ABCB19-mediated polar auxin transport modulates Arabidopsis hypocotyl elongation and the endoreplication variant of the cell cycle

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SUMMARY

Elongation of the Arabidopsis hypocotyl pushes the shoot-producing meristem out of the soil by rapid expansion of cells already present in the embryo. This elongation process is shown here to be impaired by as much as 35% in mutants lacking ABCB19, an ATP-binding cassette membrane protein required for polar auxin transport, during a limited time of fast growth in dim white light beginning 2.5 days after germination. The discovery of high ectopic expression of a cyclin B1;1-based reporter of mitosis throughout abcb19 hypocotyls without an equivalent effect on mitosis prompted investigations of the endoreplication variant of the cell cycle. Flow cytometry performed on nuclei isolated from upper (growing) regions of 3-day-old hypocotyls showed ploidy levels to be lower in abcb19 mutants compared with wild type. CCS52A2 messenger RNA encoding a nuclear protein that promotes a shift from mitosis to endoreplication was lower in abcb19 hypocotyls, and fluorescence microscopy showed the CCS52A2 protein to be lower in the nuclei of abcb19 hypocotyls compared with wild type. Providing abcb19 seedlings with nanomolar auxin rescued their low CCS52A2 levels, endocycle defects, aberrant cyclin B1;1 expression, and growth rate defect. The abcb19-like growth rate of ccs52a2 mutants was not rescued by auxin, placing CCS52A2 after ABCB19-dependent polar auxin transport in a pathway responsible for a component of ploidy-related hypocotyl growth. A ccs52A2 mutation did not affect the level or pattern of cyclin B1;1 expression, indicating that CCS52A2 does not mediate the effect of auxin on cyclin B1;1.

Keywords: Arabidopsis thaliana, hypocotyl growth, ploidy, endocycle, CCS52A.

INTRODUCTION

Plant growth and development continuously balances the fundamental processes of cell proliferation, expansion, and differentiation, each of which auxin and auxin transport helps coordinate (Santner and Estelle, 2009; Vanneste and Friml, 2009). The Arabidopsis seedling hypocotyl has proven useful for studying aspects of this coordination. Auxin in hypocotyls is mainly synthesized in the shoot apex and then transported rootward, or basipetally, where it can regulate cell expansion including differentially across the stem during tropisms (Spalding, 2013). Previous studies have shown a membrane protein of the ATP-binding cassette superfamily, ABCB19 (hereafter B19), to be necessary for 80% of the polar auxin stream in the Arabidopsis hypocotyl (Noh et al., 2001). By impairing this auxin stream, b19 mutations reduce the growth-controlling level of auxin in the hypocotyl, which renders photoreceptor-mediated inhibition of elongation more effective during de-etiolation (Wu et al., 2010a). This genetic evidence supported previous pharmacology-based conclusions about polar auxin transport promoting growth in opposition to the inhibitory effects of the cryptochrome and phytochrome photosensory systems (Jensen et al., 1997).

For decades, studies of auxin effects on stem elongation utilized excised segments of pea epicotyls, cucumber hypocotyls, and oat coleoptiles. Recently, auxin promotion of growth was also demonstrated in excised Arabidopsis hypocotyl segments (Takahashi et al., 2012). Old and new results are consistent with the view that auxin leads to apoplast acidification, which promotes the activity of wall-loosening expansin proteins, which results in a rapid increase in growth rate (Rayle and Cleland, 1992; Cleland, 1995). Remarkably, a demonstration of exogenous auxin positively affecting elongation of an intact plant stem over longer time frames may not have been achieved until a

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wick-based flow-through treatment was applied to pea plants (Yang et al., 1993). The difference between the behavior of intact plants and stem segments was classically attributed to the need to deplete auxin levels by removing the segment from its auxin supply, principally the apical meristem, before a restorative effect of exogenous auxin could be detected. In these experimental scenarios, auxin promotes cell expansion up to an optimal concentration beyond which it is inhibitory.

