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Running head: FRA1 mediates trafficking of cell wall components

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The FRA1 kinesin contributes to cortical microtubule-mediated trafficking of cell wall components

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Running head: FRA1 mediates trafficking of cell wall components

One sentence summary: The FRA1 kinesin moves rapidly and processively along cortical microtubules and contributes to the production of both primary and secondary walls by mediating membrane trafficking.

Footnote:

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ABSTRACT

The cell wall consists of cellulose microfibrils embedded within a matrix of hemicellulose and pectin. Cellulose microfibrils are synthesized at the plasma membrane while 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, FRA1. In a *fra1-5* knockout mutant, the expansion rate of the inflorescence stem is halved compared to wild type, as is the thickness of both primary and secondary cell walls. Nevertheless, cell walls in *fra1-5* have an essentially unaltered composition and ultrastructure. A functional FRA1-3GFP fusion protein moves processively along cortical microtubules and its abundance and motile density correlate with growth rate. Motility of FRA1 and CESA complexes are 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.

INTRODUCTION

The cell wall plays a vital role in the life of a plant. In growing cells, the tough but extensible primary wall determines the rate and direction of expansion and overall plant form. In differentiated cells, such as interfascicular fibers and xylem cells, the thick secondary cell wall provides strength to withstand gravity and large negative pressures. Besides mechanics, cell walls feature in essential processes such as pathogen resistance, signal transduction, and cell-to-cell communication. In addition, cell wall biomass has potential as a feedstock for biofuel production. Therefore, understanding cell wall biogenesis is important fundamentally and practically.

Cell walls consist primarily of cellulose, hemicellulose, and pectin, along with small amounts of protein. Typical of eukaryotic secretory products, hemicellulose and pectin are synthesized in the Golgi and then delivered to the extracellular space through the secretory system. Atypically, cellulose microfibrils are synthesized *de novo* at the plasma membrane by cellulose synthase (CESA) complexes, although the CESA complexes themselves are thought to be assembled in the Golgi and trafficked to the plasma membrane (McFarlane et al., 2014). How the various cell wall components are delivered to the plasma membrane and extracellular space to form a functional cell wall remains poorly understood.

Among cell wall components, cellulose has perhaps the best understood delivery process, which is influenced by cortical microtubules (Baskin, 2001; Lloyd, 2011). The cortical microtubule array organizes cellulose deposition spatially by targeting the secretion of CESA complexes (Crowell et al., 2009; Gutierrez et al., 2009) and orienting their movement (Gardiner et al., 2003; Paredez et al., 2006). What controls the delivery of other wall components is less clear. Sustained transport of organelles in plants is actin-based (Sparkes, 2011) and vesicle trafficking is generally assumed to be independent of microtubules, at least during interphase. Nevertheless, in xylem tracheary cells, cortical microtubule bands have been linked not only to cellulose guidance but also to the targeted exocytosis of hemicellulose and other matrix components (Fukuda, 1997). In seed coat cells, vesicles containing pectin associate with cortical microtubules that line the mucilage secretion pockets (McFarlane et al., 2008). In addition, in maize 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, since 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 FRA1, was shown to move rapidly and processively (*i.e.*, taking multiple steps) towards microtubule plus-ends *in vitro* (Zhu and Dixit, 2011). This makes FRA1 a candidate for sustained and active vesicle transport.

An *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, *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 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 (Figure 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 a *FRA1* transcript detectable by RT-PCR (Figure 1A). In addition, the mutant did not express detectable *FRA1* protein on an immunoblot probed with a polyclonal anti-serum against *FRA1* (Figure 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 (Figure 1C and Supplemental Figure 1A). In addition, *fra1-5* inflorescence stems were wider than wild type controls (Supplemental Figure 1B), 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 Figures 1C-1E), 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 Figures 1F and 1G). 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 Figures 1H and 1I). The reason for the relative insensitivity of the seedling organs to the loss of *FRA1* function might be due to 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, as 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 never did (Supplemental Figure 2A). The *fra1-5* stems had a reduced bending strength and bending modulus, with the latter reduced by more than a factor of two (Supplemental Figure 2B). Mechanical defects also

occurred in *fra1-5* xylem cells, which had an abnormal, collapsed appearance (Supplemental Figure 2C), similar to the appearance of the irregular xylem (*irx*) mutants that synthesize less secondary cell wall cellulose than 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 to wild type (Figures 1D and 1E). A similar decrease in wall thickness also occurred in protoxylem cells (Figures 1F and 1G) and pith cells (Figures 1H and 1I). 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 Figures 3A and 3B). Reduced cell wall thickness combined with the fewer tiers of lignified cells likely explains 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 (Figures 1J and 1K).

Using 4-week-old inflorescence stems, we next analyzed cell wall composition. At 4 weeks, the apical region is elongating in both genotypes while the basal region has stopped elongating (Supplemental Figure 1A), allowing cell wall composition to be compared between growing and non-growing 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 1). As for the neutral sugars present in the non-cellulosic polysaccharides, mutant walls had significantly more arabinose in both apical and basal stems, and more xylose in the apical but not basal stem (Table 2). 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, leads us to hypothesize that FRA1 acts to mediate overall cell wall polymer secretion.

Both cortical microtubule and cell wall organization 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 Figures 4A and 4B) and in addition, for hypocotyl cells, found that wild-type and *fra1-5* have indistinguishable rates of both growth and shortening at cortical microtubule plus-ends (Supplemental Figure 4C). Furthermore, roots of the mutant responded to oryzalin no differently than did the wild type (Supplemental Figure 4D). Thus neither microtubule organization nor dynamics appear to require FRA1 function.

To determine whether cellulose organization is disrupted in *fra1-5*, we used scanning electron microscopy (SEM) to image the innermost cell wall layer. Because *fra1-5* stems grow with decreased anisotropy, we first examined pith cells in the apical (growing) region of the inflorescence stem. Note that while the epidermis is argued to play a starring role in controlling the rate of stem elongation, pith cells command the spotlight for anisotropy (Baskin and Jensen, 2013). The innermost cell wall layer appeared highly organized in both genotypes (Figures 2A and 2B). To determine whether the genotypes differ quantitatively in wall organization, we analyzed orientation as a function of feature size in the images by using a Fourier transform-based method (Marga et al., 2005). Although the wild type had slightly more uniformly oriented cell walls than the *fra1-5* mutant for features around 20 nm, the difference was invisible by eye. Considering the 50% difference in elongation rate, cell wall organization in the two genotypes was strikingly similar (Figure 2C). Additionally, the net orientation of the cell wall fibrils, recovered in the same analysis, for each genotype averaged to 90° (*i.e.*, transverse to the long axis of the stem), although the distribution of angles was modestly wider for *fra1-5* (Figure 2D). The difference in distribution was small but statistically significant; it indicates a difficulty among cells in *fra1-5* stems to achieve a strictly transverse orientation of microfibrils. Similar patterns occur in roots growing with reduced anisotropy and have been hypothesized to weaken transverse mechanical reinforcement and hence increase radial growth (Baskin, 2005).

We next imaged interfascicular fiber cells in basal regions of the stem to study secondary cell walls. Cell wall organization among these fiber cells was surprisingly variable: some were

highly organized (Figures 2E and 2F), but others were disorganized, resembling non-growing parenchyma (Figures 2G and 2H). 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 SEM 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 (Figures 3A - 3C), indicating that the resulting fusion protein is functional. To determine whether FRA1 is motile *in vivo*, we imaged living cells with variable-angle epifluorescence microscopy (Konopka and Bednarek, 2008). In cotyledon and hypocotyl epidermis, FRA1-3GFP localized to puncta at the cortex (Figures 3E and 4A). The cortical FRA1-3GFP particles were dynamic, undergoing diffusive and directional movement (Figure 3F, Supplemental Movies 1 and 4). Directional movement of FRA1-3GFP occurred at an average velocity of 0.24 $\mu\text{m/s}$ and with a characteristic run length of 2.4 μm (Figure 3H-3I). Single molecule photobleaching analysis showed that the linearly moving FRA1-3GFP particles consist primarily of one or two FRA1 dimers (Figure 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 an *RFP-TUB6* marker (Ambrose et al., 2011) into these plants, allowing for dual-channel imaging. FRA1-3GFP moved processively and unidirectionally along cortical microtubules (Figure 3G and Supplemental Movie 2). FRA1 motility depended on microtubules but not on actin filaments, based on treatments with the microtubule-depolymerizing drug, oryzalin, or the actin depolymerizing drug, latrunculin-B (Figures 3K – 3L). FRA1 appeared 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 (Figures 3K – 3L). Imaging

in guard cells, in which the cortical array has a defined polarity (Marc et al., 1989), showed that FRA1-3GFP moved towards microtubule plus-ends (Supplemental Movie 3), 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 non-growing tissues. In hypocotyls of 4-day-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 non-growing basal region (Figures 4A and 4B, Supplemental Movies 4 and 5), even though cortical microtubules are present in this region (Supplemental Figure 5A). Likewise, FRA1 was less abundant and moved less often in non-growing 10-day-old cotyledon pavement cells compared to growing cells in 4-day-old cotyledons (Supplemental Figure 5B). These results are consistent with a role for FRA1 in sustaining high rates of cell wall secretion.

FRA1 has partially distinct functions in *A. thaliana* and rice

The putative rice FRA1 ortholog (BC12, also known as GDD1) surprisingly acts as a transcription factor, spending time in the nucleus and regulating expression of the gibberellin synthesis gene *KO2* (Li et al., 2011). To determine whether phenotypes in *fra1-5* are due to 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 Figure 6A) and, in contrast to the rice *gdd1* mutant, the dwarf phenotype of *fra1-5* was unaffected by applying gibberellin (Supplemental Figure 6B). In addition, a suite of genes involved in the synthesis and modification of cellulose, hemicellulose, pectin, and lignin (Hall and Ellis, 2013) were expressed marginally lower in *fra1-5* compared to wild type (Supplemental Figure 6C) but none of the changes were of the magnitude expected from the loss of a transcription factor. Finally, FRA1-3GFP resides in the cytoplasm (Figure 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.

FRA1 contributes neither to CESA motility within the plasma membrane nor trafficking of complexes to the membrane

The velocity of FRA1 is about 60-times faster than that of the plasma-membrane-embedded CESA complexes (Paredes et al., 2006), a difference making 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 (Paredes et al., 2006), whereas DCB immobilizes CESA complexes at the membrane (DeBolt et al., 2007). As imaged in hypocotyls, neither drug affected FRA1 motility (Figure 5A), indicating that its motility is insensitive to CESA status. Reciprocally, the sensitivity of *fra1-5* seedlings to isoxaben or DCB was unaltered (Supplemental Figure 5C) and the velocity distribution of YFP-CESA6 complexes in the plasma membrane of *fra1-5* seedlings largely matched that of the wild type (Figure 5B).

The delivery of CESA complexes from the Golgi apparatus to the plasma membrane is thought to occur via specialized vesicular compartments called either small CESA compartments (SmaCCs) (Gutierrez et al., 2009) or microtubule-associated CESA compartments (MASCs) (Crowell et al., 2009). To determine whether FRA1 transports these compartments, we imaged hypocotyls in plants co-expressing FRA1-tdTomato and YFP-CESA6 in the *fra1-5* background. The FRA1-tdTomato construct rescued the *fra1-5* phenotype, indicating that it is functional (Supplemental Figure 7). Among 56 motile FRA1-tdTomato particles, none were labeled with YFP (Figure 5C and Supplemental Movie 6). Likewise, among 30 YFP-CESA6-labeled vesicles that showed directional motility, none were labeled with tdTomato (Figure 5C and Supplemental Movie 6). In addition, the YFP-CESA6 particle density in the plasma membrane of wild type ($0.42 \pm 0.05 \mu\text{m}^{-2}$) and *fra1-5* ($0.39 \pm 0.06 \mu\text{m}^{-2}$) hypocotyl cells is statistically indistinguishable (Figure 5D). Together, these findings indicate that FRA1 does not mediate the transport and insertion of CESA complexes.

FRA1 supports secretion of cell wall components

To test whether the *fra1-5* mutation results in defects in the secretion of matrix polysaccharides, we took advantage of a new method for assaying the secretion of the pectic polymer rhamnogalacturonan-I, based on the uptake and incorporation into pectin of a fucose

analog, fucose-alkyne (Anderson et al., 2012). In this method, wall-localized fucose-alkyne is labeled by a specific reaction with the membrane-impermeable compound, Alexa 488-azide. After a 2-hour incubation with the alkyne, wild-type cell walls within the growth zone were brightly and uniformly labeled, indicating abundant secretion of this pectic moiety; in contrast, *fra1-5* cell walls were dimly and unevenly labeled (Figures 6A - 6C), consistent with a reduced rate of pectin secretion.

Membrane trafficking from the Golgi to the plasma membrane is responsible for secretion of matrix polysaccharides. To examine the Golgi apparatus, we used high-pressure freezing and freeze substitution followed by transmission electron microscopy. We examined protoxylem cells in the apical (growing) region of the inflorescence stem because their walls are strikingly thinner in *fra1-5* compared to wild type (Figure 1F-1G). Golgi bodies had fewer cisternae in *fra1-5* compared to wild type and accumulated larger vesicles in their vicinity (Figures 6D - 6G). These abnormalities are consistent with inhibited secretion in *fra1-5*.

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 (Zhong 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 appeared to be unaffected in the micrograph shown (Zhong et al., 2002). Whether this reflects the true status of *fra1-1* cell walls requires further study.

Cellulose microfibrils were reported to be disorganized in *fra1-1* fiber cells (Zhong et al., 2002) and in 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 with poorly organized textures, in both genotypes. Neither of the previous studies indicated how many cells and plants were imaged and 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 inflorescence stem pith. Since 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 due to a lower expansion rate, rather than to 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 itself 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 further 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 show diffusive motility in the cytoplasm. These molecules are probably in an autoinhibited state to prevent futile ATP consumption (Ganguly and Dixit, 2013). A sub-population 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 $\mu\text{m}/\text{sec}$ and a characteristic run length of 2.4 μm , 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 due to different ionic conditions and regulatory mechanisms present in cells. The velocity and run length of FRA1 are comparable to 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 six-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 (Paredes 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 60-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 interacting 1, 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 co-localize 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*, alkynylated fucose 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). Fucose-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 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; Boutte 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 processive 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 pervasively, 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, 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

Arabidopsis thaliana L. (Heynh), Columbia accession, was used throughout. The *fra1-5* mutant was isolated from a T-DNA insertion line (SALK_084463) obtained from ABRC (<http://abrc.osu.edu/>). Homozygous mutants were identified by using primers listed in Supplemental Table 1. 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 4x with water and planted on 1X Murashige and Skoog medium (MS, 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 qRT-PCR

Total RNA was extracted from basal internodes or rosette leaves of 4-week-old plants, DNase treated and used for cDNA synthesis using qScript cDNA supermix (Quanta BioSciences). Primers for RT-PCR and qRT-PCR are listed in Supplemental Table 1. 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 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 propidium iodide for 5 min and imaged using confocal microscopy. Cell lengths and width 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 6N 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 1.3 kb sequence upstream of the *FRA1* start codon and the full length *FRA1* cDNA followed by three tandem copies of *EGFP* cDNA. The *pFRA1::FRA1-tdTomato* construct was generated using 3kb 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 1. These constructs were ligated into the pCAMBIA 1300 vector and introduced into the *fra1-5* mutant via Agrobacterium-mediated floral dip transformation. Transgenic plants were selected using 20 µg/mL 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 *pUBQ::RFP-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 to 23°C. Seedlings were gently mounted in water between two layers of double-sided adhesive tape. For drug treatments, seedlings were incubated with either 0.1 µM isoxaben for 2 h, 5 µM DCB for 3 h, 20 µM taxol (Cytoskeleton, 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% DMSO as appropriate. Epidermal cells in the apical or sub-apical 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 100X (NA 1.45) objective and 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 number of motile FRA1-3GFP particles $\mu\text{m}^{-2} \text{s}^{-1}$. Single molecule photobleaching analysis was conducted as described earlier (Ross and Dixit, 2010). Briefly, we treated seedling with 1 mM 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. Data were collected from at least 3 cells from 3 independent seedlings.

Plants expressing *YFP-CESA6* in the *prc1-1* 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 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 μ m 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 *pUBQ::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 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 and 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 post-

fixed 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 a LEO 912 AB energy filtered transmission electron microscope (TEM) operated at 120 kV. Cell wall thickness was measured using ImageJ for the outermost two layers of interfascicular fiber cells, pith cells in the center of the stem, and cells with thick walls in the protoxylem. Cellulose was labeled with colloidal gold using enzyme-gold affinity cytochemistry as described earlier (Berg et al., 1988) and detailed in supplemental experimental procedures.

To image Golgi and vesicles, transverse sections of 4-week-old apical and basal stems were loaded in specimen carriers that contained packing buffer (100 mM PIPES-pH 6.8 and 150 mM sucrose) and ultra-rapidly frozen using a BAL-TEC HPM 010 high pressure freezer. Samples were freeze substituted over 5 days at -85°C in acetone containing 2% osmium tetroxide and 0.1% uranyl acetate and slowly thawed to room temperature, rinsed in acetone, and embedded in Spurr's resin. Thin sections were stained and imaged as described above. The diameter of vesicles within $\sim 1\text{ }\mu\text{m}$ of Golgi stacks was measured in ImageJ. The number of cisternae per Golgi was counted manually.

Scanning electron microscopy

Freshly excised segments ($\sim 5\text{ mm}$ long) from 4-week old plants were sectioned in PBS at a nominal thickness of $100\text{ }\mu\text{m}$ on a Vibratome. The sections were rinsed with 1% (v/v) Triton-X100 and dehydrated using a graded ethanol series. Dehydrated samples were critically point dried, mounted on stubs, coated with platinum ($\sim 2\text{ nm}$), 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 (~ 150 images per genotype). For each section, approximately 10 cells were imaged along a transverse transect. For quantification, the "fit-ellipse" routine was used described by Marga et al. (2005) and detailed in supplemental experimental procedures.

Metabolic labeling of pectin using fucose-alkyne

Fucose-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 disk confocal microscope (488 nm laser excitation, 525/25 emission filter) using a 63X 1.40 NA 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, 2 mM EDTA, and a protease inhibitor tablet from Roche) and total protein (~100 µg each) were separated by SDS-PAGE and transferred to 0.45 µm PVDF membrane (Thermo Scientific). Proteins were probed with FRA1 primary antibody (1: 2,000) and anti-rabbit IgG HRP secondary antibody (1: 5,000, Jackson Immuno Research). Detection was conducted using SuperSignal West Dura chemiluminescence substrate (Thermo Scientific).

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FIGURES

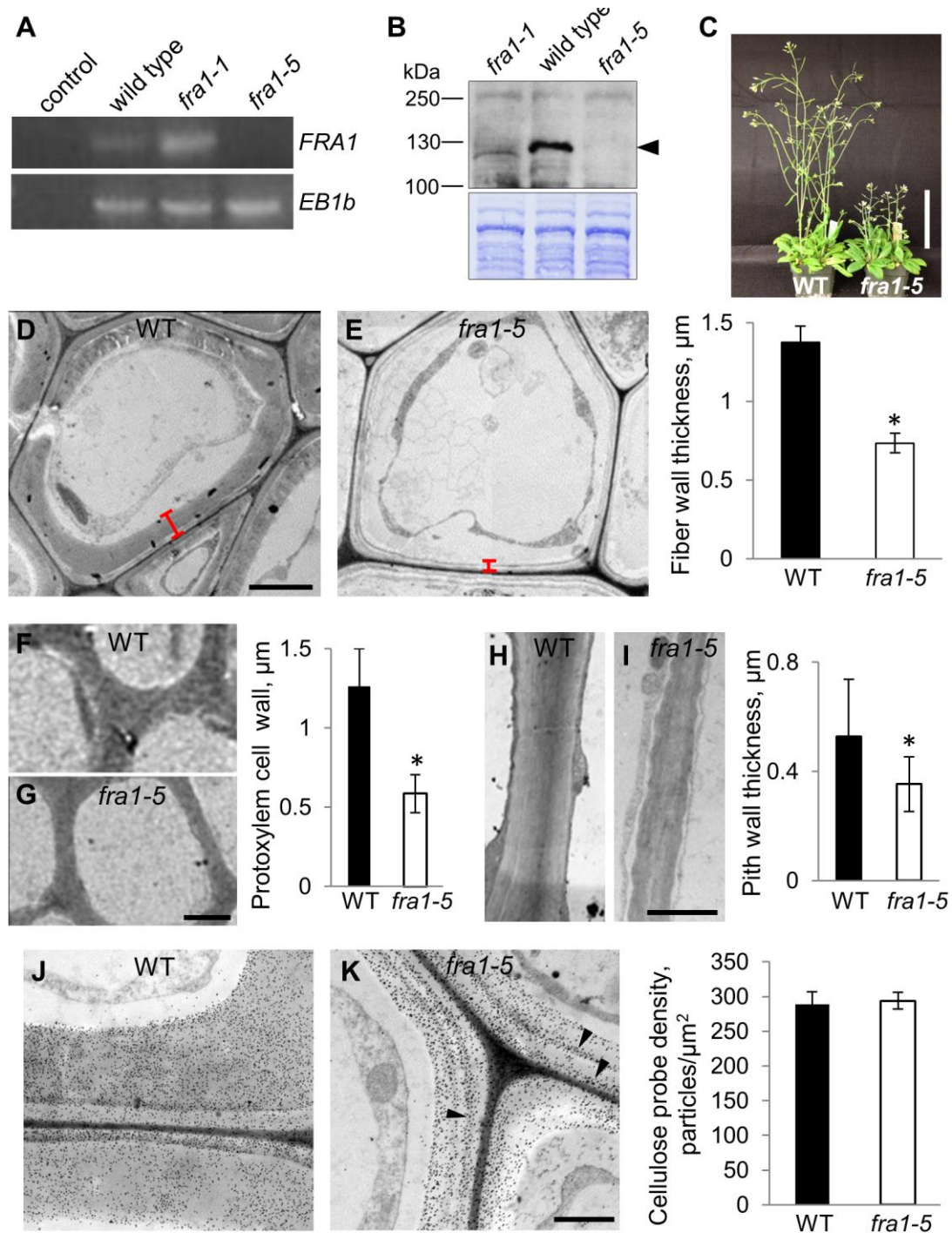


Figure 1. Phenotype of the *fra1-5* knockout mutant. All data are for 4-week old, continuously light grown plants. WT = wild type.

(A) RT-PCR analysis in rosette leaves. The control lane lacks reverse transcriptase. *EB1b* expression is used as a loading control.

(B) Immunoblot of total protein extracts probed with an anti-FRA1 antibody. The arrowhead marks the expected position of FRA1. The Coomassie stained gel is shown below.

(C) Whole plant appearance. Scale bar = 10 cm.

(D) and **(E)** Transmission electron micrographs of fiber cells in basal stems. Scale bar = 5 μm . Bars plot mean \pm SD ($n > 30$ cells). Red bars indicate the width of the cell wall.

(F) and **(G)** Transmission electron micrographs of protoxylem cells in basal stems. Scale bar = 2 μm . Bars plot mean \pm SD ($n > 40$ cells).

(H) and **(I)** Transmission electron micrographs of pith cell walls in basal stems. Scale bar = 0.5 μm . Bars plot mean \pm SD ($n > 40$ cells).

(J) and **(K)** Transmission electron micrographs of fiber cell walls labeled with gold conjugated cellobiohydrolase-2. Arrowheads point to layers of high-density labeling. Scale bar = 1 μm . Bars plot mean \pm SD ($n > 21$ cells).

Asterisks indicate significant differences between the genotypes as determined by Student's *t* test, $p < 0.01$.

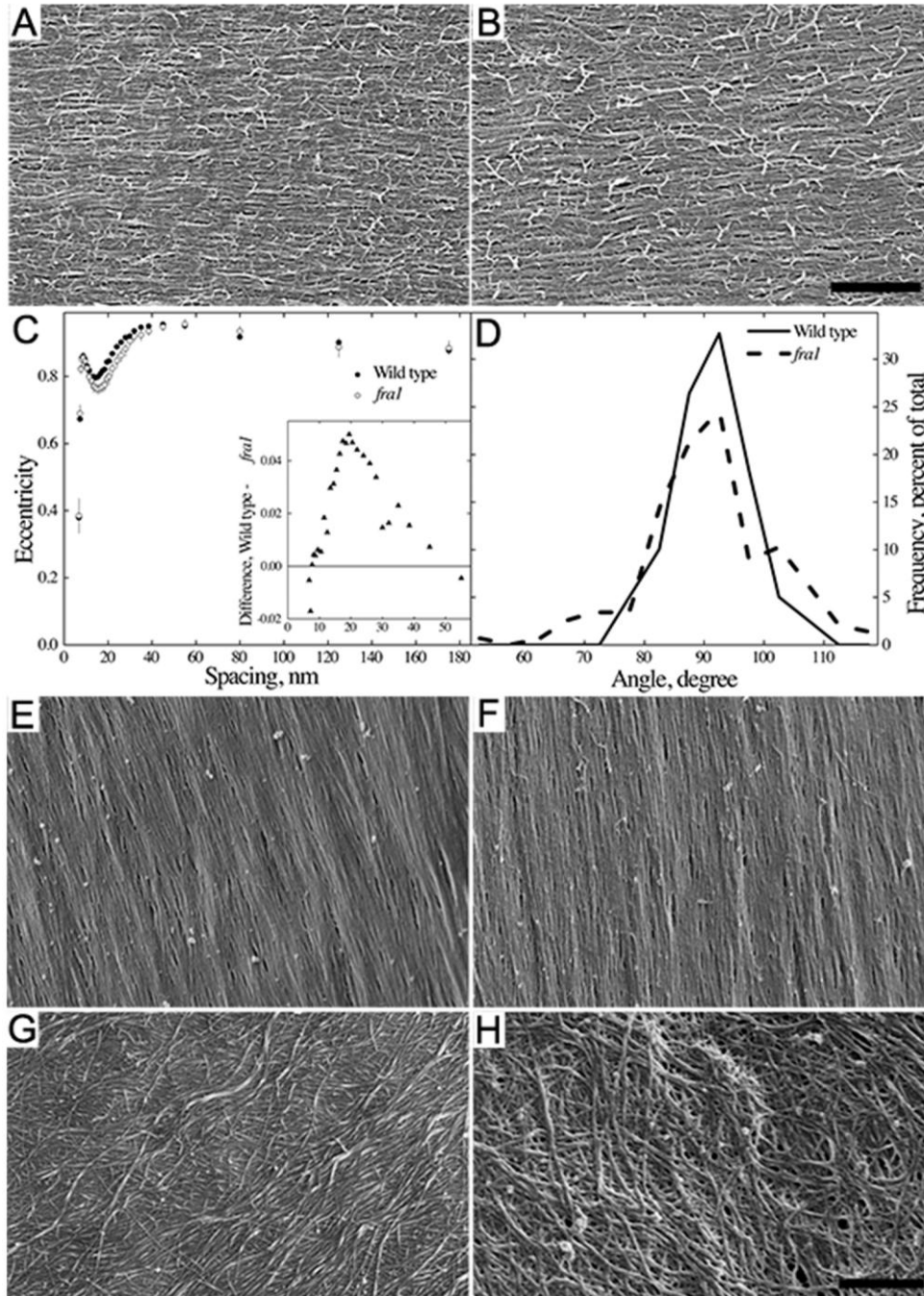


Figure 2. Cell wall organization.

(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. Scale bar = 500 nm.

(C) Eccentricity of the Fourier transform as a function of spatial frequency. A value of zero indicates complete radial symmetry and a value of one indicates perfect alignment. Symbols plot

the mean \pm SEM of 4 stems per genotype, with about 40 images per stem (~150 images total per genotype). The inset shows the difference for the curves in the main figure 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 of variance is rejected with $p < 0.001$ using Levene's test (see supplemental information).

(E) to (H) Representative images of cell wall organization in interfascicular fiber cells from the basal region of 4-week old wild-type **(E and G)** and *fra1-5* **(F and H)** inflorescence stems. Scale bar = 500 nm.

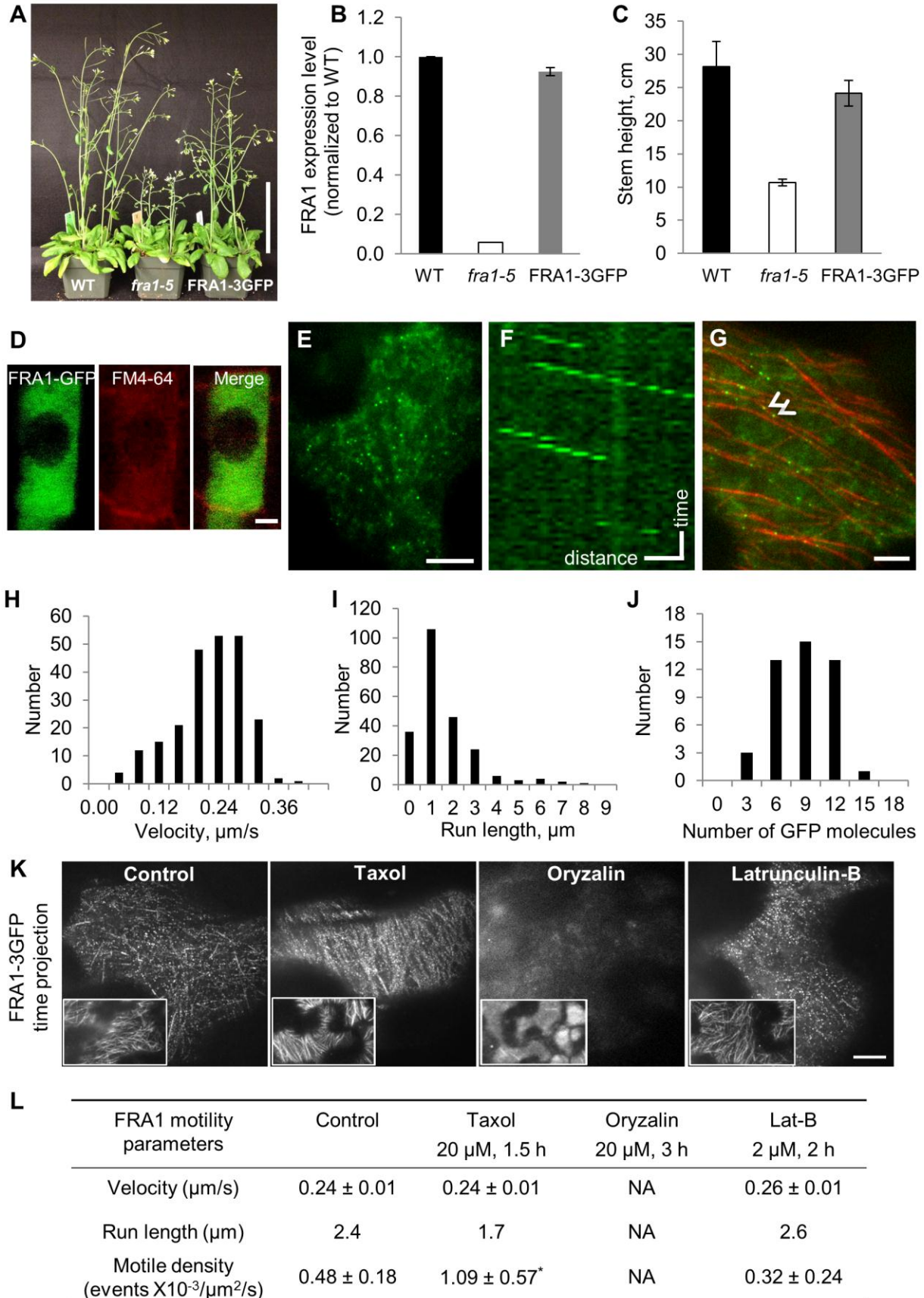


Figure 3. Motility of FRA1-3GFP *in vivo*.

(A) Overall appearance of 4-week old plants grown under continuous light. Scale bar = 10 cm.

(B) Transcript level in rosette leaves measured by qRT-PCR and normalized to the *FRA1* expression level in wild type. Values are mean \pm SEM from three biological replicates.

(C) Stem height of 4-week old plants. Values are mean \pm SD (n = 20 plants).

(D) Representative image of FRA1-3GFP (green) in a vascular cell of the root of a 4-d old seedling. FM4-64 (red) was used to label the plasma membrane. Scale bar = 2 μ m.

(E) Image of FRA1-3GFP in a cotyledon pavement cell of a 4-d old seedling (same in E - J). Scale bar = 10 μ m.

(F) Representative kymograph showing the movement of FRA1-3GFP. Diagonal lines represent motile events. Vertical bar = 20 s; horizontal bar = 1 μ m.

(G) Dual-channel image of FRA1-3GFP (green) and RFP-TUB6 (red). The arrowheads label FRA1-3GFP puncta along a cortical microtubule. Scale bar = 10 μ m.

(H) Distribution of the velocity of processive FRA1-3GFP puncta. The average velocity is 0.24 ± 0.09 μ m/s (mean \pm SD, n = 233).

(I) Distribution of the run length of FRA1-3GFP puncta. The characteristic run length ($L_{1/2}$) is 2.4 μ m (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 9 GFP fluorophores probably represent two FRA1-3GFP dimers that got photobleached prior to image acquisition.

(K) Time projections of 200 images of FRA1-3GFP from pavement cells of seedlings treated as indicated. Linear tracks in these images indicate motility of FRA1-3GFP. The insets show the cortical microtubules following the stated treatments. Scale bar = 10 μ m.

(L) Motile parameters of FRA1-3GFP from the experiments shown in (K). Values are mean \pm SD (n = 233, 107, and 87 molecules for control, taxol, and latrunculin-B, respectively). Asterisk indicates significant difference from control as determined by Student's *t* test, $p < 0.01$.

