Arabidopsis myosin XIK interacts with the exocyst complex to facilitate vesicle tethering during exocytosis

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ABSTRACT

Myosin motors are essential players in secretory vesicle trafficking and exocytosis in yeast and mammalian cells; however, similar roles in plants remain a matter for debate, at least for diffusely-growing cells. Here, we demonstrate that Arabidopsis (Arabidopsis thaliana) myosin XIK, via its globular tail domain (GTD), participates in the vesicle tethering step of exocytosis through direct interactions with the exocyst complex. Specifically, myosin XIK GTD bound directly to several exocyst subunits in vitro and functional fluorescently-tagged XIK colocalized with multiple exocyst subunits at plasma membrane (PM)-associated stationary foci. Moreover, genetic and pharmacological inhibition of myosin XI activity reduced the rate of appearance and lifetime of stationary exocyst complexes at the PM. By tracking single exocytosis events of cellulose synthase (CESA) complexes (CSCs) with high spatiotemporal resolution imaging and pair-wise colocalization of myosin XIK, exocyst subunits and CESA6, we demonstrated that XIK associates with secretory vesicles earlier than exocyst and is required for the efficient localization and normal dynamic behavior of exocyst complex at the PM tethering site. This study reveals an important functional role for myosin XI in secretion and provides insights about the dynamic regulation of exocytosis in plants.
INTRODUCTION

Exocytosis involves the production and trafficking of secretory vesicles from the Golgi to the plasma membrane (PM) where they are tethered and ultimately fuse to deliver new membrane, extracellular matrix components, and membrane-associated proteins. In plants, secretory trafficking and exocytosis coordinate the delivery of lipids, protein receptors, enzymes, polysaccharides and other molecules that are fundamental for many aspects of plant growth and survival, including cell wall biogenesis, cytokinesis, cell polarity establishment, and response to environmental stresses (Friml, 2010; McFarlane et al., 2014; Robatzek, 2014; Kim and Brandizzi, 2016; Yun and Kwon, 2017; Elliott et al., 2020).

Exocytosis is a precisely choreographed process requiring the spatiotemporal cooperation of a plethora of molecules and protein complexes, including cytoskeleton and motor proteins, Rab-family GTPases, the exocyst tethering complex, and soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs) to ensure targeted delivery of cargos to specific PM regions for secretion. The exocyst is a conserved octameric protein complex that mediates the initial tethering of secretory vesicles to the PM before SNARE-mediated membrane docking and fusion (TerBush et al., 1996; Cvrčková et al., 2012; Žárský et al., 2013; Wu and Guo, 2015; Ravikumar et al., 2017). The eight subunits comprising the exocyst complex are SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70 and EXO84. Although many of the core components in exocytosis are evolutionarily conserved and well characterized in animal and yeast models, our knowledge of the precise mechanisms and molecular players that coordinate secretion in plant cells remains sparse.

A key player in exocytosis is class V myosin, whose primary role is transport of vesicles and organelles along the actin cytoskeleton in animal and yeast cells (Rudolf et al., 2011; Hammer and Sellers, 2012). Arguably, the best characterized myosin V is budding yeast Myo2p, which plays a vital role in polarized growth through powering the transport of secretory vesicles along actin cables from the mother cell to the growing tip in the bud (Govindan et al., 1995). Myo2p transports secretory vesicles in a cargo receptor-dependent manner through interaction with the Rab
GTPase receptor SEC4 via its globular tail domain (GTD; Jin et al., 2011; Santiago-Tirado et al., 2011). SEC4 also recruits the exocyst tethering complex to the vesicle surface by direct association with the SEC15 subunit (Guo et al., 1999). The GTD of Myo2p also binds to SEC15, which is critical for localization of SEC15 to the bud tip and polarized secretion (Jin et al., 2011). Myo2p remains associated with the vesicle after it arrives at the PM tethering site until a few seconds before the vesicle fully fuses with the PM, and regulates vesicle tethering time through an unknown mechanism that is independent of its interaction with SEC15 (Donovan and Bretscher, 2012, 2015). Similarly, myosin V motors in other species interact with the homologous Rab GTPases or other cargo receptors to facilitate secretory vesicle transport (Jin et al., 2011; Lindsay et al., 2013; Vogel et al., 2015). One recent study demonstrates a role for myosin V in neuronal synapses by tethering synaptic vesicles to PM release sites in an ATPase activity-dependent manner, rather than driving vesicle transport (Maschi et al., 2018). Myosin V also contributes to vesicle tethering through Ca²⁺-dependent interaction with SNARE proteins on synaptic vesicles (Prekeris and Terrian, 1997; Watanabe et al., 2005).

Myosin V-like motors are widely present in plants and are grouped into myosin VIII and XI families (Reddy and Day, 2001). Despite well characterized roles for myosin V in secretory vesicle transport and exocytosis, there is a paucity of information about similar roles for plant myosin. Conventional wisdom maintains that the major role of plant myosin XI is to power cytoplasmic streaming and long-distance transport of organelles and vesicles (Avisar et al., 2012; Tominaga and Ito, 2015; Ueda et al., 2015). In Arabidopsis thaliana, the myosin XI family comprises 13 members, with XIK, XI1 and XI2 among the most highly expressed isoforms that function redundantly in driving intracellular motility and thereby contribute to rapid cell growth and expansion (Prokhnevsky et al., 2008; Peremyslov et al., 2010; Ueda et al., 2010; Haraguchi et al., 2018).

In addition to Rabs and the exocyst complex, numerous cargo receptors and myosin V-interacting proteins have been identified in yeast and animal cells. By comparison, the major myosin XI-binding proteins identified are plant-specific MyoB family proteins (Peremyslov et al.,
2013; Kurth et al., 2017). Surprisingly, none of the MyoBs in Arabidopsis colocalize with large organelles or secretory vesicles, but instead are associated with a specific type of endomembrane compartment that moves rapidly along actin cables to drive cytoplasmic streaming; consequently, an indirect model of myosin XI powering transport of organelles in plant cells has been proposed (Peremyslov et al., 2013; Kurth et al., 2017; Nebenführ and Dixit, 2018). Nevertheless, there is emerging evidence that myosin XI may play a role in exocytosis, at least in tip-growing cells (Orr et al., 2020). In the moss Physcomitrella patens, myosin XI interacts with a RabE GTPase that is homologous to yeast Sec4; disruption of this binding results in unpolarized growth (Orr et al., 2021). However, whether a tripartite complex of myosin XI tail domain, exocyst subunits, and Rab functions in plants to mediate secretory vesicle tethering remains to be established.

Delivery of polysaccharides and proteins to construct the cell wall provides a facile system to dissect exocytic trafficking in plant cells. Cellulose, the primary component of the cell wall, is synthesized at the cell surface by cellulose synthase (CESA) complexes (CSCs), which rely on endomembrane trafficking and exocytosis for delivery to the PM (Bashline et al., 2014; McFarlane et al., 2014). Recently, the plant exocyst complex has been implicated in mediating delivery of CSCs to the PM in primary (Zhu et al., 2018) and secondary cell wall deposition (Vukašinović et al., 2017). The exocyst complex associates with CSCs for a few seconds during the initial static phase after the vesicle has arrived at the PM, consistent with a role in tethering of CESA compartments to the PM (Zhu et al., 2018). In previous work, we showed that myosin XI is also a key player in delivery of CSCs to the PM (Zhang et al., 2019). Myosin XI regulates the rate of CSC delivery to the PM, arrives at the PM along with putative secretory vesicles and associates transiently at the docking site, and facilitates vesicle tethering or fusion. The detailed molecular mechanisms for myosin XI in vesicle tethering/fusion, however, remain unclear and whether interactions with other players, such as the exocyst complex, are required merits further investigation.

Here, we report that myosin XIK is the primary myosin isoform mediating CSC delivery, vesicle tethering, and exocytosis in Arabidopsis. Moreover, yeast two-hybrid and in vitro pull-down assays revealed direct interactions between myosin XIK GTD and several exocyst complex
subunits, including SEC5B and SEC15B. By combining genetic and pharmacological approaches with quantitative live-cell imaging of Arabidopsis lines expressing combinations of fluorescent reporters for CESA6, myosin XIK, or exocyst subunits, we showed that myosin XI influences exocyst dynamics at the PM and is required for the efficient localization or stabilization of exocyst at vesicle tethering sites during CSC delivery at the PM. Collectively, these data demonstrate the exocyst complex is an interactor of myosin XI and we propose a novel role for myosin XI in regulating exocytosis in plants.

RESULTS

Myosin XIK is a Major Isoform Involved in Cellulose Biogenesis and CESA Trafficking

Using the trafficking of CSCs as a model experimental system, Arabidopsis myosins XI were shown to play a role in vesicle tethering and/or fusion at the PM (Zhang et al., 2019). However, it remains unclear how individual myosin isoforms are involved in this process and whether any single isoform plays a predominant role. An Arabidopsis myosin xi1 xi2 xik triple-knockout mutant (xi3KO) has reduced cellulose levels and a significantly lower CSC delivery rate (Zhang et al., 2019). Myosin XI1, XI2, and XIK are among the most highly-expressed isoforms in Arabidopsis somatic cells, and XIK is the primary isoform responsible for cytoplasmic streaming and organelle transport (Peremyslov et al., 2008; Prokhnevsky et al., 2008; Ueda et al., 2010; Avisar et al., 2012; Haraguchi et al., 2018). To test whether XIK is a major player in CESA trafficking, we screened xik-2, xi1, and xi2 single gene knockout mutants (Peremyslov et al., 2010) for cellulose biosynthesis and CESA trafficking defects. Measurement of cellulose content was conducted using the trifluoroacetic acid (TFA) and acetic-nitric (AN) methods (Zhang et al., 2019). The results showed that the greatest decrease in total and crystalline cellulose levels occurred in xik-2, with the extent of reduction comparable to that in the xi3KO mutant, whereas only a slight reduction was detected in xi2 and no obvious effect was observed in xi1 (Figures 1A and 1B). To confirm the phenotype found in xik-2, a second knockout mutant allele, xik-1 (Ojangu et al., 2007), was analyzed and showed a reduction in cellulose content similar to xik-2 (Figures 1A and 1B).
To test whether XIK makes a major contribution to exocytosis, we analyzed CESA trafficking phenotypes by quantitative live cell imaging with spinning disk confocal microscopy (SDCM) in 3-d-old etiolated hypocotyl epidermal cells using xi1, xi2, xik-1, and xik-2 single mutants expressing the YFP-CESA6 in the prc1-1 homozygous CESA6-null mutant background (Paredez et al., 2006), to minimize potential trafficking artifacts caused by overexpression of CESA6. Among these lines, xi1, xi2, and xik-2 single mutants were recovered from the same cross that resulted in the previously characterized xi3KO YFP-CESA6 prc1-1 line (Zhang et al., 2019). With these reporter lines, we analyzed the density of CSCs at the PM as well as the delivery rate of CSCs to the PM by fluorescence recovery after photobleaching (FRAP) assays. Similar to our previous report, the xi3KO YFP-CESA6 prc1-1 line exhibited significantly decreased density (35%) of PM-localized CSCs and a 59% reduction of CSC delivery rate to the PM compared with wild-type siblings (Figures 1C to 1F). Among the myosin single mutants, xik-1 and xik-2 showed the most severe disruption of CSC trafficking, with CSC density decreased by ~25% and the delivery rate reduced by ~40% compared with wild-type siblings. It needs to be noted that the substantially reduced CSC delivery rate in xi3KO and xik mutants could partially result from reduced replenishment of non-bleached Golgi or other delivery compartment into the photobleached area in FRAP assays, given the significant role myosin XI plays in cytoplasmic streaming. In contrast to xik, the xi2 mutant only had a slight reduction and xi1 showed no significant reduction in either density or delivery rate assays (Figures 1C to 1F). These results indicate that XIK is a major player in delivery of CSCs to the PM and makes a substantial contribution to cellulose production. Hereafter, we use the two xik single mutant alleles to further characterize the role of Myosin XI in delivery, tethering, and fusion of vesicles at the PM.

