Cortical microtubules and physical properties of cellulose microfibrils during primary cell wall formation in *Arabidopsis thaliana*.

by

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ABSTRACT

Growth anisotropy, in which cells grow predominantly in one direction, is common in plant cells, and an essential event for plant form and function. The direction and degree of growth anisotropy are governed by the mechanical properties of the primary cell wall. When aligned in a parallel manner, cellulose microfibrils accommodate great resistance in the direction of their alignment to expansion driven by isotropic turgor pressure. Using the Arabidopsis thaliana inflorescence stem as a model system, field emission scanning electron microscopy (FESEM) analysis demonstrated that the establishment of parallel arrangement of microfibrils is closely correlated with anisotropic cell expansion. In the novel anisotropy 1 (any1) mutant allele of the primary cellulose synthase CesA1, growth defects were correlated with random cellulose microfibril patterns in some inflorescence stem tissues.

Microtubules have been considered to be the most likely candidates for controlling the orientation of cellulose microfibrils. Recent studies have indeed demonstrated a close association of the plasma membrane-localized cellulose-synthase-complexes (CSCs) that produce cellulose and cortical microtubules. Despite this close association, microtubule disruption did not cause cellulose microfibrils to lose parallel alignment in the radial and inner periclinal walls of cells in the inflorescence stem, suggesting that microtubules influence mechanical properties of cellulose microfibrils other than orientation. X-ray diffraction analysis demonstrated that cellulose crystallinity in wild-type plants declines at the growth-promoting temperature of 29°C, whereas crystallinity fails to adapt and remains high in mor1-1, the temperature-sensitive mutant whose microtubule arrays become disorganized at its restrictive temperature (29°C). This finding suggests that organized microtubules are involved in reducing cellulose crystallinity that normally accompanies increased cell expansion.

Live-cell imaging of CSCs by tracking a yellow fluorescent protein (YFP)-tagged CesA6 subunit in hypocotyl cells demonstrated that dynamic and well-
organized microtubules affect the velocity, the direction of movement, and the density of CSCs, suggesting that there is a close relationship between microtubules and CSCs. Together with the finding that microtubules also control the distribution of COBRA, a GPI-anchored wall protein that is essential for growth anisotropy, I discuss the variety of roles microtubules play in anisotropic growth.
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<th>Full Form</th>
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<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDTA</td>
<td>cyclohexane-trans-1,2-diamine-N,N,N'N'-tetraacetate</td>
</tr>
<tr>
<td>CesA</td>
<td>cellulose synthase</td>
</tr>
<tr>
<td>DCB</td>
<td>2,6-dichlorobenzonitrile</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis (β-amonithylether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>FESEM</td>
<td>field emission scanning electron microscopy</td>
</tr>
<tr>
<td>GA</td>
<td>gibberellin</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>mRFP</td>
<td>monomeric red fluorescent protein</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PIPES</td>
<td>piperazine-N,N'-bis (2-ethane-sulphonic acid)</td>
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<td>TEM</td>
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<td>THF</td>
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<tr>
<td>Tris [HCl]</td>
<td>2-amino-2(hydroxymethyl)-1,3-propanediol, hydrochloride</td>
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<tr>
<td>TUA</td>
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<td>UV</td>
<td>ultra violet</td>
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<td>YFP</td>
<td>yellow fluorescent protein</td>
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CHAPTER 1

Literature review
1.1 PLANT CELL ANISOTROPIC EXPANSION

1.1.1 Anisotropic expansion in diffusely growing cells

Cell expansion is a crucial event for plant cells to obtain the specific shape and volume related to their specialized functions, and often influences the size and shape of organs and of the entire plant. All cells are derived from undifferentiated ones that are formed in the meristems. At this stage cells are isodiametric; that is, they have equal diameters in all directions. Plant cell expansion is driven by turgor pressure, which is generated by water uptake, as a consequence of vacuolar activity. Plant cells that have defects in vacuole expansion do not expand (Schumacher et al., 1999). Despite the need for turgor pressure, plant cell expansion is in fact mediated by the extensibility of primary cell walls, which is dependent on various parameters including polysaccharide composition, enzymes and other proteins that modify these polysaccharides, and wall pH (Taiz and Zeiger, 2006). In their expansion, most plant cells undergo controlled and irreversible diffuse growth when turgor pressure exceeds the yield threshold of the cell wall.

Cell growth is often highly directional; called anisotropic growth, that is, the cell grows predominantly in one direction, forming cylindrical shapes in elongating cells of stems and roots, or undergoes more localized expansion to give irregular shapes, such as leaf epidermal cells resembling pieces of a jigsaw puzzle. Cell walls and microtubules are crucial for regulating cell growth and the control of its direction, as discussed in the following sections.

1.1.2 Arabidopsis roots, inflorescence stems, and dark-grown hypocotyls - model systems for studying growth anisotropy

The root and inflorescence stem of Arabidopsis thaliana are ideal systems to study anisotropic growth. Their expansion is highly unidirectional, with extensive longitudinal expansion and little radial expansion, resulting in hemicylindrical shapes of epidermal cells and elongated polyhedral shapes of cells in the inner tissues. The main advantage of using these organs is that the developmental stages can be
distinguished along the apical-basal axis, so that it is easy to select expanding cells for the study of anisotropic growth. As shown in Figure 1-1, both root and inflorescence stems have apical meristems where cells are formed by cell divisions. Cell expansion takes place in restricted regions of roots and inflorescence stems, called elongation zones. After cells expand by diffuse growth, they differentiate further. For example, in the root differentiation zone, epidermal cells may develop root hairs, while in the lower part of the inflorescence stem, interfascicular fibers develop.

The dark-grown hypocotyl is another ideal system to study anisotropic growth because growth is accomplished by cell elongation without cell division (Gendreau et al., 1997; Raz and Koornneef, 2001; Saibo et al., 2003). Dark-grown seedlings develop hooks in the upper region of their hypocotyls (Figure 1-1). A recent study has demonstrated that growth starts at the base of the hypocotyls and a growth (or elongation) zone gradually propagates towards the upper region (Réfregier et al., 2004). Thin walls in rapidly growing cells and thick walls in slowly growing cells show that cell wall synthesis and cell elongation are not tightly coupled in dark-grown hypocotyls (Réfregier et al., 2004; Derbyshire et al., 2007).
Figure 1-1 Experimental systems for growth anisotropy, *Arabidopsis thaliana*.

(A) Arabidopsis light-grown seedling root: The meristematic region is in the root tip. Above the meristematic region, the elongation zone and the differentiation zone are located.

(B) Mature Arabidopsis plant: The shoot apical meristem is in the top of the inflorescence stem and the elongation zone of the stem is much broader than that of the root.

(C) Arabidopsis dark-grown seedling: Dark-grown seedlings form an apical hook and the hypocotyl becomes highly elongated.
1.2 CELL WALL AND THE CONTROL OF GROWTH ANISOTROPY

1.2.1 Mechanical properties of the cell wall during anisotropic growth

The growth of the plant cell is principally dependent on the mechanical properties of the cell wall. The primary cell wall, formed in expanding cells, is extensible but is sufficiently strong to resist the forces of turgor pressure generated within the cell. Cellulose microfibrils, one of the components of the primary wall, are strong and generally inextensible reinforcing elements that are arranged in parallel order in cells that are expanding diffusely and unidirectionally (Taiz, 1984; Carpita and Gibeaut, 1993). This highly ordered arrangement restricts cell growth but allows growth to occur at right angles to the direction of cellulose microfibril orientation. In primary cell walls, cellulose microfibrils are linked together by hemicellulose polymers, which, along with pectins, comprise the wall matrix. Hemicelluloses form a strong network with cellulose microfibrils that resists the high forces of turgor pressure. To avoid mechanical failure during growth, primary walls need to be able to expand in a controlled manner. They are both extensible and plastic so that they can accommodate controlled and irreversible expansion.

The modification and reorganization of wall components leading to wall loosening, and the deposition of new wall material into the existing wall, occur in a controlled manner (Cosgrove, 2003). In anisotropic growth, wall loosening is spatially regulated such that it mainly occurs in the walls parallel to the growth axis, with the anticlinal cross walls expanding very little, if at all. For example, in Arabidopsis roots it has been shown that there is no detectable expansion in the radial direction after cytokinesis (Baskin et al., 1994), and therefore it is an excellent model system for analyzing the mechanisms of anisotropy.
1.2.2 Cellulose microfibrils

1.2.2.1 Structure and properties of cellulose microfibrils

The basis for cell wall reinforcement is cellulose, linear homopolymers of β-1,4-linked D-glucose chains that assemble into microfibrils (Figure 1-2). Herth (1983) estimated that each cellulose microfibril consists of 36 individual 1,4-β-glucan chains based on the diameter measurements of microfibrils performed by electron microscopy in *Spirogyra*. The unique feature of cellulose microfibrils is that the hydrogen bonds and hydrophobic interactions between glucan chains impart crystalline structure to microfibrils to give them insoluble, hydrophobic, chemically resistant and mechanically strong properties. Cellulose microfibrils contain crystalline regions where cellulose chains are tightly packed forming a crystalline structure, and amorphous regions where chains are less ordered.

It is likely that cellulose microfibrils consist of cellulose chains in various sizes. The degree of polymerization (DP) represents the number of glucose residues per molecule. Cellulose microfibrils are longer than the size of individual cellulose chains predicted by DP values, so that individual cellulose chains end periodically within microfibrils (Haigler, 1985; Brett, 2000). Although extraction of cellulose from the wall may lead to some degradation, previous DP analyses have demonstrated that primary wall cellulose chains have lower molecular weights and have a greater size distribution (ie, they are more polydisperse) compared to secondary wall cellulose chains (Brett, 2000). Cotton cellulose chains have been estimated to contain 2000 to 6000 glucose residues in the primary wall and around 14,000 residues in the secondary wall (reviewed in Haigler, 1985). In primary walls produced by regenerating protoplasts and suspension-cultured cells, two DP fractions have been identified: one below 500 and another fraction ranging from 2000 to 4000 (Asamizu et al., 1977; Blaschek et al., 1982).
Figure 1-2 Structure of cellulose microfibrils in the plant cell wall.

Cellulose microfibrils are synthesized by cellulose-synthase-complexes. Cellulose microfibrils contain both crystalline regions where cellulose chains are tightly packed, and amorphous regions where chains are less ordered. Cellulose is made of a 1,4-linked β-glucan chain.
1.2.2.2 Cellulose biosynthesis

Cellulose synthesis and microfibril formation are carried out by cellulose-synthase-complexes (CSCs), which by electron microscopy resemble rosettes located at the plasma membrane (Brown et al., 1996). These structures have been identified as CSCs in electron micrographs of freeze-fractured plasma membranes (Kimura et al., 1999). As shown in Figure 1-3, each rosette is believed to consist of six rosette subunits, each of which is composed of possibly 6 cellulose synthase (CesA) enzymes (Doblin et al., 2002). This model is based on the commonly accepted estimate that a primary wall cellulose microfibril contains about 36 individual 1,4-β-glucan chains, suggesting that each CesA polymerizes a single glucan chain and a single hexagonal rosette is required for producing each microfibril (Herth, 1983; Brown and Saxena, 2000). There is no evidence, however, that each rosette subunit contains six CesAs (Taylor, 2008). Each rosette subunit is predicted to contain multiple copies of three different CesA proteins as described in the next section, but the exact number of CesAs in each rosette subunit, their stoichiometry, and their specific interactions are still unknown (Taylor, 2008).

The primary substrate for cellulose synthesis is uridine diphosphate D-glucose (UDP-glucose), which is synthesized from sucrose in the cytoplasm by sucrose synthase and then utilized by CesA catalytic subunits for glucan chain elongation (Amor et al., 1995; Delmer and Amor, 1995; Doblin et al., 2002).

The rosette is thought to become displaced laterally in the plasma membrane as it generates glucan chains through the continuous polymerization of supplied precursors. This movement of the complex in the membrane might be driven by the force generated during crystallization of the polymerized product into rigid microfibrils (Heath, 1974; Herth, 1980).
Figure 1-3 A model of the cellulose-synthase-complex (CSC). (modified from Taylor, 2008).

Each CSC has a hexameric structure of six rosette subunits, observed by electron microscopy (Brown, 1996; Kimura et al., 1999).
1.2.2.3 Cellulose-synthase-complexes (CSCs)

CesA genes in plants were first identified in cotton fibers through sequence similarities to bacterial CesA (Pear et al., 1996). The Arabidopsis genome encodes 10 CesA proteins (Richmond, 2000). Direct experimental evidence of the involvement of CesAs in cellulose synthesis came from mutant analyses and it has been shown that different CesA members are expressed in different cell types. The phenotypes of Arabidopsis mutants suggest that CesA1, A3, and A6 are involved in cellulose deposition of primary wall formation (Arioli et al., 1998; Fagard et al., 2000; Burn et al., 2002; Ellis et al., 2002; Caño-Delgado et al., 2003). According to sequence similarities, gene expression patterns and genetic analyses, CesA2, A5, and A9 may partially replace CesA6 (Robert et al., 2004; Persson et al., 2007; Desprez et al., 2007). CesA4, A7, and A8 are involved in secondary wall formation (Turner and Somerville, 1997; reviewed in Taylor, 2008).

Recent evidence from genetics, co-immunoprecipitation analyses and bimolecular fluorescence complementation suggest that the CSCs in the primary wall contain at least three isoforms, CesA1 and CesA3, which are always present, and one of the CesA6-related isoforms, CesA2, A5 or A6, competing for the third position (Persson et al., 2007; Desprez et al., 2007). Wang et al. (2008) also have shown that an 840 kDa complex in detergent extracts is a likely CesA complex, according to the results that 840 kDa complexes are pulled down with each antibody against CesA1, A3, and A6. These results support the hypothesis that CSCs are composed of several CesA isoforms (Wang et al., 2008). They also have shown that 840 kDa complexes are absent at its restrictive temperature in the rsw1-1 mutant, which is an allele of CesA1, and the complexes are detected as a 420 kDa mass in the prc1-19 mutant in which CesA6 is absent (Wang et al., 2008). In secondary wall formation, it has been shown that the lack of one CesA protein in the mutant causes two other CesA proteins to be localized in ER, suggesting that CesA4, A7 and A8 are all required for the assembly of the CSC and for their proper localization at the plasma membrane (Gardiner et al., 2003).
1.2.3 Cellulose microfibrils and growth anisotropy

The orientation of cellulose microfibrils is perpendicular to the cell growth axis in expanding cells, providing a greater resistance to radial expansion than to axial expansion in response to turgor pressure. Studies using cell expansion-defective mutants provide genetic evidence for the crucial role of cellulose microfibrils in anisotropic growth. RADIAL SWELLING 1 (RSW1, CesA1) and PROCUSTE (PRC1, CesA6) are cellulose synthase catalytic subunits involved in the synthesis of cellulose microfibrils in the primary cell wall (Arioli et al., 1998; Gillmor et al., 2002; Beeckman et al., 2002; Fagard et al., 2000). Mutations in these genes reduce cellulose production and cause loss of growth anisotropy. Together with these studies, cellulose synthesis-inhibiting drug studies (Hogetsu et al., 1974; Heim et al., 1991; Sabba et al., 1999; Scheible et al., 2003) and other analyses of cellulose-deficient mutants (Sugimoto et al., 2001; Sato et al., 2001; Pagant et al., 2002; Burk and Ye, 2002, Roudier et al., 2005; Mackinnon et al., 2006) have shown that the reduced cellulose level causes defects in growth anisotropy. In addition, cellulose microfibril orientations are disordered in these cellulose-deficient mutants. These studies suggest that there may be a general linkage between the rate of cellulose production and its proper alignment, and that both of these factors are essential for controlling growth anisotropy (Wasteneys, 2004). These studies also support the model that the generation of parallel order of microfibrils is coupled to the local density of the CSCs in the plasma membrane (Emons and Mulder, 1998, 2000; Mulder and Emons, 2001).

1.2.4 Other wall components involved in cell expansion

Other components of the primary wall include pectins, hemicelluloses, and proteins. Pectins have a characteristic feature of branched, hydrated polysaccharide molecules making semi-rigid gels in the presence of Ca$^{2+}$. Successive Ca$^{2+}$ crosslinks increase the order and rigidity of pectin molecules and enhance the rigidity of growing walls that have a lower concentration of cellulose microfibrils than non-growing walls (Jarvis, 1984). Hemicelluloses are matrix polysaccharides that
have β-1,4 linked linear backbones of glucans, xylans and mannans with short side chains made of glucose, xylose, arabinose, fucose, rhamnose and glucuronic acid (Carpita and Gibeaut, 1993). These molecules bind to cellulose microfibrils tightly via hydrogen bonds. Xyloglucan is a predominant hemicellulose in dicot primary walls (Carpita and Gibeaut, 1993). A recent model of the primary wall depicts cellulose microfibrils tethered by xyloglucan chains, with pectic polysaccharides and structural proteins filling the spaces in between microfibril-xyloglucan tethers (Cosgrove, 2003). Since cell wall expansion requires that the cellulose microfibrils move apart, enzymes such as those that cut the β-1,4 glucans of xyloglucans may need to function during cell expansion to loosen the wall (Cosgrove, 2003). Expansins, xyloglucan endotransglucosylase-hydrolases (XTHs) and endo-1,4-β-glucanases are candidates for controlling wall loosening (Rose et al., 2002; Cosgrove, 2003, 2005). Other structural proteins, such as extensins, which are highly insoluble structural hydroxyproline-rich glycoproteins, may also act as regulators of cell wall expansion (Carpita and Gibeaut 1993). In addition, recent analyses of mutants with defects in cell expansion enable the identification of wall proteins that are involved in wall synthesis, loosening and maturation, such as the cobra mutants described in section 1.4.

1.3 CORTICAL MICROTUBULES AND GROWTH ANISOTROPY

1.3.1 Cortical microtubules in the expanding cells

In expanding cells, microtubules are arranged into organized, two-dimensional patterns at the cell periphery that are referred to as cortical microtubule arrays. Well-organized cortical microtubules are essential for the anisotropic growth of diffusely expanding cells. Pharmacological studies using anti-microtubule drugs and forward genetics approaches using microtubule-disorganized mutants have revealed that loss of the perpendicular orientation of microtubules relative to the cell growth axis is correlated with loss of growth anisotropy, resulting in radial swelling of the cell (Baskin et al., 1994; Baskin 2001; Bichet et al., 2001; Whittington et al., 2001; Camilleri et al., 2002; Baskin et al., 2004).
Since Ledbetter and Porter (1963) first described cortical microtubules in plant cells, cortical microtubules have been considered to be the most likely candidates to influence the organization of cellulose microfibrils in the cell wall. The first support for this is based on early electron microscopy analyses. According to transmission electron micrographs of growing cells, cellulose microfibrils are often found to align parallel to cortical microtubules (Ledbetter and Porter, 1963; Seagull, 1985). Certain characteristics of microtubules make them good candidates for playing a role in cellulose microfibril deposition. First of all, cortical microtubules lie close to the plasma membrane, which is the site of cellulose microfibril synthesis and assembly. Furthermore, connections between microtubules and the plasma membrane have been observed (Palevitz, 1982; Lancelle et al., 1986; Gardiner et al., 2001). The precise mechanism for this interaction remains to be determined, though phospholipase D (Dhonukshe et al., 2003) and the microtubule-associated protein CLASP (Ambrose et al., 2008) may be involved. The second body of support for the role of microtubules in orienting cellulose microfibrils comes from pharmacological strategies. Anti-microtubule drugs have been reported to both depolymerize microtubules and disrupt the pattern of cellulose deposition during primary wall formation (Takeda and Shibaoka, 1981; Mueller and Brown, 1982b). Based on this evidence, cortical microtubules were thought to constrain the movement of CSCs to control the orientation of cellulose microfibrils (Giddings and Staehelin, 1991), a hypothesis outlined in the cellulose-synthase-constraint model.

Two possible roles of microtubules guiding CSC movement have been proposed. One is that microtubules create channels or barriers that allow CSCs to move passively between microtubules (Giddings and Staehelin, 1988). The other is that CSCs move directly on top of the microtubules (Hasezawa and Nozaki, 1999). Recent live-cell imaging of yellow fluorescent protein (YFP) -tagged with CesA6 has demonstrated that about 60 % of YFP-CesA6 trajectories overlap with microtubules, and that CesA6 localization and movement are tightly coupled to the microtubule array (Paredez et al., 2006a). These results suggest that CSCs are not guided by passive channeling between the microtubule arrays (Paredez et al., 2006a). It has been also shown that YFP-CesA6 moves in linear tracks when microtubule polymers
are completely eliminated, suggesting that there is a self-organization mechanism or the involvement of other mechanism for the linear movement of CSCs (Paredes et al., 2006a).

There are numerous examples from earlier studies on green algae and higher plants that show that transverse alignment of microfibrils can occur independently of microtubules (Preston, 1988; Emons et al., 1992; Baskin, 2001). As an alternative model to the cellulose-constraint-model, Baskin (2001) proposed a ‘templated-incorporation’ model in which microfibrils adhere to an oriented scaffold deposited by microtubules, and established cellulose microfibrils can continue to align in the direction of the scaffold even in the absence of microtubules. As described in the next section, Himmelspach et al. (2003), have already provided evidence that template incorporation is not a feature of cellulose alignment.

1.3.2 microtubule organization 1 (mor1-1) as a good system to examine the role of cortical microtubules in anisotropic growth

The temperature-sensitive microtubule organization 1-1 (mor1-1) mutant is a good system to examine the role of cortical microtubules in anisotropic growth. Missense mutations in the microtubule-associated protein MOR1 cause temperature-sensitive disruption of cortical microtubule arrays and loss of growth anisotropy (Whittington et al., 2001). Recently, it has been shown that cells in root tips of mor1-1 have cell division defects at the restrictive temperature (Eleftheriou et al., 2005; Kawamura et al., 2006). This change in microtubule organization is fast, and reversible by simply changing the culture temperature.

A previous study using the mor1-1 allele demonstrated that microtubule disruption caused radial swelling without altering cellulose microfibril orientation (Sugimoto et al., 2003). Furthermore, by using mor1-1 and the cellulose inhibitor 2,6-dichlorobenzonitrile (DCB), Himmelspach et al. (2003) demonstrated that cellulose microfibrils can re-establish their order with neither organized microtubules nor an organized template of cellulose microfibrils. These studies suggest that
cellulose microfibril orientation is largely self-ordered and that cortical microtubules are not required for controlling the direction of cellulose microfibril deposition.

As described in section 1.2.3, reduced cellulose content is correlated with loss of cellulose microfibril orientation and loss of growth anisotropy. In the case of *mor1-1*, neither cellulose content nor microfibril orientation was altered, but growth anisotropy was defective (Sugimoto et al., 2003). This study raised the question of what might be the exact role of transverse cortical microtubule orientation in expanding cells.

### 1.3.3 Possible role of microtubules: the microtubule-length-regulation hypothesis

Anisotropic cell expansion requires well-organized transverse cortical microtubules and also requires sufficient levels of cellulose synthesis for producing ordered microfibrils. Wasteneys (2004) proposed a new model for the role of microtubules, called the microtubule-length-regulation hypothesis, which proposes that the parallel order of cortical microtubules in the direction of cellulose microfibril synthesis and their close association with the plasma membrane are required to ensure that synthesis and integrity of cellulose microfibrils is maintained. This will either generate long cellulose microfibrils or produce microfibrils that are resistant to breakage under turgor pressure (Wasteneys 2004). During expansion, wall enzymes are activated that modify the wall matrix, including xylglucans tethering microfibrils, which results in wall loosening. If the microfibrils are long, they can only move apart from one another in a direction perpendicular to their orientation, with no lateral displacement, allowing cells to grow anisotropically. If the microfibrils are short or become fragmented, however, they would move apart from each other in both the lateral and longitudinal directions, generating isotropic expansion of the cell. Wasteneys (2004) proposed two possible roles of microtubules in maintaining production of long cellulose microfibrils. One possible role is that cortical microtubules may stabilize newly synthesized microfibrils against mechanical weakness through their close linkage to the plasma membrane. In this scenario,
microtubules might contribute to assist the microfibril strength at a vulnerable stage of synthesis through mechanical interactions with plasma membrane. Another possible role is that microtubules may maintain the longevity and/or activity of the CSC. The long life-span of the CSC would produce long cellulose microfibrils.

1.4 INVOLVEMENT OF COBRA IN ANISOTROPIC CELL EXPANSION

1.4.1 The COBRA GPI-anchored protein is required for anisotropic cell expansion.

The glycosylphosphatidylinositol (GPI)-anchored protein COBRA (COB) is another key player controlling anisotropic cell expansion. Cells in the root elongation zone of cobra mutants undergo radial swelling rather than elongating longitudinally, indicating that COB is required for anisotropic growth (Schindelman et al., 2001). The cobra mutant, initially isolated in a screen for abnormally expanding roots, is a conditional mutant with phenotypes developing in the presence of high concentrations of sucrose (Benfey et al., 1993; Hauser et al., 1995). Three conditional alleles of COB, cob-1, 2 and 3, which have missense mutations, have nearly identical phenotypes. All three alleles produce swollen roots, but no apparent phenotype in the aerial regions (Schindelman et al., 2001). cob-4, 5, and 6, all T-DNA insertion lines, are null mutants, and have constitutive radially swollen root phenotypes and the aerial part of the mutant is severely stunted with thick and swollen organs (Roudier et al., 2005, Ko et al., 2006).

COB is highly expressed during rapid expansion of organs including roots and dark-grown hypocotyls (Shindelman et al., 2001; Roudier et al., 2005). Immunofluorescence labelling studies have demonstrated that COB is polarly localized in the longitudinal side of the root cells in the rapid elongation zone (Schindelman et al., 2001; Roudier et al., 2005). More interestingly, Roudier et al. (2005) found that COB in elongating cells aligned in transverse bands in a similar pattern to cortical microtubules, and that the COB transverse band pattern is microtubule-dependent.
COB is a GPI-anchored protein that is anchored to the extracellular surface of the plasma membrane and can be released to the cell wall through the cleavage of GPI-linkages by phosphatidylinositol-specific phospholipases (Schindelman et al., 2001; Roudier et al., 2005). COB contains a putative cellulose-binding domain but it has not been shown to be functional (Roudier et al., 2002, 2005). Like other GPI-anchored proteins, COB would be modified with a GPI-anchor in the ER and secreted to extracellular surface of the plasma membrane through vesicular trafficking. Cells treated with the vesicle-trafficking inhibitor, brefeldin A (BFA), had weak COB signals at the cell surface and accumulation of COB in large intracellular compartments (Roudier et al., 2005). This suggests that COB is secreted via vesicle trafficking and that the transverse band pattern reflects where COB is deposited at the extracellular surface.

1.4.2 COBRA and cellulose microfibrils

Loss of anisotropic growth in *cobra* mutants is accompanied by a decrease in cellulose content and disorganized cellulose microfibrils, suggesting that COB is involved in cellulose synthesis (Schindelman et al., 2001; Roudier et al., 2005). Since previous studies have shown that reduced cellulose content is correlated with disorganization of cellulose microfibril orientation (discussed in section 1.2.3), Roudier et al. (2005) examined whether COB was required directly for cellulose synthesis or for organizing cellulose microfibril orientation. Results using *cob-1* conditional mutants grown under restrictive conditions suggest that COB may be involved in orienting cellulose microfibrils rather than regulating cellulose synthesis (Roudier et al., 2005). In cell wall analysis of *cob-1*, however, the inner (radial) epidermal wall was used for measuring birefringent retardance and for investigating microfibril orientation by FESEM. Taking into consideration the fact that COB is most abundant in the outer epidermal cell wall and apparently excluded from the radial and anticlinal walls (Roudier et al., 2005; described in Chapter 6), this interpretation needs to be re-examined to understand COB’s function (Wasteneys and Fujita, 2006).
1.5 AIMS AND SIGNIFICANCE OF THIS PROJECT

Determining the direction of cell growth requires both well-organized microtubules and cellulose microfibrils aligned in a highly ordered manner as cells expand. During primary wall formation, the parallel order of the innermost layer of cellulose microfibrils generates great resistance to isotropic turgor pressure, thereby contributing to establishing and maintaining growth anisotropy. Little is known, however, about the alignment of cellulose microfibrils in dividing cells, in the newly formed walls, and in the anticlinal cross walls where little expansion occurs. To further explore the control of cellulose microfibril alignment related to microtubules and growth anisotropy, cellulose microfibrils in different faces of the wall, cell types, and developmental stages are documented using the Arabidopsis inflorescence stem as a model system by field emission scanning electron microscopy (FESEM), as described in Chapter 2.

Previous studies have demonstrated that microtubules or pre-existing cellulose microfibrils are not required for controlling cellulose microfibril orientation (Sugimoto et al., 2003; Himmelspach et al., 2003). These studies suggest that one possible role of microtubules in expanding cells is to maintain the structure of cellulose microfibrils and/or the longevity of the CSCs to provide the cell with microfibrils that are long and strong enough to prevent expansion of the cell wall in the direction of their predominant orientation (Wasteneys, 2004). In another words, disorganized or depolymerized microtubules may alter cellulose microfibril properties. In chapter 3, the microtubule-length-regulation hypothesis proposed by Wasteneys (2004) is tested using the inflorescence stem of the mor1-1 mutant to analyze various properties of cellulose microfibrils including crystallinity by x-ray diffraction analysis, molecular weight of cellulose by gel permeation chromatography, and microfibril length by transmission electron microscopy.

