The Golgi-Localized Arabidopsis Endomembrane Protein12 Contains Both Endoplasmic Reticulum Export and Golgi Retention Signals at Its C Terminus

Caiji Gao, Christine K.Y. Yu, Song Qu, Melody Wan Yan San, Kwun Yee Li, Sze Wan Lo, and Liwen Jiang

School of Life Sciences, Centre for Cell and Developmental Biology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong, China

Endomembrane proteins (EMPs), belonging to the evolutionarily conserved transmembrane nine superfamily in yeast and mammalian cells, are characterized by the presence of a large luminal N terminus, nine transmembrane domains, and a short cytoplasmic tail. The Arabidopsis thaliana genome contains 12 EMP members (EMP1 to EMP12), but little is known about their protein subcellular localization and function. Here, we studied the subcellular localization and targeting mechanism of EMP12 in Arabidopsis and demonstrated that (1) both endogenous EMP12 (detected by EMP12 antibodies) and green fluorescent protein (GFP)-EMP12 fusion localized to the Golgi apparatus in transgenic Arabidopsis plants; (2) GFP fusion at the C terminus of EMP12 caused mislocalization of EMP12-GFP to reach post-Golgi compartments and vacuoles for degradation in Arabidopsis cells; (3) the EMP12 cytoplasmic tail contained dual sorting signals (i.e., an endoplasmic reticulum export motif and a Golgi retention signal that interacted with COPII and COPI subunits, respectively); and (4) the Golgi retention motif of EMP12 retained several post-Golgi membrane proteins within the Golgi apparatus in gain-of-function analysis. These sorting signals are highly conserved in all plant EMP isoforms and, thus, likely represent a general mechanism for EMP targeting in plant cells.

INTRODUCTION

In the endomembrane system of eukaryotic cells, secretory proteins start their journey from the endoplasmic reticulum (ER) prior to reaching the Golgi apparatus for further sorting to post-Golgi compartments, such as the trans-Golgi network (TGN) and prevacuolar compartment (PVC) (Barlowe et al., 1994). Protein traffic between the ER and Golgi apparatus is mediated by two distinct coated vesicles (i.e., coat protein complex II (COPII) and COPI), which mediate anterograde and retrograde transport, respectively (Cosson and Letourneur, 1997; Kuehn et al., 1998; Rabouille and Klumperman, 2005; Donohoe et al., 2007). Integral membrane proteins that traffic between the ER and Golgi usually contain specific sorting signals that are essential for selective packaging into COPII vesicles for ER export or into COPI vesicles for ER retrieval and Golgi retention (Barlowe, 2003; Beck et al., 2009).

In mammals and yeast, various ER export signals have been identified, including the diacidic motif (DXE) of vesicular stomatitis virus glycoprotein, the dihydrophobic (LL) motif of ERGIC-53, and the diaromatic motif (FF, YY) of 46-kD Endomembrane Protein Precursor (Emp46p) (Nishimura and Balch, 1997; Nufer et al., 2002; Sato and Nakano, 2002). All of these typical ER export signals are found to reside on the cytosolic regions of transmembrane proteins that interact with COPII vesicles (Barlowe, 2003). Similarly, the dilysine motif, KXXX, is one of the best known sorting signals required for retrograde Golgi-to-ER transport of several type I membrane proteins that interact with COPII vesicles (Cosson and Letourneur, 1994; Schröder et al., 1995; Harter and Wieland, 1998; Gomez et al., 2000). Recently, the semiconserved Phe-Leu-Ser-like motifs were identified as Golgi retention signals in the cytoplasmic tails (CTs) of glycosyltransferases, a group of Golgi-resident integral membrane proteins in yeast (Tu et al., 2008). These motifs were shown to interact with COPI vesicles via Vps74p to maintain the steady state Golgi localization of the glycosyltransferases, thus providing the best known molecular mechanism of Golgi retention of membrane proteins in eukaryotic cells (Tu et al., 2008).

Several studies have been performed to analyze the targeting mechanisms of integral membrane proteins in plant cells. The transmembrane domain (TMD) and CT of binding protein 80 kD (BP-80), a type I integral membrane protein belonging to the vacuolar sorting receptor (VSR) family of proteins (Paris et al., 1997; Li et al., 2002), were shown to be essential and sufficient for its correct targeting to the PVC, which has been identified as a multivesicular body (MVB) in plant cells (Jiang and Rogers, 1998; Tse et al., 2004, 2006; Robinson et al., 2008). In addition, the length of the TMD may affect the subcellular localization of an integral membrane protein, as green fluorescent protein (GFP) fusions with different TMD sequences of 17, 20, or 23 amino acids in length showed distinct subcellular localization to the ER, Golgi, and plasma membrane (PM), respectively (Brandizzi et al., 2002). Similarly, the TMD length and cytosolic...
tail have been shown to be important for proper Golgi localization of glycosyltransferases, a group of type II integral membrane proteins in plant cells (Saint-Jore-Dupas et al., 2006). Similar to yeast and mammalian membrane proteins, various ER export signals, such as the diacidic DXE motif and dibasic motif, have been identified in different classes of transmembrane proteins in plant cells (Hanton et al., 2005; Yuasa et al., 2005; Mikosch et al., 2006; Schoberer et al., 2009; Zelazny et al., 2009). In addition, the dixysine motif present in the Arabidopsis thaliana p24 (At p24) homolog has also been shown to bind with the COP1 subunit to maintain the ER localization of p24 (Contreras et al., 2004b; Langhans et al., 2008). More recently, the rice (Oryza sativa) SECRETORY CARRIER MEMBRANE PROTEIN1 (SCAMP1), a polytopic PM protein with four TMDs (Lam et al., 2007a, 2007b, 2008; Law et al., 2012), was shown to reach the PM via an ER-Golgi-TGN-PM pathway in tobacco (Nicotiana tabacum) Bright Yellow-2 cells (Cai et al., 2011). Interestingly, both its cytosolic sequences and TMDs regulated the proper trafficking steps from ER to PM owing to the presence of various signals within the SCAMP1, including the ER export signal in the cytosolic N terminus, the Golgi export signal within the TMD2-TMD3, and the TGN-PM targeting signal in TMD1 (Cai et al., 2011). However, the underlying mechanisms remain elusive.

Endomembrane proteins (EMPs) belong to a family of integral membrane proteins with nine TMDs, a large luminal N terminus, and a short (10 to 17 amino acids) C terminus located in the cytosol. Three EMP members are found in Dictyostelium discoideum (Phg1A to Phg1C) (Cornillon et al., 2000) and Saccharomyces cerevisiae (Tmn1 to Tmn3) (Froquet et al., 2008) and four members in human (TM9SF1 to TM9SF4) (Lozupone et al., 2009). Loss of Phg1A function in slime mold D. discoideum led to a defect in cellular adhesion and inefficient phagocytosis (Cornillon et al., 2000); similarly, Phg1b played a synergistic but not redundant role in controlling cellular adhesion and phagocytosis (Benghezal et al., 2003). EMps in yeast were also found to be required for cell adhesion and filamentous growth under nitrogen starvation (Froquet et al., 2008). Interestingly, the human EMP protein, TM9SF4, was highly expressed in human malignant melanoma cells from metastatic lesions but was undetectable in healthy human tissues and cells, thus serving as a marker for malignancy (Lozupone et al., 2009). In addition, studies on the subcellular localization of EMps in yeast and human cells yielded different conclusions. For example, myc-tagged human EMP p76 was found predominantly in the endosomes (Schimmöller et al., 1998), and an N-terminal GFP-tagged human EMP (GFP-TM9SF4) was also shown to colocalize with the Rab5 protein in the early endosome (Lozupone et al., 2009). By contrast, yeast EMP Yer113c was shown to localize to the Golgi apparatus (Huh et al., 2003), whereas a C-terminal GFP-tagged yeast EMP (TMN2-GFP) was recently shown to localize to the endosome and vacuole (Aguilar et al., 2010).

