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The APC/C E3 ligase remains active in most post-mitotic *Arabidopsis* cells and is required for proper vasculature development and organization

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Selective protein degradation via the ubiquitin-26S proteasome is a major mechanism underlying DNA replication and cell division in all eukaryotes. In particular, the APC/C (anaphase promoting complex or cyclosome) is a master ubiquitin protein ligase (E3) that targets PDS1/SECURIN and cyclin B for degradation allowing sister chromatid separation and exit from mitosis, respectively. Interestingly, it has been found that the APC/C remains active in differentiated neurons in which the E3 ligase regulates axon growth, neuronal survival and synaptic functions. However, despite these recent findings, the role of APC/C in differentiated cells and the regulation of its activity beyond cell division is still poorly understood. Here, we investigate the activity and function of APC/C in the model plant *Arabidopsis thaliana*. We used cyclin reporter constructs to follow APC/C activity during plant development and found that this E3 ligase remains active in most post-mitotic plant cells. Strikingly, hypomorphic mutant lines, in which the APC/C activity is reduced, exhibited several developmental abnormalities, including defects in cotyledon vein patterning and internode elongation leading to a characteristic broomhead-like phenotype. Histological analyses revealed an increased amount of vascular tissue, most notably xylem and lignified sclerenchyma, indicating a role for APC/C in plant vasculature development and organization.

KEY WORDS: *Arabidopsis*, Cell cycle, Endoreduplication, Ubiquitin, Vasculature

INTRODUCTION

Regulation of protein stability through the ubiquitin proteasome system (UPS) is considered to be a major mechanism underlying many different cellular and organismal processes, such as: cell division; DNA repair; quality control of newly produced proteins; and regulation of developmental pathways, of important parts of immune defense and, in plants, of light and phytohormone signal transduction (Ciechanover et al., 2000; Pickart, 2001; Smalle and Vierstra, 2004). Degradation via the UPS is a two-step process: the protein is first tagged by covalent attachment of ubiquitin and subsequently degraded by a multicatalytic protease complex called the 26S proteasome. The transfer of ubiquitin to an internal lysine residue in the target protein substrate requires an ubiquitin protein-ligase (E3). As E3 enzymes specify the substrates, they play the most important role in the ubiquitylation reaction. Two E3s dominate DNA duplication and cell division: the SCF and the anaphase promoting complex/cyclosome (APC/C).

The APC/C is the largest E3 known so far. In vertebrates, it is composed of 12 subunits (Peters, 2002), whereas, in yeast, 13 subunits have been described (Yoon et al., 2002). The functions of individual subunits are still unclear, although a structural model of its organization has been proposed (Thornton et al., 2006). The minimal ubiquitin ligase module of the APC/C comprises APC2, a distant member of the cullin family and the RING-finger protein APC11. The APC/C also contains three essential subunits with tetratricopeptide repeat (TPRs) protein-protein interaction domains: APC3/CDC27, APC8/CDC23 and APC6/CDC16. One of them (APC3/CDC27) has been implicated in binding the activating subunit CDH1 (Vodermaier et al., 2003; Kraft et al., 2005). Phosphorylation of these subunits during mitosis is required to activate the APC/C (Rudner and Murray, 2000; Kraft et al., 2003). Finally, the APC10/DOC1 subunit, which is characterized by the presence of a DOC domain, is important for substrate recognition and/or extending the poly-ubiquitin chain on a substrate (Carroll and Morgan, 2002; Carroll et al., 2005; Passmore et al., 2003). In addition to its core components, the APC/C requires co-activator proteins such as CDC20/FIZZY or CDH1/FIZZY-RELATED, which activate the APC/C sequentially during the cell cycle. CDC20 and CDH1 also bind to substrates, suggesting that they recruit substrates to the catalytic center of the E3 complex (Vodermaier, 2001).

During the past decade, much attention has been paid on the role of the APC/C in regulating cell cycle transitions. The APC/C is mainly required to induce progression and exit from mitosis by mediating proteolysis of different cell cycle regulators, including PDS1/SECURIN and cyclin B. Degradation of PDS1/SECURIN is required for sister chromatid separation (Uhlmann et al., 2000; Yanagida, 2000), whereas the destruction of cyclin B triggers the inhibition of CDK1 activity and, as a consequence, induces different cell processes such as disassembly of the mitotic spindle, chromosome decondensation, cytokinesis and reformation of the nuclear envelope (Murray and Kirschner, 1989; Luca et al., 1991; Gallant and Nigg, 1992; Holloway et al., 1993; Surana et al., 1993). In all eukaryotes, the instability of B-type cyclins is conferred by a small degenerated but conserved motif of nine amino acids RxxLxxIxN located in the N-terminal region of these proteins and known as the destruction box (Dbox) (Glotzer et al., 1991). Deletion or point mutation of the Dbox inhibits cyclin proteolysis (Brandeis and Hunt, 1996; Yamano et al., 1998; Genschik et al., 1998).

In addition to PDS1/SECURIN and mitotic cyclins, many other important cell cycle proteins were proved to be targets of APC/C in yeast and mammalian cells. Among them, the kinesin-related protein XKID involved in chromosome movements (Funabiki and Murray,

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2000; Levesque and Compton, 2001), two motor proteins (KIP1 and CIN8) (Gordon and Roof, 2001; Hildebrant and Hoyt, 2001) and the mitotic spindle-associated protein ASE1 are implicated in central spindle formation and cytokinesis (Juang et al., 1997; Yamashita et al., 2005). The APC/C remains also active during G1 to restrain the accumulation of the mitotic cyclins, but its inactivation is required for timely S-phase entry (Irniger and Nasmyth, 1997).

More surprising was the discovery that the APC/C is expressed and remains active in differentiated vertebrate cells, such as neuron (Gieffers et al., 1999). Unexpected neurobiological functions orchestrated by APC/C^{CDH1} have since been deciphered, ranging from axon growth, neuronal survival and synaptic functions (reviewed by Kim and Bonni, 2007). Thus, SnoN, a co-transcriptional repressor of the TGFβ signaling pathway, which is involved in elongation of neuron axons in developing cerebellum, is a target of APC/C^{CDH1} (Stroschein et al., 2001; Wan et al., 2001; Stegmüller et al., 2006). Another transcriptional regulator targeted in a Dbox-dependent manner by neuronal APC^{CDH1} is the helix-loop-helix protein Id2 (inhibitor of DNA binding 2) (Lasorella et al., 2006). Finally, APC^{CDH1} regulates synaptic function through the Dbox-containing substrate Liprin-α in flies (van Roessel et al., 2004). A postsynaptic role for the APC/C in the restriction of glutamate receptor abundance has also been described in worm (Juo and Kaplan, 2004). However, despite these recent findings, the number of identified APC/C substrates in differentiated cells remains limited and the regulation of its activity, beyond cell division, is still poorly described.

In the model plant *Arabidopsis thaliana*, single-copy genes encode counterparts of all known vertebrate APC/C subunits, except for APC3/CDC27 (Capron et al., 2003a). Nine APC/C activators have also been identified: six CDC20 and three CCS52/CDH1 isoforms. Several reports support a role for plant APC/C in the regulation of the cell cycle. Thus, mitotic cyclins are degraded in a Dbox-dependent manner (Genschik et al., 1998) and their degradation is required for the reorganization of mitotic microtubules to the phragmoplast and for proper cytokinesis (Weingartner et al., 2004). Furthermore, *Arabidopsis apc2* (Capron et al., 2003b), *apc6/NOMEGA* (Kwee and Sundaresan, 2003) loss-of-function mutants are impaired in megagametogenesis after the first mitotic division. In addition, in *Medicago* species, CDH1-type activators are involved in endoreduplication, a modified cell cycle during which DNA continues to be duplicated in the absence of mitosis (Cebolla et al., 1999; Vinardell et al., 2003). Finally, the removal of CDC27B/HBT APC/C subunit in *Arabidopsis* root or leaf sectors revealed defects in both cell division and endoreduplication (Serralbo et al., 2006).

Here, we have used two different cyclin reporter constructs to probe APC/C E3 ligase activity during plant development and particularly in various differentiated cell types. Strikingly, we found that the APC/C remains active in most post-mitotic cells. Second, we engineered different APC/C hypomorphic *Arabidopsis* transgenic lines and found that a reduction in the activity of this E3 ligase leads to multiple developmental abnormalities. Among them, the cellular organization of the inflorescence stems revealed increased amounts of vascular tissues.

MATERIALS AND METHODS

Gene constructs

35S::cyclin-GUS constructs

The N-terminal mitotic cyclin domains used in this report have been described previously (Genschik et al., 1998). The fragments carrying the coding regions of tobacco cyclins with native or mutated Dbox motifs were cloned into pBluescript II SK^+ (Stratagene) vector in frame with the β-Glucuronidase (GUS)-coding sequence. Subsequently, the *Bam*HI-*Sac*I fragments were subcloned into the binary pBI121.1 vector (Clontech), replacing the GUS reporter gene.