Previous studies using YFP-tagged CesA6 have described a close relationship between microtubules and CSC movement (Paredez et al., 2006a). There is still a lack of direct evidence that YFP-CesA6 particles represent actual CSCs and not vesicles containing YFP-CesA6 for secretion at the plasma membrane. To obtain supporting evidence that YFP-CesA6 particles are actual
CSCs producing cellulose microfibrils, I use an actin-disrupting drug that arrests cytoplasmic streaming to examine the effect on YFP-CesA6 movement. Since CSCs are enzyme complexes that are affected by temperature, the control of temperature during imaging is required to examine the velocity of YFP-CesA6 particles accurately. The YFP-CesA6 velocity is examined at 21°C and 29°C. To examine if the movement and velocity of YFP-CesA6 particles are affected in the mor1-1 mutant, a comparison of the movement and velocity of YFP-CesA6 particles in wild type and mor1-1 at 21°C and 29°C by spinning disc confocal microscopy is described in Chapter 4.

CesA1 is one of the cellulose synthase subunits playing a role in primary wall formation. In chapter 5, I describe allele-specific phenotypes of the any1 mutant, a novel allele of CesA1. Compared with other CesA1 mutant alleles, any1 has a relatively mild phenotype that enables examination of its phenotype to gain more understanding of CesA1 involvement in cellulose biosynthesis in mature plants. I examine growth, cellulose microfibril orientation, and cellulose crystallinity of the any1 inflorescence stems.

The COBRA (COB) GPI-anchored protein is required for anisotropic cell expansion and cellulose synthesis. As a GPI-anchored protein, COB is predicted to follow a secretion path and be positioned at the outer surface of the plasma membrane. In Chapter 6, COB subcellular localization is further examined by transmission electron microscopy on high-pressure-frozen, immunogold-labelled wild-type roots. Roudier et al. (2005) found that the COB transverse band pattern is microtubule-dependent in the elongating root. To investigate the role of microtubules in COB deposition, I examine the distribution of COB in microtubule-drug treated roots and in the mor1-1 roots, along with epitope-tagging of COB.

Finally, my general conclusions are presented in Chapter 7.

My specific research objectives were
1. To document the cellulose microfibril orientations in various cell face and types, which include both dividing and elongating cells to examine the relationships
between cellulose microfibrils, orientations, microtubules, and growth anisotropy in the Arabidopsis inflorescence stem as a system;

2. To test the hypothesis that disorganized microtubules affect the properties of cellulose microfibrils, by comparing these properties in wild type and the *mor1-1* mutant;

3. To test the hypothesis that disorganized microtubules affect the movement and velocity of CSCs using YFP-CesA6 to compare CSC movement in wild type and the *mor1-1* mutant;

4. To investigate allele-specific phenotypes of the constitutive any1 CESA1 mutant allele to interpret CesA1 functions in the elongating cells of inflorescence stems;

5. To examine the subcellular localization of COBRA (COB), how microtubules are involved in the distribution of COB protein, and to assess the specificity of the COB antibody by examining COB's distribution in transgenic plants expressing an epitope-tagged version of COB.
CHAPTER 2

Cellulose microfibril organization and the development of the Arabidopsis thaliana inflorescence stem
2.1 INTRODUCTION

Primary cell walls, which are formed in growing cells, are composed of cellulose microfibrils, hemicelluloses, pectins and secreted proteins. Cellulose microfibrils are strong and inextensible elements that give mechanical strength to the wall. The arrangement of newly synthesized cellulose microfibrils, deposited in the innermost layer of the cell wall, is thought to determine the growth direction during diffuse expansion (Preston, 1974; Richmond et al., 1980; Richmond, 1983; Taiz, 1984; Carpita and Gibeaut, 1993). Previous electron microscopy work described the cellulose microfibril alignment in various types of the plant cells, but only in limited regions of the wall (Palevitz and Hepler, 1976, Hardham et al., 1980; Seagull and Heath, 1980; Vesk et al., 1996). In Arabidopsis, orientation of cellulose microfibrils has been described in various cell types by polarized laser light microscopy on stained tissues with Congo Red, which is a fluorescent dye that binds to cellulose microfibrils (Kerstens and Verbelen, 2003). Another method is to measure birefringence retardation on thin sections of tissues using a polarized-light microscope (Baskin et al., 1999). Both of these methods, however, provide a preferential mean or net orientation of cellulose microfibrils through all layers of the wall and do not measure the orientation of the most recently deposited microfibrils.

A technique to view the innermost layer of cellulose microfibrils in intact organs was developed by Sugimoto et al. (2000). The cell wall preparation for field emission scanning electron microscopy (FESEM) removes the cytoplasmic content from the cryo-planed tissue and the innermost layer of cellulose microfibrils is exposed (Sugimoto et al., 2000). This method has subsequently been widely used to examine the orientation of cellulose microfibrils in the roots of various Arabidopsis mutants (Sugimoto et al., 2000, 2001, 2003; Burk et al., 2002; Pagant et al., 2002; Wiedemeier et al., 2002; Himmelspach et al., 2003, Baskin et al., 2004; Roudier et al., 2005), in dark-grown hypocotyls (Réfregier et al., 2004; MacKinnon et al., 2006), in fiber cells (Burk and Ye, 2002; Zhong et al., 2002), in pith cells (Burk and Ye, 2002; Zhong et al., 2005), and in parenchyma cells (Burk and Ye, 2002). Compared to roots and dark-grown hypocotyls, the inflorescence stem is a large organ, which is easy to prepare by cryo-planing, so that cellulose microfibrils can be observed in
various cell types, from epidermal layers to inner cell layers, and from recently divided cells to mature cells.

Despite some previous studies describing cellulose microfibril orientation in the most recently deposited cell wall layer, this information is lacking for many cell types or for specific walls within these cells including anticlinal cross walls where relatively little expansion occurs, newly synthesized walls, junctions of walls, cells undergoing division, developing guard cells, and trichomes. The inflorescence stem is an ideal organ for FESEM analysis, which allows us to analyze the orientation of cellulose microfibrils in relation to growth anisotropy and to the organization of microtubules. To understand the relationship between cellulose microfibril arrangement, microtubule organization, and growth anisotropy, I describe in this chapter FESEM analysis of the upper region of the Arabidopsis inflorescence stem, where cells are produced prior to undergoing rapid elongation.
2.2 MATERIALS AND METHODS

2.2.1 Plant materials and growth condition

The Arabidopsis (Arabidopsis thaliana) wild type Columbia ecotype was used to examine the cellulose microfibril orientation in various cell types found in inflorescence stems. Seeds were surface sterilized in a mixture of 50 % (v/v) ethanol and 3 % (v/v) hydrogen peroxide for 2 min. After rinsing in sterilized water, seeds were planted on nutrient-solidified Hoagland’s plates (2 mM KNO₃, 5 mM Ca(NO₃)₂, 2 mM MgSO₄, 1 mM KH₂PO₄, 9 0μM EDTA, 46 μM H₃BO₄, 9.2 μM MnCl₂, 0.77 μM ZnSO₄, 0.32 μM CuSO₄, 0.11 μM MoO₃, 3 % (w/v) sucrose and 1.2 % (w/v) Bacto Agar (Difco Laboratories). Plates were sealed with surgical tape (Micropore, 3M, USA), and held vertically in a wooden rack. After 3-4 days in a cold room, plates were moved to a growth cabinet under constant light (80-100 μmol/m²/s) at 21°C.

2.2.2 Cell wall preparation for the inflorescence stem

The upper part of the inflorescence stems was excised with a razor blade into 5 mm lengths, fixed in 0.5 % (v/v) glutaraldehyde and 4 % formaldehyde made up in PME buffer (25 mM PIPES, 0.5 mM MgSO₄, and 2.5 mM EGTA, pH 7.2) for 20 min under vacuum and for another 30 min without vacuum, and rinsed three times for 10 min in PME buffer. Prior to cryo-planing, samples were cryoprotected in 25 % and 50 % (v/v) DMSO in PME buffer for 10 min each. The stem piece was placed on a pin-head with tissue-tek embedding media (Tissue-tek OCT compound, Sakura) and frozen in liquid nitrogen. To perform cryo-planing of the stem, a glass knife and cryo-chamber were maintained at -120°C. The surface of the stem was sliced off with a glass knife set at a 6° angle with a speed of 0.6 mm/sec on a cryo-ultra-microtome (Ultracut T ultramicrotome with Leica EM FCS attachment, Leica). The remaining portion of the stem on the pin was thawed in 50 % (v/v) DMSO in PME buffer and rinsed in PME buffer. In order to extract cytoplasmic materials, cryo-planed stems were incubated either in 0.1 %, 0.5 % or 1.0 % NaClO solution for 10
min on a rotary shaker, followed by three 10 min washes in distilled water. The stem samples were incubated with 2 % OsO₄ for 1 h at room temperature and washed in distilled water three times for 10 min. After the dehydration steps with an ethanol series (30, 50, 70, 95, and 100 % three times, 20 min each step), samples were critical point dried in a Autosamdri 815B critical point dryer (Tousimis) using CO₂ with a purge time of 15 min. The cut surface of the samples was placed upward, mounted on aluminum stubs with double-sided sticky carbon tape, and then coated with Pt/Pd (80/20) at 20 mA to a 5 nm thickness (High resolution sputter coater 208HR, Cressington).

2.2.3 Cell wall preparation for leaf trichomes

Rosette leaves were fixed with 4 % formaldehyde in 1xPME for 15 min under vacuum, washed in 1xPME three times, and then embedded in 2 % low-melting temperature agarose. Agarose blocks with leaves were sectioned with a vibratome (VT1000, Leica) at speed 3, frequency 10, and 80 µm thickness. Cytoplasmic materials were extracted with either 0.1, 0.5, or 1 % NaClO for 20 min and washed in distilled water. Leaf sections were then incubated with 2 % OsO₄ for 1 h at room temperature and washed in distilled water three times for 10 min. Following dehydration with an ethanol series (30, 50, 70, 95, and 100 % three times, 20 min each step) and critical point drying (Balzers 020), the samples were mounted on carbon stubs with double-sided sticky carbon tape, and coated with gold at 25 mA for 225 sec (Nanotech). To give more conductivity around the sample, silver paste was placed around the sample.

2.2.4 Field emission scanning electron microscopy (FESEM)

Cellulose microfibrils were observed with a Hitachi S4700 SEM set at 3 kV and 10 µA current, 1 kV or 5 kV for trichome cellulose microfibrils. High-resolution images were taken with an upper detector and with a working distance between 5 to
6 mm. Objective and beam apertures were set at 3 and 2 respectively. Samples from more than three individual plants were viewed.
2.3 RESULTS

2.3.1 Cellulose microfibrils in the upper part of inflorescence stems

The cells that make up the inflorescence stem contribute to the establishment and maintenance of stem growth by expanding longitudinally. Epidermal cells in the Arabidopsis thaliana inflorescence stem elongate more than 80 times their original length (Suh et al., 2005). The epidermal layer in the upper part of the inflorescence stems contained polygonally shaped cells that have yet to undergo rapid expansion, and cell plates were evident in some cells, indicating that they were undergoing cytokinesis at the time of fixation (Figure 2-1A and B). FESEM images revealed densely packed microfibrils overlain by many short, crooked and interconnected strands that had microfibrillar appearance (Figure 2-1C to G). These short disorganized strands were likely to be the most recently synthesized cellulose microfibrils for two reasons. First, they often displayed free ends. Second, they were less abundant when stems were fixed by rapid freezing instead of chemical fixation (data not shown). These observations suggest that during the early stages of chemical fixation, some cellulose is produced or that the chemical fixation process modifies the most recently synthesized cellulose so that it dissociates from the densely packed microfibril lamella to generate tangled loose ends. These tangled strands were typically observed and abundant in cells from the upper region of the stem but mostly absent in older cells.

The orientation of the long, densely packed microfibrils beneath the crooked microfibrillar strands was analyzed. In cells judged to be dividing by the presence of cell plates, microfibrils were oriented at a wide variety of angles with no predominant orientation (Figure 2-1C and D). In contrast, in polygonally shaped cells lacking cell plates, cellulose microfibrils were aligned transversely to the stem’s growth axis (Figure 2-1E to G). Taken together, this suggests that the transverse orientation of cellulose microfibrils is well established in young epidermal cells prior to rapid elongation, and that parallel microfibril order is rapidly lost during mitosis.
Figure 2-1 Cellulose microfibril orientations in epidermal cells of the upper part of inflorescence stems.

Arrows indicate stem growth axis.

(A) Longitudinal section of the inflorescence stem.
(B) Epidermal cells. Cell (C) and (D) were divided by a cell plate.
(C) and (D) Relatively random orientation of cellulose microfibrils.
(E) to (G) Transverse orientation of cellulose microfibrils.

Scale bars = 100 µm in (A), 5 µm in (B), 500 nm in (C) to (G).
2.3.2 Cellulose microfibrils at the cell plate and anticlinal cross walls

Cells partitioned by cell plates were often observed in the epidermal and cortex layers in the upper region of inflorescence stems. Most of the cell plates I observed were inserted perpendicularly to the stem growth axis, which will later form anticlinal cross walls. The extraction procedure retained cellulose microfibril meshworks in the newly synthesized cell plates (Figure 2-2A and B), and these microfibrils were often connected to microfibrils in the inner periclinal and radial walls (Figure 2-2C). Variations in the appearance of cell plates, from simple meshworks (Figure 2-2A) to meshworks interspersed with more solid cell wall materials (Figure 2-2B to F), probably represent different stages of cell plate and cross wall development.

These observed cell plates give rise to the anticlinal cross walls between cells. Cellulose microfibril deposition patterns at the transverse anticlinal walls have not previously been documented. Cellulose microfibrils at the anticlinal wall of cells from the upper region of the stems showed tangled strands and random orientations (Figure 2-3A). In older cells, the tangled strands were absent and cellulose microfibril orientations were varied among the cells (Figure 2-3B to D). Patches of parallel microfibrils were aligned in variable orientations in some cells (Figure 2-3 B), while patches of parallel microfibrils and an underlying parallel texture that was transverse to the radial axis of the stem were observed in other cells (Figure 2-3C and D).
**Figure 2-2** Cellulose microfibrils at cell plates.

Asterisks indicate the area from which the higher magnification images were taken.

(A) Cell plate showing meshworks of cellulose microfibrils.
(B) Meshworks of cellulose microfibrils interpersed with solid wall material.
(C) In the area between the cell plate and the inner periclinal wall, some cellulose microfibrils are linked between the cell plate and the inner periclinal wall as shown by arrows.
(D) Higher magnification image of (B).
(E) Meshworks with more solid wall material.
(F) Higher magnification image of (E).

Scale bars = 5 µm in (A) and (B), 500 nm in (C) to (F).
**Figure 2-3** Cellulose microfibrils at anticlinal walls.

Asterisks indicate the area from which the higher magnification images were taken.

(A) Random cellulose microfibrils underneath of the entangled strands in a young cell.

(B) to (D) Absence of the entangled strands in older cells and variations in microfibril texture. Patches of parallel microfibrils in various orientations (B), patches of parallel microfibrils and underlying microfibrils that are transverse to the radial axis of the stem and patches of microfibrils in various orientations (C) and (D).

Scale bars = 500 nm.
2.3.3 Cellulose microfibril orientation in different tissue layers

To examine if cellulose microfibril orientations are consistent throughout the different tissue layers (Figure 2-4A), cellulose microfibrils were observed in epidermal cells (Figure 2-4B), cortex cells in the first (Figure 2-4C), second (Figure 2-4D), and third layers (Figure 2-4E), and parenchymatous cells in the first (Figure 2-4F) and second layers (Figure 2-4G). Cellulose microfibrils underneath the short disorganized microfibril strands were consistently transverse to the stem’s growth axis in all tissue and cell layers examined (Figure 2-4B to G) with the exception of cells undergoing division, as indicated by the presence of a forming cell plate.

2.3.4 Cellulose microfibril orientation at the radial walls

Cellulose microfibrils were transversely oriented in radial walls (Figure 2-5A and B). Some cellulose microfibrils appeared to continue from one cell face to another as shown in Figure 2-5C at the junction of radial, anticlinal and inner periclinal walls, suggesting that CSCs can continue to synthesize cellulose around the corners of cells.

2.3.5 Cellulose microfibrils in pit fields

Pit fields were often observed in inner periclinal, radial and anticlinal walls and in all types of the cells. Pit fields often had a distinctive circular shape of cellulose microfibrils (Figure 2-6A and B).
Figure 2-4 Cellulose microfibril orientations in the cells from each different cell layers of the inflorescence stem.

(A) Longitudinal section of the inflorescence stem.
(B) to (G) Images of cellulose microfibrils in the cells from each cell layer of (A). Transverse orientation of cellulose microfibrils in epidermis (B), first cortex layer (C), second cortex layer (D), third cortex layer (E), first layer of parenchymatous cells (F), second layer of parenchymatous cells (G).

Scale bars = 30 µm in (A), 500 nm in (B) to (G).
Figure 2-5 Cellulose microfibrils at radial walls.

Asterisks indicate the area from which the higher magnification images were taken. Arrowheads indicate pit fields.

(A) and (B) Transverse cellulose microfibrils in the radial wall with pit fields. (C) Cellulose microfibrils in the corner of the cell show continuation of cellulose microfibrils from the inner periclinal wall.

Scale bars = 1 µm.
Figure 2-6 Cellulose microfibril arrangement surrounding pit fields.

(A) Clusters of pit fields each of which is surrounded by a circular microfibrillar structure.
(B) High-magnification image of individual pit fields showing the circular arrangement of microfibrils.
2.3.6 Cellulose microfibrils in guard mother cells and guard cells

Many pit fields were observed in the wall dividing epidermal cells from guard mother cells or meristemoids (Figure 2-7A and B). Developing guard cells, prior to stomatal pore formation, had a random orientation of cellulose microfibrils (Figure 2-7C), while mature guard cells had a radial orientation of cellulose microfibrils (Figure 2-7D). Dense accumulation of cellulose microfibrils was observed around the pore area (Figure 2-7E), but no microfibrillar structures were observed at the outer surface of the pore (Figure 2-7F).

2.3.7 Cellulose microfibril orientation in leaf trichomes

Trichomes are huge single epidermal cells more than 500 µm in length. To overcome the difficulty of sectioning these three-dimensional structures, I embedded layers of leaves in agarose blocks and obtained sections using a vibratome. Due to the big single cell without any supporting tissue layers, trichomes, after FESEM preparation, were often distorted (Figure 2-8A and C). Cellulose microfibrils in the stalks and branches of trichomes were longitudinally oriented, parallel to the cell’s growth axis (Figure 2-8B and D).
Figure 2-7 Cellulose microfibrils in guard cells and guard mother cells.

Asterisks indicate the area from which the higher magnification images were taken.

(A) Many pit fields in the wall between the epidermal cells and the meristemoid, which gives rise to the guard cell lineage.
(B) Pit fields and cellulose microfibrils in the wall between the epidermal cell and the meristemoid.
(C) Random cellulose microfibrils in a developing guard cell.
(D) Radial orientation of cellulose microfibrils in a mature guard cell.
(E) Cellulose microfibrils close to the pore.
(F) No cellulose microfibrils were observed in the outer wall of the pore.

Scale bars = 1 µm in (A), (B), (D), 500 nm in (C), (E), (F).
Figure 2-8 Cellulose microfibrils in leaf trichomes.

White boxes indicate the area from which the higher magnification images were taken.

(A) The stalk of a trichome after vibratome sectioning to remove the tip.
(B) High-magnification image of the inner wall surface of the stalk shown in (A). Cellulose microfibrils were aligned in parallel to the cell long axis.
(C) One of the branches of a trichome.
(D) High-magnification images of the branch (C). Cellulose microfibrils were aligned parallel to the growth axis.

Scale bars = 50 µm in (A), 1 µm in (B) and (D), 10 µm in (C).
2.4 DISCUSSION

2.4.1 Establishing transverse orientation of cellulose microfibrils

In the upper part of inflorescence stems, epidermal cells with polygonal shapes, except those undergoing division, were transversely oriented relative to the stem’s growth axis, suggesting that transverse cellulose microfibril patterns are established prior to the elongation phase. Similar results have been reported in Arabidopsis roots where disc-shaped cells in the root cell division zone have predominantly transverse orientation of cellulose microfibrils to the root growth axis (Sugimoto et al., 2000), and in leaf primordia (Verbelen and Kerstens, 2000) and very young leaves (Kerstens and Verbelen, 2003) where spherically shaped epidermal cells have orientations that are transverse to the leaf growth axis.

In contrast to the transverse parallel order of cellulose microfibrils in most cells, those cells presumed to be undergoing cell division by the presence of cell plates showed no preferred cellulose microfibril orientation. The loss of parallel cellulose microfibril orientation in cells undergoing division can be interpreted in different ways. Reduced cellulose synthesis has been correlated with loss of parallel microfibril orientation (Sugimoto et al., 2001) so it is possible that cellulose synthesis is reduced during mitosis. Alternatively, assuming that cellulose synthesis continues throughout mitosis, the absence of cortical microtubules during this phase could account for the altered deposition pattern, although this has previously been shown to not generate randomly oriented cellulose microfibrils (Sugimoto et al., 2003; Baskin et al., 2004) or to change the parallel tracking of fluorescently tagged CesAs (Paradez et al., 2006a). Finally, it is possible but unlikely that in the absence of cellulose synthesis, microfibrils deposited transversely prior to mitosis are passively reoriented into random patterns during mitosis.
2.4.2 Cellulose microfibril orientation at the cell plate

So far FESEM is the only tool able to examine the texture of cellulose microfibrils at the cell plate. The thin layers of cellulose microfibrils organized in meshworks were often observed in the cell plates or newly formed cross walls dividing epidermal and cortex cells. The process of cell plate formation has previously been studied using transmission electron microscopy (Samuels et al., 1995). Little is known, however, about cellulose microfibril synthesis and orientation in the cell plate and early cross wall. The present study demonstrates that there is no predominant orientation of cellulose microfibrils at the cell plate. The presence of microfibrils linking the cell plate with radial and periclinal walls also suggests that cellulose-synthase-complexes (CSCs) initiated at the cell plate can continue to produce cellulose microfibrils when they reach the junction with the existing parental walls or vice versa. During formation of a new cell wall within the cell plate, cell wall polysaccharides, especially callose, are actively deposited prior to cellulose deposition (Kakimoto and Shibaoka, 1992; Samuels et al., 1995). The immunogold labelling with anti-callose antibody may provide answers if the patches of solid wall materials shown in Figure 2-2C are callose.

Previous TEM observations during cell plate formation demonstrated that cell wall assembly starts with the disappearance of the phragmoplast microtubules, which are oriented perpendicular to the cell plate (Samuels et al., 1995). The formation of meshworks of cellulose microfibrils in the cell plate is thus likely to occur in the absence of microtubules.

2.4.3 Cellulose microfibril orientations at the transverse anticlinal walls where microtubules may not be present

Cellulose microfibril orientation was transverse in the longitudinal walls of epidermal cells, while more or less random in the transverse anticlinal walls, similar to the patterns observed in the transverse cell plates that give rise to these walls. In contrast to longitudinal (periclinal and radial) walls, transverse anticlinal walls undergo relatively little expansion unless the inflorescence stem increases in
Cortical microtubules are often not observed at the anticlinal face of elongating cells. Variations in microfibril orientation in anticlinal walls may therefore reflect different developmental stages of anticlinal walls. Anticlinal walls could be an example of cortical microtubule-independent cellulose microfibril deposition.

2.4.4 Cellulose microfibril arrangement in pit fields

Previous studies on freeze-fractured membranes have shown clusters of pit fields containing circular structures about 150 nm in diameter (Mueller and Brown, 1982a, 1982b). My FESEM analysis showed that the diameter of the circular structures in Arabidopsis was similar to those from maize shown by Mueller and Brown, suggesting that the size of plasmodesmata is the same in these different plants. Compared to the analysis of freeze-fractured membranes, FESEM provided a more detailed arrangement of microfibrils in these structures, showing a circular arrangement of microfibrils with radially arranged microfibrils between the circular microfibrils and surrounding microfibrils. The distinctive circular structures often observed around pit fields imply that CSC movement is carefully coordinated at the site of plasmodesmata formation.

2.4.5 Cellulose microfibril organization during stomatogenesis

FESEM analyses demonstrated that cellulose microfibrils are aligned radially in mature guard cells, consistent with previous studies (Ziegenspeck, 1938; Singh and Srivastava, 1973; Palevitz and Hepler, 1976; Galatis, 1980; Busby and Gunning, 1984). In contrast, cellulose microfibrils were aligned randomly in developing guard cells prior to pore formation. These results support the idea that the radial pattern of cellulose microfibrils contributes to the shaping of the pore (Busby and Gunning, 1984). Co-alignment between radial microtubule arrays and cellulose microfibrils has been shown in guard cells (Palevitz and Hepler, 1976). Lucas et al. (2006) reported that microtubule arrays are radial in developing guard cells prior to pore formation, which does not correlate with random cellulose microfibrils observed here.
by FESEM. These results suggest that microfibril orientation may not be always co-aligned with microtubule orientation during stomatogenesis.

Accumulation of cellulose microfibrils around the stomatal pore explains locally distinctive areas that may contribute to the thickening of the wall around the pore observed by TEM (Palevitz and Hepler, 1976; Busby and Gunning, 1984; Zhao and Sack, 1999). The absence of microfibrillar structures at the outer surface of the pore could indicate the presence of cuticle (Zhao and Sack, 1999). Abundant pit fields in the wall dividing guard mother cells and meristemoids from the epidermal cells suggest that cell-cell communication via plasmodesmata with neighboring cells occurs actively in these cells.

2.4.6 Cellulose microfibril orientation in trichomes

FESEM analysis on trichomes revealed longitudinally oriented cellulose microfibrils in both the stalks and branches, which is consistent with results obtained when Congo Red-stained trichomes were observed by polarized laser confocal microscopy (Kerstens and Verbelen, 2003). I attempted to observe cellulose microfibril orientation along the trichome at different developmental stages, but this was difficult to ascertain after sectioning because of distortions to the shape of trichomes prepared for FESEM. More modifications for the sample preparations are required for viewing cellulose microfibrils in these huge single cells with complex-shapes.
CHAPTER 3

Cortical microtubules and the mechanical properties of cellulose in wild-type and mor1-1 inflorescence stems
3.1 INTRODUCTION

Anisotropic cell expansion, in which cells grow predominantly in one direction, is a crucial event for plant cells to establish the proper shape and volume that reflects their functions, and often influences organ and entire plant morphology. This process requires both well-organized cortical microtubules and cellulose microfibrils, the parallel arrangement of which in the cell wall allows expansion perpendicular to the direction in which they are deposited (Wasteneys, 2000; Baskin, 2001; Sugimoto et al., 2003; Wasteneys, 2004).

The growth of the plant cell is principally dependent on the mechanical properties of the cell wall. The primary cell wall in expanding cells is thin and semi-rigid, yet sufficiently strong to resist the forces of turgor pressure generated within the cell. Cellulose microfibrils are strong and inextensible reinforcing elements that are arranged in parallel with one another in expanding cells to determine the cell growth axis (Preston, 1974; Richmond et al., 1980; Richmond, 1983; Taiz, 1984; Carpita and Gibeaut, 1993). Inhibition of cellulose synthesis with certain herbicides causes loss of growth anisotropy (Hogetsu et al., 1974; Heim et al., 1991; Sabba et al., 1999; Scheible et al., 2003). Also, many cellulose-deficient mutants, such as radial swelling 1 (Arioli et al., 1998), korrigan (Lane et al., 2001; Nicol et al., 1998; Sato et al., 2001), kobito (Pagant et al., 2002), and cobra (Schindelman et al., 2001; Roudier et al., 2005), show defects in growth anisotropy.

In recent years, it has become clear that sufficient cellulose biosynthesis is required for the production of well-ordered cellulose microfibrils. Analysis of cellulose-deficient mutants reveals that reduced cellulose content is coupled with disordered cellulose microfibrils, resulting in swelling of the cells (Sugimoto et al., 2001; Sato et al., 2001; Pagant et al., 2002; Burk and Ye, 2002; Roudier et al., 2005; MacKinnon et al., 2006). These studies suggest that there may be a general linkage between the rate of cellulose production and its optimal alignment, and that both of these factors are essential for controlling growth direction (Wasteneys, 2004). In contrast, there seems no such correlation in other cases, such as fiber cells in the fragile fiber 1 (fra1) mutant (Zhong et al., 2002).
Since 1963, when Ledbetter and Porter first described coincident orientation of cortical microtubules and cellulose microfibrils, cortical microtubules have been considered as the most likely candidates to influence the deposition and organization of cellulose microfibrils in the cell wall. According to transmission electron micrographs of growing cells, cellulose microfibrils are often found to align parallel to cortical microtubules (Ledbetter and Porter, 1963; Seagull, 1985). Disruption of cortical microtubules has revealed that loss of oriented microtubules perpendicular to the growth axis is correlated with radial swelling of the cell (Baskin et al., 1994). Many microtubule-disorganized mutants also have radial swelling phenotypes. They include fass/ton (McClinton and Sung, 1997; Camilleri et al., 2002), mor1-1 (Whittington et al., 2001), and bot1/fra2/erh3/lue1 (Bichet et al., 2001; Burk et al., 2001; Webb et al., 2002; Bouquin et al., 2003). Early studies have shown that disruption of cortical microtubules using anti-microtubule drugs is also correlated with disruption of the pattern of cellulose deposition and radial swelling of the cell (Takeda and Shibaoka, 1981; Mueller and Brown, 1982b).

