The *Arabidopsis* ESCRT protein–protein interaction network

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Abstract In yeast, endosomal sorting of monoubiquity-lated transmembrane proteins is performed by a subset of the 19 "class E vacuolar protein sorting" proteins. The core machinery consists of 11 proteins that are organised in three complexes termed ESCRT I-III (endosomal sorting complex required for transport I-III) and is conserved in eukaryotic cells. While the pathway is well understood in yeast and animals, the plant ESCRT system is largely unexplored. At least one sequence homolog for each ESCRT component can be found in the *Arabidopsis* genome. Generally, sequence conservation between yeast/animals and the *Arabidopsis* proteins is low. To understand details about participating proteins and complex organization we have performed a systematic pairwise yeast two hybrid analysis of all *Arabidopsis* proteins showing homology to the ESCRT core machinery. Positive interactions were validated using bimolecular fluorescence complementation. In our experiments, most putative ESCRT components exhibited interactions with other ESCRT components that could be shown to occur on endosomes suggesting that despite their low homology to their yeast and animal counterparts they represent functional components of the plant ESCRT pathway.

Keywords ESCRT · *Arabidopsis* · Protein–protein network · Endosomal sorting · MVB · Protein trafficking

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Introduction

The endosomal sorting complex required for transport (ESCRT) has been identified in yeast on the basis of its function in sorting of monoubiquitylated transmembrane proteins into the internal vesicles of multivesicular bodies (MVB) for subsequent delivery to the vacuole (Babst et al. 2002a, b; Katzmann et al. 2001). MVB sorting is executed by the concerted action of four protein complexes, ESCRT-0-III (Slagsvold et al. 2006; Hurley and Emr 2006; Babst 2005). Whereas ESCRT-I-III are conserved among eukaryotes, the upstream ESCRT-0 complex is only present in *Opistokontha* and therefore absent in plants (Leung et al. 2008; Winter and Hauser 2006). Three recent publications have identified a new fourth component of the *S. cerecisiae* ESCRT-I complex, Mvb12. Mvb12 does not seem to be evolutionarily conserved as no homologs can be found in multicellular organisms (Oestreich et al. 2007; Chu et al. 2006; Curtiss et al. 2007). However, a fourth component of ESCRT-I has also been reported in *C. elegans*. It has two human homologs and was named MVB12 although it is lacking recognizable sequence similarity to yeast Mvb12 (Morita et al. 2007a; Shi et al. 2007). Neither the yeast nor the human type of MVB12 proteins is present in plants (Schellmann and Pimpl 2009). In the process of ESCRT sorting, monoubiquitylated cargo is thought to be passed from one ESCRT subcomplex to the next (conveyor belt) starting with its recognition by the UIM

(ubiquitin interacting motifs) containing Vps27/Hse1 dimer (ESCRT-0) (Bilodeau et al. 2002, 2003). ESCRT-0 recruits the heterotetrameric ESCRT-I complex consisting of the UEV (ubiquitin E2 variant) domain containing protein Vps23 and Vps28, Vps37 and Mvb12 from the cytosol to the endosomal membrane (Katzmann et al. 2001, 2003). The C-terminus of Vps28 is required for interaction with the N-terminus of Vps36, a member of the ESCRT-II complex comprising Vps36, Vps22 and Vps25 (Kostelansky et al. 2006; Teo et al. 2004, 2006). Finally, the cargo is concentrated in certain membrane regions of the endosome by members of the ESCRT-III, which is composed of four small coiled-coil proteins sufficient to induce inward budding of the internal vesicles (Babst et al. 2002a; Babst 2005; Wollert et al. 2009). Prior to invagination, the ubiquitin moiety is removed from the cargo by the deubiquitylase Doa4 (Swaminathan et al. 1999; Amerik et al. 2000; Dupre and Haguenauer-Tsapis 2001) and the ESCRT components are disassembled from the endosomal surface by the AAA ATPase Vps4/SKD1 (Scheuring et al. 1999, 2001; Babst et al. 1997, 1998; Finken-Eigen et al. 1997; Yoshimori et al. 2000).

Recently, a function of ESCRT in cytokinesis of multicellular organisms has come into focus (Carlton and Martin-Serrano 2007; Morita et al. 2007b; Spitzer et al. 2006). In animals, different ESCRT proteins are recruited to the midbody by the centrosomal protein Cep55 and function at the abscission stage (Carlton and Martin-Serrano 2007; Morita et al. 2007b) whereas in plants a role in regulating the tubulin cytoskeleton was discussed based on genetic interactions of the *Arabidopsis* TSG101 homolog *ELCH (ELC)* and the tubulin folding cofactor A *KIESEL* (Spitzer et al. 2006).

Apart from the *elc* related cytokinesis phenotype, little is known about function, architecture and mechanisms of ESCRT in plants. Three publications have shown that with exception of ESCRT-0 sequence homologs of all ESCRT core components are present in the *Arabidopsis* genome (Mullen et al. 2006; Spitzer et al. 2006; Winter and Hauser 2006). The characterisation of the plant Vps4 homolog AtSKD1 has revealed a role in the biogenesis of MVBs as in cells expressing dominant negative AtSKD1, larger MVBs that contain less internal vesicles are formed (Haas et al. 2007).

That ESCRT is likely to be involved in sorting of transmembrane proteins into the vacuole was shown recently. The double mutant of *chmp1a chmp1b*, two proteins that in yeast act as regulators of Vps4, leads to missorting of the auxin transporter PIN2 to the tonoplast instead of the vacuolar lumen, resulting in developmental defects and finally lethality (Spitzer et al. 2009).

