EXO70A1-Mediated Vesicle Trafficking Is Critical for Tracheary Element Development in Arabidopsis

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Exocysts are highly conserved octameric complexes that play an essential role in the tethering of Golgi-derived vesicles to target membranes in eukaryotic organisms. Genes encoding the EXO70 subunit are highly duplicated in plants. Based on expression analyses, we proposed previously that individual EXO70 members may provide the exocyst with functional specificity to regulate cell type– or cargo-specific exocytosis, although direct evidence is not available. Here, we show that, as a gene expressed primarily during tracheary element (TE) development, EXO70A1 regulates vesicle trafficking in TE differentiation in Arabidopsis thaliana. Mutations of EXO70A1 led to aberrant xylem development, producing dwarfed and nearly sterile plants with very low fertility, reduced cell expansion, and decreased water potential and hydraulic transport. Grafting of a mutant shoot onto wild-type rootstock rescued most of these aboveground phenotypes, while grafting of a wild-type shoot to the mutant rootstock did not rescue the short root hair phenotype, consistent with the role of TEs in hydraulic transport from roots to shoots. Histological analyses revealed an altered pattern of secondary cell wall thickening and accumulation of large membrane-bound compartments specifically in developing TEs of the mutant. We thus propose that EXO70A1 functions in vesicle trafficking in TEs to regulate patterned secondary cell wall thickening.

INTRODUCTION

The tracheary elements (TEs) of xylem tissues in terrestrial plants are elongated and terminally differentiated cells with spiral or pitted secondary cell wall (SCW) thickening (Lucas et al., 2013). The major role of TEs is to transport water and minerals from roots to shoots. TEs are formed through a series of sequential events including cell fate determination, cell elongation, patterned SCW deposition, and programmed cell death (PCD) (Fukuda, 1997; Turner et al., 2007). Exocytosis is expected to occur during patterned SCW thickening in order to insert cellulose synthase complexes into the plasma membrane and to allow deposition of SCW materials, such as hemicellulose and lignin, at specific sites of the cell wall after the cessation of cell elongation (Fukuda, 1997). PCD occurs during the final stage of TE development removes all intracellular contents and transverse cell walls between adjacent TEs, allowing for unimpeded water movement within mature TEs (Bollhöner et al., 2012). Using in vitro TE differentiation systems of Zinnia elegans and Arabidopsis thaliana, two NAC (for NAM, ATAF1,2, CUC2) domain transcription factors, VASCULAR-RELATED NAC-DOMAIN6 and 7, and a transcriptional repressor, VND-INTERACTING2, have been identified as key regulators for TE development (Milioni et al., 2001; Kubo et al., 2005; Yamaguchi et al., 2008, 2010, 2011). A recent study showed that active Rho GTPase ROP11 is able to recruit the MICROTUBULE DEPLETION DOMAIN1 protein to the SCW pits in developing TEs, causing local cortical microtubule disassembly and subsequently preventing the deposition of SCW materials to the pits (Oda and Fukuda, 2012).

In eukaryotic organisms, polarized exocytosis relies on an evolutionarily conserved exocyst complex that consists of eight subunits: SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70, and EXO84. In budding yeast (Saccharomyces cerevisiae), SEC5, SEC6, SEC8, SEC10, SEC15, EXO70, and EXO84. In budding yeast (Saccharomyces cerevisiae), SEC5, SEC6, SEC8, SEC10, SEC15, and EXO84 are associated with vesicles, while SEC3 and EXO70 are localized to the target membranes (Finger et al., 1998; Boyd et al., 2004). Exocysts act downstream of Rho, Rab, and Rab small GTPases to tether secretion vesicles to the target membranes (Robinson et al., 1999; He and Guo, 2009; Wu et al., 2010). As expected, mutations of SEC3 and EXO70 in yeast lead to accumulation of secretory vesicles at the budding site (He et al., 2007a).

In many species, such as yeast (Saccharomyces cerevisiae) and humans (Homo sapiens), each exocyst subunit is encoded by a single gene (Chong et al., 2010). In plants, although most of these exocyst subunits are encoded by one or a few genes, genes encoding for EXO70 are duplicated greatly, with 13 in moss (Physcomitrella patens), 15 in grape (Vitis vinifera), 23 in Arabidopsis,
and 47 in rice (Oryza sativa; Cvrčková et al., 2012). Several reports suggest that exocysts in plants are also involved in regulating polarized exocytosis. In Arabidopsis, mutations of SEC5, SEC6, SEC8, and SEC15a cause defects in pollen germination, pollen tube growth, and pollen transmission (Cole et al., 2005; Hála et al., 2008). Mutations of EXO84B, which encodes a cell plate–localized protein, lead to dwarfed plants with defective cytokinesis and accumulation of vesicles in leaf epidermal cells (Fendrych et al., 2010). In maize (Zea mays), a mutation in SEC3 leads to defective root hair elongation (Wen et al., 2005). However, in Arabidopsis, although SEC3A–green fluorescent protein has a cell plate localization during cytokinesis, in the tips growing root hairs it forms immobile punctate structures that are evenly distributed over the cell surface, suggesting that it is unlikely involved in polarized secretion (Zhang et al., 2013).