While many studies have shown that there is a correlation between the orientation of cortical microtubules and cellulose microfibrils in expanding cells, there have been some exceptions (Preston, 1988; Emons et al., 1992; Baskin, 2001). Recent studies using the temperature-sensitive microtubule organization 1-1 (mor1-1) mutant and the microtubule-depolymerizing drug oryzalin demonstrated that microtubule disruption accompanied radial swelling, without altering the cellulose microfibril orientation (Sugimoto et al., 2003). Himmelspach et al. (2003) demonstrated that cellulose microfibrils were able to re-establish transverse parallel order with neither organized microtubules nor an organized template of cellulose microfibrils by using mor1-1 and the cellulose inhibitor 2,6-dichlorobenzonitrile (DCB). These studies suggest that cellulose microfibril orientation is largely self-ordered and that cortical microtubules are not essential for orienting the deposition of cellulose microfibrils. Furthermore, disorganized microtubules in mor1-1 did not affect cellulose content, indicating that loss of growth anisotropy was not caused by a defect in cellulose production in this mutant (Sugimoto et al., 2003). These studies
raised the question of what might be the exact role of transverse cortical microtubules in expanding cells.

Wasteneys (2004) proposed a new model in which cortical microtubules might influence the length and strength of cellulose microfibrils. Well-organized transverse cortical microtubules with close linkage to the plasma membrane where cellulose synthesis occurs may stabilize newly synthesized microfibrils against mechanical weakness. Cellulose microfibrils are made of linear homopolymers of β-1,4-linked D-glucan chains that are assembled into microfibrils that include both crystalline and non-crystalline regions. The degree of cellulose crystallinity is one of the factors that influences the mechanical properties of cellulose microfibrils. Crystalline cellulose stabilized by hydrogen bonds and van der Waal’s forces between adjacent glucan chains, gives chemical resistance and mechanical strength to microfibrils (Williamson et al., 2002). Another factor that may contribute to the mechanical properties of cellulose microfibrils is the degree of cellulose polymerization (DP), which refers to the number of monomeric glucose residues per cellulose molecule. DP is not correlated with native microfibril lengths. It is thought that glucan chains may end randomly within microfibrils since cellulose microfibrils are longer than the chain length estimated from DP (Brett, 2000). Increasing the frequency of glucan chain termination is likely to weaken the overall strength of a microfibril (Wasteneys and Fujita, 2006).

X-ray diffraction analysis can be used to measure the degree of cellulose crystallinity. On the other hand, X-ray diffraction has been the main technique used to study the crystal structure in plant cell walls since it avoids the damage associated with isolation and purification of cellulose (French, 1985). On the other hand, it has been suggested that the relatively small extent of crystalline cellulose in the primary wall may make it challenging to obtain detailed crystallinity information by X-ray diffraction analysis (Newman et al., 1996; Newman, 2004; Sturcová et al., 2004). Although there are potential problems with the primary wall, X-ray diffraction analysis should be a useful approach to compare relative values of the degree of cellulose crystallinity among different genotypes.
The inflorescence stem of *Arabidopsis thaliana* is an ideal system to study the relationship between microtubules and cellulose microfibril properties. Expansion in the stem is highly anisotropic, with extensive longitudinal expansion and relatively little radial expansion, resulting in a cylindrical organ shape (Chapter 2). Compared to other cylindrically shaped organs, the inflorescence stem has a more extensive elongation zone that allows us to obtain a sufficient amount of material for cellulose analysis.

In this chapter, using the Arabidopsis inflorescence stem as a model system, I have tested the hypothesis that microtubules may influence the physical properties of cellulose microfibrils (Wasteneys, 2004). Despite the fact that growth anisotropy is reduced, and that microtubules are disrupted and short in *mor1-1*, I show that cellulose microfibril orientation and cellulose content are normal in *mor1-1* inflorescence stems, as previously described for roots of the *mor1-1* mutant. Cellulose crystallinity is one of the factors that may influence the mechanical properties of microfibrils. X-ray diffraction analysis demonstrates that cellulose crystallinity in the wild type declines when stems undergo more rapid growth at 29°C, *mor1-1*’s restrictive temperature, while cellulose crystallinity in *mor1-1* remains at a high level, suggesting that cellulose crystallinity is controlled in order to define the mechanical properties of the cell wall during changing growth conditions, and that organized microtubules are involved in regulating cellulose crystallinity. To further investigate the relationship between microtubule organization and cellulose crystallinity, I compare the crystallinity among other microtubule-related mutants, such as *mor1-2* (Whittington et al., 2001), *botero1 (bot1)* (Bichet et al., 2001) and *RIC1-OX3* (Fu et al., 2005). The *mor1-2* mutant is another conditional allele of *MOR1*, which shows microtubule disorganization at restrictive temperature (Whittington et al., 2001). The *bot1* mutant has a mutation in the p60 subunit of the katanin microtubule severing protein, and shows aberrant microtubule organization (Bichet et al., 2001). Overexpression of RIC1, the ROP (plant-specific Rho GTPase) effector, induces microtubule bundling and well-ordered microtubules (Fu et al., 2005). I also describe experiments in which samples from the primary wall are prepared for analysis of molecular mass distribution of cellulose molecules by gel
permeation chromatography and in which individual cellulose microfibrils are isolated for the measurement of their length.
3.2 MATERIALS AND METHODS

3.2.1 Plant materials and growth condition

The Arabidopsis (*Arabidopsis thaliana*) homozygous *mor1-1* mutant (Whittington et al., 2001), which had been backcrossed eight times, and wild-type segregants from this backcross, were used throughout this study. Other microtubule-related mutant lines included *mor1-2* (Whittington et al., 2001), *botero1* (*bot1*) (Bichet et al., 2001), and *RIC1-OX3* (Fu et al., 2005). Plants were grown on Hoagland’s media in agar plates and placed in a 21°C growth cabinet after an initial cold treatment as described in Chapter 2. Ten day-old seedlings were transferred to soil and grown at 21°C in a growth cabinet as described in Chapter 2.

3.2.2 Measurement of inflorescence stem growth, cell length and width

3.2.2.1 Measurement of stem growth

Once the length of inflorescence stems of wild type and *mor1-1* grown at 21°C reached 5 to 8 cm in length, plants were marked at 1 cm intervals from the top and hereafter referred to as sections A, B and C respectively. Plants grown at 21°C for 1 day or 29°C for 1 day were used for the measurement of total length of each region. Average lengths of each region were then calculated from data on twenty inflorescence stems of wild type, *mor1-1* and other mutant lines. Standard deviations were calculated, and means were compared by the independent Student’s t-test for samples with unequal variance at a significance level of 0.05.

3.2.2.2 Measurement of cell length and width of epidermal cells

Epidermal peels from the three apical zones of wild-type and *mor1-1* inflorescence stems were obtained with forceps from the plants grown at 21°C, marked into three sections of 1 cm each and then grown either for 1 day or for 2 days at 29°C. A minimum of three peels from each region from five individual plants was collected and stained with 0.01 % calcofluor to visualize the cell wall and
mounted in Citifluor AF1 antifade agent. Fluorescent images were collected with a Zeiss Axiovert 200M inverted microscope equipped with an AxioCamHR camera (Carl Zeiss, Germany). A total of 285 cells in each region from five plants were measured for length and width, using Zeiss AxioVision image analysis software. The epidermal cells next to guard cells were not measured. Means between data points were compared by the Student’s independent t-test for samples with unequal variance at a significance level of 0.05.

3.2.3 Immunofluorescence labelling of microtubules in the epidermal cells of inflorescence stems

3.2.3.1 Preparation of inflorescence stem material

Inflorescence stem preparation for immunofluorescence labelling was performed using methods modified from Kazama and Mineyuki (1997) and Sugimoto et al. (2000). The A and B regions of wild-type and mort1-1 inflorescence stems grown at 29°C for 24 h were excised with scissors, fixed in 0.5 % (v/v) glutaraldehyde and 4 % formaldehyde made up in PME buffer (25 mM PIPES, 0.5 mM MgSO$_4$, and 2.5 mM EGTA, pH 7.2) for 1 h at 29°C, and rinsed three times for 10 min in PME buffer. The excised stem was placed on double-sided sticky tape that was taped on to a glass slide, and longitudinal sections of the stem were made with a razor blade. Excised longitudinal sections were affixed to the 0.2 % (w/v) polyethyleneimine-coated cover slip, with the outer surface of the stem side facing down, and both ends of the section taped with double-sided sticky tape. To obtain a single epidermal layer, the longitudinal section was treated with 0.5 % (w/v) Pectolyase Y-23 (MP Biomedicals, USA) in PME buffer with 0.4 M mannitol and 1 % (w/v) BSA for 10 to 20 min at room temperature. Overlying xylem and cortex cells were mechanically removed by gentle stroking with an eyelash attached at the end of a thin rod under the dissecting microscope. Debris from released cells was gently washed away with PBS. The epidermal layer attached to the cover slip was then immersed in pre-cooled 100 % methanol at -20°C for 10 min, and washed with PBS three times. Autofluorescence from free aldehydes derived from glutaraldehyde
fixation was reduced with 1 mg/ml NaBH$_4$ in PBS for 20 min. 50 mM glycine in PBS (IB: incubation buffer) was used for the following washing processes. To reduce nonspecific antibody binding, samples were incubated in 1 % BSA in IB for 30 min and incubated with mouse anti-α-tubulin (clone B512 diluted 1:1000; Sigma-Aldrich) in IB at 4°C overnight. After primary antibody incubation, samples were rinsed in IB three times for 10 min each on the shaker, and incubated with 5 % non-immune Goat serum for 30 min to reduce nonspecific antibody binding. For the secondary antibody, samples were incubated at 37°C for 3 h with Alexa Fluor 488 conjugated goat anti-mouse antibody (Molecular Probes) diluted 1:200 in 1 % non immune-goat serum and IB. After rinsing in PBS three times for 10 min, the double-sided sticky tape was removed from the sample and the epidermal strip was mounted in Citifluor antifade agent. The sample was observed by confocal laser scanning microscopy as described below.

3.2.3.2 Confocal laser scanning microscopy

The Alexa-488 fluorescent images of immunolabelled samples were collected with an upright AxioImager M1 microscope (Carl Zeiss, Germany) equipped with a Zeiss PASCAL Excite two channel scan head, using the 488 nm line from an argon laser, with 488 nm dichroic filter and a 505 nm emission filter, along with a 63 x NA 1.4 oil-immersion lens and Kalman averaging (N=2). Images were recorded with LSM software (Carl Zeiss, Germany) and processed with ImageJ software (http://rsb.info.nih.gov).

3.2.4 Measurement of cellulose microfibril angle

3.2.4.1 Cell wall preparation

The apical region of the inflorescence stems grown at 29°C for 1 day was used for the measurement of cellulose microfibril orientation in wild type and mort1-1. Cell wall preparation was performed as described in Chapter 2.
3.2.4.2 Field emission scanning electron microscopy (FESEM)

The FESEM analysis was performed as described in Chapter 2. Images of each stem sample and epidermal cell were taken at low magnification to record the apical and basal orientation of stem, and the cell long axis. High magnification images at 35 K and 70 K magnification were obtained mid-way along the cell. For some cells with pit fields, high magnification images were taken from several areas to avoid the area around pit fields.

3.2.4.3 Measurement of cellulose microfibril angle

A 5x7 array of reference points was set on each image taken at 35 K magnification, and the orientation of cellulose microfibrils at each reference point was measured relative to the cell long axis using ImageJ. A total of 420 cellulose microfibrils from twelve epidermal cells (15 to 35 µm in cell length) from four stems in wild type and mor1-1 were examined.

3.2.5 Measurement of cellulose crystallinity

3.2.5.1 Sample preparation for cellulose crystallinity

Arabidopsis plants were grown as described in the Chapter 2. The A and B regions of inflorescence stems grown at 21°C and 29°C for 1 day were excised with scissors, and pressed between sheets of paper by placing a weight on top to flatten and to dry them for approximately 1 month to avoid shrinkage of the tissue that occurs when drying is too rapid. Desiccation was important to exclude water from the sample prior to X-ray diffraction analysis. For the other mutant lines, plants grown at 21°C were marked at 1 cm increments from the top of the stems and the A and B regions collected after 1 day, flattened and dried as described above.
3.2.5.2 X-ray diffraction

X-ray diffraction patterns were recorded using a Bruker AXS Advance D8 X-ray diffractometer equipped with X-ray generator (Bruker, USA). The CuKα radiation was generated at an accelerating voltage of 40 kV with a current of 40 mA. A total of ten flattened dried stems was placed vertically in the sample holder. X-ray diffraction patterns were collected from the area between the A and B regions. Diffraction intensities were counted at 0.1° steps between 3 to 40° in the 2θ angle range. A complete scan took 200 sec. After data collection, the diffraction data from 3.9 to 36.9° in the 2θ angle range were integrated using GADDS software (Bruker, USA) and the output data were further analyzed.

3.2.5.3 Calculation of cellulose crystallinity

The collected data were normalized and resolved by employing a crystallinity calculation program based on the Vonk method (Vonk, 1973).

3.2.5.4 Calculation of width of cellulose crystallite

The width of the cellulose crystallite was calculated from the breadth of the X-ray diffraction peak using the Scherrer formula (Scherrer, 1918).

3.2.6 Molecular mass distribution of cellulose molecules

3.2.6.1 Preparation of α-cellulose

Arabidopsis plants were grown as described in the Chapter 2. The A and B regions of inflorescence stems grown at 21°C and 29°C for 1 day were excised with scissors, and were oven-dried at 90°C for 1 day. Dried stems were ground using a Wiley mill to pass through a 40-mesh (1 mm x 1 mm) screen, and were placed in an envelope made of filter paper. Ground stems in the Soxhlet were extracted with 150 ml of acetone (HPLC grade, Fisher) for 24 h. After evaporating the samples, sequential lignin and hemicellulose extraction were performed using a
microanalytical method modified from Yokoyama et al. (2002). Ground materials were suspended in 4 ml of distilled water overnight in a vial, and then warmed to 90°C with a heat block. The reaction was initiated by adding 1 ml of sodium chlorite solution (400 mg of 80 % sodium chlorite, 4 ml of distilled water, 0.4 ml of acetic acid). An additional 1 ml of sodium chlorite solution was added every half hour to a total of 3 ml, and the samples were placed in a cold-water bath. The resulting holocellulose was transferred to a 50 ml Falcon tube and thoroughly washed 5 times with 50 ml distilled water. After removal of distilled water, 10 ml of 17.5 % sodium hydroxide was added to the tube and the reaction left for 30 min, then 10 ml of distilled water was added. The sample was macerated for 1 min and left for another 29 min. After a total reaction time of 60 min, the sample was washed thoroughly with 50 ml distilled water 8 times. The neutralized α-cellulose was then oven-dried at 90°C and the yield determined. The α-cellulose fraction was ground with a mortar and pestle and then kept in a phosphorous pentoxide dessicator for 1 week prior to the derivatization reaction.

3.2.6.2 Cellulose derivatization reaction

The molecular weight distribution of cellulose in wild type and mor1-1 was obtained by gel permeation chromatography (GPC) analysis of tricarbanilate derivatives. Carbanilization of the cellulose was performed using a method modified from Schroeder and Haigh (1979) and Wood et al. (1986). About 3 mg of dried α-cellulose was weighed in the test tube, and 1 ml of anhydrous pyridine was added. After the mixture in the test tube was warmed to 80°C in a heat block, 250 µl of phenyl-isocyanate was added. The test tube was sealed with a Teflon cap and wrapped with parafilm. The derivatization reaction was carried out at 80°C for 48 h, resulting in a yellowish gel solution. The reaction mixture was cooled to room temperature and 1 ml of methanol (HPLC grade, Fisher) added to terminate the reaction. The reaction mixture was then transferred to a round-bottomed flask with 10 ml of iso-octane (HPLC grade, Fisher). The pyridine, methanol and iso-octane were removed by evaporation under vacuum at 40°C for 20 min, and at room
temperature for 40 min. The resulting viscous yellow liquid was dissolved in a total of 2 ml tetrahydrofuran (THF, HPLC grade, Fisher). Solutions of cellulose tricarbanilate in THF were analyzed as soon as possible after preparation.

3.2.6.3 Gel permeation chromatography (GPC)

Cellulose tricarbanilate in THF was filtered through a 0.45 µm filter and analyzed on an Agilent 1100 HPLC system (Agilent Technologies, USA) using Waters Styrage HR2 and HR5 columns with a weight range of 500 to 20,000, and 50,000 to 4,000,000 (Waters, USA). THF was used as the eluting solvent at a flow rate of 1 ml/min. The tricarbanilated cellulose in the eluate was detected using a UV spectrophotometer at a wavelength of 254 nm.

The calibration curve was generated from the elution profiles of six monodisperse polystyrene standards with molecular weights of 5x10^4, 1x10^5, 3x10^5, 6x10^5, 9x10^5, and 1.8x10^6. Polystyrene standards were dissolved in THF and filtered through a 0.45 µm filter. The polystyrene standards were run in parallel with the samples. The average molecular weight and polydispersity were calculated using ChemStation GPC Data Analysis Software Rev. B.01.01 (Agilent Technologies, USA).

3.2.7 Observation of cellulose microfibrils with transmission electron microscopy (TEM)

3.2.7.1 Isolation of cellulose microfibrils from cell wall extracts

Epidermal peels were obtained from the top 4 cm of primary stems and the top 2 cm of lateral stems of wild type. Cell wall materials were extracted as described in Carpita et al. (2001) and Peng et al. (2000) with slight modifications. Collected epidermal peels in 100% ethanol were heated to 65°C and incubated for 1 h. After rinsing in water, peels were homogenized in 1% SDS in 50 mM Tris [HCl] buffer (pH 7.0). Samples were centrifuged at 5000 g for 10 min at room temperature, and the supernatant removed. The pellets were washed in water,
centrifuged and kept in water at 4°C. This cell wall material was used for further extraction.

Two protocols for isolating cellulose were carried out. Protocol 1 for the extraction of pectins and xyloglucans was as described by Carpita et al. (2001) and Peng et al. (2000). Cell wall materials were incubated in 100 mM cyclohexane-trans-1,2-diamine-N,N,N',N"-tetraacetate (CDTA) in 20 mM potassium phosphate buffer (pH 7.0) at room temperature for 11 h to extract pectins. After centrifuging the mixture, the supernatant was discarded, the pellet was washed in water and centrifuged three times. Then the pellet was incubated in 0.34 M NaClO₂ with 65 mM acetic acid for 1 h at 65°C. The supernatant was decanted and the pellet was washed in water. The pellet was incubated with 3 mg/ml NaBH₄ in 0.1 M NaOH for 1 h, washed in water, and further incubated with 1 mg/ml NaBH₄ in 4 M KOH for 1 h to extract xyloglucans. After washing the pellet in water, the pellet was incubated in acetic acid-nitric acid-water (8:1:2) at 100°C for 1 h (Updegraff, 1969). The pellet was washed in water and kept at 4°C for negative staining.

Protocol 2 was as described by Lai-Kee-Him et al. (2002). The cell wall material was incubated in 5 % KOH at room temperature for 48 h. The pellet was obtained by centrifugation, and washed in water three times. The pellet was then suspended in 50 mM acetic acid with 1.7 % sodium chlorite (pH 5.0) and heated at 80°C for 16 h. Insoluble material was recovered after centrifugation as a pellet, and washed with water eight times. Xyloglucans were removed by extraction with 3 % NaOH for 18 h at 4°C, and insoluble cellulose was recovered by centrifugation, washed with water and kept at 4°C for negative staining. Some insoluble cellulose material was used for further incubation with 2.5 N HCl at room temperature for 30 min, plus boiling for either 5 min or 30 min, followed by centrifuging to remove the supernatant, washing the pellet in water, and negative staining. Insoluble cellulose material was also stained with 0.01 % calcofluor for 10 min and observed with a Zeiss Axiovert 200M inverted microscope equipped with an AxioCamHR camera (Carl Zeiss, Germany).
3.2.7.2 Negative staining of isolated cellulose

The droplet of cellulosic material in water was mounted on 0.25 % formvar-coated nickel 200-mesh grids. After the removal of excess water, the grids were stained with 2 % aqueous uranyl acetate for 5 min and observed by transmission electron microscopy. The samples on grids were viewed on a Hitachi H7600 PC-TEM (Hitachi, Japan) at an accelerating voltage at 80 kV. Photographs were taken using an ATM Advantage HR digital CCD camera (Advanced Microscopy Techniques, USA).
3.3 RESULTS

3.3.1 Growth of wild-type and *mor1-1* inflorescence stems

The Arabidopsis inflorescence stem undergoes extensive longitudinal growth and has a well-defined elongation zone that is large enough to obtain a sufficient amount of primary cell wall material for studying the properties of cellulose in rapidly expanding cells. To examine if the *mor1-1* inflorescence stems exhibit defects in growth anisotropy at restrictive temperature as described previously for *mor1-1* roots and other organs (Whittington et al., 2001; Sugimoto et al., 2003), I measured the growth of *mor1-1* inflorescence stems at this mutant’s restrictive temperature of 29°C. The stems grown at 21°C were marked at 1 cm intervals from the top, and then plants were left at 21°C or transferred to 29°C (Figure 3-1A). Analysis of elongation rates at 21°C and 29°C for 1 day demonstrated that high temperature caused an increase in elongation rate in both wild type and *mor1-1* (Figure 3-1B and C), but that the *mor1-1* stem elongation rate was significantly less than that of the wild type at 29°C for 1d (Figure 3-1C). The top part of the stem, in particular the A region, was in the rapidly elongating zone, and the B and C regions fell within the late elongation zone, and stopped elongation after 2 day at 29°C (Figure 3-1C and D). Compared to the diameter of wild type, *mor1-1* inflorescence stems were significantly wider in the A region and slightly wider in the B and C regions after 1 day at 29°C (Figure 3-2). Cross sections from the inflorescence stem in the B region demonstrated that there were no altered cell files or incomplete walls in the interior tissues (Figure 3-3A to D). Left-handed stem twisting was also observed in *mor1-1* after 1 day at 29°C (Data not shown).
**Figure 3-1** Elongation rate of wild-type and *mor1-1* inflorescence stems at 21°C and 29°C.

Values are means ± SD for 20 plants. Asterisks indicate significant differences (Student t-test: p < 0.0001).

(A) Schematic drawing of *Arabidopsis thaliana*. Stems grown at 21°C (5 – 8 cm stem height) were marked at 1 cm intervals from the top, and plants were moved to 29°C.

(B) Elongation rate of each region of the stem in wild type and *mor1-1* grown at 21°C for 1 day.

(C) Elongation rate in wild type and *mor1-1* grown at 29°C for 1 day.

(D) Elongation rate in wild type and *mor1-1* grown at 29°C between day 1 and day 2 after the temperature shift.
Figure 3-2  Diameter of wild-type and mor1-1 inflorescence stems.

Values are means ± SD for 10 plants. Asterisk indicates significant differences (Student t-test: p < 0.05).
Figure 3-3  Transverse sections of wild-type and *mor1-1* inflorescence stems grown at 21°C until 5 to 8 cm in height, and then at 29°C for 1 day.

Inflorescence stem from the lower part of region B grown at 29°C for 1 day were embedded in 1.5 % agarose, hand sectioned, and stained with 1 % toluidine blue. Images are not from the region where X-ray diffraction analysis was conducted, which was further up the stem in the region of rapid elongation.
3.3.2 Cell length and width of epidermal cells in wild-type and mor1-1 inflorescence stems

Inflorescence stem growth in the elongation zone is primarily dependent on the extent of longitudinal expansion of cells making up the stem. To examine if reduced stem elongation in mor1-1 was compensated by increased radial cell expansion, I measured and compared the length and width of epidermal peels of the inflorescence stem in wild type and mor1-1 grown at 21°C, 29°C for 1 day and 2 days. Clusters of guard cells were often observed in the A region of epidermal peels in mor1-1 inflorescence stems at 29°C, but no effect on guard cell morphology was observed (Figure 3-4).

At 21°C, the mor1-1 cells were slightly longer than wild-type cells, and the temperature shift to 29°C caused shorter cells in mor1-1 than in wild type (Figure 3-5A to C). As for cell width, mor1-1 cells were wider than wild-type cells at 21°C (Figure 3-6A), and continued to be wider than wild-type cells after the temperature shift to 29°C (Figure 3-6B and C). Reduced elongation and increased cell width in mor1-1 after 2 days at 29°C revealed that the temperature shift caused a defect in anisotropic cell expansion (Table 3-1).
**Figure 3-4**  Epidermal peels from region A in wild-type and *mor1-1* inflorescence stems after growth at 29°C for 1 day.

Epidermal peels were stained with 0.1% calcofluor and viewed with microscope. Arrows indicate clusters of guard cells.
Figure 3-5  Length of epidermal cell from wild-type and mor1-1 inflorescence stems grown at 21°C until stems reached 5 to 8 cm in height (A), and then at 29°C for 1 day (B) and 2 days (C).

Epidermal peels were made from each region of inflorescence stems and stained with 0.01 % calcofluor. Values are means ± SD for 285 cells in each region from 5 plants. Asterisks indicate significant difference (Student t-test: p < 0.05).
Figure 3-6  Epidermal cell width in wild-type and *mor1-1* inflorescence stems grown at 21°C until stems reached 5 to 8 cm in height (A), 29°C for 1 day (B) and 2 days (C).

Values are means ± SD for 285 cells in each region from 5 plants. Asterisks indicate significant differences (Student t-test: p < 0.05).
Table 3-1 Fold increases of cell length and width after the temperature shift to 29°C for 2 days relative to those at 21°C.

Values are mean ± SD for 285 cells in each region of 5 stems. Asterisks indicate significant difference (Student t-test: p < 0.05).

<table>
<thead>
<tr>
<th>Cell length</th>
<th>wild type</th>
<th>mor1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5.2 ± 1.1</td>
<td>4.4 ± 1.0 *</td>
</tr>
<tr>
<td>B</td>
<td>2.4 ± 0.3</td>
<td>1.9 ± 0.6 *</td>
</tr>
<tr>
<td>C</td>
<td>1.4 ± 0.4</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell width</th>
<th>wild type</th>
<th>mor1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.3 *</td>
</tr>
<tr>
<td>B</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>C</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>
3.3.3 Cortical microtubules in wild-type and \textit{mor1-1} inflorescence stems

Cortical microtubules in the \textit{mor1-1} root elongation zone lose their transverse orientation and become shorter at restrictive temperature (Sugimoto et al., 2003). To test if microtubule disruption occurs also in the \textit{mor1-1} inflorescence stem, I performed immunofluorescence labelling of microtubules in epidermal cells of both wild-type and \textit{mor1-1} inflorescence stems that were grown at 29°C for 1 day. In the cells from the early elongation zone, corresponding to the A region of wild-type stem, the cortical microtubules were locally parallel to each other but not uniformly transverse (Figure 3-7A), and microtubules in the cells from the late elongation zone, corresponding to the B region of the wild-type stem were well-aligned but in oblique orientations that varied in handedness from one cell to the next (Figure 3-7C). The \textit{mor1-1} cortical microtubules appeared to be shorter and more randomly oriented compared to microtubules in equivalent wild-type cells (Figure 3-7B), but those in the late elongation zone of the \textit{mor1-1} inflorescence stem appeared to be not as short as ones in the early elongation zone, and were generally oriented in parallel but oblique orientations (Figure 3-7D).
Figure 3-7  Immunofluorescence labelling of microtubules in epidermal cells of wild-type and mor1-1 inflorescence stems at 29°C for 1 day.

Microtubules in epidermal cells from region A of wild type (A) and mor1-1 (B), region B of wild type (C) and mor1-1 (D).

Scale bars = 20 μm.
3.3.4 Cellulose microfibril orientation in wild-type and mor1-1 inflorescence stems

I examined the orientation of cellulose microfibrils mostly from the inner periclinal walls of epidermal cells of inflorescence stems in wild type and mor1-1 grown at 29°C for 1 day using FESEM. The surface of epidermal cells at the top part of the inflorescence stem was cryo-planed longitudinally and the cytoplasmic contents extracted. The epidermal cells of the inflorescence stems in wild type and mor1-1 are shown in Figure 3-8A and B, and the most recently deposited layer of cellulose microfibrils of the corresponding epidermal cells are shown in the high-magnification FESEM images (Figure 3-8C and D). In both wild type and mor1-1, cellulose microfibrils were well aligned. Quantitative measurement of microfibril angles demonstrated that the majority of cellulose microfibrils in wild type and mor1-1 were aligned transverse to the major cell growth axis (Figure 3-9), and there was no significant difference in the mean values (p > 0.05). An F-test showed that the wild type had more variations in cellulose microfibril orientation than mor1-1 (F > F critical). Plotting the mean angle of cellulose microfibril orientation and standard deviations in each cell demonstrated that the wild type cellulose microfibril orientation was more variable than in mor1-1 (Figure 3-10).
Figure 3-8  Cellulose microfibrils in epidermal cells of wild-type and *mor1-1* inflorescence stems after growth at 29°C for 1 day.

