Expression and Functional Analyses of *EXO70* Genes in Arabidopsis Implicate Their Roles in Regulating Cell Type-Specific Exocytosis[1][W][OA]

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During exocytosis, Golgi-derived vesicles are tethered to the target plasma membrane by a conserved octameric complex called the exocyst. In contrast to a single gene in yeast and most animals, plants have greatly increased number of *EXO70* genes in their genomes, with functions very much unknown. Reverse transcription-polymerase chain reactions were performed on all 23 *EXO70* genes in Arabidopsis (*Arabidopsis thaliana*) to examine their expression at the organ level. Cell-level expression analyses were performed using transgenic plants carrying β-glucuronidase reporter constructs, showing that *EXO70* genes are primarily expressed in potential exocytosis-active cells such as tip-growing and elongating cells, developing xylem elements, and guard cells, whereas no expression was observed in cells of mature organs such as well-developed leaves, stems, sepals, and petals. Six *EXO70* genes are expressed in distinct but partially overlapping stages during microspore development and pollen germination. A mutation in one of these genes, *EXO70C1* (At5g13150), led to retarded pollen tube growth and compromised male transmission. This study implies that multiplications of *EXO70* genes may allow plants to acquire cell type- and/or cargo-specific regulatory machinery for exocytosis.

As an essential function for plant growth and development, exocytosis involves the fusion of Golgi-derived vesicles with the target plasma membrane to release vesicle contents into the extracellular space. Virtually all plant cell growth, for instance, in elongating root cells and developing leaf pavement cells, is in a polarized manner (i.e. more in some cell facets than in others). Exocytosis is a multistep process regulated by independent but synergistically coordinated components. The interaction between exocytotic vesicles and the plasma membrane before fusion is initiated by a process called vesicle tethering that requires the exocyst complex originally identified in *Saccharomyces cerevisiae* (Novick et al., 1980; Bowser and Novick, 1991; Bowser et al., 1992). The subsequent fusion of secretory vesicles with the plasma membrane is catalyzed by the SNARE complex (Lipka et al., 2007).

The evolutionarily conserved exocyst complex consists of eight subunits, SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70, and EXO84, ranging in size from 70 to 144 kD in yeast (TerBush and Novick, 1995; TerBush et al., 1996; Guo et al., 1999). Biochemical studies reveal the presence of a single member of each subunit per complex, which yields complexes of 834 and 743 kD for yeast and rat, respectively (Hsu et al., 1996; TerBush et al., 1996). In both yeast and metazoa, the exocyst complex is localized at the active exocytosis sites in polarized growing cells (Finger et al., 1998; Mostov et al., 2003). For example, in nonpolarized Madin-Darby canine kidney epithelial cells, the exocyst complex is located in the cytosol, while upon initiation of local calcium-dependent cell-cell adhesion, the complex is rapidly recruited to the lateral membrane, an area of active exocytosis (Grindstaff et al., 1998; Lipschutz et al., 2000; Kreitzer et al., 2003; Oztan et al., 2007).

Insight into the recruitment of different exocyst subunits to exocytosis sites has been obtained in yeast through genetic and biochemical analyses (for review, see He and Guo, 2009). Although all subunits in the complex eventually accumulate at sites of active exo-

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cytosis, they are recruited by different mechanisms. Sec3 is believed to be the subunit that marks sites of exocytosis in the plasma membrane independent of the actin cytoskeleton and the other subunits of the exocyst, whereas localization of EXO70 to the target sites is partially dependent on F-actin (Finger et al., 1998; Boyd et al., 2004). The other six subunits are localized to exocytic vesicles and depend on actin for their delivery to the sites of exocytosis (Boyd et al., 2004). Phosphatidylinositol 4,5-bisphosphate located in the inner leaflet of the plasma membrane recruits SEC3 and EXO70 to the plasma membrane (He et al., 2007; Zhang et al., 2008). Thus, it is likely that SEC3 and EXO70 first associate with phosphatidylinositol 4,5-bisphosphate to establish a polarized localization on the target membrane and then interact with the rest of the exocyst components on the arriving secretory vesicle to tether it to the target membrane.

Plant exocytosis starts at cytokinesis when the newly formed cell plate is initiated between two anaphase nuclei, with the help of a cytoskeletal structure called the phragmoplast (Staehelin and Hepler, 1996). This is essentially comparable to the extension of the existing cell wall during cell elongation and tip growth. Prior to exocytosis, the secretory vesicles have to be targeted to the right location of the cell, tethered, and docked there, as in yeast and mammals. The actin cytoskeleton delivers the Golgi bodies with the secretary vesicles to the plasma membrane (Ketelaar et al., 2003; Collings et al., 2006), at least for vesicles containing the cellulose synthases (Crowell et al., 2009; Gutierrez et al., 2009). All eight corresponding exocyst subunits have been identified in silico in the Arabidopsis (*Arabidopsis thaliana*) genome: one for *SEC6* and *SEC8*, two for *SEC3*, *SEC5*, *SEC10*, and *SEC15*, three for *EXO84*, and 23 for *EXO70* (Eliáš et al., 2003; Synek et al., 2006; Žárský et al., 2009; Chong et al., 2010; Zhang et al., 2010). Yeast two-hybrid experiments reveal a similar but also distinct manner of interaction among different exocyst subunits in plants (Hála et al., 2008). Immunocytochemical analysis showed that SEC6, SEC8, and EXO70A1(At5g03540) are colocalized in tips of growing pollen tubes, which seems to be similar to the polarized localization to the sites of secretion of the exocyst complex in yeast and animals (Hála et al., 2008). Mutations of both *SEC3* in maize (*Zea mays*) and *EXO70A1* in Arabidopsis cause a defect in root hair elongation (Wen and Schnable, 1994; Wen et al., 2005), whereas mutations of *SEC3*, *SEC5*, *SEC6*, *SEC8*, or *SEC15a* in Arabidopsis lead to defects in pollen germination and pollen tube growth (Cole et al., 2005; Hála et al., 2008). One of these *EXO84* genes in Arabidopsis is involved in secretion processes during cytokinesis (Fendrych et al., 2010).

In contrast to a single copy of the *EXO70* gene in yeast, fungi, and most animal genomes, a striking feature of plants is the multiplication of *EXO70* genes: 13 in *Physcomitrella patens*, 23 in Arabidopsis and *Populus trichocarpa*, and 41 in rice (*Oryza sativa*), with functions very much unknown (Eliáš et al., 2003; Synek et al., 2006; Chong et al., 2010). Phylogenetic analysis showed that these *EXO70* genes in plants can be divided into three clades and nine subclades. The amplification appears to be generated through ancient duplications in a common ancestor as well as subsequent duplications in different plant lineages (Chong et al., 2010). *EXO70A1* in Arabidopsis is the only member in this family that has been studied through GUS-based expression analysis and knockout analysis, revealing its roles in hypocotyl and root hair elongation and the recognition between stigma and pollen (Synek et al., 2006; Samuel et al., 2009).

