Autophagy and Vesicle Trafficking in Arabidopsis: Emerging Roles of Gamma Secretase Complex Subunits and AP4 Complex

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ABSTRACT

Presenilins are the catalytic component of the γ-secretase complex which was first identified in the genetic screens of the patients suffering from Alzheimer's disease. Macroautophagy is a major route that encompasses the degradation of the cell components and damaged proteins, lipids and carbohydrates to recycle nutrients in response to starvation. Here we report that Arabidopsis presenilin double mutant *ps1/ps2* shows defective clearance of autophagosomes in root cells when autophagy is induced through sucrose starvation. In addition to being susceptible to the sucrose starvation, mutant root cells have altered expression levels of important autophagy-related genes. Mutant root cells show differential protein accumulations under sucrose starvation leading to inefficient clearance of proteins. Biochemical and cell biological approaches combined with autophagy inhibitors such as wortmannin and concanamycin-A suggested aberrant degradation of autophagosomes within the lytic plant vacuoles. Taken together, our data suggest an involvement of the γ-secretase complex and/or presenilins in plant autophagy. Presenilin enhancer-2 (PEN2) is another subunit of the γ-secretase complex which was first discovered in a genetic study involving *C. elegans*. It is required for the γ-secretase complex activity and undertakes the endoproteolysis of presenilins. Here, we demonstrate that AtPEN2 vesicles are very sensitive to latrunculin-B, an F-actin depolymerizing drug, which suggests the role of the actin cytoskeleton in the motility of these vesicles. Moreover, AtPEN2 partially localizes with DsRED-FYVE, a PI3P reporter, which is specifically localized with the dynamic and highly motile late endosomal compartments and has been implicated in the tip growth. Furthermore, the phenotypic analysis of *pen2* mutant reveals reduced primary root growth compared to the wild type seedlings. Collectively, our results indicate possible roles of AtPEN2 in regulating tip growth and protein trafficking pathways in Arabidopsis. However, studies on other possible functions of AtPEN2 in signal transduction and stress responses are still required.

Adaptor protein (AP) complexes are conserved throughout eukaryotic organisms and are vital for protein sorting among various post-Golgi pathways by recognizing specific cargo protein motifs. Among the five AP complexes (AP1-AP5), AP4 is the most poorly understood. In animals, AP4 has recently been recognized as

a regulator of autophagy through mediating the export of ATG9, a core autophagy protein from the trans-Golgi to promote autophagosome formation. Here we have performed an analysis of Arabidopsis mutants lacking different subunits of AP4 in connection with autophagy. We report that the YXXØ motif is conserved in the ATG9 protein of Arabidopsis which is required for its recognition by AP4 complex. Moreover, a colocalization study reveals that AP4 complex localizes with ATG9 in the *Nicotiana benthamiana* leaf epidermal cells. Besides showing sensitivity towards dithiothreitol (DTT), an ER stress inducer, the mutants of AP4 complex accumulate ATG8, a structural component of autophagosomes. Taken together, we propose that the Arabidopsis AP4 complex may interact with ATG9 and play a role in its transport to the phagophore assembly site similar to animals and in addition to the missorting of proteins, defective autophagy is also responsible for the phenotypic abnormality of AP4 mutants.

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INTRODUCTION

Plants have limited locomotion capacities and cannot move to the same extent as animals to deal with environmental challenges. This makes them vulnerable to both biotic and abiotic stress conditions. In modern times, large scale cultivation of crops is the basis for feeding the ever-increasing population of the world. In the years to come, increased demand for food and a more challenging environment for plant growth is expected. Consequently, it is vital to understand the intricacies of plant growth and survival at the cellular and molecular levels. Plants have evolved mechanisms to adapt to the changes in climate and environment and these responses are controlled by various signaling pathways. These pathways upon stimulation lead to protein abundance of various key proteins besides other effects. It occurs through the mechanisms that implicate changes in the transcription and translation as well as rates of protein degradation. This thesis addresses some of the important issues in this important facet of plant growth.

1.1 Autophagy

Autophagy (self-eating) is an intracellular degradation system that encompasses the delivery of cellular cargo such as molecular complexes, protein aggregates, infectious agents and damaged organelles to the vacuole (or the animal counterpart, the lysosome). The delivered cargo is then degraded inside the vacuole and the breakdown products are then exported from the vacuole and recycled for other uses. There are different types of autophagy pathways described in different species such as chaperone-mediated autophagy, microautophagy, and macroautophagy. In plants, however, only micro and macroautophagy have been described (Figure 1). In microautophagy, the vacuolar membrane invaginates to engulf the cytoplasmic cargo and pinches off to release it inside the vacuole where it is degraded (Müller *et al.,* 2000). In this doctoral thesis, my focus was only on the macroautophagy pathway which will hereafter be referred to as autophagy.

Autophagy is characterized by the formation of a double membrane compartment called autophagosome around the cargo that needs to be recycled and is sent to the vacuole for degradation (Figure 1). The cargo is delivered to the vacuole through a fusion of the outer membrane of the autophagosome with tonoplast of vacuole and the vacuolar hydrolases degrade it. It is a highly conserved process in eukaryotes and involves core machinery of approximately 40 autophagy (ATG) related genes. In plants, most of the ATG genes are encoded by the gene families and their transcript levels increase upon autophagy induction. Autophagosome formation requires three complexes (Figure 1) i.e. Atg1/ULK1, VPS34 (VACUOL SORTING COMPLEX)/PI3K (phosphoinositide-3-kinase) complex and the ATG9 complex. Besides, a variety of proteins are required for autophagy completion such as small GTPases (guanosine triphosphatases), coat complexes, tethering factors and SNARE's (Soluble NSF Attachment Protein Receptor).

Atg1/ULK1 complex is located at the phagophore assembly site (PAS)/pre-autophagosomal structure. Phagophore is a double membrane that encloses and isolates the cytoplasmic components during autophagy. Although the composition of the Atg1 complex varies among eukaryotes, it mainly consists of Atg1 kinase, Atg13, Atg11 and Atg101 and It is located at the PAS (Li et al, 2014; Suttangkakul *et al.,* 2011). Activation of the Atg1/ULK1 complex requires kinases that sense nutrient availability and environmental stresses. These include TOR (Target of Rapamycin) and SnRK1 (Snf1-RELATED PROTEIN KINASE 1) protein kinases. TOR negatively regulates autophagy and under nutrient-rich conditions, activated TOR hyperphosphorylates the ATG13 and prevents its binding to Atg1 to avoid the complex assembly and prevents autophagy. Under starvation, TOR activity is inhibited which dephosphorylates ATG13 and promotes the Atg1/Atg13 complex assembly together with other factors (Atg17, Atg11 in yeast and Atg101 in mammals and probably also in plants) that leads to autophagy activation.

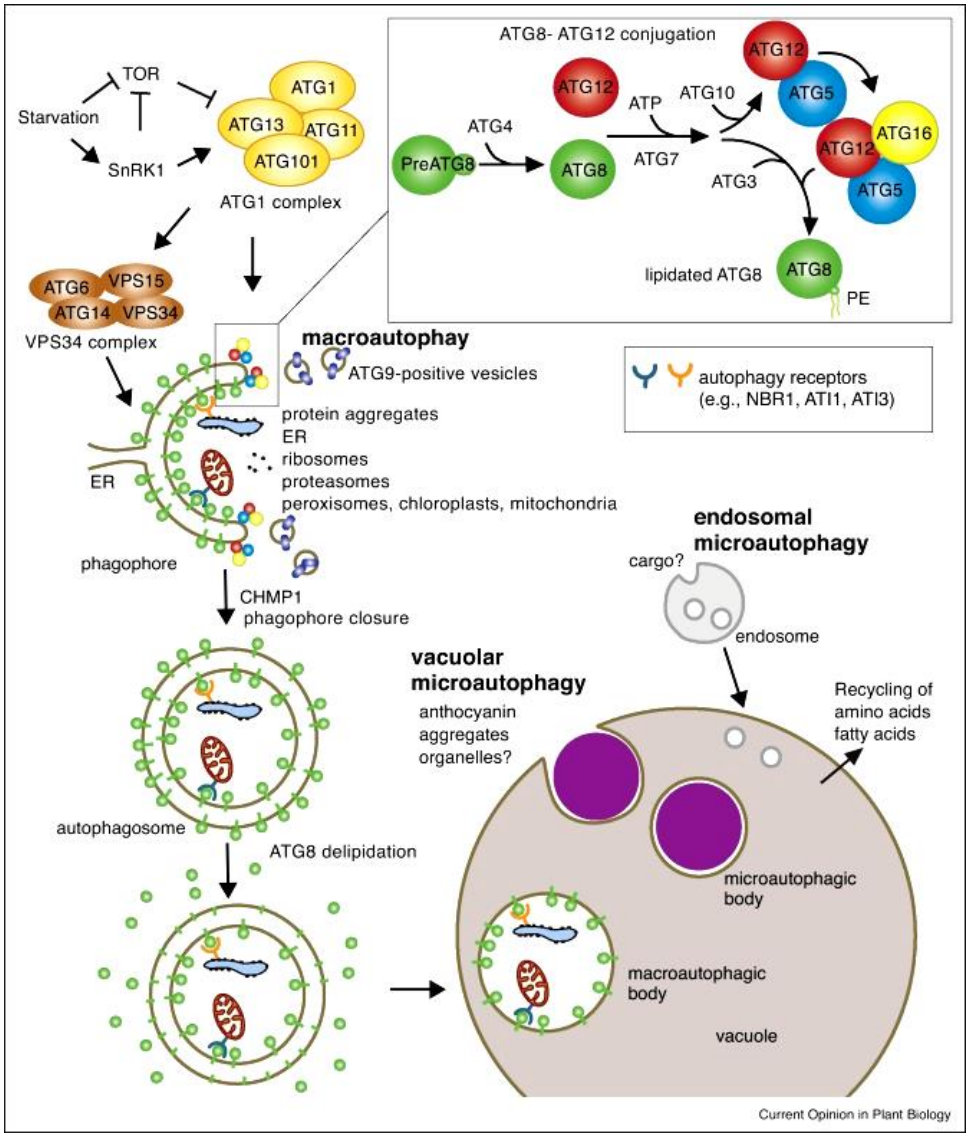

Figure 1: Three major routes of the autophagy pathway. Macroautophagy, vacuolar microautophagy, and endosomal microautophagy together with the main proposed molecular events encompassing macroautophagy (Ding *et al.,* 2018).

The next step is decorating the phagophore with ATG8 which is at the core of the autophagic machinery and is extensively used to study autophagy. Decoration of the phagophore involves conjugation of ATG8 with PE (phosphatidylethanolamine) and that of ATG12 with ATG5. ATG8 first undergoes proteolytic processing by ATG4 whereby its C-terminal glycine residue is exposed (Seo *et al.,* 2016). Then ATG3, ATG7 and a ligase complex consisting of ATG12-ATG5-ATG16 transfer ATG8 to the PE molecules in the growing phagophore (Figure 1). ATG12 and ATG5 conjugation

also requires ATG7 and conjugating enzyme ATG10 and can be seen at the margins of the phagophore. In plants, CHMP1 (CHARGED MULTIVESICULAR BODY PROTEIN1), is needed for the closure of the autophagosomes (Spitzer *et al.,* 2015). Once formed, autophagosomes are transferred to the vacuole and fuse with tonoplast by means of v-SNARE mechanism and deliver the contained molecule as an autophagic body. The autophagic body is then degraded with the action of acidic hydrolases. These enzymes are inhibited by the action of concanamycin A (ConA), an H^+-ATPase inhibitor which makes it an effective tool to study autophagy.

In addition to interacting with various adaptors for autophagosome formation, ATG8 interacts with a variety of receptors that deliver cargo to the phagophore. Most important of them are AIMs (ATG8 interacting motif) containing proteins such as ATG6, ATG1, and ATG11. NBR1 (Neighbor of BRCA1) is another important protein that promotes plant defenses through acting as xenophagy receptor (removal of intracellular pathogens through autophagy) [Zhou *et al.* 2013]. TSPO (TRYPTOPHAN-RICH SENSORY PROTEIN/TRANSLOCATOR) is another ATG8 interacting protein that upregulates under abiotic stresses (Vanhee *et al.,* 2011). Other plant-specific autophagy receptors include ATI1 and ATI3a which are required for the autophagic turnover of plastids and ER components (Michaeli *et al.,* 2014).

Studies with *Arabidopsis thaliana* and other plant species have shown that a mechanistically very identical ATG mediated autophagic system exists in the plant kingdom just like mammals and yeast (Li *et al.,* 2012). In plants, autophagy is crucial under natural senescence, under carbon and nitrogen limiting conditions. The more specific roles of autophagy in plants include removal of chloroplast components (piecemeal chlorophagy), ER domains (reticulophagy), peroxisomes (pexophagy), protein aggregates (aggrephagy) and intracellular pathogens (xenophagy) [Ding *et al.,* 2018].

1.2 The Gamma-Secretase Complex

γ-Secretase belongs to a group of so-called intramembrane-cleaving proteases (I-CLiPs) required for regulated intramembrane proteolysis (RIP) along with site-2 proteases (S2P), signal peptide peptidases (SPP) and rhomboids (Sannerud & Annaert, 2009). RIP is an evolutionarily conserved process required for degrading transmembrane protein fragments on one hand (Kopan and Ilagan 2004; Lichtenthaler *et al.,* 2011) and signal transduction on the other hand (Lal & Caplan, 2011). The γ-Secretase complex is a multisubunit cleaving protease and is implicated in the cleavage of a variety of transmembrane substrates such as amyloid precursor protein (APP) and Notch. Research shows that γ-secretase mediated aberrant cleavage of APP and Notch leads to Alzheimer's and cancer respectively. This makes γ-secretase an important molecular target and various inhibitors and modulators are currently in clinical investigations for both diseases (Gertsik *et al.,* 2015).

There are over 90 substrates of γ-secretase and the list continues to grow (Haapasalo & Kovacs, 2011). γ-secretase is involved in the processing of these substrates to produce distinct amino and carboxy termini with distinct functions ranging from signaling to cell adhesion and transcriptional regulation to cytoskeletal dynamics (Gertsik *et al.,* 2015). Despite many years of intensive studies, the structure, function and precise localization of the γ-secretase and its subunits remains somewhat controversial. It has been proposed to localize in most of the endomembrane compartments including cis-Golgi, Golgi, trans-Golgi, ER, plasma membrane, lysosomes, and endosomes, etc. (Annaert *et al.,* 1999; Pasternak *et al.,* 2003; Réchards *et al.,* 2003; Jutras *et al.,* 2005; Fukumori *et al.,* 2006; Fassler *et al.,* 2010; Smolarkiewicz *et al.,* 2014).

Biochemical studies have shown that γ-secretase complex comprises of a core catalytic subunit called presenilin (PS) and three other non-catalytic subunits required for its activity namely presenilin enhancer-2 (PEN2), nicastrin (NCT) and anterior pharynx defective-1 (APH-1) [Francis *et al.,* 2002; Parks & Curtis, 2007]. All of these subunits are vital for the activity of γ-secretase and the loss of any of these leads to the loss of its function (Edbauer *et al.,* 2003). Genes that code for the γ-

secretase subunits are evolutionarily highly conserved and are found in metazoans and higher plants but are missing in fungi. It has even been reported to function in evolutionarily distinct organisms like the moss, *Physcomitrella patens* and slime mould *Dictyostelium discoideum* (Khandelwal *et al.,* 2007; McMains *et al.,* 2010). In Arabidopsis, the components of γ-secretase were first described in a doctoral thesis wherein the focus was set on understanding the complex formation and identification of the substrates (Walker 2010). Later on, Smolarkiewicz *et al.,* (2014) reported that all of the γ-secretase subunits also exist in *A. thaliana* and are active at the transcriptional level. Through multiple sequence alignments, they also described that all of the amino acid motifs essential for the activity of the γ-secretase complex are conserved in plant homologues. Moreover, they also found that all of these subunits co-localize to endomembrane system and also interact with each other in Arabidopsis leaf protoplasts which is consistent with the evidence from animal studies (Smolarkiewicz *et al.,* 2014).

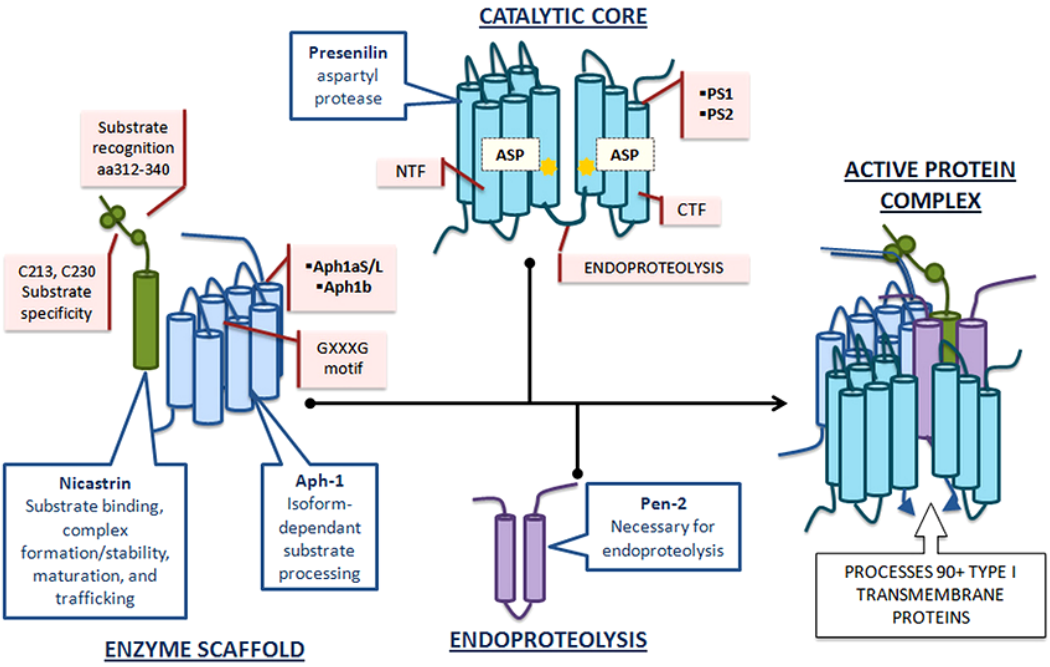

Figure 2: Formation of the γ-Secretase complex and possible roles of its subunits (Gertsik *et al.,* 2015).

It is widely accepted that all the γ-secretase complex subunits are synthesized in the ER. It is synthesized by the stepwise assembly of APH-1, nicastrin, presenilin and PEN2 (Figure 2). First, N-glycosylated nicastrin comes and together with Aph1 forms an enzyme scaffold or a subcomplex. Next, a full-length presenilin comes and binds with this subcomplex. Lastly, PEN2 joins the complex and presenilin undergoes endoproteolysis releasing presenilin-NTF/CTF (N/C terminal fragments) to activate the enzyme complex.

Nicastrin is a large protein consisting of a single transmembrane domain (Figure 2) and four amino acid residues in the one-third proximal end of its TMD important for interaction with other components of the complex (Capell *et al.,* 2003). Nicastrin is itself catalytically inactive but together with APH-1, it provides a scaffold for the γ-secretase complex. It also functions in substrate and binding site recognition (Dries *et al.,* 2009). APH-1 has seven TMDs and Its GXXXG motif present in the fourth TMD is particularly vital for the γ-secretase complex assembly through intramembrane helix-helix interactions. In mammalian cells, a mutation in Gly 122 of APH-1 to aspartic acid leads to a loss of function phenotype rendering it unable to associate with γ-secretase (Lee *et al.,* 2004). In humans, there are two APH-1 genes (APH-1a and APH-1b) and APH1a exists as two C terminal splice forms (Shirotani *et al.,* 2004). Presenilins (PSs) are aspartyl proteases and the catalytic component of the γ-secretase complex which must be endoproteolysed for its activity. Human presenilins (HsPS1 & HsPS2) were first identified in the genetic screens for mutations causing early-onset familial Alzheimer's disease (FAD) [Sherrington *et al.,* 1995; Levy-Lahad *et al.,* 2015]. Most of the genomes studied contain two homologues of presenilins i.e. PS1 and PS2 and have up to 67% amino acid sequence homology. Presenilin (PS) has 9 transmembrane domains and the catalytic residues reside on the TMDs 6 and 7 (Figure 2) [Tolia *et al.,* 2008; Li *et al.,* 2013]. In metazoans, PS is produced as a full-length holoprotein, an unstable version that undergoes endoproteolysis between TMD 6 and TMD 7. Endoproteolysis which is required for the activity of γ-secretase releases NTFs and CTFs of PS which function together as a stable heterodimer (Podlisny *et al.,* 1997). PEN2 is the smallest subunit and contains two TMDs (Figure 2). It consists of 101 amino acids and its TMDs share no homology with any of the known family

members. PEN2 is required for the endoproteolysis of PS and ultimately for the activity of the γ-secretase complex (Mao *et al.,* 2012). The significance of PEN2 in endoproteolysis of PS was evidenced in one study by Takasugi *et al.,* (2003) where a knockdown mutant of PEN2 through RNAi resulted in decreased NTF & CTF levels of PS and the over-expression of PEN2 in *PEN2* deficient cells recovered the PS fragments. In addition, PEN2 contributes to stabilize the γ-secretase complex and plays an important role in its overall proteolytic activity.

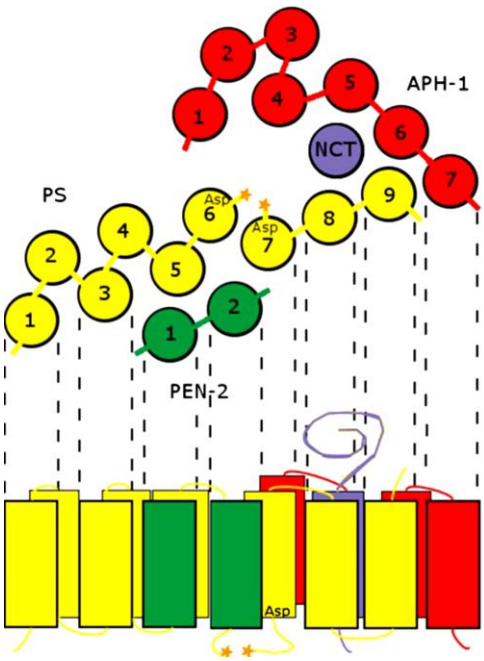

Figure 3: The γ-secretase complex subunits. The four subunits of the active γ-secretase complex: presenilin (PS, yellow) with catalytic aspartyl residues (Asp), nicastrin (NCT, violet), anterior pharynx defective 1 (APH-1, red) and presenilin enhancer-2 (PEN2, green) [Smolarkiewicz *et al.,* 2013].

1.3 The Multifunctional Nature of Presenilins

Beyond their role in γ-secretase complex regulation, presenilins are also involved in a variety of highly conserved γ-secretase independent cellular functions. They contain distinct protein binding domains and undergo post-translational modifications such as endoproteolysis, caspase cleavage, phosphorylation, and ubiquitination which regulate their function and interaction with other proteins (Gudey *et al.,* 2014). As previously mentioned, all the γ-secretase complex components and their amino acid motifs are evolutionarily conserved in mammals as well as plants. *Physcomitrella patens* mutated for presenilins (*Ppps*) showed abnormal growth, reduced chloroplast movement and defective endocytosis (Khandelwal *et al.,* 2007). Exogenous expression of γ-secretase subunits in *A. thaliana* resulted in impairment

of vacuolar trafficking (Smolarkiewicz *et al.,* 2014). These studies indicate that the predominant role of presenilins is not connected with the γ-secretase dependant protease activity instead of its evolutionary function in endocytosis and vesicle trafficking (Duggan *et al.,* 2016).

Presenilins and γ-secretase indirectly take part in protein trafficking since several γ-substrates are involved in protein transport such as mammalian sortilin-related receptor and Vsp10p sorting receptor (Nyborg *et al.,* 2006). Other studies report that presenilins also participate in protein trafficking, a function independent of γ-secretase related proteolytic activity. For instance, presenilins interact with Rab11, a vesicular trafficking protein (Dumanchin *et al.,* 1999) and neurons lacking PS lead to reduced trafficking of TrkB and EphB receptors required for the neuronal survival by regulating neuroprotective functions of these receptor systems (Barthet *et al.,* 2013). Besides, axonal transport is regulated by PSs by their interaction with glycogen synthase kinase-3 beta (GSK-3β), thus influencing kinesin-1 and dynein function in transport (Dolma *et al.,* 2013). Multiple evidence indicates a direct role of presenilins in regulating calcium homeostasis (Figure 4). It has been found to create calcium pores and its interaction with calcium channels like sarco/ERCa2+-ATPase (SERCA) pump and the inositol triphosphate receptor (InsP3R) has also been evidenced (Figure 4) [Oh *et al.,* 2012; Nelson *et al.,* 2011].

