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Interaction studies of HvVPS24 within the Barley ESCRT-III complex

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1 Abstract

The Endosomal Sorting Complex Required for Transport (ESCRT)-III has been studied in plants and has key roles in protein sorting, membrane fission and in multivesicular body (MBV) formation. Barley seeds, which were examined in this project, are efficient and requested platforms in molecular farming in terms of production of high quality recombinant pharmaceutical proteins. Moreover barley seeds are used as a nutrition source for human and animals. The members of ESCRT-III were identified and their localization and putative function have been characterized in barley endosperm. It has been shown that the localization of recombinant expressed ESCRT-III barley proteins such as HvSNF7a, HvVPS24 and HvVPS60a are different between aleurone and subaleurone layers of barley endosperm. It has been also reported that the localization may differ during the various developmental stages.

The aim of this study was to identify the interactions of HvVPS24 with ESCRT-III components like HvSNF7, HvVPS60, HvVPS4 and a putative seed storage protein HvHinb2. The project was designed using yeast two-hybrid (Y2H) and biomolecular fluorescence complementation (BiFC) assay to test the protein-protein interactions between these proteins. BiFC was performed in *Nicotiana benthamiana* leaves and analyzed by confocal microscopy.

The examined HvVPS24 does interact lightly with HvSNF7 but no HvVPS24 homodimerization was observed. According to our yeast two-hybrid and BiFC analysis there is no HvVPS24 interactions with other components of HvESCRT-III. In further studies Co-IP assays should be performed to identify the HvVPS24/HvSNF7/HvVPS60 partners in Barley endosperm.

2 Zusammenfassung

Die Endosomal Sorting Complex Required for Transport (ESCRT)-III Maschinerie wird in Pflanzen studiert und spielt Schlüsselrollen in der Sortierung von Proteinen und der Membran Fission aber auch in der multivesikulären Kompartmentbildung.

Gerste dient als Nahrungsquelle für Menschen und Tiere. In der Wissenschaft wird dieses Getreide auch als Modellpflanze und für die Produktion von rekombinanten Proteinen verwendet.

Die Gerste ESCRT-III Mietglieder wurden identifiziert und erste Lokalisierungstudien bzw. ihre putativen Funktionen wurden in Gerste Samen studiert. Die Lokalisierung von rekombinant exprimierten ESCRT-III Proteinen (wie HvSNF7a, HvVPS24 und HvVPS60a) ist zwischen Aleurone und Subaleurone unterschiedlich und ändert sich auch während der Entwicklung.

Ziel dieser Masterarbeit war es, die Interaktionen von HvVPS24 mit ESCRT-III Komponenten wie HvSNF7, HvVPS60, HvVPS4 und dem putativen Speicherprotein HvHinb2 zu identifizieren. Die Interaktionen wurden mit dem Hefe-Zwei Hybrid System und der Bimolekularen Fluoreszenzkomplementation identifiziert. Die Bimolekulare Fluoreszenzkomplementation wurde in Blättern von *Nicotiana benthamiana* ausgeführt und die Interaktionen wurden mittels Konfokal Mikroskope untersucht.

Das untersuchte HvVPS24 interagiert leicht mit HvSNF7, zeigt aber keine HvVPS24 Homodimerisierung. Es wurden keine weiteren Interaktionen zwischen HvVPS24 und anderen Komponenten von HvESCRT-III beobachtet. In einer weiterführenden Arbeit sollte ein Ko-Immunopräzipitation durchgeführt werden, um Interaktionspartner von HvVPS24/HvSNF7/HvVPS60 zu entdecken.

3 Introduction

3.1 Barley

Barley **(***Hordeum vulgare* L.) is a member of grass family and a major of cereal grain, which is used in malting industry as a source of fermentable material for beer, animal fodder and barley bread as a healthy food for human. Moreover, barley is among world's earliest domesticated crop plants, which was sequenced in 2012. Its small diploid genome (5000 Mbp) is containing 14 nuclear chromosomes (Mayer et al. 2012). Nowadays barley plant is also used as a model plant for the production and accumulation of recombinant proteins and for molecular farming (Hensel et al. 2015; Mrizova et al. 2014). The endosperm is a storage seeds tissue, which can collect huge amounts of storage metabolites for molecular and biochemical studies (Muntz 1998). In this study we analyzed the barley ESCRT-III complex.

3.2 The Cereal Endosperm & ESCRT III in cereal (seeds)

Cereal endosperm has fascinated researchers due to the economic importance; it is a main source of food and nutrition for human and animal (Olsen 2004). Furthermore it has a short growth and maturation span which makes it a good model system for biological plant studies (Hilscher et al. 2016; Young and Gallie 2000). The major part of cereal seeds fills with endosperm that consists of four different layers: starchy endosperm, transfer cells in basal layer, an epidermal aleurone and some embryo surrounding cells (Olsen 2001). A programmed cell death starts through the developmental processes in cereal endosperm, in which hallmarks of apoptosis begin. Therefore in a fully maturated seed there is dead inner starchy endosperm which is surrounded by some living cell layer in aleurone to produce enzyme for germination (Olsen 2004; Young and Gallie 2000; Arcalis et al. 2014). The endomembrane system experience some morphological changes for mobilizing of storage proteins during germination in cereal seeds (Ibl and Stoger 2014). There are three main categories of storage proteins in seeds including prolamins, albumins and globulins. Protein storage vacuoles (PSVs) accumulate albumins and globulins (Galili 2004; Shewry, Napier, and Tatham 1995). The main point is the ability of specialized storage organelles in seeds that helps protein accumulation with a high concentration in a relative small compartment (Stoger

et al. 2005; De Jaeger et al. 2002; Arcalis et al. 2014). There are two routes for seed storage proteins (SSPs) to arrive their destinations: prolamins aggregate in ER-derived protein bodies (PBs) while soluble globulins and albumins cross over ER and Golgi to PSVs (Ibl and Stoger 2012). The species, tissue layers and time point are important factors in storage protein transportation in cereals (Ibl and Stoger 2012; Zheng and Wang 2014). Considerable endomembrane reorganization during development leads to extensive and complex SSP transport in to the endosperm (Hoh et al. 1995; Ibl and Stoger 2012; Wang et al. 2010). Figure 1 shows the dynamic morphology of protein storage vacuoles (PSV) from three different layers of barely endosperm containing aleurone, subaleurone and starchy endosperm at the central part (Ibl et al. 2014). At the initiate stage of development large PSVs were formed in subaleurone in contact with ER. During endosperm development, PSVs first shrink followed by fusion of PSVs containing protein bodies. During late development protein bodies degenerate and lose their membrane in central starchy endosperm and starch granules become more prominent (Ibl et al. 2014).

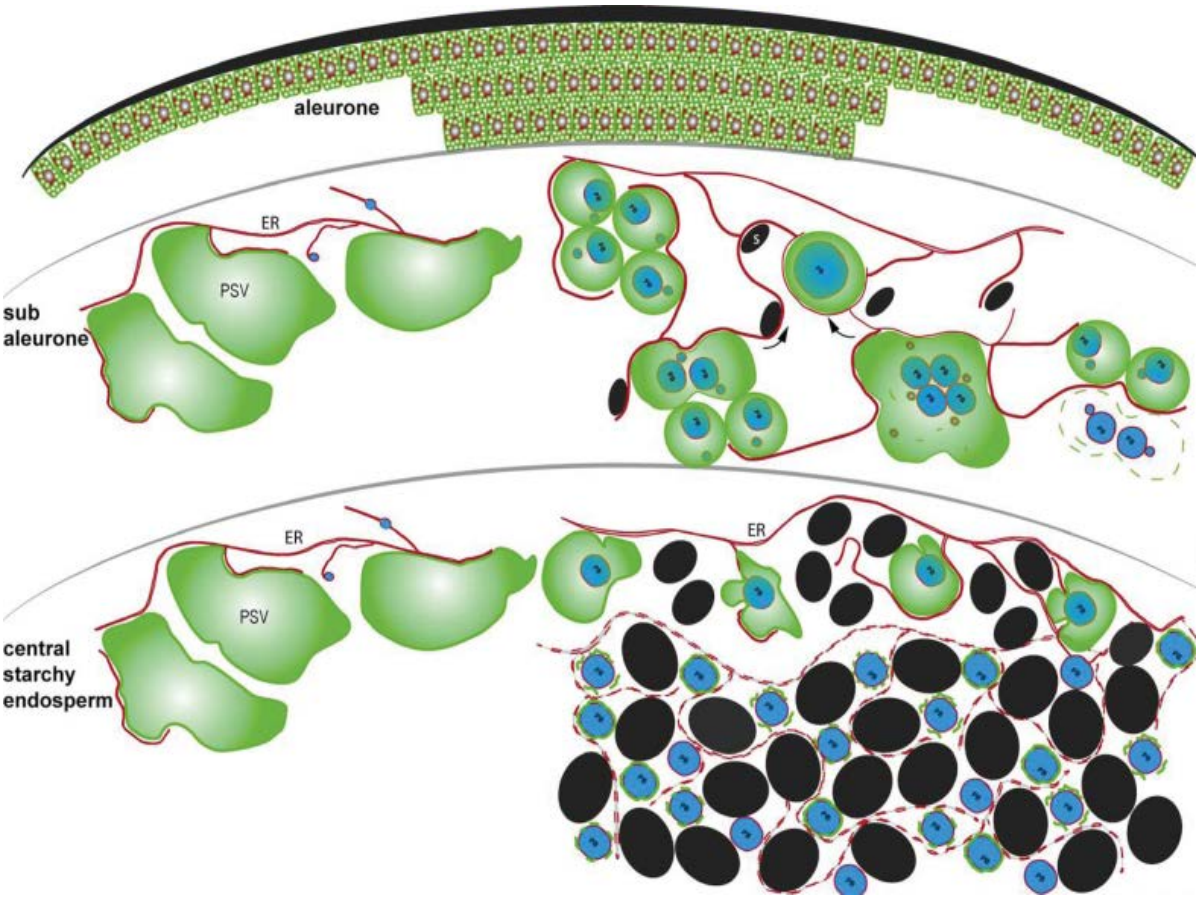


Figure 1.This scheme shows the destiny of protein storage vacuoles in three different layers of barley seeds. Green color is small PSV, red color is ER and blue color shows protein bodies (Ibl et al. 2014)**.**

3.2.1 Endosomal trafficking in plants

Cells always receive so many signals from inside and outside and cells must response continuously to the all stimuli. Endocytosis and endosomal trafficking are important cellular mechanisms to control the dynamics and turnover of plasma membrane proteins containing transporters, receptors and enzymes for cell wall biosynthetic (Paez Valencia, Goodman, and Otegui 2016). Generally the vesicular trafficking system is based on transport of proteins and lipids to their destination, for instance in vacuoles, lysosomes and multivesicular bodies for degradation or in the case of secretion and recycling to the plasma membrane (Gruenberg and Stenmark 2004; Russell, Nickerson, and Odorizzi 2006; Katzmann, Odorizzi, and Emr 2002). Endocytosis in plant cells occurs mainly at plasma membrane and at the cell plate. At the plasma membrane extracellular components are internalized and form endocytic vesicles. Endosomes as membrane-bound compartments have four classes such as: early, recycling, intermediate and late endosomes. If endocytic vesicles release from membrane and fuse with

endosomes, early endosomes will form. Trans-Golgi network (TGN) in plant functions as an early endosome which take up the endocytic cargo (Dettmer et al. 2006; Lam et al. 2007). Late endosomes are consequences of fusion of early endosome with vacuoles and lysosomes for plasma membrane degradation, showed in figure 2 (Paez Valencia, Goodman, and Otegui 2016). Additionally, late endosomes regulate the transport of new-synthesized vacuolar proteins form Golgi to the vacuole (Spitzer et al. 2009). ESCRT machinery is a part of endosomal trafficking system and the MVB sorting pathway (Fig. 2).

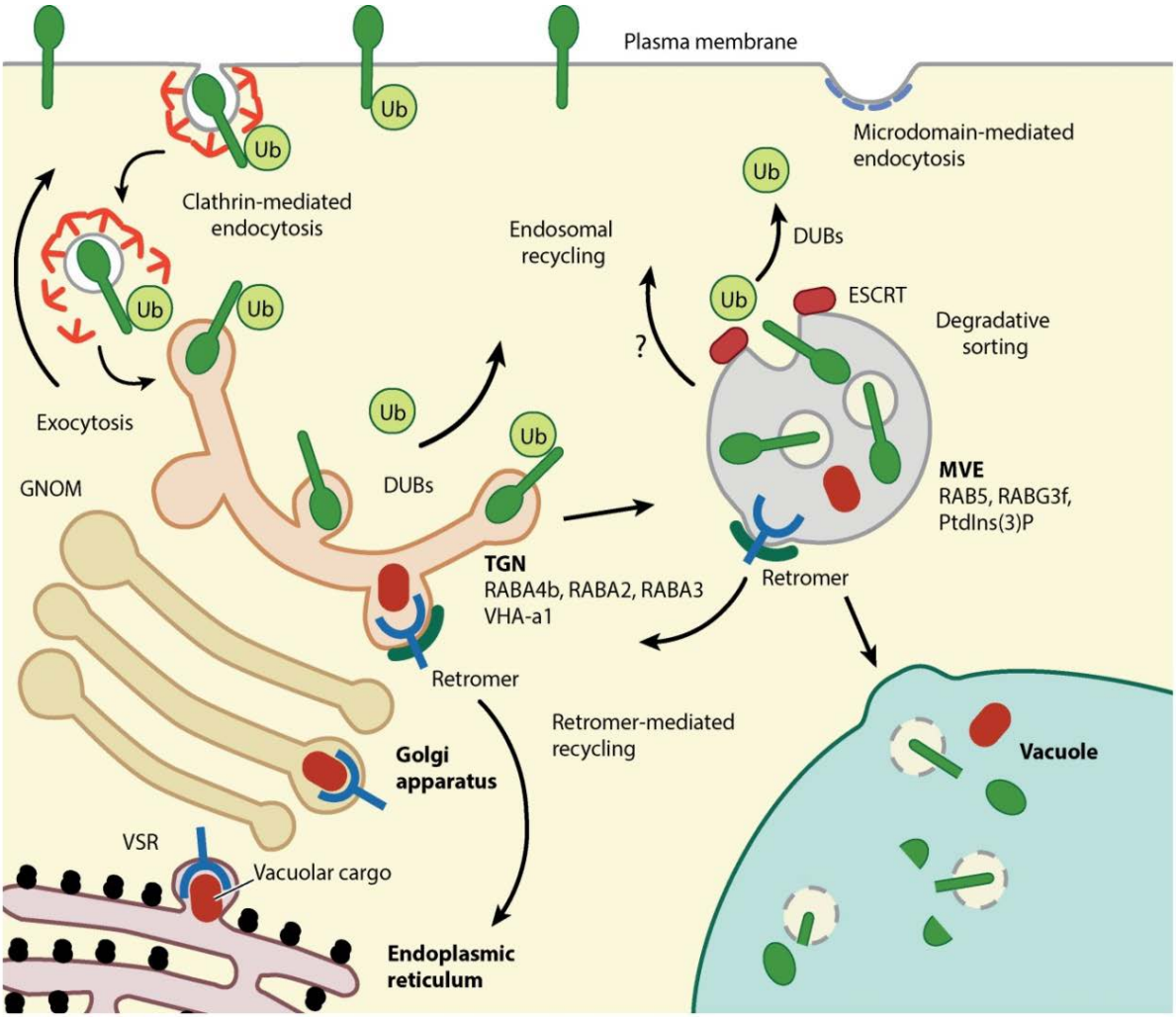


Figure 2. This overview scheme shows endocytic and endosomal trafficking system in plants (Paez Valencia, Goodman, and Otegui 2016).

