Pausing of Golgi Bodies on Microtubules Regulates Secretion of Cellulose Synthase Complexes in *Arabidopsis*

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Plant growth and organ formation depend on the oriented deposition of load-bearing cellulose microfibrils in the cell wall. Cellulose is synthesized by plasma membrane–bound complexes containing cellulose synthase proteins (CESAs). Here, we establish a role for the cytoskeleton in intracellular trafficking of cellulose synthase complexes (CSCs) through the in vivo study of the green fluorescent protein (GFP)-CESA3 fusion protein in *Arabidopsis thaliana* hypocotyls. GFP-CESA3 localizes to the plasma membrane, Golgi apparatus, a compartment identified by the VHA-a1 marker, and, surprisingly, a novel microtubule-associated cellulose synthase compartment (MASC) whose formation and movement depend on the dynamic cortical microtubule array. Osmotic stress or treatment with the cellulose synthesis inhibitor CGA 325'615 induces internalization of CSCs in MASCs, mimicking the intracellular distribution of CSCs in nongrowing cells. Our results indicate that cellulose synthesis is coordinated with growth status and regulated in part through CSC internalization. We find that CSC insertion in the plasma membrane is regulated by pauses of the Golgi apparatus along cortical microtubules. Our data support a model in which cortical microtubules not only guide the trajectories of CSCs in the plasma membrane, but also regulate the insertion and internalization of CSCs, thus allowing dynamic remodeling of CSC secretion during cell expansion and differentiation.

INTRODUCTION

Anisotropic cell expansion drives plant growth and organ formation and depends primarily on the properties of the cell wall (Baskin, 2005). The load-bearing component of the plant cell wall consists of cellulose microfibrils, which play a key role in generating differential resistance to turgor pressure to allow anisotropic expansion (Green and Selker, 1991). Shortly after the discovery that cellulose microfibrils are deposited in precisely oriented layers during development (Probine and Preston, 1961, 1962), it was proposed that long cytoplasmic elements sensitive to colchicine could direct the orientation of cellulose microfibrils in the wall (Green, 1962). The discovery of cortical microtubules soon afterward, and the observation of their parallel alignment with cellulose microfibrils, gave rise to the microtubule-microfibril alignment hypothesis (Ledbetter and Porter, 1963). Subsequently, numerous studies reported correlations in the orientation of cortical microtubules and cellulose microfibrils (reviewed in Baskin, 2001). However, the alignment hypothesis became increasingly controversial as evidence for absence of correlation was found in certain cell types (Emons, 1982; Himmlersbach et al., 2003; Sugimoto et al., 2003).

In vascular plants, cellulose is synthesized by a plasma membrane–localized cellulose synthase complex (CSC), recognizable as a hexameric rosette structure with a diameter of 25 to 30 nm (Mueller and Brown, 1980) and a cytoplasmic component ~45 to 50 nm in diameter (Bowling and Brown, 2008). The only known component of the CSC is the cellulose synthase catalytic subunit (CESA) (Kimura et al., 1999). Identification of the genes encoding CESAs in higher plants (reviewed in Somerville, 2006) and advances in live-cell imaging techniques permitted visualization of fluorescently tagged CSCs and microtubules simultaneously in living cells and provided powerful evidence that cortical microtubules define the trajectories of CSCs (Paredes et al., 2006). Experimental evidence presented by Paredes et al. lends strong support to the microtubule-microfibril alignment hypothesis. Nevertheless, the mechanism by which microtubules guide CSC trajectories remains unknown and represents an important focus of current research.

The importance of the actin cytoskeleton in cell expansion in non-tip-growing cells is only beginning to be revealed (Smith and Oppenheimer, 2005). Wightman and Turner (2008) recently showed that actin participates in secondary cell wall synthesis by delivering organelles containing fluorescently labeled CESA to bands of secondary cell wall thickenings in developing xylem cells. Thus, the role of actin in cellulose synthesis seems to be related to the intracellular trafficking of cellulose synthases, but this needs to be further confirmed.
While it is generally accepted that the microtubule cytoskeleton influences patterns of deposition of cellulose, the actin and/or microtubule cytoskeleton may also influence the rate of cell wall synthesis by regulating the delivery of cell wall–synthesizing enzymes to the plasma membrane. The regulation of cellulose synthesis activity through cytoskeleton-dependent intracellular trafficking has remained largely unexplored. Here, we studied the intracellular trafficking of CESA3, an essential enzyme for primary cell wall cellulose synthesis. We demonstrate that Golgi bodies carrying CSCs are distributed throughout the cell via the acto-myosin system and that Golgi bodies pause on cortical microtubules for the targeted delivery of CSCs to the plasma membrane. Under conditions of growth cessation or treatment with a cellulose synthesis inhibitor, CSCs are internalized into compartments that show movement dependent on cortical microtubule array dynamics. Together, our data establish novel roles for the actin and microtubule cytoskeleton in regulating cellulose synthesis in the primary cell wall.

RESULTS

CSC Density and Trajectories Are Developmentally Regulated

To examine regulation of the localization of CESA proteins, we used stably transformed *Arabidopsis thaliana* lines expressing green fluorescent protein (GFP)-tagged CESA3 fusion protein under the control of the CESA3 endogenous promoter. This construct has been shown to complement the cesa3 mutant phenotype (Desprez et al., 2007). We studied GFP-CESA3 on the outer face of epidermal cells in 3-d-old etiolated hypocotyls, in which epidermal cells show an acropetal developmental gradient (Gendreau et al., 1997). Cells near the apical hook elongate slowly and deposit a thick, external cell wall, cells in the middle of the hypocotyl elongate rapidly, and cells at the base are fully elongated (Refregier et al., 2004). As described previously, discrete GFP–CESA3 particles were found aligned at the cell surface (Figure 1A; Desprez et al., 2007). These particles exhibited steady, bidirectional movement in the focal plane of the plasma membrane (Figures 1B and 1C; see Supplemental Movie 1 online). Immunogold labeling of cryosections confirmed the plasma membrane localization of the GFP-CESA3 surface particles (Figure 1D), leading us to conclude that these particles represent active CSCs in the plasma membrane. CSC density was highest in cells in the apical hook and decreased toward the base of the hypocotyl (Figure 2). The orientation of CSC trajectories and cortical microtubules visualized using a GFP fusion to *Arabidopsis* α-tubulin6 (GFP-TUA6) varied from longitudinal to transverse in the apical hook and hypocotyl top (Figures 2A and 2B), becoming strictly longitudinal at the base of the hypocotyl (Figures 2C and 2D). Notably, the densities of cortical microtubules and CSCs were also correlated (Figure 2).