The duplication of EXO70 genes in plants has received much attention (Elías et al., 2003; Synek et al., 2006; Chong et al., 2010; Zhang et al., 2010; Cvrčková et al., 2012). Because expression of these genes in Arabidopsis is diverse and tightly associated with differentiating cells, we proposed previously that individual EXO70 members may provide the exocyst a functional specificity to regulate cell type– or cargo-specific exocytosis. EXO70C1 that is expressed in guard cells and pollen grains functions in pollen tube growth (Li et al., 2010). Expression of both EXO70B2 and EXO70H1 is upregulated by treatment with an elicitor peptide, ELF-18, derived from a bacterial elongation factor. Mutations of these two genes lead to enhanced susceptibility to Pseudomonas syringae, thereby implicating roles in plant–pathogen interaction (Pecenková et al., 2011). Recently, it has been shown that a plant U-box type ubiquitin ligase 22 targets EXO70B2 for a pathogen-associated molecular pattern in Arabidopsis (Stegmann et al., 2012). EXO70E2 has been implicated in a recently described exocytotic organelle, named EXPO, with unknown functions (Wang et al., 2010).

Microarray and RT-PCR analyses showed that EXO70A1 is expressed in most organs tested except for mature pollen (Synek et al., 2006; Li et al., 2010). Functions of EXO70A1 have been implicated in elongation of hypocotyls, stigmatic papillae, and root hairs (Synek et al., 2006), in pollen–stigma interaction during self-incompatibility response (Samuel et al., 2009), in cell plate formation (Fendrych et al., 2010), in pectin deposition in seed coats (Kulich et al., 2010), and in auxin polar transport in root epidermal and cortical cells (Or dová et al., 2013). Using polyclonal antibodies directed against EXO70A1, SEC6, and SEC8 from Arabidopsis, Hála et al. (2008) showed that these proteins are colocalized to the apex of growing pollen tubes in tobacco (Nicotiana tabacum). Kitashiba et al. (2011) showed that overexpression of EXO70A1 neither abolishes nor weakens self-incompatibility in Arabidopsis. Our studies using in situ hybridization and transgenic plants carrying a construct with EXO70A1 promoter fused with β-glucuronidase (pEXO70A1:GUS) previously (Li et al., 2010) and in this study showed that EXO70A1 is not expressed in root hair and cortical cells, pollen grains, pollen tubes, stigmatic papillae, and seed coats, nor during cytokinesis, but primarily in developing TEs. These results prompted us to investigate the role of EXO70A1 in TE development.

Through genetic, physiological, and cell biological examinations in Arabidopsis, we show that EXO70A1 regulates a step between Golgi vesicle production and vesicle fusion during TE development. Mutations of EXO70A1 in Arabidopsis led to nearly sterile and dwarfed plants with reduced cell and organ expansion, decreased water potential, and compromised hydraulic transport. Histological studies revealed that exo70a1-1 mutants exhibited aberrant xylem with an altered pattern of SCW thickening. Most of these aboveground phenotypes can be rescued by grafting onto a wild-type root stock, while the grafting of a wild-type shoot to the mutant root stock did not rescue the short root hair phenotype. Using cryo-fixation and electron microscopy, we showed that mutation of EXO70A1 led to altered SCW thickening and accumulation of large membrane-bound vesicles specifically in developing TEs. We thus propose that EXO70A1 acts in cell type–specific exocytosis to regulate TE formation.

RESULTS

EXO70A1 Is Primarily Expressed in Developing TEs

Using in situ hybridization, in transgenic plants carrying a GUS reporter construct, we showed previously that EXO70A1 is expressed during TE development (Li et al., 2010). In this study, we performed further analyses on pEXO70A1:GUS transgenic plants and confirmed that, in all tissues examined, GUS expression was observed specifically in differentiating TEs (Figure 1). Within the vascular bundles of roots (Figures 1A and 1B), cotyledons (Figure 1C), and leaves (Figure 1D), weak GUS expression was first observed in elongated TEs before patterned SCW thickening became visible (Figures 1A and 1C, white arrowheads), and GUS expression became stronger when patterned SCW thickening formed (Figures 1A to 1D, black arrowheads). GUS expression was most abundantly observed when patterned SCW thickening was distinct (Figures 1A and 1C, black arrows) and disappeared gradually afterwards. No GUS expression was seen in mature TEs (Figures 1B to 1D, white arrow). The association of GUS expression with developing TEs was observed in all organs examined: young hypocotyls (see Supp. Figure 1A online), inflorescence stems (see Supp. Figure 1B online), and pedicels (see Supp. Figure 1C online). Although defective root hair elongation, pollen rehydration, and seed coat pectin deposition have been reported in exo70a1 mutants (Synek et al., 2006; Samuel et al., 2009; Kulich et al., 2010), we did not detect GUS expression in root hairs, pollen grains, stigmatic papillae, or seed coats in the stages we examined.

Mutations of EXO70A1 Lead to Dwarfed and Nearly Sterile Plants with Reduced Water Potential

To elucidate the function of EXO70A1, two independent insertion lines from the SALK collection (www.Arabidopsis.org), namely, exo70a1-1 and exo70a1-2, with T-DNAs inserted in the first intron and the sixth exon, respectively, were examined. Consistent with results published previously, phenotypes of these two alleles are similar to each other (Synek et al., 2006). Therefore, in the following studies, we focused on exo70a1-1, as it has been shown to be a null allele (Synek et al., 2006). Homozygous exo70a1-1 plants were dwarfed and nearly sterile with smaller leaves, compared with the wild type (Figure 2A; see Supplemental
in exo70a1-1 leaves (Figure 2E). These studies suggest a possible defect in hydraulic transport in the mutant.

We measured the mineral content in aboveground tissues (mixed harvested samples of leaves, stems, flowers, and siliques) using inductively coupled plasma–mass spectrometry (ELAN DRC-e; Perkin-Elmer) and found no apparent differences between exo70a1-1 and the wild type (see Supplemental Figure 3 online), suggesting the mutant phenotype is unlikely to be caused by nutritional defects.

