Exo70E2 is essential for exocyst subunit recruitment and EXPO formation in both plants and animals

Yu Ding[a,*], Juan Wang[a,*], John Ho Chun Lai[a,b], Vivian Hoi Ling Chan[a], Xiangfeng Wang[a], Yi Cai[a,†], Xiaoyun Tan[c], Yiqun Bao[c], Jun Xia[b], David G. Robinson[d], and Liwen Jiang[a,e]

[a]School of Life Sciences, Centre for Cell and Developmental Biology and State Key Laboratory of Agrobiotechnology, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China; [b]Division of Life Science, Division of Biomedical Engineering and State Key Laboratory of Molecular Neuroscience, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China; [c]College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China; [d]Department of Plant Cell Biology, Centre for Organismal Studies, University of Heidelberg, D-69120 Heidelberg, Germany; [e]Shenzhen Research Institute, Chinese University of Hong Kong, Shenzhen 518057, China

ABSTRACT **In contrast to a single copy of Exo70 in yeast and mammals, the *Arabidopsis* genome contains 23 paralogues of Exo70 (AtExo70). Using AtExo70E2 and its GFP fusion as probes, we recently identified a novel double-membrane organelle termed exocyst-positive organelle (EXPO) that mediates an unconventional protein secretion in plant cells. Here we further demonstrate that AtExo70E2 is essential for exocyst subunit recruitment and for EXPO formation in both plants and animals. By performing transient expression in *Arabidopsis* protoplasts, we established that a number of exocyst subunits (especially the members of the Sec family) are unable to be recruited to EXPO in the absence of AtExo70E2. The paralogue AtExo70A1 is unable to substitute for AtExo70E2 in this regard. Fluorescence resonance energy transfer assay and bimolecular fluorescence complementation analyses confirm the interaction between AtExo70E2 and Sec6 and Sec10. AtExo70E2, but not its yeast counterpart, is also capable of inducing EXPO formation in an animal cell line (HEK293A cells). Electron microscopy confirms the presence of double-membraned, EXPO-like structures in HEK293A cells expressing AtExo70E2. Inversely, neither human nor yeast Exo70 homologues cause the formation of EXPO in *Arabidopsis* protoplasts. These results point to a specific and crucial role for AtExo70E2 in EXPO formation.**

Monitoring Editor
Akihiko Nakano
RIKEN

Received: Oct 15, 2013
Revised: Nov 26, 2013
Accepted: Nov 27, 2013

INTRODUCTION

Exocyst is an evolutionarily conserved multisubunit tethering factor composed of eight proteins: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Sztul and Lupashin, 2006; Yu and Hughson, 2010). Originally described as a protein complex that captures and guides secretory vesicles to the plasma membrane (PM) before cognate soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)–mediated membrane fusion (TerBush *et al.*, 1996), exocyst is now known to participate in a number of polarized exocytotic processes, such as bud formation in yeast, epithelial polarity establishment, neurite outgrowth, and ciliogenesis, as well in vesicle recycling from endosomes, cytokinesis, and autophagosome formation (He and Guo, 2009; Das and Guo, 2011; Heider and Munson, 2012; Liu and Guo, 2012). Although there is little sequence similarity among the various exocyst subunits, they have some structural features in common: they are all rod shaped and contain tandem helical bundles, each of which is composed of three to five α-helices (Munson and Novick, 2006; Yu and Hughson, 2010). In addition, most exocyst subunits can interact with multiple subunits in the

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-10-0586) December 4, 2013.

Address correspondence to: Liwen Jiang (ljiang@cuhk.edu.hk).

*These authors contributed equally to this work.

†Present address: Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, MA 02114.

Abbreviations used: BiFC, bimolecular fluorescence complementation; EXPO, exocyst-positive organelle; FRET, fluorescence resonance energy transfer assay; PM, plasma membrane.

exocyst complex (Liu and Guo, 2012). Under electron microscopy the exocyst complex displays a Y-shaped morphology with a 30 × 13 nm stalk and two 6 × 15 nm arms branching away from the stalk (Hsu *et al.*, 1998; Segui-Simarro *et al.*, 2004).

The first studies on the subcellular localization of exocyst subunits indicated that they were not uniformly distributed. For mammalian cells, Moskalenko *et al.* (2003) proposed that three subunits were present on the membrane of the secretory vesicle (Sec15, Sec10, and Exo84), and the other five (Sec3, Sec5, Sec6, Sec8, and Exo70) were attached to the PM, with the assembly of the two subcomplexes being mediated by a small GTPase, RalA. In contrast, the first investigations on yeast cells suggested that Sec3p and Exo84p were characteristic of exocytic domains of the PM (Finger *et al.*, 1998; Boyd *et al.*, 2004; Zhang *et al.*, 2005). More recent studies, however, point instead to the presence of Exo70p and Sec3p at the sites of exocytosis, with the other subunits being present on the surface of the secretory vesicle (He *et al.*, 2007; Hutagalung *et al.*, 2009). In both yeast and mammalian cells, recruitment of these two subunits to the PM is achieved through an interaction between polybasic sequences at the N-terminal (for Sec3) or C-terminal (for Exo70) domains and phosphatidylinositol 4,5-biphosphate (PI(4,5)P_2) located in the inner leaflet of the PM (He *et al.*, 2007; Liu *et al.*, 2007; Zhang *et al.*, 2008). The Sec6 subunit has been identified as the binding partner for the PM-localized SNARE syntaxin Sec9 (Songer and Munson, 2009; Morgera *et al.*, 2012).

Exocyst homologues have also been found in plants (Elias *et al.*, 2003; Hala *et al.*, 2008; Wang *et al.*, 2010b). Of interest, there are 23 paralogues of Exo70 in *Arabidopsis*, the most abundant form being Exo70A1 (Synek *et al.*, 2006; Zarsky *et al.*, 2009), which, together with Sec3, Sec6, and Sec8, is localized to the tips of growing pollen tubes and root hairs (Cole *et al.*, 2005; Wen *et al.*, 2005; Hala *et al.*, 2008). In accord with its expected role in exocytic processes, the plant exocyst complex plays an essential role in cell wall and cell plate formation (Fendrych *et al.*, 2010; Kulich *et al.*, 2010; Li *et al.*, 2013), and fluorescent protein–tagged exocyst subunits have recently been detected as discrete punctae at the plant PM that are distinct from endocytic sites (Wang *et al.*, 2010b; Fendrych *et al.*, 2013; Zhang *et al.*, 2013).

Owing to the amplification of Exo70 subunits, it was proposed that plant cells do not have a single exocyst complex (Cvrčková *et al.*, 2012) and that different exocyst complexes participate in different physiological processes. In agreement with this, there is evidence for the participation of the exocyst in plant–pathogen interactions (Pecenkova *et al.*, 2011) and more recently in autophagic transport to the vacuole (Kulich *et al.*, 2013). Another example is the exocyst-positive organelle (EXPO), which is a novel exocytic structure discovered through transient expression experiments with a fluorescently tagged version of Exo70E2 (Wang *et al.*, 2010b). This organelle was also recognized in normal cells by immunofluorescence and immunogold electron microscopy using Exo70E2 antibodies. EXPOs were present as discrete structures in the cytoplasm but were also visualized fusing to the PM. In having two boundary membranes, EXPO resembles the autophagosome but did not colocalize with Atg8e, a marker for autophagosome formation, and its numbers seemed to be unaffected by starvation. Based on the lack of effect of standard inhibitors (brefeldin A, wortmannin), the relationship of EXPO to the secretory or endocytic pathways is unclear. On the other hand, there is evidence supporting a role for EXPO in stress-related unconventional protein secretion (Wang *et al.*, 2010b; Ding *et al.*, 2012; Krause *et al.*, 2013).

In the present study we investigate the membrane recruitment of some of the other exocyst subunits in relation to Exo70E2. When expressed alone, fluorescently tagged versions of Sec5a, Sec6, Sec8, and Sec10 gave rise to cytosolic signals. However, when they are expressed together with Exo70E2, punctate signals are observed that colocalize with the Exo70E2 signals. This observation is confirmed and extended by triple-labeling experiments suggesting that the formation of EXPO involves the recruitment of several if not all of the exocyst subunits. Of interest, some of the other Exo70 paralogues are not capable of being recruited to EXPO. These (Exo70A1, Exo70B1, Exo70E1, and Exo70H1) remain strongly cytosolic, although in coexpression experiments Exo70E2 can be successfully recruited. Heterologous expression experiments performed with an animal cell line and with human and yeast Exo70 homologues point to the specific and crucial role of AtExo70E2 in EXPO formation.

RESULTS

Exo70E2 is required for the membrane recruitment of a number of exocyst subunits

When transiently expressed in *Arabidopsis* protoplasts both green fluorescent protein (GFP)– and red fluorescent protein (RFP)–tagged versions of AtExo70E2 localize as discrete punctae at the plasma membrane and in the cytoplasm. Coexpression of both tagged versions produces completely overlapping signals for GFP and RFP (Supplemental Figure S1). As previously described, we interpret the fluorescent punctae as representing EXPO (Wang *et al.*, 2010b; Ding *et al.*, 2012). Using the protoplast transient expression system, we therefore assayed for the ability of other *Arabidopsis* exocyst subunits to be recruited to EXPO. We expressed these subunits either singly or together with Exo70E2. In single expression, fluorescently tagged Sec5a, Sec6, Sec8, and Sec10 all gave rise to diffuse signals throughout the cytoplasm (Figure 1, a, e, i, and m). However, when coexpressed with Exo70E2, all were corecruited with Exo70E2 to the EXPO sites (Figure 1, b–d, f–h, j–l, and n–p). The same result was obtained with Sec3a and Sec15b (Supplemental Figure S2). That the recruitment of Exo70E2 serves as a nucleus for the recruitment of other exocyst subunits was underlined by triple-expression experiments. These showed perfect colocalization of fluorescent punctae when Exo70E2 was expressed together with Sec6 and Sec8 (Figure 2, a–d) or with Sec6 and Sec10 (Figure 2, e–h).

Exo70E2 is required for the recruitment of other, but not all, Exo70 subunits

We performed single and coexpression experiments with Exo70E2 and a number of other Exo70 subunits. Of these, GFP-tagged Exo70A1 and Exo70B1 gave rise to punctate signals in addition to a diffuse cytosolic background. Nevertheless, positive corecruitment with Exo70E2 was observed with these two Exo70 paralogues (Figure 3, a–d and e–h). In contrast, both Exo70E1 and Exo70H1 produced only diffuse cytosolic signals but did recruit to EXPO when expressed together with Exo70E2 (Figure 3, i–l and m–p). Such corecruitment phenomena can also be seen with some other Exo70 paralogues (Supplemental Figure S3). Exo70 paralogues that produced only diffuse cytosolic signals and failed to be recruited to EXPO by coexpression with Exo70E2 were Exo70A3 (Figure 4, a–d), Exo70C1 (Figure 4, e–h), Exo70D1, D2, and D3 (Figure 4, i–l; Supplemental Figure S4, a–h), Exo70F1 (Figure 4, m–p), and Exo70H2, H4, H6, and H8 (Supplemental Figure S4, i–x). Exo84c also did not corecruit with Exo70E2 but nevertheless did give rise to fluorescent punctae (Supplemental Figure S5, a–d).