The Arabidopsis genome contains 12 EMP isoforms (termed EMP1 to EMP12 in this study), but little is known about their protein subcellular localization and function in plants. As a first step to study the biology of EMPs in plants, we performed studies on the subcellular localization and targeting mechanisms of EMP12 in Arabidopsis. We demonstrated that both endogenous EMP12 and GFP-EMP12 fusion (as detected by EMP12 and GFP antibodies, respectively) were localized to the Golgi apparatus in wild-type and transgenic Arabidopsis plants and in transiently expressed Arabidopsis protoplasts. We further showed that the cytosolic C terminus of EMP12 contained both ER export and Golgi retention signals that interact with COP11 and COPI subunits, respectively. In addition, this Golgi retention signal of EMP12 trapped several post-Golgi membrane proteins within the Golgi apparatus in a gain-of-function analysis using transiently expressed Arabidopsis protoplasts. It seems that these targeting features of EMP12 are conserved among the plant EMps for their correct targeting to the Golgi apparatus in plant cells.

RESULTS

EMP12 Is a Golgi-Localized Protein with Multiple TMDs

All 12 isoforms of Arabidopsis EMP proteins (termed EMP1 to EMP12 in this study; see Supplemental Figure 1 online) share high similarity at the amino acid level and in predicted topology. All At EMps are predicted to have a large luminal N-terminal domain, followed by nine TMDs and a short CT (Figure 1A). Eleven At EMP members (excluding EMP1) are predicted to have a signal peptide (SP) in their N terminus. As a first step to understanding the biology of EMps in plants, we analyzed the subcellular localization and trafficking route of EMP12 in Arabidopsis.

We first used a GFP fusion approach by making a GFP-EMP12 construct in which a SP-GFP was used to replace the SP of EMP12 (Figure 1B). To test if this new fusion maintains the identical topology as EMP12, we performed a protease protection assay using microsomes isolated from Arabidopsis cells expressing the GFP-EMP12 fusion, followed by immunoblot analysis using GFP antibody (Figure 1C). The N terminus of GFP-EMP12 was found to be resistant to protease digestion because the 47-kD GFP-TMD1 fragment remained intact in the presence of trypsin, whereas the 75-kD full-length GFP-EMP12 fusion did not (Figure 1C, lanes 1 and 2), indicating the luminal location of the GFP tag as predicted (Figure 1A). The reliability of such a protease protection assay was further verified by an identical experiment using a GFP fusion of the type I integral membrane protein GFP-VSR2 as a control, in which the luminal GFP remained intact upon protease digestion (Figure 1C, lanes 4 and 5; Cai et al., 2011).

To study the subcellular localization of EMP12 and GFP-EMP12, we next generated EMP12 antibodies and transgenic Arabidopsis plants expressing GFP-EMP12 for immunoblot analysis. As shown in Figure 2A, GFP antibody detected a single protein band around 75 kD, likely representing GFP-EMP12 in the cell membrane (CM) fraction but not the cell-soluble (CS) fraction of the transgenic Arabidopsis seedlings expressing GFP-EMP12, whereas no such protein band was detected in wild-type plants. By contrast, anti-EMP12 antibodies detected the putative 50-kD endogenous EMP protein band in the CM fraction of both wild-type and GFP-EMP12 plants (Figure 2A, lanes 6 and 8, asterisk) as well as the GFP-EMP12 fusion (lane 8,
A subcellular fractionation study using a continuous sucrose gradient and wild-type Arabidopsis cells showed that anti-EMP12 had an identical distribution pattern as the cis-Golgi marker anti-Man1 (Tse et al., 2004; Lam et al., 2007b), thus indicating a possible Golgi localization of EMP12 (Figure 2B). To further prove this, confocal immunofluorescence labeling was next performed in root cells of transgenic Arabidopsis plants expressing GFP-EMP12. As shown in Figure 2C, the punctate GFP signals of GFP-EMP12 were fully colocalized with anti-EMP12. In addition, anti-EMP12 was found to fully colocalize with the cis-Golgi marker yellow fluorescent protein (YFP)-SYP32 but was largely separated from the PVC marker YFP-ARA7 (Geldner et al., 2009) in these transgenic Arabidopsis root cells (Figure 2C), indicating that both endogenous EMP12 and GFP-EMP12 fusion are localized to the Golgi apparatus in Arabidopsis cells. Thus, GFP-EMP12 can be used to study the subcellular localization and trafficking of EMP12 in plant cells.

To further confirm the Golgi localization of EMP12 and GFP-EMP12 proteins from the above confocal immunofluorescence study, we next performed immunogold electron microscopy (EM) using either EMP12 or GFP antibodies on ultrathin sections prepared from high-pressure frozen/freeze-substituted root tips of wild-type or transgenic GFP-EMP12 Arabidopsis plants. As shown in Figure 3, in wild-type Arabidopsis root cells, anti-EMP12 labeled the Golgi apparatus in which the majority of the gold particles (>80%) were found in the cis- and medial-Golgi (Figure 3A). Similar patterns of Golgi localization were also obtained in GFP-EMP12–transformed Arabidopsis root cells using anti-GFP antibodies (Figure 3B). In addition, Golgi labeling with EMP12 or GFP antibodies was specific, as little labeling was seen in other organelles, including TGN, MVB, and mitochondria in these experiments (Figure 3, Table 1). Taken together, these confocal immunofluorescence and immunogold EM studies demonstrated that EMP12 and GFP-EMP12 localized to the Golgi apparatus with preferential localization to the cis- and medial-Golgi in Arabidopsis cells.

We next wanted to test if the established transient expression system of Arabidopsis protoplasts (Miao and Jiang, 2007) could be used to study the trafficking of GFP-EMP12. Consistent with the above immunofluorescence and immunogold EM studies in transgenic Arabidopsis root cells (Figures 2 and 3), GFP-EMP12 was found to be fully colocalized with the cis-Golgi marker.
Man1-mRFP (for monomeric red fluorescent protein; Tse et al., 2004; Lam et al., 2007b) when they were transiently coexpressed together in Arabidopsis protoplasts (Figure 4A). By contrast, GFP-EMP12 was largely separated from the TGN marker mRFP-SYP61 and the PVC marker mRFP-VSR2 in Arabidopsis protoplasts (Figures 4B and 4C). In addition, when the ER-to-Golgi traffic was blocked by overexpression of Sec12p, a Sar1p-specific guanosine nucleotide exchange factor for COPII vesicle formation (Pimpl et al., 2003; Cai et al., 2011), both GFP-EMP12 and Man1-mRFP were trapped and colocalized within the ER (Figure 4D), indicating that GFP-EMP12 reached the Golgi via the COPII-dependent ER-to-Golgi sorting route, a result consistent with the Golgi localization of Man1-mRFP (Langhans et al., 2008).

