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Auxin modulates the transition from the mitotic cycle to the endocycle in *Arabidopsis*

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SUMMARY

Amplification of genomic DNA by endoreduplication often marks the initiation of cell differentiation in animals and plants. The transition from mitotic cycles to endocycles should be developmentally programmed but how this process is regulated remains largely unknown. We show that the plant growth regulator auxin modulates the switch from mitotic cycles to endocycles in *Arabidopsis*; high levels of TIR1-AUX/IAA-ARF-dependent auxin signalling are required to repress endocycles, thus maintaining cells in mitotic cycles. By contrast, lower levels of TIR1-AUX/IAA-ARF-dependent auxin signalling trigger an exit from mitotic cycles and an entry into endocycles. Our data further demonstrate that this auxin-mediated modulation of the mitotic-to-endocycle switch is tightly coupled with the developmental transition from cell proliferation to cell differentiation in the *Arabidopsis* root meristem. The transient reduction of auxin signalling by an auxin antagonist PEO-IAA rapidly downregulates the expression of several core cell cycle genes, and we show that overexpressing one of the genes, *CYCLIN A2;3* (*CYCA2;3*), partially suppresses an early initiation of cell differentiation induced by PEO-IAA. Taken together, these results suggest that auxin-mediated mitotic-to-endocycle transition might be part of the developmental programmes that balance cell proliferation and cell differentiation in the *Arabidopsis* root meristem.

KEY WORDS: Auxin, Mitotic cell cycle, Endocycle, Cell expansion, Cell differentiation

INTRODUCTION

The development of multicellular organisms requires mechanisms that pattern cell proliferation and cell differentiation with temporal and spatial precision. The requirement of such patterning strategies is, for example, evident in plant root meristems, where stem cells undergo cell division and differentiation in a highly ordered fashion (De Veylder et al., 2007; Dello Ioio et al., 2008a). It is now well established that the plant hormone auxin forms concentration gradients in developing plant organs and that different cellular auxin levels are translated into distinct cellular responses, such as cell proliferation, cell expansion and cell differentiation (Benjamins and Scheres, 2008; Vanneste and Friml, 2009). Recent studies have demonstrated that both local auxin biosynthesis and intercellular auxin transport contribute to the establishment and maintenance of active auxin gradients (Vanneste and Friml, 2009; Zhao, 2008). Central players of the auxin polar transport are the group of auxin efflux carrier proteins encoded by the *PIN* gene family (Galweiler et al., 1998; Paponov et al., 2005). Genetic analyses of multiple *pin* mutants illustrate that the combinatory actions of several PIN proteins play pivotal roles in producing auxin gradients in the *Arabidopsis* root meristem, with the maximum concentration being located in the quiescent centre and young columella cells (Blilou et al., 2005). These *pin* mutants also display smaller root meristems, demonstrating the requirement of active auxin distribution in maintaining the root meristem (Blilou et al., 2005). Other key regulators crucial for the development of *Arabidopsis* root meristems are the members of the *PLETHORA* (*PLT*) gene family, which encode AP2-domain transcription factors, and which also operate in a concentration-dependent manner to maintain the balance between cell proliferation and cell differentiation (Aida et al., 2004; Galinha et al., 2007). The PLT proteins act redundantly to control the expression of the *PIN* genes (Galinha et al., 2007) while, in turn, several PIN proteins together modulate the expression of the *PLT* genes (Blilou et al., 2005), pointing to a robust feedback mechanism to sustain the dynamic auxin gradients. Recent studies have also uncovered that another class of plant hormones, the cytokinins, acts antagonistically to auxin to stimulate cell differentiation in the *Arabidopsis* root meristem (Dello Ioio et al., 2007). The proposed model suggests that this process is mediated through the AHK3/ARR1 two-component signalling pathway downregulating *PIN* expression and thus normal auxin transport (Dello Ioio et al., 2008b; Ruzicka et al., 2009).

Cellular auxin levels are first perceived by the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN-SIGNALING F BOX (AFB)-class of F-box proteins that function as auxin receptors (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). At high concentrations, auxin is thought to stabilise the interaction between TIR1/AFB and AUXIN (AUX)/INDOLE-3-ACETIC ACID (IAA) transcriptional repressors that constitute the core auxin signalling pathway together with auxin response factor (ARF) transcription factors (Mockaitis and Estelle, 2008). The auxin-dependent stabilisation of TIR1/AFB-AUX/IAA interaction promotes the ubiquitination of AUX/IAA by $SCF^{TIR1/AFB}$ E3 ligases and their subsequent proteolysis, resulting in an activation of ARF transcription factors and thus an up- or downregulation of their target gene expression. As auxin concentrations decrease, AUX/IAA become more stable and increase their interactions with ARF to repress their transcriptional activities. What remains to be established is how these relatively short auxin signalling cascades mediate so many different cellular responses, including cell proliferation, cell expansion and cell differentiation, which presumably involve the activation or repression of completely different sets of genes and subsequent cellular activities.

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The *Arabidopsis* genome encodes 29 AUX/IAA and 23 ARF (Mockaitis and Estelle, 2008), and the current challenge is to test how far numerous different combinations of AUX/IAA-ARF heterodimers, potentially formed under different developmental or environmental conditions, can account for the diverse transcriptional responses to auxin.

Another key cellular event that often accompanies the switch from cell proliferation to cell differentiation in growing plant organs is the transition from the mitotic cell cycle to an alternative cell cycle, called the endoreduplication cycle or endocycle. Cells undergo sequential phases of DNA replication and cell division in the mitotic cycle, whereas in the endocycle cells replicate genomic DNA without intervening cell divisions, thus leading to an increase in the total nuclear DNA content, ploidy, within a cell. In *Arabidopsis*, most postembryonic cells, except those in meristems, guard cell lineages or meiotic tissues, endoreduplicate several times and their ploidy levels usually reach 16C to 32C (Galbraith et al., 1991). Exact functions of endoreduplication have not been established, but the correlation that is often found between the level of endocycles and cell size has suggested that an increase in ploidy by endocycling plays an important role in cell expansion and/or cell differentiation (Inze and De Veylder, 2006; Nagl, 1978; Sugimoto-Shirasu and Roberts, 2003). Ectopic induction of endocycling in *Arabidopsis* meristem cells causes severe developmental defects, such as small and abnormally shaped leaves (Verkest et al., 2005), clearly demonstrating that tight controls over the mitotic-to-endocycle transition are prerequisites for normal postembryonic plant development. As in all other eukaryotes, the progression of mitotic cycles in plants is controlled by cyclin-dependent kinases (CDKs) and their interacting partners, cyclins (CYCs), which regulate the kinase activity of CDKs. Plants possess two classes of CDKs that directly control the cell cycle, CDKA, which has functional orthologues in yeasts, and CDKB, which has been identified only in the plant kingdom. Previous studies implicate CDKA for both the G1-to-S and the G2-to-M transition, whereas CDKB appears to function only for the G2-to-M transition (Inze and De Veylder, 2006). The switch from the mitotic cycle to the endocycle usually involves the downregulation of CDK activities at the G2-to-M transition to skip mitoses while still permitting DNA replication (Edgar and Orr-Weaver, 2001; Larkins et al., 2001). Accumulating evidence suggests that the downregulation of both CDKA and CDKB is vital to promote this transition in plants (De Veylder et al., 2007; Inze and De Veylder, 2006). Recent molecular and genetic studies have identified several key regulators, such as E2F/DEL transcription factors and Kip-related proteins (KRPs) that modulate the expression and/or activities of these mitotic CDKs, respectively (De Veylder et al., 2007; Inze and De Veylder, 2006). Given that cells often transit into endocycles at well-defined time points and positions during plant development (Beemster et al., 2005), the decision of endocycle initiation is likely to be developmentally programmed. The candidates that can provide such developmental cues include various plant hormones, and one of the most direct ways in which they might work is that such upstream regulators modulate the expression and/or activities of the core cell cycle regulators to initiate the endocycle. To date, however, very little is established about the identities these upstream regulators and how they function in the cell cycle transition.