3.3 ESCRT Machinery

Endosomal Sorting Complex Required for Transport (ESCRT) is a conserved machinery in eukaryotes which is well studied in mammals and yeast. Plant ESCRT orthologs were first described in 2006 (Winter and Hauser 2006). ESCRT plays part in degradation of cargo protein (plasma membrane proteins), formation of multivesicular bodies (MVBs), cytokinesis, regulation of endosomal recycling, receptor-mediated endocytosis, receptor signaling from endosome and also autophagy in plant cells (Muller et al. 2007; Cardona-Lopez et al. 2015) Moreover plant ESCRT machinery is involved in some cellular processes such as embryo differentiation, auxin transportation and protein ubiquitination for vacuole degradation (Otegui and Spitzer 2008).

ESCRT has four multi-protein subcomplexes such as ESCRT-0, -I, -II, and -III and some associated proteins (Richardson et al. 2011). ESCRT-0 (VPS27) concentrates ubiquitinated cargo proteins at the endosomal membrane and recruits ESCRT-I (VPS23, VPS28 and MVB12) that recruits ESCRT-II (VPS22, VPS36, VPS25) (Richardson et al. 2011). ESCRT-III (VPS20-VPS32, SNF7 and VPS2-VPS24) is recruited by ESCRT-II and facilitates membrane deformation, membrane scission and protein sequestration (Figure 3) (Winter and Hauser 2006; Richardson et al. 2011). ESCRT III regulates the MVB formations and is important for membrane remodeling (Ibl et al. 2012; Gruenberg and Stenmark 2004).

In this study we focused on the barley ESCRT-III machinery and its associated proteins.

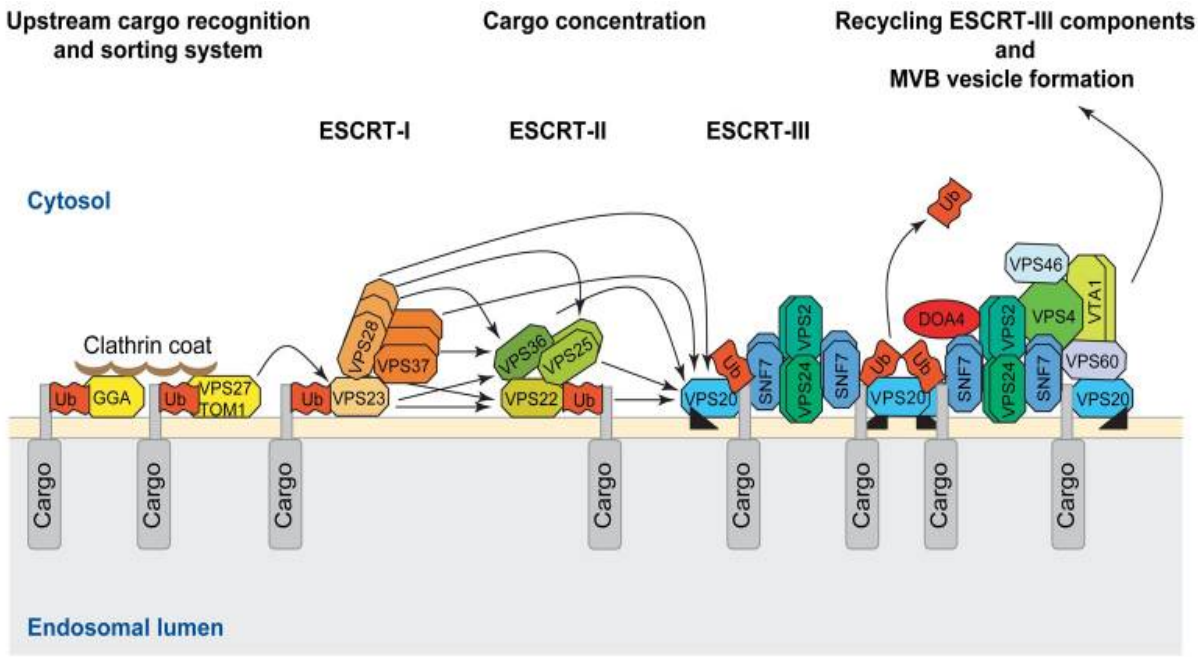


Figure 3. Model of the ESCRT-dependent protein sorting in the MVB vesicle formation pathway.

This figure summarizes the cargo sorting system of endosome via series of ubiquitin- and clathrin-binding proteins and also formation of all 3 ESCRT complexes, their subcomlexes and all interactions between them (Winter and Hauser 2006)

3.3.1 ESCRT-III and its interaction partners

The most conserved part of ESCRT machinery, ESCRT-III, consists of four protein subunits (VPS2, VPS24, VPS20, SNF7) which are transiently assembled on endosomes (Winter and Hauser 2006). VPS2-VPS24 forms together a subcomplex as well as VPS20-SNF7. Their positively charged N-terminus domain (125 amino acids) has function in membrane interaction and oligomerization in comparison with their C-terminus acidic domain which inhibits self-oligomerization. SNF7 domain made up of 160 amino acids which has function in protein-protein interaction (Shen et al. 2003). VPS60 (CHMP5), VPS46 (CHMP1) and VPS4 are known as associated, non-classical ESCRT-III proteins (Winter and Hauser 2006). After binding the ESCRT-III subunits with endosomal membranes, there are two conformation possibilities, a cytoplasmic, closed, inactive structure versus an endosomal, open active state (Babst et al. 1997). If VPS25 and VPS20 of ESCRT-II recruit ESCRT-III, its activation leads to MVB formation. VPS20 also recruits SNF7, caused its homo-oligomerization. Then SNF7 recruits VPS24 an ESCRT-III adaptor protein to stabilize its

filament and recruiting an enzyme for deubiquitinatation of the cargo protein. For complete assembly of ESCRT-III VPS24 recruits VPS2 at the endosome. In class E compartment VPS2.1 and VPS24.1 also interact with the SH3 domain of STA3 (AMSH3) as a deubiquitinating enzyme (Katsiarimpa et al. 2014). Class E compartments are abnormal endosomes which are formed by ESCRT dysfunction in yeast due to blocking of cargo sorting system (Babst et al. 1997; Russell et al. 2012) In case of ESCRT-III disassembly inhibition by SKD1 AMSH3 protein interacts with VPS2.1 and VPS24.1 of ESCRT-III (Katsiarimpa et al. 2011). Accessory proteins like VPS4 play important roles in ESCRT-III machinery. VPS4 acts as a multimeric mechanoenzyme that interacts with ESCRT-III components via MIT domain at the N-terminus which identifies C-terminal MIM (MIT interacting motifs) domain of other ESCRT-III components. VPS60 interacts directly with another accessory protein Vta1 (Vps twenty-associated-1) via binding the MIM domain on the ESCRT-III subunits to two MIT (microtubule-interacting and trafficking) domains at own N terminus (Lata et al. 2008). Vta 1 also induces VPS4 ATPase activity and leads to form a supercomplex. There is AtSKD1 expression in all Arabidopsis tissues that plays roll in MVB formation and localizes in cytoplasm (Haas et al. 2007). VPS4 maybe has a function in fission and pinching off the luminal vesicles because overexpression of SKD1 in Arabidopsis leads to form a large hybrid endosomal/vacuolar structure. Even blocking of ATPase activity of SKD1 is lethal (Haas et al. 2007). In plant cells SKD1 helps the vacuolar protein trafficking to keep the shape of large central vacuole. VPS24 is a class-E vacuolar protein sorting which has effect on compartimentalization of phosphatidylinositol 3, 5-biphosphate-dependent endosome. AtVPS2.2 was localized into the vesicles at plasma membrane and also nucleus that is shown by GFP fluorescence localization studies (Ibl et al. 2012).

There is almost interaction between all ESCRT-III subcomplexes. VPS4/SKD1 in Arabidopsis interacts with ESCRT-III proteins in different ways, for example with SNF7.1/B and SNF7.2/A, with VPS46.2, with VPS20.1/2 and further with VPS60.1/A and VPS60.2/B (Shahriari et al. 2011; Cai et al. 2014; Ibl et al. 2012). Moreover, interaction between SNF7.2/A and SNF7.1/B was shown. According to the results of Y2H studies an interaction between VPS24.1/A and VPS60/1, VPS60.2/B and also between VPS2.2 and VPS4 was observed which I summarized in figure 4.

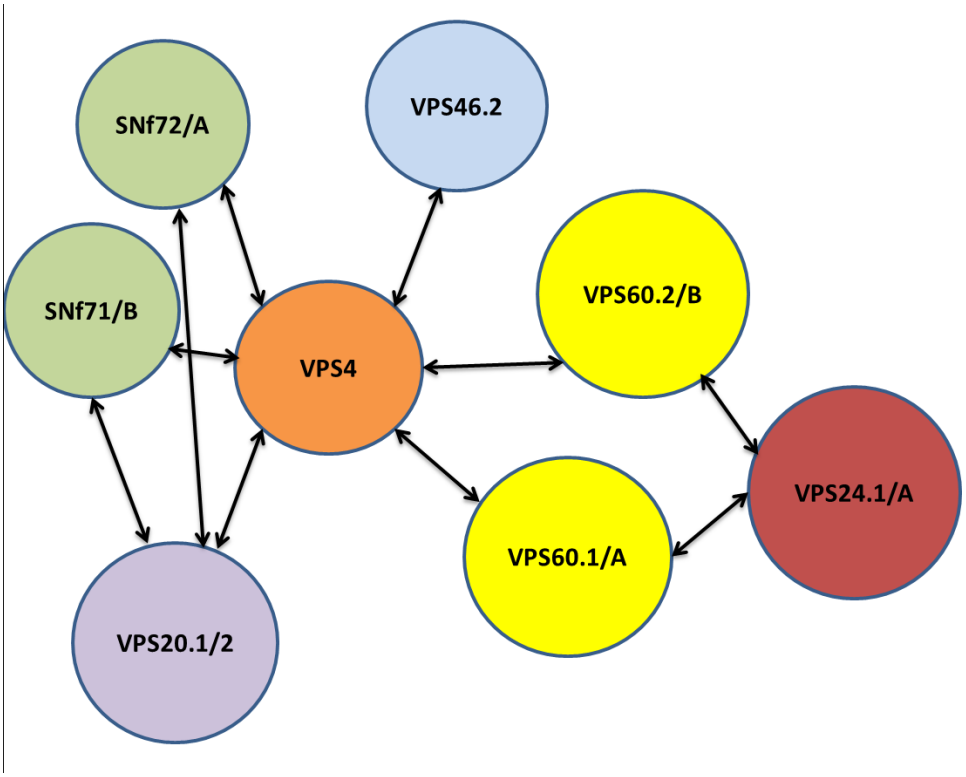


Figure 4. Interaction scheme of plant ESCRT-III components.

3.4 Hordoindolines (Hinb) in barley seeds

Wheat puroindolines as cysteine-rich basic hydrophobic proteins group on the starch surface and play an important role in grain texture and malting quality (Beecher et al. 2002). There are two significant puroindoline proteins (a) and (b) which have a particular tryptophan-rich sequence motif. In the absence of puroindoline (a) or mutation of puroindoline b wheat grain become harder than normal and it cannot bind the granule surface (Takahashi et al. 2010). In a previous Co-IP experiment from a transgenic actin::VPS24mCherry barley line HvHinb2 is precipitated with HvVPS24 (Verena Ibl, personal communication). Therefore we decided to include Hinb2 in this protein interaction study.

3.5 Methods of protein-protein interaction

Protein-protein interactions are involved in almost all cellular mechanisms such as vesicle budding, trafficking and fusions, gene regulation, signaling pathways and receptor dimerization (Xing et al. 2016). The achievement of gene fusion and protein labeling with fluorescence probes prepared a novel technological approach in protein-protein interaction studies (Braun and Gingras 2012). To find the best method for protein-protein interaction studies considering some points and issues is needed. Some interactions are transient for example or not strong enough to be detected. Moreover kinetics, thermodynamics, stoichiometry or cofactors can affect the detection system. In terms of expression control a strong promoter, which helps fusion proteins to overexpress, is needed but it may lead to artificial results. Controls are very important in protein-protein interaction studies: to detect a reliable positive interaction the best negative control would be the identical construct but with mutation in the interacting domain. Non-native environmental conditions can dramatically influence the protein expression, translation, post translational modifications and even lifespan and turnover of a protein which may manipulate positive interaction analysis (Xing et al. 2016). There are some protein-protein interaction methods which have been used frequently in plant sciences including yeast-two-hybrid, split-ubiquitin system, biomolecular fluorescence complementation (BiFC), föster resonance energy transfer (FRET), split-luciferase assay and co-immunoprecipitation (Co-IP). In this master study I tried to characterize the interactions of the barley ESCRT-III complex with the focus on VPS24 using yeast two-hybrid assay (Y2H) and bio-molecular fluorescence complementation (BIFC) in transiently transformed *Nicotiana benthamiana* cells.

3.5.1 Yeast Two-Hybrid (Y2H)

The principle of Y2H is based on properties of Gal4 protein that have DNA-binding domain and Gal4 activation domain as a transcriptional activator (Xing et al. 2016). When these two domains with fusion proteins interact together reporter genes will be expressed (figure 5).

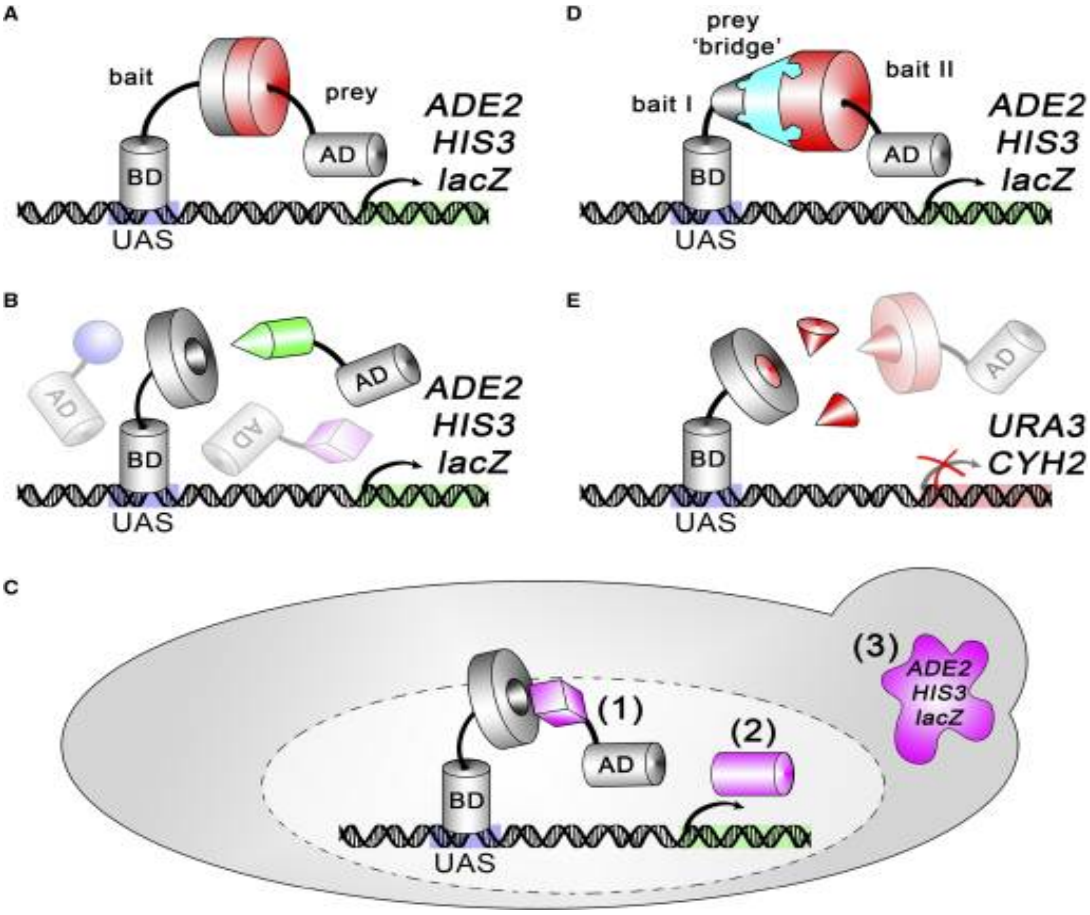


Figure 5. The Yeast Two-hybrid and its modifications.

