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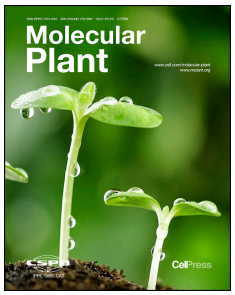

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N-linked glycosylation of the p24 family protein p24δ5 modulates retrograde Golgi-to-ER transport of K/HDEL ligands in *Arabidopsis*

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Running title: p24δ5 glycosylation and retrieval of ER proteins

Short summary:

Arabidopsis p24 protein p24δ5 is *N*-glycosylated in its GOLD domain. This post-translational modification is important for its coupled transport with p24β2 at the ER-Golgi interface, for its interaction with the K/HDEL receptor ERD2 and for retrograde transport of ERD2 and K/HDEL ligands from the Golgi apparatus back to the ER.

ABSTRACT

The K/HDEL receptor ERD2 mediates the transport of soluble endoplasmic reticulum (ER) resident proteins containing a C-terminal K/HDEL signal from the Golgi apparatus back to the ER via COPI (COat Protein I)-coated vesicles. Sorting of ERD2 within COPI vesicles is facilitated by p24 proteins. In particular, Arabidopsis p24δ5 has been shown to interact directly with ERD2 (via its luminal GOLD -GOLgi Dynamics- domain) and with COPI proteins (via its cytoplasmic C-terminal tail) at the acidic pH of the Golgi apparatus. Several members of the p24 family in mammals and yeast have been shown to be glycosylated, but there was no previous report on glycosylation of *Arabidopsis* p24 proteins and on the role of the sugar moiety in p24 function. Here we show that the *Arabidopsis* p24 protein p24δ5 is *N*-glycosylated in its GOLD domain. In addition, we show that this post-translational modification is important for its coupled transport with p24β2 at the ER-Golgi interface, for its interaction with the K/HDEL receptor ERD2 and for retrograde transport of ERD2 and K/HDEL ligands from the Golgi apparatus back to the ER.

Keywords: K/HDEL receptor, COP(Coat Protein) I, p24 proteins, N-glycosylation

INTRODUCTION

Retrieval of soluble endoplasmic reticulum (ER)-resident proteins (containing a C-terminal K/HDEL signal) from the Golgi apparatus occurs via their interaction with the K/HDEL receptor ERD2 (Lewis et al. 1990; Semenza et al. 1990). ERD2 proteins, bound to K/HDEL ligands are then packaged into COP(COat Protein)I vesicles for their retrograde transport back to the ER, where ligands are released. To facilitate this transport, receptor-ligand interactions are pH-dependent, being optimal at acidic pH and very low at neutral pH. According to this, the prevailing model assumes that K/HDEL ligands interact with their receptors at the acidic pH of the Golgi apparatus and are released at the neutral pH of the ER (Munro and Pelham, 1987; Lewis et al. 1990; Lewis and Pelham, 1992; Wilson et al. 1993; Scheel and Pelham, 1996; Dancourt and Barlowe, 2010).

p24 proteins are single-spanning type-I (N-terminal luminal) membrane proteins which cycle between the ER and the Golgi apparatus via COPI and COPII vesicles (for a recent review, see Pastor-Cantizano et al. 2016). Their luminal region contains a GOLD (GOLgi-Dynamics) domain, suggested to be important for their interaction with putative cargos (Ananthraman and Aravind, 2002), and a coiled-coil domain, which allows the oligomerization of p24 proteins (Figure 1A). Their single transmembrane domain is followed by a short cytosolic tail that contains sorting signals for binding COPI and COPII subunits, allowing their efficient sorting within COPI or COPII vesicles, respectively (Pastor-Cantizano et al. 2016). p24 proteins can be classified, by sequence homology, into four subfamilies (alpha, beta, gamma and delta) (Pastor-Cantizano et al. 2016). Arabidopsis p24 proteins from the delta subfamily have been shown recently to facilitate the incorporation of ERD2 proteins into COPI vesicles via a direct interaction with the K/HDEL receptor ERD2, which is also pH-dependent, being optimal at acidic pH and very low at neutral pH (Montesinos et al. 2014).

When newly synthesized glycoproteins enter the lumen of the ER, an oligosaccharyltransferase (OST) associated to the translocon catalyzes the transfer of an oligosaccharide precursor to specific asparagine residues which are part of a consensus site for *N*-linked glycosylation (Asn-X-Ser/Thr, where X can be any amino acid except proline) (Bañó-Polo et al. 2011). The subsequent

action of glycosyltransferases and glycosidases in the ER and the Golgi apparatus modify the *N*-linked sugars of glycoproteins. Plant *N*-linked glycans can be classified in high-mannose-type and complex-type (Strasser, 2016). The enzyme endoglycosidase H (EndoH) cleaves high-mannose and some hybrid *N*-linked glycosylation modifications, but not complex oligosaccharides from *N*-linked glycoproteins (Maley et al. 1989). While glycosylated proteins entering the *cis*-Golgi are sensitive to EndoH treatment, the acquisition of complex oligosaccharides only occurs in the *medial*- and *trans*-Golgi and renders EndoH resistant proteins. Protein glycosylation may contribute to their folding, stability, transport and/or interaction with other molecules; therefore, it might be important for their function (Moremen et al. 2012; Hebert et al. 2014; Strasser, 2016).

In the case of p24 proteins, there are conflicting results about their glycosylation pattern. p24α2 (a p24α subfamily member) has been shown to be glycosylated. However, it was found to be sensitive to EndoH treatment in HeLa cells (Fullerkrug et al. 1999), suggesting that it does not reach the *medial*- and *trans*-Golgi, but EndoH resistant in rat liver (Dominguez et al. 1998; Lavoie et al. 1999) and human embryo kidney (HEK) cells (Liu et al. 2015). Furthermore, p24γ3 (a p24γ subfamily member) has also been shown to be glycosylated, in contrast to p24γ4 (p24γ subfamily) and p24β1 (p24β subfamily) in HeLa cells (Fullerkrug et al. 1999). These are examples of the different glycosylation status found among p24 proteins, even for the same subfamily members. In the case of p24δ1 (p24δ subfamily), it was found to be glycosylated in chondrocytes (Osiecka-Iwan et al. 2014) but not in HeLa cells (Fullekrug et al. 1999). Remarkably, none of these reports investigated whether glycosylation of p24 proteins may have functional implications in mammalian cells. In yeast, a novel p24δ isoform, Rtr6, is the only p24 protein that has been found to be *N*-glycosylated, and this glycosylation was proposed to be important to modulate cargo specificity (Hirata et al. 2013).

To our knowledge, glycosylation of p24 proteins in plants has not yet been reported. In contrast to mammalian and yeast cells, plants contain only p24 proteins from the beta and delta subfamilies. In particular, *Arabidopsis* contain 9 members of the delta subfamily, which can be divided, attending to sequence

homology, into two different subclasses (p24δ-1 and p24δ-2), and 2 members of the beta subfamily (Chen et al. 2012; Montesinos et al. 2012; Pastor-Cantizano et al. 2016).

Sequence analysis of p24 proteins from the delta subfamily using the NetNGlyc Server (http://www.cbs.dtu.dk/services/NetNGlyc/) predicted that *Arabidopsis* p24 proteins from the delta-1 subclass (including p24δ3-6) contain in their GOLD domain a consensus sequence for *N*-linked glycosylation (Figure 1A), precisely located at the same position when their sequences are aligned, which is not present in Arabidopsis p24 proteins from the delta-2 subclass (including p24δ7-11) (Supplemental Figure S1). Therefore, we aimed to investigate whether Arabidopsis p24 proteins from the delta-1 subclass were indeed glycosylated and if this glycosylation may have functional implications, in particular with respect to their role in sorting ERD2 within COPI vesicles for retrieval of K/HDEL ligands from the Golgi apparatus back to the ER.