Recruitment of Sec6 by Exo70E2 also occurs when using an endogenous promoter

To counteract the argument that the transient expression of Exo70E2 is artifactual through the use of the 35S promoter, we generated an

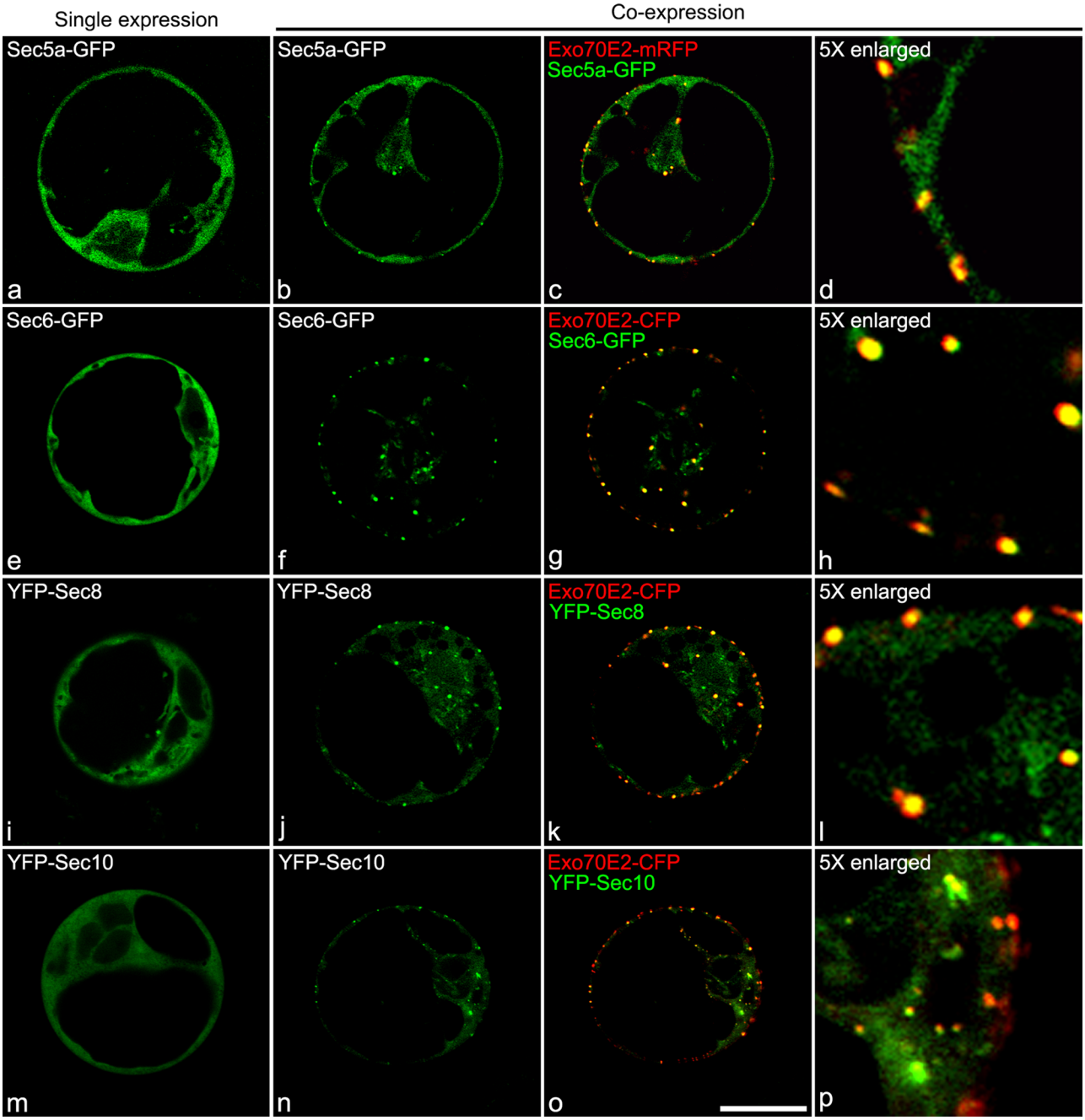

FIGURE 1: Recruitment of Sec exocyst subunit proteins to Exo70E2-positive organelles (EXPOs) by Exo70E2. When expressed individually in *Arabidopsis* protoplasts, GFP/YFP-tagged Sec exocyst proteins give rise to a cytosolic pattern. However, after coelectroporation as indicated, the different Sec exocyst proteins show in addition a punctate pattern and are colocalized with fluorescent protein–tagged Exo70E2. Bar, 20 μm.

Exo70E2-GFP construct under the control of the native Exo70E2 promoter. As seen in Figure 5, a–d, a similar density of overlapping fluorescent punctae for Sec6 and Exo70E2 was obtained with the native promoter as previously seen with the 35S promoter (compare with Figure 1, e–h).

Exo70A1 is unable to recruit other exocyst subunits

In addition to Exo70E2, we tested one of the other *Arabidopsis* Exo70 paralogues in terms of its ability to corecruit other exocyst subunits. Although, as mentioned, Exo70A1-GFP gave rise to fluorescent punctae, these did not lead to a corecruitment of Sec6, Sec8, or Sec10 when coexpressed (Figure 6, a–d, e–h, and i–l). Instead, the signals for Sec6, Sec8, and Sec10 remained diffuse and cytosolic.

Fluorescence resonance energy transfer assay and bimolecular fluorescence complementation confirm interactions between Exo70E2 and other exocyst subunits

Because colocalization of fluorescence signals is an indicator but not proof of molecular interactions between two partners in a complex,

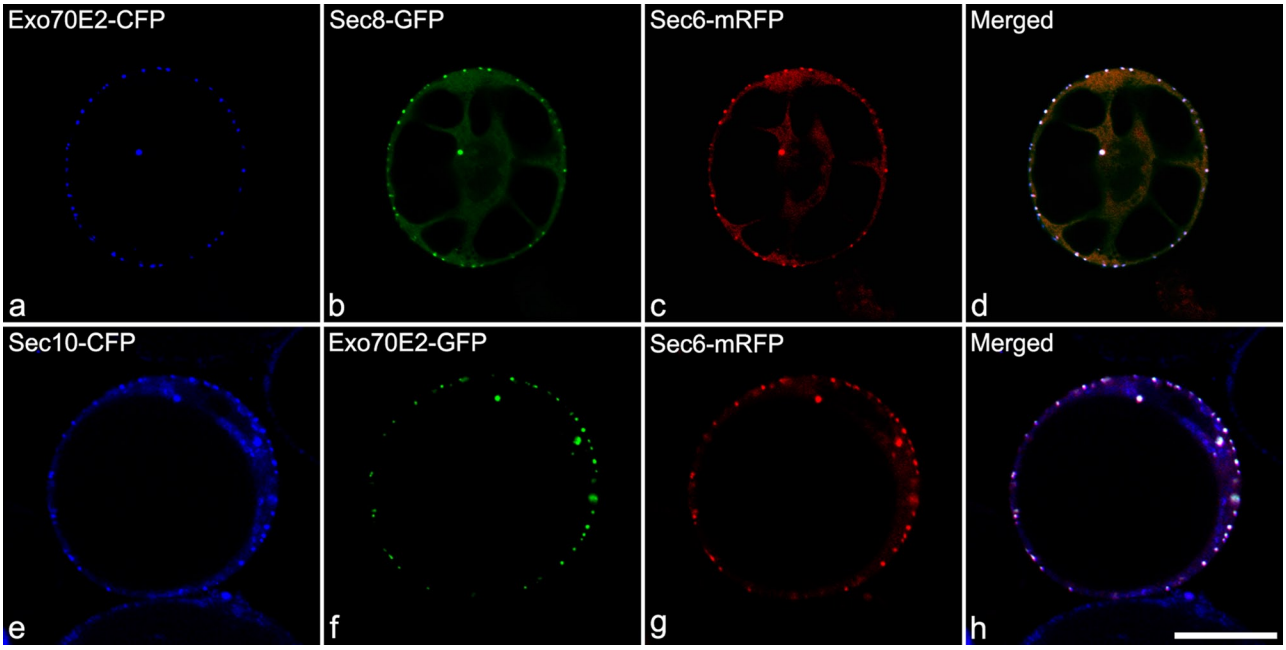

FIGURE 2: Multiple recruitment of Sec exocyst proteins to the same EXPO by Exo70E2. *Arabidopsis* protoplasts were coelectroporated with fluorescent protein–tagged Exo70E2 and different Sec exocyst proteins as indicated. After 13–16 h of expression, the protoplasts were observed in a CLSM. Bar, 20 μm.

we performed both fluorescence resonance energy transfer assay (FRET) and bimolecular fluorescence complementation (BiFC) on the putative interactions between Exo70E2 and other exocyst members. For the FRET analysis, protoplasts were observed in the confocal laser scanning microscope (CLSM) after ~15 h of coexpression of the two fluorescent constructs and subjected to spectral FRET analysis. As can be seen from Figure 7A, a–h, we selected for this analysis two colocalizing fluorescent punctae. In each case there was a high degree shifting of fluorescence signal intensity from the emission peak of cyan fluorescent protein (CFP) to the emission peak of yellow fluorescent protein (YFP), which is a positive indicator of protein–protein interaction (Shah *et al.*, 2001). For the BiFC analysis, positive fluorescence signals were obtained after coexpression of the different exocyst partners when tagged with the split BiFC constructs YC and YN (Figure 7B, a–d). As controls, we performed transient expressions with different combinations of the individual exocyst subunit with one or the other free BiFC reagent (Supplemental Figure S6, a–i). Although different Sec proteins can be positively recruited by Exo70E2 (Figure 1 and Supplemental Figure S1), not all of them can directly interact with Exo70E2 (Table 1). Of interest, some of them behaved as bridges between Exo70E2 and other exocyst members (Table 1).

Arabidopsis Exo70E2 can also induce EXPO formation in animal cells

We introduced AtExo70E2 into cultured human embryo kidney cells (HEK293A cell line) in an attempt to see whether an EXPO-like structure could be induced by its overexpression. As detailed in Supplemental Figure S7, full-length AtExo70E2-GFP can be successfully expressed in HEK293A cells, its presence being detectable by both GFP and E2 antibodies in Western blots of total cell extracts. When transiently expressed in HEK cells, AtExo70E2-GFP showed a punctate distribution (Figure 8A, a and b). Of interest, overexpression of human Exo70-GFP in HEK cells also gave rise to large fluorescent punctae, although fewer in number (Figure 8A, e and f). However, when coexpressed with AtExo70E2-mRFP, there was no overlap with the punctate human Exo70-GFP signals (Figure 8A, i–l). We also expressed yeast Exo70-GFP in HEK cells but only observed a diffuse cytosolic fluorescence (Figure 8A, g and h).

Neither human nor yeast Exo70 can induce EXPO in plant protoplasts

To determine whether nonplant Exo70 homologues could induce EXPO in *Arabidopsis* protoplasts, we performed the appropriate heterologous transient expression experiments both in the presence and the absence of AtExo70E2. Neither when expressed singly (Figure 8B, m–p) nor when coexpressed together with AtExo70E2 (Figure 8B, q–x) was EXPO formed from the nonplant Exo70 homologues, although punctate fluorescent AtExo70E2 signals were still produced.

EXPO induced by AtExo70-GFP expression in HEK cells does not colocalize with standard organelle markers

We performed immunofluorescence with antibodies directed against a number of standard organelle markers on HEK cells expressing AtExo70E2-GFP. The fluorescent punctae obtained did not overlap with any standard organelle markers for early endosomes (Figure 9, a–d), the Golgi apparatus (Figure 9, e–h), late endosomes or lysosomes (Figure 9, i–l and m–p), or autophagosomes (Figure 10, a–h).

