Plant growth is sustained by two complementary processes: biomass biosynthesis and cell expansion. The cell wall is crucial to both as it forms the majority of biomass, while its extensibility limits cell expansion. Cellulose is a major component of the cell wall and cellulose synthesis is pivotal to plant cell growth, and its regulation is poorly understood. Using periodic diurnal variation in Arabidopsis thaliana hypocotyl growth, we found that cellulose synthesis and cell expansion can be uncoupled and are regulated by different mechanisms. We grew Arabidopsis plants in very short photoperiods and used a combination of extended nights, continuous light, sucrose feeding experiments, and photosynthesis inhibition to tease apart the influences of light, metabolic, and circadian clock signaling on rates of cellulose biosynthesis and cell wall biomechanics. We demonstrate that cell expansion is regulated by protein-mediated changes in cell wall extensibility driven by the circadian clock. By contrast, the biosynthesis of cellulose is controlled through intracellular trafficking of cellulose synthase enzyme complexes regulated exclusively by metabolic signaling related to the carbon status of the plant and independently of the circadian clock or light signaling.

INTRODUCTION

Plant growth is sustained by two complementary processes: biomass biosynthesis, resulting in dry matter accumulation, and cell expansion accompanied by water uptake leading to irreversible increases in size. Both processes are tightly linked to the cell wall, a structure that encases all plant cells. A major proportion of plant biomass is in the cell wall and large fluxes of photosynthetic products are directed to cell walls in growing cells (Wang et al., 2013; Boex-Fontvieille et al., 2014; Haigler et al., 2001). On the other hand, plant cell expansion is limited by cell wall extensibility (Cosgrove, 1986, 2016a). The cell wall is thus a key player in cell expansion, morphology, and size as well as plant biomass accumulation.

Cell expansion is controlled by changes in cell wall properties, which have been extensively studied under different conditions (Cosgrove and Li, 1998; Schopfer et al., 2002; Suslov et al., 2009; Peaucelle et al., 2013). More specifically, cell expansion is linked to cell wall extensibility, which can be defined as the ability of the cell wall to increase in surface area irreversibly during growth (Cosgrove, 2016a). Cell wall extensibility is regulated through selective breakage and formation of links between wall polymers driven by loosening and tightening proteins secreted by the cell (Cosgrove, 2016b). By contrast, how different external and internal cues control cell wall synthesis is only starting to be unraveled (Bischoff et al., 2011; Boex-Fontvieille et al., 2014). To protect expanding cell walls from excessive thinning and failure during growth, their synthesis and expansion need to be coordinated (Voxeur and Höfte, 2016). While several reports have observed that these processes can occur independently of each other (Edelmann et al., 1989; Edelmann and Fry, 1992; Refrégier et al., 2004; Derbyshire et al., 2007), the principles behind such uncoupling are not clear.

How cell wall synthesis is controlled and whether it is coordinated with cell expansion can be investigated in plants growing in conditions of alternating light and darkness during 24-h cycles, whereby light, temperature, and metabolic resource supply fluctuate. In these conditions, dicot plants exhibit diurnal variation in expansion growth. Studies on diurnal growth dynamics (Wieze et al., 2007; Yazdanbakhsh et al., 2011; Apelt et al., 2015) have shown that the periodic patterns are generated through the interplay between light...
signaling and an internal timing mechanism known as the circadian clock (Nozue et al., 2007; Nusinow et al., 2011) and that the patterns persist in constant-light, constant-temperature conditions for extended periods. However, the mechanistic basis for these growth variations at the level of cell wall synthesis and cell expansion remains unclear, and it is unknown to what extent the synthesis of cell wall varies with changing growth rates and what regulatory inputs impact on it. In addition, how the cell wall extensibility modulates these rapid changes in growth and to what extent cell wall biosynthesis and cell expansion are coordinated as growth rates vary remain largely unknown.

Any diurnal pattern observed in a plant may be the confluence of separate responses to light, metabolic, and endogenous cues. Untangling these effects requires an experimental system where all three can be manipulated separately but all remain functional. Cell wall biosynthesis relies on carbon newly fixed through photosynthesis during the day and remobilized from starch reserves at night (Smith and Stitt, 2007; Stitt and Zeeman, 2012; Sulpicie et al., 2014). When days are very short, the limited starch reserves accumulated during the day are only sufficient to maintain respiration at night (Gibon et al., 2009; Sulpicie et al., 2014), leaving little for biosynthesis, while still allowing a circadian rhythm to be maintained in a natural diurnal cycle. These conditions offer an excellent experimental system to explore the regulatory inputs that control growth rates at the level of cell wall synthesis and remodeling.