RESULTS

Arabidopsis p24δ5 is *N*-glycosylated *in vitro*

N-glycosylation of proteins at the ER-lumen depends on proper targeting to the ER-translocon mediated by the presence of signal sequences at the N-terminal end of the glycosylated protein (Whitley and Mingarro, 2014). Both the signal peptidase (SP) and OST enzymes are adjacent to the ER-translocon, SP to cleave off the signal sequence and OST to glycosylate the polypeptide (Johnson and van Waes, 1999). Thus we parsed p24δ5 sequence to test the presence of a signal sequence on SignalP predicting algorithm (Petersen et al. 2011). The algorithm predicted a cleavage site between Ala27 and Ile28 (Figure 1A). Furthermore, an *N*-linked glycosylation site was predicted at Asn86 (Figure 1A).

To investigate these post-translational modifications of *Arabidopsis* p24 proteins, we first used an *in vitro* translation cell-free assay in the presence of dog pancreas microsomes. In this system, the microsomes provide all of the membrane insertion and glycosylation components (i.e., the translocon complex, including the SP and the OST enzymes). *In vitro* translated [^{35}S]Met/Cys-labeled p24δ5/P2 fusions were analyzed by SDS-PAGE and autoradiography. The reporter P2 domain is the extramembrane C-terminal domain from the bacterial *leader peptidase* (Lep) that carries an *N*-glycosylation site extensively used to report membrane translocation (Figure 1A) (Martinez-Gil et al. 2011; Saurí et al. 2009; Peiró et al., 2014). In the absence of microsomes, protein translations rendered polypeptides containing full-length p24δ5 (including its native signal sequence) and the P2 domain (Figure 1B, lane 1). When translation experiments were performed in the presence of microsomes, a modest alteration in the electrophoretical mobility was observed (Figure 1B, lane 2). It should be noted that the combined effect of signal sequence cleavage plus modification of one glycosylation site would generate polypeptides with an expected molecular mass similar to the observed molecular mass of the chimera expressed in the absence of microsomes. The presence of the sugar moiety was confirmed by endoglycosidase H (EndoH) treatment, a highly specific enzyme that cleaves *N*-linked oligosaccharides (see above). After EndoH treatment the molecular mass of the protein chimera decreased by about 2.5 kDa (Figure 1B, lane 3), in good agreement with single-glycosylation (Martinez-Gil et al. 2010). To identify the glycosylation acceptor site modified in these *in vitro* experiments, native Asn86 was mutated to non-acceptor glutamine (N86Q). Translation of p24δ5(N86Q)/P2 mutant in the presence of microsomes yields lower molecular mass species, originated from signal sequence cleavage (Figure 1B, lane 5), as proved after EndoH treatment (Figure 1B, lane 6). These results also suggest that p24δ5/P2 fusions insert into biological membranes with its native type I orientation (i.e., N-terminal lumenal), since the glycosylation site present in the P2 domain was never modified.

To verify p24δ5 membrane topology, protein translations were treated with proteinase K (PK). Digestion with PK degrades membrane protein domains located exclusively towards the cytosol, while membrane-embedded or

microsome luminally exposed domains remain protected. PK treatment of p24δ5 samples translated in the presence of microsomes produced a slightly lower molecular weight band corresponding to the luminal and transmembrane regions of p24δ5 upon PK digestion of the C-terminus of the protein (approximately twelve residues, 205-216) (Figure 1C, compare lanes 3 and 4). Parallel experiments were performed with constructs were p24δ5 sequence was followed by the first 50 amino acids from P2 (p24/50P2) to accentuate electrophoretical mobility differences (Figure 1C, compare lanes 5 and 6). Altogether these *in vitro* experiments demonstrate that p24δ5 is glycosylated and properly oriented into microsomal membranes.

Arabidopsis p24δ5 is N-glycosylated *in vivo*

We next investigated further the glycosylation of *Arabidopsis* p24 proteins of the delta subfamily *in vivo*. As mentioned in the introduction, the N-linked glycosylation site of p24 proteins of the delta-1subclass is not present in the proteins of the delta-2 subclass (Supplemental Figure S1). As representatives of p24 proteins from the delta-1 and delta-2 subclasses, we selected p24δ5 and p24δ9, respectively, since we had generated specific antibodies against these two proteins and have already analyzed their trafficking properties in plants (Montesinos et al. 2012, 2013, 2014). To investigate putative glycosylation of endogenous p24 proteins we used tunicamycin (Tm), a chemical that prevents *N*-linked glycosylation (Ericson et al. 1977). Arabidopsis protoplasts were incubated in the absence or presence of tunicamycin and protein extracts from these protoplasts were analyzed by SDS-PAGE and Western blotting with antibodies against the N-terminus of p24δ5 or p24δ9 (Montesinos et al. 2012). As shown in Figure 2A, p24δ5 migrated faster upon tunicamycin treatment (~ 2,5 kDa), consistent with the *in vivo* modification of a single *N*-glycosylation site. In contrast, we could not detect a shift in the mobility of p24δ9. To analyze the impact of *N*-glycosylation in the trafficking of p24δ5, we used transient expression experiments in protoplasts of its RFP-tagged version, RFP-p24δ5 (Langhans et al. 2008) or the mutant version of this chimera where the critical Asn residue in position 86 was changed to Gln (N86Q), RFP-p24δ5(N86Q). We

first investigated the electrophoretic mobility of the RFP-p24δ5(N86Q) mutant compared to that of the wild-type version. Protein extracts were obtained from protoplasts expressing RFP-p24δ5 or the RFP-p24δ5(N86Q) mutant and used for pull-down experiments using an RFP-trap assay, as described previously (Montesinos et al. 2012, 2013, 2014). Pull-downs were analyzed by SDS-PAGE and Western blotting with RFP antibodies. As shown in Figure 2B, the RFP-p24δ5(N86Q) mutant migrated faster than wild-type RFP-p24δ5 (compare lanes 1 & 3), suggesting that transiently expressed RFP-p24δ5 is also *N*-glycosylated at Asn86, as endogenous p24δ5. We next performed tunicamycin treatment of Arabidopsis protoplasts upon transient expression of RFP-p24δ5 or RFP-p24δ9. As shown in Figure 2B, RFP-p24δ5 migrated faster upon tunicamycin treatment (compare lanes 1 & 2), which suggests proper *N*-glycosylation, as for endogenous p24δ5. In contrast, we could not detect a shift in the mobility of RFP-p24δ9 (Figure 2B, lanes 5 & 6). Pull-downs were next treated in the absence or presence of EndoH. As shown in Figure 2C, EndoH treatment produced a shift in the electrophoretic mobility of RFP-p24δ5 to a position identical to that of RFP-p24δ5 upon tunicamycin treatment or to that of the RFP-p24δ5(N86Q) mutant (compare lanes 2 & 3), suggesting that glycosylation of Asn86 is responsible of the change in electrophoretic mobility of RFP-p24δ5. Moreover, the mobility of the RFP-p24δ5(N86Q) mutant was not changed upon tunicamycin (Figure 2B, lanes 3-4) or EndoH treatment (Figure 2C, lanes 3-4), indicating that RFP-p24δ5 does not contain any other *N*-glycosylation site. In addition, the mobility of RFP-p24δ9 was not changed upon EndoH treatment (Figure 2C, lanes 5-6), as it happened upon tunicamycin treatment, which indicates that p24δ9 is not glycosylated.

p24δ5 glycosylation is required for its coupled transport with p24β2 at the ER-Golgi interface

We next investigated whether glycosylation of p24δ5 is important for its steady-state subcellular localization. To this end, we transiently expressed in tobacco mesophyll protoplasts wild-type RFP-p24δ5 or the RFP-p24δ5(N86Q) mutant, which cannot be glycosylated, and analyzed its steady-state-localization by

confocal laser scanning microscopy (CLSM). As shown in Figure 3 (A-C), the RFP-p24δ5(N86Q) mutant mostly localized to the ER network, as wild-type RFP-p24δ5. This suggests that the ER localization of p24δ5 at steady-state is not dependent on its *N*-linked glycosylation status.

