The Fragile Fiber1 Kinesin Contributes to Cortical Microtubule-Mediated Trafficking of Cell Wall Components1[OPEN]

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The cell wall consists of cellulose microfibrils embedded within a matrix of hemicellulose and pectin. Cellulose microfibrils are synthesized at the plasma membrane, whereas matrix polysaccharides are synthesized in the Golgi apparatus and secreted. The trafficking of vesicles containing cell wall components is thought to depend on actin-myosin. Here, we implicate microtubules in this process through studies of the kinesin-4 family member, Fragile Fiber1 (FRA1). In an fra1-5 knockout mutant, the expansion rate of the inflorescence stem is halved compared with the wild type along with the thickness of both primary and secondary cell walls. Nevertheless, cell walls in fra1-5 have an essentially unaltered composition and ultrastructure. A functional triple green fluorescent protein-tagged FRA1 fusion protein moves processively along cortical microtubules, and its abundance and motile density correlate with growth rate. Motility of FRA1 and cellulose synthase complexes is independent, indicating that FRA1 is not directly involved in cellulose biosynthesis; however, the secretion rate of fucose-alkyne-labeled pectin is greatly decreased in fra1-5, and the mutant has Golgi bodies with fewer cisternae and enlarged vesicles. Based on our results, we propose that FRA1 contributes to cell wall production by transporting Golgi-derived vesicles along cortical microtubules for secretion.

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microtubules, at least during interphase. Nevertheless, in xylem tracheary cells, cortical microtubule bands have been linked to not only cellulose guidance but also, the targeted exocytosis of hemicellulose and other matrix components (Fukuda, 1997). In seed coat cells, vesicles containingpectin associate with cortical microtubules that line the mucilage secretion pockets (McFarlane et al., 2008). In addition, in maize (Zea mays) roots, vesicles bind cortical microtubules densely (Tian et al., 2004). These and other observations indicate that cortical microtubules might serve as roadways for trafficking secretory vesicles.

If microtubules are tracks, then the engines are kinesins, because seed plants lack dyneins (Zhu and Dixit, 2012). Kinesins are molecular motors that move along microtubules and transport various cargo, including organelles, vesicles, and proteins. Kinesins have proliferated in plant lineages, and many are expressed during interphase, which is surprising given that long-distance organelle motility is thought to be actin based. Recently, a particular plant kinesin of the kinesin-4 family, called Fragile Fiber1 (FRA1), was shown to move rapidly and processively (i.e. taking multiple steps) toward microtubule plus ends in vitro (Zhu and Dixit, 2011). This makes FRA1 a candidate for sustained and active vesicle transport.

An Arabidopsis (Arabidopsis thaliana) partial loss-of-function mutant, fra1-1, was reported to have altered cellulose organization in fiber cells, despite having evidently undisturbed cortical microtubule organization (Zhong et al., 2002). Those results suggested a function for FRA1 in cell wall organization rather than secretion, and FRA1 has been proposed to link motile CESA complexes in the plasma membrane to cortical microtubules (Zhong et al., 2002; Lloyd and Chan, 2004; Zhu and Dixit, 2011). Strengthening this suggestion, a null mutant of the FRA1 ortholog in rice (Oryza sativa), brittle culm12 (bc12), also was reported to have disorganized sclerenchyma cell walls (Zhang et al., 2010). However, the motility of FRA1 in vivo and its relationship to CESA complexes remain unknown.

We have reexamined FRA1 function in cell wall formation. Because the originally characterized allele, fra1-1, is predicted to give rise to a nearly full-length protein, we characterized a transfer DNA (T-DNA)-induced knockout mutant, fra1-5. Using this allele as well as imaging a functional FRA1-3GFP fusion protein, we show here that FRA1 is involved in membrane trafficking that contributes to delivery of cell wall polysaccharides, such as pectin. We propose that FRA1 drives the movement of vesicles containing cell wall cargo along cortical microtubules to facilitate their secretion.

RESULTS
The fra1-5 Knockout Mutant Has Decreased Elongation

The previously studied ethyl methanesulfonate (EMS)-induced fra1-1 allele is predicted to cause a 28-amino acid deletion (Zhong et al., 2002) and did not ablate FRA1 expression (Fig. 1A). To obtain a null allele, we identified a T-DNA insertion line (SALK_084463), in which the T-DNA resides in the second exon of the FRA1 gene. This mutant lacked an FRA1 transcript detectable by reverse transcription (RT)-PCR (Fig. 1A). In addition, the mutant did not express detectable FRA1 protein on an immunoblot probed with a polyclonal antiserum against FRA1 (Fig. 1B), confirming that it is a knockout. Because Zhong et al. (2002) mentioned three other EMS alleles, we designated the T-DNA mutant fra1-5.

The fra1-5 inflorescence stems elongated significantly more slowly than those of the wild type and ceased growth at about the same time, giving rise to a large difference in final stem length (Fig. 1C; Supplemental Fig. S1A). In addition, fra1-5 inflorescence stems were wider than wild-type controls (Supplemental Fig. S1B), indicating a decreased degree of expansion anisotropy. The alteration of organ length and width was recapitulated closely in the alterations of the lengths and widths of pith cells (Supplemental Fig. S1, C–E), indicating that the morphological phenotypes observed can be plausibly accounted for by defective expansion.

Rosette leaves and siliques of the fra1-5 mutant were also substantially shorter than those of the wild type (Supplemental Fig. S1, F and G). Interestingly, fra1-5 seedlings had only slightly shorter hypocotyls in dark-grown seedlings and slightly shorter roots in both light- and dark-grown seedlings (Supplemental Fig. S1, H and I). The reason for the relative insensitivity of the seedling organs to the loss of FRA1 function might be because of genetic redundancy with a pair of FRA1 homologs present in the genome.

The fra1-5 Mutant Has Mechanically Weaker Stems and Irregular Xylem

The inflorescence stems of fra1-5 were fragile during handling, which has been reported for fra1-1 (Zhong et al., 2002). To quantify mechanical properties of the stem, we performed three-point bending assays, because these tests are prone to fewer artifacts than breakage tests. During these experiments, fra1-5 stems always broke, whereas wild-type stems did not (Supplemental Fig. S2A). The fra1-5 stems had a reduced bending strength and bending modulus, with the latter reduced by more than a factor of two (Supplemental Fig. S2B). Mechanical defects also occurred in fra1-5 xylem cells, which had an abnormal, collapsed appearance (Supplemental Fig. S2C), similar to the appearance of the irregular xylem mutants that synthesize less secondary cell wall cellulose than the wild type (Turner and Somerville, 1997).