A: Interactions between BD and AD of Gal4 to activate reporter genes like ADE2, HIS3 and lacZ. B: Green pin-like shape binding partner. C: False positive interaction in Y2H. D: Extension of Y2H by a third protein as a bridge. E: Reverse Y2H to detect inhibited proteins 1: Unrelated prey fusion binds to the bait. 2: transcriptional activator. 3: Enzyme that affected the selection pressure. URA3: selection marker (Xing et al. 2016)**.**

3.5.2 Biomulecular Fluorescence Complementation (BiFC)

Another useful, simple and reliable technique of protein-protein interaction within living cells used in this project is BiFC. BiFC was used first time in E. coli: GFP was split into 2 domains N-terminal and C-terminal of non-fluorescence, after interaction the fused fragments can refold and again fluorescence (Magliery et al. 2005). This method is mostly based on

complementary association of fluorescent fragments of a fluorophore which are fused to the putative interaction partner (figure 6) (Kerppola 2009). Moreover, in case of multiple protein interactions multicolor BiFC can be used to visualize the interactions at the same time within the same cell (Grinberg, Hu, and Kerppola 2004; Hu and Kerppola 2003). Even though BiFC assays provide a very sensitive and high resolution visualization system to detect protein-protein interactions, there is always risk of false positive if the split fragments reassemble autonomously (Magliery et al. 2005; Kerppola 2009). In some cases an irreversible reassembly occurs because of overexpression of the split fusion protein (Xing et al. 2016). The level of protein expression, quality of protein folding and turnover rate can influence by the complex formation and time consuming detection procedure (Xing et al. 2016; Kerppola 2009). For designing an effective and reliable BiFC system, the size of promoter, the location of tag, type of fluorophore and most importantly appropriate controls should considered (Grefen and Blatt 2012). The principle of this method is mainly based on constitutive over-expression of fusion proteins by a 35s promoter- of split YFP which all provided by the pSPYNE and pSPYCE vectors (Walter et al. 2004). In this study N-terminal and c-terminal of YFP (yellow fluorescence protein) are fused with the HvESCRT-III proteins. If split proteins interact with each other the extended fluorescence will detected by confocal microscope.

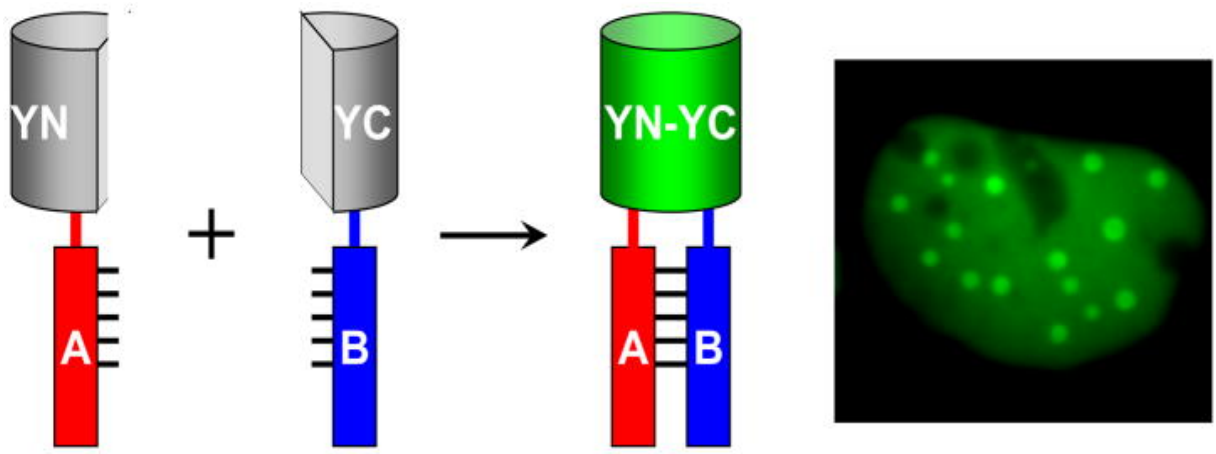


Figure 6. Fundemental basics of biomolecular fluorescence complementation assay.

The red and blue colors show putative interaction partners which fused to the N-terminus and C-terminus fragments of a flourophor. In case of protein interaction fluorescent fragments correlate together and produce fluorescent (Kerppola 2009).

4 Aim of study

Cereal endosperm is examined as a highly specialized tissue for protein storage; its application is also important in molecular farming, human and animal feeding.

As described, the huge and conserved subcomlex of ESCRT machinery (ESCRT-III) drives the MVB sorting pathway. In previous studies, Barley *(Hordeum vulgare)* ESCRT-III orthologs were identified. First localization studies of barley ESCRT-III showed ESCRT-III HvVPS60a localized dependently to the cell layer.

Thus a highly specific characterisation study of other ESCRT-III components in different layers of barley endosperm is required. First step is the invistigation of putative interactions between different ESCRT-III components. So the aim of this study was to identify any interactions between HvVPS24 and other members of ESCRT-III (HvVPS60, HvVPS4, HvSNF7 and HvHinb2).

5 Material and Methods

5.1 Bacterial growth conditions and media

Following strains were used: E.coli OmniMAX 2T1R (life technologies) and Agrobacterium Tumefaciens GV3101:: pMP90 pSOUP (a gift from Helene Persak).
E.coli overnight culture was grown at 37 °C in liquid LB medium (0.5% (w/v) yeast extract, 1% (w/v) peptone, 512 mM NaCl) with shaking (180 rpm on rotary platform) or on LBplates containing 1.5% (w/v) agar. Appropriate amounts of antibiotics were added in both conditions following table 5. Agrobacterium *tumefaciens* was grown in liquid YEB medium (Beef extract 5g, yeast extract 1g, tryptone 5g, saccharose 5g, $MgSO_4$ x 7 H_2O 0,5g, in 500ml dH_2O).YEB plates containing 15 g agarose with appropriate antibiotics were incubated as well for 12-60 hours at 28 °C.

5.2 Heat shock transformation of competent E. coli

Competent E.coli cells (OmniMAX 2T1R) were prepared after Inoue protocol by Dr. Julia Hilscher. 50 µl of competent E. coli cells were thawed on ice and approximately 50 ng/ µl of plasmid DNA were added. Samples were mixed and incubated on ice for 30 minutes. A heat shock stress was prepared with 40 seconds of 42 °C followed by 2 minutes chilling on ice. 450 µl of LB medium was added and samples were incubated at 37 °C on a shaker for 40-60 minutes. Bacteria were harvested by a centrifuge step of 4000 rpm for 1minute. The pellet was resuspended in 80 µl of LB than 50 µl and 25 µl plated on selective medium.

5.3 Preparation of electro-competent A. tumefaciens

3-5 ml of YEB medium (including an appropriate amount of antibiotics) was inoculated by a single A. tumefaciens colony and incubated overnight at 28°C on shaker and were added as

well. 1ml of the overnight culture was added in 10 ml of YEB (including an appropriate amount of antibiotics) for about 24 hours to get an OD_{600} of 0.5. The culture was transferred into a 50 ml falcon on ice and centrifuged at 5000 rpm for 10 minutes. The pellet was resuspended in cold and sterile dH_2O, pellet was washed 2 times more and resupended finally in 25 ml dH_2O. Pellet was washed again in 10 ml and subsequently in 3 ml of cold and sterile 10% (w/v) glycerol. Cells were stored at -80 °C in 50 µl aliquots.

5.3.1 Transformation of electrocompetent A. tumefaciens by gene pulser

50 µl aliquots of competent A. tumefaciens were thawed on ice, mixed with 100 ng DNA plasmid and transferred to cuvettes. Before electroporation special cuvettes were washed and sterilized first with dH2O and then with 1% TritonX-100 and 0.25 N HCl each step for 30 minutes and finally sprayed with 96% ethanol and left to dry. Electroporation was done in BIO-RAD MicroPulser Electroporation system at 2.45 kV, 25 µF and 200 ohm. 4.4 ms of impulse was applied to agro bacteria and 900 µl of YEB medium was added gently. Samples were incubated one hour at 28 °C on shaker and finally plated on selective plates.

5.4 Polymeras chain reaction (PCR) with Taq polymerase

PCR reactions were prepared in total volumes of 10 µl or 20 µl with appropriate amount of DNA template, about 50-100 pg plasmid DNA and 10-20 ng DNA genome. PCR reaction contained 1× PCR buffer (10 mM Tris/HCl pH 8.5, 50 mM KCl, 1.5–3 mM $MgCl_2$, 0.15 % TritonX-100), 200 µM dNTPs, 10 pmol of forward and reverse primer, 0.5 U of *Taq* polymerase (table 3). 30-35 cycles of PCR were applied in this order: first 3 minutes of initial denaturation step at 95 °C then 30 seconds of extra denaturation step of 95 °C in each cycles, were continued by 30 seconds of an annealing step at 50-60 °C depends on primer length and were terminated by 30 seconds to 2 minutes of an extension step at 72 °C.

5.5 Cloning

5.5.1 Plasmid DNA mini preparation from E. coli:

Mini preparation was done with plasmid mini-prep purification kit (cat. #DP01-300). DNA pellet was resuspended in 50 µl of dH_2O and stored at -20°C.

5.5.2 Plasmid DNA midi preparation from E. coli

NucleoBond® Xtra Midi (Plus) and Maxi (Plus) kit (from macherey nagel Inc.) was used for plasmid DNA isolation. Pellet was resuspended in 100 µl of dH_2O and stored at -20°C.

5.5.3 Digestion reaction by restriction enzymes

Vectors and gene of interest were cut by restriction enzyme with Thermo Scientific Fast Digest protocol (table 2). Plasmids were incubated at 37°C for 15 minutes and enzymes were inactivated by heating for 10 minutes at 65°C if necessary. The molar amount of DNA was used to get certain cutting number of enzymes.

5.5.4 Dephosphorylation of sticky-end termini of plasmid

Simultaneous vector linearization and dephosphorylation protocol from thermo Scientific was used to inhibit the vector re-ligation by alkaline phosphatase.

5.5.5 DNA ligation

Quick Ligation ^{TM} kit (from New England Biolabs) was used for ligation. Samples were prepared in 20 µl volume of 10 µl 2X ligation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10

mM Dithiothreitol 1 mM ATP, pH 7.5), 1:3 molar ratio of vector:insert about 50 ng DNA of both, appropriate amount of dH_2O and 1mM T4 DNA ligase (table 2). Reaction was incubated at room temperature for 1 hour.

5.5.6 Sequencing

To check the correction of cloned sequences 40-100 ng/ µl of DNA was provided in different tubes with appropriate amount of forward and reverse primers (table 3). The mixture was sent to Microsynth Austria GmbH. The sequencing results were analyzed by DNA Star software/Seq Man.

5.6 Agarose gel DNA electrophoresis

Size of the gel pores depends on sample size, 0.8-2% (w/v) agarose gels were prepared in 1× TAE (40 mM Tris, 5.71% (v/v) acetic acid, 1 mM EDTA) and 0.5 µg/ml of extra ethidiumbromide was added. Samples were combined with 6× loading solution (Fermentas; 10 mM Tris/HCl pH 7.6, 0.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol, 60% (v/v) glycerol, 10mM EDTA). Gels were run in 1× TAE at 80 V speed for approximately 20 minutes. GeneRulerTM 1kb DNA Ladder (from Thermo scientific) was used as a marker.

5.6.1 DNA extraction from agarose gel

Gel slice containing DNA was cut under the UV light and stored at -20 °C overnight. QIAEX® II Gel Extraction kit (QIAGEN) was used to isolate DNA from agarose gel and pure DNA was eluted in 30 µl of elution buffer or in the same amount of dH_2O and stored in -20 °C.

5.7 Yeast two-hybrid

5.7.1 Yeast transformation:

A pre-culture was prepared as following: 3 ml of YPD media (yeast-extract 5 g, Trypton 10 g, glucose 10 g) was inoculated with a single yeast colony taken by a yellow tip and incubated at 28°C over-night on a shaker. The main culture was made from 15 ml of YPD media inoculated by 1 ml of pre-culture and incubated at 28°c for further 3 hours on a shaker. Main culture was centrifuged at 3000 rpm for 5 minutes at 20°C and supernatant was discarded. Pellet was washed with TE buffer (10mM Tris/HCL pH 7.5, 1mM EDTA pH 8.0). After centrifugation 110 µl LA-buffer (Li-Acetat*2H2O 1.02 g, in 100 ml TE-buffer pH 7.5) pro transformation was added directly on the pellet and mixed by shaking. Both DNA interaction partner were pipetted into eppendorf tubes (40-60 µg) and 100 µl of LA-yeast mixture was added in each Eppendorf tube. After 5 minutes of incubation at room temperature, 300 µl of PEG4000 (50% Roth) was added and mixed very well by pipetting up and down. Samples were incubated for 1 hour at room temperature and40 µl of DMSO (100%) was added. A heat-shock was performed where samples were incubated at 42°C for 10 minutes and put immediately on ice for 5 minutes. Samples were centrifuged at 2000 rpm for 3 minutes and supernatant was discarded by pipetting. One step washing was done with 1ml TE buffer. Pellet was re-suspended in 80 µl TE buffer and plated on –LW plates (yeast nitrogen base 3.35 g, Drop-out I 0.77 g, glucose 10 g, bacteria agar 10g, adenine 0.29 g, Kp-buffer 5ml) and incubated at 30°C for 2 days.

5.7.2 Interaction studies of yeast on selection plates

A single colony was picked from the transformation plate and plated on –LW plate and incubated overnight at 30°C. Single colonies were plated on selection plate of –LWH (yeast nitrogen base 3.35 g, Drop-out II 0.77 g, uracil 0.07 g, glucose 10 g, bacteria agar 10g, adenine 0.29 g, Kp-buffer 5ml) and –LWH including1mM AT (3 amino-1,2,4-trizole) and –LWH containing 3mM AT.

5.8 Yeast protein extraction and western blot

5.8.1 Extraction:

3 ml of yeast overnight culture (YPD medium) was prepared. OD_{600} was measured to get approximately the same amount of yeast from all samples. Samples were centrifuged at 3000 rpm for 1 minute. Pellet was weighted and the same amount of quarry sands was added. Pellet was frizzed in liquid nitrogen and mechanically crashed by mill to soft powder. 1:1 amount of Laemmli buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8) was added.

5.8.2 SDS page:

Samples were shortly vortexed and boiled at 95 °C for 5 minutes. Cell extract was centrifuged at high speed for 1 min and 20 µl of supernatant was loaded on SDS (sodium dodecyl sulfate) gel. Gel was run in 1 X running buffer (10 X buffer: 30.0 g of Tris base, 144.0 g of glycine, and 10.0 g of SDS in 1000 ml of H_2O PH=8.3) at 200 V for 45 minutes.

5.8.3 Semi-dry blotting:

BioRad Semi-Dry Trans-Blot Cell was used to transfer protein from gel to western nitrocellulose blotting membrane (from Amersham™ Protran® Supported). Gel was placed direct on the nitrocellulose membrane covered by 3 layers of whatmann paper pre socked in semi-dry buffer (48 mM Tris, 39 mM glycine, 20% methanol, 0.04% SDS). Membrane was run at18 V for 30 minutes.

5.8.4 Western blotting:

Membrane was washed twice with TBST (tris-buffered saline (TBS) and Polysorbate 20 also known Tween 20) each time for 10 minutes and then incubated in 5% milk (2.5 g milk in 50 ml PBST) for 1 hour. After two times washing with TBST, membrane was incubated in primary antibodies (table 6) with appropriate dilution at room temperature for 2 hours or at 4 °C overnight. 2 washing steps with TBST were applied and membrane was incubated with secondary antibodies for 1 hour (table 7). Last washing steps were 2 times in TBST for 5 minutes. For signal development, membrane was incubated in the ROCHE BM Chemiluminescence Western Blotting Substrate (POD) (Code: 11500694001) for 1 minute and also in BCIP®/NBT Alkaline Phosphatase Substrate (SIGMAFAST™ BCIP®/NBT tablet from SIGMA-Aldrich company) for 10 minutes.