In this study we demonstrate that TIR1-AUX/IAA-ARF-mediated auxin signalling acts as a repressor of the mitotic-to-endocycle transition in *Arabidopsis*. We show that mutations in auxin biosynthesis, transport or signalling all lead to an early transition into endocycling. Our physiological experiments using exogenous auxin and its antagonists demonstrate that lowering auxin levels is sufficient to promote an entry into endocycles both in planta and in vitro cell culture systems. Reduced auxin signalling rapidly downregulates the expression of several mitotic cycle genes and their upregulation can partially override the premature cell differentiation under low auxin conditions. These data strongly suggest that transcriptional repression of the mitotic cycle regulators constitutes an early cellular response to modified auxin signalling with downstream consequences in cell cycle transition and cell differentiation.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis mutants and transgenic lines used in this study were provided by Eva Benkova [*monopteros* (*mp*) mutants (Hardtke and Berleth, 1998), *bodenlos* (*bdl*) mutants (Hamann et al., 2002), *auxin resistant 2-1* (*axr2-1*) mutants (Nagpal et al., 2000), *HS:axr3-1* lines (Knox et al., 2003), and *pinoid* (*pid*) mutants (Friml et al., 2004)], Hiroo Fukuda [*vascular network 3* (*van3*) mutants (Koizumi et al., 2005)], Yunde Zhao [*yucca 1* (*yuc1*) *yuc4 yuc10 yuc11* mutants (Cheng et al., 2007)], Ben Scheres [Histone 2B (H2B)-YFP and LTI6B-GFP lines (Campilho et al., 2006)], Dirk Inze [*CDKA;1pro:GUS* lines (Hemerly et al., 1993)], Takashi Aoyama [*CYCA2;3pro:GUS* and CYCA2:3-GFP lines (Imai et al., 2006)], and Tatsuo Kakimoto [*isopentenyltransferases 3* (*ipt3*) *ipt5 ipt7* mutants (Miyawaki et al., 2006)]. The transgenic lines with *CDKB2;1pro:GUS* or *CYCB1;1-GUS* were previously described by Adachi et al. (Adachi et al., 2006) and Breuer et al. (Breuer et al., 2007). The *mp*, *bdl* and *van3* mutants were in Lansdberg *erecta* (L*er*) ecotype and *axr2-1*, *HS:axr3-1*, *pid*, *yuc1 yuc4 yuc10 yuc11* and *ipt3 ipt5 ipt7* mutants were in Columbia (Col) ecotype. Seeds were surface sterilized in 70% ethanol for 1 minute, then in 20% (v/v) sodium hypochlorite for 5 minutes, and rinsed three times in sterile water. Sterilized seeds were plated on Murashige and Skoog (MS) media supplemented with 1% (w/v) sucrose and 0.5% Gelrite. After cold treatment in the dark for 2 days, seeds were incubated under continuous light at 22°C. For the ploidy and cell-size analysis of leaves, seedlings were grown on horizontally placed plates. For the ploidy and cell-size analysis of roots, seedlings were grown on vertically placed plates.

Arabidopsis MM2d cells were cultured in MS media supplemented with 200 mg/l potassium dihydrogenphosphate, 100 mg/l myo-inositol, 1 mg/l thiamine hydrochloride, 3% (w/v) sucrose, and 0.2 mg/l 2,4-D (pH 5.8). The MM2d cell culture was incubated at 27°C in continuous darkness and subcultured every 7 days.

For chemical treatments, 1-naphthaleneacetic acid (NAA), BH-IAA [also referred to as probe-8 in Hayashi et al. (Hayashi et al., 2008b)], PEO-IAA and 6-Benzylaminopurine (6-BA) were dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 1 mM, 100 mM, 50 mM and 0.1 mM, respectively, and diluted into the MS medium.

To induce transient expression of *axr3-1* from the heat shock promoter, transgenic plants harbouring the *HS:axr3-1* construct were first incubated at 22°C on the MS media. At 4 days after germination, plants were transferred to the 40°C growth chamber for 1 hour and returned to the 22°C growth chamber thereafter. This 1-hour heat-shock treatment was applied every 12 hours and plants were harvested for observation at various time points from 2 days after the first heat-shock treatment.

Ploidy measurement

Ploidy levels were measured using the ploidy analyzer PA-I (Partec) as described previously (Sugimoto-Shirasu et al., 2002). Briefly, nuclei were released in Cystain extraction buffer (Partec) from fresh cotyledons or true leaves by chopping lightly with a razor blade, filtered through a CellTrics filter (Partec), and stained with Cystain fluorescent buffer (Partec). At least 5000 nuclei isolated from ~30 cotyledons or true leaves were used for each ploidy measurement. Flow cytometry experiments were repeated at least three times for each genotype using independent biological replicates.

Microscopy

For the visualization of nuclei in cotyledons, 7-day-old seedlings were fixed following the protocol described by Sugimoto et al. (Sugimoto et al., 2000) and stained with 4′,6-diamidino-2-phenylindole (DAPI). Root nuclei were

visualised as above or by using transgenic plants harbouring H2B-YFP fusion protein constructs. Nuclei in the *Arabidopsis* MM2d cell culture were fixed in methanol:acetic acid (3:1) and stained with DAPI. Root meristem organisations were visualised using seedlings stained with 5 mg/ml propidium iodide or in transgenic plants harbouring LTI6B-GFP fusion protein constructs. Samples were observed using either an Olympus BX51 fluorescence microscope or a Carl Zeiss LSM510 META confocal laser microscope. To estimate the initiation of endocycling and cell expansion in the root meristem, three to six confocal optical sections, which were collected at approximately 1-μm intervals using identical confocal settings, were merged. Surface areas of individual nuclei and lengths of individual cells within same epidermal cell files were measured using ImageJ software (NIH).