The Cytosolically Exposed C-Terminal Region Contains Essential ER Export Signals for the Trafficking of EMP12 from the ER to the Golgi

Since GFP-EMP12 is fully colocalized with the endogenous EMP12 (as detected by anti-EMP12) to the Golgi apparatus in Arabidopsis cells (Figure 2) or with the Golgi marker in Arabidopsis protoplasts (Figure 4), we thus used Arabidopsis protoplasts to study the targeting of GFP-EMP12. Since little is known about the Golgi targeting of EMPs with nine TMDs, we first tested whether the large luminal N terminus or the short cytosolic C terminus are essential for Golgi localization of GFP-EMP12 by generating two truncated constructs, GFP-EMP12 (∆N) and GFP-EMP12(∆C), in which either the luminal N terminus

Figure 2. Golgi Localization of Endogenous EMP12 and GFP-EMP12 Fusion in Arabidopsis.
(A) Characterization of EMP12 antibody. Proteins were isolated from wild-type (WT) or transgenic Arabidopsis plants expressing GFP-EMP12, followed by SDS-PAGE and immunoblot analysis using GFP or EMP12 antibodies. Both anti-GFP and anti-EMP12 recognized the full-length GFP-EMP12 fusion (lanes 4 and 8), whereas anti-EMP12 also detected the endogenous EMP12 protein in both wild-type and transgenic Arabidopsis plants (lanes 6 and 8). Arrowhead and asterisk indicate GFP-EMP12 fusion and endogenous EMP12, respectively.
(B) Subcellular fractionation analysis of endogenous EMP12. The microsomes prepared from wild-type Arabidopsis (At) cells were fractionated by ultracentrifugation on a continuous (25 to 50%) Suc gradient. Proteins extracted from individual fractions were probed with anti-EMP12, anti-Man1 (Golgi marker), and anti-VSR (PVC marker) as indicated.
(C) Golgi localization of EMP12 and GFP-EMP12 fusion in transgenic Arabidopsis plants. Confocal immunofluorescence labeling showing full colocalization of anti-EMP12 with GFP-EMP12 (panel 1) or Golgi marker YFP-SYP32 (panel 2) in root cells of transgenic Arabidopsis plants expressing these two XFP fusions but distinct localization from the PVC marker YFP-ARA7 in root cells of a transgenic Arabidopsis plant (panel 3). The insets represent a three times enlargement of a randomly selected area. DIC, differential interference contrast. Bars = 10 μm.
or the cytosolic CT was deleted, respectively. As shown in Supplemental Figure 2 online, upon transient expression in *Arabidopsis* protoplasts, GFP-EMP12(DN) remained in the Golgi as it colocalized with the Golgi marker, indicating that the N terminus of EMP12 was not essential for its Golgi localization. By contrast, CT deletion resulted in ER localization of EMP12 because GFP-EMP12(DC) fully colocalized with the ER marker Calnexin-mRFP upon their coexpression, indicating the possible existence of an ER export signal at the CT of EMP12.

So far, various ER export signals have been identified at the cytoplasmic part of integral membrane proteins. Best known examples are the diacidic motif DXE within the vesicular stomatitis virus glycoprotein cytosolic tail, the dihydrophobic motifs (FF, YY, LL, or FY) found in the p24 family proteins, and the Tyro-based signals identified in the tail sequence of ERGIC53/Emp47p family proteins (Nishimura and Balch, 1997; Nufer et al., 2002; Sato and Nakano, 2002; Langhans et al., 2008). Alignment analysis of CTs of *Arabidopsis* EMPs indicates the existence of a putative dihydrophobic motif and Tyr residue in all At EMPs (see Supplemental Figure 1 online). To find out if the putative dihydrophobic motif and Tyr residue in the EMP12 CT (SNLFVRRIYRNKCD) are important for the ER export of EMP12,
we performed mutagenesis analysis on the dihydrophobic or Tyr motifs of GFP-EMP12 CT (Figure 5A). Mutations of dihydrophobic combined with Tyr motifs totally block the ER export of GFP-EMP12, which was fully colocalized with the ER marker Calnexin-mRFP (Figure 5B). Interestingly, the solo Tyr mutation only partially inhibited the ER export of GFP-EMP12 (Figures 5C and 5D). However, the combined mutations of Tyr with one hydrophobic amino acid, Phe or Val, effectively blocked the ER export of GFP-EMP12 (Figures 6A and 6B), indicating that the dihydrophobic residue synergistically combined with Tyr to drive the ER export of EMP12. This conclusion was further confirmed by double mutation of the dihydrophobic motif, Phe and Val, that only partially blocked the ER export of EMP12 as they colocalized with both Golgi and ER markers (Figures 6C and 6D); however, single mutation on one hydrophobic amino acid, Phe or Val, had no effect on the ER export of EMP12 (Figures 5E and 6E). Mutations of other amino acids besides the dihydrophobic or Tyr residues did not affect the ER export of GFP-EMP12 (see Supplemental Figure 3 online). Taken together, these results support the conclusion that both the hydrophobic motif and Tyr residue in the cytosolic tail play essential roles in the ER export of EMP12.

Transmembrane cargos usually possess a binding affinity with Sar1–Sec23–Sec24 prebudding complexes through their ER export signals in order to be included in COPII vesicles for ER export (Bi et al., 2002; Barlowe, 2003). We next wanted to find out if COPII subunits interact with the ER export motifs in the EMP12 CT using in vitro peptide binding experiments (Contreras et al., 2004b). The soluble fractions isolated from Arabidopsis protoplasts expressing YFP-Sec24 were used as a source of COPII subunits (Hanton et al., 2009) for binding with columns conjugated with synthetic peptides of EMP12 CT or its mutation on the dihydrophobic/Tyr residues. As shown in Figure 7, the expressed YFP-Sec24 was retained in the column with the EMP12 CT peptides (lane 3), but such binding was dramatically reduced in the mutated column (lane 4), indicating that the dihydrophobic/Tyr residues of EMP12 CT preferably bound to the COPII Sec24 subunit.

Figure 4. Golgi Localization of GFP-EMP12 in Arabidopsis Protoplasts.

GFP-EMP12 was coexpressed with various known organelle markers as indicated ([A] to [C]) in Arabidopsis protoplasts, followed by confocal imaging at 12 to 14 h after transfection, whereas overexpression of Sec12p was used to inhibit the ER export of GFP-EMP12 and Man1-mRFP (D). Bars = 10 μm.