5.9 Bimolecular fluorescence complementation (BiFC)

5.9.1 Syringe infiltration of Nicotiana benthamiana leaves with BiFC constructs

5ml of YEB (including appropriate antibiotics) was inoculated with transformed agrobacterium and incubated at 28°C for 2 days. Cells were centrifuged at 5000 g for 5 min and the pellet was washed with 1ml of infiltration buffer (10mM MES pH 5.7, 10mM $MgCl_2$, 100µM acetosyrengone). The cells were washed again two times in 500µl of infiltration buffer. OD 600 (optical density) was adjusted to 0.3. Interaction partner were mixed 1:1 and suspension was injected into the abaxial surface of mature leaves (4-week-old n-Bentamiana plants) by a syringe. Plants were incubated at 23°C 16 hours light for 2-5 days.

MKK4_SPYCE pGWR	Andrea Pitzschke #920
MPK3_SPYNE pGWR	Andrea Pitzschke #559

Table 1. Vectors (Plasmids were a gift from Andrea Pitzschke)

Usage	Enzyme name	Name of company
Restriction digestion	EcoRI, SalI, NcoI, NotI, BamHI	Thermo Fisher
PCR enzymes	Dream Taq, Pfu Polymerase	Thermo Scientific
Ligation	Quick T4 Ligase	New England Biolabs

Table 2. List of Enzymes used for cloning

Primer Name	Sequence 5´-3´	Tm	Usage
m13-F	GTAAAACGACGGCCAGTG	58	Sequencing from vectors
m13_R	GGAAACAGCTATGACCATG	56	Sequencing from vectors
pA35S-R	GCTCAACACATGAGCGAAAC	60	35S terminator
HvSNF7.1_NcoI-F	ATACCATGGATGTTCAACAGGTTATTTG	76	Cloning into BiFC
HvSNF7.1_NotI-R	AGCGGCCGCcCAATGCCATTTCAGCTTG	90	Cloning into BiFC
HvVPS60_NcoI-F	ATACCATGGATGAAGAAGATCTTTG	68	Cloning into BiFC
HvVPS60_NotI-R	AGCGGCCGCcGGTACGAATTGACGCGTG	89	Cloning into BiFC
HvVPS24_SalI-F	GTCGACATGGAGGCGGTGAAGA	76	Cloning into BiFC
HvVPS24_SalI-R	GTCGACCGACCTCACTTTGGCCAG	84	Cloning into BiFC
HvVPS4-BamHI-F	GGATCCTCATGTACAGCAACTTCAAGGAGC	64	Cloning into BiFC
HvVPS4-SalI-R	GTCGACGCCCTCCTCGCCAAACTCC	64	Cloning into BiFC
HvHinb-NcoI-F	CCATGGATGAAGACCTTATTCCTCCTAG	62	Cloning into BiFC
HvHinb1-BamHI-R	GGATCCCCAGTAATAGCCGCTAGGGA	62	Cloning into BiFC
HvHinb2-BamHI-R	GGATCCCCAGTAATAGCCACTAGGGA	58	Cloning into BiFC

Table 3. List of primers used for BiFC

Primer Name	Sequence 5´-3´	Usage
pGADT7_SNF7-F	GCT GAC ATA TGA ATA TGT TCA ACA GG	Cloning into Y2H
pGADT7_SNF7-R	GAG GCC TCC ATG GCC TCA CAA T	Cloning into Y2H
pGADT7_VPS60-F	TCA TAT GTT AAA TAT GAA GAA GAT CTT TGG G	Cloning into Y2H
pGADT7_VPS60-R	GAC CCG GGA GAA TGT ATT AGG TA	Cloning into Y2H
pGADT7_VPS24-F	TTC TGC TGA CAT ATG AAT TAT ATG GAG GC	Cloning into Y2H
pGADT7_VPS24-R	AGG CCT CCA TGG CCT ATC ACG A	Cloning into Y2H
pGADT7_Hinb-EcoRI-F	GAATTCATGAAGACCTTATTCCTCCTAG	Cloning into Y2H
pGADT7_Hinb1-BamHI-R	GGATCCTCACCAGTAATAGCCGCTAG	Cloning into Y2H
pGADT7_Hinb2-BamHI-R	GGATCCTCACCAGTAATAGCCACTAG	Cloning into Y2H

Table 4. List of primers used for Y2H

E. coli

Selection for	Antibiotic	Stock solution	Solvent	Working concentration
pCR®4-TOPO® [1]	Ampicillin	100 mg/ml	dH2O	100 mg/l
pCR®4-TOPO® [1]	Kanamycin	100 mg/ml	dH2O	50 mg/l
pPZP211, pPZP221 [2]	Spectinomycin	50 mg/ml	dH2O	100 mg/l
pBIC20 [3]	Tetracyclin	15 mg/ml	50% EtOH	15 mg/l

A. tumefaciens

Selection for	Antibiotic	Stock solution	Solvent	Working concentration
pTiC58ΔT-DNA [4]	Gentamycin	50 mg/ml	dH2O	50 mg/l
A. tumefaciens genomic background C58 [4]	Rifampicin	25 mg/ml	DMSO	100 mg/l
pBIC20 [3]	Kanamycin	100 mg/ml	dH2O	50 mg/l
pPZP211, pPZP221 [2]	Spectinomycin	50 mg/ml	dH2O	100 mg/l

Table 5. List of antibiotics used for bacterial selection

1: Invitrogen; 2: (Hajdukiewicz et al, 1994); 3: (Meyer et al, 1996); 4: (Koncz & Schell, 1986)

Name	Biological source	clone	Mfr. No.	Isotype	Dilution factor
Anti-HA-Peroxidase, High Affinity	from rat	clone 3F10, monoclonal	Roche	IgG1	1:500
GAL4 Antibody (DBD) : sc-577	From rabbit	polyclonal	Santa Cruz biotechnology	IgG	1:2000

Table 6. List of primary antibodies used for western blot

Name	Biological source	clone	Mfr. No.	Dilution factor
Anti-Mouse IgG	From goat	polyclonal	Sigma-Aldrich	1:5000

Table 7. Secondary antibody used for western blot

6 Results

6.1 Yeast Two-Hybrid analysis of Barley ESCRT-III proteins

Gal4 binding domain and activation domain vectors of ESCRT-III proteins for yeast two-hybrid were cloned by Doris Abraham (bachelor student), HvHinb2-BD and HvHinb2-AD were cloned by me (figure 7 and 8). Vectors contain T7 promotor, activation or binding domain of Gal4, gene of interests (HvVPS24, HvVPS60, HvSNF7, HVVPS4 and HvHinb2), a multiple cloning site (MCS) and a kanamycin resistance sequence. DNA sequences of HvESCRT-III were cloned into GAL4 activation domain (GAL4-AD) and into GAL4 DNA-binding domain (GAL4-BD), respectively. To test the proper expression of vectors, each of the AD/BD ESCRT fusion were expressed individually in yeast. Already known Arabidopsis ESCRT-III interacting proteins (personal communication, kindly provided by Dr. Marie-Theres Hauser) were used as positive controls for the Y2H experiments. Transformation was done 4-6 times in 2 separate biological replicates for all interaction partners of HvESCRT-III. In parallel, the expression levels of most HvESCRT-III identified by western blot of extracted protein from transformed yeast. The growth rates of co-transformed yeast were compared on selections medium (-LWH) without and with 1mM- AT and 3mM-AT. Positive interactions were categorized then in weak, medium or strong groups. Generally, we observed always a dense yeast growth on selection plates without AT in comparison to weaker yeast growth on 1mM-AT and no growth on –LWH containing 3mM AT. First, the homo-dimerization and autoactivation of HvVPS24 was checked (figure 9). AD/BD and HvVPS24-AD/BD as negative controls show no interactions and no yeast growth on –LWH containing 3mM AT. HvVPS24-BD/AD shows a strong auto-activation and a high growth rate even on 3mM AT selections medium and consequently a false positive interaction was detected on HvVPS24-AD/HvVPS24-BD (figure 9, table 8). In case of Y2H interactions of HvVPS24 and HvSNF7 a weak growth ratio of HvVPS24-AD/HvSNF7-BD were seen on the 3mM-AT selection medium. Contrary, all negative controls such as VPS24-AD/BD, SNF7-BD/AD and AD/BD have no growth on 3mM AT (figure 10, table 9). Thus, we suggest a light interaction between HvVPS24 and HvSNF7. Negative control of VPS60-BD/AD was examined and an auto-activation was observed. Moreover, no interaction was seen between HvVPS60 and

HvVPS24, other negative controls showed no positive signal (figure 11, table 10). HvVPS24 and HvVPS4 show no positive Y2H interaction results. As negative controls show no yeast growth the correctness of this experiment was proofed (figure 12, table 11). Hordoindoline as a putative storage protein of barley endosperm, which was pulled down in a CO-IP experiment with HvVPS24 together is included in this study. Y2H Homo-dimerization of HvHinb2showed no significant yeast growth on LB selection medium containing 3mM AT and also between HvVPS24-AD/HvHinb2-BD no interaction was observed (figure 13, table 12). In all Y2H studies below a positive control of AtVPS60-AD/AtVPS24-BD was included. Figure 15 shows a general overview of all HvESCRT-III Y2H interactions.

