New Phytologist

Research

The Arabidopsis exocyst subunit SEC3A is essential for embryo development and accumulates in transient puncta at the plasma membrane

Ying Zhang[1], Richard Immink[2], Chun-Ming Liu[3], Anne Mie Emons[1] and Tijs Ketelaar[1]

[1]Laboratory of Cell Biology, Wageningen University, 6708 PB Wageningen, the Netherlands; [2]Bioscience, Plant Research International, Wageningen University and Research Center, 6708 PB Wageningen, the Netherlands; [3]Institute of Botany, Chinese Academy of Sciences, 100093 Beijing, China

Author for correspondence:
Tijs Ketelaar
Tel: +31 317 484329
Email: tijs.ketelaar@wur.nl

Received: *18 January 2013*
Accepted: *19 February 2013*

New Phytologist (2013)
doi: 10.1111/nph.12236

Key words: Arabidopsis, exocyst, exocytosis, green fluorescent protein, localization, plasma membrane, SEC3, secretion.

Summary

- The exocyst is a protein complex that is essential for polarized secretion in mammals and fungi. Although the exocyst is essential for plant development, its precise function has not been elucidated. We studied the role of exocyst subunit SEC3A in plant development and its subcellular localization.
- T-DNA insertional mutants were identified and complemented with a SEC3A-green fluorescent protein (GFP) fusion construct. SEC3A-GFP localization was determined using confocal microscopy.
- *sec3a* mutants are defective in the globular to heart stage transition in embryogenesis. SEC3A-GFP has similar cell plate localization to the other plant exocyst subunits. In interphase cells, SEC3A-GFP localizes to the cytoplasm and to the plasma membrane, where it forms immobile, punctate structures with discrete lifetimes of 2–40 s. These puncta are equally distributed over the cell surface of root epidermal cells and tip growing root hairs. The density of puncta does not decrease after growth termination of these cells, but decreases strongly when exocytosis is inhibited by treatment with brefeldin A.
- SEC3A does not appear to be involved in polarized secretion for cell expansion in tip growing root hairs. The landmark function performed by SEC3 in mammals and yeast is likely to be conserved in plants.

Introduction

Exocytosis is a fundamental process for cells of all eukaryotic organisms. It is required for the secretion of extracellular materials and for the enlargement of the plasma membrane. In plant cells, exocytosis is also crucial for cell wall formation and cell elongation, which are coupled processes. Vesicle fusion for anisotropic plant cell elongation occurs in a non-random pattern, which implies that the sites of exocytosis must be temporally and spatially controlled. This could occur through directed Golgi body transport by the actin cytoskeleton and the targeting of Golgi vesicles to specific sites of the plasma membrane and/or by tethering and docking of the vesicles to a specific membrane domain before exocytosis. In yeast, the exocyst is involved in vesicle tethering before exocytotic vesicle membrane fusion with the plasma membrane. As almost all plant cells expand in a polar fashion, by either axial or tip growth, a local tethering complex could be important for the determination of the orientation of cell expansion.

In budding yeast, bud growth requires polarized exocytosis. An octameric protein complex, termed the exocyst, has been identified that serves as a tethering factor for exocytotic vesicles in the bud tip (TerBush *et al.*, 1996). Polarized localization of the exocyst is essential for polarized secretion during bud formation (Hsu *et al.*, 2004; Munson & Novick, 2006; Zhang *et al.*, 2008; Songer & Munson, 2009).

The exocyst consists of SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70 and EXO84 (Hsu *et al.*, 1996; Kee *et al.*, 1997; Eliás *et al.*, 2003; Li *et al.*, 2010; Zhang *et al.*, 2010). Budding yeast SEC3 is considered to be a landmark protein for polarized exocytosis, as it is localized to the plasma membrane at which exocytosis will occur and is involved in the recruitment of the other exocyst subunits that reside in the cytoplasm or are associated with vesicles at this location (Finger *et al.*, 1998; Wiederkehr *et al.*, 2003; Hutagalung *et al.*, 2009). It is thought that the polarized localization of SEC3 is mediated by interactions with phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) in the inner leaflet of the plasma membrane (He *et al.*, 2007; Liu *et al.*, 2007; Zhang *et al.*, 2008) and with Rho family GTPases (He & Guo, 2009). The actin cytoskeleton that is essential for the delivery of the vesicle-associated subunits is not involved in the positioning of SEC3 (Finger *et al.*, 1998; Wiederkehr *et al.*, 2003; Hutagalung *et al.*, 2009).

Relative to budding yeast, the role of the exocyst in plant cells remains unclear, although it could serve as a key component for

the regulation of polarized exocytosis during cell expansion. All exocyst subunits are conserved in plants, and a plant-specific amplification of EXO70 genes has occurred during evolution, resulting in 23 EXO70 genes in Arabidopsis and 39 in rice (Eliás *et al.*, 2003; Synek *et al.*, 2006; Li *et al.*, 2010). The expression patterns of these EXO70 genes are different, but they are all expressed in cells that are actively dividing or expanding (Li *et al.*, 2010), supporting the idea that the exocyst is involved in the regulation of polarized exocytosis in plant cells.

Mutations in plant exocyst subunits that are encoded by one or two genes (SEC5, SEC6, SEC8, SEC15) cause defects in pollen germination and tube growth (Cole *et al.*, 2005; Synek *et al.*, 2006; Hála *et al.*, 2008), suggesting an important function of the exocyst during plant cell tip growth. Mutations in the exocyst subunits that are encoded by more than two copies of genes (EXO84 (three genes) and EXO70 (23 genes)) cause growth defects of different degrees (Synek *et al.*, 2006; Samuel *et al.*, 2009; Fendrych *et al.*, 2010; Kulich *et al.*, 2010; Pecenková *et al.*, 2011). Plant homologues of SEC3 have only been studied in maize. A mutation in the SEC3 encoding gene *ROOTHAIRLESS1* results in the failure of correct root hair elongation (Wen *et al.*, 2005). However, the expression patterns of maize *SEC3* genes are not known, which makes it difficult to interpret this result. In Arabidopsis, SEC3 is encoded by two nearly identical genes in a tandem arrangement (Eliás *et al.*, 2003). This has hampered the genetic analysis of SEC3 function in Arabidopsis. Interestingly, in Arabidopsis, the ICR1 (interactor of constitutive active ROP1) adaptor protein interacts with SEC3, which provides a putative link to activated ROP (Rho of plants) GTPases (Lavy *et al.*, 2007). As ROP GTPases serve as intracellular polarity markers (Yang, 2008), this link suggests that exocyst recruitment could be (partially) ROP mediated.

Hála *et al.* (2008) have shown by immunocytochemistry that SEC6, SEC8 and EXO70A1 are enriched in the apex of growing tobacco pollen tubes, which is consistent with a role in polarized exocytosis. In tobacco Bright Yellow 2 (BY-2) suspension cultured cells, transient expression of Arabidopsis SEC5A, SEC15A, SEC15B and EXO84B fused to green fluorescent protein (GFP) resulted in fluorescent, globular structures in the perinuclear cytosol (Chong *et al.*, 2010), whereas immunofluorescence revealed that the intracellular localization of EXO70 proteins differs depending on the isoform; they show either a cytoplasmic organization or localize to smaller or larger compartments with different degrees of co-localization with snares specific for early endosomes, late endosomes and the trans Golgi network (Chong *et al.*, 2010; Wang *et al.*, 2010). Distinct structures were also observed by Samuel *et al.* (2009) in stigma cells expressing red fluorescent protein (RFP):EXO70A1. On opening of the flowers, the RFP:EXO70A1 fluorescence relocated to the cell cortex, suggesting that EXO70A1 localization depends on the developmental stage. During cytokinesis, the Arabidopsis exocyst subunits SEC6, SEC8, SEC15B, EXO70A1 and EXO84B localize to the early cell plate, whereafter their localization on the cell plate disappears (Fendrych *et al.*, 2010). Exocyst subunits reappear on the division wall for a short period after completion of cytokinesis (Fendrych *et al.*, 2010).

Recent research has revealed that the exocyst subunits EXO84B, EXO70A1, SEC6 and SEC8 form distinct foci at the plasma membrane, with lifetimes ranging from 9.3 (EXO70A1) to 13.3 s (Fendrych *et al.*, 2013). Co-localization studies have revealed that both EXO84B and SEC6 are present in 37% of these foci (Fendrych *et al.*, 2013). As this study was carried out by variable-angle epifluorescence microscopy, only the plasma membrane localization was studied, and it was not investigated whether these exocyst subunits also localize to vesicles. Wang *et al.* (2010) showed that EXO70E2 localizes to discrete punctate structures at the plasma membrane and in the cytosol of Arabidopsis cells and tobacco BY-2 suspension cultured cells. The compartments at the plasma membrane are contained by two membranes, both of which are EXO70E2 decorated, and secretory. On secretion, the inner membrane is expelled into the apoplast. These organelles, which do not co-localize with any known organelle, have been named EXPO (exocyst-positive organelles; Wang *et al.*, 2010). For a better understanding of the localization of different exocyst subunits and their assembly into multicomponent complexes, additional work is needed.

Here, we report the characterization of the *SEC3A* gene in Arabidopsis. Disruption of the *SEC3A* gene is embryo lethal, with defects in the acquisition of embryo polarity. During cytokinesis, SEC3A-GFP localizes to the early cell plate and to the completed division wall, respectively, similar to other exocyst subunits (Fendrych *et al.*, 2010). In interphase cells, SEC3A-GFP localizes to the cytoplasm and to the plasma membrane, where it forms immobile, punctate structures with discrete lifetimes. In tip growing root hairs, the puncta localize over the whole cell surface, and the amount of puncta does not decrease strongly in fully grown cells. Inhibition of exocytosis causes a strong reduction in the density of the puncta. As the density of SEC3A-GFP puncta does not depend on (the location of) cell expansion, this indicates that SEC3A mediates a type of exocytosis not related to cell growth. Our data show that the polar localization of SEC3A-GFP in the cell cortex occurs only during and just after cytokinesis, and that SEC3A-GFP puncta are evenly spread throughout the cell cortex in interphase cells.

Materials and Methods

Plant material, plant transformation and growth

All *Arabidopsis thaliana* (L.) Heynh. lines used had a Col-0 background. Plants were grown in a growth room at 22C with a 16-h light and 8-h dark photoperiod. Arabidopsis transformation was performed by floral dipping as described by Clough & Bent (1998). Seeds were surface sterilized as described by Ketelaar *et al.* (2004), followed by stratification at 4°C for 2 d. Seeds were grown on plates containing half-strength Murashige and Skoog (MS) salts (Duchefa, Haarlem, the Netherlands) and 0.7% Phyto agar (Duchefa), pH 5.7, or in biofoil slides, as described by Ketelaar *et al.* (2004), for fluorescence microscopy, with the exception that we used 0.16% (w/v) Hoagland's medium (Sigma-Aldrich, Zwijndrecht, the Netherlands), supplemented with 1% sucrose (Duchefa) and 0.7% Phyto agar (Duchefa), pH 5.7.