Field emission scanning electron micrograph of an epidermal cell layer from upper part of the stem in wild type (A) and *mor1-1* (B). Higher magnification micrograph showing cellulose microfibrils at the cytoplasmic face of inner epidermal cell walls from wild type (C) and *mor1-1* (D).

Asterisks indicate the area from which the higher magnification images were taken, (C) from (A), (D) from (B).
A 5x7 array of reference points was set on each image, and the orientation of cellulose microfibrils situated on each reference point was measured relative to the cell long axis. A total of 420 cellulose microfibrils were measured for orientation from 12 epidermal cells (15 to 35 µm cell length) from 4 stems in wild type (90 ± 18 degree) and mor1-1 (87 ± 15 degree).

A Wilcoxon rank sum test showed that there is no significant difference between mor1-1 and wild type in terms of mean cellulose microfibril orientation (p = 0.09 > 0.05).

An F-test showed that cellulose microfibril orientation in wild type is more variable than that of mor1-1 (F= 1.49 > F critical).
Figure 3-10 Variations in cellulose microfibril orientation in 12 epidermal cells of wild-type and mor1-1 inflorescence stems after 1 day at 29°C.

Each plot represents means and standard deviation from 35 cellulose microfibril orientation measurements in one cell.
3.3.5 Degree of cellulose crystallinity in wild-type and *mor1-1* inflorescence stems

At restrictive temperature, *mor1-1* elongating cells in the stem appear to have defects in anisotropic cell expansion despite having well-aligned cellulose microfibrils. Given that the cellulose microfibrils act as reinforcing elements in the wall, mechanical properties of the cellulose microfibrils are predicted to be altered in *mor1-1*. Cellulose crystallization helps to hold adjacent cellulose chains together and contributes to the strength of the microfibrils. The top part of the stems, including A and B regions at either 21°C or 29°C for 1 day, were dried and ten flattened stems were stacked together and analyzed for crystallinity by X-ray diffraction. The diffraction pattern of dried stems generated a peak near 22 degree of two theta (Bragg angle) corresponding to the 002 reflection of cellulose I (Figure 3-11).

To examine if the crystallinity in *mor1-1* is altered, the method of Vonk (Vonk, 1973) was employed to calculate the degree of cellulose crystallinity. X-ray diffraction analysis demonstrated that although there was no significant difference in cellulose crystallinity between wild type and *mor1-1* at 21°C, crystallinity was significantly higher in *mor1-1* at 29°C (Figure 3-12). Interestingly, cellulose crystallinity in *mor1-1* was the same at 21°C and 29°C, while cellulose crystallinity in wild type decreased at 29°C. Similarly, using the bottom part of the stem, below the C region where stem growth is almost complete (Figure 3-1C and D), changes in cellulose crystallinity were evaluated in wild type and *mor1-1* at 29°C for 1 day. The degree of cellulose crystallinity in wild type was lower at 29°C, but there was no significant difference between wild type and *mor1-1* either at 21°C or 29°C (Figure 3-13).
Figure 3-11 X-ray diffraction pattern of cellulose in inflorescence stems.

The growing regions of 10 inflorescence stems were dried for X-ray diffraction analysis. The diffraction peak around 22 degrees represents the 002 peak of cellulose. The degree of cellulose crystallinity was calculated by subtracting background signals and drawing an amorphous curve according to the Vonk method (Vonk, 1973, see Appendix A).
Figure 3-12  Cellulose crystallinity in the growing regions of inflorescence stems in wild type and mor1-1 after growth at 21°C until stems reached 5 to 8 cm in height and then at 29°C for 1 day.

Values are means ± SD for 10 sets of 10 dried stems. Asterisks indicate significant differences (Student t-test: p < 0.05).
Figure 3-13  Cellulose crystallinity from the non-growing lower region of inflorescence stems in wild type and mor1-1 at 21°C until stems reached 5 to 8 cm in height and then at 29°C for 1 day.

The lower part of stems was below region C. Values are means ± SD for 8 sets of 10 dried stems.

There was no significant difference in crystallinity between wild type and mor1-1 at 21°C or after 1 day at 29°C.
3.3.6 Width of cellulose crystallite in wild-type and mor1-1 inflorescence stems

Crystalline cellulose has multiple crystal planes. The width of the cellulose crystallite represents the crystal dimension perpendicular to the respective crystal plane (the 002 plane in this study). The width of the cellulose crystallite was calculated from the breadth of the x-ray diffraction peak using the Scherrer formula (Scherrer, 1918). Relative comparison of the width of cellulose crystallite showed that there was no significant difference between wild type and mor1-1 at 21°C and at 29°C for 1 day (Figure 3-14).

3.3.7 Degree of cellulose crystallinity in other microtubule-related mutants

The high degree of cellulose crystallinity in mor1-1 at restrictive temperature suggests that microtubule dynamics and organization contributes to cellulose polymer crystallize, which adjusts in response to temperature. Other microtubule-related mutants and transgenic lines, including mor1-2, bot1, and RIC1-OX3 were also used to measure inflorescence stem growth rate and cellulose crystallinity. The growth of mor1-2 and bot1 inflorescence stems was significantly reduced (Figure 3-15A), and cellulose crystallinity of mor1-2 and bot1 were higher than that of wild type (Figure 3-15B). On the other hand, the RIC1-OX3 inflorescence stem had similar growth (Figure 3-15A), but lower cellulose crystallinity compared to wild type (Figure 3-15B).
Figure 3-14 Cellulose crystallite width in wild-type and mor1-1 inflorescence stems at 21°C or after 1 day at 29°C.

Values are means ± SD for 8 sets of 10 dried stems.
Figure 3-15  Inflorescence stem growth rates (A) and cellulose crystallinity (B), mutants and transgenic lines having altered microtubule organization.

Values are means ± SD for 10 sets of 20 dried stems for stem growth rate and 10 dried stems for crystallinity. Asterisks indicate significant differences compared to wild type (Student t-test: p < 0.05).

Inflorescence stems grown at 21°C (5 to 8 cm stem heights) were marked at 1 cm intervals from the top of the stem, and growth continued at 21°C, the mor1-2 plants were grown at 29°C.
3.3.8 Cellulose content in wild type and mor1-1

Previous studies have measured cellulose content in young seedlings by gas chromatography-mass spectrometry (GC-MS) analysis of trifluoroacetic acid (TFA) - insoluble cellulose fractions and showed that mor1-1 is similar to that of wild type (Sugimoto et al., 2003). Preliminary data of cellulose content analysis by GC-MS analysis on the TFA-insoluble cellulose fraction of growing regions of inflorescence stems demonstrated that mor1-1 inflorescence stems also have a similar amount of cellulose compared to wild type (Himmelspach, unpublished data). To test if cellulose content is not altered in mor1-1 inflorescence stems, the amount of α-cellulose from the growing region of inflorescence stems was quantified. Preparation of α-cellulose removes all glucose derived from non-cellulosic components, including sucrose and hemicellulose, with acid and strong base treatment, thus α-cellulose is considered to be the insoluble glucose derived from the cellulose polymer. The proportion of dried α-cellulose in dried inflorescence stem tissues was 5.9 ± 0.9 % in wild type and 6.5 ± 1.2 % in mor1-1 at 29°C. A Student t-test showed that there was no significant difference between wild type and mor1-1 cellulose content (Figure 3-16).
Figure 3-16  α-cellulose content measured from material obtained from the growing regions of wild-type and mor1-1 inflorescence stems.

Values are means ± SD for 3 different batches of collected samples. α-cellulose was isolated from the growing regions of inflorescence stems after the extraction of soluble carbohydrates. α-cellulose content (%) indicates proportion of dried α-cellulose weight per total weight of dried inflorescence stems. There was no significant difference in α-cellulose content between wild type and mor1-1 at 21°C and 29°C (Student t-test: p > 0.05).
3.3.9 Molecular size distribution of cellulose in wild type and mor1-1

Cellulose microfibrils are comprised of cellulose chains of different lengths. An analysis of the molecular size distribution of cellulose molecules provides the average molecular weight and distribution patterns of different sizes of cellulose molecules. The \( \alpha \)-cellulose derived from oven-dried inflorescence stems grown at 21°C and 29°C for 1 day was isolated. Following extraction of the lignin and hemicellulose, the \( \alpha \)-cellulose from the growing region formed hard pellets, while the \( \alpha \)-cellulose from the non-growing region was of fine fibrillar structure. Derivatization reactions with the hard pellets from the actively growing region, rich in primary wall material, did not undergo complete derivatization. The size distribution curve of cellulose molecules had 2 peaks, 1 peak in the low molecular weight range, and 1 peak at higher molecular weight (Figure 3-17A). Derivatization reactions after grinding the \( \alpha \)-cellulose resulted in an orange solvent colour and a distribution curve with 1 peak (Figure 3-17B). Despite the ability of this assay to measure the average molecular weight values of cellulose, no consistent trends were obtained through the tests, thus it was not possible to compare the values.
Figure 3-17  Molecular mass distribution of derivatized $\alpha$-cellulose from the growing regions of wild-type inflorescence stems.

(A) Molecular mass distribution of $\alpha$-cellulose. A hard pellet of $\alpha$-cellulose was used for the derivatization reaction.
(B) Molecular mass distribution of ground $\alpha$-cellulose.
3.3.10 Isolation of cellulose microfibrils from the cell walls of wild-type and \textit{mor1-1} inflorescence stems

Cellulose microfibril length cannot be measured using FESEM images. It is hard to distinguish where the termini of cellulose microfibrils are. To measure cellulose microfibril length, cell wall material was obtained from the growing region of inflorescence stems, and cellulose microfibrils were isolated using two methods. One method was modified from Carpita et al. (2001) and Peng et al. (2000) in which cellulose was extracted by the Updegraff method (Updegraff, 1969). No fibrillar structures, however, were observed. A second method, modified from Lai-Kee-Him et al. (2002), was employed. After removal of xyloglucans, negative staining showed tangled fibers (Figure 3-18). I attempted to disentangle fibers by longer acid treatments, but such treatments appear to break up the cross-linking of cellulose chains, resulting in loss of visible material.
Figure 3-18  Negative staining of extracted cellulose materials from epidermal peels of wild-type inflorescence stems.

Cellulose materials were obtained after subsequent extractions of pectin and xyloglucan, and incubation with 2.5 N HCl for 1 h.
3.4 DISCUSSION

3.4.1 Growth anisotropy and microtubule organization are defective, but cellulose microfibrils are well ordered in the *mor1-1* inflorescence stem grown at restrictive temperature

In this study, the epidermal cells in the growing region of *mor1-1* inflorescence stems were found at restrictive temperature to be significantly shorter and wider than those of wild type, accompanied by slower elongation rates and an increase in diameter of inflorescence stems, indicating that *mor1-1* mutants have partially impaired growth anisotropy. Immunofluorescence labelling of microtubules demonstrated that the microtubules in the epidermal cells of the *mor1-1* inflorescence stem were short and disorganized only in the early elongation zone, unlike in the roots where short and disorganized microtubules were previously observed throughout the elongation zone (Sugimoto et al., 2003). Quantitative measurement of microfibril angles relative to the growth axis demonstrated that both the wild type and *mor1-1* had well-aligned, transversely oriented cellulose microfibrils in the elongation zone, consistent with the results described in *mor1-1* roots (Sugimoto et al., 2003). Cellulose content was not altered in the *mor1-1* inflorescence stems, as previously shown in young seedlings (Sugimoto et al., 2003). The results I obtained from *mor1-1* inflorescence stems were similar to those of previous analysis of *mor1-1* roots, thus I hypothesized that the mechanical properties of cellulose microfibrils may be altered, rendering them unable to restrict the direction of cell growth, even though the cellulose microfibrils are still well ordered (Wasteneys 2004), and tested this hypothesis in *mor1-1* inflorescence stems.

3.4.2 Cellulose crystallinity measurement by X-ray diffraction analysis

Cellulose crystallinity is one of the physical properties of microfibrils that may affect mechanical properties of cellulose microfibrils and the cell wall. I measured the degree of cellulose crystallinity by calculating the ratio of the sum of the relative integrated intensities and the total intensity of X-ray diffraction signals. The growing
regions of inflorescence stems, specifically in the area between the A and B regions, were used for X-ray diffraction analysis to perform relative comparisons of the degree of cellulose crystallinity among mutants. The width of cellulose crystallite in dried samples was about 7 nm (Figure 3-14), which was a large enough crystallite to apply the Vonk method to calculate crystallinity (Krässig, 1993). Previous studies with solid-state NMR reported that the cross-sectional dimensions of cellulose crystallites isolated from the primary wall of Arabidopsis leaves were approximately 3 nm (Newman et al., 1996). The relatively large crystallite width measured by X-ray diffraction analysis may be a result of the sample preparation, in which no extraction method was employed, or dependent on which crystal plane of cellulose crystallite was used to determine the crystalline width. Due to the differences in the way of sample preparation, analytical instruments, and also the organ used for the analysis (leaves and stems), my results can not be directly compared to previous results.

Hemicellulose and lignin may contribute to the amorphous signal components that must be removed from the X-ray diffraction signal to determine cellulose crystallinity (Thygesen et al., 2005). There would be a correlation between cellulose content and cellulose crystallinity if the amorphous signal components are derived from hemicellulose and lignin. Wild-type stems showed slightly higher cellulose content and a lower degree of cellulose crystallinity at 29°C compared to that measured at 21°C (Figure 3-12 and 16). It is unlikely that the crystallinity values I observed here reflect differences in the amount of wall matrix or other wall components that may contribute the amorphous signal components.

By using growing region of inflorescence stems, X-ray diffraction analysis have demonstrated that cellulose crystallinity is reduced during rapid growth in primary wall, and that dynamic and well-organized microtubules are involved in the decline in cellulose crystallinity, as discussed in section 3.4.4. and 3.4.5.

3.4.3 Mechanical properties of mor1-1 cellulose microfibrils

Previous mor1-1 studies led to the hypothesis that organized microtubules might influence the length and strength of cellulose microfibrils (Wasteneys 2004).
attempted to isolate individual cellulose microfibrils and measure their length, but isolation of individual cellulose microfibrils was technically challenging, as the microfibrils tend to get entangled with each other (Figure 3-18). The Isogai group has reported that oxidation of hard wood-derived cellulosics by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO) enables loosening of adhesion between cellulose fibrils (Saito et al., 2006, 2007). TEMPO-mediated oxidation of cellulose followed by mechanical treatments to disintegrate oxidized cellulose fibers may be another approach to prepare samples for the measurement of cellulose microfibril length, and should be considered.

Cellulose crystallinity remained high in mor1-1 after shifting to the restrictive temperature. Crystalline cellulose is chemically stable and physically strong. Thus, the cellulose microfibrils with a high degree of cellulose crystallinity produced in mor1-1 at restrictive temperature when microtubules are disorganized may not be weak. The degree of cellulose polymerization (DP) still has to be considered before it is possible to draw any conclusion on the likely mechanical properties of cellulose microfibrils. DP would provide the information about the frequency of glucan chain termination within microfibrils that may affect the overall strength of a microfibril (Wasteneys and Fujita, 2006). I attempted to measure the DP on derivatized α-cellulose, but α-cellulose from primary walls formed hard pellets, which complicated the derivatization reactions, resulting in inconsistent results.

Although I was not able to obtain evidence that proves or disproves the hypothesis proposed by Wasteneys (2004), in the following sections I discuss the control of cellulose crystallinity as it relates to the mechanical properties of the wall and anisotropic growth, and the role microtubules play in controlling cellulose crystallinity.

3.4.4 Control of cellulose crystallinity during rapid anisotropic cell expansion

Results from X-ray diffraction analysis showed a decrease in cellulose crystallinity that accompanied rapid growth at higher temperature in wild type (Figure
3-1 and 3-12), suggesting that cellulose crystallinity is adjusted as the rate of cell expansion changes in order to define the mechanical properties of the cell wall.

The changes in the degree of cellulose crystallinity shown here need to be assessed in the context of two current structural models of cellulose microfibrils. In one model, the crystalline and amorphous domains are organized within the cellulose microfibrils. This current model for cellulose microfibrils is still not proven, but suggests that xyloglucans are entrapped within amorphous regions of cellulose microfibrils. This model has been supported experimentally by analyses of xyloglucan fractions after differential extractions of the cell wall (Pauly et al., 1999). Xyloglucans also bind non-covalently to the cellulose surface, resulting in cross-linking of adjacent microfibrils (Hayashi 1989; McCann et al., 1990). According to this model, the increase in amorphous cellulose synthesized during rapid cell expansion in wild type may provide more sites for non-cellulosic wall polysaccharides and proteins to modify structures including xyloglucan linkages to cellulose microfibrils.

Another model of cellulose crystallinity reflecting the cellulose microfibril structure is that the outer parts of the microfibrils are composed of amorphous cellulose, which is surrounded by hemicellulose, and the inner parts are composed of crystalline cellulose (O’Sullivan, 1997; Brett, 2000; Davies and Harris, 2003). Thicker microfibrils tend to have a higher percentage of crystalline cellulose than thinner ones (O’Sullivan, 1997). If a high degree of cellulose crystallinity is reflected by an increase in cellulose microfibril diameter, then microfibrils may be thick in wild type at 21°C and thinner at 29°C.

It has been suggested that each cellulose microfibril contains one cellulose crystallite in the cross-dimension (Davies and Harris, 2003). Cellulose crystallite width is not altered in wild type after the temperature-shift (Figure 3-14), suggesting that microfibril diameter may be not altered. Although diameter of cellulose microfibrils derived from Arabidopsis callus tissue was estimated to be 3 nm by atomic force microscopy (Davies and Harris, 2003), it is technically challenging to determine the diameter of cellulose microfibrils from extracted cellulose, as they would be influenced by the chemical processes during extraction. It is also
problematic to estimate the diameter of cellulose microfibrils from FESEM images. The cellulose microfibrils are coated with platinum, and the thickness of the platinum coating is variable depending on the surface structure, and therefore the apparent diameter of microfibrils could be misleading.

3.4.5 Organization of cortical microtubules and the control of cellulose crystallinity

Disorganized microtubules in mor1-1 at 29°C caused cellulose crystallinity to remain high even after the temperature-shift from 21°C, a treatment that leads to a decrease in cellulose crystallinity in wild type (Figure 3-12), suggesting that well-organized and dynamic microtubules are involved in the decline in cellulose crystallinity during rapid cell expansion. Microtubule dynamics is one of the parameters that may affect cellulose synthesis, through the close association of microtubules with the plasma membrane where cellulose synthesis occurs. Paredez et al. (2006a) reported a close correlation between microtubules and the trajectories of YFP-tagged CesA6, one of the subunits of cellulose-synthase-complexes. Recent live cell imaging of microtubule dynamics in wild-type and mor1-1 leaf epidermal cells demonstrated that microtubule growth and shrinkage rates increased as temperature increased in wild type, and that microtubule growth and shrinkage rates in mor1-1 were less than that of wild type both at 21°C and 31°C. Furthermore, the microtubules in mor1-1 tend to pause more frequently and for longer times at 31°C (Kawamura and Wasteneys, 2008). Despite a slight but significant reduction in microtubule dynamics in mor1-1 at 21°C, microtubule spatial organization appears to be normal. Apparently the slight reduction in microtubule dynamics in mor1-1 at 21°C is insufficient to generate any change in cellulose crystallinity. At restrictive temperature, the combination of drastically reduced microtubule growth and shrinkage, the increased frequency of pause and the relative time microtubules spend in the pause state, the reduced microtubule length, and the disruption of spatial organization may all contribute to the inability of cellulose crystallinity to decline in the mor1-1 mutant.
Other microtubule-related mutants, *mor1-2* (Whittington et al., 2001) and *bot1* (Bichet et al., 2001), which have previously been shown to have disorganized microtubules, also had a higher degree of cellulose crystallinity as well as reduced inflorescence stem growth relative to wild type (Figure 3-15). The *mor1-2* mutant is another conditional allele of *MOR1* (Whittington et al., 2001), which shows a reduced stem growth and a high crystallinity similar to that of *mor1-1*.

Although both *bot1* and *mor1* mutants have defects in microtubule organization and a high degree of cellulose crystallinity, it has been shown in the *lue1* mutant, an allele of *BOTERO* (p60 subunit of the katanin microtubule severing protein, AtKSS), that the loss of katanin microtubule severing activity increases the expression of gibberellin (GA) biosynthesis genes, and expression levels of AtKSS is regulated by the GA level (Bouquin et al., 2003), while *mor1-1* microarray data did not show altered expression level of GA biosynthesis genes (A. Walia, unpublished data). It has not been shown if GA levels are altered in the *lue1* mutant, but it is suggested that low levels of GA in the mutant may up-regulate GA biosynthesis genes (Wasteneys and Collings, 2006), according to the fact that GA levels negatively regulate the expression of GA biosynthesis genes (Meier et al., 2001; Raventos et al., 2000). GA has been suggested to promote wall loosening and GA’s effect on wall extensibility has been shown in aerial tissues such as cucumber hypocotyls (Katsumi and Kazama, 1978), pea stems (Cosgrove and Sovonick-Dunford, 1989), and Avena shoots (Montague, 1995). The increased crystallinity and reduced growth anisotropy in *bot1/lue1* may be influenced by aberrant microtubule organization and/or defects in wall loosening due to altered GA levels.

The arrangement of cellulose microfibrils in the *bot1* mutant, unlike those in *mor1-1* is disordered. The characterization of *fra2* mutant, another allele of *BOTERO*, demonstrated that the orientation of cellulose microfibrils was aberrant in elongating parenchyma cells (Burk and Ye, 2002), and reduced cell wall thickness in *fra2* parenchyma cells and fibers suggests that normal cell wall biosynthesis is impeded in the *fra2* mutant (Burk and Ye, 2002). In contrast, the *mor1-1* mutation does not affect cellulose microfibril orientation or cellulose content. These results suggest that cellulose crystallinity is controlled by some aspect of microtubule
Previous studies of plants overexpressing the ROP effector RIC1 \((RIC1-OX3)\) demonstrated that cortical microtubules were heavily bundled in \(RIC1-OX3\) pavement cells, and that transient RIC1 overexpression induced microtubule bundling and well-ordered microtubules (Fu et al., 2005). \(RIC1\) overexpression inhibited lobe formation in pavement cells, resulting in the formation of elongated narrow cells (Fu et al., 2005). Unlike other microtubule-related mutants, the \(RIC1-OX3\) inflorescence stems grew as wild type, and the degree of cellulose crystallinity was lower than that of wild type (Figure 3-15). Although the results from \(mor1\) and \(bot1\) mutants showed an inverse correlation between the degree of cellulose crystallinity and growth rate, the lower crystallinity but normal growth in \(RIC1-OX3\) compared to wild type suggests that cellulose crystallinity is not necessarily dependent on growth rate, and that microtubule dynamics and/or organization is involved in the control of cellulose crystallinity.

These results generated further questions. How are microtubules involved in controlling cellulose crystallinity? What is the mechanism by which cells produce a high degree of crystalline cellulose when microtubules are disorganized? The current model suggests that crystallization of cellulose chains occurs after extrusion of cellulose chains from the outer membrane (Saxena and Brown, 2005). Although no protein has been shown to be directly involved in the crystallization process in vascular plants, KORRIGAN, a membrane-localized \(\beta-1,4\)-glucanase, is suggested to play a role in removing non-crystalline glucan chains that fail to incorporate into microfibrils (Mølhøj et al., 2002). Considering topology, it is unlikely that microtubules can affect directly the crystallization process, but may be affecting it indirectly through their association with plasma membrane-localized cellulose-synthase-complexes, or through the deposition of non-cellulosic wall components that interact with cellulose.

Lai-Kee-Him et al. (2002) demonstrated that cellulose microfibrils synthesized in vitro have a higher degree of cellulose crystallinity than endogenous cellulose from suspension cultured cells and bacterial cellulose. They suggest that the
absence of other wall polysaccharides helps microfibrils to grow without restraint, and to contain fewer structural defects. Synthesis of Acetobacter cellulose in a hemicellulose-rich or pectin-rich medium resulted in a greater proportion of amorphous cellulose (Atalla et al., 1993; Whitney et al., 1995). Preliminary results of a microarray study on mor1-1 at its restrictive temperature showed no remarkable changes in expression pattern of wall-related biosynthesis enzymes, but some changes in expression of genes encoding wall-related proteins and wall modification enzymes, such as 3-fold reduction in proline-rich extensin-like family protein, a hydroxyproline-rich glycoprotein family protein, and a wall-associated kinase after 2 h at 31°C, a 3.6-fold increase in xyloglucan endotransglycosylase (XET) after 4 h at 31°C (A. Walia, unpublished data). These results suggest that the amount of wall polysaccharide may not be altered in mor1-1 but instead that the wall may be modified through XETs and a reduction in wall-related protein levels.

Altered microtubule organization affects deposition of wall components, such as the COBRA (COB) GPI-anchored protein (Roudier et al., 2005). COB is released from the plasma membrane and distributed in the wall, where it is predicted to bind to cellulose (Roudier et al., 2005). Disruption of microtubules causes the transverse band pattern of COB to dissipate (Roudier et al., 2005). In mor1-1 at 29°C, COB is diffusely distributed as microtubules are disorganized, as described in Chapter 6. A recent microarray analysis of the Arabidopsis inflorescence stem indicates that COB is highly expressed, especially in the epidermal peels (D. Bird and L. Samuels, personal communication; Suh et al., 2005). A higher degree of crystalline cellulose in mor1-1 at 29°C may be produced as a result of the absence of other wall polysaccharides or altered distribution of proteins such as COB.

An analysis of wall compositions and wall modification enzyme activity in the mor1-1 mutant should lead to a greater understanding of the role of organized microtubules in the cellulose crystallization process in relation to other wall polysaccharides.
CHAPTER 4

Microtubule organization, movement of cellulose-synthase-complexes and microfibril deposition in dark-grown hypocotyls
4.1 INTRODUCTION

Cellulose synthesis and microfibril formation are carried out by cellulose synthase complexes (CSCs), which when viewed by electron microscopy resemble rosettes located at the plasma membrane. Each rosette is believed to consist of six rosette subunits, each of which is itself composed of possibly 6 synthetic subunits, called cellulose synthases or CesAs (Doblin et al., 2002). CSCs situated at the plasma membrane are thought to be displaced laterally as the complex generates glucan chains through the continuous polymerization of UDP-glucose precursors. Thus, the movement of the complex in the membrane is likely to be driven by the force generated during crystallization of the polymerized product into rigid microfibrils (Heath, 1974; Herth, 1980). The movement of CSCs does not need a driving force generated by the membrane or the underlying cytoskeleton, but may be influenced by membrane fluidity (Emons, 1991).

In Arabidopsis, a family of 10 CesA proteins has been identified and CesA1, 2, 3, 5, and 6 are now known to be involved in primary wall synthesis (reviewed in Somerville, 2006). Recently, Paredez et al. (2006a) produced transgenic plants in which an N-terminal citrine YFP fusion to the CesA6 protein was expressed under the control of the native CesA6 promoter in the CesA6-null procuste1-1 (prc1-1) mutant background. The fusion protein was shown to be functional through its complementation of the prc1-1 mutant phenotype, which includes radial swelling and reduced elongation of dark-grown hypocotyls (Paredez et al., 2006a).

Visualization of YFP-CesA6 demonstrated that YFP-CesA6 particles move along linear trajectories that coincide closely with fluorescently labelled microtubules (Paredez et al., 2006a). Recent studies have provided supporting evidence that YFP-CesA6 particles are present at the plasma membrane. The herbicide isoxaben, whose direct targets are assumed to be CesA3 and CesA6 (Scheible et al., 2001; Desprez et al., 2002), causes rapid disappearance of YFP-CesA6 particles from the plasma membrane (Paredez et al., 2006a; DeBolt et al., 2007b). DeBolt et al. (2007b) also reported that the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) caused accumulation of YFP-CesA6 at discrete sites, in agreement with a previous study that used freeze-fracture electron microscopy to observe rosette
accumulation in the plasma membrane of DCB-treated wheat roots (Herth, 1987). Moreover, recent studies have demonstrated that reduced YFP-CesA6 velocity correlates with a reduction in cellulose content in cells treated with morlin, which causes microtubule disorganization (DeBolt et al., 2007a), and in cellulose-deficient korrigan (kor) mutants (Paredez et al., 2008).

Despite these results, fluorescent signals appear much larger than the source object and the resolution limit of confocal microscopy is greater than the thickness of the plasma membrane. It is therefore not possible to determine the exact location of the YFP-CesA6 particles (Wasteneys; http://www.f1000biology.com/article/id/1032153), or to conclude that they are actually moving in the plasma membrane and producing cellulose microfibrils. Thus, it remains possible that the fluorescent particles described in these recent publications are cytoplasmic vesicles carrying multiple YFP-CesA6 complexes and that single complexes with fewer fluorescent subunits at the plasma membrane may generate insufficient fluorescence for detection. Linear movement of YFP-CesA6 particles has been shown in the complete absence of microtubules in cells treated with oryzalin (Paredez et al., 2006a; Debolt et al., 2007a) but this does not eliminate the possibility that the fluorescent particles are vesicles, which might be moving along actin filaments. It has been shown that fine actin filaments lie parallel to the cortical microtubules in elongating root cells (Collings and Wasteneys, 2005; Voigt et al., 2005) and that they could be closely associated with cortical microtubules (Blancaflor, 2000; Collings et al., 2006).