GFP–CESA3 Accumulates in Golgi Bodies and in the VHA-a1 Compartment

GFP-CESA3 labels ring-shaped structures with bright, peripheral punctae, previously shown to be Golgi bodies (Figure 1H; Paredez et al., 2006). Golgi bodies circulate through the cytoplasm via the actomyosin system (Sparkes et al., 2008) and are characterized by rapid, nonlinear movement (Figure 1I; see Supplemental Movie 1 online). Electron microscopy (EM) images of immunogold-labeled cryosections of hypocotyl epidermal cells confirmed that GFP-CESA3 was present at the periphery of the Golgi stack, consistent with their ring shape in confocal images (Figures 1P and 1Q). In addition to Golgi bodies, we identified a second, relatively rare population (<1 observed per cell per 10 min imaging period) of smaller, homogeneously labeled particles with the same rapid, nonlinear movement as Golgi bodies (Figures 1J and 1K). These particles were invariably labeled with the early endosome/trans-Golgi network (TGN) marker VHA-a1-mRFP (Dettmer et al., 2006) in transgenic lines expressing both markers (41 time series analyzed; Figures 1N and 1O). VHA-a1 compartments rapidly associated and dissociated with Golgi bodies (Figures 1L to 1O; see Supplemental Movie 2 online). Most often a single VHA-a1 compartment was found centered beneath a Golgi stack (Figure 1L). The occasional presence of GFP-CESA3 in the TGN was further confirmed in EM micrographs (Figures 1Q and 1R). Thus, we will refer to these particles as VHA-a1/GFP-CESA3–containing compartments.

Regulated Distribution of GFP-CESA3 in a Unique Population of Intracellular Particles

We identified a third population of small, bright particles just below the plasma membrane, which were distinguished from CSCs by their fluctuating velocities and intracellular localization (Figures 1E to 1G; Supplemental Figures 2A to 2F online). These particles were likewise distinguished from Golgi bodies and VHA-a1/GFP-CESA3–containing compartments by their smaller size and linear trajectories (Figures 1E to 1G). In basal hypocotyl cells with low CSC density, GFP-CESA3 signal was preferentially found in these bright intracellular compartments (Figure 2D). Interestingly, treatment with 500 mM mannitol to induce osmotic stress or treatment with 50 μM cycloheximide both caused a rapid decrease in CSC density and a simultaneous increase in the density of the same bright compartments (Figures 3A and 3B). To determine the response kinetics, we examined the redistribution of GFP-CESA3 under mannitol treatment in the root tip, a widely used, well-characterized system (Robinson et al., 2008) where the absence of a thick cuticle allows rapid penetration of treatment solutions. In the root tip, CSC redistribution was complete within 6 min (five independent experiments), suggesting that these bright intracellular compartments result from internalization of CSCs as opposed to a block in secretion (Figures 3C and 3D).

CGA 325’615 (CGA), a potent inhibitor of cellulose synthesis in cotton fibers (Peng et al., 2001), causes reduced crystalline cellulose content and isotropic cell expansion in *Arabidopsis* (see Supplemental Figure 3 online). CGA treatment did not affect microtubules labeled with either GFP-TUA6 or a GFP fusion to the *Arabidopsis* microtubule end binding protein1A (EB1-GFP) or actin filaments marked by an N-terminal GFP fusion to the second actin binding domain of *Arabidopsis* fimbrin 1 (GFP-FABD2) or alter the morphology of the Golgi apparatus or organelles observed by EM (see Supplemental Figure 4 online).
Figure 1. Four Distinct Localizations of GFP-CESA3 in Epidermal Cells of Etiolated Arabidopsis Hypocotyls.

(A) to (D) Particles corresponding to CSCs in the plasma membrane.
(A) Maximum projected z-stack of first time point in a series showing CSC arranged in linear tracks in the plasma membrane focal plane.
(B) Average projection of the same time series showing organized CSC trajectories.
(C) Kymograph along the trajectory indicated by a line in (B). Time is represented along the vertical axis, and a 5-min scale bar is shown at left (see Supplemental Figure 1 online for information on interpreting kymographs). CSC movement is steady and bidirectional (see Supplemental Movie 1 online).
(D) Ultrathin cryosection of epidermal cells in the upper hypocotyl of seedlings expressing GFP-CESA3, labeled with anti-GFP primary antibody and a Nanogold-coupled secondary antibody. Control labeling of wild-type seedlings was negative. Discrete CSCs are labeled in the plasma membrane. Bars = 500 nm.
(E) to (G) Small, bright, intracellular particles with fluctuating velocity (referred to as MASCs).
(E) Maximum projected z-stack of first time point in a series acquired in a cell near the apical hook; a small, bright, intracellular particle is indicated by an arrowhead.
(F) Average projection of the time series.
(G) Kymograph along the trajectory indicated in (E). The movement of the small, bright particle is along a linear track, but different from that of the surrounding CSCs. Initial unsteady translocation is followed by acceleration in the opposite direction.
(H) and (I) GFP-CESA3–labeled Golgi bodies.
(H) GFP-CESA3 is found exclusively at the periphery of Golgi bodies and is characterized by bright punctae (deconvolved image).
(I) Trajectories of four Golgi bodies indicated by colored lines overlaid on an image from the time series. Golgi body movement is nonlinear and interrupted by pauses.
(J), (K), (N), and (O) VHA-a1/GFP-CESA3–containing compartments.
(J) Image from a time series showing a GFP-CESA3–labeled compartment (arrowhead) just below the plasma membrane.
(K) The white line illustrates the trajectory of the compartment marked in (J). The movement pattern is similar to that of Golgi. The particle associates with a Golgi body before disappearing into a lower focal plane.
Time-lapse imaging of GFP-CESA3 revealed that CGA treatment resulted in clearance of CSC from the plasma membrane, concomitant with an increase in the density of small, bright intracellular compartments (Figure 3E) identical in appearance, behavior, and velocity to those observed in untreated basal hypocotyl cells and those induced by osmotic stress or cycloheximide treatment (cf. Figures 2D, 3A, 3B, and 3E; see Supplemental Figure 2G online). Mock treatment with an identical dilution of DMSO did not observably alter GFP-CESA3 localization (see Supplemental Figure 5A online).