Presenilins also regulate Wnt/β-catenin signaling pathway, by modulating the transcriptional activity of the β-catenin/Tcf-4 complex (Figure 4) [Xia *et al.,* 2001]. β-catenin is a multifunctional protein that links transmembrane adhesion proteins such as the γ-secretase substrate E-cadherin with several signaling pathways and participates in regulating transcription of various survival and anti-apoptotic pathways (Rosenbluh *et al.,* 2014; Marambaud *et al.,* 2002).

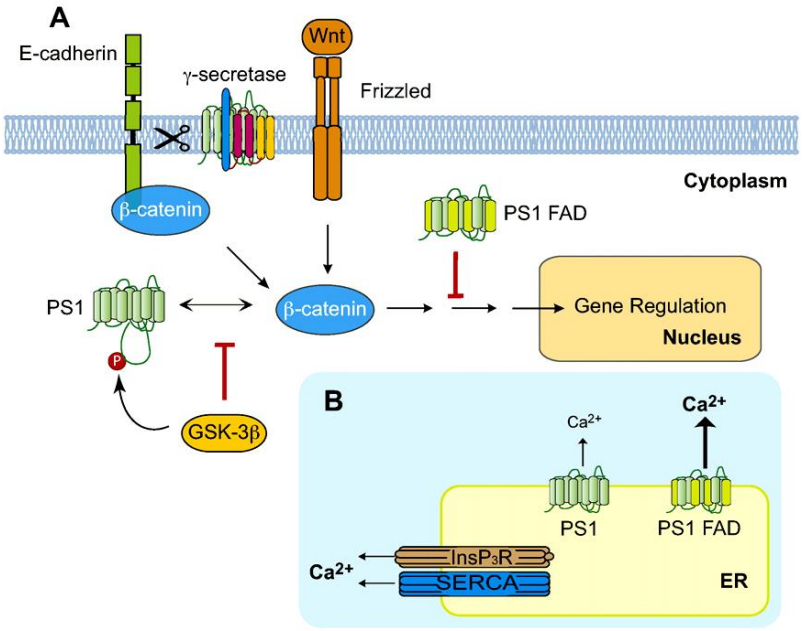

Figure 4: PS1 and γ-secretase regulate Wnt and calcium signaling. (A) The γ-secretase cleaves E-cadherin/ β-catenin complex from the cytoskeleton to release β-catenin into the cytosol, the key regulator of Wnt signaling. In the cytosol, PS1 negatively regulates β-catenin and thereby negative regulate Wnt signaling. PS1 FAD mutations or GSK3β phosphorylation of PS1 also negatively regulates the Wnt pathway. **(B)** Presenilins interact with Ca^{2+} channels such as SERCA and InsP3R and regulate Ca^{2+} levels in the ER. PS1 FAD mutations have also been reported to increase cytosolic calcium (Duggan *et al.,* 2016).

Presenilins have also been linked to apoptosis, an important process involved in the development, normal aging as well as pathogenesis of important diseases such as neurological disorders and cancer. PS1 FAD mutations showed increased susceptibility to apoptotic stimuli (Yang *et al.,* 2008). PSs have been shown to interact with apoptotic proteins such as PS1-associated protein (PSAP), FKBP38, Omi/HtrAS, and PARL and with anti-apoptotic proteins such as Bcl-2 and Bcl-XL and regulate apoptosis in γ-secretase dependent and independent manners (Alberici *et al.,* 1998). In short, presenilins have a very multifunctional nature in that they not only take part in γ-secretase dependant functions such as regulated intramembrane proteolysis which is required for cell differentiation, transcription and disease progression but also in regulating γ-secretase independent functions like endocytosis, apoptosis, Wnt signaling, calcium fluxes, and degradation.

1.3.1 Presenilins and Autophagy

Presenilins have been shown to function in autophagy through maintaining normal acidification of lysosomes and proteolysis (Figure 5). Lee *et al.,* in 2010 reported that PS1 mutations result in disrupted lysosomal acidification and thereby inhibited autophagy. They proposed that PS1 facilitates glycosylation of the vATPase V0a1 subunit, an element of the proton pump required for normal acidification of the lysosomes. OST (Oligosaccharyltransferase) is a multimeric complex in the ER that transfers a preassembled oligosaccharide to the asparagine residues on the consensus sequence asparagine-X-serine/threonine. In the cells lacking PS1, there is improper glycosylation of the V0a1 subunit resulting in poor assembly and function of the vATPase pump leading to reduced acidification of the lysosomes and ultimately defective autophagy. In contrast to this proposal, another study suggested an alternative function for the role of PS1 in the autophagy pathway. It was proposed that it's not the proton pump defects rather the Ca^{+} homeostasis defects that lead to lysosomal dysfunction (Figure 5) [Coen *et al.,* 2012]. Another study proposed that defective Ca^{+} storage and release are secondary to the lysosomal dysfunction in PS1 mutants (Lee *et al.,* 2015). It was revealed that PS1 mutation and lysosomal acidification lead to the efflux of lysosomal calcium mediated by pH-regulated transient receptor potential (TRP) cation channel mucoplin subfamily member 1 (TRPML1). However, a relatively recent study has provided a completely different explanation for the autophagy inhibition resulting from PS1 mutation whereby it was proposed that PS1 undergoes phosphorylation at Ser367 residue and facilitates autophagosome/lysosome fusion (Bustos *et al.,* 2017). This is accomplished when the phosphorylated PS1 interacts with Annexin A2 that as a result interacts with a lysosomal SNARE Vamp8. Annexin A2 helps in the binding of Vamp8 to an autophagosomal SNARE protein Stx17 to facilitate autophagosomal/lysosomal fusion. All these studies supply evidence for an essential role of PS1 in autophagy regulation but with somewhat conflicting results. However, future studies will in detail elucidate the role of PS1 in autophagy.

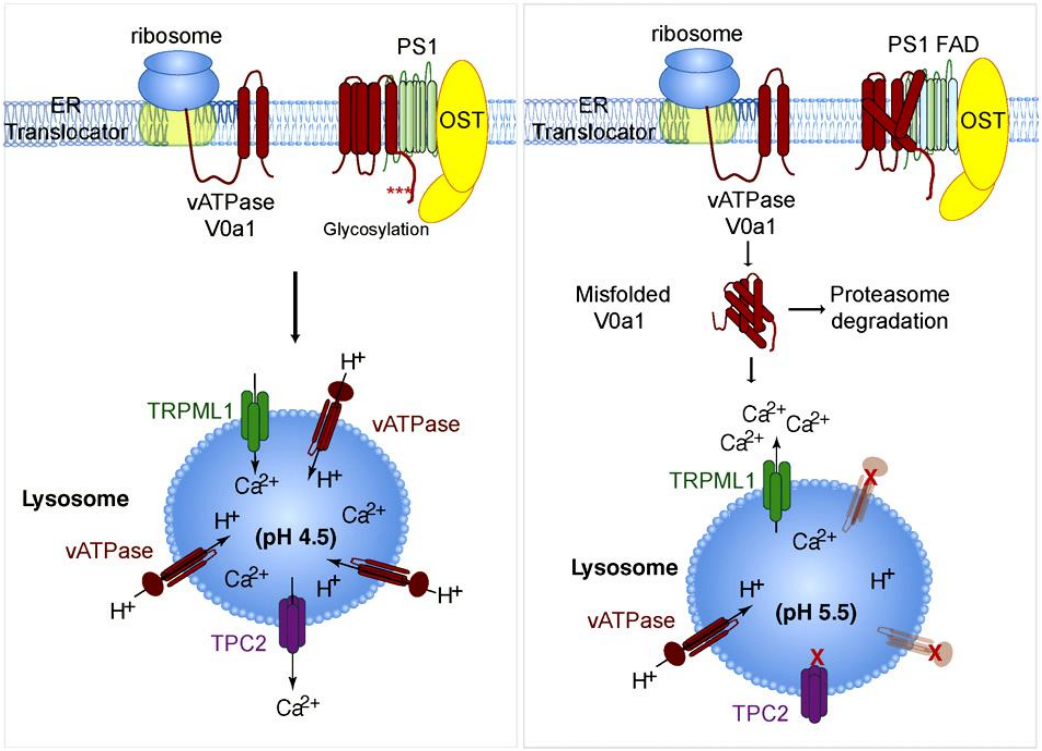

Figure 5: Presenilins, lysosomal acidification and Ca^{+} homeostasis. OST complex located in ER carries out glycosylation of the vATPase V0a1 subunit. Defects in PS1 lead to improper glycosylation resulting in misfolding and proteasome degradation of the V0a1 subunit leading to defective lysosomal vATPase function. Lysosomal acidification leads to the accumulation of autolysosomes in the cytosol. Defects in lysosomal acidification also lead to lysosomal calcium efflux through TRPML1. Increased lysosomal pH also leads to a failure of the two-pore channel (TPC) to dissociate from NAADP making it inactive and preventing lysosomal Ca+ efflux (Duggan *et al.,* 2016).

In plants, there is only one report from Arabidopsis concerning the possible role of presenilins in plant autophagy (Smolarkiewicz *et al.,* 2014). Arabidopsis contains two presenilins i.e. PS1 and PS2. It was reported that 6-week old presenilin double mutant plants (*psn1psn2*) of *A. thaliana* display accelerated chlorosis under darkness treatment. The experiment was also repeated under *in vitro* conditions where 2-week old seedlings were transferred to growth medium plates containing no nitrogen and carbon followed by transfer to dark and again the double mutant depicted enhanced chlorosis. Since darkness and starvation induce autophagy, a possible involvement of presenilins in plant autophagy was speculated.

1.4 PEN2 and Vesicular Trafficking

PEN2 (presenilin enhancer-2) was discovered in a genetic study involving *C. elegans* and is the smallest subunit of the γ-secretase complex. It is ~10 kDa protein and consists of 101 amino acids and shares no significant homology with any of the known family members (Dries & Yu, 2008). It consists of two TMDs connected through a cytoplasmic loop and an ER retention signal is localized on the TMD1 (Figure 3) [Fassler *et al.,* 2010]. PEN2 is needed for the endoproteolysis of the PS holoprotein to produce PS-NTF/CTF. In a study, RNAi mediated knockdown of PEN2 led to reduced production of PS-NTF/CTF fragments (Takasugi *et al.,* 2003). Apart from its role in PS endoproteolysis, PEN2 is also essential for the γ-secretase activity. In the mammalian cells, PEN2 knockdown resulted in an accumulation of PS holoprotein and a decreased activity of γ-secretase (Takasugi *et al.,* 2003). PEN2-/- mouse embryos showed a notch deficiency phenotype and mouse embryo fibroblasts (MEFs) cells showed no γ-secretase activity (Bammens *et al.,* 2011). However, the role of PEN2 in endoproteolysis of PS and γ-secretase activity raised an important question that whether PEN2 is needed for the activity of γ-secretase in itself or is it the PEN2 inspired proteolytic activity that is essential for its activity. To answer this, an endoproteolysis deficient mutant i.e. *ps1Δe9* was expressed into PEN2-/-MEFs and was found to have no γ-secretase (Bammens *et al.,* 2011). This revealed that PEN2 is not just important for the endoproteolysis of PS1 but also the γ-secretase activity. PEN2 regulates the γ-secretase activity at multiple levels, for instance, it regulates the composition of the complex. Overexpression of PEN2 leads to the production of more PS2 containing complexes than PS1 comprising complexes and to an increased Aβ42:Aβ40 ratio (Figure 6) [Placanica *et al.,* 2009]. All this evidence shows that PEN2 modulates the γ-secretase complex through diverse mechanisms and not just through providing stability to the complex and by carrying out endoproteolysis of PS.

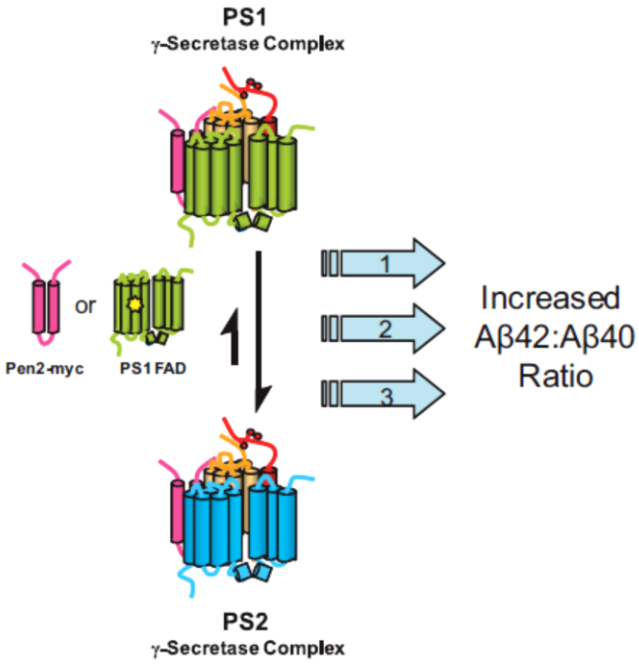

Figure 6: Proposed mechanism for the relationship between the dynamics of γ-secretase complex and Aβ42:Aβ40 ratios. PS1 and PS2 compete for cofactors PEN2, Aph1, and NCT. PS1 complex leads to a higher γ-secretase activity compared to PS2 and therefore more Aβ40 peptides are processed. However, overexpression of PEN2 or PS1 FAD mutations favours the formation of PS2 γ-secretase complexes and reduced PS1 complexes. This shift leads to a rise in Aβ42:Aβ40 ratio (Placanica *et al.,* 2009).

There is very little work done regarding the function of γ-secretase and its subunits in plants. However, Smolarkiewicz *et al.,* (2014) reported that amino acid motifs vital for the complex activity are present in all of the γ-secretase subunits in Arabidopsis including the well-conserved ER retention signal of PEN2 i.e. WLVNIFWF in the form of WFVNCFYF motif. In addition, a short NF motif is also well conserved in Presenilin at TMD4 which was recognized as a PEN2 binding site (Fassler *et al.,* 2010). This highlights that like other components of the γ-secretase, PEN2 as well is well conserved in evolutionarily distant plant species. The transient transformation of Arabidopsis leaf protoplasts showed that PEN2 shows cellular localization in the form of vesicular compartments. It co-localizes with trans-Golgi network (TGN) and pre-vacuolar compartment (PVC) and with all members of the γ-secretase such as APH-1, NCT and PS1 and PS2 (Smolarkiewicz *et al.,*2014). In animals, the ER retention is vital in the targeting of the assembled γ-secretase complex subunits to various endomembrane compartments. The TMD1 of PEN2 contains an asparagine residue which is important for its ER retention and to prevent unassembled subunits to enter the secretory pathway. To study this in plants, Smolarkiewicz *et al.,* (2014) conducted site-directed mutagenesis by substituting asparagine with leucine. Interestingly, PEN2 was found localized only in the ER as reticulate compartments unlike its usual vesicular localization and showed no

localization in PVC and TGN. The interaction study of a mutated version of PEN2 and PS2 was also carried out through FLIM measurements but surprisingly there was no interaction observed. This showed that PEN2 is required for the trafficking of presenilins although this observation needs to be further tested. Another important observation was that PEN2 localizes with ATG8 (autophagosomal marker) in the leaf protoplasts pre-treated with dark for two days to induce autophagy (Smolarkiewicz *et al.,* 2014). All of these studies present intriguing evidence about the function of PEN2 in important cellular processes but the molecular mechanisms behind these observations need to be further elucidated.

1.4.1 Intracellular Protein Trafficking Pathways in Plants

Endomembrane system of the eukaryotic cells is very elaborate, fine-tuned and is involved in the syntheses, sorting, delivery, and degradation of the macromolecules. The system is comprising of dynamic organelles such as endoplasmic reticulum, Golgi complex, trans-Golgi, endosomes, and vacuole. Endosomes are sorting organelles within the endomembrane system which act as a gateway for endocytosis of the materials and an interim compartment for the transport of macromolecules to the vacuole (Otegui & Spitzer, 2008). They are responsible for the sorting of receptors, transporters and plasma membrane (PM) proteins which are a vital regulatory process for PM structure and the ability of the cells to react to the external stimuli (Reyes *et al.,* 2011).

In plants, endosomes are classified into early endosomes (EE) which are tubular and dynamic and late endosomes (LE) which are mostly spherical and are recognized by the marker proteins particularly Rab GTPases (Huotari & Helenius, 2011). In the biosynthetic pathway, secretory proteins are synthesized in the ER lumens and exit the ER through budding of the COPII vesicles. These vesicles reach Golgi and fuse with Golgi apparatus releasing the proteins through cisternal maturation. As the maturation proceeds the trans most cisternae become TGN which is a dynamic compartment important for protein sorting. In plants, TGN assumes the function of EE and is not only an entry point for the endocytosed material but also a sorting spot for the cargo proteins coming from the Golgi and targeted to PM, cell

wall or cell plate, etc. This indicates that TGN is at the intersection of the endocytic and secretory pathways (Viotti *et al.,* 2010). After arriving at EE, the endocytosed material might be recycled back to PM through recycling endosomes (REs), maintained in EE or mature into LE also called MVB (multivesicular body). MVBs contain several sorting receptors and vacuolar proteins that are on the way to the vacuole indicating that MVBs act as intermediates in the biosynthetic trafficking en route to the vacuole (Bottanelli *et al.,* 2011). The MVB is also a PVC (pre vacuolar compartment) and is the point where the endocytic and vacuolar pathway meet. The plant endocytic pathways have been illustrated in Figure 7 (Contento & Bassham, 2012).

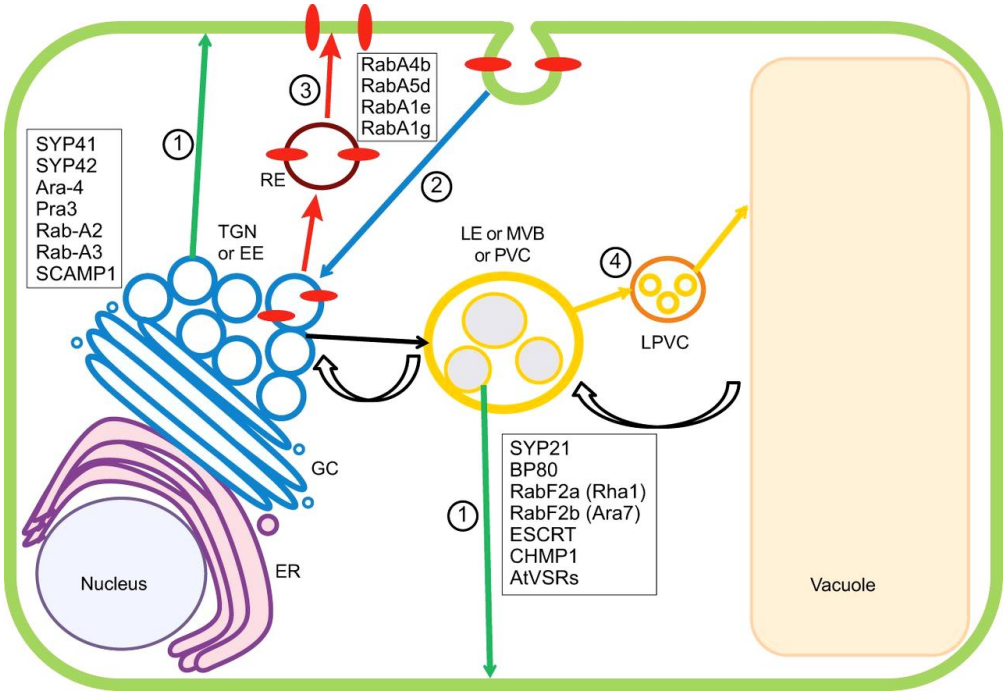

Figure 7: General overview of protein trafficking pathways in plant cells (Contento & Bassham, 2012). Pathway (1) is depicted by green arrows which indicate the biosynthetic pathway that leads to PM and passes through TGN or sometimes MVB. Pathway (2) is represented by blue arrows which is taken up by PM components (red ovals) as they are internalized into endocytic vesicles and pass through TGN. The red arrows indicate pathway (3) in which PM components can recycle back to PM through RE. The orange arrows show pathway (4) for vacuolar components that are meant for degradation in the vacuole.

1.5 Adaptor Protein Complexes

In the endocytic and biosynthetic/secretary pathways, the cargo proteins are assembled into vesicles and are conveyed to distinct target locations by vesicular trafficking. Adaptor proteins (AP) play an essential part in this process. APs bind to the sorting signal at the cytoplasmic tail of cargo, engage accessory proteins and concentrate the cargo into vesicular carriers (Park & Guo, 2014). These vesicles are then transported to the membrane of the targeted organelle. A total of 5 heterotetrameric adaptor protein complexes AP1, AP2, AP3, AP4, and AP5 have been identified and all of them are evolutionarily conserved. All of these adaptor complexes consist of two large subunits (γ/β1, α/β2, δ/β3, ε/β4, and ζ/β5), one medium subunit (μ1-5) and one small subunit (σ1-5). In AP1 to AP3, these subunits occur in multiple isoforms and each subunit of adaptor proteins performs a specific function (Park & Guo, 2014). One of the large subunits in each adaptor protein complex binds to the membrane of the donor compartment where vesicles are formed and the other large subunit recruits clathrin to the membrane (Brodsky *et al.,* 2001).

Some distinct sorting signals such as NPXY, YXXΦ, and dileucine motifs have been identified in the cytoplasmic tail of the cargo proteins (Bonifacino & Dell'Angelica, 1999). The μ subunit recognizes the tyrosine-based motif (YXXØ) in the cytoplasmic tail of the cargo proteins. The small subunits have been implicated in the stabilization of the complex. The previous decade has seen a lot of advancement in understanding the role of adaptor protein complexes. An overview of the localization and functions of the adaptor protein complexes has been shown in Figure 8. Just like animal cells, all of the adaptor protein complexes are found in plant genomes including Arabidopsis through sequence analysis. Arabidopsis genome encodes all of the subunits (adaptins) of the adaptor proteins except the sigma 5 subunit of AP5 (Hirst *et al.,* 2011). This indicates the possibility that the functions of these complexes are evolutionarily conserved but it should nevertheless be experimentally confirmed.

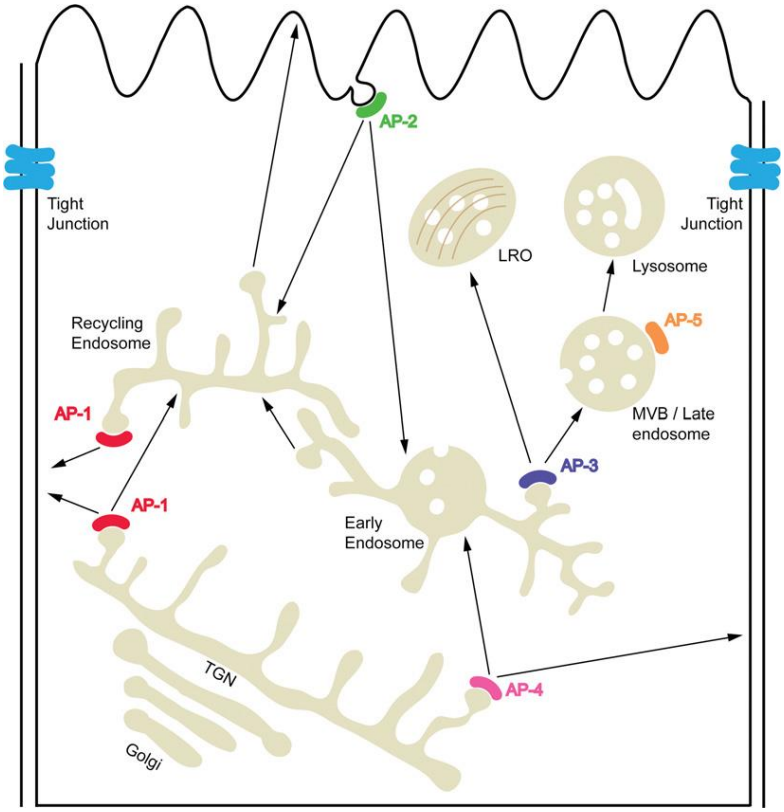

Figure 8: Trafficking and localization of the adaptor protein complexes. AP1 localizes to the TGN and RE. AP2 localizes to the plasma membrane and mediates clathrin-mediated endocytosis. AP3 is present in TGN and is involved in LRO biogenesis. AP4 is present at the TGN and plays a role in the transport of vesicles from TGN to endosomes. AP5 is localized at late endosomes and has unknown functions (Park & Guo, 2014).

