TAACTCTCTAA-3′) and R3 (5′-AAGGGATTTGAAGATTTG-3′) for *AtRNR1*; CD5 (5′-TAACTCGTGAAGAATTTGAA-3′) and CD3 (5′-TTCTGAGAGG-TTTCGTAAAA-3′) for *AtCDKB1;1*; Cy5 (5′-GGAAATCAATGCTGAAA-GAG-3′) and Cy3 (5′-TGAGAGAGAGATCTTGAA-3′) for AtCYCA2;1; KR5 (5′-GTTTCGCGTAATGGCAAAT-3′) and KR3 (5′-GCGTGAAGTCAC-AATCT-3′) for AtKRP1; and D5 (5′-TCTCAAATTTTAGGCAAGTT-3′) and D3 (5′-GGCTTCTTCTTCTTCAACTT-3′) for *AtGDH*.

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