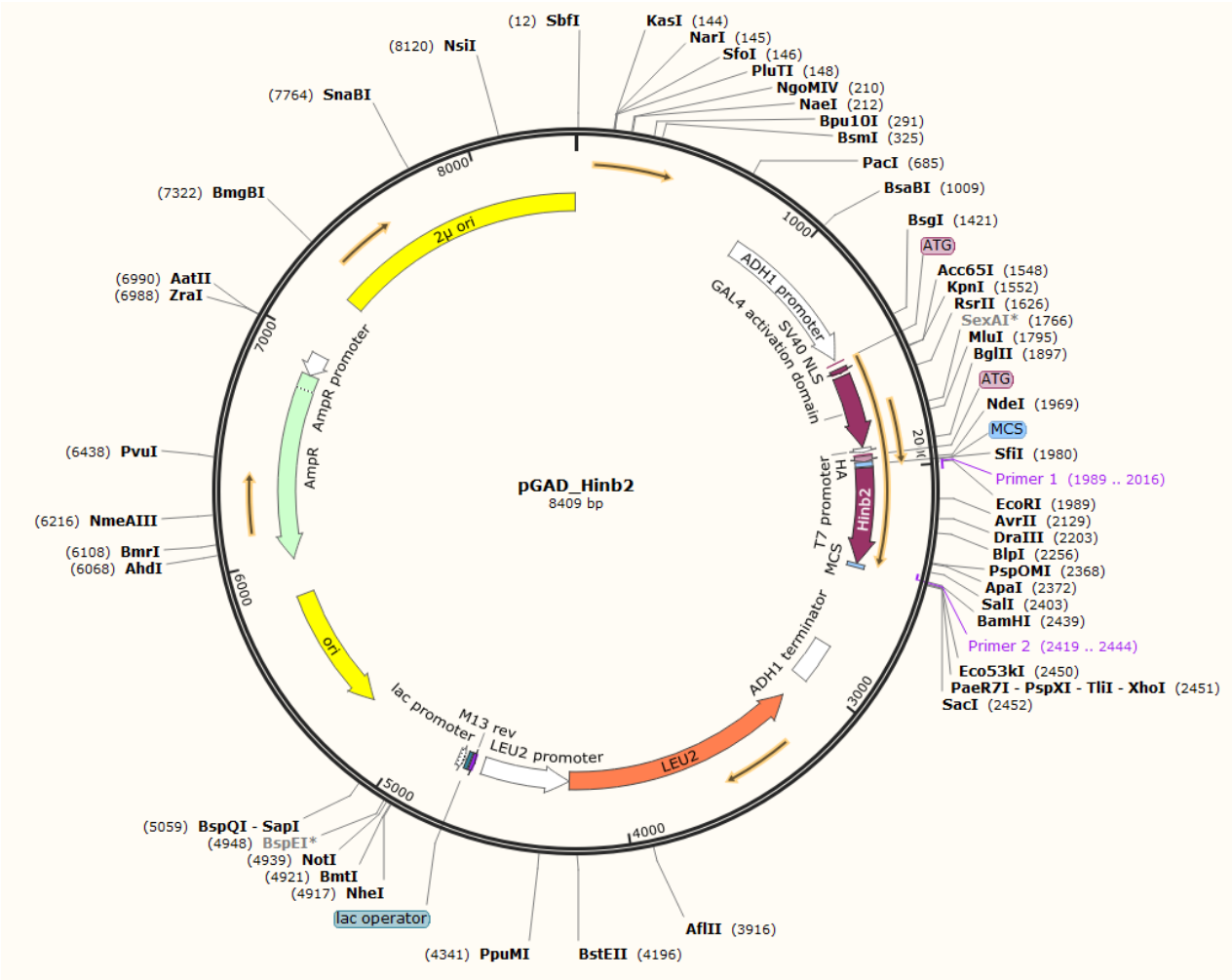


Figure 7. HvHinb2-pGAD (Gal4 activation domain) vector map

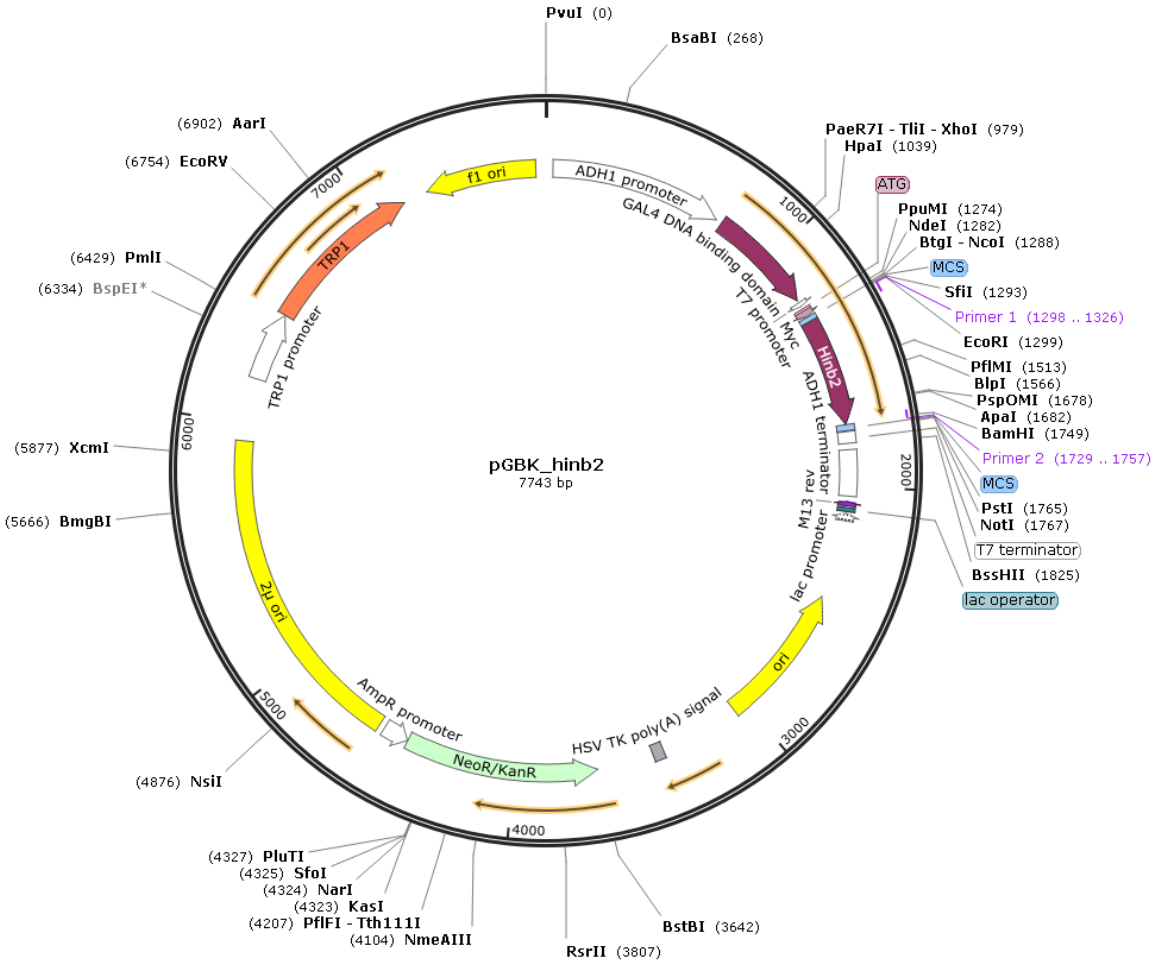


Figure 8. HvHinb2-pGBK (binding domain of Gal4) vector map

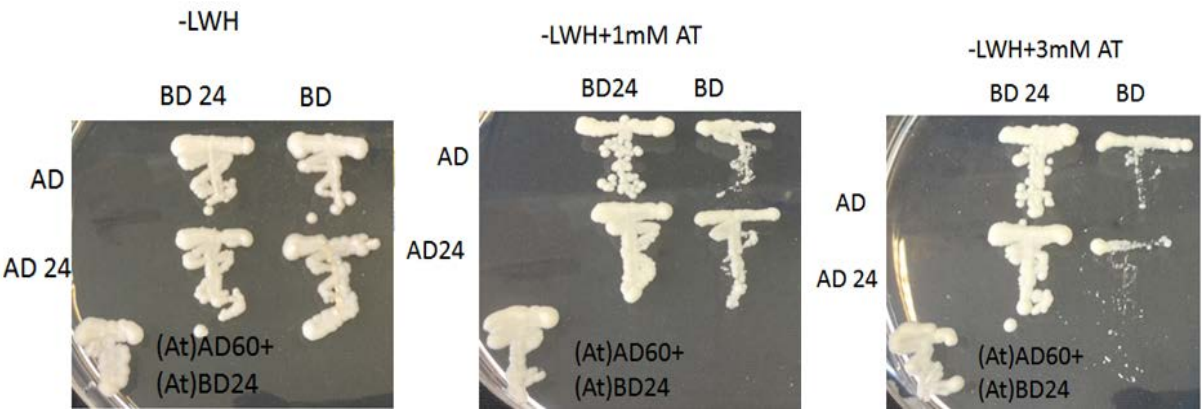


Figure 9. Homodimerization of HvVPS24 of barley in yeast.
BD shows Gal4-binding domain and AD shows Gal4 activation domain, 24 is ESCRT-III HvVPS24. Negative control shows an auto-activation of BD24/AD on 3mM AT selection medium. Positive interaction detected between AtVPS60-AD/AtVPS24-BD as positive control on 3mM AT.

	BD	**24 BD**	**(AT) 24 BD**
AD	negative control	+++ auto-activation (false positive)	
24 AD	negative	+++ auto-activation (false positive)	
(AT) AD 2			+++ positive control

Table 8. Interaction results of VPS24 with itself as a homodimer

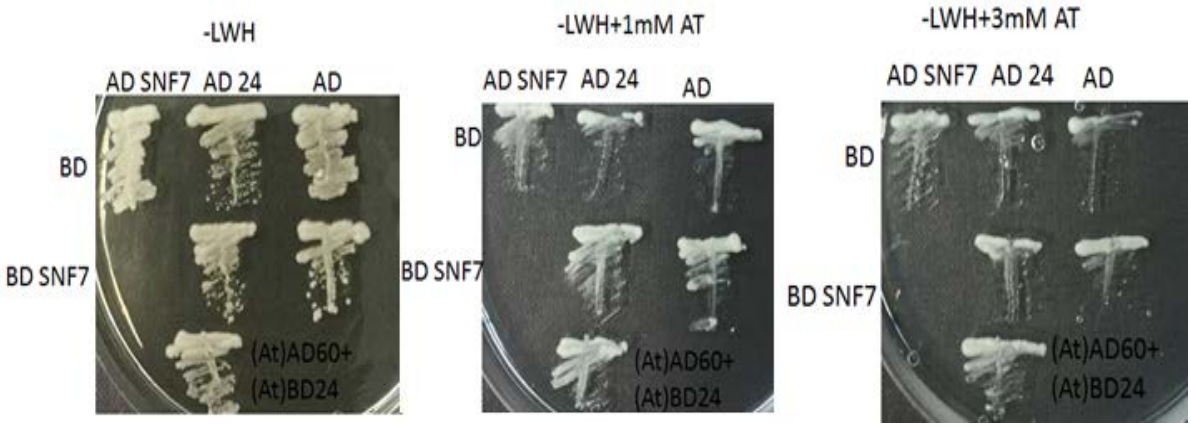


Figure 10. Interaction studies between HvSNF7 and HvVPS24.
A weak growth of HvVPS24/HvSNF7 was observed on the 3mM-AT selection plate which indicates a weak positive interaction between these two barley ESCRT-III proteins. Positive interaction detected between AtVPS60-AD/AtVPS24-BD as positive control on 3mM AT.

	BD	**SNF 7 BD**	**(AT) 24 BD**
AD	negative control	negative	
SNF7 AD	negative	negative	
24 AD	negative	+ light positive	
(AT) AD 60			+++ positive control

Table 9. A weak positive Y2H interaction of HvVPS24-AD with HvSNF7-BD

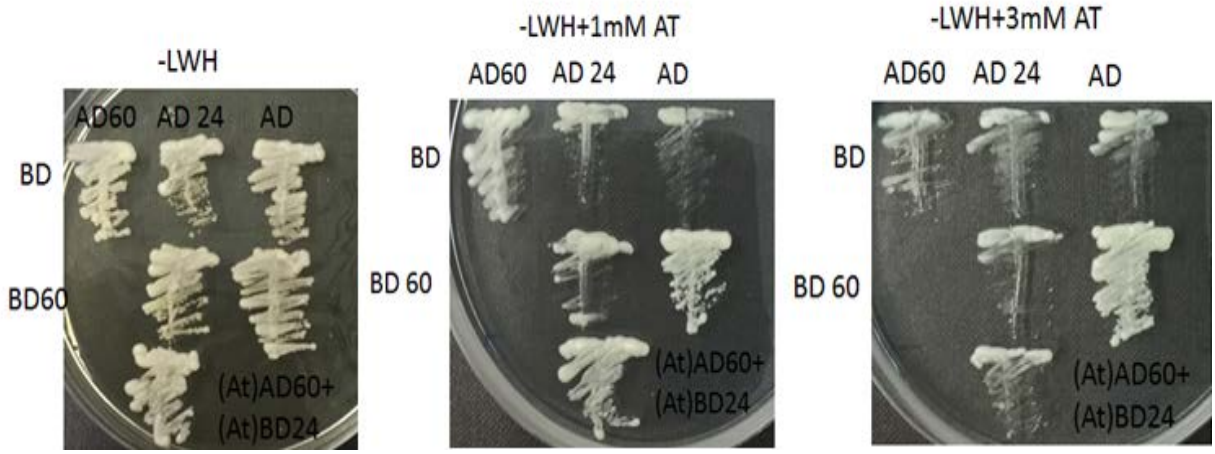


Figure 11. Y2H interaction study of HvVPS60-BD and HvVPS24-AD.

In 3 different selection mediums of –LWH, +1mM AT and +3mM AT the yeast growth was studied. Auto-activation of HvVPS60-BD/AD was detected but no interaction between HvVPS60 and HvVPS24. Positive interaction detected between AtVPS60-AD/AtVPS24-BD on 3mM AT.

	BD	60 BD	(AT)AD 60
AD	negative control	+++ auto-activation (false positive)	
24 AD	negative	negative	
(AT) 24 BD			+++ positive control

Table 10. Auto-activation of HvVPS60 and negative interaction of HvVPS60-BD/HvVPS24-AD.

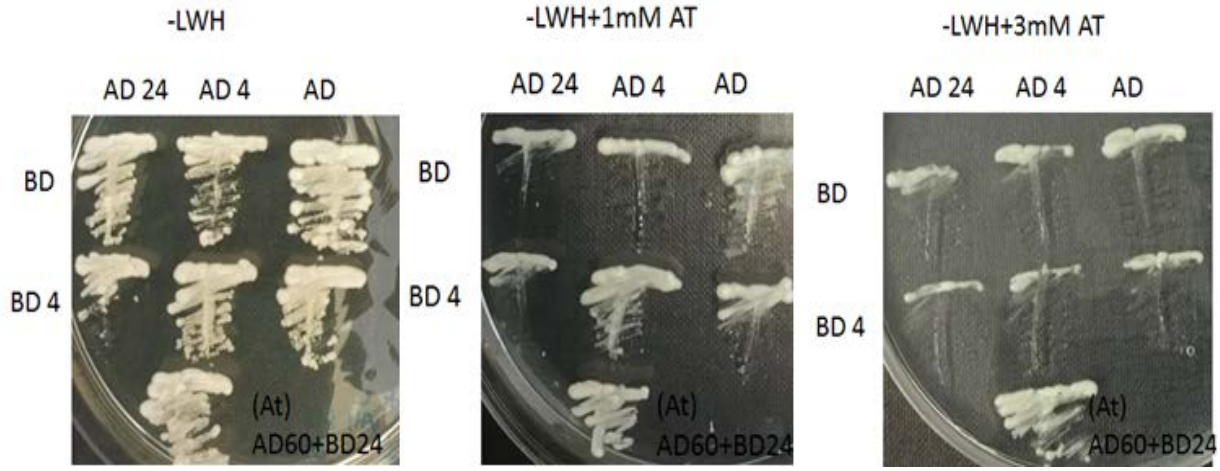


Figure 12 Interaction analysis of HvVPS24-AD with HvVPS4.
No positive interaction between HvVPS4 and HvVPS24 was detected on –LWH containing 3mM-AT selections plate. Positive interaction was detected between AtVPS60-AD/AtVPS24-BD on 3mM AT as positive a control.

	BD	4 BD	(AT) 24 BD
AD	negative control	negative	
4 AD	negative	negative	
24 AD	negative	negative	
(AT) AD 60			+++ positive control

Table 11. Negative Y2H interacting between HvVPS24 and HvVPS4.

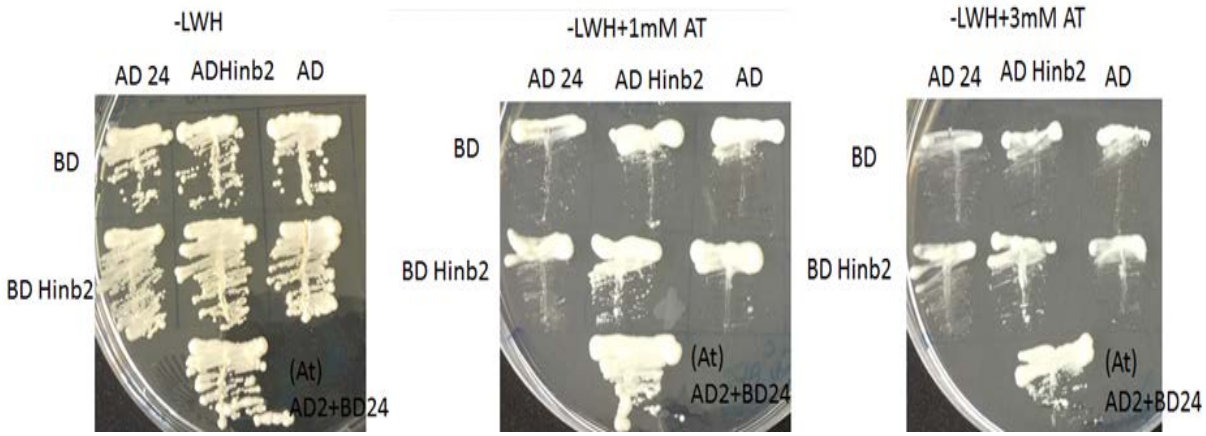
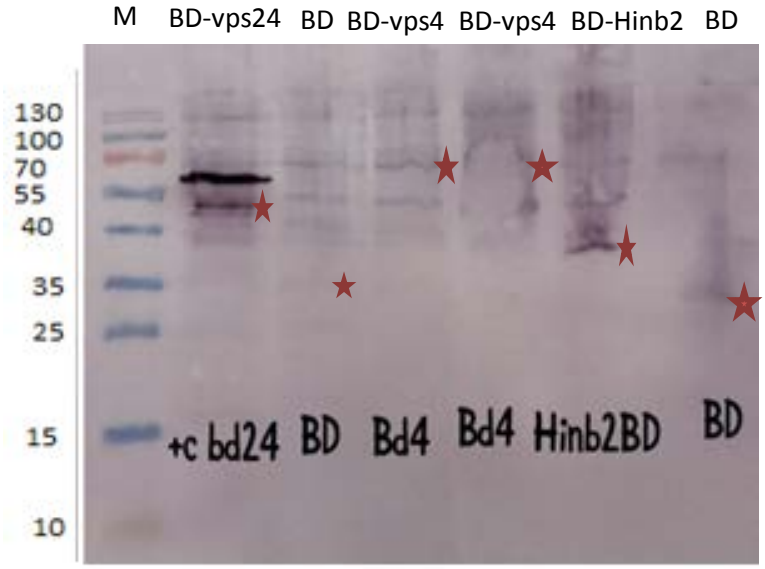


Figure 13. Homo-dimerization studies of HvHordoindoline b2 and its interaction with HvVPS24.
A light growth of yeast was observed between HvHinb2-AD/HvHinb2-BD in 3mM-AT selection medium.

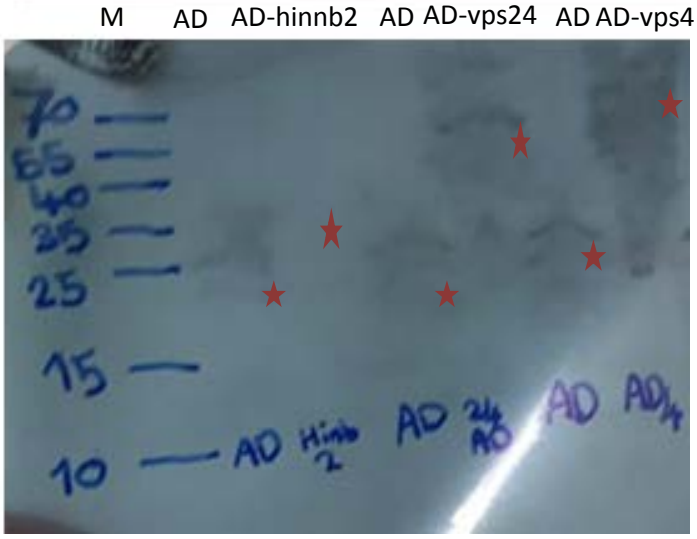
	BD	Hinb2BD	(AT)BD24
AD	negative control	negative	
Hinb2AD	negative	positive ++ ??	
24 AD	negative	light positive + ?	
(AT)AD2			+++ positive control

Table 12. Probable interaction of HvHinb2 with itself and with HvVPS24.