Electron microscopy confirms the presence of EXPO-like, double-membrane structures in HEK cells after expression of AtExo70E2

EXPOs present in wild-type BY-2 cells, and also in wild-type *Arabidopsis* root cells, have two boundary membranes (Figure 11b; see also images in Wang *et al.*, 2010; Ding *et al.*, 2012). When captured in the process of fusing with the PM, they show continuity between the outer membrane and the PM, leaving a single membrane-bordered structure containing cytosol and ribosomes in the apoplast/cell wall (Figure 11a). Thin sections cut from cryofixed/freeze-substituted

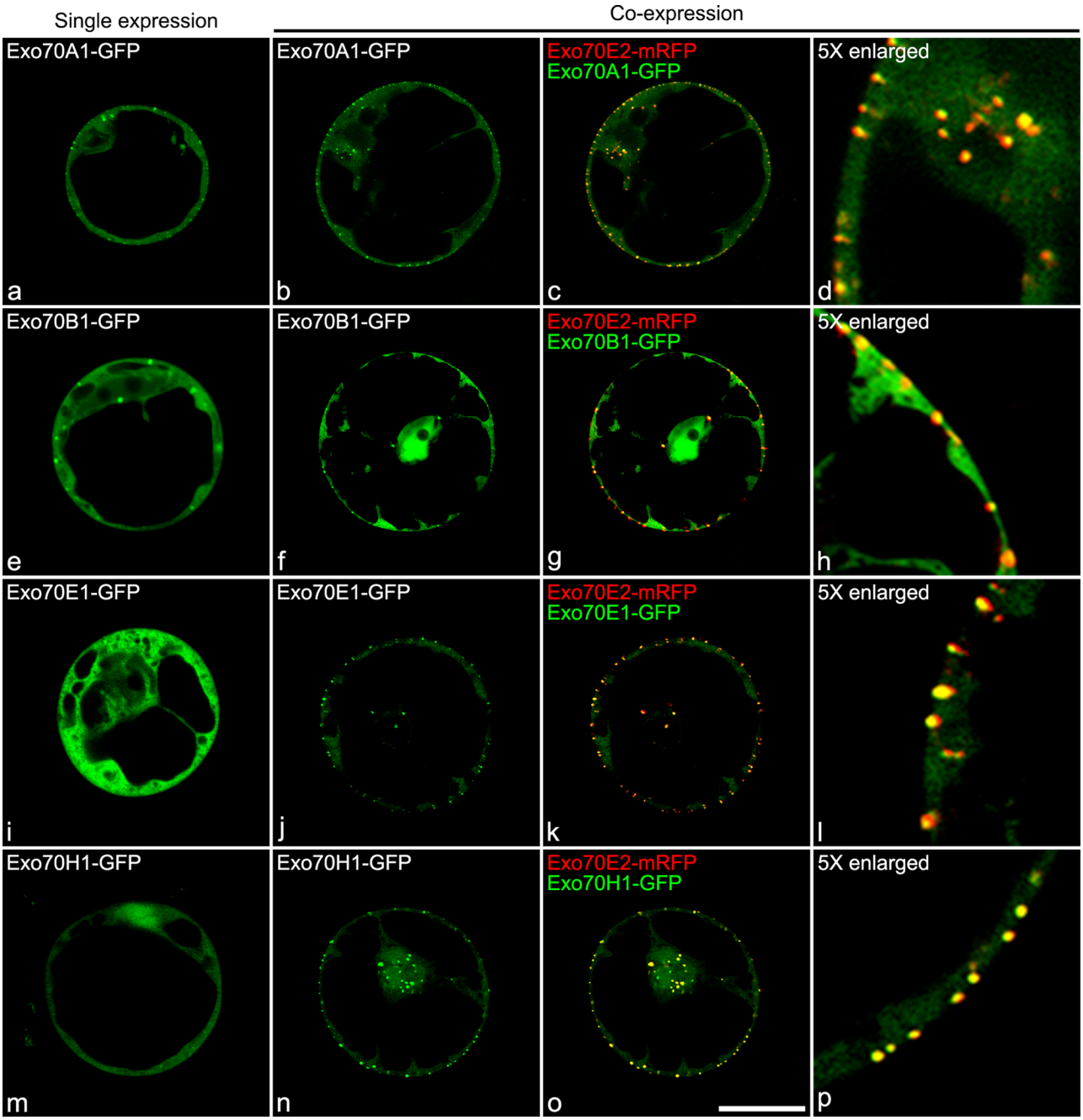

FIGURE 3: Some Exo70 exocyst proteins can be recruited to EXPO by Exo70E2. When expressed individually in *Arabidopsis* protoplasts, different Exo70 proteins gave strong cytosolic patterns. After coelectroporation with fluorescent protein–tagged Exo70E2 as indicated, these proteins show a punctate pattern and are colocalized with Exo70E2. Bar, 20 μm.

HEK cells expressing AtExo70E2 also reveal the presence of double-membraned, EXPO-like structures (Figure 11, d and e). EXPO-like membrane structures were not found in control, nontransfected HEK cells.

To confirm the identity of these structures as EXPOs, we performed immunogold labeling on HEK cells expressing AtExo70E2-GFP with both GFP and AtExo70E2 antibodies. With the GFP antibodies we obtained clusters of gold particles at the PM (Figure 11f), which resembled closely the images seen when immunogold electron microscopy was performed with AtExo70E2 antibodies for EXPO fusion in plant cells (Figure 11c; also see Figure 10 in Wang *et al.*, 2010). In addition, AtExo70E2 antibodies labeled the membrane of the EXPO-like structures (Figure 11, g–i).

DISCUSSION

Plant exocyst and the discovery of EXPO

In comparison to the situation with yeast and mammalian cells (see recent reviews by Heider and Munson, 2012; Liu and Guo, 2012), comparatively little is known about the function of the plant exocyst complex. The pioneer publications on plants were essentially proteomic in nature, establishing that plants do possess exocysts and emphasizing the unusually large number of paralogues for the Sec15

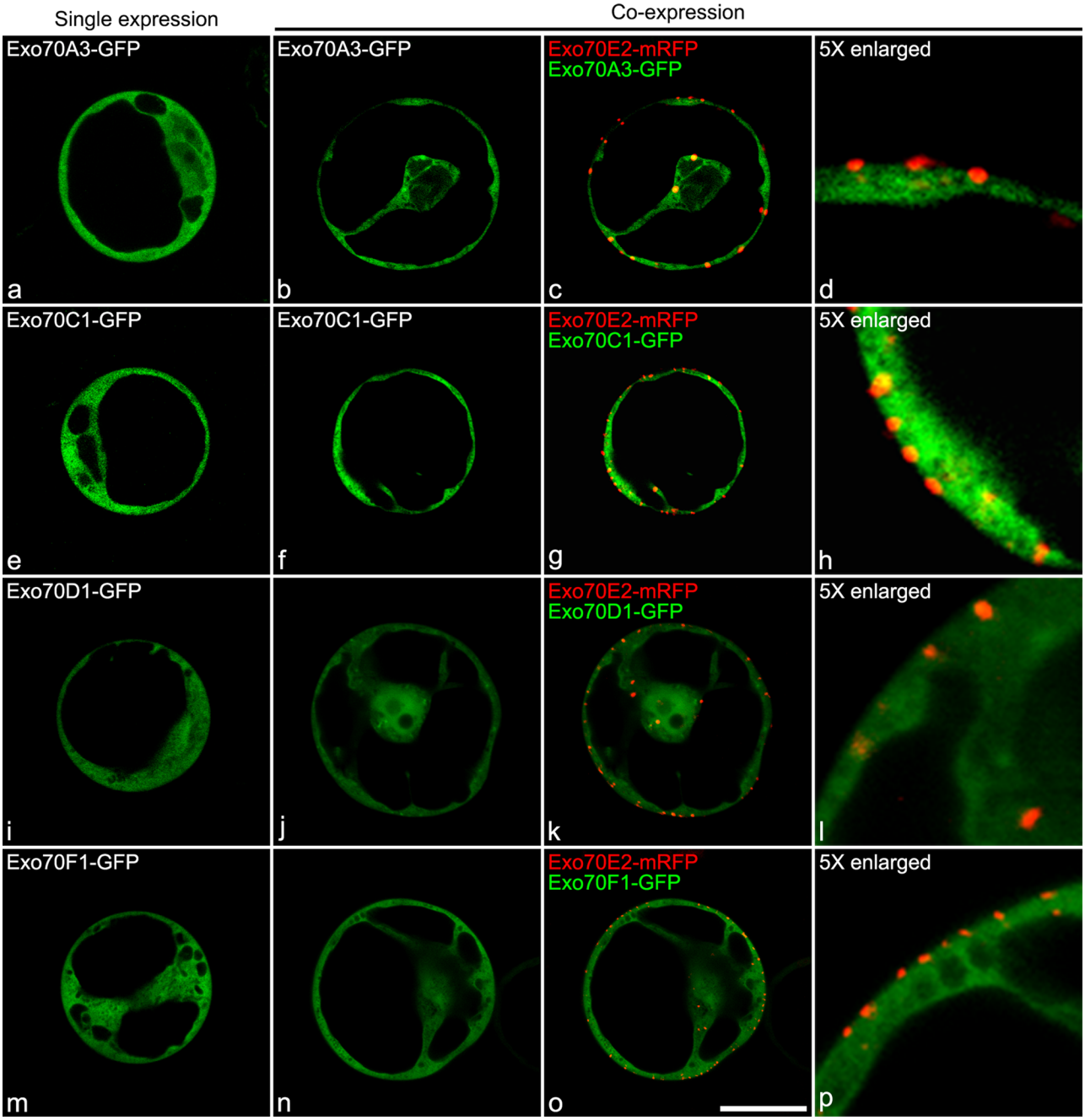

FIGURE 4: Other Exo70 exocyst proteins do not show EXPO recruitment with Exo70E2. Whether singly expressed or coexpressed with Exo70E2-mRFP in *Arabidopsis* protoplasts, a number of other Exo70 exocyst proteins give rise to only cytosolic patterns. Bar, 20 μm.

and especially Exo70 subunits (Elias *et al.*, 2003; Chong *et al.*, 2010; Zhang *et al.*, 2010; Cvrčková *et al.*, 2012). The first evidence that the plant homologues/paralogues to the yeast and mammalian exocyst subunits do actually interact with one another to form a complex was provided by Hala *et al.* (2008). Gel chromatography of a cytosolic fraction extracted from *Arabidopsis* culture cells led to the detection of a protein complex of molecular mass of ~900 kDa. Although this was higher than the predicted value for an exocyst complex (760 kDa), this behavior was also previously recorded for yeast and mammalian exocyst (Bowser *et al.*, 1992; Yeaman *et al.*, 2004). This complex reacted positively toward antibodies generated against seven of the eight exocyst subunits (all except for Exo84). Moreover, yeast two-hybrid analysis confirmed the interaction between Sec3a and Exo70A1, Sec15b, and Sec10, as well as between Sec6 and Sec8 (Hala *et al.*, 2008). A later two-hybrid screen showed interactions between Exo70B2 and Sec5a and Sec15b, and Exo70H1 and Sec3a, Sec5a, and Sec15b, as well as an interaction between Exo70B2 and Exo70H1 (Pecenkova *et al.*, 2011).

