Atypical E2F activity restrains APC/C^{CCS52A2} function obligatory for endocycle onset

Tim Lammens*†, Véronique Boudolf*†, Leila Kheibarshekan*‡, L. Panagiotis Zalmas§, Tarik Gaamouche*†, Sara Maes*†, Marleen Vanstraelen¶, Eva Kondorosi¶‖, Nicholas B. La Thangue§, Willy Govaerts‡, Dirk Inzé*†, and Lieven De Veylder*†**

*Department of Plant Systems Biology, Flanders Institute for Biotechnology and †Department of Molecular Genetics, Ghent University, 9052 Gent, Belgium; ‡Department of Applied Mathematics and Computer Science, Ghent University, 9000 Gent, Belgium; §Laboratory of Cancer Biology, Medical Sciences Division, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, United Kingdom; ¶Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Unité Propre de Recherche 2355, 91198 Gif-sur-Yvette, France; and ‖Institute for Plant Genomics, Human Biotechnology and Bioenergy, Bay Zoltan Foundation for Applied Research, 6726 Szeged, Hungary

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The endocycle represents an alternative cell cycle that is activated in various developmental processes, including placental formation, *Drosophila* oogenesis, and leaf development. In endocycling cells, mitotic cell cycle exit is followed by successive doublings of the DNA content, resulting in polyploidy. The timing of endocycle onset is crucial for correct development, because polyploidization is linked with cessation of cell division and initiation of terminal differentiation. The anaphase-promoting complex/cyclosome (APC/C) activator genes *CDH1*, *FZR*, and *CCS52* are known to promote endocycle onset in human, *Drosophila*, and *Medicago* species cells, respectively; however, the genetic pathways governing development-dependent APC/C$^{CDH1/FZR/CCS52}$ activity remain unknown. We report that the atypical E2F transcription factor E2Fe/DEL1 controls the expression of the *CDH1*/*FZR* orthologous *CCS52A2* gene from *Arabidopsis thaliana*. E2Fe/DEL1 misregulation resulted in untimely *CCS52A2* transcription, affecting the timing of endocycle onset. Correspondingly, ectopic *CCS52A2* expression drove cells into the endocycle prematurely. Dynamic simulation illustrated that E2Fe/DEL1 accounted for the onset of the endocycle by regulating the temporal expression of *CCS52A2* during the cell cycle in a development-dependent manner. Analogously, the atypical mammalian E2F7 protein was associated with the promoter of the APC/C-activating *CDH1* gene, indicating that the transcriptional control of APC/C activator genes by atypical E2Fs might be evolutionarily conserved.

CDH1 | DEL1 | E2F7 | endoreduplication

During the mitotic cell cycle, DNA that is duplicated during the S phase is divided at the M phase, so that each daughter cell produced has a genomic DNA content equal to that of its parents. In contrast, during the endoreduplication cycle, no cytokinesis occurs between rounds of DNA replication, resulting in successive doublings of the DNA ploidy level. This process occurs in a wide variety of cell types in arthropods and mammals and is particularly prominent in dicotyledonous plants (1), especially in species with a small genome and a short life cycle, in which repetitive DNA replication might support growth under conditions that require rapid development (2, 3).

Mitotic cell cycle progression and endoreduplication are linked events. Premature or delayed exit from the cell division program results in an increased or decreased DNA ploidy, respectively (4–10). Therefore, the onset of endoreduplication must be controlled precisely. At the molecular level, endoreduplication is likely achieved through elimination of the components needed to progress through mitosis (11). Predominant roles in this process are played by the anaphase-promoting complex/cyclosome (APC/C) activator genes, such as *CDH1*, *FZR*, and *CCS52A*, which have been found to promote endocycle onset and progression in human, *Drososphila melanogaster*, and *Medicago truncatula* cells, respectively (12–17). The mechanisms controlling the transcriptional activity of these genes remain unclear, however.

Over the years, it has become clear that the E2F transcriptional network acts as a key regulator in the balanced expression of many essential genes involved in proliferation and differentiation (18). Recently, a class of novel atypical E2F proteins was identified in *Arabidopsis thaliana* (E2Fd/DEL2, E2Fe/DEL1, and E2Ff/DEL3) and mammals (E2F7 and E2F8) that operate as transcriptional repressors (18, 19). Similar to the typical E2F proteins, the atypical E2F proteins bind the consensus E2F recognition sequence, but they have two DNA-binding domains and do not require a DP partner to bind DNA. In contrast to the classical E2F proteins, the physiological relevance of the novel E2Fs is less clear. E2F7 and E2F8 have been demonstrated to play a role in controlling E2F1-dependent apoptosis (20, 21), whereas in plants, atypical E2Fs operate as inhibitors of post-mitotic events; mutants of E2Fe/DEL1 display increased endoreduplication levels (22), whereas E2Ff/DEL3-deficient plants are prone to rapid cell expansion (23).

