Light-Dependent Regulation of *DEL1* Is Determined by the Antagonistic Action of E2Fb and E2Fc[1][W][OA]

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Endoreduplication represents a variation on the cell cycle in which multiple rounds of DNA replication occur without subsequent chromosome separation and cytokinesis, thereby increasing the cellular DNA content. It is known that the DNA ploidy level of cells is controlled by external stimuli such as light; however, limited knowledge is available on how environmental signals regulate the endoreduplication cycle at the molecular level. Previously, we had demonstrated that the conversion from a mitotic cell cycle into an endoreduplication cycle is controlled by the atypical E2F transcription factor, DP-E2F-LIKE1 (DEL1), that represses the endocycle onset. Here, the Arabidopsis (*Arabidopsis thaliana*) *DEL1* gene was identified as a transcriptional target of the classical E2Fb and E2Fc transcription factors that antagonistically control its transcript levels through competition for a single E2F cis-acting binding site. In accordance with the reported opposite effects of light on the protein levels of E2Fb and E2Fc, *DEL1* transcription depended on the light regime. Strikingly, modified *DEL1* expression levels uncoupled the link between light and endoreduplication in hypocotyls, implying that DEL1 acts as a regulatory connection between endocycle control and the photomorphogenic response.

Plant development occurs mostly postembryonically. It involves the production of new cells that arise at the meristems from divisions of pluripotent stem cells, followed by their successive cell cycle exit and differentiation. Due to their sessile life style, plants are exposed to changing environmental conditions and thus are continuously forced to adapt their body plan (Walter et al., 2009; Skirycz and Inzé, 2010). This plasticity requires a close connection between cell division, differentiation, and development. Several studies indicate that the core cell cycle machinery is a direct target of various developmental factors (Gutierrez, 2005; Ramirez-Parra et al., 2005; Busov et al., 2008). Correspondingly, cell division rates and cell cycle gene expression levels change upon biotic and abiotic stresses (Burssens et al., 2000; Granier et al., 2000; Kadota et al., 2004; West et al., 2004). The importance of cell cycle control during plant development is further demonstrated by the aberrant plant morphologies that result from alterations in cell cycle regulation (De Veylder et al., 2001; Wyrzykowska et al., 2002; Dewitte et al., 2003, 2007).

Over the last decades, the core cell cycle machinery has been well characterized. Upon cell cycle stimulation, cyclin-dependent kinases (CDKs) are activated that in turn relieve the repressive action of the RETINOBLASTOMA-RELATED (RBR) protein on the E2F transcription factors (Inzé and De Veylder, 2006; Berckmans and De Veylder, 2009), resulting in the transcriptional activation of hundreds of E2F target genes, which are mostly DNA replication genes (Vlieghe et al., 2003; Vandepoele et al., 2005; de Jager et al., 2009; Naouar et al., 2009). The E2F/dimerization partner (DP)/RBR pathway is highly conserved among higher eukaryotes. The structure and function of the E2F/DP proteins, as well as their cis-acting recognition site, are identical in both mammals and plants. In Arabidopsis (*Arabidopsis thaliana*), a total of six E2F factors can be subdivided into typical (E2Fa, E2Fb, and E2Fc) and atypical (DP-E2F-LIKE1 [DEL1]/E2Fe, DEL2/E2Fd, and DEL3/E2Ff). Typical E2F factors need to dimerize with a DP to gain a high DNA-binding specificity, which is not the case for atypical ones because they possess two DNA-binding domains and, hence, can bind DNA as monomers.

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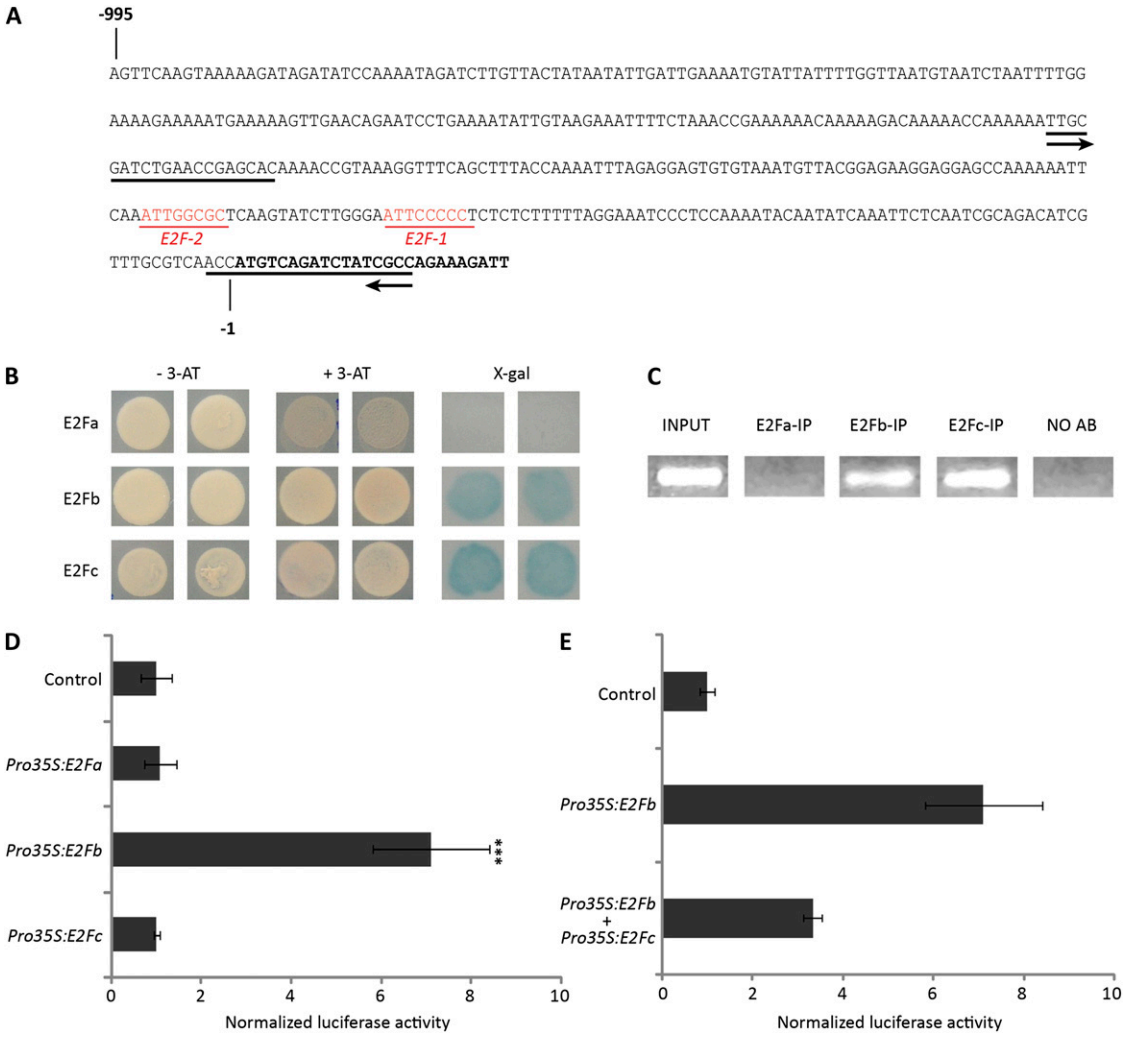

Figure 1. Interaction of E2Fb and E2Fc with the *DEL1* promoter. A, Sequence of the *DEL1* promoter with the two putative *E2F* cis-acting sites (red) and the primers used for ChIP (black arrows) indicated. B and C, E2Fb and E2Fc interaction with the *DEL1* promoter in yeast (B) and in planta (C) as shown by Y1H and ChIP, respectively. Interactions observed by Y1H are positive when both *HIS3* (grown on +3-aminotriazole [3-AT] medium) and *LacZ* (X-gal positive) expression were induced. IP, Immunoprecipitation; NO AB, no antibody. D and E, Protoplast transactivation activity assays with a *ProDEL1:fLUC* reporter construct, a *Pro35S:rLUC* normalization construct, and a *35S:E2Fa*, *35S:E2Fb*, or *35S:E2Fc* effector construct, showing stimulation of *DEL1* promoter activity by E2Fb (D) being counteracted by E2Fc (E). Luciferase activity of control cells was arbitrarily set to 1. Data are means ± SE ($n = 8$; *** $P \leq 0.001$, two-sided t test).