Myosin XIK Mediates the Exocytosis of CSCs

The xi3KO mutant has vesicle tethering or fusion defects as well as an abnormal accumulation of CESA-containing compartments in the cortical cytoplasm, likely resulting from inefficient exocytosis (Zhang et al., 2019). To investigate a role for XIK in these processes, we initially
measured the abundance of cortical and subcortical CESA compartments in \textit{xik}-1 and \textit{xik}-2. Using previously described methods (Sampathkumar et al., 2013; Zhang et al., 2019), we imaged epidermal cells from 3-d-old etiolated hypocotyls with SDCM and combined the optical sections into cortical (0 to 0.4 µm below the PM) and subcortical (0.6 to 1 µm below the PM) cytoplasm. Segmenting the cytoplasm into two regions is logical because if myosin participates in vesicle exocytosis, an abnormal CESA compartment population is more likely to be detected in the cortex close to the PM rather than in the subcortical region. Similar to observations with \textit{xi3KO}, an increased population of CESA compartments was detected in the cortical cytoplasm but not in the subcortical region of \textit{xik}-1 and \textit{xik}-2 cells compared with that in wild-type cells (Figures 2A and 2B).

To further investigate the role of XIK in vesicle secretion and test whether exocytosis defects result from loss of XIK, we performed a single CSC insertion assay using high-resolution spatiotemporal imaging with SDCM (Guitierrez et al., 2009; Zhang et al., 2019). A single CSC insertion event typically undergoes three phases of dynamic movement: a transient erratic phase, representing a vesicle approaching its PM destination for fusion; a pause phase at the PM that lasts for 1 to 2 min, representing vesicle tethering, docking and fusion; and a steady movement phase, indicative of an active cellulose-producing complex moving along a linear trajectory in the PM (Figure 2C; Supplemental Movie S1). By tracking the dynamics of new CSC insertion events, we previously showed that they can be categorized into five groups: (1) a standard event with a pause time for 1 to 2 min followed by a steady movement phase following successful CSC insertion; (2) a successful insertion event with a shorter pause time (shorter than the mean pause time in wild type minus one standard deviation); (3) a successful insertion with a longer pause time (longer than the mean pause time in wild type plus one standard deviation); (4) a failed insertion with only a shorter pause phase and no steady movement phase; (5) a failed insertion with a normal or longer pause time (Figure 2D; Zhang et al., 2019). Through analysis of kymographs, we are able to quantify pause times and the frequency of each type of insertion event. In wild type, the average pause time was 84 ± 29 s (mean ± SD, n = 131 events), similar to the previously reported value of 81 ± 27 s (Zhang et al., 2019). In contrast, in two \textit{xik} alleles and \textit{xi3KO}, a number of
events exhibited an abnormal pause time, with 15–30% of the events showing pause times longer than 120 s compared with that of 10% in wild-type cells (Figure 2E). In addition, the population with short pause times of 15–50 s also increased from 9% in wild type to 16–23% in myosin single or triple mutants (Figure 2E). Moreover, the majority of events with abnormal pause times in myosin mutants also failed to insert functional CSCs into the PM (Figure 2F). The average percentage of total failed insertion events increased from 12% in wild type to 44, 31, and 29% in xi3KO, xik-1, and xik-2, respectively (Figure 2F). This apparent reduced effect on CESA6 single particle insertion events compared with FRAP delivery data (Figure 1F) could be because the latter experiments are influenced by reduced cytoplasmic streaming under myosin inhibition conditions that reduces bulk delivery of fluorescent CSCs to the bleached area. The altered CSC pause time and failed PM insertion likely result from failed vesicle tethering after initiation and/or normal tethering but failed membrane fusion. These results indicate that XIK plays a role in the tethering or fusion step during the exocytosis of CSC-containing vesicles at the PM.

Consistent with a role for myosin XI in vesicle tethering, we showed previously that functional YFP-tagged XIK displayed transient colocalization with tdTomato-CESA6 during the first 3 s of the pause phase during CSC insertion at the PM (Zhang et al., 2019). Given that tethering is likely to last for ~10 s at the beginning of the pause phase as indicated by the colocalization of exocyst tethering complex with CSCs (Zhu et al., 2018), we speculate that myosin and exocyst may cooperate to achieve vesicle tethering.

Myosin XIK Interacts Directly with Exocyst Subunits

A direct interaction between the GTD of Myo2p and the exocyst subunit SEC15 has been demonstrated in budding yeast (Jin et al., 2011). To explore this relationship in plants, we tested for direct interactions using a yeast two-hybrid assay with Arabidopsis exocyst subunits as prey and the GTD of XIK as bait. Through a screen of members from all eight subunits of the exocyst complex, SEC5B strongly interacted with XIK GTD and EXO84A showed a weak interaction (Figure 3A). The direct interaction of XIK GTD with SEC5B was verified with an in vitro pull-down
assay; recombinant SEC5B cosedimented with purified GST-tagged XIK GTD but not with purified GST alone (Figure 3B). Because yeast SEC15 and SEC10 are exocyst subunits in closest proximity to secretory vesicles and because SEC15 directly interacts with Myo2p (Guo et al., 1999; Jin et al., 2011; Picco et al., 2017), we tested recombinant Arabidopsis SEC15B and SEC10 in the pull-down assay. We found that GST-XIK GTD bound to purified SEC15B but not to SEC10 (Figure 3B and 3C). Because SEC5B interacted with XIK-GTD by two complementary methods and because SEC5B was previously implicated in CESA6 trafficking (Zhu et al., 2018), we focused further experiments mainly on this exocyst subunit.

The exocyst binding site on Myo2p includes two positively charged surface-exposed residues (R1402 and K1473) that are highly conserved among the myosin V family members and implicated in direct interactions with SEC15 (Jin et al., 2011). In a homology model of myosin XIK GTD constructed using the crystal structure of Myo2p GTD as template, two residues, R1366 and R1441, were also conserved in the primary sequence as well as on the tertiary structure of XIK GTD (Figure 3D, Supplemental Figure S1). We tested whether these specific amino acids were involved in direct interactions with plant SEC5B. Single and double amino acid substitution mutations for R1366 and R1441 were generated and tested with pull-down assays. Single mutations of R1366 and R1441 significantly reduced binding to purified SEC5B and double mutations reduced binding to approximately 20% of wild-type levels (Figure 3E and 3F). A surface-exposed lysine residue, K1103, was also mutated as a negative control and had no reduction in binding to SEC5B (Figure 3E and 3F). These results revealed a conserved exocyst-binding site on XIK GTD and further verified the direct interaction of XIK GTD with exocyst subunits.

Myosin XIK Influences the Dynamic Behavior of Exocyst Foci at the PM

Given the direct interaction between XIK and the exocyst complex, we tested whether the two are functionally associated in plants. Dynamic analysis of several exocyst subunits in Arabidopsis shows localization in discrete foci at the PM (Fendrych et al., 2013; Zhang et al., 2013). We crossed a functional EXO70A1-GFP reporter (Fendrych et al., 2010) into xik-1 and xik-2 and
recovered homozygous mutant lines as well as wild-type siblings expressing EXO70A1-GFP. In addition to the genetic disruption of XIK, we also applied acute drug treatments with the myosin inhibitor pentabromopseudilin (PBP), which potently inhibits XIK-YFP motility in Arabidopsis (Zhang et al., 2019). Hypocotyl epidermal cells were imaged with high resolution variable-angle epifluorescence microscopy (VAEM), and the EXO70A1-GFP signal appeared as abundant distinct foci at the PM with a density of ~1.5 particles/µm² (Figure 4A), similar to previous reports (Fendrych et al., 2013). The overall density and distribution pattern of EXO70A1-GFP foci at the PM were similar in untreated xik cells and wild-type cells treated with PBP for 15 min, compared to untreated wild-type cells (Figures 4A and 4C; Supplemental Figure S2), suggesting that XIK is not required for the distribution of exocyst complexes at the PM.

When we tracked the dynamic behavior of the EXO70A1 foci at the PM, two distinct populations were observed: one population exhibited a stationary phase following their appearance at the PM, whereas the other population did not have a pause phase but showed short and rapid diffuse motility before disappearing from the plane of the PM (Supplemental Movie S2). We focused on the stationary population, because it has been suggested that not all exocyst foci tether a secretory vesicle and foci with a stationary phase at the PM are more likely to represent real exocytic events (Fendrych et al., 2013). A stationary exocyst particle was defined by a straight vertical line in kymographs prepared from time-lapse series (Figure 4A) that could be tracked for at least 5 frames (> 2 s). Quantification of the newly appeared stationary foci over time showed that the rate of appearance of these foci at the PM was significantly decreased by ~25% in xik and PBP-treated wild-type cells compared with untreated wild-type cells (Figure 4D; Supplemental Figure S2; Supplemental Movie S2). As the stationary foci are likely to be linked to vesicle tethering/exocytosis events (Fendrych et al., 2013), the results suggest that even though XIK activity is not required for overall exocyst localization at the PM, it may be necessary for efficient PM targeting of a subpopulation that is responsible for vesicle tethering.

We next analyzed the fluorescence intensity profiles of newly appeared stationary foci at the PM. A small fixed region of interest (ROI) that covered the centroid of an exocyst particle was
analyzed over a time course, from a few seconds before the appearance of the foci to a few seconds after the foci fully disappeared. By quantifying average fluorescence intensity from multiple events, the results showed that in wild-type cells, after the appearance of EXO70A1 particles at 0 s, the average fluorescence intensity quickly reached a high level and lasted for 6–7 s before it decreased to background level at around ~ 9 s (Figure 4B). However, in xik-2 and PBP-treated wild-type cells, the foci displayed an apparently shorter lifetime, with an average signal that peaked and then disappeared about 1–2 s earlier than in untreated wild-type cells (Figure 4B). The average lifetime of EXO70A1 foci in untreated wild type was 7.5 s, whereas in xik-2 and PBP-treated wild-type cells, the lifetime was reduced to 6.1 s and 5.7 s, respectively (Figure 4E). A similar reduction of lifetime of EXO70A1 foci was detected in the xik-1 mutant when compared with wild-type siblings (Supplemental Figure S2). These results indicate that XIK is required for maintaining normal dynamic properties of exocyst at the PM and disruption of XIK activity results in shorter exocyst lifetimes.

