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RESEARCH PAPER

Coupled transport of *Arabidopsis* p24 proteins at the ER–Golgi interface

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Abstract

p24 proteins are a family of type I membrane proteins localized to compartments of the early secretory pathway and to coat protein I (COPI)- and COPII-coated vesicles. They can be classified, by sequence homology, into four subfamilies, named p24α, p24β, p24γ, and p24δ. In contrast to animals and fungi, plants contain only members of the p24β and p24δ subfamilies. It has previously been shown that transiently expressed red fluorescent protein (RFP)–p24δ5 localizes to the endoplasmic reticulum (ER) as a consequence of highly efficient COPI-based recycling from the Golgi apparatus. Using specific antibodies, endogenous p24δ5 has now been localized to the ER and p24β2 to the Golgi apparatus in *Arabidopsis* root tip cells by immunogold electron microscopy. The relative contributions of the cytosolic tail and the luminal domains to p24δ5 trafficking have also been characterized. It is demonstrated that whereas the dilysine motif in the cytoplasmic tail determines the location of p24δ5 in the early secretory pathway, the luminal domain may contribute to its distribution downstream of the Golgi apparatus. By using knock-out mutants and co-immunoprecipitation experiments, it is shown that p24δ5 and p24β2 interact with each other. Finally, it is shown that p24δ5 and p24β2 exhibit coupled trafficking at the ER–Golgi interface. It is proposed that p24δ5 and p24β2 interact with each other at ER export sites for ER exit and coupled transport to the Golgi apparatus. Once in the Golgi, p24δ5 interacts very efficiently with the COPI machinery for retrograde transport back to the ER.

Key words: *Arabidopsis*, coat protein (COP) I, coat protein (COP) II, ER–Golgi transport, p24 proteins, secretory pathway.

Introduction

p24 proteins constitute a family of small (20–25 kDa) type I membrane proteins which in mammals and yeast localize to the endoplasmic reticulum (ER), the ER–Golgi intermediate compartment (ERGIC), the *cis*-Golgi network (CGN), or the Golgi apparatus (Stamnes *et al.*, 1995; Belden and Barlowe, 1996; Blum *et al.*, 1996, 1999; Sohn *et al.*, 1996; Nickel *et al.*, 1997; Rojo *et al.*, 1997; Dominguez *et al.*, 1998; Füllerkrug *et al.*, 1999; Gommel *et al.*, 1999; Emery *et al.*, 2000; Rojo *et al.*, 2000). They are also major constituents of both coat protein I (COPI)- (Stamnes *et al.*, 1995; Sohn *et al.*, 1996; Gommel *et al.*, 1999) and COPII- (Schimmöller *et al.*, 1995; Belden and Barlowe, 1996) coated vesicles. All p24 proteins share the same topology: an N-terminal signal sequence, a large luminal portion, which includes the GOLD (GOLgi Dynamics) and coiled-coil domains, a single transmembrane domain, and a short (12–18 amino acids) cytoplasmic C-terminus (for a review, see Strating and Martens, 2009). The coiled-coil domain of p24 proteins enables intermolecular interactions between copies of the same protein, but also between different p24 proteins (Fullerkrug *et al.*, 1999; Gommel *et al.*, 1999; Marzioch *et al.* 1999; Jenne *et al.*, 2002). Indeed it has been proposed that oligomerization is required for the proper localization of p24 proteins (Emery *et al.*, 2000; Ciufo and Boyd, 2000). The luminal GOLD domain, present in several proteins involved in Golgi dynamics, is predicted to be involved in

specific protein–protein interactions and has been postulated to interact with putative cargo proteins (Anantharaman and Aravind, 2002; Carney and Bowen, 2004).

In the past it has been customary in mammalian and yeast p24 research to use individual, sometimes unrelated names for p24 orthologues (e.g. p23, p24, p25, p26, in mammals; Erp1-6p; Emp24p, Erv25p in yeast). However, a more systematic nomenclature has now emerged, first proposed by Dominguez *et al.* (1998) and recently updated in Strating *et al.* (2009), which places p24 proteins into one of four subfamilies named p24α, p24β, p24γ, and p24δ. Whereas animals and fungi have representatives in all four subfamilies, plants have only members of the p24δ (nine in *Arabidopsis*) and the p24β (two in *Arabidopsis*) subfamilies (Carney and Bowen, 2004). Following this nomenclature, *Arabidopsis* p24 proteins have been named here with a Greek letter (to identify the subfamily) followed by a number (Fig. 1A). In the case of the delta subfamily, two non-plant members had already been called p24δ1 and 2 (Strating *et al.*, 2009). Therefore, the nine *Arabidopsis* members were named p24δ3–p24δ11 (in the order they appear in the phylogenetic tree) (Fig. 1A). In the case of the beta subfamily, there was already a p24β1 (Strating *et al.*, 2009), and thus the two *Arabidopsis* members were named p24β2 and p24β3 (Fig. 1A).

Although this classification is based upon sequence alignments for the whole protein, some specific features can be seen in the cytosolic C-terminal tails of the members of the different subfamilies. The cytoplasmic tail of most p24 proteins contains a pair of aromatic residues which has been shown to bind COPII subunits and thus may function as an ER export signal (Dominguez *et al.*, 1998; Contreras *et al.*, 2004*b*; Aniento *et al.*, 2006). Some p24 proteins also have a canonical dilysine motif which binds COPI and mediates Golgi to ER retrograde transport (Cosson and Letourneur, 1994; Letourneur *et al.*, 1994; Fiedler *et al.*, 1996; Langhans *et al.*, 2008). This is the case for mammalian p25 proteins (p24α1–p24α3), which contain a dilysine motif in the optimal position (-3,-4) for COPI binding (Teasdale and Jackson, 1996; Dominguez *et al.*, 1998). This causes these proteins to locate mainly to the ER or the ERGIC in mammalian cells (Wada *et al.*, 1991; Dominguez *et al.*, 1998; Lavoie *et al.*, 1999; Marzioch *et al.*, 1999; Emery *et al.*, 2000, 2003). Mammalian p23 (p24δ1) has an additional C-terminal residue, which brings the dilysine motif into a suboptimal position for COPI binding, and mainly localizes to the CGN (Nickel *et al.*, 1997; Rojo *et al.*, 1997, 2000; Blum and Lepier, 2008). In mammalian p24 (p24β1), lysines are replaced by arginines (p24), which do not bind coatomer (Cosson and Letourneur, 1994; Contreras *et al.*, 2004*a*). As a result, this protein does not localize to the ER but is usually found in the Golgi apparatus (Dominguez *et al.*, 1998; Blum *et al.*, 1999; Gommel *et al.*, 1999; Emery *et al.*, 2000). Interestingly, all *Arabidopsis* p24 proteins of the delta subfamily (p24δ3–p24δ11) contain a C-terminal tail with the characteristics of mammalian p25 (p24α1–p24α3) proteins, including a dilysine motif in the -3,-4 position and a diaromatic/large hydrophobic motif in the -7,-8 position. One of them, p24δ5, has been shown to localize exclusively to the ER in the steady state (Langhans *et al.*, 2008).

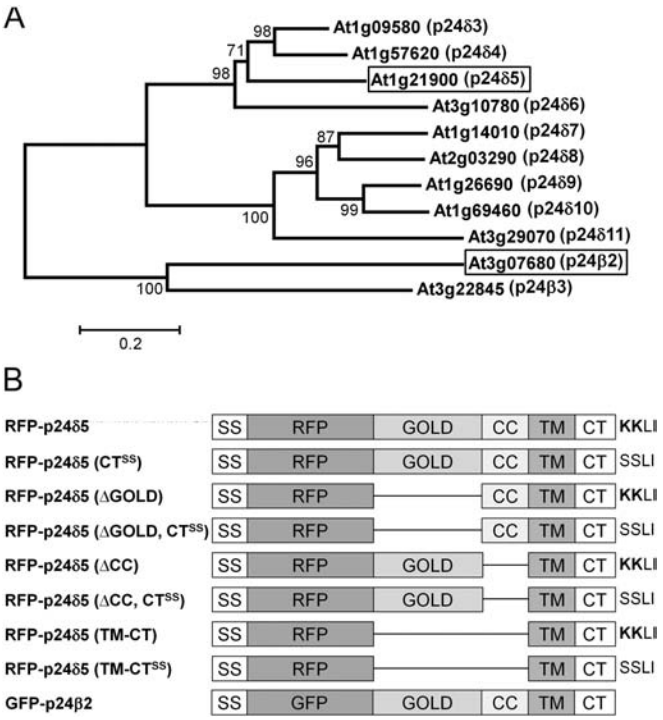

Fig. 1. The p24 family in *Arabidopsis*. (A) A phylogenetic tree containing the δ and β subfamilies of p24 proteins in *Arabidopsis*. A multiple alignment of the p24 proteins was constructed using ClustalW and the tree was generated from this alignment using the Molecular Evolutionary Genetics Analysis software (MEGA, version 5.03) with the Neighbor–Joining method. The numbers beside the branches represent bootstrap percentage based on 1000 replications. The names assigned to these proteins, following the nomenclature proposed by Dominguez *et al*. (1998), are shown next to the AGI code. The two proteins analysed in this study, p24δ5 and p24β2, are highlighted. (B) Schematic representation of the RFP–p24δ5 and GFP–p24β2 constructs used in this study, including the different domains of p24δ5 and p24β2. SS, signal sequence; RFP/GFP, red or green fluorescent proteins; GOLD, domain involved in Golgi dynamics (see text for details); CC, coiled-coil domain; TM, transmembrane domain; CT, cytoplasmic tail, which in p24δ5 contains a dilysine (KKXX) motif; CT^{SS}, cytoplasmic tail with the two lysines replaced by serines; ΔGOLD or ΔCC, deletion mutants lacking the GOLD or the CC domains; TM-CT, deletion mutant containing only the transmembrane domain and the cytosolic tail.

Numerous functions have been ascribed to p24 proteins, including quality control of protein movement through the secretory pathway (Wen and Greenwald, 1999; Belden and Barlowe, 2001), cargo protein selection and packaging into transport vesicles (Schimmöller *et al.*, 1995; Muniz *et al.*, 2000; Takida *et al.*, 2008; Castillon *et al.*, 2011; Fujita *et al.*, 2011), the formation of ER exit sites (ERESs), and the biogenesis and maintenance of the Golgi apparatus (Lavoie *et al.*, 1999; Mitrovic *et al.*, 2008; Koegler *et al.*, 2010).

These few examples indicate that p24 proteins are one of the most interesting groups of proteins involved in regulating the structure and function of the organelles of the secretory pathway. A cargo receptor function for p24 proteins has often been suggested (see, for example, Schimmoller *et al.*, 1995), but the identity of putative cargoes has remained elusive. Indeed, only two types of cargoes have been shown to interact with p24 proteins, none of them being a real soluble cargo (as originally postulated). These are glycosyl-phosphatidylinositol (GPI)-anchored proteins, both in yeast (Schimmoller *et al.*, 1995; Muniz *et al.*, 2000, 2001; Castillon *et al.*, 2011; Fujita *et al.*, 2011) and in mammals (Takida *et al.* 2008), and G protein-coupled receptors (protease-activated receptors PAR-1 and PAR-2, nucleotide P2Y receptors, and μ-opioid receptors) (Luo *et al.*, 2007, 2011).

