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RESEARCH ARTICLE

SPECIAL ISSUE: PLANT CELL BIOLOGY

Loss of *Arabidopsis* p24 function affects ERD2 trafficking and Golgi structure, and activates the unfolded protein response

Noelia Pastor-Cantizano, Cesar Bernat-Silvestre, María Jesús Marcote* and Fernando Aniento*

ABSTRACT

The p24 family of proteins (also known as the TMED family) are key regulators of protein trafficking along the secretory pathway, but very little is known about their functions in plants. A quadruple loss-of-function mutant affecting the p24 genes from the δ-1 subclass of the p24δ subfamily (*p24δ3δ4δ5δ6*) showed alterations in the Golgi, suggesting that these p24 proteins play a role in the organization of the compartments of the early secretory pathway in *Arabidopsis*. Loss of p24δ-1 proteins also induced the accumulation of the K/HDEL receptor ERD2a (ER lumen protein-retaining receptor A) at the Golgi and increased secretion of BiP family proteins, ER chaperones containing an HDEL signal, probably due to an inhibition of COPI-dependent Golgi-to-ER transport of ERD2a and thus retrieval of K/HDEL ligands. Although the *p24δ3δ4δ5δ6* mutant showed enhanced sensitivity to salt stress, it did not show obvious phenotypic alterations under standard growth conditions. Interestingly, this mutant showed a constitutive activation of the unfolded protein response (UPR) and the transcriptional upregulation of the COPII subunit gene *SEC31A*, which may help the plant to cope with the transport defects seen in the absence of p24 proteins.

KEY WORDS: p24 family, K/HDEL receptor, *Arabidopsis*, Unfolded protein response, Golgi

INTRODUCTION

p24 proteins, also known as TMED proteins, constitute a family of ∼24 kDa, type I transmembrane proteins, which have long been shown to cycle between the endoplasmic reticulum (ER) and the Golgi via coat protein I (COPI) and COPII vesicles (Pastor-Cantizano et al., 2016). They can be classified, by sequence homology, into four subfamilies, named α, β, γ and δ (Dominguez et al., 1998). The four p24 subfamilies are present in yeast and all animals. Phylogenetic analysis of vertebrate p24 proteins has shown that the p24α and p24δ subfamilies have a common origin, as it is also the case for p24β and p24γ subfamilies (Strating et al., 2009). In most vertebrates, the p24α and p24γ subfamilies have several members, while the p24β and p24δ subfamilies contain only one single member. The situation is clearly different in plants, which seem to have only members of the p24β and p24δ subfamilies. While the yeast, *Drosophila* and vertebrate p24 subfamilies have expanded independently, the plant p24δ subfamily seems to have greatly expanded independently from the fungi/animals (Strating et al., 2009). In the case of *Arabidopsis*, the δ subfamily contains nine members (named p24δ3 to p24δ11), which have been proposed to belong to two different subclasses, the δ-1 subclass (including p24δ3 to p24δ6) and the δ-2 subclass (including p24δ7 to p24δ11) (Chen et al., 2012). In addition, *Arabidopsis* contains two members of the p24β subfamily, p24β2 and p24β3 (Montesinos et al., 2012; Pastor-Cantizano et al., 2016).

Although p24 proteins have been extensively characterized in mammals, yeast and, more recently, in plants, their specific functions are still far from being understood. In addition, striking differences have been observed among different organisms. In yeast, a mutant strain lacking all p24 family members had only very mild phenotypic alterations, which has been explained by the activation of the unfolded protein response (UPR) (Aguilera-Romero et al., 2008; Belden and Barlowe, 2001). In clear contrast, the knockout of a single p24 family member (p23 or p24δ1; *Tmed10*) in mice is lethal in an early embryonic stage (Denzel et al., 2000). p24 proteins have long been proposed to be involved in transport of cargo along the secretory pathway. However, most putative p24 cargoes described so far appear to be lipid-linked or membrane proteins, rather than soluble proteins (Pastor-Cantizano et al., 2016). In particular, p24 proteins have been shown to be essential for transport from the ER to the plasma membrane of glycosylphosphatidylinositol (GPI)-anchored proteins, both in mammals and in yeast (for recent reviews see Kinoshita et al., 2013; Muñiz and Zurzolo, 2014; Muñiz and Riezman, 2016). p24 proteins have also been shown to be involved in the transport of Wnt proteins (Port et al., 2011; Buechling et al., 2011; Li et al., 2015), secreted lipid-modified glycoproteins that control animal development and G-protein-coupled receptors (GPCRs), including protease-activated receptors, nucleotide receptors and a μ-opioid receptor (Luo et al., 2007, 2011). In plants, a p24 protein named CYB (because of its accumulation in cytoplasmic bodies; it corresponds to p24δ4) has been proposed to function as a cargo receptor for ER-export of GLL23, a putative myrosinase-associated protein, probably to prevent its accumulation at the ER, where it can be detrimental for ER organization (Jancowski et al., 2014).

p24 proteins have a well-characterized function in the biogenesis of COPI vesicles from Golgi membranes and therefore in retrograde Golgi-to-ER transport. This way, they facilitate efficient retrieval of ER-resident proteins (for reviews see Popoff et al., 2011; Jackson, 2014; Pastor-Cantizano et al., 2016). It has been proposed that dimers of p24 proteins (probably including members of the p24δ and p24β subfamilies) first interact with the soluble (GDP-bound) form of ADP ribosylation factor 1 (ARF1-GDP). GDP/GTP exchange, catalyzed by guanine nucleotide exchange factors (ARF1 GEFs), then activates ARF1 and allows its dissociation from p24 proteins and its insertion into Golgi membranes. Both p24 proteins (via dilysine and diaromatic signals in their cytosolic tail) and ARF1-GTP can interact with components of the COPI complex (coatomer), which is made of seven subunits (α-, β-, β′-, γ-, δ-, ε-

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and ζ-COP), which are recruited *en bloc* onto Golgi membranes. Coatomer polymerization drives the formation of COPI vesicles (Popoff et al., 2011; Jackson, 2014; Pastor-Cantizano et al., 2016). p24 proteins can also interact with several members of the ERD2 (ER lumen protein-retaining receptor) family of K/HDEL receptors (herein, ERD2 refers to the family of proteins; ERD2a/b is specifically used when that protein is meant), which facilitates sorting of ERD2 within COPI vesicles (Majoul et al., 1998, 2001; Montesinos et al., 2014). Rather than being a passive passenger, ERD2 seems to also be part of the machinery involved in COPI vesicle formation. Binding of K/HDEL ligands to ERD2 at the acidic pH of the *cis*-Golgi (Wilson et al., 1993; Scheel and Pelham, 1996) induces ERD2 oligomerization (Majoul et al., 2001), as well as its interaction with ARF1 and ARF1 GTPase-activating protein (ARF1-GAP), which also seems to be involved in vesicle formation (Aoe et al., 1997; Majoul et al., 2001). ERD2, as is the case for ARF1 or p24 proteins, can also interact with coatomer, thus contributing to the formation of COPI vesicles (Majoul et al., 2001; Montesinos et al., 2014). In *Arabidopsis*, p24 proteins of the δ subfamily have been shown to interact both with ARF1-GDP and with coatomer subunits via dilysine and diaromatic motifs in their cytoplasmic tail (Contreras et al., 2004). More recently, we have found that p24δ5 and p24δ9 can also interact with the K/HDEL receptors ERD2a and ERD2b at the Golgi, facilitating their COPI-dependent Golgi-to-ER transport and thus retrieval of K/HDEL ligands (Montesinos et al., 2014).

p24 proteins have also been proposed to play a fundamental role in the organization of the compartments of the early secretory pathway. In particular, several reports have suggested the involvement of p24 proteins in the formation of ER export sites, the structure of the ER and the ER–Golgi intermediate compartment (ERGIC), and the biogenesis and maintenance of the Golgi (Blum et al., 1999; Lavoie et al., 1999; Denzel et al., 2000; Rojo et al., 2000; Mitrovic et al., 2008; Koegler et al., 2010). In addition, p24 proteins have received a lot of attention recently because they seem to be involved in a variety of functions relevant to animal physiology and pathology, including development, apoptosis, insulin secretion, diabetes and Alzheimer disease (for a recent review see Pastor-Cantizano et al., 2016). In contrast, very little is known about specific functions of p24 proteins in plants. In this manuscript, we have conducted a loss-of-function approach to investigate the function of p24 proteins in *Arabidopsis*. In particular, we have generated a quadruple loss-of-function mutant affecting all genes of the p24δ-1 subclass. This mutant showed a strong decrease in the protein levels of other p24 family members (including p24β subfamily members), but no obvious phenotypic alterations under standard growth conditions. However, it showed a clear alteration in the structure of the Golgi complex, an accumulation of the K/HDEL receptor ERD2 at the Golgi and increased secretion of the ER chaperone BiP (immunoglobulin heavy-chain binding protein), an HDEL ligand. In addition, this mutant showed a constitutive activation of the unfolded protein response (UPR) and the transcriptional upregulation of the COPII subunit *SEC31A*, which may explain the absence of major phenotypic alterations in the mutant.