**Mutation of EXO70A1 Leads to Reduced Cell Expansion**

Consistent with the observation from Synek et al. (2006), exo70a1-1 seedlings grown on culture plates exhibited shorter root hairs compared with the wild type (Figure 3A). In transversely sectioned roots, hypocotyls, and stems, reduced cell expansion was observed in almost all of the cell types in exo70a1-1 compared with those in the wild type (Figures 3B and 3C; see Supplemental Figures 4A to 4F online). The diameters of individual TEs in both protoxylem and metaxylem in transversely sectioned exo70a1-1 roots were smaller than those in the wild type (Figures 3D and 3E).

In addition, extra cell division was observed in both pericycle and ground tissue in exo70a1-1 (Figures 3D and 3E, arrowheads). The exo70a1-1 plants also showed smaller floral organs. In fully opened stage 13 flowers, the lengths of sepals, petals, and pistils in the wild type were measured as 161.8 ± 138.48 μm, 1,762.36 ± 249.06 μm, and 1,835.65 ± 119.51 μm, respectively; while those in exo70a1-1 were 556.12 ± 126.09 μm, 688.85 ± 85.69 μm, and 434.3 ± 50.83 μm, respectively (n = 15 for each measurement; P < 0.01 for every comparative pair, as determined by Student’s t test). We then examined the lengths of cells in petals excised from these flowers. Under a differential interference contrast (DIC) microscope, we observed that, although cell sizes in the upper portion of the petal in exo70a1-1 were similar to those in the wild type, cell lengths in the lower portion of the petal from exo70a1-1 were significantly reduced (Figure 3F). Such a difference was not observed in these cells prior to flower opening. Therefore, it is likely that the shorter petal phenotype observed in exo70a1-1 was caused by reduced cell elongation in this region.

The Nearly Sterile Phenotype of exo70a1-1 Was Rescued by Grafting

To examine the exo70a1-1 phenotype further, we performed grafting experiments. We switched the shoots and roots between wild-type and exo70a1-1 plants by cutting and joining shoots and roots at the hypocotyl position of 3-d-old seedlings. Grafted seedlings were maintained under sterile conditions for 7 d before being transplanted to soil. We used the naming convention “rootstock-scion” to describe grafted plants. Therefore, “W-m” is used for plants with an exo70a1-1 shoot grafted onto a wild-type root and “m-W” for a wild-type shoot grafted onto an exo70a1-1 root. “W-W” and “m-m” refer to self-grafts of the wild type and the exo70a1-1, respectively. In the W-W and m-m graftings, plants obtained were almost indistinguishable from those nongrafted ones, suggesting that the grafting did not affect their general growth and development (Figure 2A). Following transfer to soil, slightly dwarfed plants with normal seed set were...
obtained from m-W seedlings (Figure 2A), suggesting that the grafted exo70a1-1 root caused only a slight growth retardation to the wild-type shoot. By contrast, W-m grafted plants, when compared with exo70a1-1 plants, showed greatly restored growth and fertility (Figure 2A). Seed set in primary inflorescences of W-m plants was similar to that of the wild type, while in secondary branches, a reduced seed set was observed (Figure 2A, arrow), suggesting that the wild-type root is able to partially rescue the growth and fertility defects in exo70a1-1.

We next examined whether grafting could rescue the small floral organ phenotype in exo70a1-1. In the main inflorescence of W-m grafted plants, the sizes of stage 12 floral buds and stage 13 flowers were indistinguishable from those of the wild type (Figure 2B), suggesting that expansion of these organs in exo70a1-1 flowers are restored by the grafted wild-type root. However, in side branches where the nearly sterile phenotype was evident, the sizes of floral buds and flowers were about the same as those in exo70a1-1, suggesting a close connection between organ expansion and fertility. In m-W grafted plants, the sizes of floral buds and flowers showed no difference from those in the wild type (Figure 2B).

When m-W seedlings were grown on vertically cultured plates, we observed that these seedlings still exhibited short root hairs (Figure 3A). Moreover, in W-m grafted seedlings, normal root hairs were observed (Figure 3A). Thus, it is unlikely that the short-root hair phenotype was caused by disrupted nutrients or signals from the shoot.

We then measured cell lengths in the lower portion of the petal in different grafting combinations and observed that epidermal cell lengths were increased significantly in W-m grafted plants.
compared with those in homozygous \textit{exo70a1-1} (mm; Figure 3F), suggesting that elongation of these cells was restored by the wild-type rootstock. We did notice that cells in W-m and m-W grafted plants were still significantly shorter than those in the wild type (Figure 3F), indicating that the cell elongation in both graftings did not achieve the same level as that in the wild type. Since the lengths of the flowers in both grafting combinations were about the same as those of the wild type, it is plausible that extra cell divisions in petals of these grafted plants compensated for the reduced cell elongation.

It has been reported previously that the stigmatic papillae in \textit{exo70a1-1} is immature in appearance, with almost no elongation (Synek et al., 2006). We examined if grafting could rescue this phenotype. As showed in Supplemental Figure 5 online, the elongation of stigmatic papillae in open flowers of W-m grafted plants was about the same as those in W-W grafting, while those in m-m grafted had no elongation, as with those in the \textit{exo70a1-1} mutant. This result showed the W-m grafting restored the defective stigmatic papillae elongation in \textit{exo70a1-1}.

These grafting experiments together revealed that the grafted wild-type root is able to repair most defects in the aboveground organs of \textit{exo70a1-1} plants, such as cell expansion and fertility. Most likely the reduced cell expansion in \textit{exo70a1-1} is caused by defective root to shoot hydraulic transport, while the reduced root hair elongation is not caused by defects in shoots.