Despite the difficulty in the identification of the cargoes, p24 proteins have been shown recently to be involved in a variety of specific functions in animal cells, including early embryonic mouse development (Denzel *et al.*, 2000) and morphogenesis of the mouse embryo and placenta (Jerome-Majewska *et al.*, 2010), insulin biosynthesis and subsequent secretion in pancreatic beta cells (Zhang and Volchuk, 2010), or amyloid precursor metabolism and pathogenesis of Alzheimer disease (Chen *et al.*, 2006; Vetrivel *et al.*, 2007; Hasegawa *et al.*, 2010). In addition, several studies have recently been performed investigating the role of p24 proteins in the secretory pathway of *Xenopus laevis* (Strating and Martens, 2009; Strating *et al.*, 2010) and *Drosophila* (Buechling *et al.*, 2011; Port *et al.*, 2011). Therefore, p24 proteins are emerging as very important players in many cellular processes in mammalian cells dependent upon the secretory pathway. In contrast, very little work has been performed on p24 proteins in plants. Only three papers have been published so far: two examining the roles of COPI- and COPII-binding motifs in the cytosolic tail of an *Arabidopsis thaliana* p24 protein (At1g21900) (here named p24δ5), the other demonstrating that this protein is localized to the ER as a consequence of highly efficient COPI-based recycling from the Golgi apparatus (Contreras *et al.*, 2004*a*, *b*; Langhans *et al.*, 2008). Given the peculiarities of the p24 family and also the different organization of the early secretory pathway in plants, it was deemed necessary to investigate in further detail the localization and trafficking properties of *Arabidopsis* p24 proteins, as a first step towards their functional characterization.

Following up on previous studies (Contreras *et al.*, 2004*a*, *b*; Langhans *et al.*, 2008), the localization of endogenous p24 proteins in *Arabidopsis*, including that of a member of each of the two subfamilies (p24δ5 and p24β2), has now been investigated. It was found that p24δ5 localizes mainly to the ER but also to the *cis*-Golgi, while p24β2 localizes mainly to the Golgi apparatus. In addition the relative contributions of the cytosolic tail and the luminal (GOLD and coiled-coil) domains to p24δ5 trafficking have been characterized. It was found that the cytosolic tail, containing the dilysine motif, is alone sufficient for ER localization at steady state and trafficking of p24δ5 in the early secretory pathway, while the luminal domains may contribute to its transport beyond the Golgi apparatus. Finally, data are presented which suggest that p24δ5 and p24β2 interact with each other and exhibit coupled trafficking at the ER–Golgi interface.

Materials and methods

Plant material

Arabidopsis thaliana ecotype Columbia (Col-0) and T-DNA mutant plants were grown in growth chambers as previously described (Ortiz-Masia *et al.*, 2007). For immunogold electron microscopy, seedlings were grown on Murashige and Skoog (MS) medium containing 0.5% agar, and the roots were harvested after 5 d. To obtain a membrane fraction from *Arabidopsis* roots, seedlings were grown in liquid MS medium for 15 d. *Arabidopsis thaliana* cell suspension cultures (LT87) (Axelos *et al.*, 1992) were cultivated as described (Ortiz-Zapater *et al.*, 2006). Tobacco plants (*Nicotiana tabacum* var. SR1) were grown as described (Pimpl *et al.*, 2006).

Recombinant plasmid production

The coding sequence of green fluorescent protein (GFP)–p24β2 was commercially synthesized *de novo* (Geneart AG) based on the sequence of GFP and that of the *Arabidopsis* p24 protein At3g07680 (p24β2). The sequence of the fluorophore is behind the coding sequences of the p24 signal sequence (SS) and the 5' extreme end of the mature p24 coding sequence. The ΔCC, ΔGOLD, ΔCC-CT^{SS}, ΔGOLD-CT^{SS}, TMCT, and TMCT-CT^{SS} mutants of red fluorescent protein (RFP)–p24δ5 were obtained by 3' deletion of RFP–p24δ5 and synthesis of a new sequence (Langhans *et al.*, 2008). The coding sequences of XFP–p24β2/δ5 were cloned into the pBP30 vector (carrying the ^{35}S promoter, Nebenführ *et al.*, 1999) through *Bgl*II/*Not*I.

Plasmids encoding marker proteins were: RFP–p24δ5 and RFP–p24δ5-CT^{SS} (mutant1) (Langhans *et al.*, 2008), ST–yellow fluorescent protein (YFP) (Brandizzi *et al.*, 2002), Man1–RFP and Man1–GFP (Nebenführ *et al.*, 1999), GFP–HDEL (Nebenführ *et al.*, 2000), ARA6–RFP (Ueda *et al.*, 2004), GFP–BP80 (daSilva *et al.*, 2005), and ARF1(Q71L) (Pimpl *et al.*, 2003). All RFP-tagged proteins were tagged with monomeric RFP (mRFP) to prevent oligomerization. Similarly, only mGFP5 was used for GFP-tagged proteins.

Isolation of protoplasts and transient gene expression

Mesophyll protoplasts from *N. tabacum* var. SR1 leaf cells were isolated and transfected as previously described (Bubeck *et al.*, 2008). Unless otherwise stated, 1–50 μg of plasmid DNA was transfected and expressed for 20 h. Protoplasts from *A. thaliana* (LT87) cell suspension cultures were isolated as previously described (Axelos *et al.*, 1992).

Generation of antibodies

Rabbit antibodies against different *Arabidopsis* p24 proteins were generated by Eurogentec (Belgium, http://www.eurogentec.com) using as antigen peptides corresponding either to the N- or to the C-terminus of the indicated proteins: p24δ5-Ct (YLKRYFH-KKKLI), p24δ5-Nt (IWLTIPTTGG), p24β2-Nt (IRFVIDREE), and p24β2-Ct (LFERKLGMSRV). Affinity-purified antibodies (Eurogentec) were used for immunogold labelling as well as for covalent binding to magnetic beads in co-immunoprecipitation experiments (see below).

Confocal microscopy and immunofluorescence labelling

Imaging was performed using a Zeiss Axiovert LSM510 Meta confocal laser scanning microscope. At the Metadetector, the main beam splitters (HFT) 458/514 and 488/543 were used. The following fluorophores (excited and emitted by frame switching in the multitracking mode) were used: GFP (488 nm/496–518 nm), YFP (514 nm/529–550 nm), and RFP (543 nm/593–636 nm). To verify the co-localization of fluorescent signals obtained by fast frame switching, parallel observations were performed in the line switching mode. As shown in Supplementary Fig. S8 available at *JXB* online, there was no difference in the localization of the fluorescent signals, thus eliminating possible false co-localization events caused by the movement of organelles. Post-acquisition image processing was performed using the Zeiss LSM 5 image Browser (4.2.0.121) and CorelDrawX4 (14.0.0.567) or ImageJ (v.1.45m).

Immunogold electron microscopy

Root tips from *Arabidopsis* were high pressure frozen, freeze substituted, embedded, labelled, and post-stained as described (Bubeck *et al.*, 2008). Affinity-purified antibodies were used at the following dilutions: Ct-p24β2 (1:100); Nt-p24δ5 (1:10); and Ct-p24δ5 (1:10). Micrographs were taken with a JEM1400 transmitting electron microscope operating at 80 kV using a TVIPS F214 digital camera.

Preparation of membrane extracts and co-immunoprecipitation

Membrane fractions were obtained from *Arabidopsis* cell suspension cultures (LT87) or from *Arabidopsis* roots. Cells were collected by centrifugation and washed twice in homogenization buffer [50 mM TRIS-HCl, pH 7.5, 0.3 M sucrose, 10 mM KCl, 1 mM dithiothreitol (DTT), 3 mM EDTA, and a cocktail of protease inhibitors (Sigma)]. Pellets were resuspended 1:1 (v/v) in homogenization buffer and cells disrupted by sonication (4×15 s). Cell extracts were separated from unbroken cells by centrifugation (10 min at 2000 *g*). Membranes were pelleted by centrifugation of the extracts for 1 h at 150 000 *g*. *Arabidopsis* roots (from either wild-type or mutant plants) were homogenized in homogenization buffer using a mortar and a pestle, and membrane fractions were obtained as above. Membrane pellets were extracted using a lysis buffer containing 50 mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.5 mM DTT, 0.5% Triton X-100, and a cocktail of protease inhibitors (30 min at 4 °C), and extracts were obtained after centrifugation for 5 min at 10 000 *g*. Protein extracts were used for SDS–PAGE followed by western blot analysis or for co-immunoprecipitation. The intensity of the bands obtained after western blot was quantified using the Quantity One software (Bio-Rad Laboratories).

Co-immunoprecipitation experiments were performed using magnetic beads (Dynal, Invitrogen), following the recommendations of the manufacturer. Briefly, affinity-purified antibodies were covalently bound to magnetic beads (6 μg of antibody mg^{-1} Dynabeads). Then 150 μl (1.5 mg) of Dynabeads (with bound antibody) were incubated with 1 ml of protein extracts from membrane fractions obtained from cell suspension cultures (10 mg ml^{-1}) for 2 h at 4 °C. Beads were washed three times with lysis buffer and bound proteins were eluted with elution buffer (buffering salts, pH 2.8, Dynal).

Mutant characterization

Lines (Col-0 background) containing a T-DNA insertion in *p24δ4* (SAIL_664_A06, *p24δ4-1*) and *p24δ5* (SALK_016402C, *p24δ5-1*) were identified from the SALK T-DNA collection (http://signal.salk.edu/cgi-bin/tdnaexpress). They were characterized by PCR as previously described (Ortiz-Masia *et al.*, 2007). The primers used for the *p24δ4* mutant (*p24δ4-1*) were the following: 5′GGATCCACTTAGATCTCCTCAAAATTC3′ and 5′ATACTGTACCATGCGACTCTCGAG3′. The T-DNA left border primer used was 5′TTCATAACCAATCTCGATACAC3′. The primers used for the *p25δ5* mutant (*p25δ5-1*) were 5′GAAGACCATCGTTGTTCTCCGATGGC3′ and 5′TTGGTGATGAAGATTGTTCCC3′. The T-DNA left border and *Actin7* (*ACT7*, At5g09810) primers used were described previously (Ortiz-Masia *et al.*, 2007). Reverse transcription-PCR (RT-PCR) analysis of *p24δ4-1* and *p24δ5-1* mutants was performed as described (Ortiz-Masia *et al.*, 2007) to show the absence of *p24δ4* and *p24δ5* mRNA, respectively. The primers used for PCR amplification were the same as above. To generate the double mutant, the homozygous line *p24δ4-1* was crossed with the homozygous line *p24δ5-1*. Individuals homozygous for T-DNA insertion in both *p24δ4-1* and *p24δ5-1* were identified by PCR with the primers described above.