RESULTS

Expression analysis of p24 family genes in *Arabidopsis*

Several previous studies have examined the expression of a limited number of p24 proteins in specific tissues (Denzel et al., 2000; Kuiper et al., 2000; Hosaka et al., 2007; Vetrivel et al., 2008; Xie et al., 2014). To our knowledge, only three studies have examined in detail the expression of p24 family members in whole organisms, one in *Xenopus* (Rötter et al., 2002), one in *Drosophila* (Boltz et al., 2007) and another in mouse (Strating et al., 2009). These studies showed that most p24 proteins are ubiquitously expressed, although a few of them are expressed in a tissue-specific manner and show regulated expression.

In *Arabidopsis*, five out of the eleven p24 genes (*p24δ4*, *p24δ5*, *p24δ9*, *p24β2* and *p24β3*) have high or medium levels of expression in different organs, according to public microarray databases. In contrast, *p24δ6* and *p24δ11* have floral tissue-specific expression (Zimmermann et al., 2004). To verify the microarray data and to extend the study to the complete p24 family, the mRNA expression levels of all p24 genes were analyzed by RT-PCR. As shown in Fig. 1, we found that *p24δ4*, *p24δ5*, *p24δ7*, *p24δ9*, *p24δ10*, *p24β2* and *p24β3* genes are widely expressed. *p24δ3* expression was observed in all organs examined except in flowers. In contrast, expression of *p24δ6* and *p24δ8* was mostly detected in flowers and siliques, and *p24δ11* expression was only observed in flowers, with no expression found in the other organs examined. Therefore, most

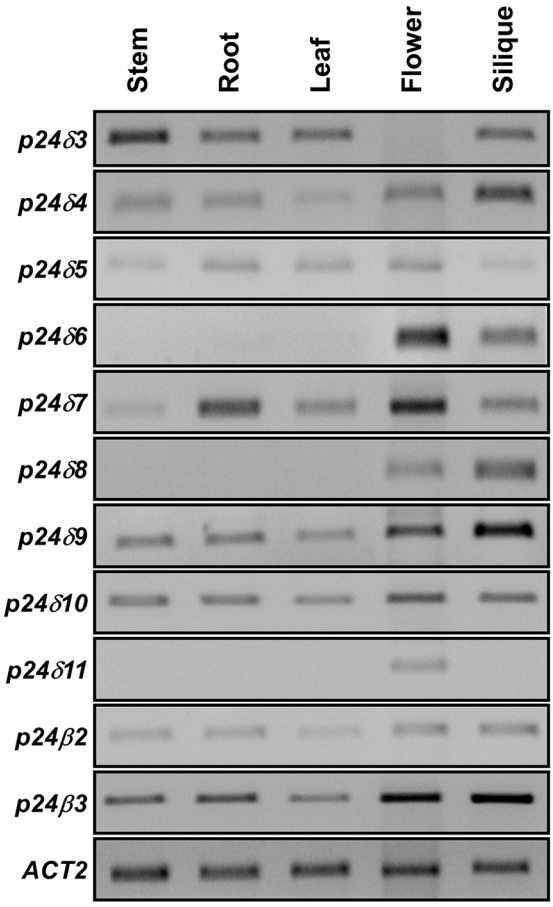

Fig. 1. Expression of the p24 family genes in *Arabidopsis* organs as determined by RT-sqPCR. Different organs from wild-type plants grown 45 days in soil were used. Total RNA was obtained from stem (between the first and the second internode), root, rosette leaves, flowers (at stage 13) and siliques (at stage 17). In the sqPCRs, gene-specific primers were used (Table S2). *ACT2* was used as a control for gene expression in the different *Arabidopsis* organs. The PCRs were performed as described in Materials and Methods and PCR samples were collected at cycle 26 for *ACT2*, at cycle 28 for *p24δ10*, *p24β2* and *p24β3*, at cycle 30 for *p24δ9*, at cycle 32 for *p24δ3*, *p24δ5*, *p24δ6*, *p24δ7* and *p24δ8*; and at cycle 34 for *p24δ4* and *p24δ11*. Results are representative of three independent experiments.

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p24 genes are widely expressed in *Arabidopsis*, which indicates that these genes may play a housekeeping function. However, the restricted expression patterns of three genes of the p24δ subfamily, *p24δ6*, *p24δ8* and *p24δ11*, may reflect specialized functions for the p24 proteins coded by these genes in floral tissues. To study the role of the p24 proteins, a reverse genetic approach was chosen. Previous results have shown that single mutants affecting two members of the p24δ-1 subclass, *p24δ4* and *p24δ5*, also had reduced protein levels of other members of the p24 family, including p24δ9 (p24δ-2 subclass) and the two members of the p24β subfamily (Montesinos et al., 2013). Therefore, we decided in this study to produce a quadruple mutant affecting all members of the p24δ-1 subclass (*p24δ3* to *p24δ6*).

Generation of a *p24δ3δ4δ5δ6* (δ-1 subclass) quadruple mutant

Previously, single knockout (KO) mutants of p24δ4 and p24δ5 were identified in our laboratory (Montesinos et al., 2012). T-DNA insertion mutants of p24δ3 and p24δ6 were now identified in the SALK collection (http://signal.salk.edu/cgi-bin/tdnaexpress), corresponding to the stock numbers GK_029E10 and GK_823G03, respectively. RT-PCR analysis confirmed that the *p24δ3* and *p24δ6* mutants lacked the full-length transcript of *p24δ3* and *p24δ6*, respectively (Fig. S1). To obtain the quadruple mutant, double mutants were first generated by crossing single mutants (Table S1). Then, triple mutants were generated by crossing double mutants sharing one allele. Finally, the *p24δ3δ4δ5δ6* quadruple mutant was generated by crossing *p24δ3δ4δ5* and *p24δ4δ5δ6* mutants. Genotype analysis of the progenies was performed by PCR in order to obtain the homozygous lines for the multiple mutants. RT-PCR analysis showed that the quadruple *p24δ3δ4δ5δ6* mutant lacked the full-length transcript of *p24δ3*, *p24δ4*, *p24δ5* and *p24δ6* (Fig. 2A). No obvious phenotypic differences were observed in the *p24δ3δ4δ5δ6* mutant when compared with wild-type plants under standard growth conditions (Fig. 2B,C), suggesting that p24 proteins of the δ-1 subclass are not necessary for growth under these conditions in *Arabidopsis*. However, we found that the *p24δ3δ4δ5δ6* mutant showed enhanced sensitivity to salt stress (Fig. 2D).

Loss of p24δ-1 proteins causes a reduction in the protein levels of other p24 family members

In a large number of previous studies where a single member of the p24 family had been deleted or knocked down, a reduction in the protein levels of other p24 proteins was observed. These studies showed that depletion of one p24 family member affects the stability of other members of the p24 family, which probably reflects the fact that these p24 proteins interact with each other in hetero-oligomeric complexes (Belden and Barlowe, 1996; Füllekrug et al., 1999; Marzioch et al., 1999; Denzel et al., 2000; Vetrivel et al., 2007; Takida et al., 2008; Koegler et al., 2010; Jerome-Majewska et al., 2010; Zhang and Volchuk, 2010; Fujita et al., 2011; Theiler et al., 2014).

In *Arabidopsis*, previous studies performed in our group showed that KO mutants or lines with a reduced expression of single p24 family members had reduced protein levels of other p24 family members (Montesinos et al., 2012, 2013). We now analyzed how the deletion of the four p24δ-1 subclass members affects the levels of other p24 proteins. To this end, protein extracts from roots of wild-type and the *p24δ3δ4δ5δ6* mutant were obtained and analyzed by western blotting with the available antibodies for p24 proteins in *Arabidopsis* (p24δ5, p24δ9, p24β2 and p24β3) (Montesinos et al., 2012). As expected, the *p24δ3δ4δ5δ6* mutant lacked p24δ5, but also showed a dramatic reduction in the protein levels of p24δ9 (p24δ-2 subclass), p24β2 and p24β3 compared with the wild-type (Fig. 3A).