We have shown previously that p24δ5 and p24β2 interact with each other at ER export sites for ER exit and coupled transport of both proteins to the Golgi apparatus (Montesinos et al. 2012). In addition, we have also shown that p24δ5 stabilizes p24β2, presumably by holding it at the ER-Golgi interface and preventing its transport to the vacuole, where p24β2 is degraded (Montesinos et al. 2013). As shown in Figure 3 (D-F), co-expression with YFP-p24β2 partially shifted the steady-state localization of RFP-p24δ5 from its typical ER pattern to punctate Golgi structures where both proteins extensively colocalized (Table 1), as described previously (Langhans et al. 2008, Montesinos et al. 2012). In those studies, we found that this effect required the coiled-coil domain in p24δ5 (Montesinos et al. 2012). We have now investigated the possible involvement of its GOLD domain and glycosylation of Asn86. As shown in Figure 3 (G-I) YFP-p24β2 did not cause any significant change in the steady-state localization of a RFP-p24δ5 deletion mutant lacking the GOLD domain, which localized almost exclusively to the ER (Figure 3, G-I). As shown in Table 1, there was a very significant decrease in the percentage of RFP-p24δ5(ΔGOLD) colocalizing with YFP-p24β2 when compared with RFP-p24δ5 (Table 1). The same happened with the RFP-p24δ5(N86Q) mutant, which contains the GOLD domain but cannot be glycosylated (Figure 3, J-L and Table 1). In addition, in the co-expression with both mutants, the fluorescence of YFP-p24β2 was much lower than that in the presence of RFP-p24δ5. A similar effect was previously observed when p24β2 was expressed alone or with a p24δ5 deletion mutant lacking the coiled-coil domain (Montesinos et al. 2012). To quantify the protein levels of YFP-p24β2 under these conditions, protein extracts were analyzed by SDS-PAGE and Western blotting. As shown in Figure 4, the levels of YFP-p24β2 were highly increased upon co-expression with RFP-p24δ5, as described previously (Montesinos et al. 2012). In contrast, the protein levels of YFP-p24β2 were lower upon co-expression with the RFP-p24δ(ΔGOLD) or the RFP-

p24δ5(N86Q) mutants, which is consistent with the images obtained by CLSM. To investigate whether this decrease in the protein levels of YFP-p24β2 in the presence of the RFP-p24δ5(N86Q) mutant might be caused by its transport to the vacuole, protoplasts were incubated in the presence of the cysteine proteinase inhibitor E-64 during protein expression. As shown in Figure 4, protein levels of YFP-p24β2 increased to levels very similar to those in the presence of RFP-p24δ5. These results suggest that *N*-glycosylation of p24δ5 at Asn86 is important to hold p24β2 at the ER-Golgi interface and to prevent its transport to the vacuole.

p24δ5 glycosylation is important for its interaction with the K/HDEL receptor ERD2

We have previously shown that p24δ5 interacts with the K/HDEL receptor ERD2a at slightly acidic pH but not at neutral pH, consistent with this interaction taking place at the Golgi apparatus but not at the ER (Montesinos et al. 2014). The interaction between p24δ5 and ERD2 was shown to require the presence of the luminal GOLD domain in p24δ5 (Montesinos et al. 2014). We have now investigated whether the interaction between both proteins is dependent upon p24δ5 glycosylation at Asn86, which as mentioned is located within its GOLD domain. To this end we performed pull-down experiments, using protein extracts from protoplasts after transient co-expression of RFP-p24δ5 or the RFP-p24δ5(N86Q) mutant and ERD2a-YFP and GFP- or RFP-trap assays, as described previously (Montesinos et al. 2014). As shown in Figure 5, RFP-p24δ5 interacted with ERD2a-YFP, while a deletion mutant of RFP-p24δ5 lacking the GOLD domain (RFP-p24δ5ΔGOLD) showed a strongly reduced ability to interact with ERD2a-YFP, as previously reported (Montesinos et al. 2014). Using the same type of experiments, we have now found that the interaction between the RFP-p24δ5(N86Q) mutant and ERD2a-YFP, was reduced by about a 50 % when compared with that of wild-type RFP-p24δ5 (Figure 5C). This suggests that p24δ5 glycosylation is important for its interaction with the K/HDEL receptor ERD2. In contrast, the RFP-p24δ5(N86Q) mutant bound COPI and COPII subunits with similar efficiency as RFP-p24δ5,

suggesting that glycosylation of p24δ5 at its luminal side does not influence binding of coat proteins by its cytosolic tail (Supplemental Figure 2).

p24δ5 glycosylation is required for shifting the steady-state distribution of the K/HDEL receptor ERD2 from the Golgi to the ER

We have previously shown that p24δ5 partially shifts the steady-state distribution of the K/HDEL receptor ERD2 from the Golgi back to the ER, an effect that required the GOLD domain from p24δ5 (Montesinos et al. 2014). We have now investigated whether this effect was p24δ5 glycosylation-dependent. To this end, we transiently co-expressed ERD2a-YFP with RFP-p24δ5 or the RFP-p24δ5(N86Q) mutant in tobacco mesophyll protoplasts and analyzed the steady-state localization of ERD2a-YFP by confocal laser scanning microscopy (Figure 6). In the absence of RFP-p24δ5, the steady-state localization of ERDa-YFP was mainly the Golgi apparatus, with only a partial ER localization (Figure 6A, quantified in Figure 6J). However, in the presence of RFP-p24δ5, there was a significant shift in the steady-state localization of ERD2a-YFP from the Golgi apparatus to the ER (Figure 6D, quantified in Figure 6J), as described previously (Montesinos et al. 2014). In contrast, co-expression with the RFP-p24δ5(N86Q) mutant did not produce a significant change in the localization of ERD2a-YFP, which mainly localized to the Golgi apparatus and partially to the ER (Figure 6G, quantified in Figure 6J), as in the absence of RFP-p24δ5 (Figure 6A). This suggests that p24δ5 glycosylation is required for shifting the steady-state distribution of the K/HDEL receptor ERD2 from the Golgi to the ER.

p24δ5 glycosylation is required for its inhibitory effect on secretion of HDEL ligands

We have previously shown that by facilitating the retrograde transport of ERD2 (bound to K/HDEL ligands) from the Golgi apparatus to the ER, p24δ5 inhibited the secretion of K/HDEL ligands, an effect that was dependent on p24δ5 GOLD domain (Montesinos et al. 2014). To investigate whether this function of p24δ5 was dependent on its *N*-linked glycosylation, we performed secretion assays in

tobacco mesophyll protoplasts upon transient co-expression of a HDEL ligand, GFP-HDEL, and RFP-p24δ5 or the RFP-p24δ5(N86Q) mutant, as described previously (Montesinos et al. 2014). GFP-HDEL mostly localizes to the ER at steady-state, but it can be partially secreted upon overexpression. Therefore, we monitored the secretion of this marker by analyzing its presence in the culture medium by SDS-PAGE and Western blotting with GFP antibodies (Figure 7A, left panels). Overexpression of GFP-HDEL also causes a partial secretion of the chaperone BiP, an endogenous HDEL ligand (Figure 7A, central panels). Co-expression of RFP-p24δ5 caused a significant inhibition of GFP-HDEL and BiP secretion. This effect required the GOLD domain in p24δ5, since a RFP-p24δ5 mutant lacking the GOLD domain (RFP-p24δ5ΔGOLD) was much less effective in inhibiting the secretion of HDEL ligands (GFP-HDEL or BiP), as described previously (Montesinos et al. 2014). Here we have found that the RFP-p24δ5(N86Q) mutant was also less effective in inhibiting the secretion of HDEL ligands (GFP-HDEL or BiP), very similar to the RFP-p24δ5ΔGOLD mutant (Figure 7A, left and central panels, quantified in Figure 7B), while control Sec-GFP remain unaffected (Figure 7A, right panels, quantified in Figure 7B). Very similar results were obtained when secretion of GFP-HDEL was monitored by quantification of GFP fluorescence in medium and protoplasts (Supplemental Figure 3). These results suggest that N-linked glycosylation of p24δ5 is essential for its function to inhibit secretion of HDEL ligands.