The Nearly Sterile Phenotype of \textit{exo70a1-1} Is Caused by Defects in Pollination

Based on defective pollen rehydration on stigmas in \textit{exo70a1-1} mutants and \textit{Exo70A1RNAi} transgenic \textit{Brassica napus} plants, it has been proposed previously that \textit{EXO70A1} is involved in the self-incompatibility response (Samuel et al., 2009). We examined whether or not this supposition is true. Although \textit{exo70a1-1} plants produced very few pollen grains, we were able to perform hand-pollination experiments with pollen from several anthers. When wild-type pollen was used to pollinate \textit{exo70a1-1} stigmas, ~45% of siliques (\(n = 20\)) were able to expand to the length of the wild-type ones after 4 d, and 10 to 30 seeds were produced in each siliqua. Similarly, when wild-type stigmas were pollinated by \textit{exo70a1-1} pollen, 65% of siliques (\(n = 20\)) were able to expand to the length of the wild-type ones, producing 20 to 40 seeds in each siliqua. When \textit{exo70a1-1} pistils were hand-pollinated with self pollen, we observed that up to 20 seeds per siliqua were produced. All progeny obtained from the self-pollination were homozygous for the T-DNA insertion, as shown by PCR-based genotyping, and exhibited \textit{exo70a1-1} phenotypes, thereby excluding the possibility of pollen contamination. Most likely the nearly sterile phenotype is caused by a mechanistic defect in pollination. Consistent with this observation, aniline blue staining of pistils from \textit{exo70a1-1} flowers 1 d after anthesis revealed almost no pollen tubes in the style (Figure 4B), while a large number of pollen tubes was observed in the style of the wild type (Figure 4A). The number of pollen tubes in the mutant style was increased significantly when \textit{exo70a1-1} stigma was hand-pollinated with self-pollen (Figures 4C and 4D). Thus, it is plausible that the nearly sterile phenotype in \textit{exo70a1-1} is caused by failure in pollination through combined defects in male and female floral organs.
Mutation of EXO70A1 Leads to Reduced Hydraulic Conductance

To address if hydraulic transport was affected in exo70a1-1, we designed an ink transport experiment. Young inflorescence stems, 2 cm in length, were excised from wild-type and exo70a1-1 plants, in which all postanthesis silica spines were removed, keeping flowers and floral buds intact. The bottom ends of the inflorescence stems were soaked in 1:5 diluted red or blue ink. Using a dissection microscope, we monitored how long it took for the ink to reach the sepals and petals. As shown in Figure 5, the ink was visible in the sepals and petals from the wild-type in –40 min, while it took –120 min to reach the flower in the exo70a1-1. Unexpectedly, neither inflorescence stems excised from the m-W grafted nor those from the W-m grafted plants showed altered hydraulic transport efficiencies compared with inflorescence stems with the same genotype from nongrafted plants, suggesting that the restored fertility and organ expansion observed in aboveground organs in exo70a1-1 after grafting are not caused by improved hydraulic conductance in inflorescence stems.

To examine if hydraulic transport is also compromised in the root of exo70a1-1 plants, we designed an ink transport experiment for roots. We monitored how long it took for the ink to move from the 2-cm-long roots to the hypocotyl under a dissection microscope. As shown in Figure 5C, the average time needed for the ink to reach the hypocotyl was 3.28 ± 0.56 h for the wild type, while it was 8.44 ± 2.13 h for the exo70a1-1 plants (n = 30; P < 0.01, as determined by Student’s t test), suggesting a compromised hydraulic transport in the root of exo70a1-1.

These studies together revealed that the hydraulic transport efficiencies in the inflorescence stems and roots of exo70a1-1 have been reduced to approximately one-third of those in the wild type.

exo70a1-1 Mutants Show Irregular TE Development

TEs in Arabidopsis exhibit highly organized patterns of SCW deposition, which is spiral-shaped in the protoxylem and pitted in the metaxylem. In addition to the aforementioned reduced inner diameter of TEs in both protoxylem and metaxylem in transversely sectioned exo70a1-1 roots (Figures 3D and 3E), we also observed that the inner diameter of TEs in exo70a1-1 was greatly reduced in transversely sectioned inflorescence stems (Figures 6A and 6B). We then examined whether the mutation of EXO70A1 led to altered TE development. In cleared samples, we observed that basic TEs were present in exo70a1-1 inflorescences. However, in stage 13 flowers, we noticed that TEs in pistils were very faint in exo70a1-1 compared with those in the wild type (Figures 6C and 6D). Similarly, TEs with variable thickness were observed in the inflorescence stems of exo70a1-1 (Figures 6E and 6F).

More detailed studies were performed at the seedling stage. We observed that TEs in exo70a1-1 cotyledons often exhibited discontinuity, with alternations of strong and weak light reflections as observed under a DIC microscope (Figures 7A and 7B). Similar defects were also observed in hypocotyls (see Supplemental Figure 6 online). The altered TE morphology was more evident in seedlings stained with basic fuchsin in which lignin-containing TEs were bright pink (Figures 7C and 7D). Confocal microscopy observations of propidium iodide–stained root samples revealed that, instead of the well-organized pits in the metaxylem of the wild type, irregular patterns of pits with variable distances between them were observed in exo70a1-1 plants (Figures 7E and 7F). Although the length of mature TEs in exo70a1-1 roots was not significantly different from those in the wild type, a significantly reduced inner diameter of TEs was observed (Table 1). The width of perforations between two neighboring TEs was also narrower in exo70a1-1 than those in the wild type (Figures 7G and 7H, Table 1). Among 47 TEs examined in exo70a1-1, five of them showed an absence of perforations.