Characterization of CGA-Induced Compartments

CGA-induced compartments were observed to move with fluctuating velocities along linear tracks (Figure 3F; see Supplemental Movie 3 online). These linear tracks could be visualized in average projections of time series, and their position was observed to change over time (Figure 3G). In image acquisitions of 10 to 15 min, individual compartments could be found that were stationary, migrating steadily at 10 to 3000 nm/min, or moving as fast as 8900 nm/min (Figure 3N; see Supplemental Movie 3 online). The linear trajectories of CGA-induced compartments contrast with the movement of intracellular compartments known to be regulated by the actomyosin system, such as Golgi bodies. To test if an intact actin cytoskeleton is required for the movement of CGA-induced compartments, we treated GFP-CESA3–expressing seedlings first with CGA and then with the actin-disrupting agent Cytochalasin D (CytD). CytD causes fragmentation of actin microfilaments and inhibits their dynamics (see Supplemental Figures 5D and 5E online; Foissner and Wasteneys, 2007). Treatment with CytD resulted in aggregation of GFP-CESA3–labeled Golgi bodies within 25 min, but no difference was found in the density or velocity distribution of CGA-induced compartments compared with CGA treatment alone (Figures 3H, 3I, and 3N).

To further study the nature of CGA-induced compartment movement, seedlings were pretreated with the microtubule-stabilizing drug taxol and then treated with taxol + CGA. The resulting CGA-induced compartments showed a density similar to that in CGA treatment alone (Figure 3J); however, the addition of taxol greatly reduced the motility of the compartments (Figures 3K and 3N). Out of 989 individual particles measured, only 11 were found with velocities equal to or greater than the mean velocity of 543 nm/min measured in CGA treatment (Figure 3N).

As a third test, seedlings were pretreated with 7 μM oryzalin, which results in depolymerization of most cortical microtubules within 4 h (see Supplemental Figures 5B and 5C online). The subsequent treatment with oryzalin + CGA resulted in reduced CSC density; however, we never observed characteristic CGA-induced compartments with normal density near the cell surface (41 cells from two independent treatments; Figure 3L). In one cell, we found a small group of aligned particles resembling CGA-induced compartments, but these particles were stationary (Figure 3M), suggesting they were associated with fragments of stable, resistant microtubules.

Based on these results, we conclude that an intact, dynamic cortical microtubule array is essential for the movement of CGA-induced compartments, and the actin cytoskeleton does not appear to have a role in their movement. Henceforth, we will refer to CGA-induced compartments as microtubule-associated cellulos synthase compartments (MASCs). To verify that MASCs observed in untreated cells were also associated with cortical microtubules, we transiently transformed cotyledons of the GFP-CESA3–expressing line with the microtubule binding domain (MBD) of the microtubule-associated protein 4 fused to monomeric red fluorescent protein (mRFP). Although plants stably overexpressing GFP-MBD and cultured on kanamycin were observed to show growth defects in one study (Granger and Cyr, 2001), MBD remains a widely used marker for microtubules in plants and yields signals highly similar to that obtained with immunolabeling of tubulin (Sainsbury et al., 2008). In the absence of any drug treatments, we observed MASCs migrating along cortical microtubules labeled by mRFP-MBD (Figure 3O; see Supplemental Movie 4 online). Intriguingly, MASC particles moving along linear tracks merge with one another, progressively increasing in intensity and cotranslocating until a split occurs (Figure 3P; see Supplemental Movie 3 online). This merging behavior is reminiscent of that observed for the DAM1 ring protein, known to move on depolymerizing microtubule ends (Westermann et al., 2006). Indeed, MASC velocities (0 to 8.9 μm/min) are within the range of velocities measured for depolymerization at leading or lagging microtubule ends (0 to 30 μm/min) and polymerization at leading ends (0 to 15 μm/min) (Shaw et al., 2003).

The Golgi Apparatus Pauses on Cortical Microtubules, and Golgi Bodies and the VHA-a1 Compartment Physically Interact with MASCs

High-frame-rate high-resolution imaging of GFP-CESA3 in combination with mRFP-MBD or VHA-a1-mRFP markers yielded several salient observations. GFP-CESA3–labeled Golgi bodies...
were observed to pause at discrete sites on microtubules (Figures 4A to 4C; see Supplemental Movie 4 online). In one representative time series, 63 Golgi bodies were tracked and pause events were quantified for each individual Golgi body. A pause event was defined as a period of immobility lasting at least 8 s. One or more pause events were observed for 48 of the 63 Golgi bodies (76%), and in each instance, the pause event occurred on a microtubule. The remaining 24% of the Golgi bodies moved rapidly in and out of frame without undergoing a pause event. We observed multiple Golgi bodies successively pausing at the same positions (45 independent time series analyzed; presented in the following section). Detailed analysis in untreated cells in the hypocotyl revealed that Golgi pauses occurred in proximity to MASCs (Figures 4D and 4E) and could last as long as 73 s (\( n = 34, \text{mean} = 22 \) s). VHA-a1 compartments were also observed to pause at MASCs, even in the absence of an associated Golgi body (seven events from five independent experiments; Figure 4D). Visible connections between MASCs and Golgi bodies or VHA-a1 compartments were prominent in CGA-, mannitol-, and cycloheximide-treated cells (see Supplemental Movies 3 and 5 online; Figure 4E).

CSC Delivery to the Plasma Membrane Depends on Interactions between the Golgi Apparatus and the Cytoskeleton

The high density of CSCs in cells near the top of the hypocotyl has thus far prevented visualization of individual CSC particle insertions (DeBolt et al., 2007b). We circumvented this problem by focusing our attention on cells closer to the hypocotyl base, where CSC density is lower (Figure 2C). Through the rigorous analysis of time series acquired in these untreated cells, we were able to identify seven CSC insertion events. Each insertion event was analyzed both manually and through kymographs to verify the absence of the CSC in the initial frames, to monitor the movement of all particles in proximity to the identified insertion site, and to measure the velocity of the newly inserted CSC. The newly inserted CSCs all began translocating with a velocity approximating 270 nm/min, as expected.