Given that SEC5B directly interacts with myosin XIK GTD in vitro, we generated a GFP-SEC5B reporter under the control of its native promoter and tested whether the dynamic behavior of SEC5B was altered upon disruption of myosin XI activity. At the PM plane of hypocotyl epidermal cells, GFP-SEC5B appeared as dense puncta that showed rapid dynamic behavior, similar to that of EXO70A1-GFP foci and previous reports (Supplemental Figure S3; Fendrych et al., 2013; Zhu et al., 2018). Similar to EXO70A1, we did not detect apparent alteration in overall density or distribution pattern of SEC5B foci at the PM in xik-2 cells or in wild-type cells after acute PBP treatment; however, the particle dynamic analysis showed a reduced rate of appearance and a shorter lifetime of stationary SEC5B foci in myosin-deficient cells (Supplemental Figure S3). Furthermore, alteration of PM dynamics was observed with a third exocyst subunit marker, SEC6-GFP (Fendrych et al., 2013), when myosin activity was inhibited (Supplemental Figure S4).

Since myosins are actin-based motors, it is likely that the cortical actin cytoskeleton is also involved in regulating exocyst dynamics at the PM. Fendrych et al. (2013) report that a 10-min treatment with the actin polymerization inhibitor latrunculin B (LatB) did not alter exocyst subunit
density and distribution at the PM, whereas prolonged treatment (1 h) resulted in aggregation and uneven distribution of exocyst subunits at the PM. Similarly, we observed that a 15-min treatment with 10 μM LatB did not change the overall density or distribution of exocyst foci at the PM; however, the stationary population was affected similar to that in xik or PBP-treated wild-type cells, with significantly reduced rate of appearance and a shorter lifetime (Supplemental Figure S4). These results suggest that both myosin XI and cortical actin are required for exocyst dynamics at the PM.

Collectively, these data indicate that the rate of appearance and lifetime of stationary exocyst foci at the PM are dependent on myosin XI and actin function, thereby supporting a functional association between myosin and exocyst in plant cells.

**Myosin XIK Transiently Colocalizes with Exocyst Foci Near the PM**

Given the apparent functional connection between myosin XI and stationary exocyst foci at the PM, we tested whether XIK and these foci colocalize. A transgenic line co-expressing XIK-mCherry (Peremyslov et al., 2013) and GFP-SEC5B under the control of their native promoters was generated and imaged with time-lapse SDCM. The co-expression line was in the xik-2 mutant background to avoid overexpression of XIK protein, which may lead to functional artifacts. Similar to previous reports, XIK-mCherry was mostly localized in the cell cortex, with a majority decorating an unknown type of endomembrane compartment that translocated rapidly along actin filaments to drive cytoplasmic streaming, and the rest was present as dynamic patches or diffuse signal in the cytoplasm (Supplemental Movie S3; Peremyslov et al., 2012; Zhang et al., 2019). We previously reported that XIK-YFP cytoplasmic patches transiently and specifically colocalized with newly arrived CSCs at the site of insertion at the PM (Zhang et al., 2019). Here, we investigated whether there was also specific association of the cytoplasmic XIK signal with newly appeared SEC5B foci at the PM. Dual-channel time-lapse imaging was performed with SDCM at 1-s intervals and only newly arrived SEC5B foci in the GFP channel that showed a stationary phase for at least 5 frames (> 4 s) were tracked. Analysis of the corresponding mCherry channel showed that a cluster of
cytoplasmic XIK-mCherry signal was frequently observed to colocalize with a new SEC5B particle during the first few seconds upon its arrival at the PM (Figure 5A; Supplemental Movie S3 and S4). Interestingly, the high frequency of spatiotemporal colocalization was mainly observed in the events when SEC5B had a longer lifetime at the PM (measured as the duration of stationary phase in kymographs). SEC5B foci are reported to have a lifetime of 8–12 s (Zhu et al., 2018), and here the mean lifetime was measured to be 10 ± 4 s in this assay (n = 202 particles). We found that 70% of the GFP-SEC5B foci with a lifetime of 8 s or longer showed colocalization with XIK-mCherry at the beginning of the stationary phase (88 of 126 particles), whereas the colocalization rate was only 41% when the GFP-SEC5B foci had a lifetime shorter than 8 s (31 of 76 particles). To confirm the specific spatiotemporal colocalization, we quantified fluorescence intensity profiles of individual stationary GFP-SEC5B foci as well as the same ROIs in the corresponding XIK-mCherry channel from time-lapse series (Figure 5C). There was significantly higher fluorescence intensity of XIK-mCherry that appeared 2 s before the arrival of a new SEC5B particle, which then peaked at 0 s and lasted for an average of 4 s after the appearance of the new foci at the PM, compared with the remainder of the time points (Figure 5C).

In addition to colocalization of XIK with SEC5B, we created a double marked line expressing XIK-mCherry and EXO70A1-GFP and observed a similar spatiotemporal pattern of colocalization which only occurred in the first few seconds upon arrival of the EXO70A1 foci (Supplemental Figure S5).

Considering that XIK-mCherry mostly appeared as clusters of diffuse, amorphous signal in the cortical cytoplasm, and to exclude the possibility that the colocalization with exocyst was due to non-specific cytoplasmic signal, we co-expressed a cytoplasmic mCherry construct with GFP-SEC5B and examined colocalization. In most cases, a constant mCherry signal was present at the same region with the newly appeared SEC5B particle throughout the time course (Figure 5B). Fluorescence intensity analysis showed that there was no significant difference of fluorescence intensity of cytoplasmic mCherry among all the time points measured in the selected ROIs that correspond to the SEC5B foci (Figure 5D). Thus, these results indicate that there was specific
spatial association of XIK with the exocyst complex near the PM, presumably during vesicle tethering.
Exocyst Localization and Lifetime at the Vesicle Tethering Site in CSC Secretion Depend on Myosin XIK

To further confirm the functional relationship between XIK and the exocyst complex in vesicle tethering, we investigated their association during exocytosis of CSCs at the PM. Previous studies show that both SEC5B (Zhu et al., 2018) and XIK (Zhang et al., 2019) transiently associate with CSCs at the beginning of the pause phase during CSC secretion; mutation of either exocyst subunits or XIK resulted in reduced CSC insertion/delivery rates or increased insertion defects, suggesting both components are critical for CSC secretion at the PM. Here, we verified the spatiotemporal association of exocyst and XIK with CSCs during the vesicle tethering step. We tested two exocyst subunits, SEC5B and EXO70A1, using double marked lines of GFP-SEC5B tdTomato-CESA6 prc1-1 and EXO70A1-GFP tdTomato-CESA6 prc1-1, as well as the colocalization of XIK with CESA6 using the double marked line of XIK-mCherry YFP-CESA6 prc1-1 xik-2. We tracked single CSC insertion events during the progression from erratic phase, pause phase, to the steady translocation phase in the double marked lines and measured the fluorescence intensity in the corresponding exocyst or XIK channels (Figure 6; Supplemental Figure S6).

Similar to the previous report (Zhu et al., 2018), we observed that in the majority of insertion events, a GFP-SEC5B particle appeared at the location of a CSC particle at the PM at the beginning of the pause phase (Figures 6A, 6C and 6D; Supplemental Movie S5), and colocalization lasted for an average duration of 11.2 ± 5 s (n = 115 insertion events). Colocalization was further confirmed by fluorescence intensity analysis over a time course in the GFP-SEC5B channel in small ROIs that correspond to the centroid of a CSC particle (Figures 6D and 6E). Significantly higher fluorescence signal was detected from 0 to 12 s after the beginning of the pause phase compared with the remaining time points (Figure 6E). The colocalization of EXO70A1-GFP with tdTomato-CESA6 exhibited a similar spatiotemporal pattern, with the association of EXO70A1-GFP signal at the beginning of the pause phase for 12.8 ± 6 s (n = 90 insertion events; Supplemental Figure S6).
We next examined the colocalization of XIK-mCherry with YFP-CESA6 during CSC secretion. Similar to our earlier study (Zhang et al., 2019), we detected transient spatial association of XIK-mCherry with YFP-CESA6 during the erratic phase, when a CSC vesicle initially arrives near the PM, as well as during the first few seconds at the beginning of the pause phase (Figures 6B to 6D). In many qualitative images, partial spatial overlap of the XIK-mCherry and YFP-CESA6 signal was observed, possibly due to the high dynamicity of the XIK-mCherry cytoplasmic signal and the time delay caused by sequential image acquisition at different wavelengths. In addition, the XIK-mCherry fluorescence often appeared as a diffuse signal or formed large clusters near the PM (Figure 6B), making detection of colocalization challenging. To provide compelling quantitative evidence for the spatiotemporal colocalization of XIK with CESA6, we performed fluorescence intensity analysis of small ROIs (Figure 6D) in the XIK-mCherry channel at the CESA insertion sites from numerous events in multiple cells. The results showed that the average signal of XIK-mCherry in the ROIs was significantly higher during the erratic phase and the first 9 s at the beginning of the pause phase than the remainder of the time points (Figure 6F), which supports the specific spatial association of XIK with CSC particles at the early time points. The reason that the XIK-mCherry fluorescence often appeared as large clusters at the CESA insertion sites could be due to association with secretory vesicle clusters or other compartments in the close vicinity.

In contrast to the observation that XIK was associated with the erratically moving vesicles in the cortex before the pause and insertion of CSCs, we failed to detect any significant colocalization of either GFP-SEC5B or EXO70A1-GFP foci with CSCs during the erratic phase (Figures 6A, 6C to 6E; Supplemental Figure S6; Supplemental Movie S5). In yeast, some models suggest that EXO70 and SEC3 directly bind to the PM and other subunits arrive at the PM subsequently by interaction with secretory vesicles (Boyd et al., 2004; Wu and Guo, 2015). Our results suggest that at least the SEC5B or EXO70A1 subunits were not pre-attached to secretory vesicles before PM tethering and fusion; rather, both subunits arrived at the CSC insertion site from the nearby cortex or membrane after the arrival of a CSC compartment and myosin XIK.
However, the temporal resolution (2-s intervals) in our study may be unable to detect transient colocalization events or sequential arrival events. Nevertheless, our results showed that during a CSC insertion event, XIK associated with a CESA compartment from the erratic phase to the initial tethering phase, whereas the exocyst appeared to arrive at a later time, only from the beginning of the tethering phase.