Yeast two-hybrid assay

The cDNAs of Arabidopsis *SEC3A*, *SEC5A*, *SEC6*, *SEC8*, *SEC10*, *SEC15A*, *SEC15B*, *EXO70A1*, *EXO70H5*, *EXO70H7*, *EXO84B* and *EXO84C* were amplified by PCR (the primer sequences are given in Supporting Information Table S1) and cloned into the pCR8/GW/TOPO vector (Invitrogen). The coding sequence of each protein in the entry clone was confirmed by sequencing and transferred into the Gateway destination vectors pDEST32 (pBD-GAL4, bait) and pDEST22 (pADGAL4, prey; Invitrogen). As a result of technical problems, SEC15A, EXO70H5 and EXO84B could not be cloned into the pDEST32 vector and SEC10 could not be cloned into the pDEST22 vector. All the constructed bait vectors were transformed into the yeast strain PJ69-4α (MATa; James *et al.*, 1996) and transformants were selected on SD (synthetic drop-out) plates lacking leucine (Leu). All the constructed prey vectors were transformed into yeast strain PJ69-4a (MATα; James *et al.*, 1996) and the transformants were selected on SD plates lacking tryptophan (Trp). Subsequently, all the baits were tested for self-activation of the yeast reporter genes, and the basal expression level of the HIS3 reporter gene was determined by titrating histidine (HIS) activity with 3-amino-1,2,4-triazole (3-AT). The addition of 10 mM 3-AT inhibited the growth of yeast in the absence of HIS, except for SEC3A, SEC10 and EXO84C. Diploid yeasts containing both bait and prey were generated by mating the two yeast strains on SD plates, followed by selection on SD plates lacking Leu and Trp. Next, the diploid yeast strains were transferred to SD plates lacking Leu, Trp and adenine (Ade), and SD plates lacking Leu, Trp and HIS supplemented with 10 mM 3-AT, respectively. The β-galactosidase assay was performed as described by Duttweiler (1996).

Identification of the *sec3a* mutant

The T-DNA insertion line SALK_145185 was obtained from NASC (European Arabidopsis Stock Centre, http://arabidopsis.info/) and verified by PCR-based genotyping. Total genomic DNA was extracted as described by Edwards *et al.* (1991). The wild-type allele was amplified using gene-specific primers (Table S1) and the insert alleles were amplified using a gene-specific primer and the T-DNA left border primer LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) and right border primer JMRB1 (5′-GCTCATGATCAGATTGTCGTTTCCC GCCTT-3′). Examples of genotyping PCR results are given in Fig. S2.

β-Glucuronidase (GUS) fusion

A 7589-bp genomic DNA fragment containing 967 bp of sequence upstream from the start codon and the full-length genomic sequence of *SEC3A* was amplified by PCR using the primers 5′-CATGGAAGCCAGAAGTCCTCTCATTTC-3′ and 5′-AA AGCCGGGACTTAGCCATCC-3′. The amplified fragment was cloned into the pDONR207 vector (Invitrogen), followed by recombination into the Gateway binary vector pMDC162 (Curtis & Grossniklaus, 2003), and transformed into Arabidopsis by the floral dip method (Clough & Bent, 1998). GUS staining was performed as described by Fiers *et al.* (2004).

Phenotypic analysis

To determine the terminal phenotype of *sec3a* embryos, seeds were excised from siliques from heterozygous plants and cleared in Hoyer's solution, as described by Liu & Meinke (1998). The seeds were then mounted and observed for defects in embryogenesis under an Eclipse 80i microscope (Nikon, Amstelveen, the Netherlands) equipped with Nomarski optics. Alternatively, immature seeds were dissected from siliques and fixed overnight in 4% paraformaldehyde and 0.25% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.2, rinsed three times in the same buffer, dehydrated in a graded ethanol series (10%, 30%, 50%, 70%, 90%, 100%) and embedded into Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany) according to the manufacturer's instructions. Three-micrometer-thick sections were cut with a rotation microtome (HM 340; Microm GmbH, Walldorf, Germany) and stained in 0.05% toluidine blue. For the investigation of tissue organization of mutant embryos, immature seeds were placed in 0.002% w/v Calcofluor White in water between a microscope slide and coverslip, and gently squashed until the embryos popped out of the immature seeds.

Genetic complementation

A 7989-bp fragment encompassing the *SEC3A* genomic DNA, including 967 bp of upstream sequence and 397 bp of downstream sequence, was amplified by PCR using the primers 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTCG GAGTTAATATGTATGCGC-3′ and 5′-GGGGACCACTT TGTACAAGAAAGCTGGGT5AAAGCCGGGACTTAGCCA TCC-3′. The amplified fragment was recombined into the vector pDONR207 (Invitrogen), followed by recombination into the Gateway binary vector pMDC99 (Curtis & Grossniklaus, 2003). The transformed plants harboring the *SEC3A* gene were selected on 20 μg ml^{-1} hygromycin-containing plates.

Construction of GFP fusion gene construct

The entry clone was recombined into the Gateway binary vector pMDC107 (Curtis & Grossniklaus, 2003). The selection and complementation analysis of transformants were conducted as described above (section on Genetic complementation).

Microscopy

SEC3A-GFP fluorescence and Calcofluor White-stained embryos were imaged using a Nikon Eclipse Ti inverted microscope connected to a Roper Scientific spinning disk system, consisting of a CSU-X1 spinning disk head (Yokogawa, Tokyo, Japan), QuantEM:512SC CCD camera (Roper Scientific, Evry, France) and a ×1.2 magnifying lens between the spinning disk head and the camera. GFP was excited using a 491-nm laser line and Calcofluor White using a 405-nm laser line.

Photobleaching was performed using the FRAP/PA system (Roper Scientific) fed into the spinning disk microscope. The 491-nm laser line was used at 100% intensity (10 ms per scan point of four pixels in diameter) to photobleach GFP.

Image analysis

All imaging processing was performed using Image J software (http://rsb.info.nih.gov/ij/). Figures were composed in Photoshop CS2 (Adobe, San Jose, CA, USA). Puncta were defined as high-intensity (> 80 gray levels above the background level in eight-bit, contrast-stretched images), immobile, circular structures with a Gaussian distribution around the pixel, with the peak intensity ranging in size between 4 and 20 pixels. For measurements of SEC3A-GFP intensity in median planes of root hairs, we defined the root hair tube as the tubular part of the root hair with a constant width. We measured the intensity of the area 5–10 μm from the base of the dome. The tip is defined as the top of the dome; we measured the intensities of a 3-μm-long membrane stretch centered around the top of the dome. For intensity measurements at the side of the dome, we measured a 3-μm-long membrane stretch centered around the center point between the top and the base of the dome.

FM4-64 staining and drug treatments

FM4-64 (final concentration, 17 μM) and brefeldin A (BFA) stock solutions were prepared in dimethylsulfoxide (DMSO). Drug treatments were performed by submerging biofoil slides into drug-containing medium for 20 min. Negative controls were treated with the same concentrations of DMSO as the drug-treated samples.

Results

Interactions between the subunits of the exocyst complex in Arabidopsis

To gain an insight into the assembly and structure of the plant exocyst, we determined the interactions between different exocyst subunits using a matrix-based yeast two-hybrid assay. Interactions between the exocyst subunits SEC3A, SEC5A, SEC6, SEC8, SEC10, SEC15A, SEC15B, EXO70H7, EXO70A1, EXO84B and EXO84C were determined. In the self-activation assessment of the baits, SEC3A, SEC10 and EXO84C showed very strong self-activation; therefore, they could not be used as baits in the interaction test. Six pairs of strong interactions were detected for which all three reporter genes were activated: SEC3A interacts with EXO70 A1 and SEC5A, SEC15B interacts with both EXO84B and EXO84C, EXO70A1 interacts with EXO84C, and EXO70H7 interacts with EXO84B (Table 1; Fig. S1). The LacZ reporter gene was activated for two pairs only: SEC5A with SEC6 and SEC5A with EXO84C; hence, these putative weak interactions need to be confirmed by alternative methods. We decided to focus on SEC3, because of its prominent role in the yeast exocyst complex.

Table 1 Interactions between exocyst subunits in Arabidopsis as determined by yeast two-hybrid assays

Protein	Interacts with
SEC3A	EXO70A1
	SEC5A
SEC5A	EXO84C (LacZ only)
	SEC6 (LacZ only)
SEC15B	EXO84B
	EXO84C
EXO70H7	EXO84B
EXO70A1	EXO84C

The Arabidopsis Genome Initiative numbers for the listed genes are At1 g47550 (*SEC3A*), At1 g76850 (*SEC5A*), At1 g71820 (*SEC6*), At3 g10380 (*SEC8*), At5 g12370 (*SEC10*), At3 g56640 (*SEC15A*), At4 g02350 (*SEC15B*), At2 g28640 (*EXO70H5*), At5 g59730 (*EXO70H7*), At5 g03540 (*EXO70A1*), At5 g49830 (*EXO84B*) and At1 g10180 (*EXO84C*).

SEC3A expresses in multiple tissues, most of which contain dividing and expanding cells

Arabidopsis possesses two genes that encode SEC3, *SEC3A* (At1 g47550) and *SEC3B* (At1 g47560), which are tandemly duplicated and share 97% sequence identity at the protein level. The ATH1 whole-genome chip is unable to discriminate between *SEC3A* and *SEC3B* expression. However, expressed sequence tags (ESTs) and a search against the Arabidopsis MPSS database revealed that both genes are expressed (Chong *et al.*, 2010). We determined the temporal and spatial expression pattern of *SEC3A* by expression of a *SEC3A* genomic-GUS gene fusion. Six independent transgenic plants expressing the *SEC3A* genomic sequence fused to GUS were examined histologically for GUS activity. In light-grown seedlings, *SEC3A* was expressed in cotyledons and root, but not in hypocotyls, and highly expressed in shoot apical meristems (Fig. 1a). In roots, *SEC3A* was highly expressed in the root tip, the vasculature (Fig. 1b) and lateral root primordia (Fig. 1b). Unlike in younger seedlings, *SEC3A* expression was observed in the hypocotyls of older seedlings (Fig. 1d). In both expanding and expanded rosette leaves, *SEC3A* expression was detected (Fig. 1e). In flowers, *SEC3A* expression was not detected in petals and sepals (Fig. 1f), but strong *SEC3A* expression was detected in the stigma (Fig. 1g), unfertilized ovules (Fig. 1g) and pollen (Fig. 1h). In embryos, *SEC3A* expression was observed from the early heart stage onwards (Fig. 1i–k). Thus, *SEC3A* expresses in various tissues of both seedlings and mature plants and preferentially in tissues containing dividing and expanding cells, such as the shoot apical meristem, root tip, lateral root primordia and developing embryos.