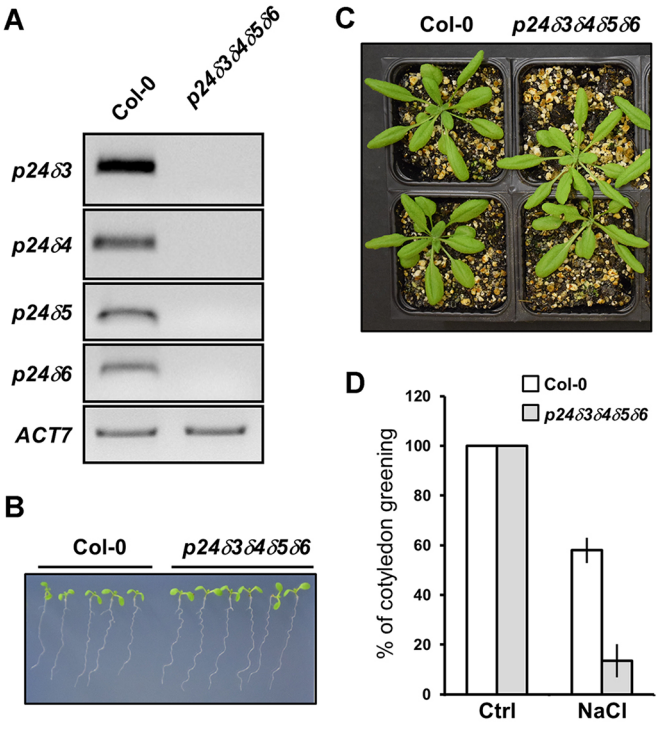

Fig. 2. Characterization of the quadruple *p24δ3δ4δ5δ6* mutant. (A) RT-sqPCR analysis to show the absence of the *p24δ-1* mRNA in *p24δ3δ4δ5δ6* mutants. Total RNA from the quadruple T-DNA insertion mutant *p24δ3δ4δ5δ6* and wild-type (Col-0) 7-day-old seedlings were used for RT-sqPCR. In the PCRs, specific primers for *p24δ3*, *p24δ4*, *p24δ5* and *p24δ6* were used (Table S1). *ACT7* was used as a control. The PCRs were performed as described in Materials and Methods, and PCR samples were collected at cycle 22 for *ACT7* and at cycle 36 for *p24δ3*, *p24δ4*, *p24δ5* and *p24δ6*. In wild-type seedlings, cDNA fragments at the expected molecular mass were amplified. In contrast, no fragments were amplified in the mutant. (B) Wild-type and *p24δ3δ4δ5δ6* mutant seedlings grown on MS agar plates for 7 days. (C) 3-week-old plants of wild-type and the *p24δ3δ4δ5δ6* mutant. (D) Phenotypic analysis of *p24δ3δ4δ5δ6* exposed to salt (NaCl) stress. Wild-type (Col-0) and *p24δ3δ4δ5δ6* seeds were sown on 0.5× MS for control (Ctrl) conditions and 0.5× MS supplemented with 160 mM NaCl in Petri plates. The percentage of seedlings with green cotyledons was calculated after 12 days. Data are mean±s.e.m. (n=120) of three independent experiments.

Indeed, these proteins were almost undetectable in this mutant. To check whether the decrease in p24 protein levels found in the mutant correlated with a decrease in mRNA levels, these were analyzed by RT-PCR. As shown in Fig. 3B, no reduction of the p24 mRNA levels was observed in the quadruple mutant for any of the p24 genes.

Structure of the compartments of the secretory pathway in the *p24δ3δ4δ5δ6* quadruple mutant

Several reports have suggested that p24 proteins may be involved in the structure and organization of the compartments of the early secretory pathway, including the formation of ER exit sites, the structure of the ER and the ERGIC, and the biogenesis and maintenance of the Golgi (for a review see Pastor-Cantizano et al., 2016). In order to investigate possible alterations of the compartments of the early secretory pathway in the *p24δ3δ4δ5δ6* mutant, marker proteins of different organelles were transiently expressed in protoplasts obtained from *p24δ3δ4δ5δ6* plants, and the steady-state location of these markers was analyzed by confocal laser scanning microscopy (CLSM). To analyze a possible alteration of the ER structure, protoplasts obtained from *p24δ3δ4δ5δ6* plants were transiently transformed with GFP–HDEL (Haseloff et al., 1997), which contains a HDEL signal that

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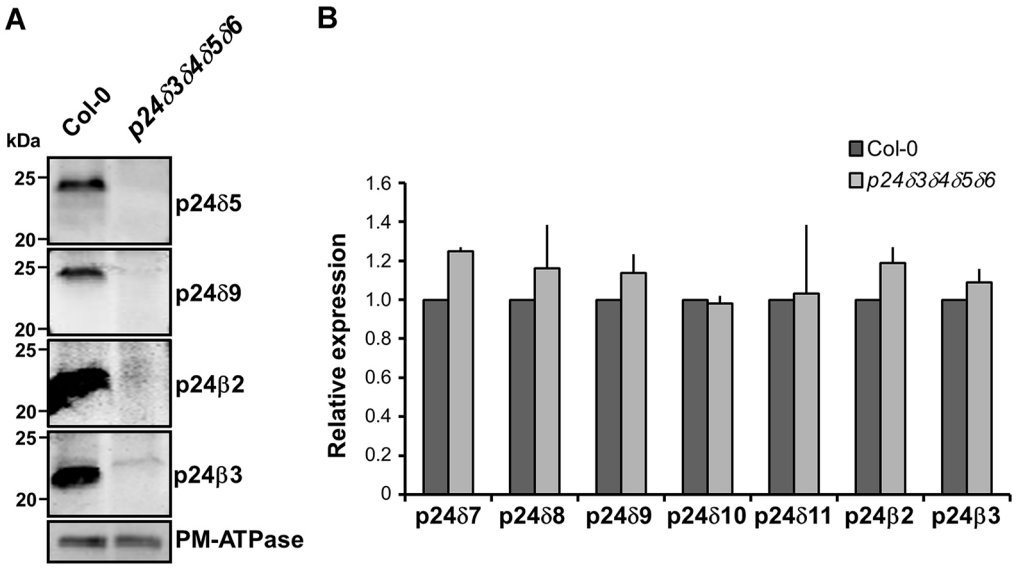

Fig. 3. p24 protein levels are interdependent. (A) Western blot analysis of protein extracts from membranes of wild-type (Col-0) and *p24δ3δ4δ5δ6* with antibodies against the N-terminus of p24δ5, p24δ9 or p24β3, or the C-terminus of p24β2. The positions of molecular mass markers are indicated on the left. A 25 µg aliquot of protein was loaded in each lane. Western blotting with an antibody against the plasma membrane (PM)-ATPase was used as a loading control. (B) RT-qPCR analysis of *p24δ-2* gene expression in *p24δ3δ4δ5δ6*. Total RNAs were extracted from 4-day-old seedlings (for *p24δ7*, *p24δ9*, *p24δ10*, *p24β2* and *p24β3*) and inflorescences of 40-day-old plants (for *p24δ8* and *p24δ11*). The mRNA was analyzed by RT-qPCR with specific primers for the corresponding genes, and normalized to *UBIQ10* gene expression (Table S3). mRNA levels are expressed as relative expression levels, and represent fold change for mutant over wild type. Values represent mean±s.e.m. of at least three biological samples.

results in retention of soluble proteins within the ER in plant cells and is used as an ER marker. As shown in Fig. 4A,B, loss of p24 proteins of the δ-1 subclass did not produce any significant change in the pattern of GFP–HDEL. This was also the case for GFP–BP80, a marker of the prevacuolar compartment (PVC) (daSilva et al., 2005), which showed its typical punctate pattern, both in protoplasts from wild-type plants and in the *p24δ3δ4δ5δ6* mutant (Fig. 4C,D). To investigate a possible alteration of the Golgi, protoplasts from the *p24δ3δ4δ5δ6* mutant were transiently transformed with mannosidase I–GFP (ManI–GFP), which localize to the *cis* side of the Golgi apparatus (Nebenführ et al., 1999). ManI–GFP showed the typical punctate pattern characteristic of normal Golgi stacks in protoplasts obtained from wild-type plants (Fig. 4E; Fig. S2). However, in protoplasts obtained from the *p24δ3δ4δ5δ6* mutant, ManI–GFP also localized to clusters of punctate structures (Fig. 4F), which suggests an alteration in the organization of the Golgi in this mutant.

To gain further insight into the traffic defects observed in the *p24δ3δ4δ5δ6* mutant at the ultrastructural level, transmission electron microscopy (TEM) analysis was performed. As shown in Fig. 5, the *p24δ3δ4δ5δ6* mutant showed a clear alteration in the Golgi, with dilated areas throughout the whole cisternae that were usually more prominent at the rim of the Golgi cisternae (Fig. 5C,D). In some cases, discontinuous cisternae were also observed (Fig. 5C,D). These results are consistent with previous results obtained upon the silencing of p24δ1 in mouse (Denzel et al., 2000). Therefore, the morphological changes in the Golgi observed in the *p24δ3δ4δ5δ6* mutant suggest that p24 proteins are involved in the maintenance of the structure and organization of the Golgi apparatus in *Arabidopsis*.

ERD2 accumulates in big intracellular structures containing a Golgi marker in the *p24δ3δ4δ5δ6* mutant

p24 proteins have long been proposed to function as cargo receptors facilitating the transport of specific cargoes between the ER and the Golgi (for a review, see Pastor-Cantizano et al., 2016). In particular, proteins of the p24δ subfamily have been shown to be involved in the retrograde transport of K/HDEL ligands bound to the K/HDEL receptor ERD2 (Majoul et al., 2001; Montesinos et al., 2014). Therefore, we have investigated whether loss of p24δ proteins affects ERD2 trafficking and localization in *Arabidopsis*.