Cellulose is a major component of plant cell walls. In contrast to other cell wall components, cellulose is produced by plasma membrane cellulose synthase (CesA) enzyme complexes (CSCs). Fluorescently labeled CSCs can be observed microscopically as moving foci in the plasma membrane of plant cells (Paredez et al., 2006). Their movement is thought to be driven by the polymerization and extrusion of cellulose chains into the cell wall, which pushes the enzyme complex in the plane of the plasma membrane, and this has been used as a proxy for the rate of cellulose synthesis. CSCs are also observed in intracellular membrane compartments thought to be involved in their trafficking and the CSCs are thought to be inactive until they reach the plasma membrane (Paredez et al., 2006; Gutierrez et al., 2009; Crowell et al., 2009). Here, we investigated the regulation of cellulose synthesis and cell wall extensibility in hypocotyls of Arabidopsis thaliana plants grown in short diurnal cycles.

RESULTS

CesA Abundance at the Plasma Membrane Exhibits Diurnal Fluctuations in Low-Carbon Conditions

To create a strong diurnal alternation between periods of growth and no growth, and to rely purely on photosynthesis-derived carbon for cell wall biosynthesis, we grew Arabidopsis seedlings in short photoperiods (4 h day/20 h night) on sucrose-free media for 7 d. It has been shown that seed storage lipids are essentially depleted as early as three days after germination (Andre and Benning, 2007; Kelly et al., 2011), and the plants must then rely on photosynthesis for their carbon supply, although it is not known whether the reserves last longer or shorter under certain conditions. We observed a fluorescently tagged subunit of the CSC, YFP-CesA6, in hypocotyl cells using spinning disc confocal microscopy as an indicator of cellulose synthesis. At the end of the night, there was a low abundance of CesA particles in the plasma membrane, with the overwhelming majority associated with intracellular membrane compartments (Supplemental Movie 1 and Supplemental Figure 1), referred to as small CesA compartments (SmaCCs; Gutierrez et al., 2009), or microtubule-associated CesA compartments (MASCs; Crowell et al., 2009), which are thought to contain inactive CSCs and to be involved in either exo- or endocytosis of the CSCs and exhibit erratic, irregular movement. CesA particles began to repopulate the plasma membrane during the light period, while the number and intensity of SmaCCs decreased. The density of plasma membrane-based particles varied significantly with time (ANOVA P < 0.001) and steadily increased to a peak at the end of the day (ED; Figure 1A; Supplemental Table 1) and declined thereafter, persisting for several hours into the night. This diurnal pattern of CesA dynamics suggests that light signaling and/or metabolic status and/or endogenous signals may regulate the secretion of CesA to the plasma membrane during the diurnal cycle.

Extended Darkness Prevents the Mobilization of CesA to the Plasma Membrane

To assess whether endogenous factors or signals regulated the observed CesA behavior, we performed an extended night experiment where, on one day, plants were kept in the dark for 4 h instead of in the light and were imaged at end of the subjective day. Here, plasma membrane CesA particle abundance did not increase significantly during the extended night (Figure 1A; Supplemental Movie 1 and Supplemental Figure 1). This suggests that the diurnal fluctuations in CesA abundance at the plasma membrane were not the result of endogenous rhythms and indicated that light signaling (i.e., photoreceptors) or the metabolic and/or signaling effects associated with photosynthesis in the light must be required to mobilize the CesAs to the plasma membrane.

Inhibition of Photosynthesis in the Light Prevents CesA Mobilization

To separate the roles of light signaling and photosynthesis, we pretreated short-day-grown seedlings with the photosynthesis inhibitor DCMU overnight and then exposed them to light in the absence or presence of exogenous sucrose to provide a carbon source independent of photosynthesis. Treatment with DCMU reduced the quantum efficiency of photosystem II to very low levels both in the presence and absence of sucrose (Supplemental Figure 2). In the absence of sucrose, CesA abundance at the plasma membrane did not increase significantly during the day after inhibition of photosynthesis by DCMU (Figure 1B; Supplemental Movie 2 and Supplemental Figure 3). However, in the presence of DCMU and sucrose, CesA complexes were mobilized to the plasma membrane despite the severe inhibition of photosynthesis. These results suggest that metabolic signaling associated with the supply of carbon triggers the mobilization of CesA complexes to the plasma membrane in the light.
A Metabolic Signal Is Necessary and Sufficient to Trigger CesA Mobilization

To test whether a favorable metabolic status alone is sufficient to mobilize CesAs to the plasma membrane, we performed the extended night experiment while transferring seedlings to media containing 2% sucrose. Plasma membrane CesA particles were abundant after 4 h of extended night (Figure 1C; Supplemental Movie 3 and Supplemental Figure 4). This increase was absent in control seedlings (2% mannitol; Supplemental Movie 3 and Supplemental Figure 4). This indicates that a favorable metabolic status is necessary and sufficient to trigger the mobilization of CesA to the plasma membrane and that a light stimulus is not required.