DISCUSSION

There are many reports indicating that p24 proteins are key players in the formation of COPI vesicles involved in retrograde Golgi-to-ER transport (for reviews see Popoff et al. 2001; Jackson, 2014; Pastor-Cantizano et al. 2016). According to the most accepted model, dimers of p24 proteins (probably involving members from the p24β and p24δ subfamilies) first interact with the GDP-bound form of ADP ribosylation factor 1 (ARF1-GDP). Nucleotide exchange factors (ARF-GEFs) then facilitate GDP/GTP exchange and thus ARF1 activation. ARF1-GTP dissociates from p24 dimers and inserts onto the Golgi membrane and both p24 proteins and ARF1-GTP interact with coatomer. Subsequently, coatomer polymerization leads to the formation of COPI vesicles (Popoff et al. 2001; Jackson, 2014; Pastor-Cantizano et al. 2016) (Figure 8). p24 proteins can also interact with the K/HDEL receptor ERD2, facilitating its sorting within COPI vesicles (Majoul et al. 1998; Majoul et al. 2001; Montesinos et al. 2014). ERD2 itself has been proposed to participate actively in COPI vesicle formation. Binding of K/HDEL ligands, which takes place at the slightly acidic pH of the *cis*-Golgi (Wilson et al. 1993; Scheel and Pelham, 1996), induces ERD2 oligomerization (Majoul et al. 20001) and facilitates its interaction with ARF1 and ARF-GAP (which is also part of the machinery involved in COPI vesicle formation) or between ARF1 and ARF-GAP (Aoe et al. 1997; Majoul et al. 2001). ERD2 can also interact with coatomer, thus contributing to the formation of COPI vesicles (Majoul et al. 2001)(Figure 8). In *Arabidopsis*, we have shown previously that two members of the p24δ subfamily, p24δ5 and p24δ9, interact with both ARF1-GDP and coatomer via a dilysine and a diaromatic motif located in their cytoplasmic C-terminal tail, which are present in all members of the *Arabidopsis* p24δ subfamily (Contreras et al. 2004) (Supplemental Figure S1). In addition, we have shown recently that *Arabidopsis* p24δ5 and p24δ9 interact with two different K/HDEL receptors, ERD2a and ERD2b (Montesinos et al. 2014). In the case of p24δ5, the interaction required its luminal GOLD domain and was optimal at acidic pH, consistent with this interaction taking place at the Golgi apparatus, as it is the case for the interaction between ERD2 and K/HDEL ligands. Acidic pH also favors the

interaction of p24δ5 with both ARF1 and coatomer (Montesinos et al. 2014). Therefore, pH-dependent interactions seem to be essential for the bidirectional transport of ERD2 and K/HDEL ligands between ER and Golgi.

In this manuscript, we have further investigated the molecular basis of the function of p24 proteins in efficient retrieval of K/HDEL ligands from the Golgi back to the ER, and analyzed the putative role in this process of *N*-linked glycosylation of *Arabidopsis* p24 proteins. We have found that p24δ5 (p24δ-1 subclass) is *N*-glycosylated in its GOLD domain (which is important for its interaction with ERD2). EndoH sensitivity indicates that p24δ5 is not modified by glycosylation enzymes of the *medial*- and *trans*-Golgi to acquire complex oligosaccharides. This would be consistent with its predominant localization at the *cis*-side of the Golgi apparatus, which we have previously shown by immunogold labeling with p24δ5 antibodies (Montesinos et al. 2012). In contrast, p24δ9 (p24δ-2 subclass) is not glycosylated. There is a previous report showing differential glycosylation of p24 proteins from the same subfamily. In particular, p24γ3 was shown to be glycosylated, in contrast to p24γ4 (Fullerkrug et al. 1999). Indeed, the γ-subfamily appears to be the most divergent within the p24 family, and it has been proposed that different p24γ proteins may be involved in cargo specificity. However, it is not clear whether the differential glycosylation status of Arabidopsis proteins from the p24δ subfamily may reflect functional differences between δ-1 and δ-2 subclasses.

We have found that *N*-linked glycosylation of p24δ5 does not change its steady-state ER localization, which was shown to be dependent on a dilysine and a diaromatic motif in its cytosolic tail that is involved in binding ARF1 and coatomer subunits (Contreras et al. 2004a; Montesinos et al. 2014). Indeed, p24δ5 glycosylation had no effect on binding of COPI and COPII subunits, which is consistent with our previous observation that p24δ5 deletion mutants lacking the GOLD domain were able to cycle between the ER and the Golgi apparatus (Montesinos et al., 2012). On the other hand, we have previously shown that p24δ5 and p24β2 exhibit coupled trafficking at the ER-Golgi interface (Montesinos et al. 2012). We have also shown that the coupled transport of both proteins required their coiled-coil domains (Montesinos et al.

2012). Recently, it has been proposed that the interaction between mammalian p24δ1 and p24β1 involves their GOLD domains (Nagae et al. 2016). In this report, we have found that the GOLD domain is indeed important for coupled transport of both proteins between the ER and the Golgi apparatus. In addition, mutation of the glycosylation sequence in p24δ5 mimicked the absence of the GOLD domain, which highlights the importance of N-glycosylation of p24δ5 for its coupled transport with p24β2 at the ER-Golgi interface.

We have previously shown that p24δ9, which is not glycosylated, can also bind to ERD2 (Montesinos et al. 2014). This would be consistent with the fact that a mutant version of p24δ5 lacking asparagine 86, which cannot be glycosylated, still bound to ERD2, although with lower efficiency (around 50 %). It is thus possible that he GOLD domain contains other determinants for the interaction with ERD2. Alternatively, binding of p24δ9 to ERD2 may be mediated by p24δ5. Indeed, we have proposed that hetero-oligomeric complexes of Arabidopsis p24 proteins may contain members from both p24δ-1 and p24δ-2 subclasses (Montesinos et al. 2013). In any case, it seems clear that glycosylation of p24δ5 (and perhaps of other p24 proteins from the δ-1 subclass) increases its ability to interact with ERD2 and therefore to facilitate its sorting within COPI vesicles and thus the retrieval of K/HDEL ligands *in vivo*, as shown by the inability of the RFP-p24δ5(N86Q) mutant to change the steady-state localization of ERD2 from the Golgi to the ER or to inhibit the secretion of HDEL ligands (including GFP-HDEL and BiP) (Figures 6 and 7).

Several reports demonstrated that glycosylation might affect protein-protein and receptor-ligand interactions (Yang et al., 1993; Huang and Tai, 1998; Wang et al., 2001; Zhang et al., 2001; Pang et al., 1999; Kamitani, 2001; van der Hoorn et al., 2005; Häweker et al., 2010). In a recent study, it was shown that *N*-glycosylation of the vacuolar sorting receptor AtVSR1 affects the binding affinity of AtVSR1 to cargo proteins and therefore vacuolar protein sorting, without affecting its targeting to the prevacuolar compartment (Shen et al. 2014). Here we show that *N*-glycosylation of p24δ5 may facilitate its ER-Golgi transport and increase its binding affinity towards the K/HDEL receptor ERD2, thus facilitating

retrieval of ER resident K/HDEL ligands, a central mechanism for ER-Golgi networking transport in plants.

METHODS

Plant material

Plants of Arabidopsis were grown in chambers under controlled conditions of temperature, 21ºC, and 16h/8h photoperiod with 16 hours of white, cold and fluorescent light (150 µE m^{-2} s^{-2}, Sylvania Standard F58W/133-T8).