1.5.1 Adaptor Protein Complex 4

Adaptor Protein complex 4 (AP4) has been most recently described and is the least understood adaptor protein complex. It is evidenced from the studies in animal systems that AP4 is localized at the TGN or the endosomal membranes and its membrane recruitment is facilitated by ARF1 (Boehm *et al.,* 2001). It facilitates the transport of the cargo proteins between TGN and endosomes in a clathrin-independent manner (Dell'Angelica *et al.,*1999). Among the major cargos sorted by AP4 include APP (amyloid precursor protein), AMPA type glutamate receptors (AMPAR) and glutamate receptor protein (Matsuda *et al.,* 2008; Yap *et al.,* 2003). Mutations in the genes coding for the subunits of AP4 result in intellectual disabilities, seizures, and microcephaly (Moreno-De-Luca *et al.,* 2011). It is

noteworthy that AP4 has also been implicated in regulating autophagy in the animal cells (Mattera *et al.,* 2017). ATG9, the only transmembrane containing core autophagic machinery protein has been identified as a specific cargo of AP4. ATG9 is exported by AP4 from the TGN to the peripheral cytoplasm where it contributes to the maturation of preautophagosomal structure (PAS) [Mattera *et al.,* 2017].

In plants, the first report about AP4 came from Arabidopsis where it was shown that AP4 is localized at the TGN and that it binds with YXXΦ motif of the pea vacuolar sorting receptor (VSR-PS1) [Happel *et al.,* 2004]. Fuji *et al.,* (2016) identified all of the four subunits of the AP4 complex and demonstrated that AP4 is involved in receptor-mediated protein sorting through recognizing VSR1 (VACUOLER SORTING RECEPTOR1). They developed a vacuolar sorting mutant library named as green fluorescent seeds (GFS) and identified gfs seeds that accumulate very high levels of VSR1. The seeds were named *gfs4*, *gfs5,* and *gfs6* which code for AP4B, AP4M and AP4S subunits of AP4 complex respectively. They also identified another mutant that encodes the fourth subunit i.e. AP4E. All of the mutants had defects in vacuolar protein sorting of 12S globulins which is a major storage protein. These subunits were also shown to build AP4 complex in-vivo which was localized at the TGN (Fuji *et al.,* 2016). By employing a proteomics approach, Pertl-Obermeyer *et al.,* (2016) demonstrated that *ap4β* mutant has defects in the sorting of aquaporins and the proteins of lipid metabolism. Another relatively recent report has shown that the AP4 mutants exhibit defects in development and protein sorting (Müdsam *et al.,* 2018). The mutants depict defective roots and hypocotyls growth and show abnormalities in male fertility and trichome morphology (Müdsam *et al.,* 2018).

MATERIALS AND METHODS

2.1 Plant Materials and Growth Conditions

2.1.1 Plant Materials

Experiments were carried out using *Arabidopsis thaliana* ecotype, Col-0 (Columbia-0) as control. Various mutant lines such as *ps1ps2* double mutant, *atg9*, *pen2*, *gfs4/ap4β, gfs6/ap4σ,* and *ap4e1/ap4ε* were employed in different experiments. In addition, GFP constructs and seeds of stably transformed lines such as AtPEN2-GFP, GFP-ATG8f, RFP-ATG8f, GFP-ATG8f/*ps1ps2*, DsRED-FYVE, MAP4-RFP, AP4-GFP, and YFP-ATG9 were used in different confocal microscopic studies. Seeds and/constructs of *ps1ps2* and AtPEN2-GFP were kindly supplied by Professor Przemysław Wojtaszek of the Adam Mickiewicz University, Poznań. Constructs of GFP-ATG8f and RFP-ATG8f was provided by Professor Viktor Žárský of the Charles University in Prague. Seeds of the GFP-ATG8f were kindly provided by Dr. Tamar Avin-Wittenberg of the Hebrew University of Jerusalem. Plasmid construct of AP4-GFP and the seeds of mutant lines of *gfs4/ap4β, gfs6/ap4σ,* and *ap4e1/ap4e* was gifted by Tomoo Shimada of the Kyoto University. YFP-ATG9 construct and *atg9* seeds were kindly provided by Professor Jiang Liwen of the Chinese University of Hong Kong. *pen2* mutant was ordered from NASC (Nottingham Arabidopsis Stock Center) and genotyped using primers listed in the primer list (Table 1). GFP-ATG8f/*ps1ps2* transgenic lines were produced by Agrobacterium-mediated transformation using floral dipping of *ps1ps2* with the construct of GFP-ATG8f (Clough & Bent, 1998) and DsRED-FYVE construct was prepared by Dr. Boris Voigt (Voigt *et al.,* 2004).

2.1.2 Seed Sterilization

Arabidopsis thaliana seeds were surface-sterilized under a laminar flow cabinet. 1% NaOCl (Sodium Hypochlorite), a bleaching agent was added into microcentrifuge tubes containing the seeds and the bleach treatment was done for up to 5 minutes

by flicking the tube from time to time. Thereafter, the bleach was discarded and 70% ethanol (EtOH) was added. EtOH treatment was carried out for 3 minutes followed by washing with sterilized distilled water (dH_2O). The washing step was repeated 5 times and later the seeds were spread out on a sterilized filter paper to let them dry before planting on the growth media.

2.1.3 Plant Growth Media

Plants were grown on half-strength (½) Murashige and Skoog (MS) media supplied by Duchefa Biochemie. It contained microelements (0.1 µM $CoCl_2.6H_2O$, 0.10 µM $CuSO_4.5H_2O$, 100 µM FeNaEDTA, 100.27 µM H_3BO_3, 5 µM KI, 100 µM $MnSO_4.H2O$, 1.03 µM $Na_2MoO_4.2H_2O$, 29.91 µM $ZnSO_4.7H_2O$), macroelements (2.99 mM $CaCl_2$, 1.25 mM KH_2PO_4, 18.79 mM KNO_3, 1.50 mM $MgSO_4$ and 20.61 mM NH_4NO_3) and vitamins (26.64 µM glycine, 554.94 µM myo-inositol, 4.06 µM nicotinic acid, 2.43 µM pyridoxine HCl and 0.30 µM thiamine HCl). To make 1 liter of ½MS media, 2.1 g of MS salt along with 10 g of sucrose (1%) was added to 1 liter of dH_2O and mixed with a magnetic stirrer. The pH of the media was adjusted between 5.7 to 5.8 using 0.1 M KOH and 5 g plant agar (Duchefa Biochemie) was added to solidify the media followed by autoclaving at 121ºC for 20 minutes. For doing carbon (C) starvation experiments, media was prepared without adding sucrose and for liquid ½MS no agar was added. For various abiotic stresses, different chemicals in appropriate amounts were added to the media before autoclaving.

2.1.4 Planting

Seeds were planted on the ½MS plates which were then incubated in the refrigerator at 4ºC for 48 hours to break seed dormancy. The plates were then placed vertically in the growth chamber which was set for long-day conditions with a 16h light/8h dark cycle at 22°C temperature with a light intensity of 175-250 µmol photons $m^{-2}s^{-1}$. After four days, the seedlings that were supposed to be subjected to stress treatments were transferred to the relevant stress containing media. For carbon starvation experiments, the 5-day-old seedlings were transferred to the solid ½MS media

plates or to 6 well cell culture plates containing liquid ½MS media, wrapped in aluminum foil and placed in a growth chamber to induce carbon (C) starvation.

To grow plants in the soil, commercially available peat moss based soil was used. The soil was treated with insecticides before planting the seeds or transplanting the seedlings into it and transferred to the growth chamber in long-day conditions (16h light/8h dark cycle). The tobacco plants (*Nicotiana benthamiana*) used for Agrobacterium-mediated transformation were germinated in the same soil under long-day conditions for up to two weeks followed by the transfer of individual plants to different pots and grown for another 2 weeks before infiltration with *Agrobacterium tumefaciens*.

2.2 Plasmid Constructs

Arabidopsis PEN2 tagged with GFP driven by 35S promoter (AtPEN2-GFP) and cloned into pSITE expression vector which has been previously described (Smolarkiewicz *et al.,* 2014). Autophagosomal markers ATG8f tagged with RFP and ATG8f tagged with GFP had been previously reported (Honig, *et al.,* 2012). The late endosomal marker i.e. DsRED-FYVE (Voigt *et al.,* 2005) and microtubule marker MBD-MAP4-DsRed (Granger & Cyr, 2001; Marc *et al.,* 1998) have also been used in the studies.

2.3 Bacterial Manipulations

2.3.1 Preparation of *Escherichia Coli* Competent Cells

To make competent cells of *Escherichia Coli* (*E. coli*) the $CaCl_2$ method was employed since calcium increases the probability of foreign DNA uptake by the cells. 3 ml of LB media was used to inoculate *E. Coli* DH5α strain and incubated overnight at 37°C with agitation at 200 rpm (revolutions per minute). The next day, 1 ml of the overnight pre-culture was inoculated in 100 ml of LB medium (primary culture) in a sterile Erlenmeyer flask. This LB broth was incubated at 37°C with gentle shaking until the absorbance reached 0.5 at 600 nm. Afterward, the culture was chilled for 10

minutes on ice. Then, it was divided into 2 pre-chilled 50 ml falcon tubes and subjected to centrifugation at 4500 rpm for 10 minutes at 4°C to spin down the bacterial cells. After removing the supernatant carefully, the cell pellet was gently resuspended in 2.5 ml of 0.1 M $CaCl_2$ solution and kept on ice for 30 minutes. Later, the contents of both falcon tubes were combined and the suspension was centrifuged again at 4500 rpm for 10 minutes at 4°C. The pallet was carefully resuspended in ice-cold 2.5 ml of 0.1 M $CaCl_2$ with 15% glycerol (v/v). The cell suspension was stored as 50 µl aliquots in 1.5 ml microcentrifuge tubes and was flash-frozen in LN_2 (liquid nitrogen) before storage in -80°C fridge.

2.3.2 Transformation of *E. Coli* Competent Cells

E. coli cells were transformed using the heat shock method. The frozen 50 µl aliquot tubes were thawed on ice before 5 µl of plasmid DNA was added into them and gently mixed by flicking the tubes. These tubes were kept on ice for 20 minutes and after that, a heat shock treatment was given in a water bath for 45 seconds at 42°C. After heat shock, the tubes were again incubated on ice for another 2 minutes. Thereafter, 500 µl of LB media without antibiotics was added into the tubes which were then incubated at 37°C on a shaker at 170 rpm for 1 hour. The cell suspension was spread on the Petri plates containing LB media and relevant antibiotics for selection of the plasmid. The Petri plates were sealed and put upside down in 37°C incubator overnight.

2.3.3 Preparation of *Agrobacterium tumefaciens* Competent Cells

A colony of *A. tumefaciens* was picked from a plate containing gentamycin (15 µg/ml) and was inoculated into 3 ml of liquid YEB medium also containing gentamycin and incubated at 28°C with shaking (200 rpm) to prepare an overnight culture. The next day, 2 ml of the overnight culture was inoculated into a 500 ml flask containing 100 ml of the YEB medium. The flask was incubated at 28°C with shaking until the OD (optical density) at 600 nm was reached to 0.5. Thereafter, the culture was divided into two pre-cooled falcon tubes and centrifuged at 4000 rpm for 15

minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 25 ml of cold 10 mM Tris/HCl with 7.5 pH. The contents of both tubes were combined and subjected to another centrifugation just as stated above. After removing the supernatant, the cells were carefully resuspended in ice-cold 25 ml of 10% glycerol (v/v). After another and final centrifugation, the cell pellet was gently resuspended in 600 µl of ice-cold 10% glycerol (v/v). The cells were divided into 50 µl aliquots into 1.5 ml centrifuge tubes, frozen in LN_2 and stored in -80°C for prolonged storage.

2.3.4 Isolation of Plasmid DNA from *E. Coli*

A mini culture was prepared by picking a single colony of transformed *E. coli* from the LB media plate and putting into a 3 ml of liquid LB medium containing a specific antibiotic for selection and incubated at 37°C overnight with agitation at 200 rpm. Plasmid DNA was isolated the following day from 1 ml of bacterial culture using Hi$^{Yield®}$ Plasmid Mini Isolation Kit supplied by SLG (Süd-Laborbedarf Gauting) using manufacturer's instructions.

2.3.5 Transformation of *A. tumefaciens* Competent Cells

A high-voltage electroporation method was used to transform the *Agrobacterium tumefaciens* cells (Mersereau *et al.,*1990). The method is based on the premise that the electric field applied to the cells increases the permeability of the cell membrane and facilitates the introduction of DNA by the cells. To perform this, a 50 µl aliquot of frozen Agrobacteria (GV3101) competent cells were thawed on ice and 3 µl of plasmid DNA was added to it and gently mixed. This mix was then carefully transferred to a pre-chilled 0.2 cm Gene-Pulse Cuvette (BIO-RAD #1652086) avoiding the air bubbles. Meanwhile, the electroporator was set to 2.0 V leaving all other settings at default (200 Ω, capacitance extender 250 µFD, capacitance 25 µFD). The cuvette was placed in proper orientation in the electroporator and electroporation was carried out. Now, 500 µl of YEB medium was added to the cuvette, mixed by pipetting up and down and transferred to a 2 ml of microcentrifuge

tube. The tube was incubated at 28°C in a shaker at 200 rpm for 2 hours. Thereafter, 100 µl of the bacteria were spread on YEB media plates containing rifampicin and other antibiotics specific for selection and incubated in a 28°C growth chamber to get the transformed *A. tumefaciens* cells.

2.4 *In-Planta* Expression Systems

2.4.1 Agrobacterium-Mediated Transient Expression in *N. benthamiana*

Transient expression of the fluorescently tagged proteins was carried out using 4-week old tobacco (*Nicotiana benthamiana)* plants grown under long-day conditions. A single colony was isolated from the plates containing transformed Agrobacteria and put into 3 ml YEB media with rifampicin and other appropriate antibiotics to prepare an overnight mini culture by incubating at 28°C with agitation at 200 rpm. The following day, 1.5 ml of the bacterial culture was put in a microfuge tube and centrifuged at 3500 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 1 ml of infiltration media (200 µM acetosyringone, 20 mM citric acid and 2% sucrose). The OD_{600} was measured and adjusted to 0.1 to 0.3 by making dilutions. The lower epidermal leaf cells of tobacco were infiltrated using the syringe method (Sparkes *et al.,* 2006). The plants were then kept in the same growth chamber for two more days before visualization with confocal laser scanning microscope (CLSM).

2.4.2 PEG-Mediated Transient Transformation of Protoplasts

Arabidopsis mesophyll protoplast isolation and the transient transformation were done according to J. Sheen (2002) with slight modifications. Leaves of 3-4 week old plants grown under long-day conditions were cut into 0.5-1 mm thin strips using razor blades. These strips were transferred to glass Petri plates containing 10 ml enzyme solution (1% cellulase, 0.2% macerozyme, 0.4 M mannitol, 20 mM KCl, 20 mM MES-pH 5.7 and 10 mM $CaCl_2$). The Petri plates were placed in a vacuum

desiccator to apply vacuum for 10-20 minutes followed by its transfer to a growth chamber on a platform shaker at 40 rpm to release protoplasts into the solution. Afterward, the same amount of W5 solution (125 mM $CaCl_2$, 154 mM NaCl, 5 mM KCl and 2 mM MES-pH 5.7) was added and the mixture was filtered with a 75 µm nylon mesh. The protoplasts were then spun down in a round bottom glass tube at 500 rpm for 10 minutes and the supernatant was removed. The pelleted protoplasts were resuspended in W5 solution, washed twice and incubated on ice for 30 minutes. During incubation, the protoplasts were counted under a light microscope using a hemocytometer, centrifuged and resuspended in MMg solution (0.4 M mannitol 15 mM $MgCl_2$ and 4 mM MES-pH 5.7) to have a final concentration of 1-2 x 10^4/ml. 20 µg plasmid DNA was introduced into 1 ml protoplast solution (1-2 x 10^4) and mixed well. 1 ml of freshly prepared 40 % (w/v) Polyethylene Glycol (PEG, Sigma-Aldrich) solution was added and the mixture was incubated for 5 minutes. Thereafter, 6 ml of W5 solution was added and mixed gently followed by centrifugation at 400 rpm for 5 minutes and the supernatant was removed. The transfected protoplasts were now subjected to two more washings with W5 followed by incubation in the 6 well plates. To induce C starvation, protoplasts were incubated in W5 solution without sucrose (0.5%) and incubated at room temperature in darkness for two days before their observation with CLSM.

2.4.3 Agrobacterium-Mediated Stable Transformation of Arabidopsis

Arabidopsis plants (both mutants and Col-0 wild type) grown under long-day conditions were stably transformed through Agrobacteria containing various vectors by floral dip method (Clough & Bent 1998). The first inflorescence shoots were removed to promote the growth of more inflorescence and well-watered plants containing many floral buds were used for transformation. A mini culture was prepared by taking 3 ml of YEB medium containing appropriate antibiotics and inoculating it with transformed Agrobacteria colony. The mini culture was incubated at 28°C overnight with shaking to let the bacteria grow. The following day, this 3 ml of the mini culture was mixed with 300 ml of fresh YEB media with antibiotics in an

Erlenmeyer flask and incubated for one day at 28°C with shaking until the OD_{600} was reached to 0.8. Thereafter, 1.5 ml of 10% Tween-20 and 300 µl of acetosyringone (1 mg/25 µl in chloroform) were added and mixed in the bacterial culture before dipping the plants. The Arabidopsis plants were now dipped gently into the bacterial culture for up to 10 seconds followed by covering them with plastic bags to maintain high humidity. The plants were then transferred to the growth chamber and put away from the direct light in order to recover them from inoculation. After one day, the plastic covers were removed and the plants were kept under long-day conditions until their harvest.

2.5 Plant Genotyping

2.5.1 Genomic DNA Isolation

To extract genomic DNA, a leaf was cut from a 4-week old plant and ground in a 1.5 ml microcentrifuge tube containing 400 µl of DNA extraction buffer (200 mM NaCl, 200 mM Tris-HCl pH 7.5 and 25 mM EDTA). After grinding, it was centrifuged at 13000 rpm for 10 minutes. Meanwhile, 715 µl of 70 % ETOH was added to another tube. The supernatant from the centrifuged sample was subsequently added to the EtOH containing a tube followed by its vigorous vortexing. Thereafter, the tube containing the sample was subjected to centrifugation at 13000 rpm for another 10 minutes followed by removal of the supernatant. Afterward, the sample was subjected to the last centrifugation at 2000 rpm for 10 minutes and any supernatant left was completely discarded. The tube containing pallet was incubated at 50°C to let the pallet dry. After drying, the pellet was resuspended in 50 µl of 10 mM Tris-HCl pH 7.5.

2.5.2 PCR Reactions

PCR (Polymerase Chain Reaction) was conducted for genotyping the mutant lines in order to determine the presence of T-DNA (Transfer DNA) insertion. A master mix was prepared such that each reaction (25 ml) contained 5 ml of Promega 5X

GoTaq® green reaction buffer, 0.5 µl of 10 mM forward primer (FP), 0.5 µl of 10 mM reverse primer (RP), 0.5 µl of 2.5 mM dNTPs, 0.1 µl of Promega GoTaq® G2 DNA Polymerase, 16.4 ml dH_2O and 2 µl of DNA. The 0.2 ml PCR tubes containing these reactions were put inside BIO-RAD MyCycler™ Thermal Cycler System and following PCR conditions were set. Denaturation was done at 95°C for 5 minutes on the initial cycle followed by 30 seconds on the rest, annealing was done for 30 seconds at a temperature set to 5 degrees less than the Tm (melting temperature) of the primers and extension was carried out at 72°C for ~1 min/kb with 5 minutes at the last cycle. The total number of cycles was set to 30.

2.5.3 Gel Electrophoresis

After running the PCR, the reactions were run on 1% (w/v) agarose gel prepared by dissolving in 1X TAE buffer (20 mM acetate, 40 mM Tris and 1 mM EDTA with pH 8.6) through boiling in a microwave oven. After boiling, once the temperature of the solution was dropped to ~50°C, the DNA stain (Roti®-GelStain, Carl Roth GmbH, Karlsruhe) was added to it. The solution was loaded onto the electrophoreses chamber and after it cooled down, the samples were directly loaded into the wells. 100 bp (base pair) or 1 kb (kilobase) GeneRuler DNA ladders (Thermo Fischer Scientific) were also loaded in one of the wells to determine the size of the DNA fragments. The gels were run for 40 minutes at 100 V (volts) and later observed under transilluminator.

2.6 Quantitative real-time PCR

2.6.1 RNA Isolation

Total RNA was isolated from the seven-days old carbon starved seedlings of Arabidopsis plants (Col-0 and *ps1ps2* double mutant) to perform validation of the protein sequencing results (Figure 13) and to determine the relative mRNA expression of various autophagy-related genes in *ps1ps2* through qPCR (Figure 14). A total of 50 mg plant sample was taken in the microcentrifuge tubes and

immediately placed on LN_2. RNA was extracted using Qiagen, RNeasy Mini Kit (Hilden, Germany) following the instructions laid out by the manufacturer. After RNA extraction, its quality and quantity were evaluated by using NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific). The integrity of RNA was also checked by running it on 1% agarose gel.

2.6.2 cDNA Synthesis

cDNA (complementary DNA) was made from the total RNA using Invitrogen™, First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's instructions with slight modifications. A reaction of 20 µl was prepared on ice in a nuclease-free 1.5 ml microcentrifuge tube and the following components were added: 1 µl (1 ng-5 µg) template RNA, 1 µl Oligo (dT) primer and 9.5 µl dH_2O. To get rid of any secondary structures, the tube was gently mixed, briefly centrifuged and incubated at 65°C for 5 minutes. After that, it was chilled on ice, briefly centrifuged and again placed on ice. Next, 4 µl of the 5x reaction buffer, 0.5 µl RNAse inhibitor, 2 µl of 10 mM dNTPs and 1 µl reverse transcriptase were added. The tube was gently mixed, briefly centrifuged and placed at 42°C for 60 minutes. The reaction was terminated by heating at 70 for 10 minutes and prepared cDNA samples were stored in -20°C refrigerator.

2.6.3 Real-time PCR Analysis

Quantitative real-time PCR (qPCR) was performed in a 48 well plate using the StepOne Real-Time PCR system (Applied Biosystems). SYBR™ green PCR master mix (Applied Biosystems, Lot. 1805518) was used as a fluorescent dye to monitor double-stranded DNA (dsDNA) synthesis. The reactions of 10 µl volume (5 µl SYBR™ Green master mix reagent, 200 nM of each gene-specific primer, 1.0 ng cDNA and sterile dH_2O) were prepared on ice. The experiment was set up on the machine with the following PCR reaction conditions, 2 minutes at 50°C on the first cycle, followed by 10 minutes at 95°C then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (annealing). The resulting PCR data was analyzed using the

StepOne software v2.1 (Applied Biosystems). All of the amplification plots were analyzed using a threshold of 2.0 to get the Ct (threshold cycle) values. These values were then normalized to the Ct values of Arabidopsis Actin2 (AT3G18780) which was used as the reference gene.