35S::APC10/APC6 constructs

Fragments of 323 bp (nucleotides 191 to 514) from the APC10-coding sequence and of 456 bp (nucleotides 1046 to 1502) from the APC6-coding sequence were cloned into the pB2GW7 [described by Karimi et al. (Karimi et al., 2002)] to generate the 35S::APC10 and the 35S::APC6 co-suppression constructs, respectively.

RNAi constructs

The vector pFGC5941 (Kerschen et al., 2004) was used to generate the RNAi constructs. The same APC10 and APC6 fragments as indicated above were cloned into the PFGC5941 vector. One sequence was cloned at the *Xho*I and *Nco*I sites. and the other was cloned in the other orientation at the *Xma*I and *Xba*I sites. These constructs generate hairpin structured RNA that will trigger RNAi silencing of APC10 and APC6, respectively.

Transgenic plants

Plant transformation was performed by the floral dip method (Clough and Bent, 1998). Wassilewskija (WS) ecotype was used to generate transgenic plants expressing cyclin-GUS fusion. We isolated a minimum of 17 T1 lines for each construct and selected at least five T3 lines on the basis of their segregation ratio for kanamycin resistance.

Columbia (Col0) ecotype was used to generate APC10 and APC6 co-suppressing lines (APC10S and APC6S), and RNAi lines for APC10 and APC6. We isolated a minimum of 15 T1 lines for each construct for further analysis.

BY2 cell culture, transformation and synchronization

The tobacco BY2 suspension culture (*Nicotiana tabacum* L. cv. Bright Yellow 2) was maintained according to Nagata et al. (Nagata et al., 1992). Transgenic BY2 cells generated by Agrobacterium-mediated transformation were synchronized and analyzed as described by Genschik et al. (Genschik et al., 1998).

GUS assays

Histochemical localization of GUS activity was performed as described by Jefferson et al. (Jefferson et al., 1987). Several independent T3 lines, at least three per construction, were assayed for GUS activity at various developmental stages, using in vitro grown plantlets. The expression patterns were qualitatively the same among these lines. Thereafter, the pictures shown in this study are typical examples of the different patterns observed.

Three-week-old in vitro grown plantlets were used for quantitative GUS assays performed with the GUS-Light kit (Tropix) in a microplate luminometer (TR717 Tropix, Applied Biosystems) according to the manufacturer's instructions. GUS activity is expressed as RLU per 5 μg proteins after 30 minutes of enzymatic reaction.

For the transgenic BY2 cells, fluorimetric GUS assay were performed according to Jefferson et al. (Jefferson et al., 1987). GUS activity is expressed as pmoles of MU produced per minute per milligram of protein.

RNA and DNA gel blotting and RT-PCR analysis

Total RNAs from plantlets were isolated as described by Verwoerd et al. (Verwoerd et al., 1989) and blotted as indicated in Criqui et al. (Criqui et al., 2002). The genomic DNA extraction was performed using the Plant DNAZOL Reagent (Invitrogen).

For RT-PCR on *Arabidopsis* leaves, RNA was extracted from 1, 2, 3 and 5 mm long leaves of a ProCycB1;1::NterCycB1;1-GUS line using the RNeasy plant mini kit (Qiagen). Total RNA (3 μg) was reverse transcribed with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The PCR reaction was carried out with 1 μl template and 20 pmoles of specific primer (details can be provided on request). For small RNA analysis, total RNA was extracted from flower buds from different RNAi APC6 lines with Tri-Reagent (Sigma). RNA gel blot analysis of low molecular weight RNA was conducted on 15 μg of total RNA as described by Akbergenov et al. (Akbergenov et al., 2006).

Flow cytometry

The method is described elsewhere (Cebolla et al., 1999).

Histological analysis

To determine cell size of rosette leaves we used the ImageJ software (http://rsb.info.nih.gov/ij/). For cytology analysis, inflorescences from stem, leaves and eight-day-old seedlings of wild-type (Col-0), APC10S and RNAi APC6 lines were fixed overnight at 4°C in a FAA buffer (3.7% formaldehyde, 50% ethanol, 5% cold acetic acid, 0.1% Triton X-100) after two infiltrations of 30 minutes each. The tissues were washed at room temperature in 1×PBS buffer (2×30 minutes), then in 50% ethanol (2×30 minutes), 70% ethanol (1 hour) and 96% ethanol (overnight). After two additional washes in 100% ethanol, the samples were successively incubated 15 minutes in 100% ethanol/histoclear mix (75%/25%; 50%/50%; 25%/75%) and 3×15 minutes in 100% histoclear. The samples were then incubated at 60°C in a histoclear/Paraplast mix (1V/1V) overnight and in 100% Paraplast for 2 days (with the solution changed 2 times per day). Tissues were embedded in 100% Paraplast and sections were cut on a microtome (Leica RM2155). Sections were then colored with Toluidine Blue and observation were done on a Nikon Eclipse E800 microscope (Champigny sur Marne, France).

RESULTS

Expression of APC/C subunits and activators during leaf development

The role of the APC/C in post-mitotic cells is still poorly understood in all organisms. Thus, we investigated whether components of the APC/C pathway are expressed in differentiated and post-mitotic cells, during the process of leaf development. We took advantage of the ProCYCB1;1::NterCYCB1-GUS reporter line (Colon-Carmona et al., 1999) to monitor cell division activity and harvested *Arabidopsis* leaves at different developmental stages, ranging from very young leaves with high cell division activity to fully expanded leaves, in which cell division activity is scarce and most cells are differentiated (Fig. 1A). This analysis includes selected subunits of the APC/C (Capron et al., 2003a) and, most importantly, different APC/C activators (CDC20 and CCS52/CDH1), as well as two specific ubiquitin-conjugating enzymes (UBC19 and UBC20). In contrast to AtCYCB1;1, all APC/C subunits tested (APC3/CDC27A, APC4, APC6 and APC10) were expressed similarly at the different leaf-stages (Fig. 1B). In particular, their transcript levels remained high in fully expanded leaves (lane 5). However, both classes of APC/C activators were expressed differentially. Under our experimental conditions, we could detect in leaves the transcripts of only two of the six CDC20-related subunits (CDC20-1 and CDC20-2). Both showed an expression profile similar to AtCYCB1;1, suggesting that they are cell cycle regulated. In contrast to the CDC20-types of activator, CCS52/CDH1-related subunits, as well as the E2 UBC19 and to a lesser extent UBC20, remained expressed in older leaves, suggesting their involvement in the function of APC/C in post-mitotic cells. These data are consistent with the recent finding that some APC/C subunits are expressed in organs with overall low cell division rate, such as siliques (Eloy et al., 2006).

Proteolysis of the chimeric cyclin-GUS proteins throughout the cell cycle

As a next step, we wished to visualize directly the APC/C activity in various tissues and during the process of plant organ development and growth. We have previously reported that a fusion protein between the N-terminal domain of either Nicta;CYCA3;1 or Nicta;CYCB1;1 tobacco mitotic cyclins and the chloramphenicol acetyl transferase (CAT) reporter protein is degraded in a cell cycle-dependent manner in synchronized tobacco BY2 cells (Genschik et al., 1998). Both cyclin N-terminal domains carry the well-characterized Dbox motif, which is one of the degrons recognized by the APC/C (Klotzbücher et al., 1996). To visualize the accumulation of these APC/C substrates in planta by histochemical analysis, we replaced the *CAT* gene by the *GUS* reporter gene (Fig. 2A). Constructs CycA-GUS and CycB-GUS carry a native Dbox motif in the cyclin N-terminal domains, whereas the two highly conserved amino acids are mutated from RxxLxx(L/I)xN to GxxVxx(L/V)xN in CycAΔBox-GUS and CycBΔBox-GUS constructs (Genschik et al., 1998). We first investigated the stability of these chimeric proteins during the cell cycle.