Transient expression of fluorescent protein–tagged exocyst subunits in tobacco BY-2 cells as a means of identifying the subcellular location of the plant exocyst complex was attempted on two occasions. Chong *et al.* (2010) reported that whereas Sec6, Sec8, and A1, B2, C2, D2, and H1/7 of the Exo70 family gave diffuse cytosolic patterns, Sec5a, Sec15a/b, and Exo84b produced large perinuclear

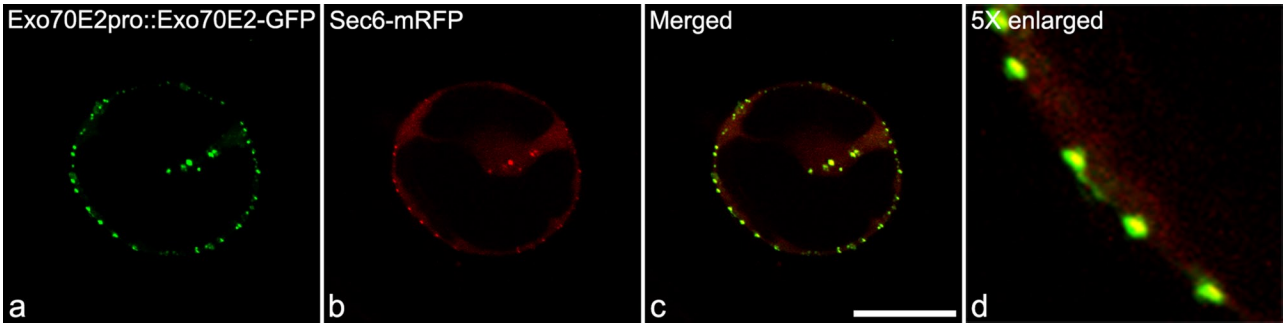

FIGURE 5: Sec6 can also be recruited to EXPO by Exo70E2 driven by Exo70E2 native promoter. Whether coexpressed with Exo70E2-GFP driven by Exo70E2 native promoter in *Arabidopsis* protoplasts, Sec6-mRFP shows a punctate pattern and is colocalized with Exo70E2. Bar, 20 μm.

aggregates. Only Exo70E2 and Exo70G1 gave rise to somewhat smaller fluorescent punctae. In our previous article dealing with exocyst subunits we concentrated on the Exo70 family and also showed that some of them (B2, D1/2, E1, and F1) gave rise to a diffuse cytosolic pattern, whereas A1, B1, and E2 produced small, discrete punctae at the cell surface and in the cytosol (Wang *et al.*, 2010b). We also showed that Exo70E2 colocalized with Exo70A1 and Exo70B1 when coexpressed. Specific Exo70E2 antibodies then allowed us to identify by immunogold electron microscopy a novel double-membraned structure, which we termed EXPO.

Recent work by Zarsky and coworkers indicates that Exo70A1, Exo70B1, and Exo70B2 are present in exocyst complexes with different functions. Whereas Exo70A1 and Exo70B2 are more involved in exocytic events (Hala *et al.*, 2008; Pecenkova *et al.*, 2011; Li *et al.*,

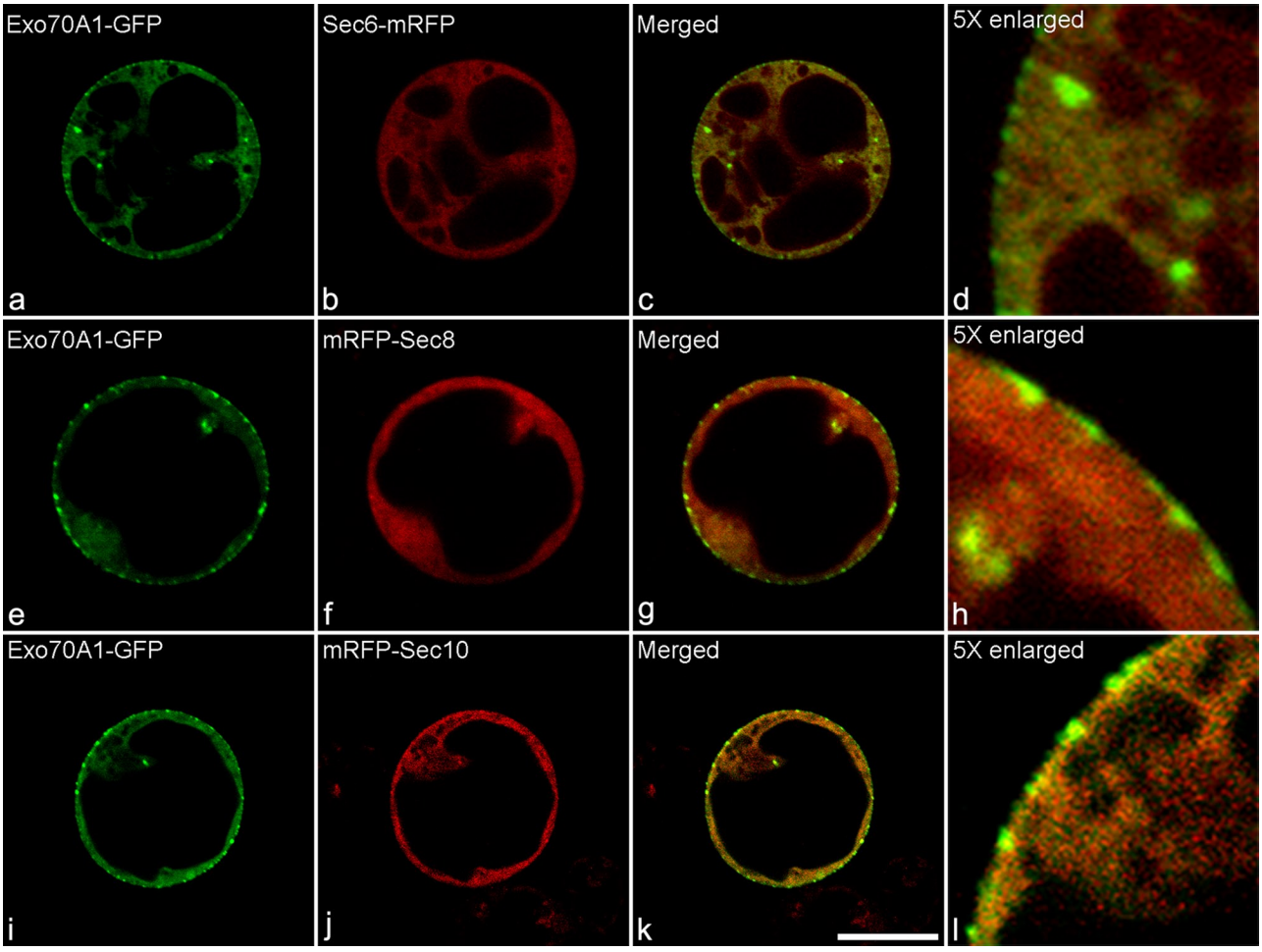

FIGURE 6: Exo70A1 cannot recruit Sec exocyst proteins to EXPO. Various Sec exocyst proteins remain cytosolic even when coexpressed with Exo70A1-GFP in *Arabidopsis* protoplasts, even though Exo70A1-GFP itself produces fluorescent punctae. Bar, 20 μm.

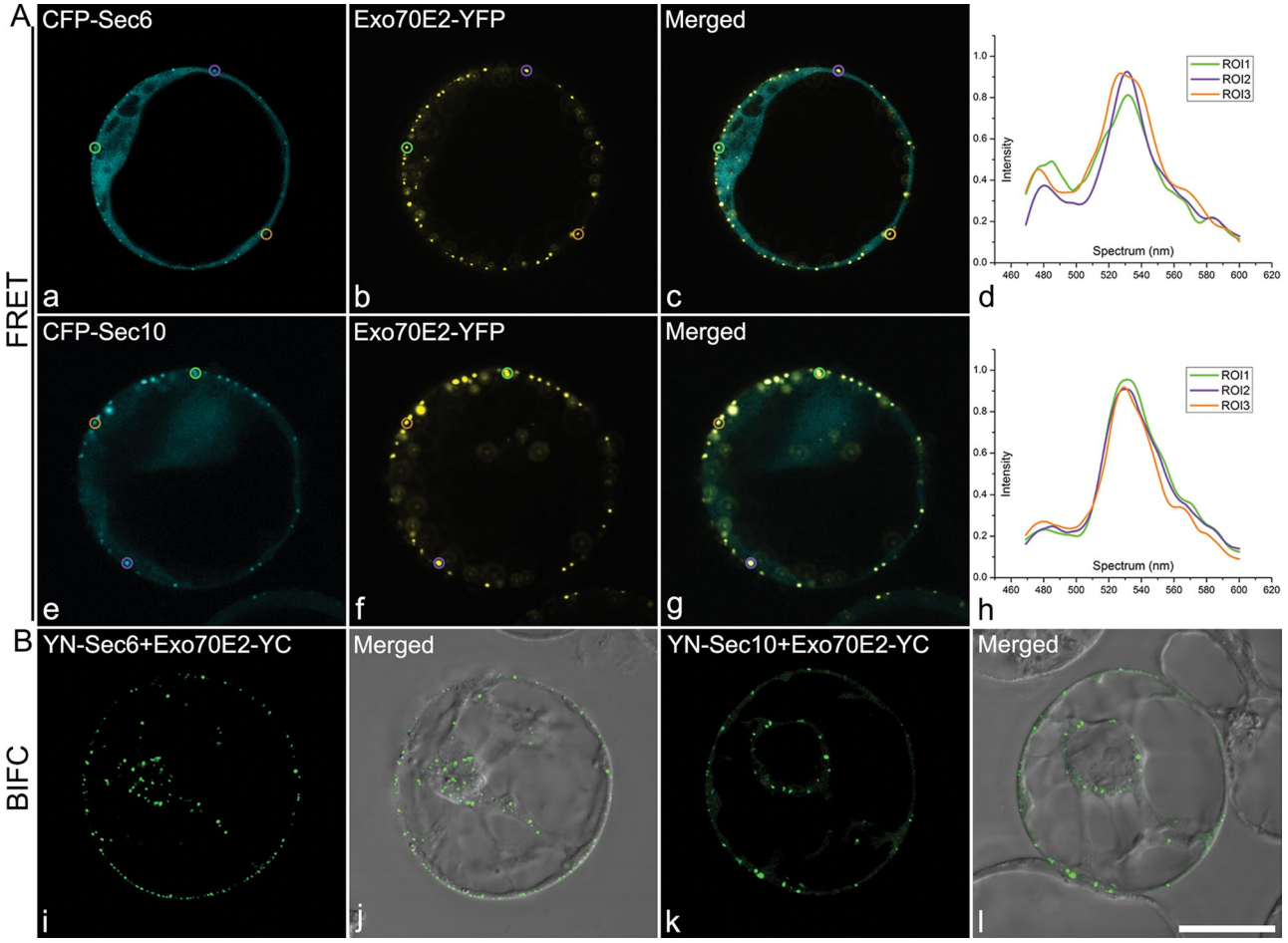

FIGURE 7: Visualization of protein–protein interactions between different exocyst subunits and Exo70E2 by spectral-FRET and BiFC. (A) *Arabidopsis* protoplasts were coelectroporated with different combinations of fusion protein constructs as indicated in a–c and e–g. After 13–16 h of expression, the protoplasts were observed in a CLSM and analyzed with spectral-FRET (d, h). (B) Epifluorescence images (i, k) and overlay images (j, l) with bright-field images from *Arabidopsis* protoplasts coexpressing different combinations of BiFC constructs as indicated. Bar, 20 μm.

2013), Exo70B1 appears to be specifically associated with an autophagic pathway leading to fusion with the tonoplast (Kulich *et al.*, 2013). In contrast to these findings, Exo70E2 localizes to the plasma membrane and to internal membranous structures (EXPOs), which do not label with the standard autophagosomal marker Atg8E (Zhuang *et al.*, 2013). As judged by the sequestration of leaderless secretory proteins, EXPOs seem to be organelles involved in unconventional protein secretion (Wang *et al.*, 2010b; Ding *et al.*, 2012; Krause *et al.*, 2013).

Exo70E2 is a key player in exocyst recruitment onto EXPO

We confirmed by protein–protein interaction analyses (FRET/BiFC) that colocalization of punctate fluorescence signals is a faithful indicator of the ability of Exo70E2 to act as a nucleator for exocyst

	Exo70E2	Sec6	Sec8	Sec10	Sec3a	Sec5a	Sec15b
Exo70E2		Y	N	Y	N	N	N
Sec6	**Y**		Y	Y	N	Y	Y
Sec8	**N**	**Y**		Y	N	Y	N
Sec10	**Y**	**Y**	**Y**		Y	Y	Y
Sec3a	**N**	**N**	**N**	**Y**		N	N
Sec5a	**N**	**Y**	**Y**	**Y**	**N**		Y
Sec15b	**N**	**Y**	**N**	**Y**	**N**	**Y**	

Bold, data from FRET analysis; not bold, data from BiFC assay.

TABLE 1: Interaction network of different exocyst subunits in plant cells.