Both E2Fa and E2Fb are transcriptional cell cycle activators, and their overproduction enhances cell proliferation (De Veylder et al., 2002; Magyar et al., 2005; Sozzani et al., 2006). As *E2Fc* overexpression inhibits cell cycle progression, E2Fc is seen as a repressor (del Pozo et al., 2002, 2006). Atypical E2Fs are considered as repressors as well because they lack a transcriptional activation domain (Lammens et al., 2009) and, in agreement, counteract the activation of E2F-responsive reporter genes (Kosugi and Ohashi, 2002b; Mariconti et al., 2002).

Previously, the atypical E2F transcription factor DEL1 had been identified as an important negative regulator of endocycle onset (Vlieghe et al., 2005; Lammens et al., 2008). The endocycle, or endoreduplication, is a variant of the mitotic cell cycle in which the genome is duplicated without cell division, resulting in polyploid cells. In Arabidopsis, endoreduplication occurs in almost all tissue types and has been suggested to play a role in cell differentiation, development, UV resistance, and metabolic potential (Grafi and Larkins, 1995; Gendreau et al., 1997; Joubès and Chevalier, 2000; Larkins et al., 2001; Vinardell et al., 2003; Beemster et al., 2005; Hase et al., 2006; Bramsiepe et al., 2010; Kaźmierczak, 2010; Radziejwoski et al., 2011). Mitotic cell cycle progression and endoreduplication are intimately linked during organ development, in which a cell proliferation phase is followed by

the onset of endoreduplication (Jacqmard et al., 1999; Joubès et al., 1999; De Veylder et al., 2001; Gonzalez et al., 2007). The switch between mitotic cell division and endoreduplication might involve the inactivation of mitotic CDK-cyclin complexes through the degradation of the cyclin moiety by the anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase that targets proteins to the 26S proteasome. The APC/C is conserved in vertebrates and plants and consists of several subunits, of which the activator subunit confers the substrate specificity. In Arabidopsis leaves, transcription of the APC/C activator *CCS52A2* is repressed by DEL1 during the mitotic cell cycle (Lammens et al., 2008). Upon transition to the endocycle, the *DEL1* transcript levels drop dramatically, triggering a peak in *CCS52A2* transcripts that marks the onset of the endocycle.

As observed for the mitotic cell cycle, the endocycle is under the control of different environmental cues. Water deficit, temperature, nutrient supply, and light all affect endoreduplication, but the molecular mechanisms linking the environment with the endocycle machinery are still largely unknown (Artlip et al., 1995; Cavallini et al., 1995; Engelen-Eigles et al., 2001; Setter and Flannigan, 2001; Cookson et al., 2006). The best-studied case is probably the response of Arabidopsis hypocotyls to dark/light treatments, in which an extra endoreduplication cycle is triggered by darkness (Gendreau et al., 1997, 1998). Similar effects can be seen in the hypocotyls of other plant species, including cabbage (*Brassica oleracea*) and pea (*Pisum sativum*; Van Oostveldt and Van Parijs, 1975; Kudo and Mii, 2004). Nevertheless, the way in which light controls the DNA ploidy level of cells is still unclear. Here, we show that both E2Fb and E2Fc antagonistically control *DEL1* expression and that *DEL1* levels are regulated by light through the balance between E2Fb and E2Fc. In *DEL1*-overexpressing and mutant hypocotyls, the connection between light and ploidy was uncoupled, which indicates that DEL1 is a mediator of the light-dependent endoreduplication in hypocotyls.

RESULTS

E2Fb and E2Fc Associate with the *DEL1* Promoter

To identify possible transcriptional regulators of *DEL1*, we analyzed its promoter with the Plant Cis-Acting Regulatory DNA Elements (PLACE) database (Higo et al., 1999). Two putative E2F-binding sites, *E2F-1* and *E2F-2*, were found (Fig. 1A). Interestingly, the presence of E2F-binding sites in the promoter of the atypical *E2F* genes was conserved within the green plant lineage (Supplemental Fig. S1). To investigate whether the E2F transcription factors associate with the *DEL1* promoter, we carried out a yeast one-hybrid (Y1H) experiment. A reporter strain was designed, harboring the *DEL1* promoter (995 bp) upstream of a *HISTONE3* (*HIS3*) selection gene and the bacterial β-galactosidase-encoding *LacZ* reporter gene. Subsequently, the binding of the three classical E2Fs to the

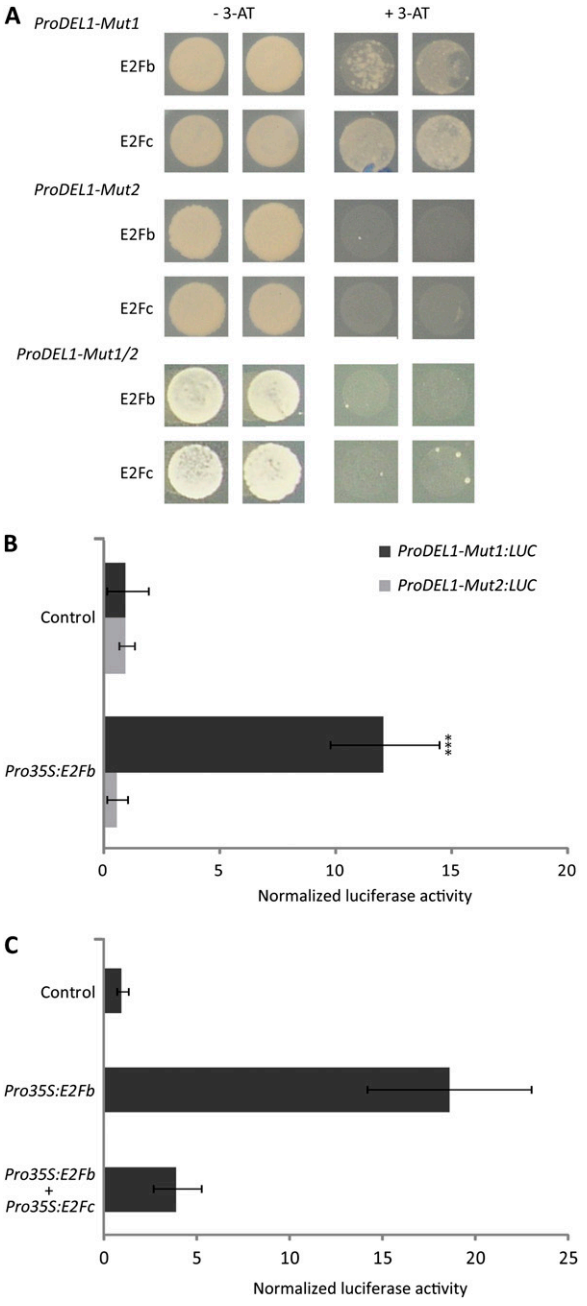

Figure 2. Competition of E2Fb and E2Fc for binding to the *E2F-2* site in the *DEL1* promoter. A, Interaction of E2Fb and E2Fc with *E2F-2* in yeast shown by Y1H. 3-AT, 3-Aminotriazole. B and C, Protoplast transactivation activity assay with a *ProDEL1:fLUC* reporter construct, a *Pro-35S:rLUC* normalization construct, and a *35S:E2Fb* or *35S:E2Fc* effector construct. An intact *E2F-2* binding site was required for activation of the *DEL1* promoter by E2Fb (B). Both E2Fb and E2Fc bound *E2F-2* in a competitive manner (C). Luciferase activity of control cells was arbitrarily set to 1. Data are means ± SE ($n = 8$; *** $P \leq 0.001$, two-sided *t* test).