We then used transmission electron microscopy (TEM) to examine the junction regions between cotyledons and the hypocotyl, where TE differentiation is active. Instead of the regularly patterned SCW thickening in the wild type (Figure 7I), we observed various shapes of SCW thickening and irregular distances between neighboring thickening within mature TEs in exo70a1-1 (Figure 7J). Furthermore, the distances between two neighboring SCW thickenings were significantly larger than those in the wild type (Table 1; n = 150, P < 0.01, as determined by Student’s t test). Also, patterned SCW thickening was often

Figure 4. In Vivo Pollen Germination.

Pollen tubes in styles were visualized under a fluorescence microscope after aniline blue staining. Bar = 100 μm for all photos.

(A) A self-pollinated wild-type Arabidopsis pistil.
(B) A self-pollinated exo70a1-1 pistil. Note that no pollen tube was observed in the style.
(C) A wild-type pistil was hand-pollinated with self pollen.
(D) An exo70a1-1 pistil was hand-pollinated with self pollen.
observed at the perforation sites in the exo70a1-1 plants (six out of 11 examined), leading to narrow perforations (see Supplemental Figure 7 online). It is interesting to note that the PCD that occurs during the final stage of TE development to remove cellular contents does not seem to be affected by the mutation of EXO70A1 (Figures 7I and 7J).

Membrane-Bound Compartments Accumulate in Developing TEs of exo70a1-1

We used high-pressure cryo-fixation in combination with TEM to examine TE development at the junction regions between cotyledons and the hypocotyl. We focused our attention on developing TEs in which the patterned SCW thickening was evident. In the wild-type plants during patterned SCW deposition in TEs, we observed Golgi vesicles with an average size of 75.1 ± 12.98 nm (n = 50; Figures 8A and 8E). However, in developing TEs of exo70a1-1 plants, we observed that Golgi bodies in the exo70a1-1 were often deformed, with an increased number of cisternae (Figure 8D). The average number of Golgi cisternae in developing TEs in wild-type plants was 3.75 ± 0.55, while the average number in exo70a1-1 plants was 5.10 ± 0.79 (n > 20, P < 0.01, as determined by Student’s t test). More strikingly, we observed abundant accumulation of larger membrane-bound compartments with an average size of 158.97 ± 46.44 nm (n = 53) in the cytoplasm of developing TEs in exo70a1-1 plants (Figures 7B and 7F), a size that is significantly different from those Golgi vesicles observed in the wild type (P < 0.01, as determined by Student’s t test). In the surrounding non-TE cells, these abnormal Golgi bodies and large membrane-bound compartments were not observed, suggesting that the defect is specific to TEs. Since these accumulated membrane-bound compartments were much larger than normal Golgi vesicles, we speculate that they either originate from fusions of multiple vesicles or that the mutation of EXO70A1 leads to the production of larger vesicles.

DISCUSSION

Instead of one gene encoding each subunit of the octomeric exocyst complex as in most animal species, genes encoding EXO70 in plants are expanded greatly for unknown reasons. As the most well-characterized member of the 23 EXO70s in Arabidopsis, the functions of EXO70A1 have been implicated in hypocotyl and root hair elongation (Synek et al., 2006), self-incompatibility (Samuel et al., 2009), cytokinesis (Fendrych et al., 2010), localized pectin deposition in seed coat (Kulich et al., 2010), and auxin polar transport in roots (Drdová et al., 2013). However, our expression studies showed that EXO70A1 is primarily expressed in developing TEs (Li et al., 2010). In this study, we showed by combined genetic, physiological, histological, and grafting experiments that EXO70A1 regulates vesicle trafficking...
and rigidity to mature TEs to prevent collapse under the high negative pressures exerted by transpiration (Fukuda, 1997, 2004; Turner et al., 2007). Patterned SCW deposition in TEs usually occurs at the lateral cell faces and is excluded from the end walls that become perforated during the final stage of TE development. Particle rosettes, now known to be cellulose synthase complexes (Kimura et al., 1999), have been observed in vesicles of differentiating TEs in *Zinnia* decades ago (Haigler and Brown, 1986), suggesting exocytosis is involved in SCW thickening (Fukuda, 1997; Ye, 2002; Turner et al., 2007).

EXO70A1 has been implicated in hypocotyl and root hair elongation (Synek et al., 2006), self-incompatibility (Samuel et al., 2009), and localized deposition of seed coat pectin (Kulich et al., 2010). The expression data used in these studies are mainly from the publicly available Genevestigator database, showing that EXO70A1 is expressed in all tissue samples examined except for mature pollen (Synek et al., 2006; Kulich et al., 2010). However, our expression analyses using pEXO70A1:GUS transgenic plants revealed that, as verified by in situ hybridization, EXO70A1 is one of two EXO70 genes in *Arabidopsis* expressed in developing TEs (Li et al., 2010). Here, we further showed that EXO70A1 is primarily expressed in developing TEs in roots, hypocotyls, cotyledons, leaves, stems, and various floral organs. Within TEs, the expression was first observed during TE elongation, then became stronger during patterned SCW thickening, and ceased completely in mature TEs. No expression was observed in root hairs, pollen grains, stigmatic papillae, or seed coats in the stages examined. Since TEs are a specialized tissue running across almost all organs, the expression profile established in whole-organ level microarray analyses is true but can be misleading when interpreted improperly. We therefore propose that, although microarrays are an efficient tool to provide a general picture for gene expression, it is critically important to use in situ hybridization and reporter constructs to elucidate the expression pattern at the cellular level when individual genes are studied.