Because XIK associates with CESA compartments in advance of exocyst subunits during exocytosis and because disruption of myosin XI activity reduces the frequency and lifetime of stable exocyst foci at the PM, we tested whether myosin activity is required for the localization and dynamics of exocyst at vesicle tethering sites during CSC delivery. We generated double marked lines expressing GFP-SEC5B tdTomato-CESA6 and EXO70A1-GFP tdTomato-CESA6 in the \( xik-2 \) homozygous background as well as wild-type siblings expressing both reporters. We also applied pre-treatment with PBP for 10 min to acutely inhibit myosin XI activity in wild-type seedlings. The CSC insertion events were tracked in double marked lines and only successful insertion events were quantified, assuming those events rely on the exocyst complex during the vesicle tethering step. The colocalization of exocyst subunits with CSC particles was examined during tethering and only exocyst foci that were continuously present for 2 frames (4 s) or longer at the insertion site from the beginning of the pause phase were considered colocalized. With the GFP-SEC5B tdTomato-CESA6 co-expression line, we detected positive association of SEC5B foci with CESA6 at the beginning of the pause phase in an average of 87% of the insertion events from 3 independent experiments. The reason that the proportion is below 100% could be due to native unmarked exocyst subunits in the transgenic lines that could not be detected, or a small proportion of observed insertion events that was not related to exocytosis. In contrast to untreated wild-type cells, the average percentage of colocalization dropped significantly to 71% and 59% in \( xik-2 \) and PBP-treated wild-type cells, respectively (Figures 7A and 7B). Reduced colocalization was further confirmed with fluorescence intensity analysis. In wild type, the average SEC5B fluorescence intensity was significantly higher from 0 to 12 s at the beginning of the pause phase compared with the rest of the time points (Figure 7C), which was consistent with the average lifetime of exocyst
measured in our earlier results and indicated a high frequency of association of SEC5B with CESA6 during the first 12 s of the pause phase. In contrast, decreased fluorescence intensity was detected in xik-2 and PBP-treated wild-type cells during the first 12 s of the pause phase (Figure 7C). In xik-2, only the 0, 2, and 6 s time points showed significantly higher fluorescence signal, and in PBP-treated wild-type cells, none of the time points tested had significantly higher fluorescence intensity compared with the other time points (Figure 7C), suggesting that there was no significant colocalization of SEC5B foci with CESA6 at the tethering phase in those cells.

The lifetime of GFP-SEC5B at CSC insertion sites was also reduced in xik-2 or PBP-treated wild-type cells, compared with that in untreated wild-type cells (Figures 7A, 7D, and 7E). In the untreated wild type, the distribution of the resident lifetimes of GFP-SEC5B revealed a non-Gaussian distribution and three subpopulations: in a majority of colocalization events (~70%), GFP-SEC5B foci were associated with CESA6 for 8–16 s (defined based on overall mean ± SD of 11.5 ± 5 s for wild type); ~20% of the events only lasted for 4–6 s, and the remaining 10% showed colocalization for 18 s or more (Figures 7D and 7E). However, in xik-2 or PBP-treated wild-type cells, the proportion of colocalization events that had a lifetime of 8–16 s was decreased by 15–20%, whereas the populations with shorter (< 8 s) or prolonged (>16 s) duration of SEC5B association were increased in myosin-deficient cells, compared with that in wild type (Figures 7D and 7E).

Because yeast EXO70 is proposed to mark secretion sites and recruit other subunits together with the secretory vesicle to the PM (Boyd et al., 2004; Wu and Guo, 2015), we examined whether the localization and dynamics of EXO70A1 were affected by myosin XI activity during CSC secretion. Using the EXO70A1-GFP tdTomato- CesA6 co-expression line, we detected a reduced percentage of colocalization of EXO70A1-GFP with CESA6 at the tethering stage in both xik-2 and PBP-treated wild-type cells, comparable to that observed for SEC5B in the previous experiment (Figure 8). The lifetime analysis of EXO70A1-GFP foci at the CSC insertion sites showed that there was an increased proportion of insertion events that had a transient exocyst association of 4 s and the lifetime peaked at 8–10 s in xik-2 and PBP-treated wild-type cells,
compared with that in wild-type cells which had a peak colocalization duration for $12–14$ s (Figure 8). The results suggest that similar to SEC5B, the association and lifetime of EXO70A1 during vesicle tethering is dependent on myosin XI.

Collectively, our data demonstrate that disruption of myosin XI reduced the localization and shortened the lifetime of exocyst subunits at vesicle tethering sites during exocytosis of CSCs. Using CESA as a model cargo, our results confirmed that plant myosins and exocyst complex cooperate to mediate vesicle tethering near the PM and that efficient localization and dynamic behavior of exocyst is dependent upon myosin XI activity, as shown in Figure 9.

DISCUSSION

Myosin XI motors power movement along the actin cytoskeleton and are major contributors to the transport and distribution of intracellular components in plant cells. In a previous study, we demonstrated a new role for myosin XI in vesicle tethering or fusion during delivery of CSCs to the PM, although the exact mechanism was unclear (Zhang et al., 2019). Here, we showed that myosin XIK, the predominant motor driving organelle transport in plant vegetative cells, participates in the vesicle tethering step of exocytosis through direct interactions with the exocyst complex via its globular tail domain (GTD). Specifically, myosin XIK GTD bound directly to exocyst subunits in vitro and a functional fluorescently-tagged XIK colocalized with multiple exocyst subunits at PM-associated stationary foci. Moreover, genetic and pharmacological inhibition of myosin activity reduced the rate of appearance and lifetime of stationary exocyst complexes, which are presumptive sites of vesicle tethering and docking during secretion. Using high spatiotemporal resolution imaging and pair-wise colocalization analysis of myosin XIK, exocyst subunits, and CESA6 in single CSC exocytosis events, we demonstrated that XIK associates with secretory vesicles earlier than exocyst and likely recruits or stabilizes the exocyst at the PM tethering site to initiate vesicle tethering. This study provides insights about the dynamic regulation of exocytosis in flowering plants as well as the role of plant myosin XI in secretion.
A Conserved Role for Myosin XI in Exocytosis

Our results reveal an evolutionarily conserved role for myosin XI that includes direct interaction with exocyst complex subunits and participation in exocytosis, similar to Myo2p in budding yeast. Myo2p delivers secretory vesicles to the growing bud and binds directly to the SEC15 subunit of exocyst through conserved amino acids at its tail cargo-binding domain (Jin et al., 2011). The Rab GTPase SEC4 binds to Myo2p as well as SEC15 and is thought to recruit SEC15 and other exocyst subunits to the secretory vesicle surface (Guo et al., 1999; Jin et al., 2011; Santiago-Tirado et al., 2011). Mutation of SEC15-binding sites on Myo2p results in failed localization of SEC15 to the growing bud tip, suggesting that Rab activity alone is not sufficient for recruiting exocyst to the vesicles and Myo2p is also required for the correct localization of exocyst during exocytosis. Consistent with the yeast model, we showed that during secretion of CSCs, myosin XIK was required for the efficient localization of SEC5B and EXO70A1 to CSC insertion sites. Combined with results that an overall reduction of membrane dwelling events of SEC5B, EXO70A1 and SEC6 were observed in xik or PBP-treated wild-type cells, and evidence that XIK colocalized with newly appeared exocyst subunits at the PM, we propose that XIK may have a general role in recruiting exocyst complex to exocytosis sites, not just for CSC trafficking.

Homology modeling and in vitro protein binding assays reveal Arabidopsis myosin XI and yeast Myo2p GTD share a conserved binding site for exocyst subunits. Here, myosin XIK GTD not only interacted with SEC15B, but also with SEC5B and exhibited a weak interaction with Exo84A in a yeast two-hybrid screen. Myosin XIK GTD did not, however, bind to another exocyst subunit, SEC10, so the interaction is not universal among all exocyst subunits. It is not known whether myosin interacts with additional exocyst subunits in other organisms. On the other hand, because plant myosin XI, exocyst and Rab gene families are all expanded in size compared to that in the yeast and mammalian cells, it is plausible that plants utilize a more complicated and specific interaction network to fulfill the needs of different cell types and trafficking pathways. In yeast, SEC15 directly interacts with the Rab SEC4 as well as Myo2p and the SEC4-Myo2p-SEC15 complex brings together secretory vesicles, motors and the exocyst complex to couple polarized
vesicle transport to exocytosis. In plants, which exocyst subunit is responsible for secretory vesicle binding and which Rab regulates exocyst dynamics remains unknown. Interestingly, Arabidopsis SEC5B also interacts with CESA6 in a yeast two-hybrid assay (Zhu et al., 2018), indicating that SEC5B may have role in secretory vesicle binding in plants. A recent study shows that Arabidopsis SEC15B interacts with STOMATAL CYTOKINESIS DEFECTIVE1 (SCD1) and SCD2, which also interact with RabE1 (Mayers et al., 2017), a close homolog of SEC4 in plants, suggesting a potential interaction network that brings together Rabs, the exocyst complex, and myosin motors in post-Golgi trafficking. Further studies are needed to uncover the functional connection and interaction specificity between myosin motors, exocyst, Rab GTPases and other players in plants.

**Dynamics of the Exocyst Complex During Vesicle Tethering**

Although recent studies show that the exocyst complex plays important roles in many secretion-related processes in plants, such as root hair and hypocotyl elongation, cell division, cell wall deposition, auxin signaling and defense response against pathogens (Synek et al., 2006; Hála et al., 2008; Fendrych et al., 2010; Drdová et al., 2013; Žárský et al., 2013; Vukašinović et al., 2017; Pečenková et al., 2020), the assembly and dynamic regulation of the exocyst complex during vesicle tethering remain enigmatic. Different models for the dynamic assembly of exocyst have been proposed in yeast and mammalian cells. In budding yeast, the EXO70 and SEC3 subunits are localized at the PM through binding with membrane lipids (Boyd et al., 2004; He et al., 2007; Pleskot et al., 2015), whereas other subunits are vesicle-bound and when the vesicle arrives at the secretion sites, the PM-bound subunits interact with the vesicle-bound population to form a holocomplex that tethers the vesicle to the PM (Mei and Guo, 2019). Plant cells may have distinct mechanisms for exocyst assembly during vesicle tethering, however. Using CESA6 as a vesicle marker, we captured single vesicle tethering events and tracked the dynamics of two exocyst subunits, SEC5B and EXO70A1. Both SEC5B and EXO70A1 appeared at the CSC insertion site coincident with stabilization of CSC vesicles at the PM and had a similar average lifetime of 11–12
s; disruption of myosin XI equally affected the localization of the two subunits at PM insertion sites. Further, neither subunit pre-associated with vesicles prior to tethering and docking. These results suggest that EXO70A1 does not pre-exist at the PM secretion site to recruit other vesicle-bound subunits as suggested in the yeast model, and neither SEC5B or EXO70A1 are delivered to the PM on secretory vesicles. Consistent with our findings, a previous study that tracked several exocyst subunits with high resolution VAEM shows that exocyst subunits reside at the PM as dense particles whose density is obviously higher than the expected number of vesicle tethering/exocytosis events, and their localization at the PM is independent of secretory vesicle or exocytosis, because brefeldin A treatment did not affect the density of exocyst foci at the PM (Fendrych et al., 2013).

Exocyst subunit density at the PM is also unaffected by perturbation of cortical actin and microtubules with short-term inhibitor treatment (Fendrych et al., 2013). Similarly, we showed that the overall density and distribution pattern of SEC5B, EXO70A1, and SEC6 foci at the PM were unchanged in xik or cells with short-term PBP or LatB treatment, even though those cells were supposed to have a reduction in overall exocytosis rate (Zhang et al., 2019). Based on these data, it is highly likely that the EXO70 and SEC3 subunits do not function as landmark proteins to recruit other subunits during vesicle tethering; instead, most plant exocyst subunits pre-exist at the PM regardless of the presence of vesicles, and are transiently recruited to the secretion sites when vesicles arrive at the PM.