The C-Terminal-Fused GFP Tag Causes Mislocalization of the EMP12-GFP Fusion to the TGN, PVC, and Vacuole

Interestingly, when GFP was fused at the C terminus of EMP12 (Figure 8A), the resulting EMP12-GFP was found to localize to
both the vacuole and punctate structures distinct from the Golgi marker Man1-mRFP (Figure 8B), but partially colocalized with the TGN marker mRFP-SYP61 and the PVC marker mRFP-VSR2 (Figures 8C and 8D) in Arabidopsis protoplasts, indicating that GFP fusion at the C terminus of EMP12 caused it to move from the Golgi to the vacuole via TGN/PVC. In addition, EMP12-GFP also passed through the ER-Golgi sorting route via the COPII vesicle en route to the vacuole because overexpression of Sec12p trapped it within the ER (Figure 8E), indicating that the C-terminal GFP fusion did not affect the ER-to-Golgi transport but caused mislocalization of EMP12-GFP to post-Golgi compartments, including TGN, PVC, and the vacuole.

**The EMP12 CT Contains a Novel KXD/E Motif for Golgi Retention**

To further find out if the EMP12 CT is essential for its Golgi retention, we used a gain-of-function approach and added an additional EMP12 CT sequence to the C terminus of EMP12-GFP (Figure 9A). Indeed, the resulting EMP12-GFP-CT was

---

**Figure 5.** The EMP12 C Terminus Contained Multiple ER Export Signals.

(A) The GFP-EMP12 C terminus mutation constructs and their subcellular localization in Arabidopsis protoplasts. CT, C terminus; NT, N terminus; T, TMD; WT, the wild type.

(B) to (E) Typical subcellular localization patterns of four selective GFP-EMP12 C terminus mutation fusions upon their coexpression with known markers (as indicated) in Arabidopsis protoplasts. Confocal images were collected from cells at 12 to 14 h after transfection. Bars = 10 μm.
found to remain in the Golgi as it colocalized with the Golgi marker Man1-mRFP but largely separated from the TGN and PVC markers upon their transient coexpression in Arabidopsis protoplasts (see Supplemental Figure 4 online). Further mutagenesis (deletion) analysis of the EMP12 CT demonstrated that the last six amino acids RNIKCD (EMP12-GFP-RNIKCD; Figure 9B) or even the last three residues KCD (EMP12-GFP-KCD; see Supplemental Figure 4 online) were sufficient to retain the EMP12-GFP within the Golgi or efficiently retrieve EMP12-GFP back to the Golgi apparatus.

Interestingly, sequence alignment analysis of the CTs of EMP homologs from Arabidopsis, rice, and maize (Zea mays) showed the presence of a highly conserved Lys in the –3 position and an acidic residue (Asp or Glu) in the –1 position (see Supplemental Figure 1D online). To test if this conserved Lys-based motif KXD/E was essential for Golgi retention, we performed point mutation analysis on this motif via substitution with Ala residues. Double point mutations on Lys and Asp (EMP12-GFP-RNIACA; see Supplemental Figure 5 online) or single mutation on Lys (EMP12-GFP-RNIACD; Figure 9C) or Asp (EMP12-GFP-RNIKCA; Figure 9D) all resulted in mislocalization of these GFP fusions to vacuole and post-Golgi PVC organelles that were largely distinct from the Golgi apparatus (see Supplemental Figures 5A to 5G online). Thus, it seems that both K and D/E in the KXD/E motif are essential for Golgi localization or retention of EMP12. In addition, the KXD/E motif must be presented at the C-terminal end to function as a Golgi retention signal, as the addition of three Ala residues at the C terminus of the Golgi-localized

Figure 6. Subcellular Localization of GFP-EMP12 Harboring Mutations on the ER Export Signals.

These various GFP fusions were coexpressed with known markers (as indicated) in Arabidopsis protoplasts. Confocal images were collected from cells at 12 to 14 h after transfection. Bars = 10 μm.
Soluble proteins extracted from Arabidopsis protoplasts expressing YFP-Sec24 were incubated with Sepharose beads conjugated with synthetic peptides corresponding to the wild type (WT; lane 3) or mutated (mut; lane 4) C terminus of EMP12 for a pull-down assay, followed by immunoblot detection of bound proteins using a GFP antibody. M, molecular weight marker in kilodaltons.

EMP12-GFP-RNIKCD caused its mislocalization to the vacuole and PVC (Figure 9E; see Supplemental Figures 5H and 5I online). The PVC-mediated degradation of these mislocalized EMP12-GFP fusions was further proved by coexpression with a constitutively active Rab5 mutant, ARA7(Q69L), which can induce PVC enlargement and trap the degradative cargo proteins within its lumen (Kotzer et al., 2004). Indeed, both EMP12-GFP and EMP12-GFP-RNIKCDAAA occurred as large round dots within the lumen of mRFP-ARA7(Q69L)-labeled ring-like structures, distinct from the punctate patterns of Golgi-localized GFP-EMP12 and EMP12-GFP-RNIKCD (see Supplemental Figure 6 online).

The detection of GFP signals in the vacuole in cells expressing GFP-tagged transmembrane proteins usually indicates the occurrence of protein degradation (Kleine-Vehn et al., 2008; Langhans et al., 2008; Foresti et al., 2010; Cai et al., 2012). To provide further evidence that these various EMP12-GFP-RNIKCD fusion mutations were degraded in vacuoles upon their expression in Arabidopsis protoplasts (e.g., Figure 9), we performed immunoblot analysis using GFP antibodies. As shown in Figure 10, upon expression in Arabidopsis protoplasts, the Golgi-localized EMP12-GFP-RNIKCD largely (>90%) remained as the full-length protein in the CM fraction, while <10% of the signal was detected as the GFP core (Foresti et al., 2010) in the CS fraction. By contrast, in cells expressing the three other constructs with a mutation on the K2010) in the CS fraction. By contrast, in cells expressing the three other constructs with a mutation on the K2010) in the CM fraction, while >50% (50 to 70%) of the detected signals were found as the GFP core in the CS fraction (Figure 8C), indicating that their turnover rate is much higher than that of EMP12-GFP-RNIKCD. Quantification analysis of these GFP signals in the CM versus CS fractions of individual proteins further confirmed this conclusion (Figure 10C). Thus, both confocal data (Figure 9) and immunoblot analysis (Figure 10) point to the same conclusion that the C-terminal KXD/E motif functions as a Golgi retention signal for EMP12.

The KXD/E Golgi Retention Motif Can Interact with COPI Vesicles

To further explore the molecular mechanism of KXD/E motif function in Golgi retention, we next used the synthetic peptides corresponding to the EMP12 CT as bait to isolate possible interacting proteins from wild-type Arabidopsis proteins in pull-down experiments, followed by protein elution, separation via SDS-PAGE, and tandem mass spectrometry (MS/MS) analysis for protein identification. In duplicate experiments, several proteins that were specifically pulled out by the EMP12 CT peptide column were identified as the COPI coat subunits (see Supplemental Figure 7 online). The COPI vesicles are the predominant means by which the membrane proteins recycle back to the ER or are retained in the Golgi (Cosson and Letourneur, 1994; Tu et al., 2008). We thus further tested whether COPI coatomers could bind with the KXD/E motif using in vitro peptide binding experiments as described previously (Contreras et al., 2004b). The soluble fraction isolated from Arabidopsis protoplasts expressing Sec21-Myc, an Arabidopsis γ-COP protein (Movafeghi et al., 1999), was used as a source of COPI coat protein in the binding experiment using the synthetic peptide of EMP12 CT or its mutants of the KXD/E motif as binding baits. As shown in Figure 11, Sec21-Myc specifically binds to the EMP12 CT as detected by anti-Myc antibody (Figure 11, lane 3), but such binding was completely abolished when the KXD motif in the CT was mutated to either ACA or ACD (Figure 11, lanes 4 and 5), while the binding ability was reduced significantly for the KCA peptide (Figure 11, lane 6), indicating that the Lys in the KXD motif played a more critical role in COPI binding. These binding results are consistent with those obtained with confocal subcellular localization and immunoblot analysis of EMP12-GFP-RNIKCD (Figures 9 and 10). Therefore, binding with the COPI coatomer was a means by which the KXD/E motif achieved the Golgi retention function.