DEL1 promoter was tested. Both E2Fb and E2Fc, but not E2Fa, bound to the *DEL1* promoter, as indicated by both auxotrophic growth on His-lacking medium and

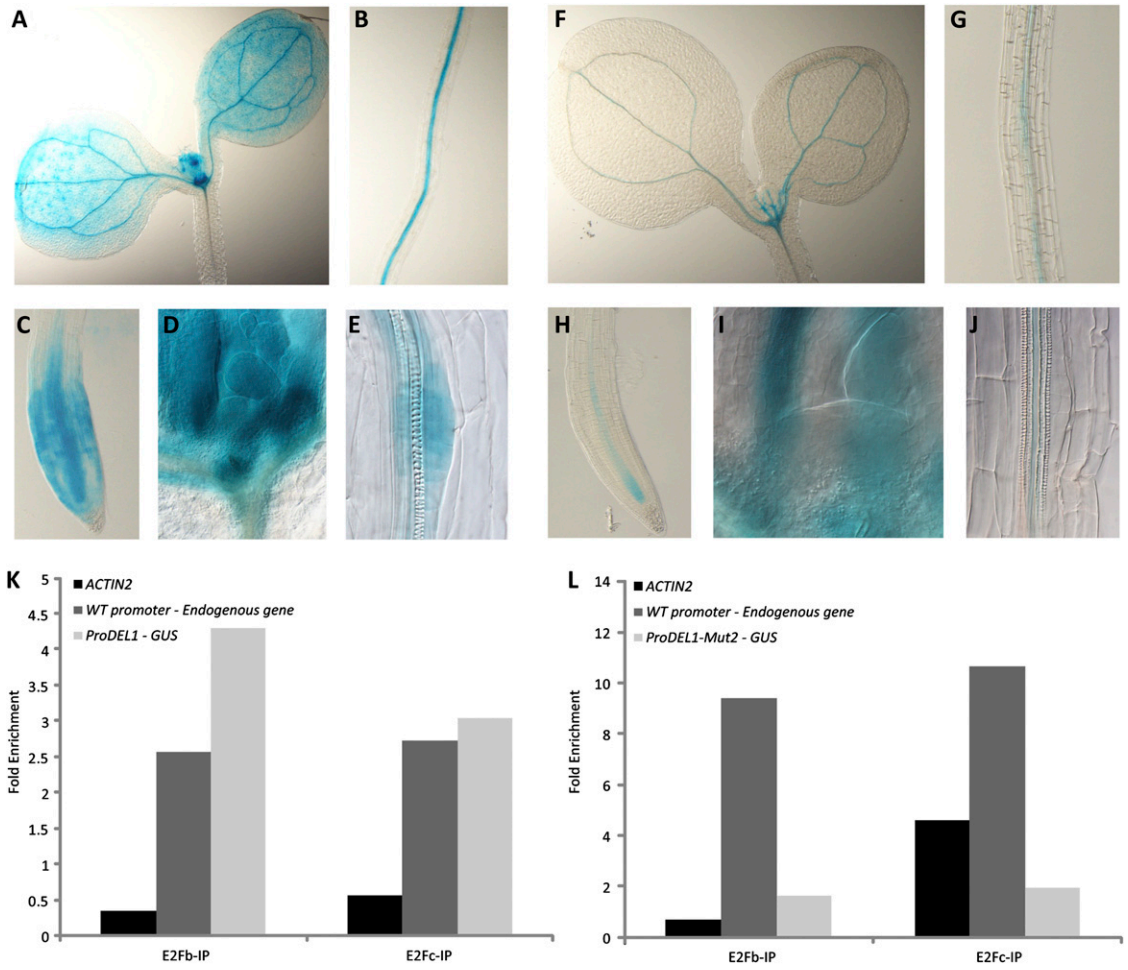

Figure 3. Requirement of *E2F-2* for *DEL1* expression in dividing tissues and binding of E2Fb and E2Fc in vivo. A to J, *ProDEL1*:*GUS* (A–E) versus *ProDEL1-Mut2*:*GUS* (F–J) expression patterns. K and L, In vivo analysis by ChIP of E2Fb (E2Fb-IP) and E2Fc (E2Fc-IP) binding to the endogenous *DEL1* and inserted *ProDEL1*:*GUS* (K) or *ProDEL1-Mut2*:*GUS* (L) promoter, with a reverse primer, specific for the endogenous *DEL1* or *GUS* gene. WT, Wild type.

activation of the *LacZ* gene (Fig. 1B). These results were confirmed by chromatin immunoprecipitation (ChIP), demonstrating the association of E2Fb and E2Fc with the *DEL1* promoter in vivo (Fig. 1C).

A transient expression assay was used to assess the effect of the different E2Fs on *DEL1* promoter activity. A *ProDEL1*:*Luciferase* construct was cotransformed with overexpression constructs for *E2Fa*, *E2Fb*, or *E2Fc*. E2Fb activated the *DEL1* promoter (Fig. 1D), but no significant effect was seen for E2Fa and E2Fc, despite its association with the *DEL1* promoter. Due to the lack of a transcriptional activation domain, E2Fc is assumed to function as a repressor either through the recruitment of chromatin-modifying enzymes or by competition for available binding sites with the active E2Fs. To test the latter hypothesis, we combined the *E2Fb* and *E2Fc* overexpression constructs in the transactivation assay. Interestingly, the presence of E2Fc diminished the activation of the *DEL1* promoter by E2Fb (Fig. 1E). Hence, *DEL1* is bound by E2Fb and E2Fc both in vitro and in vivo, whereby E2Fb transcriptionally activates the *DEL1* promoter in the absence of E2Fc.