In vitro translation cell-free assay

The full-length p24δ5 sequence was cloned in pGEM1 plasmid fused to the P2 domain of the *E. coli* leader peptidase as previously described (Martínez-Gil et al (2007) Virology 367:348-57).

PCR products adding T7 promoter at the 5' end and the appropriated reverse primer were transcribed and translated using the TNT T7 Quick for PCR DNA system (Promega) by adding 75 ng of DNA purified from PCR, 0.5 µL 35S-Met (5.5 µCi) (Perkin Elmer) and 0.25 µL of ER rough microsomes from dog pancreas (tRNA Probes, College Station, TX, USA). Samples were incubated for 90 min at 30 ℃.

Mutagenesis was done using the QuikChange kit (Agilent) following the manufacturer's protocol. DNA purification kits were from Thermo Fisher Scientific (Ulm, Germany). Oligonucleotides were purchased from Macrogen (Seoul, Korea). All the new constructs were confirmed by sequencing at Macrogen Europe Service (Amsterdam, The Netherlands).

Translation products were ultracentrifuged (100,000×g for 15 min) on a sucrose cushion, and analyzed by SDS–PAGE.

Deglycosylation assays were performed using EndoH enzyme (BioLabs®, Ref. P0702S) according to the manufacturer's protocol.

For the proteinase K protection assay, 2 µL of proteinase K (1 mg/mL) were added to the sample, and the digestion reaction was incubated for 15 min on ice. Before ultracentrifugation and SDS-PAGE analysis, the reactions were stopped by adding 1 mM phenylmethanesulfonylfluoride.

Transient gene expression and secretion assays

Arabidopsis protoplasts were isolated as described previously (Wu et al. 2009). Mesophyll protoplasts from N. tabacum var. SR1 leaf cells were isolated and transfected by electroporation as previously described (Foresti et al. 2006; Montesinos et al. 2012). For secretion experiments, we used the PEG transformation method, as described (Yoo et al. 2007).

The coding sequence of the RFP-p24δ5(N86Q) mutant was synthesized commercially *de novo* (Geneart - Life Technologies - Thermo Fisher Scientific). The Sec-GFP construct (Leucci et al. 2007) was kindly provided by Dr. G.P. Di Sansebastiano (University of Salento, Lecce, Italy). Other plasmids have been described previously: RFP–p24δ5, RFP–p24δ5(ΔGOLD) (Langhans et al., 2008; Montesinos et al., 2012); RFP–p24δ9 (Montesinos et al., 2013); GFP–HDEL (Nebenführ et al., 2000); ERD2a–YFP (Brandizzi et al., 2002).

Secretion assays were performed as previously described (Crofts et al., 1999). Briefly, protoplasts were pelleted by centrifugation (5 minutes at 800 rpm) and culture medium was collected and further centrifuged for 10 minutes at 100000 rpm and 4ºC to remove traces of protoplasts. Culture medium was concentrated 5x by methanol/chloroform precipitation of proteins. Protoplast proteins were extracted with homogenization buffer (0.3 M sucrose; 1 mM EDTA; 1 mM dithiothreitol DTT; 20 mM KCl; 20 mM HEPES pH 7.5) supplemented with 0.1% protease inhibitors (Sigma). Protoplasts and medium proteins were analyzed by SDS-PAGE and Western blotting. Secretion of GFP-HDEL, Sec-GFP or BiP was analyzed by Western blotting with antibodies against GFP (to detect GFP-HDEL and Sec-GFP) or BiP and calculated as the percentage of the amount of

these markers in the medium (extracellular) with respect to their amount in the protoplasts (intracellular), as described previously (Philipson et al., 2001). Alternatively, the amount of GFP in the culture medium and in protoplast fractions was analysed using a Victor X-3 plate Reader (ex. 485 nm – em. 535 nm) (Perkin Elmer, www.perkinelmer.com) and secretion was calculated as a percentage of the amount of GFP in the medium *versus* the amount in protoplasts.

Preparation of protein extracts, pull-down experiments and Western Blotting

Tobacco protoplasts were diluted 10-fold with W5 medium and sedimented by centrifugation. Pellets were resuspended in 1 mL homogenization buffer [50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 10 mM KCl, 1 mM DTT, 3 mM EDTA and a cocktail of plant protease inhibitors (Sigma)] and cells disrupted by sonication (3 x 5 sec). Homogenates were centrifuged for 10 min at 1200 g, the PNS was collected and incubated 30 min at 4^{o}C with 0.5 % Triton X-100. After a 5 min centrifugation at 16 000 g to remove detergent-insoluble material, PNS were used for SDS-PAGE and Western blotting or for pull-down experiments.

Pull-down experiments from tobacco protoplasts expressing RFP-tagged or YFP-tagged proteins were performed using RFP-Trap or GFP-Trap magnetic beads (Chromotek®), respectively, following the recommendations of the manufacturer, as described previously (Montesinos et al., 2013, 2014).

Protein extracts from protoplasts or medium fractions and pull-down experiments were analyzed by SDS-PAGE and western blotting using the SuperSignal West Pico chemiluminiscent Substrate (Pierce, Thermo Scientific). Western blots were analyzed using the ChemiDoc XRS+ imaging system (Bio-Rad, http://www.bio-rad.com/). Western blots in the linear range of detection were quantified using the Quantity One software (Bio-Rad Laboratories).

Antibodies against RFP and GFP were obtained from Clontech and Life Technologies, respectively. Antibodies against p24δ5 and p24δ9 (Montesinos et

al., 2012, 2013), Sec23 (Movafeghi et al., 1999) and α-COP (Harter et al., 1996) have been described previously.

Confocal microscopy

Confocal fluorescent images were collected using an Olympus FV1000 confocal microscope with 60x water lens. Fluorescence signals for GFP (488 nm/ 496-518 nm), YFP (514 nm/529-550 nm) and RFP (543 nm/593-636 nm) were detected. Sequential scanning was used to avoid any interference between fluorescence channels. Post-acquisition image processing was performed using the FV10-ASW 4.2 Viewer and ImageJ (v.1.45).

AUTHOR CONTRIBUTIONS

F.A., M.J.M. and I.M. conceived and designed the experiments. N.P-C., M.J.G-M, M.J.M., C.B. and F.A. performed the experiments. F.A., M.J.M. and I.M. wrote the paper.

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FIGURE LEGENDS

Figure 1. *Arabidopsis* p24δ5 protein is glycosylated and properly oriented into microsomal membranes. (A) Schematic representation of the p24δ5-derived proteins used in the *in vitro* translation cell-free assays. The p24δ5 is shown in green. Dark green highlights the signal sequence (SS) and the transmembrane region (TM). The GOLD and coiled-coil (CC) domains are also highlighted. The P2 domain from Lep fused *in frame* is shown in orange, with lighter orange highlighting the first 50 residues of P2. *N*-glycosylation sites are represented by Y-shaped symbols. An oligonucleotide (arrowed line) was designed to generate p24δ5/50P2 construct, which includes the first 50 residues from P2 domain fused at the C-terminus of p24δ5 sequence. **(B)** *In vitro* translation of p24δ5/P2 (lanes 1-3) and p24δ5(N86Q)/P2 (lanes 4-6) constructs in the presence (+) or absence (−) of rough microsomes (RM), followed by treatment with Endoglycosidase H (EndoH) as indicated. Radioactive molecular weight markers are shown on the left (Mw). **(C)** *In vitro* translation of wild-type p24δ5 (lanes 1, 3 and 4) and p24δ5/50P2 (lanes 2, 5 and 6) constructs in the presence (+) or absence (−) of RM, followed by Proteinase K (PK) treatment as indicated.

Figure 2. p24δ5 is N-glycosylated *in vivo*.