Figure 2. CSC Density and Trajectory Change with the Developmental Gradient along the Length of Etiolated Hypocotyls.

(A) to (D) In the left-most and central column are maximum projected z-stacks and average projections of time series, respectively, of CSCs visualized in the outer face of epidermal cells in GFP-CESA3–expressing plants. The right-most column shows maximum projected z-stacks of GFP-TUA6–labeled microtubules in the same cell types.

(A) Mixed CSC trajectories and microtubule orientations in slowly expanding cells of the apical hook. The high density of CSCs in these cells correlates with the high rate of wall synthesis.

(B) A wide range of orientations from transverse to longitudinal is found in rapidly expanding cells \(~2 \text{ mm below the apical hook.}\)

(C) Approximately 6 mm below the apical hook, in cells beginning to cease elongation, CSC density is reduced and trajectories are consistently longitudinal.

(D) CSCs are nearly absent from cells \(10 \text{ mm below the hook, and cortical microtubules are equally sparse. Small, bright, intracellular compartments, referred to as MASCs (arrowheads), are abundant in these basal hypocotyl cells. Bar} = 10 \mu \text{m.}\)
Figure 3. Small, Bright, Intracellular Compartments Represent a MASC Whose Movement Is Dependent on Cortical Microtubule Dynamics.

(A) and (B) Maximum projected z-stacks showing GFP-CESA3 intracellular distribution in Arabidopsis epidermal hypocotyl cells. The ring-shaped labeling of Golgi bodies is present in all treatments. Treatments with 50 μM cycloheximide (CHX) for 4 h (A) or 500 mM mannitol for 1.5 h (B) result in disappearance of CSCs (cf. to Figure 1A) and accumulation of MASCs.

(C) and (D) Maximum projected z-stacks showing GFP-CESA3 intracellular distribution in the root tip upon treatment with 500 mM mannitol. (C) After 2 min of treatment, CSCs are still visible in linear tracks in the plasma membrane, as they are in control treatments. (D) After 6 min of treatment, CSCs have been internalized in MASCs. In five independent experiments, MASCs formed after ~5 to 6 min.

(E), (H), (J), and (L) Maximum projected z-stacks showing the GFP-CESA3 intracellular distribution in Arabidopsis epidermal hypocotyl cells. Labeling of Golgi bodies is found in all treatments. Representative images are shown from treatments with 5 nM CGA for 5 h (E), 5 nM CGA followed by 5 nM CGA + 50 μM CytD (H), 4 μM taxol followed by 5 nM CGA + 4 μM taxol (J), and 7 μM oryzalin followed by 5 nM CGA + 7 μM oryzalin (L). In (E), (H), and (J), MASCs formed at high densities near the cell surface, as they did in the presence of 5 nM CGA treatment alone, for an equal time duration. In (L), MASCs did not form normally and are largely absent.

(F) Representative kymograph of a time series for 5 nM CGA treatment (corresponding to the region indicated by a line in [E]). Time is represented along the vertical axis, and a 10-min scale bar is shown at left (see Supplemental Figure 1 online for information on interpreting kymographs). With 5 nM CGA treatment alone, MASCs are highly motile and have inconstant velocities (cf. to Figure 1F; see Supplemental Movie 3 online).

(G) MASCs move along linear tracks that are remodeled over time. From left to right: the first time point in a series, average projection of the first 5 min of the series, average projection of the last 6 min of the series, overlay of the two average projections. The red lines and arrowheads indicate new trajectories that were not present at the 5 min time point.

(I), (K), and (M) Representative kymographs of time series for treatments with 5 nM CGA followed by 5 nM CGA + 50 μM CytD (I), 4 μM taxol followed by
for GFP-CESA3–labeled CSCs (Desprez et al., 2007). In each insertion event, appearance of new CSC particles was coincident with the pause of a Golgi body just beneath the plasma membrane (Figures 5A to 5C). In two of these events, this pause of the Golgi body resulted in the insertion of multiple particles in a row (Figure 5B).

To further study the role of the Golgi apparatus and VHA-a1/GFP-CESA3–containing compartments in CSC trafficking, we inhibited the movement of these two compartments using actin-depolymerizing agents. Incubation with CytD or Latrunculin B caused aggregation of Golgi bodies and VHA-a1/GFP-CESA3–containing compartments and led to localized reductions in CSC density (Figure 5D). Regions of the plasma membrane containing high CSC density were found exclusively above Golgi aggregates (seven independent experiments; Figures 5D to 5F).

We further explored the insertion of new CSC particles using fluorescence recovery after photobleaching (FRAP) experiments in untreated seedlings. We found that CSCs are not inserted randomly, but in linear tracks resembling those present before bleaching (Figure 5G). In these experiments, we could also observe multiple CSCs inserted one after another in rows, and these insertions were coincident with the passage of Golgi bodies (13 experiments analyzed). Based on our observations of the frequent pauses of Golgi bodies on microtubules (Figures 4A to 4C; see Supplemental Movie 4 online), the insertion of CSCs in rows along linear tracks, we hypothesized that interactions between Golgi bodies and microtubules were directing the plasma membrane delivery of CSCs.

To test if cortical microtubules define the insertion sites of CSCs, we examined the effects of treatment with oryzalin or taxol on the organization of CSCs in the plasma membrane. Complete removal of microtubules by treatment with 20 μM oryzalin resulted in a uniform distribution of CSCs that lacked row organization compared with controls (five independent experiments; Figure 5I). By contrast, microtubule stabilization through taxol treatment resulted in an enhanced alignment of CSCs (five independent experiments; Figure 5J). In light of our results, we propose that disorganization of CSC insertion in the presence of microtubule-disrupting drugs is partly due to deregulation of interactions between the Golgi/VHA-a1/GFP-CESA3–containing compartments and cortical microtubules.

**DISCUSSION**

Synthesis of the plant cell wall requires secretion of cell wall components and CSCs to specific locations at the cell periphery by motile organelles. In this study, we establish a role for the actin and cortical microtubule cytoskeletons and the Golgi apparatus in the delivery of CSCs to the plasma membrane and shed light on the mechanism of endocytosis of CSCs. A schematic model of the intracellular trafficking of GFP-CESA3 is presented in Figure 6.