In this study, we aim to obtain a complete expression profile for all 23 *EXO70* genes in the Arabidopsis genome. We performed expression analyses on these genes using semiquantitative reverse transcription (RT)-PCR and transgenic lines carrying promoter::*GUS* fusion constructs. For *EXO70A1*, we used RNA in situ hybridization to confirm its expression pattern. Our work revealed that, except for *EXO70B2* (At1g07000), for which no *GUS* expression was detected, all other 22 *EXO70* genes showed cell type-specific expression in potential exocytosis-active cells such as elongating pollen tubes and root hair cells, whereas no expression was found in mature organs such as fully developed leaves, stems, sepals, and petals. The distinct expression patterns of *EXO70* members strongly suggest their functional divergence and specificity, implying their roles in regulating cell type- and/or developmental stage-specific exocytosis. Plants with a mutation in *EXO70C1*, a member specifically expressed in mature pollen and germinated pollen tubes, showed retarded pollen tube growth, and male transmission was compromised. These data may help to predict the functions of individual *EXO70* genes and can serve as a guide for further molecular and genetic analyses.

RESULTS

RT-PCR Revealed Diversified Expression of *EXO70* Genes in Arabidopsis

Phylogenetic analyses of *EXO70* genes in plants revealed an independent gene expansion in dicots and monocots within each of the nine subclades (Supplemental Fig. S1, A–I). Although 23 EXO70 proteins encoded by Arabidopsis have similar M_r values, sequence identities between members were relatively low, ranging from 15.8% to 76.5%, suggesting a functional divergence among these family members (Supplemental Figs. S1 and S2). By aligning EXO70 proteins from rice and Arabidopsis, both highly conserved and subclade-specific amino acids were found, especially in the QR motif located near their C termini (Supplemental Fig. S3).

To obtain detailed expression data, we performed semiquantitative RT-PCR analyses on all 23 *EXO70* genes in Arabidopsis. Twelve RNA samples were pre-

pared from roots, leaves, and cotyledons of 5-d-old seedlings, cauline and rosette leaves, stems, flowers, anthers, and pistils at different stages. Except for *EXO70A2* (At5g52340) and *EXO70A3* (At5g52350), which did not give any PCR product, all 21 other genes showed expression in different tissues (Fig. 1). Six of them, *EXO70A1*, *EXO70B1* (At5g58430), *EXO70B2*, *EXO70D2* (At1g54090), *EXO70G1* (At4g31540), and *EXO70H7* (At5g59730), were expressed in all 12 samples tested. Among them, *EXO70A1*, *EXO70D2*, and *EXO70H7* appear to be constitutively expressed. Furthermore, we observed that *EXO70C1*, *EXO70C2* (At5g13990), *EXO70H3* (At3g09530), *EXO70H5* (At2g28640), and *EXO70H6* (At1g07725) were actively expressed in anthers. Three of them, *EXO70H3*, *EXO70H5*, and *EXO70H6*, were also expressed in pistils of 0.5 cm in length but not in 1-cm pistils. High levels of expression in roots were observed for *EXO70G2* (At1g51640), *EXO70H1* (At3g55150), and *EXO70H2* (At2g39380). It is worthy of note that the *ACTIN8* gene used in this study for normalization is constitutively expressed in most organs except pistils, in which a decreased level of expression has been observed (An et al., 1996). In summary, RT-PCR analyses revealed evident expression differences of *EXO70* members in various tissues of Arabidopsis.

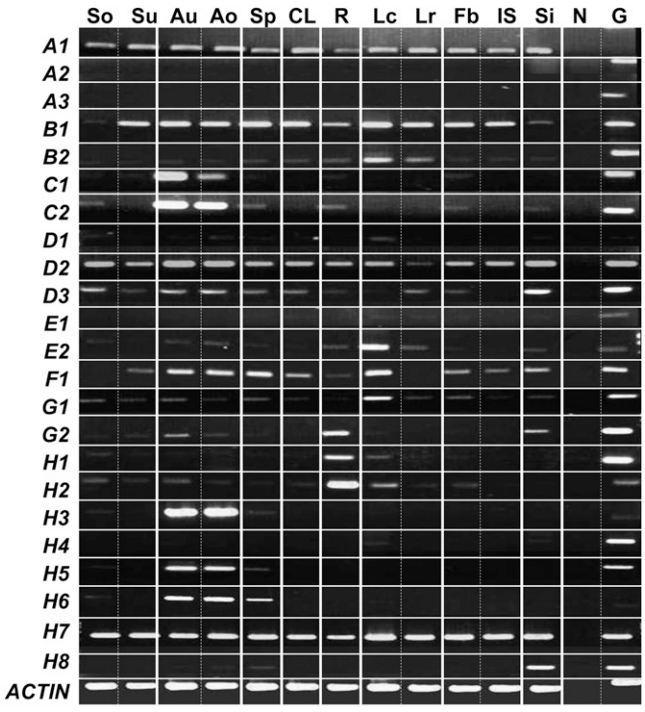

Figure 1. Expression analyses of *EXO70* genes by RT-PCR. RT-PCR was performed for 28 cycles for *ACTIN8* and 30 cycles for *EXO70* genes. So, Siliques from opened flowers; Su, siliques from unopened flowers; Au, anthers from unopened flowers; Ao, anthers from opened flowers; Sp, siliques 2 d after pollination (about 0.5 cm in length); CL, cotyledons and leaves of 5-d-old seedlings; R, roots of 5-d-old seedlings; Lc, cauline leaves; Lr, rosette leaves; Fb, flower buds; IS, internodes and stems; Si, siliques (about 1 cm in length); N, negative control; G, genomic DNA. Note that relative constitutive expression was observed in *EXO70A1*, *EXO70D2*, and *EXO70H7*. *EXO70C1*, *EXO70C2*, *EXO70H3*, *EXO70H5*, and *EXO70H6* were highly expressed in anthers but barely detectable in other organs. *EXO70G2*, *EXO70H1*, and *EXO70H2* showed a high level of expression in roots.

No *EXO70* Genes Are Constitutively Expressed

To examine the temporal and spatial expression of *EXO70* genes in the Arabidopsis genome, we generated fusion constructs carrying 5′ upstream sequences fused to the coding region of the *GUS* gene (*EXO70pro*::*GUS*). In the T0 generation, individual transgenic plants resistant to DL-phosphinothricin were screened to identify *GUS*-positive lines. Since aberrant expression patterns were occasionally observed in about 5% to 10% of transgenic lines carrying the same construct, we routinely examined 30 or more independent T1 plants to obtain a consistent expression profile. Detailed analyses were carried out in T2 plants at three developmental stages: 3-d-old seedlings, 2-week-old vegetative plants, and 3-week-old flowering plants. The basic expression patterns of these 23 constructs in transgenic plants are summarized in Table I and presented in Supplemental Figures S4 to S26. Except for *EXO70B2pro*::*GUS* transgenic plants that showed no *GUS* expression in the samples examined, for the other 22 *EXO70* genes, distinct cellular expression levels were observed. There is little doubt that *EXO70B2* is an expressed gene, as indicated by the microarray data (Supplemental Fig. S8; Chong et al., 2010), 31 ESTs found in The Arabidopsis Information Resource database, and our RT-PCR analysis. Since the distance between the start codon of *EXO70B2* and that of the oppositely orientated upstream gene At1g070010 is only 643 bp, it is likely that regulatory elements are located beyond the 1,236-bp 5′ upstream sequence used.