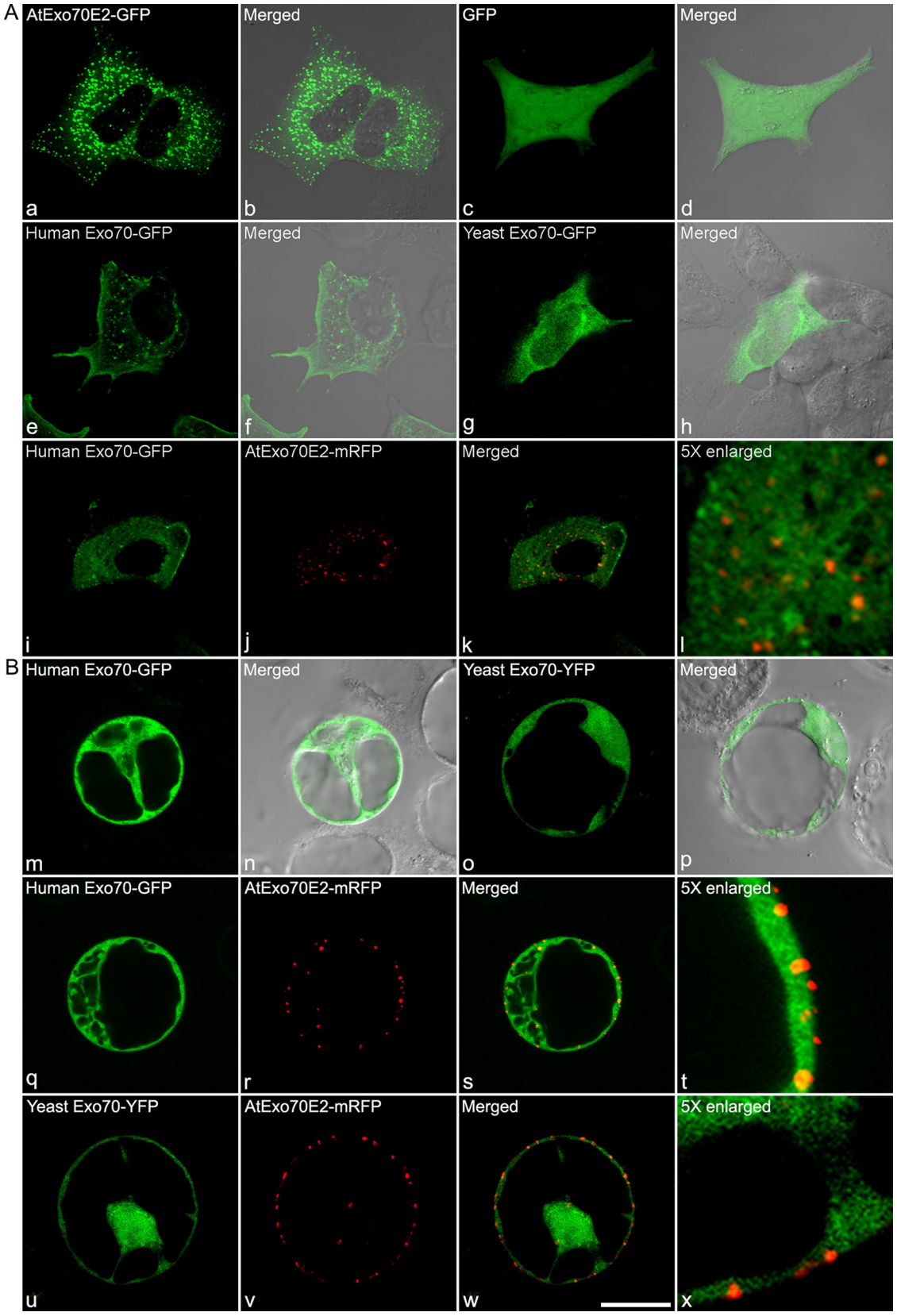

FIGURE 8: Overexpression of AtExo70E2-GFP also leads to the formation of EXPO-like structures in mammalian HEK293A cells. (A) When transiently expressed in HEK293A cells, AtExo70E2-GFP shows a punctate pattern (a). HEK293A cells transfected with free GFP (c) is included as control. (b, d) Merged differential interference contrast images. In contrast, human Exo70 labels the plasma membrane and cytosolic punctae (e), and yeast Exo70 shows only a cytosolic pattern (g). (B) Whether singly expressed or coexpressed with Exo70E2-mRFP in *Arabidopsis* protoplasts, human Exo70 (m, q, and s) or yeast Exo70 (o, u, and w) produces only cytosolic patterns. Bar, 20 μm.

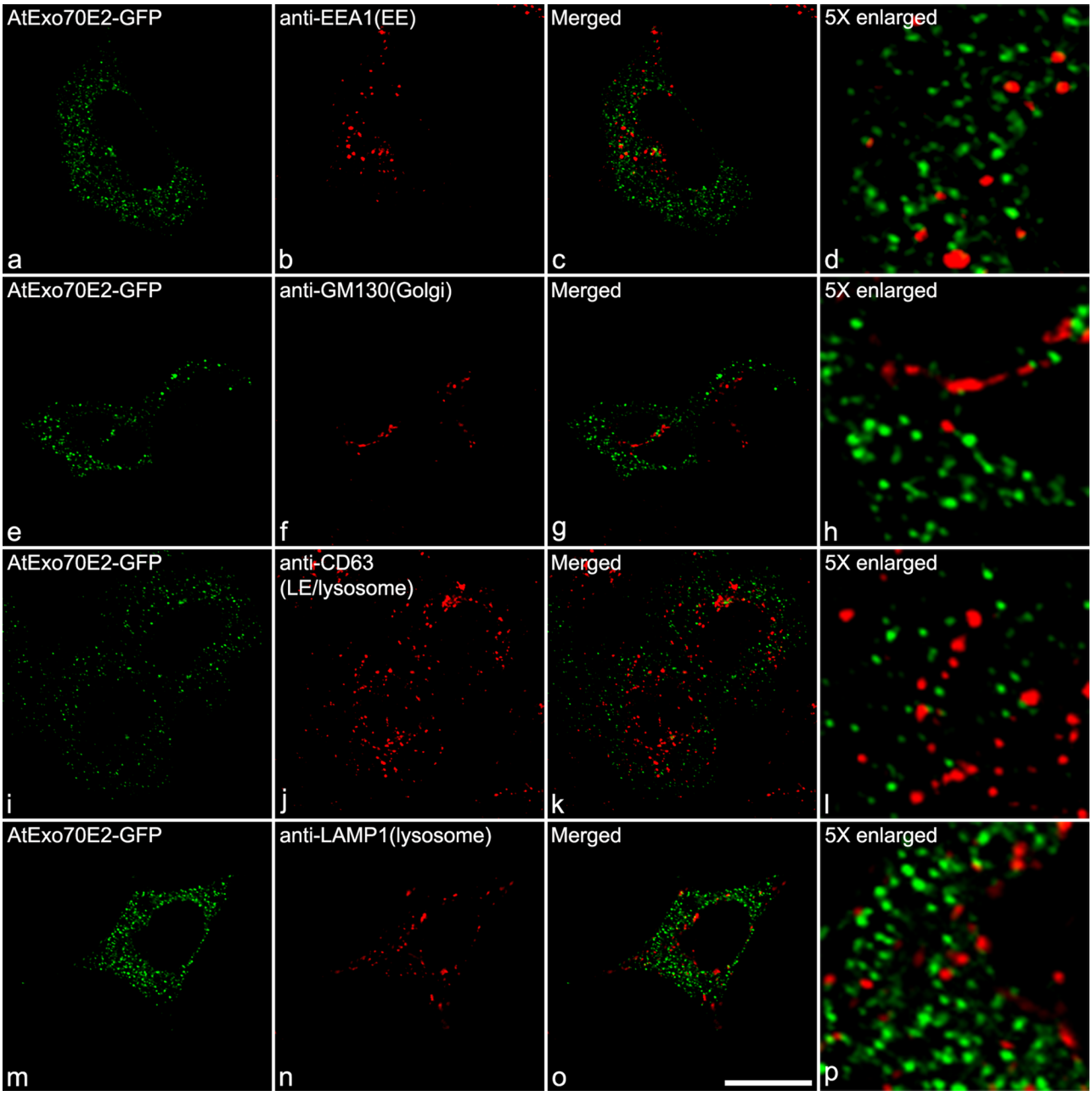

FIGURE 9: EXPO-like structures are distinct from conventional organelles in mammalian HEK293A cells. Immunofluorescence with standard organelle marker antibodies demonstrates that AtExo70E2-GFP–positive structures do not lie on the secretory or endocytic pathways. EE, early endosome; LE, late endosome. Bar, 20 μm.

assembly. As summarized in Table 2, coexpression with Exo70E2 allows for the successful recruitment of all of the Sec subfamily and about half of the other Exo70 paralogues. Of interest, although Exo84c seems to be recruited to membranes, since it does not colocalize with Exo70E2, the exocyst form binding to EXPO may lack Exo84c. In addition, Exo70A1, which features prominently in previous studies on the plant exocyst, seems to lack the ability to recruit the Sec subfamily, suggesting that Exo70E2 is the specific Exo70 paralogue for EXPO formation (Figure 12 and Supplemental Table S3).

As is the case in yeast and mammals with Exo70 (He *et al.*, 2007; Liu *et al.*, 2007), we speculate that Exo70E2 is responsible for interaction with a lipid (possibly $PI(4,5)P_2$) in the membrane of EXPO. This is depicted in Figure 12. What we do not know is whether Exo70E2 attaches the membrane with or without the interacting Sec exocyst subunits. If the latter scenario is correct, then the membrane attachment of Exo70E2 would act as a trigger for exocyst recruitment. Exo70E2-GFP signals are visible at both the plasma membrane and on EXPO (see earlier discussion; Wang *et al.*, 2010b), so at issue is whether Exo70E2 may act as a bridging molecule by binding to both membranes. This is unlikely, since in yeast, Exo70 interacts with $PI(4,5)P_2$ via basic residues clustered in the C-terminus (He *et al.*, 2007). Because Sec3 is also known to interact with $PI(4,5)P_2$ (through a polybasic domain at the N-terminus; Zhang *et al.*, 2008), it is possible that this is the binding partner in the case of fusion of EXPO with the plasma membrane.

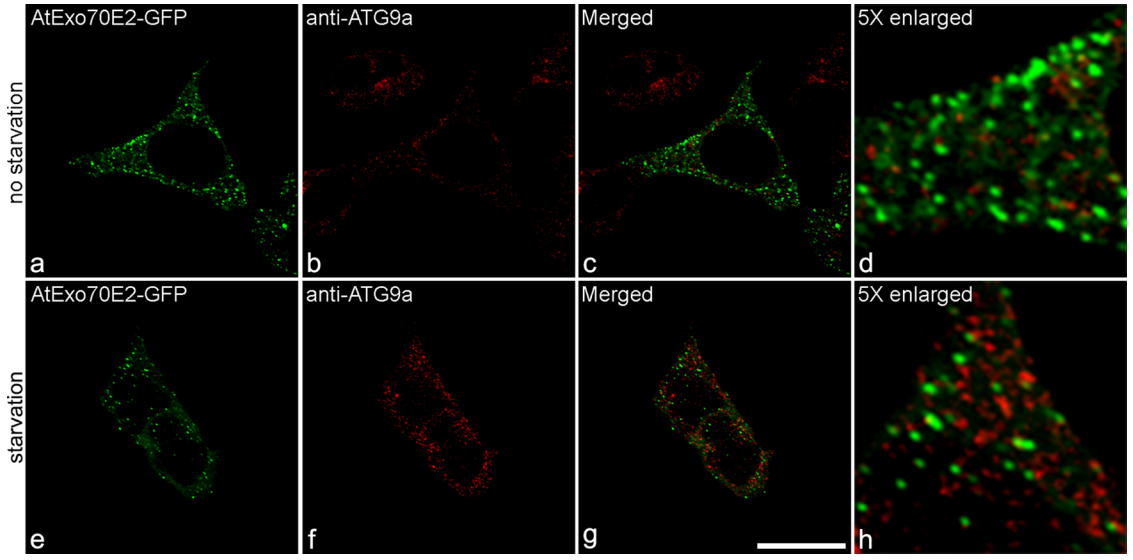

FIGURE 10: EXPO-like structures are distinct from autophagosomes at different conditions in mammalian HEK293A cells. Immunofluorescence with autophagosome marker antibody anti-ATG9a demonstrates that AtExo70E2-GFP–positive organelles are different from autophagosomes with (e–h) or without (a–d) starvation treatment. Bar, 20 μm.