**Four Distinct Localizations of GFP-CESA3**

It had been previously established using confocal microscopy that GFP-CESA3 and GFP-CESA6 (another related primary cell wall CSC isoform) are present as discrete particles at the cell surface and in punctae at the periphery of Golgi stacks (Paredes et al., 2006; Desprez et al., 2007). In this study, the plasma membrane localization of GFP-CESA3 surface particles was confirmed by EM (Figure 1D), lending further support to the idea that these motile, discrete particles represent CSCs actively synthesizing cellulose. We also confirmed the localization of GFP-CESA3 at the periphery of medial- and trans-Golgi cisternae by EM (Figures 1P and 1Q). Early reports show that the CSC is fully assembled upon arrival in the Golgi apparatus (Haigler and Brown, 1986; Rudolph, 1987). The bright punctae observed at the periphery of Golgi stacks may therefore correspond to assembled CSCs, as represented in Figure 6.

In our experiments, we were able to identify two previously unknown localizations for the CESA protein. We found GFP-CESA3 in a subset of VHA-a1 compartments (Figures 1J to 1O) and a unique compartment we refer to as a MASC (Figures 1E to 1G and 3; see Supplemental Figure 2 online). In our analyses, pausing of the Golgi apparatus was always observed prior to insertion of a new CSC in the plasma membrane, whereas VHA-a1/GFP-CESA3–containing compartments were not detected in proximity to these insertion sites (Figures 5A to 5C). Although we cannot exclude that CSCs pass through VHA-a1/GFP-CESA3–containing compartments on a time scale too rapid to resolve in our experiments, it is more likely that GFP-CESA3 passes through the VHA-a1 compartment only on the endocytic route, as shown for PIN2 (Robert et al., 2008). Since VHA-a1 labels the plasma membrane of the Golgi, the localization of CSCs in VHA-a1 compartments should therefore be analyzed in the context of the Golgi apparatus itself.

**Figure 3.** (continued).

5 nM CGA + 4 μM taxol (K), or 7 μM oryzalin followed by 5 nM CGA + 7 μM oryzalin (M). Time is represented along the vertical axis, and a 10 min scale bar is shown at left (see Supplemental Figure 1 online for information on interpreting kymographs). The kymograph in (M) represents the region indicated by a line in (L).

(N) Frequency histogram comparing MASC velocities calculated from kymographs in replicates of the experiments described in (C), (H), and (I) (CGA alone, n = 278; CGA + taxol, n = 989; CGA + CytD, n = 342). The points indicate the percentage of particles with velocities in the range indicated on the x axis. Lines have been added to facilitate visualization of each population distribution.

(O) Cotyledon cells of untreated GFP-CESA3–expressing plant transiently transformed with mRFP-MBD (red). The maximum projected z-stack (left panel) shows a GFP-CESA3-labeled MASC particle (green) marked by an arrowhead. Right panel, the average projection of the time series showing translocation of the MASC along the mRFP-MBD–labeled microtubule (see Supplemental Movie 4 online).

(P) Montage of a deconvolved time series illustrating representative merging behavior of four aligned MASCs in cells treated with 50 μM cycloheximide for 4 h. At 8.8 s, the fourth particle (from the top) merges with the third, and both continue cotranslocating. A second encounter at 19.8 s results in a rapid split of the particle. The resulting three particles then remain relatively stable in their positions.

Bars = 10 μm.
TGN (Dettmer et al., 2006), these combined data question the role of the TGN in secretion in plants.

Cellulose Synthesis Is Regulated by Rapid Internalization into MASCs

Under conditions of osmotic stress, protein synthesis inhibition, or cellulose synthesis inhibition, we find GFP-CESA3 present in a novel compartment we refer to as a MASC (Figures 3A, 3B, and 3E). Lower densities of CSCs in fully elongated basal hypocotyl cells correlated with a reduced rate of primary cell wall cellulose synthesis in these mature cells (Figure 2D). We conclude that MASCs result from internalization of CSCs, rather than a block in secretion, based on the following three observations: formation of MASCs correlates with disappearance of CSCs at the plasma membrane (Figure 2D), redistribution of CSCs into MASCs occurs within only 6 min in mannitol-treated roots (Figures 3C and 3D), and MASCs still form despite prolonged inhibition of protein synthesis using the drug cycloheximide (Figure 3A). Clathrin-mediated endocytosis has recently been shown to be a predominant endocytic pathway in plants (Dhonukshe et al., 2007). Given that the 45- to 50-nm diameter cytoplasmic domain of the CSC (Bowling and Brown, 2008) would be expected to interfere with the formation of the clathrin coat, it is likely that a different mechanism operates in the endocytosis of CSCs (Figure 6). Indeed, the reduced formation of MASCs in the presence of oryzalin suggests that microtubules may have an active role in the internalization of CSCs under CGA treatment. Oryzalin also prevented the redistribution of YFP-CESA6 into similar small, bright compartments.
in seedlings treated with Morlin, an inhibitor of CESA and microtubule dynamics (DeBolt et al., 2007a). Together, these results show that cellulose synthesis is regulated in part through the internalization of CSCs in intracellular compartments and that internalization of CSCs into MASCs is environmentally regulated. The higher abundance of MASCs in fully elongated basal hypocotyl cells compared with growing apical hypocotyl cells suggests that internalization in MASCs is also developmentally programmed.

MASC Movement Is Dependent on an Intact, Dynamic Cortical Microtubule Array

The unusual movement pattern of MASCs (see Supplemental Movies 3 and 5 online) has not been reported for any other plant

(A) to (C) Images from times series showing three examples of de novo CSC insertion in the plasma membrane focal plane by GFP-CESA3–labeled Golgi bodies in untreated seedlings. Kymographs of the time series are at the far right, with time represented along the vertical axis as indicated by the scale bars (see Supplemental Figure 1 online for information on interpreting kymographs).

(A) and (B) In the left-most image, no particle can be detected at or near the future position of insertion (corresponding to black region above the trace in the kymograph). The central image shows a Golgi body pausing beneath the plasma membrane, which can also be detected on the kymograph at right (white smear marked by arrow). In (A), a single CSC is inserted and begins moving nearly immediately, as evidenced by the angled white trace on the kymograph. In (B), three new CSC are inserted in a row and begin moving steadily (only one is kymographed).