Histochemical β-glucuronidase (GUS) staining of roots was performed as described by Ishida et al. (Ishida et al., 2009).

RESULTS

Mutations in auxin biosynthesis, transport or signalling lead to an early transition from the mitotic cycle to the endocycle

Auxin forms active concentration gradients in developing plant organs to promote various cellular processes, including cell proliferation, cell expansion and cell differentiation (Benjamins and Scheres, 2008; Vanneste and Friml, 2009). To investigate an involvement of auxin in regulating the switch from the mitotic cycle to the endocycle, we first tested whether a disruption of auxin signalling results in an impaired mitotic-to-endocycle transition. The ARF5/MP transcription factor promotes the expression of auxin response genes, and loss-of-function mutations in the *MP* gene severely disrupt auxin signalling (Hardtke and Berleth, 1998). Our flow cytometry analysis showed that at 7 days after germination, the ploidy level of approximately 40% of nuclei in cotyledons remained at 2C or 4C, whereas all other nuclei progressed through several rounds of endocycling, allowing them to reach 8C or 16C (Fig. 1A). By contrast, the proportion of both 2C and 4C nuclei was severely reduced in 7-day-old *mp* mutant cotyledons (Fig. 1A), implying an early exit from the mitotic cycle. The *mp* mutants also displayed significantly increased 16C and 32C ploidy peaks (Fig. 1A), suggesting the promotion of successive endocycling in *mp* mutants. By 14 days, *mp* mutant cells had undergone further rounds of endocycling, giving rise to 64C and 128C nuclei that are not normally present in wild-type plants at the same developmental stage (Fig. 1B). Furthermore, the ploidy distribution resulting from gain-of-function mutations of *IAA7/AXR2*, *IAA17/AXR3* or *IAA12/BDL* genes, which cause constitutive repression of auxin signalling (Hamann et al., 2002; Nagpal et al., 2000; Rouse et al., 1998), was similar to that observed in *mp* mutants, demonstrating a decrease in 2C and 4C peaks and an increase in 32C, 64C and, in some cases, 128C and 256C peaks (Fig. 1B). Consistent with these altered ploidy distributions, visualisation of DAPI-stained nuclei revealed the presence of >32C nuclei in 7-day-old cotyledon epidermal cells of *mp*, *bdl*, *axr2-1* and *axr3-1* mutants (Fig. 1C).

To further substantiate the role of auxin in mediating the switch from mitotic cycles to endocycles, we examined the ploidy distribution of plants defective in auxin biosynthesis or its transport. The flavin monooxygenases encoded by the *YUC* gene family are key enzymes involved in tryptophan-dependent auxin production (Zhao et al., 2001), and correspondingly *yuc1 yuc4 yuc10 yuc11* quadruple mutants display pleiotropic auxin deficient phenotypes (Cheng et al., 2007). Our flow cytometry revealed that *yuc1 yuc4 yuc10 yuc11* mutants displayed reduced 2C and 4C peaks and increased 64C and 128C peaks (see Fig. S1 in the supplementary material). Consistently, loss-of-function mutations in *PID* and *VAN3*, both of which result in impaired auxin distribution and response (Friml et al., 2004; Koizumi et al., 2005), caused similar ploidy alterations (Fig. 1B).

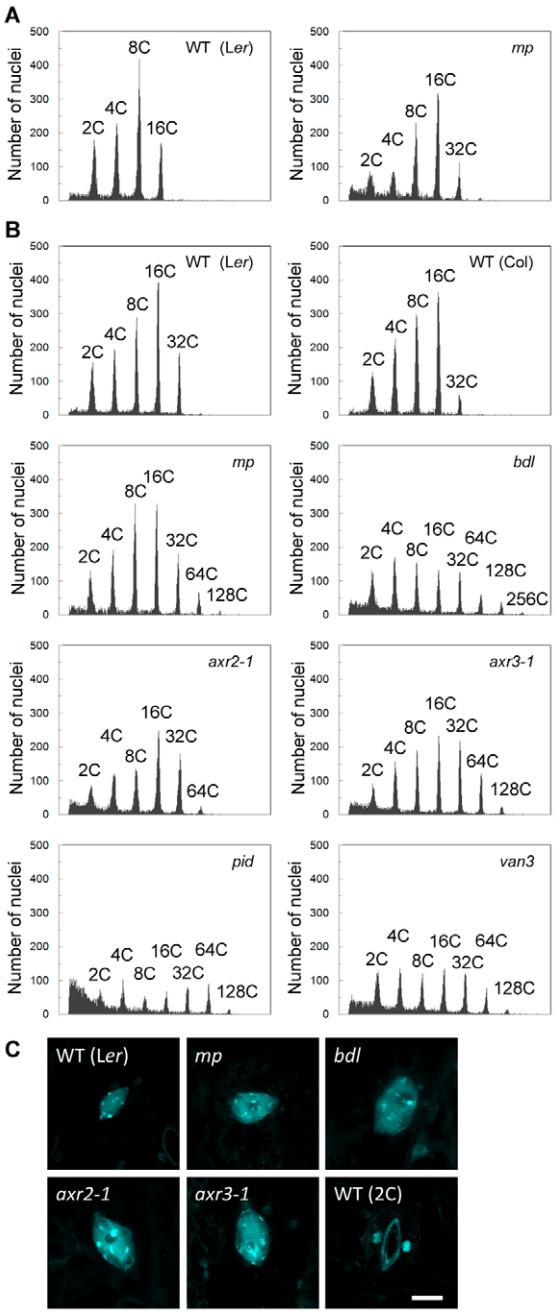

Fig. 1. Mutations in auxin signalling or transport lead to an early withdrawal from the mitotic cycle and the promotion of successive endocycles. (**A**) Flow cytometric analysis of 7-day-old wild-type (WT) L*er* and *monopteros* (*mp*, L*er* ecotype) cotyledons. (**B**) Flow cytometric analysis of 14-day-old wild-type Col, wild-type L*er*, *mp*, *bodenlos* (*bdl*, L*er* ecotype), *auxin resistant 2-1* (*axr2-1*, Col ecotype), *auxin resistant 3-1* (*axr3-1*, Col ecotype), *pinoid* (*pid*, Col ecotype) and *vascular network 3* (*van3*, L*er* ecotype) cotyledons. All flow cytometric analyses were repeated with at least three independent biological replicates and representative ploidy distributions are shown for each genotype. (**C**) Confocal imaging of DAPI-stained nuclei in 7-day-old wild-type L*er*, *mp*, *bdl*, *axr2-1* and *axr3-1* cotyledon epidermal cells. The nuclei in a pair of wild-type stomata cells represent 2C, suggesting that the nucleus shown for wild type is approximately 16C, whereas those in *mp*, *bdl*, *axr2-1* and *axr3-1* are at least 32C. Presented images show nuclei representing the most highly endoreduplicated nuclei populations for each genotype. Strongly stained dots in each nucleus correspond to centromeres. Scale bars: 10 μm.