Results

Localization of endogenous p24 proteins in Arabidopsis

In order to localize endogenous p24 proteins in *Arabidopsis*, peptide antibodies against two different members of the p24 family were generated. Since previous work (Contreras *et al.*, 2004*a*, *b*; Langhans *et al.*, 2008) was undertaken with p24δ5 (At1g21900) (Fig. 1A), this protein was chosen as one representative of the delta subfamily for antibody generation. Moreover, it has relatively high levels of expression in different tissues, according to gene expression data of public microarray repertoires (Zimmermann *et al.*, 2004). Antibodies were produced using peptides corresponding to both the N- and the C-terminus of p24δ5. In contrast to the N-terminus, the C-terminus of *Arabidopsis* p24 proteins is very similar among different members of the delta subfamily (Supplementary Fig. S1 at *JXB* online). In particular, the C-termini of p24δ3, p24δ4, and p24δ6 share 10–11 (out of 12) residues with the C-terminus of p24δ5, while sequence homology is lower in other members of the subfamily. Therefore, C-terminal p24δ5 antibodies may also recognize p24δ3, p24δ4, and p24δ6. According to public microarray databases, p24δ4 has expression levels comparable (although lower) with those of p24δ5, while p24δ6 expression is very low and tissue specific (no data are available for p24δ3). Antibodies against both the N- and the C-terminus of a p24 protein of the beta subfamily, p24β2 (At3g07680), were also generated (Fig. 1A; Supplementary Fig. S1).

p24 proteins were extracted from membranes of *Arabidopsis* cell suspension cultures or from *Arabidopsis* roots. As shown in Fig. 2A, antibodies against the N-terminus of p24δ5 recognized a protein of the expected molecular weight (24 kDa) in membranes from cell suspension cultures as well as in membranes from roots. In the case of p24δ5 C-terminal antibodies, a major band of ~24 kDa was obtained in membranes from cell suspension cultures, although a double band was detected in root membranes. To characterize the specificity of the antibodies further, available T-DNA insertion mutants for p24δ5 (*p24δ5-1*) and p24δ4 (*p24δ4-1*) were used. Both mutants were found to be knock-out mutants. In addition, the *p24δ4δ5* double mutant was obtained. Plants from the three lines resembled wild-type plants under standard growth conditions, either on vertical agar plates or on soil (Supplementary Fig. S2 at *JXB* online).

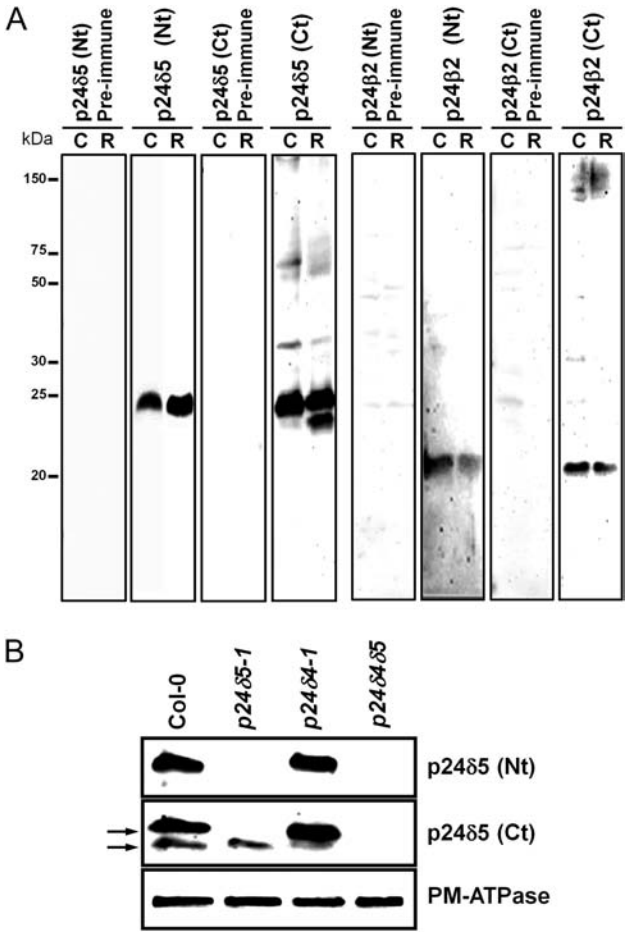

Fig. 2. Characterization of p24 antibodies. (A) Characterization of antibodies against *Arabidopsis* p24 proteins. Protein extracts were obtained from membranes of *Arabidopsis* cell suspension cultures (C) or *Arabidopsis roots* (R), as described in the Materials and methods, and analysed by SDS–PAGE and western blotting with affinity-purified antibodies against the p24δ5 N-terminus, p24δ5 C-terminus, p24β2 N-terminus, and p24β2 C-terminus, or with the corresponding pre-immune sera. (B) Western blot analysis of membranes from wild-type (Col-0) or p24 knock-out mutants (*p24δ5-1*, *p24δ4-1*, or *p24δ4δ5*) using antibodies against the Nt or the Ct of p24δ5. A 25 μg aliquot of protein was loaded in each lane. Western blot with an antibody against the plasma membrane (PM) ATPase was used as a loading control.

Western blot analysis with the Nt-p24δ5 antibody showed that p24δ5 was absent in root membranes from the *p24δ5-1* mutant or from the *p24δ4δ5* double mutant, as expected for a p24δ5 knock-out mutant, but was present (at similar levels to those in the wild type) in the *p24δ4-1* mutant (Fig. 2B). These mutants were next analysed with the Ct-p24δ5 antibody, which detected two bands in extracts from wild-type root membranes. The upper band should correspond specifically to p24δ5, as it was absent in root membranes from the *p24δ5-1* mutant or from the *p24δ4δ5* double mutant, confirming the results obtained with the N-terminal antibody, but was present in the *p24δ4-1* mutant (Fig. 2B). In contrast, the lower band was still present in the *p24δ5-1* mutant but was almost undetectable in the *p24δ4δ5* double mutant (Fig. 2B). This suggests that the lower band detected by the Ct-p24δ5 antibody corresponds to a large extent to p24δ4, and that the Ct-p24δ5 antibody does not recognize any other abundant member of the delta subfamily in *Arabidopsis* roots. Finally, antibodies against both the N- and the C-terminus of p24β2 recognized a protein with an apparent molecular weight of ~22 kDa (instead of the predicted 24 kDa) (Fig. 2A).

These antibodies were used to localize endogenous p24 proteins by immunogold labelling on sections cut from cryofixed *Arabidopsis* roots. As shown in Fig. 3A and B, the N-terminal p24δ5 antibody produced a prominent labelling on ER membranes. Occasionally, some labelling was also seen on the *cis*-Golgi or on putative COPI vesicles (Fig. 3B; see also Table 1). In contrast, the N-terminal p24β2 antibody showed significantly higher labelling at the Golgi apparatus, although some labelling could also be seen at ER membranes (Fig. 3C–E; see also Table 1). The C-terminal p24δ5 antibody was also used to localize endogenous p24δ proteins in p24δ5 (Fig. 4A, C, D) and p24δ4 (Fig. 4B, E) knock-out mutants. As stated above, the Ct-p24δ5 antibody should recognize p24δ5 (in the p24δ4 knock-out mutant) and p24δ4 (in the p24δ5 knock-out mutant). In both mutant lines, labelling was again found at ER membranes, but also a significant labelling at the *cis* side of the Golgi apparatus. The labelling obtained with the Ct-p24δ5 antibody in both mutant lines was very similar to that observed in wild-type *Arabidopsis* roots (Fig. 4F–H), suggesting that the localization of endogenous p24δ5 or p24δ4 is not significantly affected by the absence of p24δ4 or p24δ5, respectively.

The dilysine motif in the cytoplasmic tail of p24δ5 is sufficient for its trafficking in the early secretory pathway

It was previously demonstrated that transiently expressed RFP–p24δ5 shows a uniform signal distribution throughout the ER, both in tobacco mesophyll and in *Arabidopsis* protoplasts (Langhans *et al.*, 2008), as well as in BY2 cells (Langhans *et al.*, 2008) and in tobacco leaf epidermal cells (Lerich *et al.*, 2011). Whether the dilysine motif at the cytoplasmic tail of p24δ5 is sufficient for ER localization and trafficking in the early secretory pathway, or if the luminal GOLD and coiled-coil domains could also play a role has now been investigated. To this end, RFP–p24δ5 deletion mutants lacking either the GOLD or the coiled-coil domains were prepared (Fig. 1B). These constructs were used for transient expression in tobacco mesophyll protoplasts, since they represent a versatile, well-characterized, and reproducible system for transient expression (Pimpl and Denecke, 2001; da Silva *et al.*, 2006; Ribeiro *et al.*, 2008). Nevertheless, the localization of the different constructs was also confirmed in *Arabidopsis* protoplasts (see below). As shown in Fig. 5A and B, both deletion mutants localized exclusively to the ER, where they co-localized extensively with the ER marker GFP–HDEL. This suggests that the steady-state localization of RFP–p24δ5 at the ER is not dependent on the GOLD or the coiled-coil domains. A deletion mutant lacking both the GOLD and the coiled-coil domains (and thus having only the transmembrane

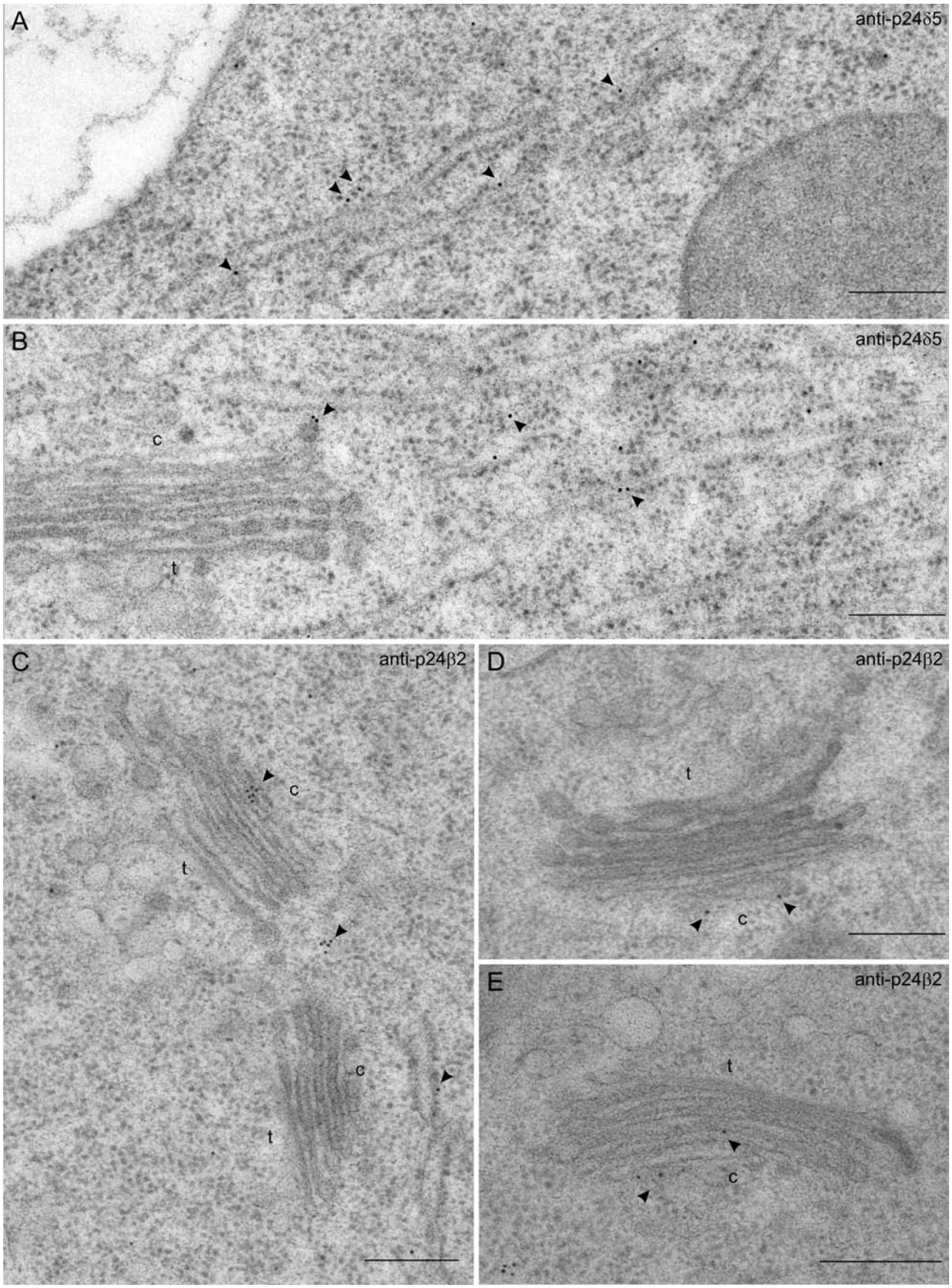

Fig. 3. Localization of p24δ5 and p24β2 by immunogold labelling on cryofixed *Arabidopsis* roots. (A and B) Labelling with antibodies against Nt-p24δ5 at the ER (A and B) and at a putative COPI vesicle (B). (C–E) Labelling with antibodies against Ct-p24β2 at the Golgi (C–E) and at the ER (C). Arrowheads point to gold particles. c, *cis*-Golgi; t, *trans*-Golgi. Scale bars=300 nm.