(C) Image 1 shows a first Golgi body pausing at a specific site marked by an arrowhead. Image 2 shows this pause does not result in an insertion event. In image 3, a new Golgi body moves into frame and pauses at the same site (arrowhead), resulting in insertion of a CSC, marked by the arrowhead in image 4. The white smears on the kymograph (marked by a gray arrow and black arrow) illustrate the pauses of successive Golgi and the newly inserted CSC that begins moving with a steady velocity.

(D) to (F) GFP-CESA3 intracellular distribution in epidermal hypocotyl cells treated with 1 μM Latrunculin B.

(D) Maximum projected z-stack in the plasma membrane focal plane shows localized reductions in CSC density.

(E) Average projection of the time series illustrates that CSC are migrating normally along linear trajectories.

(F) Maximum projected z-stack ~600 nm below the plasma membrane showing Golgi body aggregates (arrows). Positions of Golgi body aggregates correspond to the areas of highest CSC density in (A).

(G) FRAP in untreated epidermal hypocotyl cells. The prebleach image is an average of 10 frames showing CSCs arranged in obliquely oriented rows. The central image was acquired ~500 ms after bleaching the indicated area. In the average projection of the postbleach frames, newly inserted CSCs are arranged in oblique rows and migrate along linear tracks similar to those present in the prebleach image.

(H) to (J) Images of a single time point showing the organization of GFP-CESA3-labeled CSCs in epidermal hypocotyl cells.

(H) Untreated seedling showing CSCs arranged in rows.

(I) After an 8-h treatment with 20 μM oryzalin, row organization is lost and CSCs are scattered uniformly throughout the plasma membrane.

(J) A 7-h treatment with 4 μM taxol increases the level of CSC row organization.

Bars = 10 μm.

Figure 5. CSC Delivery to the Plasma Membrane Depends on Interactions between the Golgi Apparatus and the Cytoskeleton.
intracellular compartments or markers thus far, to our knowledge. The merging behavior of MASCs and their movement along linear tracks clearly distinguish them from the actomyosin-based movement of Golgi bodies (Sparkes et al., 2008) and the nonlinear movement of the VHA-a1 compartment (cf. Supplemental Movies 2 and 3 online). MASC movement is not significantly changed by the fragmentation of actin or the inhibition of cytoplasmic streaming (Figure 3I). Both the velocity distribution and the qualitative characteristics of MASC movement remain the same in controls and CytD-treated cells (Figure 3N). By contrast, the depolymerization of the cortical microtubule array or the inhibition of array dynamics by treatment with taxol has a striking effect on the formation and velocity of MASCs (Figures 3J to 3N). The fact that stabilization of microtubule array dynamics also immobilizes MASCs indicates that their movement may not be kinesin driven, but rather depends on intrinsic properties of the microtubule array dynamics itself.

Our findings lend support to the hypothesis that CSCs transiently interact with cortical microtubules, rather than merely passively diffusing between them. For the microtubule-dependent

Figure 6. Schematic Illustration of CESA Trafficking.

Objects were drawn to scale based on published data and EM micrographs obtained in this study. CSCs may be secreted directly to the plasma membrane from the Golgi apparatus or may transit rapidly through the TGN. CSC insertion is accompanied by interactions between the Golgi apparatus and cortical microtubules. We define MASCs as a microtubule-associated compartment containing the CSC, which may encompass both short-lived secretory vesicles and more stable endocytic vesicles. Although we were not able to directly observe CSC secretory vesicles, we hypothesize that they may associate with microtubules if the CSC itself is competent for interactions with the microtubule array. It is possible that CSC secretory vesicles do not associate with microtubules or that secretion occurs in the absence of a vesicular intermediate, though we find these hypotheses less likely. Once fused with the plasma membrane, CSCs are propelled by the polymerization of glucan chains into cellulose microfibrils, and their trajectories may be guided by CSC-cortical microtubule interactions of a transient nature. Internalization of CSCs is promoted under certain conditions (e.g., osmotic stress, cell growth cessation, and cellulose synthesis inhibitor treatment). Consideration of the size of the CSC rosette (~25-nm diameter; Mueller and Brown, 1980) and the CSC cytoplasmic domain (45 to 50-nm diameter; Bowling and Brown, 2008) with respect to the size of clathrin-coated vesicles (CCV) (30-nm diameter excluding coat; Dhonukshe et al., 2007) suggests that endocytosis of CSC does not occur via conventional clathrin-coated vesicle-mediated endocytosis. When internalized, CSCs show surprisingly stable associations with microtubules, which may be due to an interaction between a microtubule-associated protein and the CSC itself or a coat protein specifically recruited to MASC vesicles. Together, our results support a model in which interactions with cortical microtubules have a role both in plasma membrane delivery and internalization of CSCs. These interactions provide a regulatory mechanism for the dynamic remodeling of CSC secretion during cell expansion and differentiation.
movement of MASCs to be possible, either an interaction exists between a microtubule-associated protein and a coat protein specifically recruited to MASC vesicles or an interaction exists between a microtubule-associated protein and a component of the CSC itself. It will be interesting to determine if microtubule-associated proteins can be copurified with CSCs under CGA treatment. The discovery of proteins able to mediate interactions between CSC and microtubules will be essential to reveal the mechanism underlying microtubule-microfibril alignment in growing cells.

**Trafficking of CSCs Is Dependent on Actin and Microtubule Cytoskeletons**

Our combined results strongly support a role for the microtubule cytoskeleton in targeting CSC secretion. CSC density and cortical microtubule density are correlated during cellular differentiation in the hypocotyl (Figure 2), suggesting that cortical microtubules may be involved in regulating CSC delivery to the plasma membrane. We demonstrate that new CSC insertions are preceded by pauses of the Golgi apparatus at specific sites beneath the plasma membrane (Figures 5A to 5C). The so-called stop-and-go behavior of Golgi bodies was proposed to be due to pauses at ER export sites (Nebenführ et al., 1999); however, recent evidence suggests that ER export sites and Golgi stacks may form mobile secretory units (DaSilva et al., 2004). Our data reveal that Golgi bodies pause at cortical microtubules, and these pauses often result in insertion of CSCs at the plasma membrane (Figures 5A to 5C).