Partial disruption of microtubule organization by oryzalin treatment results in aggregated and curved trajectories of YFP-CesA6 particles (Paredez et al., 2006a), suggesting that microtubules are involved in the distribution of CSCs into linear arrays. Complete microtubule removal by oryzalin, however, resulted in uniformly dispersed particles moving in linear trajectories at oblique angles to the cell long axis (Paredez et al., 2006a). Assuming that the YFP-CesA6 particles observed in these experiments are active CSCs producing cellulose microfibrils, their aberrant trajectories due to disorganized microtubule arrays or linear trajectories at oblique angles in the complete absence of microtubules contradict previous results of
Sugimoto et al. (2003) that demonstrated how microtubule disruption at \textit{mor1-1}'s restrictive temperature or complete microtubule removal by oryzalin treatment caused radial swelling of the root without altering transverse cellulose microfibril orientation. The field emission SEM images shown in the Sugimoto et al. (2003) publication were mainly from radial and inner periclinal walls of root epidermal cells, whereas Paradez et al. (2006a) tracked YFP-CesA6 particles only at the outer periclinal face of hypocotyl epidermal cells. Moreover, the Paradez et al. (2006a) study did not investigate the orientation patterns of cellulose microfibrils under these conditions.

Despite the fact that the equivalent cell area has to be observed to determine the relationship between microtubules and cellulose microfibril patterns, the continued linear movement of YFP-CesA6 particles described by Paradez et al. (2006a) in the absence of microtubules is consistent with previous studies. By documenting the recovery of cellulose microfibril patterns in the \textit{mor1-1} mutant at restrictive temperature after first treating plants with the cellulose synthesis inhibitor DCB, Himmelspach et al. (2003) demonstrated that cellulose microfibrils can re-establish transverse parallel order without organized microtubules or an organized template of cellulose microfibrils, suggesting that oriented cellulose deposition is self-organized.

Taking together these previously published results, it remains unclear (1) whether the YFP-CesA6 particles detected are actual CSCs at the plasma membrane and not vesicle-bound CesAs, and (2) what the relationship is between microtubules, YFP-CesA6 movement, and cellulose microfibril deposition patterns.

One of the objectives of this study was to resolve the apparent contradictions between these recent studies. Specifically, the compelling correlation of microtubule distribution and the movement of fluorescent CesA6 needs to be reconciled with the contrasting data showing that loss of microtubules has relatively little effect on the pattern of cellulose microfibril deposition. To address the question of whether the YFP-CesA6 particle movement previously described could be vesicular trafficking along actin filaments and not plasma membrane-bound synthase complexes moving as a result of cellulose polymerization, latrunculin B was used to disrupt actin
filaments before following the behaviour and velocities of YFP-CesA particles. To further investigate the relationship between microtubules and cellulose deposition, I measured the velocity, trajectory and the density of YFP-CesA6 particles in the mor1-1/prc1-1 double mutant background, as well as the effects of oryzalin on YFP-CesA6 particles while monitoring temperature. I also examined the effect of temperature on the velocity of YFP-CesA6 particles when microtubules remained unperturbed. Temperature is one of the factors that affect enzyme activities. Assuming that YFP-CesA6 particles are actual CSCs, temperature increases may cause an increase in YFP-CesA6 particle velocity, which is expected to correlate with the rate of cellulose biosynthesis. Finally, FESEM analyses were performed on hypocotyls to observe cellulose microfibril orientations in equivalent cells to those used for measuring YFP-CesA6 trajectories. I discuss the properties of the outer periclinal surface of epidermal cells where YFP-CesA6 and microtubules were observed, and the role of microtubules in relation to cellulose deposition.
4.2 MATERIALS AND METHODS

4.2.1 Plant materials and growth condition

A T1 generation of YFP-CesA6 transgenic lines carrying the same construct described by Paredez et al. (2006a) was obtained from the laboratory of Dr. C. Somerville. Transgenic plants were identified by rescue of short hypocotyls of the prc1-1 mutant phenotype in the dark-grown seedlings, and also by kanamycin resistance. A T3 homozygous line was used to cross with an 8 times backcrossed homozygous mor1-1 mutant (Whittington et al., 2001). For dark-grown seedlings, plants were grown on Hoagland’s medium in 1.2 % agar in vertically held Petri plates and covered with aluminum foil. Seeds were incubated at 4°C for 4 days, and then moved to a 21°C growth cabinet. For live-cell imaging, 3 day-old seedlings were used.

4.2.2 Crossing and screening of YFP-CesA6 in mor1-1/prc1-1

4.2.2.1 Screening YFP-CesA6 in the mor1-1 mutant

A putative homozygous YFP-CesA6 segregant in the prc1-1 mutant (i.e., CesA6 null) background was obtained in the T3 generation and crossed with a homozygous mor1-1 mutant. F2 seedlings were screened for YFP-CesA6 expression in mor1-1/prc1-1 double homozygotes. I determined that unless the construct is expressed in a prc1-1 null background, fluorescent particles are not detected at the plasma membrane, suggesting that the endogenous CesA6 out-competes the YFP-tagged transgenic version. Therefore, to identify prc1-1 homozygotes expressing YFP-CesA6, 3 day-old dark-grown seedlings were observed under a spinning disc confocal microscope to screen for lines expressing YFP particles at the cell periphery. The selected seedlings were then placed onto Hoagland’s media without sucrose and grown for 2 days in the light at 21°C, then shifted to 29°C for 1 day to identify mor1-1 homozygotes by their temperature-dependent left-hand twisting root phenotype. The prc1-1 lines were also segregated in the F2 generation to be used as control lines. To genotype these putative YFP-
CesA6-expressing mor1-1/prc1-1 double homozygotes and prc1-1 segregant control lines, seedlings were grown further at 21°C until genomic DNA could be obtained from leaves for genotype confirmation.

4.2.2.2 Genomic DNA extraction and genotyping of the prc1-1 mutant background

To verify that selected lines carried the prc1-1 mutation, genomic DNA was extracted from leaf tissue using the quick DNA preparation method (Weigel and Grazebrook, 2002). To amplify the region of DNA containing the prc1-1 mutation, a 3.5 kb fragment was amplified with primers, prcF (5’-AGTGGCTGCGGATAAGAA-3’) and prcGR (5’- CCTTCACAGAAGCACC CGAA-3’) using Phusion DNA polymerase (Finnzymes, Finland). PCR products were cleaned using a QIAquick PCR purification kit (QIAGEN) and sequenced.

4.2.3 Generation of mRFP-TUB6 in YFP-CESA6, in prc1-1 and in mor1-1/prc1-1 transgenic lines

The binary vector pCambia1300 containing a CaMV35S promoter-driven mRFP fused with TUB6 was obtained from Dr. Richard Cyr (Pennsylvania State University), and transformed into Agrobacterium tumefaciens (strain GV3101) by electroporation. The transformed Agrobacteria were grown on LB media (1 % Bacto tryptone, 0.5 % Bacto Yeast Extract, 1 % NaCl, 1.2 % Bacto agar) at 28°C with 25 µg/L gentamicin and 50 µg/L kanamycin. The screened colonies were inoculated in 5 ml of LB media with antibiotics and grown overnight at 28°C on a rotary shaker at 220 rpm. This overnight culture was suspended in 200 ml fresh LB media containing both gentamicin and kanamycin and grown overnight at 28°C, while shaking at 220 rpm. The overnight culture was centrifuged at room temperature for 10 min at 3000 g and the supernatant discarded. The pellet was re-suspended twice in 5 % sucrose and centrifuged for 10 min at 3000 g. The infiltration media (5 % sucrose and 0.025 % of Silwet-77) was poured into the container and mixed with the Agrobacteria.
All siliques from 5 week-old seedlings of either the YFP-CesA6 *proc1-1* or the YFP-CesA6 *mor1-1/proc1-1* line were removed, and flower buds were dipped into the infiltration medium. Floral-dipped plants were covered with plastic wrap, kept in the dark for 1 day, and then grown in a 21°C growth chamber. T1 seeds were harvested and planted on Hoagland’s medium with 50 µg/L kanamycin. T2 seeds were grown in the dark for 3 days and screened for fluorescence in the hypocotyl. For experiments, T2 and T3 plants were used.

4.2.4 Live-cell image acquisition of YFP-CesA6

4.2.4.1 Sample preparation

Dark-grown seedlings were mounted between 22 x 22 mm cover slips and glass slides with 70 µl water and sealed with silicon vacuum grease. All tools such as glass slides, cover slips, mounting media and forceps were either pre-cooled or pre-warmed to the desired temperature of 21°C or 29°C.

4.2.4.2 Spinning disc confocal microscopy

Imaging was performed on the Quorum Wave FX system employing a modified Yokogawa CSU-10 spinning disk scan head (Yokogawa Electric Corporation; Tokyo, Japan) and a Leica DMI6000 inverted microscope equipped with a 63 x NA 1.3 glycerol lens. Citrine-YFP for YFP-CesA6 was excited with a 491 nm laser and mRFP for mRFP-TUB6 was excited with a 561 nm laser. Images were acquired using a Hamamatsu 9100-13 EM CCD camera (Hamamatsu, Japan), controlled with Volocity software (Improvision, England) through emission band filters, 528/38 for YFP, 593/40 for mRFP (Chroma Technology, Rockingham VT).
4.2.4.3 Temperature-controlled stage and objective lens heater

To keep the temperature stable around the mounted samples, a temperature-controlled stage, Bionomic controller BC-110 equipped with Heat exchanger HEC-400 (20/20 Technology Inc.), was set up to accommodate either 21°C or 29°C. An objective lens heater (Bioptechs) was used for the 29°C setting (Figure 4-1). The temperature of the sample was monitored immediately after imaging by measuring the temperature of glycerol on the cover slip.

4.2.4.4 Acquiring images

Images were taken with 600 ms exposure times for both YFP-CesA6 and mRFP-TUB6. Two-frames were taken at each time point and averaged to reduce background fluorescence from rapidly moving Golgi bodies. Live-cell imaging was carried out with a setting of 10 sec intervals over a period of 5 min.

4.2.5 Image processing

To adjust tissue translocation during imaging, the StackReg (Thévenaz et al., 1998) or Align Slice plugins for ImageJ were used to align two-frame averaged images. The images were smoothened by averaging three continuous frames using the Walking Average plugin for ImageJ, and kymograph analysis was performed using Multiple kymograph plugins from ImageJ.

4.2.6 Statistics

A set of more than 400 individual measurements of YFP-CesA6 velocity under each condition was first tested by a Shapiro-Wilk normality test to determine if the data were normally distributed. The YFP-CesA6 velocity data showed abnormal distributions under each condition. I therefore used a Wilcoxon-rank sum test to determine if the means of YFP-CesA6 velocity in different conditions were significantly different.
4.2.7 Inhibitor treatments

Latrunculin B (Calbiochem) was dissolved in DMSO to make a 20 mM stock solution. Oryzalin (Supelco) was dissolved in DMSO to make a 2 mM or 20 mM stock solution. Stock solutions were diluted in water to the indicated concentrations and DMSO adjusted to 0.1 % DMSO prior to each experiment and, in the case of temperature shift experiments, warmed to 29°C. For latrunculin B (LatB) treatments, the YFP-CesA6 line described in Paredez et al. (2006a) was grown in the dark for 3 days (2 days at 21°C and 1 day at 29°C). Seedlings were mounted in 70 µl of 20 µM LatB or 0.1 % DMSO for control treatments. Imaging was performed 10 min after mounting seedlings. For oryzalin treatments, the YFP-CesA6 prc1-1 segregant from the mor1-1 cross was used. Dark-grown 3 day-old seedlings (2 days at 21°C and 1 day at 29°C), were incubated in either oryzalin or DMSO control solution at 29°C for 3 h, and then imaging was performed.

4.2.8 Cell wall preparations on dark-grown seedlings for FESEM

Hypocotyls from dark-grown 3 day-old YFP-CesA6 prc1-1 and YFP-CesA6 mor1-1/prc1-1 seedlings that were either (1) grown for 3 days at 21°C or (2) grown for 2 days at 21°C and for 1 day at 29°C, were fixed with 4 % formaldehyde and 0.5 % glutaraldehyde and their cell walls were prepared for FESEM as described in Chapter 2.
Figure 4-1  Spinning disk confocal microscopy with a temperature-controlled stage, and an objective lens heater.

The temperature-controlled stage is set on the microscope (A) and a metal cover on top of the glass slide (B). The objective lens is warmed up with an objective lens heater (not shown in the pictures).
4.3 RESULTS

4.3.1 Effects of latrunculin B on YFP-CesA6 velocity

Due to the resolution limit of fluorescence microscopy, it is difficult to accurately determine the location of a fluorescent particle. Therefore it is not possible to distinguish between YFP-CesA6 particles being in cytoplasmic vesicles or at plasma membrane by fluorescence alone. To obtain evidence supporting the assumption that YFP-CesA6 particles are in the plasma membrane, I tested whether the YFP-CesA6 particle velocities or trajectories are independent from actin filaments, which are often reported to be in close association with microtubules in the plant cell cortex (Blancaflor, 2000; Collings et al., 2006). Seedlings were treated with latrunculin B (LatB), a drug that binds monomeric actin and leads to rapid disassembly of actin filaments. Incubation of 3 day-old dark-grown seedlings for 10 min with 20 µM LatB arrested cytoplasmic streaming (Figure 4-2A). Recovery of cytoplasmic streaming was observed after LatB was washed away (Figure 4-2B), indicating that 10 min incubation in LatB was fully reversible and did not affect cell viability. YFP-CesA6 particles continued to move along linear trajectories while cytoplasmic streaming was completely arrested. The velocity and trajectories of YFP-CesA6 were not affected by the disruption of actin filaments. In the 0.1 % DMSO control, the mean velocity of YFP-CesA6 particles was $299 \pm 85$ nm/min while during LatB treatments, the mean velocity was $295 \pm 76$ nm/min (Figure 4-3A to C).
Figure 4-2 Arrest of cytoplasmic streaming by 20 µM latrunculin B (LatB) and recovery of cytoplasmic streaming after LatB was washed away.

3 day-old dark-grown seedlings were incubated in 20 µM LatB for 10 min, and images were taken every 10 sec for 5 min. After 10 min of LatB incubation, LatB was washed away and images were taken after 1 h. Time-lapse images were taken every 10 sec for 5 min. Arrows indicate vesicles.

(A) Arrest of cytoplasmic streaming by 20 µM LatB.
(B) Recovery of cytoplasmic streaming after LatB was washed away.

Scale bar = 5 µm.
Figure 4-3  The effect of 20 µM latrunculin B on YFP-CesA6 particle velocity and trajectories.

Three day-old seedlings grown for 2 days at 21°C and for 1 day at 29°C were incubated with 20 µM LatB in 0.1 % DMSO for 10 min, and used for imaging every 10 sec over a 5 min period. Velocity of a total of 400 YFP-CesA6 particles in 18 cells from 11 seedlings was measured by kymograph analysis.

(A) YFP-CesA6 particle velocity of the seedlings incubated with 0.1 % DMSO or 20 µM LatB. Average velocity: 299 ± 85 nm/min in 0.1 % DMSO control, 295 ± 76 nm/min in 20 µM LatB). Wilcoxon rank sum test: p = 0.49 > 0.05.
(B) YFP-CesA6 trajectories in 0.1 %DMSO over 5 min.
(C) YFP-CesA6 trajectories in 20 µM LatB over 5 min.

Scale bar = 5 µm for (B) and (C).
4.3.2 Effects of temperature on YFP-CesA6 velocity

The CSC is an enzyme complex whose polymer-forming activity is likely to be affected by temperature. To test temperature effects on YFP-CesA6 particle velocity, I used a temperature-controlled stage as well as an objective lens heater set at either 21°C or 29°C. The mean velocity of YFP-CesA6 particles at 21°C was 84 ± 47 nm/min, and at 29°C was 354 ± 73 nm/min. Higher temperature was correlated with increased velocity of YFP-CesA6 particles, consistent with particle movement being driven by enzyme-mediated polymerization of glucan chains.

4.3.3 Screening of prc1-1 and mor1-1/prc1-1 expressing YFP-CesA6

To examine if microtubule organization affects the velocity and trajectories of CSCs, I crossed a YFP-CesA6-expressing prc1-1 mutant with a homozygous mor1-1 mutant, and in the F2 generation identified mor1-1/prc1-1 double homozygotes that expressed YFP-CesA6. Control lines carrying YFP-CesA6 in the prc1-1 background were also obtained from the same F2 population so that the genetic background was similar to the mor1-1/prc1-1 double mutant segregant.

Hypocotyls of 3 day-old dark-grown mor1-1/prc1-1 seedlings at 21°C look similar to rescued prc1-1, but hypocotyls of 3 day-old dark-grown mor1-1/prc1-1 seedlings grown at 21°C for 2 days and at 29°C for 1 day showed swollen epidermal cells and had a slightly open-hook (Figure 4-4).
Figure 4-4 Dark-grown seedlings of rescued *prc1-1* and *mor1-1/prc1-1* expressing YFP-CesA6 at 21°C and 29°C after 1 day.

(A) YFP-CesA6 seedlings grown at 21°C for 3 days in the dark.
(B) YFP-CesA6 seedlings grown at 21°C for 2 days and 29°C for 1 day in the dark.

Scale bar = 1mm.
4.3.4 YFP-CesA6 particle velocity in prc1-1 and mor1-1/prc1-1

According to the kymograph analyses, YFP-CesA6 particle movement was bi-directional, in agreement with a previous study (Paredez et al., 2006a). This bi-directional movement of particles was not disturbed in the mor1-1/prc1-1 background at 29°C (Figure 4-5). At 21°C, there was no difference in particle velocity between prc1-1 (mean velocity: 84 ± 47 nm/min) and mor1-1/prc1-1 (mean velocity: 71 ± 42 nm/min) (Figure 4-6A and B). Figure 4-6C and D illustrate how after 3 h at 29°C, the particle velocities increased both in prc1-1 (mean velocity: 311 ± 89 nm/min) and mor1-1/prc1-1 (mean velocity: 339 ± 84 nm/min). These differences indicate that particle velocities are significantly faster in mor1-1/prc1-1 than in the rescued prc1-1 single mutant (Wilcoxon-rank sum test: p < 0.0001). As shown in Figure 4-6E and F, a 1 day-long temperature shift to 29°C also caused the YFP-CesA6 particles to move significantly faster in mor1-1/prc1-1 (mean velocity: 410 ± 79 nm/min) than in the rescued prc1-1 line (354 ± 73 nm/min) according to a Wilcoxon-rank sum test (p < 0.0001). At 29°C, velocity was 4.2 times greater than that measured at 21°C in the rescued prc1-1 line and 5.8 times greater than at 21°C in the mor1-1/prc1-1 double mutant.

4.3.5 YFP-CesA6 particle trajectories in prc1-1 and mor1-1/prc1-1

YFP-CesA6 particle trajectories in the rescued prc1-1 line were oriented mostly transversely or obliquely relative to the cell’s predominant growth axis. Locally parallel but variable YFP-CesA6 particle trajectories within the same cell were often observed, even on hypocotyls that were recently placed in the observation chambers (Figure 4-7A). Despite the variable orientation of YFP-CesA6 trajectories, there was no correlation between YFP-CesA6 velocity and trajectory orientation (Table 4-1). In mor1-1/prc1-1, YFP-CesA6 trajectories were variable among cells but more obliquely and longitudinally curved trajectories were observed compared to that of the rescued prc1-1 line (Figure 4-7B and C).
Figure 4-5 Kymographs of YFP-CesA6 particles in rescued *prc1-1* and *mor1-1/prc1-1* at 21°C and 29°C after 1 day.

Projection of 5 min time-lapse images of YFP-CesA6 in *prc1-1* at 21°C (A) and in *mor1-1/prc1-1* at 21°C (B), in *prc1-1* at 29°C (D), and in *mor1-1/prc1-1* at 29°C (E). Kymographs of YFP-CESA6 in *prc1-1* and *mor1-1/prc1-1* at 21°C (C) and at 29°C (F).

Scale bar = 5µm
Velocity of a total of 450 particles in 14 to 20 cells from 5 to 13 seedlings was measured by kymograph analysis.

YFP-CesA6 particle velocity at 21°C for 3 days in prc1-1 (A) and mor1-1/prc1-1 (B), at 21°C for 3 days then 29°C for 3 h in prc1-1 (C) and mor1-1/prc1-1 (D), at 21°C for 2 days then 29°C for 1 day in prc1-1 (E) and mor1-1/prc1-1 (F). There are significant differences between (C) and (D), (E) and (F). Wilcoxon rank sum test: p<0.0001.

**Figure 4-6** Velocity of YFP-CesA6 particles in rescued prc1-1 and mor1-1/prc1-1 at 21°C, 29°C after 3 h, and 29°C after 1 day.
Figure 4-7 Variable YFP-CesA6 trajectories in rescued *prc1-1* and *mor1-1/prc1-1*.

Projections of 5 min time-lapse images.

(A) YFP-CesA6 trajectories in rescued *prc1-1* at 29°C. Trajectories were locally parallel but variable, from transverse to oblique orientations.

(B) Oblique YFP-CesA6 trajectories in *mor1-1/prc1-1* at 29°C.

(C) Oblique to longitudinal YFP-CesA6 trajectories in *mor1-1/prc1-1* at 29°C.

Scale bar = 5 µm.
Table 4-1 YFP-CesA6 particle velocity and trajectory orientation in prc1-1 and in mor1-1/prc1-1.

Values are means ± SD for the number of particles indicated.

<table>
<thead>
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<th>Seedling</th>
<th>Velocity (nm/min)</th>
<th>Orientation of trajectories</th>
</tr>
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<tr>
<td><strong>YFP-CesA6 in prc1-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>323 ± 50 (n=48)</td>
<td>transverse</td>
</tr>
<tr>
<td></td>
<td>377 ± 68 (n=18)</td>
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</tr>
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<td></td>
<td>353 ± 69 (n=30)</td>
<td>oblique</td>
</tr>
<tr>
<td></td>
<td>392 ± 73 (n=30)</td>
<td>oblique</td>
</tr>
<tr>
<td><strong>YFP-CesA6 in mor1-1/prc1-1</strong></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>411 ± 80 (n=22)</td>
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<td>429 ± 72 (n=20)</td>
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</tr>
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<td></td>
<td>392 ± 73 (n=32)</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
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<td>430 ± 69 (n=36)</td>
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</tr>
</tbody>
</table>
4.3.6 YFP-CesA6 particle trajectories and microtubule orientation in 
prc1-1 and mor1-1/prc1-1

To investigate the effect of microtubule organization on YFP-CesA6 trajectories, microtubules were visualized with mRFP-TUB6, while YFP-CesA6 particles were tracked in the prc1-1 mutant, and in the mor1-1/prc1-1 double mutant. Microtubule orientation in the rescued prc1-1 line was mostly transverse to oblique to the cell growth axis at 21°C and 29°C, and YFP-CesA6 trajectories followed a similar pattern to microtubules (Figure 4-8A to F). As I observed hypocotyls for longer periods of time under the microscope, microtubule orientation became more variable within and among cells. It has been described in Paredez et al. (2006a) that the blue light used for YFP excitation stimulates microtubule reorientation.

Most of the microtubules in mor1-1/prc1-1 at 21°C were arranged in parallel arrays, and their orientation (Figure 4-8G) and that of YFP-CesA6 trajectories were roughly transverse to oblique to the growth axis (Figure 4-8H). After either 3 h or 1 day at 29°C microtubules in mor1-1/prc1-1 were disorganized (Figure 4-8I and K), as were YFP-CesA6 trajectories (Figure 4-8J to L). At 21°C, YFP-CesA6 trajectories and mRFP-TUB6-labelled microtubules often overlapped in prc1-1 (Figure 4-9A to C) and in mor1-1/prc1-1 (Figure 4-9D). The degree of overlap between YFP-CesA6 trajectories and mRFP-TUB6-labelled microtubules was reduced at 29°C compared to 21°C in both the prc1-1 single (Figure 4-10A to C and 4-11) and the mor1-1/prc1-1 double mutant (Figure 4-10D to F and 4-12).
Figure 4-8 mRFP-TUB6 and YFP-CesA6 distribution in prc1-1 and mor1-1/prc1-1 at 21°C, 29°C after 3 h, and 29°C after 1 day.

Images are projections of 5 min time-lapse images recorded every 10 sec of mRFP-TUB6 (red) and YFP-CesA6 (green).

mRFP-TUB6 and YFP-CesA6 in prc1-1 at 21°C (A) and (B), after 3 h at 29°C. (C) and (D), after 1 day at 29°C (E) and (F).

mRFP-TUB6 and YFP-CesA6 in mor1-1/prc1-1 at 21°C (G) and (H), after 3 h at 29°C (I) and (J), after 1 day at 29°C (K) and (L).

Scale bar = 5µm.
Figure 4-9 Relative intensity plots of mRFP-TUB6 labelling and YFP-CesA6 trajectories in prc1-1 and mor1-1/prc1-1 at 21°C.

Plots (C and F) show relative fluorescent intensities along the line scan shown on images A, B, D and E. Images are projections of 5 min time-lapse images recorded every 10 sec of mRFP-TUB6 (red) and YFP-CesA6 (green).

(A) and (B) mRFP-TUB6 and YFP-CESA6 in prc1-1 at 21°C.
(C) Plot of a line scan of mRFP-TUB6 and YFP-CESA6 in prc1-1.
(D) and (E) mRFP-TUB6 and YFP-CESA6 in mor1-1/prc1-1 at 21°C.
(F) Plot of a line scan of mRFP-TUB6 and YFP-CESA6 in mor1-1/ prc1-1.

Scale bar = 5 µm.
Figure 4-10 Relative intensity plots of mRFP-TUB6 labelling and YFP-CesA6 trajectories in *proc1-1* and *mor1-1/prc1-1* after 1 day at 29°C.

Plots (C and F) show relative fluorescent intensities along the line scan shown on images A, B, D and E. Images are projections of 5 min time-lapse images recorded every 10 sec of mRFP-TUB6 (red) and YFP-CesA6 (green).

(A) and (B) mRFP-TUB6 and YFP-CesA6 in *proc1-1* after 1 day at 29°C.
(C) Plot of a line scan of mRFP-TUB6 and YFP-CesA6 in *proc1-1*.
(D) and (E) mRFP-TUB6 and YFP-CesA6 in *mor1-1/prc1-1* after 1 day at 29°C.
(F) Plot of a line scan of mRFP-TUB6 and YFP-CesA6 in *mor1-1/prc1-1*.

Scale bar = 5 µm.
Figure 4-11 Trajectories of YFP-CesA6 particles and mRFP-TUB6 in the prc1-1 background at 29°C over time.

Projections of 5 consecutive frames of time-lapse images, mRFP-TUB6 (red), YFP-CesA6 (green), and merged images. Triangles show areas of mRFP-TUB6 labelling over time that do not overlap with YFP-CesA6. Circles show mRFP-TUB6 labelling over time that coincides with YFP-CesA6. Arrowheads show areas of YFP-CesA6 trajectories that do not overlap with mRFP-TUB6 labelling.

Scale bar = 5 µm.
Figure 4-12 Trajectories of YFP-CesA6 particles and mRFP-TUB6 in *mor1-1/prc1-1* at 29°C over time.

Projections of 5 consecutive frames of time-lapse images, mRFP-TUB6 (red), YFP-CesA6 (green), and merged images. Triangles show areas of mRFP-TUB6 labelling over time that do not overlap with YFP-CesA6. Circles show mRFP-TUB6 labelling over time that coincides with YFP-CesA6. Arrowheads show areas of YFP-CesA6 trajectories that do not overlap with mRFP-TUB6 labelling.

Scale bar = 5 µm.
4.3.7 Density of YFP-CesA6 particles in *prc1-1* and *mor1-1/prc1-1*

To test if microtubule organization affects the density (in terms of number of fluorescent particles per area) of CSCs, YFP-CesA6 particles in a given area were manually counted in the *prc1-1* and *mor1-1/prc1-1* genotypes after a temperature shift to 29°C for either 3 h or 1 day. It was not possible to distinguish individual YFP-CesA6 particles at 21°C because of their relatively slow movement, so density data were not obtained under this condition. Particle density after 3 h at 29°C was 14.5 ± 3.2 particles/15 µm² in *prc1-1* and 12.2 ± 1.2 particles/15 µm² in *mor1-1/prc1-1* (Figure 4-13). After 1 day at 29°C, YFP-CesA6 particle density increased in both *prc1-1* (23.2 ± 3.6 particles/15 µm²) and in *mor1-1/prc1-1* (16.6 ± 3.8 particles/15 µm²). Particle density in *mor1-1/prc1-1* was significantly less than that in *prc1-1* both after 3 h and after 1 day at 29°C (Student t-test: p < 0.05).
Figure 4-13 Density of YFP-CesA6 particles in prc1-1 and mor1-1/prc1-1 at 29°C.