In this work, we have analysed the protein–protein interaction network of the putative *Arabidopsis* ESCRT core complex homologs. (Mullen et al. 2006; Spitzer et al. 2006; Winter and Hauser 2006). We show that all analysed homologs are part of the network suggesting that a functional ESCRT system exists in plants.

Materials and methods

Constructs and molecular biology

All full length cDNAs for PCR and subsequent BP reaction were obtained from RIKEN Genomic Sciences Center (Sakurai et al. 2005; Seki et al. 1998, 2002) with the exception of VPS37-1 that was obtained from the Nottingham *Arabidopsis* stock centre (NASC). ARA6 was a gift from Takashi Ueda and has been described earlier (Ueda et al. 2004; Ueda and Nakano 2002). Gene specific primers incorporating *attB1* and *attB2* sequences were purchased from Invitrogen, Karlsruhe, Germany. Details are available on request. The coding sequences of the VPS genes were amplified by PCR reactions with primers incorporating *attB1* and *attB2* sequences (Invitrogen) and introduced into the pDONR201 by BP clonase reaction according to the manufacturer's instructions (Invitrogen). The resulting pDONR201 clones were transformed into the *E. coli* strain DH5-alpha and sequenced. DNA fragments were transferred to the destination vectors pAS2.1 and pACT2.1 for Y2H analysis or to pENSG-YFP and pENSG-CFP for localisation studies by LR clonase reaction according to the manufacturer's instructions (Invitrogen). The product of recombination reaction (LR reaction) was used to transfect *Arabidopsis* protoplasts.

Cell culture, protoplasting, transfection and microscopy

Arabidopsis cells were cultured in suspension culture (Columbia ecotype; grown in MS medium supplemented with 0.5 mg/l NAA and 0.1 mg/l KIN) as described (Mathur and Koncz 1998a). Protoplasts were isolated and transformed by polyethylene glycol-mediated transfection according to (Mathur and Koncz 1998b). The transfected protoplasts were incubated at 23°C for 16 h in the dark before microscopic observation.

A LEICA TCS SP2 microscope was used for acquisition of confocal microscopic images. Adobe Photoshop was used to adjust brightness, contrast and levels of the pictures and for producing the merged pictures.

BiFC constructs

Unless mentioned otherwise, all genes were fused to the N-terminus of the split-YFP parts and were therefore

amplified without stop-codon and introduced into pDONR201. After sequencing, the resulting clones were used for LR recombination reaction into the destination vectors pSPYNE and pSPYCE (Walter et al. 2004) and subsequent transfection of 10 μg of each plasmid into *Arabidopsis* protoplasts together with 10 μg of a 35S$_{pro}$:KRP-CFP containing plasmid (Jakoby et al. 2006) as transfection control. Negative controls were carried out for each experiment by cotransforming an empty vector containing only the appropriate split-YFP part together with the respective gene:split-YFP fusion and by expression of the non-interacting transcription factors AtMYB51 (At1g18570) in pSPYNE and bHLH133 (At2g20095) in pSPYCE to exclude non-specific association caused by high local concentrations of non interacting partners (Gigolashvili et al. 2007; Lalonde et al. 2008). Experiments were performed in a reciprocal manner at least three times. In the negative controls and negative samples we could never observe YFP fluorescence.

Yeast transformation and yeast 2-hybrid

The yeast strain AH109 (James et al. 1996) was grown in standard yeast full media or selective drop-out media (Clontech, Saint-Germain-en-Laye, France) under standard conditions.

Plasmids were transformed into yeast by the LiAc transformation method (Gietz et al. 1995). Interactions were analysed on synthetic dropout medium lacking leucine and tryptophan and on synthetic dropout medium without leucine, tryptophan and histidine supplemented with 3 mM 3-aminotriazole (3-AT) (Sigma–Aldrich, Munich, Germany). SNF1 and SNF4 were used as positive controls (Fields and Song 1989). Prior to pairwise testing, autoactivation controls were performed by transfecting yeast cells with the bait vectors together with the empty prey vector. No autoactivating baits were observed. Interactions were scored as positive if the test plate contained the same colony density as the transformation control plate after 4 days (>100 colonies). On test plates scored as negative, by contrast, we did not find any colonies after 4 days. We have not checked expression of the Y2H constructs in yeast. It is therefore possible that cases without any apparent interaction (VPS24-1, VPS2-3, both directions, VPS28-2, as prey) are due to lack of expression. All experiments were repeated at least three times and performed in a reciprocal manner using the respective genes both as bait and prey. A gene excluded from our analysis is the VPS24-2 gene that according to Genevestigator (https://www.genevestigator.ethz.ch/at/) is only expressed in embryos and could not be isolated by us (Table 1).

Table 1 *Arabidopsis* ESCRT homologs used in this study

Yeast	Mammals	ESCRT	*Arabidopsis*[a]	Ident./simil. to yeast (%)[b]	AGI code[a]
Stp22 (Vps23)	TSG101	I	VPS23-1/ELCH	19/33	At3g12400
		I	VPS23-2	19/34	At5g13860
Vps28	VPS28	I	VPS28-1	32/47	At4g21560
		I	VPS28-2	31/46	At4g05000
Vps37	VPS37 A, B, C, D	I	VPS37-1	18/29	At3g53120
		I	VPS37-2	18/29	At2g36680
Vps22	EAP30	II	VPS22	24/34	At4g27040
Vps25	EAP25	II	VPS25	25/40	At4g19003
Vps36	EAP45	II	VPS36	13/24	At5g04920
Vps2	CHMP2 A, B	III	VPS2-1	26/38	At2g06530
		III	VPS2-2	19/32	At5g44560
		III	VPS2-3	18/31	At1g03950
Snf7 (Vps32)	CHMP4 A, B, C	III	VPS32-1	29/40	At2g19830
		III	VPS32-2	30/44	At4g29160
Vps20	CHMP6	III	VPS20-1	28/41	At5g63880
		III	VPS20-2	29/41	At5g09260
Vps24	CHMP3	III	VPS24-1	30/50	At5g22950
		III	VPS24-2[c]	22/40	At3g45000