Table 1. p24 immunogold labelling in wild-type Arabidopsis root cells

Antibody	Gold particles over ER	Gold particles over Golgi Stack	Gold particles over mitochondria
Nt-p24δ5[a]	48	12	6
Ct-p24β2[b]	23	57	7

[a] Number of micrographs analysed: 10.
[b] Number of micrographs analysed: 14.

domain and the cytosolic tail), RFP–p24δ5 (TM-CT) (Fig. 1B), also localized exclusively to the ER (Fig. 5C), indicating that the dilysine motif in the cytosolic tail constitutes the minimal requirement for the steady-state localization of RFP–p24δ5 to the ER. The same ER localization was obtained when these constructs were expressed in *Arabidopsis* protoplasts (Supplementary Fig. S3A–F at *JXB* online). Furthermore, this ER localization was not a consequence of overexpression, since all constructs localized to the ER even at very low expression levels (Supplementary Fig. S4). It was also tested whether GOLD or coiled-coil domains were involved in anterograde ER to Golgi transport of RFP–p24δ5. To this end, the three deletion mutants were co-expressed with the ARF1(Q71L) mutant, in order to inhibit retrograde Golgi to ER transport (Langhans *et al.*, 2008). In this case, RFP–p24δ5 deletion mutants still localized to the ER, but also to Golgi-like punctae, where they co-localized with the Golgi marker Man1–GFP (Fig. 5D–F), as was the case for RFP–p24δ5 (Langhans *et al.*, 2008). Therefore, the GOLD and coiled-coil domains do not seem to have an influence on trafficking and localization of RFP–p24δ5 in the early secretory pathway, at least when expressed individually.

The luminal domain may be involved in transport of p24δ5 beyond the early secretory pathway

In mammals, the luminal domain of p24 proteins has been proposed to play a role in trafficking of p23 (p24α) to the cell surface (Blum and Lepier, 2008). It was previously found that RFP–p24δ5 mutants lacking the dilysine motif were no longer present at the ER but were transported downstream to the pre-vacuolar compartment (PVC) and the vacuole (Langhans *et al.*, 2008). Therefore, it was decided to investigate whether the GOLD and/or the coiled-coil domains could play a role in RFP–p24δ5 trafficking in the absence of the dilysine motif. To this end, RFP–p24δ5 deletion mutants with a mutated dilysine motif (CT^{SS}) lacking either the GOLD or the coiled-coil domains were prepared (Fig. 1B). As shown in Fig. 5G, H, J, and K, none of these mutants was found at the ER. In contrast, they localized to the Golgi (as shown by co-localization with ST–YFP), the PVC (as shown by co-localization with GFP–BP80), and the vacuole lumen. The dilysine mutant lacking the GOLD domain had a more prominent vacuolar labelling than the dilysine mutant lacking the coiled-coil domain (Fig. 5G, H, J, K). This suggests that the coiled-coil domain facilitates transport of dilysine mutants to the vacuole. To quantify the contribution of the coiled-coil domain and to investigate whether transport of dilysine mutants to the vacuole correlated with their degradation, the levels of the different constructs were analysed by western blotting with an RFP antibody. As shown in Fig. 6, when compared with those of RFP–p24δ5, the levels of RFP–p24δ5 (CT^{SS}) were very low, although the same amounts of DNA were used for transient expression. These data suggest that once synthesized, RFP–p24δ5 (CT^{SS}) is degraded, probably as a consequence of its transport to the vacuole, as had been previously proposed (Langhans *et al.*, 2008). In contrast, the levels of the dilysine mutant lacking the coiled-coil domain [RFP–p24δ5 (ΔCC, CT^{SS})] were significantly higher, suggesting that a reduced transport to the vacuole may correlate with a reduced degradation (Fig. 6).

Finally, a dilysine mutant lacking both the GOLD and the coiled-coil domains (RFP–p24δ5, TM-CT^{SS}) was used. As shown in Fig. 5I and L, this was the only dilysine mutant which was partially localized to the ER, showing a partial co-localization with the ER marker GFP–HDEL. It also showed co-localization with the Golgi marker Man1–GFP and eventually with GFP–BP80 (data not shown), but was rarely seen in the vacuole lumen. Altogether, it seems that the absence of the luminal domain impairs trafficking from the ER to the Golgi, and downstream to the PVC and the vacuole. The lower vacuolar labelling (and increased protein levels) obtained in the dilysine mutant lacking the coiled-coil domain suggests that this domain is important in trafficking beyond the Golgi apparatus.

Trafficking properties of a p24 protein of the beta subfamily and coupled transport of Arabidopsis *p24 proteins*

The localization and trafficking properties of a member of the beta subfamily, p24β2 (Fig. 1A), have also been investigated. To this end, a fusion construct similar to the one used to investigate *in vivo* trafficking and localization of RFP–p24δ5, but with GFP instead of RFP, was prepared (Fig. 1B). Surprisingly, the GFP signal obtained upon expression of GFP–p24β2 appeared to be very low. However, GFP–p24β2 was not present at the ER, in contrast to RFP–p24δ5, but showed a punctate pattern, and it co-localized partially with the Golgi marker Man1–RFP, in both tobacco mesophyll (Fig. 7A–C) and *Arabidopsis* protoplasts (Fig. 7G–I), but not with the PVC marker ARA6–RFP (Fig. 7D–F).

Interestingly, when co-expressed with RFP–p24δ5, the signal of GFP–p24β2 was clearly more intense and localized to punctae. In addition, it was observed that RFP–p24δ5, which showed its typical ER pattern, also localized to the same punctae under these conditions (Fig. 8A–C). An increased ratio in the concentrations of GFP–p24β2 versus RFP–p24δ5 induced a progressive change in the localization of RFP–p24δ5, from its typical reticulate ER pattern to a mostly punctated one, and increased co-localization

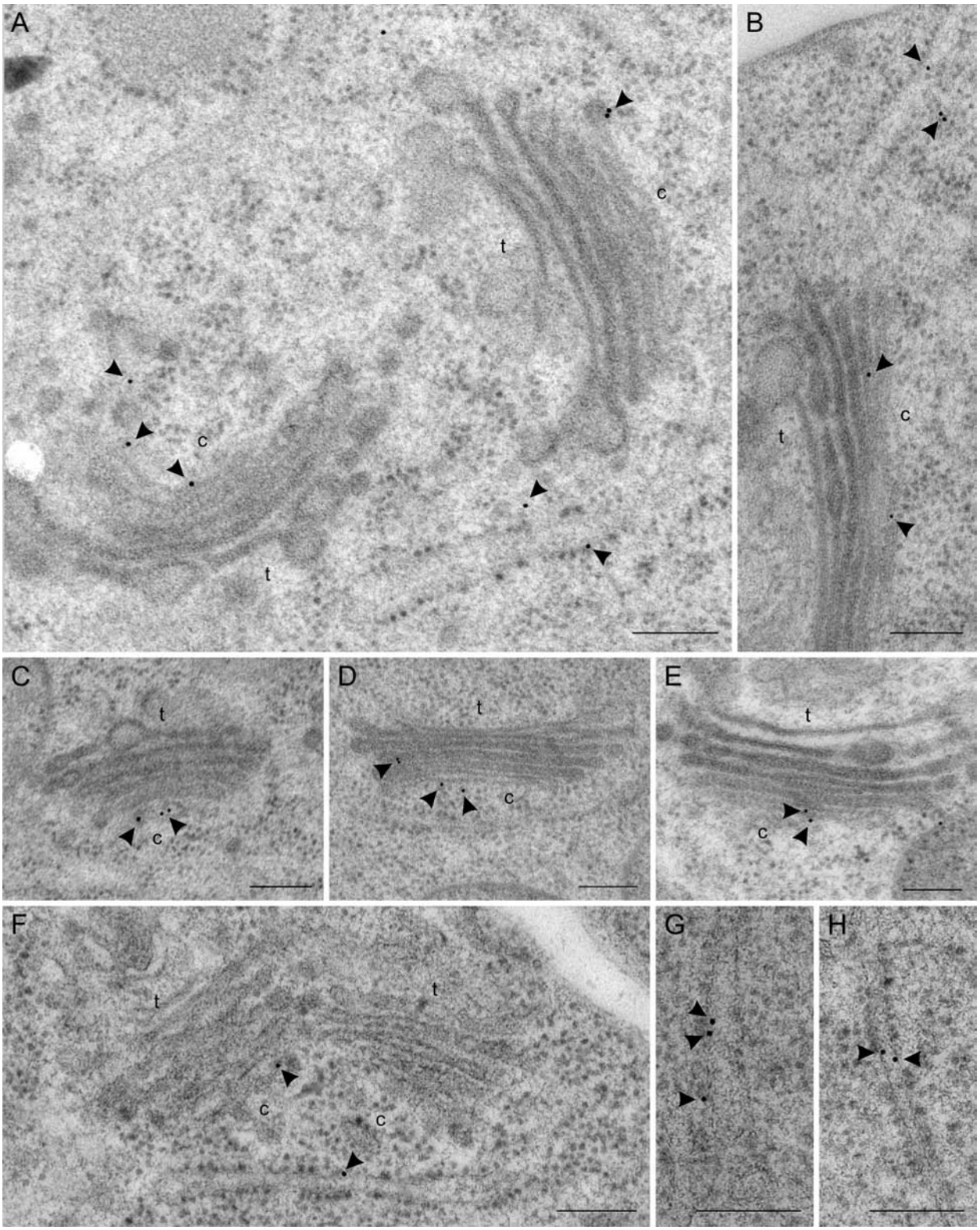

Fig. 4. Localization of p24δ proteins by immunogold labelling on cryofixed wild-type (Col-0), *p24δ5-1*, and *p24δ4-1 Arabidopsis* roots. (A, C, and D) Labelling with Ct-p24δ5 antibodies in roots from the *p24δ5-1* mutant. (B and E) Labelling with Ct-p24δ5 antibodies in roots from the *p24δ4-1* mutant. (F, G, and H) Labelling with Ct-p24δ5 antibodies in wild-type (Col-0) *Arabidopsis* roots. Arrowheads point to gold particles. c, *cis*-Golgi; t, *trans*-Golgi. Scale bars=300 nm.

between both proteins (Fig. 8D–F; Supplementary Fig. S5 at *JXB* online). This suggests that GFP–p24β2 is able to interact with RFP–p24δ5 and is transported with the latter out of the ER. This co-localization was also observed when both proteins were expressed in *Arabidopsis* protoplasts (Supplementary Fig. S3G–I).