RNKCD Functions as a Golgi Retention Motif for Post-Golgi Membrane Proteins

To find out if the Golgi retention signal RNIKCD identified in the EMP12 CT would have a similar function in other membrane proteins, we next used a gain-of-function approach by adding this motif to the C terminus of GFP fusions of two selective post-Golgi membrane proteins: SCAMP1-GFP and TPK1-GFP (Figure 12). GFP fusions with the SCAMP1 (SCAMP1-GFP or GFP-SCAMP1), an integral membrane protein with four TMDs, were localized to both the TGN and PM via an ER-Golgi-TGN-PM pathway (Lam et al., 2007b; Cai et al., 2011). Indeed, the original TGN/PM localization of SCAMP1-GFP was altered by the addition of the RNIKCD motif to the C terminus of SCAMP1-GFP because SCAMP1-GFP-RNIKCD was largely colocalized with the Golgi marker Man1-mRFP (Figure 12A, panels 1 and 2) in Arabidopsis protoplasts. A similar result was obtained when the RNIKCD motif was fused to the C terminus of GFP-SCAMP1 because the resulting GFP-SCAMP1-RNIKCD fusion was also found to be trapped in the Golgi, colocalizing with the Golgi marker Man1-mRFP (Figure 12A, panel 3), indicating that the RNIKCD motif showed similar Golgi retention function even without direct fusion with GFP. The Arabidopsis two-pore potassium channel (TPK1) protein is a tonoplast-localized potassium channel with four TMDs and a cytoplasmic N terminus and C terminus (Gobert et al., 2007; Latz et al., 2007; Dunkel et al., 2008; Isayenkova et al., 2011). As
expected, TPK1-GFP showed a typical tonoplast localization pattern that was distinct from the Golgi marker Man1-mRFP in Arabidopsis protoplasts (Figure 12B). However, when RNIKCD was added to its C terminus, the resulting TPK1-GFP-RNIKCD was found to localize to both Golgi (colocalized with the Golgi marker Man1-mRFP) and tonoplast (Figure 12B), indicating that RNIKCD partially trapped the tonoplast-localized TPK1-GFP within the Golgi. Such dual localization of TPK1-GFP-RNIKCD in the Golgi and tonoplast may be caused by the competition between the KXD/E Golgi retention signal and the tonoplast targeting signals found in TPK1 (Dunkel et al., 2008; Isayenkov et al., 2011). Thus, it will be interesting to investigate the combinatorial effect of these distinct targeting signals in determining targeting of these proteins. Taken together, the C-terminal addition of RNIKCD caused Golgi retention of two post-Golgi membrane proteins, SCAMP1-GFP and TPK1-GFP, which were originally localized in the TGN/PM and tonoplast, respectively. In addition, since most of these targeting studies were performed using the Arabidopsis protoplast transient expression system, it will be of interest to generate transgenic tobacco Bright Yellow-2 or Arabidopsis cell lines and transgenic Arabidopsis plants expressing some of the key fusion constructs (e.g., EMP12-GFP-CT, SCAMP1-GFP-RNIKCD, and TPK1-GFP-RNIKCD) for use in future studies.

**DISCUSSION**

**The Position of the GFP Tag Affects the Proper Golgi Localization of EMP12**

Reporter fusion proteins have been a useful tool for studying the targeting and subcellular localization of integral membrane proteins in plant cells. Because of the complex nature of membrane protein topology, care must be taken when

![Figure 8](image-url)
a reporter (e.g., GFP) is fused to an integral membrane protein to ensure that the fusion protein’s topology remains identical and that the targeting signals are not affected. For example, a SP-GFP was used to replace the N terminus of BP-80, a type I integral membrane protein belonging to the VSR family, to generate the GFP-BP-80 reporter that was colocalized with the endogenous VSRs to PVC/MVB (Tse et al., 2004), demonstrating the correct targeting of GFP-BP-80 and various GFP-VSR fusions in plant cells (Jiang and Rogers, 1998; Tse et al., 2004; Miao et al., 2006). In this study, we adopted a similar approach and used SP-GFP to replace the N terminus of EMP12, whereas the generated GFP-EMP12 fusion was shown to colocalize with the endogenous EMP12 proteins to the Golgi apparatus in transgenic Arabidopsis plants and protoplasts (Figures 2 to 4). Similar Golgi localization was shown for GFP fusions to three other EMPs (EMP2, EMP3, and EMP8) in Arabidopsis protoplasts (see Supplemental Figure 8 online). Such Golgi localization of EMPs in Arabidopsis is consistent with previous results obtained from an organelle proteome study using Arabidopsis culture cells (Dunkley et al., 2006). By contrast, N-terminal GFP fusion with the human EMP protein (i.e., GFP-TM9SF4) was shown to localize to early endosomes in mammalian cells (Lozupone et al., 2009). Such variation in EMP localization (i.e., Golgi versus endosome localization in plant versus mammalian cells) suggests that EMPs evolved distinct functions in different organisms.

Figure 9. The C Terminus of EMP12 Contained a Golgi Retention Signal.

(A) EMP12-GFP-CT mutation constructs and their subcellular localization in Arabidopsis protoplasts. CT, C terminus; NT, N terminus; T, TMD.
(B) to (E) Subcellular localization patterns of four selective EMP12-GFP-CT mutation fusions with or without Golgi retention signals at their CT upon their coexpression with Golgi or PVC marker in Arabidopsis protoplasts. Confocal images were collected from cells at 12 to 14 h after transfection. Bars = 10 μm.
Interestingly, C-terminal GFP fusion to EMP12 caused mis-targeting of EMP12-GFP to post-Golgi compartments and, ultimately, degradation in vacuoles (Figures 8 and 13); a trafficking pathway previously demonstrated to be mediated by PVCs (Tse et al., 2004; Miao et al., 2008). Indeed, EMP12-GFP was found to internalize into the ARA7(Q69L)-induced enlarged PVCs (Kotzer et al., 2004) prior to reaching the vacuole. The mislocalization of EMP12-GFP is likely caused by masking of the KCD Golgi retention signal within the short CT of EMP12 because C-terminal fusions of both GFP (Figure 8) and short triple peptide sequences (AAA) (Figure 9) resulted in its mislocalization to the post-Golgi compartment. Such mislocalization upon C-terminal fusion was also true for other tested EMP members, including EMP2, EMP3, and EMP8 (see Supplemental Figure 8 online), indicating a general targeting mechanism for the EMPs in Arabidopsis plants. These results support the idea that the vacuole rather than the PM is the default destination for missorted or misfolded integral membrane proteins in plant cells (Höfte and Chrispeels, 1992). In addition, our study might explain the different subcellular localizations of two EMPs in yeast: While the localization of Yer113c to the Golgi (Huh et al., 2003) is consistent with that of plant EMPs, the localization of a C-terminal GFP-tagged yeast EMP (TMN2-GFP) to the endosome and vacuole (Aguilar et al., 2010), rather than the Golgi, was likely caused by the C-terminal GFP fusion masking the Golgi retention signal as described in this study. It would thus be interesting to find out if GFP-TMN2 would localize to the Golgi apparatus in yeast in future experiments.