### Most Defects Observed in exo70a1-1 Can Be Explained by Imperfect TEs

Homozygous *exo70a1-1* plants show pleiotropic phenotypes, including dwarfing, shortened root hairs, and greatly reduced fertility (Synek et al., 2006; Samuel et al., 2009). Genetic analyses showed that pollen in *exo70a1* functions normally, while the reduced fertilization and seed set are caused by a sporophytic defect (Synek et al., 2006). We showed that both the pollen tube growth in styles and seed set were improved greatly in *exo70a1-1* when hand-assisted pollinations were performed, indicating a mechanistic defect in pollination. This is further supported by the observation that, when wild-type roots were grafted to *exo70a1-1* shoots, the mutant shoots showed an almost complete restoration of stigmatic papillae elongation and seed set.

Physiological experiments performed here revealed that *exo70a1-1* plants exhibited significantly reduced water content, water potential, and water loss efficiency. Analyses of mineral content in aboveground tissues of *exo70a1-1* plants did not show significant differences from those of the wild type. However, histological studies showed reduced cell expansion in many cell types throughout the *exo70a1-1* plant (Synek et al.,

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**Figure 6.** Defective TE Formation in *exo70a1-1*.  
(A) and (B) UV autofluorescence of transversely sectioned inflorescence stems of the wild type (A) and *exo70a1-1* (B), showing smaller TEs (arrowheads) in *exo70a1-1* compared with those in the wild-type *Arabidopsis*.  
(C) and (D) Dark-field observations of the wild-type (C) and *exo70a1-1* (D) stage 13 flowers. Note the underdeveloped TEs (arrowheads) in the pistils of *exo70a1-1* pistils compared with those in the wild type.  
(E) and (F) Abnormal TEs observed in cleared *exo70a1-1* inflorescence stems. Note the difference between TEs in the wild type (E), indicated by arrowheads) and those in *exo70a1-1* (F, arrowheads). Bars = 500 μm in (A) and (B), 40 μm in (C) and (D), and 100 μm in (E) and (F).
We showed that exo70a1-1 plants exhibit severe defects in TE development, with reduced inner cell diameter, altered patterns of SCW thickening, and partial perforations. EXO70G2 is another gene exhibiting a similar expression pattern to EXO70A1 (Li et al., 2010), which may explain why a basic xylem system, with some abnormalities, is able to form in homozygous exo70a1-1 plants. Mutations of both EXO70A1 and EXO70G2 may lead to a complete loss of SCW thickening during TE differentiation and, consequently, to an embryo- or seedling-lethal phenotype.

Using ink transport experiments, we showed that the hydraulic transport efficiencies of inflorescence stems and roots of exo70a1-1 are only approximately one-third of those in the wild type. Grafting of a wild-type root to the exo70a1-1 shoot restores most aboveground defects, such as sterility, reduced

Figure 7. Defective TE Formation in exo70a1-1 Seedlings.

(A) and (B) DIC images of cleared wild-type (A) and exo70a1-1 (B) cotyledons of Arabidopsis. Note the gaps (red arrowheads) in TEs of exo70a1-1 in contrast with continuous TEs (white arrowheads) in the wild type.

(C) and (D) Basic fuchsin staining of the cotyledon-hypocotyl junction regions from wild-type (C) and exo70a1-1 (D) seedlings. Lignin-containing xylem is stained in bright pink. Note the lightly stained gaps (red arrowheads) in TEs.

(E) and (F) Mature metaxylem from wild-type (E) and exo70a1-1 (F) roots, showing the pitted SCW pattern. Roots were stained with propidium iodide and observed under a confocal microscope. Note that pits in exo70a1-1 were irregular.

(G) and (H) Perforations (white arrows) between two adjacent TEs in wild-type (G) and exo70a1-1 (H) roots. Note that the perforation between two adjacent TEs in exo70a1-1 was incomplete.

(I) and (J) TEM images of longitudinally sectioned mature protoxylem in wild-type (I) and exo70a1-1 (J) hypocotyls. Note the irregular SCW thickenings (red arrowheads) on the side walls of TEs in exo70a1-1.

Table 1. Properties of Mature TEs

<table>
<thead>
<tr>
<th>Genotype/P Value</th>
<th>Width of Perforations (µm)</th>
<th>Inner Diameters of TEs (µm)</th>
<th>Length of TEs (µm)</th>
<th>Pit Sizes (µm²)</th>
<th>Distance between Pits (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>The wild type</td>
<td>3.16 ± 1.16 (n = 32)</td>
<td>4.70 ± 1.30 (n = 28)</td>
<td>248.42 ± 107.58 (n = 30)</td>
<td>2.56 ± 0.54 (n = 160)</td>
<td>6.15 ± 0.79 (n = 150)</td>
</tr>
<tr>
<td>exo70a1-1</td>
<td>1.46 ± 1.54 (n = 30)</td>
<td>4.16 ± 1.16 (n = 36)</td>
<td>261.92 ± 107.08 (n = 30)</td>
<td>2.10 ± 0.56 (n = 171)</td>
<td>8.46 ± 0.85 (n = 150)</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
organ expansion, and stigmatic papillae elongation. Nevertheless, ink transport experiments in grafted plants reveal that grafting a wild-type rootstock to an exo70a1-1 shoot did not give a significant improvement of hydraulic transport in the inflorescence stem. We thus believe that the complementation of aboveground defects in W-m grafted plants is a consequence of improved water supply from the wild-type rootstock, which presumably leads to improved water delivery to the exo70a1-1 inflorescence stem.