Cellulose Synthase Dynamics Are Constant if Carbon Resources Are Constantly Available

Finally, we observed plants grown in short-day conditions on media supplemented with sucrose. In these conditions, CesA abundance at the plasma membrane was high and exhibited no significant variation throughout the diurnal cycle (Figure 1D, ANOVA P = 0.07; Supplemental Movie 4, Supplemental Figure 5, and Supplemental Table 2). The speed of particle movement also did not vary significantly (Figure 1E, Kruskal-Wallis rank ANOVA, P = 0.114; Supplemental Table 3). Furthermore, extension of the night did not result in a decrease in CesA particle abundance at the plasma membrane or their speed of movement. Taken together,
the unchanging abundance and movement speed of CesA in these conditions implies that cellulose synthesis rates are constant when carbon resources are constantly available.

**Substrate Levels Do Not Underlie Diurnal Variation in Cellulose Synthase Dynamics**

The fluxes through many enzymes are limited by the supply of substrate, and it may be assumed that severe metabolic limitation may reduce the pool of substrate available to the CesA, limiting its activity or affecting its trafficking. To investigate this, we measured the levels of UDP-glucose, the substrate of CesA, throughout diurnal cycles in seedlings grown in 4-h days with and without sucrose supplementation and also in extended night conditions. Surprisingly, UDP-glucose levels did not vary significantly without sucrose supplementation (Figure 1F) despite altered CesA localization. Extension of the night by 4 h also did not significantly alter UDP-glucose levels compared with normal short-day conditions. The presence of sucrose in the growth medium significantly increased UDP-glucose levels (ANOVA, P < 0.001; Supplemental Table 4), with 48 to 78% higher levels at the end of the day and end of the night in sucrose-grown plants compared with no sucrose, respectively. However, sucrose-grown plants had similar levels of UDP-glucose at the end of the day and end of the night (Supplemental Table 4). These results suggest that the CesA dynamics are not due to substrate limitation as UDP-glucose is present at similar levels during the day, night, and extended night and implies that a signaling process might be responsible for the observed changes in CesA trafficking.

**Extension Growth Is Independent of Cellulose Synthesis**

To assess how the regulation of cellulose synthesis relates to rates of expansion growth, we monitored hypocotyl elongation in the presence of sucrose under the same short-day conditions as above using an infrared camera. Hypocotyl growth exhibited a diurnal rhythm similar to that reported in previous studies (Nozue et al., 2007; Dowson-Day and Millar, 1999), with a peak in growth at the end of the night and a slowdown during the day (Figure 2A). Growth rates varied 3.0-fold, despite constant high abundance and speed of plasma membrane CesA particles and, by implication, cellulose synthesis rates in the same conditions. This indicates that cellulose synthesis and plant expansion growth are uncoupled on short timescales.

**Protein-Dependent Cell Wall Modification Underlies Diurnal Variation in Extension Growth**

Next, we investigated whether changes in cell wall extensibility can explain the observed diurnal growth differences. We harvested hypocotyls at the end of the night (EN; fast growth) and end of the day (ED; slow growth). The frozen/thawed hypocotyls were extended in vitro using the constant load (creep) method in buffered conditions at pH 5, 6, and pH 5 with heat inactivation at 90°C (Suslov et al., 2015). At pH 5, creep rates were 2- to 6-fold higher than at pH 6, reflecting the higher activity of expansins under acidic conditions (Figure 3A). Fast-growing plants harvested at EN had 1.7- to 2.7-fold higher creep rates than slow-growing plants harvested at ED at pH 5, the difference being significant at higher loads (1600 and 2000 mg). Up to 2.0-fold difference was observed between EN and ED at pH 6, but this was significant at lower loads. There was no significant difference in creep rates in heat-inactivated hypocotyls, indicating that the observed differences at pH 5 and pH 6 are protein mediated. Thus, diurnal variation in hypocotyl growth rate is accompanied by variations in the activity of at least two groups of cell wall-loosening proteins: those active at pH 5 (high loads) and at pH 6 (low loads).

**The Circadian Clock Controls Cell Wall Modification to Control Plant Growth**

Next, we sought to investigate to what extent the diurnal variations in creep rates are controlled by the circadian clock. We did this by first entraining plants in our short-day conditions and then transferring them to constant-light conditions and recording their growth. Periodic peaks in extension growth rate continued to occur every 24 h (Figure 2B). The timing of the peak in growth rate was shifted forward by ~4 h and maximum growth now occurred at the subjective ED, while minimum growth was observed at the subjective EN, similar to what has been reported in previous

![Figure 2. Rates of Hypocotyl Extension in Arabidopsis Plants Growing Vertically on Sterile Media Containing 2% Sucrose.](image-url)
studies (Nozue et al., 2007). Growth rates were 3.6-fold higher at the subjective ED than at the start of the subjective day.

We harvested hypocotyls at subjective dawn and subjective dusk, close to the minimum and maximum elongation rate in continuous light and subjected them to creep measurements. In contrast to diurnal cycles, creep rates at pH 6 did not vary significantly between time points in continuous light (Figure 3B). However, a large, significant difference in creep rates was observed at pH 5 under 1600 and 2000 mg loads, where fast-elongating hypocotyls harvested at subjective dusk had 2.2- to 2.7-fold higher creep rates than slow-growing hypocotyls harvested at subjective dawn. Creep rates in heat-inactivated hypocotyls did not vary significantly between time points, confirming that the changes observed at pH 5 are protein dependent.