[a] Winter and Hauser (2006), Spitzer et al. (2006), Mullen et al. (2006)

[b] Spitzer et al. (2006)

[c] Not used in this study

Network analysis

Analysis of the network and graphical representation of the networks presented in Fig. 3 were done with the Java JUNG framework (http://jung.sourceforge.net/) and PAJEK (http://vlado.fmf.uni-lj.si/pub/networks/pajek/). Network representation in Fig. 1 was done with the program yED (http://www.yworks.com/en/products_yed_about.html).

Results

Establishment of a protein–protein interaction network of ESCRT components

To examine a possible functional connection between the *Arabidopsis* ESCRT homologs, we performed a systematic yeast two hybrid assay, using each gene as bait and as prey with every other putative ESCRT member (Table 2; Fig. 1).

To extend and complement the yeast two-hybrid experiments, we used bimolecular fluorescence complementation (BiFC) assays in *Arabidopsis* protoplasts. In

Fig. 1 The *Arabidopsis* ESCRT interactome. Interaction network summarizing the Y2H and BiFC interaction assays (Table 2). *Nodes* and *connecting lines* (edges) represent proteins and interactions, respectively. Sequence homologs of ESCRT-I components are shown in *grey*, ESCRT-II in *blue* and ESCRT-III in *yellow*. Details of the used genes are presented in Table 1
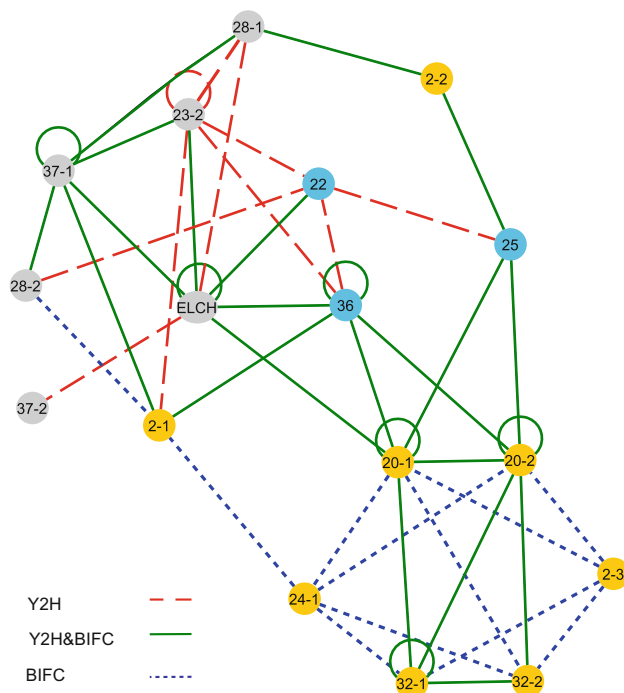
these experiments, we could reproduce most of the interactions discovered in the yeast two-hybrid assays. In addition, we found interactions that were not detected with the yeast two-hybrid approach (Table 2; Fig. 1; Supplementary Fig. 1).

As summarized in Fig. 1, all putative ESCRT components exhibited interactions with other ESCRT components suggesting that they represent a functional unit, the plant ESCRT pathway. According to the conveyor belt model, the ESCRT core consists of three subcomplexes, ESCRT-I-III, which directionally pass on the ubiquitylated cargo. Contacts between ESCRT-I and ESCRT-III are not necessary for sorting. Interestingly, we found interactions between ESCRT-I and ESCRT-III members in both assay systems (Table 2; Figs. 1, 2a–e). ESCRT-I ESCRT-III interactions had been identified in yeast previously (Bowers et al. 2004), but have never been taken into account for the molecular models of ESCRT sorting. For the mammalian system such interactions have not been reported (von Schwedler et al. 2003).

Computational network analysis

The *Arabidopsis* network shows two densely connected regions, one that contains the ubiquitin-interacting proteins of ESCRT-I and ESCRT-II and one that contains the ESCRT-III homologs (Fig. 1). To further corroborate this first but possibly subjective impression we analysed the network with computational methods that were originally developed for small scale social networks (Freeman 1977; Girvan and Newman 2002) and have been successfully applied for protein–protein interaction networks. For this purpose, every interaction was incorporated into the network regardless whether it was found by one or both interaction assays. This is justifiable as it cannot be expected that one assay detects all genuine interactions (Yu et al. 2008). Furthermore, it has recently been shown that Y2H only produces a low degree of false positive results in systematic pairwise studies (Yu et al. 2008). In addition, both the Y2H and BiFC assays performed in this study each contain a high number of internal negative controls due to the systematic approach. Therefore, we consider false positive results unlikely. Self interactions of proteins do not contribute to the network and were therefore neglected.