Cell Walls of fra1-5 Are Thin, with Minor Changes in Chemical Composition

Reduced elongation and altered mechanical properties are typically associated with defective cell wall assembly. To determine whether the fra1-5 mutant had aberrant cell walls, we examined its cell wall thickness and composition.
In transmission electron micrographs of sections from 4-week-old basal inflorescence stems, interfascicular fiber cell walls were consistently about 50% thinner in fra1-5 compared with the wild type (Fig. 1, D and E). A similar decrease in wall thickness also occurred in protoxylem cells (Fig. 1, F and G) and pith cells (Fig. 1, H and I). Evidently, FRA1 functions in the deposition of both primary and secondary cell walls.

In the basal stems of 6-week-old plants, fra1-5 had one-third fewer fiber cell tiers than wild-type stems (Supplemental Fig. S3). Reduced cell wall thickness combined with fewer tiers of lignified cells likely explain the mechanical weakness of the mutant stems.

Turning to cell wall composition, we first studied the distribution of cellulose in the cell walls of interfascicular fiber cells by staining sections with gold particles coated with cellobiohydrolase 2, a probe that binds crystalline cellulose (Berg et al., 1988). In fiber cells, the mean density of the probe was not particularly different between the genotypes; however, the staining was relatively uniform in wild-type cell walls but appeared uneven or striated in mutant walls (Fig. 1, J and K).

Using 4-week-old inflorescence stems, we next analyzed cell wall composition. At 4 weeks, the apical region is elongating in both genotypes, whereas the basal region has stopped elongating (Supplemental Fig. S1A), allowing cell wall composition to be compared between growing and nongrowing tissues as well as between genotypes. Mutant walls had similar amounts of cellulose in both apical and basal stems and a modest reduction in lignin in basal stems (Table I). As for the neutral sugars present in the noncellulosic polysaccharides, mutant walls had significantly more Ara in both apical and basal stems and more Xyl in the apical but not basal stem (Table II). Although these differences could mean that specific polysaccharides were altered in the mutant, the essentially constant composition per total cell wall mass combined with the substantial decrease in cell wall thickness lead us to hypothesize that FRA1 acts to mediate overall cell wall polymer secretion.
Both Cortical Microtubule and Cell Wall Organizations Are Essentially Unaltered in fra1-5

The original characterization of FRA1 reported that microtubule organization in fra1-1 plants was similar to that of wild-type plants (Zhong et al., 2002). We confirmed this for fra1-5 in both roots and hypocotyls (Supplemental Fig. S4, A and B) and in addition, for hypocotyl cells, found that the wild type and fra1-5 have indistinguishable rates of both growth and shortening at cortical microtubule plus ends (Supplemental Fig. S4C). Furthermore, roots of the mutant responded to oryzalin no differently than the wild type did (Supplemental Fig. S4D). Thus, neither microtubule organization nor dynamics seem to require FRA1 function.

To determine whether cellulose organization is disrupted in fra1-5, we used scanning electron microscopy to image the innermost cell wall layer. Because fra1-5 stems grow with decreased anisotropy, we first examined pit cells in the apical (growing) region of the inflorescence stem. Note that, although the epidermis is argued to play a starring role in controlling the rate of stem elongation, other cells were disorganized, resembling nongrowing parenchyma (Fig. 2, G and H). The variability precluded quantitative analysis, but cell wall organization could not be distinguished between the two genotypes qualitatively. The presence of numerous fra1-5 fiber cells with highly organized walls contradicts the previous claim that FRA1 is essential for fiber cell wall organization (Zhong et al., 2002). Taken together, the scanning electron microscopy analyses indicate that cell wall organization is unlikely to account for the mechanical weakness of fra1-5 stems.

FRA1 Moves Processively along Cortical Microtubules and Its Motility Correlates with Expansion

To understand the function of FRA1, we studied its localization and dynamics by expressing full-length FRA1 fused to a triple GFP tag under the control of the native FRA1 promoter. The FRA1-3GFP construct essentially complemented fra1-5 transcriptionally and morphologically (Fig. 3, A–C), indicating that the resulting fusion protein is functional. To determine whether FRA1 is motile in vivo, we imaged living cells with variable-angle

### Table I. Cellulose and lignin content of wild-type and fra1-5 plants

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>Cellulose</th>
<th>Lignin ABSL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical stem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>182.6 ± 9.5</td>
<td>--b</td>
</tr>
<tr>
<td>fra1-5</td>
<td>164.9 ± 1.2</td>
<td>--b</td>
</tr>
<tr>
<td>Basal stem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>421.7 ± 14.4</td>
<td>10.9 ± 1.0</td>
</tr>
<tr>
<td>fra1-5</td>
<td>402.1 ± 2.7</td>
<td>9.1 ± 0.2*</td>
</tr>
</tbody>
</table>

*Ten percent acetyl bromide soluble lignin (ABSL) is equal to 100 μg mg⁻¹AIR. bLignin in apical stems is close to the detection limit; therefore, it is not reported here.

### Table II. Neutral monosaccharide composition of wild-type and fra1-5 plants

<table>
<thead>
<tr>
<th>Tissue Source</th>
<th>Neutral Monosaccharide Composition AIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rha</td>
<td>Fuc</td>
</tr>
<tr>
<td>Apical</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>14.3 ± 0.7</td>
</tr>
<tr>
<td>fra1-5</td>
<td>14.5 ± 0.8</td>
</tr>
<tr>
<td>Basal</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>fra1-5</td>
<td>9.4 ± 0.4</td>
</tr>
</tbody>
</table>
Figure 2. Cell wall organization. A and B, Representative images of the newly deposited cell wall layer in pith cells from the apical region of 4-week-old wild-type (A) and fra1-5 (B) inflorescence stems. Bar = 500 nm. C, Eccentricity of the Fourier transform as a function of spatial frequency. A value of 0 indicates complete radial symmetry, and a value of 1 indicates perfect alignment. Symbols plot means ± SEM of four stems per genotype, with about 40 images per stem (approximately 150 images total per genotype). C, Inset shows the difference for the curves in C over a selected range of frequency. D, Distribution of the net orientation for the images used in C. Orientation per image was averaged for features between 30 and 60 nm. Homogeneity
epifluorescence microscopy (Konopka and Bednarek, 2008). In cotyledon and hypocotyl epidermis, FRA1-3GFP localized to puncta at the cortex (Figs. 3E and 4A). The cortical FRA1-3GFP particles were dynamic, undergoing diffusive and directional movement (Fig. 3F; Supplemental Movies S1–S4). Directional movement of FRA1-3GFP occurred at an average velocity of 0.24 μm s⁻¹ and a characteristic run length of 2.4 μm (Fig. 3, H and I). Single-molecule photobleaching analysis showed that the linearly moving FRA1-3GFP particles consist primarily of one or two FRA1 dimers (Fig. 3J), consistent with kinesin-driven motility in other systems (Hendricks et al., 2010).