To confirm the involvement of the circadian clock, we measured growth and estimated cell wall extensibility in the elf3 loss-of-function mutant, deficient in a key component of the Evening Complex of the circadian clock. The circadian clock in this mutant becomes arrhythmic in constant-light conditions (Nozue et al., 2007; Hicks et al., 1996; Covington et al., 2001), allowing the effects of the clock to be tested. The Ws-2 wild-type control behaved similarly to the Col-0 and exhibited periodic growth rhythms, with 10.7-fold higher growth rates at the peak compared with the trough (Figure 2C). There was no periodic variation in growth rates in the elf3 mutant after transfer to continuous light (Figure 2D), consistent with its impaired clock function (Nozue et al., 2007).

Creep measurements were conducted at pH 5, where Col-0 exhibited large changes in creep rate in continuous light. Ws-2 also showed a significant increase in creep rate between subjective dawn and subjective dusk (P = 0.015; Figure 3C). elf3 exhibited very high creep rates compared with the Ws-2 (Figure 3C); however, there was no significant difference in creep rates between subjective dawn and dusk in elf3. Thus, a functional circadian clock is required to modulate cell wall extensibility during periodic growth fluctuations and controls plant expansion growth by modulating the activity of cell wall-modifying proteins active at pH 5.

**DISCUSSION**

The ability to switch cell wall synthesis on and off should be of vital importance to plant cells given that cell wall synthesis is the largest metabolic flux in growing cells, while resource supplies to support it can be scarce and intermittent. Reports using isotope labeling

Figure 3. Cell Wall Creep Rates Exhibit Diurnal Patterns and Are Regulated by the Circadian Clock.

(A) Creep rates measured in vitro under controlled pH conditions at different loads in Col-0 wild type plants growing in 4-h-day/20-h-night diurnal cycles and harvested at EN and ED 7 d after germination.
(B) Col-0 wild-type plants grown in 4-h-day/20-h-night diurnal cycles for 4 d, transferred to continuous light, and harvested on the second day after transfer at EN and ED.
(C) Ws-2 wild-type and elf3 mutant plants grown in 4-h-day/20-h-night diurnal cycles for 4 d, transferred to continuous light, and harvested on the second day after transfer at EN and the end of the subjective day. Ws-2 and elf3 were only measured at pH 5 and under a 1600 mg load. In each creep test, a 2-mm-long hypocotyl segment of one thawed Arabidopsis seedling was extended. n = 6 to 10. Bars are SE. Letters (bold for pH 6 and italicized for pH 5) indicate significant differences (Student’s t test, P < 0.05) between EN and ED under the same load.
techniques show that the incorporation of glucose units into cell wall polymers decreases during the night in wild-type Arabidopsis plants and is abolished from ~4 h into the night in the starchless pgm mutant, showing a close temporal relationship to the depletion of sucrose in the mutant (Boxe-Fontvieille et al., 2014; Ishihara et al., 2015). These labeling experiments are in close agreement with our results on diurnal changes in cellulose synthesis estimated from CSC dynamics. Based on our data and the report from Ishihara et al. (2015), it is tempting to speculate that the production and secretion of other cell wall components, like pectin and hemicelluloses, may be regulated in a similar manner. This would perhaps not be surprising as they are also abundant components of cell walls, their synthesis is a significant resource-consuming flux in growing cells, and their deposition likewise depends on vesicle trafficking and exocytosis (McFarlane et al., 2014; Kim and Brandizzi, 2016).

It has been shown previously that cellulose synthesis is controlled by light through the action of the photoreceptor Phytochrome B (Bischoff et al., 2011), in contrast to our findings indicating that light does not play a part in diurnal CesA dynamics. However, that study focuses on and highlights the distinction between etiolated plants growing in complete and constant darkness and light-grown seedlings growing in a long photoperiod. Furthermore, there were no differences in CesA behavior between light- and dark-grown seedlings in wild-type plants, the differences only becoming apparent when a subunit of the CesA complex was mutated, resulting in slower CesA speeds in the dark. Taken together, our findings and those of Bischoff et al. (2011) suggest that there may be functional differences in the CesA populations between etiolated seedlings and those growing in the light. However, once growing in the light, the CesA population exhibits diurnal dynamics that do not depend on light directly, but respond to the metabolic effects of light on photosynthesis and carbon assimilation.