Exo70E2 expression as a signal for EXPO formation

It would seem that AtExo70E2 specifically causes EXPO formation, since overexpression of AtExo70E2 in plant cells gives rise to EXPO, whereas overexpression of human and yeast Exo70 in the same cell type does not. This is supported by the formation of EXPO in HEK293A cells, which seems to occur only when the plant Exo70 paralogue and neither human nor yeast Exo70 is expressed. Is there therefore something unique about the structure of AtExo70E2 that might explain this phenomenon? As can be seen from Figure 13A, AtExo70E2 is slightly longer than its mammalian and yeast counterparts (11 and 14 amino acids, respectively), but at the primary sequence level, AtExo70E2 shows only relatively low identity (14.6%) and similarity (33.1%) with its mammalian counterpart (Supplemental Figure S8B). The values for AtExo70A1 are slightly higher. The

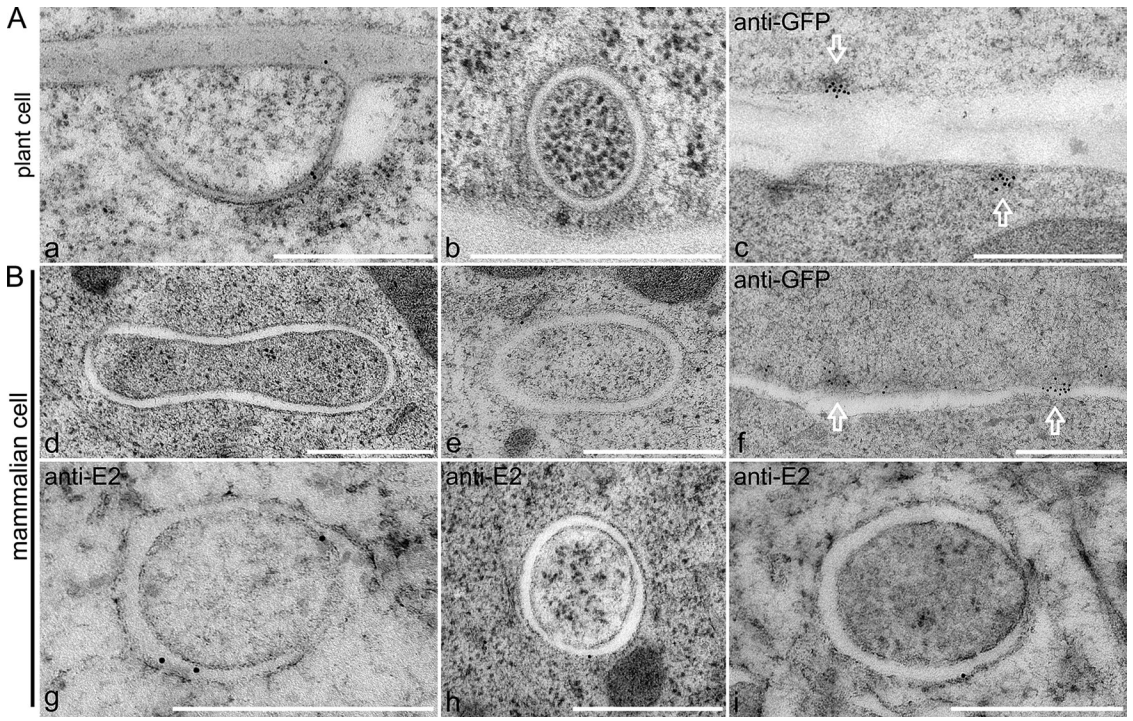

FIGURE 11: Ultrastructure of EXPO-like structures in plant cells or HEK293A cells stably expressing AtExo70E2-GFP. (A) Examples of EXPO-like structures in transgenic tobacco BY-2 cell expressing AtExo70E2-GFP (a), *Arabidopsis* wild-type root cell (b), and protein patch in transgenic *Arabidopsis* cell expressing AtExo70E2-GFP (c). (B) Profiles of EXPO-like structures in the cytoplasm from sections cut from high-pressure frozen/freeze-substituted samples of HEK293A cells expressing AtExo70E2-GFP (d–i). (f) Immunogold labeling with GFP antibodies; (g, h, and i) immunogold labeling with AtExo70E2 antibodies. Bars, 500 nm.

Protein group	Colocalization	Recruitment
Sec[a]	Y	Y
Exo84[b]	Y	N
Exo70 1[c]	Y	Y
Exo70 2[d]	N	N

Exo84c and Exo70H4 only show partial colocalization. Proteins in different groups are indicated in the remaining footnotes.
[a]Sec3a, Sec5a, Sec6, Sec8, Sec10, Sec15b.
[b]Exo84c.
[c]Exo70A1, Exo70A2, Exo70B1, Exo70B2, Exo70E1, Exo70G1, Exo70G2, Exo70H1, Exo70H3.
[d]Exo70A3, Exo70C1, Exo70D1, Exo70D2, Exo70D3, Exo70F1, Exo70H2, Exo70H4, Exo70H6, Exo70H8.

TABLE 2: Summary of recruitment function of Exo70E2 to different exocyst subunits in plant cells.

comparative values between AtExo70E2 and yeast Exo70p are even somewhat lower. There are equally low values for identity and similarity between yeast and mammalian Exo70s. However, when the predicted three-dimensional structural models for Exo70 from the three organisms are compared, there are indeed significant differences (dotted circles in Figure 13A). Although great overall similarity in regard to number and position of the α-helical domains (19 in total) exists, there is a significant difference at the N-terminus, where the first helical domain seems to be lacking in AtExo70E2. In addition, there are nonhelical extensions of the primary sequence between the helical domains H6 and H7 and between H12 and H13. Whether one or the other of these structural differences is the cause of the specific EXPO-inducing ability of AtExo70E2 remains to be seen.

The heterologous induction of EXPO in mammalian cells through the expression of a single plant exocyst subunit likely suggests that AtExo70E2 can interact with mammalian Sec exocyst subunits, but such a scenario needs further proof from experiments. Of interest, in having two boundary membranes, EXPO bears a superficial resemblance to the mammalian autophagosome and the plant autophagosome (Zhuang *et al.*, 2013), and the exocyst has been implicated in autophagosome biogenesis (Bodemann *et al.*, 2011). That mammalian autophagosomes, like EXPO, have been shown to be vehicles for unconventional protein secretion (reviewed by Pfeffer, 2010) may therefore be more than just a coincidence. It will be of interest to illustrate the distinct molecular mechanisms and possible connections between EXPO and the autophagosome in plants in future studies (Zhuang *et al.*, 2013).

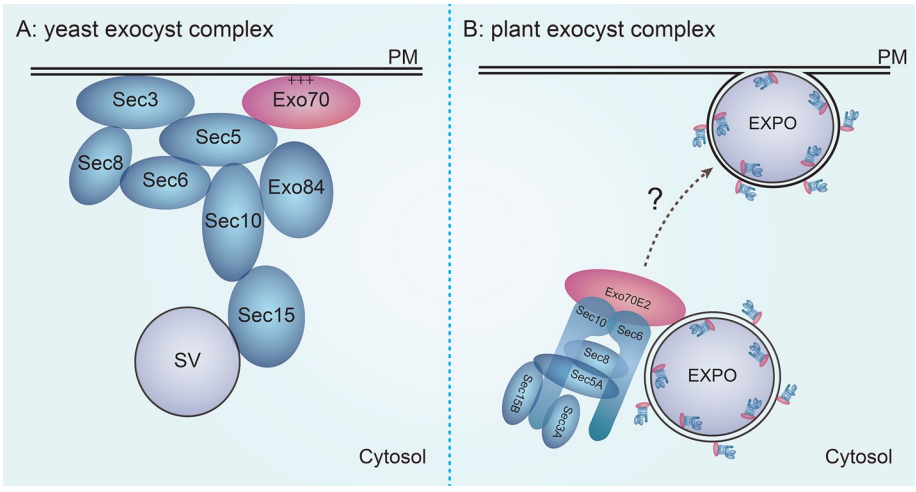

FIGURE 12: Working model for exocyst complex assembly into EXPO in plants. (A) Structure of the exocyst complex in yeast cells, showing exocyst connecting a secretory vesicle (SV) to the plasma membrane (PM). This model is modified from published results (He *et al.*, 2007). (B) Assembly, and recruitment of exocyst to EXPO directed by Exo70E2, followed by fusion of EXPO with the plasma membrane.

Perspectives

Although EXPO can be found in normal plant cells (Wang *et al.*, 2010b; Ding *et al.*, 2012), overexpression of AtExo70E2 can certainly increase their number in plant cells and their biogenesis in animal cells. These effects point strongly toward a signal function for this exocyst subunit. If, as we recently proposed, EXPO is an agent for unconventional protein secretion in plants and this process is geared up under stress conditions such as pathogen attack (Ding *et al.*, 2012; Krause *et al.*, 2013), then increased expression of endogenous Exo70E2 may be a good indicator of stress.

MATERIALS AND METHODS

Plasmid construction

Different constructs of *Exo70A1/B1/B2/D1/D2/E1/E2/F1* were generated as previously described (Wang *et al.*, 2010b). Full-length cDNAs of other exocyst subunits were amplified from *Arabidopsis* Columbia-0 leaf tissue cDNA with the primer sets listed in Supplemental Table S2. The amplified fragments were cloned into the pBI221 vector with different fluorescent tags at the N-terminus or C-terminus using specific restriction cutting sites to generate the various fusion constructs under the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator. cDNAs coding for full-length *Arabidopsis Exo70E2*, yeast *Exo70p*, or human *Exo70* were amplified from *Arabidopsis* Columbia-0 leaf tissue cDNA, the genomic DNA of budding yeast *Saccharomyces cerevisiae* BY 4743, or cDNA from human HEK293A culture cells with different primer sets listed in Supplemental Table S2. The amplified fragments were then cloned into pBI221, pEGFP-N1/C1 (Clontech, Mountain View, CA) or pDsRed-N1 (Clontech), respectively. For the *Exo70E2* promoter::*Exo70E2-GFP* construct, the *Exo70E2* promoter sequence was amplified as previously described (Li *et al.*, 2010) by using the primer set listed in Supplemental Table S2. The PCR fragment was digested with *Cla*I/*Bam*HI and then ligated into the premade pBI121 vector containing the *Exo70E2-GFP* coding sequence described previously (Wang *et al.*, 2010b) to replace the cauliflower mosaic virus 35S promoter.

Plant material and transient expression in protoplasts

Procedures for maintaining *Arabidopsis* suspension cultured cells were as described previously (Miao and Jiang, 2007). Transient expression using *Arabidopsis* protoplasts was been done essentially as in a previously established protocol (Miao and Jiang, 2007).