To characterize FRA1-3GFP motility with respect to cortical microtubules, we crossed a red fluorescent protein-β-tubulin6 (RFP-TUB6) marker (Ambrose et al., 2011) into these plants, allowing for dual-channel imaging. FRA1-3GFP moved processively and unidirectionally along cortical microtubules (Fig. 3G; Supplemental Movie S2). FRA1 motility depended on microtubules but not actin filaments based on treatments with the microtubule-depolymerizing drug oryzalin or the actin depolymerizing drug latrunculin-B (Fig. 3, K and L). FRA1 seemed to prefer moving on stable or bundled microtubules insofar as treatment with taxol, which stabilizes and bundles microtubules, increased the number of motile FRA1-3GFP particles (Fig. 3, K and L). Imaging in guard cells, in which the cortical array has a defined polarity (Marc et al., 1989), showed that FRA1-3GFP moved toward microtubule plus ends (Supplemental Movie S3), consistent with previous in vitro experiments (Zhu and Dixit, 2011).

In view of the high demand for cell wall precursors during expansion, we compared FRA1 activity in growing and nongrowing tissues. In hypocotyls of 4-d-old seedlings, cortical FRA1 puncta were abundant in the growing apical region, and those undergoing directed, persistent motility were relatively easy to find. In contrast, FRA1 puncta were scarce and rarely motile in the nongrowing basal region (Fig. 4; Supplemental Movies S4 and S5), although cortical microtubules are present in this region (Supplemental Fig. S5A). Likewise, FRA1 was less abundant and moved less often in nongrowing 10-d-old cotyledon pavement cells compared with growing cells in 4-d-old cotyledons (Supplemental Fig. S5B). These results are consistent with a role for FRA1 in sustaining high rates of cell wall secretion.

**FRA1 Has Partially Distinct Functions in Arabidopsis and Rice**

The putative rice FRA1 ortholog (Brittle Culm12 [BC12]; also known as Gibberellin-Deficient Dwarf1 [GDD1]) surprisingly acts as a transcription factor, spending time in the nucleus and regulating expression of the gibberellin synthesis gene Ent-Kaurene Oxidase (KO2; Li et al., 2011). To determine whether phenotypes in fra1-5 are caused by anomalous transcription, we used quantitative real-time PCR to assay the expression of representative genes. In fra1-5, the KO2 ortholog (Yamaguchi, 2008) was transcribed at essentially wild-type levels (Supplemental Fig. S6A), and in contrast to the rice gdd1 mutant, the dwarf phenotype of fra1-5 was unaffected by applying gibberellin (Supplemental Fig. S6B). In addition, a suite of genes involved in the synthesis and modification of cellulose, hemicellulose, pectin, and lignin (Hall and Ellis, 2013) was expressed marginally lower in fra1-5 compared with the wild type (Supplemental Fig. S6C), but none of the changes were of the magnitude expected from the loss of a transcription factor. Finally, FRA1-3GFP resides in the cytoplasm (Fig. 3D), unlike the rice ortholog, which localizes to both cytoplasm and nucleus (Zhang et al., 2010). This is consistent with a canonical nuclear localization signal being present in BC12/GDD1 but absent in FRA1 (Zhang et al., 2010). Together, these data indicate that FRA1 has diverged between the crucifer and the grass family.

**FRA1 Does Not Contribute to CESA Motility within the Plasma Membrane or Trafficking of Complexes to the Membrane**

The velocity of FRA1 is about 50 times faster than that of the plasma membrane-embedded CESA complexes (Paredez et al., 2006), a difference that makes it unlikely that FRA1 directly guides the movement of CESA complexes along microtubules. Nevertheless, to investigate the relationship between FRA1 and CESA motility, we used two cellulose synthesis inhibitors: isoxaben and 2,6-dichlorobenzonitrile (DCB). Isoxaben depletes CESA complexes from the plasma membrane (Paredez et al., 2006), whereas DCB immobilizes CESA complexes at the membrane (DeBolt et al., 2007). As imaged in hypocotyls, neither drug affected FRA1 motility (Fig. 5A), indicating that its motility is insensitive to CESA status. Reciprocally, the sensitivity of fra1-5 seedlings to isoxaben or DCB was unaltered (Supplemental Fig. S5C), and the velocity distribution of yellow fluorescent protein (YFP)-CESA6 complexes in the plasma membrane of fra1-5 seedlings largely matched that of the wild type (Fig. 5B).

The delivery of CESA complexes from the Golgi apparatus to the plasma membrane is thought to occur through specialized vesicular compartments called either small CESA compartments (Gutierrez et al., 2009) or microtubule-associated CESA compartments (Crowell et al., 2009). To determine whether FRA1 transports these compartments, we imaged hypocotyls in plants coexpressing FRA1-tandem-dimeric
Figure 3. Motility of FRA1-3GFP in vivo. A, Overall appearance of 4-week-old plants grown under continuous light. Bar = 10 cm. B, Transcript level in rosette leaves measured by qRT-PCR and normalized to the FRA1 expression level in the wild type. Values are means ± SEM from three biological replicates. C, Stem height of 4-week-old plants. Values are means ± SD (n = 20 plants). D, Representative image of FRA1-3GFP (green) in a vascular cell of the root of a 4-d-old seedling. Fei Mao4-64 (FM4-64) (red) was used to label the plasma membrane. Bar = 2 μm. E, Image of FRA1-3GFP in a cotyledon pavement cell of a 4-d-old seedling (same in E–J). Bar = 10 μm. F, Representative kymograph showing the movement of FRA1-3GFP. Diagonal lines represent motile events. Vertical bar = 20 s; horizontal bar = 1 μm. G, Dual-channel image of FRA1-3GFP (green) and RFP-TUB6 (red). The arrowheads label FRA1-3GFP puncta along a cortical microtubule. Bar = 10 μm. H, Distribution of the velocity of processive FRA1-3GFP puncta. The average velocity is 0.24 ± 0.09 μm s⁻¹ (means ± SD, n = 233). I, Distribution of the run length of FRA1-3GFP puncta. The characteristic run length (L₁₂) is 2.4 μm (n = 228). J, Distribution of the number of GFP fluorophores in motile FRA1-3GFP puncta (n = 45). The puncta that contain 6 and 12 GFP fluorophores indicate one and two FRA1-3GFP dimers, respectively. The FRA1-3GFP puncta that contain nine GFP fluorophores probably represent two
FRA1 supported secretion of cell wall components.