E2Fb and E2Fc Regulate *DEL1* Expression through Binding of the Same *E2F* cis-Acting Element

As the *DEL1* promoter holds two putative E2F sites, we wondered whether E2Fb and E2Fc might bind different cis-acting elements or compete with each other for the same binding site. To analyze the functional relevance of both detected E2F cis-acting elements, we designed constructs in which either one (*ProDEL1-Mut1* and *ProDEL1-Mut2*) or both (*ProDEL1-Mut1/2*) E2F sites were mutated. The first E2F site, 5′-ATTCCCCC-3′, was mutated into 5′-ATTCAACC-3′ (*ProDEL1-Mut1*) and the second, 5′-ATTGGCGC-3′, into 5′-ATTGAAGC-3′ (*ProDEL1-Mut2*), because previously these types of mutations had been demonstrated to impair E2F binding (Kosugi and Ohashi, 2002a; Boudolf et al., 2004). In the first experiment, the three promoter

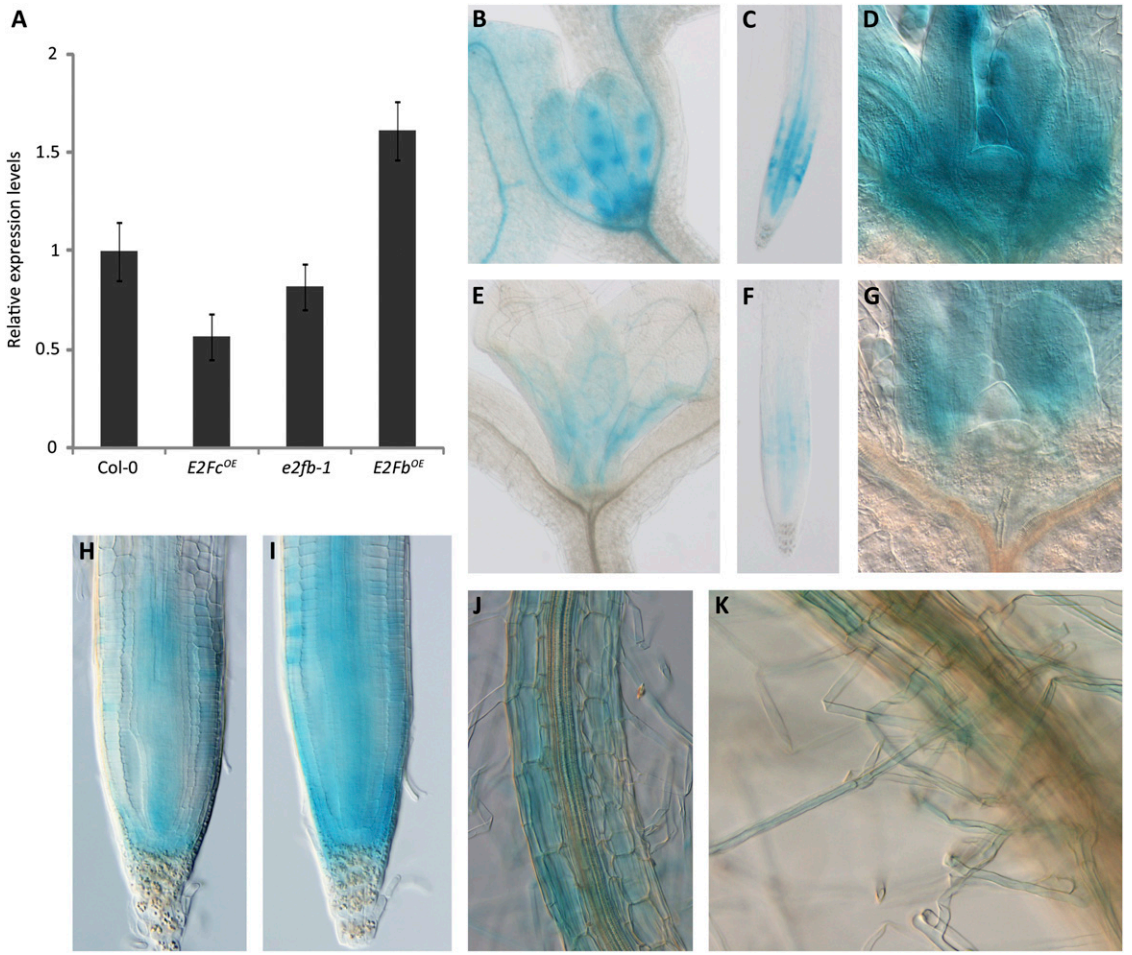

Figure 4. Changes in *DEL1* expression levels in *E2Fb* and *E2Fc* transgenic lines. A, *DEL1* expression levels in control (Columbia [Col-0]), $E2Fc^{OE}$, *e2fb-1*, and $E2Fb^{OE}$ lines. Data are means ± SD ($n = 3$). B to G, *ProDEL1*:*GUS* in Col-0 (B–D) or *e2fb-1* (E–G) background. H to K, *ProDEL1*:*GUS* in Col-0 (H) or $E2Fb^{OE}$ (I–K) background.

constructs were tested by Y1H for their ability to bind E2Fb and E2Fc. Mutation of the two sites impaired the binding of both E2Fb and E2Fc. Also the *ProDEL1-Mut2* promoter failed to interact with both E2Fb and E2Fc, as seen for *ProDEL1-Mut1/2*. By contrast, the *ProDEL1-Mut1* construct was still functional (Fig. 2A).

In a second experiment, we analyzed the mutated promoters by means of the transient activation assay. The *DEL1* promoter was activated by E2Fb only when the second E2F site was not mutated, indicating that E2Fb activates *DEL1* through binding of the *E2F-2* site (Fig. 2B). Analogously, a competition experiment with *ProDEL1-Mut1* showed that E2Fc acts as a repressor on the *E2F-2* site, in agreement with its binding preference to this site (Fig. 2C). In conclusion, E2Fb and E2Fc compete for the same E2F-binding site (*E2F-2*).

E2F-2 Mutation Decreases *DEL1* Expression in Vivo

To analyze the in vivo effect of the mutated *E2F-2* site, *ProDEL1*:*GUS* and *ProDEL1-Mut2*:*GUS* reporter constructs were transformed into Arabidopsis plants. The wild-type *DEL1* promoter was expressed in vascular and dividing tissues, including the shoot and root apical meristems (Fig. 3, A–E), confirming previous results (Lammens et al., 2008). The *E2F-2* mutation constrained GUS staining to the vascular tissues (Fig. 3, F–J) and strongly reduced the GUS activity in dividing cells of leaves, root tips, lateral root primordia, and the shoot apical meristem (Fig. 3, F–J), probably because E2Fb cannot activate the *DEL1* promoter mutated in *E2F-2*. A ChIP experiment was designed to compare binding of the endogenous promoter with that of the introduced promoter constructs. To this end, primers were constructed that amplified either the endogenous or the mutant promoter via reverse primer annealing with the *DEL1* or *GUS* gene, respectively. This experiment revealed that deletion of *E2F-2* abolished the binding of both E2Fb and E2Fc in vivo (Fig. 3, K and L).