Fendrych et al. (2013) also propose that exocyst foci at the PM represent pre-assembled complexes, although direct evidence is lacking. Several recent studies in yeast and mammalian cells suggest that exocyst can assemble into two stable subcomplexes (SEC3-SEC5-SEC6-SEC8 and SEC10-SEC15-EXO70-EXO84) and a new model shows that upon arrival of secretory vesicles to the PM, the two subcomplexes are triggered to assemble into a holocomplex to tether the vesicle to the membrane (Heider et al., 2016; Ahmed et al., 2018; Mei et al., 2018; Mei and Guo, 2019). In plants, the exocyst subunits may reside at the PM either as pre-assembled complexes, or are assembled immediately upon arrival of the secretory vesicle at the PM sites,
and in either case, the subunits are recruited to the secretion sites by myosin motors and possibly other molecular players on the vesicle surface. Further, we show that myosin XI affects the lifetime of exocyst at the PM tethering site during CSC insertion.

Our results provide the first detailed study of exocyst tethering time, as verified by the insertion of a functional cellulose-synthesizing complex into the PM. We observed, based on the distribution of lifetimes for both SEC5B and EXO70A1, that there are three types of association of exocyst during CSC insertion events. The majority have an association for 10–16 s and likely represent standard tethering events. This duration is very similar to exocyst residency time of 12–18 s during exocytosis in yeast and mammalian cells (Donovan and Bretscher, 2015; Ahmed et al., 2018), indicating a conserved vesicle tethering/fusion process in all eukaryotes. A short exocyst association of 4–6 s was also observed in ~20% of the events, likely representing a short-lived or aborted exocyst complex that failed to form a mature complex, similar to that reported for the formation of clathrin-coated pits for endocytosis (Loerke et al., 2009). Notably, we found that this short-lived population was significantly increased in cells with compromised myosin XI activity. Similarly, the average lifetime of several subunits was reduced by 1–2 s in myosin-deficient cells. Although the detailed mechanism is unclear, one possibility is that myosin XI is required for the formation of stable exocyst complexes at the PM. A third population of exocyst (~10%) has a lifetime of 20 s or longer at CSC tethering sites; however, it is unclear whether those represent prolonged or defective vesicle tethering or fusion events.

Our data showed that inhibition of myosin XI results in modest defects of exocyst localization or CSC insertion at the PM, suggesting that other players, such as Rab GTPases or cargo proteins on vesicle surface, may also coordinate exocyst recruitment and dynamic function during secretion. CESA6 interacts with SEC5B in a yeast two-hybrid assay, supporting a role for cargo proteins in recruiting or stabilizing the exocyst complex at the PM–vesicle interface (Zhu et al., 2018). A plant specific protein PATROL1 (PTL1) directly interacts with SEC10 and is required for the exocytosis of CSCs (Zhu et al., 2018). PTL1 arrives at the CSC insertion sites 1–2 s later than SEC5B and genetic disruption of PTL1 did not affect SEC5B dynamics, suggesting a role in a
later step during exocytosis. Another protein with a probable role during the fusion step of exocytosis, the Sec1/Munc18-related protein KEULE, interacts with SEC6 during cell plate formation (Wu et al., 2013). Finally, interactions between SNAREs and exocyst subunits have been demonstrated in plants (Larson et al., 2020). Collectively, these data suggest that several multiprotein complexes must cooperate in a precise spatiotemporal manner to build the macromolecular machines that execute vesicle tethering, docking, and fusion.

**An Expanded Model for Post-Golgi Trafficking Coordinated by the Cytoskeleton**

Although the actin–myosin XI transport network plays a predominant role in long-distance organelle and vesicle movement, its role in local post-Golgi or secretory vesicle trafficking is less clear (Nebenführ and Dixit, 2018). In tip-growing cells, actin and myosin XI are proposed to directly regulate the targeted delivery of secretory vesicles to the growing apex (Park and Nebenführ, 2013; Madison et al., 2015; Orr et al., 2020; Orr et al., 2021), whereas in diffusely-growing cells, a similar role has not been established. Our results from diffusely-growing Arabidopsis epidermal cells demonstrate that myosin XI and actin play a direct and active role in the final steps of secretory vesicle trafficking by recruiting the exocyst complex and mediating vesicle tethering at the PM, rather than during an early vesicle transport step (Figure 9).

Although myosin XIK was shown to be responsible for the motility of several compartments potentially involved in secretion, such as the trans-Golgi network, putative secretory vesicles, and endosomes (Avisar et al., 2012; Peremyslov et al., 2015), it is not known whether myosin binds directly to these compartments or mediates the targeted delivery of these compartments to specific PM regions for secretion. Instead, indirect or passive models are favored by some; specifically, it is proposed that vesicles and organelles move passively with the hydrodynamic flow generated by myosin XI-driven cytoplasmic streaming (Peremyslov et al., 2013; Buchnik et al., 2015; Nebenführ and Dixit, 2018). Evidence supporting these indirect models came from the study of a major group of plant-specific myosin receptors, MyoBs, which attach myosin XI to a specific
type of small endomembrane compartment to drive rapid cytoplasmic streaming (Peremyslov et al., 2013; Peremyslov et al., 2015).

Specific secretory vesicle receptors, such as Rab GTPases, that directly link myosin XI to cargo and function in secretory vesicle transport have not been identified in flowering plants. One study in Arabidopsis identified RabD1 and RabC2a using a yeast two-hybrid screen with myosin XI2 tail; however, RabC2a localizes to peroxisomes and RabD mainly mediates ER to Golgi trafficking (Zheng et al., 2005; Hashimoto et al., 2008). A recent study identified RabE as a myosin XI partner in the moss Physcomitrium (Physcomitrella) patens and their interaction is important for polarized growth (Orr et al., 2021). Further investigations are required to determine whether this interaction is conserved across different plant species and also functions in diffusely-growing cells.

A Golgi localized RabH GTPase, RabH1b, has been shown to mediate the secretion of CSCs likely through regulating Golgi to PM trafficking; however, it is not known whether this process involves interaction with cytoskeletal motors (He et al., 2018).

Finally, a wealth of evidence indicates a role for cortical microtubules and possibly kinesin motors, rather than actin and myosin, in mediating post-Golgi trafficking of secretory vesicles (Nebenführ and Dixit, 2018; Elliott et al., 2020). Cortical microtubules and a kinesin-4 motor are implicated in delivery of non-cellulosic cell wall components in Arabidopsis (Kong et al., 2015; Zhu et al., 2015). Cortical microtubules also play an important role in CSC delivery by marking insertion sites through the linker protein CELLULOSE SYNTHASE INTERACTIVE1 (CSI1) and can interact with small CSC compartments that may be responsible for delivery or recycling of CSCs at the PM (Gutierrez et al., 2009; Bringmann et al., 2012; Li et al., 2012; Lei et al., 2015). However, pharmacological removal of microtubules or genetic disruption of CSI1 has no effect on CSC delivery rate to the PM (Gutierrez et al., 2009, Zhu et al., 2018, Zhang et al. 2019), indicating that CSI1 and microtubules may only serve as landmarks for the targeting of vesicles to the cortex and are not essential for subsequent steps like tethering and fusion with the PM. In the act2 act7 mutant as well as cells treated with actin or myosin inhibitors, a substantial reduction of CSC delivery/exocytosis rate was detected; however, the preferential positioning of CSCs to cortical
microtubule sites was not affected, suggesting that the targeting of CSCs to cortical microtubules is independent of actin and myosin XI activity, precedes it in time and space, or both (Sampathkumar et al., 2013; Zhang et al., 2019).

In this study, significantly higher signal of myosin XIK coincident with CESA compartments was detected as early as 9 s before they arrive at PM insertion sites. Since the targeting of CSCs to cortical microtubules is mediated by CSI1 (Zhu et al., 2018) and disruption of actin or myosin did not affect this targeting, myosin XIK on the vesicle surface may not have a major role in vesicle transport per se. It is likely that the force generated by cytoplasmic streaming is sufficient to propel the vesicles to the cortex or PM, or the CESA-containing Golgi transported along actin by myosin XI are already in close proximity to the PM to deliver newly formed secretory compartments. Indeed, previous models ascribe an indirect role for actomyosin in CSC delivery by maintaining a uniform global distribution of Golgi in the cortical cytoplasm of plant cells (Gutierrez et al., 2009; Sampathkumar et al., 2013). Nevertheless, this study and recent work suggest that class XI myosins contribute to CSC delivery at two levels: one function is to power cytoplasmic streaming and cell-wide transport of Golgi bodies to the cortex or cortical microtubule sites, and once the delivery compartments are anchored to cortical microtubules, a second function of myosin XI is to cooperate with the exocyst complex and other players to facilitate efficient membrane tethering and fusion (Figure 9). This model is consistent with fluorescent-tagged functional myosin XIK exhibiting two major locations in cells (Peremyslov et al., 2012; Zhang et al., 2019): one population displays a “beads-on-a-string” pattern to power cytoplasmic streaming and long-distance transport of organelles along actin cytoskeleton, whereas another population is more diffusely distributed in the cortical cytoplasm and is transiently associated with secretory vesicles to fulfill a conserved role in membrane tethering and exocytosis. Collectively, our study sheds fresh light on the spatiotemporal coordination of cytoskeleton and motors in regulating post-Golgi trafficking in plants, and helps uncover the evolutionarily conserved and divergent regulation of exocytosis across kingdoms.
METHODS

Plant Materials and Growth Conditions

The Arabidopsis myosin xi1 xi2 xik triple knock-out (xi3KO) mutant and xi3KO expressing YFP-CESA6 in the homozygous prc1-1 background were characterized previously (Peremyslov et al., 2010; Zhang et al., 2019). The xi1, xi2, and xik-2 homozygous single mutant lines expressing YFP-CESA6 in the presence of prc1-1 were recovered from the same cross that resulted in the previously characterized xi3KO YFP-CESA6 prc1-1 lines (Zhang et al., 2019). The T-DNA insertion mutants xik-1 (SALK_136682), xik-2 (SALK_067972), xi1 (SALK_019031) and xi2 (SALK_055785) used in cellulose content assays were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Transgenic Arabidopsis thaliana Col-0 lines expressing EXO70A1-GFP and SEC6-GFP were described previously (Fendrych et al., 2010). To prepare GFP-SEC5B expressing lines, the Ubiquitin 10 promoter in the pUBN-GFP-DEST vector was restriction digested with Sacl and SpeI and replaced with the SEC5B native promoter (2000 bp upstream of the SEC5B start ATG) using the NEB Gibson assembly master mix kit (MA, USA) to obtain a modified pSEC5BN-GFP-DEST vector. SEC5B promoter was amplified from Col-0 genomic DNA by primers TGACCATGATTACGAATTCGAGCTCTGTATTGAAACCCAAAATAT and CTCGCCCTTGCTCACCATACTAGTTGTATCTGACTTAGATG. Then, the full-length CDS for SEC5B was cloned into the modified binary vector pSEC5BN-GFP-DEST using the Gateway system to obtain the final GFP-SEC5B expression vector. The XIK-mCherry construct, which carries a genomic copy of the XIK gene and a C-terminal mCherry fusion driven by the native promoter (Peremyslov et al., 2012), was kindly provided by Valerian V. Dolja (Oregon State University).