Since *EXO70A1*, *EXO70D2*, and *EXO70H7* showed constitutive expression in RT-PCR and microarray analysis (Fig. 1; Synek et al., 2006; Chong et al., 2010), we first examined the *GUS* expression in corresponding transgenic lines. Interestingly, all of them showed specific expression in well-defined cells at the tissue level (Supplemental Figs. S4, S12, and S25). Actually, after complete examinations through GUS assays, as summarized in Table I, we found that none of the 22 *EXO70* genes were constitutively expressed in Arabidopsis (Supplemental Figs. S4–S26).

We noticed that the expression pattern of *EXO70A1* observed in our experiment was different from what has been reported previously using an anther *GUS* reporter construct (Samuel et al., 2009). Instead of general expression in the seedlings, leaves, roots, stipules in the nodes, anther filaments, and the stigma hairs described (Samuel et al., 2009), we found a xylem-specific expression throughout the development of Arabidopsis (root, stem, leaves, and flowers) and in glands located at the bases of the cotyledons (Fig. 3, A,

Table I. *Summary of EXO70 expression in different tissues in Arabidopsis*

X, *Exo70* expression detected through GUS assays.

Cell Types	EXO70A1	EXO70A2	EXO70A3	EXO70B1	EXO70B2	EXO70C1	EXO70C2	EXO70D1	EXO70D2	EXO70D3	EXO70E1	EXO70E2	EXO70F1	EXO70G1	EXO70G2	EXO70H1	EXO70H2	EXO70H3	EXO70H4	EXO70H5	EXO70H6	EXO70H7	EXO70H8
Root meristem																							
Columella			X																				
QC														X									
Lateral root cap									X	X												X	
Epidermis								X														X	
Cortex								X						X								X	
Endodermis								X						X								X	
Stele								X						X								X	
Elongation zone																							
Epidermis						X										X							
Cortex						X								X		X							
Endodermis														X		X							
Stele	X													X		X							
Root hair zone																							
Root hair cells						X	X				X					X							
Nonhair cells						X										X							
Cortex						X										X							
Endodermis																X							X
Vascular bundles	X														X	X							
Mature roots																							
Root hair							X				X	X	X				X						
Nonhair cells							X					X	X				X						
Cortex							X							X			X		X				
Endodermis				X			X							X			X		X				X
Vascular bundles		X												X								X	
Lateral root primordia								X						X	X								
Aboveground tissues																							
Vascular bundles	X														X								
Guard cells		X				X													X				
Trichomes									X	X													
Pollen grains						X	X								X			X		X			
Pollen tubes						X									X			X		X	X		
Carpels									X	X						X			X		X	X	
Siliques													X										
Filaments																	X						
Glands	X												X	X	X								

B, E, and F; Supplemental Fig. S4). The xylem element-specific expression was restricted to the stage when banded cell wall thickenings are being formed, whereas no *GUS* expression was observed before and after this stage (Fig. 3E). Initially, we thought that the 1.7-kb upstream promoter sequence we used lacked some regulatory elements, as compared with the 2.5-kb fragment used by Samuel et al. (2009). We then fused the same 2.5-kb fragment to the *GUS* reporter gene. Transgenic plants carrying the longer promoter sequence showed identical expression patterns as those carrying the shorter one. In situ RNA hybridization confirmed that the expression of *EXO70A1* observed through the GUS assay was indeed specific (Fig. 3F). Most likely, the discrepancy is caused by a double-enhanced cauliflower mosaic virus *35S* promoter located adjacent to the *EXO70A1* promoter in the pCAMBIA1391Z vector used by Samuel et al. (2009). It has been reported previously that the active *35S* promoter in some of the pCAMBIA vectors including pCAMBIA1391Z causes significant interference to the expression patterns of the promoters analyzed (Yoo et al., 2005; http://www.patentlens.net/daisy/cambia/home.html).

Overlapping and Complementary Expression of *EXO70* in the Root Tip

Among 23 GUS-positive *EXO70* genes, 10 of them were expressed in the root tips, where none of them was constitutively expressed. *EXO70A3* was previ-

ously reported to be a potential pseudogene (Synek et al., 2006); however, our GUS assay showed that it was expressed in degenerating root cap cells (Fig. 2A; Supplemental Fig. S6). Since the root cap represents only a very small fraction of the cells in roots, it is not surprising that this expression is not detected in analyses at the organ level. As it is the only gene expressed in these cells, it will be of interest to examine if it is involved in regulating root cap-specific exocytosis (Wen et al., 2007).

All three *EXO70* genes in the D subclade, *EXO70D1* (At1g72470), *EXO70D2*, and *EXO70D3* (At3g14090), showed expression in root tips. For *EXO70D2pro*::*GUS* and *EXO70D3pro*::*GUS* transgenic plants, *GUS* expression was seen specifically in the newly formed lateral root cap initial cells in roots (Fig. 2, B and C). Interestingly, these two genes that share the highest similarity (76.5%) in Arabidopsis at the protein level also showed similar expression in trichome development in the aboveground tissues (Fig. 4, I and J; Supplemental Figs. S2, S12, and S13). It is very likely that they are functionally redundant. In transgenic *EXO70D1pro*::*GUS* plants, *GUS* expression was observed in most meristematic cells in root tips except for the root cap and the quiescent center (QC; Fig. 2D; Supplemental Fig. S11). The *EXO70D1pro*::*GUS* expression overlapped partially with that of *EXO70G1pro*::*GUS* in root meristems, except that the former showed no expression in the QC and the latter showed no expression in epidermis (Fig. 2, D and E). Since proteins encoded by these two genes share a rather low sequence identity (21%; Supplemental Fig. S2), most likely they perform different functions in root meristems. Furthermore, *EXO70H7pro*::*GUS* was also expressed in root meristems with a patched expression pattern (Fig. 2F), implying its involvement in cell cycle regulation.

Root hairs are exocytosis-active tip-growing cells. Four *EXO70* genes, *EXO70C2*, *EXO70E1* (At3g29400), *EXO70H1*, and *EXO70H2*, were expressed during root hair development (Fig. 2, G–J; Supplemental Figs. S10, S14, S19, and S20). Among them, *EXO70C2* and *EXO70E1* were expressed specifically in root hair-producing cells but not in non-root-hair cells (Fig. 2, G and H). In contrast, *EXO70H1* and *EXO70H2*, which share a high sequence identity (75%) at the protein level, appear to be coexpressed in all cells in the root hair region (Fig. 2, I and J; Supplemental Figs. S19 and S20).