DISCUSSION

FRA1 Functions in Both Primary and Secondary Cell Wall Production

Our characterization of the fra1-5 knockout mutant revealed defects in the cell walls of growing pith, protoxylem, and mature interfascicular fibers, indicating that FRA1 is important for the production of both primary and secondary cell walls. The most conspicuous cell wall defect was the loss of mass; fra1-5 cell walls were 40% to 50% the thickness of wild-type counterparts. This finding is consistent with the expression of FRA1 throughout the plant (Zhang et al., 2002; Zhou et al., 2007). In the report on the EMS-induced fra1-1 allele, cell wall thickness in fiber cells was not quantified but seemed to be unaffected in the micrograph shown (Zhang et al., 2002). Whether this reflects the true status of fra1-1 cell walls requires additional study.

Cellulose microfibrils were reported to be disorganized in fra1-1 fiber cells (Zhong et al., 2002) and rice bc12 sclerenchyma (Zhang et al., 2010). We saw disorganized cell wall textures in fra1-5 fiber cells but also observed them in the wild-type cells. We examined fiber cells in about 20 plants per genotype prepared in a dozen independent experiments and consistently saw cells with highly organized and poorly organized textures in both genotypes. Neither of the previous studies
indicated how many cells and plants were imaged, and they might, therefore, have sampled insufficiently. Nevertheless, for the wild type, the presence of disorganized wall textures among fiber cells is disquieting. Perhaps, the disorganized texture reflects cell wall delamination during sectioning, thus exposing old, intrinsically disordered regions of the wall rather than the fresh innermost layer. Regardless, many fra1-5 fiber cells had highly organized cell wall textures, and we found only minor cell wall disorganization for growing fluorescence stem pith. Because cell wall patterning is not consistently altered in fra1-5, we conclude that cell wall organization is not a primary function of the FRA1 kinesin.

### Expansion Is Impaired in fra1-5

The major morphological phenotype of fra1-5 is reduced axial expansion. All organs of the mature fra1-5 plant were shorter than the wild type, and our analysis of the inflorescence showed that this was caused by lower expansion rate rather than altered timing of growth.

The process of expansion depends on the extensibility of the cell wall. Reduced delivery of agents that weaken the cell wall, such as expansins, might account for the decreased expansion seen in fra1-5. Although it has been less explored, the provision of new cell wall polymers themselves also alters cell wall extensibility (Hoson and Masuda, 1991; Boyer, 2009). This is because a polymer within the cell wall that is bearing load might have only a limited capacity for additional deformation, whereas at the moment of its incorporation, a polymer would have considerable slack. Therefore, taking the reduced expansion and cell wall thickness together, we hypothesize that FRA1 acts to ensure efficient secretion of vesicles, which is needed to support both rapid expansion in the primary cell...
wall and the massive secondary thickening in fiber and xylem cells.

FRA1 Is a Motile Kinesin in Living Plant Cells

To the best of our knowledge, this work is the first example of single-molecule imaging of kinesins in living plants. Similar to other kinesins (Cai et al., 2007; Verhey et al., 2011), the majority of FRA1-3GFP molecules shows diffusive motility in the cytoplasm. These molecules are probably in an autoinhibited state to prevent futile ATP consumption (Ganguly and Dixit, 2013). A subpopulation of the FRA1-3GFP molecules shows directional movement along cortical microtubules. The mechanism that activates FRA1 motility is unknown but could involve binding of the C-terminal tail domain of FRA1 to cargo or phosphorylation of FRA1 (Ganguly and Dixit, 2013). The motile FRA1-3GFP molecules move in a plus end-directed manner, with an average velocity of $0.24 \mu m \cdot s^{-1}$ and a characteristic run length of $2.4 \mu m$, consistent with our in vitro motility data (Zhu and Dixit, 2011). The small differences between in vivo and in vitro FRA1 velocity and run length might be caused by different ionic conditions and regulatory mechanisms present in cells. The velocity and run length of FRA1 are comparable with other motile kinesins in eukaryotic systems (Cai et al., 2007; Verhey et al., 2011). Importantly, the ability to move long distances makes FRA1 suited for efficient transport of cargo along cortical microtubule tracks.

FRA1 motility in hypocotyl and cotyledon epidermal cells was positively correlated with growth status. FRA1 abundance and motile density were high in expanding cells and about 6-fold lower in cells that had stopped expanding. Interestingly, CESA complexes show a similar pattern of expression and activity in the hypocotyl (Crowell et al., 2009). Together, these correlations support the hypothesis that FRA1 activity is deployed specifically during stages of active wall deposition.

FRA1 Does Not Contribute to the Movement of CESA Complexes

The plasma membrane-embedded CESA complexes track cortical microtubules (Paredez et al., 2006), and these elements have been proposed to be linked by FRA1 (Zhong et al., 2002; Lloyd and Chan, 2004; Zhu and Dixit, 2011). However, our data indicate that this is unlikely. FRA1 moved about 50 times faster than plasma membrane-embedded CESA complexes, and the motility of FRA1 and CESA complexes was independent of one another. Furthermore, mutation of another protein, cellulose synthase interacting1, that is widely accepted as somehow linking CESA complexes to cortical microtubules gives rise to plants with decreases in cellulose content and increases in radial expansion that are larger than those caused by mutations in FRA1 (Bringmann et al., 2012; Li et al., 2012). Vesicular compartments carrying CESA complexes have also been observed to interact with cortical microtubules (Crowell et al., 2009; Gutierrez et al., 2009), and kinesins, such as FRA1, have been hypothesized to mediate this interaction (Crowell et al., 2010). However, we found that FRA1 does not colocalize with motile, vesicular CESA compartments, indicating that FRA1 is unlikely to mediate their association with cortical microtubules.
FRA1 Contributes to the Export of Matrix Polysaccharide

The hypothesis that the FRA1 kinesin is required to maintain high rates of secretion receives support from our observations of pectin delivery to cell walls. In fra1-5, alkynylation of Fuc was incorporated into the cell walls of rapidly elongating root epidermal cells to a lower extent and in an uneven pattern. Both characteristics resemble the lower incorporation seen for the mature zone of wild-type roots, which presumably delivers matrix polysaccharides to the epidermal apoplast at a reduced rate in the absence of either expansion or secondary cell wall thickening (Anderson et al., 2012). Fuc-alkyne predominantly labels rhamnogalacturonan-I, meaning that our data are consistent with FRA1 being important for the delivery of that pectic component to the cell wall. However, given our chemical composition analysis and analysis from another recent study (Kong et al., 2015) showing that major polysaccharides of the cell wall are present at similar proportions in both genotypes, we think that FRA1 does not handle pectin specifically but that it functions in maintaining high rates of secretion for multiple matrix polysaccharides.