In the network analysis, we focussed on two questions. Firstly, we wanted to identify components that are occupying central positions in the network and can therefore be considered key components of the pathway. For this purpose, we used the "betweenness centrality" (C_B) algorithm (Freeman 1977). With this algorithm all shortest paths along the edges (representing the interactions) between every node of the network (in this case representing the

Table 2 Summary of protein–protein interactions between *Arabidopsis* ESCRT components

Bait/N-term	Vps23-1	Vps23-2	Vps28-1	Vps28-2	Vps37-1	Vps37-2	Vps22	Vps25	Vps36	Vps2-1	Vps2-2	Vps2-3	Vps20-1	Vps20-2	Vps24-1	Vps32-1	Vps32-2
Prey/C-term																	
Vps23-1	+/+	+/+	+/-	-/-	+/+	+/-	+/+	-/-	+/+	-/-	-/0	-/0	-/-	-/-	-/0	-/0	-/0
Vps23-2	+/+	+/-	+/-	-/-	+/+	-/0	+/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/0	-/-	-/-
Vps28-1	-/-	-/-	-/0	-/-	+/+	-/0	-/-	-/-	-/-	-/-	+/+	-/0	-/0	-/-	-/-	-/-	-/-
Vps28-2	-/-	-/-	-/-	-/-	+/+	-/0	+/-	-/-	-/-	-/+	-/-	-/0	-/0	-/0	-/0	-/0	-/0
Vps37-1	+/+	+/+	-/+	-/+	+/+	-/0	-/-	-/0	-/-	-/+	-/-	-/0	-/0	-/0	-/0	-/0	-/0
Vps37-2	+/-	-/0	-/0	-/0	-/-	-/0	-/0	-/0	-/0	-/0	-/0	-/0	-/0	-/0	-/0	-/0	-/0
Vps22	+/+	-/-	-/-	-/0	-/-	-/0	-/0	+/-	-/-	-/-	+/+	-/0	-/0	-/0	-/0	-/0	-/-
Vps25	-/-	-/-	-/-	-/-	-/-	-/0	-/-	-/0	-/-	-/-	+/+	-/0	+/+	+/+	-/0	-/0	-/-
Vps36	-/+	+/-	-/-	-/-	+/+	-/0	+/-	-/0	+/+	-/+	-/-	-/-	+/+	+/+	-/-	-/-	-/-
Vps2-1	-/-	-/-	-/-	-/-	+/+	-/0	+/-	-/-	+/+	-/0	-/-	-/-	-/-	-/-	-/-	-/-	-/-
Vps2-2	-/-	-/-	+/+	-/-	-/-	-/0	-/-	-/-	-/-	-/-	-/0	-/-	-/-	-/-	-/-	-/+	-/+
Vps2-3	-/-	-/-	-/-	-/0	-/-	-/0	-/-	-/-	-/-	-/-	-/-	-/0	-/+	-/+	-/-	-/-	-/+
Vps20-1	+/+	-/-	-/0	-/-	-/-	-/0	-/-	+/+	+/+	-/-	-/-	-/+	+/+	+/+	-/+	+/+	+/+
Vps20-2	-/-	-/-	-/0	-/-	-/-	-/0	-/-	+/+	+/+	-/-	-/-	-/+	+/+	+/+	-/+	-/+	-/+
Vps24-1	-/0	-/0	-/0	-/0	-/-	-/0	-/-	-/-	-/-	-/+	-/-	-/0	-/+	-/+	-/0	-/+	-/+
Vps32-1	-/-	-/-	-/-	-/-	-/-	-/0	-/-	-/-	-/-	-/-	-/-	-/+	+/+	+/+	-/+	+/+	+/+
Vps32-2	-/-	-/-	-/-	-/0	-/-	-/0	-/-	-/-	-/-	-/-	-/-	-/+	-/+	+/+	-/+	-/+	-/0

Results from the yeast two-hybrid assays are given on the left side of each cell, results of BiFC tests are given on the right side. "+" and "−" denote positive or negative results, respectively, "0" stands for not determined. Interactions that were observed in both directions are indicated in bold. Note, the interaction of VPS23 with VPS37 in the yeast two hybrid assay has already been published (Spitzer et al. 2006). Bait/prey situation is indicated for the Y2H experiments, fusion with the N-terminal (N-term) or C-terminal (C-term) half of YFP is indicated for BiFC