(A) Arabidopsis protoplasts obtained from wild-type plants (Col-0) were incubated for 16 h in the presence or absence of 100 µg/ml tunicamycin (Tm). Membrane protein extracts were analyzed by SDS-PAGE and Western blotting using antibodies agains Nt-p24δ5 (left panel) and Nt-p24δ9 (right panel). Notice the change in electrophoretic mobility of p24δ5, but not of p24δ9, upon Tm treatment (arrowheads). 15 µg protein was loaded in each lane (30 µg for Tm-treated samples). **(B)** RFP-p24δ5, RFP-p24δ5(N86Q) or RFP-p24δ9 were transiently expressed in tobacco mesophyll protoplasts incubated in the presence or absence of 100 µg/ml Tm, as indicated. RFP-p24δ5, RFP-p24δ5(N86Q) or RFP-p24δ9 were pulled-down using RFP-trap and analyzed by SDS-PAGE and Western blotting using an antibody against RFP. Notice the difference in electrophoretic mobility between RFP-p24δ5 and RFP-

p24δ5(N86Q) (lanes 1 and 3) and the change in mobility of RFP-p24δ5 (lanes 1-2), but not of RFP-p24δ5(N86Q) (lanes 3-4) or RFP-p24δ9 (lanes 5-6) upon Tm treatment. **(C)**. Protein extracts from tobacco mesophyll protoplast expressing RFP-p24δ5, RFP-p24δ5(N86Q) or RFP-p24δ9 were pulled-down using RFP-trap and treated with or without Endo H and analyzed by SDS-PAGE and Western blotting using an antibody against RFP. Notice the change in electrophoretic mobility of RFP-p24δ5 (lanes 1-2), but not of RFP-p24δ5(N86Q) (lanes 3-4) or RFP-p24δ9 (lanes 5-6), upon EndoH treatment, although a fraction of RFP-p24δ5 remained EndoH resistant.

Figure 3. p24δ5 glycosylation is required for its coupled transport with p24β2.

Transient gene expression in tobacco mesophyll protoplast. **(A-C)** RFP-p24δ5(N86Q) (B-C) shows the typical ER pattern, as RFP-p24δ5 (A). **(D-F)** Co-expression with YFP-p24β2 (D) partially changes the steady-state localization of RFP-p24δ5 (E) from its typical ER pattern to punctate Golgi structures where both proteins extensively colocalize (merged image in F)(see also Table 1). **(G-L)** Co-expression with YFP-p24β2 (G, J) did not produce a significant change in the steady state localization of RFP-p24δ5ΔGOLD (H) or RFP-p24δ5(N86Q) (K), which mostly localized at the ER (merged images in I and L) and their co-localization with YFP-p24β2 was significantly reduced compared with that of RFP-p24δ5 (see also Table 1). Scale bars: 5 μm.

Figure 4. Transient expression of YFP-p24β2 in the absence or presence of RFP-p24δ5 and mutant versions. (A) Tobacco mesophyll protoplasts were electroporated in the absence (–DNA) or the presence of 30 μg of plasmid DNAs corresponding to YFP-p24β2, RFP-p24δ5 and mutant versions, as indicated. At 20 h post-electroporation (in the absence or presence of 50 μM E-64), protoplasts were homogenized and post-nuclear supernatants analyzed by SDS-PAGE (10 % acrylamide) and western blot analysis with antibodies against GFP (to detect YFP-p24β2) (upper panel) or RFP (to detect p24δ5 and mutant versions)(lower panel). 15 μg protein was loaded in each lane. **(B)** The protein

levels of YFP-p24β2 in lanes 2-5 were quantified as described in Material and methods. Error bars represent SD of the mean from at least three independent experiments.

Figure 5. p24δ5 glycosylation is important for its interaction with the K/HDEL receptor ERD2. (A) Pull-down of ERD2a-YFP from a post-nuclear supernatant (PNS) of protoplasts expressing ERD2a-YFP and RFP-p24δ5 or mutant versions, using a GFP-trap at pH 6.0. **(B)** Pull-down of RFP-p24δ5 or mutant versions from a PNS of protoplasts expressing these proteins and ERD2a-YFP, using a RFP-trap at pH 6.0. Bound proteins in A and B were analyzed by SDS-PAGE and Western blotting with antibodies against RFP (to detect RFP-p24δ5 or mutant versions) or GFP (to detect ERD2a-YFP). Input: 5% of the PNS used for the pull-down assay. UB: unspecific binding (using blocked magnetic particles). PD: pull-down. **(C)** Quantification of the biochemical interactions in pull-down experiments. In experiments using the RFP-trap, the amount of GFP-labeled interacting protein (PD, pull-down) was normalized to the amount of RFP-labeled protein bound to the beads, although the latter was consistently similar in the different points of the same experiments (as it was the case with the inputs). The opposite was done when using the GFP-trap. When the interaction was monitored both using the RFP-trap and GFP-trap, we obtained the average between both values. Error bars represent SD of the mean from at least three independent experiments.

Figure 6. p24δ5 glycosylation is required for shifting the steady-state distribution of the K/HDEL receptor ERD2 from the Golgi to the ER.

Transient gene expression in tobacco mesophyll protoplasts. **(A)** ERD2a-YFP mainly localized to punctate Golgi structures, while RFP-p24δ5 **(B)** and RFP-p24δ5(N86Q) **(C)** localized exclusively to the ER. **(D-F**) RFP-p24δ5 caused a partial relocalization of ERD2a-YFP (D) to the ER (blue arrowheads), although ERD2a-YFP also showed a punctate localization (ER and Golgi localization) (marged image in F). **(G-I**)RFP-p24δ5(N86Q) had no effect on the localization of ERD2a-YFP (G), which localized exclusively to punctate Golgi structures (merged image in I). Images included in the panels show the most representative pattern found for each condition according to the quantification

shown in J. Scale bars = 5 µm. **(J)** Quantification of the localization of ERD2a-YFP when it is expressed alone and co-expressed with RFP-p24δ5 or RFP-p24δ5(N86Q). A significant number of protoplasts (from at least three independent experiments), showing comparable expression levels of ERD2a-YFP and RFP-p24δ5 or RFP-p24δ5(N86Q), were analyzed per condition, using identical laser output levels and imaging conditions. Number of protoplasts analyzed per condition: ERD2a-YFP (39); ERD2a-YFP + RFP-p24δ5 (76); ERD2a-YFP + RFP-p24δ5(N86Q) (111). The localization of ERD2a-YFP was assigned as Golgi (only punctate structures, without a significant colocalization with RFP-p24δ5 or RFP-p24δ5(N86Q)), ER (mostly reticular, colocalizing with RFP-p24δ5 or RFP-p24d5(N86Q)) or ER and Golgi (both punctate and reticular), as described previously (Montesinos et al. 2014) and calculated as a percentage. Error bars represent SE of the mean.

Figure 7. p24δ5 glycosylation is required for its inhibitory effect on secretion of HDEL ligands. (A) Tobacco mesophyll protoplasts were transfected with the indicated constructs and incubated for 20 h. Total proteins from protoplasts and culture medium (concentrated 5x by methanol/chloroform precipitation), were analyzed by SDS-PAGE and Western blotting with antibodies against GFP (to detect GFP-HDEL or Sec-GFP), RFP (to detect RFP-p2δ5 and mutant versions) or BiP. Notice the difference in molecular weight between wild-type RFP-p24δ5 and mutant versions. **(B)** Quantification from at least three independent experiments as the ones shown in panel (A), with duplicated samples. Secretion of GFP-HDEL, BiP or Sec-GFP (upper panels, “Medium”) under different conditions of co-expression was calculated as a percentage of the secretion of these markers when expressed alone.