The lack of correlation between UDP-glucose levels and apparent cellulose synthesis rates is intriguing. However, it has been proposed, although never proven, that UDP-glucose may undergo metabolic channeling to the active site of CesA by sucrose synthase associated with the CesA rosette as sucrose is broken down (Haigler et al., 2000). Therefore, the relevant UDP-glucose concentration, i.e., that at the active site, may not be apparent in UDP-glucose measurements conducted on whole plant extracts, as was done here. Another possible explanation is that a metabolic signaling process, rather than substrate levels directly, is responsible for the observed CesA dynamics. While the identity of the metabolic sensor or signaling pathway responsible for the observed patterns of CesA trafficking is not known at this point, several candidate sugar signaling pathways have been reported, including sucrose/trehalose-6-phosphate signaling, and several kinases such as Target of Rapamycin, Sucrose-Nonfermenting (SNF1), and hexokinases (Moore et al., 2003; Cookson et al., 2005; Ruan et al., 2010; Dobrenel et al., 2011; Eveland and Jackson, 2012; Yadav et al., 2014). It is not known whether any of these pathways are involved in the regulation of vesicle trafficking; however, they are good candidates to explore. Mechanisms linking cellular metabolic status to the regulation of vesicular secretion through Golgi-localized lipid binding SEC14 domain proteins have been proposed in animal cells (Bankaitis et al., 2010). Arabidopsis contains a family of these proteins and they are known to regulate vesicle trafficking (Huang et al., 2016). Investigation of the involvement of these pathways in the regulation of CesA trafficking and vesicle trafficking in general may be a promising lead for future research.

Superimposed on the accumulation of cell wall material is cell expansion, resulting in increases in cell and plant size. Regulation of expansion growth rate is known to be one of the chief outputs of the plant circadian clock (Nözue et al., 2007; Dowson-Day and Millar, 1999). This regulation appears to work through the effect of the clock on the expression of growth-promoting PHOTOPERIOD-INTERACTING FACTOR (PIF) transcription factors, which further modulate auxin signaling (Nözue et al., 2011; Hornitschek et al., 2012; Seaton et al., 2015). However, the final step in this regulatory cascade is not clear. The exclusive effect of the clock on the activity of cell wall loosening proteins active at pH5 and high loads strongly suggests that expansins are the ultimate targets in the regulation of expansion growth by the clock (Cosgrove, 2016b). This is also in line with published observations that several expansin genes have rhythmic, circadian expression patterns (Harmer et al., 2000; Bläsing et al., 2005). Some of these have been implicated as targets of PIF transcription factors (Leivar et al., 2009; Hornitschek et al., 2012), as well as being directly targeted by the clock transcription factor CIRCADIAN CLOCK ASSOCIATED1 (Nagel et al., 2015). Interestingly, we observed that changes in cell wall extensibility are also partly mediated by proteins active at pH6 and under low loads in short photoperiods (Figure 3A). Their activity is not controlled by the clock (Figure 3B), but may be dependent on light signaling and/or metabolic inputs. Xyloglucan endotransglycosylase/hydrolases (XTHs) may be responsible for this activity because of their characteristic pH-optima (Maris et al., 2011) and their ability to induce wall extension under low loads (Miedes et al., 2013).

The EN versus ED differences in cell wall extensibility were abolished by heat inactivation (Figures 3A and 3B), showing that they resulted from changes in the activity of cell wall loosening proteins active at pH 5.0 and 6.0 that are present in the wall. However, the diurnal regulation of cell expansion and the contribution of these proteins to diurnal growth rhythms (Figure 2) could depend not only on their relative abundance or activity, but also on possible variations in turgor and pH. High turgor stimulates expansion by exerting a greater force on the wall, while low pH has been shown to stimulate expansion by increasing the activity of expansins. Higher turgor will favor the contribution of expansins, consistent with their more pronounced effects on creep rate under higher loads (Miedes et al., 2013; Suslov et al., 2015). Diurnal and circadian variations in turgor have been demonstrated in specialized cells such as stomata and pulvini (Hennessey and Field, 1992). Although it has never been shown to occur in Arabidopsis hypocotyls, diurnal changes in turgor there could reflect rhythmic diurnal changes in the accumulation/mobilization of osmotically active assimilates (Haydon et al., 2011) and variation in plant water status brought on by stomatal opening and closing, known to affect turgor (Ache et al., 2010; Bramley et al., 2015).

Changes in cell wall acidification may also play a role in the regulation of cell expansion. Cell wall acidification mediated by plasma membrane H+-ATPases (Falhof et al., 2016) stimulates expansins due to their low-pH optima (Cosgrove, 2016b) and is also the driving force for solute uptake to generate cell turgor.
H+-ATPases are major consumers of ATP, and their activity may be increased by light, which elevates the ATP level (Liang et al., 2016). On the other hand, light strongly up- or downregulates H+-ATPase activity via phosphorylation (Kinoshita and Shimazaki, 1999; Hohn et al., 2014). To our knowledge, no diurnal or circadian rhythms in cell wall pH have ever been demonstrated. However, there is clear evidence for circadian rhythms in auxin content in Arabidopsis shoots (Jouve et al., 1999). Auxin is known to promote cell wall acidification by activating H+-ATPases (Spartz et al., 2014), and it is therefore likely that cell wall pH variation also contributes to diurnal growth rhythms. These data show that the contribution of proteins active at pH 5.0 and 6.0 could vary depending on the phase of the diurnal growth cycle and external conditions. The role of expansins may be prevalent in conditions optimal for growth, i.e., in highly turgid cells having favorable energetic status. By contrast, the contribution of proteins active at pH 6.0 may increase in more stressful conditions.