Evidence of Golgi bodies pausing at cortical microtubules has also been observed in cells undergoing high rates of secondary cell wall synthesis. In developing xylem vessels, secondary cell wall synthesis is nonuniform and characterized by cell wall thickenings in a banded pattern. Within these bands, CSCs are more abundant in the plasma membrane (Herth, 1985), and microtubules are found in organized bundles (Hepler and Newcomb, 1964). It was recently shown that organelles labeled by YFP-CESA7, of which a subset colocalizes with the Golgi marker mannosidase I, pause at bands marked by microtubules (Wightman and Turner, 2008). These organelles appear to pause less frequently following depolymerization of the microtubule bands by oryzalin, although the statistical significance of this observation can be questioned. Thus, there is limited evidence that delivery of secondary cell wall CSCs to the plasma membrane in developing xylem vessels may also be related to the pausing of Golgi bodies on microtubules.

In plants, Golgi bodies, mitochondria, and peroxisomes are three organelles known to move along actin filaments via myosins (Sparkes et al., 2008). Although the actomyosin system may drive the movement of these organelles, there is increasing evidence showing their proper function also depends on interactions with cortical microtubules. The plant peroxisomal multifunctional protein localizes to peroxisomes and decorates the cortical microtubule array, thus mediating interactions between peroxisomes and cortical microtubules (Chuong et al., 2005). The *Arabidopsis* internal motor kinesin, Kinesin-13A, and the homologous *Gossypium hirsutum* Kinesin-13A have been shown to localize to Golgi bodies (Lu et al., 2005). *Arabidopsis* kinesin-13a mutants show aggregation of Golgi bodies in trichomes and have defects in trichome morphogenesis, suggesting a role for Kinesin-13A in regulating secretion of cell wall components by the Golgi apparatus (Lu et al., 2005). Similarly, mutants in the kinesin FRAGILE FIBER1 show alterations in cellulose deposition and reduced fiber strength (Zhong et al., 2002). Finally, cortical microtubules were found in close association with electron-dense secretory vesicles targeted to pectic mucilage pockets in *Arabidopsis* seed coat cells (McFarlane et al., 2008). Thus, the actin filament and microtubule components of the cytoskeleton are highly interdependent, and each plays an important role in secretion and organelle movement.

Our data support a model in which the actomyosin system serves to distribute Golgi bodies throughout the cell, and sites of secretion are defined through interactions with the microtubule cytoskeleton. This finding explains the diffuse, randomized appearance of CSCs at the plasma membrane following oryzalin treatment and is also supported by observations of enhanced CSC alignment under taxol treatment. In conclusion, we propose that in growing cells, cortical microtubules not only direct the trajectories of CSCs in the plasma membrane, but also regulate the delivery of CSCs to the plasma membrane and possibly their internalization. This allows the dynamic remodeling of CSC secretion patterns during the expansion and differentiation of plant cells.

**METHODS**

**Plant Material and in Vitro Growth Conditions**

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col) were provided by K. Feldman (University of Arizona, Tucson, AZ), and GFP-CESA3 and VHA-a1-mRFP lines were described previously (Dettmer et al., 2006; Desprez et al., 2007). GFP-TUA6 (Ueda et al., 1999) and EB1-GFP (Chan et al., 2003) lines were a generous gift of J. Chan (John Innes Centre, Norwich, UK). GFP-FABD2 was a gift of M. Pastuglia and has been described previously (Voigt et al., 2005). For imaging, seedlings were cultured in chambers as described (Chan et al., 2007). For hypocotyl measurements, seedlings were grown at 20°C in Petri dishes on Estelle and Somerville medium (Estelle and Somerville, 1987) without sucrose. Seeds were cold-treated at 4°C for 48 h, exposed to fluorescent white light (150 μmol m−2 s−1, True Light; Philips) for 6 h, and then wrapped in aluminum foil. The age of the seedlings was defined with respect to the end of the cold treatment.

**Hypocotyl Length Measurements**

Etiolated seedlings were fixed in 0.2% paraformaldehyde. The lengths of at least 50 hypocotyls per treatment were measured using Optimas 5.2 software as described (Refregier et al., 2004). The absorption of CGA treatment. The discovery of proteins able to mediate interactions between CSC and microtubules was essential to reveal the mechanism underlying microtubule-microfibril alignment in growing cells.

**Generation of the GFP-CESA3/RFP-VHA-a1 Transgenic Line**

Pollen from GFP-CESA3–expressing plants in *je5cesa3* background (Desprez et al., 2007) was used to fertilize plants expressing VHA-a1-mRFP (Dettmer et al., 2006). F1 seeds were collected and amplified. F2 seedlings were screened by PCR for the presence of both markers and the cesa3 mutation. F3 selected seedlings were used for imaging.

**Drug Treatments**

For hypocotyl measurements, Col-0 seeds were germinated on media containing CGA 325′615 (CGA) in the range of 0 (mock treatment DMSO
dilution) to 5 nM and measured after 4 d. For Fourier transform infrared microscopy, Col-0 seeds were germinated on media plates containing drugs at the indicated concentrations, and mutant seeds were germinated on normal media. Measurements were taken after 4 d.

For live-cell imaging, treatment and mock treatment solutions were injected into chambers using a fine-needle syringe under green light. Seedlings expressing markers to verify drug treatment efficacy were injected into chambers using a fine-needle syringe under green light. Seedlings expressing markers to verify drug treatment efficacy were

**Transient Expression in Arabidopsis Seeds**

The mRFP-MBD vector was constructed by M. Pastuglia (Institut Jean-Pierre Bourguin-Institut National de la Recherche Agronomique). The MBD of microtubule-associated protein 4 was amplified from the GFP-MBD construct (Marc et al., 1998) and cloned into Gateway vector pDONR207 (Invitrogen). To subclone the mRFP-MBD fusion downstream of the 35S promoter, pDONR207-MBD was used in an LR reaction with destination vector pH7WGR2 (Karimi et al., 2002). mRFP-MBD and GFP-CESA3 expression vectors were electroporated in Arabidopsis protoplasts. The MBD was then cleaved from the MBD after its cleavage from the MBD by using a classic method.