To this end, ERD2a–YFP (Brandizzi et al., 2002) was transiently expressed in protoplasts obtained from *p24δ3δ4δ5δ6* plants. As shown in Fig. 4G and Fig. S2, ERD2a–YFP showed its typical punctate pattern, which is characteristic of the Golgi in protoplasts obtained from wild-type plants, although it also showed some ER labeling (Fig. S2). In protoplasts obtained from the *p24δ3δ4δ5δ6* mutant, ERD2a–YFP partially localized to the ER, as judged by colocalization with RFP–calnexin (Fig. 6G–I), but mostly accumulated in big intracellular structures (Fig. 4H). These structures were also observed after 4 h treatment with cycloheximide (Fig. S3), which suggest that they are not the result of the accumulation of newly synthesized protein at ER export sites. To test for the possibility that these structures could correspond to Golgi membranes, ERD2a–YFP was transiently co-expressed with the Golgi markers ManI–RFP and sialyltransferase (ST)–RFP. As shown in Fig. S2, ERD2a–YFP partially colocalized with ManI–RFP and ST–RFP in punctate structures in protoplasts obtained from wild-type plants. In protoplasts from the *p24δ3δ4δ5δ6* mutant, ERD2a–YFP partially colocalized with ManI–RFP and ST–RFP to clusters of punctate structures (Fig. 6A–F), consistent with the alteration in the structure of the Golgi described above. This suggests that ERD2 might accumulate at Golgi membranes with an altered morphology.

To confirm that the localization pattern of ERD2a–YFP in the mutant was caused by the loss of p24 proteins from the δ-1 subclass, ERD2a–YFP was transiently co-expressed with a member of this subclass, RFP–p24δ5. As shown in Fig. 7A–C, ERD2a–YFP localized both to punctate Golgi structures but also to the ER network in protoplasts obtained from wild-type plants. This is

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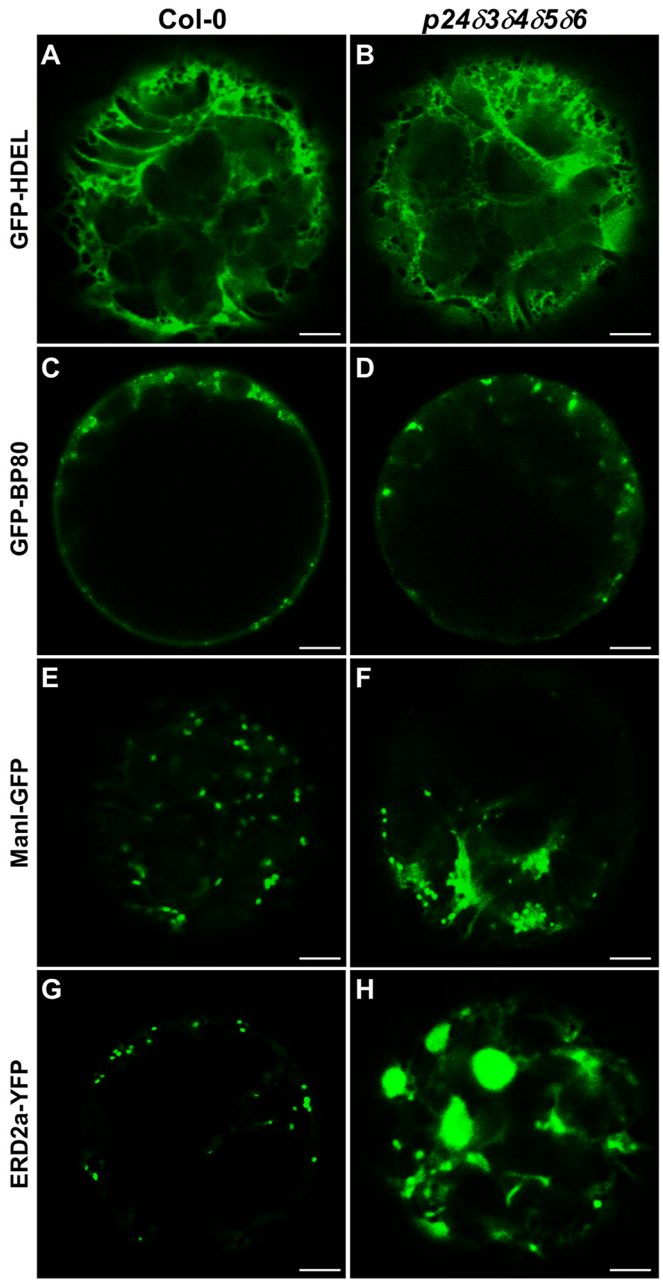

Fig. 4. Localization of organelle marker proteins in the *p24δ3δ4δ5δ6* mutant. Transient gene expression in *Arabidopsis* protoplasts obtained from wild-type (Col-0) or *p24δ3δ4δ5δ6* plants grown 4 weeks in soil. (A,B) GFP–HDEL. (C,D) GFP–BP80. (E,F) ManI–GFP. (G,H) ERD2a–YFP. Scale bars: 5 µm.

consistent with previous results obtained in tobacco protoplasts or in tobacco leaf epidermal cells where expression of RFP–p24δ5 or RFP–p24δ9 induced a partial re-localization of ERD2a–YFP from the Golgi back to the ER (Montesinos et al., 2014). A very similar pattern was observed when RFP–p24δ5 was co-expressed with ERDa–YFP in the *p24δ3δ4δ5δ6* mutant (Fig. 7D–F). These results suggest that the function of p24δ5 is enough to prevent the accumulation of ERD2a–YFP at the Golgi in the *p24δ3δ4δ5δ6* mutant.

Previous studies in animals have indicated that overexpression of HDEL ligands induced a redistribution of the human KDEL receptor from the Golgi complex to the ER (Lewis and Pelham, 1992). In *Arabidopsis*, we have previously shown that overexpression of an HDEL ligand, cherry–HDEL, also produces a partial redistribution of both ERD2a and ERD2b to the ER in tobacco leaf epidermal cells (Montesinos et al., 2014). Therefore, we investigated whether a KDEL ligand also induces the redistribution of ERD2a in the *p24δ3δ4δ5δ6* mutant. To this end, ERD2a–YFP was co-expressed with RFP–KDEL in protoplasts obtained from *p24δ3δ4δ5δ6* plants. As shown in Fig. 7G–L, ERD2a–YFP was partially redistributed to the ER upon transient expression of RFP–KDEL in protoplasts from both wild-type and *p24δ3δ4δ5δ6* mutant plants. Therefore, ERD2a–YFP localization in protoplasts from the *p24δ3δ4δ5δ6* mutant was very similar to that in protoplasts from wild-type upon RFP–KDEL or RFP–p24δ5 expression. Taken together, these results indicate that loss of p24 proteins of the δ-1 subclass causes ERD2a–YFP accumulation at the Golgi, probably due to inhibition of its retrograde Golgi-to-ER transport.

BiP secretion is increased in the *p24δ3δ4δ5δ6* mutant

In yeast, most mutants that block retrograde Golgi-to-ER transport show an increased secretion of ER-resident proteins containing an HDEL signal into the media, because the ERD2 receptor fails to retrieve the ER-resident proteins back from the Golgi apparatus (Semenza et al., 1990). This is the case of the chaperone BiP, which is efficiently retrieved from the Golgi through its interaction with the K/HDEL-receptor ERD2 by COPI vesicles. To investigate whether retrograde Golgi-to-ER trafficking of ERD2 (and thus of K/HDEL ligands) was altered in the *p24δ3δ4δ5δ6* mutant, the levels of BiP in the apoplastic fluid of leaves from wild-type plants and the *p24δ3δ4δ5δ6* mutant were analyzed by western blotting using an antibody against BiP. As shown in Fig. 8A, wild-type and mutant plants contained similar amounts of intracellular BiP, as determined by western blot analysis of total leaf extracts. However, *p24δ3δ4δ5δ6* plants secreted detectable amounts of BiP into the apoplastic fluid, in contrast to wild-type plants.

The UPR is activated in the quadruple *p24δ3δ4δ5δ6* mutant

In yeast, deletion of p24 proteins activates the UPR, which may compensate for the transport defects caused by the loss of p24 function (Belden and Barlowe, 2001; Aguilera-Romero et al., 2008). This UPR activation may be related to the fact that, in yeast, a mutant strain lacking all eight p24 family members does not show severe transport phenotypes or morphological alterations in the endomembrane system (Springer et al., 2000). Indeed, the concomitant loss of some of the p24 genes together with inositol-requiring enzyme-1 (IRE1), an UPR activator, produces a slow growth and more severe transport defects than does deletion of the p24 genes alone (Belden and Barlowe, 2001).