While we use the Arabidopsis hypocotyl as a simple model of one-directional cellular and organ growth, it is possible that this system may be unique in certain aspects and may not reflect the regulation of growth in other organs. For example, an investigation of rhythmic patterns of leaf growth and leaf movements in Arabidopsis uncovered that light was an essential factor promoting leaf expansion during the day (Dornbusch et al., 2014); however, light suppresses hypocotyl extension (Nozue et al., 2007). Hypocotyls and leaves have somewhat opposite growth responses, in that darkness, low light, or short photoperiods stimulate hypocotyl extension while inhibiting the growth and development of leaves in what is known as the etiolation response (Fankhauser and Chory, 1997). This serves to direct the plant’s resources to rapid expansion growth to “grow out” of zones of darkness or shade, e.g., when seeds germinate underground. When light is sensed by photoreceptors, hypocotyl growth is rapidly suppressed and leaf growth is stimulated to produce photosynthetic leaf area and begins to assimilate carbon. Thus, the regulation of leaf expansion growth is rather different to that of the hypocotyl. On the other hand, biosynthesis is inevitably under metabolic constraints in any cell or organ and must be regulated in accordance with the supply of available resources (Smith and Stitt, 2007). This regulation may differ slightly in green organs that fix their own carbon (e.g., leaves) compared with non-green carbon-importing cells in hypocotyls and roots; however, the constraints remain the same and unregulated biosynthesis in times of scarce supply can exhaust the supply of carbon and energy and result in death. Therefore, unlike organ-specific requirements for expansion growth in response to environmental cues, it is unlikely that hypocotyls are unique with respect to the regulation of cellular biosynthesis.

This work demonstrates that cellulose biosynthesis and cell expansion are regulated by different mechanisms, with the ensuing result that they can be uncoupled for short time periods. Of course, there cannot be complete uncoupling between the two processes, as wall expansion cannot proceed limitlessly without the synthesis and addition of new wall material to maintain the integrity of the cell wall. While we show that plant carbon status defines the amount of cellulose the cell produces, Stewart et al. (2011) showed that sucrose also affects hypocotyl expansion growth in Arabidopsis. In that study, growth became very slow, while still maintaining diurnal rhythms, in short days in the absence of external carbon similar to our conditions, while the addition of sucrose strongly stimulated expansion growth. This suggests that on longer timescales, the rate of cell expansion adjusts to the amount of cell wall material that is able to be produced. This is reminiscent of the cell wall integrity sensing mechanisms that have been shown to inhibit expansion growth in situations where cellulose synthesis is inhibited using genetic or chemical means and under conditions of stress, known to involve several receptor-like-kinases and wall-associated-kinases (Hématy et al., 2007; Wolf et al., 2012). These kinases are worthy of investigation as the possible missing piece of the puzzle in the regulation of growth in response to resource supply.

In summary, we show that cell wall synthesis and cell expansion can be uncoupled in Arabidopsis hypocotyls on short timescales. We conclude that this uncoupling is based on different regulatory mechanisms of cell wall biosynthesis and cell expansion. The biosynthesis of cellulose, and possibly other cell wall polymers, is regulated through a relatively simple one-way control mechanism involving metabolic signals associated with the carbon status of the plant. It is independent of the circadian clock or light signaling. On the other hand, cell expansion is controlled by the plant circadian clock mediating protein-dependent changes in cell wall extensibility. At the moment, these observations are only valid for the hypocotyl, which is a highly specialized organ, and for a rather extreme photoperiod. Therefore, a much closer coupling between wall synthesis and cell expansion may exist in other cell types or other conditions. Our finding that the two basic processes underlying plant growth are regulated by different mechanisms enables their independent manipulation. This could open up avenues to modulate plant growth and biomass production.

METHODS

Plant Material

YFP-CesA6 expressed under the native CesA6 promoter in the prc1-1 background was provided by D. Ehrhardt (Paredes et al., 2006), elf3-4 mutant in Ws-2 background was provided by Andrew Millar (Hicks et al., 1996).

Plant Growth

Seeds were sterilized with 70% ethanol with a drop of Triton X detergent for 30 min and washed four times with sterile water. Seeds were spread on Murashige and Skoog agar plates with 0 or 2% sucrose and stratified at 4°C for 3 d. Plates were transferred to controlled environment chambers (Percival Scientific) running on a 4-h-light/20-h-dark 20°C/18°C photoperiod and an irradiance of 100 µmol m\(^{-2}\) s\(^{-1}\) provided by fluorescent lamps during the day, and grown vertically. Confocal imaging was done on the seventh day after stratification. For continuous light treatments, plants were grown in 4-h photoperiods for 4 d and transferred to continuous low light at 20°C for 2 d. Lamps were darkened with plastic shades during continuous light to give an irradiance of 12 µmol m\(^{-2}\) s\(^{-1}\). For creep measurements, plants were harvested and frozen in liquid nitrogen on the seventh day in diurnal cycles and on the second day of continuous light.