We observed abnormal Golgi morphology and accumulation of enlarged vesicles in the fra1-5 mutant, which are characteristic symptoms of defective post-Golgi trafficking and vesicle secretion (Synek et al., 2006; Feraru et al., 2012; Boutilé et al., 2013; Li et al., 2013). Whether the accumulated vesicles in fra1-5 are destined to the plasma membrane and represent FRA1 cargo remains to be determined.

Microtubules and Oriented Cell Wall Assembly

The cell wall phenotype of fra1-5 along with the progressive movement of FRA1 and its abundance in regions of rapid growth implicate this kinesin in the process of cell wall secretion. This is surprising. Except for specialized cell types, microtubules have long been considered inconsequential for secretory vesicle delivery (Hepler and Palevitz, 1974; Steinborn et al., 2002). Certainly, phenotypes associated with the removal of microtubules do not involve notable reduction of either expansion or the amount of secreted cell wall (Shibaoka, 1972; Shibaoka and Hogetsu, 1977).

Nevertheless, when no longer governed by microtubules, cell wall assembly becomes far less patterned, and this might simplify requirements for cell wall delivery and secretion. Cortical microtubules are dense, cross linked to the plasma membrane, and bound by many enzymes; consequently, the removal of microtubules is apt to alter conditions in the cell’s cortex perversely, including changing limits on when and where vesicles may fuse with the membrane. We hypothesize that FRA1-driven transport of secretory vesicles is needed to support rapid rates of secretion where cell walls are undergoing highly patterned assembly, such as in the anisotropically expanding inflorescence pith or secondarily thickening fibers. The motor-driven transport might efficiently target vesicles to exocytotic sites positioned along cortical microtubules or otherwise, allow microtubules to guide patterned assembly around oriented cellulose microfibrils.

MATERIALS AND METHODS

Plant Material and Growth

The Arabidopsis (Arabidopsis thaliana) Columbia-0 accession was used throughout. The fra1-5 mutant was isolated from a T-DNA insertion line (SALK_084463) obtained from Arabidopsis Biological Resource Center (http://abrc.osu.edu/). Homozygous mutants were identified by using primers listed in Supplemental Table S1. Seeds of fra1-1 were obtained from Zheng-Hua Ye.

For growth on plates, seeds were sterilized with 5% (v/v) bleach for 10 min, rinsed four times with water, and planted on 1 × Murashige and Skoog medium (Caisson Laboratories). Seeds were stratified at 4°C for 2 d and then grown at 20°C under 16 h of light.

For growth in soil, seeds were grown under continuous light, 70% humidity, and 21°C after stratification at 4°C for 2 d. Continuous light was chosen, because it produced more severe developmental defects in adult fra1-5 plants than growth under the 16-h photoperiod.

RT-PCR and Quantitative Reverse Transcription-PCR

Total RNA was extracted from basal internodes or rosette leaves of 4-week-old plants, DNAse treated, and used for complementary DNA (cDNA) synthesis using qScript cDNA Supermix (Quanta BioSciences). Primers for RT-PCR and quantitative reverse transcription (qRT)-PCR are listed in Supplemental Table S1. The qRT-PCR was conducted using the SYBR Green method. Three biological replicates, each with three technical replicates, were used to estimate fold change in gene expression relative to the wild type. Actin2 was used as an internal control.

Histology

To image pith cells, 5-mm segments from apical and middle regions of 4-week-old inflorescence stems were cut longitudinally by a Vibratome into 100-μm sections. Middle plane sections were stained with 5 μg mL⁻¹ propidium iodide for 5 min and imaged using confocal microscopy. Cell lengths and widths were measured using ImageJ (http://imagej.nih.gov/ij/). To observe the lignification pattern, transverse sections of stems were stained with 1% (w/v) phloroglucinol in 6 N HCl for 5 min and imaged under a dissecting light microscope.

Generation of the FRA1-3GFP and FRA1-tdTomato Constructs and Transgenic Plants

The pFRA1::FRA1-3GFP construct was generated using a 1.3-kb sequence upstream of the FRA1 start codon and the full-length FRA1 cDNA followed by three tandem copies of enhanced green fluorescent protein (eGFP). The pFRA1::FRA1-tdTomato construct was generated using a 3-kb sequence upstream of the FRA1 start codon and the full-length FRA1 cDNA followed by tdTomato cDNA. Primers for making these constructs are listed in Supplemental Table S1. These constructs were ligated into the pCAMBIA 1300 vector and introduced into the fra1-5 mutant through Agrobacterium spp.-mediated floral dip transformation. Transgenic plants were selected using 20 μg mL⁻¹ hygromycin, and homozygous lines expressing a single copy of the transgene were used for phenotypic analysis and imaging. To determine the relationship between FRA1 motility and microtubules, the pFRA1::FRA1-3GFP line was crossed to plants expressing ubiquitin promoter-driven KFP-TUB6 (Ambrose et al., 2011), and progeny expressing both markers were selected for imaging.

Live Cell Imaging and Image Analysis

FRA1-3GFP was imaged using variable-angle epifluorescence microscopy at 22°C to 23°C. Seedlings were gently mounted in water between two layers of double-sided adhesive tape. For drug treatments, seedlings were incubated with 0.1 μM isoxaben for 2 h, 5 μM DCB for 3 h, 20 μM taxol (Cytokeleton, Inc.) for 1.5 h, 20 μM oryzalin for 3 h, or 2 μM latrunculin B (Enzo Life Science) for
2 h before mounting and imaging. For controls, seedlings were treated with either water or 0.1% (v/v) dimethyl sulfoxide as appropriate. Epidermal cells in the apical or subapical region of the hypocotyl were imaged unless otherwise indicated. GFP and RFP were excited using 2-mW, 488-nm and 2-mW, 561-nm diode-pumped solid-state lasers (Melles Griot), and images were collected using a 100× (numerical aperture 1.45) objective and a back-illuminated electron-multiplying CCD camera (ImageEM; Hamamatsu) at 1-s intervals in the GFP channel and 4-s intervals in the RFP channel for 3 min. Velocity and run length for individual motile events were measured using kymograph analysis in SlideBook 5.0 (Intelligent Imaging Innovations). Motile density was calculated as the number of motile FRA1-3GFP particles per micrometer ² per second -¹. Single-molecule photonbleaching analysis was conducted as described earlier (Ross and Dixit, 2010). Briefly, we treated seedlings with 1 ms sodium azide for 2 h to deplete cellular ATP, which caused FRA1-3GFP particles to become immobilized on cortical microtubules. The fluorescence intensity of individual FRA1-3GFP particles was then recorded over time, and clearly detectable single bleach steps were used to estimate the fluorescence intensity of a single GFP molecule. This information was used to calculate the number of GFP molecules in motile FRA1-3GFP particles as (initial fluorescence intensity of a motile particle — background fluorescence intensity) fluorescence intensity of a single GFP molecule -¹. Data were collected from at least three cells from three independent seedlings.