To investigate whether the UPR pathway was activated in the *p24δ3δ4δ5δ6* mutant, variations in the mRNA levels of BiP3 (At1g09080) and BiP1/2 (At5tg28540 and At5g42020) (Hsp70 proteins), ERDJ3A and ERDJ3B (J-domain proteins) and protein disulfide isomerase 6 (PDI6), which are well-established UPR molecular markers, were analyzed (Chen and Brandizzi, 2013). While BiP1 and BiP2 transcript levels are constitutively quite high, BiP3 is usually expressed at lower levels but is induced to a much larger extent under stress conditions and has been shown to be one of the main transcriptional targets of active bZIP60 transcription factor involved in the UPR (Iwata and Koizumi, 2005; Iwata et al., 2008; Noh et al., 2002). AtERDJ3A expression is upregulated after UPR activation; in contrast, expression of AtERDJ3B shows only a modest induction (Yamamoto et al., 2008). Finally, the expression of PDI6 is also upregulated by the UPR (Lu and Christopher, 2008; Kamauchi et al., 2005). Furthermore, as UPR activation induces the

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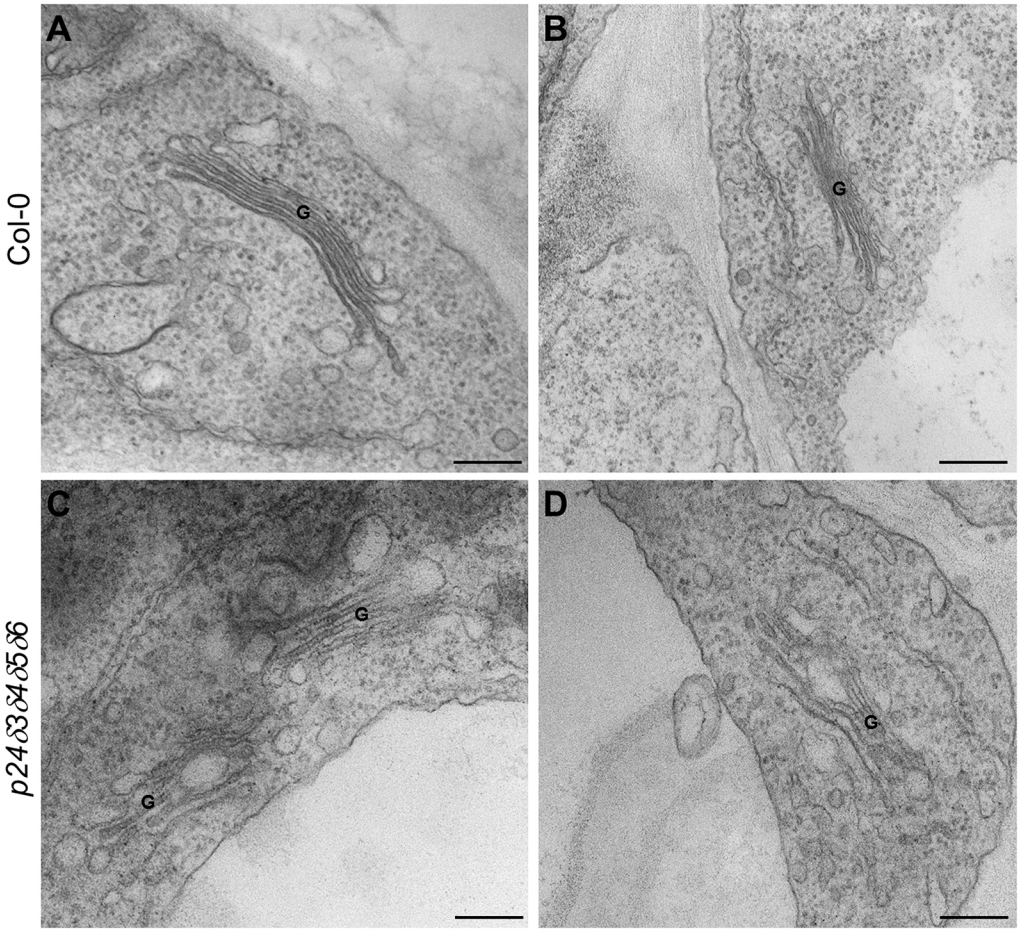

Fig. 5. Ultrastructural analysis of the *p24δ3δ4δ5δ6* mutant. Chemically fixed cotyledon cells from 4-day-old seedlings of wild-type (Col-0) (A,B) and the *p24δ3δ4δ5δ6* mutant (C,D). G, Golgi. Scale bars: 200 nm.

unconventional splicing of AtbZIP60 mRNA by AtIRE1 in *Arabidopsis* (Iwata and Koizumi, 2005; Iwata et al., 2008; Deng et al., 2011; Nagashima et al., 2011), the levels of spliced AtbZIP60 mRNA were also analyzed. As it is shown in Fig. 8B, an increase of mRNA levels for BiP3, ERDJ3A and PDI6 was observed in the *p24δ3δ4δ5δ6* mutant. These data indicate that the *p24δ3δ4δ5δ6* mutant exhibit upregulation of UPR genes in the absence of an exogenous ER stress. Interestingly, this mutant also showed increased levels of spliced bZIP60 mRNA. These results strongly suggest that the UPR is constitutively activated in the *p24δ3δ4δ5δ6* mutant.

COPII *SEC31A* gene expression is upregulated in the *p24δ3δ4δ5δ6* mutant

Recently, we have described that loss-of-function of both a gene which encodes the ARF-guanine nucleotide exchange factor involved in COPI assembly, *GNL1*, and a gene that encodes the α2-COP subunit of the COPI coat led to a strong upregulation of *SEC31A*, which encodes a subunit of the COPII coat. However, no change in the expression of the other SEC31 isoform gene (*SEC31B*) was detected (Gimeno-Ferrer et al., 2017). Since p24 proteins are key molecular players in the formation of COPI vesicles from Golgi membranes, the expression levels of *SEC31A* and *SEC31B* were analyzed in the *p24δ3δ4δ5δ6* mutant. RT-PCR analysis indicated that the expression of *SEC31A* is approximately 6-fold higher in the *p24δ3δ4δ5δ6* mutant than in wild-type (Fig. 8C). In contrast, *SEC31B* expression was not upregulated in the mutant, as it was also the case for other COPII subunit genes, including *SEC24A–SEC24C*, *SEC13A*, *SEC13B* and also *SAR1A* and *SAR1B* (Fig. S4).

DISCUSSION

p24 protein levels are interdependent

Previous studies in our group have already shown the interdependence between p24 protein levels in *Arabidopsis* (Montesinos et al., 2012, 2013). In particular, single KO mutants affecting p24δ4 or p24δ5 (p24δ-1 subclass) had similar protein levels of p24δ5 or p24δ4, respectively, but reduced (~50%) protein levels of p24δ9 (p24δ-2 subclass), p24β2 and p24β3. On the other hand, lines with reduced expression of p24β2 or p24β3 showed reduced protein levels of p24β3 or p24β2, respectively, but also reduced protein levels of p24δ5 (p24δ-1 subclass) and p24δ9 (p24δ-2 subclass). Here, we found that p24δ9 (p24δ-2 subclass), p24β2 and p24β3 protein levels are almost undetectable in the *p24δ3δ4δ5δ6* mutant. Since the reduction in protein levels does not correlate with reduced mRNA levels of the p24δ-2 subclass and the p24β subfamily genes, we hypothesize that this may be due to a decrease in protein stability. This is consistent with *Arabidopsis* p24 proteins forming hetero-oligomeric complexes, as described in other systems, probably including p24 proteins from the p24δ-1 and p24δ-2 subclasses and the p24β subfamily. Indeed, our previous results, obtained from experiments using transient expression, knockout mutants and co-immunoprecipitation, suggest that *Arabidopsis* p24 complexes involved in anterograde ER-to-Golgi transport should include p24β2, which has been shown to facilitate transport of both p24δ5 (p24δ-1 subclass) and p24δ9 (p24δ-2 subclass) from ER export sites to the Golgi (Montesinos et al., 2012, 2013). In contrast, p24 complexes involved in retrograde Golgi-to-ER transport would be expected to include p24δ proteins, which can