Double-marked lines were generated either by Agrobacterium-mediated transformation through floral dip or by crossing. The double-marked line for XIK-mCherry and EXO70A1-GFP was prepared by transforming the XIK-mCherry construct into plants expressing EXO70A1-GFP in the homozygous xik-2 mutant background. For the XIK-mCherry and GFP-SEC5B double-marked line, XIK-mCherry in homozygous xik-2 was crossed with GFP-SEC5B lines. For the double-
marked line expressing GFP-SEC5B and tdTomato-CESA6, the GFP-SEC5B was transformed into plants expressing tdTomato-CESA6 in homozygous prc1-1 background (Sampathkumar et al., 2013). The double-marked line of EXO70A1-GFP and tdTomato-CESA6 was generated by crossing. For plants co-expressing tdTomato-CESA6 and exocyst markers in the homozygous xik-2 background, EXO70A1-GFP or GFP-SEC5B was crossed with tdTomato-CESA6 in homozygous xik-2 and the F3 generation of xik-2 homozygous plants expressing both markers was recovered. For plants co-expressing XIK-mCherry and YFP-CESA6, the XIK-mCherry construct was transformed into plants expressing YFP-CESA6 in homozygous xik-2 and prc1-1 mutant background.

Arabidopsis seeds were surface sterilized and stratified at 4°C for 3 d on half-strength Murashige and Skoog medium supplemented with 0.8% agar. For light growth, plants were grown in a growth chamber with a light intensity of 120–140 μmol m⁻² s⁻¹ provided by Philips F32T8/L941 Deluxe Cool White bulbs, under long-day conditions (16 h light/8 h dark) at 21°C. For dark growth, plates were exposed to light for 4 h and then placed vertically and kept at 21°C in continuous darkness.

Live-Cell Imaging

For most experiments, epidermal cells from the apical region of 3-d-old etiolated hypocotyls were imaged, unless otherwise stated. Spinning-disk confocal microscopy (SDCM) was performed using a Yokogawa scanning unit (CSU-X1-A1; Hamamatsu Photonics) mounted on an Olympus IX-83 microscope, equipped with a 100x 1.45–numerical aperture (NA) UPlanSApo oil objective (Olympus) and an Andor iXon Ultra 897BV EMCCD camera (Andor Technology). YFP, GFP, and mCherry/tdTomato fluorescence were excited with 514-nm, 488-nm, and 561-nm laser lines and emission collected through 542/27-nm, 525/30-nm, and 607/36-nm filters, respectively. For measuring the abundance of plasma membrane (PM)-localized YFP-CESA6, time-lapse images were collected at the PM with a 2-s interval for 5 frames. For quantifying the abundance of cortical and subcortical CSC vesicles, z-series at 0.2 μm step sizes plus time-lapse with 1.6-s intervals for
10 frames were collected. For dual-wavelength imaging of GFP-SEC5B or EXO70A1-GFP with XIK-mCherry with SDCM, time-lapse images were collected at the PM focal plane with 1-s intervals for 2 min. For dual-wavelength imaging of GFP-SEC5B or EXO70A1-GFP with tdTomato-CESA6, time-lapse images were collected at the PM focal plane with 2-s intervals for 10 min. For dual-wavelength imaging of XIK-mCherry with YFP-CESA6, time-lapse images were collected at the PM focal plane with 3-s intervals for 10 min. For all dual-wavelength image acquisition, single-marked lines were tested initially to make sure there was no bleed through in each channel.

For imaging of single-marked lines of EXO70A1-GFP, SEC6-GFP and GFP-SEC5B, variable-angle epifluorescence microscopy (VAEM) was performed using a total internal reflection fluorescence (TIRF) illuminator on an IX-71 microscope (Olympus) equipped with a 150x 1.45–NA PlanApo TIRF objective (Olympus) and an EMCCD camera (ORCA-EM C9100-12; Hamamatsu Photonics). GFP fluorescence was excited with a 488-nm laser at 5% power and time-lapse images were collected at the PM focal plane with 0.5-s intervals for 1 min.

Fluorescence recovery after photobleaching (FRAP) experiments were performed as described previously (Zhang et al., 2019).

Image Processing and Quantitative Analysis

Image processing and analysis were performed with Fiji Is Just ImageJ (Schindelin et al., 2012). The YFP-CESA6 related assays, including CSC density, delivery rate, cortical and subcortical CESA compartment density, and single CSC insertion assays, were performed as described previously (Zhang et al., 2019). For CSC delivery rate analysis from FRAP images, newly delivered CSCs in the photobleached region during the first 5 min of recovery were counted. Only the particles that exhibited a linear steady movement pattern in subsequent frames were considered as functional CSCs and counted as new delivery events (Gutierrez et al., 2009). The CSC delivery rate was calculated as the number of delivery events divided by the measured area and time. Cortical and subcortical CESA compartment abundance was counted manually in 3D time-lapse series taken with 1.6-s intervals for 10 frames. The vesicles in the first frame that
displayed continuous mobility in the following frames (to be distinguished from the PM-localized CSCs which had no mobility within a short time frame) and had a size smaller than a Golgi or TGN compartment were counted as cytoplasmic CESA compartments.

For exocyst subunit dynamics assay, the density of PM-localized exocyst foci was measured with the TrackMate plugin using the first frames of the time-lapse images. The Laplacian of Gaussians (LoG) detector was used and the estimated particle diameter was 4 pixels and the threshold set to 10. Stationary exocyst foci were tracked with TrackMate using the Simple LAP tracker with the maximal linking distance and gap-closing distance set to 1 pixel, which allowed the detection of foci that had no lateral motility. The rate of appearance of stationary foci was calculated as the number of stationary foci divided by the measured area and elapsed time. For fluorescence intensity measurements of stationary foci at the PM, a fixed ROI of 3 X 3 pixels at the centroid of the foci was selected and analyzed over a time course. The first frame in which a new particle appears was set as 0 s. The fluorescence intensity of the ROI was measured from 6 frames (-3 s) prior to the first appearance of the particle to a few seconds after the foci fully disappeared. For normalization, the fluorescence intensity of the ROI in each frame was divided by the average fluorescence intensity from the first 6 frames prior to the appearance of the particle, assuming that the fluorescence in these early time points represents background signal. The lifetime of exocyst foci was measured by analysis of kymographs prepared from the time series. To avoid sampling bias, a 100 X 100 pixel² area was randomly selected at the PM and every stationary particle within the region in the time series was analyzed for lifetime. The Stack Reslice function in ImageJ was used to generate kymographs that covered the trajectories of every particle in the image stack. A stationary particle was defined as a straight vertical line in the kymograph that could be tracked for at least 5 frames (>2 s). The pixel length of the vertical lines in the kymographs was measured and converted into lifetime.

For colocalization analysis of XIK-mCherry with GFP-SEC5B or EXO70A1-GFP, individual exocyst foci were tracked at the PM plane over a time course. Only stationary foci that could be tracked for at least 5 frames (>4 s) were analyzed. For fluorescence intensity analysis, a fixed
ROI of 3 X 3 pixels at the centroid of the foci was selected and the ROI was measured from 6 frames (−6 s) prior to the first appearance of the particle (0 s) to a few seconds after the foci fully disappeared. The same ROI in the corresponding XIK-mCherry channel was measured for fluorescence intensity at every time point. For normalization, the relative fluorescence intensity of GFP-SEC5B or EXO70A1-GFP was calculated as the fluorescence intensity of the ROI in each frame divided by the average fluorescence intensity of ROIs from the 6 frames prior to the first appearance of the particle, assuming that the fluorescence in those time points represents background signal. The relative fluorescence intensity of XIK-mCherry was calculated as the fluorescence intensity of the ROI in each frame divided by the average fluorescence intensity of ROIs from the last 6 frames in the time course when the foci were fully disappeared, assuming that the myosin signal in those time points represents random fluorescence.

The colocalization analysis of CESA6 with exocyst markers or myosin XIK was performed as previously described (Zhang et al., 2019). Individual CSC insertion events were tracked from erratic phase, through the pause phase, and into the beginning of the steady movement phase. Fluorescence intensities of ROIs (mean gray value) in the corresponding exocyst or XIK channel were measured at each time point. The background fluorescence intensity was removed and the fluorescence intensity was normalized against the average intensity of ROIs from the steady movement phase as previously described (Zhang et al., 2019). The lifetime of exocyst foci at the CSC insertion site was also estimated based on the fluorescence intensity profiles of individual exocyst ROIs. The lifetime was calculated as the beginning of the exocyst signal to the time point at which half decay of the peak intensity relative to the baseline intensity occurred. For fluorescence intensity analysis of XIK-mCherry, photobleaching was corrected by dividing the mean gray value of ROIs with the mean gray value of the whole cell in each analyzed frame, and then the derived values were divided by the average intensity of ROIs from the steady movement phase for normalization.

Yeast Two-Hybrid Assay
The cDNAs encoding Arabidopsis exocyst subunits were cloned into the prey vector pGADT7. The cDNA encoding the globular tail domain (GTD) of myosin XIK (amino acids 1055-1531) was cloned into the bait vector pGBKT7. The bait and prey vectors were co-transformed into yeast AH109 strain and the positive clones were selected on SD medium lacking Trp, Leu and His and supplemented with 4 mM 3-amino-1,2,4-triazole. For negative controls, the XIK GTD bait vector was co-transformed with the empty pGADT7 vector and the empty bait vector was co-transformed with each prey vector containing the exocyst subunits.

Homology Models
The three-dimensional structure of myosin XIK GTD was predicted with the I-TASSER server (Yang and Zhang, 2015), SWISS-MODEL (Waterhouse et al., 2018), and the Phyre2 server (Kelley et al., 2015), using the Saccharomyces cerevisiae Myo2p cargo binding domain (PDB ID: 2F6H) as template. Visualization and display of modeled structures was accomplished with PyMOL Molecular Graphics System (Version 2.0 Schrödinger, LLC). Sequence alignments were conducted and displayed using JalView software (Waterhouse et al., 2009).

Protein Pull-Down Assay
The cDNA encoding the GTD (amino acids 1055–1531) of myosin XIK was amplified and cloned into pGEX-4T-3 to generate an N-terminal GST fusion. The wild-type XIK GTD GST fusion construct was used as template for site-directed mutagenesis with the Q5 Site-Directed Mutagenesis Kit (New England BioLabs) to generate single or double amino acid substitution mutations. Primers used for cloning and site-directed mutagenesis are listed in Supplemental Table S1. The full-length cDNAs for SEC5B, SEC15B and SEC10 were cloned into the pRSF-Duet vector to generate N-terminal His6 fusions. Constructs were transformed into Escherichia coli BL21 (DE3) and fusion protein expression induced with 0.2 mM isopropylthio-β-galactoside at 16°C for 20 h. The GST and GST-XIK GTD fusion proteins were extracted with Pierce Immobilized Glutathione resin (Thermo Fisher Scientific) with the protein interaction buffer (20 mM Hepes-KOH
pH 7.2, 50 mM potassium acetate, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5% glycerol, 0.5% Triton-100) as lysis and wash buffer. The His6-tagged SEC5B, SEC15B, and SEC10 fusion proteins were extracted and purified with Ni-NTA His bind resin (Novagen) following standard procedures. The freshly extracted resin-bound GST or GST-XIK GTD proteins were mixed with purified His6-tagged exocyst proteins in protein interaction buffer at a stoichiometry of 1:1 and incubated at 4°C for 1 h with shaking. For quantitative pull-down assays of SEC5B with wild-type or mutated forms of GST-XIK GTD, resin-bound GST-tagged proteins (0.2 µM) were incubated with 1.5 µM His6-SEC5B in 500 µL protein interaction buffer at 4°C for 1 h with shaking. This represents a 1.3:1 stoichiometry of XIK-GTD to SEC5B, assuming 10% full length His6-SEC5B in the purified fraction. The resin was washed 3 times with protein interaction buffer followed by boiling in SDS/PAGE sample buffer for 10 min, and then analyzed with SDS-PAGE followed by Coomassie blue staining. The protein levels in SDS-PAGE gels were quantified by densitometry using ImageJ from multiple biological repeats.