Plants expressing YFP-CESA6 in the proc131 background (Paredes et al., 2006) were crossed to fra1-5, and F3 progeny homozygous for YFP-CESA6 and fra1-5 were used for imaging. The motility of YFP-CESA6 at the cell cortex was imaged as above for FRA1-3GFP. Time-lapse images were captured at 10-s intervals using a 2-mW, 488-nm laser for 10 min. For plasma membrane YFP-CESA6 particle density measurements, single images of hypocotyl epidermal cells were captured and then cropped to select regions of the plasma membrane of cells of interest. CESA particles within the region of interest were identified as puncta with a diameter of 0.5 μm using the Imaris (Bitplane) software’s Spots tool, setting the minimum quality threshold to that automatically assigned by running the algorithm on the corresponding uncropped image. A maximum quality threshold was used to exclude spurious puncta arising from edge effects. Particle density was calculated by dividing the total remaining puncta by the plasma membrane surface area.

To determine the relationship between FRA1 and vesicular CESA compartments, a pFRA1-FRA1-tdTomato construct was transformed into plants homozygous for YFP-CESA6 and fra1-5. T2 progeny expressing both markers were selected and imaged at 1-s intervals using 1-mW, 488-nm and 2-mW, 561-nm lasers for 2 min. To quantify microtubule organization and dynamics in fra1-5, plants expressing ubiquitin promoter-driven RFP-TUB6 (Ambrose et al., 2011) were crossed to fra1-5, and F3 progeny were used for imaging. Time-lapse images were captured at 3-s intervals using a 1-mW, 561-nm laser for 5 min. The growth and shortening rates of cortical microtubule plus ends were measured using kymograph analysis in ImageJ. We noticed that the RFP-TUB6 signal is lower in fra1-5, which made it difficult to reliably score catastrophe and rescue events; therefore, these data were not quantified.

Transmission Electron Microscopy

To image cell walls, freshly excised 2-mm basal stem segments were fixed for 90 min in 2% (w/v) glutaraldehyde buffered with 0.1 M pipes buffer, pH 6.8. The tissue was then postfixed for 90 min in buffered 2% (w/v) osmium tetroxide, dehydrated, and embedded in Spurr’s resin. Thin sections were stained with uranyl and lead salts and imaged in an LEO 912 AB Energy-Tetroxide, dehydrated, and embedded in Spurr’s resin. Thin sections were stained and imaged as described above. The diameter of vesicles within approximately 1 μm of Golgi stacks was measured in ImageJ. The number of cisternae per Golgi was counted manually.

Scanning Electron Microscopy

Freshly excised segments (approximately 5 mm long) from 4-week-old plants were sectioned in phosphate-buffered saline at a nominal thickness of 100 μm on a Vibrotome. The sections were rinsed with 1% (v/v) Triton X-100 and dehydrated using a graded ethanol series. Dehydrated samples were critically point dried, mounted on stubs, coated with platinum (approximately 2 nm), and imaged in a scanning electron microscope (FEI Magellan) equipped with a field emission gun. Samples were imaged at 1 kV and 25 pA. For analysis of organization in the apical material, four sections from four independent stems per genotype were imaged (approximately 150 images per genotype). For each section, approximately 10 cells were imaged along a transverse transect. For quantification, the fit-ellipse routine was used as described by Marga et al. (2005) and detailed in Supplemental Methods S1.

Metabolic Labeling of Pectin Using Fuc-Alklyne

Fuc-alkyne-based labeling of pectin was conducted essentially as described previously (Anderson et al., 2012). The root elongation zones of labeled seedlings were imaged with a Zeiss Cell Observer SD Spinning Disc Confocal Microscope (488-nm laser excitation and 525/25 emission filter) using a 63×, 1.40 numerical aperture oil immersion objective. Z stacks of epidermal cells were collected, and cell wall-associated fluorescence intensity in maximum projections was measured using ImageJ by calculating mean pixel intensity values for individual cells.

Immunoblotting

A polyclonal antiserum was generated against a 24-amino acid peptide (P931–P954) from the FRA1 tail domain and purified using affinity chromatography against the same peptide (Epitomics). For FRA1 immunoblotting, 4-week-old light-grown shoots were first ground in liquid N2 and then homogenized in protein isolation buffer (50 mM Tris-acetate, pH 7.5, and 2 mM EDTA); a protease inhibitor tablet from Roche and total protein (approximately 100 μg each) were separated by SDS-PAGE and transferred to a 0.45-μm polyvinylidene difluoride membrane (Thermo Scientific). Proteins were probed with FRA1 primary antibody (1:2,000) and anti-rabbit IgG horseradish peroxidase secondary antibody (1:5,000; Jackson Immuno Research). Detection was conducted using SuperSignal West Dura chemiluminescence substrate (Thermo Scientific).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number At5g47820 for FRA1.

Supplemental Data

The following supplemental materials are available.

- Supplemental Figure S1. Reduced anisotropic growth of fra1-5.
- Supplemental Figure S2. Cell wall mechanical properties.
- Supplemental Figure S3. Stem lignification.
- Supplemental Figure S4. Cortical microtubule organization in fra1-5.
- Supplemental Figure S5. Motility of FRA1-3GFP in pavement cells.
- Supplemental Figure S6. Transcriptional and GA response.
- Supplemental Figure S7. Phenotype of fra1-5 plants expressing FRA1-tdTomato.
- Supplemental Table S1. Primers used in this work.
- Supplemental Methods S1. Supplemental methods.
- Supplemental Movie S1. Motility of FRA1-3GFP in a pavement cell.
- Supplemental Movie S2. FRA1-3GFP moves along cortical microtubules.
- Supplemental Movie S3. FRA1-3GFP moves toward microtubule plus-ends.
- Supplemental Movie S4. Motility of FRA1-3GFP in the apical region of a hypocotyl.
- Supplemental Movie S5. Motility of FRA1-3GFP in the basal region of a hypocotyl.
- Supplemental Movie S6. Two-color imaging of FRA1-tdTomato and CESAs-YFP.
ACKNOWLEDGMENTS

We thank Geoffrey Wasteneys (University of British Columbia) for the gift of the GFP-TUB6 tubulin line and David Ehhardt (Carnegie Institution, Stanford, CA) for the gift of the YFP-CEAS6 line.