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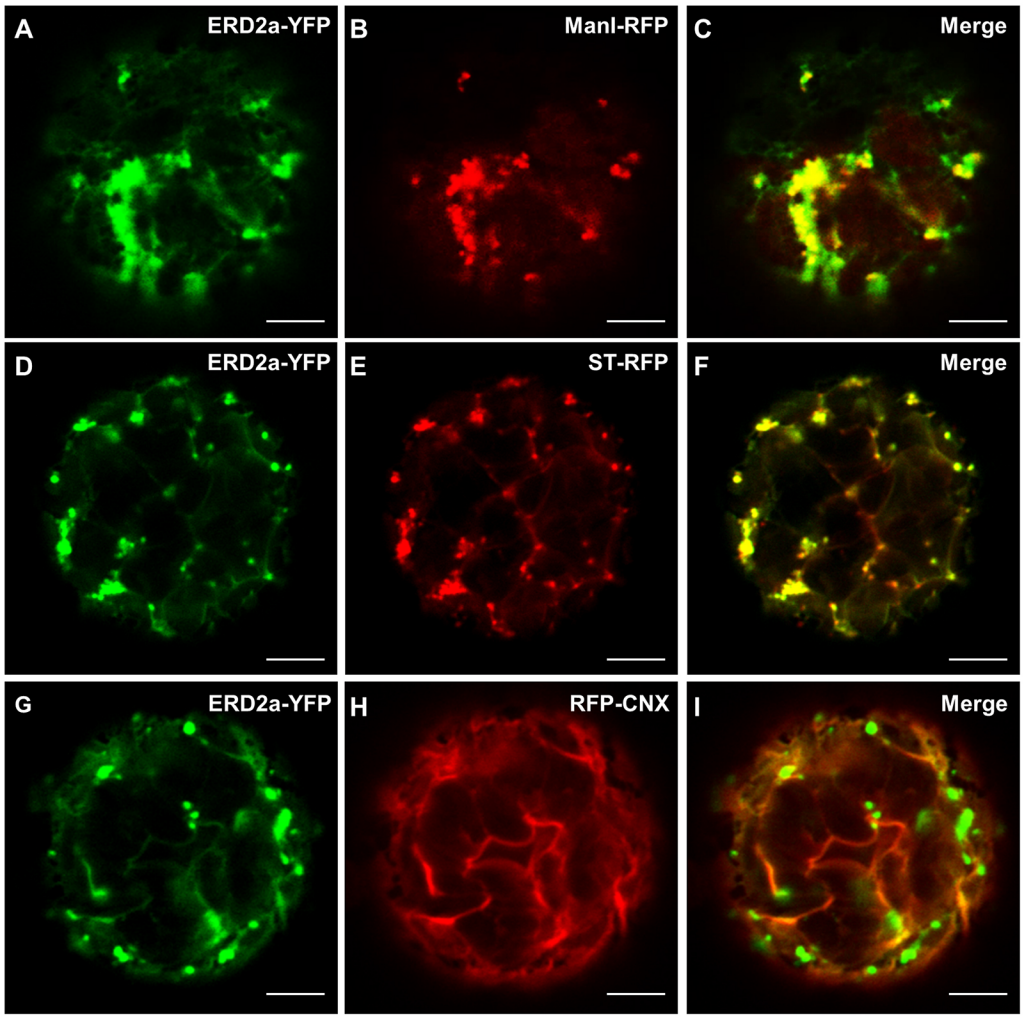

Fig. 6. ERD2 accumulates in intracellular structures containing Golgi markers in the *p24δ3δ4δ5δ6* mutant. Transient gene expression in *Arabidopsis* protoplasts obtained from *p24δ3δ4δ5δ6* plants grown for 4 weeks in soil. ERD2a–YFP (A,D,G) was co-expressed with ManI–RFP (B), ST–RFP (E) or RFP–CNX (H) (merged images in C,F and I). Scale bars: 5 µm.

interact with ARF1, COPI subunits and the K/HDEL receptor ERD2 at the acidic pH of the Golgi, thus facilitating COPI vesicle formation (Montesinos et al., 2013, 2014).

p24 proteins and structure of the Golgi

p24 proteins have been proposed to play a structural role in the organization of the compartments of the early secretory pathway, in particular the Golgi. Different results have been obtained with different p24 family members when using loss-of-function or gain-of-function approaches. Overexpression of mammalian p23 (p24δ1; TMED10) or p24 (p24β1; TMED2) has been shown to cause Golgi fragmentation and the appearance of smaller Golgi fragments (i.e. Golgi mini-stacks) (Blum et al., 1999; Rojo et al., 2000; Gong et al., 2011). Inactivation of one allele of p23 (p24δ1) in mice caused dilation of the Golgi cisternae, which was more prominent at their rim (Denzel et al., 2000). On the other hand, silencing of mammalian p25 (p24α2) or p28 (p24γ2) produced Golgi fragmentation and the appearance of Golgi mini-stacks, as observed upon overexpression of p23 (p24δ1) or p24 (p24β1) (Mitrovic et al., 2008; Koegler et al., 2010). At present it is not clear whether p24 proteins from the different subfamilies may play different roles in the organization of the Golgi. The alteration of the Golgi structure in the *p24δ3δ4δ5δ6* mutant, which showed dilated Golgi cisternae that were more prominent at their rim, is consistent with that observed upon inactivation of p23 (p24δ1) in mice (Denzel et al., 2000), and suggests that p24δ-1 proteins are important for the organization of the Golgi in *Arabidopsis*. Compromised p24 activity might contribute to this altered Golgi structure, perhaps through mislocalization of essential Golgi proteins, as previously described for conserved oligomeric Golgi (COG) complex mutants (Ungar et al., 2002).

In addition, we have shown recently that loss-of-function of the gene encoding α2-COP, a subunit of the COPI coat, also caused an alteration in the structure of the Golgi, which, when viewed using CLSM, appeared as clusters of punctate structures (Gimeno-Ferrer et al., 2017) reminiscent to what we observed in the *p24δ3δ4δ5δ6* mutant. Upon COPI depletion, the Golgi shows a reduced number of cisternae and is surrounded by a high number of vesicular profiles (Gimeno-Ferrer et al., 2017). We cannot rule out that the alteration in the structure of the Golgi upon loss of p24δ-1 proteins is due to the lack of other p24 proteins which also show dramatically reduced levels in the *p24δ3δ4δ5δ6* mutant, including p24δ9 (or perhaps other members of the p24δ-2 subclass) and p24 proteins of the p24β subfamily.

p24 proteins, ERD2 trafficking and COPI-dependent Golgi-to-ER transport

p24 proteins are key molecular players in the sorting of the K/HDEL receptor ERD2 (bound to K/HDEL ligands) within COPI vesicles for its transport from the Golgi to the ER and thus retrieval of ER-resident proteins. In animal cells, p23 (p24δ1) was shown to interact with ligand-bound ERD2 at the Golgi. Ligand binding was found to induce ERD2 oligomerization and its interaction with other

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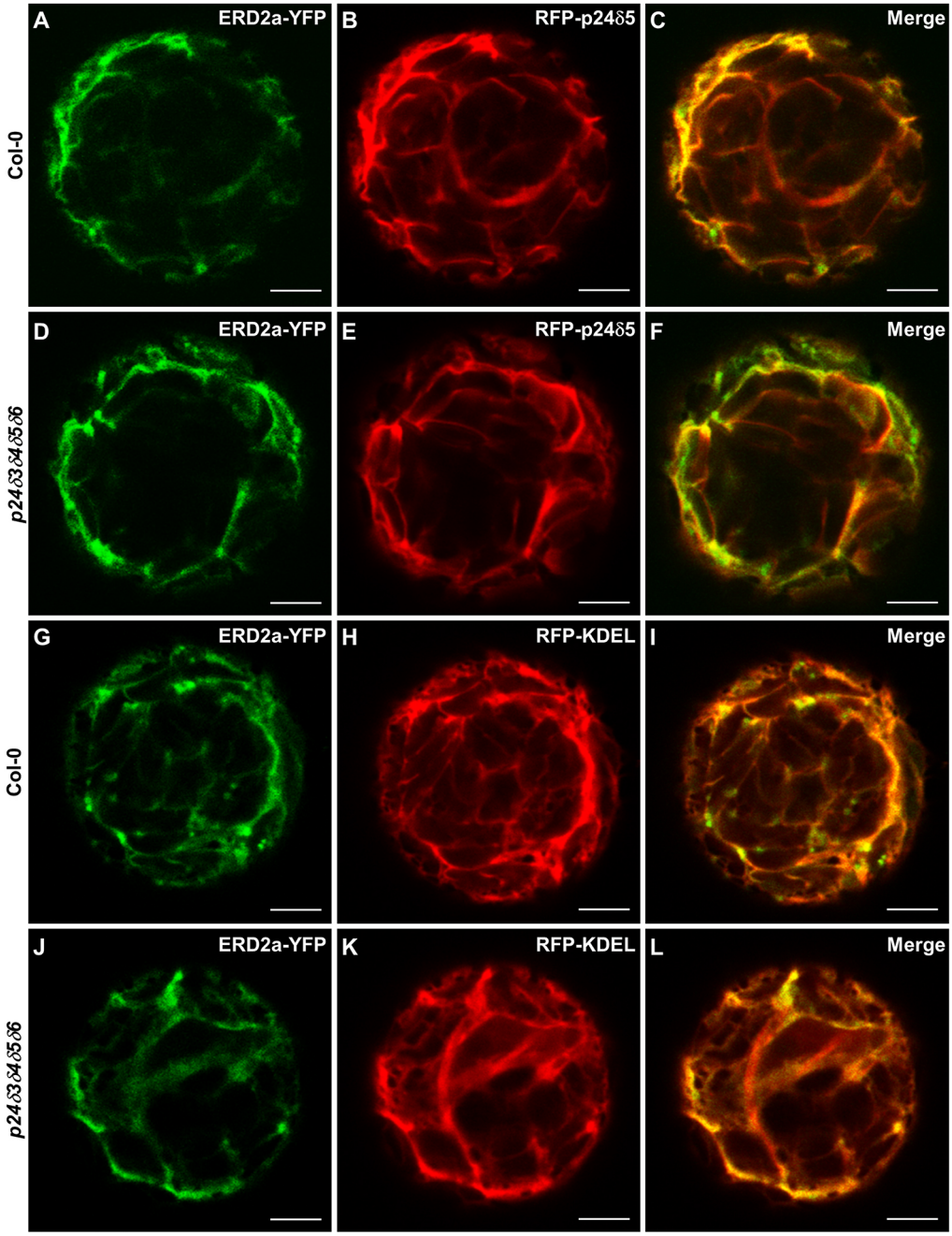