Figure 8. p24δ5 facilitates retrograde Golgi-to-ER transport of K/HDEL ligands in Arabidopsis. p24δ5 (possibly as a dimer with p24β2) can recruit ARF1 to Golgi membranes and both p24δ5 and ARF1 interact with coatomer, leading to the formation of COPI vesicles. p24δ5 can also interact (via its luminal GOLD domain) with the K/HDEL receptor ERD2. Glycosylation of p24δ5 is important for its interaction with ERD2 and therefore for sorting of ERD2 within COPI vesicles and Golgi-to-ER transport of K/HDEL ligands.

Supplemental Figure S1. Alignment of Arabidopsis p24 proteins from the delta subfamily. A multiple alignment of the p24 protein delta subfamily sequences was constructed using T-Coffee. The putative signal sequences are shown in blue font. Arabidopsis p24 proteins from the δ-1 subclass (p24δ3-δ6) contain a putative glycosylation site (highlighted in red and underlined) which is not present in p24 proteins from the δ-2 subclass (p24δ7-δ11). Dilysine and diaromatic motifs in the C-terminal tail are shown in green and navy blue, respectively. The predicted transmembrane region is underlined.

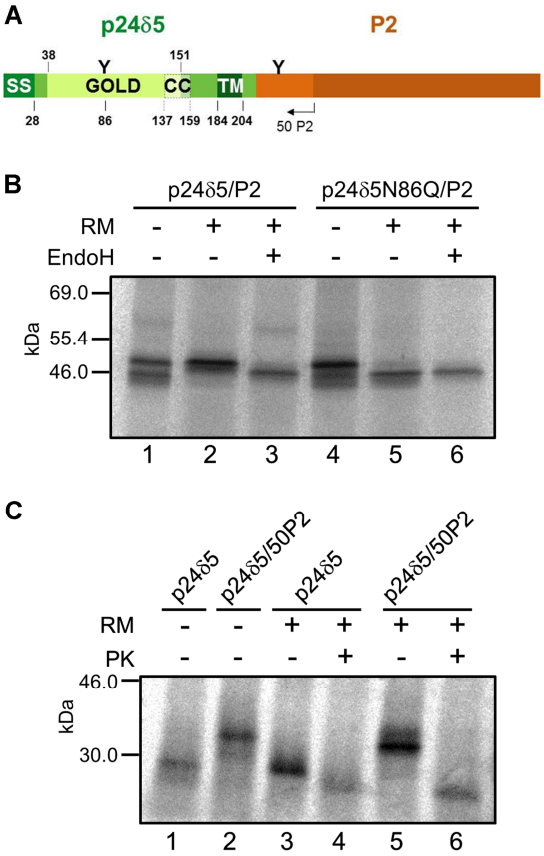
Supplemental Figure S2. p24δ5 glycosylation has no effect in binding of COPI/COPII subunits. Pull-down of RFP-p24δ5 or RFP-p24δ5(N86Q) from post-nuclear supernatant (PNS) of protoplasts expressing these proteins, using a RFP trap at pH 6.0 (to detect binding of COPI proteins) or at pH 7.5 (to detect binding od COPII subunits). Bound proteins were analyzed by SDS-PAGE and Western blotting with antibodies against the COPI subunit α-COP or the COPII subunit Sec23. Input, 5% of the PNS used for the pull-down assay. UB, unspecific binding (using blocked magnetic particles). PD, pull-down.

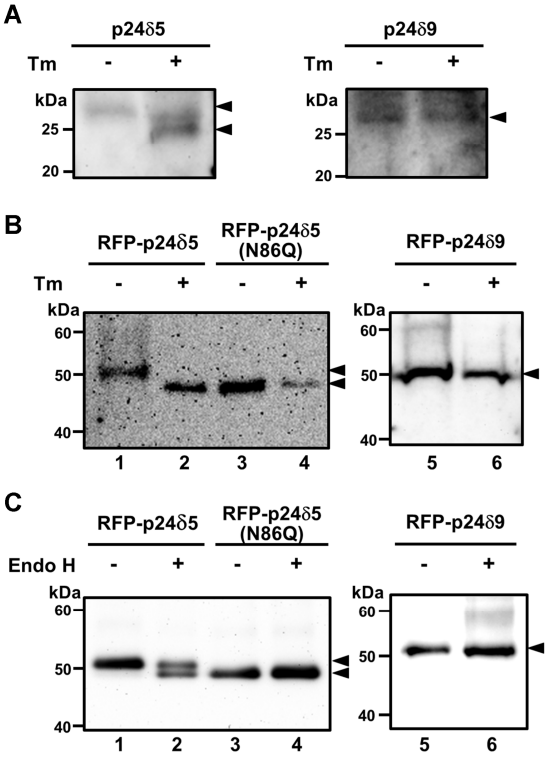
Supplemental Figure S3. p24δ5 glycosylation is required to inhibit secretion of GFP-HDEL. Tobacco mesophyll protoplasts were transfected with the indicated constructs and incubated for 20 h. GFP fluorescence (ex. 485 nm – em. 535 nm) in protoplast extracts and culture medium were analyzed using a microplate reader and secretion was calculated as a percentage of the amount of GFP in the medium versus the amount in protoplasts.