Microscopy

For live-cell imaging, seedlings expressing YFP-CesA6 were mounted on glass slides under an agarose gel pad and imaged on a Nikon Ti-E inverted
confocal microscope with a CSU-X1 Yokogawa spinning disc head, using a CFI APO TIRF 3100 N.A. 1.49 oil immersion objective, an evolve CCD camera (Photometrics Technology), and a 1.2 × lens between the spinning disc and camera. YFP was excited at 491 nm using a multichannel dichroic and an ET525/50M band-pass emission filter (Chroma Technology). Images were acquired using Metamorph online premier, version 7.5. Exposure times were 400 ms.

**Growth Imaging**

To image plant growth in real time, a modified Nikon DSLR camera was used, where the infrared cut-off filter in front of the CCD sensor was removed. A long-pass near-infrared filter was fitted to the objective so that only infrared radiation entered the camera, allowing the plants to be imaged day and night. Plants were continuously illuminated from behind with infrared light at 900 nm, shown not to stimulate plant photoreceptor systems. Images were taken every 5 min.

**Image Analysis**

Confocal space-time stacks of fluorescently labeled CesA were analyzed using custom-written scripts running in Fiji and R. For CesA particle density measurements, one fluorescent replica was a confocal time-lapse movie of 30 frames and lasting 5 min, with each movie being collected on a different plant. Each movie was analyzed using our particle tracking algorithm, detecting tens to hundreds of CesA particles in each movie. Particle density was calculated by dividing the number of detected particles by the area imaged in the movie in μm². Multiple movies were collected for each time point or treatment, and the means and se are reported in the figures.

**CesA Particle Tracking**

The first 20 frames of each stack were used for analysis. The positions of CesA particles in each frame were detected by applying a Laplacian filter with a smoothing kernel width of 1.5 pixels. The particles were then detected by finding local maxima with a noise tolerance of 800.

Tracks were detected in R. The detected particles in all frames were encoded as three-dimensional x-y-t points. Particles in the top frame (first time point) of each movie were used as starting points. Proceeding iteratively, unit vectors from each starting point to all other points in all frames were computed. Vectors with a t-direction component of at least 0.9 were then retained. This removes the majority of vectors which point to CesA particles in other parts of the image (and thus probably not the same track).

The remaining points are then expected to contain points corresponding to the track of interest (the same CesA particle moving over time) and a number of erroneous points corresponding to other CesAs moving into the search region, or noise points. Due to the very regular movement of plasma membrane CesA particles (constant speed, constant direction), the points in a track should have a very consistent direction through the x-y-t space, looking from the starting point, while the erroneous points would deviate from this direction as outliers. A median direction vector was calculated from the retained points using the L1 multivariate median (Vardi and Zhang, 2000), a measure with low sensitivity to outliers. All the points were then compared with the median direction using cosine similarity, a measure that expresses the cosine of the angle between two direction vectors. Points with a cosine similarity of at least 0.8 to the median direction were retained. The L1 median was then recalculated on the remaining points with cosine similarity of at least 0.95 were retained. This way, only points with highly similar directions are selected. These points are then treated as a track. Tracks with fewer than five points were discarded.

Each track was then centered by subtracting its mean and a line of best fit in the x-y plane was fitted by extracting the first principal component, a line which minimizes the perpendicular distances between all points and itself. The percentage of explained variance (similar to r² in least squares regression) was used as a measure of linearity for the track and tracks with linearities below 90% were discarded.

The tracks that satisfy the above criteria were then treated as a single CesA particle moving over time. For these tracks, each point was projected onto the line of best fit to infer the “true” position of the particle, treating deviations from the line as error. The position of the particle along the line was then regressed against time and the slope taken as the speed of movement along the track. The number of tracks was divided by the area of the region of interest to obtain particle density (CesA/area).

It must be noted that at a given time a proportion of SmaCCs move in a similar manner and with similar speeds to plasma membrane CesA complexes and cannot be distinguished from them with certainty in all instances. Thus, speed and density measurements are only accurate in situations where few SmaCCs are present and the vast majority of all particles are plasma membrane CesA complexes, or vice versa. In situations where SmaCCs are in high abundance, plasma membrane CesA numbers are overestimated, while speed distributions are biased toward the movement properties of SmaCCs.