Transient transfection of HEK293A cells

HEK293A cells were propagated in DMEM containing 10% heat-inactivated fetal calf serum (Invitrogen, Grand Island, NY) and 1% penicillin and streptomycin in 75-cm^2 flasks under 5% CO_2 at 37°C. For transfection, HEK293A cells were cultured in six-well

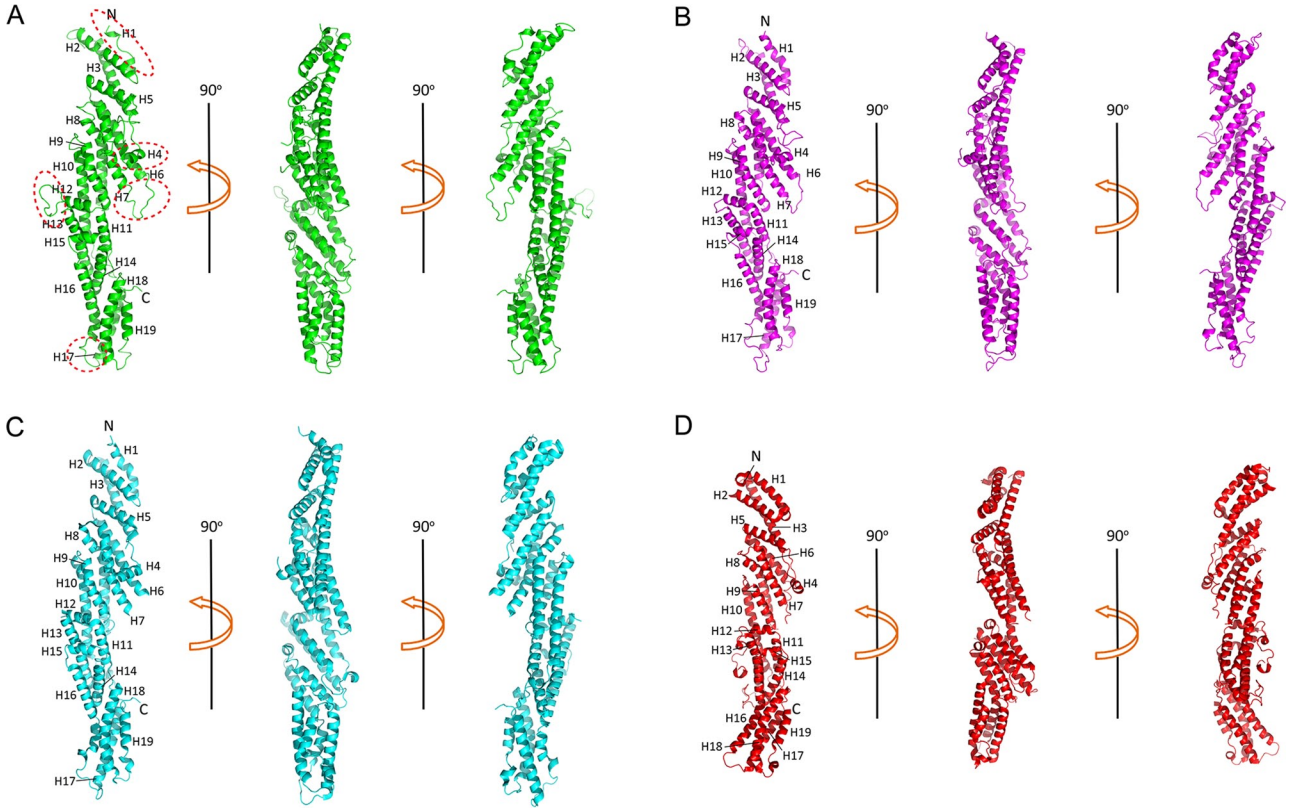

FIGURE 13: Predicted three-dimensional (3D) structures of Exo70 from different organisms. (A) The 3D structure of AtExo70E2 based on mouse Exo70 structure as a model. Potential structural differences are shown in red circles. (B) The 3D structure of AtExo70A1. (C) The 3D structure of mouse Exo70. (D) The 3D structure of yeast Exo70p. The 3D structures of mouse Exo70 (Protein Data Bank: 2PFT) and yeast Exo70p (Protein Data Bank: 2B1E) were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank. The 3D structures of *Arabidopsis* Exo70E2 and A1 were obtained from the ESyPred3D program (Lambert *et al.*, 2002). They were further analyzed using the PyMOL program (PyMOL Molecular Graphics System, version 1.3; www.pymol.org/) The structures were opened, centered, and zoomed to proper size. High-resolution photos were made by Ray command (Ray 2844 × 1422) before being saved. The structures were rotated 90 and 180° to get better views. The helix information and potential structural differences are labeled with red circles. The secondary structures of mouse Exo70 and yeast Exo70p were obtained from published data (Dong *et al.*, 2005; Moore *et al.*, 2007).

plates and transfected with plasmids using jetPEI transfection reagent (PolyPlus, New York, NY). Twenty-four hours after transfection, cells were washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde for immunolabeling and confocal imaging.

Generation of HEK293A stable cell line

Transfection of linearized AtExo70E2-GFP plasmid was carried out in 24-well tissue culture plates containing 50,000 cells using jetPEI transfection reagent. The medium was replaced 24 h after transfection by DMEM supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin and streptomycin. At 48 h after transfection, cells were fed with selective medium containing 1 mg/ml Geneticin. Then cells were fed with fresh medium every day and passaged upon reaching confluence by trypsinization. Cells were selected for at least 14 d before proceeding to cloning by limited dilution. To isolate monoclonal cell lines with highest AtExo70E2-GFP expression, cells were subcloned by seeding 50, 100, and 200 cells into three separate 96-well tissue culture plates and maintained in selective medium supplemented with 500 μg/ml Geneticin. After 1 wk, cells were trypsinized and passed to another 96-well plate, fixed in 4% paraformaldehyde, and examined under a fluorescence microscope. Subclones that gave good AtExo70E2 expression were frozen and proceeded to other rounds of subcloning until single-cell subclones that gave good AtExo70E2 expression were obtained.

Immunofluorescence studies

Fixation and preparation of AtExo70E2GFP-transformed HEK293A cells and their labeling and analysis by confocal immunofluorescence were described previously (Jiang and Rogers, 1998; Jiang *et al.*, 2000; Miao *et al.*, 2008, 2011; Wang *et al.*, 2010a; Gao *et al.*, 2012). HEK293A cells were seeded on coverslip in six-well culture plates. Cells were fixed in 4% paraformaldehyde for 10 min in room temperature, followed by methanol fixation for 5 min at −20°C. After blocking with 1% bovine serum albumin diluted in 1× PBS for 1 h at room temperature, primary antibody was added with different concentrations, and slides were incubated overnight at 4°C. Slides were then washed with 1× PBS three times, incubated with secondary antibodies at room temperature for 1 h, washed with 1× PBS three times, and subjected to confocal laser scanning microscopy. Confocal fluorescence images were collected using an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) with a 60× water lens. The settings for collecting confocal images and processing of images using Photoshop

(Adobe, San Jose, CA) were as described previously (Cai *et al.*, 2011; Shen *et al.*, 2013).

Autophagy induction of HEK293A transgenic cells expressing AtExo70E2-GFP by starvation

Starvation of HEK293A transgenic cells to induce autophagy was performed as previously described (Munafo and Colombo, 2001; Martinet *et al.*, 2006). Generally, the normal cultured cells were washed three times with PBS and then incubated in Earle's balanced salts solution at 37°C for 2 h to induce autophagosome formation.

Electron microscopy of resin-embedded cells

The general procedures for transmission electron microscopy, sample preparation, and thin sectioning of resin blocks of AtExo70E2GFP-transformed HEK293A cells were performed essentially as described previously (Tse *et al.*, 2004; Lam *et al.*, 2007, 2008). For high-pressure freezing, AtExo70E2GFP-transformed HEK293A cells were harvested by trypsinization, gently spin down, and immediately frozen in a high-pressure freezing apparatus (EM Part2; Leica, Wetzlar, Germany). Immunolabeling on HM20 sections was done using standard procedures as described previously (Tse *et al.*, 2004) with Exo70E2 antibodies or GFP antibodies and gold-coupled secondary antibodies. Sections were poststained with aqueous uranyl acetate/lead citrate and then examined with a Hitachi H7650 transmission electron microscope (Hitachi, Tokyo, Japan) with a MacroFire monochrome charge-coupled device camera (Optronics) operating at 80 kV as described previously (Tse *et al.*, 2004; Wang *et al.*, 2007; Zhuang *et al.*, 2013).

FRET

FRET assays were performed essentially as previously described, with minor modifications (Shah *et al.*, 2001; Sieben *et al.*, 2008). *Arabidopsis* protoplasts were coelectroporated with different combinations of fusion proteins. After incubating for 13 h, fluorescence images were taken using a confocal laser scanning microscope (TCS SP5 II; Leica, Japan). The fluorescence of CFP was detected at 470–500 nm and that of YFP at 530–550 nm. Fluorescence resonance energy transfer was performed by a donor-acceptor spectral shift assay. The transfected *Arabidopsis* protoplasts were excited using a 458-nm laser, and emission fluorescence was monitored between 465 and 600 nm. Image acquisition was performed by a Leica LSM 5 image browser and analyzed by Origin 8.0 (OriginLab, Northampton, MA).

BiFC

The general procedures of BiFC were basically as previously described (Waadt *et al.*, 2008). *Arabidopsis* protoplasts were coelectroporated with different combinations of fusion proteins with split YFP. Fluorescence images were taken using a confocal laser scanning microscope (Olympus FluoView FV1000).

Western blotting analysis

Proteins were isolated from different HEK293A cells and fractionated on 10% SDS–PAGE, followed by Western blotting as previously described (Cai *et al.*, 2012).

Accession numbers

Sequence data for different genes can be found in the Tair/GenBank/EMBL data libraries under the accession numbers listed in Supplemental Table S1.

ACKNOWLEDGMENTS

This work was partially supported by grants from the Research Grants Council of Hong Kong (CUHK466610, 466011, 465112, CUHK2/CRF/11G, HKUST10/CRF/12R, and AoE/M-05/12), the National Natural Science Foundation of China/Research Grants Council (N_CUHK406/12), the National Natural Science Foundation of China (31270226), and the Shenzhen Peacock Project (KQTD201101) to L.J., and also a grant from the National Natural Science Foundation of China (30970272) and a grant from the Research Fund for the Doctoral Program of Higher Education (B0201100672) to Y.Q.B.

REFERENCES

Bodemann BO *et al.* (2011). RalB and the exocyst mediate the cellular starvation response by direct activation of autophagosome assembly. Cell 144, 253–267.

Bowser R, Muller H, Govindan B, Novick P (1992). Sec8p and Sec15p are components of a plasma membrane-associated 19.5s particle that may function downstream of Sec4p to control exocytosis. J Cell Biol 118, 1041–1056.

Boyd C, Hughes T, Pypaert M, Novick P (2004). Vesicles carry most exocyst subunits to exocytic sites marked by the remaining two subunits, Sec3p and Exo70p. J Cell Biol 167, 889–901.

Cai Y, Jia T, Lam SK, Ding Y, Gao C, San MWY, Pimpl P, Jiang L (2011). Multiple cytosolic and transmembrane determinants are required for the trafficking of SCAMP1 via an ER-Golgi-TGN-PM pathway. Plant J 65, 882–896.

Cai Y, Zhuang X, Wang J, Wang H, Lam SK, Gao C, Wang X, Jiang L (2012). Vacuolar degradation of two integral plasma membrane proteins, AtLRR84A and OsSCAMP1, is cargo ubiquitination-independent and prevacuolar compartment-mediated in plant cells. Traffic 13, 1023–1040.

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

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

Cvrčková F, Grunt M, Bezvoda R, Hala M, Kulich I, Rawat A, Zarsky V (2012). Evolution of the land plant exocyst complexes. Front Plant Sci 3, 159.

Das A, Guo W (2011). Rabs and the exocyst in ciliogenesis, tubulogenesis and beyond. Trends Cell Biol 21, 383–386.

Ding Y, Wang J, Wang J, Stierhof YD, Robinson DG, Jiang L (2012). Unconventional protein secretion. Trends Plant Sci 17, 606–615.

Dong G, Hutagalung AH, Fu CM, Novick P, Reinisch KM (2005). The structures of exocyst subunit Exo70p and the Exo84p C-terminal domains reveal a common motif. Nat Struct Mol Biol 12, 1094–1100.