The constructs were introduced into tobacco BY2 cells by Agrobacterium-mediated transformation, as this cell suspension still represents one of the best and most homogenous systems to synchronize plant cells. Each synchronization experiment (as illustrated in Fig. 2B-F) was repeated at least twice by using different transgenic lines per construct. As expected, the GUS protein alone, expressed under the control of the constitutive CaMV 35S promoter, did not exhibit a significant difference in the pattern of its accumulation (Fig. 2B). However, both the CycA-GUS and CycB-GUS chimeric protein levels oscillated during the cell cycle (Fig. 2C,D) in a similar manner as previously described for their CAT variants (Genschik et al., 1998). Thus, the CycB-GUS chimeric protein accumulates during G2 and is actively degraded early in mitosis, always before the mitotic index reaches its maximum (Fig. 2D). By contrast, the accumulation pattern of the CycA-GUS protein is broader (Fig. 2C,E). The fusion protein has already accumulated during S-phase and is also degraded later in mitosis, as its level

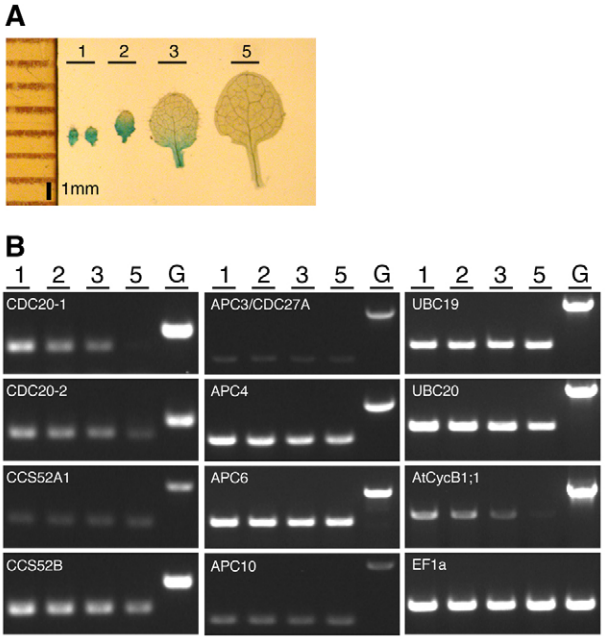

Fig. 1. CDH1-related and APC/C genes remain expressed in post-mitotic cells. (**A**) GUS staining of 1 mm, 2 mm, 3 mm and 5 mm long leaves from a ProCycB1;1::NterCycB1;1-GUS line. Only dividing cells are stained blue. (**B**) RT-PCR on RNA extracted from leaves of the ProCycB1;1::NterCycB1;1-GUS line at different developmental stages using specific primers for CDC20-1, CDC20-2, CCS52A1, CCS52B, APC3/CDC27A, APC4, APC6, APC10, UBC19 and UBC20. RT-PCR on AtCycB1;1 and EF1α are used as controls. 1, 2, 3, 5 correspond to the leaf stages and G to genomic DNA.

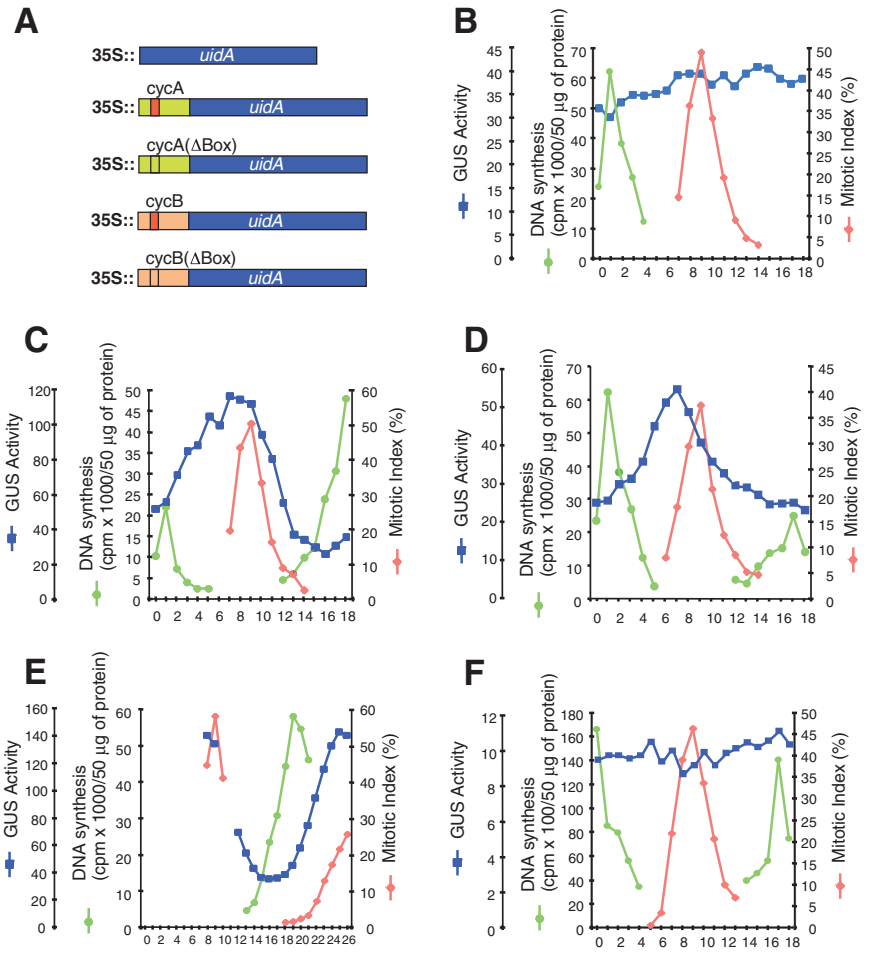

Fig. 2. CycA-GUS and CycB-GUS reporter proteins are degraded in a cell cycle-dependent manner. (**A**) Schematic structure of the constructs inserted into the binary vector pBI121 under the control of the CaMV 35S promoter. Green and orange boxes represent the N-terminal domain of the cyclin A3 and cyclin B1, respectively, and blue boxes indicate the GUS sequence. Functional Dbox is highlighted in red, in contrast to the mutated Dbox. (**B-F**) Oscillation of GUS activity (blue curves) during the cell cycle in transgenic BY2 cell lines. After aphidicolin removal, the progression through the cell cycle was monitored by 3H-thymidine incorporation (green curves) and mitotic index determination (red curves). Quantitative GUS assays presented in this figure were obtained with the following transgenic cell lines 35S::GUS (B), 35S::CycA-GUS (C,E), 35S::CycB-GUS (D) and 35S::CycAΔDbox-GUS (F).

declined always after the mitotic index pick. The differential accumulation patterns of A- and B-type cyclin reporters indicates that the plant APC/C destroys sequentially these cyclins during the cell cycle, similar to the situation in animal cells (Sigrist et al., 1995). For both constructs 35S::CycAΔDbox-GUS and 35S::CycBΔDbox-GUS, mutations inside the Dbox motif abolished the cell cycle-specific oscillations of the fusion proteins (Fig. 2F; and data not shown). From these experiments, we can conclude that these chimeric proteins can be used as tools to reflect the APC/C activity in planta, at least in the cell types in which the 35S promoter is expressed.

The APC/C remains active in most differentiated plant cells

To investigate APC/C activity in non-cycling plant cells, we generated transgenic *Arabidopsis* lines for each 35S::CycA-GUS and 35S::CycB-GUS construct and their mutated Dbox variants, as well as for the 35S::GUS control construct. Fifteen to 20 independent transformed lines were selected for each construct. The plants were further selected for a single insertion of the transgenes (see Fig. S1A in the supplementary material). In order to compare the level and spatio-temporal distribution of GUS activity in plants expressing the CycA-GUS or CycB-GUS chimeric proteins versus their Dbox variants, we retained lines expressing similar levels of transcripts of the transgenes (see Fig. S1B in the supplementary material). Despite similar mRNA levels, the GUS activity quantified by luminometry was much stronger in plant extracts expressing the non-degradable forms of the cyclin-GUS fusion proteins compared with the native forms (see Fig. S1C in the supplementary material).

The histochemical staining at different developmental stages of plants expressing the CycB-GUS chimeric protein shows a patchy distribution of stained cells, which is restricted to regions of cell division, such as in young leaves (Fig. 3A). As leaves expand, a longitudinal gradient in the frequency of GUS-expressing cells developed. GUS activity is confined to the base of the developing leaf (Fig. 3D) and thus follows the basiplastic patterns of cell cycling described by Donnelly et al. (Donnelly et al., 1999). In cotyledon (Fig. 3A) and older leaves (Fig. 3G), the GUS staining is maintained in a group of cells associated with the vascular system (primary, secondary and tertiary veins) and can also be visualized at the hydathodes (Fig. 3F). However, GUS staining was never observed in any other cell types of fully differentiated leaves, including branched trichomes (Fig. 3H). In the root, GUS staining is detected with a patchy pattern at the level of the root meristem, but not the elongation zone (Fig. 3K). CycB-GUS reporter protein accumulation was also observed in cells in the stele of the primary root (Fig. 3J). Conversely, plants expressing the CycBΔDbox-GUS protein variant show a uniform distribution of the GUS staining in the different plant tissues (Fig. 3B,E,I,L), which is similar to the 35S::GUS control plants (not shown). To further demonstrate that the absence of GUS staining in non-cycling plant cells is due to the active degradation of the fusion protein, we used MG132, an inhibitor of the 26S proteasome. Indeed, we observed the

appearance of GUS staining in 14 hours-MG132 treated CycB-GUS plants (Fig. 3C). From these experiments, we conclude that CycB-GUS reporter protein is actively degraded in non-dividing cells in a Dbox-dependent manner, indicating that the APC/C is active in most post-mitotic plant cells.

The histochemical characterization of plants expressing the CycA-GUS chimeric protein also shows a patchy distribution (Fig. 4). However, in contrast to the 35S::CycB-GUS-transformed plants, the pattern of GUS-stained cells is much broader, correlating with the stronger GUS activity measured in these lines (see Fig. S1C in the supplementary material). Specifically, the staining was found to be more pronounced in the vascular bundles of cotyledons (Fig. 4A), leaves (Fig. 4F), hypocotyls (Fig. 4C) and roots (Fig. 4G). The strong increase in the number of cells in which GUS expression is detected reflects the stability of the CycA-GUS protein during the cell cycle (Fig. 2). Indeed, the fusion may not only label mitotic cells, but also cells undergoing DNA replication and most probably also cells that are arrested in or proceed through G2 phase. However, many post-mitotic cells such as epidermis and cortex cells of the elongated primary root (Fig. 4H) showed no staining. Conversely, plants expressing the CycAΔDbox-GUS protein exhibit a GUS signal uniformly distributed in all cells (Fig. 4B,E,I).

Overall, we conclude that the APC/C is maintained active in many different post-mitotic cell types. To obtain more insight into the role of the APC/C during plant development, we generated transgenic lines with reduced APC/C activity.

Production of APC/C knockdown mutant

Because APC/C activity is essential in plants (Capron et al., 2003b; Kwee and Sundaresan, 2003; Perez-Perez et al., 2008), we decided to generate hypomorphic mutant lines using RNAi. Two subunits of the complex, APC6 and APC10 were chosen because both are encoded by single copy genes and are expressed in post-mitotic cells (Fig. 1B). We believe that this method is particularly appropriate to characterize the function of APC/C in differentiated cells, because it has been shown that RNAi is less efficient in dividing cells in meristems or in Agrobacterium-induced tumors (Voinnet et al., 1998; Dunoyer et al., 2006), and thus should permit the recovery of viable plants. To do so, we followed two strategies: first, we produced co-suppression lines in which a fragment of each gene was overexpressed under the control of the strong 35S promoter (see Fig. S2A in the supplementary material). Several independent lines were selected and the expression of the corresponding endogenous gene was tested by RT-PCR. In some of these lines, we observed a significant reduction in the expression of APC10 and APC6 (see Fig. S2B in the supplementary material). Second, the same cDNA fragments of APC10 and APC6 were cloned into the pFGC5941 vector, on both sides of the chalcone synthase intron into opposite orientation (see Fig. S2A in the supplementary material). This vector generates hairpin structured RNA that triggers RNAi silencing of APC10 and APC6, respectively. Expression level of the endogenous genes and accumulation of small RNA were tested in the selected lines (see Fig. S2C in the supplementary material). A significant reduction in the expression of the endogenous APC6 and APC10 mRNAs was observed in the RNAi lines, which correlates with a high accumulation of siRNAs (see Fig. S2C in the supplementary material).

If silencing of *APC10* and *APC6* has an effect on APC/C activity in planta, we could expect to see a higher accumulation of the artificial cyclin-GUS substrates in those mutant lines. To address this issue, we introgressed the 35S::CycB-GUS construct into two of these mutant lines: APC6S-4 and APC10S-6. Indeed,

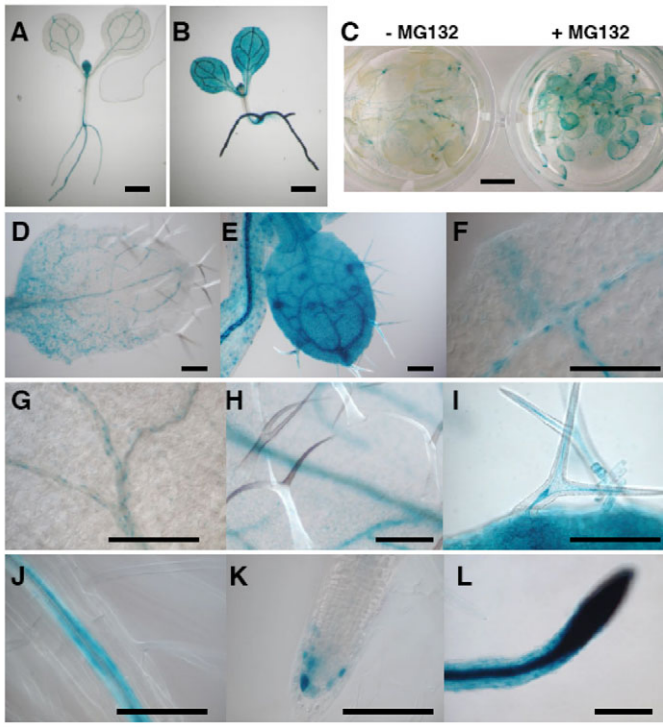

Fig. 3. CycB-GUS reporter activity indicates that APC/C is active in post-mitotic plant cells. (**A**,**B**) Histochemical GUS staining of 6-day-old 35S::CycB-GUS (A) and 35S::CycBΔDbox- GUS (B) seedlings grown in vitro. The CycB-GUS reporter protein stains only regions of cell division, whereas the CycBΔDbox-GUS reporter protein accumulates in dividing and non-dividing cells. (**C**) Histochemical GUS staining of 35S::CycB-GUS seedlings treated with 100 μM MG132 (+) or mock treated (–) for 14 hours. (**D-L**) Histochemical GUS staining of 11-day-old 35S::CycB-GUS (D,K) and 35S::CycBΔDbox-GUS (E,L) plantlets, and 21-day-old 35S::CycBGUS (F,G,H,J) and 35S::CycBΔDbox-GUS (I) plantlets grown in vitro. The pictures show representative distribution pattern of GUS staining in leaves (D,E,F,G), trichomes (H,I) and roots (J,K,L). Scale bars: 1 mm in A,B; 5 mm in C; 100 μm in D-I.

by histochemical analyses we observed a stronger accumulation of the CycB-GUS protein in the APC/C hypomorphic mutant backgrounds compared with wild type (see Fig. S3 in the supplementary material). By contrast, histochemical analyses of the Dbox-mutated version of the 35S::CycB-GUS showed no difference between wild-type and hypomorphic mutant backgrounds (see Fig. S3 in the supplementary material). This result indicates that the APC/C activity is reduced in the *APC10* and *APC6* hypomorphic mutants and that these lines are appropriate for further characterization of the role of APC/C in plant development.

APC/C hypomorphic mutants display endoreduplication defect in rosette leaves

The phenotype of the APC/C hypomorphic lines was followed during plant development. We could not detect major morphological difference at the young seedling stage between wild-type and the hypomorphic lines (Fig. 5A, upper panel). In contrast to the hobbit mutant (Willemsen et al., 1998), we could not observe any defect during the root growth (see Fig. S4A in the supplementary material). At the rosette stage, we also did not detect major differences between the wild type and APC10S and APC6S lines (Fig. 5A,

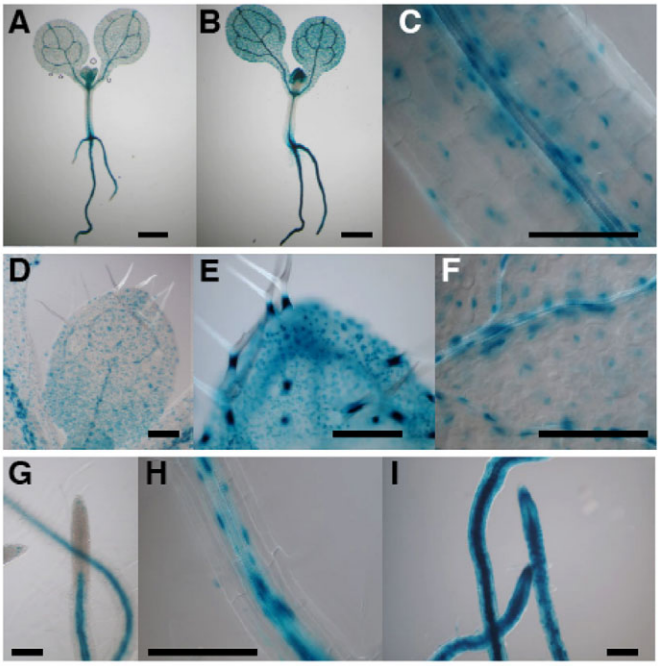

Fig. 4. CycA-GUS reporter protein shows broader GUS staining distribution. (**A**,**B**) Histochemical GUS staining of 6-day-old 35S::CycA-GUS (A) and 35S::CycAΔDbox- GUS (B) seedlings grown in vitro. (**C-I**) Histochemical GUS staining of 11-day-old 35S::CycA-GUS (D) and 35S::CycAΔDbox-GUS (E) plantlets and 21-day-old 35S::CycA-GUS (C,F,G,H) and 35S::CycAΔDbox-GUS (I) plantlets grown in vitro. The pictures show representative distribution pattern of GUS staining in hypocotyl (C), leaves (D-F) and roots (G-I). Scale bars: 1 mm in A,B; 100 μm in C-I.

bottom panel). However, several RNAi APC/C lines exhibited smaller and denser rosette and curled leaves (Fig. 5A,C). Moreover, even without major morphological differences, we noticed that in hypomorphic lines the cotyledon vein patterning is altered (Fig. 5B). In *Arabidopsis* cotyledon, the vein pattern is simple, containing a single primary vein that extends though the center of the cotyledon and additional four secondary veins that form four closed loops. In the hypomorphic mutants, the primary vein shows no defect, but in most cases, secondary veins formed only two or three loops (Fig. 5B).

Next, we compared epidermal cell size of young rosette leaves between the APC/C hypomorphic mutants and wild-type plants (Fig. 5D). We observed a reduction of cell size in the strongest RNAi APC6 lines (Fig. 5D) showing altered development at the rosette stage (Fig. 5A) and reduced leaf size (Fig. 5C). A slight, but significant, reduction was also observed in the weaker APC6S and APC10S co-suppression lines (Fig. 5D), with no leaf size phenotype. Overall, these results suggest that a strong decrease of the APC/C activity affects the cell cycle and most probably causes the observed leaf phenotype in the RNAi APC lines, whereas in the co-suppression lines, the residual APC/C activity supports an almost normal leaf developmental program.

Endoreduplication is a post-mitotic mechanism during which several rounds of DNA replication occur without cell division. The regulation of this mechanism is not well understood; however, CCS52, a CDH1-type activator of APC/C complex, has been previously involved in endoreduplication in plant cells (Cebolla et al., 1999; Vinardell et al., 2003). Therefore, we analyzed the DNA content in leaf cells of the APC hypomorphic mutants. In the strongest APC/C knockdown lines (RNAi-APC6 and RNAi-APC10), we observed an important reduction in the 8C and 16C DNA contents compared with the wild-type plants and conversely an increase of cells with 4C and 2C DNA contents (Fig. 5E), which correlates with reduced leaf cell size (Fig. 5D). Interestingly, in the co-suppression lines (APC6S and APC10S), we could already detect a similar, although weaker, phenotype on the ploidy level (Fig. 5E). This result supports a function of the APC/C in the mechanism of plant endoreduplication, as earlier suggested (Cebolla et al., 1999).

APC hypomorphic mutants are impaired in inflorescence architecture and vascular tissue organization

Strikingly, at later developmental stages, the mutant lines developed dramatic morphological aberrations during shoot elongation and inflorescence. These plants showed shorter stems and a severe shortening of the internodes causing the formation of a ‘broomhead-like’ cluster of siliques at the top of stems (Fig. 6A). This phenotype is more or less severe according to the lines, but was observed for both the co-suppression and the RNAi lines. In the case of a ‘strong broomhead’ phenotype, stem elongation is extremely reduced and the inflorescences appear at the level of the plant rosette (Fig. 6A, part e). Apart from this inflorescence phenotype, the flowers and the siliques are normal, as is the fertility of the hypomorphic lines.

To investigate in more detail the cellular organization in the inflorescence stem of the APC/C hypomorphic mutants, we performed histological analyses. Longitudinal and transverse sections of different organs were colored with Toluidine Blue (Fig. 6B; see Fig. S4B in the supplementary material). This dye stains specifically the lignified cells like xylem and secondary vascular tissues. First, the sections of 8-day-old seedling hypocotyls reveals that the APC/C hypomorphic lines present all differentiated cell types, with the epidermal, cortical and endodermal layers and the xylem and phloem in the inner stele tissue (see Fig. S4B, parts a,b in the supplementary material). This correlates with the lack of morphological abnormalities of these lines at this developmental stage. Next, we made sections of stems of 5-week-old plants, which show a ‘broomhead’ phenotype. In the upper stem, the vascular system of wild-type *Arabidopsis* is organized in discrete collateral bundles with the phloem towards the outside and the xylem towards the inside of the bundle and separated by interfascicular regions (Fig. 6B, parts a,g). By contrast, the hypomorphic mutants exhibit an increased amount of vascular tissue, most notably cambium, xylem and lignified sclerenchyma, organized in a continuous ring (Fig. 6B, parts b,h). No interfascicular fibers are distinguishable in hypomorphic mutant stem sections. In addition to the overproliferation of the cambium, we could observe enlarged lignified cells (see arrows in Fig. 6B, parts d,h), which have disorganized cell division planes (Fig. 6B, part d). Moreover, cells in the cortex and epidermis are larger in the hypomorphic mutant stems compared with wild type (Fig. 6B, parts g,h).

The secondary growth shown in the sections of the base of the stem (Fig. 6B, part e) is characterized by the presence of vascular cambium, which originates from procambium within the vascular bundles and from parenchyma cells in the interfascicular region. As in the upper part, the hypomorphic mutants exhibit an increased amount of vascular tissues (Fig. 6 B, part f). In addition to the curled leaf phenotype (see Fig. S4B, part d in the supplementary material), there is also a disorganized vascular tissue in the primary vein of the strong hypomorphic mutant (see Fig. S4B, part f in the supplementary material) compared with wild type (see Fig. S4B,

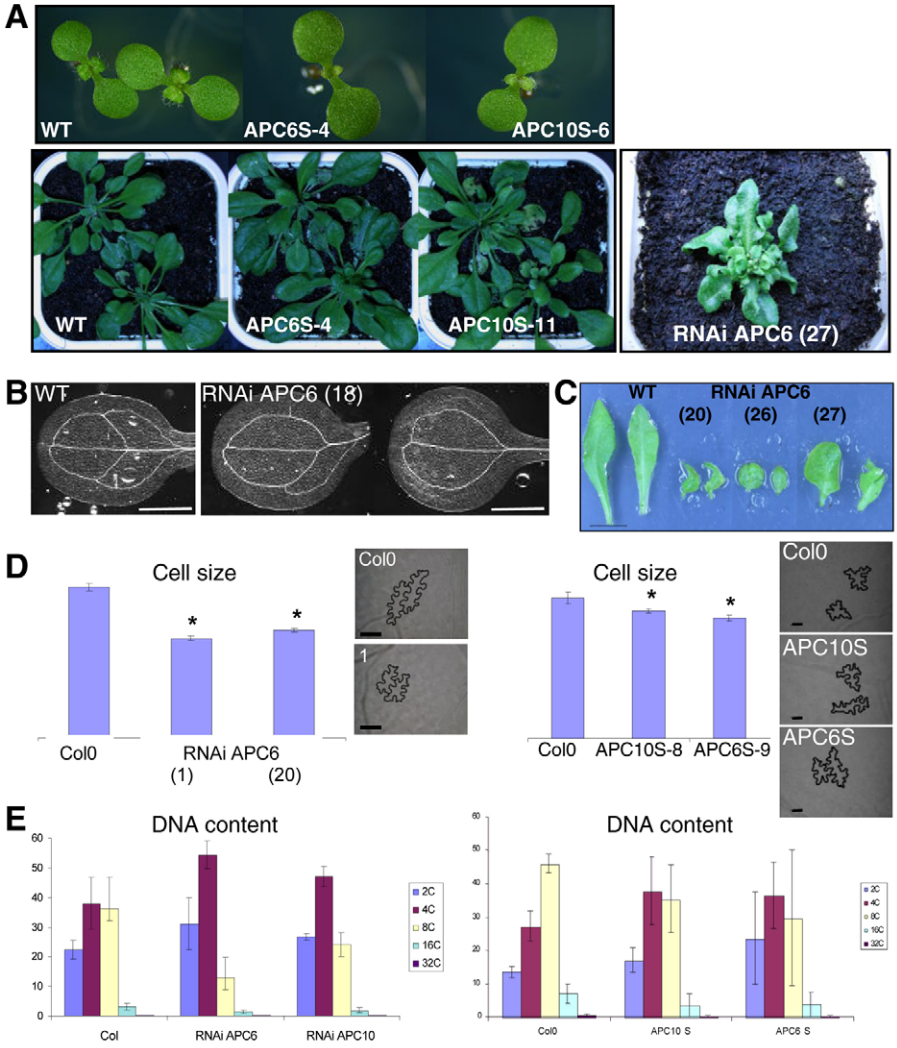

Fig. 5. APC hypomorphic mutants show various leaf developmental alterations, including shape, cell size and endoreduplication. (**A**) One-week-old seedlings of in vitro grown wild-type and APC/C hypomorphic lines (upper panel) and 3-week-old plants of wild-type and APC/C hypomorphic lines grown in soil (bottom panel). (**B**) Venation patterns of cleared cotyledons from wild-type and RNAi APC6 (18) hypomorphic line. Scale bars: 1 mm. (**C**) Adult rosette leaves from wild-type and RNAi APC6 lines. Scale bar: 1 cm. (**D**) Epidermal cell sizes were measured in rosette leaf from 3-week-old wild-type (Col0) and different RNAi APC6 hypomorphic lines (numbered 1 to 20) and from 3-week-old wild-type (Col0) and APC6S-9 and APC10S-8 hypomorphic lines. *T*-tests were performed for each value compared with wild type to determine significant differences. Asterisks indicate values for which $P<0.05$. Scale bars: 40 μm in Col0 and line RNAi APC6-1; 50 μm in Col0, APC6S and APC10S. (**E**) DNA contents were measured on cells from the fifth rosette leaf of 4-week-old wild-type (Col0) and APC6 and APC10 hypomorphic lines (RNAi APC6-1, RNAi APC10-38, APC6S-4 and APC10S-6) using flow cytometry.

part e in the supplementary material). Within this vein, vascular tissues are generally arranged with the xylem on the upper (adaxial) side of the leaf and the phloem on the abaxial side (see Fig. S4B, part e in the supplementary material). In the APC/C hypomorphic lines, some cells on the abaxial side are differentiated into xylem (see Fig. S4B, part f in the supplementary material).

VASCULAR-RELATED NAC-DOMAIN7 (VND7) is involved in the differentiation of xylem vessels (Yamaguchi et al., 2008). Its promoter drives the expression in the protoxylem and metaxylem of roots, shoots and leaves (Yamaguchi et al., 2008) (see Fig. S5A,C,E). When we introgressed the P_{VND7}::GUS reporter within the APC/C hypomorphic mutant background, we did not observe any difference in the GUS expression patterns (see Fig. S5B,D,F,G,H in the supplementary material), suggesting that cell differentiation into xylem occurs normally in these lines.

DISCUSSION

APC/C expression and activity during plant development

In this report, we investigated possible functions of the APC/C beyond its well-known role in regulating the cell cycle. Our expression analysis revealed that all APC/C core subunits tested are expressed at various leaf developmental stages, including highly differentiated cells considered to be quiescent cells in mature leaves. In contrast to this constant steady-state expression level, we observed a distinct transcription profile for the APC/C activators. Two of the six expressed CDC20-related subunits showed a characteristic cell cycle regulated expression profile when compared with AtCycB1;1, whereas the CDH1-related activators have similar expression patterns to the APC/C core subunits. This is consistent with APC/C^{CDH1} being the active complex in quiescent G0 plant cells.

To visualize the spatial and temporal APC/C activity in various plant organs, we expressed two types of cyclin-GUS reporter proteins under the control of the constitutive 35S CaMV promoter. The B-type cyclin reporter lines showed GUS stained cells essentially in tissues with high rates of cell division activity, such as in meristems, young leaves and cells in the procambium. This correlates well with G2/M stabilization of the Dbox containing reporter protein in cycling cells (Colon-Carmona et al., 1999; Donnelly et al., 1999), in which B-type cyclins and other regulators need to accumulate. Conversely, no GUS staining was associated with many differentiated cells, including mesophyll and pavement cells, guard cells and trichomes. Thus, as in mammalian neurons and hepatocytes (Gieffers et al., 1999; Wirth et al., 2004), differentiated non-dividing plant cells keep an active APC/C, despite the fact that no mitotic cyclins and most probably

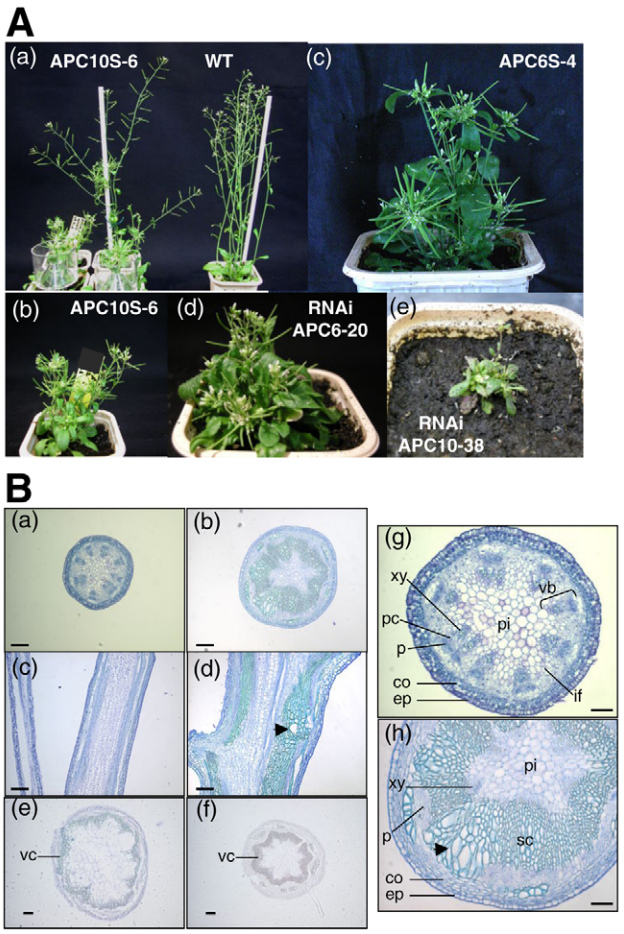

Fig. 6. APC/C hypomorphic mutants display a broomhead-like phenotype and vascular tissue disorganization. (**A**) Five-week-old plants of APC10S-6 hypomorphic line in comparison with wild type (a). Closer view of the 'broomhead' phenotype of APC10S-6 (b), APC6S-4 (c), RNAi APC6-20 (d) and RNAi APC10-38 (e) hypomorphic lines. (**B**) Transverse and longitudinal sections from the apical part of the stem from 5-week-old wild-type (a,c,g) and APC10S plants (b,d,h) stained with Toluidine Blue and viewed under bright-field illumination. pi, pith; xy, xylem; p, phloem; pc, procambium; co, cortex; ep, epidermis; if, interfascicular cells; vb, vascular bundle; sc, sclerenchyma. Scale bars: 40 μm. Transverse sections from the base of the stem from 5-week-old wild-type (e) and RNAi APC6 (f) plants stained with Toluidine Blue and viewed under bright-field illumination. vc, vascular cambium. Scale bars: 50 μm.

no other mitotic regulators are expressed. This implies that the APC/C recognizes other substrates containing a Dbox motif in post-mitotic cells.

Although the A-type cyclin-GUS reporter construct also carries a functional Dbox motif, this reporter protein showed a broader expression pattern in various plant tissues. This difference can be explained by a broader stability of cyclin A over a complete cell cycle (Fig. 2). In particular, this reporter protein is less prone to degradation during S phase in comparison with the cyclin B-GUS reporter. Thus, it is possible that the A-type cyclin-GUS lines also reveal cells that undergo endoreduplication, which occurs during differentiation in most plant organs. However, we cannot exclude other possibilities; for example, in human cells, cyclin A needs a CKS subunit of its CDK partner to be targeted by the APC/C (Wolthuis et al., 2008) and thus the N terminus of cyclin A alone may just be less efficiently degraded in some differentiated cell types.

Overall, the findings that APC/C and CDH1-related subunits are expressed in mature plant leaves and that reporter proteins caring a Dbox degron are actively degraded in differentiated post-mitotic cells suggests that the activity of APC/C is required outside of the plant cell cycle.

The APC/C in *Arabidopsis* is required for endoreduplication

Loss-of-function mutants analyses for most APC/C core subunits leads to lethality in yeast and multicellular organisms. Mice *APC2* knockouts are embryonic lethal (Wirth et al., 2004), whereas the loss of function of this gene in *Drosophila* and *Arabidopsis* leads to late larval lethality (Kashevsky et al., 2002) and female gametophyte arrest (Capron et al., 2003b), respectively. Thus, to address the function of APC/C in developing plants and in differentiated cell types, we used RNAi strategies to target two of its subunits: APC6 and APC10. It is noteworthy that RNAi, in contrast to the action of miRNAs, is less efficient in dividing cells (e.g. meristems) or in Agrobacterium-induced tumors (Voinnet et al., 1998; Dunoyer et al., 2006).

Endoreduplication is an alternative cell cycle in which at least two successive rounds of DNA replication occur without intervening mitosis. This phenomenon is observed in some animal cell types, but is much more frequent in plant cells (Edgar and Orr-Weaver, 2001; Kondorosi and Kondorosi, 2004). Interestingly, endoreduplication operates during development and in differentiated cells, such as in plant trichomes. A role for the APC/C in endoreduplication was already suspected as the function of CDH1-type activators of APC/C is required for this process in both fly and plant (Sigrist and Lehner, 1997; Cebolla et al., 1999). Hence, very recent work in *Drosophila* elucidated the role of APC/C in the process of endoreduplication (Zielke et al., 2008; Narbonne-Reveau et al., 2008). Our finding that the downregulation of APC/C activity in *Arabidopsis* leads to a significant reduction in endocycles in leaves together with the report that the loss-of-function of *Arabidopsis HBT/CDC27B* leads to a similar effect in root cells (Serralbo et al., 2006) indicates that this pathway is functionally conserved throughout evolution. Nevertheless, in fly the APC/C regulates endoreduplication by mediating Geminin oscillation (Zielke et al., 2008), but an obvious ortholog of Geminin has not yet been identified in plants (Caro and Gutierrez, 2007); thus, the mechanism how APC/C regulates endoreduplication in plants remains still unclear. Interestingly, two A-type cyclins, both containing a Dbox degron, have been involved in endoreduplication in *Arabidopsis* (Yu et al., 2003; Imai et al., 2006). Strikingly, ectopic expression of a Dbox-deficient, and thus non-degradable, cyclin CYCA2;3 significantly restrained endocycles in various plant organs (Imai et al., 2006), suggesting this cyclin as a good candidate substrate of the APC/C in the regulation of this process.

The APC/C restricts cell proliferation in the vasculature

At adult stage, *Arabidopsis* APC/C knockdown lines exhibited a severe phenotype regarding stem elongation. In some lines, stem elongation was that much reduced that all inflorescences appeared to emerge directly from the plant rosette. This phenotype is associated with overproliferation of vascular cells at the expanses

of the interfascicular tissue, which can even lead to a continuous ring-like pattern of xylem and phloem cells. Although plant and animal developmental pathways are under the control of undoubtedly different mechanisms and signals, it is intriguing that the loss of APC/C function in vertebrates results in unscheduled proliferation of some differentiated cell types. Thus, the conditional inactivation of APC2 subunit in mouse hepatocytes led those cells to re-enter the cell cycle even without any proliferative stimulus (Wirth et al., 2004). In zebrafish, the loss of APC/C activity also resulted in improper re-entry into the cell cycle of quiescent cells (Wehman et al., 2007). The mechanism by which the APC/C restrains cell division in plant vascular tissue is still unclear. Either the APC/C restrains cell cycle re-entry of G0 cells or it acts as a 'safeguard' to avoid unscheduled ongoing cell proliferation, which may result in abnormal differentiation. Whether APC/C function in vascular development is post-mitotic or still cell cycle based, this E3 ligase may act by suppressing the accumulation of extracellular or intracellular mitogens. Indeed active degradation of cyclin B1 by the APC/C^{CDH1} in post-mitotic rat neurons prevents them from cell cycle re-entering and subsequently cell death (Almeida et al., 2005). However, ectopic expression of a functional and non-destructible plant cyclin B1 leads to abnormal mitosis and many developmental abnormalities but does not induce vascular tissue overproliferation (Weingartner et al., 2004), indicating that cyclin B1 accumulation cannot explain this phenotype.

Several *Arabidopsis* mutants have been described that are affected both in the growth of inflorescence stems and ectopic vascular proliferation and/or differentiation. Among them, mutants impaired in auxin response or transport, have been reported, as auxin plays a central role in vascular patterning and differentiation (reviewed by Berleth et al., 2000; Rolland-Lagan, 2008). Overproliferation of vascular tissues occurs in plants in which the transport of auxin is chemically inhibited or by mutation of the efflux carrier PIN1 (Mattsson et al., 1999). Local auxin action probably induces genes that are required for vascular patterning. Thus, *ATHB-8*, a gene encoding a class III homeodomain-leucine zipper (HD-Zip III) transcription factor exclusively expressed in procambium tissue, is activated by auxin (Baima et al., 1995) and acts as a positive regulator of cambial cell proliferation and differentiation (Baima et al., 2001). AtHB15 is another member of the HD-Zip III gene family, strictly expressed in vascular bundles, that alters the vascular system by accelerated vascular cell differentiation from cambial cells when the transcript level is drastically reduced (Kim et al., 2005).

Interestingly, it has been reported that the 26S proteasome is involved in the vascular patterning through the regulatory subunit RPN9, a subunit of the 19S RP lid (Jin et al., 2006). RPN9-silenced tobacco plants display extra leaf vein formation with increased xylem and decreased phloem. These authors stipulated that RPN9 functions at least in part through regulation of auxin transport. It is noteworthy that mutations in the *HOBBIT/CDC27B* gene were previously reported to affect both auxin responsiveness and AXR3/IAA17 protein abundance (Blilou et al., 2002). Therefore, to investigate whether APC/C regulates auxin transport or signaling or even auxin downstream regulators during vascular patterning are important issues to address in the future.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/9/1475/DC1

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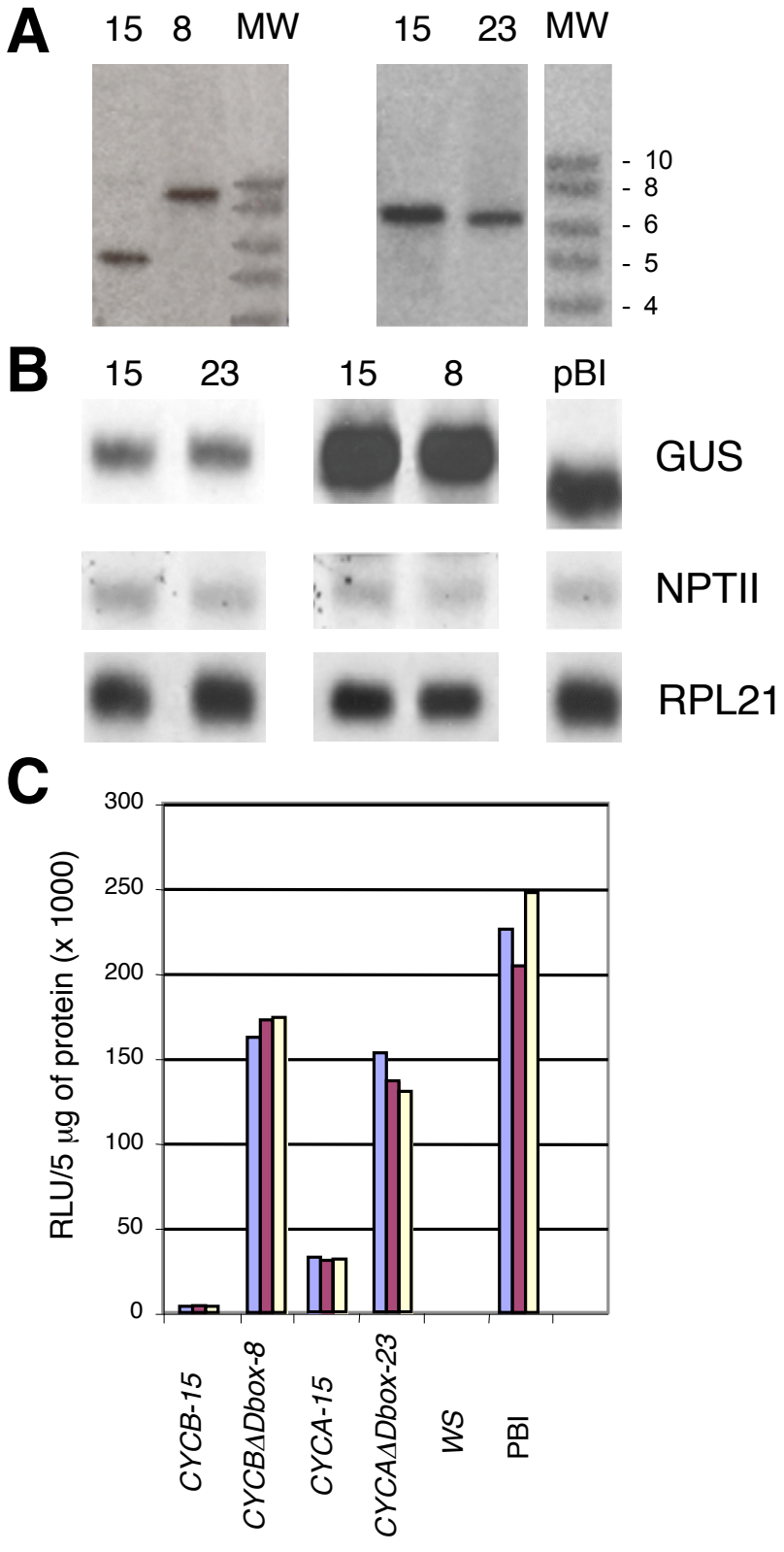
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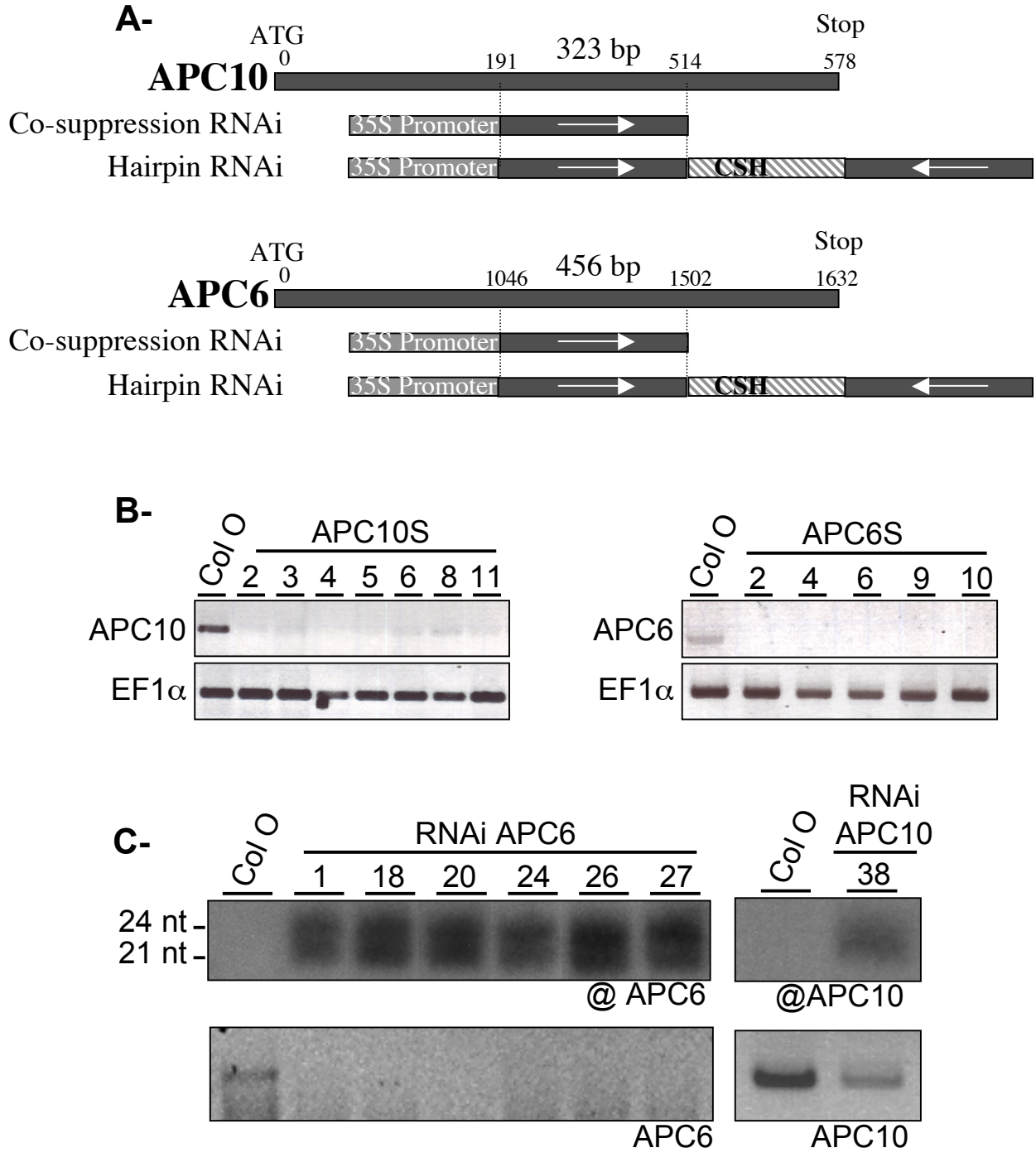
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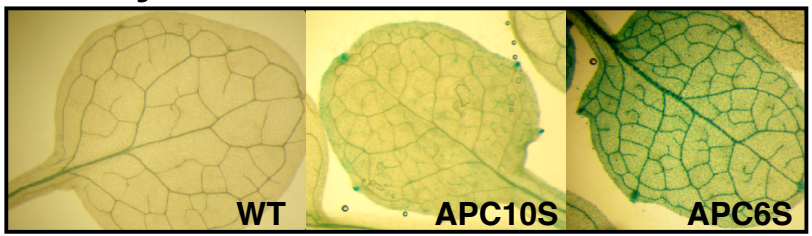