Fig. 2 BiFC detection of ESCRT-I ESCRT-III interactions. **a–d** Overlay of the BiFC signal (*green*) and the KRP-CFP transformation control (*blue*, nucleus) for BiFC interactions between ESCRT-I and ESCRT-III components. Note that the interactions are only found in the cytoplasm and on punctuate structures (*arrowheads*) but not in the nucleus. **a** ELC VPS24-1 (internal negative control/negative result); **b** ELC VPS20-1; **c** VPS28-2 VPS2-1; **d** VPS37-1 VPS2-1; **e** statistical analysis of the ESCRT-I ESCRT-III interactions. Per transfection 50 protoplast showing KRP-CFP in the nucleus were scored for presence of YFP fluorescence. All interactions were observed in three independent protoplast transfections. The interaction ELC VPS37-1 (Spitzer et al. 2006) is shown as positive control, ELC VPS24-1 is shown as internal negative control
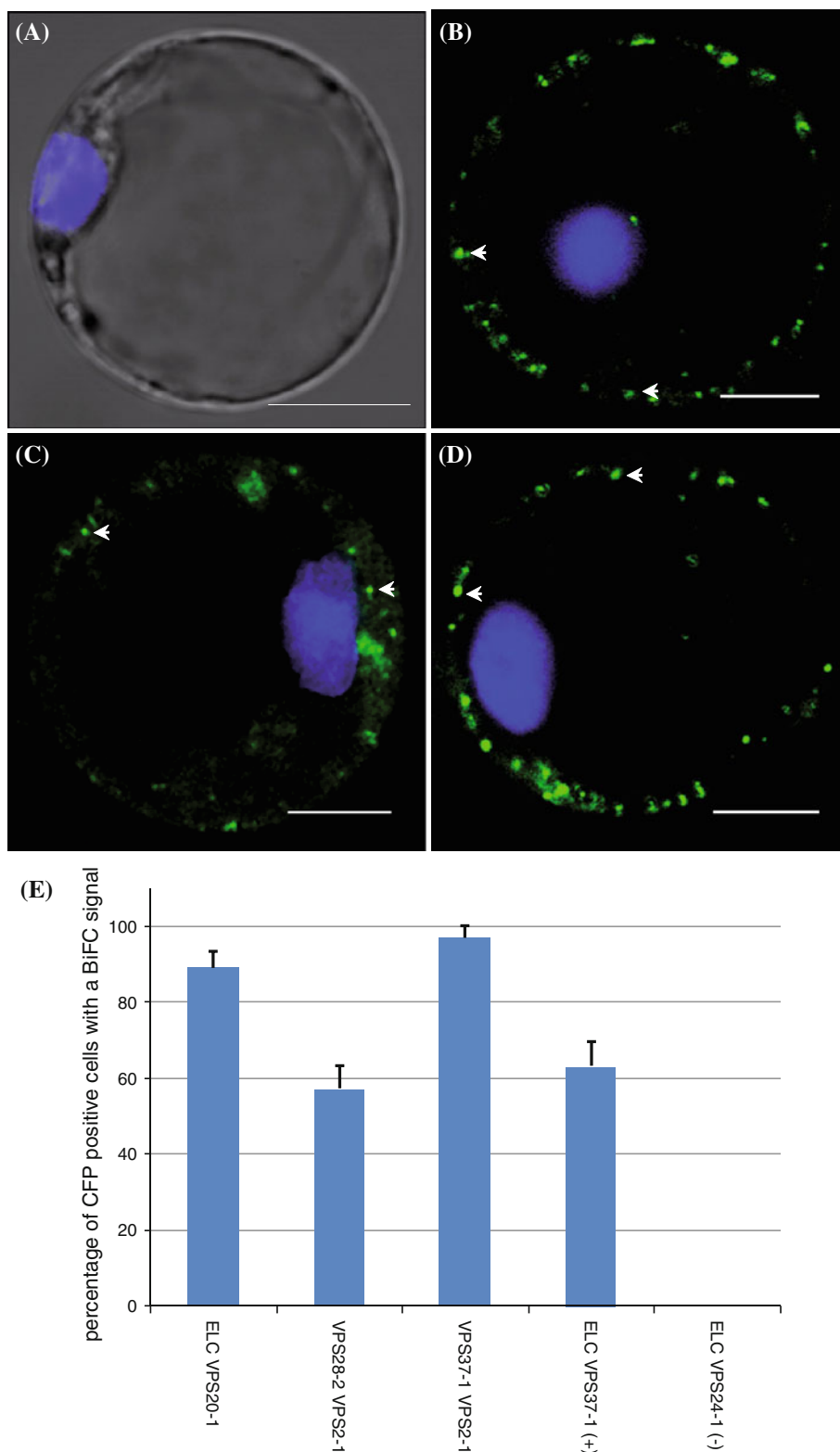
proteins) are calculated. Nodes that are crossed by many shortest paths are considered to be central. For the *Arabidopsis* ESCRT network two key components, VPS20-1 and ELCH, show the highest degree of centrality (Fig. 3a),

indicating that they might be important for assembly of the complexes or form interfaces in between subcomplexes.

Secondly, we aimed to analyse possible substructures of the network that could indicate functional subcomplexes.

(A)

(B)
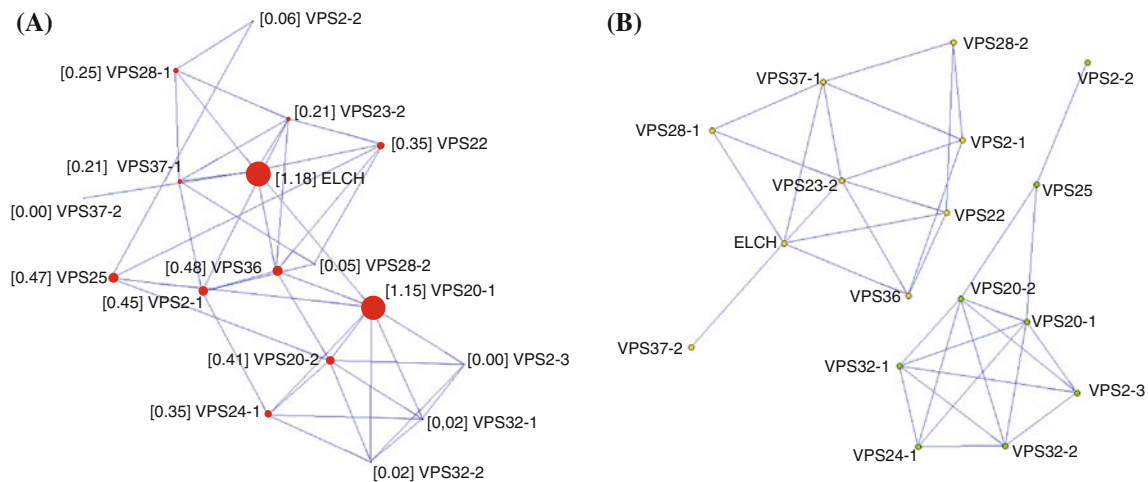
Fig. 3 Analysis of the *Arabidopsis* ESCRT network. Both graphic representations were prepared with the Kamada-Kawai Algorithm. **a** Betweenness centrality analysis. The size of the *red circles* represents the centrality of the node (absolute values are given in *square brackets*).