Expression of *EXO70* Genes during Xylem Formation

The formation of xylem elements in vascular bundles requires active secretion during progressive cell elongation, secondary cell wall thickening, and programmed cell death (Steeves and Sussex, 1989). We observed that two *EXO70* genes, *EXO70A1* and *EXO70G2*, were expressed in developing xylem elements during secondary cell wall thickening (Fig. 3; Supplemental Figs. S4 and S18), as revealed by GUS

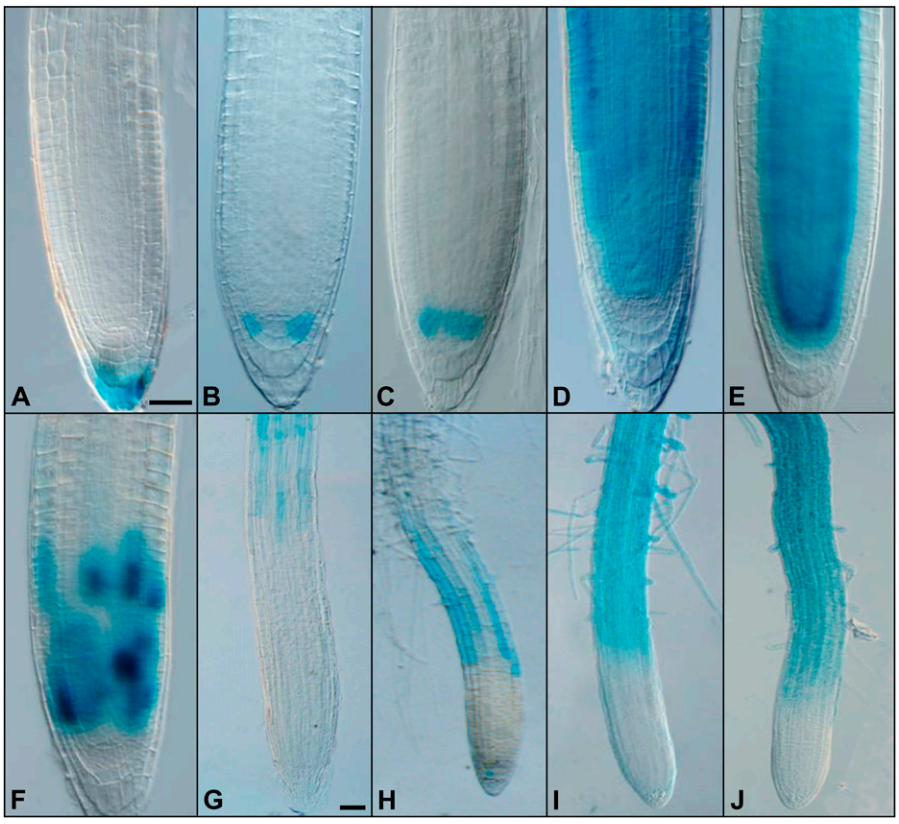

Figure 2. *EXO70* genes expressed in different cell types in root tips. A, *GUS* expression in *EXO70A3pro*::*GUS* transgenic plants was detected in the outer layer of the columella cells in 3-d-old seedlings but disappeared in 7-d-old seedlings (see Supplemental Fig. S4). B and C, For *EXO70D2pro*::*GUS* (B) and *EXO70D3-pro*::*GUS* (C), *GUS* expression was restricted to lateral root cap initial cells. D, Strong expression of *EXO70D1pro*::*GUS* was found in the root meristem, but not in the QC, root cap, and lateral root cap. E, *GUS* expression in *EXO70G1pro*::*GUS* was localized to the QC, endodermis, and inner tissues but excluded from the epidermis and root cap. F, Patched *GUS* expression was observed in the meristematic zone of plants carrying *EXO70H7-pro*::*GUS*. G and H, In *EXO70C2pro*::*GUS* (G) and *EXO70E1pro*::*GUS* (H) plants, *GUS* expression was observed specifically in root hair-producing cells in root tips. I and J, *EXO70H1pro*::*GUS* (I) and *EXO70H2-pro*::*GUS* (J) were expressed specifically in the elongation and root hair regions. Bar in A (for A–F) = 50 μm; bar in G (for G–J) = 100 μm.

Figure 3. *EXO70* genes expressed during xylem development. A and B, *GUS* expression in the *EXO70A1pro*::*GUS* transgenic line was observed in developing xylem elements in roots (A) and cotyledons (B). C and D, In the *EXO70G2pro*::*GUS* transgenic line, *GUS* expression was observed in developing xylem elements in roots (C) and cotyledons (D). E, *GUS* expression in a transgenic line carrying *EXO70A1pro*::*GUS* was observed in developing xylem elements and in glands at the bases of the cotyledons. F, RNA in situ hybridization showing the expression of *EXO70A1* in developing xylem elements and in glands at the bases of the cotyledons. Bars in A (for A and C), B (for B and D), and E (for E and F) = 50 μm.

assays. The expression specificity of *EXO70A1* was confirmed by RNA in situ hybridization (Fig. 3F). Two other *EXO70* genes, *EXO70H7* and *EXO70H8* (At2g28650), were found to be expressed in the vascular bundles well above the root hair region (Supplemental Figs. S25 and S26). Further histological analysis is needed to define the exact cell types in which they are expressed.

Expression of *EXO70* Genes in Cell Wall-Loosening Cells and Developing Trichomes

Cell wall loosening is important for many developmental processes, especially during elongation, emergence of lateral roots, guard cell formation, and detachment of the sepals and petals (Armstrong and Armstrong, 2005). Following the progression of the lateral root primordia, it is believed that enzymes are released for remodeling the cell wall of overlying and margin cells (Péret et al., 2009). The *GUS* expression in *EXO70G2pro*::*GUS* lines seems to fit this function well (Fig. 4, A–C). The expression was first observed in pericycle cells when the lateral root was initiated (Fig. 4A) and gradually moved to the margin cells when the root primordium was formed (Fig. 4, B and C). Local cell wall thickening and loosening also occur during the formation of stomata, when a gap between two neighboring guard cells is being formed (Zhao and Sack, 1999). Three *EXO70* genes, *EXO70A2*, *EXO70C1*, and *EXO70H4* (At3g09520), were expressed in developing guard cells but not in mature ones, suggesting their roles in guard cell morphogenesis (Fig. 4, D–F). Moreover, the dehiscence of sepals and petals after pollination requires the loosening of cell walls in the dehiscence zones. The *GUS* expression of *EXO70F1-pro*::*GUS* (At5g50380) and *EXO70G2pro*::*GUS* transgenic plants in this zone implicate their roles during dehiscence (Fig. 4, G and H).

Trichomes in Arabidopsis are formed from single pavement cells in the epidermis of stems, leaves and sepals. We found that two homologous *EXO70* genes, *EXO70D2* and *EXO70D3*, were expressed in developing trichomes (Fig. 4, I and J). The *GUS* expression became detectable when the trichomes started to form, and in both cases the expression was absent from mature trichomes (Supplemental Figs. S12 and S13), suggesting their functions in trichome formation.

Six *EXO70* Genes Are Expressed in Microspores and Pollen Tubes

Data compiled from microarray analysis show that *EXO70A2*, *EXO70C1*, *EXO70C2*, *EXO70G2*, *EXO70H3*, and *EXO70H5* are expressed in the stamen and pollen (Synek et al., 2006; Chong et al., 2010). Our analyses confirmed the microspore- and pollen-specific expression of *EXO70C1*, *EXO70C2*, *EXO70G2*, *EXO70H3*, and *EXO70H5* (Fig. 5, A–E; Table I) but not that of *EXO70A2* (Table I; Supplemental Fig. S5). Expression of *EXO70A2* was only observed in the stomata of leaves and vascular bundles of roots (Supplemental Fig. S5). In transgenic plants carrying *EXO70C1pro*::*GUS*, *EXO70C2pro*::*GUS*, and *EXO70G2pro*::*GUS*, strong *GUS* expression was found in mature pollen