In this article, we report that the enhanced endoreduplication levels observed in E2Fe/DEL1 knockout plants arise from a premature onset of the endocycle. Through microarray analysis and chromatin immunoprecipitation (ChIP), we identified the APC/C activator gene *CCS52A2* as a direct E2Fe/DEL1 target. By combining molecular and computational techniques, we demonstrated that E2Fe/DEL1 controls the endocycle onset through the temporal control of the *CCS52A2* expression during the cell cycle in a development-dependent manner. Moreover, an association of E2F7 to the *CDH1* promoter in mammalian cells was observed, suggesting that the transcriptional control of the APC/C activator genes through atypical E2Fs might be conserved across species.

Results

E2Fe/DEL1 Prevents Premature Exit From the Mitotic Cell Cycle. The atypical E2F transcription factor E2Fe/DEL1 of *Arabidopsis* has been shown to control the endoreduplication level in roots, hypocotyls, and leaves (22). Compared with control plants, the knockout plants *E2Fe/DEL1*^{KO} (*del1–1*) displayed enhanced endoreduplication, whereas in the *E2Fe/DEL1*^{OE} overexpressing lines, the DNA ploidy level was reduced. Using β-glucuronidase (GUS) reporter line, E2Fe/DEL1 transcription was detected

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**To whom correspondence may be addressed. E-mail: lieven.deveylder@psb.ugent.be.

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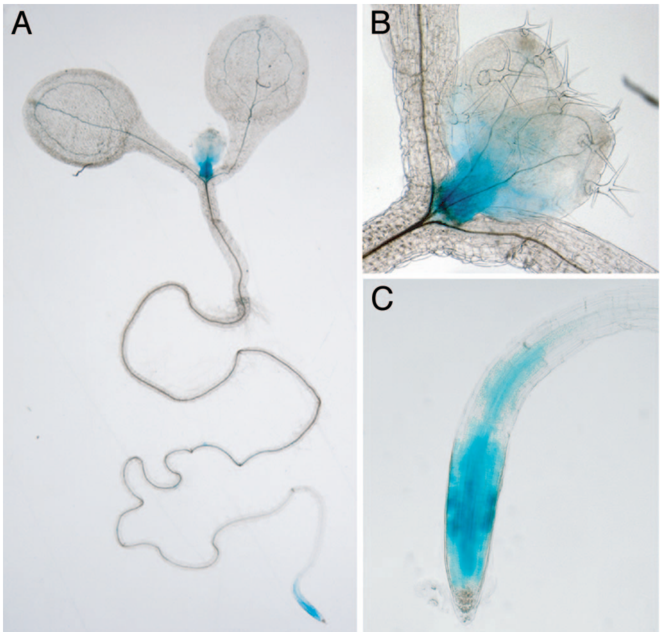

Fig. 1. Exclusive transcription of *E2Fe/DEL1* in nonendoreplicating dividing cells. (*A*) GUS activity in the shoot apical meristem, root apex, young vascular tissue, and leaf of an 8-day-old seedling. (*B*) Detail of shoot apical meristem and young leaf. (*C*) Detail of expression in the root tip.

only in dividing cells (Fig. 1), confirming previous *in situ* mRNA hybridization data (22). Therefore, we postulated that E2Fe/DEL1 operates as a repressor of the endocycle onset, preventing dividing cells from exiting the cell cycle prematurely. To test this hypothesis, we compared the timing of the endocycle onset in developing leaves of *del1–1* and control plants. Cells from the first leaf pair divide up to 9–11 days after sowing, after which they gradually exit the cell cycle and start to endoreduplicate (22, 24, 25). Correspondingly, 8-day-old leaves of both *del1–1* and control lines had an equally low endoreduplication index (EI), that is, the mean number of endoreduplication cycles per nucleus (Fig. 2*A*). In contrast to wild-type leaves, the EI of E2Fe/DEL1-deficient leaves increased significantly ($P < 0.005$) and reproducibly between day 8 and day 10 because of a rise in the cell population with an 8C DNA content. The difference in EI between wild-type and *del1–1* leaves was maintained on day 12, when wild-type leaves also began to endoreduplicate, and endured as the leaves matured. Thus, in the absence of a functional E2Fe/DEL1 protein, cells entered the endocycle more quickly. Endocycle onset and cell cycle exit are linked events. Correspondingly, *del1–1* leaf cells exited the cell cycle program prematurely [supporting information (SI) Fig. S1], eventually resulting in a reduced total cell number (16,489 ± 1322 vs. 18,666 ± 741 epidermal cells in *del1–1* and wild-type plants, respectively; $P < 0.05$). These findings were supported by analysis at the cellular level (Fig. 2*B*), showing a greater number of abaxial epidermal leaf cells with a high DNA content in 12-day-old *del1–1* leaves than in control leaves at the same age.

The APC/C Activator Gene *CCS52A2* Is an E2Fe/DEL1 Target. To examine how E2Fe/DEL1 represses mitotic exit, we compared the transcriptome of wild-type plants with that of plants that either overexpressed or were deficient in *E2Fe/DEL1* (see *SI Text*). We identified 10 genes with expression profiles that positively correlated with the endoreduplication phenotype (Fig. 2*C*; Table S1). Among these, the *CCS52A2* gene displayed the most significant changes in expression level. These changes in *CCS52A2* expression in response to altered E2Fe/DEL1 levels were confirmed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis (Fig. S2). Although the *Arabidopsis* genome encodes three atypical E2F proteins, *CCS52A2* transcripts were specifically modulated by E2Fe/DEL1, because the expression levels remained the same in

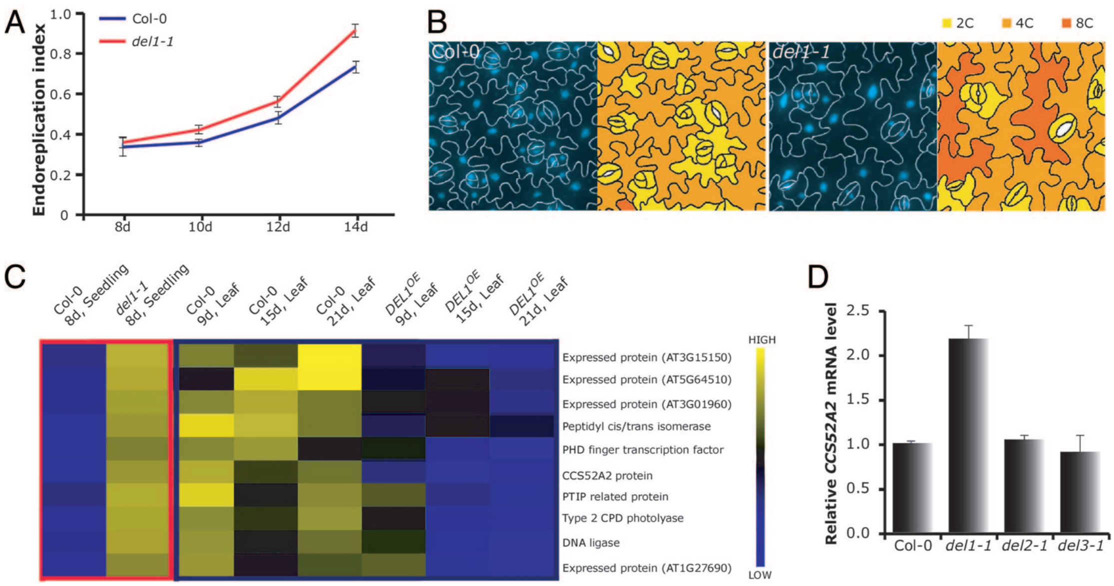

Fig. 2. Regulation of endocycle onset by E2Fe/DEL1 through control of *CCS52A2*. (*A*) Advanced onset of the endocycle in *del1–1* plants, as demonstrated by a more rapid increase in the EI (mean number of duplication cycles; for calculation, see *Materials and Methods*). Values are mean ± standard error ($n > 5$). (*B*) Ploidy maps of 12-day-old abaxial epidermal cells of wild-type (Col-0) and *del1–1* plants. 4′,6-Diamidino-2-phenylindole (DAPI) stains (left) were translated into color maps (right). (*C*) Transcript cluster positively correlated with the endoreplication phenotypes of E2Fe/DEL1^{OE} and *del1–1* plants. Data are the average of three independent experiments. (*D*) Quantification of *CCS52A2* expression in *del1–1*, *del2–1*, and *del3–1* mutants. Transcript levels were measured by real-time PCR. All values were normalized to the *ACT2* housekeeping gene. The ΔCt method was used for relative quantification of transcripts. Data are mean ± standard deviation ($n = 3$).

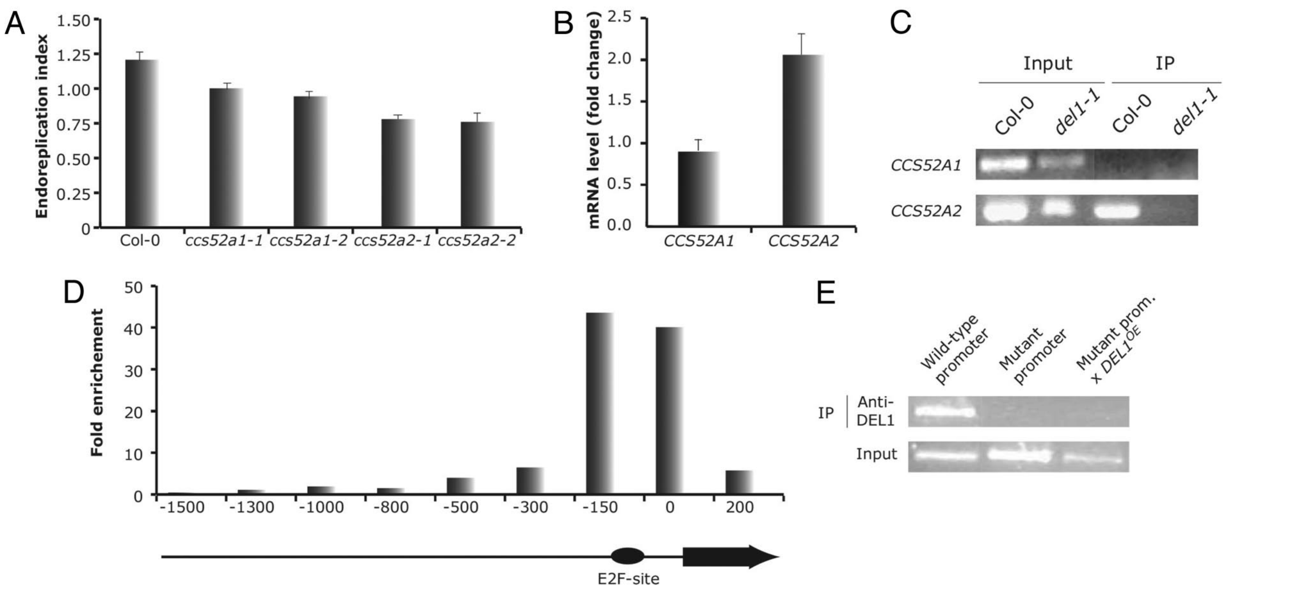

Fig. 3. E2Fe/DEL1-dependent *CCS52A2* transcription. (*A*) Effects of *CCS52A1* and *CCS52A2* knockout on the EI of mature first leaves. Values are mean ± standard deviation (SD) ($n = 3$). (*B*) *CCS52A1* and *CCS52A2* transcript levels in 8-day-old *del1–1* seedlings. Transcript levels were measured by real-time PCR. All values were normalized to the *ACT2* housekeeping gene. The ΔCt method was used for relative quantification of transcripts. Measurements were made relative to wild-type and are mean ± SD ($n = 3$). (*C*) ChIP analysis showing binding of E2F/DEL1 to the *CCS52A2* promoter *in vivo*, but not to the *CCS52A1* promoter. Data represent two independent assays. (*D*) ChIP scanning of the *CCS52A2* promoter showing the strongest E2Fe/DEL1 association around the putative E2F *cis*-acting element. (*E*) ChIP analysis illustrating that E2Fe/DEL1 binding requires a functional E2F-binding site within the *CCS52A2* promoter.

E2Fd/DEL2 knockout (*del2–1*) and E2Ff/DEL3 knockdown (*del3–1*) plants (Fig. 2*D*).

The *CCS52A2* gene encodes a putative activator of the APC/C and is related to the *Drosophila* FZR and mammalian CDH1 proteins. The *CCS52A* genes of *Medicago sativa* and *Medicago truncatula* have been shown to control the onset of endoreduplication during nodule development, and likewise, the *CDH1* and *FZR* genes regulate the mitosis-to-endocycle transition in human and *Drosophila* cells, respectively (12–14, 26). *Arabidopsis* has two *CCS52A*/*FZR*/*CDH1*-related genes, designated *CCS52A1* and *CCS52A2* (27). In analogy to their leguminous and nonplant counterparts, both *CCS52A1* and *CCS52A2* were found to control the endocycle, as indicated by the low EI of mature leaves of the knockout lines $CCS52A1^{KO}$ and $CCS52A2^{KO}$ (Fig. 3*A*; Fig. S3).

E2Fe/DEL1 Associates with the *CCS52A2* Promoter Through a Consensus E2F *cis*-Acting Element. The endoreduplication phenotypes of $CCS52A1^{KO}$ and $CCS52A2^{KO}$ plants suggest that both *CCS52A1* and *CCS52A2* may be direct target genes of E2Fe/DEL1. But only *CCS52A2* transcript levels were altered in *del1–1* plants (Fig. 3*B*). Correspondingly, ChIP assays with a specific anti-E2Fe/DEL1 antibody demonstrated that E2Fe/DEL1 associated only with the *CCS52A2* promoter (Fig. 3*C*). Locus scanning revealed that the E2Fe/DEL1-binding site within the *CCS52A2* promoter coincided with the position of a putative E2F *cis*-acting element located just upstream of the ORF of the *CCS52A2* gene (Fig. 3*D*; Fig. S4*A*). Proof that this site is required for E2Fe/DEL1 binding was provided by introducing either the endogenous *CCS52A2* promoter or an identical promoter construct with a mutated E2F *cis*-acting element into plants. The wild-type promoter fragment, but not its mutant variant, could be immunoprecipitated by the anti-E2Fe/DEL1 antibody (Fig. 3*E*), even in an $E2Fe/DEL1^{OE}$ background, implying that E2Fe/DEL1 binding requires a functional E2F regulatory sequence.

E2Fe/DEL1 Controls the Temporal Expression of *CCS52A2* During Leaf Development. The changes in *CCS52A2* transcript abundance in the E2Fe/DEL1 transgenic lines and the direct association of E2Fe/DEL1 with the *CCS52A2* promoter indicates that E2Fe/DEL1 might control the temporal expression of *CCS52A2*. Therefore, the *CCS52A2* transcript levels were analyzed in wild-type and *del1–1* plants during leaf development. *E2Fe/DEL1* mRNA levels were abundant mainly in the early stages of leaf development. In contrast, *CCS52A2* transcripts accumulated and reached a maximum at day 10, the time of cell cycle exit and onset of endoreduplication (Fig. 4*A*). Because of its low expression level before day 10, *CCS52A2* might be repressed by E2Fe/DEL1 during the dividing phase of leaf development. This hypothesis was confirmed in *del1–1* plants, in which *CCS52A2* expression levels were clearly higher during the early leaf growth stages than those of control plants (Fig. 4*B*). No significant changes in *CCS52A1* expression levels were observed (Fig. 4*C*), again indicating that E2Fe/DEL1 controls *CCS52A2* expression only.

To determine whether the increase in *CCS52A2* transcript levels in young leaves of *del1–1* plants could account for the premature onset of endoreduplication, DNA ploidy changes were analyzed in plants overexpressing *CCS52A2*. $CCS52A2^{OE}$ leaves entered the endoreduplication cycle earlier than control leaves, as indicated by the increased number of cells with a high DNA ploidy level (Fig. 4*D*), in agreement with the anticipated role of CCS52A2 as an activator of the endoreduplication program.

E2Fe/DEL1 Activity Controls Mitotic Exit by Determining the Window of *CCS52A2* Expression During the Cell Cycle. Our data suggest that E2Fe/DEL1 levels determine the timing of cell cycle exit and onset of endoreduplication by controlling when $APC/C^{CCS52A2}$ is active. To understand mechanistically how decreasing E2Fe/DEL1 levels can account for the division-to-endoreduplication transition, we mathematically modeled the cell cycle phase–dependent expression pattern of *CCS52A2* during leaf development. In a synchronized cell culture, *E2Fe/DEL1* and *CCS52A2* display complementary transcription profiles, with a predominance of *CCS52A2* expression during the G_1 and S phases (Fig. S5*B*) (28). The *CCS52A2* expression profile corresponded with the anticipated

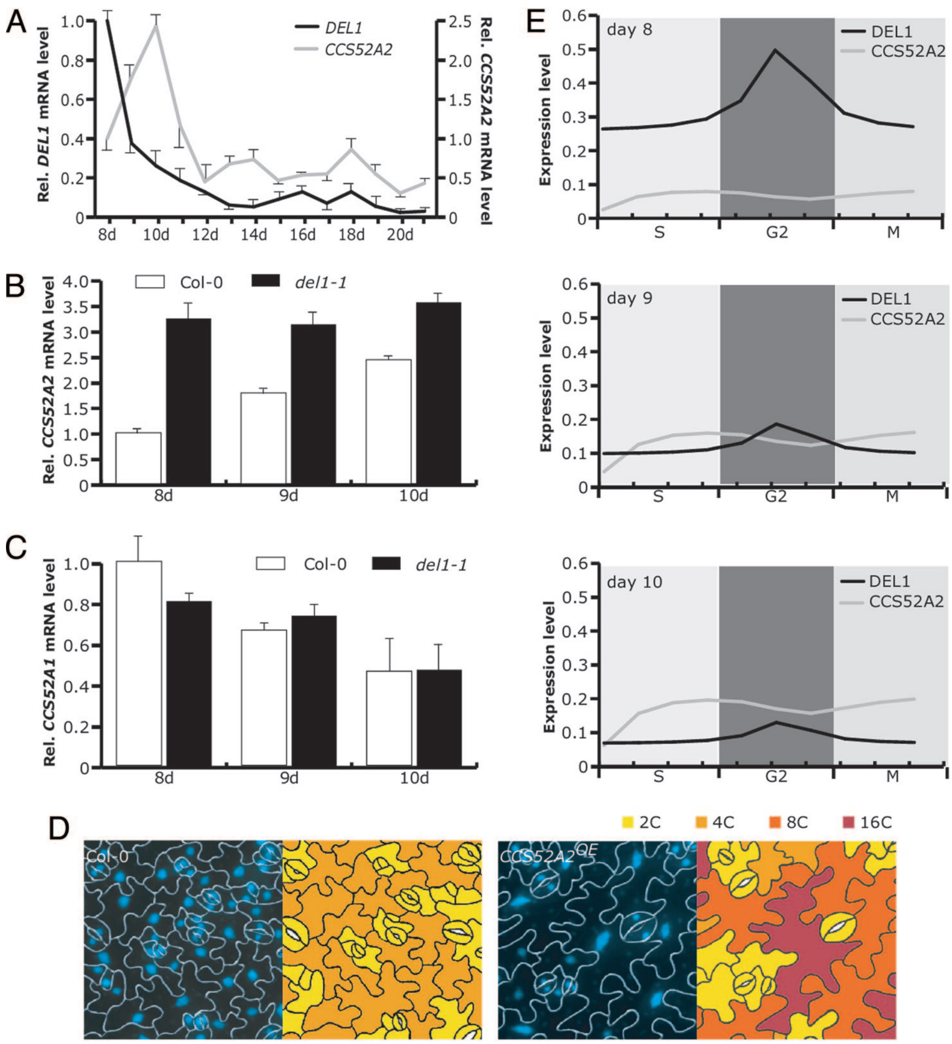

Fig. 4. Control of development-dependent expression of *CCS52A2* by E2Fe/DEL1. (*A*) Kinetics of *E2Fe/DEL1* and *CCS52A2* transcription during leaf development. Transcript levels were measured by real-time PCR. All values were normalized to the *ACT2* housekeeping gene. The ΔCt method was used for relative quantification of transcripts. Values are means ± SD ($n = 3$). Note that transcription of *CCS52A2* peaked at day 10, marking the endocycle onset. (*B* and *C*) *CCS52A2* and *CCS52A1* mRNA levels during leaf development in wild-type (Col-0) and *del1–1* mutants, respectively. Data are mean ± SD ($n = 3$). (*D*) Ploidy maps of 12-day-old abaxial epidermal cells of wild-type (Col-0) and *CCS52A2^{OE}* plants. DAPI stains (left) were translated into color maps (right). (*E*) Simulation of *CCS52A2* accumulation during leaf development showing a progressive increase in *CCS52A2* transcript levels during the S and G_2 phases.

function of its gene product in preventing premature accumulation of mitotic cyclins in interphase cells but allowing their accumulation during the late S and G_2 phases, allowing the M phase to proceed (29). E2Fe/DEL1 expression levels peaked during G_2, similar to what has been observed for its mammalian counterparts E2F7 and E2F8 (30, 31). Because cell division cannot be synchronized experimentally in a developing leaf and endoreduplication cannot be triggered in *Arabidopsis* cell cultures, we combined leaf and cell culture expression data mathematically (see SI Appendix, Modeling). This mathematical modeling permitted an *in silico* visualization of the cell cycle phase–dependent relationship between *E2Fe/DEL1* and *CCS52A2* in a developmental context. The simulation revealed that decreasing *E2Fe/DEL1* levels during leaf maturation triggered a preferential increase in *CCS52A2* transcripts during the late S-G_2 and M phases (Fig. 4*E*; Movie S1). These data suggest that E2Fe/DEL1 controls the cell cycle phase–dependent *CCS52A2* transcription profile in a developmentally dependent manner.

The Association Between Atypical E2F and the Promoter of the APC/C Activator Genes Is Evolutionarily Conserved. In analogy to *CCS52A2*, a consensus E2F *cis*-acting element was detected in the promoter region of the human *CDH1* gene (Fig. S4*B*). To investigate whether the observed control of the APC/C activator genes by atypical E2F proteins might be conserved among plants and metazoans, we evaluated the binding of the human E2F7 transcription factor to the *CDH1* promoter by ChIP analysis in human bone tissue–derived osteosarcoma cells (U2OS). As a positive control, we tested the association of E2F7 with the *E2F1* gene, recently demonstrated to be an E2F7 target gene (20, 21). No *E2F1* or *CDH1* promoter DNA was precipitated with a nonspecific antibody, but both promoters could be detected in the E2F7 immunoprecipitates (Fig. 5).

Discussion

We have demonstrated that the atypical E2F transcription factor E2Fe/DEL1 controls the onset of the endocycle through a direct transcriptional control of APC/C activity. Because E2Fe/DEL1 represses the *CCS52A2* promoter, we hypothesize that its level must drop below a critical threshold to allow sufficient accumulation of *CCS52A2* during late S and G_2 for cells to proceed from division to endoreduplication, a model suggested by the dynamic simulation of the cell cycle phase–dependent expression level of

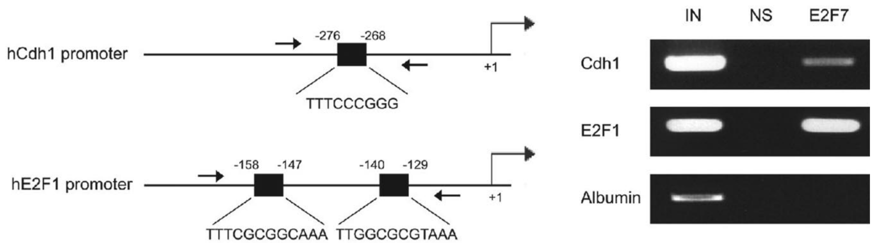

Fig. 5. Evolutionarily conserved transcriptional control of APC activator genes by atypical E2F proteins. ChIP analysis on extracts prepared from U2OS cells showed that E2F7 binds the *CDH1* promoter *in vivo*. A 10% fraction of the chromatin served as input (IN). Immunoprecipitations were carried out with an E2F7 or control antibody (NS). The E2F1 and albumin genes were used as positive and negative controls, respectively.

CCS52A2 during leaf development. The steady increase in *CCS52A2* during late S and G_2 likely counteracts the mitotic cyclin-dependent kinase (CDK) activity that builds up during these cell cycle phases (32, 33), eventually blocking the G_2 to M transition and thereby triggering endoreduplication.

No clear endoreduplication phenotype was observed in 8-day-old E2Fe/DEL1^{KO} plants (Fig. 1*A*). At this earliest developmental stage examined, the leaves were still mitotically active, corresponding to high cyclin transcription rates (25). We propose that at this stage, the cyclin production rates are so high that the cyclin abundance is insensitive to the counteracting action of the E2Fe/DEL1-controlled APC/C^{CCS52A2} activity. In contrast, when the leaf matures, the cyclin production rates decrease, and the effects of increasing *CCS52A2* levels may become apparent. The combination of decreased cyclin production rates and increased control at the protein stability level may ensure a unidirectional onset of the endoreduplication program.

Both *CCS52A1* and *CCS52A2* knockout plants displayed a reduced EI in rosette leaves, illustrating that both control the endocycle. However, in plants, E2Fe/DEL1 regulates the temporal expression of *CCS52A2* but not that of *CCS52A1*, implying that independent signaling pathways control the timing of endocycle onset and/or progression through the endoreduplication program. In *Arabidopsis*, endoreduplication is an integral part of the leaf maturation process. The presence of multiple pathways may safeguard against possible mutations in the signaling cascades that monitor the onset of differentiation, thereby protecting plants from uncontrolled cell proliferation.