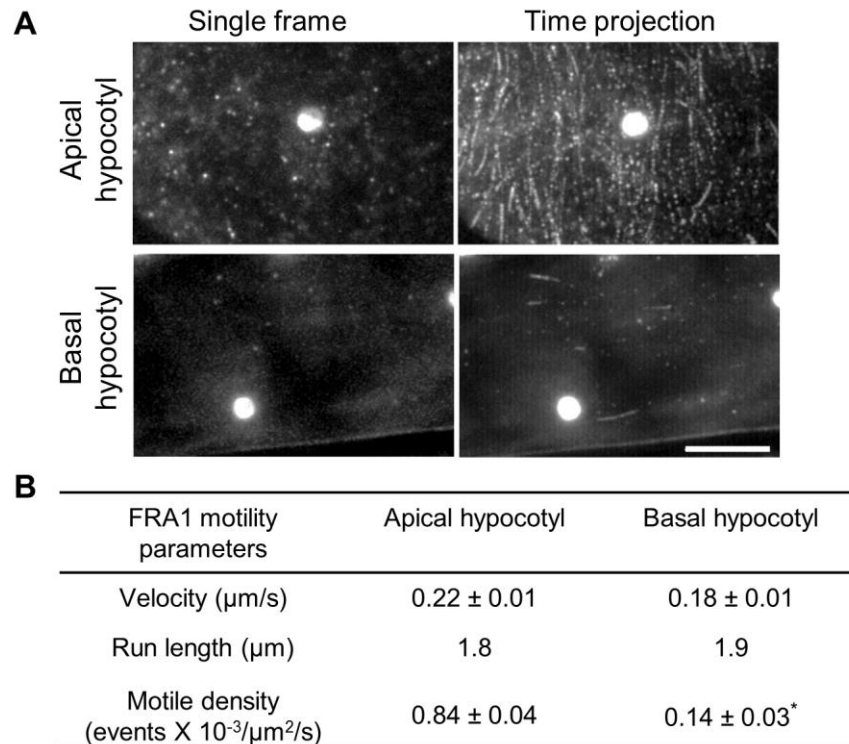


Figure 4. FRA1 motility correlates with growth.

(A) FRA1-3GFP motility assessed from image sequences. In the time projection of 200 images, motile FRA1-3GFP puncta appear as linear tracks. The bright round structure in these images is a chloroplast, imaged due to chlorophyll autofluorescence. Scale bar = 10 μm .

(B) Motile parameters of processive FRA1-3GFP puncta. Values are mean \pm SD ($n = 174$ and 55 molecules in apical and basal hypocotyl, respectively, from at least 3 seedlings). Asterisk indicates significant difference between apical and basal hypocotyl as determined by Student's t test, $p < 0.01$.

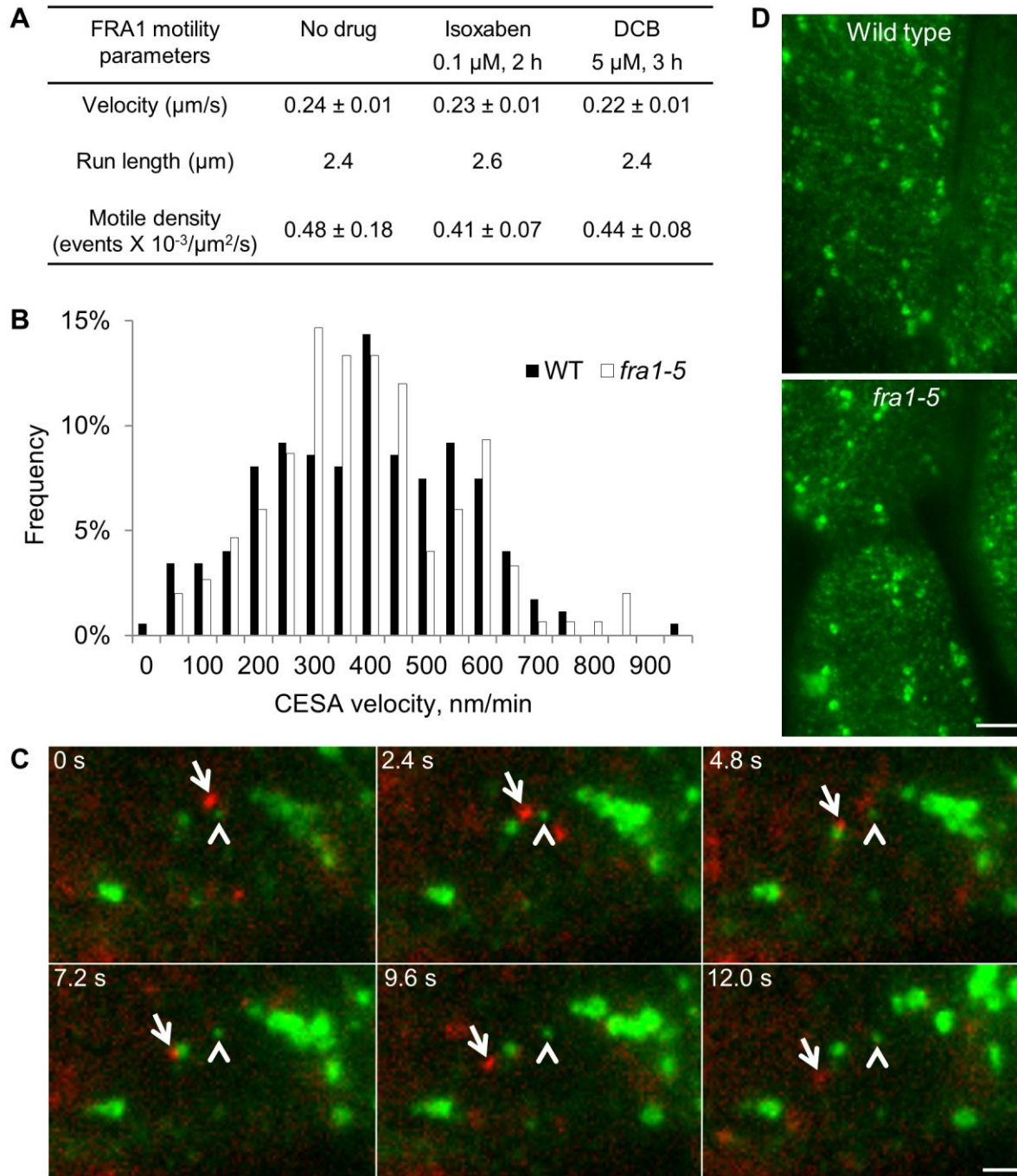


Figure 5. Relationship between FRA1 and CESA complexes.