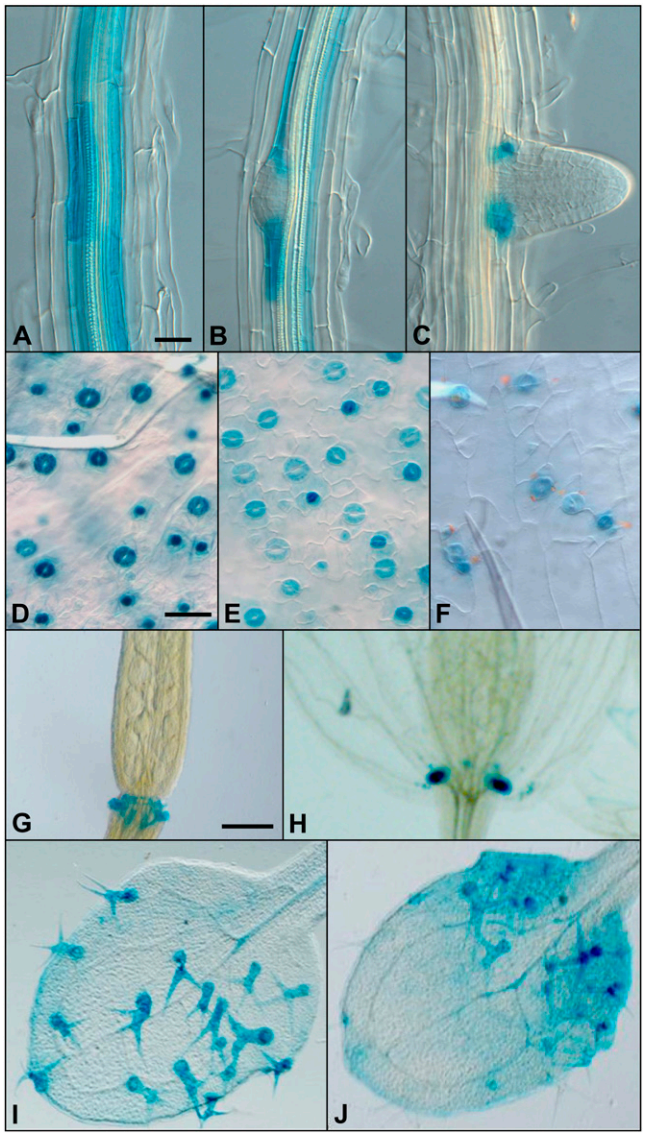

Figure 4. *EXO70* genes expressed during lateral root formation, stomata and trichome development, and dehiscence. A to C, In *EXO70G2pro*::*GUS* transgenic plants, *GUS* expression during lateral root formation was observed in pericycle cells in which the lateral roots were initiated (A) and in junction cells after the lateral root was initiated (B and C). D to F, Specific expression in developing guard cells was observed in *EXO70A2pro*::*GUS* (D), *EXO70C1pro*::*GUS* (E), and *EXO70H4pro*::*GUS* (F). G and H, For *EXO70F1pro*::*GUS* (G) and *EXO70G2pro*::*GUS* (H) transgenic plants, *GUS* expression was observed in the dehiscence zone in sepals and petals. I and J, *EXO70D2pro*::*GUS* (I) and *EXO70D3pro*::*GUS* (J) were expressed in developing trichomes but not in mature trichomes. Bars in A (for A–C) and D (for D–F) = 50 μm; bar in G (for G–J) = 500 μm.

(in stage 12 and 13 flowers; Smyth et al., 1990), while weak expression was observed in pollen tubes (Fig. 5, A–C; Supplemental Figs. S9, S10, and S18).

For *EXO70H3pro*::*GUS* and *EXO70H5pro*::*GUS* plants, *GUS* expression was comparable in mature pollen and pollen tubes (Fig. 5, D and E; Supplemental Figs. S21 and S23). *EXO70H6* is the only *EXO70* gene for which no microarray data are available. Our analysis using the *GUS* reporter construct revealed that it is only expressed in germinated pollen, not in mature pollen (Fig. 5F). The GUS staining was also observed transiently at the micropylar end of the embryo sac after pollination but absent during embryo and endosperm development (Supplemental Fig. S24). Most likely, the GUS signal was from pollen tube release instead of expression after fertilization, since no expression was observed when the transgenic plant was pollinated with wild-type pollen (data not shown).

EXO70s Expressed in Gynoecia

Among the *EXO70* genes examined, *EXO70A1*, *EXO70D2*, *EXO70H4*, *EXO70H5*, and *EXO70H7* were expressed in gynoecia (Fig. 6). In *EXO70A1pro*::*GUS* plants, the *GUS* expression was first found in median vascular bundles in stage 11 gynoecia, whereas in stage 12 gynoecia, the *GUS* expression was detected in both lateral and central vascular bundles (Supplemental Fig. S4). When the flower opened, *GUS* expression was found in the vascular bundles of the style (Fig. 6A). *EXO70D2* was the only member in our study that showed *GUS* expression in the outer and inner epidermis of the gynoecium, primarily in stage 11. At the anthesis stage, the expression was no longer detectable (Fig. 6B; Supplemental Fig. S12). For *EXO70H4pro*::*GUS*, strong *GUS* staining was localized in the transmitting tracts of gynoecia and weak *GUS* staining in the ovule (Fig. 6C).

In *EXO70H7pro*::*GUS* transgenic plants, *GUS* expression was first observed in lateral and medial vascular bundles in stage 9 gynoecia (Fig. 6D); stronger signal was observed in stage 11 gynoecia, which disappeared when the flower bud opened (Fig. 6E; Supplemental Fig. S25). For *EXO70H5pro*::*GUS* plants, *GUS* expression was detected in the embryo sac, most likely in the synergid or egg cells in stage 13 flowers (Fig. 6F; Supplemental Fig. S23). The *GUS* expression was also found in emasculated flowers, suggesting that it is unlikely to be derived from the zygote (data not shown).

A Mutation in *EXO70C1* Led to Retarded Pollen Tube Growth and Compromised Pollen Transmission

Given the cell type-specific expression patterns observed, we anticipated that mutations in *EXO70* genes might lead to defects in particular cell types where the corresponding genes are expressed, if there is no redundancy. We analyzed a line in which a *Ds* transposon was inserted into the single exon of *EXO70C1* at a position 1,123 bp downstream of the start codon. Homozygous *exo70c1* plants had short roots, were slow growing, and had low seed set. Cosegregation analysis in a population of 210 plants from a heterozygous parent shows that the *Ds* insertion was tightly linked with the phenotype. *EXO70C1* was expressed specifically in developing