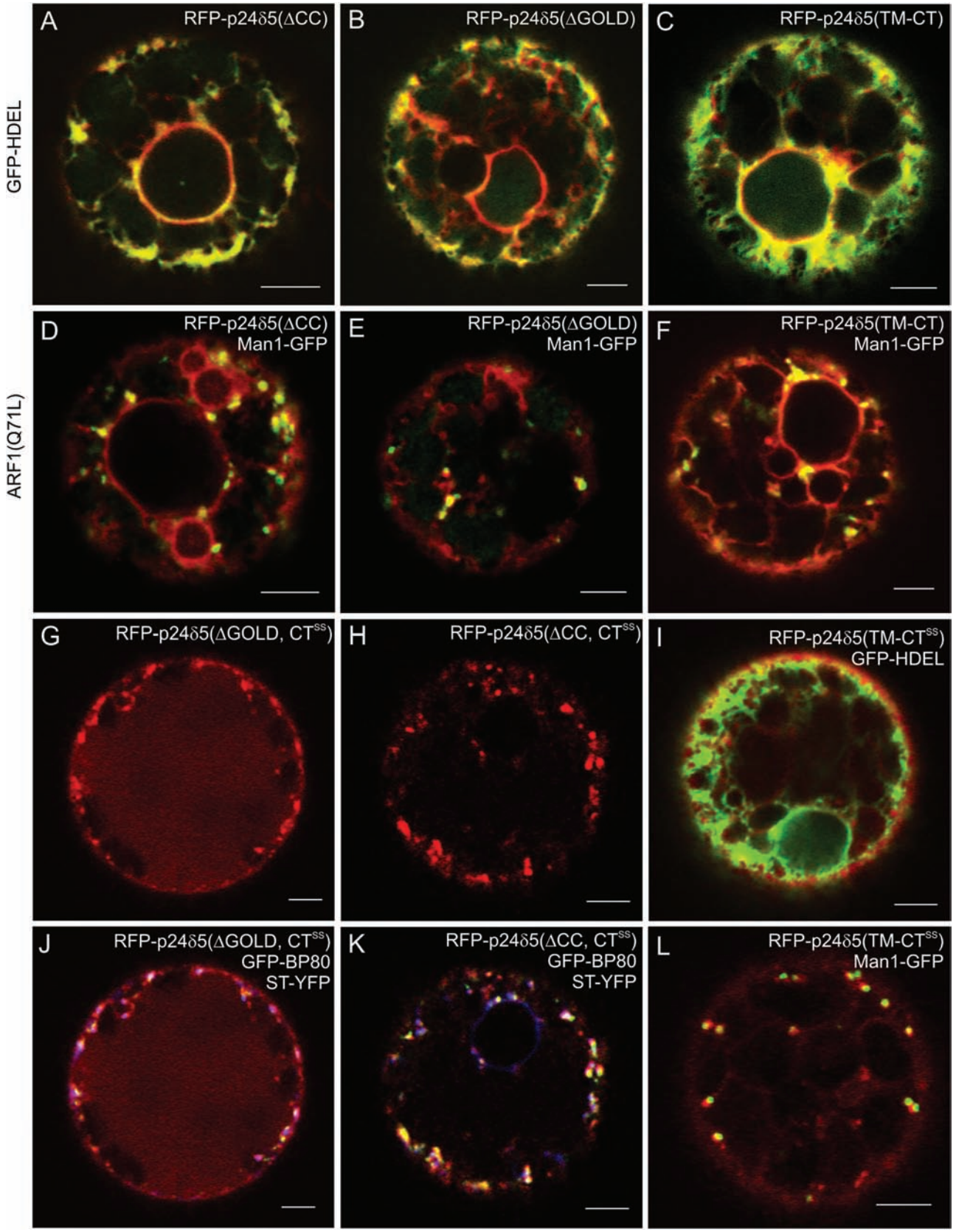

Fig. 5. RFP–p24δ5 deletion mutants localize to the ER but cycle between the ER and the Golgi, whilst mutants lacking the dilysine motif are transported to the pre-vacuolar compartment and the vacuole. (A–L) Transient gene expression in tobacco mesophyll protoplasts. (A–C) RFP–p24δ5 deletion mutants lacking the coiled-coil domain (ΔCC) (A), the GOLD domain (ΔGOLD) (B), or both (TM-CT) (C) show a typical ER pattern and co-localize with the ER marker GFP–HDEL. (D–F) RFP–p24δ5 deletion mutants lacking the coiled-coil domain (ΔCC) (D), the GOLD domain (ΔGOLD) (E), or both (TM-CT) (F) co-localize partially with Man1–GFP in Golgi-like punctae upon

To test whether p24δ5 interacts with p24β2 via coiled-coil domains, GFP–p24β2 was co-expressed with a deletion mutant of RFP–p24δ5 lacking the coiled-coil domain (Fig. 1B). In this case, almost no co-localization was detected between both proteins (Fig. 8G–I). In addition, the signal of GFP–p24β2 was weak, as it happened in the absence of RFP–p24δ5, and RFP–p24δ5 (ΔCC) showed its typical ER pattern, without punctae, even in the presence of increasing concentrations of GFP–p24β2 (Supplementary Fig. S6 at *JXB* online). This suggests that both proteins interact with each other via their coiled-coil domain and that this interaction stabilizes overexpressed GFP–p24β2. To quantify whether the protein levels of GFP–p24β2 were indeed dependent on the interaction with RFP–p24δ5, protoplasts expressing these proteins were analysed by western blotting with Ct-p24β2 or RFP antibodies. As shown in Fig. 6, GFP–p24β2 was almost undetectable when expressed alone, but its levels increased enormously upon co-expression with RFP–p24δ5, but not with RFP–p24δ5 (ΔCC). On the other hand, the protein levels of RFP–p24δ5 seem to be independent of the presence of GFP–p24β2 (Fig. 6).

In order to narrow down the identity of the compartment(s) where RFP–p24δ5 and GFP–p24β2 co-localize,

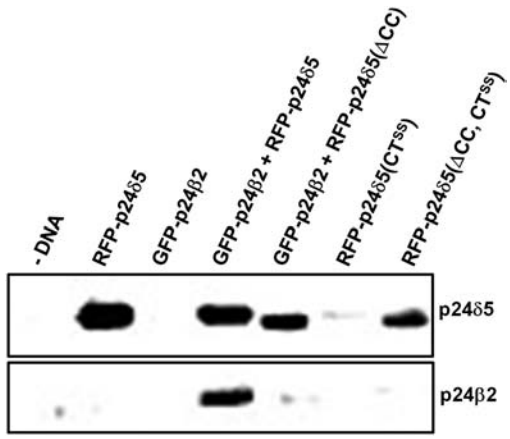

Fig. 6. Biochemical quantitation of the expression of the different constructs. Tobacco mesophyll protoplasts were electroporated in the absence (–DNA) or the presence of 30 μg of plasmid DNAs corresponding to RFP–p24δ5 (and mutant versions) and/or GFP–p24β2. At 24 h post-electroporation, protoplasts were washed, extracted in Laemmli sample buffer, and analysed by SDS–PAGE (12% acrylamide) and western blot analysis with antibodies against RFP (to detect p24δ5 and mutant versions) or the p24β2 C-terminus. Note the lower molecular weight of the RFP–p24δ5 constructs lacking the coiled-coil domain. A 30 μg aliquot of protein was loaded for each of the extracts.

RFP–p24δ5 and GFP–p24β2 were co-expressed with 6 kDa VP–CFP (cyan fluorescent protein), a putative COPII/ERES marker (Lerich *et al.*, 2011). As shown in Fig. 8J–M, a high degree of co-localization was observed between the three proteins in punctate structures, suggesting that a fraction of RFP–p24δ5 and GFP–p24β2 is present in COPII-labelled structures.

Whether the observed co-localization/interaction between RFP–p24δ5 and GFP–p24β2 correlated with their trafficking was next investigated. To this end, both proteins were co-expressed with the ARF1(Q71L) mutant, to interfere with retrograde Golgi to ER transport. This mutant expression has been shown to redistribute RFP–p24δ5 partially from the ER to Golgi-like punctae (Langhans *et al.*, 2008). However, when GFP–p24β2 was co-expressed with the ARF1(Q71L) mutant, it showed a prominent vacuolar labelling and did not co-localize with ManI–RFP (Fig. 9A–C). This suggests that GFP–p24β2 localizes to the Golgi at steady state but may also cycle between the ER and Golgi. Thus, when retrograde Golgi to ER transport is blocked, the protein is then transported by default to the vacuole. In contrast, when both RFP–p24δ5 and GFP–p24β2 were expressed together under the same conditions [ARF1(Q71L) mutant expression], GFP–p24β2 was not transported to the vacuole but co-localized in punctae with RFP–p24δ5 (Fig. 9D–F). This suggests that RFP–p24δ5 holds back GFP–p24β2 in the ER–Golgi interface. Since both proteins co-localize in punctate structures, it was speculated that some of these punctae may correspond to Golgi stacks. To check this, protoplasts were treated with brefeldin A (BFA). After 1 h treatment, a fraction of both proteins had redistributed to the ER, as is the case for standard Golgi marker proteins, for example ManI–(X)FP, which relocate to the ER under these conditions (Langhans *et al.*, 2011). In contrast, many of the punctate structures were BFA resistant (Fig. 9G–I) and could represent the fraction of both proteins present in COPII-labelled structures. However, it was possible to observe a redistribution of both proteins to the ER after 2 h of BFA treatment (Supplementary Fig. S7 at *JXB* online). Altogether, these experiments indicate that RFP–p24δ5 and GFP–p24β2 traffic together at the ER–Golgi interface.

Interaction between p24 proteins

Since the above experiments suggested an interaction between p24δ5 and p24β2, it was decided to investigate this possibility further. Previous studies where a single member of the p24 family has been deleted or knocked-down showed that the protein levels of other family members

ARF1(Q71L) mutant expression. (G and J) An RFP–p24δ5 dilysine mutant lacking the GOLD domain (ΔGOLD, CT^{SS}) shows a prominent vacuolar labelling but it also co-localizes partially in punctae with Golgi (ST–YFP; blue) or PVC (GFP–BP80; green) markers. (H and K) An RFP–p24δ5 dilysine mutant lacking the coiled-coil domain (ΔCC, CT^{SS}) shows a weak vacuolar labelling but it also co-localizes partially in punctae with Golgi (ST–YFP; blue) or PVC (GFP–BP80; green) markers. (I and L) An RFP–p24δ5 dilysine mutant with the transmembrane domain and the cytoplasmic tail (TM-CT^{SS}) co-localizes partially with the ER marker GFP–HDEL and the Golgi marker Man1–GFP. Scale bars=5 μm.