Identification of an embryo-lethal *sec3a* mutant

Using a PCR-based genotyping approach, we identified a SALK line (SALK_145185) harboring a T-DNA insertion in the first intron of the *SEC3A* (At1 g47550) gene (Fig. 2a). We performed PCR amplification of the *SEC3A* wild-type allele with primers flanking the T-DNA insertions and amplification of the mutant

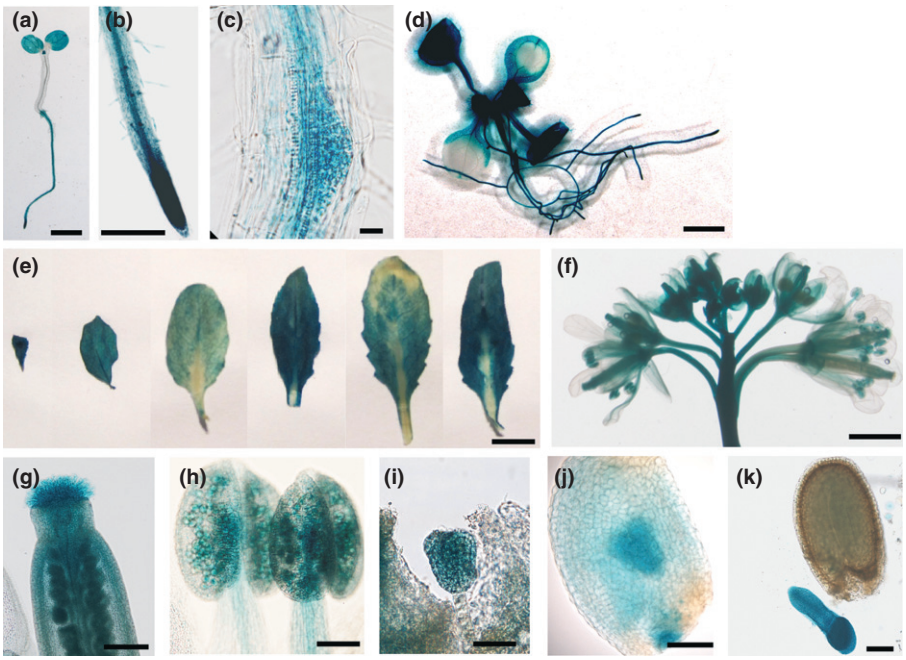

Fig. 1 Arabidopsis *SEC3A* genomic sequence: β-glucuronidase (GUS) expression pattern in seedlings and mature plants. GUS staining pattern of a 1-wk-old seedling (a), 1-wk-old root (b), a lateral root primordium (c), a 2-wk-old seedling (d), different rosette leaves of a 1-month-old plant (e), immature and mature flowers (f), an unfertilized stigma (g), developing pollen (h) and embryos of different developing stages (early heart (i), heart (j), torpedo (k)). Bars: 5 mm (a); 1 mm (b); 100 μm (c); 1 mm (d); 5 mm (e); 500 μm (f); 100 μm (g–k).

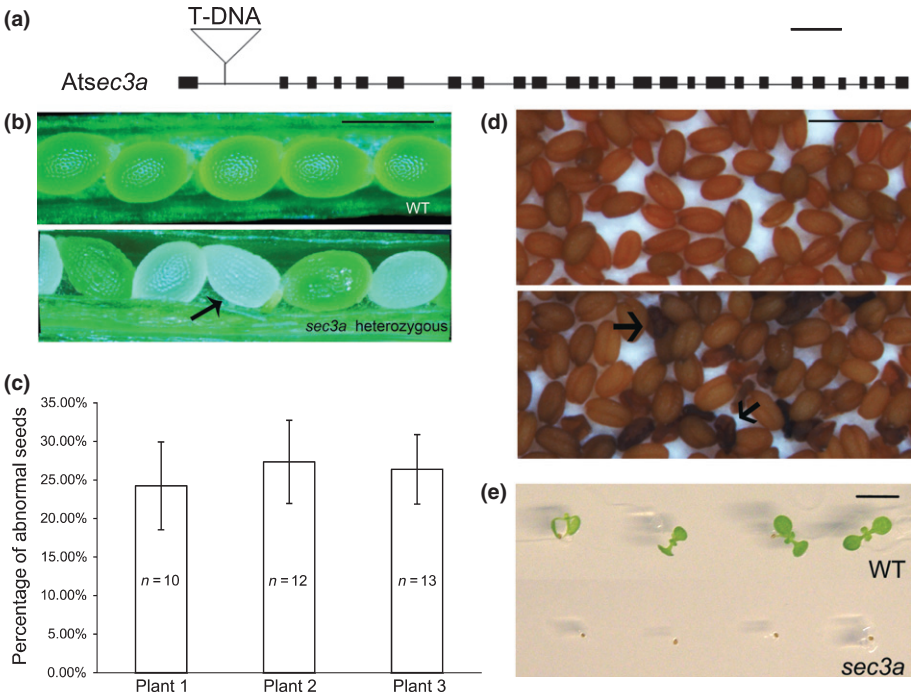

Fig. 2 A T-DNA insertion in Arabidopsis *SEC3A* causes embryo lethality. (a) Schematic representation of the *SEC3a* gene (At1 g47550). The black boxes represent exons and the T-DNA insert is located in the first intron. (b) Immature wild-type (WT) siliques show a full set of green seeds; immature *sec3a* heterozygous siliques show both green and white seeds. The arrow points to an abnormal seed. (c) In siliques of different plants that are heterozygous for *sec3a*, *c.* 25% of the seeds are abnormal. Three independent heterozygous plants were picked and at least 10 siliques from each plant were examined, as indicated in the bars of (c). The error bars indicate standard deviations. (d) Mature seeds from wild-type and *sec 3a* heterozygous siliques. *sec3a* heterozygous plants produce both seeds that appear normal and seeds that are dark-brown and wrinkled (black arrows). (e) In contrast with normal appearing seeds (top row) from plants heterozygous for *sec3a*, dark-brown wrinkled seeds (bottom row) do not germinate. This photograph shows 7-d-old seedlings. Bars: 500 bp (a); 1 mm (b, d); 5 mm (e).

sec3a allele left and right borders with one primer flanking the T-DNA insertion and the T-DNA left-border primer LBa1 and one primer flanking the T-DNA insertion and the R-DNA right-border primer JMRB1. All PCRs resulted in amplification products of the expected size, indicating that the T-DNA insertion caused no DNA rearrangements. We identified only heterozygous and wild-type plants (Fig. S2). The *sec3a* allele segregated in a ratio of 1 : 2 in progeny of selfed heterozygous plants (30.0%

wild-type : 70.0% heterozygous; $n = 350$ plants (Table 2)). As no homozygous plants were identified and the 1 : 2 segregation ratio of the *sec3a* allele is typical for embryo lethality, we tested immature siliques from heterozygous *sec3a* plants for the presence of white or otherwise abnormal seeds. Green and white seeds within single siliques were observed, whereas only green seeds were present in the immature siliques from wild-type plants (Fig. 2b). As white seeds indicate embryo lethality (Meinke & Sussex, 1979), we scored the number of white seeds from 10 siliques from three individual plants heterozygous for *sec3a*. In all these lines, *c.* 25% of seeds were white, suggesting that the *sec3a* mutation is recessively embryo lethal (Fig. 2c). The mature seeds from heterozygous and wild-type plants were also compared, showing a mixture of dark-brown, small, wrinkled seeds and normal light-brown seeds in heterozygous plants and only normal light-brown seeds in the wild-type (Fig. 2d). We tested germination by placing wrinkled seeds and normal seeds on half-strength MS plates to score the germination rate. The normal seeds all germinated, but none of the wrinkled seeds had germinated after 1 wk ($n = 300$; Fig. 2e). To determine whether, in addition to embryo lethality, the *sec3a* mutation causes gametophytic defects, we analyzed the progeny of heterozygous *SEC3A/sec3a* lines and performed reciprocal crosses between *SEC3A/sec3a* and wild-type plants. In the progeny of *SEC3A/sec3a*, the *sec3a* allele segregated as expected (Table 2). In the progeny of the reciprocal crosses, the transmission of the *sec3a* allele was not affected when *SEC3A/sec3a* was used as either pollen donor or pollen receiver (Table 2), demonstrating that gametophytic transmission of the *sec3a* allele is normal. Thus, SEC3A appears to be essential for plant development.

Embryo development in *sec3a* is arrested at the globular stage

To examine whether the white seeds in the progeny of plants heterozygous for *sec3a* were harboring aborted embryos, we investigated embryo development in these seeds. From the two-cell stage to the 32-cell stage, we could not discriminate between mutant and wild-type embryos. Unlike normally developing embryos, which become heart shaped 4 d after fertilization (Fig. 3a,b), the mutant embryos failed to undergo this developmental step (Fig. 3e,f). Mutant embryos from siliques in which the wild-type embryos had developed to the cotyledonary stage (Fig. 3c,d) were still globular, although their size and the number of cells had increased beyond the normal size and number of cells of globular embryos, and the cells of the mutant embryo appeared more rounded (Fig. 3g,h). To investigate the defects in mutant embryos in more detail, we stained the cell walls with Calcofluor White. Microscopic analysis revealed several aspects: although the suspensor looked normal, the mutant embryo proper lacked any polarity, cells were rounded and cell division occurred in random directions, whereas, in heart-shaped wild-type embryos of the same age, an epidermal cell layer had clearly developed (Fig. 4). Thus, although cell division is not inhibited, aberrant embryos apparently lack any form of differentiation and polarity.

The *sec3a* mutant phenotype is rescued by genetic complementation

Genetic complementation of the *sec3a* mutant was performed by introducing the full-length *SEC3A* genomic DNA, driven by the endogenous *SEC3A* promoter (ProSEC3A:SEC3A), into plants heterozygous for *sec3a*. In addition, we introduced a similar construct with the GFP coding region fused to the 5′ end of the *SEC3A* genomic sequence (ProSEC3A:SEC3A-GFP). We scored for white seeds in two T1 lines carrying the ProSEC3A:SEC3A construct to assess the percentage of seeds in which embryo development was arrested. In contrast with uncomplemented T1 plants that carried *c.* 25% white seeds, both lines had a strongly reduced number of abnormal (white) seeds (2.18% ($n = 1008$ seeds from 14 siliques) and 1.55% ($n = 645$ seeds from 10 siliques), respectively). Transgenic T1 plants heterozygous for the *sec3a* allele complemented with the ProSEC3A:SEC3A-GFP construct showed percentages of white seeds below 2% ($n = 7$ independent transformants). The percentages of white seeds observed were lower than that expected in T1 plants carrying one complementation construct (6.25%), indicating that the T1 plants carry more than one complementation construct. Indeed, backcrossing of these lines with the Col-0 wild-type resulted in progeny with higher percentages of white seeds in five of the seven independent lines and, after backcrossing to Col-0 twice, followed by selfing, we were able to select four lines that showed *c.* 6.25% of white seeds of lines complemented with ProSEC3A:SEC3A and ProSEC3A:SEC3A-GFP. Approximately 75% of the seedlings of these backcrossed lines carried the hygromycin resistance marker of the complementation construct. Both the complemented T1 and the backcrossed lines developed normally and contained

Table 2 Inheritance of the Arabidopsis *sec3a* allele as determined by reciprocal crosses

Natural self of *SEC3A* heterozygous	Number of progeny	Progeny genotype			Expected ratio (wild-type : heterozygous)	χ^2
		Wild-type (SEC3A/SEC3A)	Heterozygous (SEC3A/*sec3a*)	Homozygous (*sec3a*/*sec3a*)		
Reciprocal crosses	350	245	105	0	1 : 2	0.17*
Pollen source: *sec3a* heterozygous	186	100	86	0	1 : 1	0.30*
Pollen source: wild-type	214	115	99	0	1 : 1	0.27*

*P values were determined using the χ^2 test. $P > 0.05$ is different from the expected ratio.