Lowering auxin levels is sufficient to promote entry into the endocycle in postembryonic cells

Our data suggest that low levels of auxin signalling promote entry into endocycles. Given the major role that auxin plays in the cell fate specification during the *Arabidopsis* embryogenesis, however, it is also possible that impaired cell specification and/or pattern formation in mutant embryos indirectly leads to a premature exit from the mitotic cycle. To distinguish between these possibilities, we determined whether post-embryonic manipulation of auxin levels could directly modify the cell cycle transition. Externally added auxin is known to promote cell proliferation in plants. Consistently, more than 90% of 14-day-old wild-type leaf cells grown in the presence of synthetic auxin NAA (1 μM) contained 2C or 4C nuclei, whereas at least 20% of cells grown without NAA switched into the endocycle, reaching 8C or 16C (Fig. 2A). Within 2 days of removing NAA from the growth media, however, the proportion of 2C and 4C nuclei started decreasing, and by 4 days more than 30% of leaf cells possessed 8C or 16C nuclei, whereas only ~10% of cells reached 8C in leaves that remained exposed to NAA (Fig. 2B,C). These observations are consistent with our hypothesis that high auxin levels repress the transition into the endocycle; however, they do not exclude the possibility that the delayed entry into endocycling is an indirect consequence of other developmental changes caused by auxin. To test whether blocking auxin signalling directly converts mitotic cells into endocycling cells, we used *Arabidopsis* MM2d suspension culture cells, which normally possess 6C nuclei in G1 phase (Menges and Murray, 2002). As shown in Table 1, 100% of mock-treated MM2d culture cells remained in the mitotic cycle for up to 5 days, carrying only 6C and 12C nuclei. By contrast, ~10% of cells treated with an auxin antagonist BH-IAA (50 μM), which specifically blocks TIR1-mediated auxin signalling (Hayashi et al., 2008b), entered the endocycle and increased their ploidy to 24C. Consistently, our microscopic observation demonstrated that all of the cells we examined in mock-treated MM2d cell culture possessed nuclei of a relatively uniform size, which presumably corresponds to 6C or 12C, whereas a small but significant proportion (approximately 13%, n=240) of BH-IAA-treated cells possessed 24C nuclei (Fig. 3). *Arabidopsis* MM2d culture cells tend to aggregate to form large cell clusters (Menges and Murray, 2002), and cells with 24C nuclei in BH-IAA-treated cell culture were found only at the periphery of these cell clusters (Fig. 3), suggesting that the intrinsic heterogeneity of these cell culture might contribute to the relatively low frequency of 24C nuclei. Together, these results strongly suggest that lowering auxin signalling directly initiates endocycling in *Arabidopsis* postembryonic cells.

Auxin-mediated progression of endocycling is tightly coupled with cell differentiation

The final step of cell differentiation in plants is often marked by a rapid increase in cell size by cell expansion (De Veylder et al., 2007; Dello Ioio et al., 2008a). We, therefore, tested whether auxin-mediated progression of endocycling is coupled with such postmitotic cell expansion. Application of 1 μM NAA strongly repressed the expansion of *Arabidopsis* leaf mesophyll cells, and within 2 days of removing NAA from the growth media cells started to increase their size (Fig. 2D). By 6 days, cells grown in the absence of NAA were approximately three times larger than those that were left exposed to 1 μM NAA (Fig. 2D-F). Similarly, we found that BH-IAA-treated MM2d cells that had 24C nuclei had a significantly larger cell size (1037±234.5 μm^2, n=10) compared with cells with 6C or 12C nuclei (266.4±76.7 μm^2, n=42; Fig. 3). These data clearly demonstrate that auxin-dependent endocycling is tightly coupled with postmitotic cell expansion in *Arabidopsis*.

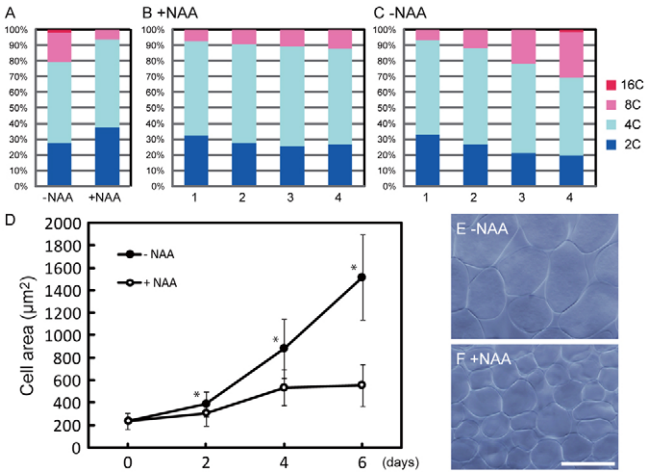

Fig. 2. Lowering the auxin level is sufficient to promote entry into the endocycle and accompanying cell expansion in the *Arabidopsis* leaf. (**A-C**) Flow cytometric analysis of (A) 14-day-old wild-type seedlings grown in the absence (–) or presence (+) of 1 μM NAA, (B) wild-type seedlings that were transferred from NAA-containing media to fresh NAA-containing media at 14 days, (C) wild-type seedlings that were transferred from NAA-containing media to NAA-free media at 14 days. Flow cytomeric analyses were performed using at least 30 sets of first true leaves harvested 1 to 4 days after transferring seedlings. The values shown represent averages of two separate experiments using independent biological replicates. (**D**) Cell surface area of leaf mesophyll cells. Wild-type seedlings were transferred from NAA-containing media to fresh NAA-containing media (open circles) or NAA-free media (closed circles). Leaf mesophyll cells in the basal region of primary leaves were photographed at 0, 2, 4 and 6 days after transfer, and their surface areas were measured using ImageJ software (NIH). Values are means ± s.d (n=40). Asterisks indicate a significant difference between treatments (P<0.005 for day 2, P<0.001 for day 4 and 6). (**E**,**F**) Differential interference contrast micrographs of leaf mesophyll cells grown in the (E) absence or (F) presence of 1 μM NAA. The images, taken after 6 days of transferring seedlings, show cell size characteristic to mesophyll cells in the basal region of primary leaves.