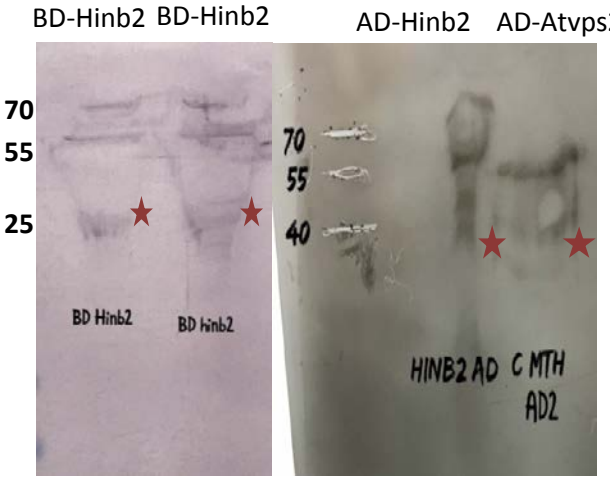


Primary antibody: Anti-HA-Peroxidase, High Affinity

Secondary antibody:Anti-Mouse IgG



Primary antibody: GAL4 Antibody (DBD): sc-577



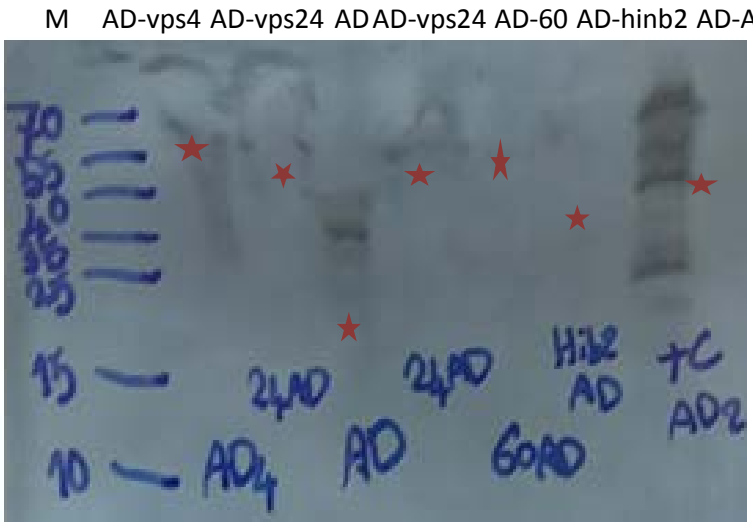
Primary antibody: anti HA

Primary antibody: anti Gal4 BD

Secondary antibody: anti mouse IgG

Primary antibody: Anti-HA-Peroxidase, High Affinity

Secondary antibody: Anti-Mouse IgG



Primary antibody: GAL4 Antibody (DBD): sc-577

Figure 14. Western blot analysis of ESCRT-III proteins from yeast extract.

Stars show the expected protein bands on membrane and film. Positive bands observed for HvVPS24-AD, HvSNF7-BD, AtVPS2-AD and AtVPS24-BD. But no clear bands detected for HvVPS4 and HvHinb2.

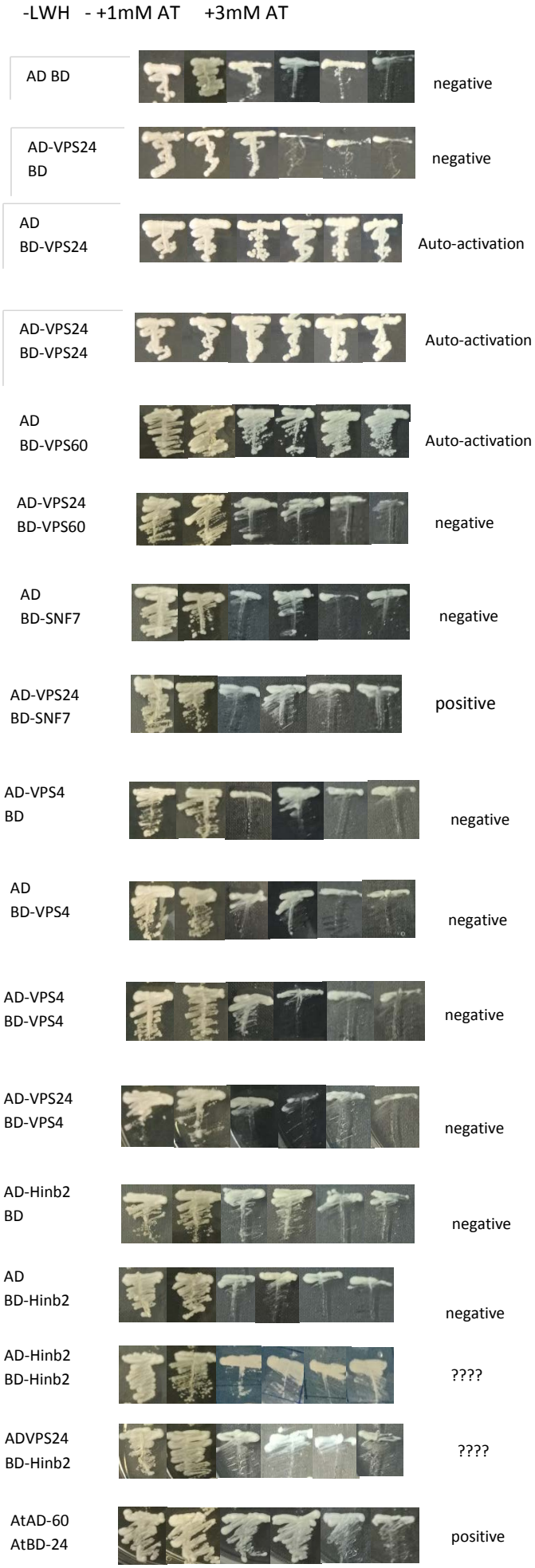


Figure 15 Over view of HvESCRT-III Y2H interactions

6.2 BiFC studies of HvESCRT-III proteins

Biomolecular fluorescence complementation assay was applied to characterize the ESCRT-III interactions of barley in a plant-based system and to evaluate the results, which were obtained from the Y2H study. To set up BiFC we cloned our gene of interest HvVPS24-SPYCE, HvVPS24-SPYNE, HvHinb2-SPYCE and HvHinb2-SPYNE into four different cloning vectors (figures 16, 17, 18, and 19). SPYCE (C-terminal YFP) and SPYNE (N-terminal YFP) vectors were kindly provided by Andrea Pitzschke´s laboratory. The vector maps contain a p35S promotor (Cauliflower Mosaic virus 35S promoter), 5´UTR (Cauliflower Mosaic virus 35S untranslated region), our genes of interest (HvVPS24, etc. and Hvhinb2), C-terminal or N-terminal sequence of YFP (SPYCE or SPYNE9, t35 (Cauliflower Mosaic virus 35S terminator), HA-tag (Human influenza hemagglutinin) or MYC-tag, M13-R,F; t35S-R, pA35S-R primer locations and MCS (multiple cloning site) and a kanamycin antibiotic resistance sequence (figure 16, 17, 18,19). All vector maps are designed by SnapGene viewer software.

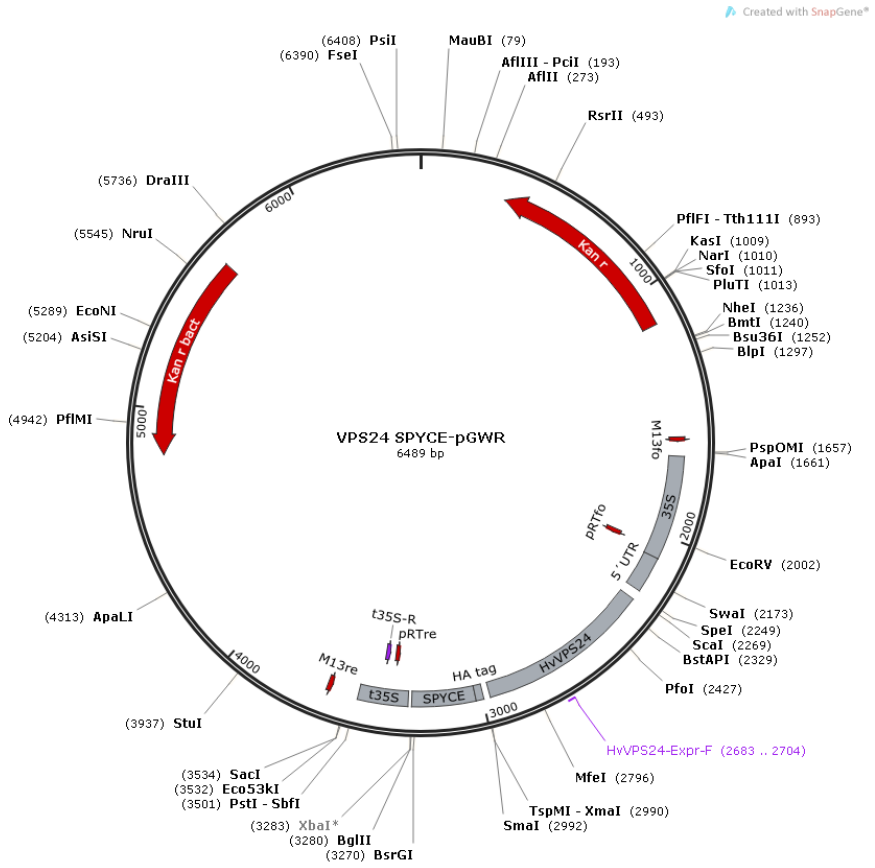


Figure 16. HvVPS24-SPYCE vector map

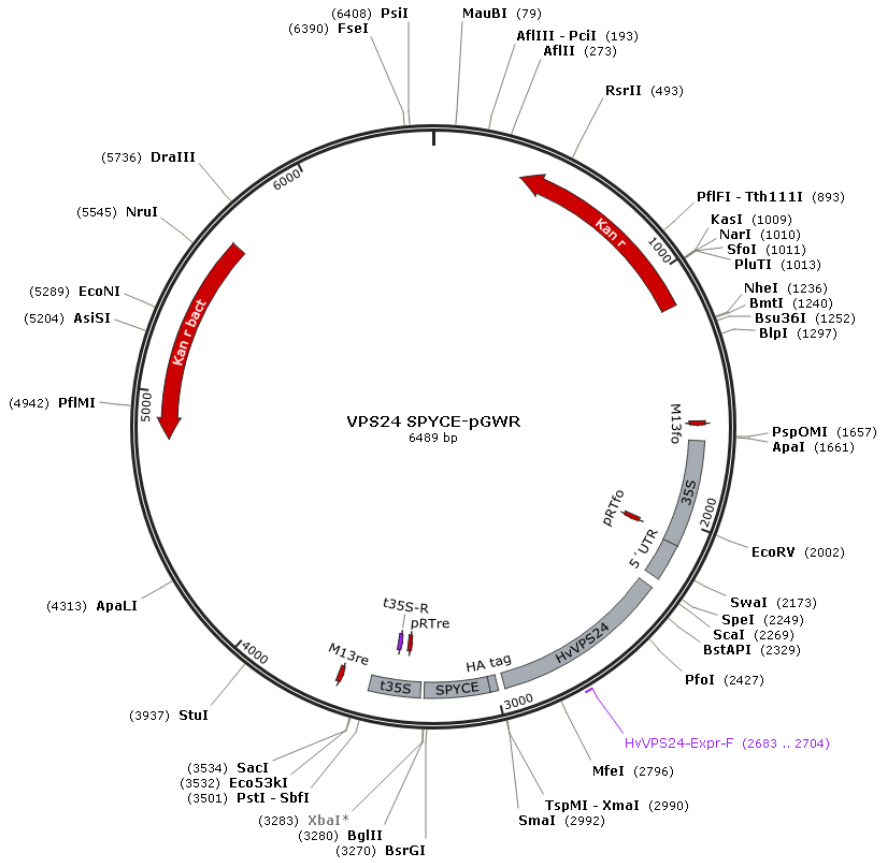


Figure 17. HvVPS24- SPYCE vector map

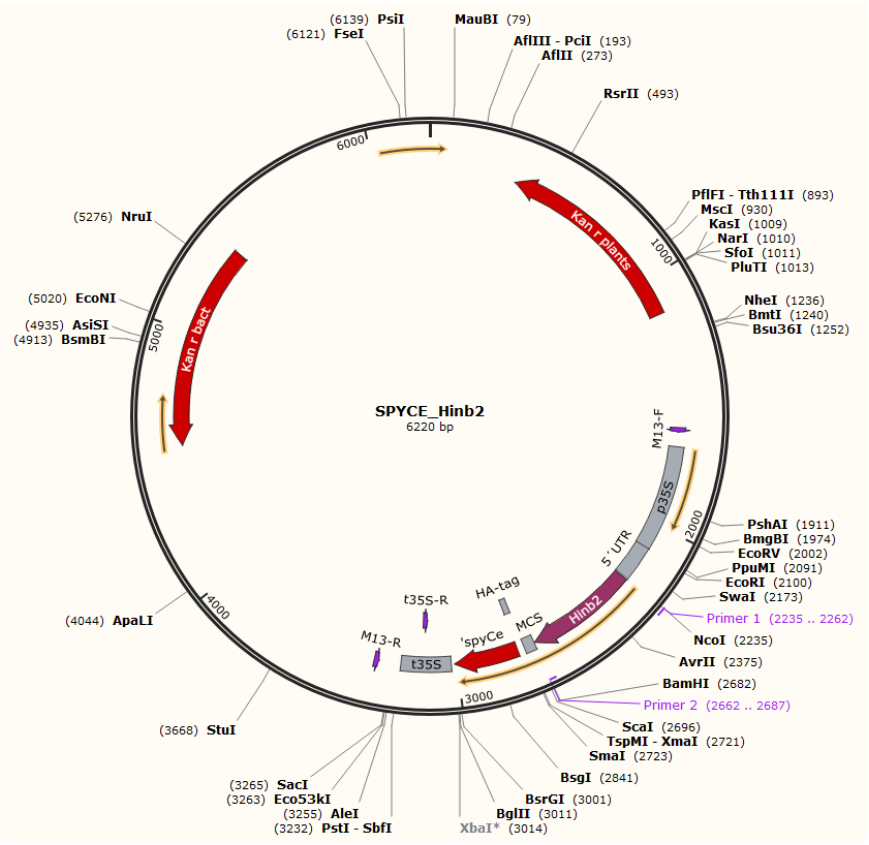


Figure 18. HvHinb2-SPYCE vector map

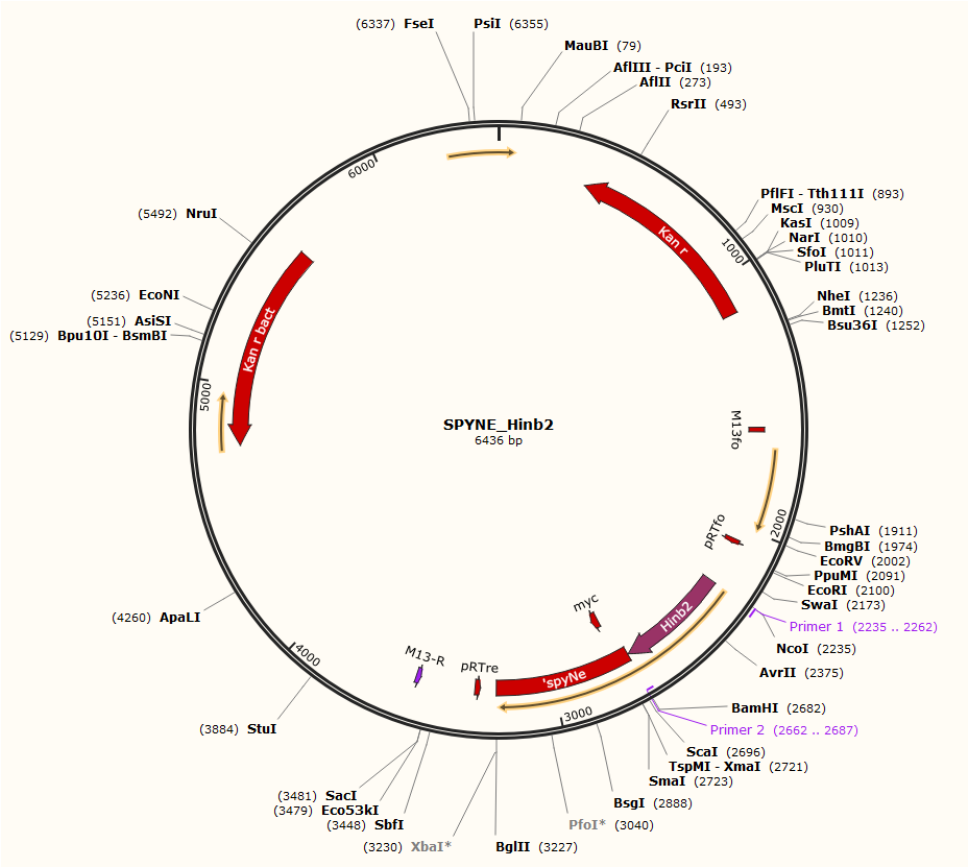


Figure 19. HvHinb2-SPYNE vector map

Different combinations of vector constructs were infiltrated in *Nicotiana benthamiana* leaves. Yellow fluorescence protein (YFP) was reconstituted in case of successful interactions between proteins of interest which were detected under the confocal microscope (Leica TCS SP5 II) after three to five days. Argon laser (wavelength, 514 nm) was used for excitation and emission of YFP was detected between 525-600 nm. Additionally, the autofluorescence was checked between 680-760 nm. Three biological replicates were examined in three to six separate experiments to get reliable results. First, we test the infiltration system and infiltrated *Nicotiana benthamiana* leaves with HvVPS24-mcherry. MCherry was excited by 543nm and observed between 600-680 nm, a red signal was detected around the plasma membrane (Figure 20 A). Moreover a co-infiltration of VPS24mCherry with all possible ESCRT-III interaction partners was prepared to check the accuracy and effectivity of infiltration method. Aco-infiltration of HvVPS24-SPYCE/HvVPS24-SPYNE with HvVPS24-mcherry was done and shown in figure 20 B. We could detect both mCherry and YFP fluorescence signals which were stimulated by different laser and different wavelength and no interference signal was observed. As negative controls we infiltrated following combinations: 1) SPYCE and SPYNE

empty vector together which showed no fluorescence signal and confirmed the validation of BiFC method (figure 21 A), 2) VPS24-SPYNE with empty SPYCE which indicates a strong falls positive signal (figure 21 D), 3) HvVPS24-SPYCE and empty SPYNE which showed interestingly only a weak background signal (figure 21 C). As it is shown in Figure 21 B (HvVPS24-SPYNE/SPYCE) and 21 D (HvVPS24-SPYCE/ HvVPS24-SPYNE) HvVPS24 mostly localized in plasma membrane and partly in nucleus. HvVPS24 Interactions of HvVPS60 with HvVPS24 are in both directions negative (figure 22 A, B). In combination of HvVPS24-SPYCE/HvSNF7-SPYNE and HvVPS24-SPYNE/HvSNF7-SPYCE weak fluorescence signals were detected that shows maybe a light interaction between HvVPS24 and HvSNF7 (figure 23 B, C). Negative control of HvSNF7-SPYCE/SPYNE was also checked and no signal was observed (figure 23 A). There is no signal detected between HvHinb2 and HvVPS24 and no homodimerization of HvHinb2 as well (figure 24). A short summary of all examined interactions is shown in table 13.