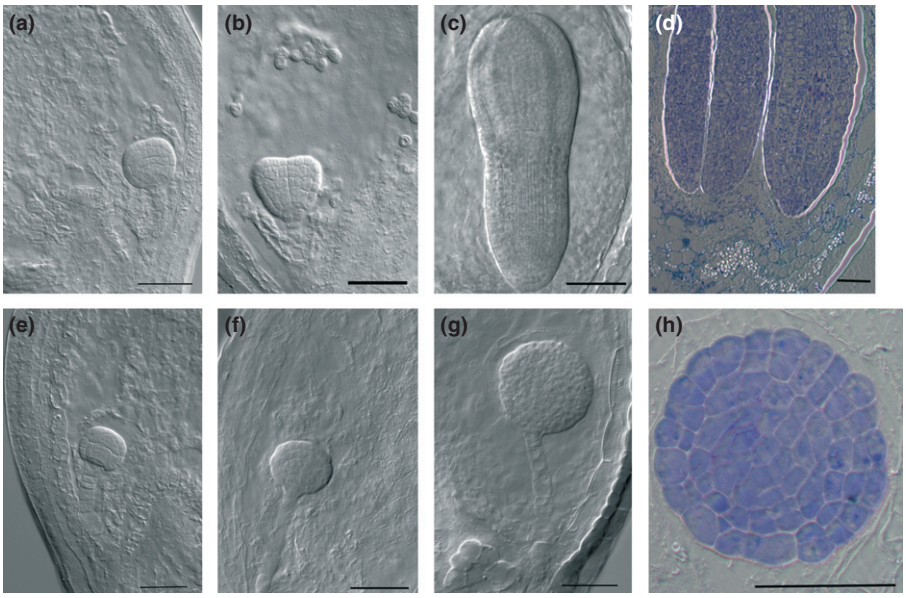

Fig. 3 Embryo development is arrested during the globular stage in 25% of the seeds of Arabidopsis plants heterozygous for *sec3a*. Wild-type (a–c) and mutant (e–g) embryos in seeds from the same silique (a and e, b and f, and c and g) were compared at the different stages of embryo development. (d, h) Toluidine blue staining of the wild-type embryo at the cotyledon stage (d) and the mutant embryo (h) of the same age and from the same silique. Bars: 50 μm (a–d) and (f–h); 100 μm (e).

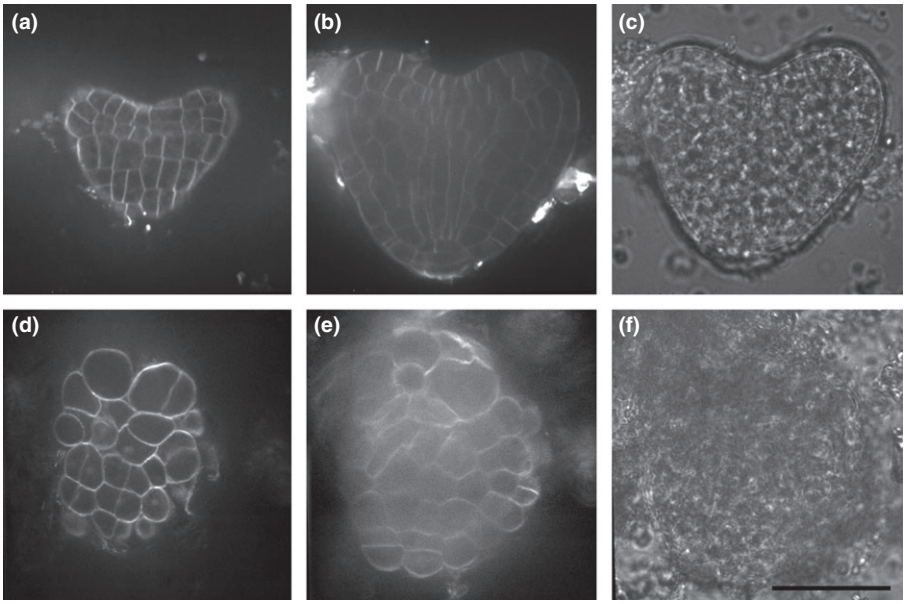

Fig. 4 Cortical (a, d) and median (b, e) confocal planes of a wild-type Arabidopsis heart-shaped embryo (a–c) and a *sec3a* embryo of the same age (d–f). *sec3a* embryos lack any polarity and fail to form a distinct epidermal cell layer.

normally developed embryos, demonstrating that the mutant phenotype is caused by the *sec3a* T-DNA insertion allele and that both ProSEC3A:SEC3A and ProSEC3A:SEC3A-GFP fully complement the mutant phenotype.

SEC3A tagged with GFP (SEC3A-GFP) is present in the cytosol and accumulates in puncta at the plasma membrane

We determined the subcellular localization of SEC3A in transgenic T2 plants carrying the SEC3A-GFP fusion construct, selected as described above, in developing embryos, root hairs and root epidermal cells. Root hairs expand by tip growth; their expansion is extremely polarized and occurs exclusively at the tip. Root epidermal cell expansion is also polar, but occurs over the whole cell facets parallel to the elongation direction of the root. We refer to this type of cell expansion as axial growth (also called intercalary or diffuse growth; for a review, see Ketelaar & Emons, 2001). We studied SEC3A-GFP localization in both expanding and fully grown cells.

In the embryos, SEC3A-GFP accumulated at the periphery of the cells and stained the cytoplasm weakly (Fig. 5). In addition to the SEC3A-GFP fluorescence, amyloplasts produced autofluorescence. This fluorescence was also present in wild-type embryos (Fig. 5a,c). Because we observed plasmolysis and relocation of the SEC3A-GFP signal within 5 min after embryo isolation, we shifted our focus to root hairs and root epidermal cells that are more suitable for live cell imaging. In both of these cell types, we observed SEC3A-GFP fluorescence evenly distributed throughout the cytoplasm and brightly fluorescent cell compartments,

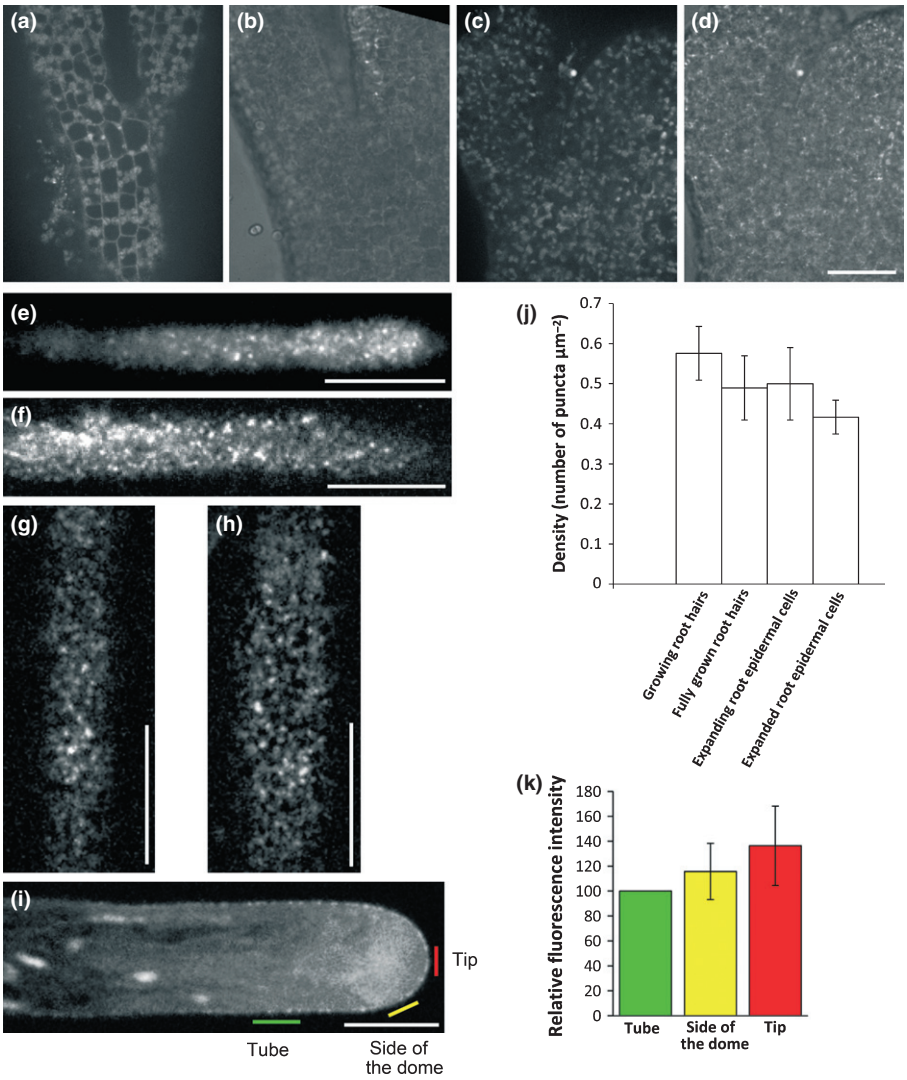

Fig. 5 Arabidopsis SEC3A-green fluorescent protein (GFP) fluorescence localizes as puncta at the plasma membrane, is evenly distributed throughout the cytosol and accumulates in cell compartments. (a–d) SEC3A-GFP localizes to the periphery of embryo cells (a). The autofluorescence of amyloplasts is also visible in a wild-type embryo (c). (b, d) The corresponding bright field images. (e–h) SEC3A-GFP is localized to the plasma membrane as discrete puncta in growing root hairs (e), in fully grown root hairs (f), in root epidermal cells of the rapid elongation zone adjacent to the meristem (g) and in fully grown root epidermal cells (h). (j) The density of puncta is $0.58 \pm 0.67\ \mu m^{-2}$ in the tube of growing root hairs relative to $0.49 \pm 0.48\ \mu m^{-2}$ in the tube of fully grown root hairs ($P = 0.30$), and $0.50 \pm 0.09\ \mu m^{-2}$ in expanding root epidermal cells relative to $0.42 \pm 0.04\ \mu m^{-2}$ in expanded root epidermal cells ($P = 0.15$). The error bars indicate standard deviations ($n > 100$ puncta per cell in four cells per sample). (i) An image of the median plane of a growing root hair shows that some of the SEC3A-GFP fluorescence is evenly distributed throughout the cytoplasm and that fluorescent puncta localize to the plasma membrane. The three areas marked by colored lines represent different regions of the cell surface that were used for analysis in (k): the tube, the side of the apical dome and the extreme apex. The different areas are defined in the Materials and Methods subsection on Image analysis. (k) The fluorescence intensities of different regions of the plasma membrane. The GFP fluorescence intensity of the plasma membrane of the root hair tube was normalized to 100 in each cell to allow the comparison of different cells ($n = 10$ cells). The fluorescence intensities of the extreme tip and the side of the dome were multiplied by the same factor as used to set the fluorescence intensity of the root hair tube of different cells to 100. The error bars indicate standard deviations. Bars: 10 µm.

which are clearly visible in Fig. 5(i). At the plasma membrane of both cell types, SEC3A-GFP fluorescence had a punctate distribution (Fig. 5e,h), similar to that observed for the other exocyst subunits SEC6, SEC8, EXO70A1 and EXO84b (Fendrych *et al.*, 2013). We did not observe clear differences in the density of the puncta in expanding and fully grown cells (Fig. 5j). As the tip of growing root hairs harbors many vesicles and is the location of cell elongation, it was important to determine the density of SEC3A puncta at this location. As it is technically challenging to measure the density of puncta in the surface of the dome of the root hair tip, because of its curvature, we measured the fluorescence intensities of membrane regions in median confocal sections (Fig. 5i). Cell expansion is fastest at the side of the dome and is slower at the tip of the dome (Shaw *et al.*, 2000). We measured the fluorescence intensities in the plasma membrane of these two areas and compared these intensities with the fluorescence intensity of the plasma membrane of the tube (Fig. 5k). Surprisingly, we did not observe significant differences in

fluorescence intensity between the different membrane areas of the expanding tip and the membrane area of the non-expanding root hair tube ($P = 0.40$ between the tube and the side of the dome, and $P = 0.11$ between the tube and the tip; ANOVA with paired samples). Thus, SEC3A puncta at the plasma membrane do not preferentially accumulate in expanding areas of cells. This suggests that SEC3A is not specifically recruited to polar exocytosis events in interphase cells.