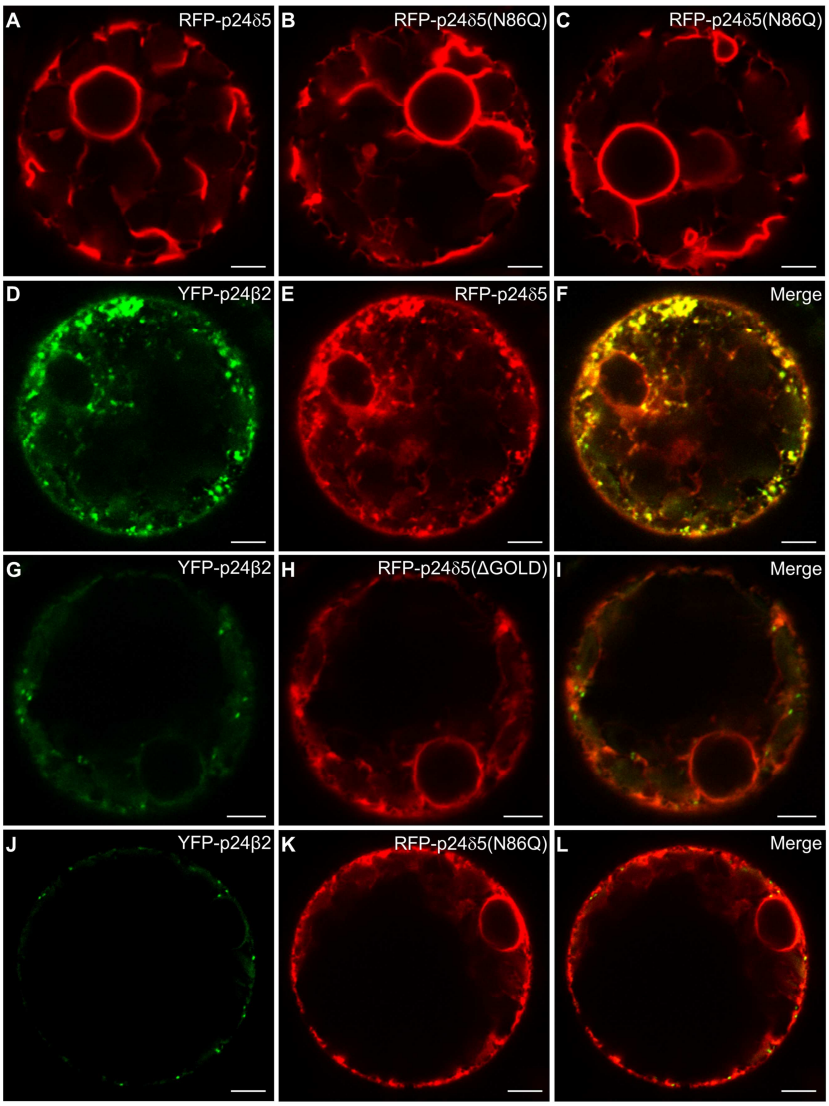
TABLE

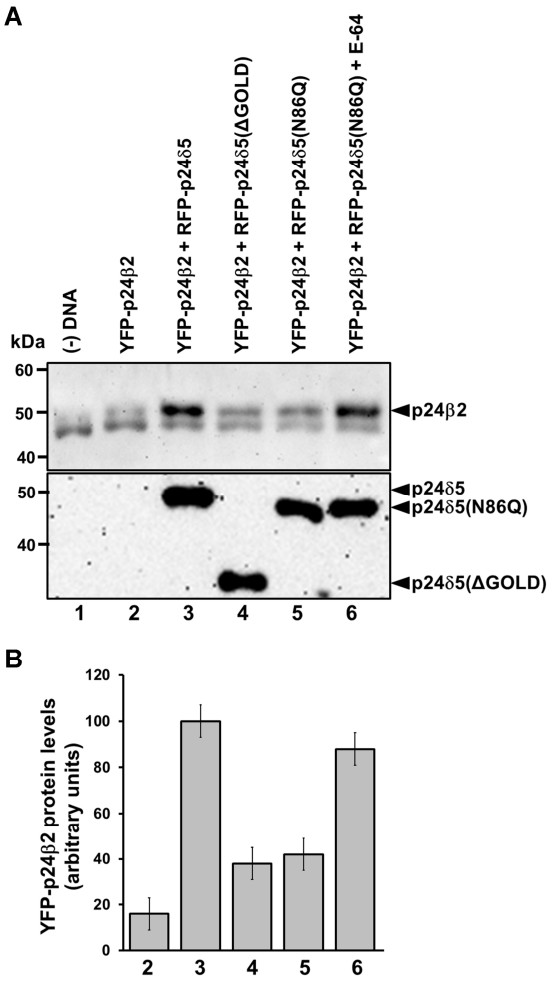
Combination of proteins		Manders coefficient	
A	**B**	**M1 (A overlapping with B)**	**M2 (B overlapping with A)**
YFP-p24β2	RFP-p24δ5	0.88 + 0.06	0.39 + 0.07
YFP-p24β2	RFP-p24δ5(N86Q)	0.83 + 0.07	0.13 + 0,06
YFP-p24β2	RFP-p24δ5(ΔGOLD)	0.79 + 0.07	0.10 + 0.06

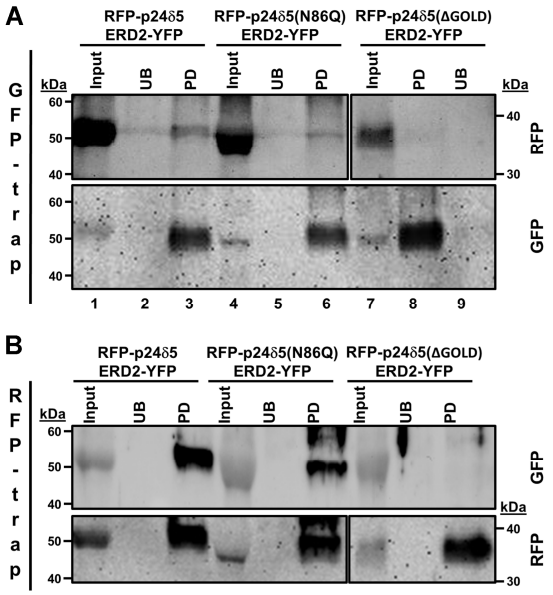
Table 1. **Co-localization of YFP–p24β2 and RFP–p24δ5 or mutant versions in co-expression experiments.** Measurements were made on 20 separate cells upon double co-expression with the indicated constructs (as shown in Figure 3) and calculated with ImageJ 1.48v and the plugin JACoP (Bolte and Cordelieres, 2006).

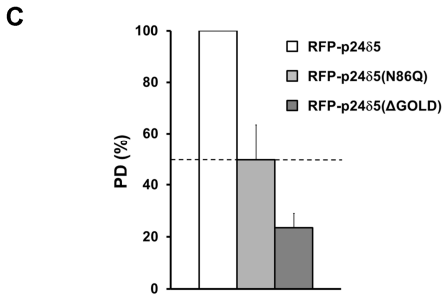


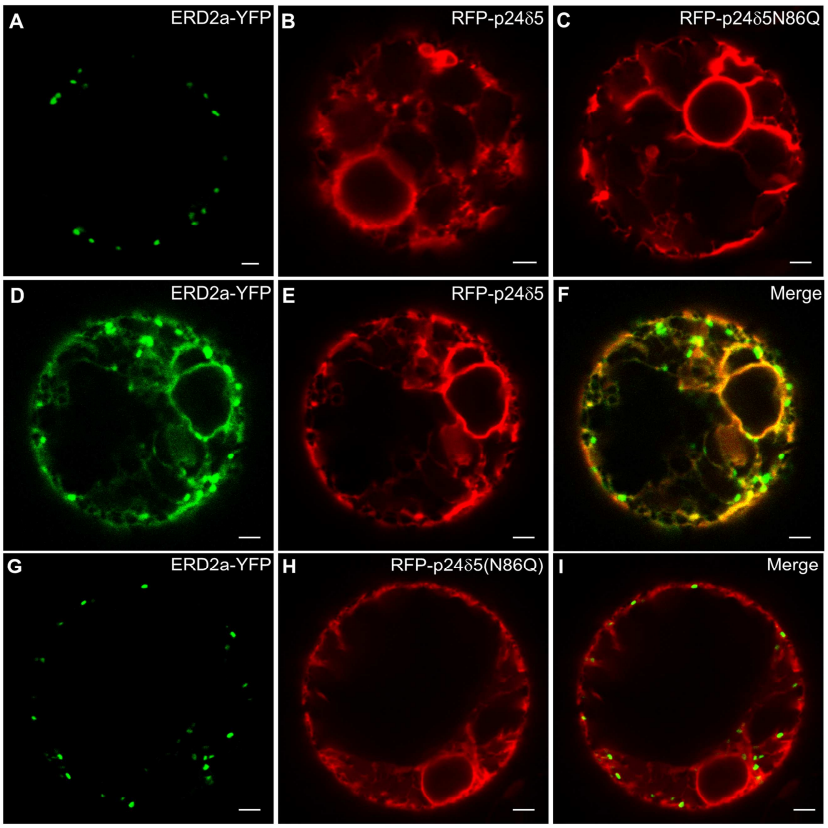


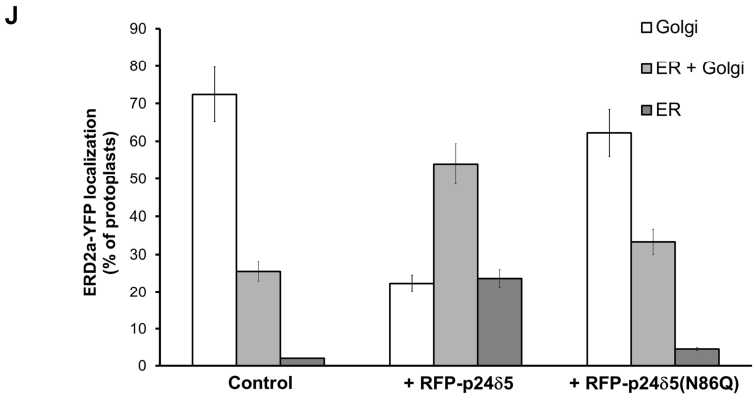









A
ERD2a-YFP
B
RFP-p24δ5
C
RFP-p24δ5N86Q
D
ERD2a-YFP
E
RFP-p24δ5
F
Merge
G
ERD2a-YFP
H
RFP-p24δ5(N86Q)
I
Merge


J
ERD2a-YFP localization
(% of protoplasts)
90
80
70
60
50
40
30
20
10
0
Golgi
ER + Golgi
ER
Control
+ RFP-p24δ5
+ RFP-p24δ5(N86Q)