Elias M, Drdova E, Ziak D, Bavlnka B, Hala M, Cvrckova F, Soukupova H, Zarsky V (2003). The exocyst complex in plants. Cell Biol Int 27, 199–201.

Fendrych M *et al.* (2010). The *Arabidopsis* exocyst complex is involved in cytokinesis and cell plate maturation. Plant Cell 22, 3053–3065.

Fendrych M, Synek L, Pecenkova T, Drdova EJ, Sekeres J, de Rycke R, Nowack MK, Zarsky V (2013). Visualization of the exocyst complex dynamics at the plasma membrane of *Arabidopsis thaliana*. Mol Biol Cell 24, 510–520.

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

Gao C, Yu CKY, Qu S, San MWY, Li KY, Lo SW, Jiang L (2012). The Golgi-localized *Arabidopsis* endomembrane protein12 contains both endoplasmic reticulum export and Golgi retention signals at its C terminus. Plant Cell 24, 2086–2104.

Hala M *et al.* (2008). An exocyst complex functions in plant cell growth in *Arabidopsis* and tobacco. Plant Cell 20, 1330–1345.

He B, Guo W (2009). The exocyst complex in polarized exocytosis. Curr Opin Cell Biol 21, 537–542.

He B, Xi F, Zhang X, Zhang J, Guo W (2007). Exo70 interacts with phospholipids and mediates the targeting of the exocyst to the plasma membrane. EMBO J 26, 5167–5167.

Heider MR, Munson M (2012). Exorcising the exocyst complex. Traffic 13, 898–907.
Hsu SC, Hazuka CD, Roth R, Foletti DL, Heuser J, Scheller RH (1998). Subunit composition, protein interactions, and structures of the mammalian brain sec6/8 complex and septin filaments. Neuron 20, 1111–1122.
Hutagalung AH, Coleman J, Pypaert M, Novick PJ (2009). An internal domain of Exo70p is required for actin-independent localization and mediates assembly of specific exocyst components. Mol Biol Cell 20, 153–163.
Jiang L, Phillips TE, Rogers SW, Rogers JC (2000). Biogenesis of the protein storage vacuole crystalloid. J Cell Biol 150, 755–769.
Jiang L, Rogers JC (1998). Integral membrane protein sorting to vacuoles in plant cells: evidence for two pathways. J Cell Biol 143, 1183–1199.
Krause C, Richter S, Knoll C, Jurgens G (2013). Plant secretome—from cellular process to biological activity. Biochim Biophys Acta 1834, 2429–2441.
Kulich I, Cole R, Drdová E, Cvrčková F, Soukup A, Fowler J, Žárský V (2010). *Arabidopsis* exocyst subunits SEC8 and EXO70A1 and exocyst interactor ROH1 are involved in the localized deposition of seed coat pectin. New Phytol 188, 615–625.
Kulich I, Pečenková T, Sekereš J, Smetana O, Fendrych M, Foissner I, Höftberger M, Žárský V (2013). *Arabidopsis* exocyst subcomplex containing subunit EXO70B1 is involved in autophagy-related transport to the vacuole. Traffic 14, 1155–1165.
Lam SK, Cai Y, Hillmer S, Robinson DG, Jiang L (2008). SCAMPs highlight the developing cell plate during cytokinesis in tobacco BY-2 cells. Plant Physiol 147, 1637–1645.
Lam SK, Siu CL, Hillmer S, Jang S, An GH, Robinson DG, Jiang L (2007). Rice SCAMP1 defines clathrin-coated, *trans*-Golgi-located tubular-vesicular structures as an early endosome in tobacco BY-2 cells. Plant Cell 19, 296–319.
Lambert C, Leonard N, De Bolle X, Depiereux E (2002). ESyPred3D: prediction of proteins 3D structures. Bioinformatics 18, 1250–1256.
Li SP, Chen M, Yu DL, Ren SC, Sun SF, Liu LD, Ketelaar T, Emons AMC, Liu CM (2013). EXO70A1-mediated vesicle trafficking is critical for tracheary element development in *Arabidopsis*. Plant Cell 25, 1774–1786.
Li SP, van Os GMA, Ren SC, Yu DL, Ketelaar T, Emons AMC, Liu CM (2010). Expression and functional analyses of EXO70 genes in *Arabidopsis* implicate their roles in regulating cell type-specific exocytosis. Plant Physiol 154, 1819–1830.
Liu J, Guo W (2012). The exocyst complex in exocytosis and cell migration. Protoplasma 249, 587–597.
Liu J, Zuo X, Yue P, Guo W (2007). Phosphatidylinositol 4,5-bisphosphate mediates the targeting of the exocyst to the plasma membrane for exocytosis in mammalian cells. Mol Biol Cell 18, 4483–4492.
Martinet W, De Meyer GRY, Andries L, Herman AG, Kockx MM (2006). In situ detection of starvation-induced autophagy. J Histochem Cytochem 54, 85–96.
Miao Y, Jiang L (2007). Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. Nat Protoc 2, 2348–2353.
Miao Y, Li H, Shen J, Wang J, Jiang L (2011). QUASIMODO 3 (QUA3) is a putative homogalacturonan methyltransferase regulating cell wall biosynthesis in *Arabidopsis* suspension-cultured cells. J Exp Bot 62, 5063–5078.
Miao Y, Li KY, Li H, Yao X, Jiang L (2008). The vacuolar transport of aleurain-GFP and 2S albumin-GFP fusions is mediated by the same pre-vacuolar compartments in tobacco BY-2 and *Arabidopsis* suspension cultured cells. Plant J 56, 824–839.
Moore BA, Robinson HH, Xu ZH (2007). The crystal structure of mouse Exo70 reveals unique features of the mammalian exocyst. J Mol Biol 371, 410–421.
Morgera F, Sallah MR, Dubuke ML, Gandhi P, Brewer DN, Carr CM, Munson M (2012). Regulation of exocytosis by the exocyst subunit Sec6 and the SM protein Sec1. Mol Biol Cell 23, 337–346.
Moskalenko S, Tong C, Rosse C, Mirey G, Formstecher E, Daviet L, Camonis J, White MA (2003). Ral GTPases regulate exocyst assembly through dual subunit interactions. J Biol Chem 278, 51743–51748.
Munafo DB, Colombo MI (2001). A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 114, 3619–3629.
Munson M, Novick P (2006). The exocyst defrocked, a framework of rods revealed. Nat Struct Mol Biol 13, 577–581.
Pecenkova T, Hala M, Kulich I, Kocourkova D, Drdova E, Fendrych M, Toupalova H, Zarsky V (2011). The role for the exocyst complex subunits Exo70B2 and Exo70H1 in the plant-pathogen interaction. J Exp Bot 62, 2107–2116.
Pfeffer SR (2010). Unconventional secretion by autophagosome exocytosis. J Cell Biol 188, 451–452.
Segui-Simarro JM, Austin JR, White EA, Staehelin LA (2004). Electron tomographic analysis of somatic cell plate formation in meristematic cells of *Arabidopsis* preserved by high-pressure freezing. Plant Cell 16, 836–856.
Shah H, Gadella TWJ, van Erp H, Hecht V, de Vries SC (2001). Subcellular localization and oligomerization of the *Arabidopsis thaliana* somatic embryogenesis receptor kinase 1 protein. J Mol Biol 309, 641–655.
Shen J, Zeng Y, Zhuang X, Sun L, Yao X, Pimpl P, Jiang L (2013). Organelle pH in the *Arabidopsis* endomembrane system. Mol Plant 6, 1419–1437.
Sieben C, Mikosch M, Brandizzi F, Homann U (2008). Interaction of the K(+)-channel KAT1 with the coat protein complex II coat component Sec24 depends on a di-acidic endoplasmic reticulum export motif. Plant J 56, 997–1006.
Songer JA, Munson M (2009). Sec6p anchors the assembled exocyst complex at sites of secretion. Mol Biol Cell 20, 973–982.
Synek L, Schlager N, Elias M, Quentin M, Hauser MT, Zarsky V (2006). AtEXO70A1, a member of a family of putative exocyst subunits specifically expanded in land plants, is important for polar growth and plant development. Plant J 48, 54–72.
Sztul E, Lupashin V (2006). Role of tethering factors in secretory membrane traffic. Am J Physiol Cell Physiol 290, C11–C26.
TerBush DR, Maurice T, Roth D, Novick P (1996). The exocyst is a multiprotein complex required for exocytosis in *Saccharomyces cerevisiae*. EMBO J 15, 6483–6494.
Tse YC, Mo B, Hillmer S, Zhao M, Lo SW, Robinson DG, Jiang L (2004). Identification of multivesicular bodies as prevacuolar compartments in *Nicotiana tabacum* BY-2 cells. Plant Cell 16, 672–693.
Waadt R, Schmidt LK, Lohse M, Hashimoto K, Bock R, Kudla J (2008). Multicolor bimolecular fluorescence complementation reveals simultaneous formation of alternative CBL/CIPK complexes in planta. Plant J 56, 505–516.
Wang H, Tse YC, Law AHY, Sun SSM, Sun Y, Xu Z, Hillmer S, Robinson DG, Jiang L (2010a). Vacuolar sorting receptors (VSRs) and secretory carrier membrane proteins (SCAMPs) are essential for pollen tube growth. Plant J 61, 826–838.
Wang J, Ding Y, Wang J, Hillmer S, Miao Y, Lo SW, Wang X, Robinson DG, Jiang L (2010b). EXPO, an exocyst-positive organelle distinct from multivesicular endosomes and autophagosomes, mediates cytosol to cell wall exocytosis in *Arabidopsis* and tobacco cells. Plant Cell 22, 4009–4030.
Wang J, Li Y, Lo SW, Hillmer S, Sun SSM, Robinson DG, Jiang L (2007). Protein mobilization in germinating mung bean seeds involves vacuolar sorting receptors and multivesicular bodies. Plant Physiol 143, 1628–1639.
Wen TJ, Hochholdinger F, Sauer M, Bruce W, Schnable PS (2005). The *roothairless1* gene of maize encodes a homolog of *sec3*, which is involved in polar exocytosis. Plant Physiol 138, 1637–1643.
Yeaman C, Grindstaff KK, Nelson WJ (2004). Mechanism of recruiting Sec6/8 (exocyst) complex to the apical junctional complex during polarization of epithelial cells. J Cell Sci 117, 559–570.
Yu IM, Hughson FM (2010). Tethering factors as organizers of intracellular vesicular traffic. Annu Rev Cell Dev Biol 26, 137–156.
Zarsky V, Cvrckova F, Potocky M, Hala M (2009). Exocytosis and cell polarity in plants—exocyst and recycling domains. New Phytol 183, 255–272.
Zhang Y, Immink R, Liu CM, Emons AM, Ketelaar T (2013). The *Arabidopsis* exocyst subunit SEC3A is essential for embryo development and accumulates in transient puncta at the plasma membrane. New Phytol 199, 74–88.
Zhang Y, Liu C, Emons AMC, Ketelaar T (2010). The plant exocyst. J Integr Plant Biol 52, 138–146.
Zhang X, Orlando K, He B, Xi F, Zhang J, Zajac A, Guo W (2008). Membrane association and functional regulation of Sec3 by phospholipids and Cdc42. J Cell Biol 180, 145–158.
Zhang X, Wang PY, Gangar A, Zhang J, Brennwald P, TerBush D, Guo W (2005). Lethal giant larvae proteins interact with the exocyst complex and are involved in polarized exocytosis. J Cell Biol 170, 273–283.
Zhuang X, Wang H, Lam SK, Gao C, Wang X, Cai Y, Jiang L (2013). A BAR-domain protein SH3P2, which binds to phosphatidylinositol 3-phosphate and ATG8, regulates autophagosome formation in *Arabidopsis*. Plant Cell, http://dx.doi.org/10.1105/tpc.113.118307.