Fig. 7. ERD2 localization in the *p24δ3δ4δ5δ6* mutant upon co-expression with p24δ5 or a K/HDEL ligand. Transient gene expression in *Arabidopsis* protoplasts obtained from wild-type (Col-0) or *p24δ3δ4δ5δ6* plants grown for 4 weeks in soil. (A–C) RFP–p24δ5 caused a partial relocalization of ERD2a–YFP to the ER in wild-type protoplasts. (D–F) RFP-p24δ5 also caused a partial relocalization of ERD2a–YFP to the ER in *p24δ3δ4δ5δ6* protoplasts. (G–L) Co-expression of ERD2a–YFP with RFP–KDEL caused a partial relocalization of ERD2a–YFP to the ER, both in wild-type (G–I) and in *p24δ3δ4δ5δ6* (J–L) protoplasts. Scale bars: 5 µm.

components involved in COPI vesicle formation, including ARF1 and ARF-GAP, thus facilitating sorting of ERD2 within COPI vesicles (Majoul et al., 2001). We have previously found that co-expression with RFP–p24δ5 induced the partial relocalization of the K/HDEL receptor ERD2 from the Golgi to the ER, suggesting a role of p24δ5 in retrograde Golgi-to-ER transport of ERD2. This effect required the luminal GOLD domain in p24δ5 as well as the dilysine motif in its cytosolic tail, which is involved in binding both ARF1 and COPI subunits (Montesinos et al., 2013, 2014). In addition, p24δ5 and p24δ9 proteins were shown to interact with two different K/HDEL receptors, ERD2a and ERD2b; this interaction was pH dependent, being maximal at acidic pH but very low at neutral pH, consistent with the interaction taking place at the Golgi (Montesinos et al., 2014). Moreover, both p24δ5 and ERD2 can interact with ARF1 and COPI subunits at the acidic pH of the Golgi, consistent with their role in COPI vesicle formation (Montesinos et al., 2014).

In this work, we have found that the loss of p24δ-1 proteins has an effect in trafficking of the K/HDEL receptor ERD2, which accumulated in big intracellular structures containing the Golgi marker ManI–GFP. Fluorescently tagged ERD2 proteins in plants have been shown to localize either to the Golgi, with a lesser level of labeling in the ER (Boevink et al., 1998; Brandizzi et al., 2002; Li et al., 2009), or be equally distributed to the ER and Golgi (Xu et al., 2012). This steady-state localization should be the consequence of the anterograde ER-to-Golgi transport of free ERD2 molecules (in COPII vesicles) and the retrograde Golgi-to-ER transport of ERD2 molecules bound to KDEL ligands (in COPI vesicles). It is thus possible that the absence of p24δ-1 proteins affects the retrograde Golgi-to-ER transport of ERD2, resulting in its higher accumulation at the Golgi. It is not clear whether this causes the alteration we have observed in Golgi morphology. Co-expression with RFP–p24δ5 restored the normal localization of ERD2a–YFP, suggesting that the

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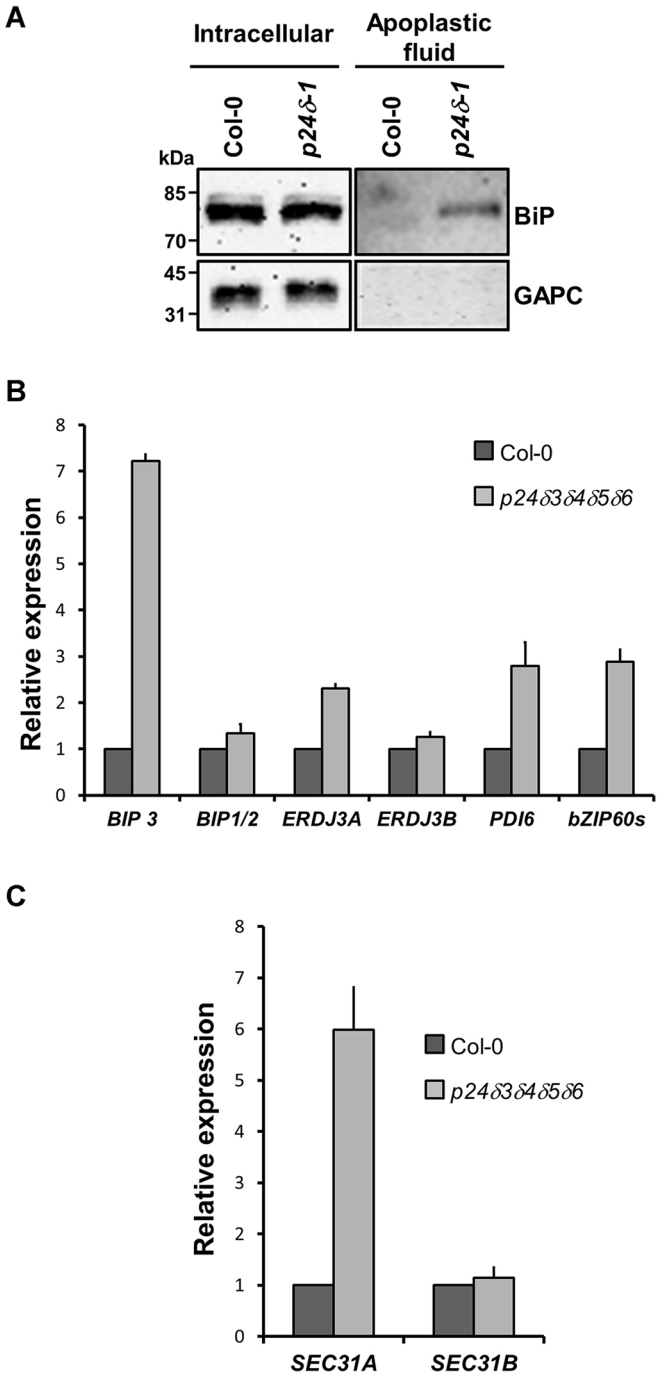

Fig. 8. The *p24δ3δ4δ5δ6* mutant shows upregulation of UPR genes and the COPII subunit *SEC31A* gene, and higher secretion of BIP. (A) Secretion of BiP. Apoplastic fluid was obtained from rosette leaves of wild-type (Col-0) and *p24δ3δ4δ5δ6* plants. As a control, total proteins were obtained from the same leaves used to obtain the apoplastic fluid. Total proteins from leaves and apoplastic fluid were analyzed by SDS-PAGE and western blotting with an antibody against tobacco BiP2 (Pedrazzini et al., 1997). The western blot analysis of glyceraldehyde-phosphate dehydrogenase (GAPDH), a cytosolic protein, was used as a control. Results are representative of three independent experiments. (B,C) Expression of UPR genes (B) and *SEC31A* and *SEC31B* (C). Total RNA was extracted from 10-day-old seedlings of wild-type (Col-0) and *p24δ3δ4δ5δ6*. The mRNA was analyzed by RT-qPCR with specific primers (Chen and Brandizzi, 2013; Lai et al., 2014; Gimeno-Ferrer et al., 2017) and normalized to *UBIQ10* gene expression (Table S3). mRNA levels are expressed as relative expression levels and represent fold changes of mutant over wild-type. Values represent mean± s.e.m. of at least three biological samples.

function of a single member of the p24δ-1 subclass is enough to compensate for the loss of the four members of this subclass. This suggests that there is functional redundancy between different p24 protein members (at least within the p24δ-1 subclass) and that the function of p24δ-1 proteins is essential for normal trafficking and localization of ERD2. Co-expression with a KDEL ligand, RFP–KDEL, also restored the normal trafficking and localization of ERD2. Previous studies in animals indicated that overexpression of HDEL ligands induced a redistribution of the human KDEL receptor from the Golgi complex to the ER (Lewis and Pelham, 1992). In *Arabidopsis*, it has been previously shown that overexpression of the HDEL ligand cherry–HDEL also produces a partial redistribution of both ERD2a and ERD2b from the Golgi to the ER in tobacco leaf epidermal cells (Montesinos et al., 2014). Since ligand binding induces ERD2 oligomerization, it is possible that the expression of a K/HDEL ligand favors the concentration of ERD2 molecules at the Golgi membrane, together with ARF1, thus increasing binding sites for COPI subunits and the formation of COPI vesicles even in the absence of p24δ-1 proteins. It is also possible that remaining p24δ-2 proteins in the p24δ-1 mutant (only p24δ9 protein levels could be analyzed) can also contribute to COPI vesicle formation, since they also contain a dilysine motif in its cytosolic tail with the ability to bind ARF1 and coatomer.