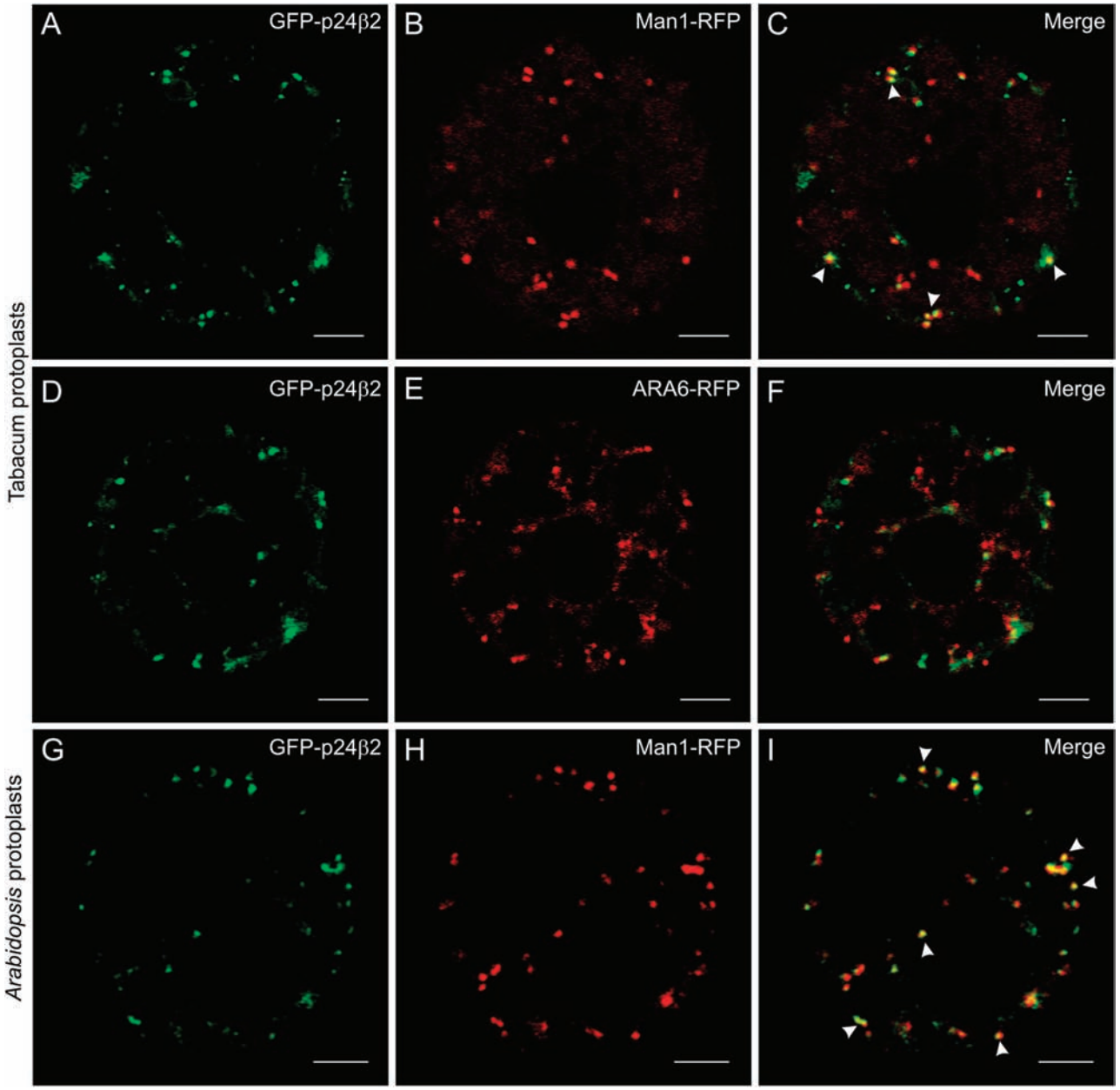

Fig. 7. Localization of GFP–p24β2. (A–F) Transient gene expression in tobacco mesophyll protoplasts. (A–C) GFP–p24β2 (A) co-localizes partially with the Golgi marker Man1–RFP (B) in punctate structures (merged image in C). (D–F) GFP–p24β2 (D) punctae do not co-localize with the PVC marker ARA6–RFP (E) (merged image in F). (G–I) Transient gene expression in *Arabidopsis* protoplasts. GFP–p24β2 (G) co-localizes partially with the Golgi marker Man1–RFP (H) in punctate structures (merged image in I). Arrowheads point to co-localizing signals. Scale bars=5 μm.

were reduced, which probably reflects the fact that those p24 proteins interact with each other in hetero-oligomeric complexes (Belden and Barlowe, 1996; Marzioch *et al.*, 1999; Denzel *et al.*, 2000; Vetrivel *et al.*, 2007; Takida *et al.*, 2008; Jerome-Majewska *et al.*, 2010; Koegler *et al.*, 2010; Zhang and Volchuk, 2010). Therefore, the protein levels of p24β2 in a T-DNA mutant lacking p24δ5 (*p24δ5-1*) were examined. To this end, protein extracts were analysed with Nt- and Ct-p24β2 antibodies. In both cases, it was found that the levels of p24β2 in the *p24δ5-1* mutant were drastically reduced (~70%) when compared with the wild type (Fig. 10A). These results indicate that the protein levels of p24β2 are dependent on p24δ5, suggesting an interaction between these proteins.

To test biochemically for a direct interaction between endogenous p24δ5 and p24β2, co-immunoprecipitation experiments were performed. To this end, affinity-purified antibodies were covalently bound to magnetic beads and were incubated in the presence of protein extracts from membrane fractions, as described in the Materials and methods. Proteins could be immunoprecipitated with the C-terminal antibodies. Under these conditions, the corresponding proteins could be

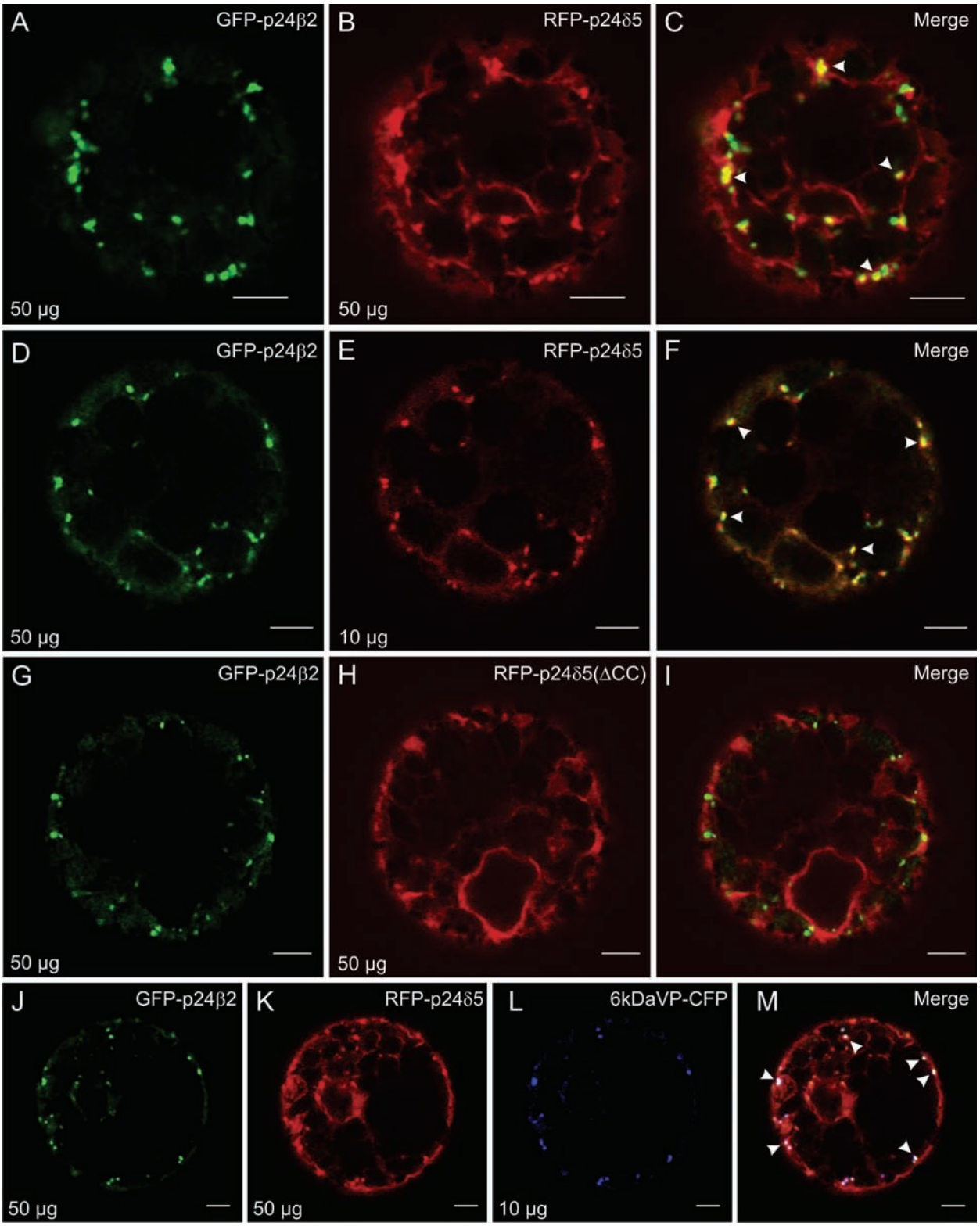

Fig. 8. GFP–p24β2 and RFP–p24δ5 co-localize in punctae via the coiled-coil domain. (A–M) Transient gene expression in tobacco mesophyll protoplasts. (A–F) GFP–p24β2 (A and D, 50 µg) and RFP–p24δ5 (B, 50 µg; E, 10 µg) co-localize in punctate structures (see merged images in C and F). (G–I) GFP–p24β2 (G) and RFP–p24δ5 (ΔCC) (H) do not co-localize (see merged image in I). (J–M) GFP–p24β2 (J) and RFP–p24δ5 (K) show extensive co-localization with 6 kDa VP–CFP (L) in punctate structures (see merged images in M). Arrowheads point to co-localizing signals. Scale bars=5 µm.

only immunoprecipitated with the C-terminal antibodies. As shown in Fig. 10B, antibodies against the C-terminus of p24δ5 immunoprecipitated p24δ5, as shown by western blot analysis with antibodies against the p24δ5 N-terminus. More importantly, immunoprecipitates containing p24δ5 also contained p24β2. The reverse was also true:

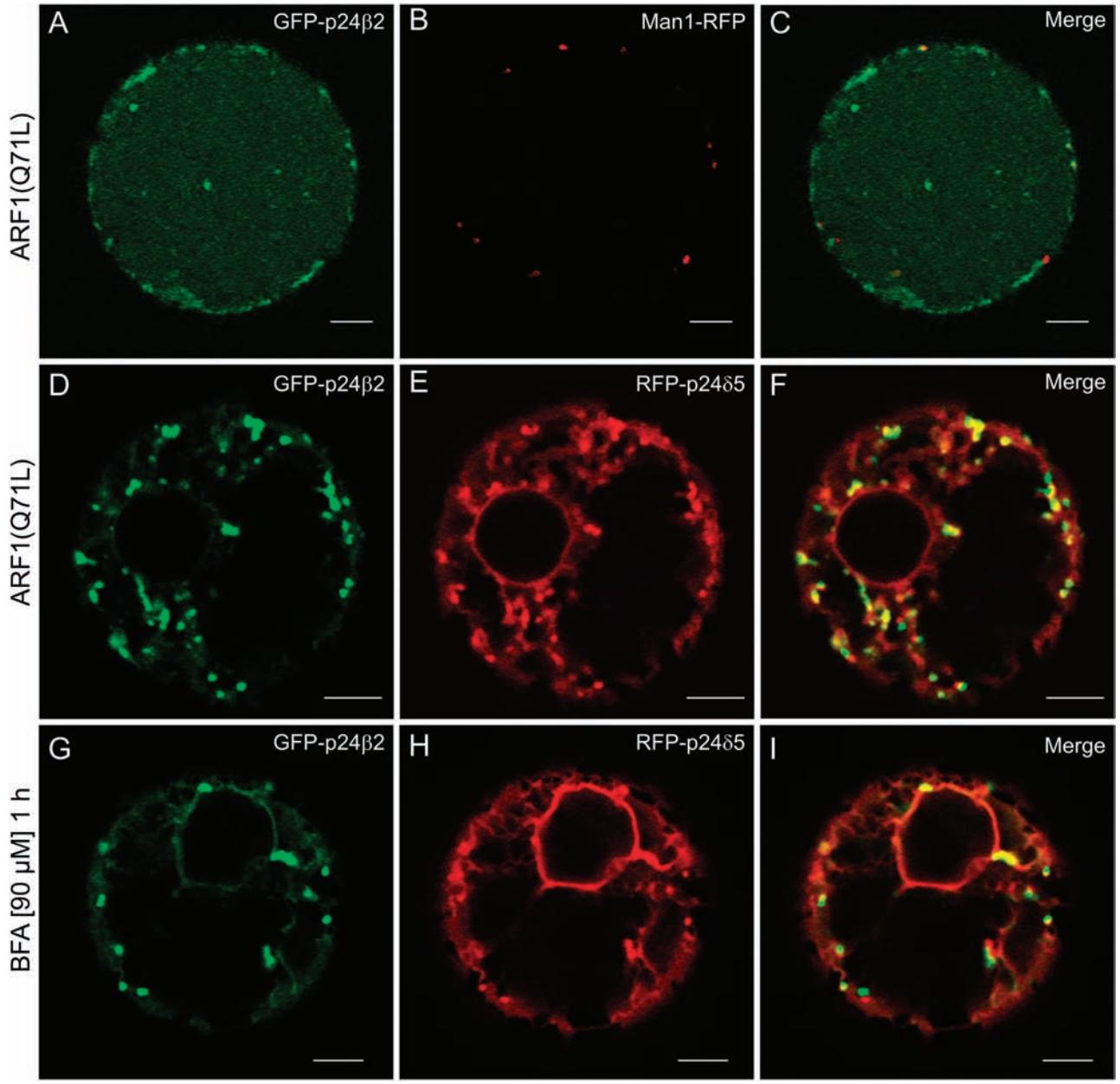

Fig. 9. Coupled trafficking of RFP–p24δ5 and GFP–p24β2 at the ER–Golgi interface. (A–F) Transient gene expression in tobacco mesophyll protoplasts. (A–C) GFP–p24β2 (A) is transported to the vacuole upon co-expression with the ARF1(Q71L) mutant and shows no co-localization with the Golgi marker Man1–RFP (B) (merged image in C). (D–F) GFP–p24β2 (D) is not transported to the vacuole upon co-expression of the ARF1(Q71L) mutant in the presence of RFP–p24δ5 (E) but both proteins co-localize in punctate structures (see merged image in F). (G–I) Treatment with BFA (1 h, 90 μM) after co-expression of GFP–p24β2 (G) and RFP–p24δ5 (H) induces a partial relocalization of both proteins to the ER, but many punctate structures still remain (merged image in I). Scale bars=5 μm.

antibodies against the C-terminus of p24β2 immunoprecipitated p24β2, and these immunoprecipitates also contained p24δ5 (Fig. 10B). These experiments again suggest that there is a direct interaction between p24δ5 and p24β2.

Discussion

The p24 family in Arabidopsis

In contrast to animals and fungi, plants posses only representatives of the p24δ and the p24β subfamilies (Carney and Bowen, 2004; Strating and Martens, 2009; Strating *et al.*, 2009). The p24α and p24δ subfamilies have a common origin, as is the case for the p24β and p24γ subfamilies. In most vertebrates, the p24α and p24γ subfamilies have expanded, whereas the p24β and p24δ subfamilies each have only a single member. Therefore, only one subfamily from each pair of evolutionarily related subfamilies (p24α/δ and p24β/γ) has expanded. This has led to the suggestion that there is a certain degree of functional redundancy within the two branches, which eliminates the need to expand both subfamilies. The fact that plants have

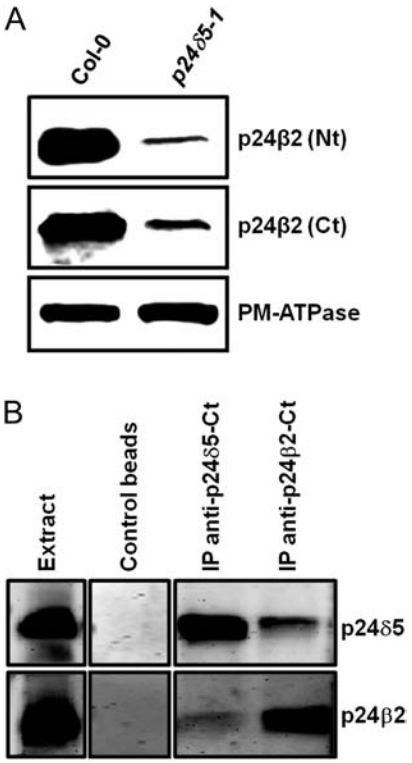

Fig. 10. Interaction between p24 proteins. (A) Western blot analysis showing the levels of p24β2 in membranes from the wild type (Col-0) or the p24δ5 knock-out mutant (*p24δ5-1*), using antibodies against the Nt or the Ct of p24β2. A 25 μg aliquot of protein was loaded in each lane. Western blot with an antibody against the plasma membrane (PM) ATPase was used as a loading control. (B) Immunoprecipitation of p24δ5 and p24β2 using affinity-purified antibodies against the Ct of both proteins or control beads, followed by SDS–PAGE and western blot with antibodies against p24δ5 (Nt) or p24β2 (Ct). The extract lane contains 20 μg of the membrane proteins used as input for the immunoprecipitation.

only members of one subfamily in each branch (p24β and p24δ) would be in support of this idea (Carney and Bowen, 2004; Strating and Martens, 2009; Strating *et al.*, 2009). In *Arabidopsis*, the delta subfamily contains nine different members (which herein have been named p24δ3–p24δ11), while the beta subfamily has only two members (here named p24β2 and p24β3). According to public microarray databases, five of them (p24δ4, p24δ5, p24δ9, p24β2, and p24β3) have high/medium levels of expression in different tissues. In contrast, other members of the delta subfamily have low and tissue-specific expression (Zimmermann *et al.*, 2004). It is thus possible that the first category includes members with a more general/housekeeping function, probably related to the maintenance of the secretory pathway, while the second category may include members with more tissue-specific functions. In this study, the focus was on the first category. To this end, p24δ5 was selected as a representative of the p24δ subfamily and p24β2 as a representative of the p24β subfamily (see Fig. 1A).

To gain some insight into the functions that p24 proteins may have in plants, T-DNA insertion mutants that were available in the SALK collection for p24δ4 and p24δ5 (no T-DNA insertion knock-out mutants for p24β2 are available in mutant collections) were characterized and also a double mutant *p24δ4δ5* was generated. The lack of a distinct phenotype in the mutants suggests that p24 proteins of the delta subfamily play redundant functions and thus multiple mutants are being obtained. In any case, the available mutants were useful to characterize the specificity of antibodies and to investigate if the levels of p24 proteins in *Arabidopsis* were interdependent.

Localization of Arabidopsis *p24 proteins*

To localize endogenous p24 proteins, specific antibodies against p24δ5 and p24β2 were used. Immunogold labelling on sections from cryofixed samples showed that endogenous p24δ5 localized to ER membranes (as revealed with both Nt- and Ct-p24δ5 antibodies) as well as to the *cis* side of the Golgi complex (more evident with Ct-p24δ5 antibodies). This localization is consistent with the localization of mammalian p25 (p24α), which is the only p24 family member which, in addition to *cis*-Golgi labelling also shows extensive ER localization (Wada *et al.*, 1991; Domínguez *et al.*, 1998; Lavoie *et al.*, 1999; Emery *et al.*, 2000, 2003). It is important to emphasize that although p24δ5 belongs to the delta subfamily, its cytosolic tail is reminiscent of that of mammalian p25 (which belongs to the alpha subfamily). This cytosolic tail has been shown to bind COPI with high affinity via a dilysine motif at the -3,-4 position (relative to the cytosolic C-terminus) (Contreras *et al.*, 2004*a*) and is important for efficient Golgi to ER retrograde transport (Langhans *et al.*, 2008). In contrast to p24δ5, endogenous p24β2 localizes mainly to the Golgi apparatus. This localization is consistent with the localization of members of the p24β subfamily, both in mammals (Domínguez *et al.*, 1998; Blum *et al.*, 1999; Gommel *et al.*, 1999; Emery *et al.*, 2000) and in yeast (Shimmöller *et al.*, 1995; Belden and Barlowe, 2001).

The localization of endogenous p24 proteins may depend, at least, on the sorting determinants present in the individual proteins as well as on the interactions with other p24 proteins. To investigate these two possibilities, RFP–p24δ5 and deletion mutants were transiently expressed, either individually or in combination with GFP–p24β2.

Sorting determinants in p24δ5

Tagging of p24 proteins with XFP between the signal sequence and the luminal domain produces constructs which faithfully reflect the trafficking dynamics and localization pattern of native p24 proteins (Blum *et al.*, 1999; Barr *et al.*, 2001; Majoul *et al.*, 2001; Gupta and Swarup, 2006; Simpson *et al.*, 2006; Blum and Lepier, 2008). Therefore, as in a previous investigation (Langhans *et al.*, 2008), the same strategy was used for all the constructs used in this study.