b Girvan-Newman clustering analysis. The *Arabidopsis* ESCRT network can be divided in two subparts mainly containing the ESCRT-III components (*green*) or the ESCRT-I/-II components (*yellow*), respectively. ESCRT-I and -II cannot be separated

For this purpose we used the Girvan-Newman algorithm (Girvan and Newman 2002). The Girvan-Newman algorithm determines all shortest paths of the network as the above mentioned C_B algorithm does. Edges that are used by many shortest paths are assigned high ranks. In a second step, the edge with the highest rank is removed from the network. The two steps are repeated until all edges are removed. Whenever removing an edge creates regions detached from the rest of the network the "modularity" is calculated, a parameter indicating the significance of the partition. Using this method, we could not find the postulated three complexes but the *Arabidopsis* network could be divided into two groups, one that among others contains the ubiquitin interacting proteins VPS23 and VPS36 and one that contains the structural ESCRT III components (Fig. 3b).

Arabidopsis ESCRT interactions occur on endosomes

The BiFC technique does not only allow to detect whether proteins interact but also to determine the intracellular localization of the interaction. The BiFC signals of the positive interactions were observed on punctuated structures. To decide whether these are Golgi stacks or endosomes we performed colocalisations with the Golgi marker G-rb (Nelson et al. 2007) and the MVB marker ARA6 (Ueda et al. 2004) (Fig. 4) in *Arabidopsis* protoplasts with one example for intra-ESCRT-I interactions (Fig. 4a, b), one example for ESCRT-I ESCRT-II interactions (Fig. 4c, d), and one example for ESCRT-I ESCRT-III interactions (Fig. 4e, f). In these experiments, no colocalisation of any BiFC interaction with the Golgi marker G-rb was found

(Fig. 4a, c, e). That the punctae represent endosomes is evident from triple transformations of protoplasts with the BiFC constructs of the respective interactors and ARA6:CFP as MVB marker (Fig. 4b, d, f). Therefore it is likely that the *Arabidopsis* ESCRT system is assembled on MVBs to mediate sorting in analogy to the mechanism in yeast and mammals.

Discussion

The ESCRT pathway in plants

The finding that the *Arabidopsis* Vps23 homolog, ELCH, shares typical features with Vps23/TSG101 such as binding to ubiquitin, endosomal localization and interaction with ESCRT-I components strongly suggested the presence of an ESCRT pathway in plants (Spitzer et al. 2006). In support of this, homologs of most yeast class E genes were identified in the *Arabidopsis* genome (Mullen et al. 2006; Winter and Hauser 2006; Spitzer et al. 2006). Our systematic pairwise analysis revealed a network of interactions integrating all tested ESCRT components into the pathway. To assemble the network, we used two reporter based methods that allow high throughput analysis. Both methods rely on the reconstitution of functional protein from two protein halves. The Y2H assay uses the DNA-binding domain of GAL4 fused to the bait protein and the GAL4 transactivation domain fused to the bait (Fields and Song 1989). Interacting bait/prey pairs reconstitute the functional transcription factor that activates transcription of the His3 gene that allows growth of yeast cells in histidine

depleted media. As yeast transformation is an easy and robust method, Y2H is particularly suited for high-throughput analysis. The main pitfalls often attributed to Y2H screens are high numbers of false positive results due to autoactivation of the reporter by the bait-protein, or so called de novo autoactivation as consequence of spontaneous mutations (Lalonde et al. 2008). In this respect, it has to be noted that a systematic analysis of pairwise Y2H interaction assays (as we have used here) has recently shown a high stringency of this method with comparably low false positive rates (Yu et al. 2008). In the BiFC method, the N- and C-terminal halves of the yellow fluorescent protein YFP are fused to the bait and prey, respectively. Upon protein interaction, the halves build a functional YFP that allows detection of the localization of the interacting proteins (Hu et al. 2002). The advantage of both methods is the in vivo situation of the assays. A further advantage of the BiFC assay is the very high sensitivity that is due to the stability of the reconstituted fluorescent protein (Magliery et al. 2005). This sensitivity on the other hand leads to the problem of unspecific reconstitution of the YFP halves, especially upon overexpression of the fusion-proteins of interest (Lalonde et al. 2008). Furthermore, the stability of the interaction does not allow claims about interaction dynamics (Magliery et al. 2005). The reliability of the network presented here is based on five arguments.

First, sequence homologs generally display similar interaction patterns. Noteworthy exceptions are the VPS2 homologs; VPS2-3 is embedded into the putative ESCRT-III structure whereas VPS2-1 and VPS2-2 are showing interactions mainly with members of the putative ESCRT-I and ESCRT-II subcomplexes. This could mean that they do not act redundantly with *VPS2-3* but serve special functions. In the *Arabidopsis* genome, four more genes are present that share similarities with the ESCRT-III subunit Vps2. They most likely represent orthologs of the yeast genes Vps46 and Vps60 that are regulators of the AAA ATPase Vps4 (Shahriari et al. 2010; Spitzer et al. 2009; Winter and Hauser 2006; Mullen et al. 2006). One could speculate that in plants a further diversification of the family has occurred. As the different position in the network is, however, mainly based on non-detectable interactions further experiments such as a thorough genetic analysis of the VPS2-like clade of small coiled-coil proteins is needed before a conclusion can be made.

Second, the systematic pairwise manner of the study supplies numerous internal negative controls for each of the found interactions in both test systems. Therefore, false positive interactions that result from systematic errors as discussed above are unlikely.