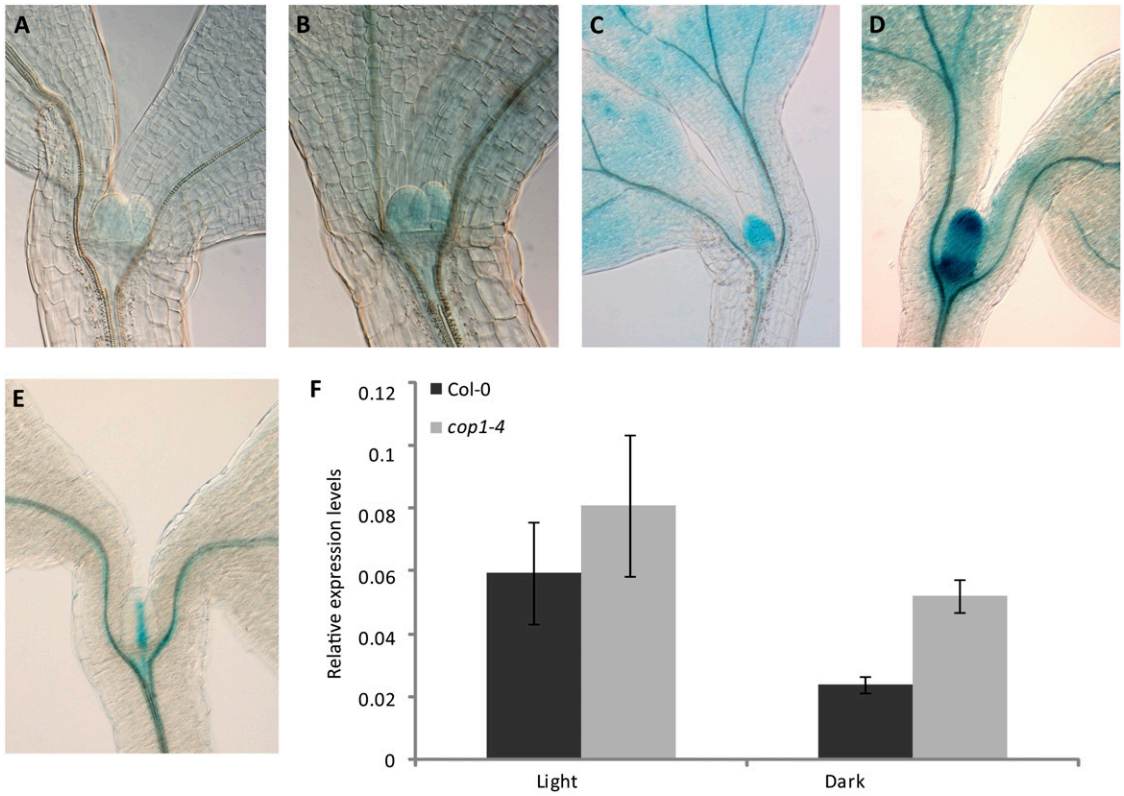

Figure 5. Dependence of *DEL1* expression levels on light and COP1. A to D, *ProDEL1*:*GUS* plants grown for 3 d in the dark (A) and exposed for 4 h (B), 24 h (C), or 48 h (D) to light. E, *ProDEL1-Mut2*:*GUS* plant switched to continuous light for 48 h after 3 d of germination in the dark. F, *DEL1* expression levels in control (Columbia [Col-0]) and *cop1-4* lines. Data are means ± SD ($n = 3$).

DEL1 Expression Levels Are Modified in *E2Fb* and *E2Fc* Transgenic Lines

As E2Fb and E2Fc bind and regulate *DEL1* promoter activity, we hypothesized that *DEL1* transcript levels would be modified in *E2Fb* and *E2Fc* transgenic lines. An E2Fb T-DNA insertion line (*e2fb-1*) was isolated and an *E2Fb* overexpression line ($E2Fb^{OE}$) was generated. The E2Fb transcript and protein levels increased and decreased in the $E2Fb^{OE}$ and *e2fb-1* lines, respectively (Supplemental Figs. S2 and S3). Although the *E2Fc* overexpression and silencing lines have been described previously (del Pozo et al., 2002, 2006), in our hands the silencing appeared unstable, and our attempts to generate such lines failed. Within the available transgenic lines, *DEL1* expression levels were down-regulated in both *e2fb-1* and $E2Fc^{OE}$ lines but increased in the $E2Fb^{OE}$ lines (Fig. 4A). The changes in transcript levels were relatively small, indicating that *DEL1* regulation by E2Fb and E2Fc might be restricted to specific tissues or conditions. To visualize in which tissues *DEL1* expression was altered, we crossed the *ProDEL1*:*GUS* reporter line with the different *E2Fb/E2Fc* transgenic lines. In the *e2fb-1* background, an overall decrease in *DEL1* promoter activity could be observed, clearly visible in the shoot and root apical meristems (Fig. 4, B–G) and closely resembling the *GUS* expression pattern of the *ProDEL1-Mut2*:*GUS* lines. In the $E2Fb^{OE}$ background, GUS staining intensified in the root apical meristem, whereas ectopic GUS staining could be seen in stretches along the root and in root hair cells (Fig. 4, H–K). In the $E2Fc^{OE}$ background, the spatial expression did not change (data not shown).

DEL1 Expression Levels Depend on Light and Are Regulated by COP1

Previously, it had been demonstrated that E2Fc protein levels are high in etiolated seedlings, whereas those of E2Fb are low, when compared with light-grown seedlings. Transfer of plants from darkness into light resulted in the degradation of E2Fc and an increase in E2Fb protein levels (López-Juez et al., 2008). As E2Fb and E2Fc protein levels were found to be light responsive and were also shown to antagonistically control *DEL1* promoter activity, we postulated that E2Fb and E2Fc might be responsible for the light-dependent regulation of *DEL1* transcription. To test this hypothesis, we examined whether *DEL1* transcription was light responsive by comparing the GUS activity of dark-grown *ProDEL1*:*GUS* plants with that of seedlings transferred from darkness to light for 4, 24, and 48 h. In dark-grown seedlings, the GUS activity was low, but it increased dramatically upon transfer to light

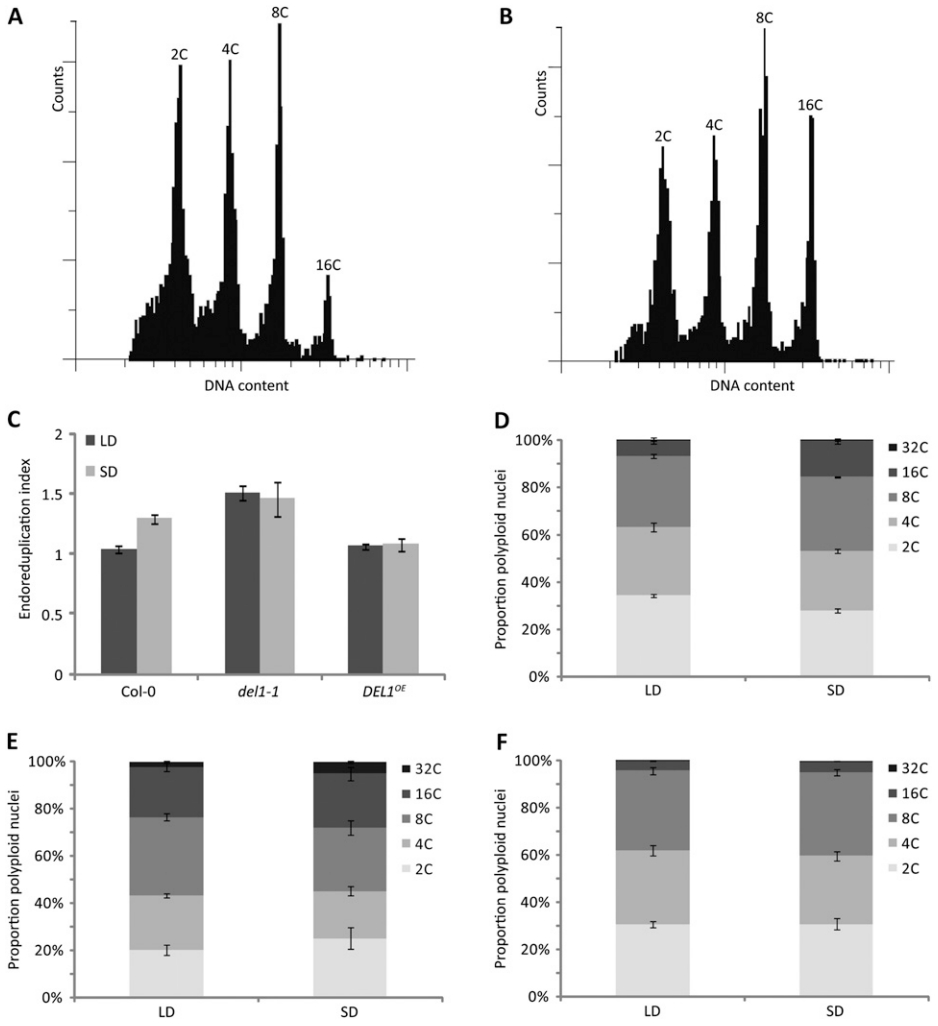

Figure 6. Influence of *DEL1* transcript levels on the response to light of hypocotyl ploidy levels. A and B, Ploidy distribution in hypocotyls of plants grown under long-day (A) and short-day (B) conditions. C, Endoreduplication index in Columbia (Col-0), *del1-1*, and $DEL1^{OE}$ lines under short-day (SD) and long-day (LD) conditions after 12 d of growth. D to F, Ploidy distribution in Col-0 (D), *del1-1* (E), and $DEL1^{OE}$ (F) lines under SD and LD conditions after 12 d of growth. Data are means ± SD ($n = 3$).