Table 1: Primers List

Genomic DNA Amplification Primers	
PEN2-F	5'-TGCCGTACAGATTTTGATTCC
PEN2-R	5'-AAGTCCATTGGCCTATTCAGG
LBb1.3	5'-ATTTTGCCGATTTCGGAAC
Primers for Gene Expression Analysis	
PEN2-F	5'-CGACCCCAGTCTGAACCCTA
PEN2-R	5'-CAGACAAGCCGAGACGATCA
PS1-F	5'-GAACTCCCAAAAATGGCGTA
PS1-R	5'-GCTTCACTTGAACATCACAAGG
PS2-F	5'-GAAATCAAAGACCCAGAAGCA
PS2-R	5'-TCAAAGTCTAGGACGAGCACA
qPCR Primers for Validation of the Genes Identified in Sequencing	
AT5G01300.1-F	5'-CGGTCACCGTTTTCAGTTCAAG
AT5G01300.1-R	5'-TGCCCTTCAATTGCGATCAG
AT1G56410-F	5'-TGATGCATCTGTCCAAAGCG
AT1G56410-R	5'-TCCGCAGCGAATTGTTTCTC
AT1G58380.1-F	5'-TGGGTTCCTTACACCAGAGTTC
AT1G58380.1-R	5'-ACCTTGGTTGCAGAAACAGC
AT3G06040.1-F	5'-ATTTCATCGGACGGCATTGG
AT3G06040.1-R	5'-TCACATCGAAAGCCGTCTTC
AT3G12650-F	5'-TGTGGAACGCGATTTGTCAG
AT3G12650-R	5'-AAATCGCCGCTCCAAGAATC
AT1G55490-F	5'-AGCAAGCGGAGCAAGATTAC
AT1G55490-R	5'-TGCTCCAACCTGAATCACAG
AT1G13060.2-F	5'-ATGAGCTGGCAAACAAGAGG
AT1G13060.2-R	5'-CCAACAGAAAGTCCCATTCC
AT1G52600.1-F	5'-AGGACAGCTTTGGCTTCATC
AT1G52600.1-R	5'-CCCATCCAACATAAGGCAAG
Actin2-F	TGCCAATCTACGAGGGTTTC
Actin2-R	CTTACAATTTCCCGCTCTGC

qPCR Primers for Autophagy Related Genes	
AtTOR-F	5'-TGAAGTCCCCCAATTAGCAC
AtTOR-R	5'-TTCGTCAGGCTCAACATCAG
ATG9-F	5'-AAGGTGTAGGCCATGTTTGC
ATG9-R	5'-CGACGAGTTGCATTATGTGG
ATG8a-F	GAACACATTGCCTCCAACTG
ATG8a-R	5'-GGAACCCATCCTCATCTTTG
ATG18a-F	5'-CAAGATCATGCTTGCTTCGC
ATG18a-R	5'-AATCACGCCGGAAAATCTCG

Table 1: A list of the primers utilized in the various analysis.

2.7 Protein Work

2.7.1 Protein Extraction

Seven-day-old etiolated seedlings were collected, weighed and put in liquid nitrogen. For total protein extraction, 50 mg of the plant material was ground in liquid nitrogen and ice-cold protein extraction buffer (50 mM Tris-pH 7.5, 20 mM NaCl, 10% [v/v] glycerol, 0.1% SDS and phosphatase protease inhibitor cocktail [Roche]) was added into it. The samples were mixed well and centrifuged at 16000g for 15 minutes at 4°C. The supernatant (total protein content) was recovered in fresh tubes. To obtain the soluble and insoluble protein fractions, the protein extraction buffer without and with 0.1% SDS was added to the samples respectively.

2.7.2 SDS PAGE

The SDS PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was carried out using 0.75 mm casts in a Hoefer™ Mighty Small™ gel system (Thermofisher Scientific). The separation gel was prepared using 12.5% acrylamide/bis solution 37:5:1 (Serva), 3 M Tris-pH 8.8, 10% SDS, 10% APS (ammonium persulphate), 0.04% TEMED (tetraethylenediamine) and dH_2O. The stacking gel was prepared using 6% acrylamide/bis solution 37:5:1, 1 M Tris-pH 6.8,

10% SDS, 10% APS, 0.1% TEMED and dH_2O. The protein samples were subjected to 4X protein loading dye (300 mM Tris-HCl, 500 mM DTT, 60% Glycerol, 10% SDS, and 0.01% bromphenol blue) and denatured at 96°C for 3 minutes. The denatured protein samples were then loaded on the gel along with an unstained protein molecular weight marker (prestained protein ladder for western blotting [PagerRuler, Thermofisher Scientific]) and ran at an appropriate voltage at room temperature until the blue dye reached the lower end of the gel. After the run was complete, the protein gel was stained with Coomassie blue staining. The gels were soaked in Coomassie solution (1.1% Coomassie Brilliant Blue R250, 20% isopropanol, 20% acetic acid and 60% dH_2O) for 30 minutes with shaking. Thereafter, the gel was destained for another 30 minutes with the destaining solution (10% isopropanol, 10% acetic acid and 80% dH_2O) until the blue background was gone. The protein identification in the bands of interest was done by Dr. Youjun Zhang in the lab of Prof. Alisdair Fernie based on a previously described method (Avin-Wittenberg *et al.,* 2005).

2.7.3 Western Blotting

In western blotting, the proteins ran on the separation gel can be subjected to detection by antibodies. The proteins were transferred to a PVDF membrane through electroblotting at room temperature for 1 hour at an appropriate voltage. Thereafter, the membrane was blocked for 30 minutes in 5% skimmed milk (Serva), Tris buffer saline (TBS) and Tween-20 (TBST: 50 mM Tris-HCl pH 7.6, 100 mM NaCl and 0.1% Tween-20). After blocking, the membrane was incubated overnight at 4°C with shaking in the anti-GFP antibody (Roche) diluted 1:2000 in TBST buffer. The following day, the membrane was subjected to 3 washings (each for 10 minutes) in TBST buffer. A 1/4000 dilution of HRP (Horseradish Peroxidase) linked anti-mouse secondary antibody (Cell Signaling Technology, #7076) made in TBST milk was prepared and the membrane was incubated in it for 1 hour at room temperature with shaking. Thereafter, the membrane was washed 3 times in TBST buffer and each time for 10 minutes. The antibody attachment places were observed using electrochemiluminescence (ECL) by soaking the membrane for 5 minutes into the

western blotting substrate solution (Serva) prepared by mixing reagents A and B in 1:1 ratio. Imaging was performed using the BIO-RAD ChemiDocTM MP imaging system.

2.8 Confocal Microscopy

Imaging was performed using the Olympus FluoView FV1000 confocal laser scanning microscope. The instrument is fitted with a diode (405 nm), argon-ion (458, 488 and 514 nm) and helium-neon (543 nm) lasers which were used to excite different fluorophores. GFP was excited at 488 nm wavelength and emission was collected between 500 to 600 nm. MDC (monodansylcadaverine) dye was excited at the wavelength of 533 nm and emitted at 508 nm while RFP and red fluorescent dye FM4-64 were excited at 543 nm and their emission was filtered between 660 and 760 nm. Roots of Arabidopsis seedlings were observed by putting on a glass slide between two glass coverslips in aqueous media and using 40X oil immersion objectives. In order to observe transient expression in *N. benthamiana*, a small piece of a leaf (1 cm^2) was cut, mounted on the cover slide with abaxial side facing up and was observed using 40X oil immersion objective. For simultaneous imaging of RFP, GFP, and FM4-64, the images were acquired by using sequential imaging mode to prevent fluorescence bleed (Hutter H. 2004). Image processing was done using Fiji software (Schindelin *et al.,* 2012) to adjust the contrast, intensity, and projection of the serial confocal sections of the images.

2.8.1 Fluorescent Dyes and Inhibitor Treatments

FM4-64 dye treatment was carried out using seven-day-old seedlings grown on ½MS media plates. The seedlings were incubated in pre-cooled FM4-64 dye solution (5 ug/ml) on ice for 5 minutes to slow down endocytosis followed by 3 washings with dH2O to remove the excess of dye. The seedlings treated with dye were put on the glass slides containing aqueous ½MS medium and were hanged vertically in glass slide staining jars for different time intervals (15, 30, 45 and 60 minutes) before observation under the confocal microscope. For BFA (Brefeldin A) treatment, the

FM4-64 stained samples were put on the slides containing ½MS solution with BFA (50 µM) and hanged in the same jars for 30 minutes before examination under the microscope. To study autophagy with MDC dye, seven-day-old carbon starved Arabidopsis seedlings were incubated in 50 µM dye solution at ambient temperature for 10 minutes followed by washing with PBS buffer (phosphate buffer saline) before subsequent observation with confocal microscopy. Latrunculin-B (Lat-B) and gamma-secretase complex inhibitor (BSM-299897) treatments were done by incubating the seven-day-old seedlings in 10 µM of Lat-B and 50 µM of BSM-299897 solutions respectively for 30 minutes.

2.8.2 Treatment with Autophagy Inhibitors

Autophagy inhibitors such as ConA (Concanamycin A) and wortmannin (Wort) were used for studying autophagy as specified in a protocol described by Jung *et al.,* (2017). Six-day-old seedlings grown on ½MS media plates were transferred to the 6 well plates containing liquid ½MS media with 1 µM ConA or 10 µM wortmannin. As a control, plates were put in the growth chamber on a shaker with gentle shaking (40 rpm) for 16 hours before observation with the confocal microscope. The seedlings subjected to C starvation treatment were transferred to 6 well plates containing ½MS medium without sucrose. These plates were enwrapped with aluminum foil and transferred to the growth chamber for 16 hours with shaking.

2.9 Hypocotyl/Root Length Measurements and Statistical Analysis

Arabidopsis seedlings were grown in the plates as described previously for seven days and were scanned using Adobe Photoshop (Adobe Systems Inc.). Hypocotyl and root length measurements were done using the Fiji software. The experiment was replicated thrice and the statistical analysis was carried out with Student's *t*-tests on the raw data using Prism GraphPad version 5.00 for the Windows (La Jolla California, USA). Results were found to be statistically different at a probability level of $P < 0.05$.

2.10 Accession Numbers

The locus numbers of major Arabidopsis genes discussed in this work include PS1 (At1G08700), PS2 (AT2G29900), PEN2 (AT5G09310), ATG8f (AT4G16520), ATG8a (AT4G21980), ATG9 (AT2G31260), ATG18a (AT3G62770), AP4β (Atg11490.1), AP4σ (At2g19790.1) and AP4ε (At1g31730.1).

RESULTS

3.1 Carbon Starved Etiolated Seedlings of *ps1ps2* have Shorter Hypocotyls and Roots

Arabidopsis etiolated seedlings were used as a model system to study the effect of carbon starvation since the only C source available to the plants is coming from the seeds which makes this system heterotrophic (Avin-Wittenberg *et al.,* 2015). Besides, there is no additional photosynthesis happening. The analysis was started by studying the phenotype of presenilins double mutant grown under carbon starvation and comparing it with wild-type Col-0. We also used ATG9 as an additional control since it is an essential component of autophagic machinery and has been reported to be involved in autophagosomes formation in Arabidopsis (Zhuang *et al.,* 2017). In the past, it has been shown that *atg* mutants display a delayed growth phenotype (Avin-Wittenberg *et al.,* 2015). Seeds were grown on ½MS medium without C source and grown in the dark for seven days. Presenilins mutant *ps1ps2* and *atg9* mutant displayed shorter hypocotyls and roots compared to the wild-type plants (Figure 9) and this difference was also statistically significant. The phenotype was recovered when the media was supplemented with an external C source. The size of the hypocotyls and roots of the mutants was similar to that of wild-type plants when grown on 1% sucrose (Figure 9A, E). These results show that the presenilins mutant displays a delayed growth phenotype when grown under C starvation just like other autophagy-related mutants and gave important evidence that autophagy is disturbed in this mutant line.

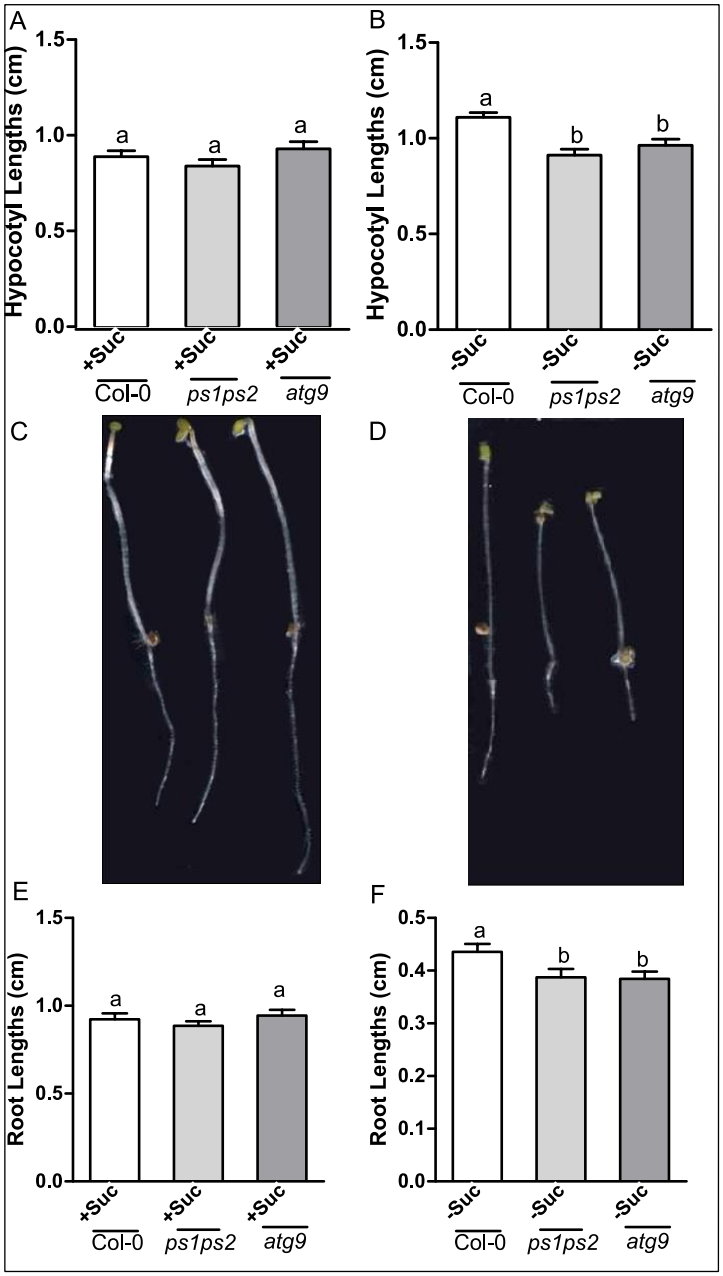

Figure 9: Etiolated seedlings of *ps1ps2* and *atg9* mutants grown under C starved condition showed significantly shorter hypocotyls and roots compared to the wild type seedlings. (A, E) Measurement of the hypocotyls and roots of *ps1ps2*, *atg9* and WT control grown under 1% sucrose using ImageJ. **(C)** Representative image of the etiolated seedling of each line grown under 1% sucrose. **(B, F)** Measurement of the hypocotyls and roots of *ps1ps2*, *atg9* and WT control grown without sucrose using ImageJ. **(D)** Representative image of the etiolated seedling of each line grown without sucrose. Data represent the mean (S.E) of three independent replicates and different letters above the error bars show significant differences compared to WT ($P < 0.05$) based on the student *t*-test.

3.2 Carbon Starvation Induces Autophagy in *ps1ps2* Mutant

Although the role of gamma-secretase complex particularly the presenilin subunit in terms of autophagy has been well studied in animals very little is known in plants. However, recently Smolarkiewicz *et al.,* (2014) showed *ps1ps2* displays enhanced chlorosis in the darkness which is an autophagy-related symptom. To further this observation and investigate the role of presenilins in autophagy. Five-day-old wild type (WT) Col-0 and *ps1ps2* seedlings grown on ½MS were subjected to sucrose/C starvation media for two days under dark to induce autophagy. For control, the seedlings were kept growing on ½MS media under standard growth conditions. Autophagy was observed in the root cells of the seedlings by staining with MDC dye. MDC dye stains acidic compartments and autophagosomes which as well are acidic bodies and it has been tested for use in mammals (Munafo & Colombo, 2001). *ps1ps2* showed a high number of autophagosomes under sucrose/C starvation compared to wild type (Col-0) plants (Figure 10D). Remarkably, *the ps1ps2* seedlings showed a higher number of autophagosomes even under the normal growth conditions (Figure 10B). This gave a first hint about the defectiveness of autophagy in the root cells depleted with presenilins.

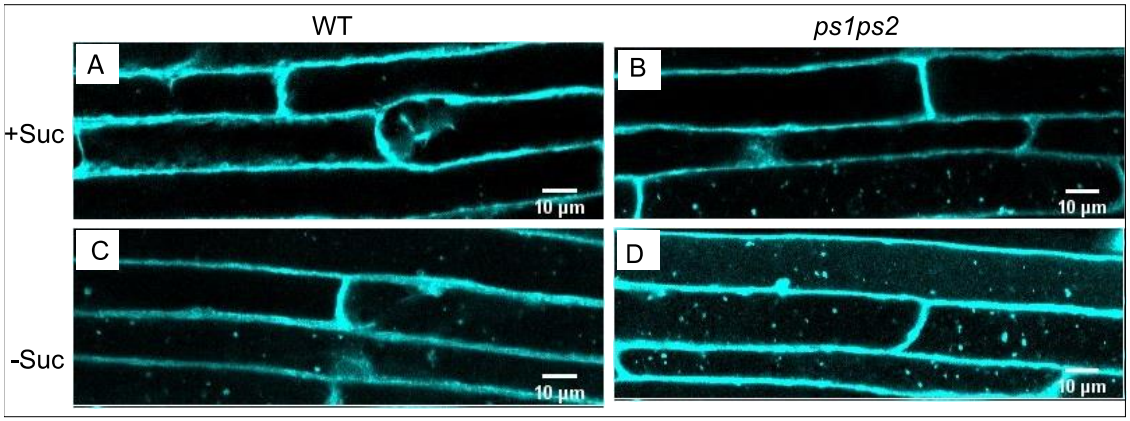

Figure 10: Autophagy induction in *ps1ps2* double mutant under sucrose starvation. (A, B) Root cell images of seven-day-old ½MS grown seedlings of WT and *ps1ps2* under standard growth conditions. **(C, D)** WT and *ps1ps2* grown without sucrose for two days (C starvation), *ps1ps2* showing a visibly higher number of autophagosomes in the root cells.

3.3 Presenilins Mutant has Higher Number of Autophagosomes

In order to further confirm that the *ps1ps2* mutant has a high level of basal autophagy under normal growth conditions, we followed another approach by isolating protoplasts from the leaves of WT and *ps1ps2* mutant plants. These protoplasts were transformed with autophagosomal marker i.e. GFP-ATG8f which is an extensively used marker to follow autophagy. The presence of more than three visible autophagosomes in a protoplast determines active autophagy (Yang *et al.,* 2016). Previously, autophagy in *ps1ps2* was observed by using MDC dye (Figure 11) which is although a reasonable tool to study autophagy but has some drawbacks associated with it such as its lack of specificity. Following the transient transformation of the leaf protoplasts, we observed a visibly higher number of GFP-ATG8f tagged autophagosomes in *ps1ps2* protoplasts compared to WT even in the transient system as is evident from Figure 11 and it was consistent with our previous observation using MDC dye (Figure 11). Although, this experiment was useful to get insights about autophagy in *ps1ps2* mutant, nevertheless, further studies were necessary to be performed using the stably transformed lines.

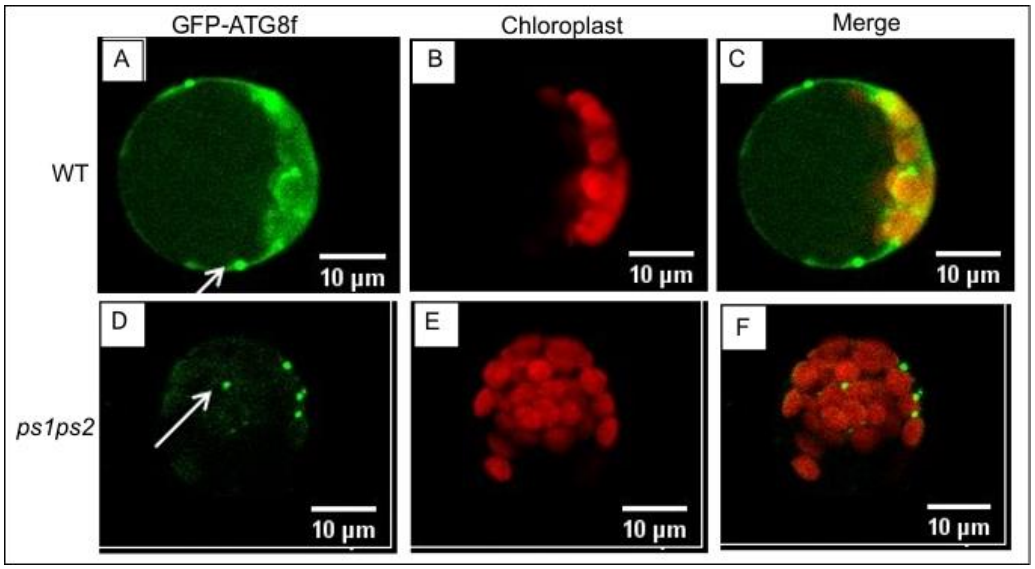

Figure 11: Leaf protoplasts of *ps1ps2* mutant have an abnormal accumulation of autophagosomes. Transient transformation of GFP-ATG8f in the leaf protoplasts of WT and *ps1ps2* mutant observed by confocal microscopy. **(A, D)** GFP tagged autophagosomes appear as green puncta and are indicated by the arrows. **(B, F)** show chloroplast and **(C, F)** merge respectively.

3.4 Presenilins Malfunction Results in Abnormal Accumulation of Autophagosomes in the Root Cells

Autophagosome formation is a conserved process and ATG8 is a widely used and reliable autophagosomal marker to study autophagy in Arabidopsis. In plants, GFP-ATG8 fusion makes it easier to visualize autophagosomes which appear as puncta. To confirm the previous results of (Figures 10 & 11) and further study a possible functional role of presenilins in autophagy, GFP-ATG8f was transformed into the *ps1ps2* mutant line through stable transformation. Root cells of seven-day-old ½MS grown seedlings of GFP-ATG8f/*ps1ps2* and GFP-ATG8f as control were observed under CLSM. Surprisingly, in the *ps1ps2* mutant background, there was a huge buildup of autophagosomes as reflected by an increase in the GFP-ATG8f labeled puncta (Figure 12). The GFP-ATG8f used as control had clear cells with very few autophagosomes. This defective accumulation of autophagosomes in *ps1ps2* suggested an important role of presenilins in autophagosome clearance and is consistent with previous observations (Figures 10 &12).

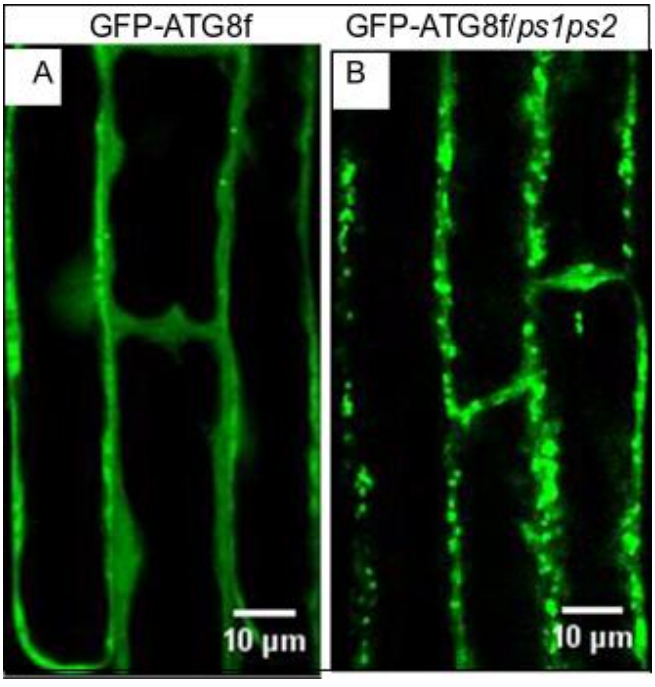

Figure 12: Subcellular expression of GFP-ATG8f in WT and *ps1ps2*. Images are from the root cells of seven-day-old seedlings grown under standard growth conditions in ½MS media plates as observed by CLSM.