SEC3A-GFP forms discrete, immobile puncta with a lifetime of seconds at the plasma membrane

To determine the behavior over time of the SEC3A-GFP puncta at the plasma membrane, we photobleached the SEC3A-GFP that was already present and analyzed the recovery of fluorescence over time. We studied the behavior of individual SEC3A-GFP puncta at the plasma membrane of tubes of growing (Fig. 6a) and fully grown (Fig 6b) root hairs, and the plasma membrane of the outer periclinal face of expanding (Fig. 6c; Movie S1) and fully grown (Fig. 6d; Movie S2) root epidermal cells. In all of these cell types, new, immobile fluorescent SEC3A-GFP puncta appeared at the plasma membrane after photobleaching. When puncta first appeared, their fluorescence was low but detectable. Over time, their fluorescence intensity increased until it reached a maximum intensity, whereafter the fluorescence intensity gradually decreased until it disappeared completely (Fig. 6e). The characteristic behavior of SEC3A-GFP puncta is illustrated in Fig. 6(f).

To check whether there are differences in the lifetime of the puncta between different cell types and/or between growing and fully grown cells, we measured the lifetimes of the SEC3A-GFP puncta in growing and fully grown root epidermal cells and root hair tubes (Fig. 6g). We collected image sequences with 2-s intervals between sequential images, determined the amount of frames during which each punctum was present and used these values to calculate an average lifetime. Within individual cells, lifetimes were highly variable, ranging from 2 to 40 s. The average lifetime of SEC3A-GFP puncta was 6.3 ± 2.7 s in the tube region of growing root hairs ($n = 81$ from three cells), 6.6 ± 3.4 s in the tube region of fully grown root hairs ($n = 113$ from five cells), 6.7 ± 3.6 s in expanding root epidermal cells ($n = 86$ from four cells) and 12.6 ± 8.3 s in fully grown root epidermal cells ($n = 127$ from five cells). The average SEC3A-GFP punctum lifetime in the growing root hairs was no different from that in fully grown root hairs ($P = 0.49$). The lifetime of puncta in expanding and expanded root epidermal cells is significantly different ($P = 0.001$), which can be accounted for by the small population (10.2%) of puncta with long lifetimes (> 24 s) in fully expanded root epidermal cells (Fig. 6g).

SEC3A-GFP is not delivered to the plasma membrane by the Golgi system

To test whether SEC3A is delivered to the plasma membrane by the Golgi system, we tested whether it associates with the vesicle pool in the apex of expanding root hairs by staining with the amphiphilic styryl dye FM4-64. It is assumed that FM4-64 decorates membranes of endocytotic vesicles rapidly and also that exocytotic vesicles are decorated rapidly (van Gisbergen *et al.*, 2008; Griffing, 2008). We did not see any SEC3A accumulation in the vesicle-rich region in the apex of growing root hairs other than the fluorescence that is present throughout the cytoplasm (Fig. 7). From this, we conclude that SEC3A-GFP does not localize to FM4-64-labeled vesicles. This suggests that SEC3A recruitment to the plasma membrane is not mediated by the Golgi system.

To investigate whether SEC3A recruitment to the plasma membrane depends on exocytosis, we applied BFA to growing root hairs and measured the density and lifetime of SEC3A-GFP puncta at the plasma membrane. BFA is a macrocyclic lactone of fungal origin that inhibits Golgi-based secretion in plants (reviewed in Robinson *et al.*, 2008). We applied a concentration range of BFA to growing root hairs. The lowest concentration of BFA that caused complete root hair growth inhibition after 2 h was 5 $\mu g\,ml^{-1}$. Occasionally, swollen root hair tips were observed, but root hairs were still alive and cytoplasmic streaming continued. The inhibition of root hair growth showed that treatment with 5 $\mu g\,ml^{-1}$ BFA successfully inhibits Golgi-based secretion in root hairs. Under these conditions, SEC3A-GFP still localized as puncta to the plasma membrane throughout the root hair tube. However, the density of the SEC3A-GFP puncta was reduced dramatically after 2 h of treatment compared with that in untreated cells ($0.23 \pm 0.05\ \mu m^{-2}$ compared with $0.58 \pm 0.67\ \mu m^{-2}$ before treatment; $P < 0.001$; Fig. 8a). After 4 h of BFA treatment, the density had decreased even further ($0.09 \pm 0.04\ \mu m^{-2}$; Fig. 8a). The SEC3A-GFP puncta still showed their characteristic increase and decrease in fluorescence intensity during their lifetime, but the average lifetime was increased when compared with control cells (10.0 ± 4.3 s in BFA-treated cells (2 h) and 6.3 ± 2.7 s in control cells; $P < 0.001$; Fig. 8b). The lifetime of SEC3A-GFP puncta did not change after longer BFA treatment (10.1 ± 5.1 s after 4 h ($P = 0.93$); Fig. 8b). Thus, the disruption of exocytotic vesicle production causes a decrease in recruitment and longer lifetimes of SEC3A-GFP puncta.

SEC3A-GFP puncta do not co-localize with cortical microtubules and the insertion of cellulose synthase (CESA) complexes into the plasma membrane

As the insertion of CESA complexes into the plasma membrane, which is likely to be an exocytotic event, occurs predominantly along cortical microtubules, we tested whether SEC3A-GFP puncta co-localized with these cortical microtubules. We crossed an mCherry-TUA5-expressing line (Gutierrez *et al.*, 2009) with SEC3A-GFP-expressing lines. The optical coverage of cortical microtubules in the analyzed cells was $30.3 \pm 0.9\%$ ($n = 3$ cells; Fig. 9a). If the SEC3A-GFP puncta appear at random locations of the cell cortex, the expected percentage of puncta co-localizing with cortical microtubules is *c.* 30%. Thirty-eight of 125 SEC3A-GFP puncta showed co-localization with cortical microtubules, which is 28.9% (Fig. 9a; Movie S3). This is not

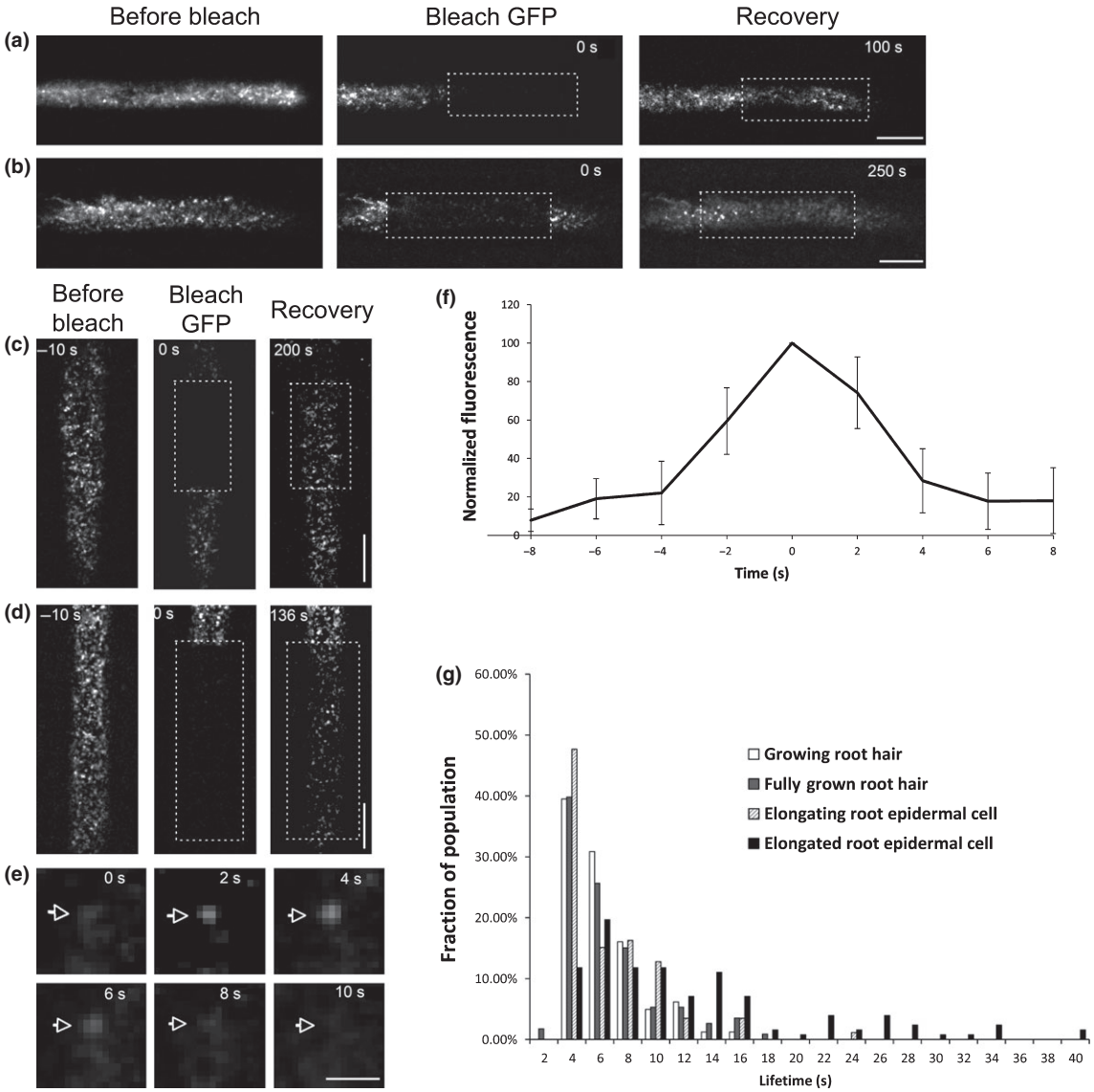

Fig. 6 Arabidopsis SEC3A-green fluorescent protein (GFP) forms discrete, immobile puncta with discrete lifetimes. (a–d) Time course of photobleaching and recovery of SEC3A-GFP at the plasma membrane of a growing root hair tube (a), a fully grown root hair tube (b), an expanding root epidermal cell (c; Supporting Information Movie S1) and a fully expanded root epidermal cell (d; Movie S2). The three consecutive images show SEC3A-GFP before photobleaching, immediately after photobleaching and the recovery of SEC3A-GFP puncta over time. Dashed boxes indicate the bleached areas. (e) An individual SEC3A-GFP punctum that appears and, *c.* 10 s later, disappears. (f) The average normalized fluorescence intensity of puncta over time ($n = 12$) from one fully expanded root epidermal cell. The peak fluorescence was centered at time 0. Bars show standard deviations. (g) A comparison of the lifetimes of SEC3A-GFP puncta in growing root hairs, fully grown root hairs, expanding root epidermal cells and expanded root epidermal cells. Bars: 10 μm (a–d); 1 μm (e).