High levels of auxin signalling prevent an early onset of endocycling and accompanying cell expansion in the *Arabidopsis* root meristem

Auxin concentration gradients direct pattern formation in *Arabidopsis* roots (Benjamins and Scheres, 2008; Vanneste and Friml, 2009). A maximum auxin concentration in the root stem-cell niche promotes cell proliferation, whereas in the region distal to the maximum, where lower auxin levels are predicted, cells expand and differentiate (Blilou et al., 2005; Sabatini et al., 1999). To test whether auxin-mediated modulation of the mitotic-to-endocycle switch accompanies these developmental transitions, we determined whether lowering auxin levels triggers an early entry into endocycling at the root meristem. Compromised auxin gradients reduce the root meristem size (Blilou et al., 2005) and, correspondingly, our confocal microscopy revealed that the first endocycle initiated closer to the quiescent centre in wild-type roots with H2B-YFP (Campilho et al., 2006) treated with either 50 μM BH-IAA (data not shown) or another auxin antagonist PEO-IAA (10 μM) which also exhibits specific inhibition of TIR1-mediated auxin signalling (Hayashi et al., 2008a) (Fig. 4). Consistently, we found that root meristem cells transited into endocycling prematurely in *axr3-1* roots (Fig. 4). In addition,

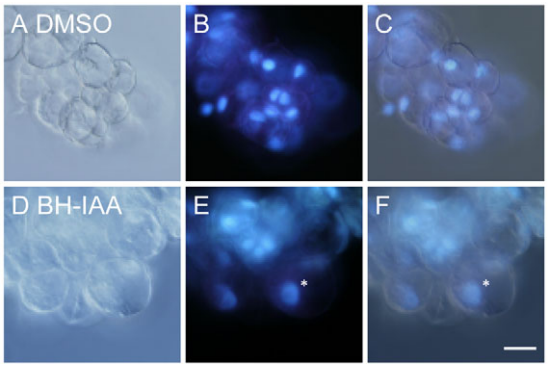

Fig. 3. Blocking auxin signalling with an auxin antagonist, BH-IAA, induces endocycling and accompanying cell expansion in the *Arabidopsis* MM2d cell culture. (**A-F**) A 3-day-old MM2d cell culture was transferred to 2,4-D-free MS media supplemented with (A-C) dimethyl sulfoxide (DMSO) and (D-F) 50 μM BH-IAA. (A,D) Application of 50 μM BH-IAA induces cell expansion in the MM2d cell culture. (B,E) Visualisation of DAPI-stained nuclei shows that a subpopulation of BH-IAA-treated cells (approximately 13%, *n*=240) contains 24C nuclei, marked by an asterisk (*), that are not present in DMSO-treated cells. (C,F) Merged images of A and D, and B and E, respectively. All images were taken after 5 days of BH-IAA application. Scale bar: 20 μm.

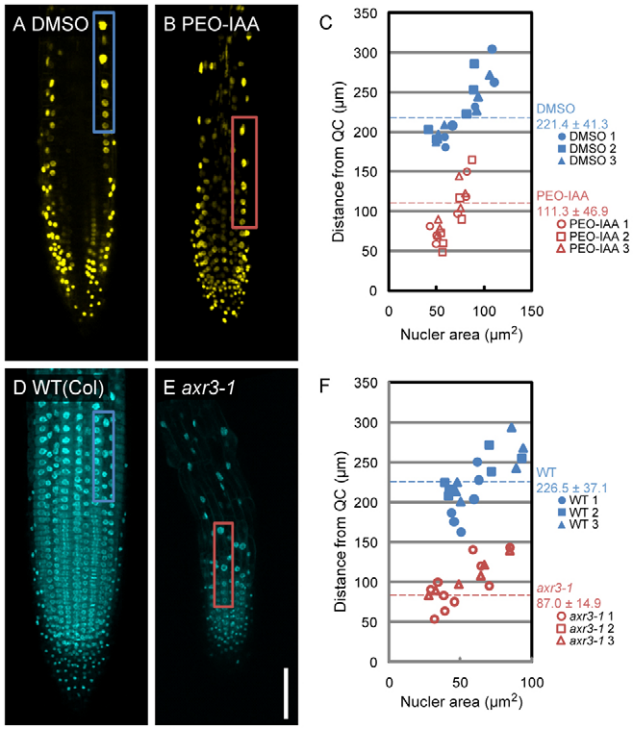

Fig. 4. Disruption of auxin signalling results in the early onset of endocycling in the *Arabidopsis* root meristem. (**A**,**B**) Confocal microscopy of nuclear surface area as visualised by H2B-YFP fusion proteins. Representative images for 6-day-old wild-type roots treated with (A) DMSO and (B) the auxin antagonist PEO-IAA (10 μM) are shown. (**D**,**E**) Confocal microscopy of DAPI-stained nuclei in 6-day-old (D) wild-type roots and (E) *axr3-1* roots. Scale bar: 50 μm. (**C**,**F**) Quantification of nuclear surface area. Nuclear area, defined as total pixel intensities of (C) H2B-YFP or (F) DAPI signals per individual nuclei, was quantified for up to six consecutive cells within the same epidermal layers (e.g. those indicated by blue or red boxes in A,B,D and E), and plotted against distance from the quiescence centre (QC). Data collected from three different biological samples (1,2,3) are shown for 6-day-old (C) DMSO- or PEO-IAA-treated wild-type roots, and (F) wild-type and *axr3-1* roots. Blue or red dotted lines indicate the average position of the first endocycles, as estimated by the sharp increase in measured nuclear area (*n*=3-11).

visualisation of the propidium iodide (PI)-stained root meristem structures showed that blocked auxin signalling induced an early postmitotic cell expansion in both PEO-IAA-treated wild-type roots and *axr3-1* roots (see Fig. S2 in the supplementary material). These cellular responses are strictly dependent on auxin deprivation, as the transient induction of dominant-negative *axr3-1* mutations by a heat shock promoter (Knox et al., 2003) was sufficient to induce premature endocycling and cell expansion (Fig. 5). These results indicate that auxin is required to prevent an early onset of endocycling and accompanying cell expansion in the *Arabidopsis* root meristem.

High levels of cytokinins promote the onset of endocycling in the *Arabidopsis* root meristem

Cytokinins regulate the root meristem size through modulating polar auxin transport (Dello Ioio et al., 2008b; Ruzicka et al., 2009). The application of cytokinins promotes an early transition from cell proliferation to cell differentiation in the *Arabidopsis* root meristem, whereas mutations in cytokinin biosynthesis or signalling delay this transition. To test whether cytokinins also impact the mitotic-to-endocycle transition, we examined whether the application of exogenous cytokinins (e.g. 6-BA) modifies the timing of endocycle onset. As shown in Fig. S3A-C in the supplementary material, 0.1 μM 6-BA induced endocycling closer to the quiescent centre compared with mock-treated wild-type roots. Conversely, entry into endocycling was significantly delayed in *atipt3 atipt5 atipt7* triple mutants, which have mutations in ATP/ADP isopentenyltransferases which are involved in the biosynthesis of cytokinins (Miyawaki et al., 2006) (see Fig. S3D-F in the supplementary material). These results suggest that cytokinins act antagonistically to auxin in mediating the mitotic-to-endocycle transition in the *Arabidopsis* root.