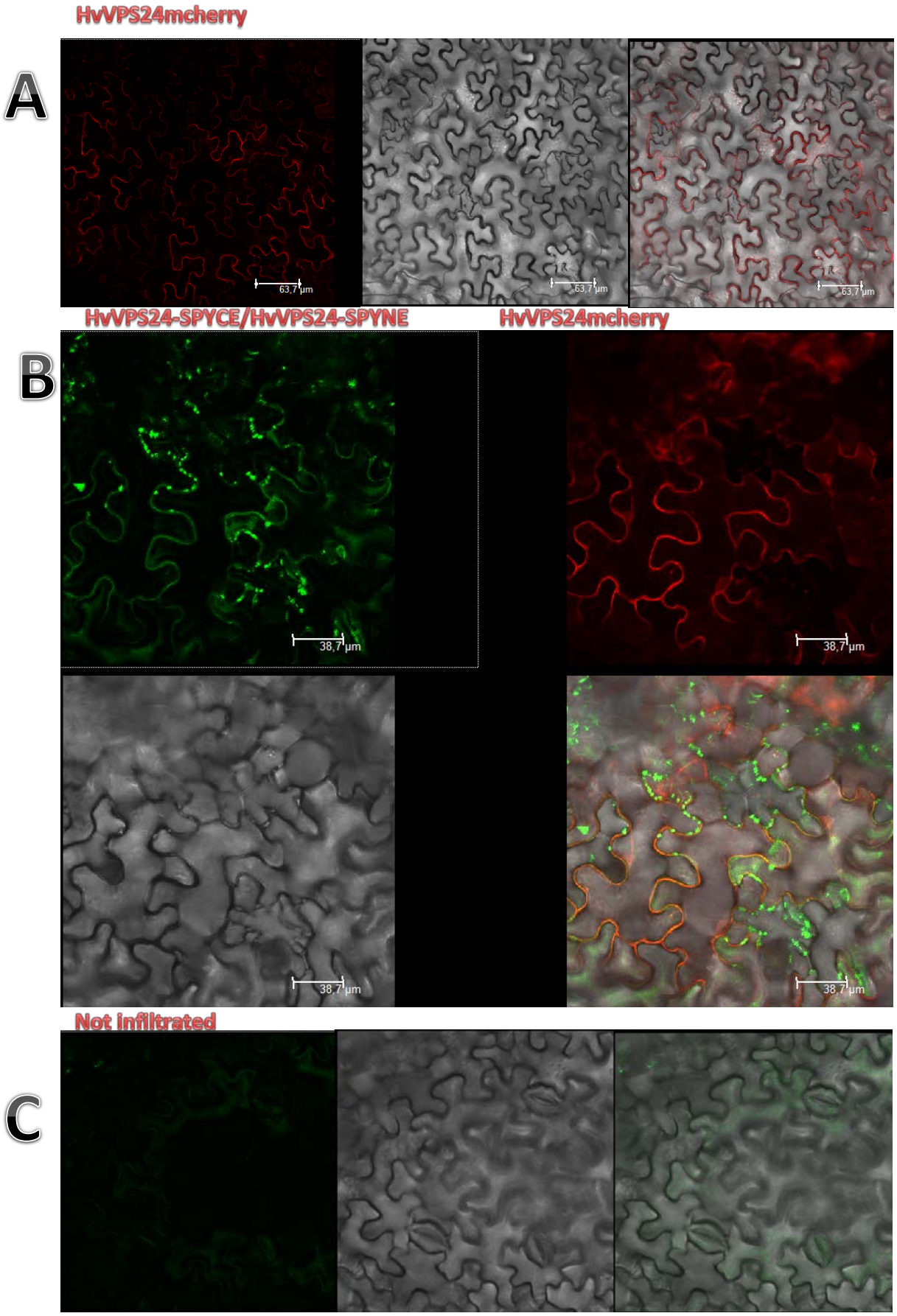


Figure 20. A: Confocal analyses of HvVPS24mcherry infiltrated *Nicotiana benthamiana* leaves

B: Co-infiltration of VPS24mcherry with HvVPS24-SPYCE/HvVPS24-SPYNE C: No infiltrated leaves

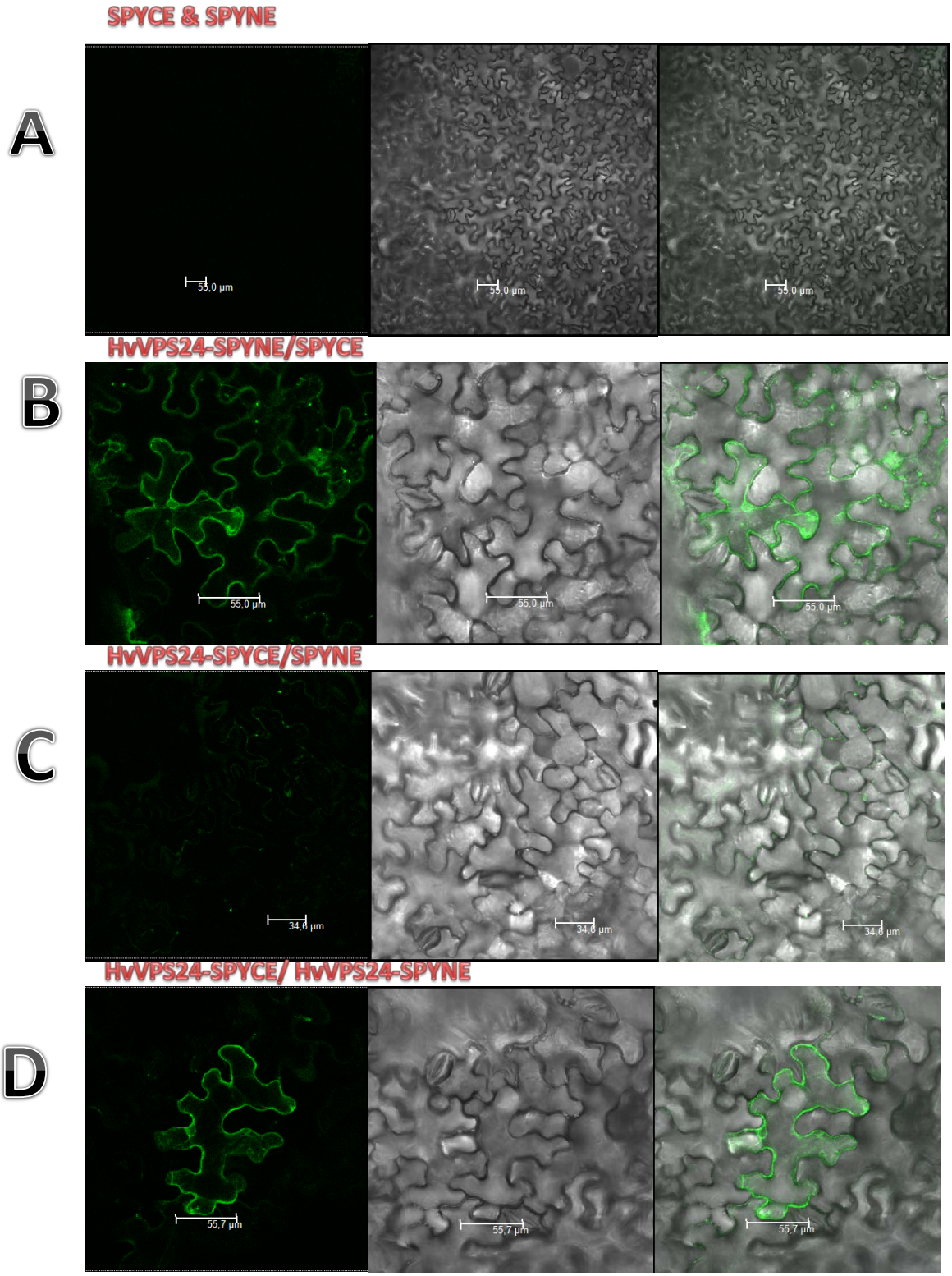


Figure 21. Confocal analyses of BiFC interaction studies of HvVPS24 in *Nicotiana benthamiana* leaves
A: BiFC visualization of SPYNE empty vector with SPYCE. No fluorescence signal was observed B: HvVPS24-SPYNE/SPYCE shows a false positive signal. C: Only a weak signal of HvVPS24-SPYCE/SPYNE was detected. D: false positive BiFC visualization of HvVPS24 homodimerization.

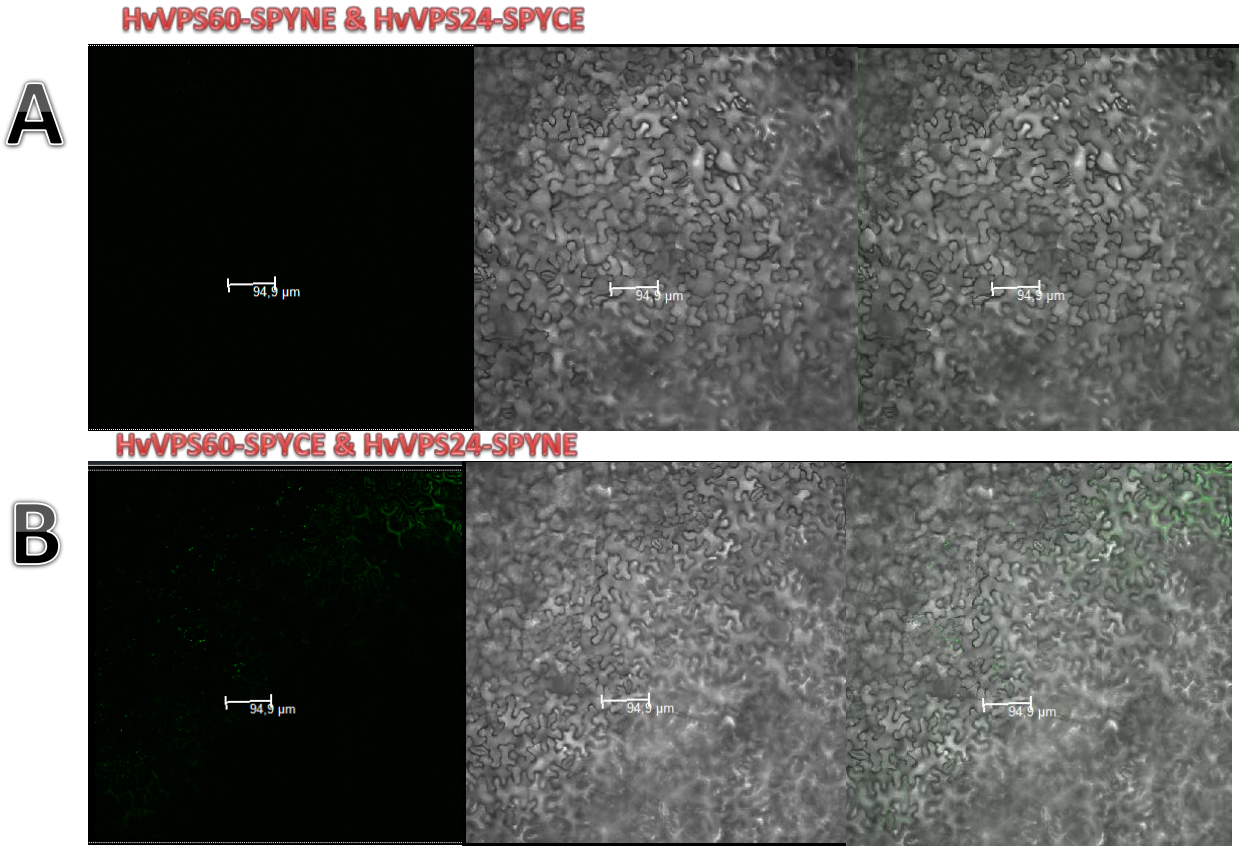


Figure 22. Confocal analyses of BiFC interaction studies of HvVPS60 and HvVPS24.
A: Interaction Studies between HvVPS60-SPYNE and HvVPS24-SPYCE and B: HvVPS60-SPYCE and HvVPS24-SPYNE. No signal and consequently no interaction were observed in both situations