The number of YFP-CesA6 particles was counted in a 15 µm² area. Values are means ± SD for 10 cells. Particle density in mor1-1/prc1-1 at 29°C was significantly less than that of the rescued prc1-1 (Student t-test: p < 0.05).
4.3.8 Effects of oryzalin on YFP-CesA6 velocity and trajectories

DeBolt et al. (2007a) reported that loss of microtubules by treatments with the microtubule-depolymerization drug oryzalin did not affect particle velocity. Their study, however, did not monitor temperature during imaging. My results on the effect of temperature on YFP-CesA6 indicated that temperature monitoring is crucial when analyzing particle velocity. To test whether YFP-CesA6 velocity remains the same in the absence of microtubules, YFP-CesA6 velocity was measured on oryzalin-treated rescued prc1-1 seedlings at 29°C. The control incubation with 0.1% DMSO for 3 h showed mRFP-TUB6 in transverse to oblique orientations relative to the cell growth axis (Figure 4-14A) and YFP-CesA6 trajectories followed similar directions (Figure 4-14B). A 3 h treatment with 20 µM oryzalin caused complete loss of microtubules (Figure 4-14C) but YFP-CesA6 particles continued to move along linear trajectories (Figure 4-14D), which is consistent with the previous studies (Paredez et al., 2006a; DeBolt et al., 2007a). The mean values of particle velocity showed that YFP-CesA6 particles moved more slowly in 20 µM oryzalin-treated cells (mean velocity: 347 ± 75 nm/min) than in the DMSO control (mean velocity: 369 ± 82 nm/min), and a Wilcoxon-rank sum test revealed that this difference was significant (p < 0.0001) (Figure 4-15A and B). Low concentrations of oryzalin for short periods of time can cause cortical microtubules to lose parallel orientation so that their spatial organization resembles that observed in mor1-1 cells at 29°C. To investigate if drug-induced microtubule disorganization also leads to an increase in the YFP-CesA6 velocity, as occurs in mor1-1, oryzalin was used at 2 µM and the temperature held at 29°C. Incubating seedlings in 2 µM oryzalin for 3 h caused microtubules as well as YFP-CesA6 trajectories to deviate from their previous parallel distribution (Figure 4-13E and F), and the mean YFP-CesA6 velocity (342 ± 72 nm/min) was slightly but significantly (Wilcoxon rank sum test: p < 0.0001) reduced (Figure 4-15C) compared to the DMSO control (mean velocity: 369 ± 82 nm/min). There was no significant difference in the YFP-CesA6 velocities between the 2 µM and 20 µM oryzalin treatments (Wilcoxon rank sum test: p > 0.05).
**Figure 4-14** Effect of oryzalin on microtubules and YFP-CesA6 trajectories.

Images are projections of 5 min time-lapse images of mRFP-TUB6 (red) and YFP-CesA6 (green).

Scale bar = 5 µm.
**Figure 4-15** Effect of oryzalin on YFP-CesA6 velocity

Velocities of total of 400 particles in 12 cells from 8 seedlings were measured by kymograph analysis. Oryzalin caused a significant reduction in YFP-CesA6 particle velocity. Wilcoxon rank sum test: \( p < 0.0001 \) for 0.1% DMSO and 2 \( \mu \)M oryzalin, 0.1 % DMSO and 20 \( \mu \)M oryzalin. There was no significant difference in YFP-CesA6 velocity between 2 \( \mu \)M and 20 \( \mu \)M oryzalin-treated cells (\( p > 0.05 \)).
4.3.9 FESEM analyses on cellulose microfibril orientation in hypocotyls of rescued prc1-1 and mor1-1/prc1-1 expressing YFP-CesA6 in dark-grown seedlings

Given that YFP-CesA6 particles are expected to be producing cellulose, the cellulose microfibrils in dark-grown mor1-1 hypocotyls at 29°C should be aligned in variable orientations that resemble the YFP-CesA6 trajectories (Figure 4-8J and L, and 4-12). Here, I focused on examining cellulose microfibril orientation at the outer periclinal wall, where YFP-CesA6 particle trajectories and microtubule orientation patterns are most easily documented. Cryoplaning hypocotyls for FESEM analysis occasionally retains the outer periclinal walls of some epidermal cells and often these are folded back so that the inner surface of the wall can be clearly observed. Microfibril orientation patterns produced at 29°C in dark-grown hypocotyls of the rescued prc1-1 line were mostly locally parallel but variable within (Figure 4-16C and D) and among the cells (Figure 4-16C, D and F). These patterns are consistent with the locally parallel but variable orientations of YFP-CesA6 trajectories observed in these cells.

In the mor1-1/prc1-1 line, cellulose microfibrils deposited at the outer periclinal wall were also locally parallel but were generally more variably oriented (Figure 4-17C and I) compared to those in the rescued prc1-1 line, but were also well ordered in some cells (Figure 4-17G). Cellulose microfibrils in radial and inner periclinal walls had transversely well-ordered orientation in both rescued prc1-1 (Figure 4-16G and H) and mor1-1/prc1-1 (Figure 4-17D and E), which is consistent with previous results described in the mor1-1 roots and inflorescence stems (Sugimoto et al., 2003; Chapter 3). Interestingly, at 21°C cellulose microfibrils in the rescued prc1-1 line were more uniformly aligned, with locally parallel but variable orientations (Figure 4-18). In comparison, the microfibrils in the mor1-1/prc1-1 line showed a greater variation in orientation patterns among the cells (Figure 4-19).
Figure 4-16 Cellulose microfibril orientation in epidermal cells of dark-grown hypocotyls of the prc1-1 line expressing YFP-CesA6 after a temperature shift to 29°C for 1 day.

(A) Three day-old dark-grown hypocotyl 2 days at 21°C and 1 day at 29°C.
(B) Epidermal cell immediately below the hook.
(C) Well-ordered cellulose microfibrils in the outer periclinal wall of the cell (B).
(D) Locally parallel but variable orientations of cellulose microfibrils in outer periclinal wall of the cell (B).
(E) Epidermal cell below the hook.
(F) Locally parallel but variable orientations of cellulose microfibrils in the outer periclinal wall of the cell (E).
(G) Transverse cellulose microfibrils at the inner periclinal wall of the cell (E).
(H) Transverse cellulose microfibrils at the radial wall of the cell (E).

Scale bars = 250 μm (A), 10 μm (B,E), 500 nm (C,D,F,G,H).
Figure 4-17 Cellulose microfibril orientation in epidermal cells of dark-grown hypocotyls of *mor1-1/prc1-1* expressing YFP-CesA6 after a temperature shift to 29°C for 1 day.

(A) Three day-old dark-grown hypocotyl 2 days at 21°C and 1 day 29°C.
(B) Epidermal cell below the hook.
(C) Locally parallel but variable orientations of cellulose microfibrils at the outer periclinal wall of the cell (B).
(D) Transverse cellulose microfibrils at the radial wall of the cell (B).
(E) Transverse cellulose microfibrils at the inner periclinal wall of the cell (B).
(F) Epidermal cell.
(G) Well-ordered cellulose microfibrils at the outer periclinal wall of the cell (F).
(H) Epidermal cell.
(I) Locally parallel but variable orientations of cellulose microfibrils at the outer periclinal wall of the cell (H).

Scale bars = 250 µm (A), 10 µm (B,F,H), 500 nm (C,D,E,G,I).
Figure 4-18 Cellulose microfibril orientation in epidermal cells of dark-grown hypocotyls of rescued prc1-1 expressing YFP-CesA6 at 21°C.

(A) Three day-old dark-grown hypocotyl at 21°C for 3 days.
(B) Epidermal cell below the hook.
(C) Transverse cellulose microfibrils at the outer periclinal wall of the cell (B).
(D) Transverse cellulose microfibrils at the radial wall of the cell (B).
(E) Transverse cellulose microfibrils at the inner periclinal wall of the cell (B).
(F) Epidermal cell.
(G) Locally parallel but variable orientations of cellulose microfibrils in outer periclinal wall of the cell (F).

Scale bars = 250 µm (A), 10 µm (B,F), 500 nm (C,D,E,G).
Figure 4-19 Cellulose microfibril orientation in epidermal cells of dark-grown hypocotyls of mor1-1/prc1-1 expressing YFP-CESA6 at 21°C.

(A) Three day-old dark-grown hypocotyl at 21°C for 3 days.
(B) Epidermal cell below the hook.
(C) Variable cellulose microfibril orientations at the outer periclinal wall of the cell (B).
(D) Transverse cellulose microfibrils at the radial wall of the cell (B).
(E) Epidermal cell.
(F) Locally parallel but variable orientations of cellulose microfibrils at the outer periclinal wall of the cell (E).
(G) Relatively more variable orientations of cellulose microfibrils at the outer periclinal wall of the cell (F).
(H) Epidermal cell.
(I) Transverse cellulose microfibrils at the outer periclinal wall of the cell (H).
(J) Transverse cellulose microfibrils at the inner periclinal wall of the cell (H).

Scale bars = 500 µm (A), 10 µm (B,E,H), 500 nm (C,D,F,G,I,J).
4.4 DISCUSSION

4.4.1 YFP-CesA6 particles may be present in the plasma membrane producing cellulose

In this study, I have collected evidence supporting the idea that the YFP-CesA6 particles detected near the cell surface are located in the actual CSCs producing cellulose microfibrils, and not merely vesicles containing YFP-CesA6 particles. First, I demonstrated that motility of the YFP-CesA6 particles continues in the absence of microtubules or actin filaments so does not depend on cytoskeletal motor activity. In addition, the velocity of the fluorescent particles increased greatly at higher temperatures, which is consistent with the CSCs generating motility by enzymatic activity. Finally, FESEM analysis of microfibrils at the inner surface of the outer periclinal walls identified locally parallel but variable orientation patterns, which are predicted by the YFP-CesA6 trajectories observed by spinning disk confocal microscopy. Despite the lack of direct evidence, these results suggest that the YFP-CesA6 particles are present in active CSCs at the plasma membrane and are able to rescue the prc1-1 phenotype by restoring the normal production of cellulose.

4.4.2 YFP-CesA6 velocity and density are affected by temperature

Both short and long incubations at the higher temperature of 29°C caused velocity and also the density of YFP-CesA6 particles to increase (Figure 4-5 and 4-10). Despite a 4.2 fold increase in YFP-CesA6 particle velocity after the temperature shift, previous analysis of cellulose content has shown that cellulose content increases only slightly at high temperature (Sugimoto et al., 2003; Chapter 3; Figure 3-15). Rapid cell expansion, however, is facilitated at high temperature, so any increase in particle velocity, and therefore cellulose production will be compensated for by the faster growth rate.

The fact that an 8°C temperature shift quadrupled the YFP-CesA6 velocity suggests that there is a faster supply of precursors to the CSCs. Previous studies on the effect of temperature on cellulose synthesis have demonstrated similar
increases over 10°C temperature ranges in cotton fibers. The apparent $Q_{10}$ values calculated by measuring incorporation of $[^{14}C]$ glucose into cellulose were 4.28 over an 18 to 28°C temperature range in cultured cotton ovules and fibers (Roberts et al., 1992), and 6 over a 15 to 25°C temperature range in cotton fibers (according to Martin and Haigler, 2004, although the original data in fact suggest that the $Q_{10}$ value is about 4: Pillonel and Meier, 1985). These temperature effects on cellulose synthesis in cotton were measured during secondary wall formation, and the results demonstrate that a dramatic increase in cellulose biosynthesis occurs over a 10°C temperature range, and that this increase closely corresponds with the increased velocity of YFP-CesA6 particles that we measured over a similar temperature range.

4.4.3 Relationship between YFP-CesA6 velocity and cellulose crystallinity

After polymerization of glucan chains at the site of each rosette, the glucan chains crystallize into microfibrils. Previous studies using *Acetobacter xylinum* demonstrated that the cellulose crystallization process can limit the rate of polymerization (reviewed in Haigler, 1985). The plateau in the rate of cellulose synthesis above 28°C in cultured cotton ovules and fibers, despite the increased respiratory activity that should support faster cellulose synthesis, led the authors to consider whether, as in bacteria, crystallization is the rate-determining process in cellulose biosynthesis in higher plants (Roberts et al., 1992). The results of the previous and current chapters indicate that cellulose crystallinity is decreased but that YFP-CesA6 velocity is increased in wild type at 29°C, and that the high degree of crystallinity in the *mor1-1* mutant at 29°C is accompanied by an even more rapid movement of YFP-CesA6 particles (Figure 3-12, Figure 4-6). These results suggest that during primary wall formation at 29°C, polymerization of glucan chains continues without any feedback control from cellulose crystallization.

Previous studies on the effects of temperature on cellulose biosynthesis in higher plants have shown that as temperatures increase, cellulose synthesis at first increases, then reaches a plateau and eventually declines (Roberts et al., 1992;
Pillonel and Meier, 1985). Using the transgenic Arabidopsis system, it will be interesting in future studies to follow YFP-CesA6 particle velocity over a more extensive range of temperatures, and to correlate the measured velocities with rates of cellulose synthesis by measuring [14C] glucose incorporation, and with the degree of cellulose crystallinity.

4.4.4 YFP-CesA6 velocity is affected by microtubule organization

The temperature-controlled imaging used in the present study demonstrated that YFP-CesA6 particles move more slowly in the absence of microtubules as well as when microtubules were disorganized in oryzalin-treated cells. Recent studies using the drug morlin, which causes disorganization but not depolymerization of microtubules, also revealed that morlin caused reduced velocity of YFP-CesA6 particles, accompanied by a reduction in cellulose synthesis (DeBolt et al., 2007a). In contrast to these studies showing reduced YFP-CesA6 velocity by microtubule-related drugs, disorganized microtubules in the mor1-1 mutant caused a significant increase in YFP-CesA6 particle velocity. Despite the fact that drug studies and the mor1-1 mutant had opposite effects on YFP-CesA6 velocity, the results do support the conclusion that microtubule organization affects CSC velocity, and leave in question just what specific properties of microtubules influence YFP-CesA6 velocity. Studies of microtubule dynamics in oryzalin-treated and morlin-treated cells have demonstrated that microtubules become less dynamic (Nakamura et al., 2004; DeBolt et al., 2007a). Recent live-cell imaging of microtubules in mor1-1 has demonstrated that microtubules at 31°C become less dynamic but also tend to pause more frequently and to spend a longer time in pause, which may affect the spatial organization of microtubules (Kawamura and Wasteneys, 2008). Considering that YFP-CesA6 particles move in the fluid plasma membrane, and the fact that microtubules lie close to the plasma membrane, microtubule interaction with the plasma membrane may change membrane fluidity. In the case of the mor1-1 mutant, an increase in membrane fluidity could contribute to increasing the velocity
of CSCs, as well as generating a higher degree of cellulose crystallinity by controlling secretion of wall proteins such as COBRA as described in Chapter 6.

4.4.5 Relationship between microtubule organization and YFP-CesA6 density and trajectories

Interestingly, longitudinally curved and relatively sparse distributions of YFP-CesA6 trajectories were observed only in the presence of disorganized microtubules, and not when microtubules were completely eliminated. Together with a lower density of YFP-CesA6 particles in the mor1-1/prc1-1 line, these results suggest that microtubule organization affects the distributions of CSCs, either by controlling the insertion sites of CSCs or by controlling the sites where CSCs move. FESEM analyses on outer periclinal walls of the mor1-1/prc1-1 line after the temperature shift to 29°C for 1 day, however, did not show any remarkable differences in cellulose microfibril orientations compared to the prc1-1 line. Little correlation between YFP-CesA6 trajectories and microfibril orientations as viewed by FESEM can be interpreted as follows. First, the observed YFP-CesA6 particles may represent several CSCs moving together instead of the individual CSCs. Individual CSCs might be producing normal microfibrils. Second, the tracking of only one CSC subunit (in this case CesA6) does not show the full complement of CSCs. CesA2, CesA5, and CesA9 are partially redundant with CesA6 (Persson et al., 2007; Desprez et al., 2007), and might replace CesA6 in some of the CSCs. Third, the CSCs that have trajectories altered by disorganized microtubules may have short life spans, so that the overall microfibril orientation may look normal.

4.4.6 Cellulose microfibrils at the outer periclinal wall of epidermal cells

FESEM analyses of the rescued prc1-1 line revealed that outer periclinal walls had locally parallel but variable orientations of cellulose microfibrils, while inner periclinal walls and radial walls had well-ordered transverse cellulose microfibrils. I was not able to observe YFP-CesA6 movement in inner periclinal and radial walls
due to the depth of the focal plane. Despite the fact that CesA6 is not the only CesA involved in primary wall formation, a good agreement of YFP-CesA6 trajectories and cellulose microfibril orientations observed by FESEM in the rescued prc1-1 line suggests that the orientation of CesA movement is locally parallel but often variable at the outer periclinal wall and that it is transverse to the growth axis at the radial and inner periclinal surfaces.

The outer epidermal wall has been shown to have distinct properties compared to other walls such as greater thickness and less extensibility (reviewed in Kutschera, 2008a; 2008b). The outer epidermal wall of dark-grown Arabidopsis hypocotyls has greater thickness compared to other faces of the walls (Réfregier et al., 2004; Derbyshire et al., 2007), and has successive layers of mixed cellulose microfibril orientation (ie, polylamellated walls) (Réfregier et al., 2004), which indicates that the outer epidermal wall could have more isotropic mechanical properties compared to other walls.

Recent live-cell imaging of microtubules in the hypocotyl epidermis of light-grown Arabidopsis seedlings revealed that microtubule arrays undergo periodic shifts in orientation described as rotary movements (Chan et al., 2007), and that CesA trajectories also rotate in these cells (H. Höfte, Third conference on the biosynthesis of plant cell walls 2008). These behaviours might explain the basis for polylamellated walls at the outer epidermal surface.

4.4.7 Can variable orientations of cellulose microfibrils in the outer periclinal wall cause loss of growth anisotropy in the mor1-1 mutant?

Previous mor1-1 studies led to a conclusion that microtubules are not required for well-ordered cellulose microfibril orientation (Sugimoto et al., 2001; 2003; Himmelspach et al., 2003), based on the observations of disorganized microtubules throughout the cells and well-aligned cellulose microfibrils in the inner periclinal and radial walls. Microtubules at the radial face of mor1-1 root cells have been shown to be disorganized (Sugimoto, 2000) so, while it remains possible, it is
unlikely that hypocotyl microtubule patterns are well ordered in the radial and inner periclinal surfaces.

It is unlikely that relatively variable orientations of cellulose microfibrils at the outer periclinal wall in mor1-1 can directly cause loss of growth anisotropy. One supporting piece of evidence for this is that the rescued prc1-1 line also had locally parallel but variable orientations of cellulose microfibrils at the outer periclinal wall without any defect in growth anisotropy. The inner tissue is also likely to assist epidermal layers in generating anisotropic organ expansion as demonstrated in some inflorescence stems (reviewed in Baskin, 2005; Schopfer, 2006). Inner periclinal walls and radial walls in the mor1-1/prc1-1 line had transverse cellulose microfibrils as in the rescued prc1-1 line. It is therefore likely that other factors besides microfibril orientation alone, such as mechanical strength of the cellulose microfibrils, are involved in the control of anisotropic cell expansion, as suggested by Wasteneys (2004).
CHAPTER 5

Cellulose properties in the CesA1 mutant
\textit{anisotropy (any1)} inflorescence stem
5.1 INTRODUCTION

Cellulose microfibrils in higher plants are synthesized at the plasma membrane from a structure known as a rosette (Brown et al., 1996; Kimura et al., 1999), which contains multiple cellulose synthase (CesA) subunits (Doblin et al., 2002). Each CesA protein contains a cluster of 2 predicted transmembrane domains (TMDs), a cysteine-rich domain suggested to form zinc- or RING-finger domains to facilitate interaction with other CesAs (Kurek et al., 2002), and a highly variable region (HVR1) at the N-terminus that is not present in bacterial CesAs (Pear et al., 1996). The central domain contains the D, D, D, QxxRW motif found to be conserved in processive β-glycosyltransferases (Coutinho et al., 2003), and also contains a region that is relatively conserved between orthologues, called the class-specific region (CSR) (Vergara and Carpita, 2001). After a cluster of 6 predicted TMDs, there is a small C-terminal region (Figure 5-1).

Among the 10 CesA genes identified in Arabidopsis thaliana, CesA1 is known to be involved in primary wall formation. CesA1 null mutations are gametophytic-lethal (Persson et al., 2007). Another T-DNA insertion line, radial swelling 1-10 (rsw1-10), is a leaky allele, in which the expression level of CesA1 is reduced, and has a weak constitutive phenotype of post-embryonic radial swelling (Fagard et al., 2000). The effect of this leaky allele is inherently different from the single amino acid-substituted mutants that have been identified. Mutational analysis is a useful strategy for understanding the functional domains of CesA1 and its interactions with other CesAs by identifying mutant allele-specific phenotypes and interpreting function from the phenotype. To date, four missense mutant alleles of CesA1 have been identified. All of these mutants have a mutation in the central cytosolic domain of the CesA1 protein where the actual catalytic site of the β-glycosyltransferase enzyme is located. The first of these, radial swelling 1-1 (rsw1-1) has an alanine to valine change at amino acid residue 549 (Arioli et al., 1998). The rsw1-2 mutation causes a glycine to serine change at residue 631 (Gillmor et al., 2002), the rsw1-20 mutation substitutes asparagine for the third of three asparatic acid residues in the conserved glycosyltransferase D, D, D, QxxRW motif, and the rsw1-45 mutation exchanges lysine for glutamic acid in the residue adjacent to rsw1-20, (Beeckman et
The rsw1-1 mutant is temperature-sensitive, and shows reduced cellulose production at 31°C, causing growth and morphogenesis defects (Arioli et al., 1998; Williamson et al., 2001). The rsw1-2 mutant was later identified based on its radially swollen phenotype during embryogenesis and was found to have a greatly reduced level of crystalline cellulose at the cotyledon stage of embryo development (Gillmor et al., 2002). The rsw1-20 and rsw1-45 mutations also cause defects in embryogenesis, including reduced cellulose production and the formation of thin and incomplete cell walls (Beeckman et al., 2002). Severe phenotypes of rsw1-2, 1-20 and 1-45 suggest that the domains where these missense mutations occur are critical for CesA1 function.

The anisotropy 1 (any1) mutant was isolated from M2 lines of an ethyl methane-sulfonate (EMS)-treated population during a screen for microtubule organization-defective mutants. It was selected based on its dwarf stature and abnormal cellular morphologies characterized by loss of anisotropy (G. Wasteneys, unpublished data). There was no clear microtubule phenotype so the mutant was named anisotropy1 on the premise that the affected gene’s function appeared to play a major role in anisotropic expansion. Cryo-SEM analyses on the twice back-crossed any1 mutant demonstrated swollen and often ruptured trichomes, swollen epidermal cells of cotyledons, hypocotyls, floral organs and leaves, ectopic root hair formation and short root hairs (G. Wasteneys, unpublished data). Map-based cloning of any1 revealed that the ANY1 locus mapped close to RSW1. The F1 lines of the complementation cross of any1 and the temperature-sensitive rsw1-1 mutant showed a partial allelic complementation of the any1 phenotype by rsw1-1 at 21°C and complementation of rsw1-1 phenotype by any1 at 31°C such as intermediate root length between wild type and the any1 mutant and diminished root-swelling of rsw1-1. F2 segregation analysis revealed that rsw1 and any1 were allelic (Phenotype of rsw1-1 : F1-like : any1= 1:2:1) (G. Wasteneys, unpublished data). The sequencing of the any1 mutant demonstrated that any1 has a missense mutation that exchanges the asparatic acid residue 604 with asparagine, and transgenic expression of the CesA1 cDNA in the any1 mutant rescued the phenotype (J. Ward and G. Wasteneys, unpublished data).
Unlike other CesA1 mutant alleles that show severe phenotypes, the *any1* mutant has a relatively mild phenotype in the sense that plants are viable and can produce seeds. The *any1* root is short and appears to have abundant root hairs due to the short elongation zone (G. Wasteneys, unpublished data). FESEM analyses on *any1* root epidermal cells, however, demonstrated that cellulose microfibrils were transversely oriented (R. Himmelspach, unpublished data). To investigate the *any1* mutant phenotype further, I have examined growth rate, diameter, cellulose microfibril orientation and cellulose crystallinity in the *any1* inflorescence stem.
Figure 5-1 Schematic diagram of CesA1 protein indicating domains and sites of point mutations of CesA1 (modified from Wang et al., 2006; Arioli et al., 1998; Williamson et al., 2001).
5.2 MATERIALS AND METHODS

5.2.1 Plant materials and growth condition

The Arabidopsis (Arabidopsis thaliana) homozygous any1 (anisotropy1) mutant, which had been backcrossed eight times, and wild-type segregants from this backcrossing were used throughout this study. Plants were grown on Hoagland’s media in agar plates and placed in a 21°C growth cabinet after an initial cold treatment as described in Chapter 2. Ten day-old seedlings were transferred onto soil and grown at 21°C in a growth cabinet as described in Chapter 2.

5.2.2 Measurement of any1 inflorescence stem growth and diameter

Growth measurements of inflorescence stems were performed as described in Chapter 3. Images of inflorescence stems were taken using a stereomicroscope (Leica MZ16FA, Leica) equipped with a digital camera (DC 350 FXR2, Leica), and stem diameters were measured using Leica Application Suite (Leica) and ImageJ software. Standard deviations were calculated, and means were compared by the independent Student’s t-test for samples with unequal variance at a significance level of 0.05.

5.2.3 Cell wall preparation and FESEM

The apical regions of inflorescence stems were used to examine cellulose microfibril orientation. Cell wall preparation and FESEM were performed as described in Chapter 2.

5.2.4 Measurement of cellulose crystallinity

Cellulose crystallinity was calculated from X-ray diffraction data as described in Chapter 3.
5.3 RESULTS

5.3.1 Growth of wild-type and any1 inflorescence stems

Bolting occurred 4 weeks after germination in wild type, while it occurred 5 weeks after germination in the any1 mutant. The shape of the rosette leaves was narrow and long in any1, and plants were small (Figure 5-2A and B). The elongation rate of the any1 inflorescence stem was significantly reduced compared to that of wild type (Figure 5-3A). Compared to wild type, the diameter of inflorescence stems in any1 was normal in the elongation zone but remained significantly thinner in the region of the stem where elongation had ceased (Figure 5-3B).

5.3.2 Cellulose microfibril orientation in wild-type and any1 inflorescence stems

FESEM analyses on epidermal cells of any1 inflorescence stems demonstrated that, in contrast to the any1 root epidermal cells, where cellulose microfibril orientation is normal (R. Himmelspach, unpublished data), the orientation of cellulose microfibrils was random compared to that of wild type (Figure 5-4A to D). Cellulose microfibrils also appeared to be less densely packed in the any1 mutant. Cellulose microfibrils in the cortex cells (Figure 5-5D) and the highly elongated parenchymal cells of the inner tissue layers (Figure 5-5E) of any1 stems, however, were relatively transverse in comparison to the microfibrils in epidermal cells.

5.3.3 Cellulose crystallinity in wild type and any1

X-ray diffraction analysis demonstrated that there was no significant difference in cellulose crystallinity between wild type (69 ± 3%) and the any1 mutant (71 ± 6%) (Figure 5-6).
Figure 5-2 Growth of wild type and any1.

(A) 3 week-old plants of wild type and the any1 mutant. The rosette leaves of any1 are narrower and smaller than those of wild type.
(B) 5 week-old plants of wild type and any1 mutant.
Figure 5-3 Comparison of elongation rate and stem diameter of wild-type and *any1* inflorescence stems.

Values are means ± SD from 15 plants. Asterisks indicate significant differences as determined by a Student t-test: *p < 0.05.*

(A) Elongation rate of each region of the stem in wild type and *any1.*
(B) Stem diameters of each region of the stem in wild type and *any1.*
**Figure 5-4** Comparison of cellulose microfibril orientation pattern in epidermal cells of wild-type and *any1* inflorescence stems.

Field emission scanning electron micrographs from the epidermal cell layers from the top part of the stem in wild type (A) and *any1* (B). Higher magnification micrographs showing cellulose microfibrils at the cytoplasmic face of inner epidermal cell walls from wild type (C) and *any1* (D).

Asterisks indicate the area from which the higher magnification images were taken (C) from (A), (D) from (B).
**Figure 5-5** Cellulose microfibrils in other cell layers of *any1* inflorescence stems.

Field emission scanning electron micrographs of a longitudinal section of an *any1* inflorescence stem (A), a cortex cell (B), and a highly elongated cell in an inner tissue layer (C).
Higher magnification micrographs showing cellulose microfibrils at the cytoplasmic face of the inner epidermal cell walls from a cortex cell (D) and the highly elongated cell (E).
Cellulose microfibrils are relatively transversely oriented in the cortex cell (D), and more uniformly transverse in the highly elongated cell (E).

Asterisks indicate the area from which the higher magnification images were taken. (D) from (B), (E) from (C).
Figure 5-6 Cellulose crystallinity of in the growing regions of inflorescence stems in wild type and *any1*.

Values are means ± SD for 14 sets of 10 dried stems.