The short-root hair phenotype in exo70a1-1 is more difficult to explain. Sufficient evidence shows that exocysts are involved in polarized growth in root hairs and pollen tubes. A mutation of maize ROOTHAIRLESS1 that encodes a homolog of SEC3, another subunit of the exocyst complex, leads to severe deficiencies in root hair elongation (Wen and Schnable, 1994; Wen et al., 2005). A T-DNA insertion in EXO70C1, a gene expressed in pollen grains and pollen tubes, leads to a severe defect in pollen transmission (Li et al., 2010). However, because EXO70A1 is not expressed in root hair cells and the exocyst is expected to act in a cell-autonomous manner, it is unlikely that the short root hair phenotype is caused directly by the mutation of EXO70A1. Actually, in addition to root hairs, we observed reduced cell expansion in many other cell types in roots, stems, and floral organs. It is plausible that the short-root hair phenotype is part of the general cell expansion defects caused by reduced hydraulic transport. Consistent with this, we observed severely reduced ink transport efficiency in the root of exo70a1-1 plants. Of course, further experiments are needed in linking hydraulic transport to root hair elongation.

EXO70A1 Plays a Critical Role in Vesicle Trafficking in Developing TEs

The exocyst complex regulates the vesicle tethering process during exocytosis. As a key subunit of the exocyst complex, EXO70 in yeast interacts with Rho GTPases to regulate the polarized exocytosis and cell wall deposition (Robinson et al., 1999; He et al., 2007b; Wu et al., 2010). One thing peculiar in plants is the multiplication of EXO70 genes in the genome (Synek et al., 2006). It remains to be answered whether these duplicated EXO70 members in plants act redundantly in exocytotic events or whether individual members have evolved specialized functions in specific cell types. Our studies support the latter proposition. Expression analyses of all 23 EXO70 genes in Arabidopsis showed that individual EXO70 members are expressed diversely in differentiating cells of various cell types, and none of these genes is expressed in fully differentiated cells such as mesophyll cells in mature leaves. This supports their hypothesized roles in regulating cell type- or cargo-specific vesicle trafficking (Li et al., 2010). In this study, we showed that mutation of EXO70A1 resulted in irregular patterns of SCW thickening in TEs and abundant accumulation of large membrane-bound compartments specifically in developing TEs, although PCD, the final stage of TE differentiation, does not seem to be affected. These membrane-bound compartments, which may or may not be EXO70A1 positive, are significantly larger than the Golgi vesicles observed in the wild type (Fendrych et al., 2010). It is interesting to note that vesicles accumulated in the exo84b mutants are also much larger than the Golgi vesicles observed in the wild type (Fendrych et al., 2010). Of course, from these results, we cannot determine whether these larger membrane-bound compartments are on

**Figure 8.** Cryo-Fixation and Electron Microscopic Observations of Developing TEs.

The junction regions between cotyledons and the hypocotyl, where new TEs are continuously formed, were examined. (A), (C), and (E), the wild type; (B), (D), and (F), exo70a1-1.

(A) and (B) Developing TEs in the wild type (A) and exo70a1-1 (B). Note the large membrane-bound compartment (arrow) in exo70a1-1 compared with small vesicles in the wild type (arrowheads). Patterned SCW thickenings are marked with asterisks.

(C) and (D) Golgi bodies (arrows) in developing TEs of the wild type (C) and exo70a1-1 (D). Note the deformed Golgi bodies with an increased number of cisternae in exo70a1-1.

(E) and (F) Vesicles (arrowheads) in the developing TE of the wild type (E) compared with large membrane-bound compartments (arrows) in exo70a1-1 (F).

Bars = 0.5 μm in (A) and (B) and 0.2 μm in (C) to (F).
their way to the plasma membrane, if they originate from fusions, or if an unconventional protein secretion pathway is involved (Ding et al., 2012).

In summary, these studies allowed us to propose that EXO70A in developing TEs regulates vesicle trafficking and patterned SCW thickening during TE formation. This is in agreement with the proposed hypothesis that individual EXO70 members in plants regulate cell type–or cargo-specific exocytosis (Li et al., 2010). The requirement of such a large number of EXO70 genes in plants may be associated with dynamic functions of extracellular materials and communications between cells. Further functional characterization of other EXO70 genes is needed to build an insightful connection between exocytosis and plant development.

METHODS

Plant Materials and Growth Conditions

T-DNA insertions in exo70a1-1 and exo70a1-2 were confirmed by PCR-based genotyping with T-DNA left border primer (LBa1, 5'-TGGTTCA-CGATGGGCGGATC-3') and EXO70A1-specific primers (5'-AAAGATTTTCTCCAGTTTTGGAAT-3' and 5'-TGTTACTGTTTCGATTTTCC-3' for exo70a1-2). The selected homozygous exo70a1-1 plant was backcrossed three times with the wild-type Arabidopsis thaliana Columbia-0) to confirm the genetic linkage. For growth of seedlings on plates, seeds were gas sterilized and sown on solid media containing half-strength Murashige and Skoog mineral salts (Duchefa), 1% Suc, 0.1% MES, and 1.5% agar, pH 5.7, and kept at 4°C in the dark for 3 d prior to transfer to a growth room and being cultivated vertically under long-day conditions (16 h light, 8 h darkness) at 21°C. For growth in soil, 5-d-old seedlings on plates were transferred to pots filled with a soil-vermiculite mixture (1:1) under the same conditions.

Grafting Experiments

Grafting was performed using the method described previously (Turnbull et al., 2002) with some modifications. Wild-type and exo70a1-1 seedlings cultivated in vertical plates for 3 d were cut perpendicularly at the upper quarter of the hypocotyls using a double-sided blade and rejoined using split 1-μL pipette tips as the supporting collar. The grafted seedlings were cultured in vertical plates for another 7 d before being transferred to soil and grown as described above.