(Fig. 5, A–D). In contrast, in *ProDEL1-Mut2*:*GUS* lines, *GUS* expression was not up-regulated, even after 48 h of light treatment, except for the vascular cells (Fig. 5E).

The ubiquitin E3 ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) is responsible for the degradation of light signaling components in the dark (Deng et al., 1991; Osterlund et al., 2000). Mutants of COP1 are characterized by normal photomorphogenesis even under dark-grown conditions, due to their failure to degrade positive light signals (Deng et al., 1991). It had been previously shown that degradation of E2Fb in the dark was mediated by COP1. In dark-grown *cop1-4* mutant plants, E2Fb proteins were stabilized (López-Juez et al., 2008). To investigate whether this stabilization of E2Fb had an effect on *DEL1* expression, we compared transcript levels in dark- and light-grown wild-type and *cop1-4* mutant plants. No statistical difference in *DEL1* transcript levels could be observed between light-grown wild-type and mutant plants, but in dark-grown *cop1-4* plants, the *DEL1* expression level was statistically ($P \leq 0.01$, two-sided t test) higher than that of dark-grown wild-type plants (Fig. 5F). From these data, we conclude that the *DEL1* transcript levels are inhibited in the dark through the COP1-mediated degradation of E2Fb.

Light-Dependent Endoreduplication of Hypocotyls Depends on DEL1

As DEL1 inhibits the endocycle onset and the DNA ploidy level of hypocotyl cells depends on light (Gendreau et al., 1998; Vlieghe et al., 2005; Lammens et al., 2008), we tested whether DEL1 could be involved in the control of light-dependent hypocotyl endoreduplication. To this end, we compared the hypocotyl ploidy levels of 12-d-old seedlings grown under short-day (8 h of light) versus long-day (16 h of light) conditions. Hypocotyls of seedlings grown under short-day conditions had a statistically significantly ($P \leq 0.01$, two-sided t test) increased ploidy level, mostly due to an increase in the 8C and 16C ploidy content (Fig. 6, A and B), confirming the previously reported dependence of the DNA content on light (Gendreau et al., 1998). $DEL1^{OE}$ and *del1-1* plants,

however, reacted differently to the applied light regime. Whereas the endoreduplication index was higher in control plants under short-day than under long-day conditions, the endoreduplication level remained the same in *del1-1* plants under both light regimes. Interestingly, the endoreduplication level of the *del1-1* plants was approximately the same as that in short-day-grown wild-type plants (Fig. 6C). Conversely, both light- and dark-grown $DEL1^{OE}$ plants displayed an endoreduplication index comparable to that of long-day-grown wild-type plants (Fig. 6C). When the relative proportion of each ploidy class was considered, the proportion of 16C increased under short-day growth conditions in wild-type plants, but the ploidy distribution did not change obviously between short- and long-day-grown *del1-1* and $DEL1^{OE}$ plants (Fig. 6, D–F). These data illustrate that modified *DEL1* transcript levels uncoupled the effect of light on the endoreduplication level.

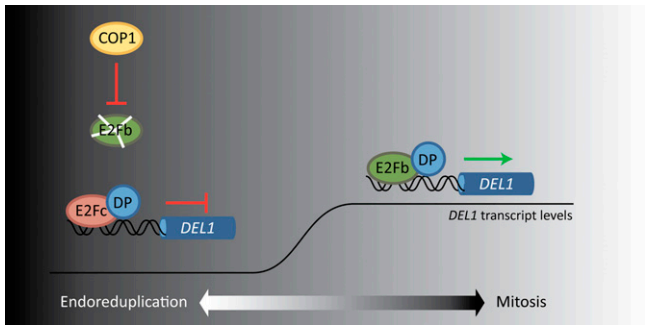

Figure 7. Model for light-controlled hypocotyl endoreduplication. In the presence of light, E2Fb activates *DEL1* expression, preventing cells from entering the endoreduplication program. Under dark conditions, E2Fb is marked by COP1 for degradation, by which E2Fc becomes the most abundant E2F binding the *DEL1* promoter. The decrease in *DEL1* transcript level allows cells to enter the endoreduplication cycle.

DISCUSSION

Both E2Fb and E2Fc Bind the *DEL1* Promoter in a Competitive Manner

Previously, we had demonstrated that DEL1 operates in mitotically dividing cells as a repressor of endocycle onset (Lammens et al., 2008). To get insight into how *DEL1* expression might be regulated, we focused on the two putative E2F cis-acting elements present in its promoter. Through Y1H and ChIP experiments, we established that both E2Fb and E2Fc, designated as classical E2Fs, bind the *DEL1* promoter, implying a transcriptional cross talk between typical and atypical E2Fs. An analogous interaction between different types of E2Fs has been observed in mammals, in which *E2F7* and *E2F8*, the mammalian homologs of the *DEL1* gene, are regulated by the classical E2F1 (Di Stefano et al., 2003; Christensen et al., 2005), indicating that the interplay between typical and atypical E2Fs is evolutionarily conserved.

E2Fb activates gene expression, whereas E2Fc, lacking a transcriptional activation domain, operates as a repressor (del Pozo et al., 2002; Mariconti et al., 2002; Magyar et al., 2005; Sozzani et al., 2006). Analysis of the two E2F cis-acting elements within the *DEL1* promoter revealed that E2Fb and E2Fc occupy the same DNA-binding site (*E2F-2*). The association of antagonistic E2Fs with the same promoter element suggests that the *DEL1* transcript levels are controlled by the relative abundance of E2Fb and E2Fc. Indeed, in the protoplast activation assays, E2Fc counteracted the activation of the *DEL1* promoter by E2Fb. In the absence of E2Fb, E2Fc was unable to repress *DEL1* activity. Analogously, no activation of the *DEL1* promoter could be observed upon deletion of the *E2F-2* site, which would have been expected if E2Fc played a role as an active repressor. Rather, a strong reduction of promoter activity in young leaves, lateral root primordia, and apical meristems was seen. As a similar decrease in promoter activity was observed in the E2Fb knockout plants, we postulate that *DEL1* expression in dividing tissues mostly depends on E2Fb and that E2Fc passively hinders *DEL1* promoter activation through occupation of the *E2F-2* binding site.