In metazoans, the APC/C activity is managed indirectly by classical E2F proteins through the transcriptional expression of Emi1 (34, 35) and CYCA (36), both of which negatively regulate *CDH1* activity. Emi1 acts as a pseudosubstrate of *CDH1*, and CYCA activates CDKs, which phosphorylate CDH1 and cause it to dissociate from the APC. Whether these control mechanisms also are operational in plants remains unclear. In contrast, the association of E2F7 to the *CDH1* promoter suggests that the control of gene expression of the APC/C activator genes by atypical E2Fs is evolutionarily conserved. Whether E2F7 controls the timing of cell cycle exit in this manner remains to be demonstrated. Significantly, in contrast to mammalian cells that undergo widespread apoptosis in the absence of E2F7 and E2F8 (20, 21), the *del1-1* lines display no signs of cell death. Treatments that cause apoptosis in mammals, such as E2F overexpression or exposure to genotoxic compounds, provoke endoreduplication rather than cell death in *Arabidopsis* (37, 38), suggesting that apoptosis and endoreduplication might represent evolutionarily equivalent response mechanisms in mammals and plants to cope with potentially harmful cells. Because endoreduplicating cells differentiate and likely do not reenter the cell cycle, the endocycle could represent a mechanism that prevents transmission of deleterious mutations into the gametophytic cells and the progeny. Such a mechanism possibly could explain the evolutionary success of endoreduplication among angiosperms that grow under environmentally harsh conditions.

Materials and Methods

Plant Material and Culture Conditions. 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/liter of 2-(*N*-morpholino)ethanesulfonic acid [MES], 10 g/liter of sucrose, and 0.8% plant tissue culture agar). The *del1–1* and *del3–1* alleles have been described previously (22, 23); *del2–1*, *ccs52a1–1*, *ccs52a2–1*, and *ccs52a2–2* are the SALK T-DNA insertion lines 093190, 083656, 001978, and 073708, respectively. The SAIL T-DNA insertion line 797-F01 represents *ccs52a1–2*. All lines were provided by the Signal Insertion Mutant Library (http://signal.salk.edu). Primers for genotyping are listed in Table S2.

Cloning. The intergenic region containing the *CCS52A2* (At4g11920) promoter was isolated by PCR (for primers used, see Table S2) and cloned into the Gateway pKm43GW vector (39). The resulting plasmid was used to mutate the E2F-binding site with a site-directed plasmid mutagenesis (for primers, see Table S2). The coding region of *CCS52A2* was amplified by PCR (for primers, see Table S2) and cloned into the Gateway pDONR221 vector by *att*B × *att*P recombination and subsequently recombined into the pH2GW7 vector by *att*L × *att*R recombination. All vectors were used to transform *Arabidopsis thaliana* (L.) Heyhn plants by the flower-dip method (40). Transgenic homozygous plants containing only one T-DNA were obtained on a selective medium.

Histochemical GUS Assay. Briefly, young seedlings were incubated in 80% acetone for 30 min. After the material had been washed in phosphate buffer, it was immersed in the enzymatic reaction mixture (1 mg/ml of 5-bromo-4-chromo-3-indolyl β-D-glucuronide, 2 mM ferricyanide, and 0.5 mM ferrocyanide in 100 mM phosphate buffer [pH 7.4]). The reaction was run at 37°C in the dark for 4 h. The material was cleared in ethanol and examined under a light microscope.

Flow Cytometry and Densitometry. Flow cytometry and densitometry (7) were used to create the DNA ploidy maps. The EI was calculated (3) from the number of nuclei of each represented ploidy level multiplied by the number of endoreduplication cycles necessary to reach the corresponding ploidy level. The sum of the resulting products was divided by the total number of nuclei.

Antibody Generation. A GST-tagged fusion protein containing the last 100 aa of the E2Fe/DEL1 protein was produced in *Escherichia coli* BL21-CodonPlus(DE3)-RIL according to standard methods. Polyacrylamide gel slices containing this recombinant protein were injected into rabbits to produce polyclonal anti-E2Fe/DEL1 antiserum.

Synchronization of MM2d Cell Suspension Culture. Aphidicolin block/release was done as described previously (41).

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