Table 1. Flow cytometric analysis of *Arabidopsis* MM2d cells treated with the auxin antagonist BH-IAA

Treatment	Days after treatment	6C	12C	24C
DMSO	0	62.2±0.9%	37.8±0.9%	0.0±0.0%
	1	66.1±2.6%	33.9±2.6%	0.0±0.0%
	2	77.0±3.4%	23.0±3.4%	0.0±0.0%
	3	77.8±0.8%	22.2±0.8%	0.0±0.0%
	4	79.8±2.5%	20.2±2.5%	0.0±0.0%
	5	81.3±2.5%	18.7±2.5%	0.0±0.0%
BH-IAA	1	52.7±7.5%*	47.3±7.5%*	0.0±0.0%
	2	52.8±5.3%*	47.2±5.3%*	0.0±0.0%
	3	53.2±2.5%*	39.1±0.2%*	7.8±2.6%*
	4	52.4±5.8%*	35.5±1.4%*	12.1±7.1%*
	5	55.6±3.0%*	34.6±1.8%*	9.8±4.0%*

Values are averages ± s.d. of three independent experiments. Asterisks (*) indicate a significant difference between DMSO- and BH-IAA-treated cells ($P<0.05$).

Transient reduction in auxin signalling rapidly downregulates the expression of mitotic cell cycle genes

To gain insight into the molecular basis underlying the auxin-mediated endocycle transition, we tested whether a transient reduction in auxin signalling modifies the expression of mitotic cell cycle genes.

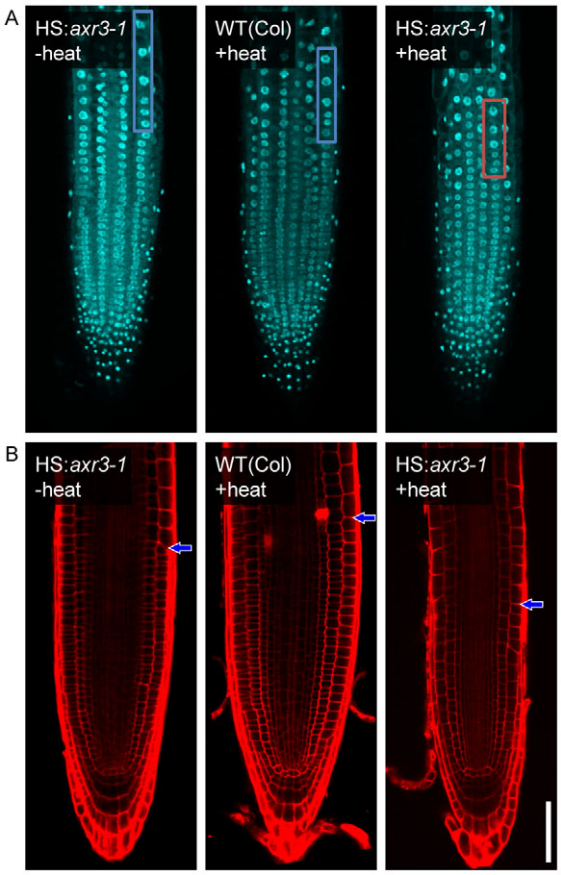

Fig. 5. Transient induction of dominant-negative *axr3-1* mutations by a heat shock promoter is sufficient to induce premature endocycling and cell expansion in the *Arabidopsis* root meristem. (**A**) Confocal microscopy of DAPI-stained nuclei in the root meristem cells. Images are stacks of 3-6 confocal optical sections that include nuclei in several adjacent epidermal cell files. Blue or red boxes indicate the position of the first endocycles, as estimated by the sharp increase in nuclear surface area. (**B**) Cellular organisation of the root meristem visualised by propidium iodide staining. Blue arrows mark the initiation of cell expansion in epidermal cell layers. Images represent *HS:axr3-1* roots without heat shock (–heat, left), wild-type roots, 2 days after heat shock (+heat, centre), and *HS:axr3-1* roots, 2 days after heat shock (+heat, right). Scale bar: 100 μm.

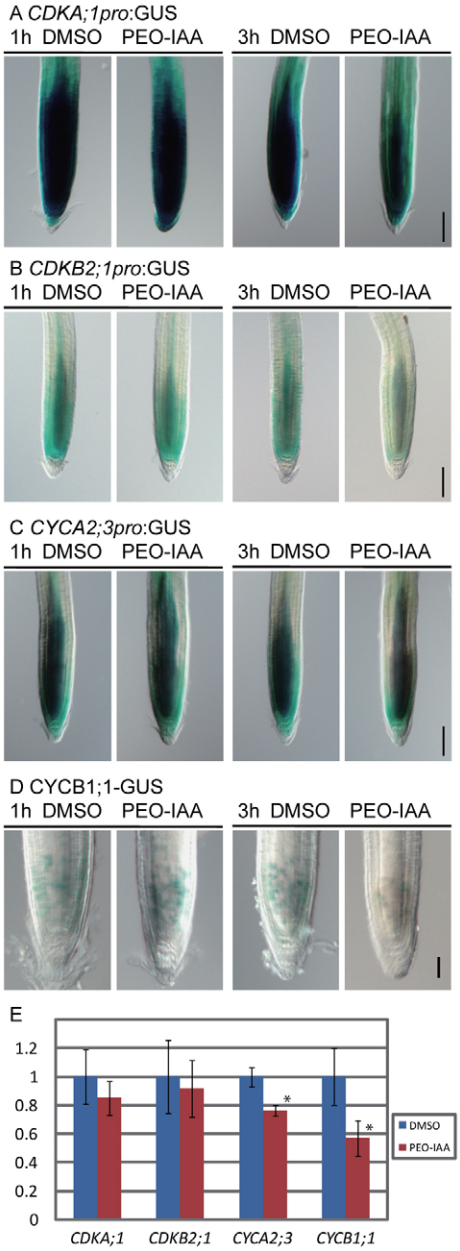

Fig. 6. Blocking auxin signalling with PEO-IAA rapidly downregulates the expression of mitotic cell cycle genes. (**A-D**) Differential interference contrast (DIC) microscopy of root meristems carrying (A) *CDKA;1pro:GUS* constructs, (B) *CDKB2;1pro:GUS* constructs, (C) *CYCA2;3pro:GUS* constructs, and (D) *CYCB1;1-GUS* constructs. Six-day-old seedlings were transferred to growth media with either DMSO or 20 μM PEO-IAA and fixed in 90% acetone after either 1 or 3 hours. (**E**) Quantitative RT-PCR of *CDKA;1*, *CDKB2;1*, *CYCA2;3* and *CYCB1;1* expression in 6-day-old roots treated with DMSO or 20 μM PEO-IAA for 3 hours. Mean values of three repeats, relative to those of DMSO-treated samples, are shown ±s.d. Asterisks indicate significant differences between DMSO- and PEO-IAA-treated roots (Student's *t*-test, $P<0.05$). Scale bars: 100 μm in A,B,C; 50 μm in D.