Cellulose Content Measurement
Five-d-old dark-grown hypocotyls were used for cellulose content assay. The alcohol-insoluble cell wall material was generated and hydrolyzed with acetic-nitric (AN) reagent or trifluoroacetic acid (TFA) based on the Updegraff method (1969) as described previously (Zhang et al., 2019). The insoluble fractions were then measured by a phenol-sulfuric colorimetric assay (Dubois et al., 1956) to determine the cellulose amount in the samples.

Statistical Analysis
One-way ANOVA with Tukey’s post hoc tests were performed in SPSS (Version 25) to determine significance among different treatments. Test results and parameters are given in Supplemental Dataset S1. Chi-square tests were used for statistical comparison of data that did not follow parametric distributions and the P values were calculated in Excel 15.32.
The X-bar and S Control Chart tests were used for the statistical analysis of the fluorescence intensity variations of XIK or exocyst markers during particle insertion/secretion processes in the colocalization analysis. The mean and standard deviation of fluorescence intensities were calculated using all data points presented in the graphs. The upper control limit (UCL) was determined based on the mean and standard deviation of all data points using the equation at a P-value < 0.05 (Montgomery, 2009):

\[ UCL = \bar{x} + 2 \left( \frac{s}{c_4 \sqrt{n}} \right) \]

The assumption is that the fluorescence intensities within the control limit represent random or background signals, while any intensity higher than the control limit was considered as significantly different, and indicates specific association of the signal at the given time point and location.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *Myosin XIK*, At5g20490; *Myosin XI1*, At1g17580; and *Myosin XI2*, At5g43900; *SEC3A*, At1g47550; *SEC5A*, At1g76850; *SEC5B*, At1g21170; *SEC6*, At1g71820; *SEC8*, At3g10380; *SEC10*, At5g12370; *SEC15A*, At3g56640; *SEC15B*, At4g02350; *EXO70A1*, At5g03540; *EXO84A*, At1g10385; *EXO84B*, At5g49830; *EXO84C*, At1g10180.

**Supplemental Data**

**Supplemental Figure S1.** Homology Model of Myosin XIK GTD Shows Conservation of the Exocyst Binding Site.

**Supplemental Figure S2.** Dynamic Behavior of EXO70A1-GFP Is Altered in xik-1.

**Supplemental Figure S3.** Dynamic Behavior of GFP-SEC5B Is Altered upon Inhibition of Myosin.

**Supplemental Figure S4.** Dynamic Behavior of Exocyst Subunits Is Altered upon Inhibition of Myosin and Actin.

**Supplemental Figure S5.** Myosin XIK Transiently Colocalizes with Stationary Foci of EXO70A1.
Supplemental Figure S6. EXO70A1 Transiently Colocalizes with CESA6 during the Vesicle Tethering Step of Secretion.

Supplemental Table S1. Primers Used for Molecular Cloning and Site-Directed Mutagenesis.

Supplemental Movie S1. A CSC Insertion Event at the PM.

Supplemental Movie S2. EXO70A1-GFP Distribution and Dynamic Behavior at the PM.

Supplemental Movie S3. Colocalization of GFP-SEC5B and XIK-mCherry Near the PM.

Supplemental Movie S4. Colocalization of GFP-SEC5B and XIK-mCherry during a Single SEC5B Arrival Event at the PM.

Supplemental Movie S5. Colocalization of GFP-SEC5B and tdTomato-CESA6 during a CSC Insertion Event at the PM.

Supplemental Dataset S1. ANOVA Results and Parameters.

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AUTHOR CONTRIBUTIONS
W.Z., L.H., C.Z., and C.J.S. designed the research. W.Z. and L.H. performed the experiments and analyzed the data. W.Z. and C.J.S. wrote the article.

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Figure Legends

Figure 1. Myosin XIK Is the Major Myosin Isoform Involved in Cellulose Biogenesis and CESA Trafficking.  
(A, B) XIK contributes to cellulose production. Ethanol-insoluble cell wall material (CWM) was prepared from 5-d-old etiolated hypocotyls of wild-type (WT) seedlings, myosin xi3KO, xik-1, xik-2, xii1, and xii2 mutants. The non-cellulosic component of CWM was hydrolyzed with 2 M trifluoroacetic acid (TFA; A) for total cellulose determination, or with acetic nitric reagent (AN; B) for crystalline cellulose determination. Cellulose content was significantly reduced in xi3KO, xik-1, and xik-2 mutants compared to that in WT, xii1, and xii2 mutants. Values given are means ± SE (n = 4; One-way ANOVA with Tukey’s post hoc test, letters [a-c] denote samples/groups that show statistically significant differences from other groups, P < 0.05).  
(C, D) XIK is necessary for the abundance of CSCs at the PM. Representative single-frame images show the plasma membrane (PM) of hypocotyl epidermal cells expressing YFP-CESA6 imaged with spinning disk confocal microscopy (C). Bar = 5 µm. Quantitative analysis shows that the density of CSC at the PM was significantly reduced in xi3KO, xik-1, xik-2, and xii2, but not in xii1 (D). Values given are means ± SE (n > 60 cells from 12 hypocotyls per genotype; One-way ANOVA with Tukey’s post hoc test, letters [a-d] denote samples/groups that show statistically significant differences from other groups, P < 0.05).  
(E, F) Loss of XIK reduces the rate of delivery of CSCs to the PM. Representative single-frame images of PM-localized CSC particle recovery after photobleaching. A region of interest at the PM was photobleached and the number of newly delivered CSCs were counted in a subarea within the region (yellow dashed box, E). Bar = 5 µm. The rate of delivery of CSCs to the PM was calculated from the total number of newly delivered CSCs during the initial 5 min of recovery divided by the measured area and time (F). The CSC delivery rate was significantly inhibited in xi3KO, xik-1, xik-2, and xii2, but not in xii1. Values given are means ± SE (n = 9–12 cells per genotype; One-way ANOVA with Tukey’s post hoc test, letters [a-d] denote samples/groups that show statistically significant differences from other groups, P < 0.05).

Figure 2. Myosin XIK Is Involved in Exocytosis of CSCs.  
(A, B) Loss of XIK results in increased abundance of cortical vesicles containing CESA6. Representative single images taken at cortical and subcortical focal planes in hypocotyl epidermal cells show cytoplasmic CESA compartments (magenta circle) in WT, xi3KO, and xik (A). Bar = 5 µm. Quantitative analysis of vesicle density shows that the number of CESA compartments was increased significantly in the cortical but not in the subcortical cytoplasm for xi3KO, xik-1, and xik-2 compared to WT siblings (B). Values given are means ± SE (n > 25 cells from 12 seedlings for each genotype; One-way ANOVA with Tukey’s post hoc test, letters [a-c] denote samples/groups that show statistically significant differences from other groups, P < 0.05).  
(C-F) XIK is necessary for CSC tethering and fusion at the PM. Representative images show a typical CSC insertion event at the PM (C). A CESA particle (yellow arrowhead) arriving in the cortex initially undergoes erratic motility, which likely represents a delivery vesicle (V) that is transported to an exocytosis site. The particle then pauses (marked as 0 s) and exhibits a static or pause phase for ~80 s in a fixed position, which likely corresponds to tethering, docking and fusion of the delivery compartment to the PM. After the CSC particle is inserted, it shows steady movement in the PM as an active complex. T: tethering proteins. Bar = 1 µm.  
(D) Representative kymographs show five categories of insertion events from left to right: standard insertion with normal pause time; insertion that has a shorter pause time; insertion that has a longer pause time; a shorter pause time and failure to insert; and, a longer pause time and failure to insert. Pause phases are marked with magenta dashed lines and steady movement phases are marked with green dashed lines. Bar = 1 µm.  
(E) Distribution of pause times during CSC insertion at the PM in WT and mutant hypocotyl epidermal cells. Values given are means ± SE from 3 biological repeats (A total of 316, 331, 392, and 356 events were measured in WT, xi3KO, xik-1, and xik-2 cells, respectively; Chi-square test,
letters [a-c] denote samples/groups that show statistically significant differences from other groups, P < 0.05).

(F) The proportion of five types of insertion events described in (D) in WT and mutant hypocotyl epidermal cells. A shorter or longer pause time was defined as the mean value (84 ± 30 s) of particle pause time in WT minus (<54 s) or plus one standard deviation (>114 s), respectively. Values given are means ± SE from 3 biological repeats (Chi-square test, letters [a-c] denote samples/groups that show statistically significant differences from other groups, P < 0.05).

![Figure 3](https://example.com/figure3.png)

**Figure 3.** The Globular Tail Domain (GTD) of Myosin XIK Interacts Directly with Exocyst Subunits.

(A) Yeast two-hybrid results using XIK GTD as bait and exocyst subunits as prey. The co-transformed colonies were grown on SD-Trp-Leu (−TL) plates or SD-Trp-Leu-His plates supplemented with 4 mM 3-amino-1,2,4-triazole (−TLH 3AT). Growth of colonies on −TLH 3AT plates when SEC5B and EXO84A were used as prey indicated interaction with XIK GTD.

(B) In vitro protein pull-down assay demonstrates direct interaction between XIK GTD and several exocyst subunits. Purified, recombinant His6-tagged SEC5B and SEC15B, but not SEC10, co-sedimented with purified XIK GTD fused to a GST tag (GST-XIK) but not with purified GST alone.

(C) Quantification of the percentage of His6-tagged SEC5B, SEC15B and SEC10 proteins that co-sedimented with GST-XIK GTD relative to the total input in each reaction evaluated by densitometric analysis of protein gels. Values given are means ± SE from 3 biological replicates (One-way ANOVA with Tukey’s post hoc test, letters [a,b] denote samples/groups that show statistically significant differences from other groups, P < 0.001).

(D) Homology model of XIK GTD using the *Saccharomyces cerevisiae* Myo2p GTD structure as template for the prediction of potential exocyst binding sites on Arabidopsis Myosin XIK. The structure displayed was generated using the I-TASSER server and additional structures using other methods and software packages can be found in Supplemental Figure S1. The predicted surface residues of XIK GTD that are homologous to the exocyst binding sites of Myo2p (R1402 and K1473) are shown in cyan. A third residue, K1103 (magenta), was chosen as a negative control for site-directed mutagenesis and protein pull-down experiments.