First, the relative contribution of the different domains of p24 proteins to their intracellular trafficking and localization

was investigated. For this project, p24δ5 was used as being representative of the family, since the sorting determinants in its cytosolic tail have previously been analysed. It was shown that the dilysine motif in the -3,-4 position was the main determinant for its ER localization. Here, the relative contributions of the luminal (GOLD and coiled-coil) domains were determined, using deletion mutants, in the presence or the absence of the dilysine motif in the -3,-4 position. The results show that neither the GOLD nor the coiled-coil domains are necessary for the ER localization of RFP–p24δ5 in the steady state or for the cycling of the protein in the early secretory pathway, at least when expresssed individually. The fact that deletion mutants having only the transmembrane domain and the C-terminal tail localize correctly to the ER, unless the dilysine motif is mutated, strongly implicates the dilysine motif in the cytosolic tail as being the main determinant for the ER localization of RFP–p24δ5, presumably by facilitating its interaction with the COPI machinery. In clear contrast, the luminal domain (in particular the coiled-coil domain) seems to be necessary for transport of p24 dilysine mutants to the vacuole lumen. Since cargoes for plant p24 proteins have not yet been identified (as has been the case for many years for their mammalian and yeast counterparts), it cannot be ruled out that a fraction of p24δ5 (or other p24 proteins) could be involved in the transport of cargo destined for the PVC or the vacuole. Alternatively, transport to the vacuole may simply be a default pathway for membrane proteins in the secretory pathway (Langhans *et al.*, 2008).

The relative contribution of the different p24δ5 domains to trafficking of the p24δ5 protein shown here is consistent with results obtained using similar GFP constructs for p23 (p24δ1) in animal cells. Blum and Lepier (2008) showed that the minimal requirement for p23 cycling within the early secretory pathway was the transmembrane domain and the cytoplasmic tail with an intact KKLIE motif. The luminal domain was expendable for cycling between the ER and the Golgi apparatus, but was necessary for trafficking beyond the Golgi apparatus, in their case to the cell surface. In another study, however, the location of a p23/p24 dimer was shown to be independent of the KKLIE motif in p23 but instead required the coiled-coil domains in both proteins (Emery *et al.*, 2000). It has to be noted that, in contrast to p23 (p24δ1), p24δ5 has a p25 (p24α)-like C-terminal tail, with high affinity for COPI (Contreras *et al.*, 2004*a*), which is responsible for its efficient Golgi to ER recycling (Langhans *et al.*, 2008). Therefore, it is likely that the sorting information contained in the cytosolic tail of RFP–p24δ5 is sufficient for its steady-state distribution in the ER. This scenario has also been proposed for mammalian p25, which normally resides in the ER, even when co-expressed with p23, p24, and p26 (Emery *et al.*, 2000).

Interactions between p24 proteins and coupled transport of p24 family members

Shuttling of p24 proteins in the ER–Golgi interface has been proposed to depend on interactions with other p24 family members (Dominguez *et al.*, 1998; Füllekrug *et al.*, 1999; Emery *et al.*, 2000). Early experiments concluded that p24 proteins form heterotetrameric complexes via their coiled-coil domains with one member of each subfamily, both in animals (p23, p24, p25, and p27) (Fullerkrug *et al.*, 1999) and in yeast (Erv25p, Emp24p, Erp1p, and Erp2p) (Marzioch *et al.*, 1999). However, the pattern of interdependence between different p24 members seen in yeast raises the possibility that the postulated p24 tetramer is a double dimer (Ciufo and Boyd, 2000). Moreover, the studies of Jenne *et al.* (2002) have shown that p24 proteins occur mostly as monomers and dimers of various compositions, depending on their subcellular location. In most cases, the best characterized dimers are the ones formed between one member of the p24δ subfamily and one member of the p24β subfamily. In yeast, Erv25 (p24δ subfamily) and Emp24 (p24β subfamily) have been shown to form a complex which is efficiently incorporated into ER-derived COPII vesicles and can exit the ER without the presence of Erp1 and Erp2, putative components of the yeast tetrameric p24 complex (Belden and Barlowe, 1996, 2001). In animals, a complex is formed between p23 (p24δ subfamily) and p24 (p24β subfamily) which can also exit the ER to be transported to the Golgi apparatus (Gommel *et al.*, 1999; Emery *et al.*, 2000). In both cases, retrograde transport of these complexes was postulated to depend on the dilysine motif present in the p24δ members: Erv25 in yeast (Belden and Barlowe, 2001) and p23 in animals (Gommel *et al.*, 1999).

Several lines of evidence suggest that *Arabidopsis* p24δ5 and p24β2 interact with each other, and that this interaction is mediated by the coiled-coil domain. First, the protein levels of p24β2 seem to be dependent on p24δ5: a knock-out mutant lacking p24δ5 showed a drastic reduction in the levels of p24β2. This is consistent with previous reports in yeast and mammalian cells showing that depletion of one member of the p24 family affects the protein levels of other family members, suggesting interaction between those proteins (Belden and Barlowe, 1996; Marzioch *et al.*, 1999; Denzel *et al.*, 2000; Vetrivel *et al.*, 2007; Takida *et al.*, 2008; Jerome-Majewska *et al.*, 2010; Koegler *et al.*, 2010; Zhang and Volchuk, 2010). Secondly, our co-immunoprecipitation data indicate that endogenous p24δ5 and p24β2 interact with each other: immunoprecipitation with p24δ5 antibodies is able to co-immunoprecipitate p24β2, and vice versa.

Transiently expressed proteins also seem to interact with each other. When expressed individually, GFP–p24β2 seems to be unstable, since the protein is hardly detectable either by confocal laser scanning microcopy or after western blot analysis. However, the levels of GFP–p24β2 increase enormously upon co-expression with RFP–p24δ5, an effect which is not seen when RFP–p24δ5 lacks the coiled-coil domain. This suggests that the interaction with p24δ5, via the coiled-coil domains, stabilizes p24β2. In contrast, transiently expressed RFP–p24δ5 seems to be stable in the absence of co-expressed GFP–p24β2, probably as a consequence of its predominant localization to the ER. In addition, the co-expression of GFP–p24β2 changes the pattern of

RFP–p24δ5 from its typical reticulate ER pattern to punctae where both proteins co-localize. This does not occur when p24δ5 lacks the coiled-coil domain.

Finally, the two proteins seem to travel together along the early secretory pathway. RFP–p24δ5 and GFP–p24β2 may be efficiently incorporated into ERESs, as suggested by their significant co-localization with the COPII marker 6 kDa VP–CFP, for transport to the Golgi. When retrograde Golgi to ER transport is inhibited by expression of the ARF1(Q71L) mutant, GFP–p24β2 is transported downstream to the vacuole, where it appears to be degraded. In contrast, when RFP–p24δ5 is co-expressed with GFP–p24β2, the latter is not transported to the vacuole, but both proteins co-localize to punctate structures. This suggests that RFP–p24δ5 is able to hold GFP–p24β2 at the ER–Golgi interface, and possibly to mediate coupled trafficking of both proteins back to the ER. Therefore, it is possible that the steady-state localization found for endogenous proteins and their stability reflects the tight balance between the levels of these two proteins (or of other members of the family).

In summary, the results presented herein are consistent with a coupled trafficking of both proteins at the ER–Golgi interface. p24δ5 and p24β2 could interact with each other at ERESs for ER exit and coupled transport to the Golgi apparatus. Once in the Golgi, p24δ5 could interact very efficiently with the COPI machinery for retrograde transport back to the ER.

Addendum

While this manuscript was undergoing review, a paper was published showing trafficking and localization of p24 proteins in *Arabidopsis* (Chen *et al.*, 2012), which overlaps with some of the results presented herein. Using transient expression in tobacco leaf epidermal cells, these authors propose a subclass-specific localization for *Arabidopsis* p24 proteins: p24δ1a–d (p24δ3–6 in this study) are found to localize exclusively to the ER, p24δ2a–d (p24δ7–11 in this study) localize to ER and Golgi, and p24β proteins localize exclusively to the Golgi apparatus. As representatives of each subclass for trafficking studies, they chose p24δ1d (p24δ6 in this study), p24δ2d (p24δ10 in this study), and p24β1 (p24β2 in this study). Surprisingly, these authors maintain that the steady-state localization of these proteins is not dependent on the position of the XFP tag. There are also significant discrepancies with the results presented here, which it is felt could be explained by this fact: p24 proteins have a very short cytosolic C-terminal tail, which contains the sorting signals for COPI/COPII binding. In the case of p24δ proteins, these signals consist of a dilysine motif at the -3,-4 position and a diaromatic motif at the -7,-8 position. Indeed, it has been shown that the position of these signals (with respect to the C-terminus) is important for optimal binding of COP subunits (Teasdale and Jackson, 1996). Accordingly, it is difficult to imagine that the presence of an XFP molecule at the C-terminus has no influence in trafficking and localization of the fusion proteins, even if they might be still partially functional. This is the reason why p24–XFP constructs used in mammalian research (Blum *et al.*, 1996; Majoul *et al.*, 2001; Simpson *et al.*, 2006; Blum and Lepier, 2008), as well as in a previous paper on plant p24 proteins (Langhans *et al.*, 2008) and in this study, have the XFP tag at the luminal N-terminus (immediately behind the signal sequence). The most striking differences between both studies can be summarized as follows. First, although transiently expressed RFP–p24δ5 localizes exclusively to the ER (as do all the members of the p24δ1 subfamily in the study by Chen *et al.*), endogenous p24δ5 (and possibly p24δ4) localizes to the ER but also to the *cis*-Golgi, suggesting that the steady-state localization of endogenous p24 proteins and their stability reflect the tight balance between the levels of the different members of the family. In this respect, the present study represents the first report on the localization of endogenous p24 proteins, through immunogold electron microscopy, in *Arabidopsis*.

Secondly, when RFP–p24δ1d (p24δ6 in this study) was co-expressed with p24β1–YFP (Ct-fusion) (p24β2 in this study), there was no change in the localization of RFP–p24δ1d. This is in marked contrast to the present co-expression data showing that GFP–p24β2 (Nt-fusion) changes the localization of RFP–p24δ5 (a close homologue of p24δ1d) and this requires the coiled-coil domain. Thirdly, Chen *et al.* showed that when p24β1–YFP (Ct-fusion) (p24β2 in this study) was co-expressed with the ARF1(Q71L) mutant, to interfere with Golgi to ER transport, the protein was still Golgi localized and only occasionally was found at the PVC (never at the vacuole). In the present study, when this protein was co-expressed with the ARF1 mutant, it was transported to the vacuole, unless it was co-expressed with RFP–p24δ5. This suggests that RFP–p24δ5 holds GFP–p24β2 at the ER–Golgi interface and may explain why transiently expressed GFP–p24β2 is not stable in the absence of RFP–p24δ5. Finally, the present study shows the first biochemical demonstration of interactions between *Arabidopsis* p24 proteins. In summary, while both studies are somehow complementary, there are a number of discrepancies which need to be resolved.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Nt- and Ct-sequences of *Arabidopsis* p24 proteins.

Figure S2. Characterization of T-DNA insertion mutants.

Figure S3. Localization of RFP–p24δ5 and deletion mutants and co-localization between GFP–p24β2 and RFP–p24δ5 or RFP–p24δ5(ΔCC) in *Arabidopsis* protoplasts.

Figure S4. RFP–p24δ5 and RFP–p24δ5 (ΔCC) localize to the ER at different expression levels.

Figure S5. Co-expression of RFP–p24δ5 and different DNA concentrations of GFP–p24β2.

Figure S6. Co-expression of RFP–p24δ5(ΔCC) and different DNA concentrations of *GFP–p24*β2.

Figure S7. A 2 h BFA treatment redistributes RFP–p24δ5 and GFP–p24β2 to the ER.

Figure S8. A comparison between frame scan and line scan modes for co-localization studies.

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