significantly different from the expected percentage ($P = 0.59$). To test the co-localization of SEC3A-GFP with CESA complexes directly, we crossed a tdTomato-CESA6-expressing line (Gutierrez *et al.*, 2009) into a SEC3A-GFP-expressing line and searched for the co-localization of SEC3A-GFP with the insertion of CESA complexes. This was performed by the photobleaching of existing tdTomato-CESA6 in the plasma membrane. We performed a co-localization analysis of newly inserted tdTomato-CESA6 with SEC3A-GFP puncta during the insertion process (both the erratic movement and static phase, as described by Gutierrez *et al.*, 2009), and found that only 2% of the CESA complex insertion events correlated with the presence of a SEC3A punctum at the same location ($n = 100$ insertion events in five cells; Fig. 9b; Movie S4). Thus, it is unlikely that SEC3A puncta are involved in exocytosis for the insertion of CESA complexes into the plasma membrane.

SEC3A-GFP transiently localizes to the early cell plate, is absent during cell plate extension and re-appears at the division wall

In mitotic cells, the exocyst subunits SEC6, SEC8, SEC15B, EXO70A1 and EXO84B are localized to the initiating cell plate, disappear during cell plate extension and reappear on the division

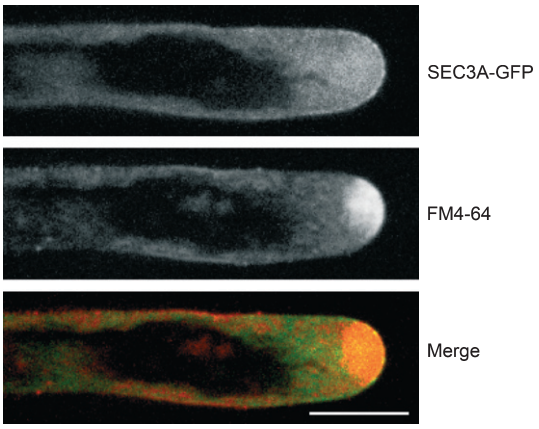

Fig. 7 Arabidopsis SEC3A-green fluorescent protein (GFP) does not decorate the pool of vesicles in the apex of growing root hairs. A median section of a growing root hair expressing SEC3A-GFP, counterstained with the membrane marker FM4-64, shows that there is no increased SEC3A-GFP signal present in the apical region in which vesicles accumulate. Bar: 10 μm.

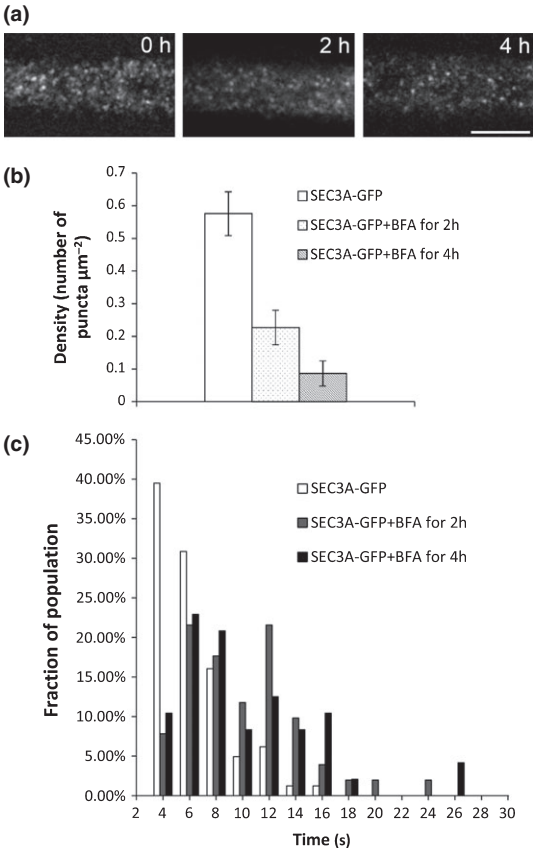

Fig. 8 The density and lifetime of Arabidopsis SEC3A-green fluorescent protein (GFP) puncta at the plasma membrane of tubes of growing root hairs are affected by the secretory trafficking inhibitor brefeldin A (BFA). (a) Representative images of the root hair cortex before and during BFA treatment. (b) The density of SEC3A-GFP puncta at the plasma membrane in growing root hairs treated with 5 μg ml^{-1} BFA. The error bars indicate standard deviations; $n > 100$ puncta in at least four cells. (c) The lifetimes of SEC3A-GFP puncta at the plasma membrane in growing root hairs treated with 5 μg ml^{-1} BFA; $n > 100$ puncta in at least four cells. Bar: 10 μm (a).

wall for some time after the completion of cytokinesis (Fendrych *et al.*, 2010). To determine whether SEC3A behaves similarly, we studied the localization of SEC3A-GFP during the course of cell plate formation in dividing cells of the Arabidopsis root meristem. In the meristematic cells in both the epidermal and cortical cell layer, SEC3A-GFP is localized to distinct structures with high fluorescence intensity in some cells (Fig. 10a). To gain an insight into the SEC3A-GFP localization, we counterstained with FM4-64, which labels developing cell plates (Dhonukshe *et al.*, 2006), and followed the SEC3A localization in cells of the root meristem over time. An accumulation of SEC3A-GFP fluorescence was associated with the appearance of the cell plate. During the centrifugal expansion of the cell plate to the parental cell walls, the SEC3A-GFP fluorescence disappeared (Fig. 10b). SEC3A-GFP accumulation during early cell plate formation lasted 173 ± 15 s ($n = 5$ cells). When cell plate formation had completed, SEC3A-GFP fluorescence started to accumulate at the division wall (Fig. 10c). The average residence time of SEC3A-GFP at the division wall was 27 ± 5 min ($n = 15$ cells from three independent root meristems). Thus, SEC3A localization during cytokinesis is similar to the localization of other exocyst subunits.

Discussion

Our results can help to decipher the role of the exocyst in general and, in particular, of SEC3A in plant development. In interphase cells, SEC3A-GFP has a cytoplasmic localization and accumulates as immobile puncta at the plasma membrane with average lifetimes of 6.3–12.6 s depending on the cell type. Although SEC3A may be involved in exocytosis, it is not involved in the insertion of CESAs into the plasma membrane, and the density of SECA-GFP puncta does not depend on (polar) cell growth in interphase cells. SEC3A-GFP localizes to the cell plate during cytokinesis, but the lack of polarity in embryos of *sec3a* mutants suggests that SEC3A performs a function beyond the formation and/or correct positioning of cell plates in obtaining polarity during embryo development.

Interactions between different Arabidopsis exocyst subunits have been identified using different techniques. Hála *et al.* (2008) used chromatographic fractionation and yeast two-hybrid assays, whereas Pecenková *et al.* (2011) used yeast two-hybrid assays and fluorescence resonance energy transfer (FRET) analysis between several subunits. Together, these results strongly suggest that exocyst composition, and probably also function, is conserved in plants. Although co-fractionation shows that different exocyst subunits are in the same complex in plants (SEC3, SEC5, SEC6, SEC8, SEC10, SEC15 and EXO70A1; Hála *et al.*, 2008), the pairwise interactions found by the yeast two-hybrid assay provide additional insight into exocyst assembly. We have identified additional interactions between different exocyst subunits: SEC3A interacts with SEC5A, SEC15B interacts with EXO84C, EXO70A1 interacts with EXO84C, and EXO70H7 interacts with EXO84B. In addition, we determined two weak interactions in the yeast two-hybrid assay: SEC5A with SEC6, and SEC5A with EXO84C.

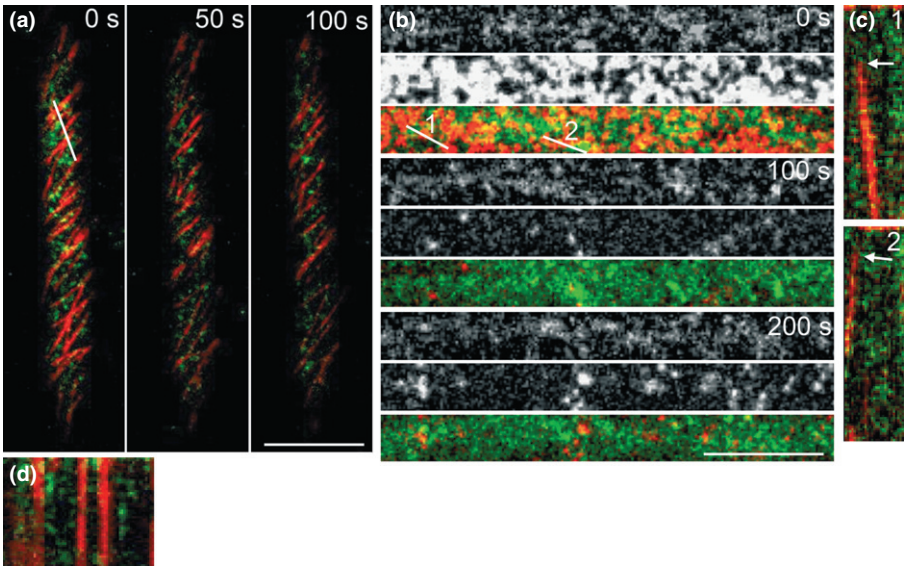

Fig. 9 Arabidopsis SEC3A-green fluorescent protein (GFP) puncta do not preferentially co-localize with cortical microtubules (CMTs) and do not co-localize with cellulose synthase (CESA) complex insertion events. Time series of a line expressing mCherry-TUA5 (red) and SEC3A-GFP (green (a); Movie S3) and time series of a line expressing tdTomato-CESA6 (middle panels and red in merged images) and SEC3A-GFP (upper panels and green in merged images (b); Movie S4). To clearly mark CESA complex insertion events, the CESA channel was photobleached after the first frame. (c) Two kymographs of CESA complex insertion into the plasma membrane and their subsequent movement over time. The arrows mark the insertion events that do not co-localize with a SEC3A-GFP punctum. (d) A kymograph of SEC3A puncta (green) and cortical microtubules (red) where no preferential co-localization is observed. Kymograph locations are marked in (a, b) by white lines. Bars: 10 μm (a); 5 μm (b).

Knowledge about interactions within the exocyst could aid in the deciphering of the exocyst structure.