Transgenic plants carrying *CDKA;1pro: GUS* constructs (Hemerly et al., 1993) or *CDKB2;1pro:GUS* constructs (Adachi et al., 2006) displayed strong GUS activities in 6-day-old *Arabidopsis* root meristems (Fig. 6A,B), reflecting their high promoter activities. We found that reducing auxin signalling by the application of 20 μM PEO-IAA led to a rapid reduction of these GUS signals. The reduced GUS activities were detectable within 1 hour of PEO-IAA treatment, and by 3 hours the signal intensities were greatly weakened throughout the meristem (Fig. 6A,B). Importantly, the root meristem size was not yet reduced by 3 hours (Fig. 6A,B), indicating that these modified GUS activities are not due to the compromised meristems. The GUS activities driven by the *CYCA2;3* promoter (Imai et al., 2006) were also strong in the root meristems but were significantly reduced within 3 hours of PEO-IAA treatment (Fig. 6C).

We also examined the effect of PEO-IAA on the accumulation level of mitotic cyclin CYCB1;1 by using transgenic lines carrying *CYCB1;1-GUS* constructs in which the expression of CYCB1;1-GUS fusion proteins is driven by the *CYCB1;1* promoter (Colon-Carmona et al., 1999). As shown in Fig. 6D, CYCB1;1-GUS signals are normally detected in a punctate pattern across the root meristem. Both the signal intensity and the number of CYCB1;1-GUS-positive

cells appeared to be comparable between mock-treated roots and PEO-IAA-treated roots within the first 1 hour of PEO-IAA treatment but by 3 hours the number of CYCB1;1-GUS-positive cells dropped substantially in PEO-IAA-treated roots (Fig. 6D). Our quantitative RT-PCR analysis, using RNA isolated from 6-day-old roots, further supported that the inhibition of auxin responses by PEO-IAA causes significant reduction in the expression of *CYCA2;3* and *CYCB1;1* genes by 3 hours (Fig. 6E). These results strongly suggest that the transcriptional and/or post-transcriptional repression of these cell cycle regulators is a part of the early cellular responses to reduced auxin signalling in the *Arabidopsis* root meristem.

Overexpression of *CYCA2;3-GFP* partially suppresses the early initiation of cell differentiation in PEO-IAA-treated roots

Recent studies using transgenic lines harbouring the β-oestradiol-inducible *CYCA2;3-GFP* overexpression construct show that CYCA2;3 is one of the key mitotic cycle regulators that suppress endocycle onset (Imai et al., 2006; Boudolf et al., 2009). Consistently, we found that both entry into endocycling and the initiation of postmitotic cell expansion were moderately delayed in 6-day-old *CYCA2;3-GFP* roots grown in the presence of 10 μM β-oestradiol from germination (see Fig. S4 in the supplementary material). To test whether the downregulation of mitotic cycle genes was responsible for the early transition into cell differentiation under low auxin conditions, we transiently induced *CYCA2;3-GFP* expression by 1-day exposure to β-oestradiol in the absence or presence of PEO-IAA. As shown in Fig. 7, 1-day incubation of 5-day-old *CYCA2;3-GFP* roots with 10 μM β-oestradiol did not modify meristem size in the absence of PEO-IAA. By contrast, 1-day co-treatment of *CYCA2;3-GFP* roots with both β-oestradiol and PEO-IAA partially overrode the premature initiation of postmitotic cell expansion induced by PEO-IAA (Fig. 7). These results support that an early transcriptional change of cell cycle genes can provide an instructive signal for cell differentiation in the *Arabidopsis* root.

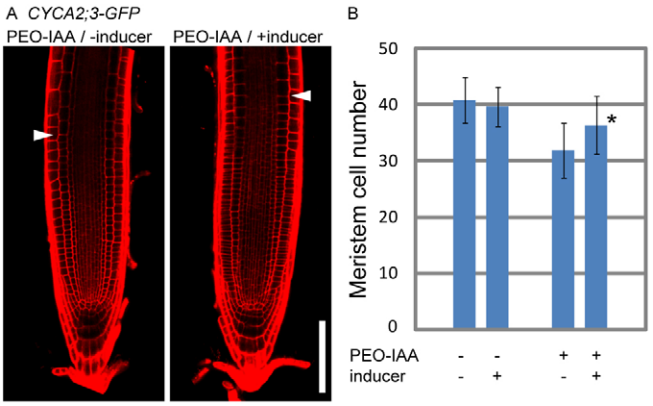

Fig. 7. An overexpression of *CYCA2;3-GFP* partially suppresses premature postmitotic cell expansion in PEO-IAA-treated roots. (**A**) Cellular organisation of the root meristem visualised by propidium iodide staining. Representative images for 6-day-old *CYCA2;3-GFP* roots are shown after a 1-day exposure to 10 μM PEO-IAA alone (–inducer, left) or to PEO-IAA plus 10 μM β-oestradiol (+inducer, right). Arrowheads mark the initiation of postmitotic cell expansion in cortex cell layers. (**B**) Quantitative analysis of meristem cell number. The number of meristem cells was counted in 6-day-old *CYCA2;3-GFP* roots after a 1-day incubation in the presence (+) or absence (–) of 10 μM PEO-IAA and/or 10 μM β-oestradiol. Data are shown as averages ± s.d. (*n*=6-8) and an asterisk indicates a significant difference between PEO-IAA-treated roots and those co-treated with PEO-IAA and β-oestradiol (Student's *t*-test, $P<0.05$). Scale bar: 100 μm.