3.5 Differential Protein Accumulation occurs in *ps1ps2*

Proteins are the degradation target of autophagic machinery (Li & Vierstra, 2012), therefore, we decided to investigate the protein content of the carbon starved *ps1ps2* mutant compared with WT plants. For this purpose, the total protein content was isolated and run on SDS gel. As expected *ps1ps2* showed higher accumulation of proteins compared to the wild-type plants (Figure 13A). This observation showed that due to the defectiveness of the autophagy pathway, the proteins were not able to recycle leading to their abnormal accumulation in the cytosol. When the same quantities of protein were separated on SDS-PAGE, some of the bands in *ps1ps2* showed higher intensity compared to wild-type plants. Especially, a band of around ~22 kDa size (marked by arrow) in *ps1ps2* was very prominent compared to the same band in wild-type which suggested a differential accumulation and degradation of some proteins in this mutant under C starvation. To get further insights that whether the protein accumulation at ~22 kDa is either present in the soluble or non-soluble fractions of the proteins, non-soluble protein fraction was extracted using extraction buffer with SDS and soluble proteins without SDS and separated on 12% SDS gel (Figure 13B). White arrow indicates that the prominent ~22 kDa band is present in *ps1ps2* double mutant in the soluble protein fraction.

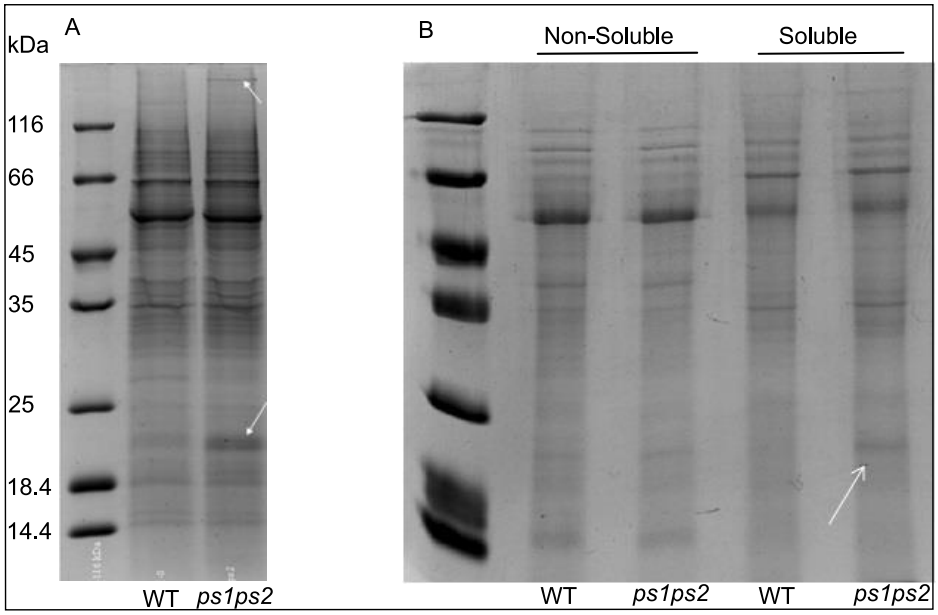

Figure 13: Separation of total protein content, as well as the soluble and non-soluble protein fraction from the seven-days-old C, starved etiolated WT and *ps1ps2* seedlings on the SDS gel. (A) Total protein content from the WT and *ps1ps2* mutant grown under C starvation media and complete darkness was isolated and run on 12% SDS gel. Arrows indicate protein bands with visibly higher protein content in *ps1ps2* compared with WT. **(B)** Separation of soluble and non-soluble protein fraction on 12% SDS gel from the seven-day-old C starved etiolated seedlings. The ~22 kDa band is present in the soluble fraction of the proteins and is indicated by an arrow.

3.6 Identification of the Selected Protein Band by Liquid Chromatography Mass Spectrometry (LCMS)

The ~22 kDa band from *ps1ps2* showing a difference in accumulation in Figure 13A along with WT band of the same size were excised followed by in-gel digestion with trypsin. The resulting peptides were identified using liquid chromatography coupled with tandem mass spectrometry (Table 2). We identified a total of 8 proteins from sequencing that accumulated in higher amounts and could be of interest for further studies (Table 2). Our proteomic analysis revealed the accumulation of chaperonin 61 beta (CPN60β) which has many known functions. It is a chloroplast localized chaperone implicated in protein folding of newly synthesized and stress denatured proteins (Peng *et al.,* 2011). Additionally, it has been found involved in proper plastid division and a mutation in CPN60β abolishes the greening of plastids and results in

reduced chlorophyll levels (Suzuki *et al.,* 2009). We were able to identify the accumulation of two endopeptidases PBE1, a 21S proteasome beta E1 protein and peptidase S24 family protein, both of these are enzymes involved in endopeptidase activity. PBE1 has been found involved in proteolysis of catabolic proteins in cells and it is responsive to cadmium (Cd) stress in Arabidopsis (Polge *et al.,* 2009). The accumulation of these proteins in *ps1ps2* mutant indicates a defectiveness in protein degradation which is a known function of autophagy. Our proteomic analysis also revealed the accumulation of PEBP which belongs to the phosphatidylethanolamine-binding protein family. We also identified another interesting protein i.e. HSP70T-1 (heat shock protein 70T-1) which is a cytosol localized chaperone. The expression of HSP70T-1 is induced by temperature extremes and rises after seed imbibition (Sung *et al.,* 2001). Two ribosomal proteins XW6 and L12 were also accumulated which implies possible defects in the ribophagy pathway.

Table 2: Identification of 22 kDa Protein Band in *ps1ps2* from Figure 13A

AGI	PEPTIDES	DESCRIPTION	MW
AT1G55490	2	CPN61B, LEN1 | chaperonin 61 beta	63.8 kDa
AT1G13060	2	PBE1 | 21S proteasome beta subunit E1	32.3 kDa
AT5G01300	2	PEBP (phosphatidylethanolamine-binding protein) family protein	17.8 kDa
AT1G56410	1	ERD2, HSP71T-1 | heat shock protein 71 (Hsp 71) family protein	68.3 kDa
AT1G58380	1	XW6 | Ribosomal protein S5 family protein	30.7 kDa
AT3G06040	1	Ribosomal protein L12/ ATP-dependent Clp protease adaptor protein ClpS family protein	20.7 kDa
AT3G12650	1	Unknown protein; Functions Unknown	26.8 kDa
AT1G52600	1	Peptidase S24/S26A/S26B/S26C family protein	20 kDa

Table 2: Mass spectra of the peptides obtained from 22 kDa band from figure 13A through in-gel digestion were matched using Mascot software and Arabidopsis protein database. The description represents the annotation of the identified proteins. AGI and MW indicate genome accession number molecular weight of proteins respectively.

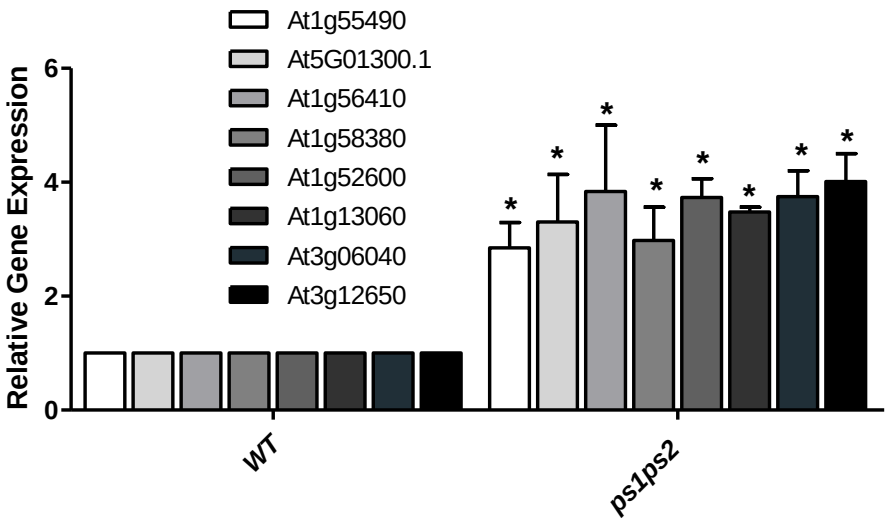

Figure 14: Relative gene expression of 8 genes observed in the ~22 kDa band from the seven-day-old C starved etiolated seedlings of WT and *ps1ps2* mutant. Data is representing relative gene expression of the said genes compared to WT whose value is set to 1. Asterisks indicate $P < 0.05$ compared to WT based on the student *t*-test.

Since there are a total of 8 proteins detected in the LCMS analysis, we wanted to further validate the protein sequencing findings at the RNA level by doing a qPCR. For this, gene-specific primes of all those 8 genes were ordered. The Col-0 and *ps1ps2* mutant seeds were grown on C starvation media for seven days. RNA of the seedlings was extracted and cDNA was synthesized. A qPCR was run using the gene-specific primers. Hereby, we show that all of the genes (At1g56410, At5g58380.1, At1g52600, At5g01300.1, At1g13060, At3g06040, At1g55490 and At3g12650) were upregulated. The results of this experiment are in accordance with the LCMS data and therefore validate that.

3.7 The Internalization of FM4-64 is Uninterrupted in *ps1ps2*

It has been shown that the subunits of the gamma-secretase complex interact with the proteins involved in trafficking. The presenilin component has particularly been implicated in trafficking, for instance, it interacts with a small GTPase Rab11 (Dumanchin *et al.,* 1999). Furthermore, it has also been reported that PS interacts with two SNARES i.e. syntaxin 5 and syntaxin 1A which are implicated in synaptic

vesicle fusion and Golgi trafficking (Jing & Prekeris, 2009). In moss *Physcomitrella patens*, the presenilins mutant *PpΔpsn* had defective internalization of FM4-64 dye and defects in cytoskeletal networks. FM4-64 is a lipophilic dye used to monitor endocytosis and vesicle trafficking in eukaryotic cells. We used this dye to monitor endocytosis in the root cells of WT and *ps1ps2* mutant seedlings to observe any possible defects in vesicle trafficking (Figure 15). Just after staining, the plasma membrane was stained bright red in both WT as well as in the *ps1ps2* mutant. After 30 minutes, there were plenty of punctated vesicles seen in the WT as well as in the mutant root cells. However, neither the number of these vesicles nor the fluorescence was different in the mutant line compared with WT. This result indicates that endocytosis remains unaffected as a result of presenilins deletion in Arabidopsis.

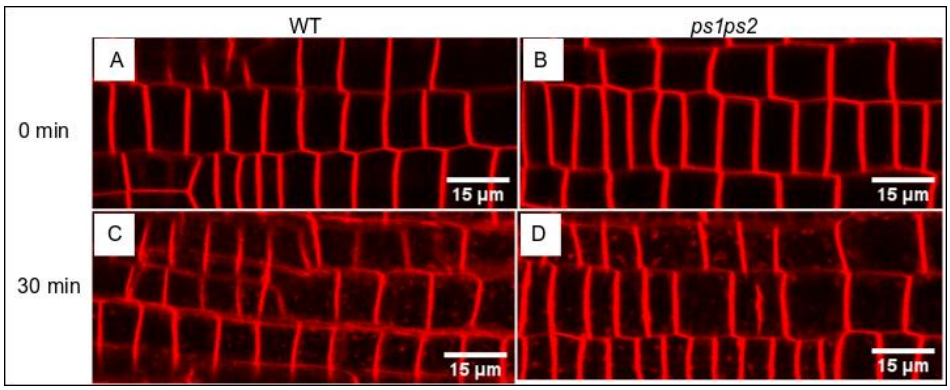

Figure 15: *ps1ps2* deficiency does not affect endocytosis in Arabidopsis root cells. (A-D) Roots of seven-day-old Arabidopsis WT and *ps1ps2* seedlings were treated with 5 µg/ml FM4-64 and the stained seedlings were observed after 30 minutes under the confocal microscope. The internalization of the dye was uninterrupted in the *ps1ps2* mutant line.

3.8 Autophagy Marker Proteins are Upregulated in *ps1ps2*

Several genes regulate autophagy in Arabidopsis and most of them are upregulated under conditions that induce autophagy transcriptionally. Since autophagy was disrupted and the autophagosome number was high in *ps1ps2*, it was tempting to check the gene expression of some well-known autophagy marker genes like *ATG9*, *ATG18a,* and *ATG8a* in *ps1ps2*. Since all these genes perform their functions in the important steps of autophagy pathways, we tested the mRNA level of these genes in

the C starved etiolated seedlings of WT and *ps1ps2* through real-time qPCR (Figure 16). ATG9 is involved in the early stages/initiation step of autophagy and is thought to deliver membrane source for the autophagosome formation in yeast (Yamamoto *et al.,* 2012). Its deficiency completely blocks autophagy in yeast, however, in mammals, it is less severely affected. ATG9 is not well characterized in plants, but a recent advanced imaging analysis has provided insights that it is involved in forming the early autophagic structures and upon autophagy induction, it interacts with the autophagic membrane (Zhuang *et al.,* 2017). The gene expression of ATG9 was found to be upregulated in the *ps1ps2* mutant line which indicated that the levels of ATG9 are increased under C starvation conditions and that there is increased formation of autophagosomes. ATG18 is another important autophagy-related molecule that is conserved from yeast to higher organisms including humans (Mizushima *et al.,* 2011). It plays an important role in starvation-induced autophagy and is needed at the early steps of autophagosome formation. Studies from yeast show that ATG18 makes a complex with ATG2 and localizes at the PAS in a PI3P dependent manner for autophagosome biogenesis (Obara *et al.,* 2008). In Arabidopsis, there is a family of ATG18 genes which comprises of 8 members. One member i.e. ATG18a is particularly sensitive to nutrient deprivation conditions and is reported to play a role in autophagosome formation. Due to these important functions, we tested ATG18a in the qPCR analysis and its expression was also upregulated under C starvation conditions in the *ps1ps2* mutant line. This also indicates the increased formation of autophagosomes in the absence of presenilins (Xiong *et al.,* 2015).

Next, we decided to check the expression of ATG genes at vesicle elongation and completion steps. ATG8 is involved at these steps which as previously described is an important autophagy marker. The soluble form of ATG8 is converted to autophagic vesicle-associated form i.e. ATG8-PE through its conjugation with PE. This process is said to be required for the phagophore elongation. We observed an increase in the transcript level of ATG8 as well which suggests that a lack of presenilins function leads to an increase in the number of autophagosomes.

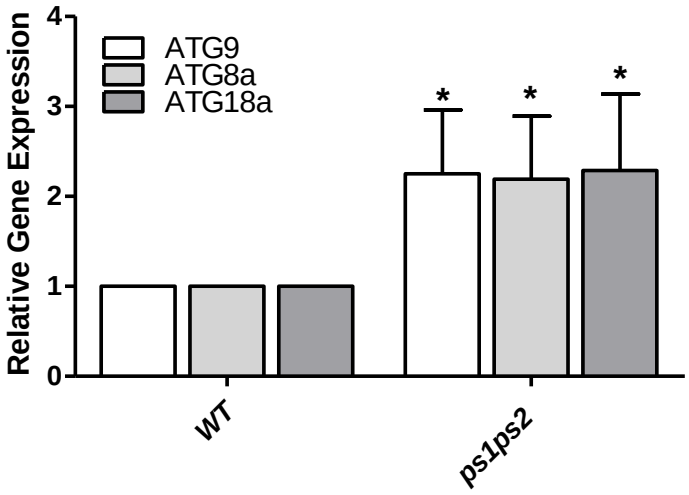

Figure 16: Relative gene expression of ATG8, ATG9 and ATG18 genes in seven-day-old C starved etiolated seedlings of WT and *ps1ps2*. Data is representing relative gene expression of the said genes compared to WT whose value is set to 1. Asterisks indicate $P < 0.05$ compared to WT based on the student *t*-test.

3.9 *ps1ps2* has Reduced Autophagy Flux under Sucrose Starvation

In order to be sure that the *ps1ps2* mutant has defective autophagy, we also carried out a GFP-ATG8f processing assay using an anti-GFP antibody to measure the autophagic flux (Figure 17). Autophagic flux gives a semi-quantitative measure of the rate of autophagosome degradation. It is based on the principle that GFP is cleaved from the GFP-ATG8f containing vesicles after they reach vacuole. Free GFP is stable in the vacuole and more the amount of free GFP the higher is the autophagic flux and vice versa. Typically, autophagy defective mutants have a reduced amount of free GFP and this assay can be used effectively in plant cells. Arabidopsis seeds expressing GFP-ATG8f and GFP-ATG8f/*ps1ps2* were grown on ½MS plates containing either 1% sucrose or no sucrose. The seedlings were grown in the dark for seven days and then collected (~50 mg) and immediately frozen in liquid nitrogen. Total proteins were extracted and separated on an SDS-PAGE gel. Immunoblot analysis was performed using an anti-GFP antibody to detect either the full-length fusion protein (GFP-ATG8f, ~45 kDa) or free GFP (~27 kDa) and the

experiment was repeated thrice. *ps1ps2* double mutant plants show low autophagic flux indicated by low levels of free GFP in the vacuole under both sucrose-containing as well as sucrose starvation media (Figure 17). *ps1ps2* double mutant has a higher amount of full-length fusion protein i.e. GFP-ATG8f (45 kDa) in the sucrose-containing media compared to the WT seedlings. This indicates that even when sucrose is present in the growth media, the autophagosomes labeled with GFP-ATG8f are abundantly present in the cytoplasm or the vacuole of the mutant. They either have difficulty in reaching the vacuole or are already in the vacuole but have a low degradation rate. However, when autophagy was induced through sucrose starvation, the amount of free GFP was increased in both WT as well as in the mutant seedlings but the amount was less increased in mutant indicating reduced autophagic flux in the later.

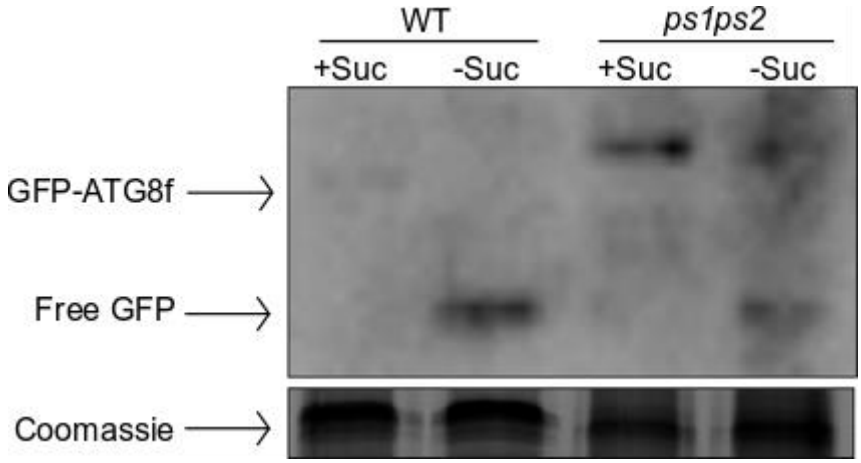

Figure 17: Western blot of seven-day-old C starved Col-0 and *ps1ps2* mutant plants using Anti-GFP antibody. *ps1ps2* mutant exhibits lower autophagic flux during C starvation.

3.10 Presenilins Mutant has Defective Autophagy at the Later Stages of Autophagic Pathway and Not at the Initiation Step

To further study autophagy in *ps1ps2* mutant, an autophagy inhibition experiment using carbon starvation and wortmannin was conducted. Wortmannin is an extensively used autophagy inhibitor because of its inhibition of vacuolar transport and autophagy. It inhibits the activity of PI3K (phosphatidylinositol 3-kinase) which is essential for the induction of autophagy (Merkulova *et al.,* 2014). Seven-day-old seedlings of GFP-ATG8f and GFP-ATG8f/*ps1ps2* transgenic plants grown vertically on nutrient-rich solid growth media were transferred to nutrient-rich liquid media with and without carbon and wortmannin in different combinations for 16 hours before observation under the confocal microscope (Figure 18). Root cells of GFP-ATG8f seedlings have almost no autophagosomes under nutrient-rich conditions (+C-Wort) when there is no wortmannin used. However, as expected GFP-ATG8f/*ps1ps2* root cells have a lot of autophagosomes under these control conditions as can be seen in Figure 18B. When seedlings were subjected to carbon starvation media (-C-Wort) for 16 hours, we could observe some autophagosomes in the GFP-ATG8f root cells which formed as a result of autophagy induction under starvation and GFP-ATG8f/*ps1ps2* still had an abundance of autophagosomes in the cytoplasm (Figure 18D). When wortmannin was used for 16h under nutrient-rich conditions (+C+Wort), there was hardly any autophagic body in the GFP-ATG8f as well as in GFP-ATG8f/*ps1ps2* which might be because of the reason that the inhibitor completely stopped the formation of autophagosomes (Figure 18E, F). Wortmannin treatment also resulted in a diffused signal of GFP-ATG8f in the cytoplasm which is a typical effect of this inhibitor. Similarly, when treated with wortmannin under starvation media (-C+Wort) for 16h it stopped the autophagosome formation in GFP-ATG8f as well as in GFP-ATG8f/*ps1ps2* (Figure 18G, H). These results suggest that the mutant has defective autophagy probably at the later steps of the autophagic pathway and not at the initiation step.

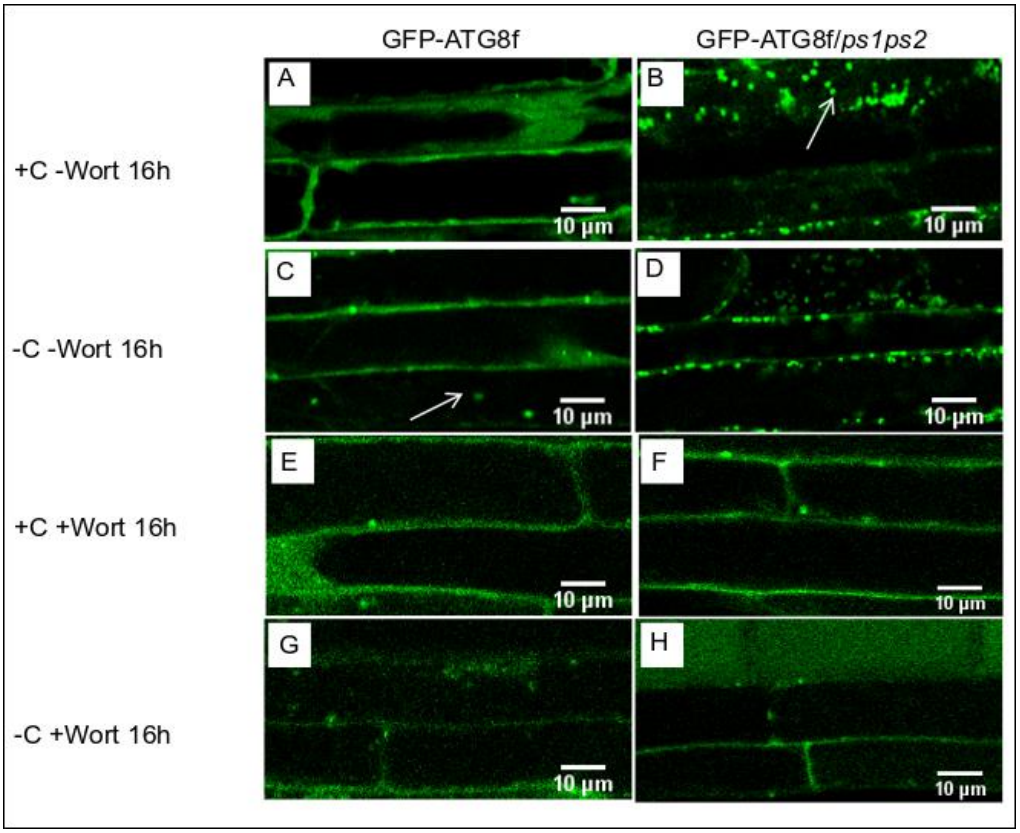

Figure 18: *ps1ps2* mutant has autophagy defectiveness at the later steps of the autophagic pathway and not at the initiation step. Seven-day-old ½MS media grown seedlings of GFP-ATG8f and GFP-ATG8f/*ps1ps2* were subjected to various treatments such as DMSO/control (A, B), C starvation (C, D), 33 µM wortmannin (E, F) and C starvation with 33 µM wortmannin (G, H) for 16 h before observation under CLSM.