Multiple Sorting Signals and Proper COPII Vesicle Function Are Involved in ER Export of EMP12

In the secretory pathway of eukaryotic cells, soluble and membrane cargo proteins are packed into ER-derived COPII vesicles for Golgi and post-Golgi transport. The COPII coat consists of

Figure 10. Immunoblot Analysis of Protoplasts Expressing the Four EMP12-GFP Fusions with the Golgi Retention Motif or its Mutation.

(A) Arabidopsis protoplasts expressing the four EMP12-GFP fusions (as described in Figures 7B to 7E) were subjected to protein isolation into CS and CM fractions, followed by protein separation via SDS-PAGE and protein gel blot analysis using GFP antibody. Arrowhead and arrow indicated the full-length EMP12-GFP fusion and its degradative GFP core, respectively. M, molecular weight marker in kilodaltons.

(B) Coomassie blue-stained gel was used as control for showing equal protein loading.

(C) The relative grayscale intensity (scale 0 to 1.0) of the two protein bands in the CM (full-length fusion) and in the CS (GFP core) fractions of individual EMP12-GFP fusions was quantified using ImageJ software, in which the combinational intensity of CM plus CS was expressed as 1.0 (or 100%).

Figure 11. The Cytoplasmic C Terminus of EMP12 Binds to COPI Subunit in a Sequence-Specific Manner.

Soluble proteins extracted from Arabidopsis protoplasts expressing Sec21-Myc were incubated with Sepharose beads conjugated with synthetic peptides corresponding to the wild-type (WT) C terminus of EMP12 (lane 3) or its various mutations (lanes 4 to 6; K587A/D589A, K587A, and D589A) for a pull-down assay, followed by immunoblot detection of bound proteins using anti-Myc antibodies. Blank Sepharose beads without peptide conjugation were used as a control. M, molecular weight marker in kilodaltons.
Figure 12. The Golgi Retention Motif of EMP12, RNKCD, Retained Post-Golgi Membrane Proteins within the Golgi.

(A) C-terminal fusion of RNKCD to SCAMP1-GFP or GFP-SCAMP1 caused their relocalization from their original PM/TGN (panel 1) to the Golgi apparatus (panels 2 and 3). Bars = 10 μm.

(B) C-terminal fusion of RNKCD to the tonoplast-localized TPK1-GFP (panel 4) partially trapped TPK1-GFP within the Golgi (panel 5). Bars = 10 μm.
Sar1, Sec23-Sec24, and Sec13-Sec31 complexes that are sequentially recruited to the ER membrane (Barlowe, 2003). Transmembrane cargo proteins usually contain ER export signals in their cytoplasmic region that interact with the COPII coat complex. Typical ER export signals identified in membrane cargo proteins in yeast, mammalian, and plant cells include the diacidic motif (D\(\times\)E), dihydrophobic motif (LL), diaromatic motif (FF, YY), dibasic motif (RR, RK), and Tyr-based motif Y\(\times\)X\(\times\)F (where \(\times\) is any amino acid, and \(\Phi\) is a bulky hydrophobic group) (Sato and Nakano, 2002; Barlowe, 2003; Hanton et al., 2005).

In this study, using both loss-of-function and gain-of-function approaches in transiently transformed *Arabidopsis* protoplasts, we also identified the dihydrophobic and Tyr-based motif, FV/Y, at the short cytosolic C terminus of EMP12 to be an ER export signal for EMPs in plants. FV and Y in EMP12 may function synergically as the ER export signals to facilitate its ER export and Golgi localization, as triple mutations of FV/Y resulted in complete ER localization, whereas double mutations of FV or a single mutation of Y resulted in dual ER and Golgi localization. Furthermore, an in vitro peptide binding assay demonstrated that triple mutations of FV/Y in the C terminus of EMP12 caused a dramatic reduction in the original interaction between the COPII coat protein Sec24 and EMP12 CT. Since the FV/Y motif is highly conserved in the C terminus of all plant EMPs, including *Arabidopsis*, rice, and maize, this motif may represent a general mechanism that regulates the ER export of EMPs in plants. In addition, when all EMP12 fusions and their mutants were expressed alone, they showed identical patterns as those coexpressed with other markers (see Supplemental Figure 9 online), thus verifying that coexpression with the organelle markers did not affect the localization of EMP12 fusions and its mutants in this study.

**The KXD/E Motif and COPI Vesicle Mediated Golgi Localization of EMP12**

In eukaryotic cells, the best-known signal-mediated Golgi retention mechanism for membrane proteins is related to COPI function. In yeast, the Golgi-localized glycosyltransferases contain the semiconserved motif (F/L)-(L/V)-(S/T) that directly interacts with the Golgi resident protein Vps74p (Schmitz et al., 2008; Tu et al., 2008). Vps74 was shown to interact with multiple subunits of the COPI coat, and Vps74 thus likely served as an adapter in mediating cargo packaging into the retrograde COPI

---

**Figure 13. Working Model of EMP12 Trafficking in Plant Cells.**

(A) Structure and topology of EMP12, GFP-EMP12, and EMP12-GFP fusions. Both EMP12 and GFP-EMP12 reached Golgi via ER. The ER export motifs FV/Y of EMP12 interacted with the COPII subunit for its transport via COPII vesicles to the Golgi apparatus for residency because of the presence of the Golgi retention motif KXD and its interaction with the COPI subunit.

(B) Pathways and signals. EMP12-GFP reached the Golgi via the ER. Since its Golgi retention motif KXD was masked by C-terminal GFP fusion, EMP12-GFP reached the vacuole via the TGN and PVC.

[See online article for color version of this figure.]
vesicles for retrograde transport in maintaining the steady state Golgi localization of glycosyltransferases (Tu et al., 2008). Similarly, the localization of Emp46p family proteins to the Golgi in yeast was achieved via direct interaction of their dilysine signals with the COPI coat (Sato and Nakano, 2002). Indeed, the dilysine motifs (KKXX or KXXXX), which are usually located in the cytoplasmic C terminus of membrane proteins, have been shown to bind with the COPI subunit, especially the γ-subunit of coatomer, in yeast, mammalian, and plant cells (Cosson and Letourneur, 1994; Harter and Wieland, 1998; Contreras et al., 2004a).