There was no significant difference in mean crystallinity between wild type and *any1* at 21°C (Student t-test: *p* = 0.3 > 0.05).
5.4 DISCUSSION

5.4.1 Growth defects and random cellulose microfibril orientation in the any1 inflorescence stems

Growth of inflorescence stems was reduced in any1. Almost no growth in the B and C regions suggests that the elongation of any1 inflorescence stems takes place in a small restricted region. The thickness of the stem was normal in the any1 elongation zone, but was thinner in the non-elongating zone compared to that of wild type. The relatively poor vegetative growth, such as narrow leaves observed in any1 may contribute to the delayed and poor development of the inflorescence stems. Short and thin inflorescence stems were also observed in CesA1 antisense plants (Burn et al., 2002). Since cell production rate is not altered in the any1 root (T. Baskin and G. Wasteneys, unpublished data), it is unlikely that tissue patterns and cell numbers are altered in the any1 inflorescence stems.

The narrow shape of the rosette leaves is a characteristic of the any1 mutant phenotype. Other alleles, rsw1-1 (Williamson et al., 2001), rsw1-10 (Hematy et al., 2007), and CesA1 antisense plants (Burn et al., 2002) all have small and rounded rosette leaves. These results suggest that the effect of the any1 mutation on leaf morphogenesis is allele-specific.

Cellulose microfibrils appeared to have no preferred orientation in the epidermal cells of any1 inflorescence stems, but their orientation was transverse in the cortex and highly elongated in the parenchyma cells of the inner tissue layers. By contrast, in the epidermal cells of the any1 root, cellulose microfibrils were found to be transversely oriented (R. Himmelspach, unpublished data). Previous gene expression patterns of CesA1 studies have demonstrated that CesA1 is expressed in all tissues including stems, roots, leaves, flowers, young seedlings and embryos (Beeckman et al., 2002; Burn et al., 2002; Hamann et al., 2004). My FESEM results suggest that the contribution of CesA1 to cellulose synthesis might vary from tissue to tissue, from organ to organ, and that other CesAs may have redundant function among tissues, organs and developmental stages.
Random cellulose microfibril orientation often correlates with cellulose content and loss of growth anisotropy (Wasteneys, 2004). The relatively mild phenotypes of the CesA1 antisense plants are accompanied by a slight but not statistically significant reduction in cellulose content (Burn et al., 2002). Cellulose content should be tested separately in the any1 mutant inflorescence stems and roots.

5.4.2 Normal cellulose crystallinity in the any1 inflorescence stems

X-ray diffraction analyses demonstrated that cellulose crystallinity in the any1 mutant was not altered in comparison to the wild type. The production of crystalline cellulose in the rsw1-1 mutant is reduced at restrictive temperature, accompanied with the disappearance of rosettes from the plasma membrane (Arioli et al., 1998). The correlation between the absence of rosettes and reduced crystalline cellulose was also observed in cotton fibers in the presence of the herbicide CGA 325’165 (W. Herth and K. Kreuz, unpublished data cited in Peng et al., 2001). Given that organized rosettes are required for producing crystalline cellulose, the normal cellulose crystallinity in any1 suggests that the organization of rosettes may be normal in the any1 mutant. Future investigations should examine the behavior of CSC movements using fluorescently labelled CesA in the any1 mutant.
CHAPTER 6

Subcellular localization of COBRA, the relationship between COBRA distribution and microtubules, and epitope-tagging of COBRA
6.1 INTRODUCTION

The COBRA (COB) protein is a key player in anisotropic cell expansion and cellulose synthesis (Shindelman et al., 2001; Roudier et al., 2005; Ko et al., 2006). The cobra mutants that have been identified so far show defects in anisotropic cell expansion, including root swelling in the cobra-1 (cob-1) conditional mutant, and constitutive root and aerial organ swelling in the cob-4 and cob-5 T-DNA insertion lines (Benfey et al., 1993; Hauser et al., 1995; Shindelman et al., 2001; Roudier et al., 2005; Ko et al., 2006).

COB is a glycosylphosphatidylinositol (GPI)-anchored protein, which contains an N-terminal signal sequence for secretion, a highly hydrophobic C-terminal region with a cleavage site for GPI-anchor attachment, two N-glycosylation sites, a putative cellulose-binding domain and a Cys-rich domain (Schindelman et al., 2001; Roudier et al., 2002). As shown in Figure 6-1, GPI-anchored proteins can be released from the anchor by phosphoinositol (PI)-specific phospholipases such as PI-phospholipase C (PLC) and phospholipase D (PLD). It has been shown by phase partitioning assay that COB is localized at the plasma membrane and is released from the plasma membrane after PI-PLC treatment (Roudier et al., 2005). COB is first expressed in the rapid elongation zone and remains at high levels in the differentiation zones of the root (Roudier et al., 2005). Immunofluorescence labelling of COB has demonstrated that COB forms a transverse band pattern relative to the axis of cell expansion (Shindelman et al., 2001; Roudier et al., 2005).

In this chapter, I first describe the results of immuno-gold labelling COB on high-pressure frozen roots to examine its subcellular localization. This work is published in Roudier et al. (2005). The same article showed that COB bands are dissipated in oryzalin-treated roots and in the microtubule-defective ton2 mutant (Roudier et al., 2005). To further examine the role of microtubules in the distribution of COB, I analyzed changes in COB distribution patterns in taxol-, and oryzalin-treated roots, and in the temperature-sensitive mor1-1 mutant. In carrying out immunofluorescence labelling of COB in the cob-4 mutant, which is a null allele, I found that an antibody that was thought to be specific to COB labels root hairs, suggesting that it cross-reacts with an epitope that is distinct to COB. Since COB is
one of a 12-member gene family, it is difficult to select isoform-specific antigenic sequences. Epitope tagging of COB was therefore performed by engineering a 4 x myc tagged version of COB. The transgene was transferred to cobra mutants, and immunofluorescence labelling of myc tag was performed on transgenic plants to re-examine the distribution pattern of COB.
Figure 6-1 Schematic diagram of the COBRA glycosylphosphatidylinositol (GPI)-anchored protein.

The GPI-anchor is a glycolipid structure that is added posttranslationally to the C-terminus of the protein in the ER. The GPI-anchored protein is secreted via the Golgi-derived secretory pathway to the outer leaflet of the cell membrane. Each GPI-anchor has a phosphoethanolamine linker, a glycan core, and a phospholipid tail. The phosphoinositol, glucosamine, and mannose residues within the glycan core can be variously modified (reviewed in Paulick and Bertozzi, 2008). GPI-anchors can be cleaved by either phospholipase D or phosphatidyl inositol (PI)-phospholipase C.
6.2 MATERIALS AND METHODS

6.2.1 Plant materials and growth condition

The Arabidopsis (*Arabidopsis thaliana*) wild type (Columbia ecotype); homozygous *mor1-1* mutants (Whittington et al., 2001), which had been backcrossed eight times; wild-type segregants from this backcrossing; homozygous *cob-1* mutants (Schindelman et al., 2001); and homozygous *cob-4* mutants screened from a heterozygous population (Roudier et al., 2005) were used throughout this study. Transgenic plants expressing GNOM (GN)-myc (Geldner et al., 2003) were used as positive controls for immunofluorescence labelling of myc. Plants were grown on Hoagland’s medium in agar plates with 3 % sucrose for *mor1-1* and *cob-4*, and with 4.5 % sucrose for *cob-1*, and placed in a 21°C growth cabinet after an initial cold treatment as described in Chapter 2.

6.2.2 Antibodies

Anti-COB polyclonal chicken IgY antibodies were obtained from the laboratory of Dr. P. Benfey, as described in Roudier et al. (2005). For immuno-gold labelling, goat anti-chicken IgG conjugated to 10-nm colloidal gold (Aurion, Netherlands) was used as a secondary antibody. For immunofluorescence labelling of COB, Alexa Fluor 488 conjugated goat anti-chicken antibody (Molecular Probes) diluted 1:500 was used as a secondary antibody. For immunofluorescence labelling of microtubules, mouse anti-α-tubulin (clone B512, Sigma-Aldrich) diluted 1:1000 was used as a primary antibody, and Fluorolink Cy5 conjugated goat anti-mouse antibody (Amersham Biosciences) diluted 1:100 was used as a secondary antibody. For immunofluorescence labelling of myc, anti-myc antibody (9E10, Santa Cruz Biotechnology, USA) diluted 1:600 was used as a primary antibody, and Alexa Fluor 488 conjugated goat anti-mouse antibody (Molecular Probes) diluted 1:100 was used as a secondary antibody.
6.2.3 Immunogold labelling of COBRA

6.2.3.1 High pressure freezing and resin embedding

Roots from 5 day-old seedlings were cut into 1.5 mm lengths with a razor blade while submerged in hexadecane. Cut root tissue was transferred into flat specimen holders filled with hexadecane and cryofixed in a high pressure freezing unit (Bal-Tech HPM010, Balzers). For LR White (London Resin Company, UK) embedding, frozen samples were put into cryogenic vials containing freeze substitution medium (0.25 % glutaraldehyde, 0.1 % uranyl acetate in acetone) and placed into a dry ice-acetone bath as described (Rensing et al., 2002). This bath was placed at -20°C for 7 days and warmed to 4°C for 4 h, then brought to room temperature. The tissue was rinsed with fresh acetone and infiltrated with LR White embedding resin according to the following schedule: drop by drop for 3 days, then 50, 75, 85% (v/v) LR White: acetone, each step for 12 h, and finally 100 % LR White resin for 36 h with resin exchanged every 12 h. Polymerization was at 60°C for 24 h. Thin sections (< 70 nm) were cut with an Ultracut E (Leica) ultramicrotome and mounted on Formvar-coated 200-mesh nickel grids.

6.2.3.2 Immuno-gold labelling and transmission electron microscopy (TEM)

All incubations of sections on the grids were carried out by placing the grids on the droplets of solution with sections side down at room temperature. Thin sections mounted on the grids were incubated for 10 min on the droplets of 10 mM NH₄Cl in TBS (10 mM Tris and 0.25 M NaCl, pH 7) to block free aldehydes. Nonspecific antibody-binding sites on the sections were blocked by incubating the sections for 30 min in 5 % nonfat dried milk in TBS. The sections were then incubated for 1 h on droplets of chicken anti-COB antibody diluted 1:100 in 0.5 % nonfat dried milk in TBS. After rinsing the sections in TBS five times, they were incubated for 1 h on droplets of goat anti-chicken IgG conjugated to 10-nm colloidal gold diluted 1:100 in 0.5 % nonfat dried milk in TBS. The sections were then rinsed in TBS five times, in distilled water twice, and air dried. Grids were stained in 2 %
aqueous uranyl acetate for 30 min and in lead citrate for 5 min, and then observed by Hitachi H7600 transmission electron microscopy (Hitachi, Japan).

6.2.4 Root tissue localization of COBRA

Longitudinal thick sections (about 0.5 µm) of the wild-type root embedded in LR White were obtained with an Ultracut E ultramicrotome. To view the root tissue during sectioning, thick sections were stained with 1% toluidine blue. For COB immunolabelling, sections were placed in a droplet of water on top of a 1 mg/ml poly-L-lysine-coated 10-well slide, and air dried. To prevent nonspecific antibody binding, the sections were blocked by incubating them for 20 min in 5 % nonfat dried milk in TBS. After washing in TBS, the sections were incubated for 1 h with chicken anti-COB antibody diluted 1:100 in 1 % nonfat dried milk in TBS. After rinsing in TBS three times for 10 min each, the sections were incubated for 1 h with goat anti-chicken Alexa 488 diluted 1:100 in 1 % nonfat dried milk in TBS. The sections were then washed in TBS twice for 10 min each, washed in PBS twice for 5 min each, and mounted in Citifluor antifade agent. Fluorescent images were collected using a Zeiss Axiovert 200M inverted microscope equipped with an AxioCamHR camera (Carl Zeiss, Germany) and acquired using Zeiss AxioVision software.

6.2.5 Immunofluorescence labelling of COBRA and microtubules in whole mounted roots

6.2.5.1 Immunofluorescence labelling

Immunofluorescence was performed using a modified method described in Sugimoto et al. (2000). Whole seedlings were fixed at room temperature for cob-1 and cob-4, at either 21°C or 31°C for mor1-1 and wild-type segregants in 0.5 % (v/v) glutaraldehyde and 4 % formaldehyde made up in PME buffer (25 mM PIPES, 0.5 mM MgSO₄, and 2.5 mM EGTA, pH 7.2) for 40 min at 31°C, and rinsed three times for 10 min in PMET (PME with 0.05 % Triton-X 100) buffer. Cell walls were partially digested with 0.5 % (w/v) Pectolyase Y-23 (MP Biomedicals, USA) in PME buffer
with 0.4 M mannitol and 1 % (w/v) BSA for 20 min at room temperature. After rinsing in PMET three times for 5 min each, seedlings were incubated with 1 % Triton-X 100 in PBS for 3 h. Seedlings were washed in PBS for 10 min, and then incubated with 1 mg/ml NaBH₄ in PBS for 20 min to reduce autofluorescence from free aldehydes derived from glutaraldehyde fixation. Seedlings were incubated with 1 % BSA in 50 mM glycine in PBS (IB: incubation buffer) for 30 min, and then incubated with primary antibody in IB at 4°C overnight. After primary antibody incubation, samples were rinsed in IB three times for 10 min each on the shaker, and incubated with 5 % non immune-goat serum for 30 min to reduce nonspecific antibody binding. Samples were incubated at 37°C for 3 h with secondary antibody in 1 % non immune-goat serum and IB. After rinsing in PBS three times for 10 min, root tips were excised and mounted in Citifluor antifade agent.

6.2.5.2 Fluorescence microscopy

Fluorescent images for double labelling of microtubules and COBRA were collected with a Zeiss Axiovert 200M inverted microscope equipped with an AxioCamHR camera (Carl Zeiss, Germany). The filter sets, YFP (excitation BP500/20, emission BP535/30) and Cy5 (excitation HQ620/60, emission 700/75) were used for Alexa Fluor 488 and Cy5 respectively.

6.2.5.3 Oryzalin and Taxol

Oryzalin (Supelco) was dissolved in DMSO solution to make a 10 mM stock solution. Taxol was dissolved in DMSO solution to make a 1 mM stock solution. A stock solution was added to Hoagland’s medium to make the indicated concentrations in 0.1 % DMSO in the solidified Hoagland’s plates.
6.2.6 Epitope-tagging of COBRA

6.2.6.1 Generation of COBRA fused with a myc-tag construct

All primer information for COB cloning was summarized in Table 6-1. A 1.5 kb fragment of the COB promoter upstream of the initiation codon was amplified by PCR from a BAC clone (MSL3, AGI-TIGR) with specific primers containing KpnI/XbaI and Sall restriction sites. The 4 x myc tag was amplified by PCR from pGWB16 vector with specific primers containing PstI and SpeI restriction sites. To insert the 4 x myc tag into the COB gene, a 1.5 kb fragment of COB cDNA from the initiation codon to the region before the GPI-anchor cleavage site, and a 100 bp fragment of COB cDNA from the GPI-anchor cleavage site to the stop codon, were amplified from a COB cDNA clone (126J6, ABRC) by PCR with specific primers containing Sall and PstI restriction sites and specific primers containing SpeI and SacI restriction sites, respectively. All fragments were subsequently cloned into pBluescript SK+ (Stratagene) and sequenced. The P_COBRA:COB tagged with 4 x myc was removed from pBluescript KS+ by PCR with specific primers containing attB1 and attB2 recombination sites for cloning into pDoner221 and put into the binary vector pGWB1 using Gateway cloning technology (Invitrogen). The construct was introduced into Arabidopsis wild-type Columbia, cob-1 homozygotes and cob-4 heterozygotes by Agrobacterium-mediated transformation (strain GV3101). Transgenic plants were screened for antibiotic resistance, and resistant T2 lines were used for testing for complementation of the mutant phenotype and for genotyping by PCR amplification to identify cob-4 homozygotes with primers containing intron 4 and 5 to avoid amplifying the transgene (Table 6-2). T3 lines were used for immunofluorescence labelling of the myc tag.

6.2.6.2 Immunofluorescence labelling of myc-tags in transgenic plants

Eight-day old seedlings were used for immunofluorescence labelling. Wild-type Columbia was used as a negative control, and transgenic plants expressing GNOM (GN)-myc (Geldner et al., 2003) were used as a positive control. Immunofluorescence labelling of myc was performed as described in section 6.2.5.1.
The Alexa-488 fluorescent images of immunolabelled samples were collected with an upright AxioImager M1 microscope (Zeiss, Germany) equipped with a Zeiss PASCAL Excite two channel scan head, using the 488 nm line from an argon laser, with a 488 nm dichroic filter and a 505 nm emission filter, along with a 63 x NA 1.4 oil-immersion lens and Kalman collection (N=2). Images were recorded with LSM software (Zeiss, Germany).
Table 6-1 Primers used for cloning \( P_{\text{COBRA}} \) : COBRA tagged with 4 x myc and for gateway system.

<table>
<thead>
<tr>
<th>DNA fragments</th>
<th>Primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>KpnI/XbaI site - ( P_{\text{COBRA}} ) –SalI site</td>
<td>5’-cagcacgtggtactcttagaattaagtttgac-3’</td>
</tr>
<tr>
<td></td>
<td>5’-ctggagaaggtgacagttttaatactctg-3’</td>
</tr>
<tr>
<td>Sall site-COBRA cDNA N terminus region-PstI site</td>
<td>5’-ctggctagtcgaccatggagtctttc-3’</td>
</tr>
<tr>
<td></td>
<td>5’-tactatactgcagcaggagcatgagc-3’</td>
</tr>
<tr>
<td>PstI site-4 x myc-Spel site</td>
<td>5’-atgtgacctgcagccattaacggtgaac-3’</td>
</tr>
<tr>
<td></td>
<td>5’-catgtactagctgcgtccgctacgtcagtc-3’</td>
</tr>
<tr>
<td>Spel site-COBRA cDNA C terminus region-Sacl</td>
<td>5’-ctatgcagactagtccagacttacc-3’</td>
</tr>
<tr>
<td></td>
<td>5’-aactctgagagctctttaggcagagaag-3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>For Gateway cloning</th>
<th>Primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>attB1-( P_{\text{COBRA}} ); COBRA tagged with 4 x myc – attB2</td>
<td>5’-ggggacaagttgtacaaaaaagcaggctcttaagatttgactgatttaag-3’</td>
</tr>
<tr>
<td></td>
<td>5’-ggggaccacattttagcagaagaagctgggtcttaggcagagaagaagaaaag-3’</td>
</tr>
</tbody>
</table>
**Table 6-2** Primers used for genotyping *cob-4* homozygous mutant background from screened transgenic plants containing $P_{COBRA}$:COBRA tagged with 4 x myc transgene.

<table>
<thead>
<tr>
<th></th>
<th>Primer pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>To amplify T-DNA region</td>
<td>LB2: 5'-'gcttcctatatctctccccaaattacat-3'</td>
</tr>
<tr>
<td></td>
<td>RP3: 5'-'acacaaagccctcttc-3'</td>
</tr>
<tr>
<td>To amplify wild-type</td>
<td>LP2: 5'-'gtaagaacctgactgacatcc-3'</td>
</tr>
<tr>
<td>COBRA region</td>
<td>RP3: 5'-'acacaaagccctcttc-3'</td>
</tr>
</tbody>
</table>
6.3 RESULTS

6.3.1 Subcellular localization of COBRA on high-pressure-frozen, immunogold-labelled Arabidopsis roots

Given that COB is GPI-anchored, its secretion is likely to be carried out via Golgi-derived-vesicle fusion and placement at the extracellular surface of the plasma membrane. To determine subcellular localization of COB using transmission electron microscopy (TEM), immunogold-labelling of COB was performed on the sections of high-pressure-frozen Arabidopsis roots.

High-pressure freezing (HPF) followed by freeze-substitution is a cryo-fixation technique that is the most reliable method available to obtain superior preservation of organelles, cells and tissues compared to conventional chemical fixation (Dahl and Staehelin, 1989). Roots from 5 day-old Arabidopsis seedlings were high pressure-frozen and immunogold-labelled. Negative controls, sections incubated without COBRA antibody and with pre-immune chicken serum, showed that almost no labelling was found on the sections, indicating that there was no nonspecific binding of 10 nm gold-conjugated secondary antibody or of chicken serum on the sections (Appendix B). By comparing the labelling patterns on the sections with different concentrations of COB antibodies, a 1:100 dilution was found to be an optimal concentration for the immunogold-labelling (Appendix B).

COB in elongating cells was associated with Golgi and abundant in the cell wall (Figure 6-2A and B), supporting the idea that its secretion is via Golgi-derived vesicles. Since COB was present at various distances from the plasma membrane, COB appears to exist both in a GPI-anchored form attached to plasma membrane and as a cleaved free protein in the wall. Serial transverse sections (Figure 6-3C and D) of epidermal cells revealed that COB was abundant in some sections and not present in others. Some outer periclinal sections showed patches of COB distribution close to plasma membrane along the outer periclinal wall (Figure 6-3E). These results are consistent with COB distribution to transverse bands observed by immunofluorescence labelling (Roudier et al., 2005; Figure 6-3A).
Figure 6-2 COB subcellular localization in high-pressure-frozen, immunogold-labelled epidermal cells.

CW: cell wall.

(A) COB distribution in tangential sections from the outer epidermis of the root elongation zone. COB is found in Golgi and in the cell wall both close to and well away from the plasma membrane.

(B) Tangential section from the outer epidermis of the root elongation zone. COB is associated with Golgi and abundant in the cell wall.

Figure 6-2A (Copy-right; Wasteneys and Fujita (2006) Establishing and maintaining axial growth: wall mechanical properties and the cytoskeleton. Journal of Plant Research 119, page 9, Figure 2, With kind permission of Springer Science+Business Media).

Figure 6-2B (Copy-right; Roudier et al. (2005) Plant Cell 17 page 1759, Figure 8, American Society of Plant Biologists).
**Figure 6-3** COB band pattern at the outer epidermis.

CW: cell wall, PM: plasma membrane.

(A) Immunofluorescence labeling of COB showing transverse band patterns at the outer epidermis in the root elongation zone.
(B) Schematic drawing of the root in longitudinal view showing the section planes from which the images in (C) and (D) were obtained.
(C) and (D) COB distribution in the root elongation zone revealed by immunogold labelling of high-pressure frozen material. Transverse sections from the same series of the outer epidermis about 350 nm apart.
(E) Outer epidermal periclinal section shows patches of COB distribution close to plasma membrane along the outer periclinal wall. Black arrows indicate the region where COB was abundant.

Figure 6-3A (Copy-right; Wasteneys and Fujita (2006) Establishing and maintaining axial growth: wall mechanical properties and the cytoskeleton. Journal of Plant Research 119, page 9, Figure 2, With kind permission of Springer Science+Business Media).

Figure 6-3B to D (Copy-right; Roudier et al. (2005) Plant Cell 17, page 1759, Figure 8, American Society of Plant Biologists).
Analysis of different section planes of the root elongation zone (Figure 6-4A) indicated that COB was abundant at the outer face of epidermal cells (Figure 6-4B) but relatively little was present in other epidermal cell faces, the inner longitudinal faces (Figure 6-4C), and the radial faces (Figure 6-4E), and that no COB was found at the anticlinal faces (Figure 6-4D) of the epidermal cells. These observations indicate that COB secretion is spatially regulated. There was anti-COB labelling in the cytoplasm near all faces, but the greatest labelling concentration was found in the cytoplasm near the outer face of epidermal cells. Longitudinal sections from the root’s cell division zone (Figure 6-5A and C) suggested that little COB was present in the cytoplasm and no COB was found in the walls of epidermal cells (Figure 6-5B), consistent with COB’s expression being up-regulated at the onset of the elongation (Roudier et al., 2005). Interestingly, anti-COB labeling was high in the outer periclinal walls of lateral root cap cells (Figure 6-5D) but very little labelling was detected in the anticlinal walls (Figure 6-5E), indicating that polar secretion of COB is also maintained in lateral root cap cells. In the root differentiation zone, very little anti-COB was detected in both the cell walls and cytoplasm of epidermal cells (Figure 6-6B), while the antibodies labelled abundant epitopes in the cell wall of root hairs (Figure 6-6C).

Immunofluorescence labelling of longitudinal sections of the whole root indicated consistent results that COB was present at high levels in outer face of elongating epidermal cells as well as of lateral root cap cells (Figure 6-7). These results, summarized in Figure 6-8, indicate that COB secretion is spatially and developmentally regulated in roots.
**Figure 6-4** COB subcellular distribution in the root elongation zone.

CW: cell wall, Epi: epidermal cell, Cortex; cortex cell.

(A) Schematic drawing of the root in longitudinal view showing the section planes from which (B) to (E) were obtained.

(B) Outer epidermal periclinal section shows abundant COB in both the cytoplasm and wall.

(C) Longitudinal periclinal section between epidermal and cortex cell shows COB in the cytoplasm but relatively little COB in the wall.

(D) Longitudinal section showing low abundance of COB in transverse anticlinal face between adjacent epidermal cells.

(E) Longitudinal radial section between adjacent epidermal cell files shows COB in the cytoplasm but relatively little COB in the wall.

Scale bars = 500 nm.

Figure 6-4A to E: (Copy-right; Roudier et al. (2005) Plant Cell 17 page 1759, Figure 8, American Society of Plant Biologists).
**Figure 6-5** COB distribution in the division zone.

CW: cell wall.

(A) Longitudinal sections from the root cell division zone showing lateral root cap cell layer and epidermal cell layer.
(B) Longitudinal periclinal section between an epidermal cell and a root cap cell shows little COB in the epidermis of the cell division zone.
(C) Longitudinal sections from the lateral root cap cells.
(D) Outer periclinal section of root cap cell shows abundant COB in the outer periclinal wall.
(E) Longitudinal section showing low abundance of COB in transverse anticlinal wall between adjacent root cap cells.
Figure 6-6 COB distribution in the differentiation zone.

CW: cell wall.

(A) Schematic drawing of the root differentiation zone in longitudinal view showing the section planes from which the images in (B) and (C) were obtained.

(B) Outer epidermal periclinal section showing that little COB is present in the outer epidermal cell wall in the differentiation zone.

(C) Transverse section of the root hair shows abundant COB in the cell wall.

Scale bars = 500 nm.
Figure 6-7 COB localization on thick sections of wild-type root cells.

(A) Longitudinal 70 µm thick section of wild-type root stained with 1% toluidine blue. 
(B) COB localization on longitudinal section of wild-type root labelled with COB-specific antibody. COB antibody labeling is abundant in the outer longitudinal walls of the epidermis as well as in root cap cells.

Scale bar = 100 µm.
COB antibody labeling was abundant in the outer epidermal periclinal wall from the elongation zone, as observed in thick sections of the root as shown in Figure 6-7. COB was relatively abundant in the cytoplasm of outer periclinal sections compared to the cytoplasm in other sections. COB was also abundant in the cell wall of lateral root cap cells and in root hairs.

**Figure 6-8** Summary of COB distribution in the root.
6.3.2 Cortical microtubules and COBRA deposition in wild type roots, microtubule-drug treated roots and *mor1-1*

The abundance of COB in the cell wall, as shown by immunogold TEM, and the separation of COB bands and microtubules to distinct focal planes by double immunofluorescence labelling suggest that the transverse COB bands observed by immunofluorescence (Figure 6-3A) are confined to the cell wall. Thick transverse COB bands in elongating cells occasionally overlapped with underlying microtubules (Roudier et al., 2005; Figure 6-9A to D). Interestingly, not all elongating cells had transverse COB bands. The frequency of cells with COB bands increased in the early elongation zone and decreased in the late elongation zone (Figure 6-9F). There was no correlation between root hair forming cell files and cells that have COB bands.

To examine the role of microtubules in COB deposition, I performed immunofluorescence labelling of microtubules and COB to quantify changes in COB distribution patterns in seedlings treated with microtubule-targeted drugs and in the *mor1-1* mutant. A 1 day treatment with the microtubule-depolymerizing drug oryzalin at 1 µM dissipated microtubules and COB bands (Figure 6-10A to C), whereas the microtubule-stabilizing drug taxol at 10 µM for 1 day caused thick COB bands following the more variable orientations of microtubules (Figure 6-10D to F), supporting a close link between COB secretion and the presence of microtubules. Taxol treatment generated a high frequency of cells that have COB bands in the late elongation zone (Figure 6-10H).
Figure 6-9 Cortical microtubules and COBRA bands in wild-type root epidermal cell.

(A) Immunofluorescence labelling of microtubules. Microtubules were transversely aligned.
(B) Immunofluorescence labelling of COBRA. COBRA bands were thicker than microtubules and transversely aligned.
(C) Merged image of (A) and (B).
(D) Zoomed image of (C). Some microtubules and COBRA were co-aligned shown in triangles, others were not.
(E) Schematic drawing of root shows each region of the root.
(F) Frequency distribution histograms showing the proportion of epidermal cells that have COBRA bands. Five roots of wild-type seedlings were observed. Values are means ± SE.

Scale bars = 10 μm.
Images are pseudocoloured.
Figure 6-10 Cortical microtubules and COB distribution in 1 µM oryzalin-treated cells and in 10 µM taxol-treated cells.

(A) Microtubules in the root epidermis after 1 day grown on 1 µM oryzalin-containing media.
(B) No COB bands were observed in oryzalin-treated cells.
(C) Merged images of (A) and (B).
(D) Microtubules in the root epidermis after 1 day grown on 10 µM taxol-containing media.
(E) COB bands similar pattern as microtubules shown in (D).
(F) Merged images of (D) and (E). Triangles show the co-alignment of microtubules and COB.
(G) Schematic drawing of root shows each region of the root.
(H) Frequency of epidermal cells that have COB bands. Seedlings were transferred to media containing DMSO or 10 µM taxol and grown for 1 day. A total of five roots for DMSO treatment and seven roots for taxol treatment was observed. Values are means ± SE.