(A) Motile parameters of processive FRA1-3GFP puncta in apical hypocotyl cells treated as indicated. Values are mean \pm SD ($n \geq 95$ molecules from at least 3 seedlings).

(B) Distribution of the velocity of YFP-CESA6 at the plasma membrane. The average velocity of YFP-CESA6 is 409 ± 173 nm/min ($n = 174$ molecules) in wild type and 430 ± 192 nm/min ($n = 150$ molecules) in *fra1-5*.

(C) Movement of FRA1-tdTomato (arrow) and YFP-CESA6-labeled vesicles (arrowhead) in hypocotyl epidermal cells. Scale bar = 2 μm .

(D) Images of plasma membrane-localized YFP-CESA6 particles in hypocotyl epidermal cells. The average particle density \pm SD in wild type ($0.42 \pm 0.05 \mu\text{m}^{-2}$, $n = 10$ cells) and *fra1-5* ($0.39 \pm 0.06 \mu\text{m}^{-2}$, $n = 11$ cells) is not significantly different as determined by Student's *t* test. Scale bar = 5 μm .

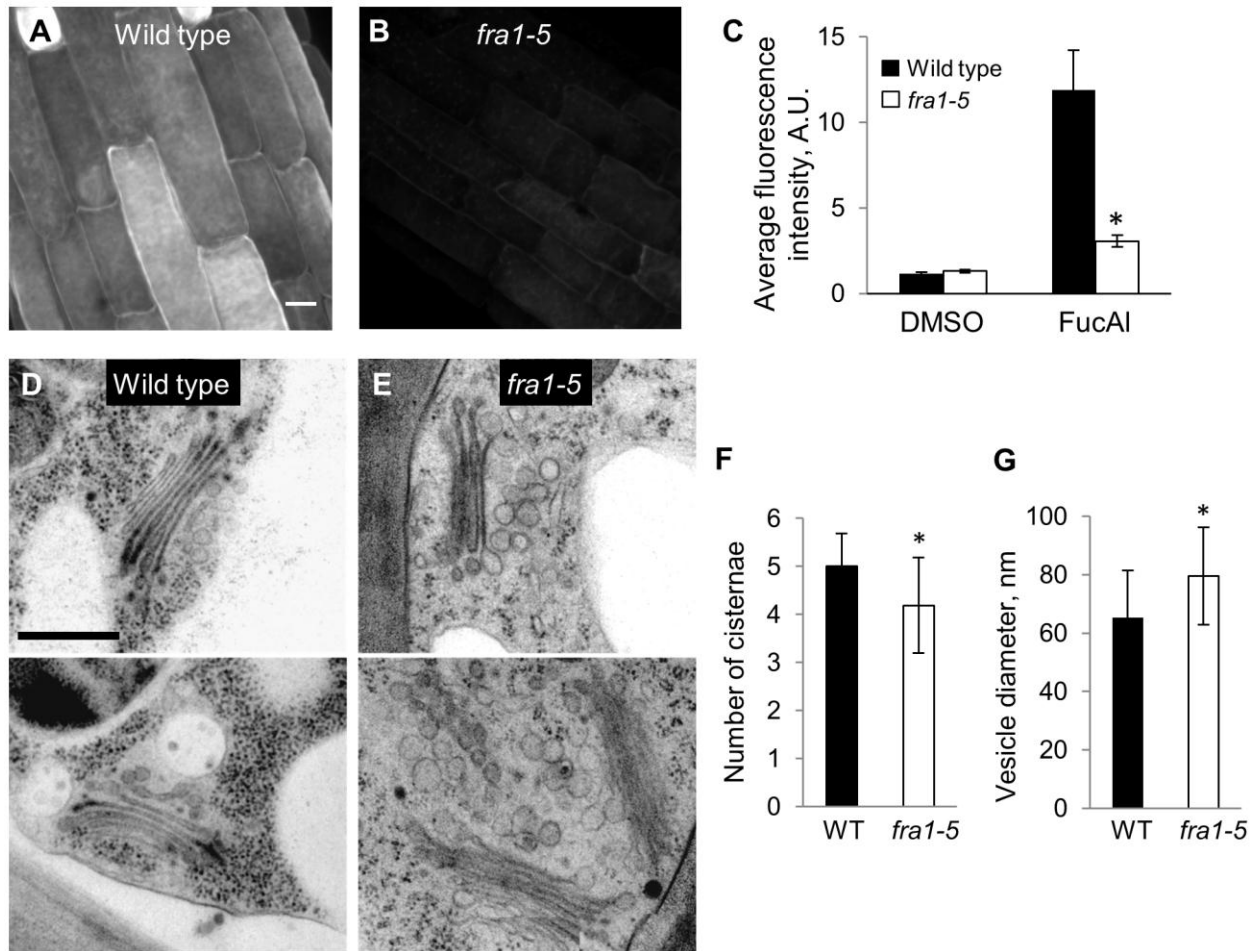


Figure 6. Secretion of pectin and endomembrane morphology.

(A) and (B) Representative fluorescence images in root elongation-zone epidermal cells of 4-d old seedlings incubated in fucose-alkyne (FucAl) for 2 h and reacted with Alexa 488-azide. Scale bar = 10 μm .

(C) Quantification of the fluorescence for the experiment shown in (A) and (B). Values are mean \pm SEM ($n \geq 6$ cells per seedling with 9 seedlings per treatment from two experiments). DMSO is used as a solvent control.

(D) and **(E)** Representative images showing Golgi and vesicle morphology in protoxylem cells of the inflorescence, prepared by high pressure cryo-fixation. Scale bar = 500 nm.

(F) and **(G)** Quantification of the number of cisternae per Golgi body ($n > 30$ Golgi from 3 plants) and the diameter of Golgi-associated vesicles ($n > 200$ vesicles from 3 plants) in wild type and *fra1-5*. Values are mean \pm SD.

Asterisks indicate significant differences between the genotypes as determined by Student's *t* test, $p < 0.001$.