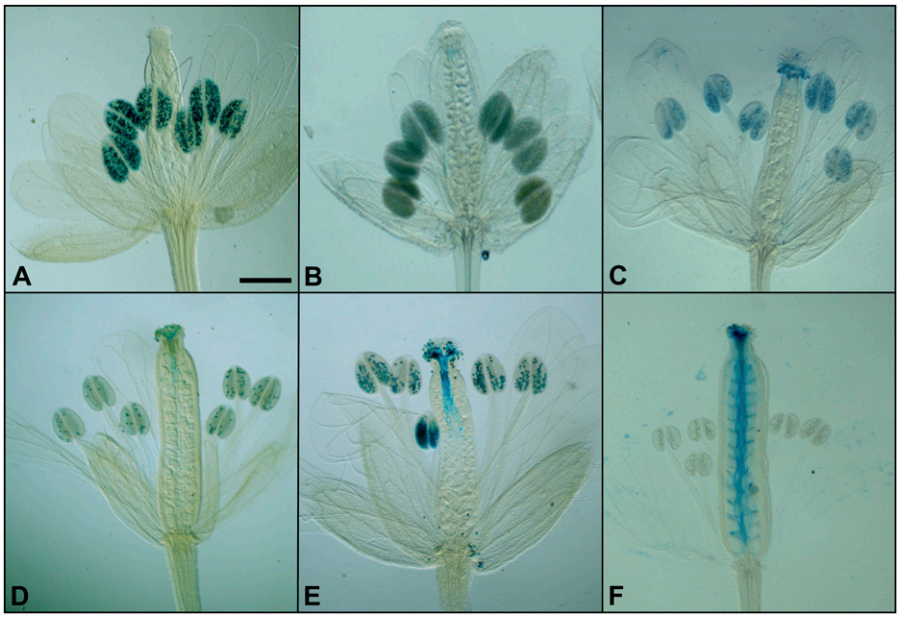

Figure 5. *EXO70* genes expressed in microspores and pollen tubes. *GUS* expression was examined in microspores and pollen using transgenic plants carrying *EXO70pro*::*GUS* constructs. A to C, *EXO70C1pro*::*GUS* (A), *EXO70C2pro*::*GUS* (B), and *EXO70G2pro*::*GUS* (C) were expressed strongly in mature pollen and weakly in pollen tubes. D and E, For *EXO70H3pro*::*GUS* (D) and *EXO70H5-pro*::*GUS* (E), *GUS* expression was observed in mature pollen and pollen tubes. F, In the *EXO70H6pro*::*GUS* transgenic line, *GUS* expression was detected in germinating pollen tubes and in embryo sacs. Bar in A (for A–F) = 500 μm.

guard cells, mature pollen, and pollen tubes. No obvious morphological changes were observed in guard cells, and the number of pollen grains per anther were normal (522.8 ± 14.7 per anther in the wild type, 526 ± 22.8 in the mutant). Staining with Alexander's stain and with 4,5-diamidino-2-phenyindole (DAPI) shows that the mutant pollen was viable and had the correct nuclear constitution (Fig. 7, A–D). However, reciprocal crosses showed that there was reduced (78%) transmission efficiency through the male, but transmission through the female was normal (Table II). We examined pollen tube growth 14 h after pollination in vivo after applying limited pollen (about 20 grains) to wild-type stigmas. With wild-type pollen, five to 10 pollen tubes were observed in the vicinity of ovules in the lower portion of the pistils (Fig. 7E, arrowheads). However, with mutant pollen, almost no pollen tubes were visible in this region (Fig. 7F), suggesting retarded pollen tube growth.

To further determine if the slow pollen tube growth caused the reduced transmission through the male, we pollinated wild-type pistils with pollen from a plant heterozygous for the mutation. F1 seeds were harvested separately from the top and bottom halves of the siliques and germinated on medium supplemented with kanamycin. Of the seedlings produced from seeds from the top half, only 39.9% were kanamycin resistant (n = 248), instead of the expected 50%, and from the bottom half, only 29% were kanamycin resistant (n = 248), suggesting that the slow growth of pollen tubes carrying the mutant *exo70c1* allele caused the male transmission defect.

DISCUSSION

The eight-subunit exocyst complex has been identified as a key player in targeted exocytosis in yeast and animals. During polarized growth of yeast and animals, the SEC3 and EXO70 of the exocyst complex first accumulate to the target membrane and then interact with other components in the complex, allowing exocytosis to occur on the target membrane (Boyd et al., 2004). A recent study shows that exocyst in plants is involved in cytokinesis and cell plate maturation (Fendrych et al., 2010). Instead of a single gene in yeast and most animal genomes, the number of *EXO70* genes in plants has expanded greatly, with reasons very much unknown (Synek et al., 2006; Chong et al., 2010; Zhang et al., 2010). Through expression analyses using RT-PCR and GUS assays in over 700 individual transgenic lines carrying one of the 23 *EXO70pro*::*GUS* constructs made, we provide a set of tissue-level expression data on *EXO70* genes in the Arabidopsis genome. We found that expression of *EXO70* genes is tightly associated with exocytosis-active cells such as tip-growing cells and differentiating cells, suggesting cell type- and/or cargo-specific exocytotic activities regulated by different EXO70 isoforms. Consistent with this speculation, a mutation in a mature pollen-specific gene, *EXO70C1*, led to retarded pollen tube growth and compromised male transmission.

EXO70 May Regulate Cell Type-Specific Exocytosis

Two hypotheses have been proposed by Synek et al. (2006) for the pronounced multiplication of *EXO70* genes in plants: (1) only some of the EXO70 proteins serve as subunits of the plant exocyst complex, whereas the others perform functions independent of the exocyst; and (2) plant cells may be endowed with a number of different exocyst forms, each with a specific EXO70 subunit and potentially a different function (Synek et al., 2006). Based on the data presented in this paper, the second hypothesis is most likely to be

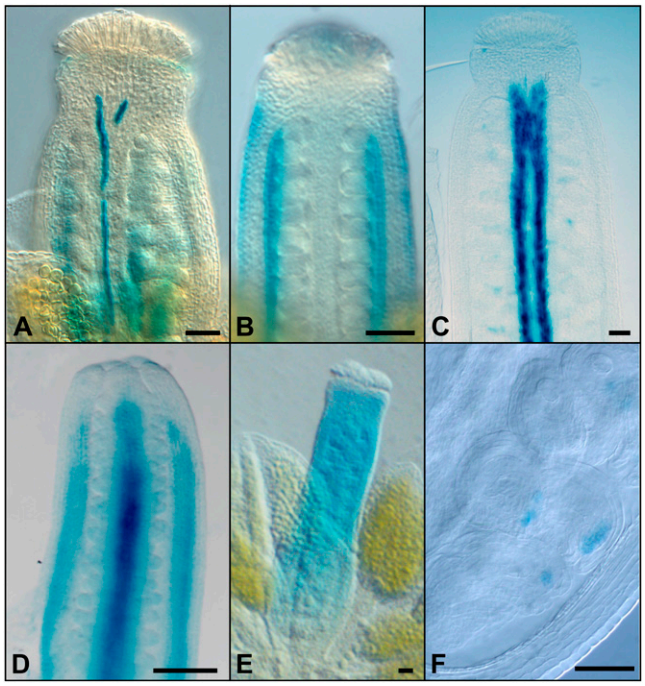

Figure 6. Five *EXO70* genes expressed in gynoecia. A, For *EXO70A1-pro*::*GUS*, *GUS* expression was observed in developing xylem elements in pistils. B, For *EXO70D2pro*::*GUS*, *GUS* expression was found in outer and inner epidermis of the pistil. C, For *EXO70H4pro*::*GUS*, strong *GUS* staining was localized in transmitting tracts of gynoecia; weak staining was presented in ovule. D and E, For *EXO70H7pro*::*GUS*, *GUS* expression was found in the vascular bundles of the carpels in stage 9 floral buds (D) and in most cells of the carpel at stage 11 (E). F, For *EXO70H5pro*::*GUS*, *GUS* expression was observed in the synergid region of the ovules at stage 11. Bars = 50 μm.

correct, for three reasons. First, the expression of different *EXO70* members is tightly associated with exocytosis-active cells, and each member was expressed specifically in one or several cell types. Second, in the tip-growing cells, root hairs, and pollen tubes, multiple *EXO70* members were expressed but with defined temporal differences, during which they may function synergistically. Third, in mature organs and tissues, *EXO70* expression was notably absent. As such, we hypothesized that the expansion of *EXO70* family members may enable plants to evolve cell type-specific exocysts, distinguished by different EXO70s, for tethering various vesicles to specific target membranes in certain cell types.