Third, most of the interactions could be observed in both test systems. Here, it has to be noted that most interactions

Fig. 4 Colocalisation of selected BiFC interactions with ARA6:CFP ▶ and the Golgi marker G-rb. **a** Colocalisation of ELC VPS37-1 BiFC with the Golgi marker G-rb; **b** colocalisation of ELC VPS37-1 BiFC with the MVB marker ARA6:CFP; **c** Colocalisation of ELC VPS36 BiFC with the Golgi marker G-rb; **d** Colocalisation of ELC VPS36 BiFC with the MVB marker ARA6:CFP; **e** Colocalisation of ELC VPS20-1 BiFC with the Golgi marker G-rb; **f** Colocalisation of ELC VPS20-1 BiFC with the MVB marker ARA6:CFP. For each cotransfection four images are shown, localisation of the respective BiFC interaction, localisation of the respective marker, an overlay with the BiFC shown in *green* and the marker in *red* and a brightfield picture (*from left to right*). Colocalisation in the overlay appears in *yellow*. Colocalising MVBs in **b**, **d**, and **f** are indicated by *arrowheads*. Non-colocalising punctae in **a**, **c** and **e** are *highlighted* by *closed* (BiFC) and *open* (marker) *arrowheads* in the respective fluorescence channel and in the overlay. *Scale bars* 10 μm

within the ESCRT-III subcomplex were not detected in the two-hybrid system. It is unclear whether this is due to masking of interaction interfaces by the used tags or whether the putative role of ESCRT-III as endosomal coat requires membranes for some interactions to occur.

Fourth, all genes tested in this work that are present in the Genevestigator database (https://www.genevestigator.ethz.ch/at/) show a largely ubiquitous expression profile indicating that they are present in the same cells at the same time (data not shown).

Finally, the observed BiFC signal is always found on endosomes, as would be expected for components of the ESCRT system. However, the colocalisation experiments have to be regarded with care, as the impact of the irreversibility of the BiFC system has to be taken into account (Magliery et al. 2005). It is possible that the interactions between the ESCRT components might have occurred at an earlier compartment, such as the trans-Golgi network, and that detection of the BiFC signal at MVBs is due to prolonged association with the membrane. Consistent with this, we found also some punctuate structures in our colocalisation experiments with the MVB marker ARA6 that only displayed the BiFC signal (Fig. 4b, f).

In most cases we additionally observed a cytosolic background that could be interpreted as preformation of the respective interaction in the cytosol, as has been shown for ESCRT-I (Chu et al. 2006; Spitzer et al. 2006). As, however, the stability of the BiFC interactions could also lead to unspecific effects upon ESCRT dismantling from the membrane, the cytosolic BiFC signal will not be discussed further.

Topology of the network

To address possible substructures and to identify central proteins in the network we used the Girvan-Newman and the C_B algorithms (Freeman 1977; Girvan and Newman 2002). We found that the *Arabidopsis* network can be divided into two parts containing either the ubiquitin-interacting proteins
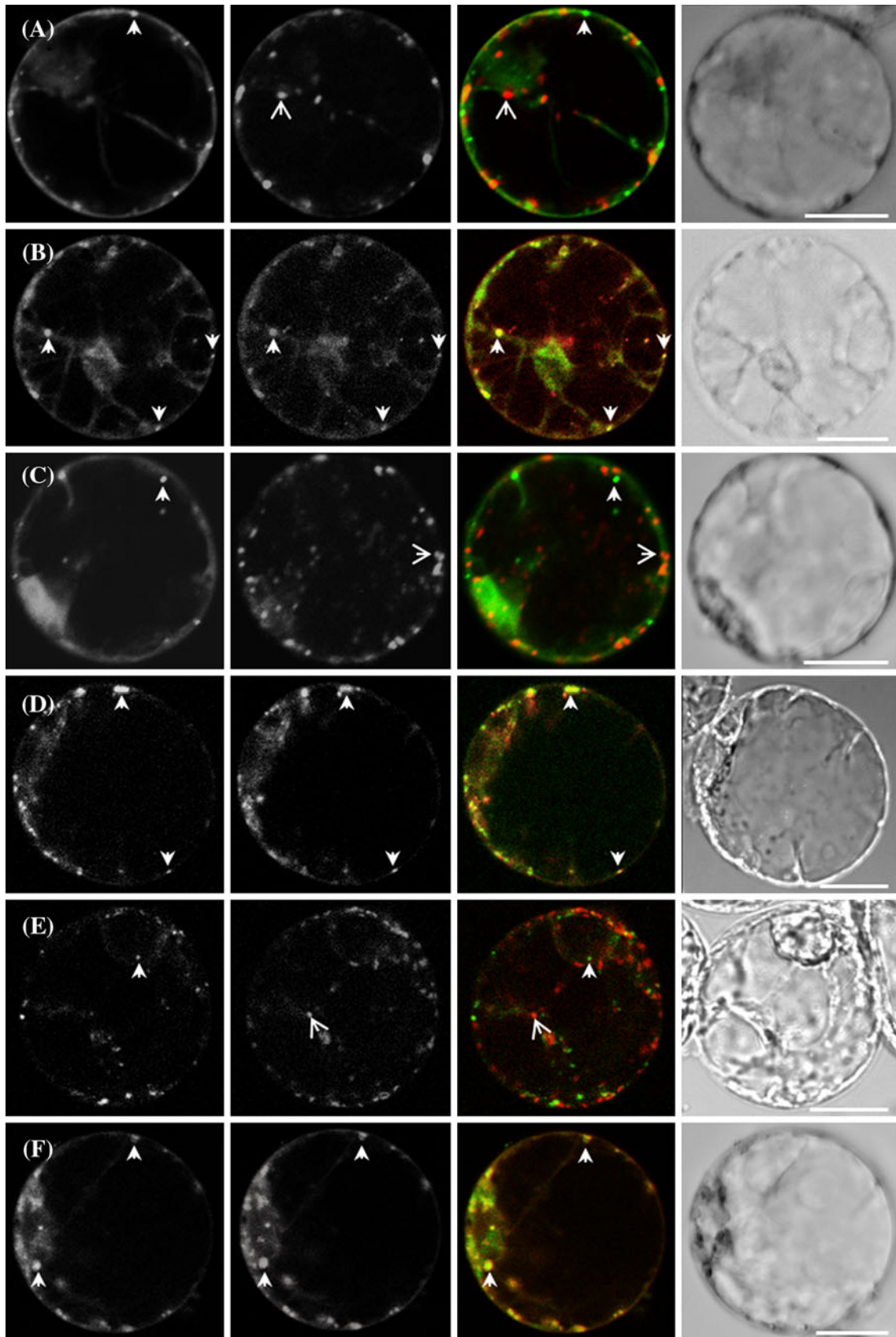
of the ESCRT-I and ESCRT-II components or the strongly interconnected ESCRT-III constituents. Interactions between ESCRT-I and ESCRT-II are mediated by a large interaction interface that is built by the ESCRT-I proteins VPS23-1, -2 and VPS28-2 and the ESCRT-II proteins VPS22 and VPS36. However, the finding that ESCRT-I components have been purified by co-immunoprecipitation with ELCH (Spitzer et al. 2006), suggests that ESCRT-I and ESCRT-II complexes can occur as separate complexes in vivo.