In addition to *DEL1*, the plant *RIBONUCLEOTIDE REDUCTASE* and *PROLIFERATING CELL NUCLEAR ANTIGEN* genes are also both regulated by repressing and activating E2Fs (Chabouté et al., 2000; Egelkrout et al., 2002). The antagonistic relationship between repressing and activating E2Fs is well described for fruitfly (*Drosophila melanogaster*). *Drosophila* contains only two E2Fs, one activator (dE2F1) and one repressor (dE2F2; Ohtani and Nevins, 1994; Sawado et al., 1998). Depletion of the activating dE2F1 inhibits the expression of G1/S-specific cell cycle genes and cell proliferation. In contrast, only a subset of the dE2F1-controlled G1/S genes is up-regulated upon mutation of dE2F2, without any clear effect on cell proliferation (Duronio et al., 1995; Cayirlioglu et al., 2001; Frolov et al., 2001; Dimova et al., 2003). Remarkably, the combined *de2f1* and *de2f2* mutations restore the cell proliferation phenotype of the single *de2f1* mutants (Frolov et al., 2001), implying that the phenotypes of deleting the activating E2F are in part due to the unchecked activity of the repressive E2F, thus hinting at an antagonistic action of dE2F1 and dE2F2. However, although larval cell proliferation is normal, *de2f1 de2f2* mutant flies are not viable because of developmental defects. Similarly, in mouse (*Mus musculus*), mutations of activator or repressor E2Fs result in tissue-specific defects in proliferation and/or development, indicating that a balance between positively and negatively acting E2Fs is important for the coordination of cell division and differentiation (Attwooll et al., 2004; Dimova and Dyson, 2005). The relative abundance of active and repressive E2Fs is probably essential for correct plant development as well, as illustrated by the strong growth and differentiation defects observed upon *E2F* overexpression or silencing

(del Pozo et al., 2002, 2006; De Veylder et al., 2002; Sozzani et al., 2006). Through the antagonistic regulation of genes involved in the cross talk between cell division and differentiation, the expression level of genes promoting or repressing differentiation might be switched on rapidly, a process important to ensure the irreversibility of cell differentiation.

Although mutation of the *E2F-2* cis-acting element within the *DEL1* promoter strongly reduced its activity, transcription was maintained in the vascular tissues. Currently, it is still unclear how *DEL1* expression is maintained in the vascular cells; however, E2F-independent transcriptional control might possibly be involved. As endoreduplicated cells rarely reenter the cell cycle, *DEL1* expression in vascular cells might represent a mechanism to keep these cells competent for division, thereby contributing to vascular thickening.

Linking Light-Dependent Regulation of *DEL1* with Hypocotyl Endoreduplication

E2Fb and E2Fc protein levels are antagonistically regulated by light. Transfer of dark-grown seedlings into light quickly stabilizes and destabilizes E2Fb and E2Fc, respectively (del Pozo et al., 2002; López-Juez et al., 2008). Competitive binding of E2Fb and E2Fc to the *DEL1* promoter suggested that *DEL1* transcription might also be controlled by light. In agreement, in the *ProDEL1:GUS* lines, the *DEL1* promoter activity was stimulated by the transition to light. E2Fb degradation during darkness is mediated by the ubiquitin E3 ligase COP1, because E2Fb protein levels are stabilized in dark-grown *cop1-4* mutant plants (López-Juez et al., 2008). Here, we could link this stabilization with an increase in *DEL1* expression in the dark. Correspondingly, the ploidy level of dark-grown *cop1-4* mutant hypocotyls matches that of light-grown wild-type plants (Gendreau et al., 1998).

As light controls *DEL1* transcript levels and endoreduplication of hypocotyls also depends on light, light might be assumed to regulate the ploidy level of hypocotyls through DEL1. Analysis of ploidy levels of $DEL1^{OE}$ and *del1-1* mutant plants grown under short-day and long-day conditions revealed that the endoreduplication index of the hypocotyls did not differ, in contrast to that of control plants, which displayed an increase in ploidy levels under short-day conditions. Thus, in *DEL1* transgenic plants, the level of endoreduplication is not coupled with the light input. Based on these data, we propose a model in which the balance between E2Fb and E2Fc controls the level of light-responsive hypocotyl endoreduplication (Fig. 7). In this model, E2Fb is the most prominent E2F under light conditions. Through occupancy of the *DEL1* promoter, it activates *DEL1* transcription, and thus endoreduplication is repressed. In contrast, in the dark, E2Fb protein levels are destabilized by COP1, allowing E2Fc to be the most abundant E2F. By displacing E2Fb from the *DEL1* promoter, *DEL1* transcription is reduced and thus endoreduplication commences.

As endoreduplication is often associated with cell growth, it is tempting to speculate that the additional endocycles of dark-grown plants might aid hypocotyl elongation in its search for light. However, no drastic effects on hypocotyl growth were observed in the various mutant and overexpression lines analyzed, with the exception of a slight reduction in the length of dark-grown *E2Fb*-overexpressing hypocotyls (Sozzani et al., 2006; B. Berckmans and L. De Veylder, unpublished data). A plausible reason for the lack of a clear growth phenotype might be that hypocotyl endoreduplication is not necessarily coupled to cell length. Indeed, plants with a defective endocycle still elongate in the dark, implying that the increase in ploidy level of etiolated seedlings contributes only marginally to the final hypocotyl length. Thus, although our work indicates how light-mediated repression of endocycles in hypocotyls could be controlled at the molecular level, the physiological role of dark-induced endoreplication remains an open question. Possibly, endoreduplication does not control the final hypocotyl length but rather the hypocotyl growth kinetics, a process that is under the influence of both the circadian clock and diurnal control (Nozue et al., 2007; Nusinow et al., 2011). Therefore, it will be important to take these regulatory pathways into account in future experiments.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown at 22°C and a 16-h photoperiod (65 μE m^{-2} s^{-1}) on agar-solidified culture medium (0.5× Murashige and Skoog medium, 0.5 g L^{-1} MES, 10 g L^{-1} Suc, and 0.8% plant tissue culture agar). Plates were incubated at 4°C for 48 h to synchronize seed germination. *ProDEL1:GUS*, *del1-1*, $DEL1^{OE}$, $E2Fc^{OE}$, and *cop1-4* had been described previously (Deng et al., 1991; del Pozo et al., 2002; Vlieghe et al., 2005; Lammens et al., 2008). The *e2fb-1* knockout line corresponded to the SALK insertion line (SALK_103138). Primers used for genotyping are given in Supplemental Table S1. For light inducibility tests of *DEL1*, *ProDEL1:GUS* seeds were exposed to white light for 30 min to induce germination before they were placed in the dark. Three days after germination, plants were transferred to continuous light (110 μE m^{-2} s^{-1}) and analyzed after 4, 24, and 48 h of light treatment or after 24 h of light switched between 24-h/24-h dark/light conditions. Transcript levels in *cop1-4* plants were determined by growing plants for 7 d in darkness or continuous light conditions (110 μE m^{-2} s^{-1}). Dark-grown plants were again exposed to 30-min light treatments to induce germination. For ploidy measurements, plants were grown in either a 16-h or an 8-h photoperiod.

Cloning and Generation of Transgenic Lines

Expression clones were obtained according to standard molecular biology protocols and Gateway technology (Invitrogen). Open reading frames (ORFs) were amplified from a cDNA template with Pfu DNA Polymerase (Promega). For promoter isolation, genomic DNA was used as a source. Primers for ORF and promoter isolation are listed in Supplemental Table S2. The pdonr221 and p4-p1r vectors were utilized as ENTRY vectors for the ORFs and promoters, respectively (Karimi et al., 2002, 2007). *Pro-35S:E2Fb* was generated by cloning the ORF of *E2Fb* in the destination vector pH2GW7. Mutation of the E2F-binding sites in the *DEL1* promoter was mediated by PCR-based mutagenesis (Fisher and Pei, 1997). Briefly, the p4-p1r ENTRY clone containing the *DEL1* promoter (995 bp upstream of ATG) was amplified with primers bearing the mutated E2F sites. After degradation of the methylated (parental) DNA with *Dpn*I (1 h at 37°C), the mutated plasmid was transformed in *Escherichia coli*, and the presence of the mutation was confirmed by sequencing. *ProMut2:GUS*

constructs were generated by cloning the mutated promoter in the pHGWFS7 destination vector. Transgenic plants were obtained with the floral dip method (Clough and Bent, 1998).