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LITERATURE CITED


Zhu et al.

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792


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**Supplemental Figure 1.** Reduced anisotropic growth of *fra1-5*.

(A) Growth curves for the inflorescence stem. Values are mean ± SD (n = 20 plants).

(B) Inflorescence stem diameter. Values are mean ± SD (n = 12 stems).

(C) Vibratome sections stained with propidium iodide showing the pith in the middle region of the inflorescence stem. Scale bar = 100 µm.

(D) Pith cell length. Values are mean ± SD (n = 70 cells from 7 stems).

(E) Pith cell width (data for the middle stem). Values are mean ± SD (n = 40 cells from 4 stems).

(F) Rosette leaves of 4-week old plants (stems were removed for clarity).

(G) Siliques of 4-week old plants. Bars plot mean ± SEM from 3 biological replicates (n > 30).

(H) Growth curves of roots and hypocotyls of light-grown wild type (filled symbols) and *fra1-5* (open symbols) seedlings. Values are mean ± SD (n = 70).

(I) Growth curves of roots and hypocotyls of dark-grown wild type (filled symbols) and *fra1-5* (open symbols) seedlings. Values are mean ± SD (n = 70).

Asterisks indicate significant differences between the genotypes as determined by Student’s *t* test, *p* < 0.05.
Supplemental Figure 2. Cell wall mechanical properties.

(A) Representative force-displacement curves from three-point bending tests on the basal inflorescence stem. Wild-type stems bend but do not break under force, while fra1-5 stems always break beyond a force of about 2N (arrow). The fra1-5 stem withstands higher force initially presumably because it is wider than wild-type stem.

(B) Mechanical properties of the basal region of the inflorescence stem. Values are mean ± SD (n = 12 plants). Asterisks indicate significant difference between genotypes determined by Student’s t test, p value < 0.001.

(C) Representative images of vascular bundles from 7-week old stems grown under a 16 h photoperiod stained with toluidine blue. Arrows point to xylem cells. Scale bar = 50 µm.
Supplemental Figure 3. Stem lignification.

(A) Representative images of phloroglucinol-stained transverse sections of 6-week old basal stems. Scale bar = 100 μm.

(B) Number of lignified cell tiers in images as shown in (A). Values are mean ± SEM from three biological replicates, each with 4 stems. Asterisk indicates significant difference between the genotypes as determined by Student’s t test, p < 0.05.
Supplemental Figure 4. Cortical microtubule organization in *fra1-5*.

(A) Immunofluorescence images of cortical microtubules in root epidermal cells in the elongation zone of 4-day old seedlings. Scale bar = 10 µm.

(B) Representative images of *pUBQ*: RFP-TUB6 in hypocotyl cells of 4-day old seedlings. The RFP-TUB6 signal tends to be lower in the *fra1-5* mutant for unknown reasons. Scale bar = 20 µm.

(C) The growth and shortening rate for cortical microtubule plus ends in wild-type and *fra1-5* hypocotyl cells. Values are mean ± SD (n > 100 from 9 cells of 3 seedlings for growth rate and n > 52 from 9 cells of 3 seedlings for shortening rate).

(D) Root length of wild type and *fra1-5* plants after 3-d growth in various concentrations of oryzalin. Values are mean ± SD (n = 20 plants per genotype for each treatment).
Supplemental Figure 5. Motility of FRA1-3GFP in pavement cells.

(A) Representative images of pUBQ:: RFP-TUB6 in apical and basal hypocotyl cells of 4-day old seedlings. Scale bar = 10 µm

(B) Single frame and time projection of 200 images of FRA1-3GFP from pavement cells of 4-d old and 10-d old seedlings. Linear tracks in the time projection images indicate motility of FRA1-3GFP. Scale bar = 20 µm.

(C) Root length after 3-d growth on the indicated concentration of isoxaben or DCB. Values are mean ± SD (n = 20 plants per genotype for each treatment; WT = wild type).
Supplemental Figure 6. Transcriptional and gibberellin response.

(A) Relative expression level of AtKO2 (At5G25900) in wild type (WT), fra1-5 and FRA1-3GFP plants. Values are mean ± SEM from three biological replicates.

(B) Growth curves of wild type (WT) and fra1-5 stems treated with either a mock solution or gibberellin (GA3) solution. Values are mean ± SEM from three biological replicates.

(C) Relative expression level of selected cell wall synthesizing or modifying genes in wild-type and fra1-5 plants. CESA3 and CESA4 are involved in cellulose synthesis in primary and secondary walls, respectively. PAL4 encodes a rate-limiting enzyme for lignin synthesis. XXT2 and XTH19 encode enzymes that are involved in synthesis and modification of xyloglucan, respectively. UXS3 and PME encode for enzymes that are involved in xylan synthesis and pectin modification, respectively. Values are mean ± SEM from three biological replicates.
Supplemental Figure 7. Phenotype of fra1-5 plants expressing FRA1-tdTomato.

Overall appearance of 5-week old plants grown under continuous light. Scale bar = 10 cm.
### SUPPLEMENTAL TABLE 1

**Primers used in this work**

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<sup>a</sup> Primers used for RT-PCR.
<sup>b</sup> Primers used for screening fra1-5 homozygous lines.
<sup>c</sup> Primers used for qRT-PCR.
<sup>d</sup> Primers used for FRA1-3GFP cloning.
<sup>e</sup> Primers used for FRA1-tdTomato cloning.
SUPPLEMENTAL METHODS

Drug treatments

For drug treatments, seeds were first grown under standard conditions that are described in the manuscript for 5 d and then transferred to plates containing oryzalin (Supelco Analytical), 2,6-dichlorobenzonitrile (DCB Sigma-Aldrich), or isoxaben (Sigma-Aldrich). Stock solutions of 10 mM were prepared in anhydrous DMSO and stored at -20°C. Root lengths were measured 3 d after growth on plates containing either drug or DMSO as control.

Three-point bending test

The mechanical strength of stems was measured using three-point bending as described previously (Paul-Victor and Rowe, 2011) using a universal testing machine (Model 5583, Instron). Briefly, the basal-most internode of 4-week-old stems was placed on two supports positioned 12.5 mm apart. A round bar, 1 mm in diameter, was lowered until it contacted the stem and force was applied to the middle of the specimen by advancing the bar at a speed of 10 mm/min. The testing machine software recorded applied force and stem displacement over time. The initial linear part of the force-displacement curves was used to calculate the bending rigidity. All stems were tested within 2 min of being cut from the plant. As this method is sensitive to the age and growth condition of the plants (Paul-Victor and Rowe, 2011), we used fra1-5 and wild type plants that were grown at the same time.