For transient expression studies, A. tumefaciens cultures were cultured overnight at 28°C and diluted to OD<sub>600</sub> = 1 in 5% sucrose with 200 μM acetoxyzirine. Infiltration of Col-0 A. tumefaciens cultures was performed as described (Marion et al., 2008).

**Spinning Disk Microscopy and Image Analysis**

During all experiments, cell viability was verified by monitoring cytoplasmic streaming. Hypocotyls of 3-d-old etiolated seedlings were analyzed on an Axiocam 200 microscope (Zeiss) equipped with a Yokogawa CSU22 spinning disk, Zeiss 100/1.4 numerical aperture oil objective, and Andor EMCCD (Kox DU 895 camera (Plateforme d’Imagerie Dynamique, Institut Pasteur, Paris, France), A 488- or 560-nm diode-pumped solid-state laser was used for excitation, and emission was collected using band-pass 488/25 and 568/25 filters (Semrock) for GFP and mRFP, respectively. Projections presented in figures were acquired with a minimum of three z-slices using a 300-nm step interval. For analysis of particle interactions, time series were acquired at a frame rate of 2 to 4 frames/second.

FRAP experiments were performed using an inverted microscope (Nikon TE2000) equipped with a cooled interline CCD detector (Roper CoolSNAP HQ2) and a piezoelectric stage driver (LVDT; Physik Instrument) (Nikon TE2000) equipped with a cooled interline CCD detector (Roper CoolSNAP HQ2) and a piezoelectric stage driver (LVDT; Physik Instrument). Images were acquired at 2 frames/second with a FRAP duration of 20 ms and an xy resolution of 130 nm/pixel (2 x 2 binning).

When required, fluorescent images were deconvolved using a classic maximum likelihood estimation deconvolution algorithm (Huygens Professional v. 3.1; Scientific Volume Imaging). The integrated morphometry analysis function in Metamorph Offline (V. 7.0; Molecular Devices) was used to detect and measure the orientations of microtubules. Particle velocities were calculated from kymographs created in Image J (W. Rasband, National Institutes of Health). Kymograph montages were generated with the help of an ImageJ plug-in developed by F. Cordelières (Plateforme d’Imagerie Cellulaire et Tissulaire , Unité Mixte de Recherche 146, Institut Curie). Image size and contrast were adjusted when needed using Adobe Photoshop CS v. 8.0.1 (Adobe Systems).

**Confocal Laser Scanning Microscopy**

The images presented in Supplemental Figures 2A to 2F online were obtained using a spectral Leica SP2 AOBS confocal microscope (Leica Microsystems). GFP-CESA3 and the sterol dye FM4-64 (Molecular Probes) were excited at 488 nm, and emission was collected at 512 to 655 nm and at 833 nm, respectively. A x63 water immersion objective (numerical aperture 1.2) and a zoom factor of eight were used to collect z-series in a 512-scan format.

**Fourier Transform Infrared Microspectroscopy**

Four-day-old seedlings were brushed between two barium fluoride windows and rinsed abundantly with distilled water for 2 min, before drying at 37°C for 20 min. For each mutant, 20 spectra were collected from hypocotyls from four independent cultures (five seedlings from each culture) as described (Mouille et al., 2003). The collected spectra were baseline and normalized as described, and statistical analysis was performed by a Student’s t test (Robin et al., 2003).

**Crystalline Cellulose Measurements**

Wild-type Col-0 plants were grown in darkness on plates containing 0.625 nM CGA or a DMSO mock treatment solution. Four-day-old seedlings were harvested and stored in 70% ethanol at 4°C. Crude cell wall extracts were prepared as described (Reiter et al., 1993) with minor changes. Seed coats were removed and plant material was incubated twice during 1 h at 70°C in 70% ethanol. Pellets were washed in methanol for 5 min and acetone for 2 min and were vacuum-dried. The dry cell wall pellets were weighed in 2 mL reaction tubes, suspended in 3 mL Updegraff reagent (acetic acid:nitric acid:water, 8:1:2 v/v), and further incubated in a boiling water bath for 30 min. Crystalline cellulose was determined as described (Scott and Melvin, 1953; Updegraff, 1969). Absorbance was determined at 630 nm (Labsystems iEMS Reader MF). Measurements were performed in duplicate using independent seed batches.

**Transmission Electron Microscopy**

Degassed seedlings were high-pressure frozen, freeze-substituted, and rehydrated (Ripper et al., 2008) before samples were infiltrated with the cryoprotectant polyvinylpyrrolidone and sucrose, mounted on a specimen holder, and frozen in liquid nitrogen for ultrathin cryosectioning as described before (Reichhardt et al., 2007). Ultrathin thawed cryosections were treated with 0.5% BSA, 1% milk powder in PBS (20 min) before labeling with rabbit anti-GFP antibodies (Torrey Pines Bioslabs; 1:500) and silver-enhanced Nanogold coupled to goat anti-rabbit IgG (1:60) (HQSilver, 8.5 min; Nanoprobe) for 60 min each.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: CES3A, Atilg05170; VHA-a1, At1g28520; EB1a, At3g47690; TUA6, At4g14960; MBD from MAP4, M72414; and FABD2 from At FIM1, At4g26700.

**Supplemental Data**

The following materials are available in the online version of this article.
Supplemental Figure 1. Image Analysis Methods.

Supplemental Figure 2. Localization of GFP-CESA3 following CGA Treatment in FM4-64–Labeled Cells.

Supplemental Figure 3. CGA Causes Specific Reductions in Crystalline Cellulose and Inhibits Cellular Elongation.

Supplemental Figure 4. CGA Does Not Have an Observable Effect on the Cytoskeleton or Golgi.

Supplemental Figure 5. Effects of Drug Treatments Used in This Study.

Supplemental Movie 1. CSCs Move Bidirectionally with Constant Velocities.

Supplemental Movie 2. GFP-CESA3–Labeled Golgi Bodies and the VHA-1a1 Compartment Rapidly Associate and Dissociate.

Supplemental Movie 3. CGA-Induced MASCs Have a Wide Range of Velocities and Merge with One Another along Linear Tracks.

Supplemental Movie 4. GFP-CESA3–Labeled Golgi Bodies Pause on Microtubules.

Supplemental Movie 5. Physical Interactions Occur between Mannitol-Induced MASCs and GFP-CESA3–Labeled Golgi Bodies.

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