Y1H

Yeast strain YM4271 and destination vectors pDEST-MW1 and pDEST-MW2 were obtained from Bart Deplancke (Ecole Polytechnique Fédérale de Lausanne; Deplancke et al., 2004). For the Y1H cDNA library screen, the *DEL1* and mutated *DEL1* promoters (each 995 bp upstream of ATG) were cloned in pDEST-MW1 and pDEST-MW2 vectors, creating transcriptional fusions between the promoters and the *HIS3* and *LacZ* gene, respectively. Yeast reporter strains were designed as described previously (Deplancke et al., 2004). All handling and transformation of yeast were done according to the Yeast Protocol Handbook (Clontech).

Real-Time PCR

RNA was extracted with the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from 1 μg of total RNA with SuperScript III reverse transcriptase (Invitrogen) and analyzed on a LightCycler 480 apparatus (Roche Diagnostics) with the SYBR Green I Master kit (Roche Diagnostics), according to the manufacturer's instructions. All individual reactions were done in triplicate. Primers used are listed in Supplemental Table S3. For the *DEL1* expression analysis in *E2Fb* and *E2Fc* transgenic lines and confirmation of *E2Fb* transcript levels in *e2fb-1* and *E2Fb*^{OE}, values were normalized to the *ACTIN2* (AT3G46520) housekeeping gene. UBQ10 (AT4G05320) and PP2AA3 (AT1G13320) were used to analyze transcript levels in *cop1-4* mutant plants.

Histochemical and Histological Analyses

GUS staining was done as described (Lammens et al., 2008). For microscopic analysis, samples were cleared by mounting in 90% lactic acid or in a chloral hydrate solution (25 g of chloral hydrate in 10 mL of 30% glycerol). Samples were analyzed with a light microscope and differential interference contrast microscopy (Olympus BX51).

ChIP

ChIP experiments were carried out as described (Bowler et al., 2004), with minor modifications. One gram of 8-d-old plants was harvested and immersed in 1% formaldehyde under vacuum for 10 min. Gly was added to a final concentration of 0.125 M, and incubation was continued for 5 min. After washing, the nuclei were isolated and cross-linked DNA/protein complexes were fragmented by sonication with a Bioruptor Next Gen (Diagenode), resulting in fragments of approximately 500 bp. After centrifugation (16,000*g*), the supernatant was precleared with 40 μL of salmon sperm DNA/protein A agarose (Millipore). Of the supernatant, 10 μL was used as input, while the remainder was divided into three samples that were treated with 10 μL of anti-E2Fb, 10 μL of E2Fc, or without antibody. The samples were incubated overnight. Immunoprecipitates were collected with 40 μL of salmon sperm DNA/protein A agarose (Millipore) and subsequently eluted from the beads. All bead-containing samples were centrifuged at 1,000*g*. Proteins were de-cross-linked, and DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. Pellets were resuspended in 40 μL of Tris-EDTA buffer (0.05 M Tris-HCl and 0.02 M EDTA [pH 8]). The concentration of DNA purified by ChIP was measured with the Quant-iT ds-DNA Assay Kit HS (Invitrogen), and each sample was diluted for quantitative PCR at the same starting concentration. The SYBR Green I Master kit (Roche Diagnostics) was used for all quantitative PCRs, with ACTIN2 as a negative control. The approach used to analyze the quantitative PCR data was percentage input, with values calculated by $100 \times 2^{(\text{threshold cycle [Ct] (Input)} - \text{Ct (Immunoprecipitate)})}$. Primers are listed in Supplemental Table S3.

Flow Cytometer

Hypocotyls of 12- and 21-d-old plants were harvested, frozen in liquid nitrogen, and kept at −70°C until analysis. Plant material was chopped in 200 μL of Cystain UV Precise P Nuclei extraction buffer (Partec) and supplemented with 800 μL of staining buffer. The mix was filtered through a 50-μm filter and read through the CyFlow MB flow cytometer (Partec). The nuclei were analyzed with the CyFlow flow cytometer and FloMax software (Partec).

Transient Expression Assays

Transient expression was assayed as described (De Sutter et al., 2005). Briefly, protoplasts were prepared from a Bright Yellow-2 tobacco (*Nicotiana tabacum*) cell culture and cotransfected with a reporter plasmid containing the firefly luciferase (fLUC) reporter gene driven by *ProDEL1*, *ProDEL1-Mut1*, *ProDEL1-Mut2*, or *ProDEL1-Mut1/Mut2*, a normalization construct expressing *Renilla* luciferase (rLUC) under the control of the cauliflower mosaic virus *35S* promoter, and effector constructs. For the fLUC reporter constructs, the pEN-L4-PROMOTER-R1 vector (PROMOTER representing *ProDEL1*, *ProDEL1-Mut1*, *ProDEL1-Mut2*, or *ProDEL1-Mut1/Mut2*), also used for cloning Y1H vectors, was recombined together with pEN-L1-fLUC-L2 by multisite Gateway LR cloning with pm42GW7 (Karimi et al., 2007). For the effector constructs, pEN-L1-ORF-R2 (ORF either *E2Fb* or *E2Fc*) was used to introduce the ORFs by Gateway LR cloning into p2GW7. For each experiment, 2 μg of each plasmid was used, and the total effector amount in each experiment was equalized with the p2GW7-GUS mock effector plasmid. After transfection, protoplasts were incubated overnight and then lysed. fLUC and rLUC activities were determined with the Dual-Luciferase reporter assay system (Promega). Variations in transfection efficiencies and technical errors were corrected, normalizing fLUC by the rLUC activities.

Protein Gel Blotting

Proteins were extracted from 8-d-old plants. Samples were collected, ground in liquid nitrogen, and homogenized in cold homogenization buffer HB (25 mM Tris-HCl [pH 8], 5 mM EDTA, 1 mM β-mercaptoethanol, 15 mM $MgCl_2$, 85 mM NaCl, 0.1% Tween 20, and one protease inhibitor tablet per 50 mL [Complete; Roche Diagnostics]). The homogenate was centrifuged twice for 15 min at 15,000*g* at 4°C. Protein concentrations were determined by the Bradford Protein Assay (Bio-Rad). After equal amounts of protein extracts had been loaded, protein gel blotting was carried out according to standard procedures with E2Fb as primary antibody at a dilution of 1:500 and an anti-rabbit antibody (GE Healthcare) diluted 1:10,000 as a secondary antibody. Proteins were detected with the Western Lightning detection kit (Pierce) according to the manufacturer's instructions.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Conservation of *E2F*-binding sites in the *DEL1* promoter within the green plant lineage.

Supplemental Figure S2. Molecular characterization of *e2fb-1*.

Supplemental Figure S3. Confirmation of *E2Fb*^{OE} lines by reverse transcription-PCR and western-blot analysis.

Supplemental Table S1. Primers used for genotyping.

Supplemental Table S2. Primers used for cloning.

Supplemental Table S3. Primers used for quantitative reverse transcription-PCR.

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