In yeast, most mutants affecting retrograde Golgi-to-ER transport show an increased secretion of ER-resident proteins (Semenza et al., 1990), and this appears to be also the case for BiP, an HDEL ligand, which showed enhanced secretion in the *p24δ3δ4δ5δ6* mutant. In yeast, deletion mutants of some p24 proteins also showed an increased secretion of Kar2p/BiP (Elrod-Erickson and Kaiser, 1996; Marzioch et al., 1999; Belden and Barlowe, 2001). It has been shown that in addition to a defect in retrograde transport, the activation of the UPR in yeast mutants may also contribute to Kar2p/BiP secretion (Belden and Barlowe, 2001). As the UPR is activated in the *p24δ3δ4δ5δ6* mutant, it cannot be ruled out that the same happens in this mutant.

p24 proteins and the UPR

The absence of phenotypic alterations under standard growth conditions in the *p24δ3δ4δ5δ6* mutant is consistent with results obtained in yeast, where a mutant strain lacking all the eight members of the yeast p24 family grew identically to wild-type, although it showed delayed transport of GPI-anchored proteins (Springer et al., 2000). However, it cannot be ruled out that the *p24δ3δ4δ5δ6* mutant still contains low levels of p24δ-2 proteins which may play redundant functions with that of p24δ-1 proteins and thus allow normal growth.

In yeast, UPR activation may help to alleviate the transport defects caused by the loss of p24 function (Aguilera-Romero et al., 2008). UPR is triggered by ER transmembrane sensors on ER stress. IRE1 is the only identified stress sensor in yeast and is conserved in all eukaryotes. In addition to the IRE1 pathway, other UPR pathways have been identified in animals and plants (Chen and Brandizzi, 2013). One transcription factor has been identified as a splicing target of IRE1 (AtbZIP60 in *Arabidopsis*). In *Drosophila*, loss of p24 function also activates an ER stress response and it seems to be activated by nuclear factor (NF)-κB proteins instead of by splicing of Xbp1 (the homolog of AtbZIP60) (Boltz and Carney, 2008). In mammals, the knockdown of p24β1 in mouse or knockdown of p24δ1 in rat cells does not activate the splicing of Xbp1 (Jerome-Majewska et al., 2010; Zhang and Volchuk, 2010). Our results suggest that, in *Arabidopsis*, the loss of p24δ-1 proteins

activates at least the IRE1–bZIP60 pathway of the UPR and it may act as a compensatory mechanism that helps the plant to cope with the trafficking defects. In the future, it would be interesting to study whether other *Arabidopsis* UPR pathways are also involved in the UPR activation in the *p24δ3δ4δ5δ6* mutant.

We have previously shown that a knockout mutant affecting the α2-COP subunit of COPI (*α2-cop*) showed a strong upregulation of *SEC31A* but not of *SEC31B* (Gimeno-Ferrer et al., 2017). This is consistent with increasing evidence indicating that specific expression patterns in COPII subunit isoforms in *Arabidopsis* may reflect functional diversity (Chung et al., 2016). Interestingly, *SEC31A* was also strongly upregulated in *gnl1*, a mutant of the ARF-GEF GNL1 involved in COPI assembly (Gimeno-Ferrer et al., 2017). Here, we showed that deletion of p24δ-1 proteins, the third major molecular player involved in COPI vesicle formation also causes the upregulation of *SEC31A*. Therefore, loss of function of the three major components involved in COPI vesicle formation seems to produce a specific increase in the expression levels of the COPII subunit *SEC31A*. This could be also related to the activation of the UPR in the *p24δ3δ4δ5δ6* mutant, as *SEC31A* is also upregulated by the UPR pathway mediated by IRE1 (Nagashima et al., 2011; Song et al., 2015). In the *p24δ3δ4δ5δ6* mutant, the induction of *SEC31A* might enable efficient packaging of specific cargo proteins into anterograde vesicles or simply increase the overall capacity of anterograde transport to compensate for the trafficking defects caused by the loss of p24δ-1 function.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana (ecotype Col-0) was used as wild type. T-DNA insertion mutants of *p24δ3*, *p24δ4*, *p24δ5* and *p24δ6* used in this study are listed in Table S1. All mutants were obtained from the Nottingham Arabidopsis Stock Centre. *A. thaliana* plants were grown in growth chambers as previously described (Ortiz-Masia et al., 2007). The T-DNA insertion mutants were characterized by PCR. The primers used are included in Tables S1 and S2.

To study whether salt tolerance was affected in the *p24δ3δ4δ5δ6* mutant, seeds of wild-type (Col-0) and *p24δ3δ4δ5δ6* were sown on Murashige and Skoog (MS) plates containing 160 mM NaCl. Plates were transferred to a controlled growth chamber after cold treatment in the dark for 3 days at 4°C. After 12 days, the rates of cotyledon greening were scored. Seeds harvested from Col-0 and *p24δ3δ4δ5δ6* plants grown under the same conditions and at the same time were used.

RT-PCR

Total RNA was extracted from different plant organs and seedlings by using a Qiagen RNeasy plant mini kit, and 3 µg of the RNA solution was reverse-transcribed using the maxima first-strand cDNA synthesis kit for quantitative RT-PCR (Fermentas) according to the manufacturer's instructions. Semi-quantitative PCRs (sqPCRs) were performed on 3 µl of cDNA template using the kit PCR Master (Roche). The sequences of the primers used for PCR amplifications are included in Table S2.

Quantitative PCR (qPCR) was performed by using a StepOne Plus machine (Applied Biosystems) with SYBR Premix Ex Taq TM (Tli RNaseH Plus) (Takara) according to the manufacturer's protocol. Each reaction was performed in triplicate with 100 ng of the first-strand cDNA and 0.3 µM of primers for all the genes and 0.9 µM for *SEC31A* in a total volume of 20 µl. The specificity of the PCR amplification was confirmed with a heat dissociation curve (from 60°C to 95°C). Relative mRNA abundance was calculated using the comparative Ct method according to Pfaffl (2004). Primers used for qPCR are listed in Table S3.

Isolation and transformation of *Arabidopsis* protoplasts

To obtain mesophyll protoplasts from *Arabidopsis* plants (4-week-old rosette leaves), the Tape-*Arabidopsis* Sandwich method was used, as described previously (Wu et al., 2009). Protoplasts were isolated from 4-week-old *Arabidopsis* rosette leaves of wild-type and *p24δ3δ4δ5δ6* plants. For transient expression, we used the polyethylene glycol (PEG) transformation method, as described previously (Yoo et al., 2007). Plasmids encoding marker proteins used were: GFP–HDEL (Haseloff et al., 1997), RFP–KDEL (Di Sansebastiano et al., 2015), Man1–GFP/RFP (Nebenführ et al., 1999), ERD2a–YFP (Brandizzi et al., 2002), GFP–BP80 (daSilva et al., 2005), RFP–calnexin (Künzl et al., 2016), RFP–p24δ5 (Langhans et al., 2008). ST–RFP was provided by Peter Pimpl (University of Tübingen, Tübingen, Germany).

Preparation of protein extracts and western blotting

To obtain a membrane fraction from *Arabidopsis* roots, seedlings were grown in liquid MS medium for 20 days. *Arabidopsis* roots (from either wild-type or mutant plants) were homogenized 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)] using a mortar and a pestle, and 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***. Membrane proteins were extracted from the pellets 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 quantification was performed with a Bradford assay (Bio-Rad Laboratories GmbH, Munich, Germany). Protein extracts were used for SDS-PAGE followed by western blot analysis. Protein samples were separated by electrophoresis on 14% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher and Schuell). Before blotting, membranes were stained with Ponceau-S solution (Sigma) to show loading of the protein samples. Membranes were probed with antibodies against p24δ5, p24δ9, p24β2 or p24β3 (at 1:500 dilution; Montesinos et al., 2012) and developed by ECL (enhanced chemiluminescence; GE Healthcare) as previously described (Montesinos et al., 2013). Western blots were analyzed using the ChemiDoc XRS+ imaging system (Bio-Rad).

Isolation of apoplastic fluid from leaf tissue

For the isolation of apoplastic fluid from leaf tissue, we followed the vacuum infiltration-centrifugation technique with leaves from 4-week-old plants, as described previously (Joosten, 2012).

Transmission electron microscopy

For electron microscopy, seedlings were grown on MS medium containing 1% agar, and the seedlings were harvested after 4 days. Chemical fixation of cotyledons was performed according to Osterrieder et al. (2010). Ultrathin (70 nm) sections were cut on a Microtome Leica UC6, stained with uranyl acetate and lead citrate and observed with a JEM-1010 (JEOL) transmission electron microscope.

Confocal microscopy

Imaging was performed using an Olympus FV1000 confocal microscope with a 60× water lens. Fluorescence signals for GFP (488 nm, band-pass 496–518 nm), YFP (514 nm, band-pass 529–550 nm) and RFP (543 nm, band-pass 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 3.1 Viewer and coreldrawx4 (14.0.0.567) or ImageJ (version 1.45 m) (Abramoff et al., 2004).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.J.M., F.A.; Investigation: N.P.-C., C.B.-S., M.J.M., F.A.; Writing - original draft: N.P.-C., M.J.M., F.A.; Writing - review & editing: M.J.M., F.A.; Supervision: M.J.M., F.A.; Project administration: M.J.M., F.A.; Funding acquisition: M.J.M., F.A.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.203802.supplemental

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