Despite recent major advances in understanding auxin receptor-mediated signaling (Bargmann and Estelle, 2014) and the participation of small auxin up-regulated RNAs in auxin action (Chae et al., 2012; Spartz et al., 2012), it is still not clear how auxin controls hypocotyl growth. Unlike the growth of leaves or roots, which depends on the production of new cells by a tightly controlled cell cycle (De Veylder et al., 2007; Gutierrez, 2009; Desvoyes et al., 2014), hypocotyl cells rarely undergo mitosis except in the few epidermal cells that produce stomata (Gendreau et al., 1997). Post-germination growth is essentially a manifestation of cell expansion. In the short term, the acid-growth mechanism is usually invoked to explain effects of auxin on cell expansion. Over longer time frames, polyploidy resulting from endoreplication of the nuclear genome has been linked to expansion of cells (Gutierrez, 2009). Polyploidy resulting from the endocycle variant of the cell cycle is found in most mature tissues in Arabidopsis (Galbraith et al., 1991). It positively correlates with cell size (Melaagno et al., 1993; Kondorosi et al., 2000; Sugimoto-Shirasu and Roberts, 2003) and is influenced by environmental factors such as light (Gendreau et al., 1998). The present study uses mutations in the B19 transporter to impair auxin flow down the Arabidopsis hypocotyl as a tool to investigate the coupling between auxin, hypocotyl elongation, and the endocycle.

RESULTS

Auxin transport regulates the nuclear endocycle in hypocotyl cells

The surprising observation that instigated the present study was very strong and ectopic expression of a mitosis marker gene, proCYCB1;1:CYCB1;1-GUS (Colón-Carmona et al., 1999) in hypocotyls of b19-1 mutants (Figure 1). This marker is used to visualize the pattern of endogenous CYCB1;1 which accumulates during the G2 to M phase of the cell cycle (Shaul et al., 1996). Instead of the signal being restricted to the shoot apical meristem and scattered locations within the young cotyledons as is typical of the wild type (Figure 1a), b19-1 hypocotyls displayed a strong signal throughout the seedling, particularly in the upper hypocotyl (Figure 1b). This signal is apparently distributed across the epidermis, cortex and central cylinder (Figure 1b inset). This reporter-gene phenotype was detectable

Figure 1. Auxin transport determines the expression pattern of a cycle reporter gene. Compared with WT (a), CYCB1;1-GUS expression is strongly up-regulated in the hypocotyl of b19-1 mutant seedlings (b) grown for 2.5 DAG in 10 μmol m⁻² sec⁻¹ white light. The inset shows a b19-1 hypocotyl cross-section. This phenotype also is apparent in 1-day-old seedlings (c). Auxin application rescues the reporter-gene phenotype (d, e), and the auxin transport blocker NPA partially phenocopies the mutant (f).

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as early as 1 day after germination (Figure 1c). If this cyclin promoter phenotype of b19 was related to auxin deficiency caused by the well documented defect in polar auxin transport down the hypocotyl from a major site of synthesis at the shoot apex (Noh et al., 2001; Geisler et al., 2005; Spalding, 2013), auxin treatment would be expected to restore normal CYCB1;1 reporter-gene signal patterns in the mutant. Auxin treatment indeed rescued the reporter-gene pattern, particularly faithfully when presented at the extremely low concentration of 2.5 nM or 1 µM IAA (Figure 1d,e). Moreover, treatment of the wild type with naphthylphthalamic acid (NPA), an inhibitor of polar auxin transport, caused an upregulation and abnormal spread of the reporter-gene signal, partially phenocopying the b19 mutant (Figure 1f). At the mRNA level, the reporter gene was expressed similarly in the mutant and wild type (WT 1.00 ± 0.16 versus b19 1.32 ± 0.44, P-value = 0.54) as determined by quantitative PCR. The native CYCB1;1 gene was not overexpressed in b19 relative to wild type (Figure S1). These transcript analyses indicate that the conspicuous spread of the proCYCB1;1:CYCB1;1-GUS signal is due to defective degradation of the protein mediated by the destruction box domain of the CYCB1;1 coding sequence (Colón-Carmona et al., 1999). The endogenous CYCB1;1 protein is expected to share this reporter signal pattern.