3.11 Autophagy in *ps1ps2* Root Cells: Effects of Concanamycin-A and Wortmannin

The presence of autophagosomes in high numbers even under control conditions in *ps1ps2* suggests that autophagy is defective in this mutant line, but there is still a likelihood that in these plants the fusion/delivery of autophagosomes to the vacuole is inhibited which leads to an abnormal accumulation of autophagosomes in the cytoplasm. To rule out this possibility, in addition to the wortmannin treatment done in the previous experiment (Figure 18), a concanamycin-A (ConA) treatment experiment was performed (Figure 19). ConA is an inhibitor of V-ATPase which maintains the vacuolar pH keeping it acidic. When added exogenously, the inhibitory

effect of ConA on V-ATPase raises the pH of the vacuole. Consequently, the vacuolar hydrolases cannot act under high pH conditions leading to a buildup of autophagic bodies inside vacuole thus allowing us to monitor autophagic flow (Yoshimoto *et al.,* 2004). When seven-day-old seedlings of GFP-ATG8f and GFP-ATG8f/*ps1ps2* were grown under nutrient-rich (NR) growth conditions and the root cells were observed by confocal microscopy, there were no autophagosomes in WT but there was already a high level of autophagy in the cells of *ps1ps2* (Figure 19A, B). When treated with ConA, we observed a huge buildup of autophagosomes in the cells of both WT as well as *ps1ps2* seedlings indicating that the autophagic bodies were able to reach the vacuole (Figure 19C, D). When only wortmannin was used, the GFP-ATG8f signal appeared to be diffused in the cytoplasm in both WT as well as *ps1ps2* root cells and the autophagosomes were almost nonexistent. Finally, when wortmannin was used in combination with ConA, it markedly diminished the effect of ConA (Figure 19G, H) which indicates that the PI3 kinase activity is vital for the efficient delivery of the autophagosomes to the vacuole which corroborates our previous results (Figure 18). These results clearly indicate that the ATG8f associated autophagosomes reach the vacuole.

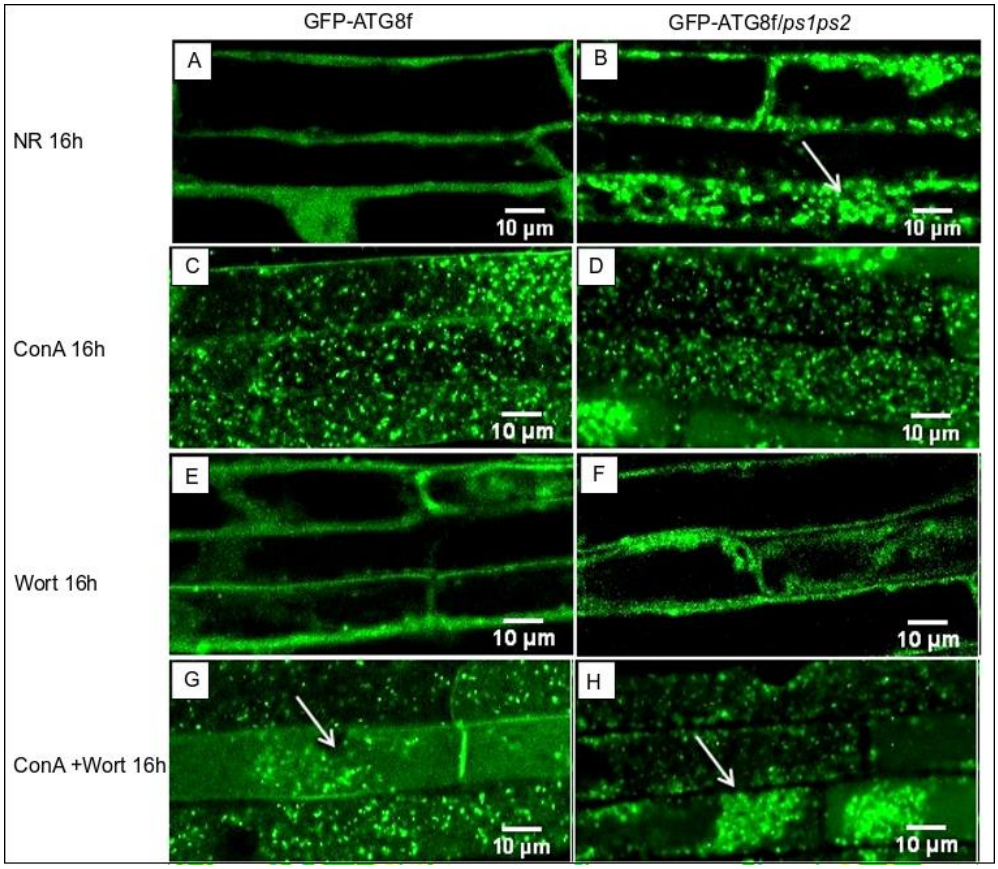

Figure 19: Autophagosomes reach the vacuole in *ps1ps2* mutant. Seven-day-old ½MS media grown seedlings of GFP-ATG8f and GFP-ATG8f/*ps1ps2* were subjected to various treatments such as nutrient-rich media NR with DMSO/control (A, B), 1 µM ConA (C, D), 33 µM wortmannin (E, F) and 1 µM ConA in combination with 33 µM wortmannin (G, H) for 16 h before observation under CLSM.

3.12 GFP-ATG8f is Delivered to the Vacuoles in *ps1ps2* Mutant

FM4-64 labels the plasma membrane and is taken up by the cell via endocytosis. After being endocytosed, it labels the tonoplast and possibly also goes into the vacuole together with the vacuolar membrane. To further confirm whether the autophagosomes in *ps1ps2* go into the vacuole, we performed an FM4-64 uptake study in both GFP-ATG8f as well as in GFP-ATG8f/*ps1ps2* seedlings before treating with C starvation and ConA (Figure 20). As can been seen from Figure 19, there is a pronounced buildup of the GFP-ATG8f as well as that of GFP-ATG8f/*ps1ps2* inside the vacuole. This indicates that the GFP-ATG8f labeled structures are delivered to the vacuole in the presenilins mutant.

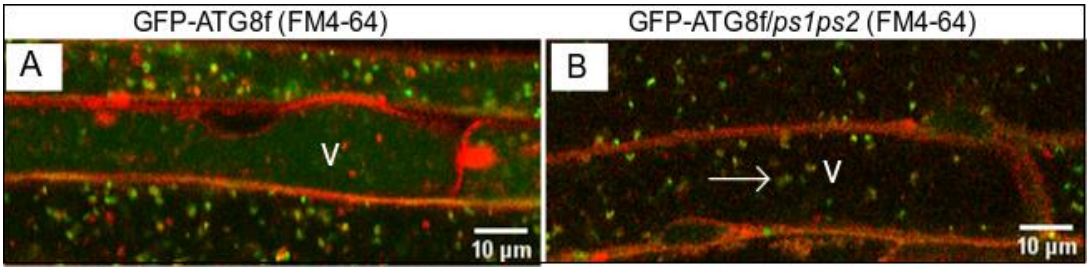

Figure 20: GFP-ATG8f labeled autophagosomes are delivered to the vacuole in the presenilins mutant. FM4-64 was applied to the seven-day-old seedlings for 1 hour which was followed by transfer of the ATG8-GFP and GFP-ATG8f/*ps1ps2* to the C starvation medium containing ConA for another 6 hours before observation with CLSM. "v" indicates vacuole.

3.13 AtPEN2 Bodies are Distinct from Classical Autophagosomes

Previously it has been shown that AtPEN2-GFP is partially localized with autophagy marker protein i.e. ATG8 in the Arabidopsis leaf protoplasts (Smolarkiewicz *et al.,* 2014). Through the transient transformation of the leaf protoplast, it was shown that about 80% of cells show clear co-localization of AtPEN2 with autophagosomes and the rest of 20% of cells show no co-localization. It might mean that AtPEN2 is linked with autophagy. To confirm the role of AtPEN2 in plant autophagy, we transiently co-expressed AtPEN2-GFP and ATG8f-RFP in the tobacco leaf epidermal cells. The transformed tobacco plants were pre-treated with darkness for two days to induce autophagy before confocal microscopy analysis. Surprisingly, as depicted in Figure 21, AtPEN2-GFP vesicles were distinct from ATG8f-RFP in structure and generally did not co-localize with autophagosomes. However, only a small portion of the AtPEN2-GFP was associated with ATG8f at some point.

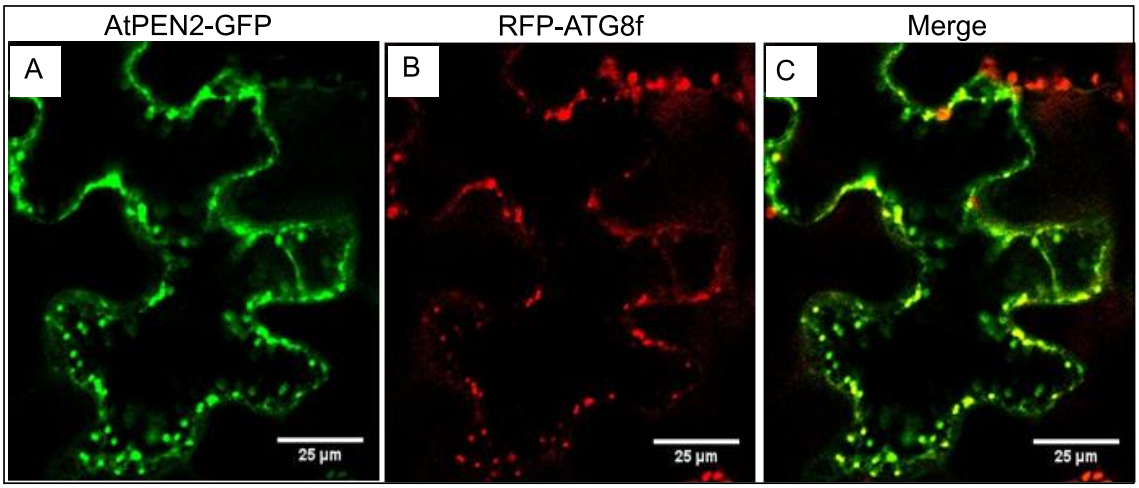

Figure 21: RFP-ATG8f, a marker for autophagosomes is not co-localized with the AtPEN2-GFP vesicles in the transient transformation assay. (A) AtPEN2-GFP (B) RFP-ATG8f (C) Merge showing partial co-localization.

3.14 Motility of AtPEN2 is Independent of Microtubules

Since the AtPEN2-GFP labeled vesicles are motile and we know that the motility of vesicles is dependent on actin and microtubule bundles of the cytoskeleton (Langford, G. M. 1995). Therefore, it would be interesting to see if the mobility of AtPEN2 is dependent on the microtubules. For this purpose, the MAP4-RFP which is a marker for microtubules was transiently co-expressed with AtPEN2-GFP in *N. benthamiana* leaf epidermal cells. AtPEN2-GFP failed to localize with microtubule bundles (Figure 22). To further explore the role of microtubules in AtPEN2-vesicle motility, oryzalin, a microtubule disrupting reagent was employed. Five-day-old seedlings of Arabidopsis AtPEN2-GFP were treated with 10 µM oryzalin solution for 30 minutes before observation with the confocal microscope. There was no effect observed in the motility of AtPEN2 vesicles further confirming that they are not associated with microtubules and their movement is independent of the later (Figure 22).

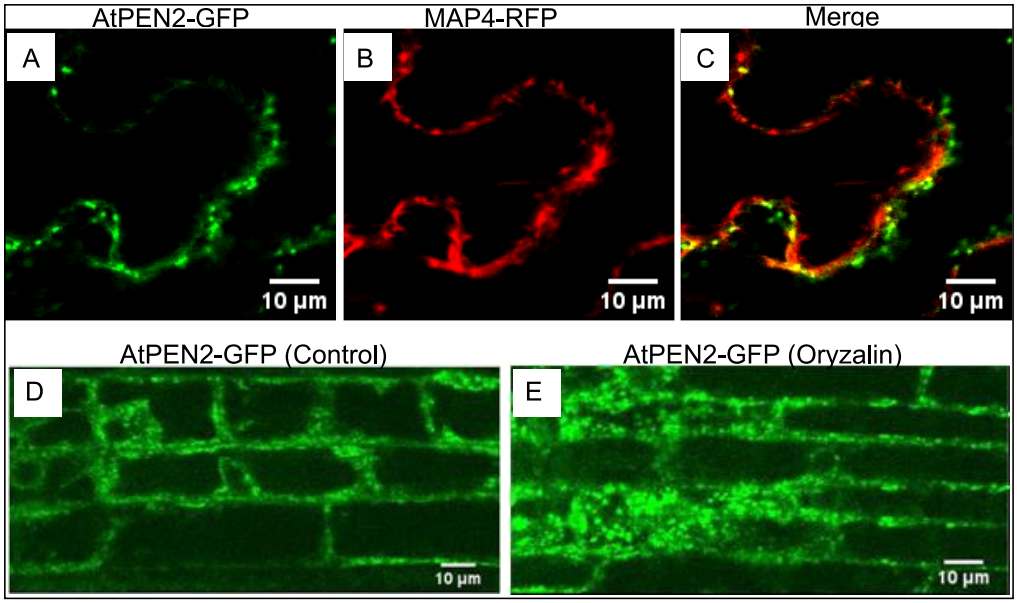

Figure 22: AtPEN2-GFP does not localize with MAP4-RFP in epidermal leave cells of *N. benthamiana* and the movement of AtPEN2-GFP vesicles is independent of the microtubule cytoskeleton. (A) AtPEN2-GFP (B) MAP4-RFP (C) Merge showing no co-localization. (D, E) seven-day-old ½MS media grown seedlings of AtPEN2-GFP treated with and without oryzalin for 30 minutes before observation under CLSM.

3.15 Motility of AtPEN2-GFP is Actin Cytoskeleton Dependent

Similar to microtubules, the actin cytoskeleton is also important for the motility of vesicles. Latrunculin-B (Lat-B) is an actin-depolymerizing agent that disrupts F-actin assembly and thereby inhibits the motility of vesicles in the cells. Arabidopsis roots expressing AtPEN2-GFP were treated with 10 µM Lat-B for 30 minutes and observed under a spinning disk confocal microscope. Lat-B stopped the movement of AtPEN2-GFP positive vesicles within 30 minutes of treatment and also changed their shape indicating that actin is important for their mobility and AtPEN2 is sensitive to Lat-B treatment (Figure 23).

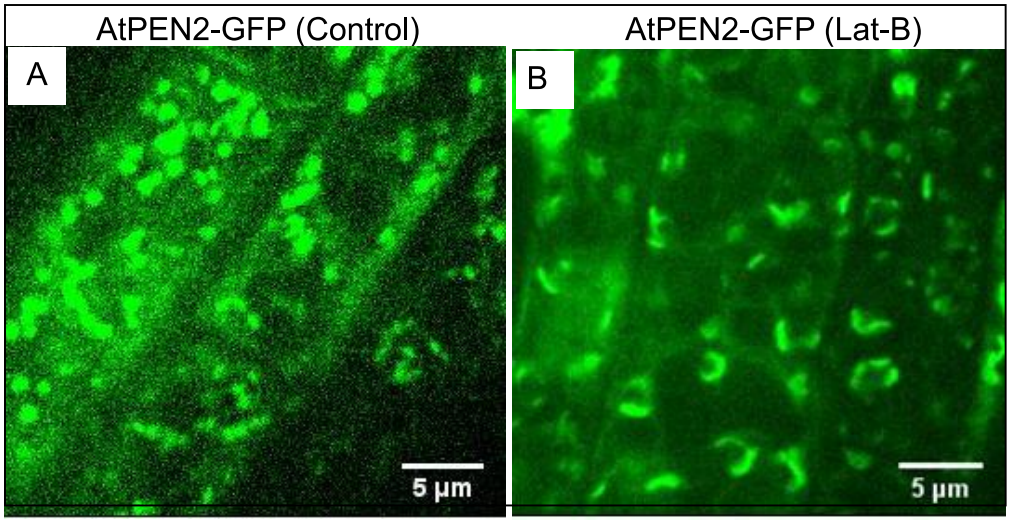

Figure 23: Lat-B stops the movement of AtPEN2-GFP. Seven-day-old ½MS grown seedlings of AtPEN2-GFP were subjected to Lat-B treatment for 30 minutes before observation under a spinning disk confocal microscope. (A) Control (B) Lat-B treated Arabidopsis root cells.

3.16 AtPEN2-GFP Partially Co-localizes with Late Endosomes

AtPEN2-GFP has previously been reported to be absent from the early endocytic pathway (Walker, 2010), therefore, I wanted to check its presence in the late endocytic pathway. FYVE domain-containing marker binds to the PI3P (phosphoinositol-3-phosphate) in the membranes of late endosomes (Voigt *et al.,* 2005). AtPEN2-GFP and DsRed-FYVE constructs were transiently co-expressed in the tobacco (*Nicotiana benthamiana*) leaf epidermal cells (Figure 24). Confocal microscopy imaging revealed that DsRED-FYVE, a marker for late endosomes showed partial localization of AtPEN2 and FYVE domain-containing protein in tobacco leaf epidermal cells. Both AtPEN2 and FYVE are highly motile compartments and in the tobacco leaf cells, they were seen to interact at some point and then leave each other which might mean they exchange some molecule.

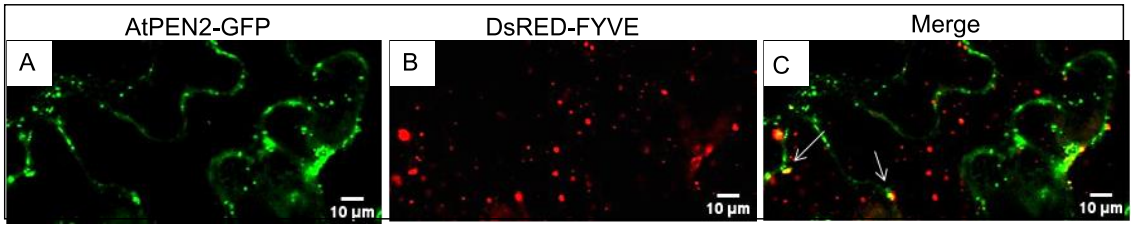

Figure 24: AtPEN2-GFP partially localizes with DsRED-FYVE in epidermal leave cells of *N. benthamiana*. (A) AtPEN2-GFP (B) DsRed-FYVE (C) Merge showing partial co-localization as indicated by arrows.

3.17 Effect of Gamma Secretase Inhibitor on AtPEN2-GFP

We tested an inhibitor (BSM-299897) of the gamma-secretase complex with the purpose to get further insights into the function of this complex in plants. The inhibitor has been successfully tested in animals before. To test the inhibitor, seven-day-old AtPEN2-GFP expressing plants were treated with 50 µM for 30 minutes in dark. The root cells treated with the inhibitor changed the localization of AtPEN2-GFP signal with more signal at the cross walls (Figure 25). This shows that the inhibitor affects the complex activity also in the plant cells.

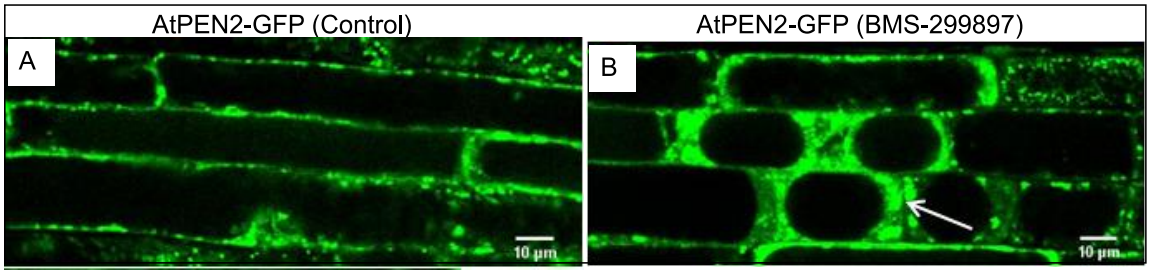

Figure 25: Gamma secretase inhibitor (BSM-299897) changes the localization of AtPEN2-GFP signal. (A) Control (B) BSM-299897 treated Arabidopsis root cells showing relocalization of the AtPEN2-GFP signal.

3.18 Genotyping of AtPEN2 Mutant

The Arabidopsis AtPEN2 mutants have already been described in a doctoral thesis by Walker (2010). They phenotyped two SALK mutants for AtPEN2 i.e. *pen2-1* and *pen2-2* for the lines Salk_128110 and Salk_140461 respectively. Homozygous *pen2-1* mutant plants showed stunted growth and failure to produce functional inflorescence (Walker 2010). However, when it was tried to complement the *pen2.1* phenotype with ATPEN2-GFP it did not complement the phenotype. On the other hand, *pen2-2* mutant did not show any gross morphological differences when exposed to various abiotic stresses. Due to this ambiguity, we also decided to analyze the *pen2* mutant. We ordered the SLAK_140461 line which is *pen2-2* mutant line according to Walker (2010). The mutant was genotyped using the primers listed in the primer list (Table 1). When grown on ½MS media, the homozygous *pen2* mutant always displayed a reduced primary root growth as can been seen in Figure 26. When exposed to various abiotic stress treatments such as hormonal (GA, ABA, IAA), ER stress (DTT), salt stress, etc., no significant change occurred in the growth of the *pen2* mutant. This might mean that the reduced primary root length of the mutant is independent of the treatments used.

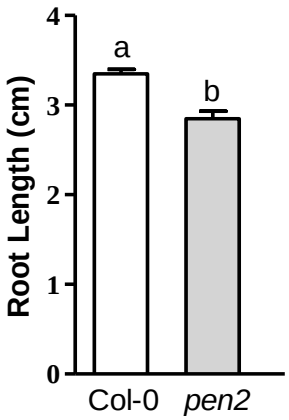

Figure 26: Root lengths in *pen2* mutant are significantly shorter than in WT seedlings when grown under normal growth conditions. Measurement of the hypocotyls and roots of *pen2* and WT control grown under ½MS media using ImageJ. Data represent the mean (S.E) of two independent replicates and letters above the error bars show significant differences compared to WT (*P < 0.05)* based on the student *t*-test.

3.19 ATG9 Motif Crucial for Binding with AP4 is Conserved in Plants and Animals

Recent studies from animal models have highlighted an important role of adaptor protein 4 complex in autophagy by its interaction with ATG9A (Mattera *et al.,* 2017; Davies *et al.,* 2018; De Pace *et al.,* 2018). By employing various functional and biochemical approaches, Mattera *et al.,* (2017) reported that ATG9A is a physiological cargo of AP4. ATG9A is the only transmembrane domain-containing protein in animals and plants among the core ATG machinery. It has been reported that ATG9 cycles between TGN/early endosomes and preautophagosomal structures where it provides membrane to the growing autophagosomes in animals as well as in plants (Imai *et al.,* 2017; Zhuang *et al.,* 2017). The interaction between ATG9A and AP4 is mediated by YXXØE (YQRLE) motif present on the N terminal cytosolic tail of ATG9A (Mattera *et al.,* 2017). In order to verify if this sequence is also conserved in other organisms including plants, we carried out multiple sequence alignments using amino acid sequences of ATG9A homologues from *A. thaliana*, *Caenorhabditis elegans*, *Physcomitrella patens*, *Zea mays,* and *Homo Sapiens*. The YQRLE sequence in the N terminal cytoplasmic tail of ATG9 is required for binding with AP4 seems to be quite well conserved (Figure 27). This indicates that ATG9 and AP4 interaction is conserved in evolutionarily distinct organisms.

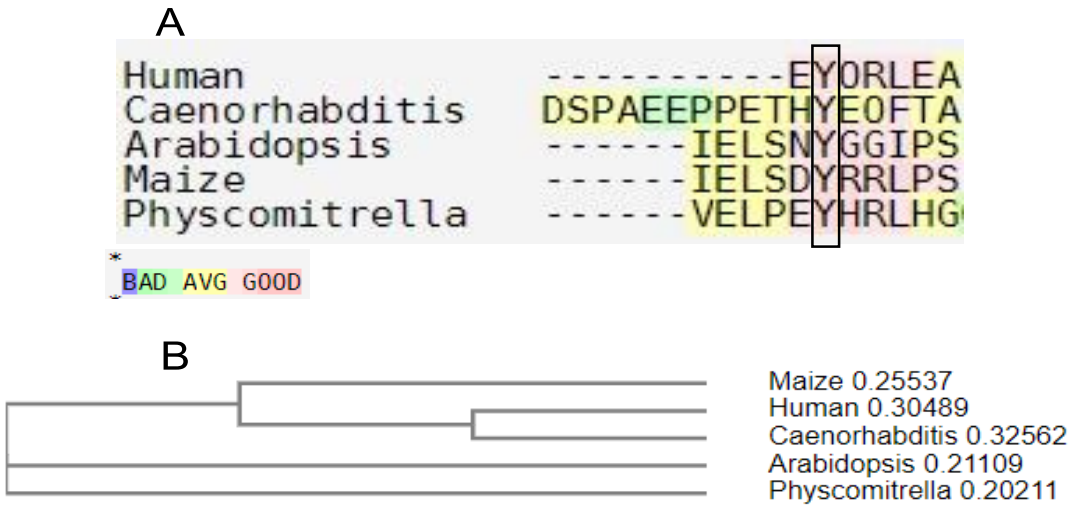

Figure 27: Sequence alignments of amino acid motifs known to be crucial for binding with AP4 in different species. **(A)** YQRLE motif in ATG9 **(B)** Cladogram was created based on the amino acid sequence alignments for ATG9.