Stem bending rigidity \((EI; \text{N mm}^2)\) was calculated as \(EI = (L^3) (c/48)\), where \(L (\text{mm})\) is the distance between the two supports and \(c\) is the slope of the force-displacement curve \((\text{N mm}^{-1})\). In this equation, \(I (\text{mm}^4)\) is the second moment of area of the stem and was calculated as \(I = \pi r^4 / 4\), where \(r (\text{mm})\) is the average radius of the stem. Young's modulus or bending modulus...
\( (E; \text{N/mm}^2) \) was derived from the above equation by \( E = EI/I \). Bending strength \((\sigma)\) is calculated as \( \sigma = (L) \left( \frac{F_{\text{max}}}{\pi r^3} \right) \), where \( F_{\text{max}} \) is the maximal force withstood by a stem.

**Xylem morphology**

Freshly excised 2 mm basal stem segments were fixed for 90 min in 2% (w/v) glutaraldehyde buffered with 0.1 M pipes buffer, pH 6.8. The tissue was then post-fixed for 90 min in buffered 2% (w/v) osmium tetroxide, dehydrated and embedded in Spurr’s resin. Thin sections were stained with 0.01% aqueous toluidine blue, rinsed with water three times and imaged using a wide field light microscope.

**Detailed procedure for enzyme-gold affinity cytochemistry**

Thin sections of fixed and stained basal stem segments were mounted on nickel grids, blocked 30 min at room temperature with 0.5% cold water fish gelatin (G7765, Sigma-Aldrich) buffered with 50 mM citrate, pH 5, and labeled with probe suspended in blocking buffer for 1 h at room temperature. Grids were rinsed three times in buffer, then in water and imaged in a LEO 912 AB energy filtered transmission electron microscope (TEM) operated at 120 kV.

**Detailed procedure for quantification of cell wall organization from SEM images**

For the “fit-ellipse” routine (Marga et al., 2005), an 850 pixel x 850 pixel (3.1 µm x 3.1 µm) window was placed in the center of each image and the fast Fourier transform obtained. This was converted to a binary image choosing a low threshold to include all information in the transform, an ellipse was fitted to the transform and ellipse parameters recorded. The threshold was incremented by two gray levels, a new binary image obtained, the ellipse fitted and
parameters recorded, and this was iterated until the area within the transform was less than 200 pixels. Here we plotted the eccentricity of ellipse as a function of the length of the major axis, which was converted to a spatial frequency (i.e., “spacing”) within the image. To characterize orientation, we averaged the orientation of the ellipse between spacings of 30 to 60 nm where the texture was most uniformly aligned. Homogeneity of the distributions was tested by Levene’s test (Levene, 1960) which is generally considered robust to departures from normality of the tested distributions. Note that similar distributions of orientation were produced by two other entirely independent image analysis methods (data not shown).

**Cell wall biochemical analysis**

Cell wall composition was analyzed separately for basal and apical stem samples. For basal specimens, we used the basal-most internode of 4-week-old plants. For apical samples, we used 3 cm segments from the top of the inflorescence stem. As the stems in the fra1-5 mutant elongate more slowly, apical tissue was harvested from wild type and fra1-5 stems that were the same height (7 cm). Samples were dried and analyzed for crystalline cellulose, neutral sugars, and lignin were described previously (Foster et al., 2010a, b).

**Detailed procedure for immunofluorescence staining of cortical microtubules**

Four-day old light-grown seedlings were fixed in 1.5% (v/v) formaldehyde (Sigma-Aldrich) and 0.5% (v/v) glutaraldehyde (Alfa Aesar) made up in PEMT buffer (50 mM pipes, 2 mM EGTA, 2 mM MgSO4, 0.05% [v/v] Triton X-100, pH 7.2) for 40 min in 6 well-plate. After rinsing in PEMT buffer three times for 10 min each, the specimens were digested with 0.1% pectinase (Macerozyme, Sigma-Aldrich) in PEM buffer (50 mM pipes, 2 mM EGTA, 2 mM
MgSO₄, pH 6.8) supplemented with 0.4 M mannitol for 30 min and rinsed in PEM buffer three times. Samples were then dehydrated with methanol for 20 min at -20°C and rehydrated in PBS at room temperature for 10 min. Autofluorescence caused by free aldehydes from glutaraldehyde fixation was reduced with 1 mg/mL NaBH₄ in PBS for 20 min, followed by the treatment with 50 mM glycine in PBS for 30 min. Seedlings were subsequently incubated with anti-β-tubulin primary antibody (Clone Tub2.1, 1 : 1,000 dilution, Sigma-Aldrich) and anti-Mouse-FITC secondary (1 : 400 dilution, Sigma-Aldrich) and mounted in antifade reagent (Invitrogen) before imaging the root epidermal cells in the elongating region using confocal microscopy.
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL MOVIE LEGENDS

**Supplemental Movie 1.** Motility of FRA1-3GFP in a pavement cell.
Time-lapse imaging of FRA1-3GFP at the cell cortex using VAEM. Images were captured at 1 s intervals for 150 seconds. The yellow arrow heads track representative motile FRA1-3GFP puncta.

**Supplemental Movie 2.** FRA1-3GFP moves along cortical microtubules.
Time-lapse imaging of FRA1-3GFP and RFP-TUB6 at the cell cortex using VAEM. Images were captured at 1 s intervals in the GFP channel and 4 s intervals in the RFP channel. The yellow arrow heads track representative FRA1-3GFP puncta (green) moving along cortical microtubules (red).

**Supplemental Movie 3.** FRA1-3GFP moves towards microtubule plus-ends.
Time-lapse imaging of FRA1-3GFP in guard cells. The majority of FRA1-3GFP puncta move away from the stomatal pore. This pattern of movement is consistent with microtubule plus-end-directed motility because cortical microtubules in guard cells form a radial array with their plus-ends oriented away from the stomatal pore.

**Supplemental Movie 4.** Motility of FRA1-3GFP in the apical region of a hypocotyl.
Time-lapse imaging of FRA1-3GFP in apical hypocotyl epidermal cells using VAEM. The abundance and motility of FRA1-3GFP is high in these cells.

**Supplemental Movie 5.** Motility of FRA1-3GFP in the basal region of a hypocotyl.
Time-lapse imaging of FRA1-3GFP in basal hypocotyl epidermal cells using VAEM. The abundance and motility of FRA1-3GFP is low in these cells.

**Supplemental Movie 6.** Two-color imaging of FRA1-tdTomato and CESA6-YFP.

Time-lapse imaging of FRA1-tdTomato (red) and CESA6-YFP compartments (green) in hypocotyl epidermal cells using VAEM. The blue arrow heads track representative motile FRA1-tdTomato puncta.