Functions of *EXO70* Genes in Tip Growth

Polarized cell growth is a common phenomenon in plants, occurring in virtually every cell type but abundantly during tip growth. Root hairs and pollen tubes are tip-growing plant cells. Since cell elongation is local and abundant in these cells, tip growth requires the establishment of a narrow growth site at the plasma membrane and the continuous and abundant targeting of Golgi-derived vesicles to this site and their fusion with the plasma membrane (Miller et al., 1997; Yang, 1998; Cole and Fowler, 2006; Emons and Ketelaar, 2009; Žárský et al., 2009). The essential function of the exocyst in tip growth in plants has already been demonstrated by genetic studies, although the exact role of the exocyst has not been elucidated yet. Mutations of *SEC3* in maize and *EXO70A1* in Arabidopsis lead to impaired root hair elongation (Wen et al., 2005; Synek et al., 2006). Mutations of *SEC3*, *SEC5*, *SEC6*, *SEC8*, and *SEC15a* in Arabidopsis dramatically affect both pollen germination and pollen tube elongation (Cole et al., 2005; Hála et al., 2008). In examining transgenic lines carrying *GUS* reporter constructs, we found that several *EXO70* members were coexpressed in tip-growing cells. During the growth of root hairs, *EXO70H1* and *EXO70H2* were found to be expressed in all cells in the root hair regions, whereas *EXO70C2* and *EXO70E1* were specifically expressed in trichoblasts, the root hair-forming cells. Associated with pollen development and pollen tube growth, six *EXO70* genes, *EXO70C1*, *EXO70C2*, *EXO70G2*, *EXO70H3*, *EXO70H5*, and *EXO70H6*, were expressed in overlapping but slightly different patterns, suggesting their roles in executing different vesicle-trafficking processes. Additional analyses will be needed to determine where these EXO70 proteins are localized subcellularly and how they function in tip-growing cells.

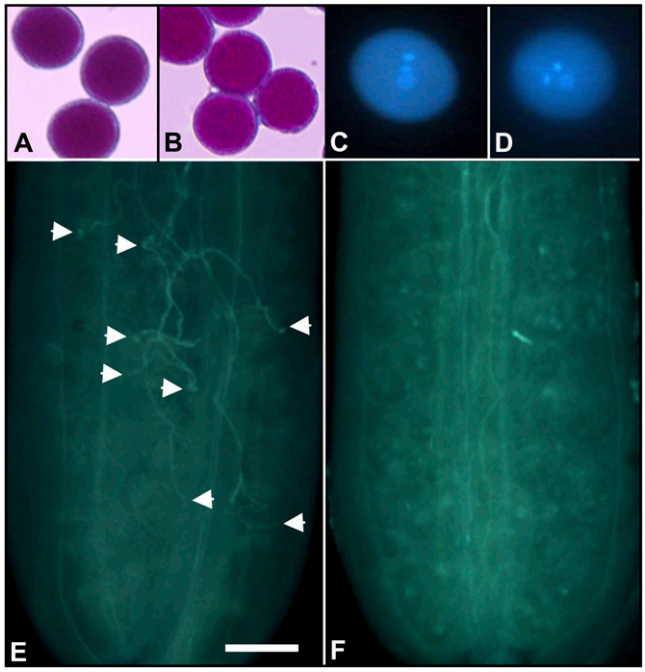

Figure 7. Mutation in *EXO70C1* caused compromised pollen tube elongation. A and C, Wide-type pollen grains. B and D, Mutant pollen grains. A and B, Alexander's staining. C and D, DAPI staining. E and F, Lower portions of wild-type pistils stained with decolorized aniline blue 14 h after pollination with wild-type (E) or *exo70c1* (F) pollen. Bar in E = 26 μm for A and B, 21 μm for C and D, and 100 μm for E and F.

Table II. *Transmission analyses of the Ds insertion by crossing an EXO70C1/exo70c1 plant with the wild type*

A heterozygous single insertion Ds line, *EXO70C1/exo70c1*, was used as male or female to cross with wild-type plants. F1 seeds obtained were plated under kanamycin selection to calculate the transmission efficiency.

Backcrosses	No. of Progeny Obtained	Kanamycin Sensitive	Kanamycin Resistant	Transmission Efficiencies	
		%			%
Wild type as male	347	50.7	49.3	Female	100
Wild type as female	648	61.0	39.0	Male	78

EXO70 May Regulate Cell Differentiation in Plants

Delivery of secretory vesicles to discrete plasma membrane domains is critical for establishing and maintaining cell polarity and for cell differentiation (Moskalenko et al., 2001). We observed that a large collection of *EXO70* genes were expressed during cell division and elongation, which hints to their roles in regulating functional specification in these cells. During root development, the expression of *EXO70D1*, *EXO70D2*, *EXO70D3*, *EXO70G1*, and *EXO70H7* was found in different cell types of the root meristem, whereas *EXO70G2* was expressed during lateral root initiation. In aboveground organs, *EXO70E1* was expressed in young cotyledons; *EXO70A2*, *EXO70C1*, and *EXO70H4* were coexpressed in developing stomata cells; *EXO70D2* and *EXO70D3* were coexpressed during trichome formation. Associating with reproductive development, *EXO70C1*, *EXO70C2*, *EXO70G1*, *EXO70G2*, *EXO70H3*, and *EXO70H5* were coexpressed in anthers; *EXO70A1*, *EXO70D2*, *EXO70H4*, *EXO70H5*, and *EXO70H7* were expressed in different tissues of the carpels; *EXO70H2* was expressed in the junction between anther and filament; whereas *EXO70F1* and *EXO70G2* were expressed in the dehiscence zone of the sepal and petals. Throughout plant development, two genes, *EXO70A1* and *EXO70G2*, were expressed transiently in developing xylem elements when spiral cell wall thickening was occurring. As such, it appears that at the tissue level, the expression of several *EXO70* genes was tightly associated with cell differentiation. The functions in which these *EXO70* members participate may include defining the orientation of cell elongation, building the local thickening of the cell walls, loosening the cell wall, and pattern formation in general but may also be related to the specific substance secreted locally or inserted into the plasma membrane.

Using antibodies, Hála et al. (2008) demonstrated that *EXO70A1* is localized at the apex of growing pollen tubes of tobacco (*Nicotiana tabacum*). This, however, is different from the data we obtained using *GUS* reporter lines and in situ hybridization, showing that *EXO70A1* was specifically expressed in developing xylem elements and axillary glands. We suspect that the antiserum raised against EXO70A1 may have interacted with other EXO70 members that are present in the pollen tubes.