SEC3A in cytokinesis

sec3a null mutants are embryonically lethal, which differs from defects in other exocyst subunit mutants that have been described (Cole *et al.*, 2005; Synek *et al.*, 2006; Samuel *et al.*, 2009; Kulich *et al.*, 2010; Pecenková *et al.*, 2011). The successful transmission of the *sec3a* allele from the male and female gametophytes to the progeny allows us to study exocyst functioning in somatic development, which is not possible in exocyst mutants that are gametophytically lethal. The failure of the *sec3a* mutant embryo to develop from globular to heart shape shows that embryo polarization is not established correctly in the *sec3a* mutant. Although exocyst subunits localize to the cell plate (Fendrych *et al.*, 2010; our results), the defective *sec3a* embryos show that cell plate formation is not disrupted. It is also unlikely that the exocyst plays a role in determining the orientation of cell divisions, as defects during embryo development only arise after the initial divisions that occur normally. Moreover, in the fass mutant, in which the orientation of cell division and cell expansion is disrupted, this does not interfere with embryonic pattern formation and cell polarity (Torres-Ruiz & Jürgens, 1994). The *sec3a* mutant phenotype resembles that of the gnom mutant, with the difference that embryo development in the *sec3a* mutant arrests earlier than in the gnom mutant (Mayer *et al.*, 1991). As the complete lack of polarity in the gnom mutant is caused by defects in PIN cycling during polar auxin transport (Geldner *et al.*, 2003), it is possible that SEC3A plays a role in polar auxin transport. As the non-lethal exocyst mutants, exo70A1 and sec8, display defects in PIN cycling (Drdová *et al.*, 2013), SEC3A may function in PIN localization during embryo development. In addition, SEC3A interacts with ICR1, which could transduce ROP-mediated signaling to the exocyst (Lavy *et al.*, 2007; Bloch *et al.*, 2008); the exocyst could function as a ROP effector. ROP signaling has been implicated in many developmental processes, ranging from cell morphogenesis and differentiation to polar positioning of PIN proteins and the polarization of plant cell divisions (Yang & Fu, 2007; Humphries *et al.*, 2011; Nagawa *et al.*, 2012).

Localization and behavior of SEC3A-GFP puncta at the plasma membrane of interphase cells

SEC3A is cytoplasmic and localizes transiently to the plasma membrane in puncta, with an average lifetime varying from 6.3 to 12.6 s. There is no difference in the density of puncta between the growing root hair tip, the non-growing tube and the fully grown hair, and no difference between expanding and fully grown root epidermal cells. Thus, it is unlikely that SEC3A has a role in the exocytosis of cell wall matrix polysaccharides, unless the SEC3A-GFP puncta serve as an anchoring location for the remainder of the exocyst. In this case, although evenly spaced, SEC3A-GFP puncta in the plasma membrane may only mediate exocytotic events when interaction with the exocyst subunits on the exocytotic vesicle occurs. SEC3A could be compared with a door handle, always present, which requires an actor to open the door. This is unlikely as the other exocyst subunits, SEC6, SEC8, EXO70A1 and EXO84B, have a similar localization pattern (Fendrych *et al.*, 2013). In addition, the non-polar localization of

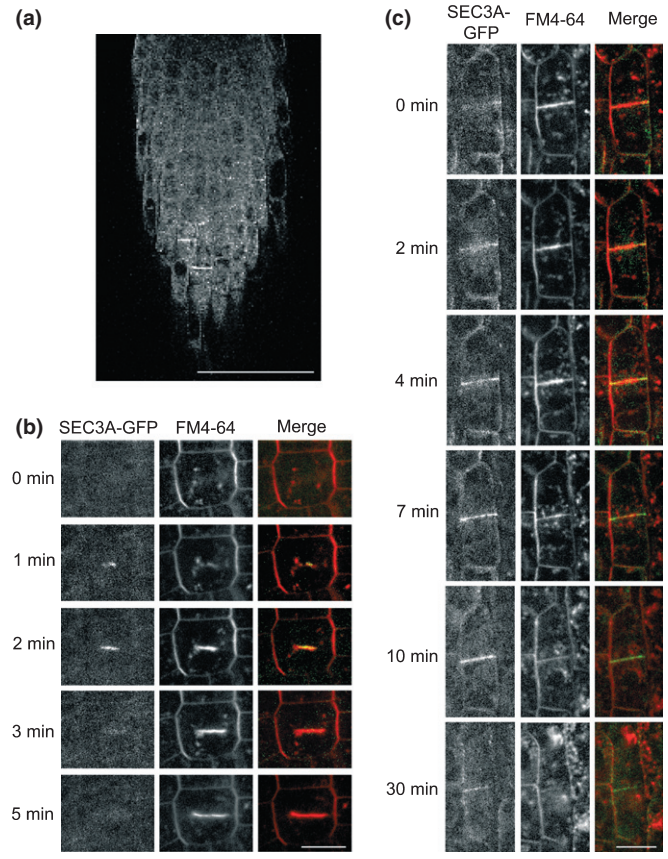

Fig. 10 Arabidopsis SEC3A-green fluorescent protein (GFP) localizes to early cell plates, is absent during later stages of cell plate development and reappears on the division wall after cytokinesis is complete. (a) SEC3A-GFP localizes to distinct cross walls in the root meristem. (b) SEC3A-GFP decorates the cell plate during its early stages and disappears during later stages of cell plate expansion in a root cortical cell. Cell membranes are counterstained with FM4-64. (c) After completion of cytokinesis, SEC3A-GFP accumulates at the division wall. Bars: 50 μm (a); 10 μm (b, c).

SEC3A differs from the situation in budding yeast, where SEC3 localizes to plasma membrane areas in which polarized exocytosis occurs, for example presumptive bud sites, the tips of budding cells and the mother–daughter cell neck during cytokinesis, where it plays a role in tethering secretory vesicles (Finger *et al.*, 1998). In addition, in growing tobacco pollen tubes, Hála *et al.* (2008) showed the accumulation of SEC6, SEC8 and EXO70A1 in the apical region where expansion occurs. Unlike the polar localization of SEC3 in yeast and the polar localization of exocyst subunits in tip growing pollen tubes, SEC3A-GFP does not show polarized localization on the plasma membrane of polarly expanding plant cells.

EXO70E2 localizes to discrete punctate structures that are present both in association with the plasma membrane and in the cytosol. This is different from the localization of SEC3A-GFP which does not localize to punctate structures in the cytoplasm. The EXO70E2-containing compartments in the cytoplasm were termed EXPO, as they did not co-localize with any conventional organelle (Wang *et al.*, 2010). Unlike EXO70E2, SEC3A-GFP only localizes to discrete puncta at the plasma membrane, and not in the cytoplasm. In budding yeast, SEC3A functions as a landmark protein at the plasma membrane, where most other exocyst subunits (SEC5, SEC6, SEC8, SEC10, SEC15 and EXO84) are recruited via attachment to exocytotic vesicles (Zhang *et al.*, 2008; Hutagalung *et al.*, 2009). Budding yeast EXO70 localizes to the plasma membrane but, unlike SEC3, its localization is dependent on the actin cytoskeleton (Hutagalung *et al.*, 2009). This is consistent with the localization of EXO70A1 to the plasma membrane (Fendrych *et al.*, 2013) and EXO70E2 to both EXPOs and plasma membrane puncta in plant cells.

Plasma membrane-localized SEC3A-GFP puncta have discrete lifetimes. The fluorescence intensity during the lifetime of a punctum can be divided into three phases: first, it gradually increases; it then remains constant for a short time span and, finally, it decreases gradually. This suggests the recruitment of multiple SEC3A proteins over time from the cytosol, followed by a gradual dissociation of SEC3A proteins in the final phase. The yeast exocyst is thought to consist of single or, at most, a few proteins per subunit (Munson & Novick, 2006). As the puncta in the plasma membrane of interphase cells are unlikely to represent single SEC3A-GFP proteins, our results show that the plant exocyst either consists of multiple SEC3A proteins or that multiple exocyst-mediated events occur in one punctum. The decrease in density of SEC3A-GFP puncta during BFA treatment suggests that the presence of SEC3A at the plasma membrane is dependent on the presence of Golgi-derived vesicles or vesicle-associated factors, for example other exocyst subunits. Interestingly, the lifetime of SEC3A-GFP puncta is shorter than that of the other exocyst subunits that display this localization. In comparable cell types, elongating root epidermal cells, the lifetime of SEC3A-GFP puncta (6.7 ± 3.6 s) is almost 3 s shorter than that of GFP-EXO70A1 (9.3 s) and almost 7 s shorter than that of GFP-SEC8 (13.3 s; Fendrych *et al.*, 2013). The short SEC3A lifetime compared with that of other exocyst subunits suggests that it is unlikely that SEC3A is recruited to the plasma membrane before the other exocyst subunits, and draws its role as a landmark protein into question. To be conclusive, thorough co-localization studies between SEC3A and the other exocyst subunits should be performed.

As discussed above, the similar, uniform density and similar lifetimes of SEC3A-GFP puncta at the plasma membrane suggest that SEC3A mediates an exocytotic event that is not related to (polarized) cell expansion. The only type of exocytosis that occurs over the cell surface, which can currently be detected using fluorescence microscopy, is that of CESA into the plasma membrane (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009). We did not find co-localization between SEC3A and CESA insertion into the plasma membrane. Therefore, SEC3A does not appear to be involved in the insertion of CESA, but may mediate exocytosis of the cell wall matrix via Golgi vesicles. However, we have not been able to show this directly and cannot exclude the possibility that SEC3A mediates exocytotic events not related to cell wall formation, for example the insertion of trans-membrane receptors or ion channels into the plasma membrane. Pecenková *et al.* (2011) have shown the involvement of Exo70B2 and Exo70H1 in plant

defence against pathogens. The insertion of receptors into the plasma membrane by an exocytotic mechanism during pathogen defence could be mediated by the SEC3A-GFP puncta.

Acknowledgements

This work was supported by the Ministry of Science and Technology of China (SQ2012CC057223). Y.Z. was funded by Wageningen University Sandwich Fellowship P2310. T.K. thanks Viktor Zárský (Charles University, Prague, Czech Republic) for sharing unpublished data and great discussions.

References

Bloch D, Hazak O, Lavy M, Yalovsky S. 2008. A novel ROP/RAC GTPase effector integrates plant cell form and pattern formation. *Plant Signaling and Behaviour* **3**: 41–43.

Chong YT, Gidda SK, Sanford C, Parkinson J, Mullen RT, Goring DR. 2010. Characterization of the *Arabidopsis thaliana* exocyst complex gene families by phylogenetic, expression profiling, and subcellular localization studies. *New Phytologist* **185**: 401–419.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**: 735–743.

Cole RA, Synek L, Zárský V, Fowler JE. 2005. SEC8, a subunit of the putative Arabidopsis exocyst complex, facilitates pollen germination and competitive pollen tube growth. *Plant Physiology* **138**: 2005–2018.

Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof YD, Schumacher K, Gonneau M, Hofte H, Vernhettes S. 2009. Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. *Plant Cell* **21**: 1141–1154.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology* **133**: 462–469.