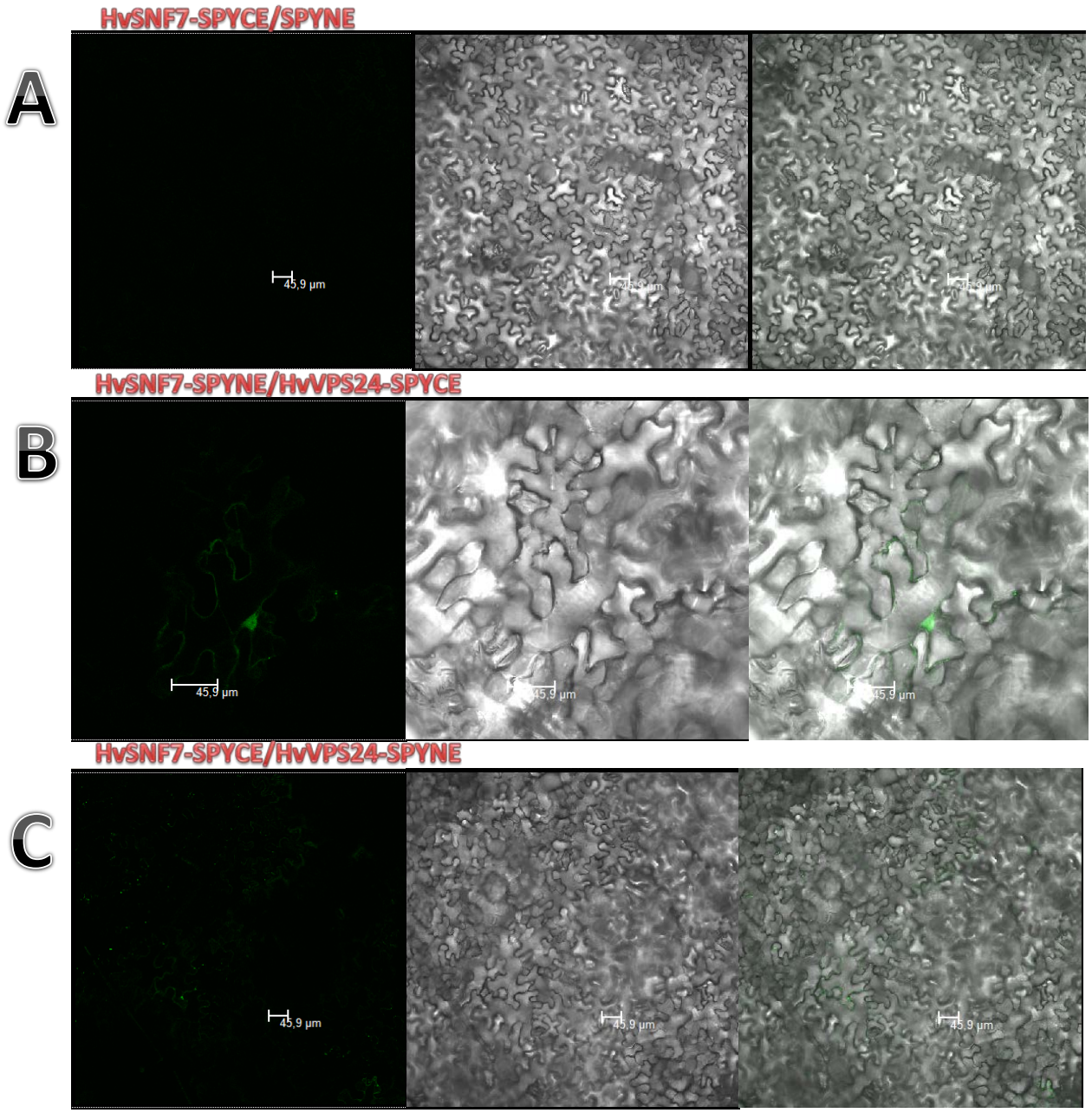


Figure 23. Confocal analyses of BiFC interaction studies of HvVPS24/HvSNF7
A: Interaction study of HvSNF7-SPYCE/SPYNE shows no signal. B: Weak signal of HvSNF7-SPYNE/HvVPS24-SPYCE. C: positive signal was detected also in other direction HvSNF7-SPYCE/HvVPS24-SPYNE.

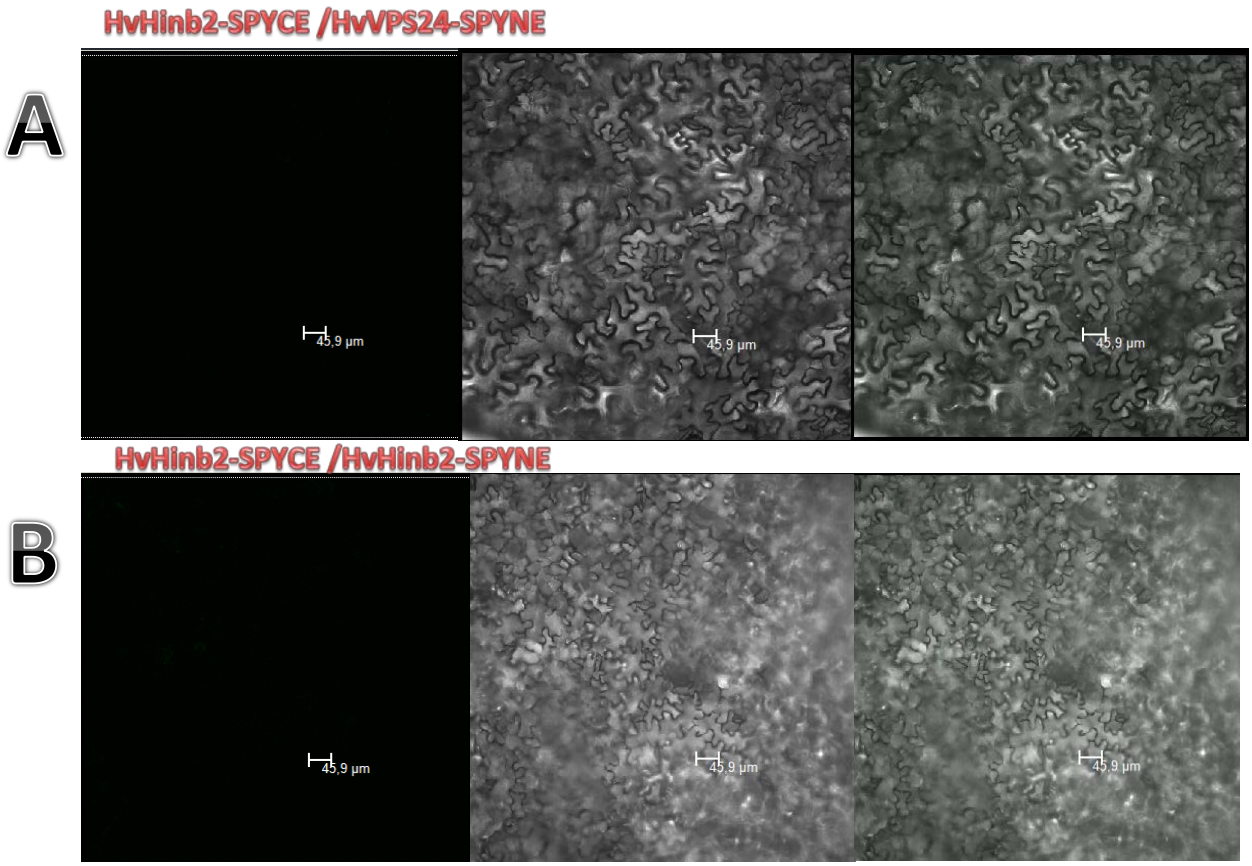


Figure 24. Confocal analyses of BiFC interaction studies of HvHinb with HvVPS24
A: No flourescence signal of HvHinb2-SPYCE /HvVPS24-SPYNE. B: no homodimerization of HvHinb2.

VPS24		**VPS60**		**SNF7**		**Hinb2**	
CE+NE	negative	**CE24+NE60**	negative	**CE7+NE**	negative	**CEhinb+NEhinb**	negative
CE+NE24	positive	**CE60+NE24**	negative	**CE24+NE7**	positive	**CE+NEhinb**	negative
CE 24+NE	negative			**CE7+NE24**	positive	**CE24+NEhinb**	negative
CE24+NE24	positive					**CE Hinb+ NE24**	negative

Table 13. Overview of BiFC interactions of HvESCRT-III proteins

7 Discussion

Concerning to the organization and trafficking pathways, the endosomal system in plants seems to be more complex than in yeast or mammals (Otegui and Spitzer 2008; Robinson, Jiang, and Schumacher 2008). Plant ESCRTs were first studied in the model plant Arabidopsis thaliana where in addition to ESCRT interaction, localization and mutant analyses the expression of ESCRT components were described by micro-array data (Richardson et al. 2011; Korbei et al. 2013; Isono and Kalinowska 2017; Otegui and Spitzer 2008; Spitzer et al. 2006). Recently, ESCRTs also start to be studied in cereals, for example in rice, maize and barley (Zhu et al. 2016; Hilscher et al. 2016; Tian et al. 2007).

Considerable attempt has been applied in yeast, mammals and Arabidopsis to develop yeast two-hybrid protein-protein interactions for the ESCRT machinery (Nikko and Andre 2007; Richardson et al. 2011). Here we employed the yeast two-hybrid system to characterize the barley ESCRT-III interactome. The translation abundance of large number of ESCRT proteins is not so efficient in vitro, we were just able to detect some translated ESCRT-III proteins after yeast two-hybrid assay by western blot such as HvVPS24-AD, HvSNF7-BD, AtVPS2-AD and AtVPS24-BD. But there were no clear bands detected for HvVPS4 and HVHinb2 proteins.

Negative controls were carried out concerning to evaluate the autoactivation of binding and activation domains of Gal4. Positive controls from Arabidopsis ESCRT-III-interacting-proteins such as AtVPS60-AD/AtVPS24-BD, AtVPS2-AD/AtVPS24-BD and AtVPS24-AD/AtVPS24.1-BD, which were kindly provided by Dr. Maria-Theres Hauser, confirmed the accuracy of our yeast-two-hybrid assay.

Although there is a strong interaction and yeast growth rate of our positive control of AtVPS24-AD with AtVPS24.1-BD as homodimer, no self-interaction and homodimerization of HvVPS24 could observe in this study. A strong autoactivation of HvVPS24 fused with binding domain (HvVPS24-BD) limited this yeast-two-hybrid. Of course the lack of barley VPS24 self-interaction in this study does not preclude a homodimerization of HvVPS24. Unlike to the previous yeast two-hybrid studies of Richardon et al. (Richardson et al. 2011), who showed some interactions between VPS24.1/A and the ESCRT-III associated protein VPS60.1/A and VPS60.2/B, we found no interactions between HvVPS24 and HvVPS60. Also no interactions were observed in this Y2H study between HvVPS24 and HvVPS4, in contrast

to the results reported by Richardson et al. in Arabidopsis (Richardson et al. 2011). Based on our yeast two-hybrid results no homodimerization of HvVPS4 was detected, there is no other scientific report in case of VPS4 homodimer in plant ESCRT.

Richardson et al. found no interaction between SNF7A/B with putative components of the Arabidopsis ESCRT-III complex VPS24A. Here we observe also no interaction between VPS24 and SNF7 unlike to the results of our BiFC assay which is discussed below. However the probable lack of this interaction between Arabidopsis VPS24A and SNF7 may reflect again the limitation of yeast two-hybrid assay, or maybe there are some plant-specific diversity in ESCRT-III assembly (Richardson et al. 2011). Additionally, the growth rate colonies containing SNF7 was not strong on LB plate in comparison with other transformed colonies and cells seemed not to be in good growth condition.

In terms of HvHinb2 interactions there are publications available. In this yeast-two-hybrid study a light yeast growth rate of HvHinb2-AD/HvHinb2-BD on selection medium containing 3mM AT was reported, that might be a homodimerization of HvHinb2. There were no other interactions detected between HvHinb2 with HvVPS24, HvVPS60, HvVPS4 and HvSNF7.

Biomolecular fluorescence complementation (BiFC) assay in *Nicotiana benthamiana* leaves was applied in this study to reinforce and reevaluate the protein-protein-interactions of barley ESCRT-III components. BiFC has a prominent advantage in comparison with Yeast two-hybrid assay in this study; the reason behind is the ability of BiFC assay to express and translate the plant-based protein in a plant background system that makes it more appropriate than yeast-two-hybrid.

To validate the results of the BiFC assay a suitable negative control has a key role. In our study the perfect negative control would have been the mutated interaction domain of HvVPS24. This would have included bioinformatic work, ordering the mutated barley gene and cloning procedures and was subsequently not possible to carry out during this thesis. Negative controls were executed in order to evaluate the unspecific background fluorescence of leave tissue because sometimes at high expression levels (35s promoter) free YFP fragments associate non-specifically and produce background fluorescence. Little yellow autofluorescence was detected in no infiltrated leaves between the cell components itself. This results show that our analysis were all safe and unaffected.

In terms of BiFC assay there are so many publications, but limited information concerning western blot that proof the expression of BiFC proteins is available. Here, we could not detect the expression of ESCRT-III proteins by western blot after the BiFC assay. The reason behind is maybe the expression level was not strong enough to be detected on the western blot or the fused proteins were degenerated during the protein extraction process.

It has been suggested in the publications that the best time point to check the protein-protein-interactions in BiFC system is three days after infiltration, before the saturation or completion of all potential interactions in cells happens (Xing et al. 2016; Grefen et al. 2010). In this study we checked the interactions between day three and day five after infiltration, though we got the best and clear interaction signals at the day five.

According to the ESCRT protein characterization studies by Richardson et al. (Richardson et al. 2011) an interaction was reported between VPS24 and SNF7A during their assembly on the endosomal membrane. We also observed a weak interaction between VPS24 and SNF7 in our BiFC assay that seems to show the association of VPS24 with ESCRT-III in barley.

There are few *in vivo* localization studies of ESCRT in seeds and cereal endosperm available because of technical complexity. Hilscher et al. have shown the localization studies of HvSNF7a, HvVPS24 and HvVPS60 in various layers of developing barley. They used monomeric fluorophore and rice actin promoter to transform and express ESCRT-III components (Hilscher et al. 2016). However, we could not precisely report the localization of HvVPS24 and other ESCRT-III components in this study, due to overexpression of 35S-promoter. But HvVPS24 is mainly observed at the plasma membrane and also around the nucleus. HvVPS24 localization study of barley endosperm showed that transiently overexpressed HvVPS24-mEosFP localized mainly cytosolic but also at agglomerations. The latter observation demonstrates the importance of stoichiometry in ESCRT-III assembly (Lin et al. 2005).

Localization studies of HvVPS60a in barley endosperm showed cytosolic localization and at small agglomerations in aleurone layer. It may indicate different roles of HvVPS60 in protein sorting and also targeting the ubiquitinated proteins to the vacuole (Hilscher et al. 2016). In Arabidopsis it has been illustrated the dynamic expression and function of ESCRT proteins in different tissues, organ and under different treatment. Therefore further cellular and molecular studies of ESCRT-III are required separately in every single tissue.

Re-localization studies of HvVPS24 to the plasma membrane in barley endosperm introduced the importance of steady-state of ESCRT-III association. ESCRT components are cytosolic proteins and they are recruited to the membrane. It seems that their binding at the membrane is a critical factor to associate and produce functional machinery. Generally ESCRT-III in barley endosperm seems to be transient complex with various functions in different layer that is provided with some regularly assembly/disassembly cycles (Hilscher et al. 2016).

A Co-immunoprecipitation assay was planned in this study to reinforce the results of the 2 interaction methods (yeast-two-hybrid and BiFC) used in this thesis. Actin::SNF7mEos was previously used to prepare transgenic barley lines and no interaction was seen by the Co-IP of HvVPS24mCh with other ESCRT proteins. Therefore it would be good to check the Co-IP of other fused HvESCRT-III proteins like SNF7 to evaluate this result. Unfortunately the mEos antibody did not work properly in order to recognize actin::SNF7mEos in barley endosperm. Thus, Co-IP could not be performed.

8 Conclusion

Our results of our Y2H and BiFC assay reconfirm some known plant ESCRT-III interactions. Taken together it seems that barley VPS24 does not stably interact with other barley ESCRT-III proteins but it may collaborate and cooperate transiently in some cases with ESCRT-III subunits for instance with SNF7. Thus, barley ESCRT seems to be an adjustable and dynamic machinery in order to a spatial- temporal expression and interactions with associated proteins and itself.

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10 Appendix

Abbreviations

AAA-type: ATPases associated with a variety of cellular activities

ABA: abscisic acid

AMSH: associated molecule with the SH3 domain of STAM

BiFC: bifluorescence complementation analyses

CHMP: chromatin modifying protein, renamed charged multivesicular body

Co-IP: Co-immuno precipitation

EE: early endosome

ESCRT: endosomal sorting complex required for transport

FRET: Fluorescence resonance energy transfer

HIV: human immunodeficiency virus

ILV: intraluminal vesicles

LIP: LYST interacting protein

LYST: lysosomal trafficking regulator

MIT: microtubule interacting and trafficking molecule domain

MVB: multivesicular bodies

PI3P: phosphatidylinositol-3 phosphate

PSV: protein storage vacuole

STAM: signal transducing adaptor molecule

SKD: Suppressor of K+ transport growth defect

SNF: sucrose non-fermenting

TNG: trans-Golgi networkTOM1: target of Myb1

VPS: vacuolar protein sorting

VTA1: VPS twenty associated

Y2H: yeast two-hybrid

µl: microliter

ml: milliliter

°: degree symbol

°C: degree Celsius

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