In summary, the diversified expression patterns and their associations with exocytosis-active cells observed in this study suggest that the increased numbers of *EXO70* genes in plants may contribute to cell type-specific subfunctionalization, possibly to regulate many exocytotic activities, such as cytokinesis, cell expansion, cell wall thickening, tip growth, and intercellular signaling during cell differentiation. Given the relatively low degree of conservation at the protein level among these 23 members (15.8% to 76.5%; Supplemental Fig. S2), specialization in protein functions may have evolved in EXO70s to add a new dimension of regulation in polarized secretion. In addition, the large numbers of *EXO70* genes in terrestrial plants might be associated with the divergent exocytosis functions needed to establish an extensive extracellular matrix and to maintain different types of polarized growth. In particular, cell type-specific exocysts could have evolved in plants through the expansion of *EXO70* genes, while in yeast and animal cells, the specificity may have been sufficiently executed by proteins such as small GTPases (Guo et al., 2001; Wu et al., 2010). With these data in hand, genetic, biochemical, and subcellular localization analyses are needed to elucidate how different EXO70 members function in a cell type- and/or cargo-specific manner and what kind of exocytotic activities are involved. One further question to be answered is whether different EXO70-containing exocysts regulate the destination of the secretion.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotypes Columbia-0 mostly and Lansberg *erecta*, as specified below, were used in all experiments. Seeds resulting from the *Agrobacterium tumefaciens*-mediated floral dip transformation (Clough and Bent, 1998) were gas sterilized in a desiccator for 1 h with 100 mL of commercial bleach (4% NaClO) mixed with 3 mL of concentrated HCl. Seed dormancy was broken by 3 to 4 d of cold treatment at 4°C. For growing adult plants, 5-d-old seedlings were transferred from solid medium containing half-strength Murashige and Skoog salts (Duchefa), 1% Suc, 0.1% MES, and 1.5% agar, pH 5.7, to pots with a soil:vermiculite mixture (1:1) and grown under long-day conditions (16 h of light, 8 h of darkness) at 21°C ± 1°C. For the selection of transgenic plants, 30 μg L^{-1} DL-phosphinothricin (Duchefa) was added to the medium described above. An insertion line, CW841908 (in Lansberg *erecta* background), was obtained from the Nottingham Arabidopsis Stock Centre, with a *Ds* transposon inserted into the coding region of *EXO70C1*.

Expression Analysis through RT-PCR

For semiquantitative RT-PCR analysis of *EXO70* expression in Arabidopsis, fresh samples of different organs were harvested from Columbia-0 plants.

RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), and cDNA was synthesized with an iScript cDNA Synthesis Kit (Bio-Rad). RT-PCR on *ACTIN8* (At1g49240; primer pair 5′-GCGGTTTTCCCCAGTGTTGTTG-3′ and 5′-TGCCTGGACCTGCTTCATCATACT-3′) was used to normalize the cDNAs for equal concentrations. Primer pairs used to determine their expression levels for each *EXO70* gene are listed in Supplemental Table S1. RT-PCR was repeated twice.

Construction of Reporter Constructs and Transformations

The 5′ upstream sequences (1,004–2,246 bp before the start codons, depending on the length of the intergenic region) of *EXO70* were amplified by PCR from Columbia-0 genomic DNA, cloned into pDONR221 (Gateway System; Invitrogen), and then introduced into the pBGWSF7 binary vector (Karimi et al., 2002), in which the *EXO70* promoter was placed in front of a *GUS* coding sequence. All primer pairs used to amplify the promoter sequences and the sizes of their products are listed in the Supplemental Table S2.

Expression Analyses Using *EXO70pro*::*GUS* Transgenic Plants

Expression using GUS assays in transgenic Arabidopsis plants carrying reporter constructs was first examined in 30 or more independent T1 plants to identify lines with a consistent expression pattern. Detailed analyses were performed in the T2 generation. GUS assay was routinely performed as described previously by Fiers et al. (2004). Roots and flowers were cleared after GUS staining following the protocol of Sabatini et al. (1999) before examination with a Leica microscope equipped with Nomarski optics.

RNA in Situ Hybridization

For probe labeling, a 302-bp *EXO70A1* cDNA fragment was amplified using primer P1 (5′-AATTAATACGACTCACTATAGGGCGCAGGTCAGAAGCCAAGG-3′) in combination with P2 (5′-AATTAATACGACTCACTATAGGGGCAAGTCTTAGTGACTCTC-3′), both of which contain the T7 RNA polymerase binding sequence at their 5′ ends, and cloned into pGEM T-Easy vector (Promega) to produce pT-A1. After sequence verification, the antisense RNA probes and sense RNA probes were transcribed in vitro with P1 and P2 using T7 RNA polymerase, respectively, and labeled using the digoxigenin RNA labeling kit (Roche). Seedlings were fixed with 37% formaldehyde, 5% acetic acid, and 50% alcohol. Seven-micrometer-thick paraffin sections were used for hybridization. Prehybridization, hybridization, washing, antibody staining, and signal detection were performed as described previously (Shi et al., 2005). The signal was observed with a Leica microscope equipped with Nomarski optics.

Genetic and Histological Analyses

To examine the pollen transmission efficiency, wild-type and *EXO70C1/exo70c1* plants were emasculated and pollinated reciprocally with each other. The seeds obtained were plated under 50 mg L^{-1} kanamycin, and seedlings were counted 10 d after germination. To examine in planta pollen germination, wild-type and *exo70c1* pollen were used to pollinate wild-type plants, and the pistils were examined 14 h later using aniline blue staining as described before (Procissi et al., 2003). The Alexander and DAPI staining of mature pollen was performed as described (Huang et al., 1986; Park et al., 1998).

Supplemental Data

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

Supplemental Figure S1. Phylogenetic analysis of EXO70 proteins in different organisms.

Supplemental Figure S2. Homology matrix tree of 23 EXO70 protein sequences.

Supplemental Figure S3. Alignment of the QR motifs of EXO70s from rice and Arabidopsis.

Supplemental Figure S4. Expression pattern of *EXO70A1*.

Supplemental Figure S5. Expression pattern of *EXO70A2*.

Supplemental Figure S6. Expression pattern of *EXO70A3*.

Supplemental Figure S7. Expression pattern of *EXO70B1*.

Supplemental Figure S8. Expression pattern of *EXO70B2*.

Supplemental Figure S9. Expression pattern of *EXO70C1*.

Supplemental Figure S10. Expression pattern of *EXO70C2*.

Supplemental Figure S11. Expression pattern of *EXO70D1*.

Supplemental Figure S12. Expression pattern of *EXO70D2*.

Supplemental Figure S13. Expression pattern of *EXO70D3*.

Supplemental Figure S14. Expression pattern of *EXO70E1*

Supplemental Figure S15. Expression pattern of *EXO70E2*

Supplemental Figure S16. Expression pattern of *EXO70F1*.

Supplemental Figure S17. Expression pattern of *EXO70G1*.

Supplemental Figure S18. Expression pattern of *EXO70G2*.

Supplemental Figure S19. Expression pattern of *EXO70H1*.

Supplemental Figure S20. Expression pattern of *EXO70H2*.

Supplemental Figure S21. Expression pattern of *EXO70H3*.

Supplemental Figure S22. Expression pattern of *EXO70H4*.

Supplemental Figure S23. Expression pattern of *EXO70H5*.

Supplemental Figure S24. Expression pattern of *EXO70H6*.

Supplemental Figure S25. Expression pattern of *EXO70H7*.

Supplemental Figure S26. Expression pattern of *EXO70H8*.

Supplemental Table S1. Primers used for the RT-PCR analysis of the expression of *EXO70* genes.

Supplemental Table S2. Primers used for amplifying *EXO70* promoters for GUS fusion.

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