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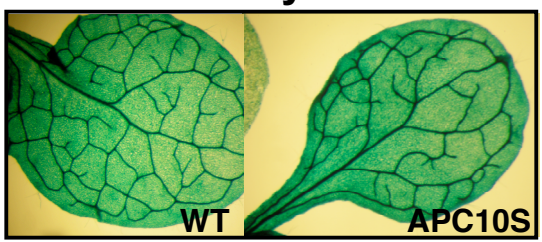

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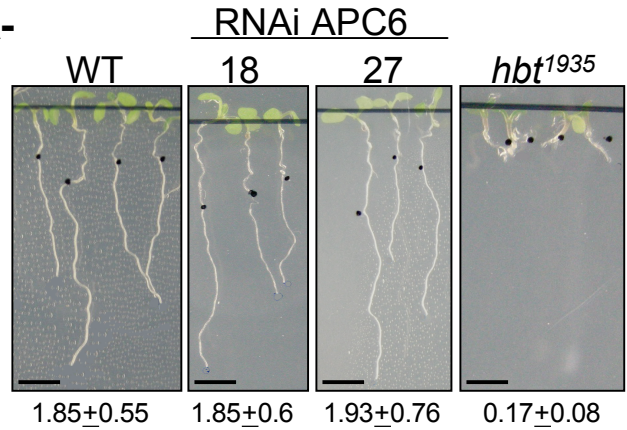
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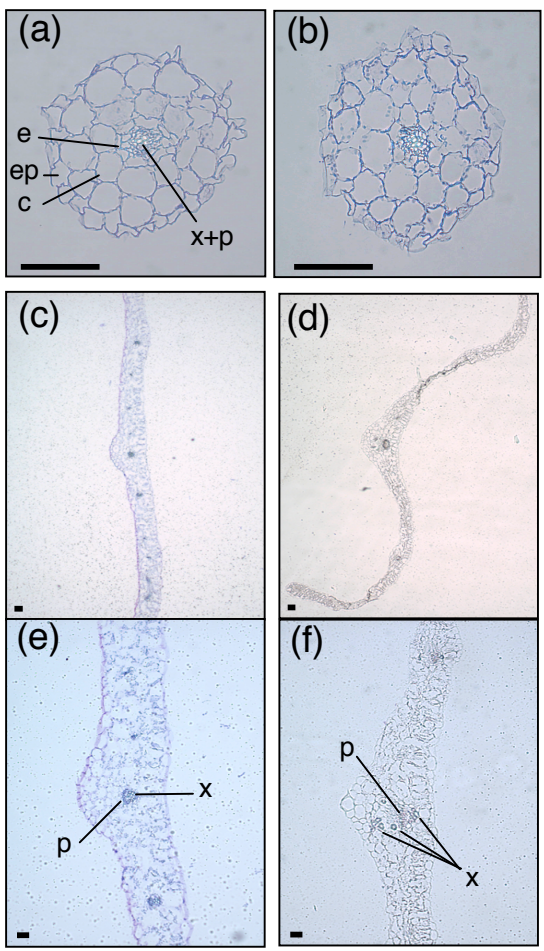
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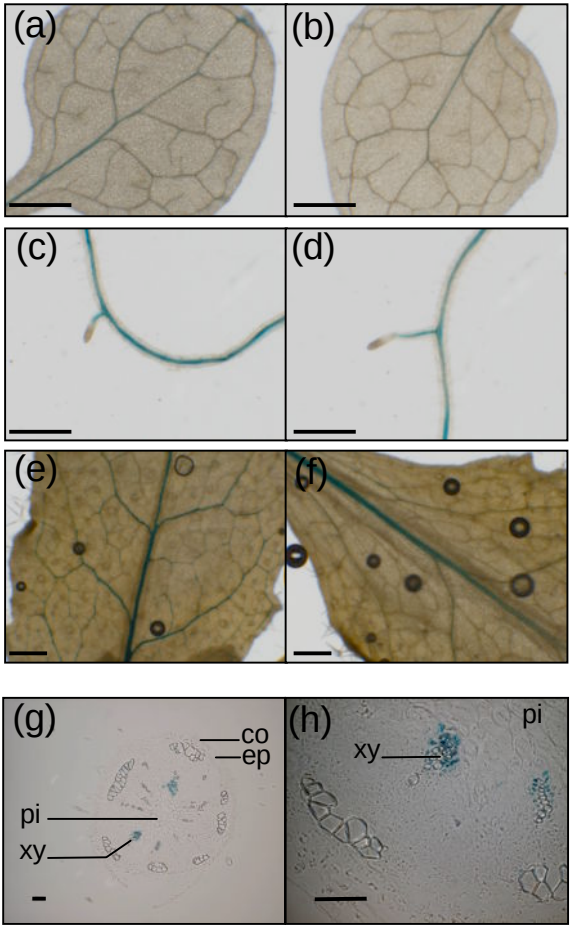
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