Scale bars= 10 µm. Images are pseudocoloured.
COB distribution patterns were documented in *mor1-1* roots over the course of microtubule disorganization for 2 h, 4 h, 18 h, and 24 h at restrictive temperature. Epidermal cells in wild type roots for 2 h at 31°C showed well-organized microtubules (Figure 6-11A) and transverse COB band patterns (Figure 6-11B), whereas cells in *mor1-1* roots showed short and disorganized microtubules (Figure 6-11C) and relatively faint and less tightly concentrated bands (Figure 6-11D). The frequency of epidermal cells with COB bands was reduced in *mor1-1* (Figure 6-11F) after 4 h at 31°C. Near the differentiation zone of *mor1-1* roots treated for 18 h at 31°C, where cell files were obviously twisted (Figure 6-12A), some epidermal cells had only very faint and diffuse COB bands (Figure 6-12B), while other cells in the elongation zone with short disorganized microtubules (Figure 6-12D) had almost no COB bands (Figure 6-12C and E). After 24 h at 31°C, almost no COB bands could be detected in *mor1-1* roots.

When *mor1-1* seedlings were transferred back to permissive temperature to allow microtubules to recover, COB bands were detected again. After 2 h at 21°C following a 1 day incubation at 31°C some root epidermal cells still had disorganized microtubules and no COB bands (Figure 6-13A to C), while others had organized microtubules and COB bands (Figure 6-13D to F).
**Figure 6-11** Cortical microtubules and COB distribution pattern in wild-type and *mor1-1* root epidermis 2 h after the temperature-shift to 31°C.

(A) Well-organized cortical microtubules in wild-type root epidermis.
(B) COB bands in wild-type root epidermis.
(C) Short and relatively disorganized microtubules in *mor1-1* root epidermis.
(D) Relatively faint COB bands in *mor1-1* root epidermis.
(E) Frequency distribution histograms showing proportion of epidermal cells that have COB bands. Five roots of wild type and *mor1-1* 4 h after temperature-shift to 31°C were observed. Values are means ± SE.

Scale bars = 20 µm.
**Figure 6-12** Cortical microtubules and COB distribution patterns in *mor1-1* root epidermis 18 h after temperature-shift to 31°C.

(A) Immunofluorescence labeling of COB in *mor1-1* root 18 h after the temperature-shift. White boxes show the area from which the images in (B) and (C) were obtained.

(B) High magnification image from the beginning of the root differentiation zone shows faint COB but thick band-like labellings.

(C) High magnification image from elongation zone shows no COB-specific bands in the cells.

(D) Short and disorganized microtubules in *mor1-1* root epidermis.

(E) No COB bands in the same area as (D).

Scale bars = 100 µm in (A), 20 µm in (D) and (E).
Figure 6-13 Recovery of microtubule organization and COB band pattern in *mor1-1* root epidermis after 2 h at 21°C after the temperature-shift to 31°C for 1 day.

There were variations in the cells. Some were not fully recovered from the temperature shift (A) to (C), and others appeared to be fully recovered (D) to (F).

(A) Microtubules were still relatively disorganized.
(B) No COBRA bands were observed.
(C) Merged images of (A) and (B).
(D) Microtubules were relatively organized.
(E) COBRA bands were observed.
(F) Merged images of (D) and (E).

Scale bars = 10 µm.
Images are pseudocoloured.
6.3.3 COBRA labelling in root hairs of wild type and cob-4 null mutant

Immunofluorescence labelling demonstrated that COB or some non-specific epitope is arranged in helical or punctate patterns in wild-type root hairs (Figure 6-14A to C). The preparation of roots for immunofluorescence labelling involves incubating the root with the wall degrading pectolyase enzyme mixture. The same helical COB patterns were observed in root hairs without pectolyase treatment (Figure 6-14D), indicating that pectolyase treatment did not affect labelling pattern of root hairs. To examine if this labelling pattern on root hairs is COB-specific, anti-COB labelling was also performed on the cob-4 null mutant. Anti-COB did not label expanding cells of the cob-4 root (Figure 6-15A), but did labelled root hairs in the same helical or punctate patterns seen in wild-type root hairs (Figure 6-15B). Importantly, COB expression is not detected in the cob-4 mutant by RT-PCR and western blotting (Roudier et al., 2005), suggesting that the anti-COB antibody is instead cross-reacting with distinct non-COB epitopes in the root hair cell wall. COB belongs to a multigene family (Roudier et al., 2002) and COBRA-like (COBL)1, 2 and 4 can not be ruled out as carrying epitope that the antibody may recognize, based on the amino acid sequence similarities (Table 6-3).
Figure 6-14 COB pattern in wild-type root hairs by immunofluorescence labeling.

(A) COB labeling only in root hairs, not in epidermal cells.
(B) Higher magnification image of (A). COB labelling formed was helical band pattern or punctate pattern.
(C) Young root hairs with thick band pattern of COB.
(D) COB helical bands or punctate patterns in root hairs were observed in the roots that were not treated with pectolyase.
Figure 6-15 Immunofluorescence labeling of COB in the cob-4 mutant.

(A) Immunofluorescence labelling of COB in cob-4 root shows swollen cells and no COB band pattern in the root epidermis.
(B) Helical pattern of labelling in cob-4 root hairs.
Table 6-3 Amino acid sequence similarities of COBRA epitope used for generating antibodies between COBRA and COBRA-like (COBL) family by using TAIR BLAST search.

Identities indicate the percentage of amino acids that are identical. Positives indicate the percentage of amino acids that are not identical but similar properties.

<table>
<thead>
<tr>
<th>Name</th>
<th>Locus</th>
<th>Identity (%)</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COBL1</td>
<td>At3g02210</td>
<td>67</td>
<td>80</td>
</tr>
<tr>
<td>COBL2</td>
<td>At3g29810</td>
<td>64</td>
<td>78</td>
</tr>
<tr>
<td>COBL4</td>
<td>At5g15630</td>
<td>57</td>
<td>70</td>
</tr>
</tbody>
</table>
6.3.4 Epitope-tagged COBRA in *cobra* mutants

To assess the specificity of the COB antibody and to re-examine COB distribution, I generated a 4 x myc epitope-tagged version of COB. I used 1.5 kb of the COB 5’ upstream sequence as a native promoter fused with COB cDNA, in which a 4 x myc-tag was inserted approximately 100 bp upstream of the 3’ end of the gene. I chose this location for the myc-tagging to avoid interfering with the ER transit signal peptide sequence at the 5’ end, as well as a critical cleavage site for the GPI-anchor attachment at the 3’ end (Figure 6-16). The construct was transformed into wild type, the *cob-1* conditional mutant and the *cob-4* null mutant.

Wild-type transgenic plants did not show any obvious phenotype (Figure 6-17A). A total of 11 individual T2 lines of *cob-1* transgenic plants complemented *cob-1* phenotype on high sucrose (4.5 %) containing medium, and they were selfed to obtain T3 lines. In the T3 generation, only 1 line complemented the *cob-1* root swelling phenotype (Figure 6-17B and C), but root growth in this line was slow. The transgene was not able to complement *cob-4* null phenotype (23 T2 lines were examined). Immunofluorescence labelling using an anti-myc antibody demonstrated that transgenic plants in *cob-1* (Figure 6-18D) had a lower fluorescence intensity than in the positive control (GN-myc line, Figure 6-18C), but had a higher fluorescence intensity than the negative control (wild type, Figure 6-18A; wild type without myc antibody incubation, Figure 6-18B), indicating that transgenic plants expressed the myc tag. Punctate labelling on the surface of the epidermal cells was observed in the non-transgenic wild-type root negative control (Figure 6-19A), indicating that the anti-myc antibody cross-reacted with other epitopes. The positive control, GN-myc did not have punctate labelling on the root surface (Figure 6-19B), but had diffuse labelling in the epidermal cell cytoplasm (data not shown). Strong cytosolic background staining with myc-antibody has been previously described (Geldner et al., 2003). In transgenic *cob-1* roots, no COB band pattern was detected in elongating epidermal cells even though the *cob-1* phenotype was restored (Figure 6-19C).
Figure 6-16 Epitope-tagging of COB with 4 x Myc tag.

COB contains putative N-terminal signal sequence (green), putative cellulose-binding site (light blue), cys-rich domain (yellow), N-glycosylation sites (pink), and predicted cleavage site for the GPI-anchor substitution (red). The myc-tag was inserted upstream of the C-terminal GPI-anchor cleavage site (blue). Several amino acids were added due to the insertion of the myc-tag in the middle of the gene (grey).
Figure 6-17 Eight day-old seedlings of wild type, COB-myc in wild type, cob-1 and COB-myc in cob-1 transgenic lines.

(A) wild type and wild type with COB-myc grown on Hoagland’s medium containing 3 % sucrose.
(B) cob-1 and COB-myc in cob-1 grown on Hoagland’s medium containing 3 % sucrose for 6 days and 4.5 % sucrose for 2 days.
(C) Higher magnification image of (B). The cob-1 seedlings have swollen root tips and some of seedlings of COB-myc in cob-1 rescued the cob-1 swollen root tip phenotype indicated by the arrowheads.
Figure 6-18 Immunofluorescence labelling of myc-tags on transgenic plant roots.

All images were taken at the same exposure time to compare the fluorescence intensity among roots.

(A) wild-type root.
(B) Immunolabelling without myc primary antibody treatment on wild-type root.
(C) GN-myc root.
(D) COB-myc in cob-1 root.
(E) COB-myc in wild-type root.

Scale bars = 100 μm.
Figure 6-19 Immunofluorescence labelling of myc-tags on the root surface of transgenic plants.

(A) wild-type root surface, showing non-specific punctate labeling of the myc antibody.
(B) GN-myc at root surface.
(C) Root surface of COB-myc transgenic plants in cob-1.

Scale bars = 20 µm.
6.4 DISCUSSION

6.4.1 COBRA subcellular localization and polar COBRA secretion in elongating root epidermal cells

Immunogold labelling of high pressure-frozen roots demonstrated that COB was associated with the Golgi apparatus and abundant in cell walls at various distances from the plasma membrane, indicating that after its secretion via Golgi-derived vesicles, that COB exists as a cleaved free protein. It has been shown that COB is cleaved by PI-PLC and released from the plasma membrane (Roudier et al., 2005). While the membrane localization of COBRA has been demonstrated by phase partitioning in calli extracts (Roudier et al., 2005), the abundance of COB within the cell wall demonstrated by immuno-gold labelling suggests that the GPI-anchored form is temporary, and that the PI-specific phospholipases may play an active role in releasing COB from the plasma membrane in elongating epidermal cells. It is also possible that the epitope may be masked when COB is anchored to the plasma membrane. Several studies have suggested that GPI-anchors may influence protein conformation by interacting with the plasma membrane (reviewed in Paulick and Bertozzi, 2008). Other studies using antibody specific for GPI-anchored protein showed that the binding affinity of the same antibody is greatly reduced after the lipid tail was removed by PLC or PLD (Barboni et al., 1995; Butikofer et al., 2001). Conversely, antibodies raised against a soluble form of GPI-anchored protein without a GPI-anchor reacted poorly with the GPI-anchored form of the protein (Butikofer et al., 2001). These results suggest that the GPI anchor may affect the conformation of proteins and that this may change the binding affinity of antibodies to the protein.

6.4.2 Role of cortical microtubules in COBRA deposition

Based on the fact that immunolabelled COB and microtubules were observed in distinct focal planes, transverse COB bands are likely to represent COB present in the cell wall. Dissipation of COB bands was observed after 24 h of oryzalin
treatment (Roudier et al., 2005; Figure 6-10). It has been suggested that long treatments with microtubule-drugs block mitosis or disturb the cell division plane, resulting in the loss of axialization (Wasteneys, 2004; Wasteneys and Collings, 2004). The dissipation of COB after oryzalin treatment for 24 h could therefore be a secondary effect of impaired axialization and may not be the direct effect of microtubule disruption. It has been shown that mor1-1 has cell division defects at restrictive temperature (Eleftheriou et al., 2005; Kawamura et al., 2006) but that mor1-1 for a short period of time at restrictive temperature may not be affected by cell division defects. Short and disrupted microtubules in mor1-1 for 2 h at restrictive temperature induced faint COB bands and locally diffuse COB distribution patterns, supporting a close link between COB secretion and the presence of organized microtubules.

The presence of organized microtubules may determine the secretion sites for COB or may restrict the site of COB secretion as physical obstacles when vesicles carrying COB fuse with the plasma membrane. I expected to see both microtubules and COB in the root sections from high-pressure frozen material, but microtubules were rarely detected. Different resin embedding and sectioning at different orientations were not successful for improving the frequency of observing microtubules. It may be worthwhile to obtain samples from other tissues such as growing hypocotyls and inflorescence stems to observe both microtubules and COB, since microtubules are rarely observed in Arabidopsis root sections prepared for TEM (R. Himmelspach, personal communication). Alternatively, adding tannic acid to the fixative may help preserve microtubules, as described in Ishida et al. (2007).

It has been also proposed that microtubules may be involved in the regulation of an endosomal population containing cell wall-related enzymes, according to the results that motility of a GFP-KORRIGAN1 containing compartment was inhibited after a 10 min treatment with oryzalin (Robert et al., 2005). The role of microtubules in the secretion pathway therefore remains to be determined.
6.4.3 Assessment of the specificity of the COBRA antibody

The fact that there was a helical pattern of immunofluorescence labelling on cob-4 root hairs but no labelling in epidermal cells indicates that the COB antibody cross-reacts with other epitopes present in root hairs. Immunogold labelling demonstrated that the gold labelling was abundant in walls of root hairs, indicating that the helical pattern of immunofluorescence labelling in root hairs represents epitopes that are present in the wall. This helical pattern of labelling is quite unique, and no report has been published related to this localization pattern in root hairs. Root hairs undergo tip growth, in which only the tip region grows rapidly, and microtubule orientations are found to be longitudinal to the growth axis (Van Bruaene et al., 2004). The epitope recognized by the COB antibody would be interesting to characterize. Possible candidates are COBL1, 2 and 4 based on the sequence similarity of the peptide used for generating the COB antibody, but recent gene expression analyses demonstrated that COBL1 is expressed in the columella and stele, COBL2 is expressed in the columella, and COBL4 is expressed in the xylem (Brady et al., 2007). COBL4 is therefore not likely a candidate for the epitope because it has been demonstrated that COBL4 is involved in secondary wall formation (Brown et al., 2005). Since COBL1, 2, and 4 are not expressed in the root hairs, the epitope recognized by the COB antibody may be some unrelated protein or even a polysaccharide epitope.

To re-examine the distribution pattern of COB, COB tagged with the 4 x myc epitope was generated. The transgene did not complement cob-4 null allele, but partially complemented the cob-1 conditional allele. The 3’ flanking region seems to be required to get high expression of COB, suggesting that it contains an enhancer or is required to stabilize the transcript (F. Roudier, personal communication). Thus, low levels of transgene expression may explain its inability to complement the cob-4 phenotype. Alternatively, the myc-tag may be interfering with the function of COB. Immunofluorescence labelling of myc demonstrated a higher intensity of labelling in transgenic plants compared to the negative control, but no band pattern in the epidermis. The lines that restore cob-1 root tip swelling phenotype also grew slowly, at rates similar to cob-1, suggesting that the transgene product was not fully
functional. Since the seedlings grew slowly, the strong swelling phenotype of *cob-1* was not observed in transgenic plants. The fact that fluorescence intensities among the lines that show and do not show the *cob-1* phenotype were similar indicates that COB-4 x myc is expressed in transgenic plants but is not rescuing the *cob-1* phenotype. To obtain a high level of transgene expression, the 3’ flanking region will need to be added to the construct (F. Roudier, personal communication), and then transformed to *cobra* mutants to test if the phenotype is restored.
CHAPTER 7

Conclusions and future directions
7.1 SUMMARY OF MAJOR FINDINGS OF THIS THESIS

In this thesis, I investigated the role cortical microtubules play in controlling the chemical and physical properties of cellulose microfibrils during cell growth. In particular, through the use of mutants and drugs that alter microtubule dynamics and activities, I examined the interplay between cortical microtubule organization and the mechanisms of both synthesis and crystallinity of cellulose microfibrils. I further explored the cellulose microfibril orientation and crystallinity using a cellulose synthase-defective mutant. Finally, I demonstrated that microtubules control the subcellular distribution of the GPI-anchored protein COBRA, which plays a major role in the mechanical properties of expanding cells. The major findings are summarized below.

1. The Arabidopsis inflorescence stem as an experimental system was established for studies on the microtubule basis for growth anisotropy (Chapter 2, 3).
2. Cells undergoing division show no preferred cellulose microfibril orientation (Chapter 2).
3. There is no predominant orientation of cellulose microfibrils at the cell plate (Chapter 2).
4. Cellulose microfibrils are transversely oriented at the radial and inner periclinal walls of epidermal cells, while microfibrils are locally parallel but variably oriented or well ordered at the outer periclinal wall. There are variations in microfibril orientations in the anticlinal walls, which are possibly developmental stage-dependent (Chapter 2, 3, 4).
5. Cellulose crystallinity is reduced during rapid growth at high temperature (Chapter 3).
6. Dynamic and well-organized microtubules are involved in the decline in cellulose crystallinity that normally accompanies increased cell expansion (Chapter 3).
7. Motility of YFP-CesA6 particles seen at the cell periphery does not depend on the presence of microtubules or actin filaments (Chapter 4).
8. The velocity of YFP-CesA6 particles at the cell surface is affected by temperature (Chapter 4).

9. The velocity of YFP-CesA6 particles at the cell surface is slightly but significantly increased in the mor1-1 mutant at restrictive temperature, but significantly reduced in oryzalin-treated cells (Chapter 4).

10. The any1 allele of CesA1 shows random cellulose microfibril orientations in some tissues, but normal cellulose crystallinity in the inflorescence stem (Chapter 5).

11. COBRA deposition is spatially and developmentally regulated, and microtubules are involved in determining this deposition pattern (Chapter 6).

7.2 CELLULOSE MICROFIBRILS AND MICROTUBULES DURING ANISOTROPIC GROWTH

7.2.1 Cellulose microfibril organization in inflorescence stems

Extensive FESEM analysis on inflorescence stems has provided a new finding that cells undergoing division lack the transverse orientation of cellulose microfibrils to the stem growth axis. This suggests several possibilities. Cellulose synthesis might be reduced during mitosis, or microfibrils could become disorganized passively during mitosis. Alternatively, microfibrils might be synthesized in random orientations since cortical microtubules are not present during mitosis. FESEM analysis also demonstrated that there is no predominant orientation of cellulose microfibrils in the forming cell plate or in mature anticlinal walls where microtubules are not present. Cellulose microfibrils in radial and inner periclinal walls of epidermal cells are consistently transverse to the cell growth axis, while microfibrils at the outer periclinal walls are locally parallel but variable or well-ordered depending on the cells (Chapter 2,3, and 4). Given that cellulose microfibrils are major reinforcing elements that determine growth anisotropy in the wall, the variations in the arrangement of cellulose microfibrils in each wall may be reflected in variation in the resistance of the wall to stress.
7.2.2 Cellulose crystallinity is adjusted as cells expand, and microtubules are required for controlling cellulose crystallinity

One of the major findings from this work is that cellulose crystallinity is adjusted as cells expand, and dynamic and organized microtubules are required for the decline in cellulose crystallinity during rapid cell expansion (Chapter 3). It has been shown by \textit{in vitro} adsorption experiments that cellulose crystallinity affects the binding capacity of xyloglucans, and specifically that the binding capacity of xyloglucans is high when cellulose has a low degree of crystallinity (Chambat et al., 2005). During rapid cell expansion, cellulose crystallinity may decline so that xyloglucan association with microfibrils may be increased. Conversely, microtubules may control xyloglucan secretion that in turn regulates the crystallinity of cellulose. The high degree of cellulose crystallinity in \textit{mor1-1} measured at high temperature may reduce xyloglucan association with microfibrils, and this is likely to be counterproductive for maintaining anisotropic wall properties. Other properties of cellulose microfibrils, such as their overall length, the length of individual cellulose chains, and diameter have to be analyzed in \textit{mor1-1} at the restrictive temperature to test if the microfibrils are short or mechanically weak as suggested by Wasteneys (2004). I attempted to measure these parameters but it was technically challenging. For future work, it would be interesting to examine the relationship between cellulose crystallinity and the activities of wall modifying enzymes such as XTH in wild type and \textit{mor1-1}, and to examine if the xyloglucan/cellulose ratio is altered in \textit{mor1-1}.

7.3 RELATIONSHIP BETWEEN MICROTUBULES AND CELLULOSE-SYNTHASE-COMPLEXES (CSCs)

7.3.1 High temperature, microtubule dynamics and organization affect the velocity of CSCs

In Chapter 4, I showed supporting evidence that YFP-CesA6 particles are likely functional subunits of actual CSCs producing cellulose microfibrils. High temperature increased the velocity of YFP-CesA6 particles. It would be interesting
to examine the effect of temperature on the velocity of YFP-CesA6 in greater detail and to correlate the velocity with the rate of cellulose synthesis by measuring $[^{14}\text{C}]$ glucose incorporation.

The intriguing result of Chapter 4 is that YFP-CesA6 particles move faster in \textit{mor1-1} at restrictive temperature compared to wild type, but velocity is reduced in oryzalin-treated cells. Nevertheless both treatments generate less dynamic and disorganized microtubules (Kawamura and Wasteneys, 2008; Nakamura et al., 2004). Together with the fact that CSCs move in the fluid membrane, future investigations should examine whether microtubule dynamics and organization affect the precursor supply to CSCs and the fluidity of the plasma membrane. Several studies have demonstrated that lipids are involved in cellulose synthesis. Peng et al. (2002) identified the lipid sitosterol-β-glucoside as the primer for initiation of cellulose biosynthesis. Cellulose synthesis activity has been shown in the detergent-resistant membranes (DRMs) from hybrid aspen cell suspension cultures (Colombani et al., 2004). DRMs are enriched in certain lipids such as cholesterol and sphingolipid, and in specific proteins including GPI-anchored proteins (Borner et al., 2003, 2005; Mongrand et al., 2004; Morel et al., 2006). Despite the lack of evidence for the presence of such lipid microdomains in vitro, the involvement of microtubules in cellulose synthesis and COBRA distributions may be closely related and mediated by the changes of composition and environment of the plasma membrane.

### 7.3.2 Disorganized microtubules cause altered trajectories of YFP-CesA6 particles.

In Chapter 4, I showed that in \textit{mor1-1}, the YFP-CesA6 trajectories became longitudinally curved and dispersed in some cells when microtubules were disorganized, while FESEM analysis showed little effect on cellulose microfibril orientation in comparison to wild type. To correlate the trajectories of fluorescently labelled CesA markers and microfibril orientations by FESEM, the following future studies should be considered: (1) Examine by TEM whether the YFP-CesA6
particles I have observed represent individual CSCs or clustered CSCs; (2) Use other CesA markers to determine how representative CesA6 is as a reporter of CSC movement; (3) Develop a protocol that retrieves the outer epidermal surface so that cellulose microfibril orientations can be reliably measured relative to cell growth axis.

7.4 WHAT IS THE ROLE OF ORGANIZED CORTICAL MICROTUBULES IN GROWTH ANISOTROPY?

In this thesis, I showed that dynamic and well-organized microtubules are required for controlling cellulose crystallinity (Chapter 3), that microtubules affect velocity, trajectories, and density of CSCs (Chapter 4), and that microtubules affect COB distribution patterns (Chapter 6). These results suggest that (1) microtubules influence cellulose crystallinity and CSC movement through their close association with the plasma membrane where cellulose microfibrils are produced, (2) microtubules determine either secretion sites of CSCs or the area where CSCs move, (3) the presence of organized microtubules may provide or restrict the secretion sites of COB, and (4) cortical microtubules may influence the degree of cellulose crystallinity, which occurs on the opposite side of the plasma membrane, by controlling secretion of wall proteins such as COB and/or polysaccharides.

7.5 THE any1 MUTANT

A relatively weak constitutive phenotype in the any1 mutant compared to other missense mutations suggests that the any1 mutation in CesA1 may not affect CesA1 enzyme activity critically (Chapter 5). I showed that in the any1 mutant, cellulose microfibril orientation is random in the epidermis but transverse in cortex and inner tissues of the inflorescence stem and in the root epidermis, suggesting that CesA1 may be partially redundant in some tissues and organs. Another finding is that the degree of cellulose crystallinity is normal in the any1 inflorescence stems. Future work will compare cellulose content in the aerial parts and roots, and examine the behaviour of fluorescently labelled CesAs in any1.
7.6 COBRA AND GROWTH ANISOTROPY

In Chapter 6, I showed that COB is abundant in the outer periclinal wall of the root epidermis and that the COB band pattern is dependent on the presence of microtubules. These results suggest that COB’s membrane-anchored GPI linkage may be temporary, and that there is a close link between COB secretion and the presence of organized microtubules.

Previous COBRA studies demonstrated that COB is required for cellulose synthesis (Schindelman et al., 2001; Roudier et al., 2005). COB belongs to a COBRA-like (COBL) multigene family consisting of 12 members in Arabidopsis encoding GPI-anchored proteins with putative cellulose-binding domains (Roudier et al., 2002). Gene expression analyses have demonstrated that COB gene family members are expressed in a tissue, cell-type, and developmental stage-specific manner, and at least one COBL gene is expressed within every tissue and cell type (Roudier et al., 2002; Brady et al., 2007). Recent studies have demonstrated that mutations in COBL4 (irregular xylem 6) (Brown et al., 2005), in the rice ortholog of COBL4 (Li et al., 2003) and in the maize ortholog of COBL4 (Ching et al., 2006; Sindhu et al., 2007) are correlated with significant reductions in the amount of cellulose in secondary walls. These results suggest that COBL proteins may have similar functions to COB, and general roles in cellulose synthesis in a cell-specific manner, not only during cell expansion but also in secondary walls.

COB may cross-link cellulose microfibrils and maintain structure and production of cellulose microfibrils by preventing the breakage of newly formed microfibrils or by regulating the activity of CSCs, leading to sufficiently long and strong cellulose microfibrils for cells to maintain growth anisotropy (Wasteneys, 2004). COB encodes a putative cellulose-binding domain, yet the homology to cellulose-binding domains is weak, and cellulose-binding assays did not show any binding (Roudier et al., 2002; Roudier et al., 2005).

It also remains possible that COB may play a structural role in the wall. In yeast, many GPI-anchored proteins are predominantly localized in the cell wall, and a few are mainly localized at the plasma membrane (reviewed in Orlean and Menon, 2007). It has been demonstrated that GPI-anchored cell wall proteins (GPI-CWPs)
are cross-linked to $\beta_{1,6}$-glucan, and then the glucan portion of GPI-CWP-$\beta_{1,6}$-glucan is cross-linked to $\beta_{1,3}$-glucan and chitin in yeast (reviewed in Orlean and Menon, 2007). These results suggest that GPI-anchored proteins in cell walls can be structural components of the cell wall. In plants, the outer epidermal wall is thought to be the major load-bearing structure of the organ during cell growth (Kutschera, 1989). Abundant COB distribution to the outer epidermal wall during rapid expansion makes it a candidate for having a more direct function in wall mechanical properties (Wasteneys and Fujita, 2006).
REFERENCES


Desprez, T., Juraniec, M., Crowell, E.F., Jouy, H., Pochylova, Z., Parcy, F.,


**Kazama, H., and Mineyuki, Y.** (1997). Alteration of division polarity and


KORRIGAN endo-1,4-beta-glucanase to cellulose synthesis and cytokinesis in Arabidopsis. Plant Physiology 126, 278-288.


Montague, M (1995) Gibberellic acid promotes growth and cell wall synthesis in Avena internodes regardless of the orientation of cell expansion. Physiologia
Plantarum 94, 7-18.


Orlean, P., and Menon, A.K. (2007). GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love


cambial cell cytokinesis in pine seedlings preserved by cryofixation and substitution. Protoplasma 220, 39-49.


expansion. Plant Physiology 130, 538-548.


**Arabidopsis thaliana.** Protoplasma **215**, 116-127.


APPENDICES

Appendix A: Cellulose crystallinity calculation.

Cellulose crystallinity was calculated by using the Vonk method (Vonk, 1973). First of all, the background diffraction signal was subtracted by drawing a tangential line to the data plots shown in the first graph. In the second graph, the amorphous curve (black) was drawn to fit the diffraction pattern (pink). After a linear regression analysis was carried out in the third graph, the crystallinity value was obtained.
Appendix B: Negative control and concentrations of COBRA antibodies for immuno-gold labelling.

CW: cell wall, V: vacuole

(A) and (B) Negative control. Immunogold labelling of COB without primary COB antibody incubation (A), and with pre-immune chicken serum (B).

(C) to (E) Immunogold labelling of COB with different concentrations of COB primary antibody. Incubation with COB antibody at 1:200 dilution (C), 1:100 dilution (D), and 1:50 dilution (E).

Scale bars = 500 nm.
Appendix C: List of publications and presentations.

Some of the material in this chapter has appeared in the following publications:


Some of the material from this chapter has been included in the following conference presentations.