In the C_B analysis, we found that the ESCRT-I component ELCH has the highest degree of centrality. In this respect, the interaction network differs between plants and yeast. In yeast, interactions of ESCRT-I with ESCRT-II and ESCRT-III are made by Vps28 binding to Vps22 and Vps36 (ESCRT-II) and Vps20 (ESCRT-III) (Bowers et al. 2004). By contrast, in plants and similarly in mammals (von Schwedler et al. 2003), the interaction between the complexes appears to be based on the interactions between VPS23, VPS22, VPS36 and Vps20 [Vps20 not shown in mammals (von Schwedler et al. 2003)].

Interaction between ESCRT-I and ESCRT-III

We found a number of interactions between putative ESCRT-III and ESCRT-I components. Two of these are the above mentioned VPS2-1 and VPS2-2 that possibly have special functions. One interaction (ELCH VPS20-1) is present between components that are integral to their respective ESCRT complex. This interaction could be observed in both the BiFC and the yeast two-hybrid system. As mentioned above, an interaction between ESCRT-I and Vps20 from ESCRT-III has also been found in the yeast network (with Vps37 and Vps28). Therefore, one can assume that this interaction is mechanistically meaningful. In yeast, Vps20 is the first ESCRT-III component recruited to the endosomal membrane. For the classical conveyor belt model such an interaction is not only unnecessary but would even short-circuit the belt. The model is largely based on genetic epistasy experiments in yeast that seem to suggest a successive action of the ESCRT subcomplexes (Babst et al. 2002b). This view is not well supported by biochemical data. The affinities of Vps36 and Vps23 for ubiquitin, for example, are in a similar range (K_D 0.1–0.3 μM) making it difficult to explain, how the cargo can be passed on directionally (Raiborg and Stenmark 2009). Structural data also seem to contradict the model. The Vps23 and Vps36 ubiquitin binding domains are separated by the long rigid ESCRT-I stalk, suggesting that ubiquitin transfer is unlikely to occur within one copy of the ESCRT complex (Raiborg and Stenmark 2009). Therefore, in the yeast field currently alternative models are discussed. In 2007, the so called concentric ring model

has been proposed (Nickerson et al. 2007). In this model, the ESCRT-0 components Vps27 and Hse1 nucleate the assembly of the ESCRT complexes on the endosomal membrane. The ESCRT-I and -II complexes are arranged in a ring surrounding ESCRT-0 and are in turn surrounded by the ESCRT-III components (Nickerson et al. 2007).

In a recent publication, a new model has been introduced (Wollert and Hurley 2010): ESCRT-0 is not structurally embedded in the ESCRT complex but acts upstream in cargo clustering. Thus, in other organisms this function could be performed by other molecules like the TOM1 homologs (Blanc et al. 2009). ESCRT-I and -II together induce bud formation and confine the cargo. ESCRT-III then forms a collar around the neck of the budding vesicle thereby mediating scission (Wollert and Hurley 2010). Here, ESCRT-I and ESCRT-II mechanistically act at the same level and ESCRT-I and ESCRT-III interactions would not only be possible but even beneficial for the stability of the whole complex. Taken together, our data are difficult to explain with the older models and support the model formulated by Wollert and colleagues (Wollert and Hurley 2010).

Conclusions and outlook

Analysis of protein–protein interaction networks is a useful tool to study complex formation and gain initial clues about biochemical mechanisms. However, the approach is limited by its lack of time resolution. Therefore, to further understand the plant ESCRT system thorough analysis of subcellular localisations of ESCRT components and the kinetics of ESCRT mediated vacuolar sorting cargo in the respective mutants will be necessary. The main prerequisite for such experiments will be the identification of an ESCRT cargo as sorting marker. It will be therefore important to find and characterise suitable sorting markers. Good candidates are already available with PIN1, PIN2 and AUX1 that are mislocalised in a *chmp1a chmp1b* background (Spitzer et al. 2009) or BRI1 and BOR1 that have recently been shown to be sorted into the intraluminal vesicles of MVBs (Viotti et al. 2010).

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