3.20 AP4 Co-localizes with ATG9 in Tobacco Leaf Epidermal Cells

As mentioned above, ATG9 is the only TMD containing protein among ATGs and cycles between TGN and the PAS and plays a key role in autophagosome formation. Zhuang *et al.,* (2017) for the first time provided comprehensive analysis of the structure and function of Arabidopsis ATG9. They reported that in Arabidopsis, both N and C termini of ATG9 are exposed to the cytosol in a manner similar to the yeast or mammals. Through subcellular localization studies, they showed that ATG9 is present in the proximity with TGN and that the ATG9 vesicles may be derived from the Golgi. In plants, AP4 complex has been shown to localize at the TGN and mediates sorting and trafficking to the plasma membrane and the pre vacuolar compartment (Pertl-Obermeyer et al 2016; Fuji *et al.,* 2016). In animals, several recent reports show that ATG9 is the physiological cargo of the AP4 complex (Mattera *et al.,* 2017, De Pace *et al.,* 2018; Davies *et al.,* 2018). The interaction is mediated by recognition of the YXXØE motif (YQRLE) in the N terminal of ATG9 by the μ subunit of AP4 (Mattera *et al.,* 2017). Through the export of ATG9 from the TGN to the surface of cytoplasm and PAS site, AP4 complex functions in the maintenance of autophagic function. Keeping in view these recent developments about the interaction of AP4 with ATG9, we were also interested to see if AP4 also

localizes with ATG9 in plants. For this purpose, YFP-ATG9 and AP4-GFP were transiently co-transformed in the tobacco leaf epidermal cell and interestingly, both fluorescently tagged proteins showed complete localization (Figure 28). This observation indicates that AP4 may be involved in the transport of the ATG9 but it needs to be tested with further experimental analysis.

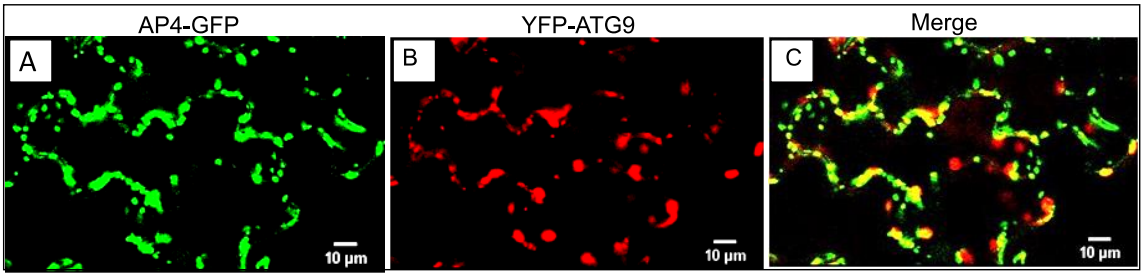

Figure 28: AP4µ-GFP localizes with YFP-ATG9 in the leaf epidermal cells of *N. benthamiana* in the transient transformation assay. (A) AP4µ-GFP (B) YFP-ATG9 (C) Merge showing complete co-localization.

3.21 AP4 Mutants Accumulate Autophagy Marker

It has previously been demonstrated that membrane trafficking is closely related with the autophagic pathway (Kulich *et al.,* 2013). The autophagic pathway recycles the cellular constituents by forming double-membrane compartments called autophagosomes and ATG8 is the structural component of these structures. Based on the previous observations, there was a possibility of autophagy defectiveness in the AP4 mutant lines, we analyzed the steady-state levels of ATG8 in these lines (Figure 29). ATG8 accumulated in all of three mutant lines which indicates that the mutant lines are defective in autophagy and that AP4 is potentially involved in autophagy.

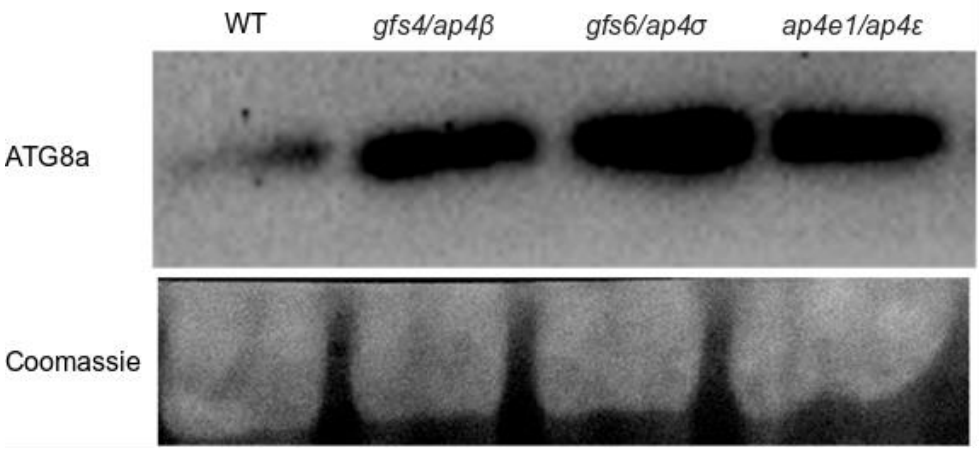

Figure 29: AP4 mutants accumulate autophagosomal marker. Total protein extracts from wild-type (WT), *gfs4/ap4β, gfs6/ap4σ, ap4e1/ap4ε* grown under long-day conditions were subjected to immunoblotting using an anti-ATG8 antibody. Coomassie was used as a loading control. The experiment was repeated twice.

3.22 ER-Stress Mediated Autophagy Induction Affects the Growth of Arabidopsis AP4 Mutants

Müdsam (2018) described that the mutants for two out of four AP4 subunits namely *gfs4/ap4β* and *gfs5/ap4μ* show reduced hypocotyl and root growth under normal as well as under sucrose starvation growth conditions and this defective growth was attributed to the reduced cell expansion. DTT is a classical ER stress inducer and interferes with protein folding by inhibiting N linked glycosylation and by blocking the disulfide bond formation (Martínez & Chrispeels, 2003). It has also been implicated as autophagy inducer in plants and under the influence of DTT, the portions of ER are recycled to the vacuole through the macroautophagy pathway (Liu *et al.,* 2012). Moreover, Zhuang *et al.,* (2017) proposed that ATG9 might be involved in ER-phagy due to tight coherence between autophagosome structure and the ER membrane. Since our results suggest that AP4-GFP localizes with ATG9 and might be involved in its export from the TGN in a manner similar to animal cells, we decided to test the effect of DTT on AP4 mutant lines namely *gfs4/ap4β*, *gfs6/ap4σ,* and *ap4e1/ap4ε*. We tested the mutants for two subunits which were not tested before i.e. *gfs6/ap4σ* and *ap4e1/ap4ε* and another *gfs4/ap4β* which was tested already by Müdsam (2018). Seven-day-old seedlings of WT and AP4 mutants grown on ½MS medium

were transferred to the ½MS media plates containing 0mM and 1mM DTT and allowed to grow for another one week (Figure 30). Under the control condition, the growth of mutant lines tested was significantly shorter than that of WT seedlings and these results are also consistent with those of Müdsam (2017). Under 1mM DTT or ER stress induction, the growth of both WT as well AP4 mutants was negatively affected and the mutant lines had significantly shorter growth of primary roots (Figure 30). Our data hints that the reduced growth of AP4 mutants under ER stress might be correlated with defective autophagy.

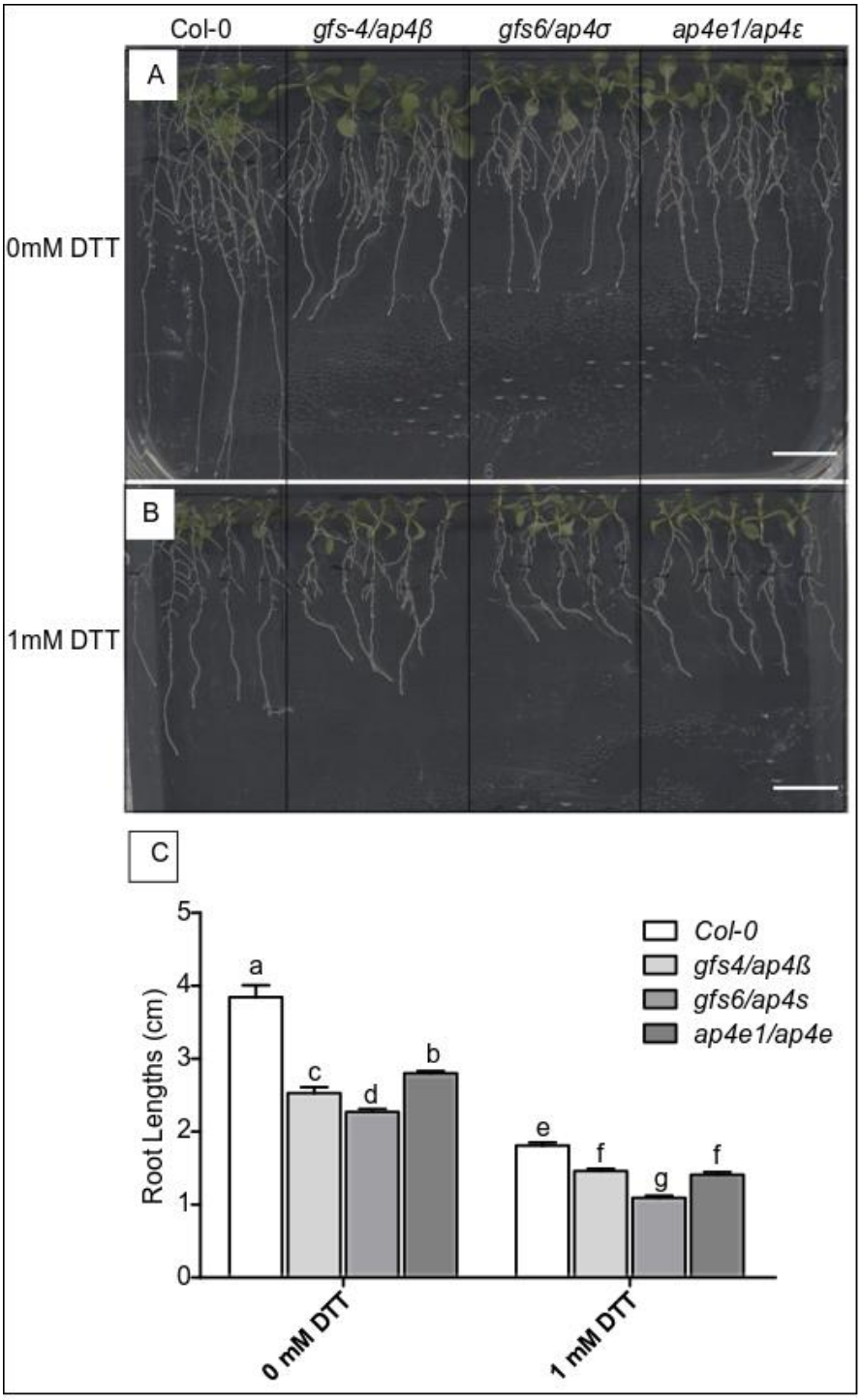

Figure 30: Seedlings of AP4 mutants grown with or without DTT showed significantly shorter roots in comparison to the wild type seedlings. (A, B) Image showing seedlings of each line grown under normal growth media and media supplemented with 1mM DTT. **(C)** Measurement of the roots of AP4 mutants i.e. *gfs4/ap4β*, *gfs6/ap4σ*, *ap4e1/ap4ε* and WT control grown with and without DTT using ImageJ. Data represent the mean (S.E) of two independent replicates and different letters above the error bars show significant differences compared to WT ($P < 0.05$) based on the student *t*-test.

3.23 AP4μ-GFP Accumulates in the Core of BFA Compartments

The subcellular localization of AP4 has already been described in animal cells and is reported to be localized at the TGN (Hirst *et al.,* 1999). In plants, it as well has been confirmed that the fluorophore fusions of AP4μ and AP4β are localized specifically at the TGN (an endosomal compartment) [Fuji *et al.,* 2016; Müdsam *et al.,* 2017]. The authors confirmed this by showing a localization with FM4-64 which labels the endomembrane compartments in addition to performing a series of co-localization experiments by employing well established TGN markers (Fuji *et al.,* 2016). To better substantiate the above results, we also stained the roots of seven-day-old Arabidopsis seedlings stably expressing AP4μ-GFP with FM4-64. As expected, the AP4μ-GFP vesicles co-localized with FM4-64 compartments of the early endocytic pathway (Figure 31A-C). Next, FM4-64 stained roots of AP4μ-GFP were stained with BFA which is a fungal toxin known to inhibit ARF-GTPases. BFA treatment of Arabidopsis seedlings causes an accumulation of BFA compartments in the root cells. The core of these compartments is made up of the TGN and is surrounded by remnants of Golgi stacks (Satiat-Jeunemaitre & Hawes, 1994). After the treatment with BFA, AP4μ-GFP puncta were re-localized and accumulated in the core of BFA compartments (Figure 31D-F). This result further confirmed that AP4μ-GFP is localized at the TGN/early endosomes and that its localization is affected by BFA treatment.

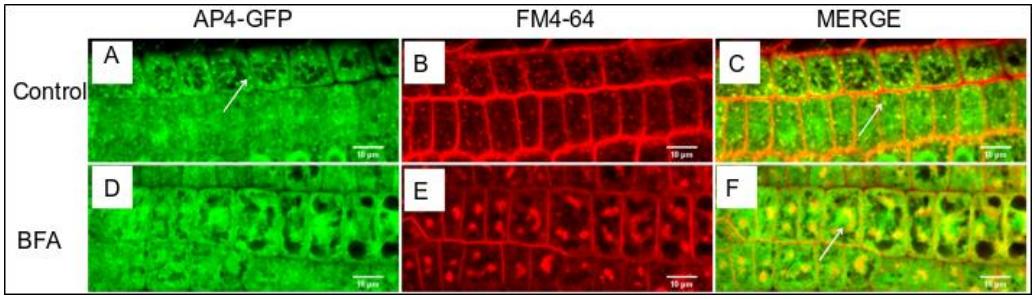

Figure 31: AP4μ-GFP accumulates in the core of BFA compartments. (A-C) Arabidopsis root cells of stably expressing AP4μ-GFP were analyzed with confocal microscopy. AP4μ-GFP localized with the early endocytotic compartments labeled with FM4-64. (D-F) After BFA treatment, both FM4-64 and AP4μ-GFP accumulated in the core of the BFA compartments as indicated by the arrow.

DISCUSSION

4.1 Arabidopsis Presenilins

4.1.1 Presenilins Respond to Carbon Starvation

Presenilins are the central and catalytic subunit of the gamma-secretase complex and are ubiquitous proteins known to function in important cellular processes. These are evolutionarily conserved proteins throughout metazoa and there has been considerable research done in animal models to understand their functions especially their role in the development of Alzheimer's disease. In animals, presenilins have particularly been implicated in autophagy and are considered a critical mediator of the lysosome mediated protein degradation (Neely *et al.,* 2011). In plants, there has been little or no work done on presenilins concerning autophagy. In this work, we report that presenilins are important for the normal functioning of the autophagic pathway in plants as well and that their absence can lead to defective autophagy and ultimately adverse effects on plant growth and development.

Autophagy is a highly regulated process with crucial roles in cell regulation, maintenance of amino acid levels and mitigating toxic effects of the proteins prone to accumulation. Over the last decade, there has been accumulating evidence about the important role of autophagy under nutrient-limited conditions (Chung *et al.,* 2010; Izumi al. 2010). Eukaryotes require high carbohydrate levels to synthesize macromolecules and generate energy. Low energy leads to retarded growth because carbon (C) is retained by the cells to survive and maintain core functions of life. In Arabidopsis, the availability of C has a significant effect on growth and development in both seedling as well as adult stages. Carbon starvation is one of the well-characterized phenotypes of autophagy-related mutants and the relationship between autophagy, protein degradation, and amino acid recycling under nutrient-limited conditions has already been described (Izumi *et al.,* 2013; Avin-Wittenberg *et al.,* 2015). The previous study of our collaborators showed that two-week seedlings of Arabidopsis presenilin double mutant (*ps1ps2*) exhibit enhanced chlorosis under carbon/nitrogen starved media combined with darkness (Smolarkiewicz *et al.,* 2014).

Since starvation and darkness are known to induce autophagy, we took this as a starting point to further study this process in the *ps1ps2* double mutant. We also carried out a C starvation experiment to determine the phenotypic response of the *ps1ps2* mutant. However, in our C starvation system, we took etiolated seedlings grown on media without supplemented sucrose as a C starvation system. This system gives an advantage of studying autophagy at the early seedling establishment where nutrients are acquired through reserve mobilization (Avin-Wittenberg *et al.,* 2015). We observed a clear delayed growth phenotype for *ps1ps2* mutant where it displayed shorter growth of roots and hypocotyls (Figure 9) and this phenotype is also in line with the previous report that autophagy defective mutants have delayed growth when grown on media without C (Avin-Wittenberg *et al.,* 2015).

Autophagy induction by stress conditions results in an increase in the formation of autophagosomes in the cells (Xiong at al. 2005). Since the *ps1ps2* mutant showed the phenotype of an autophagy defective mutant under C starvation we wanted to observe autophagosomes in the root cells. We used established methods such as MDC staining and GFP-ATG8 labeling to observe autophagosomes in the root cells of *ps1ps2* (Figure 10 & 12). Protoplasts of WT and *ps1ps2* mutant plants were also transiently transformed with GFP-ATG8f to monitor autophagy (Figure 11). We demonstrated that *ps1ps2* had an abnormal build-up of autophagosomes which indicates that presenilins have a vital role in the process of autophagy in multiple cell types. This observation is very similar to the one made in animals where it was shown that knockout of presenilins leads to a buildup of autophagosomes in the mouse embryonic fibroblasts (MEFs) and neuroblastomas (N2A) [Neely *et al.,* 2011]. The increase in autophagosomes in *ps1ps2* mutant might mean either an increase in autophagic activity or a disturbance in the degradation of autophagosomes late in the pathway at the vacuole.

4.1.2 Presenilins Regulate Protein Trafficking in Arabidopsis

Proteins are the degradation target of the autophagic machinery, therefore, we analyzed the total protein content of the C starved presenilin mutant as compared to wild type. Furthermore, previously it has been shown that Arabidopsis ATG mutants

have differential protein accumulation and have a reduction in free amino acids when grown under C starvation (Avin-Wittenberg *et al.,* 2015). Interestingly, the total protein content of *ps1ps2* was turned out to be higher than the wild type seedlings particularly a band of ~22 kDa and it suggested a possible disruption in the normal protein degradation in this mutant (Figure 13). This finding is also similar to the one reported by Neely *et al.,* (2011) whereby they conducted pulse-chase assays in PSDKO (Presenilin-1 and -2 double knockout) MEFs to monitor the breakdown of long-lived proteins. The authors reported that PSDKO had a significant reduction in proteolysis and attributed this to the dysfunction of downstream steps of the autophagic pathway. These similarities between the response of Arabidopsis presenilin mutant with MEF presenilin mutants suggest that presenilins play similar roles in animals as well as in plants and function identically in these evolutionarily distinct species. Proteomic analysis of the ~22 kDa band suggested that differential protein accumulation occurred in presenilin mutant during C starvation and revealed the accumulation of some interesting proteins (Table 2). Among these proteins were PBE1, a 21S proteasome beta E1 protein and serine peptidase protein of S26A, both of which are enzymes involved in peptidase activity. Although we did not measure the endopeptidase activity in the C starved *ps1ps2* mutant but over-accumulation of these proteins suggests a possible impairment in the endopeptidase activity in presenilin mutant. Surprisingly, a few years ago another study had reported a higher endopeptidase activity in the ATG mutants under nitrogen starvation during the later stages of growth before reaching senescence (Guiboileau *et al.,* 2013). This is contrary to what we conclude from our observation, however, this inconsistency might be due to the age of the plants observed. Guiboileau *et al.,* (2013) worked with plants that were 30 to 75 days old while we observed seven-day-old etiolated seedlings. Moreover, the difference is not just in the age but also in the experimental conditions, in our experiment the seedlings are growing in a heterotrophic system while the older plants are autotrophic. In animals, some of the peptidases known as cathepsins including the ones from the serine group have been reported as autophagy regulators. They undertake cleavage of the peptide bonds of the autophagy substrates and maintain autophagic flux (Kaminskyy & Zhivotovsky, 2012). It is also worth noting that asparagine endopeptidase (AEP) has been

described as an autophagy regulator. Its deficiency in mice resulted in abnormal activation of PI3K resulting in the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-bisphosphate PIP3 (Lan & Guo, 2015). An increase in PIP3 and a decrease in PIP2 led to impaired autophagic-lysosomal biogenesis and ultimately defective autolysosomal clearance. This is remarkably similar to the Arabidopsis presenilin double mutant *ps1ps2* which is also defective in autophagosome clearance. In addition to endopeptidases, *ps1ps2* also accumulated peptides of ribosomal proteins XW6 and L12 (Table 2). The over-accumulation of these proteins suggests the malfunction of ribophagic activity in *ps1ps2* mutant. Another interesting protein identified in our proteomic analysis was chaperonin 61 beta (CPN61β) which is a type 1 chaperonin involved in the proper folding of the newly synthesized proteins (Horwich *et al.,* 2007). It has many known functions such as it is needed for the folding of NdhH, a subunit of chloroplast NADH dehydrogenase-like complex (NDH) [Peng *et al.,* 2011]. Additionally, CPN61β and CPN61α are vital for the formation of the plastid division apparatus (Suzuki *et al.,* 2009). These proteins maintain plastid division by means of regulating the FtsZ ring dynamics, FtsZ is required for dividing plastids into equal-sized daughter plastids (Yang *et al.,* 2008). An accumulation of chloroplast division protein CPN61β in the C starved *ps1ps2* mutant suggests that FtsZ dynamics might be disrupted in this mutant resulting in the improper plastid division. This assumption is further supported by a previous observation by our colleagues that *ps1ps2* exhibits enhanced chlorosis when exposed to dark under C starvation conditions (Smolarkiewicz *et al.,* 2014). However, this aspect needs to be determined with further experimentation. We also identified HSP70T-1 (heat shock protein 70T-1) which is a cytosolic chaperone and upregulates under temperature extremes and after seed imbibition (Sung *et al.,* 2001). It is also noteworthy that HSP70T-1 has an actual molecular weight of 68.3 kDa but it was identified in ~22 kDa region and this was a similar case for other proteins identified in our analysis. A possible explanation for this can be that a partial degradation of these proteins occurs in the *ps1ps2* mutant. *ps1ps2* mutant also accumulated PEBP (phosphatidylethanolamine-binding protein) family protein which in humans has been implicated in the development of various cancers (Table 2). Recently, PEBP1 has also been reported to negatively

regulate starvation-induced autophagy through interaction with LC3 (microtubule-associated protein1 light chain 3β) which functions in autophagosome biogenesis in mammalian cells (Noh *et al.,* 2016). It has been reported that PEBP1 contains an LIR (LC3 interacting region) motif important for binding to LC3. Overexpression of PEBP1 suppressed autophagy through activation of MTORC1 (mechanistic target of rapamycin complex 1) and its disruption promoted starvation-induced autophagy (Noh *et al.,* 2016). Since PEBP proteins are evolutionarily conserved therefore they can be expected to play similar roles in plants as well. In Arabidopsis, autophagosome formation requires conjugation of ATG8 with phosphatidylethanolamine (PE) and an overaccumulation of PEBP family protein in C starved *ps1ps2* mutant suggests the involvement of this protein in regulating autophagy in Arabidopsis. Collectively, our results indicate that *ps1ps2* shows an increased stress response under energy deprivation as compared to wild type.