Hypocotyls are not expected to display mitotic markers because cell divisions after embryogenesis are restricted to the few scattered epidermal cells forming stomata and rarely in the central cylinder (Gendreau et al., 1997). Therefore, the auxin-dependent CYCB1;1 reporter phenotype in an organ consisting almost entirely of non-dividing cells was considered an important phenomenon to understand. We began by determining if mitosis was occurring ectopically where the reporter was active. The number of cortical cells in a file the length of the hypocotyl was the same for wild type (33 ± 2; n = 20 seedlings) and b19-1 (33 ± 1; n = 20 seedlings). The mean number of stomata along one flank of a b19-3 hypocotyl (1.7 ± 0.5, n = 7) was lower (P < 0.05) than the wild-type value (3.5 ± 0.5, n = 8). Thus, high ectopic expression of a mitotic marker in b19 was not associated with increased frequency of cell division. An alternative hypothesis, that CYCB1;1 misregulation indicates an effect on the endocycle, a variant of the cell cycle in which doubling of the nuclear genome is not followed by cytokinesis (De Veylder et al., 2011), was tested first by measuring nuclear size in cortical cells of the upper hypocotyl because ploidy and nuclear size may be correlated (Jovtchev et al., 2006). Figure 2a shows representative b19-1 nuclei marked by H2B–YFP in cortical cells of the upper hypocotyl. They appeared smaller than wild-type nuclei. The probability distribution functions fitted to manual measurements of wild-type and b19 nuclear sizes from confocal microscopy images differed to a statistically-significant degree (P < 0.01) as determined by a Kolmogorov–Smirnov test, and showed that nuclei from b19 hypocotyls on average were smaller than wild type (Figure 2b). The b19 distribution displayed less evidence of peaks corresponding to higher ploidy levels than wild type. Therefore, ploidy levels were more directly examined by measuring nuclear DNA content in populations of b19 and wild-type nuclei by flow cytometry. Figure 3(a) shows a representative frequency histogram of the fluorescence signals from a population of upper-hypocotyl nuclei measured by flow cytometry. The peaks clearly distinguish the different
ploidy classes for individual nuclei. From the histograms it was determined that nuclei isolated from the upper region of b19 hypocotyls are more likely to have two copies of the genome (2C) and less likely to have 8C than the wild type (Figure 3b). A double mutant in which the related ABCB1 gene is also disrupted (b1 b19), which more severely inhibits polar auxin transport in hypocotyls (Noh et al., 2001), displayed a slightly stronger ploidy phenotype as evidenced by the lack of the highest detectable 32C category of nuclei. The TWD1 gene encodes an immunophilin protein that is required for proper trafficking of B1 and B19 to the plasma membrane (Bouchard et al., 2006). Morphologically, b1 b19 and twd1 mutants are almost indistinguishable due to missing or mislocalized ABCB proteins, respectively (Wu et al., 2010b). Figure 3(b) shows that the ploidy distributions in twd1-1 nuclei are essentially indistinguishable from those isolated from b1 b19 double mutants. A formula can convert the class distributions shown in Figure 3(b) to the average number of nuclear doublings that have occurred in the populations of nuclei, a metric known as the cycle value or endoreplication index. Interpretation of this value assumes no mitosis, which was verified in the cortex where we expect most of the sampled nuclei originate. Figure 3(c) shows cycle values calculated from the results shown in Figure 3(b) to capture the low ploidy phenotype of the auxin-transport mutants in a single value. Figure 3(d) shows that treatment of intact hypocotyls with 2.5 nM IAA did not affect the cycle value of wild-type nuclei but completely rescued the b19-3 nuclear defect. Because evidence of mitosis was not observed, these cycle value differences can be interpreted as differences in rates of endoreplication.

Thus, impaired polar auxin transport through the hypocotyl results in misregulation of the CYCB1;1 reporter gene and lower endocycle activity.

The molecular basis of the auxin transport effect on the endocycle

A survey of cell-cycle gene expression was conducted in wild-type and b19 upper hypocotyls to learn more about the effects of altered auxin transport on the molecules controlling mitosis exit and endocycle entry (Lammens et al., 2008). Quantitative PCR was used to measure the mRNA levels of 16 cyclin genes expected to be relevant in hypocotyls based on expression data (http://bar.utoronto.ca/efp/cgi-bin/efp-Web.cgi). Of the cyclin genes tested, CYCD4;1 and CYCD4;2 were significantly down-regulated in b19 (Figure S1a). Similar results were obtained in a second allele of b19 in a different ecotype background (Figure S1b). These gene expression phenotypes were rescued by 2.5 nM IAA (Figure S1b). In Arabidopsis, these two cyclins form active kinase complexes with CDKA;1 that promote G1/S and G2/M transitions (De Veylder et al., 2011). The abnormally high activity of the CYCB1;1 reporter and the lower ploidy in b19 may result from reduced CYCD4 levels, which appear to be the result of reduced auxin transport. The onset of endoreplication requires the multisubunit E3 ubiquitin ligase known as the anaphase-promoting complex/cyclosome (APC/C) to degrade mitotic cyclins (Cebolla et al., 1999; Zheng et al., 2011). In Arabidopsis, these two cyclins form active kinase complexes with CDKA;1 that promote G1/S and G2/M transitions (De Veylder et al., 2011). The lower ploidy and the fewer stomata in b19 may result from reduced CYCD4 levels, which appear to be the result of reduced auxin transport.

The onset of endoreplication requires the multisubunit E3 ubiquitin ligase known as the anaphase-promoting complex/cyclosome (APC/C) to degrade mitotic cyclins (Cebolla et al., 1999; Zheng et al., 2011). In Arabidopsis, three CELL CYCLE SWITCH 52 proteins known as CCS52A1, CCS52A2 and CCS52B form part of the APC/C and influence its activity and substrate specificity (Fülöp et al., 2005; De Veylder et al., 2011). The abnormally high activity of the CYCB1;1 reporter and the lower ploidy in b19 hypoco-
tyls (Figures 1–3) are possibly the result of low levels of the CCS52 activators and therefore low APC/C activity in the mutant. To investigate this potential explanation, we measured mRNA levels of CCS52A1, CCS52A2 and CCS52B in upper-hypocotyl sections. Only CCS52A2 expression was detected in the wild type. Its mRNA level was significantly lower in b19 and wild-type seedlings treated with NPA (Figure 4a). Treatment with IAA (2.5 nm or 1 μM) rescued the CCS52A2 expression phenotype of the b19 mutant. The auxin-insensitive mutant axr3-1 also displayed lower CCS52A2 expression. The CCS52A2 protein level was also lower in b19 and NPA-treated wild-type seedlings, as determined by measuring GFP fluorescence in nuclei of transgenic plants expressing a previously characterized functional CCS52A2–GFP fusion under the control of its native promoter (Vanstraelen et al., 2009). As a control, the H2B–YFP fluorescent histone (Boisnard-Lorig et al., 2001) was not affected by loss of B19 or NPA treatment (Figure 4b,c). Collectively, these results indicate that polar auxin transport controls CCS52A2 expression in a manner that may explain the CYCB1;1 reporter phenotype observed in b19. A genetic test of this hypothesis was arranged by crossing the proCYCB1;1:CYCB1;1–GUS reporter into a ccs52a2 mutant. Figure 5 shows the pattern of CYCB1;1 accumulation visualized by GUS staining was clearly not affected by the ccs52a2 mutation. A phenocopy of the b19 CYCB1;1 pattern was not observed. The result indicates that lowering CCS52A2 levels is not sufficient to cause ectopic overexpression of CYCB1;1. A linear pathway in which auxin delivered by ABCB19-dependent polar transport promotes the CCS52A2-dependent E3 ligase that degrades CYCB1;1 to restrict its expression pattern is not supported.

Hypocotyl elongation, auxin transport, and ploidy

The relationship between ABCB19-dependent auxin transport, hypocotyl elongation, and cycle value was tested here by acquiring digital time-lapse images and analysis utilizing the HYPOTrace software tool (Wang et al., 2009), following the methods Wu et al. (2010a) used to study the connections between ABCB19-dependent auxin transport and the photoregulation of hypocotyl elongation. Figure 6(a) shows that wild-type and b19 seedlings differed in growth rate most significantly between 2.5 and 3 days after germination, which was also the period of fastest hypocotyl elongation. These results were used to design image-based assays of growth rate specifically over the 2.5–3 days time period to determine the effects of auxin. A dose–response analysis demonstrated that nanomolar concentrations of auxin in the medium contacting the seedlings rescued the slow hypocotyl growth phenotype of b19 seedlings (Figure 6b). Higher concentrations of IAA were inhibitory to both genotypes as previously documented (Collett et al., 2000). A primary role of auxin in the control of hypocotyl growth is due to defective auxin transport. (a) qPCR analysis of CCS52A2 expression in WT (Col-0) and b19 seedlings. Error bars indicate standard error (n = 3 biological replicates). Significant differences between WT and mutants or WT with NPA treatment are indicated with * (P-value < 0.05).

Figure 4. Lower CCS52A2 expression in b19 upper hypocotyls is due to defective auxin transport. (a) qPCR analysis of CCS52A2 expression in WT (Col-0) and b19 seedlings. Error bars indicate standard error (n = 3 biological replicates). Significant differences between WT and mutants or WT with NPA treatment are indicated with * (P-value < 0.05).

(b) Confocal microscopy analysis of nuclear fluorescence intensity of CCS52A2–GFP and the histone-based nuclear marker H2B–YFP in the cortex of upper hypocotyls of Col-0 or b19-3 treated with or without 1 μM NPA. Same chromophore has the same confocal setting. The backgrounds are chloroplasts.

(c) Quantification of fluorescence signal in nuclei like those shown in (b). Error bars indicate standard error of the mean (n = 14–20).
of this phase of peak hypocotyl growth rate was further supported by showing that axr2 and axr3 auxin signaling mutants were impaired similar to or even more than b19 mutants (Figure 6c), consistent with previously published hypocotyl end-point measurements (Nagpal et al., 2000). If lower ploidy caused by reduced auxin flow to the growth zone reduces hypocotyl elongation then mutating CCS52A2 while leaving B19 intact would be expected to reduce hypocotyl growth rate. Figure 6c shows this to be the case. Furthermore, the low growth rate of a ccs52a2 mutant could not be rescued by auxin, though high auxin (1 μM) had its normal inhibitory effect (Figure 6c). Apparently, low concentrations of auxin promote growth by a mechanism that involves CCS52A2 but the inhibitory effects of micromolar auxin occur independently of CCS52A2.

A ccs52a2 b19-3 double mutant was created. Figure 6c shows its phenotype to be consistent with the sum of the two constituent components. Nanomolar levels of auxin promoted elongation to a small extent, presumably by rescuing the b19 genetic component. It appears that auxin promotes hypocotyl growth through CCS52A2-dependent and CCS52A2-independent pathways.

Figure 7(a) co-plots cycle value and growth rate data both obtained 2.5-3 DAG with wild-type and b19-3 seedlings with or without auxin, and data from an axr3 mutant, all in the Col-0 genetic background. The data show a striking rescue of b19 ploidy (cycle value) and growth rate by 2.5 μM auxin. Clearly, the nuclear endocycle and growth rate in the auxin-transport mutant are both auxin-limited. Suppling auxin at a level high enough to restore the deficiency but low enough to avoid inducing inhibition allowed this response to be quantified. The wild type is not auxin deficient and therefore did not respond with respect to either the cycle value or growth rate. The auxin-insensitive axr3 mutant has a cycle value and growth rate pair similar to an auxin-deficient mutant (b19). A logarithmic relationship was obtained between ploidy (cycle value) and growth rate when the latter was measured over a period of development spanning from 2 to 5 days after germination and the former was altered by mutations, chemical treatments, or light (Figure 7b).

**DISCUSSION**

The extraordinarily high level and spatial spread of the CYCB1;1-GUS cell cycle marker in b19 hypocotyls was the original observation that instigated this study. The widely used CYCB1;1-GUS reporter gene includes the D-box domain of the cyclin fused to the GUS coding sequence. It faithfully reports mitotic events in otherwise wild-type plants because the D-box is sufficient for recognition and degradation at the appropriate phase of the cell cycle by the APC/C ubiquitin ligase complex, which would be expected to contain one of the CCS52A substrate-recognition and activating proteins (Peters, 2002; Capron et al., 2003; Marrocco et al., 2009). For example, CYCA2;3 cyclin is inactivated by an APC/C that contains CCS52A1 (Boudolf et al., 2009). The present work used a ccs52a2 mutant to test the analogous hypothesis that CYCB1;1 is inactivated by an APC/C containing CCS52A2. The results did not support the hypothesis (Figure 5). Expression data did not identify an alternative CCS52 family member to play the role apparently ruled out for CCS52A2. Perhaps in the absence of CCS52A2, CCS52A1 is induced to control CYCB1;1 and thereby provide an explanation for the results in Figure 5. Supporting this possibility is that an APC/C containing CCS52A1 was shown to degrade CYCB1;1 and promote the endocycle in Arabidopsis trichomes (Kasili et al., 2010). Alternatively, auxin may suppress CYCB1;1 in hypocotyls, thereby promoting the endocycle, by a pathway that does not involve cell-cycle switch proteins such as CCS52A2.

The present work does not provide a molecular-level explanation of the wide-spread CYCB1;1-GUS phenotype in b19, except that it is a consequence of auxin deficiency. What appears to be a similar ectopic spread of the same CYCB1;1-GUS reporter is induced by ultraviolet-B radiation in Arabidopsis hypocotyls, a treatment that also suppresses hypocotyl elongation (Biever et al., 2014). However, the effect of ultraviolet-B radiation on CYCB1;1

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**Figure 5.** The expression of CYCB1;1-GUS is independent of CCS52A2. GUS staining of CYCB1;1-GUS in the hypocotyl of WT (a), b19-3 (b) and ccs52a2 (c) mutant seedlings grown in 10 μmol m \(^{-2}\) sec \(^{-1}\) white light at 2.5 DAG.
expression is explained by an increase in transcription (Biever et al., 2014) whereas the auxin-deficiency effect on CYCB1;1 reported here cannot be explained by increased CYCB1;1 mRNA (Figure S1) and is probably a result of decreased protein degradation. Thus, the effects of ultraviolet-B radiation and impaired auxin transport on CYCB1;1 in hypocotyls are probably not mechanistically related.

The results presented here demonstrate that B19-mediated auxin transport affects CCSS2A2 levels and endo-cycle activity in the upper hypocotyl (Figure 8), but the molecular mechanism is not yet clear. Transcript analysis of many cell-cycle regulators indicated that CYCD4 genes

Figure 6. Auxin transport is required for hypocotyl elongation.
(a) High-resolution time courses of hypocotyl growth for 48 h beginning 2.5 days after germination under 10 μmol m⁻² sec⁻¹ white light. Images were automatically collected every 10 min and processed by a custom image analysis program. Error bars indicate standard error of the mean (n = 6–8 individuals for each genotype/treatment).
(b) Response of growth rate during the period of fastest elongation (2.5–3 days after germination) to dose of exogenous IAA in WT (Col-0) and b19-3 seedlings (n > 20 per genotype/treatment).
(c) Nanomolar auxin restores low hypocotyl elongation of b19-3 but not ccss2a2 mutants to WT (Col-0) levels (n > 20 per genotype/treatment).

Figure 7. Positive relationships between cycle value and hypocotyl elongation rate.
(a) Hypocotyl elongation rate determined by computational analysis of images acquired over 12 h beginning with 2.5 DAG seedlings, and cycle value of nuclei extracted from the upper region of the hypocotyl. Auxin completely rescued the low growth/low cycle value of the b19 mutant. The genotypes used were in the Col-0 background.
(b) Average growth rate obtained by measuring hypocotyl length from digital images acquired at 2.5 days and 5 days after germination, plotted as a function of cycle value in the indicated genotypes and treatments. The genotypes used were in the Ws background.

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Figure 8. Structure of pathways connecting polar auxin transport to control of the endocycle and growth in the Arabidopsis hypocotyl that are consistent with the presented results.

should be considered in future studies of the mechanism. The lower transcript levels of CYCD4;1 and CYCD4;2 in b19 mutants were restored to wild type by auxin treatment (Figure S1). Kono et al. (2007) showed that knockout and overexpression of CYCD4 genes had major effects on cell division and differentiation in the epidermis of the Arabidopsis hypocotyl. Perhaps auxin regulation of CYCD4 genes will be found to affect the endocycle and B19-dependent hypocotyl growth.

Regardless of the mechanism linking reduced CCS52A2 levels and reduced ploidy, there is a clear quantitative relationship between the endocycle and hypocotyl cell expansion (Figure 7). This is not the first evidence of a link between the endocycle and hypocotyl growth rate. Gendreau et al. (1998) used light quality and photomorphogenesis mutants to conclude that the photocontrol of hypocotyl elongation is exerted in part by controlling the endocycle. A defect in cell expansion in bin4 mutant with a severe defect in endoreplication can be partially overcome by drug-induced tetraploidization (Breuer et al., 2007). Other relevant research includes the finding that CCS52A2 overexpression lines tend to have larger leaves with higher ploidy levels while mutation of CCS52A2 causes dwarf plant and lower ploidy in leaves (Lammens et al., 2008; Liu et al., 2012).

Insufficient auxin availability due to impaired rootward transport is sufficient to explain the low levels of CCS52A2 in b19 hypocotyls (Figure 8). Resupply of auxin at nanomolar or below concentrations rescues b19 phenotypes, which may seem surprising given that auxin typically acts in the 0.1–1 μM range. However, the defect to be rescued in this case is the supply of the hormone to the deficient cells, which apparently use the nanomolar supply to create the appropriate internal concentrations using the proteins and thermodynamic gradients that govern auxin movement (Spalding, 2013). In fact, nanomolar treatments may be more effective at rescuing auxin deficiencies than micromolar concentrations because they may be less likely to trigger effects on transporter trafficking, internalization, and degradation (Friml, 2010).

In the present study, high auxin rescued the CYCB1;1 reporter phenotype and low ploidy of b19 but not the hypocotyl growth phenotype. Instead, high auxin suppressed hypocotyl growth. Even the slow growth of cc-s52a2 hypocotyls was inhibited more by high auxin (Figure 6c). Therefore, promotion of growth by nanomolar concentrations of auxin requires CCS52A2 but the inhibitory action of high auxin does not.

Although the experiments reported here were performed in an organ mostly lacking cell division, the link between auxin transport, the endocycle, and cell expansion may be very relevant to the shoot and root apical meristems, as these are sites of high auxin and cell-cycle activity (Ishida et al., 2010). In Arabidopsis, both CCS52A1 and CCS52A2 are reportedly required for root meristem maintenance (Vanstraelen et al., 2009). CCS52A2 controls meristem size by promoting endocycle and mitotic exit in the elongation zone. In contrast, CCS52A2 is expressed at the distal part of the root meristem and required to maintain the stem cell identity in the quiescent center. CCS52A2 is also required for shoot apical meristem maintenance by controlling mitotic activity (Liu et al., 2012).

Decades of auxin studies led to the acid-growth hypothesis in which wall acidification due to H+-ATPase activation promotes cell expansion at least in part by putting the wall-loosening expansin proteins in a permissive environment. Auxin activation of the H+-ATPase in Arabidopsis involves phosphorylation (Takahashi et al., 2012) over a time frame best measured in minutes. The experiments reported here have a different context. Rather than a mechanism that responds to a change in auxin distribution such as following tropic stimuli, the mechanism explored here relates more to a longer term steady state in which auxin flow down the hypocotyl coordinates cell-cycle activity to adjust ploidy as an element of growth control. A comprehensive understanding of hypocotyl elongation during seedling development will require integration of short term and steady state growth control mechanisms.

**EXPERIMENTAL PROCEDURES**

**Plant material**

The following Arabidopsis thaliana genotypes were used: wild-type plants of ecotypes Ws and Col-0; mutant b19-1, b1b19 (Noh et al., 2001); b19-3 (Lewis et al., 2007); twd1-1 (Wu et al., 2010b); axr2-1 and axr3-1 (Leyser et al., 1996); cc-s52a2 (Vanstraelen et al., 2015).
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2009). Construction of proCYCB1:1:CYCB1;1-GUS, which contains the promoter and the destruction box (D-box) of CYCB1;1 fused to the uidA gene, was described by Colón-Carmona et al. (1999). CCS52A1-GFP and CCS52A2-GFP constructs driven by native promoters, are previously characterized (Vranstraelen et al., 2009).

H2B–YFP was described by Boisnard-Lorig et al. (2001). All seedlings were grown on half-strength Murashige and Skoog medium under continuous 10 μmol m⁻² sec⁻¹ white light at 25°C. In the case of IAA or NPA treatment, the chemicals were included in the agar medium to which seeds displaying an emerged radicle were transferred, typically 1 day after a 2-day stratification period.

Confocal microscopy

Confocal microscopy was performed on a Zeiss LSM 510 laser scanning confocal microscope (Oberkochen, Germany) containing DAPI. Next, 2 ml more solution was added to the samples and the suspensions passed through 30 μm CellTrics filter (Partec). The ploidy was measured with BD LSR II flow cytometer using Lightwave Xcyte laser with 20 mW at 355 nm (BD Biosciences, San Jose, California, USA). In each run, average 5000 events were counted at a speed of 500 events min⁻¹. DAPI fluorescence histogram (Figure S1) was used to calculate ploidy levels using FlowJo software. The cycle value was calculated as described (Barow and Meister, 2003).

Flow cytometric analysis

Nuclei suspensions were prepared from average of 20–25 upper hypocotyls per sample, collected from 2.5 to 3 DAG seedlings using a surgical razor. Then tissues were chopped for 2–3 min in a drop of CyStain UV Ploidy solution (Partec GmbH, Germany) containing DAPI. Next, 2 ml more solution was added to the samples and the suspensions passed through 30 μm CellTrics filter (Partec). The ploidy was measured with BD LSR II flow cytometer using Lightwave Xcyte laser with 20 mW at 355 nm (BD Biosciences, San Jose, California, USA). In each run, average 5000 events were counted at a speed of 500 events min⁻¹. DAPI fluorescence histogram (Figure S1) was used to calculate ploidy levels using FlowJo software. The cycle value was calculated as described (Barow and Meister, 2003).

Hypocotyl length measurements and time course of hypocotyl growth

Growth rates presented in Figures 6 and 7 were measured from time-lapse digital images but by different methodologies depending on the goal. The extended time course of growth rate presented in Figure 6(a) was generated by tracking the growth of events on the goal. The extended time course of growth rate presented in Figure 6(b) related to the growth rate of cell cycle arrest initiated by photodimer accumulation.

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REFERENCES

Barow, M. and Meister, A. (2003) Endopolyploidy in seed plants is differentially correlated to systematics, organ, life strategy and genome size. Plant, Cell Environ. 26, 571–584.

Biewer, J.J., Brinkman, D. and Gardner, G. (2014) UV-B inhibition of hypocotyl growth in etiolated Arabidopsis thaliana seedlings is a consequence of cell cycle arrest initiated by photodimerization. J. Exp. Bot. 65, 2949–2961.

Boisnard-Lorig, C., Colon Carmona, A., Bauch, M., Hodge, S., Doerner, P., Bancharel, E., Dumas, C., Haseloff, J. and Berger, F. (2001) Dynamic analyses of the expression of the HISTONE-YFP fusion protein in Arabidopsis show that syncytial endosperm is divided in mitotic domains. Plant Cell, 13, 495–509.

Boudolf, V., Lammens, T., Boruc, J. et al. (2009) CDKB1;1 forms a functional complex with CYCA2;3 to suppress endocycle onset. Plant Cell, 21, 3060–30612.

Breuer, C., Stacey, N.J., West, C.E., Zhao, Y., Chory, J., Tsukaya, H., Azumi, Y., Maxwell, A., Roberts, K. and Sugimoto-Kondorosi, E. (2007) ccs52 is required for endoreduplication in Arabidopsis. Plant Cell, 19, 3655–3668.

Capron, A., Okresz, L. and Genschik, P. (2003) First glance at the plant APC/C, a highly conserved ubiquitin-protein ligase. Trends Plant Sci. 8, 33–38.

Cebolla, A., Vinardell, J.M., Kiss, E., Olah, B., Roudier, F., Kondorosi, A. and Kondorosi, E. (1999) The mitotic inhibitor ccs52 is required for endoreduplication and ploidy-dependent cell enlargement in plants. EMBO J. 18, 101–109.
Kono, A., Umeda-Hara, C., Adachi, S., Nagata, N., Konomi, M., Nakagawa, K., Geisler, M., Blakeslee, J.J., Bouchard, R. (2010) Subcellular trafficking of PIN auxin efflux carriers in auxin transport. *Eur. J. Cell Biol.* 89, 231-235.

Fulop, K., Tariq, A., Zalmas, L. (2011) Hormonal interactions in the control of Arabidopsis hypocotyl elongation. *Plant Physiol.* 154, 1850-1859.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, Jovtchev, G., Schubert, V., Meister, A., Barow, M. and Schubert, I. (1997) Auxin transport is required for hypocotyl growth in Arabidopsis thaliana.* Curr. Opin. Plant Biol.* 10, 646-652.

Collett, C.E., Harberd, N.P. and Leyser, O. (2000) Hormonal interactions in the control of Arabidopsis hypocotyl elongation. *Plant Physiol.* 124, 553-562.

Colón-Carmona, A., You, R., Haimovitch-Gal, T. and Doerner, P. (1999) Spatiotemporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. *Plant J.* 20, 503-508.

Desvoyes, B., Fernández-Marcos, M., Sequeira-Mendes, J., Otero, S. and Gutiérrez, Z. (2014) Looking at plant cell cycle from the chromatin window. *Front. Plant Sci.* 5, 389.

Friml, J. (2010) Subcellular trafficking of PIN auxin efflux carriers in auxin transport. *Eur. J. Cell Biol.* 89, 231-235.

Galbraith, D.W., Harkins, K.R. and Knapp, S. (1995) Auxin and cell elongation. In *Plant Hormones: Physiol- ical, Biochemical and Molecular Biology* (Davies, P.J., ed.), Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 214-227.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, Jovtchev, G., Schubert, V., Meister, A., Barow, M. and Schubert, I. (1997) Auxin transport is required for hypocotyl growth. *Plant Physiol.* 116, 455-452.

Jovtchev, G., Schubert, V., Meister, A., Barow, M. and Schubert, I. (2006) Nuclear DNA content and nuclear and cell volume are positively correlated with the number of endoreduplication cycles. *Cytogenet. Genome Res.* 114, 77-82.

Ishida, T., Adachi, S., Yoshimura, M., Shimizu, K., Umeda, M. and Sugimoto, K. (2010) Auxin modulates the transition from the mitotic cycle to the endocycle in Arabidopsis. *Development.* 137, 63-71.

Jensen, P.J., Hangarter, R.P. and Estelle, M.A. (1997) Auxin transport is required for hypocotyl elongation in light-grown but not in dark-grown Arabidopsis. *Plant Physiol.* 116, 455-452.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, J.C. (2010) SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana. *Cytogenet. Genome Res.* 126, 82-84.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, J.C. (2010) SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana. *Cytogenet. Genome Res.* 126, 82-84.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, J.C. (2010) SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana. *Cytogenet. Genome Res.* 126, 82-84.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, J.C. (2010) SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana. *Cytogenet. Genome Res.* 126, 82-84.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, J.C. (2010) SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana. *Cytogenet. Genome Res.* 126, 82-84.

Kasili, R., Walker, J.D., Simmons, L.A., Zhou, J., De Veylder, L. and Larkin, J.C. (2010) SIAMESE cooperates with the CDH1-like protein CCS52A1 to establish endoreplication in Arabidopsis thaliana. *Cytogenet. Genome Res.* 126, 82-84.