Microscopy

GUS assays were performed as described previously (Li et al., 2010). For semithin sections, 10-d-old seedlings were fixed in a modified formalin-acetic acid solution (Liu et al., 1993) and embedded in LR White resin as described in the manufacturer’s manual (London Resin Company). One-micrometer-thick sections were cut with a microtome and stained using periodic acid and Schiff’s reagent (Sigma-Aldrich) for polysaccharides or toluidine blue for the general cellular structure (Sigma-Aldrich). For lignin visualizations, 7-d-old seedlings were cleared and stained with 0.01% basic fuchsin as described previously (Dharmawardhana et al., 1992). For observation of TEs, roots of 7-d-old seedlings were stained with 0.5 μg/mL propidium iodide solution (Sigma-Aldrich) for 5 min and then visualized under a confocal scanning microscope (Leica SP5). For observation of pollen tubes, 10 h after pollination, pistils were excised and fixed in a cold ethanol/acetic acid (3:1) solution for 1 to 3 h and then washed three times with distilled water, followed by incubation in 8 M NaOH overnight. These pistils were then washed in distilled water at least three times for 1 h each and then stained in 0.1% aniline blue in 0.1 M K3PO4-KOH buffer for 30 min and observed under a fluorescence microscope (Leica DM4500B).

Cryo-Fixation and Electron Microscopy

Excised samples of the cotyledon-hypocotyl junction regions from 3-d-old seedlings were rapidly frozen using a High-Pressure Freezing Machine (Leica EM PACT2 with Leica EM RTS) at 2050 bars and stored under liquid nitrogen. Samples were freeze substituted in acetone containing 2% OsO4 and 1% distilled water at −140°C. The temperature was gradually raised to −90°C over 2 h. The substitution was then performed according to following procedure: −90°C for 48 h, −60°C for 24 h, and −30°C for 24 h. After warming to 0°C, samples were washed three times in acetone and allowed to reach room temperature. These samples were then infiltrated with gradually increasing percentages of Spurr’s resin (EMS) in acetone and embedded into flat modules. The resin was hardened by incubation at 70°C for 12 h. Section was performed with a microtome (Leica UC6), and observation was performed under a Philips–FEI Tecnai20 transmission electron microscope.

Physiological Assays

Water potentials were measured in 5-week-old plants using a vapor pressure osmometer (HR-33T, Wescor) by following the manual provided by the supplier. Water content was calculated by subtracting the weight of fully expanded, dried leaves from the fresh weight of the same leaves. For calculation of water loss rates, fully expanded leaves were excised and placed in a Petri dish and allowed to air dry. These leaves were weighed at different time points.

The hydraulic conductance assay was performed in stems using an ink transport experiment. The young inflorescence stems, 2 cm in length, were excised from 5-week-old wild-type and exo70a1-1 plants, in which all postanthesis silicles were removed, keeping flowers and floral buds. The bottom ends of the stems were soaked in 1:5 diluted red ink (Pelikan). Under a dissection microscope, we monitored how long it took for the ink to reach the first flower or floral bud.

To measure the hydraulic transport in roots, two agarose blocks were prepared using half-strength Murashige and Skoog salts with 1% Suc and 1.5% agarose, one with 2:5 diluted blue ink (Pelikan) and another one without ink. These blocks were placed 1 mm apart in a 5-cm Petri dish. Eight-day-old wild-type and exo70a1-1 seedlings (the length of primary roots is between 2.2 and 2.4 mm) were laid onto the surfaces of these blocks, with the root tip (−2 mm) on the ink block, and the rest of the seedling on the non-ink block. The distance of the ink transport was −2 cm for all roots. We monitored how long it took for the ink to reach the hypocotyl under a dissection microscope.

For assisted pollinations, wild-type and exo70a1-1 floral buds were emasculated and pollinated with pollens from wild-type and exo70a1-1 plants in different cross combinations, and seed sets were counted after 2 weeks.

Measurements of Mineral Content

Minerals in aboveground tissues of 50-d-old wild-type and exo70a1-1 plants were extracted and quantified using inductively coupled plasma-mass spectrometry as described by Gong et al. (2003).

Accession Numbers

The Arabidopsis Genome Initiative locus identifier for EXO70A1 is At5g03540. T-DNA insertion lines, exo70a1-1 (Salk_014826) and exo70a1-2 (Salk_135462), were obtained from the ABRC.
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. EXO70A1 Is Expressed in Developing TEs.

Supplemental Figure 2. exo70a1-1 Plants Exhibit Dwarf, Reduced Organ Size and Nearly Sterile Phenotypes.

Supplemental Figure 3. Mineral Contents in Aboveground Tissues from Wild-Type and exo70a1-1 Plants.

Supplemental Figure 4. Reduced Cell and Organ Expansion in exo70a1-1.

Supplemental Figure 5. Defective Stigmatic Papillae Elongation in exo70a1-1 Can Be Rescued by W-m Grafting.

Supplemental Figure 6. Defective TE Development in exo70a1-1 Hypocotyls.

Supplemental Figure 7. Patterned SCW Thickening at the Perforation Site in exo70a1-1.

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AUTHOR CONTRIBUTIONS

S.L. and M.C. performed most of the research, and S.L. drafted the article. D.Y. carried out grafting experiments. S.R. performed some expression and ink transport analyses. S.S. carried out high-pressure freezing and freeze substitution. L.L. carried out electron microscopy analysis. T.K. and A.-M.C.E. supervised part of this project and revised the article. C.-M.L. designed the experiment, supervised the study, and revised the article.

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EXO70A1-Mediated Vesicle Trafficking Is Critical for Tracheary Element Development in Arabidopsis

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