Dhonukshe P, Baluška F, Schlicht M, Hlavacka A, Šamaj J, Friml J, Gadella TWJ. 2006. Endocytosis of cell surface material mediates cell plate formation during plant cytokinesis. *Developmental Cell* **10**: 137–150.

Drdová EJ, Synek L, Pečenková T, Hála M, Kulich I, Fowler JE, Murphy AS, Zárský V. 2013. The exocyst complex contributes to PIN auxin efflux carrier recycling and polar auxin transport in Arabidopsis. *Plant Journal.* **73**: 709–719.

Duttweiler HM. 1996. A highly sensitive and non-lethal beta-galactosidase plate assay for yeast. *Trends in Genetics* **12**: 340–341.

Edwards K, Johnstone C, Thompson C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acid Research* **19**: 1349.

Eliás M, Drdová E, Ziak D, Bavlnka B, Hála M, Cvrcková F, Soukupová H, Zárský V. 2003. The exocyst complex in plants. *Cell Biology International* **27**: 199–201.

Fendrych M, Synek L, Pecenková T, Drdová EJ, Sekeres J, Rycke RD, Nowack MK, Zársky V. 2013. Visualization of the exocyst complex dynamics at the plasma membrane of *Arabidopsis thaliana*. *Molecular Biology of the Cell* **24**: 510–520.

Fendrych M, Synek L, Pecenková T, Toupalová H, Cole R, Drdová E, Nebesarová J, Sedinová M, Hála M, Fowler JE *et al.* 2010. The Arabidopsis exocyst complex is involved in cytokinesis and cell plate maturation. *Plant Cell* **22**: 3053–3065.

Fiers M, Hause G, Boutilier K, Casamitjana-Martinez E, Weijers D, Offringa R, van der Geest L, van Lookeren Campagne M, Liu CM. 2004. Mis-expression of the CLV3/ESR-like gene *CLE19* in Arabidopsis leads to a consumption of root meristem. *Gene* **327**: 37–49.

Finger FP, Hughes TE, Novick P. 1998. Sec3p is a spatial landmark for polarized secretion in budding yeast. *Cell* **92**: 559–571.

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G. 2003. The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* **24**: 219–230.

van Gisbergen PA, Esseling-Ozdoba A, Vos JW. 2008. Microinjecting FM4-64 validates it as a marker of the endocytic pathway in plants. *Journal of Microscopy* **231**: 284–290.

Griffing LR. 2008. FRET analysis of transmembrane flipping of FM4-64 in plant cells: is FM4-64 a robust marker for endocytosis? *Journal of Microscopy* **231**: 291–298.

Gutierrez R, Lindeboom JJ, Paredez AR, Emons AM, Ehrhardt DW. 2009. Arabidopsis cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. *Nature Cell Biology* **11**: 797–806.

Hála M, Cole R, Synek L, Drdová E, Pecenková T, Nordheim A, Lamkemeyer T, Madlung J, Hochholdinger F, Fowler JE *et al.* 2008. An exocyst complex functions in plant cell growth in Arabidopsis and tobacco. *Plant Cell* **20**: 1330–1345.

He B, Guo W. 2009. The exocyst complex in polarized exocytosis. *Current Opinion in Cell Biology* **21**: 537–542.

He B, Xi F, Zhang X, Zhang J, Guo W. 2007. Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. *EMBO Journal* **26**: 4053–4065.

Hsu SC, TerBush D, Abraham M, Guo W. 2004. The exocyst complex in polarized exocytosis. *International Review in Cytology* **233**: 243–265.

Hsu SC, Ting AE, Hazuka CD, Davanger S, Kenny JW, Kee Y, Scheller RH. 1996. The mammalian brain rsec6/8 complex. *Neuron* **17**: 1209–1219.

Humphries JA, Vejlupkova Z, Luo A, Meeley RB, Sylvester AW, Fowler JE, Smith LG. 2011. ROP GTPases act with the receptor-like protein PAN1 to polarize asymmetric cell division in maize. *Plant Cell* **23**: 2273–2284.

Hutagalung AH, Coleman J, Pypaert M, Novick PJ. 2009. An internal domain of Exo70p is required for actin-independent localization and mediates assembly of specific exocyst components. *Molecular Biology of the Cell* **20**: 153–163.

James P, Halladay J, Craig EA. 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144**: 1425–1436.

Kee Y, Yoo JS, Hazuka CD, Peterson KE, Hsu SC, Scheller RH. 1997. Subunit structure of the mammalian exocyst complex. *PNAS* **94**: 14438–14443.

Ketelaar T, Anthony RG, Hussey PJ. 2004. Green fluorescent protein-mTalin causes defects in actin organization and cell expansion in Arabidopsis and inhibits actin depolymerizing factor's actin depolymerizing activity *in vitro*. *Plant Physiology* **136**: 3990–3998.

Ketelaar T, Emons AMC. 2001. The cytoskeleton in plant cell growth: lessons from root hairs. *New Phytologist* **152**: 409–418.

Kulich I, Cole R, Drdová E, Cvrcková F, Soukup A, Fowler J, Zárský V. 2010. Arabidopsis exocyst subunits SEC8 and EXO70A1 and exocyst interactor ROH1 are involved in the localized deposition of seed coat pectin. *New Phytologist* **188**: 615–625.

Lavy M, Bloch D, Hazak O, Gutman I, Poraty L, Sorek N, Sternberg H, Yalovsky S. 2007. A novel ROP/RAC effector links cell polarity, root-meristem maintenance, and vesicle trafficking. *Current Biology* **17**: 947–952.

Li S, van Os GM, Ren S, Yu D, Ketelaar T, Emons AM, Liu CM. 2010. Expression and functional analyses of EXO70 genes in Arabidopsis implicate their roles in regulating cell type-specific exocytosis. *Plant Physiology* **154**: 1819–1830.

Lindeboom J, Mulder BM, Vos JW, Ketelaar T, Emons AM. 2008. Cellulose microfibril deposition: coordinated activity at the plant plasma membrane. *Journal of Microscopy* **231**: 192–200.

Liu CM, Meinke DW. 1998. The titan mutants of Arabidopsis are disrupted in mitosis and cell cycle control during seed development. *Plant Journal* **16**: 21–31.

Liu J, Zuo X, Yue P, Guo W. 2007. Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. *Molecular Biology of the Cell* **18**: 4483–4492.

Mayer U, Torres Ruiz RA, Berleth T, Miséra S, Jürgens G. 1991. Mutations affecting body organization in the Arabidopsis embryo. *Nature* **353**: 402–407.

Meinke DW, Sussex IM. 1979. Embryo-lethal mutants of *Arabidopsis thaliana*. A model system for genetic analysis of plant embryo development. *Developmental Biology* **72**: 50–61.

Munson M, Novick P. 2006. The exocyst defrocked, a framework of rods revealed. *Nature Structural and Molecular Biology* **13**: 577–581.

Nagawa S, Xu T, Lin D, Dhonukshe P, Zhang X, Friml J, Scheres B, Fu Y, Yang Z. 2012. ROP GTPase-dependent actin microfilaments promote PIN1 polarization by localized inhibition of clathrin-dependent endocytosis. *PLoS Biology* **10**: e1001299.

Pecenková T, Hála M, Kulich I, Kocourková D, Drdová E, Fendrych M, Toupalová H, Zárský V. 2011. The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant–pathogen interaction. *Journal of Experimental Botany* **62**: 2107–2116.

Robinson DG, Langhans M, Saint-Jore-Dupas C, Hawes C. 2008. BFA effects are tissue and not just plant specific. *Trends in Plant Science* **13**: 405–408.

Samuel MA, Chong YT, Haasen KE, Aldea-Brydges MG, Stone SL, Goring DR. 2009. Cellular pathways regulating responses to compatible and self-incompatible pollen in Brassica and Arabidopsis stigmas intersect at Exo70A1, a putative component of the exocyst complex. *Plant Cell* **21**: 2655–2671.

Shaw SL, Dumais J, Long SR. 2000. Cell surface expansion in polarly growing root hairs of *Medicago truncatula*. *Plant Physiology* **124**: 959–970.

Songer JA, Munson M. 2009. Sec6p anchors the assembled exocyst complex at sites of secretion. *Molecular Biology of the Cell* **20**: 973–982.

Synek L, Schlager N, Eliás M, Quentin M, Hauser MT, Zárský V. 2006. AtEXO70A1, a member of a family of putative exocyst subunits specifically expanded in land plants, is important for polar growth and plant development. *Plant Journal* **48**: 54–72.

TerBush DR, Maurice T, Roth D, Novick P. 1996. The exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. *EMBO Journal* **15**: 6483–6494.

Torres-Ruiz RA, Jürgens G. 1994. Mutations in the *FASS* gene uncouple pattern formation and morphogenesis in Arabidopsis development. *Development* **120**: 2967–2978.

Wang J, Ding Y, Hillmer S, Miao Y, Lo SW, Wang X, Robinson DG, Jiang L. 2010. EXPO, an exocyst-positive organelle distinct from multivesicular endosomes and autophagosomes, mediates cytosol to cell wall exocytosis in Arabidopsis and tobacco cells. *Plant Cell* **22**: 4009–4030.

Wen TJ, Hochholdinger F, Sauer M, Bruce W, Schnable PS. 2005. The *roothairless1* gene of maize encodes a homolog of *sec3*, which is involved in polar exocytosis. *Plant Physiology* **138**: 1637–1643.

Wiederkehr A, Du Y, Pypaert M, Ferro-Novick S, Novick P. 2003. Sec3p is needed for the spatial regulation of secretion and for the inheritance of the cortical endoplasmic reticulum. *Molecular Biology of the Cell* **14**: 4770–4782.

Yang Z. 2008. Cell polarity signaling in Arabidopsis. *Annual Review of Cell and Developmental Biology* **24**: 551–575.

Yang Z, Fu Y. 2007. ROP/RAC GTPase signaling. *Current Opinion in Plant Biology* **10**: 490–494.

Zhang X, Orlando K, He B, Xi F, Zhang J, Zajac A, Guo W. 2008. Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. *Journal of Cell Biology* **180**: 145–158.

Zhang Y, Liu CM, Emons AM, Ketelaar T. 2010. The plant exocyst. *Journal of Integrative Plant Biology* **52**: 138–146.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Pairwise interactions between Arabidopsis exocyst subunits detected by yeast two-hybrid assays.

Fig. S2 Genotyping of the Arabidopsis SALK_145185 (*SEC3A*) T-DNA insertion line.

Table S1 Primer sequences

Movie S1 The behavior of individual Arabidopsis SEC3A-GFP puncta at the plasma membrane of the outer periclinal face of an expanding root epidermal cell.

Movie S2 The behavior of individual Arabidopsis SEC3A-GFP puncta at the plasma membrane of the outer periclinal face of a fully grown root epidermal cell.

Movie S3 Arabidopsis SEC3A-GFP puncta do not preferentially co-localize with cortical microtubules (CMTs). Time series of a line expressing mCherry-TUA5 (red) and SEC3A-GFP (green).

Movie S4 Arabidopsis SEC3A-GFP puncta do not co-localize with cellulose synthase (CESA) complex insertion events. Time series of a line expressing tdTomato-CESA6 (red) and SEC3A-GFP (green).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.