(E) In vitro protein pull-down assay between SEC5B and wild-type (WT) or mutated forms of XIK GTD. The single or double amino acid mutations of R1366 and R1441 on XIK GTD showed reduced binding with His6-SEC5B in vitro, when compared with WT XIK GTD or the K1103A mutant.

(F) Quantification of His6-SEC5B protein levels by densitometric analysis of gels from multiple independent protein pull-down assays as shown in (E). The single or double mutation of R1366 and R1441 resulted in significantly reduced binding with SEC5B in vitro. Values given are means ± SE from 6 biological replicates (One-way ANOVA with Tukey’s post hoc test, letters [a-d] denote samples/groups that show statistically significant differences from other groups, P < 0.001).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Inhibition of Myosin Activity Alters the Dynamic Behavior of EXO70A1 at the PM.

(A) Representative single frame images show distribution of exocyst subunit EXO70A1-GFP at the PM in 3-d-old etiolated hypocotyl epidermal cells imaged with variable-angle epifluorescence microscopy. Kymographs reveal the presence of stationary EXO70A1-GFP foci in time-lapse series. There were fewer stationary foci in xik-2 and cells treated with PBP for 15 min, and their lifetime appeared shorter compared to WT cells. Dashed lines indicate where the kymographs were generated. Bar = 5 μm.

(B) Quantitative analysis of fluorescence intensity for newly appearing stationary foci of EXO70A1-GFP at the PM. The zero timepoint was defined as the first frame in which a new particle appears. The fluorescence intensity was normalized against the average fluorescence intensity from the first 6 frames prior to the appearance of the particles. Values given are means ± SE (n = 71, 88 and 78 particles in untreated WT and xik-2 cells and PBP-treated WT cells, respectively).
(C–E) Quantitative analysis shows that the density of total EXO70A1-GFP foci remains similar in untreated xik-2 cells and PBP-treated WT cells (C); however, the rate of appearance (D) and lifetime (E) of stationary foci were significantly reduced compared with that in WT cells. Values given are means ± SE (n = 20–30 cells from 10 seedlings per genotype or treatment; for lifetime assay, n = 71, 88 and 78 particles in WT, xik-2 and PBP-treated cells, respectively; One-way ANOVA with Tukey’s post hoc test, letters [a, b] denote samples/groups that show statistically significant differences from other groups, P < 0.001).

Figure 5. Myosin XIK Transiently Colocalizes with Stationary Foci of SEC5B at the PM. (A) Representative time series show arrival of a GFP-SEC5B particle (white boxes, green in merged images) at the PM that was colocalized with XIK-mCherry (magenta) in an etiolated hypocotyl epidermal cell imaged with spinning disk confocal microscopy. The images and corresponding kymographs show that colocalization occurred transiently in the first few seconds upon arrival of the GFP-SEC5B particle. Bars = 1 µm. (B) Representative time series show localization of a GFP-SEC5B particle (white boxes, green in merged images) and cytoplasmic mCherry (magenta) signal near the PM. The images and corresponding kymographs show that the mCherry signal was constantly present at low levels throughout the entire time course without showing any specific association with the GFP-SEC5B particle. Bars = 1 µm. (C, D) Quantitative analysis of fluorescence intensity for newly appearing stationary foci of GFP-SEC5B at the PM in the GFP channel and the corresponding XIK-mCherry (C) or cytoplasmic mCherry signal (D) in the mCherry channel. There was significantly higher fluorescence intensity of XIK-mCherry that peaked at 0 s and lasted for 4 s after the appearance of new GFP-SEC5B particles. In contrast, there were no significant changes in fluorescence intensity of cytoplasmic mCherry that correspond to newly arrived GFP-SEC5B particles. Values given are means ± SE (For intensity assay in C, n = 71 particles; for intensity assay in D, n = 78 particles; the X-bar and S Control Charts were used for statistical comparison of timepoints, *P < 0.05).

Figure 6. Myosin XIK and Exocyst Subunits Colocalize with CESA6 during the Vesicle Tethering Step of Secretion. (A) Representative images of a time series collected from the cortical cytoplasm of a hypocotyl epidermal cell show that GFP-SEC5B (magenta) colocalized with a newly arrived CSC vesicle (white boxes, green signal in merged images) during the first few seconds of the pause phase, but not during the erratic phase or steady movement phase. Bar = 1 µm. (B) Representative time series shows that diffuse XIK-mCherry (magenta) signal was associated with a newly arrived CSC vesicle (white boxes, green signal in merged images) during the erratic phase and the first few seconds of the pause phase, but not during the steady movement phase. Bar = 1 µm. (C) Representative kymographs demonstrate transient colocalization of GFP-SEC5B (magenta) with tdTomato-CESA6 (green), as well as XIK-mCherry (magenta) with YFP-CESA6 (green) at the beginning of the pause phase (yellow arrowheads). Bar = 1 µm. (D) A small ROI (3 × 3 pixels) was selected at the centroid of a single CSC vesicle or particle from the representative images shown in (A, B). The ROI, defined by the presence of CESA6, was tracked in both channels during the erratic phase, pause phase, and the steady movement phase. The ROI in the GFP-SEC5B channel or the XIK-mCherry channel was extracted for analysis of fluorescence intensity. (E, F) Quantitative analysis of mean fluorescence intensity of GFP-SEC5B (E) or XIK-mCherry (F) in ROIs as shown in (D) from multiple insertion events in different epidermal cells. The fluorescence intensity of the ROI in each frame was divided by the mean fluorescence intensity of the whole cell in the same frame to correct for photobleaching. The derived values were then divided by the average intensity of ROIs from the steady movement phase for normalization,
assuming that any signal associated with an actively translocating CSC represents a random event. Values given are means ± SE (For the GFP-SEC5B intensity assay, n = 40 insertion events, because the duration of the erratic phase varied among different insertion events, the sample sizes at −6 s, −4 s, and −2 s were 12, 10, and 21, respectively; for the XIK-mCherry intensity assay, n = 63 insertion events, and the sample sizes at −9 s, −6 s, and −3 s were 19, 31, and 49, respectively; the X-bar and S Control Charts were used for statistical comparison of timepoints; *P < 0.05).

Figure 7. Disruption of Myosin Activity Results in Reduced Colocalization of SEC5B with CESA6 and Altered SEC5B Tethering Time at the PM during CSC Secretion.
(A) Representative kymographs show colocalization of GFP-SEC5B (magenta) and tdTomato-CESA6 (green) at the beginning of the pause phase (yellow arrowheads) during CSC insertion events. Cells were treated with mock (0.5% DMSO) or 10 µm PBP for 10 min prior to dual-channel time-lapse imaging. In xik-2 or PBP-treated cells, the lifetime of GFP-SEC5B appeared shorter or there was no SEC5B foci colocalized with the CESA6 particle at the pause phase. Bar = 1 µm. 
(B) Quantitative analysis shows that the percentage of colocalization between SEC5B and CESA6 at the beginning of the pause phase was greatly reduced in xik-2 and PBP-treated cells. Values given are means ± SE (n = 3 biological repeats; A total of 154, 130, and 134 CSC insertion events were tracked in WT, xik-2 and PBP-treated cells, respectively; One-way ANOVA with Tukey’s post hoc test, letters [a-c] denote samples/groups that show statistically significant differences from other groups, P < 0.05).
(C) Quantitative analysis of mean fluorescence intensity of GFP-SEC5B in ROIs that correspond to the centroid of a CSC particle during the erratic phase, pause phase, and the steady movement phase. Values given are means ± SE (Data represent one biological repeat; n = 44, 43, and 37 insertion events in WT, xik-2 and PBP-treated cells, respectively; the X-bar and S Control Charts were used for statistical comparison of timepoints; *P < 0.05).
(D, E) Distribution of lifetimes of GFP-SEC5B foci at the vesicle tethering sites during secretion. The distribution of lifetimes was further grouped into three subpopulations (E) based on overall mean ± SD (11.5 ± 5 s) measured in wild type with the same data shown in (D). Values given are means ± SE (n = 3 biological repeats; A total of 115, 99, and 73 SEC5B foci were measured in untreated WT and xik-2 cells and in PBP-treated WT cells, respectively; Chi-square test, letters [a, b] denote samples/groups that show statistically significant differences from other groups, P < 0.05).

Figure 8. Disruption of Myosin Activity Results in Reduced Colocalization of EXO70A1 with CESA6 and a Shorter EXO70A1 Tethering Time during CSC Secretion.
(A) Representative kymographs show colocalization of EXO70A1-GFP (magenta) and tdTomato-CESA6 (green) at the beginning of the pause phase (yellow arrowheads) during CSC insertion events. Cells were treated with mock (0.5% DMSO) or 10 µm PBP for 10 min prior to dual-channel time-lapse imaging. In xik-2 or PBP-treated cells, the lifetime of EXO70A1-GFP appeared shorter or no Exo70A1 foci colocalized with the CESA6 particle at the pause phase. Bar = 1 µm. 
(B) Quantitative analysis shows that the percentage of colocalization between EXO70A1 and CESA6 at the beginning of the pause phase was significantly reduced in xik-2 and PBP-treated wild-type cells. Values given are means ± SE (n = 3 biological repeats; A total of 141, 162, and 96 CSC insertion events were tracked in untreated WT and xik-2 cells and in PBP-treated WT cells, respectively; One-way ANOVA with Tukey’s post hoc test, letters [a-c] denote samples/groups that show statistically significant differences from other groups, P < 0.05).
(C, D) Distribution of lifetimes of EXO70A1-GFP foci at the vesicle tethering sites during secretion. The distribution of lifetimes was further grouped into three subpopulations (D) with the same data shown in (C). Values given are means ± SE (n = 3 biological repeats; A total of 120, 97, and 69 EXO70A1 foci were measured in untreated WT and xik-2 cells and in PBP-treated WT cells,
respectively; Chi-square test, letters [a, b] denote samples/groups that show statistically significant differences from other groups, P < 0.05).

**E** Quantitative analysis of mean fluorescence intensity of EXO70A1-GFP in ROIs that correspond to the centroid of a CSC particle during the erratic phase, pause phase, and the steady movement phase. Values given are means ± SE (Data represent one biological repeat; n = 45, 35, and 23 insertion events in WT, xik-2 and PBP-treated cells, respectively; the X-bar and S Control Charts were used for statistical comparison of timepoints; *P < 0.05).

**Figure 9.** Roles for Myosin XI in Delivery of CSCs to the PM.
Myosin XI mediates CSC delivery at two levels: One group of motors is responsible for the cell-wide transport and distribution of CESA-containing Golgi; another group is transiently associated with secretory vesicles potentially through interactions with an unknown receptor and a Rab GTPase. Once a secretory vesicle is anchored along cortical microtubules through an interaction between CESA and CSI1, the myosin XI and possibly a Rab GTPase on the vesicle surface are required for the recruitment and stabilization of exocyst complex subunits at the PM site for membrane tethering and fusion. Vesicle exocytosis is also facilitated by interactions between CSI1, PTL1, and exocyst subunits, as shown previously (Zhu et al., 2018).
Diagram showing the process of vesicle formation and movement in the cell. Key components include Golgi apparatus, vesicle, plasma membrane, PTL1, CSC, Myosin XI, Rab, GTPase, actin complex, microtubule, and unknown receptor.
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