DISCUSSION

Auxin mediates the mitotic-to-endocycle transition

We have demonstrated that auxin has a physiological role in coordinating the developmental transition from the mitotic cycle to the endocycle in *Arabidopsis*. Our model (see Fig. S5 in the supplementary material) predicts that high auxin levels, e.g. at the proximal root meristem, serve to repress endocycles, thus promoting the mitotic phase. By contrast, as cells depart from the meristem, cellular auxin levels are lowered and thus the repression is lifted, allowing cells to switch into endocycles. Our genetic and physiological data suggest that the mitotic-to-endocycle transition is mediated by the TIR1-AUX/IAA-ARF-dependent auxin signalling pathway. That auxin promotes the mitotic cycle is well established but what is striking in our finding is that depleting auxin signals does not simply block the mitotic cycle but also leads to the initiation of an alternative cell cycle, i.e. the endocycle, thus still permitting plant organs to grow as a whole. This role of auxin as a repressor of endocycling has not been demonstrated in the plant organ context before; the only previous study that has reported similar effects of auxin is in the cell culture context, where depletion of 2,4-D from the culture media induced endocycling and a corresponding elongation of tobacco Bright Yellow-2 cells (Magyar et al., 2005). Our study not only supports their finding but further demonstrates that this function of auxin is vital for programming postembryonic plant development. Interestingly, Magyar et al. (Magyar et al., 2005) found that auxin positively regulates the stability of E2FB transcription factors but that this auxin action does not depend on the TIR1-mediated auxin signalling.

Physiological roles of auxin in promoting cell proliferation have been extensively studied in various plant species but surprisingly little is established about the molecular mechanisms underlying these responses. For example, the application of exogenous auxin promotes the expression of many cell cycle genes (John, 2007), but it is not known whether some members of the AUX/IAA-ARF transcription factors directly control their expression in response to auxin. Previous studies have shown that auxin is also involved in regulating the stability of E2F proteins (del Pozo et al., 2002; Magyar et al., 2005), which suggests that part of the auxin-dependent cell cycle control might rely on their post-transcriptional modification. However, how direct this regulation is in relation to the existing auxin signalling pathway is not established. How auxin controls the mitotic-to-endocycle transition at the molecular level also remains unclear but our data suggest that transient inhibition of auxin signalling by PEO-IAA rapidly downregulates the expression of key mitotic cycle genes. Importantly, reduced activities of these genes have been implicated for endocycle initiation (Andersen et al., 2008; Verkest et al., 2005; Imai et al., 2006; Boudolf et al., 2009) and the promoter sequence of *CDKB2;1* contains a putative auxin responsive element (Vanneste et al., 2005), which raises the possibility that transcriptional repression of these cell cycle regulators by AUX/IAA-ARF constitutes a part of the primary responses to altered auxin levels. We have recently identified a novel SUMO E3 ligase HIGH PLOIDY2 (HPY2) that functions in the PLT-dependent pathway to mediate endocycle onset and meristem development in *Arabidopsis* (Ishida et al., 2009). It is thus plausible that HPY2-dependent sumoylation constitutes part of the developmental programme linking auxin signalling and downstream cell cycle progression.

It is intriguing that all of the auxin mutants we have tested also exhibit enhanced endoreduplication phenotypes with their ploidy reaching up to 256C, indicating that the termination of endocycling is an active process requiring regulatory mechanisms and that auxin signalling is involved in this control. The *hpy2* mutants also display similar ploidy phenotypes, i.e. reduced 2C and 4C peaks and increased 32C, 64C and 128C peaks (Ishida et al., 2009), suggesting that the entry into and exit from endocycling might be linked processes. Finally, we need to add that our data are also consistent with the idea that endoreduplication occurs 'by default' in plant organs and that cell proliferation is promoted only where stimulating signals, such as auxin, are provided (e.g. at the root meristem). Further elucidation of the molecular basis governing the mitotic-to-endocycle transition should help us to uncover how plants use these two alternative cell cycles during their postembryonic development.

Involvement of other plant hormones in the endocycle control

Auxin signalling intersects with signalling pathways mediated by other plant hormones and, in particular, recent elegant studies have begun to unravel the molecular basis underlying the multilevel crosstalk between auxin and cytokinin in the *Arabidopsis* root meristem. The current model suggests that cytokinins counteract the effects of auxin by downregulating the expression of auxin transport genes, thus promoting cell expansion/differentiation (Dello Ioio et al., 2008b; Ruzicka et al., 2009). Consistently, our data show that cytokinins act antagonistically to auxin in mediating the endocycle transition. Although this model seems to fit well for *Arabidopsis* root development, existing data also suggest that auxin and cytokinin are not the only developmental signals mediating endocycle control in other plant organs. For example, ethylene and gibberellin have been suggested to control endocycle progression in the *Arabidopsis* hypocotyl (Gendreau et al., 1999). We found that several of the auxin mutants we have examined, including *yucca* quadruple mutants that give strong ploidy phenotypes in cotyledons, do not display major ploidy alterations in hypocotyls (T.I. and K.S., unpublished), suggesting that endocycle control in *Arabidopsis* hypocotyls does not require inputs from auxin signalling. Therefore, the upstream signalling pathways involved in endocycle control might differ in various plant organs and it is likely that different combinations of plant hormones take part in this control.

How does cell cycle transition link with the initiation of cell expansion and cell differentiation?

This study shows that the auxin-mediated switch into endocycling is tightly coupled with the developmental transition from cell proliferation to cell expansion and/or cell differentiation. Our data suggest that the downregulation of mitotic activities precedes by far the modification of cell expansion/differentiation under low auxin conditions and that these cell cycle changes can have downstream consequences in cell expansion/differentiation. There are also other examples reported where cell cycle genes provide instructive signals for cell expansion/differentiation (Walker et al., 2000; Verkest et al., 2005; Churchman et al., 2006; Boudolf et al., 2009). It is thus tempting to speculate that the dose-dependent control of the two alternative DNA replication cycles, mitotic cycles and endocycles, is part of the regulatory mechanisms that translate auxin gradients into distinct cellular responses – cell proliferation and cell expansion – in developing plant organs. We need to note that the correlations between ploidy levels and plant cell size are not absolute (Inze and De Veylder, 2006; Sugimoto-Shirasu and Roberts, 2003), and we cannot rule out the possibilities that low auxin triggers endoreduplication and cell expansion independently, or that endoreduplication is a downstream consequence of cell expansion. However, in the context of endocycle or cell expansion initiation, we think these are both less likely situations, as existing evidence points to a very tight coupling between endoreduplication onset and the initiation of cell expansion in various plant cell types (Inze and De Veylder, 2006; Sugimoto-Shirasu and Roberts, 2003), although there are some cases in which final ploidy levels are uncoupled from final cell sizes (Beemster et al., 2002; Gendreau et al., 1998). Likewise, there are numerous experimental data reported for ploidy-dependent cell expansion (e.g. Sugimoto-Shirasu et al., 2005; Breuer et al., 2007; De Veylder et al., 2002), whereas almost no examples have been found so far for cell expansion-induced endoreduplication.

In summary, this study unveils a novel function of auxin in plant cell cycle control and provides a new molecular framework in which to further investigate how growth factors mediate cell differentiation during postembryonic development of multicellular organisms.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.035840/-/DC1

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