In this study, we demonstrated that the KXD/E motif, which occurs within the last six amino acids of the C terminus of EMP12, functioned as a Golgi retention signal that regulates the Golgi localization of EMP12 via its interaction with the COPI vesicle. This conclusion is supported by several lines of experimental evidence. First, mutation of the KCD residues caused mistargeting of EMP12. Second, blockade of this cytosolic signal by C-terminal GFP or triple peptide AAA fusion resulted in mislocalization of the fusion protein. Third, this motif interacted specifically with the COPI subunit. Last, when attached to other post-Golgi membrane proteins, the motif functioned as a Golgi retention signal that retained these proteins largely within the Golgi. Similar to the ER export signal, this KXD/E Golgi retention motif is also highly conserved within the cytosolic C terminus of all plant EMPs, indicating a general mechanism of Golgi retention for EMPs in plants. Thus, this KXD/E motif represents a newly identified Golgi retention signal for multiple TMD proteins in eukaryotes.

Interestingly, although the KXD/E motif in EMPs is different from the dilysine motif KKXX or KXXXX identified in single TMD proteins, the Lys in the −3 position of these motifs is highly conserved, indicating the COPI binding ability of the KXD/E motif because the Lys in the −3 position of KKXX or KXXXX has been shown to play a more vital role in COPI binding (Harter and Wieland, 1998; Hardt and Bause, 2002). Indeed, results from the in vitro peptide binding assay and confocal study demonstrated that the Lys in the −3 position of the KXD/E motif played a more critical role in COPI binding and Golgi retention of EMP12, whereas the last acidic residue played a minor role, indicating that the Lys-based motif can also function in Golgi retention of multiple TMD proteins in plant cells.

Both p24 (Contreras et al., 2004b) and EMP (this study) are shown to interact with the COPI vesicle. However, p24 is recycled back to the ER (Langhans et al., 2008), whereas EMP is trapped in the Golgi, as demonstrated by both confocal immunofluorescence and immunogold EM in this study, in which both endogenous EMP12 and the GFP-EMP12 fusion were found to localize mainly to the medial- and cis-Golgi cisternae. A previous EM tomography study (Donohoe et al., 2007) suggests the existence of distinct COPI populations in plant cells: The COPIa vesicles that bud exclusively from cis-Golgi cisternae may mediate the recycling transport to the ER, whereas the COPIb vesicles that bud exclusively from medial- and trans-Golgi cisternae may mediate the retrograde transport of Golgi resident proteins (Donohoe et al., 2007). Since EMP12 proteins show mainly medial- and cis-Golgi localization in this study, we are not sure if EMP12 would achieve its Golgi localization by interacting with COPIb vesicles in Arabidopsis cells. It would therefore be interesting to test the possible distinct functions of KXXX and KXD/E motifs in Golgi-ER recycling (for p24) or Golgi retention (for EMP12) by swapping the two COPI interaction motifs of p24 and EMP for subsequent dynamics and subcellular localization studies.

METHODS

Plasmid Construction

The full-length cDNA of EMP12 was amplified and cloned into the pBI212 backbone for construction of the GFP fusion and generation of transgenic plants using floral dip (Clough and Bent, 1998). All EMP12 mutant constructs used for transient expression in protoplasts were PCR amplified and cloned into the pBl221 backbone containing the cauliflower mosaic virus 35S promoter, GFP coding sequence, and the nopaline synthase terminator (Miao et al., 2008). The SP sequence for making the SP-GFP-EMP12–linked constructs was derived from barley (Hordeum vulgare) proaleurain (Jiang and Rogers, 1998) or from the native signal sequence of EMP12 based on the SignalP 3.0 Server prediction (http://www.cbs.dtu.dk/services/SignalP/). The protein topology was predicted using TMHMM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM). The primers used to generate various EMP12 constructs are listed in Supplemental Table 1 online. All constructs were confirmed by restriction mapping and DNA sequencing.

Plant Materials and Transient Expression in Protoplasts

Procedures for generating transgenic Arabidopsis thaliana plants expressing XFP fusion proteins and maintaining Arabidopsis suspension cultured cells were described previously (Clough and Bent, 1998; Jiang and Rogers, 1998; Tse et al., 2004; H. Wang et al., 2010; J. Wang et al., 2010). Transient expression using protoplasts derived from Arabidopsis suspension culture cells was essentially performed according to our established protocol (Miao and Jiang, 2007; Lam et al., 2009; Wang and Jiang, 2011).

Immunofluorescence Study and Confocal Microscopy

Fixation and preparation of Arabidopsis roots for immunofluorescent labeling and confocal analysis were performed as described (Sauer et al., 2006). Fixed roots were incubated with anti-EMP12 antibody at 4 μg/mL overnight at 4°C for immunolabeling and were then probed with Alexa 568 goat anti-rabbit IgG (Invitrogen) secondary antibody for confocal observation. Expression of the fluorescent fusion proteins and observation of their subcellular localization in protoplasts were usually performed at 12 to 14 h after transient expression. For each experiment or construct, more than 50 individual cells were observed for confocal imaging that represented >80% of the cells showing similar expression levels and patterns. Images collected from 10 individual cells were used for the data process and analysis. All confocal images were captured using the Olympus FV1000 laser scanning confocal system (http://www.olympusconfocal.com/). The GFP signal was visualized with excitation at 488 nm and emission at 500 to 550 nm (using dual-channel observations to avoid possible crosstalk between two fluorophores. All images were prepared using Adobe Photoshop as described previously (Jiang and Rogers, 1998; Tse et al., 2004; H. Wang et al., 2010; J. Wang et al., 2010).

EM Study

The general procedures for transmission EM sample preparation, thin sectioning, and immunogold labeling were performed essentially as
In 0.1% uranyl acetate at followed by subsequent freeze substitution in dry acetone containing d-old wild-type and transgenic GFP-EMP12 (Shen et al., 2011; Wang et al., 2011; Wang et al., 2012). Root tips of 7-d-old Arabidopsis plants were cut and immediately frozen in a high-pressure freezer (EM PACT2; Leica), followed by subsequent freeze substitution in dry acetone containing 0.1% uranyl acetate at −85°C in an AFS freeze substitution unit (Leica). Infiltration with HM20, embedding, and UV polymerization were performed stepwise at −35°C. Immunogold labeling was performed with GFP or EMP12 antibodies at 40 μg/mL and gold-coupled secondary antibody at a 1:50 dilution. Transmission EM examination was done with a Hitachi H-7650 transmission electron microscope with a charge-coupled device camera (Hitachi High-Technologies) operating at 80 kV.

**Antibodies, Protein Preparation, and Immunoblot Analysis**

Synthetic peptide (GeneScript), CLRNDYAKYAREDD, corresponding to the cytosolic region between the TMD1 and TMD2 of EMP12 was conjugated with keyhole limpet hemocyanin and used to immunize rabbits at the Laboratory Animal Services Center of the Chinese University of Hong Kong. Antibodies were affinity purified using cyanogen bromide-activated Sepharose 4B column (Sigma-Aldrich) conjugated with the peptides. Myc antibody was purchased from Santa Cruz. Alexa 568 goat anti-rabbit IgG was purchased from Invitrogen.