**Biomechanics Measurements**

Cell wall extensibility was estimated by the creep method in which frozen/thawed hypocotyls were extended under a constant load. On the seventh day after germination, each Arabidopsis thaliana seedling was transferred to a 2-mL Eppendorf test tube, which was closed and plunged into liquid nitrogen. The frozen seedlings were stored at −20°C and extended in creep tests within 2 weeks after freezing. Freeze/thawing disrupts the cells while leaving the cell wall intact, making the measurements independent of turgor variations, while the constant load resembles the action of turgor on the cell wall and allows the force applied to the wall to be controlled. Cell wall heat inactivation was performed at 90°C for 3 min as described by Suslov et al. (2015). The optimization of the heat inactivation procedure is detailed in Supplemental Figure 6. For each cell wall creep (time-dependent deformation) measurement, one thawed Arabidopsis seedling was secured in a custom-built constant-load extensiometer (Suslov and Verbelen, 2006) such that a 2-mm-long hypocotyl segment located 1.5 mm below the cotyledons was placed between the clamps of the setup. This segment was then preincubated in a buffer (20 mM MES-KOH, pH 6.0, or 20 mM Na-acetate, pH 5.0) in the relaxed state for 2 min, after which a constant load (1200, 1600, or 2000 mg) was applied, and cell wall extension was recorded for 15 min in the same buffer as during the preincubation. Conducting the measurements in buffered conditions eliminates the role of wall pH variations that may occur in vivo. The pH of buffers used corresponds to typical values in the plant apoplastic space (Felle, 2001). The early (0–5 min after loading) cell wall deformation is known to contain large viscoelastic components having no direct relation to cell wall extensibility (Suslov and Verbelen, 2006). This deformation was taken into account to calculate the length of extending cell wall samples at 5 min after loading (Miedes et al., 2013). The average creep rate was then calculated during the interval 5 to 15 min after loading using the formula \( \ln L_{15\text{min}} - \ln L_{5\text{min}} \times 100\% \), where \( L_{15\text{min}} \) and \( L_{5\text{min}} \) indicate the length of an extending hypocotyl segment at 15 and 5 min after loading, respectively, and \( T \) is the time during which the average creep rate is calculated (10 min) (Miedes et al., 2013).

**Metabolite Analysis**

Plants were grown as described above and harvested at relevant time points by quenching in liquid nitrogen. Approximately 200 seedlings were
pooled per biological replicate sample. Material was homogenized while frozen in a ball mill (Retsch) and aliquoted into Eppendorf tubes. Samples were extracted in a 3:7 chloroform/methanol solution. UDP-glucose was measured using reverse-phase liquid chromatography-tandem mass spectrometry as described by Arrivault et al. (2009).

Statistical Analysis
All statistical analysis was performed in R. Time courses of particle densities were tested for a significant effect of time using one-way parametric ANOVA, testing the null hypothesis that particle densities do not vary over time. Time courses of CesA speed were tested for a significant effect of time using the nonparametric Kruskal-Wallis rank test as the CesA speed distributions were not normally distributed, testing the null hypothesis that CesA speed does not vary over time. Pairwise comparisons of particle densities and creep rates were conducted using Student’s t test. Null hypotheses were rejected below the 0.05 P value threshold.

Supplemental Data
Supplemental Figure 1. Inhibition of photosynthesis by DCMU.
Supplemental Figure 2. Heat inactivation of cell wall proteins under different conditions.
Supplemental Figure 3. Cellulose synthase localization follows a diurnal pattern in living Arabidopsis cells growing in very short days without exogenous sucrose.
Supplemental Figure 4. Cellulose synthase localization to the plasma membrane in the light requires active photosynthesis or an alternative carbon source.
Supplemental Figure 5. A favorable metabolic status is necessary and sufficient to promote localization of cellulose synthase to the plasma membrane.
Supplemental Figure 6. Arabidopsis seedlings synthesize cellulose constantly under a favorable metabolic status.
Supplemental Figure 7. Speed of moving particles in seedlings grown on medium without exogenous sucrose and imaged throughout a diurnal cycle or after a 4-h extension of the night.

Supplemental Table 1. Variation in CesA particle densities over a diurnal cycle and an extended night in seedlings grown in the absence of sucrose.
Supplemental Table 2. Variation in CesA particle densities over a diurnal cycle and an extended night in seedlings grown in the presence of 2% sucrose.
Supplemental Table 3. Variation in CesA particle speed over a diurnal cycle and an extended night in seedlings grown in the presence of 2% sucrose.
Supplemental Table 4. Variation in UDP-glucose content over a diurnal cycle and an extended night in seedlings grown in the presence and absence of 2% sucrose.
Supplemental Movie 1. Cellulose synthase localization follows a diurnal pattern in living Arabidopsis cells growing in very short days without exogenous sucrose.
Supplemental Movie 2. Cellulose synthase localization to the plasma membrane in the light requires active photosynthesis or an alternative carbon source.
Supplemental Movie 3. A favorable metabolic status is necessary and sufficient to promote localization of cellulose synthase to the plasma membrane.
Supplemental Movie 4. Arabidopsis seedlings synthesize cellulose constantly under a favorable metabolic status.

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