4.1.3 Arabidopsis Presenilins Function in the Degradation Steps of Autophagy Pathway and Not in the Initiation

A prominent feature of the *ps1ps2* mutant is an increase in the number of autophagosomes compared to WT even under normal growth conditions which are evidenced by the increase in GFP-ATG8f labeled puncta (Figure 12). An increase in the autophagosome number indicates an increased amount of autophagy. Given this fact, we pondered whether there is a change in the expression of some key ATG genes in *ps1ps2* under C starvation which are involved in the initiation or vesicle elongation steps. All three of the ATG genes included in the analysis i.e. ATG9, ATG18a and ATG8a were found to be up-regulated (Figure 16). ATG9 is involved at the initiation step of autophagy and delivers membrane source for the autophagosome formation in yeast and in plants, it is required to make the early autophagic structures (Yamamoto *et al.,* 2012). ATG18a is an important molecule needed for autophagosome biogenesis which localizes at PAS and is particularly sensitive to nutrient starvation conditions. ATG8a bears the central position in autophagic vesicle elongation through its conjugation with PE. An increase in the gene expression of all of these ATG genes reflects an increase of autophagy in

ps1ps2. We also carried out a GFP-ATG8f processing assay to measure the autophagic flux of C starved *ps1ps2* seedlings. *ps1ps2* mutant exhibited a lower flux as revealed by a small amount of free GFP (Figure 17). During disease condition or autophagy dysfunction, an increase in autophagosomes is observed in cells. Therefore, it is plausible to assume that the loss of presenilins in Arabidopsis causes a disease state of autophagy which increases autophagosomes. Moreover, the observation that C starvation leads to differential accumulation of proteins in *ps1ps2* seedlings also validates our assumption that the loss of presenilins leads to a disease state of autophagy in Arabidopsis. The reduced autophagic flux in *ps1ps2* also indicates that the loss of presenilin function does not completely stop autophagy instead renders the system inefficient by disturbing autophagy after the formation of autophagosomes.

Keeping in view the malfunction in the autophagic pathway in the presenilin mutant, we wanted to know at what stage of the pathway does this dysfunction happen. Presenilin mutant showed the ability to increase the number of autophagosomes under C starvation and it confirmed that the formation of normal autophagosomes was functional in the mutant. However, we went further to investigate autophagy in GFP-ATG8f/*ps1ps2* in the subcellular level using some established inhibitors of the autophagy pathway such as wortmannin and concanamycin A. Wortmannin, an inhibitor of PI3kinase completely stopped autophagosome formation in *ps1ps2* as well as in WT both under nutrient-rich conditions as well as under C starvation (Figure 18). This observation confirmed that the presenilin mutant has defective autophagy pathway and the defect is at the later stages of the pathway and not at the initiation step. Conconamycin A is an inhibitor of vacuolar hydrolases and prevents autophagosome degradation in the vacuole. Its treatment resulted in a huge buildup of the autophagosomes in the vacuoles of both WT as well as *ps1ps2* cells (Figure 19). This showed that autophagosomes are indeed able to reach the vacuole and there is no problem in their fusion with the vacuole. The FM4-64 uptake study further confirmed the claim about the delivery of autophagosomes to the vacuole in *ps1ps2* cells (Figure 20). This claim is further strengthened by the observation that when *ps1ps2* seedlings were treated with wortmannin for 16 hours, the autophagosomes which are always present in huge

number in the knockout mutant were also disappeared (Figure 19). A possible explanation can be that wortmannin stopped the formation of new autophagosomes and the already existing autophagosomes reached the vacuole and degraded there. This may also mean that there is another pathway active which is responsible for the autophagosome delivery to the vacuole or their degradation in the absence of presenilins. It also favors our claim of the disease state of autophagy in *ps1ps2* whereby the autophagy is not completely stopped and instead becomes inefficient as a result of presenilin deficiency. However, further experimentation and analysis need to be done to better explicate the role of presenilins in plant autophagy.

As previously mentioned, there has been considerable work done in animal models to understand the function of presenilins in mammalian autophagy, however, some findings were conflicting. For instance, Lee *et al.,* (2010) demonstrated that the autophagosome buildup in the presenilin-1 knockout mutant is due to defective lysosomal acidification. Soon after, Neely *et al.,* (2001) found that there is no defect in the acidification of lysosomes, but they referred this discrepancy to different cells types used in their study than the study of Lee *et al.,* (2010). Two years later, another study reported that the autophagy turnover, vesicle pH or lysosomal function are unaffected in the mice and mammalian presenilin single and double knockout mutants (Zhang *et al.,* 2012). A relatively recent study attributed a new function to presenilin-1 which is its role in facilitating autophagosome fusion with vacuole in mouse brain cells (Bustos *et al.,* 2017). At this stage, based on our observation and in the light of knowledge obtained from animal studies, it is evident that presenilins function in a much-complicated manner and multiple mechanisms are underway to efficiently clear autophagosomes from the vacuoles. A recent study in Arabidopsis has characterized the interactome of two γ-secretase complex subunits i.e. AtPEN2 and PS2 through affinity purification and co-immunoprecipitation studies. The analysis revealed seven interacting partners of PS2 which were mainly comprised of integral membrane proteins including one R-SNARE protein i.e. PHYL1.1 (Skrzypczak *et al.,* 2019). Of note is PHYL1.1, mainly because SNARE proteins are critical for most of the vesicle fusion events including autophagosome-vacuole fusion. It would also be intriguing to discover the interacting partners of PS1 and it is possible that these groups of proteins may regulate autophagic degradation in

Arabidopsis cells by presenilins. One such important interacting partner can be Bax-inhibitor-1 (BI-1) which is an evolutionarily conserved cell death inhibitor and provides cytoprotection against various stimuli. Recently, it has been described that BI-1 forms a stable complex with presenilin-1 in a γ-secretase complex independent manner (Wu *et al.,* 2019). Interestingly, this interaction is evolutionarily highly conserved indicating the co-evolution of both proteins in eukaryotes (Wu *et al.,* 2019). Interestingly, BA-1 has been found to interact with ATG6, a core autophagy-related gene in Arabidopsis and regulates cell and autophagy (Xu *et al.,* 2017). Presenilins mutation leads to a loss of γ-secretase activity and because secretase is required for processing various substrates that regulate many downstream events, a possible mechanism by which autophagy is regulated. In its entirety, our data suggest an important role of Arabidopsis presenilins in the management of protein turnover through mediating autophagosome-vacuole interaction or maintaining vacuolar function. However, more research needs to be done to comprehend the precise mechanism of presenilins function in plants.

4.2 Arabidopsis PEN2

PEN2 or presenilin enhancer-2 is a subunit of the γ-secretase complex and plays an essential role in the complex activity. It undertakes the endoproteolysis of presenilins to generate the N and C terminal fragments (Takasugi *et al.,* 2003). Since it is an intrinsic part of the γ-secretase complex, therefore, a loss of its activity leads to a failure of the γ-secretase function. As mentioned before, the knowledge about the function of PEN2 in plants is very limiting. The little available information comes from the work on Arabidopsis and is mainly about its subcellular localization and expression (Walker 2010; Smolarkiewicz *et al.,* 2014). It has been previously reported that AtPEN2 localizes with the autophagosomal marker ATG8 in Arabidopsis leaf protoplasts (Smolarkiewicz *et al.,* 2014). Since we were keen to investigate the role of γ-secretase complex subunits in autophagy, therefore, we tested the effect of some autophagy inducing stresses such as C starvation and NaCl on the expression of stably transformed AtPEN2-GFP (data not shown). However, we did not see any change in the expression pattern of AtPEN2 under the

effect of these stresses. We then decided to find out the colocalization of AtPEN2-GFP with RFP-ATG8f and for this purpose, we co-expressed both of these constructs in tobacco leaf epidermal cells. We observed that AtPEN2-GFP vesicles were distinct from ATG8f-RFP in structure and generally did not co-localize with autophagosomes (Figure 21). However, a small portion of the AtPEN2-GFP was indeed associated with ATG8f at some point which is in conformity with the previous observation of Smolarkiewicz *et al.,* (2014). Nevertheless, further work is needed to understand this interaction.

Actin and microtubule cytoskeleton is essential for intracellular vesicle and organelle movement. Lack of colocalization studies with microtubule marker MAP4-RFP revealed no localization of AtPEN2 with microtubules (Figure 22). It was further confirmed by the treatment with oryzalin, a microtubule inhibitor which also did not influence the motility of AtPEN2-GFP. However, we found that AtPEN2-GFP positive vesicles were very sensitive to latrunculin-B, an F-actin depolymerizing drug (Figure 23). Spinning disk confocal images revealed that Lat-B treatment not just stopped the movement of AtPEN2-GFP but also changed the shape of these vesicles. Previous research shows that F-actin filaments play a critical role in protein trafficking from the Golgi complex to the vacuole (Kim *et al.,* 2005). The transport of two Arabidopsis vacuolar reporter proteins i.e. sporamin (Spo-GFP) and aleurain like protein (AALP-GFP) were affected as a result of Lat-B treatment. Subcellular localization studies disclosed that these proteins are localized as punctate stains at the Golgi complex. Moreover, *A. thaliana* vacuolar sorting receptor (At-VSR) which is normally localized at the PVC was also found accumulated at the Golgi complex after Lat-B treatment (Kim *et al.,* 2005). AtPEN2 has been reported to localize at the Golgi complex (Walker 2010) and a subset of it has also been shown to localize at the TGN and PVC (Smolarkiewicz *et al.,* 2014). A change in the expression pattern of AtPEN2-GFP under the influence of Lat-B not only confirms the role of F-actin in the movement of these vesicles but also suggests their possible involvement in the trafficking pathways between Golgi complex and vacuole. Moreover, a recent interactome analysis of the γ-secretase subunits in Arabidopsis suggested that AtPEN2 interacts with secretory proteins which suggest its involvement in protein trafficking (Skrzypczak *et al.,* 2019). F-actin has been implicated in polarized tip

growth in eukaryotic cells whereby it mediates the focusing of vesicles at the cell tip (Bibeau *et al.,* 2018). In a colocalization study, we found that AtPEN2-GFP partially localizes with DsRED-FYVE in the tobacco leaf epidermal cells (Figure 24). DsRED-FYVE is a PI3P reporter which is specifically localized with the dynamic and highly motile endosomal compartments and hence recognized as a late endosomal marker (Voigt *et al.,* 2005). This late endosomal marker has also been implicated in tip growth and interestingly, the motility of these endosomes was reported to be dependent on dynamics and polymerization of actin (Voigt *et al.,* 2005). Based on these similarities, we propose that AtPEN2 might play a role in tip growth through mediating vesicle trafficking. This suggestion is further supported by the phenotypic analysis of the *pen2* mutant whereby it displayed a reduced root growth (Figure 26), however, it needs to be confirmed with further experimentation and analysis.

In Alzheimer's disease (AD), the deposition of amyloid β protein (Aβ42) accounts for the onset and progression of the disease (Selkoe 2001). Particularly, mutations in the genes APP, PS1, and PS2 result in an increased formation of Aβ42 leading to the development of AD. Many gamma-secretase inhibitors (GSIs) have been employed to inhibit the production of Aβ42 and are considered an important tool to treat AD. Keeping in view the effectiveness of GSIs, we also tested the effect of a potent gamma-secretase inhibitor i.e. BSM-299897 on the AtPEN2-GFP line to study its effect on plant gamma-secretase activity (Figure 25). The inhibitor treatment changed the expression of AtPEN2-GFP which became more concentrated at the cross walls. This result showed that BSM-299897 affects the assembly of the gamma-secretase complex in Arabidopsis. Although we did not use this inhibitor in further experiments it would potentially prove an important tool to study the gamma-secretase complex activity in plant cells. Taken together, our work suggests that AtPEN2 is an essential constituent of the gamma-secretase complex and plays an important role in protein trafficking.

4.3 Arabidopsis Adaptor Protein Complex 4

Adaptor protein complex 4 (AP4) is one of the five AP complexes which are conserved throughout eukaryotic organisms and have been implicated in protein

trafficking. In animals, the interest in AP4 had risen from the studies where the mutations in genes coding for subunits of AP4 were identified in the patients with hereditary spastic paraplegias (HSPs) [Verkerk *et al.,* 2009]. The AP4 deficiency syndrome results in microcephaly, intellectual disability and growth retardation (Moreno-De-Luca *et al.,* 2011). Later work revealed that AP4 complex and autophagy are directly linked when ATG9 was identified as a physiological cargo of AP4 (Mattera *et al.,* 2017; Davies *et al.,* 2018; De Pace *et al.,* 2018; Ivankovic *et al.,* 2017). This interaction is carried out by the recognition of YXXØE (YQRLE) motif in the N terminal tail of ATG9 by μ4 subunit of AP4 (Mattera *et al.,* 2017). The authors of these studies attributed these defects to the mistrafficking of ATG9 by AP4 which leads to a defective function of ATG9. Among the components of ATG machinery, ATG9 is a unique protein not just because it is the only transmembrane domain-containing protein but also because it recycles between TGN and peripheral compartments of the cell. In yeast, ATG9 is exported into Golgi vesicles by ATG23 and ATG17 and in mammalian cells, AP4 is responsible for the export of ATG9 from the TGN (Yamamoto *et al.,* 2012; Mattera *et al.,* 2017). In plants, information about ATG9 is very limited, however, a recent imaging analysis has provided a profound understanding of the role of ATG9 in the formation of early autophagosomal structures (Zhuang *et al.,* (2017). It has been reported that in Arabidopsis, both N and C termini of ATG9 are exposed to the cytosol in a manner identical to the yeast or mammals. Through subcellular localization studies it has been shown that ATG9 is present in the proximity with TGN and that the ATG9 vesicles may be derived from the Golgi which is similar to the animal counterparts (Zhuang *et al.,* (2017). Overall, it was demonstrated that ATG9 regulates autophagosome progression from the ER in Arabidopsis.

Recent reports in Arabidopsis have illuminated an essential role of AP4 complex in subcellular protein sorting in plants (Pertl-Obermeyer *et al.,* 2016; Fuji *et al.,* 2016; Müdsam *et al.,* 2018). Arabidopsis AP4 is also comprising of four essential subunits as their animal counterparts encoded by four different genes namely μ4 (*AP4M1*), σ4 (*AP4S1*), ε (*AP4E1*) and β4 (*AP4B1*) [Fuji *et al.,* 2016]. Moreover, it has been demonstrated that Arabidopsis AP4 is also localized at the trans-Golgi and recognizes tyrosine-based motif YXXΦ present in the cytosolic tail of proteins

required for their sorting (Fuji *et al.,* 2016). Importantly, we also demonstrated that BFA treatment induces accumulation of AP4 in the core of the BFA compartments which further confirms that AP4 is localized at the TGN or early endosomes (Figure 31). Mutants for the subunits of AP4 were reported to exhibit defects in protein sorting, defective root and hypocotyl growth, abnormalities in male fertility and trichome morphology (Müdsam *et al.,* 2018; Fuji *et al.,* 2016). It was also reported that AP4 mutants have significantly reduced growth when grown on the media without sucrose and this observation was attributed to defects in meristem activity and cell elongation (Müdsam *et al.,* 2017). However, under nutrient starvation and as a result of the missorting of proteins, autophagy is induced in the context of arrested and reduced cell growth. Sucrose starvation causes accumulation of proteins and reduced amino acid synthesis in the cells of ATG mutants leading to defects in cellular homeostasis (Avin-Wittenberg *et al.,* 2015). In the light of the studies from animals combined with the above-mentioned abnormalities of the AP4 mutants and their similarity with the ATG mutants, we proposed that autophagy is defective in these mutant plants.

By carrying out multiple sequence alignments of ATG9 homologues from various species including Arabidopsis we provided a piece of evidence that YXXØ motif is well conserved in evolutionarily distinct organisms (Figure 27). This conserved sequence might also result in a similar interaction between ATG9 and AP4 in Arabidopsis and possibly other plants as it does in human or mice cells. This finding was further compounded by our observation that ATG9 and YFP-AP4 co-localize in the tobacco leaf epidermal cells (Figure 28). Since the N terminus of Arabidopsis ATG9 protein is also facing the cytosol, it may contain the sorting information required for interaction with the AP4 complex. Multiple sequence analysis from evolutionarily distinct plant and animal species showed that YXXØ motif is very good conserved in ATG9. Particularly, in maize, the YXXØ motif in ATG9 i.e. YRRL was very similar to that of human i.e. YQRL which suggests the evolutionary pressure to conserve this motif in evolutionarily divergent species. Since AP4 seems to play a critical role in protein sorting as described in the previous reports, it can be assumed that defects in AP4, in turn, lead to missorting and accumulation of proteins. These accumulated proteins need to be degraded through

the autophagy pathway of which ATG9 is an essential component. In AP4 deficient cells, ATG9 might not be exported to the site of phagophore assembly leading to defective autophagy. However, further investigation will be needed to better understand how this interaction between AP4 and ATG9 takes place in plants.

The mutants of AP4 subunits used in this study i.e. *gfs4/ap4β, gfs6/ap4σ, ap4e1/ap4ε* showed an accumulation of ATG8, a structural component of autophagosomes which further supports our proposal about autophagy defects in these mutants (Figure 29). DTT is an inducer of ER stress which interferes with protein folding and is used as autophagy inducer in plants. Zhuang *et al.,* (2017) proposed that ATG9 might be involved in ER-phagy due to tight coherence between autophagosome structure and the ER membrane. Since our results indicated that AP4-GFP localizes with ATG9 and might be involved in its export from the TGN like animal cells, we tested the effect of DTT on AP4 mutant lines and interestingly, all of these mutant lines showed sensitivity towards DTT which was reflected as significantly shorter growth of primary roots. Because DTT induces ER stress and ATG9 has been demonstrated to be involved in ER-phagy, our observation about the sensitivity of AP4 mutants to DTT reflects a specific role of AP4 complex in plant autophagy.

Taken together, our study has proposed that Arabidopsis AP4 might interact with ATG9 and play a role in its transport to the phagophore assembly site similarly to the situation in animals. Besides the missorting of proteins, defective autophagy might also be responsible for the phenotypic abnormality of AP4 mutants. Further investigations are needed to understand the link between the AP4 complex and plant autophagy.

CONCLUSIONS

Our study characterizes the important roles of Arabidopsis presenilins in plant macroautophagy. We have reported adverse consequences of ablating presenilins in the clearance of autophagosomes in root cells when autophagy is induced through sucrose starvation. In addition to being susceptible to the sucrose starvation, mutant cells have altered expression levels of important autophagy-related genes. Presenilin mutant root cells show dysfunction in the turnover of proteins and organelles. Immunoblotting and microscopy techniques provide evidence about the aberrant degradation of autophagosomes within the lytic plant vacuoles. Collectively, our data propose an involvement of the presenilins and/or γ-secretase complex in plant autophagy. However, the mechanisms that underlie this novel aspect of presenilin biology needs to be further investigated in plants. Our work also demonstrates that the motility of Arabidopsis PEN2, another component of the gamma-secretase complex, is dependent on the actin cytoskeleton and proposes its role in protein trafficking. Moreover, the localization of PEN2 with the late endosomal marker combined with the reduced primary root growth of the *pen2* mutant suggests a role for PEN2 in the tip growth in Arabidopsis. Our results implicate the roles of AtPEN2 in regulating tip growth and protein trafficking pathways in Arabidopsis. Nevertheless, studies on other possible functions of AtPEN2 in signal transduction and stress responses are still required.

In this study, we have proposed the involvement of AP4 complex in plant autophagy by performing analysis of Arabidopsis mutants lacking different subunits of AP4. We report that the YXXØ motif is conserved in the ATG9 protein of Arabidopsis which is required for the recognition by AP4. Moreover, a colocalization study reveals that AP4 localizes with ATG9 in the tobacco leaf epidermal cells. Besides showing sensitivity towards DTT, an ER stress inducer, the mutants of AP4 subunits accumulate ATG8, a structural component of autophagosomes. We present evidence that Arabidopsis AP4 may interact with ATG9 and play a role in its transport to the phagophore assembly site similar to animals and in addition to missorting of proteins, defective autophagy is also responsible for the phenotypic abnormality of AP4 mutants. In animals, a role for AP4 in autophagy has already

been established whereby ATG9 has been identified as a cargo of the AP4 complex. To our knowledge, this is the first report which shows that the AP4 complex plays an important role in plant autophagy. Nonetheless, further studies are required to better elucidate the connections between AP4 subunits and ATG9 and their role in autophagy.

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APPENDIX

8.1 Abbreviations

AALP	Arabidopsis Aleurain Like Protein
AD	Alzheimer Disease
AEP	Asparagine Endopeptidase
AIMs	ATG8 Interacting Motifs
Aβ	Amyloid β protein
AP	Adaptor Protein
APH-1	Anterior Pharynx Defective-1
APP	Amyloid Precursor Protein
APS	Ammonium Persulphate
AP4	Adaptor Protein Complex 4
ATG	Autophagy related
bp	base pair
BI-1	Bax inhibitor-1
BFA	Brefeldin A
C	Carbon
Cd	Cadmium
cDNA	Complementary DNA
CHMP1	CHARGED MULTIVESICULAR BODY PROTEIN 1
CLSM	Confocal Laser Scanning Microscope
Col-0	Columbia-0
CPN60β	Chaperonin 61 beta
Ct	Threshold Cycle
CTF	C Terminal Fragment
dH_2O	distilled Water

dsDNA	double-stranded DNA
EE	Early Endosomes
ECL	Electrochemiluminescence
EtOH	Ethanol
E. coli	*Escherichia Coli*
FAD	Familial Alzheimer's Disease
GFS	Green Fluorescent Seeds
GSI	Gamma Secretase Inhibitor
GTPases	Guanosine Triphosphatases
HRP	Horseradish Peroxidase
HSPs	Hereditary Spastic Paraplegias
HSP70T-1	Heat Shock Protein 70T-1
InsP3R	Inositol Triphosphate Receptor
I-CLiPs	Intramembrane Cleaving Proteases
LatB	Latrunculin B
LC3	Microtubule associated Protein 1 Light Chain 3β
LCMS	Liquid Chromatography Mass Spectrometry
LE	Late Endosomes
LIR	LC3 Interacting Region
MEFs	Mouse Embryonic Fibroblasts
MDC	Monodansylcadaverine
MS	Murashige and Skoog
MTORC1	Mechanistic Target of Rapamycin Complex 1
MVB	Multi Vesicular Body
NaOCl	Sodium Hypochlorite
NCT	Nicastrin
NDH	NADH Dehydrogenase-like complex
NTF	N Terminal Fragment

N2A	Neuroblastomas
OD	Optical Density
OST	Oligosaccharyltransferase
PAS	Phasgophore Assembly Site
PBE1 | 21S	Proteasome Beta Subunit E1
PBS buffer	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PEBP	Phosphatidylethanolamine Binding Protein
PEG	Polyethylene Glycol
PEN2	Presenilin Enhancer 2
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-bisphosphate
PI3K	Phosphoinositide-3-Kinase
PI3P	Phosphoinositol-3-Phosphate
PM	Plasma Membrane
PS	Presenilin
PSAP	PS1-Associated Protein
PSDKO	Presenilin-1 and -2 Double Knockout
PVC	Pre-Vacuolar Compartment
qPCR	Quantitative PCR
RE	Recycling Endosomes
RIP	Regulated Intramembrane Proteolysis together with
RPM	Revolutions Per Minute
SERCA	Sarco/ERCa2+-ATPase
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SNARE	Soluble NSF Attachment Protein Receptor
SnRK1	Snf1-RELATED PROTEIN KINASE 1

Spo	Sporamin
SPP	Signal Peptide Peptidases
S2P	Site-2 Proteases (S2P)
TBS	Tris Buffer Saline
TEMED	Tetraethylenediamine
TGN	trans-Golgi Network
Tm	Melting Temperature
TMD	Trans Membrane Domain
TOR	Target of Rapamycin
TPC	Two Pore Channel
TRP	Transient Receptor Potential
TSPO	TRYPTOPHAN-RICH SENSORY PROTEIN/TRANSLOCATOR
T-DNA	Transfer DNA
GSK-3β	Synthase Kinase-3 Beta
V	Volts
VPS34	VACUOLER SORTING COMPLEX 34
VSR	Vacuolar Sorting Receptor
Wort	Wortmannin
WT	Wild Type

8.2 List of Figures

8.3 List of Tables

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