For the subcellular fractionation study, microsomal membrane fraction was first isolated from the wild-type Arabidopsis suspension culture cells via fractionation on a 25 to 50% continuous Suc gradient according to the published procedure (Tse et al., 2004). These fractions were then probed with anti-VSR, anti-Man1, and anti-EMP12 antibodies. To prepare cell extracts from protoplasts, transformed protoplasts were first diluted threefold with 250 mM NaCl and then harvested by centrifugation at 100g for 10 min, followed by resuspension in lysis buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, and 25 μg/mL leupeptin. The protoplasts were further lysed by passing through a 1-ml syringe with needle and then spun at 600g for 3 min to remove intact cells and large cellular debris, the supernatant total cell extracts were then centrifuged at 100,000g for 30 min at 4°C; the supernatant and pellet were assigned as soluble and membrane fractions, respectively. Proteins were separated by SDS-PAGE and analyzed by immunoblotting. Quantification of the relative gray-scale intensity in immunoblot analysis was done with ImageJ software v1.45 according to the procedure (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/).

**In Vitro Binding Assay of COPI and COPII Coat Proteins to Sorting Motifs**

In vitro pulldown and peptide binding assay were performed according to previously published procedures (Contreras et al., 2004b; Suen et al., 2010). Synthetic peptides (GeneScript) corresponding to the C-terminal cytosolic tail of EMP12 were coupled, via their N-terminal NH₂ group, to cyanogen bromide-activated Sepharose 4B (3 mg peptides per mL of beads) according to the standard procedure. The coupling reaction was quenched by incubation with 1 M Gly, pH 8.0, at room temperature for 2 h. Peptide coupling efficiency was monitored by measuring the absorbance at 280 nm.

For the peptide binding assay, the soluble fractions, which were extracted from the wild-type protoplasts, the protoplasts transfected with the COPI coat-Myc fusion (Sec21-Myc), or the COPII coat-GFP fusion (Sec24-GFP), were diluted with 2× lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulphonyl fluoride, and 50 μg/mL leupeptin) and were then incubated with Sepharose beads coupled with the peptides for 4 h at 4°C in a top to end rotator. After incubation, the beads were washed five times with 1× lysis buffer and then eluted by boiling in reducing SDS sample buffer. Proteins were separated by SDS-PAGE and subjected to liquid chromatography-MS/MS analysis or immunoblotting using appropriate antibodies.

Sample preparation and MS were performed as previously described (Wu et al., 2009). Briefly, the gel was silver stained after SDS-PAGE separation, and the interesting protein bands were cut out for in-gel trypsin digestion. The peptides were extracted from the trypsin-digested gel for MS analysis with a matrix-assisted laser desorption ionization-time of flight/time of flight tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems). Mass data acquisitions were piloted by 4000 Series Explorer Software v3.0. The MS and MS/MS data were loaded into the GPS Explorer software v3.5 (Applied Biosystems) and searched against the National Center for Biotechnology Information nonredundant database (released on March 5, 2010) with species restriction to Arabidopsis by Mascot search engine v2.1 (Matrix Science).

**Topology Analysis and Protease Protection Assay**

Microsome isolation and protein topology analysis were performed as described with some modifications (Abas and Luschnig, 2010; Cai et al., 2011). Basically, the protoplasts expressing GFP-EMP12 or GFP-VSR2 were diluted with 250 mM NaCl and harvested by centrifugation. The harvested protoplasts were then lysed in the extraction buffer (40 mM HEPES-KOH, pH 7.5, 1 mM EDTA, 10 mM KCl, and 0.4 M Suc) by passing through a 1-ml syringe with a needle several times. After centrifugation at 600g for 3 min to remove intact cells and large cellular debris, the supernatants were further centrifuged at 100,000g for 30 min at 4°C. The pellet was assigned as the microsomal-enriched membranes and was resuspended in the extraction buffer to be divided into three parts with equal volume for protease protection assay with three distinct conditions: without trypsin, 100 μg/mL trypsin and 100 μg/mL trypsin with 1% Triton X-100. After incubation at 37°C for 30 min, the samples were pelleted for protein extraction and immunoblot analysis using GFP antibody.

**Accession Numbers**

The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are EMP2 (At5g25100), EMP3 (At2g24170), EMP8 (At1g14670), EMP12 (At1g10950), Sec21 (At4g34450), and Sec24 (At4g07100).

**Supplemental Data**

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

**Supplemental Figure 1.** Plant EMP Proteins.

**Supplemental Figure 2.** The C Terminus of EMP12 Was Essential for ER Export.

**Supplemental Figure 3.** Subcellular Localization of GFP-EMP12 with Triple Mutations at Its C Terminus.

**Supplemental Figure 4.** Subcellular Localization of EMP12-GFP with the Golgi Retention Motif Fused to the C-Terminal End.

**Supplemental Figure 5.** Subcellular Localization of EMP12-GFP with C-Terminal Fusion of Mutated Golgi Retention Motif.

**Supplemental Figure 6.** EMP12-GFP Reached the Vacuole via the PVC for Degradation.

**Supplemental Figure 7.** EMP12 C Terminus Interacted with COPI Subunits.

**Supplemental Figure 8.** Subcellular Localization of N-Terminal or C-Terminal GFP Fusions with EMP2, EMP3, and EMP8 in Arabidopsis Protoplasts.

**Supplemental Figure 9.** Localization Patterns of Singly Expressed Various EMP12 Fusions and Their Mutants in Arabidopsis Protoplasts.

**Supplemental Table 1.** Primers Used in This Study.
ACKNOWLEDGMENTS

We thank Akihiko Nakano and Takashi Ueda (University of Tokyo, Japan) for providing the cDNA encoding ARA7(Q69L). This work was supported by grants from the Research Grants Council of Hong Kong (CUHK466309, CUHK466610, CUHK466011, CUHK2/CRF/11G, and HKBU1/CRF/10) and Chinese University of Hong Kong Schemes A/B/C to L.J.

AUTHOR CONTRIBUTIONS

L.J. supervised the project. C.G. and L.J. designed the experiments. C.G., C.K.Y.Y., S.Q., M.W.Y.S., K.Y.L., and S.W.L. performed specific experiments and analyzed data. C.G. wrote the article draft, and L.J. revised the article.

Received January 19, 2012; revised March 12, 2012; accepted April 18, 2012; published May 8, 2012.

REFERENCES


The Golgi-Localized *Arabidopsis* Endomembrane Protein12 Contains Both Endoplasmic Reticulum Export and Golgi Retention Signals at Its C Terminus
Caiji Gao, Christine K.Y. Yu, Song Qu, Melody Wan Yan San, Kwun Yee Li, Sze Wan Lo and Liwen Jiang

*Plant Cell*; originally published online May 8, 2012; DOI 10.1105/tpc.112.096057

This information is current as of October 29, 2017

Supplemental Data /content/suppl/2012/04/24/tpc.112.096057.DC1.html

cTOCs Sign up for cTOCs at: http://www.plantcell.org/cgi/alerts/ctmain
CiteTrack Alerts Sign up for CiteTrack Alerts at: http://www.plantcell.org/cgi/alerts/ctmain
Subscription Information Subscription Information for *The Plant Cell* and *Plant Physiology* is available at: http://www.aspb.org/publications/subscriptions.cfm

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY